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A biosensor based on the self-entrapment of glucose oxidase within biomimetic silica nanoparticles induced by a fusion enzyme

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ABSTRACT

We constructed a fusion protein (GOx–R5) consisting of R5 (a polypeptide component of silaffin) and glucose oxidase (GOx) that was expressed in *Pichia pastoris*. Silaffin proteins are responsible for the formation of a silica-based cell matrix of diatoms, and synthetic variants of the R5 protein can perform silicification *in vitro* [1]. GOx secreted by *P. pastoris* was self-immobilized (biosilicification) in a pH 5 citric buffer using 0.1 M tetramethoxysilane as a silica source. This self-entrapment property of GOx–R5 was used to immobilize GOx on a graphite rod electrode. An electric cell designed as a biosensor was prepared to monitor the glucose concentrations. The electric cell consisted of an Ag/AgCl reference electrode, a platinum counter electrode, and a working electrode modified with poly(neutral red) (PNR)/GOx/Nafion. Glucose oxidase was immobilized by fused protein on poly(neutral red) and covered by Nafion to protect diffusion to the solution. The morphology of the resulting composite PNR/GOx/Nafion material was analyzed by scanning electron microscopy (SEM). This amperometric transducer was characterized electrochemically using cyclic voltammetry and amperometry in the presence of glucose. An image produced by scanning electron microscopy supported the formation of a PNR/GOx complex and the current was increased to 1.58 $\mu A\,cm^{-1}$ by adding 1 mM glucose at an applied potential of −0.5 V. The current was detected by way of PNR-reduced hydrogen peroxide, a product of the glucose oxidation by GOx. The detection limit was 0.67 mM (S/N = 3). The biosensor containing the graphite rod/PNR/GOx/Nafion detected glucose at various concentrations in mixed samples, which contained interfering molecules. In this study, we report the first expression of R5 fused to glucose oxidase in eukaryotic cells and demonstrate an application of self-entrapped GOx to a glucose biosensor.

1. Introduction

Immobilized biomolecules are more useful than their soluble counterparts in an industrial enzyme system owing to their stability and ease of handling. Enzymes can be immobilized by non-covalent adsorption, covalent bonds, entrapment, and cross-linking [2]. Biosilicification is an entrapment method that utilizes biological extracts (including protein and carbohydrates). It is related to the type of silicification that occurs in diatoms, sponges, and grass [3]. Biomimetic synthesis has become of interest recently because it can take place under mild conditions with greater ease [4]. Silaffin is a polycationic peptide (R_x) that is present in diatoms and induced nano-structured silica precipitates [5]. Synthetic variants of the R5 protein (a repeating unit of silaffin polypeptides) can perform silicification *in vitro* [1]. Several groups have used silaffin to induce biosilicification, which can immobilize fusion enzymes [6,7]. Our group also demonstrated that silaffin-fused proteins (Green Fluorescence Protein, GFP-R_x) were biosilicificated at lower concentrations that were 14–17 times less than those of synthetic polypeptides (R_x) [8]. In addition to the requirement of less protein to induce silicification, an enzyme fused with a silaffin protein takes advantage of the composite sol–gel electrode, leading to increased sensitivity due to voids or less dense film, biocompatibility without any formation of covalent bonds to the enzyme, and good adhesion to the supporting electrode [9,10]. Here, we present the expression of the novel fusion protein glucose oxidase–R5 (GOx–R5) in *Pichia pastoris*, and an application involving the self-immobilization of the enzyme on an electrode, which can function as a biosensor.

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This process functions by taking advantage of the simple and rapid immobilization of the enzyme, which does not need an additional catalyst for silicification, similar to that of poly-L-lysine [11,12] and lysozyme [13,14], or any treatment such as electrodeposition [15].

Glucose oxidase (GOx) is one of many enzymes used in biosensors due to its high enzymatic activity. GOx catalyzes the oxidation of glucose to gluconolactone and the subsequent hydrolysis of gluconolactone to gluconic acid. It was demonstrated that the following materials can increase the conductivity of GOx: carbon nanotubes (CNT) [16–18], platinum and silica nanoparticles [19], tetrathiafulvalene (TTF) [20], and Tm_2O_3 nanoparticles [21]. Moreover, electrochemically prepared poly(neutral red) (PNR) can mediate the electron transfer from glucose to a cathode [22–25]. Neutral red (NR) has a phenazine structure, similar to that of flavins, which can mimic NADH dehydrogenase [24]. To overcome the handicap of the limited GOx production by *P. pastoris* in the laboratory, we used PNR as an electron shuttle to accelerate the electron transport between the electrodes and chemicals produced by glucose oxidase.

Unmodified silica is highly hydrophilic [26,27]. The entrapment of GOx by biogenic silica production creates a favorable environment for accessing diffused glucose and H_2O_2. In situations in which enzymes are lipophilic, such as the case with lipases, siloxane can be applied to enhance the level of hydrophobicity using alkylsilanes [28]. Additionally, a sol–gel matrix can improve the chemical and thermal stability of enzymes [6,7,13,27].

The primary goal of this study was to design and express a glucose oxidase–R5 fusion protein in *P. pastoris*. To demonstrate a possible application of the GOx–R5 fusion protein, we used self-entrapment characteristics of GOx–R5 to immobilize GOx on an electrode, which could function as a biosensor.

2. Materials and methods

2.1. Strains, plasmid construction, and transformation

The GOx gene was cloned from *Aspergillus niger* KCTC 6278, which was obtained from the Korean Collection for Type Cultures (KCTC). *A. niger* KCTC 6278 was cultured on potato dextrose agar (PDA, BD, USA). The GOx gene was cloned into yeast expression plasmid pGAPZαC (Invitrogen, USA) and expressed in wild-type *P. pastoris* X-33 yeast (Invitrogen).

Chromosomal DNA from *A. niger* KCTC 6278 was extracted with the PowerSoil® DNA Isolation Kit (Mo Bio, USA). The GOx gene from *A. niger* KCTC 6278 was cloned into pGAPZαC using the methods reported previously [29]. Briefly, the GOx gene was amplified by polymerase chain reaction (PCR) with primers pGO1f (5′-CCT TTC CTC TCT CAT TCC CTC A-3′) and pGO1r (5′-AAT GCC CTT GTT TGG TAG TAA T-3′). The resulting PCR product was re-amplified with PCR primers pGOef (5′-ATC CAT CGA TGA GCA ATG GCA TTG AAG CCA GCC TCC T-3′) and pGOer (5′-ATT AGC GGC CGC CTG CAT GGA AGC ATA ATC TTC CAA GAT AG-3′), where pGOef and PGOer contained restriction enzyme sites for Cla I (ATCGAT) and Not I (GCGGCCGC), respectively. The second PCR product and pGAPZαC were digested with Cla I and Not I, and ligated using the LigaFastTM Rapid DNA Ligation System (Promega, USA). pGAPZαC containing the GOx gene was named pGAP-GOx.

The R5 peptide, Ser-Ser-Lys-Lys-Ser-Gly-Ser-Tyr-Ser-Gly-Ser-Lys-Gly-Ser-Lys-Arg-Arg-Ile-Leu, was attached to the C-terminus of GOx. For the construction of the fusion enzyme, the GOx gene in pGAP-GOx was modified with a polyhistidine (HHHHHH) sequence (5′-CAT CAT CAT CAT CAT CAT-3′) and R5 (5′-TCT TCT AAG AAG TCT GGT TCT TAC TCT GGT TCT AAG GGT TCT AAG AGA AGA ATC TTG-3′), at the N-terminus and C-terminus of GOx gene, respectively. Gly-Gly-Gly-Ser (GGGS; 5′-GGT GGT GGT TCT-3′) was used as a linker peptide in an attempt to reduce steric interference and was inserted between the polyhistidine, R5 and the GOx genes. Insertion of the polyhistidine-GGGS sequence into the N-terminal region of the GOx gene was accomplished using a 64 bp DNA fragment which was amplified with primers HGf (5′-GAG GCT GAA GCA TCG CAT CAT CAT CAT CAT CAT GGT GGT GGT TCT G-3′) and HGr (5′-TGC CAT TGC TCA TCG ATC CAG AAC CAC CAC CAT GAT GAT G-3′). After digestion of the 64 bp PCR product and pGAP-GOx with Cla I (ATCGAT), the two DNA fragments were ligated and the resulting plasmid was named pGAP-HGOx. Insertion of GGGS-R5 into the C-terminus of the GOx gene contained in pGAP-HGOx was accomplished using a 107 bp fragment of DNA, which was amplified with the primers LR5f (5′-TCC ATG CAG GCG GCC GCC GGT GGT GGT TCT TCT TCT AAG AAG TCT GGT TCT TAC TCT GGT TCT AAG G-3′) and LR5r (5′-AGA AAG CTG GCG GCC CCT CAC AAG ATT CTT CTC TTA GAA CCC TTA GAA CCA GAG TAA GAA CCA GAC-3′). The 107 bp PCR product and pGAP-HGOx were digested with Not I and ligated. The ligation product was named pGAP-HGOxR5 (Fig. 1). Ligations of the digested PCR products and pGAP-GOx or pGAP-HGOx were accomplished using an In-FusionTM 2.0 Dry-Down PCR Cloning Kit (Clontech, USA). The plasmid pGAP-HGOxR5 was expected to express a polypeptide having the following sequence, HHHHHH-GGGS-GOx-GGGS-SSKKSGSYSGSKGSKRRIL.

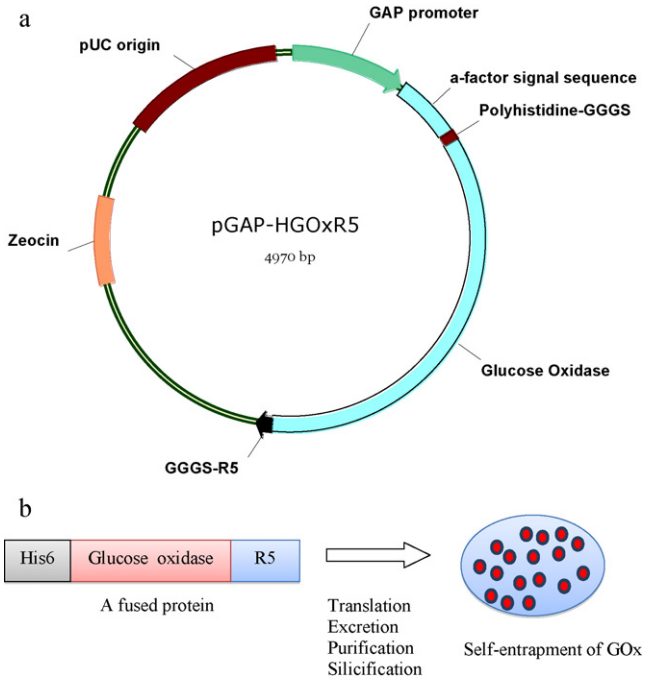

Fig. 1. (a) Structure of the yeast expression plasmid pGAP-HGOxR5, designed for the production of the GOx–R5 fusion protein in *P. pastoris*. (b) The overall scheme to design, express, and self-entrap the fusion protein.

The plasmid pGAP-HGOxR5 was transformed into *P. pastoris* X-33 to obtain a transformant expressing GOx–R5. Yeast transformations were performed using electroporation (Gene Pulser II System, Bio-Rad Laboratories, USA). Transformants were randomly selected after growth on a selective YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) containing 300 mg/l Zeocin (0.03%, w/v).

Expressed GOx–R5 was purified using an IMAC HyperCel (Pall, USA) that was the sorbent for immobilized metal affinity chromatography, according to the manufacturer's instructions, and used for the measurement of enzymatic activity and silicification.

2.2. Recombinant protein expression and purification

P. pastoris was aerobically incubated at 150 rpm in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) fructose) and sealed with a silistopper at 30 °C for improved air permeability. Fructose was used instead of glucose for GOx–R5 collection because GOx can oxidize glucose added to the media as a carbon source.

After 72 h of cultivation, the activity of GOx excreted from *P. pastoris* reach a plateau (data not shown), and the supernatant was collected from the pelleted biomass after centrifugation. GOx–R5 was harvested from the supernatant using the QIA*express*® Ni-NTA Fast Start kit (Qiagen, Valencia, CA), which can purify recombinant 6-His-tagged proteins.

2.3. Evaluation of GOx activity

The activity of glucose oxidase was kinetically analyzed by continuous spectrophotometric rate determination. Peroxidase reacts with hydrogen peroxide produced by GOx in the presence of glucose, resulting in a colorimetric change of *o*-dianisidine. After GOx (0.02 mL) was injected into a reaction solution (0.58 mL, 0.17 mM *o*-dianisidine and 1.72% (w/v) glucose), the increase in absorbance was measured by a UV–vis spectrophotometer (UV mini-1240, Shimadzu, Japan) at 500 nm, after which it was continuously monitored by an interfaced personal computer loaded with UV Probe 2.20 software (Hiroshima, Japan). The quantity of GOx produced by *P. pastoris* was measured using a NanoDrop in protein assay mode (Thermo Scientific, Wilmington, DE, USA).

2.4. Preparation of the GOx immobilized electrode

Before modification, a bare graphite rod electrode (GR, 6.0 mm diameter) was cut and polished with 0.05 μm alumina powder on a microfiber cloth. The working

surface area was 0.28 cm^2, and the side surface was sealed with paraffin film to prevent contact with the solution.

Poly(neutral red) can be prepared after reactive radicals are electrically formed and react with one another [22]. PNR was formed on the GR by cycling applied potentials between −1.0 and +1.0 vs. Ag/AgCl at a scan rate of 50 mV/s for 20 cycles in 1 mM neutral red (Sigma Aldrich Korea, Korea), 0.05 M potassium phosphate buffer, and 0.1 M KNO_3 [22]. The next day, 20 μL of GOx–R5 (14 unit/mL min) was dropped onto the PNR and air dried. Biosilicification carried out using 25 μL of citric buffer (0.1 M, pH 5) and 5 μL 1 M TMOS (tetramethyl orthosilicate, Sigma Aldrich Korea, Korea) upon adsorption of GOx–R5/PNR. Preliminary data suggests that TMOS did not affect GOx activity (data not shown). Nafion (5%, w/t, in alcohol, Sigma Aldrich Korea, 4 μL) was used to coat the surface of the PNR to prevent its sudden release from the GR. The modified electrode was stored at 4 °C in phosphate buffer (pH 7) and warmed to room temperature prior to use.

2.5. Electrochemical measurements

Cyclic voltammetry and amperometric measurements were carried out using a Potentiostat/Galvanostat (273A, Princeton Applied Research, Princeton, NJ) controlled by the commercial software package Power Suite. A VC-2 voltammetry cell (BASi, West Lafayette, IN) with a coiled platinum wire and an Ag/AgCl electrode (saturated with KCl) were used as the counter electrode and reference electrode, respectively. The biosensor was operated at −500 mV (vs. Ag/AgCl) in a flow system containing equal volumes of potassium phosphate buffer (0.1 M, pH 6.2) and KCl (0.1 M) for amperometric detection at room temperature. The corresponding current was recorded every 3.6 s by an interfaced personal computer.

2.6. Morphological characterization

The surface morphology of the working electrode was analyzed by a field emission scanning electron microscope at 10 kV before and after the fusion enzyme was immobilized (Nova NanoSEM 200 scanning electron microscope, FEI Company). A specimen coated in PNR and a specimen coated with PNR/GOx–R5 (silicificated GOx on PNR) were prepared on separate graphite rods, and the upper portions were analyzed after cutting and fixing with an adhesive tape holder.

3. Results and discussion

3.1. Excretion of glucose oxidase fused with R5 and biosilicification

Glucose oxidase was excreted from *P. pastoris*, reaching its highest activity after 72 h of growth (data not shown). The amount of GOx immobilized by biosilicification was extrapolated from the measurement of the activity of GOx in the sol–gel matrix after biosilicification. It was dependent on the pH of the buffer solution used for biosilicification. Biosilicificated GOx was most abundant at pH 5 (0.25 unit/mg), compared to the amounts at pH 6 (0.12 unit/mg) and at pH 7 (0.07 unit/mg) and also it was greater in the citric buffer than in the phosphate buffer (0.02 unit/mg). Expressed GOx only in *P. pastoris* (not containing any fused protein) flocculated at pH 7–8 but not at pH 5. Silicification occurred only with fused GOx with R5 in the presence of TMOS at pH 5.

Prokaryotes accumulate the fusion protein in the intracellular environment, whereas eukaryotes secrete it as a foreign protein. We took advantage of the expression in yeast and easily purified the fusion protein without the need for cell lysis. The main advantage of utilizing the fusion protein is that the enzyme can be immobilized without an additional treatment. Furthermore, the metal ion complex-forming peptide $(His)_6$ fused with GOx–R5 was directly retrieved from the culture medium.

We previously reported the expression of a GFP-R_x chimera in *Escherichia coli* [8]. GFP can function as a monomer, but the full dimeric structure of GOx is needed for it to function. Additionally, GFP was originally derived from a prokaryote, whereas GOx was derived from a eukaryote. GOx activity was not observed in a GOx/R5 fusion protein which was not connected by a GGGS linker. R5–GOx fusion may inhibit the activity of GOx as a result of steric hindrance generated by R5-forming dimers, blocking its active site. In this study, GGGS was inserted between GOx and R5 to eliminate any steric hindrance.

3.2. SEM image of the surface of the working electrode

To identify the formation of biosilicification by the fusion enzyme, GOx–R5, the surface of the electrode was examined by scanning electron microscopy. However, instead of a glassy carbon electrode, a graphite rod was used as an electrode (a template for biosilicification) due to ease of specimen preparation. Neutral red was well polymerized on the surface of the graphite rods by an electrochemically induced radical reaction, and it was coated onto the end of electrode (Fig. 2a). After a drop of the GOx–R5 in 0.1 M TMOS on PNR, GOx was immobilized by biosilicification. Immobilized GOx was typical spherical particles form on the PNR (Fig. 2b). The size of the silica particles was approximately 200–500 nm, as shown in Fig. 2b. GOx fused with R5 was immobilized efficiently and became entrapped on the PNR (Fig. 2c).

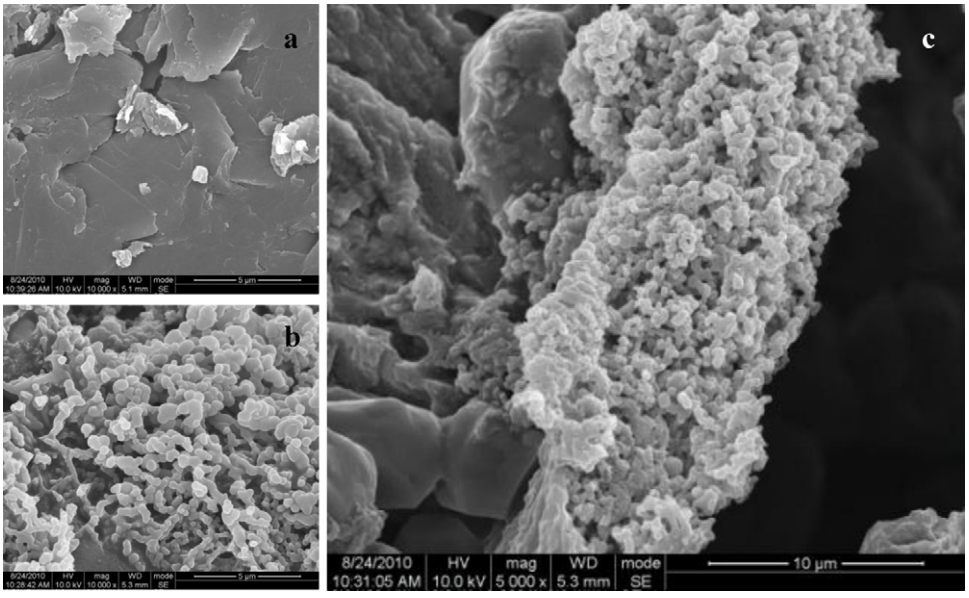

Fig. 2. A surface image taken by scanning electron microscopy before GOx immobilization on neural red polymers (a), a top view after GOx–R5 fusion protein induced silicification (b), and side view (c).

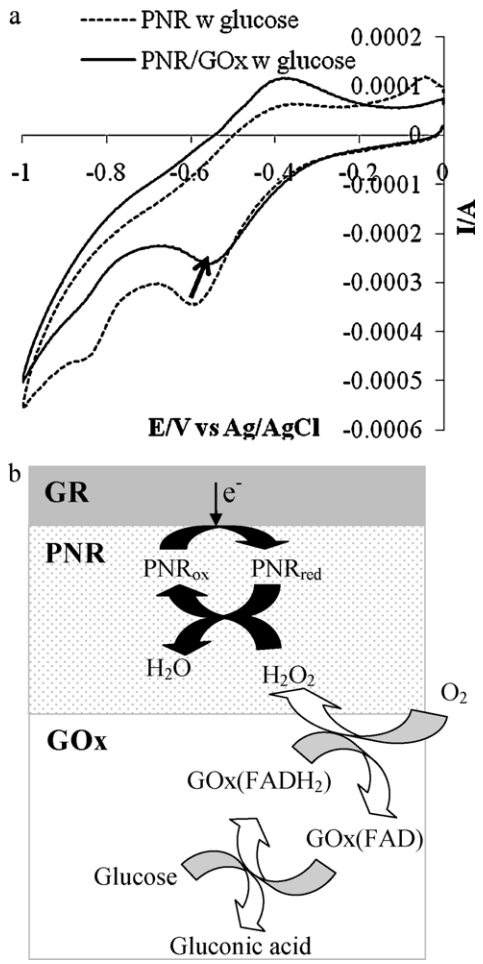

Fig. 3. (a) The cyclic voltammogram of PNR/GR (dashed line) and GOx/PNR/GR (unbroken line) in 20 mM glucose and 0.05 M KCl, 0.05 M pH 6.2 phosphate buffer. The reduction peak shifted due to the reduction of H_2O_2. (b) A scheme representing the reaction mechanism occurring at the GOx/PNR/GR electrode, which allowed for hydrogen peroxide detection.

3.3. Role of PNR in glucose oxidation

Co-silicification of GOx and carbon nanotubes (CNT) did not produce cyclic voltammetry peaks in the relevant potential range (data not shown), unlike in a previous study [13]. The overall dose of GOx was limited because excreted GOx expressed with fused R5 protein from cultured *P. pastoris* was used. To aid electron transport, poly(neutral red) was applied to the GR, and a well-defined redox peak was identified, as shown in Fig. 3a.

Two reactions involving PNR are possible, as shown in Eqs. (1) and (2) [22].

$$\text{anode}: \quad PNR_{ox} + \text{glucose} \xrightarrow{GOx} PNR_{red} + \text{gluconic acid} \tag{1}$$

$$\text{cathode}: \quad PNR_{red} + H_2O_2 \rightarrow PNR_{ox} + H_2O \tag{2}$$

In the presence of glucose, the cathodic potential of a GR coated with PNR shifted when GOx was immobilized on the PNR coat. In our GR/PNR/GOx system, the electrons flow from an electrode to PNR, as shown in Eq. (2) and Fig. 3b. The electrode served as the cathode, and after PNR_{ox} was reduced, PNR_{red} was re-oxidized by H_2O_2 reduction to H_2O. The electrode responded to H_2O_2, a product produced by GOx. A potential of −0.5 V vs. Ag/AgCl was fixed for the amperometric detection of glucose. This potential is similar to the redox potential of the cofactor FAD/$FADH_2$ and similar to the potential we observed with a GOx/Tm_2O_3/Nafion/GC electrode

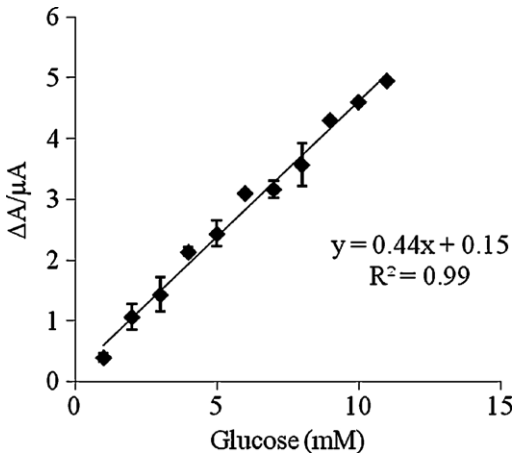

Fig. 4. Amperometric response of the GOx/PNR/GR electrode to successive additions of glucose solution to 8 mL of buffered solution (0.05 M KCl and 0.05 M pH 6.2 phosphate buffer).

(−0.48 V) [21]. This potential was also similar to that observed with a GC electrode modified with graphene (−0.49 V) [30].

3.4. Amperometric determination of currents associated with glucose

A current change was observed on the GR/PNR/GOx electrode with successive additions of glucose at a working potential of −0.5 V vs. Ag/AgCl. The reduction current of H_2O_2 reached a steady state within 15 s. The amperometric current response for glucose at the GOx/PNR/GR electrode was linear, with a range from 1 mM to 11 mM glucose (Fig. 4) and a correlation coefficient of 0.99 ($n = 3$). From the regression equation, the sensitivity and the detection limit of the GOx/PNR/GR electrode were determined to be 1.58 μA/mM cm^2 and 0.67 mM (S/N = 3), respectively. The range of blood glucose level in healthy people is 4–8 mM while that in diabetic patients is much wider, at 2–30 mM [31]. The linear response range of the GOx/PNR/GR electrode was from 1 to 11 mM, thus satisfying the requirements for use as a biosensor for normal blood glucose detection.

The biosensor functioned by using poly(neutral red) to detect peroxide, a product of glucose oxidase by GOx. PNR successfully enhanced the electron flow despite the fact that only a small amount of GOx became immobilized (14 unit/mL min). Biosilification occurred efficiently when R5–GOx was pre-dried on PNR after the addition of TMOS, however; the insulating matrix contained silica particles, which limited the detection ability. Co-silicification with a conducting material such as a carbon nanotube (CNT) [13] may be applied to increase the sensitivity. Recently, Ivnitski et al. showed the mild immobilization of GOx with CNT and demonstrated widely applicable bioelectrocatalysis [13]. Generally, commercially available GOx has been used in the study of GOx biosensors, and it is highly concentrated (e.g., 160 unit/mg, GOx from Sigma Aldrich). However, we gained GOx (ca. 14 unit/mg) from a *P. pastoris* culture. Due to the limitation of amount of GOx concentrated from the *P. pastoris* culture and because of the limited immobilization efficiency (a fraction of immobilized GOx to the expressed GOx/R5), activity of GOx upon co-silicification with CNT was not observed. Therefore, highly concentrated-fused enzymes may be applicable to co-silicification with CNT in future research.

3.5. Interference test to observe substrate selectivity

Two common substances that interfere with glucose determination are ascorbic acid (AA, 50–70 μM at physiological levels [32])

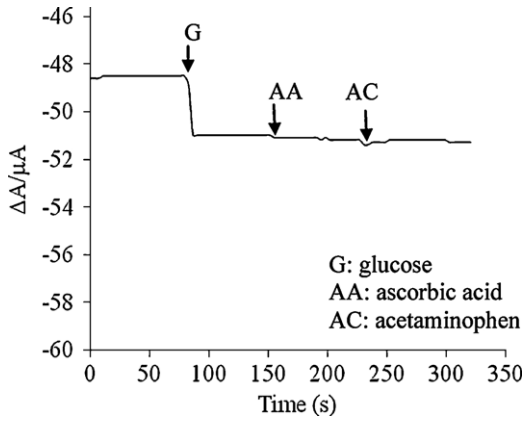

Fig. 5. Interference study using ascorbic acid (0.1 mM) and acetaminophen (0.05 mM) to monitor glucose (5 mM) by exposing the biosensor (PNR/GOx–R5).

and acetaminophen (AC, 60–130 μM at physiological levels [32]), both of which are electroactive in blood. The interference effects of AA (0.1 mM) and AC (0.05 mM) are negligible when glucose is found at concentrations of 5 mM (4–7 mM in normal healthy blood [33]) (Fig. 5). Acetaminophen has low solubility and was prepared at a relatively low concentration. At lower negative potentials, ascorbic acid (oxidation potential −0.05 V vs. Ag/AgCl, at pH 7.4) and acetaminophen (0.26 V) are not oxidized [34]. The lower potential (−0.2 V) allowed for the selective detection of glucose and eliminated interfering substances, while higher potentials could not eliminate interfering substances with covalently immobilized GOx on carbon nanotubes (+0.4 V) [35].

4. Conclusions

We designed a fusion protein containing R5, a protein that controls silicification, and an enzyme, glucose oxidase. To the best of our knowledge, this is the first report describing a R5-linked silicification protein with an enzyme from a eukaryotic organism. To test the potential application of the GOx–R5 fusion protein, a biosensor was prepared to determine the glucose concentration. A bilayer configuration was used on the electrode's surface. A current increase 1.58 μA cm^{-1} after adding 1 mM glucose at an applied potential of −0.5 V was observed.

The self-entrapment characteristics of the GOx–R5 fusion protein can be used to immobilize enzymes. Our findings suggest that this novel enzyme immobilization technique can be applied to various types of biomolecules. This technique may also be applied to enzymes and proteins already used in engineering for additional purposes, such as silicification.

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References

[1] Knecht MR, Wright DW. Functional analysis of the biomimetic silica precipitating activity of the R5 peptide from *Cylindrotheca fusiformis*. Chem Commun 2003;24:3038–9.
[2] Bornscheuer UT. Immobilizing enzymes: how to create more suitable biocatalysts. Angew Chem Int Ed 2003;42:3336–7.
[3] Patwardhan SV, Clarson SJ, Perry CC. On the role(s) of additives in bioinspired silicification. Chem Commun 2005:1113–21.
[4] Luckarift HR, Spain JC, Naik RR, Stone MO. Enzyme immobilization in a biomimetic silica support. Nat Biotechnol 2004;22:211–3.
[5] Kröger N, Deutzmann R, Sumper M. Polycationic peptides from diatom biosilica that direct silica nanosphere formation. Science 1999;286:1129–32.
[6] Marner IIWD, Shaikh AS, Muller SJ, Keasling JD. Enzyme immobilization via silaffin-mediated autoencapsulation in a biosilica support. Biotechnol Prog 2009;25:417–23.
[7] Chien L-J, Lee C-K. Biosilicification of dual-fusion enzyme immobilized on magnetic nanoparticle. Biotechnol Bioeng 2008;100:223–30.
[8] Nam DH, Won K, Kim YH, Sang BI. A novel route for immobilization of proteins to silica particles incorporating silaffin domains. Biotechnol Prog 2009;25:1643–9.
[9] Florescu M, Barsan M, Pauliukaite R, Brett CMA. Development and application of oxysilane sol–gel electrochemical glucose biosensors based on cobalt hexacyanoferrate modified carbon film electrodes. Electroanalysis 2007;19:220–6.
[10] Walcarius A, Mandler D, Cox JA, Collinson M, Lev O. Exciting new directions in the intersection of functionalized sol–gel materials with electrochemistry. J Mater Chem 2005;15:3663–89.
[11] Tian F, Wu W, Broderick M, Vamvakaki V, Chaniotakis N, Dale N. Novel microbiosensors prepared utilizing biomimetic silicification method. Biosens Bioelectron 2010;25:2408–13.
[12] Buiculescu R, Hatzimarinaki M, Chaniotakis N. Biosilicated CdSe/ZnS quantum dots as photoluminescent transducers for acetylcholinesterase-based biosensors. Anal Bioanal Chem 2010:1–7.
[13] Ivnitski D, Artyushkova K, Rincón RA, Atanassov P, Luckarift HR, Johnson GR. Entrapment of enzymes and carbon nanotubes in biologically synthesized silica: glucose oxidase-catalyzed direct electron transfer. Small 2008;4:357–64.
[14] Ramanathan M, Luckarift HR, Sarsenova A, Wild JR, Ramanculov EK, Olsen EV, et al. Lysozyme-mediated formation of protein-silica nano-composites for biosensing applications. Colloids Surf B Biointerfaces 2009;73:58–64.
[15] Jia W-Z, Wang K, Zhu Z-J, Song H-T, Xia X-H. One-step immobilization of glucose oxidase in a silica matrix on a Pt electrode by an electrochemically induced sol–gel process. Langmuir 2007;23:11896–900.
[16] Ivnitski D, Branch B, Atanassov P, Apblett C. Glucose oxidase anode for biofuel cell based on direct electron transfer. Electrochem Commun 2006;8:1204–10.
[17] Liu Y, Wang M, Zhao F, Xu Z, Dong S. The direct electron transfer of glucose oxidase and glucose biosensor based on carbon nanotubes/chitosan matrix. Biosens Bioelectron 2005;21:984–8.
[18] Gavalas VG, Law SA, Christopher Ball J, Andrews R, Bachas LG. Carbon nanotube aqueous sol–gel composites: enzyme-friendly platforms for the development of stable biosensors. Anal Biochem 2004;329:247–52.
[19] Ren X, Meng X, Tang F, Zhang L. Biosensor enhanced by glucose oxidase biomimetic membrane containing the platinum and silica nanoparticles. Mater Sci Eng C 2009;29:2234–8.
[20] Asav E, Akyilmaz E. Preparation and optimization of a bienzymic biosensor based on self-assembled monolayer modified gold electrode for alcohol and glucose detection. Biosens Bioelectron 2010;25:1014–8.
[21] Li Y, Gao Y, Zhou Y, Liu Y, Liu J. Glucose oxidase-Tm_2O_3 nanoparticle-modified electrode for direct electrochemistry and glucose sensing. J Electroanal Chem 2010;642:1–5.
[22] Pauliukaite R, Ghica M, Barsan M, Brett C. Characterisation of poly(neutral red) modified carbon film electrodes; application as a redox mediator for biosensors. J Solid State Electrochem 2007;11:899–908.
[23] Pauliukaite R, Chiorcea Paquim AM, Oliveira Brett AM, Brett CMA. Electrochemical EIS and AFM characterisation of biosensors: trioxysilane sol–gel encapsulated glucose oxidase with two different redox mediators. Electrochim Acta 2006;52:1–8.
[24] Ghica M, Brett C. Development of novel glucose and pyruvate biosensors at poly(neutral red) modified carbon film electrodes. Application to natural samples. Electroanalysis 2006;18:748–56.
[25] Qu F, Yang M, Chen J, Shen G, Yu R. Amperometric biosensors for glucose based on layer-by-layer assembled functionalized carbon nanotube and poly (neutral red) multilayer film. Anal Lett 2006;39:1785–99.
[26] An D, Wang Z, Zhao X, Liu Y, Guo Y, Ren S. A new route to synthesis of surface hydrophobic silica with long-chain alcohols in water phase. Colloids Surf Physicochem Eng Aspects 2010;369:218–22.
[27] Braun S, Shtelzer S, Rappoport S, Avnir D, Ottolenghi M. Biocatalysis by sol–gel entrapped enzymes. J Non-Cryst Solids 1992;147–148:739–43.
[28] Reetz MT, Zonta A, Simpelkamp J, Rufinska A, Tesche B. Characterization of hydrophobic sol–gel materials containing entrapped lipases. J Sol-Gel Sci Technol 1996;7:35–43.
[29] Yamaguchi M, Tahara Y, Nakano A, Taniyama T. Secretory and continuous expression of *Aspergillus niger* glucose oxidase gene in *Pichia pastoris*. Protein Expr Purif 2007;55:273–8.
[30] Shan C, Yang H, Song J, Han D, Ivaska A, Niu L. Direct electrochemistry of glucose oxidase and biosensing for glucose based on graphene. Anal Chem 2009;81:2378–82.
[31] Heller A. Implanted electrochemical glucose sensors for the management of diabetes. Annu Rev Biomed Eng 1999;1:153–75.
[32] Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG. Goodmann and Gilmans's the pharmacological basis of therapeutics. 9th ed. New York, NY: McGraw-Hill; 1996.
[33] Mathews CK, Holde KEV. Biochemistry. Reading, MA: Benjamin/Cummings; 1990.
[34] Arai G, Shoji K, Yasumori I. Electrochemical characteristics of glucose oxidase immobilized in poly(quinone) redox polymers. J Electroanal Chem 2006;591:1–6.
[35] Lin Y, Lu F, Tu Y, Ren Z. Glucose biosensors based on carbon nanotube nanoelectrode ensembles. Nano Lett 2003;4:191–5.