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# Electroformation of giant liposomes in microfluidic channels

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**Abstract**
We present an on-chip method to produce various types of giant liposomes using electroformation in microfluidic channels. These channels were sandwiched between glass slides coated with indium tin oxide (ITO) electrodes. Giant liposomes were formed inside the channels by applying an ac voltage. Important characteristics of the obtained liposomes were investigated quantitatively. We found that 54% of the liposomes produced by electroformation had diameters exceeding 10 $\mu$m and that 90% of the liposomes did not enclose extra liposomes inside themselves. Using two microfluidic channels, we found that giant liposomes with nano/micrometre-sized materials encapsulated were formed simultaneously on a chip.

**Keywords:** giant liposome, electroformation, microfluidic channels

(Some figures in this article are in colour only in the electronic version)

## 1. Introduction

This paper describes a method for simultaneously producing various types of giant liposomes on a single chip using an electroformation method in microfluidic channels. Liposomes are synthetic lipidic containers that have a similar membrane structure to a cell membrane and whose size ranges from 10 nm to 100 $\mu$m. Among the various sized liposomes, micrometre-sized liposomes (over 10 $\mu$m in diameter) are specifically called giant liposomes or giant unilamellar vesicles (GUVs) [1]. An advantage of giant liposomes over smaller liposomes is that they can be directly observed by conventional microscopy. Therefore, giant liposomes research provides information as to the structure and physical properties of individual liposomes in real time [2–4]. Another advantage of giant liposomes is that they have a size comparable to that of cells and have cell-like membranes; they can be used as artificial cells [1, 5]. Furthermore, since giant liposomes can encapsulate nano- or micro-functional materials, they can also be used as micro-sized containers for studying reactions and the transportation of bio-functional materials (e.g., reagents, DNA and proteins) [6–8].

Several preparation methods of giant liposomes have been developed [9]. The gentle hydration method is one of the conventional methods used to produce liposomes [10, 11], and this method involves the spontaneous swelling of dried lipid films in aqueous solutions. Another method called the electroformation method was developed later by Angelova and Dimitrov [12, 13]. This method involves applying external electric fields to cause the swelling of lipid films, probably due to the electro-osmotic vibrations of the medium, leading to the formation of unilamellar giant liposomes with relatively narrow distribution [14].

In this research, we aim to establish a preparation technique for several types of unilamellar giant liposomes using small amounts of lipids, reactants, or reagents on a chip. Our approach is to use an electroformation method within micro-sized fluidic channels. Previous work by Estes *et al* has reported electroformation of giant liposomes in millimetre-sized flow channels [15–17]. The flow channels allowed changes to buffers after the formation of giant liposomes. We used micrometre-size fluidic channels for preparing liposomes in the current research because: (i) extremely small amounts of reactants or reagents are needed to prepare liposomes in

  

a microfluidic device—this feature is particularly important when the reactants or reagents are expensive, and (ii) many channels can be produced on a single chip. Therefore several different types of liposomes (e.g., liposomes having different lipidic compositions or including different reagents) were prepared simultaneously on a single device.

We performed two experiments to prepare giant liposomes using microfluidic channels. In the first experiment, we focused on forming giant liposomes in a single channel using electroformation or gentle hydration methods. This experiment aims to evaluate the size distribution of liposomes and to determine the usefulness of liposomes as micro-containers by counting the number of liposomes with several/no liposomes inside; the liposomes without small liposomes inside themselves are able to encapsulate bio-functional materials efficiently. The characteristics of liposomes produced on a chip were analysed quantitatively. Numeral data are extremely useful to describe both the differences and similarities between different preparation methods, in addition to qualitative data (e.g. photographs [18, 19]). A confocal microscope was used in the experiments because measuring the diameters of liposomes and counting the number of liposomes with small liposomes inside can easily be performed. This results in the ability to obtain clear cross-sectional images using a confocal microscope as compared to using a phase contrast or fluorescence microscope [18–20].

In the second experiment, we used two microfluidic channels to assess the feasibility of preparing various kinds of giant liposomes efficiently by electroformation on a single chip. We demonstrated giant liposomes with nanometre or micrometre-sized beads encapsulated as bio-mimic materials were produced simultaneously in the same microfluidic device.

## 2. Materials and methods

### *2.1. Lipid film preparation*

Two types of phospholipids, egg yolk L-$\alpha$-phosphatidyl choline (EPC) 0.9 mg ml$^{-1}$ and L-$\alpha$-phosphatidic acid (EPA) 0.1 mg ml$^{-1}$ (Sigma-Aldrich), were diluted with a mixed solution of chloroform and methanol (2:1 in volume). They were stained with fluorescent DiI (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbo-cyanie perchlorate, ex/em: 549/564 nm, Molecular Probes). The lipid solution was prepared under a nitrogen atmosphere to prevent lipid oxidation.

We used a droplet deposition technique [13] to produce lipid films, even though uniform lipid films could be prepared by a spin-coating method using a lipid solution [16]. Advantages of the droplet deposition technique over the spin-coating method are that (i) it is simple; a spin coater is not required, (ii) only a small amount of the lipid solution is needed, and (iii) different types of lipid can be deposited on the same substrate. We used indium tin oxide (ITO) glass plates as the substrate (ITO thickness: 200 nm, resistance: 10 Ω/sq, custom coated by Shintoku-Glass, Japan). The lipid solution was evaporated under nitrogen, and then stored in a vacuum chamber for at least 2 h to completely evaporate the organic solvent. The average number of lipid bilayers was calculated from the amount of lipid and the surface area of the drop; we produced approximately 20 bilayers in our experiment.

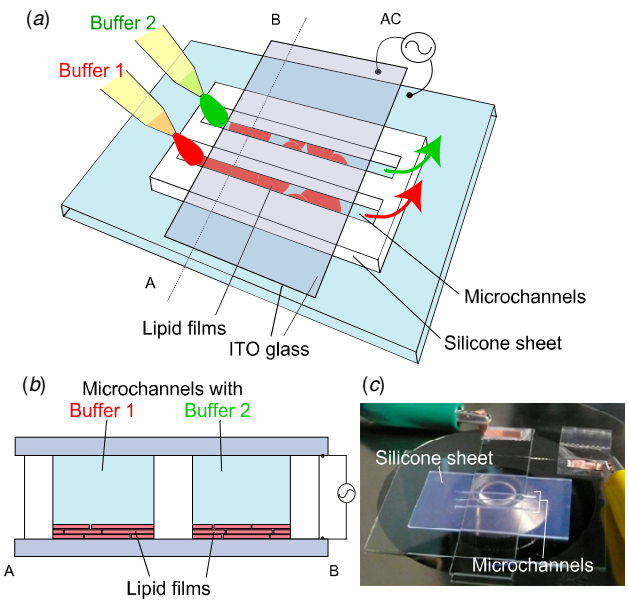


**Figure 1.** (*a*) Schematic of electroformation in a microfluidic device. (*b*) Sectional view along A–B. (*c*) Photograph of the device (in this case, width, depth and length of the microfluidic channels are 300 $\mu$m, 500 $\mu$m and 20 000 $\mu$m, respectively).

### *2.2. Microfluidic device preparation*

We used two kinds of devices for the liposome preparation. The first device was used to quantitatively evaluate the characteristics of the liposomes obtained. The device was based on a structure presented in previous research [13] and was produced using a polymethylvinylsiloxane sheet (silicone rubber sheet, GE Toshiba Silicones Co. Japan). The channel (30 mm × 60 mm × 1 mm) was simply sandwiched by two ITO glass plates (40 mm × 80 mm × 0.55 mm). A benefit of using ITO as an electrode is transparency; thus the formation of giant liposomes can easily be observed using a conventional microscope.

The second device was used to simultaneously produce various types of giant liposomes using the electroformation method on a single chip. As shown in the illustration of the microfluidic device in figure 1, thin lipid films were dried on the ITO glass plate (30 mm × 40 mm × 0.12–0.55 mm) by the method described above, and two parallel microfluidic channels formed in the silicone sheet were then sandwiched between the ITO glass plates. These micro-sized fluidic channels were easily produced using very simple methods; these methods are available in any lab and do not require any specific microfabrication process (e.g., photolithography). A channel was formed by cutting out the silicon sheet (500 $\mu$m in thickness) with two attached razor blades. Therefore, the width of the channel was determined by the width of the two blades. The mean width of each channel was 300 $\mu$m, and the volume of the channel was about 2.7 $\mu$l. Using this method, we produced three parallel microchannels, as shown in figure 1(*c*). Also, many microfluidic channels were easily reproduced within an error width of ±9%. As an alternative method, we made microfludic channels by aligning small strips of the silicone sheets at regular intervals on the substrate. These regular intervals were formed by using a

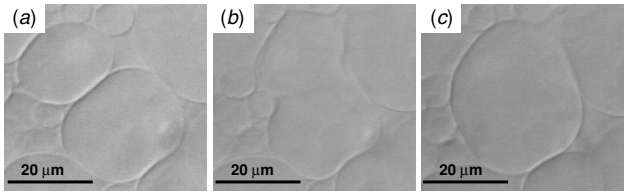


**Figure 2.** DIC microscopic images of liposomes (*a*) before, (*b*) during and (*c*) after the fusion of two liposomes when an ac field (1.5 V and 10 Hz) was applied for electroformation.

micro-thick spacer (we sandwiched the spacer between two strips on the substrate and then removed the spacer, so the strips could be aligned with micro-sized intervals). The error width of the channels produced using this method was ±26% when the mean width was 280 $\mu$m.

### *2.3. Liposome preparation*

For the electroformation method, after deionized water (Milli-Q system; Millipore, Japan) was introduced gently into the channels by a capillary force, an ac signal (sinusoidal - wave, 0.5 V peak-to-peak, 10 Hz) was applied to each ITO glass plate for a few seconds using a function generator (Agilent 33120A, Agilent Technology, US). The voltage was then raised to 1.5 V peak-to-peak and maintained for 2 h. The liposomes only formed inside the microchannels. For the gentle hydration method, the preparation was performed using the same conditions as for the electroformation method except for the application of the electric field.

### *2.4. Microscopic observation*

The giant liposomes were observed by using an inverted, differential interference contrast (DIC) and fluorescence microscope (IX-70, Olympus, Japan) equipped with cameras (C2741-79 Hamamatsu Photonics Co. or DXC-C33 Sony Co., Japan) and video recording equipment and analysed using image processing software (AquaCosmos, Hamamatsu Photonics Co., Japan). Fluorescence images were taken with a double band green and yellow excitation filter (U-DM-FI/TR, Olympus Co., Japan). A confocal microscope (CSU 22, Yokokawa Elect. Co., Japan) was also used for the cross-sectional observation of the liposomes. Diameters of the liposomes were measured by using Image-Box software (Library Co., Japan).

## 3. Results and discussion

### *3.1. Characterizing giant liposomes in microchannels: electroformation versus gentle hydration*

We noticed that the lipid films vibrated immediately after the application of the ac field in the electroformation method. We also noticed that some liposomes were formed within a few minutes. As indicated in the sequential images of the fusion process shown in figure 2, two liposomes fused almost instantaneously to form a large vesicle. Such dynamic fusion processes were observed frequently within the first 20–30 min. In contrast, this fusion process was not observed

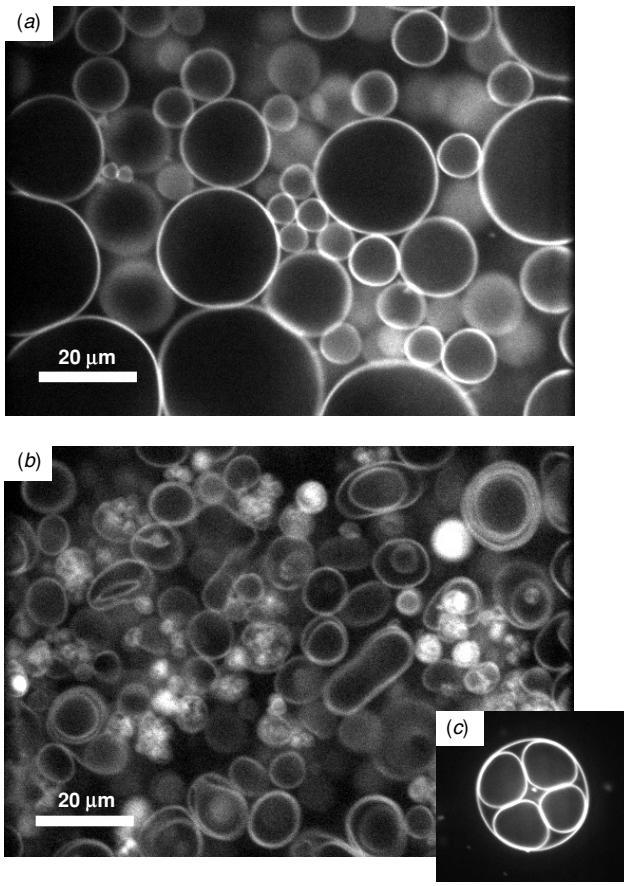


**Figure 3.** Confocal images of liposomes obtained (*a*) by electroformation after 2 h of an applied ac field (10 Hz and 1.5 V), and (*b*) and (*c*) by gentle hydration 2 h after having introduced deionized water (pH = 6.0). Image (*c*) shows an individual liposome containing four smaller liposomes.

in liposomes produced using the gentle hydration method. The liposomes were formed spontaneously when the dried lipid films were hydrated and swollen in aqueous conditions due to the presence of hydrophobic terminations in the lipid structure. The presence of small liposomes inside large liposomes was observed in this preparation. It took more than 2 h to produce many giant liposomes using the gentle hydration method, while it took tens of minutes using the electroformation method.

Figure 3(*a*) shows confocal fluorescence images of liposomes produced by electroformation in the channel. The white circular lines represent cross-sectional views of lipid membranes. For comparison, liposomes produced using the gentle hydration method are also shown in figures 3(*b*) and (*c*). The images were taken 2 h after applying the ac field in the electroformation method and 2 h after adding the buffer in the gentle hydration method. Unlike the images shown in figure 3(*c*) where four extra small liposomes were enclosed in a large liposome as a result of the gentle hydration method, no large liposomes with smaller liposomes inside as a result of electroformation are shown in figure 3(*a*). Figure 4(*a*) shows the number of liposomes that contained several/no liposomes. About 90% of the electroformed liposomes did not contain any extra small liposomes. These results indicate that giant

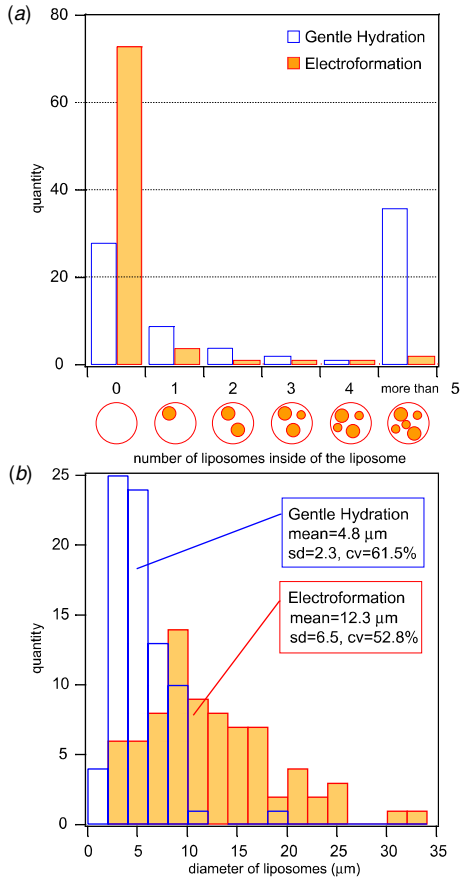


**Figure 4.** (*a*) Number of liposomes with several/no liposomes inside and (*b*) liposome diameter distribution. Results were obtained by observing the cross-sectional images of produced liposomes at their maximum diameter for 80 randomly chosen liposomes. Liposomes with diameters less than 1 $\mu$m were not counted.

liposomes formed by electroformation are more suitable to use to encapsulate bio-functional materials than to be used as reactors or containers.

Figure 4(*b*) shows the distribution of liposome diameters. The diameters were measured from cross-sectional images of the liposomes collected from the channel using a confocal microscope at $\times$60 magnification. The estimated error of the measurement was about $\pm$2 pixel; a pixel is equivalent to $\pm$0.2 $\mu$m when the magnification is $\times$60. As a result, the mean liposome diameters produced by gentle hydration or electroformation were 4.8 or 12 $\mu$m, respectively. About 54% of the electroformation liposomes were larger than 10 $\mu$m, in comparison, only about 6% of the liposomes formed by the gentle hydration method were larger than 10 $\mu$m. This size is probably due to the fusion of liposomes during the electroformation process (figure 2), resulting in the production of larger liposomes.

### *3.2. Electroformed giant liposomes in double microfluidic channels*

Solutions with nanometre- or micrometre-sized (200 nm or 1 $\mu$m in diameter) polystyrene beads (Bangs Laboratories, Inc.) at a concentration of $10^9$ particles ml$^{-1}$ were introduced into each channel (figure 5(*a*)). In this case, the channels were made by aligning several small strips of the silicon sheets, as explained in section 2.3. The width of the channel was about 360 $\mu$m with a volume of 5.4 $\mu$l. Giant liposomes enclosing nano- or micro beads were simultaneously formed inside each channel (figures 5(*c*)–(*e*)). As illustrated in figure 5(*b*), we believed that the beads went through the moisturized and separated lipid films, and then became entrapped inside the giant liposomes when they were formed. The number of beads inside the liposomes could not be controlled, with many beads remaining unencapsulated after the process.

As shown in figure 6(*a*), we also attempted to mix 500 nm diameter metal-bound polyimine beads (details of their fabrication are described elsewhere [21]) with a mixed lipid and organic solvent instead of mixing the beads in an aqueous solution. Figure 6(*b*) shows that many more beads were encapsulated in giant liposomes and that the beads seemed to be easily encapsulated inside the liposomes. This method

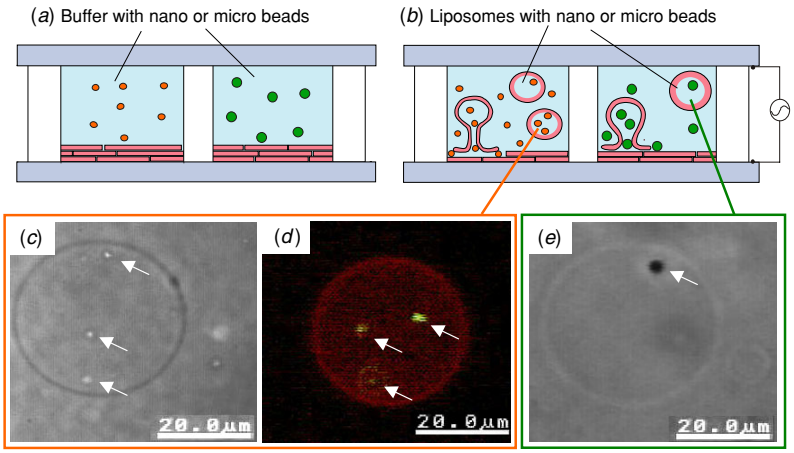


**Figure 5.** (*a*) Nanometre- or micrometre-sized polystyrene beads were added with degassed water and introduced into each channel. After an ac field was applied (*b*) giant liposomes enclosing these beads were produced in each channel. (*c*) Three 200 nm beads, (*d*) two green-fluorescent 200 nm beads and (*e*) an individual 1 $\mu$m bead were encapsulated in giant liposomes. Arrows indicate beads.

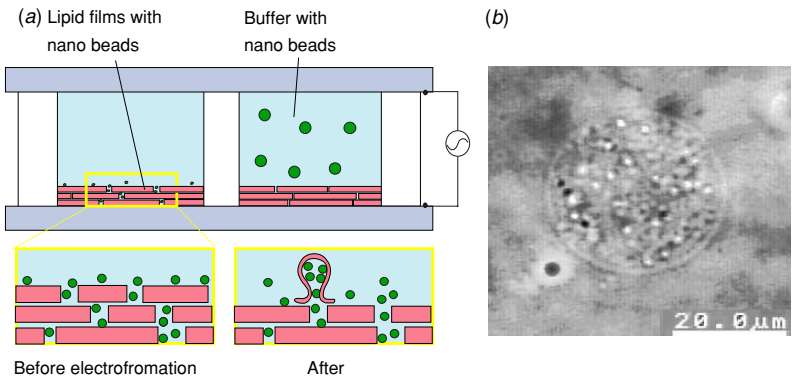


**Figure 6.** (*a*) Schematic view in which beads of 500 nm diameter were mixed with the lipid solution. (*b*) Many beads were encapsulated in a giant liposome by electroformation.

is a useful way to mix beads in the lipid solution to efficiently produce liposomes with beads. Note that this method is limited because of the compatibility of organic solvent used.

## 4. Conclusion

Using the electroformation method, we efficiently produced giant liposomes without enclosing extra smaller liposomes inside the giant liposomes. The proposed fabrication process of the microfluidic channels is extremely simple and quick; therefore can be easily used by researchers not familiar with microfabrication processes. The electroformation method with microfluidic channels allowed us to prepare giant liposomes having different lipidic components and/or encapsulating different types of micro/nano materials with only using a minute amount of solution on a chip. We believe that this technique will be useful in various research fields, including membrane physics, membrane protein analysis, microreactors and artificial cell studies.

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