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# Evaluation of concanavalin A–mannose interaction on the electrode covered with collagen film

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**Abstract**

An electrode covered with a lectin/collagen film was constructed to investigate whether the film was usable as a reaction field of binding between the lectin and sugar. The protein–sugar binding on cell surface plays an important role to various physiologic processes. The film is considered to be a cell surface, due to its biocompatibility. The immobilization of concanavalin A (Con A) which is one of proteins was attempted by an electrostatic interaction of the protonated functional groups of film to the negative charged Con A. The merit of this immobilization is that the interaction hardly causes any changes in the protein structure. Because Con A recognizes mannose moiety, the mannose was labeled with an electroactive compound. The binding was estimated from the changes of the electrode response based on the holding of electroactive moiety in the binding site of Con A to the mannose moiety. However, the electrode responses of glucose and galactose labeled with the same substance did not change. The result shows that Con A is immobilized on the film and combines with labeled mannose. Therefore, it is clear that the collagen film is suitable as the reaction field to evaluate the protein–sugar binding.


*Keywords:* Lectin; Voltammetry; Collagen; Label

## 1. Introduction

Collagen is one of the screoproteins and most abundant form of natural protein found in the body of animals and human. In mammals, collagen constructs 70% of skin and 90% of fiber component on coria layers. Recently, 19 different types of collagen based on the structure of amino acid have been found in human. These are grouped into seven classes (I–VII), for example, type I is major element of skin, bone, and tendon, where else type II forms cartilage. All types of the collagen are triple-helical structure and mainly consist of glycine, proline, and hydroxyproline residues. Arginine and lysine residues are also characteristic in the collagen. The role of collagen is to form and retain internal organs. Due to the substance's biocompatibility to human being, it is applied to artificial skin, blood vessel and suture thread of operative treatment. Furthermore, collagen is frequently used to coat titanium plate [1] and silicon rubber [2], which are implanted in body. To further extend the application of collagen, the use of film formed collagen is encouraged. Yamauchi et al. has proposed the film cross-linked by disulfide [3] and multiplayer film mixed calcium phosphate [4]. Modifications of growth hormone and bovine serum albumin to collagen film were also useful in ensuring that a medication gradually dissolves in the body [5,6]. On the other hand, sensors in which an enzyme were immobilized on the film was designed [7,8].

Binding between protein and its ligand relates to various physiologic processes, and often occurs at cell surface. To investigate the binding, approaches using lipid bilayer membrane [9] and Langmuir–Blodgett film [10] are generally carried out. Furthermore, the binding is evaluated by using biomaterial in which a protein having molecular recognition function is immobilized. We have proposed a method to investigate the streptavidin–biotin interaction at an electrode covered with chitin film [11]. It is expected that this method

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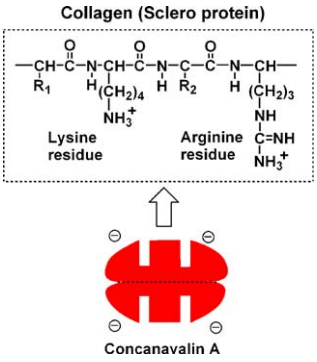


Fig. 1. Principle of immobilization of concanavalin A (Con A) on the collagen flim surface.

can be applied to examine the protein–ligand binding which happens at the cell surface.

Lectin is glycoprotein which combines with a sugar residue that has a specific structure. Lectin contributes to cytogenesis, cell attachment, fertilization and immunization. Because sugar chain exists on hematocyte and cell surface, the lectin–sugar binding is related to the cell aggregation and metabolism of hormone [12]. Therefore, a number of studies concerning the binding between lectin and sugar have been reported. For example, the affinity constant of lectin–sugar binding was determined by capillary electrophoresis [13]. Biosensor was also developed at a Pt electrode with multilayer thin film of lectin and enzyme combining mannose [14]. Afonso et al. evaluated the binding between sugar chain and lectin immobilized on glass slide with MALDI [15].

In this study, an electrode covered with a lectin/collagen film was constructed to investigate whether the lectin immobilized on the film combined with a sugar moiety. The lectin and the collagen film were considered to be a receptor at the cell surface and to be a reaction field for event of lectin–sugar binding. Concanavalin A (Con A), which is lectin from jackbean, recognizes mannose moiety. The Con A consists of four subunits, and its isoelectric point is 5.0. In weak acid solution, the immobilization of Con A on the collagen film surface is achieved by an electrostatic interaction between the protonated amino acid residues of the film and the negative charged Con A. The merit of this method is that the structure of protein could be hardly changed (Fig. 1). In the previous study, mannose labeled with an electroactive compound was used to establish a homogeneous assay to the Con A–mannose binding [16]. The Con A–mannose binding was evaluated from the changes in the electrode response of labeled mannose. The reagent is assumed to be a substitute of a sugar chain and also becomes a powerful probe in the heterogeneous system (Fig. 2).

## 2. Experimental

### 2.1. Reagents

Collagen film was supplied from KOEN Co. Ltd., Japan. Daunomycin, mannose, galactose, and glucose were from Wako Pure Industries, Japan. Concanavalin A, soybean agglutinin (SBA), and wheat germ agglutinin (WGA) were purchased from Sigma. Phosphate buffer (pH 6.0) of 0.1 M with $KH_2PO_4$ of 0.1 M and NaOH of 0.1 M was used as incubation solution and as a supporting electrolyte in electrochemical measurement. High quality nitrogen gas was used for deaeration of measurement solution. All reagents were of analytical reagent grade.

### 2.2. Apparatus

All voltammetric measurements were carried out using CV-50W analyzer (Bioanalytical Systems Inc. (BAS)). A glassy carbon electrode (Model No. 11-2012, BAS) was used as a working electrode. Before measurements, the electrode was polished with 1.0, 0.3, and 0.05 μm alumina (Baikoski International Corp., Charlotte, NC). A counter electrode was a platinum wire, and an Ag/AgCl electrode (Model No. 11-2210, BAS) was used as a reference electrode. All potentials

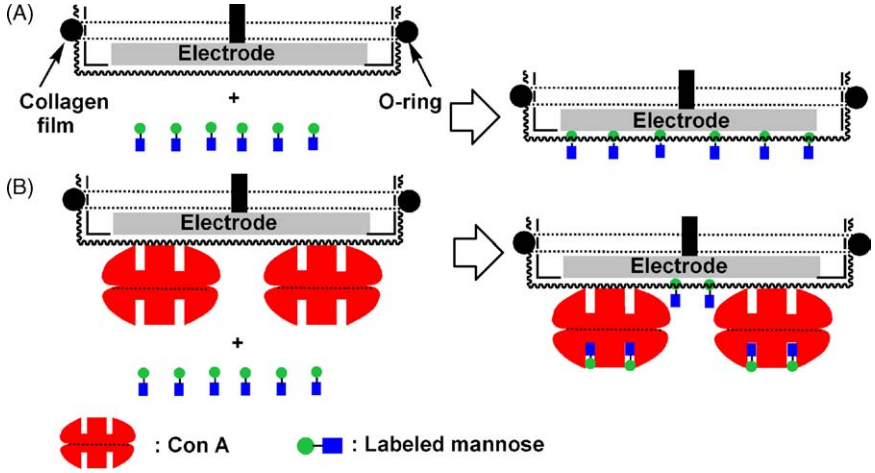


Fig. 2. Evaluation of Con A–mannose binding. (A) Labeled mannose without Con A, and (B) labeled mannose with Con A.

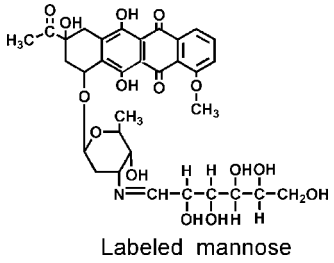


Fig. 3. Structure of labeled mannose.

were measured against the Ag/AgCl electrode. The pH of buffer solution was measured with a Horiba D-21 pH meter. A UV 1240 mini recording spectrophotometer (Shimadzu, Tokyo, Japan) was used for measurements of visible spectra of daunomycin and sugar labeled with daunomycin.

### 2.3. Preparation of labeled mannose

Labeled mannose was prepared by reaction between daunomycin and mannose for 24 h at 4 °C in 0.1 M phosphate buffer solution (pH 8.0). The structure was shown in Fig. 3. The solution was spotted on a sheet of thin-layer chromatography (silica gel alumina sheet, MERCK) in 1-methylpropanol:ammonia water = 30:10% (v/v). After developing, the labeled mannose was stripped from the sheet and collected. The reagent was dissolved in ethylalcohol, and the solution was centrifuged to exclude silica gel. The concentration of the labeled mannose was determined on the basis of the mole absorbance efficiency of daunomycin at 490 nm [13]. Glucose and galactose were labeled under the same procedures.

### 2.4. Procedure

Collagen film (1.0 cm × 1.0 cm) was mounted on a polished electrode with a rubber O-ring. To immobilize Con A to the collagen film, the electrode was immersed with stirring for about 1 h in 10 ml of 0.1 M phosphate buffer (pH 6.0) with Con A. Next, the electrode was removed to 0.1 M phosphate buffer (pH 6.0) without Con A and immersed in the solution with labeled mannose. Then, a potential at −1.0 V was applied to the electrode for 5 min with stirring. After a rest period of 15 s, an oxidation response of labeled mannose was recorded by scanning the potential between −1.0 and 0.0 V with differential pulse voltammetry (scan rate, 5 mV $s^{-1}$; pulse amplitude, 50 mV; sample width, 2 ms; pulse width, 50 ms; pulse period, 200 ms).

## 3. Results and discussions

### 3.1. Membrane permeability of labeled mannose to collagen film

Voltammograms of $5 \times 10^{-7}$ M labeled mannose at a glassy carbon electrode in 0.1 M phosphate buffer (pH 6.0)

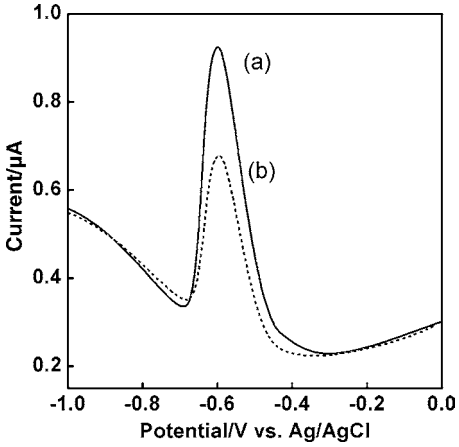


Fig. 4. Voltammograms of labeled mannose at the electrode without and with films. (a) Without collagen film, and (b) with collagen film. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode in 0.1 M phosphate buffer (pH 6.0) containing $5.0 \times 10^{-7}$ M labeled mannose.

were shown in Fig. 4. An oxidation peak appeared at about −0.60 V is due to reoxidation of daunomycin moiety. Although a peak was observed at more positive potential, the oxidation peak at negative side was selected because of high sensitivity. Next, the relations between the peak current of labeled mannose and pH in supporting electrolyte were examined at the electrode. Within pH ranges of 5.0 and 8.0, the highest peak current was obtained at about 6.0. The permeability of labeled mannose to the collagen film was also investigated in 0.1 M phosphate buffer (pH 6.0). The current value at the electrode covered with the film was 70% of that measured at a bare electrode. On the other hand, voltammograms of $5 \times 10^{-7}$ M daunomycin were measured at the electrode with and without collagen films. The peak current obtained at the electrode with the collagen film was about 75%, compared to that obtained at a plain electrode. Therefore, it was found that the membrane permeability of labeled mannose hardly decreased.

### 3.2. The response of labeled mannose at the electrode covered with Con A/collagen film

Peak current of daunomycin was measured at an electrode covered with collagen film. The electrode was immersed for 1 h in 0.1 M phosphate buffer containing $8.0 \times 10^{-7}$ M Con A. After the electrode was removed to 0.1 M phosphate buffer without Con A, the electrode was incubated with stirring for 1 h in the solution containing $5 \times 10^{-7}$ M daunomycin. When the peak current of daunomycin was recorded, the decrease of the peak current was not observed. Peak current of $5 \times 10^{-7}$ M labeled mannose was also measured under the same conditions except for the concentration of Con A. The concentration of Con A in the solution was changed from 0 to $8.0 \times 10^{-7}$ M. As the results, the peak current decreased with increasing concentration of Con A (Fig. 5). In addi-

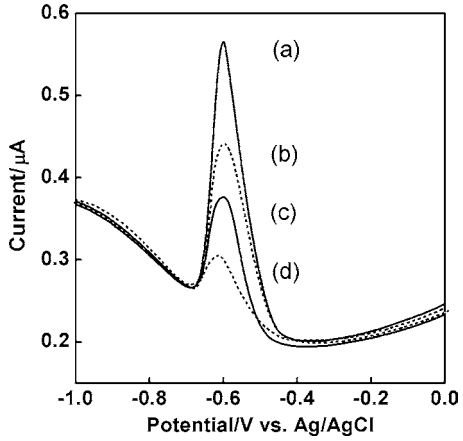


Fig. 5. Voltammograms of labeled mannose at the electrode covered with collagen film in the presence of Con A. (a) 0 M Con A, (b) $2.0 \times 10^{-7}$ M Con A, (c) $4.0 \times 10^{-7}$ M Con A, and (d) $8.0 \times 10^{-7}$ M Con A. The electrode was immersed with stirring for 1 h in 0.1 M phosphate buffer (pH 6.0) containing Con A. Next, $5 \times 10^{-7}$ M labeled mannose was added to 0.1 M phosphate buffer (pH 6.0) without Con A, and the electrode was incubated for 1 h with stirring in the solution. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode.

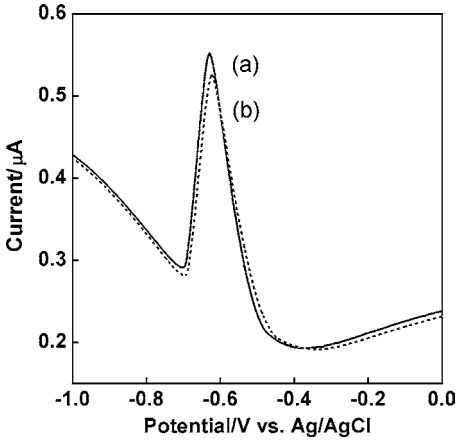


Fig. 7. Voltammograms of labeled mannose and SBA at the electrode covered with collagen film. (a) $5.0 \times 10^{-7}$ M labeled mannose, and (b) $5.0 \times 10^{-7}$ M labeled mannose + $4.0 \times 10^{-7}$ M SBA. The electrode was immersed with stirring for 1 h in 0.1 M phosphate buffer (pH 6.0) containig SBA. Next, $5 \times 10^{-7}$ M labeled mannose was added to 0.1 M phosphate buffer (pH 6.0) without SBA, and the electrode was incubated for 1 h with stirring in the solution. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode.

tion, the pH of the solution containing $8.0 \times 10^{-7}$ M Con A which the electrode covered with chitin film was immersed in was investigated in the ranges of 6.0–8.0 (Fig. 6). The pH of measurement solution was identical to that of solution for immobilization of Con A. The peak current at pH 6.0 decreased to 20% compared with the peak current obtained in a solution without Con A. However, the peak current at pH 8.0 was about 85%. When labeled glucose or labeled galactose was used, the peak current did not decrease. Consequently, this phenomenon is due to that Con A is immobilized

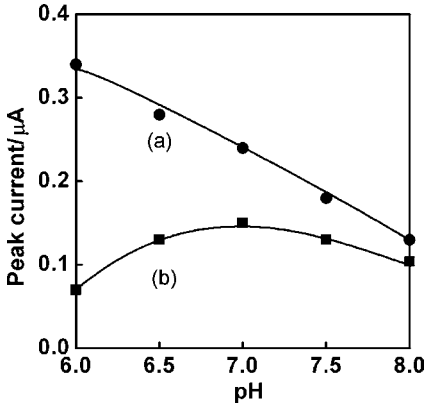


Fig. 6. Relations between the peak current of labeled mannose and the pH of solution for immobilization of Con A. (a) 0 M Con A, and (b) $8.0 \times 10^{-7}$ M Con A. The electrode was immersed with stirring for 1 h in a solution for immobilization of Con A. Next, the electrode was removed to a measurement solution without Con A. Labeled mannose of $5 \times 10^{-7}$ M was added to the solution, and the electrode was incubated for 1 h with stirring. The pH of measurement solution was identical to that of solution for immobilization of Con A. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode.

to the collagen film based on an electrostatic interaction, and the labeled mannose is captured in the binding site of Con A.

### *3.3. Interaction between labeled mannose and other lectin at the electrode covered with collagen film*

After the electrode covered with collagen film was immersed for 1 h in 0.1 M phosphate buffer with SBA or WGA, the electrode was removed to a solution without the lectin. The electrode was incubated with stirring for 1 h in the solution containing $5 \times 10^{-7}$ M labeled mannose. The peak current of labeled mannose was similar to that of only labeled mannose at an electrode covered with collagen film (Figs. 6 and 7). The reason for this is that SBA and WGA do not have the binding site to mannose moiety. On the other hand, it is expected that the lectins are immobilized at the collagen film under the conditions. The investigations are described below.

### *3.4. Amount of Con A immobilized at the surface of collagen film*

To estimate the amount of Con A immobilized on the collagen film surface, two experiments were carried out. First, an electrode covered with the film was immersed in 0.1 M phosphate buffer with $8.0 \times 10^{-7}$ M Con A for 1 h. The electrode was removed from the solution, and $5.0 \times 10^{-7}$ M labeled mannose was added to the solution. After mixing the labeled mannose and Con A in the solution for 1 h, the peak current was recorded at a plain electrode. If Con A is immobilized at the collagen film, the concentration of Con A in the solution

should decrease. Second, $5.0 \times 10^{-7}$ M labeled mannose and $8.0 \times 10^{-7}$ M Con A were mixed in 0.1 M phosphate buffer for 1 h. Next, the peak current was measured at the electrode. The peak current of the first experiment was higher than that in the second experiment. Therefore, it is expected that Con A was immobilized on the film surface. Calibration curve of Con A to the peak current of $5 \times 10^{-7}$ M labeled mannose at the plain electrode was made, and the amount of Con A immobilized on the surface of collagen film was determined from the difference of electrode response obtained from these experiments. The value was about $3.0 \times 10^{-9}$ mol/cm$^2$.

### *3.5. Interaction between labeled galactose and SBA on the collagen film*

An electrode covered with collagen film was immersed for 1 h in 0.1 M phosphate buffer (pH 6.0) containing SBA and was removed to 0.1 M phosphate buffer (pH 6.0) without SBA. The electrode was incubated with stirring for 1 h in the solution containing $5 \times 10^{-7}$ M labeled galactose. The peak current observed was similar to a peak current of a solution with only $5 \times 10^{-7}$ M galactose at an electrode covered with collagen film. This fact shows that SBA is not immobilized on the collagen film. On the other hand, the voltammogram was measured at a plain electrode after the $5 \times 10^{-7}$ M labeled galactose and $4 \times 10^{-7}$ M SBA were mixed for 1 h in the solution. The peak current drastically decreased in comparison with that of only labeled galactose. That is, the labeled galactose has an interaction with SBA. From the results, it is considered that SBA is not immobilized on the collagen film because the isoelectric point of SBA is 6.0. For WGA (p*I* 9.0), the immobilization to the collagen film is difficult by using this same method.

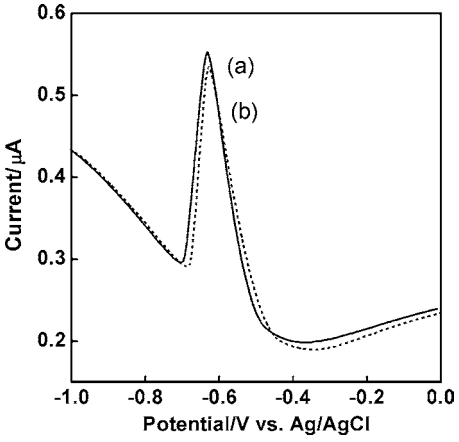


Fig. 8. Voltammograms of labeled mannose and WGA at the electrode covered with collagen film. (a) $5.0 \times 10^{-7}$ M labeled mannose, and (b) $5.0 \times 10^{-7}$ M labeled mannose + $4.0 \times 10^{-7}$ M WGA. The electrode was immersed with stirring for 1 h in 0.1 M phosphate buffer (pH 6.0) containig WGA. Next, $5 \times 10^{-7}$ M labeled mannose was added to 0.1 M phosphate buffer (pH 6.0) without WGA, and the electrode was incubated for 1 h with stirring in the solution. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode.

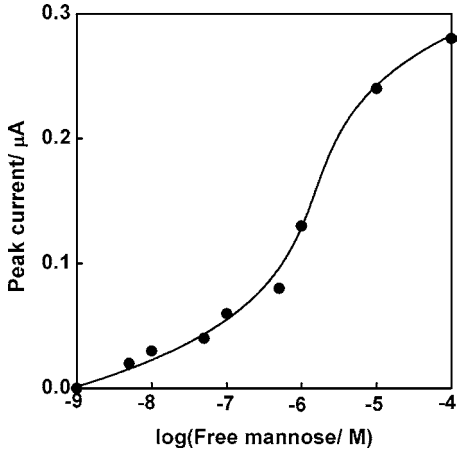


Fig. 9. Detection of free mannose based on the competitive reaction. The electrode was immersed with stirring for 1 h in 0.1 M phosphate buffer (pH 6.0) containig $8 \times 10^{-7}$ M Con A. Next, $5 \times 10^{-7}$ M labeled mannose and free mannose were added to 0.1 M phosphate buffer (pH 6.0) without Con A, and the electrode was incubated for 1 h with stirring in the solution. Measurements were carried out with DPV, after a potential at −1.0 V for 5 min was applied to the electrode.

### *3.6. Detection of mannose based on the competitive reaction to the limited binding sites of Con A between free mannose and labeled mannose*

Detection of free mannose was carried out on the basis of the competitive reaction to the limited binding sites of Con A between free mannose and labeled mannose at an electrode covered with a Con A/collagen film. When free mannose at various concentrations was incubated in the solution with $5 \times 10^{-7}$ M labeled mannose, the peak current of labeled mannose increased with increasing the concentration of free mannose (Fig. 8). This phenomenon is due to the occupation of binding site by free mannose. The relative S.D. at $1 \times 10^{-7}$M free mannose was about 4.8% ($n = 5$). This result also showed that Con A was immobilized at the collagen film. Thus, the collagen film is suitable as a support immobilizing a protein. Furthermore, this system is effective to monitor the binding between protein and its ligand (Fig. 9).

## 4. Conclusion

In this study, interaction between Con A and mannose labeled with an electroactive compound was evaluated at an electrode covered with a collagen film. Con A could be immobilized on the surface of collagen film by an electrostatic interaction between the protonated arginine and lysine residues of the film and the negative charged Con A. This immobilization hardly changes the structure of Con A, and the film brings a pseudo ambience such as cell surface. The binding was investigated from the changes of electrode response of labeled mannose. This approach can be considered as a new method to evaluate the interaction between biological molecules. Consequently, this concept will contribute towards the devel-

opment of sensor in monitoring protein–ligand interaction in vivo.

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