Microfluidic trapping of giant unilamellar vesicles to study transport through a membrane pore

T. Robinson, P. Kuhn, K. Eyer, and P. S. Dittrich

Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich 8093, Switzerland

(Received 7 February 2013; accepted 10 July 2013; published online 26 July 2013)

We present a microfluidic platform able to trap single GUVs in parallel. GUVs are used as model membranes across many fields of biophysics including lipid rafts, membrane fusion, and nanotubes. While their creation is relatively facile, handling and addressing single vesicles remains challenging. The PDMS microchip used herein contains 60 chambers, each with posts able to passively capture single GUVs without compromising their integrity. The design allows for circular valves to be lowered from the channel ceiling to isolate the vesicles from rest of the channel network. GUVs containing calcein were trapped and by rapidly opening the valves, the membrane pore protein α -hemolysin (α HL) was introduced to the membrane. Confocal microscopy revealed the kinetics of the small molecule efflux for different protein concentrations. This microfluidic approach greatly improves the number of experiments possible and can be applied to a wide range of biophysical applications. © 2013 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4816712>]

I. INTRODUCTION

The cell membrane regulates all transport of biomolecules into and out of the cytosol. This lipid membrane also contains a myriad of receptors that transduce an environmental signal into the cell which triggers an appropriate response. For this reason, biological membranes are studied intensively in order to understand these processes and therefore aid drug discovery as well as answer fundamental biological questions. In this context, giant unilamellar vesicles (GUVs) present an excellent model system for cell membranes and have become an important tool in biophysical research.^{1,2} Given the range of available model membrane systems (e.g., monolayers, black lipid membranes (BLMs), supported planar bilayers, and liposomes) GUVs are often the preferred choice. Planar supported bilayers, for example, are known to suffer from artifacts due to interactions with the required solid substrate. GUVs, on the other hand, do not require such a support and are considered more biologically relevant.³ Their applications are numerous and include membrane protein research, artificial cells, membrane fusion, drug discovery and investigations of nanotubes.⁴ Using multiple lipid species has even allowed observations of lipid domain behaviour and formation,⁵ therefore giving valuable insights into the behaviour of lipid rafts thought to exist in cell membranes.

There are various ways to prepare GUVs, with electroformation being the most favourable due to the unilamellarity of the vesicle membranes and the fast production time.^{6,7} Once formed, they can be investigated using standard optical microscopy and even spectroscopic techniques such as fluorescence correlation spectroscopy.⁸ The disadvantage, however, is the difficulty in handling these delicate objects. A common approach is to allow GUVs with a more dense fluid inside (e.g., sucrose) to sediment onto a glass surface in a less dense solution (e.g., glucose) where they can be observed. However, this makes it difficult to supply reagents without flushing the GUVs away. A functionalized surface can solve this problem by biochemically tethering the vesicles but typically requires the addition of artificial lipids to the membrane which is not always appropriate.^{9,10} We recently solved this problem using a protocol that allows the immobilisation of unmodified lipid enveloped objects (including liposomes,

bacteria, and virus particles).¹¹ The drawback of using molecular linkers, however, is the need for additional experimental steps for surface modifications (e.g., microcontact printing). Moreover, they are not always practical for some applications as the bonds are not sufficiently robust when subjected to the high flow rates (and thus high shear stress) within flow-cells often used in GUV experiments.

Another method to spatially confine GUVs is to manipulate them using micropipette aspiration. While useful for investigating membrane fusion,¹² membrane line tension,¹³ and curvature driven lipid and protein sorting,^{14,15} micropipette aspiration has its limitations: (i) the vesicles must be under constant tension which could affect the outcome of the experiment; (ii) only one or two vesicles can be held simultaneously therefore limiting the throughput; (iii) rapid and homogeneous fluid exchange is not possible due to the required bulk environment.

Microfluidic systems offer an alternative way to confine vesicles using laminar flow as demonstrated by Deschamps *et al.*¹⁶ This method can be used to study the dynamics of GUVs subjected to flow but is not suitable for studies requiring rapid fluid exchange. Our approach solves the above issues by using a microfluidic device with multiple hydrodynamic traps situated within the channels.¹⁷ Hydrodynamic trapping of single mammalian cells has been reported previously,¹⁸ but despite being similar in size, cells and vesicles possess different elasticities and membrane stabilities. Cells usually retain their shape under shear stress, whereas GUVs can deform which is not always desirable.¹⁹ Hence, trapping using these regular microfluidic cell traps is not efficient as GUVs are lost or pass through geometric traps easily. We solved this issue by diverting the fluid flow around single traps in a circular chamber after the GUVs have been trapped (Fig. 1). The traps are made from two polydimethylsiloxane (PDMS) posts positioned adjacently and are able to spatially confine single GUVs. The device consists of an array of chambers each containing a trap and therefore allowing up to 60 vesicles of a similar size to be immobilized simultaneously.

As an additional feature, we integrated “donut” shaped valves positioned above the traps that can be lowered by pressurizing a separate microchannel network. This allows isolation of each trapped vesicle from the rest of the channel network, leading to a defined microenvironment where the GUV is under no shear stress. By exchanging the solution inside the chip and subsequently opening the valves, we can perform fast kinetic studies with negligible concentration gradient of the added reagent. To demonstrate the feasibility of using the device for membrane biophysical studies, we encapsulated calcein within GUVs and using the donut valves we

FIG. 1. Microfluidic device design. (a) Schematic showing a trapped GUV isolated by a donut valve. (b) Enlargement showing the fluid flow being diverted around the trap in the presence of a GUV, before the donut is lowered. (c) Bright-field image showing 30 of the 60 micro-chambers with a height of 20 μm . Fluidic isolation by the donuts is demonstrated using blue and red food dye at various dilutions. Scale bar: 500 μm . (d) Schematic cross-section illustrating the washing step and the function of the valves.

added the membrane pore protein alpha hemolysin (α HL). This permitted rapid fluid exchange which allowed repeated observations of kinetics as the small molecule was transported across the membrane and released into the donut volume.

II. MATERIALS AND METHODS

A. Chemical reagents

Sphingomyelin (SM), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabama, USA). Calcein was purchased from Fisher Scientific AG (Wohlen, Switzerland). Texas Red[®] labeled streptavidin, fluorescent beads, membrane probe DiI and PBS buffer were all obtained from Invitrogen. α -hemolysin from *Staphylococcus aureus* and bovine serum albumin (BSA) were purchased from Sigma Aldrich. PDMS and curing agent (Sylgard 184) were purchased from Dow Corning (MI, USA). SU-8 2010 & 2015 photoresist was obtained from Microchem Corp (MA, USA). Methanol and chloroform were from Acros Organics.

B. GUV preparation

GUVs were created by electroformation.⁷ SM, DOPC, and Chol were dissolved in chloroform/methanol (9:1 v/v) in a molar ratio of 0.4:0.4:0.2 and at a concentration of 1 mM. DiI was added at a concentration of 1 μ M and 2.5 μ l of the mixture was deposited on a conductive indium tin oxide (ITO) slide (15–25 Ω /sq, Sigma-Aldrich). This was repeated in 12 locations separated by a 1.5 mm thick silicone rubber spacer to maximize the yield of vesicles. The lipid film was then dried in a vacuum overnight and hydrated with MilliporeTM filtered water containing 100 μ M calcein or 200 nM Texas Red labeled streptavidin. The chambers were sealed by a second ITO slide and held within a custom-built heating device set to 60 °C. GUVs were formed by applying 0.7 V at a frequency of 10 Hz for 4 h using a function generator (HMF2525, Hamag). After applying 1 V at 4 Hz for 30 min to detach the vesicles from the surface, harvesting was achieved by careful pipetting. GUVs were stored at room temperature and used within 48 h.

C. Microfluidic chip fabrication

The microfluidic chips were fabricated in PDMS and consisted of a two-layer design.²⁰ The upper layer was used as a control layer, while the bottom layer served as the fluid layer. Fabrication of the device was achieved using multilayer soft lithography as previously described.¹⁷ Briefly, master forms at heights of 10 and 20 μ m were produced on a silicon wafer using SU-8 2010 or 2015 by exposure to UV light source through a chrome or film mask respectively. PDMS was prepared by mixing oligomer and curing agent at a ratio of 10:1 and cured on the first master form at 80 °C for 3 h. For the bottom layer, the PDMS mixture was spin-coated at 2000 rpm onto the second master form to a height of approximately 40 μ m and cured at 80 °C for 1 h. The upper layer was cut to size and holes were punched with a 1 mm diameter biopsy puncher (Miltex, PA, USA). Both layers were then oxidized by an air plasma, quickly aligned under a microscope and bonded overnight at 80 °C. After punching fluidic access holes with a 1.5 mm biopsy puncher (Miltex, PA, USA), the microchannels were completed by bonding the PDMS to a glass slide using a plasma cleaner (PDC-32 G, Harrick, NY, USA).

D. Chip preparation and operation

Before introduction of GUVs, the bottom layer channels were filled, via centrifugation, with 2% (w/v) BSA solution in PBS to coat the channels. This prevents vesicle rupture upon contact with the walls. After 30 min, the BSA was exchanged with Millipore filtered water using a syringe-pump (neMESYS, cetoni, Germany). The same pump was used to draw the GUV solution and reagents through the fluid channels during the experiments. To close the donut valves,

3 bars of pressure were applied to the upper layer with a custom-built pressure control instrument.

E. Microscopy

Wide-field microscopy was performed with an inverted microscope (IX70, Olympus) equipped with a mercury lamp and a 40x/0.65 NA air objective lens. Images were recorded with an EMCCD camera (iXon DV887, Andor). Fluorescence signals were monitored using appropriate optical filter sets for DiI and calcein. Optically sectioned microscopy was performed using a confocal laser scanning microscope (Axiovert 200 M, Zeiss) with a 63x/1.4 NA oil immersion objective lens. Calcein fluorescence was recorded using 488 nm Argon ion laser line, HFT UV/488/535/633 dichroic, 505 nm long-pass filter. DiI and Texas Red fluorescence was recorded using 561 nm diode laser, HFT 405/561 dichroic, and 575 nm long-pass filter.

III. RESULTS AND DISCUSSION

A. Trapping of GUVs and fluidic exchange

The device contains 60 chambers fabricated in PDMS with posts acting as hydrodynamic traps positioned at the centre. The chambers are split between 8 separate channels (Fig. 1) each containing a circular valve shaped like a donut. These “donuts” can be lowered by pressurizing a second PDMS control layer, above the main fluidic layer, to isolate the trap from the rest of the microchannel network.¹⁷ The hydrodynamic traps consist of two posts which, when unoccupied, allow the fluid flow to pass freely between. When occupied, the flow is diverted around the structures allowing the captured object to remain confined. Introducing a solution of GUVs through the device at a total flow rate of $5 \mu\text{L min}^{-1}$ for 20 min resulted in $95 \pm 2\%$ occupancy of the traps ($n=3$) (see supplementary material²¹ Fig. S1 for a fluorescence image of the entire array). A similar result could also be achieved for more dilute vesicle solutions by using lower flow rates and longer time periods.

GUVs were formed in a solution containing fluorescent calcein in order to encapsulate the small molecule for subsequent membrane transport studies. Figure 2(a) shows a trapped GUV between the two PDMS posts in a flow rate of $0.625 \mu\text{L min}^{-1}$ with calcein fluorescence visible both inside and outside. The solution was then exchanged for Millipore filtered water to demonstrate the ability to remove the surrounding reagent and monitor the same vesicle (Figs. 1(d) and 2(b)), a task which would be difficult to achieve without the precise fluidic control that microfluidic systems can offer. Due to the hydrophilic nature of calcein, it does not permeate

FIG. 2. Trapping of GUVs. (a) Wide-field fluorescence image of a single GUV trapped hydrodynamically by the posts (black). The surrounding calcein solution (green) is diverted around the trap. Fluid flow lines are indicated by the dashed lines. (b) The fluid was then exchanged for water without removing the calcein filled GUV. (c) Bright-field image of the donut (grey scale) overlaid with a fluorescence image of the GUV. The membrane is stained with DiI (red). Scale bars: 20 μm . (d) 3-D rendering (using confocal micrographs and ImageJ) of the calcein fluorescence showing minimum deformation of the trapped vesicle.

the membrane and remains encapsulated. All the donuts were then closed to isolate the GUVs in a 600 pl volume as shown in Figures 1(d) and 2(c).

Once trapped, the GUVs remain stable for long time periods (at least 12 h) even when subjected to high flow rates (see supplementary material²¹ for a movie with 2 μm diameter fluorescent beads flowing at 1, 5, 20, and 50 $\mu\text{l min}^{-1}$ resulting in linear flow rates of 2, 25, 63, and 147 mm s^{-1}). Due to the diversion of the flow around the occupied traps, no significant deformation or rupture of the GUVs was observed for flow rates up to 50 $\mu\text{l min}^{-1}$. A 3-D rendering of the calcein shows how the spherical shape of an example trapped GUV is maintained (see Fig. 2(d) as well as supplementary material²¹ Fig. S2 for optically sectioned images of the membrane and a movie showing the 3-D rendering). Being able to withstand high flow rates is important for applications requiring fast kinetic measurements or studying the effects of shear stresses. Without prior treatment of the microchannels with BSA, contact with the PDMS traps would lead to vesicle rupture. Therefore, coating of the channel walls with BSA provided a necessary barrier between the PDMS/glass and lipid membrane to stabilize the GUVs for the duration of the experiments.

Whilst electroformation offers a high yield of unilamellar vesicles within a few hours, one of the issues with the technique is the wide size distribution of the GUVs that are created (Fig. 3(a)). Techniques such as extrusion-dialysis can be used to produce suspensions of monodisperse vesicles and could in theory be used with unilamellar vesicles.²² The advantage of using a microfluidic approach is that a prior filtering process is no longer required as the device allows passive capturing of giant vesicles of a similar size. With a channel height of 20 μm , the average diameter of the trapped vesicles was $14.0 \pm 4.7 \mu\text{m}$ due to the dimensions of the microfluidic traps used in this study. Vesicles larger than $\sim 20 \mu\text{m}$ were not trapped for long time periods as the flow was not fully diverted around them. A histogram of the GUV diameters on-chip (Fig. 3(a)) reveals the existence of two populations of ~ 8 and 17 μm (shown in Figs. 3(b) and 3(c), respectively). The smaller population reflects vesicles, which are trapped between the posts, and the larger population shows those which are trapped in front of the posts. In order to capture a single population, the design was modified so that the gap between the posts was reduced to $\sim 1 \mu\text{m}$ and the channel height was reduced to 10 μm . This single population exhibited an average diameter of $5.8 \pm 1.4 \mu\text{m}$ with no vesicles being trapped between the posts (Fig. 3(d)).

FIG. 3. Histograms of GUV sizes. (a) Before being introduced into the device, the GUVs have a wide size distribution with an average diameter of $25.1 \pm 23.6 \mu\text{m}$ (blue) ($n_{\text{off-chip}} = 483$). After flushing through the device, the trapped GUVs have a more narrow size distribution with an average diameter of $14.0 \pm 4.7 \mu\text{m}$ (green) ($n_{\text{on-chip } 20 \mu\text{m}} = 64$) for 20 μm high channels and $5.8 \pm 1.4 \mu\text{m}$ (black) ($n_{\text{on-chip } 10 \mu\text{m}} = 53$) for 10 μm high channels. Representative vesicles of 8, 17 and 6 μm diameter are shown in (b), (c), and (d) respectively. Scale bar: 5 μm .

We believe that the trapped size distribution could be further tuned using this approach simply by changing the height of the channels and the dimensions of the traps.

B. Small molecule transport by α HL pore formation

To demonstrate the feasibility of using GUVs trapped in microchannels, we investigated the pore formation of α HL and subsequent transport of calcein across the membrane. Once inserted in a lipid bilayer, α HL forms a heptameric pore that allows small molecules like calcein to pass through the membrane.²³ The solution outside the donuts was exchanged with an aqueous solution of α HL. With a total flow rate of $5 \mu\text{l min}^{-1}$, the donuts were opened and closed for 2 s to rapidly expose the GUV to the membrane protein. Confocal microscopy was then used to monitor the fluorescence intensity of the calcein within three separate vesicles over time for different concentrations of α HL on a single device. To ensure transport and not membrane lysis was occurring, a control experiment was performed by adding α HL to a vesicle containing labeled streptavidin. This large protein cannot diffuse through the membrane pore and remained encapsulated whilst the small calcein molecules passed through (see Fig. S3 in the supplementary material²¹). This demonstrates that the α HL was not causing vesicle lysis.

Two processes govern the release of calcein from the vesicles: (i) the diffusion to and incorporation of α HL into the membrane and (ii) diffusion of calcein across the membrane due to a concentration gradient. Figure 4(a) shows an image series after the addition of $50 \mu\text{g/ml}$ α HL, where calcein diffuses out of the GUVs and the calcein fluorescence decreases

FIG. 4. α HL pore formation. (a) Confocal fluorescence images of calcein being released from a GUV with $50 \mu\text{g/ml}$ α HL. Scale bar: $10 \mu\text{m}$. (b) Kinetics of calcein release for 0, 0.5, 2.5, and $50 \mu\text{g/ml}$ α HL using 4 separate vesicles. The control without α HL showed no calcein release. The number of individual experiments at a given α HL concentration was, $n = 3$. Error bars are calculated from the standard deviation.

accordingly. It should be noted that the released calcein was diluted by a factor of ~ 100 into the donut volume so that the background fluorescence was negligible. This experiment was repeated with 0, 0.5, and 2.5 $\mu\text{g/ml}$ αHL using different vesicles to demonstrate multiple experiments on a single device. These GUVs all had the same diameter of 14 μm in order to maintain the same surface area for comparable results. The fluorescence signals from the GUVs at each 30 s time interval were averaged, normalized, and plotted against time (Fig. 4(b)). The absence of the membrane protein served as a control, and the amount of calcein within the GUV remained constant indicating that photobleaching had not occurred. The calcein release is governed by the diffusion/incorporation the protein. For 0.5 $\mu\text{g/ml}$ αHL , the slope of the calcein release becomes steeper over time, suggesting that more pores are formed on the surface as the free protein diffuses from the chamber to the GUV surface. With higher concentrations of free protein, the number of pores increases faster and therefore the efflux of calcein occurs more rapidly. These time scales agree with those of other studies of small molecule release.^{24,25} The clear advantage of using microfluidics for monitoring membrane transport kinetics is the speed at which analytes can be added to the surrounding solution. Fast mixing times can be achieved with stopped flow measurements but this traditional approach is limited to bulk measurements and cannot directly monitor the interior of the liposomes. Moreover, due to the precise positioning of the immobilised giant vesicles, automated imaging could easily be implemented.

IV. CONCLUSIONS

We have presented a microfluidic solution for handling and investigating GUVs. Microfluidic technology has become more accessible over the past 10 years therefore we believe the method presented here offers an elegant alternative to the more conventional biophysical tools with clear advantages such as positioning, isolation, fluid exchange and parallelization. Being able to reliably trap and isolate single GUVs (without modification to the lipid or interior density) and quickly exchange buffer solutions for membrane analysis is of great interest and could be used for range of applications including encapsulated enzymatic reactions, ligand-membrane protein binding, and drug screening. This design could also be combined with on-chip formation of vesicles^{24,26–28} for a complete integrated system for studying membranes.

ACKNOWLEDGMENTS

We gratefully acknowledge funding from the European Research Council (ERC Starting Grant No. 203428-2, μLIPIDS) and Merck Serono. We would also like to thank Christoph Bärtschi for constructing the electroformation device, Heinz Benz for the pressure control unit, the LMC for use of the confocal microscope and the clean room facility FIRST at ETH Zurich. Thanks to Simone Stratz for use of the 10 μm high chip.

¹Y. -H. M. Chan and S. G. Boxer, *Curr. Opin. Chem. Biol.* **11**, 581–587 (2007).

²S. F. Fenz and K. Sengupta, *Integr. Biol.* **4**, 982–995 (2012).

³N. Kahya, D. Scherfeld, K. Bacia, B. Poolman, and P. Schwille, *J. Biol. Chem.* **278**, 28109 (2003).

⁴P. Walde, K. Cosentino, H. Engel, and P. Stano, *ChemBioChem* **11**, 848 (2010).

⁵D. Lingwood and K. Simons, *Science* **327**, 46–50 (2010).

⁶M. I. Angelova and D. S. Dimitrov, *Faraday Discuss. Chem. Soc.* **81**, 303 (1986).

⁷M. I. Angelova, S. Soleau, P. Meleard, J. Faucon, and P. Bothorel, *Prog. Colloid Polym. Sci.* **89**, 127–131 (1992).

⁸N. Kahya, D. A. Brown, and P. Schwille, *Biochemistry* **44**, 7479 (2005).

⁹D. Stamou, C. Duschl, E. Delamarche, and H. Vogel, *Angew. Chem., Int. Ed.* **42**, 5580 (2003).

¹⁰S. Li, P. C. Hu, and N. Malmstadt, *Biophys. J.* **101**, 700 (2011).

¹¹P. Kuhn, K. Eyer, T. Robinson, F. I. Schmidt, J. Mercer, and P. S. Dittrich, *Integr. Biol.* **4**, 1550–1555 (2012).

¹²Y. Sun, C.-C. Lee, and H. W. Huang, *Biophys. J.* **100**, 987 (2011).

¹³A. Tian, C. Johnson, W. Wang, and T. Baumgart, *Phys. Rev. Lett.* **98**, 208102 (2007).

¹⁴B. Sorre, A. Callan-Jones, J.-B. Manneville, P. Nassoy, J.-F. Joanny, J. Prost, B. Goud, and P. Bassereau, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5622 (2009).

¹⁵A. Tian and T. Baumgart, *Biophys. J.* **96**, 2676–2688 (2009).

¹⁶J. Deschamps, V. Kantsler, E. Segre, and V. Steinberg, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 11444–11447 (2009).

¹⁷K. Eyer, P. Kuhn, C. Hanke, and P. S. Dittrich, *Lab Chip* **12**, 765 (2012).

¹⁸D. Di Carlo, N. Aghdam, and L. P. Lee, *Anal. Chem.* **78**, 4925 (2006).

- ¹⁹H. Nuss, C. Chevillard, P. Guenoun, and F. Malloggi, *Lab Chip* **12**, 5257–5261 (2012).
- ²⁰M. Unger, H. Chou, T. Thorsen, A. Scherer, and S. Quake, *Science* **288**, 113 (2000).
- ²¹See supplemental material at <http://dx.doi.org/10.1063/1.4816712> for an image of entire array with trapped vesicles, optically sectioned images of a trapped GUV, control experiment of streptavidin filled GUV, a movie of a trapped GUV in an aqueous flow at 1, 5, 20, and 50 $\mu\text{l min}^{-1}$ with 2 μm diameter fluorescent beads, and a movie of a 3-D rendering of a trapped GUV.
- ²²T. F. Zhu and J. W. Szostak, *PLoS ONE* **4**, 5009 (2009).
- ²³L. Song, M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, and J. E. Gouaux, *Science* **274**, 1859 (1996).
- ²⁴S. Matosevic and B. M. Paegel, *J. Am. Chem. Soc.* **133**, 2798 (2011).
- ²⁵J. C. Stachowiak, D. L. Richmond, T. H. Li, A. P. Liu, S. H. Parekh, and D. A. Fletcher, *Proc. Natl. Acad. Sci. U.S.A* **105**, 4697 (2008).
- ²⁶D. van Swaay and A. deMello, *Lab Chip* **13**, 752 (2013).
- ²⁷S.-Y. Teh, R. Khnouf, H. Fan, and A. P. Lee, *Biomicrofluidics* **5**, 044113 (2011).
- ²⁸S. Ota, S. Yoshizawa, and S. Takeuchi, *Angew. Chem., Int. Ed.* **48**, 6533–6537 (2009).