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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Heat-induced formation of single giant unilamellar vesicles

Céline Billerit,[a] Ilona Wegrzyn,[a] Gavin D. M. Jeffries,[a] Paul Dommersnes,[a,b] Owe Orwar[a] and Aldo Jesorka*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Giant unilamellar vesicles (GUVs) are an excellent model system for the investigation of lipid membranes, the study of membrane proteins and ion channels in a biomimetic environment, and in the creation of artificial cells. Here, we describe a novel method for the preparation of GUVs from single multilamellar liposomes by means of directed infrared laser heating. Our method generates individual unilamellar vesicles at selected locations, not only from natural and artificial lipid mixtures containing negatively charged lipids, but also from preparations of single lipids, such as neutral phosphatidylethanolamine. The presented method provides a new efficient resource for giant vesicle research and offers an alternative to the electroformation and de/rehydration techniques.

Introduction

Liposomes are self-assembled structures that are widespread in nature and can be artificially prepared. Since the pioneering work by Bangham et al.[1] liposomes have been used as a model system to reveal the behaviour of cell membranes. These biological containers are of current interest in several fields such as; biochemistry,[2] molecular biology,[3] and the cosmetics industry.[4] Recent advances in preparation and size control, have further facilitated liposome application within artificial cell research and strengthened their potential for use as drug delivery systems.[5]

Among spherical thin-shelled membrane compartments (liposomes), which are known to exist over a large size range from ~ 20 nm to several hundred µm, giant unilamellar vesicles (GUVs) are a subset of unilamellar vesicles and they are defined by their characteristic dimension being larger than 1 µm. They can be readily observed in aqueous media using light microscopy, where their size allows for modulation using micromanipulators and microinjections. At present, GUVs are extensively studied within the fields of biomimetic chemistry,[6-8] biomembrane physics,[9, 10] and in the field of artificial protein synthesis.[11, 12]

Protein synthesis, being a crucial step for constructing a more realistic cell-model, requires exquisite control over the localized environmental conditions within the GUV, a key component of which being modulation of gene expression inside such vesicles. Although this step seems currently difficult to reach, it is apparent that progress in this and other fields of giant vesicle research, strongly depends on whether reliable methods for the reproducible preparation of giant vesicles are available.

Recently Walde et al.[13] summarized and compared the different known methods to obtain GUVs. In brief there are three widely used protocols for GUVs preparation, each with advantages and disadvantages. The lipid film hydration method is very simple, but requires a few percent of negatively charged lipids, and the yield of GUVs is variable. In contrast, electroformation procedure has high reproducibility, but does not work well with charged lipids in the mixture and requires an aqueous phase of low ionic strength. Finally the water/oil emulsion method has a high encapsulation efficiency and high yield of vesicles nonetheless the possible presence of oil in the vesicle membrane might significantly affect the physical properties of the lipids in the bilayer or properties of reconstituted membrane proteins.[13, 14] New methods for the formation of unilamellar vesicles have recently emerged utilizing microfluidic devices, enabling the ability to encapsulate with high yield, however these devices necessitate special equipment and expertise.[15, 16]

Methods for the preparation of GUVs strongly depend on the lipid composition used, often requiring a mixture of different lipids. This is especially pertinent when using neutral lipids such as phosphatidylethanolamine and phosphatidylcholine, which are the chief components of cell membranes. Generation of liposomes with these neutral lipids, using the lipid film hydration method, often proves difficult, as adjacent lamellae in the film cannot exhibit sufficient repulsion to separate from each other. [17-19] Moreover, all of these described preparation methods are bulk procedures, on-demand generation of single GUVs in a location of interest has thus far proven difficult to achieve.

In this study we report a novel procedure for preparing single GUVs from pure lipids and from lipids mixtures at selected surface locations. We describe an optical heating method and discuss the results, based on the influence of elevated temperature

[a]*Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-412 96 Göteborg, Sweden. Fax: +46 31 772 6120; Tel: +46 31 772 6112; E-mail: aldo@chalmers.se*

[b]*Matières et Systèmes Complexes, Université Paris Diderot, 75013 Paris, France. E-mail: paul.dommersnes@univ-paris-diderot.fr*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

on lipid bilayers, which is known to increase membrane permeability, membrane defects, as well as induce lipid phase transitions. We found that by means of infrared laser heating, surface-adhered multilamellar vesicles (MLVs) can undergo temporary structural transformations, resulting in the formation of stable GUVs. After generation, these GUVs remain connected to the surface-adhered MLVs.

The MLV provides a lipid reservoir that is essential for constructing nanotube-vesicle networks (NVNs). Networks of vesicles interconnected via lipid nanotubes, have been demonstrated in such areas as, modeling exocytosis,[20] and in the study of biochemical processes within confined and dynamic geometries.[21] As the NVNs are biomimetic, it is possible to incorporate biological cells within the network construct, enabling greater control and understanding of communication pathways and inter-cellular transport mechanisms.[22]

This preparation method of GUVs at selected surface locations provides a new approach for the fabrication and utilization of surface-based single vesicle arrays. Such arrays have application in; the transport of membrane proteins, investigating communication pathways, and following enzymatic activity.

Material and methods

Membrane composition of the vesicles

Soybean Polar Lipid Extract (SPE), L-α phosphatidylethanolamine (PE) (18:0/20:4c5,8,11,14 is the predominant species) , L-α phosphatidylinositol, L-α phosphatidic acid, L-α phosphatidylcholine (PC) (16:0/18:2c9,12 is the predominant species), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids and Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red DHPE) was purchased from Sigma-Aldrich.

We selected four different membrane compositions to prepare giant liposomes using the infrared heating method; natural (1), artificially created (2) (using known compositional makeup percentages from the natural), isolated phospholipid (3) and a mixture of two neutral phospholipids (4). These compositions are as follows, 1) 99% of SPE consisting of phosphatidylethanolamine 22.1%, phosphatidylinositol 18.4%, phosphatidylcholine 45.7 %, phosphatidic acid 6.9 %, other 6.9%, with 1% of Texas Red DHPE. 2) Artificially generated SPE (ASPE) with the same composition as (1), omitting the 6.9% unknown components. 3) 99% of PE with 1% of Texas Red DHPE. 4) 60% PC and 39% of PE with 1% of Texas Red DHPE.

Vesicle preparation

A suspension of small unilamellar vesicles (6 mg/ml) was prepared for each lipid composition.[23] This was then further processed using the dehydration-rehydration method described by Criado and Keller[24] with modifications,[25] for the preparation of MLVs. For the dehydration step, a 7 µl droplet of this suspension was placed on a glass coverslip and dehydrated in a vaccuum dessicator for 10 min. When the lipid film was dry, it was rehydrated using a buffer solution containing 5 mM Trizma base, 30 mM K_3PO_4, 30 mM KH_2PO_4, 0.5 mM NaEDTA, balanced to pH 7.8 with 2 M phosphoric acid. After the vesicles were formed, they were carefully transferred to the appropriate surface for analysis.

Surfaces fabrication

Borosilicate coverslips (#1, VWR) were thoroughly cleaned and plasma treated using microwave generated oxygen plasma (Tepla Plasma Batch System 300), at a power of 250W for 2 min. We prepared 2 different types of surfaces;

i) SU-8 surfaces; cleaned glass coverslips were spin coated (3000 rpm, 1 min) with SU-8 5 photoresist (Microchem) and heated on a hot plate (65˚C for 5 min and 95˚C for 5 min). After cooling to room temperature (RT), the surfaces were UV illuminated (5 mW/cm^2, 20 s) followed by post-exposure baking on a hot plate (65˚C for 5 min and 95˚C for 5 min) to allow for crosslinking of the photopolymer.

ii) Gold surfaces; a titanium adhesion layer (thickness 0.5 nm) and a gold layer (thickness 2 nm) were deposited onto cleaned glass coverslips, using a DC magneteron, sputtering at a deposition rate of 5 Å/s and 20 Å/s, respectively. The surfaces were used immediately after fabrication.

Microscopy and fluorescence

A confocal laser scanning microscopy system (Leica TCS SP2 RS), with either a HCX PL APO CS 40x 1.25 or a HCX PL APO BL 63x 1.40 oil immersion objective, was used for the acquisition of confocal fluorescence micrographs. The 594 nm line of a He/Ne laser was used for excitation of Texas Red DHPE, and the emission was collected by a photomultiplier tube, between 600-700 nm using the AOBS module.

Heating system

The vesicle system was heated using direct introduction of IR-B radiation through an optical fiber. A 1470 nm semiconductor diode laser (HHF-1470-6-95, Seminex) driven with an 8 A power source (4308 Laser Source, Arroyo Instruments) was used in combination with a 50 µm core diameter, 0.22 NA optical fiber (Ocean Optics). The fiber was prepared by removing the outer sheath cladding, followed by carefully cutting and polishing using a fiber cleaning kit (Ocean Optics). The heating system was calibrated using a temperature-dependent fluorescent dye Rhodamine B with the aid of a microfluidic device.[26, 27] The laser heating was located around at 40-50 µm from the vesicle and the volume being targeted and efficiently heated was approximately equal to 1 nL.

Formation of networks

NVNs were constructed using a microelectroinjection technique which is described elsewhere.[25] The electroinjection was controlled by a micro-injection system (Eppendorf Femtojet) and a pulse generator (Digitimer Stimulator DS2A). Micropipettes used for injection and for the formation of the networks, were prepared from borosilicate capillaries (GC100TF-10, Harvard Apparatus) by extrusion using a CO_2-laser-puller (model P-2000, Sutter Instrument Co.). A silver wire was used in the surrounding medium as the counter electrode.

Results

We have demonstrated the heat-induced formation of GUVs from

4 different membrane compositions: SPE, ASPE, PE and PC/PE. Stability, usability and lamellarity of these GUVs were investigated by means of nanotube-vesicle networks construction and by fluorescence intensity comparison.

Heat-induced formation of GUVs from SPE lipids

By locally increasing the temperature of a MLV with directed laser radiation, we observed the formation of GUVs from SPE MLVs deposited on SU8 surfaces. Figure 1 shows the progressive formation of GUVs during the heating process. During this process the MLVs appear to maintain their structural stability, until a threshold temperature is reached. This temperature was empirically measured to be approximately 45-50˚C, at which point some fluctuations within the MLV began to become visible, specifically on the side of the vesicle closest to heating source. Successively, several unilamellar vesicles connected to the MLV began to appear (Fig. 1B). These membrane structures grew continuously during heating and typically either collapsed, or merged to form a single GUV. Upon removal of the heating source, the newly formed GUVs maintain their volume and remain connected to the MLV (Fig. 1C). The apparent size change of the MLV observed in the confocal images, is due to the sectioning nature of confocal microscopy, and is not a resulting volume change. To better image the GUV at its mid section the focal plane was adjusted, resulting in a raised section of the MLV to be analysed. This focal plane adjustment was made when analysing all GUVs in subsequent data sets.

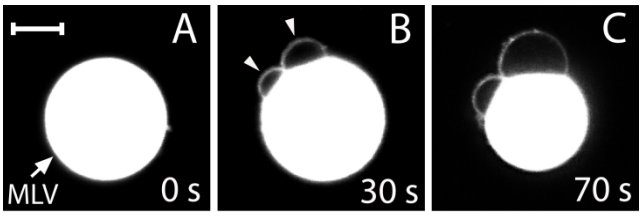

Figure 1: Heat-induced formation of GUVs from SPE. Confocal laser scanning micrographs of MLV prepared from 99% SPE and 1% Texas Red DHPE and deposited on SU-8 coated glass surface. (A) MLV before heating. The scale bar represents 5 μm. (B) MLV after 30 s of heating at 45-50˚C. Formation of unilamellar vesicles starts to appear on one side (indicated by the white arrows). (C) After heating, the formed GUVs remain connected to the MLV. The micrographs were recorded using a 63x objective.

During the dehydration-rehydration procedure, SPE lipids spontaneously form a mixture of multilamellar and giant unilamellar vesicles. The composition of lipids and other constituents, such as cholesterol, in lipid extracts and mixtures is known to have pronounced effects on properties such as surface charge, membrane permeability,[28] protein-lipid interactions[29] and others. A potential problem for many GUV applications, particularly in conjunction with gene expression and enzyme activity based protocols, is the 6.9% content of unknown constituents. Therefore in addition we prepared an artificial SPE mixture, having the same lipid composition as SPE, and evaluated the heat stimulation method on the MLVs produced from the mixture with respect to the formation of GUVs.

Heat-induced formation of GUVs from artificial SPE lipids

We likewise applied laser heating on ASPE-MLV deposited on SU-8 surfaces. No apparent difference in the heating process and the response of the MLV was observed (data not shown); beginning at 35˚C we observed rapid fluctuations on the side of the MLV facing the heating source, followed by the creation of several unilamellar vesicles, which finally merged to form one or more GUVs.

It is well known that the formation of GUVs from zwitterionic lipids by the dehydration-rehydration method is difficult to achieve. Therefore, several additional procedures in dehydration-rehydration have been devised in order to increase interlamellar repulsion, such as by addition of charged lipids[30] or by addition of nonelectrolytic monosaccharides.[31] To investigate the applicability of the directed laser heating method to obtain GUVs from such lipid sources, we prepared zwitterionic PE and PC liposomes by the dehydration-rehydration method and investigated their response to the thermal treatment.

Heat-induced formation of GUVs from PE lipids

Using the dehydration-rehydration procedure on PE lipids, with the aim to generate GUVs connected to MLVs, typically very few unilamellar containers are obtained. However, when SPE and artificial SPE compositions are used, the majority of MLVs have GUVs attached. This confirms previous findings that lipid composition is a critical factor affecting the yield of formation of GUVs and the degree of bilayer separation.

For the heat induced formation of GUVs, a sample of MLVs solution was deposited onto a gold surface. At a temperature of approximately 35-40˚C, the MLVs rapidly exhibited a change in morphology.

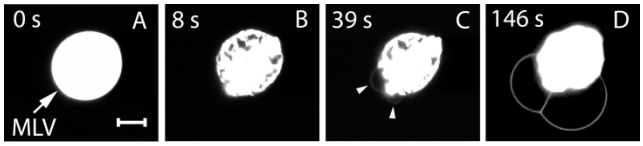

Figure 2: Heat-induced formation of GUVs from PE. Confocal laser scanning micrographs of MLV prepared from PE and 1% Texas Red DHPE on gold coated glass surface. (A) MLV before heating. Scale bar represents 5 μm. (B) Compression of the MLV after 8 s of heating. (C) Fluctuations and formation of unilamellar vesicles (white head arrow), which grow and combine. (D) After prolonged heating, GUVs are stable and remain surface-adhered as well as connected to the MLV. This image was exposure adjusted to aid in the display of GUVs. This adjustment did not affect the appearance of the MLV. The micrographs were recorded using a 63x objective.

In the recorded data series, the MLVs appear to contract and exhibit an internal rearrangement of lipid bilayers, accompanied by the appearance of microstructures. This apparent contraction creates the impression of pockets or compartments within the MLV (Fig. 2B). With continued heating at 35-40˚C, similar to the other lipid systems, several unilamellar vesicles appear (Fig. 2C) and merge to form GUVs firmly connected to the MLV. After the heating source is removed, the internal structure of the MLV appears to return to its original state, while the newly generated GUVs remain (Fig. 2 D).

Heat-induced formation of GUVs from PC lipids

Unlike PE, phosphatidylcholine lipids did not form MLVs with the dehydration-rehydration procedure. Different phosphatidylcholine lipids were investigated (DOPC, DMPC, and PC). For these lipids, only multivesicular and some unilamellar

vesicles were observed with an absence of MLVs (data not shown). These types of liposomes could not be used to generate GUVs with directed laser radiation and natively were not applicable to nanotube-vesicle network techniques. To address this problem, mixtures of PC/PE were prepared. We found that 40% of PE was required to allow for the formation of MLVs by using the dehydration-rehydration procedure.

For the heat induced formation of GUVs, a sample of MLVs solution from the PC/PE mixture was deposited onto a borosilicate coverslip. At a temperature approximately 45-50˚C, some fluctuations within the MLV became visible and several GUVs connected to the MLV appeared (data shown in supplementary information).

Fabrication of a linear network using a heat-induced GUV

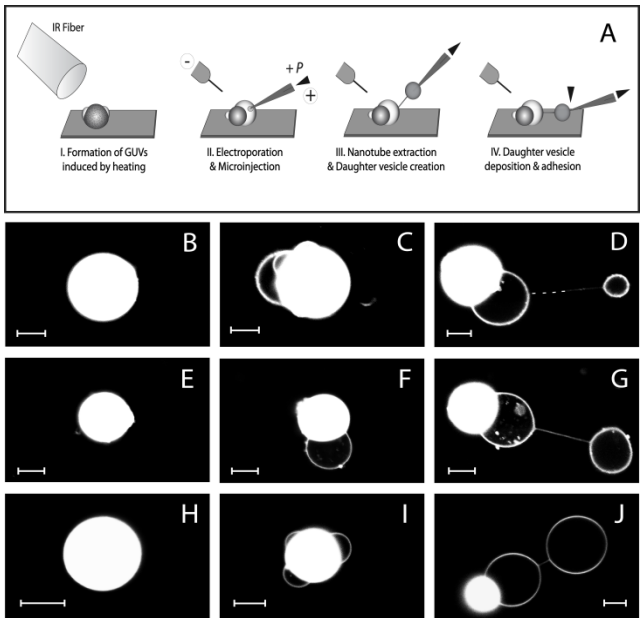

Figure 3: Fabrication of a linear Nanotube-Vesicle Network, and formation from a single GUV/MLV by laser heating of MLV with subsequent application of a microelectroporation/injection/manipulation protocol. (A) Schematic drawings of GUVs formation by heating following by the fabrication of a linear network from a single GUV/MLV. (I) Heating of the MLV deposited on the surface and formation of GUVs connected to the MLV. (II) Electroporation and penetration of a vesicle membrane with a micropipette. (III) A nanotube is extracted and a daughter vesicle connected to the mother vesicle is formed at the micropipette tip. (IV) Deposition of the daughter vesicle on the surface. (B-D) Confocal laser scanning micrographs showing the formation of GUVs after laser heating of MLV prepared from 99% SPE + 1% Texas Red DHPE, and the linear nanotube-vesicle network fabricated thereof. All scale bars represent 10 μm. (B) MLV before heating. (C) GUVs formed and connected to MLV. (D) Nanotube (white dash lines) and daughter vesicle connected to the mother vesicle. (E-F) Confocal laser scanning micrographs showing the formation of GUVs after laser heating of MLV prepared from 99% ASPE + 1% Texas Red DHPE and the linear nanotube-vesicle network created thereof. (E) MLV before heating. (F) GUV formed and connected to MLV. (G) Nanotube and daughter vesicle connected to the mother vesicle. (H-I) Confocal laser scanning micrographs showing the formation of GUVs after laser heating of MLV prepared from 99% PE + 1% Texas Red DHPE and the linear nanotube-vesicle network created thereof. (H) MLV before heating. (I) GUVs formed and connected to MLV. (J) Nanotube and daughter vesicle connected to the mother vesicle. The micrographs were recorded using a 40x objective.

To verify the stability of heat-induced GUVs, a nanotube-vesicle network was fabricated. First, the described laser heating procedure was applied to MLVs from SPE, ASPE and PE lipids to form GUVs. Subsequently, GUVs were electroporated, buffer was injected and nanotubes were extruded, followed by the application of a positive pressure, to generate daughter vesicles (Fig. 3 A). As shown in figure 3B-J, NVNs were successfully created using a heat-induced GUV from SPE, ASPE and PE lipids. This procedure demonstrates that the GUVs can be micromanipulated, electroporated and utilized for established microelectroinjection techniques.[32]

Estimation of GUV lamellarity

For a quantitative estimation of the lamellarity of both the GUVs formed during the dehydration-rehydration procedure and the GUVs formed through directed laser heating of MLVs, we utilised a fluorescence measurement technique. The membranes were visualised by incorporating 1% of a red fluorescent probe Texas Red DHPE within the membrane composition.

In total 18 GUVs were analyzed; 9 GUVs obtained by dehydration-rehydration procedure and 9 GUVs obtained by directed laser heating. Images were formed using a laser scanning confocal microscope, while maintaining the fluorescent excitation intensity and the confocal acquisition settings, for all the GUVs under observation. A typical example is shown in figure 4, where the dashed lines denote the Regions Of Interest (ROIs) for analysis.

The ROIs were used to isolate sections of the membrane, chosen at locations away from any interference of the MLV, for intensity analysis. These regions were selected at three points distributed about the vesicle, to obtain a representative sample. Through analysis, and subsequent subtraction, of the background directly surrounding the vesicle, the accurate value for the membrane intensity could be measured, and an average calculated for the vesicle membrane, using all three ROIs.

To ensure that the analysis was made on isolated membrane regions, line scans were made perpendicular to the membrane within each of the ROIs. Using a full width at two-third max approach (two-third max used to overcome the contribution of the increase of fluorescence intensity inside the vesicles), the membrane intensity distribution, which would indicate the presence of small in-plane attached objects and out of focus bound vesicles, was found to be the same for both directed laser heating formed and dehydration-rehydration GUVs. Through a population study of the vesicles created, using the analysis scheme proposed by Akashi et al,[30] we were able to elucidate that the GUVs generated by directed laser heating, were observed to have the same lamellarity as those GUVs occurring from the dehydration-rehydration scheme. As shown by Akashi et al, the GUVs formed vary in their lamellarity. Using a similar technique we were able to deduce that from our sample preparation we generated a distribution of lamellarities, with the majority having a unilamellarity, but distinct populations were observed with bi- and tri-lamellarity (data shown in supplementary information).

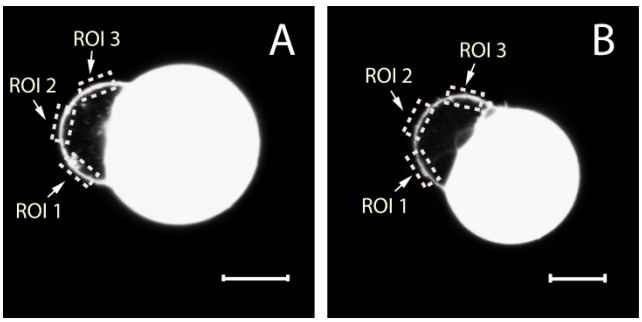

Figure 4: Confocal laser scanning micrographs of GUV connected to MLV prepared from SPE and 1% Texas Red DHPE and deposited on SU-8 coated glass surface. Dashed line rectangles represent the 3 ROIs chosen for mean intensity comparison. Two scale bars represent 10 μm. (A) Example of 1 GUV obtained by dehydration-rehydration procedure. (B) Example of 1 GUV obtained by laser heating. The micrographs were recorded using a 63x objective.

Discussion

For research on unilamellar phospholipid membrane containers, electroformation or lipid film hydration are typically the preparation methods of choice. Electroformation is the popular choice due to its high efficiency and reproducibility of morphology and lamellarity, whereas lipid film hydration is often used when simplicity and mild preparation conditions are required. Lipid film hydration also generates MLVs associated GUVs, which is a requirement for NVN preparations.

For both methods, the lipid composition is considered to be a critical parameter for obtaining giant vesicles. With electroformation, GUVs do not form when a large percentage of charged lipids are present, while with lipid film hydration, a certain amount of charged lipids is required to promote membrane separation for GUVs formation.

In this present study, we developed a novel procedure using directed laser heating, for the formation of GUVs from MLVs with lipid compositions of commonly used natural (SPE) or artificial lipid mixtures (ASPE) containing a percentage of charged lipids, as well as from neat zwitterionic PE and from a PC/PE mixture.

Generally, mixtures of multilamellar and unilamellar liposomes obtained by means of the dehydration-rehydration procedure, are not necessary well located on the sample surface, frequently surrounded by many other liposomes or situated at the border of the area accessible under the microscope. This lack of sample positioning fidelity can be a limiting factor for vesicle micromanipulations and for single vesicle experiments. In contrast, heat-induced formation of GUVs using directed laser heating allows selection of a conveniently positioned GUV on the sample surface.

We have yet to fully investigate the possible detrimental effects of IR light absorption by vesicles. The possibility that a fraction of the affected lipids might be damaged cannot be excluded, even though the temperatures reached in a typical experiment are moderate (35-50˚C). The ability to fabricate nanotube-vesicle networks from heat-induced GUV provides experimental evidence about the sufficiently high integrity of the membranes generated by this novel method. GUVs formed by means of our laser heating technique are stable on the time scale of a typical NVN experiment, and can be subjected to micromanipulation and microelectroinjection techniques.

In this study, we assessed the lamellarity of the individual liposomes by fluorescence intensity comparison. We demonstrated that SPE liposomes obtained spontaneously by dehydration-rehydration procedure and by laser heating method had the same distribution of fluorescence intensities, indicating the generation technique does not significantly influence the vesicle lamellarity. This distribution strongly suggests the formation of a majority of giant unilamellar liposomes, but also the formation of bi- and tri-lamellar liposomes.

Although some explanations have been proposed in the past,[33] the mechanism of vesicle formation using laser heating still remains uncertain. It is known however, that temperature has an influence on lipid behaviour such as; membrane permeability,[34] membranes defects[35] and phase transition.[36]

By spreading the membrane material of a MLV completely as monomolecular film onto a hydrophobic surface,[37] and measuring the total area, we experimentally estimated the inter-bilayer distance in MLVs as between 10-15 nm.[38] This is in agreement with literature values of 4 to 16 nm[39] and 4 to 40 nm,[40] respectively for inter-bilayer spacing of surfactant bilayers. This allows estimating the number of bilayer shells in an onion-like MLV as on the order of several hundred layers. In a GUV, any osmotic imbalance will be rapidly relaxed, but the many layers in an onion shell like vesicle could still lead to a non-equilibrium osmotic stress situation with compartments of higher osmotic pressure trapped in the MLV.[35, 41] However, it is well established that individual bilayers in MLV can be interconnected by "passage" or "handle" defects.[35, 42, 43] Pockets of high osmotic pressure might still exist locally. Since the surface density of these defects increases sharply with temperature,[35] such isolated pockets could connect and become accessible to in-flowing aqueous medium.

Furthermore, membrane permeability for water[34] and ions[44] increases with the temperature. The reason for this increased permeability is the enhanced area fluctuations that lead to a maximum in the lateral compressibility.[45] Nagle and Scott[46] proposed that the changes in lateral compressibility lead to a facilitation of pore formation, since the increased compressibility lowers the work necessary to create a membrane defect. The mechanism for how such an increased compressibility would promote vesicle formation has yet to be elucidated. Clearly, lipid conformation can be altered by temperature; this creates fluctuations and allows a possible water influx between bilayers.

The progressive formation of GUVs from SPE, ASPE and PC/PE mixtures during the heating process, did not result in visible alterations of MLV structure, but slight membrane fluctuations were observed. This result can likely be explained by the coexistence of different lipid species. SPE, ASPE and PC/PE liposomes are composed of different lipids, with varying behaviours according the temperature. In the experiments with SPE and ASPE and PC/PE liposomes, the increase of temperature may affect only certain species of lipids, creating perturbations within the MLV structure. The complexity of membrane compositions and the incomplete understanding of the thermotropic behaviour of the individual components, make it difficult to identify the specific lipid species participating in the observed fluctuations. In contrast, PE liposomes display a

morphological change near 39˚C within a few seconds. These morphological changes suggest that the lipids phase transition generates a new self-assembled structure. These findings are in agreement with the transition temperature of PE found in literature.[47] Indeed, at 32˚C PE has a phase transition from the lamellar to the hexagonal phase. This could explain the MLV structural changes at approximately 39˚C allowing water flux between the bilayers.

We cannot exclude GUV formation via mechanisms where the volume of the vesicle is conserved. Formation of GUVs could occur without the influx of water from the surrounding medium, if water is expelled from the inter-bilayer space and collected in a budding unilamellar membrane compartment. This could occur in conjunction with the lipid phase transition mentioned above. This phenomenon would be associated with a decrease in MLV radius, e.g. by 25% for a 50% volume reduction. We were not able to observe such a reduction in MLV size with our confocal setup. Such measurements are not easy to conduct, since shape and surface adhesion of MLVs cannot be kept constant. In particular, small changes in diameter on the order of a few μm would be very difficult to quantify. The irreversibility of GUV formation does not allow for the distinction between possible formation mechanisms, but is consistent with the argument that pockets of higher osmotic pressure become accessible and relax. Even if the number of pores or defects is reduced, or the lipid phase transition is reversed upon cooling, these reservoirs of high electrolyte content cannot be restored.

Conclusion and outlook

We have developed and herein described an easily applied and highly efficient method for the formation of single GUVs from MLVs in selected locations by means of directed laser heating. The use of a 1470 nm semiconductor laser, originally developed for optical communications purposes, in conjunction with a narrow optical fiber, provides a cheap and portable scheme for heating single vesicles without the need for extraneous optical components or a specially equipped optical microscope. A distinct advantage of this technique is the ability to create GUVs from a variety of lipid compositions, in particular different mixtures of lipids, but also pure lipids such as the neutral PE. Moreover, the possibility to generate GUVs at selected locations is advantageous for surface-based single vesicle experiments, especially when generating cell/liposome interconnections, and for nanotube-vesicle network fabrication. Pipette assisted transfer of single MLV to desired locations on micropatterned surfaces has been reported,[37] which allows for convenient localisation of a NVN on a microscope cover slide.

The generation of vesicles from spin-coated or spread lipid films using directed laser heating, would provide an exquisitely controllable alternative to AC field electroformation of GUVs, while increasing the versatility in sample handling. We have proposed mechanisms for the heat-induced formation of GUVs, however a more detailed study, with emphasis on pure negatively charged lipids and other commonly used compositions of artificial lipid mixtures, will explore the possibility of using directed laser heating as a universal formation technique.

Acknowledgements

This work was supported by grants from the European Research Council, the Knut and Alice Wallenberg Foundation, and Swedish Science Council. We also extend thanks to Ilja Czolkos for providing data concerning the total surface area of MLVs.

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