Cell-free protein expression systems in microdroplets: Stabilization of interdroplet bilayers

Mark S. Friddin, Hywel Morgan, and Maurits R. R. de Planque[a)]
Electronics and Computer Science and Institute for Life Sciences, University of Southampton, Southampton SO17 1BJ, United Kingdom

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Cell-free protein expression with bacterial lysates has been demonstrated to produce soluble proteins in microdroplets. However, droplet assays with expressed membrane proteins require the presence of a lipid bilayer. A bilayer can be formed in between lipid-coated aqueous droplets by bringing these into contact by electrokinetic manipulation in a continuous oil phase, but it is not known whether such interdroplet bilayers are compatible with high concentrations of biomolecules. In this study, we have characterized the lifetime and the structural integrity of interdroplet bilayers by measuring the bilayer current in the presence of three different commercial cell-free expression mixtures and their individual components. Samples of pure proteins and of a polymer were included for comparison. It is shown that complete expression mixtures reduce the bilayer lifetime to several minutes or less, and that this is mainly due to the lysate fraction itself. The fraction that contains the molecules for metabolic energy generation does not reduce the bilayer lifetime but does give rise to current steps that are indicative of lipid packing defects. Gel electrophoresis confirmed that proteins are only present at significant amounts in the lysate fractions and, when supplied separately, in the T7 enzyme mixture. Interestingly, it was also found that pure-protein and pure-polymer solutions perturb the interdroplet bilayer at higher concentrations; 10% (w/v) polyethylene glycol 8000 (PEG 8000) and 3 mM lysozyme induce large bilayer currents without a reduction in bilayer lifetime, whereas 3 mM albumin causes rapid bilayer failure. It can, therefore, be concluded that the high protein content of the lysates and the presence of PEG polymer, a typical lysate supplement, compromise the structural integrity of interdroplet bilayers. However, we established that the addition of lipid vesicles to the cell-free expression mixture stabilizes the interdroplet bilayer, allowing the exposure of interdroplet bilayers to cell-free expression solutions. Given that cell-free expressed membrane proteins can insert in lipid bilayers, we envisage that microdroplet technology may be extended to the study of *in situ* expressed membrane receptors and ion channels. © *2013 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4791651]

I. INTRODUCTION

Microdroplet technology enables high-throughput compartmentalization and miniaturization of chemical and biochemical assays as pl-to-μl aqueous volumes contained by a continuous oil phase.[1–5] One of the most complex droplet-scale reactions demonstrated to date is the expression of proteins by transcription of a DNA plasmid and subsequent translation of the protein-encoding RNA by a ribosomal cell extract.[6] The main advantage of this "cell-free" method for protein production is that there is no requirement for a molecular biology infrastructure, whereas its main drawback, the high cost of commercial ribosomal extracts, can be offset by performing the expression in microdroplets. Recent examples include the expression of

[a)]Author to whom correspondence should be addressed. Electronic mail: mdp@ecs.soton.ac.uk.

fluorescent proteins, a reductase enzyme, and the membrane-associated protein MreB in pl droplets-in-oil.[7,8]

Once biomolecules have been enclosed in a droplet compartment, additional reagents can be supplied by merging the original droplet with a second droplet that carries the desired molecules.[9] For example, in the case of cell-free protein expression, it can be advantageous to delay the introduction of the DNA plasmid until the droplet with the ribosomal extract is at a defined position in an array, or to replenish the molecules that supply metabolic energy after a certain period of protein expression.[10] Droplet merging with concomitant content mixing is achieved when two droplets are brought into contact with sufficient force. Although it is challenging to manipulate the relative position of specific droplets with conventional flow-driven microfluidics,[11] this can be readily achieved with digital microfluidic platforms, where droplets are moved over a two-dimensional array of planar microelectrodes by electrokinetic forces.[12–14]

To prevent spontaneous merging of droplets that are in close proximity, the oil phase is supplemented with a surfactant that forms a monolayer at the aqueous-oil interface.[15] This droplet coating is also important because biomolecules can accumulate and unfold at this interface when they are in direct contact with the oil phase.[16–18] A wide range of surfactants are used in droplet microfluidics, including fluorinated surfactants for fluorinated oils,[15] but it is also possible to employ natural lipids, the main structural component of the cell membrane.[18] Phospholipid molecules assemble at an oil-water interface with their apolar acyl chains in the oil phase and the polar lipid headgroup in the aqueous phase, thus presenting a biocompatible membrane-mimicking surface to droplet-enclosed biomolecules. Interestingly, it has been shown that when two lipid-monolayer coated droplets are brought into contact, the oil between the droplets is expelled and a lipid bilayer is formed.[19–24] The ability to form interdroplet bilayers that mimic a cell membrane offers the prospect of extending droplet technology to the study of membrane proteins, which constitute the target of approximately 50% of all pharmaceutical drugs.[25]

A large number of membrane proteins have been successfully expressed with macroscale cell-free expression reactions,[26,27] and it has also been demonstrated that the expressed proteins are incorporated in lipid bilayers, added to the reaction mixture as ~50-200 nm diameter unilamellar liposomes.[28–32] Hence, it can be expected that membrane proteins expressed in a microdroplet are able to insert in an interdroplet bilayer, which would open the way to membrane protein studies, including drug screening, with droplet-based fluorescence efflux or electrophysiology assays.[33,34] Bayley and co-workers were indeed able to demonstrate interdroplet bilayer incorporation of the small potassium ion channel Kcv, directly from the cell-free mixture in which the protein was expressed. However, they noted that the lifetime of the interdroplet bilayer, formed by mechanically bringing two droplets into contact with a micromanipulator, was significantly reduced in the presence of the cell-free mixture, resulting in merging of the two droplets.[35] It should be noted that interdroplet bilayers are generally considered to be more stable than aperture-suspended bilayers, but lipid bilayers for ion channel electrophysiology studies are normally not exposed to complex biomolecular mixtures with high protein concentrations.[36]

To explore the potential of microdroplet technology for *in situ* expressed membrane proteins, we have systematically investigated the stability of interdroplet bilayers formed by electrokinetic manipulation of two droplets on planar microelectrodes—representing a basic unit of a digital microfluidic platform—in the presence of three different commercial cell-free protein expression mixtures. We have found that interdroplet bilayers are destabilized by exposure to cell-free reaction mixtures, with bilayer lifetimes of only several minutes or less, depending on the supplier. The protein-rich lysate fractions are the main contributors to this destabilization, and selected pure proteins and polymers at higher concentrations perturb the interdroplet bilayer as well. However, the addition of lipid vesicles to the cell-free expression mixture, especially in combination with a 10-fold dilution of the mixture, stabilizes the interdroplet bilayer. This work thus shows that microdroplet technology has the potential to enable the study of membrane proteins obtained by cell-free expression in interdroplet bilayers.

II. MATERIALS AND METHODS

A. Chemicals and biochemicals

Asolectin lipid extract from soybean (~25% phosphatidylcholine), albumin from bovine serum (BSA), lysozyme from egg white, poly(ethylene glycol) 8000 (PEG 8000), and *n*-decane (≥99%) were obtained from Sigma-Aldrich (MO, USA). The synthetic phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG) were purchased from Avanti Polar Lipids (AL, USA).

B. Microelectrode array fabrication

Devices were fabricated on 6 in. glass wafers as described by Aghdaei *et al.*,[21] with minor modifications on the photoresist application. Briefly, electrode structures were formed by patterning an evaporated Ti/Pt layer (10/200 nm thick) by ion-beam milling. Wafers were diced into 20 × 20 mm chips and these were cleaned with fuming nitric acid, acetone, and isopropanol prior to overnight baking at 150 °C. A piece of Kapton tape (RS Components, UK) was applied to protect the contact pads before spin coating of TI Prime adhesion promoter (MicroChem Corp, MA, USA) at 3000 rpm for 20 s and baking at 120 °C for 2 min. Two layers of the negative photoresist SU8 2000.5 (MicroChem Corp, MA, USA) were then deposited by spin coating at 6000 rpm for 30 s, followed by soft-baking at 95 °C for 1 min. The SU8 was cross-linked by flood exposure to UV light for 10 s at an intensity of 11.85 mJ/cm^2, followed by post-exposure baking at 95 °C for 2 min, and by hard baking at 150 °C for 20 min. This procedure produced a 0.5 μm thick SU8 film, which served as the dielectric that is required for electrokinetic droplet manipulation on planar electrodes.[12–14] Finally, a plastic reservoir was glued onto the surface of the microelectrode array to contain the lipid-decane solution. Dimensions of the microelectrodes and a schematic cross-section of the device layers are shown in Figures 1(j)–1(l).

C. Interdroplet bilayer formation

The microelectrode chip with reservoir (shown in Figure 1(j)) was mounted onto a dual axis goniometer stage (Thorlabs, NJ, USA) inside a Faraday cage and connected to a signal generator, the output of which was amplified using a wideband amplifier and monitored using an oscilloscope. The reservoir was filled with 200 μl of either asolectin lipids in decane (20 mg/ml) or 1:1 (w/w) DOPC/POPG lipids in decane (4 mg/ml). Subsequently, a 2 kHz sinusoidal waveform with a peak-to-peak intensity of 10 V was applied to the two pairs of outer electrodes, (1-1′) and (4-4′), shown in Figure 1(k). A 2-μl droplet of sample or of pure buffer solution (150 mM KCl, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES buffer), pH 7.4) was placed inside the two opposing outer electrodes (as described in Aghdaei *et al.*[21]), which then drew the droplets towards the middle of the electrode array. As the droplets reached the centre of the device, both pairs of outer electrodes were turned off and the inner electrode pairs (2-2′) and (3-3′) were activated by applying 3 V at 2 kHz. Prior to the droplets making contact, both pairs of outer electrodes were also activated to hold the droplets stationary, while the hydrated agar-coated tip of a 250-μm diameter Ag/AgCl electrode, after extensive rinsing with pure buffer solution, was inserted into each droplet. The outer electrodes were then turned off, allowing the droplets to gently come into contact. Once contact was made, the applied potential was slowly reduced to 0 V and the inner electrodes were turned off.

An Axon Axopatch 200B amplifier and an Axon Digidata 1440 A digitizer (Molecular Devices, CA, USA) were used to take both capacitance and voltage-clamped bilayer current recordings using a CV-203BU headstage (Molecular Devices, CA, USA). Current measurements were obtained with a holding potential of 100 mV, a 5 kHz low-pass 8-pole Bessel filter, and a sampling rate of 50 kHz. For presentation purposes, the current traces were then digitally filtered using a 1 kHz low-pass filter. The capacitance was measured by applying a linear voltage ramp under conditions (triangle waveform; 500 Hz frequency, 1 mV peak-to-peak, i.e., ±1 V/s voltage ramp), where the measured current response (e.g., 100 pA) can be interpreted as the bilayer capacitance (e.g., 100 pF). Prior to measuring a new sample, an interdroplet bilayer was formed

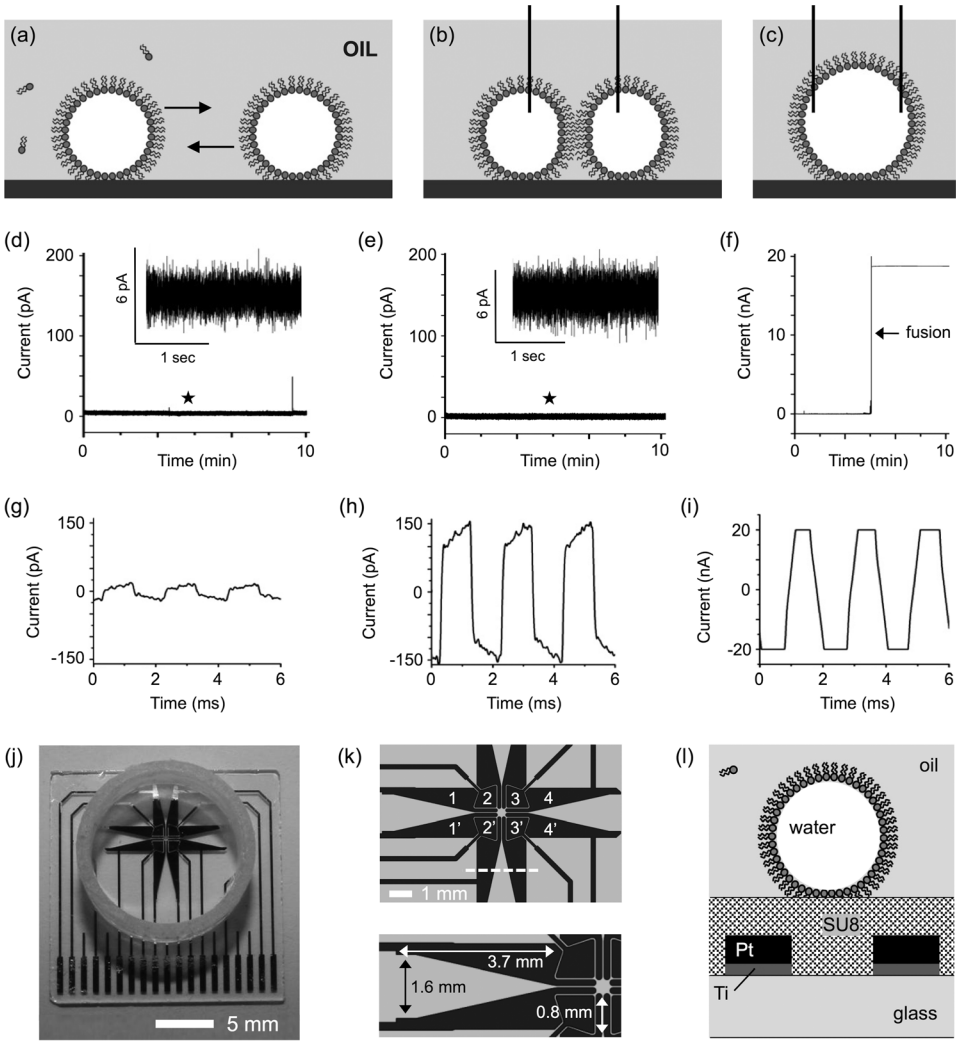

FIG. 1. Formation (a)–(c), current measurements (d)–(f), and capacitance measurements (g)–(i) of interdroplet lipid bilayers formed using a planar microelectrode array (j)–(l). The schematic diagrams (a)–(c) depict that (a) aqueous droplets submerged in a lipid-containing oil phase acquire a lipid monolayer, (b) when two droplets are brought into contact, the oil between the lipid monolayers is expelled and a lipid bilayer is formed between the droplets, and (c) when an excessive contact force is applied or when the interdroplet bilayer is destabilized by the presence of membrane-perturbing components in the aqueous phase, the lipid bilayer breaks and the droplets merge into one larger droplet. The electrical characteristics of this interdroplet bilayer can be measured by placing an electrode in each droplet, and representative current traces (d)–(f) and capacitance traces (g)–(i) are shown for three different scenarios: (d) and (g) the droplets are in close proximity but have not yet expelled the interdroplet oil layer, (e) and (h) a defect-free interdroplet bilayer has formed, and (f) and (i) the interdroplet bilayer has failed during the measurement, resulting in an open circuit and saturation of the amplifier (20 nA cut-off). The insets are an expansion of the region of the main trace marked with an asterisk. Note that bilayer failure is apparent from both current and capacitance measurements, whereas interdroplet bilayer formation is only confirmed by an appreciable capacitance value, as calculated from the current response to a triangle-shaped voltage input. The droplets are brought into contact by electrokinetic forces on a micro-electrode array (j)–(l) as described in Ref. 21: (j) microelectrode array with oil reservoir, (k) expanded view of the two pairs of outer (1-1′ and 4-4′) and two pairs of inner (2-2′ and 3-3′) electrodes, and (l) schematic cross-section (dashed line in (k)) of the various layers of the device: 1 mm glass substrate, 10 nm titanium and 200 nm platinum electrodes, 500 nm SU8 dielectric, and ∼5 mm of oil-lipid solution containing a ∼1.5 mm diameter aqueous droplet (not to scale).

between two droplets of pure buffer solution, the capacitance was verified and the bilayer current was measured for 30 min. When no current events indicative of bilayer perturbation were observed and the bilayer did not fail within the 30-min timeframe, the buffer droplets were removed from the electrode array (i.e., manually guided to the edge of the reservoir) and the new sample droplet was introduced by pipetting on the array. When this was not the case, which

indicates that bilayer-perturbing molecules were retained from the previous experiment at a significant concentration, the Ag/AgCl electrodes were recoated with fresh agar and the oil reservoir was emptied, washed, and refilled with fresh lipid-oil solution. Electrode re-coating and reservoir cleaning were also performed after every 5-10 experiments, i.e., at least at a daily basis.

D. Cell-free expression mixtures and reference samples

The L1130 *E. coli* T7 Extract System for Circular DNA (Promega, WI, USA), the EasyXpress Protein Synthesis Kit (Qiagen Ltd, Crawley, UK), and the Expressway Cell-Free *E. coli* Expression System (Invitrogen Life Technologies, CA, USA) were individually prepared as directed by the manufacturer, with the exception that the RNase-free water was supplemented with 150 mM KCl, 10 mM HEPES, pH 7.4, and that no DNA was supplied. It should be noted that these systems differ in the way the T7 RNA polymerase is supplied: premixed with the S30 lysate in the L1130 and EasyXpress systems, but separately supplied in the Expressway system. For experiments with specific fractions of these expression systems, the total amount of the fraction (e.g., the lysate component) was kept constant and the omitted fractions were replaced with an equal volume of buffer (150 mM KCl, 10 mM HEPES, pH 7.4). Unilamellar vesicles were prepared from a dried film of DOPC/POPG (1:1, w/w) lipids. The film was rehydrated with 1 ml of 150 mM KCl, 10 mM HEPES, pH 7.4, giving a suspension of multilamellar vesicles. Unilamellar vesicles were obtained by passing a freeze-thawed suspension 21 times through a 200 nm polycarbonate filter in a mini extruder (Avanti Polar Lipids, AL, USA). The vesicles were stored at 10 °C and used within 24 h. Polymer and proteins at different concentrations were all prepared in buffer solution (150 mM KCl, 10 mM HEPES, pH 7.4).

III. RESULTS AND DISCUSSION

A. Cell-free expression mixtures and specific reaction components

Bilayer capacitance and current measurements, made possible by the insertion of an electrode into each droplet, enable verification of interdroplet bilayer formation, estimation of the bilayer size, and assessment of the integrity of the bilayer (Figures 1(a)–1(i)). For 2-μl droplets of buffer solution that are brought into contact on the microelectrode array, the initial capacitance is typically 60 pF, which increases with time to 150−300 pF (Figure 1(h)). Assuming a specific bilayer capacitance of 0.5 μF/cm^2,[21] this is indicative for the presence of an interdroplet bilayer with a diameter of 195−276 μm. The bilayer current is stable with a peak-to-peak noise of ∼6 pA after 1 kHz filtering, confirming that the bilayer contains no defects (Figure 1(e)). Lower current noise can be obtained with smaller droplet volumes, which give rise to interdroplet bilayers of smaller area with reduced capacitance.[35,37] In our experiments, bilayers formed from asolectin lipids, a natural lipid mixture extracted from soybean that because of its many different lipid species could act as an effective matrix for a wide range of membrane proteins, were stable in the presence of buffer solution. Specifically, out of 34 independent experiments, 28 bilayers had a lifetime of at least 30 min (measurements were terminated after 30 min because these bilayers can have lifetimes of several hours), while the average lifetime of the six bilayers that failed within this 30-min timeframe was 19 min. Bilayers from the synthetic lipids DOPC and POPG were also stable, with 8 out of 9 independent experiments yielding bilayers that were still intact after 30 min.

Bilayer current recordings with a cell-free expression mixture in one droplet and buffer solution in the second droplet are shown in Figures 2(a)–2(c). It can be seen that the bilayer current is elevated, with a current baseline at ∼5−15 pA and with occasional bursts of short-lived current spikes (<20 ms lifetime and up to 80 pA amplitude). Similar irregular current bursts or current steps have also been observed for lipid bilayers in contact with pure buffer solution when the lipids are near their phase transition temperature[38,39] and for bilayers in the presence of silica nanospheres,[40,41] and have been attributed to lipid packing defects. Our electrical recordings thus indicate that the structural integrity of the bilayer is compromised by exposure to each of the three cell-free expression mixtures. Bilayer rupture, observed as saturation of the

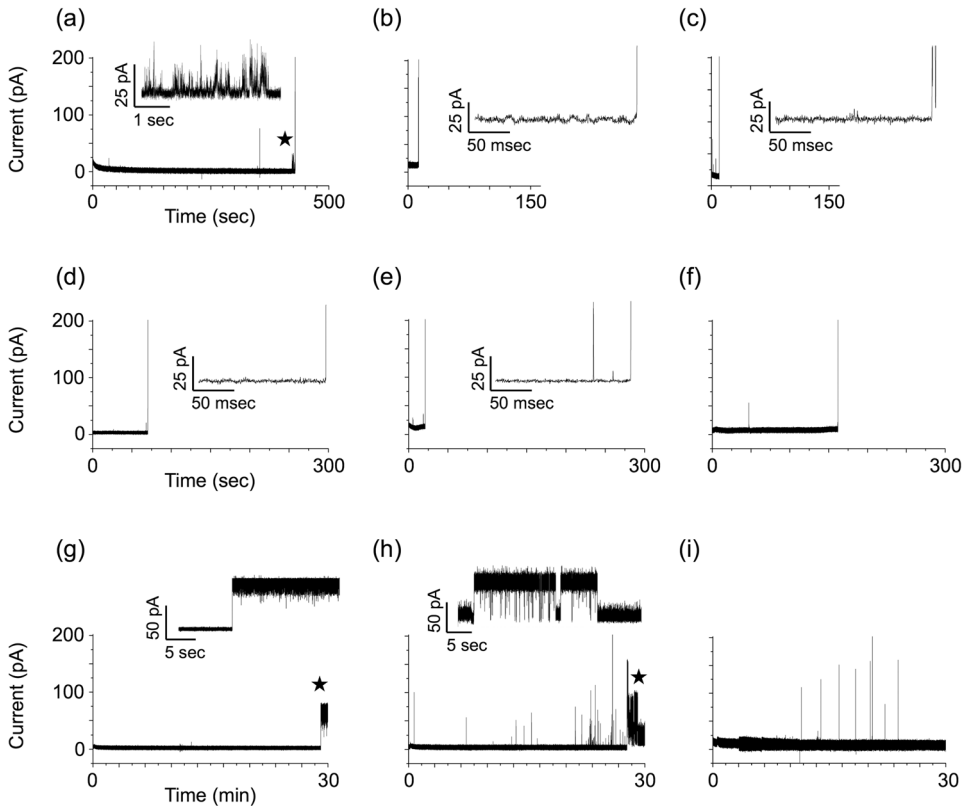

FIG. 2. Interdroplet bilayer current traces for three commercial cell-free reaction mixtures and for selected components. (a)–(c) Current traces for L1130 (a), EasyXpress (b), and Expressway (c) complete reaction mixtures. (d)–(f) Current traces for L1130 (d), EasyXpress (e), and Expressway (f) lysate fractions, supplemented with buffer solution. (g)–(i) Current traces for L1130 (g), EasyXpress (i), and Expressway (j) metabolic energy supply fractions, supplemented with buffer solution. An abrupt rise in current at the end of a trace indicates bilayer failure (a)–(f); corresponding insets show details of the current trace immediately before bilayer failure. When the bilayer remains intact for the duration of the experiment (g)–(i), the insets are an expansion of the region of the main trace marked with an asterisk.

current amplifier, is usually preceded by more intense leakage current spikes of up to 0.5−1.0 nA. Based on eight or more independent experiments, the lifetime of the interdroplet bilayers of asolectin lipids is on average reduced to 8 min in the presence of the L1130 mixture, and to less than 30 s for the EasyXpress and Expressway solutions. Similar observations were made for the L1130 mixture with DOPC/POPG lipid bilayers (data not shown). Hence, the lifetime of interdroplet bilayers in the presence of cell-free expression mixtures based on S30 lysates[42] is too short to allow experiments with *in situ* expressed membrane proteins.

To identify the cause of the bilayer instability, which is unlikely to result from an osmotic imbalance because it is also observed when the mixtures are present in both droplets (data not shown), we investigated the various components that make up the cell-free protein expression mixtures (see Table I). It is the S30 cell lysate, extracted from *E. coli* cell cultures, that contains the actual ribosomal machinery for translation of mRNA.[42–44] This is supplemented with T7 RNA polymerase for transcription of the user-supplied DNA to mRNA, and with a system

TABLE I. Different fractions of commercial cell-free protein expression kits.[a]

L1130 (Promega)	T7 S30 extract	S30 premix	Amino acids		
EasyXpress (Qiagen)	*E. coli* extract	Reaction buffer			
Expressway (Invitrogen)	*E. coli* extract	Reaction buffer	Amino acids	Feed buffer	T7 enzyme mix

[a]These separately supplied components, named according to the supplier, are to be mixed with a DNA template and supplemented with pure water or with a buffer solution to obtain a functional protein expression solution.

for metabolic energy generation (referred to as "premix" or "reaction buffer"), for example, based on pyruvate or creatine kinase enzymes,[10] to sustain the transcription and translation reactions. Because the various suppliers divide the key biomolecules over these premixed component fractions in different ways, we visualized the protein content of these various fractions by gel electrophoresis. Figure 3 shows that each of the three lysate fractions contains a large number of <150 kDa proteins, which is consistent with previous reports.[45] A band with a molecular weight corresponding to T7 RNA polymerase is visible for the Expressway enzyme mix, whereas the remaining component fractions are either devoid of protein or the protein concentration is too low to be detected by staining with Coomassie Blue.

To investigate whether the bilayer destabilization by the cell-free mixtures is due to a high protein concentration, we looked at the effect of the lysate fractions alone. The lysates were diluted with buffer solution to achieve the same concentration as in the total expression mixture, and subsequently one 2-μl droplet of diluted lysate was placed in the oil reservoir and brought into contact with a 2-μl droplet of buffer solution using the electrode array. As shown in Figures 2(d)–2(f), the lysates did not give rise to bursts of bilayer current spikes, but bilayer destabilization was evident from the decreased bilayer lifetime. In contrast to the total mixtures (Figs. 2(a)–2(c)), the average lifetime of the lysates by themselves was different for the three suppliers: ~3 min for Expressway lysate, ~2 min for L1130 lysate and <30 s for EasyXpress lysate. The absence of pronounced current spikes and the significantly increased bilayer lifetime observed, respectively, with the L1130 lysate and the Expressway lysate, suggest that it is not just the protein-rich lysate that contributes to the bilayer destabilization by the complete cell-free expression solutions.

The "energy mixture" fractions, which contain the components for metabolic energy generation, and sometimes also T7 RNA polymerase (Table I), were also investigated at a dilution corresponding to the concentrations in the total mixture. The bilayer current traces in Figures 2(g)–2(i) show low-intensity current spikes (up to 30 pA) as well as large current steps (~75 pA amplitude with a lifetime of several seconds), which are not evident for the total cell-free expression mixtures or for the lysate fractions. Despite these bilayer perturbations, the interdroplet bilayer remained intact for 30 min for all the three energy mixtures, at which point the measurement was terminated. It should be noted that the other fractions of the cell-free mixtures, such as the amino acids and the T7 RNA polymerase (Table I) did not give rise to any bilayer perturbations or reduced bilayer lifetimes, and a control experiment with DNA plasmid also resulted in a bilayer current similar to that observed for pure buffer solution. Hence, both the lysate and energy-supplying fractions contribute to the bilayer destabilization observed for

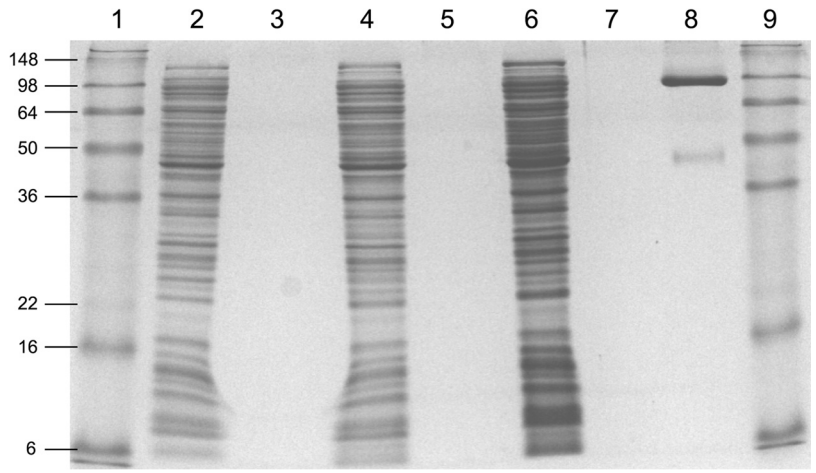

FIG. 3. Gel electrophoresis analysis of the protein content of various components of cell-free expression mixtures. The lanes are loaded as follows: (1) protein ladder with apparent molecular weights in kDa, (2) L1130 lysate, (3) L1130 reaction buffer, (4) EasyXpress lysate, (5) EasyXpress reaction buffer, (6) Expressway lysate, (7) Expressway reaction buffer, (8) Expressway T7 enzyme mixture, and (9) protein ladder. The 15% SDS-PAGE gel was run at 150 V for 75 min in Tris-glycine buffer and stained with Coomassie Blue.

the total cell-free mixtures, even though the vast majority of the proteins is contained in the cell lysate fractions (Figure 3). It is of interest that the lysates give rise to abrupt bilayer failure even though the pre-failure current is indicative of a non-perturbed bilayer, while the energy mixtures, the composition of which is well-defined but proprietary, appear to compromise the structural integrity of the bilayer but not to the point of bilayer rupture.

B. Pure proteins and polymers

Interdroplet bilayers have been used for a variety of purposes, including formation of droplet networks with rectifier properties,[46] characterization of bilayer-embedded ion channels,[21,47] and drug release studies,[48] where the droplets contained only low concentrations of biomolecules or proteoliposomes. A notable exception is the work of Bayley and co-workers, who exposed interdroplet bilayers to a L1130 cell-free mixture with an expressed potassium channel.[35] Although single-channel activity of this ion channel could be observed, the bilayer was found to be relatively unstable, which the authors hypothesized to be caused by the presence of PEG or of residual *E. coli* lipids in the S30 lysate.[35] To address the influence of high protein concentration, PEG polymers, and lipids on the stability of interdroplet bilayers, we investigated solutions of BSA, lysozyme, and PEG 8000. The addition of lipids is discussed in Sec. III C.

Cell-free expression mixtures based on S30 cell lysates contain proteins at a concentration of $\sim$15 mg/ml,[49,50] which is estimated to be equivalent to $\sim$0.1−1 mM. We, therefore, investigated a positively charged and a negatively charged protein, BSA and lysozyme, respectively, at a concentration of 3 mM and compared this with the much lower concentrations of 3 μM and 3 nM, which is within the typical range for interdroplet bilayer experiments with ion channels such as α-hemolysin. The current traces for bilayers in between a droplet with a nM protein concentration and a droplet of pure buffer solution, depicted in Figures 4(a) and 4(c), show a stable baseline as observed for pure buffer (Fig. 1(e)), and the bilayer remains intact for 30 min, after which the measurement is aborted. Similar results were obtained at a concentration of 3 μM (data not shown). At the higher concentration of 3 mM, the two proteins have a different effect on the bilayer, as shown in Figures 4(b) and 4(d). The negatively charged BSA causes a small number of current spikes prior to failure of the bilayer, which happens on average after $\sim$60 s (n = 5), whereas the positively charged lysozyme causes a large number of high-amplitude (up to 1.2 nA) bilayer current spikes, but these do not lead to bilayer failure within the 30-min measurement period (n = 3). Thus, the effects of a high concentration of BSA and lysozyme are, respectively, reminiscent of the effect of cell lysates and energy mixtures (Fig. 2). We speculate that the current fluctuations observed with lysozyme indicate electrostatic interactions with the overall negatively charged asolectin bilayer, but we cannot explain the rapid bilayer rupture observed for BSA, although it should be noted that osmotic effects are unlikely to be dominant because the bilayer lifetime is even shorter when 3 mM BSA is present in both droplets (data not shown).

The polymer PEG 8000 is added to cell-free reaction mixtures as a molecular crowding agent to compensate for the dilution of the pure S30 lysate, typically to a concentration of 4%−5% (w/v).[43,49] We measured the bilayer current in the presence of 0.1% or 10% PEG 8000 on one side of the interdroplet bilayer and pure buffer solution on the other side. As shown in Figures 4(e) and 4(f), current spikes were observed for both polymer concentrations, but occurred more frequently, as prolonged current bursts, and with a higher amplitude (up to 800 pA) for the higher PEG concentration. However, for both polymer concentrations, the bilayer would not break within 30 min (n = 3). Overall, we can conclude that proteins or PEG at the high concentrations typical for cell-free expression mixtures do destabilize interdroplet bilayers, either by inducing transient defects manifested as current spikes or by bilayer failure, which is observed as current saturation and droplet fusion. However, elevated current baselines following current steps were not observed.

C. Stabilization of interdroplet bilayers

When cell-free expression of membrane proteins would first be performed in an isolated droplet coated with a biocompatible lipid monolayer, this droplet could subsequently be brought

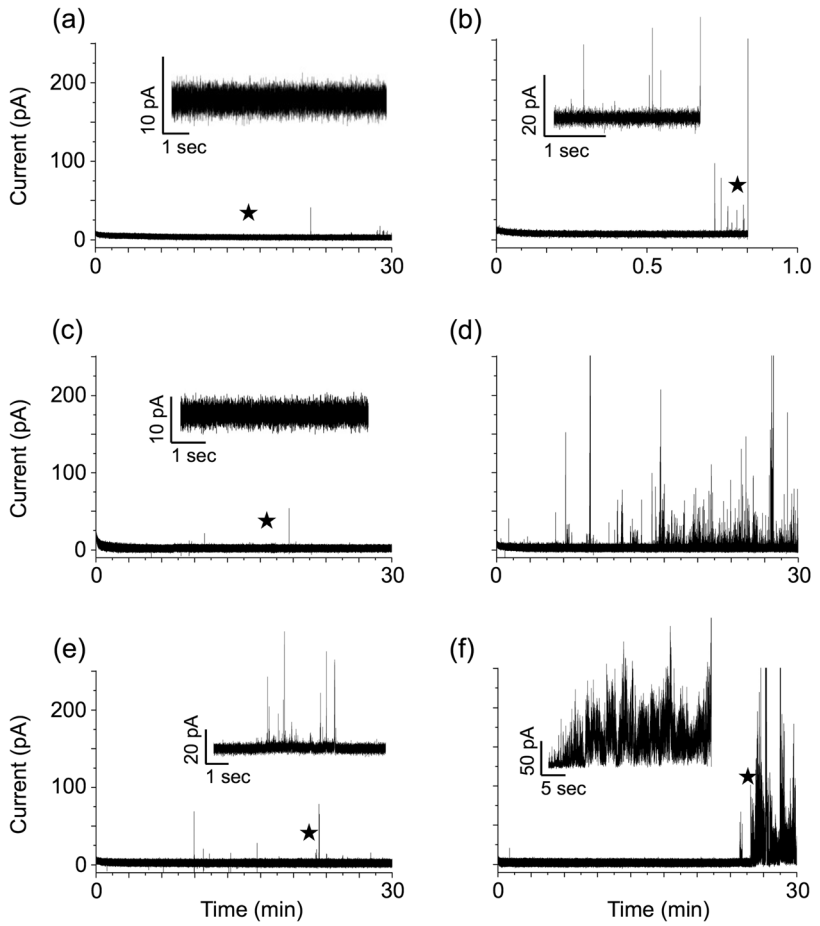

FIG. 4. Interdroplet bilayer current in the presence of various concentrations of pure proteins and polymers. (a) 3 nM bovine serum albumin; (b) 3 mM bovine serum albumin; (c) 3 nM lysozyme; (d) 3 mM lysozyme; (e) 1% (w/v) poly(ethylene glycol) 8000; (f) 10% (w/v) poly(ethylene glycol) 8000. The insets are an expansion of the region of the main trace marked with an asterisk. Note that the large current fluctuations observed at the higher polymer (f) and lysozyme (d) concentrations indicate bilayer instability without bilayer failure, whereas the presence of 3 mM albumin (b) causes the bilayer to break within 1 min.

into contact with a second droplet to form an interdroplet bilayer that enables membrane protein insertion. To subsequently perform an assay on the bilayer-embedded proteins, for example, a fluorescence flux experiment or current measurements with putative channel-targeting drugs in the second droplet,[35] the bilayer is required to be structurally sound and should be stable for at least 20−30 min. We addressed the bilayer stability in the presence of cell-free expression mixtures in two ways: dilution of the cell-free mixture and addition of lipid vesicles. The latter approach also serves to establish whether bilayer destabilization is a result of residual lipid molecules in the S30 lysates, as discussed above.

The rationale of supplementing the cell-free mixture with lipid vesicles is that additional lipid surface is provided, effectively lowering the concentration of undesirable membrane-associating molecules at the interdroplet bilayer. Also, when membrane proteins are expressed by cell-free expression (e.g., in glass vials), vesicles are commonly added to prevent aggregation of the produced proteins and to achieve protein incorporation in the vesicle bilayer for subsequent proteoliposome assays.[28–32] We supplemented the L1130 cell-free mixture with 0.05, 0.5, or 5 μg lipid (DOPC/POPG = 1:1 (w/w)) per μl, which corresponds, respectively, to concentrations of 200-nm lipid vesicles of 0.2, 2.0, or 20 nM. Figures 5(a)–5(c) shows that the dominant features of the bilayer current in the presence of vesicle-supplemented cell-free expression mixture are current steps of up to 25 pA. Once the current has increased following a current step, the current noise increases significantly and appears as downward spikes, suggesting that there is a tendency for

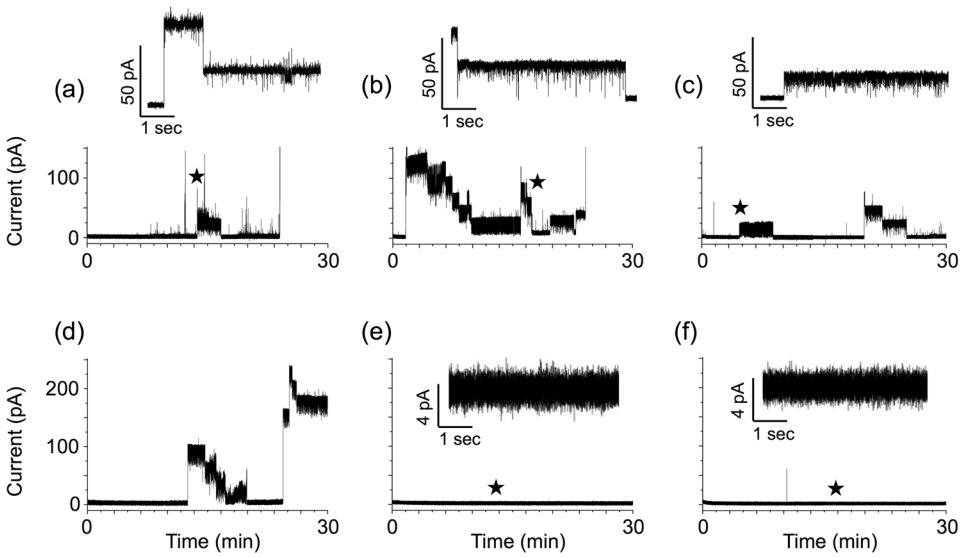

FIG. 5. Interdroplet bilayer current measurements in the presence of non-diluted and diluted L1130 expression mixture, supplemented with lipid vesicles. (a)–(c) Standard, non-diluted, L1130 mixture with 0.2 nM (a), 2.0 nM (b), and 20 nM (c) lipid vesicles. (d)–(f) Tenfold diluted L1130 mixture with 0.2 nM (a), 2.0 nM (b), and 20 nM (c) lipid vesicles. The insets are an expansion of the region of the main trace marked with an asterisk. Dilution in combination with vesicle addition stabilizes the interdroplet bilayer.

the bilayer to seal the defects that give rise to the elevated current. The current spikes are also observed in the non-supplemented expression mixtures (Figs. 2(a)–2(c)), but in the presence of the vesicles, the average lifetime of the bilayer is significantly increased, from ~2 min (see above) to 22−28 min (n = 3 for each lipid concentration). This lifetime is sufficiently long for various membrane protein assays, but the observed current steps indicate the bilayer will, to some extent, be permeable to ions, which can interfere with ion channel experiments.

It is interesting that these relatively well-defined current transitions are observed when lipid vesicles are added to the cell-free mixture, which could indicate that the current steps in the non-modified mixtures are indeed caused by residual *E. coli* lipids in the S30 cell lysates.[35] We hypothesize that these steps correspond to the fusion of vesicles with the interdroplet bilayer, with each vesicle carrying a large number of surface-associated molecules with bilayer perturbing properties, which gradually diffuse away after a vesicle-bilayer fusion event. In this scenario, increasing the vesicle concentration would reduce the amount of material associated with each vesicle and would thus cause less bilayer perturbation, but in our experiments, we could not increase the amount of lipids while maintaining a homogeneous mixture. Instead, we lowered the concentration of expression-associated molecules by diluting the L1130 expression mixture 10-fold with buffer solution. This dilution by itself, without added vesicles, improved the bilayer stability significantly, enabling an average lifetime of 21 min (n = 6), with only a few brief (~2 ms) elevations in the baseline (200−400 pA) prior to bilayer failure (data not shown). Hence, a 10-fold reduction in the concentration of all the components of the cell-free mixture mitigates the destabilization effect. In the presence of 0.2 nM vesicles, distinct bilayer current steps with associated elevated bilayer currents were apparent for the diluted expression mixture, but at higher vesicle concentrations the current baseline was identical to that observed for pure buffer solution (Figures 5(d)–5(f)), indicating that the interdroplet bilayer is free of defects, and that the bilayers remained intact during the 30-min measurements. Dilution in combination with vesicle addition, thus, enables a stable interdroplet bilayer as a matrix for insertion of cell-free expressed membrane proteins.

IV. CONCLUSIONS

Interdroplet bilayers, and oil-suspended lipid bilayers in general, are typically exposed to dilute protein or proteoliposome samples.[20,36] This study shows that, when brought into direct

contact, lipid monolayer microdroplets-in-oil can also form interdroplet bilayers when a droplet contains a complex cell-free expression solution. The lifetime of these lipid bilayers is short (minutes or less), which is attributed to bilayer perturbation by the proteins in the lysate and energy-supply fractions, but the membranes can be stabilized by the addition of lipid vesicles to the expression mixture. Moreover, in combination with a 10-fold dilution of the mixture, sufficiently stable defect-free bilayers are obtained. Since cell-free expressed membrane proteins are known to insert into vesicles/liposomes[28–32] and proteoliposome fusion is a means of membrane protein delivery to lipid bilayers,[36,51] we expect our stabilization strategy to be compatible with incorporation of membrane proteins into interdroplet bilayers. Guiding lipid-coated droplets in digital microfluidic platforms[12–14] would enable on-chip initiation and incubation of protein expression reactions, followed by the formation of interdroplet bilayers by gradually reducing the distance to droplets that contain reagents for functional assays with bilayer-incorporated membrane proteins. Further advances in this area could thus extend the manifold applications of microdroplet technology to the study of membrane receptors and ion channels, including pharmaceutical drug screening, where the protein-expressing droplet could be sequentially brought into contact with a series of drug-carrying droplets, as demonstrated by Bayley and co-workers[35] by manual droplet positioning.

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