Disposable all-solid-state pH and glucose sensors based on conductive polymer covered hierarchical AuZn oxide

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1. Introduction

One of the most serious diseases worldwide is diabetes mellitus, which causes various complications (Zimmet et al., 2001; Gale, 2001; Brownlee, 2001). Therefore, monitoring blood glucose levels with a simple procedure is helpful to reduce the risk of complications associated with diabetes. Over the years, numerous enzymatic and non-enzymatic glucose biosensors have been studied for various applications, such as healthcare, industry, environmental use. Among these, electrochemical glucose biosensors have been extensively studied and commercialized as portable devices for point-of-care diagnosis. To date, commercially available glucose sensors are mostly employed as a second generation type of sensor (Heller and Feldman, 2008; Wang, 2008). These sensors have an adequate dynamic range and robustness for the blood glucose detection; however, ascorbic acid, dopamine, and acetaldehyde compounds, which are oxidized around the glucose detection potential, can cause interference (Vashist et al., 2011). Otherwise, the potentiometric method can avoid interferences from oxidizing species in blood samples and the electronic device should be simple. Potentiometric glucose sensors have been developed in various types, such as magnetic nanoparticles (Yang et al., 2014), pH sensitive membranes (Liao et al., 2007; Luo et al., 2004), ion selective electrodes (Koncki, 2007), and boronic acid derivatives (Shoji and Freund, 2002). However, their sensitivity, selectivity, biocompatibility, response time, and cost still need to be improved. Thus, it is necessary to develop a robust, simple, and low-cost disposable potentiometric glucose and pH sensors for the point-of-care diagnosis.

pH measurements are highly important for clinical, environmental, and food industry applications. To monitor pH values, glass electrodes have mostly been used due to their good stability, long lifetime, and reliable pH measurements. However, glass pH electrodes have disadvantages for measuring biological samples, such as their high-cost, inflexibility, and difficulty to miniaturize. Thus, different types of pH sensors, including all-solid-state pH sensors that consist of polymers containing ionophores (Kwon et al., 2007) or metal/metal oxides (Karyakin et al., 1999), were developed to overcome the above problems. Of these, metal oxide pH sensors have received much attention due to their advantages, including easy miniaturization and adaptation to extreme measuring environments. It is still challenging to discover new sensing materials that display ideal responses to the pH levels of biological samples and also have high stability, reproducibility, and accuracy, and especially are of low-cost.

Nanostructured metals and metal oxides are become important
in various fields (Solanki et al., 2011). Of these, hierarchical structures (dendritic structure) consisting of main stems and many branches have received much attention due to their large surface-area-to-volume ratios, high activities, and catalytic efficiencies (Hermanson et al., 2001). Moreover, some bimetallic dendrite materials have also been studied for developing sensing materials (Noh et al., 2012; Noh et al., 2014) by even only a few research groups. AuZn oxide has not been reported for potentiometric pH and glucose sensors use to date, while many other metal oxides have been examined for pH sensor design (Kurzweil, 2009). However, metals or metal oxides in hierarchical structures have often shown somewhat disadvantages in physical strength, fouling effect, and less biocompatibility to enzymes. Thus, we have tried to form a stable functionalized-conductive polymer on the hierarchical structure to make the metal layer physically stable, to reduce the fouling effect, and to enhance biocompatible to enzymes through the chemical bonding between the polymer layer and enzymes. In addition, it probably provides a synergetic effect on the response to the pH change of the metal alloy oxide layer with an acidic group of polymer. Of various polymers, polypyrrole, polyaniline, polythiophene, and polyterthiophene have received much attention due to their potential applicability to batteries, electrochromic devices, solid-state pH sensors, and biosensors (Hwang et al., 2010; Shonaïke and Advani, 2003). Although some conventional conductive polymers, such as polyaniline and poly-pyrrole have been used as diverse sensing substrates to date (Ahuha et al., 2007), these are unstable in air and aqueous solutions, resulting in poor sensor performance. Therefore, it is crucial to use highly stable conductive polymers, such as polyterthiophene, as long-life sensor substrates. In addition to their stability, the modification of polymer structures with functional groups of -COOH and -NH2 is very attractive for electrochemical sensing substrates, because various molecules (enzyme, protein, and DNA) can be stably attached to the polymer layer through formation of amide bonds (Lee and Shim, 2001; Kim and Shim, 2013; Rahman et al., 2008; Chandra et al., 2013). Thus, we developed a glucose biosensor using an all-solid-state pH sensor fabricated by combining alloy oxide with a very stable conducting polymer bearing benzoic acid groups, since it was expected to enhance the stability, the proton transfer efficiency, and enzyme binding property.

In the present study, a functionalized terthiophene monomer was synthesized and polymerized on a dendritic AuZn alloy oxide to combine the catalytic properties of bimetals and polymers, which was characterized for a disposable all-solid-state pH sensor in the first stage. The final sensor probe obtained after immobilization of GOx on the conductive polymer/AuZn oxide layer through the amide bond formation was examined for its application as a glucose sensor, and the sensor layer was characterized using electrochemical and surface analysis methods. The analytical variables for glucose detection were optimized, and the performance and reliability of the proposed disposable sensors were examined using whole blood samples.

2. Experimental section

2.1. Materials

HAcuCl4·3H2O, ZnCl2, Na2SO4, NaOH, ascorbic acid (AA), acetaminophen (AP), and dopamine (DA) were purchased from Sigma–Aldrich Chemical Co. (USA). 2,2′:5′,5′′-terthiophene-3′-p-benzoic acid (TBA) was synthesized according to a previous report (Kim et al., 2012). Di(propylene glycol) methyl ether, tri(propylene glycol) methyl ether, N-hydroxy succinimide (NHS), 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), and glutaraldehyde (GA, Grade II, 25% v/v aqueous solution) were purchased from Sigma Co. (USA). Glucose oxidase (GOx, 166 U/mg) was purchased from Toyobo Co. (Japan). Glucose, fructose, lactose, mannose, xylose, and Nafion (5%) were purchased from Sigma Co. (USA). All of the aqueous solutions were prepared in doubly distilled water obtained from a Milli-Q water-purification system (18 MΩcm). We prepared standard buffer solutions (universal buffer) at different pH values, which were controlled by adding 0.2 M NaOH to stock solutions of 0.0286 M citric acid, 0.0286 M KH2PO4, 0.0286 M boric acid, and 0.0286 M diethyl barbituric acid.

2.2. Instruments

The experiments were performed using two different types of the electrode system. One is used for electrochemical preparation and characterization of sensing layers (AuZnOx, and pTBA), where carbon, Ag/AgCl (in saturated KCl), and Pt wire were used as working, reference, and counter electrodes, respectively. Second, the all-in-one screen printed carbon electrode (SPCE) was used as a disposable all-solid–state sensor strip for the determination of pH and glucose level, modified carbon (area = 0.07 cm2), Ag/AgCl, and mere carbon were used as working, reference, and counter electrodes, respectively (Fig. S1). To make all-solid-state reference electrode, 5% polyvinyl alcohol (PVA) containing saturated KCl was drop-coated onto the Ag/AgCl layer and dried at 40 °C for 5 min. Next, 5% Nafion was drop-coated onto PVA (sat’d KCl)-coated Ag/AgCl and dried at 100 °C for 1 h (Kwon et al., 2007). Amperograms, linear sweep voltammograms (LSVs), and cyclic voltammograms (CVs) were recorded using a potentiostat/galvanostat, (Kosentech Model PT-1, S. Korea), EG&G PAR Model PAR 273 A, and a potable type glucose meter (One Touch ultra™, Lifescan Inc.). The potential difference between the probe and the reference electrode was measured using a 16 channel pH-Ion meter (Model KST101B, Korea). Electrochemical impedance spectroscopy was performed using EG&G Princeton Applied Research, PARSTAT 2263 at an open-circuit voltage from 100 kHz to 10 mHz, at a sampling rate of five points per decade. Scanning electron microscopy (SEM) images were obtained using a VEGA 3 SB (Tescan Inc, USA). X-ray photoelectron spectroscopy (XPS) experiments were performed using a VG Scientific ESCALAB 250 XPS spectrometer with a monochromated Al Kα source with charge compensation at KBSI (Busan, S. Korea).

2.3. Fabrication of the sensor probe

The fabrication process of the sensor probe is presented in Scheme 1. The AuZn alloy dendrite was prepared by electro-deposition in 0.1 M Na2SO4 containing 30 mM HauCl4, 4H2O and 30 mM ZnCl2. The preparation conditions were optimized to show the best performance. The applied potential was –0.5 V with a deposition time of 200.0 s. The electrochemical oxidation of the AuZn dendrite surface was performed in 0.1 M NaOH using linear sweep voltammometry from 0.0 to 1.5 V until a stable voltammogram was obtained. Next, the pTBA layer was electrochemically polymerized (Lee et al., 2010) on the AuZn oxide (AuZnOx) layer deposited on the SPCE (AuZnOx/SPCE). The modified electrode was dipped into 1.0 mM TBA, which was prepared in 1:1 di(propylene glycol) methyl ether and tri(propylene glycol) methyl ether. Then, the polymer was formed on the electrode through three potentials cycling from 0.0 to 1.5 V (Ag/AgCl) at a scan rate of 0.1 V/s in 0.1 M PBS (pH 7.4). The pTBA/AuZnOx layer was first tested as a pH sensor. To prepare the glucose sensor, pTBA/AuZnOx was immersed for 3 h in 0.1 M PBS (pH 7.4) containing 10.0 mM EDC and NHS to activate the benzoic acid groups on the pTBA layer. Next, the electrode was washed with a buffer solution and subsequently incubated in 0.1 M PBS (pH 7.4) containing 6.0 mg/mL FAD-glucose.
oxidase (GOx) for 3 h. GOx was immobilized on pTBA bearing a benzoic acid group through the formation of amide bonds, followed by the EDC-NHS activation step. To prepare an enzyme cluster solution of GOx, 0.55 mg of ammonium sulfate was added to 1.0 mL of PBS containing 6.0 mg GOx, and GA was added to a concentration of 0.5% to crosslink the precipitated enzyme molecules. The GOx modified electrode was incubated in an enzyme precipitation solution for 3 h. The GOx cluster layer was immobilized on GOx-pTBA by chemical cross-linking. Finally, 1.0 μL of 1.0% NaFion was dropped onto the GOx-pTBA/AuZnOx and dried at room temperature. The final sensor probe was evaluated as a glucose sensor.

3. Results and discussion

3.1. Characterization of the pTBA/AuZnOx layer

The electrochemical preparation of AuZnOx was investigated using chronoamperometry and cyclic voltammetry. An increase in the current was observed when a deposition potential of −0.5 V applied, indicating formation of AuZn dendrite on the SPCE surface (inset of Fig. 1A). After formation of the alloy layer, as shown in Fig. 1A, the CVs were recorded for the AuZn dendrite from −1.0 to 1.1 V at a scan rate of 0.1 V/s in 1.0 M NaOH. CV showed that AuZn dendrite exhibited two pairs of redox peaks at −0.62/−0.69 and 0.13/0.65 V, which corresponds to Zn, and Au, respectively. These results indicated that the AuZnOx was successfully formed on the SPCE. The pTBA layer was formed through electropolymerization of the monomer, where it was drop-coated onto the AuZnOx in 0.1 M PBS (pH 7.4). An oxidation peak appeared at approximately 1.1 V in the first anodic scan from 0.0 to 1.4 V, which was responsible for the monomer oxidation immediately forming a polymer film. Another redox peak of Au was additionally observed at 0.9/0.29 V (Fig. S2). AuZnOx with and without the pTBA layer were examined for pH responses and compared with the performance of the commercial glass pH sensor. Next, the enzyme was immobilized on the pTBA layer of the sensor. Each layer of these sensors was evaluated with XPS (Fig. S3).

3.2. Impedance analysis of the sensor probe

Electrochemical impedance spectroscopy (EIS) was performed to investigate the surface characteristics of each layer in a 0.1 M PBS (pH 7.4). Fig. 1B shows Nyquist plots obtained for each layer of the bare SPCE (black), AuZnOx (red), pTBA/AuZnOx (blue), and NaFion/GOx-pTBA/AuZnOx (green). The experimental data corresponding to each layer were fitted with a Randle circuit (inset of Fig. 1B). The experimental data corresponding to each layer were fitted with a Randle circuit (inset of Fig. 1B). The Rct (Rp1 + Rp2) values were obtained using Zview 2 impedance software. In this case, the Rct value of the bare SPCE was 699.11 kΩ, while AuZnOx on the SPCE lowered the Rct value to 156.22 kΩ, making it 4.5 times lower than that of the bare SPCE. This indicated that the AuZnOx layers cause an increase in the electrode surface conductivity. However, the Rct values of pTBA/AuZnOx and NaFion/GOx-pTBA/AuZnOx showed an increase in charge-transfer resistance to 380.07 and 604.25 kΩ, respectively. This shows that pTBA and GOx were successfully immobilized onto the AuZnOx layer and the probe layer modifications impeded the electron-transfer reaction.

The morphology of the sensor surface was also investigated using SEM. As shown in Fig. 1C-a, the microstructure of the alloy dendrite shows homogeneous growth in a hierarchical structure with a multi-branched tree form. Energy dispersive X-ray spectroscopy (EDXS) analysis was conducted to determine the composition of the AuZnOx (Fig. S4A). The ratio of the atomic component of Au and Zn was determined to be 51.88, and 48.12, respectively. In addition, the distribution of Au and Zn was investigated using time-of-flight secondary ion mass spectroscopy (TOF-SIMS) in a positive mode. As shown Fig. S4B, the TOF-SIMS images were obtained for (a) Au3+ (m/z 590.90) and (b) Zn2+ (m/z 130.84) and (c) overlaid Au3+ and Zn2+ ions. It was clearly observed that both of Au and Zn were distributed homogeneously in
hierarchical structure. Fig. 1C-b shows an image of pTBA/AuZnOx, where the reduced pore size of the hierarchical structure can be observed. The image of GOx covered on pTBA/AuZnOx did not show any hierarchical structure, but displayed a very rough surface, as shown in Fig. 1C-c. The Nafton coating on GOx-pTBA/AuZnOx (Fig. 1C-d) showed a smoother surface morphology compared with that shown in Fig. 1C-c.

3.3. pH response

In order to compare the pH response of the sensor layer, AuZnOx, pTBA/AuZnOx, and Nafton/GOx-pTBA/AuZnOx were examined the performances of the probes in the pH range of 1–14. The response potential shifted towards more negative values as the solution pH increased, indicating the probe surface interacted with protons. As shown in Fig. 2A, the linear calibration plot of pTBA/AuZnOx was obtained with an ideal Nernstian slope of 59.2 ± 0.5 mV/pH between pH 2 and 13 at 25 °C. The slopes of the pH responses for AuZnOx and Nafton/GOx-pTBA/AuZnOx were 51.6 ± 0.3 and 41.9 ± 1.6 mV/pH, respectively (Fig. S5). For Nafton/GOx-pTBA/AuZnOx, the slope of the pH response was reduced by 29.0% compared with that of pTBA/AuZnOx-modified SPCE, because the thick enzyme layer formed by the chemical binding of GOx onto the pTBA layer/AuZnOx may have disturbed the movement of H⁺ from the bulk of measuring solution to the surface of pTBA/AuZnOx. Consequently, pTBA/AuZnOx showed the best pH sensing performance compared with AuZnOx and Nafton/GOx-pTBA/AuZnOx. The pTBA with a weak acidic property was used to enhance the pH sensitivity (59.2 ± 0.5 mV/pH) of the AuZnOx layer by a synergistic effect. The polymer layer can protect the...
alloy surface from foreign species and an additional enzyme binding sites. To evaluate the deprotonation of the benzoic acid groups of pTBA, voltammetric characterization was performed using a pTBA modified probe in different pH solutions containing 10 mM ferricyanide (negative charge) (Gao et al., 2013). As shown in Fig. 2B, a pH-dependent titration curve was obtained by measuring the current response of ferricyanide with the pTBA-modified electrode. In the low pH region (pH < 5), the current response of ferricyanide did not change because benzoic acid of pTBA was uncharged due to protonation. However, the current response of ferricyanide ions significantly decreased in the pH region greater than 5, where ferricyanide ions cannot enter the diffusion layer on the sensor surface because of the electrostatic repulsion between the deprotonated benzoic acid groups of pTBA and the anions. The pKa value of pTBA was determined to be 5.6 using the titration curve. This showed that introducing the polymeric pH-sensitive layer (pTBA) on the AuZnOx surface is promising for enhancing pH sensing performance.

The reliability of the pH sensor was investigated in terms of reversibility, repeatability, stability with storage time, and reproducibility. As shown in Fig. 2C, the reversibility and the repeatability of sensor probes were determined by measuring the potential at various pH values (pH 4.1, 6.0, 8.0, and 10.2) in both directions (acid to base and base to acid) using the same sensor probe and measuring each slope six times. The slopes obtained for acid to base and base to acid directions were determined to be 58.5 ± 0.87 and 59.2 ± 0.05 mV/pH, respectively. This result revealed a small hysteresis with a difference of 0.7 mV in the slope, which is a good indicator of reversibility and repeatability. The stability with storage time and the reproducibility of the pH sensor were examined by measuring the potential in the pH range from 4 to 6 using different sensor probes each day (Fig. 2D). The disposable pH sensor probes were stored at room temperature. After 15 days, the sensor retained 96.9% of its initial potential response, indicating that it can be used for more than 15 days at room atmosphere. The reproducibility, expressed as the relative standard deviation (RSD) of the pH sensor was determined to be 2.0% at pH 6 using five different electrodes.

Prior to the analysis of real samples, the potential of Nafton/PVA (sat'd KCl)-coated Ag/AgCl was examined in chloride solutions (NaCl) of different concentrations. The potential of Nafton/PVA (sat'd KCl)-coated Ag/AgCl remained constant in the NaCl concentration range of 100 µM–0.01 M, where the potential variation from electrode to electrode was 7.1 ± 0.8 mV in 0.1 M NaCl. The pH measurements of healthy human saliva and urine samples were performed using pTBA/AuZn oxide and a commercial pH meter (Orion glass pH electrode). 10 µL of the sample was loaded onto the pTBA/AuZnOx probe, and then measuring the potential change. The pH values of saliva and urine using a disposable pTBA/AuZn oxide electrode were 6.8 ± 0.1 (saliva) and 5.7 ± 0.1 (urine). Using the glass pH electrode, the pH values were 6.8 ± 0.02 (saliva) and 5.7 ± 0.01 (urine). These results show good agreement between the proposed pH sensor and the commercial pH glass electrode.

3.4. Glucose detection and optimization of analytical variables

The experimental variables affecting the sensor performance were examined in terms of pH, temperature, and humidity in a glucose solution of 200 mg/dL at 15–40 °C and 20-80% relative humidity. The effect of pH on the glucose detection response was...
tested between pH 5.8 and 8.3 (Fig. 3A). The sensor response was enhanced as the pH increased from 5.8 to 7.4, but it decreased at pH values greater than 7.4. The maximum response was observed for glucose at pH 7.4. As shown in Fig. 3B, the effect of temperature on the glucose response gradually increased with increasing temperature from 15 to 45 °C, and it decreased as the temperature increased to greater than 45 °C. This phenomenon was caused by both the potential dependence of the Nernst slope and the enzyme activity changes at different temperatures. For the practical purpose of glucose analysis for in vitro blood samples, all subsequent experiments were performed at 25 °C. For humidity (Fig. 3C), the sensitivity of the sensor was steady between 20% and 80%; however, there was a small increase of 4.2 and 2.6% in the sensitivity at 40% and 60% humidity, respectively. As a comparison experiment, the Nafion/GOx-pTBA/AuZnOx and Nafion/GOx/AuZnOx were evaluated in terms of their stability and sensitivity for glucose under the optimum conditions as shown in the Fig. S6. The Nafion/GOx-pTBA/AuZnOx shows the enhanced sensitivity and stability in the continuous measurement of glucose, compared with that of Nafion/GOx/AuZnOx.

Interference in the glucose response was observed using 200 mg/dL glucose containing interfering species, including ascorbic acid (AA, 5 mg/dL), acetaminophen (AP, 15 mg/dL), dopamine (DA, 5 mg/dL), and other mono-saccharides, such as, mannose (10 mg/dL), lactose (10 mg/dL), xylose (10 mg/dL), and fructose (30 mg/dL), as shown in Fig. 3D. The potentiometric response of glucose containing other monosaccharides was 1.2% lower than that of glucose without mono-saccharides. Thus, the monosaccharides did not interfere for glucose detection, since GOx is highly selective for glucose. In the presence of 200 mg/dL glucose containing DA, AP, and AA, the glucose response decreased by approximately 2.7%; however, this is not interfering for glucose detection within the measuring error range.

3.5. Calibration plot for glucose analysis

The sensing principle of the potentiometric glucose sensor is based on an enzymatic reaction catalyzed by GOx. GOx catalyzes glucose to produce gluconolactone and hydrogen peroxide. Then, gluconolactone is spontaneously converted to gluconic acid in the presence of water (at neutral pH), where the gluconate and the proton products are generated. Thus, the fluctuation in the H⁺ concentration by the enzyme reaction can be used to detect glucose. The potentiometric response time of the disposable sensor was studied at a glucose concentration of 200 mg/dL, as shown in Fig. 4A. The response potential surged from 0 mV to 32.8 mV within 0.5 s in a steady-state response. As shown in Fig. 4B, the calibration curves for glucose detection were obtained at different concentrations of glucose in 0.1 M PBS (pH 7.4). The proposed sensor was very adequate at monitoring glucose levels in the blood as a tool for point-of-care diagnosis, although the sensor was comparable with other enzymatic and non-enzymatic potentiometric sensors (Table S1). The response shows a dynamic range from 30 to 500 mg/dL. The linear dependence yielded a regression equation of $\Delta E (\text{mv}) = (1.80 \pm 0.485) + (0.15 \pm 0.002) [C] (\text{mg/dL})$ with a correlation coefficient of 0.998. The detection limit was determined to be 17.23 ± 0.32 mg/dL from blank noise signals (95% confidence level, $k=3$, $n=10$). The reproducibility was examined using ten different sensors, and a relative standard deviation of ± 5.15% at a glucose concentration of 200 mg/dL was achieved. The storage lifetime of the sensor was examined by measuring a glucose sample concentration of 200 mg/dL with
three different sensor probes each day. The sensitivity of the sensors retained 95% of their initial sensitivity for up to 30 days (Fig. 4C). The prepared sensor probe can be stored for up to one month at room temperature.

3.6. Blood sample analysis

The reliability of the proposed sensor was evaluated by determining the glucose levels in whole blood (capillary blood) samples obtained from seven volunteers (n=7). 10 μL of capillary blood was loaded onto the Naﬁon/GOx-pTBA/AuZnOx probe, and then measuring the potential change. Before analyzing the real samples, a calibration curve was constructed using standard artificial blood at three different glucose levels because of the reducing matrix effect of hematocrit in whole blood. In this case, the result yielded a regression equation of $\Delta E (\text{mV}) = (19.50 \pm 0.63) +$...
ranging from 30 to 500 mg/dL with a detection limit of 0.32 mg/dL (k = 3, n = 10). Furthermore, the sensor was inert to interfering species, such as acetaminophen, ascorbic acid, dopamine, and other small organics, because the potentiometric method does not require applying any anodic potential. The pH and glucose sensors were successfully applied to measure human saliva, urine (for pH measuring) and whole blood samples (for glucose measuring), which were in excellent agreement with the commercially available pH (Orion glass pH electrode) and glucose (One Touch ultra™, Lifescan Inc.) meters, respectively.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.12.002.

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