Paper-fluidic electrochemical biosensing platform with enzyme paper and enzymeless electrodes

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A B S T R A C T
A miniaturized paper-based microfluidic electrochemical enzymatic biosensing platform was developed and the effects of fluidic behaviors in paper substrate on electrochemical sensing were systematically investigated. The biosensor is composed of an enzyme-immobilized pure cellulose paper pad, an enzymeless screen-printed electrode (SPE) modified with platinum nanoparticles (PtNPs), and a pair of clamped acrylonitrile butadiene styrene (ABS) plastic holders to provide good alignment for stable signal sensing. The wicking rate of liquid sample in paper was predicted, using a two-dimensional Fickian-diffusion model, to be $1.0 \times 10^{-2}$ cm$^2$/s, and was verified experimentally. Dip-coating was used to prepare the enzyme-modified paper pad (EPP), which is amenable for mass manufacturing. The EPP retained excellent hydrophilicity and mechanical properties, with even slightly improved tensile strength and break strain. No significant difference in voltammetric behaviors was observed between measurements made in bulk buffer solution and with different sample volumes applied to EPP beyond its saturation wicking volume. Glucose oxidase (GOx), an enzyme specific for glucose (Glc) substrate, was used as a model enzyme and its enzymatic reaction product $\text{H}_2\text{O}_2$ was detected by the enzymeless PtNPs-SPE in the presence of ambient electron mediator $\text{O}_2$. Consequently, Glc was detected with its concentration linearly depending on $\text{H}_2\text{O}_2$ oxidation current with sensitivity of $10.5 \mu\text{A} \text{nM}^{-1} \text{cm}^{-2}$ and detection limit of 93 $\mu\text{M}$ (at $S/N = 3$). The biosensor can be quickly regenerated with memory effects removed by buffer additions for continuous real-time detection of multiple samples in one run for point-of-care purposes. This integrated platform is also inexpensive since the EPP is easily stored, and enzymeless PtNPs-SPEs can be used multiple times with different EPPs. The green and facile preparation in bulk, excellent mechanical strength, well-maintained enzyme activity, disposability, and good reproducibility and stability make our paper-fluidic biosensor platform suitable for various real-time electrochemical bioassays without any external power for mixing, especially in resource-limited conditions.

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1. Introduction

Paper is a thin, light, flexible, disposable, and biodegradable porous medium composed of bonded hygroscopic cellulose fibers. It is easy to store, transport, and manipulate and is available in various engineered forms with different functional groups on cellulose fibers [1]. Paper can also serve as a filter to remove unwanted particulates from samples depending on the pore size. Use of paper eliminates moving parts or external connections for sample handling and allows for easy and inexpensive on-site point-of-care analyses. It can also be used as a chromatographic support to elute and separate analytes [2] and to pre-concentrate certain analytes through a series of repetitive addition-drying procedures [3]. Therefore, paper and its derivatives serve as environmentally friendly analytical platforms, especially for low-volume immunogenic and enzyme-based bioassays [1].

Paper-based analytical methods have been achieved with infrared (IR) spectroscopy [4], colorimetric assays [1,5], electrochemiluminescence (ECL) [6], mass spectrometry (MS) [7],...
chemiluminescence [8] and surface enhanced Raman spectroscopy [9]. Using paper-fluidic devices in conjunction with electroanalytical sensors is a new trend in routine analytical chemistry [10, 11] due to the combined advantages of miniaturization, low-cost, high sensitivity and ease of operation. Cellulose materials such as paper and cotton can be easily modified with conducting carbon nanotubes and used for detection of ions [12] and proteins [13]. Paper has also been popular in enzyme assays and enzyme paper test strips have been on the market for a long time. In fact, oxidative enzymes such as laccase and peroxidase with different immobilization strategies are also widely used for biobleaching of virgin pulp and recycled secondary fiber to increase both mechanical and optical qualities of paper [14, 15]. Enzymes are used in many electrochemical sensing devices [16]. Nie et al. fabricated microfluidic paper-based electrochemical devices for detecting heavy metals and glucose (Glc) [10], and Dungchai et al. [17] used paper-fluidic sensor with printed Prussian Blue electrode by measuring H2O2 to detect Glc, lactate, and uric acid. These paper-fluidic devices were fabricated by photolithography or patterning by wax printing along with enzyme spotting so that the complicated immobilization and storage procedures for enzyme electrodes are largely avoided. However, most devices cannot afford real-time continuous or repetitive measurements, which are important in medical diagnostics such as diabetes [18].

Herein we demonstrate a simple and inexpensive integrated paper-fluidic electrochemical biosensing platform for resource-limited settings using glucose oxidase (GOx) as a model enzyme with ambient O2 as co-substrate to detect Glc. We also present paper fluidic behaviors in electrochemical sensing for the first time, to our knowledge, by simulation and measurements. GOx is specific for oxidation of β-D-glucose which drives mutarotation of α anomer to β and thus catalyzes all Glc in the solution [19]. Enzyme-modified paper pads (EPPs) were prepared as chemically-selective sensing substrates by dip-coating, which is amenable for large-scale manufacturing. After enzyme modification, the EPPs retained their hydrophilicity and flexibility and even exhibited slightly improved mechanical properties. Appropriate enzyme immobilizations in a porous substrate offer such advantages as prevention of enzyme precipitations, inhibitions, distortions and possible interactions with external surfaces [20]. Screen-printed electrode (SPE) modified by platinum nanoparticles (PtNPs) served as the sensing element. PtNPs are more selective for enzymeless detection of H2O2, the enzymatic reaction product. Use of enzymeless PtNPs-SPE together with EPP is advantageous over enzyme electrodes, as it avoids complicated enzyme immobilization procedures and surface chemistry. Our integrated device also affords low-volume sensing applications, different from normal solid-state electrodes. Further, our inexpensive enzymeless PtNPs-SPEs require less stringent storage conditions, making them either single use (i.e., disposable) or repeated use by replacing only the EPP.

The PtNPs-SPE exhibited a voltammetric behavior independent of the sample volume, when exceeding the saturation volume of the paper substrate, and its amperometric response was a typical diffusion-limited current signal. The PtNPs-SPEs can be regenerated to eliminate memory effects by buffer additions. This biosensor platform can be used for other assays by using different enzymes such as cholesterol oxidase and alcohol oxidase.

2. Experimental

2.1. Chemicals

Dihydrogen hexachloroplatinate (IV) hexahydrate (H2PtCl6·6H2O) was obtained from Alfa Aesar and GOx (Type X, activity > 100 U mg⁻¹, from Aspergillus niger) was purchased from MP Biomedicals. Methylene blue and filtered 10× PBS stock solution were acquired from Fisher Scientific. The buffer was diluted to 1× solution upon use with a final phosphate concentration of 11.9 mM at pH 7.4 and was used throughout the study unless otherwise noted. All other chemicals were of analytical grade and used as received. Doubly distilled Milli-Q water (>18.2 MΩ) was employed throughout. Whatman grade 1 chromatography paper (thickness = 180 μm per manufacturer) made of pure cellulose was used as the paper-fluidic substrate for analytical filtering, separation, and sensing. SPEs with integrated three-electrode configuration, featuring graphic carbon working (surface area of ~7.07 mm²) and counter electrodes, and Ag/AgCl pellet reference electrode, were obtained from Zensor Co.

2.2. Modification of SPEs and paper

SPEs were modified by immersing them in N2-purged precursor solution of 2.0 mM H2PtCl6 and 0.5 M KCl. After 10 min of stabilization, PtNPs were electrochemically deposited onto the working electrode at a constant potential of −0.4 V for 100 s. The PtNPs-SPEs were rinsed with PBS and dried at room temperature. Whatman 1 cellulose paper pad (10 mm × 10 mm) was soaked in GOx, PBS solution (120 U of enzyme activity) for 2 h at 4 °C, dried overnight and stored in a refrigerator before use. The enzyme was immobilized and stored in paper through physisorption and hydrogen bonding between cellulose fibers and enzymes, similar to filtration [21] and spotting [1] approaches, while mild drying at 4 °C helps to optimally retain the enzyme activity. After drying, the color of the EPP turned from white into light yellow due to the adsorbed GOx. Another EPP treated similarly but in PBS served as control. For real-time consecutive amperometric measurements, 20 μL (this sample volume was determined as adequate from wicking tests and electrochemical experiments) PBS buffer was typically added to wick the EPP beforehand for a stable baseline current as it established good electrical contacts between the EPP and electrode surfaces.

2.3. Fabrication of integrated apparatus

The apparatus was fabricated to hold SPEs and the EPP in place during testing (Fig. 1) from an acrylonitrile butadiene styrene (ABS) sheet by three-dimensional (3-D) milling (iModela IM-01, Roland
Co). It consists of a bottom plate with an engraved groove to hold SPE and a top plate with a 3-mm diameter hole positioned over the sensing area of SPEs to introduce samples. The EPP is sandwiched between the top plate and SPEs. The top and bottom plates are clamped together with a binder clip to align and hold SPEs and EPP in place.

2.4. Liquid fluidic behaviors in paper

Two-dimensional (2-D) diffusion experiments were conducted by taking images with a high-speed camera (HS EX-FH100, Casio, Japan) using a F5.7 lens at 11.9 × optical zoom at 420 frames/s with 224 × 168 pixel resolution. The diffusive transport of water in paper was also modeled assuming that the sole driving force is capillary pressure. Theoretically, diffusion of water can be described either by the Lucas-Washburn or the Fickian approaches [22–24]. We chose the Fickian approach because of its simplicity. The effective diffusivity (D_eff) of water in paper was used to describe wicking behavior including the capillary effect. To simulate the radial diffusion, the Python FiPy package (A Finite Volume Partial Differential Equation Solver from Python Software Foundation) was used [25]. Aqueous drop was assumed to be initially concentrated in the center over a 5.5-mm diameter circle, where the sample is introduced. The size of the initial water droplet was estimated based on the experimental images. A zero-flux (i.e., Neumann) boundary condition was imposed along the edges of computational domain. The boundary of the wetted region was tracked with simulation time. The threshold value for the wetted region and paper boundary was set as 1% of the initial water concentration.

2.5. Surface and mechanical properties measurements

Surface characteristics of paper and SPEs were examined by scanning electron microscopy (SEM) using LEO 1530 Gemini FESEM (Carl Zeiss, USA). The hydrophilicity of modified and unmodified surfaces of SPE and paper was determined by measuring their static water contact angle using an optical contact angle measuring system (OCA 15, Data Physics).

Mechanical properties of unmodified and enzyme-modified paper were determined via uniaxial tensile tests in a material testing machine (Insight®, MTS Systems Corp., Eden Prairie, MN, USA). Paper pads were gripped using a tensile test clamp to allow a sample test area of 1 cm × 1 cm; the crosshead speed was set at 1 mm/s. In addition, a water-soaked and dried paper pad was also tested to compare the effect of enzyme modification on the mechanical properties against that of water soaking.

2.6. Electrochemical characterizations and sensing performances

All electrochemical characterizations were performed on CHI 660D. Electrochemical Workstation (CH Instruments, TX, USA) using three-electrode configuration. Glucose was detected indirectly by sensing H2O2 produced in enzymatic reactions with consumption of ambient O2 co-substrate. This sensing mechanism is similar to that employed in the first generation of biosensors, except that no enzyme electrode was used:

\[ D – glucose + O_2 \rightarrow H_2O_2 + \text{ gluonic acid} + H_2O\]

The electrochemical oxidation of H2O2 in our study involves three steps [26]: 1. binding of H2O2 to oxidized Pt surface sites to form a surface complex, 2. internal electron transfer to reduce the surface site back to Pt, and 3. in situ electrochemical oxidation to recover oxidized Pt sites. This reaction is popularly used in many enzyme-based biosensors.

3. Results and discussion

3.1. Liquid fluidic behaviors in paper

The liquid fluidic behaviors in porous paper, such as absorption, wicking, and retention, are critical for paper-fluidic electrochemical sensing. Images of water droplet diffusion over time using a simple 2-D water penetration model are shown in Fig. 2A. From these images, time vs. liquid droplet traveling distances were determined and plotted in Fig. 2B. The liquid drop spread in both horizontal and vertical directions at about equal speeds, and as expected the travel speed decreased with time. It took about 0.5 s for water droplets to travel 1 cm, which is the size of the EPP used in our study. The temporal spread of water in the paper pad determined via simulation assuming D_eff = 1.0 × 10^−2 cm²/s is presented in Fig. 2C. The calculated diameters of the wetted regions are comparable to those determined experimentally, as shown in Fig. 2D (P = 0.96 with 95% confidence level from ANOVA test). Thus, we estimated the D_eff value in our case is 1.0 × 10^−2 cm²/s, which is somewhat less than 1.6–2.0 × 10^−2 cm²/s reported for in-plane diffusivity of moisture in paper [27]. The discrepancy could be attributed to various factors, including the paper itself. Cellulose fibers in paper swell when they come into contact with water, caused by fiber-water interaction, until their water holding capacity is reached. The structure and molecular arrangement of the fibers change during swelling, leading to change in the capillary pore radius, porosity and permeability of paper, depending on the material crystallinity. Swollen fibers lead to larger pores, which further increase the water holding capacity. However, this effect and the effects of paper thickness and gravity are neglected. In reality, based on concentration-dependent diffusion in Fick’s model, the wicking speed could be somewhat different from the actual value, but the concentration effect is not taken into account for simplicity. Therefore, the calculated D_eff value can reasonably represent the penetration of water in our 2-D system.

3.2. Surface and mechanical properties

SEM images of the unmodified SPE show typical flake-like graphitic carbon sheet particles, which make up the working electrode (Fig. 3A). They are covered with some blurry irregular materials, which might be polymeric binders, forming a webbed surface but not fully occupying the interstices. These rather porous and rough surface structures directly correlate to large electroactive sites and fast electron transfer in general, with the exposed edge-plane-like sites and defects as sole origins of electron transfer [28]. After modification, spherical PtNPs, 100–150 nm in diameter, are seen homogeneously deposited throughout the porous carbon network as electrocatalysts (Fig. 3B). SEM images of unmodified and enzyme-modified paper are shown in Fig. 3C and 3D, respectively. Unmodified pure cellulose paper has an extremely low ash content of <0.06% according to the vendor and a porous randomly-felted micro-scale fiber network (diameter ~10.3 μm) with loose and hollow structures between the bonded microfiber aggregates which account for its great water-absorbing ability [29]. The enzyme immobilization does not appear to have significantly altered the overall morphology and the nature of the cellulosic fibrous network. Enzyme appears to have attached to the fiber surface uniformly, forming a thin composite cladding around the fibers and occupy the hollow inter-fiber space due to the excellent biocompatibility of cellulose [30]. This compact structure and the bonding between cellulose fibers and enzyme could be the reasons for the improved mechanical properties of EPPs compared to their water-soaked counterparts.

The contact angle of bare carbon SPEs was 105.9° and that of PtNPs-SPE was 75.5° (Fig. 4A and B), which indicates a considerable
increase in hydrophilicity due to modification of SPEs with PtNPs. This affords efficient sensing in an aqueous environment when samples are delivered to the electrode surface using EPP by facilitating improved electrical contact. Cellulose paper is well known for its hydrophilicity, which is unaffected by enzyme immobilization due to the hydrophilic nature of the enzyme (Fig. 4C and D). Therefore, EPP is capable of storing enzyme, ‘mixing’ it with sample, and delivering the enzymatic reaction products by capillary force to the sensing region with comparable properties as native paper.

Mechanical properties of paper were investigated by tensile strength tests which give information between applied tensile load and material deformation, as shown in the stress–strain (σ–ε) curves (Fig. 5A). It indicates EPP is tougher, as measured by the area under the σ–ε curve until failure, than the unmodified and water-soaked and dried papers. The EPP was superior in terms of Young’s modulus (E = σ/ε), as an indicator of material stiffness to resist elastic deformation (Fig. 5B). Meanwhile, EPP also has the widest reversible elastic deformation and irreversible plastic deformation ranges and largest yield strength, with larger ultimate tensile strength and strain at break than native and water-soaked and dried papers. These findings of improved mechanical properties of EPP compared to unmodified and water-soaked and dried papers are consistent with previous reports that water-soaking can decrease the tensile strength and tear resistance of paper [29] and treating with oxidative enzymes such as peroxidase, xylanase and laccase (in our case, GOx) results in improved mechanical properties [31,32]. Water absorption causes plasticization of cellulose fibers, relaxing and weakening of inter-fiber bonding and loss of any water-soluble adhesives as seen by the weight loss in water-soaked and dried paper, though the decrease in mechanical properties is not likely to depend on the quantity of water used for soaking [29]. The improvement in mechanical properties after enzyme immobilization might be caused by the synergistic effect of hydrogen bonding between cellulose fibers and enzymes as well as fiber oxidation owing to increased fiber surface carboxylic acid groups [32].

3.3. Electrochemical properties

Cyclic voltammetry (CV) of PtNPs–SPEs in 1.0 M H2SO4 exhibited characteristic peaks of hydrogen adsorption and desorption along with the formation of Pt oxide and oxide reduction from crystalline Pt (Fig. 6A). This typical behavior of Pt is very different from that in unmodified carbon electrode (Fig. 6A inset), indicating PtNPs were successfully synthesized on the carbon support. Therefore, PtNPs–SPEs have a large electrochemically active surface area for electrocatalysis, as estimated from the area (charge) under the voltammetric curve. The electrical resistance and the dielectric constant of paper vary with moisture content, which can greatly influence paper-fluidic electrochemical detection. Low water content will not only hinder samples being transported to the SPEs sensing surface for detection but also prevent the paper from being a ‘beaker’ to facilitate

Fig. 2. (A) Time-dependent photographs taken by high-speed camera. The ruler shows a scale of 0.5 mm. (B) Vertical or horizontal length average of the droplet travels vs. time. (C) Temporal expansion of water at time = 0, 0.05, 0.25, 0.5, 0.75, and 1.3 s. The size of paper is in cm and color contrast represents the boundary of 1% of initial concentration. (D) Comparison of simulation and experimental data.
Fig. 3. SEM images of (A) Bare SPEs (B) PtNPs-modified SPEs (inset: higher magnification) (C) unmodified paper (D) enzyme-modified paper.

Fig. 4. Water contact angles of (A) bare SPEs (B) PtNPs-modified SPEs (C) unmodified paper (D) enzyme-modified paper.
good electrical contact. In preliminary paper-based electrochemical tests, using sample volume less than sufficient to saturate the paper pad resulted in low or no current measurements presumably due to poor electrical contact and high electrical resistance, even though a uniaxial vertical force is applied over the ABS device to improve electrical contact. Therefore, a wicking test was conducted using methylene blue solution to visualize the minimum sample volume to saturate the paper pad (data not shown). It was determined at least 20 μL liquid is required to completely wick the paper pad, and to achieve a stable current signal by wetting the electrode. However, this does not mean the paper pad is actually saturated. The actual maximum liquid retention value can be much larger because porous cellulose fibers can swell substantially in water, especially in compressed condition [33]. Water as a dipole molecule can also be absorbed outside the paper via hydrogen bonding from out-of-plane H and O atoms on the sugar molecules. Liquid might also be stored in the space between the two ABS holders. Therefore, our device can actually ‘hold’ more liquid. When tested with paper pads wetted with different volumes of PBS, PtNPs–SPEs exhibited voltammetric behaviors similar to that in bulk solution in a beaker (Fig. 6B). This observation is consistent with the results reported previously, which showed that diffusion is similar in paper and bulk, and mass–limited electron transfer from redox reactions is not slowed in paper compared to that in bulk solution [10]. Therefore, while 20 μL of sample is sufficient for our analytical device, excess volume does not apparently affect the electrochemical behavior.

3.4. Enzymeless H₂O₂ sensing in paper

Electrocatalytic activities of the unmodified and PtNPs-modified SPEs against H₂O₂ were tested by CV in PBS with and without H₂O₂ using the paper substrate (Fig. 7A). Unmodified SPEs displayed a faint current signal (Fig. 7A inset b), whereas PtNPs-modified SPEs yielded a substantially large oxidation current against only 1/10 concentration of H₂O₂ (Fig. 7A b), confirming that PtNPs are good enzymeless electrocatalysts sensitive to H₂O₂. Noticeably, the oxidation current signal of PtNPs–SPEs in PBS-containing H₂O₂ (b) is comparable to that of a two-step addition (c), i.e., adding PBS followed by PBS containing H₂O₂ after complete wicking. The wicking of liquid in paper is slower when wet than when dry. Probably the first PBS addition occupies the porous structure of dry paper near the sensing region and quickly diffuses out to the edges away from the sensing region. The second addition with H₂O₂ can thus easily ‘wash away’ the previously added PBS and occupy the pores near the sensing region. As a result, it yielded comparable oxidation current signals. When another drop of PBS with the same H₂O₂ concentration was added subsequently (d), it generated a slightly higher, but not twice, signal due to temporarily high local concentration, usually known as the concentration effect. It is caused by the retention of H₂O₂ in the sensing region from the previous addition. This concentration effect has an upper limit. The concentration effect due to repetitive sample additions and retentions is an important characteristic of paper-based sensing [3]. Meanwhile,
the current signal also decreases slowly with time by diffusion following Fick's second law.

Amperometric i–t curves of bare and PtNPs-modified SPEs are compared in Fig. 7B. PtNPs-SPEs (a) produced a much larger oxidation current signal than bare SPE (b) even at 1/10 of analyte concentration, which is consistent with the CV results. It is obvious that the current at PtNPs-SPEs increases dramatically after $H_2O_2$ addition and decreased slowly with time, resembling typical non-steady state diffusion, similar to the Cottrell behavior of diffusion-limited condition from a still solution in a conventional
electrochemical cell. Furthermore, it takes a fairly long time (~40 s) to register signal peak due to slow energy-free diffusion within the EPP and slow electron transfer at the electrode–analyte interface. The amperometric current responses towards H$_2$O$_2$ oxidation followed a linear dependence ($R^2 = 0.998$) (Fig. 7B inset) and the detection limit and sensitivity are calculated to be 2.4 µM and 19.2 µA·mM$^{-1}$·cm$^{-2}$, respectively.

For practical applications, real-time and regenerative detections preferably without external mixing are highly desirable. Real-time detection was evaluated by consecutively introducing PBS with or without H$_2$O$_2$ (Fig. 7C). The current signal rises immediately after H$_2$O$_2$ injection, reaches the maximum value with the highest local concentration on SPE surface and decays gradually due to diffusion. Moreover, the current signal is fairly smooth and flow-induced current fluctuation is barely seen, indicating paper-fluidic is an efficient, energy-free and stable approach to deliver liquid sample for electrochemical sensing. Apart from the fast and sensitive response, the recovery of the sensor by introducing buffer was also rapid. The signal returns to a new baseline level within 30 s. However, an apparent memory effect can be observed probably caused by the retention of previous samples in the paper network. As the mobility of liquid is slower in wet paper, one buffer introduction might not be sufficient to ‘wash off’ the previous samples leading to the comparatively higher baseline. The buffer addition also caused an amperometric spike before rapid decay (Fig. 7C inset), which is likely caused by a sudden increase in local diffusion rate after addition. This phenomenon is similar to that in a flow injection analysis (FIA) system, where oxidation current increases with increasing flow rate [34]. In spite of some memory effect, repeated injections of equal H$_2$O$_2$ concentrations yielded fairly reproducible oxidation current values (relative standard deviation, RSD = 9.3%), demonstrating its potential for multiple measurements.

From Fig. 7A, the concentration effect could have given inaccurate larger signals with repetitive additions of the same concentration of analytes. The result is further confirmed by repetitive addition of the same H$_2$O$_2$ concentration (Fig. 7D). Pure PBS buffer did not cause significant increase in current but only shows a quickly disappearing temporary current spike (first arrow) generated from local diffusion rate change. Following the first introduction of H$_2$O$_2$, which caused a drastic current rise, the second and third injections led to small continuous increases and non-linear responses corresponding well to the observation of concentration effect in CVs (Fig. 7A). As demonstrated above, this effect has an upper limit with the maximum plateau current maintained at the same level (Fig. 7E). Further additions from the fourth addition on no longer triggered a concentration effect. Even after several additions, the paper-fluidic biosensor still showed acceptable ability to recover and the second PBS addition (Fig. 7D, third arrow) can completely regenerate the sensor by returning the current to its original level and thus eliminating the memory effect quickly. The second detection was highly reproducible and still exhibited concentration effects in the first three introductions. These results prove that our integrated paper-fluidic electrochemical platform can be used for continuous and repetitive detections.

3.5. Glucose sensing

Sensing performance of our biosensor was assessed by CVs in PBS solutions containing various amounts of Glc (Fig. 8A). When samples with Glc are added, a strong oxidation current (b–g) with voltammetric behavior similar to that of H$_2$O$_2$ oxidation (Fig. 8A) was obtained, confirming that it is due to oxidation of the enzymatic reaction product, H$_2$O$_2$. The oxidation current increased linearly with increasing Glc concentration at +0.4 V ($R^2 = 0.994$) with a detection limit of 9.3 µM (at $S/N = 3$) and a dynamic working range of 0.5–3.0 mM. The sensitivity was determined to be 10.5 µA·mM$^{-1}$·cm$^{-2}$. These performances are better than some other integrated Glc devices at resource-limited settings. For example, the detection limits of paper-ITO-Prussian Blue platform [35], paper-fluidic device with flow-injection [36] and printed electrodes on paper [17], were 0.1, 0.2, and 0.21 mM, respectively. The indicator of enzyme-substrate reaction kinetics, the apparent Michaelis–Menten constant ($K_{m}^{app}$), was calculated according to the electrochemical version of Lineweaver-Burk double reciprocal equation to evaluate the affinity and biological reactivity of the enzyme-functionalized paper to the substrate [37]; 

$$\frac{1}{I} = \frac{1}{max+(K_{m}^{app})} \frac{1}{C_{Glc}}$$

In many cases of enzyme immobilization, distortion of the enzyme could happen due to the interaction with the support, causing impoverishment of enzyme properties [20]. The $K_{m}^{app}$ of our biosensor was 0.8 mM which is less than that for most enzyme electrodes. The other reported values include 0.95 mM for layer-by-layer assembly on carbon nanotubes [38], 2.2 mM for N-doped carbon nanotubes [39], 7.8 mM for the enzymes immobilized on chitosan-ionic liquid-AuNPs [37], 10.5 mM for reduced graphene sheet-AuPd NPs [40], and 12.4 mM for nano Co phthalocyanine [41]. Therefore, in our system the enzyme maintains good substrate affinity and activity towards Glc after it had been immobilized in the biocompatible cellulose network, showing advantages of using enzyme paper with enzymeless electrodes.
The amperometric response was much larger when used with EPP than with unmodified paper pad, even when the latter was tested with a sample containing five times as much Glc as the former (Fig. 8B). It confirmed that the strong current signal comes from oxidation of the enzymatic reaction product H$_2$O$_2$, rather than direct Glc oxidation. The minor current increase observed with unmodified paper pad is attributed to non-enzymatic Glc oxidation in Pt nanostructures [42]. Notably it takes longer to achieve the current signal during Glc detection (200 s) than during H$_2$O$_2$ detection, owing to the enzymatic reaction on the EPP and delivery of the produced H$_2$O$_2$ in the EPP to the sensing region.

A major advantage of the dip-coating approach we used for enzyme immobilization on paper pads is its potential for mass production. A batch of 10 EPPs we prepared using 5 mL enzyme solution with maintained the enzyme activity giving a satisfactory RSD of 7.6%, tested with 1.0 mM Glc addition through amperometric responses. However, it should be noted that for mass production of more batches, the concentration of the enzyme solution should be maintained constant making up for the enzyme being adsorbed during the process. We also examined how well the enzyme activity is preserved in the EPP during storage. Amperometric responses to 1.0 mM Glc from the same batch of EPPs were estimated after 2 week storage in a Petri dish at 4 °C and the oxidation current was 96.5% of the freshly prepared one. A previous study had suggested that EPPs can retain their activity even after 30 days of storage at 25 °C without excluding air [1]. The stability of the enzyme immobilized in paper can be further improved by lyophilization for storage and by the addition of some preserving chemicals. These results suggest that our biosensor is easy to mass produce and can well preserve the enzyme activity during storage. The paper–fluidics preparation and detection approaches used in this study can be readily extended for sensing applications involving other enzymes.

4. Conclusions

We investigated the paper-fluidic behaviors both by simulation and measurement and described their effects on electrochemical sensing using an integrated portable biosensing device with enzyme assay for Glc detection. Our paper-fluidic electrochemical enzymatic biosensor distinguishes itself from conventional standard enzyme electrode systems through the use of enzyme-modified paper pads (EPPs) in conjunction with enzymeless PtNPs-modified SPE. The EPPs retained their hydrophilicity and even exhibited slightly improved mechanical properties; they also afforded rapid sample delivery and mixing via capillary forces for enzymatic reactions without requiring an external power supply. The ABS holders used in the device helped to ensure decreased evaporation, stable alignment between the EPP and SPE and a close contact between the liquid sample and PtNPs-SPE by supplying a constant hydrodynamic compression pressure for rapid wicking, faster and larger liquid absorption ability. The facile fabrication, ease of operation, stability, and miniaturization of this device make our system suitable for various enzymatic biosensing applications.

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References

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