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Synchronized Reagent Delivery in Double Emulsions for Triggering Chemical Reactions and Gene Expression

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Microfluidic methods for the formation of single and double emulsion (DE) droplets allow for the encapsulation and isolation of reactants inside nanoliter compartments. Such methods have greatly enhanced the toolbox for high-throughput screening for cell or enzyme engineering and drug discovery. However, remaining challenges in the supply of reagents into these enclosed compartments limit the applicability of droplet microfluidics. Here, a strategy is introduced for on-demand delivery of reactants in DEs. Lipid vesicles are used as reactant carriers, which are co-encapsulated in double emulsions and release their cargo upon addition of an external trigger, here the anionic surfactant sodium dodecyl sulfate (SDS). The reagent present inside the lipid vesicles stays isolated from the remaining content of the DE vessel until SDS enters the DE lumen and solubilizes the vesicles' lipid bilayer. The versatility of the method is demonstrated with two critical applications chosen as representative assays for high-throughput screening: the induction of gene expression in bacteria and the initiation of an enzymatic reaction. This method not only allows for the release of the lipid vesicle content inside DEs to be synchronized for all DEs but also for the release to be triggered at any desired time.

for drug testing and discovery, directed evolution, antibody screening, sequencing, and many more.^[2–5] Moreover, there are numerous examples where droplet microfluidics was employed for material studies and chemical synthesis.^[6–8] However, droplet microfluidics has limitations; during long-term incubation, droplets suffer from volume reduction due to water diffusion out of the droplet into the oil, leading to changes in concentration of the encapsulated species. Furthermore, many applications require the addition of compounds to the droplet at a defined time point, which is achieved by droplet fusion,^[9–11] triple emulsions,^[12] or picoinjection.^[13] These droplet manipulation steps require complicated setups or control by imaging and address each droplet individually, resulting in a decreased throughput. Moreover, the addition of molecules leads to a volume change and, hence, to the dilution of the encapsulated components.

1. Introduction

Droplet microfluidics is one of the most promising fields for high-throughput screening as it allows the isolation and study of individual cells within a small, nanoliter volume, compartmentalized by an oil phase.^[1] Microfluidics-based methods facilitate rapid generation of monodisperse droplets with the possibility of performing single-cell assays or even single-molecule assays

The encapsulation of cells within gel microbeads partially overcomes these limitations.^[14] The use of gel microbeads makes it possible to address all entrapped cells at a selected time point, with massive parallelization capabilities.^[15] Moreover, the gel matrix provides the necessary stability for flow cytometric analysis.^[16,17] Nevertheless, more advanced systems for studying molecules secreted by cells or performing chemical reactions require additional means to prevent leakage of the studied molecules and cross contamination between microbeads. This can be achieved, for example, via chemical bonds^[18,19] or by isolating beads using a hydrophobic phase, de facto forming an emulsion.^[20,21] The flexibility of gel compartmentalization is therefore hindered by the necessity for supplementary immobilization protocols or the need for an additional immiscible phase, which has to be removed prior to flow cytometric analysis.

Another promising approach for compartmentalization uses water-in-oil-in-water double emulsions (DEs).^[22–24] In contrast to single emulsions, DEs are compatible with routine high-throughput screening instruments such as fluorescence-activated cell sorters (FACS).^[25–27] This convenient use of standard instruments makes DEs an ideal tool for screening large variant libraries.^[28–31] Compared to single emulsions, decreasing the oil phase to a thin shell surrounding the droplet also simplifies the access to individual DE vessels from the surrounding environment and the transfer of molecules through the oil shell can be partially regulated.^[32] Previous studies show that small molecules such as fluorescein diacetate or anhydrotetracycline can pass the oil shell barrier and reach individual droplets, allowing for

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monitoring cell viability or for induction of gene expression.^[33,34] Recent studies further illustrate the use of double-core DEs to delay reactant delivery. The fusion of the two compartments by applying an electric field triggers the reaction start; however, the method requires to address each DE droplet individually.^[11,35,36] Therefore, most high-throughput applications rely on the reactants already present in the DE vessel during the encapsulation, leading to an immediate reaction.

In this work, we present a novel versatile technique for the synchronized and time-controlled reagent delivery into DE vessels. Unlike previously developed techniques, this method allows us to address all DEs simultaneously at any desired time point. We employ large unilamellar vesicles (LUVs or liposomes) as tight carriers for compounds, encapsulated in DE vessels. The addition of sodium dodecyl sulfate (SDS), which can permeate across the thin oil shell of the double emulsion, causes the solubilization of the liposome membrane and subsequent cargo release.^[37] The membrane rupture and successive delivery of the encapsulated reactant, triggering the reaction start, can therefore be precisely controlled. We employ the technique for initiating an enzymatic reaction by releasing the substrate, and for the induction of gene expression in bacteria during cultivation in DEs. The analysis of the DE droplets is carried out via flow cytometry and fluorescence microscopy.

2. Results

The protocol developed for surfactant-mediated cargo delivery in double emulsions consists of three parts (**Figure 1**): 1) encapsulation by double emulsion generation, 2) cargo release, and 3) reaction and downstream analysis. DEs encapsulating liposomes and reactants are generated on a single poly(dimethylsiloxane) (PDMS) chip via two successive emulsification steps (**Figure 1**; **Figure S1**, Supporting Information). The first emulsion is formed using fluorinated oil (oil phase), and Tris buffer with 3% poly(vinyl) alcohol (PVA) is used as an outer buffer for the second emulsification step.

Each DE droplet serves as an individual reaction vessel with a diameter of about 20 μm with a shell of fluorinated oil with a thickness of $\approx 2 \mu\text{m}$. The encapsulated liposomes were prepared by extrusion^[38] and have a diameter of about 130 nm (**Figure S2**, Supporting Information). They contain phosphatidylcholine (POPC), cholesterol (Chol) and 2 mol% of polyethylene glycol (PEG)-linked phosphoethanolamine (DSPE-PEG2000) to prevent aggregation. After DE generation and collection, the anionic surfactant SDS is added to the outer buffer. The subsequent permeation of SDS inside of DEs and its interaction with liposomal membranes trigger the release of the liposomal cargo and the reaction start. In this work, we assessed reaction kinetics by measuring the resulting fluorescence signal on an automated microscope or by flow cytometry. For the time-resolved analysis of single DE vessels, we used a microfluidic chip with 614 hydrodynamic traps and pressure valves previously developed by our research group^[39] (**Figure 1**, bottom left; **Figure S1**, Supporting Information). The hydrodynamic traps and pressure valves were designed to capture individual DE vessels and to maintain their position during SDS supply and subsequent long-time measurements by automated microscopy.

Figure 1. Conceptual sketch of the protocol for surfactant-mediated cargo delivery in double emulsions (DEs). Liposomes and their cargo are co-encapsulated with reactants in DEs formed on a microfluidic device by two emulsification steps. 1) Isolating reagents in liposomes allows for their physical separation from other reagents within the same DE vessel. 2) The addition of SDS to the outer buffer leads to SDS permeation inside DEs and to the solubilization of the liposomal membrane. 3) The subsequent liposomal cargo release triggers the start of a reaction within the DE vessels. The side panels illustrate two independent analytical workflows. A microfluidic device with so-called hydrodynamic traps, here depicted as blue triangles, allows the monitoring of time-resolved changes of fluorescence in DEs via microscopy (left panel). DEs can also be analyzed at high throughput by FACS instruments at discrete time points (right panel).

For the high-throughput analysis of large DE populations, we optimized the method for measurements with commercially available FACS instruments (**Figure 1**, bottom right). In this case, SDS was added to the collected DEs in bulk, and the DEs' fluorescence was measured after different incubation times.

2.1. Characterization of the Cargo Release Kinetics

To characterize the synchronized release of liposomal cargo, we prepared liposomes containing a self-quenching concentration ($10 \times 10^{-3} \text{ M}$) of the fluorescent dye sulforhodamine B (SRB), SRB-liposomes. The rupture of SRB-liposomes upon the addition of SDS (5 g L^{-1}) leads to SRB dilution and, thus, to an immediate increase of the fluorescence in the entire DE vessel (**Figure 2A**).

To monitor the release over time, we immobilized individual DE vessels on the microfluidic device. The DEs were incubated overnight in parallel on two microfluidic chips, with and without SDS. **Figure 2B** shows time-lapse images of selected time points for DEs incubated at 30°C with a continuous supplement of SDS after 2 h of incubation. The distinct increase in fluorescence in these microscopic images confirms the rupture of the SRB-liposomes and SRB release. No visible increase is observed for the control condition, without SDS supplement (**Figure S3**, Supporting Information), demonstrating the stability of liposomes inside DEs.

Figure 2. Characterization of the synchronized release of liposomal cargo. A) Scheme representing the experimental workflow. DE vessels encapsulating liposomes with SRB at self-quenching concentrations are immobilized on a microfluidic platform containing hydrodynamic traps. The addition of SDS (5 g L^{-1}) to the outer buffer leads to the rupture of the SRB-liposomes and the release of their cargo, hence an increase of fluorescence signal. B) Microscopic images of trapped DEs at selected time points for DEs perfused with SDS at 30°C . Scale bar: $25 \mu\text{m}$. C) Kinetics of the SDS-mediated cargo release triggered after 2 h. The yellow curves with standard deviations represent the mean fluorescence value in DEs ($n = 120$) for two studied temperatures: 30 and 37°C . The red arrow indicates the time point at which SDS was supplemented. The gray curves with standard deviations represent the control condition (without SDS supplement) at 30 and 37°C .

We observe that the addition of SDS results in a decrease of the oil shell thickness (Figure S4, Supporting Information), presumably due to micelle formation of SDS with encapsulated oil. Nevertheless, we do not find any indication of reduced stability of the DEs during the observation time frame (more than 12 h).

The mean SRB fluorescence measured for individual DEs incubated at two different temperatures is depicted in Figure 2C. The results show that the cargo release started immediately after the addition of SDS for both temperatures. The data also confirm the effect of temperature on the fluidity of lipid membranes and, therefore, on the cargo release's kinetics. Incubation at a lower temperature (30°C) results in slower rupture of the liposomes and, hence, in a decelerated release of the cargo molecules. At 37°C , we observe a very sharp fluorescence increase indicating that about 2/3 of the release occurs within the first hour. In the control condition (no SDS addition), no fluorescence increase is observed over the incubation time for both temperatures.

2.2. Triggered Enzymatic Reaction in DEs Followed by Downstream Analysis by Flow Cytometry

Next, we demonstrate the triggering and analysis of an enzymatic reaction in DEs. For this purpose, we chose the hydrolysis

of fluorescein di- β -D-galactopyranoside (FDG) to fluorescent fluorescein, catalyzed by the enzyme β -galactosidase (β -gal). The enzyme was separated from the substrate by isolating FDG in liposomes (FDG-liposomes). FDG-liposomes were prepared by extrusion as described above^[38] using a solution of $0.75 \times 10^{-3} \text{ M}$ FDG containing 0.75% dimethyl sulfoxide (DMSO).

Following the DE generation, the DE vessels were collected and incubated in a flask shaker at 37°C either in the initial outer buffer (control) or in a buffer containing SDS to rupture the FDG-liposomes. From three tested SDS concentrations, we selected the optimal condition (0.1 g L^{-1} SDS) to achieve substrate delivery without inhibition of the enzymatic activity (Figure S5, Supporting Information). Following the cargo release, the FDG hydrolysis by β -gal was monitored by measuring the increase in fluorescence signal (Figure 3A).

The DE populations were analyzed at three discrete time points ($t_1 = 0 \text{ min}$, $t_2 = 4 \text{ h}$, and $t_3 = 22 \text{ h}$) using a flow cytometer. Homogeneous DEs could easily be discriminated from oil droplets or irregular DEs, e.g., DEs with two inner aqueous compartments (Figure 3B), and their fluorescence further analyzed. In Figure 3C, we compare the fluorescence distribution between DEs incubated in the initial outer buffer (top panel) and DEs incubated in SDS (bottom panel). For both conditions, the peak at t_1 displays the fluorescence intensity in DEs prior to the incubation. The peaks shift after 4 h (t_2), and this shift is

Figure 3. Triggered enzymatic reaction in DEs demonstrated for the hydrolysis of fluorescein di- β -D-galactopyranoside (FDG) to highly fluorescent fluorescein by β -galactosidase (β -gal). A) Scheme representing the experimental workflow. After generation using a microfluidic device, DEs co-encapsulating the enzyme β -gal and FDG-containing liposomes (FDG-liposomes) are collected in a tube and incubated in a buffer containing SDS. SDS permeates the DEs and ruptures the FDG-liposomes, thus releasing FDG and triggering the start of the enzymatic reaction. The incubated DEs are analyzed at discrete time points using a commercially available flow cytometer ($t_1 = 0$ h, $t_2 = 4$ h, and $t_3 = 22$ h). B) Flow cytometer light scatter gates of DE sample displaying 6000 events randomly sampled (top panel). Flow cytometer light scatter gates of homogeneous DEs from the selected DE subpopulation (bottom panel). C) Histograms displaying the fluorescence intensity of DEs incubated without SDS (top panel) and with SDS (bottom panel) at three different time points: $t_1 = 0$ h (gray), $t_2 = 4$ h (blue), and $t_3 = 22$ h (green). $n > 4800$.

attributed to the conversion of un-encapsulated FDG, i.e., FDG present outside the liposomes at the encapsulation time since it occurs in all conditions.

Further experiments in 96-well plates confirm this hypothesis (Figure S6, Supporting Information). After 22 h (t_3), the fluorescence intensity measured in DEs incubated with SDS further increased up to 100-fold the initial signal, confirming the release and conversion of the substrate. In the control condition (without SDS), no shift is observed compared to t_2 . In summary, the method allowed first the production of a large batch of DE vessels, second the synchronized release of the substrate, and finally the fast analysis in a conventional cytometer.

2.3. Bacterial Cultivation and Induced Gene Expression inside DEs

Bacteria are extensively used as host organisms in high-throughput screening applications such as directed evolution and drug discovery. DE vessels are a valuable tool to screen multiple conditions^[40] with the limitation that all compounds need to be encapsulated at the time of DE production. Here, we show that bioactive compounds such as inducers for gene expression in bacteria can be delivered at a separate time point. We first confirmed that SDS at a concentration of 5 g L^{-1} has no detrimental effect on bacterial viability. We encapsulated a well-characterized laboratory *Escherichia coli* strain (K-12 MG1655) constitutively producing superfolder green fluorescent protein (sfGFP). During incubation at 37°C with and without SDS, we monitored the increase of sfGFP fluorescence, which correlates to the bacterial biomass increase (Figure 4A; Figure S7,

Figure 4. SDS addition does not affect bacterial growth. A) Scheme representing the experimental workflow. DEs encapsulating *E. coli* constitutively expressing sfGFP were trapped on a microfluidic device and incubated with or without SDS addition (5 g L^{-1}) to the outer buffer. The bacterial growth was monitored via fluorescence intensity increase using an inverted microscope. B) Bacterial growth curves monitored in DEs. The curves represent the mean fluorescence intensity in DEs with 95% confidence intervals (gray: without SDS, green: with SDS, $T = 37^\circ\text{C}$, $n = 25$). C) Box plots of the sfGFP fluorescence at $t = 430$ min for both conditions ($T = 37^\circ\text{C}$, $n = 25$). No significant difference is found between the two distributions ($p < 0.05$).

Figure 5. Triggered gene expression in DEs. A) Scheme illustrating triggered gene expression in bacteria. DEs co-encapsulating *E. coli* cells and IPTG-containing liposomes (IPTG-liposomes) are incubated in a buffer containing SDS. The permeation of SDS inside the DEs leads to the gradual rupture of IPTG-liposomes, triggering the expression of *sfGFP* in bacteria. B) Histograms displaying the fluorescence intensity of DEs incubated without and with SDS analyzed by flow cytometry after 18 h. $n > 900$. C) Microscopic images of trapped DEs at selected time points for DEs containing IPTG-liposomes without SDS (left panel) and with SDS (right panel) in the outer buffer ($T = 37^\circ\text{C}$, scale bar: $25\ \mu\text{m}$). D) Kinetics of *sfGFP* production in DEs following IPTG-liposomes rupture. The curves represent the mean fluorescence intensity in DEs with 95% confidence intervals. The curves were recorded under three different conditions: with IPTG-liposomes and with SDS (green), with IPTG-liposomes, and without SDS (yellow), and without IPTG-liposomes and with SDS (beige) ($T = 37^\circ\text{C}$, $n = 50$).

Supporting Information). For both conditions, bacteria grew until reaching a plateau after about 200 min (Figure 4B). To compare the biomass in DEs between the two conditions, *sfGFP* fluorescence of individual droplets was plotted for the last incubation time point, $t = 430\ \text{min}$ (Figure 4C). There is no significant difference between the two distributions (Mann–Whitney U test, $p < 0.05$), confirming that the SDS supply had no significant effect on bacterial growth. This conclusion was further corroborated by visual observations; no visible difference in bacterial morphology or cell proliferation was identified between the two conditions (Figure S8, Supporting Information).

In the next step, we applied the presented reagent delivery method to the induction of gene expression. We selected isopropyl β -D-1-thiogalactopyranoside (IPTG), a broadly used inducer, to induce *sfGFP* gene expression in a recombinant *E. coli* strain DH5 α . Liposomes containing IPTG (IPTG-liposomes) at $0.5\ \text{m}$ were prepared and co-encapsulated with bacterial cells ($\text{OD}_{600} \approx 0.4$) in Luria Bertani (LB) medium in DE vessels. The DEs were analyzed using both flow cytometry and fluorescence microscopy to observe bacterial induction, i.e., *sfGFP* production (Figure 5A). For flow cytometric analysis, the DEs were incubated in batch in a buffer with and without SDS, and analyzed at three different time points (0, 4, and 18 h after production).

Based on flow cytometry data (Figure S9, Supporting Information), we can differentiate DE vessels containing fluorescent cells from empty vessels or vessels containing nonfluorescent cells. Both DE populations, incubated with and without SDS, show an increase of fluorescence after 4 h due to induced and basal *sfGFP* production, respectively.

The difference between the two populations further increased and is clearly visible in the flow cytometry data after 18 h (Figure 5B). To further characterize the kinetics of the *sfGFP* production of both conditions, we incubated DEs on a microfluidic chip (Figure 5C). DEs co-encapsulating bacteria and IPTG-liposomes were generated as well as DEs encapsulating only bacteria as an additional negative control (Figure 5D). High fluorescence levels indicating successful gene expression induction are observed in DEs encapsulating IPTG-liposomes and incubated with SDS. The endpoint fluorescence levels reached in these DE vessels are significantly higher than the fluorescence levels reached in the control conditions (Mann–Whitney U test, $p = 1.5 \times 10^{-8}$ for DEs without SDS and $p = 8.6 \times 10^{-6}$ for DEs without IPTG-liposomes). An increase in fluorescence following a similar trend can be observed for both control conditions. The end-point fluorescence values do not exhibit significant differences for these two conditions (Mann–Whitney U test, $p < 0.05$). These results confirm that the increase in fluorescence intensity is rather given

by basal sfGFP production and bacterial biomass increase over the cultivation period, and is not due to the induction of the *sfGFP* gene expression.

3. Conclusion

In summary, we developed a novel technique for the synchronized release of liposomal cargo in nanoliter DE vessels and illustrated the versatility of this method using different reaction systems and analytical tools. We showed the possibility of performing an enzymatic reaction and the induction of gene expression in bacterial cells within DE vessels. Our examples covered different fields involving biocatalyst and living cells to challenge the limits of our method. Each assay was studied using an analysis method addressing its particular requirements. Thereby, the optimized workflows for continuous measurements in a microfluidic device as well as high-throughput flow cytometric analysis at discrete time points were presented.

Compared to existing reagent delivery techniques, typically based on harsh changes of conditions (e.g., pH change, use of lasers, temperature shock, and volume changes), our method shows improved compatibility with biological systems. Indeed, we demonstrated that the surfactant concentration required for liposome rupture is not detrimental to bacterial growth or enzymatic activity. Beside the possibility of delaying and synchronizing the reagent delivery, the ability to isolate one component allows the multiplexing and parallelization of experiments.

It should be noted that this method is suitable to delay the delivery of hydrophilic, membrane-nonpermeable compounds. However, as suggested by previous works, small amphiphilic compounds are likely to permeate oil shells^[33,34] or artificial cell-like membranes^[41] and thus can be supplied to the reaction vessels directly from the outer buffer. Therefore, the proposed strategy represents a flexible complementary approach for a large number of compounds, for which the direct permeation through the oil shell would not be applicable.

We believe that the multiplexing capabilities of the system could be further enhanced and specifically tailored for each application. In particular, variations in lipid content would open the possibility of using more than one type of liposomes and delivering multiple reagents in parallel or at different time points.

We are convinced that the technique can be tuned for many applications beyond the scope of the presented assays (e.g., chemical synthesis, antibiotic susceptibility testing, and optimization of microbial biosynthesis). In particular, the possibility of phenotype–genotype linkage provided by the encapsulation of the reaction in double emulsions and the compatibility with high-throughput flow cytometric analysis make this technique particularly suited to in-vivo- or in-vitro-directed evolution.

4. Experimental Section

Chemicals and Material: PDMS (Sylgard 184) was purchased from Dow Corning. Tris was purchased from Bio-Rad. SDS was purchased from abcr. PVA, LB medium, β -galactosidase, kanamycin sulfate, IPTG (article number: 15502), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG2000

PE, article number: 880120C) were purchased from Sigma–Aldrich. 1-Palmityl-2-oethylphosphatidylcholine (POPC, article number: 850457), and cholesterol (article number: 700000) were purchased from Avanti Polar Lipids. Hydrofluoroether (HFE 7500) with 5% 008-FluoroSurfactant was purchased from Ran Biotechnologies. Ampicillin sodium salt was obtained from Glentham Life Sciences. SRB and FDG were purchased from Thermo Fischer. The solutions were delivered to the microfluidic devices from 1 mL BD Plastipak syringes (VWR, BD Plastipak Luer-lock), with flow rates controlled by neMESYS syringe pumps (Cetoni). The connection between the microfluidic device and the syringes was made through polytetrafluoroethylene (PTFE) tubing (ID = 0.56 mm, Adtech Polymer Engineering) for the formation of DEs and PTFE tubing (ID = 0.86 mm, PKM SA) for the perfusion of DEs trapped on the second microfluidic device. The tubing/syringe interface consisted of a precision dispenser needle, 23 gauge (Metcal); the tubing/microfluidic device interface consisted of a metal pin (New England Small Tube, NE-1310-03, 0.025" OD $\times$ 0.013" ID $\times$ 1.00" length). The microfluidic pressure valves were actuated with a custom-made pressure control system. An MFCS-8C pressure control unit was used from Fluigent for the PVA coating of the devices.

Bacterial Strains: To confirm that the bacterial growth inside DEs is not affected by the presence of SDS, the well-characterized laboratory *E. coli* strain K-12 MG1655 (pSEVA271-*sfGFP*) was used.^[42] For the constitutive production of sfGFP, this strain was transformed with plasmid pSEVA271-*sfGFP* (kanamycin resistance, pSC101 origin of replication, lab collection) carrying the *sfGFP* gene. The plasmid was constructed based on plasmid pSEVA271,^[43] by adding a gene for sfGFP under control of a constitutive promoter, BioBrick part BBa_J23100.

The possibility of inducing gene expression in bacteria was tested using the bacterial strain *E. coli* DH5 α transformed with plasmid pKQ-*sfGFP* (ampicillin resistance, pBR322, origin of replication, lab collection) carrying the *sfGFP* gene. The plasmid was constructed based on plasmid pKQV4,^[44,45] by adding a gene for sfGFP under control of the promoter Ptac, whose expression can thus be induced by addition of IPTG to the bacterial culture and monitored via fluorescence increase.

Fabrication of Two Microfluidic Platforms: For double emulsion formation, the devices were fabricated by pouring PDMS (1:10 ratio of curing agent to polymer) onto an SU-8 master mold (prepared on a 4 in. silicon wafer) and curing it at 80 °C for 3 h. In parallel, a layer of $\approx$ 50 μ m of PDMS was spin-coated onto thin glass coverslips (Menzel Microscopes #1.5) and cured at the same conditions. The devices were bonded to the PDMS-coated glass slide after piercing inlets and outlet using a biopsy puncher (Electron Microscopy Sciences, diameter = 0.5 mm). The device has four inlets corresponding to the outer aqueous phase (OA), the oil phase (OP), the inner aqueous phases (IA1 and IA2), and one outlet. The OA and outlet channels were selectively coated with a 2.5% PVA to make them hydrophilic following the protocol described by Deshpande et al.^[46]

For trapping and monitoring of double emulsions, we used a similar microdevice as described before, including the fabrication protocol.^[39] The devices contained two channel systems on top of each other, separated by a thin PDMS layer (<50 μ m thick). The top part (fabricated as described above) contained a network of circular channels serving as a pressure valve system. The DEs were introduced into the wide bottom channel containing the hydrodynamic traps. This channel part was prepared by spin-coating uncured PDMS onto an SU-8 master mold and curing it at 80 °C for 1 h. The two layers were aligned and bonded together using a thin layer of the curing agent. After piercing the inlet and outlet using a biopsy puncher (Integra Miltex; diameter = 1 mm), the two-layer chips were bonded to a PDMS-coated glass slide as described above. The devices were coated with a 2.5% PVA solution for 5 min. The PVA was removed by a stream of deionized (DI) water and the devices were cured at 120 °C for 1 h.

Large Unilamellar Vesicles (LUVs or Liposomes) Preparation: SRB-liposomes were prepared by extrusion.^[38] Briefly, a mixture of POPC, Chol, and DSPE-PEG2000 at a ratio of POPC:Chol:DSPE-PEG2000 of 48:48:2 mol% in chloroform was dried by evaporation using a rotary evaporator. The dried mixture was rehydrated in a solution of 10×10^{-3} M SRB for at least 4 h. Following rehydration, the lipid suspension was

subjected to ten freeze/thaw cycles in a liquid nitrogen bath followed by a water bath at 60 °C, respectively, to reduce the liposome multilamellarity. The lipid suspension was then extruded 11 times through a membrane with a pore size of 100 µm using a Mini Extruder (Avanti Polar Lipids). To remove the un-encapsulated SRB, the SRB-liposomes were dialyzed using a dialysis cassette with a 20 kDa molecular cutoff (Thermo Scientific, Slide-A-Lyzer Dialysis Cassettes (Molecular weight cut-off: 20k)) against Tris buffer with matching osmolarity. The size distribution of the liposomes was measured by dynamic light scattering (Zetasizer Nano ZSP) (Figure S2, Supporting Information).

IPTG-liposomes were prepared by extrusion as described above using a solution of 500×10^{-3} M IPTG for the lipid rehydration.

FDG-liposomes were prepared by extrusion as described above using a solution of 0.75×10^{-3} M FDG containing 0.75% DMSO for the lipid rehydration.

Bacterial Cultivations: The bacterial strains were precultured overnight from cryo-stock in 2 mL LB medium supplemented with antibiotics for plasmid maintenance (50 µg mL⁻¹ kanamycin sulfate or 100 µg mL⁻¹ ampicillin sodium salt). Following this, 40–50 µL of the preculture was transferred in fresh LB medium with appropriate antibiotics and cultivated (shaking incubator-Minitron, Infors HT; shaking speed = 220 rpm; 37 °C) until they reached the beginning of the exponential growth phase, OD₆₀₀ corresponding to ≈0.2–0.4. Before the encapsulation in double emulsions, bacterial cells were centrifuged (Eppendorf MiniSpin centrifuge, 2 min, 3000 rpm) and collected in fresh media with a final OD₆₀₀ of ≈0.7.

Production, Trapping and Monitoring of DEs: The inner aqueous solutions (IA1, IA2), oil, and outer aqueous solution (OA) were introduced into the microfluidic device at flow rates between 0.5 and 5 µL min⁻¹, adjusted to achieve stable formation of DEs. The double emulsion production was monitored using an inverted Olympus microscope (IX71) and a high-speed camera (Phantom VEO). The double emulsions were collected at the outlet through a tubing leading to a collection tube (1.5 mL). The oil was, in all experiments, HFE 7500 with 2% 008-FluoroSurfactant. The solutions used for the different experiments are listed in Table S1 (Supporting Information).

The experiments with time-resolved monitoring of the produced DEs were performed using a microfluidic device with trapping arrays and microfluidic valves. Prior to the experiment, the top and bottom channel systems of the device were prefilled by centrifugal force. The microfluidic valves' system was filled with water and the trapping array with the solution corresponding to the outer buffer used in the experiment. The device was mounted on a motorized, inverted microscope (Nikon Ti-Eclipse) with an environmental box for temperature control. The temperature was set to 30 or 37 °C prior to the experiment and maintained constant during the measurements. After the introduction and trapping of the DEs, the round-shaped valves were partially closed by applying about 1 bar N₂. This pressure was sufficient to isolate individual trapping sites and to supply the trapped DEs with the SDS-containing solution. The chips were perfused during the entire duration of the experiment with the outer buffer or a buffer containing SDS at a flow rate of 0.7 µL min⁻¹. For the delayed rupture of SRB-liposomes, 85 µL of the outer buffer solution was aspirated in the tubing connected to the device inlet (the tubing was connected to the syringe containing 5 g L⁻¹ SDS). Considering the perfusion flow rate of 0.7 µL min⁻¹, the SDS-containing solution reached the DEs after 2 h of monitoring. The trapping sites were imaged for 15 h (4 h in 15 min intervals, 11 h in 30 min intervals) using a Hamamatsu Orca Flash 4 camera. The images were acquired using bright field and fluorescence microscopy through a 10× objective (Nikon, Plan Fluor). A Lumencor Spectra X light-emitting diode (LED) was used as a light source for fluorescence excitation. For each fluorophore, a combination of the appropriate optical filters and dichroic mirrors was used. SRB was imaged using a green LED (25% of the maximum intensity) using a 549/15 excitation filter, a 562 dichroic, and a 593/40 emission filter with an exposure time of 200 ms; for the delayed cargo release, 55% LED light intensity and 100 ms exposure were used. The sfGFP was imaged using a cyan LED (30% of the maximum intensity) using a 475/28 excitation filter, a 495 dichroic, and a 525/50

emission filter with 100 ms exposure time. The images were acquired using the Nikon Perfect Focus System and the NIKON NIS-Elements Advanced Research software.

For DE incubation and flow cytometric measurements, with the characterization of the enzymatic assay, the double emulsions were incubated in 1.5 mL Eppendorf tubes containing either 3% PVA in 0.35 M Tris buffer or 0.1 g L⁻¹ SDS in 0.35 M Tris buffer in an oven at 37 °C. The double emulsions were analyzed at different time points using a BD Fortessa analyzer.

Data Evaluation Microscopy: In the characterization of the cargo release kinetics, the mean gray values were obtained for each individual DE and for every imaged time point. Each data set was subtracted from the initial gray value to suppress the influence of the background and the initial fluorescence of the self-quenched dye. The obtained data were processed and plotted using OriginLab 2019 software including the statistical analysis. Standard deviations were used as descriptive statistics, in order to visualize the variations of fluorescence between individual DEs (originating from the initial SRB-liposome load and the differences in DEs' size).

For the evaluation of bacterial viability in the presence of SDS, the mean gray values for the two DEs populations, with and without SDS, were obtained for each DE droplet and for all time points using a circular region of interest surrounding each particular DE. The obtained data were processed and plotted using OriginLab 2019 software, with 95% confidence intervals used as descriptive statistics. The growth curves with confidence intervals allowed the visualization with precision of the mean, providing information regarding time-resolved fluorescence changes for DEs containing heterogeneous bacterial population. Statistical comparison of the two conditions for endpoint measurement was performed; the data obtained for the control condition and in the presence of SDS at the last time point of the incubation were first tested for normal distribution using the Shapiro–Wilk test. Two distributions were tested using the Mann–Whitney *U* test to evaluate the difference in the two populations (significance: $p < 0.05$).

In the induction of gene expression in the encapsulated bacteria, the mean gray value for each DE at every time point was obtained. The initial fluorescence of the DE (background fluorescence given by the experimental setup and/or basal sfGFP production by the bacteria) was subtracted from the values obtained at the following time points. The mean values and statistics were calculated after the subtraction. In cases where the initial fluorescence was below the applied threshold, the first measurable value was subtracted. If the fluorescence did not reach the threshold value at any of the following time points, this point was not included in the calculation of the mean value for that particular time point. The final *n*, considering all conditions and all time points, ranged from $n = 40$ to $n = 50$. We used confidence intervals (confidence level of 95%) as descriptive statistics. In the statistical comparison of the tested conditions for endpoint measurement, the data obtained for the bacterial gene induction in DEs (with IPTG-liposomes and SDS) and control conditions (with IPTG-liposomes, without SDS; without IPTG-liposomes, with SDS) at the last time point of the incubation were tested for normal distribution using the Shapiro–Wilk test and using the Mann–Whitney *U* test to evaluate the difference in all the populations (significance: $p < 0.05$).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.S. and P.J. contributed equally to this work. A.S., P.J., and P.S.D. developed the concept and wrote the manuscript; A.S. and P.J. performed the experiments and analyzed the data; N.N. optimized the liposome formation protocol to the project needs and prepared the different liposome populations; and S.S. provided the recombinant bacterial strains and guidance for the application of the presented cargo delivery system in microbiology. All authors discussed the results and commented on the manuscript. All authors approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. In addition, the data has been uploaded on <https://zenodo.org/record/5004700#.YNBAfEyxU2w>.

Keywords

double emulsions, droplet microfluidics, liposomes, reagent delivery

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