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A simple method for the preparation of small unilamellar vesicles

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A simple and quick method for the preparation of small unilamellar vesicles (SUV) was developed. SUV are spontaneously formed by swelling of the specially prepared phospholipid film in water/buffer. Normally, large multilamellar vesicles (MLV) are formed when a phospholipid film is dissolved in water. To prevent the formation of multilamellar structures we used the slightly charged phospholipids which exhibit infinite swelling while the formation of large structures was prevented by the deposition of the phospholipid film on the support with small surfaces. These two requirements were met by mixing a small amount of ionic detergent into phospholipid which was deposited on microcrystals. The size and size distribution of the produced vesicles depend on the size and homogeneity of the microcrystals. When 1.5 wt% of cetyltetramethylammonium bromide (CTAB) in egg yolk phosphatidylcholine was deposited on zeolite X microcrystals with crystallite sizes of approx. 0.4 μm a homogeneous population of vesicles with average diameter 21.5 nm was obtained.

Phospholipid vesicles can be prepared by many different techniques [1,2]. The most frequently used are sonication [3] or French press extrusion [4] of MLV, removal of detergent from phospholipid-detergent mixed micelles by dilution [5], chromatography [6], dialysis [7], or absorption [8], injection of phospholipid dissolved in the organic solvent into aqueous solution under controlled conditions [9], cyclic titration of dispersions of phospholipid mixed with charged lipids [10], or depleting the organic phase from water in oil emulsion of phospholipid and water in an organic phase [11].

All these techniques require rather demanding laboratory equipment and operations. Therefore our aim was the spontaneous formation of SUV, resembling the spontaneous formation of MLV, only by swelling of the phospholipid film in water.

It was suggested that vesicles are formed by bending and vesiculation of small bilayered phospholipid flakes [12] which are formed by different preparation techniques in a different way [13].

When isoelectric phospholipid film is swelling in water/buffer the bunches of lamellae peel off and close upon themselves to protect the exposed hydrocarbon chains at their edges from water and large MLV are formed [14]. However, if LUV are desired repulsion between the lamellae has to be induced. This can be done by charging the surfaces of the lamellae because charged bilayers exhibit infinite swelling [15–18]. To reduce the size of lamellae (bilayered phospholipid flakes which peel off when the system is swelling) one must reduce the dimensions of the support on which the film is deposited. This can be done by replacing the round bottomed glass flask with a support offer-

Abbreviations: SUV, small unilamellar vesicles; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; PC, phosphatidylcholine; CTAB, cetyltetramethylammonium bromide.

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ing much smaller surfaces. This was achieved in the following way.

We deposited egg yolk phosphatidylcholine (egg yolk PC) mixed with 1.5 wt% of cetyltetramethylammonium bromide (CTAB) in $CHCl_3/CH_3OH$ on various supports. Besides blank samples (egg

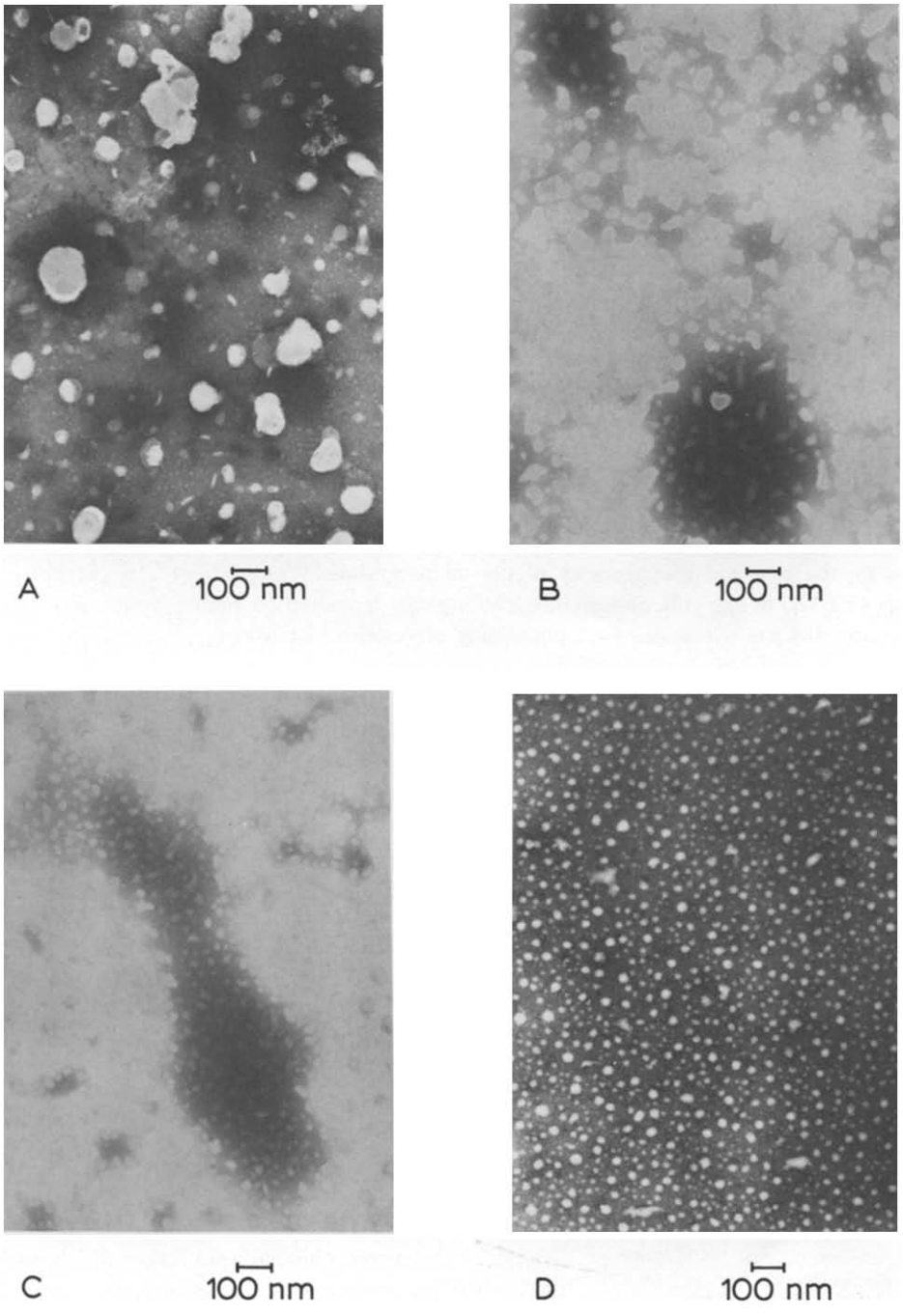

Fig. 1. Negative staining EM micrographs of vesicles formed by resuspending the egg yolk PC film with 1.5 wt% CTAB deposited on A: glass wool (Sample III), B: silica gel (Sample IV), C: zeolite ZSM 5 (Sample V) and D: zeolite X (Sample VI) in water. The samples were stained with 2% potassium phosphotungstate.

yolk PC alone on the round bottomed flask, Sample I and egg yolk PC/CTAB on the round bottomed flask, Sample II) we deposited this mixture on glass wool (Sample III), silica gel powder (Sample IV) and the microcrystals of zeolite ZSM5 (Sample V, size of crystallites 3 μm) and zeolite X (Sample VI, size approx. 0.4 μm). For additional reference, pure egg yolk PC was deposited on zeolite X (Sample VII). Normally 2–5 mg of the phospholipid mixture (1.5 wt% of CTAB) was deposited on 0.5–1 g of the support. After removal of the organic phase (overnight at 10^{-2} torr) the system was resuspended by shaking or stirring in distilled water or 5 mM NaCl to the concentration of 1–2 mg/ml. The vesicle solution was decanted (to increase the yield and save time a desk top centrifuge can be used). The losses due to adsorption of water on silica gel and zeolites are negligible (powders were handled at normal humidity) but wetting of the powder results in losses less than 5 wt% of the weight of the added powder. Losses due to the adsorption of phospholipid on the supports are 10–20% in the case of glass wool, zeolite ZSM 5, approx. 70% in the case of silica gel, and above 95% in the case of zeolite X.

After the support was removed the solutions of vesicles were investigated. All were turbid with the turbidity (naked eye and laser beam) decreasing in the order I $\gg$ VII > III $\approx$ II $\gg$ IV > V $\gg$ VI. The values of the absorbance (A) at 330 nm are shown in Table I. The concentration of phosphorus in these solutions was also determined to monitor the losses due to the absorption of egg yolk PC on the support and to normalize the measured A (see Table I).

The produced vesicles were also investigated by negative staining electron microscopy (EM) and gel permeation chromatography (GC). To increase the resolution of GC we used Sephacryl S1000 gel medium [19]. The EM micrograph of the egg yolk PC dispersion (Sample I) shows typical multilamellar structures [14] which are very large and heterogeneous in size. Sample II shows big multilamellar structures and also some SUV [17]. The micrographs of samples III–VI are shown in Figs. 1A–D, respectively. The histograms of the corresponding samples, which were counted on different grids of different sample preparations, are shown in Figs. 2A–D. The histograms of egg yolk PC/CTAB film on glass wall (Sample II) and egg yolk PC on zeolite X (Sample VII) also are shown in Fig. 2. E and F, respectively, while the histogram of I is not shown because the extremely heterogeneous sample has particles with diameters with diameters from approx. 0.1 to approx. 10 μm [14]. Figs. 1 and 2 show that with the decreasing size of the support the size of the produced vesicles also decreases while the homogeneity of the produced vesicles increases. These results were confirmed by gel chromatography. The elution profiles are shown in Fig. 3 and the radii of the vesicles calculated from the calibration curve, are listed in Table I. The shapes of the curves give also some qualitative estimates of the size distribution of vesicles although the linewidth cannot be a quantitative measure for the size distribution of particles [20]. However, the elution profiles of

TABLE I

SOME PHYSICAL AND CHEMICAL PROPERTIES OF THE PRODUCED VESICLES

EYC, egg yolk PC; EM, electron microscopy; GC, gel permeation chromatography.

Property support	Sample I EYL glass wall	Sample II EYL/CTAB glass wall	Sample III EYL/CTAB glass wool	Sample IV EYL/CTAB silica gel	Sample V EYL/CTAB zeolite ZSM	Sample VI EYL/CTAB zeolite X	Sample VII EYL zeolite X
$2r$ (nm) (EM)	100–10000	80–500	30–200	50	25.0	21.5	25–100
$2r$ (nm) (GC)	> 300	–	–	90–20	29	22	–
Homogeneity	no	no	no	bad	intermediate	good	no
A_{330nm}	2.53	1.116	1.038	0.099	0.329	0.014	1.99
C_{EYL}(mg/ml)	2.1	1.64	1.53	0.31	1.50	0.07	–
A/C_{EYl}	1.2	0.68	0.68	0.32	0.22	0.20	–

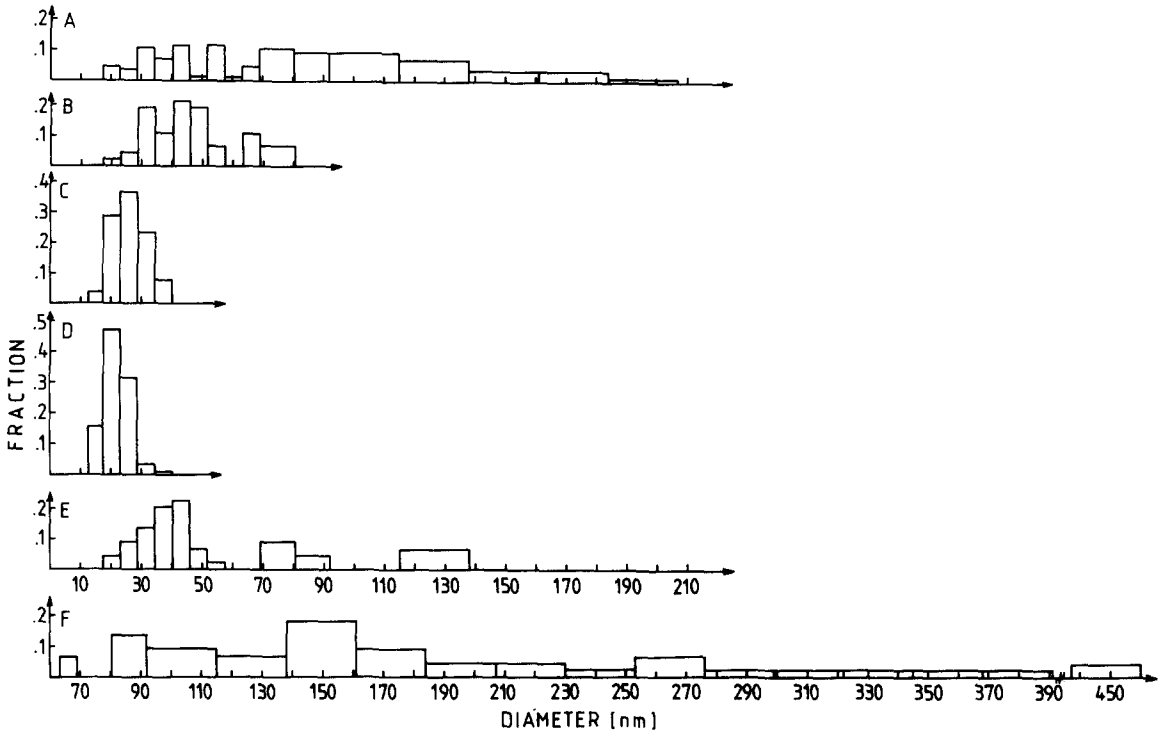

Fig. 2. The histograms of size distribution of the samples III (A), IV (B), V (C) and VI (D); E: sample VII (egg yolk PC deposited on zeolite X) and F: sample II (phospholipid film on glass wall).

samples I, IV, V and VI show that the vesicles of samples V and VI are rather small and homogeneous, sample IV is a mixture of large and small vesicles while sample I has practically only large structures (most of the phospholipid material actually stayed on the top of the column and even clogged it). SUV (samples V and VI) are stable at least for several days at 20°C as confirmed by constant A readings and reproducible EM micrographs.

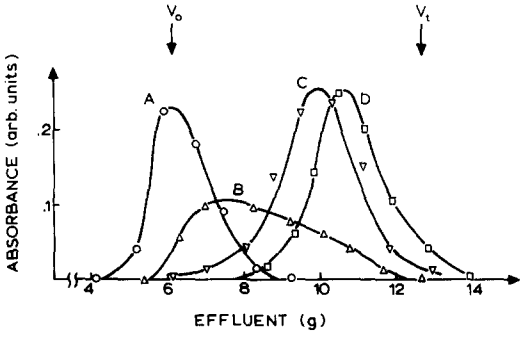

Fig. 3. Elution patterns on the Sephacryl S1000 column (39 × 0.7 cm, flow rate 6 ml/h, presatured with egg yolk PC) for four preparations: A, sample I (egg yolk PC on glass wall); B, sample IV (phospholipid film on silica gel); C, sample V (zeolite ZSM 5); and D, sample VI (zeolite X).

It is probable that many characteristics of the produced vesicles could be varied by changing of the support for the deposited phospholipid film. In addition to the size (and homogeneity) of the support the hydrophobicity of the support could also be varied. In our case the hydrophobicity of all supports is rather similar except of the zeolite X where a large ratio Al_2O_3/SiO_2 makes it even more hydrophilic than other materials which are composed predominantly from SiO_2.

The main characteristics of the support must be chemical inertness towards all substances used in the preparation process, small size of the microcrystals and low adsorption capacity. The last requirement is not satisfactory in using zeolite X which has a very large adsorption capacity (size of cages approx. 1.3 nm, channels approx. 0.74 nm) [21]. Below the saturation limit of adsorption (approx. 1 mg egg yolk PC/1.8 mg zeolite X) the yield is very low ($\leqslant 5\%$) while above this limit it

begins to increase. However, the fraction of LUV and MLV, which also are at lower ratios negligible, begin to increase indicating that the lipid film has coated several microcrystals. Zeolite ZSM5 has smaller channels (0.5–0.55 nm and no cages) [21] and this results in larger yield (about 70–80%). The use of zeolite A (channels 0.42 nm, cages 1.14 nm) and sodalite (channels 0.22 nm, cages 0.6 nm) [21] also results in larger yields (about 40 and 55%, respectively). Preliminary results show that vesicle populations are heterogeneous in size probably due to the undefined size of the support or slightly higher values of pH in these suspensions. On the other hand, amorphous silica powders cannot be used due to their thixotropic behaviour while we have not yet been able to obtain small and uniform crystals of quartz.

Changes may also be expected by changing the swelling behaviour of the phospholipid film, which can be varied from finite to infinite, depending on the surface charge [15–18].

To investigate the permeability properties we also have used γ-Al_2O_3 for support (size of crystallites approx. 1 μm) where larger yield (approx. 80%) enables trapped volume and NMR measurements. These samples show wide size distribution; besides SUV there are also LUV and some MLV, as determined by EM and phase contrast optical microscopy. ^1H high-resolution NMR spectrum of these vesicles is shown in Fig. 4. The addition of paramagnetic shift reagents (3 mM $EuCl_3$, 3 mM $PrCl_3$) does not split the $N(CH_3)_3$ signal. This indicates that vesicles are permeable to cations what can be expected from the presence of detergent. Trapped volume measurements by ESR (water soluble spin label and bleaching agent $[Cr(C_2O_4)_3]^{-3}$), show, on the other hand, that vesicles are impermeable to this anion. The measured value of the trapped volume 0.67 litre per mol of phospholipid, which corresponds to the average diameter of approx. 35 nm of the hypothetic monodisperse sample, cannot be used to calculate the vesicle size because the vesicles are rather heterogeneous and a few bigger structures were also observed by optical microscopy. The permeability of vesicles induced by CTAB within the bilayers can be eliminated by mixing charged phospholipids instead of ionic detergents, into the bilayer. Permeability studies of mixed egg yolk PC/phosphatidic acid vesicles [22,23] show that their permeability is similar to egg yolk PC vesicles prepared by sonication.

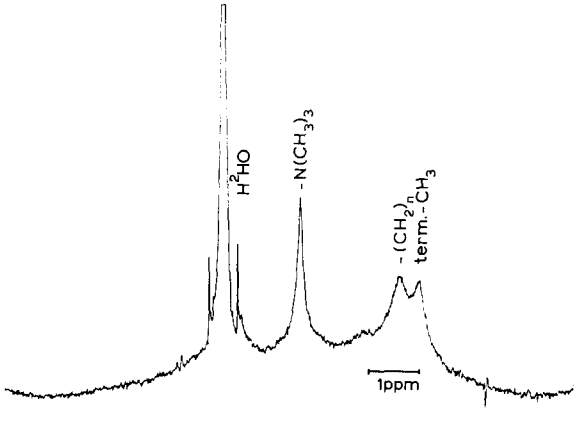

Fig. 4. 90 MHz ^1H-NMR of vesicles (6 mM egg yolk PC with 1.5 wt% CTAB) which were obtained by swelling of the lipid film deposited on Al_2O_3 (10 mg of lipid was deposited on 0.5 g Al_2O_3) and resuspended with 1.3 ml ^2H_2O).

In addition to the novelty, simplicity and rapidness of this method for the preparation of vesicles, the results obtained also shed some light on the mechanism of the vesicle formation.These results are in agreement with the proposed model where a bilayered phospholipid flake is an intermediate structure in the vesicle formation process [12,13]. Because the surface area of the support was several times larger than the area of the phospholipid film we can assume that the phospholipid film was deposited more or less as a monolayer. The swelling and peeling of the film produced bilayered phospholipid flakes which bend and close upon themselves due to their instability at the edges. The results of this work, considering the size and homogeneity of the produced vesicles, are in qualitative agreement with the proposed model. On the basis of these findings and with proper variation of the experimental parameters LUV could also be probably produced.

Besides their permeability to cations the produced vesicles are similar to SUV prepared by sonication or by detergent (sodium cholate) removal. The advantage of these two techniques are higher concentrations of the produced vesicles. The yield of egg yolk PC (mass of resuspended egg yolk PC as SUV/mass of deposited egg yolk PC) is in the case of zeolite X very low but with

another support it can increase. The advantage of this method is its simplicity and therefore avoidance of sonication, organic solvents, detergents (by the use of charged phospholipid), or pH changes what makes it very suitable for the drug or genetic material encapsulation. The subjects of our present studies are to increase the yield, increase the concentration of vesicles and additional physicochemical characterizations of the spontaneously formed SUV.

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References

1 Szoka, F.C. and Papahadjopoulos, D. (1980) Annu. Rev. Biophys. Bioeng. 9, 467–508
2 Hauser, H. (1982) Trends Pharm. Sci. 3, 274–277
3 Huang, C. (1969) Biochemistry 8, 344–352
4 Lichtenberger, D., Freire, E., Schmidt, C.F., Barenholz, Y., Felgner, P. and Thompson, T.E. (1981) Biochemistry 20, 3462–3467
5 Razin, S. (1972) Biochim. Biophys. Acta 265, 241–296
6 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322–331
7 Baron, C. and Thompson, T.E. (1975) Biochim. Biophys. Acta 382, 276–285
8 Gerritsen, W.J., Verkleij, A.J., Zwaal, R.F. and Van Deenen, L.L. (1978) Eur. J. Biochem. 85, 255–261
9 Batzri, S. and Korn, E.D. (1973) Biochim. Biophys. Acta 298, 1015–1019
10 Hauser, H. and Gains, N. (1982) Proc. Natl. Acad. Sci. USA 79, 1683–1687
11 Szoka, F.and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198
12 Lasič, D.D. (1982) Biochim. Biophys. Acta 692, 501–502
13 Lasič, D.D. (1986) J. Theor. Biol., in the press
14 Bangham, A.D. and Horne, R.W. (1964) J. Mol. Biol. 8, 660–668
15 Krzewycki, T.G., Tardieu, A. and Luzzati, V. (1969) Mol. Cryst. Liq. Cryst. 8, 285–291
16 Hauser, H. (1984) Biochim. Biophys. Acta 712, 37–50
17 Hauser, H., Gains, N. and Lasič, D.D. (1983) in Physics of Amphiphiles (Corti, M. and Di Giorgio, V., eds.), pp. 648–662, North Holland, Amsterdam
18 Hauser, H. and Lasič, D.D. (1985) in Lecithin and Health (Paltauf, F. and Lekim, D., eds.), pp. 221–231, Semmelweis Verlag, Hoya
19 Nozaki, Y., Lasič, D.D., Tanford, C. and Reynolds, J.A. (1982) Science 217, 366–367
20 Lasič, D.D. (1986) J. Colloid Interface Sci., 110, 282–286
21 Breck, D.W. (1974) Zeolite Molecular Sieves, pp. 1–771, J. Wiley and Sons, New York
22 Gains, N. and Hauser, H. (1983) Biochim. Biophys. Acta 731, 31–39
23 Hauser, H., Gains, N. and Müller, M. (1983) Biochemistry 22, 4775–4781