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Artificial-enzyme gel membrane based biosurveillance sensor with high reproducibility and long-term storage stability

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Summary

We propose that the most sophisticated strategy for primary biosurveillance is to exploit structural commonality through the detection of biologically relevant phosphoric substances. A novel assay, an artificial-enzyme membrane was designed and synthesized for sensor fabrication. This artificial-enzyme catalyzes the hydrolysis of the diphosphoric acid anhydride structure. This structure-selective, albeit not molecule-selective, catalytic hydrolysis was successfully coupled with amperometric detection. Since the catalytic reaction produces a dephosphorylation product (PO_4^{3-}), it can be reduced by an electrode potential of -250 mV vs. Ag/AgCl. Owing to the structural selectivity of the artificial-enzyme membrane, the sensor can detect biological phosphoric substances comprehensively that have the diphosphoric acid anhydride structure. The sensor successfully determined various biological phosphoric substances at concentrations in the micromolar (μM) to millimolar (mM) range, and it showed good functional stability and reproducibility in terms of sensor responses. This sensor was used to detect *Escherichia coli* lysed by heat treatment, and the response increased with increasing bacterial numbers. This unique technique for analyzing molecular commonality can be applied to the surveillance of biocontaminants, *e.g.*, microorganisms, spores and viruses. Artificial-enzyme-based detection is a novel strategy for practical biosurveillance in the front line.

Keywords

artificial-enzyme membrane, bio-surveillance, dephosphorylation, electrochemical detection, PO_4^{3-}, poly acrylamide

Introduction

Biosurveillance is a key way to have secure condition in the front line of the food industry, pharmaceutical industry, and social security. Undoubtedly, biosurveillance is becoming an important aspect of hygiene maintenance, *e.g.*, in food production processes,[1,2] clinical medicine,[3,4] and biological hazard security, as in fighting bioterrorism.[5-7] These different applications of biosurveillance should be satisfied by *in situ* monitoring methods based on detecting biological phosphoric substances (ATP, ADP, AMP, pyrophosphate, deoxyphosphate, etc.). In the case of ATP assay, a high-sensitivity bioluminescence assay based on the luciferin/luciferase enzymatic reaction is gaining popularity, since ATP can be employed as a detection marker for living microorganisms.[8-10] However, ATP cannot be detected in dead or quiescent organisms. Moreover, spores and viruses have little or no intracellular ATP.[11,12] Thus, an ATP-specific analysis is inadequate for a comprehensive, simultaneous survey of biocontaminants. For a biosurveillance sensor to detect simultaneously a large variety of biocontaminants (*e.g.,* live or quiescent microorganisms, spores, pollen, and viruses) and with sufficiently broad selectivity, it must be capable of detecting, in addition to ATP, many other biologically relevant phosphate-containing substances.

For biosurveillance applications in the above-mentioned fields, some biosensors have certain advantages, notably the Smart *In Situ* assay.[13,14] However, the enzyme exhibits

strict molecular selectivity. To detect multiple targets, selectivity for common structural elements is required. In addition, a practical biosurveillance sensor needs to have functional stability and reproducibility. To ensure the novel selectivity of enzyme, either a genetically engineered enzyme or artificial enzyme must be used. We chose to develop an artificial-enzyme membrane as part of a novel biosurveillance sensor. Artificial enzymes are advantageous in that selectivity and stability can be designed.[15-17] We designed, synthesized, and evaluated an artificial-enzyme membrane, a type of polymer metal matrix for use as a sensor application. The metal complex cavity is a key component that ensures catalytic activity.[16] Many enzymes contain metal complexes as active centers or catalysis-related components of the protein structure. In our artificial-enzyme membrane, the Cu(II) complex is employed as the active center for the catalytic hydrolysis of the diphosphoric acid anhydride structure.[16] The membrane form also acts as a comprehensive molecular selection layer for biological phosphoric substances. This type of membrane can be used to fabricate a sensor device on an electrode or other type of transducer.[16,17] Previously, we constructed an artificial-enzyme membrane that consisted of two different polymer chains and a coordinated metal ion. However, the efficacy of membrane formation was poor; its characteristic of swelling in solution was too flexible to have good reproducibility. Therefore, modifications to the molecular design of the membrane form were required.

To fabricate biosensors with good reproducibility, several research groups have developed methods for enzyme immobilization on an electrode.[18-24] Peteu *et al*. developed a glucose micro-biosensor with good reproducibility that consisted of immobilized glucose oxidase copolymerized with acrylamide.[24] This glucose sensor was drift-free and showed little deterioration of response over 72 hours.

In the present study, the structure of the artificial-enzyme membrane was stabilized by copolymerization with acrylamide. The artificial-enzyme membrane based sensor showed a good reproducibility in term of sensor responses, with the sensor performing duplicate assays without calibration. In the present study, this improved artificial-enzyme based sensor was tested for its applicability to biosurveillance.

Experimental

Reagents

Poly-L-histidine hydrochloride (MW 6,700) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Poly (sodium-4-styrene sulfonate) (MW 70,000) was purchased from Aldrich Chemical Corp. (Milwaukee, WI, USA). ATP, ADP, AMP, pyrophosphate, copper(II) chloride dihydrate, sodium acetate, 30 % (w/v) acrylamide solution, ammonium persulfate (APS) and *N,N,N′,N′*-tetramethyl-ethylenediamine (TEMED) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HEPES, MES, and CHES were purchased from Dojindo (Kumamoto, Japan). The water used was deionized and passed through a Milli-Q water purification system (Millipore Co. Bedford, MA, USA). The actual pH levels of the buffer solutions were determined with a pH Meter (F-22; Horiba, Kyoto, Japan). The percentages of the reagents given in this paper represent mass percent, unless otherwise stated.

Synthesis of the artificial-enzyme membrane

We designed and synthesized the artificial-enzyme membrane as follows: 20 μmol (monomer units) of poly-L-histidine hydrochloride were dissolved in 10 ml of HEPES buffer (10 mM, pH 7.4). Then, copper (II) ions were added to the solution to a final concentration of 10 mM, and the mixture was reacted for 1 hour to form

polyhistidine-Cu^{2+} complexes. After coordination, 20 μmol (monomer units) of poly (sodium-4-styrene sulfonate) were added to the coordinative mixture, and allowed to form polyion complexes with the shrunken polyhistidine-Cu^{2+} complexes. The synthesized artificial enzyme was centrifuged and washed repeatedly with HEPES buffer (10 mM, pH 7.4), to remove free copper (II) ions. Finally, the collected artificial enzyme was dissolved in 1.0 ml of HEPES buffer (10 mM, pH 7.4).

Preparation of a biological phosphate sensor device with the artificial-enzyme membrane

The synthesized artificial-enzyme membrane was mixed with an equivalent volume of 12% acrylamide solution (pH 7.4) with initiator (TEMED, 10% APS), and the mixture was polymerized in the space between two glass plates. The thickness of the membrane was maintained at 120 μm with a spacer. The synthesized artificial-enzyme membrane was immersed in 0.1 M HEPES buffer (Figure 1A). The artificial-enzyme membrane was attached to a glassy carbon electrode surface (3 mm in diameter; BAS, Tokyo, Japan) (Figure 1B), and dried at room temperature. The dry form of the artificial-enzyme membrane sensor can be stored at room temperature for more than 3 months. The sensor electrode was re-swollen in 0.1 M HEPES buffer that contained 0.1 M KCl (pH 7.4) for 1 hour before use. This glassy carbon disk electrode was coated

with the 6 % polyacrylamide-artificial-enzyme membrane gel sheet and used as the sensor electrode.

Electrochemical evaluation of biological phosphoric substances using the artificial-enzyme membrane biosensor

Electrochemical measurements of biological phosphoric substances were performed with a three-electrode system using the artificial-enzyme membrane coated on a glassy carbon electrode (sensor device), an Ag/AgCl electrode (reference electrode), and a Pt plate electrode (counter electrode). The substances were measured amperometrically at an applied potential of -250 mV vs. Ag/AgCl using an electrochemical analyzer (HZV-100; Hokutodenko, Tokyo, Japan). Cyclic voltammograms of the artificial-enzyme membrane-coated electrode were acquired at a potential sweep speed of 10 mV sec^{-1}. The buffer solution used for sensing was 0.1 M HEPES (pH 7.4) that contained 0.1 M KCl. *Escherichia coli* BL21 (DE3) was used as the model microorganism for biosurveillance with the artificial-enzyme membrane biosensor. *E. coli* were cultured in Luria-Bertani (LB) medium for 8 hours at 37^{o}C, and then collected by centrifugation. The bacteria were diluted in distilled water and pretreated by heating at 95^{o}C for 2 min, to release biological phosphoric substances from the cells.

Results and Discussion

The basic design of the artificial enzyme consisted of a metal coordinative polymer, copper ions, and counter polymer(s) with functional residues.[16] In the artificial-enzyme structure, numerous metal-coordinated nano-cavities were accumulated as the active site for catalytic activity. In previous study, the weak efficiency of membrane formation proved problematic for the design of an artificial enzyme, which had the characteristic of readily swelling in solution, which mean that it was too flexible to have good reproducibility.

To prepare an artificial-enzyme membrane with good reproducibility, the basic structure of the artificial enzyme was stabilized by copolymerization with acrylamide. The synthesized membrane was also expected to act as an effective molecular sieve for biological phosphoric substances to the sensor electrode owing to the hydrophobicity of the polymer. Achieving the correct thickness is also very important for artificial-enzyme membrane reproducibility. In the present study, copolymerization was performed between glass slabs to ensure the accuracy of the gap distance. Using the new molecular design and synthetic process, a stable artificial-enzyme membrane was prepared with good reproducibility aspects (Figure 1A). This artificial-enzyme membrane was employed in the fabrication of a biosurveillance sensor for detection of molecular commonality among biological phosphate substances. The artificial-enzyme membrane

was placed on a glassy carbon electrode surface, which was used as the sensor device (Figure 1B).

The ATP responses measured by artificial-enzyme membrane sensors with various concentrations of polyacrylamide in the membranes are shown in Figure 2. The sensor response current decreased according to the concentration of acrylamide, which ranged from 6% to 15%. The initial current response rate also decreased depending on the acrylamide concentration. These results indicate that diffusion of the substrate (ATP) to the electrode surface through artificial-enzyme membrane is controlled by the degree of acrylamide polymerization. The low-concentration (<3%) acrylamide gel membrane formed a weak membrane on the electrode surface, and it was not expected to act effectively as a molecular sieve, since the network structure of the membrane was loose. Based on the above considerations, an artificial-enzyme membrane that contained 6% acrylamide was chosen to detect biological phosphoric substance in the present study.

Figure 3A shows the cyclic voltammograms of the sensor device in HEPES buffer with or without pyrophosphate. A cathodic current peak was observed with the artificial-enzyme membrane sensor in the pyrophosphate solution. In contrast, no current peak was noted for the sensor in the pure buffer solution or with the acrylamide membrane electrode (without the artificial enzyme). It appears that dephosphorylation of pyrophosphate occurs on the electrode surface. This dephosphorylation produces

PO_4^{3-} at the interface between the artificial-enzyme membrane and the electrode surface. The cyclic voltammograms indicated that the PO_4^{3-} was reduced to HPO_3^{2-} by the electrode potential. Curves a, b, and c in Figure 3B show the cyclic voltammograms of the sensor in 10 mM solutions of AMP, ADP, and ATP, respectively. A cathodic current peak was observed at negative potential vs. Ag/AgCl for each solution of biological phosphoric substance. These cathodic current peaks increased with increases in the number of the phosphate groups. These results indicate that the response current of artificial enzyme based sensor is derived from electrochemical reduction of the dephosphorylation product (PO_4^{3-}).

In general, electrochemical measurements of phosphate ions are difficult, especially in aqueous solutions. In the present sensor device, the thin membrane of the artificial enzyme is carried out on the electrode surface as a molecular recognition and catalytically active site, and the hydrophobic nano-cavity in the artificial enzyme is formed by a metal ion and the functional polymer residue.[15-16] Thus, the dephosphorylation product (PO_4^{3-}) is accumulated in the hydrophobic nano-cavity at the artificial-enzyme membrane-electrode surface interface, and electrochemical evaluation of PO_4^{3-} is performed with high sensitively before the PO_4^{3-} is protonated in the solution.

The artificial-enzyme membrane exhibits a pH-dependence for the dephosphorylation

catalytic activity, with a maximal catalytic activity at neutral pH.[16] At high pH levels, the nano-cavity structure of the artificial-enzyme membrane is deformed because the artificial enzyme is composed of a polyion-ion complex. The electrostatic interactions between the anion and cation polymer are weakened by a non-protonated imidazole residue in poly-L-histidine (cation polymer) at high pH in solution. The catalytic activity is also reduced at low pH in solution, since the coordination of the copper ion with poly-L-histidine is reduced by the protonated imidazole ligand.[16] Therefore, we studied the sensor properties under different pH conditions. Figure 4 shows the sensor responses for 100 μM ATP at each pH level in solution. The sensor responses showed high sensitivity at neutral pH, and were less sensitive at high and low pH levels. These data resemble those obtained for the catalytic activity of the artificial enzyme.[16] Our present results strongly indicate that the sensor response is dependent upon the dephosphorylation catalytic activity of artificial-enzyme membrane.

Since biological entities, such as enzymes, antibodies, and receptors, have optimal temperatures for their activities, a biosensor that employs a biological element has to be used at the appropriate temperature. In addition, these biosensors require calibration for accurate sensing. In contrast, an artificial enzyme generally is not readily inactivated by changes in the reaction temperature. As shown in Figure 5, the sensor response increased linearly with the temperature of sensing. The sensor response also achieved

good reproducibility. In particular, the sensing data at 25^{o}C showed good reproducibility for the sensor responses, with a coefficient of variation that within 3.0%. In general, a biosensor for which the coefficient of variation of the sensor response exceeds 10% cannot be used for any practical application. Moreover, when the coefficient of variation of the sensor response is within 3%, there is no need to calibrate the sensor for each sensing event. The coefficient of variation for the sensor response of a general biosensor is typically within 10%, and the value for an excellent biosensor should be within 5%. Therefore, our results show that the artificial-enzyme biosensor can be utilized without calibration at every sensing event. Moreover, this sensor can detect a biological phosphoric substance (ATP) under severe conditions, *e.g*., at 50^{o}C or 10^{o}C. Thus, the present sensor is not subject to strict restrictions with respect to its operating temperature.

In general, a biosensor that uses an enzyme must be stored in a freezer or refrigerator, to prevent deactivation or degradation by proteases, heat, etc. Thus, the storage stability of artificial-enzyme membrane sensor was examined (Table 1). The dry form of artificial-enzyme membrane sensor was stored at room temperature for 1 week or for 3 months. All the sensors showed functional stability during long-term storage, and the sensor responses were within 99% after 3 months. This high-level stability can be attributed to the physical and chemical improvements brought about by

artificial-enzyme membrane copolymerization with acrylamide. As a result, the artificial-enzyme membrane was hardly denatured, and the membrane was not exfoliated from the electrode surface. This long-term storage stability also means that the sensor can be used without calibration in every sensing.

Using the sensor electrode, the dephosphorylation product (PO_4^{3-}) derived from the hydrolysis of ATP can be determined at an electrode potential of -250 mV vs. Ag/AgCl. The artificial-enzyme membrane sensor was used to determine ATP concentrations, and the obtained responses were linear in the 1 μM to 200 μM range (Figure 6). The sensor also gave responses to all biological phosphoric substances that have the diphosphoric acid anhydride structure, and the extent of the sensor response was dependent upon the number of phosphates. However, the sensor did not give responses to mono-phosphate, NADH, and FAD, which do not contain the phosphoric acid hydride structure with a terminal group.[16] Therefore, this sensor provides sensing of molecular commonality for all molecules that contain a phosphoric acid hydride structure with a terminal group, and it is expected to be applicable to practical one-stop biosurveillance.

We have devised a practical application in biosurveillance for the artificial-enzyme membrane sensor. In Figure 7, the present sensor detected *E. coli*, and the response increased with increasing bacterial numbers. The sensor can also be used to detect in stable fashion biological phosphoric substances in culture media, as the sensor matrix is

not digested by naturally occurring proteases. In addition, large contaminating substances (e.g. proteins, peptides, and large nucleic acids) in the culture medium are excluded by the molecular sieving property of the membrane matrix. In contrast, small molecules can reach the active center of artificial-enzyme membrane on the electrode. The detection limit of the sensor device for bacteria (*E. coli*) is 1.0 $x10^{-8}$ bacteria ml^{-1}, which is not sufficient for biosurveillance, although applied studies have not yet been completed. However, it can be expected that sensor devices of this type will be developed for biosurveillance in the fields of food production, HACCP, and clinical testing.

Conclusions

The present artificial-enzyme membrane based biosensor is a unique tool for next-generation biosensing technology. Most importantly, common structure selectivity (molecular commonality) is a novel concept in biocontaminants analysis. The comprehensive selectivity of the artificial enzyme based sensor may open new fields to sensor applications. Furthermore, artificial enzyme based biosensors have certain advantages over biosensors fabricated with native enzymes, such as resistance to protease digestion, high reproducibility (making calibration unnecessary), and long-term storage properties.

Our synthesized artificial-enzyme membrane demonstrates comprehensive molecular selectivity for biological phosphoric substances. The catalytic activity of the artificial enzyme is coupled to amperometric detection on an electrode surface. This artificial-enzyme membrane based sensor device can be used to detect biological phosphates at the micromolar level of sensitivity. The greatest advantages of this sensor are that it can be stored at room temperature for a long time (3 months) without loss of activity, and that it does not need to be calibrated because the sensor matrix is functionally stable and shows excellent reproducibility of the sensor response (coefficient of variation within 3%). Therefore, this biosensor may be useful in different settings, *e.g.*, biotrace sensing can be conducted accurately to detect all biological

phosphoric substances.

Acknowledgment

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Table 1 Storage stability of the artificial-enzyme membrane sensor. All the data for the coefficient of variation values are within 3%. The preservation terms are: 1) after synthesis (0 day); 2) 1week (7 days); and 3) 3 month (90 days).

Time	Existence of sensor response [%]
0 day	100.00
1 week	97.86
3 month	99.43

Figure legends

Figure 1. Construction of artificial-enzyme membrane biosensor. (A) The artificial-enzyme membrane contains 6 % polyacrylamide. The thickness of the membrane is 120 μm. (B) Artificial-enzyme membrane biosensor. The membrane was immobilized by sticking on, drying, and re-swelling processes.

Figure 2. ATP responses measured using a sensor device coated with different concentrations of polyacrylamide in the sensor matrix. The thickness of each sensor matrix is 120 μm. ATP was added at the time-point indicated by the arrow, and the final concentration of ATP in the solution was 100 μM. The polyacrylamide concentrations (% by weight) are: a,15%; b,12%; c, 9%; d, 6%; and e, 3% respectively.

Figure 3. Cyclic voltammograms of phosphate in 0.1 M HEPES buffer (pH 7.4) at a sweep rate of 50 mV/sec. (A) a, 10 mM Pyrophosphate evaluated by the polyacrylamide membrane (no catalyst) cording sensor; b, HEPES buffer (0 mM pyrophosphate) evaluated by artificial-enzyme membrane sensor; c, 10 mM pyrophosphate evaluated by artificial-enzyme membrane sensor. (B) Each biological phosphate (10 mM) was evaluated by the artificial-enzyme cording sensor. a, AMP; b, ADP; c,ATP.

Figure 4. The pH-dependence of artificial-enzyme membrane sensor response. The pH levels were set using the following buffers (0.1 M): Acetate buffer (pH 5.0); MES buffer (pH 6.0, pH 6.5); HEPES buffer (pH 7.0, pH 7.4, pH 8.0); and CHES buffer (pH 9.0).

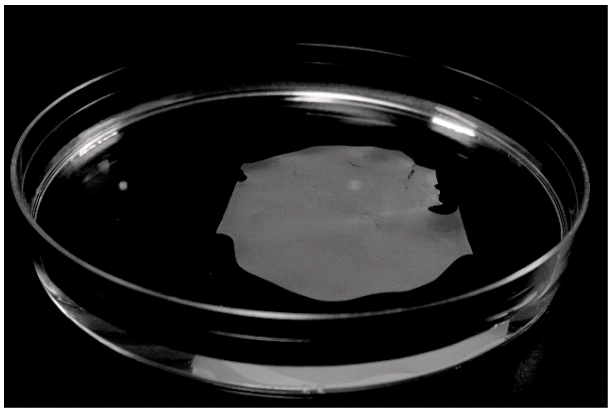
Figure 5. Temperature-dependence of ATP detection by the artificial-enzyme membrane sensor.

Figure 6. Electrochemical evaluation of the ATP concentration in 0.1 M HEPES buffer (pH 7.4) by the artificial-enzyme membrane sensor.

Figure 7. Detection of *E. coli* using an artificial-enzyme membrane sensor. The arrow indicates the time of addition of the bacterial sample to the sensing system. a, 1.5×10^9 bacteria ml^{-1} a detected using a bare electrode (negative control); b, 1.5×10^9 bacteria ml^{-1} detected by the artificial-enzyme membrane sensor; and c, 3.0×10^9 bacteria ml^{-1} detected by the artificial-enzyme membrane sensor.

1

(A)



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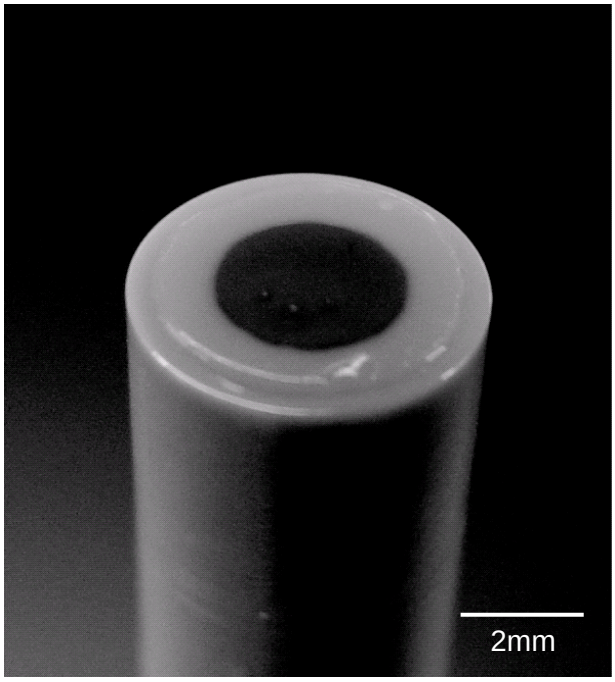
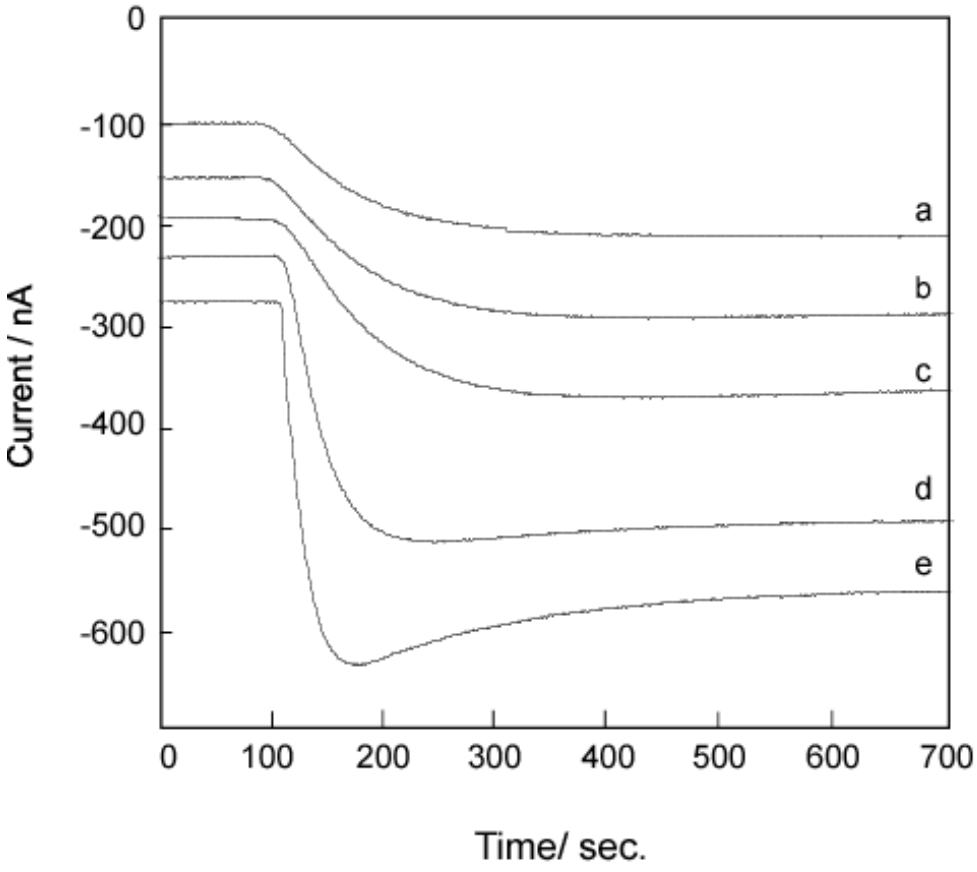


Figure 1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-200
-300
-400
-500
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b
c
d
e
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100
200
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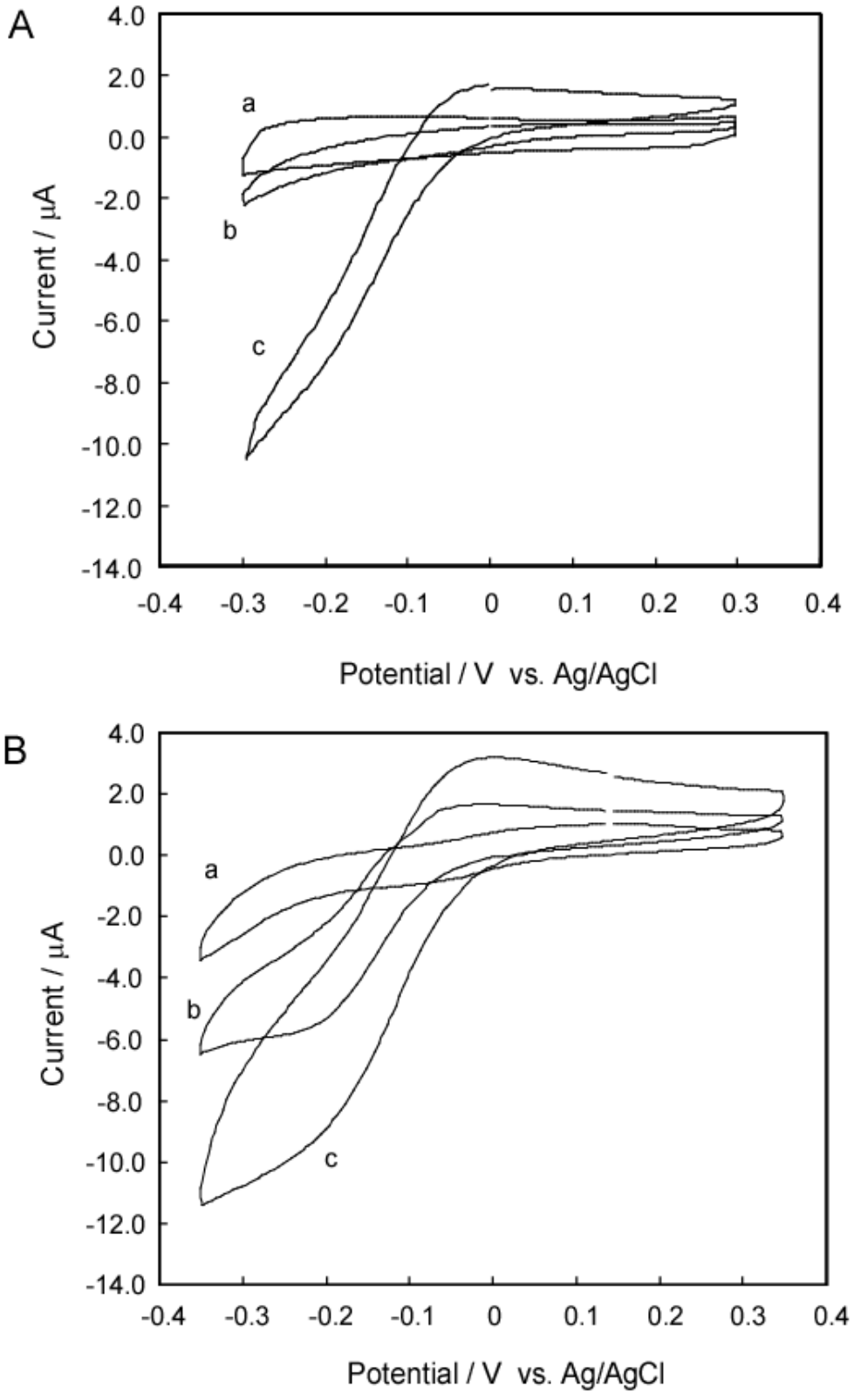
Time/ sec.

Figure 2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Current / µA
a
b
c
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-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
Potential / V vs. Ag/AgCl
B
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
Current / µA
a
b
c
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Potential / V vs. Ag/AgCl

Figure 3

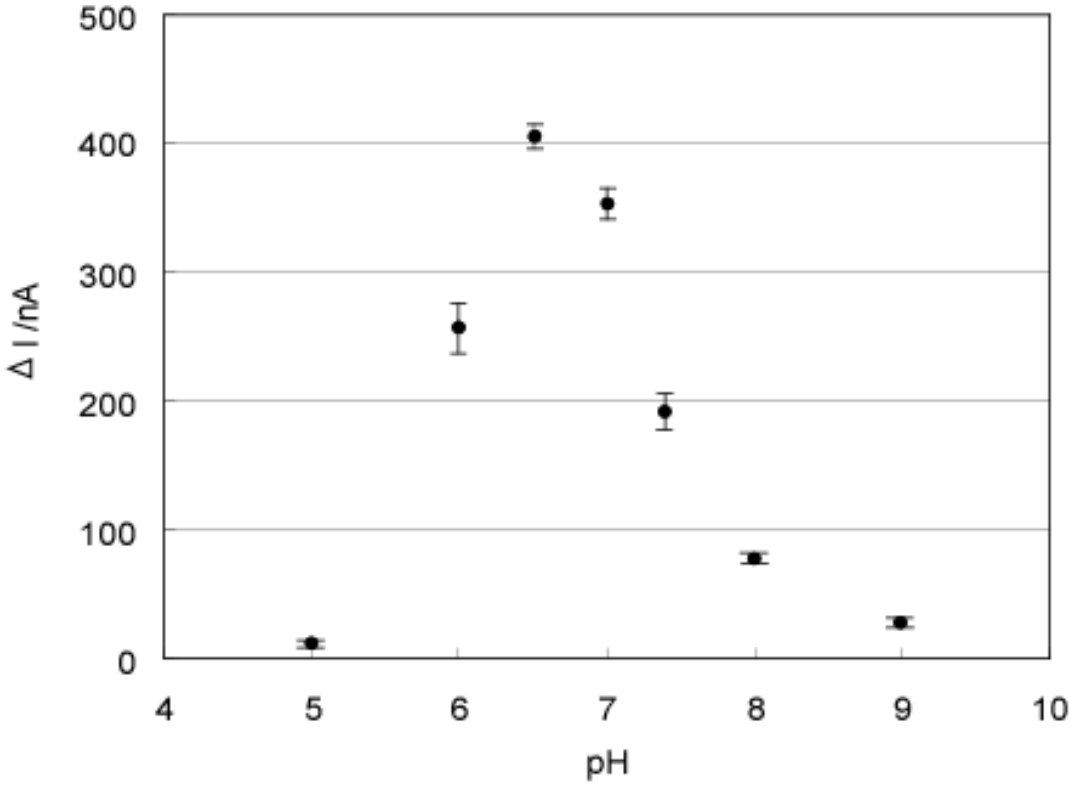


Figure 4

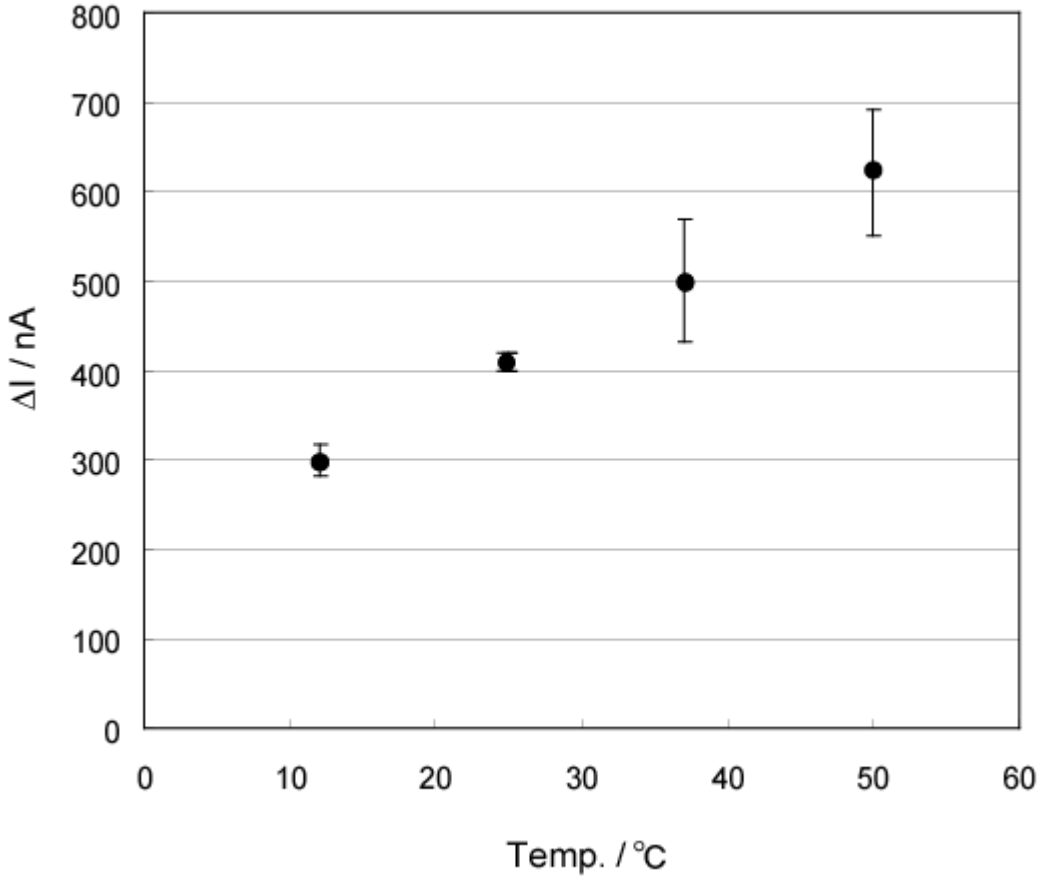
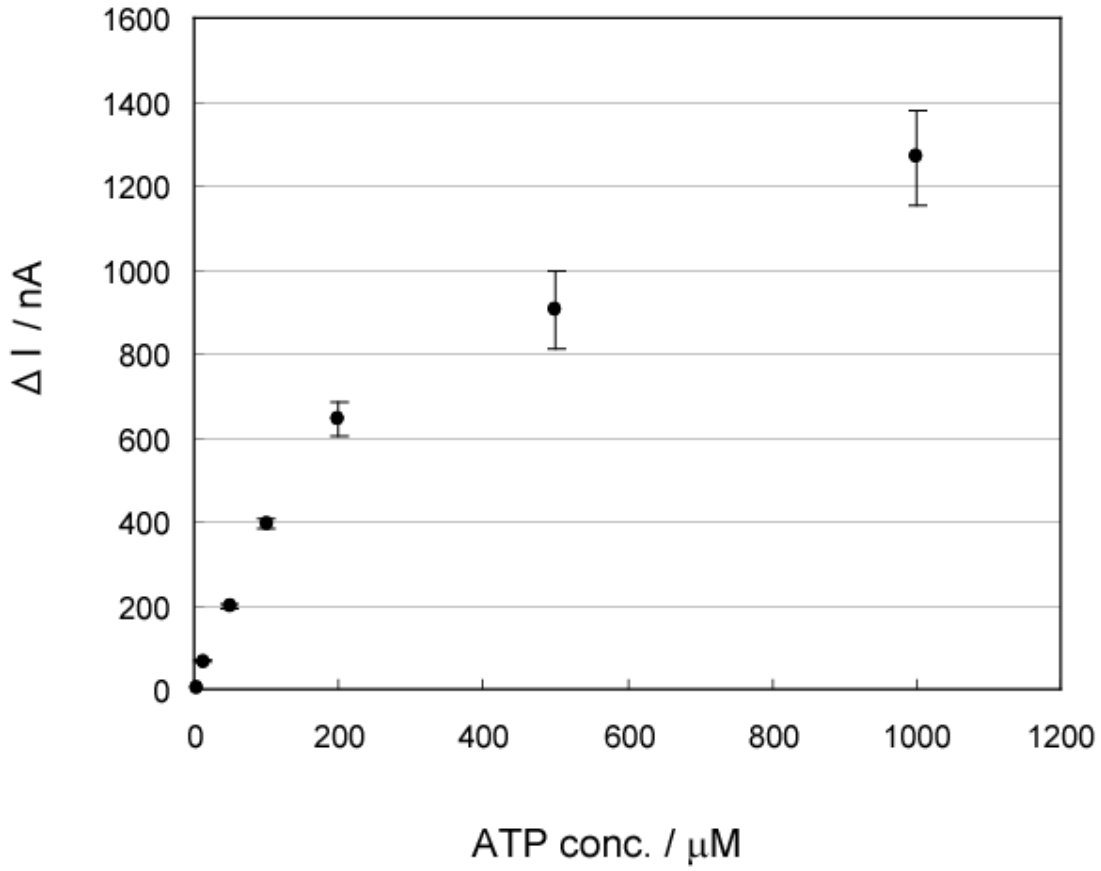


Figure 5


1600
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Δ I / nA
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200
400
600
800
1000
1200
ATP conc. / μM

Figure 6

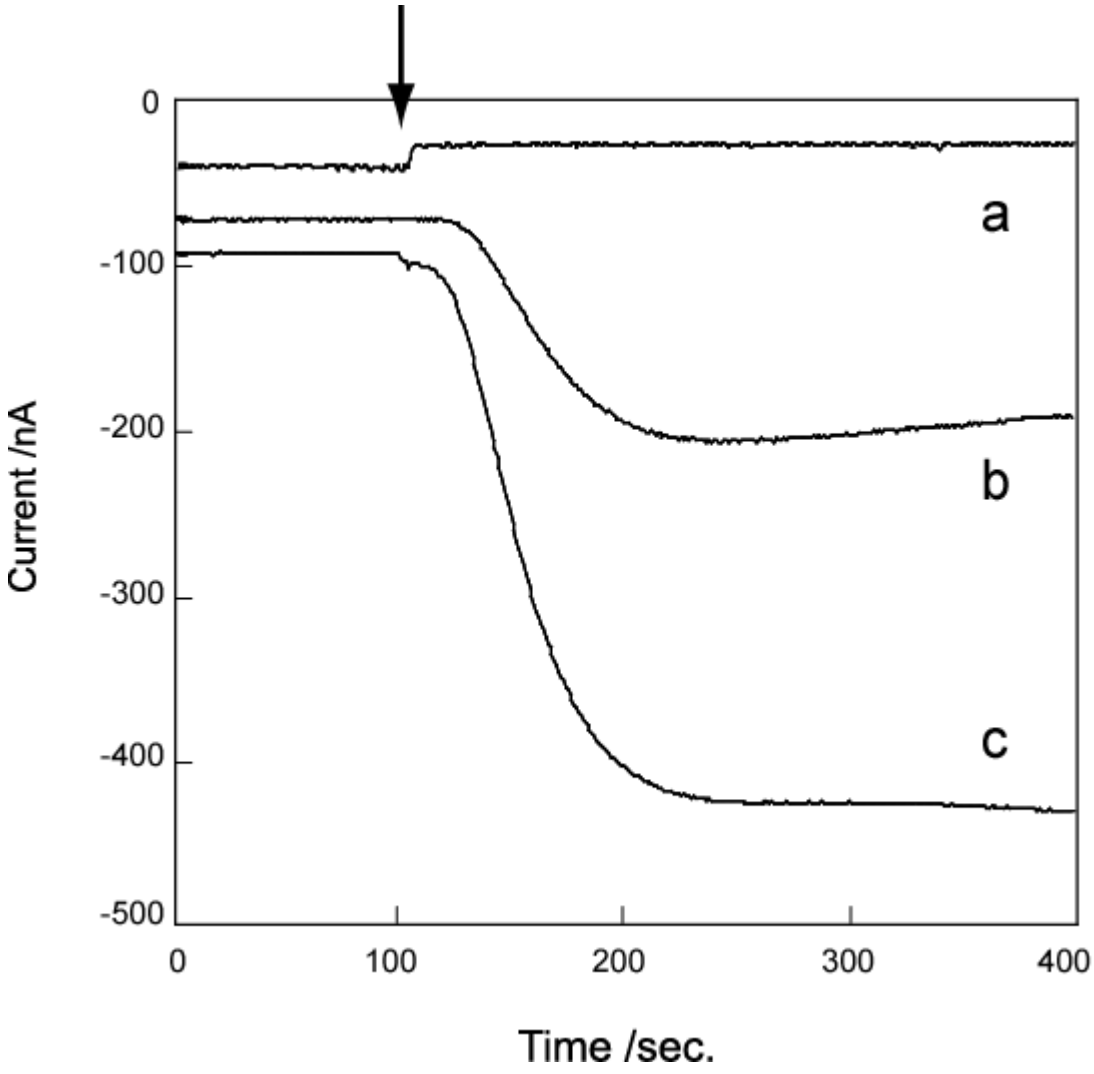

Figure 7

Graphical Contents entry

The molecular commonality detection based artificial-enzyme membrane sensor is a novel strategy to perform practical bio-surveillance, and the sensor shows good functional stability and reproducibility in the sensor response.