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# $Ca^{2+}$-mediated interaction between negatively charged and neutral liposomes

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In the present work it is shown that large unilamellar lecithin/cholesterol liposomes are able to sequester small negatively charged liposomes in the presence of divalent cations. Evidence is presented suggesting that the sequestration occurs via the formation of membrane invaginations transformed further into intraliposomal vesicles.

Liposome; Divalent cation; Intermembrane interaction

## 1. INTRODUCTION

Recently, we showed that lecithin/cholesterol LUVs (7:3, M/M) can sequester polynucleotides in the presence of $Me^{2+}$ [1]. Both biochemical and microscopic data indicate that the sequestered material is located within the vesicles derived from LUV membrane invaginations [1]. Here we describe the interaction between the LUVs and negatively charged SUVs under similar conditions.

## 2. MATERIALS AND METHODS

Egg yolk PC and PE were prepared according to [2–4]. All other chemicals used were of analytical grade. Br-R-N-O was a generous gift of Dr. L.B. Volodarsky. NBD-PE was synthesized according to Struck et al. [5]. PE-R-N-O was prepared by reacting PE with Br-R-N-O [1].

LUVs were prepared from DPPC/cholesterol (7:3, M/M) by using a reverse-phase evaporation technique [6]. SUVs were made from PC/DCP (7.5:2.5, M/M) by sonication [7] and, if required, passed over a Sephadex G-75 column to remove non-incapsulated material

RET was detected using donor and acceptor membranes containing 1% of NBD-PE or Rd-DPPE, respectively, as described in [6]

In binding experiments, LUVs (0.21 mM DPPC) were incubated with [$^{32}$P]ATP-containing SUVs (0.225 mM PC) in 10 mM Tris-HCl (pH 7.6)/150 mM sucrose at various $CaCl_2$ concentrations for 5 min at 20°, after which LUVs were either pelleted by centrifugation at 10,000 × *g* for 5 min or made 20 mM in EDTA and washed 3–4 times by centrifugation and resuspension in 10 mM Tris-HCl (pH 7.6)/110 mM sucrose/20 mM EDTA. The efficiency of SUV adsorption and irreversible binding by LUVs was calculated as a percentage of LUV-associated $^{32}$P-radioactivity in the former and latter cases, respectively. The efficiency of SUV aggregation was measured as a percentage of SUVs pelleted under the same conditions in 10 mM Tris-HCl (pH 7.6) in the absence of LUVs.

In electron microscopy experiments, SUVs (1.5 mg/ml) and LUVs (1.5 mg/ml) prepared in 10 mM sodium acetate (pH 4.7) were mixed (1:3) and 5 μl aliquots applied to carbon-coated discharged microscopic grids. Thirty seconds later, excess liquid was blotted and replaced with 4–5 μl of 1% uranyl acetate which was removed in 30 s by blotting. The preparations were air-dried and examined in a JEM-100C microscope.

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*Abbreviations.* LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; $Me^{2+}$, divalent metal ions; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DCP, dicetyl phosphate; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; Rd-DPPE, *N*-(lissamine rhodamine B sulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; Br-R-N-O, 4-bromomethyl 2,2,3,5,5-pentamethylimidazolidin-1-oxyl; NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, CF, carboxyfluorescein; ESR, electron spin resonance; EM, electron microscopy; RET, resonance energy transfer

## 3. RESULTS

To study the interaction of neutrally charged LUVs with negatively charged particles, SUVs of PC/DCP mixture (7.5:2.5, M/M) were used. The presence of PC was required to diminish the fusion and rearrangement of SUVs in the presence of $Ca^{2+}$ ions [8,9]. The integrity of SUVs at 10 mM $CaCl_2$ was confirmed by using the CF fluorescence dequenching assay at the intravesicular CF concentration of 50 mM [10]. No CF release was observed under these conditions (not shown). Therefore, the aggregation of SUVs observed by an increase in the incubation mixture turbidity after the addition of greater than 2 mM $CaCl_2$ (Fig. 1a) had little if any effect on SUV integrity.

Once initiated at the concentration of $CaCl_2$ over 5 mM, the aggregation partially persisted, decreasing by about 90%, after the addition of EDTA to the final concentration of 20 mM. The fractionation of SUVs by

chromatography on Sephacryl S 1000 combined with EM revealed a $Ca^{2+}$-dependent increase in the average SUV diameter from 27 to 40,5 nm. The resonance energy transfer assay with the mixture of SUVs containing 1% of either NBD-PE or Rd-DPPE [6] has shown significant fusion of SUV membranes coincident with the aggregation (not shown).

The incubation of SUVs with LUVs was carried out in medium allowing precipitation of LUVs but not SUVs by centrifugation. The adsorption of SUVs on LUVs, detected by SUVs co-precipitation, was strictly $Ca^{2+}$-dependent (Fig. 1b) and inhibited by EDTA (not shown). However, EDTA added after $Ca^{2+}$ could not completely dissociate LUV-SUV complexes. A small fraction of the SUVs was found in the pellet even after extensive washing of the LUVs with EDTA-containing buffer (Fig. 1c). The irreversible binding of SUVs correlated with SUV adsorption (Fig. 1b,c) and was inversely dependent on SUV aggregation (Fig. 1a,c). Similar isotherms for SUV aggregation, adsorption and irreversible binding were obtained using other divalent metal ions, in particular uranyl ions (Fig. 1d,e and f, respectively).

The irreversible binding of SUVs caused no changes in the self-quenching of intravesicular CF fluorescence, which ruled out the possibility of fusion between LUVs and SUVs under the experimental conditions. No membrane fusion has been detected by a resonance energy transfer assay with NBD-PE-labeled LUVs and Rd-DPPE-labeled SUVs (not shown; [6]).

The binding experiments with spin-labeled SUVs [11] showed that, once irreversibly bound, SUVs were poorly accessible to ascorbate ions during short-term incubation (Fig. 2). Low accessibility, if any, of the irreversibly bound SUVs to small molecules in the me-

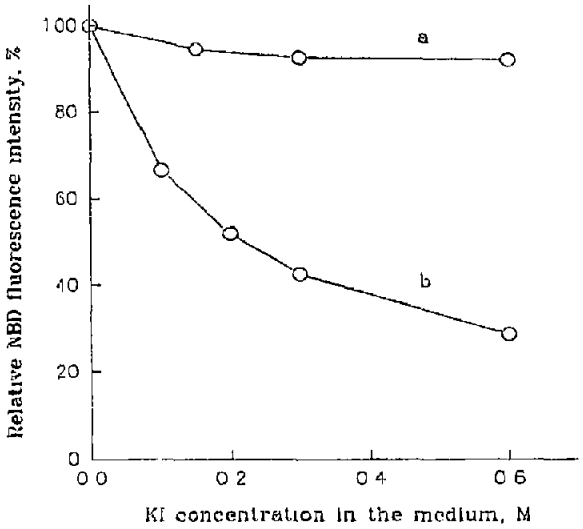


Fig. 2. Accessibility of irreversibly bound SUVs to $I^-$-ions. Fluorescently labeled PC/DCP/NBD-PE SUVs (74.5:25:0.5, M/M) were incubated with LUVs and then washed out as described in section 2. (a) The LUVs were resuspended in 10 mM Tris-HCl (pH 7.6)/150 mM sucrose to the final lipid concentration of 0.2 mM and titrated with 5 M KI. NBD fluorescence was detected at 535 nm (excitation wavelength = 468 nm) with a Hitachi MPF-4 instrument. (b) The initial SUVs were analyzed at the same lipid concentration.

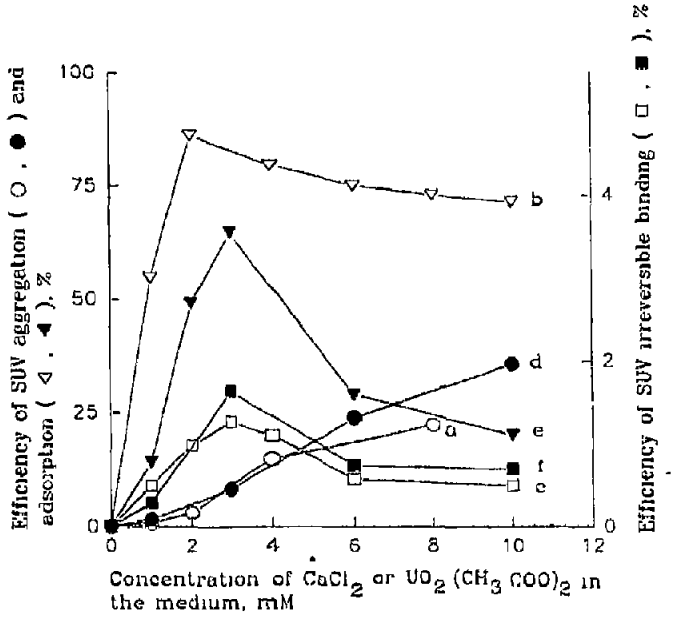


Fig. 1. Aggregation (a and d) of SUVs and adsorption (b and e) and irreversible binding (c and f) of SUVs by LUVs in the presence of $CaCl_2$ (open symbols) or uranyl acetate (closed symbols) were assessed as described in section 2.

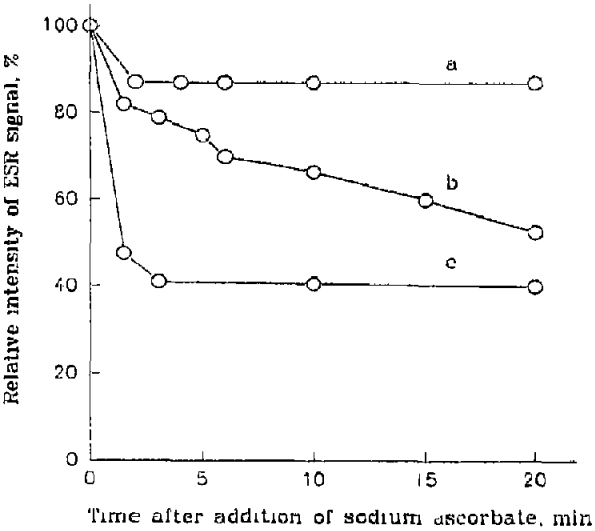


Fig. 3. Accessibility of irreversibly bound SUVs to ascorbate ions. PC/DCP/PE-R-N-O SUVs (74:25:1, M/M) were incubated with LUVs and then washed out as described in section 2. The LUVs were resuspended in 10 mM Tris-HCl (pH 7.6)/150 mM sucrose/10 mM sodium ascorbate to the final lipid concentration of 0.2 mM and the ESR signal was measured in an ER-200-SRC Bruker instrument at 9.74 MHg (a). The initial SUVs (b) and spin-labeled DPPC/cholesterol/PE-R-N-O LUVs (69.30.1, M/M) (c) were analyzed at the same lipid concentration.

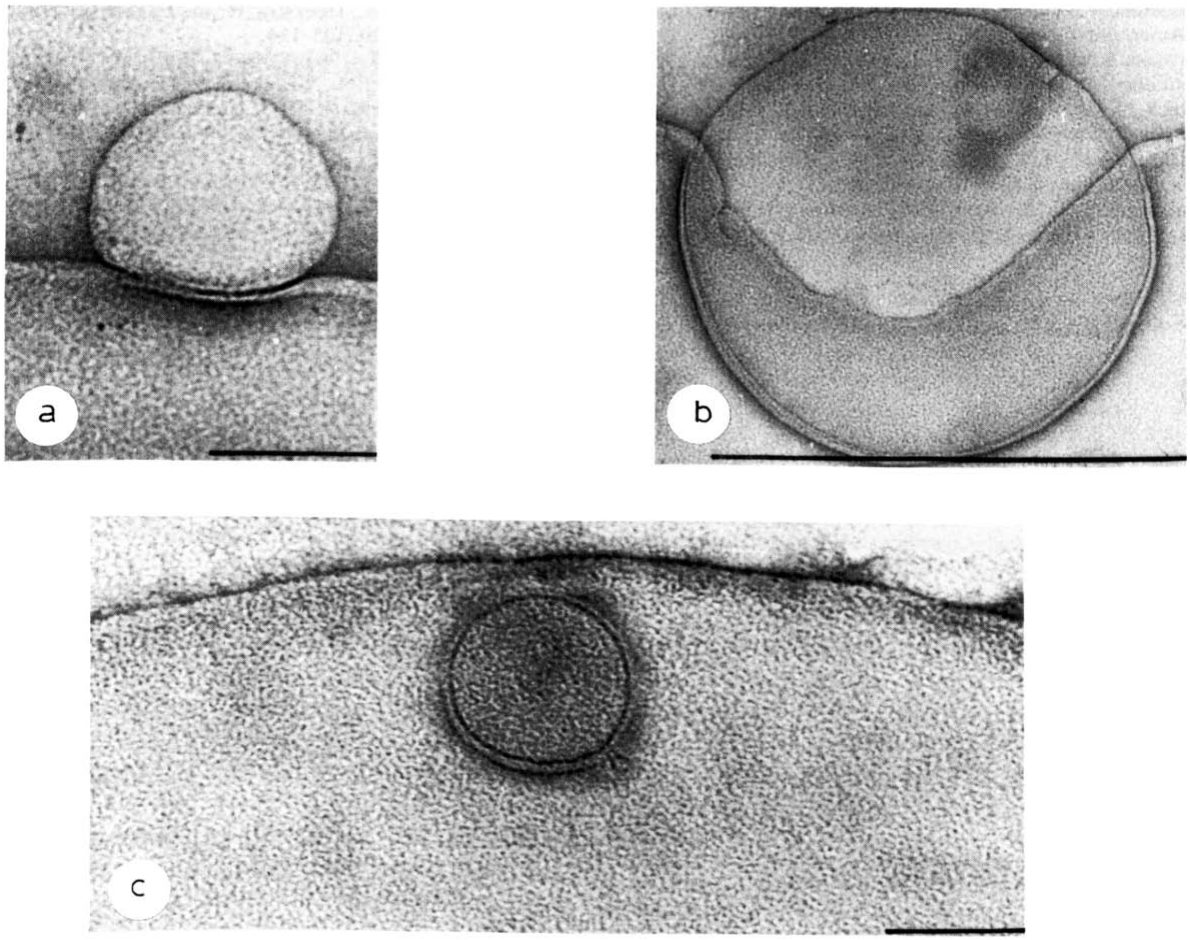


Fig. 4. Micrographs of uranyl ions-mediated SUV/LUV complexes. SUVs were observed on the LUV surface (a), within LUV membrane invaginations (b), and apparently in the LUV inner volume encoated by an additional membrane (c). Bar = 0.03 μm.

dium was also observed with NBD-labeled SUVs [12] probed with iodide ions (Fig. 3). These results strongly suggest the location of irreversibly bound SUVs within the LUVs. An alternative explaination might be dehydration of the SUV surface regions interacting with LUVs. However, it seems highly unlikely since no $Me^{2+}$-mediated dehydrated contacts between phospholipid membranes in the presence of EDTA have been so far observed with a variety of experimental systems.

It has been shown by using a Tb/dipicolinic acid assay [13] that sequestration of SUVs is not associated with LUV fusion. The intensity of terbium fluorescence, which was 50–100 times lower than that derived by co-sonicating $Tb^{3+}$- and dipicolinic acid-containing LUVs, was independent of the order of adding $CaCl_2$ and EDTA. Similarly, no fusion of LUVs associated with SUV sequestration has been detected by using the RET technique ([6]; not shown).

Electron microscopy showed that the SUVs incubated with LUVs in the presence of uranyl ions were either (i) adsorbed on the LUV surface (Fig. 4a), (ii) sequestered within membrane invaginations (Fig. 4b), or (iii) apparently located within the LUVs being encoated with an additional membrane (Fig. 4c). It is likely that the described structures might appear in this order in the course of SUV internalization.

## 4. DISCUSSION

The results in this study indicate that neutrally charged LUVs can sequester negatively charged vesicles as well as polynucleotides in a $Me^{2+}$-dependent way. In both systems, the sequestration is similarly affected by a number of different factors (concentration of $Me^{2+}$, LUV aggregation etc.), which suggests a common sequestration mechanism [1,6]. Generally, one might expect that multisite binding of a particle with a phospholipid membrane would be an essential prerequisite for its translocation across the membrane.

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