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Electroformation of giant liposomes from spin-coated films of lipids

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Abstract

This paper describes spin-coating of solutions of lipids and using the resulting thin films for electroformation of giant liposomes. Spin-coating made it possible to generate uniform films of lipids with controllable thickness over large surfaces (>25 cm^2) of indium tin oxide. Establishing a range of thicknesses optimal for electroformation (25–50 nm), we demonstrate formation of giant liposomes from lipids (such as asolectin, phosphatidylserine, and phosphatidylglycerol) that do not readily form giant liposomes from traditional, droplet-derived films. We compared liposomes from a spin-coated film of lipids to liposomes formed from traditional droplet-derived films and found that spin-coated films produced larger (by factor of 2–5) and more abundant liposomes than droplet-derived films of lipids. Electroformation from spin-coated, homogenous lipid films of optimal thickness provided a reproducible way to obtain liposomes with diameters that are predominantly larger than 30 μm over the entire surface of formation.

Keywords: Electroformation; Giant liposome; Spin-coating; Lipid film; Vesicles

1. Introduction

Giant liposomes are large (>10 μm diameter), spherical enclosures consisting of one or more phospholipid bilayers surrounding an aqueous center [1,2]. These vesicles are useful models for studying membranes of cells, as vesicle size is comparable to cellular dimensions and allows for examination of phospholipid bilayers under a light microscope [1]. Applications of giant liposomes include: (1) studying osmotic stress on membranes [3]; (2) examining membrane curvature and elasticity under varying conditions [4,5]; (3) forming "nano-fluidic" networks [6,7]; (4) studying interactions and distances between lipid membranes and surfaces [8]; (5) recording activity from reconstituted ion channels [9–11]; (6) making micro-scale bioreactors [12,13]; (7) delivering enclosed drugs [14]. In our group, we are using liposomes to form planar lipid bilayers on microfabricated pores [15], and this procedure requires the formation of a high concentration of liposomes that are as large as possible (ideally >20 μm) [16,17]. Also, Yamashita et al. crystallized proteins within giant liposomes, and the formation of many, large liposomes is desirable for this procedure [12].

Electroformation is a very useful technique for generating giant liposomes. Originally developed by Angelova and Dimitrov, this method employs ac fields to form liposomes from a film of lipids deposited onto the surface of an electrode (platinum wire or indium tin oxide (ITO)) [18,19]. Electroformation succeeds in the formation of giant liposomes that are predominantly unilamellar [3,19,20].

Several parameters affect the process of electroformation including the peak-to-peak voltage and frequency of the applied ac field and the duration of electroformation. Another important factor is the thickness of the film of lipids [18,19]. Estimates of the appropriate thickness range from 5 to 10 lipid bilayers (approximately 30–60 nm) [18,19], 30–50 lipid bilayers [1,21], or 10–50 bilayers (approximately 8–45 nm) [22,23].

These estimates are, however, not based on direct measurements of the actual thickness of the lipid film. Traditionally, liposomes form on electrodes of ITO (or platinum) from films of lipids created by depositing a droplet (2.5 μL) of

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a solution of lipids onto the surface of the electrode [3,19]. The estimated thickness of the film results from a calculation that takes into account the amount of lipid deposited and the area of the dried droplet. This calculation assumes that the film of lipids from the deposition of a droplet is homogenous [19,22]. The lipid films from dried droplets, however, span a wide range of thicknesses across the area of the lipid spot [19]. This range has the advantage that it usually results in successful formation of giant liposomes, as some regions of the film have suitable thicknesses [18]. Other regions of the film, however, are too thick or too thin and thus exhibit either no growth of liposomes, the formation of vesicles with diameters <10 μm, or aggregates of lipids that do not form liposomes.

While electroformation is a very successful technique for forming giant liposomes, certain lipids do not readily form liposomes through electroformation. Electroformation from films of pure phosphatidylserine or pure phosphatidylglycerol is difficult or impossible [22], and we found no reports of electroformation of giant liposomes from asolectin lipids, a natural lipid mixture widely used in lipid bilayer experiments [24].

Ideally, electroformation would occur from uniform films of lipids of optimal thickness that span the entire surface of ITO. In this case, liposome growth would occur not from localized spots of lipids but rather across the entire surface of formation, thus increasing the number of resulting giant vesicles. Also, a uniform film would allow for the direct measurement of thicknesses of lipid films that result in successful formation of giant liposomes.

Here we employ spin-coating, a technique commonly used in photolithography [25–27], to produce thin, macroscopically homogenous films of lipids on a surface of ITO. Previous studies have employed spin-coated films of lipids for the preparation of supported lipid bilayers [28–30]. Here we measure the thicknesses of spin-coated films of lipids to determine the optimal range for electroformation, and we employ these films to form liposomes from lipids and lipid mixtures that do not readily form giant liposomes from droplet-derived spots, such as asolectin, 100% 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS), and 100% 1-palmitoyl-2-oleoyl-*sn*-glycero-[phospho-*rac*-(1-glycerol)] (POPG). We compare growth of liposomes from spin-coated films to the growth of liposomes from the droplet-deposition technique, discuss parameters affecting the thickness of spin-coated films of lipids, and provide a method for the formation of films of lipids of desired thickness.

2. Experimental

2.1. Spin-coating of solutions of lipids

We used a Speedline Technologies (Franklin, MA) Model P-6000 to spin-coat solutions of lipids at speeds between 50 and 1200 rpm (Fig. 1). Solutions consisted of either asolectin lipids (Fluka, Switzerland), mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-[phospho-*rac*-(1-glycerol)] (POPG), pure POPG, pure L-α-phosphatidylcholine (egg, chicken) (eggPC), and pure 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) (all from Avanti Polar Lipids, Alabaster, AL) dissolved in 100% chloroform (Acros, NJ), 95% chloroform/5% acetonitrile (HLPC grade, Fisher Scientific), 95% chloroform/5% methanol, or 90% chloroform/10% methanol (Burdick & Jackson, Muskegon, MI). We prepared all solutions freshly every day, and during the day we stored lipid solutions at –20 °C. After the formation of the films of lipids, we dried the films under vacuum (−740 Torr) for 1–2 h to remove traces of solvent.

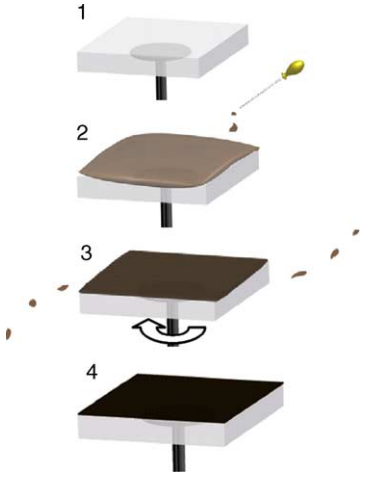

Fig. 1. Spin-coating procedure for generating thin films of lipids. (1) The substrate was placed onto the rotary platform of the spin-coater and was held tightly by a vacuum, (2) the entire substrate was covered with lipid solution, (3) spinning (50–1200 rpm) was initiated ∼2 s after lipid deposition and (4) after 4–10 min, spinning was stopped, leaving a homogenous lipid film on the substrate surface.

We deposited the solutions of lipids (typically 0.6–0.8 mL) onto four types of substrate: 25 mm × 25 mm × 1.1 mm glass with a coating of conductive ITO; 50 mm × 50 mm × 1.1 mm glass with a coating of conductive ITO (both from Delta Technologies, Stillwater, MN); silicon wafers (Silicon Sense, Nashua, NH) with a 3 nm layer of SiO_2, cut into 25 mm × 25 mm squares; silicon wafers with a 120 nm coating of ITO on top of a 3 nm layer of SiO_2, cut into 25 mm × 25 mm squares [31]. We cleaned all substrates prior to deposition of solution by rinsing the surface with methanol and drying under a stream of argon (repeat twice). We cleaned the glass substrates with surfaces of ITO with the following protocol: 1× H_2O rinse; 2× ethanol (95%, VWR International) rinse and dried under a stream of argon; sonication for 10 min with glass slides in a solution of 50% methanol/50% chloroform; 2× methanol rinse and dried after each rinse under a stream of argon.

As an alternative method to spin-coating, we tested dip-coating to form films of lipids. We submerged a

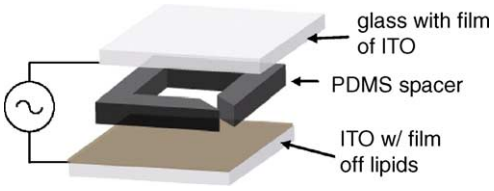

Fig. 2. Electroformation set-up. Two surfaces of ITO (one or both with a film of lipids) were separated by a PDMS spacer (∼1.6 mm thick) forming a chamber for electroformation. Two binder-clips (Officemate International Corp., Edison, NJ) held together the two plates and the spacer. The chamber was filled with water, and an ac field (typically 1.7 V peak-to-peak) was applied to the surfaces of ITO to initiate electroformation.

50 mm × 50 mm glass slide with a coating of ITO in a solution of lipids (3.75 mg mL^{-1} in 95% chloroform/5% acetonitrile) for ∼5 s, lifted the glass plate out of the solvent, and immediately held the plate vertically to allow for excess solution to drop off of the slide. The plate remained in this vertical position for approximately 2 min to allow for the evaporation of solvent and formation of a lipid film across the surface of ITO.

2.2. Electroformation of liposomes

We formed liposomes from films of lipids using a configuration similar to that first described by Angelova et al. (Fig. 2) [19]. A rectangular frame created from poly(dimethylsiloxane) (PDMS) [32] (Sylgard 184 Silicone, Dow Corning, Midland, MI) served as a spacer of thickness ∼1.6 mm between two opposing glass substrates each coated with ITO (with one or both ITO surfaces having a film of lipids and with both ITO surfaces facing each other). We filled the chamber with de-ionized water (18.2 MΩ cm) through a hole in the PDMS spacer and immediately applied a 1.7 V (peak-to-peak), 10 Hz ac field to the ITO electrodes using a function generator (National Instruments PCI-6711 High-Speed Analog Output Board using LabView). Liposomes formed quickly (10 min), but we allowed electroformation to continue for at least 1.5 h until liposomes grew to large sizes over most of the surface of ITO.

We observed liposomes during electroformation using a Nikon Eclipse TE 2000-U inverted microscope using a 10× and 20× objective with extra-long working distance in phase-contrast mode. We captured images of liposomes using a Photometrics CoolSnap HQ camera from Roper Scientific (Trenton, NJ), and image analysis software (Metamorph from Universal Imaging Corporation, Downington, PA) allowed for determination of the diameter of liposomes.

2.3. Characterization of films of lipids

In addition to ellipsometry and atomic force microscopy (AFM) studies (see below), we used visual inspection of films of lipid to assess the homogeneity of the film across the entire surface of the substrate. While films of lipids on glass substrates with coatings of ITO appeared transparent, tilting of the glass substrate in white light revealed distinct reflected colors from the films of lipids. We used a Nikon SMZ 1500 stereomicroscope fitted with a color camera (Evolution MP Color from Media Cybernetics, Silver Spring, MD) to capture color images of lipid films [33].

We measured thicknesses of films of lipids using a model VASE ellipsometer (J.A. Woollam Co. Inc., Lincoln, NE). For each film we took single-scan ellipsometry measurements from five different regions of the film (corresponding to middle, top, left, right, and bottom of the substrate) to obtain a thickness representative of the entire film. These five measurements provided an average and standard deviation for the thickness of each film. For silicon wafers coated with ITO, we performed ellipsometry measurements both before and after formation of the film of lipids because the thickness of the layer of ITO varied significantly between each 25 mm × 25 mm section cut from the ITO-coated wafer. We used standard propagation of error to compute final average and standard deviation values for film thickness.

To examine the topography of films of lipids, we used a NanoScope IIIa atomic force microscope (AFM) from Digital Instruments (Woodbury, NY). Scanning the maximum region accessible by the device (13.5 μm × 13.5 μm), AFM provided images of spin-coated films and droplet-derived spots of lipids (using a soft tip (UltraSharp Non-Contact Cantilever, MikroMasch, Madrid, Spain) with resonance frequency of 371.014 kHz in tapping-mode). Image analysis software flattened the topographic images to produce representations of lipid contours of a spin-coated film of lipids.

3. Results and discussion

3.1. Formation of lipid films with homogenous surface coverage

We used spin-coating to deposit thin films of lipids onto glass slides and silicon wafers covered with a film of ITO and onto silicon wafers with a layer of 3 nm SiO_2. To obtain lipid films with homogenous coverage, we spread the lipid solution over the entire substrate surface and initiated spinning ∼2 s after deposition [34]. During the spin-coating procedure, excess lipid solution ejected from the surface, and the solvent evaporated, leaving behind a thin film of lipids.

Solutions of lipids in 95% chloroform/5% acetonitrile produced the most uniform films by spin-coating. Initially, we attempted to spin-coat lipids in solutions of 90% chloroform/10% methanol, which represents the solution most commonly used in droplet-deposition experiments [19]; however, these solutions did not effectively wet the ITO surfaces, leaving behind non-uniform, thin films with thicknesses ≪20 nm after spin-coating. Changing the solution to 95% chloroform/5% methanol did improve film formation during spin-coating, but visible regions of non-homogeneity (especially near the center of the plate) comprised ∼40% of the film. We replaced methanol with acetonitrile, assuming that the more

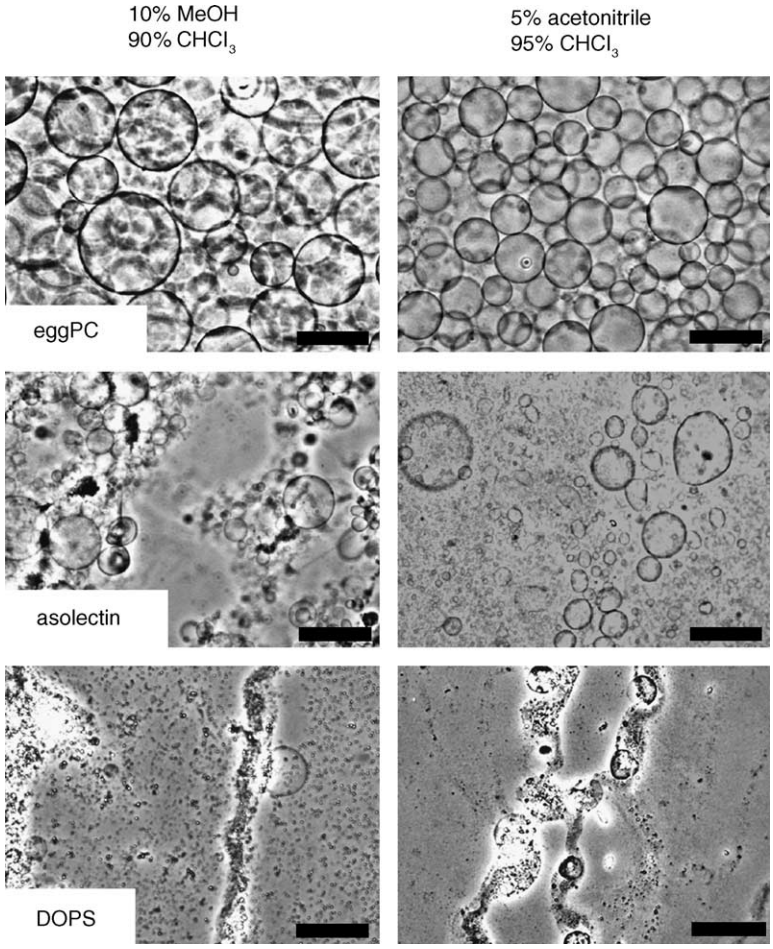

Fig. 3. Comparison of liposomes formed using the traditional droplet-deposition technique with different solvents. Films of lipids were formed by depositing 2.5 μL droplets of 1.0 mg mL^{-1} lipids (eggPC, asolectin, or DOPS) dissolved in 10% MeOH/90% chloroform or 5% acetonitrile/95% chloroform onto ITO. Micrographs in the same row represent the same composition of lipids, and micrographs in the same column indicate the same solvent. Scale bars = 50 μm.

polar acetonitrile [35] would wet the hydrophilic surfaces of ITO or SiO_2 more effectively. We obtained the following results with spin-coating of lipids in different ratios of chloroform/acetonitrile: 100% chloroform left behind non-uniform, thin films; 95% chloroform/5% acetonitrile provided an effective balance between dissolving the lipids and wetting the substrate and allowed for the formation of thin, uniform films of lipids with controllable thickness; 90% chloroform/10% acetonitrile resulted in films with apparent non-uniformities. Throughout this work we used lipid solutions in 95% chloroform/5% acetonitrile for spin-coating.

To test whether 95% chloroform/5% acetonitrile as a solvent would also yield better results using the traditional droplet-deposition technique, we formed liposomes from films of various lipids and compared the results to films made from the same lipids in 90% chloroform/10% methanol (Fig. 3). Using 3 μL droplets of 1.0 mg mL^{-1} lipids (eggPC, asolectin, or DOPS), we found that fewer aggregates of lipid formed from films deposited from 5% acetonitrile, especially for liposomes of asolectin. Liposomes from eggPC deposited from 5% acetonitrile formed in less abundance than liposomes from eggPC deposited from 10% methanol; in the latter case, liposomes stacked on top of each other to form vertical "layers". Few liposomes formed from films from either solvent using DOPS. While the use of acetonitrile appeared to improve homogeneity of deposited droplets (fewer aggregates of lipids), it did not noticeably improve the formation of liposomes formed by using the droplet-deposition technique.

3.2. Macroscopic uniformity of lipid films

We examined the uniformity of films on ITO surfaces by inspecting the color of reflected light from the sample (Fig. 4). Thin films reflect different wavelengths of light depending on the thickness of the film [36]. Films formed at low spin-speeds (<200 rpm) varied in color and showed rings (Fig. 4D) because the spin-speed was too slow to eject excess solution. Films formed from high spin-speeds (>1000 rpm) showed some regions of uniform color but also contained areas with no apparent films of lipids. In contrast, films from intermediate spin-speeds (300–800 rpm) contained large regions (∼90% of the entire surface) of the same color (Fig. 4B), indicating that spin-coating can create areas of homogenous thickness. The color of these uniform films ranged between

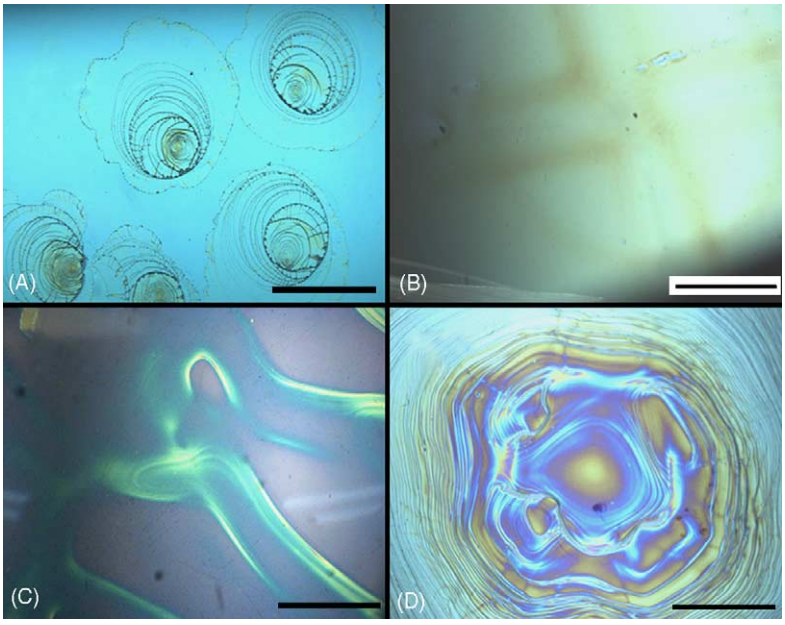

Fig. 4. Comparison of the homogeneity of lipid films formed by different methods on ITO. The optical micrographs of reflected light from films of lipids show: (A) non-homogenous film formed by the evaporation of five droplets (volume: 2.5 μL) of 0.5 mg mL^{-1} asolectin lipids in 90% chloroform/10% methanol; (B) homogenous film (center of the ITO plate) formed from 2.5 mg mL^{-1} asolectin lipids in 95% chloroform/5% acetonitrile spin-coated at 300 rpm; (C) non-homogenous film formed by dip-coating an ITO plate (50 mm × 50 mm) into 3.75 mg mL^{-1} asolectin lipids in 95% chloroform/5% acetonitrile; (D) non-homogenous film from a spin-speed (100 rpm) that was too slow to eject excess lipid solution (2.5 mg mL^{-1}). Scale bars = 4.0 mm.

light green, green-gold, gold, blue, and purple depending on the thickness of the films of lipids.

The traditional method of deposition of droplets of lipid onto ITO plates, by contrast, formed isolated regions of varying thickness of the lipid film (Fig. 4A). After evaporation of the solvent, the drops left behind concentric rings of lipids (so-called coffee-ring effect [37]). Several groups have used techniques (e.g. using a glass rod) to spread lipid solution over the surface of ITO [38,39], to improve homogeneity and increase the area of the lipid film; however, the thickness of the resulting film cannot be controlled with this approach.

We also compared spin-coated films to dip-coated films of lipids (Fig. 4C) and found that dip-coating produced less homogenous films than spin-coating. Although dip-coating did result in the formation of lipid films across the entire surface of ITO, solvent evaporated at different rates while the solution of lipids dripped from the vertically held slide; consequently, streaks of lipid film of differing thicknesses formed on the dip-coated ITO surface (Fig. 4C).

3.3. Average thickness of spin-coated films of lipids

We used ellipsometry to measure the thickness of films of asolectin lipids on ITO surfaces covering silicon wafers. Fig. 5 shows spin-curves for concentrations of 5.0, 2.5, and 1.0 mg mL^{-1} asolectin in 95% chloroform/5% acetonitrile. First-order exponential decay functions ($y = y_0 + Ae^{-(x/\lambda)}$) fitted all curves for spin speeds >200 rpm. We also examined the thickness of asolectin films as a function of initial lipid concentration when spun at a constant speed of 600 rpm (Fig. 5 inset). The thickness of lipid films was proportional to the initial concentration of lipids. Using the exponential spin-curves and the dependence of the film thickness on the lipid

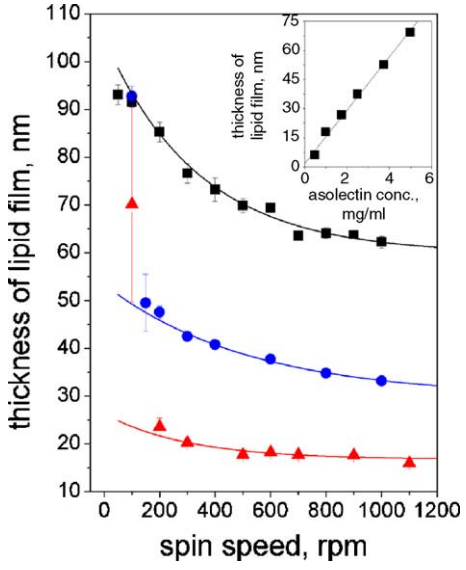

Fig. 5. Thickness of lipid films as a function of spin-speed at three different concentrations of asolectin lipids: (▲) 1.0 mg mL^{-1}; (●) 2.5 mg mL^{-1}; (■) 5.0 mg mL^{-1}. The error bars reflect the standard deviation of measured thicknesses from five different regions of the films of lipids. The solid lines represent best curve fits using first-order exponential decay functions. Inset: thickness of films as a function of concentration of asolectin lipids, spin-coated at a constant speed of 600 rpm. The data were fitted linearly: film thickness (nm) = 13.8 (nm (mg/mL)$^{-1}$) × concentration of asolectin (mg/mL) ($N = 6$, $R^2 = 0.99$).

Table 1
Success of liposome formation from various lipids as a function of the thickness of lipid films

Lipid	Concentration (mg mL^{-1})	Thickness of film (nm)	Quality of liposomes[a]
Asolectin	1.0	17 ± 2	–
Asolectin	2.5	30 ± 3	+
Asolectin	3.75	49 ± 3	++
Asolectin	5.0	63 ± 1	0
DOPS	3.75	44 ± 5	0/+
EggPC	3.75	37 ± 4	++
POPG	3.75	44 ± 6	+
90% POPC/10% POPG	2.5	21 ± 5	0
90% POPC/10% POPG	3.0	26 ± 5	++
90% POPC/10% POPG	3.75	32 ± 4	++

All lipid films were obtained by spin-coating solutions of lipids at 600 rpm.
[a] We define quality of liposomes as: (-) little or no liposome growth; (0) some regions of liposome growth (e.g. Fig. 7D); (+) large regions of liposome growth; (++) growth of liposomes with a majority of diameters ≥30 μm across most of the surface (e.g. Fig. 7A).

concentration, lipid films of desired thickness could be obtained reproducibly (Table 1).

3.4. Thickness of films as a function of composition of lipid mixture

We prepared films from solutions of different lipids to investigate the effect of the lipid composition on the thickness of the films. Different solutions resulted in films with thicknesses from 32 to 49 nm from the same initial concentration of solution of lipids (3.75 mg mL^{-1}) and spin-speed (600 rpm) (Table 1). To investigate the effect of varying headgroup and charge of the lipids on the thickness of spin-coated lipid films, we varied the ratio of POPC and POPG in the mixture of lipids and measured the resulting thicknesses of films of lipids. Films formed from lipids containing 99% POPG/1% POPC were ∼30% thicker than films containing 80% POPC/20% POPG. Therefore, the thickness of films depends on the lipid composition; achieving a desired film thickness requires optimization of the lipid concentration for different lipid mixtures. We found, however, that spin-coating solutions of lipids with concentrations of 3.75 mg mL^{-1} at 600 rpm generally produced homogenous films that were effective for electroformation (Table 1). Also, once optimized for a given lipid mixture, spin-coating provides a way of obtaining desired thicknesses of lipid films with a precision of a few nanometers (Fig. 5 inset).

3.5. Microscopic uniformity of lipid films

We performed AFM analysis of lipid films spin-coated onto surfaces of SiO_2 to characterize the microscopic uniformity of the films. AFM images (Fig. 6) show that macroscopically homogenous spin-coated films (e.g. Fig. 4B and C) consist of closely spaced aggregates of lipids at the micro-scale. These aggregates generally have the same height (Fig. 6B), thus appearing macroscopically as a film of

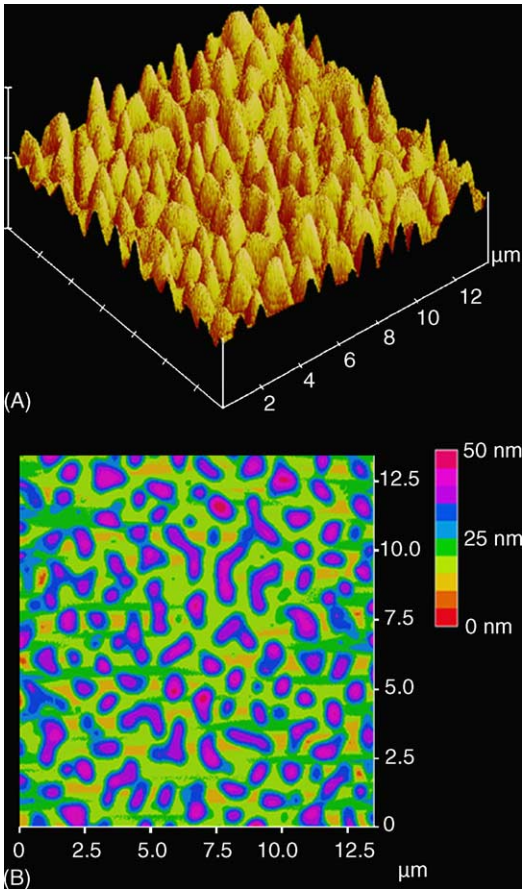

Fig. 6. Atomic force microscopy (AFM) images of spin-coated lipid films. These films were formed from a solution of 2.5 mg mL^{-1} asolectin spin-coated at 600 rpm. Image (A) depicts three-dimensional microscopic contours of the film of lipids across the entire region scanned (13.5 μm × 13.5 μm). In (B), the contours of the lipid aggregates are shown by color contrast indicating the uniform height of the highest and lowest regions of the film.

uniform thickness. AFM analysis performed on asolectin films formed on three different substrates created under the same conditions yielded almost identical topographic images. These results are in agreement with findings by Muller-Buschbaum et al. [40], who reported significant roughness of spin-coated films of polystyrene using chloroform as a solvent; solvents of high vapor pressure (volatility) did not allow for sufficient diffusion to form microscopically uniform films [40]. Presumably, the same mechanism applies to spin-coated films of lipids from 95% chloroform/5% acetonitrile.

We also performed AFM on films created from droplet-derived lipid films on SiO_2 (e.g. Fig. 4A). The maximum region observable by AFM was a 13.5 μm × 13.5 μm area. This limited region could not depict the non-uniform sections in the droplet area, namely the concentric rings extending from the droplet center, since the rings themselves were 30–100 μm wide. Instead, AFM images showed smooth lipid contours over the scanned region that appeared more homogenous on the micro-scale than the lipid aggregates

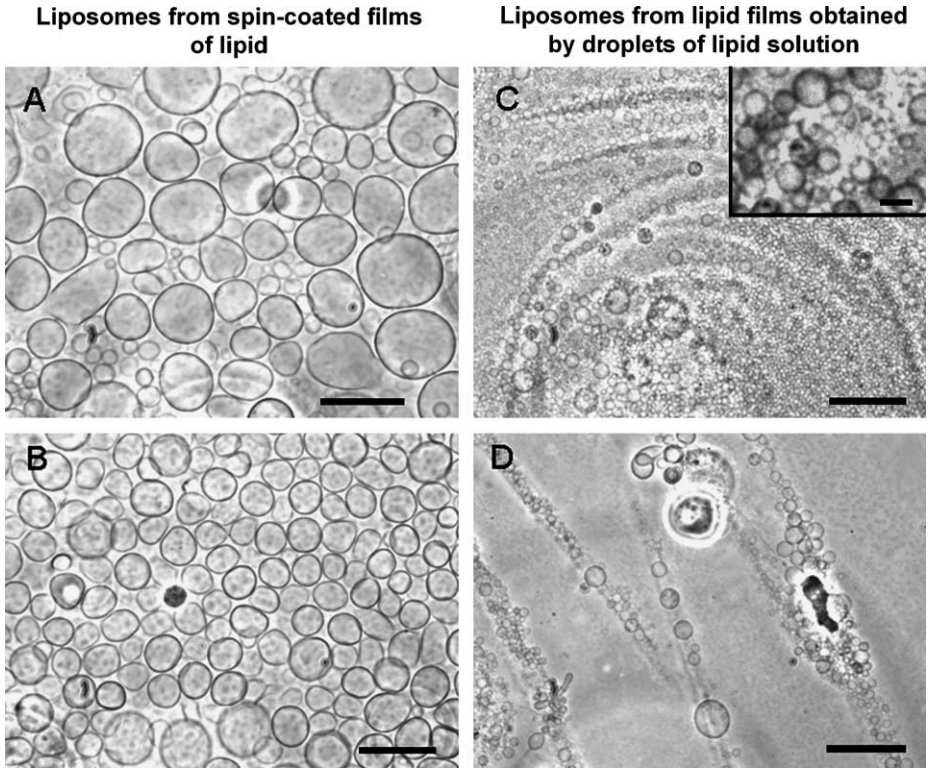

Fig. 7. Phase-contrast images of liposomes formed on ITO surfaces. The column on the left (A, B) shows liposomes grown from two different regions of a spin-coated film of asolectin. The column on the right (C, D) shows liposomes grown from two different regions of a spot of asolectin formed by evaporation of solvent from a droplet of lipid solution. Inset in (C) shows an expanded image of a region in the lower left part of C (scale bar of inset = 10 μm). The experimental conditions and duration (1.5 h) of liposome growth were identical for spin-coated and droplet-deposited films. Note that spin-coated films of lipids result in the formation of liposomes with average diameters 2–5 times larger than liposomes formed from droplet-derived films. Asolectin was chosen here to illustrate the benefit of electroformation from spin-coated vs. droplet-derived film for certain lipid mixtures. Liposomes from eggPC, on the other hand, grow very well from droplet-derived films (see Fig. 3). Scale bars = 75 μm.

of spin-coated films. Scanning three different films from droplets, however, showed strongly varying thicknesses (75, 45 and 20 nm) of the lipid films depending on whether the scanned area was close to the center or to the edge of the dried droplet of lipid. These differences in thickness confirm the macroscopically visible variability in thicknesses of films created from evaporation of droplets of lipid solution.

3.6. Formation of giant liposomes from spin-coated films of asolectin lipids

Using electroformation, we compared growth of liposomes from a spin-coated film of asolectin lipids to growth from spots of asolectin lipids formed by droplets (Fig. 7). We spin-coated a solution of 3.75 mg mL^{-1} asolectin onto a glass slide with a layer of ITO at 600 rpm. The resulting thickness of the lipid film was 53 ± 3 nm. On another ITO surface we deposited five separate droplets (2.5 μL) of 0.5 mg mL^{-1} asolectin in 90% chloroform/10% methanol. We used these two ITO plates as the top and bottom electrodes of the electroformation set-up (Fig. 2) to compare both methods of liposome formation under the same experimental conditions.

Liposomes from the spin-coated lipid film formed over the entire surface of the ITO electrode and grew to larger sizes than liposomes grown from the droplet-derived film. We measured the average diameters of liposomes shown in Fig. 7; liposomes grown from spin-coated films of asolectin exhibited average diameters of 34.5 ± 20.0 μm (Fig. 7A, $N = 88$) and 31.1 ± 6.5 μm (Fig. 7B, $N = 146$), while liposomes grown from droplet-derived films had average diameters of 5.7 ± 3.1 μm (Fig. 7C, $N = 448$, examining only the lower left quadrant) and 6.6 ± 3.7 μm (Fig. 7D, $N = 477$). In general (except for eggPC), liposomes from the spin-coated lipid films exhibited average diameters that were a factor of 2–5 larger than liposomes from droplet-derived films and grew from the entire surface of the ITO electrode.

One disadvantage of the spin-coating procedure is that it requires more lipids than the droplet-deposition technique. For a substrate with an area of 25 cm^2, ∼3 mg of lipids were used for spin-coating. In comparison, the droplet-deposition technique requires only ∼2 μg of lipids per droplet. To cover an area of 25 cm^2, ∼100 droplets would be needed (see Fig. 4A). Therefore, spin-coating requires approximately 15 times more lipids per area of substrate than the droplet-deposition technique.

3.7. Optimal thickness of lipid films for electroformation of liposomes

To determine optimum conditions for electroformation, we tested liposome growth from different thicknesses of lipid films and from different mixtures of lipids (Table 1). Lipid films with thicknesses of 25–50 nm produced large (>30 μm) liposomes over ∼90% of the surface for both asolectin and 90% POPC/10% POPG and were optimal for liposome growth. This range of thicknesses corresponds to 30–60 layers of lipid [41,42]. Thicknesses of lipid regions in droplet-derived spots of lipids ranged from 0 to 70 nm (from AFM); some areas of these spots presumably exhibited thicknesses of 25–50 nm, and these regions seemed to grow liposomes of size comparable to liposomes from spin-coated films. Other regions of the droplet-derived spots of lipid, however, exhibited film thicknesses outside the optimal range, and electroformation resulted in small liposomes (<10 μm, see Fig. 7C), sporadic lipid aggregates, or regions of no growth. We observed similar artifacts when growing liposomes from films of lipids generated by dip-coating, due to variations in thickness across these films.

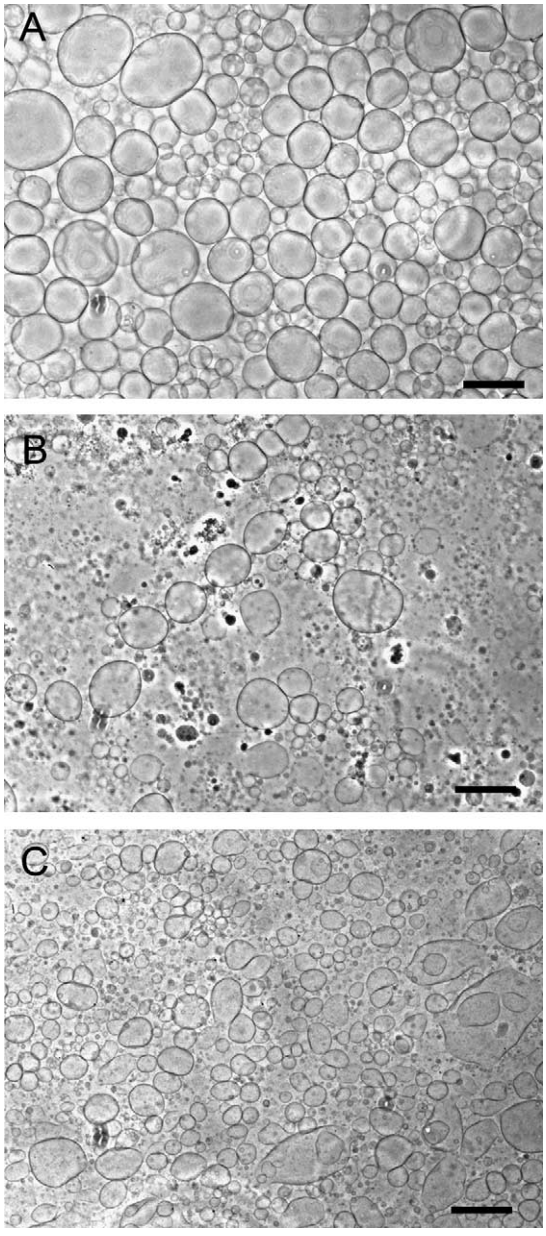

Fig. 8. Phase-contrast images of giant liposomes formed from the following lipids: (A) 100% eggPC; (B) 100% DOPS; (C) 100% POPG. Scale bars = 100 μm. Lipid solutions were spin-coated at 600 rpm from initial concentrations of 3.75 mg mL^{-1}.

3.8. Growth of giant liposomes from uniform films of optimal thickness

Using optimal thicknesses of lipid films, we formed liposomes from several different mixtures of lipids (Table 1). Spin-coated films of eggPC produced exceptionally large liposomes (average diameter 49.2 ± 24.1 μm, $N = 218$) across the entire surface of ITO (Fig. 8A). Most notably, however, we formed giant liposomes from two solutions with 100% negatively charged lipids: phosphatidylserine (we used DOPS) and POPG. Spin-coated films of pure DOPS produced large liposomes (50–100 μm) (Fig. 8B) but also resulted in regions of no liposome growth and in smaller liposomes that appeared to be multilamellar. Spin-coated films of pure POPG resulted in giant liposomes over most of the surface of formation, with sizes ranging from 10 to 100 μm (Fig. 8C). Previous reports indicate difficulties in forming liposomes using electroformation from these two lipids. The successful formation of liposomes from spin-coated films of pure DOPS and POPG demonstrates the effectiveness of growing liposomes from a macroscopically uniform lipid film of optimal thickness.

4. Conclusions

Spin-coating solutions of lipids generates uniform lipid films over large areas. Optimizing the concentration of the lipid solution and the spin-speed makes it possible to control the thickness of films with a precision of a few nanometers. Spin-coating affords a reproducible procedure to obtain films with a thickness of 25–50 nm, which is optimal for electroformation. These films make it possible to generate liposomes that are on average 2–5 times larger than liposomes formed with standard techniques and allow for the formation of liposomes from lipids that do not readily form giant liposomes with standard techniques.

There are several drawbacks of spin-coating.

(1) This technique requires access to a spin-coater.
(2) Compared to the amount of lipid used for the droplet-deposition technique, spin-coating requires an increased amount of lipid solution to cover the surface of ITO.
(3) Different mixtures of lipids generate different spin-curves; thus, obtaining precise thicknesses of films

requires optimizing the conditions of spin-coating for a given lipid mixture.

Despite these drawbacks, spin-coating offers an effective technique for producing homogenous films of lipids that form many, exceptionally large liposomes through electroformation from a variety of lipid mixtures including pure phosphatidylserine and phosphatidylglycerol.

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References

[1] F.M. Menger, M.I. Angelova, Acc. Chem. Res. 31 (1998) 789.
[2] F.M. Menger, J.S. Keiper, Curr. Opin. Chem. Biol. 2 (1998) 726.
[3] L. Mathivet, S. Cribier, P.F. Devaux, Biophys. J. 70 (1996) 1112.
[4] E. Farge, P.F. Devaux, Biophys. J. 61 (1992) 347.
[5] W. Rawicz, K.C. Olbrich, T. McIntosh, D. Needham, E. Evans, Biophys. J. 79 (2000) 328.
[6] A. Karlsson, M. Karlsson, R. Karlsson, K. Sott, A. Lundqvist, M. Tokarz, O. Orwar, Anal. Chem. 75 (2003) 2529.
[7] M. Davidson, M. Karlsson, J. Sinclair, K. Sott, O. Orwar, J. Am. Chem. Soc. 125 (2003) 374.
[8] P. Fromherz, V. Kiessling, K. Kottig, G. Zeck, Appl. Phys. A 69 (1999) 571.
[9] E. Lopez-Alonso, J. Canaves, M. Arribas, A. Casanova, J. Marsal, J.M. Gonzalez-Ros, C. Solsona, Neurosci. Lett. 196 (1995) 37.
[10] G. Riquelme, E. Lopez, L.M. Garcia-Segura, J.A. Ferragut, J.M. Gonzalez-Ros, Biochemistry 29 (1990) 11215.
[11] B.U. Keller, R. Hedrich, W.L. Vaz, M. Criado, Pflugers Arch. 411 (1988) 94.
[12] Y. Yamashita, M. Oka, T. Tanaka, M. Yamazaki, Biochim. Biophys. Acta 1561 (2002) 129.
[13] D.T. Chiu, C.F. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B.P. Modi, A. Moscho, R.A. Garza-Lopez, O. Orwar, R.N. Zare, Science 283 (1999) 1892.
[14] F. Nacka, M. Cansell, P. Meleard, N. Combe, Lipids 36 (2001) 1313.
[15] M. Mayer, J.K. Kriebel, M.T. Tosteson, G.M. Whitesides, Biophys. J. 85 (2003) 2684.
[16] C. Schmidt, M. Mayer, H. Vogel, Angew. Chem. Int. Ed. 39 (2000) 3137.
[17] M. Mayer, S. Terrettaz, L. Giovangrandi, H. Vogel, Functional analysis of ion channels: planar patch clamp and impedance spectroscopy of tethered lipid membranes, in: J.M. Cooper, A.E.G. Cass (Eds.), Biosensors: A Practical Approach, 2nd ed., Oxford University Press, Oxford, 2003, p. 153.
[18] M.I. Angelova, D.S. Dimitrov, Faraday Discuss. (1986) 303.
[19] M. Angelova, S. Soleau, P. Meleard, J.F. Faucon, P. Bothorel, Prog. Colloid Polym. Sci. 89 (1992) 127.
[20] L.A. Bagatolli, T. Parasassi, E. Gratton, Chem. Phys. Lipids 105 (2000) 135.
[21] The technique for estimating the number of layers of lipids was not specified; therefore, we could not determine the thickness of the film of lipids.
[22] P. Bucher, A. Fischer, P.L. Luisi, T. Oberholzer, P. Walde, Langmuir 14 (1998) 2712.
[23] Previous studies used differing models for computing the number of layers of lipids from the calculated thickness of film, thus resulting in differing ranges for appropriate number of layers of lipids.
[24] W.R. Schlue, W. Hanke, Planar Lipid Bilayers, Academic Press, London, 1993.
[25] D.B. Mitzi, L.L. Kosbar, C.E. Murray, M. Copel, A. Afzali, Nature 428 (2004) 299.
[26] F.S. Ligler, B.M. Lingerfelt, R.P. Price, P.E. Schoen, Langmuir 17 (2001) 5082.
[27] E.M.A. Pereira, D.F.S. Petri, A.M. Carmona-Ribeiro, J. Phys. Chem. B 106 (2002) 8762.
[28] U. Mennicke, T. Salditt, Langmuir 18 (2002) 8172.
[29] H. Amenitsch, M. Rappolt, C.V. Teixeira, M. Majerowicz, P. Laggner, Langmuir 20 (2004) 4621.
[30] A. Terheiden, C. Mayer, K. Moh, B. Stahlmecke, S. Stappert, M. Acet, B. Rellinghaus, Appl. Phys. Lett. 84 (2004) 3891.
[31] Tim Lin provided us with silicon wafers coated with ITO and performed sputtering of ITO using the following parameters: base pressure = 4.1×10^{-6} Torr; gas = argon at 7 mTorr; RF power = 400 W; deposition thickness = ~120 nm; annealing temperature = 540 °C for 10 min.
[32] Y.N. Xia, G.M. Whitesides, Angew. Chem. Int. Ed. 37 (1998) 551.
[33] To create a proper angle of incident light for reflection of light from the film of lipid, we placed one edge of the glass substrate onto a stack of wedges of PDMS (~8 mm high) on a glass slide and rotated the slide until the stereomicroscope captured light from the ceiling lamps reflecting off of the film.
[34] This incubation period of ~2 s improved the wetting of the substrates with the 95% chloroform/5% acetonitrile solution that contained the lipids.
[35] J.N. Lee, C. Park, G.M. Whitesides, Anal. Chem. 75 (2003) 6544.
[36] J. Mathe, J.M. di Meglio, B. Tinland, Langmuir 17 (2001) 6736.
[37] R.D. Deegan, O. Bakajin, T.F. Dupont, G. Huber, S.R. Nagel, T.A. Witten, Nature 389 (1997) 827.
[38] P. Ratanabanangkoon, M. Gropper, R. Merkel, E. Sackmann, A.P. Gast, Langmuir 18 (2002) 4270.
[39] A.L. Bernard, M.A. Guedeau-Boudeville, L. Jullien, J.M. di Meglio, Langmuir 16 (2000) 6809.
[40] P. Muller-Buschbaum, J.S. Gutmann, M. Wolkenhauer, J. Kraus, M. Stamm, D. Smilgies, W. Petry, Macromolecules 34 (2001) 1369.
[41] We estimated the number of layers of lipid using a model in which the films of lipids consisted of rod-shaped lipid molecules lying flat on the surface of the substrate. Layers of these rod-shaped lipid molecules stacked on top of each other, and the height of each layer equalled the height of the lipid headgroup. We used 60 $Å^2$ as the cross-sectional area of each headgroup (diameter of headgroup = 8.74 Å) and found the resulting number of layers of lipid by dividing the thickness of the film by the diameter of the headgroup.
[42] R.B. Gennis, Biomembranes: Molecular Structure and Function, Springer-Verlag, New York, 1989.