ANALYTICAL SCIENCES SEPTEMBER 2009, VOL. 25

Notes

Application of 4-Iodophenol-enhanced Luminol Chemiluminescence to Direct Detection of Horseradish Peroxidase Encapsulated in Liposomes

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4-Iodophenol was applied to an enhancer in the direct detection of horseradish peroxidase (HRP) encapsulated in liposomes by using luminol chemiluminescence (CL). Luminol, 4-iodophenol and hydrogen peroxide permeate into the inner phase of liposomes containing HRP, resulting in the progress of 4-iodophenol-enhanced luminol CL catalyzed by HRP in liposomes. The CL intensity observed in liposomes was a factor of 150 greater than that observed in a lipid-free bulk solution. The detection limit in the direct detection of HRP encapsulated in liposomes was sensitive by a factor of 30 compared with that in a lipid-free bulk solution. 4-Iodophenol effectively functioned as an enhancer in HRP-catalyzed luminol CL in liposomes.

(Received July 8, 2009; Accepted July 27, 2009; Published September 10, 2009)

Introduction

Liposomes are spherical vesicles composed of phospholipids that have an aqueous volume enclosed by lipid bilayers.[1] Recently, liposomes encapsulating such enzymes as alkaline phosphatase[2] and β-galactosidase[3] have been employed as markers for enzyme-immunoassay (EIA) for obtaining signal-enhancement and higher sensitivities.

Horseradish peroxidase (HRP) is also used as a marker enzyme in EIA and can be sensitively detected by a luminol chemiluminescence (CL) method.[4] However, there have been no reports on the encapsulation of HRP in liposomes. We then prepared HRP-encapsulated liposomes by an extrusion technique.[5] The number of HRP molecules encapsulated in liposomes was about 1200 HRP molecules per liposome. HRP encapsulated in liposomes was detected by a luminol CL method after the release of HRP from liposomes, accomplished by lysis of liposomes with Triton X-100. However, the dissolution of the HRP concentrated in the aqueous interior of liposomes into the bulk solution might cause a dilution of HRP, resulting in a decrease in the sensitivity of HRP. Therefore, the direct detection of HRP encapsulated in liposomes without lysis of liposomes could be desirable for improving the sensitivity of HRP.

In the course of our studies on the direct detection of HRP encapsulated in liposomes by a CL method, we found that luminol and hydrogen peroxide (H_2O_2) permeate into the inner phase of liposomes to initiate HRP-catalyzed luminol CL with H_2O_2.[6] The intensity of light emission observed in HRP-catalyzed luminol CL in liposomes was a factor of seven greater than that observed in a lipid-free bulk solution. We then applied the luminol CL method for the direct determination of HRP encapsulated in liposomes prepared by an extrusion technique. The detection limit in the direct detection of HRP encapsulated in liposomes was more sensitive by a factor of 3 compared with that in HRP-catalyzed luminol CL in a lipid-free bulk solution. On the other hand, the intensity of light emission in HRP-catalyzed luminol CL markedly increases by adding the substrate that works as an enhancer.[7] Though numerous derivatives of phenol act as substrates, 4-iodophenol is the most popular enhancer in HRP-catalyzed luminol CL.[8]

In the present work, 4-iodophenol was applied to an enhancer in HRP-catalyzed luminol CL in liposomes for improving the sensitivity in the direct detection of HRP encapsulated in liposomes.

Experimental

Reagents and solutions

Egg yolk phosphatidylcholine (PC; molecular weight, 773) was purchased from Nichiyu Liposome Co. (Tokyo, Japan). DL-α-Phosphatidylglycerol dimyristoyl (PG), cholesterol and 4-iodephenol were obtained from Wako Chemicals Co. (Tokyo, Japan). Luminol purchased from Kanto Chemicals (Tokyo, Japan) was a guaranteed-grade reagent. Horseradish peroxidase (HRP; Type VI) was bought from Sigma Chemical Co. All other chemicals used were guaranteed-grade 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 spectrophotometrically with an ε_{403} value of 1.02×10^5 M/cm.[9] 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

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

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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 (MLV). The MLV suspensions in the flask were frozen at –196°C by gently shaking the flask in liquid nitrogen for 1 min and left to thaw at 25°C for 35 min. The procedure of freezing and thawing was repeated three times. Large unilamellar vesicle (LUV) suspensions thus prepared were extruded through two stacked polycarbonate filters with a pore size of 1000 nm, using a LiposoFast-Basic extruder (Avestin Inc.). The LUV suspensions 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 every 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 with inductively coupled plasma atomic emission spectrometer equipped with an ultrasonic nebulizer (ICPS-1000IV, Shimadzu, Japan).[10] The amounts of HRP encapsulated in VET_{1000} were determined as previously described.[6]

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 CL measurements of HRP encapsulated in VET_{1000}, a 50-μl portion of HRP-encapsulated VET_{1000} suspensions, a 50-μl portion of H_2O_2 solution and a 100-μl portion of the buffer solution were added into a glass cuvette (19 mm i.d. × 22 mm) in a luminometer (BLD-100HU, Tohoku Electronic Industrial). Next, a 200-μl portion of luminol solution containing 4-iodophenol 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).

In the CL measurements of HRP dissolved in lipid-free bulk solution, a 50-μl portion of a 1.0 μM HRP solution, a 50-μl portion of H_2O_2 solution and a 100-μl portion of the buffer solution were placed in the glass cuvette in the luminometer. Next, a 200-μl portion of a luminol solution containing 4-iodophenol was injected into the cuvette with the injector, and the CL reaction was initiated.

Results and Discussion

Characterization of HRP-encapsulated VET_{1000}

In order to increase the trapping efficiency of HRP encapsulated in VET_{1000}, we prepared HRP-encapsulated VET_{1000} by both freezing-thawing and extrusion techniques. 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 578 ± 150 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 36%. Triplicates agreed with less than 1.5% from the mean. The trapping efficiency of HRP in VET_{1000} prepared by both freezing-thawing and extrusion techniques was a factor of 3.5 greater than that prepared by extrusion technique alone.

Effect of 4-iodophenol on HRP-catalyzed luminol CL in VET_{1000}

In 4-iodophenol-enhanced luminol CL, native HRP reacts initially with H_2O_2 to form compound I as a HRP-intermediate [reaction (1)].[11]

$$\text{Native HRP} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

In the presence of both luminol and 4-iodophenol (IP) as a substrate, 4-iodophenol reduced HRP-intermediates in place of luminol through the following reactions, since the reduction potential of 4-iodophenol is greater than that of luminol [reactions (2) and (3)].[12]

$$\text{Compound I} + \text{IP} \longrightarrow \text{Compound II} + \text{IP}^{-\bullet} \quad (2)$$

$$\text{Compound II} + \text{IP} \longrightarrow \text{Native HRP} + \text{IP}^{-\bullet} \quad (3)$$

4-Iodophenol radicals ($IP^{-\bullet}$) thus formed react with luminol (LH^-) to form luminol radicals ($L^{-\bullet}$) [reaction (4)].

$$LH^- + IP^{-\bullet} \longrightarrow L^{-\bullet} + IP \quad (4)$$

Luminol radicals react with dissolved oxygen to yield endoperoxide (LO_2^{2-}) [reaction (5)]. LO_2^{2-} then decomposes to yield an electronically excited 3-aminophthalate dianion (AP^{*2-}) [reaction (6)], which returns to the ground state to emit light [reaction (7)]:

$$2L^{-\bullet} + O_2 \longrightarrow L + LO_2^{2-} \quad (5)$$

$$LO_2^{2-} \longrightarrow AP^{*2-} + N_2 \quad (6)$$

$$AP^{*2-} \longrightarrow AP^{2-} + h\nu \quad (7)$$

The formation rate of luminol radicals in the presence of 4-iodophenol remarkably increases compared with that in the presence of luminol alone, thus resulting in the increase in the amount of light emission. Therefore, the permeation rate of 4-iodophenol into the inner phase of liposomes should be the same as that of luminol and H_2O_2.

On the other hand, the permeation rate of luminol, 4-iodophenol and H_2O_2 into the inner phase of VET_{1000} could be related to the membrane composition of VET_{1000}, because the membrane permeability depends on the lipid compositions. Cholesterol is known to reduce the fluidity of hydrocarbon chains in the lipid

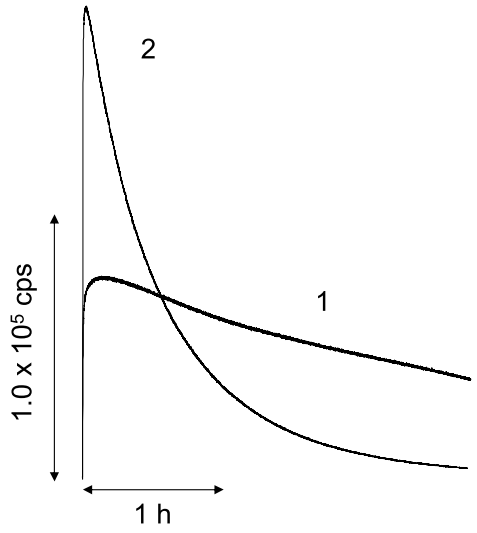

Fig. 1 Typical CL response curves in the direct detection of HRP encapsulated in VET_{1000}. Curve 1, detection of HRP in the absence of 4-iodophenol. Conditions for the CL measurements: [HRP] = 4.0 nM, [H_2O_2] = 100 μM, [luminol] = 400 μM. Curve 2, detection of HRP in the presence of 4-iodophenol. Conditions for the CL measurements: [HRP] = 4.0 nM, [H_2O_2] = 100 μM, [4-iodophenol] = 200 μM, [luminol] = 400 μM. All the reagent concentrations are initial concentrations.

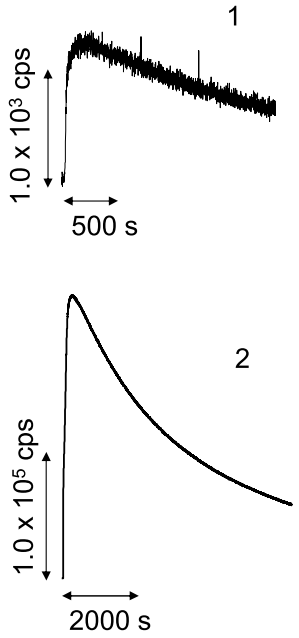

Fig. 2 Typical CL response curves both in lipid-free bulk solution and in VET_{1000} at the optimal concentrations. Curve 1, detection of HRP dissolved in the lipid-free buffer solution. Conditions for the CL measurements: [HRP] = 100 pM, [H_2O_2] = 10 μM, [4-iodophenol] = 200 μM and [luminol] = 30 μM. Curve 2, direct detection of HRP encapsulated in VET_{1000}. Conditions for the CL measurements: [HRP] = 100 pM, [H_2O_2] = 1.0 mM, [4-iodophenol] = 100 μM and [luminol] = 300 μM. All the reagent concentrations are initial concentrations.

bilayer of membranes by cholesterol-phospholipid interaction.[13] Previously, we investigated the effect of the concentration of cholesterol on the membrane permeation *via* use of HRP-catalyzed eosin Y CL in VET_{1000}.[14] The concentration of cholesterol was investigated in the range 30 – 45 mol%, since the effect of cholesterol on membrane fluidity appears remarkably above 30 mol% of cholesterol in liposomes.[15] The permeation rate of eosin Y was maximal at 30 mol% of cholesterol in VET_{1000}. Therefore, the lipid composition of VET_{1000} was thus chosen to be a PC:PG:cholesterol molar ratio of 6:1:3.

First, the direct detection of HRP encapsulated in VET_{1000} was performed in the absence of 4-iodophenol. A 200-μl portion of 400 μM luminol solution was added into the mixtures of a 50-μl portion of HRP-encapsulated VET_{1000} suspensions, a 50-μl portion of 100 μM H_2O_2 solution and a 100-μl portion of the buffer solution. The concentration of HRP dissolved in the bulk solution after lysis of HRP-encapsulated liposomes with Triton X-100 was determined according to the procedure. A typical CL response curve is shown in Fig. 1 (curve 1). Light emission appeared after the CL reaction started, and reached its maximum intensity at 8.3 min, after which the intensity of light emission gradually decreased.

Next, the direct detection of HRP encapsulated in VET_{1000} was performed in the presence of 4-iodophenol. A 200-μl portion of 400 μM luminol solution containing 200 μM 4-iodophenol was added into the mixtures of a 50-μl portion of HRP-encapsulated VET_{1000} suspensions, a 50 μl-portion of 100 μM H_2O_2 solution and a 100 μl-portion of the buffer solution. The concentration of HRP dissolved in the bulk solution was the same as that in the CL measurement in the absence of 4-iodophenol. A typical CL response curve is shown in Fig. 1 (curve 2). Light emission appeared after the CL reaction started, and reached its maximum intensity at 3.9 min, after which the intensity of light emission decreased. The initial rate of HRP-catalyzed luminol CL in the presence of 4-iodophenol was 2.2 times faster than that in the absence of 4-iodophenol. The initial rate of the CL reaction was obtained as the slope of the CL response curves immediately after the CL reaction started. In addition, the CL intensity in the presence of 4-iodophenol was 2.3 times greater than that in the absence of 4-iodophenol. The maximum light emission is referred to as the CL intensity. These results indicate that 4-iodophenol permeates across the liposomal membrane and acts as an enhancer in HRP-catalyzed luminol CL in VET_{1000}.

Analytical results and parameters

In subsequent studies, the optimum conditions for the concentrations of H_2O_2, luminol and 4-iodophenol were determined by measuring the CL response curves, in which only one peak appears due to the peroxidase cycle[6] and the CL intensity is maximum.

First, the effect of the H_2O_2 concentration on the CL response curve in the lipid-free bulk solution was examined in the range of 6.0 – 100 μM using a 100 pM solution of HRP prepared in the lipid-free buffer solution. Below 10 μM of H_2O_2, only one peak appeared in the CL response curves. The optimum concentration of H_2O_2 was thus determined to be 10 μM. The dependence of the 4-iodophenol concentration on the CL intensity was examined in the range of 10 to 300 μM. The CL intensity had a broad maximum at 200 μM of 4-iodophenol. The optimum concentration of 4-iodophenol was thus determined to be 200 μM. We next examined the effect of the luminol concentration on the CL intensity in the range of 10 to 100 μM. The CL intensity had a broad maximum at 30 μM of luminol. 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 600 μM to 10 mM. The concentration of HRP dissolved in the bulk solution after the lysis of HRP-encapsulated liposomes with Triton X-100 was 100 pM. Below 1.0 mM of H_2O_2, only one peak appeared in the CL response curves. The optimum concentration of H_2O_2 was thus chosen to be 1.0 mM. The dependence of the 4-iodophenol concentration on the CL intensity was examined in the range of 10 μM to 1.0 mM. The CL intensity had a broad maximum at 100 μM of 4-iodophenol. The optimum concentration of 4-iodophenol was thus determined to be 100 μM. Next, we examined the effect of the luminol concentration on the CL intensity in the range of 10 μM to 1.0 mM. The CL intensity had a broad maximum at 300 μM of luminol. The optimum concentration of luminol was thus chosen to be 300 μM.

CL response curves both in VET_{1000} and in lipid-free bulk solution were measured under the optimum concentrations thus established. Light emission reached its maximum intensity at 4.5 min in lipid-free bulk solution (Fig. 2, curve 1) and at 3.9 min in VET_{1000} (Fig. 2, curve 2), respectively. The CL intensity observed in VET_{1000} was 150 times greater than that in the lipid-free bulk solution. The enhancement of the CL intensity in VET_{1000} could be explained in terms of the concentration effect of HRP in VET_{1000}. The trapping efficiency of HRP in VET_{1000} was 36%, suggesting that the concentration of HRP encapsulated in VET_{1000} is three times greater than that of HRP dissolved in the bulk solution after lysis of HRP-encapsulated VET_{1000}. In addition, the difference in the optimal concentrations of H_2O_2 and luminol is probably attributable to the differences in the CL intensity between in VET_{1000} and in lipid-free bulk solution.

The calibration curve of HRP dissolved in a lipid-free bulk solution was prepared. The logarithmic calibration curve was linear from the detection limit of 30 pM to 3.0 nM with a slope of 1.20 and a correlation coefficient (R^2) of 0.994. 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 were 2.1% for 1.0 nM HRP.

Next, VET_{1000} suspensions containing HRP collected by gel filtration were diluted arbitrarily for preparing calibration curves for the direct detection of HRP encapsulated in VET_{1000}. The concentration of HRP was determined after lysis of HRP-encapsulated VET_{1000} with Triton X-100. The logarithmic calibration curve was linear from the detection limit of 1.0 pM to 100 pM with a slope of 1.20 and a correlation coefficient (R^2) of 0.998. The relative standard deviations of the CL intensity for five experiments were 2.2% for 10 nM HRP. The detection limit for the direct detection of HRP encapsulated in VET_{1000} was improved by a factor of 30 compared with that of the detection of HRP dissolved in lipid-free bulk solution.

In conclusion, 4-iodophenol permeates the liposomal membranes to function effectively as an enhancer in HRP-catalyzed luminol CL in liposomes. When HRP-encapsulated liposomes are applied to labels in EIA, 4-iodophenol-enhanced luminol CL is effective in the direct detection of HRP encapsulated in liposome.

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