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Direct Determination of Horseradish Peroxidase Encapsulated in Liposomes by Using Luminol Chemiluminescence

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Horseradish peroxidase (HRP) encapsulated in liposomes was directly detected by using luminol chemiluminescence (CL) with H_2O_2 without lysis of liposomes. At a low concentration of H_2O_2, the initial rate of HRP-catalyzed luminol CL in liposomes was slower than that of HRP-catalyzed luminol CL in a lipid-free bulk solution. The decrease in the initial rate of the CL reaction in liposomes was due to the membrane permeation of luminol and H_2O_2. At a high concentration of H_2O_2, the initial rate of the CL reaction in liposomes was the same as that in a lipid-free bulk solution. The CL measurement conditions in both a lipid-free bulk solution and in liposomes were optimized in the concentrations of luminol and H_2O_2 by measuring the CL response curves, in which only one peak appeared and the CL intensity was maximal. The CL intensity observed in HRP-catalyzed luminol CL in liposomes was a factor of seven greater than that observed in a lipid-free bulk solution. The CL intensity was dependent on the amount of HRP-encapsulated liposomes used. The detection limit in the direct detection of HRP encapsulated in liposomes was sensitive by a factor of 3 compared with that in HRP-catalyzed luminol CL in a lipid-free bulk solution.

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Introduction

Liposomes are spherical vesicles composed of phospholipid bilayers surrounding an aqueous volume. Liposomes have been extensively used as model systems of biological membranes and drug-delivery systems.[1] Recently, much interest has been shown concerning liposomes as signal-enhancement agents, since thousands of small marker molecules, such as calcein[2] and carboxyfluorescein[3] can be encapsulated in the aqueous interior. Antibodies labeled with liposomes containing marker molecules have been employed for immunoassays. On the other hand, the encapsulation of macromolecules inside liposomes has been carried out using proteins and enzymes.[4-6] Enzyme-containing lipid vesicles have been applied to two main areas: in the medical or biomedical field, and in the cheese ripening process.[7]

Horseradish peroxidase (HRP) is widely used as a marker molecule in enzyme-immunoassays. We prepared previously HRP-encapsulated liposomes composed of phospholipids and cholesterol.[8] 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 the immunodotblotting of rabbit IgG.[9] The detection of HRP encapsulated 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, the release of HRP from liposomes might cause a reduction in the amount of light emission, since the HRP concentrated in the aqueous interior of liposomes is dissolved in the bulk solution by the lysis of liposomes, thus resulting in the dilution of HRP. Therefore, the development of a method that enables the direct detection of HRP encapsulated in liposomes without the lysis of liposomes is desirable for improving the sensitivity in enzyme-immunoassays when used with HRP-encapsulated liposomes as labels.

In the course of our studies on the direct detection of HRP encapsulated 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.[10] Light emission observed in HRP-catalyzed fluorescein CL in liposomes was remarkably greater than that observed in free HRP-catalyzed fluorescein CL in a lipid-free bulk solution. The intensity of light emission observed in fluorescein CL in liposomes was dependent on the concentration of H_2O_2. We then applied HRP-catalyzed fluorescein CL in liposomes for the determination of H_2O_2.[11]

In the present work, we investigated HRP-catalyzed luminol CL in both lipid-free bulk solution and in liposomes, in order to clarify the effect of the reaction medium on the initial rate and the intensity of light emission in HRP-catalyzed luminol CL. In addition, the detection limit in the direct detection of HRP encapsulated in liposomes was compared with that in the detection of HRP dissolved in a lipid-free bulk solution.

Experimental

Reagents and solutions

Egg yolk phosphatidylcholine (PC; molecular weight, 773) was obtained from Nichiyu Liposome Co. (Tokyo, Japan). DL-α-phosphatidylglycerol dimyristoyl (PG) and cholesterol were purchased from Wako Chemicals Co. (Tokyo, Japan). Luminol obtained from Kanto Chemicals (Tokyo, Japan) was guaranteed to be grade reagent. HRP (Type VI) was bought from Sigma Chemical Co. All other chemicals used were guaranteed-grade

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reagents, and were used without further purification.

A 100 μM solution of HRP was prepared by dissolving a compound with 10 mM Tris–HCl–buffered saline (pH 8.0). The concentration of HRP was determined spectrophotometorically with an ε_{403} value of 1.02×10^5 M/cm.[12] Working solutions of HRP were prepared by serial dilution with the buffers. All solutions used were prepared with water from a Millipore Milli-Q water purification system.

Preparation of HRP-encapsulated liposomes by an extrusion technique

A mixture of PC, PG and cholesterol (24 μmol PC, 4 μmol PG, 12 μmol cholesterol) in chloroform was used to prepare a lipid film. The mixture in chloroform was placed in a 100-ml round-bottomed flask. Chloroform was removed by rotary evaporation at 30°C under reduced pressure and a stream of nitrogen gas, forming a lipid film on the wall of the flask. After at least 2 h *in vacuo*, a 1.0-ml portion of a 100 μM HRP solution was added into the flask. All of the contents were extensively mixed with a Vortex stirrer for 15 min, thus resulting in the formation of HRP-encapsulated multilamellar vesicles (MLVs). The MLVs were extruded through two stacked polycarbonate filters with a pore size of 1000 nm, using a LiposoFast-Basic extruder (Avestin Inc.). The MLVs were subjected to 20 passages through a single filter. A VET_{1000} indicated that liposomes were extruded through a polycarbonate filter of 1000 nm pore size.

The separation of HRP-encapsulated VET_{1000} from free HRP was performed by gel filtration using a Sepharose 4B column (15 mm i.d. × 300 mm). The column was equilibrated with 10 mM Tris–HCl–buffered saline (pH 8.0) at 25°C, and the flow rate was 12 ml/h. A 1.0-ml portion of HRP-encapsulated VET_{1000} suspensions was applied to the column. The eluent from the column was collected for each 5 min with a fractional collector (Pharmacia). HRP-encapsulated VET_{1000} collected by the column were stored at 4°C in a refrigerator.

The amounts of HRP-encapsulated VET_{1000} eluted from the column were determined by measuring phosphorus by inductively coupled plasma atomic emission spectrometry with an ultrasonic nebulizer (ICPS-1000IV, Shimazu, Japan).[13] The amounts of HRP encapsulated in VET_{1000} were determined as follows. A 125-μl portion of HRP-encapsulated VET_{1000} suspensions, a 125-μl portion of 3.0 mM luminol solution and a 125-μl portion of the buffer solution were added into the glass cuvette. Next, a 125-μl portion of 160 mM Triton X-100 was added into the cuvette. After the lysis of HRP-encapsulated VET_{1000} with Triton X-100, HRP dissolved in the bulk solution was detected by injecting a 500-μl portion of H_2O_2 into the mixture. The concentration of HRP was determined by applying the CL intensity obtained from the CL response curve to the calibration curve prepared previously. When HRP-encapsulated VET_{1000} was used in the CL measurements, the concentration of HRP was an indication of the concentration of HRP dissolved in the bulk solution after lysis with Triton X-100.

The size distribution of HRP-encapsulated VET_{1000} was estimated with a fiber-optics particle analyzer (FPAR 1000, Otsuka Electronics, Japan).

Measurement of CL response curves

In the measurements of HRP-catalyzed luminol CL in lipid-free bulk solution, a 125-μl portion of a 1.0 μM HRP solution, a 125-μl portion of H_2O_2 solution and a 250-μl portion of the buffer solution were placed in a glass cuvette (19 mm i.d. × 22 mm; internal volume, 6.23 ml) in a luminometer. Next, a 500-μl portion of a luminol solution was injected into the cuvette with the injector, and the CL reaction was initiated. Light emission was detected by a photomulitiplier, and the CL response curves were displayed on a personal computer. The intensity of light emission in CL response curves was indicated by counts per second (cps). The luminometer equipped with a photon-counting head (Hamamatsu H6180-MOD) was constructed in our laboratory.

In the measurements of HRP-catalyzed luminol CL in VET_{1000}, a 125-μl portion of HRP-encapsulated VET_{1000} suspensions, a 125-μl portion of H_2O_2 solution and a 250-μl portion of the buffer solution were added into the glass cuvette in a luminometer. Next, a 500-μl portion of luminol solution was injected into the cuvette with the injector and the CL reaction was initiated.

Results and Discussion

Characterization of HRP-encapsulated VET_{1000}

The size distribution of HRP-encapsulated VET_{1000} was measured by collecting the HRP-contained fractions eluted from the column. The size distribution of HRP-encapsulated VET_{1000} was in the range from 276 to 1516 nm, and the mean diameter ± standard deviation was 585 ± 29 nm. The trapping efficiency of HRP encapsulated in VET_{1000} was determined as the mole ratio of HRP encapsulated in VET_{1000} to HRP in the mixture of free HRP and HRP encapsulated in VET_{1000} before separation by the column. The concentration of HRP in VET_{1000} was determined by mixing the fractions containing HRP-encapsulated VET_{1000}. Three successive experiments were accomplished for determining the trapping efficiency. The average value of the trapping efficiency of HRP in VET_{1000} was 10.3%. Triplicates agreed with less than 1.5% from the mean.

The number of HRP molecules encapsulated in VET_{1000} was estimated from such experimental quantities as the mean diameter of HRP-encapsulated VET_{1000} and the molar concentration ratio of HRP to lipid. The relation between these quantities is given as follows:[14,15]

$$\text{Number of HRP/liposome} = (\text{HRP concentration/lipid concentration}) \times N_{\text{tot}}$$

The total number of lipid molecules per liposome (N_{tot}) is given by the following equation for spherical unilamellar vesicles of radius Rv, a bilayer thickness of t and an average area per lipid molecule of A:[16]

$$N_{\text{tot}} = (4\pi Rv^2/A) + \{4\pi(Rv - t)^2/A\}$$

The Rv value was obtained from the mean diameter of HRP-encapsulated VET_{1000}. The bilayer thickness (t) was assumed to be 45 Å.[17] The average area per lipid molecule (A) was calculated using values of 71, 44 and 19 Å^2 for PC,[18] PG[19] and cholesterol,[18] respectively, weighted by the mole fraction of each component. The average value of area per lipid molecule (A), obtained for VET_{1000}, was 63 Å^2/lipid. Then, the N_{tot} value was calculated, and its value was 3.6×10^6. Next, the concentrations of HRP and lipid in HRP-encapsulated VET_{1000} were determined by the CL method and ICP-AES, respectively. The number of encapsulated HRP molecules per VET_{1000} was calculated based on these values. VET_{1000} encapsulated about 1200 HRP molecules per liposome.

Typical CL response curves observed in HRP-catalyzed luminol CL in a lipid-free bulk solution and in VET_{1000}

HRP-catalyzed luminol CL was examined in a lipid-free bulk

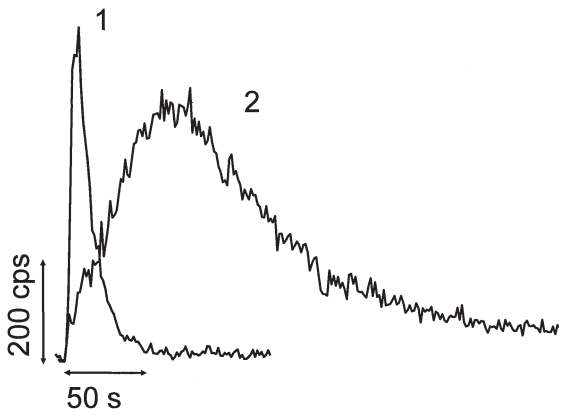

Fig. 1 Typical CL response curves in the detection of HRP dissolved in a lipid-free bulk solution and in the direct detection of HRP encapsulated in VET_{1000}. Curve 1, detection of HRP dissolved in lipid-free bulk solution. Curve 2, direct detection of HRP encapsulated in VET_{1000}. Conditions for the CL measurements: [HRP] = 100 nM, [luminol] = 50 μM, [H_2O_2] = 1.0 μM. All the reagent concentrations are initial concentrations.

solution. The concentration of HRP was prepared in the same way as that dissolved in the solution after the lysis of HRP-encapsulated liposomes with Triton X-100, according to the procedure. A typical CL response curve is shown in Fig. 1 (curve 1). Light emission appeared rapidly after starting the CL reaction, and reached its maximum intensity, 10 s after which the intensity of light emission decreased rapidly. The maximum light emission is referred to as the CL intensity.

Next, HRP-catalyzed luminol CL in VET_{1000} was performed according to a procedure in which a 500-μl portion of 50 μM lumoniol solution was added into the mixtures of a 125-μl portion of VET_{1000} suspensions, a 125-μl portion of 1.0 μM H_2O_2 solution and a 250-μl portion of the buffer solution. The concentration of HRP dissolved in the bulk solution after the lysis of HRP-encapsulated liposomes was determined according to the procedure. A typical CL response curve is shown in Fig. 1 curve 2). Light emission gradually appeared after the CL reaction started, and reached its maximum intensity at 65 s, after which the intensity of light emission gradually decreased. The initial rate of HRP-catalyzed luminol CL in VET_{1000} was remarkably slower than that of HRP-catalyzed luminol CL in a lipid-free bulk solution. The initial rate of the CL reaction was obtained as the slope of the CL response curves immediately after the CL reaction started.

In this reaction, luminol exists in monoanion and dianion forms under the assay conditions. These luminol anions could permeate across the liposomal membrane because organic ions are known to be membrane-permeable. However, organic ions with lower charge exhibit higher permeability. Thus the major species of luminol that permeates across the liposomal membrane is most likely to be the monoanion. On the other hand, hydrogen peroxide apparently has even higher permeability than luminol monoanion because of its small molecular size and electrostatic neutrality. Then, in the initial period of the CL reaction, the concentration of hydrogen peroxide in the inner phase of liposomes is probably in large excess over that of luminol. Furthermore, the permeation of luminol from the inner to the outer phase can be negligible, because luminol should be rapidly consumed in the CL reaction. Therefore, the CL reaction in HRP-encapsulated VET_{1000} during the initial period can be a pseudo-first-order reaction.[20] The initial rate of luminol CL in HRP-encapsulated VET_{1000} can be expressed by

$$dI/dt = \Phi_{CL} k_{app} A [\text{luminol}]_o P / V_{in},$$

where I is the intensity of light emission, k_{app} is the apparent rate constant of the CL reaction, Φ_{CL} is the quantum yield of CL, $[\text{luminol}]_o$ is the total concentration of luminol, P is the permeability coefficient of luminol, and A and V_{in} are the total surface area and internal volume of VET_{1000}, respectively. Therefore, the decrease in the initial rate of the CL reaction in VET_{1000} could be attributable to the membrane permeation of luminol, since we previously confirmed that the activity of HRP remained throughout the encapsulation process by measuring the catalytic activity of HRP for a luminol CL.[8] As can be seen in Fig. 1, the reaction time in VET_{1000} was remarkably longer than that in the bulk solution. The result was also explained in terms of the membrane permeation of luminol. That is, luminol gradually permeats into VET_{1000}, and is oxidized catalytically by HRP with H_2O_2 to emit CL. Therefore, the reaction time of HRP-catalyzed luminol CL in VET_{1000} could be dependent on both the membrane permeation rate and the CL reaction rate of luminol in VET_{1000}.

On the other hand, the total CL emission obtained by integrating photon emission observed in the CL reaction in VET_{1000} increased remarkably compared with that in a lipid-free bulk solution. This is probably due to both the localization of HRP in VET_{1000} and the increase of Φ_{CL} in the CL reaction in VET_{1000}. The trapping efficiency of HRP in VET_{1000} was 10.3%, suggesting that the concentration of HRP encapsulated in VET_{1000} is about one tenth greater than that of HRP dissolved in the bulk solution after the lysis of HRP-encapsulated VET_{1000}. Therefore, the enhancement of the total CL emission observed in VET_{1000} could be due to an increase in the concentration of HRP by the localization of HRP in VET_{1000}. On the other hand, the increase in Φ_{CL} in HRP-catalyzed luminol CL in VET_{1000} is still not clear.

Effect of the H_2O_2 concentration on the CL response curves observed in HRP-catalyzed luminol CL in lipid-free bulk solution and VET_{1000}

In the direct detection of HRP in VET_{1000} by the fluorescein CL method, the CL intensity was dependent on the concentration of H_2O_2.[10] We then measured the CL response curves in both a lipid-free bulk solution and in VET_{1000} at a high concentration of H_2O_2. The CL measurement was made according to a procedure in which a 6.0 μM H_2O_2 solution was used. A typical CL response curve in a lipid-free bulk solution is shown in Fig. 2 (curve 1). CL emission occurred instantaneously just after initiation of the CL reaction. Two peaks appeared in the CL response curve in which first peak reached its maximum height in 4 s, and then began to decay rapidly. The maximum height of second peak occurred after 75 s from initiation of the reaction. On the other hand, light emission in HRP-catalyzed luminol CL in VET_{1000} appeared rapidly after starting the reaction, and reached its maximum intensity at 25 s, after which the intensity of the light emission gradually decreased, as shown in Fig. 2 (curve 2). Only one peak appeared in the CL response curve.

The differences in the CL response curves at a high concentration of H_2O_2 may be interpreted as follows. HRP is generally accepted to catalyze the oxidation of luminol through the enzymatic cycle illustrated in Fig. 3.[21] The numbers in parentheses show the effective oxidation level of iron in HRP intermediates. Most of luminol (LH_2) exists as LH^- at pH 8.0 to participate as an electron donor in the HRP reaction. Luminol reacts with such HRP-intermediates as compounds I to form

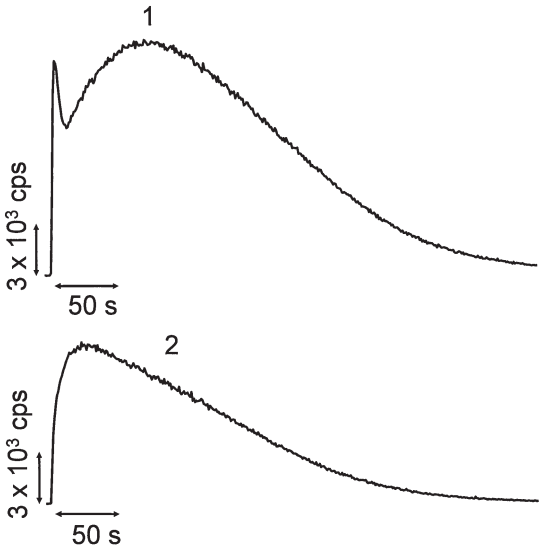

Fig. 2 Typical CL response curves at the high concentration of H_2O_2. Curve 1, detection of HRP dissolved in lipid-free bulk solution. Curve 2, direct detection of HRP encapsulated in VET_{1000}. Conditions for the CL measurements: [HRP] = 100 nM, [luminol] = 50 μM, [H_2O_2] = 6.0 μM. All the reagent concentrations are initial concentrations.

compound II and luminol radicals ($L^{-\cdot}$). Compound II, thus formed, reacts with luminol to yield native HRP and luminol radicals. This enzymatic cycle is called a peroxidase cycle. Luminol radicals generated from the peroxidase cycle react with dissolved oxygen to yield endoperoxide (LO_2^{2-}) [reaction (1)]. LO_2^{2-} then decomposes to yield an electronically excited 3-aminophthalate dianion (AP^{*2-}) [reaction (2)], which returns to the ground state to emit light [reaction (3)]:[21]

$$2L^{-\cdot} + O_2 \longrightarrow L + LO_2^{2-} \quad (1)$$
$$LO_2^{2-} \longrightarrow AP^{*2-} + N_2 \quad (2)$$
$$AP^{*2-} \longrightarrow AP^{2-} + h\nu \quad (3)$$

On the other hand, when excess amounts of H_2O_2 are present in the reaction mixture, compound II is oxidized by H_2O_2 to form compound III (ferrous-O_2 complex), which is less reactive than compounds I and II.[22] Previously, we found that compound III reacts with an electron donor, such as umbelliferone, to produce compound I and umbelliferone raical.[23] The reaction rate of compound III with umbelliferone was remarkably slower than that of compound I with umbelliferone.

Therefore, the results as shown in Fig. 2 may be interpreted as follows. HRP-catalyzed luminol CL in a lipid-free bulk solution proceeds rapidly upon the addition of H_2O_2, thus resulting in an increase in light emission. However, compound II formed by peroxidase cycle could react with excess H_2O_2 to increase the concentration of compound III, and then decrease in light emission, thus resulting in the appearance of the first peak. The reaction rate (k_4) of compound II with H_2O_2 is remarkably slower than that (k_1) of native HRP with H_2O_2.[24] Compound III thus formed reacts with luminol to yield luminol radicals. Luminol radicals react with oxygen to yield an electronically excited 3-aminophthalate dianion (AP^{*2-}), which returns to the ground state while emitting. The reaction rate (k_5) of compound III with luminol could be remarkably slower than that (k_2 and k_3) of compounds I and II with luminol. Therefore, the second peak could appear slowly in HRP-catalyzed luminol CL in a lipid-free bulk solution, as shown in Fig. 2 (curve 1).

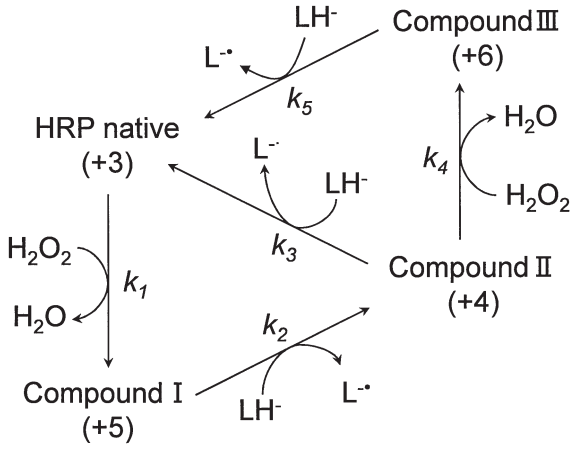

Fig. 3 Enzymatic cycle of HRP in terms of various intermediate states. The numbers in parentheses denote the effective oxidation numbers of HRP.

In contrast, only one peak appeared in the CL reaction in VET_{1000}. This is because the concentration of H_2O_2 in VET_{1000} is lower than that in a lipid-free bulk solution by the liposomal membrane permeation of H_2O_2. In addition, the concentration of HRP encapsulated in VET_{1000} is about one tenth greater than that of HRP dissolved in the bulk solution. Therefore, the concentration ratio of H_2O_2 to HRP in the CL reaction in VET_{1000} could be lower than that in the lipid-free bulk solution, thus resulting in suppressing the formation of compound III and in the appearance of only one peak in the CL reaction in VET_{1000}.

Analytical results and parameters

In subsequent studies, the optimum conditions for the concentrations of H_2O_2 and luminol were determined by measuring the CL response curves, in which only one peak appears due to the peroxidase cycle and the CL intensity is maximum.

First, in the HRP-catalyzed CL reaction in the lipid-free bulk solution, the effect of the H_2O_2 concentration on the CL response curve was examined in the range of 0.2 – 2.0 μM using a 1.6 μM solution of HRP prepared in the lipid-free buffer solution. Below 2.0 μM of H_2O_2, only one peak appeared in the CL response curves. The CL intensity linearly increased with increasing in the concentration of H_2O_2 in the range of 0.2 to 1.0 μM. The optimum concentration of H_2O_2 was thus determined to be 1.0 μM. We next examined the effect of the luminol concentration on the CL intensity in the range of 10 to 300 μM. Two peaks appeared below 10 μM of luminol. The CL intensity linearly decreased with an increase of the luminol concentration in the range of 30 to 300 μM. The optimum concentration of luminol was thus determined to be 30 μM.

Next, the dependence of the H_2O_2 concentration on the direct detection of HRP encapsulated in VET_{1000} was examined in the range of 0.2 to 300 μM. The concentration of HRP dissolved in the bulk solution after the lysis of HRP-encapsulated liposomes with Triton X-100 was 1.6 μM. The CL intensity had a broad maximum at 6.0 μM of H_2O_2. The optimum concentration of H_2O_2 was thus chosen to be 6.0 μM. Next, we examined the effect of the luminol concentration on the CL intensity in the range of 30 to 300 μM. The CL intensity had a broad maximum at 50 μM of luminol. The optimum concentration of luminol was thus chosen to be 50 μM.

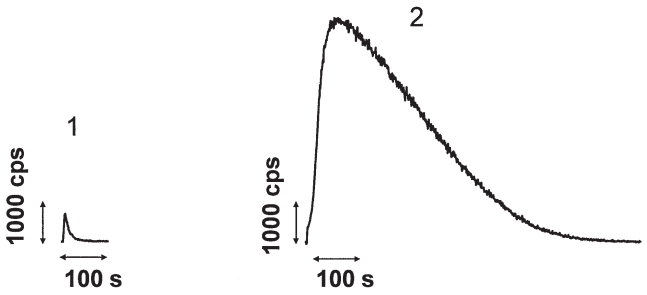

Fig. 4 Typical CL response curves in both lipid-free bulk solution and in VET_{1000} at the optimal concentrations of luminol and H_2O_2. Curve 1, detection of HRP dissolved in the lipid-free bulk solution. Conditions for the CL measurements: [HRP] = 100 nM, [luminol] = 30 μM, [H_2O_2] = 1.0 μM. Curve 2, direct detection of HRP encapsulated in VET_{1000}. Conditions for the CL measurements: [HRP] = 100 nM, [luminol] = 50 μM, [H_2O_2] = 6.0 μM. All the reagent concentrations are initial concentrations.

The optimal concentrations of H_2O_2 and luminol in the CL reaction in VET_{1000} were greater than those in the CL reaction in lipid-free bulk solution. This is because the peroxidase cycle in the CL reaction in VET_{1000} could need a higher concentration of H_2O_2 and luminol compared with those in a lipid-free bulk solution due to the liposomal membrane permeation of luminol and H_2O_2. CL response curves both in VET_{1000} and in lipid-free bulk solution were measured under the optimum conditions. The results are shown in Fig. 4. The CL intensity and the total intensity of light emission in the CL reaction in VET_{1000} was remarkably greater than those in the lipid-free bulk solution. This is because of the difference in the optimal concentrations of H_2O_2 and luminol used.

The recommended procedure described in the experimental section was established based on the optimum conditions. First, the calibration curve of HRP dissolved in a lipid-free bulk solution was prepared by measuring the CL intensity. The logarithmic calibration curve was linear from the detection limit of 3.0 to 100 nM with a slope of 0.899 and a correlation coefficient (R^2) of 0.999. The detection limit for HRP was defined as the concentration of HRP that produced a CL intensity equal to triple the standard deviation of the blank intensity counted in the mixture containing no HRP. The relative standard deviations of the CL intensity for five experiments was 4.2% for 40 nM HRP.

Next, VET_{1000} suspensions containing HRP collected by gel filtration were diluted arbitrary for preparing calibration curves for the direct detection of HRP encapsulated in VET_{1000}. The concentration of HRP was determined after the lysis of HRP-encapsulated VET_{1000} with Triton X-100. The logarithmic calibration curve was linear from the detection limit of 1.0 to 100 nM with a slope of 0.950 and a correlation coefficient (R^2) of 0.999. The relative standard deviations of the CL intensity for five experiments were 4.4% for 40 nM HRP. The detection limit for the direct detection of HRP encapsulated in VET_{1000} was improved by a factor of 3 compared with that of the detection of HRP dissolved in lipid-free bulk solution. In addition, the CL intensity in the CL reaction in VET_{1000} was a factor of seven-times greater than that in the lipid-free bulk solution.

In conclusion, the direct detection of HRP encapsulated in VET_{1000} was made by using luminol CL with H_2O_2 by considering the permeation of luminol and H_2O_2 into the inner phase of liposomes. The CL intensity observed in the direct detection of HRP encapsulated in VET_{1000} was remarkably increased compared with that observed in the detection of HRP dissolved in the lipid-free bulk solution. When HRP-encapsulated liposomes are used as labels in enzyme-immunoassays, the direct detection of HRP by using luminol CL could be more effective compared with the CL method in which HRP encapsulated in liposomes is detected after the lysis of liposomes using lytic agents.

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