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Bending rigidity, capacitance and shear viscosity of giant vesicle membranes prepared by spontaneous swelling, electroformation, gel-assisted and phase transfer methods: a comparative study

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Abstract

Closed lipid bilayers in the form of giant unilamellar vesicles (GUVs) are commonly used membrane models. Various methods have been developed to prepare GUVs, however it is unknown if all approaches yield membranes with the same elastic, electric, and rheological properties. Here, we combine flickering spectroscopy and electrodeformation of GUVs to measure, at identical conditions, membrane capacitance, bending rigidity and shear surface viscosity of palmitoylcholine (POPC) membranes formed by several commonly used preparation methods: thin film hydration (spontaneous swelling), electroformation, gel assisted swelling using polyvinyl alcohol (PVA) or agarose, and phase-transfer. We find relatively similar bending rigidity value across all the methods except for the agarose hydration method. In addition, the capacitance values are similar except for vesicles prepared via PVA gel hydration. Intriguingly, membranes prepared by the gel assisted and phase-transfer methods exhibit much higher shear viscosity compared to electroformation and spontaneous swelling, likely due to remnants of polymers (PVA and agarose) and oils (hexadecane and mineral) in the lipid bilayer structure.

1 Introduction

Membranes play a central role in living systems: all cells are encapsulated by membranes; membranes divide the eukaryotic cell into compartments to sequester specific cellular functions; membranes are the sites where many cellular machineries carry out their tasks.¹⁻⁵ In vitro membrane system such as the giant unilamellar vesicle (GUV), which is a cell-

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Conflicts of interest

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sized closed lipid bilayer provides a well-defined model to assay membrane properties and investigate the membrane biophysics at a fundamental level.⁶⁻⁸ Several methods exist to form GUVs.^{7,9,10} The oldest reported method, gentle hydration or spontaneous swelling,¹¹ relies on the hydration and continuous swelling of dry lipid films deposited on a solid substrate (e.g glass or Teflon) to form GUVs. The method itself is simple but time consuming, requiring up to around 12-24 hours or more. The electroformation technique introduced by *Angelova et al.*¹² significantly sped up the process by applying a uniform AC electric field, thus enabling GUVs formation in 1-2 hours. However, the difficulty to grow GUVs in highly saline conditions/buffer solutions,^{13,14} and the risk of lipid oxidation limits the use of this technique.¹⁵⁻¹⁷ With the advent of bottom-up approaches in synthetic biology,¹ several other methods have been proposed to create complex membranes such as asymmetric bilayers: gel assisted methods, phase-transfer/inverted emulsion method, phase reverse evaporation, microfluidics, continuous droplet interface crossing encapsulation (cDICE) and fusion of liposomes.¹⁸⁻²⁵ Given the library of available methods to form GUVs, it is unknown if all the methods yield lipid bilayers with the same material properties, or whether oxidation, residual oils, solvents, or polymers, modify the membrane thereby impacting biophysical studies.

In this work, we compare three fundamental properties, namely bending rigidity, capacitance and shear viscosity, of lipid bilayers formed by four popular methods: spontaneous swelling, electroformation, gel assisted swelling and phase-transfer method. Our study is summarized in Figure 1. For each method we adopted the optimized protocol recommended in the literature and the solution conditions were kept identical for all methods in order to isolate the effect of the preparation approach. The aforementioned properties were measured using two popular non-invasive techniques, flickering spectroscopy and electrodeformation.²⁶⁻³⁰

2 Materials and Methods

2.1 Materials

GUVs were formed from palmitoylcholine (POPC) purchased from Avanti Polar Lipids (Alabaster, AL). Polyvinyl alcohol (PVA), $M_w = 145$ kDa was purchased from Merck, Germany. Agarose (A5030, ultra low gelling temperature), hexadecane (H6703), mineral oil (M5904), sucrose and glucose were obtained from Sigma Aldrich, USA. HPLC water (22934 grade) was purchased from Fisher Scientific, USA.

2.2 Preparation methods

2.2.1 Spontaneous swelling (thin film hydration)—Thin film hydration or spontaneous swelling is one of the first methods developed to form GUVs.^{7,10,11,31,32} Vesicles grow from hydrated bilayer stacks due to a competition between osmotic and intermolecular forces. The method is popular due to the non-interference of external fields such as electric fields that limit preparation to low salinity conditions only. Here, we adopt the optimized preparation protocol suggested by *Akashi et al.*³¹ Initially, a 20 ml glass vial is rinsed with isopropanol, chloroform and water in the same order. 50 μ l of 6 mM solution of POPC in chloroform is diluted in 200-300 μ l of chloroform in a 20 ml vial. Nitrogen stream is blown over the lipid solution while it is mechanically swirled to facilitate solvent

evaporation. The vial with deposited lipid film is stored under vacuum for 3 hours. The film is then hydrated with 2 ml 500 mM sucrose solution containing 0.3 mM NaCl and placed at 60 °C in an oven for 12 hours. 20 μ l of harvested GUV solution is diluted in 100 μ l 450 mM sucrose + 60 mM glucose solution and placed in a chamber assembled from cover slides. To avoid vesicle adhesion to the cover slides prior to introducing the vesicles the slides were incubated with 10 mg/ml bovine serum albumin (BSA, Sigma) for 15 mins and then rinsed with pure water.

2.2.2 Electroformation—In order to facilitate the swelling process, electric fields have been utilized to improve GUVs yield.¹² In this approach, dried lipids are spread on two electrodes, and hydrated in the presence of electric fields to enhance the bilayers separation and closing into vesicles. The mechanism underlying the GUV formation using electroformation is still an active topic of research. Most commonly it is accepted that the alternating electric field induces electroosmotic flows to separate and bend the bilayers to form closed membranes.^{12,33-39} There are two choices for electrodes that are typically used: Indium Tin oxide (ITO) coated glass slides and Platinum (Pt) wires. Different material properties (adhesion forces to lipids, conductivity of electrodes, electrochemical properties) and geometry (flat glass ITO slides vs cylindrical Pt wires) in these two variations could potentially influence the GUV formation process.

Indium tin oxide electrodes: The glass slides (50 mm \times 50 mm ITO slides with 50 Ohms resistance, Delta technologies, USA) are cleaned with acetone and isopropanol and triple rinsed with bi-distilled water. The stock solution of POPC in chloroform is diluted to 6 mM from which 7-10 μ l of the solution is spread on the conductive sides of each slides using gas-tight glass syringe (Hamilton, USA). The lipid-coated slides are stored in dessicator for 3 hours to evaporate all organic solvents. Then the slides with facing conductive sides are assembled to sandwich a 2 mm thick Teflon spacer and are clipped together. Through a hole in the Teflon spacer, the chamber is gently filled with 2 ml of 500 mM sucrose solution in 0.3 mM of NaCl to avoid film disruption. Next, the conductive side of ITO is connected to a signal generator (Agilent Technology, USA) for 2 hours at 50 Hz and voltage 1.5 V_{pp} using copper tapes (3M, USA). After electroformation, the vesicles are smoothly aspirated using 1 ml micropipette tips (Eppendorf, Germany). The GUV harvest is diluted for analysis in a similar way like the spontaneous swelling method.

Platinum wires: For electroformation with Platinum (Pt) electrodes, the wires can be placed vertically or horizontally.^{35,36,38} Here, we utilize the horizontal configuration in order to monitor the vesicles during the electroformation process using a microscope. A home built device consisting of a PVC (polyvinyl chloride) chamber to house the Pt wires was used. The Pt wires are removable from the PVC chamber for cleaning purposes. Coverslips are attached at the bottom of chamber using vacuum grease. Slowly 5-10 μ l of lipid solution is spread on both sides of the Pt wires. The device is placed in a dessicator for 3 hours to evaporate all solvents. The chamber is slowly filled with 2 ml of 500 mM sucrose in 0.3 mM of NaCl solution and the Pt wires are directly connected to signal generator (Agilent Technology, USA) for 2 hours at 50 Hz and voltage 1.5 V_{pp} . The vesicles are smoothly

aspirated using 1 ml micropipette tips (Eppendorf, Germany). The GUV harvest is diluted for analysis in a similar way like the spontaneous swelling method.

2.2.3 Gel Assisted Methods—In these methods, polymer based substrate is used to accelerate vesicle growth. Vesicle formation is assisted by buffer influx below the bilayer through the porous polymer substrate to speed up hydration.^{7,35} We use agarose and polyvinyl alcohol (PVA), which are the two most popular polymer gel templates for vesicle formation.^{23,24}

PVA gel: This protocol is adapted and optimized from *Weinberger et al.*²⁴ First, 22 mm × 50 mm microscope cover slides and Teflon spacers are cleaned with HPLC water, isopropyl alcohol and acetone in the same order. 5 % (w/w) solution of PVA is prepared by constantly stirring PVA in water at 90°C. Next, 300 μ l of 5% PVA (w/v) in water solution is spread to form thin films without any bubbles. The coverslips are placed at 50°C in the oven for 45 mins to dry the film. Once the gel is dried, a Hamilton syringe, washed with chloroform, is used to spread 7-8 μ l of POPC lipid solution evenly onto the surface of the PVA gel at room temperature. The slides are placed in a vacuum for 3 hours at room temperature. Clean Teflon spacers are placed onto the glass slide and secured. The chamber is filled with 2 ml of 500 mM sucrose solution containing 0.3 mM NaCl and sealed with a coverslip. After 30 minutes, the chamber is gently tapped few times and vesicles are smoothly aspirated using 1 ml pipette tips (Eppendorf, Germany). The vesicles are diluted for analysis in a similar way like the spontaneous swelling method.

Agarose: The method of growing of GUVs from agarose films is adapted from *Horger et al.*²³ 22 mm × 50 mm microscope cover slides and Teflon spacers are cleaned with HPLC water and isopropyl alcohol. 1% (w/v) of agarose is prepared in HPLC water above the polymer melting temperature T_m , 52 °C. 300 μ l of agarose solution is drawn up with a pipette and spread onto the glass surface. Slides are placed in an oven at 40°C for 2 hours. Once the gel is dried, a Hamilton syringe is cleaned with chloroform and used to spread 7-8 μ l of POPC lipid solution onto the surface. Slides are placed in a vacuum for 3 hours. Clean Teflon spacers are then placed on top of the cover slides and secured. The chamber is filled with 2 ml of 500 mM sucrose solutions with 0.3 mM NaCl. The GUV harvest is diluted for analysis in a similar way like the spontaneous swelling method.

2.2.4 Phase-transfer method—The phase-transfer method is a two step process, which offers the possibility to encapsulate material inside the GUV. In the first step, a lipid monolayer (outer leaflet of the bilayer membrane) is formed between the aqueous solution, which would constitute the vesicle suspending medium, and oil. Next, water-in-oil emulsion droplets covered with a lipid monolayer, which would become the inner membrane leaflet, and containing the sugar solution that becomes the interior vesicle solution are passed through the first monolayer via gravitational or centrifugal forces.⁹

Several factors (sugar density gradients, centrifugal force, volume of inner solution, incubation time, type of oil, humidity) can influence the final yield of GUVs.⁴⁰ Here, we utilize the optimized experimental protocol by *Moga et al.*⁴⁰ The same procedure is used to prepare GUVs from either mineral oil or hexadecane.

Lipids in oil preparation: Initially the lipid-oil mixture is formed by coating the dried lipid film on a 5 ml round bottom glass tube (Fisher Scientific, USA) by evaporating all the chloroform from 100 μl 6 mM POPC solution under Nitrogen gas. The tube is stored under vacuum for 1-2 hours to evaporate any leftover solvent. 1.5 ml of mineral oil/hexadecane is added to the tube under low humidity conditions (less than 10 %) inside Atmosbag to reach a final lipid in oil concentration of 400 μM . Note that for every lipid-oil preparation step, we open a new 5 ml oil bottle to minimize contamination from humidity. To improve the lipid solubilization in oil, the solution is sonicated for two hours and later incubated overnight at room temperature.

Surface treatment of micro-centrifuge tubes: The 1.5 ml microcentrifuge tubes (Eppendorf Safe-Lock microcentrifuge tubes, T9661) are incubated for 1 hour with 200 μl of 2 mg/ml BSA solution. This is an important step to avoid adhesion and eventual bursting of GUVs. The tubes are then washed 3 times with glucose solution.

Phase-transfer process: Initially 200 μl of 510 mM glucose solution is added to the surface treated microcentrifuge tubes. Next, 100 μl of lipids-oil solution is added on top of glucose solution. The entire setup is incubated for 1-2 hours for homogeneous formation of interfacial monolayer of lipids at the oil-water interface (outer vesicle leaflet). In another 1.5 ml microcentrifuge tube 250 μl of lipid-oil solution is added. To this tube, 10 μl of 500 mM sucrose solution in 0.3 mM NaCl is added and mechanically agitated using tube rack 2-3 times. Note that too much agitation can lead to small emulsion droplets, and eventually, small vesicles. An aliquot of 125 μl of the emulsion is pipetted on top of the lipid monolayer. The tubes are centrifuged for 3 mins at 1800 rpm. Next, we punch a hole at the bottom tip of microcentrifuge using a needle and harvest only 100 μl GUV solution with a 1 ml syringe (BD, USA) to prevent oil contamination from top layer. The vesicles are diluted on cover slips for analysis in a similar way like the spontaneous swelling method.

2.3 Characterization methods

2.3.1 Bending rigidity—Bending rigidity of membranes can be measured by a variety of methods.⁴⁵ Here, we chose the flickering spectroscopy due to its non-invasive data collection and well developed statistical analysis. It can be implemented using either confocal or phase contrast microscopy. We select the latter to avoid the use of guest molecules in the bilayer such as fluorescent markers. A disadvantage of the method is that it requires visible fluctuations which means that very stiff or gel phase membranes cannot be probed. The details of the method can be found in Refs.^{26,27,30}. In essence, using a camera at 60 frames per second (fps) (Photron SA1, USA) and optical microscope (phase contrast Zeiss A1, Germany) a time series of fluctuating vesicle contours imaged at the equatorial cross section is recorded. The fluctuating contour, $r(\phi)$, is decomposed in Fourier modes, $r(\phi) = R(1 + \sum_q u_q(t) \exp(iq\phi))$, where R is the average radius of the vesicle. The mean square amplitude of the fluctuating Fourier modes, u_q , depends on the membrane bending rigidity κ and the tension σ , $\langle |u_q|^2 \rangle \sim \frac{k_B T}{\kappa(q^3 + \bar{\sigma}q)}$, where $k_B T$ is the thermal energy (k_B is the Boltzmann constant and T is the temperature), and $\bar{\sigma} = \sigma R^2 / \kappa$. The integration time effect of the camera is minimized by acquiring images at a high shutter speed of 200 μs per

image. At least 10,000 images are recorded for each vesicle for good statistics. Since we are interested in bending rigidity measurements, only vesicles with low tension value in the range $10^{-8} - 10^{-10}$ N/m are chosen. This results in a small crossover mode, $q_c = \sqrt{\sigma}$, to a regime where the shape fluctuations are dominated by bending rigidity.

2.3.2 Membrane Capacitance—Membrane capacitance is measured using steady state electrodeformation as detailed in Refs.^{28,41} The vesicle shape with inside-outside conductivity ratio $\lambda = \frac{\lambda_i}{\lambda_o} < 1$ varies with the frequency (1-100 kHz) of an applied AC uniform electric field. At low frequencies, the shape is a prolate ellipsoid. As frequency increases, the ellipsoid aspect ratio ν decreases. At a frequency f_c , the shape becomes a sphere ($\nu = 1$). Further increase of the frequency results in oblate ellipsoidal shape $\nu < 1$. The critical frequency, f_c depends on the membrane capacitance C_m ^{42,43}

$$f_c = \frac{\lambda_i}{2\pi RC_m} \frac{1}{\sqrt{(1-\lambda)(3+\lambda)}} \quad (1)$$

Hence, the membrane capacitance, C_m can be determined from the experimentally measured critical frequency for the prolate-oblate transition during a frequency sweep. The electrodeformation method to measure membrane capacitance has the advantage of being non-invasive, high throughput, probe-free and able to measure membrane capacitance of a wide range of compositions and phase state.^{28,41} The only limitation with the method is that it cannot be used to measure capacitance for charged membranes (a detailed review can be found elsewhere⁷). The same pros and cons apply to the transient electrodeformation method for measuring membrane viscosity²⁹.

2.3.3 Membrane Viscosity—We implement the transient electrodeformation of GUVs to measure membrane viscosity.²⁹ To summarize, the method involves measuring the initial deformation rate of a vesicle as an AC electric field is applied at a particular frequency. High speed imaging of the increase of the vesicle aspect ratio, ν , is done at 1-2 kfps. The linear slope of the aspect ratio as a function of time depends on membrane viscosity as

$$\nu = 1 + \frac{t}{t_{ehd}} \left(3 \frac{p(\omega)}{(55 + 16\chi_m)} \right) \quad (2)$$

where $1/t_{ehd} = \epsilon E_0^2 / \eta$ is the characteristic rate-of-strain imposed by the electric field and $\chi_m = \eta_m / \eta R$ is the dimensionless surface viscosity η_m , η is the viscosity the solution inside and outside the vesicle, E_0 is the electric field strength amplitude and $p(\omega)$ is forcing field function detailed out in *Faizi et al.*²⁹ The apparent viscosities are measured at different frequencies in the range 0.1-1 kHz. The zero-frequency viscosity is obtained by extrapolating a linear fit of the viscosity vs frequency data. Electric field of 8 kV/m (strain rate 50 s^{-1}) produces a good range of data in the linear initial slope.

2.3.4 Statistical Analysis—Statistical testing is performed using ANOVA testing for multiple comparison analyses. All data are expressed in terms of mean \pm standard deviation,

and the number of independent replicates is expressed in the figure captions. The following conventions for statistical significance are used throughout the paper: n.s, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

3 Results and Discussion

GUVs were prepared under the same conditions to avoid effects on the bilayer properties due to variations in factors such as concentration and type of sugar and salt in the suspending solution, buffers, solution and lipid asymmetry, concentration of fluorescent lipids in the membrane, etc.^{26,44-50} At least 10-15 vesicles were analyzed for every GUV preparation divided in 2-3 batches. In all methods the vesicles were prepared such that the internal solution contains 500 mM sucrose and 0.3 mM NaCl. This high sugar concentration was imposed by the implementation of the phase transfer method which does not yield high amounts of good quality vesicles at low sugar concentrations⁴⁰. After harvesting, 20 μl vesicle solution was diluted with 20 μl of a slightly higher osmolarity solution, e.g., 450 mM sucrose + 60 mM glucose, to deflate the vesicles for flickering spectroscopy and electrodeformation experiments. This combination of sugars minimizes effects of gravity on vesicle shape, thereby ensuring vesicles are quasispherical⁴⁸. Note that with the phase-transfer method the vesicles are formed with 510 mM glucose outside. For every vesicle preparation method, the same vesicle population is utilized for material property measurements. For viscosity and bending rigidity measurements, the harvested vesicles are diluted without any salt outside. For capacitance measurements, 0.6 mM NaCl is added outside. Fluorescent markers were not added to label the membranes as these are known to modify membrane properties, e.g., some dyes cause photo induced lipid oxidation.^{27,49}

Figure 2 shows the GUVs formed by different methods. The GUVs were diluted with 510 mM glucose solution on cover slips. The vesicles were imaged using phase contrast microscopy after 2 hours to allow for sedimentation. As seen on Figure 2a, spontaneous swelling produces some GUVs with defects such as lipid clump and aggregation, while the other methods yield GUVs that appear defect-free. Some vesicles also had multilayer membranes or nested vesicles, see Figure 2a (blue arrows). These observations are consistent with the results from Refs.^{31,51} The spontaneous swelling method was chosen as a control experiment although electroformation can serve as an alternative control method.

3.1 Bending Modulus

Bending rigidity of bilayers reflects the energy cost to change membrane curvature, which involves compression and expansion of the inner and outer monolayer leaflets, respectively.^{52,53} This physical property plays an instrumental role in cellular processes involving membrane remodeling.^{45,54} Figure 3 shows the box plot for bending rigidity of POPC bilayers measured with flickering spectroscopy. The bending rigidity obtained with electroformation (ITO) in this study is $25.5 \pm 2.6 k_B T$ in agreement with the literature values, $25 - 28 k_B T$,^{26,44,45,50} and in the lower region of this range, consistent with evidence for membrane softening by sugars^{46,55}. We found similar bending rigidity values with four other preparation methods: spontaneous swelling, electroformation (Pt) and phase-transfer method (hexadecane and mineral oil) with statistically insignificant values as obtained by

ANOVA test. The results with PVA show an average value $27.3 \pm 5.1 k_B T$ similar to the one obtained for the spontaneous swelling method, however, with a much wider spread, 20-37 $k_B T$. This suggests that the PVA-gel assisted hydration leads to higher variability in membrane bending rigidity. These findings are consistent with the study from *Dao et al.*⁶ where modification in another elastic property, the stretching modulus, was observed at similar hydrating conditions. Previously *Moga et al.*⁴⁰ and *Elani et al.*⁴⁴ also found no differences between bending rigidity of GUVs formed from electroformation and phase-transfer method. This rules out effects of the oils used here in modifying elastic properties such as bending rigidity.

About 30% of the defect-free vesicles formed by spontaneous swelling demonstrated a reduced mean squared amplitude of the shape fluctuations, see Figure 4, and thus a higher bending rigidity (note that these data were not included in Figure 3). The bending rigidity values are almost twice the average value, see Figure 4b. The stiffening is likely due to formation of multilamellar membranes.

Agarose-prepared GUVs also exhibited a slightly higher bending rigidity, $30.2 \pm 2.2 k_B T$, compared to the rest of preparation methods, see Figure 3. Previously *Lira et al.*⁵⁷ revealed encapsulation of agarose in GUVs in the form of gel-like network. The encapsulated agarose would arrest the thermally driven vesicle shape fluctuations and thus effectively increase the apparent bending rigidity.

3.2 Capacitance

Action potentials and electromotility depend on the bilayer capacitance, which controls ionic currents through the membrane.^{58,59} Knowledge of the capacitance value is thus needed in order to understand cell electrophysiology. The specific capacitance can be estimated from the permittivity, ϵ , and thickness, d , of the bilayer as $C_m = \epsilon/d$. Changes in the dielectric properties or thickness of the bilayer, e.g., due to impurities introduced during membrane preparation, can thus affect the values of the measured capacitance. In fact, *Vitkova et al.*⁵⁵ have demonstrated that sugars increase the membrane capacitance likely due to membrane thinning and changes in dielectric permittivity. Figure 5a shows the typical frequency dependent deformation of a GUV at fixed field strength in a uniform AC electric field. The GUV shape changes from a prolate to an oblate ellipsoid at a critical frequency related to the membrane capacitance, see Eq 1. Figure 5b shows the box plot for membrane capacitance obtained for POPC bilayers prepared from seven different methods. Membrane capacitance of bilayers prepared by spontaneous swelling and electroformation (ITO and Pt) are similar to each other suggesting similar dielectric constants and membrane thickness. Interestingly, the membrane capacitance obtained for bilayers formed by the PVA method is lower than the value for bilayers formed by spontaneous swelling/electroformation method. Given the similar bending rigidity found by us and the decrease in the stretching modulus observed by *Dao et al.*⁵⁶ for membranes prepared by PVA method compared to electroformation (ITO), there is one plausible explanation of this observation. In order to satisfy the thin plate model,^{60,61} $\kappa \sim Kd^2$, (where K is the stretching modulus) and the membrane capacitance relation, $C_m = \epsilon/d$, a decrease in capacitance and stretching modulus at constant bending rigidity value suggests an increase in thickness of the membrane. Assuming a fixed electrical

permittivity constant, a reduction from $58 \mu F/cm^2$ to $41 \mu F/cm^2$ (in this study) between electroformation and PVA method would mean a 41 % increase in membrane thickness ($d_{PVA} = 1.41 d_{ITO}$). Since we obtained same bending rigidity values, the expected stretching modulus ratio would be $K_{PVA}/K_{ITO} = (d_{ITO}/d_{PVA})^2 \sim 0.5$. This is in a reasonable agreement with the reported data of *Dao et al.*⁵⁶ from micropipette aspiration experiments where $K_{ITO} \sim 160 \text{ mN/m}$ and $K_{PVA} \sim 90 \text{ mN/m}$. Phase emulsion methods demonstrate a much wider spread in the data strongly indicating the effects of residual oil in the membrane. The presence of oil in the membrane could possibly be detected by means of fluorescent markers. However, such molecules might have different partitioning between the bulk and the membrane compared to that of the used oils, thus disproportionately reflecting the residual oil concentration in the membrane. A more suitable approach might be the use of bulk methods such as mass spectrometry (although oil droplets in the sample might jeopardize the measurement). Yet another approach, could be the application of polarity-sensitive dyes such as Laurdan as recently demonstrated for GUVs prepared PVA-assisted swelling⁵⁶.

3.3 Bilayer Viscosity

Lipids in bilayer membranes are held together by non-covalent bonds allowing for molecules to move freely in-plane (along the membrane). Membrane fluidity is essential for the lateral transport of biomolecules such as cholesterol, lipid rafts and proteins in physiological processes.^{4,62-64} To the best of our knowledge, there is no study comparing the viscosity of bilayer membranes with same lipid composition but produced by different methods. There is only a limited information about membrane fluidity from *Dao et al.*⁵⁶ who compared molecular diffusivity in POPC and diblock copolymer bilayers prepared by PVA and electroformation method. This study found that PVA-formed membranes are more viscous than the electroformed ones, likely due to entrapment of PVA in the membranes. However, it is not trivial to deduce viscosity from diffusivity because of probe dependence.⁶⁵

Here, we have utilized the recently developed non-invasive technique, the transient electrodeformation of GUVs,²⁹ to directly measure shear viscosity. Figure 6a shows the deformation curves of POPC GUVs prepared by spontaneous swelling, electroformation, gel assisted method (PVA) and phase-transfer method (MO) with different initial rates. The membrane viscosity obtained from these deformation curves is given in Figure 6b and summarized in Table 1. We found no difference between membrane viscosity of lipid bilayers prepared from spontaneous swelling and electroformation, $\eta_m \sim 8 \text{ nPa.s.m}$. However, membranes prepared by gel assisted methods (PVA and agarose) exhibit significantly higher membrane viscosities compared to the classical methods of electroformation (ITO) and spontaneously swelling. Bilayers of agarose-formed GUVs are more viscous ($36.7 \pm 23.9 \text{ nPa.s.m}$) compared to PVA-formed GUVs ($17.9 \pm 13.1 \text{ nPa.s.m}$) suggesting that remnants of agarose gel in the bilayer affect membrane rheological properties more severely. Leftover of agarose gel in the vesicle lumen compared to PVA GUVs can also lead to extra dissipation.

Next, we examined more closely how the gel impurity modifies the membrane viscosity. Previously, it has been shown that aqueous solutions of agarose and PVA behave as shear

thinning fluids: the viscosity decreases with increasing shear rate.^{66,67} The stress generated by the electric field shears the membrane with a characteristic rate $\dot{\gamma} = 1 / t_d = \epsilon E_0^2 / \eta$. Modulating the field amplitude thus enables us to vary the shear rate in a wide range and to probe if bilayers behave as Newtonian (with shear-rate independent viscosity) or non-Newtonian fluids. Increasing E_0 from 1 to 50 kV/m at a given frequency increases the effective shear rate from 1 s^{-1} to 2000 s^{-1} . Figure 6c shows that the bilayers behave as Newtonian fluids, since viscosity does not change with the shear rate, even though the gel impurities increase the overall viscosity.

GUVs formed by the phase-transfer method (mineral oil and hexadecane) also demonstrated membrane viscosities higher by almost an order of magnitude, 70-80 nPa.s.m compared to the control methods. This strongly suggests that residual oil in the bilayer modifies the material's rheology.⁶⁸⁻⁷⁰

4 Conclusions

In this work, we have compared the material properties of POPC bilayers in the form of GUVs prepared from commonly used protocols: spontaneous swelling, electroformation, gel assisted and phase-transfer method. The results are summarized in Table 1. Probing material properties provides a straightforward means to monitor any method based modulation. Using flickering spectroscopy and electrodeformation, we compared bending rigidity, membrane capacitance and shear viscosity to determine if all the methods yield membranes with same properties. We chose spontaneous swelling as a control method, however, with similar material properties obtained with electroformation, either could serve as a control method. Although the gel assisted and the phase-transfer methods offer unprecedented advantage to grow GUVs rapidly in physiological buffers, we found evidence that gel remnants or residual oil alter bilayer properties especially the shear surface viscosity. A higher sugar concentration was chosen for all GUV preparation methods primarily due to low yield obtained with phase-transfer method at a low sugar concentration (see Moga et al⁴⁰). As a word of caution, sugars (i) can interdigitate in the membrane,⁷¹ (ii) might interfere with agarose (because of the 'sugary' structure of the polymer), and (iii) might be the source of impurities in the system⁷². However, since the final solution conditions across the membrane of the vesicles were identical in all the preparation methods, we believe the presence of sugars imposed by the phase-transfer method, does not change our comparative conclusions regarding the properties of the membranes prepared by the different methods. We also acknowledge that GUV preparation protocols vary slightly from lab to lab and we have adopted protocols suggested from literature that yield high quality vesicles without visible defects. The data presented in this study would help in more informed decision in the choice of preparation method for GUVs in respect to rheology of bilayer and mobility of biomolecules in synthetic cell studies.

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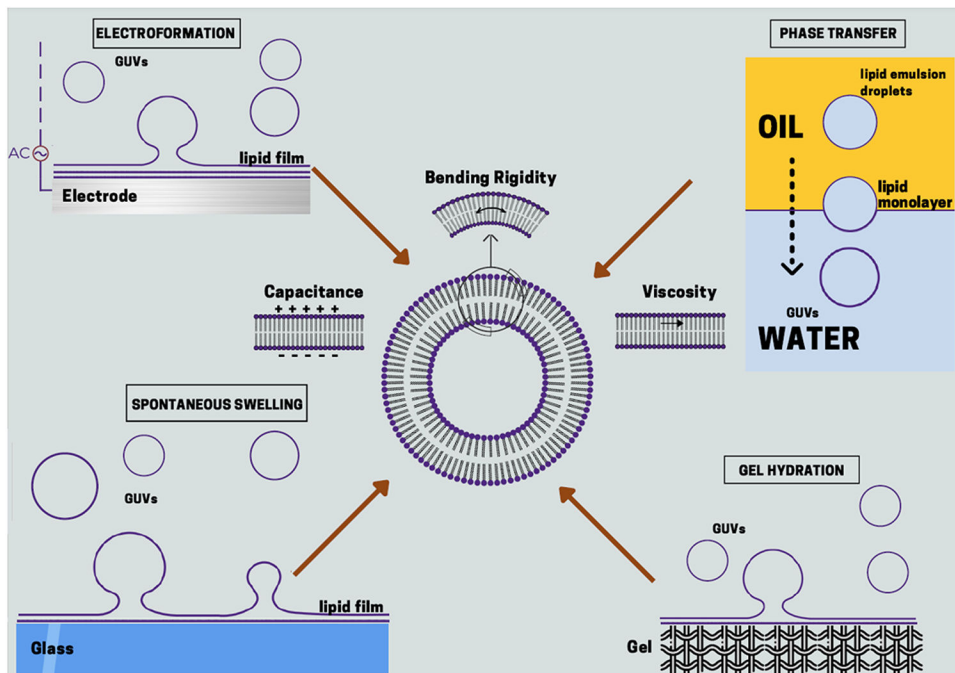


Figure 1: Summary of the preparation methods chosen for this study. The methods are spontaneous swelling, electroformation, gel hydration and phase-transfer method. The material properties probed in this study are bending rigidity, capacitance and shear viscosity of bilayer membranes.

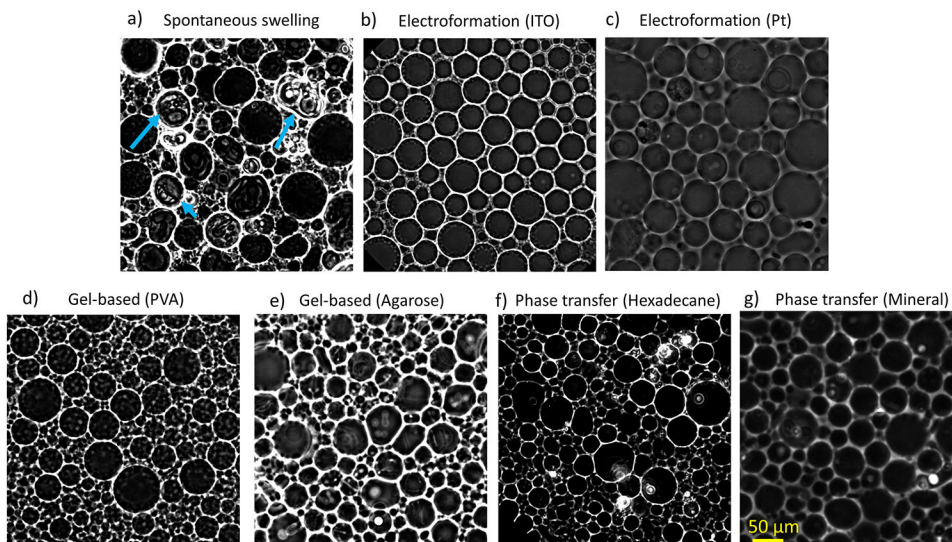


Figure 2: Phase contrast images of POPC GUVs produced from different preparation methods. 20 μl of harvested GUVs containing 500 mM sucrose and 0.3 mM NaCl inner solution were diluted in 510 mM glucose solution. The vesicles were imaged after 2 hours of sedimentation time. a) Spontaneous swelling. Blue arrows indicate lipid clumps or debris. b) Electroformation (ITO) c) Electroformation (Pt wire) d) Gel Assisted method (PVA) e) Gel assisted method (Agarose) f) Phase-transfer method (hexadecane) g) Phase-transfer method (mineral oil).

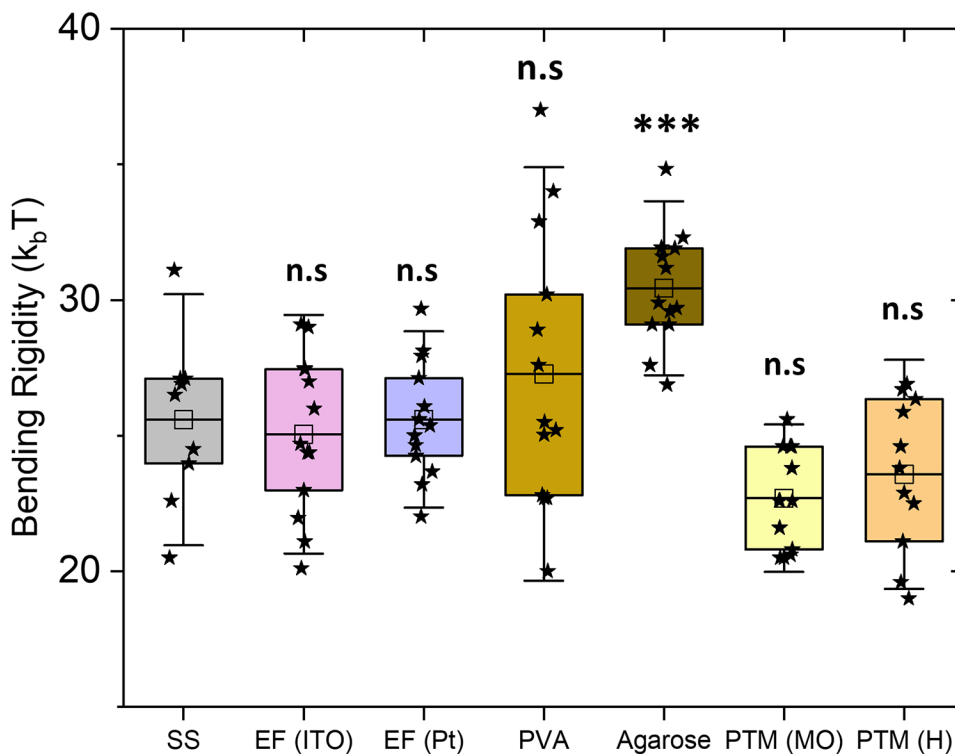


Figure 3: Bending rigidity of bilayers measured with flickering spectroscopy of GUVs prepared by the seven GUV preparation methods. The box-plot represents the standardized distribution of data based on first quartile (Q1), mean, third quartile (Q3), and the error bars represent 1.5 Standard Deviation. The abbreviations in the figure are as follows, SS: spontaneous swelling, EF: electroformation, PVA: polyvinyl alcohol, PTM (MO): phase-transfer method (mineral oil) and PTM (H): phase-transfer method (hexadecane). The open squares represent the mean values. ANOVA comparisons test compared to spontaneous swelling which is set as control. $n > 10$ vesicles were probed, *** $p < 0.001$, ** $p < 0.01$, n.s $p > 0.05$.

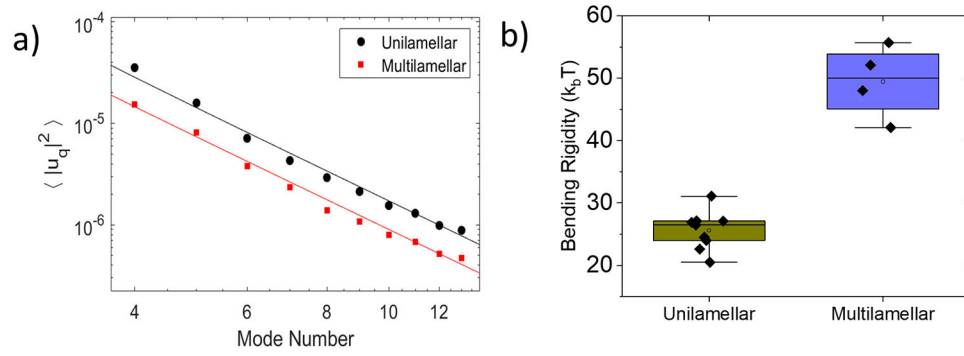


Figure 4:

a) Comparison of the mean squared amplitude of the membrane undulations of unilamellar and multilamellar vesicles prepared by spontaneous swelling b) Box-plot figure for bending rigidity values.

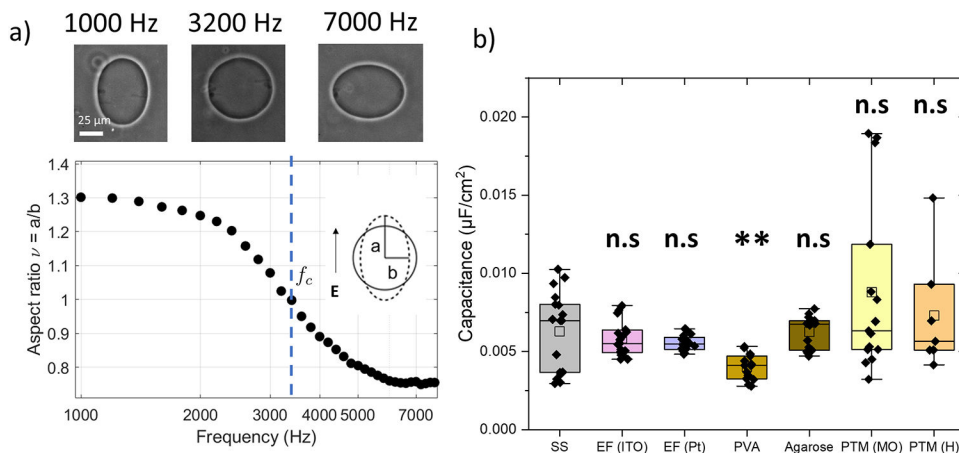
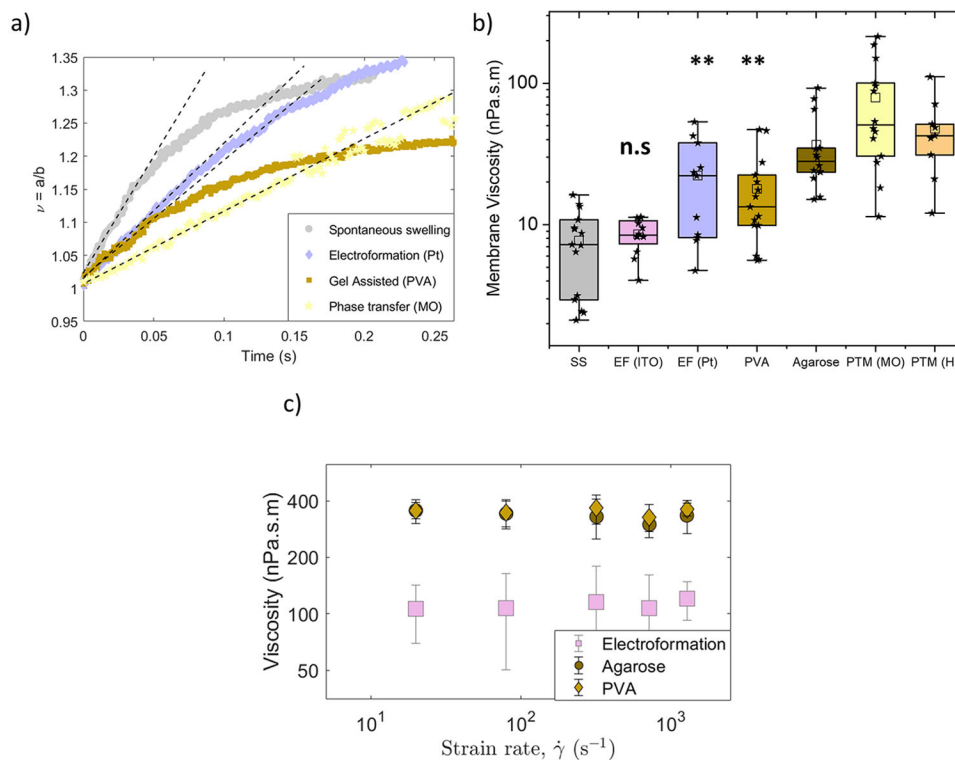


Figure 5:

a) Typical plot of membrane morphology in presence of AC electric field at different frequency at 8 kV/m and inner conductivity 40 $\mu\text{S}/\text{cm}$ and outer conductivity 60-80 $\mu\text{S}/\text{cm}$. b) The box-plot represents the standardized distribution of membrane capacitance based on five numbers minimum value, first quartile (Q1), mean, third quartile (Q3), and maximum value. The abbreviations in the figure are as follows, SS: spontaneous swelling, EF: electroformation, PVA: polyvinyl alcohol, PTM (MO): phase-transfer method (mineral oil) and PTM (H): phase-transfer method (hexadecane). The open square represents the mean value. ANOVA comparisons test compared to spontaneous swelling which is set as control. $n > 10$ vesicles were probed, *** $p < 0.001$, ** $p < 0.01$, n.s $p > 0.05$.

**Figure 6:**

a) Vesicles made of POPC by different preparation methods deform at a different rate indicating different membrane viscosity. The field strength and frequency are 8 kV/m and 0.1 kHz. The solid lines correspond to the theoretical fit with Eq. 2. b) Box-plot figure of shear viscosity values of bilayers obtained with transient electrodeformation for seven different GUW preparation methods. The box-plot represents the standardized distribution of data based on minimum value, first quartile (Q1), mean, third quartile (Q3), and maximum value. The open square represents the mean value. The abbreviations in the figure are as follows, SS: spontaneous swelling, EF: electroformation, PVA: polyvinyl alcohol, PTM (MO): phase-transfer method (mineral oil) and PTM (H): phase-transfer method (hexadecane). ANOVA comparisons test compared to spontaneous swelling which is set as control. ***p 0.001, **p 0.01, n.s p 0.05 c) Viscosity dependence on electric field strength, or equivalently strain rate $\dot{\gamma} = \epsilon E_0^2 / \eta$.

Table 1:

Membrane bending rigidity, capacitance and shear viscosity of POPC bilayers obtained from different preparation methods at 25 °C and determined in this study. Bending rigidity was measured with flickering spectroscopy and membrane capacitance and viscosity was measured with the electrodeformation method. The abbreviations in the table are as follows, SS: spontaneous swelling, EF: electroformation, PVA: polyvinyl alcohol, PTM (MO): phase-transfer method (mineral oil) and PTM (H): phase-transfer method (hexadecane).

Method	$\kappa (k_B T)$	$C_m (\mu F/cm^2)$	η_m (nPa.s.m)
SS	25.0 ± 3.1	0.63 ± 0.26	7.72 ± 4.6
EF (ITO)	25.5 ± 2.6	0.58 ± 0.11	8.57 ± 2.3
EF (Pt)	25.5 ± 2.1	0.55 ± 0.05	22.2 ± 16.3
PVA	27.3 ± 5.1	0.41 ± 0.08	17.9 ± 13.2
Agarose	30.2 ± 2.2	0.63 ± 0.11	36.7 ± 23.9
PTM (MO)	22.7 ± 1.7	0.87 ± 0.55	79.11 ± 63.8
PTM (H)	23.6 ± 2.8	0.72 ± 0.37	47.7 ± 29.8