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Characterization of natural chitosan membranes from the carapace of the soldier crab *Mictyris brevidactylus* and its application to immobilize glucose oxidase in amperometric flow-injection biosensing system

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Abstract

This study investigated characteristics of a chitosan membrane from the carapace of the soldier crab *Mictyris brevidactylus* intended *to* construct an amperometric biosensor. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used in this study to characterize these chitosan membranes intended for constructing enzymatic biosensors. Chitosan membranes suffering various durations (>10 min) of deacetylation had small charge-transfer resistances (<7.88 kΩ) but large double-layer capacitances (>0.55 μF). They were found in EIS where both the solution resistance and Warburg impedance upon electrode interface were almost independent of the durations and degree of deacetylation. The degree of deacetylation and the thickness of chitosan membranes were also determined. Membrane thickness was slightly dependent with the duration but degree of deacetylation was slightly dependent on the duration. Chitosan membranes with various thicknesses suffered various durations of deacetylation, but this did not influence their electrochemical characteristics. The chitinous membrane was covalently immobilized with glucose oxidase (EC 1.3.4.3) and then attached onto the platinum electrode of a homemade amperometric flow cell. Sensor signal was linearly related to glucose concentration (r=0.999 for glucose up to 1.0 mM). The system was sensitive (S/N>5 for 10 μM glucose) and reproducible (CV<1.3% for 50 μM glucose, n=5).

Keywords: Chitosan; Chitin; Soldier crab; Electrochemical impedance spectroscopy; Enzyme immobilization; Biosensor

1. Introduction

Soldier crabs are common intertidal sandy-shore crabs in the tropical and subtropical Indo-Pacific regions. These small crabs reside in estuary lagoon areas with certain special environmental characteristics [1]. Unlike other crab species, soldier crabs do not attack each other and generally live in high population densities, exceeding 200 m^{-2} for the Taiwanese soldier crab, *Mictyris brevidactylus* [2]. Our lab therefore considered the species a potential natural resource of chitinous materials, the major components of crustacean shell [3].

Chitin is a structural amino polysaccharide in the exoskeletons of arthropods; the linear biopolymer of β(1–4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose is biodegradable and biocompatible, and is a promising material with various applications [4]. Chitosan, the N-deacetylated derivative of chitin, is even more useful, especially in biomedicine [5,6]. With additional functional groups on the cellulose-like insoluble skeleton, chitinous materials (chitin and chitosan) have been used as a scaffolding matrix for enzyme immobilization [7,8]. The electrochemically inert

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materials can be cast on the electrode surface to establish a biosensor [9,8,10,11].

The chitinous exoskeletons of the Taiwanese soldier crabs were purified in our laboratory. The paper-thin contact lens-sized chitinous membranes from the dorsal parts of their carapaces were found to provide an ideal support for protein immobilization, particularly for electrochemical biosensing [3]. The membranes suffered pretreatment processes (e.g., demineralization, deproteinization, deacetylation...) in batch can be used without laborious membrane casting processes.

This work investigated chitinous membranes with various degrees of deacetylation (DD) via electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) to assess their electrochemical characteristics for constructing enzymatic biosensors. Furthermore, their physical properties were also investigated to assess the durability of membranes and relationships to conditions of deacetylation and electrochemical responses. To further expand the practical use of the versatile biomaterial, the present approach also integrated the bioactive membrane into an amperometric flow-injection analytical system.

2. Experimental

2.1. Chemicals and reagents

Hydrochloric acid, sodium hydroxide, acetone, phenol and phosphoric acid (85%, w/v) were sourced from Union Chemicals, Taiwan. Potassium ferrocyanide was purchased from Katayama Chemical (Japan). Glutaraldehyde was from Wako Pure Chemical, Japan as 25% (w/v) aqueous solution and stored at 4 °C. Glucose oxidase (EC 1.1.3.4 from *Asperigillus niger*) and peroxidase (EC 1.11.1.1. from Horseradish) were from Sigma Chemical as Type X-S (250 U mg^{-1} solid) and Type II (158 U mg^{-1} solid) enzyme powder, respectively. The enzymes were stored at −20 °C. Nafion™ 117 (5% solution, w/v), glucosamine hydrochloride and N-acetylglucosamine were purchased from Wako Pure Chemical. 4-aminoantipyrine was from Nacalai Tesque, Japan. Other chemicals were analytical grade and were used as received. Deionized water (<1 μS cm^{-1}) was used throughout this work.

2.2. Preparation of chitinous membranes from carapace of the soldier crab

Fifteen solder crabs were rinsed with water, and then added to 300 ml of 1 N HCl and soaked for 20 min at room temperature. The acid waste was decanted, and soaked in the same solution for a further 3 h to completely remove the calcium carbonate deposited on the exoskeleton. This demineralization process was performed under mild-power sonication (40 KHz, 10 W/L) in a thermo-controlled bath sonicator (T760DH, Elma, Taiwan).

The acid waste was decanted, and the crabs were then rinsed with water. The demineralized crabs were added into 300 ml of 1 N NaOH, and then soaked for 5 min, 20 min or 3 h under sonication. Finally, the alkali waste, mainly comprising crab meat proteins, was decanted. The crabs treated with the previous process were then added into 300 ml of 1 N NaOH for 30 min under sonication at 40 °C for approximately 24 h. This deproteinization process was repeated three times to yield clear chitinous membranes.

The prepared chitinous membranes were rinsed several times with acetone to remove the red pigment on the lags. Chitinous membranes were deacetylated via heterogeneous alkaline in 50% NaOH solution at a ratio of 20 in chitin-to-solution at 100 °C for 0∼90 min. Fig. 1(a) illustrates the final product of the chitinous membranes.

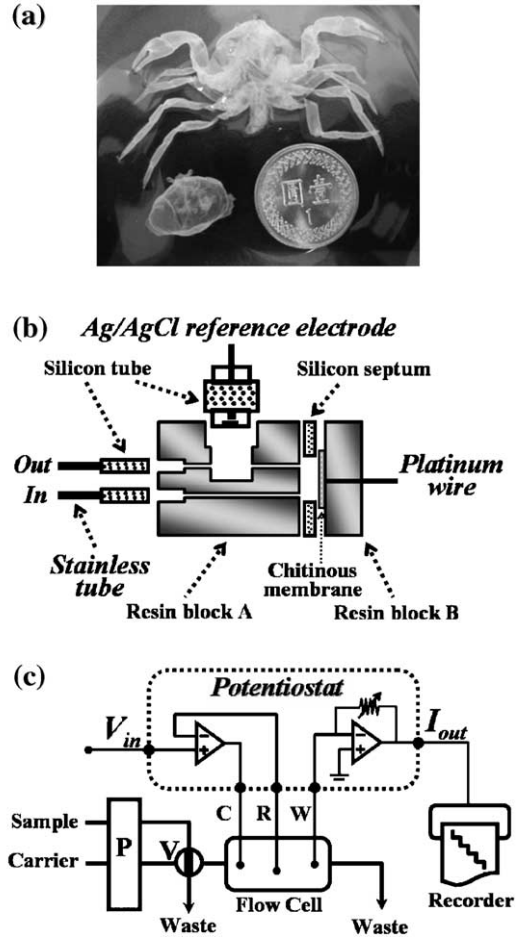

Fig. 1. Construction of the biosensor. (a) Purified chitinous membrane of a Taiwanese soldier crab (upper, ventral view). The membrane cut from the carapace was used for enzyme immobilization and sensor construction (lower left, dorsal view). Also shown in the lower right of the photo is a coin of 2.0 cm in diameter. (b) Schematic diagram of the amperometric flow cell (side view). (c) Schematic diagram of the experimental setup. C represents counter electrode (a stainless tube); R, reference electrode (an Ag/AgCl electrode); W, working electrode (a platinum wire); V, injection valve; P, peristaltic pump; V_{in}, voltage input (0.6V vs. working electrode); I_{out}, current output (oxidative current was converted into positive voltage.).

2.3. Determination of degree of deacetylation in chitinous membranes

The degree of deacetylation (DD) in the chitinous membranes was determined using an UV spectrophotometry, as proposed in our previous study [3] and was revised in a recent work [12]. Chitinous materials were dissolved with 85% phosphoric acid with the aid of assisted by a thermal-controlled sonicator (Elmer; 40 kHz, 10 W/L, 60 °C) for approximately 60 min. The resulting solutions were diluted with deionized water to a suitable extent (0.5 mg/ml chitinous materials) for measuring absorbance in a wavelength of 210 nm (Jasco V-530 system). Degree of Deacetylation was determined using a proposed equation in previous work [12].

2.4. Measurement of membrane thickness

Chitosan membranes were stored in deionized water before measurement to ensure sufficient polymer swelling. Chitosan thickness was measured using a micron micrometer (Mitutoyo, Japan) fitted with a ratchet stop. Every measurement was repeated three times.

2.5. Enzyme immobilization

Prior to the experiment, alkaline glutaraldehyde solution (5%, v/v) was freshly prepared by mixing 1 ml of 25% aqueous glutaraldehyde with 4 ml of carbonate buffer (0.2 M, pH 10.0). Chitinous membranes from the crabs' carapaces were immersed in the alkaline glutaraldehyde solution for ca. 2 h to activate the surface amino groups. After being rinsed with deionized water, the glutaraldehyde-treated chitinous membrane was immersed into enzyme solution (0.1 mg glucose oxidase in 2 ml of 0.1 M phosphate buffer, pH 6.8) and kept at 4 °C for 16 h. The chitinous enzyme membrane was then rinsed with the mentioned phosphate buffer, cut into suitable size (4 mm in diameter) and stored at 4 °C until use.

2.6. Apparatus and procedures

A three-electrode cell was used with an Ag/AgCl/3MNaCl (BAS) as the referenced electrode, a platinum wire as the counter electrode, and a glassy carbon (3 mm diameter) as the working electrode. The glassy carbon electrodes were wet-polished prior to use on a polishing cloth with alumina particles (1 μm diameter). Moreover, the slurry that accumulated on the electrode surface was removed using deionized water. The chitosan membrane was tightly wrapped on the electrode surface via a silicon O-ring with an appropriate dimension fitted to the electrode for electrochemical characterization. Cyclic voltammetry was performed in phosphate buffer solution containing 10 mM of $K_3[Fe(CN)_6]$ at a scan rate of 50 mV s^{-1}. The scan range was from −0.3 to +0.7 V. Cyclic voltammetry was performed using an Autolab PGSTA-30 digital potentiostat/galvanostat, using GPES 4.9 software for the electrochemical measurements (Eco Chemie BV, the Netherlands). An FRA module with FRA software 4.9 (Eco Chemie BV) was used for impedance measurement. The impedance spectra were recorded within the frequency range 0.1∼100 k Hz. Notably, the amplitude of the applied sine-wave potential in each case was 50 mV, while the DC potential was +0.3 V in the presence of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ as the redox couple. The phosphate buffer solution (pH 6.8) provided a background electrolyte in all experiments. All experiments were conducted at ambient temperature (25±2 °C).

2.7. Amperometric flow cell

The flow cell was composed of two resin blocks separated by a silicon septum (1 mm in thickness); these components were hold securely together by two sets of bolt and nut (Fig. 1b). On the surface of Resin block B is the platinum working electrode (1 mm in diameter) that was polished over 0.6 μm aluminum powder. Resin block A contains pathways (the inlet and outlet) for the carrier stream and a socket for Ag/AgCl reference electrode (RE-4, BAS). The stainless outlet-pipe (1 mm i.d. tube for HPLC) served as the counter electrode for 3-electrode amperometric measurement. Carrier solution flowed upward through the oval opening (2.5 mm width × 1.5 cm length, as the electrochemical reaction chamber) of the silicon septum.

2.8. Electrode modification procedure

Platinum electrode of the flow cell was modified as following. On the surface of the electrode was layered with 20 μl of methanolic Nafion solution (0.5%, w/v), the solvent was evaporated in air for ca. 1 h. The coating procedure was repeated if necessary. The chitinous enzyme membrane (4 mm in diameter) was attached on the Nafion-coated electrode and was fasten by the oval opening (2.5 mm in diameter) of the septum through the assembling process of flow cell.

2.9. Flow-injection analytical system

The assembled electrochemical biosensing flow cell was incorporated into a flow-injection manifold (Fig. 1c). With a controllable peristaltic pump (SMP-23 S, Tokyo Rikakikai Co., Japan), carrier solution (0.1 M phosphate buffer, pH 6.8) was continuously driven (typically, 1 ml min^{-1}) through a six-port rotary injection valve (Model 5020, Rheodyne) and then the flow cell. Sample solution was introduced into the carrier stream *via* the sample loop (ca. 100 μl) of the injection valve. Amperometric signal (0.6 V *versus* Ag/AgCl) was continuously monitored with a potentiostat (CV-1, BAS) and a chart-pen recorder (101 A-1875, Cole-Parmer).

2.10. Enzymatic glucose assay by colorimetric measurement

Glucose oxidase (1 mg) and peroxidase (1 mg) was dissolved in 100 ml of freshly prepared Trinder's reagent containing 250 μl of phenol, 0.1 g of 4-aminoantipyrine, 50 ml of 0.1 M phosphate buffer (pH 6.8) and 50 ml of deionized water. Into a test tube was added sequentially with 1 ml of sample solution (containing 0.01–0.1 mg of glucose) and 2 ml of the above enzyme mixture. After 10 min, absorbance at 500 nm was measured with a spectrophotometer (V-530, Jasco, Japan).

2.11. Preparation of sample solutions for glucose determinations

Commercial soft drinks made by lactate fermentation were diluted with phosphate buffer (0.1 M, pH 6.8) to suitable extent and then filtered (Whatman No. 1 ashless filter paper) before the analysis.

3. Results and discussion

3.1. Thickness and degree of deacetylation of chitosan membranes

Physicochemical or mechanical properties of membranes mounted on electrode surfaces might influence the performance and utilization of biosensors or chemical sensors. For example, the durability and sensitivity of a biosensor based on the electrochemically amperometry principle are influenced by the toughness and hydrophilicity of the membrane, respectively. It is a popular method for preparing chitosan membranes for biosensing applications that film casting with chitosan in acetic acid solution. The main sources of the chitosan include commercial powder reagents with specified high values of DD. The main shortcomings of this chitosan are membrane durability and facility of biosensor construction because the casting chitosan membrane could not be tightly attached to the electrode surface to provide a contact with the aqueous solutions. Furthermore, chitosan membranes prepared from acetic acid are also difficult to handle since they are brittle when dry and tend to roll up on contact with water. Previous study [3] showed that chitosan membranes from Taiwanese soldier crabs provide a convenient material for sheathing this polymer film upon the surface of commercial rod-type electrode. The physicochemical characteristics are examined in this section.

According to qualitative observations, chitosan membranes suffered longer duration of deacetylation was more flexible and might rupture in this work. Table 1 shows that the mean and variation of thickness in chitin membranes (sample 1, suffered no deacetylation process) are significantly less than in chitosan membranes that suffered with different durations (10∼90 min) of deacetylation. It indicated that chitin membranes originally obtained from the Taiwanese soldier crab in this study were uniform in thickness. The pretreatment time of the deacetylation process influenced the thickness but not the DD of chitinous membranes in this study. For pretreated chitosan membranes, the thickness was inversely proportional to the deacetylation duration. It is consistent with a previous report [13] demonstrating that the crystallinity and swelling index of chitosan (molecular weigh is ca. $7.4 * 10^5$) membranes are proportional and inversely proportion to the degree of deacetylation, respectively. The hydrophilic groups, hydroxyl and amino groups, on the backbone of chitosan, are known to cause chitosan membrane swelling. The increased deacetylation duration produces higher amino group content on the backbone of chitosan, which appears to improve the hydrophilicity and reduce the membrane thickness (shown in Table 1). Increasing duration of deacetylation, improves the hydrophilicity of membranes caused by the amino groups on the chitosan backbone [13] and then reduces the thickness of chitosan membranes. The thinning of chitosan is followed by an improvement in the hydrophilicity because of the degree of deacetylation (or duration of deacetylation), which was determined using a micrometer and by inspection. Furthermore, hydrophilic chitosan membranes were indirectly observed to become soft and transparent as they undergo deacetylation. Comparatively, the uniformity of thickness for chitosan membranes is strongly influenced by the deacetylation process.

Table 1
The degree of deacetylation (DD), thickness and CV parameters of chitinous membranes depend on pretreatment duration of deacetylation process

Sample number	Pretreatment duration (min)	DD (%)	Thickness (μm)		CV parameters	
			Mean	Variation	ΔVpp (V)	Ipp (mA)
1	0	5	33.67	2.52%	0.179	0.1478
2	10	21	44.08	25.77%	0.218	0.0947
3	20	27	43.75	17.03%	0.278	1.0776
4	40	24	41.17	19.58%	0.337	0.2932
5	60	24	38.92	18.12%	0.317	0.9274
6	90	34	32.92	13.56%	0.357	0.0827

Each data of CV parameters is the average of 3 repeated experiments with a standard deviation smaller than 3%.

Chitosan membranes with higher DD are facilitated to be as substrates mounted on electrodes for enzyme immobilization and concentration of negative-charged electrochemical mediators used in amperometry biosensors. The deacetylation process slightly influenced the thickness of the chitosan membranes. However, the DD was almost independent of the pretreatment duration of deacetylation. Furthermore, chitosan membrane durability is moderated as the duration of the deacetylation process increases. Given the practicability of constructing enzyme-immobilized biosensors (such as, in our previous study), chitosan membranes suffering 40∼60 min of deacetylation might provide suitable materials for developing enzyme-immobilized

biosensors according to our experiments. According our observations and experiences, the durability of chitosan membranes from Taiwanese soldier crabs suffering long duration of deacetylation is not good, but is still far better than that prepared by film casting with chitosan solution which was prepared by chitosan powder dissolved in acetic acid. Although chitosan membrane thickness variation was enhanced due to the deacetylation, this did not necessarily influence the electrochemical characteristics on the interface of electrodes. This assumption is dealt with below.

3.2. Cyclic voltammetry of chitosan membranes

Fig. 2 illustrates the CV curves of the $K_3[Fe(CN)_6]$ redox probe, dissolved in phosphate buffer (pH 6.8), on a bare glassy carbon electrode (curve a), on a chitosan membrane prior (curve b) and subsequent (curve c) to an EIS test. The curve of the bare glassy carbon electrode is a typical curve of a diffusion-controlled quasi-reversible redox process. The chitosan-mounted glassy carbon electrode before the EIS test produced small peak-to-peak separation and clear reduction in amperometric response. Curve b in Fig. 2 shows a CV response of chitosan membrane contact with electrolyte within 5 s. The redox probes ($K_3[Fe(CN)_6]$) were not yet be preconcentrated into chitosan membrane at this moment. The current peaks of CV would be increased after 5 min of incubation for preconcentrating redox probes (shown in curve C of Fig. 2). Furthermore, both the peak-to-peak separation and the amperometric response after were larger than before the EIS test. The large decrease in the amperometric response and slight increase in the peak-to-peak separation in curve B of Fig. 2 showed that the interfacial electron-transfer between the redox probe and the glassy carbon surface was mostly blocked before redox probes were concentrated into membrane, and the electrochemical double-layer on the electrode surface was almost changeless. These results implied that a chitosan membrane inhibits the electron-transfer of the redox probe when electrode only just contacts electrolyte and concentration process also just begin. The glassy carbon electrode covered with chitosan membrane posterior to the EIS test demonstrated a lager amperometric response and peak-to-peak separation than before the EIS test (curve C in Fig. 2). These experimental results might suggest that chitosan membranes with amino groups with positively-charged amino groups have a pre-concentration capacity to negative-charge of redox probe used in this work.

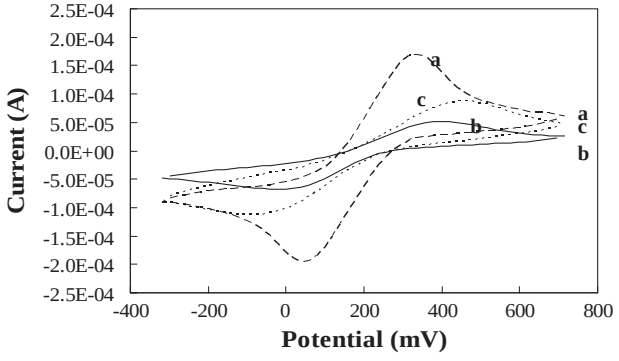

Fig. 2. Cyclic voltammograms for chitosan membranes onto a bare glassy carbon electrode in 10 mM $K_4Fe(CN)_6$ in phosphate buffer (pH 6.8). Scan rate=50 mV/s. (a) Bare glassy carbon electrode; (b) chitosan membrane immediately contact with electrolyte within 5 s; (c) chitosan membrane after 5 min of incubation.

Table 1 also lists the peak-to-peak separation potential (ΔV_{pp}) and the peak-to-peak current (I_{pp}) of cyclic voltammograms of chitinous membranes before EIS tests. The peak-to-peak separation potential was increased in chitosan membranes that suffered deacetylation of various durations. However, the relationship between the peak-to-peak separation potential and DD value was unclear. This phenomenon was also noted in other subsequent to EIS tests (not shown here). The I_{pp} values in samples 3, 4 and 5 (with DD values in the range 24~27%) significantly exceeded those in natural chitin (sample 1, DD value=5%). Chitosan members underwent short (sample 2, 10 min) and long (sample 6, 90 min) deacetylation. No predictive relationship between the peak-to-peak current of cyclic voltammograms and the duration of deacetylation is observed for chitosan membranes with various DD values, perhaps because of the irreproducible O-ring-based attachment of chitosan membranes to glassy carbon electrode. Suitable degrees of deacetylation (such as 24~27% herein) for chitosan membranes may enhance electrochemical responses of redox probes in the membranes. The higher I_{pp} values from samples 3 and 5 reveal a higher concentration of the redox probe in the membranes or a higher Faradic current between solid/liquid interfaces. These results imply that an advanced analytical method is necessary for investigating this natural material, in addition to the CV technique used in this study. Therefore, EIS was used to qualitatively and quantitatively assess chitosan membranes in the following section.

3.3. Electrochemical impedance spectroscopy of chitosan membranes

Electrochemical impedance spectroscopy is an effective method for examining the interfacial properties of electrodes, especially to modified surfaces, and is frequently used for realizing electrochemical transformation and processes associated with conductive supports [14,15]. Electrode mounted chitosan membranes are expected to adjust the interfacial electron-transfer characteristics (capacitance and resistance) on the electrode surface. Therefore, in this study, the EIS also served as the electrochemical analysis method in addition to CV. The complex impedance can be presented as the sum of the real, $Z_{re}(\omega)$, and imaginary, $Z_{im}(\omega)$, components that originate primarily from the resistance and capacitance of the cell, respectively. The general electronic equivalent circuit (Randles and Ershler model [16]) includes the ohmic resistance of the electrolyte solution (R_s), the Warburg impedance (W), resulting from ion diffusion from

the bulk electrolyte to the electrode interface, the double-layer capacitance (C_{dl}), and charge-transfer resistance (R_{ct}), which exists when the electrolyte solution contains a redox probe. Here R_s and W denote the bulk properties of the electrolyte solution and diffusion features of the redox probe in the solution, respectively.

Chitosan is a basic polymer with an intrinsic pKa value of ~6.5 (primary amines), and DD independence [17]. It indicates that chitosan membrane is protonated and positively charged in the condition used in this work. The chitosan membrane mounted on electrode may promote the redox probe $Fe(CN)_6^{4-/3-}$ to the surface owing to the positive charge in chitosan. Fig. 3 illustrates the results of EIS in the form of Nyquist plots (Z_{im} versus Z_{re}) on glassy carbon electrode (curve a), a chitin-mounted electrode (curve b) and a chitosan-mounted electrode in the presence of the redox probe (curve c). The curve shape of the electrochemical impedance spectra had the same form as the theoretical model, including a semicircle portion that appeared at higher frequency range corresponding to the electron transfer-limited process, and followed by a linear part, characterizing the lower frequency attributable to the diffusion-limited process. The semicircle diameter corresponds to the electron transfer resistance (R_{ct}) on the electrode surface. Table 2 illustrates that the charge transfer resistances in chitin membrane (sample 1) and the chitosan membrane that suffered just 10 min of deacetylation (sample 2) are considerably higher than the bare glassy carbon electrode and other chitosan-mounted electrodes. This phenomenon may occur because when the chitin or chitosan membrane has low DD, the tight layer significantly blocked the redox probe to the electrode surface. The EIS data agreed with the observations of cyclic voltammetry.

Table 2 lists the parameters of EIS for various chitosan membranes. The solution resistance (R_s) and Warburg impedance (W) are almost independent of the DD of chitosan membrane. The effect of the membrane DD affects the double layer capacitance (C_{dl}) and charge transfer resistance (R_{ct}). For natural chitin membrane that underwent no deacetylation process (sample 1, DD~5%), the double layer capacitance and charge transfer resistance were considerably lower and higher, respectively, than in other cases. Variance in the double layer capacitance (C_{dl}) was insignificant for membranes that underwent over 10 min of deacetylation (sample 2~6, DD> 21%). And charge transfer resistances (R_{ct}) would be close as membranes suffered over 20 min and less than 90 min of deacetylation (sample 3~5, DD: 24~27%) The result also complied with the results of CV, in which the peak-to-peak separation in natural chitin membrane without pretreatment was considerably smaller than in other natural chitin membranes. Those results indicated that chitosan membranes with various DD other than natural chitin membrane (sample 1) can enhance double layer capacitance, which may be induced by the precencentration of negative-charge redox probes in chitosan membranes with high value of DD. Charge transfer resistances in natural chitin membrane (sample 1) and chitosan membrane that underwent only 10 min of deacetylation process were significantly higher than the resistances in other membranes. Table 2 demonstrated that sample 3, which underwent 20 min of deacetylation process, had a minimum charge transfer resistance (R_{ct}=4.12 kΩ) that was closer to that of blank electrode had a lower Warburg impedance than samples that had undergone over 40 min of deacetylation (samples 4, 5 and 6). The CV results in Table 1 demonstrated that sample 3 underwent a maximal Faradic reaction (I_{pp}). Additionally, charge transfer resistances in samples 3, 4 and 5 were substantially less than those in other membranes. This result agreed with the results of CV experiments (Table 1 presents the I_{pp} values). The Warburg impedance (W in Table 2) was almost independent of the pretreatment time and DD, meaning that hydrophilic chitosan membranes will not block the mass transfer nearby the electrode interface. It will be facilitated to be a substrate used to immobilize enzyme for constructing amperometry biosensors.

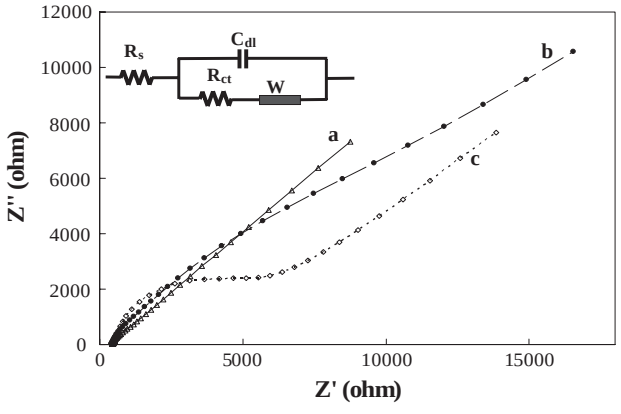

Fig. 3. Nyquist plot of the glassy carbon electrode (a), chitin (b) and chitosan (c) membranes in 10 mM $K_4[Fe(CN)_6]$ solution (phosphate buffer pH 6.8). Electrode diameter: 3 mm; applied potential: +0.3 V vs. Ag/AgCl; amplitude: 10 mV; frequency range: 10000~0.1 Hz. The insert diagram shows the equivalent circuit for the electrochemical impedance spectroscopy. Detail descriptions refer text.

Table 2
The EIS parameters obtained from different DD of chitinous membranes on glassy carbon electrodes

Sample number	Pretreatment duration (min)	DD (%)	Rs (kΩ)	Cdl (μF)	Rct (kΩ)	W (mΩ)
Blank	–	–	0.330	0.372	4.000	7.667
1	0	5	0.381	0.067	12.100	7.608
2	10	21	0.398	0.524	10.270	6.696
3	20	27	0.444	0.707	4.120	7.485
4	40	24	0.503	0.547	5.040	8.110
5	60	24	0.429	0.693	5.570	8.691
6	90	34	0.495	0.740	7.880	8.592

Rs: solution resistance; Rct: electron-transfer resistance; Cdl: double-layer capacitance; W: Warburg impedance. Each data of EIS parameters is the average of 3 repeated experiments with a standard deviation smaller than 3%.

3.4. Consideration of chitosan membranes for constructing biosensors

Chitosan membranes from the carapace of the soldier crab *M. brevidactylus* were developed for constructing an amperometric glucose sensor immobilized with glucose oxidase in our preliminary investigation [3]. The natural chitosan membrane was utilized as a substrate for the enzyme immobilization in biosensors. Previous investigation demonstrated the application potential and advantages of this material for enzymatic biosensors. In this study, EIS results demonstrated that chitosan membranes have suitable electrochemical characteristics for developing enzyme-immobilized biosensor in conditions appropriate deacetylation processes. The original chitin membrane that has undergone no deacetylation is unsuitable for use as a substrate of enzyme immobilization for amperometry sensing owing to the extremely high charge transfer resistance. The blocking of charge transfer markedly reduces the amperometry responses of biosensors used in the original membrane with low DD. Furthermore, the chitin membrane that had not undergone deacetylation (DD only around 5%) has fewer positively-charged units in its polymer substrate. This feature is unfavourable to enzyme immobilization regardless of the physical adsorption or chemical modification of specific biochemical molecules because of small quantities of positively-charged functional groups. These analytical results here also are consistent with the considerations and practical processes involved in constructing an enzymatic biosensor for glucose in previous investigation [3].

Although preparing chitosan membranes from soldier crab is laborious and consumes large quantities of solvents, the work-loading, economic efficiency and environmental influence can be improved through batch-based mass production. The chitosan membranes that directly utilize natural sources proposed in this work, unlike spin-or dip-coating chitosan solution on the electrode surface, have good durability and are easy to handle in constructing enzymatic sensors on the rod-type electrodes generally used in laboratories. The chitosan membranes can be easily removed from and re-mounted on electrodes and also preserved in appropriate retaining solutions (e.g., such as, phosphate buffer, pH 6.8). The reusability and convenience of these membranes means that they have potential for developing biosensors using rod-type electrodes.

3.5. Enzymatic biosensor based on chitosan membranes

3.5.1. Enzyme immobilization

Glucose oxidase was covalently immobilized on the surface amino groups of the chitinous membrane; its Michaelis constant (10.6 mM) was similar to the previous study [3] and reported values [9,10]. The membrane surface turned slightly reddish and yellowish as described previously [3]; the bioactive enzyme membrane was durable for months.

3.5.2. Dose-dependent biosensor responses

Injected segments (100 μl) of sample solution were delivered into the electrochemical flow cell by the carrier stream. Catalyzing by immobilized glucose oxidase, β-D-glucose was oxidized rapidly by dissolved oxygen on the outer surface of the chitinous membrane. The evolved hydrogen peroxide diffused through the chitinous membrane and finally reached the positively polarized (0.6 V *versus* Ag/AgCl) platinum electrode.

The carrier (0.1 M phosphate buffer, pH 6.8) served as both the pH buffer and supporting electrolytes for the electrochemical bioanalytical system. After filling with the carrier solution, the conductance between platinum and Ag/AgCl electrode of the flow cell was around 30 μS as measured with a conductometer (2 Vpp, 1 kHz); the calculated IR drop by 1 μA was below 35 mV. Potential (600 mV) can therefore be accurately imposed on the electrochemical system.

Oxidative currents of hydrogen peroxide were monitored, and typical FIAgrams were recorded. The sample throughput was higher than 15 determinations per hour when carrier flow rate was set around 1 ml min^{-1}. For convenience, peak heights were taken for quantification. Calibration curve (Fig. 4) passed through the origin with linear dynamic range up to mM range ($r=0.999$). Noise was reduced by applying a built-in low-pass filter of the potentiostat (time constant=1 s), and glucose concentration lower then 10 μM can be determined with S/N ratio higher than 5. The relative standard deviations ($n=5$) for determination of 50 μM glucose were less than 1.3%.

3.5.3. Effects of ascorbate

Ascorbate is the chief problematic compound for an amperometric biosensor. The compound will be oxidized on a polarized platinum surface with biased potential (+0.1 *versus* Ag/AgCl) lower than the overpotential needed for oxidizing hydrogen peroxide. Therefore, interference removing strategies cannot be omitted when considerable amount of ascorbate coexists in the sample matrix.

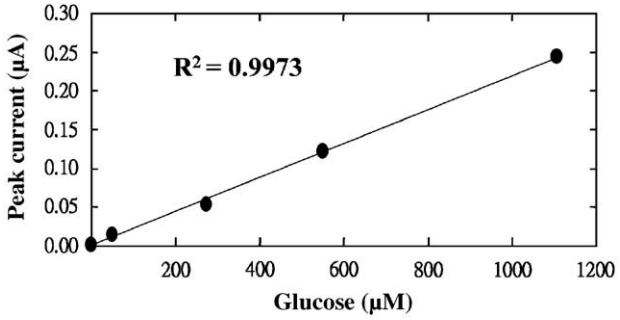

Fig. 4. Calibration curve of the flow-injection biosensing system. Peak heights were taken for the analysis.

Chitinous membrane from the soldier crab did somewhat restrict the diffusion of ascorbate, and the sensor response (the peak oxidative current) to glucose was about twice as to ascorbate when the signals of solutions containing equal concentration (0.5 mM) of the analytes were compared (Fig. 5b). The pH value (6.8) used in the present system was optimized in the previous investigation [3] for a better selectivity against ascorbate.

3.5.4. Effects of flow rate

Effects of carrier flow rate on both the signals of glucose and ascorbate were compared (Fig. 5a). Unfortunately, no obvious improvement in selectivity can be achieved by adjusting carrier flow rate (Fig. 5b). Flow rate of 0.15 ml min^{-1} was considered to be the best condition for sensor's sensitivity. However, flow rate of 0.7 ml min^{-1} is the choice by considering both the sensitivity and sample throughput (Fig. 5c).

Studies on the baseline reversion time revealed additional information of the selective permeation property of the chitinous membrane. Compared with hydrogen peroxide (the product of GOD-catalyzed reaction), larger chemicals such as ascorbate are generally slower in mass transfer through a selective membrane. Broader signal profiles (longer baseline reversion time) were therefore observed for ascorbate.

3.5.5. Effects of Nafion-coating

To further improve the selectivity, Nafion™ was coated on the platinum electrode surface [18] prior to the attachment of the chitinous enzyme membrane. The interfering current signal from 0.5 mM ascorbate was below 5%, which was considerably lower than that without Nafion-coating (20% estimated from Fig. 5a), of the value from the same concentration of glucose. The FIAgrams for glucose were similar to those obtained without Nafion-coating. The anionic Nafion membrane and the cationic chitosan membrane were compatible and worked in concert for interference removal.

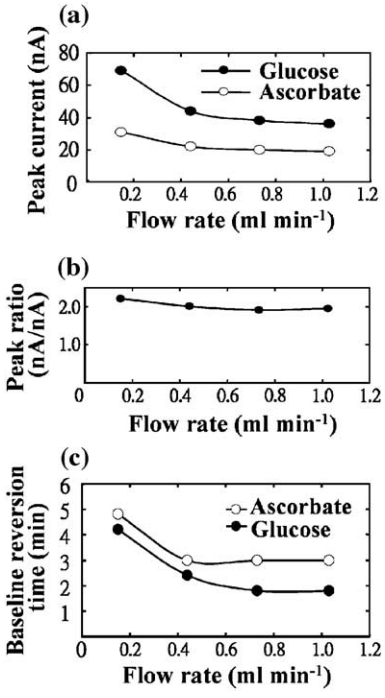

Fig. 5. Effects of flow rate on sensor responses to 0.5 mM glucose and ascorbate. (a) Effects on peak heights of FIAgrams. (b) Effects on ratio (glucose/ascorbate) of the peak heights. (c) Effects on baseline reversion time.

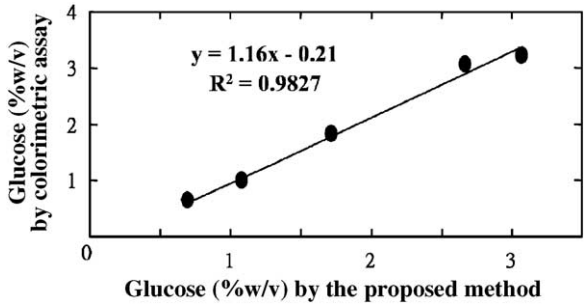

Fig. 6. Comparison of analytical results obtained by colorimetric enzymatic assay and those with the proposed biosensing system.

3.5.6. Evaluation of the accuracy of the biosensing system

Data obtained by the Nafion-coated biosensor were compared with those determined by a Trinder's reagent-based enzymatic assay. Since ascorbate reacts also with hydrogen peroxide, higher concentrations of the reactants (4-aminoantipyrine and phenol) and the catalysts (glucose oxidase and peroxidase) were added into the assay mixture to reduce the side reaction. The analytical results for 5 commercial soft drinks correlated well as expected (Fig. 6).

4. Conclusion

Chitosan membrane prepared from Taiwanese soldier crab is a thin, tough and durable membrane that can be directly mounted on rod-type electrode without film casting. The degree of deacetylation of chitosan membranes increased considerably with various deacetylation durations in the consistency of the thickness of chitosan membranes depends on the deacetylation process. The membrane thickness slightly influences the toughness and electrochemical behaviour on the electrode interface. The EIS and CV test results demonstrated that chitosan membranes that suffered an appropriate deacetylation process provide a suitable substrate for immobilizing enzymes for biosensor construction owing to their good electrochemical characteristics and excellent mechanical properties.

The membrane itself even acted as the permeation barrier for interference removal, biosensing devices of practical uses were therefore easily developed. As revealed by the present study, chitinous membrane from the soldier crab is certainly a promising versatile biomaterial; other biomedical applications are currently under investigations. Beside its

beautiful look and harmless behaviour, the creature does have potential economic values and deserves to be protected.

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