Electrochemical Recognition of *Candida albicans*

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An antibody to *Candida albicans* was covalently immobilized on a membrane prepared from cellulose triacetate, 1,8-diamino-4-aminomethyloctane, and glutaraldehyde. The antibody bound membrane was set between a couple of glass cells containing 32 mM KCl and 8 mM KCl respectively. The membrane potential was measured with Ag/AgCl electrodes. Then, the membrane was reacted with *C. albicans* and its membrane potential was measured again. The membrane potential changed toward the positive side. On the other hand, the membrane showed no response to *Escherichia coli*, *Bacillus subtilis*, *Bacillus licheniformis*. However, it responded to *Saccharomyces cerevisiae*, though membrane potential shifted toward the negative side. Analysis of the membrane potential and the microscopic observation of the immunoreaction suggested that the membrane has a strong affinity to *C. albicans*. The shift of the membrane potential due to the reaction with *C. albicans* was correlated with the concentration of *C. albicans* in the range of 10^4 – 5×10^5 cells·cm⁻³.

1 Introduction

Recently the measurement of *Candida albicans* has become an important item in clinical analysis. *C. albicans* is found in human body even in normal conditions. However, unusual increase is observed when the immunological function is diminished by the administration of antibiotics, adrenocortical hormones, anti-tumor reagents, and immunosuppressive reagents^{1–3}. Increase of *C. albicans* induces various malfunctions or diseases. Therefore, the concentration of *C. albicans* in blood is an important indicator of the immunological function, which is important for the control of administration schedule of reagents mentioned above.

The measurement of *C. albicans* is performed by visual inspection of antibody-antigen (*Candida* species) aggregation on a glass plate. However this conventional method requires skillful techniques and hardly gives quantitative results. Moreover it is not useful for the automated continual measurement of the yeast. Therefore more simple and quantitative method for the determination of *C. albicans* is required.

The authors previously showed that antibody bound membrane is feasible for the selec-

tive determination of specific proteins, lipids, peptides, and hormones. The amount of bound substances were estimated either by potentiometric or by amperometric method. The potentiometric method is based on the membrane potential shift resulting from charge density change or ionic permeability change due to the adsorption of substances on the membrane^{4–6}. The amperometric method is also sensitive and used for the determination of various substances^{7–9}.

In this study, the potentiometric method is applied to the immunoassay of *C. albicans*. Since *C. albicans* has negative charge, the adsorption of the cells on the antibody-bound membrane may induce a considerable change of membrane potential. Feasibility of the antibody-bound membrane as the immunosensor for *C. albicans* is described.

2 Experimental

2.1 Materials

Anti-*Candida* antibody (*Candida* check No. 1, IgG fraction) was obtained from Sanko Pure Chemicals Co. (Tokyo). Cellulose triacetate, 1,8-diamino-4-aminomethyloctane, 70% glutaraldehyde were obtained from Eastman Kodak Co. (Rochester, N.Y.), Asahi Kasei (Tokyo), Tokyo Kasei (Tokyo) respectively. *Candida albicans*, *Bacillus subtilis*, *Bacillus licheniformis*, *Escherichia coli*, *Saccharomyces cerevisiae* were cultured according to conventional methods described previously. Other reagents were of analytical grade or

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laboratory grade. Distilled water was used throughout the experiments.

2.2 Preparation of antibody bound membrane

Cellulose triacetate (250 mg) was dissolved in 5 cm³ dichloromethane and 150 mm³ 1,8-diamino-4-aminomethyloctane was added and stirred for 20 min. Then 50 mm³ of 70% glutaraldehyde was added to the mixture and stirred for 20 min. The resulting homogeneous solution was spread on a glass plate and stood at 20°C under a dried atmosphere for 2 days. The membrane formed on the glass plate was cut into small sheets and peeled off the glass plate. After washed with distilled water, the membrane was reacted with 1% glutaraldehyde for 1 hr at 25°C in a phosphate buffer solution (0.1 M, pH 7.0). One sheet of the membrane was set on a watch glass and 200 mm³ of antibody solution (10 mg protein·cm⁻³) was put on the membrane. The glass was covered with another watch glass and reacted for overnight at 4°C. Then the membrane was incubated in 0.1 M glycine solution (pH 7.7) at 25°C for 30 min in order to block residual aldehyde groups. The antibody-bound membrane was washed with 0.5 M NaCl for 30 min and 0.1 M phosphate buffer solutions for 30 min and stored at 4°C.

2.3 Measurement of membrane potential

Figure 1 shows an experimental setup. Antibody-bound membrane was set between the glass cells and a couple of Ag/AgCl electrodes were inserted in each compartment. About 5 cm³ of 32 mM and 8 mM of KCl solutions were added in the compartment I and II respectively. The electrode inserted into the compartment II was put to earth and the membrane potential was measured. The potential difference of the electrodes (ΔE_b) was detected with an electrometer (Hokuto Denko Co., Model HE-101A) and displayed on a recorder.

When pH dependence of the membrane potential was examined, 1 mM citrate solutions (pH 3.0, 4.6, 6.0) containing KCl (32 mM or 8 mM) and 1 mM phosphate buffer solutions (pH 7.0, 8.0) containing KCl (32 mM or 8 mM) were used as electrolytes for membrane potential measurement.

2.4 Immunoreaction

KCl solution in compartment I was replaced

Fig. 1 Schematic diagram for electrochemical recognition system of microorganisms

(a): Compartment I, (b): Compartment II, (c): Antibody bound membrane, (d): Silicone rubber, (e): Ag/AgCl electrodes, (f): Water bath, (g): Stirring bar, (h): Magnetic stirrer, (i): Electrometer, (j): Recorder

by a phosphate buffer solution containing microorganisms. On the other hand, the compartment II was filled with a phosphate buffer solution (0.1 M, pH 7.0). After the reaction at 32°C for 30 min, the membrane was washed with 0.5 M NaCl for 15 min and 0.1 M phosphate buffer (pH 7.0) for 15 min.

2.5 Measurement of membrane potential after the immunoreaction

After washed, the glass cells were filled with 32 mM and 8 mM KCl solutions respectively, and the membrane potential (ΔE_a) was measured by the electrodes. The membrane potential changes ($\Delta E_a - \Delta E_b$) were plotted against the microbial concentration.

2.6 Microscopic observation of immunoreaction

The antibody used in the present study is known to react with at least 7 species of yeast. Therefore, the reactivity of the bound antibody to microorganisms applied in the present experiments was checked by a visual test using a microscope.

Microbial suspension was dropped on a slide glass and observed with a microscope ($\times 200$). Then 10 mm³ of the antibody was dropped with microsyringe. Agglutination due to antigen-antibody reaction was observed.

3 Results and Discussion

3.1 Membrane potential caused by *C. albicans*

Before the immunoreaction, the membrane potentials were 28–32 mV. After the reaction with *C. albicans* (10^5 cells·cm⁻³), the membrane potential shifted about 2 mV toward the positive side. The difference of the membrane potentials before and after the reaction ($\Delta E_a - \Delta E_b$) was plotted against the *C. albicans* concentration as shown in Fig. 2. The membrane potential difference increased with increasing cell concentration in the range 10^4 – 5×10^5 (cells·cm⁻³). No change of the membrane potential was observed below 10^4 (cells·cm⁻³). Therefore, the order estimation of *C. albicans* concentration was possible in the range of 10^4 – 5×10^5 (cells·cm⁻³).

Fig. 2 Correlation curve between *C. albicans* concentration and membrane potential change

3.2 Membrane potential change caused by various microorganisms

Bacteria observed in blood are classified into gram negative bacteria, gram positive bacteria and others (including yeast such as *C. albicans*). These bacterial groups are considerably different from each other in their cell surface structures and antigenic groups. Therefore, several bacteria belonging to each group were applied to the present system, in order to examine the selectivity of the antibody bound membrane.

When *E. coli* (gram negative) was applied, no change in the membrane potential was

Fig. 3 Correlation curves between other microorganisms and membrane potential changes

Dotted lines show correlation curves obtained in Fig. 2

observed, as shown in Fig. 3 (a). When *B. subtilis* or *B. licheniformis* (gram positive) was applied, the membrane potential shifted about 1 mV toward the positive side. However, the extent of the change was independent of the cell concentration in the range 10^4 – 5×10^5 (cells·cm⁻³) (Fig. 3(b), (c)). This might be due to non-specific adsorption of these microorganisms.

In contrast, the membrane potential shifted toward the negative side, when *S. cerevisiae* was applied to the system (Fig. 3(d)). The extent of the membrane potential change increased with increasing the cell concentration in the range 10^4 – 5×10^5 (cells·cm⁻³).

Since *C. albicans* and *S. cerevisiae* belong to the same family, they might have similar antigenic structures located on the surface of their cell wall. Therefore, at least one of these antigenic structures is common for both strains. Thus the antibody to *C. albicans* might respond to *S. cerevisiae*.

3.3 pH dependence of the membrane potential

The antibody bound membrane has amino groups or carboxyl groups. The electric charge density changes with pH conditions. Therefore, the membrane potential was measured at various pH. As shown in Fig. 4, the

Fig. 4 pH profile of membrane potential
(○): Before reaction, (Δ): After reaction with *C. albicans*, (●): Membrane potential difference; [(Δ)—(○)].

membrane potential was constant below pH 4.6, while it gradually decreased above pH 4.6.

After the reaction, the pH profile of the membrane potential shifted about 1 mV toward the positive side, though the overall profile was almost similar to that obtained before the reaction. Therefore, the charge of the bound microorganisms was slightly affected by pH.

3.4 Dependence of ionic strength on the membrane potential

The dependence of the membrane potential (ΔE) on the electrolyte concentration is approximated by the following equation when uni-uni valent electrolyte solutions are separated by a charged membrane, and the electrolyte concentration is very high relative to the charge density¹⁰⁻¹³⁾

$$\Delta E = \frac{RT}{F} \left\{ -\tau \ln r + \left(1 + \frac{1}{r} \right) \frac{1 - \tau^2}{2} \frac{\theta^*}{C_2} + \dots \right\}$$

where $r = C_1/C_2$; $\tau = (\omega_+ - \omega_-)/(\omega_+ + \omega_-)$;

$$\theta^* = X \cdot (\gamma_+ \gamma_- / k_+ k_-)^{1/2};$$

C_1, C_2 are the electrolyte concentration ($C_1 > C_2$); ω_+, ω_- are the molar mobilities of cation and anion in the membrane; X is charge density in the membrane ($X > 0$); γ_+, γ_- are activity coefficients of cation and anion in the membrane; k_+, k_- are the distribution coefficients of cation and anion at the membrane/solution interface. Therefore, a plot of the membrane potential (ΔE) versus reciprocal electrolyte concentration ($1/C_2$) yields a straight line, and the gradient and the inter-

Fig. 5 Dependence of reciprocal concentration of KCl on the membrane potential
(●): Before reaction, (○) After reaction with *C. albicans*.

cept at the ordinate ($1/C_2 \rightarrow 0$) give values of τ and θ^* .

Figure 5 shows the dependence of the membrane potential of the antibody bound membrane before and after the reaction with *C. albicans*. An approximated straight line was obtained in the range $1/C_2 < 40$. From the gradients and the intercepts of the line at $1/C_2 = 0$, τ and θ^* were estimated. θ^* did not change due to the reaction with *C. albicans* as shown in Table 1, while τ increased about 50%. Therefore, the membrane potential change was ascribed responsible for the change in the ionic permeability. *C. albicans* has about several μm diameter. On the other hand, average pore size of the membrane is smaller than several hundreds $\text{\AA}$. Therefore, adsorption of *C. albicans* might diminish the effective area for ion permeation.

Table 1 Analytical results of the membrane potential change due to the reaction with *C. albicans*

	θ^* (mol·cm ⁻³)	$\tau = \frac{\omega_+ - \omega_-}{\omega_+ + \omega_-}$
Before reaction	4.8×10^{-9}	0.226
After reaction	4.7×10^{-9}	0.301

On the other hand, analytical results obtained above suggested that the negative charge of *C. albicans* was lost due to the immunoreaction. When the membrane was reacted with *S. cerevisiae*, the membrane

Fig. 6 Dependence of reciprocal concentration of KCl on the membrane potential

(●); Before reaction, (○): After reaction with *S. cerevisiae*

potential showed a different profile as shown in Fig. 6. An approximated straight lines were derived by the least square method from the points measured under the conditions $1/C_2 < 62.5$. From the gradients and the intercepts at $1/C_2=0$, τ and θ^* were calculated as sum-

Table 2 Analytical results of the membrane potential change due to the reaction with *S. cerevisiae*

	θ^* ($\text{mol} \cdot \text{cm}^{-3}$)	$\tau = \frac{\omega_+ - \omega_-}{\omega_+ + \omega_-}$
Before reaction	1.7×10^{-9}	0.226
After reaction	0.2×10^{-9}	0.431

marized in Table 2. The value of τ increased in a similar manner to the case of *C. albicans*. In sharp contrast, however, the charge density also changed in this case. The decrease of charge density was possibly due to the addition of negative charge of *S. cerevisiae*^{14,15} adsorbed on the membrane. It is not clear why *C. albicans* lost its negative charge while *S. cerevisiae* maintained it.

3.5 Microscopic observation of the immunoreaction

The antibody used in the present study is known to react with several strains of yeast other than *C. albicans*. Therefore, the antigenicity of *S. cerevisiae* was checked by the

Fig. 7 Microscopic observations of immunoreaction ($\times 200$)

(a): *C. albicans* before reaction, (b): *S. cerevisiae* before reaction, (c): *C. albicans* after reaction with antibody, (d): *S. cerevisiae* after reaction with antibody

conventional method. *C. albicans* or *S. cerevisiae* suspension was dropped on a glass plate and observed with a microscope (Fig. 7(a), (b)). Then, 10 mm³ of antibody was added to each suspension. Within 2–3 min, aggregation occurred as shown in Fig. 7(c) and (d). The rate of aggregating reaction depends upon the concentration of antibody and number of antigenic structures on the cell surface. The results of Figs. 7(c) and (d) demonstrate that *C. albicans* has much more antigenic groups to antibody used than *S. cerevisiae*.

4 Conclusion

The immunosensor system proposed could recognize *C. albicans* in a sample solution. The measurement was based on the determination of membrane potential change which might be caused by the ionic permeability change. The use of specific antibody considerably improves specificity of biosensors. The results obtained demonstrate the feasibility of this system as the electrochemical determination of other microorganisms in biological fluids.

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現在、大学または研究所での電気化学に関する研究は大体次の通りである。国立ソウル大学校では透明 SnO₂ 半導体修飾電極における各種電気化学反応機構の解明、ポーラログラフィーによる基礎測定、アルミニウム陽極酸化皮膜のガス通過特性、電気化学的腐蝕、防蝕および金属析出機構に関する研究を行っている。漢陽大学校(ソウル)では不飽和脂肪族アルコール類または芳香族カルボン酸類の有機電解反応、各種メッキ浴の添加剤の開発およびその電気化学的挙動、析出ニッケルメッキ層の析出機構の解明、鉛-カルシウム合金類または黄銅類などの腐蝕メカニズムの究明、2次電池特性の改良などの研究が進行中であり、今後半導体電極による光電気化学に関する研究も計画中である。延世大学校(ソウル)と西江大学校(ソウル)ではニッケルおよび各種金属表面の酸化メカニズムなどを光と電気化学的測定法により究明する研究を行っている。高麗大学校(ソウル)と蔚山工科大学(蔚山)ではイオン選択性電極の開発とその基礎的特性などの研究が進行している。仁荷大学校(仁川)では電解

過塩素酸生成反応機構の解明および無機化合物の酸化還元反応の研究が進んでいる一方、金属の電解析出および電解精製なども研究中である。慶北大学校(大邱)では非水溶液中でのポーラログラフィーによる基礎測定、海洋大学(釜山)では海水などの水溶液中での金属の防蝕を電気化学的に研究しており、東亜大学校(釜山)も有機電解を行っている。大学以外の韓国科学技術院(ソウル)には電気化工研究室があり、現在リチウム-塩素高温電池の開発をアメリカの Battelle 研究所のチームと共同研究を組んでいる。一方、腐蝕問題の解決も行っているが一次半導体電極(おもに TiO₂ など)を用いた光電気化学による水の分解を行ったこともある。国立工業試験院(ソウル)ではポーラログラフィーなどを用いる基礎研究と各種電気メッキによる金属の析出機構、メッキ方法の改良などの研究を、また韓国機械金属研究所(慶南昌原)でも表面処理研究チームが電気メッキに関する研究が活発に行なわれている。

(漢陽大学校工科大学教授 李 柱性 (Ju-Seong Lee))