CHAPTER NINE

TXTL-based approach to synthetic cells

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Contents

Abstract

Cell-free transcription–translation (TXTL) has recently emerged as a versatile technology to engineer biological systems. In this chapter, we show how an all *E. coli* TXTL system can be used to build synthetic cell prototypes. We describe methods to encapsulate TXTL reactions in cell-sized liposomes, with an emphasis on the composition of the external solution and lipid bilayer. Cell-free expression is quantitatively described in bulk reactions and liposomes for three proteins: the soluble reporter protein eGFP, the membrane proteins alpha-hemolysin (AH) from *Staphylococcus aureus*, and the mechanosensitive channel of large conductance (MscL) from *E. coli*.

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Abbreviations

AH	alpha-hemolysin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
eGFP	enhanced green fluorescent protein
MscL	mechanosensitive channel of large conductance
P28	*E. coli* promoter corresponding to sigma factor 28
P70	*E. coli* promoter corresponding to sigma factor 70
RNAP	RNA polymerase
TXTL	cell-free transcription–translation

1. Introduction

Recent innovations in synthetic biology have increased the prospects for the bottom-up construction of synthetic cells (Rideau, Dimova, Schwille, Wurm, & Landfester, 2018). Though the goal of such work is to create artificial life in the laboratory, such an ambitious project requires and spurs the development of new technologies. The synthesis of life also encourages biologists, chemists, and physicists to tackle the question of "what *is* life," or, put another way, what does it mean for an organism to be considered alive (Periyasamy, Gray, & Kille, 2013)? Although a consensus answer to this question remains elusive, most would agree that cellular life relies on a synergy between information (the ability to process molecular instructions stored in polymers), metabolism (the ability to collect chemical and/or physical energy to synthesize molecular building blocks), and self-organization (the ability to assemble molecules into active machines)—the three of which are necessary for replication and evolution (Fig. 1). The synthesis of unicellular analogs in the laboratory generally follows one of three approaches. Constructing *protocells* explores the origin of life through the assembly of compartments made of prebiotic components such as fatty acids. Protocells are based on primitive metabolism schemes and self-assembly mechanisms with little to no focus on an information component (Mansy & Szostak, 2009; Sole, 2009). *Artificial cells* merge synthetic and natural components, perhaps someday leading to what can be called orthogonal life (Hammer & Kamat, 2012; Kamat, Katz, & Hammer, 2011). The *minimal cell* approach consists of synthesizing a cell using natural molecular components and containing the minimum set of genes for self-maintenance and self-reproduction.

There are two separate but complementary paths to build a minimal cell: top-down construction strips the genome of a living cell to a near-minimal set of genes responsible for life (Gibson et al., 2010); bottom-up construction involves creating a set of genes from scratch, encapsulating

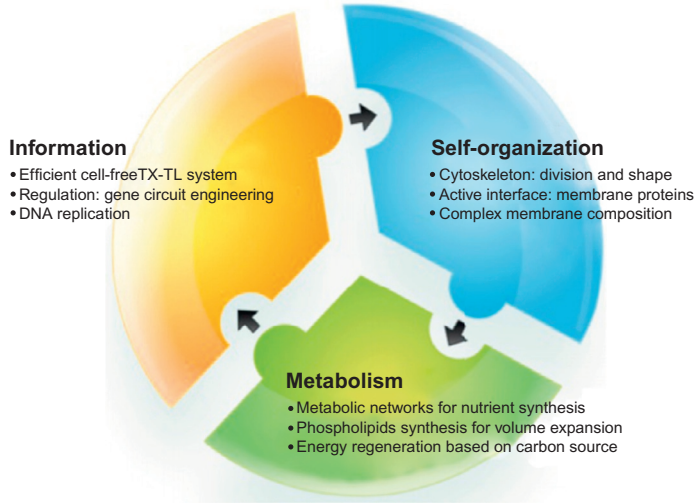

Fig. 1 Representation of the three indispensable parts to a minimal cell: information, metabolism, and self-organization. Some of the most challenging obstacles are listed for each part. Information: a powerful TXTL platform capable of executing complex gene circuits is a lasting goal. DNA replication would need to be incorporated. Self-organization: cytoskeletal proteins of structure and division would need to be expressed while providing the physical asymmetries necessary for vesicle budding and cell division. Though work on membrane proteins and membrane composition is furthered by this chapter, there is a degree of complexity lacking in both areas. Metabolism: nutrient synthesis is a process to be continuously improved upon as it is the basis for the other two tenets. Phospholipid synthesis is a highly complex problem to reconstitute in vitro and is fundamental when considering the growth of the cell or the budding of daughter cells. Energy regeneration has been improved, although the metabolism for a self-replicating container would need to be incredibly robust and thus more work is needed. *Reprinted with permission from Caschera, F., & Noireaux, V. (2014). Integration of biological parts toward the synthesis of a minimal cell.* Current Opinion in Chemical Biology, 22*, 85–91.*

and expressing them in cell-sized compartments. In this chapter, we describe the bottom-up synthesis of minimal cells using cell-free transcription–translation (TXTL). TXTL stands out as a technology to build minimal cells because it enables the construction of liposomes that are genetically programmable, thus seeding the trio of information–metabolism–self-assembly. TXTL is highly versatile, providing researchers with tools to perform activities as diverse as prototyping CRISPR technologies (Marshall, Garamella, Noireaux, & Pierson, 2018; Marshall, Maxwell, et al., 2018; Maxwell, Jacobsen, Marshall, Noireaux, & Beisel, 2018), testing gene circuits (Garamella, Marshall, Rustad, & Noireaux, 2016; Karzbrun,

Tayar, Noireaux, & Bar-Ziv, 2014; Niederholtmeyer et al., 2015), or imparting mechanosensitivity into a synthetic cell (Majumder et al., 2017). Cell-free expression has the edge on in vivo studies in that TXTL reactions can be carried out in hours as opposed to days for in vivo experiments. They also offer a greater degree of control over experimental design as the only genes expressed are those the experimenter programs. TXTL kits are easy to use and commercially available, with transcription booted up either from the T7 RNA polymerase or the RNA polymerase from *E. coli*. Herein we describe a minimal cell approach based on a highly versatile TXTL system that uses *E. coli* TX (core RNA polymerase and sigma factor 70) and TL molecular machineries for gene expression (Fig. 2) (Garamella et al., 2016). TXTL reactions are prepared first in test tube and then encapsulated into cell-sized liposomes. The DNA programs added to the reactions before encapsulation are assembled using standard molecular cloning techniques and typically consist of regulatory elements and genes cloned into plasmids.

There are many methods to encapsulate complex solutions. Lipid hydration techniques can yield a monodisperse population of unilamellar vesicles, although the size distribution ($<1\,\mu m$) is often too small to image using fluorescence microscopy techniques (Pereira de Souza, Stano, & Luisi, 2009). Microfluidic devices offer control over encapsulation efficiency and compartment size, though the techniques are not readily accessible to novice scientists (Damiati, Mhanna, Kodzius, & Ehmoser, 2018). Both techniques struggle in the presence of solutions as complex as something like TXTL.

The emulsion transfer method (Pautot, Frisken, & Weitz, 2003), shown in Fig. 3, for producing liposomes has been an effective technique for encapsulating TXTL reactions for well over a decade (Ishikawa, Sato, Shima, Urabe, & Yomo, 2004; Noireaux & Libchaber, 2004). Although this method does not yield a homogeneously sized population, this can be advantageous in experiments studying how protein systems, such as those in charge of cell structure or division, behave at varying scales. The lipid composition of the bilayer membrane can be tuned using this technique, as we show in this chapter. It also offers simple solutions to alter the environment in which the liposomes reside. These liposomes are stable in test tubes and persist on a microscope cover slide for several days, allowing one to monitor the behavior of proteins on long timescales and allowing endpoint expression of DNA programs. The emulsion transfer method produces cell-sized liposomes 1–50 μm in diameter, which is readily viewable using simple light microscopes. Lastly, the barrier to entry for this technique is low relative to the others discussed, with nearly all of the equipment accessible in an introductory biology or chemistry laboratory.

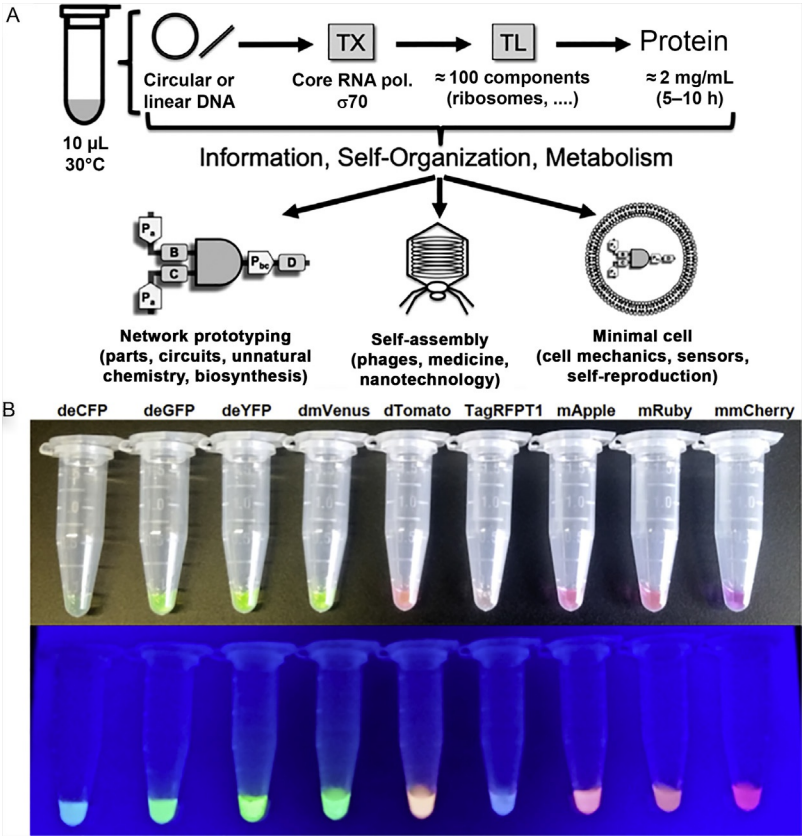

Fig. 2 Overview of the all *E. coli* TXTL system's capabilities and applications. (A) This TXTL platform is highly flexible and suited for applications at molecular (network prototyping), supramolecular (self-assembly), and cell-sized scales (minimal cell, microfluidics). (B) Nine reporters expressed in TXTL batch-mode reactions, imaged under visible (*top*) and ultraviolet light (*bottom*).

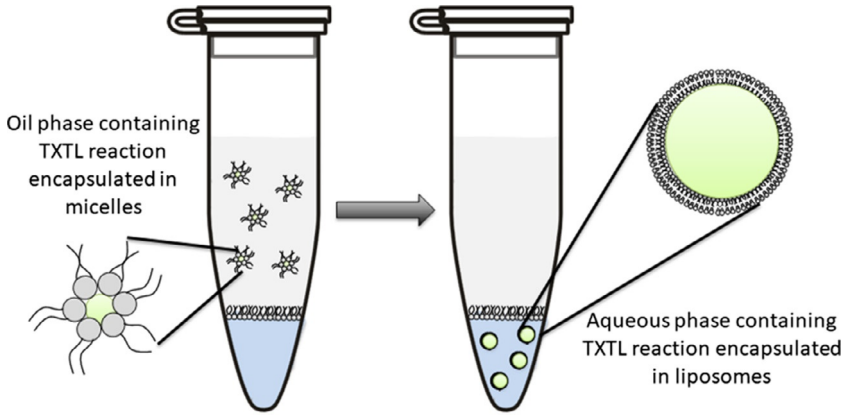

Fig. 3 Illustration of the emulsion transfer method to produce liposomes. Before centrifugation (*left*), a TXTL reaction is emulsified in an oil saturated with phospholipids. It is added atop an aqueous feeding solution. During centrifugation, the liposomes are formed by passing through the interface oil-feeding solution. After centrifugation (*right*), the oil phase is depleted of micelles and liposomes now reside in the aqueous feeding solution, immediately ready for observation via microscopy.

2. TXTL protein expression

2.1 Expressing proteins using the *E. coli* RNA polymerase and sigma factor 70

TXTL reactions are composed of an *E. coli* lysate, salts, and buffers that provide both the 20 amino acids and an ATP regeneration system. The lysate contains the TX machinery (e.g., *E. coli* core RNA polymerase, RNAP, and sigma factor 70) and the TL components (e.g., ribosomes and tRNA). The TX speed in a cell-free reaction is between 5 and 10 nucleotides per second. To initiate transcription, RNAP and sigma factor 70 (σ70) form a holoenzyme to bind to the promoter, in this case P70a, specifically recognized by σ70. Because TX is based on RNAP-σ70 from *E. coli*, the TX repertoire is composed of hundreds of regulatory elements present in *E. coli* and other bacteria. The other six *E. coli* sigma factors are not present in the lysate, and therefore must be expressed and used with their specific promoters. Translation is carried out at a rate of roughly two amino acids per second by the same ribosomal machinery present in *E. coli*. Reactions are incubated at 29°C (the optimal temperature) for roughly 12 h, depending upon the composition of the circuit and what is being expressed. The volume of reactions is critical in TXTL as cell-free reactions require oxygen.

For this reason, bulk reactions carried out in test tubes are typically 12 μL and kinetics carried out in well plates are 2–5 μL, depending on the particular well plate. Recently, TXTL has been miniaturized to allow high-throughput TXTL reactions in volumes as low as 0.5 μL using automated liquid handlers, specifically the Labcyte Echo 550 (Marshall, Garamella, et al., 2018). This modern approach conserves resources and allows researchers to more efficiently analyze the experimental parameter space for any genetic circuit they can design.

The primary source of information in TXTL reactions is DNA, both plasmid and linear. Plasmid DNA is preferable in TXTL reactions. Linear DNA, degraded by the RecBCD complex present in the extract (Klocke, Garamella, Subramanian, Noireaux, & Franco, 2018; Michel-Reydellet, Woodrow, & Swartz, 2005), can be readily used in TXTL reactions, though protein expression is weaker (Marshall, Maxwell, Collins, Beisel, & Noireaux, 2017). As seen in Fig. 4, a reporter protein like eGFP is expressed at a concentration of more than 50 μ*M* in a batch-mode reaction, which corresponds to an amplification factor of 10^4, with respect to the concentration of plasmid DNA added (~5 n*M* plasmid DNA). There are several methods to measure protein expression in TXTL reactions, with the easiest being the use of a fluorescent reporter protein. Upon proper folding, reporter proteins like GFP (green), YFP (yellow), or RFP (red) emit well-defined spectra when

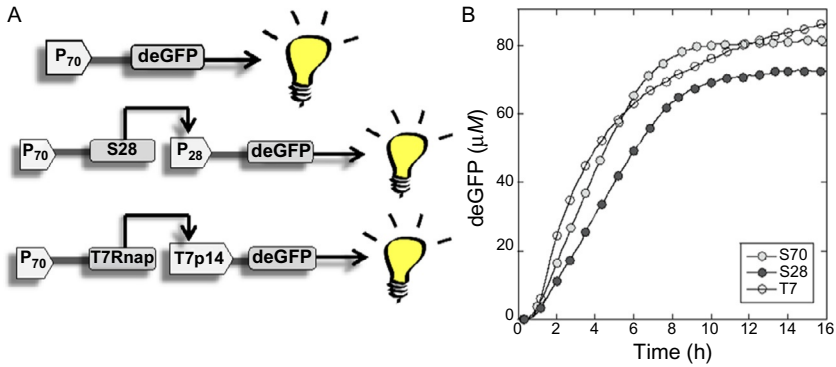

Fig. 4 Design and characteristics of three genetic circuits expressing deGFP. (A) Circuit diagrams to express deGFP through promoters specific to σ70, σ28, and T7 RNAP. (B) Kinetics of expression for 5 n*M* P70a-deGFP; 0.5 n*M* P70a-S28 with 15 n*M* P28a-deGFP; and 0.2 n*M* P70a-T7rnap with 2 n*M* T7p14-deGFP (all plasmid DNA). *Adapted with permission from Garamella, J., Marshall, R., Rustad, M., & Noireaux, V. (2016). The all* E. coli *TX-TL toolbox 2.0: A platform for cell-free synthetic biology.* ACS Synthetic Biology, *5, 344–355.*

excited by the appropriate wavelength of light. We most commonly use a truncated version of eGFP, what we call deGFP (Garamella et al., 2016), that is more translatable in TXTL but retains the properties that make eGFP so popular: fast folding, well-defined spectra, and enhanced stability. Reporter proteins are preferable to luciferases as they do not require the addition of luciferin to emit light and can be used quantitatively for kinetics.

2.1.1 Equipment list

- 1.5 mL Tubes, Denville C2170
- Denville Vortexer 59A
- Denville Mini Mouse 2 centrifuge
- 29°C Incubator, Denville mini incubator I2200-H
- −80°C Freezer, Revco
- Biotek Synergy H1 plate reader
- 384-Well plates, Nunc, flat bottom, #242764
- MyTXTL sold by Arbor Bioscience (Arbor Bioscience #507024)

2.1.2 Buffers and chemicals

- Type 1 water (PURELAB Option-Q DV 25, ELGA)
- P70a-deGFP

2.1.3 Protocol

1. Remove the MyTXTL tube from −80°C and thaw at room temperature.
2. Once the prepack reaction is thawed, add the necessary volume of plasmid DNA to achieve the desired concentration (5 n*M* for P70a-deGFP).
3. Complete to 90 μL with autoclaved Type 1 water. Vortex gently and spin down.
4. Split the 90 μL solution into seven different Eppendorf tubes containing 12 μL.
5. Incubate the 12 μL reactions at 29°C for 8–12 h or overnight.
6. After 8–12 h, carefully pipette 10 μL of each reaction into a single well of a 384-well plate.
   a. It is important here to pipette the reaction without causing the formation of small bubbles.
   b. Measure the fluorescence in a plate reader measuring fluorescence at 485 nm excitation and 528 nm emission.

Notes:

1. The TXTL system used in this work is commercially available from Arbor Biosciences under the name of myTXTL.

2. If the source of water is Type 1, an autoclave is not absolutely necessary. It is recommended to use autoclaved water when possible.
3. Plasmids are stable at room temperature, though we use −20°C for long-term storage.

2.2 Expressing proteins in transcription cascades (T7p14 and S28)

While the ability to express protein from one plasmid is simple and makes TXTL more accessible, it does not offer much in the way of gene regulation. Furthermore, certain genes of interest—MscL, a protein used by *E. coli* to respond to osmotic stress (Majumder et al., 2017)—are toxic to *E. coli* cells and cannot be amplified using the housekeeping promoter. By making use of transcription cascades, we are able to overcome both of these obstacles (Fig. 5). It is a method to amplify a plasmid containing a gene cloned under the T7 promoter as it is relatively silent in *E. coli* (Studier & Moffatt, 1986). We also use the *E. coli* promoter P28a specific to the flagellar sigma factor, sigma 28, as cells grown in optimal conditions do not express sigma 28 (Studholme, Buck, & Nixon, 2000). Amplifying plasmids with a P28a or T7 promoter is done using either JM109 (Promega) or DH5alpha (NEB) cells. For plasmids using the P70a promoter, we prepare our own KL740 competent cells. Since P70a originates from the lambda phage Cro promoter with the two operators sites OR2 and OR1 overlapping the −10 and −35 sequences, and KL740 overexpresses the lambda repressor, cI, we are able to amplify toxic plasmids in *E. coli*. To increase complexity, repressors can be expressed simultaneously and degradation tags can be applied to any part of the pathway. ClpXP, an AAA + protease from *E. coli*, is present in the extract and readily acts on degrons such as ssrA or ompA. The *E. coli* interferase MazF can be added to increase and to adjust the mRNA inactivation rate (Shin & Noireaux, 2010). The T7p14 cascade requires the production of T7 RNAP by way of the P70a-T7rnap plasmid, while the P28a cascade requires the production of sigma 28 via the P70a-S28 plasmid. Due to the ease of amplification, along with promoter strength and selectivity, these cascades are the two most commonly used in our system.

2.2.1 Buffer and chemicals

- Type 1 water
- P70a-S28
- P28a-deGFP
- P70a-T7rnap
- T7p14-deGFP

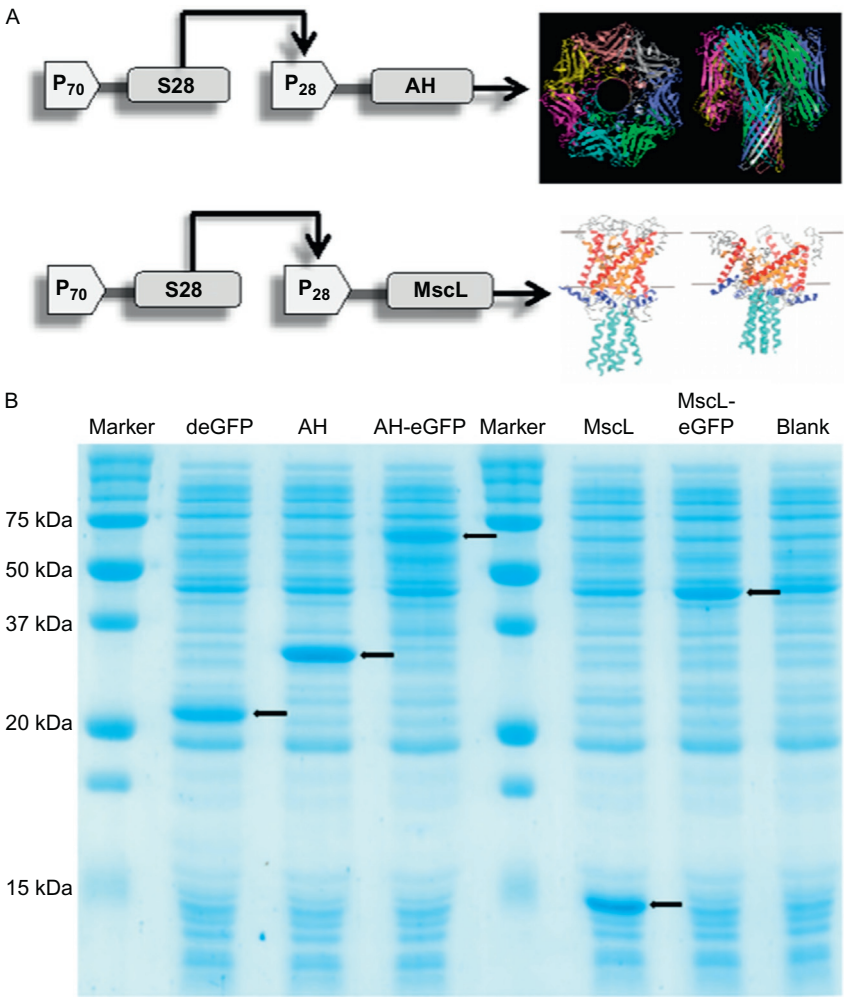

Fig. 5 Expression of AH and MscL in TXTL. (A) Schematic of AH and MscL expressed through the σ28 cascade. (B) SDS-PAGE gel showing the production of deGFP (27 kDa), AH (33 kDa), AH-eGFP (61 kDa), MscL (14 kDa), and MscL-eGFP (41 kDa). The "blank" shows the background proteins present in a TXTL reaction containing no plasmid DNA. *Images adapted with permission from EMBL-EBI Protein Data Bank in Europe and Deplazes, E., Louhivuori, M., Jayatilaka, D., Marrink, S. J., & Corry, B. (2012). Structural investigation of MscL gating using experimental data and coarse grained MD simulations.* PLoS Computational Biology, 8, *e1002683.*

2.2.2 Protocol for cascades

1. The preparation of the reaction is the same as described above except for the change in plasmid concentrations.
2. To execute the sigma 28 cascade, 0.5 n*M* of P70a-S28 and 2 n*M* of P28a-deGFP are added into the mix.
3. To execute the T7 cascade, 0.1 n*M* of P70a-T7rnap and 1 n*M* of T7p14-deGFP are added into the mix.

3. TXTL encapsulation in liposomes

3.1 Adjusting the feeding solution during encapsulation of a CFR (cell-free reaction) (P70a-deGFP) into liposomes using the emulsion transfer method

There are potentially many ways to encapsulate a TXTL reactions in lipid vesicles, including microfluidic devices, lipid film hydration, and the emulsion transfer method. In this chapter we describe liposomes prepared using the emulsion transfer method. The technique is conceptually a simple one, the steps being emulsification of the TXTL reaction into an oil saturated with dissolved phospholipid, followed by centrifugation of the emulsion into an aqueous solution (the outer solution) to produce unilamellar liposomes. Both the outer solution and the composition of the phospholipid membrane can be readily altered. Due to the importance that local environment can have on bacterial life, it is imperative to have the ability to alter the environment we introduce our synthetic cell to. From the perspective of the mechanical construction of a synthetic cell, the environment would dictate the type of functionality the cell would require. If the environment is nutrient rich or nontoxic, passive membrane pores, such as alpha-hemolysin (AH), could be included to give the cell the ability to take in resources from its surroundings or allow by-products to be excreted. If changes in osmotic pressures are expected, the cell would need a way to respond to these changes. This could either be addressed via the expression of cytoskeleton proteins to maintain structural integrity or a mechanosensitive pore like MscL (mechanosensitive channel of large conductance) to allow the flux of molecules either into or out of the cell.

We use three different outer solutions in our research depending on the goal of the experiment as the type of outer solution used greatly affects gene expression (Fig. 6). The first and simplest solution (F1) is a mixture of phosphate-buffered saline (PBS) and glucose. PBS has a physiological pH where myTXTL functions efficiently (pH 7–8), while glucose serves as a simple molecule to vary the osmotic pressure. Since the encapsulated solution is complex, it is necessary to balance the osmotic pressure of the inner solution with that of the outer solution. This easily made solution allows for synthetic cell experiments even in the most rudimentary laboratory environments. The second outer solution (F2) is more complex as it involves tailoring the components of the exterior solution to match the most readily accessible components of myTXTL. A Tris-buffered solution of magnesium-glutamate,

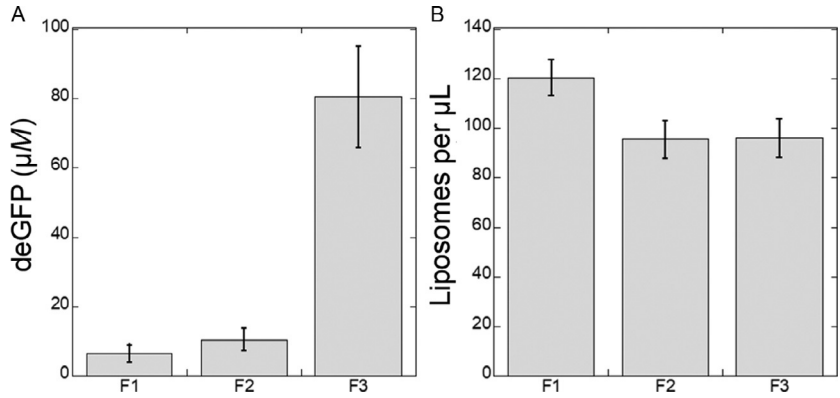

Fig. 6 Total reporter protein synthesis and concentration of liposomes with respect to variable feeding solutions. (A) deGFP expression for TXTL reactions containing 5 n*M* P70a-deGFP encapsulated in liposomes residing in three different feeding solutions. Protein production increases as more nutrients are added to the feeding solution. (B) Typical concentrations of liposomes produced using the emulsion transfer method for each feeding solution described herein. F1: 300 m*M* glucose, 1 × PBS. F2: 33% S30 buffer, 90 m*M* K-Glu, 5 m*M* Mg-Glu, 35 m*M* maltodextrin, and 1.5% PEG. F3: 33% S30 buffer, 18 × energy buffer, 3 m*M* amino acid buffer, 90 m*M* K-Glu, 5 m*M* Mg-Glu, 35 m*M* maltodextrin, and 1.5% PEG.

potassium-glutamate, PEG, and maltodextrin is used to feed the myTXTL reaction while simultaneously matching osmotic pressure conditions. This requires the addition of these chemicals as well as an autoclave, making it more difficult to prepare for the novice scientist. Finally, in the third outer solution (F3), the energy and amino acids buffers present in the myTXTL reaction are added to make a true and balanced feeding solution. This solution is identical to a myTXTL reaction with the replacement of the lysate with a buffered solution. We use this solution in experiments where we want to feed the interior reaction to increase the overall protein production. This solution is the most difficult to prepare and the cost ($3000–4000) to prepare the ATP regeneration buffer alone makes this solution out of reach for labs that do not specialize in cell-free protein expression.

3.1.1 Equipment list

- Bench-top centrifuge, Eppendorf Centrifuge 5415D
- Eppendorf tubes, Denville C2170
- Glass vials for storage, 4 mL glass vials with Teflon-lined caps, Avanti 600460

- Emulsification tubes, Fisherbrand™ 7 mL Borosilicate Glass Scintillation Vial 0333726
- Denville Vortexer 59A
- Cover slides, Fisherbrand 12-545-D
- Cover seals, 125499
- pH meter
- Autoclave

3.1.2 Buffers and chemicals

- Egg PC (Phosphatidylcholine), Avanti #840051
- Chloroform
- Liquid paraffin, Wako #128-04375
- Magnesium-glutamate, Sigma-Aldrich #49605
- Potassium-glutamate, Sigma-Aldrich #G1149
- Maltodextrin, dextrose equivalent 4.0–7.0, Sigma-Aldrich #419672
- PEG 8000, Sigma-Aldrich #P2139
- Tris, Fisher #BP154-1
- 10 × PBS, Sigma-Aldrich #P5493
- Glucose, Sigma-Aldrich #G5500

3.1.3 Protocol for buffer preparations

1. F1:
   a. Using a vortexer, dissolve glucose in Type 1 water at a concentration of 1 *M*.
      i. Do not autoclave as glucose can caramelize.
   b. Combine Type 1 water, glucose solution, and 10 × PBS at appropriate volumes to achieve a 1 × PBS solution with 200–400 m*M* glucose.
2. F2:
   a. Prepare a 1 *M* solution of Mg-Glutamate in Type 1 water using an autoclave.
   b. Prepare a 3 *M* solution of K-Glutamate in Type 1 water using an autoclave.
   c. Prepare a 40% weight by volume solution of PEG 8000 in Type 1 water using an autoclave.
   d. Prepare a 2 *M* solution of Tris in Type 1 water using an autoclave.
   e. Prepare S30 buffer by combining 14 m*M* Mg-Glu and 150 m*M* K-Glu in Type 1 water. Buffer to pH 7.2 with Tris 2 *M* solution and autoclave.

f. Prepare a 500 m*M* solution of maltodextrin by vortexing in an Eppendorf tube with Type 1 water.
  i. Maltodextrin is a polymer with a nonuniform molecular weight. However, TXTL reactions are optimized assuming the molecular weight is 360.31 g/mol.
g. In the complete F2, the S30 buffer comprises 33% solution volume, e.g., 30 μL of a 90 μL solution is composed of the S30 buffer.
h. To a given volume of S30 buffer, add K-Glu, Mg-Glu, maltodextrin, and PEG to final concentrations of 90 m*M*, 5 m*M*, 35 m*M*, and 1.5% w/v, respectively. Complete to 100% volume with Type 1 water.

3. F3:
a. To F2, add 3 m*M* amino acid mixture and 18 × energy buffer, both as described in Garamella et al. (2016).

3.1.4 Emulsion transfer method for preparing liposomes

1. Obtain a solution of lipids suspended in $CHCl_3$ and/or CH_3OH.
a. Solubility depends on the lipid. For Egg PC, resuspend at 50 mg/mL in $CHCl_3$.
b. Pipette as quickly as possible as $CHCl_3$ reacts with plastic.
c. Store in an airtight glass vial with a Teflon-lined cap.
  i. You can also use Teflon tape if a Teflon-lined cap is not readily available.
  ii. Stable ~1 month at −20°C.

2. Prepare a solution of lipids dissolved in mineral oil/liquid paraffin.
a. Pipette 500 μL oil into a microcentrifuge tube.
  i. This can be done by eye since the oil is too viscous to precisely pipette.
b. Quickly pipette the lipid/chloroform solution into the oil.
  i. Depending on the lipid, add lipid/chloroform into the oil at 0.5–4 mg/mL. For Egg PC, 1.5 mg/mL (20 μL at 50 mg/mL and ~2 m*M* final) is optimal.
c. Vortex at max until the oil/chloroform solution is well mixed.
  i. Put on a heat block at 55°C with the tube cap open to evaporate off the chloroform.
    1. 1 h per 20 μL chloroform.
    2. Cover loosely with foil to block light.
d. Once oil is at room temperature, it is ready to use.

3. Prepare the emulsion
   a. Pipette 350 μL oil + lipid solution into a 7 mL glass vial.
   b. Add 6 μL reaction solution into the oil.
      i. Reaction solution is the mixed cell-free reaction before overnight incubation as described in Section 2.
   c. Emulsify by vortexing at max speed for ~5 s.
4. Prepare the emulsion/feeding solution stack in a microcentrifuge tube.
   a. Pipette 20 μL feeding solution into the bottom of a microcentrifuge tube.
   b. Add 100–200 μL of the emulsion prepared in step 3 on top of the feeding solution.
      i. Do this carefully so as not to disturb the feeding solution such that the feeding solution and emulsion do not mix.
5. Centrifuge in a bench-top centrifuge for 10 min at 1500 × *g*.
6. After centrifugation, remove as much oil as possible without disturbing the aqueous solution now containing your liposomes.
7. Using a gel-loading tip (very thin pipette tip), remove the aqueous solution.
   a. We usually remove 10 μL of the 20 μL from the bottom of the tube.
8. Onto a glass coverslip, pipette 0.5–1.5 μL volume droplets.
   a. Smaller droplets are better if you intend to monitor the liposomes kinetically as there is less volume for the liposomes to move through.

3.2 Altering the membrane composition of liposomes using the emulsion transfer method

Beyond changing the environment in which the synthetic cell resides, we also want to consider the lipid composition of the bilayer. As the interface between an organism and its surroundings, the lipid bilayer is the physical boundary hosting the molecular mechanisms to exchange nutrients and ions with the cellular exterior. Therefore, the makeup of this intersection is critical. The literature suggests soluble and membrane proteins interact or respond to specific lipids, domains, or global bilayer properties (Ces & Mulet, 2006). Different lipids have different spontaneous curvatures, affecting structure, stability, and rigidity (Sohlenkamp & Geiger, 2016). This likely plays an important part in the function of the MreB and FtsZ protein families in *E. coli*. Some membrane proteins require specific lipids or lipid environments to properly fold and others likely provide structural and

functional support to transmembrane proteins. Indeed, the gating properties of MscL are affected by the bilayer composition (Nomura et al., 2012). Almost all cells extensively regulate the surface charge potential of the lipid bilayer as this can influence headgroup packing and protein function (Wikstrom et al., 2009). The fluidity of the membrane, strongly influenced by simple molecules like cholesterol, affects localization of membrane-bound proteins (Cooper, 1978).

We describe methods to create liposomes using L-α-phosphatidylcholine (Egg PC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DOPG) and detail differences in liposome yield based upon the composition of the lipid membrane (Fig. 7). Our technique for creating these vesicles is simple and hinges on the emulsion transfer method to create liposomes. Using lipids dissolved in an organic solvent, we are able to suspend lipids mixtures by evaporating the solvent from the lipid/solvent/oil mixture. This step is slightly variable as the temperature of the mixture should be held above the melting temperature or transition temperature of any single lipid in the mixture. In this way, we are able to adjust the lipid composition in our synthetic cell experiments to monitor the effects on permeability, gene expression, or membrane protein activity.

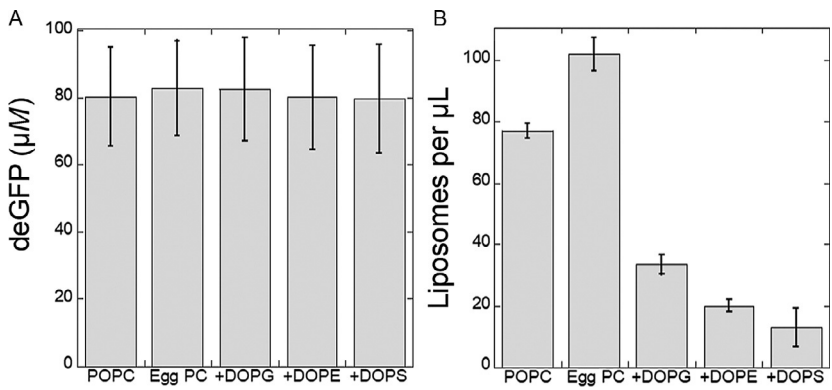

Fig. 7 Total reporter protein synthesis and concentration of liposomes as a function of membrane composition. (A) deGFP expression for TXTL reactions containing 5 n*M* P70a-deGFP encapsulated in liposomes with membranes composed of POPC, Egg PC, 9:1 Egg PC:DOPG, 9:1 Egg PC:DOPE, 9:1 Egg PC:DOPS (molar parts). (B) Typical concentrations of liposomes produced using the emulsion transfer method with membranes composed of POPC, Egg PC, 9:1 Egg PC:DOPG, 9:1 Egg PC:DOPE, 9:1 Egg PC:DOPS.

3.2.1 Chemicals

- Egg PC, Avanti #840051P
- POPC, Avanti #850457P
- DOPG, Avanti #840475P
- DOPE, Avanti #850725P
- DOPS, Avanti #840035P

3.2.2 Protocol for making liposomes composed of Egg PC and DOPG

1. The preparation of the feeding and reaction solutions is the same as described above
2. Resuspend both Egg PC and DOPG in $CHCl_3$ at 50 mg/mL
   a. Pipette $CHCl_3$ as quickly as possible as $CHCl_3$ reacts with plastic
3. Store in an airtight glass vial
   a. Stable ~1 month at −20°C
4. Pipette 500 μL oil into a microcentrifuge tube
   a. This can be done by eye since the oil is very viscous
5. Quickly pipette the lipid/chloroform solution into the oil
   a. The lipid solution should be made based on the final molar ratio. In this case, the Egg PC and DOPG lipid solutions have been prepared at 64 and 63 m*M*, respectively. For a 1:1 mixture at 2 m*M* total lipid concentration, add 7.81 μL Egg PC and 7.94 μL DOPG
6. Vortex at max until the oil/chloroform solution is well mixed
   a. Put on a heat block at 55°C with the tube cap open
      i. 1 h per 20 μL chloroform
      ii. Cover loosely with foil to block light
      iii. The temperature should be higher than the highest melting temperature of any one lipid in the mixture. Increasing the temperature does not adversely affect the evaporation step
7. Once oil is at room temperature, it is ready to use
8. This technique can be applied to the other lipids listed above in the same manner
9. The number of liposomes produced is greatly affected by the lipid composition

3.3 Encapsulating and monitoring membrane proteins in liposomes using the emulsion transfer method

Finally we approach the problem of adding functionality to a synthetic cell. Cells need the ability to respond to their environments to uptake nutrients or

deal with physical stressors (Berube & Bubeck Wardenburg, 2013; Booth & Blount, 2012). In this way, self-assembly is coupled to information and metabolism as stimuli encountered at the interface between the cytoplasmic interior and exterior can induce a response from the synthetic cell. Using AH (gene ID: 3920722), a heptameric pore-forming beta-barrel toxin released by the bacterium *Staphylococcus aureus*, we increase membrane permeability and nutrient exchange, in turn providing a boost to gene expression (Fig. 8). Furthermore, by expressing MscL (gene ID: 947787), a pore-forming protein in *E. coli* able to open in response to physical force, we impart mechanosensitivity to a synthetic cell design scheme (Fig. 9). We have added both of these proteins to a synthetic cell scheme using myTXTL and the emulsion transfer method.

3.3.1 Encapsulation of MscL, MscL-eGFP, AH, and AH-eGFP

1. The protocols above describe the procedure to prepare the reaction solutions and to perform the emulsion transfer method to produce liposomes.
2. As AH and MscL need to be expressed via cascades, use the following concentrations for the S28 and T7 cascades:
   a. S28: 0.2 n*M* P70a-S28, 5 n*M* P28a-AH/MscL
   b. T7: 0.1 n*M* P70a-T7rnap, 1 n*M* T7p14-AH/MscL
3. To monitor the activity of these proteins, either use a fusion protein such as AH-eGFP or MscL-eGFP to measure localization at the membrane or include in the reaction solution a fluorescent dye that can be monitored to leak from the liposomes. We recommend FITC-UTP and 3 kDa TRITC-dextran for AH and MscL, respectively.

3.3.2 Preparation of AH protein for increased nutrient feeding

1. Using the protocol described in Section 2.2, express AH under a T7 cascade
2. Combine the reaction solution containing the expressed AH in a single tube
3. Add 4 U of restriction enzyme *Kas*I and incubate 1 h at 37°C
   a. Be sure to use an enzyme that will cut P70a-T7rnap but not whatever plasmid you intend to express in liposomes
4. Aliquot by 4 μL and store at −80°C
5. To use in liposomes to promote nutrient exchange between interior reaction solution and exterior feeding solution, add to the reaction solution at 30 ×

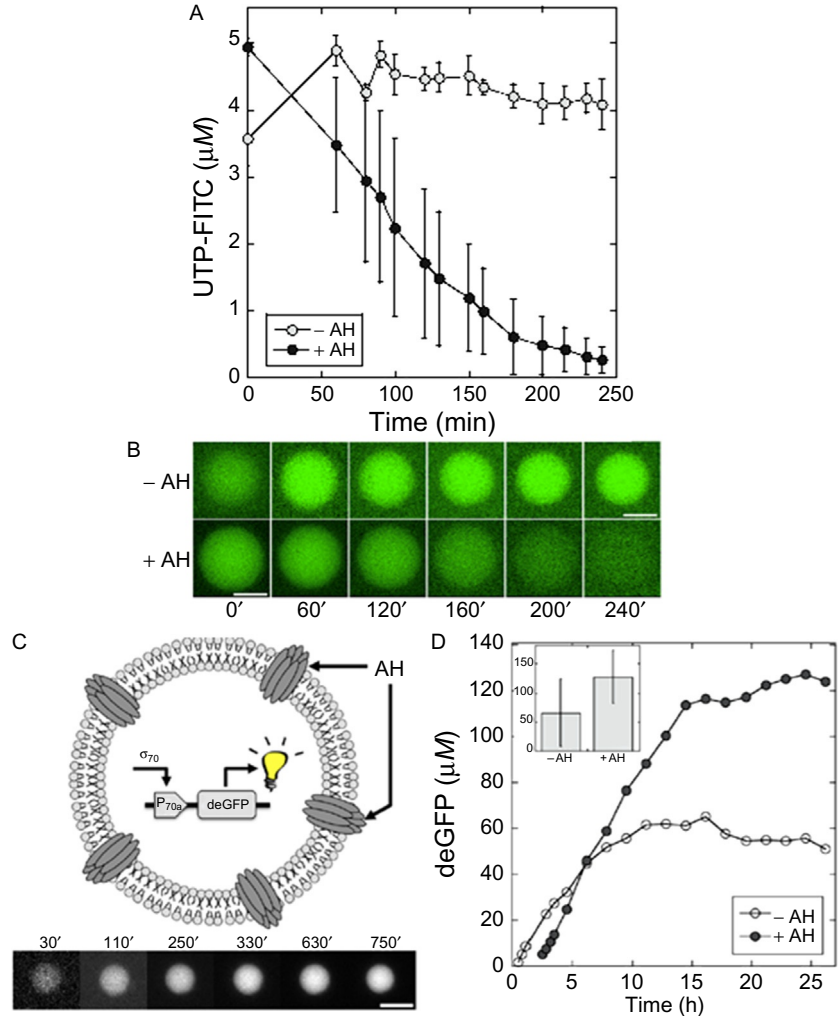

Fig. 8 Functionality of the pore-forming protein alpha-hemolysin (AH) in Egg PC liposomes. (A) Kinetics of the leak of UTP-FITC from liposomes with and without AH. (B) Fluorescence images of liposomes leaking UTP-fluorescein at different times (minutes) for two different liposomes: with AH (2 μ*M*) added to the reaction (8 μm diameter) and without AH (6 μm diameter). Scale bars: 4 μm. (C) Schematic showing a cell-free reaction (plasmid P70a-deGFP) inside a liposome. The toxin (AH) was added to the encapsulated reaction. *Below*: a series of photos showing deGFP fluorescence intensity (time in minutes, scale bar: 10 μm), when AH (2 μ*M*) was added to the reaction. (D) Kinetics of expression of deGFP (P70a-deGFP) inside liposomes (liposome of diameter 11.5 μm) with AH (2 μ*M*) and without AH (liposome of diameter 7.3 μm). *Inset*: statistics of deGFP fluorescence after 12 h of incubation with and without AH. *Reprinted and adapted with permission from Garamella, J., Marshall, R., Rustad, M., & Noireaux, V. (2016). The all* E. coli *TX-TL toolbox 2.0: A platform for cell-free synthetic biology*. ACS Synthetic Biology, *5, 344–355.*

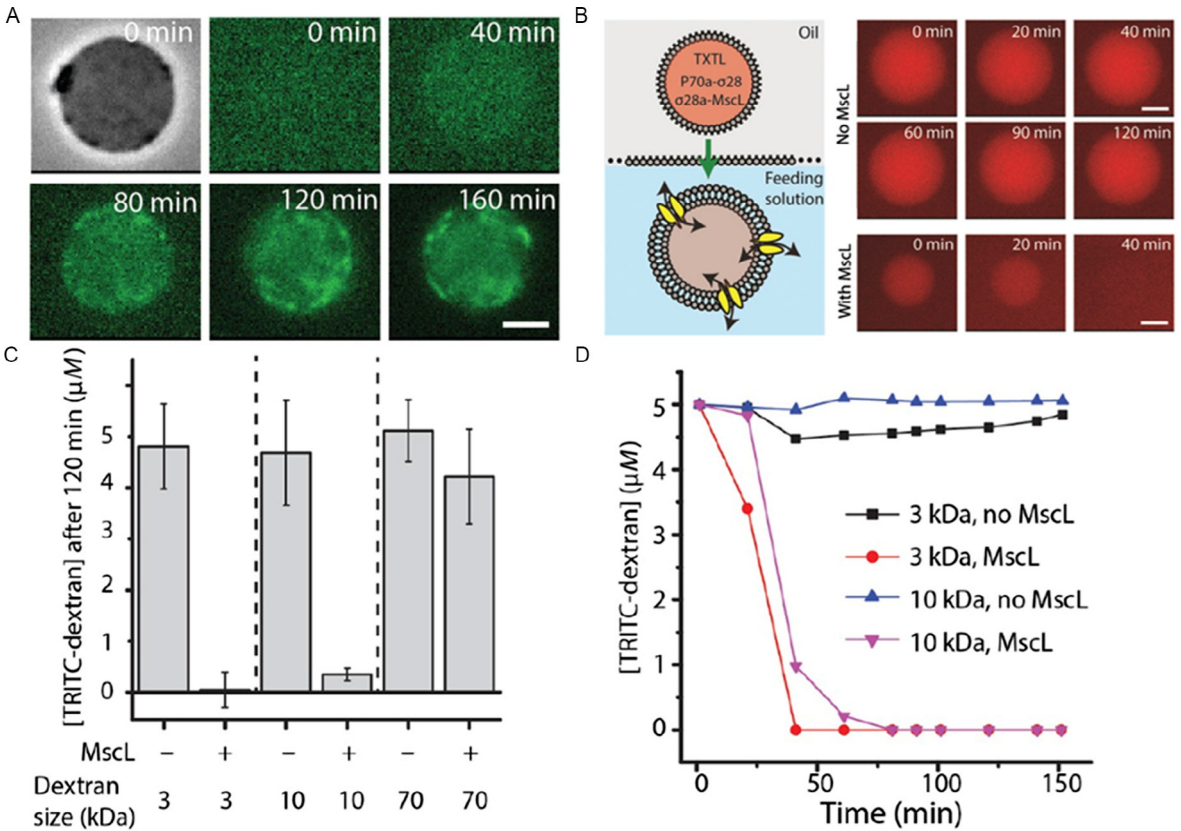

Fig. 9 See figure legend on opposite page

4. Concluding remarks

The protocols we have developed in this chapter are the basis to prepare liposomes loaded with TXTL reactions. We describe techniques allowing researchers to encapsulate a robust protein synthesis platform into liposomes with tunable membrane compositions. These liposomes can be imparted with membrane pores that offer nutrient feeding or mechanosensitivity, giving them the ability to respond to their environment, which can itself be adjusted. The combination of these abilities are valuable tools for not only novel scientific discovery, but also in fields such as drug discovery and drug delivery.

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Conflict of interest

The Noireaux laboratory receives research funds from Arbor Biosciences, a distributor of the myTXTL cell-free protein synthesis kit.

Fig. 9 Functionality of the mechanosensitive channel of large conductance (MscL) in Egg PC liposomes. (A) Fluorescence images of MscL-eGFP being expressed in liposomes over 160 min (0.2 n*M* P70a-S28 and 5 n*M* P28a-MscL-eGFP). Scale bar: 5 μm. (B) *Left*: Schematic of the encapsulation of a cell-free reaction containing 3 kDa TRITC-dextran (5 μ*M*), P70a-S28 (0.2 n*M*), and P28a-MscL (5 n*M*) into liposomes via the water-in-oil emulsion transfer method. *Right*: Fluorescence images of liposomes containing 3 kDa TRITC-dextran over a 2-h period with only P70a-S28 added to the cell-free reaction. Fluorescence images of liposomes containing 3 kDa TRITC-dextran over a 40-min period with P70a-S28 and P28a-MscL added to the cell-free reaction. Scale bar: 5 μm. (C) Bar graph of average TRITC-dextran concentration at 0 and 120 min. 5 μ*M* TRITC-dextran encapsulated in liposomes at $t = 0$. Sizes of dextran are 3, 10, and 70 kDa. − or + denote the absence or presence of MscL. (D) Liposome kinetics of 3 and 10 kDa TRITC-dextran concentration in the presence or absence of MscL. *Reprinted with permission from Majumder, S., Garamella, J., Wang, Y. L., DeNies, M., Noireaux, V., & Liu, A. P. (2017). Cell-sized mechanosensitive and biosensing compartment programmed with DNA.* Chemical Communications (Cambridge, England), 53, *7349–7352.*

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