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Controlling the contents of microdroplets by exploiting the permeability of PDMS†

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A microfluidic device capable of exploiting the permeability of small molecules through polydimethylsiloxane (PDMS) has been fabricated in order to control the contents of microdroplets stored in storage wells. We demonstrate that protein precipitation and crystallization can be triggered by delivery of ethanol from a reservoir channel, thus controlling the protein solubility in microdroplets. Likewise quorum sensing in bacteria was triggered by delivery of the auto-inducer N-(3-oxododecanoyl)-l-homoserine lactone (OdDHL) through the PDMS membrane of the device.

Introduction

Microdroplets in microfluidics are now established as a format for carrying out chemical and biological experiments at the pico- to nanolitre scale.1–5 An individual water-in-oil droplet compartment serves as the equivalent of the conventional test tube. Droplets can be rapidly produced, processed and interrogated for high-throughput experiments while they are moving through microchannels.6 Alternatively, interrogation of droplet arrays in which droplets are held static gives high quality data, allowing even single cell or single molecule measurements.7–9 The droplet boundary can be set-up to prevent transfer processes (e.g. by diffusion) and cross-contamination can be minimized through the judicious choice of surfactant, its concentration and other added components10–13 or by using fluorinated surfactant/oil systems.8,14–16

Several formats for the addition of reagents to initiate or terminate a process have been developed. Individual droplets in a moving droplet stream can be merged or reagent added e.g. by electrocoalescence,17–20 destabilization of the droplet interface by surfactant,21–23 or by channel surface patterning.24

The delivery of reagents to static droplet arrays presents different challenges. It has been shown that static droplets can be fused by electrocoalescence18,25 or by a laser-induced thermocapillary force.26,27 However, these methods require side-by-side positioning of droplets and the degrees of freedom over the amount of reagent to be delivered are limited by the proximity of neighboring droplets.

The work described in this paper demonstrates the ability to manipulate the contents of droplets based on the permeability of the PDMS of the device. The compatibility of PDMS with organic solvents has previously been studied by comparing the swelling ratio of PDMS in various solvents and their solubilities.28 The ability of hydrophobic small molecules (exemplified by Nile red and quinine) to permeate PDMS has been described.29 For example, the permeability of PDMS has been exploited in the removal of trace organic compounds from an aqueous sample.30,31 There have also been attempts to reduce the permeability of PDMS to small molecules by pre-adsorbing bovine serum albumin (BSA) to the surface,32 by coating the PDMS with silane33 and by using a large excess of cross-linking reagents.34 In addition, the adsorption of a hormone onto PDMS was studied in a microfluidic device.35 We now address the potential of the permeability of PDMS for initiating multiple processes in droplets.

In order to utilize the diffusion of small molecules through PDMS to microdroplets trapped in resting positions, a microfluidic device was built by multilayer soft lithography.36 This device contained fluid supply channels (reservoir channel) below the droplets (Fig. S1, ESI†) from which small molecules can be supplied for diffusion across the PDMS membrane, and eventually transported into the trapped droplets.

In this work two processes that are influenced by small molecule stimuli were probed, namely protein crystallization and the control of bacterial gene expression by quorum sensing molecules. Both processes have been previously addressed in microdroplets.23,37–41 We now report that ethanol and quorum sensing molecules can be supplied into droplets in a controlled...
fashion via the permeable PDMS membrane from the reservoir channel. Delivery of ethanol is shown to trigger protein precipitation and crystallization by addition of a co-solvent that lowers the protein solubility, thus creating a supersaturated solution and increasing the crystallization propensity. Delivery of a small molecule (an auto-inducer of quorum sensing) triggers a cellular response that is measured indirectly by expression of a reporter gene encoding a fluorescent protein.

**Materials and methods**

**Device design**

The device contains an array of 2000 wells in the storage area (Fig. S1, ES†). The droplet deposition is controlled by inlet valves and occurs via the channels shown in Fig. S1a†. Small molecules are administered after the wells have been filled with droplets via a reservoir channel that is separated from the wells by a thin PDMS membrane (15 μm, Fig. S1b†). Droplets were stable over 12 hours as assessed by visual inspection. No fusion or break-up of droplets was observed as a result of the addition of solutes in the reservoir channels.

**Device fabrication**

The device was drawn with AutoCad (AutoDesk) and photolithographic masks were fabricated on transparent plastics (Circuit Graphics, Essex, UK). A positive photoresist (AZ-9260, AZ Electronic Material) was used to build the valve channels and negative photoresists (SU8-2025, SU8-2007 Microchem Inc.) were employed to fabricate the flow channel, wells and valve-reservoir channel. A commercially available PDMS kit (Sylgard 184, Dow Corning) composed of a pre-polymer and a cross-linker was used in the recommended weight ratio of 10 : 1. Two masters were required to fabricate the double-layered microfluidic device. A thin PDMS layer was manufactured onto the face of the master imprinted onto the PDMS slab. Injection holes were punched through the PDMS with lure stub adapters to insert tubings that deliver the fluid into the device. In order to fabricate the control channel and the reservoir in the device, a thin PDMS layer was manufactured onto the face of the PDMS slab covering them with a glass substrate. This thin layer was formed by spinning liquid PDMS onto the second master. The wafer was cured at 85 °C for 5 minutes. After alignment and assembly of the first PDMS slab on the second master, the device was heated again at 85 °C for 25 minutes. The resulting flexible silicone rubber was removed, leaving relief features from the master imprinted onto the PDMS slab. Injection holes were punched through the PDMS with lure stub adapters to insert tubings that deliver the fluid into the device. In order to fabricate the control channel and the reservoir in the device, a thin PDMS layer was manufactured onto the face of the PDMS slab covering them with a glass substrate. This thin layer was formed by spinning liquid PDMS onto the second master. The wafer was cured at 85 °C for 5 minutes. After alignment and assembly of the first PDMS slab on the second master, the device was heated again at 85 °C for 30 minutes to enhance adhesion between two PDMS layers. Injection holes were punched to insert tubing. The resulting PDMS slab was sealed against a glass slide after plasma treatment. CYTOP (Asahi Glass company) was coated on the flow channels to prevent the water sticking onto the PDMS walls.

**Device operation**

Aqueous droplets were formed in fluorinated oil (FC-40, Fluorinert™) previously mixed with surfactants (2% w/w; EA-surfactant, Raindance Technologies) to prevent the coalescence of droplets. The reservoir constructed underneath the wells supplied small molecules and organic solvents to droplets through the PDMS membrane. At the same time the reservoir maintained the volume of stored droplets from water evaporation of microdroplets. Gene expression in cells was induced at a constant temperature of 30 °C.

**Optical detection**

Fluorescence images were taken on an inverted microscope (IX71, Olympus) using a collimated LED light source (M455L2-C1, Thorlab) for widefield illumination operated in epifluorescence mode. In order to monitor large numbers of arrayed droplets, the device was mounted on a computer-controlled motorized stage (H117 ProScan II, Prior Scientific) that moved the device in a pre-determined pattern. To minimize photobleaching of green fluorescent protein (GFP) produced in cells, droplets were illuminated only during the acquisition by means of a computer-controlled LED illumination unit (M455L2-C1, Thorlab) using the same objective (UPLSAPO 40X2, Olympus). An EMCCD camera (Xion™, Andor Technologies) was used to acquire images, which were saved to the computer for offline analysis. Automatic acquisitions and image analysis were performed using softwares written in LabView (National Instruments). The fluorescence of GFP was measured from the integration of all green foci above the droplet background.

**Materials**

Chemicals were obtained from Sigma-Aldrich unless otherwise noted.

**Protein crystallization**

The initial concentration of protein was 59.4 mg ml⁻¹ dissolved in 0.2 M sodium chloride, 0.05 M sodium acetate (pH 4.5).

**Cell preparation**

*Escherichia coli* (E. coli) cells (DH5α) harboring a pMHLAS plasmid were grown and diluted to an OD₆₀₀nm=0.08, suspended in LB-media containing ampicillin (30 μg ml⁻¹) with 15% v/v of Percoll and loaded into a syringe (Hamilton, Gastight, 250 μl), the second identical syringe containing LB-media with various concentrations of OdDHL. Cells were injected into the microfluidic device in 1 : 1 volume ratio with OdDHL solutions and emulsified with fluorinated oil (FC-40, Fluorinert™) mixed with EA-surfactant in a flow focusing device (Fig. S1a†).

**Results and discussion**

**Protein crystallization**

Protein crystallization is an activated process due to the energy barrier that prevents crystals below a certain size from growing. To grow crystals effectively, it is necessary for protein solutions to initially be highly supersaturated leading to the formation of many small crystal nuclei that subsequently grow into large crystals at lower levels of supersaturation.

In this study a highly supersaturated solution of lysozyme was achieved by supplying ethanol via the reservoir to droplets...
containing proteins stored in the wells in the microfluidic device. Fig. 1b–e shows the process of crystal growth in microdroplets. Initially droplets contained a high concentration of lysozyme, approximately equivalent to the maximal solubility in water (59 mg ml\(^{-1}\)) in 0.2 M sodium chloride, 0.05 M sodium acetate, pH 4.5) (Fig. 1b and S2a†). When neat ethanol was supplied via the reservoir channels, the permeability of PDMS\(^{38}\) resulted in an increase of the ethanol content in the droplet. The solubility of lysozyme in ethanol is about 300-fold lower (0.2 mg ml\(^{-1}\))\(^{49}\) than it is in aqueous solution,\(^{48}\) so the addition of ethanol reduced the solubility of the protein and created a supersaturated solution, at which point the protein precipitated in minutes (Fig. 1c and S2b†). Such precipitation is frequently observed in highly supersaturated protein solutions, which is in non-equilibrium state, and is thought to provide seeds for crystallization.\(^{37,49}\)

Although supersaturation is required to nucleate seed crystals, a subsequent lower degree of supersaturation is necessary for crystal growth.\(^{50}\) In the next step for protein crystallization, the reservoir content was changed to water. The ethanol in the droplets was exchanged via the PDMS for the water supplied from the reservoir channel, thus lowering the ethanol content in droplets and dissolving the precipitate over a period of hours (Fig. 1d and S2c†) to allow crystal growth thereafter. The images shown in Fig. 1 illustrate this process of transforming many small crystals into fewer, but larger, crystals over 39 hours (Fig. 1e).\(^{51}\) Some conventional crystallization approaches rely on irreversible kinetic processes to partially decouple crystal nucleation and growth,\(^{52}\) which are difficult to control and optimize. Microdialysis methods permit independent control of nucleation and growth\(^{53}\) and this has been implemented in microfluidics.\(^{37,54}\) Our experiments show that protein crystal nucleation and growth can be independently manipulated by reversibly controlling the protein solubility in microdroplets.

In order to further demonstrate the control of ethanol content in droplets, various concentrations of ethanol were introduced into the reservoir channel and the time until protein precipitation was measured. For example, precipitation took 20 minutes in 66.7% v/v ethanol in the reservoir channel, but using 100% ethanol in the reservoir channel decreased the precipitation time to only 4 minutes (Fig. 1a). As the diffusion coefficient of ethanol\(^{48}\) in PDMS is 1.7 \(\mu\)m\(^{2}\) s\(^{-1}\) and the membrane thickness is about 15 \(\mu\)m (Fig. S1b, ESI†), we estimate that it takes \(\sim\)2 minutes for ethanol to cross the PDMS membrane based on the diffusion equation, \(t = d^2/D\), where \(t\) is the time, \(d\) the membrane thickness and \(D\) the diffusion coefficient. Therefore, the difference in time until precipitation as a function of the ethanol concentrations can be ascribed to differences in the ethanol flux across the PDMS membrane from reservoir to droplets since higher concentrations in the reservoir would enhance the ethanol sorption into the PDMS. It should be noted that ethanol-induced swelling of the PDMS would reduce the heights of the channels and this distorted the shape and diameter of the droplets, making it impossible to quantify the ethanol concentration in the droplets (Fig. 1c and S2b†). No protein precipitation was observed when concentrations of ethanol in the reservoir were lower than 50% v/v.

**Fig. 1** (a) The time until the precipitation of protein depends on the concentration of ethanol introduced into the reservoir. The plot shows the time until protein precipitation could be observed in droplets as a function of the ethanol content in the reservoir channel. (b) A stable protein solution of lysozyme stored in wells at the beginning of the experiment. (c) Precipitation occurred 6 minutes after the reservoir was filled with 100% ethanol. Ethanol transported from the reservoir to the droplets, increasing ethanol contents in droplets thereby lowering the protein solubility. (d and e) After the reservoir channel was filled with pure water, ethanol continued to evaporate from droplets and was replaced with water. This increased the solubility of the protein in the microdroplets and the precipitate dissolved (d) and crystals were subsequently formed over 39 hours (e). The size of protein crystals in droplets varied between 5–30 \(\mu\)m diameter. A time lapse movie shows this process (see the ESI†).

The observed permeability of PDMS to ethanol prompted us to investigate whether small organic signaling molecules might be delivered to cells entrapped in microdroplets. We used the ability of cells to respond to auto-inducers (AI) during quorum sensing (QS). Over the last decade, many species of bacteria have been shown to respond to self-produced QS signals.\(^{55–60}\)

In contrast to QS in vivo, which responds to endogenously produced AI, in this work the signaling molecule was supplied exogenously and detected using a genetically reconstituted version of the las QS system of *Pseudomonas aeruginosa* (expressed in a heterologous host, *E. coli*).\(^{55,58,61}\) Here, exogenously supplied \(N\)-(3-oxododecanoyl)-L-homoserine lactone (OdBHL) was sensed by plasmid-borne LasR, which subsequently activated the expression of green fluorescent protein (GFP) (Fig. 2a).

**Fig. 2c** shows GFP production in encapsulated cells triggered by the presence of OdBHL (10 \(\mu\)M) in the reservoir channels, suggesting OdBHL diffused readily through the PDMS membrane. In contrast, in the absence of an external trigger, cells did not produce GFP, showing that the plasmid-based reporter system used is sufficiently tightly controlled to yield no appreciable GFP expression under these conditions (Fig. S3†).

The total production of GFP in each droplet varied considerably, giving rise to a 20-fold difference in measured...
fluorescence values around an average of $1.3 \times 10^4$ RFU after 12 hours (Fig. 3a). The significant differences in GFP production between droplets can be ascribed to different initial cell occupancies in droplets as a consequence of Poissonian encapsulation of cells and the variation in expression levels in individual cells at a given concentration of auto-inducer. The latter may be related to variations in the plasmid copy number in each cell as well as cell division on the timescale of the experiment.

Concentration dependence of OdDHL delivery

We next examined the OdDHL concentration dependence of GFP expression in this system. GFP fluorescence was measured from droplets encapsulating cells in which a range of concentrations of OdDHL had been added at the time of droplet formation (Fig. 3c). The time course of GFP expression showed the onset to be around one hour after droplet formation. The lag time between droplet formation and observation of GFP fluorescence was delayed slightly more, presumably reflecting the time required for the OdDHL to diffuse across the membrane and to accumulate in the droplets. In both cases, GFP production increased over the next few hours and displayed saturation, especially at the higher concentrations of OdDHL tested. The magnitude of the GFP signal was strongly dependent on the OdDHL concentration but reached a maximum when the concentration was above 0.1 μM (Fig. 4a). Notably, the average signal intensity at the saturation point was comparable, irrespective of the delivery method employed, the fraction of droplets producing GFP differed. When OdDHL was directly mixed with cells in droplets, the fraction of droplets producing GFP started to increase at 0.01 μM (only four out of 959 droplets) and saturated around 1 μM (approximately 80% of cell-containing droplets) (squares in Fig. 4b). By contrast, when the auto-inducer was delivered through the PDMS membrane, the fraction of droplets producing GFP slowly started to increase from 0.5% at 0.1 μM to 60% when saturation was reached at 100 μM (circles in Fig. 4b). This difference can be ascribed to differences in the actual amount of OdDHL available to cells due to the slow diffusion of OdDHL from reservoir to droplet, thus limiting GFP expression. The dependence of OdDHL concentrations is similar to experiments using plate reader detection (Fig. S4†).

RFU at concentrations over 0.1 μM OdDHL. While the final averaged amount of GFP was independent of the delivery method employed, the fraction of droplets producing GFP differed. When OdDHL was directly mixed with cells in droplets, the fraction of droplets producing GFP started to increase at 0.01 μM (only four out of 959 droplets) and saturated around 1 μM (approximately 80% of cell-containing droplets) (squares in Fig. 4b). By contrast, when the auto-inducer was delivered through the PDMS membrane, the fraction of droplets producing GFP slowly started to increase from 0.5% at 0.1 μM to 60% when saturation was reached at 100 μM (circles in Fig. 4b). This difference can be ascribed to differences in the actual amount of OdDHL available to cells due to the slow diffusion of OdDHL from reservoir to droplet, thus limiting GFP expression. The difference in the fraction of cells expressing GFP (dashed lines in Fig. 4b) suggests that only ~1% of the OdDHL introduced in the reservoir is delivered into the droplet through the membrane.

The reservoir channels constructed under the wells allow the sequential addition of different molecules to droplets. This can be used to modulate cellular responses. The initial droplet and reservoir contents were 100 nM OdDHL and water, respectively. Around 3.5 hours after droplet formation, the reservoir content...
was changed to 10 mM N-heptanoyl-L-homoserine lactone (HHL) instead of pure water (circle in Fig. 4c). HHL is a quorum sensing molecule involved in the RaiI/RaiR circuit in *Rhizobium leguminosarum*. Shorter chain homoserine lactones such as HHL are very weak activators of LasR that compete with OdDHL and inhibit QS.

Although the bacteria were already ‘switched on’ by OdDHL that was co-compartmentalized during droplet formation, the introduction of HHL to the reservoir resulted in a slow down in GFP production after about 5 hours (Fig. 4c). This suggests that HHL was delivered to stored droplets from the reservoir and antagonized the action of OdDHL. The delay between the introduction of HHL and the cellular responses is presumably due to the slow diffusion process of molecules across the membrane, which is consistent with the delay time observed in Fig. 3b.

Conclusions

We have shown, using two very different experimental systems, that delivery of small molecules through PDMS membranes allows the contents of stored droplets in a microfluidic device to be manipulated. Small molecules delivered to droplets were shown to trigger processes as diverse as protein crystallization or quorum sensing in bacteria.

This approach does not require additional device features for droplet fusion such as synchronized lasers or electrodes. Furthermore, the delivery through PDMS is open to all molecules that are soluble in PDMS. The broad correlation of calculated log *P* values and the solubility parameter (Fig. S5†) can be used to estimate their permeability. A drawback of this approach is that it is difficult to estimate the concentration of small molecule that diffuses through the PDMS. This is further compounded because the ethanol swells the PDMS to make the channel height unpredictable and the observables in these experiments were not linearly proportional to the amount of compound accumulated in droplets. It may be possible to get some estimate of the concentration of solutes delivered to droplets by measuring the permeability of fluorescent dye in PDMS, which has a similar log *P* value to OdDHL or HHL. Despite this limitation, the approach clearly has applications in areas where a threshold concentration is required to trigger a process or where longer term change in a droplet content is required.

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