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Chitosan–ferrocene film as a platform for flow injection analysis applications of glucose oxidase and *Gluconobacter oxydans* biosensors

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ABSTRACT

Chitosan–ferrocene (CHIT–Fc) hybrid was synthesized through covalent modification and its electrochemical properties in immobilized form were studied by using cyclic voltammetry. The hybrid film exhibited reversible electrochemistry with a formal potential of +0.35 V (vs. Ag/AgCl) at pH 5.5. The Fc in CHIT matrix retained its electrocatalytic activity and did not diffuse from the matrix. This redox-active hybrid was further employed as a support for immobilization of glucose oxidase (GOx) and whole cells of *Gluconobacter oxydans* using glutaraldehyde on a glassy carbon electrode (GCE). The experimental conditions were optimized and the analytical characteristics of enzyme and microbial biosensors were evaluated for glucose in flow injection analysis (FIA) system. Under optimized conditions, both enzyme and microbial biosensors exhibited wide linear ranges for glucose from 2.0 to 16.0 mM and from 1.5 to 25.0 mM, respectively. Moreover, the biosensors have the advantages of relatively fast response times, good reproducibility and stability in FI mode. It was demonstrated that CHIT–Fc provides a biocompatible microenvironment for both bioctalysts and an electron transfer pathway. Additionally, integration of the enzyme and microbial biosensors into the FIA system has several advantages including capability of automation and high throughput at low cost. This promising redox hybrid can be utilized as an immobilization matrix for biomolecules in biosensor systems.

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1. Introduction

Chitosan (CHIT) is synthesized by the partial deacetylation of chitin, the most abundant polysaccharide after cellulose in the world [1]. CHIT is a biocompatible biopolymer consisting of D-glucosamine and N-acetyl-D-glucosamine units linked in a β(1 → 4) fashion [2]. CHIT is a cationic and soluble polysaccharide under acidic conditions due to the amino groups of the D-glucosamine residues. Additionally, CHIT possesses unique physico-chemical characteristics such as excellent membrane forming ability, good biocompatibility, high permeability towards water, adhesion capability, non-toxicity, high mechanical strength and ease of chemical modifications [2,3]. Because of this unique combination of properties, CHIT has been extensively studied as an immobilization material for the fabrication of biosensors, sensors and immunosensors [4–6]. Unfortunately, it has very low electron transfer efficiency. To overcome this limitation, redox mediators can be bound covalently to the biopolymer [7,8].

Among various redox mediators, ferrocene (Fc) and its derivatives have attractive properties (including relatively low molecular mass, rapid responses to many electroactive substances, being pH-independent, stability in both oxidized and reduced forms at low potential and having fast electron transfer) that make them the most widely used class of mediators in the development of chemically modified electrodes [9–11]. The covalent linking of Fc group to CHIT can prevent leakage, the principal problem of low-molecular-weight mediators [12]. Furthermore, this strategy can combine biocompatibility and redox efficiency. The Fc groups will facilitate the electron transfer between immobilized biocatalyst and electrode surface. Therefore, more sensitive and stable biosensors can be obtained.

CHIT–Fc hybrids have been used as a functionalized matrix in electrochemical biosensors to immobilize various enzymes such as glucose oxidase (GOx), horseradish peroxidase (HRP) and laccase [13–15].

The integration of biosensors into flow injection analysis (FIA) has a wide variety of applications [16–20]. In the FIA system, a biosensor is exposed to the substrate for a short time yet in batch

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analysis the biosensor is kept immersed in the substrate solution for a long time [21]. Subsequent to the measurements, the reaction products are removed as the continuous flowing stream passes through the detector and the contamination risk is prevented [22]. Thus, the stability of the biosensor is preserved for a long time. In addition, compared to the batch systems, the FIA systems offer the advantage of working with small volumes of the substrate, thereby reducing cost and waste [21,23]. Also, FIA is capable of being automated and allows high sample throughput. This system is able to inject a constant sample volume into a continuously flowing carrier solution followed by quantification at a detector device [24]. All these properties of the FIA system lead to highly sensitive and reproducible results.

In this study, we attempted to present the advantages of covalently linked chitosan–ferrocene (CHIT–Fc) hybrid, and its use as a functionalized biopolymer to immobilize two types of biocatalysts. GOx, an oxidoreductase widely used for D-glucose quantification [25], and whole cells of *Gluconobacter oxydans*, contain membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) specific for D-glucose [26], were employed as biosensing elements. They were immobilized on the CHIT–Fc matrix by cross-linking via glutaraldehyde to form hybrid films on the surface of glassy carbon electrodes (GCE). The proposed biosensors were optimized and characterized for glucose detection using cyclic voltammetry and a FIA system. The results showed that this immobilization technique was effective in preventing the leakage of both enzyme and microorganisms. Furthermore, the Fc group improved the electron transfer efficiency of the biopolymer film, which provides a biocompatible microenvironment around the enzyme and bacterial cells. The present amperometric FIA-based biosensors exhibited good analytical characteristics including wide linear ranges, fast response times, sufficient stability and reproducibility towards the quantification of glucose.

2. Materials and methods

2.1. Materials

Chitosan (CHIT, from crab shells, minimum 85% deacetylated) was purchased from Fluka (Steinheim, Germany). Sodium cyanoborohydride ($NaCNBH_3$, 95%) was obtained from Merck (Darmstadt, Germany). Ferrocene carboxaldehyde (Fc-CHO, 98%), glucose oxidase (GOx, from *Aspergillus niger*, EC 1.1.3.4, 50 units/mg), glutaraldehyde solution (25%, v/v) and D-glucose were obtained from Sigma–Aldrich (Dorset, UK). Commercial enzyme assay kit [27] (Glucose MR, Cat. no. 1129010) was acquired from Cromatest (Barcelona, Spain). All other chemicals were of analytical grade. All solutions were prepared with double distilled water.

2.2. Apparatus

Electrochemical measurements were performed with a PalmSens Electrochemical Instrument (Palm Instruments, Houten, The Netherlands). Cyclic voltammetric (CV) experiments were performed in a reaction cell (10 mL) at room temperature using a conventional three-electrode system. A GCE (3.0 mm diameter) was used as a working electrode (Bioanalytical Systems, Inc. BAS). An Ag/AgCl electrode (3 M KCl, Metrohm, Switzerland) and a platinum electrode (Metrohm, Switzerland) were used as reference and counter electrodes, respectively.

All amperometric experiments were carried out using the FIA system, comprises a peristaltic pump (FIAtron, Oconomovoc, WI, USA) equipped with Teflon tubing (0.50 mm inner diameter), for carrying 0.9 mL buffer solution per minute, an eight-port injection valve (FIAtron, Oconomovoc, WI) with a 100 μL sample injection loop, a laptop connected PalmSens potentiostat, for electrochemical measurements, and a cross-flow cell with three electrodes (Supplementary Fig. S1). A GC dual electrode (3.0 mm diameter and 1.0 mm electrode gap), an Ag/AgCl electrode and a Pt wire (CHI 130, CH Instruments, Austin, US) were used as working, reference and counter electrodes, respectively. Colorimetric assays were performed with a Pharmacia LKB Novaspec II spectrophotometer (LKB Biochrom, England). ^{1}H NMR spectra were recorded with a Varian AS 400 Mercury instrument. As solvent, 2.0% CF_3COOD in D_2O was employed. Chemical shifts (δ) were given in ppm. FT-IR-ATR spectra were recorded on a Perkin Elmer Spectrum 100 series. ICP-MS measurements were performed using an Agilent 7500 CE ICP-MS for element-specific detection.

2.3. Synthesis of chitosan–ferrocene derivatives

Ferrocene bound chitosan (CHIT–Fc) was synthesized by following the reported procedure [28]. Briefly, CHIT (75.0 mg, 0.39 mmol NH_2) was dissolved in 15 mL of 0.1 M acetic acid solution. Fc-CHO (39.0 mg, 0.18 mmol) was dissolved in 15 mL of methanol and added to the first solution. After stirring the reaction mixture at room temperature for 1 h, $NaCNBH_3$ (23.0 mg, 0.36 mmol) was added to the mixture and stirred for 24 h (Scheme 1). When 5.0% NaOH was added, the orange precipitates formed. Subsequently, the orange products were separated by filtration and washed with distilled water and methanol. Finally, the products were first dried in air for a few hours and then dried under vacuum. The degree of substitution (DS) of ferrocenyls was determined by ^{1}H NMR spectroscopy. DS was calculated by integration of the peak area of ferrocenyl protons (δ = 4.22–4.38, 0. 99H), H^2 of GlcN and *N*-alkylated GlcN residues (δ = 3.09, 0. 71H).

^{1}H NMR (2% CF_3COOD in D_2O) for CHIT–Fc (DS = 0.16): δ = 1.98 (s, 0.23H, N*H*Ac of GlcNAc), 3.09 (s, 0.71H, H^2 of GlcN and *N*-alkylated GlcN), 3.63–3.81 (m, 3.15H, FcCH_2N, H^2 of GlcNAc and $H^{3,4,5,6}$ of GlcN, GlcNAc and *N*-alkylated GlcN), 4.22–4.38 (m, 0.99H, Cp-*H* of Fc).

The total Fe content in the CHIT–Fc polymer was determined by ICP-MS and found to be 6.2 wt.%.

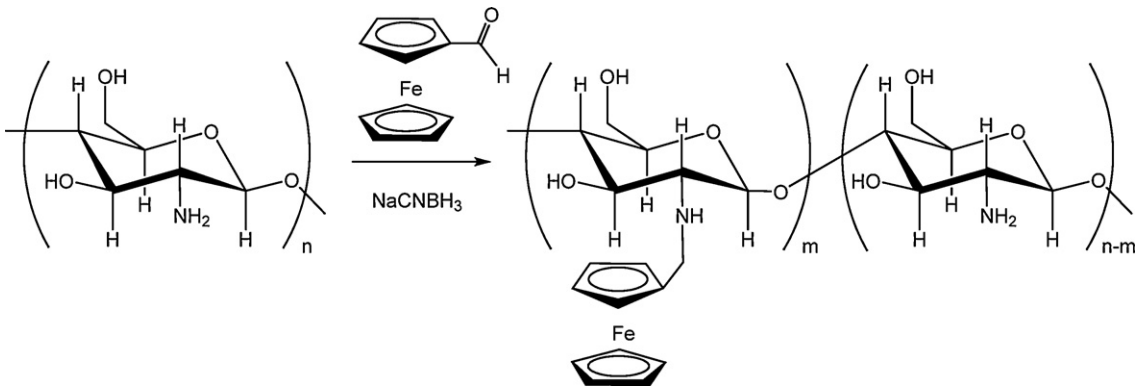
2.4. Cultivation of G. oxydans

The strain of *G. oxydans* DSMZ 2343 was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and stored in the media containing (g/L): D-glucose, 100; yeast extract, 10; calcium carbonate, 20; agar, 20. Cells were cultivated on a rotary shaker (175 rpm, 28 °C), in 250 mL Erlenmeyer flaks in 50 mL media containing (g/L): D-glucose, 5.0 and yeast extract 5.0. Bacterial growth was determined by measuring absorbance at 600 nm. After cells reached late exponential phase, they were separated by centrifugation (4500 rpm, 10 min), resuspended in 0.9% NaCl solution and recentrifuged. The collected cells were used for biosensor preparation [29–31].

2.5. Preparation of CHIT–Fc/GOx and CHIT–Fc/G. oxydans biosensors

Before each modification, the GCE was wet polished with 1.0 and 0.3 mm alumina slurry, respectively, to produce mirror-like surface, then rinsed thoroughly with distilled water. It was finally cleaned by ultrasonication in ethanol and twice-distilled water for 5 min.

Two distinct biological recognition elements, either GOx or *G. oxydans*, were used for electrode preparation. Initially, 1.0 wt.% CHIT–Fc solution was prepared by dissolving 2.5 mg of CHIT–Fc in 250 μL of 1.0 wt.% acetic acid with stirring for 1 h at room temperature [32]. Daily prepared CHIT–Fc solutions were used for biosensor



Scheme 1. Synthesis of ferrocene covalently bound chitosan derivatives.

construction. Approximately 17 μL of CHIT–Fc solution was spread onto the mirror-like GCE surface. Subsequently, 5.0 μL of 1.0% glutaraldehyde in sodium phosphate buffer (50 mM, pH 7.5) was dropped on the polymer coated electrode surface for cross-linking.

To prepare the enzyme electrode, 5.0 μL of GOx (equal to 125 U or 2.5 mg) in sodium acetate buffer (50 mM, pH 5.5) was placed on the CHIT–Fc, 1.0% glutaraldehyde containing GCE and allowed to dry at room temperature for 90 min. The electrode was washed with distilled water.

To immobilize whole cells of *G. oxydans*, 5.0 μL of *G. oxydans* suspension (a cell titer of 2.72×10^9), prepared by using sodium phosphate buffer (50 mM, pH 6.0), was spread over the CHIT–Fc, 1.0% glutaraldehyde containing GCE and allowed to dry at room temperature for 90 min. The electrode was washed with distilled water. Fresh cells were used to construct microbial biosensors. All biosensors were daily prepared unless otherwise stated. Scheme 2 shows the schematic representation of the stepwise fabrication process of the biosensor.

2.6. Measurements

Firstly, the electrochemical behavior of modified GCE without any biological component was examined by cyclic voltammetry (CV). CV measurements were performed in 50 mM pH 5.5 acetate buffer (10 mL) and reversible signals were recorded between 0.5 V and 0.0 V at different scan rates from 5.0 to 150 mV s^{-1}.

All amperometric measurements were carried out in a flow cell at room temperature in 50 mM either pH 5.5 sodium acetate or pH 6.0 sodium phosphate buffer (depending on the used biological component). According to the CV results, +0.35 V (vs. Ag/AgCl) was selected as the applied potential between the working and reference electrodes for amperometric measurements. In the amperometric experiments steady state currents (nA), proportional to glucose concentration, were measured at the applied potential of +0.35 V (vs. Ag/AgCl). Glucose standard solutions and samples were used as substrates. The mean values with their standard deviations and variation coefficients were calculated.

2.7. Sample application

A cherry juice and a coke were purchased from a hypermarket and the glucose contents of samples were determined by GOx biosensor. The samples were degassed and diluted 1:20 in pH 5.5 sodium acetate buffer. Daily constructed calibration curves were utilized for the determination of the glucose concentrations of real samples. In addition, as a reference method, a commercial enzyme assay kit based on spectrophotometric Trinder reaction [27] (Cromatest, Glucose MR, Cat. no. 1129010) was used for independent glucose analysis. In this reaction, the glucose is oxidized to D-gluconate by GOx with the formation of hydrogen peroxide. In the presence of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AAP) is oxidized by hydrogen peroxide to form a red quinoneimine dye proportional to the glucose concentration in the sample.

3. Results and discussion

3.1. Characterization of CHIT–Fc

CHIT–Fc biopolymer, is a supporting material, was synthesized in a two-step procedure proposed by Yang et al. [28]. In the first step, a Schiff base was formed by the reaction between the aldehyde group of Fc-CHO and the amino group of CHIT. In the second step, the Schiff base was reduced by $NaCNBH_3$. The formation of CHIT–Fc was confirmed by ^1H NMR, ICP-MS and FT-IR-ATR analysis. ^1H NMR results were consistent with the reported data [28], which indicated that the DS of ferrocenyls was 0.16. This value shows that 16 out of 100 NH_2 groups on CHIT polymer reacted with ferrocenyl moieties. The total Fe concentration of the CHIT–Fc biopolymer was found to be 6.2 wt.% by ICP-MS measurements. CHIT showed characteristic peaks at around 1654 and 1596 cm^{-1}, which are assigned to the amide bands of CHIT in the FT-IR-ATR spectra [28]. We observed these peaks at 1658 and 1594 cm^{-1} in both CHIT and CHIT–Fc polymers (Fig. 1). Due to the *N*-alkylation of the glucosamine unit with Fc-CHO, the intensity of the peak at 1594 cm^{-1}, for primary amine N—H bending, was decreased. The characteristic C=O peak of Fc-CHO at 1679 cm^{-1} was also disappeared in the CHIT–Fc spectra. In addition, another new absorption band at 816 cm^{-1}, for Cp C—H, in

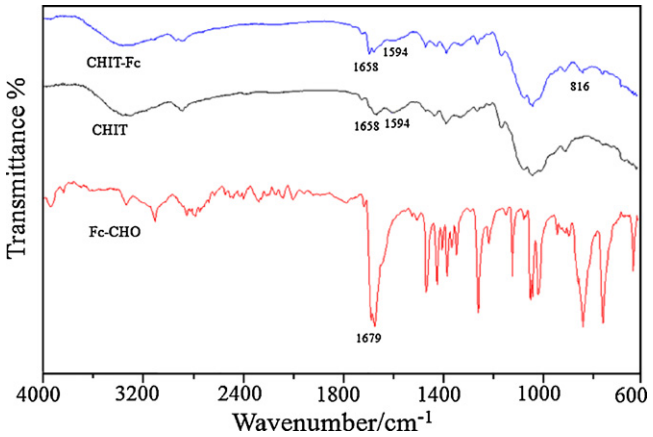
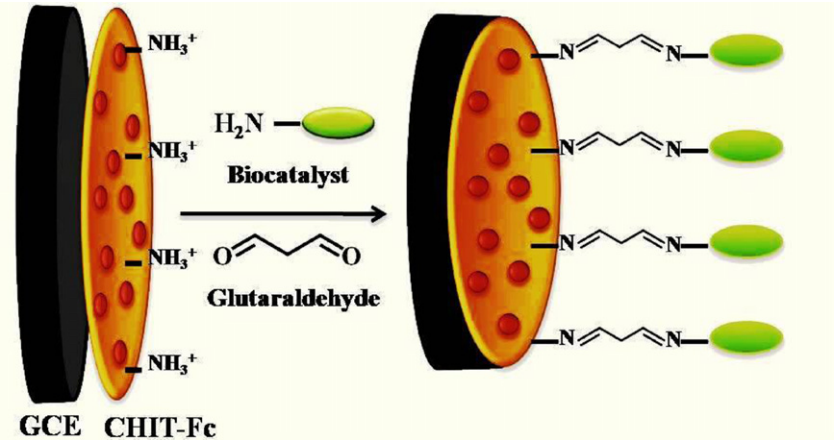


Fig. 1. FT-IR spectra of CHIT–Fc, CHIT and Fc-CHO.



Scheme 2. The stepwise fabrication process of the biosensor.

the spectra also indicates that the ferrocenyls exist in the CHIT–Fc biopolymer.

3.2. Electrochemical characterization of CHIT–Fc electrode

In order to investigate the electrochemical activity of CHIT–Fc film on a GCE, CV experiments were performed in 50 mM pH 5.5 acetate buffer. CHIT–Fc electrode was prepared using 1.0% glutaraldehyde solution (Fig. 2). The well-defined cyclic voltammograms showed two reversible redox peaks, which were attributed to the oxidation and reduction of Fc group. As the scan rate increased from 5.0 to 150 mV s^{-1}, the CV peak currents increased as well, while the cathodic and anodic peak potentials were nearly the same. Both cathodic and anodic peak currents were linearly proportional to the square root of scan rate, which proves diffusion-controlled electrode process. Similar behavior was observed for a ferrocene-containing nanocomposite chitosan [15]. The average value of the cathodic and anodic peak potentials gave the formal potential ($E^{\circ\prime}$) of 0.35 V (vs. Ag/AgCl), was the applied potential for amperometric experiments.

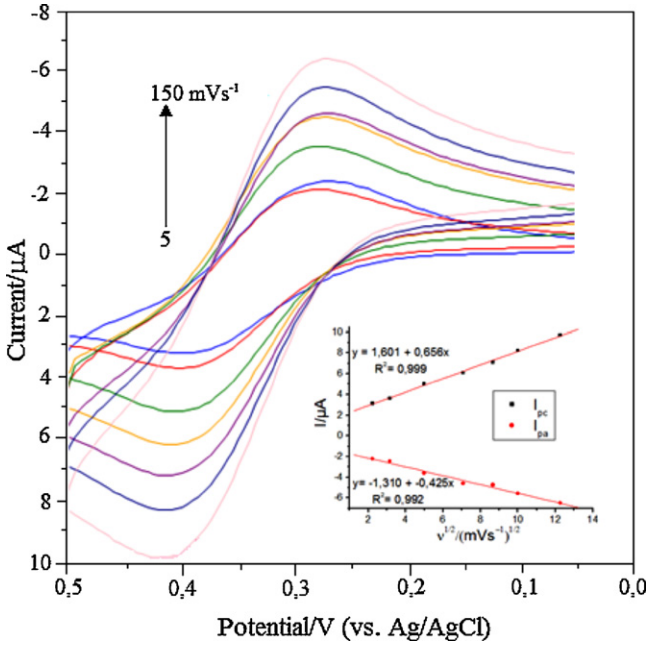


Fig. 2. Cyclic voltammograms of the CHIT–Fc/Glutaraldehyde modified GCE in acetate buffer (50 mM, pH 5.5) at scan rates of 5, 10, 25, 50, 75, 100, 150 mV s^{-1}. Inset: Plot of the cathodic (I_{pc}) and anodic (I_{pa}) peak currents vs. square root of scan rate.

The electrolyte solutions were not deoxygenated prior to the measurements because dissolved oxygen did not interfere with the electrochemical response of the mediator and CHIT–Fc electrode gave almost the same response in aerated and nitrogen-purged buffer [33].

3.3. CHIT–Fc/GOx modified GCE

3.3.1. Optimum experimental conditions

The buffer pH is an influential parameter for enzymes to sustain their maximum activity and catalysis efficiency [34]. The effect of buffer pH on the biosensor response was examined amperometrically by injecting 8.0 mM glucose into the carrier solution (Supplementary Fig. S2). The buffer solutions tested were 50 mM sodium acetate over the pH range 4.5–5.5 and 50 mM sodium phosphate over the pH range 6.0–6.5. The highest response was observed at pH 5.5. The responses tended to decrease at pH values higher than 5.5 so the biosensor with GOx gave maximum response at pH 5.5, was selected as the optimum carrier solution for other experiments. The optimal pH value of free GOx was reported as 5.5 as well [35,36]. This is attributed to that the microenvironment of the enzyme wasn't affected by the immobilization.

The redox-active material, CHIT–Fc, enhances electron transfer efficiency between the redox center of GOx and the surface of the working electrode. The enzymatic process occurs according to the following reaction [37]:

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Gluconolactone} + \text{GOx(FADH}_2\text{)}$$

$$\text{GOx(FADH}_2\text{)} + 2\text{CHIT–Fc}^+ \rightarrow \text{GOx(FAD)} + 2\text{CHIT–Fc} + 2\text{H}^+$$

$$\text{CHIT–Fc} \leftrightarrow \text{CHIT–Fc}^+ + \text{e}^-$$

where CHIT–Fc and CHIT–Fc^+ represent reduced and oxidized forms of the redox polysaccharide, GOx(FAD) and GOx($FADH_2$) are the oxidized and reduced forms of GOx.

According to the catalytic reaction above, the biosensor performance is related to the enzyme amount. Hence, the effect of the amount of immobilized GOx on the amperometric response of biosensor was investigated in a FIA system (Fig. 3). The response gradually increased with increasing enzyme loading from 50 U to 125 U. When GOx was further increased to 250 U, lower response was observed because GOx molecules became too rigid to react with Fc and the substrate diffusion was reduced. 125 U of GOx was selected for subsequent experiments.

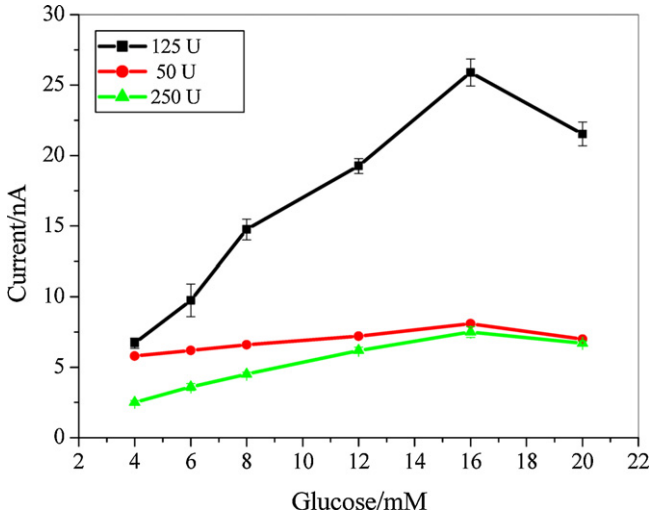


Fig. 3. Effect of GOx amount on the current responses to glucose standard solutions. Acetate buffer (50 mM, pH 5.5) was used as a carrier at flow rate of 0.9 mL min^{-1}. Applied potential was +0.35 V vs. Ag/AgCl. The error bars correspond to the standard deviation for three replicates.

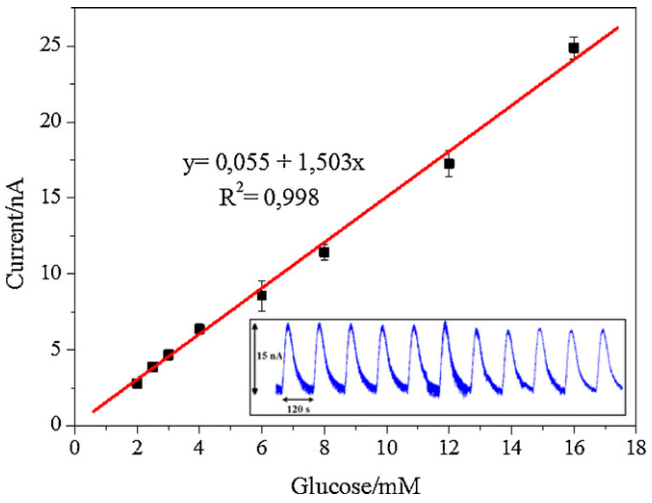


Fig. 4. Calibration curve of the CHIT–Fc/GOx biosensor (with 125 U GOx) in FIA mode. The other conditions are as in Fig. 3. Inset shows the amperometric responses of the GOx biosensor to 8.0 mM glucose.

In order to enhance the stability of the biosensor, the enzyme GOx was cross-linked with glutaraldehyde to CHIT–Fc via its amino groups. Glutaraldehyde has been previously used as a cross-linker for covalent immobilization of HRP on a chitosan film [14]. In this study, to determine the effect of glutaraldehyde concentration on the amperometric response by the FIA system, 0.5, 1.0, 2.5 and 5.0% glutaraldehyde solutions were used for constructing the amperometric enzyme biosensor. These biosensors were subjected to 8.0 mM glucose (Supplementary Fig. S3). The maximum response observed when 1.0% of glutaraldehyde was used. Higher concentrations of glutaraldehyde resulted in a decreased response. This was due to the high cross-linking between the enzyme and CHIT–Fc leading to enzyme denaturation and the loss of enzyme flexibility necessary for substrate binding [38]. Moreover, one enzyme reacted with many glutaraldehyde molecules which lowered the permeability of GOx. Using 0.5% of glutaraldehyde for biosensor preparation caused unstable CHIT–Fc/GOx films. Thus, 1.0% of glutaraldehyde was selected to provide stable and sensitive CHIT–Fc/GOx biosensors.

3.3.2. Analytical characteristics

The current response of the CHIT–Fc/GOx biosensor was investigated after injecting glucose standard solutions into the carrier solution under optimized conditions. The biosensor reached its maximum steady-state response in ~20 s. As illustrated in Fig. 4, the calibration plot was linear in the glucose concentration range from 2.0 to 16.0 mM. This linear response range is wider than the ranges for the glucose biosensor based on covalent immobilization of ferrocene on a chitosan modified GCE (0.8–4.0 mM) [39] and the glucose sensor prepared using ferrocene modified polysiloxane/chitosan composites (1.0–6.0 mM) [40]. The limit of detection (LOD) of our biosensor was 0.565 mM (S/N = 3) which is inferior to that of CHIT–Fc/MWNTs/GOx modified electrode (6.5×10^{-3} mM) [37]. Lower LOD values can be obtained by entrapping nanomaterials in CHIT–Fc matrix.

The reproducibility of CHIT–Fc/GOx biosensor was studied by making 10 consecutive injections of 8.0 mM glucose. The calculated coefficient of variation was 4.3% for injections with a single biosensor. Moreover, the fabrication reproducibility of three electrodes, which were independently prepared, was examined (Fig. 5). The operational stability was also tested by monitoring the peak currents for repeated injections of 8.0 mM glucose solution over a period of 7.2 h. After about 208 injections, the biosensor lost 13% of its initial activity. The storage stability of the enzyme biosensor was monitored for 14 days. The biosensor was stored in pH 5.5 acetate buffer solution at 4 °C when not in use. The current response to the injection of 8.0 mM glucose standard was measured intermittently (every 2–3 days). After 14 days the biosensor kept 82% of its original current response. The good reproducibility and stability of the biosensor can be attributed to the good biocompatibility and the enhanced electron transfer ability of CHIT–Fc biopolymer which provides a suitable microenvironment to retain the activity of the cross-linked enzyme. Additionally, by using glutaraldehyde, the optimal distance between GOx and Fc for efficient electron transfer and the high performance of the enzyme were obtained.

3.3.3. Sample application

In order to prove the applicability of the biosensor for real sample analysis, glucose concentration in beverages was investigated utilizing a calibration method. Coke and cherry juice samples were diluted 1:20 in 50 mM pH 5.5 sodium acetate buffer, used as a carrier. Three injections were carried out for each sample. The glucose concentrations of coke and cherry juice samples were found to

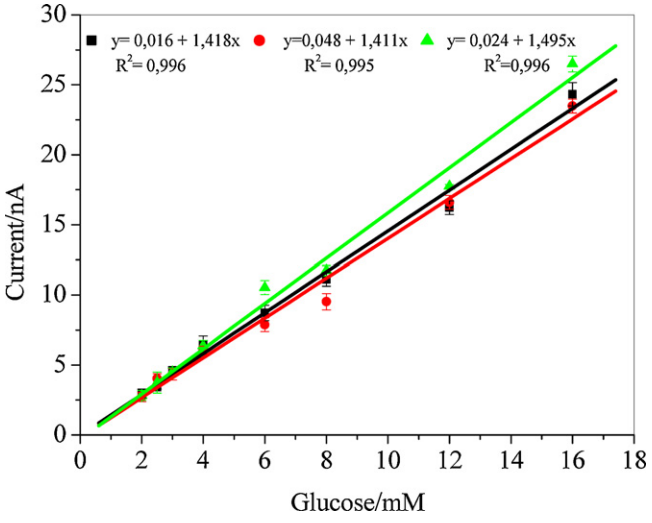


Fig. 5. The fabrication reproducibility of three CHIT–Fc/GOx biosensors. The other conditions are as in Fig. 4.

be 5.99 ± 0.38 and 4.25 ± 0.21 g/100 mL, respectively. These results were similar to 5.42 ± 0.28 and 4.36 ± 0.15 g/100 mL, respectively, which were obtained with a commercial enzyme assay kit based on spectrophotometric Trinder reaction [27]. Good recovery yields, which were 110% for coke and 97% for cherry juice, indicated that the proposed biosensor provides an accurate method for the measurement of glucose in real samples.

3.4. CHIT–Fc/G. oxydans modified GCE

3.4.1. Optimum experimental conditions

In addition to GOx, *G. oxydans* was also chosen as a biocatalyst to evaluate the electrocatalytic performance of the CHIT–Fc modified GCE. Since, the effect of medium pH is important to the response of the intact cells immobilized on the CHIT–Fc film, the working buffer pH was optimized (Supplementary Fig. S4). The buffer solutions of 50 mM sodium acetate pH 5.5 and 50 mM sodium phosphate pH 6.0, 6.5, 7.0 were used as a carrier. The peak current responses to 10 mM glucose were investigated. The maximum peak current of the microbial biosensor was observed at pH 6.0. As previously reported, the membrane-bound GDH directly oxidizes D-glucose at an optimal acidic pH around 6.0 [41]. Thus, 50 mM sodium phosphate buffer pH 6.0 was selected as the optimum for subsequent experiments.

G. oxydans is a gram-negative bacterium belonging to the family Acetobacteraceae and an obligatory aerobe, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor [42]. These cells possess a wide variety of membrane bound dehydrogenases such as GDH, gluconate DH and 2-ketogluconate DH [42]. The porin proteins of the outer membrane allow the diffusion of small molecules, like substrates and mediators, into the cell [29,43]. The PQQ-dependent GDH of *G. oxydans* offers a fast and efficient way to catalyze the direct oxidation of D-glucose at the surface of the cytoplasmic membrane [44]. The catalytic process taking place on the surface of the bacterial biosensor can be expressed as follows:

$$\text{Glucose} + \text{GDH(PQQ)} \rightarrow \text{Gluconolactone} + \text{GDH(PQQH}_2\text{)}$$

$$\text{GDH(PQQH}_2\text{)} + 2\text{CHIT–Fc}^+ \rightarrow \text{GDH(PQQ)} + 2\text{CHIT–Fc} + 2\text{H}^+$$

$$\text{CHIT–Fc} \leftrightarrow \text{CHIT–Fc}^+ + \text{e}^-$$

The amperometric response of the microbial biosensor is dependent on the viable cells immobilized on the CHIT–Fc matrix. The effect of cell amount on the current response was examined by injecting glucose solutions in the concentration range of 2.5–25.0 mM (Supplementary Fig. S5). As expected, the current response initially increased, reached a maximum at a cell titer of 2.72×10^9 and then decreased with increased cell loading. The decrease at high cell loading can be attributed to the diffusion problem. This trend is similar to that observed for other microbial biosensors [45]. The cell titer of 2.72×10^9 was selected for further experiments.

Another parameter that affects the biosensor response is glutaraldehyde concentration. Four microbial biosensors were prepared using different concentrations of glutaraldehyde (0.5, 1.0, 2.5 and 5.0%) and tested with 10 mM glucose solution in the FIA system (Supplementary Fig. S6). The best current response was obtained when 1.0% glutaraldehyde concentration was used for cross-linking. Therefore, 1.0% of glutaraldehyde was used for subsequent experiments.

3.4.2. Analytical characteristics

Under optimized conditions, the CHIT–Fc/*G. Oxydans* biosensor was calibrated using glucose standards, with triplicate injections of each concentration. The biosensor reached 95% of the steady-state current in 70 s. The response time is faster than that of previously reported mediated *G. oxydans* biosensor (2–3 min) [31]. The upper limit of the linearity was about 25.0 mM (Fig. 6) of glucose concentration, which is higher than those reported for CHIT/*G. oxydans* (1.0 mM), CHIT-carbon nanotube (CNT)/*G. oxydans* modified GCE (1.0 mM) [46] and an osmium redox polymer mediated *G. oxydans* modified gold electrode (2.0 mM) [29]. The microbial biosensor had a detection limit of 0.797 mM (S/N = 3).

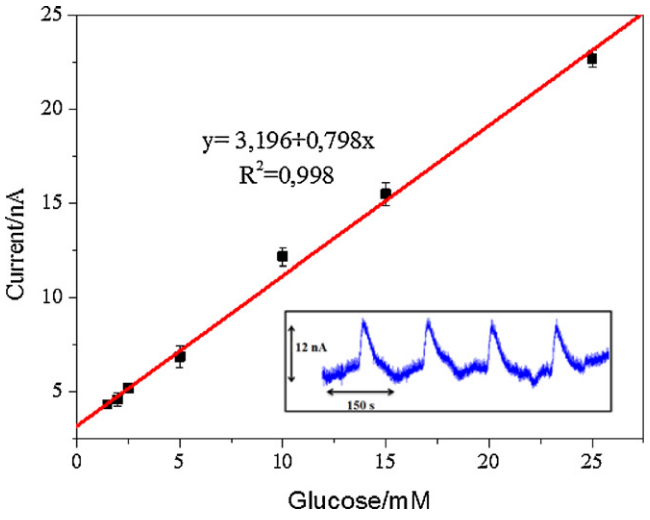


Fig. 6. Calibration curve of the CHIT–Fc/*G. oxydans* biosensor in FIA mode. Phosphate buffer (50 mM, pH 6.0) was used as a carrier at flow rate of 0.9 mL min^{-1}. The error bars correspond to the standard deviation for three replicates. Inset shows the amperometric responses of the microbial biosensor to 10 mM glucose.

The reproducibility was tested with 10 repetitive injections of 10 mM glucose. The variation coefficient was found to be 4.8%. In addition, the operational stability of the CHIT–Fc/*G. oxydans* biosensor was evaluated. A total of 72 injections were performed over a period of 3 h. The biosensor retained 85% of its original response after 72 successive injections. This stability is better than other mediated *G. oxydans* biosensors [29]. These data indicated that covalent attachment of the redox mediator to the polymer matrix results in an appropriate biosensing platform for the fabrication of whole cell biosensors. The redox-active hybrid material, which has a three-dimensional network structure, provides good reproducibility and stability. Consequently, the satisfactory characteristics make this microbial biosensor a promising route for the development of novel biosensor devices and fuel cells.

4. Conclusion

In this study, Fc was successfully linked covalently to CHIT and the resulting biopolymer film, CHIT–Fc, on a GCE showed a reversible redox process at $E^{\circ\prime}$ = 0.35 V (vs. Ag/AgCl). The CHIT–Fc film was demonstrated for the fabrication of CHIT–Fc/GOx and CHIT–Fc/*G. oxydans* biosensors, which showed well-defined amperometric responses towards glucose in a FIA system. The redox film not only provides a favorable environment for biomolecules but also mediates electron transfer between biomolecules and electrode. Both biosensors displayed wide linear response ranges, good stability and reproducibility.

The results also demonstrated the possibility of applying enzyme and bacterial biosensors in FIA mode for automated and high-throughput analysis at low cost.

In order to improve the sensitivity, nanomaterials will be entrapped in CHIT–Fc matrix and studied in the future. This promising redox biopolymer can offer a route to build novel biosensing

platforms that have both biocompatibility and enhanced electron transfer ability. Therefore, it can facilitate the development of new biosensors and bioelectrochemical devices.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.05.020.

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