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ORIGINAL PAPER

An electrochemical signal switch–based (on–off) aptasensor for sensitive detection of insulin on gold-deposited screen-printed electrodes

Samet Şahin[1,2] · Şevval Kaya[2] · Zafer Üstündağ[3] · Mustafa Oguzhan Caglayan[1]

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Abstract

Insulin hormone is of great importance for many diseases, especially for diabetes management. Therefore, different detection strategies have been used for sensitive and fast detection of insulin in physiological conditions. In this study, an electrochemical signal switch aptasensor for sensitive detection of insulin has been developed based on methylene blue (MB)–modified insulin-specific aptamer immobilized on gold-deposited screen-printed electrodes. The aptamer fabrication parameters of aptamer, blocker and insulin incubation times, were optimized using the response surface method as 180 min, 60 min, and 25 min, respectively. Optimized values were then used to fabricate aptasensor, and analytical parameters were calculated using square wave voltammetry. The calibration of the aptasensor was performed based on the current difference calculated by subtracting the respective current values obtained for the MB-probe's on and off positions. The linear working range and limit of detection of the aptasensor were calculated as 25–150 pM and 18.5 pM, respectively. The relative standard error and accuracy of the aptasensor were 9.5% and 6.4%, respectively. The interference study showed no significant interfering substances except for uric acid, and the stability of the sensor was good for 10 days keeping 92% of its initial performance. The developed aptasensor showed promising results for easy and sensitive insulin detection in physiological conditions.

Keywords Aptasensor · Insulin · Biosensor · Square wave voltammetry · Diabetes · Electrodeposition

Introduction

Diabetes is a severe chronic and metabolic disease that affects millions of people globally and poses an even greater danger for future generations [1]. According to the World Health Organization, the number of people diagnosed with diabetes has increased fourfold in the last 40 years resulting in 422 million diabetic people worldwide [2]. Therefore, diagnosis and monitoring of diabetes play a vital role in public health. Glucose is by far the most widely known biomarker for diabetes management and billions of dollars spent on research and innovation for glucose sensing [3]. However, significantly less money and effort have been spent on other clinically relevant biomarkers such as insulin, which can be significantly important. Insulin is mostly known for insulin resistance, which occurs when our body becomes resistant to insulin. In this condition, our blood sugar goes up accordingly, causing various conditions such as type 2 diabetes, hypertension, glucose intolerance, and dyslipidemia [4, 5].

Several methods are used for the detection of insulin in laboratories such as enzyme-linked immunosorbent assays, radioimmunoassays, high-performance liquid chromatography, electrochemiluminescence, and fluorescence [6–9]. However, such methods require complex assay procedures, expensive equipment, long test durations, and experienced technical staff to conduct the tests. Therefore, developing low-cost, fast, and sensitive detection strategies for insulin becomes very important for the biomedical industry. The detection of insulin poses a more complicated challenge than glucose as its physiological concentration is around 25 mIU/L (0.86 ng/mL or 150 pM) [10]. Furthermore, it is a peptide hormone whose structure and function make

✉ Samet Şahin
samet.sahin@bilecik.edu.tr

[1] Department of Bioengineering, Faculty of Engineering, Bilecik Şeyh Edebali University, Bilecik 11230, Turkey

[2] Department of Biotechnology, Bilecik Şeyh Edebali University, Bilecik 11230, Turkey

[3] Department of Chemistry, Kütahya Dumlupınar University, Kütahya 43100, Turkey

it difficult to be utilized in many conventional biosensor formats. A recent review on insulin detection's future reveals that electrochemical aptasensors are one of the most promising candidates for point-of-care detection of insulin [11]. Aptasensors have attracted many researchers for the detection of many various biomarkers for health and environmental monitoring [12–15]. However, the number of studies that utilize aptamers for the detection of insulin is limited and even less for the use of electrochemical techniques.

Electrochemical aptasensors have the advantages of high specificity, low cost, and high stability compared to widely used antibodies [16, 17]. Therefore, more biomarkers such as insulin are needed to be investigated for their use as electrochemical aptasensors. The investigation of insulin-binding aptamer has been reported by Yoshida et al., and promising aptasensors have been designed for the detection of insulin until then [18]. Several different electrochemical techniques such as voltammetry, electrochemical, impedance spectroscopy, and electrochemiluminescence have been utilized to develop insulin aptasensors [19–25]. Reports of the first attempts to develop insulin detection methods using the electrochemical approach date back to 1989 [26], and a brief review of electrochemical approaches for insulin detection is given in Table S1.

Among these sensors, only a couple of studies demonstrated the use of a signal switch–based detection strategy by either using affinity binding and release of methylene blue (MB) redox probe or incorporating two redox probes (ferrocene and MB) for dual signal switching [23, 25].

Optimization of aptasensor fabrication parameters such as aptamer, blocker, and target analyte incubation times is critical in achieving sensitive and reproducible results. However, such studies have not been demonstrated extensively, especially for insulin detection using Au surfaces. There is a wide range of different experimental conditions for different incubation times of aptasensor fabrication [27, 28]. Therefore, in this study, the aptamer modification processes on the Au surface are optimized using the response surface method (Design-Expert 12 (trial version)). It was aimed to achieve a maximum performance aptasensor with the minimum detection time possible. To achieve this goal, aptamer, blocker, and insulin incubation times were selected as independent variables for the model, and electrochemical sensor response was used as an output signal. An electrochemical signal switch aptasensor was developed with an MB-modified insulin-selective aptamer [18] using Au-deposited screen-printed electrodes. The proposed aptasensor has a simple two-step fabrication and simple detection mechanism using single-probe electrochemistry based on square wave voltammetry (SWV). This approach with optimized aptasensor parameters stands out as promising for highly sensitive and fast detection of insulin from physiological body fluids.

Experimental

Materials

The chemicals used in this study were obtained from Sigma-Aldrich at analytical grade and used as received unless otherwise specified. Ultra-pure water (UPW) at 18.2 MΩ cm was used for all aqueous solutions and washing steps. All electrochemical experiments were performed with Ivium Potentiostat (Ivium Technologies, Netherlands) using screen-printed electrodes SPEs (DRP-110, working electrode surface area of 0.126 cm^2) at 23 ± 3 °C. SPEs were obtained from Metrohm AG (Switzerland). The working electrode, counter electrode, and reference electrode were carbon, carbon, and silver paste, respectively. SPEs were pre-treated before use to remove impurities on the working electrode surface and obtain reproducible results using the linear sweep voltammetry technique (LSV) (between 0 and −2 V at 20 mV s^{-1} scan rate). The methylene blue (MB)–modified insulin aptamer probe (MB-aptamer) specific to insulin was purchased from Ella Biotech (Germany) as SH- $(CH_2)_6$ – GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC—AttoMB2.

Preparation of aptamer-modified SPEs

Pre-treated SPEs were coated with Au using the electrodeposition technique using a modified procedure from previously published studies in the literature [29]. Figure 1 shows the schematic illustration of aptasensor fabrication steps for insulin detection. Briefly, 200 µL of 1 mM chloroauric acid $[AuCl_4]^-$ solution in 0.1 M KCl was dropped on SPE, and 15 cycles of cyclic voltammetry (CV) at 50 mV s^{-1} between 0 and − 1.5 V were applied. A visible Au layer was formed after the electrodeposition process, and the resulting electrode was denoted as SPE/Au. SPE/Au electrodes were then washed with UPW and dried before any further modification. Ten microliters of 10 µM MB-aptamer solution in 0.1 M phosphate-buffered saline at pH 7.4 (PBS) was dropped on the working electrode and allowed to bind to the Au via disulfide bond formation. The aptamer-modified SPE/Au electrodes were then washed with PBS following by a blocking step with 1 mM 6-mercapto-1-hexanol (MCH) solution in PBS to block the unoccupied Au surface. MB-aptamer final electrodes were denoted as SPE/Au/Apta and used for insulin detection or kept at 4 °C before use.

Optimization and characterization of aptamer-modified SPEs

Design-Expert 12 (trial version) was used to optimize the three important fabrication parameters for the preparation

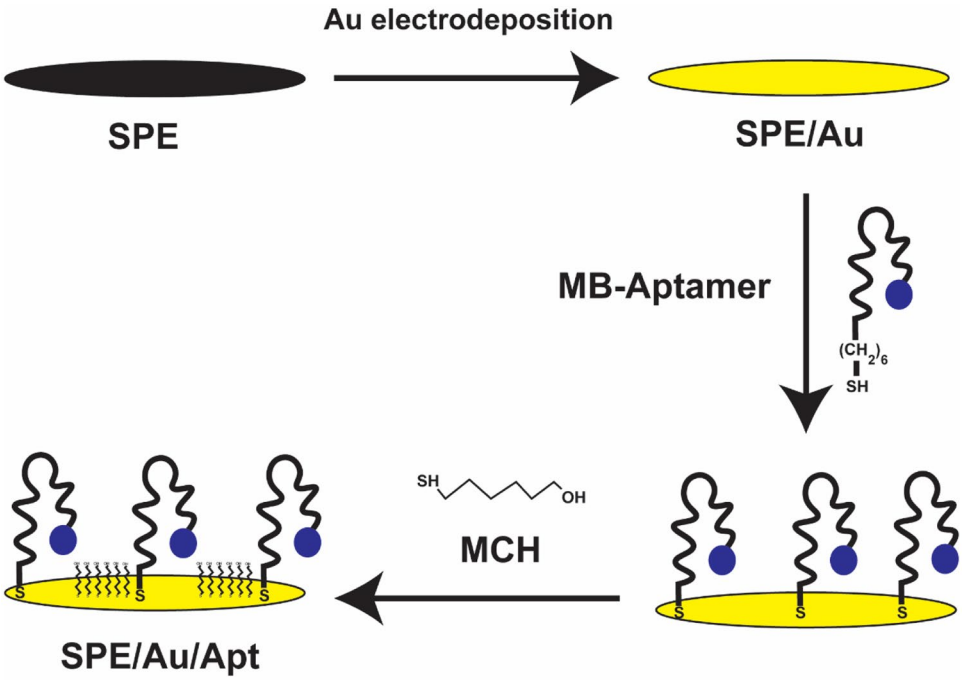

Fig. 1 The schematic illustration of aptasensor fabrication steps for insulin detection

of SPE/Au/Apta aptasensor configuration. The central composite design approach was used to design the experiments, and response surface method (RSM) was used to optimize the aptamer, MCH, and insulin incubation times using the current difference between the baseline peak current. The input parameter for the model was the peak current obtained from MB oxidation associated with the concentration of insulin. The model has been established using the software and was later used to optimize the aptamer, MCH, and insulin incubation times for the aptasensor. It was aimed to obtain the minimum insulin incubation time with a minimum standard deviation for a quick test and higher peak current change as a response for a more sensitive aptasensor design. The optimized parameters were used in the aptamer calibration and validation experiments.

Aptasensor calibration and validation

SPE/Au/Apta electrodes were first tested in PBS using SWV between −0.8 V and −0.3 V (10-mV pulse amplitude, 25-Hz frequency, and 1-mV potential step) without insulin incubation to obtain a baseline peak current value (aptasensor "on" signal). Then, they were incubated with insulin concentrations of 20 pM, 50 pM, 100 pM, and 150 pM (in PBS) for 25 min (optimized value) for aptamer insulin interaction. After the incubation of insulin, the electrodes were washed with PBS to remove unbound insulin molecules. Insulin-modified electrode was finally tested in PBS using square wave voltammetry (SWV) between −0.8 V and −0.3 V (10-mV pulse amplitude, 25-Hz frequency, and 1-mV potential step). The aptasensor response was recorded as the change in peak current values between no insulin and various insulin concentrations. The calibration curve was obtained using the peak current change vs insulin concentration. Calibration experiments were repeated 4 times using different electrodes and represented with sample standard deviation error bars. The limit of detection (LOD) was calculated using 3σ based on the highest σ value at a 95% confidence level.

Interference studies were performed using potential interfering substances that are either structurally mimic insulin or are present in the blood for a potential blood insulin test application. Streptavidin, glucose, thrombin, and uric acid were chosen at 50 pM, 1.5 mM, 0.5 µM, and 0.15 mM concentrations, respectively. After taking the baseline measurement, each interfering substance was mixed with 50 pM insulin and incubated on an aptasensor for 25 min. The electrodes were then washed with PBS and tested. The interference experiments were repeated 3 times ($N=3$) and represented with sample standard deviation error bars. Finally, the reproducibility experiments were performed for at least 5 samples. The electrode's stability was then tested by storing the electrode at 4 °C for 10 days and evaluating the sensor response in terms of its reference value.

Results and discussion

Preparation and characterization of modified electrodes

The electrodeposition of Au on SPEs has been performed using CV in 0.1 M KCl for 15 cycles between 0 and −1.5 V (vs Ag/Ag^+), as shown in Fig. 2A. The peaks observed in Fig. 2A follow the typical behavior of $[AuCl_4]^-$ electrolysis [29]. The cathodic peaks are observed around −0.5 V (vs Ag/Ag^+) and −0.8 V (vs Ag/Ag^+) corresponding the reduction of Au^{3+} to Au^+ and Au^+ to Au^0, respectively [30]. As the number of scans applied, corresponding cathodic peak current values are decreased, indicating Au's successful single-step electrodeposition on SPE (SPE/Au). Figure 2B shows CVs for different stages of SPE modifications, supporting the Au modification on SPEs showing more reversible peaks than the bare SPE. SPE/Au/Apta electrodes were also tested in PBS using SWV to prove the MB-aptamer's successful immobilization. Figure 2C shows that a definitive peak was observed around 0.5 V (vs. Ag/Ag^+) corresponding to the oxidation of MB. Figure 2D also shows the picture of SPE and its components before (i) and after (ii) Au modification process with a yellow layer visible to the naked eye.

SEM and EDX analyses were performed to investigate the formation of Au layers on SPEs. Figure 3 shows the SEM images of SPE electrodes before (A) and after (B) Au electrodeposition processes confirming the formation of

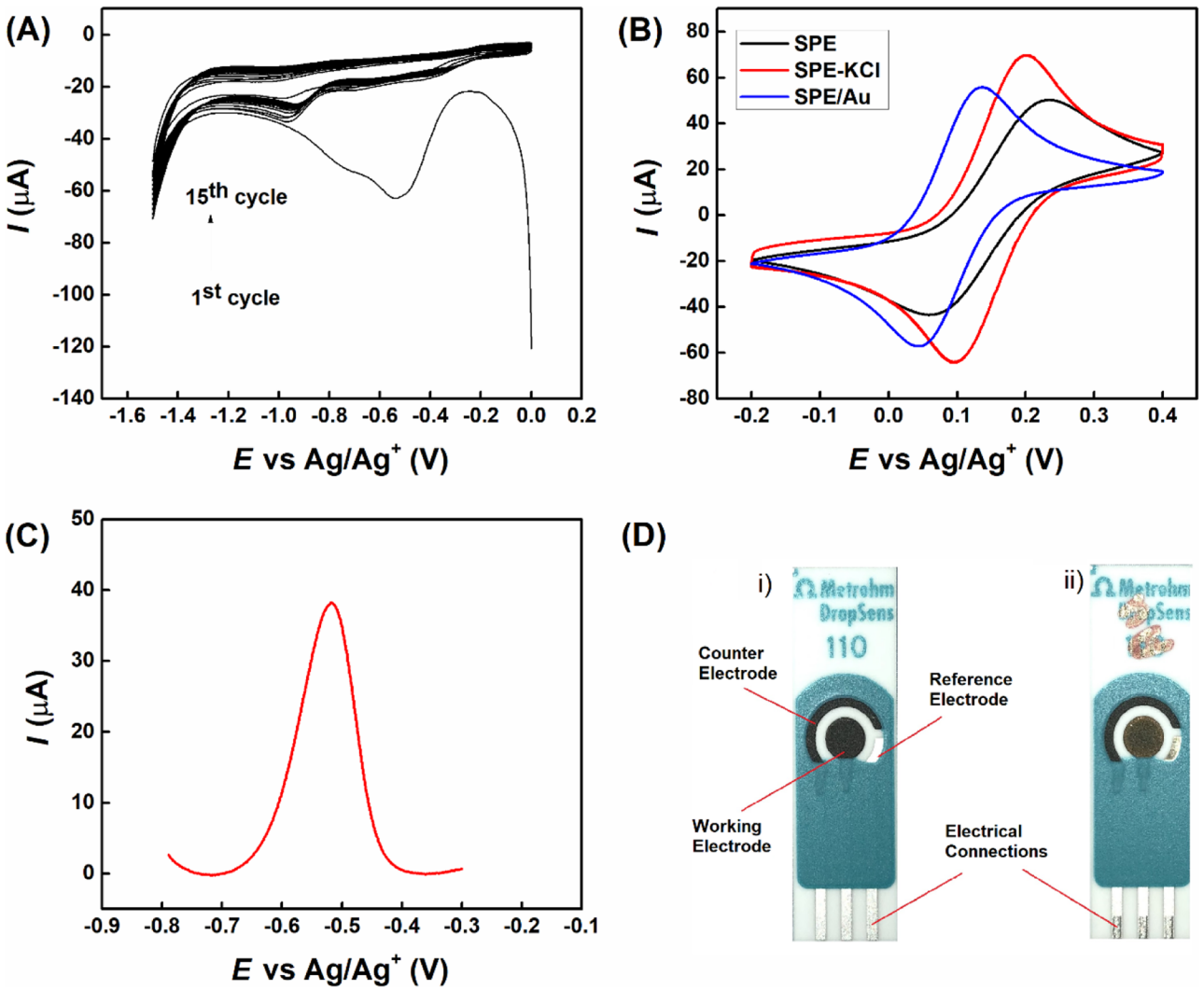

Fig. 2 **A** CVs for the electrolysis of 1 mM $[AuCl_4]^-$ in 0.1 M KCl at 50 mV/ for 15 cycles. **B** CVs of the different stages of SPE modification at 50 mV/s. **C** SWV scan for SPE/Au/Apta electrode in 0.1 PBS (pH 7.4) at 1 mV step potential. **D** photos of SPEs before (i) and after (ii) Au electrodeposition

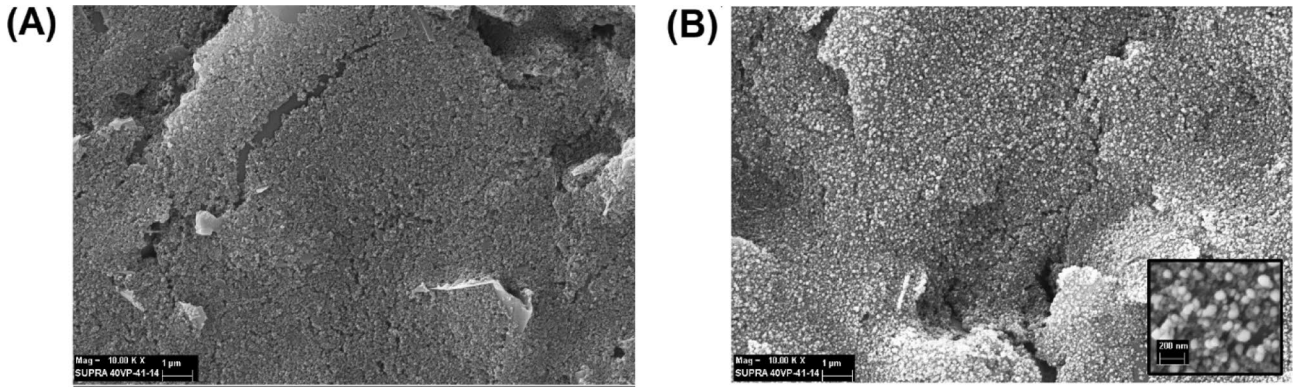

Fig. 3 SEM images of SPEs **A** before and **B** after Au electrodeposition

Au layers in the form of nanoparticles. EDX analysis also revealed the elemental composition of SPE and SPE/Au by weight. It was determined that the bare SPE electrode contains C (70.5 ± 5.1%) and O (4.2 ± 0.9%). On the other hand, SPE/Au electrode contains Au (98.3 ± 6.7%) and O (1.7 ± 2.4% O), similar to previously reported values [31]. Therefore, EDX analysis has also confirmed the formation of the Au layer on SPEs.

Figure 4 shows the schematic representation of the aptasensor working principle and aptasensor response to insulin. As seen from Fig. 4A, the aptasensor is "on" when there is no insulin, so electron transfer between the electrode and MB probe allows a current response. On the other hand, the current is hindered by incubating with insulin; thus, the aptasensor is in an "off" position. This "on–off" type of behavior indicates that the electron transfer distance can depend on the number of insulin molecules bound to the aptamer; therefore, it could be calibrated to quantification the amount of insulin in a sample.

Optimization of electrode modification parameters

To achieve the best aptasensor performance that provides minimum detection time, RSM was applied using Design-Expert software. The aptamer, MCH, and insulin incubation times were optimized to achieve the design goals. The model analysis showed that quadratic inverse transformation was the best fit for the experimental data. ANOVA results obtained from Design-Expert software and the model equation are given in Table S2. The ANOVA results showed that the model's p value and individual model parameters were found to be < 0.05 and < 0.1, respectively. ANOVA results show that the model was significant for the experimental data. Figure 5 shows the

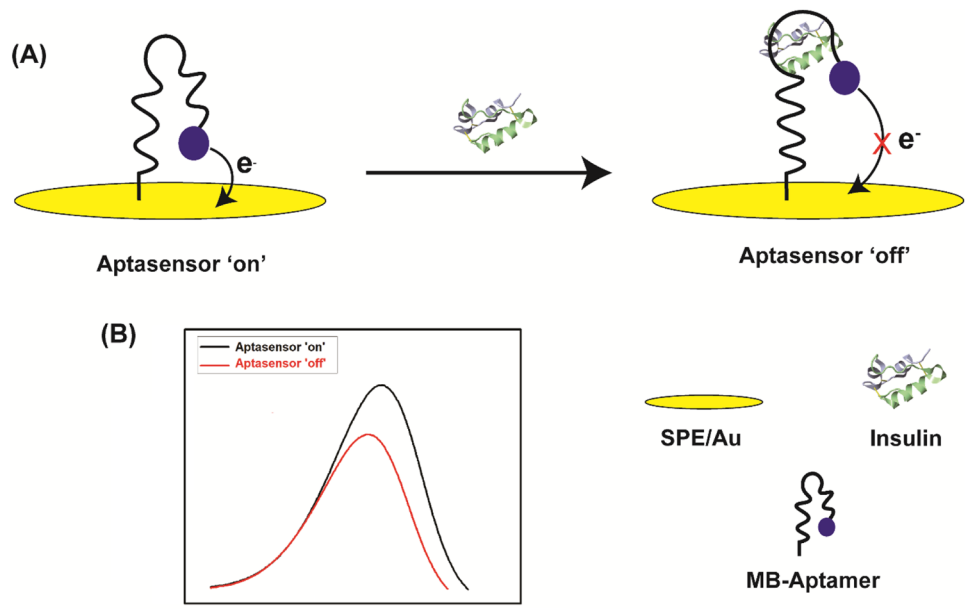

Fig. 4 **A** Schematic representation of aptasensor working principle and **B** aptasensor on–off signal response to insulin

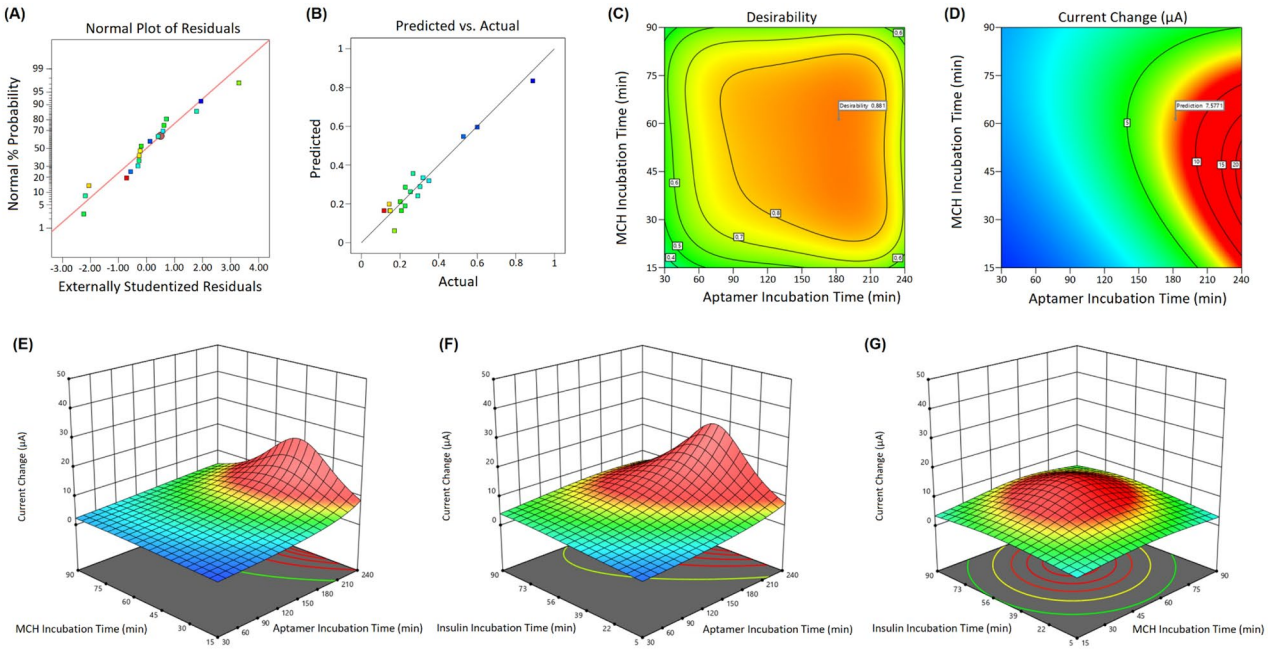

Fig. 5 Optimization results of the aptasensor preparation using RSM. (**A**) Normal plot of residuals (**B**) Predicted vs. Actual graph (**C**) Desirability (**D**) Aptamer incubation time, MCH incubation time and current change relationship (**E**), (**F**), and (**G**) 3D graphs showing the relationship of aptamer, MCH, and insulin incubation times with the current change response of the aptasensor (The plots were created using Design-Expert 12 (trial version) program)

optimization results obtained from RSM analysis. Residual analysis and predicted versus actual values plots can be seen from Fig. 5A, B, demonstrating a good correlation of the experimental data with the model. 3-D model analysis shows that there are optimum values between insulin and MCH incubation times as well as aptamer and insulin incubation times. The insulin incubation time seems to be optimum at 40 min to get the highest sensor response, whereas MCH and aptamer incubation times are 60 and 180 min, respectively (Fig. 5E–G). On the other hand, the current change seems to be decreasing with insulin incubation time after reaching its highest value. This could be due to irregular accumulations restricting the reach of insulin and deformation of the surface components in this case aptamer-MB complex. Similar behavior was also reported in the literature [32]. It was believed that 40-min insulin incubation time is longer than desired for a quick test. Therefore, further optimization has been used to minimize the time of insulin incubation time and the error of the model parameters using desirability in addition to RSM. The desirability is a transformed estimated response into a scale-free value which shows how desirable the optimized response is according to the objectives of the optimization (such as to maximize, minimize or obtain the target value of a response) [33]. In this case, the optimized parameters for the aptasensor fabrication were chosen for aptamer, MCH, and insulin incubation times as 180 min, 60 min, and 25 min, respectively, with a desirability value of $d = 0.88$ (Fig. 5C, D).

Aptasensor calibration and validation

Various amounts of insulin ranging from 20 to 150 pM were incubated on SPE/Au/Apta electrodes prepared using optimized parameters to obtain the calibration curve and the analytical performance of the aptasensor. The linear detection range was chosen between 20 and 150 pM based on the average blood insulin concentration. Twenty-picometer, 50-pM, 100-pM, and 150-pM insulin in PBS were incubated for 25 min on SPE/Au/Apta electrodes and washed with PBS to remove unbound insulin. The electrodes were then tested using SWV, as shown in Fig. 6. The aptasensor response showed a different change in peak current values; therefore, the peak current change was used as the calibration parameter.

After the aptasensors were tested with SWV, the change in peak current density values was plotted against insulin concentrations. Figure 7A shows the calibration curve of the developed aptasensor. The more insulin caused higher peak current change based on the "on–off" aptamer working principle. The calibration curve showed a linear response ($R^2 = 0.999$) for the given range with a LOD value of

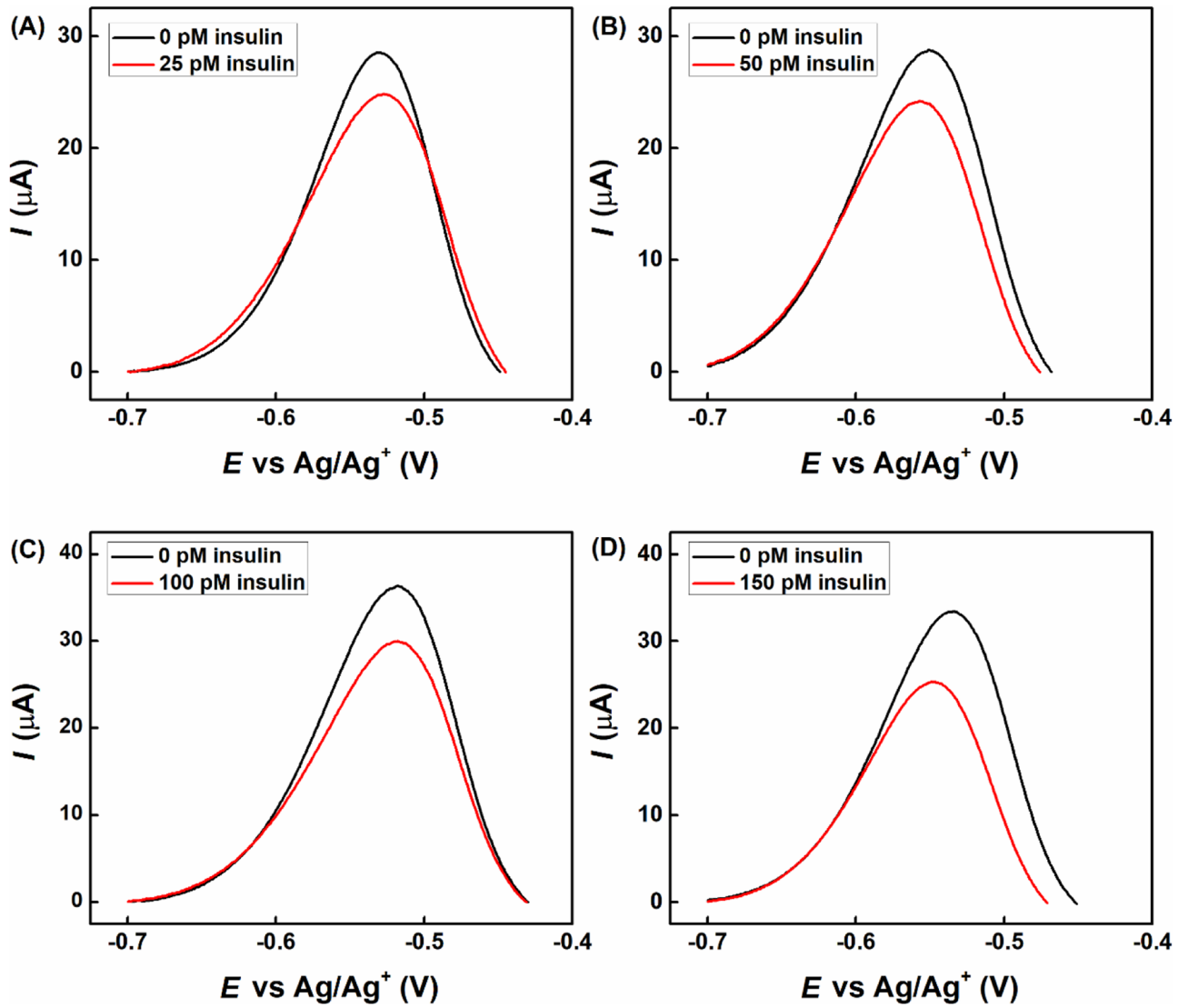

Fig. 6 SWV curves for SPE/Au/Apta and SPE/Au/Apta/Ins electrodes for **A** 25 pM, **B** 50 pM, **C** 100 pM, and **D** 150 pM in 0.1 PBS (pH 7.4)

18.5 pM for 4 individually prepared electrodes. A summary of the analytical parameters is given in Table 1.

The developed aptasensor provides a fast and mostly test compared to conventional methods as well as most similar studies where insulin aptamer has been used as a detection probe [34]. Although the LOD value of the aptasensor is not the lowest reported to date, it can provide accurate detection of insulin for blood samples (if diluted 2 to 3 times based on the average blood insulin levels reported in the literature [10]). To the best of the authors' knowledge, this study is the first-time demonstration of the optimization of aptasensor preparation methods based on a model; therefore, sensitive insulin detection can be achieved in less than 30 min.

Figure 7B shows the effect of several interfering substances on aptasensor response when incubated with 50 pM insulin. All the interfering substances show little to no effect on aptasensor performance except uric acid. Uric acid showed about 1.7 times more peak current change than 50 pM insulin response. This interference was specifically chosen as it is one of the most important interfering substances for electrochemical blood testing, yet it has not been widely demonstrated in studies for insulin detection. However, it has been previously reported that uric acid is one of the most important endogenous interference alongside ascorbic acid and C-protein [35]. This is an important design parameter to consider for further steps of the aptasensor development that should be noted. The relative standard deviation and accuracy of the aptasensor were calculated as 9.5% and 6.4%, respectively. The stability of the aptasensor was also calculated based on the % change in the response of the aptasensor after 10 days of refrigerated storage. The change in aptasensor response after 10 days was found to be 8%

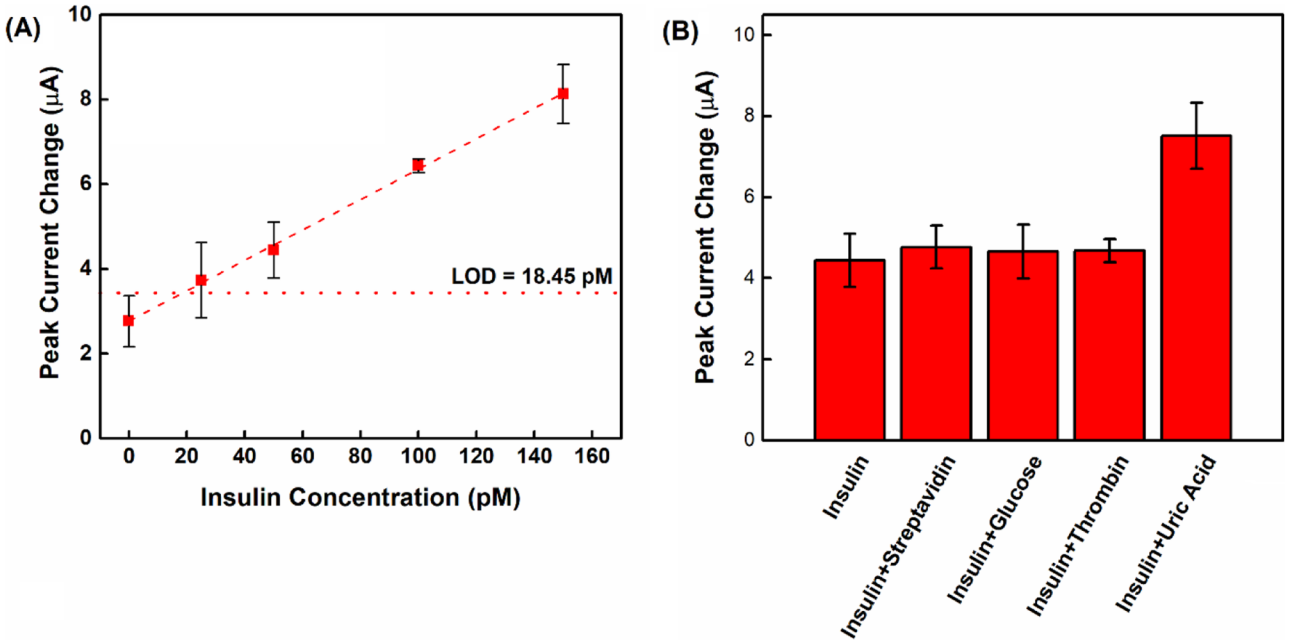

Fig. 7 **A** Calibration curve of the developed aptasensor, **B** the effect of interfering substances on aptasensor response. Error bars are sample standard deviations for 4 individually prepared electrodes

Table 1 The analytical parameters for the aptasensor

Analytical parameters	Values
Linear range	25–150 pM
Linear equation	$y = 2.7643 + 0.0359x$
Standard error of slope,±	0.0008
Standard error of intercept,±	0.0645
R^2	0.999
LOD, pM	18.5

(4.1 ± 1.1 μA, $N = 3$) compared to the first-day response of the aptasensor for 50-pM insulin, which shows that the aptasensor can maintain its performance for a reasonable storage time at this preliminary development stage.

Conclusion

Herein, an electrochemical signal switch aptasensor for sensitive detection of insulin has been developed using disposable SPEs. To the best of the authors' knowledge, the first-time comprehensive optimization of thiol-modified aptamer modification and aptasensor fabrication steps are presented using RSM. The developed model was then used to optimize the aptasensor parameters for the fast response and sensitive detection of insulin. The design of experiments provided the values for best aptasensor performance while optimizing the important application parameters such as minimizing the detection time for the aptasensor while maintaining the highest current signal change for insulin detection at a high desirability level. Signal switch–based detection was achieved using MB-modified insulin specific aptamer with a LOD value of 18.5 pM for a linear detection range of 25–150 pM. The developed aptasensor showed good relative standard error and accuracy as well as not significantly affected by potential interferences. Furthermore, the stability of the aptasensor for 10 days showed promising results, maintaining 92% of the initial aptasensor performance. The developed aptasensor could be used for the sensitive and fast detection of insulin from physiological body fluids in a clinical setting using minimally invasive methods without the need for complicated laboratory equipment.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10008-022-05133-x.

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Declarations

Conflict of interest The authors declare no competing interests.

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