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KOBE UNIVERSITY

Doctoral dissertation

Development of sensitive immunoassay and efficient affinity-screening methods utilizing liposomes

リポソームを利用した高感度免疫測定法および高効率スクリーニング法の開発

Yoichi KUMADA

Graduate school of science and technology
Kobe University, 2005

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Chapter 1

Introduction

In recent years, the number of biological analytes to be detected has increased, especially, in the research areas of clinical assays, proteomics, environmental pollutants, and genetically-modified crop (GM crop), where not only the high throughput, but also more sensitive quantification methods are required. As another example, in 2002, about 1.25 million samples of bovine brain were checked out in Japan to pick out bovine spongiform encephalopathy (BSE) infected cows. Consequently, 5 positive samples were detected although 44 samples were false-positive. With development of the recombinant protein technology and clinical assays, for example, rapid detection methods of proteins with high sensitivity and signal intensity have become essential. Therefore, many detection methods have been proposed and developed to satisfy these requirements. Among these quantification and detection methods, immunoassay methods utilizing the antigen-antibody interaction have following advantages.

- Since purification of samples is not essential, almost all samples can be directly assayed.
- The specific immunointeraction can recognize structure of antigen with high selectivity.
- Small volume of samples (10~ 100 μl) are required.
- Detection limit is much lower than other methods because of higher selectivity and affinity of the immunointeraction.
- Antibodies against targets can easily be obtained by immunization of antigen or cultivation of hybridoma cells.
- Many kinds of biological substances, such as proteins, poly saccharides, hormones, peptides, cytokines and so on, can be detected.

- Various assay formats and signal detection methods can be available depending on characterization of samples.

The immunointeraction between antigen and antibody shows higher affinity and selectivity, because the binding affinities are in the range of $1 \times 10^6 - 1 \times 10^{12}$ (M^{-1}) and are strongly affected by small changes in the antigen structure. Therefore, many kinds of specific antibodies and assay kits are already commercialized to detect target substances with high sensitivity and selectivity.

1.1 Enzyme immunoassay (EIA)

In 1960, Yallow and Berson developed a homogeneous radio immunoassay (RIA) utilizing radioisotope-conjugated antigen to quantify the insulin concentration in human serum. Enzyme immunoassay (EIA) utilizing enzyme-conjugated antigen or antibody was invented in 1970s as a harmless detection method than RIA. In EIA, instead of radioisotopes, enzymes, such as horseradish peroxidase (HRP), calf intestine alkaline phosphatase (CIAP), and bacterial β-galactosidase (β-GAL) are covalently conjugated with antigens or antibodies as markers to detect analytes. Since it was found that many antibodies and antigens could be immobilized on solid supports with retaining their binding activities in 1970s (Engvall and Perlamann, 1971), heterogeneous EIA methods have been developed. Especially, enzyme-linked immunosorbent assay (ELISA) is the most popular among many EIA methods. In comparison with homogeneous ones, various assay formats can be available depending on characteristics of samples and research purposes, although several washing steps are necessary to remove free or nonspecifically adsorbed antibody (antigen) molecules in ELISA (B/F separation). **Figure 1-1** schematically shows several assay formats in ELISA.

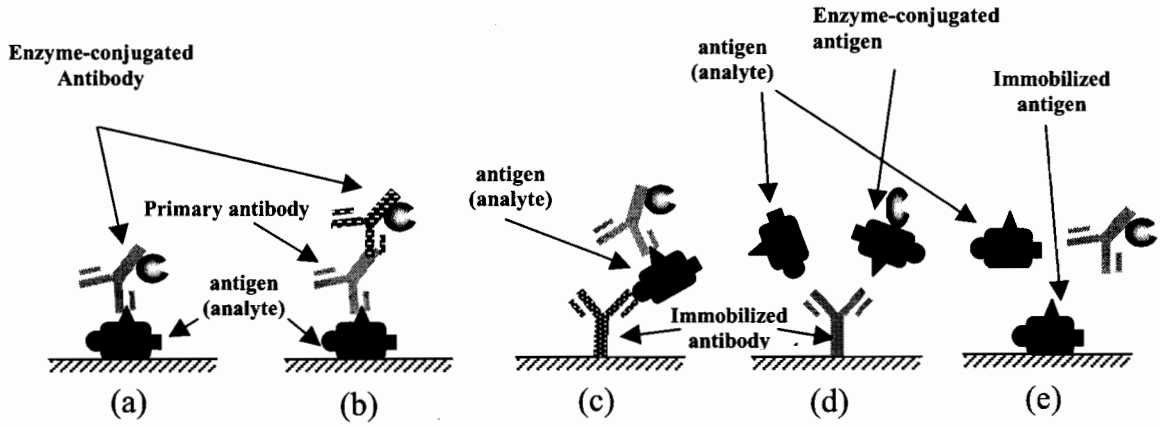


Figure 1-1 Scheme of several assay formats in ELISA

(a) Non-competitive direct method
(b) Non-competitive indirect method
(c) Non-competitive sandwich method
(d) Competitive direct method (enzyme-conjugated antigen)
(e) Competitive direct method (enzyme-conjugated antibody)

Figures 1-1 (a) ~ (c) show the non-competitive ELISA methods of direct, indirect and sandwich-type methods, respectively. In the cases of (a) and (b), analyte molecules are directly immobilized on the solid support by physical adsorption or covalent coupling. In the case of (c), analyte molecules are captured on the solid surface by pre-immobilized antibody and detected by another antibody labeled with an enzyme. **Figures 1-1** (d) and (e) show two kinds of competitive ELISA. These are effective methods for detection of substances of small sizes such as hormones and peptides. In these methods, immobilization of target molecules on the solid support is not required. As shown in **Figure 1-2**, the signal intensity obtained increases with increase in the analyte concentration in non-competitive ELISA, while it decreases with the analyte concentration in competitive ELISA.

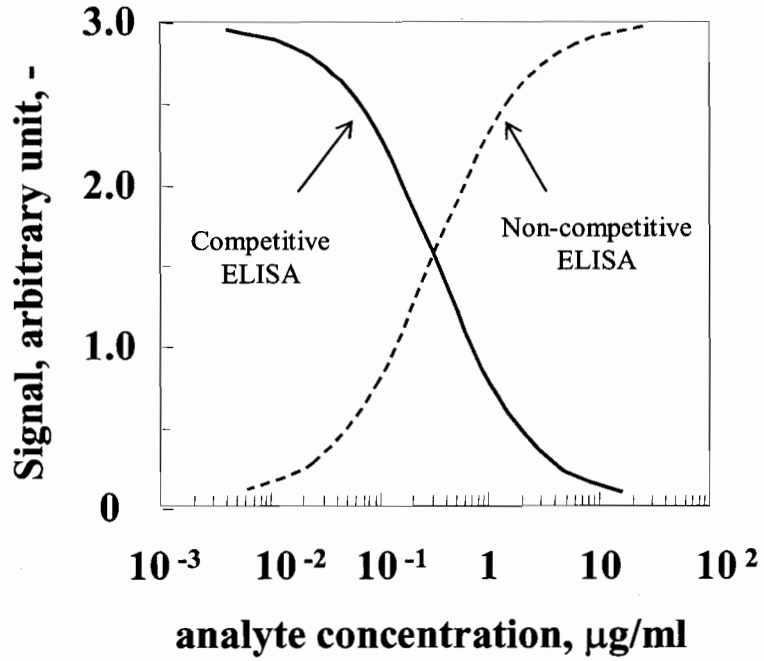


Figure 1-2 Signal intensity change in competitive and non-competitive ELISA

1-1-1 Solid supports in EIA

In EIA, 96 wells microtiter plates made of polystyrene are widely used as solid supports to immobilize analytes and measure signal intensity. Hydrophobic membranes such as PVDF and nitrocellulose are also useful supports in EIA, since they have higher binding capacities of protein analytes. Therefore, they have been used as supports for not only detection of target proteins, but also analysis of protein localization in Western blotting and colony-lift immunoassay. Recently, they have been used as supports for high throughput assay, because analytes can be immobilized with a high density on small areas of membranes, and thus many assay formats can be designed. In comparison with immunoassay methods using microtiter wells, however, special signal detection

methods are required to quantify analytes on the membrane with high sensitivity.

1-1-2 Alternative strategies for sensitive rapid and high throughput immunoassay

Many kinds of supports, signal detection methods, labeled markers, and antibodies were proposed to achieve more sensitive, rapid and high throughput measurements. However, most of them had not satisfied conflicting between the sensitivity and running time or running cost. In EIA, one antibody molecule can be conjugated with a few enzyme molecules as marker, and thus the signal obtained from the antibodies attached on solid surface is limited. Higher signal intensities in EIA could be obtained by utilizing more sensitive enzymes or avidin-biotin interaction, but its running time or cost significantly increased.

For above requirements, immunoassay methods using liposomes (Liposome immunoassay, LIA) have been proposed. A liposome particle composed of phospholipids and other lipids, can encapsulate a large number of many marker molecules, and the particles size can easily be controlled. By applying these characteristics of liposomes, sensitive, rapid and high throughput detection methods could be developed.

1.2 Liposome immunoassay

Liposomes were discovered as synthetic vesicles constituting phospholipid bilayers in 1965. (Bangham et al., 1965) Chemical or biological molecules, such as fluorophores, enzymes, oligonucleotides or drugs, can be immobilized on liposomal membrane and/or encapsulated in inner water phase. Initial researches using liposomes mainly focused on the properties of phospholipid membranes. (Kinsky and Nicolotti, 1977) Recently, liposomes are widely applied to drug or gene delivery systems. (Gregoriadis and Florence, 1993) Immunoliposomes were developed to clarify localization of specific proteins and to deliver drugs to target cells. To apply immunoliposomes in these fields, a wide variety of preparation and conjugation methods have been developed. These

methods are very useful when liposomes are used as analytical tools in immunoassay.

1.2.1 Characteristics of immunoliposomes

(a) Liposome preparation

Both preparation methods and phospholipid compositions significantly affect the characteristics of liposomes produced. As shown in **Figure 1-3**, liposomes are classified into four types depending on their sizes and lamellar structure. Multi-lamellar vesicles (MLVs) are generally constituted 5 – 20 lipid bilayers and their sizes cover a range from several hundred nm to several μm. Small unilamellar vesicles (SUVs), which are prepared from multi-lamellar vesicles (MLVs) by supersonic treatment, have average diameters of 60 nm ~ 100 nm. Large unilamellar vesicles (LUVs) are generally obtained by extruding MLVs several times through a piece of polycarbonate membrane with uniform pore sizes (100 nm – 1 μm). Giant unilamellar vesicles (GUVs) with over 1 μm average diameter are directly prepared from lipid thin film by supplying an appropriate electric field. In addition, as shown in **Figure 1-4** and **Table 1-1**, membrane characteristics including its fluidity and surface property can be controlled by changing lipid compositions.

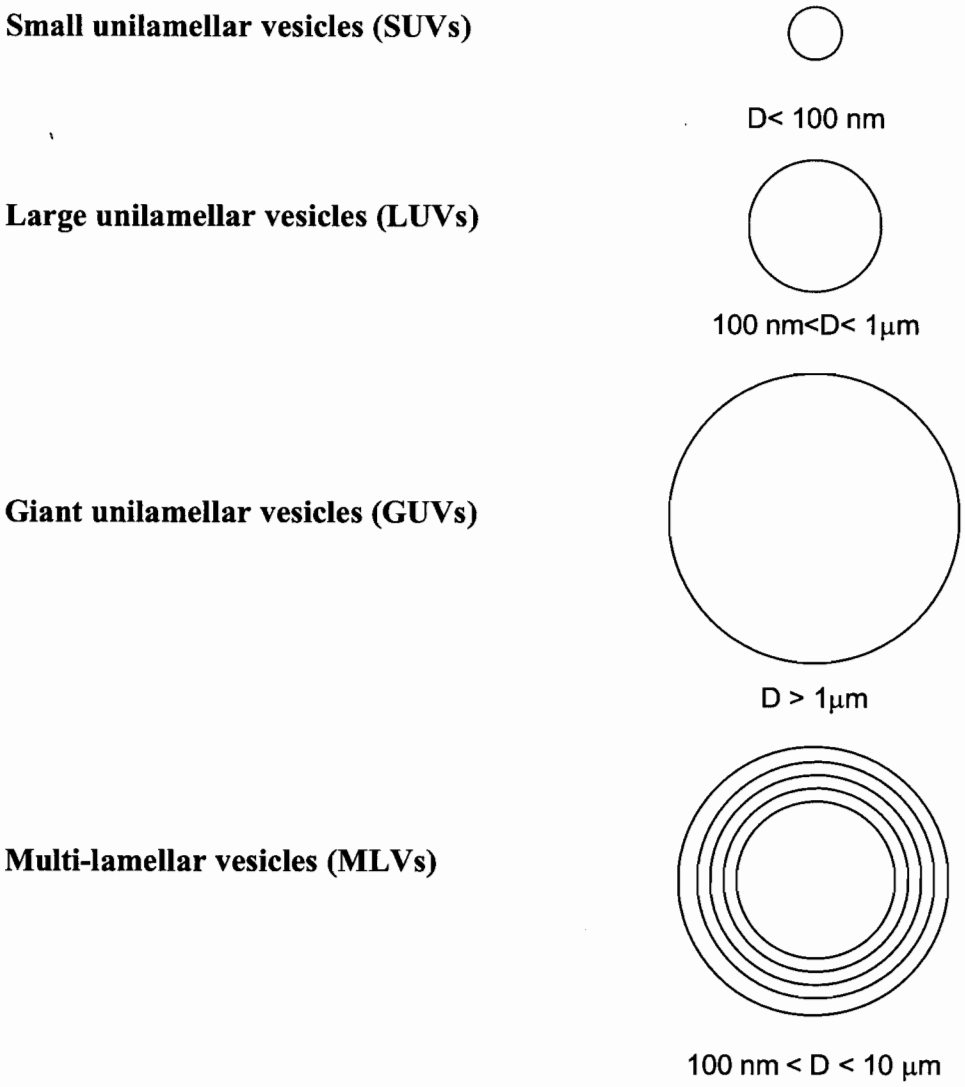
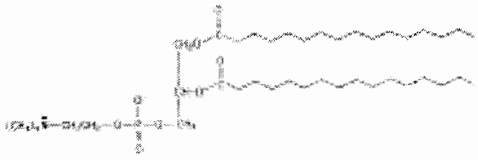
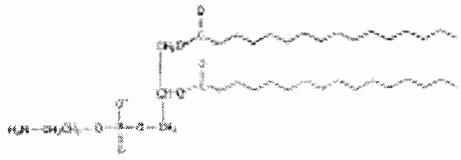


Figure 1-3 Liposome size and lamellar structure

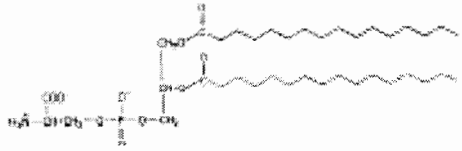
Phosphatidyl coline (PC)



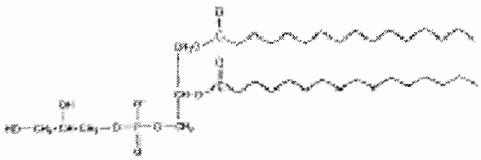
Phosphatidyl ethanolamine (PE)



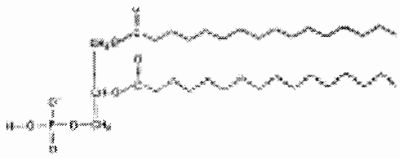
Phosphatidyl serine (PS)



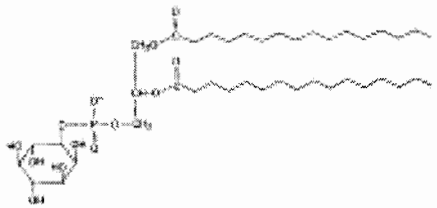
Phosphatidyl glycwrol (PG)



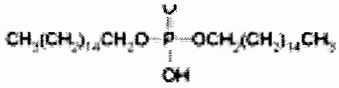
Phosphatidic acid (PA)



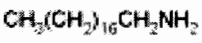
Phosphatidyl inositol (PI)



Dicetylphosphate (DCP)



Stearyl amine (SA)



Cholesterol (Chol)

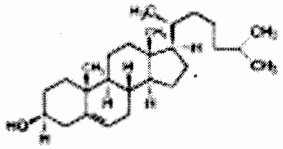


Figure 1-4 Phpspholipid and other components used for liposome preparation

Table 1-1 Characteristics of liposome components

(a) Phase trandition temperature of diacyl phosphatidyl coline

Diacyl phosphatidyl coline (PC)	Phase transition temperature (°C)
DioleoylPC	-22
DimyristoylPC	23
Dipalmitoyl PC	41
Distearoyl PC	55

(b) Charge of lipids

Lipid	Charge of head group
PA	-
PG	-
PS	-
DCP	-
SA	+

Liposomes with higher stability and capacity of marker encapsulation are suitable for immunoassays. They also show less undesirable interactions against other proteins and solid supports. Therefore, LUVs mainly composed of saturated phospholipids such as DPPC and DMPC, are often used for these purposes. (Rongen et al., 1994, Jones et al., 1993)

(b) Immobilization of antigen or antibody on liposome membrane

Many kinds of immobilization methods of antigen or antibody molecules on liposome membrane were reported. To introduce these molecules on liposome membrane, functional groups of protein and lipid component of liposomes are coupled with heterobifunctional cross-linkers. The functional groups can be amine (in phosphatidylethanolamine and most protein), thiol (in Fab' fragments), or hydroxy groups (in glycolipids and glycoprotein). To conjugate antibody (antigen) molecules, thiol group is often introduced as a reactive group with cross-linkers such as SPDP. (Hashimoto et al., 1986, Weckenmann et al., 1988, Shahum and Therien, 1989, Frost, et al., 1990) These cross-linkers contain a succinimidyl group, which reacts under mild conditions with amino groups of antibody (antigen) molecules. Reacted molecules are activated by reduction using DTT. By using 2-iminothiolane, thiolated molecules can be produced directly and efficiently without any reduction. (Jue et al, 1978)

A maleimide group is also introduced to amino group on antibody (antigen) molecules, or lipids with several commercially available anchor molecules, such as SMCC (Scwendener et al., 1990), MBS (Kim and Lim, 1993), or SMPB (Martin and Papahadjopoulos, 1982, Bredehorst et al., 1986, Loughrey et al., 1990). These linkers contain one succineimidyl group and one maleimide group. The maleimide group is reactive towards the thiol group. **Figure 1-5** schematically shows one immobilization method of protein onto liposome membrane. Maleimide group is introduced into phosphatidylethanolamine (Maleimide-PE) by using SMPB. Maleimide-PE is mixed with other

liposome components including phosphatidylcoline (PC) to prepare maleimide-labeled liposomes (activated liposomes). On the other hand, hetero-bifunctional crosslinkers, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) or 2-iminothiolane introduces SH groups into protein molecules. Consequently, SH-protein molecules are irreversibly and covalently coupled on liposome membrane after mixing.

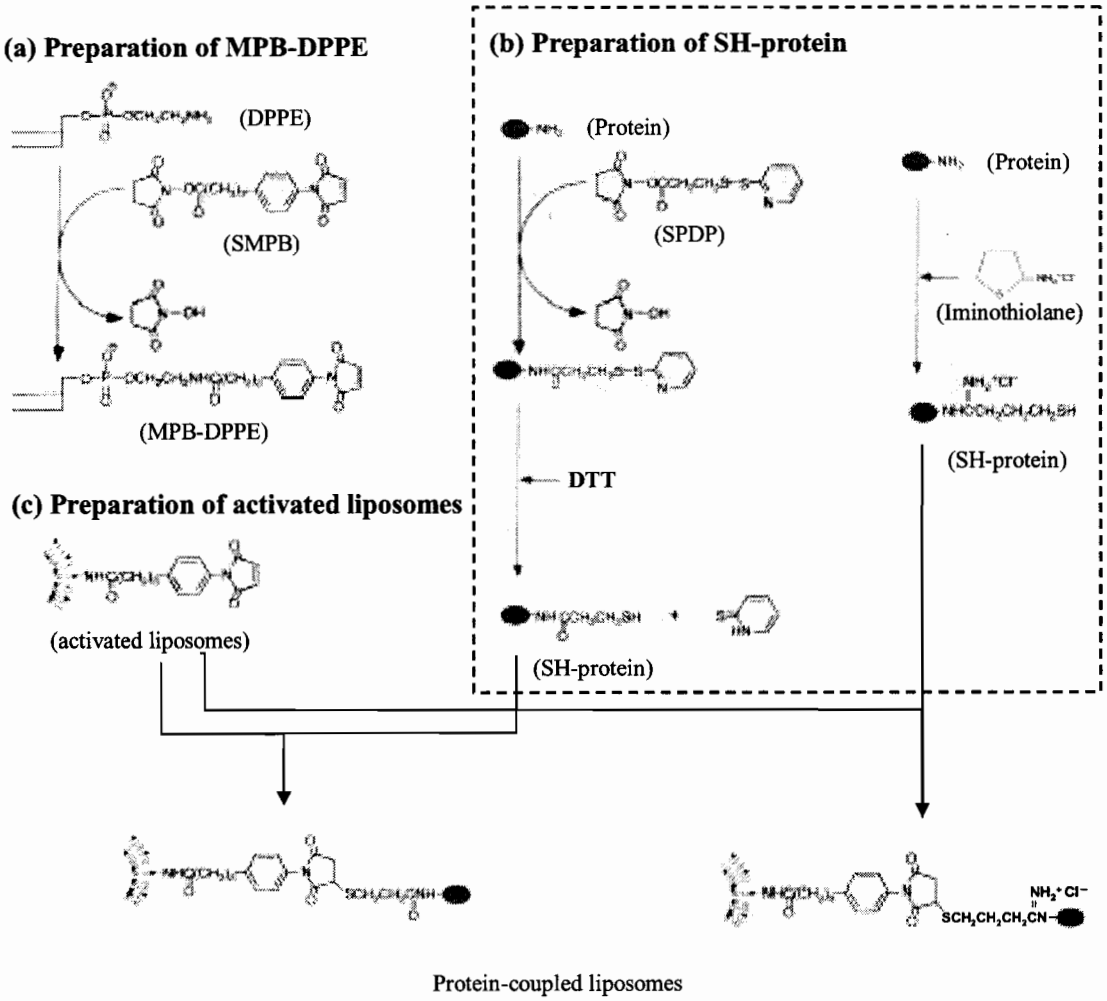


Figure 1-5 Protein immobilization on liposome membrane

1.2.2 Conventional liposome immunoassay (LIA)

Liposome immunoassays (LIA) using antibody- (or antigen-) immobilized liposomes (immunoliposomes) have been developed for rapid detection of analytes with higher sensitivity and signal intensity as alternative methods to EIA. They are classified into homogeneous and heterogeneous immunoassays similar to EIA.

(a) Homogeneous LIA

In homogeneous LIA, the membrane lysis reactions by the complement system, toxins, or enzymes are utilized for measurement of the analyte concentration. Among several methods, liposome immune lysis assays (LILA) based on complement-mediated LILA by using immunoliposomes (Hosada and Yasuda, 1989, Kim and Lim, 1993) and cytolysin-mediated liposome immunoassays (CyMLIA) based on melitin-mediated LILA by using unlabeled liposomes (Litchfield et al 1984, Freytag and Litchfield, 1984) are widely used. In the former LILA, antibody or antigen concentrations can rapidly be detected utilizing membrane lysis reaction through the classical pathway of the complement system, which is initiated by recognition of antigen-antibody complex on liposome membrane. Kishimura et al. reported measurements of also the complement activity by using complement-mediated LILA. In CyMLIA, antigen as an analyte and antibody are incubated together with the melitin-conjugated antigen and liposomes containing marker molecules. (Kishimura et al., 1989)Capture of the melitin-conjugated antigen molecules by the antibody reduces the cell lysis activity of melitin. Therefore, with increase of the analyte concentration, the amount of released marker molecules with increases. In homogeneous LIAs, washing operations and B/F separation are not necessary, and thus analytes can rapidly be detected only by mixing and incubation. For some cases, however, the alternative pathway of complement may increase background signal in LILA, and higher signal/noise (S/N) ratio may not be obtained.

(b) Heterogeneous LIA

In heterogeneous LIA, antigen or antibody-immobilized liposomes (immunoliposomes) are utilized instead of enzyme-labeled antibody in ELISA. Liposome immunosorbent assay (LISA) based on ELISA was developed to obtain higher sensitivity and signal intensity than that in ELISA. In contrast with homogeneous LIA such as LILA and CyMLIA, LISA needs several incubation and washing steps and thus longer time to detect analyte. In comparison with heterogeneous EIA, however, the heterogeneous LIA has a serious disadvantage. In heterogeneous LIA, immunoliposomes have to be lysed by detergent to release encapsulated maker molecules. Released marker molecules disperse to outside of lysed liposomes. Therefore, LIA could not be applied to immunoblotting assays using hydrophobic membrane, because strong signals could not be obtained from analyte blotted area on membrane.

1.3 Selection methods of targets from combinatorial libraries

In drug design, proteomics, and clinical diagnosis, it is important to detect specific and selective interactions such as protein-protein, peptide-protein, DNA-protein, and so on. Recently, a large number of combinatorial libraries have been proposed and produced in genetic engineering fields to design new functional proteins, analyze specific interactions of biomolecules and screen specific antibodies against target molecules. (Stephen and George, 1988, Gianni et al., 1999, Wilson and Finlay, 1997) At same time, many kinds of screening methods from these libraries have also been developed. Among many library systems, combinatorial proteins or peptides displayed by filamentous M13 phages containing phagemid vectors are the most common system, because of effective infection and release properties of M13 phage and easy rescue of phagemid vectors with helper phage. For example, phage libraries displaying human monoclonal antibodies were prepared from human spleen cDNA, and play important roles for screening of specific antibodies targeting cancer cells in vitro, and generation of human monoclonal antibodies as a kind of drugs for cancer therapy. The positive phages, which display specific proteins or peptides against target substances, could be selected from many kinds of candidates by biopanning procedures. As shown in **Figure 1-6**, the biopanning procedures, which consist of adsorption of phages in a library to a target molecule, recovery of the adsorbed phages, infection and amplification of the phages in host cells and phagemid rescue, have to be repeated several times to obtain desirable candidates, because an initial number of phages displaying candidate molecules is small, and recovered phages contain many fakes nonspecifically adsorbed even after recovering. Zhuang et al. (2001) proposed a simulated kinetic model of phage-protein interaction and an optimization strategy of biopanning procedures to screen desirable positive phages with minimum efforts. In spite of these efforts, however, it is still difficult to obtain positive phages with higher efficiency, especially, in libraries having larger repertory.

1.3.1 Conventional biopanning procedures

In biopanning procedures, incubation and washing steps have to be repeated similar to heterogeneous immunoassay procedures, and plastic tubes and microbeads are widely used for adsorption of target molecules in phage screening. In biopanning procedure, the following factors may lead to inefficient outputs. 1) The surface area available for adsorption, and thus the adsorption capacity, is limited by a geometrical shape. 2) Denaturation of target molecules on plastic surface may cause incorrect recognition of the molecules by phages. 3) Blocking procedures are essential to reduce nonspecific adsorption of phages. 4) Some phages may be specifically adsorbed on blocking agents. For example, as phage libraries contain phages targeting such as blocking proteins or other components, they can not be removed during the washing step. Because of above reasons, selection of positive phages against targets has been sometimes failed even though phage library contains our desirable candidates. Therefore, efficient biopanning procedures to select our desirable candidates are required.

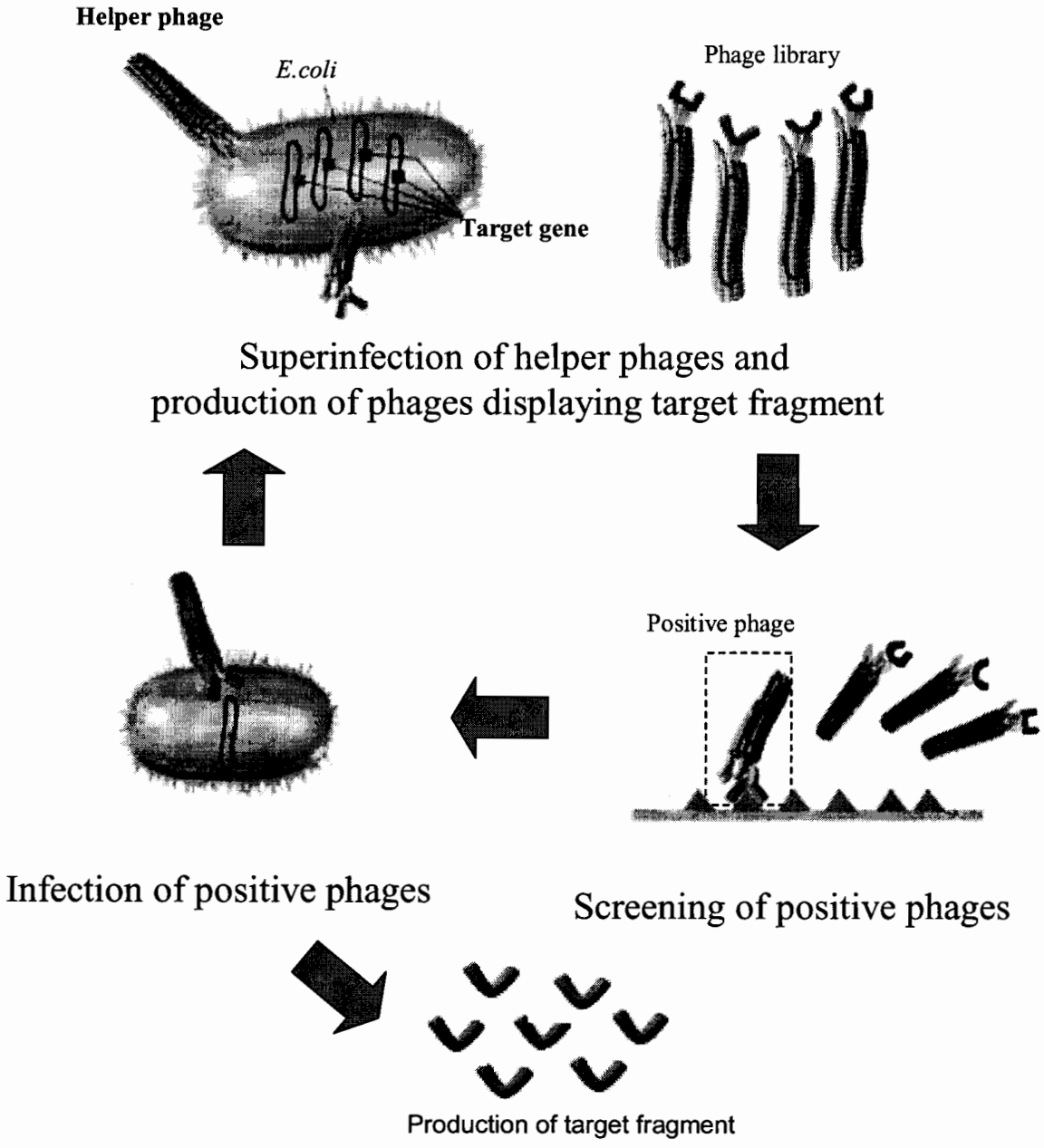


Figure 1-6 Selection of positive phages by biopanning procedures

1.3.2 Novel biopanning procedures by use of protein coupled liposomes

Protein-coupled liposomes (proteoliposomes) have been used for drug and gene delivery, liposome immunoassays and artificial models of cells. (Pedroso et al., 2003, Rongen et al., 1997, Eanes, 1989) To solve the above problems in biopaninng procedures, we propose a biopanning method of phage-displayed libraries utilizing proteoliposomes as adsorbents. Proteoliposomes have the following characteristics. 1) The surface area available for adsorption can be controlled by the size and amount of liposomes used. 2) Proteins may be coupled on the surface of phospholipid membrane with minimum denaturation, and membrane-bound proteins are also effectively incorporated into the membrane. Jespersen et al. (2000) successfully selected monoclonal antibodies against membrane receptors by biopanning utilizing proteoliposome-immobilized tube. 3) The hydrophilic nature of the surface of phospholipid membrane can reduce nonspecific adsorption of phages without blocking operations. 4) A phage library should contain very few phages specific to the components in liposome membrane, because they would be adsorbed specifically on *E.coli* membrane before their secretion.

Screening from peptide libraries displayed by phages could be a promising way to obtain affinity ligands and antagonists against target proteins and to clarify sequential epitopes of antibodies, if the intrinsic interaction between the targets and peptides can be measured.

1.4 Research objectives of this work and strategies

Although many kinds of LIA methods have been proposed and reported as above, many points to be solved, are still remaining to apply LIA kits to practical use. Therefore, in **Part I** of this thesis, I studied characteristics of liposome immunosorbent assay (LISA), especially, about the effects of the number of antibody on liposome membrane (Chapter 2), liposome diameter (Chapter 3) and preservation condition (Chapter 4) on the sensitivity and signal intensity in LISA. I also proposed a liposome immunoblotting assays utilizing hydrophobic membranes as supports to apply LIA to various assay formats such as micro-array, Western blotting and colony-lift immunoassay. (Chapter 5 and 7) In Chapter 6, the relationship between analyte blotted area and obtained signal density in liposome immunoblotting assay was clarified to optimize it for high throughput assay format. In Chapter 8, enhanced chemiluminescent (ECL) reaction was used for more sensitive detection, and effects of some surfactants on the enzyme activity, nonspecific adsorption and sensitivity in liposome immunoblotting assay were investigated.

Biopanning procedures have been utilized for screening of specific proteins or peptides against target substance from phage libraries. Especially, these procedures are popular and useful to isolate more specific monoclonal antibodies for immunoassay or cancer therapy. However, depending on screening conditions, library sizes, or other factors, selection of positive phages against targets has been sometimes failed even though phage library contains our desirable candidates. Therefore, in **Part II**, to develop a more efficient screening method from phage libraries, I developed an alternative biopanning procedure utilizing protein-coupled liposomes. In Chapter 9, adsorption characteristics of nonspecific M13 phages to liposomes of different compositions was investigated, and the performances of biopanning utilizing liposomes coupled with an anti-peptide antibody and plastic tubes coated with the antibody for screening antigen peptides from an octapeptide-displayed phage library were compared.

Part I Development of liposome immunoassay with high sensitivity and signal intensity

Chapter 2

Binding characteristics of immunoliposomes to immobilized antigens and development of LISA

2.1 Introduction

The specific recognition of molecules between antigen and antibody is widely used in detection and separation of trace amounts of biomaterials, and also in medical diagnosis. Among many immunoassay methods depending on the immunointeraction, enzyme-linked immunosorbent assay (ELISA) is currently most popular. Many modified methods of ELISA have been proposed to increase the sensitivity and to amplify the signal in measurements. Liposomes encapsulating markers can be used for these purposes in place of labeled secondary antibodies, because one liposome can couple many antibody (or antigen) molecules on its surface and can contain a larger amount of a marker than a labeled antibody. (Singh and Carbonell, 1996) Multipoint interaction between antigen and antibody may increase their binding affinity (avidity) in comparison with ELISA.

In the previous work, liposomes encapsulating coenzyme β-NAD^+ as a marker were prepared and used in liposome immune lysis assay (LILA) with use of complement. (Miyagawa et al., 2000) Conjugated redox reactions involving β-NAD^+ accumulated a colored material and attained high detection signals. In this chapter, I used this type of liposomes coupled with antibodies or antigens for liposome immunosorbent assay (LISA) in direct and sandwich methods. Those liposomes encapsulated coenzyme β-NAD^+ as a marker, resulting in amplification of the signal. The sensitivity and signal of LISA were compared with those of the conventional ELISA.

2.2 Materials and Methods

2.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque), dipalmitoylphosphatidylethanolamine (DPPE) (Sigma Aldrich), cholesterol (Chol) (Wako Pure Chemical Industries, Ltd.), dicetylphosphate (DCP) (Sigma Aldrich) and succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce) were used for liposome preparation. As a marker encapsulated in liposomes, β-nicotinamide-adenine dinucleotide, oxidized form (β-NAD^+) (Oriental Yeast Co., Ltd.) was used. N-succinimidyl 3-(2-pyridyl-dithio)propionate (SPDP) was purchased from Amersham Pharmacia Biotech, dithiothreitol (DTT), octyl-β-D-glucopyranoside from nacalai tesque, D-glucose 6-phosphate disodium salt (G6P), glucose-6-phosphate dehydrogenase (G6P-DH) from Oriental Yeast Co., Ltd., 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) from Dojindo, anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP from Cappel Products Co., anti-human IgM antibody (goat) and human IgM from Chemicon Int. Inc. Anti-α-chymotrypsinogen A antibody and anti-human IgM antibody were prepared by the previously reported method. (Katoh and Terashima, 1994) Anti-human IgM antibody (rabbit), anti-rabbit IgG antibody (goat) and α-chymotrypsinogen A (Sigma Aldrich) were coupled on liposomes. All other chemicals were of reagent grade.

Buffers used in this work were prepared as follows:

Tris-HCl (Tris(hydroxymethyl)aminomethane 6.06 g/L, pH=8.0), PBS (NaCl 9.0 g/L, KCl 0.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g/L, KH_2PO_4 0.2 g/L, pH=7.4) and MES (2-(N-morpholino) ethanesulfonic acid 4.27 g/L, NaCl 8.77 g/L, pH=6.0).

Microtiter plates of 96 flat-bottom wells (Coster 9017, Corning Inc.) were used for LISA and ELISA. Two types of solutions, phosphate buffered saline (PBS) and PBS containing 0.05 vol%

Tween 20 (PBST) were used for washing of plates.

2.2.2 Preparation of antibody or antigen-coupled liposomes

(a) Preparation of activated liposomes containing MPB-DPPE and encapsulating β-NAD^+

DPPE was activated to N-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidyl-ethanolamine (MPB-DPPE) by SMBP as previously reported. (Kishimura et al., 1989) Small unilamellar vesicles (SUVs) prepared by the sonication method (Huang, 1964) were used as antibody- or antigen-coupled liposomes. A lipid mixture containing DPPC (10 μmol), Chol (10 μmol), DCP (1 μmol) and MPB-DPPE (0.5 μmol) in chloroform was dried to a thin lipid film on the inside wall of a pear-shaped flask by using a rotary evaporator at 42℃ or above under reduced pressure. After repeating the evaporation, the lipid film was dried overnight under reduced pressure in a desiccator. Then, 4 ml of 300 mM β-NAD^+ in Tris-HCl was added to be encapsulated as a marker, and the flask was shaken by a vortex mixer at around 50℃ for about 10 min to peel off the lipid film and form multilamellar vesicles (MLVs). To obtain SUVs, the suspension of MLVs was sonicated with a probe-type sonicator (UD 201, TOMY) for 20~30 min at 40 W with cooling. The liposomes thus prepared were separated from untrapped β-NAD^+ by gel chromatography with a Sephadex G-25 (Amersham Pharmacia Biotech) column equilibrated with PBS.

(b) Modification of antibody or antigen for coupling to liposomes

A heterobifunctional cross-linking reagent SPDP was employed to combine proteins covalently with the activated lipid molecules. Proteins were modified by SPDP as previously reported. (Kishimura et al., 1989)

(c) Preparation of antibody or antigen-coupled liposomes

A suspension of activated liposomes was mixed with a modified protein solution and left overnight at room temperature. Uncoupled protein was removed by gel chromatography with a

Bio-Gel A-15m (Bio-Rad Laboratories) column equilibrated with PBS. Coupling efficiencies of modified proteins to activated liposomes were 20% ~ 40% of the mixed proteins. Antibody- or antigen-coupled liposomes obtained were stored at 4℃.

The concentrations of DPPC and protein were measured after addition of 20 μl of 10 % deoxycholic acid sodium salt to 100 μl of each sample. The concentration of DPPC in the liposome suspension was measured by the cholineoxidase method, using Phospholipids C-Test Wako (Wako Pure Chemical Ind. Ltd.). The concentration of protein coupled on liposomes was measured by DC-protein assay (Bio-Rad Laboratories).

2.2.3 Sandwich assay by LISA and ELISA

Sandwich LISA protocol is as follows. Wells were coated with 50 μl/well with 10 μg/ml of antibody or antigen in PBS for 24 h at 4 °C in most experiments. After washing with PBS, all wells were coated for 2 h with 300 μl/well of 5-fold diluted Blocking Solution (nacalai tesque) to decrease non-specific adsorption. After washing with PBS, samples were added to the wells (50 μl/well) and incubated for 1 h at room temperature. After washing with PBS, 50 μl/well of appropriately diluted anti-human IgM antibody (rabbit)-, anti-rabbit IgG antibody (goat)- or α-chymotrypsinogen A-coupled liposomes in PBS was added to the wells and incubated for 1 h. After washing with PBS, liposomes adsorbed on the surface of wells were completely lysed with addition of 50 μl of 21.4 mM octyl-glucopyranoside. The reaction mixture (80 μl) dissolved in PBS containing 0.5 mM $MgCl_2$, 0.4 mM G6P, 4.05 x 10^6 mU/L of G6P-DH, 0.032 mM 1-methoxy PMS and 0.4 mM WST-1 was added to each well to develop WST-1 formazan in the conjugated redox reactions involving β-NAD^+, as shown in **Figure 2-1**. After 100 min the absorbance was measured at 405 nm by a microplate reader (Model 550, Bio-Rad Laboratories). The absorbance of WST-1 formazan measured at 405 nm corresponded to the amount of lysed liposomes. In direct LISA, the first coating step of antibody or antigen was

skipped. In sadwich ELISA, 50 μl/well of anti-rabbit IgG antibody (goat)-HRP x2000 diluted in PBST was used after addition of samples and washing. After washing with PBST, color was developed using 100 μl/well of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (0.3 mg/mL in 0.1 M citrate buffer containing 0.01% H_2O_2, pH 4.1). The absorbance was recorded by the microplate reader at 405 nm.

2.2.4 Determination of association constants

Anti-α-chymotrypsinogen A antibody (rabbit) or anti-human IgM antibody (rabbit) was coupled on CNBr-activated Sepharose 4B, and the adsorbent was packed in a column of 1.26 cm i.d. After equilibration with PBS, antigen dissolved in PBS was supplied to the column. The absorbance of the effluent solution was measured at 280 nm with a spectrophotometer (UV-1600, Shimadzu). From the breakthrough curve obtained, the adsorption capacity was calculated by subtracting the amount of the antigen in the dead space.

From the adsorption equilibrium, the effective association constant K_0 and the heterogeneity index σ for the antibodies were estimated by assuming that the free energy of antigen-antibody combination can be described by the normal distribution function, according to Pauling *et al.*:

$$y=1-\frac{1}{\sqrt{\pi}}\int_{-\infty}^{\infty}\frac{e^{-\alpha^2}}{1+K_0Ce^{\alpha\sigma}}d\alpha \qquad (1)$$

$$\alpha=\ln(K/K_0)/\sigma \qquad (2)$$

where C is the liquid phase concentration of free antigen; K : the association constant; σ : the heterogeneity of K; and y : the fractional saturation of antibody.

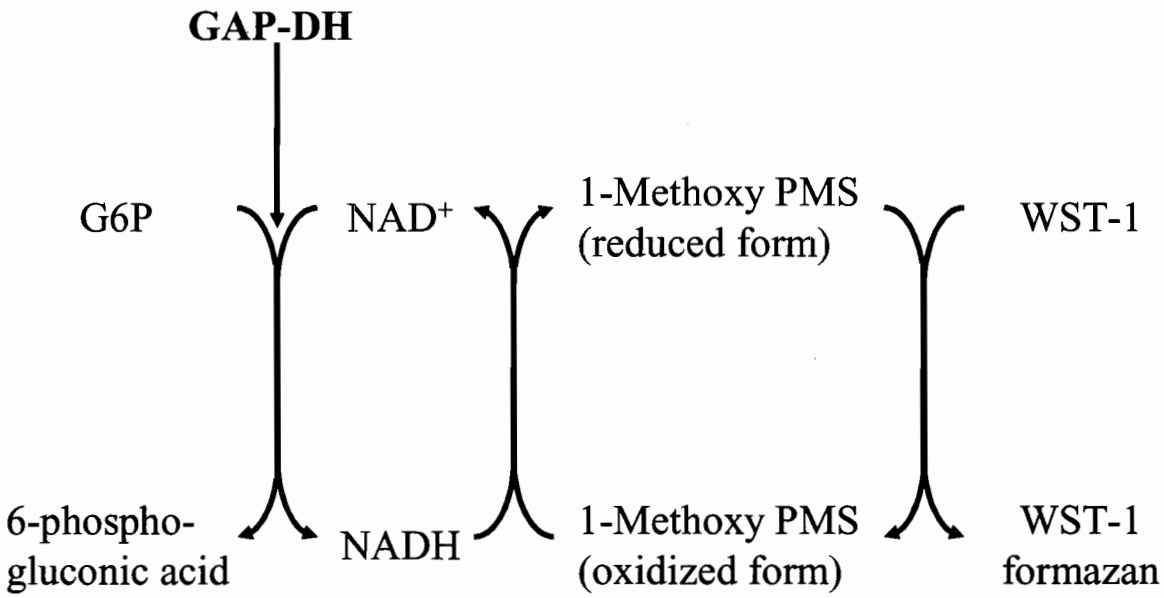


Figure 2-1 Reactions producing WST-1 formazan

2.3 Results and Discussion

2.3.1 Comparison between direct and sandwich LISA

Figure 2-2 shows the effects of the concentration of anti-rabbit IgG antibody (goat) adsorbed on the surface of each well of the microtiter plate. After coating the wells with specific concentrations of anti-rabbit IgG antibody, the wells were blocked from nonspecific adsorption by Blocking Solution, and samples containing anti-α-chymotrypsinogen A antibody were added as stated in Materials and Methods. In direct LISA samples were adsorbed without addition of anti-rabbit IgG antibody. With increase in the concentration of the antibody used in coating both the signal and sensitivity increased higher than those of direct LISA above 2 μg/ml. Above 10 μg/ml the similar behaviors were observed. Thus, in most experiments wells were coated with 10 μg/ml of antibody or antigen dissolved in PBS.

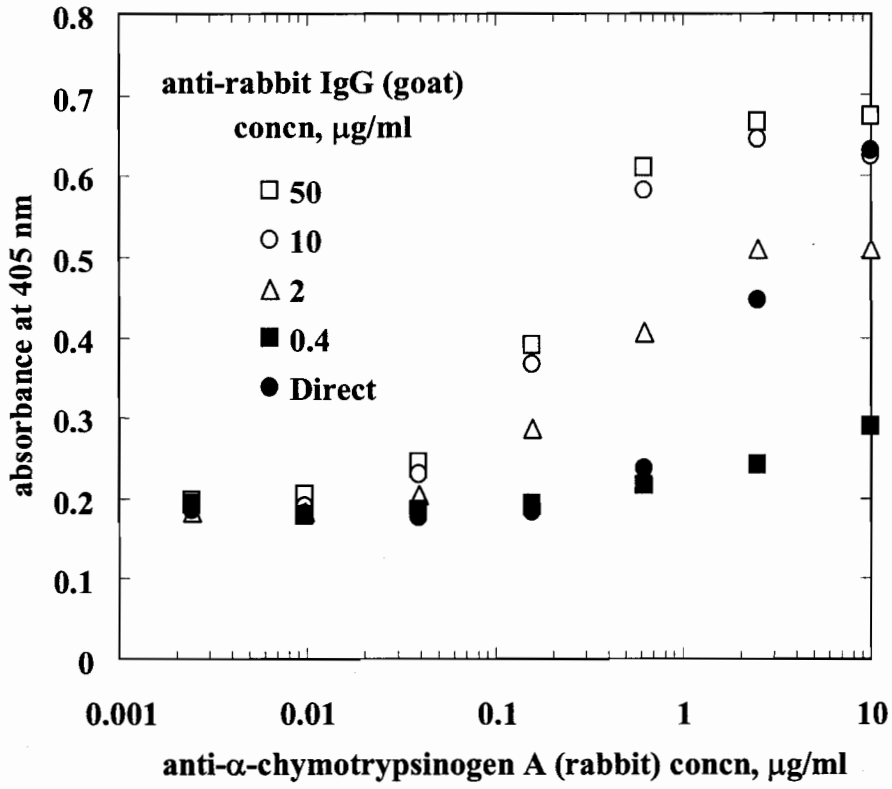


Figure 2-2 Effects of coating concentration of anti-rabbit IgG (goat) in LISA using α-chymotrypsinogen A coupled liposome

sample : anti-α-chymotrypsinogen A (rabbit)
liposome concn : 25 nmol/ml
coupled α-chymotrypsinogen A : 15.6 mmol/mol-DPPC

The absorbance at 405 nm observed as the background was not high only by washing with PBS, and this means that the nonspecific adsorption of liposomes is low. The lysis of liposomes in washing steps by PBS was less than 4 % (data not shown).

Figures 2-3 (1) and **2-3 (2)** compare the results of measurement of human IgM and anti-α-chymotrypsinogen A antibody (rabbit) by sandwich LISA with those of direct LISA. In both cases the sensitivity of the sandwich LISA was about 16 times higher. The sensitivities of the two systems were almost same, since the association constants of anti-human IgM antibody (rabbit)-human IgM and anti-α-chymotrypsinogen A antibody (rabbit)-α-chymotrypsinogen A were the same order, 5.0×10^{-7} M^{-1} and 9.2×10^{-7} M^{-1}, respectively. These results show that sandwich LISA is effective for increasing the sensitivity and signal, and that both antibody-coupled liposomes and antigen-coupled liposomes can be used for detection in LISA.

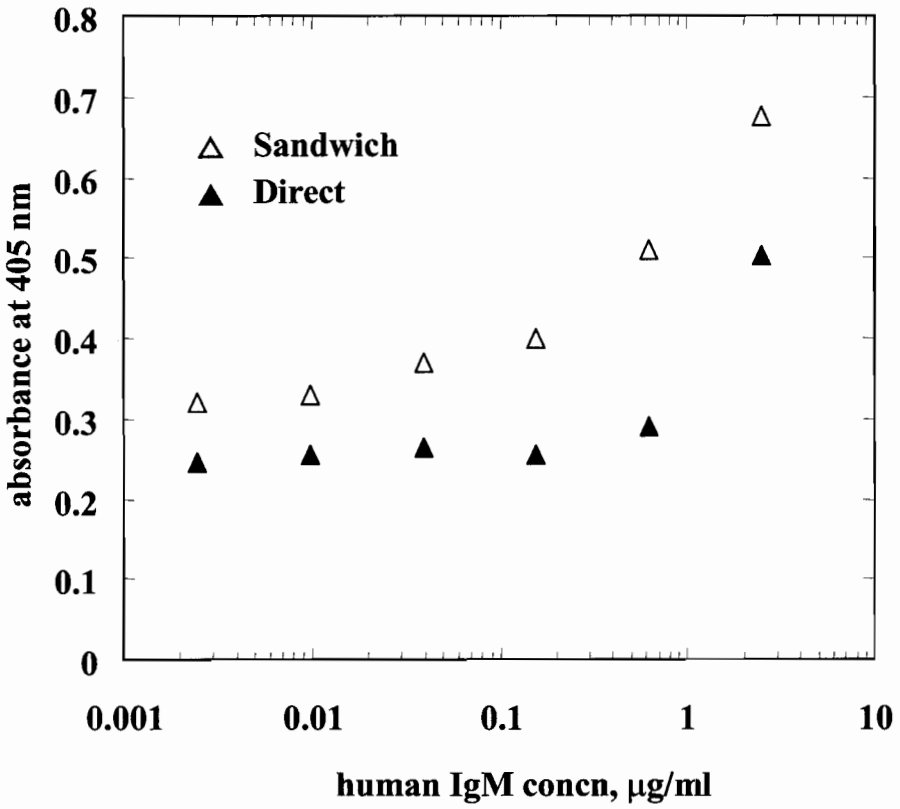


Figure 2-3 (1) LISA using anti-human IgM antibody (rabbit) coupled liposomes

coating : anti-human IgM antibody (goat) 10 μg/ml
sample : human IgM
liposome concn : 25 μg/ml
coupled anti-human IgM antibody (rabbit) : 1.95 mmol/mol-DPPC

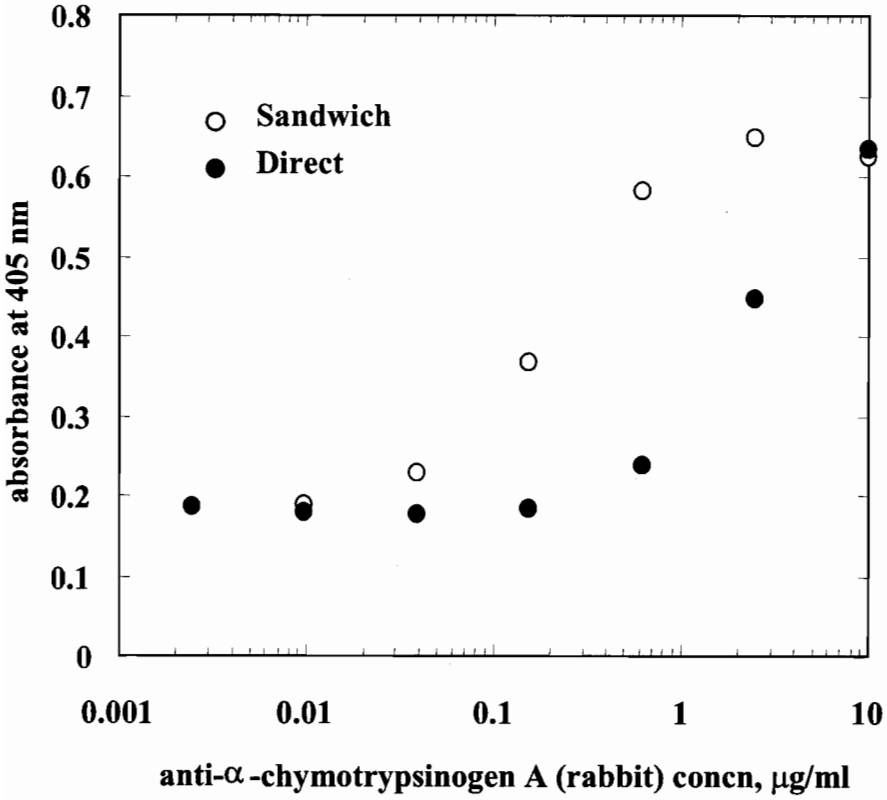


Figure 2-3 (2) LISA using α-chymotripsinogen A coupled liposomes

coating : anti-rabbit IgG antibody (goat) 10 μg/ml
sample : anti-α-chymotrypsinogen A (rabbit)
liposome concn : 25 μg/ml
coupled α-chymotrypsinogen A : 15.6 mmol/mol-DPPC

2.3.2 LISA with general secondary antibody-coupled liposomes

In the above systems, however, liposomes corresponding to each target have to be prepared. By using anti-IgG antibody-coupled liposomes, for example, this problem can be avoided. **Figures 2-4 (1)** and **2-4 (2)** show the results of measurement of anti-human IgM antibody by LISA by use of anti-rabbit IgG antibody (goat)-coupled liposomes and by ELISA with anti-rabbit IgG antibody (goat)-HRP as a secondary antibody. By this method LISA can have wide applicability to many systems similar to ELISA. LISA shows higher signals at low concentrations of anti-human IgM antibody, and suited for detection in these ranges of the concentration.

One liposome particle used in **Figure 2-4 (1)** couples three to four molecules of IgG on its surface as average, and thus multi-molecule coupling of IgG to antigens can be possible. This may cause a higher affinity to antigen (avidity) than that of single-molecule coupling. We prepared three kinds of liposomes, which couple one, three and 22 molecules of IgG (anti-human IgM antibody (rabbit)) as average, and compared the signals obtained at the same concentration of IgG added to wells. On each well of the microtiter plate, 0.25 μg/ml of human IgM was adsorbed. After washing, liposomes were added and washed again. Color was developed by the reactions shown in **Figure 2-1**. **Figure 2-5** shows the absorbance at 405 nm, which corresponds to the amount of liposomes adsorbed. Above 1 μg/ml of anti-human IgM antibody added in each well, the absorbances of the liposomes coupling larger numbers of molecules of IgG were higher at the same concentration of anti-human IgM antibody added, in spite of less number of the liposomes added in each well. This is because the liposomes coupling a large number of IgG can show a higher affinity for adsorption, which may be caused by multi-molecule coupling of liposomes to antigens. This can be one reason of the high sensitivity of LISA.

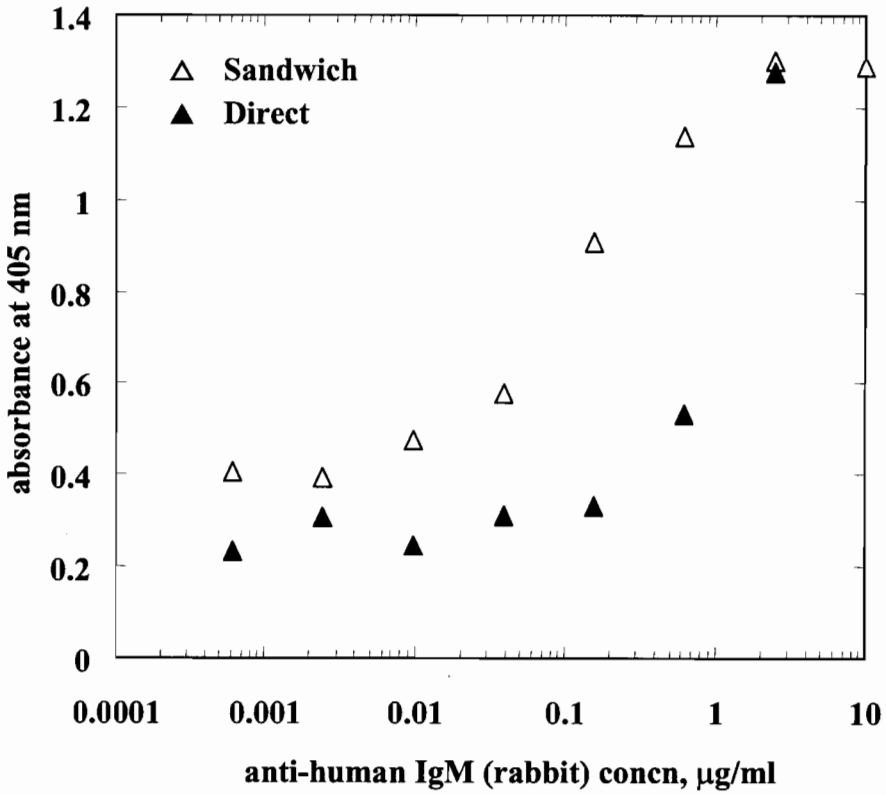


Figure 2-4 (1) LISA using anti-rabbit IgG antibody (goat) coupled liposomes

coating : human IgM 10 μg/ml
sample : anti-human IgM antibody (rabbit)
liposome concn : 61.5 nmol/ml
coupled anti-rabbit IgG antibody (goat) :
1.08 mmol/mol-DPPC

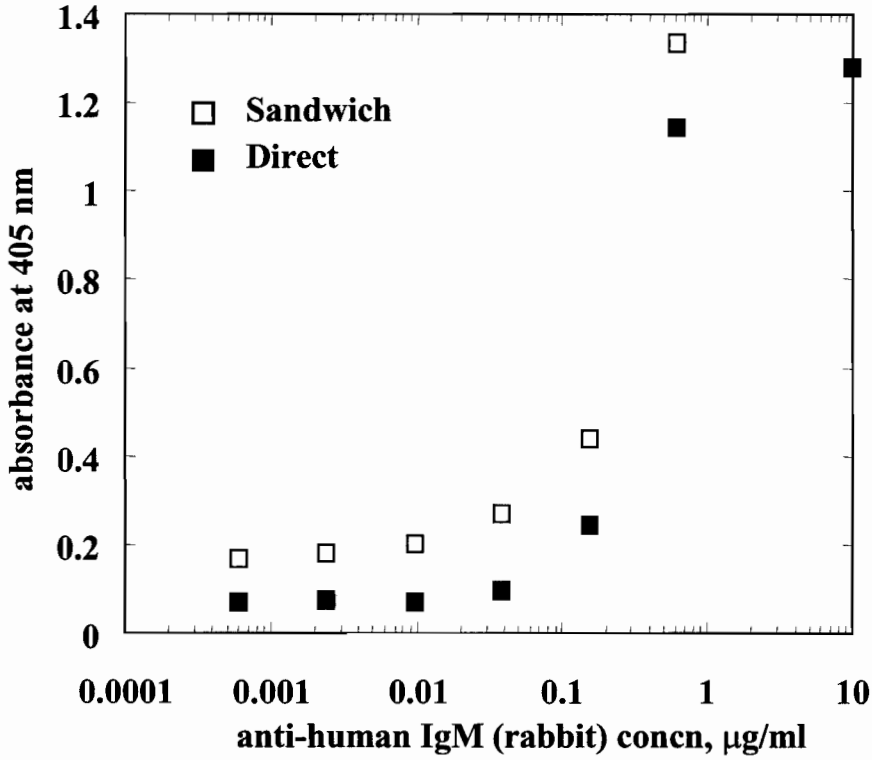


Figure 2-4 (2) ELISA using anti-rabbit IgG antibody (goat) -HRP

coating : human IgM 10 μg/ml
sample : anti-human IgM antibody (rabbit)

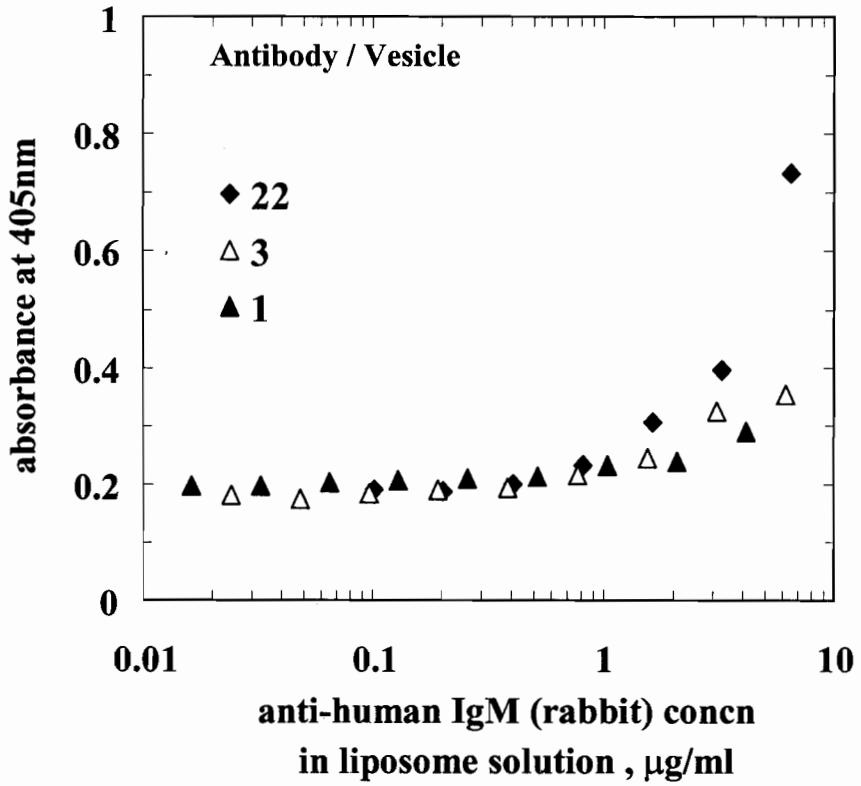


Figure 2-5 Comparision of LISA using liposomes coupled with different numbers of anti-human IgM antibody (rabbit) molecule

coating : human IgM 0.25 μg/ml
◆ : 1.37mmol/mol-DPPC
△ : 0.16mmol/mol-DPPC
▲ : 0.07mmol/mol-DPPC

2.4 Conclusion

By use of liposomes encapsulating β-NAD^+, liposomes can contain a large amount of β-NAD^+, and the color development is accumulated by the conjugated reactions including β-NAD^+. Liposomes coupling several molecules may have a high avidity to antigens. Thus, LISA can show the high signal and the high sensitivity for detection compared with ELISA, especially at lower concentration ranges of samples.

Nomenclature

C	=	liquid phase concentration of free antigen	[mol/m^3]
K	=	association constant	[m^3/mol]
K_0	=	average association constant for polyclonal antibody	[m^3/mol]
y	=	fractional saturation of antibody	[-]
σ	=	heterogeneity of K	[-]

Chapter 3
Effects of liposome diameter on signal intensity in liposome imunosorbent assay

3.1 Introduction

In recent years, the number of biological analytes to be detected has been increased, since the proteomics, environmental pollutions, and genetically-modified crop (GM crop) has been focused. Therefore, the high sensitive and rapid quantification methods are required, because the level of their concentration is getting decrease. Enzyme immunoassay (EIA) is widely used as one of analytical method utilizing immunological interactions between antigen and antibody. Especially, enzyme-linked immunosorbent assay (ELISA) is the most popular one in EIA, although many incubation and washing steps are required, and its sensitivity is limited by the number of labeling agents conjugated with an antibody molecule. In ELISA, the specific antibody was conjugated with a small number of enzyme molecules and a part of antibody might be inactivated by cross-linking reaction. Therefore, many modified methods have been proposed for detection of trace amounts of analytes with higher sensitivity and signal intensity. In Chapter 2, I reported the characteristics of liposome immunosorbent assay (LISA), in which antibody-coupled liposomes encapsulating marker molecules (immunoliposomes) were used as a secondary antibody instead of enzyme-conjugated antibody. (Kumada et al., 2001) LISA provided higher sensitivity in comparison with that in ELISA, because an immunoliposome could bind to antigen molecules immobilized on the plastic plate by multipoint interactions. (Avidity)

In this chapter, the effects of the liposome diameter and the number of coupled antibody on one vesicle were investigated to detect analytes are detected with higher sensitivity and signal intensity in LISA.

3.2 Materials and Methods

3.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque), dipalmitoylphosphatidyl-ethanolamine (DPPE) (Sigma Aldrich), cholesterol (Chol) (Wako Pure Chemical Industries, Ltd.), dicetylphosphate (DCP) (Sigma Aldrich) and succinimidyl-4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce) were used for liposome preparation. As a marker enzyme encapsulated in liposomes, horseradish peroxidase (HRP, 100 U/mg, grade III, TOYOBO) was used, and 2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) was used as substrates. N-succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) was purchased from Amersham Pharmacia Biotech, dithiothreitol (DTT) from nacalai tesque, anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP from Cappel Products Co., human IgM from Chemicon Int. Inc. Anti-rabbit IgG antibody (goat) was coupled on liposomes. Anti-human IgM antibody (rabbit) was prepared by the previously reported method. (Katoh and Terashima, 1994) All other chemicals used were of reagent grade.

Buffers used in this work were prepared as follows:

PBS (NaCl 9.0 g/L, KCl 0.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g/L, KH_2PO_4 0.2 g/L, pH=7.4) and MES (2-(N-morpholino) ethanesulfonic acid 4.27 g/L, NaCl 8.77 g/L, pH=5.5). Blocking solution (pH 7.2, nacalai tesque) and skim milk solutions in PBS were used for blocking and washing, respectively. 96-well microtiter plate (Coster 9017, medium binding) was purchased from Corning.

3.2.2 Preparation of antibody-coupled liposomes

DPPE was activated to N-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidyl-ethanolamine (MPB-DPPE) by SMBP as previously reported (Kishimura *et al.*, 1989). A lipid mixture containing DPPC (20 μmol), Chol (20 μmol), DCP (2 μmol) and MPB-DPPE (1 μmol) in

chloroform was dried to a thin lipid film on the inside wall of a pear-shaped flask by using a rotary evaporator at 42°C or above under reduced pressure. The lipid film was dried overnight under reduced pressure in a desiccator. Then, 6 ml of 5 mg/ml HRP in PBS was added to be encapsulated as a marker, and the flask was shaken by a vortex mixer at around 50°C for about 10 min to peel off the lipid film and form multilamellar vesicles (MLVs). To obtain small unilamellar vesicles (SUVs), the suspension of MLVs was sonicated with a probe-type sonicator (UD 201, TOMY) for 20~30 min at 40 W with cooling at 4°C (Huang, 1964). Large vesicles were prepared by use of an extruder (Thermobarrel Extruder, Lipex Biomembrane Inc.) from MLVs. (Mayer et al., 1986) The liposomes thus prepared were separated from untrapped HRP by gel chromatography with a Bio-Gel A-15m (Bio-Rad Laboratories) column equilibrated with PBS.

A heterobifunctional cross-linking reagent SPDP was employed to combine seconaly antibody covalently with the activated lipid molecules. Anti-rabbit IgG antibody was modified by SPDP as previously reported. (Kishimura et al., 1989) A suspension of activated liposomes was mixed with a modified antibody solution and gently agitated overnight at room temperature. Uncoupled antibody was removed by gel chromatography with the Bio-Gel A-15m column equilibrated with PBS. Antibody-coupled liposomes obtained were stored at 4°C. The HRP activity was about 90 % of the initial value, and HRP released from liposomes was less than 5 % after 4 weeks.

The average diameters of antibody-coupled liposomes were measured by the differencial light scattering analysis (DLS 7000, Photal Otsuka Electronics). The diameter of SUVs was 60 nm and those of large vesicles prepared by use of membranes with pore diameters of 100 nm, 200 nm and 400 nm were 130 nm, 200 nm and 350 nm, respectively. The concentrations of DPPC and protein were measured after addition of 10 μl of 10 % deoxycholic acid sodium salt to 100 μl of each sample. HRP was inactivated by heating at 90 °C for 10 min. The concentration of DPPC in the liposome suspension was measured by the cholineoxidase method, using Phospholipids C-Test Wako (Wako

Pure Chemical Ind. Ltd.). The concentration of protein coupled on liposomes was measured by DC-protein assay (Bio-Rad Laboratories).

3.2.3 Liposome immunosorbent assay (LISA) and enzyme-linked immunosorbent assay (ELISA)

In direct immunoassay, 50μl of antigen solutions with appropriate concentration were added to wells in a microtiter plate. The plate was incubated overnight at 4 °C. After 3 times washing with PBS, these wells are blocked with the 5 times diluted Blocking solution at 37 °C for 2 hours. After 3 times washing with PBS, in LISA, 50 μl of 3-5 μg/ml immunoliposome suspension, which contained 0-1% skim-milk, was added. The plate was incubated for 1 hour at room temperature, and after 6 times washing with PBS, 100 μl substrate solution, which contained 0.3 mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline -6-sulfonic acid) diammonium salt (ABTS) and 0.01 % H_2O_2 in citrate buffer (pH4.0), was added to each well so that color was developed for detection of antigen. After 30 minutes of incubation, the absorbance in each well was measured at 405 nm by a microplate reader (Model550, Bio-rad Laboratories). In ELISA, anti-rabbit IgG antibody (goat)-HRP conjugates were used for the detection of antigen instead of immunoliposomes.

In indirect type immunoassay, 5 μg/ml of the primary antibody solution in PBS was added into the antigen-coated and blocked well. After 6 times washing with PBS, immunoliposomes or antibody-HRP conjugates are utilized as secondary antibodies, and the color was developed as stated above.

In sandwich type immunoassay, 10 μg/ml of antigen solution was coated in each well. After blocking, the primary antibody solutions with different concentration were used as samples. The color in each well was developed by using immunoliposome or HRP-antibody as stated above.

3.3 Results and Discussion

3.3.1 Coupling condition and efficiency of antibody on liposome membrane

To obtain immunoliposomes showing high avidity to antigen molecules, the number of antibody molecules on one vesicle must be controlled. Therefore, the effects of reaction conditions on the coupling efficiencies of antibody to liposome membrane were investigated. **Figure 3-1** shows time course of the coupling reaction. The amount of coupled antibody increased with reaction time and reached to a plateau after 6 hours. **Figure 3-2** shows the effects of the antibody /lipid ratio on the amount of coupled antibody. The amount of coupled antibody increased gradually with the molar ratio. On the other hand, the diameter of liposomes little affected the coupling efficiencies, because these liposomes formed unilamellar vesicles and thus, the same amount of MPB-DPPE was exposed on the surface. Therefore, by controlling the molar ratio of antibody to liposome, immunoliposomes coupled with a suitable number of antibody molecules on their membrane can be obtained.

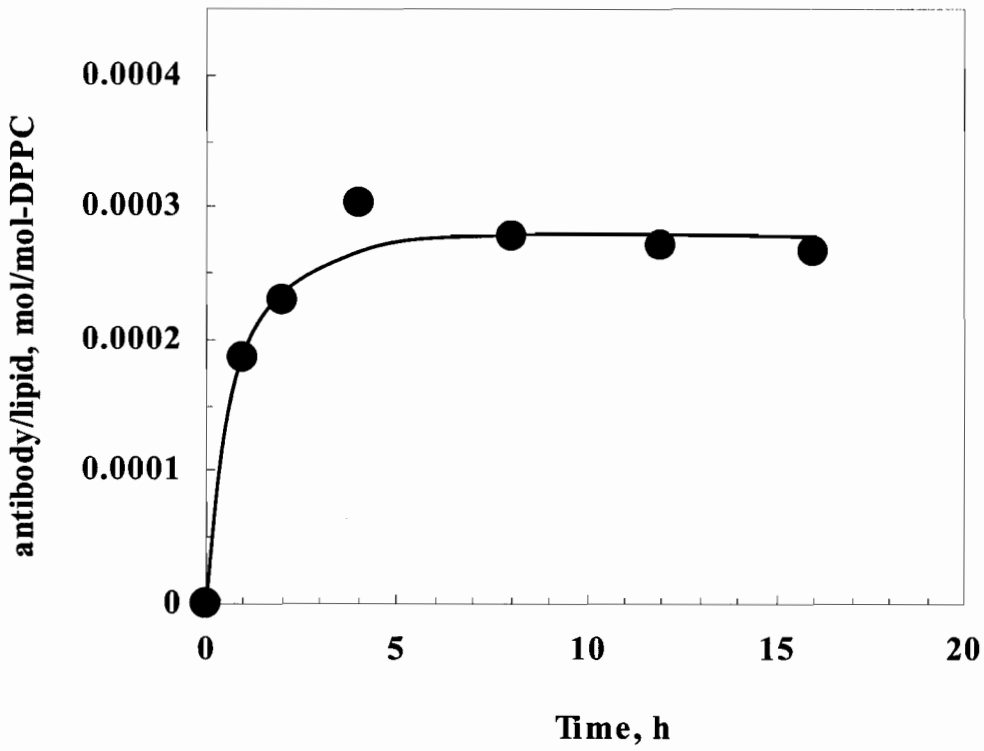


Figure 3-1 Time course of antibody coupling on liposome membrane

Antibody/lipid ratio; 0.48 mmol/mol

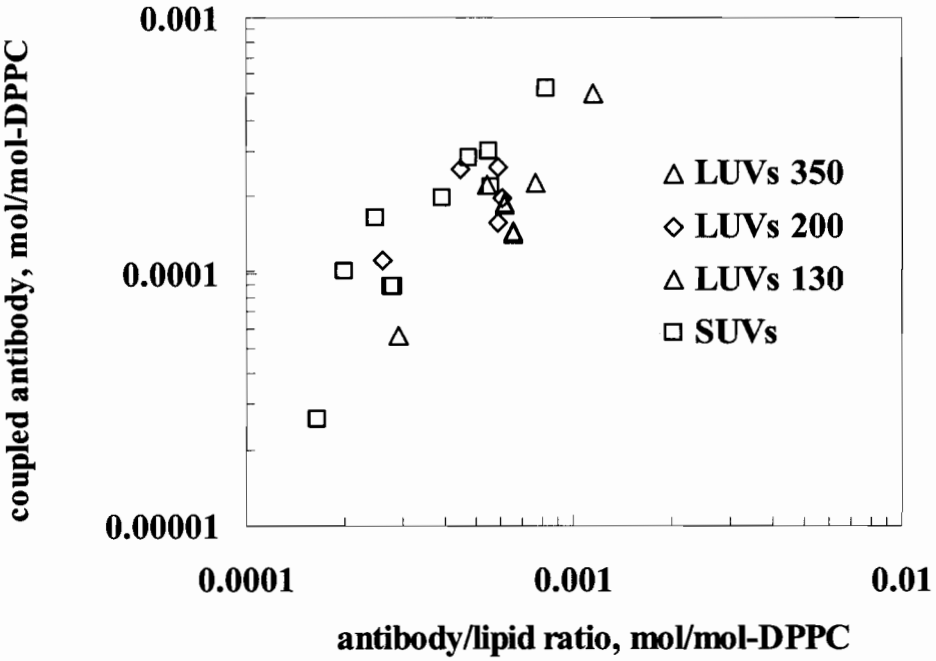


Figure 3-2 Effect of antibody/lipid ratio on coupling efficiency

3.3.2 Permeation of ABTS through liposome membrane

A substrates ABTS is one of substrates permeable through liposome membrane. As shown in **Figure 3-3**, almost the same intensity of signals was obtained from the liposomes encapsulating HRP under the different concentrations of Tween 20. Therefore, in this LISA, the lysis step of liposomes to release encapsulated marker molecules is not necessary, when substrates permeable through liposome membrane was used.

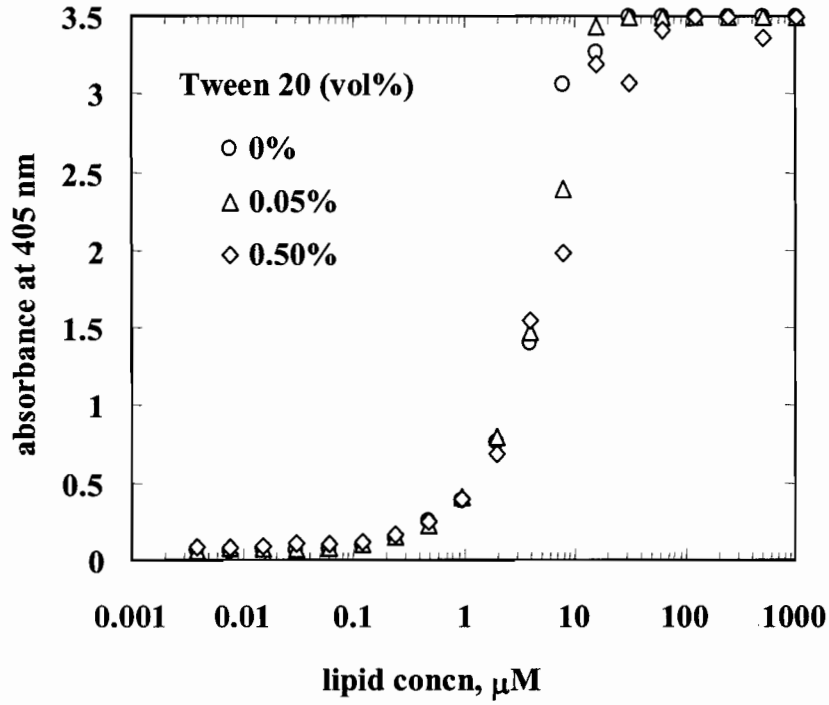


Figure 3-3 Color development with and without lysis of liposomes

Diameter of liposomes, 350 nm
Tween 20 concn, ○: 0 vol%, △: 0.05 vol%, ◇: 0.5 vol%

3.3.3 Effect of nonspecific adsorption of immunoliposomes in LISA

Since the sizes of immunoliposomes are larger than that of HRP-conjugated antibody, liposomes nonspecifically adsorbed on plastic surface might cause high background signals. To prevent nonspecific adsorption of immunoliposomes, the effect of the concentration of skim milk in liposome suspensions on the amount of nonspecific adsorption was studied in LISA of human IgM. As shown in **Figure 3-4**, the background signals decreased with increasing the skim milk concentration, and 0.1% skim milk in PBS was effective to keep the background signal low and to detect with the high S/N ratio even by use of immunoliposomes of 350 nm diameters.

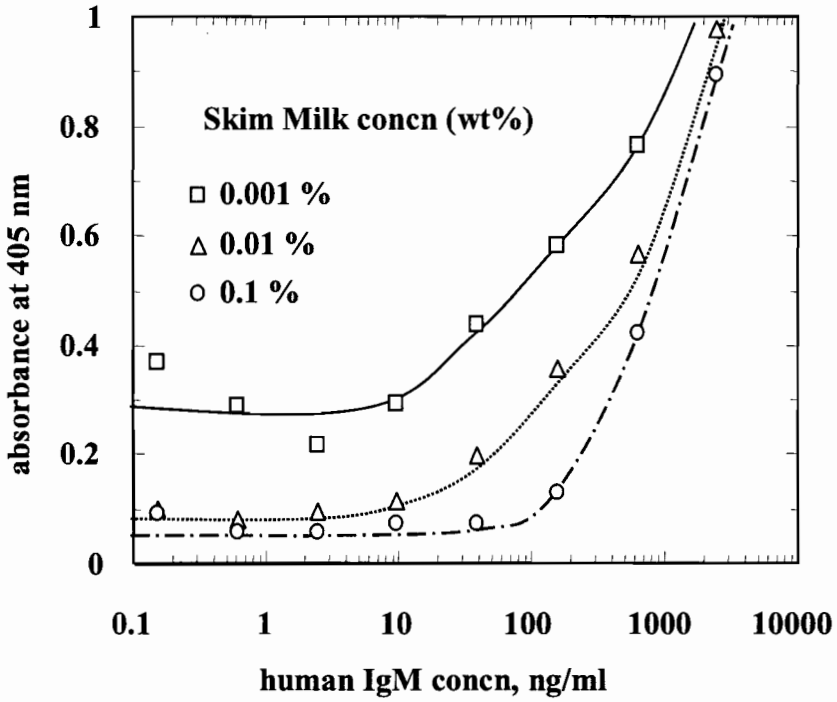


Figure 3-4 Effect of concentration of blocking reagent on nonspecific adsorptioin of liposomes

Sample, human IgM
Immunoliposome, anti-human IgM antibody (rabbit) coupled liposome encapsulating HRP
Antibody concn in immunoliposome solution, 10 μg/ml
Skim Milk concn, □: 0.001 wt%, △: 0.01 wt%, ○: 0.1 wt%
Diameter of liposomes, 350 nm
Coupled antibody, 0.39 mmol/mol-DPPC

3.3.4 Signal intensity from liposomes of different diameters

Immunoliposomes with large diameter can encapsulate large amounts of marker molecules. To study the effects of the liposome diameter on the signal intensity, 4 types of liposomes, which are 3 LUVs with average diameters of 130, 200, 350 nm and SUVs with 60 nm, were prepared. **Figure 3-5** compares the absorbance obtained from the same number of the liposome. By use of the

larger liposomes, the high intensity of signals was obtained, while the number of encapsulated HRP molecules seemed to slightly decrease with increasing the diameter. These liposomes could be stored at 4°C for at least 1 month without any activity change.

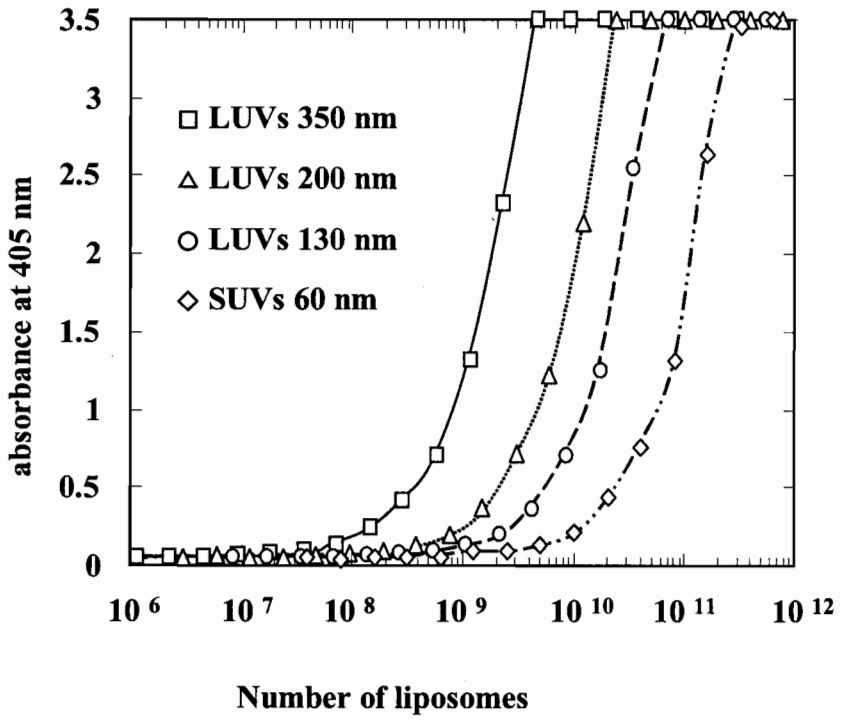


Figure 3-5 Signal intensity of liposomes with different sizes

Diameter of liposomes, □: 350 nm, △: 200 nm, ○: 130 nm, ◇: 60 nm,

Figure 3-6 shows the signals detected in LISA of anti-human IgM antibody by use of the immunoliposomes with different diameters. By use of immunoliposomes with 350 nm, the analyte, anti-human IgM antibody (rabbit) could be detected with the highest signal intensity. Since the immunoliposomes can encapsulate larger amounts of marker, they give higher signals. Further, they can couple many antibody molecules on the membrane, the sensitivity also increase by multipoint interactions between antibodies on immunoliposome and the analyte as previously reported. (Kumada et al., 2001)

3.3.5 Comparison of sensitivity and signal intensity in immunoassay by using immunoliposome and antibody-HRP conjugates

To compare the performances of LISA and ELISA, the sensitivity and signal intensity were measured under the optimal conditions for each case. In ELISA, 2000-times diluted antibody-HRP conjugate was used as a secondary antibody, since the similar output was obtained at higher concentrations. **Figures 3-7 and 3-8** show the results in indirect and sandwich immunoassays, respectively. In both cases, LISA utilizing antibody-coupled LUVs with 350 nm showed highest signal intensities. The ng/ml order of the analytes could be detected in LISA with 50 ~ 100 times higher than those in ELISA.

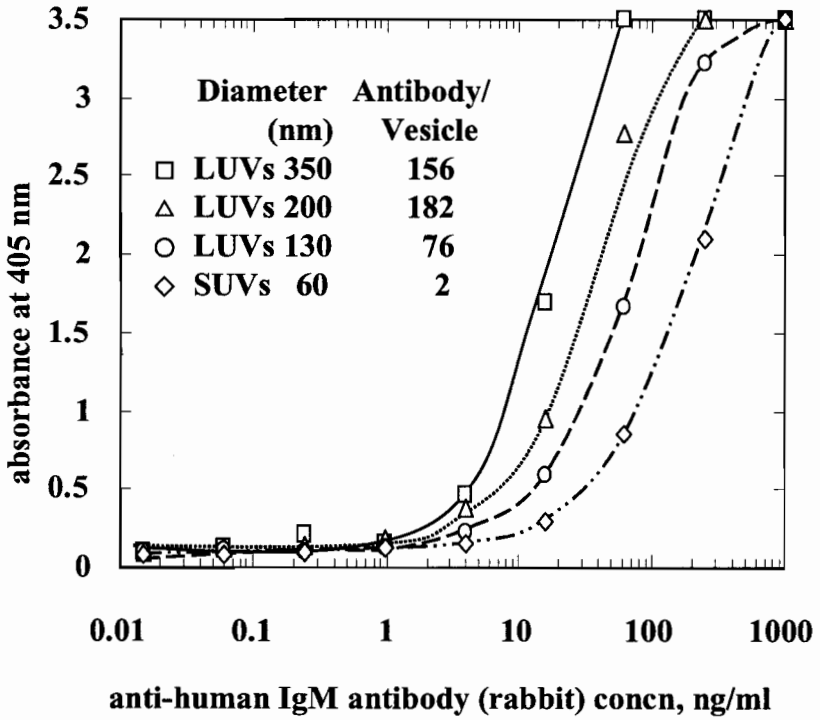


Figure 3-6 LISA using liposomes coupled with different numbers of antibody molecules

Coating antigen, human IgM concn, 10 μg/ml
Sample, anti-human IgM antibody (rabbit)
Immunoliposome, anti-rabbit IgG antibody (goat) coupled liposome encapsulating HRP
Antibody concn in immunoliposome solution, 3 μg/ml
Skim Milk concn in immunoliposome solution, 0.1 wt%
Diameter, □: 350 nm, △: 200 nm, ○: 130 nm, ◇: 60 nm
Coupled antibody, □: 0.14 mmol/mol-DPPC, △: 0.51 mmol/mol-DPPC, ○: 0.54 mmol/mol-DPPC, ◇: 0.06 mmol/mol-DPPC

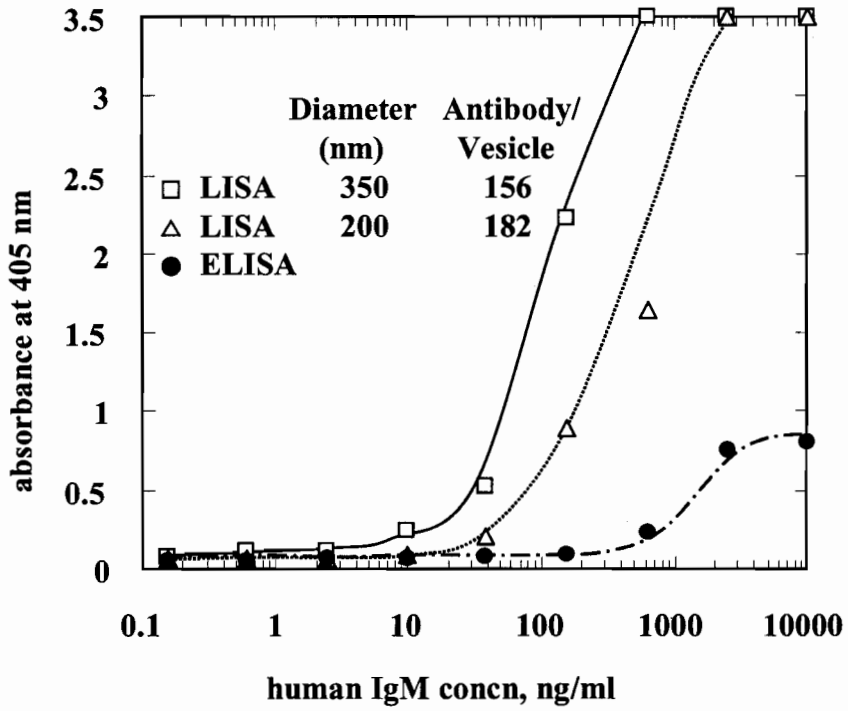


Figure 3-7 Comparison of LISA with ELISA in indirect method

Sample, human IgM
1st antibody, anti-human IgM antibody (rabbit) concn, 5μg/ml
LISA
Anti-rabbit IgG antibody (goat) coupled liposome encapsulating HRP
Antibody concn in immunoliposome solution, 5 μg/ml
Skim Milk concn in immunoliposome solution, 0.1 wt%
Diameter, □: 350 nm, △: 200 nm,
Coupled antibody, □: 0.14 mmol/mol-DPPC, △: 0.51 mmol/mol-DPPC
ELISA
HRP-conjugated anti-rabbit IgG antibody (goat)
Enzyme-conjugated antibody concn, 3 μg/ml
Tween20 concn in enzyme-conjugated antibody solution, 0.05 vol%

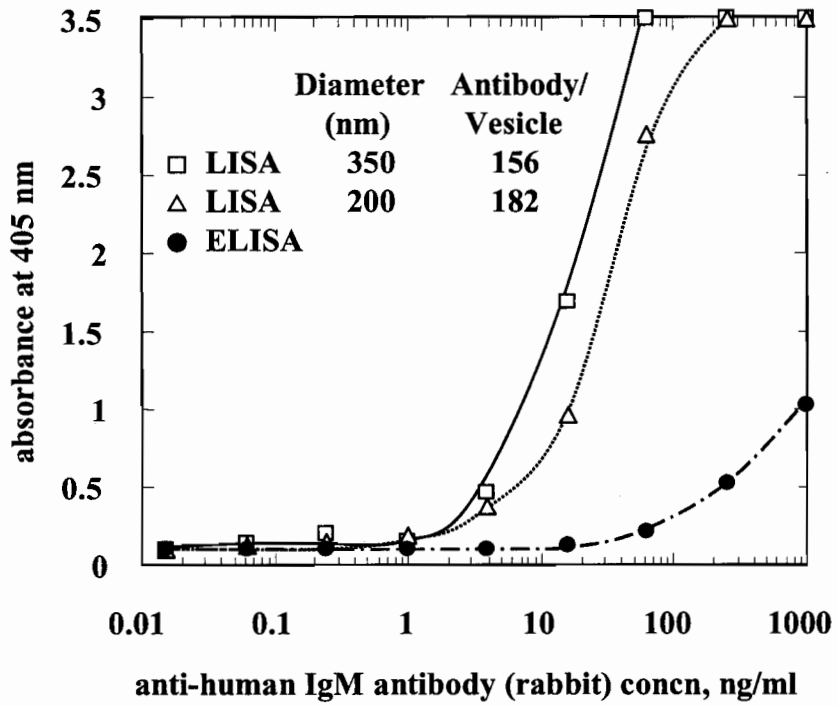


Figure 3-8 Comparison of LISA with ELISA in sandwich method

Coating antigen, human IgM concn, 10 μg/ml
Sample, anti-human IgM antibody (rabbit)
LISA
Anti-rabbit IgG antibody (goat) coupled liposome encapsulating HRP
Antibody concn in immunoliposome solution, 3 μg/ml
Skim Milk concn in immunoliposome solution, 0.1 wt%
Diameter, □: 350 nm, △: 200 nm
Coupled antibody, □: 0.14 mmol/mol-DPPC, △: 0.51 mmol/mol-DPPC
ELISA
HRP-conjugated anti-rabbit IgG antibody (goat)
Enzyme-conjugated antibody concn, 3 μg/ml
Tween20 concn in enzyme-conjugated antibody solution, 0.05 vol%

3.4 Conclusion

To obtain high sensitivity and signal intensity in LISA, immunoliposomes of large unilamellar vesicles (LUVs) were effective, because they could encapsulate a large amount of marker and couple many antibody molecules on their membrane surface. In addition, nonspecific adsorption of immunoliposomes on plastic support could be significantly reduced by use of 0.1 % skimmilk in PBS as a dilution buffer. Consequently, about 50-100 times higher sensitivity and signal intensity could be obtained in LISA in comparison with those in conventional ELISA. Therefore, LISA can be one of promising methods for detection of trace amounts of analytes in clinical diagnostics and detection for environmental pollutants.

Chapter 4
Effect of preservation conditions on immunoliposome function in immunoassay

4.1 Introduction

Recently, functional liposomes immobilizing protein molecules on phospholipid membrane or inside vesicles (proteoliposomes) have been focused in the clinical, analytical and cosmetic fields. Especially, antibody coupled liposomes (immunoliposomes) have been utilized not only as drug carriers for drug delivery system (DDS) but also as specific markers for liposome immunoassay (LIA). (Rongen H A H et al., 1997) In comparison with conventional EIA utilizing enzyme-antibody conjugates, LIA utilizing immunoliposomes has many advantages. (Kumada et al., 2002 a) Large unilamellar vesicles have higher capacity for encapsulation of enzyme molecules, and can bind antigen with multipoint interaction. Consequently, liposome immunosorbent assay (LISA) shows much higher sensitivity and signal intensity compared to traditional enzyme-linked immunosorbent assay (ELISA). For practical application of liposome immunoassay, functions of immunoliposomes must be kept for a long period. Proteins and microorganisms are stored below 0 ^{o}C in glycerol solution, and retain their activities. Crew L M et al. found that trehalose and other saccharides were effective for liposome lyophilization and avoid liposome fusion or aggregation by their anti-freezing effect. (Crew L M et al., 1985) Since immunoliposomes used in LIA must have two functions, binding ability of antibody and catalytic activity of encapsulated enzyme, for signal detection, preservation conditions should keep both activities.

In this chapter, we studied the effects of additives, such as saccharides and glycerol, on the stability of immunoliposomes under refrigerated or lyophilized states.

4.2 Materials and Methods

4.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque), dipalmitoylphosphatidyl-ethanolamine (DPPE) (Sigma Aldrich), cholesterol (Chol) (Wako Pure Chemical Industries, Ltd.), dicetylphosphate (DCP) (Sigma Aldrich) and succinimidyl-4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce) were used for liposome preparation. As a marker enzyme encapsulated in liposomes, horseradish peroxidase (HRP, 100 U/mg, grade III, TOYOBO) was used, and 2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) was used as substrate. DPPE was activated to N-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidylethanolamine (MPB-DPPE) by SMBP as previously reported. (Kishimura et al., 1989) Anti-rabbit IgG antibody (goat) was purchased from ICN Cappel and coupled on liposomes. HRP-conjugated anti-rabbit IgG (goat) was purchased from ICN Cappel. All other chemicals used were of reagent grade.

In immunoassay, PBS was used for plate washing and 4 times diluted Blocking One (nakarai tesque) with distilled water was used as a blocking reagent. Blocking One 10 times diluted with PBS was used for dilution of antibody or immunoliposomes.

4.2.2 Preparation of antibody-coupled liposomes encapsulating HRP

Antibody-coupled liposomes containing HRP (immunoliposomes) were prepared according to the method stated in Chapter 5. (Kumada et al., 2002 b) The phospholipid and coupled antibody concentrations of immunoliposome suspensions were determined by use of Phospholipid C-Test Wako (Wako Pure Chemical) and DC-protein assay (Bio-Rad Laboratories).

4.2.3 Preparation of freezed and lyophilized immunoliposomes

Four kinds of solutions containing one of additives, which were glucose, sucrose, trehalose, and glycerol, were added in the immunoliposome suspensions to form 1 % solutions of the additive. These suspensions were kept at – 20 °C to prepare freezed immunoliposomes (FLs).

To obtain lyophilized liposomes (LLs), these suspensions were dried in a lyophilizer under vacuum for 6 h. When FLs were used for immunoassay, they were thawed at room temperature without agitation.(Freeze-thawed liposomes, FTLs) In the case of LLs, distilled water was added to form the same concentration of liposome suspensions before lyophilization and stirred gently. (Lyophilize-dissolved liposomes, LDLs)

4.2.4 Measurement of aggregation of FTLs and LDLs

To evaluate liposome aggregation, FTLs and LDLs were centrifuged at 20,000 g for 2 min, and the supernatant containing liposomes without any aggregation was recovered. The DPPC concentration in the supernatant was measured with Phospholipid-C Test Wako. The aggregation ratio was determined by following equation:

$$\text{Aggregation ratio (\%)} = 100 \times (C_0 - C_s) / C_0$$

where C_0 and C_s are the DPPC concentrations in the initial liposome suspension and in supernatant, respectively.

4.2.5 Liposome immunosorbent assay (LISA) and enzyme-linked immunosorbent assay (ELISA) for measurement of overall activity of immunoliposomes

Rabbit IgG was used as a model analyte in immunoassay. 100 μl of 100 ng/ml rabbit IgG

in PBS was incubated in each well of a microplate at 37 °C for 3 h. After 3 times washing with PBS, the wells were blocked with 4 times diluted Blocking One 37 °C for 3 h. After 3 times washing, in LISA, 100 μl of immunoliposome suspension of 5.6 μg-antibody/ml-dilution buffer was added to each well, and the plate was incubated at room temperature for 1 h. In ELISA, 100 μl of 1000-times diluted antibody-HRP conjugates solution was used instead of the immunoliposome suspension. After 6 times washing with PBS, the color in each well was developed by use of substrate solution which contained 0.3 mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 0.01 % H_2O_2 in citrate buffer (pH4.0). After developing for 30 min, the absorbance at 405 nm in each well was measured by a microplate reader (Model 550 Bio-rad).

From measured absorbance, the overall activity of immunoliposome was determined by the following equation:

$$\text{Overall activity} = (A_i - A_{i0})/ (A_l - A_{l0})$$

where A_i and A_{i0} are the absorbances obtained by use of immunoliposome in presence and absence of antigen, respectively, and A_l and A_{l0} are these by use of HRP-labeled antibody.

4.3 Results and Discussion

4.3.1 Effect of freeze and freeze-dry operation on liposomal aggregation

Figure 4-1 shows the aggregation ratio of FTLs and LDLs. After freeze-thawing operation, liposomes were well dispersed without any aggregation in all FTL solutions. In the case of LDLs, however, the liposomes significantly aggregated without additive, whereas glucose, sucrose and trehalose as the additives were effective to reduce their aggregation. Glycerol, which has been used as one of popular stabilizers to maintain the activities of enzymes and antibodies, was little effective.

Therefore, these results suggested that about 80 % of LDLs could be kept dispersed state by addition of these additives.

4.3.2 Overall activity of FTLs and LDLs for immunoassay

Figure 4-2 shows the overall activity of FTLs and LDLs used in immunoassay. Without additives, the activity of FTLs decreased to 80 % of the initial activity, while no activity remained in LDLs. However, almost 100 % activity could be retained in the case of FTLs with 1 % glucose or sucrose. In addition, these additives were also effective in the case of LDLs, and the remaining activities of LDLs were 30-40 % of the initial activity. These activity changes seemed to be caused by loss of marker molecules inside liposomes, because signal intensities dramatically decreased after lyophilize-dissolving operation. Therefore, these additives might act as stabilizers of phospholipid bilayer.

4.3.3 Activity change of FTLs during repeated freeze-thaw operations

The activity change of FTLs containing 1% glucose was monitored during repeated freeze-thawing operations. As shown in **Figure 4-3**, after the first freeze-thaw operation, over 90 % overall activity of FTLs was retained, and then the activity gradually decreased with repeated operations. It was suggested that antibody molecules on liposomes might be inactivated by repeated freeze-thawing operations, because the obtained signal intensity of A_{i0} did not significantly decrease. Anyway, repeated freeze-thaw operations should be avoided to keep their overall activity to detect target antigen.

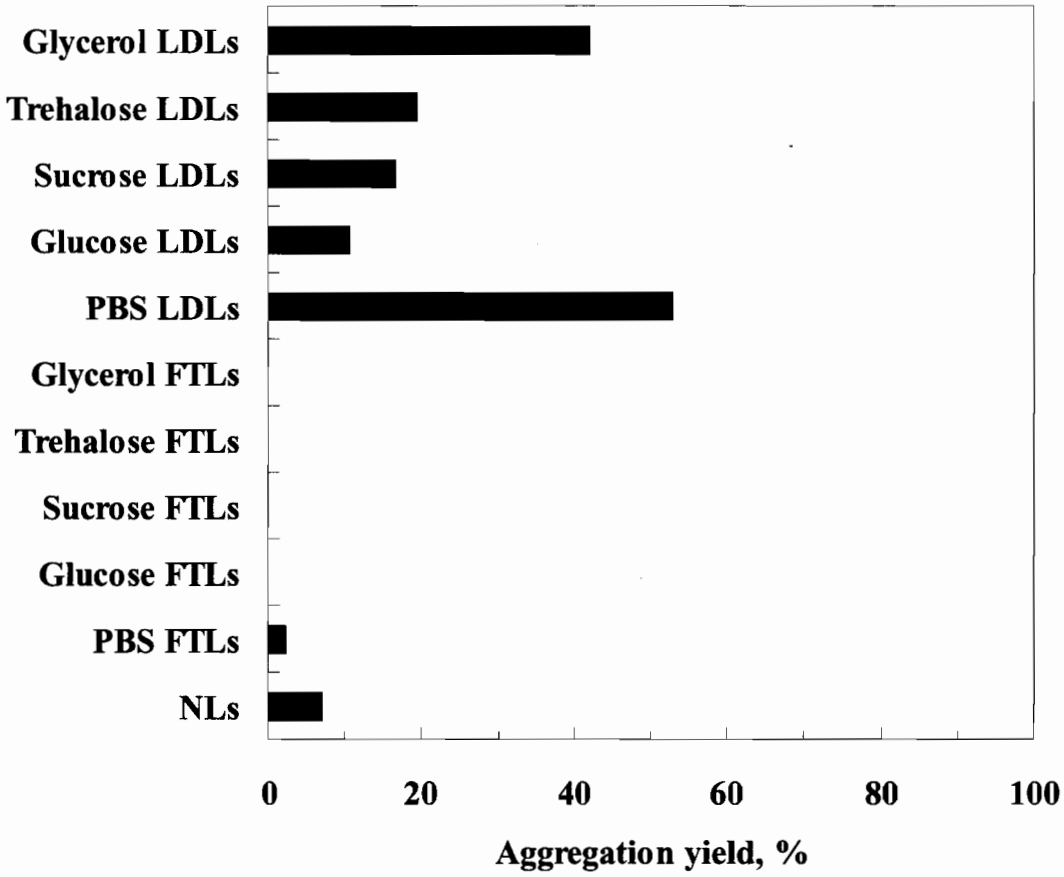


Figure 4-1 Aggregation ratio of immunoliposomes after freeze-thawing or lypphilize-dissolving operations

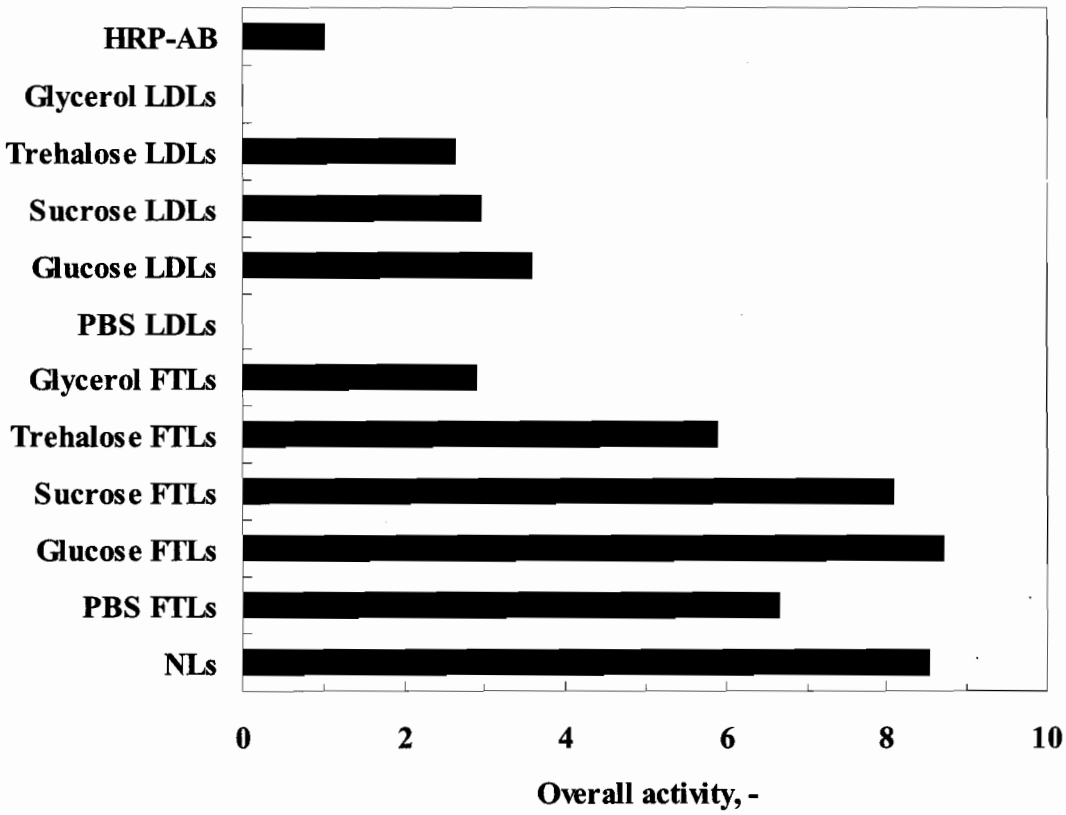


Figure 4-2 Overall activity of immunoliposomes after freeze-thawing or lyophilize-dissolving operations

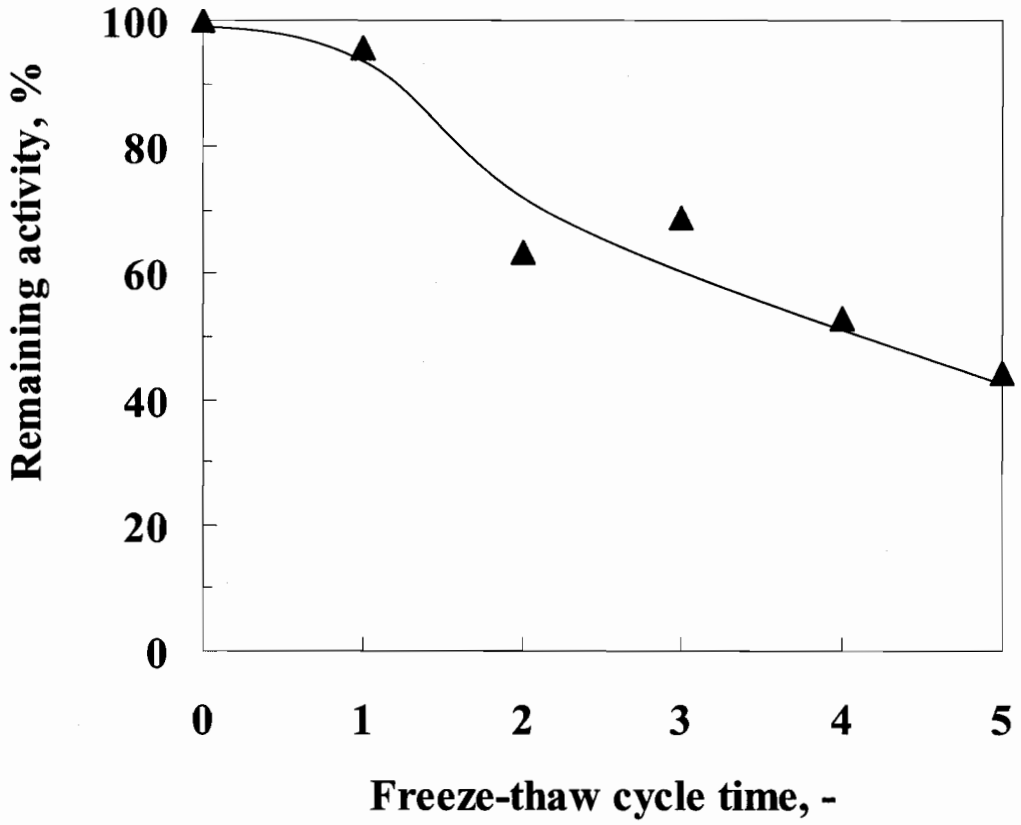


Figure 4-3 Activity change of FTLs contained in glucose solution during repeated freeze-thaw operations

4.3.4 Effect of temperature for LLs preservation on overall activity of LDLs

Figure 4-4 shows the effects of temperature on preservation of LL's overall activity. During about 1 month, LLs in presence of glucose was stable at 4°C, while their activity dramatically decreased at room temperature. The activities of antibody and HRP and bilayer structure were unstable at room temperature. Therefore, LLs in glucose solution could be stored stably at around 4°C.

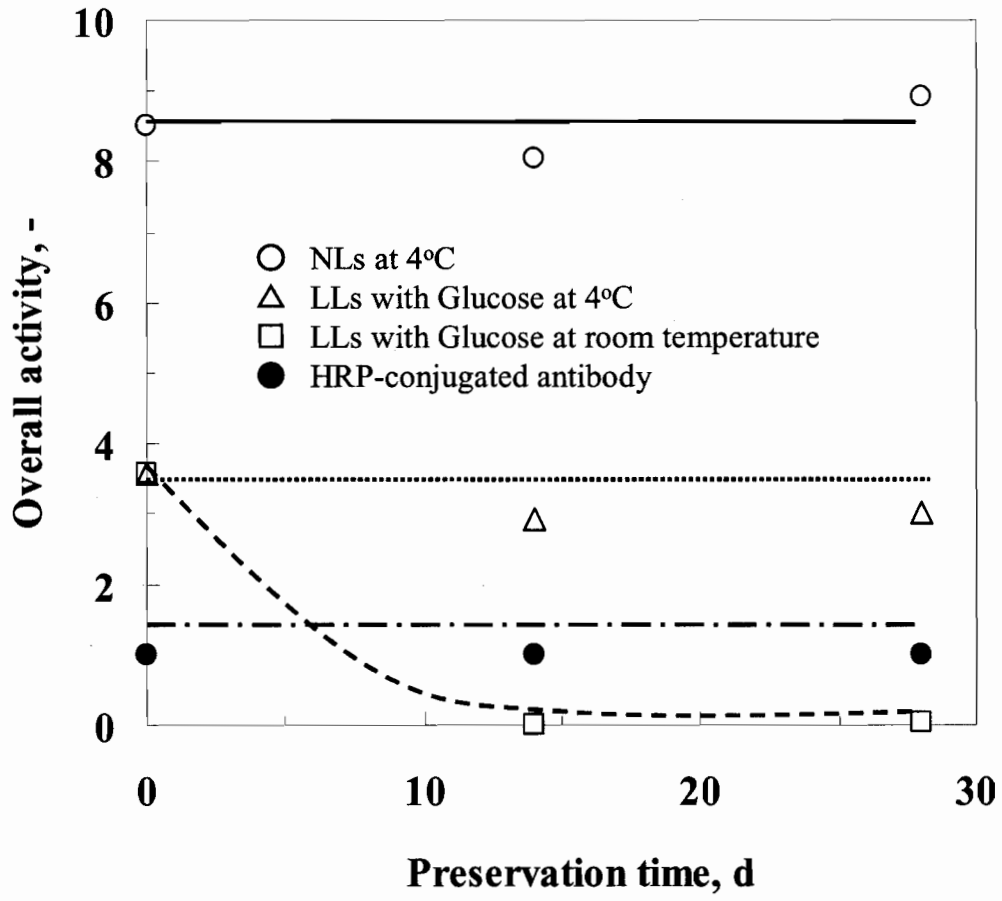


Figure 4-4 Effect of temperature for preservation of LLs containing glucose on overall activity of LDLs

4.3.5 Activity change of FTLs and LDLs suspension at 4 °C preservation

FTLs and LDLs were preserved at 4°C for 28 days to observe their activity change. As shown in **Figure 4-5**, the untreated immunoliposomes kept the initial activity during about 1 month, while the activity of FTLs without additive gradually decreased. The activity of LDLs with glucose was maintained at least for 14 days, and then they were slightly decreased. LDLs with saccharides could keep their full activity.

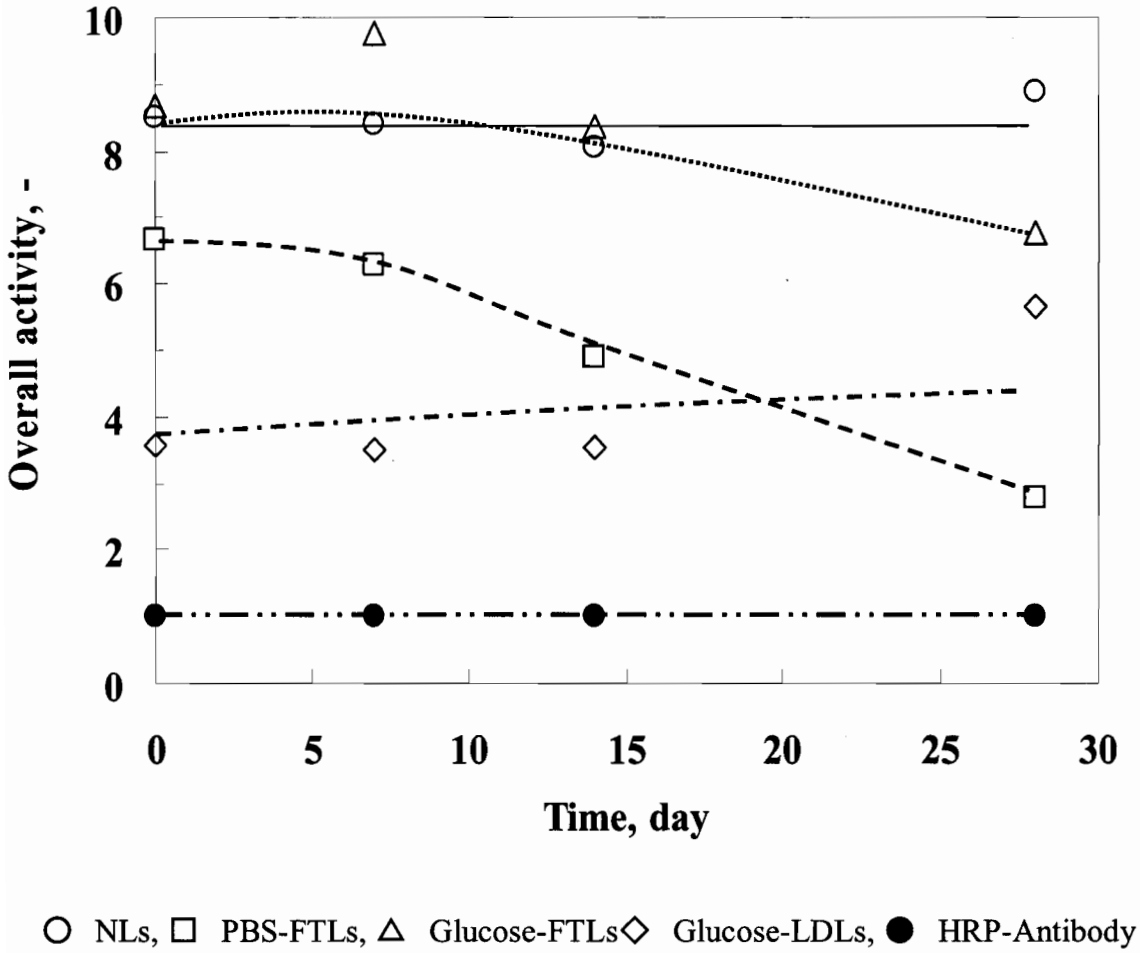


Figure 4-5 Activity changes of FTLs and LDLs containing 1% glucose solution at 4 °C

4.4 Conclusion

Immunoliposomes without additives were significantly unstable against freeze-thawing or lyophilize-dissolving operation and their overall activities decreased to 80 % and 0 %, respectively. In the presence of glucose as an additive during freeze-thawing, almost all immunoliposomes retained their activities. They maintained their activity at least for 2 weeks at 4°C, while their activity without the additives gradually decreased. Repeated freeze-thaw operations extensively decreased their activities because of incubation of antibody. The activities of lyophilize-dissolved liposomes (LDLs) were also kept 30-40 % of initial activity in the presence of glucose. Thus, glucose is the most effective to retain the overall activities of FTLd and LDLs.

Chapter 5
Development of liposome immunoblotting assay using a substrate-forming precipitate inside immunoliposomes

5.1 Introduction

The specific and strong immunological interaction between antigen and antibody has widely been used in assay methods of many biological materials. In recent years, the great increase in the number of samples for detection, characterization and screening of proteins in proteomics, clinical assay and bioindustries requires a rapid and automated immunoassay method. Although enzyme-linked immunosorbent assay (ELISA) is the most popular immunoassay among many methods based on the immunological interaction, it requires many repeated incubation and washing steps for separation of bound and free antibodies and antigens. Therefore, this method is too time consuming to apply to these purposes and is not able to handle a large number of samples.

Membrane blotting analyses, such as immunoblotting assay and colony lift immunoassay, may afford automated micro-array methods, but their sensitivities are limited by the amount and signal strength of labeling agents. For example, only a few enzyme molecules can be coupled on one molecule of antibody used for detection in immunoblotting assay. Many modified methods have been proposed to increase the sensitivity and to amplify the signal in measurements. Immunoliposomes encapsulating markers can be used for these purposes in place of labeled antibodies, because one liposome can couple many antibody (or antigen) molecules on its surface and can contain a larger amount of a marker than a labeled antibody. (Rongen et al., 1997, Singh and Carbonell, 1996) Multipoint interaction between antigen and antibody could increase their

binding affinity (avidity) in comparison with ELISA as stated in Chapter 2. (Kumada et al., 2001)

To increase the sensitivity, however, antibody-coupled liposomes in immunoblotting assays were not successfully used, because the lysis step of liposomes in development of signals caused spread of colored products out of liposomes. Thus, clear signals were not obtained. By use of a substrate which can permeate through the membrane of liposomes, the lysis step can be avoided, but a colored product can still diffuse out of liposomes. (Chapter 3, Kumada et al., 2002 a) To overcome this problem, in this chapter, a substrate which can permeate through the membrane of antibody-coupled liposomes and form a colored product precipitating inside the liposomes was used for liposome immunoblotting assay. The precipitates accumulated in the liposomes and could be detected at the positions where the liposomes coupled with a target in blotted samples. The sensitivity of this liposome immunoblotting assay was compared with that of the conventional immunoblotting assay using labeled antibody. The effects of the diameter of antibody-coupled liposomes and the pore size of membranes used for blotting were studied.

5.2 Materials and Methods

5.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque), dipalmitoylphosphatidylethanolamine (DPPE) (Sigma Aldrich), cholesterol (Chol) (Wako Pure Chemical Industries, Ltd.), dicetylphosphate (DCP) (Sigma Aldrich) and succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce) were used for liposome preparation. As a marker enzyme encapsulated in liposomes, horseradish peroxidase (HRP, 100 U/mg, grade III, TOYOBO) was used, and 4-chloro-1-naphthol and 2,2'-azino-bis(3-ethylbenzothiazoline-6-

sulfonic acid) diammonium salt (ABTS) were used as substrates, and the former is converted to a colored precipitate by HRP. N-succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) was purchased from Amersham Pharmacia Biotech, dithiothreitol (DTT) from nacalai tesque, anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP from Cappel Products Co., human IgM from Chemicon Int. Inc. Anti-rabbit IgG antibody (goat) was coupled on liposomes. Anti-α-chymotrypsinogen A antibody (rabbit) and anti-human IgM antibody (rabbit) were prepared by the previously reported method. (Katoh and Terashima, 1994) All other chemicals used were of reagent grade.

Buffers used in this work were prepared as follows: PBS (NaCl 9.0 g/l, KCl 0.2 g/l, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g/l, KH_2PO_4 0.2 g/l, pH=7.4) and MES (2-(N-morpholino) ethanesulfonic acid 4.27 g/l, NaCl 8.77 g/l, pH=5.5). Blocking solution (pH 7.2, nacalai tesque) and 1.0 % and 0.1 % skim milk solutions in PBS were used for blocking and washing. Membranes used for blotting were PVDF membranes with pore sizes of 450 nm (Immobilon P, Millipore) and 200 nm (Immuneblot, Bio-Rad Laboratories).

5.2.2 Preparation of antibody-coupled liposomes

DPPE was activated to N-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidyl-ethanolamine (MPB-DPPE) by SMBP as previously reported. (Kishimura et al., 1989) A lipid mixture containing DPPC (20 μmol), Chol (20 μmol), DCP (2 μmol) and MPB-DPPE (1 μmol) in chloroform was dried to a thin lipid film on the inside wall of a pear-shaped flask by using a rotary evaporator at 42 °C or above under reduced pressure. The lipid film was dried overnight under reduced pressure in a desiccator. Then, 6 ml of 5 mg/ml HRP in PBS was added to be encapsulated

as a marker, and the flask was shaken by a vortex mixer at around 50 °C for about 10 min to peel off the lipid film and form multilamellar vesicles (MLVs). To obtain small unilamellar vesicles (SUVs), the suspension of MLVs was sonicated with a probe-type sonicator (UD 201, TOMY) for 20~30 min at 40 W with cooling at 4°C. (Huang, 1964) Large vesicles were prepared by use of an extruder (Thermobarrel Extruder, Lipex Biomembrane Inc.) from MLVs. (Mayer et al., 1986) The liposomes thus prepared were separated from untrapped HRP by gel chromatography with a Bio-Gel A-15m (Bio-Rad Laboratories) column equilibrated with PBS.

A heterobifunctional cross-linking reagent SPDP was employed to combine seconaly antibody covalently with the activated lipid molecules. Anti-rabbit IgG antibody was modified by SPDP as previously reported. (Kishimura et al., 1989) A suspension of activated liposomes was mixed with a modified antibody solution and gently agitated overnight at room temperature. Uncoupled antibody was removed by gel chromatography with the Bio-Gel A-15m column equilibrated with PBS. Antibody-coupled liposomes obtained were stored at 4 °C. The HRP activity was about 90 % of the initial value, and HRP released from liposomes was less than 5 % after 4 weeks.

The average diameters of antibody-coupled liposomes were measured by the differencial light scattering analysis (DLS 7000, Photal Otsuka Electronics). The diameter of SUVs was 60 nm and those of large vesicles prepared by use of membranes with pore diameters of 100 nm, 200 nm and 400 nm were 130 nm, 200 nm and 350 nm, respectively.

The concentrations of DPPC and protein were measured after addition of 10 μl of 10 % deoxycholic acid sodium salt to 100 μl of each sample. HRP was inactivated by heating at 90 °C for 10 min. The concentration of DPPC in the liposome suspension was measured by the

cholineoxidase method, using Phospholipids C-Test Wako (Wako Pure Chemical Ind. Ltd.). The concentration of protein coupled on liposomes was measured by DC-protein assay (Bio-Rad Laboratories).

5.2.3 Liposome and conventional immunoblotting assays

Two immunoblotting assays are schematically shown in **Figure 5-1**. Samples (20 μl) containing different concentrations of human IgM (0 - 10 μg/ml) were blotted on the PVDF membrane (pore size 200 nm or 450 nm). After washing with PBS, the membrane was blocked with Blocking solution (nacalai tesque) to avoid non-specific adsorption. The membrane was washed with 1 % skim milk-PBS and was incubated in an anti-human IgM antibody (rabbit) solution of 3 μg/ml in 1 % skim milk-PBS for 1 h at room temperature. Bovine serum albumin of the same concentration also showed similar effects for preventing non-specific adsorption.

In the liposome immunoblotting assay, the membrane was washed with 1 % skim milk-PBS and incubated with anti-rabbit IgG antibody (goat)-coupled liposomes in 0.1 % skim milk-PBS diluted to a concentration of 1 μg/ml of the antibody for 1 h. After washing with PBS, a substrate solution containing 0.5 mg/ml 4-chloro-1-naphthol, 0.015 % H_2O_2 and 17 % methanol in PBS was added. This substrate can permeate through the phospholipid membrane of liposomes and was converted to colored precipitates of 4-chrolo-1,2-naphthoquinone and its polymer produced by radical reaction. Color was developed for 30 min. As shown in **Figure 5-1**, the colored product from 4-chloro-1-naphthol by HRP precipitates and accumulates in the liposomes. Thus, all the product does not diffuse out of the liposomes and can be detected *in situ*.

In the conventional immunoblotting assay, the membrane was washed with 1 % skim

milk-PBS and incubated with anti-rabbit IgG antibody (goat)-HRP diluted 2000 times in PBS-Tween 20 (0.05 %, 2 μg/ml specific antibody) for 1 h. After washing with PBS, color was developed by the same procedure stated above. In this case a part of the product is adsorbed onto the membrane and detected.

Photos of colored circles of blotted samples were taken by a camera (Coolpix 950, Nikon), and the lightness of the circles on photos was determined based on the Lab-mode by use of a computer.

5.2.4 Western blot technique by use of antibody-coupled liposomes

Samples of several concentrations (0.01 μg/ml - 10 μg/ml) of α-chymotrypsinogen (15 μl, diluted 1:3 with sample buffer) were applied to SDS-PAGE and blotted to the PVDF membrane (pore size, 450 nm) with conventional procedures. After blocking with Blocking solution and washing with 1 % skim milk-PBS, the membrane was incubated with anti-α-chymotrypsinogen antibody (rabbit) solution (5 μg/ml in 1 % skim milk-PBS) for 1 h. After thorough washing with 1 % skim milk-PBS, color was developed with anti-rabbit IgG antibody-coupled liposomes containing HRP or anti-rabbit IgG antibody (goat)-HRP by the similar procedure to the above immunoblotting assays.

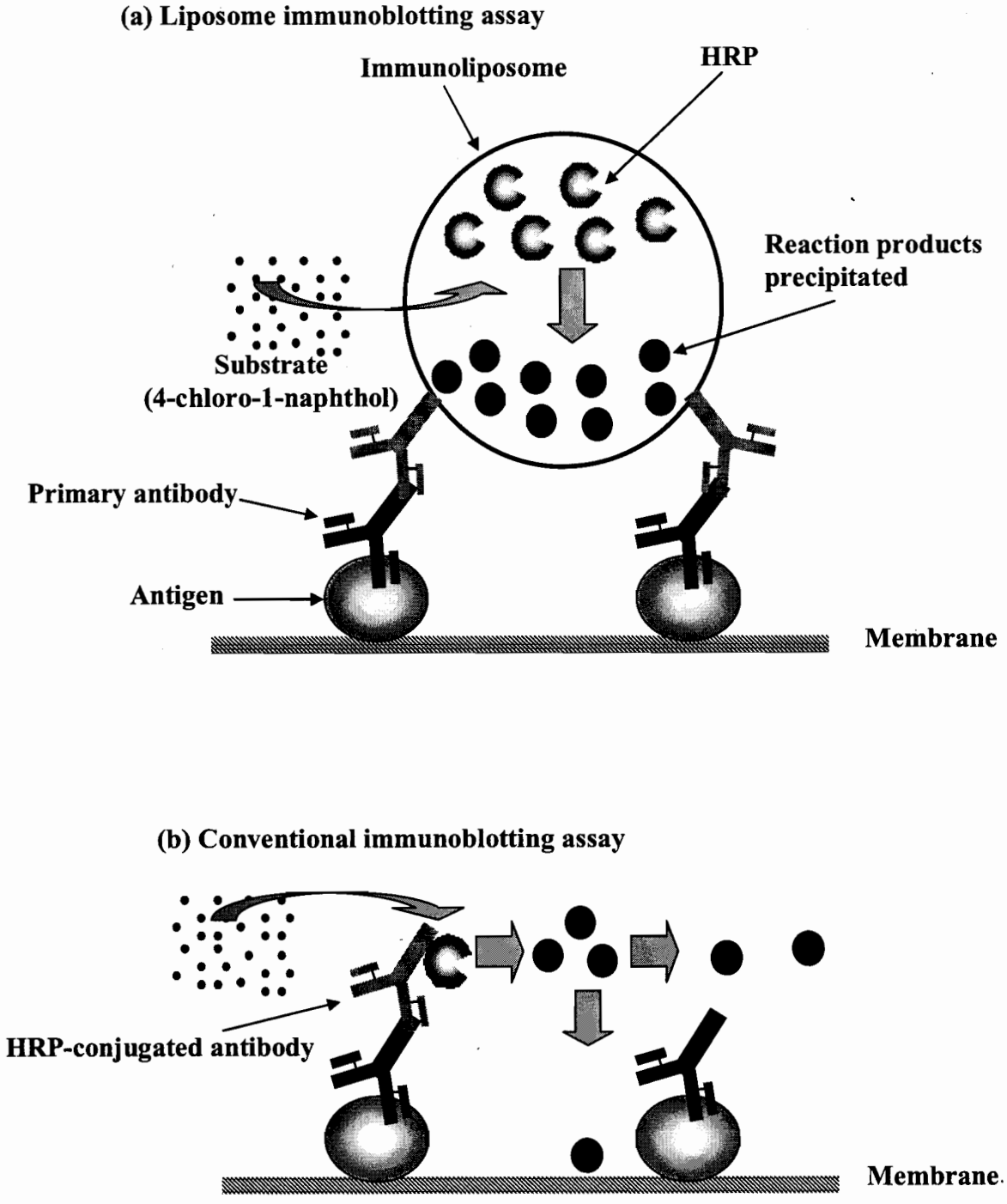


Figure 5-1 Mechanisms of color development in liposome immunoblotting assay and conventional immunoblotting assay

5.3 Results and Discussion

5.3.1 Effect of permeability of products through liposome membrane

Two substrates of HRP, 4-chloro-1-naphthol (4-CN) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were used for detection of coupled liposomes by developed color. Both of them are permeable into liposomes, but the product of the former forms precipitate and is no longer permeable through the phospholipid membrane of liposomes.

Figure 5-2 shows substrate solutions after color development with mixing liposomes containing HRP or anti-rabbit IgG antibody (goat)-HRP for 15 min. As shown in the middle row of **Figure 5-2**, aggregates of the colored product from 4-CN were sedimented by centrifugation at 9,200 g in the case of 4-CN and anti-rabbit IgG antibody (goat)-HRP, but the product encapsulated in liposomes was not sedimented at this centrifugal force. Naturally, no precipitate was observed by use of ABTS.

Centrifugation at 600,000 g, where liposomes were sedimented, made the supernatant clear in the case of 4-CN and liposomes. This shows that the product was retained in liposomes in this case, while the colored product diffused to the supernatant in the case of ABTS and liposomes. Thus, immunoblotting assay by use of ABTS as a substrate was impossible because of spreading of the product. Formation of precipitate of the colored product is critical to accumulate it inside liposomes.

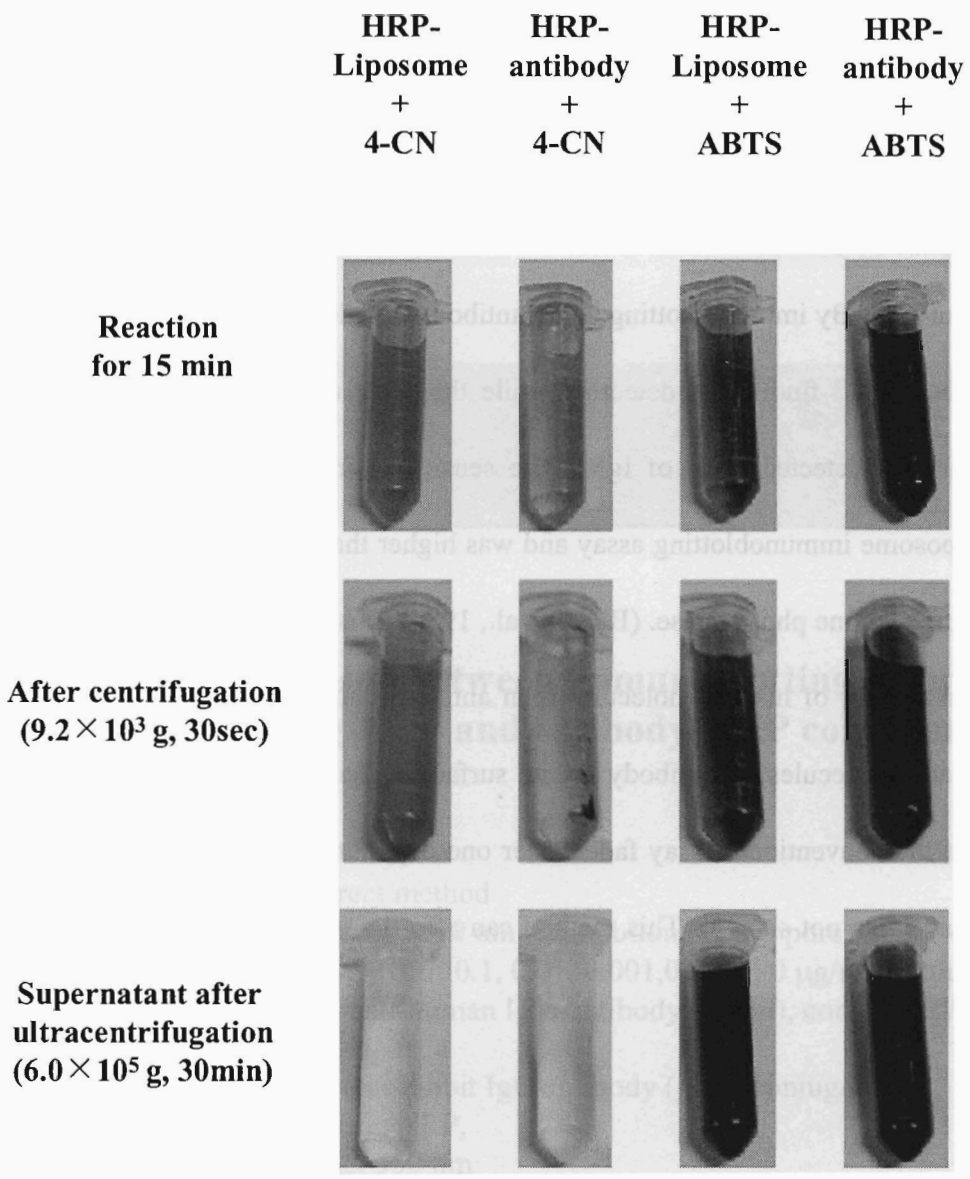


Figure 5-2 Difference in states of products formed by reaction with HRP

5.3.2 Comparison between liposome and conventional immunoblotting assays

Figure 5-3 compares the sensitivity of these two immunoblotting assays of human IgM from 0.0001 to 10 μg/ml. The applied sample volume was 20 μl, and the PVDF membrane with the pore size of 450 nm and antibody-coupled liposomes with the diameter of 350 nm were used. Non-specific adsorption of liposomes and labeled antibody were very low, as shown by the results of the sample without IgM. By immunoblotting using antibody-coupled liposomes containing HRP, 0.02 ng of IgM (about 0.02 fmol) was detected, while the conventional immunoblotting using antibody-HRP conjugates detected 2 ng of IgM. The sensitivity increased about two orders of magnitude by the liposome immunoblotting assay and was higher than the maximum sensitivity of Western blotting using alkaline phosphatase. (Blake et al., 1984) This is because that liposomes can contain much larger amount of marker molecules than antibody molecule and that one liposome vesicle can bind many molecules of antibody on its surface. (Chapter 2, Kumada et al., 2001) Further, the color of the conventional assay faded after one day, but it was stable in the liposome immunoblotting assay (data not shown). This method can afford a membrane micro-array method with a high sensitivity.

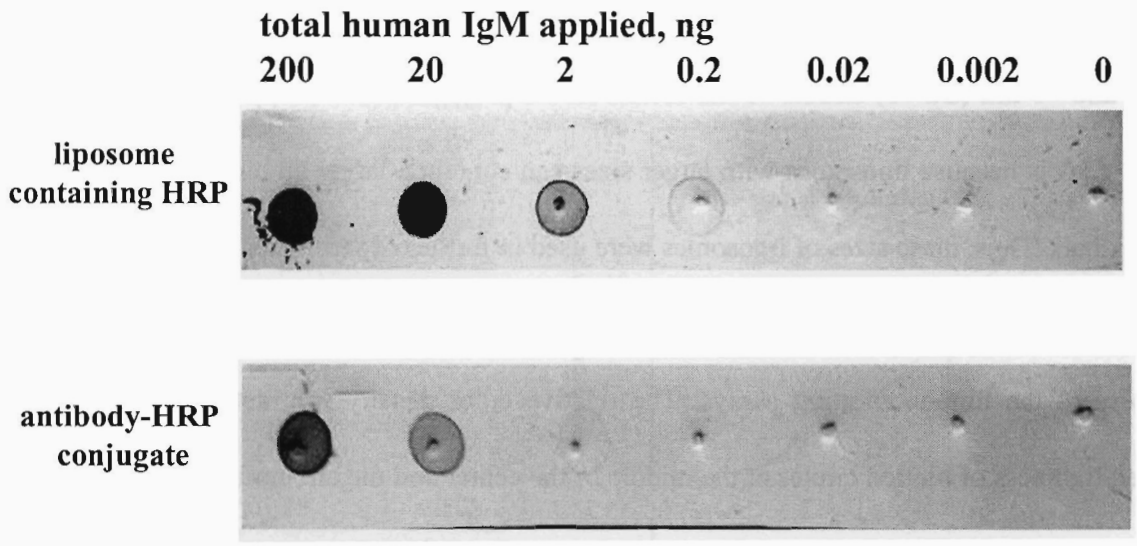


Figure 5-3 Comparison between immunoblotting assays using liposomes containing HRP and antibody-HRP conjugates

Assay system: Indirect method
Pore size of membrane: 450 nm (Immobilon P, Millipore)
Sample: human IgM, 10, 1, 0.1, 0.01, 0.001,0.0001, 0 μg/ml * 20μl
Primary antibody: anti-human IgM antibody (rabbit), concn 3 μg/ml

Immunoliposome: anti-rabbit IgG antibody (goat) conjugated
liposome encapsulating HRP,
diameter of liposome, 350 nm
antibody concn, 1 μg/ml
Coupled antibody, 0.14 mmol/mol-DPPC

Enzyme-conjugated antibody: anti-rabbit IgG antibody (goat) -HRP,
2000 times dilution

5.3.3 Effects of pore size of membrane and diameter of liposomes

Liposomes with diameters of 200 nm and 350 nm showed higher sensitivities than those of 130 nm and 60 nm (SUVs) similar to the results in the previous work (Chapter 3, Kumada et al., 2002 a). This is because liposomes with larger sizes can contain a larger amount of HRP and show higher signals. Thus, these sizes of liposomes were used in further experiments.

Figures 5-4 and 5-5 compare the effect of the pore size of blotting membranes on the sensitivity of the immunoblotting assays. The relative color density was determined from the averaged lightness of blotted circles at the middle of the center and the circumference of the circle. Two types of PVDF membranes with the pore sizes of 200 nm and 450 nm and immunoliposomes having the diameter 350 nm, which is intermediate between the two pore sizes. Blotting on the PVDF membrane with the pore size of 450 nm gave about 10 times higher sensitivity than that with 200 nm. In the membrane with 450 nm pores, liposomes with diameters of 350 nm can penetrate into the membrane pores to bind with target protein adsorbed inside the pores. Increase in the amount of adsorbed liposomes improved the sensitivity and signal strength. On the other hand, in the membrane with 200 nm pores, liposomes can be adsorbed only on the surface of the membrane. The sensitivity of the conventional immunoblotting assay was much lower than those of the liposome immunobloting assay and little affected by the pore sizes of the membranes, as shown in **Figure 5-5**. Although labeled antibody can penetrate into the pores because of much smaller size than the pore sizes, they can couple small amount of labeling agent.

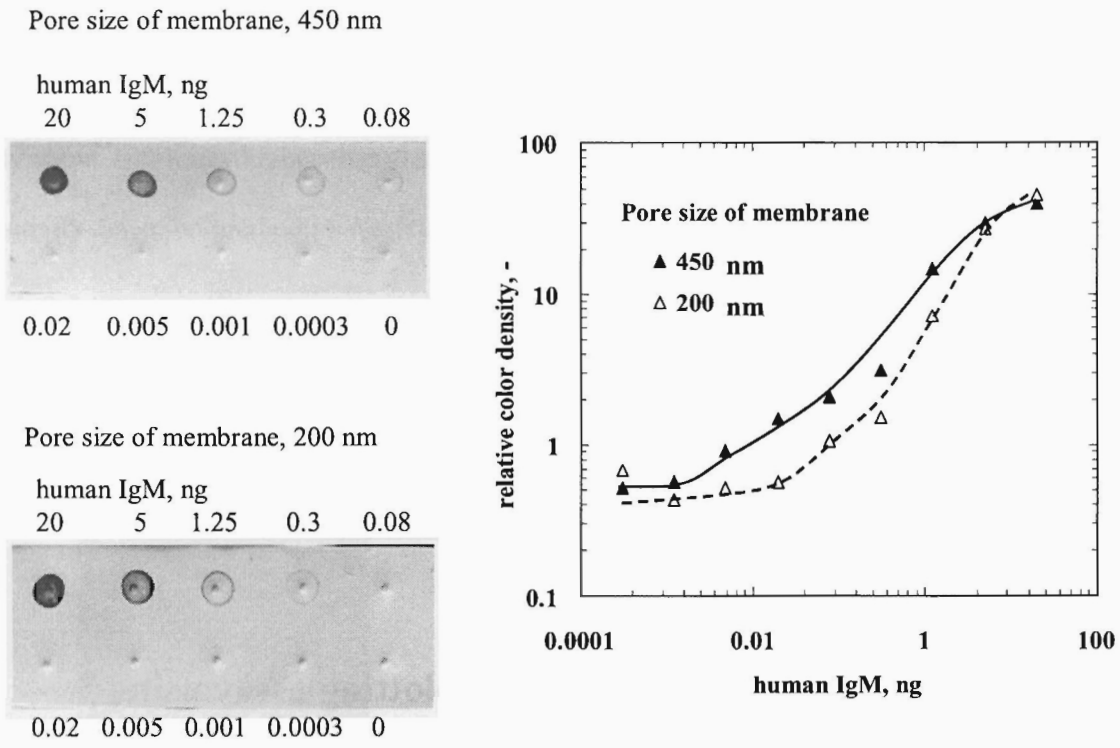


Figure 5-4 Liposome immunoblotting assay using membranes with pore size of 450 nm and 200 nm

Assay system, Indirect method
Pore size of membrane, 450 nm (Immobilon P, millipore), 200 nm (Immune blot, Bio-rad laboratories)
Sample, human IgM, 20 μl
Primary antibody, anti-human IgM antibody (rabbit), concn 3 μg/ml

Immunoliposome, anti-rabbit IgG antibody (goat) coupled liposome encapsulating HRP
Diameter of liposome, 350 nm
Antibody concn, 1 μg/ml
Coupled antibody, 0.14 mmol/mol-DPPC,

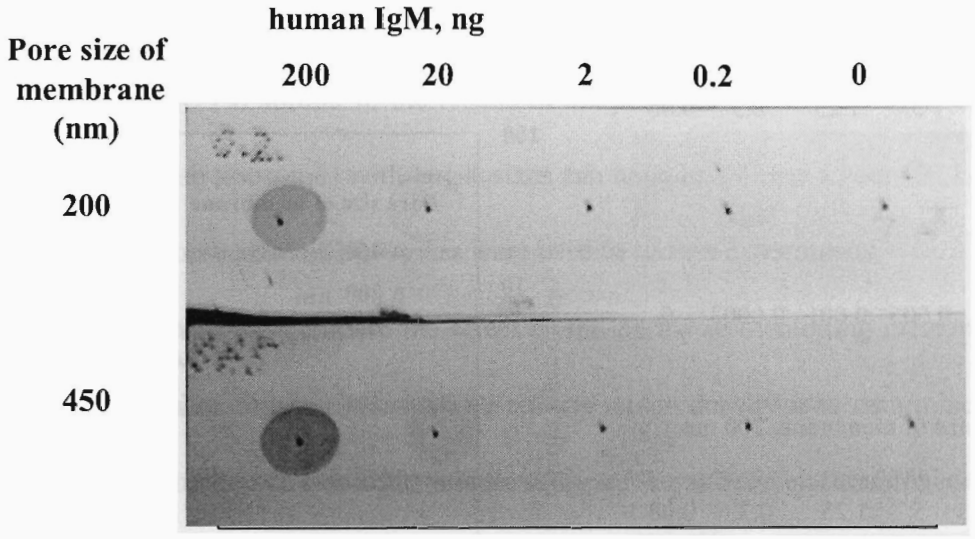


Figure 5-5 Conventional immunoblotting assay using membranes with pore size of 200 nm and 450 nm

Assay system: Indirect method
Pore size of membrane: 200 nm (Immune Blot, Bio-Rad) and 450 nm (Immobilon P, millipore)
Sample: human IgM, 20 μl

Primary antiboby: anti-human IgM antibody (rabbit), concn 3 μg/ml

Enzyme-conjugated antibody: HRP-conjugated anti-rabbit IgG antibody (goat), 2000 times dilution

5.3.4 Western blot technique with liposomes

Figure 5-6 shows the results of Western blotting of α-chymotrypsinogen developed with antibody-coupled liposomes containing HRP and antibody-HRP conjugates. The former gave the sensitivity about 100 times higher than that of the latter and immunoliposomes can give a simple and high sensitivity detection method in Western blotting.

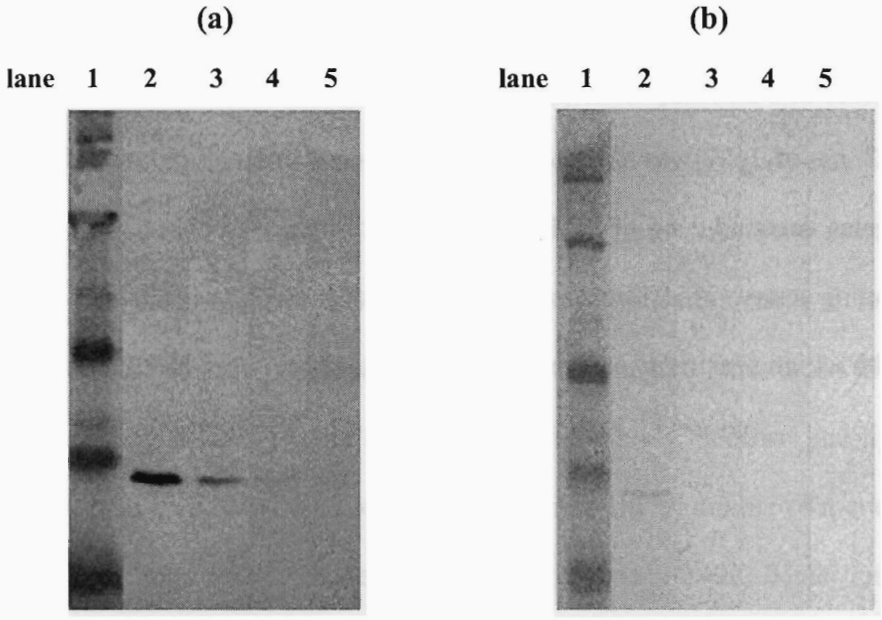


Figure 5-6 Comparison of Western blotting using liposomes containing HRP and antibody-HRP conjugates

Assay system: Indirect method
Pore size of membrane: 450 nm (Immobilon P, Millipore)
Sample: α-chymotrypsinogen A
lane1: Marker, lane2: 37.5, lane3: 3.75, lane4: 0.375, lane5: 0.0375 ng
Primary antibody: anti-α-chymotrypsinogen A antibody (rabbit),
concn 5 μg/ml

(a) Immunoliposome: anti-rabbit IgG antibody (goat) conjugated liposome encapsulating HRP, antibody concn, 1 μg/ml
Coupled antibody, 0.14 mmol/mol-DPPC
(b) Enzyme-conjugated antibody: anti-rabbit IgG antibody (goat) -HRP, 2000 times dilution

5.4 Conclusion

In this chapter, a new immunoblotting assay, which uses antibody-coupled liposomes containing horseradish peroxidase, was investigated. A substrate 4-chloro-1-naphthol permeated through the phospholipid membrane of the antibody-coupled liposomes and formed a colored product precipitating inside the liposomes. The precipitates accumulated in the liposomes and could be detected at the positions where the liposomes coupled with a target in blotted samples. Combination of liposomes with average diameter of 350 nm and a PVDF membrane with a pore size of 450 nm, 0.02 ng of IgM was detected, while the conventional immunoblotting using antibody-HRP conjugates detected 2 ng of IgM. The sensitivity increased about two orders by the liposome immunoblotting assay. This liposome immunoblotting assay gives a simple detection method of proteins with a high sensitivity, as well as high sensitivity Western blotting assay.

Chapter 6
Relationship between sample blotted area and obtained signal density in liposome immunoblotting

6.1 Introduction

In recent years, the requirement for rapid, automatic and sensitive detection methods for biological materials has increased because of the increase in the number of biological samples and decrease in the concentration to be detected in clinical, environmental and bioindustrial fields. The specific immunological interaction between an antigen and an antibody has been widely used in these detection methods. Among them, the liposome immunosorbent assay (LISA) utilizing immunoliposomes encapsulating markers is a promising detection method for trace amounts of biological materials. It has higher sensitivity and signal intensity than those of the enzyme-linked immunosorbent assay (ELISA) using enzyme-labeled antibody, because one liposome can couple many antibody molecules on its surface and can contain a larger number of marker molecules than a labeled antibody. (Singh and Carbonell, 1996, Rongen et al., 1997) Thus, multipoint interaction between an antigen and an antibody can increase the overall binding affinity (avidity) in comparison with the affinity of monovalent interaction and higher signal intensity is obtained from a large number of marker molecules encapsulated inside liposomes. (Chapter 2, Kumada et al., 2001, Chapter 3, Kumada et al., 2002 a)

In Chapter 5, we developed a liposome immunoblotting assay utilizing a color developing reaction, which is also useful for Western blotting and colony lift immunoassay. (Kumada et al., 2002 b) This method may be applied to microassay systems, in which the amounts and volumes of samples applied are smaller than those in conventional immunoblotting assays, but this may decrease the sensitivity and signal intensity. To clarify the performance of this liposome immunoblotting assay in microassays, we studied the effects of the sample volume blotted on

PVDF membrane on the sensitivity and signal intensity in this method and in a conventional immunoblotting assay.

6.2 Materials and Methods

6.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque, Kyoto), dipalmitoylphosphatidylethanolamine (DPPE) (Sigma Aldrich, St. Louis, MO, USA), cholesterol (Chol) (Wako Pure Chemical, Osaka), dicetylphosphate (DCP) (Sigma Aldrich) and *N*-succinimidyl 4-(*p*-maleimidophenyl)-butyrate (SMPB) (Pierce, Rockford, IL, USA) were used for liposome preparation. As a marker enzyme encapsulated in liposomes, horseradish peroxidase (HRP, 145 U/mg, grade III; Toyobo, Osaka) was used, and 4-chloro-1-naphthol (4-CN) (Bio Rad Laboratories, Cambridge, MA, USA) and hydrogen peroxide were used as substrates of HRP for the color developing reaction. Anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP were purchased from Cappel Products (Irvine, CA, USA). Human IgM used as a model analyte was obtained from Chemicon Int. (Temecula, CA, USA). Anti-human IgM antibody (rabbit) used as a primary antibody was prepared by the previously reported method. (Katoh and Terashima, 1994) Hydrophobic PVDF membrane with a pore size of 450 nm (Immobilon P; Millipore, Bedford, MA, USA) was used for blotting. All other chemicals used were of reagent grade.

6.2.2 Preparation of antibody-coupled liposomes

Antibody-coupled liposomes composed of a lipid mixture of DPPC (20 μmol), Chol (20 μmol), DCP (2 μmol) and activated DPPE (1 μmol) were prepared by the previously reported method. (Kumada et al., 2002 b) The amount of HRP released from prepared liposomes was less than 5 % of the initial amount after 4 weeks, and HRP activity was maintained at about 90 % of the

initial value. The average diameter of antibody-coupled liposomes measured by differential light scattering analysis (DLS 7000; Photal Otsuka Electronics, Osaka) was 350 nm.

6.2.3 Sample blotting and immunoblotting assay

Samples (2, 5, 10 and 20 μl) containing different concentrations of human IgM were blotted on the PVDF membrane using a microplotter (402 syringe pump and 221-XL liquid handler, Gilson, WI, USA). After washing with PBS for 10 min, the membrane was blocked with Blocking solution (nacalai tesque) to avoid nonspecific adsorption. The membrane was washed with PBS for 10 min and incubated in an anti-human IgM antibody (rabbit) solution (3 μg-specific antibody/ml) in 1% casein-PBS for 1 h at room temperature.

In the liposome immunoblotting assay, the membrane was washed three times with 1% casein-PBS and incubated with anti-rabbit IgG antibody (goat)-coupled liposomes (1 μg-specific antibody/ml) in 1% casein-PBS for 1 h at room temperature. After washing three times with 1% casein-PBS, a substrate solution containing 0.5 mg/ml 4-chloro-1-naphthol, 0.015% H_2O_2 and 17% methanol in PBS was added, and color was developed for 30 min.

In the conventional immunoblotting assay, the membrane was washed with 1% casein-PBS and incubated with 2,000-fold diluted anti-rabbit IgG antibody (goat)-HRP in PBS containing 0.05 % Tween 20 for 1 h at room temperature. After washing, color was developed by the same procedure as in the liposome immunoblotting assay.

Photographs of colored circles of blotted samples were scanned using a scanner (EPSON TWAIN 5; Epson, Tokyo), and the lightness of the circles on the image was determined using Lab mode by Adobe Photoshop. The signal intensity was obtained by dividing the difference of the lightness of a circle and that of the background by the area of the circle.

6.3 Results and Discussion

6.3.1 Blotted sample amount and signal densities in liposome immunoblotting assay

Figure 6-1 shows the photoimages of colored circles obtained by blotting different concentrations (0.0024 μg/ml – 20 μg/ml) and volumes (2, 5, 10 and 20 μl) of human IgM solutions in the liposome immunoblotting and conventional assays. The liposome immunoblotting assay yielded higher sensitivity and signal intensity. In the conventional assay, changing the dilution ratio of anti-rabbit IgG-HRP from a 2000-fold dilution to a 1000-fold or 500-fold dilution did not affect the signal density or sensitivity. **Figure 6-2** shows the signal density plotted against the total amounts of human IgM blotted on PVDF membranes. As shown in the case of the liposome immunoblotting assay, the signal density should increase with decreasing sample volume at an equal amount of blotted IgM, because the concentration of IgM blotted on PVDF membranes increased. Although the blotted area was not proportional to the blotted volume, as shown in **Table 6-1**, this increase in the signal density is understandable. In the conventional immunoblotting assay, however, the signal densities observed were almost the same at an equal amount of blotted IgM, irrespective of the sample volume. These differences might be due to the followings. In the liposome immunoblotting assay, 4-CN, the substrate of HRP, can permeate through the liposome membrane and form colored precipitates accumulating in the liposomes without losses of the signal intensity, while a portion of the colored products may be lost by diffusion into the substrate solution in the conventional immunoblotting assay. The fraction of loss increased with decreasing sample volume.

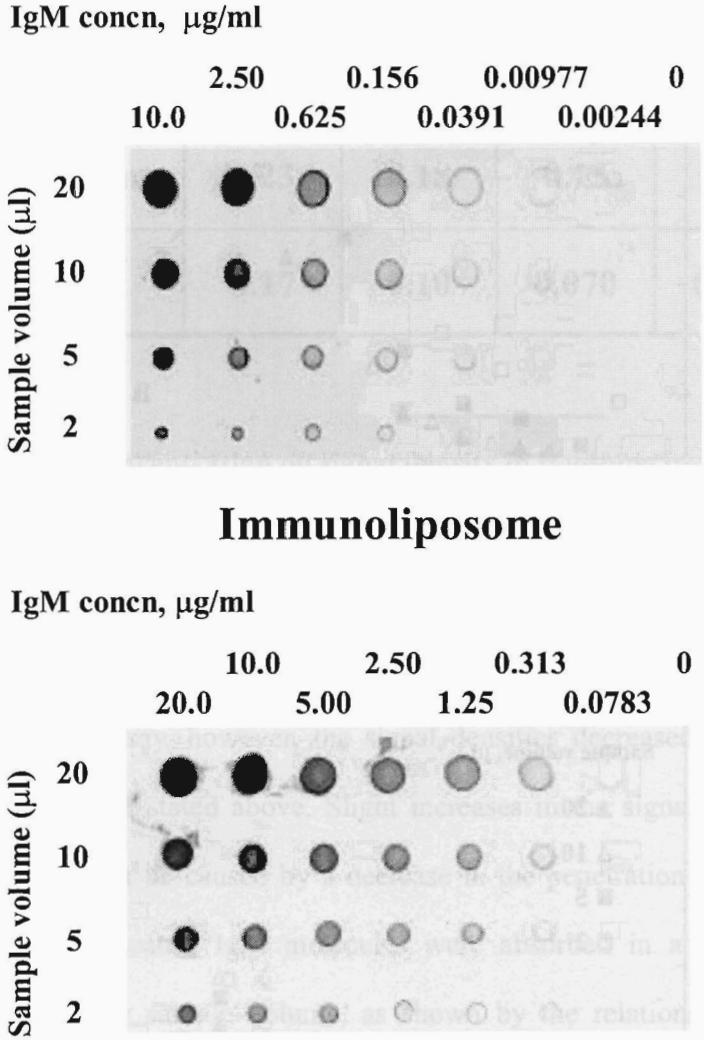
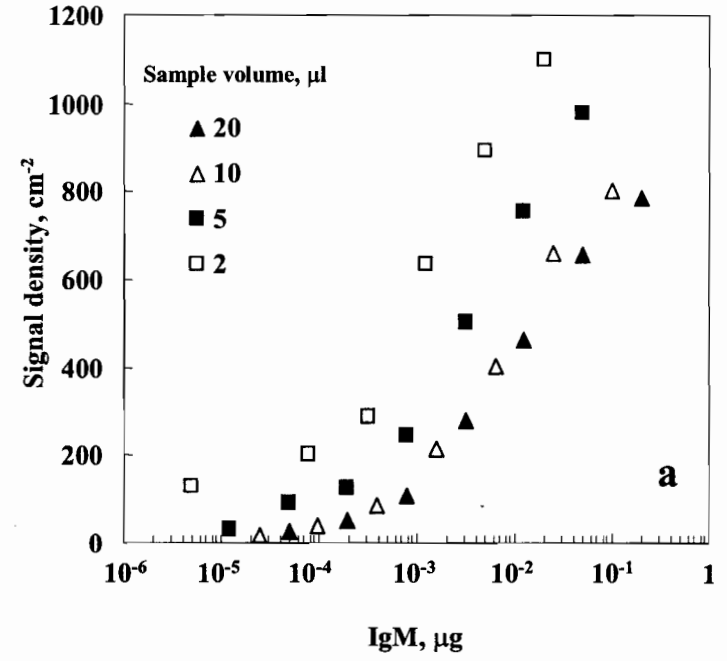


Figure 6-1 Liposome and conventional immunoblotting assays with different concentrations of IgM and sample volumes

Analyte: human IgM.
Primary antibody: 3.0 μg/ml anti-human IgM antibody (rabbit).
Secondary antibody:
liposomes coupled with anti-rabbit IgG antibody (goat), 1.0 μg-antibody/ml,
2000 -fold diluted anti-rabbit IgG antibody (goat)-HRP.



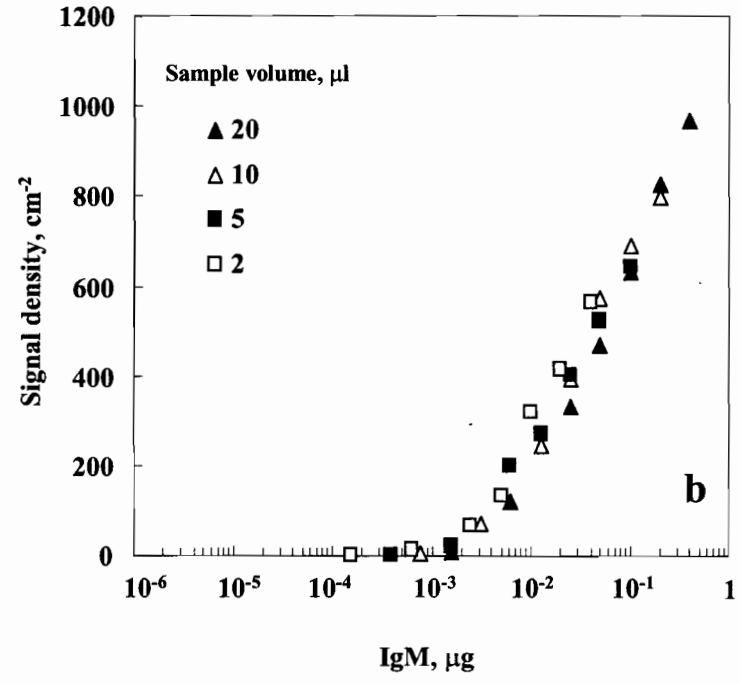


Figure 6-2 Effects of sample volume and IgM amount on sensitivity and signal density in liposome and conventional immunoblotting assays

(a) liposome immunoblotting assay and (b) conventional immunoblotting assay

Table 6-1 Average radius and area of blotted sample

Sample volume, μl	20	10	5.0	2.0
Average radius, cm	0.23	0.18	0.15	0.12
Average blotted area, cm^2	0.17	0.10	0.070	0.045

6.3.2 Effect of analyte concentration on signal density in liposome immunoblotting assay

Figure 6-3 shows the signal density plotted against the concentration of human IgM. At the same concentration, the signal densities obtained in the liposome immunoblotting assay were almost the same or increased slightly with decreasing blotted sample volume (from 20 to 2 μl). In the conventional immunoassay, however, the signal densities decreased with decreasing sample volume for the same reason stated above. Slight increases in the signal density in the liposome immunoblotting assay might be caused by a decrease in the penetration depth of blotted samples into PVDF membranes, because IgM molecules were absorbed in a shallow layer of PVDF membranes with decreasing sample volume, as shown by the relationship between the sample volume and the blotted area in **Table 6-1**. This facilitated accessibility of liposomes to adsorbed IgM in membrane pores.

These results showed that the liposome immunoblotting assay is suitable for the detection of trace amounts and small volumes of analytes in microassay systems.

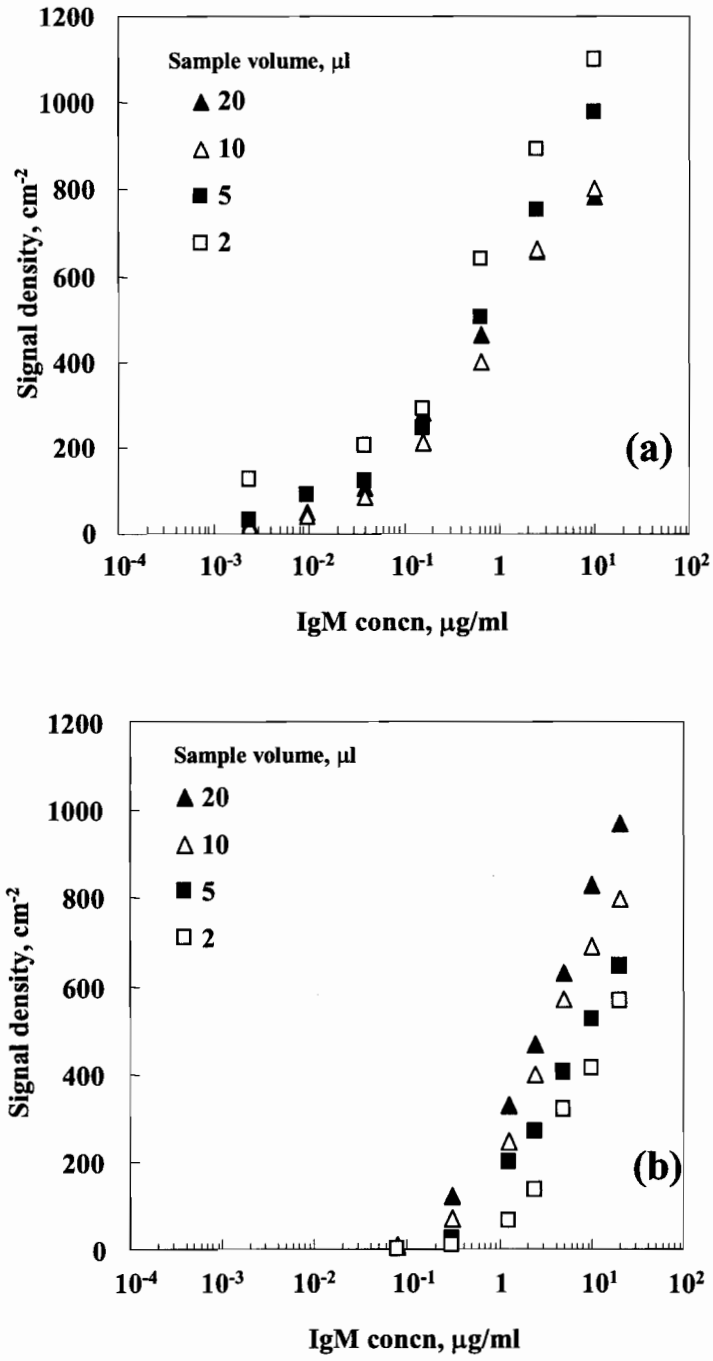


Figure 6-3 Effects of sample volume and IgM concentration on sensitivity and signal density in liposome and conventional immunoblotting assays

(a) liposome immunoblotting assay and (b) conventional immunoblotting assay.

6.4 Conclusion

To clarify the applicability of a liposome immunoblotting assay to microassay systems, the effects of the sample volume blotted on PVDF membrane on the sensitivity and signal intensity were studied and compared with those in a conventional immunoblotting assay utilizing enzyme-labeled antibody. In the liposome immunoblotting assay, signal intensities per unit area (signal density) were almost the same or increased slightly with decreasing blotted sample volume (from 20 to 2 μl) at the same concentration of analyte (human IgM) adsorbed on PVDF membrane. A substrate, 4-chloro-1-naphthol, for color development formed colored precipitates inside the liposomes. Thus, the colored signal was detected without loss of intensity. On the other hand, in the conventional immunoblotting assay, signal densities decreased with decreasing sample volume at the same concentration of analyte. This is caused by partial losses of the colored signal into the substrate solution by diffusion, because the fraction of loss increases with decreasing sample volume. These results show that the liposome immunoblotting assay is a promising method for the detection of analytes blotted in small areas, because its sensitivity is higher than that of the conventional blotting assay.

Chapter 7
Application of immunoliposomes to colony lift immunoassay

7.1 Introduction

The specific and strong immunological interaction between antigen and antibody has widely been utilized in immunoassays for detection of many biological materials. In recent years, the great increase in the number of samples for detection, characterization and screening of proteins in proteomics, clinical assay and bioindustries requires a high throughput and automated immunoassay method. Membrane blotting analyses including colony lift immunoassay may afford automated micro-array methods. Especially, colony lift immunoassay is useful for not only detection of pathogenic bacteria such as O157:H7, *Listeria monocytogenes* and so on, but also rapid screening of desirable transformants expressing target recombinant protein from huge libraries. (Ingram et al., 1998, Carroll et al., 2000) Although immunoblotting assays utilizing enzyme-conjugated antibodies are widely used in colony lift immunoassay to detect target colonies, their signal strengths are limited by the amount of conjugated enzymes. If the sensitivity of immunoblotting assay is increased, small amounts of target protein secreted from small colonies would be detected. Thus, it would be possible to analyze a larger number of colonies. Therefore, many modified methods have been proposed to increase the sensitivity and to amplify the signal in immunoblotting assays.

I have proposed and demonstrated a liposome immunoblotting assay method utilizing antibody-coupled liposomes (immunoliposomes) encapsulating HRP. In comparison with the conventional immunoblotting assay, this method showed advantages to detect trace amounts of a target antigen with 100 times higher sensitivity. (Chapter 5, Kumada et al., 2002 b) Moreover, in the immunoblotting assay, the signal intensity did not decrease with decreasing in the volume of blotted samples because of accumulation of colored precipitates in liposomes. (Chapter 6, Kumada

et al., 2004)

In this chapter, this immunoblotting assay was applied to colony lift immunoassay, and blotting methods to obtain higher signal intensity in comparison with that in the conventional method were investigated.

7.2 Materials and Methods

7.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque), dipalmitoylphosphatidylethanolamine (DPPE) (Sigma Aldrich), cholesterol (Chol) (Wako Pure Chemical Industries, Ltd.), dicetylphosphate (DCP) (Sigma Aldrich) and succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce) were used for liposome preparation. DPPE was activated to N-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidylethanolamine (MPB-DPPE) by SMBP as previously reported. (Kishimura et al., 1989) As a marker enzyme encapsulated in liposomes and a substrate for color development, horseradish peroxidase (HRP, 100 U/mg, grade III, TOYOBO) and 4-chloro-1-naphthol (4-CN) were used, respectively. 2-Iminothiolane·HCl (Pierce) was used for immobilization of antibody to MPB-DPPE in phospholipid membrane. Anti-His 6 antibody (rabbit) was purchased from BETHYL. Anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP were purchased from Cappel Products Co. All other chemicals used were of reagent grade.

Blocking solution (pH 7.2, nacalai tesque) was used for a blocking operation. To prevent nonspecific adsorption of antibody or immunoliposomes, 1.0 % casein-PBS was used as a buffer for each incubation step and washing step. Hydrophobic and hydrophilic PVDF membranes were purchased from Millipore. The hydrophobic one (Immobilon P, pore size: 450 nm) was used as an adsorbent of a target secreted from colonies in colony lift assay. The hydrophilic membrane

(Durapore R membrane filter) was inserted between the hydrophobic membrane and colonies to prevent direct adsorption of cells and debris on the surface of the hydrophobic membrane.

7.2.2 Preparation of antibody-coupled liposomes encapsulating HRP

Anti-rabbit IgG antibody-coupled liposomes encapsulating HRP (immunoliposomes) were prepared according to the method previously reported. (Chapter 5, Kumada et al., 2002 b) Briefly, a lipid thin film composed of DPPC (20 μmol), Chol (20 μmol), DCP (2 μmol) and MPB-DPPE (1 μmol) was peeled off by shaking with 6 ml of 5 mg/ml HRP in PBS at around 50°C to form multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were prepared from MLVs by use of an extruder (Thermobarrel Extruder, Lipex Biomembrane Inc.) and a polycarbonate membrane (Whattman) with 400 nm pore size (Mayer et al, 1986). For preparation of freeze-thaw LUVs (FT-LUVs), LUVs, prepared by the above method, were freezed in liquid nitrogen and thawed at 50 °C for 10 times, and were uniformed by the extrusion method with the membrane of 400 nm pore size. To obtain small unilamellar vesicles (SUVs), a suspension of MLVs was sonicated with a probe-type sonicator (UD 201, TOMY) for 20~30 min at 40 W with cooling. The liposomes prepared were separated from untrapped HRP by gel chromatography with a Bio-Gel A-15m (Bio-Rad Laboratories) column equilibrated with PBS. The anti-rabbit IgG antibody (goat) was mixed with the liposome suspension to give the final concentrations of 200-300 μg-antibody/ml and ca. 2 μmol-DPPC/ml, and 2-iminothiolane-HCl, which conjugates antibody molecules to activated liposomes through sulfhydryl groups, was added in 5-fold excess of antibody. The mixture was gently agitated overnight at room temperature. Uncoupled antibody was removed by gel chromatography with the Bio-Gel A-15m column equilibrated with PBS. Antibody-coupled liposomes obtained were stored at 4 °C. The concentrations of DPPC and coupled antibody were measured by using Phospholipids C-Test Wako (Wako Pure Chemical Ind.

Ltd.) and DC-protein assay (Bio-Rad Laboratories), respectively.

7.2.3 Model antibody and *Escherichia coli* strain

Recombinant *Escherichia coli* BL21 pLysS (Novagen), which contained pBBA-2187scFv to express anti-bisphenol A single chain antibody, was kindly provided by Dr. H. Ookawa. As shown in **Figure 7-1**, the scFv was expressed as a fusion protein, which had pel B reader peptide and His 6-tag at the N- and C-termini of scFv, respectively, and was transported to periplasmic space or secreted from cells.

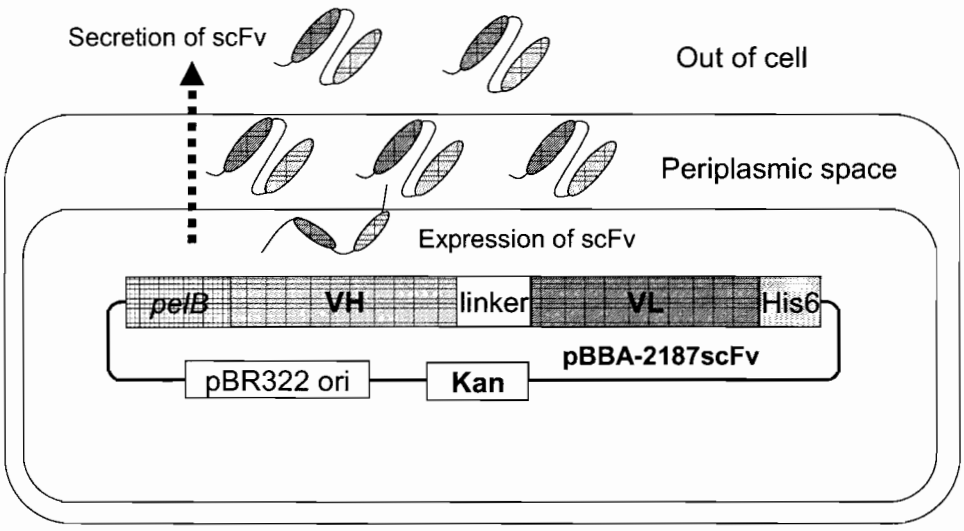


Figure 7-1 Scheme of scFv expression in *E. coli* and secretion

7.2.4 Microbial cultivation on selective agar plate

The precultured recombinant cells were appropriately diluted with sterile water to form 100-500 colonies per plate and inoculated on a 2 x YT selective agar plate containing 30 mg/L kanamycin, 34 mg/L chloramphenichol and 0.1 mM IPTG. The inoculated plate was incubated to form colonies with diameter of 1.0 – 2.0 mm at 37°C. (about 10-14 h)

7.2.5 Blotting of secreted scFv on antigen-coated hydrophobic membrane and detection by immunoblotting assay

7.2. 5. 1 Pretreatment of hydrophobic membrane with antigen

After a hydrophobic membrane was soaked with methanol, it was washed with PBS for 15 min with gentle shaking. Then, it was incubated in 0.5 μg/ml of bisphenol A-rabbit serum albumin conjugate in PBS for 1 h to immobilize the antigen (bisphenol A) in the membrane. After washing with PBS for 10 min, it was incubated with Blocking solution for 1 h to reduce non-specific adsorption. After washing with PBS, residual water on the membrane was drained off. It was used for blotting of active scFv secreted from cells forming colonies.

7. 2. 5. 2 Blotting strategies of secreted scFv on membrane

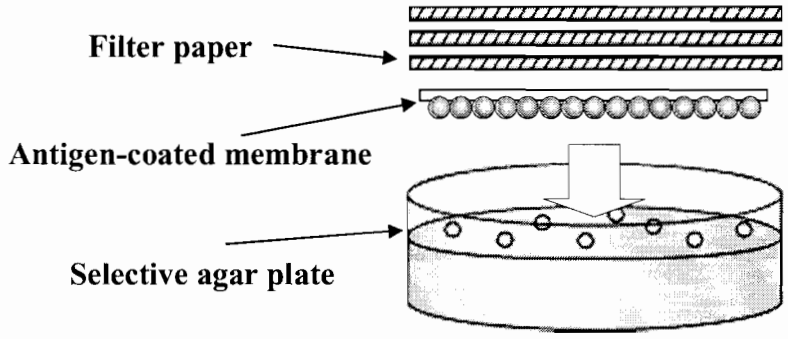
(a) Direct blotting of scFv on antigen-coated membrane

As shown in **Figure 7-2 (a)**, the antigen-coated hydrophobic membrane was carefully put on the plate, on which several hundred colonies were grown, to reduce residual air bubbles between agar plate and the antigen-coated membrane. Three pieces of filter paper were put on it to adsorb medium containing secreted scFv and were incubated for 30 min at room temperature. Then, it was washed for 30 min in 1 % casein PBS, which was exchanged to fresh one at 10 minute-interval.

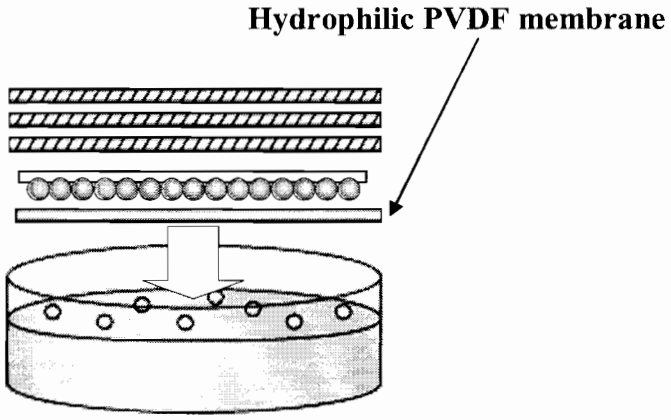
(b) Indirect blotting of scFv on antigen-coated membrane

Figure 7-2 (b) schematically shows an indirect blotting method, in which the hydrophilic

membrane was inserted between a selective agar plate and the antigen-coated hydrophobic membrane to prevent direct contact of cells with the antigen-coated membrane.



(a) Direct blotting method



(b) Indirect blotting method

Figure 7-2 Direct and Indirect blotting method of functional scFv expressed on selective agar plate

(a) Direct blotting method and (b) Indirect blotting method

7. 2. 5. 3 Immunoblotting assay utilizing immunoliposome or HRP-conjugated antibody

The blotted membrane was incubated in an anti-His 6 antibody solution (0.5 μg-antibody/ml in PBS containing 1% casein) for 1 h, and then was washed as stated above. In the liposome immunoblotting assay, the membrane was incubated in an immunoliposome suspension (1μg-antibody/ml containing 1 % casein) for 60 min. In the conventional immunoblotting assay, the membrane was incubated in a 2000-fold diluted solution of HRP-conjugated anti-rabbit IgG antibody (goat) containing 0.05 % Tween 20 for 1 h. After washing with 1 % casein-PBS, color was developed for 30 min with a substrate solution containing 0.5 mg/ml 4-chloro-1-naphthol, 0.015 % H_2O_2 and 17 % methanol in PBS.

The developed membranes were scanned by an image scanner (GT9700F, EPSON), and converted to digital images based on the Lab-mode by use of a computer. To determine the signal density, the lightness per unit area of colored circles was averaged over 5-10 colored spots. The lightness of back grounds was also averaged similarly.

7.3 Results and Discussion

7.3.1 Adsorption characteristics of scFv on antigen-coated hydrophobic membrane

7. 3. 1. 1 Direct blotting method with antigen-coated membrane

To detect active scFv, the antigen-coated membrane was used to blot scFv secreted from colonies on the plate by the direct transfer method stated above. **Figures 7-3 (a) and (b)** compare the results of colony lift immunoassay by use of HRP-conjugated antibody (a) and immunoliposome (immuno-LUVs) (b). In the liposome blotting assay, edges of blotted scFv secreted from colonies showed high signal intensity, whereas in the conventional blotting assay signals obtained were weak but almost uniform. It was suggested that immunoliposomes could not recognize the primary antibody inside pores of the membrane, because cells adsorbed on the surface of the membrane plugged openings of pores and physically prevented interaction between the antigen and liposomes.

7. 3. 1. 2 Indirect blotting method with antigen-coated membrane

To prevent adsorption of cells directly on the membrane, the indirect blotting method was applied to the liposome immunoblotting assay, and compared with the conventional assay. As shown in **Figure 7-4 (b)**, signals of uniform intensity were obtained from circles blotted in the liposome blotting assay, and their intensities were higher than those in the conventional assay (**Figure 7-4 (a)**). In the indirect blotting method, the immunoliposomes could react with the primary antibody inside pores of the membrane without physical hindrance, although the amount of scFv adsorbed on the hydrophobic membrane might be slightly decreased by insertion of the hydrophilic membrane.

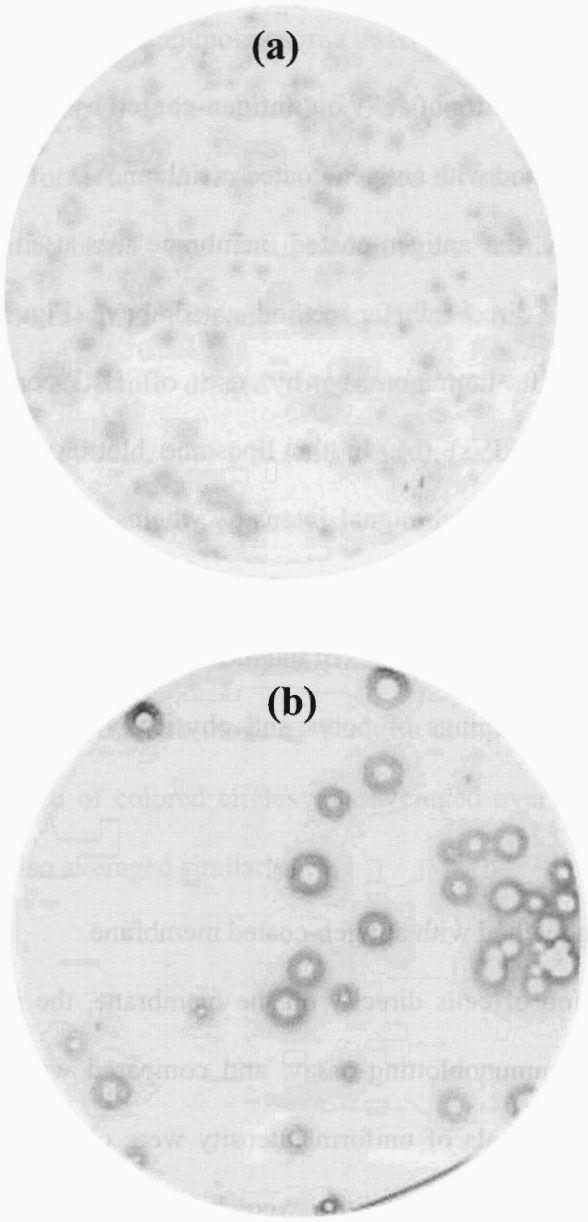


Figure 7-3 Direct colony lift immunoassay by using HRP-labeled antibody and immunoliposome

(a) HRP-labeled antibody and (b) Immunoliposome

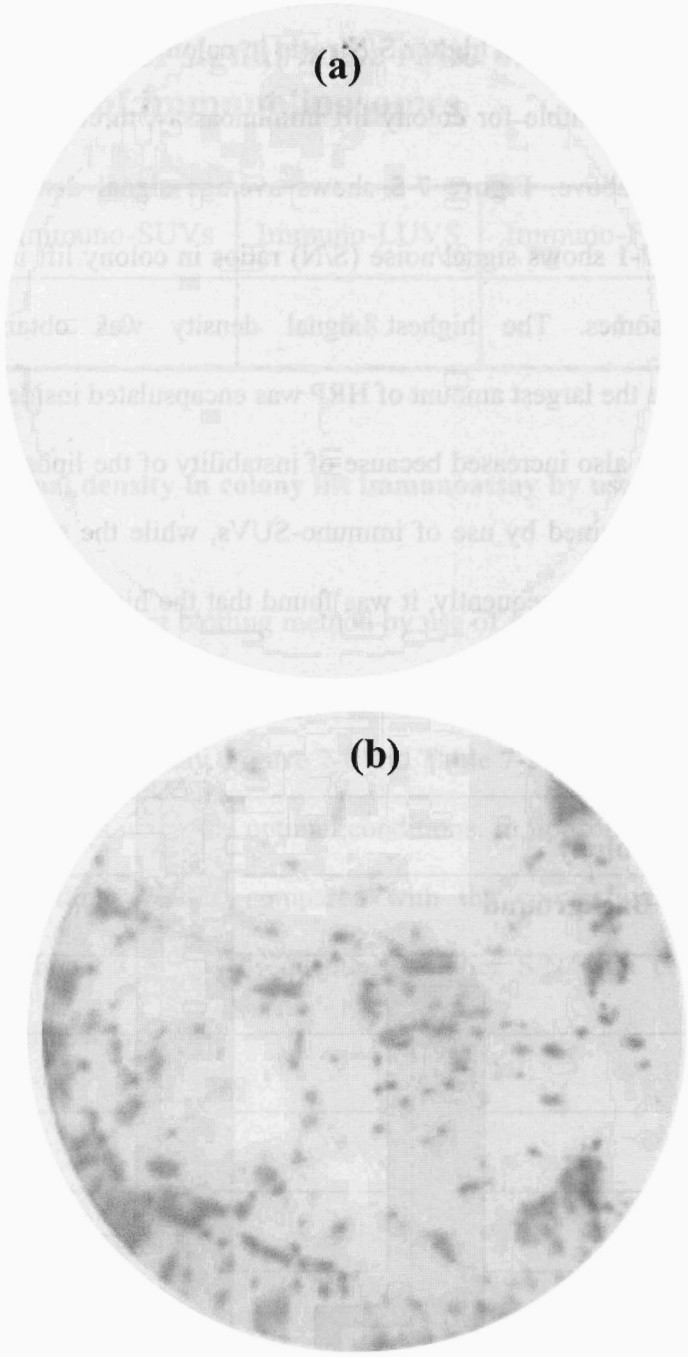


Figure 7-4 Indirect colony lift immunoassay developed by HRP-conjugated antibody and Immunoliposome

(a) HRP-conjugated antibody and (b) immunoliposome

7.3.2 Suitable liposomes to obtain higher S/N ratio in colony lift immunoassay

To obtain liposomes suiatble for colony lift immunoassay, three kinds of immunoliposomes were prepared as stated above. **Figure 7-5** shows average signal densities from colonies and background, and **Table 7-1** shows signal/noise (S/N) ratios in colony lift immunoassay with three types of immunoliposomes. The highest signal density was obtained by use of the immuno-FT-LUVs, since the largest amount of HRP was encapsulated inside the vesicles. However, the background level was also increased because of instability of the liposomes. The lowest signal of the background was obtained by use of immuno-SUVs, while the signal density from colored circles was also the lowest. Consequently, it was found that the highest signal/noise ratio could be obtained by use of immuno-LUVs.

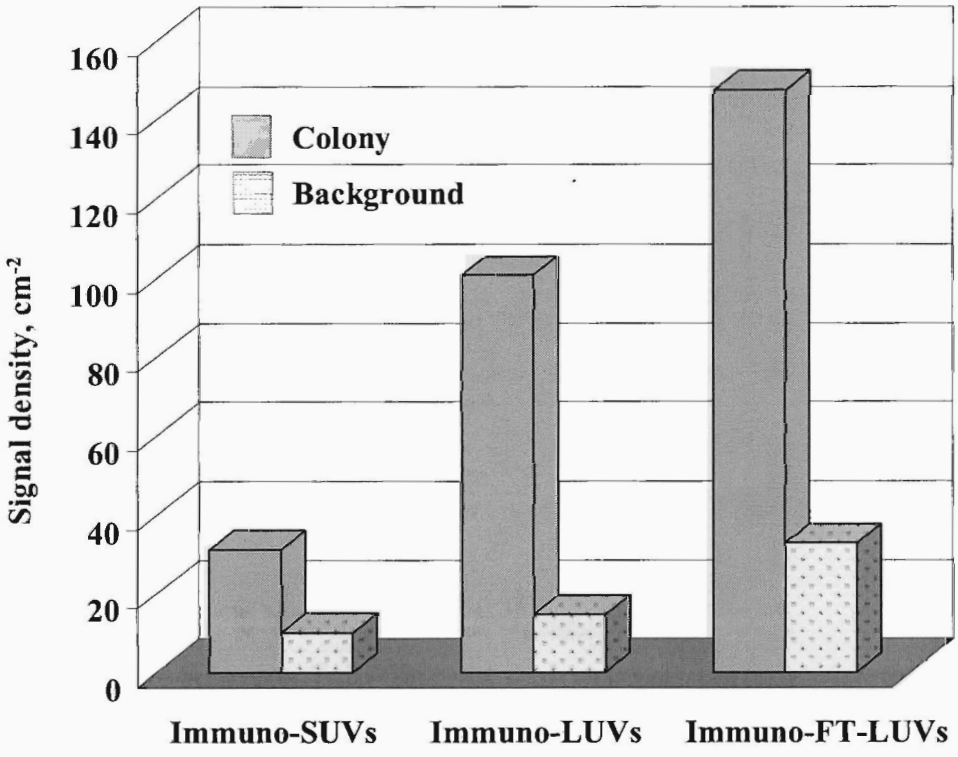


Figure 7-5 Comparison of signal density obtained by using three kinds of immunoliposomes

Table 7-1 Comparison of signal/noise ratio obtained by use of three kinds of immunoliposomes

	Immuno-SUVs	Immuno-LUVS	Immuno-FT-LUVs
Signal/Noise ratio	3.0	6.8	4.5

7.3.3 Comparison of signal density in colony lift immunoassay by use of immuno-LUVs and HRP-conjugated antibody

As shown above, the indirect blotting method by use of immuno-LUVs gave the high S/N ratio in liposome immunoblotting assay, while the direct blotting method obtained the high signal density in the conventional blotting assay. **Figure 7-6** and **Table 7-2** compare the signal density and S/N ratio of these blotting assays under the optimal conditions. In liposome immunoblotting assay, the signal density was 3 times higher, compared with the conventional assay, while their background levels were almost same. Consequently, a higher S/N ratio could be attained for detection of colonies secreting active scFv.

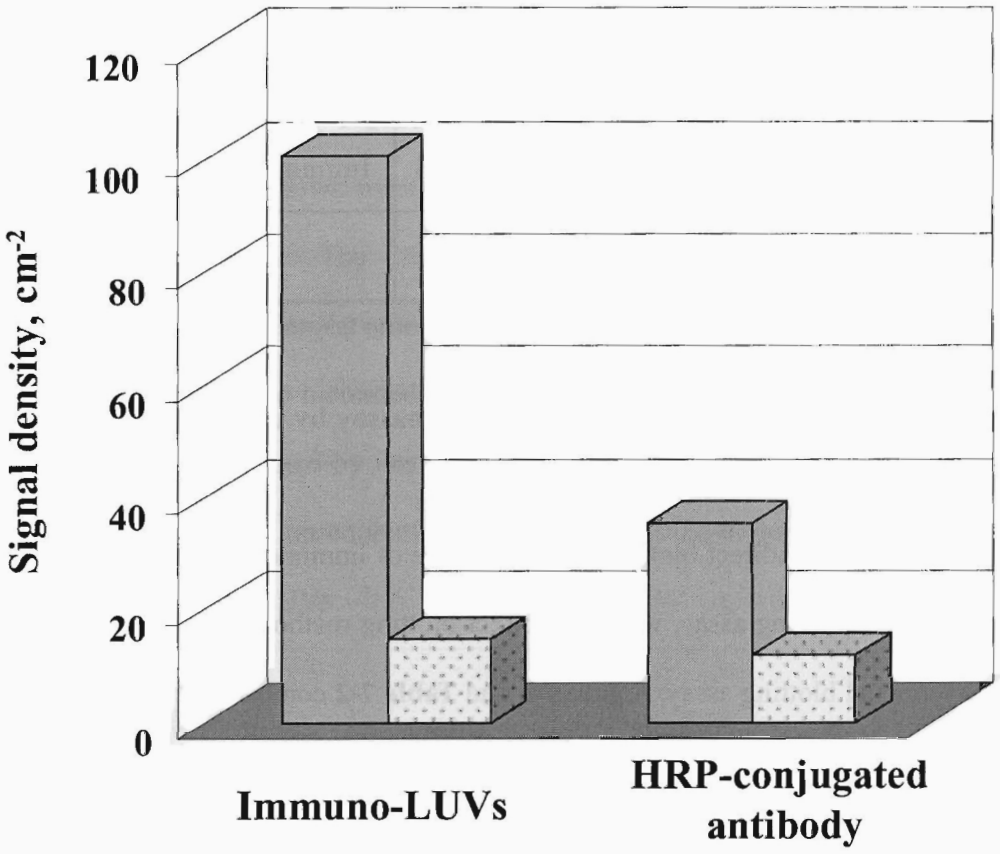


Figure 7-6 Comparison of signal density obtained by immuno-liposome and HRP-labeled antibody under optimal condition

Table 7-2 Signal/noise ratio by use of Immuno-LUVs and HRP-antibody

	Immuno-LUVs	HRP-antibody
Signal/Noise ratio	6.8	2.9

7.4 Conclusion

In this chapter, we applied the liposome immunoblotting assay for screening of colonies secreting active scFv by colony lift assay. Uniform and high density signals were observed from blotted membranes with the indirect blotting and detection with immuno-LUVs. The immuno-LUVs gave the highest S/N ratio, which was 2 times higher than that in the conventional blotting assay in a colony lift assay. Thus, liposome immunoblotting assay is useful method to screen colonies expressing target protein with higher sensitivity.

Chapter 8
Liposome immunoblotting assay utilizing enhanced chemiluminescent reaction improved by surfactant

8.1 Introduction

In recent years, rapid, automatic, and sensitive detection methods for biochemical substances and their interactions have been required because of increasing number of biological samples in the clinical, environmental, and bioindustrial fields. Enzyme-linked immunosorbent assay (ELISA) using enzyme-conjugated antibody is the most popular detection method including separation of free and antigen-bound antibodies (B/F separation) and can be utilized for a wide variety of analytes. On the other hand, membrane blotting assays based on the immunodetection such as Western blotting and colony lift immunoassay, have also been developed and often used to investigate localization of analytes. These methods can be improved in operations for rapid and automatic detection method by application of the micro-array technology. In micro-array systems, their sensitivity might decrease because small amount of analyte can be applied. Therefore, more sensitive detection methods will be essential in these systems. Immunoliposomes encapsulating markers can be used for these purposes in place of labeled antibodies, because one liposome can couple many antibody (or antigen) molecules on its surface and can contain a larger amount of a marker than a labeled antibody. (Rongen et al., 1997; Singh and Carbonell, 1996) Thus, multipoint interaction between antigen and antibody can increase an overall binding affinity (avidity) in comparison with the affinity of monovalent interaction. (Chapter 2, Kumada et al., 2001) Moreover, higher signal intensity was obtained from a large number of marker molecule encapsulated inside liposomes. (Chapter 3, Kumada et al., 2002 a) We developed a liposome immunoblotting assay, in which substrates are permeable through the phospholipid membrane and form precipitates inside liposomes. (Chapter 5, Kumada et al., 2002 b) In enzyme immunoassay methods, higher signal

intensity and S/N separation are usually obtained by use of chemiluminescence reactions in comparison with color developing reactions. Among them the enhanced chemiluminescent (ECL), in which chemiluminescence emitted by oxidized luminol catalyzed by horseradish peroxidarse is considerably enhanced and stabilized in the presence of a phenol derivative and a surfactant, is the most popular one. (Thrope et al., 1986, Larry et al., 1996) In this study, I developed a more sensitive liposome immunoblotting assay utilizing the ECL reaction. The sensitivity of this liposome immunoblotting assay was compared with that of the conventional immunoblotting assay using a labeled antibody.

8.2 Materials and Methods

8.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque, Kyoto, Japan), dipalmitoylphosphatidylethanolamine (DPPE) (Sigma Aldrich, St Louis, MO, USA), cholesterol (Chol) (Wako Pure Chemical Ind. Ltd., Osaka, Japan), dicetylphosphate (DCP) (Sigma Aldrich) and *N*-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB) (Pierce, Rockford, IL, USA) were used for liposome preparation. As a marker enzyme encapsulated in liposomes, horseradish peroxidase (HRP, 100 U/mg, grade III, TOYOBO, Tokyo, Japan) was used. Luminol (nacalai tesque), *p*-iodophenol (Wako Pure Chemical Ind. Ltd.) and hydrogen peroxide were used as substrates of HRP for ECL reaction. *N*-succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) was purchased from Pierce, and dithiothreitol (DTT) from nacalai tesque. Anti-rabbit IgG antibody (goat) and anti-rabbit IgG antibody (goat)-HRP were purchased from Cappel Products Co. (Bedford, MA, USA). Human IgM as a model analyte was obtained from Chemicon Int. Inc. (Bedford, MA, USA). Anti-human IgM antibody (rabbit) as a primary antibody was prepared by the previously reported method . (Katoh and Terashima, 1994) A hydrophobic PVDF membrane with pore sizes of 450 nm

(Immobilon P, Millipore, Bedford, MA, USA) was used for blotting. All other chemicals used were of reagent grade.

Buffers used in this work were prepared as follows:

PBS (NaCl 9.0 g/L, KCl 0.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 2.9 g/L, KH_2PO_4 0.2 g/L, pH=7.4), TBS (NaCl 9.0 g/L, KCl 0.2 g/L, tris-hydroxymethyl aminomethane 2.4 g/L, adjusted to pH 8.5 by conc HCl) and MES (2-(N-morpholino) ethanesulfonic acid 4.27 g/L, NaCl 8.77 g/L, pH 5.5). Blocking solution (pH 7.2, nacalai tesque) and 1.0 wt % casein-PBS (or casein-PBS containing Tween 20) were used as blocking and washing solutions, respectively.

8.2.2 Preparation of antibody-coupled liposomes

DPPE was activated to *N*-[4-(p-maleimidophenyl) butyryl] dipalmitoylphosphatidylethanolamine (MPB-DPPE) by SMBP as previously reported (Kishimura *et al.*, 1989). A lipid mixture containing DPPC (10 μmol), Chol (10 μmol), DCP (1 μmol) and MPB-DPPE (0.5 μmol) in chloroform was dried to a thin lipid film on the inside wall of a pear-shaped flask by using a rotary evaporator above 42 °C under reduced pressure. The lipid film was dried overnight under reduced pressure in a desiccator. Then, 3 cm^3 of 5 mg/cm^3 HRP in PBS was added to be encapsulated as a marker, and the flask was shaken by a vortex mixer at around 50°C for about 10 min to peel off the lipid film and form multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were prepared by use of an extruder (Thermobarrel Extruder, Northern Lipids Inc. Vancouver, BC, Canada) from MLVs. (Mayer et al., 1986) The liposomes thus prepared were separated from untrapped HRP by gel chromatography with a Bio-Gel A-15m (Bio-Rad Laboratories, Hercules, CA, USA) column equilibrated with PBS.

A heterobifunctional cross-linking reagent, SPDP was employed to combine secondary antibody covalently with the liposomes containing activated lipids. Anti-rabbit IgG antibody was

modified by SPDP as previously reported. (Kishimura et al., 1989) A suspension of activated liposomes was mixed with a modified antibody solution and gently agitated for 6-12 hours at room temperature. Uncoupled antibody was removed by gel chromatography with the Bio-Gel A-15m column equilibrated with PBS. Antibody-coupled liposomes obtained were stored at 4°C.

HRP released from liposomes was less than 5 % after 4 weeks, and HRP activity was kept about 90 % of the initial value. The average diameter of antibody-coupled liposomes was measured by the differential light scattering analysis (DLS 7000, Photal Otsuka Electronics, Osaka, Japan). The diameter of LUVs prepared by use of a polycarbonate filter with pore size of 400 nm was 350 nm. The concentrations of DPPC and antibody in liposome suspensions were measured after addition of 0.01 – 0.1 cm^3 of 10 % deoxycholic acid sodium salt to 0.1 cm^3 of each sample. Heating at 90°C for 10 min inactivated HRP. The concentration of DPPC in liposome suspensions was measured by the cholineoxidase method using Phospholipids C-Test Wako (Wako Pure Chemical Ind. Ltd.). The concentration of antibody coupled on liposome membrane was measured by DC-protein assay (Bio-Rad Laboratories).

8.2.3 ECL reaction catalyzed by HRP and measurement of chemiluminescence intensity

A substrate solution containing 0.056 mM luminol, 0.34 mM *p*-iodophenol and 1.9 mM hydrogen peroxide in TBS (pH 8.5) (or TBS containing surfactant or glycerol) was used for ECL reaction. (Thorpe and Kricka, 1986) To measure the enzyme activity in HRP solutions or immunoliposome suspensions, 0.05 cm^3 of a solution containing substrates in double quantity was mixed with 0.05 cm^3 of the sample in a microtiter well. Then, the microtiter plate was set in a dark box equipped with a cooled CCD camera (FAS-1000, TOYOBO), which accumulated chemiluminescent light for 30 min. After 30 min, the total amount of chemiluminescent light emitted from each well was evaluated as the relative brightness in a range of 0-800 or 0-60000. The

maximum brightness in this range was assigned as white color, and the average brightness in a center circle (1.2 mm radius) of a taken picture of each well was determined by use of Adobe Photoshop.

8.2.4 Liposome and conventional immunoblotting assays

A PVDF membrane was soaked in pure methanol and then incubated in PBS for 10 min. Sample solutions (0.02 cm^3) containing different concentrations of human IgM (0 - 1 $\mu g/cm^3$) were blotted on the PVDF membrane. After washing with PBS for 10 min, it was blocked with Blocking solution to avoid non-specific adsorption. Again, it was washed with PBS for 10 min and was incubated in an anti-human IgM antibody (rabbit) solution (3 μg-specific antibody/cm^3) containing 1 % casein-PBS for 1 h at room temperature.

In the liposome immunoblotting assay, the membrane was washed with 1 % casein-PBS and was incubated in an anti-rabbit IgG antibody (goat)-coupled liposome suspension (1 μg-specific antibody/cm^3) containing 1 % casein-PBS for 1h at room temperature. After washing with 1 % casein-PBS (or 1% casein-PBS containing Tween 20), it was incubated in TBS (pH 8.5) (or Tween20-TBS) for 10 min to adjust the pH value inside immunoliposomes, and transferred into the substrate solution. After incubation for 30 sec, it was immediately put on a filter paper soaked in the substrate solution in the dark box, and chemiluminescent light was accumulated by the CCD camera. The relative brightness was determined from analyte-blotted area (about 2.4 mm radius) as stated above.

In the conventional immunoblotting assay, the membrane was incubated with anti-rabbit IgG antibody (goat)-HRP containing 1% casein-PBS (or 1% casein-PBS containing Tween 20) for 1 h at room temperature. After washing, signal was developed by the same procedures in the liposome immunoblotting assay. The signal development systems of these immunoblotting assays are

schematically summarized in **Figure 8-1**. In liposome immunoblotting assay, the substrates (luminol, *p*-iodophenol and hydrogen peroxide) can permeate through the phospholipid membrane and emit chemiluminescent light catalyzed by HRP.

8.3 Results and Discussion

8.3.1 Effect of surfactants on ECL reaction

Figure 8-2 shows the total amount of chemiluminescent light emitted from substrate solutions containing 0.1% surfactant or glycerol. Tween 20, Tween 80, TritonX-100, sodium dodecyl sulfate (SDS) and sodium deoxycholate (DOC) were used as surfactants. The amount of chemiluminescent light emitted from the substrate solutions containing Tween 20 and TritonX-100 was about 3.7 times that from the solution without surfactants although other surfactants were also effective for enhancement of the ECL reaction. Without surfactants, it was reported that radicals produced by reaction of enhancer with oxygen radicals mainly inactivated HRP molecules. (Kapeluich et al., 1997) In my experiments, the intensity of chemiluminescence rapidly decreased to less than 10 % of the initial value after 10 min without surfactants, while it decreased only to 90 % with 1% Tween 20. These surfactants might interact with the surface of HRP molecules and protect them from attack of radicals.

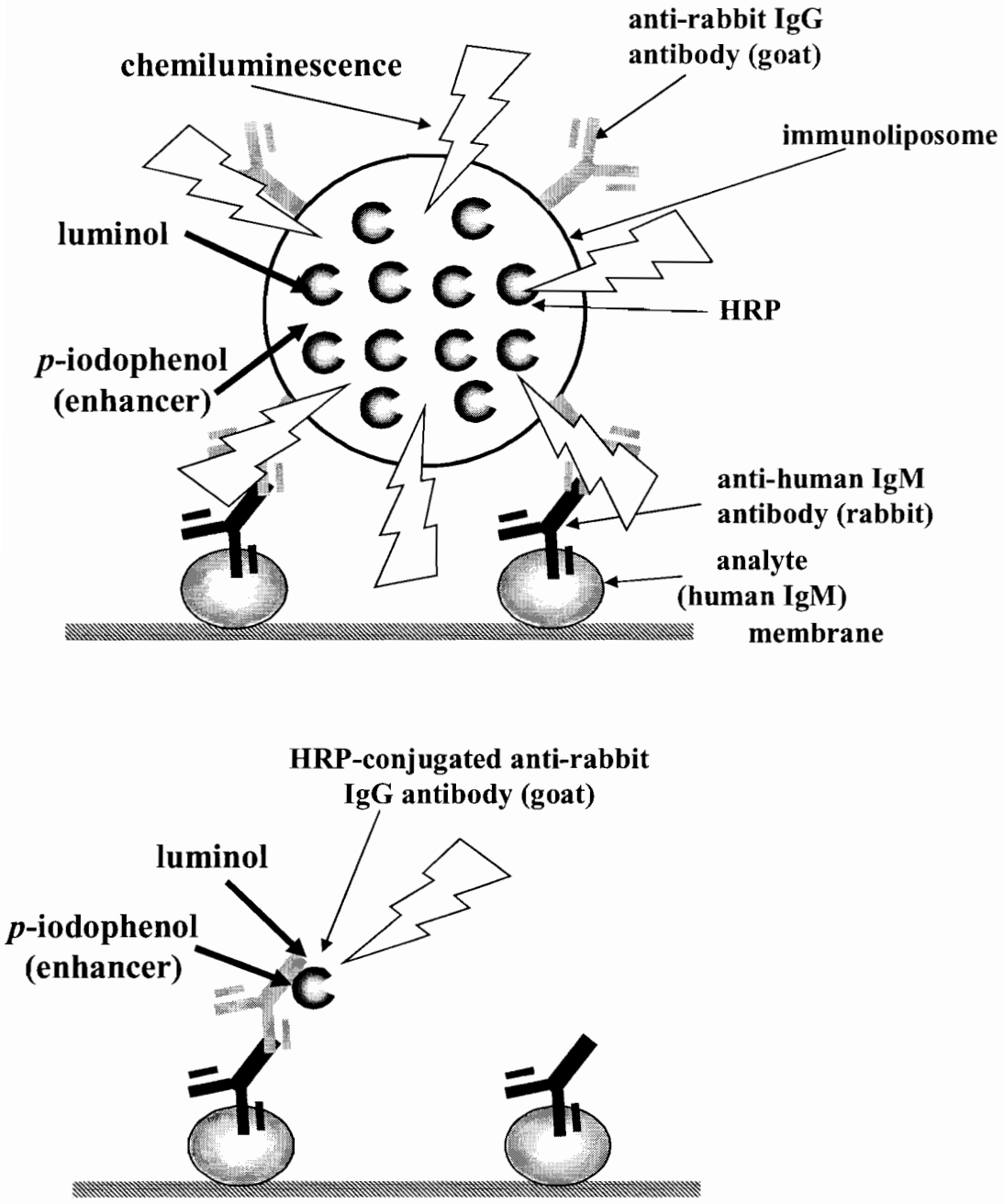


Figure 8-1 Scheme of signal development by use of ECL reaction in liposome immunoblotting assay and conventional immunoblotting assay

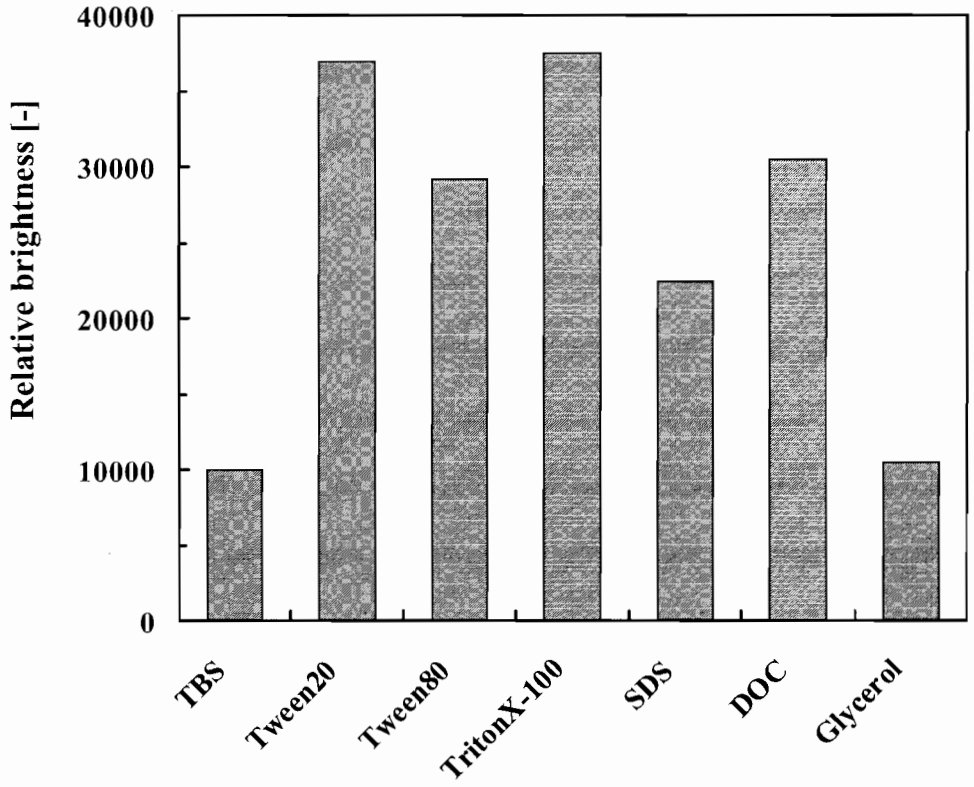


Figure 8-2 Effect of Tween 20 and other surfactants on ECL reaction

HRP concn: 6.25 ng/ml
Surfactants and glycerol concn in TBS (pH 8.5): 0.1 %,
SDS, DOC: wt %, Tween 20,Tween 80, TritonX 100, glycerol: vol %
Reaction time: 30 min

8.3.2 ECL reaction using immunoliposomes and application to liposome immunoblotting assay

The effect of the Tween 20 concentration on the chemiluminescence amount was investigated by use of the HRP solutions and immunoliposome suspensions. **Figsures 8-3** (a) and (b) show the

relative brightness obtained from HRP solutions and liposome suspensions, respectively. In the range from 0.001% to 1% of Tween 20, total amounts of chemiluminescent light increased under both conditions. In liposome suspensions reached 5-8 times those without Tween 20. At 10 % Tween 20, HRP molecules, however, might be denatured, and the chemiluminescent light decreased.

The relative brightness of the liposome suspensions from 0 to 1 % of Tween 20 was lower than that of the HRP solution. This may be caused by the resistance of the liposome membrane for transfer of substrates. Further, the protecting effect of Tween 20 from the attack by radicals against HRP was prominent in the liposome solutions because radicals might exist at a high concentration inside liposomes.

The effect of Tween 20 concentration in liposome immunoblotting assay was studied by use of the substrate solutions containing different concentrations of Tween 20. (0.0001-1 %) **Figure 8-4** shows a picture taken by the CCD camera (a) and the total amount of chemiluminescent light from the blotted samples (b). Under these conditions, clear chemiluminescence signals could be detected from blotted circles on the membrane. The relative brightness of chemiluminescence increased with the concentration of Tween 20 in the substrate solutions. Since the supplying rate of the substrates into liposomes was lower in the case of blotted liposomes than that in the liposome suspensions, effective protection of HRP at high concentrations of Tween 20 increased the accumulated chemiluminescent light. Thus, addition of Tween 20 could increase the signal intensity in the liposome immunoblotting assay utilizing ECL.

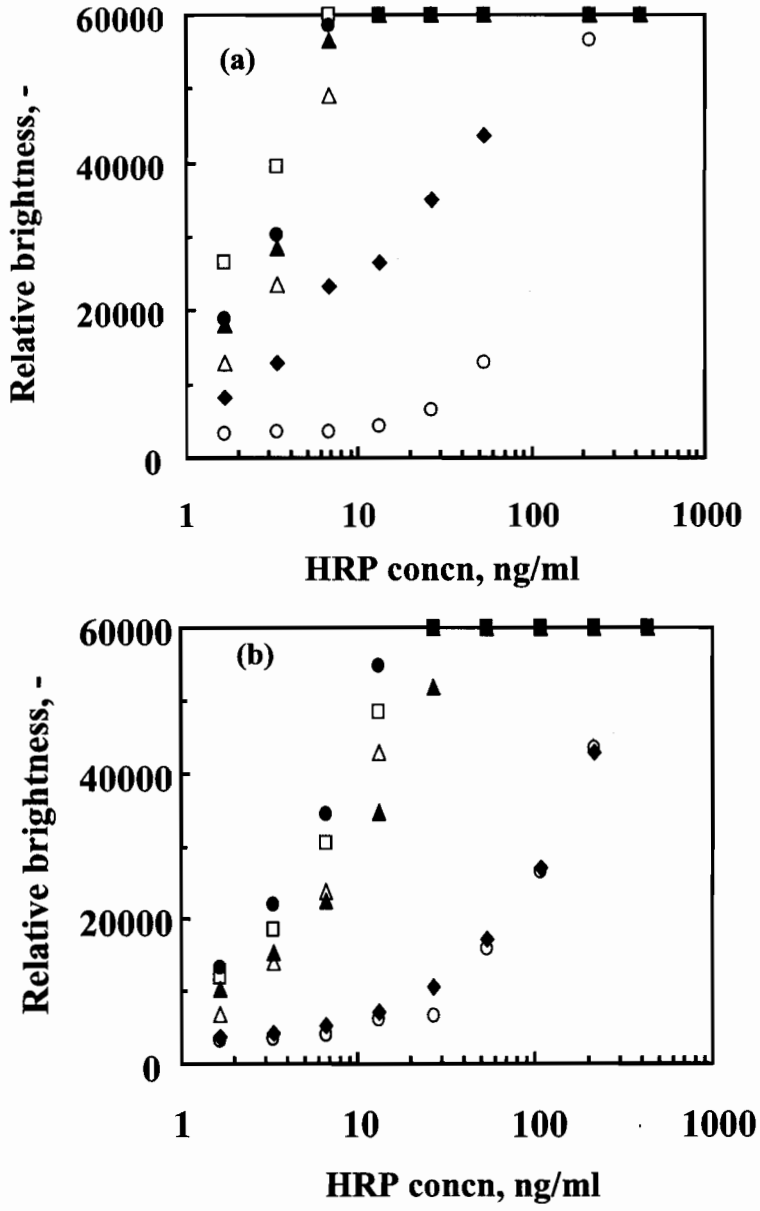


Figure 8-3 Effect of Tween 20 concentration on chemiluminescence at different concentrations of HRP

(a) HRP solution, and (b) liposome suspension

Tween 20 concn:

○ 10, △ 1, □ 0.1, ● 0.01, ▲ 0.001, ◆ 0 vol %

Reaction time: 30 min

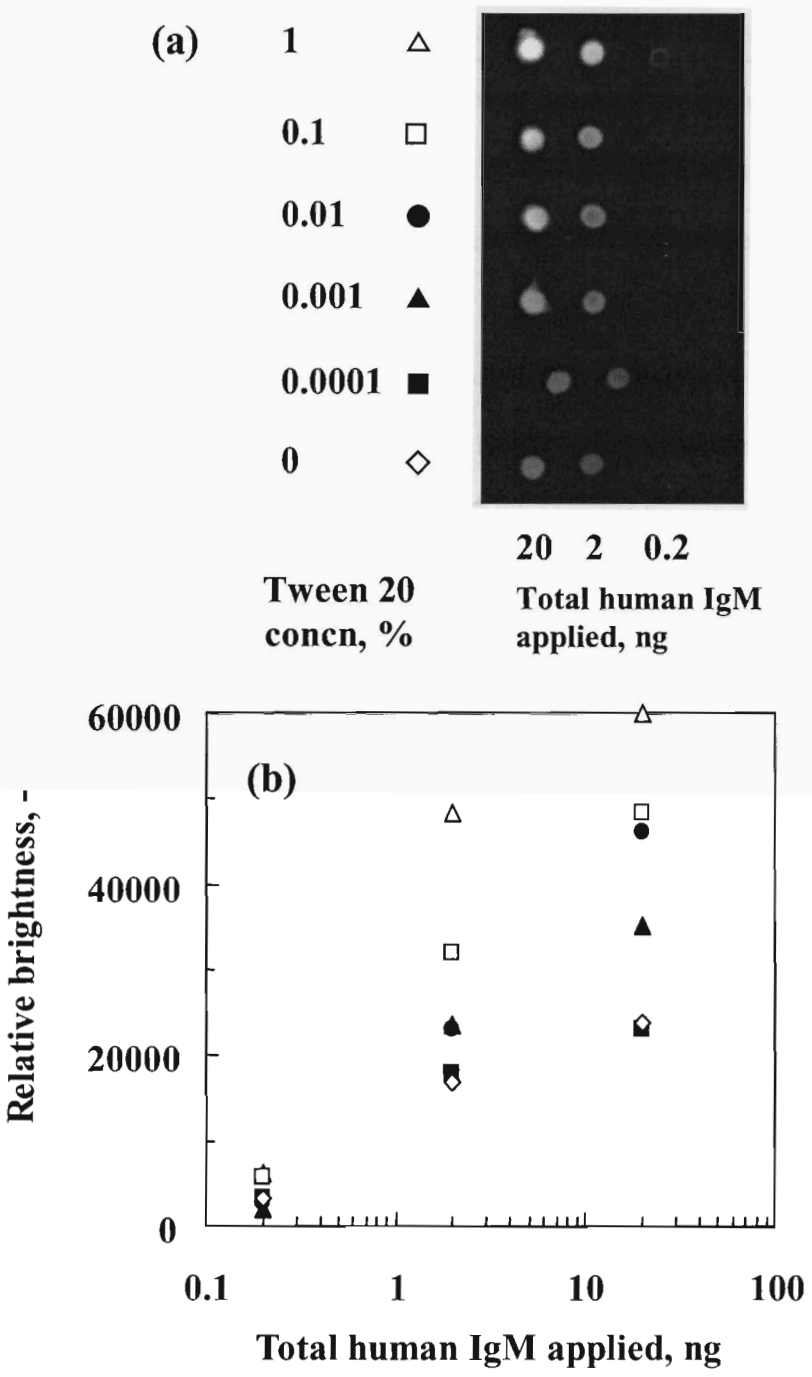


Figure 8-4 Effect of Tween 20 concentration on chemiluminescent intensity in liposome immunoblotting assay

Washing buffer: 1% casein-PBS
Buffer exchange: TBS containing Tween 20, 0-1 %
ECL reaction: Substrate solution containing Tween 20, 0-1 %
Range of relative brightness on CCD camera: 0-60000

8.3.3 Reduction of non-specific adsorption in liposome immunoblotting assay

A high signal/noise ratio is essential for highly sensitive and quantitative analysis because the detection limit is determined by the intensity of non-specific signal, i.e. background noise. In our assay washing with the 1% casein-PBS solution could not reduce non-specific adsorption of immunoliposomes effectively, and this limited the sensitivity. This non-specific adsorption might be caused by multipoint interaction of antibodies coupled on liposomes with non-specific proteins on the PVDF membrane, and a large number of HRP molecules encapsulated inside immunoliposomes. **Figure 8-5** shows the chemiluminescent images from different concentrations of IgM samples (a) and the relative brightness observed from background (b) in the liposome immunoblotting assay with washing solution containing Tween 20. By use of solutions containing 1% Tween20, the lowest chemiluminescence was observed from background. Consequently, in the liposome immunoblotting assay utilizing ECL reaction, washing, TBS and signal development solutions containing 1% of Tween 20 were suitable for reduction of non-specific adsorption.

8.3.4 Comparison of liposome immunoblotting assay with conventional immunoblotting assay

Under the optimal conditions, the liposome immunoblotting assay was compared with the conventional blotting assay. As shown in **Figure 8-6**, the sensitivity in the liposome immunoblotting assay was about 20 times that in the conventional assay, and 2 pg of human IgM as the model analyte could be detected. The value of the relative brightness from background was subtracted from those from blotted circles. In both the immunoblotting assays the reproducibility of measured intensity was ± 6 %. In comparison with the previous method utilizing colored precipitates, this method could attain 10 times higher sensitivity.

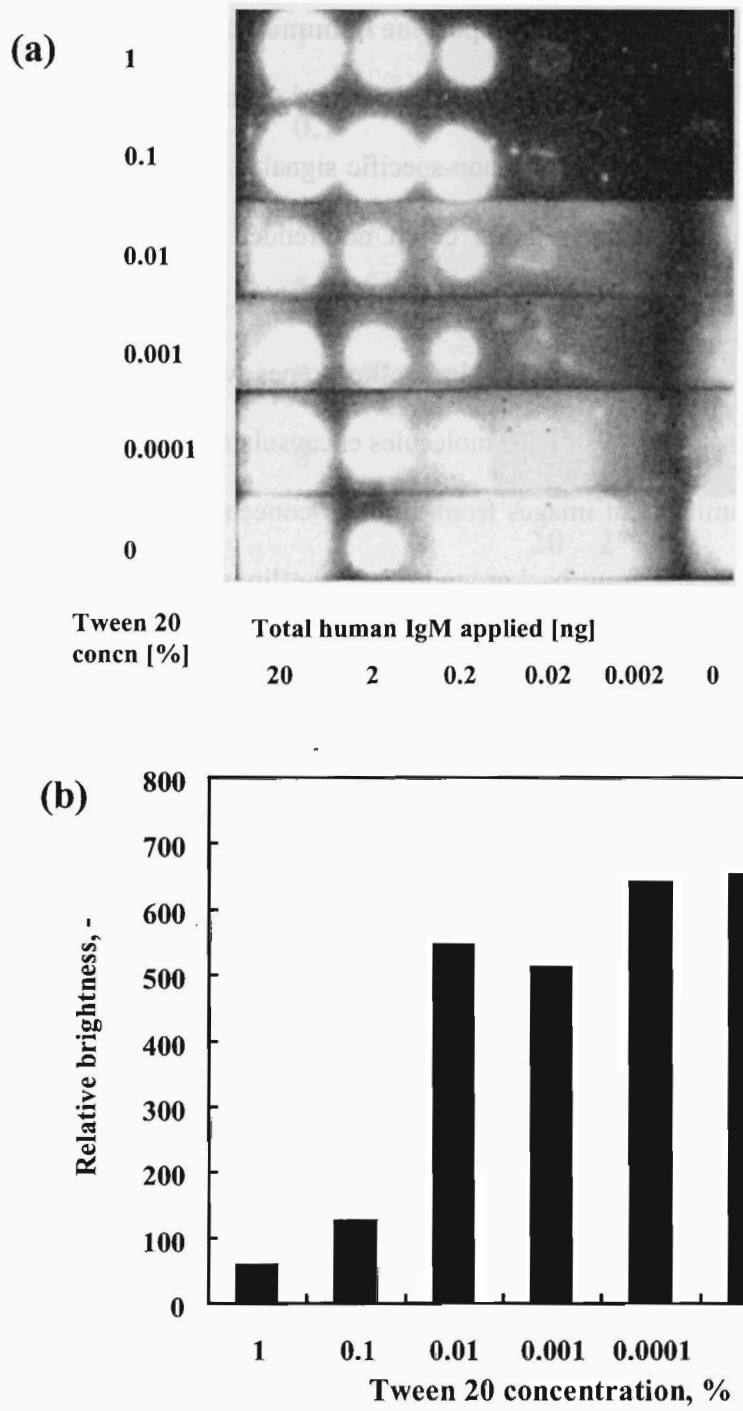


Figure 8-5 Effect of Tween 20 concentration on non-specific adsorption in liposome immunoblotting assay

Buffer exchange: TBS (pH 8.5) containing 0-1 % Tween 20
ECL reaction: Substrate solution containing 1% Tween20
Range of relative brightness on cooled CCD camera: 0-800

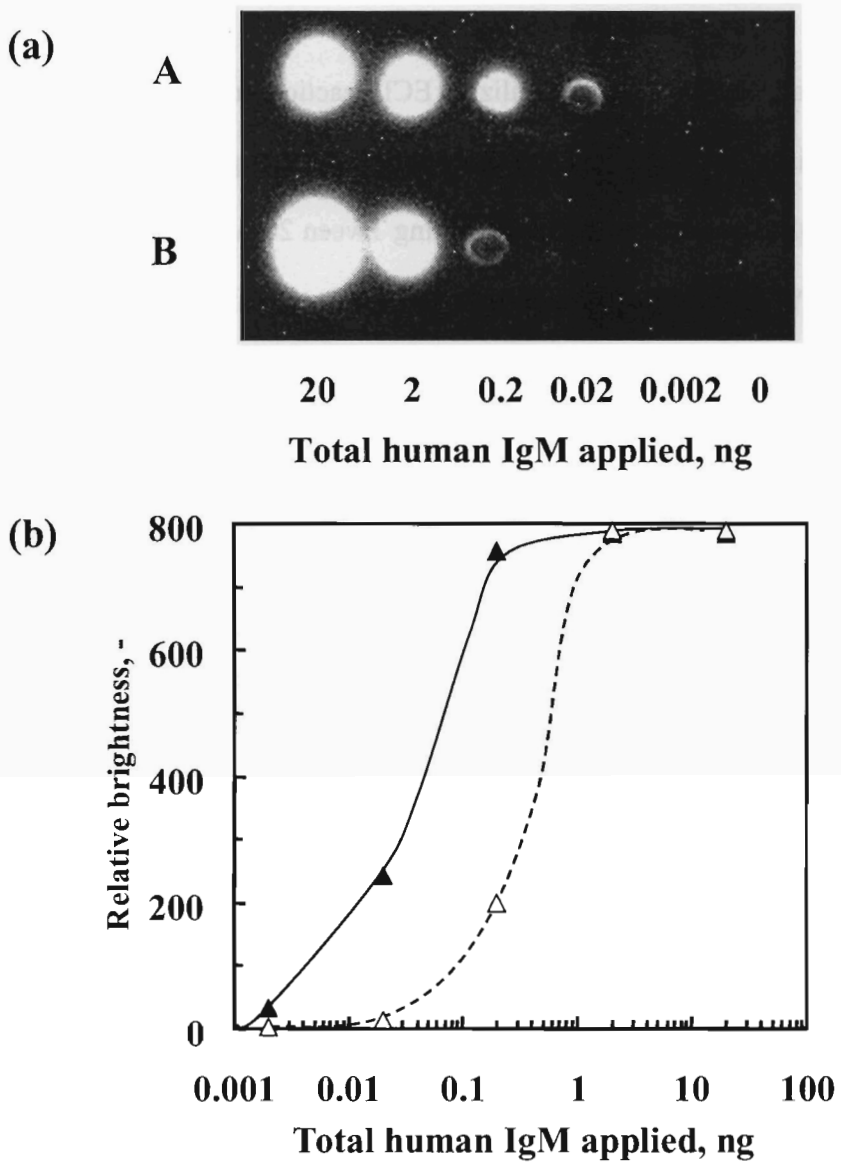


Figure 8-6 Comparison of liposome and conventional immunoblotting assays under optimal conditions

A. ▲ Liposome immunoblotting assay
B. △ Conventional immunoblotting assay

Washing buffer: 1% casein-PBS containing 1% Tween20
Buffer exchange: TBS containing 1% Tween 20
ECL reaction: Standard substrate solution containing 1% Tween 20
Range of relative brightness on CCD camera: 0-800

8.4 Conclusion

I developed the liposome immunoblotting assay utilizing ECL reaction. In a similar manner of free HRP solutions, chemiluminescent light emitted from the immunoliposome suspensions significantly increased by use of the substrates solution containing Tween 20. With the washing and substrate solutions containing 1% Tween 20, immunoliposomes non-specifically adsorbed was efficiently removed, and thus little background noise was observed. Under the optimal conditions, the liposome immunoblotting assay attained about 20 times higher sensitivity in comparison with conventional one. The liposome immunoblotting assay utilizing ECL reaction has the high sensitivity among other immunoblotting assays and can be useful for detection of trace amounts of analytes.

Part II Application of proteoliposome to affinity screening

Chapter 9
Application of protein-coupled liposomes to effective affinity screening from phage library

9.1 Introduction

In recent years, a large number of combinatorial libraries have been produced in genetic engineering fields for several purposes, such as design of new functional proteins, analysis of specific interactions of biomolecules and screening of specific antibodies against target molecules. (Stephen and George 1988, Gianni et al., 1999, Wilson and Finlay, 1997) Among them, combinatorial libraries displayed by filamentous M13 phage containing phagemid vectors are the most common system because of effective infection and release properties of M13 phage and easy rescue of phagemid vectors with helper phage. In this system, however, biopanning procedures, which consist of adsorption of phages in a library to a target molecule, recovery of the adsorbed phages, infection and amplification of the phages in host cells and phagemid rescue, have to be repeated several times to obtain desirable candidates, because an initial number of phages displaying candidate molecules is small, and recovered phages contain many fakes nonspecifically adsorbed after the panning procedures. Therefore, for effective screening of target phages in panning procedures, highly specific characteristics for adsorption of target phages and a low degree of nonspecific adsorption of other phages are required.

In biopanning procedures, plastic microplates, tubes and microbeads are widely used for adsorption of target molecules and phage screening. However, the following factors in these plastic adsorbents may lead to inefficient outputs. 1) The surface area available for adsorption, and thus the adsorption capacity, is limited by a geometrical shape. 2) Denaturation of target molecules on plastic surface may cause incorrect recognition of the molecules by phages. 3) Blocking procedures are essential to reduce nonspecific adsorption of phages. 4) Some phages may be specifically

adsorbed on blocking agents.

Protein-coupled liposomes (proteoliposomes) have been used for drug and gene delivery, liposome immunoassay and artificial models of cells. (Pedroso et al., 2003, Rongen et al., 1997, Eanes, 1989) To solve the above problems in biopaninng procedures, we propose a biopanning method of phage-displayed libraries utilizing proteoliposomes as adsorbents. Proteoliposomes have the following characteristics. 1) The surface area available for adsorption can be controlled by the size and amount of liposomes used. 2) Proteins may be coupled on the surface of phospholipid membrane with minimum denaturation, and membrane-bound proteins are also effectively incorporated into the membrane. Jespersen et al. successfully selected monoclonal antibodies against membrane receptors by biopanning utilizing proteoliposome-immobilized tube. (Jesperson et al., 2000) 3) The hydrophilic nature of the surface of phospholipid membrane can reduce nonspecific adsorption of phages without blocking operations. 4) A phage library should contain very few phages specific to the components in liposome membrane, because they would be adsorbed specifically on *E.coli* membrane before their secretion.

Screening from peptide libraries displayed by phages could be a promising way to obtain affinity ligands and antagonists against target proteins and to clarify sequential epitopes of antibodies, if the intrinsic interaction between the targets and peptides can be measured. In the present study, we investigated adsorption characteristics of nonspecific M13 phages to liposomes of different compositions, and compared the performances of biopanning utilizing liposomes coupled with an anti-peptide antibody and plastic tubes coated with the antibody by screening antigen peptides from an octapeptide-displayed phage library.

9.2 Materials and Methods

9.2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) (nacalai tesque, Kyoto, Japan), dipalmitoylphosphatidylethanolamine (DPPE) (Avanti polar lipid, AL, USA), cholesterol (Chol) (Wako Pure Chemical Ind. Ltd., Osaka, Japan), dicetylphosphate (DCP) (Sigma Aldrich, St Louis, MO, USA), stearylamine (SA) (Sigma Aldrich) and *N*-succinimidyl 4-(*p*-maleimidophenyl)-butyrate (SMPB) (Pierce, Rockford, IL, USA) were used for liposome preparation. 2-Iminothiolane·HCl (Pierce) was used for immobilization of antibody on phospholipid membrane. An octapeptide (FVNQHLCK), which corresponds to a part of the N-terminal region of insulin B chain, was synthesized and used for immunization to rabbits. (Katoh and Miura, 1999) Polyclonal anti-peptide antibody against the antigen peptide was purified from rabbit antisera with an affinity column coupled with the peptide. It could recognize not only the antigen octapeptide but also bovine insulin. Polystyrene tube used for conventional biopanning (Immunotube, Type 44474) was purchased from Nalge Nunc International (Rochester, NY, USA), and for blocking Blocking solution (nacalai tesque) was used. Bovine insulin was conjugated with bovine serum albumin via glutaraldehyde crosslinking (Insulin-BSA) and used for competitive enzyme-linked immunosorbent assay (ELISA). All other chemicals used were of reagent grade.

9.2.2 *Escherichia coli* strain

Escherichia coli XL1-Blue, which has the Tet^R F plasmid and the chromosomal genotype containing *endA1*, *hsdR17*, *supE44*, *thi-1*, *recA1*, *gyrA96* and *relA1*, was purchased from Stratagene and was used as a host for vector constructions and generation of peptide-displayed M13 phage particles.

9.2.3 Vector constructions and Transformation

Figure 9-1 schematically shows construction of an expression vector pR8Δ198MM for preparation of a random octapeptide-displayed phage library and its signal cleavage site. The DNA fragment which contains ribosome binding site (RBS), *pelB* reader peptide including *Nco* I restriction site, and cloning sites (*Not* I and *Xho* I sites) for insertion of truncated gIIIp gene (amino acid position 198-406) was amplified by PCR. The amplified fragment was inserted at the position between *Xba* I and *Bam* HI site of pT7Blue phagemid vector. The pT7Blue derivative, pT7MM was digested with *Not* I and *Xho* I. The truncated gIIIp gene was amplified from M13mp19 vector as a template DNA with primers (5'-CTCTCTGCGGCCGCATTATTTGTTTGTGAATATCAAGGCC-3' and 5'- CTCCTCGAGTTAAGACTCCTTATTACGCAGTATGTT -3'). After cleavage of the fragment and pT7MM with *Not* I and *Xho* I, they were ligated by T4 DNA ligase (pΔ198MM). The random octapeptide genes were prepared by one cycle PCR with two types of synthesized single strand DNA coding 5'-CGGCCATGGCC$(NNK)_8$GCGGCCGCTTACGCCAAGCTCTAAT-3'and 5'-GTATTAGAGCTTGGCGTAAGCGGCCGC-3', where N is dA, dT, dG or dC; K is dT or dG. The pΔ198MM and random octapeptide genes were digested by *Nco* I and *Not* I for directional insertion. The pR8Δ198MM was constructed by ligation of the digested pΔ198MM and the random octapeptide genes. The pR8Δ198MM was transformed into *E.coli XL1-Blue* by electroporation (2.5 kV, 25μF and 200Ω, GENE PULSER, Bio-rad) and 1.2 x 10^5 numbers of independent colonies were obtained. The $(NNK)_8$ sequence on pR8Δ198MM was translated to random octapeptides, since the host *XL1-Blue* is characterized as the suppressed strain of the amber stop codon (TAG) (Li et al., 1998).

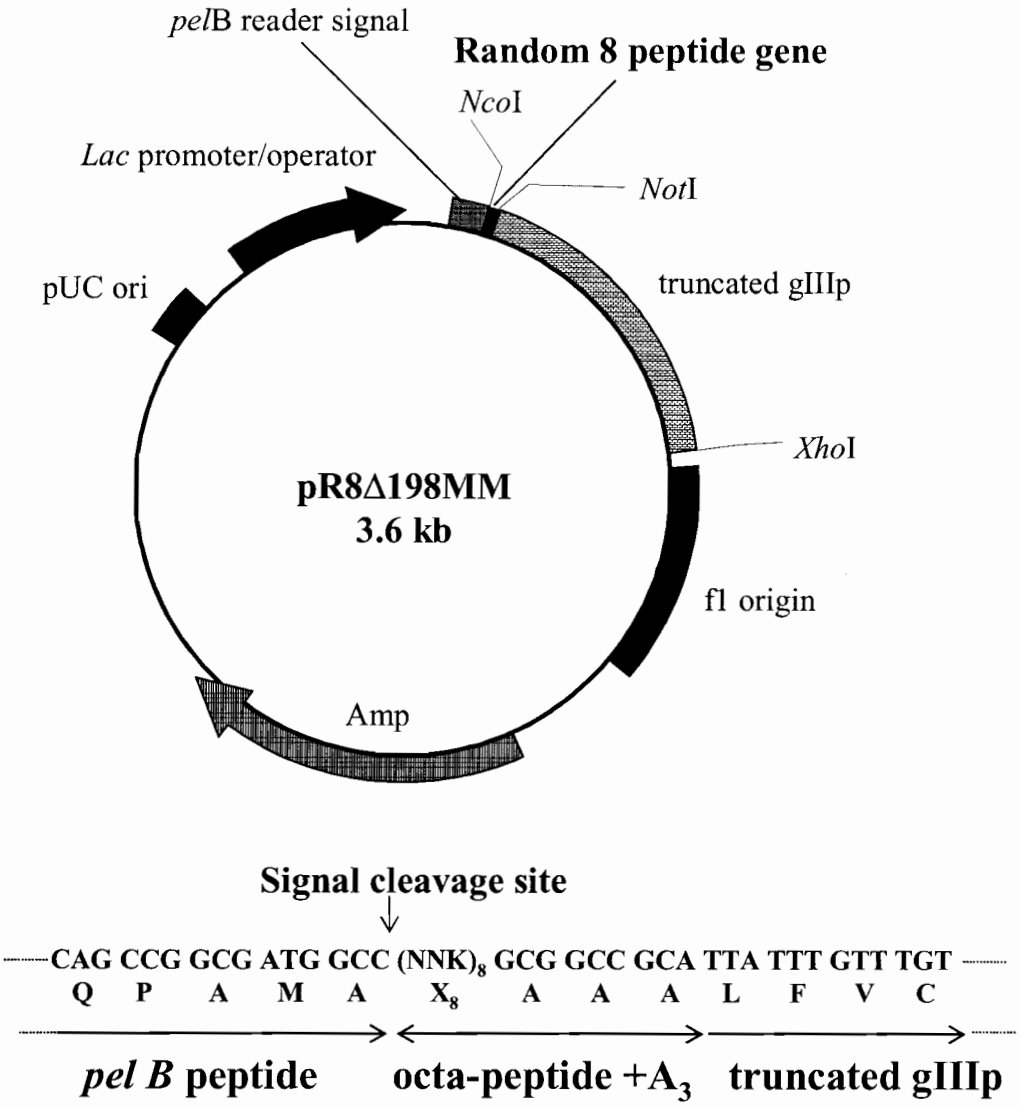


Figure 9-1 Construction of octapeptide-displayed phage library and signal cleavage site of fusion protein

9.2.4 Cultivation and generation of octapeptide-displayed M13 phages encapsulating phagemid DNA

The transformant was inoculated in 50 ml of a 2 x YT medium (yeast extract 10 g/L, trypton 16 g/L, NaCl 5 g/L, pH 7.0) containing 50 μg/ml ampicillin (Amp) and 12.5 μg/ml tetracycline (Tet) in a 500 ml-flask and shaken at 37°C at 250 rpm. At OD_{600} of 0.5, helper phage VCSM13 (Stratagene, CA, USA) was added at multiplicity of infection (MOI) of 20, and the culture cells were incubated for 30 min at 37°C without shaking. Cells were harvested by centrifugation at 5000 x g for 10 min and transferred to a fresh culture medium 2 x YT containing Amp and Tet and 70 μg/ml kanamycin (Kan). The cells were shaken again for 12 -16 h under the condition stated above. After centrifugation twice at 9,000 x g for 15 min, the supernatant containing octapeptide-displayed M13 phages was recovered as an octapeptide phage library (R8Δ198MM) and stored at 4°C until use. If necessary, phages were concentrated by PEG precipitation or diluted with PBS (NaCl 16 g/L, KCl 0.2 g/L, $Na_2HPO_412H_2O$ 2.9 g/L, KH_2PO_4 0.2 g/L, pH 7.2). The phage titer (colony forming unit) in the supernatant was determined from the number of colonies of precultured and infected *XL1-Blue* cells on 2 x YT selective agar plate containing Amp and Tet. Helper phage VCSM13 used as a model of nonspecific phage was prepared by a standard protocol provided from Stratagene.

9.2.5 Preparation of activated liposomes and immobilization of antibody to liposomes

DPPE was activated to *N*-[4-(*p*-maleimidophenyl) butyryl] dipalmitoylphosphatidylethanolamine (MPB-DPPE) by SMBP as previously reported. (Kishimura et al., 1989) The lipid mixture containing DPPC (10 μmol) and other lipids (1-10 μmol) in chloroform was dried to a thin lipid film on the inside wall of a pear-shaped flask (50 ml) using a rotary evaporator above 42°C under reduced pressure. For protein coupling on liposomes, 0.5 μmol MPB-DPPE was added in the lipid mixture. The lipid film was dried overnight under reduced pressure in a desiccator. Then, 3 ml of PBS was added, and the flask was

shaken at around 50°C to peel off the lipid film and to form multilamellar vesicles (MLVs). Activated liposomes prepared were purified twice by centrifugation for 2 min at 15,000 x g and washing with PBS at 4°C. The affinity purified anti-peptide antibody dissolved in PBS was mixed with the activated liposome suspension to give the final concentrations of 200-300 μg-antibody/ml and ca. 2 μmol-DPPC/ml, and 2-iminothiolane-HCl, which conjugates antibody molecules to activated liposomes through sulfhydryl groups, was added in 5 fold excess of antibody. The mixture was incubated with gentle stirring for 12-16 h at room temperature. Antibody-coupled liposomes were purified three times by centrifugation for 2 min at 23,500 x g and washing with PBS at 4 °C. Pellet was resuspended in PBS and stored at 4°C until use. The concentrations of DPPC and antibody in liposome suspensions were measured after addition of 10 - 100 μl of 10 % deoxycholic acid sodium salt to 100 μl of sample. The concentrations of DPPC and coupled antibody in liposome suspensions were determined by the cholineoxidase method using Phospholipids C-Test Wako (Wako Pure Chemical) and by DC-protein assay (Bio-Rad Laboratories, CA, USA), respectively. The average diameters of antibody-coupled liposomes were measured by the differential light scattering analysis (DLS 7000, Photal Otsuka Electronics, Osaka, Japan). The number of vesicles was counted by use of a hemocytometer with microscope. Antibody-coupled liposomes obtained were stored at 4 °C for at least one month without loss of binding activity.

9.2.6 Adsorption of nonspecific M13 phage on liposomes made of different lipid compositions

Nonspecific phage (VCSM13) was added in a micro-centrifuge tube (1.5 ml) containing MLVs (200 nmol-DPPC/ml) made of different compositions of lipids in PBS + 10 vol% Blocking solution or an Immunotube containing PBS + 10 vol% Blocking solution to form 1 ml of a suspension of 2.6 x 10^9 pfu/ml. The mixture was incubated for 30 min at 25 °C. In the case of MLVs, they were centrifuged at 20,000 x g for 2 min at 4 °C, and 990 μl of the supernatant was

carefully discarded. The pellet was resuspended in 1 ml of fresh PBS. The centrifugation and washing operations were repeated 10 times. To reduce phages nonspecifically adsorbed on the tube wall, at the tenth washing operation, the suspended liposomes in PBS were transferred in a new micro-centrifuge tube and centrifuged. The Immunotube was washed 10 times with PBS containing 0.05 % Tween 20 (PBST). In both cases using the MLVs and Immunotube, adsorbed phages were eluted by addition of 200 μl of 0.1N HCl after the removal of the supernatant. Then, the titer (plaque forming unit) of eluted phage (VCSM13) in the solution neutralized with 100 μl of 2 M Tris-HCl buffer (pH 8.0) was determined by use of precultured *XL1-Blue* dispersed in 3.5 ml of 2 x YT soft agar and selective agar plate containing Tet. After incubation at 37°C for 16 hours, numbers of plaque were counted and averaged in duplicate experiments.

9.2.7 Biopanning selection of phages displaying peptides specific to anti-peptide antibody from library

In this study, two types of panning strategies were used in liposome biopanning. (Strategy L1) MLVs coupled with 10 μg of the antibody were used as adsorbents for each panning round. (Strategy L2) MLVs coupled with 100 μg of the antibody were used at first round, and 10 μg-antibody for successive rounds. In conventional method using Immunotube, the wall of Immunotube was coated with antibody molecules by use of 1 ml of PBS containing 25 μg/ml of the anti-peptide antibody. After 10 times washing with PBS, it was blocked with Blocking solution and used for panning.

A solution (1 ml) containing octapeptide-displayed M13 phages (R8Δ198MM, about 1- 4 x10^8 cfu/ml) in 10 vol% Blocking solution was incubated with the antibody-coupled MLVs for 30 min at 25°C. Centrifugation and washing steps were repeated 10 times in the same way described above, and adsorbed phages were eluted with 200 μl of 0.1N HCl. After neutralization with 2M Tris-HCl,

they were infected into precultured *XL1-Blue*. Infected cells grew up to OD 0.5-1.0 in 2 x YT (Amp, Tet). After superinfection of VCSM13 (MOI=20), recovered phages were produced in fresh 2 x YT medium (Amp, Tet, Kan). The supernatant containing phages was recovered. The obtained phages were concentrated by PEG precipitation, and used for the second round of selection. This panning operation was repeated 4 times. The initial and recovered phage titers (colony forming unit) in each panning round were determined in duplicate with precultured *XL1-Blue* and selective plates containing Amp and Tet.

9.2.8 DNA sequencing

After infection of recovered phages to *XL1-Blue* cells, a portion of cells were inoculated on 2 x YT selective agar plate (Amp, Tet) to form a single colony. The 16 - 20 colonies were randomly chosen and their DNA sequences coding octapeptide were analyzed. They were determined by a genetic analyzer (310 Genetic Analyzer, Applied Biosystems) according to standard protocol from the supplier, after PCR with a primer (5'-CCCCCCCCCTCTAGAAATAATTTT- GTTTAA-3') and Big dye terminator cycle sequencing kit (Applied Biosystems).

9.2.9 Binding affinity of selected peptides against anti-peptide antibody

Two kinds of octapeptides, which showed the highest homology to the antigen peptide sequence in each method, were synthesized (BEX Corporation) and were used in determination of the binding affinity by competitive ELISA. A microtiter plate was incubated with 100 μl of 10 μg/ml Insulin-BSA in PBS to immobilize insulin molecules on its surface of solid. After washing with PBS, the plate was blocked with 4 times diluted Blocking solution. After washing, the sample solutions containing 5 μg/ml of the anti-peptide antibody, different concentrations of the octapeptide and 10 % Blocking solution were incubated at room temperature for 1 hour. After

washing, 100 μl of 2000 times diluted HRP-conjugated anti-rabbit IgG antibody (goat) solution was added and incubated for 1 hour at room temperature. After washing, color was developed by addition of 100 μl of substrate solution composed of 3 mg/ml 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS) and 0.01 % H_2O_2 in 0.1 M citrate buffer. The absorbance of developed signal was detected at 405 nm by a microplate reader (Bio-rad laboratories Model 550). The values of IC_{50} were determined from duplicate experiments as the peptide concentrations showing 50 % inhibition of the interaction between the antibody and Insulin-BSA.

9.3 Results and Discussion

9.3.1 Effects of lipid composition of liposomes on adsorption of nonspecific M13 phage

To reduce adsorption of nonspecific M13 phage in biopanning, we studied the effects of lipid compositions of liposomes on adsorption of M13 phage. Four types of MLVs were prepared; MLVs composed of (a) DPPC : Chol : DCP = 10 : 10 : 1, (b) DPPC : DCP = 10 : 1, (c) DPPC : Chol : SA = 10 : 10 : 1 and (d) DPPC : SA = 10 : 1. Liposomes (a) and (b) have negative charges on the surface of vesicles, and the others have positive charges. Cholesterol was utilized to increase the membrane stability and fluidity of the liposomes at room temperature. Liposomes (c) did not disperse well in PBS and were not used for measurement of phage adsorption. The adsorption characteristics of these liposomes were studied under the same procedures for biopanning by use of nonspecific phage VCSM13 (2.6 x 10^9 pfu/ml), and the recovered phage titer was determined. As shown in **Figure 9-2**, the liposomes (b) DPPC : DCP = 10 : 1 showed significantly low adsorption of nonspecific phage among other types of liposomes. These results indicate that nonspecific adsorption of M13 phage to the liposomes is mainly caused by electrostatic interactions between the surface charges, because major surface proteins of M13 phage are negatively charged at neutral pH .

(Armstrong et al., 1983, Makowski, 1984) Without any blocking operations, nonspecific adsorption of M13 phage to the liposomes (b) can be reduced to the adsorption level comparable with the Immunotube coated with blocking reagent.

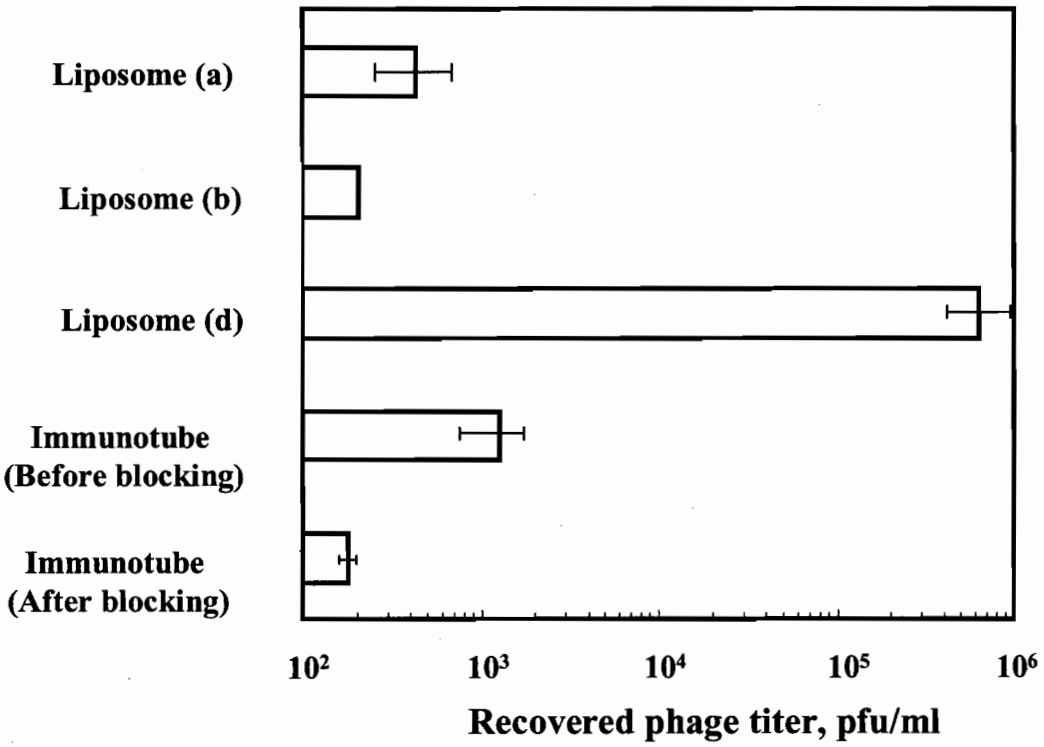


Figure 9-2 Adsorption of nonspecific phage M13 on liposomes and Immunotube

Liposome concentration; 200 nmol-DPPC/ ml
Initial titer of VCSM13; 2.6 x 10^9 pfu/ ml

9.3.2 Adsorption characteristics of antibody-coupled MLVs and antibody-coated Immunotube

Activated liposomes composed of DPPC : DCP : MPB-DPPE = 20 : 2 : 1 were coupled with the anti-peptide antibody via iminothiolane as described in Materials and Methods.

Table 9-1 shows the characteristics of antibody-coupled MLVs (AB-MLVs) prepared for panning operation. When 10 μg of the antibody was used, it was immobilized on the surface of 1.9 x 10^6 vesicles of MLVs, which total surface-area was 1.8 cm^2, 38 % of the surface area of Immunotube.

Table 9-1 Characteristics of MLVs coupled with 10 μg of antibody

Coupled antibody	**67 pmol (10 μg)**
Phospholipid	**170 nmol-DPPC**
Number of vesicles	**1.9 x 10^6**
Average diameter	**5.6 ±1.0 μm**
Antibody molecule on a vesicle	**2.0 x 10^7**
Surface area	**1.8 cm^2 (Tube 4.8 cm^2)**
Immobilized density of antibody	**36 pmol/cm^2**

Figure 9-3 shows phage titers recovered from the library (R8Δ198MM) with use of AB-MLVs or antibody-coated tube (AB-Tube). MLVs (DPPC/DCP) showed low titer of adsorption against not only nonspecific phage (VCSM13) but also the phage library. The presence of MPB-DPPE in vesicles (activated MLVs) did not affect the adsorbed titer. In spite of the smaller surface area, the number of phages recovered from the library with AB-MLVs containing 10 μg of the antibody was about 10 times that with AB-Tube. By increasing the amount of the antibody to 100 μg, the number of recovered phages increased about 10 times. These results may indicate that recognition of peptides displayed on phages by the antibody coupled on MLVs is more specific and natural than by the antibody adsorbed on the wall of Immunotube.

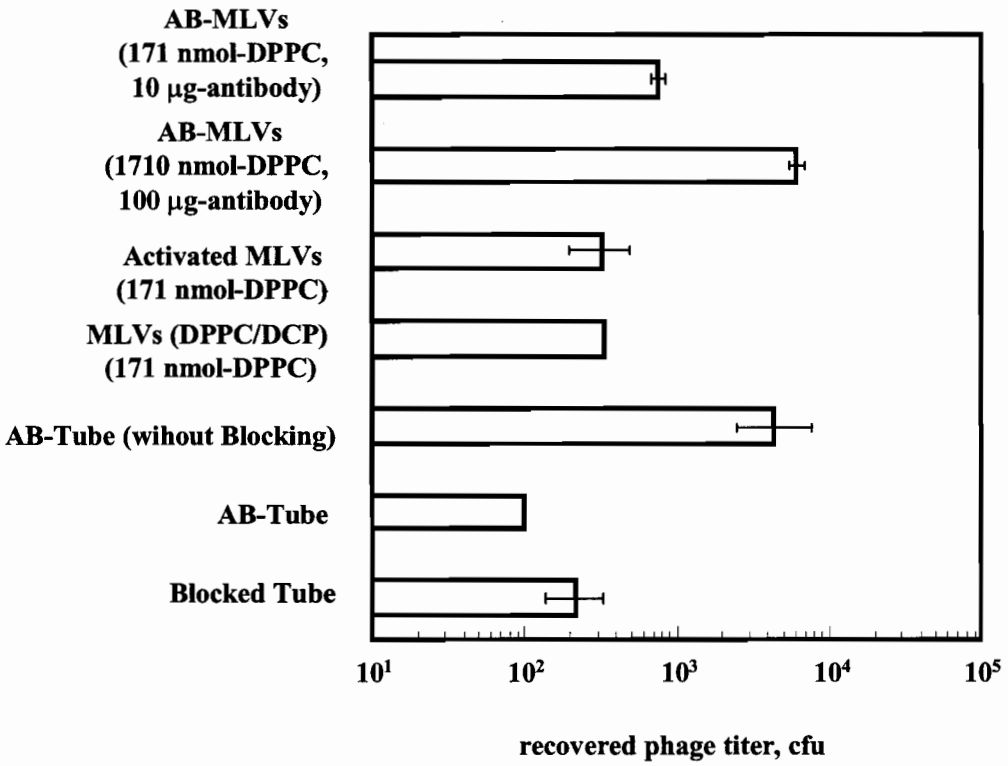


Figure 9-3 Adsorption characteristics of phage displaying peptide library on MLVs and Immunotube

R8Δ198MM: Initial titer, $4x10^8$ cfu

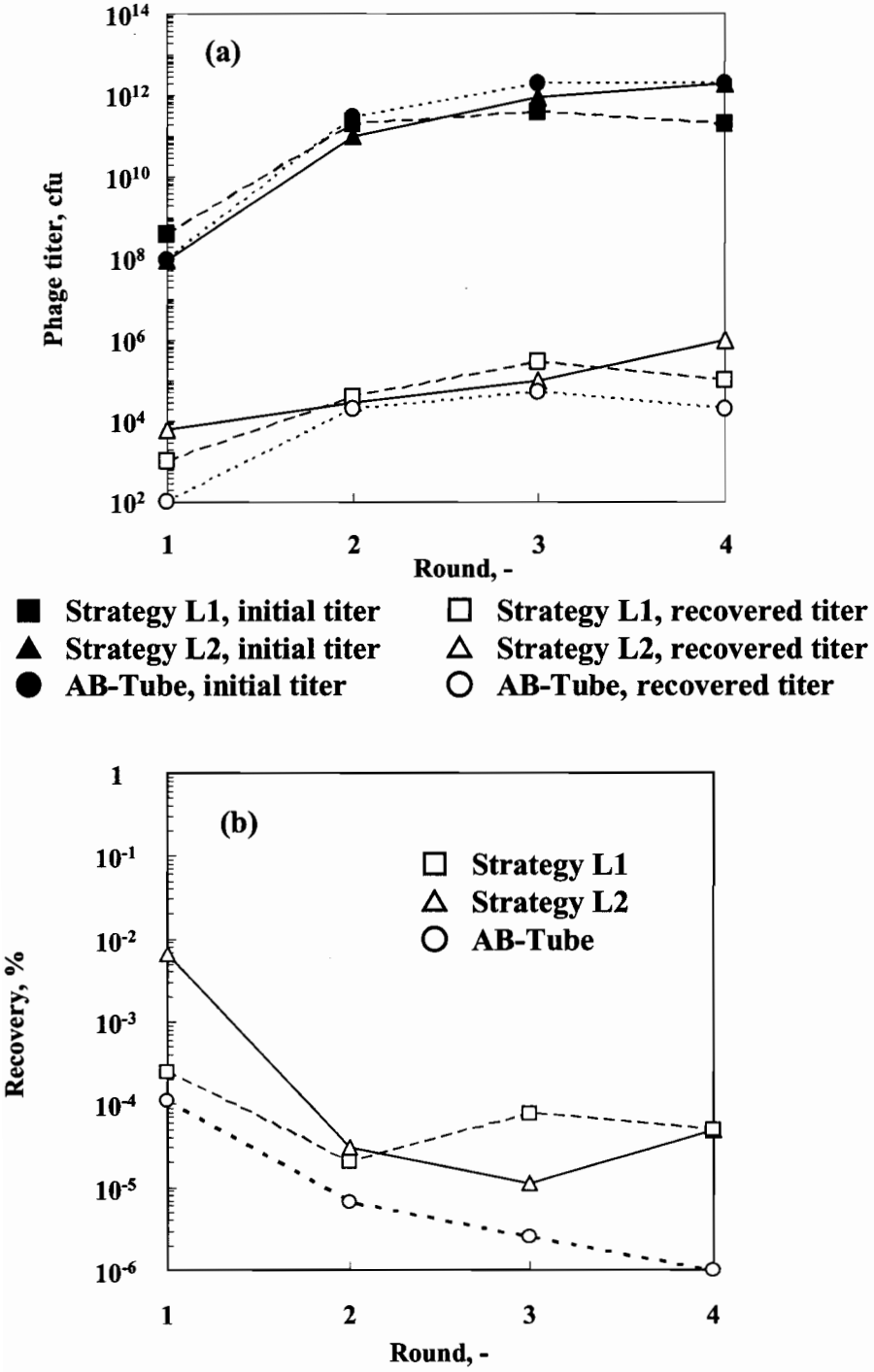
9.3.3 Biopanning selection of phages displaying specific peptides from library

Figure 9-4 (a) shows initial (closed keys) and recovered (open keys) phage titers of each round and **Figure 9-4 (b)** shows the recovery, which is calculated from the ratio of recovered titer to initial titer. In biopanning with Strategy L1 and Strategy L2, the recovery increased at round 3 and round 4, respectively, while it gradually decreased in successive rounds with AB-Tube.

After 4 rounds of biopanning, amino acid sequences displayed by 16 – 20 colonies recovered from the library were determined by DNA sequencing. **Table 9-2** shows sequences of peptides which have more than two amino acids at the same position with the antigen octapeptide (FVNQHLCK). With Strategy L1, all recovered phages displayed homological peptides, especially in the N-terminal region from 1 to 3 or 4 positions. With Strategy L2, another peptide showing homology at 5 and 6 positions (VKCDHLTR) was obtained with 40 % probability, together with similar peptides obtained with Strategy L1. With AB-Tube only three peptides showed homological sequences among 20 colonies.

Figure 9-5 shows percentage of phages displaying homological peptides in each round. Octapeptides, which have overlapped sequences to that of the antigen peptide at least at two positions, were defined as homological peptides. With biopanning using AB-MLVs, the percentage of phages displaying homological peptides was much higher than that with AB-Tube from earlier stages of biopanning and reached up to 60-100 % after 4 rounds of biopanning. On the other hand, only 15 % of recovered phages displayed homological peptides with use of AB-Tube. Interestingly, with Strategy L2, the recovered efficiency after 4 rounds was lower than that with Strategy L1. This indicates that larger surface area may increase recovery of targets showing lower affinity and/or non-specific phages from the library in the first round, although higher binding capacity is effective to select targets from large size of libraries. By use of a larger numbers of AB-MLVs, therefore, wide varieties of targets might be obtained, while the recovery of specific targets decreases. The

lower recovery in the conventional biopanning using AB-Tube may be caused by slight denaturation of antibody molecules adsorbed on polystyrene wall and changes in binding characteristics. It was reported that protein immobilized on plastic plate was denatured and showed changes in binding affinity (Friguet et al., 1984). Plastic or magnetic beads are often utilized as supports to increase the concentration of target protein, but they might suffer similar problems. Thus, biopanning using protein-coupled MLVs is one of promising methods for screening of phages expressing peptides showing specific interactions.



(a) Initial and recovered titer, and (b) Recovery

Figure 9-4 Recovered phage titer and recovery in biopanning selection

Table 9-2 Homological sequences of selected peptides against antigen octa-peptide after 4 times panning

Strategy L1, 100 % (16/16)

	1	2	3	4	5	6	7	8	Percentage, %
L1-1	F	V	N	Q	D	T	T	C	75
L1-2	F	V	N	V	E	Q	S	C	6
L1-3	F	V	N	H	Q	D	H	H	6
L1-4	F	V	N	V	S	C	P	H	6
L1-5	F	V	N	D	Y	P	A	R	6

Strategy L2, 60 % (12/20)

	1	2	3	4	5	6	7	8	Percentage, %
L2-1	V	K	C	D	H	L	T	R	40
L2-2	F	V	N	C	V	S	P	H	15
L2-3	F	T	N	W	C	G	G	D	5

AB-Tube, 15 % (3/20)

	1	2	3	4	5	6	7	8	Percentage, %
T-1	F	C	V	Q	Y	M	R	V	5
T-2	H	V	C	Q	P	L	L	L	5
T-3	F	E	V	I	R	Y	C	F	5

Antigen peptide

F	V	N	Q	H	L	C	K

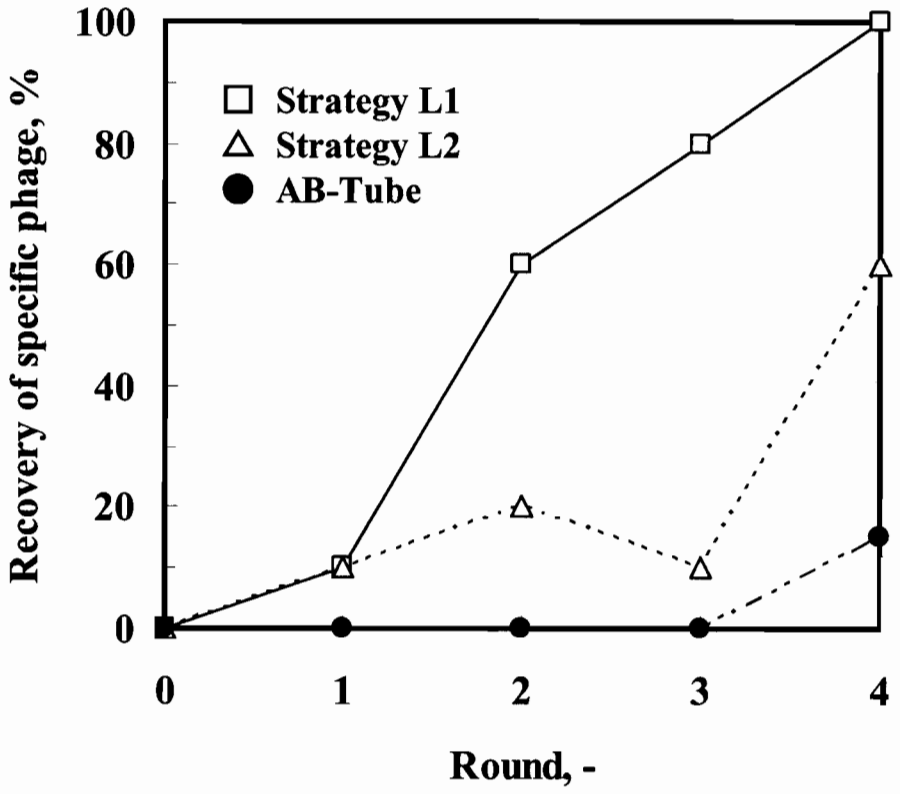


Figure 9-5 Percentage of recovered phages displaying homological peptides

Number of colony selected,
Round 1-Round 3: 10 colonies (strategy1, strategy2, AB-Tube)
Round4: 16 colonies (strategy 1), 20 colonies (strategy 2, AB-Tube)

9.3.4 Binding characteristics of antibody to isolated octapeptide

Two kinds of peptides selected by AB-MLVs and AB-Tube, which showed the highest homology in each method, were synthesized and used as a competitor in competitive ELISA. **Figure 9-6** shows the result of competitive ELISA with the octapeptide L1-1, T-2, the antigen peptide or bovine insulin. The octapeptide L1-1 inhibited the interaction between antibody and Insulin-BSA with comparable affinity (IC_{50}; 50 μM) to that of the antigen peptide, while IC_{50} value of the peptide T-2 was over 1 mM. Thus, these results indicated that the target-immobilized MLVs as a support could successfully select more specific candidates with smaller number of panning rounds.

This method utilizing target proteins coupled on liposomes is very effective to select specific peptides against antibody, enzyme, and other proteins from random peptide libraries, and might be applied also to selection of monoclonal antibodies against target antigens from reconstituted antibody libraries (Huang et al., 2003, Hoogenboom et al., 1998). For further improvements of this method, it is necessary to optimize liposome size, density of target protein on liposome membrane, total concentration of target molecules and other factor. Development of an effective B/F separation method such as magnetic separation method would make this method more effective.

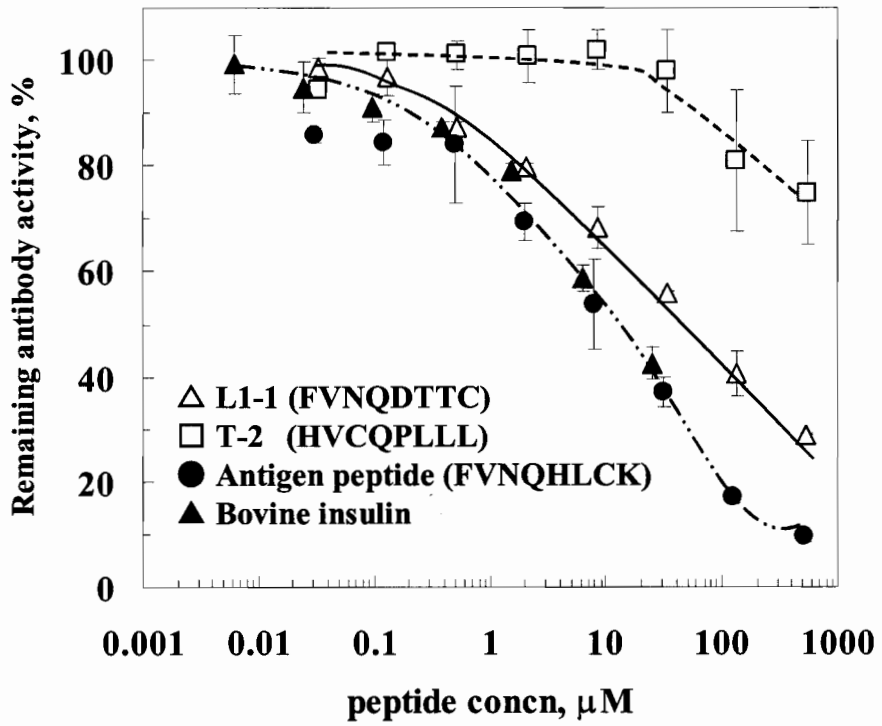


Figure 9-6 Inhibition of antibody binding by homological peptides in competitive ELISA

9.4 Conclusion

In this study, we clarified the characteristics of antibody-coupled liposomes as adsorbents for effective biopanning operation. Antibody-coupled MLVs consisted of DPPC : DCP : MPB-DPPE = 20 : 2 : 1 could significantly reduce the nonspecific adsorption of VCSM13 phages. To compare performances of AB-MLVs and AB-tube as adsorbents in biopanning, phages displaying octapeptides specific to the anti-peptide antibody against the peptide antigen (FVNQHLCK) were screened from the octapeptide-displayed phage library. After 4 rounds of biopanning, all selected phages displayed peptides homological to the antigen peptide by use of AB-MLVs, while only 15 %

of the recovered phages displayed homological peptides in the conventional biopanning. The selected octapeptide by use of AB-MLVs exhibited almost comparable binding affinity to that of the antigen peptide against the anti-peptide antibody. Thus, proteoliposomes are useful as adsorbents in biopanning.

General conclusion

The number of biological analytes to be detected has increased, especially, in the research areas of clinical assays, proteomics, environmental pollutants, and genetically-modified crop (GM crop), where not only the high throughput, but also more sensitive quantification methods are required. Since antibodies have high selectivity and affinity to recognize specific antigens, immunoassay by using the immunological interactions has been utilized for these purposes. Although enzyme immunoassay (EIA) is the most popular one, its sensitivity is limited by the number of labeling agents conjugated with an antibody molecule. Therefore, many modified methods of EIA have been proposed to increase the sensitivity and to amplify the signal in measurements. To detect lower concentration of analyte with higher sensitivity and signal intensity in immunoassay, two ways of improvements should be considered; i.e. developments of alternative immunoassay methods, in which a larger amount of marker molecules can be immobilized, and isolation methods of antibodies with higher selectivity and affinity.

In this study, by using protein-coupled liposomes, a high sensitive immunoassay method for high throughput detection and an efficient screening method for isolation of specific protein from library were developed.

Part I

In **Part I**, the liposome immunoassay (LIA) was developed and optimized to detect lower concentration of analytes with higher sensitivity and signal intensity. Especially, in **Chapters 1-4**, effects of the number of antibody molecules, liposome sizes, and preservation strategies on the sensitivity and signal intensity in liposome immunosorbent assay (LISA) were studied. In **Chapters 5-8**, I proposed and demonstrated a membrane blotting assay by use of immunoliposomes. The liposome immunoblotting assay was applied to colony lift immunoassay, Western blot analysis, and

microblotting assay in these chapters.

In **chapter 2**, effects of the number of antibody molecule coupled on a liposome vesicle on sensitivity in LISA was investigated. Several antibody molecules coupled on liposome membrane could show a high avidity to antigens by multipoint interactions. Therefore, LISA could show the higher sensitivity compared with ELISA, especially at lower concentration ranges of samples.

In **Chapter 3**, to obtain higher sensitivity and signal intensity in LISA, I investigated effects of liposome diameter of unilamellar vesicle on the signal intensity in LISA. Higher signal intensity could be obtained by use of immunoliposomes of larger unilamellar vesicles, because they could encapsulate a large amount of marker molecules and couple many antibody molecules on their membrane surface. In addition, nonspecific adsorption of immunoliposomes on plastic support could be significantly reduced by use of 0.1 % skim milk in PBS as a dilution buffer. Consequently, about 50-100 times higher sensitivity and signal intensity could be obtained in LISA, compared with those in conventional ELISA. Therefore, LISA can be one of promising methods for detection of trace amounts of analytes.

In **Chapter 4**, for preservation of immunoliposomes, effects of several saccharides as additives on stability of immunoliposomes after freeze-thawing or lyophilize-dissolved operation were investigated. The immunoliposomes without additives were significantly unstable against freeze-thawing and lyophilize-dissolving operation and their overall activities decreased to 80 % and 0 %, respectively. In the presence of glucose as an additive during freeze-thawing, almost all immunoliposomes retained their activities. After thawing them, they maintained their activity at least for 2 weeks at 4 °C, while their activity without the additives gradually decreased. Repeated freeze-thawing operations extensively decreased their activities because of antibody inactivation. The activities of lyophilize-dissolved liposomes (LDLs) were also kept 30-40 % of the initial activity in the presence of glucose. Thus, glucose is the most effective to retain the overall activities of

freeze-thawing liposomes (FTLs) and LDLs.

In **Chapter 5**, a new membrane blotting assay using immunoliposomes encapsulating HRP was developed. A substrate 4-chloro-1-naphthol permeated through the phospholipid membrane of the antibody-coupled liposomes and formed a colored product precipitating inside the liposomes. The precipitates accumulated in the liposomes and could be detected at the positions where the liposomes coupled with a target in blotted samples. Under the optimal condition, 0.02 ng of IgM was detected by use of immunoliposomes, while the conventional immunoblotting assay using antibody-HRP conjugates detected 2 ng of IgM. The sensitivity increased about two orders of magnitude by the liposome immunoblotting assay. This liposome immunoblotting assay gives a simple detection method of proteins with a high sensitivity, as well as high sensitivity Western blotting assay.

In **Chapter 6**, to clarify the applicability of a liposome immunoblotting assay to microassay systems, the effects of the sample volume blotted on PVDF membrane on the sensitivity and signal intensity were studied. In the liposome immunoblotting assay, signal intensities per unit area (signal density) were almost the same or increased slightly with decreasing blotted sample volume (from 20 to 2 μl) at the same concentration of analyte (human IgM), because the colored signal was detected without loss of intensity. On the other hand, in the conventional immunoblotting assay, signal densities decreased with decreasing sample volume. This is caused by partial losses of the colored signal into the substrate solution. These results show that the liposome immunoblotting assay is a promising method for the detection of analytes blotted in small areas, because its sensitivity is higher than that of the conventional blotting assay.

In **Chapter 7**, we applied the liposome immunoblotting assay for screening of colonies secreting active scFv by colony lift assay. Uniform and high density signals were observed from blotted membranes with the indirect blotting and detection with immunoliposomes. The immunoliposome gave the highest S/N ratio, which was 2 times higher than that in the conventional

blotting assay in a colony lift assay. Thus, liposome immunoblotting assay is useful method to screen colonies expressing target protein with higher sensitivity.

In **Chapter 8**, we developed the liposome immunoblotting assay utilizing ECL reaction. In a similar manner of free HRP solutions, chemiluminescent light emitted from the immunoliposome suspensions significantly increased by use of the substrates solution containing Tween 20. With the washing and substrate solutions containing 1% Tween 20, immunoliposomes non-specifically adsorbed was efficiently removed, and thus little background noise was observed. Under the optimal conditions, the liposome immunoblotting assay attained about 20 times higher sensitivity in comparison with conventional one. The liposome immunoblotting assay utilizing ECL reaction has the high sensitivity among other immunoblotting assays and can be useful for detection of trace amounts of analytes.

Part II

In **part II**, I proposed and demonstrated an alternative biopanning procedure by use of liposomes coupling target protein molecules (proteoliposomes) for efficient screening of positive phages from a phage library. Biopanning procedures have been utilized for screening of specific proteins or peptides against target substance from phage libraries. Especially, these procedures are popular and useful to isolate more specific monoclonal antibodies for immunoassay or cancer therapy. However, depending on screening conditions, library sizes, or other factors, positive phages displaying specific protein molecules have not always been selected.

In **Chapter 9**, I clarified adsorption characteristics of liposomes forming multi-lamellar vesicles (MLVs) to nonspecific phages (VCSM13) for effective biopanning operation. Antibody-coupled MLVs (AB-MLVs) consisted of DPPC : DCP : MPB-DPPE = 20 : 2 : 1 could significantly reduce the nonspecific adsorption of VCSM13 phages. To compare performances of

AB-MLVs and antibody-immobilized tube (AB-tube) as adsorbents in biopanning, phages displaying octapeptides specific to an anti-peptide antibody against a peptide antigen (FVNQHLCK) were screened from the octapeptide-displayed phage library. After 4 rounds of biopanning, all selected phages displayed peptides homological to the antigen peptide by use of AB-MLVs, while only 15 % of the recovered phages displayed homological peptides in the conventional biopanning. The selected octapeptide by use of AB-MLVs exhibited almost comparable binding affinity to that of the antigen peptide against the anti-peptide antibody. Thus, proteoliposomes are useful as adsorbents in biopanning.

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Part II

Chapter 9

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