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Spreading of giant liposomes on anisotropically patterned substrates

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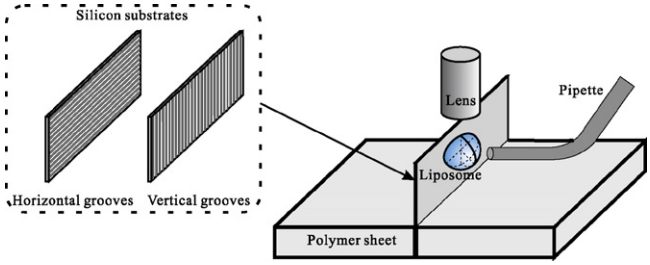
HIGHLIGHTS

- This work provides a new method to study liposome shape transformation behaviors.
- Characterization of the evolution of liposome shapes with time is enabled.
- Effect of surface patterns is studied, which lays a foundation for further studies.

GRAPHICAL ABSTRACT



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ABSTRACT

The dynamic spreading of giant liposomes on anisotropically patterned substrates is investigated experimentally on vertically fixed spreading substrates. The giant liposomes are prepared through electroformation method. The substrates with anisotropic grooves on the surfaces are fabricated on silicon wafers by photolithography and dry etching. The gravitational force of giant liposomes is negligible. The spreading is investigated dynamically, and the evolution of the contact radius, specifically in the directions parallel and perpendicular to grooves, is traced. The effect of surface patterns on spreading is studied and the anisotropy in spreading behaviors is characterized by the contact angle difference and liposome elongation.

1. Introduction

Liposomes, also referred to as lipid vesicles, can be spontaneously formed from lipid bilayers [1]. Bilayers in liposomes exist with the hydrophilic "head" facing aqueous solutions and the hydrophobic "tails" lining up away from water. According to the size of liposomes, there are giant liposomes, large liposomes, and small liposomes with their diameters larger than 10 μm, in several hundred nanometers, and of several tens of nanometers, respectively. Liposomes can be used as drug delivery vehicles and biomimetic reactors [2,3]. Besides, liposomes, especially giant liposomes whose size is similar to real cells, can serve as models to study cell behaviors [4,5]. Most of these applications are related to the spreading of liposomes, one of the shape transformations of lipid bilayers.

Spreading is a universal phenomenon in nature, and it is intrinsically about surfaces and interfaces. Not only normal liquids, but biological systems can be involved in such interfacial phenomena. The wetting and spreading of a biological fluid interface is often considered in the research of drug delivery, biomaterials processing, and tissue engineering [6,7]. Fluid interfaces, playing a crucial role in cellular or physiological behaviors, are

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formed by biological membranes which can be categorized as soft matter and modeled by liposomes. Thus, it is of great significance to investigate the spreading of liposomes. On the one hand, it can help in the understanding of the physics behind the spreading phenomenon; on the other hand, it can promote further applications of liposomes in the field of biomedical engineering.

It is known from the studies on the spreading of liquid droplets that the easiest way to study spreading is to measure the contact angles that the droplet makes with the substrate and to trace the evolution of droplet shapes. However, it is difficult to realize such experiments for liposomes. The reasons are (i) liposomes can only be seen under an optical microscope and the top view of the microscope makes the direct measurement of contact angles impossible and (ii) liposomes suspend in aqueous solutions, and it is not so easy to manipulate them as to control the dispensing of liquid droplets. Due to these restrictions, there were not many studies on the spreading of liposomes until the emergence of some practical instruments and special optical microscopes, such as micropipettes [8,9], the reflection interference contrast microscope (RICM) [10,11], the evanescent wave light scattering microscope (EVLSM) [12], and the evanescent wave-induced fluorescence (EWIF) microscope [13–15], which enabled the manipulation and observation of individual liposomes. However, the pure micropipette aspiration method is more like a method to study the adhesion and de-adhesion phenomenon, rather than to study the whole spreading process. With the RICM and EWIF techniques, the changes of contact angles and the evolution of liposome shapes cannot be directly observed. In addition, the use of RICM and EWIF microscopes will add complexity and difficulty in operation and increase cost to researchers. Thus, a method which will enable the study under normal optical microscopes is demanded.

With an optical microscope, a side view of adhered liposomes could provide more information than a top view when studying the shape changes during dynamic spreading of liposomes. Abkarian et al. [16,17] did their study by tilting the microscope by 90° and successfully obtained side views of adhered liposomes. Their work provides an option for the study of liposome spreading. However, it requires a large space and an ingenious design of the system to tilt the microscope. For more common applications, another option to achieve side views of adhered liposomes was reported in our previous work [18]. In our strategy, the spreading of giant liposomes has been successfully studied under a normal optical microscope by letting liposomes spread on vertically fixed substrates. The design and realization of these studies is under the condition that the gravitational force is negligible. If the gravitational force is remarkable, such as when the density of the solution inside of the lipid membrane is much larger than that outside of the membrane, the gravitational effect must be included when analyzing the spreading phenomenon [19]. With our experimental system, the spreading process as well as the evolution of liposome shapes can be observed in real time. The contact angles and liposome dimensions can be measured directly from the images taken by the CCD camera. It should be noted that there is no special requirement of the optical microscope and the observation system. It is an easier and more practical way to study liposome spreading, comparing with the other techniques. As an extension to our previous work on the spreading of liposomes on smooth substrates [18], this work studies the liposome spreading on anisotropically patterned substrates. The effect of surface patterns on spreading is studied, and the anisotropy in spreading is characterized.

The structure of the paper is as follows. The preparation of giant liposomes is briefly introduced in Section 2. The spreading of giant liposomes is in Section 3, including the fabrication of anisotropically patterned substrates and the spreading experiments. The results obtained from the experiments are also discussed in Section 3. The conclusions are drawn in Section 4, which ends the paper.

2. Electroformation of giant liposomes

Giant liposomes were used in this work to facilitate the observation of spreading process under an optical microscope. They were prepared through electroformation method which was accomplished under an AC electric field [20,21]. The electroformation device is schematically shown in Fig. 1. And the materials used in the electroformation and the electroformation procedure are briefly introduced as follows.

The mixture of synthetic lipids 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti, USA) were used. The molecular structures of these two lipids are shown in Fig. 2. The stock lipid solutions were diluted with chloroform to the concentration of 1 mg/ml. The mixture was prepared by mixing the diluted lipid solutions at the volume ratio of 1:9 (DOPA:DOPC). The lipid mixture was deposited onto the conductive surface of a piece of indium tin oxide (ITO) coated glass plate (ITO coating thickness: 1200–1600 Å, resistance: 1–15 Ω, Delta Technologies, USA) under nitrogen. The plate was then put into a vacuum oven and maintained for at least 6 h to completely remove the organic solvent.

The glass plate with dry lipids on the ITO coated surface was assembled with another piece of ITO coated glass plate to form a formation chamber, and the two pieces of glass plates were separated by a silicone spacer (Sylgard® 184 Silicone Elastomer Kit, Dow Corning, USA) with the ITO coated surfaces facing each other. The chamber was connected to a function generator (Thurlby Thandar Instruments, UK) which generated an AC electric field. The AC electric field, 0.2 V peak to peak at 10 Hz, was applied to the formation chamber when the sucrose (Sigma–Aldrich, USA) aqueous solution (100 mM) was gently introduced into the chamber through an opening in the silicone spacer. Then the voltage was immediately raised to 2.0 V to promote the formation of giant liposomes. The formation of giant liposomes under the electric field was maintained for 2 h and the whole process was observed from the microscope eyepiece and recorded by the CCD camera synchronously (microscope: BX51WI, Olympus, Japan; CCD camera: QICAM, QImaging, Canada; image capturing and analysis software: Image Pro Express, MediaCybernetics, USA; video recording software: StreamPix4, Norpix, Canada). After 2 h, the formed liposomes were detached from the glass plate by decreasing the frequency to 0.5 Hz while maintaining the voltage. The liposome suspension was transferred into a plastic tube and kept in a refrigerator at 4 °C for future use.

3. Spreading of giant liposomes

3.1. Fabrication and characterization of anisotropically patterned substrates

Anisotropic patterns were realized by fabricating unidirectional parallel grooves on the surface of silicon wafers. A silicon wafer was first rinsed with DI water and spin-dried to remove the residual liquid. There are two main steps of the fabrication process: photolithography and dry etching. The complete fabrication procedure is illustrated in Fig. 3.

Standard photolithography technique was employed before etching. First, a piece of clean wafer was heated to the temperature of 100 °C to remove any moisture that might be present on the surface of the wafer. At the same time, the adhesion promoter, hexamathyldisilazane (HMDS), was applied to promote the

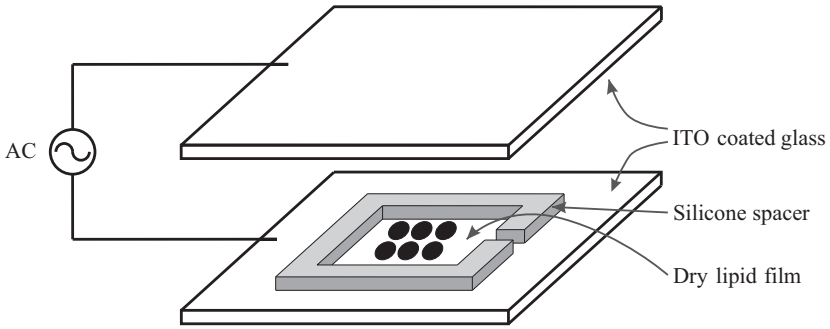

Fig. 1. Schematic diagram of the experimental setup for giant liposome electroformation.

adhesion of the photoresist to the silicon wafer. After that, a uniform thin layer of positive photoresist (AZ9260) was spin coated onto the silicon wafer. The recipe for the coating parameters, spin speed and coating time, was selected based on the desired etching depth, and the etching depth determined the coating thickness. Then the silicon wafer with the photoresist layer was prebaked at 110 °C on a hotplate for 4 min to remove the possible photoresist solvent. Second, a photomask with the desired patterns was aligned over the silicon wafer on a mask aligner and the wafer was exposed to ultraviolet light (UV light). The 5-in. photomask was made of soda lime and coated with chrome. An appropriate exposure type and the exposure time were selected according to the type of the photoresist and the thickness of the photoresist layer on the wafer. Finally, the exposed silicon wafer was developed with the developer AZ400K. It was carried out by immersing the wafer in the developer solution and gently agitating the container until the patterns could be seen clearly. The time for developing was estimated from the type of the photoresist and the coating thickness. After the development, the wafer was mildly rinsed with DI water and spin-dried to remove the residual solution.

Since positive photoresist AZ9260 was used, the exposed areas of the photoresist were soluble in the developer while the unexposed areas remained almost intact on the silicon surface. During dry etching, a thin layer of the silicon wafer, under the exposed areas and without the protection of photoresist, was removed by reactive ion etching (RIE). The etching depth is controlled by the etching time. As compared with wet etching, dry etching can result in better anisotropy of the patterns. After etching, the photoresist on the silicon wafer was thoroughly removed with acetone and rinsed with DI water. Finally, the silicon wafer with patterns on the surface was spin-dried and cut into small pieces (rectangular shape with the dimension of 4 mm × 15 mm) for the spreading experiments.

Two types of substrates were made with the grooves either parallel or perpendicular to the long edge of the substrate. According to the manner that the substrate is fixed in the spreading device, they are called "horizontal grooves" and "vertical grooves", respectively. The reason to make two types of substrates is that the spreading could not be observed or captured from the two orthogonal directions simultaneously. The spreading needs to be studied in the two directions separately. The horizontal grooves are used to study the spreading in the direction parallel to grooves while the vertical grooves are used to study the spreading in the direction perpendicular to grooves.

The dimensions of the grooves were characterized with a PLμ confocal imaging profiler (Sensofar, Spain). The width of both etched and non-etched parts was designed to be 4 μm and actually measured to be 4.5 μm and 3.5 μm averagely, respectively. The depth of the grooves was measured to be about 1.8 μm. Fig. 4 shows the two-dimensional and three-dimensional profiles of the grooves.

3.2. Spreading of giant liposomes on anisotropically patterned substrates

The spreading experiments were carried out at room temperature (24 °C) on a home-made spreading device whose fabrication has been described elsewhere [18]. The T-shaped spreading device

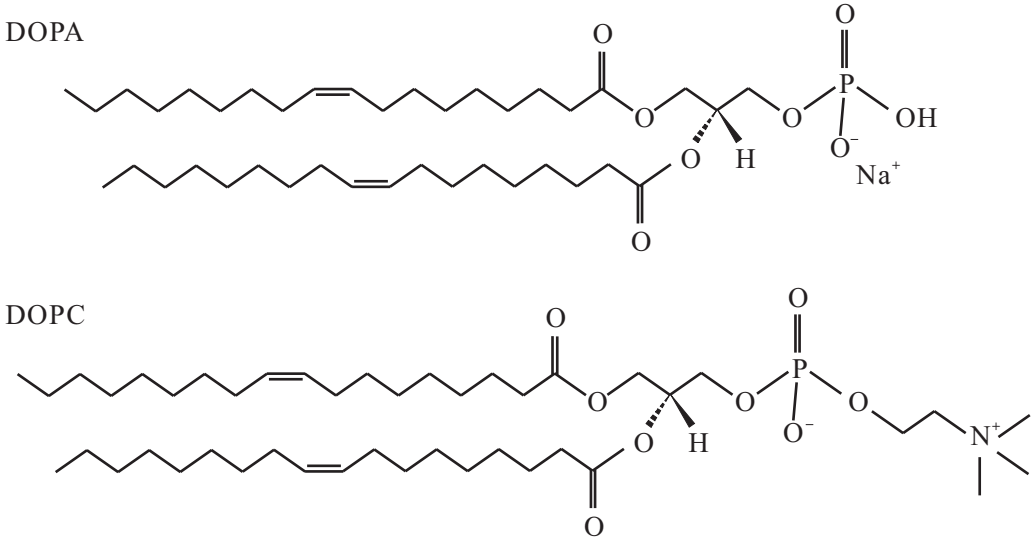

Fig. 2. Molecular structures of the lipids DOPA and DOPC.

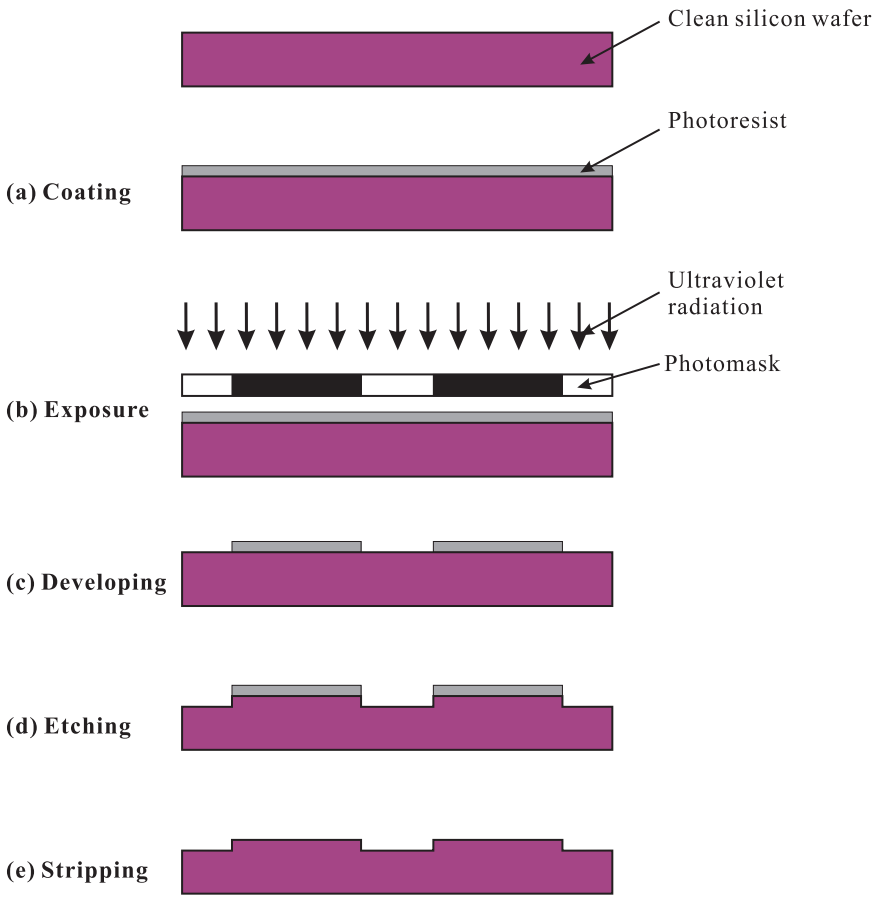

Fig. 3. Illustration of the microfabrication procedures to fabricate silicon substrates with unidirectional grooves.

is constituted by one piece of silicon substrate and two pieces of polymer sheets. In this work, the smooth silicon substrate was replaced with the patterned silicon substrate, as schematically shown in Fig. 5. The procedure of the spreading experiments on the patterned silicon substrates is similar to that on the smooth silicon substrates [18]. A small amount of the diluted liposome suspension (about 30 μl) was added along the silicon substrate under the microscope. A single liposome that appeared pure and spherical was selected and aspirated by a glass micropipette (Vacu-Tip, Eppendorf, Germany). The translational movements of the micropipette were controlled by a micromanipulator (UM-3FC, Narishige, Japan) which was fixed on the microscope stage. A microinjector (IM-6, Narishige, Japan) was used to generate the aspiration pressure. The aspirated liposome was transferred to approach to the silicon substrate until it touched the silicon surface. The micropipette was retracted immediately and the liposome spread on the substrate. The liposome spread until it reached its equilibrium state with the shape unchanged any more. The adhesion and spreading process was observed under the microscope and recorded by the CCD camera.

Fig. 6 shows the dynamic evolution of liposome shapes characterized by the change of contact radii when the liposomes spread on horizontal grooves and on vertical grooves, separately. In order to reduce the influence resulted from the size difference among different liposomes, contact radius is normalized by the initial radius of each liposome, written as $L_{\mathrm{lipo,h}}/R_{0,\mathrm{h}}$ and $L_{\mathrm{lipo,v}}/R_{0,\mathrm{v}}$, where $L_{\mathrm{lipo,h}}$ and $L_{\mathrm{lipo,v}}$ are dynamic contact radii in the parallel and perpendicular directions, respectively, and $R_{0,\mathrm{h}}$ and $R_{0,\mathrm{v}}$ are the initial radii of each liposome. Fig. 7 shows typical results of contact angles during the spreading process. The contact angles measured in this study are apparent contact angles that formed between lipid membranes and solid substrates. As shown from the experimental observations, after the liposome touches the solid surface, either on the horizontal grooves or on the vertical grooves, it quickly adheres onto the surface and starts to spread. After a short time of spreading, the contact radius increases while the contact angle decreases, and the liposome reaches its equilibrium state.

The experimental observations show that liposomes made of the mixture of DOPA and DOPC reached the equilibrium states within 5 s, which makes it challenging to fully record the details of the spreading process. Although the capturing speed of the CCD camera used in this study was at its highest value (20 fps) under the desired capturing condition, the detailed features of the spreading at the initial stage still could not be fully captured due to the fast evolution and the sheltering of the micropipette. Since the micropipette has an outer diameter of 100 μm at the tip, it may probably affect the observation of liposomes just after they touch the solid substrates. Thus, the initial stage of the spreading process cannot be experimentally characterized in this experiment.

As observed from the spreading experiments, liposomes exhibit anisotropy in spreading on the anisotropically patterned silicon substrates. Giant liposomes preferably spread along grooves, which was evidenced by the smaller equilibrium contact angles and larger dimensionless contact radii measured in the direction parallel to grooves. The average equilibrium contact angles in the two directions are $113^\circ \pm 2^\circ$ and $131^\circ \pm 4^\circ$, respectively. The typical curves in Fig. 7 show the change of contact angles with time.

When liposomes spread on anisotropically patterned substrates, they elongate along grooves. After having failed to compare with the shape of an ellipsoidal cap, it is found that the shape of the liposomes on the anisotropically patterned substrates cannot be described by a simple geometric shape, such as part of an ellipsoid.

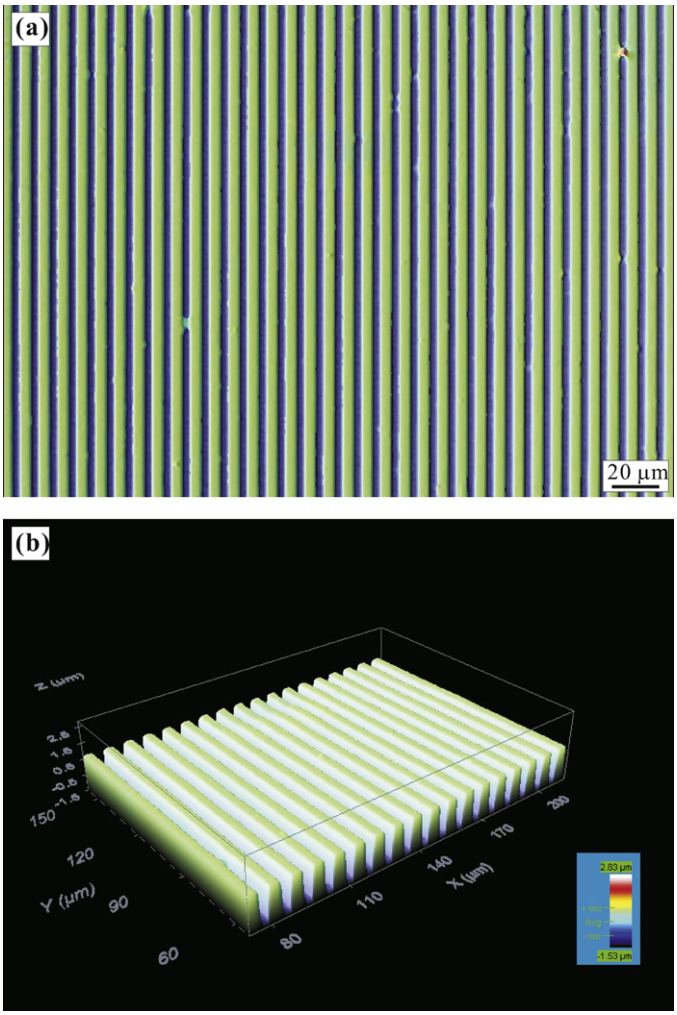

Fig. 4. Typical images of anisotropically patterned silicon substrates: (a) two-dimensional surface micrograph and (b) zoomed three-dimensional topography. Both horizontal and vertical grooves have similar groove shapes and groove dimensions.

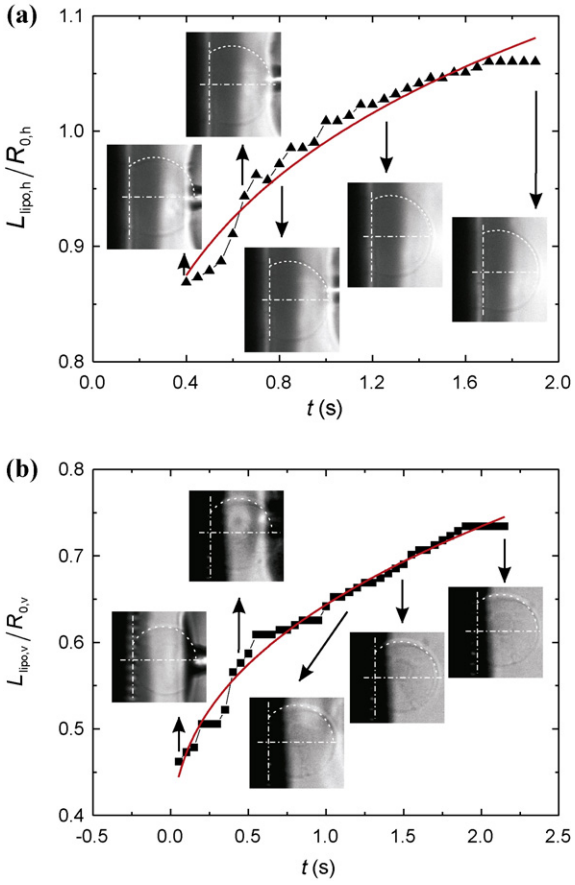

Fig. 6. Experimental results and microscopic images showing the change of the dimensionless contact radii during the spreading process. The scattered points represent the experimental results, and the continuous lines represent the curves fitted to the experimental results. Half of the liposome contour is highlighted with dashed lines in the microscopic images. (a) Spreading on the horizontal grooves (spreading in the direction parallel to grooves). The change of $L_{lipo,h}/R_{0,h}$ with t can be fitted by the relation $y = 0.991x^{0.136}$. (b) Spreading on the vertical grooves (spreading in the direction perpendicular to grooves). The change of $L_{lipo,v}/R_{0,v}$ with t can be fitted by the relation $y = 0.357 + 0.287x^{0.396}$.

To precisely describe the shape of an adhered liposome, further theoretical and numerical work is demanded [19]. However, the degree of the shape changes, such as the elongation of the adhered liposomes, can be characterized by the ratio of the average dimensionless equilibrium contact radii as shown in Eq. (1),

$$e = \frac{\overline{(L_{elipo,h}/R_{0,h})}}{\overline{(L_{elipo,v}/R_{0,v})}}, \quad (1)$$

where $L_{elipo,h}/R_{0,h}$ and $L_{elipo,v}/R_{0,v}$ are dimensionless equilibrium contact radii. The value of e was as high as 1.37 with $\overline{(L_{elipo,h}/R_{0,h})} = 1.08 \pm 0.10$ and $\overline{(L_{elipo,v}/R_{0,v})} = 0.79 \pm 0.08$, showing that liposomes were remarkably elongated by the grooves.

As shown in Fig. 6, the curves of $L_{lipo,h}/R_{0,h}$ and $L_{lipo,v}/R_{0,v}$ on the graph of L_{lipo}/R_0–t can be roughly fitted under power law. Mathematically, the evolution of the dimensionless contact radius is a function of the spreading time. Although it cannot physically

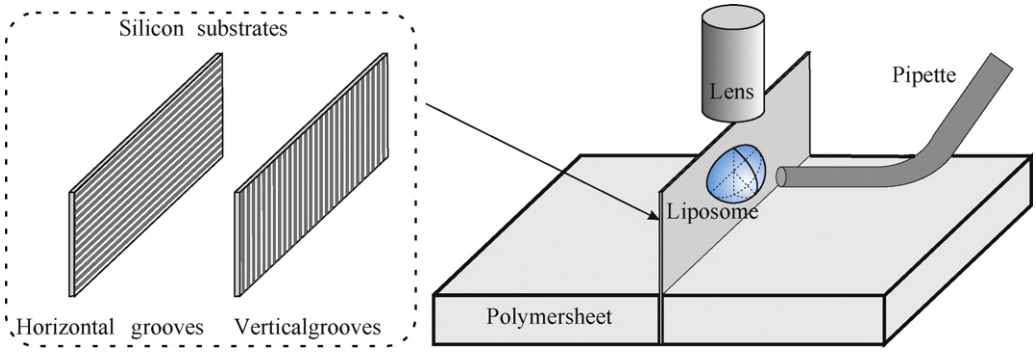

Fig. 5. Schematic diagram of the experimental setup for liposome spreading and the illustration of the two types of substrates.

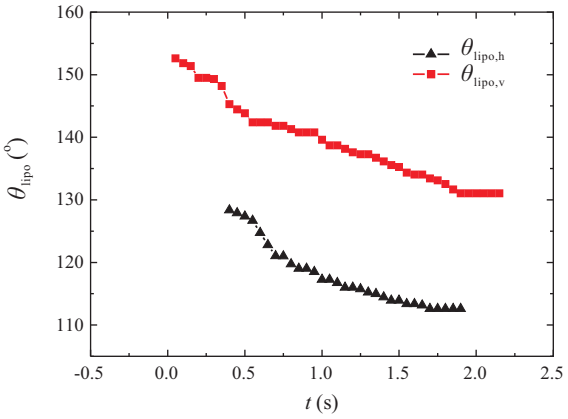

Fig. 7. Typical experimental results showing the change of contact angles during the spreading process.

describe the dependence of the dynamic contact radius on the spreading time, it helps in analyzing the spreading behaviors of giant liposomes on such solid substrates.

Comparing with the results obtained from liposome spreading on smooth substrates, the spreading behavior on the anisotropically patterned substrates is basically similar, but different in details. Liposomes spread fast in the initial period just after the spreading starts on all the substrates, and the spreading slows down with time until the liposomes reach their equilibrium states. On smooth substrates, liposomes exhibit the shape of a spherical cap with contact angles the same along the contact line. On the anisotropically patterned substrates, liposomes elongate along grooves with different contact angles in different directions. Besides, liposomes spread more smoothly in the direction parallel to grooves than in the direction perpendicular to grooves. It shows that the shape transformation behaviors of liposomes, such as spreading, can be tuned by adjusting the surface patterns of the solid substrates, which provides information for further applications of giant liposomes.

4. Conclusions

The dynamic spreading of giant liposomes on anisotropically patterned substrates has been experimentally investigated in this work. The spreading takes place on vertically fixed substrates with the gravitational force being negligible.

Liposomes spread preferably along grooves while they are trapped by the groove edges in the direction perpendicular to grooves. The difference in the equilibrium contact angles between the two directions has been found out to be 18°, and the elongation of the liposome has been characterized to be 1.37. The results show that the spreading is faster in the direction parallel to grooves, leading to the anisotropy in spreading, and the surface patterns of the solid substrates have great influence on the spreading behaviors of liposomes. This work may benefit those researchers who are interested in the anisotropic shape transformations of liposomes in relevant applications.

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