Electrochemical Flow Enzyme Immunoassay by Means of a Needle-Shaped Sampler/Reactor

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A needle-shaped sampler/reactor was developed for an electrochemical enzyme immunoassay with the direct sampling of living sample blood. This device was evaluated using IgG determination chemistry. Antibodies were immobilized on an inner wall of the sampler/reactor. Incubation for the enzyme reaction was not needed because this reactor was very small (250 μm in diameter). The analysis was conducted within 15 min in the simplest protocol including the reactor refreshment. The limit of detection was 3 pg, and 20 attomol in the most sensitive protocol. Furthermore, the sampling of a solution contained in an agar block and a whole-blood analysis were demonstrated.

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Many enzyme immunoassays (EIA) have been conducted by flow injection analysis (FIA). FIA has good reliability and repeatability. In most investigations of EIA coupled with FIA (FIEIA), the products generated on a microtiter plate have been injected into a FIA system. However, antibody-immobilized 96-well microtiter plates are usually expensive and inconvenient for small-lot sample tests, whether FIA or an exclusive absorbance/fluorometric detector is used. Another FIEIA approach is to try to make measurements with an in-line reactor, such as a mini-column, a silicone tube reactor or a flow channel. If antibodies are immobilized in the flow channel, only a very small quantity is needed, and they can be used repeatedly. Furthermore, the amount of available sample is sometimes very limited, e.g. when measuring vitellogenin of Japanese medaka Orzyzas latipes, all of the serum of 20 fishes should be gathered, or 2 μl of serum from one fish should be diluted up to 250 μl for ELISA. If a very small sample can be measured directly, a low-level analyte in individual living samples can be determined. From the viewpoint of such applications, we started to establish an electrochemical enzyme immunoassay (EIEIA). If the absolute quantity of the analyte is small, electrochemical detection is more profitable than the optical method. Recently, many microchips, or μTAS, have been studied for EIA, where only 1 nl or 100 pl is introduced into the separation channel. Before introduction into the channel, however, several-tens μl of the sample must first be put into a reservoir. It was thus decided to use a capillary combined with a sucking system as the sampler. Capillaries have been used as reactors of immune and enzyme reactions. The smallest capillary reported so far is about 20 μl (0.56 mm i.d. and 100 mm long); after incubation for the enzyme reaction, the solution was taken from the capillary once and then injected into an FIA system. In our present work, only a 5-μl capillary was adopted as a reactor. This capillary can be directly inserted into the solution sample or living sample. It has a large surface-to-volume ratio, and the solution species was expected to react well with the immobilized species. Thus, the process time was also expected to be minimized. Furthermore, the whole procedure was carried out in an in-line flow system. This simple flow analysis was demonstrated by the sandwich immunoassay of immunoglobulin G (IgG).

Experimental

Reagent

Three kinds of antigen antibody reagents were used: anti-IgG for immobilization onto the reactor, IgG as an analyte, and enzyme-labeled antibody (ALP anti-IgG). These reagents were biotin-SP-conjugated affinity pure sheep anti-mouse IgG, F(ab')2 (2.0 mg/ml), chromopure mouse IgG (whole molecule, 11.8 mg/ml) and alkaline phosphatase-conjugated affinity pure rat anti-mouse IgG (H+L) (0.8 mg/ml), all from Jackson Immuno Research Laboratory. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim. Substrate p-aminophenylphosphate (PAPP) was synthesized from p-nitrophenylphosphoric acid disodium salt (PNPP) obtained from Nacalai Tesque, and p-aminophenol (PAP) was purchased from Katayama Kagaku. The buffer solution was basically 10 mM phosphate buffer salts, pH 7.3 (PBS). The rinse solution was PBS buffer added 0.5 M NaCl. The substrate solution comprising 1 mM of PAPP was provided as an alkaline (pH 8.3) PBS solution. The removing agent comprised 0.2 M of
into the capillary at 5 μl/min for 30 min and refrigerated overnight.

The antibody immobilized capillary was used as it was, or set in a disposable injection needle 23G (o.d. 0.63 mm) or a stainless-steel tube (o.d. 0.71 mm and 0.15 mm thickness), as shown in Fig. 1 (a). The point of the capillary was adjusted to that of the metal needle, and the opposite side was connected to the flow system via a small-bore Teflon tube AWG-30 and a 1/16" PEEK tube. The base of the needle was molded with silicone glue.

Flow system and procedure

The flow system used in this experiment is shown in Fig. 1 (b). Several kinds of reagent were introduced into the capillary by just putting the capillary head into each solution and sucking it with a syringe pump. In order to prevent electrode poisoning, an electrochemical cell was connected to a 6-port valve (V1), and the solution was normally bypassed to the pump. Another 6-port valve (V2) was used, and a 20 μM Fe(CN)₆³⁻ solution was introduced while the electrode activity was occasionally checked. An electrochemical cell (EC) was arranged with dual 3-mm carbon electrodes and an Ag/AgCl reference electrode; the oxidation current was monitored with an electrochemical analyzer (ALS Chi701A, from BAS Inc.).

The volume between the capillary and the EC via V1 was made to be as small as possible, and was 14 μl.

The anti-IgG immobilized capillary was set in the flow system as shown in Fig. 1 (b). An IgG sample and 1 μg/ml ALP anti-IgG were introduced into the capillary at 5 μl/min. Next, the V1 position was switched, and PAPP was sucked through the capillary at 30 μl/min while the electrochemical signal at 0.2 V vs. Ag/AgCl was recorded. If needed, the removing agent was passed, and then another assay cycle was started repeatedly from sample introduction.

glycine with its pH being adjusted to 2.2 by adding HCl.

Sampler/reactor

A fused-silica capillary was obtained from Polymicrotechnologies. It was 0.25 mm and 0.35 mm in inner and outer diameters with a polyimide coating and cut to be 10 cm long. First, an H₂SO₄/H₂O₂ mixture (2:1) was injected into the capillary, and was then left for a few hours in order to clean the inside of the capillary. Anti-IgG was immobilized onto the inner wall of the capillary in a manner similar to that described by Yang et al. The capillary was connected to a syringe pump, and 0.5 M NaOH and water were successively sucked for 1 h and 0.5 h, respectively, at 100 μl/min. Next, the capillary was disconnected from the pump, and water was pushed out by a syringe. The capillary was then dried in a 95°C oven for 2 h. An aqueous solution of 10% 3-aminopropyltriethoxysilane was passed through the capillary for several minutes, and heated again in the oven for 30 min after removing the solution. This silane treatment was carried out twice. Next, 1% glutaraldehyde in PBS was passed through the capillary for 1 h at 100 μl/min. Streptavidin (1 mg/ml) in PBS was introduced at 5 μl/min for 20 min, and left in a refrigerator overnight. The remaining aldehyde groups were blocked by 0.5 M Tris-HCl buffer (pH 7.5) for 10 min. Finally, 40 μg/ml anti-IgG was introduced

Fig. 2 Incubation dependence on the response current. The abscissa indicates the flow stop time for incubation. The marks ■ and ○ indicate the data for a blank and 1 μg/ml IgG, and ▲ indicates the ratio of the response current to the blank current. The IgG standard or blank and 1 μg/ml ALP anti-IgG were passed for 10 min and 5 min at 5 μl/min. PAPP was then introduced and stopped for the corresponding stopping time.
Results and Discussion

Incubation for enzyme reaction

Enzyme reactions usually need some incubation, and the generated products are measured after removing the solution from a reactor.\textsuperscript{4,10} The reactor used in this work was much smaller than those previously reported, and it was impossible to operate it in the usual manner. Therefore, the product was treated in-line, and the enzyme reaction time was shortened. Thus, after the PAPP was stopped in the reactor for the enzyme reaction, the produced PAP was directly pushed out to the electrochemical cell by the running buffer. The abscissa in Fig. 2 indicates the stopping time of the substrate solution. The actual incubation corresponded to the sum of the stopping and the solution flow-through time. As can be seen from Fig. 2, the detection current increased with incubation. However, the blank current showed a similar relationship to the incubation, and the ratio of the response current to the blank current was almost constant for all stopping times. To be more exact, the ratio increased once, and then gradually decreased with an increase in the stopping time, except for a stopping time of zero. The ratio was highest when solution was not stopped. This is thought to be because only 1/3 of the flowing PAPP was stopped in the reactor. The surplus PAPP stopped in the connecting tube reacted with ALP anti-IgG slightly adsorbed on the tube wall. The ALP anti-IgG tended to adsorb onto the PEEK and Teflon tube wall, which had previously caused problems.\textsuperscript{11} Another fear was that the generated PAP might oxidize with the aging time.\textsuperscript{6} Thus, a too-long incubation was, far from getting a larger signal, undesirable. Furthermore, in the stopped mode, there were few PAPP molecules in the reactor, and they did not need much time to diffuse to the reactor wall. The flow made a perpendicular diffusion well and compensated for the shortness of the reaction time. Therefore, it was decided not to stop the substrate solution in the capillary, so that the enzyme reaction time would be equivalent to the solution passing time through the capillary. It took only 10 sec for the solution to flow through the 10-cm capillary at the optimum flow rate of 30 μl/min. In the stopped flow mode, the obtained signal was the peak of the current, and the maximum value was affected by the extent of diffusion that occurred during movement to the detector. However, when the PAPP solution was not stopped, a steady state current was obtained. The total flow system volume was also small and the electrochemical signal reached the steady state within a few minutes. This simple procedure helped to improve the repeatability, and the small bore reactor eliminated incubation because of high reaction efficiency like in a very thin wall-jet reactor.\textsuperscript{10}

Sample volume

The inner volume of the capillary, where anti-IgG was immobilized, was only 5 μl. Moreover, the sample was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Time sequence of the capillary EE1A</th>
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<tbody>
<tr>
<td>Flow rate /μl min (^{-1})</td>
<td>Prot. A</td>
</tr>
<tr>
<td>Sampling</td>
<td>5</td>
</tr>
<tr>
<td>Rinse</td>
<td>20</td>
</tr>
<tr>
<td>ALP anti-IgG</td>
<td>5</td>
</tr>
<tr>
<td>Rinse</td>
<td>20</td>
</tr>
<tr>
<td>PAPP (EA)</td>
<td>30</td>
</tr>
<tr>
<td>Rinse</td>
<td>30</td>
</tr>
<tr>
<td>Removing</td>
<td>20</td>
</tr>
<tr>
<td>Rinse</td>
<td>20</td>
</tr>
<tr>
<td>Total assay time</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^{a}\) 1 min sampling with flow and 9 min incubation in stopped mode.
sucked directly from the tip of the capillary, thus minimizing the sample volume. Figure 3 shows the relationship between the resulting current and the sample volume when 100 ng/ml IgG was introduced at 5 μl/min. After sampling, the rinse solution was flowed immediately (○), or the IgG solution was stopped in the capillary until the total number of sampling and stopping times became 10 min (□). In the latter case, the antigen-antibody reaction time was always 10 min, irrespective of the sampling volume. The plot at the sample volume of 0 μl corresponds to the blank value. In the former case, the detection current increased linearly with the sample volume. The response to low-volume sample was improved by the sample stop. As stated above, when high sensitivity was desired, the sampling volume should be large. However, the maximum sampling was only 50 μl. General samples could be measured with a 5 μl sample, namely 1 min sampling.

Calibration curves

Calibration curves for three conditions are shown in Fig. 4, and each protocol is shown in Table 1. Although these curves were obtained in different immobilized capillaries, the series of data for each calibration was obtained from the same immobilized capillary. Each blank value is shown on the left by an outline mark. In any protocol, the calibration curves had 3 or 4 order dynamic ranges, and the measurable range was largely affected by the protocol conditions. Even when the sampling volume was only 5 μl (protocol A and B), good calibration curves were obtained. As can be seen from a comparison between A and B, the current was enlarged due to incubation of the immunoreaction, especially in the low-IgG region. The sensitivities of A and B were less than that of protocol C, but their calibration curves covered the practical concentration region. In the simplest procedure, protocol A, one cycle of the assay was conducted within 15 min, including refreshment of the reactor. For protocol C, the IgG was sampled for 10 min, and the overall time was more than 50 min. However, the detection limit was estimated to be only 50 pg/ml. This corresponds to absolute quantities of 3 pg and 20 attomol.

Needle sampler

If sampling must be done by direct insertion of the antibody-immobilized capillary, it would be difficult to insert the capillary alone. Accordingly, the capillary was set in an injector needle of a stainless-steel capillary. This was demonstrated with a standard solution sample tube contained in an agar block. The detection current for this solution sample agreed with the normal standard solution within 2%. Next, it was confirmed whether this method is applicable to whole blood analysis, like Schneider et al. demonstrated. Unfortunately, the analyte which we used was IgG, and its concentration in the sample was too high. Therefore, the device could not be inserted directly into a mouse, and the IgG in mouse blood was measured after a hundred-time-dilution. The results were 40, 53 and 141 μg/ml for diluted samples with 0, 10 and 100 μg/ml of the standard addition, respectively. The results of the standard-addition samples agreed well with the theoretical values. It is expected that measurements of an individual living sample can be repeated with this sampler to monitor the analyte transition. In other words, a living sample could be monitored without killing. This would make it possible to conduct experiments on the effect of the environmental conditions and the addition of chemicals.

This device has the potential to establish a new ELISA system, as follows. After direct sampling by a needle device, several sampler/reactors are arranged in parallel and connected to a special sucking system, like a multipipette. The immunoassay is then automatically conducted with sample, reagent- and time-saving.

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References