Hydration of Polyethylene Glycol-Grafted Liposomes

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ABSTRACT This study aimed to characterize the effect of polyethylene glycol of 2000 molecular weight (PEG^{2000}) attached to a dialkylphosphatidic acid (dihexadecylphosphatidyl (DHP)-PEG^{2000}) on the hydration and thermodynamic stability of lipid assemblies. Differential scanning calorimetry, densitometry, and ultrasound velocity and absorption measurements were used for thermodynamic and hydrational characterization. Using a differential scanning calorimetry technique we showed that each molecule of PEG^{2000} binds 136 $\pm$ 4 molecules of water. For PEG^{2000} covalently attached to the lipid molecules organized in micelles, the water binding increases to 210 $\pm$ 6 water molecules. This demonstrates that the two different structural configurations of the PEG^{2000}, a random coil in the case of the free PEG and a brush in the case of DHP-PEG^{2000} micelles, differ in their hydration level. Ultrasound absorption changes in liposomes reflect mainly the heterophase fluctuations and packing defects in the lipid bilayer. The PEG-induced excess ultrasound absorption of the lipid bilayer at 7.7 MHz for PEG-lipid concentrations over 5 mol % indicates the increase in the relaxation time of the headgroup rotation due to PEG-PEG interactions. The adiabatic compressibility (calculated from ultrasound velocity and density) of the lipid bilayer of the liposome increases monotonically with PEG-lipid concentration up to ~7 mol %, reflecting release of water from the lipid headgroup region. Elimination of this water, induced by grafted PEG, leads to a decrease in bilayer defects and enhanced lateral packing of the phospholipid acyl chains. We assume that the dehydration of the lipid headgroup region in conjunction with the increase of the hydration of the outer layer by grafting PEG in brush configuration are responsible for increasing thermodynamic stability of the liposomes at 5–7 mol % of PEG-lipid. At higher PEG-lipid concentrations, compressibility and partial volume of the lipid phase of the samples decrease. This reflects the increase in hydration of the lipid headgroup region (up to five additional water molecules per lipid molecule for 12 mol % PEG-lipid) and the weakening of the bilayer packing due to the lateral repulsion of PEG chains.

INTRODUCTION

The stability and hydration of liposomes having polyethylene glycol (PEG) terminally grafted to their surface are currently receiving considerable attention. PEG is a nontoxic, water-soluble, and chemically inert synthetic polymer and is preferentially excluded from the surface of proteins (Arakawa and Timasheff, 1985), DNA (Jordan et al., 1972), and liposomes (Arnold et al., 1990). By dialysis equilibrium coupled with density measurements, it was shown that the thickness of the effective shell relatively impenetrable to free PEG around the biopolymer increases with increasing radius of gyration, R_g, of PEG, which is a function of PEG molecular weight (Bhat and Timasheff, 1992). It seems that the preferential exclusion of free PEG from the biopolymer or liposome is principally due to the steric exclusion of PEG from the hydrated surface. The gradient of the PEG concentrations between the impenetrable to free PEG layer on the liposome surface and the bulk solution leads to osmotic imbalance and changes in the thermodynamic properties and hydration of the lipids (Lehtonen and Kinnunen, 1994, 1995).

PEG grafted on the liposome surface does not cause remarkable changes in cohesive and mechanical properties of liposomes (Martin and Lasic, 1991; Needham et al., 1992) but greatly increases its biological stability, reduces leakage of the encapsulated drug, and permits achieving major advances in therapeutic application of liposomes (Woodle, 1993; Torchilin et al., 1994; Lasic and Martin, 1995; Lasic, 1996; Barenholz and Lasic, 1996). Despite intensive experimental efforts, the mechanism of stabilization of the liposomes by PEG is still a controversial subject. It was shown that attachment of PEG molecules to the surface of liposomes both strongly reduces the attractive forces and increases the repulsive ones (steric and hydration) between liposomes to each other and to cells (Kuhl et al., 1994; Kenworthy et al., 1995a,b; Lasic and Martin, 1995). The other works (Blume and Cevc, 1993; Torchilin et al., 1994) suggested that the high mobility of attached PEG molecules exerts primary control over the prolongation of the circulation time of liposomes in vivo.

Hydration plays a significant role in the thermodynamic stability of liposomes (Ulrich and Watts, 1994; Volke et al., 1994; Lasic and Martin, 1995). Thermodynamic properties of solutes, such as heat capacity, partial volume, and compressibility, are known to be sensitive to the degree and nature of solute hydration and have been used widely to estimate the hydration of proteins and amino acids (Priev et al., 1990; Sarvazyan, 1991; Kharakoz and Sarvazyan, 1993), DNA (Buckin et al., 1989, 1994; Chalikian et al., 1994), and liposomes (Buckin et al., 1979; Mitaku and

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Aruga, 1982; Barenholz et al., 1983). It was shown that hydration of polymers and biopolymers could be satisfactorily described using experimental data on the hydration of individual atomic groups.

One of the goals of this work was to investigate the thermodynamic properties of 100-nm large unilamellar liposomes (LUV) composed of mixtures of egg phosphatidylcholine (PC) and dihexadecylphosphatidic acid (DHP)-PEG^{2000} in different mole ratios and compare the degree of hydration of those liposomes. The other goal was to identify configuration and hydration of the PEG^{2000} when it is in the bulk solution and when covalently attached to the amphiphilic surface.

To meet the first goal we determined the ultrasound absorption α, sound velocity U, and density ρ, of LUV composed of egg PC and various mol % of DHP-PEG^{2000} (0, 2, 4, 6.8, 10, and 12 mol %). The measurements were performed at liposome concentrations of 0–4% and a temperature range of 3–45°C. The second goal was approached using differential scanning calorimetry, comparing hydration of PEG^{2000} in the free state and grafted on the surface of micelles.

MATERIALS AND METHODS

Lipids

The racemic DHP-PEG^{2000} was synthesized in our laboratory and was characterized by ^{31}P nuclear magnetic resonance (NMR), ^{1}H NMR, thin layer chromatography, and element analysis, as described elsewhere (Tirosh et al., 1997a).

Distearoylphosphoethanolamine (DSPE)-PEG^{2000} was a gift of SEQUUS Pharmaceuticals, Menlo Park, CA. Dihexadecylphosphatidylcholine (DHPC) was a gift of Genzyme, Cambridge, UK. Egg phosphatidylcholine (PC) and fully hydrogenated soy PC (HPC) of purity higher than 98% were obtained from Lipoid, Ludwigshafen, Germany. All chemicals were used without further purification.

Liposome preparation

Large unilamellar liposomes (LUVs) were prepared from various concentrations of egg PC and were sized to 100 nm average diameter by extrusion through a polycarbonate filter, according to an already published procedure (MacDonald et al., 1991). Several preparations were made, one composed only of egg PC (egg PC LUV), the others a mixture of egg PC containing 2, 4, 6.8, 10, or 12 mol % DHP-PEG^{2000} (DHP-PEG^{2000} LUV).

Micellar suspension preparation

DHP-PEG^{2000} or DSPE-PEG^{2000} was lyophilized overnight before preparation of micellar suspensions. The lipids were dissolved at 10% w/w in water. To obtain an optically clear solution, sonication for 1 min at 8 kHz was used.

Quantification of bound water by differential scanning calorimetry

Evaluation of bound water was done by the differential scanning calorimetry (DSC) technique using Mettler thermal analyzer model 4000. The ice-to-water fusion heat enthalpy was evaluated by the peak integral at negative temperatures around 0°C. Scanning was conducted from −30°C to 10°C at a rate of 2°C/min and was repeated three times for each sample until a plateau was reached. The amounts of water bound to free PEG^{2000} and to PEG^{2000} covalently attached to dihexadecylphosphatidic acid were calculated from the fusion enthalpy $\Delta H_{(fu)}$) of the water solutions containing PEG (or PEG-lipids) and that of water without the PEG (Barenholz et al., 1983):

$$n_h = \frac{100\ (\Delta Hf_{(fu)}\ \text{water} - \Delta H_{(fu)}\text{PEG/water})}{\Delta Hf_{(fu)}\ \text{water}\ N_{PEG}} \qquad (1)$$

where n_h is the hydration number or amount of water bound to PEG, and N_{PEG} is the moles of PEG in the solution.

Volumetric measurements

The density ρ_C of solutions at the selected solute concentration c was determined using the vibrating tube densitometer DMA-60/DMA-601 (Anton Paar, Gratz, Austria), with a precision of $\pm 3 \times 10^{-6}$ g/ml. The partial molar volume of the solute V is defined by the change in the solution volume resulting from dissolving 1 mol of solute at infinite dilution. It has been shown in the review by Zamyatnin (1984) that, for diluted solutions,

$$V = 1/\rho_0 - \lim_{c\to 0}[(\rho_C - \rho_0)/(\rho_0 c)] \qquad (2)$$

where ρ_0 is the density at 0 solute concentration (solvent density).

All solutions were degassed in vacuum for at least 12 h at 4°C before performing volumetric as well as ultrasonic measurements. The value for the density of water at different temperatures was taken from Kell (1975). The experimental error in the determination of the solute concentration was ~0.5%. For each evaluation of V three independent measurements were carried out within a concentration range of 0.4–4%.

Ultrasonic measurements

Measurements of ultrasound velocity and absorption in liposome suspensions were performed using the resonator method (Eggers and Funk, 1973) in its differential version (Sarvazyan, 1982). Due to the formation of standing waves, resonance is produced in the measuring cell at frequency f at which the distance l between the acoustic transducers in the measuring cell equals $n\lambda/2$, where n is a positive integer and $\lambda/2$ is a half-wavelength. By parallel experiments with a sample-filled and reference cell, excess ultrasonic velocity and absorption can be determined. For dilute solutions for which ultrasound velocity U does not substantially differ from that measured in the solvent, U_0 (Sarvazyan and Chalikian, 1991),

$$\Delta U = U_0 \Delta f_n / f_n \qquad (3)$$

where f_n is the frequency of the nth resonance peak of the acoustic resonator measured in the solvent.

For those solutions in which ultrasound absorption per wavelength, $\alpha\lambda$, is sufficiently high (Sarvazyan and Chalikian, 1991),

$$\Delta(\alpha\lambda) = \Delta(\pi \delta f_n / f_n) \qquad (4)$$

where δf_n is the half-power width of the resonance peak.

The acoustic cells contained 0.7 ml sample and were thermostated within ±0.01°C. The experimental error in determining relative changes in U was $\sim 1 \times 10^{-3}$% and in determining relative changes in $\alpha\lambda$ was ~2%. Temperature dependencies of U and $\alpha\lambda$ were measured by changing the temperature in steps; after setting a temperature, the samples were equilibrated and ultrasonic data were taken. The procedure was repeated for each temperature at 3, 10, 20, 30, and 45°C.

Compressibility calculation

The apparent adiabatic compressibility K, which is defined by $-(dV/dP)dV/dP$, where P is pressure applied at constant entropy, can be calcu-

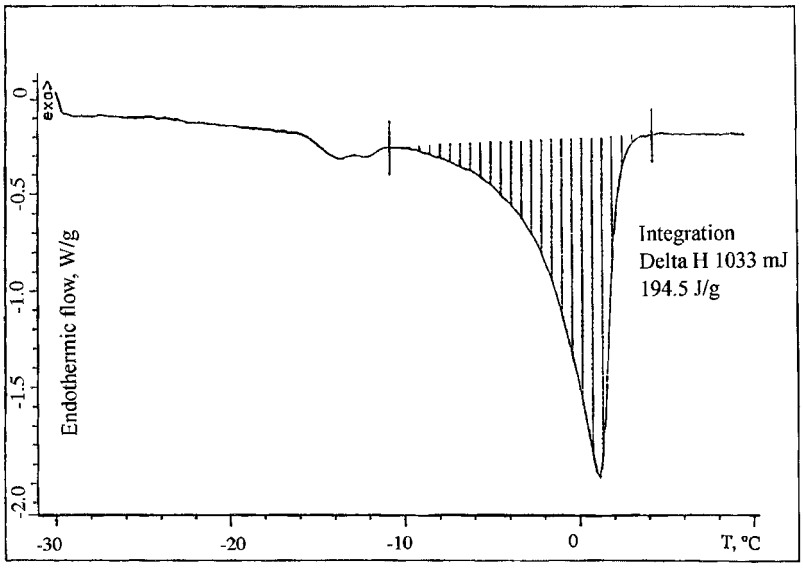

FIGURE 1 Changes of the heat profile of water ($\Delta H_{(fu)}$) in the presence of 20% PEG^{2000}. The amount of bound water was calculated from the decrease in the $\Delta H_{(fu)}$ of the free water peak (peak at −2.5°C).

lated from the relation $K = \beta V$, where β is the coefficient of adiabatic compressibility. Note that, in contrast to K (measured in ml/g-atm), β (measured in 1/atm), is independent of the apparent volume of the solute, V. β can be determined from the measured density ρ and the sound velocity U using the relation $\beta = 1/\rho U^2$. It has been shown in review by Sarvazyan (1991) that

$$K = \beta_0(2V - 2[(U_C - U_0)/(U_0 c)] - 1/\rho_0) \qquad (5)$$

For each determination of K three independent measurements of U and V were carried out within a concentration range of 0.4–4%.

RESULTS

DSC results

Increasing concentrations of PEG in water induced large changes in the DSC plot of water. Scans were performed from −30°C to 10°C for water and for solutions containing increasing concentrations of PEG^{2000}. The fusion enthalpy of water, $\Delta H_{(fu)}$ water, was 330 J/g. Twenty percent (w/w) of PEG^{2000} induced an enormous decrease in the free water peak (Fig. 1). This is an indication that water became tightly bound to the PEG. From a calibration curve (Fig. 2) of the $\Delta H_{(fu)}$ of free water versus PEG concentrations at 45% PEG, there is no free water in the solution. Based on this, the hydration number n_h or the amount of bound water to PEG was calculated. The amount of bound water to DHP-PEG^{2000} or DSPE-PEG^{2000} was estimated from three separate measurements of the $\Delta H_{(fu)}$ of 10% PEG-lipid micellar solutions. The DSC measurements of the hydration number of free and attached PEG, as well as of DHPC, are summarized in Table 1.

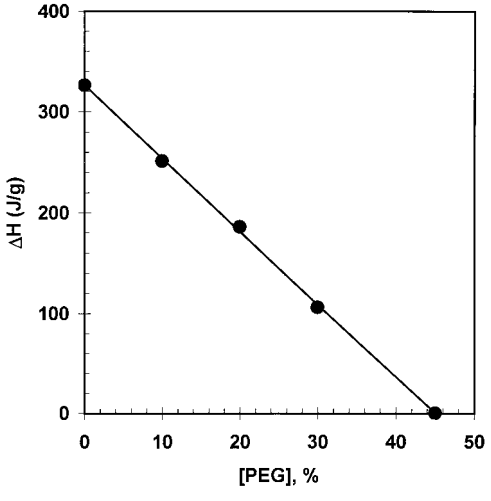

FIGURE 2 Effect of PEG^{2000} on $\Delta H_{(fu)}$ of water obtained at different concentrations of the PEG.

Volumetric and acoustic results

Ultrasound absorption, ultrasound velocity, and density of unilamellar conventional liposomes composed of a mixture of egg PC and DHP with covalently attached PEG^{2000} were measured as a function of the PEG-lipid concentration (0–12 mol %), lipid concentration (0–4%), and temperature (3–45°C).

Fig. 3 represents the behavior of density (upper), ultrasound velocity (middle), and absorption (lower) at 7.7 MHz of egg PC LUV and DHP-PEG^{2000} LUV containing 6.8 mol % of PEG-lipids in water (30°C) as a function of lipid concentration. In this system, and in all the other systems

TABLE 1 Hydration number (molecules of water per molecule of solute) of DHPC liposomes and of PEG^{2000}, free and grafted onto the surface of micelles

	Hydration number
DHPC liposomes	11 ± 2
PEG	136 ± 4
DHP-PEG^{2000} micelles	200 ± 6
DSPE-PEG^{2000} micelles	210 ± 6

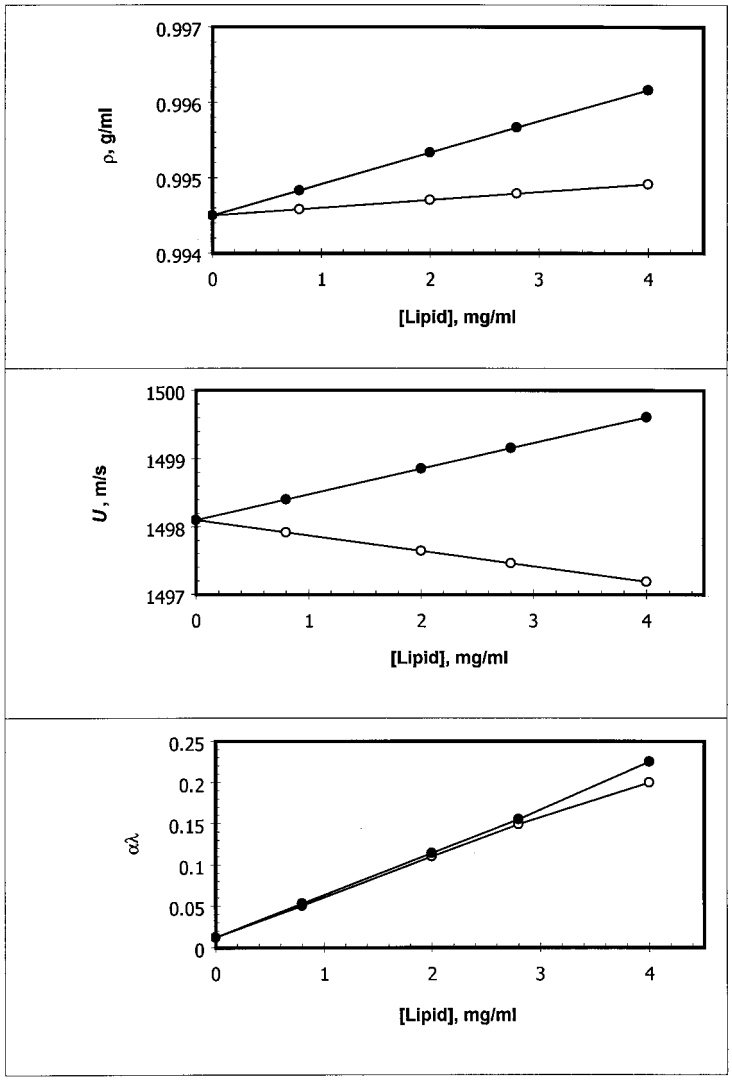

FIGURE 3 Density (*top*), ultrasound velocity (*middle*), and absorption (*bottom*), at 7.7 MHz of egg PC SUV (○) and DHP-PEG^{2000} SUV containing 6.8 mol % of PEG-lipids (●) in water (30°C) as a function of liposome concentration.

studied in this work, there was a linear relationship between density, ultrasound absorption, sound velocity, and the lipid concentrations; thus such parameters as density number $[\rho] = \lim_{c\to 0}[(\rho_C - \rho_0)/(\rho_0 c)]$, ultrasound velocity number $[U] = \lim_{c\to 0}[(U_C - U_0)/(U_0 c)]$, and molecular absorption number $[A] = \lim_{c\to 0}[(\alpha\lambda_C - \alpha\lambda_0)/c]$ can be used to characterize the thermodynamic properties of the liposomal solutions. A good linearity of the dependence of ρ, U, and $\alpha\lambda$ on c indicates that the contribution of liposome-liposome interaction to V, K, and $[A]$ is negligible.

The apparent molar volume of the egg PC liposomes at infinite dilution (partial molar volume) was 740.5 ml/mol in water solution at 30°C. The partial molar compressibility was 32.84×10^{-6} ml/mol-atm. These data are in good agreement with the literature (Barenholz et al., 1977; Buckin et al., 1979; Mitaku and Agura, 1982). Addition of PEG-lipids to the egg PC liposomes leads to a decrease in partial molar volume and compressibility due mostly to the contribution of PEG, which has a relatively small value of partial molar volume and compressibility (36.77 ml/mol and 1.5×10^{-6} ml/mol-atm, respectively, per PEG repeated unit).

The values of sound velocity number, specific volume, specific adiabatic compressibility, and molecular absorption number for the egg PC liposomes containing PEG-lipids in water solution at different temperatures are given in Table 2. Errors were estimated by taking into account uncertainties due to apparatus limitations and concentration determination. The temperature dependencies of the partial specific volumes V and adiabatic compressibilities K of PEG-modified liposomes can be approximated by second-order polynomial functions (correlation coefficient higher than 0.99).

DISCUSSION

Hydration of polyethylene glycol

Calorimetric and volumetric studies on a large variety of low molecular weight compounds indicate that solute-induced changes in the properties of water in the vicinity of a

TABLE 2 Volumetric and acoustic properties of egg PC liposomes containing DHP-PEG^{2000} at temperatures of 3–45°C

[PEG-lipid] (mol %)	Sound velocity number $[U]$ (ml/g)					Specific volume V (ml/g)					Specific compressibility K (ml/g-atm $\times 10^{-6}$)					Ultrasound absorption number $[A]$ (ml/g)				
	3°C	10°C	20°C	30°C	45°C	3°C	10°C	20°C	30°C	45°C	3°C	10°C	20°C	30°C	45°C	3°C	10°C	20°C	30°C	45°C
0	0.079	0.051	0.014	−0.018	−0.064	0.964	0.971	0.979	0.987	0.999	38.4	40.15	42.61	44.98	49.37	1.36	0.91	0.63	0.49	0.34
2	0.093	0.064	0.027	−0.005	−0.051	0.954	0.963	0.972	0.977	0.993	36.11	38.09	40.71	42.88	47.64	1.32	0.86	0.57	0.47	0.33
4	0.106	0.078	0.04	0.007	−0.036	0.952	0.955	0.966	0.968	0.988	34.55	36.06	39.01	40.95	45.89	1.37	0.89	0.62	0.46	0.33
6.8	0.129	0.096	0.057	0.023	−0.021	0.948	0.947	0.947	0.959	0.974	31.79	33.54	36.08	38.77	43.34	1.51	1.01	0.69	0.5	0.36
10	0.17	0.133	0.084	0.044	−0.007	0.932	0.939	0.938	0.949	0.965	26.23	29.23	32.43	35.98	41.25	1.79	1.28	0.85	0.54	0.38
12	0.214	0.17	0.113	0.065	0.004	0.870	0.919	0.926	0.937	0.954	15.58	23.79	28.63	33.02	39.4	2.14	1.55	1.06	0.68	0.42

Experimental errors for $[U]$, V, K, and $[A]$, are 0.001, 0.002, 0.3, and 0.02 in the corresponding units.

solute atomic group extend to a distance of ~0.4 nm, which corresponds to an average of 1–1.5 layers of water molecules (Sarvazyan, 1991; Chalikian et al., 1993). We can assume that in the case of high molecular weight compounds, hydration also consists primarily of those water molecules that contact the solute directly. Thus, the number of water molecules in the first layer around the solute can be considered as a lower limit for the hydration number n_h. n_h can be defined as the ratio of the solvent-accessible surface area of a solute, S_M, to the effective cross section of a water molecule, S_W. Thus, n_h is equal to S_M/S_W, where the effective cross section of a water molecule is 0.09 nm^2 (Chalikian et al., 1994). Our calculation of the accessible surface area of the PEG molecule from the specific volume data for PEG and its components (Harada et al., 1978; Kiyosawa, 1991) showed that approximately three water molecules are bound per PEG repeated unit, which has a solvent-accessible surface area of ~0.3 nm^2. Thus, the whole PEG^{2000} molecule, having a degree of polymerization of 46, binds ~142 water molecules. This calculation, based on volumetric studies, is in good agreement with our DSC results.

The hydration number of a PEG molecule depends on the configuration of the PEG. This was demonstrated by the DSC measurements of free PEG (a random coil configuration) compared with PEG covalently attached to the headgroup of a phospholipid (a brush configuration). Our results show that in the brush configuration PEG is more accessible to water, binding ~30% more water than PEG in the random coil configuration.

Adiabatic compressibility and specific volume of PEG-grafted liposomes

The values of specific volume, specific adiabatic compressibility, sound velocity number, and molecular absorption number given in Table 2 include the contributions of three main components: lipid bilayer, headgroup region, and PEG chains. In the case of acoustic measurements we have an additional contribution due to relaxational processes. Let us analyze all of these contributions and their relation with the hydration and conformation of the main components of the liposome.

Relaxational processes and dynamics of liposomes

As mentioned above, acoustical properties such as adiabatic compressibility and molecular absorption number are the sum of an instantaneous part, which is mainly caused by the intermolecular interactions, and a relaxational part, caused by pressure-induced changes in the structure of the system and energy dissipation:

$$K = K_\infty + K_{rel} \tag{6}$$

$$[A] = [A]_\infty + [A]_{rel} \tag{7}$$

The main relaxational processes in liposomes that are displayed at the frequencies of our ultrasonic measurements, ~7.7 MHz, are *trans-gauche* transformation with relaxation times τ on the order of 1 ns, heterophase fluctuations in liquid crystalline phase of $20 < \tau < 50$ ns, and headgroup rotation of $2 < \tau < 4$ ns (Mitaku et al., 1983; Colotto et al., 1993; Kharakoz et al., 1993).

Our measurements of ultrasound absorption number obtained in egg PC LUV containing DHP-PEG^{2000} and in egg PC LUV containing HPC are in close agreement (Fig. 4). This is due to the fact that the main relaxation process in the PEG-modified liposomes, as well as in egg PC LUV containing HPC, is the heterophase fluctuation of bilayer density. The mechanism of this relaxation was described by Kharakoz et al. (1993) and shown to be detectable acoustically even at temperatures very far from the transition temperature T_t. For egg PC, $T_t \approx -5$°C; for DHP-PEG^{2000} and for HPC, $T_t \approx 50$°C (Lichtenberg and Barenholz, 1988; Marsh, 1990; Tirosh et al., 1997b).

The PEG-induced excess ultrasound absorption $[A]_{exc}$ (difference in the ultrasound absorption number between the DHP-PEG^{2000} LUV and egg PC LUV containing HPC) is shown in Fig. 5. $[A]_{exc}$ has a distinctly higher value for PEG-lipid concentrations over 5 mol %, which points to the existence of an additional fast process in the PEG-modified liposomes. The most likely process responsible for $[A]_{exc}$ is an increase in the relaxation time of the headgroup rotation with increases in PEG-lipid concentration. This is in good agreement with the current scaling models (deGennes, 1987; Hristova and Needham, 1994) for polymers at interfaces, which predict a mushroom-brush transition in PEG conformation at 5 mol % of PEG-lipid, when PEG coils start

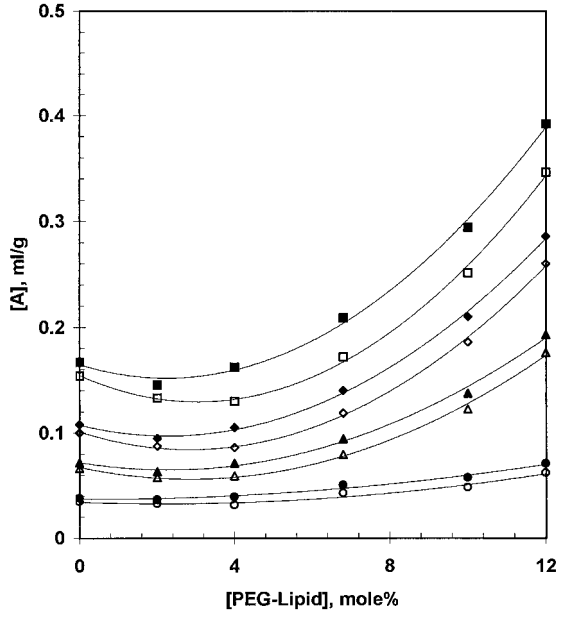

FIGURE 4 Ultrasound absorption number of DHP-PEG^{2000} LUV (solid symbols) and egg PC LUV containing HPC (open symbols) at 3°C (■), 10°C (◆), 20°C (▲), and 45°C (●).

to repel each other and extend out from the surface on which they are grafted (Fig. 6). This figure shows a schematic diagram of a PEG-grafted bilayer at low grafting concentration (mushrooms) and a PEG-grafted bilayer at high grafting concentration (brush). The theory allows one to predict the extension L of the polymer from the surface. For the mushroom regime it is given by

$$L_{\text{mush}} \cong aN^{3/5} \tag{8}$$

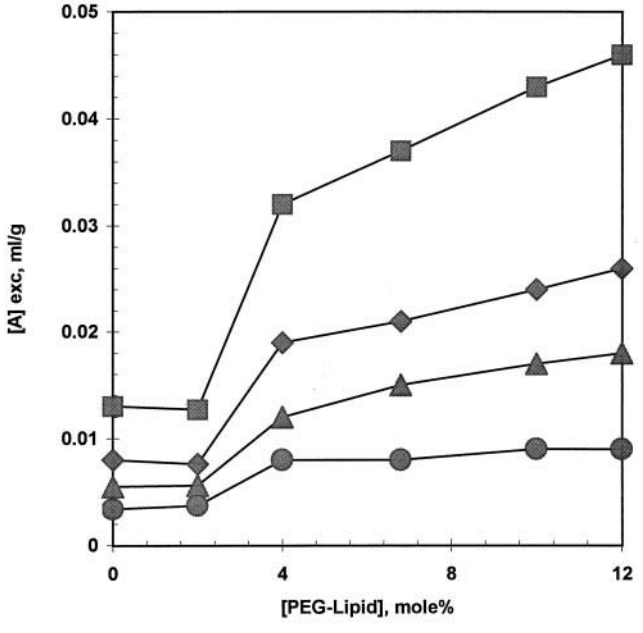

FIGURE 5 PEG-induced excess ultrasound absorption number $[A]_{\text{exc}}$ of PEG-grafted liposomes at 3°C (■), 10°C (◆), 20°C (▲), and 45°C (●).

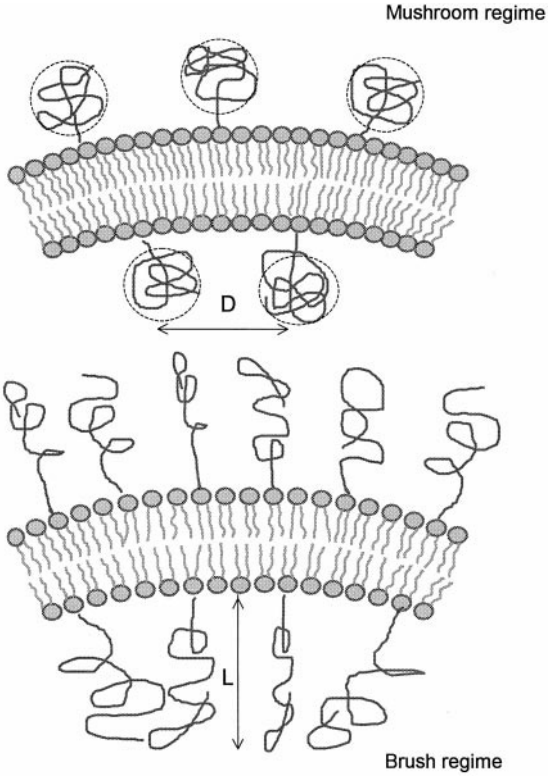

FIGURE 6 A schematic diagram of a PEG-grafted bilayer at low grafting concentration (mushrooms) and a PEG-grafted bilayer at high grafting concentration (brushes).

where a is monomer size (a = 0.35 nm for PEG^{2000}) and N is the degree of polymerization (N = 46 for PEG^{2000}). According to this equation, L_{mush} for PEG^{2000} is ~3.5 nm.

For the brush regime it is given by

$$L_{\text{brush}} \cong Na^{5/3}/D^{2/3} \tag{9}$$

where D is the distance between points of grafting of PEG.

According to this equation, L_{brush}, for example, for 7 mol % of PEG^{2000}-lipids is ~16 nm, when the distance between two points of grafting of PEG, D, is ~3.7 nm (calculated assuming homogeneous distribution of all lipids on the liposome surface). Thus, when PEG exists on the liposome surface in the brush regime it is more efficient as a repulsive barrier against the close approach of other particle surfaces, which could destabilize the liposome.

As shown by experimental studies (Needham et al., 1992; Kenworthy et al., 1995a, b; Baekmark et al., 1997), increasing the concentration of grafted PEG, as well as its molecular weight, improves the repulsive properties of the lipid bilayer surfaces, creating a denser, larger brush. With further increases in PEG, however, PEG-PEG interaction will perturb the surface and cause a transition from liposomes to micelles.

The predictions, based on free energy considerations involving lipid bilayer cohesion and lateral pressure induced by grafted polymer globules, show (Hristova and Needham, 1994) that in the case of PEG^{2000} the threshold concentra-

tion n_t, (concentration above which the vesicles will start to break down and form micellar lipid phases) is ~8 mol %. Increasing the PEG-lipid concentration above this value decreases the elastic constant and the tensile strength of the bilayer. These changes occurred in the packing of the lipid bilayer with high concentrations of grafted PEG, as verified by x-ray and calorimetric studies (Kenworthy et al., 1995a,b; Baekmark et al., 1997). Based on these and our results, it seems that for the sake of stability it is better if the grafted PEG globules barely touch and interact; i.e., they are at the borderline between mushroom and brush, which occurs at PEG^{2000}-lipid concentrations between 5 and 8 mol %.

Hydration of the lipid bilayer of the PEG-grafted liposomes

The partial volume of a solute V can be considered to be the sum of the three elements:

$$V = V_i + V_v + V_h \quad (10)$$

where V_i is the intrinsic volume of the solute atoms as determined from their van der Waals dimensions, V_v is the total free volume, including voids formed by the folding of the lipid chain ($V_i + V_v$ form the liposome core, which is free of solvent molecules), and V_h is the volume change of the solution caused by the liposome hydration.

Similarly, an instantaneous part of the adiabatic compressibility of the solute can be expressed as

$$K = K_i + K_v + K_h \quad (11)$$

where K_i is the intrinsic compressibility of lipid bilayer, K_v is the compressibility of voids in the liposomes, and K_h is the compressibility change of the solution caused by the liposome hydration. These components of liposome compressibility correspond, respectively, to the change of the volumes V_i, V_v, and V_h with pressure.

To evaluate the intrinsic compressibility of the lipid bilayer of egg PC LUV the relation between K_i and K_h was taken from Buckin et al. (1979). K_i for the lipid bilayer is $\sim 70 \pm 4 \times 10^{-6}$ ml/mol-atm at 30°C, which is 6, 45, or 60 times the K_i for protein (Kharakoz and Sarvazyan, 1993), DNA (Chalikian et al., 1994), or carbohydrate (Shilnikov et al., 1991), respectively, and corresponds to amplitudes of thermal fluctuations of atoms of ~3–3.5 nm.

The partial volume (Fig. 7) and the adiabatic compressibility (Fig. 8) of the lipid bilayer of the liposome (contribution of the relaxation processes to compressibility was calculated from the results of $\alpha\lambda$ measurements, according to Kharakoz et al., 1993) increase monotonically with PEG-lipid concentrations up to ~7 mol %. Analysis of the results shows that grafted PEG makes the lipid phase less dense (by a maximum of 1.4%) and more compressible (by a maximum of 40%). At PEG-lipid concentrations higher than 7 mol %, K and V of the lipid phase of the samples decrease, reflecting considerable increases in hydration of the lipid headgroup. This effect may be related to an increase in distance between lipid headgroups due to the repulsion of PEG chains described previously.

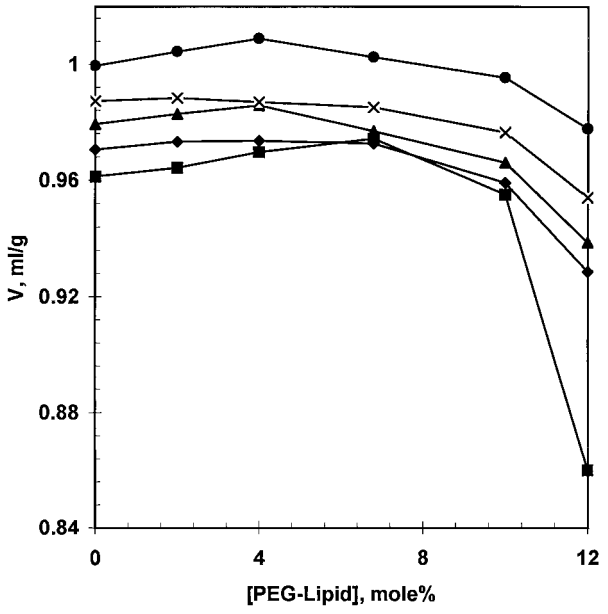

FIGURE 7 Dependence of the specific volume of the lipid bilayer on the PEG-lipid concentration at 3°C (■), 10°C (◆), 20°C (▲), 30°C (×), and 45°C (●).

To determine the hydration number for these headgroups we have applied an approach in which it is assumed that strong electrostatic headgroup-water interactions result in abolishing the anomalous properties inherent in bulk water (Kharakoz, 1989; Chalikian et al., 1993). As a result, water

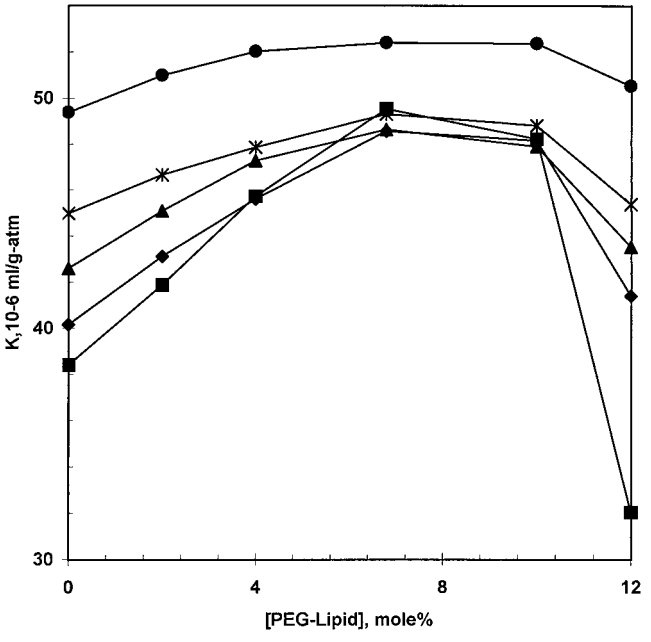

FIGURE 8 Dependence of the specific adiabatic compressibility of the lipid bilayer on the PEG-lipid concentration at 3°C (■), 10°C (◆), 20°C (▲), 30°C (×), and 45°C (●).

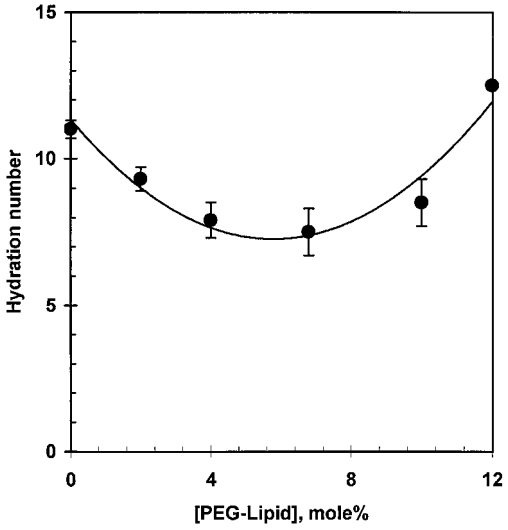

FIGURE 9 Relative changes in hydration number of the lipid bilayer of the PEG-modified liposomes (calculated from the second temperature derivative of the partial molar compressibility of lipid bilayer of the PEG-modified liposomes).

in the hydration shell of charged atomic groups exhibits a linear temperature dependence of compressibility. In other words, the second temperature derivative of compressibility of water in the hydration shell of charged groups, d^2K_h/dT^2, is equal to 0.

In this context, Eq. 11 can be differentiated, while recognizing that the temperature dependence of the intrinsic compressibility of the lipid bilayer, K_i, is negligible. In this way, the following relationship can be derived:

$$d^2K/\mathrm{d}T^2 = -n_h d^2K_w/dT^2 \qquad (12)$$

Thus, the hydration number n_h can then be evaluated if the second temperature derivative of the partial molar compressibility is determined. Analysis of the temperature dependence of the compressibility made it possible to determine quantitatively the amount of water bound to liposomes after adding different concentrations of the attached PEG (Fig. 9). These results suggest that the internal compressibility of the lipid phase is mainly contributed by the bound water. This water interacts with the lipid chain in a way that its elimination leads to a decrease in the total volume of the cavities and to enhanced intermolecular bonding.

The hydration number of the lipid bilayer has a minimum at concentrations of PEG-lipid of ~5–7 mol %. At the same concentrations of PEG-lipid, liposomes have maximal stability (Baekmark et al., 1997). This allows one to assume that hydration plays an important role in stabilization of the PEG-modified liposomes. Of course, the main reason for stabilization is related to the ability of the PEG to act as a repulsive barrier against the close approach of another surface (Martin and Lasic, 1991; Needham et al., 1992; Torchilin et al., 1994; Lasic, 1996). But the removal of water from the hydration shell of the lipid diminishes the effective size of the polar headgroup, which subsequently reduces bilayer defects, enhancing the lateral packing of the acyl chains. We assume that the dehydration of the lipid headgroup region in conjunction with the increase of the hydration of the outer layer by grafting PEG in the brush configuration is responsible for increasing thermodynamic stability of the liposomes at 5–7 mol % of PEG-lipid.

It is concluded that bound water plays a crucial role in destabilizing liposomes. Water plays a similarly crucial role in the stability of proteins, as shown by Oliveira et al. (1994) and Priev et al. (1996). It stands to reason that lipid assemblies and biopolymers are not highly stable structures, and conformational changes induced by small changes of bound water can considerably stabilize them and improve their biological functions.

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