# 行政院國家科學委員會專題研究計畫　成果報告

## 和尚蟹幾丁質皮膜的製備與產業的應用

**計畫類別：** 個別型計畫
**計畫編號：** NSC91-2313-B-002-346-
**執行期間：** 91 年 08 月 01 日至 92 年 07 月 31 日
**執行單位：** 國立臺灣大學生物產業機電工程學系暨研究所

**計畫主持人：** 陳力騏

**報告類型：** 精簡報告

**處理方式：** 本計畫可公開查詢

中　華　民　國 92 年 10 月 16 日

# Using Chitosan Membrane from the Carapace of the Soldier Crab *Mictyris brevidactylus* for Biosensor Construction

Bo-Chuan Hsieh, Tzong-Jih Cheng, Tzu-Yu Wang, and Richie L. C. Chen*

Department of Bio-Industrial Mechatronics Engineering

College of Agriculture, National Taiwan University

1, Roosevelt Road, Sec. 4, Taipei, Taiwan 106, R.O.C.

rlcchen@ccms.ntu.edu.tw

TEL: +886-2-33665330

FAX: +886-2-23627620

Running title: Soldier crab's chitosan membrane for biosensing

Keywords: chitosan, *Mictyris*, biosensor

Abstract

Glucose oxidase (EC 1.3.4.3) was immobilized on the chitosan membrane (< 0.1mm in thickness) prepared from the carapace of the soldier crab *Mictyris brevidactylus*. A glucose electrode was constructed by covering a platinum electrode (2.0mm in diameter) with the enzyme membrane. The enzyme electrode sensed glucose amperometrically (1.0 μA/mM with linear range up to 0.5mM, r=0.999) when positively imposed with 0.6V against an Ag/AgCl reference electrode. The glucose biosensor was sensitive (< 0.1μM, S/N >3), reproducible (CV for 55μM glucose < 3%, n=5), reagentless and durable for months.

## INTRODUCTION

Soldier crabs are ecologically important intertidal sandy-shore crabs in tropical and subtropical Indo-Pacific regions (Takeda et al., 1996). The unusual high population density (up to 120 crabs/$m^2$ for *Dotilla myctiroides* in Phuket, South Thailand; up to 226 crabs/$m^2$ for *Mictyris brevidactylus* in Taiwan) renders soldier crabs a potential nature resource of chitin (Bradshaw and Scoffin, 1999; Shih, 1995).

Chitin and the deacetylated derivative, chitosan, are well-known biomaterials with increasing environmental (Jeuniaux, 1986), food (Shahidi et al., 1999), pharmaceutical (Borchard and Junginger, 2001) and other biomedical applications (Madihally and Mattew, 1999; Shu et al., 2001; Singh and Ray, 1999). Different from cellulose, chitin and

chitosan have additional target functional groups for enzyme immobilization (Muzzarelli, 1980), the materials were therefore used as the supporting materials for biosensing purposes (Diamond, 1998). As the examples, Ohashi and Karube (1995) used β-chitin from squid pens of the squid *Todarodes pacificus*, and Sugawara et al. (2000) used commercialized chitin powder for sensor construction. Among those studies, membrane casting and electrode modification processes were required, which will increase the manufacturing cost for commercialization.

We are the first group to purify the versatile biomaterial from soldier crabs and founded that the paper-thin contact lens-sized chitin membranes obtained from the dorsal part of the carapace can be used directly as the supporting material for constructing a biosensor.

## MATERIALS AND METHODS

### Chemicals

Hydrochloric acid, sodium hydroxide, acetone and phosphoric acid (85%) were purchased from Union Chemical, Taiwan. Glutaraldehyde (25% aqueous solution) was from Wako Pure Chemical, Japan. Glucose oxidase (EC 1.1.3.4 from *Asperigillus niger*) was purchased from Sigma Chemical as Type X-S enzyme (250 U/mg solid). Chitin and chitosan standards (85% of deacetylation degree with about 1000 residues) were from Wako Pure Chemical, Japan. Other chemicals were of analytical grade and purchased from Nacalai Tesque, Japan. All chemicals were

used as received.

**Preparation of Chitosan Membrane from *Mictyris brevidactylus***

Alive crabs (*Mictyris brevidactylus* Stimpson, 1858) with carapace of ca. 1.5cm in diameter were collected from the intertidal sandy flats at Putai, Chiayi, Taiwan and refrigerated before use. After rinsed with tap water, whole crabs were used directly without pretreatment. Typically, 10 crabs were added into a 250ml glass flask containing 200ml of 1N HCl and were soaked for 1hr at ambient temperature to remove the calcium carbonate deposited on the exoskeleton. The demineralization process was performed under mild sonication and occasional shakings. A thermo-controlled bath-type sonicator (T760DH, Elma, Taiwan) working at 40kHz, 10W/liter, 40$^{o}$C was used throughout the study.

The acid waste solution was decanted and the crabs were rinsed with tap water. The demineralized crabs were added into another 250ml glass flask containing 200ml of 1N NaOH. After 30min of sonication, the flask was kept at 40$^{o}$C for ca. 16hr. After another 30min of sonication to disperse the solubilized materials, the alkaline waste, principally the crab meat protein lysate, was decanted. The deproteinization process was repeated about three times to obtain clear chitinous skins (or membranes).

The resulting chitinous membranes were rinsed several times with acetone to remove the red pigment on the legs and other hydrophobic materials. Immediately before the subsequent deacetylation process, a 50 %(w/v) aqueous NaOH solution was preheated in a Schott bottle at 95$^{o}$C for 1hr to remove the dissolved oxygen. Into the bottle, the decolorized chitin membranes were added and heated at 95$^{o}$C for 3hr. The resulting chitosan membranes from the dorsal part of the carapaces were

cut (1.5cm in diameter) and stored in deionized water at 4$^{o}$C.

## Measurement of Membrane Thickness

Membrane thickness was measured with a micron micrometer (Mitutoyo, Japan) equipped with a ratchet stop.

## Determination of Deacetylation Degree

Deacetylation degree of chitin or chitosan membrane was determined by first-derivative spectrophotometric method (Muzzarelli and Rochetti, 1985; Tan et al., 1998). Different from the reported approaches, samples were dissolved in phosphoric acid (typically 25 μg/ml), a solvent with negligible absorbance at the measuring wavelengths. A two wavelength (λ= 210nm & 220nm) measuring strategy (Atkins, 2001) was adapted to calculate the deacetylation degree (DD) from the following equation.

$$DD = \frac{\begin{vmatrix} D\varepsilon_{210,chitin} & DA_{210} \\ D\varepsilon_{220,chitin} & DA_{220} \end{vmatrix}}{\begin{vmatrix} DA_{210} & D\varepsilon_{210,chitosan} \\ DA_{220} & D\varepsilon_{220,chitosan} \end{vmatrix} + \begin{vmatrix} D\varepsilon_{210,chitin} & DA_{210} \\ D\varepsilon_{220,chitin} & DA_{220} \end{vmatrix}}$$

Wherein, $DA_{210}$ and $DA_{220}$ are the difference-quotients (absorbance to wavelength) obtained respectively at 210 and 220nm with 3nm data interval. $D\varepsilon_{210,chitin}$ and $D\varepsilon_{220,chitin}$ are calculated $DA_{210}$ and $DA_{220}$ of 1M chitin; $D\varepsilon_{210,chitosan}$ and $D\varepsilon_{220,chitosan}$ are $DA_{210}$ and $DA_{220}$ of 1M chitosan. The aforementioned chitin and

chitosan standards were used to calculate the above parameters. A computerized spectrophotometer (V-530, Jasco, Japan) was used.

## Enzyme Immobilization on Electrode Capped with the Chitosan Membrane

A platinum working electrode (CHI102, CH Instruments; 2.0 mm in diameter, embedded in 6.4mm O.D. plastic rod) was covered with the chitosan membrane from crab's carapace and fasten by an O-ring. The electrode was dipped into a freshly prepared glutaraldehyde solution (0.4ml of 25% aqueous glutaraldehyde was added to 1.6ml of 0.2M carbonate buffer, pH 10.0). After 2 hr, the electrode was rinsed with deionized water and dipped into an enzyme solution (0.5mg glucose oxidase in 2ml of 0.1M phosphate buffer, pH 6.8) for 16 hr at $4^{o}$C. The enzyme electrode was then rinsed with the mentioned phosphate buffer and stored at $4^{o}$C.

## Amperometric Glucose Biosensing

The enzyme electrode, together with an Ag/AgCl electrode (RE-4, BAS, USA) and a stainless counter electrode, was immersed in a beaker containing 0.1M phosphate buffer or acetate buffer (Figure 1). A cyclic voltameter (CV-1, BAS, USA) working at its constant potential mode was used to conduct the three-electrode-amperometric experiment. Referring to the Ag/AgCl reference electrode, the enzyme electrode was positively imposed with 0.6V. Sample containing glucose was added directly into the beaker with magnetic stirring. The current was *I-V* converted and monitored with a chart-pen recorder (101A-1875, Cole-Parmer, USA). Typically, the noise from the magnetic stirrer (JS-H, JII Tsann, Taiwan) was removed by applying the bulit-in low pass filter (time constant = 1s) of

the cyclic voltameter.

## RESULTS AND DISCUSSION

### Membrane Preparation and Enzyme Immobilization

Immediately upon contact with hydrochloric acid, bubbles (mainly carbon dioxide) evolved from the surface of the crabs. After about 1hr, the bubbling ceased and the exoskeletons were softened. Sonication facilitated the removal of the bubbles and increased the contact of hydrochloric acid with the exoskeletons. The meat of the crabs was separated from the exoskeletons by the aids of both the bubbling and the sonication process.

The demineralized crabs turned reddish immediately after the addition of 1N NaOH, which might be due to the conformation-associated bathochromic shift of epithelium carotenoproteins (Weesie et al., 1999; Okada et al., 1995; Matsuno, 2001). Compared with documented methods (Zentz et al., 2001), a relatively mild deproteinization temperature was adapted, which can prevent Schiff base formation reactions between the crab meat proteins and the amino groups on the chitin membrane surface. Clear chitinous membranes were obtained without manual treatments (Figure 2A, 2B).

Although it is not necessary for the following biosensing approach, the red-pigmented parts on the legs can be removed after rinsed with acetone (Figure 2C). Other parts including the claws and the carapaces are not pigmented from the beginning. The single-layered chitin membranes from the dorsal carapaces were about tens of micrometers but less than 0.1mm in thickness (Figure 2D).

After several hours of the deacetylation process, the deacetylation degrees (DD) in the carapace area were determined to be in the range of 75~85%. The chitosan (DD>40%) membranes were stable in alkaline and neutral medium but not in acidic medium. The thickness, curvature, size (ca. 1.5 cm in diameter), mechanical strength, and the hydrophilic nature of the membrane make it suitable for directly mounting on the surface of an analytical electrode. The membranes can be easily fastened on the electrode by the aid of a rubber O-ring.

After the enzyme immobilization procedure, the membrane surface turned slightly reddish and yellowish, which is partially due to Schiff base formation (between glutaraldehyde and amino groups of chitosan and enzyme molecule) and the deposited flavin molecules (the coenzyme of glucose oxidase), respectively (Figure 3).

## Glucose Biosensing

On the outer surface of the membrane, glucose was oxidized enzymatically as the following reaction:

$$\beta - D - Glu\cos e + O_2 \xrightarrow{\text{Glucose oxidase}} \delta - Gluconolactone + H_2O_2$$

The dissolved oxygen in solution was usually in excessive amount, and the reaction therefore followed pseudo-first order kinetics. The enzymatically evolved hydrogen peroxide diffused through the membrane and was oxidized on the positively polarized (0.6V *versus* Ag/AgCl) platinum electrode (Figure 3). Phosphate buffer (0.1M, pH 6.8) served as both the pH buffer and the supporting electrolytes for the electrochemical analytical system.

As revealed in Figure 4, the oxidative current responded linearly to the concentration of glucose in sample solution. The calibration curve pass through the

origin, and the linear dynamic range was up to 0.5 mM (r=0.999). While depends on the effective electrode surface area, the membrane thickness and also the enzyme activity immobilized on the membrane, the typical sensitivity was around 1 μA/mM. By applying built-in low-pass filter (1s in CV-1, BAS), the noise from the magnetic stirrer was excluded and glucose concentration lower then 0.1μM can be quantified with S/N ratio higher than 3. The relative standard deviations for 55μM glucose (n=5) were less than 3%.

## Michaelis Constant of Immobilized Enzyme

The Michaelis constant of the covalently immobilized glucose oxidase in pH 6.8 was calculated by double reciprocal plot to be 10.6 mM. The value is comparable to Sugawara's result (9.3 mM in pH 6.2) using electrostatic immobilization method (Sugawara *et. al.*, 2000).

### pH Optimization and Lifetime of the Sensor

Figure 5 shows the pH profile of the sensor response, the sensitivity was better in neutral and slightly alkaline medium. While the activity and stability of glucose oxidase from *Asperigillus niger* are better in acidic region (pH 5~6), the permeability (Shu et al., 2001; Singh and Ray, 1999) and the oxidation of hydrogen peroxide are pH-dependent and better at slightly alkaline region.

Also shown in Figure 5 is the pH dependency of sensor response to ascorbate, a common interfering substance of amperometric biosensing. Ascorbate can be easily oxidized on a positively polarized electrode, and that will result in a large oxidative current. Biosensing approaches using naked electrode generally can not tolerate the interferent to a satisfactory extent; the sensitivity ratio (glucose to ascorbate) is

usually less than 0.1 (Ohashi and Karube, 1995). The chitosan membrane from *Mictyris brevidactylus* was effective as a size-exclusion barrier; the sensitivity ratio (or selectivity factor) of the sensor was higher than 1 and about 7 at neutral and slightly alkaline pH (Figure 5). Increasing the density and activity of enzyme immobilized can further elevate the selectivity factor to a more satisfactory level.

By considering both the sensitivity and the selectivity, pH 6.8 was chosen as the working pH for the glucose sensor. Besides, a chitosan membrane will gradually dissolve in acidic environment, and the membranes were therefore more durable in neutral or alkaline environment.

Figure 6 shows the durability the glucose sensor worked and stored in the working buffer (0.1M phosphate buffer, pH 6.8). The optimum sensor response lasted for about two months, and the shelf life of sensor can be longer than four months.

## Conclusions

The chitin membrane prepared from *Mictyris brevidactylus* was proved to be useful as the supporting material for enzyme immobilization, especially for biosensing purposes. The membrane itself acted also as the permeation barrier for interferent removal, which is of merit in commercialization of the sensor. Furthermore, chitin purification process from the crabs can be conducted without manual operations, which will ease the industrialization. We are currently investigating the impedance properties, microscopic structures and other potential uses of the promising biomaterial. Other byproducts from the soldier crabs such as the crab meat protein lysates may serve as another valuable natural resource (Ferrer et al., 1996), the corresponding studies were also undertaken.

Wetland ecosystem, especially in the estuary region, is of great fertility and biodiversity. However, without economic inducements, wetland protections usually just end up with slogans. Instead of the conventional passive protection acts, environmental conservation strategies should be more constructive and would better provide concrete economic benefits. Owing to the tremendous fertility, the critical point for the conservation of wetland fauna is not to limit the consumption of certain species but to keep the entire ecosystem from intensive artificial destruction (*i.e.* Habitat protection is more crucial than utilization control).

Although the local population densities of soldier crab are possibly the highest (among other crabs) in estuarial wetland regions, their distributions are restricted in certain special environments (Bradshaw and Scoffin, 1999). The crabs are therefore easily collected and utilized but simultaneously endangered (Shih, 1995). Beside of its beautiful look and harmless behavior, the creature does have immense economical value (as revealed by the present study) and deserves to be protected. We deeply hope that the present study will facilitate the conservation of the endangered species (at least now in Taiwan) and its ecosystem, and also studies like this will open a new direction of turning biodiversity conservation actions into a more practical and aggressive mode.

REFERENCES


Atkins, P.W. (2001). In: *The elements of physical chemistry*. 3rd ed., Oxford.

Borchard, G., and Junginger, H.E. (2001). Modern drug delivery applications of chitosan. *Adv. Drug Delivery Rev.* 52:103.

Bradshaw, C., and Scoffin, T.P. (1999). Factors limiting distribution and activity patterns of the soldier crab *Dotilla myctiroides* in Phuket, South Thailand. *Mar Biol* 135:83-87.

Diamond, D. (1998). In: *Principles of chemical and biological sensors.* Wiley.

Ferrer, J., Paez, G., Marmol, Z., Ramones, E., Garcia, H., and Forster, C.F. (1996). Acid hydrolysis of shrimp-shell wastes and the production of single cell protein from the hydrolysate. *Bioresource Technol* 57:55-60.

Jeuniaux, C. (1986). Chitosan as a tool for the purification of water. In: *Chitin in nature and technology.* Muzzarelli, R.A.A., Jeuniaux, C., and Gooday, G.W. (ed.) pp. 551-570, Plenum Press, NY.

Madihally, S.V., and Mattew, H.W.T. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials* 20:1133-1142.

Matsuno, T. (2001). Aquatic animal carotenoids. *Fisheries Sci* 67:771-783.

Muzzarelli, R.A.A. (1980). Immobilization of enzymes on chitin and chitosan. *Enzyme Microbiol Technol* 2:177-184.

Muzzarelli, R.A.A., and Rochetti, R. (1985). Determination of the degree of acetylation of chitosan by first derivative ultraviolet spectrophotometry. *Carbohydr. Polym.* 5:461-472.

Ohashi, E., and Karube, I. (1995). Development of a thin membrane glucose sensor using β-type crystaline chitin for implantable biosensor. *J Biotechnol* 40:13-19.

Okada, S., Nureborhan, S.A., Watabe, S., and Yamaguchi, K. (1995). Changes in body color appearance of the black tiger prawn *Penaeus Monodon* by the varied composition of carotenoids soluble as carotenoprotein and remaining insoluble after collagenase treatment for the muscular epithelium. *Fisheries Sci* 61: 964-967.

Shahidi, F., Arachchi, J.K.V., and Jeon, Y.J. (1999). Food applications of chitin and chitosans. *Trends Food Sci Technol* 10:37-51.

Shih, J.T. (1995). Population densities and annual activities of *Mictyris brevidactylus* (Stimpson, 1858) in the Tanshui mangrove swamp of northern Taiwan. *Zoological Studies* 34:96-105.

Shu, X.Z., Zhu, K.J., and Song, W. (2001). Novel pH-sensitive citrate cross-linked chitosan film for drug controlled release. *Int J Pharmaceutics* 212:19-28.

Singh, D.K., and Ray, A.R. (1999). Controlled release of glucose through modified chitosan membranes. *J Memb Sci* 155:107-112.

Sugawara, K., Takano, T., Fukushi, H., Hoshi, S., Akatsuka, K., Kuramitz, H., and

Takana, S. (2000). Glucose sensing by a carbon-paste electrode containing chitin modified with glucose oxidase. *J Electroanal Chem* 482:81-86.

Takeda, S., Matsumasa, M., Yong, H.S., and Murai, M. (1996). “Igloo” construction by the ocypodid crab, *Dotilla myctiroides* (Milne-Edwards) (Crustacea; Brachyura): the role of an air chamber when burrowing in a saturated sandy substratum. *J Exp Mar Biol Ecol* 198:237-247.

Tan, S.C., Khor, E., Tan, T.K., and Wong, S.M. (1998). The degree of deacetylation of chitosan: advocating the first derivative UV-spectrophotometry method of determination. *Talanta* 45:713-719.

Weesie, R.J., Merlin, J.C., Degroot, H.J.M., Britton, G., Lugtenburg, J., Jansen, F.J.H.M., and Cornard, J.P. (1999). Resonance Raman-spectroscopy and quantum-chemical modeling studies of protein-astaxanthin interactions in alpha-crustacyanin (major blue carotenoprotein complex in carapace of lobster, *Homarus Gammarus*). *Biospectroscopy* 5:358-370

Zentz, F., Bedouet, L., Almeida, M.J., Milet, C., Lopez, E., and Giraud, M. (2001). Characterization and quantification of chitosan extracted from nacre of the abalone *Haliotis tuberculata* and the oyster *Pinctada maxima*. *Mar Biotechnol* 3:36-44.

FIGURE LEGENDS

Figure 1. Schematic diagram of the experimental set-up. C: counter electrode (a stainless wire); R: reference electrode (an Ag/AgCl electrode); W: working electrode (a platinum electrode); M: enzyme membrane (as detailed in Figure 3); $V_{in}$: voltage input (-0.6V *versus* working electrode); $I_{out}$: current output (Oxidative current was converted into positive voltage.). The experiments were performed at ambient temperature.

Figure 2. Chitinous membranes from *Mictyris brevidactylus*. A (Upper left): ventral view; B (upper right): dorsal view; C (lower right): dorsal view of a membrane after acetone treatment; D (lower left): dorsal view of a membrane cut from the carapace part. Indicated by the white circles in Figure 2A and 2B are the red-pigmented parts of the walking legs. Some legs were detached during the purification processes. The membranes were stored at $4^{o}$C for at least 2 months.

Figure 3. Reaction scheme on the glucose-sensing working electrode. Glucose was oxidized enzymatically outside the chitosan membrane at the expense of the reduction of molecular oxygen to hydrogen peroxide. Hydrogen peroxide diffused through the membrane and was oxidized on the surface of the positively polarized platinum electrode. The enzymatic-electrochemical relayed reactions reached a steady state within seconds.

Figure 4. Typical sensor responses to the additions of glucose solution. Glucose solution (275mM $\times$ 20μl) was added into the system (Figure 1) containing 100ml of the working buffer (0.1M phosphate buffer, pH 6.8) at the indicated time (the arrows). The oxidative current (as a voltage signal) was passed

through a low-pass filter (time constant = 1s) before recording with a chart-pen recorder.

Figure 5. Effect of pH on sensor sensitivity. pHs were controlled by 0.1M phosphate buffer (pH 6.0, 6.8, 8.0) and 0.1M acetate buffer (pH 6.0, 5.0, 4.0). There is no significant difference between the results obtained with phosphate buffer and acetate buffer at pH 6.0. Other test conditions were as Figure 4. Each data point is the average of seven repeated experiments with a standard deviation smaller than the symbol.

Figure 6. Durability of the sensor. The test conditions were as Figure 4. Each data point is the average of seven repeated experiments with a standard deviation smaller than the symbol.