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Gold nano-flowers (Au NFs) modified screen-printed carbon electrode electrochemical biosensor for label-free and quantitative detection of glycated hemoglobin

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ABSTRACT

Glycated hemoglobin (HbA1c) represents the average glucose level over the past three months and has been considered as the most important biomarker for the diagnosis of Type II diabetes (T2D). Herein, a label-free and quantitative electrochemical biosensor based on 4-mercaptophenylboronic acid (4-MPBA) modified gold nano-flowers (Au NFs) substrate was developed for the determination of HbA1c. Under optimal conditions, the linear dynamic ranges of HbA1c (5 μg/mL - 1000 μg/mL) and HbA1c% (2%–20%) by cyclic voltammetry were achieved. The electrochemical biosensor showed great detection specificity towards HbA1c and relatively stability after storage at 4 °C. This method could also be applied in human serum system which holds great potential to be applied to monitor real blood samples of diabetes patients. In human serum system, the recovery rate could reach 103.8% and 99.0%. It could achieve fast detection, the total analysis time was less than 65 min, and the detection time was less than 10 s. Moreover, in terms of fabrication process, operation procedure, detection time and cost, this technique was superior to the current HbA1c detection methods suggesting great promise for the practical clinical use in the future.

1. Introduction

Type II diabetes (T2D) is a life-long endocrine and metabolic disease. It represents one of the most important health concerns nowadays [1]. Patients with T2D have a much higher risk of severe complications such as myocardial infarction, stroke and limb amputation [2–4]. Blood glucose is the main inspection indicators of T2D. However, it is affected by many factors with large fluctuations and is not the most optimal biomarker [5,6]. Glycated hemoglobin (HbA1c) formed by nonenzymatic glycosylation of hemoglobin (Hb) is the most important long-term index reflecting the time-averaged blood glucose concentration over the previous 2–3 months which is the life span of red blood cells [7–9]. It has the advantages of better stability. Now it is considered as a promising biomarker and has become a gold standard [10] for the diagnosis of T2D [11,12].

At present, many methods have been used to detect HbA1c, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), immunofluorescence, etc. [13–19]. These HbA1c detection methods are burdened with some limitations such as availability, complexity, requirement for infrastructure and skilled personnel, costly materials, and long testing time [20,21]. Biosensors are the new developing detection technologies that can solve part of these problems [22–26]. It has long been sought to develop convenient, rapid, low-cost biosensors for T2D detection. Electrochemical biosensors can respond to biomedical substances rapidly [27,28] and are much suitable for miniaturization with rapid detection, portability and patient flexibility [27,29]. The personal glucose meter (PGM) [30] can complete one step blood glucose detection by means of enzymatic catalysis which is a label-free and quantitative detection method. Now it is the most successful example of electrochemical biosensor that is commercially

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available. Inspired by PGM, much endeavor was done to develop electrochemical biosensor for HbA1c detection. Kim [31] exploited the catalytic property of HbA1c for H_2O_2 reduction to measure the HbA1c level. The sensor construction included multiple modifications and fabrication is complicated and the dynamic range of detection spanned from 0.1 to 1.5% while it could not meet the requirement of the clinic. Chopra [32] employed an electrochemical immunoassay for HbA1c which was developed on a screen-printed gold electrode using MPBA-SAM as the capture molecule and ferrocene labeled *anti*-HbA1c as a tracer. This method needs the volume of sample up to 50 μL. Such assay is reasonably complicated to prepare, requiring washing and rinsing steps, and that such devices and the related tests are expensive due to the use of labeling antibodies and gold electrodes. All the above researches need multiple modification and labeling procedures which could not meet the requirement for sensitive and rapid detection.

Herein we proposed a novel chemically modified electrochemical biosensor for label-free and quantitative analysis of HbA1c. In order to selectively capture HbA1c, Au NFs was electrochemically deposited onto the screen-printed carbon electrode (SPCE), then 4-MPBA was attached on Au NFs with the formation of the self-assembled (SAM) by covalent attachment. Boric acid on the 4-MPBA could interact with the sugar subunit of target HbA1c.The HbA1c itself has the catalytic property. When HbA1c was added to the surface of 16-SPCE, it could produce catalytic reduction of H_2O_2 and result in specific electrochemical response which was linearly proportional to the amount of HbA1c captured on the 4-MPBA-Au NFs surface. Compared with the previous study, the obvious advantage of our strategy is that it is a label-free method which assures rapid detection with less than 5 μL samples needed; 16-SPCE could greatly lower the cost of detection and can simultaneous detection of multiple samples in one single time. It is worth noting that, the dynamic range of 2%–20% in our research could obtain a wider dynamic range which has the potential of clinical applications. It was proved that this is an ideal HbA1c detection method with well-matched sensitivity, simplicity, cost-effectiveness, and quickness.

2. Experimental

2.1. Materials and apparatus

The Hydrogen tetrachloroaurate(III) hydrate ($HAuCl_4$) was purchased from J&K Scientific Ltd. (Shanghai, China). The 4-mercaptophenylboronic acid (4-MPBA) was purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). The Potassium ferrocyanide trihydrate ($K_4Fe(CN)_6$) and hydrogen peroxide aqueous solution (30 wt %) were purchased from the Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All chemicals from commercial sources were of analytical grade. Natural Human Hemoglobin A1c (HbA1c) protein was purchased from Abcam Inc. (Cambridge, UK). Hemoglobin (Hb) was purchased from Fitzgerald Industries International Inc. (Concord, MA, USA). The buffer solutions involved in this study are as follows: samples were dissolved in 0.5% Hammarsten Casein in 1 × PBS which was equivalent to the blocking buffer. The 0.5% Hammarsten Casein in 1 × PBS was purchased from Sangon Biotech Co. Ltd (Shanghai, China). The washing buffer was 0.01 M phosphate saline (PBS) buffer (0.01 M phosphate, pH 8.5, 0.14 M NaCl, 2.7 mM KCl). All solutions were prepared using Milli-Q water (18.2 MΩ cm) from a Millipore system. Clinical serum samples were received from Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine and were provided with written informed consent. The human whole blood was pretreated by centrifugation. In the centrifugation method, the plasma was separated from 2 mL of the whole blood by centrifugation at 3000 rpm for 10 min at 4 °C. The supernatant (serum) was collected and stored at −80 °C.

Cyclic voltammetry (CV) measurement and amperometric (I-t) measurement were performed with the HSBS16× multi-channel electrochemical workstation (HuaSenXinKe (Suzhou) Nanotechnology Co. Ltd, China), which is connected to a computer. The disposable 16-channel SPCE was shown in graphical abstract. The 16 channel screen-printed carbon electrode (16-SPCE) was purchased from HuaSenXinKe (Suzhou) Nanotechnology Co. Ltd (Suzhou, China). Each electrode consists of a carbon working electrode (3 mm diameter), a carbon auxiliary electrode, and an Ag/AgCl reference electrode. An insulating layer is printed around the working area to constitute a reservoir of the electrochemical microcell.

2.2. Fabrication of the 4-MPBA-Au NFs modified SPCE

The SPCE was pretreated electrochemically to generate the Au NFs on the working electrode by running I-t with the 16-channel electrochemical detector. Au NFs were electrodeposited onto SPCE from $HAuCl_4$ solution through potentiostatic deposition. The electrodeposition conditions were as follows: deposition time, 150 s; scan rate, 100 mV/s; deposition potential, −200 mV. Then, 10 μL of freshly prepared 10 mM 4-MPBA in methanol solution (80 V/V %) was placed on to the working electrodes to immobilize at 4 °C, and washed off after 30 min. Before all electrochemical measurements, the 4-MPBA-Au NFs modified SPCE was washed by methanol solution (80 V/V %) and ultrapure water and then dried under nitrogen.

2.3. Confirmation of 4-MPBA SAM formation on the surface of Au NFs modified SPCE

The electrochemical detections were performed in 1 mM H_2O_2 containing 0.01 M PBS (pH 8.5) at room temperature. CVs were conducted at a scan rate of 100 mV/s ranging from 0 V to −0.6 V. I-t curve was fixed at −0.45 V. The electro-reduction current was measured at 60 s after the redox reaction reached a steady state.

2.4. Electrochemical biosensor for HbA1c detection

In a typical test, a 16-channel 4-MPBA-Au NFs modified SPCE was incubated at room temperature for 60 min with 5 μL drop of HbA1c samples (dilution in blocking buffer/clinical serum samples) on each electrode, followed by washing with 0.01 M PBS (pH 8.5). A 16-channel HbA1c-coated SPCE was performed in 2.5 mM $K_4Fe(CN)_6$ containing 0.1 M KCl by CV measurement at a scan rate of 100 mV/s ranging from −0.85 V to 0.85 V. After the incubation, 1 mM H_2O_2 containing 0.01 M PBS (pH 8.5) substrate was applied onto each channel of the SPCE. After that, the reactions in the 16 channels were detected simultaneously by CV measurement at a scan rate of 100 mV/s and a potential range of 0 V to −0.6 V.

3. Results and discussion

3.1. Design strategy of the HbA1c electrochemical biosensor

The disposable 16-channel electrochemical biosensor used in our experiments was referred from reported techniques [33,34]. The schematic diagram of 16-SPCE detection system was shown in graphical abstract. 16-SPCE can be applied for mass production with very low cost and high reproducibility and can achieve simultaneous detection [35,36]. The disposable 16-channel screen-printed carbon electrode (SPCE) detection platform is a multi-channel electrode array sensing device, which was able to perform simultaneous detection of multiple samples. We were able to carry out multiple measurements in single detection. The modification process of the carbon working electrode and subsequent recognition for HbA1c was shown in graphical abstract (b). Nano-noble metal materials have special physical and chemical properties which can significantly improve the performance of electrode [37]. Here, by the reduction of $HAuCl_4$ through potentiostatic deposition, the carbon working electrode of 16-SPCE was covered by a

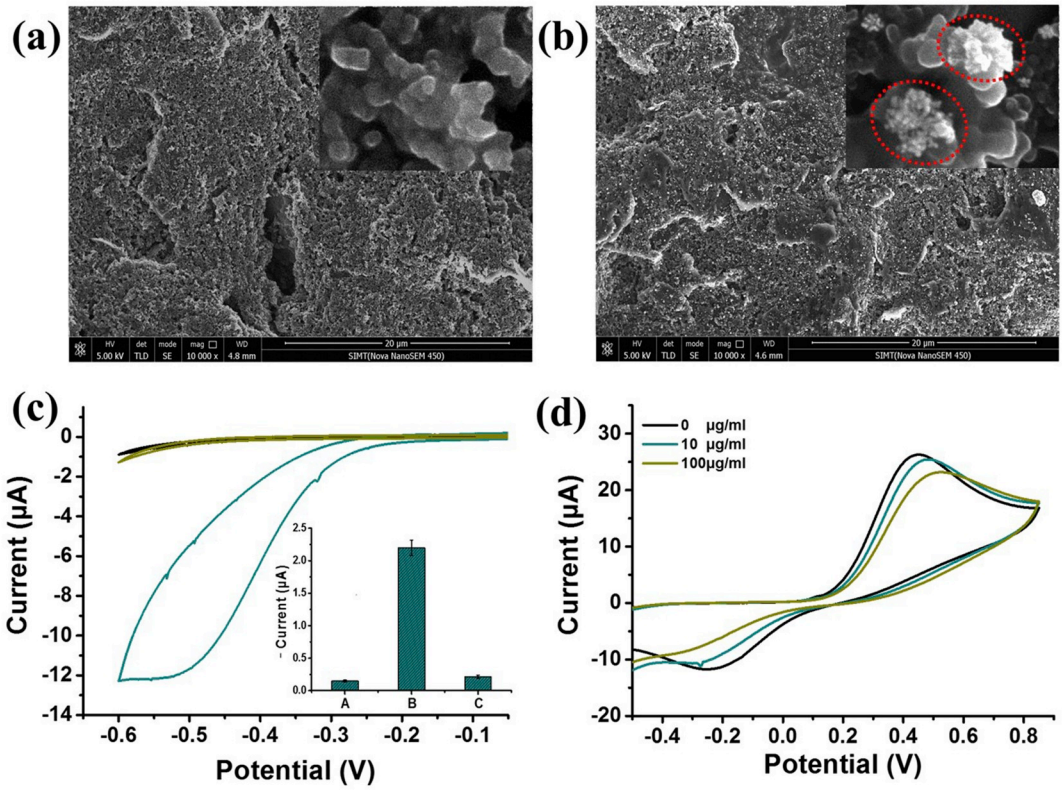

Fig. 1. The scanning electron microscopic image of the screen printed carbon electrodes before (a) and after (b) Au NFs deposition. The scale value was 20 μm. The scale value of insert image was 1 μm. (c) CVs curves of the redox on electrodes after different modification steps. Inset: Amperometric (I–T) response of the electrochemical sensor and the reduction current was recorded at 60s. Error bars represent standard deviations of at least three independents. All investigations about electrocatalysis behaviors were performed in 1 mM H_2O_2 (0.01 M PBS, pH 8.5) at room temperature. (A: bare SPCE; B: Au NFs modified SPCE; C: 4-MPBA-Au NFs modified SPCE) (d) CVs curves for the electrochemical biosensor detecting 0, 10, 100 μg/mL of HbA1c in 2.5 mM $K_4Fe(CN)_6$ containing 0.1 M KCl.

layer of Au NFs. It was reported that the boric acid group could capture target as a recognition element and detect saccharides in electrochemical biosensor [38–43]. Boric acid and a polyol can form stable complexes in aqueous solutions [44]. Then we took advantage of 4-MPBA which could selectively bind 1, 2- and 1, 3-diols of the glucose to form negatively charged boronate esters under alkaline conditions [45]. With the affinity between Au and S [46], the 4-MPBA modified self-assembled monolayer could be easily achieved. The 4-MPBA-Au NFs modified surface was rationally designed. The 4-MPBA could interact with sugar group of target HbA1c in the following quantitative experiment. As known, hemoglobin (Hb) contains four iron heme groups as an electro active center which is equivalent to horseradish peroxidase (HRP) in electrochemical detection of H_2O_2 [47]. Likewise, HbA1c could also catalyze the reduction reaction of H_2O_2 [31]. Applying this self-catalytic property of HbA1c, it can catalytically reduction H_2O_2 to bring about electrical signals by chemical signal and display a specific response in electrochemical biosensors. Consequently, the label-free electrochemical detection of HbA1c could be easily achieved.

3.2. Establishment and optimization of the 4-MPBA-Au NFs modified surface

In order to improve the detection performance, key parameters including electro-deposition time and the concentration of $HAuCl_4$ were optimized. Firstly SPCE was modified by means of potentiostatic deposition. The CV curves indicated that the peak current increased with the increasing deposition time (Fig. S1). The peak current reached maximum value at 150 s. Therefore, the electro-deposition time of 150 s was selected as the optimum time for electrode preparation. Next, the concentration of $HAuCl_4$ was optimized by electro-deposition method for the higher signal noise(S/N) ratio. It was indicated that the peak current was no longer increased at the concentration of $HAuCl_4$ of 60 μg/mL which reached the saturation (Fig. S2). Therefore, 60 μg/mL $HAuCl_4$ solutions were chosen as the optimal concentration for electrode preparation. Further, we used SEM to visualize the morphology and the size of the carbon working electrode surface after deposition. The result was shown in Fig. 1(a), the bare SPCE before deposition had a relatively smooth surface. It was obvious that a relatively dense layer of Au NFs was covered on the surface of carbon electrode after deposition (Fig. 1(b)). More clear SEM images of SPCE before and after Au NFs deposition were provided in Fig S3. This Au NFs modified carbon electrode with highly rough surface area (insert image shown in Fig. 1(b)) could improve the capture and electron transfer performance and enhance the speed of chemical response and stability.

Then 4-MPBA was immobilized on Au NFs modified electrodes by self-assembly of the Au−S bond. In order to investigate the electrochemical effect of the modified steps on the biosensor, the CV and I-t measurements were carried out. It can be shown from Fig. 1(c) that the reduction current produced by Au NFs modified electrode was significantly increased under the initial voltage of −0.45 V. The catalytic current of Au NFs modified surface was much higher than that of bare carbon electrode since the Au NFs on the electrode surface can catalyze hydrogen peroxide. With further chemical modification by 4-MPBA, it was shown that there was little difference between the current of the bare-carbon electrode and the 4-MPBA modified Au NFs electrode. It means that the modification of Au NFs had no effect on the catalysis responses of HbA1c because the catalytic site of Au NFs could be

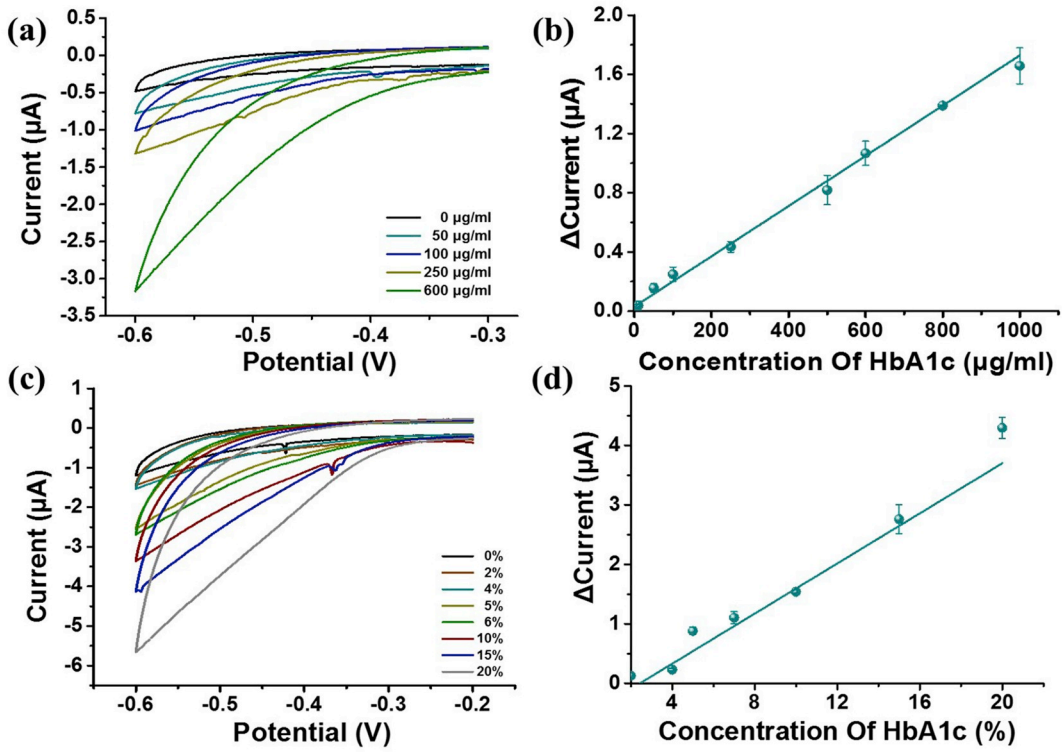

Fig. 2. CVs recorded for the reduction of H_2O_2 (1 mM containing 0.01 M PBS, pH 8.5) by the catalytic property of HbA1c (a) and %HbA1c (c). Reduction current comparison with different concentrations of HbA1c in 0.01 M PBS (pH 8.5). Calibration plot for HbA1c (b) with the ΔCV responses of 5, 10, 50, 100, 250, 500, 600, 800, 1000 μg mL^{-1} HbA1c on the HbA1c electrochemical biosensor. And calibration plot for %HbA1c (d) with the ΔCV responses of 2%, 4%, 5%, 7%, 10%, 15%, 20% HbA1c on the HbA1c electrochemical biosensor. Error bars represent standard deviations of at least three independent experiments.

blocked by 4-MPBA.

Then we set the different concentrations of HbA1c on the 4-MPBA-Au NFs modified SPCE in the $K_4Fe(CN)_6$ containing 0.1 M KCl by CV measurement. In the presence of HbA1c, it resulted in the decreased current. As shown in Fig. 1(d), with the increase of HbA1c concentration, the current signal decreased since HbA1c could impede the electron transfer. The results can clearly show that the 4-MPBA-Au NFs modified surface on the SPCE could successfully capture HbA1c which could guarantee the favorable process of the follow-up experiment.

3.3. Quantification of HbA1c based on electrochemical biosensor

The accomplishment of HbA1c quantitative determination has a guiding significance for its in-depth and accurate detection for clinical use. Here the capacity of the electrochemical biosensor we developed for the relative quantitative analysis of HbA1c was evaluated. The HbA1c were serially diluted and detected by the electrochemical biosensor. With the increase of HbA1c, the redox current increased as was demonstrated in Fig. 2(a) and Fig. 2(c). Under the optimal conditions, the value of redox peak was proportional to the amount of HbA1c in the sample. We observed a linear relationship ($\Delta Current = 1.70*10^{-3}[HbA1c] + 0.03$, $R^2 = 0.999$) towards detecting HbA1c in the concentration range from 5 μg/mL to 1000 μg/mL (Fig. 2(b)).

The relative content of HbA1c in total Hb is usually measured with the clinical range of 5–20%, and the ADA recommends a diagnostic criterion with diabetes (HbA1c ≥ 6.5%) and prediabetes (5.7% ≤ HbA1c ≤ 6.4%). To estimate the clinical applicability of the 4-MPBA-Au NFs modified electrochemical biosensor, we used this system to detect the percentage of HbA1c. As was shown in Fig. 2(d), the ΔCurrent varies linearly with HbA1c levels from 2 to 20% ($\Delta Current = 0.21*[\%HbA1c] - 0.51$, $R^2 = 0.942$) with the detection limit of 0.65%. The LOD was calculated from 3 ($S_{\Delta Current}/m$), where $S_{\Delta Current}$ is the standard error of blank and m is the slope of the calibration curve. We then combined the two linear relationships in Fig. S4 to demonstrate the intra-assay and inter-assay of the present HbA1c electrochemical biosensor, it was shown that the slope could change between the Hb/HbA1c and buffer solution system, which means the addition of extra ingredient in the detection system could change the linear relationship between the concentration (or ratio) and the signal response. The result indicated that the linear relationship should be in accordance with the detection system to make sure the precise quantification of HbA1c. Applying the self-catalytic property of HbA1c and disposable 16-channel Au NFs modified SPCE, we realized label-free and simultaneous detection of HbA1c with simple modification. Compared with the previous study (shown in Table 1), the dynamic range of APBA-pTTBA-Au NPs-covered SPCE with multiple modifications spanned from 0.1 to 1.5% while it could not meet the requirement of the clinic. The method of 3-APBA modified gold electrode needs the volume of sample up to 1000 μL. Generally , the preparation steps of these other methods in Table 1 were cumbersome, the experimental conditions were harsh, and the demands for test samples were high. The obvious advantage of our strategy is that it is a label-free method which could assure rapid detection with less than 5 μL samples needed; 16-SPCE could greatly lower the cost of detection and can simultaneous detection of multiple samples in one single time. It is worth noting that, the dynamic range of 2%–20% in our research could obtain a wider dynamic range which has the potential of clinical applications.

3.4. Specificity and stability of the HbA1c electrochemical biosensor

It is of vital importance to find out if the developed electrochemical biosensor has specificity against other interfering species. The

Table 1
Comparison among different electrochemical HbA1c detection methods.

	Sample Volume	Dynamic Range(%)	Detection Method	Bio-report Material	Sample Pretreatment	Sensitivity	References
4-MPBA-Au NFs modified SPCE	5 μL	2%–20%	Cyclic voltammetry	HbA1c	No	0.65%	This study
3-APBA modified gold electrode/Luminol CL	1000 μL (1.0 mL)	2.5%–17%	Chemiluminescence response	Luminol	No	10 ng/mL	[47]
APBA-pTTBA-Au NPs-covered SPCE	< 2 μL	0.1–1.5%	Amperometric I-T	HbA1c	No	0.052 ± 0.02%	[31]
MPBA modified gold electrode for electrochemical immunoassay	50 μL	5–16%	Cyclic voltammetry	Fc-antibody	No	N/A	[32]
3-APBA modified Interdigitated Electrode	85 μL	0.1%–8.36%	Impedance	HbA1c	Yes	0.024%	[48]
Boronate-functionalized hydrogel interface based on the competitive binding with signaling glycoprotein	40 μL	4.6%–15.2%	Colorimetry	HRP	Yes	N/A	[49]
Boronate formation on Au for an HbA1c/GOx competition assay	N/A	2.5–15%	Cyclic voltammetry	GOx & Fc	Yes	N/A	[11]

* NA – Not available in the study.
* Abbreviations: HbA1c = glycated hemoglobin; MPBA = mercaptophenylboronic acid; Au NFs = gold nano-flowers; APBA = aminophenyl boronic acid; pTTBA = poly(terthiophene benzoic acid); Au NPs = gold nanoparticles; CL = chemiluminescence; Fc = ferrocene; HRP = horse radish peroxidase; GOx = glucose oxidase.

specificity test for HbA1c has been carried out against Hb, BSA, glucose, fructose under the identical conditions. The ΔCurrent for 75 μM BSA, glucose, fructose showed significant difference, respectively, compared to that obtained for 100-fold diluted HbA1c (Fig. 3(a)). It showed that this biosensor had better discrimination ability for HbA1c than Hb, BSA, glucose, and fructose. HbA1c could catalyze the reduction reaction of H_2O_2 to produce the electrochemical signal. BSA, glucose and fructose could interact with 4-MPBA to block the boric acid recognition sites. But they could not catalyze the reduction reaction of H_2O_2 to output the response signal. However, Hb resulted in a small increase in reduction peak responses since the small amount of Hb was non-specifically adsorbed on surface of 4-MPBA-Au NFs. This result indicated that the present HbA1c electrochemical biosensor had good detection specificity towards HbA1c. The stability of 4-MPBA-Au NFs electrode was also investigated over a period of 4 days. The reduction response was determined every day. It was found that electrochemical response was relatively stable after storage at 4 °C, compared to the initial values obtained from the electrodes of the same batch (Fig. 3(b)). At the relatively low concentration of 50 μg/mL, the S/N ratio was stabilized at 1.69–1.93. This result indicated that our HbA1c electrochemical biosensor had good stability and friendly-storing in the intra-assay and inter-assay. This biosensor has a long shelf life, but we recommend using the freshly prepared biosensor.

3.5. HbA1c electrochemical biosensor for human serum sample [48–50].

To demonstrate the practical usage of the present HbA1c electrochemical biosensor, the concentration of HbA1c in diluted human blood samples from healthy adults were determined and the recovery rate experiments were conducted by the standard addition method [51]. In our experiment, blood sample was 20-fold diluted, and the samples were analyzed by our HbA1c biosensor using HbA1c linear relationship. As shown in Fig S5, the dynamic range of our detection spanned from 2% to 15%. The electrochemical current increased monotonically along with the increase of %HbA1c. The equation for the calibration curve was ΔCurrent = 0.186* [%HbA1c] - 0.251, R^2 = 0.988. With the initial ratio of 4%, the recovery rate of 4% and 6% could reach 103.8% and 99.0% (Table 2), which means this method was sufficiently accurate and could work well with serum samples. The 5% human serum improved the complexity of HbA1c samples to further demonstrate the practical usage of HbA1c electrochemical biosensor.

4. Conclusions

In summary, the Au NFs modified disposable 16-SPCE biosensor was developed. Using the catalytic property of HbA1c confirmed by voltammetry, we achieved successful application for label-free and quantitative HbA1c electrochemical bio-analysis. Our HbA1c electrochemical biosensor obtained a large dynamic range (5–1000 μg/mL and 2–20%). The proposed biosensor had good detection specificity and relatively stability. We also applied this biosensor in the serum system, and the recovery rate could reach 103.8% and 99.0%, which means this method was accurate enough. The total assay time of the biosensor was no more than 65 min and the detecting time were no more than 10 s. As a promising diagnostic platform with good sensitivity and selectivity, rapid processing period, friendliness, easy to implement, and being cost-beneficial, our method holds great potential to be applied to monitor real blood samples of diabetes patients. It also could be expanded to detect glycoprotein biomarkers of other chronic diseases, such as cancer. Currently, our method was limited in laboratory level. On the basis of this research, we hope to continue the research in depth to realize the integration and automation of the detection test system, and provide a certain reference value for the laboratory analysis and clinical measurement of HbA1c.

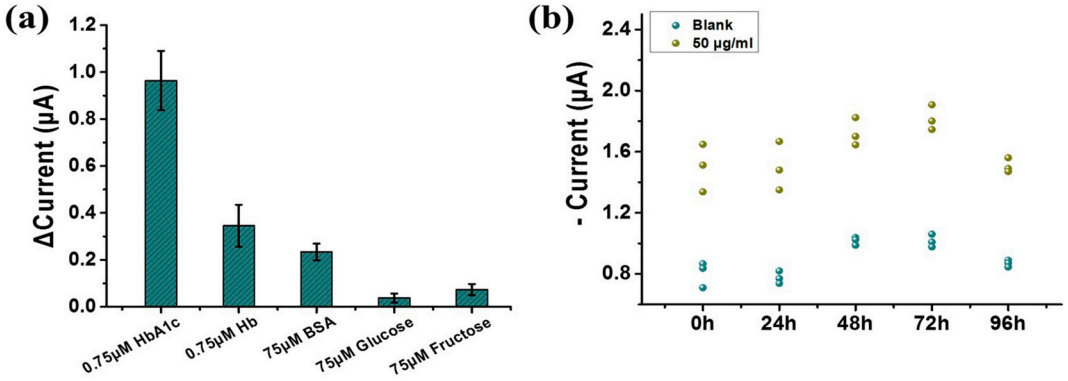

Fig. 3. (a) The specificity study of the 4-MPBA-Au NFs modified electrochemical biosensor against Hb, BSA, glucose, fructose. The detection conducted in H_2O_2 (1 mM containing 0.01 M PBS, pH 8.5). Error bars represent standard deviations of at least three independent experiments. (b) The stability study of the 4-MPBA-Au NFs electrochemical biosensors against the storage time about 0 h, 24 h, 48 h, 72 h, 96 h. The detection was conducted in H_2O_2 (1 mM containing 0.01 M PBS, pH 8.5).

Table 2
Detection of HbA1c samples in 5% human serum with the 4-MPBA-Au NFs electrochemical biosensor.

$[HbA1c]_{Initial}$ (%)	$[HbA1c]_{Added}$ (%)	$[HbA1c]_{Measured}$ (%)	Recovery (%)
4%	4%	4.15%	103.8%
	6%	5.94%	99.0%

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.03.100.

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