

Artificial Phospholipid and Glycolipid Particles, Visualization of Their Structures by Fluorescence Markers and Some Biological Properties Expressed by These Particles

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Homogeneous large ($5\text{--}30 \mu\text{m}$) lipid particles comparable in size and shape to blood cells can be obtained when excess of cholesterol is homogenized with egg phospholipids or brain glycolipids in a Dounce apparatus. The particles contain distinct structures of membranes and lipid cores as visualized by the included fluorescence markers.

When these particles were used as models in studying interactions between membranes, it was demonstrated that several properties normally pertaining to natural membranes could be exhibited by lipids alone. It could also be shown that particles containing phospholipids or glycolipids were very different in their adhesiveness to tissue culture cells and in their response to sera of various species.

When amphiphatic lipids are hand shaken in water, emulsions containing particles of myelinic structure are formed¹. Sonication of such emulsions results in very small vesicles which contain a single bilayer and an aqueous core². These vesicles, which are also called liposomes, possess many properties in common with natural membranes, but they are too small to be visible by light microscope.

I shall now describe a simple method to obtain rather homogeneous larger lipid particles which, when labeled with fluorescence markers^{3, 4}, can be readily observed by an UV-microscope. The membrane and core structures of these particles are directly detectable by the containing fluorescence. Also, some interesting biological properties pertaining to the particles will be reported and discussed.

Two types of amphiphatic lipids, one of egg phospholipids and the other of brain glycolipids, were respectively mixed with an excess of cholesterol as filler molecule. Some oleic acid to aid emulsification and fluorescence markers for visualization of the particles were also added (Table I). The mixtures, after being taken up in chloroform-methanol, evaporated to dryness and resuspended in an isotonic buffer solution (PBS), were Dounce homogenized and the particles formed were selected for homogeneous size by repeated low speed centrifugation (legend of Fig. 1). Lipid particles

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formed under these conditions ranged in sizes from 5 to $30 \mu\text{m}$. The sizes are comparable to those of erythrocytes, lymphocytes and macrophages. Dansylcerebroside³ is amphiphatic and was seen to be incorporated in the membrane structure (Fig. 1). The lipid particles contain an outer membrane and often an inner ring structure. The inner ring is either concentric or excentric to the outer membrane and sometimes two separate rings are contained in a single particle. The appearance of the inner ring suggests that it is also a membrane. If it is the case, then these lipid particles may be said to be enveloped by a double lamellar membrane. The core of the particles is made of lipid as seen by the homogeneous red or yellow fluorescence of the particles when chlorophyll or dansylsphingosine³ were added in the system. Chlorophyll and dansylsphingosine, being nonpolar, were apparently incorporated in the core structure. The P values of 0.24, 0.08 and 0.04 were obtained from fluorescence polarization measurements of the particles labeled with dansylcerebroside, chlorophyll and dansylsphingosine respectively. The high P value of dansylcerebroside particles again indicates that the molecule was aligned in an ordered structure such as that of a membrane. Also, the rather low P values of chlorophyll and dansylsphingosine particles show that the labels were simply dissolved in the lipid core. Another piece of evidence to support the present structure is that when chlorophyll (red) and dansylcerebroside (yellow) labeled particles were incubated together, the yellow fluorescence of the

Table I. Composition ratios of lipid mixtures for preparation of lipid particles.

Lipids	I	II
egg phospholipids *	1	
brain glycolipids **		1
cholesterol	2	2
oleic acid	0.2	0.2
dansylcerebroside or	0.05	0.05
chlorophyll or	0.5	0.5
dansylsphingosine ***	0.5	0.5

* Egg phospholipids were purified from the total lipid extract of egg yolk by silicic acid chromatography according to Rouser *et al.*⁵. The phospholipids are made up of phosphatidylcholine and phosphatidylethanolamine in the approximate ratio of 4 to 1.

** Brain glycolipids represented the crude cerebroside fraction of human brain prepared according to Klenk⁶. They contain cerebroside, sulfatide and ganglioside in the ratio of about 10 to 8 to 1.

*** Since chlorophyll and dansylsphingosine were apparently dissolved in the large volume of the core structure, more of them was included to obtain comparable intensity of fluorescence to dansylcerebroside which was incorporated in the membrane.



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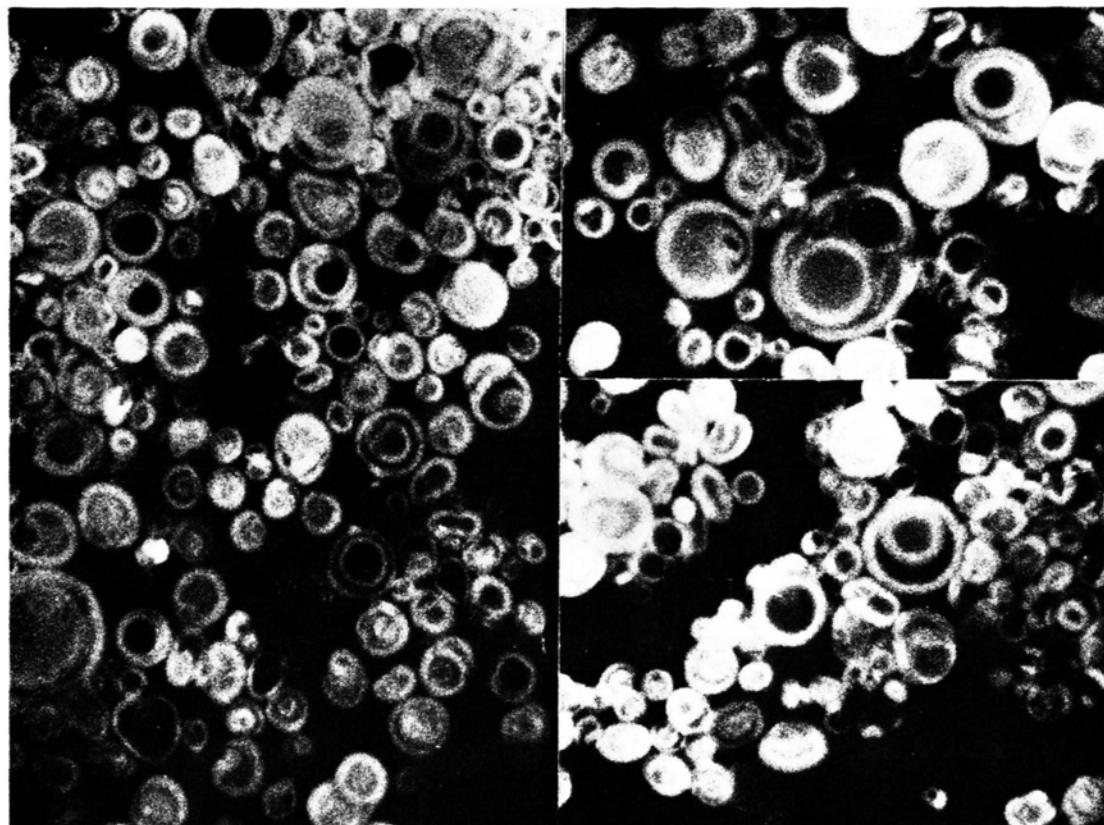
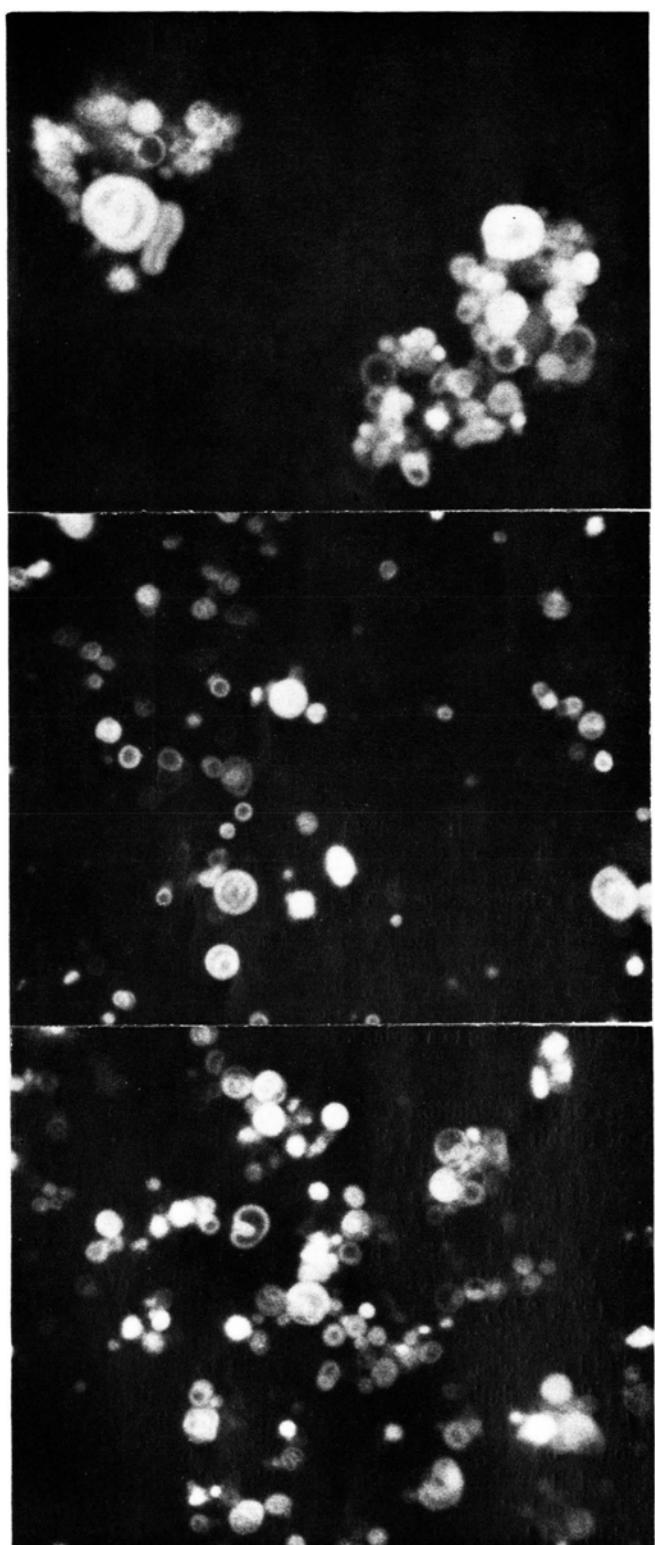


Fig. 1. Lipid particles containing amphipathic membranes and lipid cores. Left panel: glycolipid particles, right panels: phospholipid articles. $\times 1000$. To prepare particles, lipid-mixtures as listed in Table I were dissolved in warm chloroform-methanol (2:1). The solvents were then evaporated off in a rotary evaporator. The residues were taken up in about ten times the volumes of isotonic phosphate buffer (PBS) and homogenized in a Dounce apparatus (No. 93, B. Braun & Co., Melsungen) in a water bath kept at 50° . About ten up-and-down strokes were needed to ensure good homogenization. The homogenates were then centrifuged at a low speed ($200 \times g$) for one minute to remove very coarse particles which sedimented. The procedure of removing coarse particles was repeated once more. The supernatant was then centrifuged at $2000 \times g$ for 15 min, after which the supernatant, which contained very fine particles, was removed by suction. This purification procedure was repeated twice. The final sediments were then taken up in a desired volume of PBS and finely dispersed again by forcing several times through a long narrow needle (No. 18) attaching to a syringe.

latter was transferred to the membrane of the red chlorophyll particles, so that the red core was now enwrapped in an yellow membrane. In contrast, the red fluorescence of chlorophyll was not transferred to dansylcerebroside containing particles. The architecture of the artificial lipid particles described here is therefore comparable to that of chylomicrons⁷⁻⁹ or milk fat globules¹⁰⁻¹³, which are similarly made of membrane and fat core structures.

Some inherent biological properties of these particles were observed. One interesting character of the particles is their agglutinability by serum, *i.e.* both phospholipid and glycolipid particles were agglutinated when incubated with a number of normal sera at 37° for 10 min (Fig. 2 a), whereby

the ease of agglutination varied in the order of the sera of rabbit > guinea pig > calf > human > horse. However, as will be described below, agglutination of the two types of particles occur through different mechanisms. Agglutination of glycolipid particles can be inhibited by lactose and galactose at the concentration of 10 mg per milliliter of serum (Fig. 2 b), but not by glucose, mannose or glucosamine. Galactose molecules on the surface of glycolipid particles are therefore thought to participate in the agglutination reaction. The mechanism may be lectin type combination¹⁴, antigen-antibody reaction¹⁵ or enzyme-substrate complex involving galactosyl transferase¹⁶. Quite unlike glycolipid particles, agglutination of phospholipid particles is not inhibited by sugars nor by high concentrations



of (50 mg per ml of serum) phospholipid vesicles² included into the system. Therefore, it cannot be ascertained yet as to what principle is operative in the agglutination of phospholipid particles by serum.

Another noteworthy difference of the glycolipid and phospholipid particles is that glycolipid particles are very adhesive to monolayers of tissue culture cells while phospholipid particles are not. Inclusion of glycolipid particles in the culture medium of fibroblast cells, BHK cells and MDBK cells resulted in tight adhesion of these particles on the monolayers after an incubation period of 2–3 hours (Fig. 2 c). The process of adhesion occurred slower than agglutination by serum and could not be inhibited by sugars. Thus the principles of agglutination and adhesion mentioned above seem different in nature. Since the blocking of positively charged amino groups on the monolayers by glutaraldehyde completely abolished their adhesiveness to the particles, it is thought that the negative charge of the particles imparted by sulfatide and gangliosides may be at least in part responsible for the process. Mechanism of adhesion by hydrophobic interactions is excluded by ethanol extraction of the lipids of monolayers, which did not cause any change in adhesiveness of lipid particles to monolayers. In view of this phenomenon of adhesion, the glycolipid particles may be said to behave very similarly to trypsin dissociated cells described previously⁷, which could also adhere tightly to ethanol extracted monolayers but not to glutaraldehyde treated monolayers. As already mentioned, egg phospholipid particles are not at all adhesive to monolayers of cells tested. The observation demonstrates again that simple lipid particles can possess properties analogous to those of natural membranes.

The present study show that artificial lipid particles, which differ in structure from conventional liposomes, are produced when excess of filler molecule such as cholesterol is included in the system. The particles possess membranes which are in many respects similar to natural membranes. A simple variation of phospholipids vs. glycolipids in the composition of their membranes induced marked difference in their response to serum or adhesion to tissue culture cells. As compared to liposomes,

Fig. 2. Agglutination of glycolipid particles by serum and adhesion of these particles to monolayers of tissue culture cells. $\times 500$. a: Agglutination by incubation with rabbit serum at 37°C for 10 min. b: Inhibition of agglutination by including lactose (10 mg per ml of serum) in the serum. c: Adhesion of particles on monolayers of fibroblast cells after incubation at 37°C for three hours. The pictures were taken under a Zeiss UV-microscope.

the system described here has the advantage of ready detectability by an UV-microscope. The present experiment also indicates that interactions between membranes, such as expressed by agglutination or adhesion, may be mediated by lipids

alone and can operate by very different mechanisms depending on the composition of membrane lipids.

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