Coating of POPC Giant Liposomes with Hydrophobized Polysaccharide

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(Received January12, 1998; CL-980025)

POPC giant liposomes were incubated with pullulan that was hydrophobized by cholesteryl groups and labelled by a fluorescent marker (FITC). Confocal laser microscopy clearly showed coating of the outer surface of the liposomes with the polymer. This is the first and direct observation of the supramolecular assembly of an artificial cell wall onto cell-sized liposomes.

Giant liposomes (GLs) bearing micrometer dimensions are better mimicks of cells than the conventional small unilamellar vesicles, because its curvature of the membrane is smaller. They are therefore of interest for membrane protein research.[1,2]

Their formation and morphological changes have been reviewed in particular by Menger and Gabrielson.[3] The solid hydration method is useful to prepare GLs without organic solvents, as these can affect the higher order structure of membrane proteins during reconstitution. In the course of hydration, the interbilayer distance gradually increases from the solid lipid surface to yield GLs *via* intermediate states, and various morphologies. Successful formation of GLs under physiological conditions was reported by Akashi *et al.*,[4] who proposed that electrostatic repulsion between charged lipids is essential to avoid self-aggregation of the GLs. On the other hand, Magome *et al.*[5] proposed an efficient method to prepare GLs, using naturally occurring phospholipids in the presence of Mg^{2+} and suggested that is osmotic pressure rather than electrostatic repulsion was more important than electrostatic repulsion to maintain the inter-bilayer distance.

As the rehydration methods previously proposed are necessarily accompanied by major morphological changes from planar lamellar structures to closed vesicles, it is legitimate to worry about further changes, for example with budding[6,7] or with vesicle fusion.[8] Therefore, in this work, we have successfully attempted to coat the entire outside surface of the GLs with an artificial cell wall. This should prevent any self-aggregation of GLs, without introducing electrostatic or osmotic pressure effects and allow us to freely control ionic strength, buffer composition, pH, addition of inhibitors or ligands, and/or encapsulation of various water-soluble substances in the GLs.

One of us has reported that cholesteryl-bearing pullulan can coat various supramolecular assemblies such as liposomes,[9] water/oil emulsions,[10] and even plant protoplasts.[11] In this work, we used pullulan of MW ≈ 55,000 Da, bearing 1.17 cholesteryl groups per 100 monosaccharide units (coded as CHP-55-1.2).[12] A fluorescent probe, fluorescein isothiocyanate (FITC), was covalently conjugated to this modified pullulan as described earlier.[13] Two mg of this fluorescent "FITC-CHP" were suspended in 1 ml of deionized water and swelled under stirring for 12 h at 57 ℃. The resulting suspension was sonicated using a probe-type sonicator and filtered through a Millex-HV filter (pore size 0.45 m, Millipore) to yield an optically clear solution (0.2% w/w). POPC (1-palmitoyl-2-oleyol-*sn*-glycero-3-phosphatidylcholine, Sygena) GLs were prepared by the method of Menger[8] with minor modifications. One mg of POPC was dissolved in 200 l of chloroform under gentle shaking. The solution (10 - 40 l, containing 0.05 - 0.2 mg POPC) was dropped inside an O-ring (*id* 14 mm) cemented on a glass microscope slide. The chloroform was evaporated to dryness to produce a thin film of POPC. Deionized water (≈ 0.45 ml) was added to fill the inside of the O-ring, and a glass coverslip was carefully placed on the O-ring taking care to avoid trapping any air bubble. The sample-holding slide was incubated for 30 min at 26 ℃ to allow lipid hydration and formation of GLs, of known reasonable stability in an aqueous medium.[15] The slide was observed by phase contrast or epifluorescence illumination on a Carl Zeiss Axiovert 135 microscope. The images were stored and processed on a 7500/100 PowerMacintosh connected to an Argus 20 image processor. To obtain a series of confocal images, a Zeiss laser scanning fluorescence microscope (LSM 410 Invert) equipped with a Planapo oil immersion lens (63x); n_D = 1.4) was used.[16] Formation of unilamellar GLs was checked using the thinnest-contour criterion of Menger *et al.*[14] The sample was excited at 448 nm by an argon laser. The emission was cut-off with a Zeiss 515-565 nm cut-off filter.

POPC was first rehydrated with the FITC-CHP-55-1,2 solution. The excess solution was pipetted out leaving most of prematured giant vesicles partially bound to the hydrating mass of POPC solid, and then deionized water was added in order to reduce the background emission of FITC while pullulan-coated GLs were clearly observed (Figure 1). A thinnest contour balloon-like GL was selected for further examination. The ellipse-like fluorescent image provided evidence for outside coating.

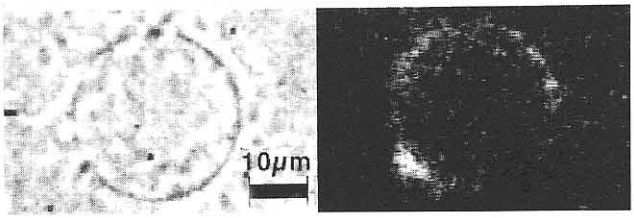

Figure 1. Phase contrast image (left) and corresponding fluorescence microscope image (right) of a GL.

Coating of pre-formed GLs was also investigated. The GLs were first prepared by rehydration with 40 l of deionized water

for 16 h, and the 0.2% FITC-CHP solution was then added (40 l) and kept for 3h. After pipetting the excess FITC-CHP solution out by the same method as described above, the GL suspension was diluted 10 times with deionized water. The surface of the multilamellar GLs observed were all coated by the polysaccharide. No penetration of FITC-CHP into the interior of the liposomes was observed (Figure 2).

The FITC-CHP-coated GLs were further examined by

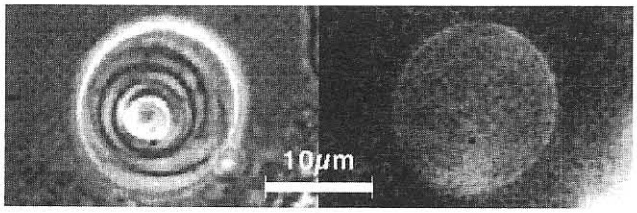

Figure 2. Phase contrast image (left) and corresponding fluorescence microscope image (right) of FITC-CHP coating of a pre-formed GL.

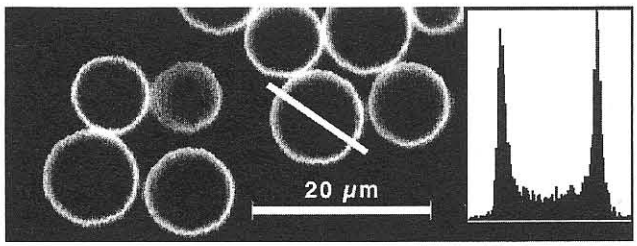

Figure 3. Confocal microscope image (horizontal cross section) of transient cluster formation of GL. The cluster of the self-aggregate disappears in minutes to separate each other. Insertion: fluorescence intensity profile of the GL along the vertical sectioning plane indicated as a bar.

confocal microscopy. Figure 3 shows the self-aggregation of GLs when POPC was rehydrated in the presence of FITC-CHP. The fluorescence intensity profile along a vertical section indicated suggests that a small amount of FITC-CHP permeated into the interior of liposome. As seen from the horizontal section, however, most of the FITC-CHP covered uniformly and completely the outmost surface of the GL. Focussing on one GL, both the vertical and the horizontal sections were ellipsoidal, showing that this GL was indeed spherical (Figure 4).

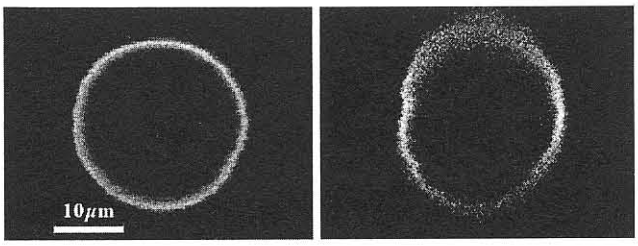

Figure 4. Confocal microscope image of a GL coated with FITC-CHP. Left; horizontal scan image and right; vertical scan image. Fluctuation of the vertical scan image was due to swimming of the liposome during the vertical scan.

The formation of the CHP-coated GLs was thus demonstrated by conventional fluorescence and by laser confocal microscopies.

These microscopic observations confirmed also that the lipophilic membrane can interact selectively the partially lipophilic CHP molecules in water.[17] The membrane interior is not only lipophilic but also strongly anisotropic. We have shown previously that cholesterol, a normal constituent of eucaryotic membranes, is indeed incorporated perpendicularly into the membrane surface, with its chain terminal buried near the interlaminar space.[18] This, and the observation of localized fluorescence described above, imply that the cholesterol units linked to pullulan are, as had been postulated earlier,[9] "nailing" the hydrophilic pullulan molecules to the outside of the membrane surface. Despite the synthetic nature of CHP, these observations are also in line with our concept of entropy-driven spontaneous complexification of vesicles as a key prebiotic mechanism.[19]

The authors express their gratitude to Dr. S. Chasserot-Golaz, Strasbourg, for her help in obtaining the confocal microscopy images.

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