

# UNIFORM SIZE LIPOSOMES ON A CHIP: OBSERVATION OF TRANSPORT KINETICS THROUGH NANOPORE MEMBRANE PROTEIN

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## ABSTRACT

In this paper, we observed transport kinetics of a fluorescent molecule through a nanopore membrane protein by using a fully-closed liposome array. The liposome array was formed on a chip by the similar protocol previously reported, where the important condition discovered was the lipid amount for each liposome increased about 30%. Since the formed liposomes are uniform size with a few picoliter volumes and stay on the substrate surface, they are suitable to follow such transport phenomena by microscopy.

## KEYWORDS

Liposome, Transport kinetics, Membrane protein, Lipid bilayer, Electrospray deposition

## INTRODUCTION

### Giant Liposomes

Lipids are the major components of biological cellular membranes, which play an important role as the boundaries of cells and subcellular organelles. Lipids autonomously form bilayer membrane structures in aqueous media because of the hydrophobic interaction between the aliphatic hydrocarbon chains. Giant liposome (or giant lipid vesicle) is one of such self-assembled structures of the lipid molecules, encapsulating an aqueous solution by a single or multiple lipid bilayer membranes with a diameter ranging between a few micrometers and a few hundred micrometers [1]. Such microcontainers find a number of applications in the fields of drug delivery, cosmetics and food products as well as the biological fundamental studies [2].

### Selective Patterning and Size Uniformity of Formed Giant Liposome

Gentle hydration and electroformation are the most common methods for the giant liposome preparation. The gentle hydration simply takes advantage of a rehydration process of a dried lipid film with an aqueous solution while the electroformation applies a low-frequency AC voltage during the rehydration [1]. Although these methods have been widely used, they both have difficulty in controlling the shape and/or the size distribution of the formed liposomes [1,3,4]. Patterning of lipids prior to the rehydration process will be one of the solutions of those problems. There have been a few techniques reported to obtain such lipid patterns, e.g. hydrophilic/hydrophobic patterning of a substrate's surface [4], a lift-off method with a patterned polymer film [5], or an application of a microcontact printing [6]. The results indicated that the selective patterning would improve the uniformity of the formed liposomes, yet neither method has successfully regulated the lipid patterning/deposition process.

In the previous work, we therefore developed an alternative method that enables the precise control of the

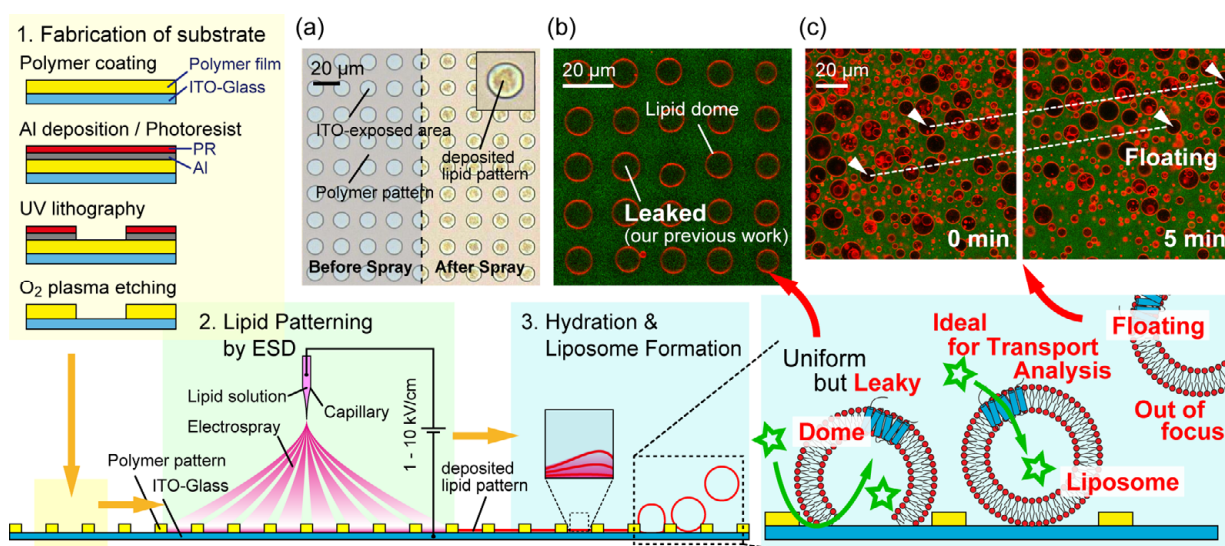


Figure 1: Conceptual diagram of the motivation of this work. Aiming for the observation of transport kinetics through a lipid membrane, it would be ideal if uniform-size fully-closed liposomes are arrayed on a substrate. Selective patterning of lipids on a polymer-ITO substrate (a) enabled to form a uniform-size liposomal-dome array, but the domes are weak in the sealing (b). On the other hand, common floating liposomes are also inadequate for time-lapse observation (c).

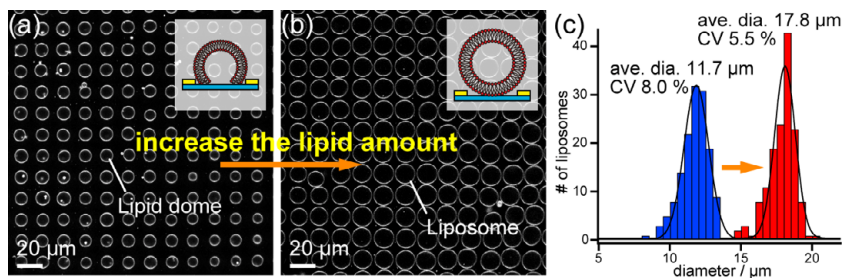


Figure 2: Microscopic images of the formed liposomal domes with the former protocol (a), and the liposome formation by increasing the lipid amount (b). The histograms of the diameter for (a: blue) and (b: red) are shown in (c).  $n = 144$ .

patterning process. The key feature of the developed method was the integration of electro spray deposition (ESD) technique and a microfabrication process [7]; By the ESD, the spray of lipids was electrically led to only the bottom of microwells which was a conductive ITO-glass slide (details in the experimental section, Fig. 1). With a simple rehydration process of these dried patterns, we succeeded in formation of giant liposomal-domes on top of the microwells with a narrow range of the size distribution and easily obtained the desired sizes of liposomal domes by changing the microwell diameter (between 5 and 30  $\mu\text{m}$  in diameter). The liposomal-dome array was a powerful platform for experiments using a fluorescent microscopy and demonstrated a ligand-binding assay of membrane protein incorporated on the array [8]. However, the liposomal domes on the platform hardly keep the interior solution (Fig. 1b), and are inadequate for the analysis of transport kinetics through the lipid membrane. In this paper, we aimed to overcome this serious drawback, i.e. to form a fully-closed liposome array with a defined size.

## EXPERIMENT

### Fabrication of Lipid Patterned Substrates

The conceptual diagram of our method is shown in Fig. 1. The method consists of two steps: 1) Fabrication process of the patterned substrate by using a common photolithography, and 2) Lipid deposition process with the ESD technique. First, a poly(chloro-*p*-xylylene) (parylene C) thin film was coated on an ITO-glass slide by a chemical vapor deposition method (PDS-2010, Specialty Coating Systems, IN, USA). Then, thin layers of aluminum and positive photoresist were respectively deposited and coated on the parylene C coated ITO-glass slides. By a standard UV-lithography process, we obtained an array of microwells (10  $\mu\text{m}$  in diameter). The ESD technique applies a relatively high DC voltage between a lipid solution filled in a thin capillary and the ITO-glass slide (i.e. target substrate) to perform a selective deposition of lipids at a conductive surface. In this paper, we used DOPC-DOPS lipid (1:1-mixture, 0.5 mg/mL) in a solvent consisting of a chloroform and acetonitrile. A rhodamine-labeled lipid was also mixed for fluorescent observations (Rhod-PE, 1 wt%). After the deposition, the samples were kept in vacuum before use.

### Observation of Fluorescent Molecule Transport through Nanopore Protein

Rehydration of the lipid was simply performed by infusion of a buffer medium to the well which was punched in a silicone-rubber sheet placed on the lipid-patterns. Same as the previous works, liposome formation completes within a several minutes [7]. We observed transport kinetics of a fluorescent molecule (calcein) through a membrane protein nanopore ( $\alpha$ -hemolysin,  $\alpha\text{HL}$ ). First, 5  $\mu\text{M}$  calcein was infused outside of the liposomes, and then ca. 500 nM  $\alpha\text{HL}$  was added to the well (see also the Results, Fig. 3). Fluorescence images were taken through the ITO-glass slide with a confocal inverted microscope (SP5, Leica Microsystems, Germany).

## RESULTS AND DISCUSSION

### Formation of a Fully-Closed Liposome Array

A fully-closed liposome array was obtained by the very similar protocol with the previous work. A typical lipid pattern by the ESD was shown in Fig. 1a. Fig. 2a shows the resulting liposomal-dome array by the former protocol: The dome diameter is close to the microwell size (see Fig. 2c, blue histogram). However, it was clarified that a part of such an array became fully-closed liposomes with the increase of the amount of the patterned lipid. By tuning the ESD condition of the lipid, we estimated that roughly 30% increase of the lipid amount gives an efficient number of fully-closed liposomes on the chip although the diameter becomes larger (Fig. 2b-c, red). Note that the increase of the amount causes the liposomes flow away, which is also problematic for microscopic observation (Fig. 1c).

### Calcein Transport into Liposomes through Nanopore Protein

As shown in Fig. 3a, the fluorescence intensity was almost zero at the inside of the liposomes, and constant before the addition of  $\alpha\text{HL}$ . Accompanied by  $\alpha\text{HL}$  incorporation and nanopore formation on the liposomal membrane, however, the intensity gradually increased by the calcein diffusion into the liposomes (Fig. 3b-d). Schematic illustrations are also presented below the images in Fig. 3.

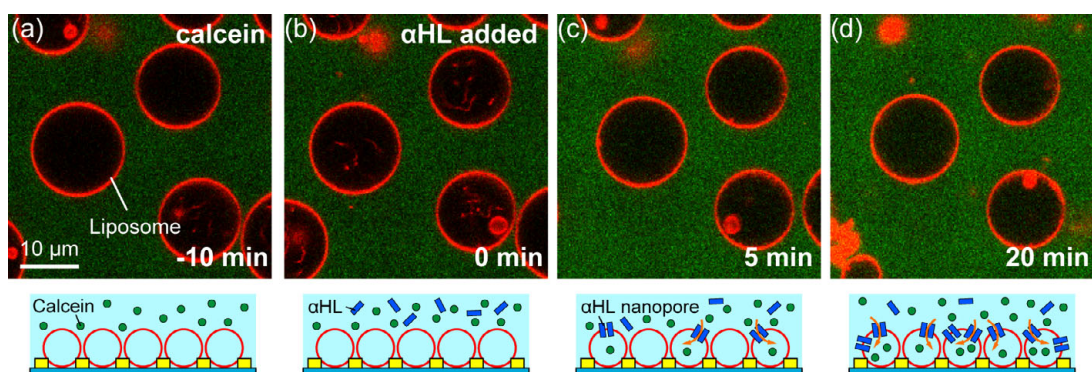


Figure 3: Transport kinetics of fluorescent molecule (calcein) through  $\alpha$ HL nanopores on the fully-closed liposomes. Time-lapse microscopic images after calcein addition (a),  $\alpha$ HL addition (b), and 5 and 20 min after the  $\alpha$ HL addition (c-d). The calcein is colored with green whereas the liposomal membrane is with red (by the rhodamine-labeled lipid). The images are merged two colors. The fluorescence intensity within each liposome increased by the calcein transport through the formed nanopore.

## CONCLUSION

In this work, we succeeded in the formation of a fully-closed liposome array on a chip with a defined size distribution by using a simple rehydration process. As demonstrated, the platform has the advantage in the time-resolved and/or statistical analyses of the molecular transport through membrane protein reconstituted in the liposomes. We consider that the developed system would find various applications from fundamental liposome studies to functional analyses of membrane proteins.

## ACKNOWLEDGMENT

The authors acknowledge the technical support provided by Ms. Utae Nose and Ms. Maiko Onuki. This work was partly supported by JSPS (Grant-in-Aid for Young Scientists B; 23710154), Japan.

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