Liposome Immobilization on Cross-Linked Polymer Gel by in situ Formation of Cleavable Covalent Bonds

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ABSTRACT: Immobilization of liposomes onto chemically modified Sephacryl gel particles by *in situ* reaction between liposome-incorporated thiols and mercapto moieties on the gel to form disulfide linkages was investigated. The immobilization occurred upon coincubation of the liposomes with the gel and completed in 48 hours. For the immobilization, both the mercapto moieties and the incorporated thiol were essential. Once immobilized, no spontaneous detachment of the immobilized liposomes was observed. The extent of the immobilization depended on both the thiol content and the ratio of the liposomes to the gel. In typical immobilization with 25 mol% 1-octanethiol, 82% of the liposomal phosphatidylcholine in the system was found associated with the gel. Decreasing the amount of the liposomes to the gel brought the immobilization close to quantitative one. Among the three different thiols examined (1-octanethiol, 1-hexadecanethiol and thiocholesterol), the extent of the immobilization was slightly higher with thiocholesterol than the alkanethiols. The immobilized liposomes were detached from the gel by treatment with dithiothreitol. Approximately 60% of fluorescent dextran derivative encapsulated in the liposomes was retained throughout the immobilization-detachment process. The gel left after the detachment was still capable of immobilizing a fresh batch of thiol-liposomes.

INTRODUCTION

Liposomes, artificial vesicles of lipid bilayer membrane, are a valuable tool in wide varieties of bio-related research fields. They have been used as a biomembrane model in researches of membrane protein functions [1, 2] or as a biocompatible microcarrier in the development of sophisticated drug delivery systems [3].

Most of those studies use large unilamellar vesicles (LUVs) or multilamellar vesicles (MLVs) [3] because of their relatively high stability and ease of preparation. However, their small size (typically 100-200nm), fragility and colloidal nature make the manipulation of those liposomes difficult. For example, one may want to prevent free diffusion of liposomes in the bulk aqueous phase and localize them in certain location.

One possible solution to the problem is to conjugate liposomes with suitable supporting material of a larger size. Particles of cross-linked hydrophilic polymer gels are excellent for this purpose. They have been widely tested as media in gel filtration technique in biochemical researches, and thus the products of stable quality are commonly available. In fact, those particles have been used for immobilization of liposomes as seen in the pioneering works of Lundahl and the coworkers. Sandberg et al. first reported immobilization of liposomes on the type of gel particles [4]. The work was extended by Yang et al. to immobilization of proteoliposomes [5] and further to chromatographic systems [6-8] in investigation of drug partitioning to lipid bilayer membrane [9-11] and protein-membrane interaction [12]. These studies clearly demonstrate usefulness of liposome-gel conjugates. In these immobilization systems, detachment of the immobilized liposomes is not intended. The design is quite logical because the intended use of the systems, such as a stationary phase in chromatographic application, demands permanent irreversible attachment of liposomes to the gels.

A system that not only stably immobilizes liposomes but also allows intentional and controlled detachment of the immobilized liposomes may provide a solution to a related but more general problem of concentrating or separating liposomes from the bulk aqueous phase. The process is essential in manipulation of liposomes such as encapsulation of substances in the aqueous interior of liposomes. Size exclusion chromatography (gel filtration) or ultracentrifugation is frequently used for the purpose although those methods are costly and time-consuming and may not be feasible in large scale.

In our previous study, cross-linked polymer gel particles were covalently bonded with hydrophobic moieties via disulfide linkage. Liposomes were immobilized on the hydrophobized gel particles using the hydrophobic moieties as anchors to liposomal bilayer membrane (Figure 1). Further, the immobilized liposomes were detached from the particles upon reductive cleavage of the disulfide bonds connecting the hydrophobic anchor to the gel [13, 14].

The results obtained in the previous study led us into further investigation of the system [15] as discussed here in detail. The proposed scheme of the immobilization-detachment of liposomes and the formation-cleavage of the disulfide linkages between the anchors and the modified gels suggests a possible alternative immobilization path (Figure 2): The same liposome-gel conjugate may be formed by interacting liposomes that contain hydrophobic thiols with cross-linked polymer gel particles that are chemically modified to possess active mercapto moieties if *in situ* formation of disulfide linkages between the liposomes and the gel is possible. The reaction should be of interest as an example of little studied covalent bond formation in the interfacial region between liposomal bilayer membrane and polymer gels. Also, this would permit the post-incorporation of thiols into pre-formed liposomes, which makes it possible to quickly test different types or amounts of the hydrophobic anchor to obtain the optimized the system.

MATERIALS AND METHODS

Materials

Sephacryl S-1000 and Sepharose 4B gels were products of Pharmacia (Uppsala, Sweden). 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2,2'-dipyridyl disulfide (2-PDS), sodium thiosulfate, sodium bicarbonate, 1,4-bis(2,3-epoxypropoxy)butane, 1-octanethiol, 1-hexadecanethiol, (ethylenedinitrilo)tetraacetic acid (EDTA), sodium hydroxide, hydrochloric acid, sodium chloride and Phospholipid Test Wako were obtained from Wako Chemicals (Tokyo, Japan). Dithiothreitol (DTT) and sodium borohydride was supplied by Kanto Chemicals (Tokyo, Japan). L-α-Phosphatidylcholine from egg yolk (egg phosphatidylcholine; eggPC) was purchased from Avanti Polar Lipids (Alabaster, AL,

USA). 5-Cholestene-3-thiol (thiocholesterol) and sodium dodecyl sulfate (SDS) were bought from Sigma (St. Louis, MI, USA). Fluorescein isothiocyanate conjugated-dextran (FITC-dex, average molecular weight of 45,000) was a product of CarboMer, Inc. (Westborough, Mo, USA).

Chemical modification of Sephacryl S-1000 gel

The preparation of chemically modified Sephacryl S-1000 gel that bears mercapto moieties capped with 2-pyridinethio groups (Py-gel) was described in the previous article [14]. The gel particles were inspected under an optical microscope (Olympus IX-50, Tokyo, Japan) before and after the reaction for absence of any physical defects that might have been caused by the modification process.

Preparation of liposomes and incorporation of thiols

For the post-incorporation of a thiol, plain liposomes were first prepared from eggPC in 0.1 M HEPES buffer (pH 7.5) using the extrusion method as described in the previous paper [14]. In a typical preparation, 15 ml (containing 30 μmol of phosphatidylcholine) of the liposome suspension was taken in a round bottom flask, and an appropriate amount of a thiol was added as 1.2 ml of an ethanolic solution (for example, 10 μmol of 1-octanethiol for 25 mol% incorporation). The mixture was then incubated for 24 hours with mild stirring under nitrogen atmosphere.

For the pre-incorporation, liposomes were prepared from a mixture of an appropriate amount of eggPC and a thiol. Typically, for the preparation of liposomes incorporating 25 mol% of 1-octanethiol, 3.85 mg of the thiol was added to 60.0 mg of eggPC dissolved in 3.0 ml of chloroform. After the removal of the solvent under reduced pressure, the obtained thin film on the flask wall was swelled in oxygen-depleted 0.1 M HEPES buffer (pH 7.5). Extrusion of the crude suspension as described in the previous paper [14] yielded liposomes containing the thiol. All the procedures were performed under nitrogen atmosphere or in oxygen-depleted conditions.

After the thiol incorporation, a part of the suspension was applied to a size exclusion chromatographic analysis (Sepharose 4B; column diameter 18 mm and length

450 mm), and the rest was used in the interaction with the modified Sephacryl gel. The amount of eggPC contained was determined as inorganic phosphate using a Phospholipid Test Wako. The scattering intensity from the fractions collected in the size exclusion chromatography and the absorbance in the phospholipid assay were monitored with a Shimadzu UV-1700 spectrophotometer (Kyoto, Japan). The fluorescence intensity from FITC-dex was measured with a Hitachi F-3000 fluorophotometer (Tokyo, Japan).

Interaction of liposomes with modified gel

In a typical experiment, 15 ml of thiol-incorporated liposome suspension (phosphatidylcholine concentration 2.0 mM) was mixed with Py-gel (typically containing 10 μmol of pyridine-capped mercapto moieties) in a test tube and incubated at an appropriate temperature (typically at 37^{o}C) for 48 hours. After the interaction, the gel particles were separated from the supernatant by centrifugation (3,000 rpm for 20 minutes). The amounts of phospholipids and the FITC-dex (when used) in the supernatants were determined. Also, a quarter of the separated gel was treated with 2.0 ml of 1.0% aqueous SDS solution for 24 hours, and the sample was subjected to phospholipid assay. The rest of the gel was washed with 3 ml of 0.10 M HEPES buffer (pH 7.5) 5-6 times using the centrifugation.

Treatment of liposome-interacted gel with dithiothreitol

The gel obtained after the interaction with the liposomes as above was incubated with 5.0 ml of a 0.1 M HEPES buffer solution (pH 7.5) containing dithiothreitol (DTT; typically 20 μmol of DTT per μmol of the anchor moieties on the gel unless noted otherwise). The amounts of the phospholipids and the FITC-dex on the gel and in the bulk aqueous phase were determined as described above. Also, a part of the bulk aqueous solution was applied to the size exclusion chromatographic analysis.

When necessary, the gel separated after the DTT treatment was further treated with a fresh batch of the DTT solution for another 24 hours, and the sample was examined for the phospholipids.

Interaction of thiol-incorporating liposomes with gels after DTT treatment

Preparation of hydrophobized Sephacryl S-1000 gel from Py-gel and 1-octanethiol, immobilization of plain eggPC liposomes onto the hydrophobized gel and detachment of the immobilized liposomes from the gel by DTT treatment were carried out as described in the previous paper [14]. Briefly, 1.0 g of the hydrophobized gel (bearing 5 μmol of the octyl moieties) was interacted with 7.5 ml of liposome suspension (containing 15 μmol of eggPC) for 24 hours and then incubated with 3.0 ml of 33 mM DTT solution for 24 hours.

Liposomes suspension (4.5 ml) containing 9.0 μmol of eggPC and 3.0 μmol of 1-octanethiol was prepared by using the pre-incorporation method, and the suspension was incubated with 0.30 g of the gel obtained as above for 24 hours at 5^{o}C. The gel was separated and treated with 1.8 ml of 33.3 mM DTT solution for 24 hours, and the amount of the released phosphatidylcholine in the bulk aqueous phase was determined.

RESULTS AND DISCUSSION

Chemical modification of Sephacryl S-1000 gel

Commercially available Sephacryl S-1000 gel was chemically modified to bear mercapto groups as previously described (Figure 2) [14]. The gel is known to possess a relatively large holding capacity for liposomes of the size used in the present study (approximately 150 nm) [5] and has also shown to be sufficiently stable to the chemical modification [4, 5]. In fact, no destruction of the gel particles was observed with an optical microscope after the modification.

The mercapto groups on the gel were capped with 2-pyridinethio groups by a reaction with 2,2'-dipyridyl disulfide (2-PDS) [16]. The capping prevents undesirable oxidation of the moieties during the storage. The amount of the mercapto groups introduced to the gel was in the range of 9-10 μmol per gram for all the gels used in the present study. The capped thiol gel (Py-gel) thus obtained can react with free thiols as previously shown [14].

Preparation of thiol-incorporating liposomes

For incorporation of thiols into liposomal membrane, two different procedures, post- and pre-incorporation, were examined. In the post-incorporation method, an appropriate amount of an ethanolic solution of a thiol was added to plain liposomes prepared from eggPC by the extrusion method [17]. Three different thiols, 1-octanethiol (C_8SH), 1-hexadecanethiol ($C_{16}SH$) and 5-cholestene-3β-thiol (thiocholesterol, CholSH) were used. Size exclusion chromatography revealed the average diameter of the thiol-incorporated liposomes as approximately 150 nm, and neither significant diversion in the size distribution from the plain liposomes nor indication of aggregates smaller than liposomes was seen. The integrity of the liposome structure was also demonstrated by the fair retention of encapsulated fluorescein isothiocyanate conjugated-dextran (FITC-dex, approximate MW 45,000) by the liposomes. The leakage of the encapsulated FITC-dex during the incorporation was less than 15% even under the severest conditions examined (25 mol% of octanethiol), and with 9 mol% thiocholesterol, only 6% of the FITC-dex was released.

In the pre-incorporation method, liposomes were prepared from a mixture of eggPC and a thiol. The obtained liposomes showed elusion profiles in size exclusion chromatography comparable with those prepared by the post-incorporation method. FITC-dex can be encapsulated during the preparation. Compared with plain liposomes, the amount of the encapsulated FITC-dex per phosphatidylcholine was comparable in the case of 9 mol% thiocholesterol, and 75% even in the case of 25 mol% octanethiol.

Interaction of gel with liposomes

Upon co-incubation of thiol-incorporating liposome suspension with the pyridine-capped gel (Py-gel), the liposomal phosphatidylcholine gradually disappeared from the bulk aqueous phase. A phospholipids assay of the gel separated from the incubation mixture revealed the phosphatidylcholine was bound onto the gel. Approximately 60% of the lipid binding occurred in 12 hours, and the proceeding of the binding ended in 48 hours (Figure 3). All the thiol incorporating liposomes examined showed the binding of the composing phosphatidylcholines to the gel following the similar time course.

The lipid bound on the gel accompanied the FITC-dex encapsulated in the

initially interacted liposomes. Typically, the loss of the encapsulated FITC-dex to the bulk phase during the lipid absorption was less than 15%. Even with liposomes containing post-incorporated octanethiol, which is expected to disturb the liposomal membrane significantly, 73% of the encapsulated FITC-Dex was still retained with the bound phospholipid on the gel after 48 hours. The good retention of the encapsulated FITC-dex indicates that most of the liposomes kept their structural integrity as vesicles throughout the immobilization process and thereafter.

Once immobilized, both the lipid and the FITC-dex firmly stayed on the gel. No phosphatidylcholine was found spontaneously liberated from the gel in 24 hours. The immobilization was so stable that the liposome-gel conjugates thus obtained can be separated from the bulk aqueous phase using gentle washing on an ordinary glass filter.

For the immobilization, both the capped mercapto moieties on gel and a thiol incorporated in liposomes are essential. No binding of liposomal phosphatidylcholine occurred with either unmodified Sephacryl gel or plain liposomes. The results suggest that the immobilization of liposomes involves the *in situ* formation of disulfide linkages by the reaction between the capped mercapto moieties and the thiol molecules.

The immobilization was examined under various conditions, and the results are summarized in Table 1. When the amount of the thiol molecules in the interacting liposomes was equivalent to the capped mercapto moieties on the gel, 82% of the liposomal phosphatidylcholine in the system was found immobilized (entry 1). By increasing the amount of the gel in the system three times, the more gel mercapto moieties became available per liposomal thiol, and moderate increase in the extent of the immobilization was observed (93%; entry 4). Meanwhile, liposome immobilization was also affected by the amount of the thiol incorporated in the liposomes. When the thiol content was reduced to approximately one third to 9%, without changing the ratio of the gel mercapto moieties to the liposomal thiol molecule, the immobilized liposomal lipid decreased significantly to 61% (entry 5). Under the conditions, the liposomes probably failed to form a sufficient number of the anchorage to hold themselves stably on the gel [14].

Compared at the thiol content of 9 mol%, the extent of the immobilization was comparable for all the three thiols (61-68%; entry 5, 9 and 12) though thiocholesterol showed slightly higher extent of the immobilization. Among the three, thiocholesterol seems to be the least intrusive to liposomal membrane: the leakage of encapsulated

FITC-dex from the liposome interior was only 8%.

Under the comparable conditions, the amount of the immobilized liposomal phosphatidylcholine was similar or only slightly larger for the post-incorporated liposomes than the pre-incorporated ones. If all the thiol molecules added in the post-incorporation method were completely localized in the outer leaflet of the liposomal bilayer membrane, the outer surface of the liposomes should have twice as much thiol moieties as the pre-incorporation. Then, the higher extent of immobilization could be expected for the post-incorporation, particularly in the case of the thiol content of 9% in which the lack of the available thiol moieties seems to cause the less efficient immobilization (see above). The absence of this expected advantage for the post-incorporation suggests the presence of equilibration in the thiol distribution between the outer and the inner leaflets of the liposomal bilayer membrane. Meanwhile, no significant difference was seen in the retention of the encapsulated FITC-dex between the two preparation methods.

The minor but noticeable leakage of the encapsulated FITC-dex during the immobilization process indicates that the integrity of the liposomal membrane was temporarily disturbed. The previous investigation of the immobilization onto the hydrophobized gel [14] showed that a certain minimum number of the anchors are required for stable immobilization of liposomes. In the present case, the immobilization of a liposome is expected to begin with initial tentative association of thiol-incorporating liposome and Py-gel through the formation of a small (but less than the required minimum) number of the disulfide linkages. If the sufficient number of additional linkages are formed in time, the liposome is to be bound firmly on the gel. However, if it fails, the liposome may dissociate leaving the anchors attached on the gel. The process may involve the forced removal of the anchors from the liposome, which should disturb the membrane integrity. Furthermore, these anchors left on the gel are eventually used in the tentative association with another liposome by inserting themselves into the liposomal membrane. This may also cause disturbance to the membrane and result in the leakage of the contents [14]. The tentative association and dissociation of liposomes should gradually accumulate hydrophobic anchors on the gel. This could promote the pre-association and thus the following formation of disulfide linkages, by bringing the two reacting sites, the thiol molecules on the liposomal membrane and the capped mercapto moieties on the gel, in proximity.

Under comparable conditions, the amount of the immobilized liposomes in the present study was similar to that observed in the immobilization of plain liposomes onto the pre-hydrophobized gel [14]. For example, upon the interaction of liposomes containing post-incorporated thiocholesterol with 1 g of Py-gel, 20.4 μmol of liposomal phospholipid was immobilized while the gel prepared by pre-reacting thiocholesterol and Py-gel could hold 21.0 μmol of the lipid from interacted plain liposomes.

During the immobilization, adhesion between free liposomes or between a free liposome to an immobilized one may proceed. This could deter the immobilization by consuming the free thiol moieties. Also, the more anchors should be required for such oligo-liposomes to be held on the gel. Under the present condition, however, no significant change indicating the inter-liposomal adhesion, such as aggregation, was noticed in the free liposome fraction at least during the early period of the immobilization. Furthermore, the high immobilization efficiency (often larger than 80%, see Table 1) proves that sufficient immobilization can be achieved with the present system even if the hindrance to the immobilization by the inter-liposomal adhesion may exist. In any event, the adhesion should be solved in the detachment process with a reducing agent (see below).

Treatment of liposome-interacted gels with dithiothreitol

Coincubation of the liposome-gel conjugates with dithiothreitol (DTT), which efficiently reduces and cleaves disulfide linkages and has frequently been used in biochemical researches [18], released the immobilized phosphatidylcholine from the gel into the bulk aqueous phase. In Figure 4, the time courses of the release in the case of the post-incorporated liposomes are shown. For all the three thiols, the time courses were similar. Approximately 90% of the release was observed in 6 hours, and the release almost ended in 12 hours.

The released phosphatidylcholine existed exclusively in the form of liposomes. In size exclusion chromatographic analysis, the supernatant separated from the gel after the DTT treatment only contained aggregates of the size comparable to the initially interacted liposomes (data not shown). By the DTT treatment, typically, 80-85% of the FITC-dex that had been retained in association with the immobilized liposomal phosphatidylcholine was also liberated from the gel. According to the result of the

size exclusion chromatography, approximately 70-80% of the liberated FITC-dex was found encapsulated in the released liposomes. The behavior of the thiol-containing liposomes so far observed in the present study indicates that most of the liposomes maintain their vesicle form and integrity throughout the immobilization and the release processes.

No release of the immobilized phosphatidylcholine occurred when the liposome-gel conjugate was treated similarly with threitol in place of dithiothreitol. Threitol lacks the two mercapto moieties in the structure of dithiotheiol and thus has no ability to cleave disulfide linkages. The result indicates that the reductive cleavage of the disulfide linkages caused the detachment of the immobilized liposomes. With these immobilization and release behaviors observed so far in the present study, one may conclude that the liposomes are immobilized indeed by *in situ* formation of the linkage by the reaction between the mercapto moieties on the gel and the thiols in the liposomal membrane.

In the release of immobilized liposomes by the DTT treatment, a small percentage (typically 20%) of the FITC-dex retained on the gel was found in the bulk aqueous phase, not encapsulated in the released liposomes. The leak suggests of perturbation to the liposomal membrane during the detachment process. This may be explained by the forced removal or "ripping" of the hydrophobic anchors from the liposomal bilayer membrane when the number of the remaining anchors becomes below the minimum after the cleavage of the majority of the disulfide linkages.

The DTT treatment leaves on the gel approximately 20% of the immobilized phosphatidylcholine and FITC-dex not liberated. Further treatment of the gel with a fresh DTT solution released only additional 1% of the initially immobilized phosphatidylcholine. The rest of the immobilized phosphatidylcholine and FITC-dex could not be detached by treating with DTT under the present conditions.

Table 2 shows the extent of the detachment of the liposomal phospholipid by the DTT treatment from various liposome-gel conjugates. The slightly higher percentage of the detachment of the phospholipid was observed for the gels obtained by immobilization of liposomes incorporating octanethiol (C_8SH) than thiocholesterol (CholSH). There was no significant difference in the detachment between the immobilized liposomes obtained with the pre- and the post incorporation methods. Together with the similarity also observed in their immobilization behavior, one may

conclude that the both liposome preparation procedures result in essentially identical immobilized liposomes. In actual applications, the post-incorporation would be preferable for its superior efficiency in the encapsulation and easy optimization of the type and the amount of the incorporating thiol.

The release behavior described above is essentially same as that seen with the liposome-gel conjugate obtained by the interaction of plain liposomes with the hydrophobized gel in the previous study [14]. The both protocols are expected to yield an identical liposome-gel conjugate (Scheme 2), and the present results indicate that this indeed is the case.

Interaction of thiol-incorporating liposomes with gels after first detachment

To further examine the present system, the gel left after the detachment was interacted again with fresh thiol-liposomes. First in the experiment, plain liposomes (no thiol incorporated) were immobilized onto the hydrophobized gel obtained from Py-gel and octanethiol as in the previous study [14]. Following treatment of the liposome-gel conjugate with DTT detached 83% of the immobilized liposomes. The gel was then coincubated with liposomes pre-incorporating 25 mol% of octanethiol.

In 24 hours at 5^{o}C, 64% of the thiol-containing liposomes were immobilized onto the gel. Following treatment of the gel with DTT liberated 95% of the newly immobilized liposomes. The results indicate that the detachment of the immobilized liposomes leaves regenerated mercapto moieties on the gel that are still capable of reacting with membrane-incorporated thiols to immobilize a fresh batch of liposomes. Although no oxidizing agent was added in particular, the oxygen dissolved in the aqueous phase seems to be sufficient to cause the formation of the disulfide linkages.

While the release is close to quantitative for the liposomes conjugated in the second immobilization, the first immobilization consistently leaves approximately 20% of the liposomal phospholipids that cannot be detached from the gel with the DTT treatment (see above). In the present case, 17% of the liposomal phosphatidylcholine remained. There seems to be two distinct types of the immobilization sites on the gel. Approximately 80% of immobilized liposomes occupy a site, in which the immobilization and the detachment of liposomes occur by the formation and the cleavage of disulfide linkages with almost quantitatively. In contrast, the other site

irreversibly traps approximately 20% of liposomal lipids. Although the exact nature of the site that causes the irreversible immobilization is not apparent, the observation obtained so far in the previous and the present studies suggests a few things. As it can be seen in the fact that the second release is quantitative, the immobilization process does not induce propagation of the site. The site or its "precursor" should already be present in the modified gel before interaction with liposomes, and the site is permanently occupied during the first immobilization. Also, the interaction is non-covalent because the phospholipids that could not be detached from the gel with DTT can be removed with sodium dodecyl sulfate [14]. The solubilization causes the destruction of large aggregate structure to small micellar aggregates. This observation suggests that the irreversibly immobilized phospholipid could exist as larger liposomes or aggregates that are trapped in the gel structure due to steric hindrance.

CONCLUSIONS

The present study revealed the basic behavior of the novel liposome immobilization system in which the binding is achieved through *in situ* formation of disulfide linkages. Throughout the studies, the observed behavior of the system is consistent with the expected immobilization-detachment scheme (Figure 2). The observation made in this study is valuable as an example of covalent bond formation in the interfacial region between liposomal bilayer membrane and cross-linked polymer gels.

The present system may be useful as a versatile support material of small liposomes to assist their manipulation such as separation of liposomes from the bulk aqueous phase. Almost quantitative binding of liposomes to gel is possible by selecting an appropriate liposome-gel ratio in the immobilization. Also, nearly quantitative detachment of immobilized liposomes is achieved by pre-conditioning of the gel with liposomes in advance. Construction of the immobilization system can be as simple as mixing a suitable thiol to pre-formed liposomes.

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Figure captions

Figure 1. Concept of immobilization and detachment of liposomes. Liposome is immobilized by hydrophobic anchors linked to polymer gel particles via disulfide linkage. Reductive cleavage of the disulfide bond releases the immobilized liposome.

Figure 2. Immobilization and detachment schemes of the system. The paths newly explored in the present study involve *in situ* formation of disulfide linkage and are indicated with bold lines.

Figure 3. Time course of liposomal phospholipid immobilization. EggPC liposomes containing 25 mol% thiols (phospholipid concentration 2 mM) were incubated with Sephacryl gels bearing 2-pyridinedithio moieties (Py-gel) at 37 ^{o}C.

Figure 4. Time course of phospholipid release from liposome-gel conjugates. The conjugates obtained from thiol-containing liposomes were incubated in dithiothreitol solutions at 37 ^{o}C.

Tables

Table 1

Immobilization of liposomal phospholipids to gels

Entry	Thiol	Incorporation method	Thiol content ()	Thiol/Py moiety (by mol)	Immobilized phospholipid ()
1	C_8	post	25	1	82
2		post	25	2/3	83
3		post	25	1/2	85
4		post	25	1/3	93
5		post	9	1/3	61
6		pre	25	1	82
7		pre	9	1/3	55
8	C_{16}	post	17	2/3	83
9		post	9	1/3	64
10		pre	17	2/3	78
11		pre	9	1/3	57
12	Chol	post	9	1/3	68
13		pre	9	1/3	59

Thiols: C_8, 1-octanethiol; C_{16}, 1-hexadecanethiol; Chol, thiocholesterol

Liposomal phospholipid concentration, 2 mM; interacted at 37^{o}C for 48 hours

Table 2

Detachment of phospholipids from gels by dithiothreitol

Thiol	Incorporation method	Immobilized Phospholipid (μmol)	Phospholipid detached (%)
C_8	post	24.7	83
		13.6	88
		10.6	82
		6.3	84
	pre	24.7	83
		12.4	88
C_{16}	post	24.1	79
		11.8	76
	pre	23.5	79
		12.8	80
Chol	post	20.4	69
		11.6	70
	pre	17.6	73
		13.2	72

Thiols: C_8, 1-octanethiol; C_{16}, 1-hexadecanethiol; Chol, thiocholesterol

Dithiothreitol /incorporated thiol = 20; 37^{o}C, 24 hours

Figure 1

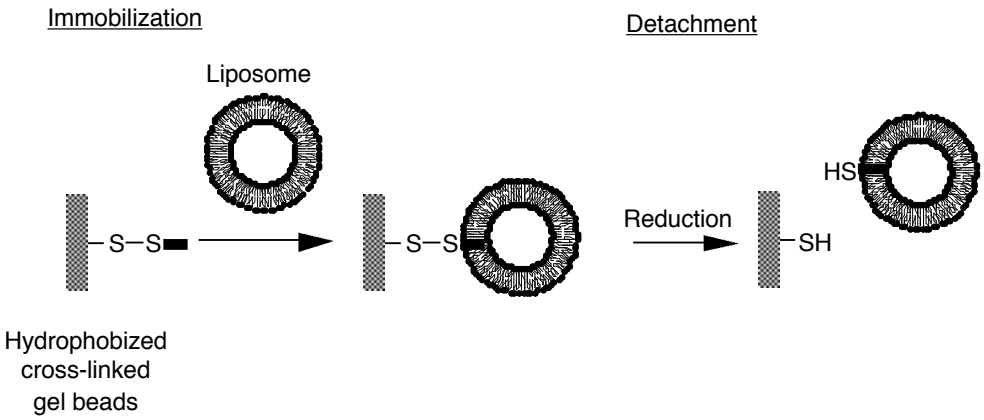
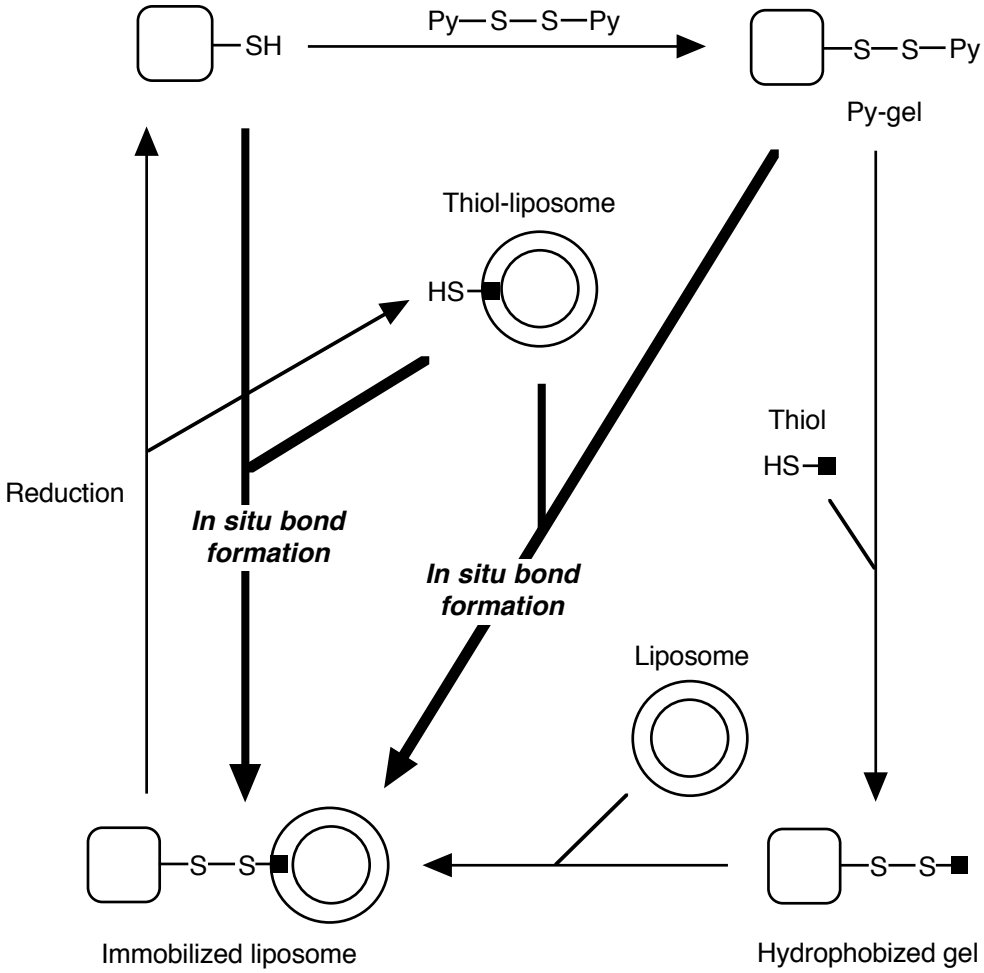

Immobilization
Liposome
S—S
S—S
Detachment
Reduction
HS
SH
Hydrophobized
cross-linked
gel beads

Figure 2



SH = Gel—$OCH_2CHCH_2O(CH_2)_4OCH_2CHCH_2SH$ (OH, OH)

Py = 2-pyridyl

Figure 3

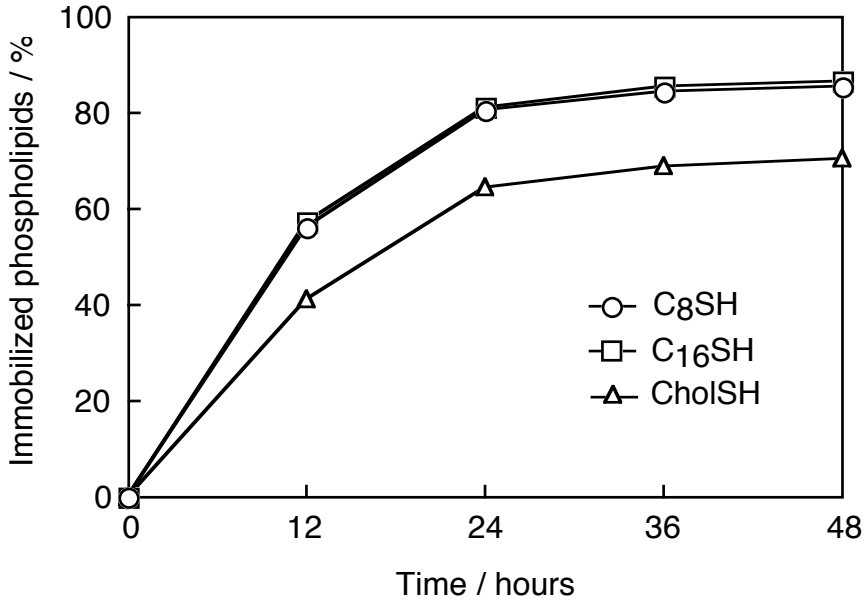
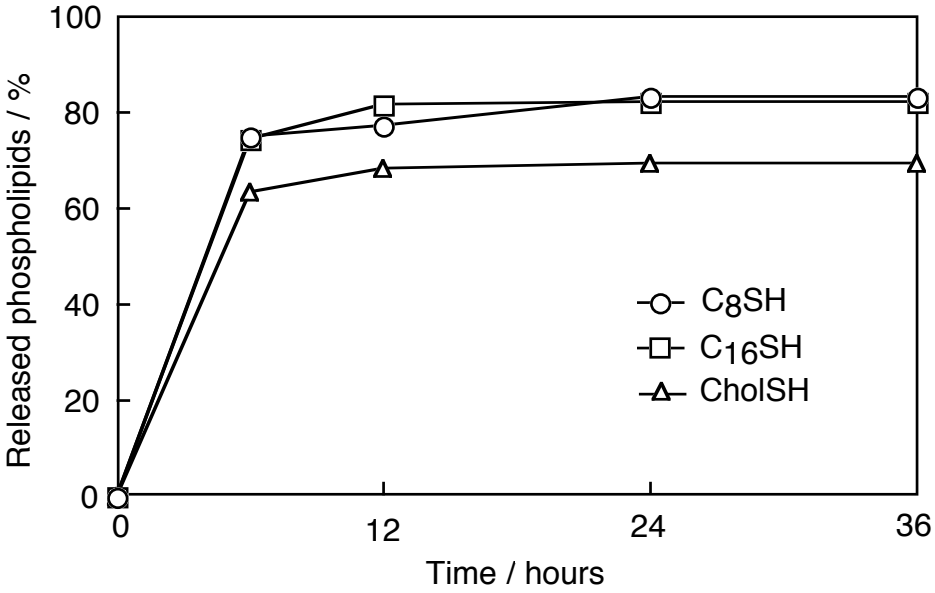

Immobilized phospholipids / %
100
80
60
40
20
0
0
12
24
36
48
Time / hours
C8SH
C16SH
CholSH

Figure 4


100
80
60
40
20
0
Released phospholipids / %
0
12
24
36
Time / hours
C8SH
C16SH
CholSH