J. Flow Injection Anal., Vol. 23, No. 2 (2006) 117–122

＜Minireview＞

Flow Immunoassay Based on Sequential Injection Using Microbeads

RuiQi Zhang, Koji Hirakawa, Masaaki Katayama, Hizuru Nakajima, Nobuaki Soh, Koji Nakano, Toshihiko Imato*

Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, Fukuoka 819-0395, Japan
*Corresponding author: Tel/Fax; +81-92-802-2889, E-mail; imato@cstf.kyushu-u.ac.jp (T. Imato)

.

Abstract

A flow immunoassay based on a combined technique of the sequential injection with the beads injection using magnetic microbeads immobilized with antigen or antibody is described. A methodology of the present immunoassay based on chemiluminescence and electrochemical detection and its application to the determination of vitelloginin (Vg) and anionic surfactant, linear alkylbenzene sulfonate (LAS) are described. Magnetic microbeads coated with agarose gel and polylactic acid were used for immobilization of an anti-Vg antibody and an anti-LAS antibody or Vg by a conventional amino coupling method. The introduction, trapping and flushing out of the magnetic microbeads in the immunoreaction cell were controlled by the magnet and the flow of the carrier solution. The protocol of the immunoassay in the immunoreaction cell, introduction of an analyte sample, the enzyme-labeled secondary antibody or antigen and a substrate solution for chemiluminescence or electrochemical detection were sequentially carried out by the sequential injection technique. A lower detection limit around ppb level was achieved for immunoassay for Vg and LAS. The time required for an analysis was ca 15 min/sample including incubation time for immunoreaction.

Keywords: Flow immunoassay; sequential injection analysis; magnetic microbeads; vitellogenin; linear alkylbenzene sulfonate.

1. Introduction

The pollution of environmental water by so-called endocrine disrupting chemical such as bisphenol A and nonylphenol etc. has created a serious environmental problem. A rapid and sensitive analytical method for monitoring such pollutants would be highly desirable. One of the reliable analytical methods is that based on gas chromatography or high performance liquid chromatography, coupled with mass spectroscopy, because these methods permit the identification of homologues and isomers. However, these methods typically involve time-consuming pretreatments of samples from the complicated matrices and expensive instrumentation. A method based on an enzyme-linked immunosorbent assay (ELISA) using a microtiter plate would be a promising method for screening large number of samples such as environmental samples. However, in general, such types of ELISA method involve many laborious and time-consuming procedures such as the washing, addition of sample and reagents.

A sequential injection analysis (SIA) technique is suitable as an analytical method for ELISA procedures, because the washing, separation of bound-free antibody and the addition of reagent solutions, etc. can be automated by using a computer-controlled syringe pump and a switching valve [1-3]. Ruzica *et al*. proposed a "beads injection technique" combined with the SIA technique, which permits use of solid particles in a flow system [4-8]. They have demonstrated that this combined technique is effective for automation of the ELISA method, where microbeads immobilized with antibody provide immunoreaction supports.

We have expanded the application of the beads injection combined with the SIA technique proposed by Ruzica *et al*. to several immunoassays by using antibody- or antigen-immobilized magnetic microbeads [9-14]. In this review, a rapid and sensitive immunoassay based on the beads injection/SIA technique for the determination of vitellogenin (Vg), which is recognized as a good biomarker for assessment of environmental water, is described. The beads injection/SIA technique is also applied to the immunoassay for the determination of linear alkylbenzene sulfonate (LAS).

2. Spectrophotometric determination of Vg using Sepharose beads immobilized with antibody [9]

Prior to start our research on flow immunoassay using microbeads, the beads injection technique using gel-type microbeads (Sephadex beads) and the jet ring cell was applied for immunoassay of Vg. An SIA system and the jet ring cell used in the work are shown in Fig. 1 and 2, respectively.

One optical fiber is used for introducing the light from the light source of tungsten lamp and the other fiber is used for detecting the transmitted light through the cell by a spectrophotometer (PC2000, Ocean Optics).

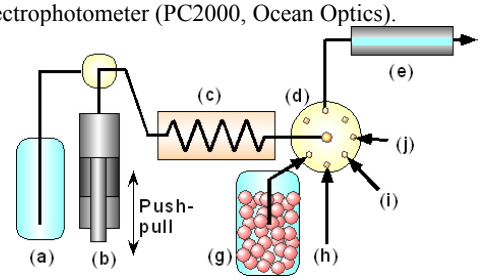


Fig. 1 SIA system combined with beads injection technique.
(a) carrier solution, (b) syringe pump, (c) holding coil, (d) selection valve, (e) Jet ring cell or immunoreaction cell, (g) beads slurry, (h) sample Vg solution, (i) enzyme labeled secondary anti-Vg antibody solution, (j) substrate solution containing color developing reagents.

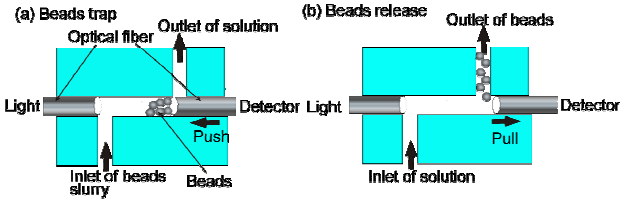


Fig. 2 Jet ring cell for beads injection technique with optical fibers for photometric detection.

For immunoassay for Vg, an anti-Vg antibody was immobilized on Sepharose beads (Sephadex G-75) via protein A and the resulting beads were introduced and trapped in the jet ring cell by using a small gap between the outlet of the cell and the one optical fiber. A sample solution of Vg, a solution of a horseradish peroxidase (HRP)-labeled anti-Vg antibody (secondary antibody) solution and a color-developing reagent solution containing *o*-phenylenediamine and hydrogen peroxide were introduced into the jet ring cell sequentially by using a sequential injection instrument (FIAlab 3000, Alitea USA). After color developing, absorbance of the solution in the jet ring cell at the wavelength of 420 nm was measured by using the couple of the optical fibers. A nearly linear calibration curve between the concentration of Vg and the absorbance was obtained in the concentration range from 10 ppb to 120 ppb Vg. However, it took more than 3 hr for the determination of one sample including 2 hr for the first immunoreaction, 1hr for the second immunoreaction and 20 min for color developing reaction. Furthermore, the present assay needs a sophisticated skill for trapping the microbeads at the gap of the jet ring cell. Therefore, we tried to use magnetic microbeads for easy tapping the beads in the immunoreaction cell by a magnet.

3. Chemiluminescence determination of Vg using magnetic microbeads immobilized with antibody [10]

Magnetic microbeads, on which surface has been coated with agarose gel, were selected because of the similarity to the Sephadex beads and were immobilized with the anti-Vg antibody by using a reaction of the epoxy group on the surface of the agarose layer on the beads with the antibody, which was introduced by a prior reaction with epichlorohydrin. A photo of the magnetic microbeads trapped with a magnet is shown in Photo 1. A reaction scheme for immobilization of the anti-Vg antibody is shown in Scheme 1.

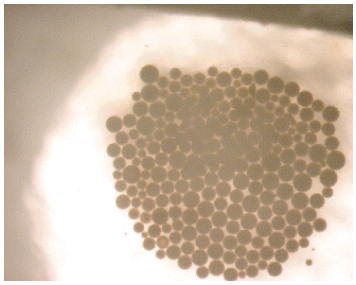
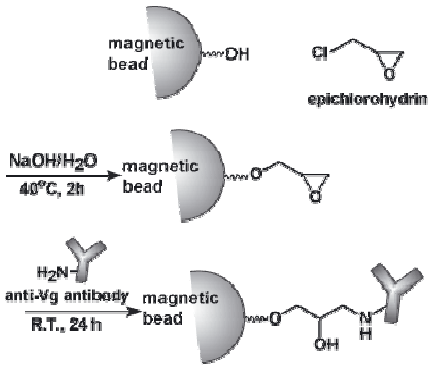


Photo 1 Magnetic microbeads coated with agarose gel.



Scheme 1 Immobilization of anti-Vg antibody on the magnetic microbeads.

A protocol of the immunoassay for Vg using the magnetic microbeads is almost the same as that using the Sepharose beads in the jet ring cell, except for chemiluminescence detection and an immunoreaction cell, which was newly constructed for trapping the magnetic microbeads. Since the magnetic microbeads used in this work is not transparent, they are not suitable for photometric detection. Therefore, a chemiluminescence detection was selected. A slurry of magnetic microbeads immobilized with the anti-Vg antibody was introduced into an immunoreaction cell equipped with a magnet beneath the cell and a photomultiplier above the cell. The beads were trapped in the immunoreaction cell by the magnet. The immunoreaction cell and its photo are shown in Fig. 3 and Photo 2, respectively. The shift to upward or downward of the magnet was controlled with a solenoid using a 24 V DC electric power source, supplied with the FIAlab 3000.

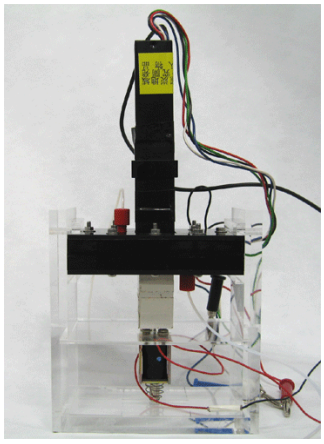


Photo 2 Immunoreaction cell for magnetic microbeads injection technique.

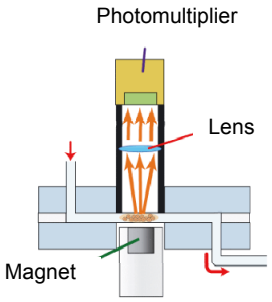
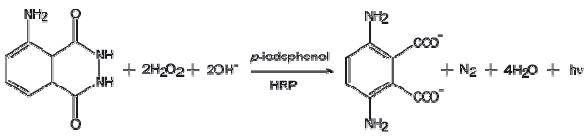
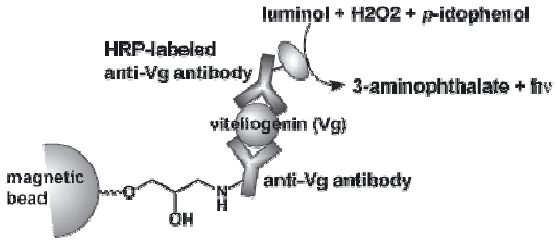


Fig. 3 Immunoreaction cell with magnet and photomultiplier.

After introduction of the magnetic microbeads, a sample solution of Vg, an HRP labeled anti-Vg antibody solution and a luminol solution containing hydrogen peroxide, *p*-iodophenol were sequentially introduced into the immunoreaction cell. In the present protocol, an incubation time for the 1st immunoreaction of the anti-Vg antibody on the magnetic beads with a sample Vg and that for the second immunoreaction of the immunocomplex of anti-Vg antibody with Vg on the magnetic beads with the HRP-labeled anti-Vg antibody in the solution were optimized. It is known that a diffusion process of a target molecule to an antibody immobilized on the solid surface is often a rate-determining step in an immunoreaction. In order to shorten the immunoreaction, in the present protocol a Vg sample solution for the 1st immunoreaction and an HRP-labeled anti-Vg antibody solution in the 2nd immunoreaction were moved forward and backward repeated at a flow rate of 2 μL/s by the syringe pump during the incubation. Thus the optimal incubation time for the first and second immunoreaction was found to be 20 min. A relatively shorter incubation time gave maximum response for determination of Vg than the immunoassay using the Sepharose beads. The final step for the determination of Vg is the chemiluminescence detection (Schemes 2 and 3). A luminol solution containing hydrogen peroxide as a substrate to HRP and *p*-iodophenol as a sensitizer was used as a chemiluminescent solution for HRP.



Scheme 2 Chemiluminescent reaction used in this work.



Scheme 3 Schematic illustration of the determination of Vg based on the sandwich chemiluminescent immunoassay on the magnetic microbeads.

The present chemiluminescence reaction is so fast and the emitted chemiluminescnce light is monitored with a photomultiplier set above the immunoreaction cell during the chemiluminescent reagent passes through the cell. The chemiluminescent responses when the luminol solution was introduced to the magnetic microbeads immobilized with the HRP-labeled antibody in the immunoreaction cell are shown in Fig. 4. As can be seen from Fig. 4, the chemiluminescence intensity increases with increasing concentrations of the HRP-labeled antibody. This suggests that the present chemiluminescence detection system with the photomultiplier has a sufficient sensitivity for the detection of ppb level of the HRP-labeled antibody.

A calibration curve for Vg shown in Fig. 5 was obtained under the optimal condition of 20 min of incubation time for the first and second immunoreactions.

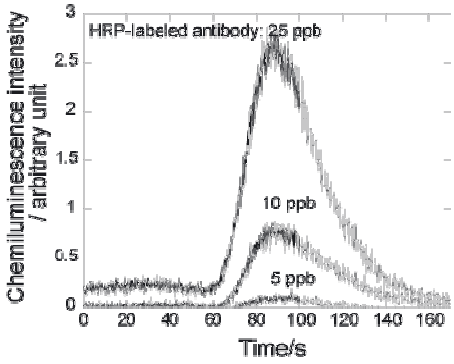


Fig. 4 Chemiluminescent responses of the microbeads immobilized with HRP-labeled anti-Vg antibody to the luminol solution.

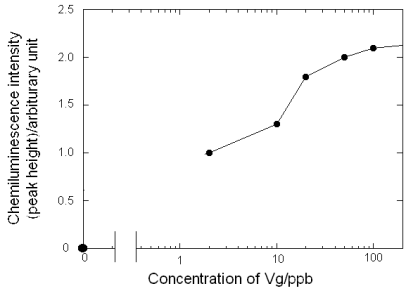


Fig. 5 Calibration curve for Vg.

4. Electrochemical determination of Vg using magnetic microbeads immobilized with antibody or antigen [11, 12]

Magnetic microbeads coated with agarose gel were used in the previous work [10]. However, this assay still required more than 20 min for incubation of the immunoreaction, which may be due to the slow diffusion of Vg or a secondary antibody in the agarose gel coated on the magnetic microbeads, because the first antibody may be immobilized in the polymer matrix of the agarose gel as well as on the surface. We found that magnetic microbeads coated with a polylactic acid showed a rapid immunoreaction rate, which may be due to the fact that the polymer layer is thin and the diffusion process of Vg or the secondary antibody in the polymer film does not seem to be a rate-determining step. Electrochemical detection has been applied to many immunoassays and sensitive detection was achieved [15-18]. In addition, electrochemical detection is as sensitive as chemiluminescence detection and could be used in conjunction with a multi-channel detection system due to its low instrument cost compared with chemiluminescence detection. So, we attempted to construct the electrochemical immunoassay system using magnetic microbeads for more rapid assay. A competitive immunoassay as well as sandwich immunoassay was compared with respect to sensitivity and analytical throughput. In order to separate the electrochemical detection cell from the immunoreaction cell, a new flow-through type immunoreaction cell was fabricated. A schematic immunoreaction cell is shown in Fig. 6.

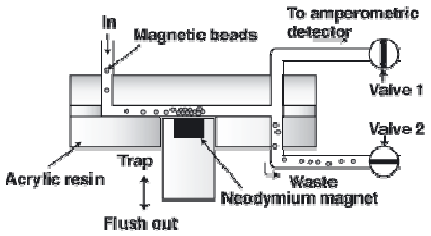


Fig. 6 Schematic flow-through type immunoreaction cell

4.1 Sandwich immunoassay

A protocol of the sandwich immunoassay based on the present method is shown schematically in Fig. 7. A slurry of magnetic microbeads immobilized with an anti-Vg antibody, which was prepared by an amino coupling reaction of the antibody with the carboxylic group on the magnetic microbeads after activation with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide and N-hydroxysuccinimide, were introduced into the immunoraction cell. After an introduction of a sample Vg solution into the immunoreaction cell, a solution of an alkaline phosphatase (AP) labeled anti-Vg antibody (secondary antibody) was introduced into the immunoreaction cell. In this case, the incubation times of the first- and second-immunoreaction were 150 sec and 200 sec, respectively. A solution of the substrate AP, *p*-aminophenyl phosphate (PAPP) was introduced into the immunoreaction cell. In this case, valve 1 in Fig. 6 is opened and valve 2 is closed. During passing the substrate solution, an enzymatic reaction shown in Fig. 8 proceeds and a product of PAPP, *p*-aminphenol (PAP) was introduced into the holding coil and was tentatively kept there. After closing valve 1 and opining valve 2, the

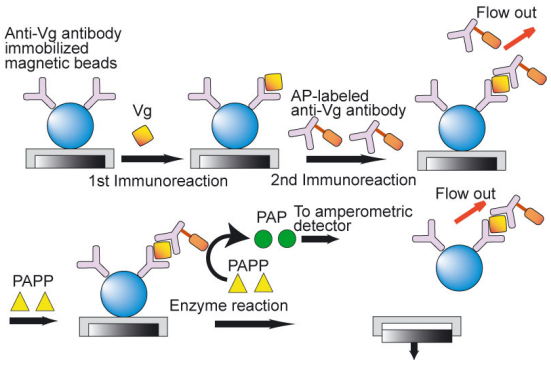


Fig. 7 Schematic protocol of the present sandwich immunoassay using magnetic microbeads immobilized with anti-Vg antibody.

microbeads in the immunoreaction cell was discharged through valve 2.

Finally, after closing valve 2 and opining valve1, the product in the holding coil was introduced into a flow cell of an amperometric detector. The oxidation current at the electrode detector, where an electrochemical oxidation of PAP to quineneimmine and its following hydrolysis reaction to *p*-benzoquinone proceed as shown in Fig. 8, was monitored with the amperometric detector. In this case, the applied potential of the working electrode was maintained at + 0.2 V and the pH of the carrier solution was adjusted to 9.0.

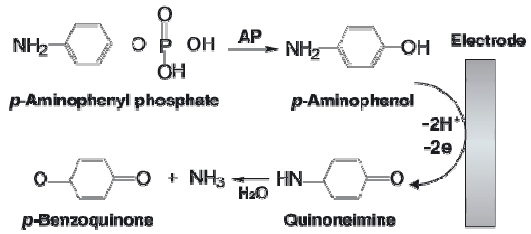
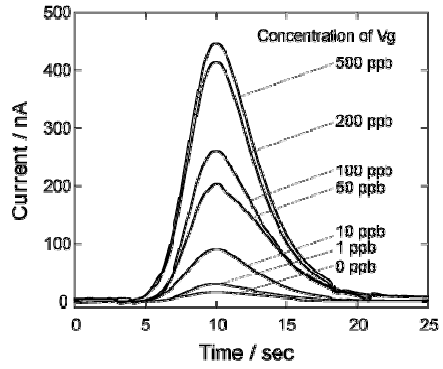


Fig. 8 Schemes for the enzyme reaction of *p*-aminophenyl phosphate (PAPP) with alkaline phosphatase (AP) and reaction of *p*-aminophenol (PAP) at the electrode.

The effect of the flow rate of the carrier solution, which transports the enzymatic product to the electrode detector, was investigated by changing the flow rate from 20 µL/sec to 200 µL/sec, because as the flow rate increases, the thickness of the diffusion layer adjacent to the electrode surface becomes thin, as a result the current increases. The peak current increased with increasing the flow rate of the carrier solution from 20 µL/sec to 100 µL/sec, as expected, and the peak current did not increase at more than 100µL/sec. Finally the flow rate at 100 µL/sec was selected in the subsequent experiments.

A calibration curve for Vg was obtained under the optimal condition that amount of the magnetic microbeads introduced into the immunoreaction cell was 0.25 mg, volume and flow rate of the Vg solution were 300 µL and 5 µL/s, volume and flow rate of the secondary antibody solution were 400 µL and 2 µL/s, and volume and the flow rate of the 10 µM PAPP solution were 300 µL and 2 µL/s, respectively. Fig. 9 (a) shows the amperometric response of the detector to PAP generated from PAPP via the enzyme reaction with AP labeled on the secondary antibody. Fig. 8 (b) shows the calibration curve for Vg, in which the peak current was plotted against the logarithm of the concentration of Vg in the sample solution. A sigmoidal curve with IC_{50}, 50% binding value, of ca 70 ppb was obtained. The lower detection limit defined as 3 times of the background signal was around 2-3 ppb.

(a)



(b)

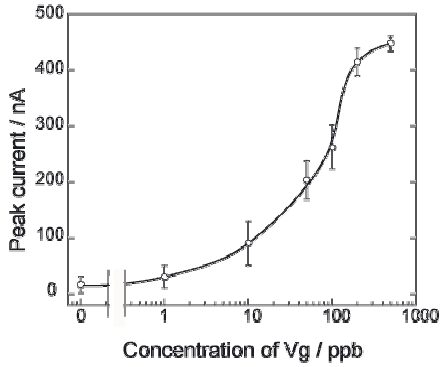


Fig. 9 (a) Amperometric signals for a Vg sample solution at various concentrations. (b) Calibration curve for Vg.

4.2 Competitive immunoassay

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 immunoreaction 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. So, we attempted to apply the SIA combined with microbeads injection technique to the competitive immunoassay of Vg.

A protocol of the competitive immunoassay based on the present method is shown schematically in Fig. 10. A slurry of magnetic microbeads immobilized with Vg, which was prepared by the same amino coupling reaction of Vg with the carboxylic group on the magnetic microbeads as the antibody-immobilization, were introduced and trapped in the immunoreaction cell. A sample Vg was preliminarily incubated in the AP-labeled anti-Vg antibody solution at a constant concentration for preparation of the sample solution. The resulting sample solution was introduced into the immunoreaction cell, where the competitive immunoreaction of the AP-labeled anti-Vg antibody in the incubation solution with Vg in the same solution and with Vg immobilized on the magnetic microbeads proceed. In this case, the incubation time for the immunoreaction of Vg on the beads with the free AP-labeled anti-Vg antibody in the solution was only 150 sec during the incubation solution passed through the immunoreaction cell. After washing the immunoreaction cell with the carrier solution to remove any nonspecifically adsorbed AP-labeled anti-Vg antibody and Vg on the beads or on the wall of the immunoreaction cell, a 10^{-5} M PAPP solution, which is the substrate to AP, was introduced into the immunoreaction cell. The time for the enzyme reaction of PAPP with AP on the beads to generate PAP was also only 150 sec during the PAPP solution passed through the

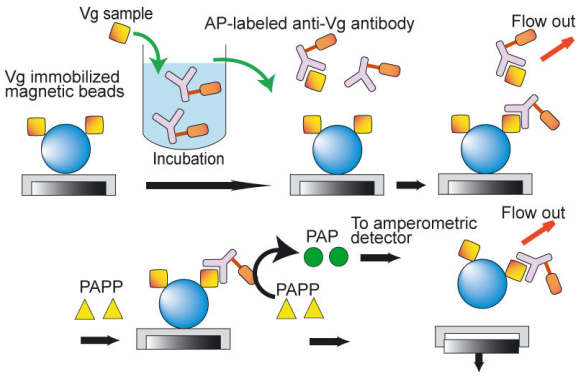
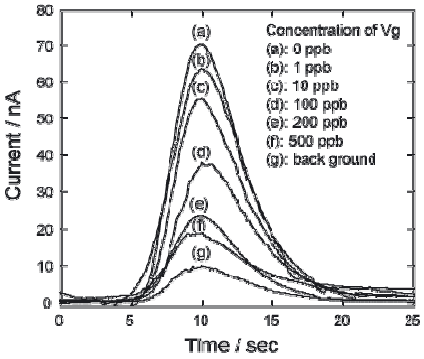


Fig. 10 Schematic protocol of the present competitive immunoassay using magnetic microbeads immobilized with Vg.

immunoreaction cell. The PAP product solution of the enzyme reaction was introduced into the holding coil and transported to the amperometric detector after flushing out the beads from the immunoreaction cell. The applied potential of the working electrode of the detector was maintained at + 0.2 V and the pH of the carrier solution was adjusted to 9.0 as the same as for the sandwich immunoassay, described in the previous section.

Amperometric responses of the detector to PAP, product of the enzyme reaction, are shown in Fig. 11 (a). A peak-shaped signal is observed and appears within as short a period as 15 sec. For measurement of the background current, the peak current was measured by the protocol without introduction of the magnetic microbeads immobilized with Vg. 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 AP, was included in the blank solution. A calibration curve for Vg is shown in Fig. 11 (b), where the peak current is plotted against the logarithm concentration of Vg in the incubation solution. 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 the previous sandwich immunoassay. However the analytical time is shorter compared to the previous method, due to the fact that the sandwich immunoreaction is omitted.

(a)



(b)

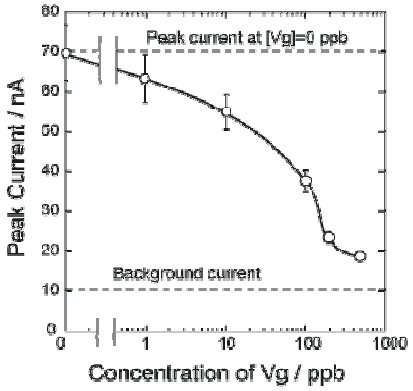


Fig. 11 (a) Amperometric signals for a Vg sample solution at various concentrations containing 500 ppb of ALP labeled anti-Vg antibody. (b) Calibration curve for Vg.

5. Chemiluminescence determination of anionic surfactants using magnetic microbeads immobilized with an antibody [13]

The pollution of environmental water by linear alkylbenzene sulfonate (LAS), which are widely used as anionic surfactants in detergents in both industry and the home, has created a serious environmental problem, such as the destruction of the hydrosphere environment for fish and other living things as well as causing human health problems due to their permeability through cell membranes [19-21]. A rapid and sensitive analytical method for monitoring such pollutants would be highly desirable. A method determining LAS based on ELISA has recently been reported as a result of the successful preparation of a monoclonal anti-LAS antibody [22]. However, in general, such types of ELISA method involve many laborious and time-consuming procedures such as the washing, addition of sample and reagents. We attempted to apply a sequential injection immunoassay using magnetic microbeads for the rapid and sensitive determination of LAS in environmental water samples, as an alternative method of ELISA.

A protocol of the competitive immunoassay for LAS based on the chemiluminescent detection is schematically shown in Fig. 12. A slurry of magnetic microbeads immobilized with an anti-LAS antibody, which were prepared the same amino coupling method as the anti-Vg antibody, were introduced into the immunoreaction cell. A LAS sample solution at various concentrations containing an HRP-labeled LAS at concentration of 500 ppb was introduced into the immunoreaction cell. A competitive immunoreaction of LAS and the HRP-labeled LAS in the sample solution with the anti-LAS antibody immobilized on the magnetic microbeads proceed during the sample solution passed through the cell. In this case, to keep the time for competitive reaction at 300 sec in the cell, a procedure of flowing 1 μL of the sample solution for 1 sec and waiting for 2 sec was repeated 100 times. This procedure can accelerate the rate of the immunoreaction compared with a conventional stopped-flow procedure (waiting for 300 sec without moving a solution), because the movement of the liquid phase diminishes the thickness of a diffusion layer adjacent to the solid phase immobilized with the antibody. After introducing the carrier solution containing Tween 20 (0.1%) to remove nonspecifically adsorbed LAS or the HRP-labeled LAS on the beads or wall of the cell, a chemiluminescent reagent solution containing luminal, hydrogen peroxide and *p*-iodophenol was introduce into the immunoreaction cell. At the same time, the chemiluminiescence intensity was measured by means of a photon counting unit. Finally, the magnet was shifted downward and the carrier solution was introduced into the immunoreaction cell to remove the used beads. New beads were introduced into the immunoreaction cell after the magnet was returned to the original position for the next measurement.

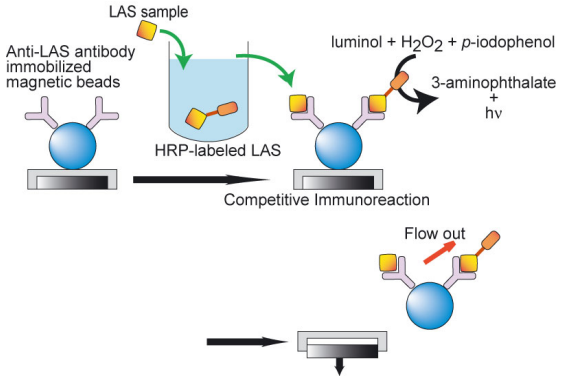


Fig. 12 Schematic protocol of competitive immunoassay for LAS using magnetic microbeads immobilized with anti-LAS antibody.

Fig. 13 (a) shows the chemiluminescence signals when 100 μL of the chemiluminescent solution containing luminol, H_2O_2 and *p*-iodophenol was introduced at a flow rate of 20 μL/s into the immunoreaction cell, where the magnetic microbeads incubated with a LAS standard solution were trapped. A peak-shaped signal is observed depending on the concentration

of LAS. The noise level of the present chemiluminescence system was less than 10^2 counts/s. The nose level for detecting chemiluminescence light has been improved by using the photon counting unit compared with a photomultiplier used for immunoassay of Vg described previously. Fig. 13 (b) shows the calibration curve for LAS, the peak height of the chemiluminescence intensity was plotted against the logarithm of the concentration of LAS. A typical sigmoid calibration curve was obtained with IC_{50}, 50 % binding value of 90 ppb. The minimum detectable concentration of LAS, which is defined as 85% inhibition, was 25 ppb. This means that the present immunoassay sufficiently satisfies the limits permitted in Japan for drinking water that contains 200 ppb LAS. Judging from the molar concentration of LAS (90 ppb = 2.6 x 10^{-10} mol/mL) and that of the HRP labeled LAS (500 ppb = 1.2 x 10^{-11} mol/mL), the binding constant of the HRP labeled LAS-anti LAS antibody complex is estimated to be larger than that of the LAS-anti LAS antibody complex.

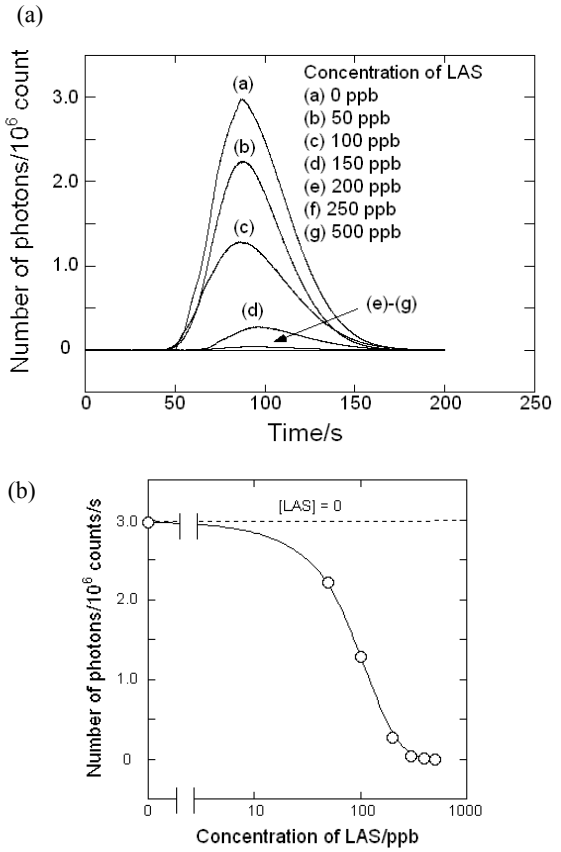


Fig. 13 (a) Chemiluminescence signals for a sample solution containing various concentration of LAS and 500 ppb HRP-labeled LAS at a fixed concentration. (b) Calibration curve for LAS.

6. Conclusion

A flow immunoassay based on a combined technique of the sequential injection with the beads injection using magnetic microbeads immobilized with antigen or antibody is described. The applicability of the proposed immunoassay method was demonstrated by the determination of Vg and LAS. Magnetic microbeads immobilized with an antibody or an antigen were used for the solid support for a competitive or a sandwich immunoreaction. Mganetic microbeads were easily handled under the flowing condition and were easily trapped in and flushed out from the immunoreaction cell equipped with a magnet, which shift up or downward was controlled by a solenoid. Chemiluminescence and electrochemical detections of products of an enzymatic reaction were utilized by coupling a photomutiplier or a photon counting unit and an amperometric detector with the immunoreaction cell. The analytical time of the present method is much shorter than that of the conventional ELISA method, which may be due to the fact that a rate-determining step of mass transfer of analyte to the surface of the magnetic microbeads is improved by use of flow technique. Magnetic microbeads have been used in separation and extraction for biologically important substances such as proteins and DNAs, taking their advantages for easy collection with a magnet. Magnetic microbeads have a potential for application to many analytical fields, such as micorarray analysis and multi-channel analysis. We continue to expand our combined technique of sequential injection with beads injection using magnetic microbeads to microarray and multi-channel analyses for realizing a real alternate to the ELISA method.

References

[1] J. Ruzicka, *Analyst*, **119**, 1925 (1994).
[2] M. Guzman, C. Pollema, J. Ruzicka, G. D. Christian, *Talanta*, **40**, 81 (1993).
[3] N. Lenghor, K. Grudpan, J. Jakmunee, B. A. Staggemeier, W. W. S. Quigley, B.J. Prazen, G. D. Christian, J. Ruzicka, R. E. Synonec, *Talanta*, **59**, 1153 (2003).
[4] D. P. Chandler, F. J. Brockman, *Trends in Anal. Chem*., **19**, 134 (2000).
[5] J. Ruzicka, A. Ivaska, *Anal. Chem*., **69**, 5024 (1997).
[6] D. A. Holman, G. D. Christian, J. Ruzicka, *Anal. Chem*., **69**, 1763 (1997).
[7] K. Jitmanee, S. K. Hartwell, J. Jakmunee, S. Jayasvasti, J. Ruzicka, K. Grudpan, *Talanta*, **57**, 187 (2002).
[8] C. H. Pollema, J. Ruzicka, G. Christian, A. Lernmark, *Anal. Chem.*, **64**, 1356 (1992).
[9] N. Soh, H. Nishiyama, K. Mishima, T. Imato, T. Masadome, Y. Asano, Y. Kurokawa, H. Tabei, S. Okutani, *Talanta*, **58**, 1123 (2002).
[10] N. Soh, H. Nishiyama, Y. Asano, T. Imato, T. Masadome, Y. Kurokawa, *Talanta*, **64**, 1160 (2004).
[11] K. Hirakawa, Masaaki Katayama, N. Soh, K. Nakanno, T. Imato, H. Ohura, S. Yamasaki, T. Imato, *Electreoanal.*, **18**, 1297 (2006).
[12] K. Hirakawa, Masaaki Katayama, N. Soh, K. Nakanno, T. Imato, *Anal. Sci.*, **22**, 81 (2006).
[13] R. –Q. Zhang, K. Hirakawa, D. Seto, N. Soh, K. Nakano, T. Masadome, K. Nagata, K. Sakamoto, T. Imato, *Talanta*, **68**, 231 (2005).
[14] R. –Q. Zhang, M. Katayama, N. Soh, K. Nakano, T. Masadome, K. Nagata, K. Sakamoto, T. Imato, Flow Analysis 10th International Conference, Abstract Book, p139 (2006).
[15] S. Farrell, N. J. Ronkainen-Matsuno, H. B. Halsall, W. R. Heineman, *Anal. Bioanal. Chem*., **379**, 358 (2004).
[16] C. A. Wijayawardhana, S. Purushothama, M. A. Cousino, H. B. Halsall, W. R. Heineman, *J. Electroanal. Chem*., **468**, 2 (1999).
[17] C. A. Wijayawardhana, H. B. Halsall, W. R. Heineman, *Anal. Chim. Acta*, **399**, 3 (1999).
[18] S. Kradtap, C. A. Wijayawardhana, K.T. Schlueter, H. B. Halsall, W. R. Heineman, Anal. Chim. Acta, **444**, 13 (2001).
[19] V. Bianchi, E. Fortunati, *Toxicol In Vitro*, **4**, 9 (1990).
[20] S. Sirisattha, Y. Momose, Y. Kitagawa, *Water Res*., **38**, 61 (2004).
[21] I. Moreno-Garrido, M. Hampel, L. M. Lubian, J. Blasco, Fresenius *J. Anal. Chem*., **371**, 474 (2001).
[22] M. Fujita, M. Ike, Y. Goda, S. Fujimoto, Y. Toyoda, K. Miyagawa, *Environ. Sci. Technol*., **32**, 1143 (1998).

(Received October 4, 2006)
(Accepted November 30, 2006)