Biophysical Chemistry 42 (1992) 213–220
Elsevier Science Publishers B.V., Amsterdam

Voltage-dependent capacitance as a probe for albumin adsorption onto a solid surface

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(Received 5 December, 1990; accepted in revised form 24 June, 1991)

Abstract

The process of adsorption of bovine serum albumin onto a platinum electrode was monitored through the measurement of a nonlinear electrochemical property. The principle of the new method is that a sinusoidal voltage source is applied to a test solution and the waveform of the output current is analyzed by Fourier transformation. It was found that the intensities of the higher harmonics in the Fourier transformation change depending on the concentration of albumin and with time. From the higher harmonics, voltage dependence of the capacitance was quantitatively evaluated. The change of the state of albumin adsorbed onto the platinum plate was also monitored from the pattern of 'crack' of adsorbed albumin by using scanning electron microscopy. These results were discussed in relation to the mechanism of bimodal adsorption of albumin.

Keywords: Bovine serum albumin; Bimodal adsorption; Electrochemical probe

1. Introduction

Although there have been many studies on the molecular process of adsorption of protein onto a solid surface, a detailed mechanism of the adsorption has not been fully clarified yet [1–10]. In order to develop artificial organs, for example, an artificial heart or blood vessels, it is most important to know the physico-chemical state of the adsorbed protein on the surface. It is rather difficult to monitor the molecular process of protein adsorption *in situ* [1,2]. We have recently reported a novel sensing method for detecting the physico-chemical state at a solution–metal interface [11]. This method is based on the principle of detecting information of the higher harmonics in the Fourier-transformation of output current under application of an external sinusoidal voltage [12–14] (See Appendices I and II). It has been demonstrated that the reproducibility of this method is excellent and that it affords us much useful information on the electrochemical system. Here, the Fourier transformation is utilized for detecting quantitatively the electrochemical nonlinearity. In this paper, adsorption of albumin has been analyzed in a quantitative manner using data of the higher harmonics of the output current. From the results of the Fourier-transformation, voltage dependence of the capac-

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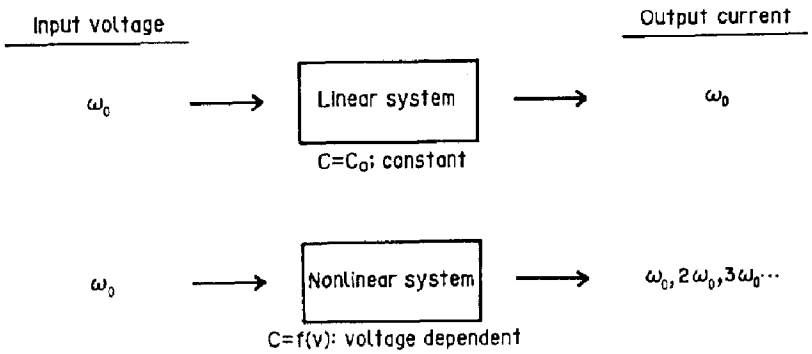

Scheme 1. Higher harmonics ($2\omega_0$, $3\omega_0$, $\cdots$) are generated in an electrochemical system with nonlinear capacitance.

itance, differential capacitance [15], has been deduced. The state of albumin adsorbed onto the platinum plate has also been studied with the aid of a scanning electron micrograph.

2. Experimental

Bovine serum albumin was obtained from Miles Laboratories, Inc. (U.S.A). The test solution consisted of 0.1369 *M* NaCl, 0.0268 *M* KCl, 0.00958 *M* Na_2HPO_4, and 0.00146 *M* KH_2PO_4 and was buffered at pH 7.4. Water was first distilled and then purified with a Millipore Milli-Q filtering system. Electrical measurements were performed in an apparatus shown in Fig. 1. Sinusoidal voltage (frequency: 1 Hz, peak-to-peak voltage: 2V) was generated by a potentiostat (HA-10 RIG, Hokuto Denko Ltd., Japan) connected to a waveform generator, Model 459 AL (Kikusui Electronics Corp., Japan). A two-electrode cell was employed and the ohmic drop arising from solution resistance in the cell was successively compensated by the positive feedback as a function of the potentiostat. The input sinusoidal voltage and the output current were successively stored in a personal computer, NEC PC-9801 (NEC Co., Ltd., Japan), and then Fourier-transformed to the frequency domain [11]. Platinum wire (length: 25 mm, diameter: 0.5 mm) was used as the working electrode and a Ag/AgCl electrode was used as the reference electrode. All measurements were performed at 25 ± 2°C. Scanning electron micrographs were obtained with a scanning electron microscope (Model S-430, Hitachi, Co., Tokyo). The albumin adsorbed onto the platinum plate was freeze-dried and then coated with gold by vacuum evaporation.

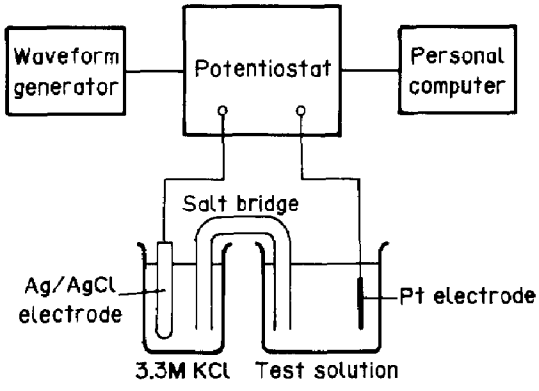

Fig. 1. Diagram of the experimental apparatus used for measuring electrochemical nonlinearity.

3. Results and discussion

3.1 Time-variation of output current for albumin solution

Figure 2a shows the output current in the albumin-free buffer solution (a-1) in 5% (w/v) albumin solution versus time (a-2, 3 and 4). Two maxima during one cycle of the sinusoidal wave are noticed in Figs. 2a-2, 3 and 4. The waveform of the output current for the buffer solution without albumin remained essentially constant for a period of more than 3 hours, we regarded the waveform of Figure 2a-1 as the control pattern. The marked change of output current in the

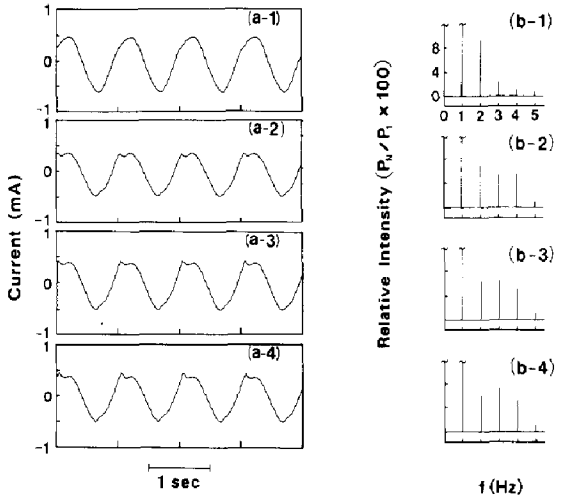

Fig. 2. The left pannel shows the waveform of the output current with the application of sinusoidal voltage (1 Hz). (a−1): the buffer solution; (a−2 to 4): 5% (w/v) albumin solution after the elapsed time of 5 min (a−2), 30 min (a−3), and 60 min (a−4). The right pannel shows the relative intensity (P_n/P_1) of the harmonics in the Fourier-transform of the output current.

albumin-containing buffer solution is attributable to adsorption of albumin molecules onto the surface of the Pt-electrode [3–5]. As the pH at the isoelectric point of bovine serum albumin is 5.3, carboxylic acid groups in albumin molecules dissociate; albumin is negatively charged under the conditions used in the present study. Figure 2b shows the relative intensity of higher harmonics to the fundamental harmonics (1 Hz) in the power spectrum of the Fourier-transformation upon the output current shown in Fig. 2a. It was found that

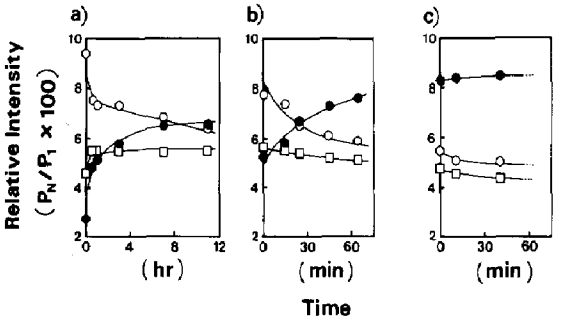

Fig. 3. Time variation of relative intensity on various concentrations of (% w/v) of albumin: (a) 1, (b) 5, and (c) 10. P_n: Intensity of the power spectrum of the nth harmonics; (○) 2nd harmonics (2 Hz); (•) 3rd harmonics (3 Hz), and (□) 4th harmonics (4 Hz).

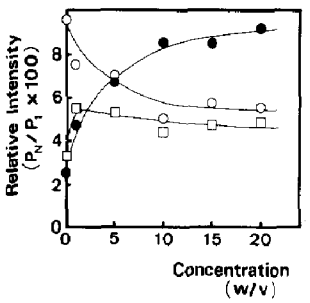

Fig. 4. Dependence of relative intensity on the concentration of albumin. These data were measured after an elapsed time of 30 minutes. P_n: Intensity of the power spectrum of the nth harmonics; (○) 2nd harmonics (2 Hz), (•) 3rd harmonics (3 Hz), and (□) 4th harmonics (4 Hz).

the intensities of the higher harmonics (2, 3, and 4 Hz) change with time (Fig. 2b-2, 3, and 4). The appearance of the higher harmonics of the Fourier spectrum is due to the electrochemical nonlinearity [11]. Even in the absence of albumin, the higher harmonics appeared significantly. This is mostly due to the electrochemical nonlinearity generated by the presence of diffuse electrical double layer of inorganic ions around the electrode surface. As is discussed in the Appendix II, diffuse electrical double layer possesses the property of the electrochemical nonlinearity in its intrinsic nature. Thus, we evaluated the higher harmonic components in the absence of albumin as the control values of the experiment. Figure 3 shows the time-dependence of the relative intensity P_n/P_1 (P_n, the intensity of the power spectrum corresponding to that of the nth harmonic; P_1, the intensities of the power spectrum of the fundamental harmonic) with various concentrations, (a) 1, (b) 5, and (c) 10% (w/v), of albumin. In 10% (w/v) albumin, the relative intensities remain essentially constant, indicating that the adsorption has been completed immediately after immersion of the electrode. In 1 and 5% (w/v) albumin solution, P_2/P_1 decreased and P_3/P_1 increased with time. The direction of the changes of the relative intensities are the same within the range of 1 to 5% (w/v) albumin solution, although the rates of the changes are apparently different. Figure 4 shows the dependence of P_n/P_1 upon the concentration of albumin after 30 minutes. It is clear that the relative intensities

change greatly in the concentration range of 0 to 10% (w/v) and the curve becomes nearly flat above 10% (w/v). These results suggest that the changes of the relative intensities reflect the time course of adsorption of albumin onto the electrode.

Under similar experimental conditions, in the classical impedance method, reproducibility is poor because of changes in the distance between the electrodes, the presence of bubbles on the electrode, and hysteresis arising from adsorption of various chemicals onto the electrode. By contrast, the reproducibility of our method was good in duplicate runs. This is because in our treatment the intensities of the power spectrum on the 2nd, 3rd, and 4th harmonics are given as normalized values relative to the intensity of the power spectrum on the first harmonics. Although the linear components showed relatively large experimental errors (ca. 20% (w/v)) on each experimental run, use of the normalization procedure reduced errors in the relative intensities to less than 5% (w/v) for all measurements in this study. In the present study, we have applied sinusoidal voltage of rather low frequency (1 Hz). The reason for this choice is that the dielectric property of the bulk solution contributes to the capacitance with higher frequency, and thus the capacitance component of the diffuse double layer near the electrode surface could be observed only with low frequencies [17].

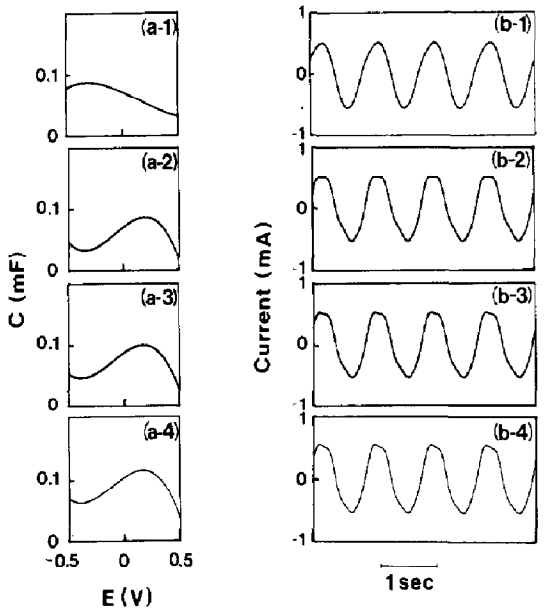

Fig. 5. (a) Capacitance–voltage curve deduced from the Fourier-transformed spectra (Fig. 2b). (b) Waveform of the output current deduced from the reversed Fourier-transformation of the spectra in the frequency domain (Fig. 2b).

3.2 Theoretical simulation

In order to clarify the relationship between the intensities of the higher harmonics and the electrochemical nonlinearity, let us discuss on a voltage-dependent capacitor as a simple nonlinear element [11], as given in eq. (1).

$$C(V) = C_0 + C_1V + C_2V^2 + C_3V^3 \tag{1}$$

When a sinusoidal voltage ($V(t) = E_1 \sin \omega_0 t$) is applied to the system of a parallel circuit with this nonlinear capacitor and a resistor, the output current is given by eq. (2). (See, for a detailed derivation, Appendix I.)

$$\begin{aligned} I(t) = {} & (E_1/R) \sin \omega_0 t \\ & + \omega_0 E_1^2 \left\{ \tfrac{1}{2} C_1 + \tfrac{1}{4} C_3 E_1^2 \right\} \sin 2\omega_0 t \\ & - \tfrac{1}{8} C_3 \omega_0 E_1^4 \sin 4\omega_0 t \\ & + \left\{ \tfrac{1}{4} C_2 E_1^2 + C_0 \right\} \omega_0 E_1 \cos \omega_0 t \\ & - \tfrac{1}{4} \omega_0 C_2 E_1^3 \cos 3\omega_0 t \end{aligned} \tag{2}$$

When the current $I(t)$ is Fourier transformed, the coefficients of the sine and cosine functions correspond to the real and imaginary components, respectively. From this relationship, it is apparent that the 2nd, 3rd, and 4th harmonics correspond to the first, second, and third derivatives, respectively, of the nonlinearity of the capacitance. Apart from the nonlinearity of the voltage-dependence of the capacitance, other phenomena such as hysteresis on the electrode and the nonlinearity of the resistance may contribute to nonlinearity in an electrochemical system. The derivation of eq. (2) has been based on the assumption that $dC/dt = 0$, and that resistance, R, is independent of the applied voltage. Figure 5 shows the simulated capacitance-voltage curve based on the experimental results of Fourier-transformation of the output current

wave. In this computer simulation, the components of the 2nd, 3rd, and 4th harmonics have been evaluated using eq. (2). This figure clearly demonstrates the relationship between the nonlinearity of the capacitance and the shape of the output current.

It should be stressed that our technique is entirely different from the classical impedance method [16]. In the classical impedance measurement, electrochemical properties have been interpreted by taking into consideration the equivalent circuit with capacitors and resistors which have only *linear* characteristics; i.e. non higher harmonics will appear, even with an electrical circuit composed of many capacitors and resistors. Nonlinear characteristics only produce the higher harmonics after application of a sinusoidal voltage with a single frequency.

3.3 Scanning electron micrograph of adsorbed albumin

In order to gain further insight into the mechanism of adsorption, we have carried out electron microscopic measurements. On examining the manner of adsorption by electron microscopy, we found that the pattern of cracks of the adsorbed layer changed markedly dependent upon the state of the adsorbed albumin. Figure 6 shows 'cracks' of albumin adsorbed onto a platinum plate. The cracks are caused by freeze-drying during preparation of the sample for the scanning electron micrograph. It is apparent that the pattern of the 'cracks' changes differs and is depending on the period of immersion of the platinum plate into the albumin solution. After an elapsed time of 5 minutes, the angle between the lines of 'cracking' is almost 90°. By contrast, after a period of 1 hour, the angle becomes nearly 120°. The difference of the pattern of the 'cracks' may depend on the surface tension and the thickness of the albumin layers. In the early stage of adsorption, albumin molecules may remain in their natural state, i.e., the adsorption is physical. Thus, when dehydration occurs, individual molecules schrink in a manner nearly independent of each other. In this situation, rectangular cracks are generated as shown in Fig. 6a. When adsorbed albumin molecules stay for a long period, they tend to be denatured, i.e., the adsorption is chemical. The peptide skeletons of denatured albumin molecules, on the strongly adsorbed albumin, may interact with each other, thereby inducing macroscopic surface tension on the layer of adsorbed albumin. Upon dehydration, cracks with 120° crossing angles are generated because of the in-

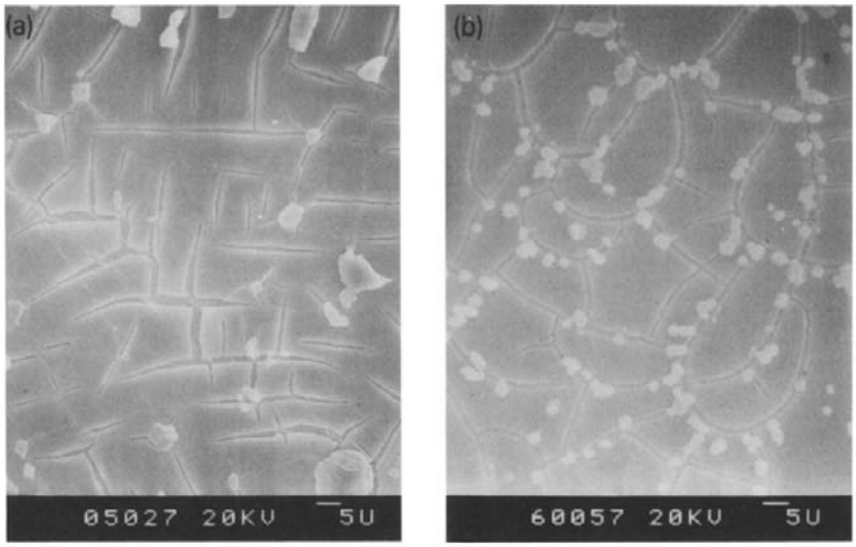

Fig. 6. Scanning electron micrograph of albumin adsorbed onto platinum plate: (a) after an elapsed time of 5 minutes, and (b) after an elapsed time of 1 hour. The conditions for (a) and (b) are the same as in the legend for Figs. 2a-2 and 2a-4, respectively.

crease of the surface tension induced throughout the layer of the albumin.

3.4 Measurement of the amount of adsorbed albumin by use of an electronic balance

In order to investigate the amount of albumin adsorbed onto the platinum plate, the time variation of the weight of the platinum plate ($20 \times 50 \times 0.1$ mm) immersed into the 5% (w/v) albumin solution was measured with a Shimadzu RNB-50 electronic balance in order to evaluate the amount of the adsorbed albumin molecules onto the platinum plate. The weight gradually increased up to ca. 1.0 mg (i.e., a surface density of 7.4×10^{-10} mol/cm^2) during 1 hour corresponding to the initial stage of adsorption (i.e., physical adsorption) after which the weight remained essentially the constant over a period of 3 hours. This result suggests that the characteristic change of the output current wave in Fig. 2 reflects the degree of the denaturation of albumin, and *not* the increase of the albumin adsorbed.

3.5 Relationship between higher harmonics and adsorption of albumin

In the present study, we have demonstrated that the voltage-dependent capacitance probe provides us with information about the nature of adsorption of albumin. Figure 7 summarizes the time-variation of the waveform of the output current, power spectrum of the Fourier transformation, and voltage-dependent capacitance. A plausible process of the manner of adsorption is also given in Fig. 7. In Fig. 7a when the Pt wire is immersed into the albumin aqueous solution, the molecules of albumin begin to be adsorbed onto the Pt surface. In this stage, the adsorbed albumin molecules do not aggregate. In Fig. 7b the adsorbed albumin molecules gradually change their conformation, corresponding to a kind of surface denaturation [1,2,6,10]. The conformational change results in albumin molecules on the surface to interact with each other. In the final stage, Fig. 7c, the surface of the electrode is fully covered with the "denatured" albumin, or with the strongly adsorbed albumin. At lower concentrations (< 1% (w/v)), the progress of adsorption is very slow, as shown in Fig. 3a. For the 5% (w/v) albumin solution, the progress of adsorption becomes fast (Fig. 3b). With the higher concentrations of albumin, more than 10% (w/v), the final stage of the adsorption (state (c)) is attained quite fast, which results in the relatively flat curves (Figs. 3c and 4). The electron microscopic observation also supports this mechanism.

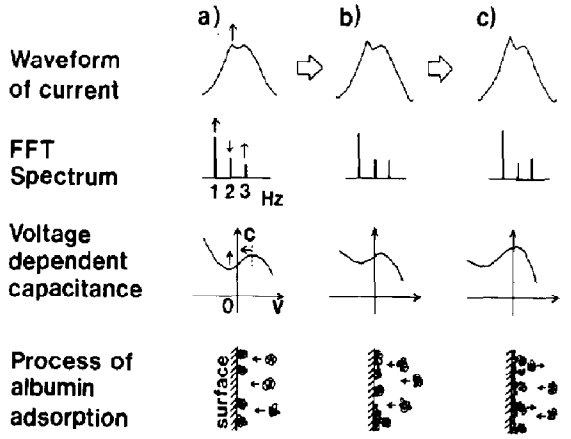

Fig. 7. Schematic representation of the relationship between nonlinear characteristics and the manner of adsorption: (a) initial stage, (b) intermediate stage, and (c) final stage.

The above mentioned scheme for albumin adsorption is compatible with the mechanism of bimodal adsorption proposed by several researchers [7,8].

In summary, it becomes clear that the characteristic change of the voltage-dependent capacitance, together with the observation of cracking of the adsorbed protein, affords us useful information on the physicochemical state of the protein adsorbed onto the solid surface.

Acknowledgments

The authors are grateful to Mr. Y. Kaneda for technical assistance. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Science.

Appendix I

Relationship between the voltage-dependent capacitance and its higher harmonic Fourier components

Suppose that an electrochemical system is modeled by a parallel circuit with a nonlinear capacitor and a linear resistor. Let us now discuss the charge Q which is expressed by eq. (A1) as a simple expansion to the fourth order with voltage V.

$$Q(V) = C_0V + \tfrac{1}{2}C_1V^2 + \tfrac{1}{3}C_2V^3 + \tfrac{1}{4}C_3V^4 \quad \text{(A1)}$$

where differential capacitance $C(V)$ is given by eq. (A2).

$$C(V) = \mathrm{d}Q/\mathrm{d}V = C_0 + C_1V + C_2V^2 + C_3V^3 \quad \text{(A2)}$$

When a sinusoidal voltage ($V = E_1 \sin \omega_0 t$) is applied to the circuit, the current through the resistor $I_R(t)$ and the current through the capacitor $I_C(t)$ are given by eqs. (A3) and (A4), respectively.

$$I_R(t) = (E_1/R) \sin \omega_0 t \quad \text{(A3)}$$

$$\begin{aligned} I_C(t) &= \mathrm{d}Q/\mathrm{d}t = (\mathrm{d}Q/\mathrm{d}V)(\mathrm{d}V/\mathrm{d}t) \\ &= C(V)(\mathrm{d}V/\mathrm{d}t) \\ &= \left(C_0 + C_1V + C_2V^2 + C_3V^3\right) \\ &\quad \times \mathrm{d}(E_1 \sin \omega_0 t) \\ &\quad /\mathrm{d}t \\ &= \left\{C_0 + C_1(E_1 \sin \omega_o t) + C_2(E_1 \sin \omega_o t)^2 \right. \\ &\quad \left. + C_3(E_1 \sin \omega_0 t)^3\right\}\omega_0 E_1 \cos \omega_0 t \\ &= C_0\omega_0 E_1 \cos \omega_0 t + C_1\omega_0 E_1^2 \sin \omega_0 t \cos \omega_0 t \\ &\quad + C_2\omega E_1^3 \sin^2\omega_0 t \cos \omega_0 t \\ &\quad + C_3\omega_0 E_1^4 \sin^3\omega_0 t \cos \omega_0 t \\ &= C_0\omega_0 E_1 \cos \omega_0 t + \left(C_1\omega_0 E_1^2/2\right) \sin 2\omega_0 t \\ &\quad + \left(C_2\omega_0 E_1^3/4\right)(\cos \omega_0 t - \cos 3\omega_0 t) \\ &\quad + \left(C_3\omega_0 E_1^4/8\right)(2 \sin 2\omega_0 t - \sin 4\omega_0 t) \end{aligned} \quad \text{(A4)}$$

The total current $I(t)$ through the voltage source becomes

$$I(t) = I_R(t) + I_C(t) \quad \text{(A5)}$$

Substituting eqs. (A2)–(A4) into eq. (A5), we obtain eq. (2) as:

$$\begin{aligned} I(t) = {} & (E_1/R) \sin \omega_0 t \\ & + \omega_0 E_1^2\left\{\tfrac{1}{2}C_1 + \tfrac{1}{4}C_3E_1^2\right\} \sin 2\omega_0 t \\ & - \tfrac{1}{8}C_3\omega_0 E_1^4 \sin 4\omega_0 t \\ & + \left\{\tfrac{1}{4}C_2E_1^2 + C_0\right\}\omega_0 E_1 \cos \omega_0 t \\ & - \tfrac{1}{4}\omega_0 C_2 E_1^3 \cos 3\omega_0 t \end{aligned} \quad \text{(2)}$$

Appendix II

Voltage-dependent capacitance in the case of electrochemical diffuse double layer

According to the Gouy–Chapman theory of a diffuse double layer of ions formed around the electrode surface, differential capacitance, C_d is given as follows [15].

$$C_d = \left(\frac{2z^2e^2\varepsilon\varepsilon_0 n^0}{kT}\right)^{1/2} \cosh\left(\frac{zeV}{2kT}\right) \quad \text{(A6)}$$

where z denotes the (signed) number of units of electronic charge, e the elementary charge, ε the dielectric constant of the medium, ε_0 the permittivity of free space, n^0 the number concentration of each ion in the bulk phase, k Boltzmann's constant, T the absolute temperature (K), and V the electrostatic potential (V).

Above equation can be expanded to a polynomial in V.

$$C_d = \left(\frac{2z^2e^2\varepsilon\varepsilon_0 n^0}{kT}\right)^{1/2} \left\{1 + \left(\frac{ze}{2kT}\right)^2 V^2 + \left(\frac{ze}{2kT}\right)^4 V^4 + \ldots\right\} \quad \text{(A7)}$$

The above equation clearly suggests that capacitive components at an electrode is generally "nonlinear" or, in other words, the capacitance is

voltage-dependent as in eq. (A1). Besides the electrochemical diffuse double layer, the voltage-dependence of the component originates from adsorption/desorption kinetics of protein at the electrode and also from time-dependent deformation of the protein.

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