研 究 速 報

A Microfluidic Device for Electrofusion of Biological Membranes

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1. INTRODUCTION

Background and objectives

Since the early 80s, the use of electric pulse techniques in biotechnology and medicine has attracted an enormous interest for a wide variety of applications such as gene transfection, hybridomas for antibody production, cell sorting, or even fundamental biophysical studies of membrane proteins. In this framework, the utilization of synthetic lipid-bilayer containers—the so-called liposomes—appears to be flexible and attractive: they are easy to prepare and to handle, they can encapsulate chemicals or artificial microstructures and their membrane is able to mimic the response of natural vesicles.

If high enough, an electric field can transiently induce the formation of tiny pores through the membrane of liposomes or cells[1]. When some of these pores, belonging to two different vesicles, are put in contact, they are likely to reconnect together. As a result, the vesicles experience a reorganization of their membranes which have been merging. A large hybrid is formed inside which the contents of the parent vesicles have been mixed together.

The device must be designed in such a way the electric field is sufficiently high to perform the membrane breakdown. Since it scales roughly as the voltage over the gap between the electrodes, the power consumption can be lowered significantly for small dimensions. Therefore, a micrometer-sized device appears to be quite suitable for industrial applications.

Our ultimate goal is then to utilize liposomes as a delivery vehicle to transfer micromachined or biological materials into cells by liposome-cell electrofusion in a MEMS device.

Protocol of electrofusion

A schematic view of the experimental protocol is depicted in Figure 1. First, the liposomes—or the cells—are introduced into the channel and an AC voltage is applied for a few seconds. This phase allows to obtain a 'pearl-chain' alignment of liposomes

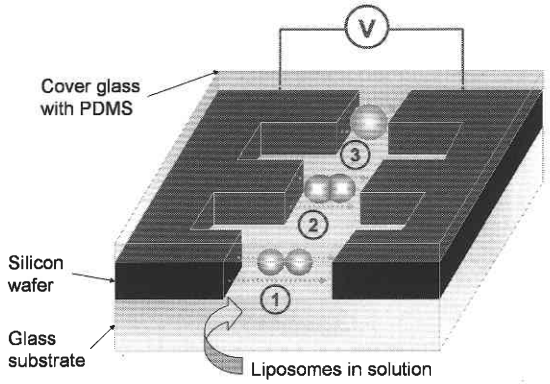

Figure 1 Schematic view of the device and protocol to fuse liposomes. Various gaps of electrodes have been designed. 1) Liposomes are aligned along the electric field lines by AC voltage. 2) DC pulses inducing high electric field perform the breakdown of membranes. 3) These membranes reconnect to form a hybrid vesicle.

along the electric field lines. Indeed, a sphere suspended in some dielectric fluid and subject to a uniform AC electric field, becomes polarized and is accordingly equivalent to an electrostatic dipole[2]. The mutual forces of interaction can be attractive and lead to chain formation where particles inevitably encounter each other. Afterwards, a series of short and strong DC pulses disrupts the membranes in the region of contact of liposomes and initiates the fusion[3].

2. MICROELECTRODE DESIGN

Requirements for electrofusion and power source coupling

Recent works have dealt with the electrofusion of liposomes using a pair of solid carbon fiber electrodes positioned by micromanipulators[4]. Our purpose is to utilize fully micromachined electrodes integrated in a microfluidic device which can perform fusion in a much more routinely way while demanding a low power level.

Our early devices consisted of gold-evaporated electrodes patterned on parylene-coated glass substrate. Surprisingly, the alignment of liposomes could be achieved but electrofusion

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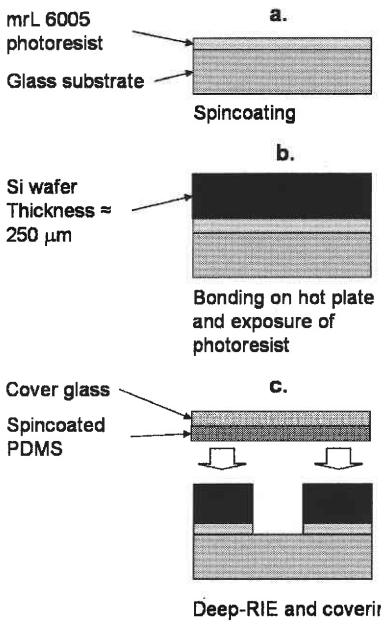

Figure 2　Fabrication process of the microelectrodes.

remained unsuccessful. It was believed that since with such a process the thickness of electrodes was very small—typically 100 nm- with respect to the liposome size, the electric field was quite non-uniform on a cross-section of the device. Consequently, the membrane disruption did not occur at the same location for two distinct vesicles that made it difficult their proper reconnection. Thus, it was thought necessary to design high aspect-ratio electrodes so that the induced electric field may be uniform across the microfluidic channel.

A straightforward method involved SOI wafer patterned by deep-RIE. Unfortunately, due to the capacitance effect of the insulated layer between electrodes and silicon substrate, the input impedance of the device became prohibitively low at the required frequency for the power source to supply enough current. In short, the device must have input impedance larger than 1 kΩ - according to the specifications of our power equipment (LF-101, Nepa Gene Company, Ltd., Tokyo) - at frequencies in the range of 100 kHz-1 MHz as demanded by the electrofusion protocol.

Fabrication process and electrical characterization

The process is shown on Figure 2. A 250μm thick silicon wafer (resistivity below 0.01Ω.cm) was bonded to a glass substrate by using negative photoresist (mrL-6005, micro resist technology). The gap between the electrodes varied from 500μm to 30μm. High aspect-ratio structures could then be efficiently fabricated without resorting to cumbersome procedures like electroplating. This technique offers the both advantages to fulfill the above requirements and to be carried out very easily. The channel was simply covered by a PDMS-coated glass slide.

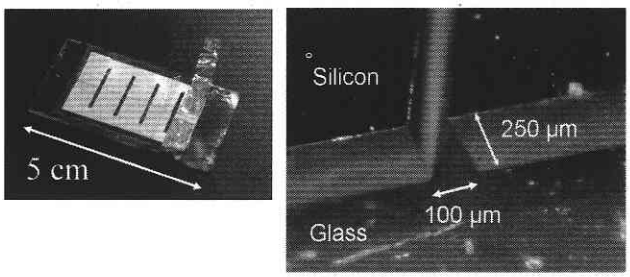

Figure 3　Photograph of the microfluidic device and close-up view of the high aspect-ratio silicon microelectrodes.

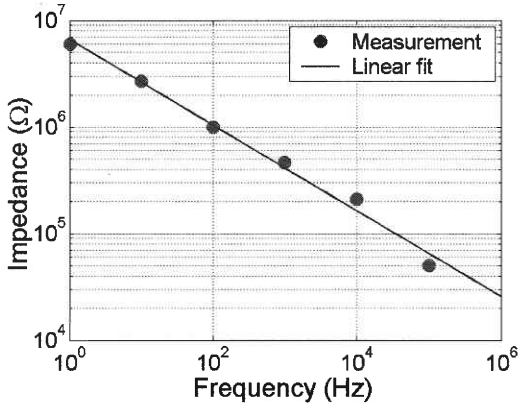

Figure 4　Measured impedance of microelectrodes with liposome solution versus frequency. At 300 kHz, it is about 40 kΩ which is compatible with the specifications of our power source.

Photographs presenting the real device are shown on Figure 3.

As highlighted previously, the electrode impedance, measured with a solution of liposomes, is of great impedance for current supply. Figure 4 demonstrates that around 300 kHz—optimal frequency for liposomes alignment—its value is 40 kΩ, i.e. fully compatible with the requirements of the power equipment. Note that the slope of impedance as a function of frequency differs from a capacitance behavior because of the complex response of liposomes in a dielectric medium.

3. LIPOSOME ELECTROFUSION EXPERIMENTS

Preparation of liposomes

There are several manners to prepare liposomes depending on the desired size and biophysical properties[5]. The so-called 'reverse-phase evaporation' procedure has been chosen because it allowed the efficient entrapping of a whole variety of materials including artificial objects. Two types of phospholipid, 0.9 mg/mL L-α-phosphatidylcholine and 0.1 mg/mL L-α-phosphatidic acid from Sigma-Aldrich, were diluted under nitrogen into a mixture of chloroform and methanol 2:1. After rough removal of the organic solvent by rotary evaporation (N-1000, Eyela, Tokyo), lipids were placed into a vacuum chamber for several hours. The buffer, 10 mM KCl and 200 mM glucose, was there-

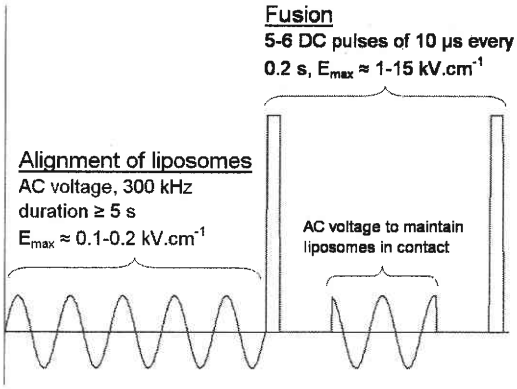

Figure 5　Voltage waveform for the electrofusion of liposomes.

after gently poured onto the thin film of dry lipids and giant unilamellar liposomes were obtained after 2 hours storage at 4°C. Note that a poorly conductive buffer was preferable to avoid electroosmotic flows during voltage application.

Voltage waveform

It has been found that the chain formation of liposomes occurred preferentially at frequency of 300 kHz for electric field intensity of 0.1–0.2 kV.cm^{-1} (Figure 5). The electric field herein is estimated as the ratio of the applied voltage to the distance between the electrodes. Five seconds were at least necessary to make the liposomes in close contact. Membrane breakdown was achieved by 5 or 6 short pulses of 10μs duration at interval of 0.2 s with electric field intensity over 5 kV.cm^{-1}. AC voltage was still applied during this phase to maintain liposomes in contact.

Figure 6 illustrates a real sequence of liposome fusion from the alignment of vesicles to the membrane reconnection subsequent to their breakdown.

Fusion yield

The experimental yield of liposome fusion obtained in a microdevice with 100μm gap electrodes is represented in Figure 7 for both large and small vesicles. The fusion yield is defined as the probability to fuse two liposomes by applying the voltage waveform described in the previous section.

This graph exhibits a threshold value on the electric field, much more clearly though for small liposomes than for large ones, at 4–5 kV.cm^{-1}. In both cases, high electric field intensities are more favorable perhaps due to the formation of more pores through the membranes. Nevertheless, over 10 kV.cm^{-1} the membrane structure may be damaged or even destroyed and accordingly, the slope of fusion yield flattens. For this reason, the efficiency never reaches 100% and it can be guessed the curve will drop at very high electric field intensities.

The fact that small liposomes fuse together less easily is not well understand yet. However, some groups have observed the successful fusion of large cells with tiny liposomes (≈200 nm in

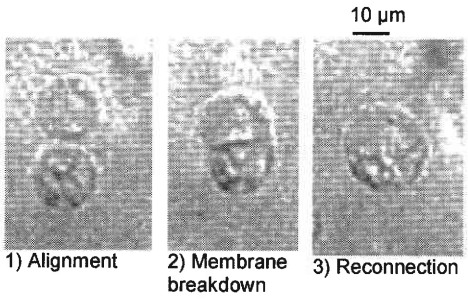

Figure 6　Experimental sequence of liposome fusion.

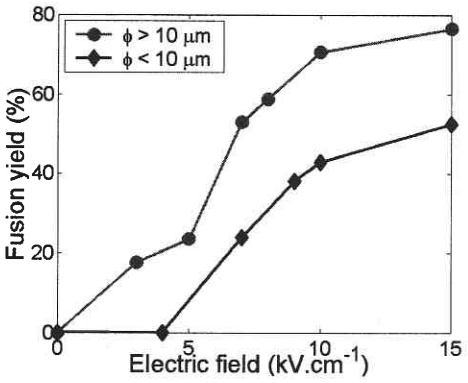

Figure 7　Experimental fusion yield as a function of the applied electric field for liposome diameters ϕ larger and smaller than 10μm.

diameter) and they have conjectured that destabilization of only one of the two membranes of the partners was sufficient for fusion[6]. Thus, provided that at least one container is large—giant liposome for instance-, it might be also possible to fuse it with smaller vesicle such as a cell. This paradigm might be important for liposome-cell fusion and for their related applications.

4. TOWARDS TRANSFER OF MICROSTRUCTURES

Electrofusion of prokaryotic cells

Very early work on cell-cell electrofusion in a micromachined system was addressed by Masuda *et al*[7]. We present here our preliminary conclusions in this field.

Experiments carried out with bulk electrodes- i.e. non-microfabricated electrodes- pointed out the difficulty to disrupt cell membrane depending on its structure. Our low fusion rate on *E. coli* bacteria suggested the membrane fluidity was a key factor. Fusion among liposomes, which had no proteins or macromolecules on their surface and which had a good fluidity, was relatively easy. But for bacterial spheroplasts, owing to the presence of cytoskeleton backside the membrane, this latter was less likely to breakdown and to reconnect with neighboring cells.

Therefore, we focused on vesicles whose the membrane was soft and fluid enough to allow pore formation and structural reorganization subsequently to electrical stimulations. Figure 8

depicts an example of electrofusion of provacuoles prepared from *E. coli*. The conditions of electrofusion were similar to liposome's ones except for more (20) and longer (90μs) DC pulses. Even though the membrane reconnection took place, the reorganization into a proper spheroplast was still hindered by the membrane stiffness.

Encapsulation and transfer of microbeads into liposomes

Within the framework of microstructure delivery from liposome to cell, the possibility of entrapping artificial materials inside a liposome and of transferring it through the membrane of another one has been assessed.

Thanks to the 'reverse-phase evaporation' protocol, we were able to prepare liposomes containing polystyrene microspheres. The buffer was this time prepared with a suspension of 0.32μm microbeads (Bangs Laboratories, Inc.) at a concentration of 6.10^8 spheres.mL^{-1}. The dry lipidic film being moisturized by that solution, lipid-bilayer vesicles spontaneously formed and entrapped some of the surrounding microbeads. Obviously, the number of microspheres per liposome could not be controlled and many of them remained unentrapped after the process.

The electrofusion of bead-loaded liposomes with empty ones is shown on Figure 9.

In this case where the solution contained a lot of dielectric microspheres, the liposomes appeared to be much more sensitive to the applied electric field. Even at a quite low voltage—equivalent to an electric field intensity of 0.5 kV.cm^{-1}-, the destruction of membranes sometimes occurred as well as the fusion of vesicles in AC phase. This amazing phenomenon is thought to arise from the field constriction created in the vicinity of dielectric microbeads and which may amplify locally the electric field. As a consequence, the nominal fusion parameters differed from the previous experiments: the alignment was achieved at 100 kHz for 0.05 kV.cm^{-1} of electric field intensity, and the fusion occurred for amplitude of 1.5 kV.cm^{-1}. The pulse durations were the same.

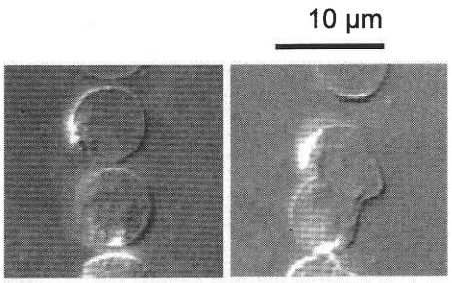

Figure 8　Electrofusion of *E. coli* provacuoles with bulk electrodes.

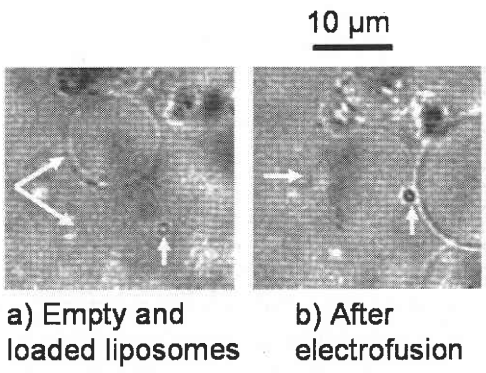

Figure 9　0.32μm microbeads transfer into an empty liposome by electrofusion with bulk electrodes.

5. CONCLUSION

A microfluidic device for the electrofusion of liposomes has been successfully designed and tested. Fusion yield can reach up 75% for large vesicles. Experiments performed with bulk electrodes have demonstrated the possibility to fuse prokaryotic cells together. However, their membrane was not soft enough to allow high fusion rates and future attempts should use other types of cells instead. That will enable thereafter to carry out artificial structure delivery since the concept has been demonstrated by utilizing liposomes. The last step will apply this protocol to our microdevice in view of efficient microstructure delivery with low power consumption.

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