Phospholipid Liposomes: Preparation, Characterization, and Uses

E. NORDMEIER,* C. ZEILINGER, and M. D. LECHNER

Department of Physical Chemistry, University of Osnabrück, 4500 Osnabrück, Germany

SYNOPSIS

Rhodopsin and cyclic guanosine monophosphat (cGMP)-dependent channel proteins are isolated from the rod outer segment disk membranes of dark-adopted bovine retinae and incorporated in liposomes, prepared by the method of detergent removal dialysis. The ion channel does not lose its transport function (release of Ca^{2+} ions by injection of cGMP) when incorporated in a liposome. Its activity depends on the degree of protein solubilization and the kind of detergent used. The highest activity is obtained by use of the detergent CHAPS. Shape, size, and size distribution of the liposomes are deduced from elastic and quasi-elastic light scattering, the liposome number density by viscometry, and the photopigment or Ca^{2+} content by optical absorbance. The liposomes are heterogeneous with respect to size and shape. Small unilamellar liposomes (R_h = 80 nm) and a narrow size distribution (U_D = 0.16) are obtained by using the detergent CHAPS. With increasing rhodopsin content per liposome, the hydrodynamic radius R_h increases and at the same time the shape of a liposome converts from a sphere to a prolate ellipsoid. The amount of entrapped Ca^{2+} per liposome reaches its maximum value when the Rhodopsin nearest-neighbor distance approaches its minimum value. This suggests an intermembrane protein–lipid–protein lattice, which serves as barriere for Ca^{2+}. The influence of temperature or total used Ca^{2+} content is less profound. Increasing temperature yields slightly smaller liposomes.

INTRODUCTION

Phospholipids are amphiphilic molecules. They consist of two parts—one easily soluble in water and the other highly insoluble, composed of up to two aliphatic chains. When put into water, these molecules organize themselves so as to minimize their free energy, building up structures such as a spherical bilayer, called a liposome. There has been considerable interest in studying such structures, in part because they represent a somewhat idealized model of a biological cell. Ion channels, which control the flow of ions inside and outside the membrane of a cell, can be isolated from the cell membrane and incorporated into a liposome membrane. Then, the behavior of the channel can be studied in a chemically defined boundary and under conditions in which external stimuli (voltage, temperature, etc.) can be varied independently. From another and a more practical point of view, liposome assays are considered as potential drug carriers, which could eventually deliver their content to special body regions, where membrane permeability would be altered by a slight change in temperature, for instance.

To assess the usefulness of liposomes, it is important to characterize a liposome suspension in terms of their shape, size, or size distribution and show how these terms change as a function of experimental conditions. Here, we report results about asolectin liposomes, which are prepared by the method of detergent–micelle formation followed by detergent removal. Proteins are incorporated into the membrane and Ca^{2+} ions are entrapped into the interior of the liposomes. Ninety-five percent of the proteins, isolated from the membrane of a bovine rod outer segment disk, are rhodopsin. One component of the other proteins is an ion channel, first detected and purified by the Department of Bio-

* To whom correspondence should be addressed.

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physics of the University of Osnabrück. It releases Ca^{2+} ions when stimulated with cyclic guanosine monophosphate (cGMP). Whereas all studies on cGMP-dependent channels have been carried out on the disks themselves, we have, for the very first time, solubilized the channel protein together with rhodopsin out of its natural boundary followed by an incorporation into a liposome. A test, where we inject cGMP to the liposome assay, will show whether the channel molecule loses or keeps up its function.

The second aim of this paper is to show how shape, size, and size distribution of the used liposomes depend on changes in the total number of proteins or Ca^{2+} ions per liposome. Additionally, the temperature and the nature of the detergent used for preparation will be varied.

Different methods are assessed as to their suitability for a routine size analysis of liposomes: (I) gel filtration, (II) electron microscopy, and (III) light scattering. The advantages and disadvantages, charged to an application of these methods, have been discussed previously.[1] Here, for purification, the method of gelfiltration is used. Size and shape analysis is performed by elastic and quasi-elastic light scattering.

MATERIALS AND METHODS

Materials

cGMP, CHAPS, arsenazo III, and asolectin (L-α-phosphatidylcholin, Type IV-S) were purchased from Sigma. The detergent octylglucosid and the ionophore A 23187 were obtained from Calbiochem. Na^+–cholat is from Serva and Mega 8 to Mega 10 from Oxyl-GmbH (Bobingen). All reagents are of at least analytical grade.

Disk Preparation

The isolation and purification procedure for rod outer-segment disk membranes is carried out according to Schnetkamp and Kaupp.[2] To set the disk membranes free from peripheric proteins such as G-proteins or phosphodiesterase, the disks are washed three times in a hypotonic buffer (10 m*M* Hepes-KOH [pH 7.4], 1 m*M* EDTA, 1 m*M* DTT) by centrifuging at 32,000 rpm for 20 min, resuspending the pellet, and repeating the procedure. DTT serves as an antioxidant, and EDTA takes away 2-valent ions. All manipulations are carried out in dim red light at a temperature of 4°C.

Disk Solubilization

Finishing the former procedure, each pellet is suspended for 30 min in a buffer containing a detergent of defined concentration, 100 m*M* KCl, 10 m*M* Hepes-KOH, pH 7.4, 2 m*M* $CaCl_2$, and 1 m*M* DTT. The suspension is then centrifuged by an airfuge at 150,000*g*. This procedure insures that all the not-solubilized material sediments. The pellet is resuspended, and the concentration of proteins in the pellet and excess is determined spectroscopically at 278 nm (one of the absorption maxima of proteins) and at 500 nm (one absorption maximum of rhodopsin) with an Aminco DM 2a photospectrometer. Finally, the degree of solubilization is estimated according to the relation

$$\text{Degree of solubilization (\%)} = \frac{A_{278}^{\text{Excess}}}{A_{278}^{\text{Excess}} + A_{278}^{\text{Pellet}}} \times 100\%$$

where A_{278}^{Pellet} or A_{278}^{Excess} designates the absorption of pellet or excess at 278 nm.

Liposome Preparation

A chloroformic solution of asolectin, first purified according to the method of Sone et al.[3] is evaporated to dryness for 1 h under reduced pressure at 25°C. The remaining lipid film is dispersed in 5.0 mL of 10 m*M* detergent buffer for 30 min. The buffer contains 100 m*M* KCl, 10 m*M* Hepes-KOH (pH 7.4), 1 m*M* DTT, $CaCl_2$ ranging from 0.5 to 10 m*M*, and proteins, obtained from the excess of disk solubilization. The content of the proteins is adjusted according to De Grip et al.[4] to 0.0–1.0 mg rhodopsin per milliliter. Then, the suspension is sonicated for 0.5 min in order to promote the formation of unilamellar liposomes. After short equilibration, the clear solution is dialyzed in a small dialysis bag (Medicell International; width 8 mm) against the detergent-free buffer for 36 h. During this time, the liposomes and proteins reassemble simultaneously. A second dialysis, lasting 12 h, is carried out against a buffer, which contains no Ca^{2+}. After this, all remaining Ca^{2+} ions should be trapped inside the liposomes.

Liposome Purification

To get useful results by elastic or quasi-elastic light scattering, it is necessary to purify a liposome suspension by gel filtration. The column material used is Sephacryl S-1000.

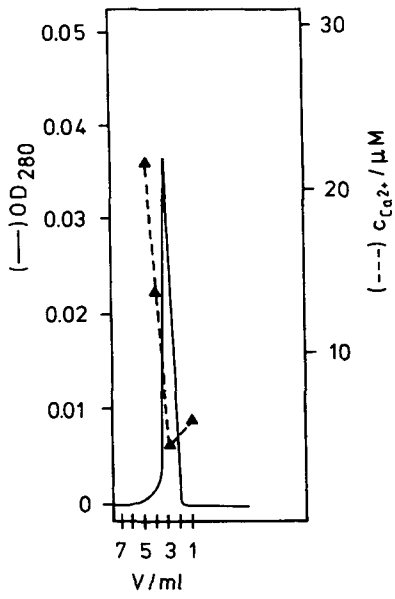

Figure 1 Gel filtration.

To prove whether the liposomes are entrapped with Ca^{2+} ions, we inject into each liposome fraction the ionophore A 23187. A typical chromatogram is shown in Figure 1. The liposome extract contains 0.065 mg mL^{-1} rhodopsin, 0.05 m*M* mL^{-1} Ca^{2+}, and 10 mg mL^{-1} asolectin. Fractions of liposomes are collected in distances of 1 mL. The left ordinate presents the optical density at 280 nm, and the right ordinate states the content of Ca^{2+} released by the ionophore A 23187. Fractions 3 and 4 contain together more than 75% of the total content of released Ca^{2+}, pointing out that fractions 1 and 2 are contaminated with lipid or protein aggregates. Thus, for light-scattering measurements, we always use fraction 4.

Calcium Measurements

Changes in the concentration of free Ca^{2+} are recorded spectroscopically using the Ca^{2+} indicator dye arsenazo III. Time-resolved changes in the absorption are recorded by an Aminco DW 2a photospectrometer in the dual wavelength mode. Artifacts due to light scattering can be eliminated by measuring the difference in absorbance at $\lambda = 650$ nm and at $\lambda = 730$ nm. Arsenazo III does not respond to changes in Ca^{2+} at 730 nm, and the decrease in transmission due to scattered light in a suspension of liposomes is almost constant for wavelengths above 600 nm.[5] Usually, 150–300 *M* cGMP or 5 μM A 23187 are injected into a volume of 2 mL liposome assay. Ca^{2+} indicating absorption changes are then calibrated by injecting a known amount of Ca^{2+} or EDTA.

The absorption change after an injection of Ca^{2+} is about 5–10% smaller in the presence of liposomes than in a solution, which contains only arsenazo III. This small decrease in the differential sensitivity of arsenazo III can be accounted for by the Ca^{2+}-buffering capacity of liposome membranes.[6]

Viscosimetry

The liposome assays are placed into an Ubbelohde viscosimeter. This is put into a constant temperature bath at 25.0 ± 0.1°C. The relative viscosity of each suspension at different dilution is determined as the ratio of the flow times of the suspension to that of solvent (buffer). Three runs of independently prepared liposome assays are made. For a given run, the relative viscosity is determined at approximately seven successive dilutions. The relative viscosity ranges from 0.040 to 0.120 with uncertainty of ±0.02, which is attributed mainly to the reproducibility of 0.3–0.5 s in flow-time measurements.

Light Scattering

Instrumentation

The apparatus for measuring both the intensity and autocorrelation function of the scattered light consists of a He–Ne laser (Spectra Physics, Model 1075/207, $\lambda = 633$ nm), a temperature-controlled scattering cell holder with a toluene index matching bath, a digital ALV-3000 autocorrelator (1026 channels), and an online data analyzer with a Peacock AT computer. Scattering angle and temperature can be varied in the range of 10–140° and 10–80°C, respectively.

Dust Removal

Aliquots of all samples are centrifuged at 3000 rpm for 20 min in order to sediment dust from suspension. The centrifuged samples are transferred into acetone-washed cylindrical quartz scattering cells of 8 mm i.d. Then, the suspensions are inspected for dust using a He–Ne laser beam. If more than a few dust particles are observed in field of view, the procedure is repeated.

Data Analysis

For each sample, the autocorrelation function $g(t)$, computed at 192 equally spaced points, $t = \tau, 2\tau, 3\tau, \cdots 192\tau$, is measured at various sampling times

τ ranging from 0.5 to 10 μs. The base-line values, B corresponding to $g(\infty)$, are calculated from the monitor channels and agree quite well with the measured values of $g(t)$ at large values of t.

In all cases, a first-, second-, and third-order cumulants analysis is performed on the first 96 points of each autocorrelation function. A second data analysis, where $g(t)$ functions, measured at various τ values, are spliced together, is not necessary, because the cumulants show only a small dependence on sampling time τ.

From the cumulants, the diffusion coefficient D_z, the nonuniformity coefficient U_D, and the skewness S are computed. D_z is related to the hydrodynamic radius R_h of the liposomes according to the Stokes–Einstein relation:

$$R_h = k_B T / 6\pi\eta D_z \qquad (1)$$

k_B is Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity.

Simultaneously to the above calculations, the intensity of the scattered light is recorded. The dependence of the intensity on the scattering angle can be used to establish the shape of the liposomes, as well as to evaluate the liposome size in terms of the radius of gyration $\langle S \rangle_z$. A typical procedure is the following[7]: One determines experimentally the particle-scattering factor $P(\theta)$, which presents the ratio of the scattering intensity I_θ at the angle θ to the incident zero-angle-scattered light intensity I_0. The form of observed particles. For a hollow sphere, infinitely thin, Oster[8] is expressed by

$$P(\theta) = [\sin(r \times q)/(r \times q)]^2. \qquad (2)$$

r is the radius of the sphere, $q = (4\pi/\lambda')\sin(\theta/2)$ is the wave vector, and λ' is the wavelength of the incident radiation in the solvent (buffer).

With regard to liposomes, the model of an ellipsoid of revolution, developed by Porod,[9] may be more realistic. It holds

$$P(\theta) = 9\pi/2 \int_0^{\pi/2} (J_{3/2}^2(V)/V^3)\cos\beta \, d\beta \qquad (3)$$

with $V^2 = q^2(a^2\cos^2\beta + b^2\sin^2\beta)$. a and b are the major and minor semiaxes of the ellipsoid. $J_{3/2}(V)$ presents the Bessel function of 3/2 *st* order. The integration of eq. (3) can be performed quite well by a Gauß quadrature.

Assuming a specific shape and size for a liposome, one can verify whether the resulting $P(\theta)$ curve coincides with the experimental one. If not, assumptions should be changed concerning the liposome assay until an agreement is reached.

RESULTS AND DISCUSSION

Degree of Solubilization of Different Detergents

Detergents, like lipids, are amphiphilic molecules. Below the critical micellar concentration (cmc), detergents, exist as monomers. A remarkable degree of protein solubilization may be expected only for concentrations above the cmc, where detergents form spherical micelles. To test this hypothesis, we use detergents, already employed with success for reconstitution experiments, namely, CHAPS (cmc = 8 mM), Na^+–cholat (cmc = 3 mM), octylglucosid (cmc = 25 mM), and Mega 9 (cmc = 24 mM). The results are shown in Figure 2. There, the degree of solubilization is plotted against the concentration of detergent. The cmc's are marked on the ordinate.

Each detergent is affected by its own individual effective range of solubilization. If we choose the cmc as base, all detergents, except Na^+–cholat, are charged with a degree of solubilization of nearly 50%. This observation indicates that the cmc hypothesis is partly true.

Reconstitution of the cGMP Channel

In the section above we found the effective range of solubilization of a detergent, but we have not proven whether the channel proteins keep out their function to release Ca^{2+} when dealt with cGMP. Additionally, we do not know, provided the channel works, how the channel activity varies with the concentration of used detergent. To clear up this uncertainty, the proteins are solubilized from the disks and then incorporated into liposomes containing entrapped Ca^{2+} ions. The detergent concentration is adjusted so that the degree of solubilization ranges from 0 to 95%. We then inject cGMP to the liposome suspension and measure the amount of released Ca^{2+} by optical absorbance.

A typical example of a reconstitution experiment is shown in Figure 3. An abrupt change in absorption is observed when cGMP is injected. The total content of released Ca^{2+} can be estimated by calibration with Ca^{2+} of a known amount. According to Figure 3, an injection of 150 μL cGMP to 0.4 mL liposome assay, prepared by 20 mM CHAPS, yields a release of 0.43 μM Ca^{2+}. A second injection of 150 μL cGMP induces no further release of Ca^{2+}, pointing out that the cGMP-sensitive Ca^{2+} pool of the channel is al-

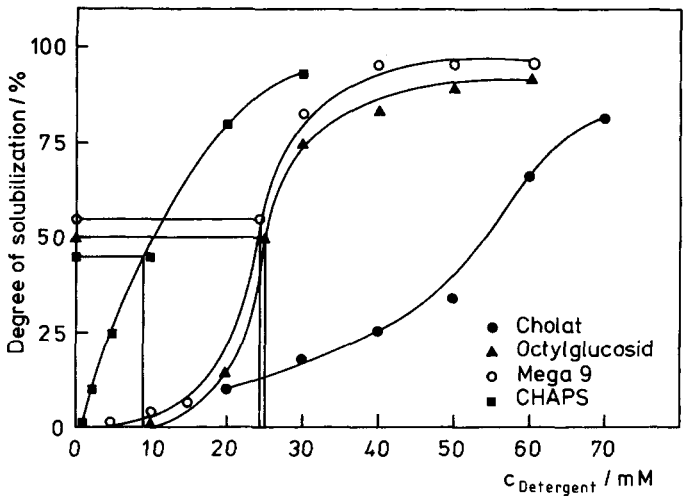

Figure 2 Protein solubilization by different detergents.

ready discharged completely after the first injection. To release the remaining Ca^{2+} ions from a liposome, we must inject a certain amount of the ionophore A 23187 to the assay. With 5 μM A 23187, 12.3 μM Ca^{2+} can be set free. This states that the Ca^{2+} amount, released by cGMP, represents only 3–10% of the Ca^{2+} content collectively entrapped to the liposomes. The ratio of the Ca^{2+} amount, maximal released by cGMP, to those, maximal released by cGMP and A 23187 together, is a measure for the activity of a reconstituted cGMP channel. The highest activity is obtained when one uses the detergent CHAPS. The activities, affected by the other detergents, are about a factor of two (Na^+-cholat) to five (Mega 9) smaller.

Next, we hold the number of liposomes per suspension constant and vary the concentration of the detergent CHAPS. The results are shown in Figure 4. The activity first increases with an increasing concentration of detergent, then the activity reaches a maximum at 10 mM, and beyond this point, the activity decreases with an increasing concentration of detergent. Finally, above 30 mM, the activity is zero. The channel loses its function.

A comparison of the activity and solubilization curve is also instructive. To get a high degree of solubilization, for instance, 75%, a detergent concentration of 20 mM CHAPS is required. The activity of the channel at this point is very small, only

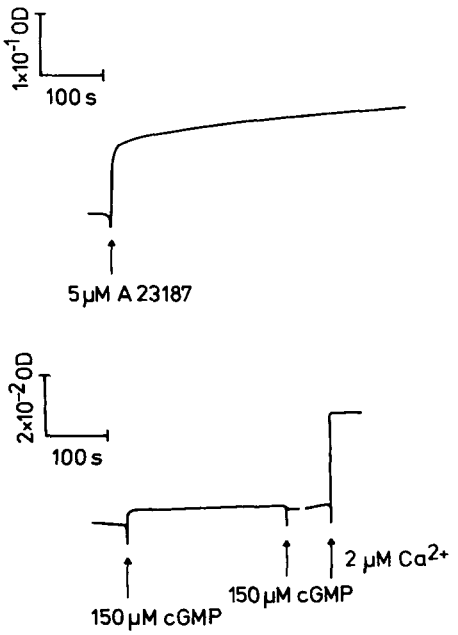

Figure 3 Ca^{2+} test.

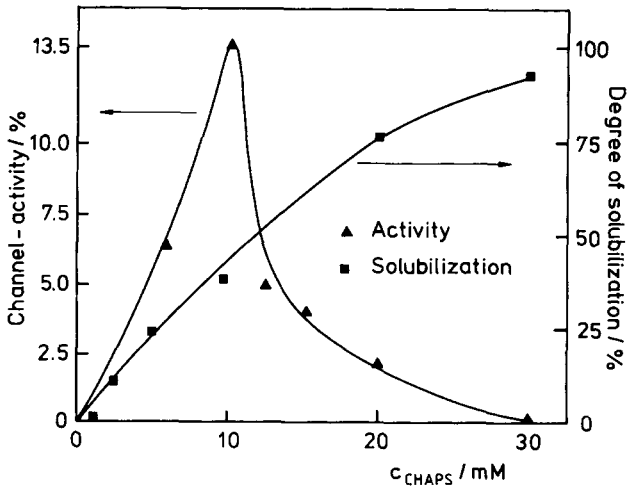

Figure 4 Interrelation between channel activity and degree of solubilization.

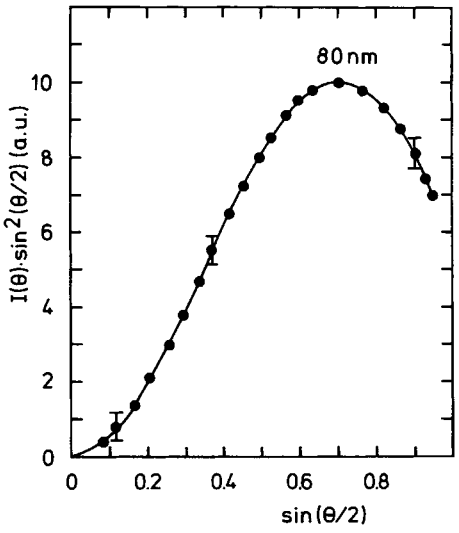

Figure 5 $I(\theta)\sin^2(\theta/2)$ vs. $\sin(\theta/2)$.

2.5%. On the other hand, at 10 mM CHAPS, the point of maximum activity, the degree of solubilization is only 45%. Thus, in summary, we conclude that (1) the cGMP channel can be reconstituted functionally by the method of detergent removal, and (2) the activity of the channel can be varied both by the degree of solubilization as well as by the concentration of detergent.

Determination of Liposome Size and Shape

Now, we present the procedure deducing statements about liposome size and shape. As an example, we use a liposome assay, prepared by 20 mM CHAPS. The liposomes are entrapped with 5 mM Ca^{2+}, but they contain no proteins. The temperature of observation is 20°C.

Figure 5 displays the results of the elastic light-scattering experiment. The ordinate is the product of the scattering intensity $I(\theta)$ and $\sin^2(\theta/2)$, at scattering angle θ. It is plotted against $\sin(\theta/2)$. For analysis, we use the hollow-sphere model, presented by eq. (2). This gives a mean outer liposome radius of $r = 80 \pm 5$ nm. However, the scattering profile can be equally well described by eq. (3), a prolate ellipsoid with an axial ratio of 0.833 and a major axis dimension of 96 nm. To distinguish these two models, we measure the mean center of mass diffusion coefficient, D_z, by means of quasi-elastic light scattering.

The normalized autocorrelation function $g(t)$ yields the reduced cumulant Γ/q^2, which has the dimension of a diffusion coefficient and may therefore also be called an apparant diffusion coefficient, D_{app}. Theory[10] on quasi-elastic light scattering proves that in the limit of $q \to 0$ ($\theta \to 0$) the first cumulant Γ is a linear function of q^2, i.e. $\lim_{q\to 0} = Dq^2$, where D is the translation diffusion coefficient. For a polydisperse polymer, D is the z-average of the true diffusion coefficient. Here, extrapolation to zero angle gives $D_z = 2.6 \pm 0.1 \times 10^{-8}$ cm^2/s as is shown in Figure 6. At larger angles, the motions of shorter sections of the liposomes are observed in the experiment. However, these motions are difficult to interpret and therefore we do not discuss the curvature of $D_{app}(\theta)$ in detail.

For prolate ellipsoids of axial ratio v and major axis a, Perrin[11] has shown that

$$D = (k_B KT/6\pi\eta a)\upsilon G(\upsilon) \qquad (4)$$

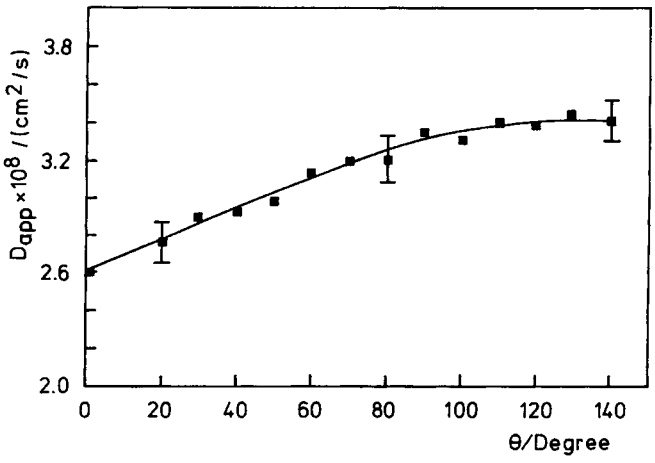

Figure 6 Diffusion coefficient D_{app} vs. scattering angle.

where $G(\upsilon) = \ln\{[1 + (1 - \upsilon^2)^{0.5}]/\upsilon\}/(1 - \upsilon^2)^{0.5}$. Using this equation, we calculate $D_z = 2.7 \times 10^{-8}$ cm^2/s for $a = 80$ nm, $\upsilon = 1$, and $D_z = 2.1 \times 10^{-8}$ cm^2/s for $a = 96$ nm and $\upsilon = 0.833$. The experiment is closer to that of a sphere. Thus, the combination of eqs. (3) and (4) shows that our liposome is better represented by a hollow sphere with an outer radius $r = 80$ nm than by a hollow prolate ellipsoid with an axial ratio of 0.833 and major axis of 96 nm.

Next, it is informative to estimate the thickness, d, of the liposome shell. For this purpose, we use the relation[12]

$$\langle S^2\rangle_z = 3/5[R_h^5 - (R_h - d)^5]/[R_h^3 - (R_h - d)^3] \quad (5)$$

where $\langle S^2\rangle_z$, calculated by $\langle S^2\rangle_z = -3\, dP(q)_z/dq^2|q = 0$,[10] presents the mean square radius of gyration. Here, $\langle S^2\rangle_z^{0.5} = 77 \pm 5$ nm. For a sphere, the hydrodynamic radius R_h and the outer sphere radius r ($r = 80$ nm) are identical. Using these values, we calculate $d = 6 \pm 2$ nm, a value that is typically for unilamellar liposomes.

Incorporation Content of Ca^{2+} per Liposome

The incorporation content of Ca^{2+} per liposome, designed by $\bar{f}$, is defined as the ratio of the Ca^{2+} amount, released maximal from a liposome by the ionophore A 23187, to one originally used to prepare the liposome. To determine $\bar{f}$, the number N of liposomes present in a sample must be known. The following handling is useful.

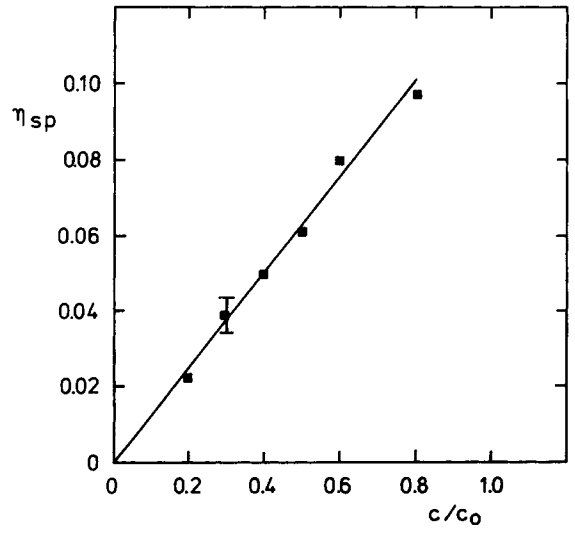

Figure 7 Specific viscosity vs. reduced liposome concentration.

Table I Dilution Experiments (Detergent: CHAPS; Ca^{2+} Amount: 5 mM; Temperature: 20°C; No Proteins)

Liposome Concentration in % of the Start Concentration	R_h/nm	U_D
100	95	0.16
75	84	0.18
50	80	0.20
25	80	0.22
12.5	80	0.20
Error	3%	10%

Figure 7 shows viscosity measurements on the liposome assay, inspected in the section above. The linear dependence of the specific viscosity η_{sp} on liposome concentration is well demonstrated in accord with the Einstein–Simha[13] equation:

$$\eta_{sp} = \nu \cdot V_h \cdot N_0(c/c_0) \quad (6)$$

Here, ν is a proportionality constant that depends on the spheroidal axial ratio υ. V_h is the hydrodynamic particle volume, N_0 or N are the number of liposomes per milliliter of sample at the start and the successive dilution, respectively, and c/c_0 is the reduced concentration. The liposome volume V_h can be calculated from the geometric parameters r or a and υ. In the limit of a sphere ($\upsilon = 1$), eq. (6) is simply

$$\eta_{sp} = 2.5 \cdot V_h \cdot N \quad (7)$$

expressing the proportionality between the specific viscosity and volume fraction of suspended liposomes. Using $r = 80$ nm and $\eta_{sp} = 0.05$, we get $V_h = (4\pi/3)r^3 = 2.14 \times 10^{-15}$ mL and $N = 9.33 \times 10^{12}$ (liposomes/mL). The Ca^{2+} amount, maximal released by A 23187, is 12.8 μM/L. The inner volume $V_i = 4\pi/3(r - d)^3$ of a liposome is 1.7×10^{-15} mL. Thus, originally, the 12.8 μM Ca^{2+} are entrapped in a volume of $N \times 10^3 \times V_i = 15.8$ mL, giving a Ca^{2+} amount per liposome of 0.81 mM/L. For liposome preparation, we have used 5 mM Ca^{2+} per liter. Using these values, the incorporation content of Ca^{2+} per liposome $\bar{f}$ is $(0.81/5) \times 100\%$, that is, 16%. Thus, the liposomes lose 84% of their Ca^{2+} content during the Ca^{2+} dialysis.

Table II Influence of Different Detergent upon Liposome Size and Shape (Ca^{2+} Amount: 5 m*M*; Temperature: 20°C; No Proteins)

Detergent	R_h/nm	S_z/nm	d/nm	$\bar{f}$/%	U_D	Shape
Mega 8	300	196	120	52	0.34	Multilamellar prolate ellipsoids
Mega 9	290	185	120	52	0.26	
Mega 10	300	180	120	52	0.22	
Octylglucosid	250	140	110	50	0.29	
Na^+—cholat	120	115	10	23	0.28	Unilamellar spheres
CHAPS	80	77	6	16	0.16	
Error	3–10%	3–10%	—	—	10%	—

Dilution Experiments

Dilution of a lipid assay with a physiological buffer can produce marked changes in the size and size distribution of micellar species. Mazer et al.,[14,15] for instance, observed at very high dilution a spontaneous micelle-to-liposome transition.

The liposome assays presented here are already diluted. However, there may be still some micelles that can be transformed into liposomes. To prove this, a liposome assay was serially diluted, by adding aliquots of liposome assay and buffer in a fixed ratio, vortex mixing for 10 s, and repeating the procedure successively using a certain volume of the previously diluted sample. The liposomes are prepared by 20 m*M* CHAPS in the presence of 5 m*M* Ca^{2+}. The results are shown in Table I.

The dilution has two effects: (1) the hydrodynamic radius R_h decreases from 95 to 80 nm, and (2) the nonuniformity coefficient U_D increases from 0.16 to 0.20. This states that there are still some micelles present that transform into liposomes by dilution. Other experiments show that the effect of dilution is generally rather small. In most cases, a dilution of 20–30% is sufficient to cast out further micelle–liposome transitions.

Effects of Different Detergents

The possibility of varying the physicochemical properties of liposomes by using different detergents for preparation are shown in Table II.

By choosing an appropriate detergent, any desired liposome with a hydrodynamic radius between 80 and 300 nm may be prepared. A narrow liposome size distribution, indicated by a small U_D value, is obtained by using the detergent CHAPS. For the other detergents, the size distribution is more heterogeneous.

Values of the shell thickness d, calculated by eq. (5), correspond with regard to Na^+–cholat and CHAPS quite well with one expected for unilamellar spherical liposomes. Against that, detergents like Mega 8 to Mega 10 or octylglucosid, which are chemical affin, yield multilamellar liposomes, shaped like a prolate ellipsoid with an axial ratio between 0.8 for Mega 8 and 0.9 for octylglucosid.

The incorporation content of Ca^{2+} per liposome $\bar{f}$ is always smaller than 100%, which states that the liposomes lose a certain part of Ca^{2+} in the course of Ca^{2+} dialysis. In fact, we calculate $\bar{f}$ = 20% for a unilamellar and $\bar{f}$ = 52% for a multilamellar liposome, a result that states decreasing Ca^{2+} loss with

Table III Influence of Ca^{2+} Amount upon Liposome Size and Shape (Detergent: CHAPS; Temperature: 20°C; No Proteins)

$C_{Ca^{2+}}$/m*M*	R_h/nm	$\langle S \rangle_z$/nm	d/nm	$\bar{f}$/%	U_D	Shape
0	80	77	6	0	0.16	
0.5	77	74	6	10	0.14	
1.0	74	72	4	13	0.14	Unilamellar sphere
2.0	72	70	4	17	0.15	
5.0	65	62	4	22	0.14	
10.0	61	58	4	27	0.15	
Error	3%	5%	—	—	10%	—

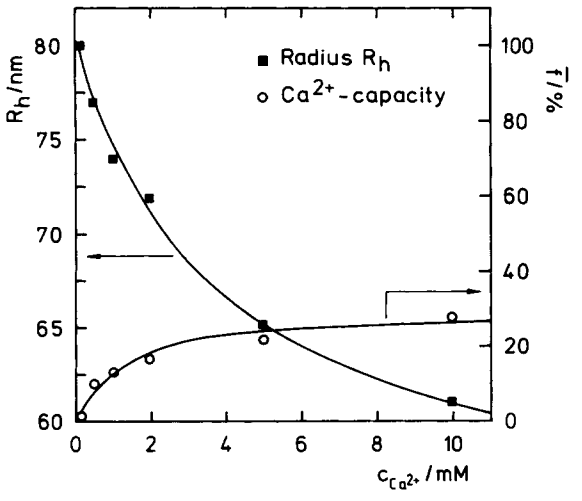

Figure 8 R_h and $\bar{f}$ vs. Ca^{2+} amount.

increasing shell thickness. On the other hand, it must be noted that the Ionophore A 23187 is not able to release all the Ca^{2+} ions entrapped into a liposome. A certain part is bounded to the membrane wall.

Finally, it should be pointed out that the detergents used here are also suitable for preparing liposomes composed of different lipids. Liposomes, containing the lipids asolectin, cholesterin, and phosphatidacid in different molar ratios, possess physicochemical properties that are rather similar to those presented in Table II, where only asolectin is used. No liposomes are obtained by detergent removal, when the lipid mixture is free of asolectin, which is shown by the fact that an injection of A 23187 induces no Ca^{2+} release. Also, a reconstitution experiment with cGMP is only then successful when asolectin is present. This observation is surprising because the natural boundary of a cGMP channel, the disk membrane, contains no asolectin. At the moment, we have no reasonable explanation for this fact.

Effects of Ca^{2+} Amount and Temperature

Marked effects upon liposome size and shape may be expected by variation of Ca^{2+} amount and temperature. To prove this, we prepare liposomes using the detergent CHAPS in the presence of different Ca^{2+} concentrations. Additionally, we vary the temperature of observation in the range of 20–65°C. The results are given in Tables III and IV.

The liposome size distribution is always narrow. Values of U_D are of the order of 0.15 and independent of the Ca^{2+} amount. In all cases, the liposomes exist as unilamellar hollow spheres with a shell thickness d of nearly 5 nm. The hydrodynamic radius R_h, shown in Figure 8, decreases slightly with an increasing amount of Ca^{2+}, and at the same time, the incorporation content of Ca^{2+} per liposome, $\bar{f}$, increases. This is consistent with the theory of Israelachvili et al.[16] stating that the electrostatic repulsion between negative-charged carboxyl groups is screened in the presence of high salt concentration. As a consequence, the liposomes contract.

As similar effect is observed with increasing temperature, where, additionally, U_D decreases. Here, it is likely that some larger liposomes decay into smaller liposomes or liposome fragments.

Effects of Protein Content

Now we report measurements on liposomes containing proteins. The proteins consist of up to 95% rhodopsin, deduced from SDS-gel electrophoresis, and 5% of other proteins, where one component is the cGMP channel. The liposomes are prepared in

Table IV Influence of Ca^{2+} Amount and Temperature upon R_h and U_D

T/°C	20		32		40		50		65	
$C_{Ca^{2+}}$/mM	R_h[a]	U_D	R_h	U_D	R_h	U_D	R_h	U_D	R_h	U_D
0.0	80	0.16	75	0.15	70	0.14	67	0.12	65	0.10
0.5	77	0.14	70	0.14	67	0.13	65	0.11	66	0.08
1.0	74	0.14	70	0.14	67	0.12	66	0.11	64	0.10
2.0	72	0.15	68	0.14	54	0.13	63	0.13	62	0.11
5.0	65	0.14	60	0.16	57	0.14	53	0.12	53	0.12
10.0	61	0.15	56	0.15	53	0.14	52	0.13	50	0.12
Error	3%	10%	3%	10%	3%	10%	3%	10%	3%	10%

[a] R_h in nm.

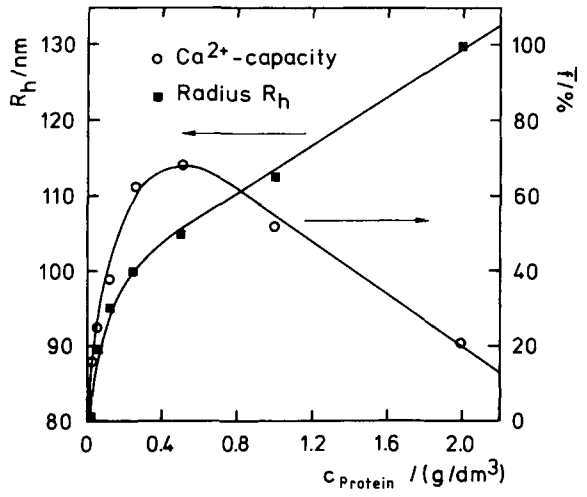

Figure 9 R_h and $\bar{f}$ vs. protein content.

the presence of 20 mM CHAPS and 5 mM Ca^{2+}. All measurements are carried out at 20°C. The results are summarized in Table V.

Both the liposome size as well as the shape depend on the content of used proteins. R_h increases with increasing protein content, and at the same time, the shape converts from an unilamellar sphere to a multilamellar prolate ellipsoid. At the highest protein content, the axial ratio v is nearly 0.82.

The incorporation content of Ca^{2+} per liposome $\bar{f}$ shows, as illustrated in Figure 9, a striking dependence upon the protein content. First, $\bar{f}$ increases with increasing protein content, then $\bar{f}$ reaches a maximum, and, at the least, $\bar{f}$ decreases slowly but continuously. Thus, first the proteins inhibit a loss of Ca^{2+} ions and then they promote a Ca^{2+} loss.

To interpret this, we determined the number, N_R, of rhodopsin molecules per liposome. From absorbance measurements at 500 nm, we get the rhodopsin content, used for liposome preparation. The extinction coefficient of rhodopsin, obtained by Shichi,[17] is $\varepsilon = 40{,}000\ M^{-1}\ cm^{-1}$, a value that is most widely accepted. Assuming the molar rhodopsin mass of 38,000 g/mol and using the number N of liposomes per assay, we calculate 10^3–10^4 rhodopsin molecules per liposome, values that are in good agreement with the literature data on affin systems.[18]

Additionally, it is instructive to calculate the surface area A of the ellipsoids inspected here. It holds that[12]

$$A = 2\pi a^2\left(1 + \frac{1}{2v^2p}\ln\frac{1+p}{1-p}\right) \qquad (8)$$

with $p = (1 - v^2)^{0.5}$, where a is the major axis of the ellipsoid and v is the axial ratio. Values of N_R, v, a, and A are summarized in Table VI.

To estimate the surface area A_R per rhodopsin molecule, called the reciprocal packing density, one must take note that the liposome jackets consist of more than one bilayer shell, where each shell is incorporated with rhodopsin. Assuming all shells possess equal reciprocal packing densities and equal thicknesses of nearly 5 nm, we calculate $d/5$ nm shells per liposome, where d presents the total thickness of liposome jacket. Then, neglecting the fact that different shells possess different radii, the mean reciprocal packing density is determined by $A_R = (A/N_R) \times (d/5\ \text{nm})$. Values of A_R are listed in Table VI. They may be used to deduce the nearest-neighbor distance l of rhodopsin molecules. For this purpose, we assume that nearest neighbors are separated by $l = 2 \times (A_R/4\pi)^{0.5}$. Values of l, derived this way, are in agreement with values of 3.5–5.6 nm, obtained by X-ray diffraction[19-20] on affin systems. Figure 10 shows that l reaches its minimal value when the incorporation content of Ca^{2+} per

Table V Influence of Protein Content upon Liposome Size and Shape (Ca^{2+} Amount: 2 mM; Detergent: CHAPS; Temperature: 20°C)

$C_{protein}$/(g/dm³)	R_h/nm	$\langle S\rangle_z$/nm	d/nm	$\bar{f}$/%	U_D	Shape
0.00	80	77	6	17	0.16	Unilamellar spheres
0.06	89	85	8	25	0.24	
0.13	95	90	10	38	0.26	
0.25	100	95	10	62	0.16	
0.50	105	100	10	68	0.16	
0.75	108	98	22	65	0.22	Multilamellar ellipsoids
1.00	115	102	35	52	0.21	
2.00	130	108	56	21	0.18	
Error	3–10%	5–10%	—	—	10%	—

Table VI Influence of Protein Content upon Geometric Parameters

$C_{Protein}/(g/dm^3)$	$N_R \times 10^{-3}$	v	a/nm	$A \times 10^{14}/m^2$	$A_R \times 10^{18}/m^2$	l/nm
0.00	—	1.0	80	8.04	—	—
0.06	2.3	1.0	89	9.95	52	4.0
0.13	4.8	1.0	95	11.30	38	3.5
0.25	9.2	1.0	100	12.60	27	2.9
0.50	18.4	0.98	116	17.40	19	2.5
0.75	27.8	0.95	123	20.70	33	3.2
1.00	37.0	0.90	126	23.50	45	3.8
2.00	72.0	0.82	152	39.10	61	4.4

liposome $\bar{f}$ reaches its maximum. This may indicate that the proteins, when they are near together, build up an intermembrane protein–lipid–protein lattice, which serves as a barrier for Ca^{2+} ions. l may be stated as a measure for the goodness of this barrier.

CONCLUSIONS

1. The cGMP channel does not lose its transport function when removed from the rod outer-segment disk membrane of bovine retinae. The activity of the reconstituted channel depends on the kind of the lipid used, the degree of protein solubilization, and the kind of detergent and its concentration used for liposome preparation. When no asolectin is used, the activity is zero. These results may become important in the future, because now it is possible to study the cGMP channel in the consensus of interaction with cellular ions or pharmacologically active drugs independent of other cellular components.

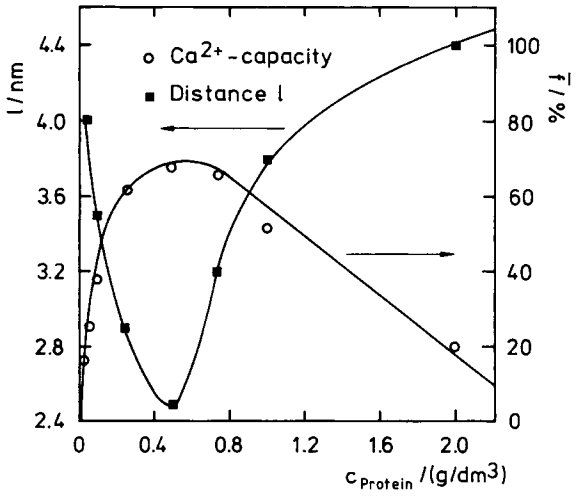

Figure 10 $\bar{f}$ and l vs. protein content.

2. Liposomes obtained by the method of detergent removal are suited for protein incorporation and for the use as drug (ion) carriers. Using different detergents, liposome size, shape, and size distribution can be varied over a wide range. The detergent CHAPS, for instance, produces a liposome population that is highly homogeneous both in size as well as in shape. Furthermore, the detergent removal technique allows the formation of liposomes that contain both saltions as well proteins of a definitive amount. The incorporation content of the ions (here Ca^{2+}) per liposome depends on the amount of ions or proteins used. It can be varied in the range of 0–65%. Other experiments, intented for the future, will show how this range can be expanded. Additionally, sensibility tests concerning time effects are planned.
3. To get a detailed analysis with regard to the physicochemical properties of a liposome population, it is indispensable to combine different techniques of measurements together. A useful combination of these is presented here. Other suitable techniques, which will be studied in the future, are electron microscopy, polarography, and small-angle neutron scattering.

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