



# A multiplexed three-dimensional paper-based electrochemical impedance device for simultaneous label-free affinity sensing of total and glycated haemoglobin: The potential of using a specific single-frequency value for analysis

Yuwadee Boonyasit<sup>a</sup>, Orawon Chailapakul<sup>b</sup>, Wanida Laiwattanapaisa<sup>c,\*</sup>

<sup>a</sup> Graduate Program in Clinical Biochemistry and Molecular Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, 10330, Thailand

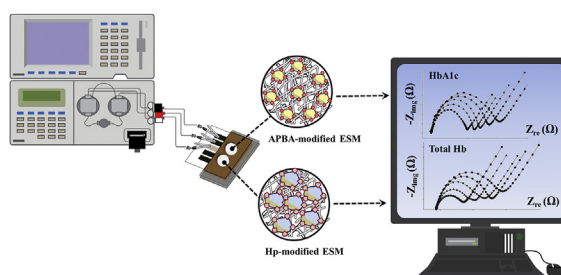
<sup>b</sup> Electrochemistry and Optical Spectroscopy Research Unit (EOSRU), Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand

<sup>c</sup> Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, 10330, Thailand

## HIGHLIGHTS

- A cost-effective 3D-PEID was developed for label-free impedimetric affinity sensing of total and glycated haemoglobin.
- Determination of the optimal binding frequency facilitates fast impedance measurements using a single frequency.
- Hp and APBA are recognition elements for selective binding of total and glycated haemoglobin, respectively.
- The proposed system meets the clinical requirements for glycaemic assessment in diabetic patients.

## GRAPHICAL ABSTRACT



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## ABSTRACT

A novel three-dimensional paper-based electrochemical impedance device (3D-PEID) is first introduced for measuring multiple diabetes markers. Herein, a simple 3D-PEID composed of a dual screen-printed electrode on wax-patterned paper coupled with a multilayer of magnetic paper was fabricated for label-free electrochemical detection. The results clearly demonstrated in a step-wise manner that the haptoglobin (Hp)-modified and 3-aminophenylboronic acid (APBA)-modified eggshell membranes (ESMs) were highly responsive to a clinically relevant range of total (0.5–20 g dL<sup>-1</sup>;  $r^2 = 0.989$ ) and glycated haemoglobin (HbA1c) (2.3–14%;  $r^2 = 0.997$ ) levels with detection limits (S/N = 3) of 0.08 g dL<sup>-1</sup> and 0.21%, respectively. The optimal binding frequencies of total haemoglobin and HbA1c to their specific recognition elements were 5.18 Hz and 9.99 Hz, respectively. The within-run coefficients of variation (CV) were 1.84%, 2.18%, 1.72%, and 2.01%, whereas the run-to-run CVs were 2.11%, 2.41%, 2.08%, and 2.21%, when assaying two levels of haemoglobin and HbA1c, respectively. The CVs for the haemoglobin and HbA1c levels measured on ten independently fabricated paper-based sheets were 1.96% and 2.10%, respectively. These results demonstrated that our proposed system achieved excellent precision for the simultaneous detection of total haemoglobin and HbA1c, with an acceptable reproducibility of fabrication. The long-term stability of the Hp-modified eggshell membrane (ESM) was 98.84% over a shelf-life of 4 weeks, enabling the possibility of storage or long-distance transport to remote regions,

\* Corresponding author.

E-mail address: [Wanida.L@chula.ac.th](mailto:Wanida.L@chula.ac.th) (W. Laiwattanapaisa).

particularly in resource-limited settings; however, for the APBA-modified ESM, the stability was 92.35% over a one-week period. Compared with the commercial automated method, the results demonstrated excellent agreement between the techniques ( $p$ -value < 0.05), thus permitting the potential application of 3D-PEID for the monitoring of the glycaemic status in diabetic patients.

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## 1. Introduction

The implementation of microfluidic paper-based analytical devices ( $\mu$ PADs) in clinical off-site diagnoses has recently emerged as a distinctive field of simple telemedicine in remote areas [1]. The multiplexed measurement of a panel of biomarkers has recently attracted considerable interest due to its great potential for monitoring patient compliance, evaluating the effectiveness of therapy, and early screening for diseases. During recent years,  $\mu$ PADs coupled with optical imaging for colourimetric detection have provided an affordable point-of-care platform for the multiplexed analysis of biomarkers, such as the transaminase assays for liver function assessment [2,3], detection of metabolic biomarkers for glycaemic control [4,5], urinalysis assays [1,6], and simultaneous detection of glucose, uric acid and lactate [7]. Alternatively, other detection methods for assaying multiple biomarkers on a single 3D- $\mu$ PAD have been demonstrated, including electrochemical [8–13], chemiluminescence [14], and electrochemiluminescence [15] techniques, most of which relied heavily on ultrasensitive immunodevices for multiplexed quantification of cancer biomarkers. To date, considering the existing paper-based electrochemical devices for measuring metabolic biomarkers, considerable efforts have been focused only on establishing a proof-of-concept work on the determination of glucose, uric acid, and lactate [16,17]. However, there has been minimal validation of  $\mu$ PADs using actual clinical specimens. Thus far the label-free impedimetric sensing of diabetes markers for the long-term assessment of glycaemic control on a single 3D- $\mu$ PAD has yet to be investigated. As an alternative to the single-analyte assays, the multiplexed 3D- $\mu$ PAD allows simultaneous measurement of multiple analytes on a single device, which provides an accurate basis for clinical diagnoses and decreases the assay time. To our knowledge, there have been no attempts to use a single three-dimensional paper-based electrochemical impedance device (3D-PEID) for measuring multiple diabetes markers.

Currently, electrochemical impedance spectroscopy (EIS)-based point-of-care diagnostic platforms for determining a panel of biomarkers have attracted great interest in the clinical assessment of early disease detection. Known as an informative and nondestructive technique for biosensing applications, EIS has an enormous potential for the label-free and ultrasensitive biomarker detection with the capability to measure multiple markers simultaneously as it can be used to study the interfacial events or diffusion effects occurring at the surface of the electrodes. Because acquisition of impedance spectra is relatively time consuming, many attempts have been made to use a single frequency value for analysis [18–22]. More recently, multiplexed sensor array designs, most of which depend on antibody-based molecular recognition, have been implemented for the determination of various inflammatory markers using a unique frequency upon binding of the target molecule to the sensor [23,24]. The biological reaction between each target and its molecular recognition element results in a unique binding frequency that is specific to each reaction. Thus, the differences in frequency signals from each reaction can be detected on a single platform due to the effective discrimination of the target binding frequencies from the others. Using this

underlying principle, by immobilising recognition elements for different biomarkers on the sensor interface, each target molecule can be measured simultaneously by monitoring their optimal binding frequency, thereby making this platform suitable for multiplexed assays of markers in a single device. The specific optimal binding frequency depends on several factors such as the sensor material, molecular recognition element, and the linkers used for immobilisation [25]. For instance, EIS in combination with technology for the management of patients with diabetes mellitus (DM) was implemented using a specific frequency for glucose-glucose oxidase binding interaction [26]. A few years later, the feasibility of EIS in detecting 1,5-anhydroglucitol levels at its optimal binding frequency was also demonstrated using the enzyme pyranose oxidase [27]. However, there are no relevant data as of yet on the label-free affinity biosensing for measuring multiple diabetes markers on a single device. Hence, a multiplexed single-sensor diabetes marker assay needs to be further developed to meet the clinical requirements for a point-of-care testing (POCT) system.

Typically, in clinical practice, the quantitative measurement of glycated haemoglobin (HbA1c) is an indispensable index for the long-term monitoring of glycaemic control in both the diagnosis and routine management of diabetes. The intensive monitoring of glycaemic status is needed to avoid diabetic complications. In general, the HbA1c level is measured as the percentage of glycated haemoglobin in the total haemoglobin (i.e., mmol mol<sup>-1</sup> or %). According to the consensus statement of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the National Glycohemoglobin Standardization Program (NGSP) on the standard interpretation norms of HbA1c values, HbA1c results are reported worldwide together with the haemoglobin value as mmol of HbA1c or a percentage of HbA1c in the total haemoglobin, respectively [28]. HbA1c analysis has been accomplished using a wide range of techniques, including mass spectrometry [29–31], electrophoresis [32], chromatography [33,34], immunoassays [35–39], electrochemistry [40–49], enzyme assays [50–53], piezoelectric sensing [54–56], and optical spectroscopy [57–59]. However, most of the aforementioned approaches require the use of highly sophisticated instruments at high operating costs by experienced personnel. Moreover, they fail to satisfy the analytical requirements of sensitivity, specificity, reproducibility, storage stability, simplicity, and portability. For these reasons, further improvements of cost-effective diagnostic POCT devices are still required for the clinical assessment of glycaemic status in diabetes patients. Thus far, there have been no reports in the literature regarding simultaneous detection of both total haemoglobin and HbA1c based on a single affinity-based sensing device. In our preceding work, the label-free boronate-modified eggshell membrane (ESM)-based affinity sensor for long-term glycaemic monitoring was first demonstrated via the cis-diol interaction between HbA1c and the boronate recognition element [60]. Using the boronate-modified sensing surface, our device could distinguish between HbA1c and non-glycated haemoglobin (HbAo). However, acquisition of impedance spectra typically required a scanning time of 15 min for the entire frequency range. Therefore, in the present work, we have further developed the affinity membrane-based

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