Anal. Chem. **2007,** *79,* 5540−5546

Molecular Commonality Detection Using an Artificial Enzyme Membrane for in Situ One-Stop Biosurveillance

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Biodetection and biosensing have been developed based on the concept of sensitivity toward specific molecules. However, current demand may require more levelheaded or far-sighted methods, especially in the field of biological safety and security. In the fields of hygiene, public safety, and security including fighting bioterrorism, the detection of biological contaminants, e.g., microorganisms, spores, and viruses, is a constant challenge. However, there is as yet no sophisticated method of detecting such contaminants in situ without oversight. The authors focused their attention on diphosphoric acid anhydride, which is a structure common to all biological phosphoric substances. Interestingly, biological phosphoric substances are peculiar substances present in all living things and include many different substances, e.g., ATP, ADP, dNTP, pyrophosphate, and so forth, all of which have a diphosphoric acid anhydride structure. The authors took this common structure as the basis of their development of an artificial enzyme membrane with selectivity for the structure common to all biological phosphoric substances and studied the possibility of its application to in situ biosurveillance sensors. The artificial enzyme membrane-based amperometric biosensor developed by the authors can detect various biological phosphoric substances, because it has a comprehensive molecular selectivity for the structure of these biological phosphoric substances. This in situ detection method of the common diphosphoric acid anhydride structure brings a unique advantage to the fabrication of in situ biosurveillance sensors for monitoring biological contaminants, e.g., microorganism, spores, and viruses, without an oversight, even if they were transformed.

The social demand for biological safety and security is currently heightened in many different fields. In the fields of hygiene, public safety including fighting bioterrorism, the detection of biological contaminant, e.g., microorganisms, spores, and viruses, is important for maintaining safety and security.[1−3] Many biosurveillance techniques have been developed thus far. Sensor technology will further contribute to the implementation of effective biosurveillance. Hygienic applications are expanding explosively, for example, in food production and hazard analysis critical control point (HACCP),[4,5] clinical activities, clinical medicine,[6,7] good manufacturing practice (GMP),[8,9] and biological hazard security, including fighting bioterrorism. In conventional biosurveillance, the plate culture method has been employed. This method is appropriate for the detection of living microorganisms, but it is time-consuming. In other cases of biosurveillance methods, specific molecular markers are traced by mean of time-consuming ex situ methods. Specific proteins located on the surface of microorganisms, spores and viruses are a good markers that show up readily in affinity-based assays allowing the recognition of the species of contaminant.[1,10] However, affinity-based assays can detect only the single contaminant species for which they are specifically designed. They cannot detect all contaminant comprehensively. At the frontline of biosurveillance, an in situ monitoring method of detecting biological contaminants without oversight is very much required. Conventional methods target specific marker molecules. This type of strategy can be used to determine specific species of biological contaminants, but it cannot be used to perform comprehensive in situ surveys for a strict and continuous surveillance. In order to perform comprehensive biosurveillance and keep a strict watch on all biological contaminants without any oversight, the modification of the base concept is probably required.

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10.1021/ac0708902 CCC: $37.00 © 2007 American Chemical Society
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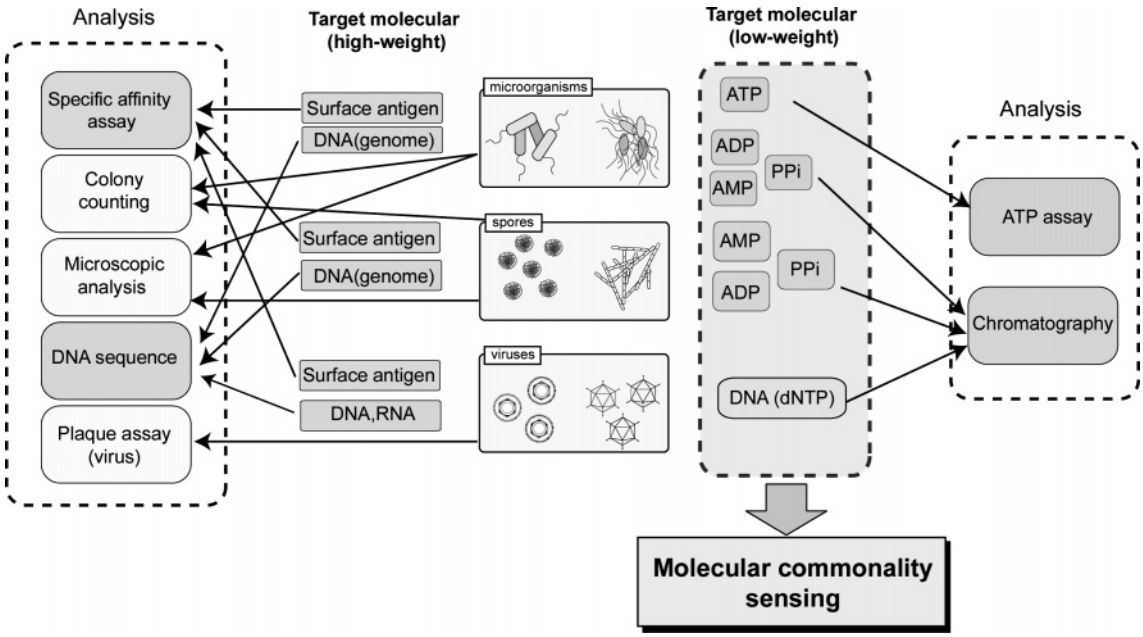

Figure 1. Hypothetical illustration of the concept of comprehensive contamination detection in biosurveillance.

The authors focused their attention on diphosphoric acid anhydride, which is a structure common to all biological phosphoric acids. Interestingly, biological nucleic acids are peculiar to all living things and include many different substances, e.g., ATP, ADP, dNTP, pyrophosphate, and so forth, all of which have a diphosphoric acid anhydride structure. In this study, substances that have diphosphoric acid anhydride structure are described as biological phosphoric substances. ATP has been employed as a marker in detection of living microorganisms.[11,12] However, any practical biosurveillance method must be able to detect all contaminants, e.g., living microorganisms (rich in ATP), dried microorganisms and spores (rich in ADP), and viruses (containing dNTP and diphosphoric acid), as detection target.

The authors took this common structure as the basis of their concept of comprehensive detection of biological contaminants in biosurveillance as illustrated hypothetically in Figure 1. To perform the comprehensive detection of contaminants, the authors have developed an artificial enzyme membrane with molecular selectivity for the structure common to all biological phosphoric substances comprehensively and studied the possibility of its application in in situ sensors for primary biosurveillance. This is the key concept of the present research, as selectivity for a structure common to many type of substances cannot be provided by natural enzymes.

In the present research, we attempted the design and synthesis of an artificial enzyme membrane that has selectivity for the diphosphoric acid anhydride structure common to molecules found in all biological organisms.

Pioneering work on the molecular design of artificial enzymes has focused on mimesis based on accurate organic synthesis.[13−25] Those early studies were oriented toward the design of both molecular selectivity and catalytic activity, but not toward specific practical tasks. There have been notable facts that still determine the design of artificial enzyme functions. For instance, Barr et al. employed Cu^{2+}−cyclodextrin complexes to induce the catalytic properties of metal and the binding characteristics of cyclodextrins.[25] These complexes catalyze the hydrolysis of phosphate triesters, and the rate of reaction has been improved spectacularly. Breslow et al. mimicked ribonuclease A in order to attach two imidazole rings to the primary face of β-cyclodextrin.[16] This mimetic enzyme specifically catalyzes the hydrolysis of a catechol cyclic phosphate carrying a 4-*tert*-butyl group. The key guideline for the design of the catalytic site of artificial enzymes has been set by these pioneering achievements.

In this study, we designed an artificial enzyme membrane with the intention of both providing selectivity for the structure common to all biological phosphoric substances and applying it in a biosensor. In our molecular design strategy, the Cu^{2+} complex cavity acts as the catalytic active site, which runs the catalytic hydrolysis reaction at the diphosphoric acid anhydride part of the substrate. The active center cavities are accumulated in the

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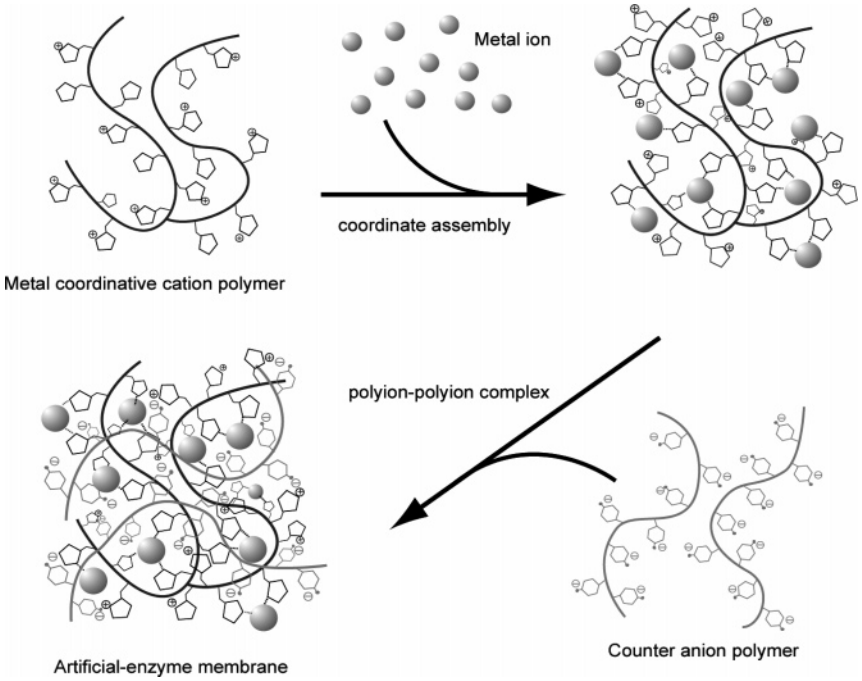

Figure 2. Schematic of the synthesis procedure of the artificial enzyme membrane through coordinative self-assembly.

membrane to ensure suitable catalytic activity in every unit section of the membrane matrix covering the electrode surface. The membrane is designed and synthesized through coordinative self-assembly among a metal coordinative cation polymer, Cu(II) ion, and a counteranion polymer. In this study, the potential characteristics of the artificial enzyme membrane were investigated kinetically and were used to construct a molecular commonality sensor for in situ biosurveillance with selectivity for molecule structure common to all biological phosphoric substances.

EXPERIMENTAL SECTION

Reagents. The water used in the present experiment was first deionized and passed through a Milli-Q water purification system from Millipore Co. (Bedford, MA). Poly(L-histidine) hydrochloride (molecular weight 6700) was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(sodium-4-styrenesulfonate) (molecular weight 70 000) was purchased from Aldrich Chemical Corp. (Milwaukee, WI). ATP, ADP, AMP, GTP, CTP, TTP, sodium tripolyphosphate, sodium pyrophosphate decahydrate, β-nicotinamide adenine dinucleotide disodium salt reduced form (NADH), flavin adenine dinucleotide disodium salt (FAD), Cu(II) chloride dihydrate, sodium acetate, ammonium dihydrogenphosphate, tetra-*n*-butylammonium hydroxide, and methanol (HPLC grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium tetrapolyphosphate was obtained from Nacalai Tesque (Kyoto, Japan). HEPES, MES, and CHES for the buffer solutions were purchased from Dojindo (Kumamoto, Japan). Cu(II) mesotetra(4-sulfonatophenyl)porphine was purchased from Frontier Scientific, Inc. (Logan, UT).

Synthesis of the Artificial Enzyme Membrane. The artificial enzyme membrane is a kind of polymer metal complex, but the molecular design is our original invention. It consists of two polymers and one metal ion.

For the present molecular design of the artificial enzyme, 20 μmol (monomer units) of poly(L-histidine) hydrochloride was dissolved into 10 mL of HEPES buffer (10 mM, pH 7.4). Then, Cu(II) ions were added to the solution to a final concentration of 10 mM and allowed to react for 1 h to form polyhistidine$-Cu^{2+}$ complexes. After coordination, 20 μmol (monomer units) of poly(sodium-4-styrenesulfonate) was added to the coordinative mixture solution and was allowed to form polyion complexes with the shrunken polyhistidine$-Cu^{2+}$ complexes. The synthesized artificial enzyme was collected by centrifugation and was repeatedly washed with HEPES buffer (10 mM, pH 7.4) in order to remove the free Cu(II) ions. Finally, the collected artificial enzyme was dissolved into 1.0 mL of HEPES buffer (10 mM, pH 7.4).

Spectral Analysis. The amount of Cu(II) ion in the artificial enzyme membrane was determined using ICP optical emission spectroscopy (Perkin-Elmer, Optima 4300DV Cyclon, Wellesley, MA). An organic elementary analyzer (Yanaco MT-5, Anatech Yanaco Corp., Kyoto, Japan) was employed to determine the amount of C, N, and H in the artificial enzyme membrane. The ESR spectra were recorded on an ESR spectrometer (Jasco, Tokyo, Japan).

The Raman spectra were acquired at an excitation of 514.5 nm under radiation from an argon ion laser (GLG2162, NEC) and were recorded on an NRS-2000 Raman spectrometer with a liquid nitrogen-cooled CCD detector (Jasco). The wave numbers of the Raman bands were calibrated with the spectrum of a silicon wafer and were reproducible to within 1.0 cm^{-1}. All spectra were recorded at room temperature (25 °C).

HPLC Analysis. The product of dephosphorylation of biological phosphoric substances effected by the artificial enzyme was analyzed by means of HPLC (Hitachi) on a Wakosil-II 3C18 RS column (10 cm × 4.6 mm, i.d. 3 μm; Wako, Osaka, Japan). The reaction mixture was prepared by adding the artificial enzyme and an adenosine phosphate (ATP, ADP, or AMP) to the buffer solution, and the reaction was initiated at that time. After the reaction, the sample was centrifuged, and the supernatant was

analyzed on the HPLC system. The mobile phase was composed of 20% methanol in water containing 20 mM ammonium dihydrogenphosphate and 5 mM tetra-*n*-butylammonium hydroxide. This mobile phase was pumped at a rate of 1.0 mL min^{-1}. The separated sample was detected with a UV detector at 260 nm. The reaction and separation were performed at room temperature (25 °C).

Electrochemical Evaluation. The electrochemical evaluation of the biological phosphoric substances was performed using three-electrode systems with an Ag/AgCl reference electrode, a Pt counter electrode, and a sensor device. The sensor device was fabricated by coating the glassy carbon electrode surface (3 mm in diameter, BAS, Tokyo, Japan) with an artificial enzyme. The electrochemical potential application and current recording were operated by a computerized electrochemical analyzer (HZV-100, Hokutodenko, Tokyo, Japan). For the electrochemical analysis, HEPES buffer (0.1 M, pH 7.4, containing 0.1 M KCl) was used as the electrolyte solution. Cyclic voltammograms of the artificial enzyme membrane-coated electrode were performed at a potential sweep speed of 10 mV s^{-1}. Sensor response was obtained and recorded at a constant applied potential of −250 mV versus Ag/AgCl.

RESULTS AND DISCUSSION

Design and Synthesis of the Artificial Enzyme Membrane. In order to obtain selectivity of molecular commonality for the common diphosphoric acid anhydride structure, the authors focused on the catalytic activity of Cu(II) complexes.[26−30] Cu(II) complexes were obtained through our peculiar process of coordinative self-assembly. The coordinatively self-assembled structure is stabilized by the formation of a polyion−polyion complex with a countercharged polymer, as illustrated in the hypothetical synthesis scheme of Figure 2.

Structural Information on the Artificial Enzyme Membrane. The synthesized artificial enzyme membrane was investigated by conventional means: element analysis and spectrum analysis through ESR and Raman spectroscopy. The Raman spectrogram indicated that the artificial enzyme was formed through coordinative self-assembly from a coordinative cation polymer (poly(L-histidine)) and Cu(II) ion. The Raman spectra of the artificial enzyme and two Cu(II) complexes, mesotetra(4-sulfonatophenyl) orphine, and deoxyglobin, are shown in Figure 3A.

The formation of a coordinative bond between the imidazole residue and the Cu(II) ion can be verified by comparing the three spectra. A peak of N−copper stretching was observed at 220 cm^{-1}, which is the characteristic Raman peak of the Cu^{2+}−histidine complex. Further structural information on the complex was obtained from the ESR spectrum, as shown in Figure 3B. The specific ESR parameters obtained were $g_{//} = 2.26$, $g = 2.06$, and

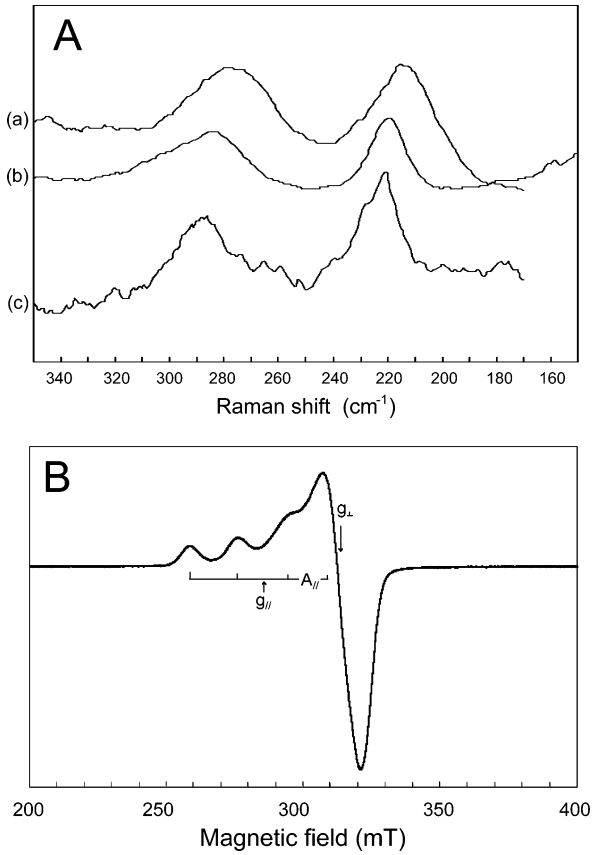

Figure 3. Spectrum investigation of the artificial enzyme structure. (A) Raman spectrum of the artificial enzyme. (a−c) show the Raman spectra of Cu(II) mesotetra(4-sulfonatophenyl)porphine, deoxyglobin, and the artificial enzyme, respectively. (B) ESR spectrum of the artificial enzyme.

$A_{//} = 17.0$ mT. Following Peisach and Blumberg,[31] the coordination position of the Cu^{2+} complex cavity was investigated. It is clear from the ESR spectrum that it is a type II Cu complex. It was verified that the coordinative position of the Cu^{2+} complex cavity was the square planar complexes of 4N or 2N2O, and structural distortion was hardly observed. The composition of the structural substances was estimated to be poly(L-histidine):Cu:poly(styrenesulfonate) = 5.1:1.0:4.3 by organic elementary analyzer and inductively coupled plasma analysis. The result strongly suggests that the artificial enzyme membrane had densely arranged Cu^{2+} complex cavities in its membrane matrix structure, which probably contributed to the prominent catalytic activity of each unit membrane area. This structure is shown in Figure 5A, which was modeled based on data from the spectrum investigation and element analysis.

Recognition of Molecular Commonality through Catalytic Dephosphorylation Activity on Biological Phosphoric Substances with the Artificial Enzyme Membrane. In order to recognize the common molecular structure, we designed the artificial enzyme membrane in such a way that it possesses a specific catalytic activity (not molecule selectivity). The catalytic activity of biological phosphoric substance dephosphorylation was investigated kinetically. We investigated the initial rate of ATP dephosphorylation by the artificial enzyme at pH 7.4 and 25 °C

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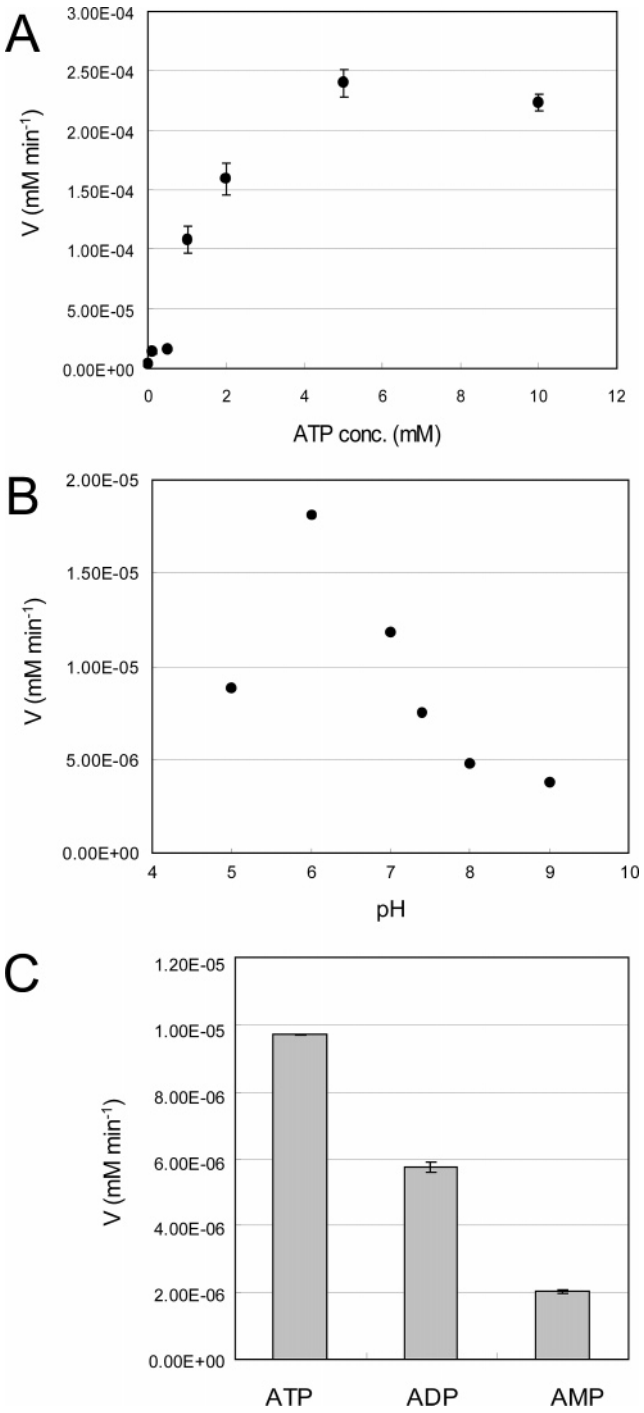


Figure 4. Catalytic dephosphorylation activity of the artificial enzyme membrane. (A) Initial velocity of ATP dephosphorylation by the artificial enzyme. (B) The pH dependence of the initial velocity of dephosphorylation of 100 μM ATP by the artificial enzyme. The reaction buffer was 0.1 M acetate buffer (pH 5.0), MES buffer (pH 6.0), HEPES buffer (pH 7.0, pH 7.4, pH 8.0), and CHES buffer (pH 9.0), respectively. (C) Initial velocity of dephosphorylation of each adenosine phosphate (100 μM; ATP, ADP, AMP) by the artificial enzyme. These initial velocities were calculated based on the HPLC analysis data.

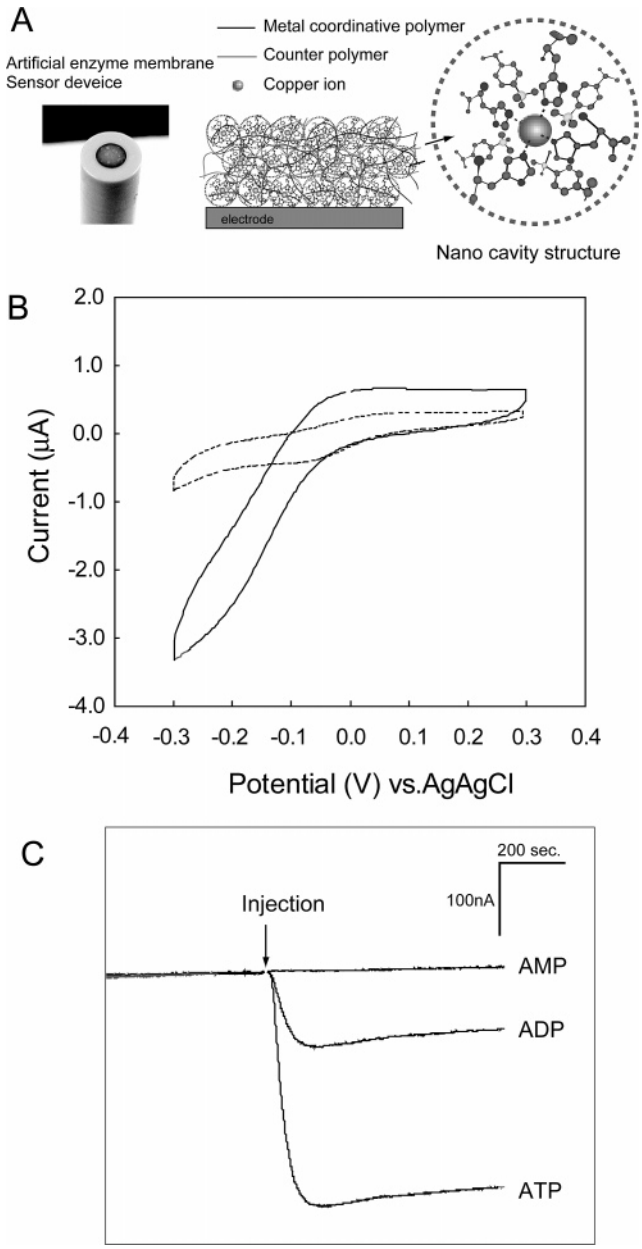
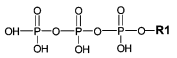
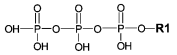
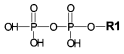
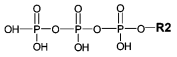
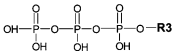
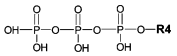
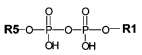
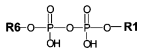
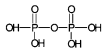
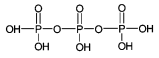
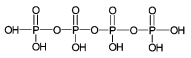


Figure 5. Structure and sensor responses of the artificial enzyme membrane-based sensor device. (A) Photograph of the artificial enzyme membrane-coated GC electrode (sensor device) and the structure model of the artificial enzyme membrane. The structure was modeled by a molecular structure modeling software (Molecular Operating Environment, MOE; Chemical Computing Group, Canada) based on data from the spectrum invest,igations and element analysis. (B) Typically, cyclic voltammograms of the sensor device in solutions with (solid line) or without (broken line) ATP. (C) Sensor response curves for adenosine phosphates (ATP, ADP, and AMP). The concentration of each phosphate is 100 μM. The sensor response was recorded at a constant potential of −250 mV vs Ag/AgCl.

by means of HPLC analysis (Figure 4A). It is clear that the artificial enzyme has a Michaelis−Menten-type catalytic activity in ATP (diphosphoric acid anhydride) hydrolysis. These kinetics can be described as the binding of the substrate ATP to the metal center in the nanocavity, to form an intermediate polymer−Cu^{2+}−ATP complex, followed by the dephosphorylation of the phosphate terminal of ATP. The kinetic parameters were estimated to be $V_{max} = 2.5 \times 10^{-4}$ mM min^{-1}, $K_m = 1.4$ mM, and $K_{cat} = 1.24 \times 10^{-5}$ s^{-1} (pH 7.4, 25 °C).

The pH of the reaction mixture has several possible effects on the structure and activity of natural enzymes. Therefore, the effect of the pH on the activity of the artificial enzyme was investigated by means of HPLC analysis. Figure 4B shows the

Table 1. Sensor Responses of the Artificial Enzyme-Based Sensor to Biological Phosphoric Substances

Biological phosphoric substances	Sensor Response (nA)	Number of phosphate	Structure[a]
ATP	291.3	3	
ATP (negative control)[b]	ND	3	
ADP	97.0	2	
AMP	– [c]	1	
GTP	476.2	3	
CTP	179.6	3	
TTP	206.9	3	
NADH	ND	2	
FAD	ND	2	
Monophosphate	ND	1	
Pyrophosphate	704.0	2	
Triphosphate	686.2	3	
Tetraphosphate	453.7	4	

[a] Concentration of each phosphoric substance is 100 μM (Final concentration). R1, adenosine; R2, guanosine; R3, cytidine; R4. thymidine; R5, nicotinamide ribose; R6, riboflavin. [b] Sensing by using polymer membrane (artificial enzyme without Cu^{2+} coordination) coated sensor device. [c] AMP can be measured by present sensor but in case of high concentration level.

initial velocity of ATP dephosphorylation catalyzed by the artificial enzyme membrane for each buffer pH. The pH was changed from 5.0 to 9.0 using 0.1 M acetate buffer, MES buffer, HEPES buffer, or CHES buffer. In general, hydrolysis is the predominant pathway, followed by OH^- as the nucleophile at high pH levels. Native enzymes have a pH optimum for their catalytic activity; this artificial enzyme showed its maximum catalytic activity at pH 6.0, just like a native enzyme.

Changes in the pH level may affect the shape of the artificial enzyme membrane or the charge properties of the substrate, so that either the substrate cannot bind to the active site or it cannot undergo dephosphorylation. The nanocavities of the artificial enzyme membrane were formed as a result of the electrostatic interaction between the cationic and the anionic polymer. Therefore, the polyion complex formed a tight structure at low-pH levels, and the intrananocavity acquired an increased hydrophobicity due to the functional polymer residue. The nanocavities were also reduced in low-pH solutions, because the coordination of the Cu(II) ion with poly(L-histidine) was reduced by the protonated imidazole ligand. Therefore, the substrates had difficulty penetrating into the nanocavities, and the phosphate terminals were not easily dephosphorylated by the OH^- in the nanocavities in low-pH solutions. In high-pH solutions, the electrostatic interactions between the anion and cation polymer were weakened by the nonprotonated imidazole residue in poly(L-histidine). As a result, the nanocavity structure of the artificial enzyme membrane was deformed and the catalytic activity decreased. In neutral solutions, the artificial enzyme showed a catalytic activity as high as that of a native enzyme. The artificial enzyme membrane in a pH 6 solution, which is the pK_a of the imidazole residue of poly(L-histidine), showed the highest catalytic ATP dephosphorylation activity. The nanocavity structure of the artificial enzyme membrane in the pH 6 solution was stable and the Cu(II) ion was able to coordinate with the OH^- in the nanocavity, which enabled the artificial enzyme membrane to show robust catalytic activity.

The artificial enzyme was designed and synthesized for the purpose of applying it to biosurveillance biosensing. Its molecular target is the structure common to all biological phosphoric substances. In the experiment, three different biological phosphoric substances were used in order to investigate the molecular selectivity of the artificial enzyme membrane for the biological phosphoric substances as well as its catalytic activity. The initial velocity of ATP, ADP, and AMP dephosphorylation catalyzed by the artificial enzyme membrane is shown in Figure 4C. The results show that the high-velocity catalytic activity was faster in the case of compounds that have more phosphate residues. This is due to the easy penetration of the phosphate terminal into the nanocavity (the active center of the artificial enzyme) in the case of longer phosphate residues. As indicated by the kinetic analysis, the artificial enzyme can catalyze the dephosphorylation of three

different biological phosphoric substances almost equally. This observation clearly indicates that the artificial enzyme membrane can be employed as the molecular recognition layer/molecular transduction layer of biosensors and that biosurveillance with a molecular commonality sensing for biological phosphoric substances can be performed as intended.

Molecular Commonality Sensor Based on the Recognition of Biological Phosphoric Substances by the Artificial Enzyme Membrane. Based on the artificial enzyme membrane, an amperometric biosensor was fabricated by way of trial, as schematically illustrated in Figure 5A. The artificial enzyme membrane in jelly form can be mounted easily on a glassy carbon electrode surface to form a molecular recognition layer/molecular transduction layer. The sensor device was preliminarily examined through electrochemical experimentation. It is very important to point out that the product of the catalytic reaction is PO_4^{3-}, which hardly exists in neutral-pH aqueous solutions because it is equilibrated immediately between the orthophosphate ion form of $H_2PO_4^-$ and HPO_4^{2-}. The two forms of orthophosphate ion are electrochemically stable and do not undergo a redox reaction under electrode potential. However, in the case of the artificial enzyme membrane-based biosensor, the reaction product, PO_4^{3-}, may accumulate between the artificial enzyme membrane layer and the electrode and can be reduced by applying electrode potential. This is clearly seen in the cyclic voltammograms of the sensor device (Figure 5B). Remarkably, a catalytic reduction current was observed in the cyclic voltammograms in the 1 mM ATP solution (Figure 5B, solid line). In contrast, no cathodic current response was observed in the absence of ATP (Figure 5B, broken line). The data indicate that the product of the artificial enzyme membrane was reduced electrochemically. With ADP, the current peak height reached two-thirds of that for ATP, because the ADP molecule has only diphosphates. It was clearly shown that the catalytic reaction of the artificial enzyme can be successfully coupled with the electrochemical reaction. In the case of the artificial enzyme-based amperometric biosurveillance sensor, sensor response can be obtained by applying a constant potential of −250 mV versus Ag/AgCl. Typical sensor responses show that the reduction current increases with the addition of adenosine phosphate (100 μM: AMP, ADP, ATP) as typical biological phosphoric substances (Figure 5C). The sensor can detect both ATP and ADP because the molecular selectivity for biological phosphoric substances, all of which have a diphosphoric acid anhydride, is comprehensive. The sensor responds to all biological phosphoric substances, but not to NADH and FAD that do not contain the phosphoric acid hydride structures with terminal group (Table 1). The amount of sensor response varies according to the number of phosphates and other structural factors. In the case of ATP, the biosurveillance sensor can determine the concentration within the nanomolar to millimolar range.

As shown in Table 1, the artificial enzyme membrane sensor provides molecular commonality sensing for all molecules that contain phosphoric acid hydride structures. This artificial enzyme-based assay is unique, in that it cannot be performed using natural enzymes. Such comprehensive molecular selectivity is not a typical for nature enzymes but this does not mean that the molecular selectivity of our artificial enzyme is too wide. The membrane form of the artificial enzyme reduces the risk of diffusion of interferences into the membrane. The molecular selectivity works within the limits of the common phosphoric acid anhydride structure.

CONCLUSION

Detection can be done in thick solutions such as serum-containing culture media. Another important point is the working potential. In the case of the artificial enzyme-based sensor, −250 mV versus Ag/AgCl is the working potential, and it is low enough to preclude most potential interferences. Both the membrane-based selectivity and the lower working potential ensure comprehensive selective sensing without disturbances.

This molecular commonality assay, which focuses on the structure common to all target molecules, represents a novel analytical concept. Especially in the case of biological safety and security applications, risk factors have to be recognized without exception. The present artificial enzyme membrane sensor can recognize living microorganisms (rich in ATP), dried microorganisms and spores (rich in ADP), and viruses (containing dNTP and diphosphoric acid), because it can detect most (such or these) biological phosphoric substances. In the trial application, the artificial enzyme-based sensor could detect microorganisms (*E. coli*). Then, we are trying detection of the contents from both spores and viruses by the sensor device. Other advantages of this artificial enzyme-based sensor are very stable for long storage (for over 3 months in dried form). The preserved sensor devise shows excellent coefficient of variation of within 3%. Therefore, the sensor matrix is very stable in its functions allowing a high level of reproducibility.

Taking advantage of the molecular commonality sensing using the artificial enzyme membrane, practical sensor devices can be developed for one-stop biosurveillance in the fields of food production, HACCP, clinical service, pharmaceutical development, GMP, and biological hazard security including fighting bioterrorism. Biological safety and security are crucial and fundamental matters for humanity.

ACKNOWLEDGMENT

This research was supported by the "Creation of Biodevices and Biosystems with Chemical and Biological Molecules for Medical Use" CREST, from the Japan Science and Technology Agency. We thank Dr. Toshiaki Kamachi for the spectral measurement of the artificial enzyme membrane using the ESR spectrometer. We also thank Mr. Shigeya Suzuki (Kikkoman Corp.) and his colleagues for the valuable information on hygiene investigation precedents.

Received for review May 1, 2007. Accepted June 3, 2007.

AC0708902