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Chemiluminescence sequential injection immunoassay for vitellogenin using magnetic microbeads

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Abstract

A rapid and sensitive immunoassay for the determination of carp vitellogenin (Vg) is described. The method involves a sequential injection analysis (SIA) system equipped with a chemiluminescence detector and a samarium-cobalt magnet. An anti-Vg monoclonal antibody, immobilized on magnetic beads, was used as a solid support for the immunoassay. The introduction, trapping and release of the magnetic beads in the flow cell were controlled by a samarium-cobalt magnet and the flow of the carrier solution. The immunoassay was based on a sandwich immunoreaction of anti-Vg monoclonal antibody (primary antibody) on the magnetic beads, Vg, and the anti-Vg antibody labeled with horseradish peroxidase (HRP) (secondary antibody), and was based on a subsequent chemiluminescence reaction of HRP with hydrogen peroxide and *p*-iodophenol, in a luminol solution. The magnetic beads to which the primary antibody was immobilized were prepared by coupling the primary antibody with the magnetic beads after an agarose-layer on the surface of the magnetic beads was epoxidized. The primary antibody-immobilized magnetic beads were introduced, and trapped in the flow cell equipped with the samarium-cobalt magnet, a Vg sample solution, an HRP-labeled secondary antibody solution 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 photomultiplier located at the upper side of the flow cell. The optimal incubation times both for the first and second immunoreactions were determined to be 20 min. A concave calibration curve was obtained between Vg concentration and chemiluminescence intensity when various concentrations of standard Vg samples (2–100 ng mL^{-1}) were applied to the SIA system under optimal conditions. In spite of a narrow working range, the lower detection limit of the immunoassay was about 2 ng mL^{-1}.

Keywords: Sequential injection; Vitellogenin; Chemiluminescence; Magnetic microbeads; Immunoassay

1. Introduction

In recent years, some man-made chemicals have been suspected to have the ability to disrupt endocrine systems of human beings as well as wildlife species [1]. Such so-called endocrine disrupting chemicals (EDCs) are thought to mimic natural hormones and thus, cause abnormal sexual development and reduce male fertility [2–4].

Hence, an analytical technique for evaluating the degree of pollution of our environment by EDCs is an important issue in public safety. One way possible solution is to determine all chemicals suspected as being EDCs. However, a large number of chemicals have been reported as EDCs and their chemical structures and environmental concentrations are diverse. Therefore, it would be difficult to evaluate the degree of pollution by detecting all EDCs species separately. Furthermore, such a strategy is not suitable for evaluating the effect of unknown EDCs, which may exist in our environment.

The risk assessment for EDCs using vitellogenin (Vg) as a biomarker would be quite useful for evaluating the degree

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of pollution especially in an aqueous environment. Vg is a female specific phospholipoprotein synthesized by female fish and should not be present in normal male fish. However, when male fish are exposed to estrogen or estrogenic EDCs, significant amounts of Vg are found in male fish. Therefore, Vg in male fish represents a biomarker for evaluating environmental risk caused by EDCs [5,6]. A risk assessment for EDCs using Vg would have great advantages because the synergistic effect to wildlife species, which is caused by exposure to both known EDCs and suspected EDCs, could be evaluated directly.

Several immunological methods for the determination of Vg have been proposed including single radical immunodiffusion [7], electroimmunoassay [8–10], radioimmunoassay (RIA) [11,12], and an enzyme-linked immunosorbent assay (ELISA) [13–15]. In particular, the ELISA method using a microtiter plate is widely used due to its safety and simplicity. Hara et al. recently reported on a highly sensitive and specific quantification of Vg based on a chemiluminescent immunoassay (CLIA) using a microtiter plate [16,17]. However, methods using microtiter plates are procedurally complicated and time-consuming because of the manual wash processes and sample-addition processes that are involved.

A sequential injection analysis (SIA) technique is a potentially promising method for the immunoassay of Vg because many of the analytical processes required for its determination can be completed automatically in a flow system. In an SIA system, for example, the introduction of several types of solutions into a flow cell equipped with a suitable detector can be strictly and freely controlled by a computer program and, as a result, a series of processes in an immunoassay could be readily automated. Furthermore, the consumption of reagents and waste solutions in SIA measurements are less than those required for FIA measurements. In our previous paper, we reported on a spectrophotometric immunoassay for carp Vg based on an SIA system equipped with a jet ring cell, in which the immunoassay was conducted using antibody-immobilized Sephadex beads [18]. A detection limit of about $5\,ng\,mL^{-1}$ was achieved for the determination of Vg by optimization of the immunoreaction among Vg, a primary and a secondary antibody. However, the precise adjustment of gap was required for trapping the antibody-immobilized beads in the jet ring cell. Magnetic beads covered with agarose gel have recently become commercially available. Since such magnetic beads are easily trapped by a magnet, they would be predicted to serve as alternate for the Sephadex beads in our previous method.

In this paper, we report on an attempt to develop a novel SIA system for the more rapid and sensitive determination of Vg using magnetic beads. A microchannel flow cell equipped with a chemiluminescence detector and a magnet was fabricated for use in this work. The handling of the magnetic beads immobilized with the anti-Vg monoclonal antibody in the SIA system and immunoreactions on the magnetic beads is described below.

2. Experimental

2.1. Apparatus and structure of flow cell

An SIA system (Fig. 1(a)) 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. The flow cell used in this study was constructed by sandwiching a silicon rubber pad 0.5 mm in thickness with a groove (2 mm × 25 mm) with two acrylic resin plates (50 mm × 100 mm × 13 mm) (Fig. 1(b)). The upper acrylic plate was inletted with a square (25 mm × 25 mm × 10 mm deep) into which a photomultiplier (Hamamatsu Photonics, H5784, Japan) was inserted. A square hole was also prepared in the lower acrylic plate and a samarium-cobalt magnet (Magnet Japan, Japan) embedded in acrylic resin was placed in this hole. The sensitivity of the photomultiplier was controlled by a control unit, C7169 (Hamamatsu Photonics, Japan) and intensity of the chemiluminescent light was converted into voltage and the signal collected at 0.1 s intervals by a data logger (Hioki, 8420, Japan).

2.2. Materials and reagents

Anti-carp Vg monoclonal antibody, standard carp Vg, and HRP-labeled anti-carp Vg antibody were purchased from Trans Genic Inc. (Kumamoto, Japan). Magnetic beads coated with agarose gel (Agarose particles-M plain, particle size: 100 μm) were purchased from Micromod (Germany). The magnetic beads were supplied suspended in a solution at a concentration of $25\,mg\,mL^{-1}$. Tris(hydroxymethyl)aminomethane, luminol, and *p*-iodophenol were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). Epichlorohydrin, hydrogen peroxide (H_2O_2), Tween20, disodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Kishida Chemical Co. Ltd. (Osaka, Japan).

2.3. Preparation of antibody-immobilized magnetic beads

There are several methods for immobilizing an antibody on magnetic beads coated with an agarose gel. In order to select an appropriate immobilization method, for a preliminary examination, the HRP-labeled antibody, the secondary antibody used in the sandwich immunoassay though, was used in these experiments, instead of the primary antibody. The reason for this is the ease of confirming whether the magnetic beads contained an attached antibody by chemiluminescent detection. One hundred twenty microliters of 2 M NaOH, 30 μL of epichlorohydrin, and 150 μL of pure water were added to 300 μL of a suspension of the magnetic beads

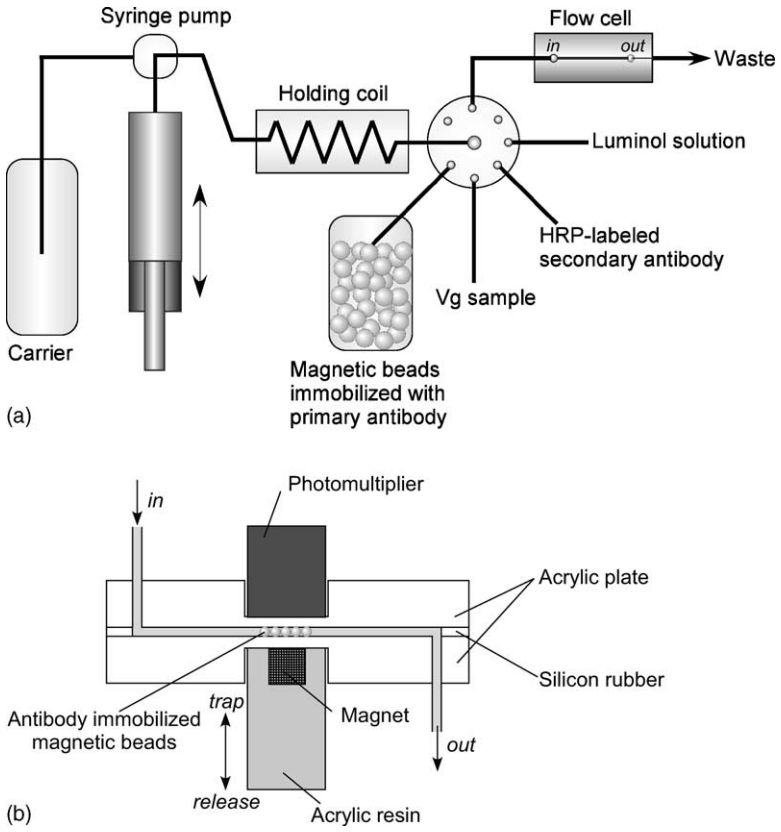


Fig. 1. Sequential injection analysis system (a) and a flow cell (b) used in this study.

(500 μg mL^{-1}) in a microtube, and the suspension was incubated for 2 h at 40 °C, after which, the magnetic beads were washed repeatedly with pure water by centrifugation. The magnetic beads were suspended in 300 μL of pure water in a microtube and 300 μL of an HRP-labeled anti-Vg antibody solution (5, 10, 20, 50 ng mL^{-1}) was added (final concentrations of the antibody: 2.5, 5, 10, 25 ng mL^{-1}) and the resulting suspensions were allowed to stand overnight for the immobilization of the HRP-labeled antibody on the magnetic beads. Since the immobilization method described above was found to be sufficient for chemiluminescent detection, an anti-Vg monoclonal antibody solution (500 ng mL^{-1}) was substituted for the HRP-labeled antibody solution for the immobilization of the primary antibody on the magnetic beads.

2.4. Observation of magnetic beads controlled by magnet in flow cell

One hundred fifty microliters of the suspension of the magnetic beads (25 μg mL^{-1}) was aspirated into a holding coil and introduced into the flow cell equipped with the samarium-cobalt magnet by switching a valve and pushing the syringe of the pump at a flow rate of 2 μL s^{-1} using the SIA instrument. A digital microscope, BS-D8000 II (Sonic, Japan) was set above the magnet position of the flow cell in place of the chemiluminescence detector to observe of the beads. After confirming that the magnetic beads were trapped in the flow cell, the behavior of the trapped beads was observed by the digital microscope when a 100 mM phosphate buffer solution (PBS) was allowed to flow at a rate of 5, 10, 20, and 50 μL s^{-1}. The behavior of trapped beads was also observed when the samarium-cobalt magnet was removed where the 100 mM PBS was flowing at a flow rate of 5 μL s^{-1}.

2.5. Chemiluminescence detection using magnetic beads immobilized with HRP-labeled antibody

In order to evaluate the performance of a chemiluminescence detector, the chemiluminescence from the reaction between the luminol and HRP, immobilized on the magnetic beads, was measured using the HRP-labeled antibody-immobilized magnetic beads prepared in Section 2.3. Two hundred microliters of a suspension of magnetic beads (188 μg mL^{-1}) was aspirated into a holding coil, and introduced into the flow cell by switching a valve and pushing the syringe of the pump at a flow rate of 5 μL s^{-1}. The trapped beads were perfused with 1 mL of a 100 mM PBS to remove unbound HRP-labeled anti-Vg antibody from the beads. Three hundred microliters of a 0.44 mM luminol solution containing 1 mM H_2O_2 and 0.8 mM *p*-iodophenol was then introduced into the flow cell, and the emitted chemiluminescent light was measured by a photomultiplier tube (control voltage, 950 mV). Finally, the samarium-cobalt

Table 1
Sequential injection protocol for the determination of Vg

Event	Sample	Volume (μL)	Flow rate (μL s^{-1})
1. Introduction of magnetic beads	Magnetic beads immobilized with the primary antibody (250 μg mL^{-1})	150	5
2. Blocking unreacted epoxy group	Tris buffer	1000	5
3. Wash	PBS	1000	5
4. Introduction of Vg sample	Vg (2–100 ng mL^{-1})	300	5
5. Incubation (20 min)	–	–	2[a]
6. Wash	PBS-T and PBS	1000 each	5
7. Introduction of the secondary antibody	HRP-labeled anti-Vg antibody (500 ng mL^{-1})	300	5
8. Incubation (20 min)	–	–	2[a]
9. Wash	PBS	1000	5
10. Introduction of luminol solution	Luminol solution (0.44 mM) containing H_2O_2 (1 mM) and *p*-iodophenol (0.8 mM)	300	5
11. Wash	PBS-T and PBS	1000 each	5

[a] The sample solution in the flow cell was moved forward and backward repeatedly at 10 s interval.

magnet was shifted downward from the flow cell and the magnetic beads were discarded by a flow of PBS. New beads were introduced into the flow cell and trapped by the samarium-cobalt magnet for the next measurement.

2.6. Chemiluminescent immunoassay for the determination of Vg using SIA system

The SIA protocol used in the determination of Vg is summarized in Table 1. One hundred fifty microliters of a suspension of the primary antibody-immobilized magnetic beads prepared in 2.3 (250 μg mL^{-1}) was aspirated into a holding coil, and introduced into the flow cell equipped with the samarium-cobalt magnet by switching a valve and pushing the syringe of the pump at a flow rate of 5 μL s^{-1}. The trapped beads were perfused with 1 mL of a Tris buffer solution, to block the unreacted epoxy group on the magnetic beads and then perfused with 1 mL of 100 mM PBS to remove unbound anti-carp Vg monoclonal antibody from the beads. Then, 300 μL of an analyte Vg solution (2, 10, 20, 50, 100 ng mL^{-1}) was introduced into the flow cell in the same manner as was used to introduce the beads. The beads in the flow cell were incubated with the Vg sample at room temperature for 5–30 min. During the incubation, the sample solution in the flow cell was moved forward and backward repeatedly at a flow rate of 2 μL s^{-1} by the syringe pump to increase the rate of the immunoreaction, which should be determined by the rate of diffusion of the analyte to the antibody immobilized on the beads. After the incubation, the beads were sequentially perfused with 1 mL of a 100 mM PBS containing 1% Tween 20 (PBS-T) and 1 mL of 100 mM PBS to remove free Vg from the beads. Three hundred microliters of a 100 mM PBS containing HRP-labeled anti-Vg antibody at a concentration of 500 ng mL^{-1} was then introduced into the flow cell and the beads in the flow cell were incubated at room temperature for 5–30 min. During the incubation, the HRP-labeled anti-Vg antibody solution in the flow cell was also moved forward and backward repeatedly at a flow rate of 2 μL s^{-1}. After the incubation, the beads were sequentially perfused with 1 mL of the 100 mM PBS-T and 1 mL of 100 mM PBS, to remove the unbound HRP labeled anti-Vg antibody from the beads. Three hundred microliters of a 0.44 mM luminol solution containing 1 mM H_2O_2 and 0.8 mM *p*-iodophenol was introduced into the flow cell, and the intensity of the chemiluminescence was measured by a photomultiplier tube (control voltage, 950 mV). Finally, the samarium-cobalt magnet was shifted downward and 100 mM PBS was introduced into the flow cell to discard the used beads. New beads were introduced into the flow cell after the magnet was returned to the original position for the next measurement.

3. Results and discussion

3.1. Handling magnetic beads in flow system using magnet

Magnetic beads have increasingly been used in the area of bioscience and medicine since the mid-1970s due to their easy handling by a magnet [19]. Applications of magnetic beads to purification or immunoassay are representative examples. Using magnetic beads for a separation in these applications is an ideal way to shorten both adsorption and separation steps [20]. Since magnetic beads are usually used in a batch system, little information is available on their behavior of in a flow system. In this study, we examined the behavior of magnetic beads in an in-house designed flow system. A digital microscope was used to observe the magnet beads. In the first step, a suspension of the magnetic beads was introduced, at a flow rate of 2 μL s^{-1}, into the flow cell equipped with the samarium-cobalt magnet. After trapping the magnet beads, a 100 mM PBS was allowed to flow in the flow cell at flow rates of 5, 10, 20, and 50 μL s^{-1}. As can be seen from Fig. 2(a)–(e), the magnet beads are completely trapped by the magnet in the flow cell and are not affected by the solution flow at the above flow rates. The behavior of the

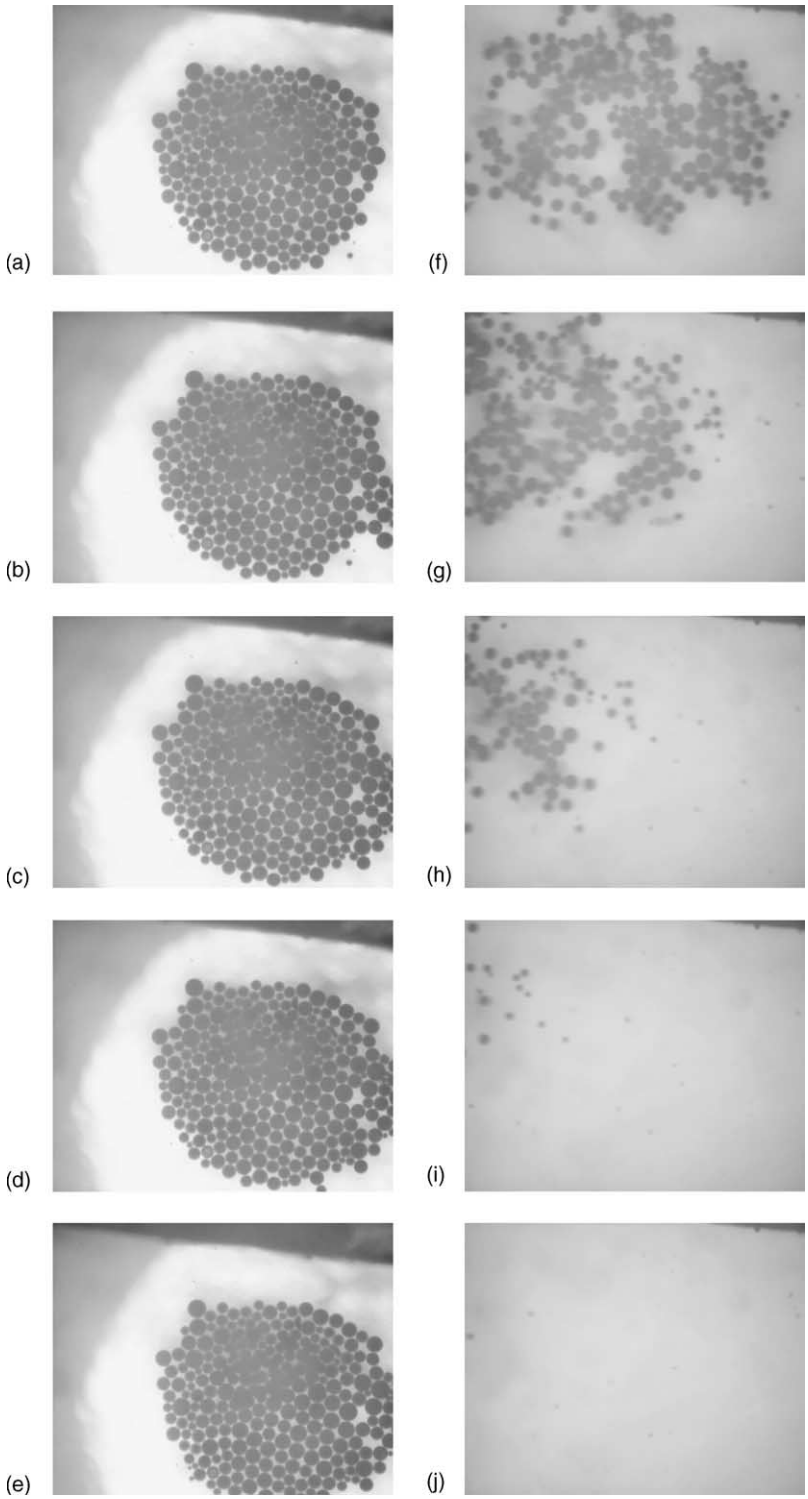
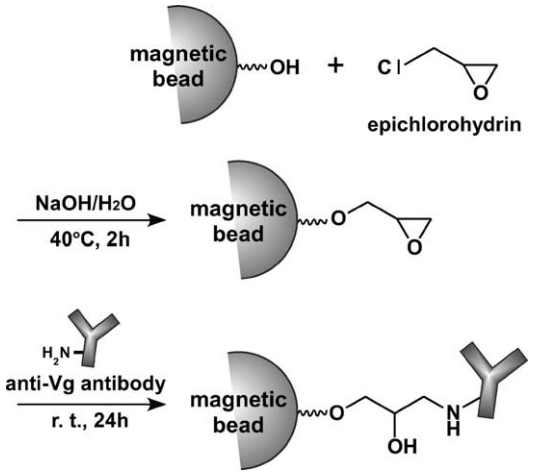


Fig. 2. Magnetic beads in the flow cell. The behavior of the magnetic beads was observed by a digital microscope when the beads were trapped with the magnet under perfusion with PBS at a various flow rates (a)–(e), and when the beads were drained by removing the magnet under perfusion with PBS at a flow rate of 5 μL s^{-1} (f)–(j). (a) Immediately after the introduction of the magnetic beads at a flow rate of 2 μL s^{-1}; (b) flow rate of 5 μL s^{-1}; (c) 10 μL s^{-1}; (d) 20 μL s^{-1}; (e) 50 μL s^{-1}; (f) 1 s later after the removing the magnet from the flow cell; (g) 2 s; (h) 3 s; (i) 4 s and; (j) 5 s.

trapped beads was next observed when the samarium-cobalt magnet was shifted downward where the PBS was allowed to flow at a rate of 5 μL s^{-1}. As shown in Fig. 2(f)–(j), the magnetic beads move out of the flow cell within 5 s after shifting the magnet downward. As a result, it can be concluded that the magnetic beads are strictly controlled in the flow system, when the samarium-cobalt magnet is used.



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

3.2. Preliminary chemiluminescence detection using magnetic beads immobilized with HRP-labeled antibody

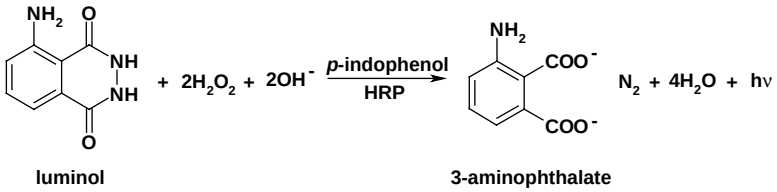
In a preliminary examination of the chemiluminescent determination of Vg, a chemiluminescent detection using the magnetic beads, on the surface of which HRP-labeled antibody was immobilized, was examined. Agarose particles-M plain are magnetic beads (Fe_2O_3) covered with a layer of agarose so that the surface of the beads can be easily modified by a number of chemical methods. In the present study, the agarose-layer was epoxidized by the treatment with an NaOH solution and epichlorohydrin, to immobilize the HRP-labeled antibody (Scheme 1).

A luminol solution containing H_2O_2 and *p*-iodophenol was selected as a chemiluminescent reagent in the present study. Luminol and H_2O_2 react to give 3-aminophthalate and chemiluminescence is produced in the presence of catalytic HRP (Scheme 2). Since the chemiluminescence in the above system is known to be enhanced by *p*-iodophenol, a sensitizer [21], *p*-iodophenol was added to the luminol solution. After the introduction of 200 μL of the suspension of magnetic beads (188 μg mL^{-1}) immobilized with the HRP-labeled antibody into the flow cell, 300 μL of a 0.44 mM luminol solution containing 1 mM H_2O_2 and 0.8 mM *p*-iodophenol was introduced into the flow cell. As shown in Fig. 3, the chemiluminescence intensity increases with increasing concentrations of HRP-labeled antibody. This suggests that the amount of HRP-labeled antibody immobilized on the magnetic beads increases with the initial concentration of HRP-labeled antibody solution where the epoxidized magnetic beads reacted. From this result, the immobilization method adopted has the potential for use in the immobilization of primary antibodies on magnetic beads.

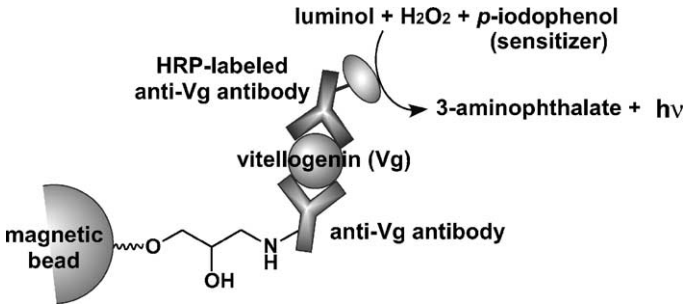
3.3. Effect of incubation time on sensitivity of chemiluminescent immunoassay for the determination of Vg

In the present method, a sandwich immunoassay was adopted for the determination of Vg, where the following two immunoreactions occur, namely, the first reaction between the primary antibody immobilized on the magnetic beads and the Vg sample, and a second reaction between the Vg sample, which is complexed with the primary antibody on the beads, and the secondary antibody labeled with HRP (Scheme 3). The incubation time for both immunoreactions would be expected to affect the sensitivity of the method. Thus, the effect of incubation time for the two immunoreactions was separately examined using a solution containing 10 ng mL^{-1} of Vg. Fig. 4 shows the time-dependence for chemiluminescent responses, where the incubation time of the first reaction was varied and that of the second reaction was kept constant at 20 min. As shown in Fig. 4, the maximum response is observed when the time for the first reaction was around 20 min. The effect of incubation time for the second reaction was examined by changing the second incubation time, where the incubation time of the first reaction was kept constant at 20 min, as shown in Fig. 5. As can be seen from Fig. 5, the maximum response is observed when the incubation time is at around 20 min as well, as in the case of the first reaction. In our previous report where antibody immobilized Sepharose beads were used in the jet ring cell, it took 2 h and 1 h, respectively—required for the first and second immunoreactions [18]. In this case, the sample solution and the secondary antibody solution were static after introduction into the jet ring cell. The shorter incubation time may be due to the fact that the diffusion of Vg or the secondary antibody to the magnetic beads was accelerated by moving the solution back and forth in the flow cell.

Generally, since it would be expected that an immunoreaction between an antigen and an antibody would proceed with an increase in incubation time, the observed



Scheme 2. Chemiluminescent reaction used in this study.



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

chemiluminescence response based on the immunoreaction should reach a plateau after the immunoreaction reaches equilibrium. However, in the case where the incubation time was longer than 20 min for the first or the second immunoreactions, the chemiluminescent responses were lower than those observed at 20 min. Although the reason for these unexpected results is unclear at this stage, the following factor may be considered as a partial explanation for the unexpected results. Namely, a part of the sample solutions containing antigen or antibody introduced into the flow cell was diluted due to diffusion to the adjacent PBS solution, because the zone of the sample solutions in the cell was moved back and forth repeatedly at 10 s intervals, in order to accelerate the immunoreaction. From an analytical point of view with respect to sensitivity, an incubation time of 20 min was selected for the first and second immunoreactions.

3.4. Calibration curve for Vg

A calibration curve for Vg was obtained according to the protocol shown in Table 1. The magnetic beads immobilized with the primary antibody were introduced into a flow cell equipped with a magnet, followed by introducing a standard Vg solution at several concentrations (2, 10, 20, 50, 100 ng mL^{-1}). After a 20 min incubation, 500 ng mL^{-1} of the HRP-labeled secondary antibody solution was introduced into the flow cell. After a second 20 min incubation, the luminol solution containing H_2O_2 and *p*-iodophenol was introduced into the flow cell, and the chemiluminescence intensity was then measured. During the incubation time, the introduced solution was moved back and forth in the flow cell. Fig. 6 shows a calibration curve for Vg obtained using the above procedures. The chemiluminescence intensity steeply increases with an increase in the concentration in concentration ranges below 20 ng mL^{-1} and was saturated at concentrations higher than 20 ng mL^{-1}. This narrow working range may be due to the fact that the amount of primary antibody immobilized on the magnetic beads is low, compared with that immobilized on the Sepharose beads reported previously. The detection limit for the determination of Vg by the present method is about 2 ng mL^{-1}. This detection limit is slightly improved compared with that for the

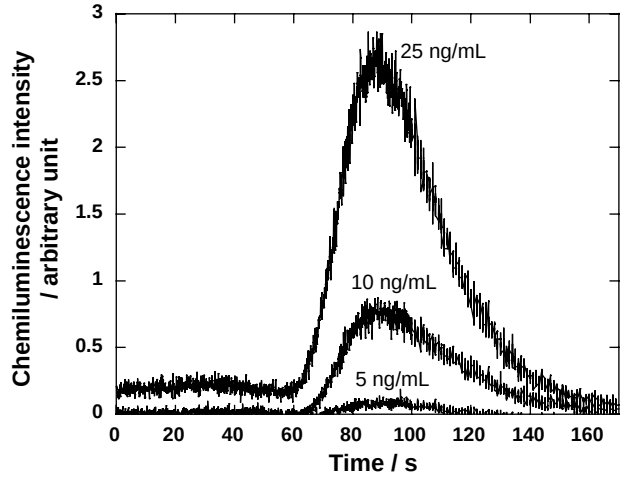


Fig. 3. Chemiluminescent responses when the luminol/H_2O_2/*p*-iodophenol mixed solution was introduced to magnetic beads immobilized with various concentrations of HRP-labeled antibody in the flow cell. The concentrations of HRP-labeled antibody used for the immobilization were 5, 10, and 25 ng mL^{-1}.

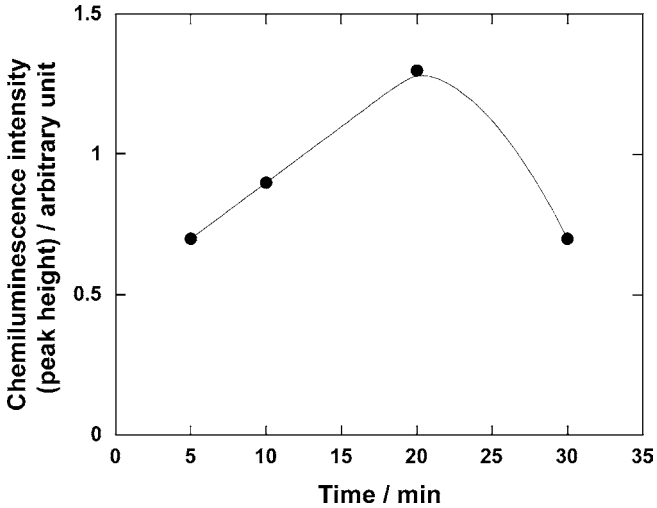


Fig. 4. Effect of incubation time on the first immunoreaction between the anti-Vg monoclonal antibody on the magnetic beads and Vg. Incubation time for the second immunoreaction between the primary antibody-Vg complex and the HRP-labeled second antibody was kept constant at 20 min. The concentration of Vg is 10 ng mL^{-1}.

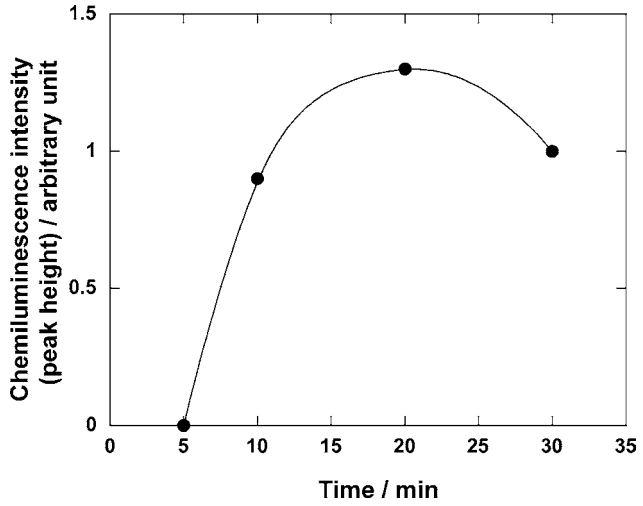


Fig. 5. Effect of incubation time on the second immunoreaction between the primary antibody-Vg complex and the HRP-labeled secondary antibody. Incubation time for the first immunoreaction between the anti-Vg monoclonal antibody on the magnetic beads and Vg was kept constant at 20 min. The concentration of Vg is 10 ng mL^{-1}.

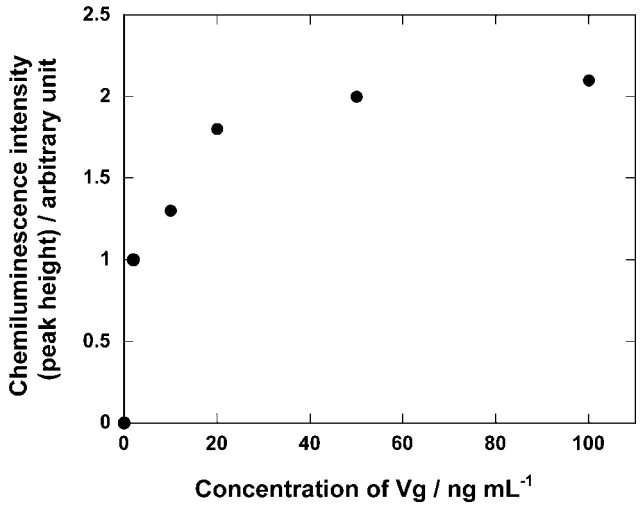


Fig. 6. Calibration curve for the determination of Vg using the present method. Incubation times for both the first and the second immunoreactions were 20 min. The concentrations of Vg are 2, 10, 20, 50, and 100 ng mL^{-1}.

determination of Vg by our previous method, which was based on spectrophotometry (5 ng mL^{-1}) [18].

4. Conclusion

A chemiluminescent immunoassay using magnetic beads in an SIA system equipped with a magnet was developed for the highly sensitive and rapid determination of carp Vg. Magnetic beads were found to be ideal for use even in a flow system, and were especially suitable for trapping securely in the flow cell by a magnet. In this study, magnetic beads immobilized with anti-Vg monoclonal antibody were prepared for the determination of Vg by a sandwich immunoassay. Since the magnetic beads are coated with agarose gel, many types of antibodies as well as specific receptors can be immobilized using a variety of chemical reactions. Therefore, magnetic beads represent new and potentially useful supports for bead injection analysis. The optimal incubation time for the first and second immunoreactions in the present immunoassay were determined to be 20 min. In the present method, a swing technique of the reagent solution in the flow cell was used. Namely, the reagent solutions were moved forward and backward repeatedly by taking advantage of the SIA. This new technique shortened the incubation time by 1/6- to 1/3-fold compared to that in our previous method, where the reagent solutions were static after their introduction into the cell. A calibration curve in a lower concentration range will be investigated in a further study.

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References

[1] T. Colborn, Environ. Health Perspect. 101 (1993) 378.
[2] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Br. Med. J. 305 (1992) 609.
[3] R. Bergstrom, H.O. Adami, M. Moher, W. Zatonski, H. Storm, A. Ekbom, S. Tretli, L. Teppo, O. Akre, T. Hakulinen, J. Natl. Cancer Inst. 88 (1996) 727.
[4] H.O. Adami, L. Lipworth, L. Titus-Ernstoff, C.C. Hsieh, U. Ahlborg, J. Baron, Cancer Causes Control 6 (1995) 551.
[5] J.P. Sumpter, S. Jobling, Environ. Health Perspect. 103 (1995) 173.
[6] T.P. Mommsen, P.L. Walsh, Vitellogeninesis and oocyte assembly, in: W.S. Hoar, D.J. Randall (Eds.), Fish Physiology XI A, Academic Press, New York, 1988, p. 347.
[7] K. Yamauchi, K. Yamamoto, Experiments on artificial maturation and femlization of the Japanese eel (*Anguilia japonica*), in: C.J.J. Richter, H.J.Th. Goos (Eds.), Reproductive Physiology of Fish, Pudoc, Wageningen, 1982, p. 185.
[8] J.C.A. Craik, Gen. Comp. Endocrinol. 35 (1978) 455.
[9] L.W. Crim, D.R. Idler, Ann. Biol. Anim. Biochem. Biophys. 18 (1978) 1001.
[10] J.L. Maitre, C. Le Guellec, S. Derrien, M. Tenniswood, Y. Valotaire, Can. J. Biochem. Cell Biol. 63 (1985) 982.
[11] D.R. Idler, S.J. Hwag, L.W. Crim, J. Fish. Res. Bd. Can. 36 (1979) 574.
[12] C.M. Campbell, D.R. Idler, Biol. Reprod. 22 (1980) 605.
[13] J. Nunez Rodriguez, O. Kah, M. Geffard, F. Le Menn, Comp. Biochem. Physiol. 92B (1989) 741.
[14] M. Kishida, T.R. Anderson, J.L. Specher, Gen. Comp. Endocrinol. 88 (1992) 29.
[15] H. Okumura, A. Hara, F. Saeki, T. Todo, S. Adachi, K. Yamauchi, Fisheries Sci. 61 (1995) 283.
[16] H. Fukada, A. Haga, T. Fujita, N. Hiramatsu, C.V. Sullivan, A. Hara, Comp. Biochem. Physiol. A 130 (2001) 163.

[17] H. Fukada, Y. Fujiwara, T. Takahashi, N. Hiramatsu, C.V. Sullivan, A. Hara, Comp. Biochem. Physiol A 134 (2003) 615.
[18] N. Soh, H. Nishiyama, K. Mishima, T. Imato, T. Masadome, Y. Asano, Y. Kurokawa, H. Tabei, S. Okutani, Talanta 58 (2002) 1123.
[19] M. Shinkai, J. Biosci. Bioeng. 94 (2002) 606.
[20] M. Shinkai, J. Wang, M. Kamihira, M. Iwata, H. Honda, T. Kobayashi, J. Ferment. Bioeng. 73 (1992) 166.
[21] G.H.G. Thorpe, L.J. Kricka, Methods Enzymol. 133 (1986) 331.