Direct Detection of Horseradish Peroxidase as a Marker Molecule Encapsulated in Liposomes via Use of Fluorescein Chemiluminescence

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A method for direct detection of horseradish peroxidase (HRP) as a marker molecule trapped in liposomes by the use of HRP-catalyzed fluorescein chemiluminescence (CL) with hydrogen peroxide has been developed. Maximum CL emission in the direct detection of HRP in liposomes increased by a factor of 13 times compared with that in the detection of HRP dissolved in lipid-free buffer solution.

Much interest has been shown in liposomes as signal-enhancement agents, since thousands of small marker molecules, such as calcein[1] and carboxyfluorescein,[2] can be trapped in the aqueous interior. Encapsulation of macromolecules inside liposomes has also been carried out using enzymes,[3–5] and antibodies labeled with liposomes containing marker molecules have been employed for immunoassays.

We previously prepared HRP-trapped liposomes, since HRP is widely used as a marker in immunoassays.[6] The number of HRP molecules encapsulated in liposomes was about 1200 HRP molecules per liposome. Biotin-tagged liposomes containing HRP were applied to labels in immunodotblotting of rabbit IgG.[7] The detection of HRP trapped in liposomes was made by a luminol CL method after the release of HRP from liposomes accomplished by the lysis of liposomes using lytic agents, such as Triton X-100. However, release of HRP from liposomes might cause a reduction in the amount of light emission, since HRP concentrated in the nanospaces of liposomes is dissolved in the bulk solution by the lysis of liposomes, thus resulting in the dilution of HRP. Therefore, a method that enables the measurement of the amount of HRP in liposomes without lysis of liposomes is desirable. However, there have been no reports on direct detection of HRP in liposomes.

In the course of our studies on direct detection of HRP in liposomes by a CL method, we found that fluorescein and hydrogen peroxide (H_2O_2) rapidly permeate into the inner phase of liposomes to initiate HRP-catalyzed fluorescein CL with H_2O_2. The maximum CL emission observed in the direct detection of HRP in liposomes was remarkably greater than that observed in the detection of HRP buffer solution in lipid-free buffer solution. Fluorescein CL can thus be applied to direct detection of HRP encapsulated in liposomes.

Egg yolk phosphatidylcholine (PC), cholesterol (Chol), DL-α-phosphatidylglycerol dimyristoyl (DMPG), and fluorescein were purchased from Wako Chemicals Co. HRP (type VI) was bought from Sigma Chemical Co. A 1.0×10^{-4} M ($1\,M = 1\,mol\,dm^{-3}$) solution of HRP was prepared by dissolving the compound with 10 mM 3-morpholinopropanesulfonic acid-buffered saline (pH 7.0).

HRP-trapped multilamellar vesicles (MLVs) were prepared by previously described procedure,[6] in which a mixture (32 μmol PC, 4 μmol DMPG, 4 μmol Chol) in chloroform and a 1-mL portion of a 1.0×10^{-4} M HRP solution were used. The HRP-trapped MLVs were extruded through polycarbonate filters with a pore size of 1000 nm to prepare unilamellar vesicles containing HRP. Samples were subjected to 20 passages through a single filter.

The separation of HRP-trapped liposomes and free HRP was performed on a Sephadex 4B column (column size, 15 mm × 300 mm). The amounts of liposomes and HRP eluted from the column were determined by measuring phosphorus and iron in each fraction tube by ICP-AES (ICPS-1000IV, Shimadzu, Japan). The HRP-trapped liposomes collected by the column were stored at 4 °C in a refrigerator.

The CL experimental procedure involved firstly pipetting a 500-μL portion of the HRP-trapped liposome suspensions into a glass cuvette in a CL detector (TDA-3A; Tohoku Denshi Sangyou Co., Ltd.). Next, a 500-μL portion of a 4.0×10^{-4} M fluorescein solution and a 500-μL portion of 1.0×10^{-4} M H_2O_2 were simultaneously injected into the cuvette. The CL reaction was initiated and the light output was detected using the CL detector. The resultant photocurrent was converted to voltage and displayed on a chart recorder.

A typical CL response curve is shown in Figure 1 (curve 1). The light emission appeared rapidly after the start of the reaction and reached its maximum intensity at 20 s. The maximum light emission is referred to as CL intensity. The results indicate that

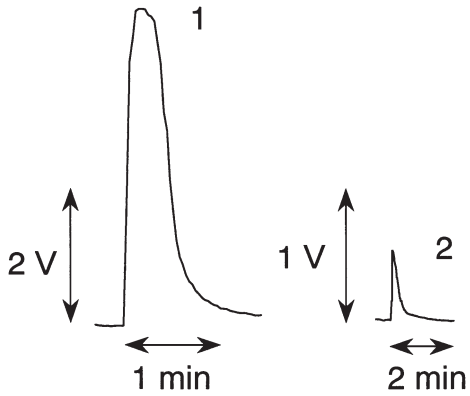

Figure 1. Typical CL response curves. 1: Direct detection of HRP encasulated in liposomes. 2: Detection of HRP dissolved in bulk solution after lysis of liposomes. Conditions for CL measurements: [HRP] = 1.6×10^{-6} M, [fluorescein] = 4.0×10^{-4} M, [H_2O_2] = 4.0×10^{-5} M.

fluorescein and H_2O_2 rapidly permeated into the inner phase of liposomes to initiate the HRP-catalyzed CL reaction.

The concentration of HRP dissolved in the bulk solution after the lysis of HRP-trapped liposomes with a 20-μL portion of 10% Triton X-100 was determined by ICP-AES. The concentration of HRP was found to be 1.6×10^{-6} M. The CL measurement was made according to the procedure in which a 1.6×10^{-6} M solution of HRP prepared in a lipid-free buffer solution was used instead of the HRP-trapped liposome suspensions. A CL response curve is shown in Figure 1 (curve 2). The CL intensity in direct detection of HRP was remarkably greater than that observed in the detection of HRP prepared in the buffer solution.

In order to determine the reason for the increase in CL intensity in direct detection of HRP, the trapping efficiency was determined as the mole ratio of HRP trapped in liposomes to HRP dissolved in liposome suspensions after extrusion through polycarbonate filters. The concentration of HRP trapped in liposomes was determined by mixing the fractions containing HRP-trapped liposomes. The average trapping efficiency in three successive experiments was 22%, suggesting that the concentration of HRP trapped in liposomes is about fifth greater than that of HRP dissolved in the bulk solution after lysis of HRP-trapped liposomes. Therefore, the enhancement of CL intensity observed in the direct detection of HRP could be due to the increase in the concentration of HRP by localization of HRP in liposomes.

The effect of Chol concentration in liposomes on the CL response curve was investigated in the range of 4–16 mM in chloroform. CL measurements were carried out under the following conditions; the total concentrations (40 mM) of PC, Chol and DMPG in chloroform were constant and the concentration of DMPG (10 mM) was constant. CL intensity linearly decreased with an increase in the concentration of Chol. CL intensity at 16 mM of Chol was about half of that at 4 mM of Chol. The total amount of light emission at 16 mM of Chol was 70% of that at 4 mM of Chol. On the other hand, the time to decay to half of the CL intensity at 16 mM of Chol increased by a factor of 1.5 compared to that at 4 mM of Chol. These results can be interpreted as follows. Chol reduces the fluidity of hydrocarbon chains in the liquid bilayer of membranes by cholesterol–phospholipid interaction when introduced into liquid-crystalline lipid bilayers in liposomes.[8] Therefore, the rate of permeation of fluorescein and H_2O_2 into liposomes could be reduced by increasing the concentration of Chol in liposomes. The optimal concentrations of PC, Chol and DMPG were determined to be 32, 4 and 4 mM, respectively.

The effect of fluorescein concentration on the CL response curve was examined in the range of 4.0×10^{-5}–4.0×10^{-3} M. Below 1.0×10^{-4} M of fluorescein, the light emission intensity gradually increased and reached maximum in 60 s. On the other hand, the light emission intensity rapidly increased after the addition of fluorescein ($>1.0 \times 10^{-4}$ M) and H_2O_2. The CL intensity was maximal at 4.0×10^{-4} M of fluorescein. The optimum concentration of fluoresein was thus determined to be 4.0×10^{-4} M.

The dependence of H_2O_2 concentration on direct detection of HRP encapsulated in liposomes was examined in the range of 5.0×10^{-8}–1.0×10^{-3} M. The CL intensity linearly increased with increase in H_2O_2 concentration in the range of 1.0×10^{-8} to 1.0×10^{-5} M and then leveled off. Next, we examined the effect of H_2O_2 concentration on CL intensity using a 1.6×10^{-6} M solution of HRP prepared in lipid-free buffer solution. CL intensity linearly increased with increase in H_2O_2 concentration in the range of 5.0×10^{-9} to 1.0×10^{-5} M and then gradually increased. Figure 2 shows the relative CL intensity-H_2O_2 concentration profiles. The relative CL intensity is defined as the ratio of CL intensity in the direct detection of HRP trapped in liposomes to that in the detection of HRP dissolved in the lipid-free buffer solution. The relative CL intensity was below 1.0 in the range of 1.0×10^{-9} to 1.0×10^{-7} M of H_2O_2. This is because the permeability of H_2O_2 through membrane of liposomes is reduced with a decrease in H_2O_2 concentration. The relative CL intensity linearly increased in the range of 1.0×10^{-6} to 1.0×10^{-4} M of H_2O_2 and then decreased. As can be seen in Figure 2, CL intensity in the direct detection of HRP in liposomes at 1.0×10^{-4} M of H_2O_2 was enhanced by a factor of 13 times compared to that in the detection of HRP dissolved in lipid-free buffer solution.

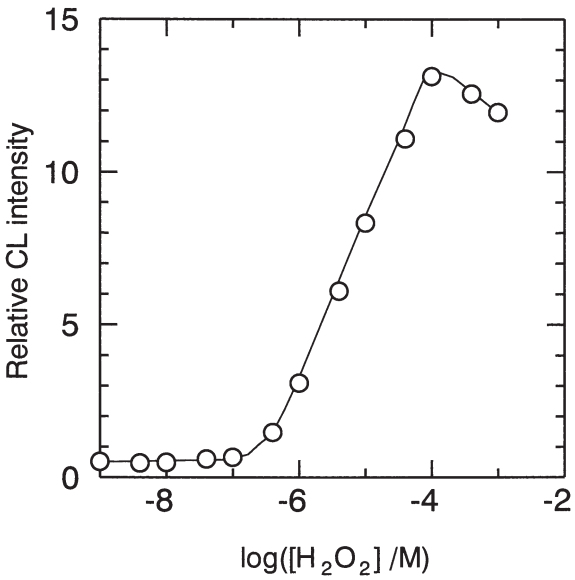

Figure 2. Effect of H_2O_2 concentration on relative CL intensity.

In conclusion, a method for direct detection of HRP trapped in liposomes by using fluorescein CL method has been developed. The sensitivity of an immunoassay using liposome trapped HRP as a label could be enhanced by using this method for direct detection of HRP trapped in liposomes without lysis of liposomes.

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