Physical properties of single phospholipid bilayers adsorbed to micro glass beads

A new vesicular model system studied by ^{2}H-nuclear magnetic resonance

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ABSTRACT Spherical supported vesicles (SSVs), a new model system consisting of single dimyristoyl phosphatidylcholine (DMPC) bilayers adsorbed to spherical glass beads with a narrow size distribution, were prepared at two different sizes (0.5 and 1.5 μm) and their physical properties were studied by deuterium nuclear magnetic resonance (^{2}H-NMR). Such SSV samples can be prepared at any desired size between 0.3 and 10 μm. The ^{2}H-NMR measurements provide evidence for a strong dependence of the spectra and the transverse relaxation times on the curvature of the SSVs in a diameter range between 0.5 and 1.5 μm. For larger SSVs (1.5 μm diameter) their powder spectra and their calculated oriented spectra are similar to those obtained for multilamellar dispersions of DMPC-d54. The lineshape of the smaller SSVs exhibits a temperature dependence which is not found in multilamellar samples.

The SSVs are stable in the liquid crystalline phase over days but irreversibly change to multilamellar vesicles in the gel state. The average thickness of the water layer between the single bilayer and the glass bead surface was estimated by ^{1}H-NMR to be 17 ± 5 Å.

INTRODUCTION

Model systems have provided fundamental information about the structure and dynamics of biological membranes. The most widely used model is the multilamellar vesicle (MLV), which results from the dispersion and swelling of phospholipids in water. In contrast to biological membranes, which usually consist of a single bilayer, MLV samples are composed of many curved bilayers whose geometrical properties depend on their preparation and on their thermal and mechanical history. There is no known reliable way of obtaining a complete description of the curvature properties of an MLV dispersion. A further shortcoming of MLVs for some types of interaction studies is that only the small fraction of lipids in the outermost bilayer is exposed directly to the bulk water. It is possible to prepare unilamellar vesicles using sonication or size extrusion (Hope et al., 1985) of multilamellar lipid dispersions. However, the small size of such vesicle preparations restricts the range of physical phenomena which they can be used to investigate. For example, the rotational diffusion correlation time of a vesicle of diameter of 100 nm in water is $\sim 10^{-4}$ s which is sufficiently short to produce appreciable motional averaging of deuterium and phosphorous NMR spectra. Vesicles of diameter comparable to the size of biological cells do not reorient fast enough to produce appreciable motional averaging of NMR spectra. Such spectra have been shown to provide valuable information about the structure and dynamics of these systems (Seelig and Seelig, 1980; Davis, 1983).

The motivation for the work reported here is to attempt to fill the obvious need in membrane biophysics research for a unilamellar model membrane system with a well-defined shape, preferably spherical, and an adjustable and narrow size distribution in the same range as biological cells. Our solution to this problem was to design a model system supported by spherical glass beads with a narrow distribution of sizes. Each bead is surrounded by a single spherical bilayer separated from the surface of the bead by a thin water layer. The advantage of using a spherical, solid support for a vesicle is its long time stability and the possibility of preselecting the size. We refer to this model system as a spherical supported vesicle (SSV). Single bilayers with a planar, solid support have been studied in more detail by a variety of optical methods (McConnell et al., 1986; Thompson and Palmer, 1988) but we are unaware of any previous work on the SSV model system. Whereas the planar systems are prepared predominantly by Langmuir-Blodgett techniques (Agarwal, 1988), we used a technique of adsorbing and collapsing highly unstable, small vesicles on glass beads. In this paper, we report first results on the basic physical characteristics of the SSVs using the technique of deuterium (^{2}H) nuclear magnetic resonance (NMR) and compare these proper-

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ties with those of well-studied MLV samples. The phospholipid chosen for this study was dimyristoyl phosphatidylcholine with perdeuterated acyl chains (DMPC-d54). The results establish the SSVs as a useful model system for membrane studies. Their basic physical properties, such as the orientational order distribution function in the liquid crystalline phase are found to be similar to that of MLVs for SSV samples of diameter of order 1 μm or greater. In addition, results on their phase behavior and on the thickness of the water layer separating the single bilayer from the glass surface are reported and discussed.

MATERIALS AND METHODS

DMPC-d54 was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The glass beads were purchased as a crude mixture with diameters between 0.2 and 20 μm from the Dragon Werke (Bayreuth, FRG).

The separation of this mixture of glass beads was accomplished by repeated application of a sedimentation procedure method. The size distribution of the separated glass beads was measured by two methods. The first method made use of dynamic light scattering (DLS) using a 1:1 glycerol/water mixture as solvent and a constrained Laplace Transform Program (CONTIN, Provencher, 1982) for the calculation of the size distribution from the photon counting autocorrelation function as described previously (Bayerl et al., 1988). In the second method, the glass beads were measured in transmission using an Axiomat microscope (Carl T. Zeiss, Inc., Thornwood, NY). The images were digitized using a CCD and an A/D converter and size analyzed in a microcomputer using homemade software. Histograms of the size distribution were calculated and compared with the DLS results. Both methods yielded a narrow, rather symmetric size distribution with a half-width of <0.1 μm. The average size obtained from the DLS was ~0.1 μm larger, probably due to z-averaging.

Preparation of spherical supported vesicle (SSV) samples

The fractionated glass beads were washed six times in excess methanol of p.a. grade (20 ml methanol were used per gram of glass beads). After each washing step, the sample was centrifuged in a desktop centrifuge until all glass beads were sedimented. The supernatant was discarded. Finally, the pellets were dried in a vacuum oven for 12 h at 80°C. The quality of surface smoothness and sphericity of the cleaned glass beads was inspected by freeze fracture electron microscopy according to procedures described elsewhere (Bayerl et al., 1988, 1990*a*). The freeze fracture replicas exhibit smooth, structureless fracture faces indicating an essentially smooth surface of the glass beads.

The DMPC-d54 was dissolved in deuterium depleted water (Sigma Chemical Co., St. Louis, MO) at a concentration of 15 mg/ml and incubated at 30°C for 30 min. The swollen DMPC-d54 dispersion was sonicated for 15 min using a Branson tip sonifier (30 W output power, pulse mode with 50% duty cycle; Branson Sonic Power Co., Danbury, CT). Use of the pulse mode and an external water bath kept the temperature of the sample below 50°C during sonication. After sonication, the sample was found to be optically transparent and was centrifuged at 5,000 g for 10 min to remove titanium dust. The resulting small unilamellar vesicle dispersion was incubated at 60°C for 2 min, and the dry glass beads (temperature 25°C) were poured into the vesicle dispersion under rigorous vortexing. Under such conditions, the unstable highly curved vesicles collapse upon contact with the glass beads and form a continuous bilayer around the glass surface. The amount of DMPC-d54 was chosen according to the total glass surface exposed to the vesicles and the amount of lipid required to cover this surface with a continous bilayer. Assuming a headgroup area per DMPC-d54 molecule of 55 Å^2 (Bayerl et al., 1990*b*) and considering the specific gravity of the glass beads of 2.5, this amount of lipid m_L required to coat glass beads of radius r and a total mass m_b with a continuous bilayer can be estimated according to:

$$m_L \approx 1.03\ 10^{-2}\ (m_b/r), \qquad (1)$$

where m_L, m_b are in grams and r is in micrometers.

After 60 s of vortexing, the glass beads-vesicle mixture was centrifuged and the supernatant was discarded. The coated glass beads in the pellet were redispersed in a fourfold excess volume of deuterium-depleted water, vortexed, and centrifuged again to remove all remaining small sonicated vesicles not adsorbed at the glass surface. This washing step was repeated a total of six times at a temperature of 25°C. As shown in Fig. 1, it causes the total ^{2}H-NMR intensity of the sample to approach its equilibrium value roughly exponentially due to the removal of excess vesicles. The latter corresponds to the amount of DMPC-d54 which can be calculated by Eq. 1 and which is tightly adsorbed onto the glass surface. The final pellet was diluted by deuterium depleted water at a glass/water ratio of 2:1 (wt/wt) and then transferred to a plastic sample tube (ϕ 10 mm) for ^{2}H-NMR measurements.

^{2}H-NMR measurements

Deuterium NMR experiments were performed at 46 MHz using a home built NMR spectometer described by Sternin (1985). All spectra were obtained using the quadrupolar echo technique with a pulse separation of τ = 50 μs and two 90° pulses of 4 μs duration. The repetition time for successive pulse sequences was 200 ms and 2,048 points were collected with a dwell time of 2 μs. If not stated otherwise, the temperature of the samples was 310 K and 50,000 scans were accumulated. All experiments were done on resonance with an eight cyclops pulse cycling sequence (Rance and Byrd, 1983), and no phase corrections were performed. The temperature dependence of the first moments of the samples was obtained by varying the temperature in a descending manner in 1–2°C

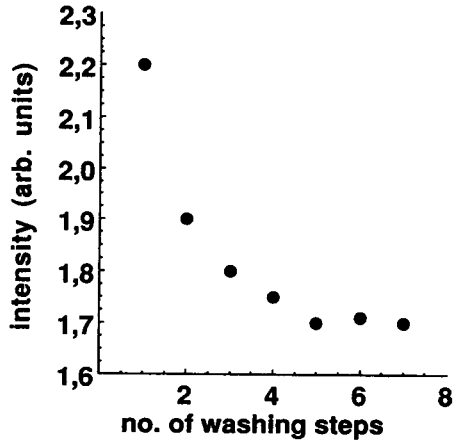

FIGURE 1 Plot of the total normalized ^{2}H-NMR intensity of a 1.5-μm SSV sample as a function of the number of washing steps applied to this sample.

steps (20 min equilibration time between each step), controlled by a temperature control unit (Bruker Instruments, Inc., Billerica, MA).

T_2^{qe} measurements were performed by increasing the separation τ between the $\pi/2$ pulses in 10 steps up to 500 μs (for MLV samples) or up to 250 μs (for SSV samples). At each separation τ, 5,000 (for MLV) or 15,000 (for SSV) scans were accumulated. Semilogarithmic plots of the normalized intensity at the top of the echo vs. 2τ yielded linear dependences for both MLV and SSV samples which gave T_2^{qe}.

RESULTS AND DISCUSSION

Order parameter distribution function and the influence of curvature

Fig. 2 shows 2H-NMR spectra, and the corresponding oriented spectra obtained from the powder spectrum by a numerical ("de-Pake-ing") procedure (Bloom et al., 1981; Sternin et al., 1983), of DMPC-d54 in multilamellar vesicles (MLV, Fig. 2, *A* and *B*) and in spherical supported vesicles (SSV) of 1.5 μm (Fig. 2, *C* and *D*) and 0.5 μm diameter (Fig. 2, *E* and *F*), respectively, in the liquid crystalline phase state. The spectral features of the large (1, 5 μm) SSV are similar to those of the MLV, although there is some broadening in the SSV spectrum. This broadening is also obvious from the "de-Paked" spectrum (Fig. 2, *B* and *D*), where the individual methylene and methyl resonances exhibit similar relative intensities and quadrupolar splittings but are less resolved for the SSV. Both MLV and SSV show a plateau region arising from the carbon numbers n = 3–9 which cannot be resolved. Hence, the flexibility along the fatty acyl chain, which can be expressed as the order parameter profile, is not significantly affected by the adsorption the bilayer to a glass surface as compared to multilamellar vesicles in the liquid crystalline phase.

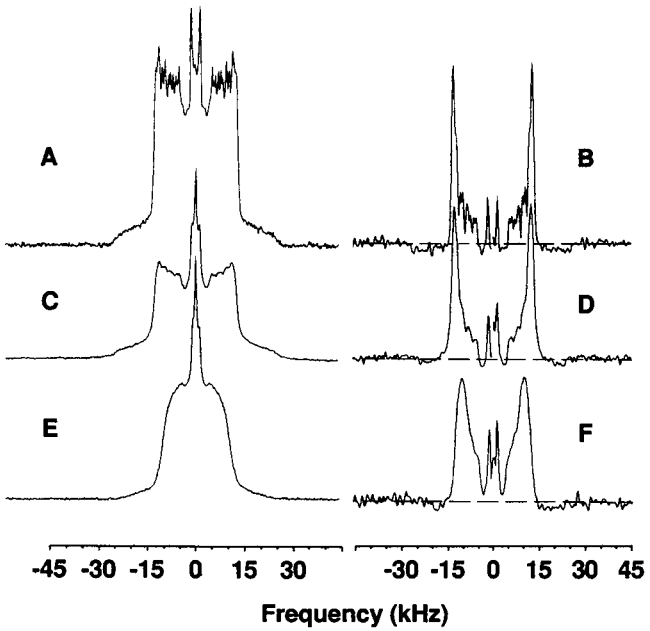

FIGURE 2 ^{2}H-NMR spectra and their corresponding oriented spectra obtained by a numerical "de-Pakeing" procedure of DMPC-d54 multilamellar vesicles (*A*, *B*), and of spherical supported vesicles (SSV) with a diameter of 1.5 μM (*C*, *D*) and of 0.5 μm (*E*, *F*) at a temperature of 310 K.

In contrast, SSVs with a diameter of 0.5 μm give ^{2}H-NMR spectra with a significantly reduced intensity of the edge region (corresponding to a 90° orientation of the normal to the membrane surface with respect to the external field $\mathbf{B}_0$), resulting in a rounding of the spectral shape near the edges (Fig. 2 *E*). Additionally, a considerable intensity contribution can be observed at the isotropic position. The "de-Paked" spectrum (Fig. 2 *F*) exhibits a 10% reduced average quadrupolar splitting of the plateau region and a broadening of the methylene resonances towards the end of the chain to such an extent that they become indistinguishable from the plateau. The only unaffected group is the terminal methyl group. Moreover, the total intensity, corresponding to the peak intensity of the echo of the 0.5-μm SSV sample is 30% lower than that of the 1.5-μm SSV sample under conditions that both samples contain the same amount of DMPC-d54. This effect can be accounted for by the differences in T_2^{qe} for the two samples, as shown below.

The transverse relaxation time (T_2^{qe}) behavior of SSV exhibits a drastic curvature dependence which will be discussed in more detail in a later paper. The value of T_2^{qe} obtained for the 1.5 μm sample (T_2^{qe} = 240 μs) is ~2.5 times higher than that obtained for the 0.5 μm sample (T_2^{qe} = 100 μs). We attribute this change of T_2^{qe} to the lateral diffusion of the phospholipids along membrane surfaces of different curvature. Such a T_2^{qe} mechanism was suggested recently by Bloom and Sternin (1987) and accounts for the observed decrease of T_2^{qe} with decreasing diameter. For DMPC-d54 MLV, a value of T_2^{qe} = 550 μs was obtained.

The T_2^{qe} measurements reported above should be treated with caution. As described in Materials and Methods, they were obtained by the standard quadrupolar echo pulse sequence, which can give systematic errors due to its incomplete refocusing of phase acquired by the spins due to the dipolar field associated with the magnetic moment of the glass bead. That this contribution to the echo decay is nonnegligible for such systems has been demonstrated in an earlier paper on the influence of diamagnetic susceptibility of SSV ^{2}H-NMR spectra. This effect could be responsible for some of the broadening of SSV spectra (Fig. 1, *C* and *D*) as well as for part of the difference in the values of T_2^{qe} obtained for MLV and SSV samples. However, the variation in T_2^{qe} with the SSV size cannot be

accounted for by this effect because the dipolar field at the surface of the glass bead is independent of its diameter (Bayerl and Bloom, 1990).

Single bilayer character of the SSVs

An important question is whether the adsorbed bilayer of SSV samples is a single one or if multilamellar SSVs can also exist. To test this, we prepared SSV samples of 1.5 μm diameter in varying excess (up to 10-fold) of DMPC-d54 of that required for a single bilayer coating according to Eq. 1 (cf. Materials and Methods). After washing these samples according to procedures described in Materials and Methods, equal volumes of these samples were measured by ^{2}H-NMR (10,000 scans for each sample). The measured total intensities, however, do not differ by >8% from the value obtained for a sample where the SSVs are prepared in 1.2-fold excess of DMPC-d54 only. Hence, these results provide evidence that SSVs always have only a single bilayer.

Isotropic signal and its temperature dependence

A salient feature of all ^{2}H-NMR spectra of SSV samples is an isotropic signal superimposed on the terminal methyl resonance (Fig. 2, *C* and *E*). This isotropic signal cannot be attributed to the glass which contains a negligible number of deuterons. Nor can it be diminished by washing of the SSV sample. The T_2^{qe} of this signal (≈700 μs) is similar to that measured for the terminal methyl group of SSV, indicating that lipids and not water account for this signal. A possible explanation is that DMPC-d54 molecules in highly curved parts of the bilayer undergo motional averaging due to lateral diffusion. Such highly curved bilayer regions could be caused by a mismatch between glass and bilayer surface as a result of the very different thermal expansion coefficients of the two substances. A possible response of the SSV bilayer to such a mismatch might be the formation of highly curved, tube like bilayer protrusions which relax the lateral pressure but minimize the area on the glass surface which is not covered by a bilayer. If true, this would mean a drastic dependence of the isotropic signal on the temperature. As shown in Fig. 3, such a dependence indeed exists. The ^{2}H-NMR spectra of the 1.5 μm diameter sample are shown at three different temperatures: 55°C (Fig. 3 *A*), 45°C (Fig. 3 *B*), and 27°C (Fig. 3 *C*).

At the lowest temperature, just 8.4°C above the gel-to-liquid crystalline phase transition of DMPC-d54 (T_m = 19.6°C), the spectrum is similar to that of an MLV sample, but the edges of the Pake doublets of the different ^{2}H nuclei on the acyl chains are less well resolved, as would be the case if they were slightly broadened. The isotropic signal accounts for only 3–4% of the total intensity. Increasing the temperature not only reduces the average quadrupolar splitting in the same way as for MLVs but also decreases the spectral intensity of the edges and increases significantly the isotropic signal (Fig. 3 *B*). Finally, the 1.5-μm SSV spectrum at 55°C (Fig. 3 *A*) resembles that obtained for a 0.5-μm SSV at 37°C (Fig. 2 *E*). At 55°C, the isotropic signal accounts for 15% of the total intensity.

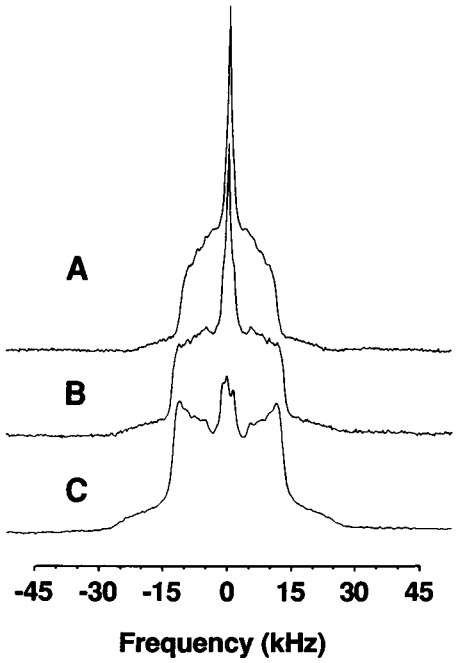

FIGURE 3 ^{2}H-NMR spectra of DMPC-d54 SSV with a diameter of 1.5 μm at 55°C (*A*), 45°C (*B*), and 37°C (*C*).

Effects of the phase transition

It should be emphasized that the temperature dependence shown in Fig. 3 is completely reversible as long as the sample is kept in the liquid crystalline phase state. However, lowering the temperature of the SSV sample below the T_m value of DMPC-d54, irreversibly changes the ^{2}H-NMR spectra of the sample. A 1.5-μm SSV sample, cooled down from 37°C to 10°C, exhibits a ^{2}H-NMR spectrum at the latter temperature indistinguishable from that of an MLV sample at 10°C. Raising the temperature again (after 3 h incubation at 10°C) to 37°C then gives a highly resolved ^{2}H-NMR spectrum typical of MLV samples as shown in Fig. 2 *A*. This indicates that the single bilayer can detach itself from the glass surface upon cooling the sample below T_m. The detached bilayer fragments spontaneously form new MLVs. Measuring the moments of the SSV spectra as a

function of the temperature upon cooling the sample gives information about the effect of the adsorption on the lipid phase behavior. As shown in Fig. 4, the first moment M_1 of the spectra, given in terms of the normalized ^{2}H-NMR line shape function $g(\omega)$ by (Davis, 1979)

$$M_1 = \int_0^\infty \omega g(\omega)\, d\omega = \langle \omega \rangle, \qquad (2)$$

increases from 0.45×10^5 s^{-1} to 1.2×10^5 s^{-1} upon traversing the T_m region. A similar change of M_1 is observed for DMPC-d54 MLV, but over a narrower temperature region. The onset temperature of the transition to the gel phase is $\approx$1.5°C higher for the SSV sample, indicating a destabilization of the liquid crystalline phase of DMPC-d54 due to the interaction with the glass surface. Heating the SSV sample after 3 h incubation at 10°C now reproduces exactly the course of M_1 obtained for MLV upon heating (Bayerl et al., 1990*a*). These results suggest that the detachment of the bilayer from the glass surface occurs during the passage from the liquid crystalline to the gel state. A likely explanation for this detachment is a disruption of the bilayer at the phase transition due to the 5% change of the molecular volume of DMPC-d54 (Knoll et al., 1985; Bayerl et al., 1990) which causes an area mismatch between the glass surface and the bilayer.

Thickness of the water layer

A further essential question about the SSV structure concerns the thickness of the water layer trapped between the single bilayer and the glass surface. The trapped water can be measured by proton (^{1}H) NMR using a paramagnetic salt such as $MnCl_2$, provided that the SSV bilayer proves sufficiently ion tight to prevent a fast permeation of manganese ions into the trapped water layer. Fig. 5 *A* shows the water signal arising from a 1.5-μm SSV sample at 30°C. Addition of $MnCl_2$ causes a fast T_2 relaxation of all protons accessible to the ions, resulting in a reduction of the ^{1}H-NMR signal intensity. Above a concentration of 12 mM $MnCl_2$ there is no further reduction of the intensity within 30 min after the salt addition (Fig. 5 *B*). As the contribution of the chain perdeuterated DMPC-d54 to the ^{1}H-NMR signal is negligible, the remaining signal intensity must arise from the water trapped between the single bilayer and the glass surface. This result provides evidence that the SSV bilayer forms a closed shell with a low permeability for manganese ions and is separated from the glass surface by a water layer. One can expect that the disruption of the bilayer at the transition from the liquid crystalline to the gel phase gives rise to the entry of manganese ions into the trapped water layer, thereby causing a complete loss of all ^{1}H-NMR signal intensity. This is the case in Fig. 5 *C*, where the sample was measured after a 5-min incubation at 10°C.

From the known volume of water (V_w), the number of glass beads (N_b) in the sample and the normalized total intensities before (I_0) and after (I_{sat}) the addition of a saturating concentration of $MnCl_2$, the average thickness d_w of the trapped water layer can be estimated according to

$$\langle d_w \rangle = [(3V_w I_{sat}/4\pi I_0 N_b) + R_b^3]^{1/3} - R_b. \qquad (3)$$

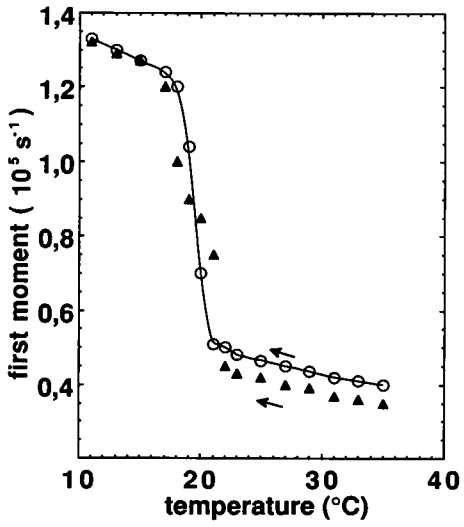

FIGURE 4 Temperature dependence of the ^{2}H-NMR first moment at the transition from the liquid crystalline to the gel phase for DMPC-d54 MLV (○) and for SSV (▲) with a diameter of 1.5 μm. The arrows indicate the direction of the temperature change.

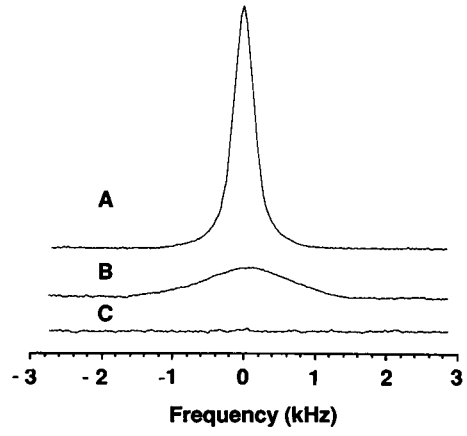

FIGURE 5 ^{1}H-NMR spectra of a 1.5-μm-diam DMPC-d54 SSV sample in a 1:1 H_2O/D_2O mixture at 310 K before (*A*) and after (*B*) the addition of 12 mM $MnCl_2$. The data in *C* were obtained a 5 min incubation of the sample at 10°C.

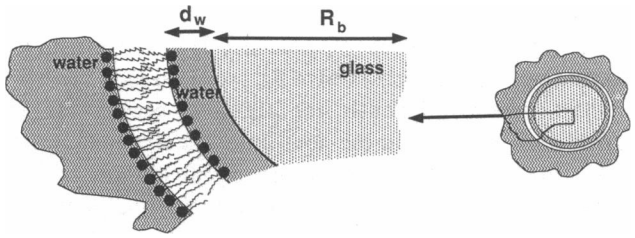

FIGURE 6 Schematic representation of a spherical supported vesicle (see text for details).

The values of N_b can be estimated from the dry weight of the beads, their specific gravity of 2.5 and their radius R_b of 0.75 ± 0.05 μm. The experiment yields a value of $R_w \approx 17 \pm 5$ Å, i.e., the single bilayer of an SSV is located near the glass surface but is distinctly separated from it. The obtained water layer thickness is in excellent agreement with that obtained very recently by specular reflection of neutrons on a DMPC bilayer adsorbed to a silica plate, where a value of $R_w = 16 \pm 5$ Å was measured in the fluid phase state (Johnson, S. J., T. M. Bayerl, D. C. McDermott, A. R. Rennie, R. K. Thomas, and E. Sackmann, to be submitted for publication). The value of R_w is in a range where repulsive hydration forces begin to dominate the total interaction energy between the glass surface and the amphiphilic lipid bilayer (Israelachvili, 1985). A schematic representation of an SSV, deducted from the results of this work, is shown in Fig. 6.

CONCLUSION

In this paper, a new model system for NMR studies of phospholipid membranes is presented and some of its basic physical properties are discussed. The major advantages of these spherical supported vesicles (SSVs) over all other vesicle preparations is their adjustable, uniform size as well as their unilamellarity. These properties of the system will help in future studies to understand the role of membrane curvature and multilamellarity on the dynamics and structure of lipid bilayers.

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