Application of a Functional Nanospace to Molecular Recognition

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Electrical and electrochemical techniques are very useful in the field of biosensing, owing to their simple handling as signal conversion units. It is necessary to develop a recognition unit for identifying a target substance with a high selectivity for the purpose of biosensing. From the viewpoint of molecular recognition, the author has studied the formation of a nanometer-sized space. This paper provides an overview of our research on biosensing using the nanometer-sized space functionally.

Introduction

Nanometer-sized spaces in a structurally regulated substance express reaction specificity. The function of a substance depends on not only its material, but also its shape, size, and placement in space. Therefore, an attention has been focused on the expression of new characteristics in the nanometer-sized space.

The author has studied the development of molecular imprinting techniques,1) which artificially form a shape-complementary cavity of the target molecule by transferring the molecular structure to a matrix (e.g., a self-assembled monolayer (SAM), conducting polymers).2–5)

Metal nanoparticles are known to express a variety of specific properties that are not found in bulk metals, such as localized surface plasmon resonance (LSPR), melting point lowering, and catalytic activities. In particular, gold nanoparticles (AuNPs) are useful for colorimetric analysis, optical labeling, and electrochemistry owing to their excellent chemical stability.6) The monodisperse AuNPs absorb a visible light in the green region of the spectrum and reflect red light (600–750 nm). Thus, the nanoparticles exhibit a reddish color. As the AuNPs aggregate, the LSPR absorption is increasingly red-shifted and eventually moves to the infrared region. Accordingly, the aggregated AuNPs show the optical properties of the bulk gold. A space exists between adjacent AuNPs in the aggregate (grain boundaries) due to the protective layer of the AuNP. Therefore, the electrical properties of the aggregation depend on the electrical conductivity and molecular size of the protective layer. It is expected that the formation of molecularly bridged AuNPs can be applied to biosensing.

This review describes the formation of the functional nanospace using these materials and their applications to molecular recognition.
Molecularly Imprinted Electrode

The “lock and key” metaphor has been explored and developed, and it has been applied to explain the mechanisms of antibiotics, enzymes, and molecularly imprinted polymers (MIP). The formation of a complementary-shaped cavity to a target essentially relies on the binding and interaction between constituents and targets (Fig. 1). Therefore, an excellent molecular recognition is provided by a complementary-shaped cavity formed in a matrix with interaction and binding sites.

Conducting polymers are also useful materials for the formation of molecular recognition sites using a template molecule as a dopant. The electrochemical properties of polypyrrole (PPy) depend strongly on the doping level and redox states. The overoxidized PPy (OPPy) at applying positive potentials has often been regarded as part of an undesirable degradation process, which led to dedoping of anionic species by a disappearance of electrostatic interactions and curing the film. Nagaoka’s group has developed a molecular imprinting technique using this method. A PPy film was deposited electrochemically onto an electrode in an aqueous solution containing pyrrole monomer and the target molecule as a dopant anion. Potential cycling was then used to overoxidize PPy. The overoxidation of PPy promoted the removal of dopants with a disappearance of the cation on the polymer backbone and led to curing of the film. This feature of PPy made it convenient to extract templates from the film and to form their complementary cavities on the OPPy film. The overoxidation process of PPy doped with a taurocholic acid (TC) was characterized using cyclic voltammetry and an electrochemical quartz crystal microbalance (EQCM) technique, as shown in Fig. 2.

![Fig. 1. Schematic illustration of a preparation procedure for a MIP.](image1)

![Fig. 2. Time profiles of the EQCM characteristics for overoxidation of a TC-doped PPy film during cyclic voltammetric activation for (a) the current, (b) the resonance frequency, and (c) the resonance resistance. The arrows in the figure indicate the reversal of the potential sweep direction. “Reprinted with permission from Ref. 17 (Copyright 2012, The Japan Society for Analytical Chemistry)”](image2)
Fig. 2a clearly shows a broad anodic peak at +0.95 V in the first positive sweep of the cyclic voltammogram of the PPy film, showing the typical irreversible response reported for a PPy film in an aqueous alkali solution. The increase in resonance frequency of 2.7 kHz, which was observed through two steps, corresponded to the extraction of 4.9 nmol of TC from the film during the dedoping process in Fig. 2b. The slight increase in the frequency at 190 s was observed without a decrease in the resonance resistance, and then we observed a drastically increase in the frequency at 230 s with a decrease in the resistance (Fig. 2c). The first and second steps in frequency are associated with the PPy oxidation process at 190 s (1.4 nmol) and at 230 s (3.5 nmol), respectively. It is noteworthy that the major frequency and resistance changes occurred simultaneously after the completion of the faradaic overoxidation process. The first step was caused by the electrostatic dedoping from the disappearance of the positive charge in the polymer backbone, whereas the second step was triggered by a physical factor due to film curing. Therefore, it is estimated that $10^{16}$ cavities cm$^{-2}$ were generated on the OPPy film. This technique, which provides excellent molecular-recognition, is very useful when the target fulfills the role of a dopant. We have successfully formed the various types of cavities of any size and shape. This method is not only useful for biomolecules (e.g., amino acids, peptides, adenosine triphosphate) but can also be applied to microorganisms.

We have adopted a more classical and direct approach to immobilize bacteria into polymer films (Fig. 3). PPy was doped with anions during the polymerization, and the insertion of bacteria was facilitated by the outer cell membranes that contained abundant negatively charged lipids, such as the lipopolysaccharides found in gram-negative bacteria. Zeta potentials of −20 to −40 mV were observed for *P. aeruginosa* and *E. coli*, and this provides experimental evidence of the dominance of the anionic residue on the cell surfaces.

The specific identification of bacteria has been achieved through precisely transferred bacterial structures onto the surface of OPPy films on a quartz crystal. The recognition of target bacteria was successfully performed in real time using a bacteria cell-imprinted OPPy film in combination with dielectrophoresis. The unique combination of both techniques made the specific detection possible at $10^5$ cells mL$^{-1}$. The observation of the movement of bacteria by using a fluorescent microscope revealed that living bacteria were being trapped vertically in the cavity created in the OPPy film. Further, the...
cavities had high selectivity and were able to discriminate between particular target bacteria.

The self-assembled monolayer (SAM) created through the Au-S binding is the simplest technique for the formation of the cavity on an electrode. To perform this procedure, a planar gold electrode was immersed in an ethanol solution containing cholesterol as a template and stearylmercaptan as a matrix (Fig. 4Aa).2,15,16

Fig. 4 (A) Model illustrations of (a) the formation of the SAM intermixed with C18SH and cholesterol on a gold substrate, and (b) the detection of cholesterol by the discharge of the redox marker. (B) Voltammograms for (a) a bare gold, (b) SAM-modified, and (c–d) molecularly imprinted SAM electrodes before (c) and after (d) immersion in an ethanolic 3 mM cholesterol solution. (b) Dependence of ΔI on the cholesterol concentration. Dependence of (c) the sensitivity and (d) the selectivity of the molecularly imprinted SAM on the number of carbon atoms in the alkanethiols. The selectivity was calculated using Eq. 2. “Reprinted with permission from Ref. 17 (Copyright 2012, The Japan Society for Analytical Chemistry)”

Voltammograms from each step of the preparation and sensing are shown in Fig. 4Ba. Cholesterol molecule is usually handled as an electroinactive species, and therefore the redox active species, ferrocyanide, is used as a redox marker for its electrochemical measurement (Fig. 4Ab). A negligible response was obtained using a modified gold electrode because its surface was completely enclosed by electroinactive species, namely stearylmercaptan and cholesterol, as represented by curve b in Fig. 4Ba. The SAM electrode was immersed in ethanol to extract the cholesterol. The extraction of cholesterol molecules restored the electrochemical activity of the SAM electrode. It also completed the creation of complementarily shaped cavities resulting from \textit{van der Waals} interactions between adjacent stearylmercaptan molecules in the tightly-packed monolayer, as represented by curve c. When there was no cholesterol molecule bound to the cavity, the redox marker diffused to the electrode surface ($I_0$). In contrast, when the cavity bound cholesterol, the redox marker did not diffuse to the electrode, and the signal ($I_r$) decreased in curve d. This response ($\Delta I$) was normalized using the following formula:

$$\Delta I = I_0 - I_r$$

where $I_0$ and $I_r$ are the oxidation peak currents of ferrocyanide at the molecularly imprinted SAM electrode before and after the recognition of cholesterol, respectively. The current is related to the cavity density for the mass-transport of the redox marker on the SAM.

Fig. 4Bb shows a plot of $\Delta I$ vs. cholesterol concentration, which exhibits a linear relationship with the coefficient of correlation at 0.9983. Moreover, this sensor had a rapid response, with a complete response time under 30 s. The length of the alkanethiol used for SAM formation was optimized for sensitivity and selectivity, as
shown in Figs. 4Bc and d, respectively.\textsuperscript{17} The selectivity ($S$) in Fig. 4Bd was calculated from:

$$S = \frac{\Delta I_{\text{cholesterol}}}{\Delta I_{\text{cholesterol}} + \Delta I_{\text{cholesterolacetate}}} \quad (2)$$

Although a greater number of carbon atoms increased the response ($\Delta I$), the highest response was obtained at around 16 carbon atoms, and $\Delta I$ decreased with an increasing number of carbon atoms greater than 18. Conversely, the SAM-modified electrode, which was prepared using shorter alkanethiols with fewer than 10 carbon atoms, represented the oxidation peak currents of ferrocyanide without extracting cholesterol molecules. This indicates that the formation of the SAM using shorter thiols allowed for the diffusion of a redox marker to the gold electrode surface because of the thickness and the low density of thiol molecules on the Au electrode. The selectivity increased with a greater number of carbon atoms, with a maximum selectivity obtained at around 18 carbon atoms. This is attributed to intermolecular van der Waals interactions, where longer alkyl chains are stronger than the shorter ones. On the other hand, the longer chain has a greater molecular length than cholesterol, may impose out of control for the extraction of cholesterol. This would keep the cholesterol in the SAM as a result of its larger size and stronger hydrophobic interactions, thereby decreasing the $\Delta I$ and selectivity.

**Nanogapped Electrodes**

The self-assembly technology is an effective bottom-up technique to assemble well-organized one- to three-dimensional structures, wherein the interparticle connections can be controlled at the single-particle level.\textsuperscript{18–21} From an application standpoint, metal nanoparticles are one of the most frequently studied inorganic materials because of their distinct combination of properties, which impart unique functionality to nanotechnology-based devices. We reported the synthesis of gold nanoparticle arrays prepared via a single-step procedure, wherein alkanethiols were self-assembled to deposit AuNPs on a plastic substrate, as shown in Fig. 5A.\textsuperscript{22,23}

Fig. 5 (A) SEM images of the AuNP array on a substrate using (a) propanethiol and (b) octanethiol as bridging molecules. (B) Dependence of the ln($R$) of the array on $n_c$. The inset represents a model of the gap ($d_{2n}$) formed between adjacent AuNPs. “Reprinted with permission from Ref. 23 (Copyright 2014, The Japan Society for Analytical Chemistry)”

SEM images of the AuNP-deposited substrate represented a uniformly covered surface without any overlap of AuNPs. These results indicate the formation of a single layer of AuNPs on the substrate. The logarithmic electrical resistivity ($R$) of the obtained AuNP layers clearly depends on the length of the gap between the AuNPs ($d_{2n}$) based on the alkyl chain, as shown in Fig. 5B. This phenomenon is considered to be the predominant mechanism of electron transport through a SAM in metal–insulator–metal (MIM) junctions.\textsuperscript{24–27} So far, several studies have concluded that the electrical properties of the MIM junction formed through a SAM between the electrodes can be determined by considering it as a parallel circuit composed of a resistor and a capacitor. Because each
AuNP is separated from the other AuNPs by alkanethiols, adjacent AuNPs electrically act as capacitors and resistors, as shown in Fig. 6.

Consequently, the equivalent \( Z_0 \) of the two AuNPs can also be expressed as a resistor \( R_0 \) and a capacitor \( C_0 \) connected in parallel, and, the entire network \( Z_{\text{total}} \) can be considered as a parallel circuit composed of a resistor and a capacitor:

\[
Z_{\text{total}} = \left( \frac{2m}{2n} \right) Z_0, \quad m, n: \text{positive integer} \quad (3)
\]

The proposed circuit scheme is substantiated by the Nyquist plot of a single hemisphere and the agreement with the DC measurements. Therefore, it is possible to discuss the electrical characteristics of the AuNPs layer formed on a substrate by considering a pair of AuNPs. The tunneling barrier dominates the electron transport in the AuNP–alkyl chain–AuNP junction, or in other terms, the resistivity, which decays exponentially with the distance according to the following equation:

\[
\ln\left(\frac{R_0}{R}\right) = -\beta d_{2n} \quad (4)
\]

where \( \beta \) is the decay constant, which reflects the strength of electronic coupling across a particular molecular bridge (tunneling coefficient), and \( d_{2n} \) is twice the length of alkanethiol \( (d_n) \) along the tunneling pathway, as shown in the inset of Fig. 5B. The slope of the plot of \( \ln(R) \) vs. \( d_{2n} \), which has a y-intercept of \( \ln(R_0) \), was found to be 8.51 nm\(^{-1}\), which is consistent with the value of \( \beta \) reported for tunneling through alkanethiols.\(^{24-26}\)

The AuNP array was modified with a single-stranded (ss) DNA probe, whose amount required for probe modification was estimated to be approximately 5.4 pmol using an electrochemical intercalator.\(^{28}\)

Once the resistance was stabilized, a tris-ethylenediaminetetraacetic acid buffer involving the sample ssDNA was added over the array for hybridization, as shown in Fig. 6. Upon sample addition, an immediate decrease of the resistance occurred with a signal-to-noise ratio above 40, followed by a steady state within 60 s. The
magnitude of the response depended on the number of mismatched base pairs (bp) in the double-stranded (ds) DNA. The largest response among the samples corresponded to the sample with the complementary strand (0.20 Ω). An increase in the number of mismatches led to a decrease in the magnitude (b–d), with the 11-bp mismatched DNA (d) showing the smallest response (0.050 Ω). It should be noted here that the resistance change was nonlinear with respect to the number of mismatches. A response was found in the complementary DNA concentration with a range of 5–100 µM (25–500 pmol). According to the results obtained from the impedance analysis, ionic migration has only minor effects on DNA sensing. Therefore, the resistance changes resulted from the conductivity of the dsDNA wire, which could be explained in terms of π overlap between adjacent base pairs. The presence of a mismatch would produce a defect in the electron transfer, which would cause a localization of electrons to reduce the electron-transfer rate. The molecular bridging that uses the nanogap makes it possible to respond to a change in the electrical characteristics because of the structural change, based on the DNA hybridization.

Conclusions

This review delineates the recent advancements in the sensing methods based on the arrangement of nanospaces. Using this approach, a molecular recognition device could be fabricated with electrodes spaced at the nanoscale. The nanoscale-designed space has enabled the detection of very small electrical signals, which could be used in highly sensitive and label-free sensing applications.

The techniques proposed in this study could further help in the realization of a high-density and high-throughput sensing platform for molecular sensors. We believe that the findings will hold the key to the future development of nano-bioscience and associated technologies. We anticipate the formation of a uniform array of nanospace and direct translation of the activity of a single molecule into an electrical signal. Some promising results have suggested that such arrangements and nano-spacing may be useful in optical applications.

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