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RESEARCH PAPER

Design and preparation of open circuit potential biosensor for in vitro and in vivo glucose monitoring

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Abstract A novel open circuit potential biosensor (OCPS) composed of a working electrode and a Ag/AgCl reference electrode was designed for in vivo continuous glucose monitoring in this work. The macroporous carbon derived from kenaf stem (KSC) was used to construct a KSC microelectrode (denoted as KSCME) which was subsequently used to load glucose oxidase (GOD) as the working electrode. The resulting GOD/KSCMEs could catalyze the oxidation of glucose directly to result in changes of the open circuit potential (V_{oc}) of the OCPS. The V_{oc} of OCPS was dependent on the glucose concentration, showing a linear range of 0.03–10.0 mM ($R = 0.999$) with a detection limit of 10 μM. In addition, the OCPS exhibited good selectivity for glucose over other common endogenous interferences. The feasibility of the proposed OCPS for glucose detection in mice skin tumors and normal tissue homogenate samples (in vitro experiment) and rat subcutaneous glucose monitoring (in vivo experiment) was also demonstrated with satisfactory results. The biosensor represents a novel example of a superficial cancer diagnostic device, and the proposed OCPS also provides new ideas for the development of a simple and highly selective device for continuous glucose sensing.

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Introduction

Glucose detection has been crucial in clinical medicine, especially in the diagnosis and monitoring of diabetes [1–6]. Recently, studies have also proved that it could be used to diagnose cancers [7] because tumors typically exhibit a greater reliance on glycolytic metabolism than normal tissues owing to hypoxia and/or inhibited mitochondrial function [8, 9]. Therefore, frequent monitoring of glucose is essential for optimal management of diabetes and avoiding its associated problems or monitoring tumors.

Many techniques have been used for monitoring glucose levels, such as near-infrared spectrophotometry [6], colorimetry [7], surface plasmon resonance [8], electrochemiluminescence [9], fluorescence [10], and electrochemistry [11–13]. Electrochemical biosensors based on biomolecules immobilized on an electrode surface are promising tools owing to their high sensitivity, good selectivity, compatibility with miniaturization, easy operation, and low cost. However, with conventional three-electrode systems, a serious problem is that many substances can undergo redox reactions under the applied voltage, which will cause great interference, especially in biological samples for in vitro and in vivo analysis.

Faced with these problems, we designed an open circuit potential biosensor (OCPS) which consists of a working electrode and a reference electrode to detect glucose according to the open circuit potential (V_{oc}) changes. The proposed OCPS has many features and benefits. First, the working electrode is made of environmentally friendly nitrogen-containing kenaf stem-derived macroporous carbon (KSC) [14, 15] which has good biological compatibility and three-dimensional (3D)

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macroporous structure. Second, in contrast to amperometric sensors, no voltage is applied to the electrodes, and all redox reactions are carried out under an extremely low V_{oc}. Thus, there might be very little interference by some contaminants. Third, it only needs one electrode to be modified, so it has better reproducibility because of the simplified modification process. Fourth, the system is self-powered by biological fluids and does not require an external energy supply; therefore, it may be miniaturized and suitable for in vivo analysis.

In the present study, we systematically studied the performance of OCPS for both in vitro and in vivo glucose detection. In the in vitro experiments, the concentrations of glucose in rat superficial tumor and normal tissue homogenates were measured by the OCPS. Simultaneously, a glucose (HK) assay kit was used to verify the accuracy of the measurements. To the best of our knowledge, this is the first report on tumor homogenate glucose detection, and we prove that the method of cancer diagnosis with the OCPS is achievable. In the in vivo study, the OCPS was used to record the changes of rat interstitial fluid (ISF) glucose over a long time. This not only proved the feasibility of the potentiometric sensor for in vivo analysis but also provided a new way of thinking about highly selective in vivo analysis, not just for glucose monitoring.

Experimental

Reagents, materials, and animals

The kenaf stem (KS) was provided by Futian farm (Ji'an, China). Paraffin liquid and graphite powder (99.95 %, 325 mesh) were purchased from Aladdin. Glucose (HK) assay kit (product no. GAHK20-1KT) and glucose oxidase (GOD, 140 units mg^{-1}) were obtained from Sigma–Aldrich. A 0.2 M phosphate buffer solution (PBS, pH 7.0) consisting of NaH_2PO_4 and Na_2HPO_4 was employed as the supporting electrolyte, and GOD solution (10 mg mL^{-1}) was prepared by using 0.2 M PBS (pH 7.0). All other chemicals were of analytical grade, and all solutions were prepared with ultrapure water (18.2 MΩ cm) purified by a Millipore-Q system. The malignant melanoma cells (A375) were obtained from the Shanghai cell bank of the Chinese Academy of Sciences (Shanghai, China). Male pathogen-free Wistar rats ($n = 21$, 200–220 g) and Kunming (KM) mice (weighing 18–22 g) were provided by Vital River Laboratory Animal Technology (Beijing, China). These animals were housed under controlled conditions (22 ± 2 °C, relative humidity (RH) 50 ± 20 %) with a natural light–dark cycle for 3 days before the experiments were carried out. Animal experiments were performed in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China.

Instruments

The morphology of the KSCs was observed by transmission electron microscopy (TEM, JEM-2100, Japan) and scanning electron microscopy (SEM). The SEM analysis was carried out using an XL30 ESEM-FEG SEM at an accelerating voltage of 20 kV equipped with a Phoenix energy dispersive X-ray analyzer (EDXA). All electrochemical experiments were performed on a CHI 750D electrochemical workstation (Shanghai, China). A two-electrode configuration was used with a Ag/ AgCl (saturated KCl) microelectrode (0.2 mm) as the reference electrode, and the bare or modified KSC paste microelectrodes (denoted as KSCMEs) as the working electrode. All *V*oc values in this work were recorded relative to the Ag/AgCl microelectrode. UV–vis absorption spectra were performed on a Lambda 35 UV–vis spectrophotometer.

Preparation of capillary filled KSCMEs

The KSC was prepared as reported in our earlier work [15]. The KSC materials were ground into powder and then mixed with paraffin liquid thoroughly using a mortar and pestle to prepare the KSC paste. The paste was then kept under vacuum (rotary pump) for 2 h to remove any adsorbed gases. It was then stored in a vial and used to make KSCMEs. Borosilicate glass capillaries (outer diameter 1.5 mm, inner diameter 0.75 mm) were pulled with a laser puller. The capillary was filled with KSC paste using a standard glass syringe from the backside of the capillary. A copper wire was inserted into the back of the capillary tube for electrical contact. The KSCME was then cleaned for 30 min in ethanol and ultrapure water, respectively. In this work, we used the soaking time of KSCME in GOD solution to optimize the response of the GOD/KSCME toward glucose. The potential responses of the KSCMEs immersed in 10 mg mL^{-1} GOD solution for 1 h, 3 h, 5 h, 8 h, 10 h, 12 h, 16 h, 20 h, and 24 h were investigated. As shown in Fig. S1 (Electronic Supplementary Material, ESM), the potential response of GOD/KSCME toward 1 mM glucose solution reaches the maximum value at 10 h. Therefore, the KSCMEs were immersed into 10 mg mL^{-1} GOD solution (pH 7.0) for 10 h at 4 °C to prepare the final electrode. The GOD/KSCME was rinsed with ultrapure water and stored at 4 °C for further use. The rinsing of ultrapure water was necessary to remove any loosely bound molecules after each modification step. For control tests, the capillary filled carbon paste MEs (CPMEs) were also prepared by similar procedures instead of KSC paste. The fabrication procedures of the KSCPMEs are illustrated in Fig. 1.

In vitro detection

The feasibility of the OCPS for glucose detection was evaluated in different mice tissue homogenate samples (mouse skin

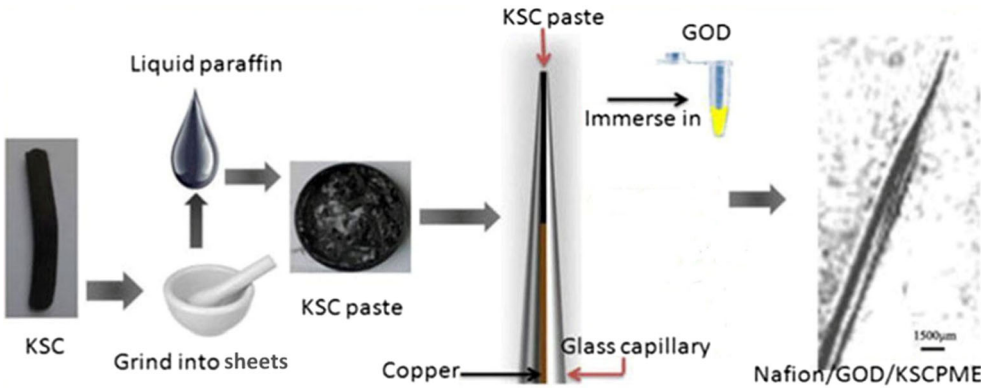

Fig. 1 Schematic illustration of the fabrication of the GOD/KSCME

tumor tissues and normal mouse subcutaneous tissues). All experimental animals were KM mice. After mice were inoculated with the malignant melanoma cells (A375) for 7 days, they were killed by cervical dislocation; the ascites was extracted under aseptic conditions, and the concentration of tumor cells was adjusted to 2×10^7/mL with sterile saline. Each mouse was inoculated subcutaneously with 0.2 mL of the tumor cell suspension under their right armpit after sterilization. Then, the inoculated mice were divided into two groups: the melanoma group and the treatment melanoma group. After the treatment melanoma group was inoculated with tumor cells for 24 h, 5-fluorouracil was given at a dosage of 20 mg/kg with intraperitoneal injection every other day over a period of 14 days. After that, the two groups and the corresponding number of normal mice were killed by cervical dislocation. The inoculated mice tumor tissues and normal mice subcutaneous tissues were removed, washed to remove residual blood, air-dried, and weighed. All tissue samples were homogenized with 0.2 M PBS (pH 7.0) (twice the tissue weight) and immediately used for the next analysis test. The results of glucose in different mice tissue homogenates determined by the developed method were compared with those obtained by the glucose (HK) assay kit (GAHK20-1KT). The detailed steps are illustrated in Fig. 3a and the results are listed in Table S1 (ESM).

In vivo detection

The rats were fasted overnight (12 h) with free access to water before the experiment. They were anesthetized with chloral hydrate (initial dose of 300 mg kg^{-1}, i.p.) with additional doses of 50 mg kg^{-1} (i.p.) as needed to maintain anesthesia and wrapped in a homeothermic blanket. Then, the GOD/KSCME was implanted under the skin of the neck, while another Ag/AgCl reference electrode was located at about 5 mm away from the working electrode and implanted under the skin. During this study, a 0.5-h time point was selected to make a glucose injection to observe the change of rat subcutaneous glucose levels. The online electrochemical detecting systems for in vivo monitoring are illustrated in Fig. 4a.

Results and discussion

Characterization of KSCs, KSC paste, and GOD/KSCMEs

SEM and TEM techniques were used to characterize the morphologies of the KSCs, KSC paste, and GOD/KSC paste. Detailed SEM and TEM images are shown in Fig. S2 (ESM). Figures S2A–D (ESM) reveal the hierarchically macroporous structure of KSCs. Figures S2E–G (ESM) show a SEM image (Fig. S2E, ESM) and TEM images of the KSC paste (Fig. S2F, G; ESM), indicating many pores ranging from 2.5 μm to 20 nm in the sheets of milled KSCs. The porous structure provided a large surface area for GOD immobilization. Figure S2H (ESM) shows the SEM image of the GOD/KSCMEs, in which the sheet-like milled KSCs dispersed on the microelectrode surface to form 3D structure. Figure S3 (ESM) shows UV–vis spectra of GOD (curve a), GOD/KSC (curve b), and KSC sheets (curve c) in a solution. The GOD shows two obvious characteristic absorption bands at about 365 and 460 nm (curve a) [16]. No characteristic absorption bands were observed for KSC (curve c). After the GOD and KSC were mixed, two characteristic adsorption bands of GOD were observed at 365 and 460 nm (curve b), suggesting that GOD/KSC still retains the integrity of the GOD biological structure and activity.

Probable mechanism of glucose measurement by OCPS

GOD, the classic biocatalyst of glucose, has a high selectivity toward the oxidation of glucose [17–21]. In the presence of GOD, O_2 could be converted into H_2O_2 accompanied by the oxidation of glucose into gluconic acid as shown by Eqs. 1–4 [5]. Meanwhile, H_2O_2 could receive electrons to be reduced into H_2O on the electrode surface (Fig. 2a). Accordingly the potential of the modified electrode changed with increasing glucose concentration, which resulted in the increasing of the *Voc* of OCPS (Fig. 2b). Therefore, a novel OCPS for the detection of glucose could be developed, and the electrochemical

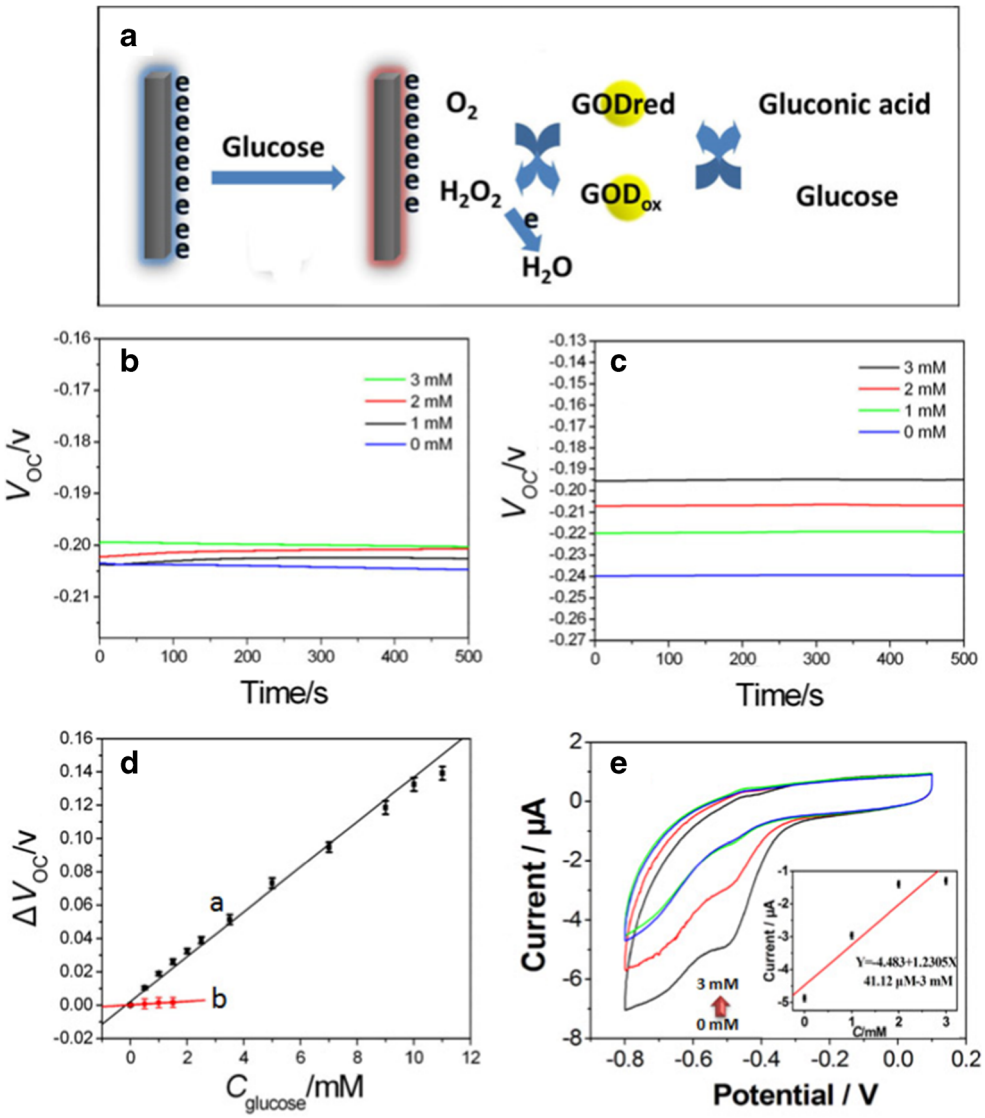

Fig. 2 (**a**) Configuration and bioelectrocatalysis mechanism of the OCPS. *Voc-t* curves obtained at (**b**) GOD/CPME and (**c**) GOD/KSCME in the absence and presence of 1.0, 2.0, and 3.0 mM glucose. (**d**) Linear relationship between ΔVoc and the glucose concentrations for (**a**) GOD/KSCME and (**b**) GOD/CPME. (**e**) CVs of the GOD/KSCME in 0.2 M O_2-saturated PBS (pH 7.0) in the presence of glucose; *inset* relationship between glucose concentration and current signal for GOD/KSCME

response mechanism of the OCPS is also illustrated in Fig. 2a.

$$\text{Glucose} + \text{GOD (FAD)} \rightarrow \text{Gluconolactone} + \text{GOD (FADH}_2\text{)} \quad (1)$$

$$\text{GOD (FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOD (FAD)} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e} \rightarrow 2\text{H}_2\text{O} \quad (3)$$

In total:

$$\text{Glucose} + \text{O}_2 + 2\text{H}^+ + 2\text{e} \xrightarrow{\text{GOD}} \text{gluconolactone} + 2\text{H}_2\text{O} \quad (4)$$

In order to investigate the possibility of glucose detection using the proposed OCPS and verify the merits of the electrode material, two kinds of working electrodes (GOD/CPMEs and GOD/KSCMEs) were prepared, and four different concentrations (0, 1.0, 2.0, and 3.0 mM) of glucose were introduced into the electrolyte solution for testing. As observed from the *Voc–t* curves in Fig. 2b and c, the *Voc* of the two aforementioned electrodes increased with the addition of glucose, which proved the validity of the proposed mechanism. As shown in Fig. S4 (ESM), the open circuit potential changed from 0 to 30 s. In order to ensure the stability of experimental results, the measurement was carried out after the glucose was added for 60 s. Meanwhile, the *Voc–t* curves of GOD/KSCME (Fig. 2c) showed a more obvious increase than that of GOD/CPME (Fig. 2b). The result could be ascribed to the fact that GOD could not be well immobilized on the bare CPME's surface, and the GOD molecules might came off when the electrode was immersed into the solution. Simultaneously, it was proven that KSC sheets had better electrocatalytic properties than carbon paste because of the porous structure. Therefore, with the addition of glucose, the significant increase of *Voc* on the GOD/KSCME might be attributed not only to GOD but also to the inherent characteristics

of KSCs such as good protein adsorption, large specific surface area, perfect conductivity, etc.

Analytical performance of the developed OCPS

Further analytical study was carried out via *Voc*–*t* curves to confirm the feasibility of quantitative determination of glucose. First, the presented OCPS (GOD/KSCME as a working electrode) was immersed in different concentrations of glucose in 0.2 M PBS (pH 7.0), and the corresponding *Voc*–*t* curves were recorded in Figs. S5 and S6 (ESM). The plot of ΔVoc versus glucose concentration is shown in Fig. 2d (curve a). There is a linear relationship between ΔVoc and glucose concentration from 0.03 to 10 mM ($R = 0.999$) with a detection limit of 10 μM. As compared with GOD/CPME (Fig. 2d, curve b) and with the result from cyclic voltammetry (Fig. 2e), it had a better sensitivity and a wider linear range. In addition, the concentration range was greater than the range of normal human blood glucose level. The reproducibility of the presented OCPS was also evaluated, and the relative standard deviation (RSD) for five repeated measurements of 1.0 mM glucose was estimated to be about 2.7 %. When the OCPS was stored at 4 °C for 15 days, the *Voc* responses in 0.2 M PBS (pH 7.0) and tissue homogenate to 2.0 mM glucose remained at 96.9 % and 95.8 % of their original values, suggesting the long-term stability of the electrode (Figs. S7 and S8, ESM).

Solutions containing five kinds of potential interfering endogenous substances (i.e., uric acid (UA), ascorbic acid (AA), sucrose, fructose, and galactose) were then tested under the same conditions to estimate the selectivity of the presented OCPS. The *Voc* of the OCPS significantly increased in response to 5.0 mM glucose, while 25.0 mM of UA, AA, sucrose, fructose, and galactose made no obvious change to the *Voc* of the OCPS (Fig. S9, ESM). The result suggested that the OCPS showed good selectivity against other relevant chemicals, which is a promising feature regarding the possibility of its application in real samples. There might be two main reasons why it had such good selectivity. First, the catalytic oxidation of glucose by GOD showed very good specificity. Second, unlike amperometric sensors, no voltage was applied to the electrodes in our system, and the redox reaction was carried out under *Voc*. Thus, there was very little interference by these contaminants.

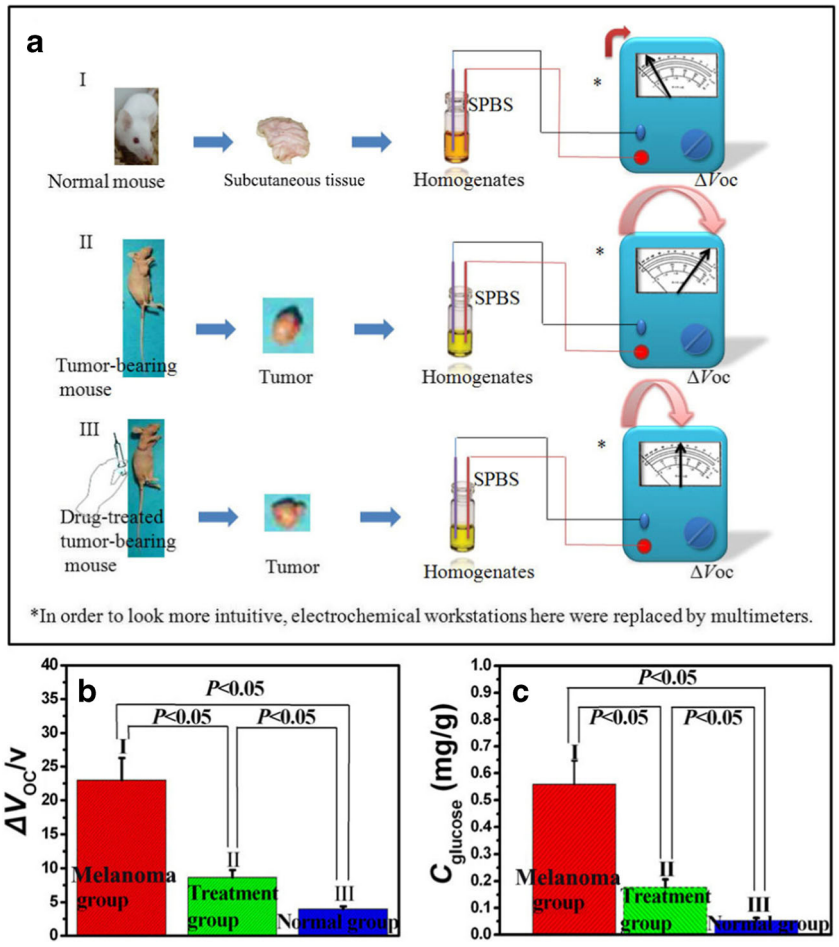

Fig. 3 (**a**) Schematic of the stepwise in vitro experiment process. (**b**) Normalized ΔVoc of the OCPS in response to glucose contents in mice melanoma group tumor tissues, treatment melanoma group tumor tissues, and normal group liver tissues homogenates. (**c**) Glucose contents in the three tissues measured by the OCPS

In vitro measurement of glucose in mouse tissue homogenates

The feasibility of the OCPS for glucose detection was evaluated in different mice tissue homogenate samples (mouse skin tumor tissues and normal mouse subcutaneous tissues) (Fig. 3a). The results were compared with the glucose (HK) assay kit, which is the classical analytical method for glucose in biological samples. Table S1 (ESM) shows that the amount of glucose in tumor tissues and normal tissues could be deduced from the *V*oc changes of the OCPS. Also, the concentrations of glucose in different homogenates determined by the OCPS were in good agreement with those estimated by the glucose (HK) assay kit. There is no doubt about the good performance of the glucose (HK) assay kit for detection of glucose; however, compared to OCPS, the commercial assay's reagents are expensive and the operation is cumbersome. Therefore, the comparison suggested that the OCPS based on the combination of KSC and GOD opens up a reliable and simple approach for monitoring biological active substances in vitro. Figure 3b, c show that the different glucose contents could be represented by the apparent changes ($p < 0.05$) in electrical potentials. Figure 3d also shows that the glucose levels in the melanoma group and the treatment melanoma group tumor tissues were nearly 10-fold ($p < 0.05$) and 3-fold ($p < 0.05$) those in the normal mice subcutaneous tissues, respectively. This phenomenon might be attributed to the fact that tumors had a greater reliance on anaerobic glycolysis for energy production than normal tissues [7]. The glucose in tumor cells would be metabolized quickly [22–24], but the concentration of glucose in tumor tissue extracellular fluid should be higher than that in normal tissue extracellular fluid because the glucose transmembrane transport by transporter protein was along the concentration gradient [25–27], i.e., both sides of the tumor cell membrane should have a large glucose concentration difference. This key discrimination of tumors from normal tissues might allow glucose to become a tumor marker for cancer diagnosis. Figure 3c also shows that after the administration of 5-fluorouracil to the tumor-bearing mice, the glucose levels of tumor tissues decreased significantly. The mechanism of the reduced drug-induced glucose demand might need to be studied further, once it is confirmed that the phenomenon was related to the damage of cancer cells. The result suggested that it was possible to determine the efficacy of the antineoplastics quickly by the OCPS. Thus, the OCPS might be a potential method for screening drugs in the future. Furthermore, owing to no power supply being required, the OCPS could also be used in a live body superficial tumor diagnosis through a multimeter.

In vivo monitoring of rat subcutaneous glucose

The excellent in vitro analytical performance of the presented OCPS in biological samples was also extended to provide a reliable platform for in vivo assay of rat subcutaneous glucose. In this work, the *V*oc of the OCPS strapped to the rat's body

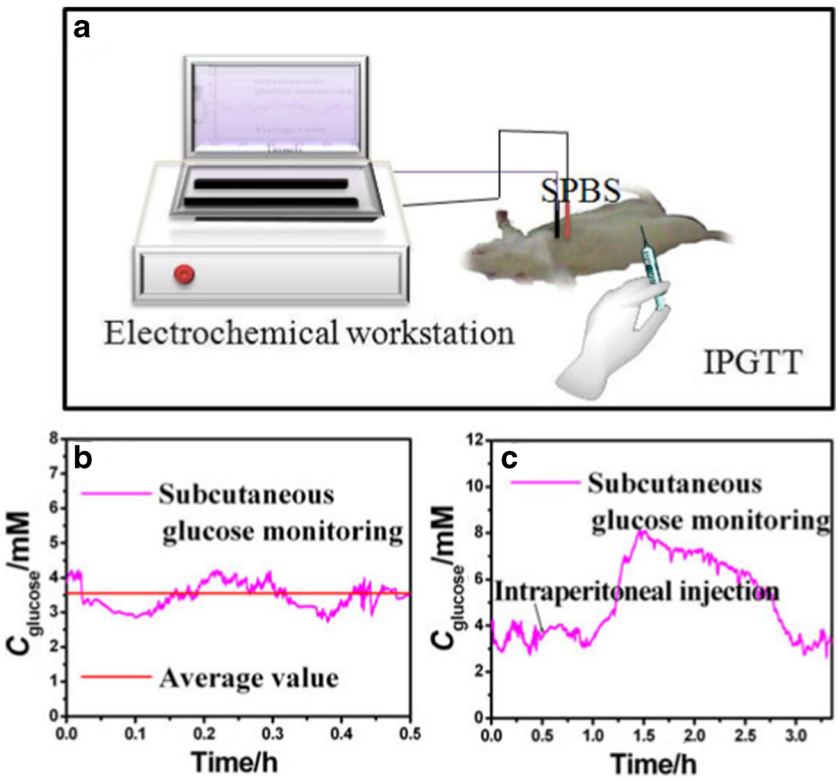

Fig. 4 (**a**) Schematic of the in vivo glucose monitoring and intraperitoneal glucose tolerance test (IPGTT). (**b**) The rat subcutaneous glucose concentrations from the potential responses continuously recorded (0–0.5 h) with the online electrochemical detecting systems with the OCPS. (**c**) The rat subcutaneous glucose concentrations from the potential responses continuously recorded (0–3.0 h) with the online electrochemical detecting systems with the OCPS; *arrow* indicates the time of the intraperitoneal injection of glucose

was continuously recorded by an electrochemical workstation (Fig. 4a). During the first 0.5 h after the implantation of the OCPS, the glucose concentrations could be found to fluctuate around 3.5 mM within a narrow range (2.8–4.2 mM) as shown in Fig. 4b; these results were consistent with the normal range of glucose in rats [28–30]. At the time point of 0.5 h after the implantation of the OCPS, a glucose solution whose concentration was 50.0 g mL^{-1} was intraperitoneally injected into the anesthetized rat (the amount injected was 2.0 g of glucose solution per 1.0 kg of body weight). From the continuous recording chart (Fig. 4c), a glucose concentration peak could be observed as a result of the absorption of glucose. One hour after the injection, this peak gradually decreased over the next 1.5 h, because of the gradual metabolism of glucose. The whole process and results proved the sensitivity and feasibility of the OCPS in glucose detection. According to the mechanism discussed above, the local oxygen concentration might be lower in cancerous tissue owing to its consumption, but the concentration will not change within the measuring time, which ensured the in vivo monitoring of rat subcutaneous glucose.

Conclusions

A simple and efficient OCPS composed of a GOD/KSCME and a Ag/AgCl (saturated KCl) reference electrode was developed for monitoring glucose. The OCPS could convert the glucose concentration into the change of *V*oc without external energy supply. The results of in vitro analysis of glucose in different homogenates proved that glucose could be a tumor marker, and the OCPS combined with a multimeter might have some practical significance in preliminary investigations of malignancies without constraints of time and without the need for a power supply. The good biocompatibility of OCPS the in vivo monitoring of glucose without an external power supply. Compared with other methods such as near-infrared spectrophotometry, colorimetry, surface plasmon resonance, electrochemiluminescence, fluorescence, glucose (HK) assay kit, etc., the OCPS has advantages of low cost, easy operation, good biological compatibility, and high selectivity. In particular, in analysis of biological samples and in vivo, the highly selective OPCS had obvious advantages, and it could eliminate the interference from a variety of components in organisms. In addition, this is the first time that OCPS has been used for in vivo analysis. Moreover, it provides a new way of thinking about highly selective in vivo analysis, not just for glucose monitoring.

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Compliance with ethical standards All experiments involving animals were in accordance with the guidelines of the National Institute of Food and Drug, Nanchang, China, and approved by the institutional ethical committee (IEC) of Jiangxi University of Traditional Chinese Medicine. This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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