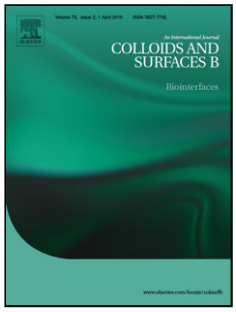

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Protein and polysaccharide-composite sol-gel silicate film for an interference-free amperometric glucose biosensor

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ABSTRACT

A novel permselective, organic-inorganic-hybrid, sol-gel silicate-film was chemically modified on an anodized platinum (Pt) electrode surface to form a selective, sensitive and interference-free amperometric glucose biosensor. This permselective hybrid sol-gel film consists of three organo-silanes [i.e., 3-aminopropyltriethoxysilane (APTES); tetraethoxysilane (TEOS); triethoxy-1H,1H,2H,2H-tridecafluoro-n-octylsilane (FAS)] and two biomacromolecules [i.e., bovine serum albumin (BSA) and a chitosan (CHIT)]. After the addition of the film to the Pt electrode, glucose oxidase (GOx) was covalently immobilized within the film with glutaraldehyde. The incorporation of the BSA and CHIT not only enhanced the permselectivity of H_2O_2 but also improved the activity of the immobilized GOx. The CHIT effectively suppressed any swelling of the film. Moreover, the conjugation of the FAS was especially effective in reducing the interference currents of AA and UA to levels less than 1/400 and 1/300 of the current of H_2O_2. The resulting organic-inorganic-hybrid sol-gel-film-based amperometric glucose biosensor exhibited rapid and sensitive responses to glucose (100 % response in < 3 s, sensitivity: 1.84 μA/mM, detection limit: 0.032 mM), and the highly selective determination of glucose was possible, even in the presence of 0.1 mM AA and UA.

Keywords: amperometric glucose biosensor, organic-inorganic hybrid sol-gel film; interference-free; permselectivity; hydrogen peroxide

1. Introduction

Highly selective, sensitive and rapid determination of hydrogen peroxide (H_2O_2) is important for the fabrication of oxidase-based biosensors, which detect H_2O_2 produced through oxidase reactions. Anodic determination of H_2O_2 with a platinum (Pt) electrode is the primary method because it is highly sensitive and convenient. However, the relatively high working potential (+0.6 V *vs.* Ag/AgCl) leave the system vulnerable to electrochemical interference caused by easily oxidizable species that are present in body samples, such as ascorbic acid (AA) and uric acid (UA). The Pt electrode oxidizes these interferents in addition to H_2O_2, which leads to a current response corresponding to a positive error. Therefore, utilizing an appropriate coating layer (i.e., permselective films) that allows the permeation of analytes (H_2O_2) but prevents the interferents from reaching the electrode surface eliminates these electrochemical interferences. Until recently, a variety of polymeric films has been employed for this purpose: e.g., cellulose acetate film [1, 2]. nafion film [3], polyion-complex films [4,5], polyelectrolyte multilayer film [6], LB film [7], and electro-polymerized films [8-10].

Sol-gel processing is an attractive methodology for the fabrication of novel molecular recognition interfaces on electrode surfaces [11, 12] because the sol-gel matrix is chemically inert, simple to prepare and has stable host properties. Additionally, the sol-gel matrix's porous structure usually provides a suitable microenvironment for the encapsulation or modification of biomolecules [13, 14]. Based on these features, a sol-gel matrix that contains immobilized enzymes, which is prepared on the electrodes surface, can be utilized to construct various electrochemical biosensors (i.e., enzyme electrodes) [15-22, 30-33, 35-41]. However, to be useful for real sample analysis, these sol-gel-film-based enzyme electrodes still require extra interference-eliminating layers

because the sol-gel film alone does not possess permselectivity toward H_2O_2 [16-20].

Recently, we have reported that the incorporation of bovine serum albumin (BSA) into the silica-based sol-gel composite film enhanced the permselectivity toward H_2O_2 against possible electrochemical interferents, such as AA and UA [23]. Two silicates [i.e., 3-aminopropyltriethoxysilane (APTES) and tetraethoxysilane (TEOS)] were used in the sol-gel matrix. The resulting BSA-composite sol-gel silicate-film was covalently attached to an anodized Pt electrode surface via a OH group [24, 25]. In this case, 1) incorporation of BSA within the sol-gel film, 2) further heat treatment (100 ˚C for 1h) and 3) cross-linking with glutaraldehyde (GA) effectively enhanced the permselectivity of H_2O_2, most likely due to a size-exclusion mechanism [23].

As an application of this bio-sol-gel film, we have developed an interference-free amperometric glucose biosensor by covalently immobilizing glucose oxidase (GOx) within the permselective sol-gel film. In addition to APTES, TEOS and BSA, we employed triethoxy-1H,1H,2H,2H-tridecafluoro-n-octylsilane (FAS) and chitosan (CHIT) as additional components of the film. The FAS effectively suppressed the interfering currents, and the CHIT was successfully prevented any swelling of the film. The resulting APTES-TEOS-FAS-BSA-CHIT-GOx-film-modified Pt electrode exhibited highly selective responses to glucose with rapid response time (100% response in < 3 s), even in the presence of possible interferents, such as AA and UA (in physiological level). This is the first report concerning an interference-free amperometric glucose biosensor using a protein-polysaccharide hybrid sol-gel silicate-film as a permselective interference-eliminating layer.

2. Experimental

2.1. Reagents and materials

Pt-wire (99.99%, 0.5 mm in diameter) was purchased from Tanaka Co. (Tokyo, Japan). 3-aminopropyltriethoxysilane (APTES) was purchased from Aldrich Chemical Co. (Milwaukee, USA). Triethoxy-1H,1H,2H,2H-tridecafluoro-n-octylsilane (FAS) was purchased from Tokyo Kasei Kogyo Ltd (Tokyo, Japan). Tetraethoxysilane (TEOS), bovine serum albumin (BSA), chitosan (CHIT), ascorbic acid (AA), uric acid (UA), 30 % hydrogen peroxide, 25 % glutaraldehyde (GA) aqueous solution and glucose oxidase (GOx, EC 1.1.3.4., 200 units/mg from *aspergillus niger*) were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and were used without further purification. Human serum was purchased from Sigma Co. (St. Louis, USA). Glucose-test-Wako (Wako Pure chemical Industries, Ltd., Osaka, Japan) was used for the spectrophotometric measurement of glucose in both beverages and serum. All other chemicals (Wako, Osaka, Japan) were analytical grade and used as received. All solutions were prepared using double-distilled deionized water.

2.2. Preparation of sol-gel film-modified Pt-wire electrode.

Pt-wire (0.5 mm in diameter, 50 mm in length) was used as the working electrode. Prior to modification with the sol-gel film, the Pt-wire was soaked in 0.1 M NaOH/ethanol solution for 2 h and then washed in an ultrasonic bath (40 kHz) (As One, US-1R, Osaka, Japan) for 5 min in distilled water. Next, the surface of the Pt-wire (25 mm length from the top end) was electrochemically cleaned in 0.5 M H_2SO_4 solution with repetitive potential scans (more than 20 times) from +1.3 V to -0.2 V at 0.1 V/s until the wave pattern characteristic of clean Pt was obtained. Finally, the Pt-wire was anodized for 10 min at +1.0 V (vs. Ag/AgCl) and washed thoroughly via ultrasonication

(40 kHz) in distilled water for 5 min. During this anodic treatment, Pt/O (Pt-OH) was introduced to the Pt surface; this oxidized layer on the Pt-surface allows the silane to be chemically coupled to the Pt surface [24, 25].

The sol-gel film-modified Pt-wire was prepared as follows: first, 10 % (w/w) APTES, TEOS and FAS ethanol solution (1 ml) were prepared separately; these solutions were combined (final volume ca. 3 ml) and were stirred (300 rpm) for 7 min at room temperature. Subsequently, 2 ml of 0.1 M phosphate buffer solution (pH 6.5), which contained both BSA (2 mg/ml) and CHIT (3 mg/ml), was added to the mixed solution and further stirred (300 rpm) at room temperature for 1 min to obtain a homogenous sol-gel precursor solution.

Next, a portion of the Pt-wire (15 mm length from the top end) was immersed into the sol-gel precursor solution in a micro-cubit. Subsequently, the sol-gel precursor solution with the immersed Pt-wire was ultrasonicated (40 kHz) for 30 min at 30 ˚C in a batch type ultrasound bath (As one, US-1R). During this treatment, the BSA and CHIT-entrapped hybrid sol-gel silicate film was chemically immobilized onto the Pt-wire surface.

Next, the Pt-wire electrode that was modified with the sol-gel film was washed via ultrasonication in ethanol (for 2 min), followed by distilled water (for 2 min), to remove any adsorbed species. The sol-gel film-modified Pt-wire was then allowed to stand in a drying oven (MOV-212F, SANYO Electric Co. Ltd., Osaka, Japan) at 100˚C for 1 h to promote silanol-condensation. This process is critical for obtaining the tightly packed three-dimensional sol-gel network structure that prohibits the approach of possible interferents (AA and UA) to the electrode surface and therefore results in the permselectivity toward H_2O_2 [23].

Finally, GOx was immobilized within the sol-gel composite film using the following protocol: the sol-gel film-modified Pt-wire was immersed in 0.1 M phosphate buffer solution (pH 6.5), which contained both GA (0.1%) and GOx (2 mg/ml), for 30 min under vacuum and for a further 20 h at ordinary room temperature and pressure. During this process, the GOx was covalently immobilized on the film through the cross-linking of the primary amino groups of GOx, APTES, BSA and CHIT.

2.3. Electrochemical measurements

Before the electrochemical measurements, the sides of the hybrid sol-gel film-modified Pt-wire were sealed with insulated parafilm, except for an 8 mm portion at the top (geometric effective surface area, ca. 12.8 mm^2). Constant-potential amperometry was carried out at an applied potential of +0.6 V *vs.* Ag/AgCl in a conventional three-electrode system composed of the sol-gel film-modified Pt electrode (working electrode), Pt-wire (auxiliary electrode, 1 mm diameter, 60 mm length) and a Ag/AgCl reference electrode (RE-1B, BAS, inner solution, 3M NaCl). Air-saturated 0.1 M phosphate buffer (10 ml, pH 6.5), which was prepared using KH_2PO_4 and K_2HPO_4, was used as the electrolyte solution. All electrochemical measurements were performed under ambient conditions with an ALS 611B electrochemical analyzer (BAS Co., Tokyo, Japan). After the steady-state background current had been obtained with continuous stirring (300 rpm) of the electrolyte solution with stirring bar (15 mm diameter), a 100 μL of aliquot of a substrate standard solution (10 mM) was added to the 10 ml electrolyte (final concentration is ca.0.1mM), and the current change was observed. Sample standard solutions for H_2O_2, AA and glucose were prepared with air-saturated 0.1 M phosphate buffer (pH 6.5). UA standard solution (10 mM) was prepared by using

air-saturated 0.1 M phosphate buffer (pH 8.0) because of low solubility in neutral pH solution.

2.4. SEM images of the hybrid sol-gel film-modified Pt-wire electrode

Scanning electron microscope (SEM) images of the bare-Pt wire and hybrid sol-gel-modified Pt wire electrode were obtained with a JSM-6330F (JEOL Ltd., Tokyo, Japan) operating at 5 kV.

2.5. Analysis of natural samples

The determination of the glucose concentration in beverage and human sera samples were carried out with the newly reported biosensor, as well as a commercially available spectrophotometric method (glucose-test kit: *o*-toluidine/boric acid method; Wako pure chemicals), and the analytical results were compared. For the biosensor measurements, the beverages samples were diluted 100 ~ 1000 times and sera samples were diluted 20 times with a 0.1 M phosphate buffer (pH 6.5); the diluted samples were filtered with a 0.45 μm membrane filter (Advantec, Dismic-3cp, Japan). After establishing a calibration curve, a 100 μl portion of a filtered sample was added and the glucose concentrations were determined via a standard calibration method. For the spectrophotometric method, the test-kit was manually used for the determination of the glucose concentration in same natural samples. Briefly, a natural sample (50 μL) was mixed with a color-generating reagent (5.0 mL) and heated to 100 ºC in water-bath for 8 min. After being cooled by flowing-water for 3 min, the absorbance was measured at 635 nm with a V-630 (Jasco Co. Ltd., Tokyo, Japan); pure water was used as a control.

3. Results and discussion

3.1. Amperometric responses to H_2O_2, glucose and possible electrochemical interferents

As described in the introduction, the primary aim of this study is the development of an interference-free, amperometric, glucose biosensor that uses a sol-gel-film-modified Pt-wire electrode. First, we compared the magnitude of the steady-state responses toward H_2O_2, AA, UA and glucose (i.e., the current difference of the steady-state current before and after the addition of samples) to those of various glucose-sensing electrodes modified with sol-gel films.

Table 1 summarizes the steady-state current responses toward H_2O_2, AA, UA and glucose (0.1 mM) measure with a variety of sol-gel film-modified Pt-wire electrodes. In all cases, GOx was immobilized within the film. A bare Pt electrode (No. 1) exhibited oxidation currents for all substrates at +0.6 V vs. Ag/AgCl. Therefore, it is clear that bare Pt-wire electrode does not allow selective determination of H_2O_2 in the presence of possible interferents. For the APTES-TEOS-GOx composite film-modified electrode (No. 2), the responses to the interferents (AA and UA) were suppressed to 10^{-8} A level. However, the response to H_2O_2 was also suppressed and the selectivity for H_2O_2 against these interferences was not sufficient. Furthermore, the response to glucose was rather small. These observations suggest that the APTES-TEOS-composite film has a densely packed three-dimensional structure, which prevents the permeation of not only the interferents but also the H_2O_2. This tendency is similar to previous results with the APTES/TEOS-film without GOx [23]. Therefore, the APTES-TEOS composite film is not suitable for use in an interference-free glucose biosensor.

In previous study, we reported that the incorporation of BSA within a

APTES-TEOS-based sol-gel film effectively enhanced the permselectivity toward H_2O_2 and against AA and UA [23]. The selectivity toward H_2O_2 and against AA and UA of the APTES-TEOS-BSA-GOx-film-modified electrode (No 3) was superior to that without BSA (No. 2); the response to glucose was larger than that without BSA. A BSA is an inert protein that possesses 30-35 reactive primary amino groups (*Lys* residues) and the co-immobilization of GOx and BSA within an alumina sol-gel matrix improved the activity of the GOx [20]. In addition, BSA is known to provide a thermo-stabilization effect on several enzymes via hydrophobic interaction [26]. Therefore, it is safe to speculate that the roles of the incorporated-BSA within the APTES-TEOS-BSA-GOx-composite sol-gel film are as follows: (1) to enhance the permselectivity of H_2O_2 and (2) to provide a suitable microenvironment to improve the activity of the immobilized GOx. However, the permselectivity toward H_2O_2 and against AA and UA of the APTES-TEOS-BSA-GOx-film (No. 3) is inferior compared with the previous APTES-TEOS-BSA film (without GOx) [23]., The lowered permselectivity of the APTES-TEOS-BSA-GOx film (NO. 3) may be caused by the gradual swelling of the film during the 21 h GOx-modification procedure because, in an analogous case, the permselectivity of the APTES-TEOS-BSA-film decreased during a 24 h storage period in buffer solution [23].

The incorporation of hydrophilic organic polymers and polyelectrolytes, such as poly(vinyl alchol), with 4-vinylpyridine derivatives into sol-gel silicate films suppress the swelling and cracking of the sol-gel films [21, 22]. A chitosan (CHIT), which is also known as non-acetylated or partially deacetylated chitin, is a natural biopolymer that possesses a primary amino group. CHIT was successfully employed as an enzyme immobilization matrix for various enzyme electrodes because it has excellent

film-forming abilities, good adhesion, biocompatibility, and high mechanical strength. [27-29]. The CHIT-blended sol-gel matrix has been successfully utilized for glucose biosensors [30-33]. The cross-linking with GA on a CHIT-polyamine film-coated electrode is effective for the selective amperometric determination of H_2O_2 in the presence of oxidizable interfering compounds, such as AA and UA [27]. Therefore, we made CHIT an additional component of the film. As expected, the permselectivity of the CHIT-blended sol-gel film (APTES-TEOS-BSA-CHIT-GOx-film) (No.4) was improved; the current ratios of H_2O_2 to AA and UA were increased almost two-fold. However because the current ratio of H_2O_2 and UA is less than 100, further improvement is still desirable.

Incorporation of a highly hydrophobic molecule (e.g., fluoroalkylsilane) into a hydrophilic polymer-film causes changes in the configuration of the polymer chains, as well as the overall morphology of the film [34]. We employed triethoxy-1H,1H,2H,2H-tridecafluoro-n-octylsilane (FAS), which possesses flexible and highly hydrophobic fluoroalkyl chain, as an additional component of the film; the effect of the FAS on the permselectivity was investigated. Interestingly, the FAS-containing sol-gel films (No. 5) demonstrated excellent permselectivity toward H_2O_2, and the magnitudes of the interference currents by AA and UA were suppressed to less than 1/480 and 1/320 to that of H_2O_2, respectively. Because FAS has a highly hydrophobic and relatively flexible fluorocarbon chain, FAS may provide highly hydrophobic regions within the film, which may lead to a tightly condensed configuration in the sol-gel polymer network that suppresses the permeation of AA and UA. In addition, the FAS-containing film (No. 5) exhibited the largest response to glucose, which is a desirable feature with regard to the aim of this study. The incorporation of a highly

hydrophobic molecule, such as FAS, enhances the hydrophobicity of the film. However, more interestingly, it can also cause an increase of hydrophilicity, which can be explained by a looser configuration of hydrophilic polymer [34]. Have a large number of hydrogen bonds in the hydrophilic polymer is beneficial to maintaining the active configuration of the enzyme molecule [22]. Based on these features, we surmise that the large response to glucose is due to the reasons that follow: The looser configuration of hydrophilic domain of the hybrid film facilitates higher enzyme loading and/or a favorable electrostatic interaction between immobilized GOx molecules and the hydrophilic residues of the film components, such as BSA and CHIT, which may enhance the apparent activity and stability of the immobilized GOx molecules. Specifically, the incorporated FAS may lead to a higher enzyme loading and/or higher enzyme activity. To elucidate the detailed mechanism, however, further investigation is still required.

3.2. Proposed permeation mechanism and surface morphology of the hybrid sol-gel film.

Mizutani et al. have reported that the permselectivity of H_2O_2 of a polyelectrolyte-based polyion complex film against possible electro-active (easily oxidizable) species was caused by a size-exclusion mechanism [4, 5]. They measured the anodic current of various electroactive species with different molecular weights using both bare and permselective-film-modified electrodes. They discussed the permselective mechanism of the film, which was based on the ratio between the currents obtained by the bare and the film-modified electrodes [4, 5]. Therefore, we have performed similar experiments to evaluate the mechanism of the permselectivity in the

new hybrid-sol-gel film (i.e., APTES-TEOS-BSA-CHIT-GOx-film). The results are displayed in Fig. 1 via plots of the current ratio versus the molecular weight of the electro-active (oxidizable) species. Overall, the current ratios for the larger molecules (the molecular weight larger than ca. 100) seem to be lower than for the smaller molecules. However, in some cases, the current ratio's order of magnitude does not necessarily correspond to the molecular size (e.g., hydrazine and H_2O_2). Therefore, we may safely assume that the permselectivity of the new hybrid-sol-gel film may originate from not only the size-exclusion mechanism, but also some of the interactions between these electro-active species and the film components (e.g., electrostatic, hydrophilic and hydrophobic interactions, hydrogen-bonding formation and van der Waals force etc.). Specifically, the three-dimensional sol-gel silanol condensation network in addition to some types of molecular interactions between the components of the film may provide the appropriate microenvironment for the selective permeation of H_2O_2 through the film.

Figure 2 displays SEM images of the surfaces of the bare-Pt electrode (A) and the APTES-TEOS-FAS-BSA-CHIT-GOx film modifying the Pt electrode (B, C). The surface of the newly developed hybrid sol-gel film (panel B) exhibits an apparent granular structure and the granular size is 0.5 to 1 μm. These structure may originate from various interactions: for example, 1) the formation of three-dimensional network via the silanol condensation by APTES, TEOS, FAS and OH groups of CHIT and BSA; 2) cross-linking reaction via primary amino groups of APTES, CHIT, BSA and GOx; 3) various molecular interactions between the film components. As observed in Fig. 2C from the cross-sectional image of the film, the thickness of this hybrid sol-gel film is estimated to be ca. 500 nm (under the conditions for SEM measurement).

3.3. Analytical characteristics of the present glucose biosensor

We subsequently evaluated the analytical properties of a Pt electrode that was modified with the APTES-TEOS-FAS-BSA-CHIT-GOx film and used as an amperometric glucose biosensor. Figure 3 displays the typical current-time curve for the sensor when the concentration of glucose increased in steps of 0.1 mM (100 μL of 10 mM glucose was successively added to 10 mL of electrolyte after a constant background measurement had been established at the points indicated by the arrows). The steady-state cathodic background current changed rapidly upon the addition of glucose and reached another steady-state current within 3 s. Because the GOx was immobilized on the film after the sol-gel film was added to the Pt electrode, the GOx may be attached to an exterior portion of the film. As a result, most of the GOx molecules would exist on an outer layer of the film. Therefore, we may reasonably assume that the diffusion of glucose, gluconolactone, O_2 and H_2O_2 surrounding the immobilized GOx is relatively smooth. Though the magnitude of the steady-state current from H_2O_2 at the film-modified electrode is significantly suppressed compared to the bare-electrode (Table 1 and Fig.1), the response time of H_2O_2 is rapid (< 3 s), which indicates a rapid permeation of H_2O_2 through the film occurs. Therefore, the H_2O_2 that was produced by the GOx reaction would penetrate the film smoothly, which results in the rapid response for glucose.

Figure 4A displays the calibration curve for the glucose obtained by the glucose biosensor. A linear relationship was obtained in concentrations ranging from 0.05 mM to 1 mM with a sensitivity (slope of the linear portion) of 1.84 μA/mM (14.4 μA mM^{-1} cm^{-2}) and a correlation coefficient of 0.9991. The lower detection limit is estimated to

be ca. 0.032 mM with a signal-to-noise ratio of 3 (noise level 20 nA). The sensitivity of the glucose biosensor is superior to other glucose sensors, which are based on alumina sol-gel/electropolymerized composite films (1.04 μA mM^{-1} cm^{-2}) [20], copolymer-modified silica sol-gels (0.6 μA mM^{-1}) [21], Pt-nanoparticle-doped sol-gel/carbon nanotubes (0.98 μA mM^{-1} cm^{-2}) [37], composite silicate sol-gel glass (0.78 μA mM^{-1}) [35], titania sol-gel films (7.2 μA mM^{-1} cm^{-2}) [16], and carbon nanotube sol-gel composite-modified graphite (0.196 μA mM^{-1}) [38]. Saturation from the linearity is observed at higher (>5 mM) glucose concentrations, which is characteristic of the Michaelis-Menten model. The relatively narrow linear range of the glucose sensor may be attributed to the limited enzyme loading, which was due to the tightly packed three-dimensional sol-gel network. To expand the linear range to a region for higher concentration, an additional outer-membrane layer would be effective [20].

The Michaelis−Menten constant (K_m^{app}), which is a typical parameter in enzymatic reaction kinetics, can be estimated via the Lineweaver−Burk equation, which is a modified version of the Michaelis−Menten equation [42]

$$1 / I_{ss} = K_m^{app} / C I_{max} + 1 / I_{max} \quad (1)$$

where, I_{ss} is the steady-state current, C is the glucose concentration, I_{max} is the maximum current, and K_m^{app} is the apparent Michaelis−Menten constant. Fig. 4B displays the Lineweaver−Burk plot based on the data in Fig. 4A. The K_m^{app} and I_{max} values that were determined from the slope and intercept of Fig. 4B are 8.2 mM and 14.6 μA, respectively. The K_m^{app} value (8.2 mM) of this biosensor is slightly larger than that for GOx immobilized in a sol-gel chirosan/silica hybrid composite film on a Prussian blue-modified-glass carbon electrode (3.2 mM) [32], a GOx-graphene-chitosan nanocomposite (4.4 mM) [29], and GOx in titania sol-gel membrane (6.34 mM) [16].

This constant is also almost comparable to GOx on a Pt nanoparticle-deposited carbon nanotube composite sol-gel-CHIT-silica hybrid film-modified glassy carbon electrode (7.9 mM) [31] and GOx on a sol-gel-derived titania/Nafion film-modified platinized glassy carbon electrode (8.4 mM) [40]; this value is smaller than that for GOx entrapped in a grafted copolymer-hybrid sol-gel film (20 mM) [21] and organically modified sol-gel chitosan film (20 mM) [33], as well as for free GOx (from *A. niger*) in solution (33 mM) [43], which suggests that this glucose biosensor possesses a higher affinity for glucose. The possible reasons for the film's smaller K_m value for GOx relative to the native GOx in solution are as follows: chemical modification of enzyme and/or some types of interactions between the film components and enzyme molecule may lead to minor structural and conformational changes in the enzyme near the active center of GOx, which may cause a decrease in the K_m value.

Importantly, the response of this new glucose biosensor was not influenced by the presence of 0.1 mM of AA and UA. As shown in Fig. 5, the calibration curve for glucose was barely influenced by the presence of 0.1 mM AA and UA, which indicates that the present glucose sensor facilitated the highly selective determination of glucose, even in the presence of physiological concentrations of AA and UA.

To evaluate the practical usage for the analysis of real samples, we measured glucose in beverages and human sera by using the present hybrid-sol-gel film-based sensor, as well as a commercially available spectrophotometric method; the results of both methods were compared. The analytical results are compared in Table 2. The measurements obtained by the sensor were agreed with those of the spectrophotometric method. Generally, when assaying body fluids, such as blood, protein adsorption can cause negative effects. Judging from the results of the glucose assays with human serum,

however, the negative effect caused by the protein adsorption is negligible, which is likely because the hybrid sol-gel film already contains proteins and polysaccharides (BSA and CHIT). Therefore, it should be emphasized that the hybrid sol-gel film has three roles; 1) immobilization of the host-matrix for the enzyme; 2) elimination of the interference caused by AA and UA; 3) protection against the fouling of the electrode. These are the most notable advantages of this protein-polysaccharide composite sol-gel film-based biosensor.

The biosensor's operational stability was assessed by measuring the successive addition of 0.1 mM glucose. After 10 additions, the electrolyte was replaced in fresh buffer and the same measurements were repeated four times. The relative standard deviation (RSD) of the 40 successive measurements was 1.7 %, which indicated that the glucose biosensor has an acceptable operational stability. The reproducibility of the electrode preparation was checked by measuring 0.1 mM glucose with different electrodes. The RSD of four different electrodes was less than 8%.

The sensor's stability toward long-term storage was examined by measuring 0.1 mM glucose and AA every two or three days for 20 days. After the measurements, the electrode was stored in 0.1 M phosphate buffer (pH 6.5) at 4 ˚C. The response to AA gradually increased during the storage period and the current for AA was 126% of initial measurement after 20 days storage, which suggests that the partial swelling and/or degradation of the film had occurred. The current response to glucose decreased to 76% after 5 days, 68 % after the 10 days, and 52% after 20 days storage. Further improvements in the film preparation conditions (e.g., the amounts of each component, reaction time, temperature, and enzyme immobilization method) may be necessary to improve the long-term storage stability. This search is now underway in our laboratory.

4. Conclusion

In this study, we developed a novel, interference-free glucose biosensor by using biomolecule-organic-inorganic hybrid sol-gel silicate film that not only contained an interference-eliminating section but also an enzyme-immobilization matrix. The glucose biosensor enabled the highly sensitive [sensitivity: 1.84 μA/mM (14.4 μA mM^{-1} cm^{-2}), detection limit: 0.032 mM], rapid (100 % response in < 3 s) and highly selective determination of glucose, even in the presence of possible interferents (0.1 mM AA and UA). Therefore, this glucose biosensor may be useful for real sample analysis. Moreover, the use of hybrid sol-gel films via this methodology can be extrapolated for various H_2O_2-producing oxidases (e.g., lactate oxidase, glutamate oxidase, etc.) in a wide range of applications.

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Table 1 Comparison of the steady-state current responses for Pt electrodes modified with various sol-gel films to hydrogen peroxide (H_2O_2), ascorbic acid (AA), uric acid (UA) and glucose.

No	Electrodes	Current response / μA [a) b) c)]				Current ratio	
		H_2O_2 (A)	AA (B)	UA (C)	Glucose	(A)/(B)	(A)/(C)
1	bare-Pt	13.83	4.19	5.76	−	2.8	2.4
2	APTES-TEOS-GOx/Pt	0.79	0.022	0.057	0.023	36	14
3	APTES-TEOS-BSA-GOx/Pt	1.52	0.016	0.032	0.171	95	47
4	APTES-TEOS-BSA-CHIT-GOx/Pt	1.31	0.011	0.018	0.158	119	73
5	APTES-TEOS-BSA-CHIT-FAS-GOx/Pt	1.92	0.004	0.006	0.194	480	320

a) Current response to 0.1 mM analytes at an applied potential of + 0.6 V vs. Ag/AgCl.

b) The average values of three preparations are listed.

c) These values contain ~ ± 10 % error.

Table 2 Analytical results for the glucose biosensor and the spectrophotometric method for the determination of the glucose concentration in beverages and human sera.

Samples	Glucose concentration (mM)	
	Glucose biosensor [a]	Spectrophotometric method [b]
Beverage 1	48	45
Beverage 2	134	130
Beverage 3	202	207
Beverage 4	218	220
Serum 1	4.6	4.4
Serum 2	5.2	5.4

a, For the biosensor analysis, the beverage samples were diluted 100 - 1000 times and the sera samples were diluted 20 times. An aliquot of suitably diluted sample (100 μL) was added to an electrolyte solution (10 mL).

b, For the spectrophotometric method, the beverage samples were diluted 10 times and sera samples were used without dilution.

Matsuhisa et al., Fig. 1

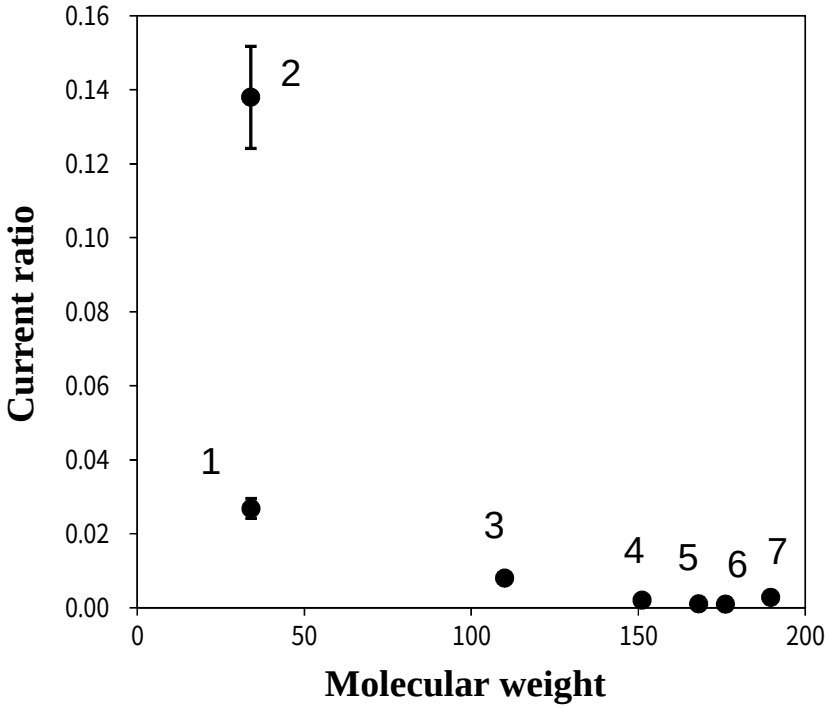

Fig. 1. The relationship between the molecular weight of the oxidizable species and the ratio between the current of organic/inorganic hybrid sol-gel film-modified Pt electrode and a bare Pt electrode. The species are (1) hydrazine; (2) hydrogen peroxide; (3) catechol; (4) acetaminophen, (5) uric acid; (6) ascorbic acid; (7) dopamine. The concentration of the species is 0.1 mM. Applied potential is +0.6 V vs. Ag/AgCl. The average values of three preparations are listed. These values contain ~ ± 10 % error.

H. Matsuhisa et al., Fig. 2

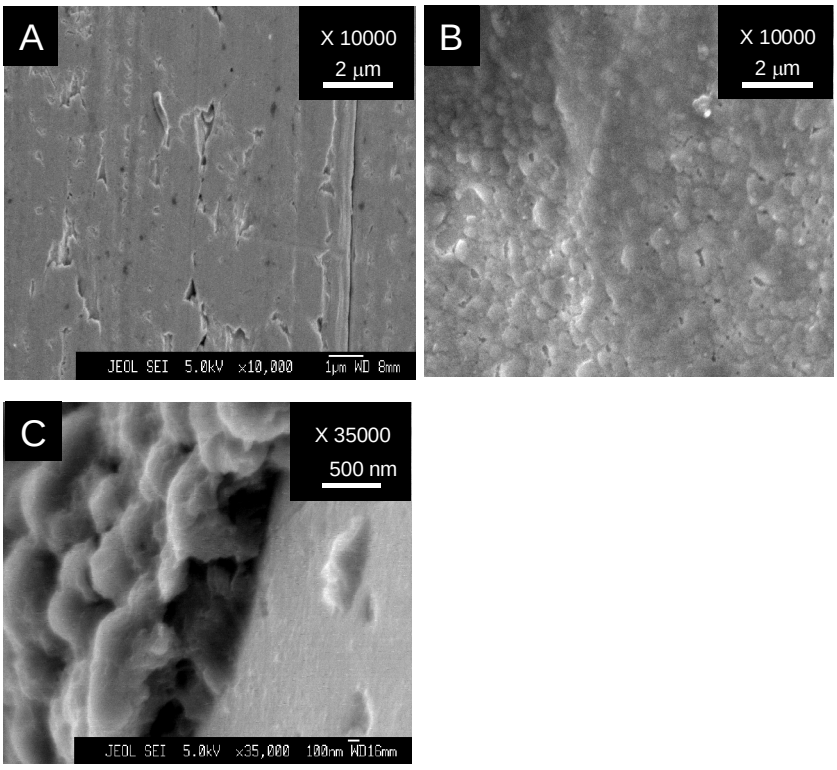

Fig. 2. SEM images of the surfaces of bare Pt-wire electrode (A) and APTES-TEOS-FAS-BSA-CHIT-GOx film-modified Pt-wire electrode (B). Cross-sectional SEM image of the APTES-TEOS-FAS-BSA-CHIT-GOx film prepared on the Pt-wire electrode via the masking method (C).

H. Matsuhisa et al., Fig. 3

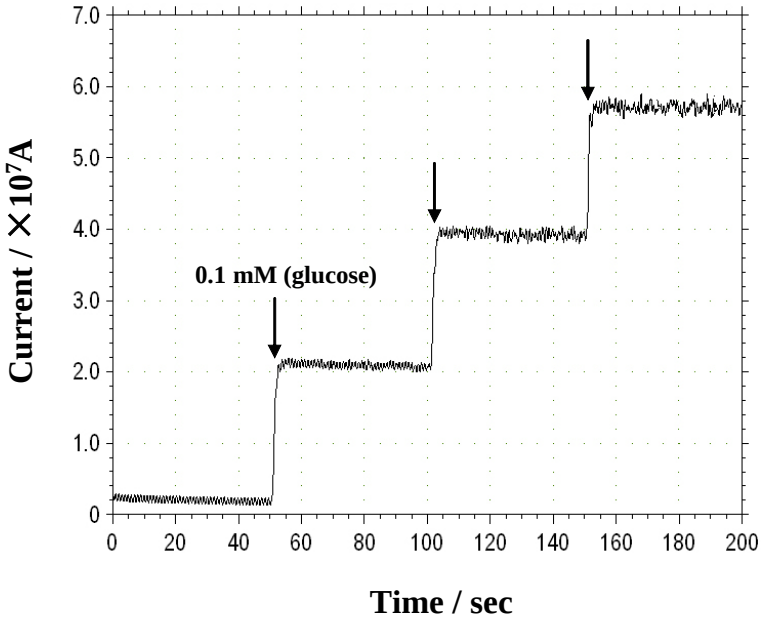

Fig. 3. Typical steady-state current−time response curve of the newly presented glucose biosensor upon successive addition of 0.1 mM glucose in air-saturated 0.1 M phosphate buffer (pH 6.5) at an applied potential of +0.60 V vs. Ag/AgCl. Sample was added every 50 s.

H. Matsuhisa et al., Fig.4A

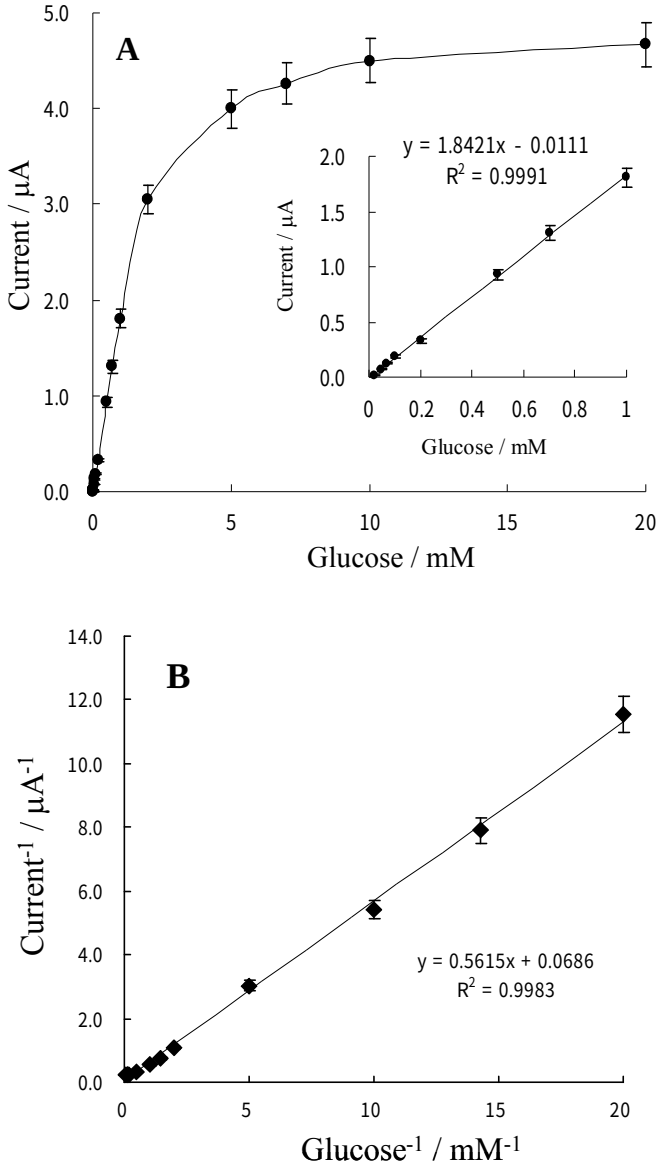

Fig. 4. (A) Calibration curve for the glucose biosensor. The measurement conditions are same as described in Fig. 3. The inset graph is an enlargement of the lower concentration range. (B) Lineweaver−Burk plot based on the data in panel A. The plotted values are the average of three measurements. These plotted values contain ~ ±8% error.

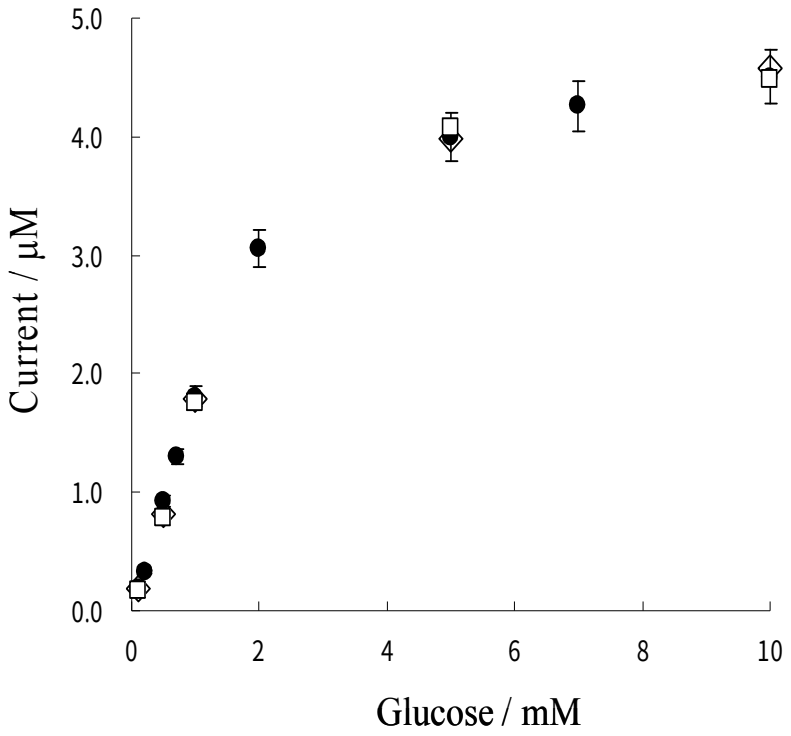

Fig. 5. Comparison of the calibration curves for the glucose sensor in the absence (●) and presence of 0.1 mM AA (□) and UA in electrolyte buffer solutions. (◇). The measurement conditions are same as in Fig. 3. The plotted values are the average of three measurements. These plotted values contain ~ ±10% error.

Highlight

- Novel protein-polysaccharide-composite silicate film was prepared on the Pt electrode surface
- The film, on which glucose oxidase was immobilized, showed permselectivity toward H_2O_2
- Highly selective interference-free amperometric glucose biosensor was developed.

*Graphical Abstract (for review)