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RESEARCH PAPER

Selective label-free electrochemical impedance measurement of glycated haemoglobin on 3-aminophenylboronic acid-modified eggshell membranes

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Abstract We propose a novel alternative approach to long-term glycaemic monitoring using eggshell membranes (ESMs) as a new immobilising platform for the selective label-free electrochemical sensing of glycated haemoglobin (HbA1c), a vital clinical index of the glycaemic status in diabetic individuals. Due to the unique features of a novel 3-aminophenylboronic acid-modified ESM, selective binding was obtained via cis–diol interactions. This newly developed device provides clinical applicability as an affinity membrane-based biosensor for the identification of HbA1c over a clinically relevant range (2.3 - 14 %) with a detection limit of 0.19 %. The proposed membrane-based biosensor also exhibited good reproducibility. When analysing normal and abnormal HbA1c levels, the within-run coefficients of variation were 1.68 and 1.83 %, respectively. The run-to-run coefficients of variation were 1.97 and 2.02 %, respectively. These results demonstrated that this method achieved the precise and selective measurement of HbA1c. Compared with a commercial HbA1c kit, the results demonstrated excellent agreement between the techniques (n=15), demonstrating the clinical applicability of this sensor for monitoring glycaemic control. Thus, this low-cost sensing platform using the proposed membrane-based biosensor is ideal for point-of-care diagnostics.

Keywords Glycated haemoglobin (HbA1c) · Diabetes mellitus · 3-aminophenyl boronic acid · Eggshell membrane · Membrane-based biosensor · Selective label-free electrochemical detection

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Introduction

The alarming increases in mortality and health care expenditures resulting from diabetes have brought about considerable efforts to make disease-related measurements outside clinical settings, especially at a patient's bedside. High blood glucose contributes significantly towards many chronic complications, e.g., atherosclerosis, kidney failure, retinopathy, and cognitive degeneration [1, 2]. The frequent monitoring of glycaemic levels is of great importance for preventing the serious complications associated with diabetes and also for delaying the clinical progression of the disease. Traditionally, glycated haemoglobin (HbA1c) has been used as a predominant biomarker for the long-term assessment of glycaemic control in clinical practice. HbA1c is irreversibly formed by a slow, non-enzymatic glycation process at one or both of the N-terminal valine residues of the β-chains of haemoglobin over a long period of time, which corresponds to the average lifespan of erythrocytes in the preceding 2–3 months. The American

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Diabetes Association (ADA) strongly recommends maintaining tight control over the level of HbA1c, with recommended values lower than 7 % and using HbA1c level as a diagnostic criterion for diabetes [3]. A quantitative measurement of HbA1c should be performed at least twice a year in patients with good glycaemic control and at least once every 3 months in individuals with poor glycaemic control [3].

Currently, the quantitative analysis of HbA1c is performed with a variety of analytical techniques, including electrophoresis [4], ion-exchange chromatography [5], boronate affinity chromatography [6], mass spectrometry [7–9], immunoassays [10–13], electrochemical detection [14–19], piezoelectric sensors [20–22], chemiluminescence [23], surface plasmon resonance [24] and surface-enhanced resonance Raman spectroscopy [25]. Among these available techniques, the major drawbacks are that such methods are rather complicated, take a long time for the analysis [10] and require the use of labelled antibodies [16], expensive equipment [20, 22] and/or sample preparation prior to the analysis [26]. Additionally, the effects of haemoglobin variants and chemically modified derivatives could also complicate these methods of HbA1c measurement [4]. Although boronate affinity chromatography and mass spectrometry appear to be unaffected by interference from haemoglobin derivatives, the high-priced cost of analysis and the need for sophisticated instruments making these methods unsuitable for routine clinical usage [7].

Due to the unique features of boronic acid binding, this compound is of interest in developing an alternative detection method to distinguish between non-glycated haemoglobin (HbAo) and HbA1c. Boronate groups are able to form stable complexes with the diol groups from glycated proteins under alkaline conditions [27]. Consequently, a boronate derivative is a critical component of an affinity biosensor for the analysis of glycosylated biomolecules. More recently, to amplify the electrochemical signal, Song et al. proposed the competition assay between HbA1c and glucose oxidase on the boronate-modified electrode surface for HbA1c determination in whole blood samples [17]. The proposed biosensor provided a linear response covering the clinical reference range found in diabetic patients (4.5–15 %). However, in contrast to the voltammetric and amperometric methods, impedimetric measurements gain the full benefits of understanding the chemical reaction mechanisms, including electron transfer, absorption, conductance, and mass transport of the redox probe [28]. Therefore, for this purpose, the use of electrochemical impedance spectroscopy for investigating chemical characteristics is highly desirable. A technique for selective HbA1c biosensing by impedance spectroscopy has been previously reported to enable the determination of HbA1c concentrations with high sensitivity [29–31]. This previously reported technique depended primarily on a self-assembled monolayer (SAM) of thiophene-3-boronic acid (T3BA) on gold electrodes. However, the dynamic detection range has yet to match the physiological range of HbA1c in real blood samples (3–13 mg mL^{-1}) [21]. Thus, improvements of the sensing interface are needed to achieve an acceptable linear range for the clinical assessment of HbA1c.

Porous fibres consisting of proteins, e.g., eggshell membranes (ESMs), have attracted much attention because of the wide potential applications of these fibres as low-cost platforms for immobilisation. Previous studies on other selective microporous membranes [32, 33] suggested the potential problems of delicate operation and high cost as important barriers for the development of membrane-based biosensors. ESM, a naturally occurring biological polymer, has a distinct property of an interconnected porous structure, making ESM a useful biomaterial to use as a template for surface modification. ESM is generally available, affordable, abundant, biocompatible and environmentally friendly. Moreover, the amines and amides on its surface present positively charged functional groups can be functionally modified [34]. Because of these properties, ESM has currently been used in several biomedical applications as a membrane for guided bone regeneration [35], a biological dressing to promote the infection-free healing of wounds [36] and a platform for protein immobilisation [37, 38].

To date, no data is currently available on label-free biosensing with boronate affinity applications. Therefore, the primary aim of this work was to investigate the possibility of using ESM as a new immobilising platform for surface modification with boronate derivatives. Such a platform can be applied to the generation of an affinity-based biosensor to identify HbA1c. In this study, a novel ESM-based analytical device for the quantitative measurement of HbA1c in patient blood samples was constructed and evaluated. The selective 3-aminophenylboronic acid (APBA)-modified ESM was constructed as a specific binding component of a device for the label-free electrochemical impedance spectroscopy measurement. The APBA plays a key role in the selective binding of HbA1c via cis–diol interactions with a boronate-recognition group. To our knowledge, this newly proposed device is the first ESM-based biosensors for determining HbA1c in blood samples. Using ESM as a new immobilising platform, our study demonstrated that ESM can be used in the development of a reliable and inexpensive device for assaying HbA1c.

Materials and methods

Reagents and chemicals

For assaying HbA1c levels, a Lyphochek® HbA1c Linearity Set and Lyphochek® Diabetes Controls were purchased from BioRad Laboratories (Hercules, CA, USA). Analytical grade chemicals were used throughout this study. APBA, 4-ethylmorpholine, sodium chloride, potassium chloride,

potassium hexacyanoferrate II, potassium hexacyanoferrate III, a glutaraldehyde solution (25 %, *w/w*), ethanolamine, sodium acetate trihydrate, potassium cyanide, sodium phosphate monobasic, sodium phosphate dibasic, potassium ferricyanide, sodium bicarbonate and haemoglobin-Ao were obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid, Brij® 35 (polyoxyethylene (23) lauryl ether detergent) and acetic acid were acquired from Merck (Darmstadt, Germany). The materials used to determine the haematocrit (Hct) values, including microhaematocrit tubes, a microcapillary reader, and a microhaematocrit centrifuge, were manufactured by Vitrex Medical A/S (Herlev, Denmark), International Equipment Company (Needham Heights, MA, USA) and Hawksley and Sons Ltd. (Sussex, England), respectively. A cyanmethaemoglobin standard solution was available from a local service provider. The commercial platinum screen-printed electrode was supplied by DropSens (Asturias, Spain). To determine the HbA1c levels in the whole blood samples, commercial in2it™ (II) A1c test kits from BioRad Laboratories (Hercules, CA, USA) were employed to validate the method based on well-established boronate affinity chromatography.

ESM preparation

Chicken eggs were purchased from the local supermarket and stored at 4 °C before use. The ESM is a double-layered membrane inside the eggshell composed of highly cross-linked proteins. According to the method described by Yeni et al. [39], to obtain the whole ESMs, eggs were soaked in absolute acetic acid at 4 °C for 18 h and the membrane was subsequently peeled off of the broken eggshell. Afterwards, the membranes were cleansed with a copious amount of deionised water before cutting into circles with a diameter of approximately 13 mm. The circular ESMs were stored in a 10-mM 4-ethylmorpholine buffer containing 0.25 M KCl and 0.1 M NaCl until further use.

To characterise the microstructure of the ESM with and without the immobilised HbA1c, a scanning electron microscope (JSM-5410LV, JEOL, Tokyo, Japan) and transmission electron microscope (TEM-2100, JEOL, Tokyo, Japan) were used to study the surface and internal structure of the ESMs. For scanning electron microscopy (SEM), the dried circular membranes were placed on a specimen stub and coated with a thin layer of gold before analysis. To prepare the ESMs for transmission electron microscopy (TEM), the membranes were initially fixed with a 2.5 % (*w/w*) glutaraldehyde solution and 1 % (*w/v*) osmium tetroxide before being dehydrated with a series of washes with ethanol at concentrations ranging from 35 to 95 %. The membranes were further immersed in propylene oxide, embedded in an epoxy resin solution, dried, and then cut to 90-nm thickness using an ultramicrotome before being placed on a copper grid. Finally, to increase the contrast level of the image, the membranes were stained with uranyl acetate and lead citrate before the TEM investigation.

Surface modification with APBA

Unless otherwise stated, for fabrication of the APBA-modified ESM, a drop of glutaraldehyde solution was placed on the surface of the membrane, which was then thoroughly washed with 10 mM 4-ethylmorpholine buffer before the addition of 2.5 mg mL^{-1} of APBA. The excess aldehyde groups were subsequently removed by rinsing with 10 mM ethanolamine followed by an additional wash. Finally, various concentrations of HbA1c were used to investigate whether HbA1c could bind to the selective sensing surface via cis–diol interactions. Each consecutive step was carried out on the same piece of platinum screen-printed electrode with the use of the electroactive redox probe. The electrochemical impedance spectroscopy measurement was conducted in a step-wise manner. The APBA-modified ESMs could be used repeatedly after washing with a regeneration buffer, 10 mM sodium acetate at pH 5, which reversed the HbA1c binding reaction. A sodium acetate buffer could be used to remove HbA1c from the APBA-modified membranes because the binding of boronate groups with diol groups has been shown to be quite unstable under acidic conditions [40].

Glutaraldehyde was used as a coupling agent and served as a homo-bifunctional crosslinker between the amine moiety of APBA and the amine groups on the surface of the ESM, as depicted in Fig. 1b. The aldehyde group is expected to attach to the amine group of APBA. Afterwards, any remaining aldehyde groups were then blocked with the ethanolamine buffer. In such a case, the specific binding between the boronic acid groups and HbA1c occurs via cis–diol interactions.

Sample preparation

Healthy participants and diabetic patients, as defined by the American Diabetes Association criteria [41], volunteered to take part in our study. Written informed consent was obtained from all the individuals before the study began. The project regarding the development of membrane-based biosensors was approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Sciences Group, Chulalongkorn University (ECCU) under approval number COA No. 057/2557. Whole blood samples were collected in vacuum blood collection tubes using tripotassium ethylenediaminetetraacetic acid (K_3EDTA) as an anticoagulant. The Hct and total haemoglobin (Hb) were quantified using the microcapillary and cyanmethaemoglobin methods (Drabkin's reagent), respectively. After measuring the Hct and total Hb, centrifugation was used to separate the plasma from cells, and then the plasma was discarded to eliminate glucose and other glycated proteins existing in the plasma.

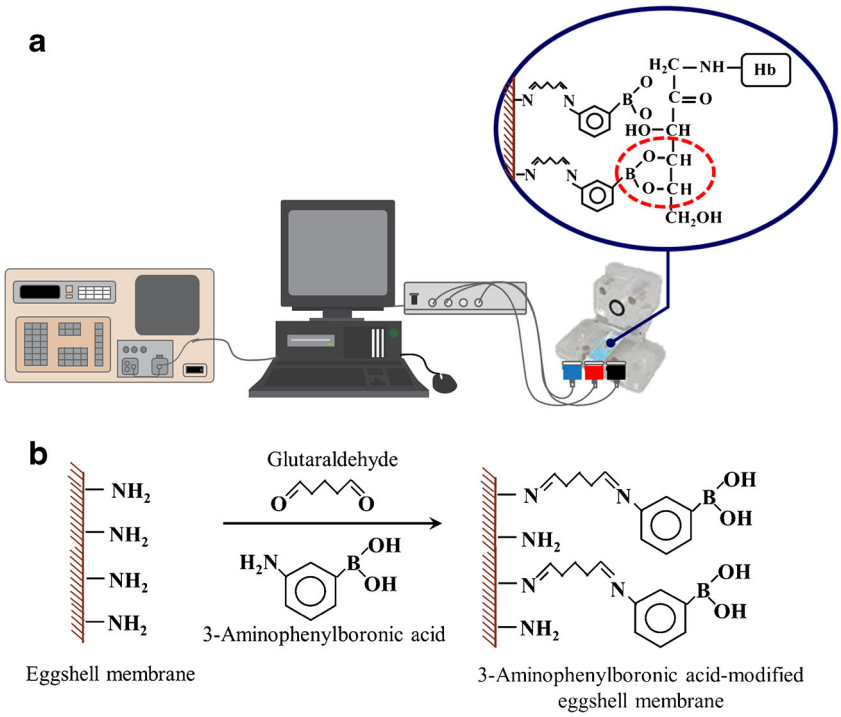

Fig. 1 Schematic diagram of the proposed ESM-based biosensor illustrating (**a**) a configuration of the label-free electrochemical impedance system setup and (**b**) a reaction scheme for immobilising APBA on the surface of ESMs

The red blood cells remaining were carefully washed three times with physiological saline (a 0.9 % sodium chloride solution) to remove the plasma completely. To prepare the red blood cell lysates, a haemolysing buffer solution (26 mM NaH_2PO_4, 7.4 mM Na_2HPO_4 and 13.5 mM KCN), as prepared according to a previous study [14], was used to lyse the red blood cells prior to the electrochemical impedance measurement.

Label-free electrochemical impedance spectroscopy system setup

The label-free electrochemical impedance spectroscopy measurement was carried out with a potentiostat/galvanostat instrument (Autolab PGSTAT30, Eco Chemie, The Netherlands) equipped with the Frequency Response Analyser system software. In this study, the impedance detection system was connected to a commercial platinum screen-printed electrode (DropSens, Asturias, Spain) for the selective electrochemical sensing of HbA1c. This new configuration is illustrated in Fig. 1a. A circle-sized APBA-modified ESM (13 mm) was well positioned on the platinum screen-printed electrode surface and covered all of the three electrodes, consisting of the counter (CE), working (WE) and reference electrodes (RE). The membrane was carefully placed over the electrode to prevent the formation of air bubbles between both layers. The whole assembly, i.e., an integrated electrode with a thin layer of ESM, an O-ring and a custom-ordered holder, was clamped together through a magnetic force. The electronic connections were accomplished by a customised designed electronic connector. The impedance measurement was conducted over a frequency range of 10 Hz to 100 kHz with an applied current potential of 10 mV. The redox ions, i.e., a 5-mM $(Fe(CN)_6)^{3-/4-}$ solution containing 0.25 M KCl and 0.1 M NaCl dissolved in 10 mM 4-ethylmorpholine buffer, were prepared and used as an electroactive probe throughout the experiment. The impedance data were fitted to an equivalent-circuit model using the NOVA 1.9 software.

Results and discussion

Surface characterisation of the ESM

The ESM, a thin film adhering inside the eggshell, is composed of three thin membranes, namely, the outer ESM, inner ESM and limiting membrane, from outside to inside [42]. In our study, the entire ESM was used as a sheet membrane, and the membrane surface that contacts the shell was called the outer surface, whereas the opposite surface was called the inner surface. Figure 2 displays scanning electron micrographs (a-d) and transmission electron micrographs (e, f) of ESMs with and without the immobilised red blood cell lysates. The surface structure was found to be different side of an ESM, as shown in Fig. 2a. The total thickness of the ESM was approximately 60–70 μm, which is in agreement with the work of Takiguchi et al. [43]. A network-like structure was observed on the ESM surface (Fig. 2b), indicating that the ESM consisted of highly cross-linked protein fibres and cavities. The fibres of the inner layer were smaller and smoother

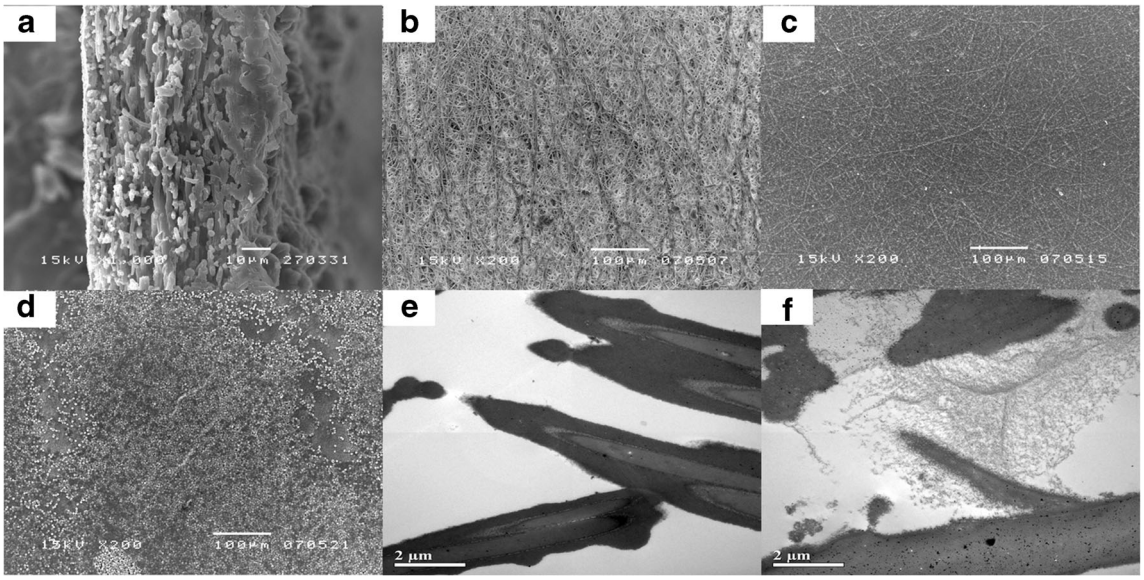

Fig. 2 SEM images of the ESM: (**a**) cross-section, (**b**) outer surface, (**c**) inner surface and (**d**) after exposure to HbA1c; and TEM images of ESM: (**e**) membrane fibres consisting of a collagen-rich core surrounded by a mantle layer and extra-fibre spaces and (**f**) after exposure to HbA1c

than those of the outer layer (Fig. 2c). After the immobilisation of APBA, the red blood cell lysates were able to adhere to the surface of the ESM, as depicted in Fig. 2d. Our analysis indicated that the red blood cell lysates were successfully immobilised on the surface of the ESM. Figure 2e presents the TEM micrograph showing that the membrane fibres are 1–4 μm in diameter and separated by extra-fibre spaces. Each fibre consists of a collagen-rich core that is surrounded by a glycoprotein-rich mantle [44]. The internal cavity of the ESM was occupied by HbA1c after exposure to the red blood cell lysates (Fig. 2f). These results implied that some components of the red blood cell lysates attached to the surface of the ESM, while other components entered into the interlacing network of ESM fibres.

HbA1c optimisation of the assay

Effect of pH

The effect of pH has been widely perceived to be the most crucial factor for affinity binding between HbA1c and the boronate groups. In general, under alkaline conditions, boronic acid is transformed to its tetrahedral anionic form, which subsequently reacts with the diol group of a glycated protein to form a cyclic ester. A pH above the pK_a value of APBA is typically considered optimal for this reaction; however, it has been suggested that determining the optimum pH for binding is not this simple [45]. Therefore, in this study, the effect of pH on binding was investigated with a 10-mM 4-ethylmorpholine buffer solution containing 0.25 M KCl and 0.1 M NaCl to maintain the pH at 8, 8.5, 9 or 9.5. As shown in Fig. 3, the normalised ratio of stimulated resistance plotted against the HbA1c concentration was greatly impacted by pH. The results revealed that the sensitivity of the HbA1c assay increased with increasing pH. Although the binding of HbA1c to the boronate complexes at pH 9.5 provided the highest sensitivity, this pH was disregarded due to the narrow linearity also obtained at this pH. Furthermore, under extremely alkaline conditions, the tertiary and quaternary structures of glycated proteins would be subject to denaturation. Thus, a pH of 8.5 was instead selected for all subsequent experiments because this pH provided a wider linear range that extended to 14 % HbA1c. At pH values close to the pK_a of APBA, the boronate group is expected to exist in an anionic form, which is able to bind specifically with a diol to form the boronate ester. Similarly, Přibyl et al. also suggested that a pH above 8 can generally be accepted as an optimum condition [22].

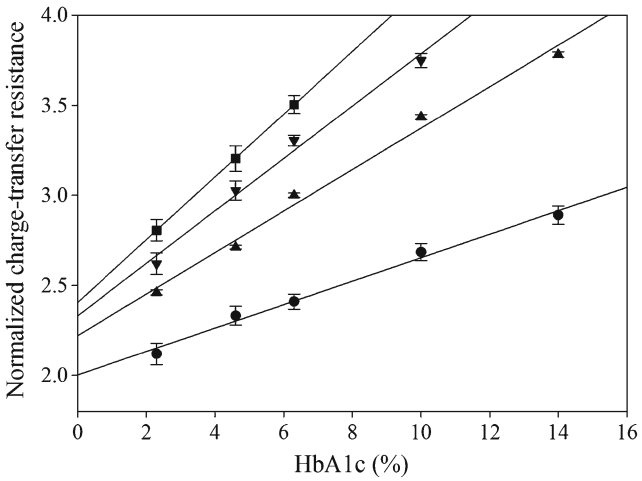

Fig. 3 The effect of pH on HbA1c binding; *circles* pH 8, *triangles* pH 8.5, *inverted triangles* pH 9 and *squares* pH 9.5

Effects of glutaraldehyde and APBA concentrations

In our system, the glutaraldehyde solution was used as a cross-linking agent to achieve covalent bonding between the amino groups of the ESMs and amine functional groups of APBA. Two levels of an HbA1c standard solution were employed to investigate the effect of glutaraldehyde concentration on the impedance signal response of the APBA-modified ESMs at a concentration of 0.25 mg mL^{-1} APBA. After 5 min of glutaraldehyde immobilisation, when normal and high levels of HbA1c were assayed, the impedance response of the membrane-based biosensor increased with increasing concentrations of glutaraldehyde, as depicted in Fig. S1A in the Electronic supplementary material. Hence, a 25 % (*w/w*) glutaraldehyde solution was the optimum condition and was selected for all subsequent experiments. In comparison, when using ESM as an enzyme immobilisation platform, higher glutaraldehyde concentrations lead to a decrease in the sensitivity of the biosensor due to the denaturation of the enzyme. In general, a 2.5 % (*w/w*) glutaraldehyde solution has been chosen as the optimal cross-linking agent for enzyme immobilisation; however, the immobilisation normally occurs over a prolonged period ranging from 30 min to 8 h, permitting long-term contact of the enzymes with glutaraldehyde [46–48]. In contrast, the method of choice in this study involved an immobilisation strategy that utilised a higher concentration of glutaraldehyde and a short contact time. This approach is not without precedent because several instances of using high concentrations of glutaraldehyde have been reported in the literature [49–51].

The concentration of APBA was also a relevant factor that directly affected the binding of HbA1c to the ESM-based biosensor. The signal response increased with an increasing concentration of APBA, as depicted in Fig. S1B in the Electronic supplementary material). However, when the concentration of APBA was higher than 0.25 mg mL^{-1}, the response reached a maximum value, indicating that the amount of APBA had achieved equilibrium. Thus, 0.25 mg mL^{-1} of APBA was used for all subsequent experiments.

Effect of incubation time

Alteration of the times for the APBA and HbA1c immobilisations on the ESMs could affect the amount of immobilised boronic acid functional groups and HbA1c molecules, respectively, on the surface of the ESMs, which are in direct proportion to the sensitivity of the membrane-based biosensors. Thus, the effect on HbA1c detection of APBA immobilisation times from 10 to 40 min was also investigated (Fig. S2A in the Electronic supplementary material). When assaying two levels of HbA1c with 0.25 mg mL^{-1} of APBA, the impedance signal increased gradually as the immobilisation time increased from 10 to 20 min and the signal reached a steady state after 20 min of incubation. Therefore, the optimum immobilisation time for APBA was determined to be 20 min. Figure S2B in the Electronic supplementary material illustrates the effect of HbA1c immobilisation time on the impedance signal obtained from the APBA-modified ESM. In this case, when assaying two levels of HbA1c, the signal response increased with an increasing incubation time and approached the maximum value after 15 min. Thus, considering a compromise between the signal response and analysis time, a 15-min incubation time was used throughout our studies.

Analytical characteristics

EIS characterisation of the sensing interface

One of the distinctive features of ESM is that its structure is composed of an intricate lattice meshwork of large and small fibres interlocking with each other, and the ESM surface is expected to contain the reactive functional groups, i.e., amines and amides, that are expected to react with APBA via a glutaraldehyde coupling agent. In our study, the inner surface of the ESM was subjected to step-wise boronate modifications with a platinum screen-printed electrode underneath. Label-free electrochemical impedance measurements were subsequently performed following each step of the surface modification. Figure 4 has Nyquist plots for the kinetic redox process, using $(Fe(CN)_6)^{3-/4-}$ as an electroactive probe, on a bare platinum screen-printed electrode and on the ESM, glutaraldehyde-activated ESM and APBA-modified ESM. Compared with the impedance data obtained from a bare electrode, a twofold increase in the charge-transfer resistance (Rct) was observed when the membrane was placed on the

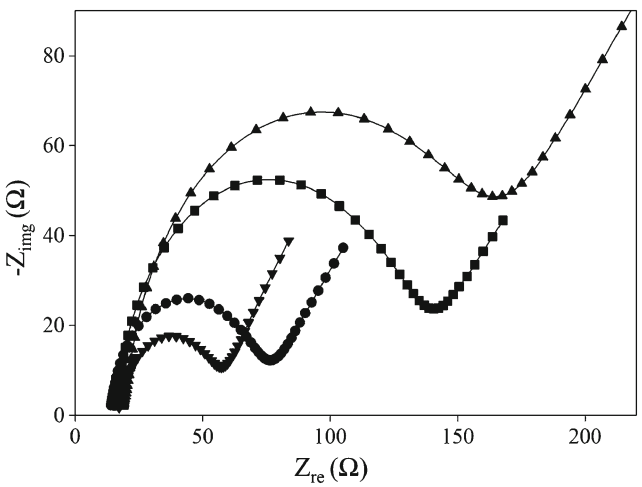

Fig. 4 Nyquist plots for the stepwise analysis of the *circles* bare electrode, *squares* ESM, *triangles* glutaraldehyde-treated ESM and *inverted triangles* APBA-modified ESM surface in the presence of a 5 mM $(Fe(CN)_6)^{3-/4-}$ redox probe in 10 mM 4-ethylmorpholine buffer (pH 8.5)

electrode. This evidence implies that the fibrous ESM immobilised on the surface of platinum electrode was blocking redox species movement. When the glutaraldehyde solution was applied, the curve broadened significantly, indicating a dramatic increase in resistance. Surprisingly, when the glutaraldehyde-activated ESM was further modified with APBA, the Rct significantly decreased, indicating neutralisation of the negative charges of the redox probes. The observed decrease in the Rct may result from the almost neutral net charge of APBA in a buffer with a pH close to the pK_a of APBA. In addition, APBA may directly bind to the surface of the ESM because the network of protein fibres in this type of membrane is composed of a collagen-rich core and a glycoprotein-rich mantle [52]. To investigate whether the impedance changes were due to the resistance of the membrane, a control experiment was also performed using the screen-printed electrode without ESM prepared in the same manner as the electrode in the proposed system. Nyquist plots for the stepwise modification of screen-printed electrodes are shown in Fig. S3 (Electronic supplementary material). The impedance response obtained from the glutaraldehyde-treated electrode was significantly decreased compared with that obtained from the bare electrode. Additionally, the glutaraldehyde-treated electrode was not responsive to the 0.25 mg mL^{-1} APBA. The impedance signals of the APBA-modified electrode were not in direct proportion to the various concentrations of HbA1c, implying that the changes in impedance of the proposed membrane-based system were due to the resistance of the membrane.

Calibration curve for the detection of HbA1c

The differential change in impedance with increasing HbA1c concentrations is clearly indicated in the Nyquist plot, as depicted in Fig. 5a. After exposure to the HbA1c, the Rct was significantly increased due to the ability of HbA1c to interact with APBA on the modified ESM and thus hinder the movement of redox species to the platinum electrode surface below the ESM. A control experiment was also carried out using an ESM without immobilised APBA, which was prepared in a manner similar to the proposed system. The impedance signal response towards HbA1c over the concentration range of 2.3 to 14 % remained unchanged compared with the baseline signal of the ESM (Fig. S4 in the Electronic supplementary material). This result confirmed that the impedance signal arose from the specific binding between the APBA-modified ESM and HbA1c via cis–diol interactions. Additionally, as shown in Fig. 5b, a significant phase shift was observed after attachment of the HbA1c and a less obvious change occurred with increasing concentrations of HbA1c. Dramatic changes in impedance were noticed at the lower frequency ranges (Fig. 5c), thereby demonstrating the sensitive response of the APBA-modified ESM towards HbA1c.

The impedance data are satisfactorily described by the behaviour of a system with the equivalent circuit shown in Fig. 5d (inset), which comprises a series of two constant-phase elements (CPEs) in parallel with two resistances (Rp) and the Warburg impedance element (W), along with the solution resistance (Rs). The good fitting results are shown in Fig. S5 (Electronic supplementary material). In this model, Rs corresponds primarily to the resistance of the electrolyte solution, whereas Rp1 and Rp2 are the Rct values that correspond to the membrane resistance and charge-transfer kinetics at the platinum screen-printed electrode, respectively. A CPE is used to describe the non-Faradic process at the membrane solution and electrode-solution layers. The CPE is defined as $Z_{CPE}=(A\omega)^{-\alpha}$, where A is a proportionality factor, ω is the angular factor and α is an exponential term with a value between 0 and 1. When the value of α is equal to 1, the CPE acts as a resistor [28]. Based on the results of fitting the electrochemical impedance data to this equivalent circuit, an increase in the membrane resistance Rp1 response is observed in the presence of increasing HbA1c concentrations. These results implied that the immobilised HbA1c could fully occupy the porous network of the ESM; therefore, the electroactive probe was not accessible to the surface of the platinum electrode, resulting in an increase in the Rct. The rate constant for the Faradic reaction of $(Fe(CN)_6)^{3-/4-}$, an electroactive probe, has been described using the electrochemical basis of the Rct, as indicated in the following equation [53]: $Rct=RT/(n^2F^2Ak^0_{app}C)$ where R is the gas constant, T is the temperature, n is the number of electrons transferred, F is the Faraday constant, A is the electrode surface area, k^0_{app} is the rate constant of the redox process and C is the concentration of the redox species. In our proposed system, the A and the k^0_{app} can be altered by the stepwise modification process.

The normalised ratio of the resistances derived from the fitted resistance values was plotted versus the various concentrations of HbA1c, as illustrated in Fig. 5d. The results revealed that the normalised response was linear up to 14 % of HbA1c, with a regression equation of $y=0.1151x+2.2216$ ($r^2=0.990$). The detection limit, defined by a signal-to-noise ratio of 3, was found to be 0.19 %, a slightly higher sensitivity than that described by Kim et al. [14]. These findings suggest that the proposed system provides sufficient sensitivity to measure the concentration of HbA1c within the required clinically relevant range of HbA1c, where 4–6 % is considered normal and covers the clinical reference range of diabetes (6.5–15 %) [3]. Compared with other electrochemical impedance measurements of HbA1c, the proposed membrane-based biosensor provides a wider linear range for assaying HbA1c [29–31]. ESM has a distinctive property. The higher the porous fibres, the higher the immobilisation surface areas, is available for boronate-binding sites. Hence, the high surface density of the available boronate groups increases the sensitivity and linearity of the proposed biosensor. With the

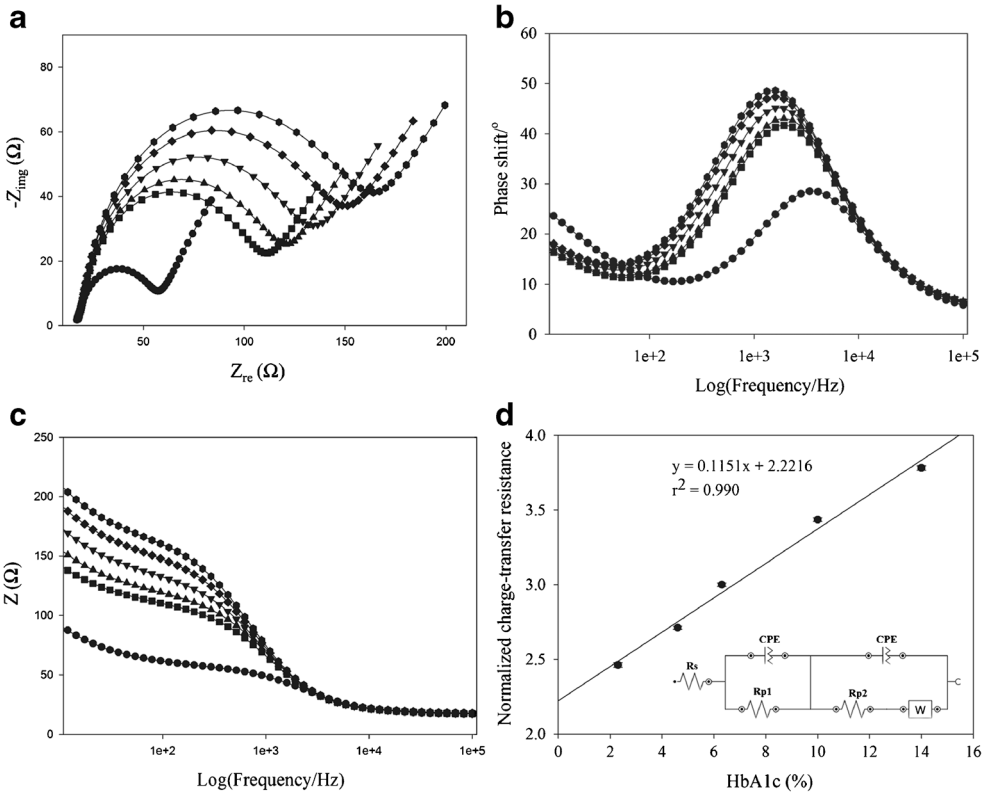

Fig. 5 Impedance data obtained from (**a**) Nyquist plot, (**b**) Bode-phase plot of the APBA-modified ESM before and after it was exposed to various concentrations of HbA1c: *circles* APBA-modified ESM, *squares* 2.3 %, *triangles* 4.6 %, *inverted triangles* 6.3 %, *diamonds* 10 % and *hexagons* 14 % HbA1c; (**c**) Bode-modulus plot and (**d**) variation of the normalised charge-transfer resistance (*Rct*) with respect to the concentration of HbA1c (%). *Inset right*, an equivalent circuit for analysing the impedance data; *Rs* solution resistance, *Rp1* membrane resistance, *Rp2* charge-transfer kinetics, *CPE* constant-phase element, *W* Warburg impedance

proposed ESM biosensor, samples can be directly tested without requiring additional sample dilution steps and avoiding pipetting errors. In addition, this label-free affinity platform provides us with a better understanding of interfacial sensing mechanisms and a great tool for glycaemic monitoring in diabetic patients, especially those in developing countries. A favourable comparison of the analytical characteristics for HbA1c determination using boronate-based electrochemical methods is provided in greater detail in Table S1 (Electronic supplementary material). Importantly, according to the standard interpretation norms of HbA1c in clinical practice, the HbA1c concentration should be expressed as the percentage of total haemoglobin (i.e., mmol mol^{-1} or %). Thus, in our studies, the HbA1c haemolysate standards (% HbA1c), which are currently used for commercial instruments, were selected as being representative of real clinical samples. As demonstrated here, the proposed membrane-based system leads to a substantial improvement in the dynamic detection range of HbA1c (up to 14 %) and also exhibits excellent sensitivity. Such capabilities make this method useful for real sample analysis and for assessing the glycaemic levels for clinical diagnosis.

Reproducibility

The reliability of the proposed membrane-based biosensor was determined with a precision assay that included two levels of HbA1c, i.e., normal (4.6 %) and diabetic (10 %) HbA1c concentrations, performed on the same day and on three consecutive days. The results revealed that the within-run reproducibility (each concentration; $n=10$) was indicated by CVs of 1.68 and 1.83 % when assaying normal and abnormal levels of HbA1c, respectively. Furthermore, the run-to-run reproducibility studies at normal and abnormal levels resulted in CVs of 1.97 and 2.02 %, respectively, assessed on three consecutive days (each concentration; $n=30$). The regeneration experiment is also demonstrated in Fig. S6 (Electronic supplementary material). Due to the reversible binding between HbA1c and boronate-recognition groups, the proposed membrane-based biosensor could be used as a reusable sensing platform. After repeated usage of the proposed membrane-based biosensor, similar signal responses were observed up to 10 cycles without losing APBA activity. Recently, Sacks et al. recommended that an intra-laboratory CV should be less than 2 % (National Glycohemoglobin Standardisation Programme (NGSP) units) because a difference of 0.5 % HbA1c between successive patient samples represented a significant change in glycaemic control [54]. Therefore, these findings clearly demonstrate that our proposed system provides an accurate assessment of HbA1c with great precision and also meets the performance goal for HbA1c measurement. Additionally, the storage stability of the screen-printed electrode covered with the boronate-modified ESM was also investigated by soaking the system in ultrapure water. Unfortunately, our results showed that the activity of APBA sensing interface was

greatly diminished after storing the electrode for a few days. The decrease in signal response could be due to detachment of the boronate group from the ESM. Thus, future studies on the storage stability of the boronate-modified ESM will be needed to be investigated.

Selectivity study

For clinical purposes, a whole blood specimen is the most complex matrix because there are many interfering molecules present, including endogenous (unconjugated bilirubin, glucose, glycated proteins or high hypertriglyceridaemia) and exogenous substances (commonly prescribed drugs and supplements). Considering the underlying principles of the boronate affinity measurement method, this analytical concept is based on a unique structural characteristic of HbA1c. The boronate-recognition group is able to covalently bind to the diol group of any glycated protein or sugar. Accordingly, the method described here may not be specific only to HbA1c but may also bind to glucose, glycated albumin, and other glycated proteins interfering in whole blood samples. However, in our study, these endogenous interfering substances present in plasma were negligible because these substances were completely removed from the red blood cells by centrifugation and washing with physiological saline. As stated earlier, the red blood cell lysates were prepared before being subjected to the impedance analysis. Therefore, to evaluate the selectivity of the proposed membrane-based biosensor for the determination of HbA1c in authentic blood samples, the use of HbAo was one possible way to investigate whether the non-glycated protein could interfere with the specificity of the proposed assay. The experiment was carried out utilising the boronate-modified ESM prepared in a manner similar to that for HbA1c determination, as described in the 'Materials and methods', but HbAo was used instead. Compared with the signal response of boronate-modified membranes, the impedance data in the response towards HbAo remained unchanged over the concentration range of 10 to 20 g dL^{-1} (data not shown). These results imply that our proposed membrane-based system is able to very precisely determine the HbA1c content in authentic blood samples, indicating the clinical applicability of the present method to monitor glycaemic levels in diabetic individuals.

Most importantly, for the correct interpretation of HbA1c measurements in clinical practice, the analytical interference of genetic variants, i.e., HbS, HbC, HbD, HbF and HbE and chemical derivatives of haemoglobin, i.e., carbamyl-Hb and pre-HbA1c, is of particular note when guaranteeing the reliability of the results. However, to our knowledge, compared with the other available methods for HbA1c determination, the boronate affinity binding method is generally considered to be less affected by the presence of haemoglobin variants and modified derivatives [55, 56]. Recent data obtained from a comparison between the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference method and boronate affinity method showed that boronate affinity method was not affected by the presence of most common haemoglobin variants [56]. However, the characteristics of the patient population should be carefully considered during the selection of HbA1c assay method, including the prevalence of haemoglobin variants. Additional physiological factors, such as severe iron-deficiency anaemia, haemolytic anaemia or any conditions that directly affect the erythrocyte lifespan, should be considered as potential restrictions for interpreting HbA1c assay results.

Assay comparison

Fifteen red blood cell lysate samples obtained from non-diabetic and diabetic volunteers were analysed for HbA1c levels using the proposed membrane-based system in parallel with the current commercially available method. All of the

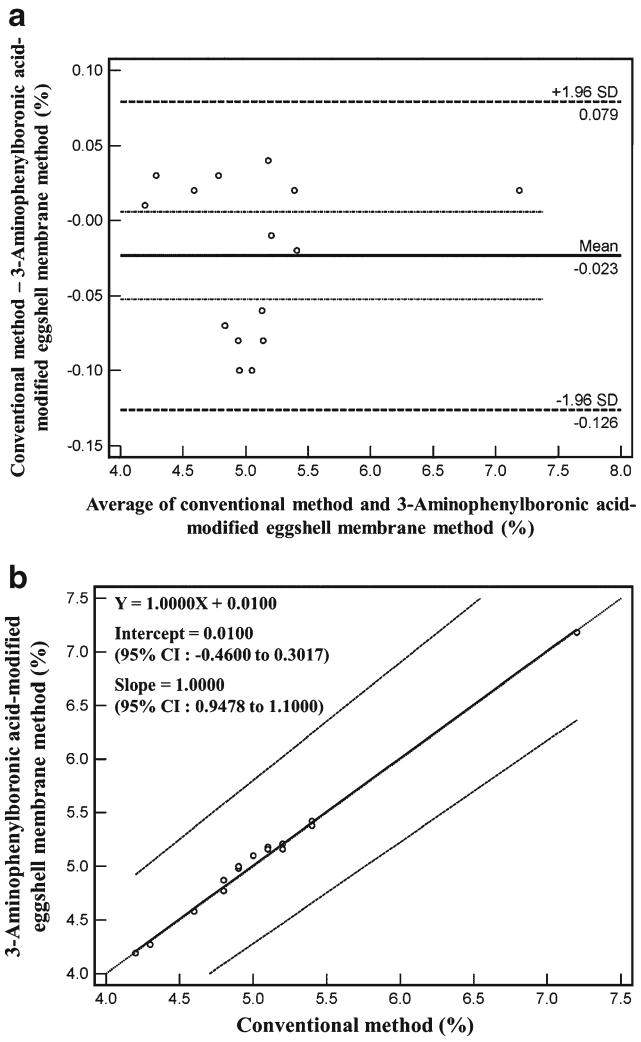

Fig. 6 Comparison of the proposed ESM-based analytical system and the current commercially available method for HbA1c measurement using (**a**) a Bland–Altman bias plot and (**b**) Passing–Bablok regression analysis

samples were analysed for haemoglobin and haematocrit values, which were determined to be within the ranges of 11–18 g dL^{-1} and 30–47 %, respectively. The agreement and correlation between the two approaches were assessed using the Bland–Altman bias plot and a Passing–Bablok regression analysis, respectively. The results revealed a reliable relationship between the proposed membrane-based system and the commercially available method within an agreement interval of ±1.96 SD. As demonstrated in Fig. 6a, these results suggest that the two methods could be used interchangeably. The results derived from our system were highly correlated with those obtained using the commercially available kit, with a Passing–Bablok regression equation of $y=1.0000x+0.0100$, as shown in Fig. 6b. Based on a 95 % confidence interval, the values of the y-intercept (0.0100) and the slope (1.0000) were trustworthy and covered a range of −0.4600 to 0.3017 and 0.9478 to 1.1000, respectively. In other words, these data demonstrate a good agreement between these two methods, and the proposed ESM-based method provides an alternative approach to monitoring HbA1c levels in authentic blood samples. In this case, the glycaemic status of each individual can be determined.

Conclusions

The proposed ESM-based biosensor was remarkably selective in determining HbA1c levels because this method was successfully applied to the analysis of authentic samples via a label-free electrochemical impedance measurement, in which the results demonstrated good agreement with the commercially available affinity method. The results showed in a stepwise process that the boronate-modified ESM was highly responsive to a wide range of HbA1c levels, indicating that this method is suitable for the clinical monitoring of glycaemic control. A novel APBA-modified ESM also provides a cost-effective biosensor for the diagnosis of diabetes, which is highly desirous in under-developed countries. Without the use of other detection labels, such as antibodies, dyes, or fluorescent materials, the proposed system has the merit of great simplicity. With the utilisation of an affinity membrane-based biosensor, this method has the potential for continuously monitoring glycaemic levels in diabetic patients and can be applicable to determining the presence of other glycated proteins, e.g., glycated albumin, found in plasma.

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