Analysis of the shape fluctuations of reconstituted membranes using GUVs made from lipid extracts of invertebrates

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Summary
Changes in the physical properties of the lipid matrix of cell membranes have repeatedly been proposed to underlie stresses associated with e.g. drought, cold and xenobiotics. Therefore, the ability to experimentally monitor such properties is central to the fundamental physiological understanding of adaptive changes. Here, we test the analysis of shape fluctuations in membranes composed of lipid extracts from two soil invertebrates, and show that theories and experimental approaches previously developed for simpler liposomes may be applied directly to reconstituted membrane lipids. Specifically, we show how the bending rigidity of giant unilamellar liposomes of lipid extracts can be determined precisely. We suggest that future measurements of this parameter could elucidate mechanisms of adaptive processes such as changes in lipid composition and accumulation of protective osmolytes.

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Introduction
Adaptive changes in the composition of membrane phospholipids that aid to preserve appropriate fluidity of the cell membrane under changing temperatures or during dehydration is a common phenomenon in ectothermic animals, known as “homeoviscous adaptation” or “homeophasic adaptation” (Hazel and Williams, 1990; Kostal, 2010). In fully functioning cells, membranes are in a fluid, liquid-crystalline phase, but when biological membranes are cooled or dehydrated sufficiently their function may be impaired through changes in microdomain structure or the occurrence of gel phase regions. Such changes may lead to a loss of selective properties (Hazel and Williams, 1990), intracellular metabolites and ions, and a damaging uptake of sodium and calcium. Organisms must therefore adapt to low temperature and dehydration by adjusting the properties of the cell membrane. This is usually accomplished through regulation of the lipid composition (Kostal, 2010) and/or accumulation of osmo- and cryoprotectants including polyols and sugars (Storey, 1997; Crowe et al., 1984).

Most studies of membrane adaptation are based on analysis of membrane lipid composition. However, this chemical parameter only provides circumstantial evidence of changes in the physical properties of the lipid matrix. There is therefore an urgent need for methods that can provide robust measures of physical properties of real membranes or semi-natural membranes mimicking the complex membrane lipid composition of the studied organisms as closely as possible.

The membrane can be considered as an infinitely thin surface that undergoes different deformations, such as the shearing deformation, the area expansion/compression, as well as the bending deformation. The membrane responses associated to these collective motions are characterized by a few parameters that quantify these membrane mechanical properties. Among these different moduli, the most sensitive is the bending rigidity, whose order of magnitude is the thermal energy, and which reflects the stiffness of the membrane against bending deformations and plays a role in shaping up cells and organelles (Dimova et al., 2006).

Bending rigidity can be estimated by Vesicle Fluctuation Analysis (Henriksen et al., 2004; Bouvrais, 2012) and this parameter has proven to be highly sensitive to various changes, since the bending elastic modulus is acknowledged to depend strongly on the membrane thermodynamic state and composition as well as the composition of the surrounding aqueous solution. For example, the bending rigidity was shown to depend on the temperature decrease when approaching the fluid-to-gel main phase transition (Fernandez-Puente et al., 1994), where the strong decrease of the bending modulus explains the anomalous swelling of the lipid bilayers near the phase transition temperature. The sterol content influence on the bending
Rigidity of membrane liposomes

from the lipid fraction during the extraction procedure, the missing quantity (as determined for separate samples (supplementary material Table S1)) was introduced as a given volume of cholesterol solution in chloroform (Avanti lipids) to get cholesterol molar ratio of 9.0% and 17.4% for F. candida and E. albida extracts, respectively. Then, the solvent was removed from the mixed lipid samples using rotary evaporator, the obtained dry film being subsequently hydrolyzed using some MillQ water (Millipore, Bedford MA, USA). Multilamellar vesicles (MLVs) were readily obtained by gentle agitation and the dispersion was sonicated in order to obtain SUVs using the Misonix sonicator 3000. The sonication lasted 30 minutes with the power fixed at 3 W successively on for 10 seconds and off for 5 seconds to prevent heating of the sample. The sonicated dispersion was then centrifuged and filtered using a 0.2 µm filter (sterile cellulose acetate membrane) to remove metal particles released by the tip of the sonicator.

Finally, GUVs were prepared using the electroformation method following the published protocol (Pott et al., 2008). SUVs deposits at a lipid concentration of about 0.1 mg/ml were made on the electrodes (6 spots of 2 µl on each electrode) using the sonicated dispersion prepared as described previously. The water from the deposits was partially evaporated during 3–4 hours under reduced pressure by introducing the electrodes in a desicator and during this step the electrodes were protected from light to prevent lipid damages. Then, the electrodes were immersed in pure water previously introduced in a glass cell. An electric field was applied to the electrodes using a waveform generator (Agilent 33120A 15 MHz function), and the protocol of electroformation being adjusted to the medium following the given recommendations (Pott et al., 2008). Vesicles with a diameter between 10 and 50 µm were visible on the electrodes after a few hours as illustrated in Fig. 1.

Flickering technique

Vesicles, after having been detached from the electrodes at the end of the electroformation protocol, were observed directly in the electroformation cuvette, which was placed in a custom-made temperature-controlled chamber holder at 20°C. Vesicles were visualized using a phase contrast microscope (Axiovert S100 Zeiss, Göttingen, Germany), equipped with a ×40/0.60 objective (440865 LD Acroplan), so that the vesicle two-dimensional contour could be seen in the focal plane of the objective. A CCD Camera (SONY SSCDC50AP) was used to record a series of 15,000 pictures at a rate of 25 frames per second with a video integration time of 4 milliseconds. The video image sequences of the GUV thermal fluctuations were analyzed using several custom-made software to perform contour extraction, contour cleaning and fluctuations analysis procedures described previously (Mitov et al., 1992). The bending rigidity was determined by a precise analysis of the statistical distribution of vesicle contours based on a simple Fourier decomposition of the angular correlation function as described previously (Méleré et al., 2011). For a given system, the bending elastic modulus, κ, represented an average of measurements amongst a population of 8 to 12 vesicles, whose diameters were between 20 and 50 µm. Dynamic analysis of the flicker spectrum of the amplitudes of decomposition of the angular autocorrelation function in the Legendre polynomial basis enabled us to determine the temporal correlation function. Additional details on the flickering technique regarding both theory and experiments are described in supplementary material Fig. S1 and elsewhere (Bouvrais, 2012).

Results and Discussion

Our results show that the static behavior of the thermal fluctuations observed in equilibrium for simple giant vesicles is indeed found also for more complex membranes obtained from full organism extracts, which allows us to determine accurate bending rigidities. Fig. 2 shows that static analysis of the flicker spectrum of GUVs made from membrane extracts leads to behaviors similar to the ones observed for one-component GUVs (data not shown). For a steady-state vesicle, i.e. vesicle presenting fluctuations that are stable in time as shown in Fig. 2a, we found that the distributions of Fourier amplitudes of the contour fluctuations are Gaussian with no mode correlation as displayed in Fig. 2b.

Thus, we were able to determine a bending rigidity value following the same procedure as the ones used for simple membranes. As shown in Fig. 3a, the distributions of the amplitudes of decomposition of the angular autocorrelation function in the cosine basis behave exponentially as expected for one-component GUVs. An exponential fit of these distributions for each mode number n led to the parameter An that is the slope of the exponential decay. Subsequently, the
fitting procedure (Méléard et al., 2011) was applied, as displayed in Fig. 3b, resulting in the determination of both the bending rigidity and the membrane tension associated to the giant vesicle that was studied.

Our observations indicate that this complex system (GUV made from membrane extracts) behaves roughly like simple membranes (only one or two lipid components), so that the same method of characterizing is apparently applicable, i.e. first a parameterization of the exponential distributions of the amplitudes for each mode number and then a final fitting procedure leading to the value of the bending modulus. Thus, the same lessons drawn from the analysis of simple systems can be carried out to complex systems, making the flickering technique a universal basic diagnostic tool.

Comparison with simple membranes
Being able to determine a bending rigidity from the flickering measurements was the first requirement to use the flickering technique as a biophysical tool for complex systems. However, if such a determined value is not precise enough or too dispersed

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Fig. 2. Illustrations of the fluctuation characteristics of a GUV (radius 19.8 μm) made from Enchytraeus albidus membrane lipid extracts. (a) Spectrum of the amplitudes of decomposition of the angular autocorrelation function in the Legendre polynomial basis for the mode 5. (b) Experimental distribution for the Fourier amplitudes of the contour fluctuations, α^5 or β^5, that can be parameterized by a Gaussian fit represented in red line. The mode 5 has been chosen randomly among the mid-range modes (4–10), which have good statistics and are not limited by the video integration time.
due to the large variety in lipid species present in the membrane extracts, it becomes useless. We therefore compared bending rigidity of one-component lipid bilayers widely used in model studies and giant vesicles made from whole-body extracts of invertebrates. Fig. 4 shows bending rigidity of POPC, *F. candida* and *E. albidus* GUVs. Although the absolute bending rigidity is not comparable, it is evident that bending rigidity measurements can be obtained for GUVs made from whole-body extracts with at least the same precision as for POPC GUVs. We found that the precision of the bending rigidity is so high that we could distinguish a difference in the bending modulus of the two invertebrates investigated, namely $20.50 \pm 0.19 \text{kBT}$ for *F. candida* and $21.25 \pm 0.36 \text{kBT}$ for *E. albidus*. The differences in bending rigidity values obtained from studies of these two invertebrates can be compared with the one obtained from the natural egg PC equal to $16.32 \pm 1.48 \text{kBT}$ (Angelova et al., 1992).

For comparison, it is worthwhile noting that small variations in the length and composition of the acyl chains result in changes in the bending rigidity that are of a few kBT, bending rigidity being equal to $32.15 \pm 1.48 \text{kBT}$ and $37.10 \pm 2.23 \text{kBT}$ for DMPC (14 C atoms in the acyl chains) and DPPC (16 C atoms in the acyl chains), respectively (Fernandez-Puente et al., 1994). Also, the cholesterol content can have significant effect on the bending rigidity (Henriksen et al., 2004).

**Dynamics of the shape**

The shape fluctuations of vesicles can be considered as thermal fluctuations around a stable equilibrium state in a viscous (over-damped) environment. Therefore, the relaxation dynamics of the shape excitations is characterized by an exponential decay. This can be experimentally obtained by the measurement of the temporal auto-correlation function of the amplitudes of a given mode $n$. The relaxation dynamics is mainly determined by the nature of the energy dissipation mechanism involved. An important contribution stems from the viscous dissipation in the solvent, which according to Milner–Safran theory (Milner and Safran, 1987) gives rise to a mono-exponential decay of the temporal auto-correlation function for a simple one-component membrane. This is indeed found to be the case for more complex systems, i.e. membrane extracts of soil-living organisms, as illustrated in Fig. 5a at early times ($\leq 1$ second). At later times the instrumental limitations prevent the observation of a relaxation time explaining these oscillations in the temporal correlation function.

The relaxation time $\tau$ for simple systems is expected to depend on the mode number ($n$) and to scale as $\tau \sim n^{-3}$ (Milner and Safran, 1987). For these membrane extracts, as shown in Fig. 5b, it is found that the relaxation time also depends on the mode number; however, the power-law decay is considerably lower. This suggests that the embedding solvent does not provide the dominant energy dissipation mechanism for the membranes. An alternative dissipation mechanism put forward (Evans et al., 1992) is the inter-monolayer friction of the lipid bilayer, which will produce a $\tau \sim n^{-2}$ dependence (Seifert and Langer, 1993),

![Fig. 3. Illustrations of the fitting procedure for a GUV made from *Enchytraeus albidus* membrane lipid extracts of radius equal to 19.8 µm. (a) Experimental distributions for the amplitudes of decomposition of the angular autocorrelation function in the cosine basis, $\xi^5$, that can be parameterized by an exponential fit represented in red line. (b) Dependence of $\Delta^5$ as a function of the mode number (close dots) up to the 11th mode and the resulting fit in full line giving $\kappa = 24.48 \text{kBT}$ and $\sigma = 28.85$.](image)

![Fig. 4. Individual bending rigidity measurements of POPC GUVs (●), and reconstituted GUVs made from membrane lipid extracts of *Folsomia candida* (■) and *Enchytraeus albidus* (▲), represented in close symbols, while the resulting averages with the standard deviations are shown with empty symbols.](image)
closer to the observed behavior. However, the conformational dynamics of complex lipid systems is still an unexplored research field with many new dissipation phenomena to be investigated.

In this paper we have demonstrated that the machinery of the membrane shape fluctuation analysis developed for simple systems can be applied for more complex bilayers. This allows precise measurements of the bending rigidity of reconstituted membranes made from whole-organism lipid extracts, and we suggest that such data can contribute to a fundamental understanding of homeoviscous membrane adaptations and other theories on membrane stress and adaptations. It is remarkable and valuable that the present method enables the precise measurement of well-defined physical characteristics like the bending rigidity using “average” membrane lipids representing a sub-population of a species. Other commonly used biophysical measures (e.g. main transition temperatures using DSC, or H1-NMR spectroscopy) would fail to provide such results when using vesicles composed of very heterogeneous membrane lipids as we have done in our study (Packham et al., 1981).

Current work in our laboratory addresses pollution-induced change in cold tolerance that is mediated by changes in membrane rigidity due to direct interactions between pollutant molecules and membranes. Further, bending rigidity measurements can be used as direct evidence for membrane-related differences in cold tolerance of individuals of the same species originating from contrasting thermal environments, and how this interacts with effects of cryoprotectants interacting with membranes. Our ongoing research has shown that bending rigidity measurements have sufficient resolution to show differences in functional membrane characteristics between enchytraeid species from cold versus temperate environments. The present study therefore opens up for better interpretation of membrane-related studies of thermal biology and ecotoxicology.

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Fig. 5. (a) Normalized temporal correlation function for mode 5 for a GUV made from Enchytraeus albidus membrane lipid extracts, whose decay is exponentially fitted at early times (solid line). The corresponding decay constants (relaxation times) for the exponentials are depicted in (b) as a function of mode number and scale as $\tau \sim n^{-1.67}$ (solid line).

Competing Interests
The authors have no competing interests to declare.

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