Changing mechanical properties of lipid vesicle bilayers investigated by linear viscoelastic measurements

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(Received 20 May 1992)

The complex viscosity of lipid vesicle dispersions as a function of frequency was monitored as the vesicles aged. Of the two relaxation processes that occur in fresh vesicle dispersions, the first (i.e., longest) relaxation time did not shift, while the second (i.e., shortest) relaxation time proved to increase dramatically in time. The first relaxation process pertains to an entropic relaxation process in which redistribution occurs of a shear-disturbed distribution of hard spheres. The second relaxation time pertains to vesicle deformation where the surface shear modulus μ of the bilayer plays a dominant role. We will show that μ decreases several orders of magnitude as the vesicles age. With increasing age, a third relaxation process became measurable. It is inferred that this pertains to vesicle deformation where the surface dilatational modulus κ plays a dominant role. We found some boundary values for the surface shear modulus, surface dilatational modulus, and the curvature modulus for fully aged bilayers. The process responsible for the changed bilayer mechanical properties is the peroxidation of the lipids. This process causes unsaturated lipids to break and modify, imposing many changes on the constitution of the bilayer.

PACS number(s): 68.10.Et, 82.70.Kj, 62.15.+i, 82.65.Yh

I. INTRODUCTION

In a previous article [1] we have shown that surface viscoelastic properties of lipid bilayers can be deduced by carrying out linear viscoelastic measurements on dispersions of lipid bilayer vesicles in water. Measurement of the complex viscosity η^* of lipid vesicle dispersions as a function of frequency reveals typically two relaxation times. One relaxation process, taking place at the higher frequencies, hereafter called the second relaxation process, pertains to vesicle deformation in shear flow in which the surface shear elasticity modulus μ dominates. The modulus μ occurs in shear deformation of the surface, and so with area conservation. By applying an expression that could essentially be derived from Oldroyd's theory for the viscoelasticity of dispersions of deformable capsules [2], we could explain the dependency that the second relaxation time τ_2 shows on radius and effective viscosity of the dispersion. This way we could determine the magnitude of μ and the surface shear viscosity ζ . In Oldroyd's theory another elasticity constant is incorporated [2]. This constant, the dilatational modulus κ , applies to deformations that do not conserve area. Comparing literature values of κ [e.g., $\kappa=0.14\pm0.02$ N/m (Ref. [3])] that are given for fresh egg-yolk lipid bilayers, with the measured μ in Ref. [1], we expect the μ - and κ -governed relaxation times to lie one to two decades apart. Considering this, for fresh lipid bilayers, the κ -dominated relaxation time is not observable with our apparatus. This relaxation time would amount to $\sim 10^{-9}$ s [1], and would not be measurable with our instruments, which can only measure relaxation times up to $\sim 10^{-7}$ s [1].

Linear viscoelastic measurements on aged lipid vesicle dispersions showed that the second relaxation time had increased substantially. In the present article it is established that this increase can be explained by a decrease of

the magnitude of μ . If the κ -dominated relaxation time shifts in the same way as the μ -dominated relaxation time, it could become visible in the frequency range where we can measure the complex viscosity.

In Sec. II we present some background of the various relevant theories and experimental data, first with respect to the relaxation transition in η^* due to an entropic elasticity and, after that, with respect to the processes pertaining to droplet deformation dynamics. In Sec. III preparation and characterization of the vesicles will be treated and in Sec. IV the measuring apparatus is discussed. Results can be found in Sec. V and a discussion in Sec. VI.

II. THEORETICAL AND EXPERIMENTAL BACKGROUND

A. Hard-sphere behavior

Recently, the linear viscoelastic behavior of dispersions of silica hard-sphere particles was investigated [4]. The complex viscosity found indicated the occurrence of a relaxation process that could be understood in terms of the redistribution of the particles that are disturbed in shear flow. It is of entropic origin for perfectly hard spheres. Macroscopically, this process gives rise to an elasticity of the dispersion: an entropic elasticity. The complex viscosity of hard-sphere dispersions could be characterized with a relaxation time, relaxation strength, and the infinite-frequency viscosity η'_∞ . Recently, it has been found [1] that linear viscoelastic measurements on lipid vesicle dispersions show the existence of the same entropic relaxation process. This is hereafter called the first relaxation process (the second one, at higher frequencies, pertains to capsule deformation, governed by surface shear elasticity). In both analyses, Eqs. (1a) and (1b),

from the theories of passive linear viscoelastic systems [5], were used to describe the dependency of the complex viscosity on the angular frequency ω . In general, the real and imaginary parts $\eta'(\omega)$ and $\eta''(\omega)$ of the complex viscosity $\eta^*(\omega)$ [$\eta^*(\omega) = \eta'(\omega) - i\eta''(\omega)$] are related to the angular frequency ω as follows:

$$\eta'(\omega) = \eta'_\infty + \sum_{p=1}^N \frac{G_p \tau_p}{1 + \omega^2 \tau_p^2} \quad (1a)$$

and

$$\eta''(\omega) = \sum_{p=1}^N \frac{\omega G_p \tau_p^2}{1 + \omega^2 \tau_p^2}, \quad (1b)$$

with G_p the p th relaxation strength and τ_p the p th relaxation time. The complex viscosity *versus* frequency behavior, as far as the entropic elasticity is concerned, could be described well with a certain series of intrarelaxed relaxation times τ_p , such that in Eqs. (1a) and (1b), τ_p equals $\tau_1 p^{-2}$, where τ_1 is the longest relaxation time and G_p (the relaxation strength of the p th process in the series) is constant (G). The series runs up till N . In Ref. [1], N has been taken as 100. The values of τ_1 and G (scaled with temperature, radius, and external viscosity) that were found for the first transition in the complex viscosity for vesicle dispersions proved to be the same as those found for hard-sphere silica dispersions at the same volume fractions. As we usually find more than one transition in the complex viscosity of vesicle dispersion, in Eqs. (1a) and (1b) more sets of intrarelaxed relaxation processes must be added to account for the other transition(s). We have to distinguish between viscosity levels between the various transitions. Steady-state viscosity ($\omega \rightarrow 0$) is denoted by η'_0 for both vesicle and hard-sphere dispersions. The real part of the complex viscosity after the first (hard-sphere) transition is denoted by $\eta'_{1,\infty}$ for vesicle dispersions. For hard-sphere dispersions, it is denoted by η'_∞ . It was found [1] that for hard-sphere and vesicle dispersions, the value for the real part of the complex viscosity after the hard-sphere transition was the same for the same volume fraction. The real part of the complex viscosity of vesicle dispersions after the second transition is denoted by $\eta'_{2,\infty}$, and so forth.

Krieger and Dougherty [6] have established an empirical relation for hard-sphere dispersions between the viscosity and the volume fraction ϕ . For vesicle dispersions, for the viscosity between the first and second transition, we can give

$$\eta'_{1,\infty} = \eta_0 (1 - k_p \phi)^{-[\eta'_{1,\infty}] / k_p}, \quad (2)$$

in which η_0 is the solvent viscosity, k_p is the reciprocal of the maximum volume fraction (here to be regarded as a fitting parameter), and $[\eta'_{1,\infty}]$ is the intrinsic viscosity of hard-sphere dispersions and equals 2.5. For measurements of $\eta'_{1,\infty}$ on vesicles and η'_∞ on silica particles, it proved that Eq. (2) could be used very satisfactorily to describe the relation between $\eta'_{1,\infty}$ (or η'_∞) and ϕ ; in Ref. [1], the value of k_p was determined to be 1.21.

B. Deformation relaxation

Oldroyd [2] has derived an expression for the complex viscosity of a dispersion of thin-walled capsules in a fluid. He defined a two-dimensional constitutive equation for the thin capsule wall, accounting for interfacial tension, surface shear viscoelasticity, and surface dilatational viscoelasticity. As interfacial tension of a lipid bilayer is negligible, it is not mentioned further in this paper. So, for the bilayer the two-dimensional stress tensor $\mathbf{T}$ within the capsule wall equals

$$\mathbf{T} = (\kappa + i\omega\sigma) \text{tr} \mathbf{E} \mathbf{I} + 2(\mu + i\omega\zeta) \bar{\mathbf{E}}, \quad (3)$$

with $\mathbf{E}$ the infinitesimal strain tensor, $\bar{\mathbf{E}}$ its deviatoric part, $\mathbf{I}$ the metric tensor, $i^2 = -1$, ω the angular frequency, μ the surface shear modulus, ζ the surface shear viscosity, κ the surface dilatational modulus, and σ the surface dilatational viscosity. To this equation can be added a two-dimensional pressure term which is omitted here as it is not relevant in the following. Solving the problem of the force balance over the bilayer and the continuous velocity field anywhere in the fluid, it was found that two relaxation and two retardation times characterized the linear viscoelastic behavior of the dispersion. The processes in the bilayer pertaining to surface shear, surface dilatation, and to another cause of surface elasticity (i.e., bending) are depicted in Fig. 1.

1. Surface shear deformation relaxation

In Ref. [1] we have shown a second transition to be present in the complex viscosity of vesicle dispersions. This pertains to vesicle deformation in which the effect of the surface shear modulus μ appears. In his theory of deformable capsules [2], Oldroyd presented an equation with which this capsule deformation relaxation time τ_2 pertaining to surface shear of the capsule wall could be related to radius, internal and external viscosities, the surface shear elasticity modulus μ , and the surface shear viscosity ζ :

$$\tau_2 = \frac{(23\eta_{\text{in}} + 32\eta_{\text{out}})a_m + 16\zeta}{16\mu}, \quad (4a)$$

FIG. 1. Deformation processes of the bilayer: (a) Surface shear and (b) surface dilatation (view perpendicular to the plane; circles model the section of a lipid molecule); (c) bending (side view of plane).

in which a_m is the distance from the center of the capsule to the middle of the bilayer, η_{in} is the viscosity of the encapsulated phase, and η_{out} the viscosity of the continuous phase. Oldroyd has derived this result for dilute systems, where η_{out} is the viscosity of the ambient fluid around a vesicle, and so without the influence of other vesicles. In concentrated systems, the viscosity of the ambient fluid is influenced by the presence of other particles. We therefore substitute for η_{out} a value that closely reflects the dissipative nature of the surrounding fluid for the relevant relaxation process, being the effective viscosity $\eta_{2,eff}$ of the fluid as a whole. The subscript “2” indicates the second process as the relevant one. If deformations of the vesicles are not coupled, this is allowed. For the value of $\eta_{2,eff}$ we take the real part of the complex viscosity at an angular frequency equal to the inverse of the second relaxation time. The viscosity of the encapsulated phase can be set equal to η_0 , the buffer viscosity. So for finite concentrations, Eq. (4a) reads

$$\tau_2 = \frac{(23\eta_0 + 32\eta_{2,eff})a_m + 16\zeta}{16\mu}. \quad (4b)$$

An interfacial tension γ that might also influence the relaxation time [replace μ with $(\mu + \frac{3}{2}\gamma)$] has not been included, as, according to sources in the literature, the interfacial tension of lipid bilayers is very small ($\gamma \ll 10^{-3}$ N/m [3,7]). Equation (4b) shows that if the second relaxation time is measured for vesicles with varying radius and varying $\eta_{2,eff}$ (i.e., varying volume fraction), a straight line is obtained in a plot of τ_2 versus $(23\eta_0 + 32\eta_{2,eff})a_m$, of which the slope gives $1/16\mu$, and the ordinate cutoff gives ζ/μ . The disadvantage of this method is that measurements have to be carried out for a number of combinations of a_m and/or ϕ to be able to investigate the mentioned linear relationship (as done in Ref. [1]).

From the theory that Oldroyd has presented, we can derive a relation between the intrinsic viscosity $[\eta'_{2,\infty}]$ at frequencies larger than $1/\tau_2$ (but smaller than $1/\tau_3$, in case a third relaxation process should take place) and the surface shear viscosity ζ , independent of the second relaxation time:

$$\zeta = \frac{a_m}{16} \frac{\frac{2}{5}[\eta'_{2,\infty}](55\eta_0) - 7\eta_0}{1 - \frac{2}{5}[\eta'_{2,\infty}]} \quad (5)$$

Note that this equation is only derived for infinite dilutions. The intrinsic viscosity $[\eta'_{2,\infty}]$ has been estimated using the Krieger-Dougherty relation [compare with Eq. (2)]:

$$[\eta'_{2,\infty}] = -k_p \frac{\ln(\eta'_{2,\infty}/\eta_0)}{\ln(1 - k_p\phi)} \quad (6)$$

2. Surface dilatation deformation relaxation

The surface shear elasticity pertains to deformation at constant area of the bilayer. The theory by Oldroyd [2] also includes surface dilatational elasticity, where the area does not remain constant. This elasticity is

governed by the surface dilatational modulus κ . If the capsule relaxation time τ_2 pertaining to surface shear is much larger than the capsule relaxation time τ_3 pertaining to surface dilatation, the relation between τ_3 and the radius, bulk viscosities, and bilayer surface mechanical properties is given by

$$\tau_3 = \frac{32\sigma\zeta + (A_3\zeta + B_3\sigma)a_m + D_3E_3a_m^2}{\kappa(32\zeta + B_3a_m)}, \quad (7)$$

in which σ is the surface dilatational viscosity in the bilayer and

$$A_3 = 4(12\eta_{3,eff} + 13\eta_0), \quad (8a)$$

$$B_3 = 2(32\eta_{3,eff} + 23\eta_0), \quad (8b)$$

$$D_3 = 3\eta_{3,eff} + 2\eta_0, \quad (8c)$$

$$E_3 = 16\eta_{3,eff} + 19\eta_0. \quad (8d)$$

Equation (7) has been adapted similarly to finite volume fractions as in Eq. (4b). The effective viscosity $\eta_{3,eff}$ is the real part of the complex viscosity at angular frequency $1/\tau_3$.

As will be seen, in some cases we have to take into account the three above-mentioned relaxation processes to analyze the observations. The equation that we can then use to analyze the dependency of the measured complex viscosity on the angular frequency can be written as

$$\eta'(\omega) = \eta'_{3,\infty} + \sum_{p=1}^N \frac{G_{1,p}\tau_{1,p}}{1 + \omega^2\tau_{1,p}^2} + \sum_{i=1}^K \frac{G_{2,i}\tau_{2,i}}{1 + \omega^2\tau_{2,i}^2} + \sum_{j=1}^L \frac{G_{3,j}\tau_{3,j}}{1 + \omega^2\tau_{3,j}^2} \quad (9a)$$

and

$$\eta''(\omega) = \sum_{p=1}^N \frac{\omega G_{1,p}\tau_{1,p}^2}{1 + \omega^2\tau_{1,p}^2} + \sum_{i=1}^K \frac{\omega G_{2,i}\tau_{2,i}^2}{1 + \omega^2\tau_{2,i}^2} + \sum_{j=1}^L \frac{\omega G_{3,j}\tau_{3,j}^2}{1 + \omega^2\tau_{3,j}^2}. \quad (9b)$$

The viscosity $\eta'_{3,\infty}$ is the limit viscosity for $\omega \rightarrow \infty$ after the third transition. In Eqs. (9a) and (9b) both the first and second types of intrarelaxed relaxation process are described with a series of relaxation times, as done in Ref. [1]. In each case, 100 times are used. For the third relaxation process, only one time will be used, which is discussed in Sec. V B 2.

III. STRUCTURE, PREPARATION AND AGING OF THE VESICLES

The studied dispersions consist of spherical capsules that contain water and which are dispersed in water [see Fig. 2(a)]. The walls of these capsules consist of a bilayer of lipids whose hydrophilic head groups are in contact with the aqueous environment and whose hydrophobic tails constitute the bilayer core. The bilayer's thickness is approximately 5 nm. The vesicles' bilayers in this study consist of a mixture of mainly unsaturated phosphatidyl

FIG. 2. (a) Basic structure of lipid bilayer vesicles and (b) structure of the phosphatidyl choline molecule. R_1 and R_2 are hydrocarbon chains.

choline lipids, with varying hydrocarbon chain lengths extracted from egg yolk [egg-yolk lipid (EYL)] [see Fig. 2(b)]. At the temperature of 10°C where the presented experiments were carried out, the bilayer is in a liquid-crystalline state. The lipids were purchased from Lipid Products Ltd., U.K. The vesicle dispersions that were used in this study are the same as those that were used in Ref. [1]. They were made by means of a detergent removal method. With this method, in a round-bottomed flask, the lipid together with a charge carrier (which is to be incorporated in the bilayer to prevent the vesicles from coagulating) is dissolved in an organic solvent (a mixture of methanol and chloroform). We have used the charge-carrier cholesteryl hemisuccinate (tris salt), purchased from Sigma Chemical Company, in a 10% mole fraction. Subsequently, the solvent is evaporated, and a film is formed on the inside of the flask and dried thoroughly. Then, a phosphate buffer is added (pH 7.4, osmolarity 0.26) in which a well-defined quantity of detergent has been dissolved. Upon dissolving the film in the soapy buffer, a clear solution of so-called mixed micelles forms. Dialysis of the mixed-micelle solution, in which process the detergent is removed, will result in the formation of vesicles that are typically unilamellar and uniformly sized. A small choice of detergents is available for dialysis, of which the *n*-alkyl β -D glucopyranosides are the most versatile. It has been shown [8,9] that on changing the ratio of two of those detergents (differing in alkyl length), one can adjust the radius of the vesicles continuously between 40 and 100 nm. This method has been used in Ref. [1] to prepare vesicles of radii 60, 65, 70, and 106 nm. The detergents *n*-heptyl (and *n*-octyl) β -D glucopyranoside were purchased from Serva, Germany. In Table I the preparation conditions are summarized. To be able to carry out linear viscoelastic measurements, the vesicle dispersions, which have a volume fraction of only

TABLE I. Preparation conditions for the used vesicle samples: L , lipid concentration (mmol/ml); L/D , molar lipid to detergent ratio for the detergents; Oc., *n*-octyl β -D glucopyranoside; Hep., *n*-heptyl β -D glucopyranoside; a , the vesicle hydrodynamic radius.

L	L/D Oc.	L/D Hep.	a (nm)
0.013	0.6	0.2	60
0.013	0.53	0.24	65
0.013	0.4	0.35	70
0.013	0.25		106

5%–8% after preparation, have to be concentrated (to 25%–50%). An Amicon ultrafiltration device has been used to this effect.

The size of the vesicles have been determined with dynamic light scattering, with which the radius can be estimated to within 5%. The volume fraction ϕ can be obtained from the steady-state viscosity η'_0 of the vesicle dispersion: Experiments have shown that for hard-sphere dispersions a very clear relationship exists between η'_0 and ϕ (Ref. [10]). It has also been shown that this is equally valid for dispersions of vesicles [1]. Therefore, the volume fraction of vesicle dispersions has been determined from their η'_0 .

The lipids that constitute the bilayer, as mentioned, are largely unsaturated. They are [11]: 16:0 (a 16-C hydrocarbon chain and 0 unsaturated bonds) by 32%, 16:1 by 2%, 18:0 by 12%, 18:1 by 36%, 18:2 by 13%, and 20:4 by 5%. As these lipids are exposed to peroxides, radicals, or freak radiation and oxygen, they will react to form a vast and complex number of products. The process by which lipids are oxidized is called peroxidation (see, e.g., Ref. [12]). Peroxidation can be subdivided into three stages: initiation, propagation, and termination. Peroxidation starts by initiation. Agents are, e.g., transition-metal ions, oxygen, hydroperoxides, light, x-ray and γ radiation. In this process, the hydroxyl radical is an important initiator. When it reacts with an unsaturated lipid, a radical is formed on one of the carbon atoms that are part of a double bond in the alkyl chain. In the propagation step, reaction of this radical with oxygen can result in cleavage of the carbon chain [13]. The complexity of the aged bilayer mainly arises during the propagation step due to the many possible ways in which the lipid radical can react, and the possible isomerizations that can take place [13]. Together with the many possible ways by which the radical reaction can be terminated (including cross-linking of lipid radicals [14]), it becomes clear that the bilayer after aging has become very complex indeed. Refer to Fig. 3. Eventually, newly formed in bilayer will be amphiphilic molecules of various lengths, hydrophilic molecules of various lengths, hydrophobic molecules of various lengths, and even gases. Some of these molecules will now be soluble in the surrounding buffer as well as in the bilayer. Also, the degree of unsaturation will have been reduced. To appreciate the complexity of the peroxidation and its secondary reactions, see the literature (e.g., Refs. [12–15]).

FIG. 3. The changes in the bilayer as a result of lipid peroxidation.

IV. LINEAR VISCOELASTIC MEASURING APPARATUS

The linear viscoelastic measurements were carried out with instruments developed in our laboratory: four torsion pendula and a nickel tube resonator. With each of the four torsion pendula, a harmonic shear flow can be applied to a fluid. The frequencies are approximately 72, 283, 686, and 2335 Hz. With the nickel tube resonator, frequencies can be applied to a fluid in the range of 4 to 235 kHz. Of these instruments, the cylindrical (solid or hollow) bodies are electromagnetically forced in oscillatory movement with the shear wave propagating radially away from the cylindrical body. This causes a fluid to be harmonically sheared. The motion of the body is electromagnetically monitored. When a fluid surrounds the body, the moment exerted by the fluid on the body influences the equation of motion of the body. The transfer function is detected while the body is immersed in the liquid under investigation. From change in the resonance frequency and bandwidth compared to those measured in air, using the equation of motion, follows the complex viscosity of the fluid. The magnitudes of the oscillations are so small that the experiment is linear. We have checked this and noticed that the complex viscosity found, did not change by changing the amplitude of the input signal. This is important as only with linear measurements can the complex viscosity found be interpreted quantitatively. The instruments are discussed in detail by Blom and Mellema [16] (torsion pendula) and Oosterbroek *et al.* [17] (nickel tube resonator).

V. MEASUREMENTS AND RESULTS

A. Previous results revisited

Before we give the results on the aging measurements, we point out the necessity of a reanalysis of the results in Ref. [1], we were able to do after the aging experiments were done. There, the second relaxation time τ_2 was plotted *versus* the value of $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$ for a number of vesicle dispersions characterized by different volume fractions (leading to different values for $\eta_{2,\text{eff}}$) and different radii. This resulted in a straight line with ordinate cutoff. From the slope and the ordinate cutoff could be established the value for the surface shear modulus μ and the surface shear viscosity ζ . It was pointed out in Ref. [1] that the analysis was hampered by the fact that the tran-

sition in $\eta^*(\omega)$ took place near the high-frequency edge of our measuring window. However, as the onset of the transition could be clearly seen, the fitted parameters to our data were regarded with confidence. During the aging experiments, the second relaxation time increased, the high-frequency transition in the complex viscosity became fully visible, and the longest in the second series of relaxation times τ_2 could be established with high accuracy. It was then found out that systematic shifts of τ_2 occur when more and more data points are left out at the high-frequency region of the complex viscosity *versus* frequency plot. Apparently, the fit formula does not perfectly match the shape of the complex viscosity *versus* frequency curve as we measure it. From the aging experiments we were able to establish the correction factor needed to account for the incomplete measurement of the transition in the complex viscosities in Ref. [1]. In Fig. 4(a) can be seen the relationship between the corrected τ_2 and $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$. It proves that the straight line has an ordinate cutoff of $(-9 \pm 32) \times 10^{-9}$ s and a slope of $30 \pm 3 \text{ Pa}^{-1} \text{ m}^{-1}$, leading to

$$\mu^0 = (2.1 \pm 0.2) \times 10^{-3} \text{ N/m} \quad (10a)$$

and

$$\zeta_{\text{max}}^0 = 1.3 \times 10^{-10} \text{ N s/m} \quad (10b)$$

These values pertain to fresh EYL bilayers, which is denoted by a superscript "0." The ζ_{max}^0 corresponds to a 95% confidence, i.e., twice the standard deviation. Now

FIG. 4. (a) Corrected τ_2 value vs $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$ for the 11 vesicle dispersions used in Ref. [1]. (b) Corrected plot of the apparent surface shear viscosity vs the volume fraction for the 11 vesicle dispersions used in Ref. [1] (· · · · ·, ζ^0 from Fig. 3(a); — — —, ζ_{max}^0 , from Eq. (10b).

the question arises if μ^0 and $\zeta_{\max}^0$ can be compared to literature values. Since we measure dynamic quantities (bilayer mechanical properties at high frequencies), they may differ from steady-state values. This can be seen from the value of $\zeta_{\max}^0$ in Eq. (10b), which is unequal to literature values cited in Ref. [1] which are steady-state values [18–20]. Furthermore, it is stated in the literature that the surface shear modulus of liquid-crystalline bilayers equals 0 [21,22], clearly differing from our measured value for μ^0 [Eq. (10a)]. The reasoning behind these apparent differences is that time scales in steady-state experiments are slow enough to allow ample time for relaxation of any correlated displacement of lipids in the bilayer to occur. What we are dealing with here, however, are experiments that are so fast that there is insufficient time for the space-correlated displacement of the lipids to disappear. Therefore, measurement of nonzero shear moduli is possible. A calculation makes this assumption plausible. The translational diffusion coefficient of lipids in a liquid-crystalline bilayer is on the order of 1×10^{-12} m²/s (Ref. [23]). It therefore follows that in an experiment of typical time scales of $< 1 \times 10^{-6}$ s that we are dealing with here, the root-mean-square distance that a lipid can travel is on the order of 0.3 nm. For the same reason, as diffusion can only take place to a very small extent within the time scale of our experiment, less dissipation occurs compared to steady-state experiments, and, therefore, the surface shear viscosity may well be decreased and approaching 0. In the discussion (Sec. VI D), this subject will be dealt with further.

As we observed the methodological difficulty that the inferred longest relaxation time depends on the difference between the real relaxation time and the shortest applied experimental time, a dependency of the viscosity level at infinite frequencies ($\eta'_{2,\infty}$) on this difference is also expected. In Ref. [1], values were presented of the apparent surface shear viscosity, established with Eqs. (5) and (6). However, as these values were derived from the value of $\eta'_{2,\infty}$, a correction is also needed for the dependency of ζ_{app} on volume fraction, a dependency that was investigated in Ref. [1]. The result of this revised relation is shown in Fig. 4(b). It appears that the value of ζ_{app} falls within the values $\zeta \pm 2\Delta\zeta$ deduced from Fig. 4(a). The value for ζ_{app} that can be derived from Eqs. (5) and (6) can therefore be regarded as a rough estimate for the value of ζ .

It was mentioned in Ref. [1] that the curvature modulus can be incorporated in the equation by Oldroyd [Eqs. 4(a) and (b)] and that to $(16\mu^0)$ can be added

$$24K_c^0/a_m^2(6 - C_0a_m + \frac{1}{2}C_0^2a_m^2),$$

with C_0 the spontaneous curvature. This was deduced by noticing similarities of some theories (Refs. [24,25]) to the one by Oldroyd. We can, therefore, look at the dependency that the data points in Fig. 4(a) show on a_m , as has been done in Ref. [1]. No dependency seems present, but we can analyze it more quantitatively. As done in Ref. [1], a plot of

$$[(23\eta_0 + 32\eta_{2,\text{eff}})a_m + 16\zeta]/\tau_2 - 16\mu$$

versus $1/a_m^2$ should yield a straight line through the origin with slope proportional to K_c . The proportionality constant depends on the choice for the spontaneous curvature. The quantitative result, indeed, is that there is hardly any dependency on a_m , so that K_c^0 is very small, but, due to errors in $\eta_{2,\text{eff}}$, τ_2 , μ^0 , and ζ^0 , the error in K_c^0 is substantial. With spontaneous curvature equal to 0,

$$K_{c,\max}^0 = 3 \times 10^{-19} \text{ J}, \quad (10c)$$

and, with the spontaneous curvature equal to the actual curvature ($C_0 = -2a_m^{-1}$),

$$K_{c,\max}^0 = 2 \times 10^{-19} \text{ J}. \quad (10d)$$

The maximum value of K_c^0 , indicated in Eqs. (10c) and (10d), is the average plus twice the standard deviation. The value of $K_{c,\max}^0$ is therefore given with 95% confidence.

B. The aging experiments

Linear viscoelastic measurements in the frequency range 70 Hz to 235 kHz on freshly made lipid vesicle dispersions show two relaxation processes. The first one pertains to an entropic relaxation, occurring also in hard-sphere dispersions. The second relaxation process pertains to deformation relaxation. The temperature, as in Ref. [1], was 10.0°C. Measurements were carried out with torsion pendula and a nickel tube resonator. The steady-state viscosity was measured with the low-shear Contraves viscometer. The phosphate buffer in which the vesicles are kept has a pH of 7.4, an osmolarity of 0.26 and a viscosity at 10.0°C of 1.35 mPa s. During storage, the dispersion was cooled to 4°C and kept in a nitrogen atmosphere.

In remeasuring the vesicle samples that were used in the previous study [1], it became clear that large shifts with aging had occurred for the second relaxation time, while the first relaxation time had not changed within experimental error.

The analysis of the measured complex viscosity as a function of frequency has been carried out with the aid of a computer fit program that uses Eqs. (9a) and (9b) to match values for $\eta'_{3,\infty}$, τ_1 , G_1 , τ_2 , G_2 , τ_3 , and G_3 to the measurements. As long as two series of intrarelated relaxation processes have been taken into account, the third series is omitted in Eqs. (9a) and (9b), and $\eta'_{3,\infty}$ in Eq. (9a) replaced by $\eta'_{2,\infty}$.

The first transition does not change systematically with age. See Fig. 5 or Table II. Since τ_1 depends on a_m^3 , we conclude that the vesicles preserve their size and their spherical shape in good approximation. This was confirmed by dynamic light-scattering measurements.

1. The relaxation pertaining to surface shear

The remeasurements of the vesicle dispersions have been carried out during one year at intervals of one to two months. See Fig. 5 for an illustration of the dramatic effect aging has on the appearance of the complex viscosity with regard to the position of the second transition. From Table III one can get a more quantitative idea of

FIG. 5. The relative complex viscosity for the 70-nm vesicle dispersion for five ages. Age (in days): 10 (—), 43 (— —), 85 (— · —), 127 (— · —), and 172 (· · ·). The viscosities are divided by the buffer viscosity η_0 at 10°C: 1.35 mPa s.

the shifts involved. For the first 175 days, the increase can be approximated as an exponential relation between τ_2 and t [t is the age (in days)]:

$$\tau_2(t) = \tau_2(0) \times 10^{t/87} = \tau_2(0)e^{t/38}. \quad (11)$$

After prolonged aging, the deformation transition pertaining to surface shear elasticity has shifted to lower frequencies to such an extent that it started to overlap the first (hard-sphere) transition. The analysis of the complex viscosity is in such case very hard to carry out, as it is not possible to distinguish between the hard-sphere effect and the capsule deformation effect due to μ . The parameters

to be fitted for the first and second processes are therefore hard to establish. However, we have seen that the hard-sphere transition did not shift significantly with age. Therefore, from the measurements on fresh and intermediately aged vesicles, in which the first and second transitions were sufficiently separated, an average was taken for the first relaxation time and strength. These were incorporated in the fit formula as constants during the fit procedure to the data from the progressively aged vesicles. That the radius, even for progressively aged vesicles, had remained the same could be checked to within 5% by dynamic light scattering.

As follows from Eq. 4(b), for each τ_2 value we have a linear relation between μ and ζ :

$$\mu = \frac{(23\eta_0 + 32\eta_{2,\text{eff}})a_m}{16\tau_2} + \frac{\zeta}{\tau_2}, \quad (12)$$

in which $\eta_{2,\text{eff}}$ in this series-of-times process, is defined as

$$\eta'_{2,\infty} + \frac{2}{3}(\eta'_{1,\infty} - \eta'_{2,\infty}).$$

As the second relaxation time shifts dramatically with age, a plot of μ versus ζ shifts as well. This is shown for the 70- and 106-nm aging vesicles in Figs. 6(a) and 6(b), respectively. In these plots can also be indicated the values of μ^0 and ζ_{max}^0 for fresh vesicles [see Eqs. (10a) and (10b)]. From the data we have so far, no conclusion can be drawn as to how μ and ζ change upon aging of the bilayer. However, we can restrict the possibilities by indicating limits to ζ as the bilayer ages.

TABLE II. Characteristics for the first relaxation process. In the first two columns, the radius of the vesicles is given, with the number of the remeasurement. The viscosities are divided by the buffer viscosity η_0 at 10°C (1.35 mPa s). The first relaxation time τ_1 is the longest in a series of times with which this process is described [see Eqs. (9a) and (9b)].

a (nm)	Reameas. No.	Age (days)	$\frac{\eta'_0}{\eta_0}$	ϕ (%)	τ_1 (10^{-3} s)	G_1 (Pa)	$\frac{\eta'_{1,\infty}}{\eta_0}$
60	1	3	4.4	33	0.58 ± 0.05	1.82 ± 0.14	3.1
	2	4	4.0	32	0.54 ± 0.05	1.67 ± 0.16	2.9
	3	79	3.8	32	0.51 ± 0.06	1.68 ± 0.20	2.8
	4	128	3.8	32	0.55 ^a	1.72 ^a	2.8
	5	233	4.0	32	0.55 ^a	1.72 ^a	2.9
65	1	16	3.8	31	0.76 ± 0.08	0.98 ± 0.10	2.9
	2	54	3.6	31	0.48 ± 0.05	1.71 ± 0.16	2.6
	3	163	3.5	31	0.97 ± 0.19	0.70 ± 0.20	2.7
	4	242	3.4	30	0.74 ^a	1.1 ^a	2.5
	5	352	3.7	31	0.74 ^a	1.1 ^a	2.7
70	1	10	6.8	39	1.70 ± 0.11	1.32 ± 0.08	4.2
	2	43	6.5	39	1.70 ± 0.11	1.25 ± 0.08	4.0
	3	85	6.5	38	1.54 ± 0.14	1.42 ± 0.12	3.9
	4	127	6.6	39	1.47 ± 0.15	1.89 ± 0.20	3.3
	5	172	6.6	39	1.88 ± 0.39	1.48 ± 0.37	3.3
106	1	17	4.2	33	2.99 ± 0.41	0.27 ± 0.03	3.3
	2	66	4.2	33	3.84 ± 0.49	0.24 ± 0.03	3.1
	3	122	4.0	32	1.97 ± 0.16	0.62 ± 0.05	2.6
	4	168	3.9	32	2.75 ± 0.47	0.47 ± 0.09	2.4
	5	248	3.7	32	2.9 ^a	0.4 ^a	2.4
	6	358	3.9	32	2.9 ^a	0.4 ^a	2.6

^aFor some very old samples the first relaxation time and strength are taken as constants: averages of τ_1 and G_1 for fresher samples.

TABLE III. Characteristics for the second relaxation process. In the first two columns, the radius of the vesicle is given with the number of the remeasurement. The viscosities are divided by the buffer viscosity η_0 at 10°C (1.35 mPa s). The second relaxation time τ_2 is the longest in a series of relaxation times with which this process is described [see Eqs. (9a) and (9b)]. The effective viscosity $\eta_{2,\text{eff}}$ is defined as $[\eta'_{2,\infty} + \frac{2}{3}(\eta'_{1,\infty} - \eta'_{2,\infty})]$ for this series-of-relaxation-time process.

a (nm)	Remeas. No.	Age (days)	ϕ (%)	$\frac{\eta'_{1,\infty}}{\eta_0}$	τ_2 (s)	G_2 (Pa)	$\frac{\eta'_{2,\infty}}{\eta_0}$	$\eta_{2,\text{eff}}$ (mPa s)
60	1	3	33	3.1	$5.4 \pm 0.5 \times 10^{-7}$	$2.0 \pm 0.3 \times 10^3$	1.9	3.7
	2	44	32	2.9	$2.5 \pm 0.1 \times 10^{-6}$	$3.7 \pm 0.2 \times 10^2$	1.8	3.4
	3	79	32	2.8	$8.7 \pm 0.7 \times 10^{-6}$	$8.0 \pm 0.6 \times 10^1$	2.0	3.5
	4	128	32	2.8	$8.0 \pm 1.4 \times 10^{-5}$	4.6 ± 0.8	2.3	3.6
	5	233	32	2.9	$7.3 \pm 1.5 \times 10^{-5}$	7.4 ± 1.0	2.3	3.6
65	1	16	31	2.9	$7.1 \pm 0.6 \times 10^{-7}$	$1.2 \pm 0.2 \times 10^3$	1.9	3.4
	2	54	31	2.6	$2.2 \pm 0.2 \times 10^{-6}$	$3.0 \pm 0.2 \times 10^2$	1.8	3.2
	3	163	31	2.7	$9.7 \pm 1.9 \times 10^{-5}$	5.5 ± 0.7	2.1	3.4
	4	242	30	2.5	$3.5 \pm 0.7 \times 10^{-5}$	8.1 ± 1.8	2.1	3.2
	5	352	31	2.7	$7.2 \pm 1.1 \times 10^{-5}$	6.2 ± 0.9	2.2	3.4
70	1	10	39	4.2	$6.6 \pm 0.3 \times 10^{-7}$	$3.2 \pm 0.2 \times 10^3$	1.6	4.5
	2	43	39	4.0	$1.3 \pm 0.1 \times 10^{-6}$	$1.5 \pm 0.1 \times 10^3$	1.6	4.4
	3	85	38	3.9	$4.0 \pm 0.2 \times 10^{-6}$	$4.3 \pm 0.2 \times 10^2$	1.9	4.3
	4	127	39	3.3	$1.3 \pm 0.1 \times 10^{-5}$	$7.5 \pm 0.4 \times 10^1$	2.1	4.0
	5	172	39	3.3	$8.0 \pm 1.9 \times 10^{-5}$	8.1 ± 1.1	2.5	4.1
106	1	17	33	3.3	$8.2 \pm 0.3 \times 10^{-7}$	$1.8 \pm 0.1 \times 10^3$	1.5	3.0
	2	66	33	3.1	$2.2 \pm 0.1 \times 10^{-6}$	$6.2 \pm 0.2 \times 10^2$	1.5	3.5
	3	122	32	2.6	$1.2 \pm 0.1 \times 10^{-5}$	$6.1 \pm 0.4 \times 10^1$	1.7	3.1
	4	168	32	2.4	$8.4 \pm 2.3 \times 10^{-5}$	3.7 ± 0.8	2.0	3.1
	5	248	32	2.4	$5.9 \pm 1.4 \times 10^{-5}$	4.4 ± 1.0	2.1	3.1
	6	358	32	2.6	$1.3 \pm 0.3 \times 10^{-4}$	2.9 ± 0.5	2.1	3.3

The determination of an upper limit can be approached in three ways. First, as mentioned in Sec. II B 1, there is in principle a way of establishing the surface shear viscosity of the bilayer independently from the relaxation time. According to Oldroyd's theory, the intrinsic viscosity $[\eta'_{2,\infty}]$ after the transition due to the surface shear elasticity at infinite dilutions is related to ζ [Eq. (5)]. The problem is to estimate $[\eta'_{2,\infty}]$: We use Eq. (6) with k_p for hard spheres. As has been discussed at the beginning of this section, the value for ζ_{app} that follows can be regarded as a rough estimate for ζ . We therefore can regard the upper limit of this rough estimate as an upper limit for ζ . In Figs. (6a) and (6b), these upper limits are indicated for all ages (+, thin). In Table IV, for each measurement, this upper limit is given (ζ_{max}^i).

There is a second way of establishing an upper limit for ζ . The following inequality holds:

$$[\eta'_{2,\infty}] \equiv \lim_{\phi \rightarrow 0} \frac{\eta'_{2,\infty} - \eta_0}{\phi \eta_0} \leq \frac{\eta'_{2,\infty} - \eta_0}{\phi \eta_0} \equiv [\eta'_{2,\infty, \phi \neq 0}]. \quad (13)$$

Consequently, substituting $[\eta'_{2,\infty, \phi \neq 0}]$ for $[\eta'_{2,\infty}]$ in Eq. (5) yields an upper limit for ζ as long as the $[\eta'_{2,\infty, \phi \neq 0}]$ value to be substituted remains smaller than 2.5, because the plot $\zeta([\eta'_{2,\infty}])$ is discontinuous there. The calculated upper limits in this way are ζ_{max}^{ii} in Table IV, and also given in Figs. (6a) and (6b) (+, bold).

With regard to the third way of estimation to determine an upper limit for ζ for fully aged bilayers, we must recollect the changes that occur in the bilayer upon aging: Lipids are peroxidised and break up, leading to

FIG. 6. Relations between μ and ζ for the aging of the (a) 70-nm and (b) 106-nm vesicle dispersions. (a) Age in days: 10 (—), 43 (—), 85 (—), 127 (—), and 172 (· · ·). (b) Age in days: 17 (—), 66 (—), 122 (—), 168 (—), 248 (—), and 358 (· · ·). Indicated are the values of μ and the upper limit of ζ for fresh vesicles (—) and some upper limits of ζ as aging progresses: (+, thin) established with Eqs. (5) and (6), (+, bold) established with Eqs. (5), and (13), and the vertical dotted line, from the literature.

TABLE IV. Minimum and maximum values of some bilayer mechanical properties during aging: (i) via Eqs. (5) and (6), (ii) via Eqs. (5) and (13), (iii) via the literature, and (iv) via Figs. 6(a) and 6(b), and similar figures for the 60- and 65-nm vesicle dispersions (not given).

<i>a</i> (nm)	Remeas. No.	Age (days)	$\xi_{\max}^i$ (10^{-10} N s/m)	$\xi_{\max}^{ii}$ (10^{-10} N s/m)	$\xi_{\max}^{iii}$ (10^{-10} N s/m)	$\mu_{\min}^{iv}$ (N/m)	$\mu_{\max}^{iv}$ (N/m)
60	1	3	4.7		20	1.0×10^{-3}	2.0×10^{-3}
	2	44	4.4	75	20	2.1×10^{-4}	3.9×10^{-4}
	3	79	7.8		20	6.0×10^{-5}	1.7×10^{-4}
	4	128	17		20	3.8×10^{-6}	2.5×10^{-5}
	5	233	18		20	5.5×10^{-6}	2.9×10^{-5}
65	1	16	6.5		20	7.0×10^{-4}	2.0×10^{-3}
	2	54	5.5		20	2.4×10^{-4}	5.2×10^{-4}
	3	163	13		20	4.3×10^{-6}	2.2×10^{-5}
	4	242	18		20	2.1×10^{-5}	8.8×10^{-5}
	5	352	23		20	6.4×10^{-6}	4.0×10^{-5}
70	1	10	1.9	5	20	1.0×10^{-3}	1.5×10^{-3}
	2	43	1.9	5	20	5.7×10^{-4}	6.8×10^{-4}
	3	85	3.1	27	20	1.8×10^{-4}	2.6×10^{-4}
	4	127	4.6		20	3.7×10^{-5}	8.0×10^{-5}
	5	172	8.5		20	6.0×10^{-6}	2.3×10^{-5}
106	1	17	2.8	5	20	1.0×10^{-3}	1.3×10^{-3}
	2	66	3.2	6	20	4.3×10^{-4}	6.0×10^{-4}
	3	122	5.5	21	20	6.7×10^{-5}	1.2×10^{-4}
	4	168	14		20	7.0×10^{-6}	3.4×10^{-5}
	5	248	16		20	1.0×10^{-5}	5.0×10^{-5}
	6	358	20		20	5.0×10^{-6}	2.5×10^{-5}

shorter but more saturated molecules (Sec. III). According to experiments given in the literature, the viscosity of a bilayer will increase when the lipids constituting the bilayer become more saturated [26]. Egg-yolk lipid (see manufacturer's data in Sec. III) is largely composed of a mixture of 16-C and 18-C phosphatidyl choline in which double bonds are present. In the literature we find that compared to the microviscosity of EYL bilayers, the microviscosity of bilayers consisting of a mixture of 16-C and 18-C saturated phosphatidyl cholines (DPPC and DSPC, respectively) is 15 times higher [27–29]. The DPPC-DSPC bilayer would roughly correspond to an EYL bilayer in which all the double bonds have become saturated. It is clear that in the present case, where the constituting molecules have also become shorter (in itself leading to decreased viscosity [23]), the microviscosity for the DPPC-DSPC bilayer can be regarded as an upper limit. If we assume that this factor of 15 can also be used for ξ measured by us at high frequencies, we find for EYL bilayers: $\xi_{\max} = 15 \times 1.3 \times 10^{-10}$ [Eq. (10b)] = 2×10^{-9} N s/m [indicated in Table IV ($\xi_{\max}^{iii}$) and in Figs. 6(a) and 6(b) (vertical dotted line)].

Concerning the lower limits for ξ , we have no choice but to set these at 0. As can be seen in Figs. 6(a) and 6(b) the upper and lower limits for μ are now fully defined. In Table IV they are given as derived from plots in Figs. 6(a) and 6(b) and from such plots for the aging 60- and 65-nm vesicle dispersions (not given here). The function $\mu(\xi)$ has error boundaries, which are not indicated in these figures, but which have been taken into account in the determination of the margins for the μ values for each age.

It can be seen that with the additional information, the

possible ways in which μ (and ξ) change on aging, are very much restricted. On aging, the surface shear modulus μ decreases by two orders of magnitude. In Figs. 6(a) and 6(b), the shifts of the rough estimates for the upper limits of ξ suggest that, eventually, the surface shear viscosity increases. A similar effect is found in the literature for steady-state ξ values for bilayers in which peroxidation has taken place: There the microviscosity increases slightly (Refs. [14] and [30–34]).

In Fig. 7 the change in μ on aging is plotted for all four aging vesicle samples. One exponential relation has been fitted to the data for the 65-, 70-, and 106-nm vesicle dispersions for ages less than 175 days. The 60-nm vesicle dispersions were not taken into account. This will be discussed later (Sec. VI D). It follows that

$$\mu(t) = \mu^0 e^{-t/(38 \pm 2)} \quad (14)$$

FIG. 7. The magnitude of μ as a function of the age for all four remeasured vesicle dispersions: 60 nm ($\circ$), 65 nm ($\blacktriangle$), 70 nm ($\bullet$), 106 nm ($\blacksquare$).

for the first 175 days. After that, $\mu(t)$ levels off to a constant value:

$$\mu^\infty = (2.0 \pm 1.1) \times 10^{-5} \text{ N/m}.$$

Comparing with

$$\mu^0 = (2.1 \pm 0.2) \times 10^{-3} \text{ N/m},$$

it follows that μ decreases two orders of magnitude.

As we have mentioned in Sec. V A the formula by Oldroyd can be adjusted for the influence of the curvature modulus K_c . For zero spontaneous curvature,

$$\tau_2 = \frac{(23\eta_0 + 32\eta_{2,\text{eff}})a_m + 16\xi}{16(\mu + 9K_c/a_m^2)}. \quad (15)$$

Thus, strictly speaking, we can only say that the changes in τ_2 upon aging are due to changes in the value of $(\mu + 9K_c/a_m^2)$ if we assume the spontaneous curvature of the bilayer to be zero. For fresh bilayers, it was concluded that the curvature modulus did not contribute (much) to the value of $(\mu + 9K_c/a_m^2)$ [1]. It might just be that the influence of K_c increases as the vesicles age, and the second relaxation time changes from a μ -pertaining to a K_c -pertaining time. However, in that case, we would begin to find a dependency on the vesicle radius for the value of $(\mu + 9K_c/a_m^2)$ for older vesicles. We have no indication for this, as can be seen in Fig. 7. Looking at Fig. 7, we see that the decrease levels off at approximately the same value for all vesicle radii.

Let us justify more quantitatively the negligibility of the influence of K_c as aging progresses. The bilayer mechanical properties for fully aged bilayers are denoted with superscript “ ∞ .” For the measurements on the nine dispersions which have aged most [for which $(\mu^\infty + 9K_c^\infty/a_m^2)$ has already seemed to level off], we have plotted the value of $(\mu^\infty + 9K_c^\infty/a_m^2)$ versus $1/a_m^2$ and fitted a straight line through the points. The result is

$$\mu^\infty = (2.0 \pm 1.1) \times 10^{-5} \text{ N/m}, \quad (16a)$$

$$K_c^\infty = (-1.7 \pm 5.9) \times 10^{-21} \text{ J}.$$

Indeed, no large contribution of K_c is present. From the value of K_c^∞ , we can give an upper limit with 95% confidence of K_c^∞ ($K_c^\infty + \text{twice the standard deviation}$), which is

$$K_{c,\text{max}}^\infty = 1 \times 10^{-20} \text{ J}. \quad (16b)$$

If we compare this value with $K_{c,\text{min}}^0$, which we can find in the literature (the smallest reported value for the curvature modulus for fresh EYL bilayers can be found in Ref. [7], where K_c equals $0.4\text{--}0.5 \times 10^{-20} \text{ J}$), we see that the curvature modulus decreases significantly upon aging: by at least a factor of 4.

2. The relaxation pertaining to surface dilatation

As the vesicles grew older and the second relaxation could be measured at lower frequencies, a new relaxation seems to enter the measurable frequency regime, as can be seen in Figs. 8(a) and 8(b). The data concerning this relaxation can be found in Table V. Analysis was carried out with Eqs. (9a) and (9b), with the third process now added. For this last process only one relaxation time was incorporated because the limited measuring accuracy makes more refined analyses pointless. In our view, our measurements warrant the analysis of the data with a third relaxation process, because omitting it gives much worse fits. However, we stress that, statistically seen, the incorporation of a third relaxation process is a borderline case, i.e., one to two standard deviations difference.

As has been discussed in Sec. II B 2, Oldroyd has predicted that a second deformation relaxation (κ -governed) process should in principle be present. The second deformation relaxation time is a function of the internal and external viscosities, the vesicle radius, and the bilayer surface mechanical properties κ , σ , and ξ , as shown in Eqs. (7) and (8). The external viscosity can again be replaced by the effective viscosity, i.e., the real part of the complex viscosity, at time scales corresponding to the second deformation relaxation time (τ_3). From the literature (e.g., Refs. [35–37]), no conclusive evidence could be obtained as to the magnitude of the surface dilatational

TABLE V. Characteristics for the third relaxation process, for those remeasurements that show three transitions. In the first two columns are shown the radius of the vesicle and the number of the remeasurement. The viscosities are divided by the buffer viscosity η_0 at 10°C (1.35 mPa s). The third relaxation time τ_3 is the only time with which this process is described. The value for $\eta_{3,\text{eff}}$ is defined as $\eta'_{3,\infty} + \frac{1}{2}(\eta'_{2,\infty} - \eta'_{3,\infty})$ for this single-time relaxation process. In the last column we have κ_{min} , calculated from Eq. (17b). The error margins arise from the errors in τ_3 .

a (nm)	Remeas. No.	Age (days)	ϕ (%)	$\frac{\eta'_{2,\infty}}{\eta_0}$	τ_3 (s)	G_3 (Pa)	$\frac{\eta_{3,\infty}}{\eta_0}$	$\eta_{3,\text{eff}}$ (Pa s)	κ_{min} (10^{-4} N/m)
60	4	128	32	2.3	$6.3 \pm 1.7 \times 10^{-7}$	442 ± 180	2.1	2.9	3.1 ± 0.8
	5	233	32	2.3	$5.1 \pm 1.6 \times 10^{-7}$	610 ± 250	2.0	2.9	3.8 ± 1.2
65	3	163	31	2.1	$6.7 \pm 1.3 \times 10^{-7}$	490 ± 140	1.9	2.7	3.4 ± 0.7
	4	242	30	2.1	$4.4 \pm 1.5 \times 10^{-7}$	730 ± 410	1.9	2.7	5.2 ± 1.8
	5	352	31	2.2	$5.9 \pm 1.7 \times 10^{-7}$	470 ± 200	2.0	2.8	3.8 ± 1.1
70	5	172	39	2.5	$7.2 \pm 2.8 \times 10^{-7}$	270 ± 150	2.4	3.3	3.4 ± 1.3
106	4	168	32	2.0	$1.3 \pm 0.2 \times 10^{-6}$	200 ± 30	1.8	2.6	2.8 ± 0.4
	5	248	32	2.1	$8.9 \pm 1.6 \times 10^{-7}$	330 ± 70	1.8	2.6	4.2 ± 0.8
	6	358	32	2.1	$8.7 \pm 1.7 \times 10^{-7}$	310 ± 80	1.9	2.7	4.3 ± 1.6

FIG. 8. The relative complex viscosity for (a) the 128-day-old-60-nm vesicle dispersion and (b) the 168-days-old 106-nm vesicle dispersion. Three relaxation processes are clearly seen. The viscosities are divided by the buffer viscosity η_0 at 10°C: 1.35 mPa s.

viscosity in steady-state experiments. Therefore, the only information we have about σ is the lower limit: $\sigma=0$. If we substitute this lower limit for σ in Eq. (7), we get the lower limit of κ :

$$\kappa_{\min} = \frac{A_3 \zeta a_m + D_3 E_3 a_m^2}{\tau_3 (32 \zeta + B_3 a_m)} \quad (17a)$$

The values for A_3 , D_3 , and E_3 can be found in Eqs. 8(a)–8(d). There, $\eta_{3,\text{eff}}$ is the effective viscosity of a single-time process. It is defined as $\eta'_{3,\infty} + \frac{1}{2}(\eta'_{2,\infty} - \eta'_{3,\infty})$. It can be calculated from Eq. 17(a) that κ is minimal for the minimal ζ (which is 0). Therefore,

$$\kappa_{\min} = \frac{D_3 E_3 a_m}{\tau_3 B_3} \quad (17b)$$

These values were calculated for the cases where three relaxation processes were visible (see Table V). We can now give a lower limit of κ with 95% confidence. The fully aged bilayer to which this quantity pertains is denoted by a superscript “ ∞ ”:

$$\kappa_{\min}^{\infty} = 1.8 \times 10^{-4} \text{ N/m} \quad (18)$$

VI. DISCUSSION

A. Fit to the relaxation transitions

The first point that we want to bring forth is the justification of an assumption that we made in Ref. [1]. It

FIG. 9. The relative complex viscosity for the 85-day-old 70-nm vesicle dispersion (—), a fit to the data in which a series of times is incorporated for the second transition (· · · ·), a fit to the data for which only one relaxation time for the second transition is incorporated. The viscosities are divided by the buffer viscosity η_0 at 10°C: 1.35 mPa s.

was suggested there that there was no reason to assume that the μ -governed deformation relaxation process should be described with only one time, rather than with a series of times. No way of verifying this was available from the measurements, as the second relaxation process could only be partially measured. Nevertheless, it was assumed that the process was to be described better with a series of relaxation times. During aging, the transition in the complex viscosity pertaining to this process shifted to lower frequencies and fell more clearly into the available measurable frequency regime. Figure 9 shows that the above-mentioned assumption was a realistic one. The $\omega^{-1/2}$ behavior fits the second relaxation process as well as the first one. Indeed, description with a single-time second relaxation process, as can be seen in the same figure, is much less satisfactory.

B. A further check on the model

We prepared a 105-nm egg-yolk lipid vesicle dispersion, let it age sufficiently in order to increase τ_2 , and measured τ_2 as a function of volume fraction (changing $\eta_{2,\text{eff}}$). The result can be seen in Fig. 10. The plot gives the values for μ and ζ for vesicles that are 30 days old. It shows that a linear relationship between τ_2 and $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$ remains valid for high volume frac-

FIG. 10. The value of τ_2 vs $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$ for nine dilutions of a 105-nm EYL vesicle dispersion to check Oldroyd's relation and find μ and ζ for intermediately aged vesicles.

tions (the range of volume fractions is 29% to 57%). We consider it as a justification of our assumption that no coupling occurs between the vesicles' deformations. Consequently, the viscosity of the ambient fluid of the droplets can indeed be taken as $\eta_{2,\text{eff}}$. From this plot of τ_2 versus $(23\eta_0 + 32\eta_{2,\text{eff}})a_m$ for nine different dilutions can be derived accurately the values for μ and ζ at this stage of aging (the age is denoted by a superscript "30"):

$$\mu^{30} = (7.6 \pm 0.4) \times 10^{-4} \text{ N/m} . \quad (19a)$$

$$\zeta_{\text{max}}^{30} = 9.5 \times 10^{-11} \text{ N s/m (95\% confidence)} . \quad (19b)$$

It can be seen that μ has indeed already decreased significantly [compare with Eq. 10(a)]. As for fresh bilayers, again, a very small ζ_{max} is found.

C. Causes for changes in surface viscoelastic properties

1. Viscosity

It has been mentioned in Sec. III that after peroxidation of the lipid bilayer, a vast array of different molecules is formed as lipids are broken and modified. So, large changes have occurred in the composition and buildup of the bilayer. The changes can be divided into five groups: (1) The hydrocarbon chains have become shorter, (2) the hydrocarbon chains will have become markedly more saturated, (3) material may have (temporarily) disappeared from the bilayer, (4) the hydrocarbon interior of the bilayer may have become (slightly) more polar as hydrophilic fragments may have been formed upon reaction of broken lipids with oxygen, and (5) the head-group area may now contain more small-sized head groups, as oxygenated long-chain fragments may now act as amphiphilic molecules. This may also cause a change in surface charge (density).

The microviscosity in a progressively aged bilayer has increased somewhat with respect to the microviscosity of a fresh bilayer (see Refs. [14] and [30–34]), as observed in this article. The fluidity of a bilayer decreases as the partial specific volume of the constituents decreases [26]. The partial specific volume is defined as the bilayer volume increase per added volume of lipid molecule. The changes in the membrane's microviscosity may be due to some of the following changes in membrane structure:

- (1) An increase in the number of smaller, largely hydrophobic molecules in the bilayer interior.
- (2) An increase in the degree of saturation in the bilayer.
- (3) Increasing opportunities for hydrogen bonding at the surface because oxidized dissociation products may now act as amphiphilic molecules.
- (4) Cross-linking between lipid radicals.

According to the literature, we may expect those structure changes to (1) decrease (smaller average molecular weight) [23], (2), increase (progressive saturation) [38,39], (3) increase (hydrogen bonding) [27,39–42], and (4) increase (cross-linking) [14] the microviscosity. This complex of partly counteracting influences may lead to a small increase in the microviscosity after aging, as is observed in the steady-state microviscosity [14,30–34].

These influences may also increase the value for our ζ for high-frequency experiments, as is suggested from the shift of the upper limit for ζ established from $\eta'_{2,\infty}$ [Figs. 6(a), and 6(b)].

2. Elasticity

In the literature, many theoretical and experimental studies give information about the curvature modulus K_c in relation to composition. It turns out that for lipid bilayers and surfactant layers studied at long time scales, a small increase in the number of shorter molecules (as compared to the basic lipid or surfactant molecules in the surface) decreases K_c (Refs. [43–49]). The reason for this decrease is that the magnitude of K_c is largely determined by the increasing amount of conformational freedom of the hydrocarbons (see Fig. 3). The lesser freedom there is, the larger K_c is. The decrease can be more than a factor of 10. Only for low K_c do polar head-group interactions influence the bilayer elasticity [50]. See Fig. 11 for the typical decrease of K_c as the fraction of short-chain molecules in a layer increases [from the literature (Ref. [48])]. We assume that this figure also represents the typical dependency of the modulus on composition in dynamic experiments. We have already concluded that the curvature modulus decreases at least a factor of 4 upon aging of the bilayer (see Sec. VB2), so this is in agreement with the literature. On a mechanical level, the elasticity moduli μ , κ , and K_c are related by the Young modulus and the Poisson ratio. In our case, they have to be regarded as complex quantities to account for energy dissipation within the bilayer. In that case, there are more parameters necessary to express the relations between the various elasticity moduli, so that no quantitative comparison of (the decrease of) K_c and μ can be carried out. Qualitatively seen, however, as the magnitude of both K_c and μ is a function of the lipids' head-group and tail interactions, their decrease upon aging is probably mutually related.

Concerning the relation between the decrease of the surface shear elasticity modulus and the molecular changes within the bilayer, we can give the following analysis. Suppose the measured surface shear elasticity modulus μ is proportional to the number N of bonds within the bilayer ($\mu \sim N$). If the decrease of the number

FIG. 11. Typical decrease of the curvature modulus (arbitrary units) at long time scales as the fraction of smaller-sized molecules in a (bi)layer increases (from the literature [48]).

of bonds per unit time dN/dt is proportional to the number of bonds present, we have

$$\frac{dN}{dt} \sim -\lambda N, \quad (20)$$

with λ a reaction constant. This model would correspond to a reaction mechanism where the number of (unsaturated bonds in the) lipid molecules is the rate-determining step. Consequently, the number of bonds will decrease exponentially:

$$N \sim e^{-\lambda t}. \quad (21a)$$

Then, the elasticity modulus will also decrease by this exponential. Taking account of boundary conditions as regards the surface shear modulus at ages 0 and ∞ , we can write

$$\mu(t) = (\mu^0 - \mu^\infty) e^{-\lambda t} + \mu^\infty. \quad (21b)$$

This corresponds to Eq. (14), as $\mu^0 \gg \mu^\infty$.

The composition of an aged vesicle sample was compared to the composition of fresh lipid. This was done by high-pressure liquid chromatography. With the used column, the molecules are separated according to their polarity. The constituents were analyzed with aid of UV spectroscopy. Qualitatively, it proved that the number of different molecules present in the aged sample had increased dramatically with respect to the fresh sample. Constituents in the aged bilayer had increased as well as decreased polarity compared to the fresh sample. This is in accordance with the statements in Sec. VI C 1 about the expected constitution of the bilayer after peroxidation. Quantitative analysis is more difficult, and was not pursued because we are only interested in the qualitative observation that the number of (different) molecules in the aged bilayer has increased, in order to explain the decrease of the elasticity moduli.

D. Final comments

We must point out the effects of two uncertainties in the analysis of the complex viscosity on the result presented in Eq. (18). First, the dependency of the number of intraconnected relaxation times in the series in Eqs. (9a) and (9b) on the fitted (longest) relaxation time of the third process (only one relaxation time was used, although more could in principle be included). Only the low-frequency part of the transition could be measured. We have observed in Ref. [1] that, in such a case, taking a series of 100 times instead of 1 time changes the fitted (longest) relaxation time to an extent well within the statistical uncertainty of this value. This, therefore, is not much of a problem. Second, measuring only part of the relaxation transition, as we do for the third relaxation time, leads to higher values of the relaxation time, as discussed in Sec. V A. So, estimates for the relaxation times are expected to be too high. As we can only calculate lower limits to κ , a lower value for the relaxation time would lead to higher κ values, and the value from Eq. (17b) would still be a lower value, and the analysis remains valid.

If we look at Fig. 7, we see that the 60-nm vesicles age slightly quicker than the others. The value of μ for these

vesicles' bilayers reaches the same level after prolonged aging as it does for the other vesicles. In preparing more EYL vesicle samples, it was noticed that the speed with which the samples age can vary. This did not depend on the temperature or on the amount of light that the samples were subjected to. Nor did it depend on the amount of transition-metal ions as all used water was demineralized and the buffer salts used were all of analytical grade. Slight variations in the amount of oxygen present in the water could explain the effect.

We have pointed out already that the bilayer surface mechanical properties μ and ζ may be frequency dependent. As is the case for bulk material, the viscosity and storage modulus of a two-dimensional material can depend on the frequency. The bilayer may be modeled by a spring-and-dashpots model, in first approximation as a parallel combination of a dashpot and a dashpot-and-spring in series [Fig. 12(a)]. The physical background to the representation of the bilayer, as done in this figure, has been mentioned in Sec. V A: The time scale of the experiment determines to what extent lipid translational diffusion within the bilayer can take place and therefore, to what extent the bilayer acts as an elastic material. The equations describing the dependency of μ and ζ on the frequency resemble Eqs. (1a) and (1b):

$$\zeta(\omega) = \zeta_\infty + \frac{\mu_\infty \tau_b}{1 + \omega^2 \tau_b^2}, \quad (22a)$$

$$\mu(\omega) = (\mu_0 + \frac{\mu_\infty \omega^2 \tau_b^2}{1 + \omega^2 \tau_b^2}). \quad (22b)$$

In Eq. (22b) the term μ_0 is present if a spring is added in parallel to the dashpot ζ_∞ [as indicated in Fig. 12(a) by the dotted lines]. The time scales are indicated by subscripts, "0" for zero frequency (long time scales), and " ∞ " for high frequencies (very short time scales). In Eqs. (22a) and (22b), τ_b is the internal relaxation time of the material of the lipid bilayer, considered as a loose piece. Furthermore, ζ_∞ is the bilayer surface shear viscosity at infinite frequencies, μ_0 the bilayer surface shear elasticity modulus at zero frequency, and $\mu_0 + \mu_\infty$ the surface shear modulus at infinite frequencies. In steady-state experiments, reported in the literature, the quantity μ_0 is taken as 0 for liquid-crystalline bilayers. Therefore, we don't have to take into account a finite μ_0 .

FIG. 12. (a) Representation of the bilayer surface as a viscoelastic material by a spring-and-dashpot model and (b) qualitative plot of the dependency of μ and ζ on ω . See Eqs. (22a) and (22b).

in the model [Fig. 12(a)] and in Eq. (22b). The surface shear viscosity in steady-state experiments equals

$$\xi_0 = \xi_\infty + \mu_\infty \tau_b. \quad (23)$$

In Fig. 12(b) a schematic diagram is given of the dependency of the mechanical quantities of surface shear on frequency. It gives the behavior of the mechanical system of Fig. 12(a). From Fig. 12(b) it can be seen that if we carry out linear viscoelastic experiments on vesicle dispersions and want to interpret the results as if the elastic properties $\mu(\omega)$ is constant, we need to be at high enough frequencies ($\omega > \tau_b^{-1}$). Via Eq. (23) we can estimate the time scales τ_b at which the internal relaxation of the bilayer takes place. We can only carry out this calculation for fresh EYL bilayers, as we know the literature value of ξ_0^0 pertaining to those bilayers only [$\xi_0^0 \sim 12 \times 10^{-10}$ N s/m (Ref. [20])]. Further, $\xi_{\infty, \max}^0 = 1.3 \times 10^{-10}$ N s/m [Eq. (10b)] and $\mu_\infty^0 = 2.1 \times 10^{-3}$ N/m [Eq. (10a)]. The superscript 0 indicates fresh bilayers. From these values, we can calculate a rough value of the internal relaxation time of the fresh bilayer: $\tau_b^0 = 6 \times 10^{-7}$ s. If we look at Fig. 4(a), we see that the measured relaxation times τ_2 of the capsules are shorter than the internal relaxation time of the bilayer τ_b^0 . This is

also the case for slightly aged vesicles. For those vesicles, the steady-state surface shear viscosity is observed to increase [14,30–34]. This increase is estimated from the increase of ξ_∞^{30} by a factor 2.5 with respect to ξ_∞^0 [from Figs. 5(a) and 5(b)]. So, ξ_0^{30} is estimated as $2.5 \times \xi_0^0$, yielding $\xi_0^{30} \approx 30 \times 10^{-10}$ N s/m. The surface shear modulus at this age is $\mu_\infty^{30} \approx 7 \times 10^{-4}$ N/m, and τ_b^{30} can be calculated to be approximately 4×10^{-6} s. In Fig. 10, all measured τ_2 values are seen to be smaller than τ_b^{30} .

VII. CONCLUSION

In conclusion we can say that our studies (this one and Ref. [1]) provide a corroboration of the effects predicted by Oldroyd. Thus, the theory can also be used to deduce the change of surface mechanical properties due to aging, and probably also for changes in, e.g., temperature, composition, pH, or electrolyte concentration.

ACKNOWLEDGMENT

The authors wish to thank Professor F. W. Wiegler for valuable discussions.

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- [1] J. B. A. F. Smeulders, C. Blom, and J. Mellema, *Phys. Rev. A* **42**, 3483 (1990).
 - [2] J. G. Oldroyd, *Proc. R. Soc. London Ser. A* **232**, 567 (1955).
 - [3] R. Kwok and E. A. Evans, *Biophys. J* **35**, 637 (1981).
 - [4] J. C. van der Werff, C. G. de Kruif, C. Blom, and J. Mellema, *Phys. Rev. A* **39**, 795 (1989).
 - [5] W. Flügge, in *Viscoelasticity* (Springer-Verlag, Berlin, 1975).
 - [6] I. M. Krieger and T. J. Dougherty, *Trans. Soc. Rheol.* **3**, 137 (1959).
 - [7] J. F. Faucon, M. D. Mitov, P. Méléard, I. Bivas, and P. Bothorel, *J. Phys. (Paris)* **50**, 2389 (1989).
 - [8] H. G. Weder and O. Zumbühl, in *Liposome Technology*, edited by G. Gregoriadis (CRC, Boca Raton, FL, 1984).
 - [9] R. A. Schwendener, M. Asanger, and H. G. Weder, *Biochem. Biophys. Res. Commun.* **100**, 1055 (1981).
 - [10] J. C. van der Werff and C. G. de Kruif, *J. Rheol.* **33**, 421 (1989).
 - [11] Manufacturer's data: Lipid Products Ltd., U.K.
 - [12] J. M. A. Kemps and D. J. A. Crommelin, *Pharmaceutisch Weekblad* **123**, 457 (1988).
 - [13] E. N. Frankel, *Prog. Lipid Res.* **23**, 197 (1985).
 - [14] R. C. Bruch and W. S. Thayer, *Biochim. Biophys. Acta* **733**, 216 (1983).
 - [15] G. Lercker, *Fat Production and Consumption*, Vol. 131 of *NATO Advanced Study Institute, Series A* (Plenum, New York, 1987), p. 269.
 - [16] C. Blom and J. Mellema, *Rheol. Acta* **23**, 98 (1984).
 - [17] M. Oosterbroek, H. A. Waterman, S. S. Wiseall, E. G. Altena, J. Mellema, and G. A. M. Kip, *Rheol. Acta* **19**, 497 (1980).
 - [18] E. Wu, K. Jacobson, and D. Papahadjopoulos, *Biochem.* **16**, 3936 (1977).
 - [19] E. Wu, K. Jacobson, F. Szoka, and A. Portis, *Biochem.* **17**, 5543 (1978).
 - [20] E. S. Wu, P. S. Low, and W. W. Webb, *Biophys. J.* (Abstr.) **33**, 109a (1981).
 - [21] E. A. Evans and R. Skalak, in *Mechanics and Thermodynamics of Biomembranes* (CRC, Boca Raton, FL, 1980).
 - [22] E. Evans and D. Needham, in *Physics of Amphiphilic Layers*, edited by J. Meunier, D. Langevin, and N. Boccaro (Springer-Verlag, Berlin, 1987).
 - [23] C. G. Wade, *Structure and Properties of Cell Membranes*, edited by G. Benga (CRC, Boca Raton, FL, 1985).
 - [24] M. B. Schneider, J. T. Jenkins, and W. W. Webb, *J. Phys. (Paris)* **45**, 1457 (1984).
 - [25] E. van der Linden, D. Bedeaux, and M. Borkovec (private communication).
 - [26] M. Shinitzky and P. Henkart, *Intern. Rev. Cytology* **60**, 121 (1979).
 - [27] B. R. Lentz, Y. Barenholz, and T. E. Thompson, *Biochem.* **15**, 4521 (1976).
 - [28] B. R. Lentz, Y. Barenholz, and T. E. Thompson, *Biochem.* **15**, 4529 (1976).
 - [29] U. Cogan, M. Shinitzky, G. Weber, and T. Nishida, *Biochem.* **12**, 521 (1973).
 - [30] O. M. Panasenko, A. I. Deyev, I. B. Deyeva, O. A. Azizova, and Yu. A. Vladimirov, *Biophys.* **30**, 889 (1985).
 - [31] G. E. Dobretsov, T. A. Borschevskaya, V. A. Petrov, and Yu. A. Vladimirov, *FEBS Lett.* **84**, 125 (1977).
 - [32] C. Richter, *Chem. Phys. Lipids* **44**, 175 (1987).
 - [33] T. Ohyashiki, T. Ohtsuki, and T. Mohri, *Biochim. Biophys. Acta* **861**, 311 (1986).
 - [34] T. Ohyashiki, T. Ohtsuki, and T. Mohri, *Biochim. Biophys. Acta* **939**, 383 (1988).
 - [35] N. F. Djabbarah and D. T. Wasan, *Chem. Eng. Sci.* **37**, 175 (1982).

- [36] H. C. Maru and D. T. Wasan, *Chem. Eng. Sci.* **34**, 1295 (1979).
- [37] E. A. Evans and R. M. Hochmuth, in *Current Topics in Membranes and Transport* (Academic, New York, 1978), Vol. 10, pp. 1–64.
- [38] M. Shinitzky and Y. Barenholz, *Biochim. Biophys. Acta* **515**, 367 (1978).
- [39] M. Shinitzky, in *Physiology of Membrane Fluidity*, edited by M. Shinitzky (CRC, Boca Raton, FL 1984), Vol. 1, Chap. 1.
- [40] G. Lenaz and G. P. Castelli, in *Structure and Properties of Cell Membranes*, edited by G. Benga (CRC, Boca Raton, FL, 1985).
- [41] Y. Lange, in *Handbook of Lipid Research: The Physical Chemistry of Lipids*, edited by D. M. Small (Plenum, New York, 1986), Chap. 13.
- [42] F. Hirata and J. Axelrod, *Science* **209**, 1082 (1980).
- [43] I. Szleifer, D. Kramer, A. Ben-Shaul, D. Roux, and W. M. Gelbart, *Phys. Rev. Lett.* **60**, 1966 (1988).
- [44] J. M. Di Meglio, M. Dvolaitzki, and C. Taupin, *J. Phys. Chem.* **89**, 871 (1985).
- [45] S. T. Milner and T. A. Witten, *J. Phys. (Paris)* **49**, 1951 (1988).
- [46] H. P. Duwe, J. Kaes, and E. Sackmann, *J. Phys. (Paris)* **51**, 945 (1990).
- [47] C. R. Safinya, E. B. Sirota, D. Roux, and G. S. Smith, *Phys. Rev. Lett.* **62**, 1134 (1989).
- [48] I. Szleifer, D. Kramer, A. Ben-Shaul, W. M. Gelbart, and S. A. Safran, *J. Chem. Phys.* **92**, 6800 (1990).
- [49] L. Paz, J. M. Di Meglio, M. Dvolaitzki, R. Ober, and C. Taupin, *J. Phys. Chem.* **88**, 3415 (1984).
- [50] H. N. W. Lekkerkerker, *Physica A* **159**, 319 (1989).