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Sequential injection chemiluminescence immunoassay for anionic surfactants using magnetic microbeads immobilized with an antibody

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Abstract

A rapid and sensitive immunoassay for the determination of linear alkylbenzene sulfonates (LAS) is described. The method involves a sequential injection analysis (SIA) system equipped with a chemiluminescence detector and a neodymium magnet. Magnetic beads, to which an anti-LAS monoclonal antibody was immobilized, were used as a solid support in an immunoassay. The introduction, trapping and release of the magnetic beads in the flow cell were controlled by means of a neodymium magnet and adjusting the flow of the carrier solution. The immunoassay was based on an indirect competitive immunoreaction of an anti-LAS monoclonal antibody on the magnetic beads and the LAS sample and horseradish peroxidase (HRP)-labeled LAS, and was based on the subsequent chemiluminscence reaction of HRP with hydrogen peroxide and *p*-iodophenol, in a luminol solution. The anti-LAS antibody was immobilized on the beads by coupling the antibody with the magnetic beads after activation of a carboxylate moiety on the surface of magnetic beads that had been coated with a polylactic acid film. The antibody immobilized magnetic beads were introduced, and trapped in the flow cell equipped with the neodymium magnet, an LAS solution containing HRP-labeled LAS at constant concentration and the luminol solution were sequentially introduced into the flow cell based on an SIA programmed sequence. Chemiluminescence emission was monitored by means of a photon counting unit located at the upper side of the flow cell by collecting the emitted light with a lens. A typical sigmoid calibration curve was obtained, when the logarithm of the concentration of LAS was plotted against the chemiluminescence intensity using various concentrations of standard LAS samples (0–500 ppb) under optimum conditions. The time required for analysis is less than 15 min.

Keywords: Sequential injection; Anionic surfactants; Chemiluminescence; Magnetic microbeads; Immunoassay

1. Introduction

The pollution of environmental water by linear alkylebenzene sulfonates (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 [1–3]. A rapid and sensitive analytical method for monitoring such pollutants would be highly desirable. One of the official methods for the determination of LAS is based on a spectrophotometric solvent extraction using a methylene blue dye [4,5]. The use of an organic solvent in this method may not meet the requirement of green chemistry and sustainable chemistry, because waste derived from the organic solvent might lead to additional pollution. Gas chromatography or high performance liquid chromatograph, coupled with mass spectroscopy is the most reliable analytical method for determining anionic surfactants as well other environmental pollutants, because these

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methods permit the identification of homologues and isomers of LAS [6–8]. However, these methods typically involve time-consuming pretreatments of samples and expensive instrumentation. A method for determining LAS by an enzyme-linked immunosorbent assay (ELISA) based on spectrophotometric, fluorimetric or chemiluminescence detection using a microtiter plate would be a promising method for screening large number of samples such as environmental samples. A method determining LAS based on ELISA has recently been reported as a result of the successful preparation of a monoclonal anti-LAS antibody [9]. 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 [10–12]. Ruzicka et al. proposed a "beads injection technique" combined with the SIA technique [13–16]. They developed a quite unique so-called jet ring cell, which can trap microbeads, permitting an immunoreaction to proceed on the microbeads. In our previous paper, we reported on a spectrophotometric immunoassay for vitellogenin (Vg) based on an SIA system equipped with a jet ring cell, in which the immunoassay was conducted using antibody-immobilized Sephadex beads [17]. In our subsequent paper, we utilized antibody-immobilized magnetic microbeads instead of the Sephadex beads for the determination of Vg, because of their ease of trapping in an immunoreaction cell by a magnet [18]. Prior to our publication, Ruzicka and Christian reported on an immunoassay based on SIA using immunomagnetic beads [19]. An analytical method using magnetic microbeads has many advantages, in that they have a large surface area, in which many ligands can be immobilized, ease of separation from the liquid phase by a magnetic field [20]. Many applications of magnetic microbeads to enzyme-based biosensors [21,22], immunoassays [23–26], genosoensors [27] have been reported. An electrochemical immunoassay based on a microelectrode using magnetic microbeads has been also proposed by Heineman et al. [28–31]. In this paper, we wish to report on an attempt 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.

2. Experimental

2.1. Apparatus and flow cell

An SIA system was constructed from 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. The total volume larger than the 1000 μL of a holding coil is used. The flow cell used in this study was constructed from two acrylic resin plates (55 mm × 100 mm × t 1 mm^t), one of which has a groove (3 mm × 40 mm × 0.5 mm) and the other does not. 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 additional two back colored acrylic resin plates (55 mm × 100 mm × t 10 mm), which contained 1 mm^ϕ holes for solution inlet and outlet. The inlet of the cell was connected to the outlet of the switching valve with Teflon tube (length 20 cm, i.d. 0.5 mm). A square hole was prepared in the upper acrylic resin plate, to accommodate a photon counting unit (Hamamatsu Photonics, C8855, Japan), which was controlled by a PC with Windows software for C8855 (Hamamatsu Photonics, Japan). Chemiluminescence light was collected using a lens (15 mm^ϕ, f_b: 16 mm), which was set at a distance 15 mm from the flow cell. The light intensity of the chemiluminescence was measured by counting the number of photons produced in 100 ms. The photon counting data were transmitted to the PC. A square hole was also prepared in the lower acrylic resin plate to accommodate a neodymium magnet (3 mm^ϕ, Magnet Japan, Japan) embedded in an acrylic resin was placed in this hole.

2.2. Materials and reagents

The anti-LAS monoclonal antibody and horseradish peroxidase (HRP)-labeled LAS were purchased from Japan Environchemical (Japan). Magnetic beads coated with agarose gel (Agarose particles-M plain, particle size: 100 μm) and beads coated with polylactate polymer (OLA-particles-M COOH, particle size: 100 μm) were purchased from Micromod (Germany). The magnetic beads were supplied suspended in a solution at a concentration of 25 mg mL^{-1} for the former beads and 10 mg mL^{-1} for the latter. Luminol and *p*-iodophenol were purchased from Wako Chemical Co. Ltd. (Japan). The other reagents were obtained commercially and were used without further purification.

2.3. Preparation of antibody-immobilized magnetic beads [32]

A 200 μL of 10 mg mL^{-1} slurry of magnetic microbeads (PLA-particles-M COOH), the surface which contained carboxylic acid groups, was placed in a test tube and washed with a phosphate–citric acid buffer (pH 8) and the total final volume of the slurry was set 600 μL. A 200 μL of 0.2 M *N*-hydroxysuccinimide (NHS) solution and 200 μL of 0.8 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were added to the test tube and the resulting mixture was incubated for 30 min at room temperature. The beads were then washed with the above phosphate–citric acid buffer and the final volume of the slurry was set 200 μL. A 200 μL aliquot of the 500 ppb of anti-LAS antibody solution was added to the test tube and resulting

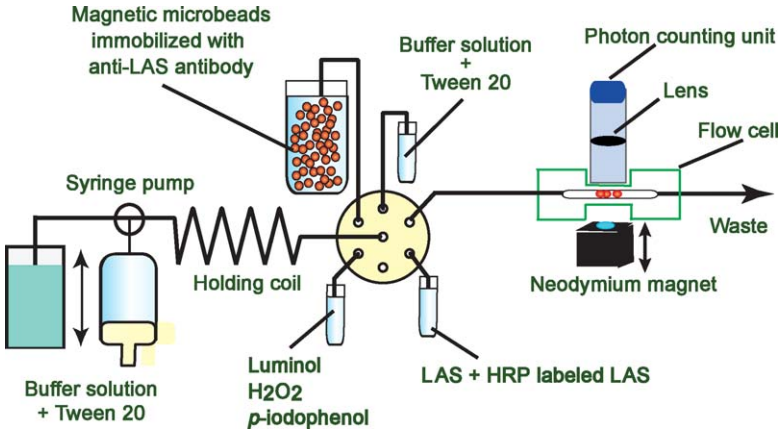

Fig. 1. Schematic flow diagram of the sequential injection system using magnetic microbeads.

slurry was incubated for 12 h at 4 °C in a refrigerator. After the incubation, the beads were washed with the phosphate–citric acid buffer several times and the final volume of the slurry set 1000 μL with the same buffer.

2.4. Evaluation of immobilization method to magnetic beads

For the selection of the immobilization method of anti-LAS antibody to the magnetic microbeads, we attempted to immobilize HRP instead of the anti-LAS antibody on the surface of the agarose coated-magnetic beads, because the amount of immobilized HRP can be easily evaluated by conventional colorimetry. The protocol for the immobilization and evaluation of the amount of HRP immobilized with magnetic beads is as follows:

(a) Amino coupling method [32]

A 20 μL slurry of agarose-coated microbeads (25 mg mL^{-1}) was placed in a microtube and a 4 M NaOH solution (40 μL) and a 4 M bromoacetic acid solution (40 μL) were added to the microtube and the resulting slurry was incubated for 16 h at room temperature. The microbeads were several times washed with a phosphate–citric acid buffer (pH 8.0) and the final volume of the slurry was kept at 120 μL with the same buffer solution. A 0.2 M NHS solution (40 μL) and a 0.8 M EDC solution (40 μL) were then added to the microtube and resulting slurry was incubated for 30 min at room temperature to activate the carboxylate groups on the microbeads. The microbeads were washed with the above buffer solution and an appropriate volume of an HRP solution was then added to the microtube and the final volume was kept at 200 μL. Five slurry samples containing HRP at final concentrations of 10, 40, 50, 100 and 200 ppb were prepared. The resulting slurry was incubated for 30 min in order to immobilize HRP on the surface of the activated ester on the microbeads. The beads were then washed with a Tris–HCl buffer solution (pH 8.0) to deactivate the unreacted NHS ester on the microbeads. After allowing the microbeads to stand in the Tris–HCl buffer solution for 30 min, they were washed several times with a phosphate–citric acid buffer solution (pH 5.2) and the final volume of the slurry was kept at 50 μL. A 2 mg mL^{-1} *o*-phenylendiamine (OPD) solution (400 μL) and a 20 mM H_2O_2 solution (50 μL) were added to the microtube for color development and the resulting slurry was incubated for 30 min at 25 °C. After the incubation, 450 μL of the supernatant of the slurry was placed in the other microtube and 450 μL of a 1.5 M H_2SO_4 solution was added to the microtube to terminate the color developing. The absorbance of the resulting solution was measured at a wavelength of 490 nm.

(b) Epoxy coupling method using chloromethyloxirane [38]

A 20 μL aliquot of a slurry of agarose-coated microbeads (25 mg mL^{-1}) was placed in a microtube and a chloromethyloxirane solution (30 μL), a 2 M NaOH solution (120 μL) and deionized water (430 μL) were added to the microtube and the resulting slurry was incubated for 2 h at 40 °C with shaking. After the incubation, the supernatant was removed and the beads were washed with water and finally with a phosphate–citric acid buffer (pH 8.0). An appropriate volume of an HRP solution was added to the microtube and the final volume was kept at 200 μL with the above buffer solution. Five slurry samples containing HRP at final concentrations of 10, 40, 50, 100 and 200 ppb were prepared. Subsequent procedures and measurement of absorbance were the same as in (a).

(c) Epoxy coupling method using ethylene glycol diglycidyl ether (EGDE) [34]

A 20 μL aliquot of a slurry of agarose-coated microbeads (25 mg mL^{-1}) was placed in a microtube and an EGDE (20 μL) and 0.2 M Na_2CO_3 solution (180 μL) were added to the microtube and the resulting slurry was incubated for 12 h at 40 °C with shaking. After the incubation, the supernatant was removed and beads were washed with water and then with a phosphate–citric acid buffer (pH 8.0). An appropriate volume of an HRP

solution was added to the microtube and the final volume was kept at 200 μL with the above buffer solution. Five slurry samples containing HRP at final concentrations of 10, 40, 50, 100 and 200 ppb were prepared. Subsequent procedures and measurement of absorbance are the same as in (a).

2.5. Chemiluminescent immunoassay for the determination of LAS using SIA system

The SIA protocol used in the determination of LAS is summarized in Table 1. After washing the Teflon tubing and the flow cell with a carrier solution of Tris–HCl buffer (pH 8.4) containing 0.1% Tween 20 (TBS-T buffer) [35], a 50 μL aliquot of the slurry of magnetic microbeads immobilized with the anti-LAS antibody was aspirated into the holding coil. The slurry was then introduced into the flow cell equipped with the magnet by pushing the syringe pump at a flow rate of 5 μL/s and the magnetic beads were then trapped in the flow cell. A 100 μL aliquot of a sample solution containing LAS at various concentrations from 0 to 500 ppb and an HRP-labeled LAS solution at concentration of 500 ppb was aspirated into the holding coil and then introduced into the flow cell. In this case, to keep the time for competitive reaction between the anti-LAS antibody on the beads and LAS or the HRP-labeled LAS at 300 s in the flow cell, a procedure of flowing 1 μL of the sample solution for 1 s and waiting for 2 s was repeated 100 times. This procedure can accelerate the rate of the immunoreaction compared with a conventional stopped-flow procedure (waiting for 300 s 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. A 500 μL aliquot of the TBS-T buffer was then introduced into the flow cell at a flow rate of 5 μL/s in order to remove nonspecifically adsorbed LAS or the HRP-labeled LAS on the beads or wall of the flow cell. A 100 μL aliquot of a chemiluminescent reagent solution containing 0.5 mM luminol, 4 mM H_2O_2 and 0.4 mM *p*-iodophenol was aspirated into the holding coil and was introduced into the flow cell at a flow rate of 20 μL/s. At the same time, the chemiluminescence intensity was measured by means of a photon counting unit. Finally, the magnet was shifted downward and 2000 μL of the TBS-T buffer was introduced into the flow cell to remove the used beads. New beads were introduced into the flow cell after the magnet was returned to the original position for the next measurement. An addition of Tween 20 to the Tris–HCl buffer is effective to reduce non-specific adsorption of the LAS-HRP and also effective to flush out the magnetic microbeads from the flow cell. The flow cell is made of polyacrylic resin and surface of which is somewhat hydrophobic. The magnetic microbeads tend to adsorb on the flow cell due to its hydrophobic feature. However, the Tris–HCl buffer containing Tween 20 makes the surface of the flow cell hydrophilic and the beads can be flushed out smoothly by the buffer containing Tween 20.

3. Results and discussion

3.1. Evaluation of immobilization method to the magnetic microbeads

In a competitive immunoreaction of an antibody with a target antigen and a labeled antigen, a lower detection of the target antigen would be expected to depend on the amount of the antibody immobilized on the solid matrices. Many methods have been reported for the immobilization of an antibody on a solid matrix [32–34,38]. Three of the more commonly used immobilization methods were compared using the agarose-coated magnetic microbeads. To more easily evaluate the methods, HRP was used instead of the anti-LAS monoclonal antibody, as described in Section 2.

Fig. 2 shows the amount of HRP immobilized on the magnetic microbeads, which was incubated with the HRP solutions the concentration range from 10 to 200 ppb. In this case, the extinction coefficient for the absorbance of the OPD in oxidized form was converted to HRP (assuming a molecular weight of 4.2×10^4 Da) concentration using the same procedures as described in Section 2. As a result, the extinction coefficient of $\varepsilon_{490\,nm} = 6.7 \times 10^{-3}\,ng^{-1}\,mL\,cm^{-1}$ was used

Table 1
Sequential injection protocol for the determination of LAS

Event	Sample	Volume (μL)	Flow rate (μL/s)
Wash	Tris–HCl buffer (pH 8.4) + Tween 20 (0.1%)	500	20
Introduction of magnetic microbeads	Slurry of magnetic microbeads immobilized with antibody (1.5 mg mL^{-1})	50	20
Trapping the magnetic microbeads with magnet	Slurry of magnetic microbeads immobilized with antibody (1.5 mg mL^{-1})	50	5
Introduction of the LAS solution containing LAS-HRP (incubation time: 5 min)	0–200 ppb LAS + 500 ppb LAS-HRP in Tris–HCl buffer (pH 8.4) + Tween 20 (0.1%)	100	1
Wash	Tris–HCl buffer (pH 8.4) + Tween 20 (0.1%)	500	5
Introduction of luminol solution	Luminol solution (0.5 mM) containing H_2O_2 (4 mM) and *p*-iodophenol (0.4 mM)	100	20
Discharge the magnetic microbeads		2000	50

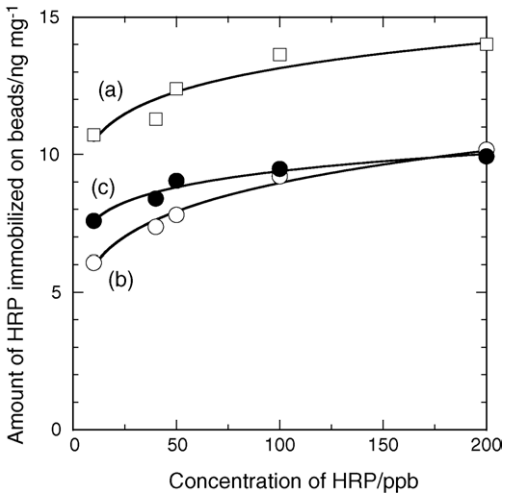

Fig. 2. Amount of HRP immobilized on magnetic microbeads by amino coupling and epoxy coupling methods: (a) amino coupling method, (b) epoxy coupling method using epichlorohidorine and (c) epoxy coupling method using ethylene glycol diglycidyl ether.

for the concentration of HRP in the final solution described in Section 2. The amino coupling method (a) appeared to be superior to the epoxy coupling methods (b) and (c) with respect to the amount of HRP immobilized on the magnetic microbeads. The concentration of HRP for incubation does not have any significant effect on immobilization efficiency. In subsequent experiments, the amino coupling method was utilized. Since, after this examination, we were able to obtain magnetic microbeads coated with polylactate, the surface of which is already a carboxylic acid, immobilization by this amino coupling method was adopted for the polylactate coated microbeads for the immobilization of anti-LAS antibody, as described in Section 2.3. The molecular weight and the molecular shape between HRP and the anti-LAS antibody are different and the activity of HRP and anti-LAS antibody after immobilization on the magnetic microbeads may be different. So, the converting the above data for immobilization of HRP to the immobilization of the anti-LAS antibody may not be adequate. However, we can estimate active site on the present magnetic microbeads for immobilization is at least 2×10^{-13} mol/g beads.

3.2. Optimization of chemiluminescent reagents

A luminol solution containing H_2O_2 and *p*-iodophenol was selected as a chemiluminescent reagent [36,37]. Chemiluminescence light is emitted when luminol reacts with H_2O_2 to give 3-aminophthalae in the presence of catalytic amounts of HRP. Since *p*-iodophenol is known to act as a sensitizer of chemiluminescence, *p*-iodophenol was added to the luminol solution. The optimum conditions for the highest sensitivity of chemiluminescence was examined using a two-channel flow injection system, where a chemiluminescent solution containing luminol, H_2O_2 and *p*-iodophenol in Tris–HCl buffer solution (pH 8.4) was pumped from one channel at a flow rate of 20 μL/s and a Tris–HCl buffer (pH 8.4) was pumped from the other channel at the same flow rate. A concentration of one of the three components was changed and the others were fixed. A 200 ppb HRP solution (100 μL) was injected from the Tris–HCl buffer channel. The coil length to the chemiluminescence detector was 20 cm, which is the same as the SIA system described in Section 2. Fig. 3 shows the concentration dependency of the three components of the luminol solution on chemiluminescence light intensity. The plots are the average of three times measurements but are somewhat scattered except for the effect of luminol. However, the tendency could be obtained from these results. The concentrations of H_2O_2, luminol and *p*-iodophenol in the chemiluminescent solution were determined to be 4, 0.5 and 0.4 mM, respectively, from the present experiments.

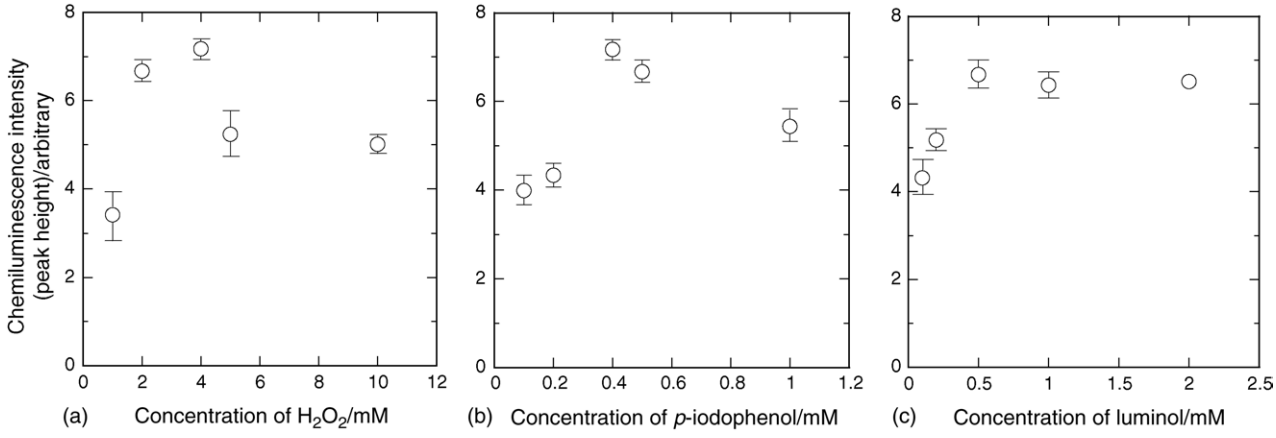

Fig. 3. Effect of composition of the chemiluminescent solution on the sensitivity of chemiluminescence intensity. (a) Varying concentrations of H_2O_2 with the concentrations of luminol and *p*-iodophenol fixed at 0.5 mM and 0.5 mM, respectively. (b) Varying concentrations of *p*-iodophenol with the concentrations of H_2O_2 and luminol fixed at 4 and 0.5 mM, respectively. (c) Varying concentrations of luminol with the concentrations of H_2O_2 and *p*-iodophenol fixed at 2 mM and 0.5 mM, respectively.

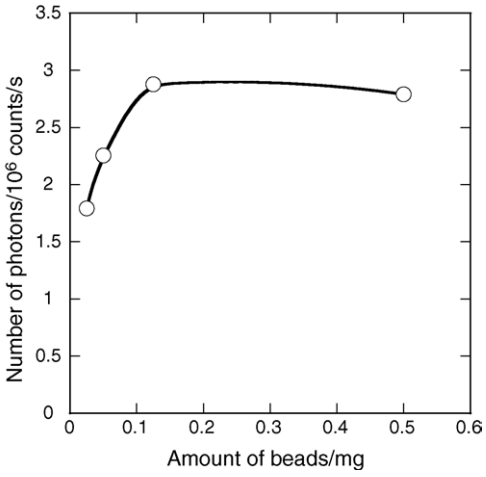

Fig. 4. Effect of the amount of magnetic microbeads introduced into the flow cell on chemiluminescence light intensity. Slurry concentration: 25 mg mL^{-1}. Volume of slurry injected: 10–100 μL. The chemiluminescent solution: 4 mM H_2O_2, 0.5 mM luminol and 0.4 mM *p*-iodophenol. The sample solution: 400 ppb HRP-labeled LAS solution (100 μL).

3.3. Optimization of amount of magnetic microbeads introduced into the flow cell

The amount of magnetic microbeads introduced into the flow would be expected to affect the sensitivity of the immunoassay, because the amount of anti-LAS antibody immobilized on the magnetic beads is proportional to the amount of microbeads present. The effect of the amount of magnetic microbeads was examined by introducing a slurry of the magnetic beads into the flow cell and the measuring the intensity of the chemiluminescence light. A 10–100 μL of a slurry of microbeads at a concentration of 25 mg mL^{-1} was introduced into the flow cell and the magnetic beads were then trapped in the flow cell. A 400 ppb of an HRP-labeled LAS solution (100 μL) was then introduced into the flow cell and the intensity of the chemiluminescence light was measured using the photon counting unit. The light intensity is expressed by normalizing the number of photons counted in 1 s. In Fig. 4, the number of photons is plotted as a function of the amount of magnetic beads, which was calculated from the concentration of the slurry and the volume of slurry introduced into the flow cell. As can be seen from Fig. 4, the number of photons increases steeply with increasing amount of microbeads and reaches a constant values for a amounts of microbeads above 0.15 mg. This saturation phenomena seems to be strange because the larger the amount of microbeads are trapped in the flow cell, the larger amount of HRP-labeled LAS would be bound to the anti-LAS antibody on the microbeads. Therefore, the number of photons should be proportional to the amount of microbeads trapped in the flow cell. However, an observation of the microbeads trapped in the flow cell by the magnet can explain this saturation phenomena. The magnet used in this work is Ø 3 mm in diameter and can trap a limited number of magnetic beads, because of its effective area. The microbeads used in this work had an averaged diameter of about 100 μm, therefore about 10^3 magnetic beads were calculated to be trapped on a magnet with a diameter of 3 mm$^{\phi}$. Even when a slurry containing 0.5 mg is introduced into the flow cell, the excess microbeads over 0.35 mg may be not trapped by the magnet and may flow away from the flow cell. Even when the excess amount of the microbeads can be trapped in multi layers, only the chemiluminescence from the microbeads of the top layer may be effective. To enhance the chemiluminescence light, a microbead with smaller diameter and a flow cell with a larger surface area for detecting the chemiluminescence light may be useful. In the subsequent experiments, 0.125 mg of magnetic microbeads was introduced into the flow cell.

3.4. Calibration for LAS under the optimized conditions

A calibration curve for LAS was obtained according to the SIA protocol shown in Table 1 under optimized flow conditions. After the introduction of 0.125 mg of magnetic microbeads immobilized with an anti-LAS antibody into the flow cell, 100 μL of a standard LAS solution at several concentrations (0, 50, 100, 150, 200, 250 and 500 ppb) containing 500 ppb HRP-labeled LAS was introduced. As pointed out in our previous paper, the incubation time for an immunoreaction between an antigen in a solution and an antibody on a solid matrix affects the total analytical time. This is because the mass transfer rate of the antigen to the antibody on the solid matrix is generally a rate limiting process. In order to enhance this mass transfer rate, in our previous paper, a 100 μL of an antigen solution was repeatedly moved back and forth in the flow cell at 10 s intervals. As a result, the incubation time for the immunoreaction was dramatically shortened by 1/3 to 1/6 [17,18]. This can be successfully achieved by the fact that the magnetic microbeads were completely trapped by the magnet even under flow conditions. However, swinging the antigen solution may diffuse the solution, i.e., the antigen solution may be diluted by the adjacent carrier solution. In the present procedure, a 100 μL of the antigen solution, LAS and HRP-labeled LAS mixed solution, was introduced into the flow cell by the procedure that 1 μL of the antigen solution was pumped for 1 s at a flow rate of 1 μL/s and then stopped for 2 s and this procedure was repeated 100 times. The incubation time by this procedure is 5 min.

Fig. 5(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 flow 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. Fig. 5(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

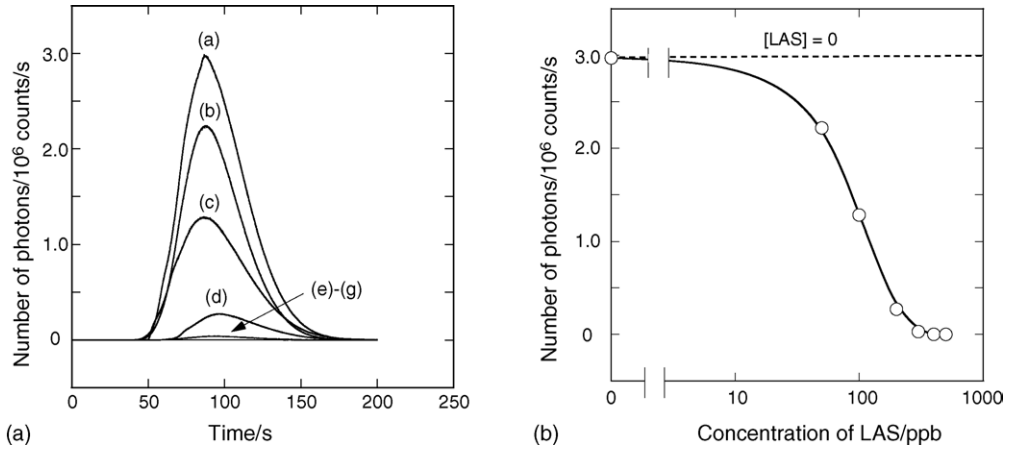

Fig. 5. (a) Chemiluminescence signals for a sample solution containing various concentrations of LAS and 500 ppb HRP-labeled LAS at a fixed concentration. Conditions: amount of magnetic microbeads introduced into the flow cell: 0.125 mg (50 μL of a slurry), Chemiluminescent solution: 0.5 mM H_2O_2, 4 mM luminol and 0.5 mM *p*-iodophenol. Concentration of LAS: (a) 0, (b) 50, (c) 100, (d) 150, (e) 200, (f) 250, (g) 500 ppb in 500 ppb HRP-labeled LAS solution. Volume of sample solution containing LAS and HRP-labeled LAS: 100 μL. (b) Calibration curve for LAS. Conditions are the same as (a).

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 = 2.6×10^{-10} mol/mL) and that of the HRP-labeled LAS (500 ppb = 1.2×10^{-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.

4. Conclusion

A chemiluminescent immunoassay using magnetic microbeads in an SIA system equipped with a magnet was proposed for the highly sensitive and rapid determination of LAS. The analytical time for the proposed immunoassay was less than 15 min including 5 min of incubation time for a competitive immunoreaction for one sample. The short analytical time can be realized by using a flow technique that a sample solution repeatedly flows and stops in a short period. This flow technique is due to the use of magnetic microbeads in an SIA system. The proposed method should be validated against the currently accepted analytical method and the tested with environmental sample. The application of the proposed method to the determination of LAS in actual environmental water samples is currently underway.

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References

[1] V. Bianchi, E. Fortunati, Toxicol. In Vitro 4 (1990) 9.
[2] S. Sirisattha, Y. Momose, Y. Kitagawa, Water Res. 38 (2004) 61.
[3] I. Moreno-Garrido, M. Hampel, L.M. Lubian, J. Blasco, Fresenius J. Anal. Chem. 371 (2001) 474.
[4] D.C. Abbott, Analyst 87 (1962) 286.
[5] L.S. Clesceri, A.E. Greenberg, R.R. Trussel, Standard Methods for the Examination of Water and Wastewater, 17th ed., American Public Health Association, Washington, DC, 1989 (Section 5540C).
[6] M.L. Trehy, W.E. Gledhill, R.G. Orth, Anal. Chem. 62 (1990) 2581.
[7] A. Nakae, K. Tsuji, M. Yamanaka, Anal. Chem. 52 (1980) 2275.
[8] M. Kikuchi, A. Tokai, T. Yoshida, Water Res. 20 (1986) 643.
[9] M. Fujita, M. Ike, Y. Goda, S. Fujimoto, Y. Toyoda, K. Miyagawa, Environ. Sci. Technol. 32 (1998) 1143.
[10] J. Ruzicka, Analyst 119 (1994) 1925.
[11] M. Guzman, C. Pollema, J. Ruzicka, G.D. Christian, Talanta 40 (1993) 81.
[12] N. Lenghor, K. Grudpan, J. Jakmunee, B.A. Staggemeier, W.W.S. Quigley, B.J. Prazen, G.D. Christian, J. Ruzicka, R.E. Synovec, Talanta 59 (2003) 1153.
[13] D.P. Chandler, F.J. Brockman, Trends Anal. Chem. 19 (2000) 134.
[14] J. Ruzicka, A. Ivaska, Anal. Chem. 69 (1997) 5024.
[15] D.A. Holman, G.D. Christian, J. Ruzicka, Anal. Chem. 69 (1997) 1763.
[16] K. Jitmanee, S.K. Hartwell, J. Jakmunee, S. Jayasvasti, J. Ruzicka, K. Grudpan, Talanta 57 (2002) 187.
[17] N. Soh, H. Nishiyama, K. Mishima, T. Imato, T. Masadome, Y. Asono, Y. Kurokawa, H. Tabei, S. Okutani, Talanta 58 (2002) 1123.
[18] N. Soh, H. Nishiyama, Y. Asono, T. Imato, T. Masadome, Y. Kurokawa, Talanta 64 (2004) 1160.
[19] C.H. Pollema, J. Ruzicka, G.D. Christian, Anal. Chem. 64 (1992) 1358.
[20] S. Solé, A. Merkoçi, S. Alegret, Trends Anal. Chem. 20 (2001) 2.
[21] A. Günther, U. Bilitewski, Anal. Chim. Acta 300 (1995) 117.
[22] R. Kindervater, W. Kunnecke, R. Schmid, Anal. Chim. Acta 234 (1990) 113.
[23] A.L. Ghindilis, R. Krishnan, P. Atanasov, E. Wilkins, Biosens. Bioelectron. 12 (1997) 415.
[24] S. Solé, S. Alegret, F. Céspedes, E. Fàbregas, Anal. Chem. 70 (1998) 1462.
[25] M. Santandreu, S. Solé, E. Fàbregas, S. Alegret, Biosens. Bioelectron. 13 (1998) 7.

[26] M. Dequaire, C. Degrand, B. Limoges, Anal. Chem. 71 (1999) 2571.
[27] M.I. Pividori, A. Merkoçi, S. Alegret, Biosens. Bioelectron. 15 (2000) 291.
[28] S. Farrell, N.J. Ronkainen-Matsuno, H.B. Halsall, W.R. Heineman, Anal. Bioanal. Chem. 379 (2004) 358.
[29] C.A. Wijayawardhana, S. Purushothama, M.A. Cousino, H.B. Halsall, W.R. Heineman, J. Electroanal. Chem. 468 (1999) 2.
[30] C.A. Wijayawardhana, H.B. Halsall, W.R. Heineman, Anal. Chim. Acta 399 (1999) 3.
[31] S. Kradtap, C.A. Wijayawardhana, K.T. Schlueter, H.B. Halsall, W.R. Heineman, Anal. Chim. Acta 444 (2001) 13.
[32] J. Turkova, J. Chromatogr. B 722 (1999) 11.
[33] S. Sole, A. Merkoci, S. Alegret, Trends Anal. Chem. 20 (2001) 2.
[34] N. Shimizu, K. Sugimoto, J. Tang, T. Nishi, I. Sato, M. Hiramoto, S. Aizawa, M. Hatakeyama, R. Ohba, H. Hatori, T. Yoshikawa, F. Suzuki, A. Oomori, H. Tanaka, H. Kawaguchi, H. Watanabe, H. Handa, Nat. Biotechnol. 18 (2002) 877.
[35] N. Kuroda, R. Shimoda, M. Wada, K. Nakashima, Anal. Chim. Acta 403 (2000) 131.
[36] B. Cercek, K. Roby, L. Cercek, J. Biol. Chem. 9 (1994) 273.
[37] L.J. Kricka, X.Y. Ji, J. Biol. Chem. 11 (1996) 137.
[38] K. Kasai, I. Matsumoto, M. Beppu, Affinity Chromatography, Tokyo Kagaku Dojin, 1991, Chapter 7 (in Japanese).