Local and transient permeation events are associated with local melting of giant liposomes†

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We reveal that the gel to fluid phase transition causes spherical membrane vesicles to release a finite number of molecules in several consecutive and localized events. By locally melting Giant Unilamellar lipid Vesicles (GUVs), using an optically trapped gold nanoparticle (AuNP) as a local heat source, we establish a local phase transition on the spherical GUV membrane clearly visualized using a phase sensitive fluorescent marker. We measure transient permeation events through this transition zone visualized as de-quenching of calcein as it escapes the interior of the GUV. Since biological membranes share several features with melting membranes, like nanoscale domain formation and critical density fluctuations, similar passive membrane transport could potentially be abundant in living cells.

Introduction

Membrane transport in cells can take place through well regulated channel proteins allowing ions and molecules to be transported across the barrier provided by the membrane. The lipid matrix provides the structural scaffold for membrane proteins but has itself a range of interesting mechanical properties that can significantly influence the conductance of transmembrane proteins.† Patch clamp measurements on protein free membranes have revealed that membranes undergoing a phase transition can exhibit significant porosity to ions.‡ This enhanced porosity has been linked to nanoscale membrane phase behavior in cells since biological membranes can exist near a phase transition and contain co-existing nanoscale domains having different degrees of lipid order.²⁻⁵⁻⁶ The lipid packing at the interfaces separating different domains is compromised and moreover, strong thermal fluctuations have been predicted at domain interfaces by Monte-Carlo simulations.⁷ Strong membrane fluctuations have been measured in both lipid monolayers⁸ and plasma membrane vesicles⁹ which confirms that biological membranes exist near a critical thermodynamic state. Model membrane systems having well defined phase transition temperatures display particularly pronounced permeability changes across the phase transition.⁴⁻¹² This has been exploited to facilitate transport across nanoscale lipid vesicles to fuel encapsulated proteins with ATP in a controlled manner¹¹ and in biomedical research to thermally release drugs encapsulated in lipid nanocarrier vesicles.¹²

In studies using Black Lipid Membranes (BLMs), a lipid film is painted across an aperture using lipids dissolved in organic solvents. Hence the membrane can be influenced by the remaining traces of organic solvents or by edge effects where the bilayer is attached to the aperture wall. Moreover, these experiments do not resemble closed biological compartments like liposomes, tubes and plasma membranes found in living cells. Ensemble measurements on permeability performed on nanoscale lipid vesicles have also shown permeability increase near the lipid phase transition which has been directly correlated with the differential calorimetric heat profile of the membrane used to measure the temperature dependent phase transition.⁴ However, permeability measurements performed on ensembles of lipid vesicles provide limited information regarding the nature or mechanism of the leakage. Single vesicle measurements using fluorescent markers encapsulated within the lumen can offer a way to visualize the time scale and magnitude of the transport. However, due to rapid diffusion of molecules in water it is difficult to detect fluorescent molecules leaving a lipid vesicle. Therefore, information regarding the nature or mechanism of the permeability has so far not been obtained from such experiments.

Here we present an assay which uses a novel combination of fluorescent markers and local melting of a Giant Unilamellar lipid Vesicle (GUV) that allows efficient detection of single and localized leakage events in GUVs undergoing a local phase transition. The local melting of the GUV is achieved by optically trapping a gold nanoparticle¹³ near the GUV membrane as shown schematically in Fig. 1a. The AuNP becomes heated due to absorption of light and consequently radiates heat.¹³⁻¹⁴ The GUV starts to leak when a region of the membrane is melted and a phase transition zone is established on the GUV membrane. To efficiently detect any single leakage events we encapsulate calcein within the GUVs at self-quenched concentrations¹⁷ which then during transport across the membrane becomes

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Calcein encapsulation was achieved by incubating the GUVs briefly with the fluorophores near the phase transition temperature or alternatively incubating the GUVs with calcein for at least 12 h at 5 °C. By incubating 85 mM calcein (adjusted to pH = 7.2 using NaOH) with a GUV suspension containing 600 mM sucrose, we achieved self-quenching concentrations within the GUVs.

Calcein quenching controls

Measurements of calcein intensity showed that at concentrations exceeding 1 mM quenching of calcein emission occurs, see ESI, Fig. S2.† Moreover, bleaching experiments of calcein trapped within GUVs revealed a rise in intensity followed by an exponential decrease, confirming that quenching of calcein took place within GUVs, see ESI, Fig. S3.†

Sample preparation

Samples were prepared by using clean glass coverslips incubated with either 1 g L⁻¹ Bovine Serum Albumin (BSA) or 1 g L⁻¹ α-casein for 10 min to passivate the glass against severe adhesion. Subsequently, the chamber was washed at least 5 times with phosphate buffered saline (PBS) containing 400 mM NaCl to remove BSA in solution. A small amount of the GUV solution containing calcein was added to the chamber and GUVs were allowed to sink to the bottom of the chamber before the chamber was carefully washed with PBS buffer containing 400 mM NaCl to dilute the extravesicular calcein.

Experimental setup

Optical trapping and imaging was performed on a Leica SP5 confocal scanning microscope with an optical trap coupled to the back port. Citrate stabilized AuNPs having diameters of 80 nm (purchased from British Biocell International, BBI) were optically trapped using a near infrared laser (λ = 1064 nm, Spectra Physics J201-BL-106C) focused through a high numerical aperture Leica objective (Water Immersion, PL APO NA: 1.2, 63 ×). The microscope was equipped with a piezoelectric stage (PI 731.20, Physik Instrumente, Germany) which facilitated nanoscale precision in the lateral movement of the GUV with respect to the optical trap.

Experimental procedure

To achieve efficient trapping, we adjusted the glass thickness collar according to the optimal values reported in ref. 20. A temperature increase of ~10 K could be obtained a few micrometers from the particle surface, which is sufficient to melt GUVs having a phase transition of Tm = 33 °C. The necessary distance to the GUV to induce leakage depended on the temperature in the imaging chamber which was kept at 22–30 °C and was not seen to affect the nature of the leakage. Controlled local melting was achieved by moving the GUV towards the trapped gold nanoparticle at a speed of 100 nm s⁻¹. Fluorophores were excited by λex = 488 nm (calcein and di-4-ANEPPDHQ), λex = 594 nm (TR-DHPE) and λex = 514 nm was used for imaging the gold nanoparticles in reflection mode.

Experimental

GUV preparation

GUVs prepared by electroformation were made from saturated lipids, 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, 850330) containing 0.3 mol% 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Invitrogen, T1395MP, Texas Red® DHPE) or 0.5 mol% di-4-ANEPPDHQ (Invitrogen, D36802) to visualize the bilayer.
using an acousto optical beam splitter. Image acquisition was performed with 111 ms per frame.

**Data analysis**

Images were analyzed using Matlab (The Mathworks, Inc.). Intensities higher than the background were quantified in regions 5 μm away from the nearest point of the GUV membrane for consecutive images to produce a time series. The peaks in the resulting intensity curves were found by using the cubic spline function in Matlab. As release events were observed to not only release molecules towards the trapped particle, the location of the leakage relative to the vector connecting the vesicle and the trapped particle, was quantified. The angular direction of the event was determined from the calcein signal by fitting a Gaussian to the polar intensity average, centered on the vesicles. This resulted in peak angles with fit errors below 1 degree.

Fitting of diffusion kinetics was done in Matlab. First eqn (1) was analytically integrated over a lateral region corresponding to the experimental Region of Interest (ROI) and subsequently a Matlab function, lscurvefit.m, was used to perform a fit using the distance to the ROI as a fitting parameter. Since the site of release was typically only slightly out of focus we chose to use the nearest distance to the GUV as an initial guess. A graphical presentation of the position of the ROI with respect to the GUV is given in the ESI, Fig. S8a.

**Results and discussion**

Leakage events, like the one in Fig. 1b, were transient and the intensity a few micrometers away from the GUV decayed after ca. 1 s. This decay was not due to depletion of fluorophores in the GUV since several consecutive leakage events could be detected for the same GUV. We verified that the GUV membrane in fact did melt due to the presence of the AuNP by adding a phase sensitive fluorescent dye, di-4-ANEPPDHQ, which specifically incorporates into the fluid part of the membrane, as shown in Fig. 1c and ESI S5.† As shown in Movie 1 and Fig. S5, the extent of the fluid region is sensitively dependent on the distance between the trapped AuNP and the GUV membrane. This is expected since the temperature has been theoretically shown to scale with the inverse distance as $1/D$, to a point-like heating source.† No leakage events were observed when the trapping laser with no AuNP was focused near the GUV membrane, as shown in Fig. 1d.

The point of release of calcein appears localized as judged from the shape of the intensity distribution shown in Fig. 2b. The plotting of contour lines with constant intensity gave circular contours having centers close to the membrane, see ESI, Fig. S6.† Events occurring slightly out of focus resulted in circular contour lines being centered slightly within the GUV. To quantify the lateral localization of the events on the GUV, we analysed the spatial distribution of the intensity to localize the event with respect to the line connecting the center of the GUV and the trapped nanoparticle, as shown in Fig. 2a and b. The dashed line in Fig. 2a is the lateral localization of the event and is expressed as an angle $\theta$ separating the dashed and the solid line in Fig. 2a. The resulting distribution of angles for all events is plotted in Fig. 2c. The localization of events is evidently not centered around $\theta = 0^\circ$ but rather distributed over a range of lateral angles. Notably, the events having $\theta \sim 0^\circ$ might still be slightly out of focus and hence have a vertical angle larger than zero. Events that are in focus, and have $\theta \sim 0^\circ$, occur in the region being nearest the trapped AuNP and hence are exposed to the highest temperatures. Therefore, the measured distribution of angles away from zero in Fig. 2c shows that thermal energy is not the only factor triggering release. Instead this strongly suggests that a phase transition occurring within an annular region on the GUV surface, as indicated schematically.
in Fig. 1a, is needed to facilitate transport across the membrane. This conclusion is supported by control experiments carried out with GUVs composed of DOPC lipids which are in a fluid phase at all experimental temperatures \(T_m = -17 \, ^\circ\text{C}\), as shown in the ESI, Fig. S7a and b.† Local heating of GUVs made from DOPC showed no leakage when heated under similar conditions as GUVs made from DCLPC as shown in Fig. S7a and Movie 2, ESI.† By incubating fluid phase GUVs with calcine for 30 min in a microscope chamber resulted in negligible leakage (see ESI, Fig. S7b†). However, GUVs made from DCLPC were porous after incubation for 1 min at \(T = 33 \, ^\circ\text{C}\) as shown by the calcine influx (see ESI, Fig. S7c†).

To measure the lifetime of the permeation events we quantified the intensity in a ROI at a distance of \(r_{\text{ROI}} = 5 \, \mu\text{m}\) from the membrane of a GUV. As shown in Fig. 3a, the resulting intensity curve reveals that several consecutive permeation events can exist for the same GUV.

This shows that the decay in intensity is not due to depletion of calcine in the GUV but rather due to closing of the bilayer followed by dilution of the fluorophores by diffusion.

To obtain a quantitative estimate of the opening time of the bilayer we compared the measured intensity evolution with calculations based on diffusion from an instantaneous point source. The transport of molecules from the point of release on the GUV, to a ROI placed at a distance \(r_{\text{ROI}}\) away from the GUV, is found by integrating the intensity within the ROI and comparing with a theoretical model of diffusion in which \(N_0\) molecules are released instantaneously from a point source in three dimensions. This dynamics is governed by Fick’s second law:²⁴

\[
N(x, y, z, t) = \frac{N_0}{8(\pi D t)^{3/2}} e^{-x^2+y^2+z^2/4Dt}
\]  

(1)

where \(D = 200 \, \mu\text{m}^2 \, \text{s}^{-1}\) is the diffusion constant for calcine in water²⁵ and \(t\) is the time elapsed since the release of the \(N_0\) molecules. Since the GUVs in our work are \(\sim 20 \, \mu\text{m}\) or larger, we account for the fact that molecules diffuse out into a half space, with a reflecting boundary condition, and the right side of eqn (1) is multiplied by a factor of two.²⁴ In Fig. 3a we quantify the integrated intensity over a ROI placed near a GUV that was being locally melted. Fitting of Fick’s second law to the data, using the distance from the ROI to the point of release as a fitting parameter, is shown for two examples in Fig. 3b and c, respectively.

The theoretical curve predicts a peak in the intensity at \(\sim 100 \, \text{ms}\) for both events whereas the data reveal a peak between 100 ms and 200 ms which is also an upper limit for the lifetime of the permeation event.

The peak intensities measured in Fig. 3b and c clearly indicate diffusion from a short lived permeation event since a decay in the intensity after ca. 150 ms must indicate that an initial rate of release has started to decrease or has become zero. However, for a few events we measured the time from the onset of the curve to the peak to be significantly longer (several seconds). This cannot be explained by an uncertainty in determining the distance to the site of release. As shown in the ESI, Fig. S8, an error in the distance, \(r_{\text{ROI}}\), of several micrometers affects the position of the peak in a time of less than 100 ms.

To get an upper estimate of the pore lifetime we use the fact that the intensity or concentration, \(C\), measured at some distance away from the GUV will continue to rise asymptotically as long as the pore stays open, according to the equation:²⁴

\[
C = -\frac{q}{4\pi D r} \text{erfc} \left( \frac{r}{2\sqrt{Dt}} \right)
\]

(2)

where \text{erfc} is the complementary error function, \(r\) is the distance to the point of release on the GUV, \(D\) is the diffusion constant and \(t\) denotes the time. The parameter \(q\) is the rate of release and is assumed to be constant in this equation. The concentration of molecules over time at a distance of \(r_{\text{ROI}} = 5 \, \mu\text{m}\) away from the GUV is plotted in the inset of Fig. 4a and is found to increase monotonically due to a constant rate of release. A decrease in the

![Fig. 3](Image)

**Fig. 3** Transient leakage events fitted to diffusion dynamics based on Fick’s second law. (a) Integrated intensity detected in a ROI placed near the GUV. (b) Assuming a point source instantaneous release of calcine molecules at the GUV membrane we fit the intensity curve to Fick’s second law, using the distance as a fitting parameter. (c) Similar analysis as in (b) but on a later event taken from the trace shown by the arrow from (a).
measured intensity, as shown by the green squares in Fig. 4a, indicates either that the rate of efflux has started to decrease or the pore has completely closed. According to eqn (1), an instantaneous pore with \( \tau_{\text{pore}} = 0 \) s gives a peak in the signal at \( t = \tau_{\text{travel}} = \frac{r_{\text{ROI}}}{6D} \) (the black solid curve in Fig. 4a) which signifies the time it takes for the molecules to diffuse the distance corresponding to \( r_{\text{ROI}} \). We therefore quantify the time separating the two peaks in Fig. 4a which we define as the pore lifetime, \( \tau_{\text{pore}} \), and the resulting pore lifetimes are plotted in Fig. 4b.

The release events are clearly transient in nature and the distribution of pore lifetimes spans from \( \sim 100 \) ms up to 1 s but with occasional events lasting up to several seconds. We note that the pore lifetimes quantified in Fig. 4b can be slightly overestimated since some release events occur slightly out of focus, see sketch in the ESI, Fig. S8a.† The position of the peak in the intensity curve scales with distance as \( \tau_{\text{peak}} = \frac{r_{\text{ROI}}}{6D} \) but for the distance used in this project (\( r_{\text{ROI}} \sim 5 \) \( \mu \)m) the uncertainty in determining the exact distance to the site of release results in an uncertainty of \( \sim 20 \) ms in determining the position of the peak (see ESI, Fig. S8b†), which is well below the time resolution of 111 ms in our experiments.

The energy needed to expand the membrane to form a hydrophilic pore critically depends on the physical properties of the membrane. Membranes existing in a fluid or gel phase have low lateral compressibility and the thermal fluctuations are not sufficient to create such pores. However, near the phase transition lipid bilayers exhibit dramatic softening,7 and consequently have a high compressibility, and thermally driven bilayer expansion requires less energy. Interestingly, we measure a higher chance of observing release of molecules away from the line connecting the heating particle and the center of the GUV, as shown in Fig. 2. This indicates that release is not only occurring in the warmest region where the thermal energy is highest, but also close to the annular region indicated in Fig. 1a where the membrane undergoes a phase transition. This annular region closely resembles domain interfaces which are ubiquitous in natural membranes. Domain interfaces have been found to exhibit strong fluctuations in Monte Carlo simulations4 and domain interfaces in lipid monolayers have been experimentally shown to exhibit critical fluctuations.9 Strong fluctuations combined with the interfacial mismatch between gel and fluid domains existing in this region could well facilitate transient release of molecules as observed here.

The structure of pores occurring in pure lipid membranes has so far not been resolved. However, two distinct types of pores have been suggested in the literature based on the amphipathic nature of the lipids.26 Firstly, hydrophilic pores in which lipids have their head groups facing the pore could facilitate transport across the membrane without increased hydration of the hydrophobic membrane interior. Such thermally activated pores would have a finite lifetime since line tension around the rim of the pore would act to close the pore. Hydrophilic pores can arise from lateral density fluctuations without rearrangement of the lipids. Lateral fluctuations are significantly enhanced during a melting transition27 and hence the permeation rate is expected to increase. Thermally activated pores would naturally close by relaxation of the resulting lateral membrane compression.26 Therefore, the relaxation time of a lipid bilayer will determine the pore lifetime in the case of a thermally activated expansion of a pore. The relaxation time has been found to scale with the heat capacity of lipid bilayers and hence a dramatic increase in relaxation times has been measured in melting lipid bilayers.27 The pore lifetimes found here are approximately an order of magnitude lower than the relaxation times measured in multilamellar lipid bilayers. However, it is well known that unilamellar liposomes have a broader and lower phase transition heat capacity profile than cooperative multilamellar bilayer systems which could explain the shorter pore lifetimes measured in our system.

**Conclusion**

We have presented a novel assay that facilitates observation of transient leakage events induced by localized heating of a gel
phase lipid membrane. Our results demonstrate for the first time that transient leakage events can occur in spherical membranes unaffected by organic solvents, patch clamp pipettes, or other scaffolding geometries that could potentially influence the permeability of the membrane. By inducing local phase transitions in a GUV we obtained a co-existing state of a gel and fluid membrane in the same vesicle as visualized using a phase sensitive membrane dye. Leakage was induced only in GUVs that were locally melted and not in fluid phase vesicles that were locally heated. With time scales of \(\sim 100 \) ms these opening events in the bilayer have a similar time scale as the opening of ion channels in cellular membranes, thus underlining the potential importance of passive permeability in cellular membranes in which nanoscale ordered and disordered domains exist in abundance. Our assay can easily be applied to test for permeability and dynamic phase behavior in other more complex and more biologically relevant membrane compositions displaying liquid ordered and liquid disordered phase coexistence.\(^8\) Also, a similar strategy can be employed to assess the effect of local heating on cell membranes which is highly relevant to the expanding field of localized laser induced hyperthermal therapy using specially designed metallic nanostructures.\(^9\) Finally, the mechanism of permeation in novel biomimetic polymers and protein capsules could well be investigated using a similar local heating assay to thermally activate changes in the material packing of heat sensitive protein–polymer cell-like protosomes.\(^18\)

**Conflict of interest**

The authors declare no competing financial interest.

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**References**


Supporting Information

Paper: Local and Transient Permeation Events are Associated with Local Melting of Giant Liposomes

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Figure S1 Example of a burst shown by 3 consecutive images. Membrane is labeled red using Texas red DHPE and calcein is green. The trapped 80 nm AuNP (white dot) is imaged by reflection microscopy. Upon leakage the calcein dilutes and de-quenches and results in a burst of intensity. Scale bar 10 μm.

Figure S2 Self quenching of calcein versus concentration measured in a microscope chamber using confocal detection. (a) The intensity increases approximately linearly up to 1 mM. (b) To avoid saturation in the image we changed the imaging settings for calcein concentrations higher than 1 mM and continued to measure intensities for concentrations up to 20 mM. The intensity versus concentration curve starts to become increasingly nonlinear as the concentration exceeds 1 mM. The data represent 20
independent experiments. All intensities are collected at the same height relative to the bottom glass surface corresponding to the z-position where the intensity was highest.

Figure S3 Calcein quenching control. Two GUVs, denoted by A and B respectively, containing different concentrations of calcein clearly indicated by the different appearance of the interior signal. GUVs containing highly quenched calcein have a darker region near the center (GUV A) whereas GUVs containing un-quenched calcein have uniform lumen intensity (GUV B). Graphs show resulting bleaching curves of the two GUVs in the image. The bleaching of the GUV containing quenched calcein results in an increase followed by a decrease in the measured intensity, red curve. Bleaching of GUV B, results in a typical exponentially decaying bleaching, blue curve. Scale bar is 35 μm.

Figure S4 Quantification of intensity inside ROIs placed near the GUV membrane. (a) The ROI was placed a distance of $r_{ROI} = 5 \mu m$ away from the nearest point on the membrane where the release of calcein could be observed. (b) The effect of placing a ROI at different distances from the GUV (as depicted in (a)), results in Intensity curves having different localization of the intensity peak. The successive curves represent the integrated intensity in ROIs placed 10 pixels apart as depicted in (a).
Figure S5 Local melting of two GUVs using an optically trapped gold nanoparticle as a nanoscopic heat generator. The AuNP is trapped in the space between two GUVs and the proximal membrane regions facing the nanoparticle undergo a gel to fluid phase transition as shown in Move 1. The fluid regions exhibit a stronger fluorescent intensity due to increased intercalation of the lipophilic fluorophore, di-4-ANEPPDHQ, into the fluid part of the membrane. The trapped AuNP (indicated by the yellow arrow) is translated through the gap between the GUVs to demonstrate how the location of the locally melted regions depends on the position of the trapped particle.
Figure S6 Contour plot of a typical burst showing concentric half-circles of constant intensity which are centered near the membrane. The intensity from the interior of the GUV has been removed by subtracting with the image just prior to the release.

Figure S7 Permeability controls of GUVs. (a) Control with DOPC and optically trapped AuNP. An 80 nm AuNP is optically trapped and brought into vicinity of a GUV made of DOPC lipids (T_{m} = -17 °C) and 0.3% TR-DHPE. 1 mM (non-quenched) calcein is mixed with the solution surrounding the GUV. No calcein influx was measured after moving the AuNP close to the membrane (see Movie 2). The optical trapping power was 450 mW. (b) Control showing that fluid phase membranes exhibit minimal porosity. Calcein leakage through fluid phase DOPC vesicles after incubation for 30 min was minimal. The concentration in this experiment is 1 mM calcein outside the vesicles. (c) DC_{12}PC GUVs after incubation at T_{m} for 1 min with 1 mM calcein.
Figure S8 Effect of distance to a point source on the localization of the intensity peak as predicted by the solution to Fick’s second law, eq. 1 in the paper. (a) Schematics showing the distance for a leakage site positioned out of focus and the distance corresponding to the shortest distance between the ROI and the GUV, $r_{ROI}$. (b) By changing the distance from 5 μm to 13 μm the position of the intensity peak only shifts ca. 80 ms showing that a large uncertainty in the distance translates into a small uncertainty in the location of the peak which is smaller than the experimental time resolution.

Movie Legends

**Movie 1** A local phase transition induced by optically trapping a gold nanoparticle ($d = 80$ nm) near two GUVs made from DC$_{15}$PC. The phase transition occurs at $T_m \sim 33^\circ$C and the fluid part of the membrane is visualized using the potentiometric dye di-4-ANEPPDHQ which partitions strongly into the fluid phase of the membrane.

**Movie 2** Control showing that no leakage occurs when locally heating a GUV in fluid phase. An optically trapped $d = 80$ nm gold nanoparticle is used to locally heat the GUV which is made of DOPC lipids (phase transition $T_m = -17^\circ$C).