

Enhanced Sensor Capability of Juxtaposed β -Cyclodextrin Rings in TiO_2 Ultrathin Matrix as Determined by Cyclic Surface-Polarization Impedance Measurement

Do-Hyeon Yang, Myung-Jong Ju, Aya Maeda and Seung-Woo Lee*

Graduate School of Environmental Engineering, The University of Kitakyushu,
1-1 Hibikino, Kitakyushu 808-0135, Japan

(Received January 31, 2008; accepted May 8, 2008)

Key words: cyclodextrins (CDs); molecular imprinting; ultrathin TiO_2 layer; cyclic surface-polarization impedance (cSPI)

The present study was focused on the sensitive detection of organic substances by electrochemical impedance spectroscopy (EIS), i.e., cyclic surface-polarization impedance (cSPI) measurement that detects the difference in interactions between the electrode and chemicals in aqueous phase. For cSPI measurement, Pt electrodes were modified with three different types of self-assembled monolayer (SAM) of -OH, -COOH, and -pyridine (Py) terminal groups. Among them, the Pt/SAM-OH electrode was additionally modified with an ultrathin TiO_2 gel layer and a 2:1 complex of β -cyclodextrin (β -CD) and bisphenol A (BPA), and then the BPA moiety was removed by washing in methanol. A highly sensitive and selective detection of BPA was achieved by the synergic effect of molecular imprinting and host-guest interaction in the ultrathin TiO_2 layer. The resulting BPA-imprinted TiO_2/β -CD film showed a linear increase in ΔR_p at -0.1 V for BPA of 2×10^{-8} to 2×10^{-6} M. However, BPA was barely detected on the SAM-modified electrodes at low concentrations, although their sensor responses were markedly enhanced at a concentration of 2×10^{-6} M, compared with that of the bare Pt electrode. The current approach suggests the simple concept and use of sensor films for the sensitive detection of various organic molecules including environmental pollutants.

1. Introduction

The role of chemical sensors is expanding owing to increased concern on safety and security. Recently, we have developed a chemical sensor on the basis of the concept of a new type of electrochemical impedance spectroscopy (EIS) called cyclic surface-polarization impedance (cSPI) measurement.⁽¹⁻³⁾ The electrochemical impedance on

*Corresponding author: e-mail: leesw@env.kitakyu-u.ac.jp

solid metal electrodes in water is given by the “frequency-dependent” capacitance rather than the “ideal” capacitance. This behavior is usually called “capacitance dispersion” or a constant phase element (CPE).^(4,5) The electrode surface is not ideal and has atomic-scale heterogeneity such as “surface disorder” and specific anion adsorption.^(6,7)

CPE impedance also depends on the adsorption and desorption of chemical substances to and from the electrode surface, respectively. Therefore, the adsorption and desorption of chemical substances can be detected by measuring the electrochemical impedance under a dynamically controlled electrode potential. The interaction between adsorbed molecules and the electrode surface is strongly affected by surface polarization. Consequently, impedance changes due to an electrode potential change represent chemical sensor outputs, and a broad range of chemical substances can be identified from the CPE impedance-voltage curve obtained by the cSPI method.

Molecular imprinting provides a very general class of sensing matrices that include predesigned recognition sites.^(8,9) Over the past ten years, we have studied molecular imprinting for the recognition of organic molecules in ultrathin TiO₂ gel films that were fabricated by the surface sol-gel process.⁽¹⁰⁻¹⁴⁾ Willner *et al.* have recently developed sensor devices associated with the ion-sensitive field-effect transistor (ISFET) by imprinting achiral and chiral aromatic compounds in TiO₂ thin films.⁽¹⁵⁻¹⁷⁾ The usefulness of the TiO₂ gel matrix is enhanced by combining useful host compounds,^(18,19) since polyhydroxyl compounds such as dendrimers, cyclodextrins and polyrotaxanes, which can act as effective receptors, are readily incorporated.⁽²⁰⁾ In our previous work, the sensing ability of cyclodextrins immobilized on a TiO₂ gel layer was demonstrated via surface imprinting of bisphenol A.⁽¹⁸⁾ In the present study, we explore a further potential of this receptor design by the cSPI method, compared with that using self-assembled monolayers. In particular, cSPI measurement offers useful information about the chemical and physical interactions between chemical substances and the electrode surface. In this study, we have successfully applied this technique to the convenient detection of the proton transfer of hydroxyl aromatic compounds trapped in cyclodextrin cavities.

2. Experimental

2.1 Materials

Diethylstilbestrol, 4-nonylphenol, bisphenol A (BPA), and β -estradiol were purchased from Tokyo Kasei Kogyo, Japan. 2-Mercaptoethanol, 3-mercaptopropionic acid, and 4,4'-dithiodipyridine were purchased from Kanto Chem., Japan. Titanium (IV)-*n*-butoxide (Ti(O-*n*Bu)₄) and β -cyclodextrin (β -CD) were purchased from Kishida Chem., Japan. All these chemicals were guaranteed to be of reagent grade, and used without further purification. All the other chemicals used were of reagent grade and obtained from commercial sources, and used as purchased. Deionized water (18.3 M Ω -cm) was obtained by reverse osmosis followed by ion exchange and filtration (Nanopure Diamond, Barnstead, Japan). The chemical structures of the template and guest molecules used in this study are shown in Fig. 1.

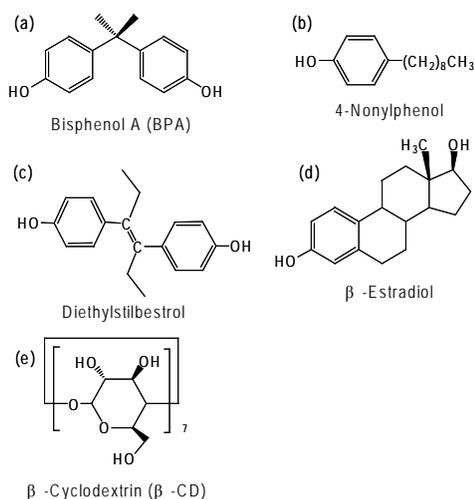


Fig. 1. Chemical structures of host and guest molecules used in this study.

2.2 *cSPI measurement*

The measurement setup is shown in Fig. 2(a). An alternating input voltage was generated by a function generator (Yokogawa, FG110) and superimposed to the electrode potential controlled by a potentiostat in order to measure the electrode impedance. The amplitudes and phase difference between the input and output signals were measured with a lock-in amplifier (NF Electronic Instruments, LI 5640). The impedance of the electrode was calculated from the ratio of the amplitude and the difference in phase between the input voltage and the output current. The measurement was performed using a computer through GPIB.

As shown in Fig. 2(b), the working electrode (WE) was attached to the acrylic board through a 0.2-mm-thick silicone rubber with a 2-mm-diameter hole. The WE and counterelectrode (CE) were a polycrystalline Pt plate (10 mm \times 20 mm and 0.5 mm in thickness) and a Pt wire, respectively. The reference electrode (RE) was an Ag/AgCl electrode with saturated KCl solution. The supporting electrolyte solution was 10 mM KCl. All experiments were carried out at room temperature.

Details of the cyclic surface-polarization impedance (*cSPI*) measurement have been described in previous studies.⁽¹⁻³⁾ Figure 2(c) shows a complex plane plot of electrode impedance. Each impedance was measured by scanning both frequency and electrode potential to determine interfacial capacitance and resistance as functions of frequency and electrode potential. First, the impedance spectra from 260 to 35 Hz were measured to estimate the characteristic CPE impedance and solution resistance. The real R_p and imaginary X_p parts of CPE impedance are given as $R_p = \text{Re } Z(f_L) - \text{Re } Z(f_H)$ and $X_p = -\{\text{Im } Z(f_L) - \text{Im } Z(f_H)\}$, where f_L and f_H are the measured lower- and higher-frequency points, respectively. $\text{Re } Z$ and $\text{Im } Z$ indicate the real and imaginary parts of the interfacial impedance Z , respectively. $\text{Re } Z(f_H)$ is regarded as the solution resistance (R_s). A simple equivalent circuit of this system was assumed to be a series circuit of R_s and Z_{CPE} , as shown in Fig. 2(d), where $Z_{\text{CPE}} = R_p - jX_p$. Although the electrode potential was swept

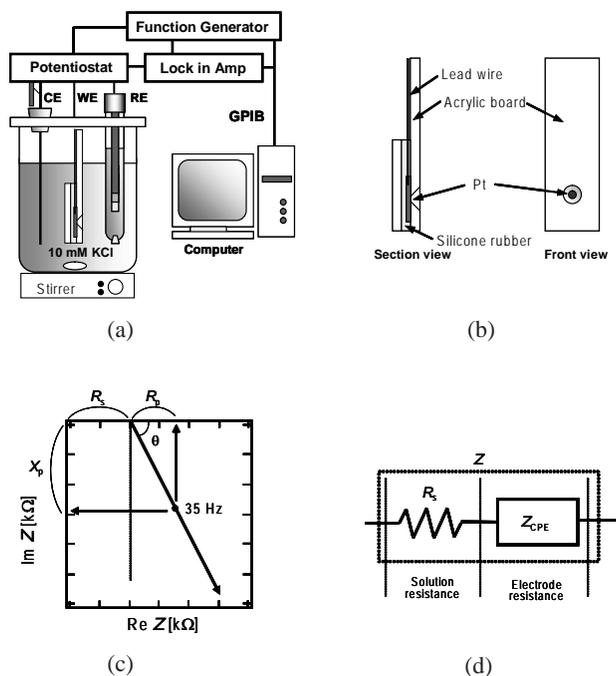


Fig. 2. (a) Measurement setup, (b) arrangement of working electrode, (c) a complex plane plot of electrode impedance, and (d) an equivalent circuit of the system.

from 0.70 to -0.30 V in 0.05 V steps, the electrochemical impedance was measured at $f_L = 35$ Hz and $f_H = 260$ Hz to evaluate R_p and X_p . The R_p - E and X_p - E curves are considered to be associated with the state of the inhomogeneous surface and the adsorption of chemical substances onto the platinum electrode, respectively, where E is electrode potential. ΔR_p denotes the changes in R_p with the addition of a chemical substance to 10 mM KCl solution. The ΔR_p - E and ΔX_p - E curves are respectively called the response profiles of ΔR_p and ΔX_p hereafter. The chemical features of the analytes, such as surface-active functional groups, flexible side chains, aromatic structure and pH, affect the response profile of impedance on electrode potential. Accordingly, pattern analyses of profile changes lead to the sensing of a broad spectrum of chemical substances.

2.3 Modification of electrodes

Pt electrodes were cleaned by polishing with alumina slurry (Buehler number 3, 0.05 μm particle size), subjected to sonication for 30 min in deionized water, and then exposed to the flame of a Bunsen burner. After this cleaning treatment, the Pt electrodes were modified with three different types of self-assembled monolayer (SAM), namely, -OH, -COOH, and -pyridine terminal groups using 2-mercaptoethanol, 3-mercaptopropionic acid, and 4,4'-dithiodipyridine, respectively. The three different SAMs were prepared by immersing the Pt electrode in a 10 mM solution of a corresponding thiol or disulfide

compound in ethanol for about 6 h at 25°C. Then, the Pt electrode was rinsed with ethanol and dried in a stream of N₂ gas.

On the other hand, a mercaptoethanol-modified Pt electrode was immersed in 100 mM Ti(O-*n*Bu)₄ in toluene/ethanol (1:1) for 10 min at 25°C, rinsed thoroughly with ethanol to remove physically adsorbed Ti(O-*n*Bu)₄, and then subjected to hydrolysis in deionized water for 1 min and drying by N₂ gas flushing. Finally, the electrode was immersed in an aqueous solution of a 2:1 β-CD/BPA complex (10 and 5 mM each) for 1 h at 40°C, rinsed with deionized water, and then dried in N₂ gas. The SAM-modified and β-CD-modified Pt electrodes were used as working electrodes for cSPI measurement.

3. Results and Discussion

3.1 SAM effects for BPA detection

Bisphenol A (BPA), an estrogenic compound, is used in the manufacture of polycarbonate plastic and epoxy resins. It is ubiquitous in the environment and found in many food and beverage containers, including baby bottles.^(21–24) Recently, Soto *et al.* reported that bisphenol A has been one factor causing the increase in breast cancer incidence over the past 50 years.⁽²⁵⁾ It was also noted that urine samples from 95% of the human subjects contained the chemical. Unfortunately, its low-concentration detection below 1 ppb is actually difficult using conventional analysis methods.

Figure 3 shows the cSPI responses (ΔR_p) to BPA on a bare Pt electrode and SAM-modified Pt electrodes. Since the response tendencies of the solution resistance (R_s) and imaginary part of the electrode impedance are similar to that of ΔR_p , in this study, we mainly discussed the sensor response of ΔR_p . In general, CPE is caused by the microscopic roughness and atomic-scale heterogeneity of the electrode surface. The adsorption (and desorption) of molecules to the electrode surface is influenced by the polarity and roughness of the surface. The cSPI method makes it possible to elucidate the adsorption and desorption of chemical substances to and from the electrode surface,

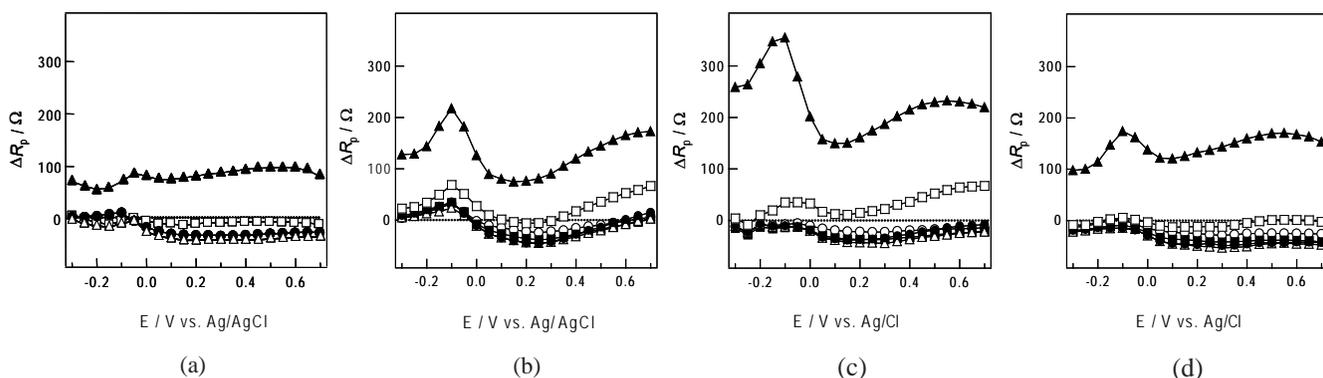


Fig. 3. cSPI responses (ΔR_p) to BPA on bare Pt electrode (a) and SAM-modified Pt electrodes ((b), (c), and (d)): (b) Pt/SAM-OH, (c) Pt/SAM-COOH, and (d) Pt/SAM-Py. [BPA] = (○) 2×10^{-8} M, (■) 6×10^{-8} M, (●) 2×10^{-7} M, (□) 1×10^{-6} M, (▲) 2×10^{-6} M.

respectively, via the evaluation of the CPE characteristics. It is expected that the adsorption of a comparatively large molecule like BPA has a significant influence on the CPE behavior. Moreover, the CPE change may be affected by the functional groups of adsorbed chemical substances.

As can be seen in Fig. 3(a), the characteristic response to BPA on the bare Pt electrode appeared only at a higher concentration of 2×10^{-6} M. In general, benzene and other aromatic compounds, in the absence of specific functional groups, are adsorbed irreversibly onto the Pt surface,⁽¹⁻³⁾ owing to the interaction between the aromatic ring and the Pt surface by π orbital overlap.^(26,27) In this case, the impedance change symmetrically increases except at approximately -0.1 V at the pzc (potential of zero charge) of Pt. In contrast, BPA with two hydroxyl groups in a molecule shows no noticeable change in the given electrode potential. It is evident that the adsorption of BPA is not altered significantly by the electrode potential. The ΔR_p of BPA shows two small unique peaks at around -0.1 V and 0 to 0.7 V (broad). They are supposed to be induced by O-Pt bond formation^(3,28) and the weak π orbital overlap between the aromatic ring and the Pt surface, respectively (see Fig. 4(a)).

Surface modification is one of the useful methods of improving the detection limit of sensor devices. The cSPI responses to BPA on the SAM-modified Pt electrodes, Pt/SAM-OH, Pt/SAM-COOH, and Pt/SAM-Py, are shown in Figs. 3(b)–3(d), respectively. Unfortunately, their response changes are not noticeable in the lower concentration range of 2×10^{-8} to 6×10^{-7} M. However, the sensor response was markedly enhanced at a concentration of 2×10^{-6} M, compared with that of the bare Pt electrode. In particular, a significant increase at around -0.1 V was observed in the Pt/SAM-OH and Pt/SAM-COOH electrodes. This indicates that the interaction of BPA molecules with the electrode surface was improved by the introduction of surface functional groups. Apparently, BPA molecules are much strongly bound on the surface by hydroxy and carboxy functionalities than by pyridine, as illustrated in Fig. 4. Additionally, the broad peak due to the π orbital overlap at 0 to 0.7 V was observed in all the modified samples. This result suggests that a complex π - π interaction, such as the stacking between BPA aromatic rings besides the above-mentioned π orbital overlap, occurs on the electrode surface.

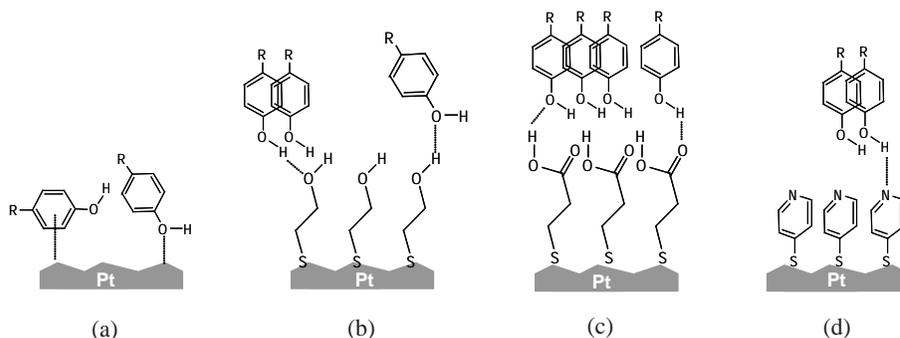


Fig. 4. Adsorption images of hydroxyl molecule on bare Pt electrode (a) and SAM-modified Pt electrodes ((b), (c), and (d)): (b) Pt/SAM-OH, (c) Pt/SAM-COOH, and (d) Pt/SAM-Py.

3.2 Surface modification using β -CD/BPA complex

As described above, the nature of the electrode surface is critical for the specific and selective detection of a given molecule. To obtain an electrode surface with high specificity to aromatic compounds, we conducted specific modification using β -CD that forms inclusion complexes with aromatic compounds using its hydrophobic cavity.^(29,30) The surface modification was conducted using a β -CD/BPA complex on a TiO_2 gel layer. First, a 2:1 β -CD/BPA complex was prepared by mixing β -CD and BPA in water in a 2:1 molar ratio upon stirring for more than 3 h at room temperature. It was then immobilized on a TiO_2 ultrathin layer, and the template BPA was removed by washing with methanol. After the template removal, the assembled β -CD molecules provide a specific binding site for the template and the hydroxyl groups of β -CD are bound onto the TiO_2 gel layer, as shown in Fig. 5.

Figures 6(a) and 6(b) show the cSPI responses to BPA on Pt electrodes of unmodified TiO_2 and BPA-imprinted TiO_2/β -CD films. The TiO_2 gel film prepared via the surface sol-gel process can have hydrophilic and hydrophobic moieties (Ti-OH, Ti-O-Ti) and the TiO_2 gel surface is expected to be capable of binding some BPA. However, the TiO_2 film showed no appreciable cSPI change in the given concentration range, although its response pattern is slightly different from that of the bare Pt electrode (Fig. 6(a)).

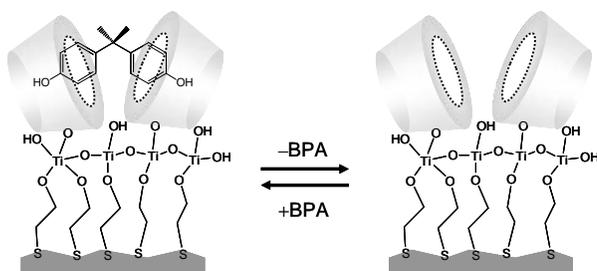


Fig. 5. Schematic illustration of molecular imprinting due to surface modification of a 2:1 β -CD/BPA complex on TiO_2 gel layer.

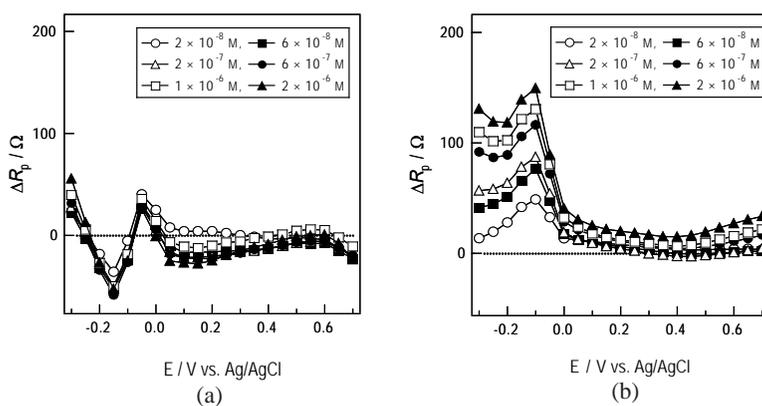


Fig. 6. cSPI responses to BPA on Pt electrodes of (a) TiO_2 and (b) BPA-imprinted TiO_2/β -CD films.

On the other hand, the response profile of BPA on the imprinted $\text{TiO}_2/\beta\text{-CD}$ film shows a characteristic peak at around -0.1 V of the electrode potential (Fig. 6(b)). This film can detect BPA molecules at 2×10^{-8} to 2×10^{-6} M. This response pattern may be attributed to the formation of an inclusion complex of BPA towards the immobilized $\beta\text{-CD}$ molecules, because the response to the TiO_2 film is barely detected. We already demonstrated the surface imprinting of the BPA template by $\beta\text{-CD}$ molecules in a different potential range of -0.5 to 0.5 V.⁽¹⁸⁾ It was expected that the sensor responses at -0.1 V might be attributed to the nonspecific adsorption via hydrogen bonding between BPA hydroxyl groups and the electrode surface. However, the impedance change at around -0.1 V seems to be influenced by isolated protons from the trapped BPA molecules, as expected with the SAM-modified Pt electrodes. This fact suggests that specific binding sites for BPA molecules, which were introduced via the imprinting of the $\beta\text{-CD}/\text{BPA}$ complex on a TiO_2 gel layer, play a role of improving the proton transfer from the trapped BPA molecules to the electrode through the imprinted $\beta\text{-CD}$ molecular cavities.

3.3 Selectivity of BPA-imprinted $\text{TiO}_2/\beta\text{-CD}$ film

Figure 7 shows the response profiles of the BPA-imprinted $\text{TiO}_2/\beta\text{-CD}$ film for structurally related guest molecules such as 4-nonylphenyl, diethylstilbestrol, and β -estradiol (Figs. 7(a)–7(c), respectively). As compared with BPA in terms of response, all guest molecules showed no noticeable change at around -0.1 V of the electrode potential. They showed two similar broad response peaks at -0.3 to -0.05 V and -0.05 to 0.7 V. These response patterns in the positive potential range are similar to those on the SAM-modified Pt electrodes, which may be attributed to the π - π interaction of nonspecifically adsorbed guest molecules. Interestingly, none of these broad responses are observed with BPA. From these impedance results, we can conclude that BPA can be discriminated from other structurally related guest molecules using a BPA-imprinted $\text{TiO}_2/\beta\text{-CD}$ film. The binding site apparently possesses structurally confined contours juxtaposed to the BPA template, and the host-guest interaction of two $\beta\text{-CD}$ s with two

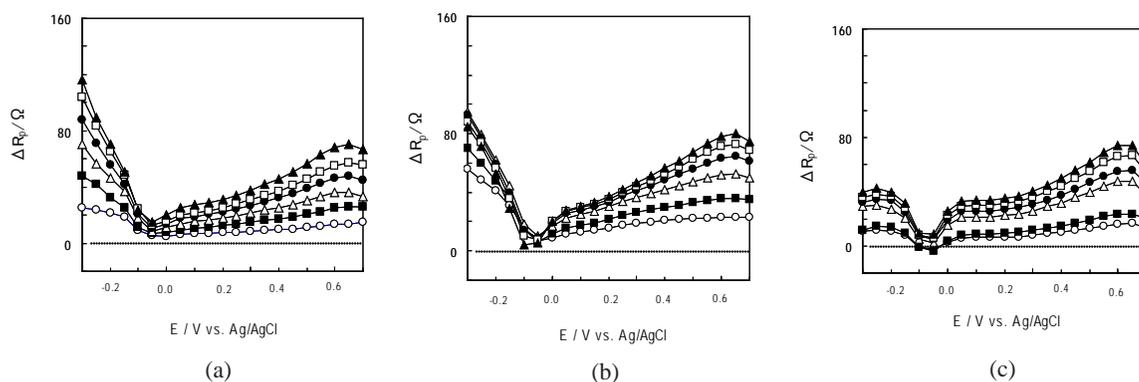


Fig. 7. Response profiles for structurally related guest molecules on BPA-imprinted $\text{TiO}_2/\beta\text{-CD}$ film: (a) 4-nonylphenyl, (b) diethylstilbestrol, and (c) β -estradiol. [Guest] = (\circ) 2×10^{-8} M, (\blacksquare) 6×10^{-8} M, (Δ) 2×10^{-7} M, (\bullet) 6×10^{-7} M, (\square) 1×10^{-6} M, and (\blacktriangle) 2×10^{-6} M.

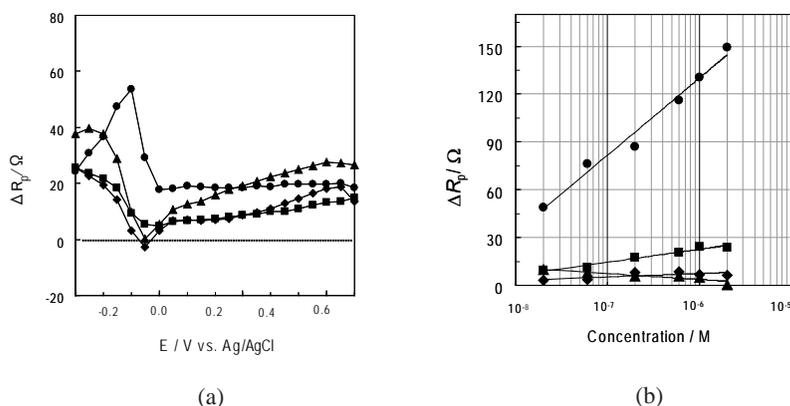


Fig. 8. (a) Response profiles (2×10^{-8} M) and (b) concentration dependences of ΔR_p (-0.1 V) of guest molecules on BPA-imprinted $\text{TiO}_2/\beta\text{-CD}$ film: (●) BPA, (■) 4-nonylphenyl, (▲) diethylstilbestrol, and (◆) β -estradiol.

phenyl groups in a BPA molecule enhances binding efficiency.

Figure 8(a) shows a comparison of response profiles for BPA and related guest molecules at a very low concentration of 2×10^{-8} M. The imprinted film reveals a specific response towards BPA at an electrode potential of -0.1 V compared with other structurally related guest molecules. Figure 8b shows the concentration dependence of ΔR_p for guest molecules at an electrode potential of -0.1 V. The imprinted film can detect BPA molecules in the entire concentration range of 2×10^{-8} to 2×10^{-6} M, showing a linear increase in ΔR_p in proportion to log concentration. The detection limit is estimated to be less than 1×10^{-8} M (at ppb). The selective detection for BPA can be achieved by a synergic effect of the host-guest interaction with $\beta\text{-CD}$ molecules and the presence of an imprinted cavity.

4. Conclusion

For the purpose of the sensitive detection of aromatic compounds, a novel electrochemical method based on cyclic surface-polarization impedance (cSPI) was combined with a molecular imprinting technique. The prepared imprinted sites have multiple recognition features: the inclusion capability and imprinting effect by templated $\beta\text{-CD}$ molecules on titania matrices. These two different binding features can be mutually supplemented in one binding site, thus leading to the formation of highly efficient binding sites. In particular, the current system was efficient for the detection of isolated protons from BPA and guest molecules could be detected with high sensitivity and selectivity. The imprinting approach using $\beta\text{-CD}$ units and TiO_2 gel layer has a much higher potential for practical applications than that using self-assembled monolayers. This simple concept for receptor design would be effective for the sensitive detection of various organic molecules including environmental pollutants.

Acknowledgments

This work was supported by MEXT via the Kitakyushu Knowledge-based Cluster Project. We are very grateful to Dr. K. Hayama, Prof. K. Hayashi, and Prof. K. Toko for their technical support with cSPI measurements.

References

- 1 M.-J. Ju, K. Hayashi and K. Toko: *Sens. Mater.* **13** (2001) 169.
- 2 M.-J. Ju, K. Hayama, K. Hayashi and K. Toko: *Sens. Actuators B* **89** (2003) 150.
- 3 K. Masunaga, K. Hayama, T. Onodera, K. Hayashi, N. Miura, K. Matusumoto and K. Toko: *Sens. Actuators B* **108** (2005) 427.
- 4 G. J. Brug, A.L.G. Van Den Eeden, M. Sluyters-Rehbach and J. H. Sluyters: *J. Electroanal. Chem.* **176** (1984) 275.
- 5 U. Rammelt and G. Reinhard: *Electrochim. Acta* **35** (1990) 1045.
- 6 Z. Kerner and T. Pajkossy: *Electrochim. Acta* **46** (2000) 207.
- 7 T. Pajkossy: *Solid State Ion* **94** (1997) 123.
- 8 G. Wulff: *Angew. Chem. Int. Ed. Engl.* **34** (1995) 1812.
- 9 B. Sellergren: *Molecularly imprinted polymers: Man-made mimics of antibodies and their applications in analytical chemistry* (Elsevier, Amsterdam, 2001).
- 10 S.-W. Lee, I. Ichinose and T. Kunitake: *Langmuir* **14** (1998) 2857.
- 11 W. Jones and C.N.R. Rao: *Supramolecular Organization and Materials Design* (Cambridge University Press, Cambridge, 2002, pp. 172–213).
- 12 T. Kunitake and S.-W. Lee: *Anal. Chim. Acta* **504** (2004) 1.
- 13 S.-W. Lee, D.-H. Yang and T. Kunitake: *Sens. Actuators B* **104** (2005) 35.
- 14 D.-H. Yang, S.-W. Lee and T. Kunitake: *Chem. Lett.* **34** (2005) 1686.
- 15 M. Lahav, A. B. Kharitonov, O. Katz, T. Kunitake and I. Willner: *Anal. Chem.* **73** (2001) 720.
- 16 M. Lahav, A. B. Kharitonov and I. Willner: *Chem. Eur. J.* **7** (2001) 3992.
- 17 S. P. Pogorelova, A. B. Kharitonov, I. Willner, C. N. Sukenik, H. Pizem and T. Bayer: *Anal. Chim. Acta* **504** (2004) 113.
- 18 D.-H. Yang, M.-J. Ju, A. Maeda, K. Hayashi, K. Toko, S.-W. Lee and T. Kunitake: *Biosens. Bioelectron.* **22** (2006) 388.
- 19 M.-J. Ju, D.-H. Yang, N. Takahara, K. Hayashi, K. Toko, S.-W. Lee and T. Kunitake: *Chem. Comm.* (2007) 2630.
- 20 I. Ichinose, T. Kawakami and T. Kunitake: *Adv. Mater.* **10** (1998) 535.
- 21 A. V. Krishnam, P. Starhis, S. F. Permush, L. Tokes and D. Ferdman: *Endocrinology* **132** (1993) 2279.
- 22 D. Feldman: *Endocrinology* **138** (1997) 1777.
- 23 F. S. Vom Saal, P. S. Cooke, D. L. Buchanan, P. Palanza, K. A. Thayer, S. C. Nagel, S. Parmigiani and W. V. Welshons: *Toxicol. Ind. Health.* **14** (1998) 239.
- 24 W. C. Hoyle and R. Budway: *Environ. Health Perspect.* **105** (1997) 570.
- 25 T. J. Murray, M. V. Maffini, A. A. Ucci, C. Sonnenschein and A. M. Soto: *Reprod. Toxicol.* **23** (2007) 383.
- 26 F. Montilla, F. Huerta, E. Morallon and J. L. Vazquez: *Electrochim. Acta* **45** (2000) 4271.
- 27 M. Saeyns, M. F. Reyniers, G. Marin and M. Neurock: *J. Phys. Chem. B* **106** (2002) 7489.
- 28 N. Saliba, J. Wang, B. A. Bansenauer and B. E. Koel: *Surface Sci.* **389** (1997) 147.
- 29 M. Singh, R. Sharma and U. C. Banerjee: *Biotech. Adv.* **20** (2002) 341.
- 30 A. Douhal: *Chem. Rev.* **104** (2004) 1966.