Sensing and Bio-Sensing Research 4 (2015) 96–102

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

On-chip highly sensitive saliva glucose sensing using multilayer films composed of single-walled carbon nanotubes, gold nanoparticles, and glucose oxidase

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ARTICLE INFO

Keywords:
On-chip
Disposable
Nano-biosensor
SWNT
Gold nanoparticles
Real-time

ABSTRACT

It is very important for human health to rapidly and accurately detect glucose levels in biological environments, especially for diabetes mellitus. We proposed a simple, highly sensitive, accurate, convenient, low-cost, and disposable glucose biosensor on a single chip. A working (sensor) electrode, a counter electrode, and a reference electrode are integrated on a single chip through micro-fabrication. The working electrode is functionalized through a layer-by-layer (LBL) assembly of single-walled carbon nanotubes (SWNTs) and multilayer films composed of chitosan (CS), gold nanoparticles (GNp), and glucose oxidase (GOx) to obtain high sensitivity and accuracy. The glucose sensor has following features: (1) direct electron transfer between GOx and the electrode surface; (2) on-a-chip; (3) glucose detection down to 0.1 mg/dL (5.6 μM); (4) good sensing linearity over 0.017–0.81 mM; (5) high sensitivity (61.4 μA/mM-cm^2) with a small reactive area (8 mm^2); (6) fast response; (7) high reproducibility and repeatability; (8) reliable and accurate saliva glucose detection. Thus, this disposable biosensor will be an alternative for real time tracking of glucose levels from body fluids, e.g. saliva, in a noninvasive, pain-free, accurate, and continuous way. In addition to being used as a disposable glucose biosensor, it also provides a suitable platform for on-chip electrochemical sensing for other chemical agents and biomolecules.

1. Introduction

Being able to rapidly and accurately detect glucose levels in biological environments is of crucial importance to human health, especially in the condition of diabetes mellitus [1,2]. In the past several decades, various methods have been developed to measure glucose concentrations, including optical approaches (infrared (IR) spectroscopy, fluorescence spectroscopy, Raman spectroscopy, optical polarization rotation measurement, photo-acoustic probes, and surface plasmon resonance) [3,4], MEMS affinity sensing [5] and electrochemical methods [6,7]. Optical measurement approaches normally require very expensive instruments, significant processing time and highly trained professions; and affinity sensing method can run into nonspecific binding issues. It is the electrochemical glucose sensing that is most studied and demonstrated with high sensitivity, good accuracy, high selectivity, fast response time, low cost and many other outstanding properties [6,8].

Glucose sensing dates back to 1841 when glucose levels were measured through urine, but the correlation between urine and plasma glucose was later found inconsistent [9]. Until now, the monitoring of blood glucose levels has been the only recognized and widely used method for diagnosis and management of diabetes. However, users have to prick their fingers multiple times a day to use these devices, which are a major problem for young children and result in negative consequences for disease management. Finger pricking can also cause transient discomfort, bruise, fainting and blood-borne infection. A noninvasive and simple technique for diagnosis and monitoring of diabetes is thus very desirable. With a direct correlation between blood glucose and salivary glucose, it is possible to simply apply salivary glucose measurements to monitor individual's health conditions [10,11]. Hence, monitoring of salivary glucose levels can be an alternative prediagnostic method for diabetics and a health indicator for any individuals.

We have developed a simple, highly sensitive, accurate, convenient, low-cost, and disposable glucose biosensor on a single chip. It can effectively and reliably determine glucose concentrations in saliva. It is fabricated using micro-fabrication and LBL assembly procedures.

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http://dx.doi.org/10.1016/j.sbsr.2015.04.006
2214-1804/

The effective immobilization of enzymes onto the electrode surface has been one of the main factors that affect the sensing performance of an enzyme biosensor [12]. LBL assembly technique, among many enzyme immobilization methods, is proven to be a simple and effective method to prepare multilayer films containing the enzymes enabling good uniformity, stability, reproducibility and remarkable sensitivity [13,14].

Carbon nanotubes (CNTs) have been widely utilized as components for nanoscale electronic devices and biosensors due to their high electrocatalytic property, their ability to promote electron transfer, and their high thermal capacity. The CNTs-modified electrodes are reported to allow direct electron transfer (DET) to glucose oxidase [15,16]. The intrinsically hydrophobic carbon nanomaterials can decrease the bioactivity of GOx [17], however, functionalized carbon nanotubes have been proven to improve the glucose sensing performance and to facile direct electron transfer between GOx and the electrode surface [16,18].

Chitosan (CS), a linear polysaccharide, has a repeating hexosamide residue unit of one amino group and two hydroxyl groups permitting chemical modifications. It is nontoxic, biocompatible and economic. Chitosan is widely applied to immobilize biomolecules, especially in the assembly of enzymes and fabrication of amperometric biosensors, due to its excellent film forming and adhesion abilities, and its easiness for chemical modifications [19,20].

Gold nanoparticles (GNp) are very attractive in constructing electrochemical biosensors due to their unique physical and chemical properties. In particular, GNp are applied in electrode functionalization to catalyze electrode chemical reactions and conduct DET, and thus to increase the sensitivity of biochemical detection [21]. They are used as one negatively charged nanomaterial for electrostatic adsorption in LBL process. GNp not only increase the surface area to allow more enzyme to be immobilized, but also provide a mild microenvironment and give the biomolecules more freedom in orientation [22].

Tremendous work has been done to develop highly selective and sensitive glucose electrochemical biosensors, as reviewed by Professor Yao's group [23]. Glucose biosensors functionalized with CNTs or GNp have the ability to linearly detect glucose down to very low levels [24–29]. Chitosan, or polymers like poly(diallydimethylammonium chloride) (PDDA), poly(ethylenedioxithiophene) (PEDOT), has been commonly used in enzyme immobilization or as a linker molecule in the fabrication of amperometric biosensors [6,24,25,27–30]. However, we have developed an accurate and reliable saliva glucose sensor using direct electron transfer enabled by using SWNT.

Our on-chip electrochemical sensing device contains at least one working electrode, a counter electrode and a reference electrode. It is manufactured through several micro-fabrication procedures. The metal for all electrodes is Pt, which is widely applied for glucose sensing [20]. Pt provides significant advantages, such as much better conductivity, signal stability, and analytical response, over the other electrode materials, like Au, Ti, Ag [31,32]. Jin et al. have also proved that Pt's deposition onto the gold electrode can increase the electrocatalytic properties of the electrodes for glucose oxidation [33]. Pt can also be used for reference electrode [34]. Thus, it is highly feasible to integrate three Pt electrodes onto one single chip to realize on-chip electrochemical sensing. Further, this single chip can not only be used for glucose detection, but also provides an innovative platform for on-chip electrochemical sensing of other chemicals and biomolecules.

2. Experimental design and procedure

The chemicals and facilities used are listed here, and more importantly, we introduce the sensor fabrication and functionalization procedures. The sensor functionalization procedure can be modified to assemble other enzymes or antibodies in building a platform for on-chip electrochemical sensing of a wide range of chemicals and biomolecules.

2.1. Reagents and apparatus

Glucose oxidase (GOx, 17,300 units/G solid) from Aspergillus niger, gold nanoparticles (GNp, 20 nm diameter), chitosan (CS), poly(allylamine) (PAA, 20 wt% solution in water), acetate buffer solution (pH 4.65), D-(+)-Glucose, phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma Aldrich. Carboxyl (COOH) groups functionalized single-walled carbon nanotube suspension (SWNT, diameter: 1–2 nm; length: 2–5 μm, 4000 mg/L in distilled (DI) water with ~5–7 wt% COOH groups at the end) was purchased from Brewer Science Company. Dulbecco's phosphate-buffered saline (DPBS, no calcium, no magnesium) was purchased from Life Technologies.

Silicon wafers (diameter 3″, boron doping, ⟨100⟩ orientation, resistivity 0–100 Ω, thickness 406–480 μm, one-side polished) were purchased from University Wafer; and Platinum Pellets (1/8″ Diameter × 1/8″ Length, Per Gram, 99.99% pure) were purchased from Kurt J. Lesker Company.

Facilities used in Gorge J. Kostas Nanoscale Technology and Manufacturing Research Center include wet bench wafer cleaning system, Bruce furnace 7355B (oxidation), Nanospec thickness measurement machine, Brewer/laurell spinner, Quintel 4000 mask aligner, Unaxis ICP etch (Plasma Therm 790), Electron-beam deposition system, Micro automation 1006 dicing saw, and Supra 25 SEM.

All cyclic voltammetry (CV) and amperometric measurements were performed in PBS (0.1 M, pH7.4) at room temperature (~23 °C) using the DY2113 mini potentiostat from Digi-Ivy company. The adapter between sensor and the potentiostat was purchased from DropSens Company. A Supra 25 Scanning Electron Microscope (SEM) was employed for the surface morphological characterization of SWNT, GNp and GOx on the sensor electrode reactive area.

2.2. Device fabrication

Fabrication of the disposable glucose biosensor is described in this section. It includes micro-fabrication of the sensor chip and LBL assembly for electrode modification.

The on-chip electrochemical sensing device contains at least one working electrode, a counter electrode and a reference electrode (one possible electrode configuration – Fig. 1a). The small rectangle (purple) marks out the reactive area on the working electrode while the larger one (blue) indicates where sample drops on. One such device- S2D2 is of size $20 \times 10\ mm^2$ with the reactive area $32\ mm^2$. It can be manufactured through microfabrication (Fig. 1b). Starting from a pre-cleaned silicon wafer (~500 μm thick), the surface is oxidized in wet atmosphere (Bruce Furnace 7355B) at 1100 °C for 40 min to form a 0.5 μm thick SiO_2 layer as the insulator layer. Then, photolithography is conducted to create the pattern of desired microelectrodes using MICROPOSIT S1813 photoresist. 200 nm thick Pt (Platinum) with 20 nm thick Cr (Chromium) adhesive layer is deposited on the surface through E-beam evaporation. After lifting off the extra Pt by acetone, isopropyl alcohol (IPA) and DI water and dicing the wafer into small chips ($20 \times 10\ mm^2$), the electrode system is present on each chip.

The glucose biosensor is fabricated through a LBL assembly of SWNT and multilayer films composed of CS–GNp–GOx (Fig. 1c) [35]. The CS–GNp–GOx unit can be repeated several times to

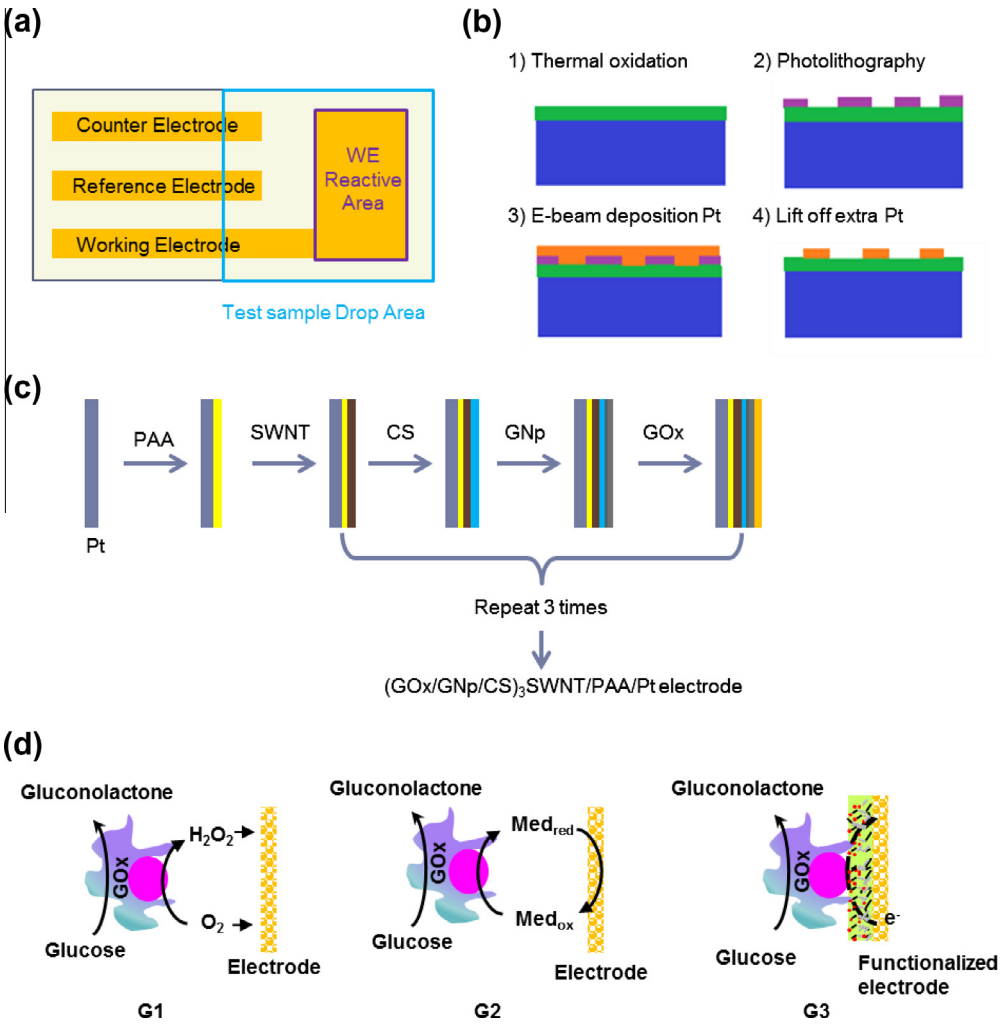

Fig. 1. (a) Schematic diagram of the electrochemical system and the sensing area on a chip; (b) micro-fabrication procedures of the microelectrodes on a silicon wafer; (c) schematic diagram of the modification procedure of the sensor electrode; (d) schematic diagram of the three enzymatic glucose detection mechanisms, presented as the first (G1), second (G2), and third (G3) generation glucose sensors.

form a multilayered coating. The optimal number of layers is between 3 and 6 enabling the best sensing performance.

There are three types of enzymatic glucose sensors based on the mechanisms of electron transfer methods between the enzyme and the electrode surface (Fig. 1d). G1 glucose sensors rely on the use of natural oxygen and the generation and detection of hydrogen peroxide, and G2 ones use artificial mediators that shuttle electrons between the flavin adenine dinucleotide (FAD, cofactor of GOx) and the electrode surface. G3 glucose sensors assemble the enzyme on the functionalized electrode permitting a low operating potential which is close to that of the redox potential of the enzyme, therefore, the electron is transferred directly from GOx to the electrode via the active site of the enzyme. Moreover, the low operating potential provides the sensor with a very high selectivity, eliminating the possible interferences from ascorbic acid, uric acid or some other similar-structure compounds (they are also not a concern for saliva samples). In our sensor fabrication, the platinum electrode was first modified with SWNT, then GOx was LBL assembled onto the electrode surface. Thus, when the functionalized electrode was potentiostated positive to the redox potential of GOx, the reduced form of GOx was reoxidized at the electrode while the oxidizing current was measured directly from the functionalized electrode.

3. Results and discussion

3.1. Sensor characterizations

Poly (allylamine) (PAA) was adsorbed onto the Pt electrode surface non-covalently with its protonated amino [36]. SWNT with carboxyl groups at both ends connected PAA and chitosan through covalent bonds. SWNT, represented by the short white lines (about several hundred-nm long), was successfully assembled on the electrode surface (Fig. 2a). Despite some white dots, which were believed to be aggregated SWNTs or small contaminations, the overall coating uniformity was very good. GNp was assembled on CS through electrostatic interaction [37] and was uniformly distributed with minor amounts of aggregation (diameter of GNp is 20 nm) (Fig. 2b). SWNT could be clearly observed underneath the GNp layer, and the dark flocculent areas were potential chitosan molecules connecting SWNT with GNp. Finally GOx was adsorbed onto the surface of GNp through the $-NH_2$ groups; while the negatively-charged characteristics allowed GOx to connect to the polycationic CS by electrostatic adsorption. With one layer of GOx assembled on the electrode surface there were some scattered molecular clusters probably because of insufficient GNp sites for GOx molecules to bind with or space repulsive force between GOx molecules (Fig. 2c). After assembling two more layers of

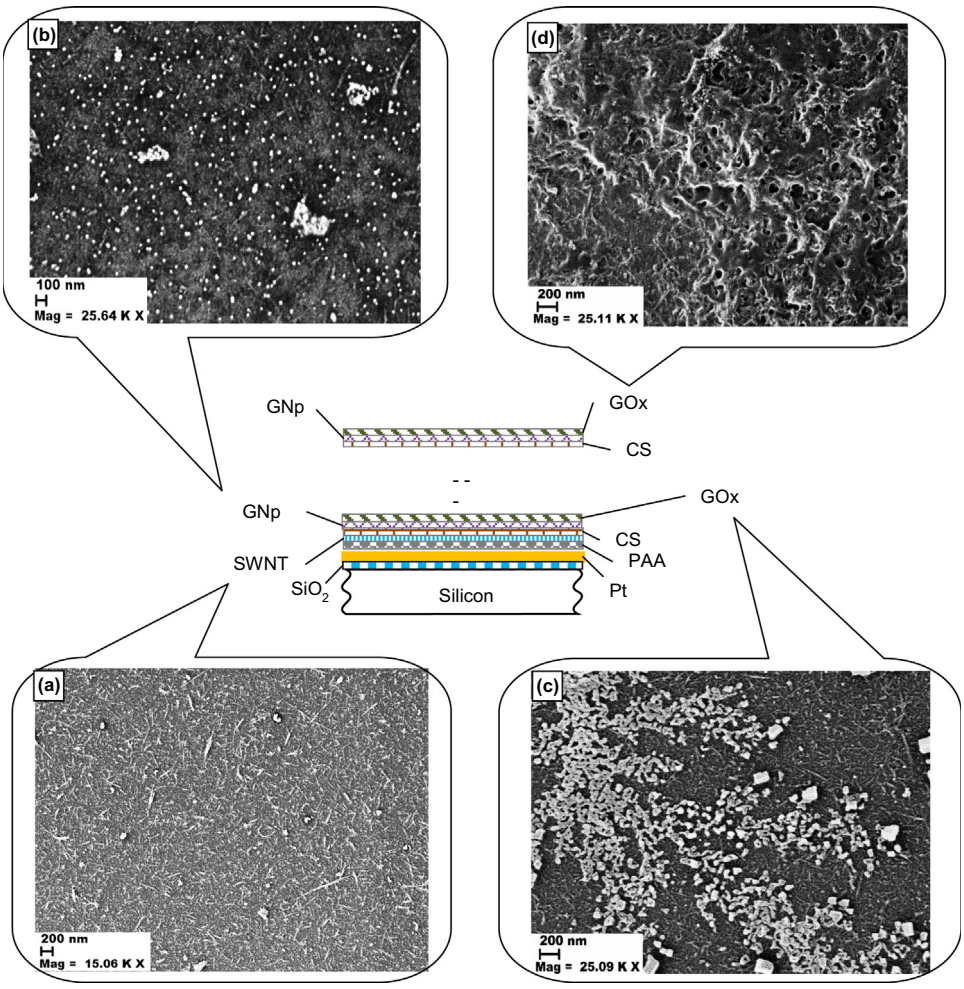

Fig. 2. Cross-section of the functional layers on the sensor electrode and SEM images of (a) one layer of SWNT; (b) one layer of SWNT/GNp; (c) PAA/SWNT/CS/GNp/GOx film; (d) PAA/SWNT/$(CS/GNp/GOx)_3$ film on the sensor electrode surface.

GOx, the accumulated layers have successfully covered the entire reactive area and the pattern was well guided by the SWNT underneath (Fig. 2d). The reason glucose oxidase can be fully assembled on the electrode surface is because each subsequent chemical/-molecule can penetrate through the already formed structure matrix filling in the gap areas where the former layer of glucose oxidase has not reached. Thus, after three layers' coating, glucose oxidase has fully covered the entire reactive area.

Our glucose sensor detects glucose levels by keeping track of the electrons directly transferred through GOx to the electrode surface. The charge transfer complex formed by the functional layers of SWNT–CS–GNp between glucose oxidase and the electrode surface has permitted direct electron transfer between the active center of glucose oxidase and the functionalized electrode. The realization of direct electrical communication of GOx with the electrode surface depends on the distance between the redox-active cofactor (FAD) and the electrode surface. Besides, native GOx alone is unable to transfer electrons to conventional electrode surfaces due to the conformational change of the enzyme required for direct electron transfer which can result in loss of enzymatic activity. In our study, we have sought to establish an appropriate balance between the enzymatic and electrochemical activities which is highly affected by the electrode functionalization materials and enzyme assembly methods. By selecting materials capable of facilitating direct electron transfer, e.g., SWNT, and GNp, and optimizing the enzyme assembly sequence and amount, we have achieved high sensitivity and accuracy of glucose sensing in both PBS solutions and saliva samples.

3.2. Glucose sensing

Cyclic voltammetry (CV) measurements were carried out to detect glucose of different concentrations in PBS buffer solutions (Fig. 3). Each 200 μL sample was dropped onto the sensing area (Fig. 1a). Then the cyclic voltammetry of the functionalized electrode was conducted with the voltage between WE and RE ranging from −0.4 V to 0.4 V at a scan rate of 50 mV/s. A steady-state calibration curve generated from the CV tests at applied potential 0.2 V was shown in Fig. 3b. We did not apply the working potential at which the peak current can be obtained, but instead we selected the working potential at which highest sensitivity and linearity can be reached. Moreover, we used lower potential to eliminate the interference resulted from other chemicals such as ascorbic acid oxidized at high current and to increase the selectivity [38,39]. Current response increased with the elevation of glucose concentration, and tended to reach a saturation value at high glucose concentration where all active sites of GOx were taken up. It agreed with the characteristics of Michaelis–Menten kinetics. The reason our applied potential was different from other research groups is due to the difference in the sensor functionalization method and the reference electrode material (ours is Pt while others is Ag/AgCl or SCE). A linear sensing ability at least over

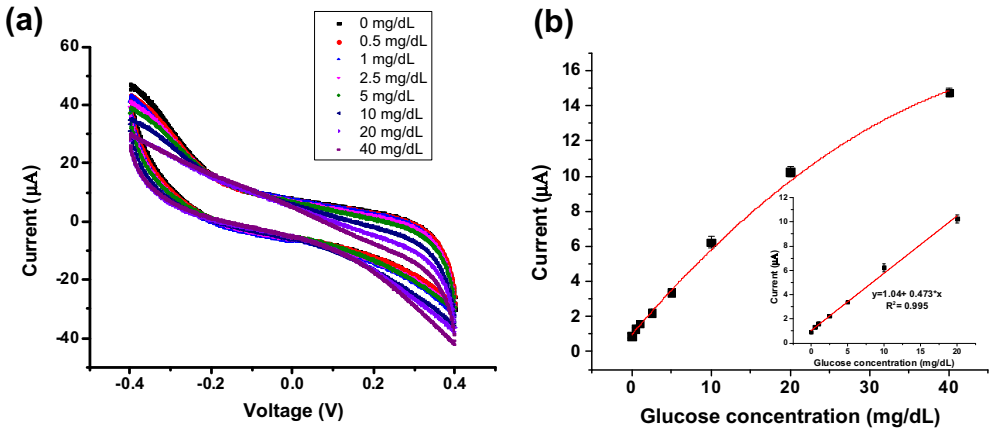

Fig. 3. Glucose sensing using the on-chip electrochemical sensing system S2D2 by, (a) cyclic voltammetry of Pt electrode modified with PAA/SWNT/(CS/GNp/GOx)$_3$ layers obtained in 0–40 mg/dL glucose solutions with the voltage between WE and RE ranges from −0.4 V to 0.4 V at a scan rate of 50 mV/s; (b) steady-state calibration curve of the PAA/SWNT/(CS/GNp/GOx)$_3$ functionalized Pt electrode at applied potential 0.2 V with linear detection range shown in the inset. Error bars = ± standard deviation and n = 3.

the range of 1–20 mg/dL (0.017–1.11 mM) with a sensitivity – 26.6 μA/mM-cm^2 and correlation coefficient of 0.995 was revealed (insert of Fig. 3b).

We compared the amperometric test results at 20 s point and at a time window of 18–21 s (Fig. 4a and b). Four individual sensors were used to measure each glucose concentration. There was much bigger data variance of the current recorded at 20 s point (larger error bars) than that of the integrated current between 18 s and 21 s. It is very likely caused by the infirm connection between sensor and potentiostat, interferences by the motions of the operator or possible data noises. Thus, compared to data at single time point, the integration one demonstrated better repeatability with excellent linearity over 0.5–20 mg/dL. Thus, we continued using current integration at applied potential – 0.2 V as our data analysis method.

Furthermore, we microfabricated another two types of sensing chips with (1) the same configuration but with ¼ size of the original one (system 0.25); (2) a round configuration (system r) (configuration in Table 1) and decorated each with GOx using the same fabrication procedures. The aim was to prove the reproducibility of our fabrication procedures, and more importantly, to accomplish higher sensitivity with much smaller size and less sample volume. The required sample volume has been reduced greatly from 200 μL to 40 μL for system 0.25 devices. The round shape device with a 12 mm^2 reactive area was chosen because one drop of glucose sample is sufficient to cover the whole electrochemical sensing area which makes the test preparation more convenient. The glucose sensing performance of these different sensor systems was summarized in Table 1. Reducing the sensor size can greatly increase the sensitivity due to the increased electron transfer efficiency. System r provided the highest sensitivity for glucose detection. The reason is very likely to be the much higher electron transfer efficiency achieved by the much shorter distances between the working and reference electrodes and between the working and counter electrodes, and the more uniform electron distribution as well.

Beyond detection of glucose in PBS buffer solution, we compared one healthy young subject's blood glucose measured by blood glucose meter and saliva glucose measured by our sensors at fasting state on different days (Fig. 5a). The study was approved by Institutional Review Board (IRB) of Northeastern University Human Subject Research Protection. The subject was required to fast overnight without drinking/eating anything (except water) after 10 pm prior to the test dates. There is a clear constant correlation between blood glucose and saliva glucose at fasting state of this individual on different days. Saliva glucose level as an indication of individual heathy conditions has been studied using other detection methods (Fig. 5b) [10,40–44], and our preliminary result – healthy young subject's fasting saliva glucose at a range of 0.8–1.05 mg/dL is in agreement with it. This can not only proves our sensors effectively and reliably determine glucose levels in saliva, but also reveals a great potential of using saliva analysis for both noninvasive diagnosis of diabetes and glucose monitoring [45].

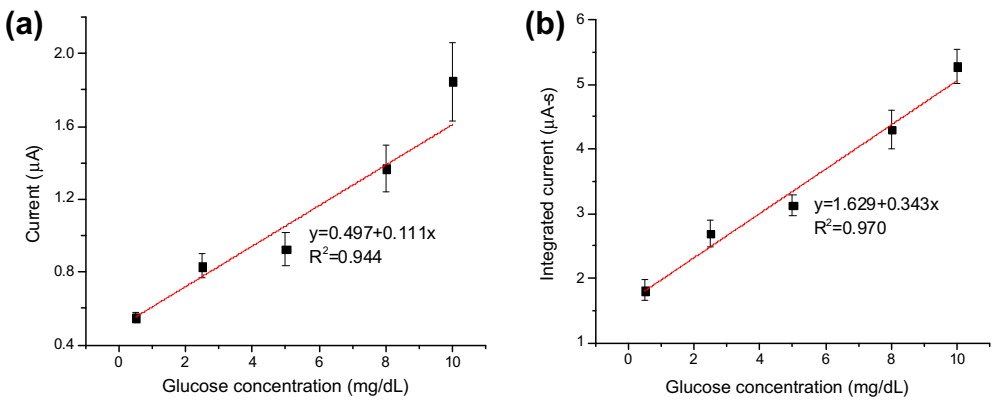

Fig. 4. Amperometric measurements of different-concentration glucose solutions at applied potential 0.2 V with (a) output current at 20 s; (b) integrated current in 18–21 s after data processing. Error bars = ±standard deviation and n = 4.

Table 1
Sensing performance of different sensor configurations.

	Configuration	Size (mm^2)	Reactive area (mm^2)	Sample size (μL)	Detection limit (μM)	Linear detection range (mM)	Sensitivity (μA/mM-cm^2)
System S2D2		200	32	200	11.1	0.017–1.11	26.6
System 0.25		50	8	40	5.6	0.017–0.81	61.4
System r		200	12	100	16.7	0.027–0.56	69.9

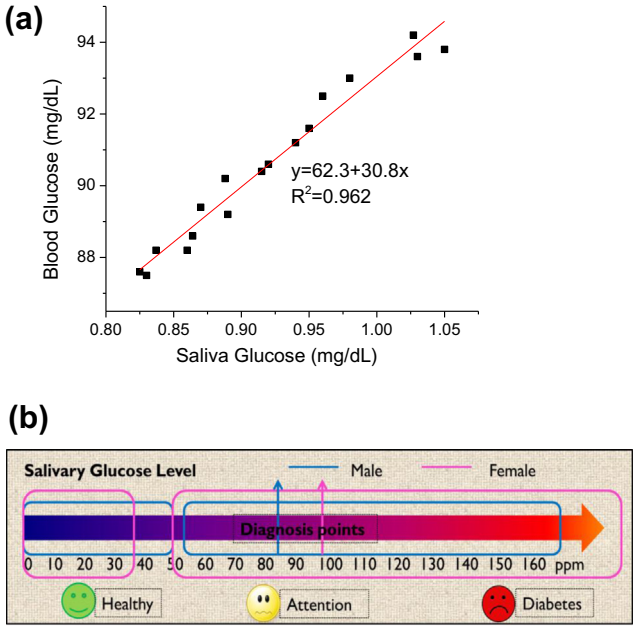

Fig. 5. (a) Correlation between blood glucose and saliva glucose when a healthy subject at fasting state; (b) summarized saliva glucose levels indicating individuals' health conditions from literature.

4. Conclusion

In summary, we have developed a simple and economic on-chip electrochemical sensing system containing at least one working electrode, a counter electrode and a reference electrode. It is currently used as a disposable nano-biosensor for glucose detection with the sensor electrode functionalized with SWNT and multilayered film composed of CS–GNp–GOx. The most important features are: (1) direct electron transfer between GOx and the electrode surface; (2) on-a-chip; (3) glucose detection down to 0.1 mg/dL (5.6 μM); (4) good sensing linearity over 0.017–0.81 mM; (5) high sensitivity (61.4 μA/mM-cm^2) with a small reactive area (8 mm^2); (6) fast response; (7) high reproducibility and repeatability; (8) reliable and accurate saliva glucose detection. It can also be further miniaturized and maintain high reproducibility and repeatability. It is an alternative for real time tracking of glucose levels from body fluids, e.g. saliva, in a noninvasive, pain-free, accurate and continuous way. Furthermore, this on-chip electrochemical system is a platform suitable for on-chip electrochemical sensing of other chemical agents and biomolecules.

Conflict of interest

None declared.

Acknowledgments

The project was supported partially by Keyghobad Ventures LLC under Grant No. 0731102, and our research work was conducted at the Gorge J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University.

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