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Citation	Analytical Sciences, 21(6), 701-704 https://doi.org/10.2116/analsci.21.701
Issue Date	2005-06
Doc URL	https://hdl.handle.net/2115/71701
Type	journal article
File Information	Anal.sci.21-701.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

ANALYTICAL SCIENCES JUNE 2005, VOL. 21

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Notes

Determination of Peroxidase Encapsulated in Liposomes Using Homogentisic Acid γ-Lactone Chemiluminescence

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Homogentisic acid γ-lactone (HAL) chemiluminescence (CL) was applied to the determination of horseradish peroxidase (HRP) encapsulated in liposomes. HRP was detected after the lysis of HRP-trapped liposomes with Triton X-100. CL response rate, detection limit and linear range of calibration curve for HRP in HAL CL were compared with those in *p*-iodophenol (*p*-IP)-enhanced luminol CL. Maximal light emission in HAL CL appeared more rapidly compared to that in *p*-IP enhanced luminol CL, thus resulting in remarkable reduction of CL measurement time. The detection limit for HRP in HAL CL was the same as that in *p*-IP-enhanced luminol CL. The linear range of calibration curve for HRP in HAL CL was improved by a factor of 50 compared with that in *p*-IP-enhanced luminol CL. From these results, it was found that HAL CL were superior to *p*-IP-enhanced luminol CL for the determination of HRP encapsulated in liposomes.

(Received February 16, 2005; Accepted April 11, 2005)

Liposomes have been employed as a signal-enhancement agent in immunoassays, since thousands of small marker molecules such as calcein[1,2] and carboxyfluorescein[3,4] can be encapsulated in the aqueous interior. The increase in the number of marker molecules conjugated to an antibody can be a lower detection limit. The encapsulation of macromolecules such as enzymes inside liposomes were also performed with alkaline phosphatase[5,6] and glucose oxidase.[7] An antibody labeled with liposome encapsulating those enzymes has been employed for immunoassays. The marker molecules can be determined by spectrophotometric and fluorometric methods after the lysis of liposomes using lytic agents such as Triton X-100.

We prepared previously horseradish peroxide (HRP)-trapped vesicles by an extrusion technique (HRP-trapped VETs).[8] The number of HRP molecules encapsulated in the VETs was about 1200 HRP molecules per VET. The activity of HRP remained throughout the encapsulation process. Next, HRP-trapped VETs were coupled to anti-rabbit IgG using *N*-hydroxy-succinimide ester palmitic acid as a component of VETs.[9] *p*-Iodophenol (*p*-IP)-enhanced luminol chemiluminescence (CL) was applied to the detection of HRP dissolved in the bulk solution after the lysis of HRP-trapped VETs with Triton X-100. The CL intensity per antibody in the detection of HRP encapsulated in the antibody-coupled VETs was 125-times greater than that of HRP conjugated directly to the antibody. In addition, HRP-trapped VETs containing biotinylated dipalmitoylphosphatidylethanolamine were prepared by an extrusion technique and were applied to labels in immunodotblotting of rabbit IgG.[10] In the previous investigation, we used *p*-IP-enhanced luminol CL for the detection of HRP owing to their high sensitivity. However, in *p*-IP-enhanced luminol CL, the time needed for maximal light emission to appear increased remarkably for the concentrations of HRP near the detection limit. In addition, the linear range of calibration curve for HRP was narrow in *p*-IP-enhanced luminol CL. On the other hand, we found previously that CL response appeared immediately for the detection of HRP using homogentisic acid γ-lactone (HAL) CL in an aqueous dimethyl sulfoxide solution.[11]

In the present study, HAL CL were applied to the determination of HRP to improve CL measurement time and to extend the linear range of HRP concentrations in the calibration curve.

Experimental

Materials

Egg-yolk phosphatidylcholine (PC), DL-α-phosphatidylglycerol dimyristoyl (DMPG), cholesterol (Chol), *p*-iodophenol (*p*-IP) and horseradish peroxidase (HRP) were purchased from Wako Pure Chemicals Co. (Japan). Luminol was purchased from Kanto Chemicals (Japan). Homogentisic acid γ-lactone (HAL) was bought from Tokyo Kasei Kogyo Co. 3-Morpholino-propanesulfonic acid (Mops) were obtained from Dojindo Laboratories (Japan). The concentration of PC was calculated by use of a molecular weight of 765.[12] All other chemicals used were guaranteed-grade reagents and were used without further purification.

A 0.1 mM solution of HRP was prepared by dissolving the compound with 0.1 M Mops-buffer solution (pH 7.0) in HAL CL. In *p*-IP-enhanced luminol CL, 10 mM Tris–HCl buffer solution (pH 8.0) was used in place of Mops-buffer solution. A stock solution of 5.0 mM HAL was prepared by dissolving HAL in dimethyl sulfoxide (DMSO). All solutions used were prepared with water from a Millipore Milli-Q water purification system.

All of the reagent concentrations reported and shown in the figures are initial concentrations.

Preparation of liposomes encapsulated HRP by an extrusion technique

A mixture of 32 μmol PC, 4.0 μmol DMPG and 4.0 μmol Chol in chloroform was used to prepare a lipid film. The

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procedure for the preparation of multilamellar vesicles (MLVs)-encapsulated HRP was the same as that previously reported.[8] Next, HRP-trapped MLVs were extruded through polycarbonate filters with 1000 nm pore size. The filters were mounted in LiposoFastTM-Basic (Avestin Inc.) fitted with two 0.50 ml Hamilton syringes. We subjected samples to 20 passes through a single filter. The notation VET_{1000} indicates liposomes extruded through polycarbonate filters with 1000 nm pore size.

Separation of HRP-trapped VET_{1000} from free HRP was performed on a Sephadex G-200 column according to a procedure described previously.[8] HRP-trapped VET_{1000} collected by the column were stored at 4°C in a refrigerator.

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

CL measurement of HRP released from HRP-trapped VET_{1000}

The experimental procedure in HAL CL consisted in pipeting a 60 µl portion of HRP-trapped VET_{1000} suspensions, a 200 µl portion of a 1.0 mM H_2O_2 solution and a 60 µl portion of 0.1 M Triton X-100 solution into a plastic cuvette (15 mm i.d. × 15 mm: internal volume 500 µl) in a luminometer (BLD-100HU, Tohoku Denshi Sangyo Co). Next, a 80 µl portion of a 5.0 mM HAL solution was injected into the cuvette with the injector and the CL reaction was initiated. The final volume percent of DMSO was 20% (v/v). Light emission was detected by a photomulitiplier. The resultant photon current was converted to counts per second (cps), whose value was displayed on a personal computer.

The experimental procedure in *p*-IP enhanced luminol CL consisted in pipeting a 50 µl portion of HRP-trapped VET_{1000} suspensions, a 100 µl portion of a 0.1 mM H_2O_2 solution and a 50 µl portion of 0.1 mM Triton X-100 solution into the cuvette. Next, a 200 µl portion of a solution containing 30 µM luminol and 0.2 mM *p*-IP was added into the cuvette and the CL reaction was initiated.

Results and Discussion

Characterization of HRP-trapped VET_{1000}

The size distribution of HRP-trapped VET_{1000} was measured by collecting the HRP-contained fractions eluted from the column. The size distribution of HRP-trapped VET_{1000} was in the range from 252 nm to 1264 nm, and the mean diameter was 608 nm. The relative standard deviation of mean diameter for HRP-trapped VET_{1000} was 3.8% at three successive measurements. The trapping efficiency of HRP in HRP-trapped VET_{1000} was determined as the mole ratio of HRP encapsulated in the VET_{1000} to HRP in the mixture of free HRP and HRP encapsulated in VET_{1000} before the separation by the column. The concentration of HRP in the VET_{1000} was determined by mixing the fractions containing HRP-trapped VET_{1000}. The trapping efficiency was 13%. The result suggests that the concentration of HRP encapsulated in the VET_{1000} is about an eighth greater than that of HRP dissolved in the bulk solution after the lysis of HRP-trapped VET_{1000} with Triton X-100.

The number of HRP molecules encapsulated in the VET_{1000} was estimated from such experimental quantities as the mean diameter of HRP-trapped VET_{1000} and the molar concentration ratio of HRP to lipid. The relation between these quantities is given as follows:[13,14]

Number of HRP/liposome = (HRP concentration/lipid concentration) × N_{tot}.

The total number of lipid molecules per liposome (N_{tot}) is given by the following equation for spherical unilamellar vesicles of radius (R_v) with bilayer thickness (t) and an average area per lipid molecule (A):[15]

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

The R_v value was calculated from the mean diameter of HRP-trapped VET_{1000}. The bilayer thickness (t) was assumed to be 45 Å. The average area per lipid molecule (A) was calculated using values of 71, 44 and 19 Å^2 for PC, DMPG, and cholesterol, respectively, weighted by the mole fraction of each component. The average value of area per lipid molecule (A) obtained for the VET_{1000} was 63 Å^2/lipid. Then, N_{tot} value was calculated and its value was 3.6×10^6. Next, the concentrations of HRP and lipid in HRP-trapped VET_{1000} were determined by ICP-AES. The number of encapsulated HRP molecules per VET_{1000} was calculated based on these values. The VET_{1000} encapsulated about 1600 HRP molecules per liposome.

We confirmed previously that the activity of HRP remained throughout the encapsulation process by measuring the catalytic activity of HRP for a luminol CL.[8]

Typical CL response curves observed in HAL and p-IP-enhanced luminol CL

The CL measurements of HRP dissolved in the bulk after the lysis of HRP-trapped VET_{1000} with Triton X-100 were made according to the procedure. Total mole amount of PC, DMPG and Chol dissolved the bulk after the lysis were estimated by taking into account the dilution factor of HRP-trapped VET_{1000} prepared according to the procedure. The optimum concentrations of CL reagents in HAL CL and in *p*-IP-enhanced luminol CL were determined by measuring the light emission to be maximal under optimum conditions. Typical CL response

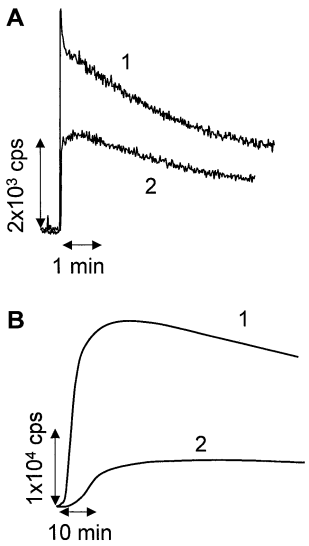

Fig. 1 Typical CL response curves for the detection of HRP dissolved in the bulk solution after the lysis of HRP-trapped VET_{1000} with Triton X-100 in HAL CL and *p*-IP-enhanced luminol CL. A, HAL CL: curve 1, [HRP] = 50 pM, total lipids = 5.5 pmol; curve 2, [HRP] = 20 pM, total lipids = 2.2 pmol; conditions for CL measurements, [HAL] = 5.0 mM, [H_2O_2] = 1.0 mM, [Triton X-100] = 0.1 M, aqueous-20% DMSO solution. B, *p*-IP-Enhanced luminol CL: curve 1, [HRP] = 30 pM, total lipids = 3.2 pmol; curve 2, [HRP] = 20 pM, total lipids = 2.3 pmol; conditions for CL measurements, [luminol] = 30 nM, [H_2O_2] = 100 nM, [*p*-IP] = 200 nM, [Triton X-100] = 1.0 mM.

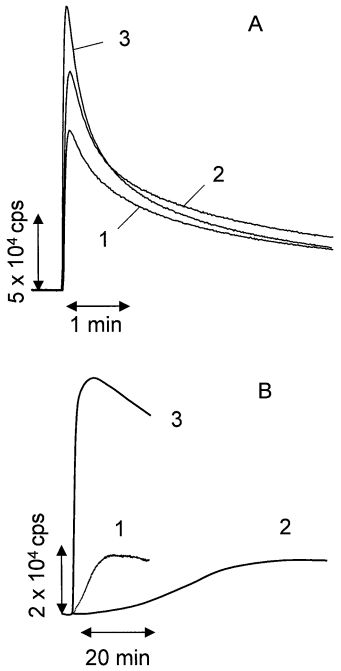

Fig. 2 Typical CL response curves. A, HAL CL; B, *p*-IP-enhanced luminol CL. 1, Detection of HRP in the absence of Triton X-100 and lipids; 2, detection of HRP in the presence of Triton X-100; 3, detection of HRP in the presence of both Triton X-100 and lipids. Conditions for HAL CL measurements: [HRP] = 20 nM, [HAL] = 5.0 mM, [H_2O_2] = 1.0 mM, [Triton X-100] = 0.1 M, total lipids = 2.2 nmol, aqueous–20% DMSO solution. Conditions for *p*-IP-enhanced luminol CL measurements: [HRP] = 60 pM, [luminol] = 30 nM, [H_2O_2] = 100 nM, [*p*-IP] = 200 nM, [Triton X-100] = 1.0 mM, total lipids = 65 pmol.

curves are shown in Fig. 1. In HAL CL (Fig. 1, A), light emission appeared immediately after the start of the reaction, and reached its maximum intensity, after which the light emission decayed gradually. The maximum light emission is referred to as the CL intensity. The time reached at CL intensity was about 20 s near the detection limit of HRP.

On the other hand, in *p*-IP-enhanced luminol CL (Fig. 1, B), the light emission increased gradually after the initiation of the CL reaction and eventually reached its maximum intensity. The time needed for the maximum light emission to appear was about 30 min near the detection limit of HRP. The CL measurement time in *p*-IP-enhanced luminol CL increased remarkably compared to that in HAL CL. The differences in CL response time between HAL CL and *p*-IP-enhanced luminol CL could be attributed to the differences in the reaction rate of HAL and *p*-IP with intermediates of HRP such as compounds I and II.[11]

Effect of Triton X-100 and lipids on CL intensity

In order to investigate whether a reaction medium can function effectively for HAL CL, we added Triton X-100 and lipids to the reaction mixture. Typical CL response curves in HAL CL are shown in Fig. 2-A. Triton X-100 acted as a CL enhancer for HAL CL. The critical micelle concentration (cmc) of Triton X-100 is not known in aqueous–DMSO solution, though cmc of Triton X-100 was 0.22 – 0.23 mM in water alone. The result shown in Fig. 2-A indicated that Triton X-100 could be formed in micelle in the experimental conditions. The presence of both Triton X-100 and lipids is more effective than

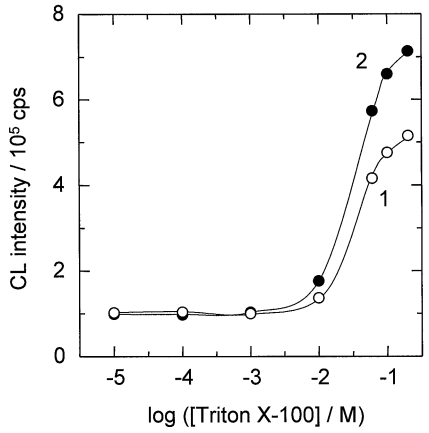

Fig. 3 Effect of Triton X-100 concentration on CL intensity in HAL CL. 1, CL intensity in the presence of Triton X-100; 2, CL intensity in the presence of both Triton X-100 and lipids. Conditions for CL measurements: [HRP] = 20 nM, [HAL] = 5.0 mM, [H_2O_2] = 1.0 mM, total lipids = 2.2 nmol, aqueous–20% DMSO solution.

that of Triton X-100 alone. The CL enhancement in the presence of both Triton X-100 and lipids could be attributable to the formation of mixed micelles of Triton X-100 and lipids.

Typical CL response curves observed in *p*-IP-enhanced luminol CL are shown in Fig. 2-B. The CL response rate in the presence of Triton X-100 (Fig. 2-B2) decreased remarkably compared to that in water alone, though the CL intensity in the presence of Triton X-100 was almost the same as that in water alone. This result suggests that Triton X-100 acts as an inhibitor for HRP in *p*-IP-enhanced luminol CL. On the other hand, as can be seen in Fig. 2-B3, the CL response rate and the CL intensity increased remarkably in the presence of both lipids and Triton X-100. These results could be explained based on the formation of mixed micelles of lipids and Triton X-100, thus resulting in the decrease in the action of Triton X-100 as an inhibitor for HRP.

Effect of Triton X-100 concentrations on CL intensity

The dependence of the CL intensity in HAL CL upon the concentration of Triton X-100 was examined in the absence of VET_{1000} suspensions. The CL intensity–Triton X-100 concentrations profile in the absence of lipids is shown in Fig. 3 (curve 1). The CL intensity increased above 10 mM Triton X-100. The increase in the CL intensity could be explained by taking into account the formation of micelles of Triton X-100.

Next, the dependence of the CL intensity upon the concentrations of Triton X-100 was examined in the range of 10 μM – 0.1 M Triton X-100 in the presence of VET_{1000} containing no HRP. A 0.1 M Triton X-100 solution was the maximal available concentration if one takes into account the solubility of Triton X-100. Figure 3 (curve 2) shows the CL intensity–Triton X-100 concentrations profile in the presence of lipids. When the concentration of Triton X-100 was below 1.0 mM, the CL intensity was about the same as that in water alone. The increase in the CL intensity commenced above 10 mM Triton X-100. The reason for the increase in the CL intensity above 10 mM Triton X-100 could be the formation of mixed micelle of Triton X-100 and lipids; the mixed micelles enhanced the CL intensity more effective compared to micelles of Triton X-100 alone. The optimum concentration for Triton X-100 was chosen to be 0.1 M.

In *p*-IP-enhanced luminol CL, the dependence of the CL

intensity upon the concentration of Triton X-100 was also examined in the range of 10 μM – 10 mM Triton X-100 (results not shown). The CL intensity was about the same as that in water alone below 10 μM. The increase in the CL intensity commenced above 10 μM Triton X-100. The CL intensity increased with increasing Triton X-100 concentrations between 10 – 100 μM, after which the CL intensity was constant. Thus, the optimum concentration for Triton X-100 was chosen to be 1.0×10^{-2} M.

Analytical results and parameters

The calibration curves for HRP dissolved in the bulk after the lysis of HRP-trapped VET_{1000} with Triton X-100 were prepared under the optimum conditions thus established. The logarithmic calibration curve in HAL CL was linear over the range from the detection limit of 20 pM up to 5.0 nM with a slope of 1.02 and a correlation coefficient (R^2) of 0.994. The relative standard deviation of the CL intensity for five successive experiments in HAL CL was 3.0% at 50 pM HRP. The detection limit was defined as the concentration of HRP that produced the CL intensity equal to triple the standard deviation of the blank intensity in the mixture containing no HRP. On the other hand, in *p*-IP-enhanced luminol CL, the logarithmic calibration curve was linear over the range from the detection limit of 20 pM up to 100 pM with a slope of 1.47 and a correlation coefficient (R^2) of 0.996. The relative standard deviation of the CL intensity for five successive experiments in *p*-IP-enhanced luminol CL was 1.9% at 60 pM HRP.

The detection limit and relative standard deviation for HRP in HAL CL were almost the same as those in *p*-IP-enhanced luminol CL. However, the linear range of calibration curve for HRP in HAL CL increased by factors of 50 compared with that in *p*-IP-enhanced luminol CL.

In conclusion, a rapid CL method was developed for the determination of HRP encapsulated in liposomes by use of HAL CL. HAL CL was excellent in the CL response rate and the linearity of calibration curve for HRP was better than that for *p*-IP-enhanced luminol CL.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research (B) (No. 15350038) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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