A method for an efficient formation of giant vesicles of neutral phospholipids in microarrays using a microplotter equipment

Juan R Avendaño-Gomez1, Obed A Solis-Gonzalez1,*, Haydee González-Martínez2 and Miguel A Vega-Cuellar3

1 Sección de Estudios de Posgrado e Investigación, Escuela Superior de Ingeniería Química e Industrias Extractivas, Instituto Politécnico Nacional, Av. Instituto Politécnico Nacional S/N, 07738, San Pedro Zacatenco, Cuidad de Mexico, México
2 Centro de Nanociencias y Micro y Nanotecnologías, Instituto Politécnico Nacional, Av. Luis Enrique Erro, 07738, Ciudad de México, México
3 Escuela Nacional de Ciencias Biológicas, Programa de Doctorado en Nanociencias y Micro y Nanotecnologías, Instituto Politécnico Nacional, Av. Luis Enrique Erro, 07738, Ciudad de México, México

* Author to whom any correspondence should be addressed.
E-mail: asolis30@hotmail.com

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Abstract
A methodology which provides a high efficiency of giant vesicle formation was established using the gentle hydration method and a microplotter equipment. The method consists of preparing a mixture of zwitterionic egg yolk phosphatidylcholine/additive in solution and printing a number of droplets onto a glass substrate, which immediately dry after deposition. Then, gentle hydration of these micro-sized thin-films provides a high amount of giant liposomes, per microsized film. Several cases were studied by varying different compounds as additives (i.e., non-electrolytes and electrolytes) at different molar ratios, lipid to additive, in order to find the optimal conditions. Optical and confocal microscopies were employed to characterize vesicle formation. Studies indicate that the kosmotropic salt KH₂PO₄ at 1:10 molar concentration, EggPC to salt, is the most effective in vesicle production. Abundant liposome formation can be observed in a short time, about 5 min upon hydration. The osmotic pressure is the driven force to produce giant liposomes in our experiments, which is generated by dissolving the additive among two lipid lamellar phases in water. In salt experiments, the osmotic pressure strength is mainly determined from the ion-specificity effect (i.e., the Hofmeister effect) rather than the concentration of the salt. The use of a salt as additive provides giant unilamellar vesicles (GUVs). The microplotter protocol provides benefits such as a facile, efficient and rapid way to prepare GUVs in mild conditions (i.e., free of solvents).

1. Introduction
Phospholipids are amphiphilic molecules which possess hydrophobic tails and a hydrophilic head group. These molecules can be self-assembled into giant liposomes when dissolve commonly in polar solvents such as water. Typical giant vesicles exhibit larger sizes than 1 μm with the advantage of being able to be observed in an optical microscope. These spherical microstructures share common features with cell membranes, e.g. size, curvature and the bilayer structure, apart that phospholipids are the main constituent of biomembranes. Therefore, their bilayer properties have been correlated and used as a simplified model of cells (Fenz and Sengupta 2012). Also, since giant vesicles are self-closed systems which may entrap active biological molecules in both within the bilayer (Witkowska et al 2018) and into the vesicle interior (Tsai et al 2011), they can also be used as micro-sized containers for studying enzymatic reactions (Shohda et al 2015). Then, efficient methodologies for giant liposome production are desirable.
There exist several approaches for giant vesicle preparation. The natural swelling (Mueller et al 1983) (also known as the gentle swelling method) is one the simplest technique which consists on addition of water to a dry film and, after a certain time, giant vesicles are formed. Also, a widely used technique in this regard is the electroformation method (Angelova and Dimitrov 1986). First introduced in 1986 by Angelova et al this technique consists on applying an electric field over electrodes, previously impregnated with a phospholipid and immersed into an aqueous solution. This process leads to the separation of bilayers by the electro-osmotic effect. However, these techniques are slow in vesicle production (e.g., from hours to several days of hydration) and by-products of vesicle formation (e.g., lipid aggregates, nest vesicles and tethers) are commonly found in different degrees, especially in the gentle swelling method (Rodriguez et al 2005). Also, all the above protocols use organic solvents, usually chloroform, to dissolve phospholipids and form a thin film on a solid surface.

There exist different strategies of liposome preparation which are based on alternative approaches, for example, preparation of an emulsion (i.e., water–in-oil emulsion) or formation of vesicles with the help of a microfluidic device. In the first case, the process of the inverted emulsion technique begins when water droplets, which lipids form a monolayer at its surface, are immersed into an oil phase (Pautot et al 2003). The bilayer is generated when a droplet emigrates from the organic into an aqueous phase and passes through a lipid monolayer located at the oil/water interface. In the second case, giant vesicles are obtained by applying a small pulse to a planar lipid membrane. As a result, the membrane detaches and forms a vesicle. These pulses are carried out using a microfluidic jetting device (Funakoshi et al 2007). However, both methods are limited in high throughput formation and commonly these methods provide either larger (e.g., microfluidic jetting device) or smaller (e.g., inverted emulsion method) vesicles when compared with sized cells.

A microcontact printing instrument (or microplotter) is a known tool for accurately depositing isolated volume of fluids or continuous lines that can feature simple or complex geometrical figures (Microplotter™ II, Manual 2015) (Larson et al 2004). This technology is based on ultrasonic/contact-surface approach which allows direct deposition on different substrates of a wide range of materials, including solutions containing metals (Robinson et al 2011), nanoparticles (Molazemoshosseini et al 2017), or enzymes (Mao et al 2014), among others. This instrument can be used as a cost-effective microfabrication technology for the development and application of various materials.

Since many of the current techniques for giant unilamellar vesicle (GUV) preparation do have limitations (e.g., long time preparation process and the use of organic solvents), developing new techniques can be helpful for the scientific community. In the current study, a successful methodology of giant liposome formation of neutral phospholipids is described using a microarray instrument. This method is based on the gentle hydration protocol of dry films. In such a protocol, GUV formation of neutral molecules has an intrinsic difficulty since they requires an input of energy (Lasic 1988). Contrary to electric charged lipids which can generates an electric repulsion between lamellar phases, which may lead to spontaneously GUV formation (Hauser 1984). Our methodology consists on preparing a mixture of phospholipid/additive (i.e., an electrolyte or non-electrolyte compound) dissolves in water. Then, we use the microplotter to deposit by contact controlled sample volumes over a glass substrate in a predetermined patterning, usually arrays of $20 \times 3$ (i.e., length x width with $100 \mu m$ of separation among droplets). Each droplet deposited onto a glass slide corresponds to a micro-sized film or lipid disc which in turns represents a stack of lipid bilayers and molecules of additive within the lamellar structures. Upon hydration, the dissolved additive generates a large osmotic repulsive force that provokes bilayer separation, which is unilamellar in nature (Yamada et al 2007). The ability of each combination of lipids with either an electrolyte or non-electrolyte compound was assessed to generate vesicles and to quantify vesicle defects. Optical and confocal laser microscopies were employed to study and characterize experimental results. The aims of this study are to provide an efficient, straightforward and rapid procedure to prepare giant liposomes from neutral phospholipids in mild conditions using a microarray equipment.

2. Materials and methods

2.1. Materials

Egg yolk L-α-Phosphatidylcholine (EggPC, 81.9%, MW = 768 g mol$^{-1}$) was acquired from Sigma-Aldrich. In some experiments Pluronic® F127 (M$n$ = 13787 Da, PDI = 1.26) was used alongside with EggPC in sample preparation. F127 is commercially available from Sigma-Aldrich. A triblock copolymer F127 is comprised of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) blocks: PEO$_{109}$PPO$_{71}$PEO$_{109}$ (the subscripts indicate the degrees of polymerization). As a fluorescent marker Nile Red was used (purity grade for microscopy) from Sigma-Aldrich. D-(+)-Glucose ($\geq$99.5%, MW = 180.16 g mol$^{-1}$) was also acquired from Sigma-Aldrich. Monopotassium phosphate (KH$_2$PO$_4$, 99.4%, MW = 136.09 g mol$^{-1}$) and potassium iodide (KI, 99.8%, MW = 166.00 g mol$^{-1}$) were purchase from J.T. Baker. All aqueous solutions EggPC/additive were prepared...
with deionized water (18.2 MΩ·cm) (Easy Pure II, Thermo Scientific). Hydration of printed glass slides was carried out with deionized water.

2.2. Sample preparation solutions for the microarray equipment
The procedure consists on preparing a mixture of EggPC with either a non-ionic or ionic compound. The EggPC/additive samples were dissolved in water at 10 wt% EggPC and were used different molar concentrations EggPC to additive, commonly 1:1 and 1:10. All samples were prepared by a vigorous overnight stirring in a vial and sonicated per one hour prior to the microcontact printing process. Samples with and without sonication (just stirring) provide similar results regarding vesicle quantity and quality (images in supporting information). Then we decided to applied sonication to sample preparation since there are not significant variations in results. The ultrasonication bath was kept about 15 °C–25 °C since overheating the sample may cause phospholipid degradation. The samples in our experiments can be referred to as (molar ratio = EggPC: additive): case (i) EggPC, case (ii) EggPC/glucose (1:1, 1:10 and 1:20), case (iii) EggPC/glucose (1:10) + 1% mol F127, case (iv) EggPC/KI (1:1 and 1:10) and case (v) EggPC/KH₂PO₄ (1:1 and 1:10). In case (iii), F127 molar percent was relative to EggPC.

The addition of a macromolecule such as the F127 triblock copolymer in the sample formulation of EggPC/sugar (case iii) has a similar function than a PEGylated lipid is introduced into a precursor lipid film (Shohda et al 2015). In this case, both the PEGylated lipid inserted into the lamellar phase and sugar act synergistically to promote interlamellar repulsion in a hydrated lipid film, which provide an efficient vesicle formation (Shohda et al 2015). We applied this concept to our experiments. In our case, F127 would have a similar function than the PEGylated lipid. The hydrophobic block (PPO) of F127 can be inserted into the lipid hydrocarbon tails and the PEO chains can be facing out the bilayer surface, when lamellar phase is formed, as studies suggest (Firestone et al 2003). In this way, additives such as F127 and glucose may help promoting vesicle formation.

2.3. Microarray equipment methodology
The microarray process was performed employing a SonoPlot® GIX Microplotter™ II (Middleton, WI) (figure 1, left-side). In this equipment the dispenser comprises a piezoelectric and a glass device, with a diameter tip of 50 μm (figure 1, right-side) (Microplotter™ II, Manual 2015) (Larson et al 2004). The dispenser position was controlled by the SonoGuide software (version 2.60) which allowed a precise manipulation in the X-Y-Z Cartesian coordinates, with 5 μm resolution. The printing feature was previously specified using the SonoDraw software (version 2.23). The phospholipid ink solution (i.e., EggPC/additive/water) was suctioned into hollow glass needle via a capillary automated process and injected picolitre volumes by contact onto a microscope slide (Knittel Glass, 24 × 50 mm). This micro-contact printing process generates finite circular films and forms a patterning of dry lamellar stacks of lipid bilayers (i.e., lamellar phases are the most stable structure at high lipid concentrations). Picolitre volumes dry after few seconds of being deposited onto a solid surface at room temperature (Talbot et al 2012). Typical arrays were comprised of 20 × 3 (i.e., length x width) with 100 μm of separation. Before experiments, the glass substrates were previously cleaned with MilliQ water and, afterwards, dried under a nitrogen stream since it was noticed that dust may reduce vesicle formation. Glass slides were used once. All experiments were conducted at room temperature.

Figure 1. Left-side: the SonoPlot® instrument used in our experiments. Right-side: A schematic illustration of sample microinjections onto a glass substrate and the different components of the dispenser system: (a) ultrasonic vibrations, (b) piezoelectric device, (c) hollow glass needle, (d) sample solution (EggPC/additive(s)), (e) droplet sample (micro-sized circular film) and (f) glass slide.
2.4. Optical and confocal microscope observation methodologies

The glass slide containing dried-microsized films (or discs) was placed on an inverted optical microscope (Nikon Eclipse Ti-U; NY U.S.A.) stage and was swollen with water at room temperature. Giant liposomes were visible using either 60× or 40× magnification with differential interference contrast (DIC) technique. The microscope was equipped with a digital camera (Nikon DS-Ri1; NY U.S.A.) which was employed to capture images of giant vesicles. The ImageJ software (National Institutes of Health, U.S.A.) was employed to process and analyze optical micrographs. After glass slide printed preparation with the microarray equipment, these samples can be stored in a dry environment (i.e., desiccator) up to about 10 days for future experiments. In this way, printed glass slides can be immediately used. This significantly reduces the overall time of sample preparation for vesicle production since GUVs can be obtained after about 5 min upon hydration (section 3.3).

The time evolution of vesicle formation and the lipid disc thickness were determined by confocal microscopy experiments. For fluorescent images, a confocal laser scanning microscope (CLSM) (XL-LSM 710 NLO Zeiss; Germany) was employed with an Argon laser source (514 nm). An EggPC/KH₂PO₄ (1:10) sample was vigorous mixed with a Nile Red aqueous solution in an amber vial and, then, printed onto a glass slide. In this way, the lipophilic fluorescent dye was mixed mainly in the hydrocarbon parts of the lipid sample and might be characterized by confocal microscopy. EggPC/KH₂PO₄ (1:10) was used for confocal experiments since this sample was the most effective for obtaining giant liposomes. Then, the thickness of the micro-sized film at the Z-direction was measured using the Zen 2009 software tool. Addition of water to the printed glass slide samples removed the dye content from microsized-lipid disc. Then, it was necessary to hydrate the samples with a Nile Red aqueous solution in order to generate fluorescent vesicles. Fluorescent images were obtained almost immediately after the dye solution addition and were observed by CLSM (section 3.3). The dye buffer was previously prepared with Nyle Red and acetone solution (1 mg/ml) follow by a 1:100 dilution concentration of the dye into water.

Figure 2 illustrates the methodology for GUV formation using the microarray equipment.
3. Results

3.1. Effect of glucose on giant liposome formation

Hydrations were carried out onto glass slides composed of EggPC/glucose dry droplets at various glucose concentrations. For comparison, a glass slide sample without any additive (EggPC only, case i) was also hydrated (refer to section 2.2 for a classification and description of experiments in several cases). Images in figures 3(a)–(b) represent different hydrated lipid disc of the same sample, i.e., case (i). At first sight, these images seem to be different; however, the common characteristic is a small bilayer separation from the lipid bulk phase after a relative long time, i.e., there were no spherical vesicle formations. This is the main feature in case (i) samples. Hydration provokes that circular films slightly expand in size due to water permeate through lamellae. After a certain time, hydration can induce a partially bilayer separation from the bulk lipid phase (figure 3(a)) and can also yield semi-spherical vesicle formation (figure 3(b)). Probably, the bilayer separation in figures 3(a), (b) was induced by a repulsive force in the form of membrane fluctuations since this type of phenomena was observed in microscope images. Whatever the case, both cases of bilayer separation and semispherical vesicle formations are a proof that, apparently, the gentle hydration of the reference sample has a limited swelling behavior. An inspection of several EggPC printed slides did not provide any evidence of spherical liposome formation at conditions here described.

In the case of EggPC/glucose sample upon water addition at 1:1 molar concentration EggPC to glucose (figure 4(a)), a very inefficient and, most of the time, no formation of giant vesicles was seen per lipid disc. At 1:10 and 1:20 (figures 4(b), (c), respectively) giant liposome production increases. However, vesicle quantity is still low. In general, the characteristics in these samples (1:10 and 1:20) are: a low yield with sizes of 2–25 μm. Also, liposomes with structural defects such as nest vesicular structures (also known as multi-vesicular vesicles) and vesicles containing lipid debris are commonly detected at these molar concentrations. Apparently, increasing glucose concentration from 1:10 to 1:20 did not have an effect in vesicle production since giant liposomes were identified with similar features in quantity. Hydration of EggPC/glucose/F127 microsized films did not improve results about increasing giant liposome production in comparison with glucose experiments without F127 (case ii). In case iii, there is a relatively few vesicle formation and diameters are commonly found in the range of 2–22 μm (figure 4(d)). Also, the same vesicle defects, as case ii, remained such as nest vesicles and lipid aggregates. A more detail description of the size distribution and vesicle defects can be found in section 3.4.
increment of F127 molar concentration (i.e., 1 mol% EggPC) makes the solution quite viscous (i.e., F127 provokes gelation of the sample due to the high affinity of F127 to water). Therefore, sample concentrations higher than 1 mol% F127 are not affordable to be used with the microarray equipment. It is worth mentioning that samples containing glucose at 1:10 or higher molar concentration seem to affect the structural integrity of the lipid bulk phase since addition of water immediately ‘dissolves’ circular lipid films, as can be seen in figures 4(b)–(d) with not lipid remnants. Therefore, probably for this reason, all these samples may have similar characteristics. Contrary, low (1:1, figure 4(a)) or no-glucose (EggPC only, figures 3(a), (b)) content in samples provide lipid remnants which are typically observed in optical microscopy images.

In summary, increasing monosaccharide molar concentration from 10 to 20 molar ratio did not make any visible difference in vesicle quantity. Liposomes will be formed in a low percentage and within a range of about 2–25 μm of size. In contrast, if there is a small amount of monosaccharide (1:1), hydration of circular lipid films will provide sporadically formation of liposomes. Also, high and low glucose concentrations in samples seem to follow a pattern. For example, at low concentration (or not addition of glucose) addition of water does not dissolve the lipid film whereas, at high concentration, the lipid disc is dissolved.

3.2. Effect of salts on giant liposome formation

Two different salts (i.e., KI and KH₂PO₄) were used as additives in EggPC sample preparation at various molar ratios. Hydration of EggPC/KI (1:1) films (case iv) results in formation of few vesicles with sizes of about 5 μm alongside with small lipid fragments (figure 5(a)). Increasing the molar ratio of KI, at 1:10 molar concentration (figure 5(b)), giant vesicles are larger in size (5–40 μm) and, in general, with less visible structural defects than previous described methodologies (case ii–iii). Also, after water swelling, lipid remnants can be observed as
circular structures at the background in micrographs (e.g., figure 5(b)). These lipid residues facilitate vesicle surface attachment to the glass substrate. However, yield is still low, similar to glucose samples at 1:10 or 1:20.

The use of a salt such as KH$_2$PO$_4$ improves substantially the efficiency of liposomes at 1:10 molar ratio (figure 5(d)). Usually after a certain short hydration time, giant vesicles typically stay in large quantities on the surface, or close to the surface, of the lipid disc remnant and other vesicles detach from the lipid phase. On the other hand, at 1:1 molar concentration (figure 5(c)) the liposome quantity considerably decreased. A kosmotropic salt such as KH$_2$PO$_4$ seems to have an extensive effect on bilayer separation at low and high molar concentrations, according to what was observed with optical microscopy. For example, EggPC/KI at 1:10 and EggPC/KH$_2$PO$_4$ at 1:1 have similar results about the amount of vesicle formation regardless that KH$_2$PO$_4$ is used in a considerable lower concentration. These results will be discussed in more detail in section 4.2.

3.3. Confocal laser scanning microscopy studies

Confocal microscopy was employed in order to provide a better insight of the vesicle formation process. In experiments, images were acquired every 15 s for a total time of 7 min 15 s. Some representative states of the hydration process are presented in figures 6(a)–(f) (for a video version, consult supplementary material). According with the micrographs, giant liposomes begin to form almost instantly since the image at time zero is few seconds after sample hydration with a Nile Red buffer. This dye diffused and immediately attached to lipophilic bilayers of liposomes. After few seconds of hydration, first vesicles are visible with the characteristic of small sizes (<10 μm) in figure 6(a). Approximately the first 3 min, the system was dynamic because the ratio of

Figure 6. Confocal images that depict the evolution of the vesicle formation process upon hydration: (a) 0 s, (b) 1 min 15 s, (c) 2 min 30 s, (d) 4 min (e) 5 min, (f) 6 min 45 s. (g) A dry droplet optical sectioning in X-Y and X-Z (inlet) directions. The optical section in the X-Z direction provides the film thickness. (h) It is the zoom-out of figure 5(d). In CLSM experiments, liposomes were prepared with EggPC/KH$_2$PO$_4$ (1:10) sample and Nile red as fluorochrome. For a detail description of sample preparation process, see section 2.4.
vesicle formation increased and more and large vesicles were seen. At this stage, vesicle fusion is possible since a number of liposomes are in close contact between each other (Cevc and Richardsen 1999). After about 3 min upon hydration, the ratio of vesicle formation seems to decrease and, 5 min after, according with the evidence of images, decreases even more. In about 5 min, the system seems to stabilize since quantity and size of vesicles considerable decrease the rate of growth.

It should be noticed that figure 6(h) shows a portion of the total vesicle population of the lipid film. The other part may be detached and flowed away from the film, as was observed in a series of different planes in confocal images (results in supplementary material). Finally, a fluorescent image of EggPC/KH$_2$PO$_4$ (1:10) sample, which was printed into a glass slide, can be found in figure 6(g). As one can see, the Z coordinate provides the film thickness. According with the measurements, the lipid disc thickness is 2.3 μm and the diameter is 64.4 μm.

3.4. Size distribution and vesicle defects
A size distribution of giant liposomes was obtained by counting on and measuring individual diameters of vesicles from three different printed glass slides and, in each glass slide, vesicles from multiple lipid disc trials were measured using the ImageJ software. It is worth mentioning that the efficiency in vesicle production may significantly vary between each sample preparation. Indeed, in some cases, hydration of the printed glass slide sample did no provide observable vesicles, specifically in glucose and KI samples. This may be a result of the variability feature of the gentle hydration method (Tsumoto et al 2009). However, in EggPC/KH$_2$PO$_4$ (1:10)
samples, the high yield was apparently homogenous in all samples tested. For this reason, the total liposome population (N) varies between KH$_2$PO$_4$ and all other samples.

Results of size distribution of EggPC with glucose or F127 or KI are presented in the form of a histogram in figure 7. EggPC/glucose + F127 and EggPC/glucose samples provide a similar vesicle distribution with approximately 2–20 μm as minimum and maximum values. These samples have a mean liposome diameter of 7.3 μm and 8.9 μm, respectively. In the case of EggPC/KI (1:10), this produces a larger mean size (20.6 μm) than glucose content samples since vesicle diameter can reach up to about 45 μm. Considerably increasing the vesicle diameter.

In the same way in figure 8, the mean liposome diameter was 18.8 μm for EggPC/KH$_2$PO$_4$ (1:10) sample. Most vesicles are within a range of 5–30 μm which represents 87.5% of the population, giant liposomes between 30–80 μm represents 11.8% and vesicles with sizes below 5 μm represents 0.7% of the total population (N = 391). These results indicate that the microplotter technique mainly produce vesicles of 5–30 μm of diameter. In each sample trial, the largest vesicle was smaller than the corresponding lipid disc size providing an upper limit of liposome size. However, the average value of dry discs was 79.6 (±15.8) μm in experiments and since there is a dispersion of vesicle sizes per one hydrated lipid disc, the microcontact printing technique provides a limited control over the vesicle diameter.

Also, we determined commonly vesicle defects observed in micrographs. Results are depicted in table 1. A half of the total vesicle population of EggPC/glucose (1:10) sample contain defects. There is a slightly decrease in total defects for EggPC/glucose (1:10)+F127 (36.4%) and this defects decrease even more for EggPC/KI sample (22%). In the case of EggPC/KH$_2$PO$_4$ (1:10), nest of vesicles (also known as multi-vesicular vesicles) are found in traces (about 1.8%) whereas lipid fragments inside vesicles are found in 7.1% of the total vesicle population analyzed. Other defects such as tethers or quasi-spherical liposomes were not observed. Then, 91% of the vesicle population obtained by the microcontact printing technique is devoid of significant defects, which surpasses commonly film-hydration techniques such as gentle hydration (40%) or electroformation (80%) (Rodriguez et al 2005). Therefore, hydration of samples containing a salt additive (i.e., KI or KH$_2$PO$_4$) provide both a larger vesicles size and a reduce percentage of vesicle defects when compared with glucose samples at the same conditions of molar concentration.

### 4. Discussion

In the reference sample (case i), the dry lipid films are comprised of zwitterionic phospholipids without additive. The hydration of these films provides a limited swelling which was insufficient to unbind lamellar phases. Commonly in this case it is required long hydration times, in the order of days, for allowing mainly membrane fluctuations to form multilamellar vesicles. Liposome formation stems from neutral charged lipids, such as phosphatidylcholine, needs an energetic contribution (e.g., in the form of sonication or osmotic pressure, among others) into the system in order to create an infinite interbilayer spacing, and, as a result, bilayer unbinding and unilamellar vesicle formation (Lasic 1988). This energy dissipation into the system is necessary since, thermodynamically, unilamellar liposome formation do not occupy the lowest state energy (i.e., they do not form spontaneously) (Lasic 1988). Therefore, the simple gentle hydration of lipid discs, without any additive, was insufficient to promote liposomes. However, micro-sized circular films containing a salt additive provide the necessary threshold energy in the way of osmotic pressure to promote GUVs (Yamada et al 2007).

The factors that generate an increment in the osmotic pressure determine the process efficiency in vesicle formation. For example, a constant temperature, osmosis is influenced by the type of additive (i.e., an ionic or non-ionic compound) and additive molar concentration, as described in the van’t Hoff equation (section 4.2). In the following sections, it will be analyzed the effect of the additive on osmotic pressure and the important factor of the Hofmeister effect in an efficient production of vesicles.

| Sample                  | Multi-vesicular vesicles (%) | Lipid fragments inside vesicles (%) | N   |
|-------------------------|------------------------------|-----------------------------------|-----|
| EggPC/glucose (1:10)    | 21.4                         | 28.6                              | 42  |
| EggPC/glucose (1:10)+F127 | 18.2                         | 18.2                              | 55  |
| EggPC/KI (1:10)         | 4.9                          | 17.1                              | 41  |
| EggPC/KH$_2$PO$_4$ (1:10) | 1.8                          | 7.1                               | 395 |
4.1. No electrolyte additives
In case ii, low quantities of glucose (1:1) as additive seems to be inefficient in liposome formation since bilayer separation structures, which were also observed in simple hydration of EggPC sample, were commonly observed in optical images. However, at 1:10 molar ratio giant vesicles begin to be observed. This data points to a threshold energy barrier where the osmotic pressure effectively takes effect and begins to promote the ‘unbinding’ bilayer process. This idea is reinforced when hydration of EggPC/glucose (1:0.75) sample (micrographs in supporting information) presented aggregates of lower free energy than vesicles, i.e., myelin aggregates (lamellae in the form of cylindrical elongated tubes). These long thin strings are evidenced of kinetically trapped structures below the threshold energy for vesicle formation. Myelin figures are commonly found alongside with unilamellar vesicles since myelin structures form spontaneously, i.e., they required less energy than GUVs (Battaglia et al 2007).

All EggPC/saccharide samples (i.e., case ii-iii) when compare with EggPC/salt samples (specifically KH$_2$PO$_4$) have a poor yield. These results may be due to the minor osmotic pressure strength of glucose, when compare with electrolyte compounds (see section 4.2). Also, an increment in lamellae permeability may have an impact in the osmotic effect in detriment of vesicle formation. It is well known that the liquid-gel phase transition temperature (Tm) in phospholipid bilayers can be altered by decreasing water content (Crowe et al 1998). For example, EggPC bilayers have a Tm of about −7 °C when they are hydrated, which increase until about 70 °C in anhydrous conditions. At room temperature and in dry conditions, EggPC bilayers are in the gel phase state. Then, when bilayers are rehydrated, they pass again through to the liquid-crystalline phase in the presence of excess bulk water (Crowe et al 1998). In this phase transition process, the integrity of the lipid membrane structures can be altered. For instance, according with literature using vesicles as a model to study membrane permeability, EggPC liposomes when they were dry presented a complete leakage compound entrapped into the vesicle interior when they were rehydrated (Wolkers et al 2004) (Crowe et al 1997). This can be counterintuitive to what it is commonly known of the protective effect of sugars on lipid membranes, which may decrease Tm and avoid permeability. However, monosaccharides such as glucose are no effective in membrane protection and disaccharides are highly efficient. For instance, trehalose and sucrose may reduce leakage in drying/rehydration of EggPC membranes (Sun et al 1996). In fact, nature has provided us some examples in membrane protection. Anhydrobiosis (i.e., desiccation-tolerant) organisms have a large quantity of disaccharides (i.e., sucrose and trehalose) that enable preservation of the structural integrity of cell membranes in completely anhydrous conditions (Oliver et al 1998). Our methodology of vesicle formation involves a drying/rehydration process (i.e., dry droplets deposited onto the glass slide and, then, addition of water) of lipid lamellae that may generate an increment in permeability and may contribute to reduce the osmotic pressure difference. Consequently, vesicle formation can decrease as was observed in our experiments with saccharides.

Another parameter to analyze is the effect of glucose on lipid lamellar phase. For example, we could not see any remnant of lipid lamellar phase after water hydration in sample formulations of EggPC/glucose (1:10–20, case ii). On the other hand, samples with low concentration of glucose (i.e., 1:1), or no glucose content (e.g., salts or EggPC only), we did not observe a destabilizing effect of lamellar structure since lipid disc remnants could be seen after hydration. This pattern of results suggests that glucose seems to destabilize lamellar structure on detrimental of vesicle formation. Previous experimental results have indicated that glucose may destabilize the lipid lamellar phase, at high concentrations, and consequently, affect osmotic pressure efficiency, which may inhibit liposome production (Tsumoto et al 2009). Therefore, the effect of glucose on lipid lamellar phase and membrane permeability can be considered the main factors of a poor vesicle formation with monosaccharide experiments.

4.2. Electrolyte additives
An efficient giant liposome production was obtained using KH$_2$PO$_4$ as additive. A higher production of vesicles, when compared with glucose, can be due to both an effective increment in inducing osmotic pressure and to the effect of ions in reducing lamellar permeability. In the first case, according with the van’t Hoff equation ($\pi = iCRT$, where $i$ = van’t Hoff factor, $C$ = molar concentration, $R$ = ideal gas constant and $T$ = absolute temperature) and at the same molar concentration and temperature, a larger osmotic pressure depends on a larger amount of particles. Ionic compounds have larger van’t Hoff factors (e.g., $i = 2$ for KH$_2$PO$_4$) than sugar compounds (i.e., $i = 1$ for glucose), which mean that salts have a higher osmotic pressure than saccharides (acid/base reaction of H$_2$PO$_4$ with water is not considered here; it can be neglected). In our experiments, for example, if we compare glucose versus KH$_2$PO$_4$ vesicle production, a high yield is favored by KH$_2$PO$_4$ rather than glucose at the same experimental conditions (i.e., concentration and temperature). On the other hand, the specificity of cation interaction with lipid bilayers may reduce membrane permeability and, consequently, afford a greater liposome production. Studies have indicated that cations such as K’ provide a tight phospholipid
packing in membranes (Redondo-Morata et al 2012), which may result in decreasing the permeability and, consequently, increasing osmotic pressure.

Results clearly indicate a different efficiency in vesicle formation of ionic compounds employed as additives. For example, at the same molar ratio (e.g., 1:10), KI sample provides a much lower giant liposome quantity than KH$_2$PO$_4$ sample. This dissimilarity in salt results stems from the intrinsic ionic properties among the two salts. According with literature, studies indicate that different types of ions influence in different degrees lamellar phase separation in neutral lipids (Hishida and Seto 2011). These results can be attributed to the Hofmeister effect which is based on how the ion specificity of salts modifies the physical behavior of a wide range of phenomena in colloidal science. These phenomena follow a trend known as the Hofmeister series which basically depends on the intrinsic characteristics of each ion in the series. Commonly, anions exhibit a more marked effect than cations (Zhang and Cremer 2006). For example, the effect of anions dominates the Hofmeister effect on giant liposome formation prepared by the oil/emulsion method instead of cations (Hadorn et al 2011). In our experiments, H$_2$PO$_4$ represents a strongly hydrated anion, which is classified as a kosmotrope or also known as water structure maker (induce the salting-out effect), and I$^-$ represents a weakly hydrated anion, which is classified as a chaotrope or also known as water structure breaker (induce the salting-in effect) (Kang et al 2020). Studies have demonstrated that strong water hydrated (i.e., hydrophilic) anions induce a large osmotic pressure (Cannon et al 2012). Therefore, H$_2$PO$_4$ anion has the characteristic of more hydrated than I$^-$. Consequently, this kosmotropic ion provides a stronger and more effective osmotic pressure effect than I$^-$ ion in our system; hence, the differences in efficiency in vesicle formation. Also, comparative observations of both ionic and non-ionic additives suggest that the specific type of additive is more important than the molar concentration in inducing abundant vesicle formation, at conditions here described in experiments.

In a system of uncharged lipid bilayers in aqueous solution with a gradient of concentration with the exterior, there exist four interactions which can be divided into attractive forces (Van der Waals (VdW)) and repulsive forces (hydration, undulation and osmotic pressure) (Yamada et al 2007) (Leontidis et al 2007). In general, the osmotic pressure can be considered the main repulsive force in the unbinding process of neutral lipids bilayers (Yamada et al 2007) since the hydration force decays exponentially with distance and is effective up to about 10 Å of interlayer spacing (Petrache et al 2006). Also, in our experiments with KH$_2$PO$_4$, undulation or steric forces are ineffective to promote bilayer separation since studies have shown that monovalent cations (such as K$^+$) make more rigid the phosphatidylcholine bilayer (i.e., the membrane elasticity decreases) (Redondo-Morata et al 2012). The undulation force is inversely proportional to elasticity and, as a result, the effect of steric repulsive force decreases. According with our methodology, a proposed mechanism of vesicle formation is presented in figure 9 even though the mechanism is not completely resolved. We can divide into (Yamada et al 2007) (Lasic 1988): (a) a dry lamellar structure formation, (b) the swollen and unbinding lamellar process due mainly to the osmotic pressure difference induces by the salt and (c) the closure of lamellar into spherical structures and generation of giant vesicles.

The rapid formation of giant liposomes with this technique can be accredited to both the micrometer surface area of the film and the addition of a salt. One of the advantage in using the microarray methodology to produce liposomes is that by reducing the phospholipid spreading size, lipid discs of about 60 μm in diameter and 2 μm in thickness greatly increase the surface area to volume ratio. In this condition, the rate of osmosis increases as well. Since the osmotic pressure is the main driving force in the unbinding process of neutral lipids bilayers.
(Yamada et al 2007), the combination of the microsized film and the osmotic pressure difference, which is induced by the additive, highly promotes bilayer unbinding in a brief period of time (e.g., giant liposomes were observed after 5–10 min after hydration). The steady-state of the system is rapidly reached, as observed in confocal experiments.

5. Conclusions

Taking advantage of the microarray/patterning technology at the microscale, the microplotter equipment provides a number of benefits. The primary advantages of this technique are the fast production of GUVs, the absence of organic solvents in the production process and high yield in vesicle formation. The instrument ability to depositing small size films contributes in part to the rapid formation of giant liposomes (i.e., five minutes upon hydration), when compared with common hydration film techniques (e.g., natural swelling and electroformation). Printed glass slides can be stored up to 1 week in a dry environment and be used without further preparation. This contributes to make vesicles rapidly, about 5 min after water hydration. Also, the microplotter process does not involve any organic solvent in vesicle preparation since microsize films favor water evaporation almost instantly after they are printed onto the glass slide. Contrary to most film hydration techniques that need an organic solvent, in one part of the process, to provide an efficient lipid-spreading film onto a solid surface. Also, since this technique commonly allows liposomes to be surface attached, they can be easily localized and individually manipulated by specialized equipment. Consequently, this methodology is appropriate for ‘in situ’ studies in biophysics and life science where giant liposomes need to be immobilized and avoids further methodologies for surface attachment after vesicle formation (Witkowska et al 2018). Future work would be targeted for trapping biological relevant molecules such enzymes into the vesicle interior. Encapsulation of enzymes can derive to conduct reactions inside giant vesicles, i.e., giant vesicles as micro-bioreactors.

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Declaration of interests

The authors declare no conflict of interests.

Authorship contribution statement

Juan R Avendaño-Gomez: Writing–review and editing, Resources and Funding acquisition. Obed A Solis-Gonzalez: Conceptualization, Methodology, Validation of experiments/results, Conducting experiments, Formal analysis of results and Writing–original draft. Haydee Gonzalez-Martinez: Conducting experiments and Resources. Miguel A Vega-Cuellar: Conducting experiments.

ORCID iDs

Juan R Avendaño-Gomez: https://orcid.org/0000-0002-0402-7169
Obed A Solis-Gonzalez: https://orcid.org/0000-0002-5151-1308

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