*บทความวิจัย*

# การตรวจสอบการดูดซับของโปรตีนโบวีนซีรัมอัลบูมิน และเคซีนบนพื้นผิวทองโดยใช้เทคนิคอิเล็กโตรเคมีคอล อิมพีแดนซ์สเปกโตรสโคปี

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## บทคัดย่อ

งานวิจัยนี้จะทำการเปรียบเทียบการดูดซับ (adsorption) ของโปรตีนโบวีนซีรัมอัลบูมิน (bovine serum albumin, BSA) และเคซีน (casein) บนพื้นผิวทองเพื่อนำไปใช้เป็นโปรตีนปิดพื้นผิว (blocking protein) สำหรับการป้องกันการจับกันแบบไม่จำเพาะ (non-specific binding) โดยจะทำการวัดปริมาณโปรตีนที่ดูดซับบนพื้นผิวทองด้วยเทคนิค surface plasmon resonance (SPR) ไซคลิกโวลแทมเมตทรี (cyclic voltammetry) และอิเล็กโตรเคมีคอลอิมพีแดนซ์สเปกโตรสโคปี (electrochemical impedance spectroscopy) ผลการทดลองพบว่าการดูดซับของโปรตีนสามารถวิเคราะห์ได้โดยใช้รูปแบบของ Langmuir model ซึ่งบ่งชี้ว่าโปรตีนโบวีนซีรัมอัลบูมินและเคซีนมีการดูดซับพื้นผิวทองเป็นแบบสุ่ม (random orientation) และเป็นชั้นเดียว (monolayer) นอกจากนี้ พื้นผิวที่มีโปรตีนโบวีนซีรัมอัลบูมินและเคซีนจะถูกนำมาทดสอบการป้องกันการจับกันแบบไม่จำเพาะจากแบคทีเรีย *Escherichia coli* K12 (*E. coli* K12) ทั้งนี้โปรตีนที่มีความเหมาะสมสำหรับการป้องกันการจับกันแบบไม่จำเพาะจะถูกนำไปใช้สำหรับการพัฒนาไบโอเซนเซอร์สำหรับการตรวจวัดแบคทีเรียต่อไป


**คำสำคัญ:** โบวีนซีรัมอัลบูมิน เคซีน อิเล็กโตรเคมีคอลอิพีแดนซ์สเปกโตรสโคปี การดูดซับโปรตีน

ภาควิชาฟิสิกส์ คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

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# Investigation of Bovine Serum Albumin and Casein Adsorption on Gold Surface by Using Electrochemical Impedance Spectroscopy

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## ABSTRACT

Adsorptions of bovine serum albumin (BSA) and casein on a gold surface were compared in order to use as a blocking protein for prevention of non-specific binding. The adsorptions of BSA and casein were investigated though surface plasmon resonance (SPR), cyclic voltammetry (CV) technique and electrochemical impedance spectroscopy (EIS). The Langmuir model can be fitted the results of adsorbed protein. This indicates that the adsorbed BSA and casein on gold surface were randomly immobilized as a monolayer on the gold surface. The adsorbed BSA and casein surface will be evaluated to prevent the non-specific binding on the surface using *Escherichia coli* K12 (*E. coli* K12). The suitable blocking protein will be chosen as a blocking protein for further development of bacteria detection based EIS immunosensor.

**Keywords:** bovine serum albumin, casein, electrochemical impedance spectroscopy, protein adsorption

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## Introduction

The interaction between proteins and solid surface is a fundamental phenomenon. This information is an important in novel applications such as biotechnology, medical devices and biosensors. In biomaterial, protein adsorption is the process for imbedding or implanting biomaterial device into tissue [1]. In biotechnology, the mechanism of protein adsorption is the great value for understanding protein/surface interaction. Moreover, the ability to monitoring protein adsorption will benefit in nanotechnology and bio-nanotechnology [2]. In biosensor, protein adsorption is crucial for immobilization of protein or biomolecules on the transduce surface [3].

Bovine serum albumin (BSA) is a globular protein with a molecular weight about 66.5 kDa. BSA has an elliptical shape with dimension of $3 \times 4 \times 4$ nm [4]. For casein protein, it has a subunit α-s1, α-s2, β, and κ casein. The approximate casein composition of milk is (g/L): α-s1 (12-15); α-s2 (3-4); β (9-11); and κ (2-4). Their molecular weight for α-s1, α-s2, β, and κ casein are 22, 25, 24, and 19 kDa, respectively [5, 6]. BSA and casein are widely used as a blocking agent for immunological techniques, such as enzyme-linked immunosorbent assay (ELISA) [7]. The blocking agent is coated onto the surface to prevent non-specific binding on to the surface. The adsorbed BSA or casein surface normally form monolayer on the surface and alter the surface properties to prevent other protein or biological molecules from adsorbing on the surface. Therefore, the information of BSA and casein adsorption on the surface is important for using in biosensors.

A various methods have been used for investigation of protein adsorption on the different surface: fourier transform infrared (FT-IR) [9], UV-visible spectroscopy [10], piezoelectric quartz crystal sensor [10], surface plasmon resonance (SPR) sensor [11], electrochemical impedance spectroscopy (EIS) [12], optical waveguide spectroscopy [13], ellipsometry [14], internal total reflection fluorescence [15], X-ray photoemission electron microscopy (X-PEEM) [16], and polarization modulation infrared reflection adsorption spectroscopy (PM-IRRAS) [17]. Among these techniques, EIS and SPR are directly able to investigate the protein adsorption on the surface without labeling molecule. Moreover, the surface can be easily prepared for using in these techniques.

EIS technique is a sensitive surface in which the technique can measure the change of biomolecules on the electrode surface. This technique provides an information in the changes of capacitance and resistance occurring at the electrode surface. EIS technique records the data in range of frequencies using small amplitude of alternating current and provides the electron-transfer resistance ($R_{et}$) reflected the amount of adsorbed biomolecules on the electrode surface without labeling molecule.

SPR is a label-free optical biosensor technique for measuring biomolecular interactions. SPR consists of a sensitive surface, upon which measurement is based on a change in the refractive index at the interface between metallic and dielectric layers. SPR exploits an evanescent field that exponentially decays along the surface of a metal layer and penetrates into the sample layer by approximately 200-300 nm. The advantage of this technique is that it can measure a change in the effective refractive index on the surface without the requirement of labeled molecules and also provides information on the interaction of the biomolecules on the surface in real time.

In this research work, we report the adsorption of BSA and casein protein on the gold electrode surface for further use in biosensor surface. The adsorption of BSA and casein on the surface was characterized by SPR, cyclic voltammetry (CV) and EIS techniques. Both surfaces were further tested for non-specific binding from *Escherichia coli* K12 (*E. coli* K12).

## Materials and methods

### Instrument and electrode

CV and EIS techniques were performed by using a μ-Autolab with FRA2 (ECO Chemie, Netherlands). Gold screen printed electrode (AuSPE) was purchased from DropSense (Spain). Detail of electrodes include a gold working electrode (disk-shaped, 12.6 $mm^2$), a gold counter electrode, and a silver pseudo-reference electrode. The electrodes are screen-printed on ceramic substrate and curing at low temperature.

### Reagents and solution

BSA, casein, $Na_2HPO_4$ and $K_3Fe(CN)_6$ were purchased from Sigma-Aldrich (Singapore). $K_4Fe(CN)_6$ was purchased from Riedel-de Haen (Gernany). $NaH_2PO_4$ was purchased from Merck (Thailand). Other chemicals were obtained as a reagent grade and were used without further purification.

### CV and EIS measurement

All the experiment were carried out at room temperature (25 ± 1°C). CV and EIS measurement were carried out in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe solution. 5 mM of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, 5 mM in each component and prepared in 0.1 M KCl, were used as redox probe for CV and EIS measurement. CV was performed in the -0.25 to 0.5 V range at a scan rate 100 mV/s for monitoring of protein adsorption. Impedance measurement was recorded in range from 0.1-10,000 Hz of frequency. The alternating voltage was constant

at 10 mV in amplitude. The impedance results were presented in a form of Nyquist plot. It plots between real part and imaginary part of resulted impedance. The Nyquist plots were fitted with proper equivalent circuit model using the facility of NOVA software v. 10.0 (ECO Chemie, Netherlands). The amount of protein adsorption was measured as the change of the electron-transfer resistance ($\Delta R_{et}$) according to the equation:

$$\Delta R_{et} = R_{et\ (adsorbed\ protein)} - R_{et\ (gold\ surface)}$$

where $R_{et\ (adsorbed\ protein)}$ is the electron-transfer resistance value measured after protein adsorption on the gold electrode and $R_{et\ (gold\ surface)}$ is the electron-transfer resistance value before protein adsorption on the surface.

**Surface plasmon resonance imaging**

SPR imaging setup is a home-made unit equipped with a seven-channel flowcell. The seven-channel flowcell was made of polydimethylsiloxane (Sylgard 184 silicone elastomer kit, Dow corning, USA) using a precision aluminium molding technique. The details of SPR imaging setup were similar to that reported previously [18]. Gold coated on BK7 (SPR chip with one inch in diameter) with an adhesive layer of titanium and 50 nm thick layer of gold was purchased from Ssenns (The Netherland).

**Protein adsorption**

The AuSPE surface was cleaned by rinsing with deionized water (DI water), ethanol, DI water, and dry with nitrogen gas. Phosphate-buffer solution consisted of 19 ml of 0.1 M $Na_2HPO_4$ and 81 ml of 0.1 M $NaH_2PO_4$ in 100 ml of DI water. BSA and casein were prepared at various concentrations (2.5, 5, 10, 50, 100, 500, and 1,000 μg/ml, respectively) in phosphate-buffer. 40 μL of BSA and casein were then dropped on the gold working electrode for 40 min of incubation time. The electrode was rinsed with phosphate-buffer for washing unbound or loosely bound protein on the surface. The adsorbed proteins on gold electrode were then measured by using the CV and EIS technique in the presence of redox probe.

In case of SPR measurement, phosphate-buffer was used as a running buffer with a flow rate 10 μL/min at room temperature. 100 μL of proteins (BSA and casein) at different concentrations were passed over the gold surface. The amount of adsorbed protein was recorded as the change of percent reflectivity (Δ%R) at 20 min after the injection of proteins.

**Bacteria preparation**

*E. coli* K12 was cultured overnight in Luria-Bertani broth (LB) and incubated at 30°C with shaking at 120 rpm. The culture was centrifuged at 5,000 rpm for 10 min. The cell pellet was then washed twice with phosphate-buffer saline (PBS; 137 mM NaCl, 3 mM KCl, 20 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, pH 7.4) and resuspended in PBS at the final concentration of $10^8$ cfu/ml.

**Effect of pH**

BSA and casein protein at 1.0 mg/ml were dissolved in acetate buffer (10 mM $C_2H_3NaO_2$, pH 5.0), PBS pH 7.4, and carbonate buffer (10 mM $NaHCO_3$, pH 9.0). Both proteins were incubated on the AuSPE surface for 40 min and then washed with PBS buffer. The adsorbed protein surfaces were then measured the impedance as described above.

## Results and discussion

**CV and EIS measurement of protein adsorption**

Figure 1A shows the cyclic voltammograms for BSA absorption on the gold electrode surface. As expected, the adsorbed BSA on the gold surface resulted in a decrease in the peak current corresponding to the concentration of BSA. The peak current rapidly decrease at the low concentration of BSA as well as at 2.5-50 μg/ml of BSA concentration. At 100 μg/ml of BSA, the peak current is a saturation of BSA concentration. Moreover, the interaction between protein and gold surface can be classified as hydrophobic interaction, ionic bonding, hydrogen bonding, and van der Waal interaction [19]. Usually, the adsorbed protein is randomly immobilized on the surface.

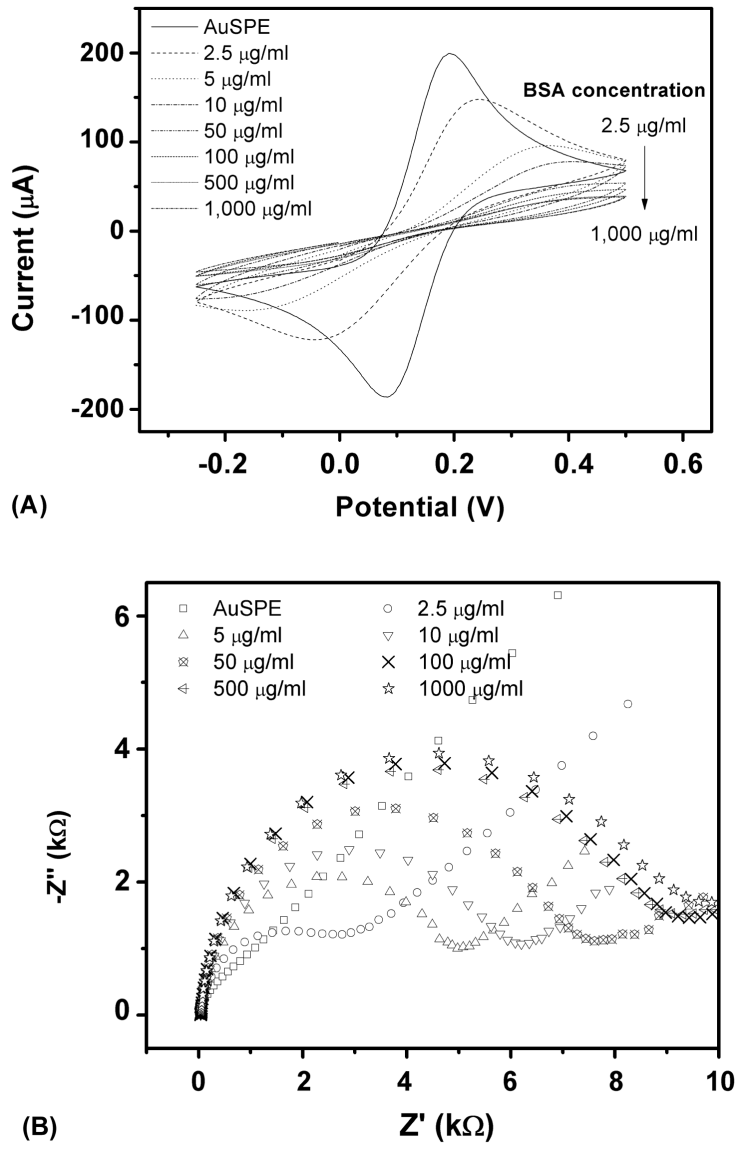


**Figure 1** (A) CV measurement of BSA adsorption on the gold surface. (B) EIS measurement of BSA adsorption on the gold surface.

Figure 1B shows the obtained Nyquist plot for BSA adsorption on the gold surface. The AuSPE or bare gold surface showed the expected electron-transfer process with a diffusion limiting step and the adsorption of BSA on the surface resulted in the expected increase in the semi-circle curve. When the BSA physically adsorbed on the surface, the penetration of redox probe was reduced yielding the increase of the semi-circle curve. This suggest that the EIS technique can be used for investigation of protein adsorption on the electrode surface. This result is consistent with the change of peak current observed by CV technique.

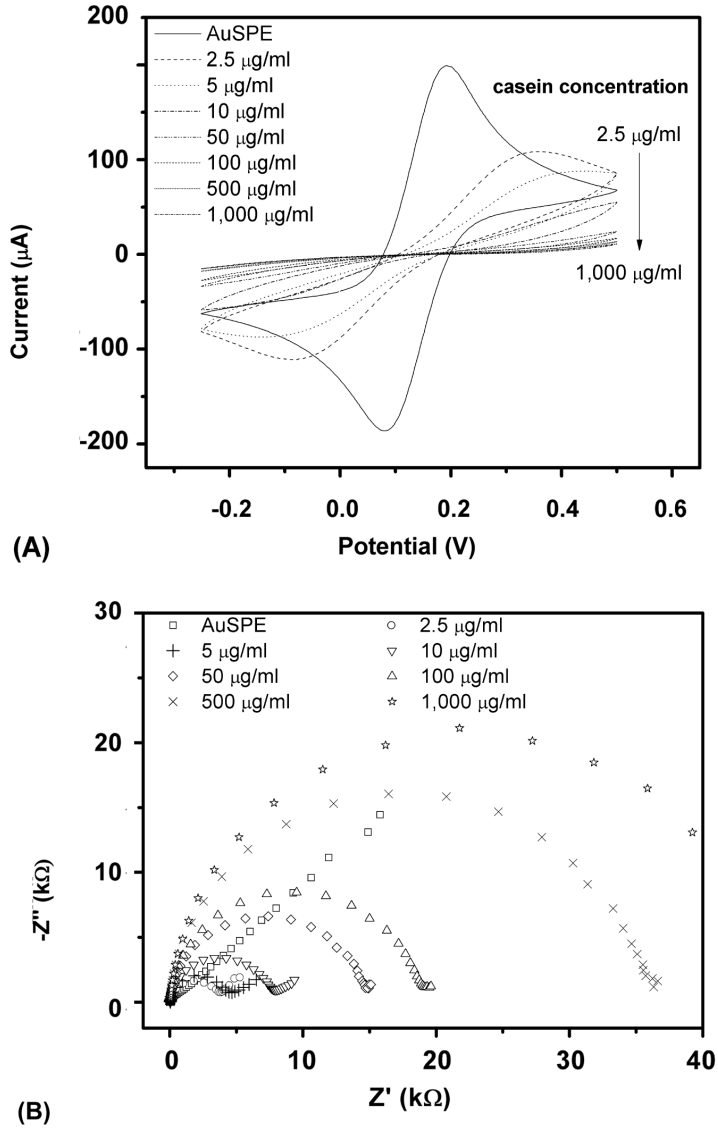


**Figure 2** (A) CV measurement of casein adsorption on the gold surface. (B) EIS measurement of casein adsorption on the gold surface.

A similar experiment of CV and EIS techniques was performed for casein adsorption on the gold surface. Figure 2A and Figure 2B show the cyclic voltammogram and Nyquist plot for casein adsorption at different concentrations. For CV techniques, the current peaks decrease according to the adsorption of casein on the surface. The results of electron-transfer resistance (Figure 2B) are consistent to the change of current peaks. These results suggest that the electron-transfer is impeded by the adsorbed casein on the electrode surface. The amount of electron-transfer resistance for casein adsorption shows higher than that of BSA adsorption due to the different size and smaller size of casein. The size of casein components is very

heterogeneous with many molecules smaller than 30 kDa [5, 6]. Thus, it is densely packed on the electrode surface. Therefore, the adsorbed casein shows the larger semi-circle curve than that of adsorbed BSA.

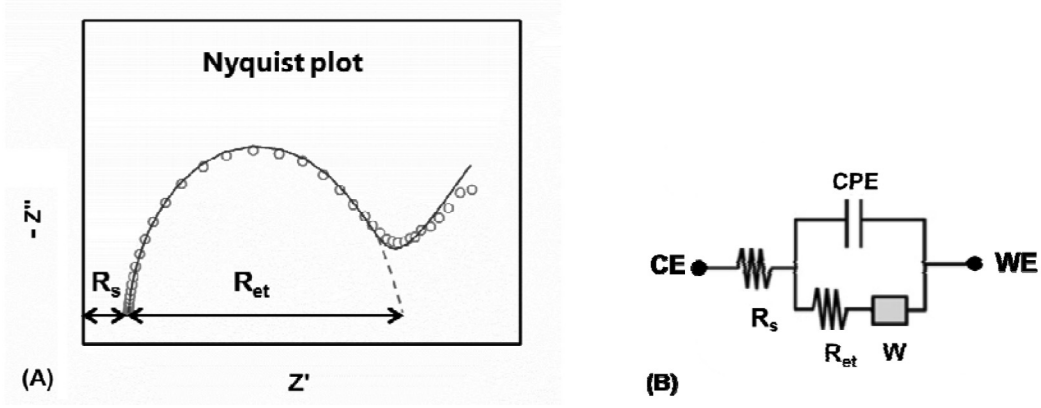


**Figure 3** (A) Nyquist plot (open-circle) and fitting with Randle circuit model (line). (B) Equivalent circuit of Randle model for fitting the experimental result.

In the Nyquist plot (simulation in Figure 3A), the semi-circle obtained at high frequencies corresponds to the faradaic electron-transfer at the interface of electrode. The data at low frequencies provide the information about the diffusion process for the transport of redox probe in the electrolyte at the electrode surface. The Nyquist plot are reveal the physical meaning by fitting with Randle equivalent circuit model as seen in Figure 3B. In the equivalent circuit model, $R_{et}$ is the electron-transfer resistance, CPE is the constant phase element, $R_s$ is the bulk property of solution resistance, and *W,* Waberge impedance, represents the bulk property of diffusion feature of redox probe in the solution. The CPE reflects the inhomogeneous and defect area of the layer on the surface [20]. The $R_{et}$ depend on dielectric features at the interface of electrode [21]. The $R_{et}$ is thus very sensitive to the electrode surface modified with a protein layer. The adsorbed protein on the surface retards the interfacial electron-transfer kinetics and consequently increases the electron-transfer resistance. Therefore, the amount of adsorbed protein on the electrode surface can be reflected by the electron-transfer resistance.

**Comparison between EIS and SPR techniques for protein adsorption**

Figure 4A shows the relationship of protein concentrations and the change of electron-transfer resistance ($\Delta R_{et}$) obtained by EIS technique. The amount of adsorbed protein can be found to be a concentration dependent as shown in Figure 4A. It can be seen that the $\Delta R_{et}$ signal of adsorbed BSA increased as well as increasing of BSA concentration and reached the saturation at 100 μg/ml of BSA concentration. For adsorbed casein, it reached the saturation at 500 μg/ml of casein concentration and the $\Delta R_{et}$ of adsorbed casein surface was 5-folds than the adsorbed BSA surface. In addition, the process of adsorbed protein was described by model of Langmuir isotherm [22]. This model can be written as the following:

$$\frac{C}{Q} = \frac{1}{bQ_m} + \frac{1}{Q_m} C$$

Where Q is the amount of adsorbed protein, $Q_m$ is the maximum of adsorbed protein at equilibrium (monolayer coverage), $C$ is the concentration of protein, and b is the adsorption to desorption ratio or equilibrium constant for adsorption process. The plot between C/Q and C should be linear relationship with slope $1/Q_m$ and intercept $1/bQ_m$.

The $\Delta R_{et}$ increased as well as increasing of protein concentration. This imply that the adsorbed protein prevents the electron of redox probe transfer to the electrode surface. The 100 μg/ml of BSA shows the saturation point for protein adsorption on the surface. In case of casein, the saturation point was 500 μg/ml. As described above, due to the smaller size of casein, the density of casein on the surface was higher than the density of BSA. The $\Delta R_{et}$ signal of adsorbed casein was 5-folds than adsorbed BSA surface.

Figure 4B shows the result obtained by SPR imaging technique. The adsorbed BSA on the surface was similar to the result obtained from EIS technique. The saturation of adsorbed BSA and casein was 100 μg/ml. The amount of adsorbed casein on the surface was twice when compared to the adsorbed BSA. The different of obtained signal from casein adsorption may be the different technique for measuring the protein adsorption.

The SPR signal and EIS signal obtained from BSA adsorption were fitted well with Langmuir model yielding the 0.9990 and 0.9987 of $R^2$, respectively (Figure 4A, 4B). For the casein adsorption, the Langmuir model can be fitted well for the SPR signal with 0.9989 of $R^2$. For Langmuir fitting of EIS signal, the $R^2$ was 0.9716 for casein adsorption. The maximum of adsorbed BSA and casein detected by EIS technique were 8,631 ohm and 50,000 ohm, respectively. By using SPR technique, the maximum of adsorbed BSA and casein were 10.61 Δ%R and 17.36 Δ%R, respectively. This results suggest that the adsorbed protein was randomly immobilized and formed a monolayer of protein on the gold surface.

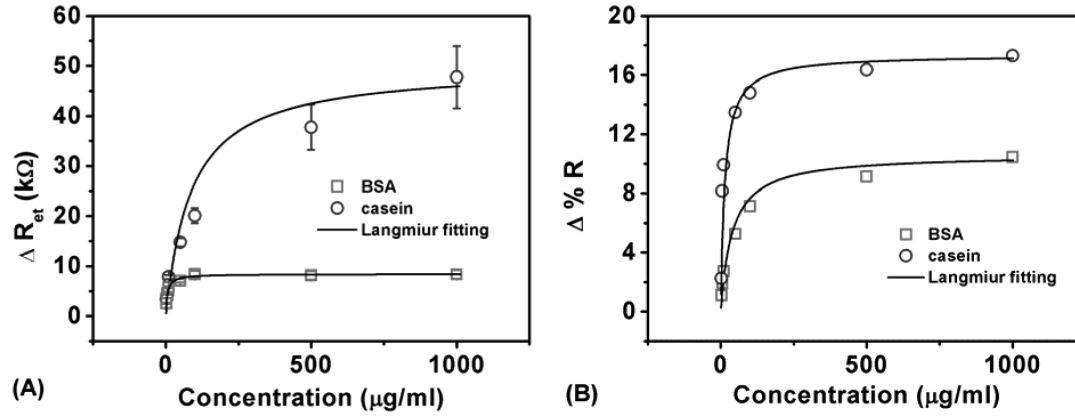


**Figure 4** The relationship between protein concentrations and response signal from EIS, graph A, and SPR techniques, graph B, respectively. The blue circle and the red square represent real data of casein and BSA, respectively. The black line is the Langmiur model fitting.

**Effect of pH**

The pH of solution affects to the change of protein structure involving to the protein isoelectric point (pI). pI is a pH in which net charge of protein is zero. Thus, amount of adsorbed protein on the surface also depends on the pH of solution. Figure 5 shows the relationship between the change of electron-transfer resistance and pH value of protein solution at 1.0 mg/ml of protein concentration. At this concentration, the adsorbed BSA and casein formed monolayer on the surface. The pH of solution effected on the value of Ret reflect to the adsorbed protein on the surface. The structure of protein was changed at different pH buffer, resulting from the adsorbed protein on the surface. The pI of BSA and casein was 4.7 [23] and 4.6 [5, 6], respectively. The pH of BSA presence solutions (pH 5, 7.4 and 9) was greater than pI of BSA resulting the protein formed a negative charge. The $\Delta R_{et}$ value of BSA surface slightly decreased for the higher pH buffers and the pH 5.0 buffer showed the highest adsorption. Similarly, the net charge of casein was negative charge. However, the $\Delta R_{et}$ value of casein surface was different at different pH buffers and the pH 7.4 buffer was highest for casein adsorption. Thus, these results suggest that pH 7.4 buffer should be used as a dilution buffer for protein adsorption.

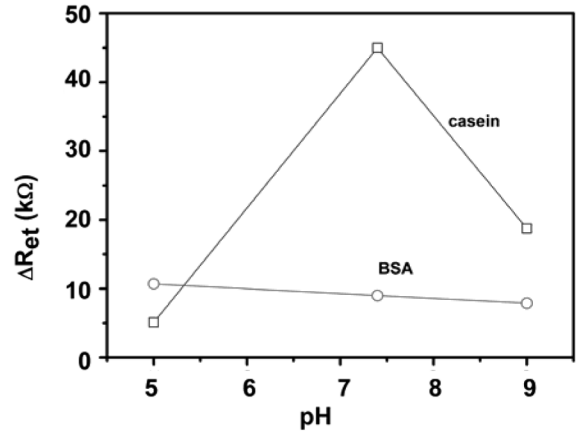


**Figure 5** Relationship between the change of electron-transfer resistance and pH value for protein adsorption.

**Non-specific binding to *E. coli* K12**

Both blocking surfaces, adsorbed BSA and casein, were tested to non-specific binding by using *E. coli* K12 as a model system. Figure 6 shows the Nyquist plot resulting to the non-specific binding signal of *E. coli* K12 on the blocking surface. The concentrations of BSA and casein on the surface were 1.0 mg/ml. The amount of adsorbed BSA on the surface was 6,150 ohm (seen in semi-circle in Figure 6, Blank-BSA surface). The $10^8$ cfu/ml of *E. coli* K12 was then incubated and measured the faradic impedance. The semi-circle curve was slightly decrease and still the same shape (*E. coli* K12-BSA surface). The value of $R_{et}$ was 6,020 ohm in the step of *E. coli* K12 incubation. This result suggest that the BSA can prevent the adsorbed bacteria on the blocking surface. In case of adsorbed casein surface, the amount of casein (1.0 mg/ml) was 11,800 ohms as shown in blank-casein surface. After incubation of bacteria, the semi-circle curve (*E. coli* K12-casein surface) was similar before the incubation. The $R_{et}$ value was 11,400 ohm in the step of *E. coli* K12 incubation. This imply that the adsorbed casein surface can also prevent non-specific binding for adsorption of bacteria on the surface. However, the gold surface without blocking protein should be carried out to adsorb bacteria in order to confirm the non-specific binding of blocking protein surface. Furthermore, the experiment should be performed in the different types of bacteria.

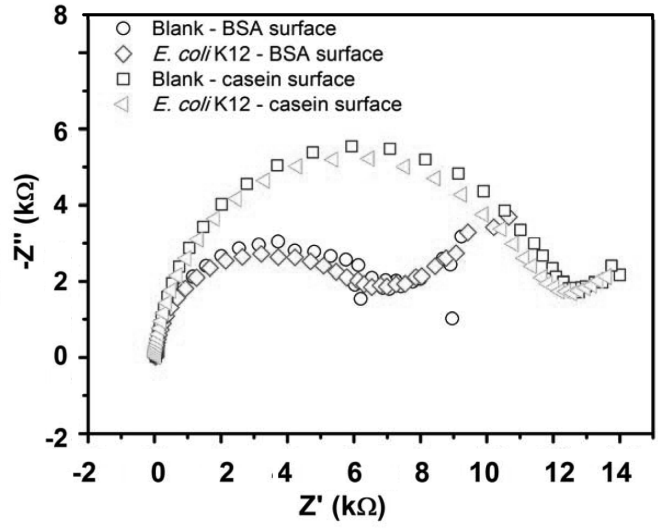


**Figure 6** Non-specific binding of *E. coli* K12 on the adsorbed protein surface. Nyquist plots show the adsorbed BSA surface, *E. coli* K12-BSA surface, adsorbed casein surface, and *E. coli* K12-casein surface.

## Conclusion

The result of protein adsorption obtained from EIS method corresponds to that of SPR method. The saturation point of protein adsorption was 100 μg/ml of BSA and 500 μg/ml of casein for the adsorption on the surface using EIS technique. The dilution solution for casein and BSA shows the highest adsorption at pH 7.4 of PBS and pH 5.0 of acetate buffer, respectively. Furthermore, the adsorbed BSA and casein can be used as a blocking protein to prevent the non-specific binding signal from *E. coli* K12 on the gold surface. This information is further used in the development of biosensor for the detection of bacteria.

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