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A novel technique for preparation of monodisperse giant liposomes

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A novel technique for the preparation of monodisperse giant liposomes has been developed based on a combination of micro-patterning of ITO glass slides with lipid solution and electroformation. The average diameter of the produced liposomes is determined by size of the micro-pattern features.

Liposomes, normally consisting of an aqueous core encapsulated within one or more bilayer membranes of phospholipid molecules, have great potential as drug and macromolecule delivery vehicles in medicine and are already widely used in cosmetics and pharmaceutical formulations.[1,2] They can be produced by various techniques including lipid swelling,[3] electroformation[4] and membrane extrusion.[5] Recently, Pautot *et al.*[6] have developed an emulsion-transfer method for the production of asymmetric liposomes in which the phospholipid compositions of the inner and outer monolayers of the bilayer membrane can be independently controlled. In addition to liposome variations based on lipids, a range of vesicular structures can be synthesised using assembly of polymeric amphiphiles.[7–11] Over the last 10 years, giant vesicles have proved to be extremely useful tool for studying lipid membrane mechanics and various inter-membrane interactions.[12] However, most methods[12] for preparation of giant vesicles produce polydisperse samples of sizes ranging between 5 and 150 μm.

Here we report a novel preparation method for monodisperse giant vesicles (suitable for direct visualisation using fluorescence microscopy) based on a combination of microcontact printing plus electroformation as shown in Fig. 1. Microcontact printing is used to deposit a micro-pattern of lipid "patches" onto an indium tin oxide (ITO) electrode surface, which forms the base of an electroformation cell. Application of an AC field across the cell causes liposomes to grow simultaneously on each patch of lipid and the final result is an array of monodisperse giant liposomes that can be detached from the electrodes. The liposome size can be controlled by: (i) the size of the pattern features and the spacing between them and (ii) by stopping the liposome growth after a fixed interval of time. A detailed description of the methodology is presented below.

Microcontact printing of lipid solutions on ITO glass slides was done by using PDMS (polydimethylsiloxane) stamps prepared in the following way: micro-patterns of square holes in glass were produced by laser ablation.[13] The micro-patterned glass substrates were replicated with Sylgard 184 (Dow Corning) with PDMS-to-cross linker ratio 10 : 1. Fig. 2 shows a typical image of a PDMS stamp prepared by this method. The PDMS stamps were made hydrophilic by surface oxidation with piranha solution (conc. H_2SO_4 and 30% H_2O_2 3 : 1 (v/v)) for 30 s at 40 °C, rinsing several times with deionised water and treatment with 10% APTES (3-aminopropyltriethoxysilane) in water for 2 hours at 50 °C. The hydrophilic stamps were stored in milli-Q water prior to the microcontact printing. Lipid "ink" solution consisted of 1 mg l^{-1} DMPC (dimyristoyl-phosphatidylcholine, Avanti Polar Lipids, Inc.) stained with an amphiphilic fluorescent dye, lissamine-rhodamine (Avanti Lipids), 2 mol% with respect to DMPC. ITO slides coated with 20 nm indium tin oxide (30 × 30 × 1 mm, resistance 80 Ω mm^{-2}, from Agar Scientific) were pre-cleaned with acetone and dried under nitrogen before printing. The PDMS stamp was inked with the lipid solution for 30 s, wiped on dry paper and pressed

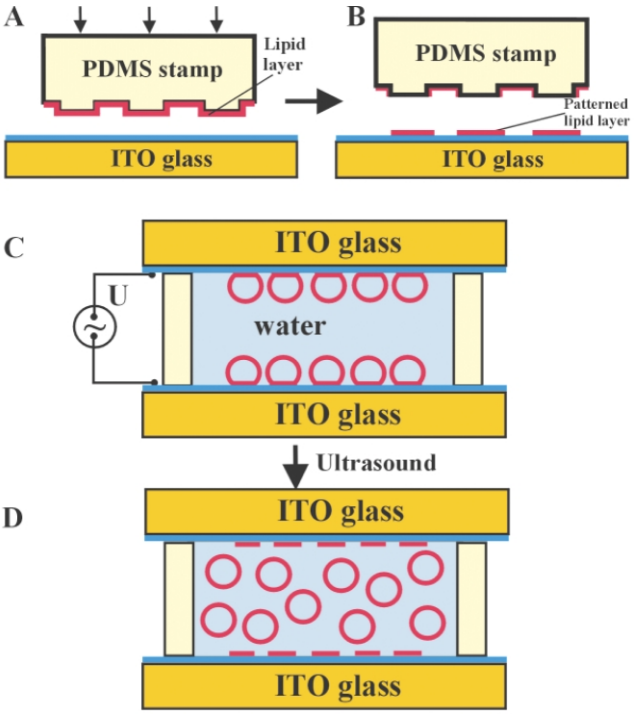

Fig. 1 The preparation of monodisperse giant liposomes by micro-contact printing technology involves the following stages: (A) and (B) the lipid (stained with a fluorescent dye) is deposited on an ITO glass by using a PDMS stamp of square grid pattern. (C) The liposomes are grown on the ITO slide in water by electroformation in AC field. (D) The monodisperse liposomes are released from the ITO slide after application of ultrasound (ultrasonic bath).

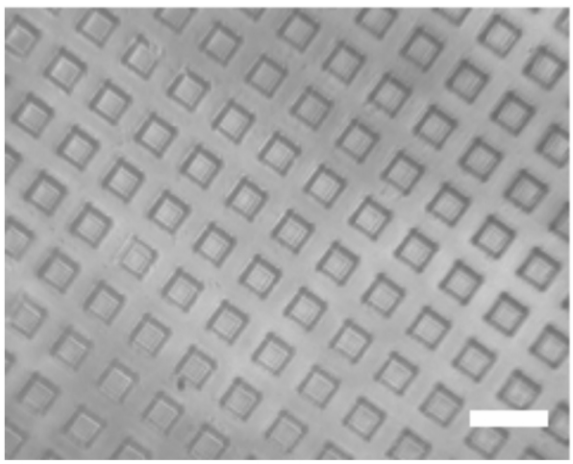

Fig. 2 Optical micrograph of a PDMS stamp used for microcontact printing of lipid solution on ITO glass slides. Square size is 10 μm and square-to-square distance is 8 μm (bar is 20 μm).

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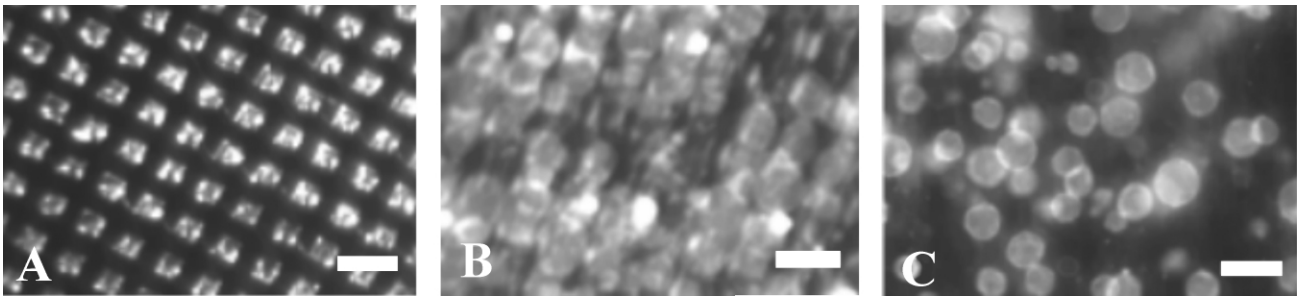

Fig. 3 (A) Microcontact printing of ITO glass surfaces with a hydrophilised PDMS stamp. The array of square phospholipid patches is imaged by fluorescent microscopy. (B) ITO surface during AC field application in 0.1865 M glucose solution (pH 5.76). Liposomes are seen to sprout on the square pattern after 1 h of application of AC field. (C) Liposomes detach after application of ultrasound. (Bar is 20 μm).

with a light touch on the ITO glass slide. After printing, the ITO-glass slides were stored in a vacuum chamber for 45 minutes (10 Torr) before the electroformation. The electroformation cell[12] was made of two ITO glass slides, separated by a PDMS spacer of thickness 1 mm (Fig. 1C). The experiment was done both with one or two ITO slides patterned with lipid solution. The cell was filled with 100 μl aqueous solution of glucose (0.1865 M, pH = 5.76) and an AC field of frequency 10 Hz and voltage 0.5 V (peak to peak) was applied to the electrodes. The temperature of the cell was kept at 45 °C (well above the main phase-transition temperature of DMPC of 24 °C) during the electroformation and the liposome growth was observed by fluorescence microscope using a filter set appropriate for lissamine rhodamine dye. Fig. 3A shows a typical fluorescence image of the stained lipid pattern on the ITO immediately before the electroformation. Note that the thickness of lipid deposit is not uniform over the square patches. Fig. 3B presents a fluorescent image of the surface of the ITO slide after approximately 1.5 hours of electroformation. One can see that giant liposomes are growing in a square array corresponding to the positions of the lipid patches on the ITO glass surface. Our attempts to detach the liposomes from the surface of the ITO slide by applying a low frequency (1 Hz) AC field were unsuccessful normally leading to breaking most of the liposomes on the ITO slide. We succeeded in releasing a large portion of giant liposomes from the ITO surface into the bulk by immersing the electroformation cell into an ultrasonic bath (Model F5300b, Decon laboratories limited) for 2 minutes at 18 °C (Fig. 1D). A typical image of the released giant liposomes floating free in the glucose solution is shown in Fig. 3C. We found that the size of the produced liposomes is very similar (slightly bigger, diameter = 13 ± 4 μm) to the size of the lipid squares on the patterned ITO surface. From the microscope images we concluded that a large portion of the liposomes are unilamellar. The prepared liposomes were found to be stable for a few days at room temperature.

The mechanism of formation of liposomes of similar size on each of the square patches of lipid deposit was revealed by observing the growth of the liposomes. If was found that initially a number of smaller polydisperse liposomes sprout on each square patch of lipid and they coalesce with each other as they grow within this limited space. Finally, only one giant liposome per lipid patch (square) survives, and since all patches of lipid on the patterned surface grow vesicles at the same conditions, this results in an array of attached liposomes of relatively narrow size distribution. Since the coalescence between liposomes happen when they are compressed against each other within a limited space, it is essential that the lipid patches are spaced from each other by a distance sufficient to prevent the coalescence of neighbouring liposomes. The latter mechanism becomes possible since the liposomes do not detach spontaneously from the lipid patches on the surface. However, by raising the pH and/or adding charged lipid (*e.g.* phosphatidyl glycerol) to the lipid ink we encountered conditions where liposomes detach early in the process of their electroformation. This does not produce samples of relatively narrow size distribution. In a separate experiment, we also left the liposomes on the patterned surface to grow to a size bigger than the distance between the squares which lead to coalescence between liposomes originating from different lipid patches and the formation of a polydisperse sample of liposomes. As such extended coalescence progresses, the biggest liposomes reach a size of over 100 μm at which they become unstable and break up. These observations serve to emphasise that the growth process should be stopped well before such overlapping occurs in order to produce a liposome population of narrow size distribution.

In summary, we have designed a novel method for the preparation of relatively monodisperse giant liposomes using a combination of microcontact printing of lipid ink on electrode surfaces with the well known electroformation procedure.[4,12] The mechanism of producing liposomes of similar size relies on multiple coalescence of growing liposomes within the patches of lipid deposit on the electrode surface produced by microcontact printing technique. The method was found to work well for DMPC but we expect that it is also applicable for the production of monodisperse vesicles of other materials, including polymersosmes.[12]

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