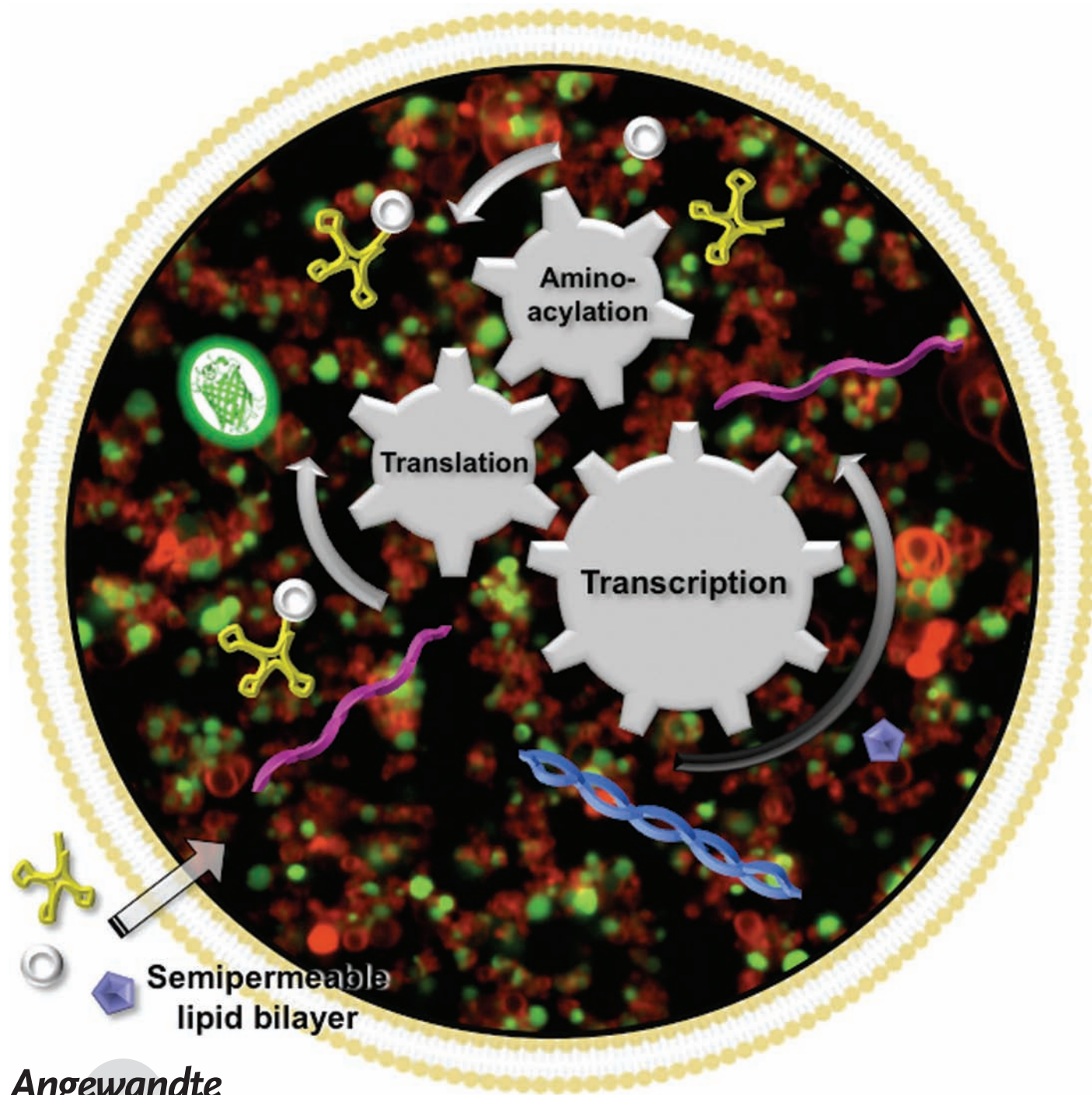


Triggered Gene Expression in Fed-Vesicle Microreactors with a Multifunctional Membrane**

Zohreh Nourian, Wouter Roelofsen, and Christophe Danelon*



With the aim of constructing an artificial cell that has organizational and functional properties mimicking those of living systems, it is now possible to assemble liposomes that enclose biochemical reaction networks as complex as the whole transcription and translation machinery capable of synthesizing proteins from a DNA template.^[1–6] Further progress towards the development of a minimal cell critically depends on technological advances to efficiently encapsulate in vitro gene expression systems within cell-sized ($>1\ \mu\text{m}$) liposomes.^[7] Ideally, the vesicle formation method should be compatible with any buffer and membrane compositions to tailor the container properties, for example, for promoting selective exchange of matter with the environment or to facilitate vesicle handling and characterization. However, current techniques to meet those demands are inadequate and technological challenges for the assembly of a semi-synthetic minimal cell remain.

A variety of liposome preparation methods have been employed for compartmentalized gene expression: lipid film swelling,^[1] mechanical lipid film resuspension,^[2] freeze-dried empty liposomes (FDEL),^[3] ethanol injection,^[4] water-in-oil emulsion transfer,^[5] and microfluidic-based devices.^[6] The lipid film swelling method offers unique advantages to create large and giant ($>10\ \mu\text{m}$) liposome-based bioreactors with almost unrestricted choice in membrane components, thus making it a popular approach to produce vesicles with various membrane functionalities, such as surface positioning,^[8] targeting and selectively delivering cargo to live cells,^[9] and temperature-responsive permeability.^[10] However, this method suffers from several deficiencies including 1) a low yield of formed liposomes in physiological buffers, 2) lack of compatibility with microliter volume handling during lipid film rehydration, and 3) low encapsulation efficiency of macromolecules compared to the emulsion-based or FDEL methods. Consequently, the benefits provided by the lipid film swelling method remain largely unexploited in the context of artificial cell assembly.

To date, in all studies of protein biosynthesis inside liposomes, phospholipids containing 16 or 18 carbon atoms were used,^[1–6,11] hence rendering bilayer permeability very low under nonstressed conditions. Two approaches have been presented to create an active interface with the environment: 1) to generate membrane defects under osmotic pressure and 2) to incorporate transmembrane ion channels in the liposome membrane.^[5a] Both methods enabled the exchange of

low-molecular weight solutes with the surrounding feeding environment and thus increased the production time of a cell-extract-based expression system as compared to that in sealed vesicles. Although the influence of charged and PEG-bearing lipids on the yield of protein synthesis has been investigated,^[3e,g] the role of the length and saturation level of the lipid carbon chain—with the associated temperature- and pressure-dependent bilayer permeability—to regulate molecular diffusion across the vesicle boundary remains unexplored.

Herein, we describe the use of a porous matrix-assisted method to produce large and giant liposomes encapsulating a minimal gene expression system by lipid film swelling. We reasoned that the increased active surface area provided by submillimeter glass beads may lead to a higher concentration of liposomes generated within inter-bead cavities, while enhancing encapsulation of the multiple components (about 40 different proteins) by promoting membrane reactivity. A similar technique has shown increased encapsulation efficiency of small vesicles by larger ones.^[12] Moreover, we for the first time report on the use of short dimyristoylated, 14 carbon atom acyl chain phospholipids, to create semipermeable membranes that make possible the uptake of nutrients from the outside feeding environment, while keeping the macromolecules entrapped inside the liposome. We exploited the capability of the lipid film swelling method to accommodate a variety of membrane constituents for integrating multiple functionalities: biotin-PEG lipids (PEG = poly(ethylene glycol)) for liposome immobilization on neutravidin-coated surfaces and vesicle stabilization through the PEG spacer, negatively charged lipids to improve swelling, TRITC-conjugated lipids (TRITC = *N*-(6-tetramethylrhodaminethiocarbamoyl)) for membrane localization using fluorescence imaging, and lipids of different phase transition temperatures (T_m) for tuning membrane permeability as a response to temperature and osmotic pressure.

For the in vitro gene expression system, we used the PURE system reconstituted solely from purified enzymes and cofactors including *Escherichia coli* ribosomes for translation, the T7 RNA polymerase for transcription, and others for aminoacylation and energy regeneration.^[13] In addition to this enzymatic mix, the PURE system comprises a feeding solution that contains the nutrients—mainly nucleotides and amino acids—as well as tRNAs. As compared to cell extracts, the PURE system is made of well-defined elements and it is therefore preferred as a constructive paradigm of a minimal cell.

Figure 1 illustrates the main experimental steps to produce surface-tethered protein-synthesizing liposome microreactors. Briefly, lipid-coated beads are prepared by organic solvent evaporation under continuous rotation in a round-bottom glass flask and can be stored under nitrogen gas at -20°C for several weeks. The equivalent of about $10\ \mu\text{L}$ of lipid-coated beads are transferred into a reaction tube, gently packed, and the lipid film is rehydrated for 1–2 h with $2\text{--}10\ \mu\text{L}$ of a solution containing the enzyme mix and a linear DNA template encoding for an autofluorescent protein. As repetitive liposome freezing and thawing is recognized to break lipidic multilamellar structures and enhance encapsulation efficiency,^[14] the reaction tube is then subjected to four freeze-

[*] Z. Nourian,^[†] W. Roelofsen,^[†] Dr. C. Danelon
Department of Bionanoscience, Kavli Institute of Nanoscience,
Delft University of Technology
Lorentzweg 1, 2628 CJ, Delft (The Netherlands)
E-mail: c.j.a.danelon@tudelft.nl
Homepage: <http://christophedanelonlab.tudelft.nl>

[†] These authors contributed equally to this work.

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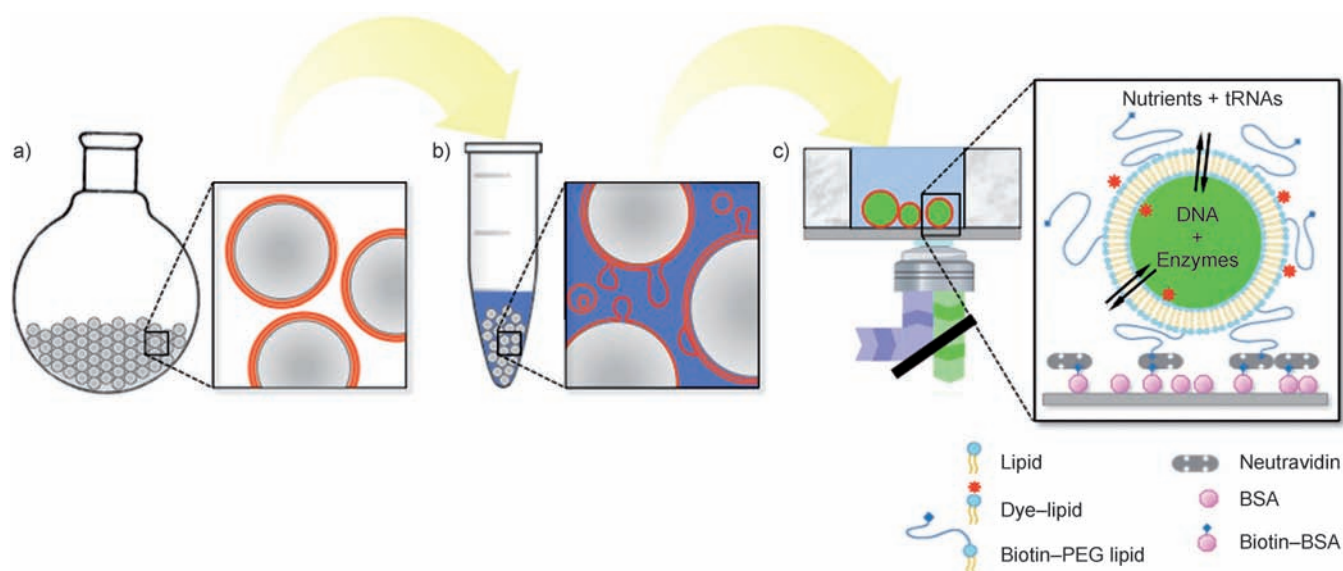


Figure 1. a) 200 μm glass beads are mixed with lipid-containing organic solvent in a round-bottom flask; b) lipid-coated glass beads are transferred into a reaction tube, the lipid film is rehydrated in the presence of a DNA template and the enzyme mix of the PURE system; c) the liposome-containing solution is transferred into a PDMS chamber for surface immobilization on a microscope coverslip using biotin-neutravidin recognition sites.

thaw cycles (see below). Next, the vial is tilted and gently rotated to unpack the glass beads and release the liposomes. Then, 1–2 μL of the vesicle-containing solution is transferred into a poly(dimethylsiloxane) (PDMS) chamber mounted on a microscope coverslip precoated with neutravidin for liposome immobilization. At this stage, no expression is possible, as the nutrients and tRNAs have not been supplemented yet. Finally, the surface-tethered vesicles are diluted in—or the external solution is exchanged with—the feeding solution, such that outside protein synthesis is inhibited, and are incubated at 37°C.

In a first series of batch reactor measurements, we mimicked the conditions applied during vesicle preparation and examined the influence of the temperature and freeze-thaw cycles, two potentially denaturing parameters, on PURE system activity. Figure 2a shows the time courses of emGFP synthesis at different temperatures applied for liposome formation or protein synthesis. All curves resulting from kinetic measurements exhibit three distinct regimes: a lag phase, a linear increase, and a slow (almost saturated) regime. An increase in temperature accelerates the rate of emGFP production and reduces the expression period. The total amount of synthesized proteins is not monotonic in temperature, as at 45°C the maximal fluorescence intensity is substantially decreased. To investigate the effect of freeze-thawing, reaction tubes containing the enzyme mix and DNA templates underwent different numbers of freeze-thaw cycles prior to incubation in the presence of the feeding solution, and the yield of emGFP production was evaluated by performing end-point fluorescence measurements (Figure 2b). The PURE system retains about 60 % of its activity after applying four cycles; we found that this number offers the best compromise between the amount of synthesized proteins and the yield of immobilized unilamellar liposomes.

Using the protocol described in Figure 1, we prepared liposomes with different lipid compositions, all comprising 0.1 mol % of DSPE-PEG-biotin for vesicle stabilization and surface immobilization, and 0.5 mol % of DHPE-TRITC for liposome localization: DMPC/DMPG 4:1 (termed DM liposomes; phase transition temperature, $T_m \approx 23^\circ\text{C}$), DPPC/DPPG 4:1 (DP liposomes; $T_m \approx 41^\circ\text{C}$), and DOPC (DO liposomes; $T_m \approx -20^\circ\text{C}$). To determine if the presence of DH- and DS-containing lipids affects the T_m value, we performed calcein efflux measurements with DM and DP liposomes and found a slight shift of about +1 °C from that of pure DMPC or DPPC vesicles (Figure S2 in the Supporting Information). Liposomes were generated by lipid film swelling above the T_m , at 30°C for DM (unless indicated otherwise) and DO liposomes, and at 45°C for DP liposomes. As shown in Figure 2 and 3, unilamellar and multilamellar vesicles with sizes ranging from < 1 μm to about 20 μm could be formed and immobilized at high density with all liposome compositions tested. While large and giant unilamellar vesicles are also observed, most liposomes have internal bilayer structures that form subcompartments.

We firstly investigated gene expression in surface-tethered DM liposomes. mRNA and protein syntheses can be triggered inside individual vesicles by external supply of nutrients and tRNAs as shown in Figure 2c,e with two different fluorescent proteins. How does internal resource allocation take place? Two distinct effects are likely to influence membrane permeability and enable liposome feeding. First, diluting enzyme-loaded vesicles with the feeding solution leads to an osmotic pressure because of the difference of osmolarity between the inside and outside of the liposomes, which generates membrane defects (see the Supporting Information). Secondly, when temperature is close to the lipid bilayer T_m , molecular diffusion, including

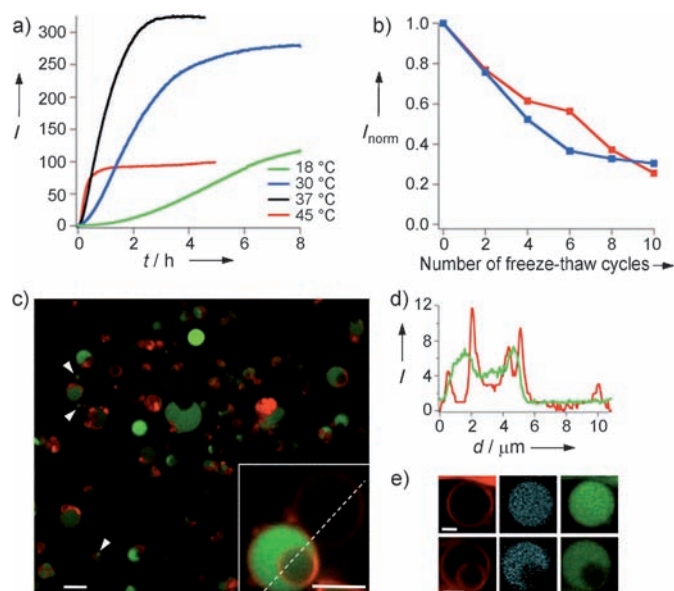


Figure 2. a) Time course of emGFP expression in batch reaction at different temperatures; b) end-point fluorescence measurements of emGFP synthesis in batch reactions after different numbers of freeze-thaw cycles, data points from two independent experiments are shown; c) typical fluorescence confocal micrographs of surface-tethered DM liposomes (red) expressing emGFP (green); swelling was performed at 30 °C for 2 h and liposomes were imaged after 23 or 5 h (inset) incubation at 37 °C; arrowheads indicate submicrometer liposomes expressing emGFP; scale bars: 5 μ m; d) intensity plots of TRITC (red) and emGFP (green) along the dotted line in Figure 2c, inset; e) dual gene expression assay in surface-immobilized DM liposomes; TRITC (red), CFP (cyan), and emGFP (green); 250 ng of CFF- and emGFP-encoding DNAs were coencapsulated and liposomes were imaged after 2 h incubation at 37 °C; scale bars: 2 μ m.

that of nucleotide triphosphates,^[15] across the vesicle membrane is enhanced. For the reactions to occur, liposomes are incubated at 37 °C, that is, far above the T_m . Therefore, temperature-assisted permeation across DM membrane is unlikely under these conditions. It is remarkable that the initial osmotic stress is not too large for bursting the liposomes but large enough to generate membrane defects that can accommodate the diffusion of nutrients and tRNAs, while maintaining the DNA template, the enzymes/cofactors, and the synthesized proteins trapped (see the Supporting Information).

Heterogeneity in the expression levels is high between individual vesicles for all analyzed time points and no obvious size dependence could be observed. As exemplified in Figure 2c,e with DM vesicles, subcompartments with different expression levels are frequently observed and compartment-specific fluorescence intensities can be quantified (Figure 2d). We then sought to identify whether DNA concentration was limiting the yield of liposomes that express the fluorescent proteins. We performed a dual gene expression assay that consists of coencapsulating two different DNA molecules, each encoding for a specific colored fluorescent protein. Figure 2e shows three representative liposomes that express either both or none of the cyan and green fluorescent

proteins (CFP/GFP), hence suggesting that, at least for $>1 \mu$ m vesicles/subcompartments, other components than DNA are not enclosed at sufficient concentration to enable gene expression. On the contrary, synthesis of either CFP or GFP would have indicated that DNA concentration is a limiting factor for the whole reaction.

To better understand and further extend the microreactor properties, several lipid compositions and incubation conditions were tested, and gene expression was quantified at four different points in time after addition of the feeding solution. DP vesicles mainly composed of phospholipids that contain C_{16} acyl chains are in the liquid ordered phase at 37 °C and fail to express fluorescent proteins (Figure 3a). As shown in Figure 2a, gene expression in a batch reactor ceases after about 30 min and leads to low production at 45 °C. To discriminate between a deleterious effect on the PURE system activity when forming DP liposomes at such a high temperature and a reduced permeability of the DP bilayer, we also swelled DM liposomes at 45 °C. Not only can GFP-synthesizing liposomes be observed (Figure 3c), the expression significantly increases for more than 5 h (Figure 3d). These results indicate that the DP membrane acts as a barrier for nutrient and tRNA uptake. In addition, enzymatic activity in semipermeable DM liposomes can partly be “rescued” and gene expression can be prolonged for longer time periods

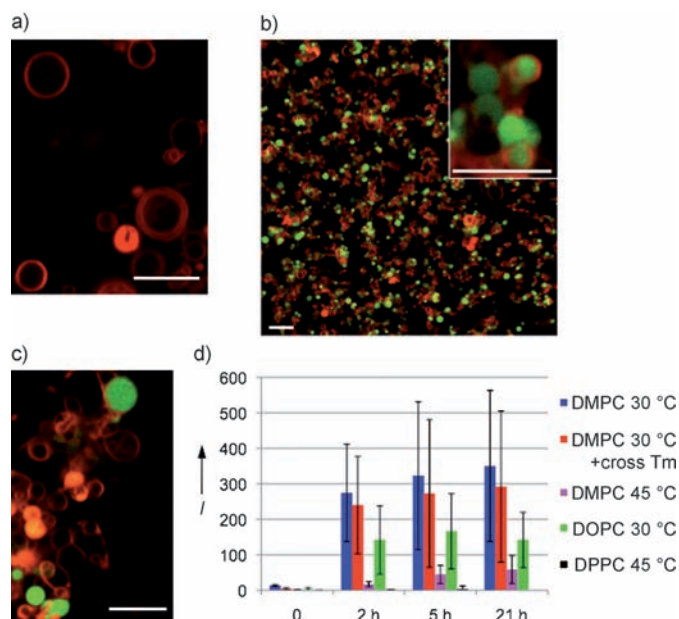


Figure 3. a–c) Fluorescence confocal micrographs of surface-immobilized liposomes diluted with the feeding solution and incubated at 37 °C for emGFP synthesis; a) DP liposomes after 5 h incubation; the image is a montage obtained on the same sample from two different fields of view under identical acquisition settings; b) DO liposomes after 21 h incubation; c) DM liposomes generated at 45 °C, immobilized and incubated for 21 h prior imaging; d) single liposome quantification of emGFP intensity at 0, 2, 5, and 21 h incubation times in the feeding solution; the main lipid in the liposome composition and the swelling temperature are indicated; to cross the T_m in DM liposomes, one temperature cycle (37 °C to 4 °C and back, thus the T_m is crossed twice per cycle) was applied between the time points; scale bars: 10 μ m.

than that in batch mode reactions. DO liposomes made of unsaturated C₁₈-chain phospholipids are in the liquid disordered phase at 37°C and successfully express GFP (Figure 3b). Note that the average size of immobilized liposomes is smaller than that of DM and DP vesicles. The highly heterogeneous expression level observed between individual liposomes for all conditions tested is featured in Figure 3d by the large standard deviation. No further increase of protein synthesis could be observed when crossing the T_m in DM and DP liposomes (Figure 3d and Figure S4 in the Supporting Information).

We established a simple methodology to produce highly concentrated lipid vesicles loaded with a minimal transcription and translation machinery. Additionally, it raises the possibility to engineer an active interfacial membrane platform for surface immobilization, fluorescence localization of liposomes and internal membranous structures, and for controlling molecular exchange through pressure- and temperature-sensitive bilayer permeability. To eliminate external synthesis and trigger gene expression inside surface-positioned liposome microreactors, we performed in situ buffer exchange with an osmolarity-mismatched feeding medium. We found that short C₁₄-chain phospholipids are particularly suited for compartmentalized gene expression with a semi-permeable boundary.

Our system offers a unique possibility to analyze compartmentalized gene expression over long time periods in an ensemble vesicle array,^[8] down to the single-molecule level. Protein-synthesizing vesicles could be integrated in microfluidic devices with potential for further miniaturization and interesting bioanalytical applications for pharmaceutical and medical screening. The field of applications can be extended beyond cell-free gene expression in confined space. Our approach is ideally suited for investigating thermodynamics and kinetics of other complex multiprotein reaction networks in a cell-sized reaction chamber with triggered membrane permeability. Thus, our methodology has the potential to transcend the applicability of liposome research to the development of vesicle-based systems with a high degree of complexity necessary to create semisynthetic minimal cells.

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