Liposome Immobilization on Polymer Gel Particles by in situ Formation of Covalent Linkages

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In situ formation of disulfide linkages between liposomes and Sephacryl gel particles both of that were bearing mercapto moieties was demonstrated. The reaction led to immobilization of the liposomes on the gel. The immobilized liposomes could be detached by reductive treatment of the liposome-gel conjugate, and the resulting gel was still capable of further immobilizing liposomes.

Conjugation of liposomes, artificial vesicles of lipid bilayer membrane, with other materials such as polymers has been actively studied in quests for novel materials with advanced functionality. Such conjugation has most often been achieved by first modifying a polymer with hydrophobic moieties, that work as anchors to liposomal bilayer membrane.

Logical alternative to this approach is first modifying liposomal surface with reacting groups. A following reaction of the groups with the corresponding ones on a polymer should achieve the conjugation of the two. However, such direct formation of covalent bonds between a lipid membrane component and a polymer, especially, a rigid cross-linked one, has been little studied. An insight into such a reaction should be valuable in development of liposome-polymer conjugates in general.

In the present communication, we tried in situ formation of disulfide linkages between liposomes and Sephacryl S-1000 gel particles both of that were bearing mercapto moieties. The successful reaction should lead to immobilization of the liposomes on the rigid cross-linked polymer gel. Such immobilized liposomes on cross-linked polymer gels[1,2] were used, for example, in estimation of drug partition as a cell membrane model.[3,4] We previously reported the immobilization of liposomes by hydrophobic anchor moieties conjugated via disulfide linkages and the detachment by reductive cleavage of the linkages (Figure 1).[5] The successful in situ reaction should yield the same liposome–gel conjugate and significantly benefit its use in practical applications by opening a door to a repetitive use of the relatively costly gel.

Sephacryl S-1000 gel was chemically modified to bear mercapto groups (9–10 μmol per gram of the swelled gel) as previously described.[5] The mercapto moieties were further converted to pyridinedithio groups by a reaction with 2,2′-dipyridyl disulfide (2-PDS) to avoid undesirable oxidation (Figure 1; Py-gel). No noticeable destruction of the gel particles was observed.

Plain liposomes were first prepared from egg phosphatidylcholine (eggPC) in 0.1 M HEPES buffer (pH 7.5) by the extrusion method.[6] The liposomes (15 ml, containing 30 μmol of eggPC) were mixed with an appropriate amount of 1-octanethiol or 1-hexadecanethiol dissolved in ethanol (1.2 ml) and incubated for 24 h. The incorporation was also performed on eggPC liposomes encapsulating fluorescein isothiocyanate conjugated dextran (FITC-Dex; approximate MW 45000). The structural integrity of the liposomes after the thiol incorporation (thiol-liposomes) was confirmed by gel filtration with a Sepharose 4B column.

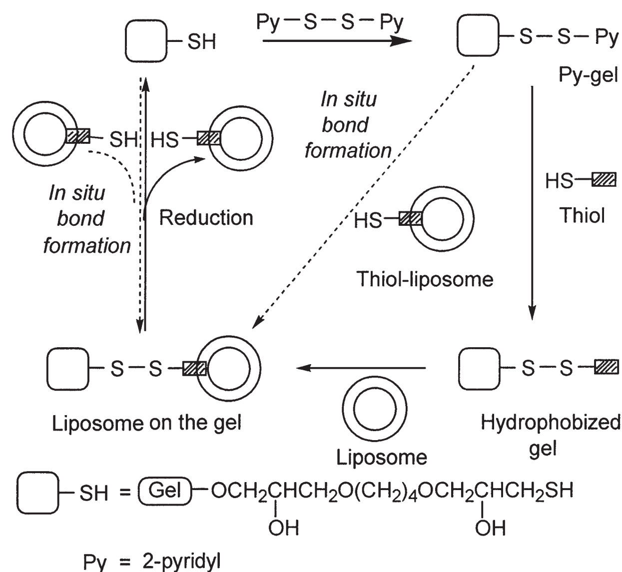

Figure 1. Immobilization and detachment of liposomes on a cross-linked polymer gel. Dotted arrows indicate in situ formation of disulfide linkage.

We first examined interaction of the thiol-liposomes with Py-gel. In a typical experiment, thiol-liposomes (PC concentration 2 mM) were mixed with wet Py-gel (1.0 g) and incubated at 37 °C. Approximately 50% of the liposomal PC was adsorbed to the gel in 12 h upon the coincubation, and the adsorption was almost completed in 24 h (Figure 2). Under the conditions, the

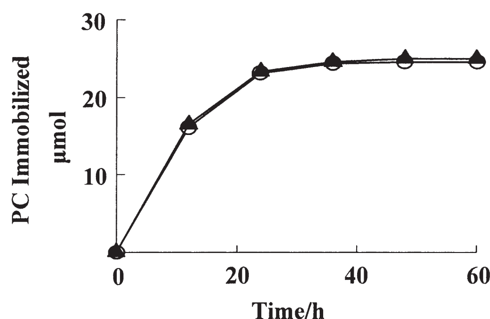

Figure 2. Immobilization of thiol-liposomes to Py-gel. Total phosphatidylcholine (PC), 40 μmol; thiol content, 25 mol% for C_8 (○) and 17 mol% for C_{16} (▲); 37 °C.

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progress of the adsorption was slower than that observed for plain liposomes immobilizing to the hydrophobized Sephacryl gel (approximately 50% of the adsorption occurred in 6 h; unpublished data).[5] No attachment of the liposomal PC was observed either with unmodified Sephacryl gel or with plain liposomes. The result is consistent with the idea that the adsorption of the PC should be based on the in situ formation of disulfide linkages.

The liposomal PC adsorbed on the gel accompanied the FITC-Dex initially encapsulated in the thiol-liposomes. The retention of the FITC-Dex suggests that the PC was immobilized without losing the initial structural integrity of liposomes.

The immobilization of the PC and the FITC-Dex on the gel was firm and stable. No spontaneous release of the PC or only marginal liberation of the FITC-Dex from the gel was observed in 24 h. In fact, the immobilization was so stable that the gel could be gently washed on a glass filter without noticeable detachment of the liposomal PC.

The extent of the immobilization of liposomal PC depended on two factors (Table 1). By decreasing the ratio of the liposomal thiol to the pyridine-capped mercapto moieties (SH/Py-moiety ratio), the immobilization became almost quantitative (93%). The thiol content in the liposomes also showed a significant effect. With 9 mol%, the extent of the immobilization was reduced to 61% although the SH/Py-moiety ratio was approximately same to the case of the 93% immobilization. Sufficient density of the mercapto moieties on the liposomal surface seemed to be necessary for efficient immobilization. Under the conditions examined, the maximum amount of the liposomal PC immobilized on the Py-gel was 2.47 per Py-moiety. No significant difference in the immobilization behavior between 1-octanethiol and 1-hexadecanethiol was observed.

Table 1. Adsorption of liposomal lipid on Py-gel[a]

Thiol	Thiol content in liposome/mol%	Thiol/Py moiety[b]	Immobilized phospholipids/%
C_8	25	1.00	82
	25	0.66	83
	25	0.33	93
	9	0.29	61
C_{16}	17	0.39	80
	17	0.62	83
	9	0.29	64

[a]Lipid concentration 2 mM; 37 °C after 48 h.
[b]Liposomal thiol/pyridine-capped mercapto moieties on the gel.

The gels with immobilized liposomal PC was then placed under the reductive conditions. Typically, the gel (1.0 g) was incubated in the HEPES buffer containing dithiothreitol (DTT) in large excess (20 equivalents to the amount of the Py-moieties initially present on the gel). In 6 h, 80–85% of the immobilized PC was released into the bulk aqueous phase, and the release was almost completed in 12 h. At the same time, the FITC-Dex immobilized on the gels with the PC was also liberated. Gel filtration of the supernatant separated after the DTT treatment revealed that the released PC was present exclusively as liposomes and that 70–80% of the liberated FITC-Dex was encapsulated. The majority of the immobilized PC was most likely to be immobilized on the gel as liposomes.

Table 2. Release of adsorbed liposomal phospholipid[a]

Thiol	Adsorbed lipid per Py moiety	Lipid released /%
C_8	2.47	83
	1.66	83
	0.84	85
C_{16}	2.41	76
	1.56	79

[a]Dithiothreitol per Py moiety, 20; 37 °C after 24 h.

Table 2 depicts some of the typical detachment behaviors. For all the gels that were immobilizing liposomes (liposome–gel conjugates) obtained under the various conditions examined, the extent of the detachment could not exceed 80–85%. Approximately 15–20% of the immobilized liposomes remained undetachable by the DTT treatment on the gel. The higher detachment efficiency than the previous report (50–60%)[5] is mainly due to improvement in the experimental procedure.

In various aspects, including the time course and the percentage of the undetachable liposomes, the release behavior observed for the liposome-gel conjugates obtained from the thiol-liposomes and Py-gel was quite similar to that of the plain liposomes immobilized on the hydrophobized gels (unpublished data). In our present scheme, the two different immobilization procedures should yield the same liposome–gel conjugate (Figure 1), and this observation is consistent of the idea.

We further proceeded to the investigation of the interaction between thiol-liposomes and the gel that was left after the detachment reaction with DTT. After the detachment of 83% of the immobilized liposomes, the gel was again coincubated with a fresh batch of liposomes incorporating 25 mol% of 1-octanethiol for 24 h at 5 °C. By the interaction, 64% of the liposomes were immobilized to the gel. Following DTT treatment released liposomes that corresponded to 94% of the newly immobilized ones, proving the immobilization by in situ formation of the disulfide linkages between the thiol-liposomes and the free mercapto moieties on the gel. The almost quantitative release in the second immobilization-detachment cycle suggests the presence of two different types of the sites on the gel; one for permanent immobilization of liposomes and the other for quantitative detachment. The successful repetition of the cycle further supports the validity of our scheme of the immobilization and the detachment of liposomes on cross-linked polymer gels.

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