Biochimica et Biophysica Acta 860 (1986) 525–530
Elsevier

BBA 73252

Osmotic swelling of unilamellar vesicles by the stopped-flow light scattering method. Elastic properties of vesicles

Shao-Tang Sun [a], Alain Milon [b], Toyoichi Tanaka [a], Guy Ourisson [b,*] and Yoichi Nakatani [b]

[a] *Department of Physics and Center for Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139 (U.S.A.) and* [b] *Laboratoire de Chimie Organique des Substances Naturelles, associé au CNRS, Université Louis Pasteur, Centre de Neurochimie, 67084 Strasbourg (France)*

(Received May 13th, 1986)

Key words: Unilamellar vesicle; Osmotic shock; Stopped-flow; Light scattering; Elastic property

Unilamellar vesicles of L-α-dimyristoylphosphatidylcholine have been prepared by the ether injection technique. Gel filtration on Sephacryl S1000 was used to obtain fractions of narrow polydispersity, of radius from 300 to 600 Å. Dynamic light scattering was used to determine the change in size of these vesicles in response to an osmotic pressure drop, and its dependence on vesicle size. The amplitude of swelling ($\Delta R/R$) is linearly proportional to the osmotic pressure difference across the bilayer. We have determined the elastic area stretching modulus using a theory of membrane elasticity: it depends on the vesicle radius in the range of size studied. Vesicles having radius smaller than 400 Å show little or no swelling.

Introduction

Vesicles are widely used as models of biological membranes. Although a variety of physicochemical techniques have been used to study phospholipid bilayers, there was no direct method to evaluate their mechanical properties. Some theoretical studies on the elasticity of bilayers have been published recently (for a review, see Ref. 1). For example, the condensing effect of cholesterol can be shown clearly by isothermal surface tension measurements of monolayers, but its rigidifying effect in bilayers was supported only by indirect experiments such as fluorescent probe measurements. Furthermore, the surface tension method mentioned above cannot be used to evaluate the mechanical effect of naturally occurring transmembrane substances such as carotenoids [2] and integral membrane proteins. Very recently, an elegant, direct method was reported by Kwok and Evans [3], to determine the elasticity of giant vesicles (10 μm). We describe here a novel method to measure directly the mechanical properties of small vesicles with 300–600 Å of radius, by studying the swelling of the vesicles in response to osmotic pressure. This method has been used in a recent paper published in this journal [4], in which we have only indicated the conclusions which we have now to substantiate.

It is well-known that vesicles behave like perfect osmometers [5]. The kinetics and the amplitude of swelling yield important information on the viscoelastic properties of the bilayers. The technique of dynmaic light scattering was used to measure the increase of vesicle size resulting from the osmotic difference across the membrane. The very high sensitivity of the technique allows determination of vesicle radius with an accuracy of ±1 Å, which is needed for the quantitative char-

* To whom correspondence should be addressed.
Abbreviation: DMPC, L-α-dimyristoylphosphatidylcholine.

acterization of vesicle swelling in response to osmotic pressure jumps.

Materials and Methods

Reagents

L-α-Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, Birmingham, AL and used without further purification. The purity of the lipid was checked by silica-gel thin-layer chromatography (TLC) with chloroform/methanol/water (65 : 35 : 4, v/v) as eluent. Sephacryl S1000 was obtained from Pharmacia, Piscataway, NJ. The water was 'ultrapure water' from Millipore (Bedford, MA). All other reagents were of analytical grade.

Preparation of vesicles

Unilamellar vesicles (300–600 Å) were prepared by the ether injection method [6]. Typically, a diethyl ether/methanol solution (9 : 1, v/v, 64 ml) of DMPC (3 mg/ml) was injected through a Hamilton syringe into 60 ml of a buffer: 10 mM Tris-HCl/1 mM Na_2EDTA/5 mM NaN_3/150 or 350 mM LiCl (pH 7) (buffer A); Na_2EDTA is added to prevent aggregation induced by bivalent cations, and NaN_3 to inhibit bacterial growth. Lithium chloride was chosen to induce osmotic pressure because phospholipid bilayers are particularly impermeable to Li^+ [7]. The injection temperature was 70°C, and the injection rate was 20 ml/h. The resulting solution was dialysed against 1 l of the same buffer for 3 h and then against 2 l of the same buffer overnight to remove the remaining solvent (tubing for dialysis: Spectrapor 2, Spectrum Medical Industries, Los Angeles, CA). The solution was then concentrated to 5 ml in an ultrafiltration cell (Amicon stirred cell) with an XM50 Amicon membrane. Immediately after concentration, the vesicle solution was fractionated by gel filtration over Sephacryl S1000 [8] which had been previously saturated with the phospholipid by filtration of a vesicle solution. The above sample (5 ml) was eluted at 4°C over a 500 ml column (100 × 2.5 cm); eluent buffer, A; void volume, 240 ml; elution peak, 330 ml; flow rate, 40 ml/h, fractions collected, 3 ml. The fractions collected were always kept at 4°C. The size of the vesicles was shown to be stable for at least 1 month. The concentration of phospholipid in each fraction was determined by phosphorus analysis [9].

Measurement of vesicle size

The vesicle size was determined by dynamic light scattering, on an apparatus described elsewhere [10] in a homodyne configuration at 90° scattering angle (scattering vector $k = 2.31 \cdot 10^5$ cm^{-1}). To obtain diffusion coefficients, the autocorrelation function was analyzed with five different fits [10], which gave the same value for the vesicle size, within 2 Å. One of them was systematically used; the autocorrelation function was fitted by

$$G(t) = A \exp(-2\Gamma t - \Delta t^2) + B$$

where A, B, Γ and Δ are optimized parameters. From the decay parameter Γ the hydrodynamic radius R was calculated [11], assuming the vesicles to be hard spheres; the parameter Δ is a measure of the polydispersity of the sample [11,12]. Our samples have been shown by direct comparison to be nearly as monodisperse as a dilute suspension of standard polystyrene spheres [13]. Corrections for the dependence of the viscosity and refractive index on temperature and salt concentration were included in the program. Some measurements have been run at small angles. At 30° scattering angle, we usually found a value 5% larger than at 90° for the radius. This difference is fairly small and we carried out most of the experiments at 90°.

The temperature was 25°C and was controlled to 0.1°C. An equilibration time of 15 min was allowed before each measurement. The measurements had a reproducibility of ±1 Å (0.2%) for the sample and of ± 5Å (1%) for independent samplings of the same fraction. The concentration dependence of the vesicle size was shown to be negligible at the concentrations used in the present study.

Typical swelling experiment

After gel filtration, each fraction was diluted to 0.02 mg/ml. This solution was directly filtered through a 0.5 μm filter (Millex SR) into one compartment of a two-compartment cell for fluorescence work (Hellma). The other compartment

was filled with the same volume of buffer of varying concentration. The two compartments were carefully mixed, and 15 min later the size was again measured. For swellings of less than 5% of radius change, the size after swelling was always stable at least for 1 h; when checked, it was even found unchanged after about 15 h. For larger swellings, the size after swelling was not stable and progressively decreased, which is probably due to the leakage of the salt through the expanded weakened membrane.

Results and Discussion

Gel filtration

The elution profile (Fig. 1) shows that Sephacryl S1000 provides an appropriate size range for the present study ($K_{av} = 0.5$). Adsorption of lipids on the column seems important. At the end of the filtration ($V = 400$–500 ml) the size increases gradually. We believe that this is due to a slow release of large vesicles initially adsorbed on the gel.

Swelling experiments

Theoretically, it should be possible to study both swelling and shrinking of vesicles by mixing the vesicles with either hypoosmolar or hyperosmolar solutions. In fact, preliminary experiments have shown that the shrinking behaviour is more complex: we observed that, for small osmotic shocks, there is no shrinking of the vesicles, and for higher pressures applied to the membrane, the shrinking is not reversible. Thus, DMPC vesicles show a strong resistance to area change by compression. The DMPC vesicle, in its equilibrium structure, is known to consist of closely packed hydrocarbon chains, and therefore the repulsion between the chains is highly sensitive to compression [14,15]. This situation, however, might be highly dependent on the composition of the vesicle.

In the present paper, we have limited our study to swelling; we have studied the phenomenon for vesicles of radius from 300 to 600 Å. This experiment was very delicate, and it has been possible to carry it out only because of the high quality of our dynamic light-scattering apparatus, and with a very monodisperse sample; but even in the best case it was impossible to prevent a certain scattering of the data: to get a 0.5% accuracy on $\Delta R/R$ it is necessary to measure R with a 0.25% accuracy, i.e., to measure a radius of 500 Å with a reproducibility close to 1 Å.

Fig. 2 shows the results for vesicles with five

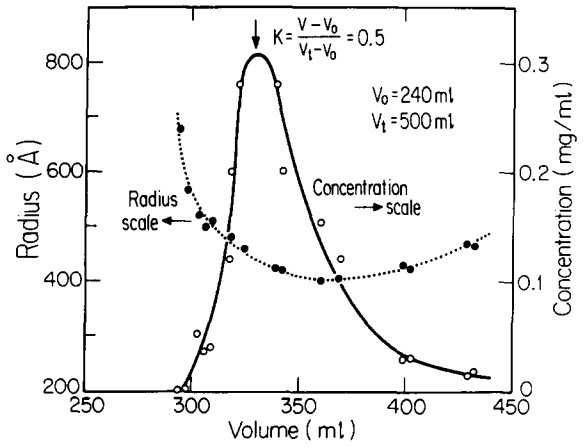

Fig. 1. Elution profile of the filtration on Sephacryl S1000; V_0 = void volume of the column; V_t = total volume; fraction collected = 3 ml; elution rate = 40 ml/h; volume of sample eluted = 5 ml (approx. 100 mg phospholipid). Plot of the radius (●·····●) and the concentration (○——○) of each fraction.

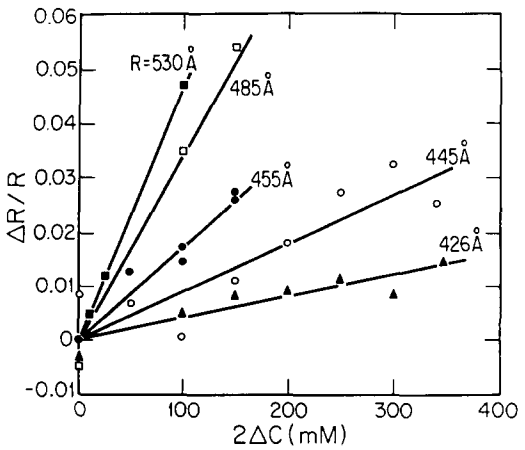

Fig. 2. Relative swelling of the vesicles ($\Delta R/R$) plotted versus $2\Delta c$ (the difference of salt concentration between the solutions which are mixed) for different preparations of initial radius 426 (▲), 445 (○), 455 (●), 485 (□), 530 (■) Å. The samples $R = 426$ Å and $R = 445$ Å were prepared in 350 mM LiCl, the others in 150 mM LiCl.

different sizes. We plotted the ratio $\Delta R/R$ vs. 2 Δc, the difference between the salt concentrations of the two solutions before mixing for each size (Δc is the difference of the concentration inside and outside just after mixing). The size dependence of the slope of $\Delta R/R$ vs. 2 Δc is shown in Fig. 3.

The swelling is related to the osmotic shock applied to the membrane and the variation of $\Delta R/R$ with Δc can be reasonably fitted to a straight line.

Theoretical considerations

We assume the vesicle to be a sphere of radius R, the osmotic pressure to be small, the membrane to be elastic and impermeable to solutes [3,5]. Mechanically the elasticity of a thin membrane is derived from two sources [1,16–18]: (a) the free energy change due to the deformation or stretching of the membrane as a flat surface; (b) the free energy change associated with the curvature change or bending of the membrane. In our experiment the effect of bending can be ignored and the potential energy per unit area $U(R)$ is, according to Israelachvili [1]

$$U(R) = (1/2)\cdot k\cdot(a-a_0)^2/a_0^2 + \gamma \qquad (1)$$

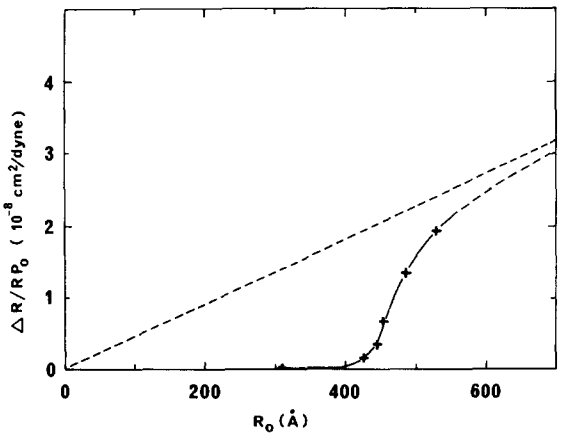

Fig. 3. Plot of the relative change in radius per unit osmotic pressure, $\Delta R/R_0P_o$ vs. inital radius, R_0. The straight line (in dotted line) represent the theoretical behaviour according to Eqn. 3, taking an arbitrary value for k. + + +, experimental data; − − −, hypothetical asymptotic behavior

where a_0 is the area per molecule in the tension-free state, and a is the area per molecule after swelling, k is the isothermal elastic area compressibility modulus and γ is a constant.

For small radius changes, Eqn. 1 can be rewritten as

$$U(R) = 2k\cdot(R/R_0-1)^2 + \gamma \qquad (2)$$

where R_0 is the initial radius of the vesicles and R is the radius after swelling.

During the swelling of the vesicle, the forces exerted on a tiny patch of membrane of area $A = 4\pi R^2$ are:

(1) the force due to the osmotic pressure, P_o

$$AP_o = A\nu\phi RT\Delta C$$

where the osmotic pressure $P_o = \nu\phi RT\Delta c$; Δc is the difference of salt concentration across the bilayer, ν is the number of ions per molecule of solution, ϕ is the osmotic coefficient, R is the gas constant, and T is the temperature. In the range of concentrations used in this work, ϕ lies always between 0.9 and 1; we shall assign it value 1. The variation of internal concentration due to swelling has been shown to be negligible within the precision of our experiments.

(2) the force due to the membrane elasticity

$$AP = A\,\partial U/\partial R$$

where P is the pressure exerted by the membrane.

(3) the friction resulting from the permeation of water through the membrane

$$Af\,\partial R/\partial t$$

where f is the reciprocal of the membrane water permeability. These three forces are perpendicular to the membrane.

During swelling, the total force on the patch must be zero. Therefore,

$$-Af\,\partial R/\partial t - A\,\partial U/\partial R + AP_o = 0 \qquad (3)$$

From Eqn. 2

$$\partial U/\partial R = 4k(R/R_0-1)/R_0 \qquad (4)$$

From Eqns. 3 and 4,

$$\partial R/\partial t + \left(4k/fR_0^2\right)R = P_o/f + 4k/fR_0 \quad (5)$$

The solution of Eqn. 5 is

$$R = R_0 + \left(R_0^2 P_o/4k\right)\left(1 - \exp\left(-4kt/fR_0^2\right)\right) \quad (6)$$

Eqn. 6 can be rewritten as

$$R - R_0 = \Delta R(1 - \exp(-t/\tau)) \quad (7)$$

with

$$\Delta R = R_0^2 P_o/4k \quad (8)$$

$$\tau = R_0^2 f/4k \quad (9)$$

Eqn. 8 can be rewritten as

$$\Delta R/R_0 = R_0 P_o/4k \quad (10)$$

Kwok and Evans [3], to measure elastic area compressibility modulus of giant vesicles, have used the following equation

$$T = k(a - a_0)/a_0 \quad (11)$$

where T is the isotropic tension.

In the case of osmotic swelling

$$T = A \cdot (\partial U/\partial A) = A \cdot (\partial U/\partial R) \cdot (\partial R/\partial A) = R \cdot P_o/2 \quad (12)$$

and

$$(a - a_0)/a_0 = 2\Delta R/R_0 \quad (13)$$

Eqns. 11, 12 and 13 lead to the same Eqn. 10. Both approaches therefore lead to the same conclusion.

This simple theoretical model shows the rate of the swelling to be exponential with time and $\Delta R/R_0$ to be linearly proportional to Δc. The first conclusion has already been reported [4] and the second one is in agreement with our present experiments (Fig. 2). However, the dependence of $\Delta R/R_0 P_o$ on R_0 is not described properly by Eqn. 10 (Fig. 3).

For each radius, one can calculate k from the results of Fig. 2, using Eqn. 10 (Table I). The order of magnitude compares well with the result of Kwok and Evans [3]: $k = 140$ erg/cm^2 (for egg phosphatidylcholine bilayers and $R > 10$ μm).

TABLE I

RELATION BETWEEN ISOTHERMAL ELASTIC AREA COMPRESSIBILITY (k) AND VESICLE RADIUS (R)

$10^6 \cdot R_0$ (cm)	4.26	4.45	4.55	4.85	5.30
k (erg/cm^2)	660	306	165	89	68

These experimental results show that, at small radius, there is a deviation from the theoretical curve derived from a simple elastic model. A more microscopic theory would probably be required to explain these results.

It is of interest to note the work of Schindler [19] about the equilibrium surface pressure of the monolayer formed at the air/water interface of a vesicle suspension. This pressure depends on the exchange of lipids between the vesicles and the monolayer [20], which in turn is affected by the capacity of the vesicles to accept deformation when coming into contact with the monolayer. Schindler has shown that, for vesicles with radius smaller than 500 Å, the exchange is limited; we have shown that precisely at this size range the vesicles become non-deformable.

Acknowledgements

Work supported partially by NSF DMR 81-19295. A.M. is grateful for support granted by the Ecole Normale Supérieure de Saint-Cloud. The authors wish to thank Dr. S. Candau for valuable discussions.

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