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Handheld impedance biosensor system using engineered proteinaceous receptors

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Abstract We put forward an impedometric protein-based biosensor platform suitable for point-of-care diagnostics. A hand-held scale impedance reader system is described for the detection of corresponding physiochemical changes as the immobilized proteins bind to the analyte molecules in the proximity of the microfabricated electrodes. Specifically, we study the viability of this approach for glucose biosensing purposes using genetically engineered glucokinase as receptor proteins. The proposed reagent-less biosensor offers a high sensitivity of 0.5 mM glucose concentration level in the physiologically relevant range of 0.5 mM to 7.5 mM with less than 10 s response time.

Keywords Protein-based biosensor · Impedometric readout system · Microfabricated gold electrodes · Glucokinase · Glucose

1 Introduction

Protein based biosensors offer low costs and good selectivity suitable for operation in a wide array of life science applications such as environmental monitoring, food quality control and blood analysis for the measurement of specific molecules including bio-available heavy metals and maltose or glucose (Bontidean et al. 1998; Zhou and Cass 1991; Yi Jae Dae et al. 2008). Among these applications, blood glucose monitoring is very important for millions of diabetes patients. The development of an accurate continuous glucose monitoring (CGM) system is the key challenge in a closed-loop artificial pancreatic system to control insulin level for type 1 diabetes suffering from loss of the insulin-producing beta cells in the pancreas (Kristensen et al. 2004). To date several companies have successfully commercialized highly accurate glucose monitoring systems (e.g. FreeStyle Lite®, Abotte Inc.) mostly using disposable electrodes incorporated with glucose oxidase enzyme which catalyzes the oxidation of glucose (Salimi et al. 2007). As the consequence of this catalytic enzymatic reaction, the concentration of glucose is obtained by measuring the released charges. However, a CGM should be performed through a reagent-less method. Human glucokinase enzyme can be an ideal candidate for this purpose. Glucokinase (ATP:D-glucose 6-phosphotransferase) plays a pre-eminent role in the regulation of hepatic glucose metabolism and is considered as the glucose sensor of the insulin secreting pancreatic islet β-cells (Aoki et al. 2005; D'Auria et al. 2002). This enzyme selectively binds D optical isomer of glucose but with a low affinity and a characteristic sigmoidal saturation curve which contributes to its role as a glucose sensor. Significant conformational change of glucokinase from super open to fully closed states upon glucose binding reported in the literature (Kamta et al. 2004). Other research groups have studied the catalytic activation of human glucokinase by substrate binding-residue contact involved in the binding of D-glucose to the super-open form and conformational transitions (Molnes et

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al. 2008). This large conformational change of glucokinase as a result of substrate binding leads to a change in overall dipole of glucokinase enzyme (Fig. 1).

Glucose biosensors can also be employed as models to develop a variety of biosensors for different applications by exploiting a wide range of selective affinities related to various proteins (Staiano et al. 2005). In this direction, the emphasis of this paper is placed on the design and implementation of an impedometric readout system incorporated with glucokinase to measure the glucose concentrations. Figure 2 illustrates the impedometric glucose biosensor consisting of glucokinase proteins immobilized on gold microelectrodes. The thin layer of proteins plays the role of a recognition element that binds to glucose molecules in the proximity of sensing gold electrodes. This binding event is monitored using Electrochemical Impedance Spectroscopy (EIS).

EIS is a well established technique routinely used in biological or chemical laboratories and is based on measuring the behavior in which a sinusoidal electrical current or voltage signal at different frequencies is conducted through the sample under test. The impedance measurement is used for a variety of applications for detecting DNA (Li et al. 2007), virus (Cho et al. 2007), and glucose and lactate (Rub et al. 2009). There are many table-top EIS systems available from Applied Biophysics Inc. and ACEA Biosciences Inc. that can be used for multi-sample analysis. Also, many companies have made available electronic microsystems (e.g. USB-6281, National Instruments and AD5933, Analog Devices) that can be employed for impedometric biosensing applications. AD5933 has recently been reported for many applications including health monitoring and single cell analysis (Seunghee et al. 2005; Kao 2006; Sherman et al. 2005). In this paper, we employ AD5933 device by developing the required software and hardware design for real-time hand-held impedance measurement system that is also scalable for multi-sample analysis.

The remainder of this paper is organized as follows. The next section provides a background on recent efforts made for glucose monitoring through electrical technique using hexokinases. The focus of Section 3 is placed on design and synthesis of sensing probe. In Section 4, we describe the proposed high-throughput EIS sensing system. The experimental procedures and testing results are provided in Section 5. This section is followed by a conclusion in Section 6.

2 Related works

The conformational change of proteins upon binding with corresponding molecules can be detected through a variety of solid-state transduction devices including piezoelectric, capacitors, cantilevers, and Ion Selective Field-Effect transistor (ISFET) (Park et al. 2009). As reported by Park et al. (2009), ISFET is a good candidate to measure the surface charge of protein layer exposed to target molecules. In that work, an ISFET device was implemented through 0.5 micron CMOS process (Kim et al. 2004) incorporated with maltose binding protein. The functionality of this technique was successfully demonstrated by showing a significant change in the drain current of ISFET exposed to the maltose in PBS buffer. However, the pH sensitivity of ISFET devices may deteriorate the performance of this sensing system in monitoring different concentrations of target molecules.

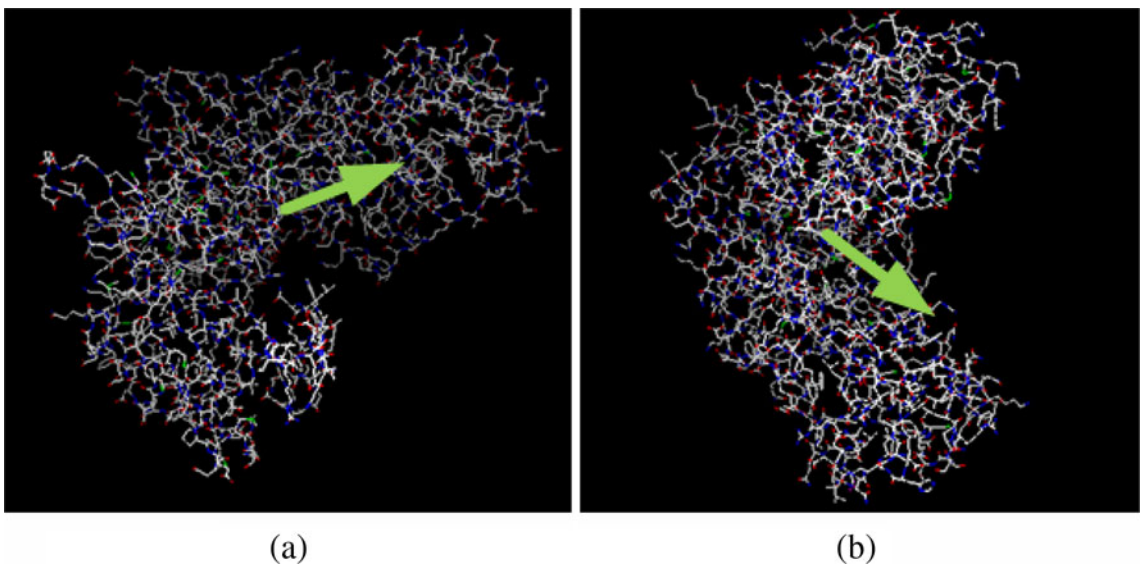

Fig. 1 Illustration of (**a**) open and the (**b**) closed form of human glucokinase enzyme

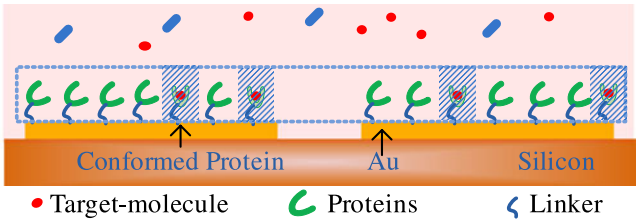

Fig. 2 Illustration of a hybrid biosensing system consisting of recognition element and microelectrodes

The physiochemical changes that occur in response to the binding event between glucokinase (GLK) and glucose can be measured through an impedometric technique as previously reported by Trifiro (2003) and Lumbroso et al. (2007). In that work, wild-type and mutant glucokinase (GK) was covalently coupled on a screen printed carbon electrodes which was previously used for several other applications including DNA detection and bacteriophage based bacteria sensor (Shabani et al. 2008; Marquette et al. 2006). Previous efforts for glucose detection using GLK through impedance spectroscopy were successful in high glucose concentrations in the range of 100 mM glucose. Herein, further modifications are applied to the sensing system and recognition element to make it suitable for glucose monitoring in physiological relevant ranges. To this end, gold inter-digitized electrodes implemented through traditional microfabrication procedures are employed instead of carbon electrodes. These microelectrodes are then chemically treated to couple the GLK on gold surface. The second step toward a portable protein-based biosensor is taken by employing a low cost impedance reader system. The focus of this paper is on the design and implementation of a portable biosensor system using commercial off-the-shelf available devices.

3 Protein-based sensing probe

In this section, we describe the protein-based sensing electrode consisting of microfabricated gold electrodes and the protein based recognition element.

3.1 Interdigitated gold electrodes

The extraordinary affinity of gold electrodes makes them very suitable substrate for several bio-electrochemical monitoring applications including glucose sensors (Hsu et al. 2009). Additionally, the microscale gold electrodes can be simply implemented through conventional microfabrication procedures. Herein, a sputtering process is employed to create inter-digitized electrodes on a silicon substrate. At the first step, a thin adhesion layer of titanium with the thickness of 20 nm is deposited on silicon followed by a 200 nm thick gold layer.

3.2 Immobilization of glucokinase on gold electrode

Alkanethiol Self-Assembled Monolayer (SAM) on gold is highly reproducible and well-characterized to generate well-defined organic surfaces with highly alterable chemical functionalities displayed at the exposed surface (Ulman 1996; Tao and Bernasek 2007). SAMs have been widely used for studying biological and chemical processes, molecular interactions, electrochemistry and electronic applications (Fendler 2001). In this work we used octane dithiol assembled monolayer on gold coupled with nitrilo-triacetic acid Ni^{2+} linker (Fig. 3(a)) which showed strong affinity for histidine-tagged recombinant human glucokinase enzyme (Fig. 3(b)).

Self assembled monolayer of 1, 8-octanedithiol was synthesized by using the technique (Fig. 4) reported in (Chidsey et al. 1990). Prior to the synthesis, the surface of the gold coated electrode was washed with piranha solution (mixture of 66% concentrated sulphuric acid and 33% hydrogen peroxide) for 5 min. Then the surface was rinsed thoroughly with deionized water, absolute ethanol and dried under nitrogen gas. After every coupling, thorough washing was carried out with deionized water and absolute ethanol to ensure complete removal of physisorbed material. Nickel was added to the maleimide-C3-NTA 20 min prior to coupling with His-tagged glucokinase enzyme. In contrast, the required experimental procedures for surface preparation are described in the following steps (1–10):

Step 1: The electrodes were cleaned using a mixture of H_2SO_4 and 33% H_2O_2 in a 3:1 ration and then the electrodes were rinsed thoroughly with de-ionized water followed by absolute ethanol. The electrodes thereafter dried under nitrogen gas.

Step 2: Self assembled monolayers of 1,8-octanedithiol are created on Au electrodes by immersing the gold substrates in a 1 mM ethanol solution at ambient condition overnight.

Step 3: SAM coated electrodes were rinsed thoroughly with ethanol in order to remove any physio-absorbed material.

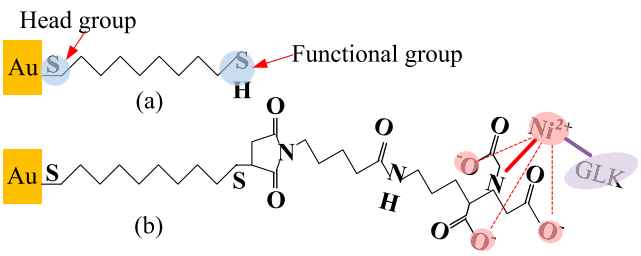

Fig. 3 Linker: (**a**) SAM of octanedithiol on Au (**b**) SAM of octanedithiol attached with melemide-NTA_Ni^{2+} linker which link His-tag glucokinase

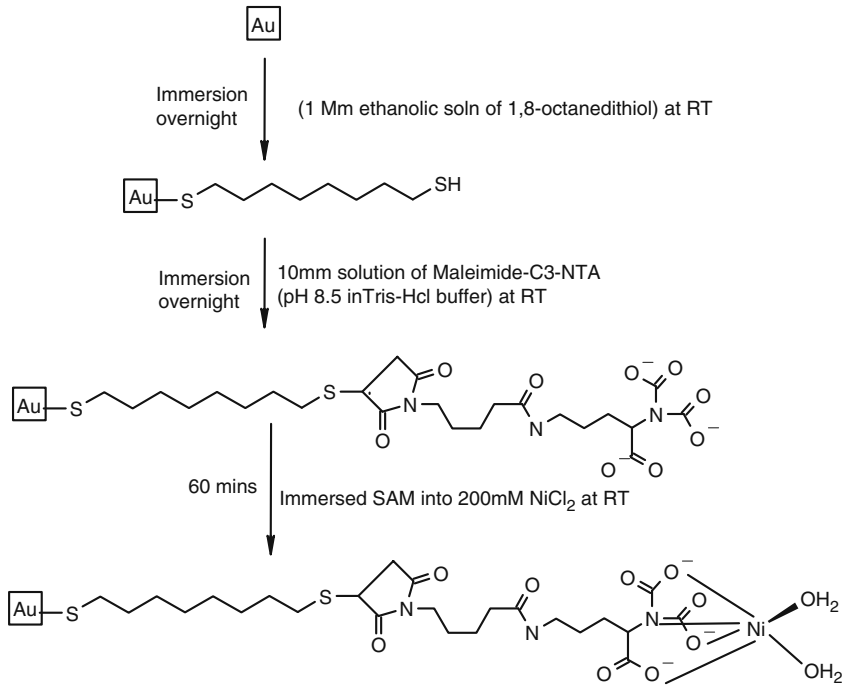

Fig. 4 Surface reaction scheme showing attachment of NTA functional groups on to the free sulfhydryl groups of 1,8 octane-dithiol SAM on gold electrode

Step 4: SAM coated electrodes were immersed into a 10 mM solution maleimide-C3-NTA (pH 8.5 in Tris–HCl buffer) overnight, in order to perform the coupling reaction of maleimide-C3-NTA and a sulfhydryl surface.

Step 5: Electrodes with NTA ligand were rinsed with deionized water and methanol in order to remove any physio-absorbed material.

Step 6: Electrodes with NTA ligand were immersed in 200 mM $NiCl_2$ in order to load nickel cations (Ni^{++}).

Step 7: Electrodes with Ni^{++} on top of surface were rinsed with deionized water to remove the residual metal and ion solution.

Step 8: His-tagged GLK proteins are coordinated to Ni^{++} when the surface treated electrode in step 7 is immersed in 100 mM of solution.

Step 9: The created sensing electrode are cleaned with deionized water and buffer.

Step 10: The implemented protein-based sensor should be freshly preserved in the buffer.

3.3 Protein based recognition element

As already mentioned, binding of glucose with GLK induces a conformational change which can be detected through electrical or optical techniques (Ghafar-Zadeh et al. 2009). As shown in Fig. 2, the linked proteins onto gold electrodes are exposed to a solution including glucose molecules. In fact, the glucose-induced conformational change of the GLK protein induces a difference in its dielectric property which in turn can be detected through an impedance sensing system.

4 Miniaturized impedance spectroscopy

The frequency domain measurement techniques are typically employed to detect the impedance values of working electrodes in two different interactions—before and after introducing sample onto the sensing electrode. The frequency-domain measurements of impedances (e.g. Bode and Nyquist plots) provide much information about the electrical modeling of the sensing electrodes and the sample. After a sufficient time of exposing electrode to the sample, the associated information with sensing electrodes is extracted to reveal the presence of specific molecules or cells in the test sample. However, this information does not give any estimation of response time and other transition issues which are important for continuous monitoring applications. In this section, we describe our proposed handheld measurement system which can efficiently be employed for both frequency and time domain analysis.

4.1 System description

The proposed measurement system consists of three different components: impedance coder (IMC), impedance reader chip (IRC) and impedance decoder (IMD). As shown in Fig. 5, IRC is employed to convert the impedance variation of a single electrode to digital values. IMC is a microelectronic system dedicated to read the impedance values of a large array of sensing electrodes and then generate an impedance

signal from the sensing electrodes. Special codes are added to the impedance signal in order to indentify the location of sensing electrode in the array. This impedance signal is thereafter converted to digital values which are transferred into computer through IRC for decoding and signal processing purposes. The recorded data is broken into several parts through IMD algorithm where each part relates the impedance values to a specific electrode.

In this work, among several commercially available IRCs, we use a low cost device—AD5933 provided from Analog Devices Inc. As shown in Fig. 5, a sinusoidal voltage signal with certain amplitude and frequency is generated through AD5933 and then injected in the unknown impedance (Z_{AB}) in between nodes A and B. It should be mentioned that the AD5933 is a high accurate impedance converter system featuring a 12-bit, 1 MSPS, analog-to-digital converter (ADC). The frequency generator allows an external complex impedance to be excited with a known frequency. This chip can be used for many applications including electrochemical analysis, bioelectrical impedance analysis, impedance spectroscopy, complex impedance measurement, corrosion monitoring and protection equipment, biomedical and automotive sensors, proximity sensing, nondestructive testing, material property analysis. The impedance of sensing gold electrodes are sampled by AD5933, a Discrete Fourier Transform (DFT) is processed and the real and imaginary data can be extracted at each output frequency.

The computer program allows the user to select the desired amplitude and the range of frequencies. The entry data are commanded into digital system in order to generate the analog signal using Digital to Analog Converter (DAC). This analog signal is then amplified through an op-amp based inverter. The amplitude and phase of amplified signal is proportional to Z_{AB}. The off-chip potentiometer (R_P) or other electrical elements can be used to calibrate the system accordingly. In the case of this paper, a pure resistor is used to avoid any change in the phase and amplitude of impedance signal. As seen in Fig. 5, an analog-to-digital converter (ADC) is used to convert the analog signal into digital signal. The real and imaginary components of each impedance sample are obtained through the signal processing unit. Furthermore, a special protocol-I^2C is generated along with the serial data and transferred to computer through USB connector. All the circuit elements described here as parts of IRC are integrated on a printed circuit board which is commercially available as EVAL-5933EB from Analog Devices Inc (2009).

4.2 Impedance coding system and decoding algorithm

Let us assume that there are N electrodes with different impedance values (Z_1, Z_2 ... Z_N) which are individually selected by activating the corresponding switches (S_1, S_2 S_N). The outputs of a binary counter (Q_1, Q_3...Q_{2N-1}) can simply scan the switches and electrodes. Therefore, a time-variant impedance $Z_{AB}(t)$ between node A and B $\{Z_{AB}(t_1)= Z_1$, $Z_{AB}(t_2)=Z_{2,....}$ $Z_{AB}(t_N)=Z_N\}$ is obtained where t_i is the i^{th} $(0\leq i\leq N)$ sampling time. In order to recognize the corresponding electrode to these impedance values, it is required to embed a certain impedance value in between each two electrodes. This impedance can simply be a resistor with a value equal to R_{Min} while always $Z_i>R_{Min}$ for any i between 0 and N. Consequently, the transition from one electrode to another electrode can simply be identified by the computer. On the other hand, in order to recognize the first electrode of array, another resistor (R_{Max}) with a constant value should be added while its value is always higher than any Z_i. If the recorded impedance associated to E_i during a sampling period (Δt) is repre-

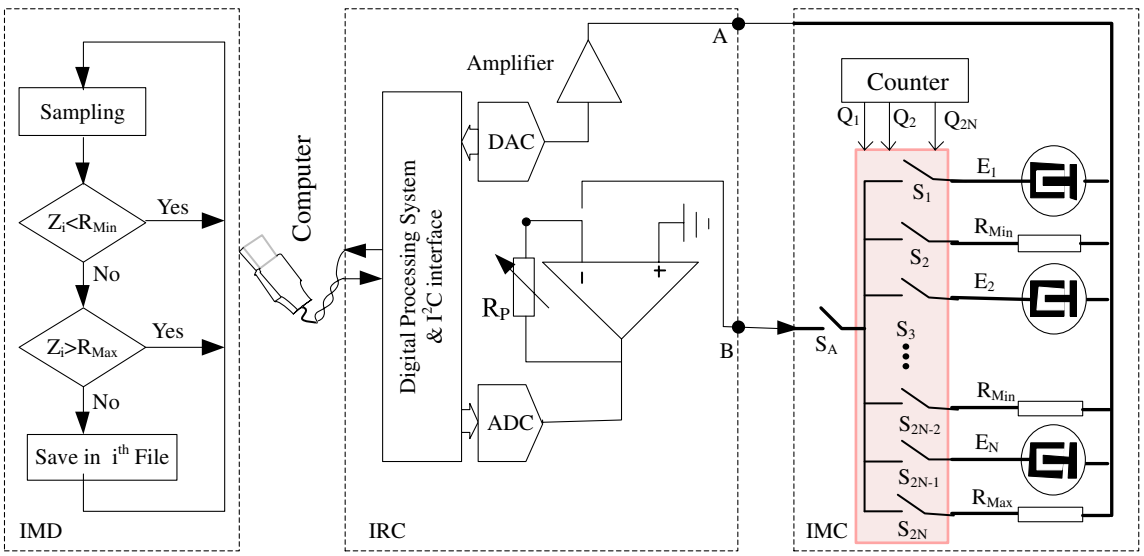

Fig. 5 Illustration of proposed system including IRC, IMC and IMD

sented by Z_i, Z_{AB} can be represented in relation (1) at a certain frequency f=f_0 as shown in Fig. 6 for N=4.

$$Z_{AB}(0 < t < T, f_0) = \begin{cases} R_{Max} & 0 < t \le \Delta t \\ Z_1 & \Delta t < t \le 2\Delta t \\ R_{Min} & 2\Delta t < t \le 3\Delta t \\ Z_2 & 3\Delta t < t \le 4\Delta t \\ R_{Min} & 4\Delta t < t \le 5\Delta t \\ & \vdots \\ Z_N & T - \Delta t < t \le T \end{cases} \quad (1)$$

Based on above discussion, in order to take k samples from each electrode, the minimum number of clock pulses applied to the counter chip is equal to (2N+1)×k. Therefore, the minimum required time (T) to scan the electrode array is (2N+1)×k×ς where ς is the period of applied clock pulse. As already mentioned, Z_{AB} is obtained in a certain frequency, therefore, by changing the frequency from one recorded string to another one, the corresponding impedance of each electrode over a range of frequencies are obtained as follows

$$Z_{AB}(0t \le (M+1) \times T, f_0 \le f \le f_M) = \begin{cases} Z_{AB}(0t \le T, f_0) \\ Z_{AB}(Tt \le 2T, f_1) \\ \vdots \\ Z_{AB}(M \times Tt \le (M+1) \times T, f_M) \end{cases} \quad (2)$$

where f_i is the applied frequency for i=1, 2,...M. The IMD module is designed to (1) enter the require data (e.g. Δt, k, the selected electrodes, range of frequency), (2) record the impedances, (3) sort the recorded data, and (4) display the impedance over a range of times or frequencies. As shown in Fig. 5, the IMD algorithm searches for R_{Min} and R_{Max} values in order to separate the impedance values of different electrodes over a range of frequencies (f_1–f_M). The impedance of the selected electrodes can be displayed over time in a designed Windows (operating system based program) through the developed IMD. In this work, the IMC board implemented through digital discrete devices including CD4148 and CD4817, however, for a large array of electrodes, the same method is applied to realize an IMC using microcontroller and other devices to decrease the number of discrete devices.

5 Experimental procedures and results

5.1 Measurement set-up

An array of gold electrodes is microfabricated on a silicon wafer; the wafer is then diced to separate the electrodes. Each electrode is adhesively bonded on a pre-prepared printed circuit board (PCB) carrier. The electrodes are wire bonded on the pads realized on PCB. The conductors on PCB are encapsulated with epoxy using direct-write technique (Ghafar-Zadeh et al. 2007) in order to protect the conductors from direct contact with solution. Based on direct-write technique, a fugitive ink is deposited on the electrode prior to epoxy encapsulation. This ink assures that the epoxy does not cover the electrode and after epoxy curing it will be removed at moderate temperature and light vacuum as described in (Ghafar-Zadeh et al. 2007). One end of each PCB is immersed in the solution with a certain glucose concentration while the other end is connected to the proposed hand-held system. Figure 7 shows the proposed system (Fig. 7(a)), impedance reader (Fig. 7(b)), array of four sensing electrodes (Fig. 7(c)), illustration of bonded gold electrodes on PCB and encapsulated with epoxy (Fig. 7(d)), optical microscopic image of an electrode (Fig. 7(e)) and sensing electrode bonded on PCB (Fig. 7(f)).

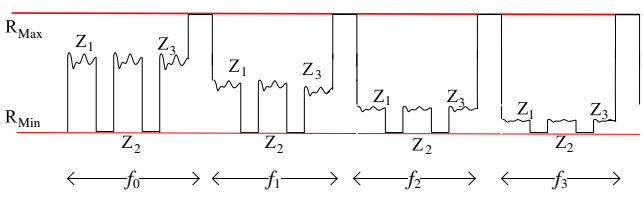

Fig. 6 Analog impedometric signal over time

5.2 Genetically engineered glucokinase

Glucokinase (GLK) was prepared as previously described (Trifiro 2003). Cloning of human GLK was accomplished by reverse transcription of mRNA followed by PCR (RT-PCR) and cloning into pcDNA3 (a mammalian cell expression vector), pGEX-KG and pET15b (for bacterial expression of a (GST)-GLK or His-tagged-Glk fusion proteins) vectors. All GLK clones have been tested in DNA transfection studies in COS cells and all have near normal enzyme activity. pcDNA3 Wild-type GLK were subsequently subcloned into pET-15b (His-GLK) bacterial expression vectors using appropriate restriction enzymes. These plasmids introduce a polyhistidine metal affinity tag to the N-terminus of GLK

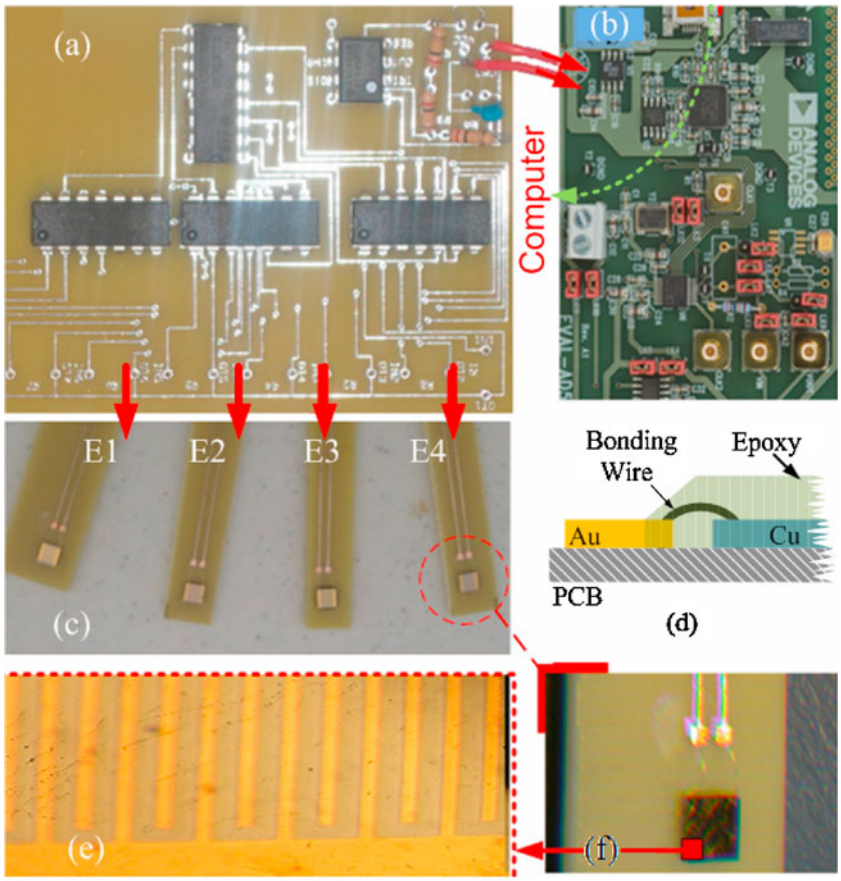

Fig. 7 Measurement Setup: (**a**) proposed IMC, (**b**) employed IRC, (**c**) microelectrode arrays, (**d**) illustration of wire bonded electrodes encapsulated with epoxy, (**e**) microscope image of microelectrode and (**f**) wire bonded electrode

and allow for one-step purification of GLK from bacterial lysates (Fig. 8) using Ni++ columns. Bacterial expression constructs express Wild-type GLK proteins in large quantities (100–200 μg). Ni2+-affinity purification produces large quantities of >95% pure His-GLK using a simple procedure

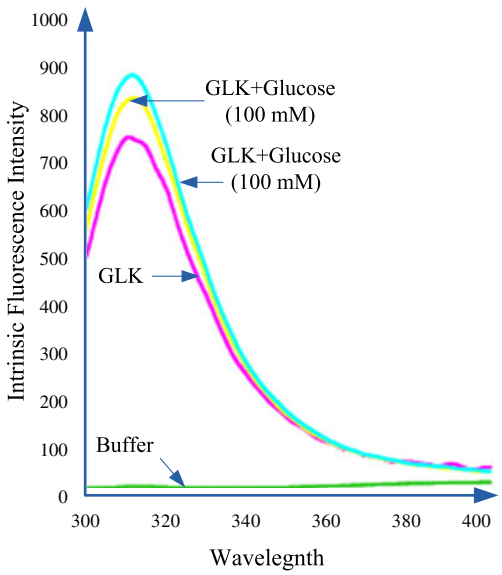

Fig. 8 Glucokinase fluorescence analysis

with a yield of 100 μg of protein/250 ml culture. Wild-type His-GLK is eluted from the Ni2+ affinity column with 250 mm imidazole and dialyzed overnight. We have performed intrinsic fluorescence experiments with GLK to document and verify glucose-induced conformational changes, which is an absolute requirement for impedance biosensing to be successful. Purified wt N-terminal His-Tagged GLK (5 μg) with enzyme specific activity of ≈200 U/mg were used in a 10×10 mm cuvette After the addition of 100 mM glucose, intrinsic fluorescence spectroscopy was performed (excitation wavelength 285 nm; maximum fluorescence 312 nm). There is a 20–25% increase in maximal intrinsic fluorescence at 312 nm with the addition of 100 mM glucose, reflective of GLK conformational change. This is similar to native wt GLK and allows us to conclude that N-terminal manipulation of GLK does not introduce any untoward affects of the protein.

5.3 Surface preparation

As described in Section 3, the surface preparation was performed by applying the biochemical processes described in ste1 to step 7. Figure 9 shows the viability of SAM on gold electrode using the cyclic voltmetery electrochemical measurement. Based on this figure, the SAM process is

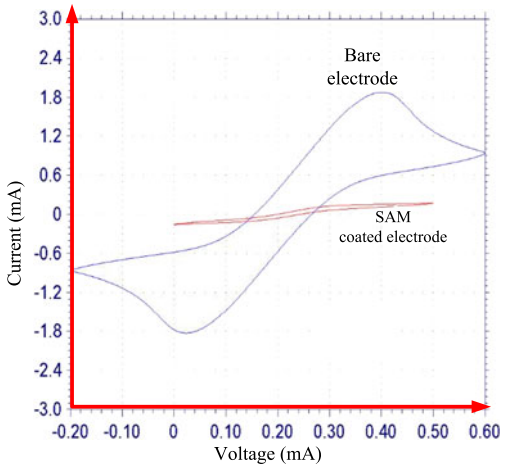

Fig. 9 Cyclic voltammetry curve to demonstrate the presence of SAM on gold

properly performed and the Sam layer created on the op of gold electrode. The measurement results described in the next section are in good agreement with the immobilization of GLK which was performed in the steps 4–7 described in Section 3.2.

5.4 Impedometric measurement results

The functionality of proposed system was tested using four electrodes (Fig. 7(c)). The corresponding impedances are separated through the algorithm described in Section 4. The impedance values of electrodes (Z_1, Z_2, Z_3 and Z_4) is always lower than UIR (the upper values of impedances) and higher than LIR (Lower values of impedance). The impedance variation of bio-sensing electrodes is measured for different concentration of glucose in PBS buffer. Each testing tube includes a buffer solution with a certain glucose concentration. The bio-sensing electrode is immersed in each tube to monitor the corresponding impedometric changes.

In this section, the measurements in frequency-domain, and then in time-domain are demonstrated as follows. The impedance variation of electrodes over a range of frequencies (kHz) is measured for different glucose concentrations (Fig. 10 (a)). It should be mentioned that IRC provided by Analog Device can only grantee the linearity of measurement system for the frequencies less than 100 kHz. This is why the proposed biosensing system is operated only in frequencies lower than maximum limit. The repeatability of system is also demonstrated in the Fig. 10(b). This figure shows the impedance variations of biosensing electrodes over time at f≈ 30 kHz for different concentrations (0.5 mM, 1 mM, 2.5 mM, 5 mM, 7.5 mM) where the measurement electrode is rapidly removed from a testing tube and immersed in another one. Based on these testing results, glucose can be sensed in the physiologically relevant range of 0.5 mM and 7.5 mM and also, a change in glucose concentration occurs in less than 10 s. Additionally, in order to reveal the repeatability of biosensor, another experiment was performed at the same frequency and the same electrode using different glucose concentrations as shown in Fig. 10(c). As seen in this figure by replacing the buffer instead of glucose solution, the impedance returns to its initial value. As already mentioned, in order to show the testing results for different glucose concentrations, the electrode were immersed in testing tubes and rapidly removed and inserted into another test solution. During this testing, the electrodes were not in the test solution for a short duration and therefore the impedance measurements shows ambiguous response in the highlighted regions in Fig. 10(c) and Transition Point (TP) in Fig. 10(b).

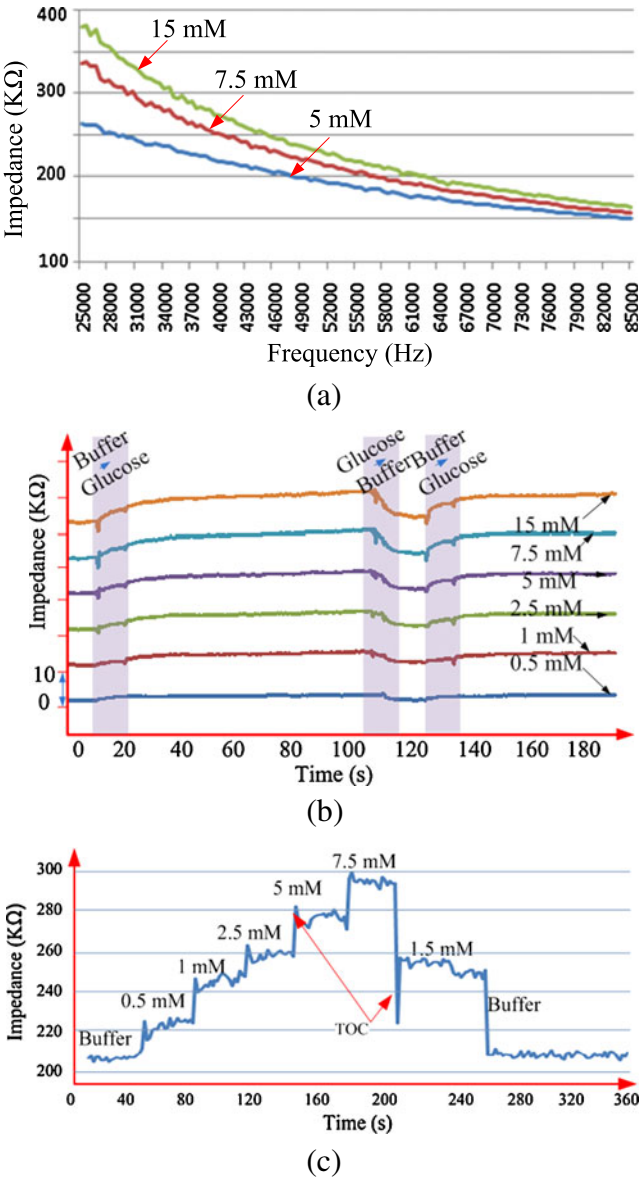

Fig. 10 Measurement results: (**a**) frequency-domain (**b**) time-domain and (**c**) continuous-time measurement results for various concentrations of glucose

6 Conclusion

In this paper, a low cost and portable protein based biosensor was presented for continuous glucose monitoring. A low-cost impedance based readout system was developed using a commercially available impedance reader. We described the applicability of this system for biological applications by monitoring different concentrations of glucose through a protein recognition element. The glucokinase proteins are immobilized on gold electrodes through a self assembly monolayer interface. The experimental results demonstrated in this paper are in a good agreement with the discussed issues throughout the paper. The proposed reagent-less and replenishable technique with microelectronic integrated sensor is well suited for working towards implantable glucose sensors.

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