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# Electrochemical Immunoassay for Vitellogenin Based on Sequential Injection Using Antigen-immobilized Magnetic Microbeads

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A rapid and sensitive immunoassay for the determination of vitellogenin (Vg) is described. The method involves a sequential injection analysis (SIA) system equipped with an amperometric detector and a neodymium magnet. Magnetic beads, onto which an antigen (Vg) was immobilized, were used as a solid support in an immunoassay. The introduction, trapping and release of magnetic beads in an immunoreaction cell were controlled by means of the neodymium magnet and by adjusting the flow of the carrier solution. The immunoassay was based on an indirect competitive immunoreaction of an alkaline phosphatase (ALP) labeled anti-Vg monoclonal antibody between the fraction of Vg immobilized on the magnetic beads and Vg in the sample solution. The immobilization of Vg on the beads involved coupling an amino group moiety of Vg with the magnetic beads after activation of a carboxylate moiety on the surface of magnetic beads that had been coated with a polylactate film. The Vg-immobilized magnetic beads were introduced and trapped in the immunoreaction cell equipped with the neodymium magnet; a Vg sample solution containing an ALP labeled anti-Vg antibody at a constant concentration and a *p*-aminophenyl phosphate (PAPP) solution were sequentially introduced into the immunoreaction cell. The product of the enzyme reaction of PAPP with ALP on the antibody, *p*-aminophenol, was transported to an amperometric detector, the applied voltage of which was set at +0.2 V *vs*. an Ag/AgCl reference electrode. A sigmoid calibration curve was obtained when the logarithm of the concentration of Vg was plotted against the peak current of the amperometric detector using various concentrations of standard Vg sample solutions (0 – 500 ppb). The time required for the analysis is less than 15 min.

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## Introduction

The pollution of environmental water by so-called endocrine disrupting chemicals (EDCs) such as bisphenol A and nonylphenol has created a serious environmental problem for fish and other organisms in the hydrosphere.[1-3] Vitellogenin (Vg), a specific phospholipoprotein synthesized by female fish, has been recognized as a good biomarker for assessing the pollution of environmental water by measuring Vg in male fish, because males could only contain Vg if they had been exposed to estrogenic EDCs.[4] Several immunoassays of Vg have been reported[5-8] and an enzyme-linked immunosorbent assay (ELISA) is the most popular method currently in use.[9-11] A kit for the determination of Vg based on an ELISA method has been commercialized.[12] However, in general, such ELISA methods involve laborious and time-consuming procedures such as washing and the addition of sample and reagents. A more rapid and simpler alternative to the ELISA method would be desirable.

A sequential injection analysis (SIA) technique is suitable as an analytical method for ELISA procedures, because the washing, the separation of the bound-free antibody and the addition of reagent solutions *etc*. can be automated by means of a computer-controlled syringe pump and a multi-position valve.[13-15] In our previous paper, we described the use of the SIA technique for the determination of Vg based on immunoassay using anti-Vg antibody-immobilized magnetic microbeads, taking advantage of the ease with which they can be trapped in an immunoreaction cell by a magnet.[16] In this case, a sandwich immunoassay based on chemiluminescence detection was utilized. The sandwich immunoassay has the advantages that it has a good selectivity to Vg, because Vg was recognized by both the primary and the secondary antibodies, and it has good sensitivity because the calibration curve has a positive slope against the concentration of an analyte. However, the procedure is somewhat tedious because two immunoreactions are required, *i.e*. the immunoreactions of the primary antibody with Vg and of the resulting antibody-Vg complex with a secondary antibody. If an antigen, Vg in this case, could be immobilized on the magnetic beads, no immunoreaction with the secondary antibody would be required, and thus the immunoassay would be simpler and more rapid. In this paper, we wish to report on an attempt to simplify the SIA based immunoassay of Vg, which we reported previously, using electrochemical detection instead of chemiluminescence detection. As electrochemical immunoassays based on a microelectrode using magnetic microbeads have been reported,[17-20] the electrochemical detection would be expected to be easily applicable to our proposed SIA immunoassay using magnetic microbeads.

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## Experimental

*Apparatus and immunoreaction cell*

An SIA system was constructed from an immunoreaction cell, an amprometric detector (BS-1, dual potentiostat, BAS, USA), an SIA instrument, FIAlab-3000 (Alitea USA, USA), and a personal computer, which served to control the SIA instrument using the FIAlab software program for Windows. A diagram of the SIA system is shown in Fig. 1. Holding coil-1, the volume of which was larger than 1000 μL, was placed between a syringe pump and a multi-position valve to hold an aspirated sample. Holding coil-2 was placed between an immunoreaction cell and the amperometric detector to hold the enzyme product prior to its transport to the amperometric detector. The immunoreaction cell used in this study was constructed from two acrylic resin plates (55 mm × 100 mm × 1 mm$^t$), of which one contained a groove (3 mm × 40 mm × 0.5 mm). The plates were fused by pressing at 130°C for 3 h. The cell volume is about 60 μL. This composite plate was sandwiched between two additional acrylic resin plates (55 mm × 100 mm × 10 mm$^t$), which contained 1 mmϕ holes for a solution inlet and two outlets. The inlet of the cell was connected to the outlet of a multi-position valve with a Teflon tube (length 20 cm, i.d. 0.5 mm). One of the outlets was connected to a flow-through cell of the amperometric detector and the other outlet was for a waste of the used beads. Two-way valve-1 was set between the outlet of the immunoreaction cell and the flow-though cell of the amperometric detector. Two-way valve-2 was placed at the end of the other outlet of the immunoreaction cell. A square hole was prepared in the lower acrylic resin plate to accommodate the neodymium magnet (3 mmϕ, Magnet Japan, Japan) embedded in an acrylic resin that was inserted into this hole.

*Materials and reagents*

The anti-Vg monoclonal antibody and Vg were purchased from Transgenic Co., Ltd. (Japan). Magnetic microbeads coated with polylacetate polymer (PLA-particles-M COOH, particle size: 100 μm) were purchased from Micromod (Germany). The magnetic microbeads were supplied as suspended in a solution at a concentration of 10 mg mL$^{-1}$. A kit for alkaline phosphatase (ALP) labeling to an antibody was purchased from Dojindo Laboratories (Japan). *p*-Aminophenyl phosphate (PAPP) and *p*-nitrophenyl phosphate (PNPP), a substrate for ALP, were obtained from Wako Pure Chemical Co., Ltd. (Japan). The other reagents were obtained commercially and were used without further purification.

*Preparation of Vg-immobilized magnetic beads*[21–23]

Two hundred microliters of a 10 mg mL$^{-1}$ slurry of magnetic microbeads (PLA-particles-M COOH), the surface of which contained carboxylic acid groups, were placed in a test tube and washed with a phosphate–citric acid buffer (pH 8.0); the total final volume of the slurry was set at 1200 μL. Four hundred microliters of a 0.2 M *N*-hydroxysuccinimide (NHS) solution and 400 μL of a 0.8 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) solution were added to the test tube; the resulting mixture was then incubated for 30 min at room temperature. The beads were then washed with the above phosphate–citric acid buffer several times. The final volume of the slurry was set at 1500 μL. A five hundred microliter aliquot of a 2000 ppb Vg solution was added to the test tube. The resulting slurry was incubated for 30 min at room temperature for immobilization of Vg on the magnetic microbeads. After the incubation, the beads were washed with a Tris–HCl buffer solution (pH 7.4) to deactivate the unreacted NHS ester on the microbeads. The final volume of the slurry was set at 2000 μL with the same buffer.

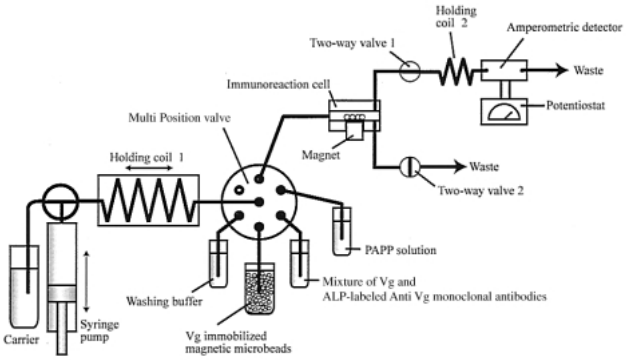


Fig. 1 Schematic flow diagram of the sequential injection system using magnetic microbeads.

*Evaluation of the amount of Vg immobilized on magnetic microbeads*

Five hundred microliters of a 1.0 mg/ml slurry of Vg immobilized magnetic beads were placed in a microtube and washed with a 25 mM Tris–HCl buffer (TBS-T buffer) and a 25 mM Tris–HCl buffer containing 0.1% BSA. Three hundred microliters of an ALP-labeled anti-Vg monoclonal antibody solution, which was diluted to appropriate concentrations (50, 100, 200, 500, 100, 1500 ppb) with a 25 mM Tris–HCl buffer containing 0.1% BSA, was added to the microtube. This tube was shaken in a thermostated bath at 25°C for 30 min. The slurry of the magnetic beads was washed with the TBS-T buffer and then with a glycine–HCl buffer (pH 10.3) and was then allowed to stand in a thermostated bath at 37°C for 5 min. The final volumes of the slurry were set at 500 μL with the glycine–HCl buffer. A five hundred microliter aliquot of a 5.5 mM PNPP solution, which was diluted with the glycine–HCl buffer, was added to the microtube to initiate the enzyme reaction of ALP-labeled on the anti-Vg monoclonal antibody for 40 min in the thermostated bath at 25°C with shaking. This enzyme reaction was terminated by adding 500 μL of a 1 M NaOH to the microtube. The final volume in the microtube was 1500 μL. The magnetic beads in the tube were removed using a magnet and the absorbance of the supernatant material at 405 nm was measured with a spectrophotometer.

*Preparation of ALP labeled anti-Vg monoclonal antibody and verification of labeled ALP on anti-Vg monoclonal antibody*

Labeling ALP on the anti-Vg monoclonal antibody (100 μg) was carried out by using an ALP labeling kit (Dojindo Laboratories), according to the manufacturer's instructions.[24] The resulting ALP labeled anti-Vg monoclonal antibody was diluted to appropriate concentrations (1.0, 2.0, 10, 20 ppb) with a glycine–NaOH buffer (pH 10.3). A five hundred microliter aliquot of the diluted ALP-labeled anti-Vg monoclonal antibody solution was placed in a microtube and the tube was allowed to stand in a thermostated bath at 37°C for 5 min. A five hundred microliter aliquot of a 5.5 mM PNPP solution, which was prepared using the glycine–NaOH buffer, was added to the microtube to initiate the enzyme reaction of ALP labeled on the anti-Vg monoclonal antibody with PNPP. The absorbance of the mixed solution at a wavelength of 405 nm, which is the maximum wavelength of the product of the enzyme reaction, *p*-nitrophenol (PNP), was measured at 5 min intervals.

Table 1 Protocol for the sequential injection analysis for the determination of Vg

| Event | Sample | Volume/μL | Flow rate/μL $s^{-1}$ |
|---|---|---|---|
| 1 Wash | HCl–Tris buffer (pH 8.0) + Tween 20 (0.1%) | 1900 | 100 |
| 2 Aspiration of magnetic microbeads | Slurry of magnetic microbeads immobilized with Vg (1.0 mg/ml) | 100 | 10 |
| 3 Introduction and trapping the magnetic microbeads with magnet | Slurry of magnetic microbeads immobilized with Vg (1.0 mg/ml) | 100 | 5 |
| 4 Aspiration of the Vg solution containing ALP-labeled anti Vg antibody (Pre-incubation time: 30 min) | Vg (0 – 500 ppb) + ALP labeled anti-Vg antibody (500 ppb) in HCl–Tris buffer (pH 8.0) + BSA (0.1%) | 300 | 100 |
| 5 Introduction of the Vg solution containing ALP-labeled anti Vg (Pre-incubation time: 30 min) | HCl–Tris buffer (pH 8.0) + BSA (0.1%) | 300 | 2 |
| 6 Wash | HCl–Tris buffer (pH 8.0) + Tween 20 (0.1%) | 900 | 5 |
| 7 Aspiration of the PAPP solution | 10 μM PAPP solution prepared with HCl–Tris buffer containing KCl and $MgCl_2$ (pH 9.0) | 300 | 100 |
| 8 Introduction of the PAPP solution and transport the product of enzyme reaction, PAP, to holding coil 2 | 10 μM PAPP solution prepared with HCl–Tris buffer containing KCl and $MgCl_2$ (pH 9.0) | 300 | 2 |
| 9 Discharge the magnetic microbeads | Carrier solution (HCl–Tris buffer containing KCl and $MgCl_2$ (pH 9.0)) | 1600 | 100 |
| 10 Transport of the solution held in holding coil 2 to an amperometric detector | Carrier solution (HCl–Tris buffer containing KCl and $MgCl_2$ (pH 9.0)) | 900 | 100 |

*Electrochemical detection of product of enzymatic reaction of ALP with p-aminophenyl phosphate (PAPP)*

In the present method, a substrate, PAPP for ALP, which was labeled on the anti-Vg monoclonal antibody, was converted to *p*-aminophenol (PAP) by the enzymatic reaction of ALP. To select the applied voltage for an amperometric detector for the detection of PAP, 300 μL of a standard solution of PAP, at various concentrations (0.1, 0.5, 1.0, 5.0 μM) was injected into the SIA system. A peak-shaped current signal obtained by the amperometric detector was recorded on a chart recorder.

*Electrochemical immunoassay for the determination of Vg using the SIA system*

The SIA protocol used in the determination of Vg is summarized in Table 1. The present immunoassay consists of three steps, as shown in Fig. 2: (1) the immunoreaction of the ALP-labeled anti-Vg antibody with Vg in the incubation solution and that of free ALP-labeled anti-Vg antibody in the incubation solution with Vg on the magnetic microbeads; (2) the enzyme reaction of the ALP labeled on the anti-Vg antibody on the magnetic microbeads with the substrate of ALP, PAPP; (3) the electrochemical detection of the product of the enzyme reaction, PAP, with the amperometric detector.

After the teflon tubing and the immunoreaction cell were washed with a carrier solution of 25 mM TBS-T buffer, a 100 μL aliquot of the slurry of magnetic microbeads (1.0 mg $mL^{-1}$) immobilized with Vg was aspirated into holding coil-1. The slurry was then introduced into the immunoreaction cell equipped with a magnet by pumping the carrier solution using the syringe pump at a flow rate of 5 μL/s and the magnetic beads were then trapped in the immunoreaction cell. A three hundred microliter aliquot of a Vg sample solution at various concentrations from 0 ppb to 500 ppb containing an ALP-labeled anti-Vg monoclonal antibody at a fixed concentration of 500 ppb, which was preliminarily incubated at room temperature for 30 min, was aspirated into holding coil-1 and then introduced into the immunoreaction cell at a flow rate of 2 μL/s. An immunoreaction of the free ALP labeled anti-Vg monoclonal antibody in the incubation solution with Vg on the magnetic microbeads proceeded during the period in which the

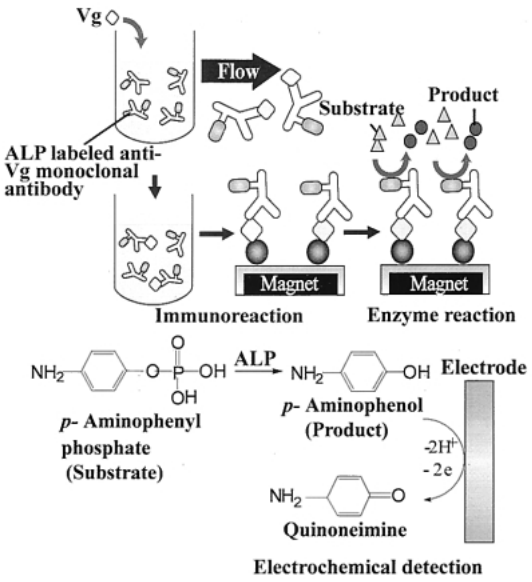


Fig. 2 Schematic protocol of the present immunoassay consisted of immunoreaction, enzyme reaction and electrochemical detection.

mixed solution flowed into the immunoreaction cell. A nine hundred microliter aliquot of the TBS-T buffer was then introduced into the immunoreaction cell at a flow rate of 5 μL/s in order to remove any nonspecifically adsorbed ALP-labeled anti-Vg monoclonal antibody on the beads or on the wall of the immunoreaction cell. A three hundred microliter aliquot of a $10^{-5}$ M PAPP solution was aspirated into holding coil-1 and was introduced into the immunoreaction cell at a flow rate of 2 μL/s, for the enzyme reaction of PAPP with ALP to proceed on the microbeads. The time for the enzyme reaction between PAPP and ALP on the microbeads was calculated to be 150 s from the flow rate and the volume of introduced sample. In this case, two-way valve-1 was opened (two-way valve-2 was closed) and a solution containing the product of the enzyme reaction was transported to holding coil-2 by flowing an additional 800 μL of the carrier solution. The magnet was shifted downward and

two-way valve-2 was opened (two-way valve-1 was closed) and 800 μL of TBS-T buffer was introduced into the immunoreaction cell at a flow rate of 100 μL/s to remove the used beads by repeating the introduction of the TBS-T buffer solution twice. Finally, after two-way valve-1 was opened (two-valve-2 was closed), the solution of the enzyme product (PAP) in holding coil-2 was transported to an amperometric detector, the applied potential of which was kept at +0.2 V *vs*. an Ag/AgCl reference electrode, by flowing 900 μL of the TBS-T carrier solution at a flow rate of 100 μL/s. New beads were introduced into the immunoreaction cell after the magnet was returned to the original position for the next measurement.

## Results and Discussion

*Verification of labeling ALP on the anti-Vg monoclonal antibody*

PNPP is enzymatically hydrolyzed by ALP to give PNP, a colored product with a maximum absorbance at a wavelength of 405 nm. The evidence that ALP was successfully labeled on the anti-Vg monoclonal antibody was provided using this enzymatic reaction. An ALP labeled anti-Vg monoclonal antibody solution at various concentrations (1.0, 2.0, 10 and 20 ppb), was mixed with a 5.5 mM PNPP solution at the same volume in a microtube and the absorbance of the mixed solution was monitored. Figure 3 shows the time-course for the absorbance of the mixed solution. As can be seen from Fig. 3, the absorbance increases with increasing time and is dependent on the concentration of the ALP-labeled anti-Vg antibody. The slope of a plot of absorbance *vs*. time at the initial part indicates the rate of the present enzyme reaction, *i.e*. the activity of ALP. The slope for various concentrations of ALP-labeled anti-Vg monoclonal antibody is nearly proportional to the concentration of ALP in the incubation solution. This verifies that the anti-Vg monoclonal antibody was labeled with ALP. According to the manufacture's instructions of an ALP labeling kit,[24] one or two molecules of ALP are introduced into a target antibody. If one molecule of ALP is assumed to be introduced into one molecule of the anti-Vg monoclonal antibody, the relative activity of ALP labeled on the anti-Vg monoclonal antibody was estimated to be 122 (units/mg-ALP labeled antibody) from the molar absorptivity of the enzyme reaction product, PNP ($\varepsilon = 1.8 \times 10^4$ L cm$^{-1}$ mol$^{-1}$).[25] The estimated activity of ALP labeled on the anti-Vg antibody was about one-third of the activity of free ALP in the ALP labeling kit used for labeling in the present work.[26] This lower activity may be due to the fact that the labeling procedure induces a structural change of ALP.

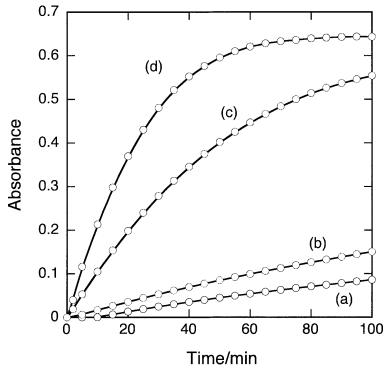


Fig. 3 Time-course for the absorbance of ALP labeled anti-Vg antibody solution after the addition of a PNPP solution. Initial concentration of the ALP labeled anti-Vg antibody: (a) 1.0 ppb, (b) 2.0 ppb, (c) 10 ppb, (d) 20 ppb. Initial concentration of the PNPP solution: 5.5 mM. The PNPP and antibody solutions were prepared by the same glycine–NaOH buffer solution (pH 10.3). Absorbance was measured after mixing two solutions at the same volume (500 μL).

*Amounts of Vg immobilized on the magnetic microbeads*

In order to estimate the amounts of Vg immobilized on the magnetic microbeads, we incubated the magnetic microbeads immobilized with Vg with the ALP-labeled anti-Vg antibody solution at various concentrations (50 – 1500 ppb). The ALP-labeled anti-Vg antibody binds to Vg immobilized on the magnetic microbeads and the fraction of Vg on the magnetic microbeads bound with the ALP-labeled anti-Vg antibody is expected to increase with an increase in the concentration of the ALP labeled anti-Vg antibody in the incubation solution. Finally, Vg on the magnetic microbeads is completely bound with the ALP labeled anti-Vg antibody. The amount of the ALP-labeled anti-Vg antibody bound to the microbeads is taken as proportional to the amount of Vg immobilized on the magnetic microbeads, if the ALP-labeled anti-Vg antibody is assumed to bind to Vg on the magnetic microbeads at the molar ratio 1:1. Therefore, when the activity of ALP on the magnetic microbeads is measured under the condition that the Vg-immobilized magnetic microbeads are completely bound with the ALP-labeled anti-Vg antibody, the amount of Vg on the magnetic microbeads can be estimated from the activity of ALP. The activity of ALP on the magnetic microbeads after incubating with various concentrations of the ALP labeled anti-Vg antibody was measured, as described in the experimental section. The absorbance of the product of enzyme reaction, PNP, is related to the activity of the ALP on the anti-Vg antibody; preliminarily, the relationship between the absorbance of PNP and the activity of ALP was obtained experimentally, as described in the previous section.

Figure 4 shows the absorbance of PNP formed by the enzyme reaction of PNPP with the ALP on the magnetic microbeads, which were incubated with the ALP labeled anti-Vg antibody solution for 40 min. As can be seen from Fig. 4, the absorbance of PNP increases with an increase in the concentration of the ALP-labeled anti-Vg antibody and reaches a constant value (0.065 A.U.) at concentrations higher than 1000 ppb. This indicates that all Vg immobilized on the magnetic microbeads is completely bound to the ALP labeled anti-Vg antibody, as expected. The absorbance of 0.065 A.U. corresponds to a PNP solution at the concentration of $3.6 \times 10^{-6}$ M, which was calculated from the molar absorption coefficient of PNP ($\varepsilon = 1.8 \times 10^4$ L mol$^{-1}$ cm$^{-1}$).[25] Since the final volume in the microtube was 1500 μL, the amount of PNP generated in the microtube during the incubation time of 40 min is calculated to be $5.4 \times 10^{-3}$ mol. Thus the generation rate of PNP in μmol/min, *i.e*. the enzyme activity of the ALP-labeled anti-Vg antibody bound to Vg on the magnetic microbeads, is $1.36 \times 10^{-4}$ units. The amount of the ALP-labeled anti-Vg antibody bound to the magnetic microbeads is calculated to be $1.1 \times 10^{-6}$ mg, from the fact that the activity of the ALP labeled on the anti-Vg antibody is 122 unit/mg-antibody, as estimated in the previous section. Since the weight of the magnetic microbeads in the microtube is 0.5 mg, the ALP labeled anti-Vg antibody is calculated to be $2.2 \times 10^{-6}$ mg-ALP labeled antibody/mg-bead. Since the molecular weight of the ALP-labeled anti-Vg antibody is estimated to be 250 kDa, assuming that the molar ratio of ALP to the anti-Vg antibody is 1:1, the amount of the ALP-labeled anti-Vg antibody, which is bound to Vg immobilized on the magnetic microbeads, is estimated to be $8.9 \times 10^{-15}$ mol/mg-bead. Therefore, the amount of Vg immobilized on the magnetic microbeads is estimated to be 3.3 ng/mg-bead, assuming that

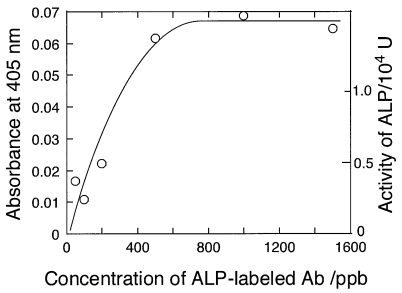


Fig. 4 Relationship between the absorbance of PNP solution and the concentration of ALP labeled anti-Vg antibody. Conditions of immunoreaction: amount of Vg immobilized beads, 0.5 mg; ALP labeled anti-Vg antibody, 50 – 1500 ppb, 300 µL; pH, 9.0. Condition of enzyme reaction: concentration of PNPP, 5.5 mM; pH, 10.3; incubation time, 40 min; amount of beads, 0.5 mg. Absorbance of PNP after enzyme reaction was measured at 405 nm.

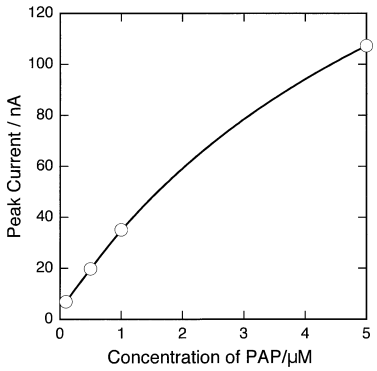


Fig. 5 Calibration curve for PAP obtained by the SIA system. Sample solution: PAP solution prepared by a Tris–HCl buffer containing KCl and $MgCl_2$ (pH 9.0). Injection volume: 300 µL. Carrier solution and flow rate: Tris–HCl buffer containing KCl and $MgCl_2$ (pH 9.0), 100 µL/s. Applied voltage of amperometric detector: +0.2 V *vs*. an Ag/AgCl reference electrode.

the molecular weight of Vg is 380 kDa. This value is about one-hundredth of the amount of an antibody of linear alkylbenzene sulfonate immobilized on the same type of the magnetic microbeads, as reported previously.[27]

*Electrochemical detection of the enzyme reaction product of p-aminophenyl phosphate (PAPP)*

In the present immunoassay, PAP, the product of the enzyme reaction of PAPP with ALP, was electrochemically measured in the final sequence. For the sensitive detection of PAP by an amperometric detector, the applied voltage of the working electrode and the flow rate of the PAP solution to the detector are important parameters. Since unreacted PAPP may be present after an enzyme reaction of PAPP with ALP on the antibody, if PAPP is electroactive and its oxidation voltage for PAP is overlapped with that of PAPP, unreacted PAPP may interfere with the detection of PAP. After a preliminary investigation of the hydrodyamic voltammetry for PAP and PAPP, the applied voltage of the working electrode was selected at +0.20 V *vs*. an Ag/AgCl reference electrode by compromising the sensitivity to PAP and the background current from PAP, which may be an impurity in PAPP or from hydrolyzed PAP. The flow rate of a sample solution containing PAP for an amperometric detector affects the sensitivity of PAP, because as the flow rate increases, the thickness of the diffusion layer adjacent to the working electrode becomes thin; as a result, the current increases. In this work, the flow rate of the carrier solution that transports the sample solution stored in holding coil-2 to the detector was set at 100 µL/s, as fast as possible. The calibration curve for PAP obtained by the same SIA system as shown in Fig. 1 is shown in Fig. 5. As can be seen from Fig. 5, a PAP solution at the micromolar level was able to be determined with a good linearity against the concentration under the conditions used.

*Calibration for Vg*

A calibration curve for Vg was obtained according to the SIA protocol shown in Table 1. After the introduction of 0.1 mg of magnetic microbeads immobilized with Vg into the immunoreaction cell, 300 µL of a standard Vg solution at several concentrations (0, 50, 100, 150, 200, 250 and 500 ppb) containing 500 ppb ALP-labeled anti-Vg monoclonal antibody, which solution was preliminarily incubated for 30 min, was introduced into the immunoreaction cell at a flow rate of 2 µL/s. At this protocol, an unreacted ALP-labeled anti-Vg monoclonal antibody with Vg in the incubation solution binds to Vg immobilized on the microbeads during flowing into the immunoreaction cell. The reaction time in the immunoreaction cell was about 150 s calculated from the flow rate of the carrier solution and the volume of the incubation solution introduced. For the next sequence in the enzyme reaction, 300 µL of a PAPP solution was allowed to flow at a flow rate of 2 µL/s to keep the enzyme reaction time at 150 s. Since 0.1 mg of the Vg-immobilized magnetic microbeads were introduced into the immunoreaction cell, the activity of ALP is estimated to be $2.7 \times 10^{-4}$ unit if Vg on the microbeads is completely bound with the ALP-labeled anti-Vg antibody. Then, $4.5 \times 10^{-4}$ µmol of PAP would be produced in 300 µL of the introduced PAPP solution in the period of 150 s, if activity of ALP to PNPP is the same as that to PAPP. Thus, the concentration of PAP would be about 1.5 µM and this concentration level is within the range measurable by the present amperometric detector, judging from Fig. 5. Indeed, as shown in Fig. 6(A), amperometric signals depending on the concentration of Vg were obtained by the SIA protocol shown in Table 1. A peak-shaped signal is observed and appears within as short a period as 15 s. For measurement of the background current, the peak current was measured by the protocol without the introduction of the magnetic microbeads immobilized with Vg in Table 1 (without events 2 and 3). About 10 nA of peak current was observed as the background current. This may be due to the fact that a small amount of PAP, which may have arisen from an impurity or from hydrolyzed PAPP without ALP, was included in the blank solution. Figure 6(B) shows the calibration curve for Vg, where the peak current was plotted against the logarithm of the concentration of Vg in the sample solution. A sigmoidal calibration curve was obtained with $IC_{50}$, 50% binding value of *ca*. 100 ppb. The minimum detectable concentration of Vg, which is usually defined as 85% inhibition, was 10 ppb. This value is slightly higher than that obtained by a previous chemiluminescence SIA method using Vg monoclonal antibody immobilized magnetic microbeads.[16] However the analytical time is shorter compared to the previous method, due to the fact that the sandwich immunoreaction is omitted.

## Conclusion

An electrochemical immunoassay for the sensitive and rapid determination of Vg using magnetic microbeads immobilized

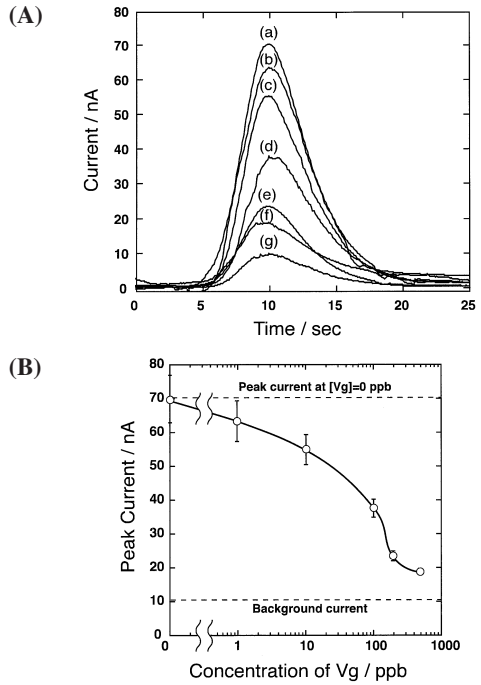


Fig. 6 (A) Amperometric signals for a Vg sample solution at various concentrations containing 500 ppb of ALP labeled anti-Vg antibody at a fixed concentration. Concentration of Vg in the sample solution: (a) 0, (b) 1, (c) 10, (d) 100, (e) 200, (f) 500 ppb, (g) background. Immunoreaction condition: amount of magnetic microbeads introduced into the immunoreaction cell, 0.1 mg (100 µL of a slurry); volume and flow rate of sample solution containing Vg and ALP labeled anti-Vg antibody in HCl–Tris buffer (pH 8.0) + BSA (0.1%), 100 µL and 2 µL/s. Enzyme reaction condition: PAPP solution, $1.0 \times 10^{-5}$ M (Tris–HCl buffer containing KCl and $MgCl_2$, pH 9.0); volume and flow rate, 300 µL and 2 µL/s. Electrochemical detection: carrier solution, Tris–HCl buffer containing KCl and $MgCl_2$, pH 9.0; flow rate of the carrier solution, 100 µL/s; applied voltage of amperometric detector, + 0.2 V *vs*. Ag/AgCl reference electrode. (B) Calibration curve for Vg. Conditions are the same as Fig. 6 (A).

with Vg in an SIA system equipped with a magnet is described. In this method, microbeads immobilized with Vg were used instead of microbeads immobilized with an anti-Vg antibody as in our previous paper.[16] The sandwich immunoreaction step can be omitted and the protocol for the immunoassay becomes simpler. Thus, the analytical time of the proposed immunoassay was reduced to shorter than 15 min for one sample. In this method, an electrochemical detection was used instead of the chemiluminescence detection used in our previous research.[16] Instrumentation of the electrochemical detection may be more economical when the present method is applied to a multichannel detection system by using a mutichannel flow cell with multi-detectors. One of the advantages of the present method is that the Vg-immobilized magnetic microbeads may be more easily regenerated by dissociation of the immunocomplex with the ALP-labeled antibody and may be reused compared with the magnetic microbeads immobilized antibody. This is because, in the case where the antibody is immobilized on the beads, the antibody might be deactivated when the immunocomplex with Vg is dissociated by a dissociating solution. The Vg-immobilized magnetic microbeads have not been characterized yet. A further study of the electrochemical determination of Vg based on SIA using magnetic microbeads is currently underway.

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