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Highly sensitive and stable zwitterionic poly(sulfobetaine 3,4-ethylenedioxythiophene) (PSBEDOT) glucose biosensor

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KEYWORDS: glucose sensor, anti-fouling, zwitterionic materials, poly(sulfobetaine 3,4-ethylenedioxythiophene)

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Abstract

A zwitterionic poly(sulfobetaine 3,4-ethylenedioxythiophene) (PSBEDOT) based glucose biosensor was fabricated via encapsulating glucose oxidase (GOx) in a one-step electro-polymerization method. Integrating conductivity and hydrophilic properties, PSBEDOT provided a great framework for GOx immobilization and stabilization. The anti-fouling, high-sensitivity, and long-term stable properties of the PSBEDOT-GOx make it a promising platform for long-term and continuous glucose monitoring.

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Determination of glucose concentration is important in many fields, including food industry,[1, 2] environmental monitoring,[2] biotech industry, and clinical applications,[3, 4] especially for diabetes which is one of the leading causes of death and disability in the world. Due to the sustained prevalence of diabetes, significant efforts have been devoted to developing a facile processed, stable, low cost, and accurate glucose biosensor. Electrochemical enzymatic biosensor, especially the amperometric method,[5, 6] is a prevalent method for glucose detection due to its high sensitivity and selectivity. Numerous electrochemical enzymatic biosensors have been made by directly immobilizing oxidoreductases and mediators on several types of electrode surfaces; however, they usually suffer from an unstable response due to a poor electron transfer between the electrode and the enzyme[5] and also due to the fast loss of enzyme activity.[6] Recently, conducting polymers have been utilized as an enzyme-immobilization matrix since it provides 3-dimentional electrically conducting network and thus leads to rapid electron transfer to the electrode surface.[7] While there are several ways of immobilizing enzymes in a conducting polymer matrix, entrapping the enzymes simultaneously during electro-polymerization of monomer in a single step process is a more attractive method due to its simplicity and its ability to control of the film thickness and enzyme loading. Several types of conducting polymers, including polyaniline (PANi),[8-11] polypyrrole (PPy),[12-14] and polythiophene[15, 16] have been deposited electrochemically with glucose oxidase (GOx) onto metal electrodes to construct the glucose biosensor probes. Among conducting polymers, poly(3,4-ethylenedioxythiophene) (PEDOT) has attracted significant attention since it offers high stability,[17] ability to be electropolymerized with low oxidation potential,[18] even in aqueous medium,[19] and excellent conductivity.[20] Po-Chin Nien *et al* reported an amperometric glucose biosensor that entrap GOx in PEDOT film in a single electro-polymerization step.[21] With ferrocene as a mediator, the current retains 80% of the original one after 18 days, but no storage

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condition was provided in the paper. Michael A. Invernale *et al* also fabricated a microneedle electrode for glucose sensing with the same single-step electro-polymerization method,[22] which shows the potential for painless sampling. However, it only showed good stability with 95% activity within 7 days when stored either in air or in PBS. The most common and severe problems for current enzymatic glucose biosensor are the insufficient long-term stability attribute to the intrinsic nature of the enzyme and relatively low sensitivity in complex biological fluids, such as human blood, due to the non-specific protein adsorption and cell attachment, originating from hydrophobic or charged surface of conventional conducting polymers.

A continuous glucose sensor with long stability, high specificity, and high selectivity, which can provide a reliable monitoring under complex conditions, is urgently needed for diabetic management. Recently, many studies have demonstrated that zwitterionic materials can effectively resist cell attachment and protein adsorption and thus are suitable for complex media applications,[23-26] such as a biosensor for blood glucose monitor[27-30]. Wei Yang *et al.* reported zwitterionic poly(carboxybetaine methacrylate) (PCBMA) hydrogels on glucose sensor surfaces shows an ultra-low fouling background to maintain a highly stable response even in a complex medium such as human blood serum.[31] Also, PCBMA can improve stability with the retention of binding affinity or bioactivity of PCBMA-trypsin conjugate.[32] Although PCBMA based glucose sensor has great improvement in blood glucose monitor, it is still limited by several issues: (1) most current zwitterionic materials only offers good biocompatibility for biosensor but sacrifices the electrochemical property for the conductive substrate due to the non-conductive polymer backbone;[33-35] (2) the fabrication of glucose biosensor is complicated and time consuming since the conductive component generally needs to mix or covalently bind with the biocompatible but non-conductive moieties. To address the issues of non-conductive zwitterionic polymers, Bin Cao

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and his colleagues developed two zwitterionic conjugated polymers: poly(sulfobetaine 3,4-ethylenedioxythiophene) (PSBEDOT) and poly(carboxybetaine thiophene) (PCBTh). PSBEDOT and PCBTh can conduct ions, provide excellent antifouling properties via zwitterionic side chains, and conduct electrons via conjugated backbones.[36, 37] Both polymers show a great potential for electrochemical biosensing.

We hypothesized that zwitterionic conjugated polymers can provide a more suitable environment to stabilize the enzyme due to its hydrophilicity and hydration nature and prevent biofouling. Herein, as the scheme 1 showed, we utilized PSBEDOT, which integrate both the conducting and antifouling properties,[36] as a matrix for entrapping GOx onto a platinum electrode in a single electro-polymerization step. The PSBEDOT-GOx electrode was expected to carry several desired properties for an enzyme-based electrochemical biosensor, including long stability, high selectivity and sensitivity, short response time, accurate measurement, stable response in complex medium due to superior anti-fouling properties, and enhanced electrical conductivity within one-step facile process. The surface morphology and roughness, protein adsorption, response time, chronoamperometry properties, and stability in dry condition, PBS, and human blood plasma of the PSBEDOT glucose biosensor were systematically investigated in this work.

PSBEDOT-GOx electrode was conveniently synthesized via a one-pot electro-polymerization process. SBEDOT monomers were electro-polymerized at the interface of a Pt electrode and the SBEDOT solution that also contains 1 mg/mL GOx. GOx was encapsulated in PSBEDOT network, when PSBEDOT deposit on the Pt electrode surface. After the 30s of polymerization, a uniform, dark-blue film as the PSBEDOT-GOx electrode was formed on the Pt electrode. Electro-deposition of highly water-soluble monomers has been a challenged issue. Firstly, water soluble monomers are very limited. Secondly, the produced polymers or oligomers cannot deposit on the surface due to

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their high solubility in aqueous phase. Unique properties of PSBEDOT includes the high solubility of the monomer in water and the ease of deposition of the polymer. Zwitterionic sulfobetaine side chain significantly increases the solubility of the monomer and electrostatic interactions among sulfobetaine sidechains of PSBEDOT lead to the deposition of the polymer on the electrodes. Although the unmodified 3,4-ethylenedioxythiophene (EDOT) has been used for numerous biomedical applications because of the water solubility of EDOT monomer and the hydrophilicity of PEDOT, the solubility of EDOT in water is not high. At the concentration of 80 mM, it will take over 12 hours to partially dissolve EDOT. These properties of SBEDOT allow people to use environmental-friendly aqueous-base process and provide the freedom to further optimize the sensors.

PSBEDOT functions as a three-dimensional conductive polymer matrix for GOx encapsulation and as well as an electrical mediator for transducing the signal produced via redox reaction of glucose. In any polymeric GOx-based glucose biosensor, glucose needs to diffuse through the polymer matrix and bind to GOx, which converts the glucose to D-glucono-1,5-lactone and produces hydrogen peroxide. In PSBEDOT-GOx sensor, hydrogen peroxide was further oxidized at the platinum surface at +0.56V potential relative to Ag/AgCl/Sat'd KCl. The current due to the oxidation of hydrogen peroxide was detected and can be related to the concentration of glucose. In this process, the retention of GOx bioactivity plays a key role for the behavior of the enzyme-based glucose sensor. It has been reported that the activity of immobilized enzyme depends upon surface area, porosity, roughness, hydrophilic property of conducting polymer matrix, and electro-polymerization condition.[2] Hence, the immobilization of the enzyme, surface morphology, and roughness of the PSBEDOT-GOx electrode were firstly characterized by fluorescence microscope, scanning electron microscope (SEM), and surface profiler in our study. PEDOT-GOx

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electrode was prepared under the same electro-polymerization condition as a reference. To confirm the encapsulation of the enzyme, GOx was labeled with fluorescein isothiocyanate (FITC) as a tracer. As shown in Fig. s1(a), bare PSBEDOT films showed very low fluorescent signal under the GFP channel. PSBEDOT-GOx surface showed strong fluorescent signal, indicating the immobilization of FITC-labeled GOx on the surface of the PSBEDOT electrode (Fig. s1(b)). The surface morphology and roughness of PSBEDOT-GOx electrode surface were then characterized using SEM and surface profiler, respectively. After GOx was encapsulated by PSBEDOT, the topography of the surface was changed considerably, and the PSBEDOT-GOx surfaces showed quite smooth morphology due to the loading of GOx (Fig. s2(a,b)). SEM results were consistent with the fluorescence microscope results. From Table 1, the surface roughness measurement also indicated a smoother and thinner surface after GOx encapsulation. The root mean squared of surface roughness (Rq) decreased from 353.21 ± 17.07 nm (PSBEDOT) to 306.19 ± 38.92 nm (PSBEDOT-GOx) and the thickness decreased from 1.36 ± 0.06 μm (PSBEDOT) to 0.74 ± 0.07 μm (PSBEDOT-GOx). As shown in Fig. s2(b), PSBEDOT-GOx surfaces are rougher and thinner (Rq = 306.19 ± 38.92 nm, T = 0.74 ± 0.07 μm from Table 1) when compared to PEDOT-GOx surfaces (Fig. 2(d); Rq = 255.79 ± 18.99 nm, T = 0.94 ± 0.09 μm from Table s1). The rougher surface provides a higher surface area to load GOx. The thinner surface reduces the distance for better mass transport for glucose to diffuse to GOx and for hydrogen peroxide to reach Pt surface,[2] thus increasing the sensor sensitivity and reducing response time.[38] Also, the pores on the PSBEDOT-GOx film were more compact than PEDOT-GOx surface, which may have reduced the leaching of the enzyme from the surface.

Non-specific protein adsorption remains a major barrier to the development of stable and sensitive glucose biosensor, especially for continuous glucose monitoring, since non-specific protein

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adsorption increases noise-signal ratio, leads to enzyme-activity loss, and blocks the electrode surface.[26, 39, 40] In this study, the amount of protein adsorbed on PSBEDOT-GOx surface was quantified by electrochemical quartz crystal microbalance (eQCM). The protein adsorption of PEDOT-GOx surface was also evaluated. During the protein adsorption experiments, the frequency change of the oscillating quartz crystal was monitored over time. As showed in Fig. 1(a), for fibrinogen adsorption, the frequency shifted about ~25 Hz for PSBEDOT-GOx surface after the flow of protein solution and PBS that washed away loosely adsorbed protein. Fibrinogen adsorption on PSBEDOT-GOx surface was 7.5% compared to that on PEDOT-GOx surface (Δf = 335 Hz). For 100% human blood plasma adsorption (Fig. 1(b)), the frequency shift increased to 1100 Hz for PSBEDOT-GOx surface, which is 8.4% compared to that of PEDOT-GOx surface (Δf = 13000 Hz). The results indicated that GOx encapsulation did not compromise the anti-fouling property of PSBEDOT surface,[36] and it still showed excellent protein resistance both for fibrinogen and 100% blood plasma compared to PEDOT-GOx surface. Our results suggest that PSBEDOT-GOx electrode has significant advantages for complex medium or *in-vivo* applications because of its anti-fouling property.

The amperometric response of the PSBEDOT-GOx glucose biosensor to the successive addition of glucose (from 0.1 mM to 50 mM) with a sampling time of 60 s at each concentration, was measured (Fig. 2). PEDOT-GOx glucose biosensor was also tested under the same condition. Three electrodes were prepared and studied for each type of glucose biosensor and the sensors show good repeatability and consistency. The response time, which is defined as the sensing current reached 95% of the steady-state current (disturbance ≤ 20 nA), was 4~ 8 s for PSBEDOT-GOx glucose sensor and 8~12 s for PEDOT-GOx glucose sensor. The steady current was recorded for each glucose concentration, and the curve for the relationship between current response and glucose

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concentration was plotted in Fig. 3. It was observed that both PSBEDOT-GOx and PEDOT-GOx sensors showed a great linear relationship (R^2>0.97) between current and glucose concentration at the intermediate glucose concentration range from 1 mM to 20 mM, and both curves became non-linear asymptotic at the higher glucose concentrations (> 20mM, data not shown). However, only PSBEDOT-GOx sensor displayed better linearity (R2=0.9874) at the lower glucose concentration range from 0.1 mM to 0.5 mM whereas PEDOT-GOx did not respond noticeably to glucose. The average sensitivity calculated for PSBEDOT-GOx and PEDOT-GOx sensor are 12.63 μA cm^{-2} mM^{-1} and 7.54 μA cm^{-2} mM^{-1}, respectively at the glucose range of 1 mM to 20 mM. The results indicate that PSBEDOT-GOx has higher sensitivity than the reported PEDOT-GOx glucose sensor.[15, 21, 41] It is worth mentioning that the enzyme concentration for PSBEDOT-GOx in electro-polymerization solution is 1 mg/mL in our work, which was much less than other glucose biosensors (10 mg/mL) reported previously.[22, 41, 42] In another word, PSBEDOT glucose biosensor was more economic to achieve higher sensitivity. The average sensitivity for PSBEDOT-GOx and PEDOT-GOx sensor at the lower glucose range (0.1 mM to 0.5 mM) was 110.64 μA cm^{-2} mM^{-1} and 2.63 μA cm^{-2} mM^{-1}, respectively. The sensitivity of PSBEDOT glucose sensor at low glucose concentrations (< 0.5 mM) is much higher than other reported glucose sensors including PANi-based hydrogel,[34] high surface area Pt-polypyrrole,[38] and carbon nanotubes.[43] The exceptional high sensitivity is contributed to the excellent anti-fouling property and high roughness of PSBEDOT-GOx surface, resulting high enzyme activity, improved H_2O_2 electrooxidation efficiency,[38] and no disturbance on the electrode surface to impede glucose transport and reaction from solution to the enzyme.

The most challenging issue for enzyme-based biosensors is their limited long-term stability, especially for continuous glucose monitoring due to the instability of the enzyme. Hence, the

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long-term stability of PSBEDOT-GOx and PEDOT-GOx glucose biosensor was investigated under both dry (in an empty vial) or wet (PBS) conditions at room temperature. The performance of PSBEDOT-GOx glucose sensor was not significantly varied by any of these storage conditions and the current signal was kept almost 100% of original current either in dry or wet condition after 21 days storage, which is much longer than most of conducting polymer based enzymatic glucose biosensors reported in the literature.[21, 44] However, only less than 38% and 35% signal left for PEDOT-GOx sensor under dry (Fig. 4(b)) and wet (Fig. 4(d)) conditions, respectively. The signal of PSBEDOT-GOx sensor disappeared after 77 days under the dry condition and 70 days for the wet condition while the signal of PEDOT-GOx sensor diminish to 0 after 30 days and 49 days. The result shows PSBEDOT glucose sensor has much better stability than PEDOT glucose sensor under both dry and wet conditions. The exceptional long-term stability could be attributed to 1) the compact morphology and hydrophilicity characteristic of PSBEDOT surface provides a much better matrix to reduce enzyme activity loss and leaching, which are the main causes of the sensor failure;[2, 45] 2) the ultra-low fouling property of PSBEDOT-GOx surface reduces the electrode fouling (often called electrode passivation) that causes sensor deactivation;[45] 3) the zwitterionic polymer has been proved that it can increase the protein stability.

One goal of the glucose biosensor is for continuous real time *in-vivo* monitoring that can provide maximal information for diabetes patients to make fast and optimal therapeutic interventions (i.e., insulin delivery). We firstly investigated the performance of PSBEDOT-GOx sensor in 100% human blood plasma. After the initial blood contact, the average sensitivity was 10.54 μA cm^{-2} mM^{-1} and 5.33 μA cm^{-2} mM^{-1} for PSBEDOT and PEDOT glucose sensor, respectively, at the glucose concentration range between 1 mM to 20 mM. After the initial contact, the sensitivity of PSBEDOT-GOx and PEDOT-GOx decreased 16.5% and 29.3% compared to PBS. However, the

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sensitivity for PSBEDOT-GOx sensor remains exceptionally high at lower glucose concentration range between 0.1 mM to 0.5 mM in human blood plasma, which is 124.57μA cm^{-2} mM^{-1}. Meanwhile, both the sensitivity and linearity (as shown in Fig. 4(e)) of PSBEDOT glucose sensor did not decline significantly (within 10%) after stored in human blood plasma over 14 days, which was better than the reported zwitterionic PCBMA hydrogel based glucose sensor.[34] However, for PEDOT-GOx sensor, more than 50% of signal was lost only after 7 days (Fig. 4(f)). In human blood plasma, more than 50% of signal remained for PSBEDOT-GOx after 3 weeks; however, the signal of PEDOT-GOx sensor was completely lost after 3 weeks. The excellent sensitivity and long-term stability of PSBEDOT sensor was also attributed to the ultra-low fouling property of the PSBEDOT surface that decreases the loss of enzyme from the surface and non-specific adsorption leads to electrode fouling and failure. We believe the performance of PSBEDOT-GOx can be further improved by improving PSBEDOT anti-fouling property and reducing the leaching of the enzymes. Our results indicate that PSBEDOT with both zwitterionic property and conductivity can improve the enzyme stability/activity for biosensing applications.[32]

In this work, a zwitterionic PSBEDOT-GOx glucose sensor was fabricated in a facile single-step procedure by encapsulating GOx into PSBEDOT network during electro-polymerization process and PSBEDOT-GOx sensor demonstrated high sensitivity for low glucose concentration measurement and much better stability for both dry and wet conditions when compared to PEDOT-GOx glucose sensor. The significantly improved stability and sensitivity of PSBEDOT-GOx sensor is due to the strong hydration of zwitterionic side chain, which stabilizes the enzyme, and the rougher surface for GOx immobilization and ultra-low fouling property to minimize the contact of biological molecule and the electrode surface. PSBEDOT-GOx shows much higher stability in 100% human blood plasma, and the current signal remains over 90% after

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stored in human blood plasma 14 days. Via this study, PSBEDOT demonstrates good potential for electrochemical bio-sensing applications since it may significantly increase the performance and service life, minimize foreign body reaction, and improve biocompatibility.

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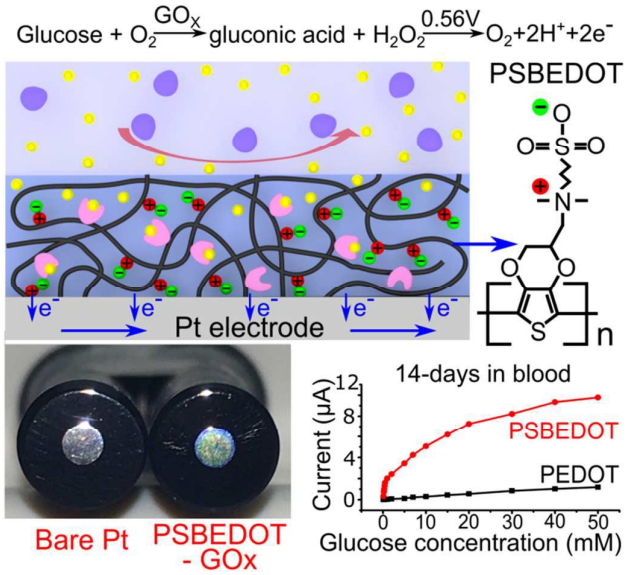

Scheme 1: The Scheme of zwitterionic poly(sulfobetaine 3,4-ethylenedioxythiophene) (PSBEDOT) glucose biosensor

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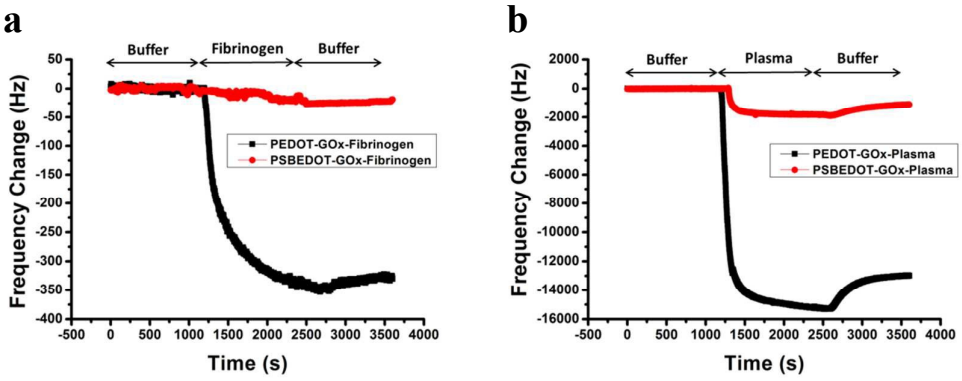

Figure 1. eQCM frequency shift of fibrinogen (a) and human blood plasma adsorption (b) on the PSBEDOT-GOx (Red) and PEDOT-GOx surfaces (Black).

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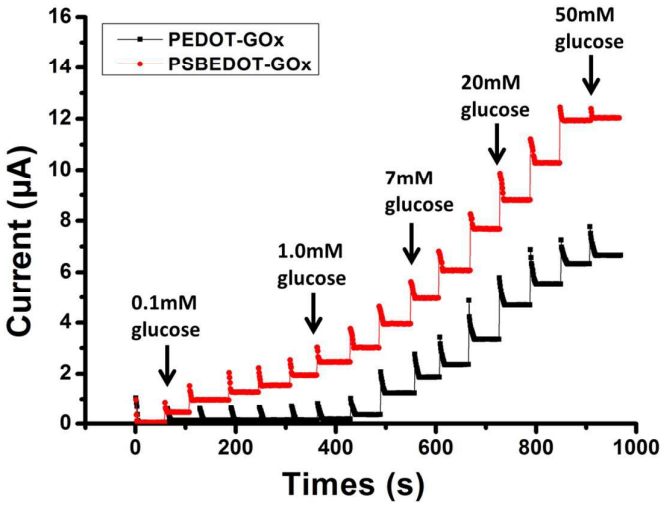

Figure 2. Amperometric response of the PSBEDOT-GOx (Red) and PEDOT-GOx (Black) biosensors to successive addition of glucose in PBS; the concentration of glucose was increased from 0.1 mM to 50 mM.

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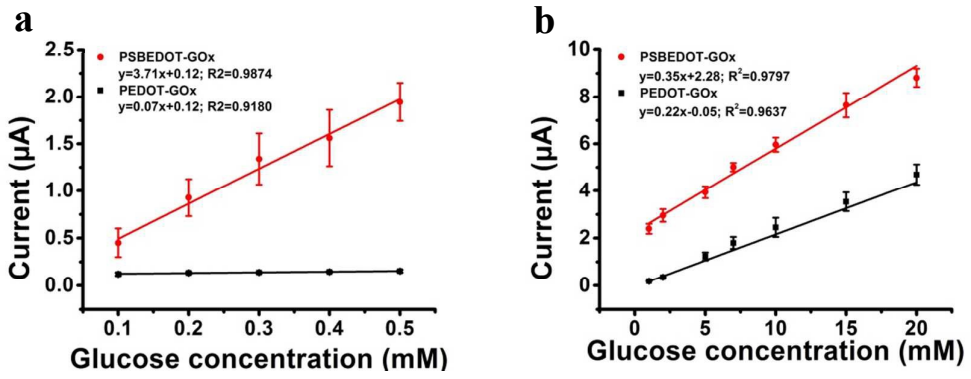

Figure 3. The corresponding calibration plot showing amperometric response vs. glucose concentration: (a) 0.1 mM to 0.5 mM; (b) 1 mM to 20 mM. Replicates=3.

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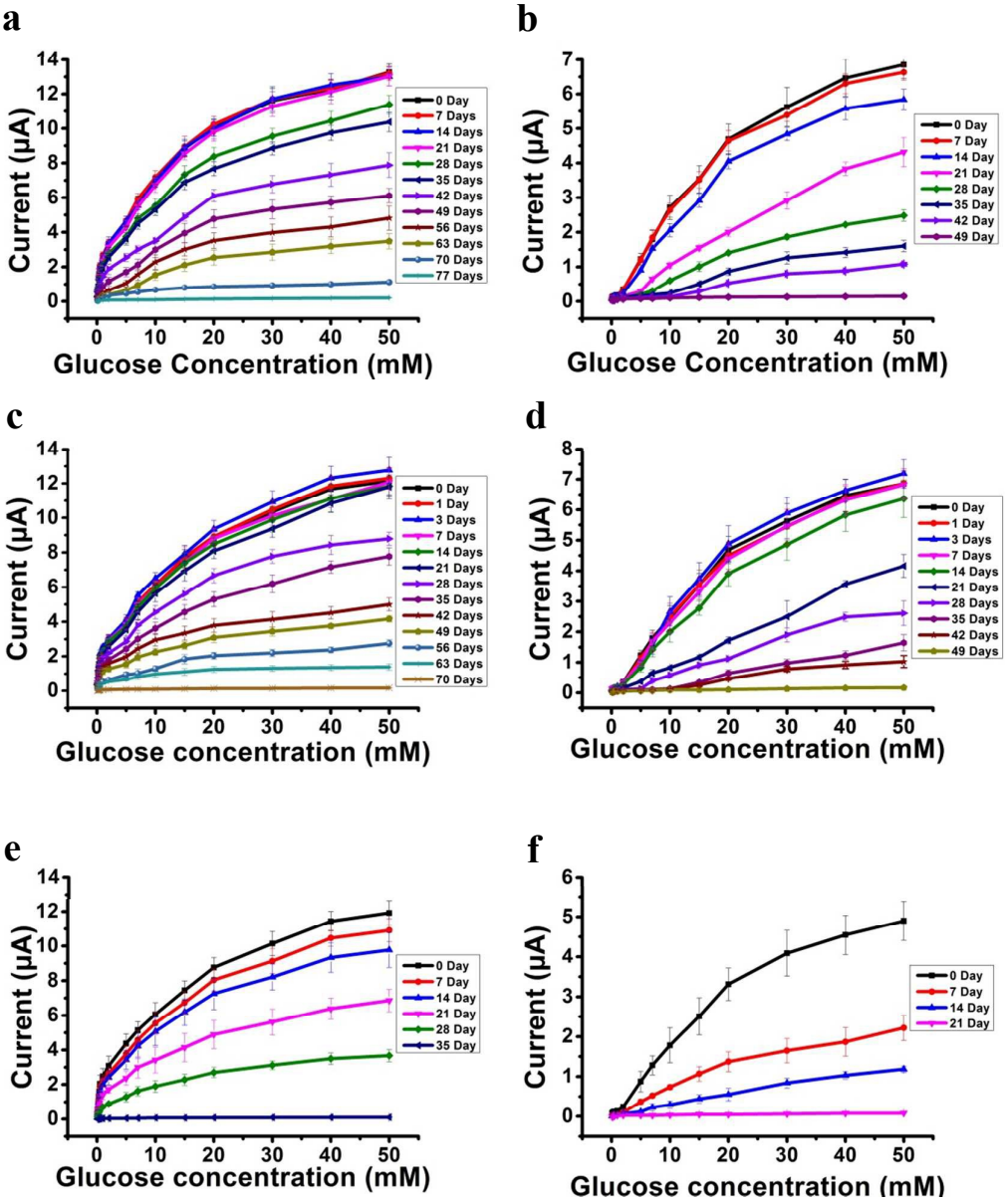

Figure 4. The response of PSBEDOT-GOx (a) and PEDOT-GOx (b) sensors to glucose from 0.1 mM to 50 mM after the long-term storage under the dry condition. The response of PSBEDOT-GOx (c) and PEDOT-GOx (d) sensors to glucose from 0.1 mM to 50 mM after the long-term storage under the wet condition. Current response of PSBEDOT-GOx (e) and PEDOT-GOx (f) glucose sensors incubated in 100% human blood plasma at 0, 7, 14, 21, 30 and 35 days until current signal lost completely as a function of glucose concentration (0.1 mM - 50 mM). Replicates=3.