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Notes

Application of Horseradish Peroxidase-Encapsulated Liposomes as Labels for Immunodotblotting

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Liposomes are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous volume. Recently, attention has focused on liposomes as a signal-enhancement agent, since a number of marker molecules can be encapsulated in their aqueous interior. An antibody labeled with liposome encapsulating enzyme, such as glucose oxidase, has been employed for immunoassays.[1] On the other hand, HRP-conjugated liposomes were prepared by covalently attaching HRP to the outside of the lipid bilayer of liposome.[2,3] However, the preparation of HRP-conjugated liposomes was tedious and time-consuming, since several reaction steps are required for linking HRP covalently to the surface of liposomes. In order to hold simply HRP in liposomes, we examined how to encapsulate HRP into the aqueous interior of liposomes prepared by an extrusion technique (VETs).[4] In addition, the HRP-encapsulated VETs were coupled covalently to anti-rabbit IgG (antibody-tagged VETs).[5] The detection of HRP encapsulated in the antibody-tagged VETs was made by a luminol chemiluminescent (CL) method. On the other hand, biotin-tagged molecules are currently more universal as a marker in immunoassays compared to antibody-tagged marker molecules.

In the present study, the HRP-encapsulated liposomes containing biotinylated dipalmitoylphosphatidylethanolamine were prepared by an extrusion technique (biotin-tagged VETs). In addition, biotin-tagged VETs were applied to labels in immunodotblotting of rabbit IgG.

Experimental

Materials

Egg-yolk phosphatidylcholine (PC), DL-α-dimyristoyl-phosphatidyl-glycerol (DMPG), cholesterol (Chol), biotin-conjugated goat anti-rabbit IgG, biotin-labeled HRP and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries (Japan). Horseradish peroxidase (HRP) (type VI) was bought from Sigma Chemical Co. Rabbit IgG was obtained from Immuno-Biological Industries (Japan). Nitrocellulose membrane filter (type TM-2) was obtained from TOYO ROSHI Co. (Japan). All other chemicals used were guaranteed-grade reagents and were used without further purification. All solutions used were prepared with water from a Millipore Milli-Q water purification system.

Preparation of biotinylated liposomes containing HRP by an extrusion technique (biotin-tagged VETs)

A mixture of 18 μmol PC, 4 μmol DMPG, 16 μmol Chol and 2 μmol biotinylated dipalmitoylphosphatidylethanolamine (BPE) in chloroform was used to prepare a lipid film. BPE was synthesized as previously described.[6] The procedure preparing for multilamellar vesicles (MLVs) containing HRP was the same as that previously reported.[5] Next, biotinylated MLVs containing HRP were extruded through polycarbonate filters with 1000 nm pore size. The filters were mounted in LiposoFast™-Basic (Avestin Inc.) fitted with two 0.50 ml Hamilton syringes.[7] We subjected samples to 20 passes through a single filter, and prepared biotinylated vesicles containing HRP (biotin-tagged VETs).

Separation of the biotin-tagged VETs from free HRP was performed on a Sephadex G-200 column according to a procedure described previously.[5] The biotin-tagged VETs collected by the column were stored at 4˚C in a refrigerator.

Immunodotblotting of rabbit IgG

A 2 μl portion of rabbit IgG solution was added dropwise to a nitrocellulose membrane (5 × 5 mm). The concentration of rabbit IgG was in the range from 0 to 5.0 mg/ml. The membrane blot was air-dried thoroughly and washed for 10 min with a phosphate buffer saline solution (PBS) 3 times. A 3% w/v solution of BSA was added to the blot. The blot was incubated for 2 h at 37˚C and the filter washed for 10 min with PBS 3 times. Next, the blot was incubated successively with a 5 μl portion of a solution containing 10 μg of biotinylated anti-rabbit IgG (goat), a 5 μl portion of a solution containing 10 μg of streptavidin and a 1 ml portion of a solution containing 25 nmol of the biotin-tagged VETs. In each step, the blot was incubated for 1 h at 37˚C and washed for 10 min with PBS 3 times.

Measurement of HRP released from the biotin-tagged VETs by a luminol CL method

After immunodotblotting, the blot was placed into a glass cuvette in a CL detector (TD-3A, Tohoku Denshi Sangyo Co., Ltd.) equipped with an automatic injector (Model 500, Nichiryo Co.). A 500 μl portion of the buffer solution and a 10 μl portion of 10% Triton X-100 solution were added to the cuvette. Next, a 500 μl portion of a solution containing 50 mM luminol, 10 mM hydrogen peroxide and 50 mM *p*-iodophenol was added into the cuvette with the injector. The CL emission was monitored as a function of time with a photomultiplier tube. The resultant photocurrent was converted to a voltage, the value of which was displayed on a chart recorder.

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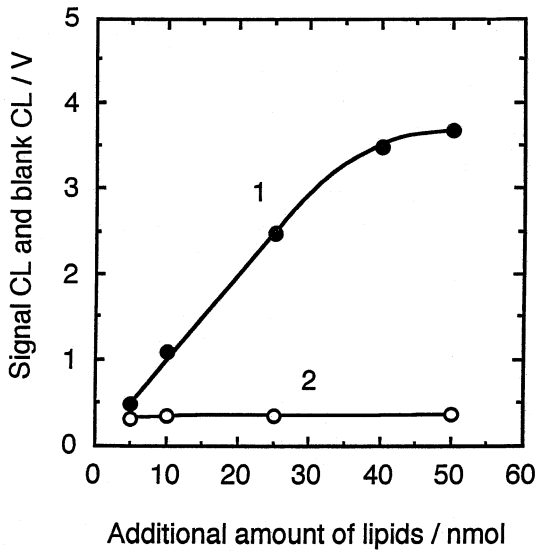

Fig. 1 Effect of an additional amount of the biotin-tagged VETs on the signal CL and blank CL. Curve 1, 1.0 μg of IgG was added dropwise to the membrane; Curve 2, no addition of IgG to the membrane. Conditions for the CL measurements: [luminol] = 50 mM, [H_2O_2] = 10 mM, [*p*-iodophenol] = 50 mM.

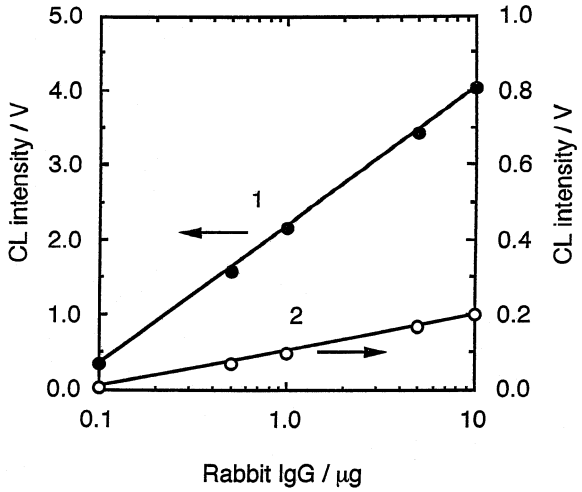

Fig. 2 Calibration curves for rabbit IgG. Curve 1, the biotin-tagged VETs containing 25 nmol lipids was added to the blot; Curve 2, biotin labeled HRP was added to the blot. Conditions for the CL measurements: [luminol] = 50 mM, [H_2O_2] = 10 mM, [*p*-iodophenol] = 50 mM.

Results and Discussion

Optimum conditions for the detection of rabbit IgG

First, we investigated the effect of the amount of the biotin-tagged VETs on the detection of rabbit IgG in immunodotblotting. The biotin-tagged VETs collected according to the procedure were diluted with Mops-buffered saline. The total amount of the liposome components was in the range from 5 to 55 nmol. A solution containing 1 μg of rabbit IgG was added dropwise to the membrane. Next, the biotinylated anti-rabbit IgG solution and the streptavidin solution were added successively to the blot. Finally, a different amount of the biotin-tagged VETs was added to the blot. After the lysis of the biotin-tagged VETs with Triton X-100, HRP released from the biotin-tagged VETs was measured by a luminol CL method.

The CL emission reached its maximum after about 5 min, and gradually decreased after 20 min from initiation of the CL reaction. The signal CL was defined as the maximum height of the CL emission. The dependence of the signal CL upon the amount of biotin-tagged VETs is shown in Fig. 1 (curve 1). The signal CL increased with increasing the amount of the biotin-tagged VETs. The optimum amount of the biotin-tagged VETs was thus chosen to be 25 nmol of total lipids, since the signal CL increased linearly up to 25 nmol of total lipids.

Next, a different amount of the biotin-tagged VETs was added to the membrane in the absence of rabbit IgG. After washing the membrane for 10 min with PBS three times, CL measurements were made after the addition of Triton X-100. Faint CL emission was observed due to the nonspecific adsorption of the biotin-tagged VETs on the membrane. The maximum height of the CL emission observed in the absence of rabbit IgG was referred to as the blank CL. The dependence of the blank CL on the amount of biotin-tagged VETs is shown in Fig. 1 (curve 2). The blank CL was independent of the amount of the biotin-tagged VETs added.

Analytical results and parameters

The calibration curve of rabbit IgG on the use of the biotin-tagged VETs as a label is shown in Fig. 2 (curve 1). The ginal CL in the presence of rabbit IgG was corrected for the blank CL in the absence of rabbit IgG. These background-corrected CL values are referred to as the CL intensity. The calibration curve was linear over the range from the detection limit of 0.1 μg to 10 μg of rabbit IgG. The detection limit for rabbit IgG was defined as the amount of rabbit IgG that produced a signal CL equal to threefold that of the blank CL. The relative standard deviation for five successive experiments was 4.2% at 1.0 μg of rabbit IgG.

Next, the enhancement of the CL intensity upon using the biotin-tagged VETs was confirmed by comparing with that of a biotin labeled HRP as a label, in which HRP was coupled to the antibody by a streptavidin-biotin bond. The biotin-labeled HRP was added to the mixture after bonding biotinylated anti-rabbit antibody to rabbit IgG on the blot. The calibration curve is shown in Fig. 2 (curve 2). The calibration curve was linear over the range from the detection limit of 0.1 μg to 10 μg of rabbit IgG. The relative standard deviation for five successive experiments was 2.8% at 1.0 μg of rabbit IgG. As can be seen in Fig. 2, the CL intensity on the use of the biotin-tagged VETs was 20-times greater than that of the biotin labeled HRP.

On the other hand, the detection limit for rabbit IgG upon using the biotin-tagged VETs was the same as that of the biotin-labeled HRP. This is because the blank CL due to the non-specific adsorption of the biotin-tagged VETs was markedly greater than that of the biotin-labeled HRP.

In order to elucidate the difference in the CL intensities in both labels, the average number of HRP molecules conjugated to the antibody was determined as the ratio of HRP molecules conjugated to the antibody to the antibody molecules used. HRP molecules conjugated to the antibody were determined by measuring the CL intensity. The activity of HRP encapsulated in the biotin-tagged VETs was found to be the same as that of the biotin-labeled HRP by a comparison of the CL response curves observed in both labeles containing the same concentration of HRP. As a result, the average numbers of HRP molecules conjugated to the antibody in the biotin labeled HRP

and the biotin-tagged VETs were 17 and 375 HRP molecules per antibody, respectively. Therefore, the difference in the CL intensities between the biotin-labeled HRP and the biotin-tagged VETs could be explained in terms of the difference in the average numbers of HRP molecule conjugated to the antibody.

When the biotin-tagged VETs were used as a marker in immunoassays of rabbit IgG, the CL intensity increased remarkably compared to the biotin-labeled HRP used. On the other hand, the detection limit was equal in both labels. These results suggest that the biotin-tagged VETs could be effective as a label in immunoassays using a cooled charge-coupled device camera or densitometry with film as a detection system in which the increase of the light emission is preferred to accurately judge the concentration of the analyte.

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