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PVDF-Nafion nanomembranes coated microneedles for *in vivo* transcutaneous implantable glucose sensing

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ABSTRACT

We demonstrate that microporous PVDF membranes sandwiched between multiple layers of nanomaterials can be used for continuous monitoring of glucose level *in vivo*. This is achieved by coating needle electrodes with Polyaniline nanofiber, Platinum nanoparticles, glucose oxidase enzyme and porous layers, successfully fabricated with layer-by-layer deposition. Nanoparticles incorporated into conductive Polyaniline nanofibers resulted in high surface to volume ratio and electrocatalytic activity for glucose enzyme. A composite coating membrane of porous PVDF and nano-sphere Nafion limited the glucose transportation and increased the lifetime of *in vivo* measurements. The glucose biosensor exhibited a sub-microamperometric output current, fast response time of less than 30 s and a sensitivity of 0.23 μA/mM. The linear sensing range in terms of glucose concentration was from 0 to 20 mM. Implantable experiments using mice models showed excellent response to the variation of blood glucose concentration while maintaining biocompatibility with the surrounding tissues. The sensitivity was shown to remain within 10% close to initial sensitivity within the 7 days of continuous monitoring, and maintain at 70% of the initial sensitivity within 21 days.

1. Introduction

Continuous glucose sensing with reliable *in vivo* performance is expected to improve glucose concentration regulation and thus reduce the number of complications related to diabetes mellitus (Battelino et al., 2012, 2011; Pfeiffer, 1989; Renard, 2002). Due to insufficient accuracy and reliability of non-invasive glucose sensors, minimally invasive sensors are the most practical option for implantable glucose sensing (Shamoon and Group, 1995; Periasamy et al., 2011; Vashist, 2012). Therefore, the Minimization of implantable devices is a practical necessity for reducing the risk of infection and the volume of blood loss (Schmelzeisen-Redeker et al., 2013). Nanostructure modified glucose sensing electrodes exhibit many attractive characteristics such as large active surface, enhanced sensitivity and decreased volume (Gerard et al., 2002; Pan et al., 2012). Miniature invasive glucose sensors after prolonged exposure *in vivo* could not maintain excellent performance as *in vitro* due to either interfering materials or lack of oxygen. The glucose concentration in blood sample is almost 100 fold higher than oxygen concentration, causing the unreliable nature of miniature invasive glucose sensors.

With nanomaterial modified sensors, oxygen consumption per area is higher than conventional sensors. Therefore, the oxygen deficiency problem becomes more serious and leads to a narrow sensing range due to the reaction current saturation at high glucose concentration (Cui et al., 2007; Zhai et al., 2013). At the same time, poor selectivity and bio-incompatibility limit the practical application of nanomaterials in glucose sensing. Various coating membranes have been developed to improve sensor performance. Nafion membrane has been used to prevent interference caused by anionic substances and protein adhesion (Zhang et al., 1994). Porous polymer membranes have also been developed to limit glucose permeability (Koschwanez et al., 2008). While single layer is insufficient to serve multiple demands for implantable glucose sensors, multiple coatings will lead to loss of enzyme activity during prolonged processing procedures.

Polymer phase separation has been investigated because it creates porous morphology with variable pore size and three-dimensional (3D) structures. Controllable morphology ensures that the technology can be used in sensing, energy harvesting and packing (Chen et al., 2014; Eswaraiah et al., 2011; Sharma et al., 2011). In this paper, we report a facile manufacture of composites with 3D porous Polyvinylidene fluoride (PVDF) membrane and nano-sphere Nafion membrane that overcame aforementioned obstacles. This work establishes the first proof of concept that PVDF-Nafion composite layer manufactured by phase separation

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can be used as a selectively permeable membrane. In the outer layer, asymmetric porous structure of PVDF and Nafion was developed to promote high selectivity and permeability. Direct film deposition and pore-formation in room temperature on electrode surface ensured this technology suitable for maintaining high enzymatic activity. In the inner layer, nanoparticles and conductive polymer were used to promote the sensitive detection of glucose. This work introduces a novel method that achieved a balance between high sensitivity and selectivity by utilizing nanoparticle catalyst and nanoporous filtration.

2. Design and experiment

2.1. Principle and design

Fig. 1 showed the layer by layer structure of the fabricated sensor electrode. The implantable electrode in this work serves as puncture needle with an ultra-small diameter (0.18 mm), adequate bendability and reliable stiffness. The submillimeter diameter of the subcutaneous part of sensor electrode caused minimal invasion to patient skin. Gold and Pt nanoparticles decorated on the needle served to enhance conductivity between enzyme layer and electrode. The electrode exhibited a high catalytic efficiency to the oxidation of hydrogen peroxide. Polyaniline nanofibers decorated with Pt particles worked as supporting structures by trapping enzymes. Nanoscaled mats exhibited a high surface area, which allowed for rapid diffusion of the sensing target.

The reaction catalyzed by glucose oxidase yields gluconic acid and hydrogen peroxide (Gough et al. 2010, 1985). The hydrogen peroxide reacts electrochemically on the Pt electrode. Reactions were detailed below:

$$\text{glucose} + O_2 + H_2O \xrightarrow{Gox} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (2)$$

According to the reaction equation, insufficient oxygen for the glucose reaction will lead to an inaccurate current. The detected current from the oxidation of hydrogen peroxide was proportional to the oxygen concentration not the glucose concentration (Tatsuma et al., 1989). Two layers of biocompatible coating were introduced to limit the amount of glucose. Porous PVDF layer filtered out excessive glucose to lower the actual glucose concentration reaching the enzyme layer, which extended the linear range. The nano-sphere Nafion layer functioned as a selective filter to control diffusion and eliminate interference.

2.2. Experiment

Transcutaneous needles for glucose measurements were made of stainless steel with diameter of 0.18 mm, and worked as a mechanical supporting substrate. The sensor nanoparticle layers were fabricated by layer-by-layer electrodeposition as shown in Fig. 1. Prior to deposition, the needle was polished with a metallographic sandpaper to remove oxide film and rinsed in acetone and deionized water. The electrodeposition of Au was carried out in 0.06 M $HAuCl_4$, 1.1 M Na_2SO_3, and 0.3 M Na_2HPO_4 solution under 1 V potential for 60 s. Then, the electrodeposition of Pt nanoparticles layer onto the Au layer was carried out in 0.5 M HCl solution containing 2.5 mg/ml H_2PtCl_6 and 1.85 mg/ml $Pb(CH_3COO)_2$ at 3 V for 180 s with constant magnetic stirring. Next, the needle was subjected to repeating potential scanning (in the range of −0.4 V to 1.0 V) in 0.5 M H_2SO_4 solution containing 0.2 M aniline (Bartlett and Birkin, 1994). During the potential scanning, the electropolymerization of aniline occurred on the surface of the Pt layer and a dark green Polyaniline nanofiber porous film was formed. Pt electrodeposition was repeated in order to enhance catalytic efficiency.

Enzyme immobilization was achieved by applying 650 mV potential on the needle for 20 min. Negatively charged glucose oxidase (GO*x*) were electrostatically entrapped in the porous structure of Polyaniline film in the GO*x* solution (60 mg/ml in 0.5 ml deionized water). Sensor was then dipped into glutaraldehyde crosslink solution to stabilize the enzyme. Finally, the sensor was dried in air at 4 °C for 8 h.

Balanced solvent composites were selected to achieve a desirable structure within one phase separation deposition. The electrodes were first dip-coated with 12 wt% PVDF in 75% Dimethylformamide (DMF)/25% Methyl Ethyl Ketone (MEK) solution (v/v). Before dipping with Nafion as a secondary membrane, the electrodes coated with PVDF solution were evaporated in dry nitrogen environment for 8 min. During evaporation in the dry chamber, MEK partly evaporated from the film surface, leading to a dense surface created by high polymer surface concentration in the top layer. After dip coating with Nafion 117 solution (Sigma-Aldrich), the electrodes were kept in a chamber of 90% relative humidity at room temperature (25 °C) for 6 h to allow complete phase separation between solvent and non-solvent. Phase separation method in humid environment led to multilayer stacking of pores in PVDF membrane and nanosphere formation in Nafion membrane. Coating-less bare sensors and sensors only coated with conventional nafion layer were also prepared for comparison purpose.

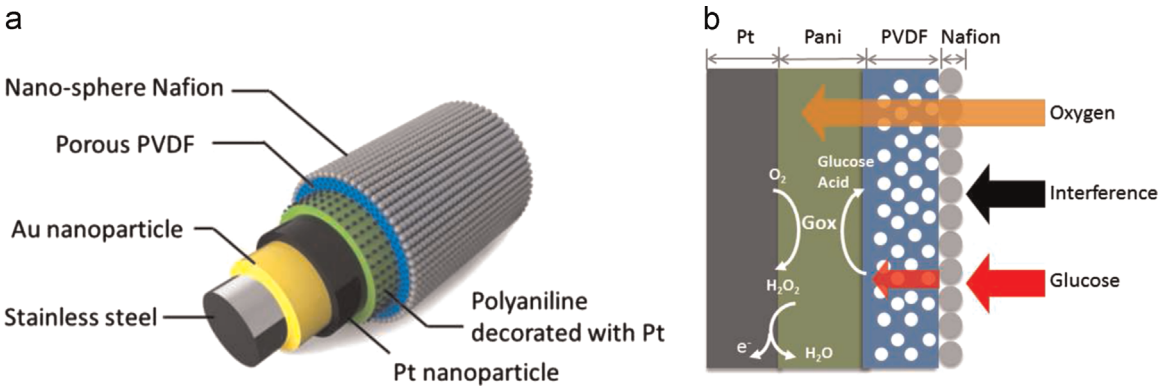

Fig. 1. Illustration of the glucose sensing needle. (a) Layered nanostructures, and (b) principle of operation.

3. Results and discussion

3.1. Material characterization of sensor electrodes

The composition and morphology of the sensor electrode were examined with scanning electron microscopy (SEM) in Fig. 2. Fig. 2a showed the gold particle diameter was less than 500 nm. Particles were uniformly covered over the needle surface. The Pt nanoparticles over the gold particles layer can be observed from Fig. 2b. When using this electrodeposition method for fabricating Pt particles layer, the size and density of particles were easily controlled by varying the electrodeposition conditions such as the electrolyte concentration, the deposition current and time. Uniform Pt nanoparticles on the sensor electrode and optimal electrochemical performance were obtained when 3 V, 180 s and 2.5 mg/ml H_2PtCl_6 were used as the electrodeposition conditions. Pt particles with 100 nm diameter clustered into vertical arrays providing more space for Polyaniline. EDS (Energy Dispersive Spectroscopy) analysis in Fig. S1 of nanoparticles decorated electrode after Gold and Pt electrodeposition showed characteristic peaks corresponding to both elements. Fig. 2c showed the porous Polyaniline film consisting of nanofibers. Nanofibers interconnected to form a mats structure, allowing enzyme immobilization. A cross section of the porous PVDF-Nafion layer was shown in Fig. 2d. This layer consisted of a top nano-sphere Nafion layer and a bottom micro-porous PVDF layer. Nano-sphere with 300 nm diameter and micro-pores of 3–5 μm diameter can be observed in the image. Pores and spheres were formed within the developing film due to the mixing of condensed water and different polymer solutions. Such structures represented solid–liquid demixing and crystallization-dominated precipitation, respectively (Ho et al., 2004). Porous structure with a nano-sphere top layer created using one-time deposition avoided multiple dipping and subsequent loss of enzyme activity.

3.2. Calibration and in vitro characterizations

All electrochemical measurements were performed on a CHI260 electrochemical workstation at room temperature (25 °C). Phosphate buffer saline (PBS, 0.2 M) was employed as the supporting electrolyte for *in-vitro* tests.

The porous layer modified sensor and non-coated sensor were tested with glucose solution in PBS to evaluate amperometric response to the concentration of glucose at a potential of 0.65 V. Both tests used a platinum wire as reference electrode. After the stabilization of the initial current, glucose solution was added into stirring PBS. The glucose concentration in solution was increased by 2 mM during each step. Fig. 3a showed the current *versus* glucose concentration plots, ranging from 0 to 20 mM glucose, which covers the glucose concentration range of the blood samples from diabetic patients. Both sensors displayed high sensitivity and response time within 30 s. The Pt nanoparticles and porous Polyaniline film on the sensor electrode provided large surface area, good conductivity, and catalytic activity. The differences between the current responses of two sensors to the addition of glucose were then characterized. When the glucose solution concentration increased from 0 to 20 mM, the current measured from a PVDF-Nafion coated sensor changed from 0.41 μA to 5.05 μA, corresponding to a current sensitivity of 232 nA/mM. In a similar test protocol, current measured from a non-coated device changed from 0.52 μA to 7.1 μA with poor linearity (1580 nA/mM in low concentration, 35 nA/mM in high concentration). Test results

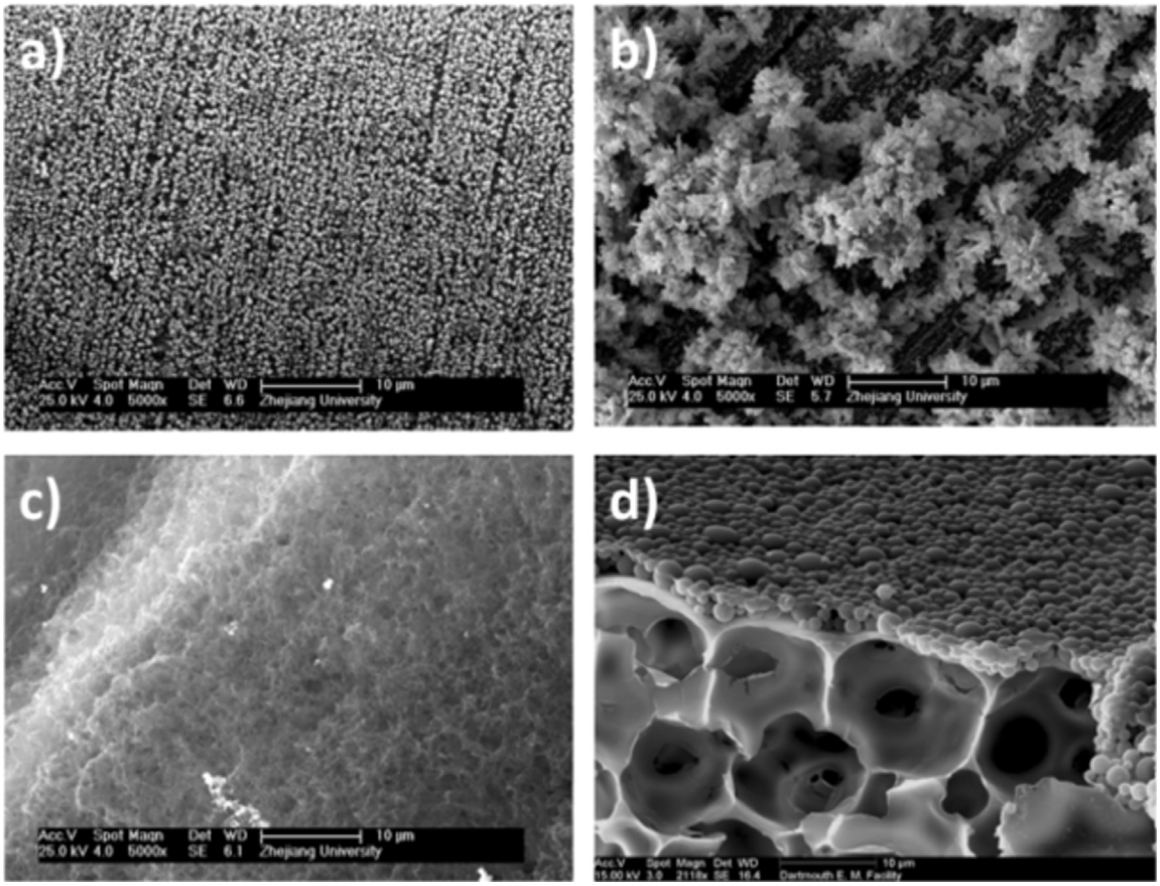

Fig. 2. SEM of nanoparticles and porous PVDF-Nafion structure. (a) Gold nanoparticles. (b) Pt nanoparticles. (c) Porous Polyaniline layer with Pt nanoparticles. (d) Porous PVDF film with nano-sphere of Nafion on top.

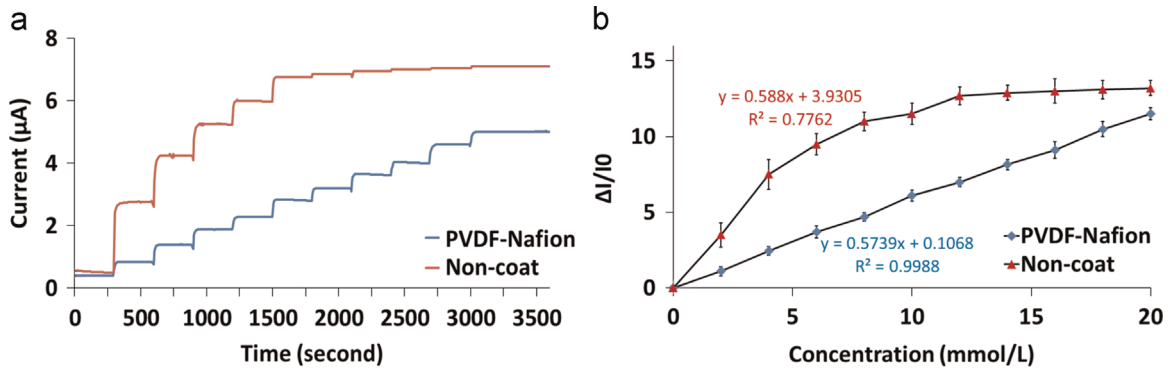

Fig. 3. Characterization of PVDF-Nafion coated sensor. (a) Amperometric response to successive injection of glucose into stirring PBS. (b) Sensitivity of sensors without coating, with Nafion coating and with PVDF-Nafion coating.

implied that PVDF-Nafion coated device showed better linearity and current sensitivity than a non-coated sensor. In Fig. 3b, the coated sensor exhibits a linear dynamic range from 0 mM to 20 mM in an *in-vitro* test (R^2 coefficient 0.9988). The R^2 value of the non-coated sensor is 0.776. PVDF-Nafion porous coating could achieve a linear sensing range of concentration covering the usual blood glucose levels.

The single step amperometric response curves of the glucose sensors with and without coating were obtained by varying the glucose concentration from 4 to 6 mM as shown in Fig. 4. This range of glucose concentration was selected because it is approximately the mean range glucose concentration in humans. The results exemplified a better performance in response current after coating the sensor with PVDF-Nafion. As expected, the sensors with coating materials had a slower response time to reach equilibrium current than bare sensors. The response time is defined as the time it takes to reach 95% of the maximum current change (I_2–I_1). The response time of a bare sensor was 17 s, whereas that of the coated sensor was 30 s. The added physical barrier of the porous layers caused this increase in response time. For the implantable application, this delay was tolerable sacrifice when compared to the wider sensing range achieved. The use of the negatively charged Nafion film in the form of a nano-sphere layer coating on the enzyme electrode and glucose diffusing porous membranes prevented interfering effects of anionic body chemicals.

The amperometric responses of possible interfering reactions on nanoporous layer coated electrode have also been studied. Such interfering materials include ascorbic acid (AA), uric acid (UA) and L-Cysteine (Cys) could be easily oxidized at a relative positive potential. The experiment was carried out by adding 0.5 mM glucose

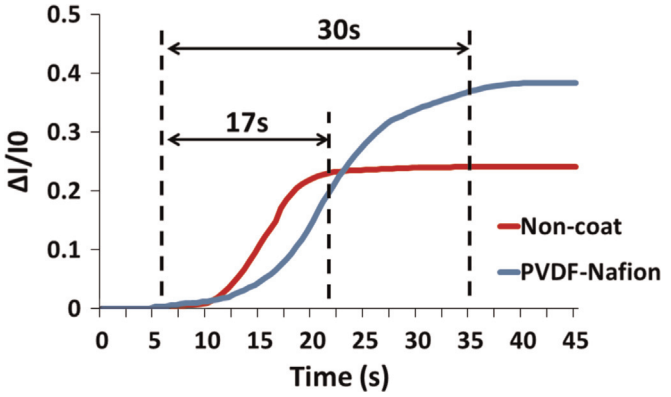

Fig. 4. Amperometric response of the PVDF-Nafion coated sensor to single injection of glucose into PBS with subsequent mixing, for sensors without coating and with PVDF-Nafion coating.

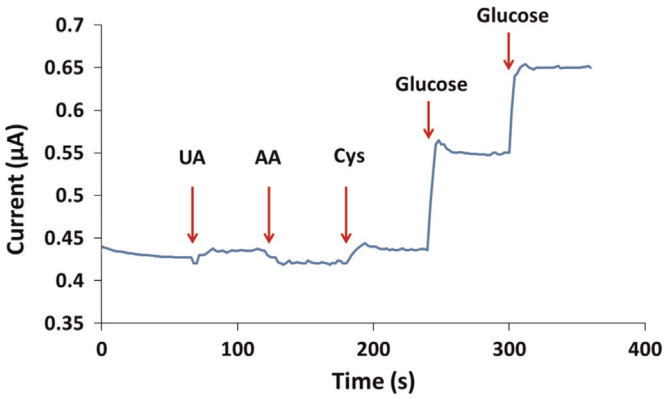

Fig. 5. Current–time response curves in PBS with successive additions of 0.5 mM glucose (twice), 0.1 mM UA, 0.1 mM AA and 0.1 mM Cys under 0.65 V electrode potential.

(twice) and 0.1 mM UA, 0.1 mM AA, 0.1 mM Cys interfering agents successively into a constantly stirring PBS at a fixed potential of 0.65 V. The glucose solutions were added first to examine the interactions between UA, AA and Cyt with hydrogen peroxide. The corresponding result is shown in Fig. 5. The porous layer coated electrode did not give significant response to the interference species of UA, AA and Cys while maintaining high sensitivity to glucose. The test results showed the combination of PVDF and Nafion membrane could achieve reliable permselectivity.

3.3. *In vivo experiments using implantable devices*

To evaluate the *in vitro* stability of the sensors under constant voltage potential, PVDF-Nafion coated and non-coated group (each group used four sensors) were continuously applied with 0.65 V voltage in bovine serum with 4 mM glucose for 21 days at 37 ± 0.5 °C. A sensitivity calibration test was taken every 1–2 days. The glucose concentration in solution was increased by 2 mM during each step to 20 mM final concentration. The same test was performed at the beginning of the experiment and marked as initial sensitivity. The relative sensitivity was calculated as test sensitivity divided by initial sensitivity. As shown in Fig. 6, the coated sensor's sensitivity remained within 90% of the initial sensitivity in the first week. On day 21, the sensitivity dropped to 75% of the initial sensitivity. The non-coated sensor sensitivity dropped below 50% of initial value after 7 days stored in the same test environment. Nano-sphere Nafion with porous PVDF as a flexible substrate showed more reliable performance than convention thin film morphology.

Male Sprague–Dawley mice (300 g) were anaesthetized using isoflurane and secured on flat surface. PVDF-Nafion coated sensor

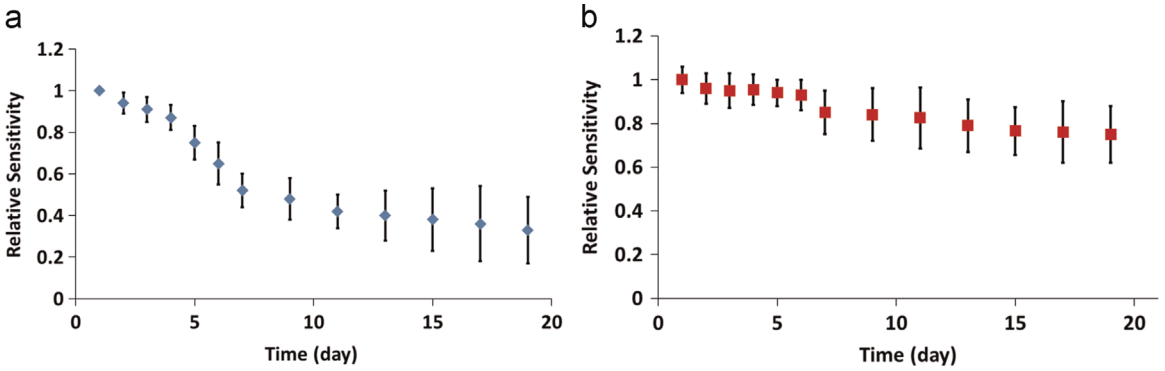

Fig. 6. (a) Relative sensitivity stability test of the conventional non-coated sensor stored in bovine serum. (b) Relative sensitivity stability test of the porous PVDF-Nafion coated sensor stored in bovine serum.

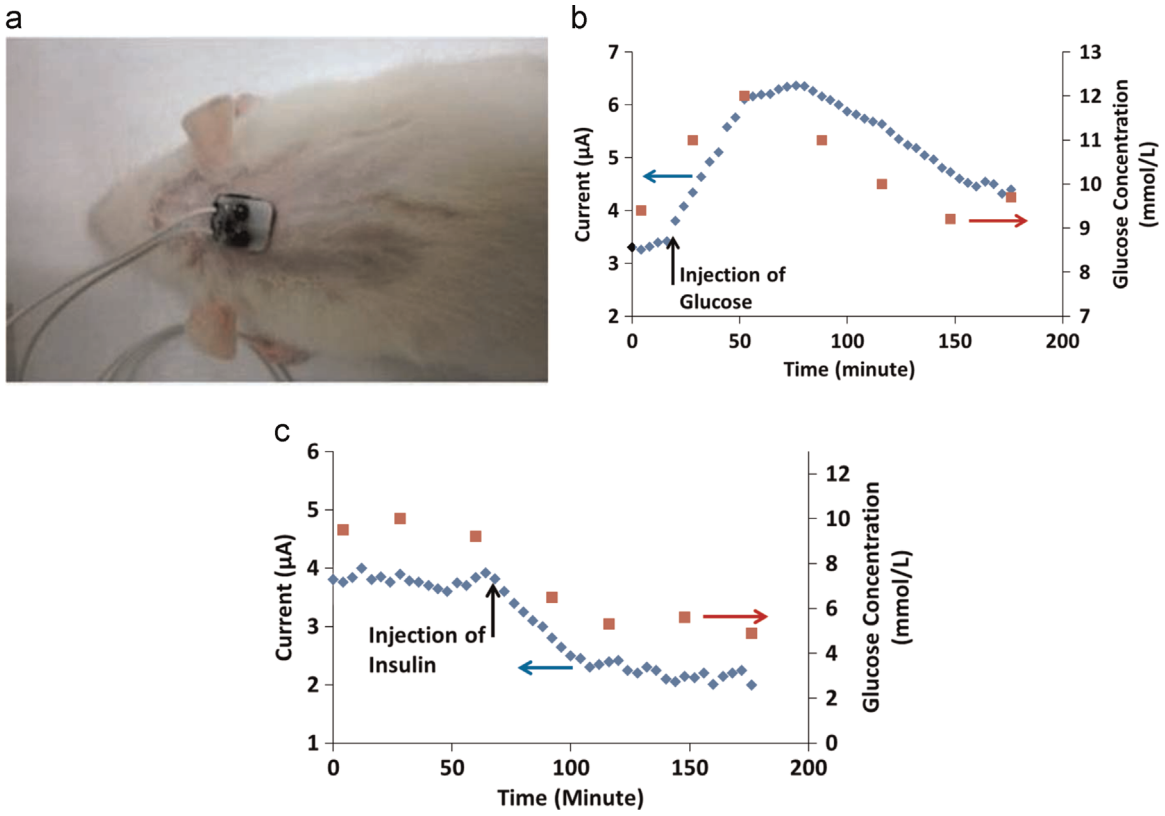

Fig. 7. *In Vivo* continuous monitoring of Glucose concentration in mice. (a) Image of transcutaneous glucose sensor attachment to mice back. (b) Implanted sensor current response to glucose injection and tail vein blood test. (c) Implanted sensors response to insulin injection and tail vein blood test.

electrode and platinum reference electrode were implanted 1 cm lateral to the spinal cord and between the scapulars as shown in Fig. 7a. All devices were sterilized using gamma irradiation (16 Gy) before implantation. Sensors were soaked in PBS for one hour before implantation to shorten the stabilization period post-implantation. We observed a rapid signal reduction after implantation, signal showed less fluctuation and became stable four hours faster than un-soaked sensors. The sensors were gently slid beneath the skin and mechanically secured by plastic adapter with electrical connection. The response to glucose of the explanted sensors was tested and recorded without further calibration. Discrete blood glucose measurements were performed in parallel from the tail vein pricks using glucose testing strips. Glucose concentrations from tail vein blood were compared with sensor output following each major change in blood glucose level throughout the entire experiment. Sensor responses to intraperitoneal glucose solution injection were shown in Fig. 7b. Response current followed the blood glucose trend closely. Sensor current reached a peak 20 min after 300 mg glucose injection. Sensor responses to insulin injection (1 U/kg) were shown in Fig. 7c. Senor current declined rapidly after injection and maintained in low level in the following time. Similar drop was observed in tail blood test. In a series of experiments where the glucose level was increased, the sensor output current was increased by 38% corresponding to the blood glucose increased 30%. In the insulin injection test, blood glucose decreased 42% while sensor current deceased 47%. With the notice of the raw current signal, both comparisons indicated that the implanted sensor could monitor the blood glucose changes. Hematoxylin and Eosin stained sections of excised sensor surrounding tissue were showed in Fig. S2. The inflammatory cells were stained as purple, while connective tissue was stained as pink. A minor inflammatory response was showed around the sensor needle three days after implantation. Predominant polymorphonuclear leukocytes (PMNs)

with monocytes and macrophages were observed and a dense connective tissue layer (fibrous capsule) surrounded the periphery of the sensor. Stained section images showed the inflammatory response was controlled under a minimal level due to the biocompatibilities of PVDF and Nafion coating materials.

Conventional electrochemical glucose sensors *in vivo* performance are influenced by multiple factors including interference in human biological mediums, oxygen dependence and biofouling on contact surface. Key strategies to overcome these limitations often require multi-layer coating on working electrode to eliminate interference, limit glucose mass and maintain biocompatibility. Each individual layer of coating procedure will significantly increase the electrical resistance and lowering the enzyme activity of the sensor. The PVDF-Nafion coating can potentially provide those desired multifunction in one process minimizing the number of coating layers and reducing of the sensor sensitivity. On the other hand, nanomaterial decoration will maximize sensitivity of the bare sensing layer for the final sensor design requiring additional coatings. In this work, nanoparticles work with PVDF-Nafion porous layer to maintain high sensor sensitivity, combined with the wider sensing linearity range, promises better sensor performance.

4. Conclusions

We demonstrate that a combination of nanostructured layers coated electrodes enabled a new generation of minimally invasive glucose sensors suitable for transcutaneous implantation. Nanoparticle material with porous coating was proven as an effective combination that improved sensor performance. The PVDF-Nafion coating membrane provided an optimal balance between oxygen and glucose transport to the sensing layer. Specifically, with an operational life of over three weeks, the glucose biosensor embedded with a porous membrane exhibited a sub-microamperometric current response within 30 s and a detection limit of 0.1 mM with excellent reproducibility. *In vivo* validation in mice models, the sensor current change was proportional to the tail blood glucose concentration level. Layer-by-layer manufacturing method utilized different functional materials to control glucose diffusion and eliminate interference for promising sensitivity and selectivity. The combination of nanoparticle and porous structure has great potential in implantable device applications related to diabetes management as well as other medical diagnosis.

Acknowledgment

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2014.05.063.

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