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Effect of surface modification on aggregation of phospholipid vesicles

(liposome/macrophage/phagocytosis/proton magnetic resonance/drug delivery system)

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ABSTRACT **Phospholipid vesicles have been extensively investigated because of their usefulness as models for biological membranes and their potential application as carriers for drug delivery. However, preparations of small sonicated vesicles tend to aggregate and fuse (on storage at room temperature and at 4°C), resulting in significant changes in turbidity, rate of uptake by macrophage, and proton NMR linewidths. By modification of the surface of phospholipid vesicles with charged groups such as β-aminogalactose that extend significantly from the vesicle surface, it is possible to obtain preparations that are stable for >7 days.**

Phospholipid vesicles have been extensively investigated because of their usefulness as models for biological membranes and their potential application as carriers for drug delivery (1–4). We have recently found that modification of the surface of distearoyl phosphatidylcholine vesicles by specific synthetic glycolipid determinants can affect the rate of uptake of these vesicles by mouse peritoneal macrophage *in vitro* and the differential tissue distribution of these vesicles *in vivo* in mice (5–9). However, small sonicated vesicles are thermodynamically unstable, and the properties of these vesicles can change significantly in the temperature range at which phase transitions occur (10–12). In particular, small sonicated vesicles tend to aggregate and fuse below the phase transition temperature, resulting in an increase in vesicle size as a function of time (13–16). As these changes will ultimately affect the practical usefulness of phospholipid vesicles for drug delivery, we report in this paper studies of the effect of surface modification on the aggregation and fusion of phospholipid vesicles and on the rate of uptake of these vesicles by mouse peritoneal macrophage.

MATERIALS AND METHODS

Materials. L-α-Distearoyl phosphatidylcholine ($Ste_2PtdCho$) from Calbiochem and cholesterol (Chol) from Sigma were used without further purification. Mannosyl, aminomannosyl, and aminogalactosyl derivatives of Chol [6-(5-cholesten-3β-yloxy)hexyl 1-thio-α-D-mannopyranoside (ManChol), 6-(5-cholesten-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-α-D-mannopyranoside (NH_2ManChol), and 6-(5-cholesten-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-β-D-galactopyranoside (NH_2GalChol), respectively] were synthesized at Merck. [*oleate*-1-^{14}C]Cholesteryl oleate (specific activity, 51 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear.

Newborn calf serum, medium-199, and penicillin/streptomycin were purchased from Microbiological Associates (Los Angeles, CA) and plastic Petri dishes (35 × 10 mm) were obtained from Falcon. D_2O (99.8% D) was purchased from Aldrich.

Preparation of Liposomes. Small unilamellar vesicles were prepared according to the method of Mauk and colleagues (5–9). Briefly, a lipid mixture was prepared by mixing $Ste_2PtdCho$, Chol, NH_2ManChol (or NH_2GalChol), and A_{23187}, 2:0.5:0.5:0.004 (mol/mol) or as otherwise specified. The mixture was dried in vacuum overnight and then probe sonicated in phosphate-buffered saline, pH 7.4. ^{14}C-Labeled cholesteryl oleate was included as a marker for the lipid phase. After sonication, annealing, and low-speed centrifugation, the vesicles were passed over a Sephadex G-50 column equilibrated with phosphate-buffered saline. Total phospholipid was determined by phosphorus assay using the method of Marinetti *et al.* (17).

Proton Magnetic Resonance Measurements. Vesicle samples were prepared in D_2O at pD 7.8 (≈pH 7.4) in phosphate-buffered saline for PMR measurements. All PMR spectra were taken on a Brucker WM 500 spectrometer equipped for proton resonance at 500 MHz. All spectra were obtained with 8000 data points, and computer-aided signal averaging was used to enhance the signal-to-noise ratio. The temperature of the sample was controlled by a BVT 1000 variable-temperature unit and was maintained at either 25°C or 37°C.

Cultivation of Mouse Peritoneal Mononuclear Phagocytes. Cells from the peritoneal cavity of unstimulated male Swiss–Webster mice (25–30 g) were harvested as described (9).

Measurements of Phagocytosis. Freshly prepared (or aged) liposomes were added to the Petri dish cultures to an activity of ≈15,000 cpm (≈30 μg of P), and phagocytosis was measured as described (9).

RESULTS

Turbidity. As turbidity increases when vesicles aggregate or fuse (18, 19), light scattering techniques were used to monitor changes in vesicle preparations stored at various temperatures. Fig. 1*A* shows the change in turbidity of $Ste_2PtdCho$ vesicles stored at 4°C and at 23°C. A plot of the initial rates for these turbidity changes as a function of temperature indicates that the initial rates undergo a sharp change at ≈11°C (Fig. 1*B*).

Fig. 2 *A–E* shows the changes in turbidity of various vesicle preparations on storage at room temperature. The turbidity appears to reach a maximum after 4 days of storage for all of the vesicle preparations examined. When the same vesicle preparations were stored at 4°C, the results shown in Fig. 2 *F–J* were obtained. The turbidity changes are more rapid at lower tem-

Abbreviations: PMR, proton magnetic resonance; $Ste_2PtdCho$, L-α-distearoyl phosphatidylcholine; Chol, cholesterol; ManChol, 6-(5-cholesten-3β-yloxy)hexyl 1-thio-α-D-mannopyranoside; NH_2ManChol, 6-(5-cholesten-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-α-D-mannopyranoside; NH_2GalChol, 6-(5-cholesten-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-β-D-galactopyranoside.

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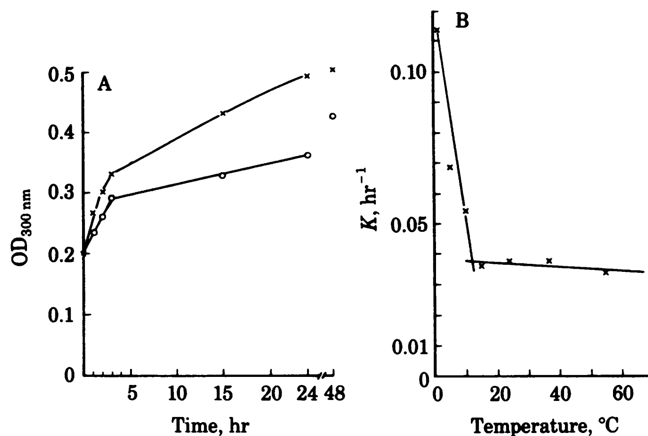


FIG. 1. Turbidity of $Ste_2PtdCho$ vesicles. (A) At room temperature. Path length, 1 cm; vesicles at 0.85 mg/ml in H_2O. X, stored at 4°C; ○, stored at room temperature. (B) Dependence of initial rate of turbidity change on temperature.

perature and reach a maximum value after 24 hr for all of the vesicle preparations studied. It is of interest that vesicles modified with β-mannose are more stable than those modified with α-mannose and vesicles modified with aminogalactose (β-linkage) are more stable than vesicles modified with aminomannose (α-linkage) both at room temperature and at 4°C as determined by turbidity measurements.

Vesicle Uptake by Macrophage. The uptake of freshly prepared aminomannose- or aminogalactose-modified vesicles by mouse peritoneal macrophage at 37°C is significantly faster than the uptake of unmodified $Ste_2PtdCho$/Chol vesicles and of $Ste_2PtdCho$/Chol vesicles modified, for example, by mannose or stearylamine (9). As shown in Fig. 3 A–J, the rate of uptake of unmodified $Ste_2PtdCho$/Chol vesicles by mouse peritoneal macrophage at 37°C changes significantly with time when the vesicles are stored for up to 1 week at room temperature. The rate of uptake of vesicles modified with α- or β-mannose or stearylamine also changes significantly with storage. However, the rate of uptake of vesicles modified with aminomannose or aminogalactose is essentially constant with storage at room temperature.

As shown in Fig. 3 F–J, the rate of uptake of unmodified

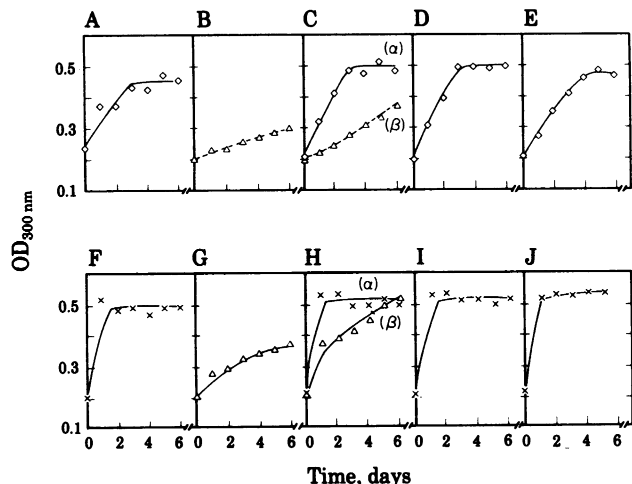


FIG. 2. Changes in turbidity of various vesicle preparations on storage at room temperature (Upper) and at 4°C (Lower). Vesicles (≈0.9 mg/ml in H_2O) were modified by aminomannose (A and F), aminogalactose (B and G), mannose (C and H; ◇ and ×, α; △, β), $Ste_2PtdCho$ (D and I), or stearylamine (E and J).

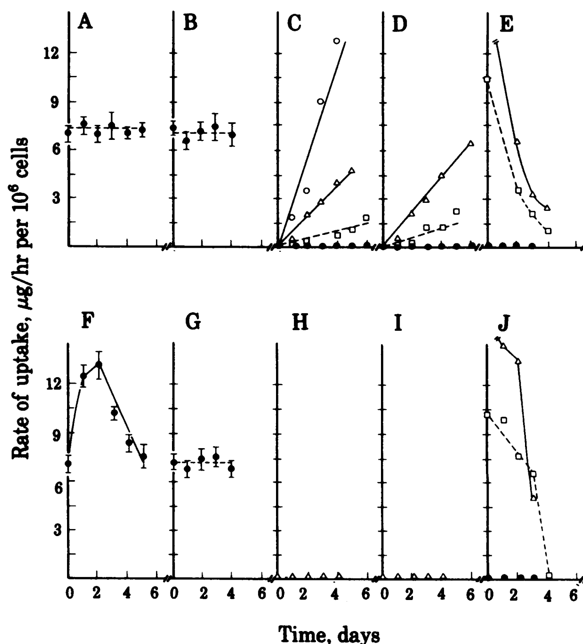


FIG. 3. Changes in rate of uptake of various vesicle preparations by mouse peritoneal macrophage on storage at room temperature (Upper) and at 4°C (Lower). Vesicles were modified by aminomannose (A and F), aminogalactose (B and G), α-mannose (C and H), $Ste_2PtdCho$ (D and I), or stearylamine (E and J). □, Rate × 1/5; △, rate × 1/10; ○, rate × 1/50.

$Ste_2PtdCho$/Chol vesicles and of vesicles modified with α- or β-mannose was too small to be observed under the conditions of our experiment, even after storage for several days at 4°C. The rate of uptake of aminomannose-modified vesicles first increased and then decreased with storage, while the rate of uptake of aminogalactose-modified vesicles remained unchanged on storage at 4°C. The rate of uptake of stearylamine-modified vesicles appeared to decrease with storage at 4°C.

NMR of Vesicle Preparations. The proton NMR spectra of the choline methyl groups for various vesicle preparations are shown in Fig. 4. For unmodified vesicles, this signal is broadened when vesicle preparations are stored for 1 day at 4°C. The proton resonance of aminomannose-modified vesicles shows

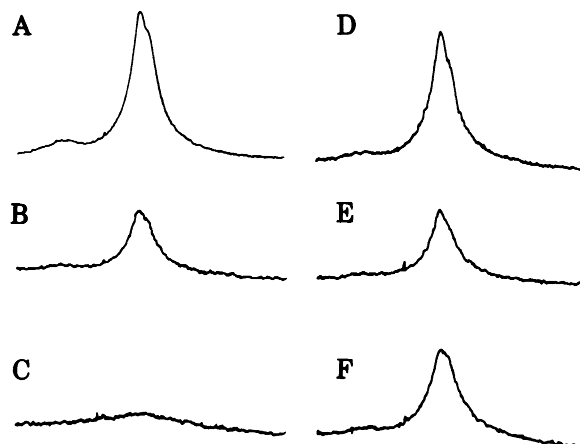


FIG. 4. Proton NMR spectra (500 MHz) of choline methyl groups in unmodified (A–C) and aminomannose-modified (D–F) vesicle preparations. (A and D) Freshly prepared vesicles. (B and E) Vesicles stored at room temperature for 1 day. (C and F) Vesicles stored at 4°C for 1 day. Probe temperature was maintained at 25°C.

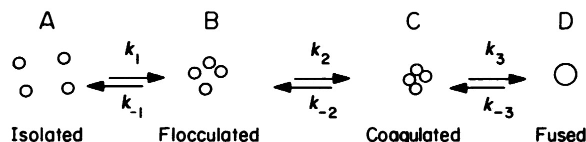
very small or no changes with storage for 1 day at either room temperature or 4°C. However, with longer storage at 4°C, the proton resonance of the choline group of aminomannose-modified vesicles is significantly broadened, as shown in Fig. 5. The proton resonances for the choline methyl groups of β-aminogalactose-modified vesicles are essentially unchanged with storage at room temperature or 4°C for up to 5 days, as shown in Fig. 6.

DISCUSSION

Small sonicated pure $Ste_2PtdCho$ vesicles have been reported to be unstable at temperatures below the phase transition temperature and are converted to larger single-bilayer vesicles on long-term storage (13, 14). The presence of fatty acid lysophosphatidylcholine components (20) or surface active agents such as alamethicin (19) in the vesicle bilayer tends to enhance the rate of vesicle–vesicle fusion. However, the presence of 1,3-distearoylglycero-2-phosphatidylcholine or Chol in the 1,2-distearoylglycero-3-phosphatidylcholine vesicles stabilizes the preparations (14). Annealing the $Ste_2PtdCho$ or DPPC suspension above its transition temperature is also reported to stabilize the vesicles and prevent fusion (16).

Peterson and Chan (18) have studied the aggregation of vesicles and pointed out that isolated vesicles are probably only found in dilute samples just after sonication. We have also noted that vesicles can exist in various states of aggregation such as flocculates or coagulates. Flocculates are stable aggregates of vesicles formed with surface-to-surface separations of 50–100 Å, while coagulates are stable aggregates formed with surface-to-surface separations of the order of 5–10 Å. A qualitative representation of the potential energy of interaction between a pair of vesicles as a function of the separation of their surfaces is given in Fig. 7 (18, 20). The detailed shape of the potential function depends on vesicle size, composition, and surface charge and on solvent composition and ionic strength (20). When the surfaces of individual vesicles are sufficiently close, fusion presumably can occur.

As light scattering, rate of uptake by macrophage, and proton NMR spectra are expected to be dependent on the state of vesicle aggregation and the rates at which various aggregates are formed are expected to depend on the nature of vesicles surface, it is of interest to explore whether a straightforward kinetic model is sufficient to describe the complex effects that occur on vesicle storage at various temperatures. A simple model of the aggregation process can be obtained by assuming that the isolated vesicles (state A) interact to form flocculated vesicles (state B) that, in turn, can react to form coagulated vesicles (state C) that, finally, react to form fused vesicles (state D). With this kinetic scheme,



the concentrations of the various species could change in a general way over time as shown in Fig. 8.

With this approach, the turbidity results can be described as follows: the light scattering (per mole of phospholipid) is expected to be substantially larger for aggregates of vesicles (states B, C, and D) than for isolated vesicles (state A). The increase in turbidity on vesicle storage either at room temperature or at 4°C is thus expected to depend primarily on the rate constants k_1 and k_{-1} and to be insensitive to the relative concentrations of states B, C, and D. The results shown in Fig. 2 suggest that k_{-1} decreases with decreasing temperature, consistent with what would be expected from the appearance of the secondary minimum in the potential function shown in Fig. 7 (21, 22).

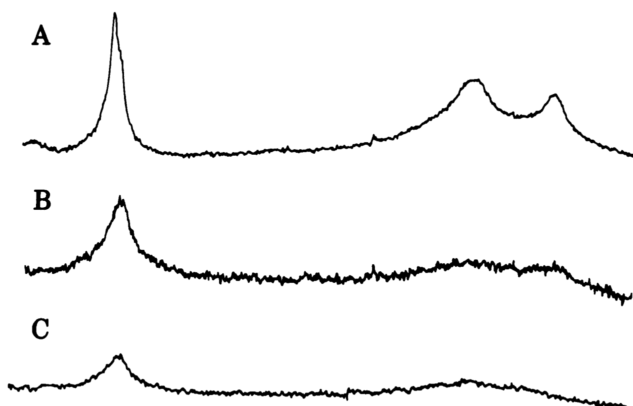


FIG. 5. Proton NMR spectra of aminomannose-modified vesicles. Vesicles were stored at room temperature for 2 days (*A*), at 4°C for 2 days (*B*), or at 4°C for 6 days (*C*). Probe temperature was maintained at 37°C.

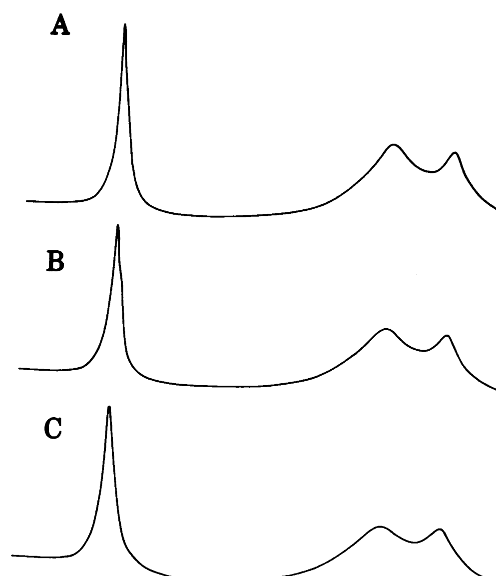


FIG. 6. Proton NMR spectra of β-aminogalactose-modified vesicles. Vesicles were stored at room temperature for 1 day (*A*), at room temperature for 5 days (*B*), or at 4°C for 5 days. Probe temperature was maintained at 37°C.

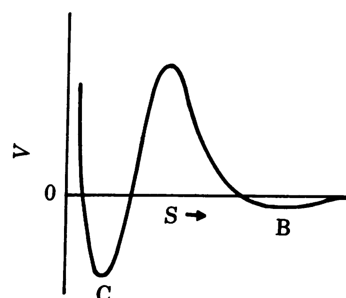


FIG. 7. Potential energy of interaction, *V*, of pairs of vesicles as a function of the separation, *S*, of their surfaces, where state B is the secondary minimum (flocculation) and state C is the primary minimum (coagulation).

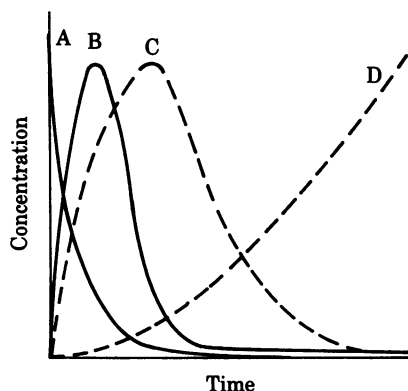
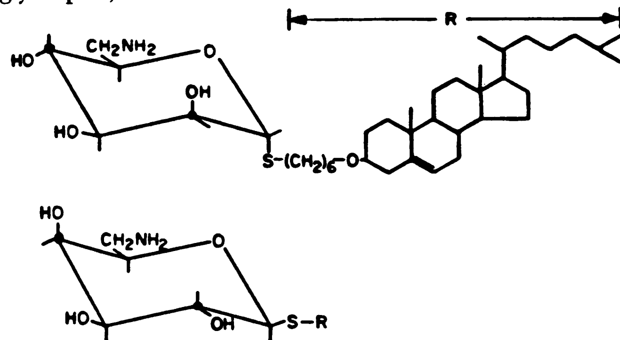


FIG. 8. Changes in concentration of various species with time. Vesicles: A, isolated; B, flocculated; C, coagulated; D, fused.

If it is assumed that the rate of uptake of phospholipid vesicles by mouse peritoneal macrophage is greater (per mole of phospholipid) for state C (coagulated vesicles) than for states A, B, or D (9), then the unusual results shown in Fig. 3 can also be described with this simple approach. For example, the increase in rate of uptake of unmodified vesicles and vesicles modified with α-aminomannose on storage at room temperature would be dependent primarily on the concentration of species B and k_2. As the reaction from state B to state C requires activation energy (see Fig. 7), k_2 would be expected to decrease with decreasing temperature. The essentially constant rate of uptake of aminomannose- and aminogalactose-modified vesicles on storage at room temperature would occur if k_{-1} were large and the vesicles remained primarily in state A on storage at room temperature for the period of observation. At 4°C, however, k_{-1} will be less than at room temperature. In this case, the concentration of state B could build up and, depending on k_2, the concentration of state C could also increase. For aminomannose-modified vesicles, k_2 could be sufficiently large to give a significant increase in the concentration of state C during the first half of the period of observation, followed by conversion from state C to state D. The average rate of uptake would decrease once again as the concentration of state D increased. For aminogalactose-modified vesicles, k_2 is not sufficiently large at 4°C to allow this sequence of events to occur. The behavior of stearylamine-modified vesicles would be consistent with the suggestion that k_2 is sufficiently large to give a significant concentration of state C and that the decrease of uptake over the period of observation is the result of conversion from state C to state D.

It has been shown (18) that the width of the PMR signal of the choline group of phospholipids in vesicle structures is broadened as the vesicle size increases. However, the formation of flocculates or coagulates would not be expected to change the linewidth significantly. The broadening of the proton NMR linewidth of the choline group thus reflects an increase in the proportion of fused vesicles of larger size in the mixture of states. The NMR results shown in Fig. 4 thus indicate that the sequence of reactions to produce fused vesicles occurs more rapidly for unmodified vesicles than for aminomannose-modified vesicles. However, when aminomannose vesicles are stored at 4°C for 6 days, a significant change in linewidth occurs, indicating extensive reaction to form state D, consistent with the change in rate of uptake by these vesicles by macrophage shown in Fig. 3 *F–J*. The proton NMR linewidths for aminogalactose-modified vesicles are essentially unchanged, even with storage at 4°C for 5 days, indicating minimum conversion to state D, consistent also with the lack of change of rate of uptake by macrophage with storage at 4°C.

These observations indicate that surface modifications can have a significant effect on the quantitative features of the potential energy of interaction of phospholipid vesicles. These changes in turn would be expected to affect the relative rates of the various steps in the aggregation process. Both α-aminomannose and β-aminogalactose are expected to carry a positive charge at neutral pH. The experimental results suggest that the presence of charge extended from the surface of the vesicle results in a shallower secondary minimum and a greater barrier between the secondary and primary minima in the potential function with a resulting increase in k_{-1} and reduction in k_2. Molecular models indicate that, for these synthetic glycolipids,



the α-D-mannopyranosyl group can assume a conformation parallel to the vesicle surface while the β-D-galactopyranosyl ring is constrained to a position somewhat more perpendicular to the vesicle surface. This steric difference probably is responsible for the enhanced stability of aminogalactose-modified vesicles. Although stearylamine-modified vesicles are also expected to be positively charged at neutral pH, the amine group would not be expected to extend significantly from the vesicle surface and thus the presence of the charge would be much less effective in changing the shape of the potential function.

The change of turbidity with time and temperature is also significantly different for α- and β-mannose-modified vesicles (see Fig. 2). These changes also presumably reflect the effect of the orientation of the carbohydrate substituent with respect to the vesicle surface. The β-mannose substituent, which would be expected to extend further from the vesicle surface, presumably reduces the depth of the secondary minimum, thus reducing the concentration of state B available for conversion to states C and D.

It is clear that a variety of changes can occur in vesicle preparations during storage. These changes give rise to apparently complex changes in phenomena such as the rate of uptake of phospholipid vesicles by macrophage. However, by modification of the surface of phospholipid vesicles, for example, by charged groups that extend significantly from the surface of the vesicle, it may be possible to obtain preparations that have adequate stability for practical applications such as drug delivery.

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