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Assembly of MreB Filaments on Liposome Membranes: A Synthetic Biology Approach

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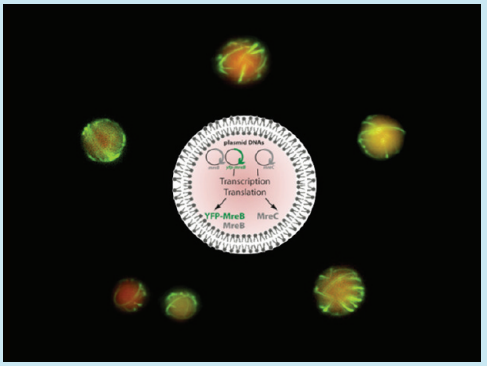
Supporting Information

ABSTRACT: The physical interaction between the cytoskeleton and the cell membrane is essential in defining the morphology of living organisms. In this study, we use a synthetic approach to polymerize bacterial MreB filaments inside phospholipid vesicles. When the proteins MreB and MreC are expressed inside the liposomes, the MreB cytoskeleton structure develops at the inner membrane. Furthermore, when purified MreB is used inside the liposomes, MreB filaments form a 4−10 μm rigid bundle structure and deform the lipid vesicles in physical contact with the vesicle inner membrane. These results indicate that the fibrillation of MreB filaments can take place either in close proximity of deformable lipid membrane or in the presence of associated protein. Our finding might be relevant for the self-assembly of cytoskeleton filaments toward the construction of artificial cell systems.



KEYWORDS: *bacterial cytoskeleton MreB, cell-free gene expression, vesicle bioreactor, artificial cells*

Phospholipid bilayer membranes, the physical boundary of living cells, are two-dimensional self-organized structures with many cellular functions.[1] The shape of eukaryotic cells, for instance, is determined by the interaction of a large number of proteins with cytoskeletal structures at the cell membrane. To understand the coordinated cytoskeleton membrane structure, the reduction of its complexity is essential. One route to understand this complex set of interactions and functions is to use a reductionist bottom-up approach to reconstruct cellular functions *in vitro*.[2−4] Actin bundles and microtubules have been polymerized inside synthetic liposomes using such a constructive cell-free approach.[5−13]

The reconstruction of bacterial cytoskeleton *in vitro* has been less explored, partly because the presence of cytoskeleton proteins in bacteria such as MreB and FtsZ was identified only recently.[14−16] The bacterial proteins MreB and FtsZ are homologous to the eukaryotic actin and tubulin respectively. Their polymerized filaments, however, have unique properties distinct from filamentous actin and microtubules. *In vitro*, the MreB monomers from *Escherichia coli* (*E. coli*) polymerize into thin and short nanofilaments of 100 nm in length and 1 nm in diameter.[17] *In vivo*, MreB helical and ring filament structures are formed by interacting with the membrane proteins MreC and MreD at the inner membrane of the bacterium.[18,19] Similar protein−membrane interactions have been observed for FtsZ and other bacterial cytoskeleton proteins.[20,21] These observations motivated us to reconstruct MreB structures at the inner membrane of synthetic liposomes.

The reconstruction of genetically programmed simple architectural models for cell division and cell shape regulation inside phospholipid vesicles is one of the challenges to synthesizing artificial cells.[22,23] In this Letter, we show that the bacterial cytoskeleton MreB can be reconstituted inside phospholipid vesicles using a transcription−translation cell-free expression system. The constructed MreB cytoskeleton shows filamentous structure at the inner membrane of liposomes when MreB and MreC are expressed inside the vesicles. In addition, we show that a MreB protein, engineered to be attached to the membrane with an amphipathic peptide, can form thick bundled structures through its interaction with the membrane in the absence of MreC.

Cell-Free Gene Expression and Polymerization of YFP-MreB in Synthetic Liposomes. The goal of this study is to reconstruct a simplified *E. coli* MreB cytoskeleton inside phospholipid vesicles. Recent technical advances with vesicle bioreactors enable encapsulating the complete cell-free expression reaction (CFER) inside liposomes.[22,23] This artificial cell system was used to express the pore-forming protein α-hemolysin, which assembles nanometer-size pores into the phospholipid bilayer.[22,24] The CFER contains all of the components necessary for transcription and translation. The genes of interest are cloned into individual plasmids, which are added to the CFER.

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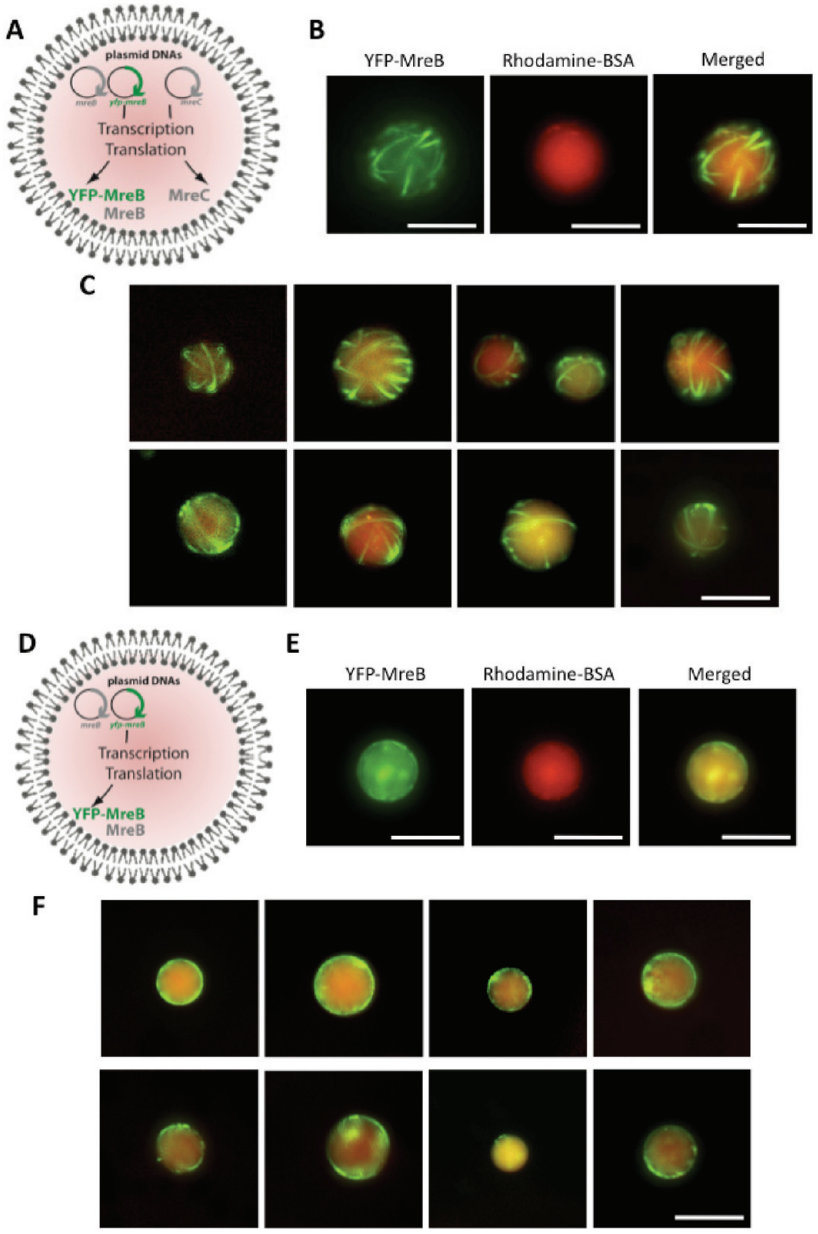

Figure 1. Cell free expression of YFP-MreB in the liposome. (A) Scheme of cell-free gene expression of MreB, YFP-MreB, and MreC in a phospholipid vesicle. (B, C) Co-expression of MreB, YFP-MreB, and MreC and cytoskeleton formation. The fluorescence of YFP (green) shows the MreB on the liposome surface forming filamentous structures in the presence of MreC, while rhodamine BSA (red) shows the isolation of the liposome from outside feeding; 2.5 nM YFP-MreB plasmid, 1.25 nM MreB plasmid, and 1.25 nM MreC plasmid were used. (D) Scheme of cell-free gene expression of MreB and YFP-MreB in a phospholipid vesicle. (E, F) The fluorescence of YFP shows no filamentous structure without MreC; 2.5 nM YFP-MreB plasmid and 2.5 nM MreB plasmid were used. Scale bar: 10 μm.

One of the advantages of cell-free expression (CFE) is the possibility to express multiple proteins simultaneously and at different levels by adjusting the concentration of each plasmid. This is particularly useful for this study because the MreB cytoskeleton involves multiple proteins. In this work we used a recently developed efficient cell-free gene expression system from *E. coli*.[25,24]

We first expressed concurrently a set of three proteins: MreB, YFP-MreB, and MreC (Figure 1A). MreC is a membrane protein that interacts with MreB.[14,18,19] The CFER and the plasmids encoding the three proteins were encapsulated inside phosphatidylcholine vesicles (see Methods).

We observed the formation of MreB filaments at the inner membrane of the liposomes after approximate 12 h of gene expression (Figure 1B and C). The filamentous structures formed in the liposomes exhibited a flexible polymer mesh. This cytoskeleton structure was observed in the vesicle whose diameter was smaller than 15 μm, whereas no structure was built in vesicles larger than 15 μm. The MreB filaments were assembled at the geodesic of the spheroid lipid vesicle, which suggests that MreB filaments grow under the minimum bending energy.

Next we examined the expression of MreB in the absence of MreC. MreB and YFP-MreB were expressed concurrently inside the liposomes (Figure 1D). We found that in the absence of MreC, MreB formed small clustered aggregations (Figure 1E and F). The polymerization of MreB into filamentous

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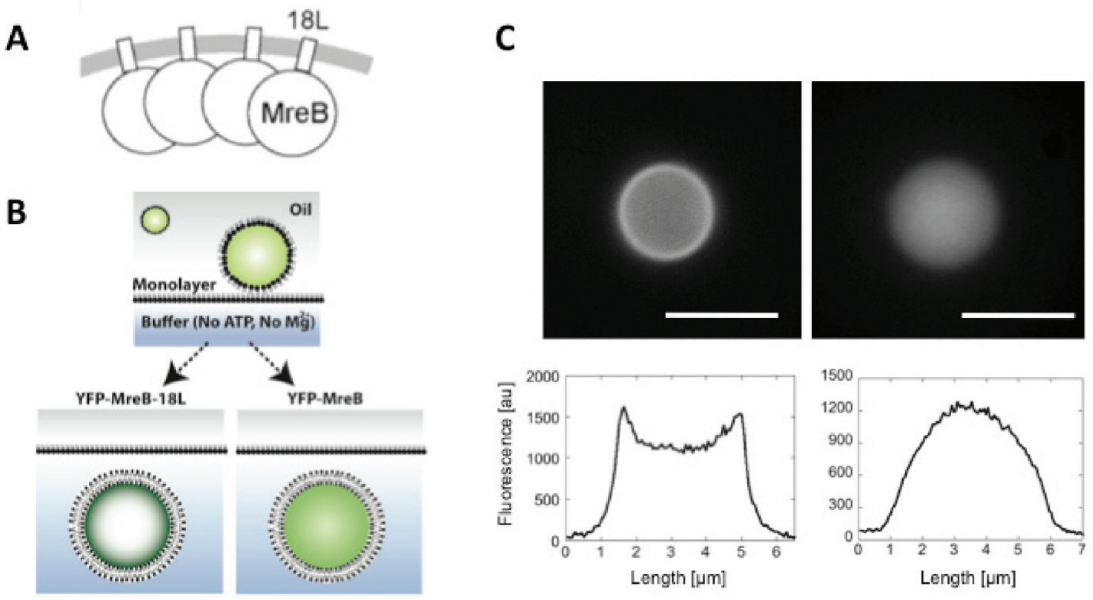

Figure 2. Design of engineered MreB-18L. (A) Diagram of the structure of MreB-18L. (B) Scheme of the experiment carried out in liposomes. The fusion protein YFP-MreB interacts with the phospholipid membrane via the amphipathic peptide 18L. Large unilamellar vesicles are formed by centrifugation of microdroplets through lipid monolayer at the interface between oil and water phases.[22] (C) Left: Localization of YFP-MreB-18L at the inner surface of vesicles (top). Line scan showing the brighter edge (bottom). Right: YFP-MreB, stays in the cytoplasm of liposome (top) and the line scan shows no edge effect (bottom). Scale bar: 5 μm.

structures in CFE inside liposomes requires the simultaneous expression of MreC.

Design and Polymerization of MreB in Artificial Vesicles. Another strategy to reconstruct a simplified bacterial cytoskeleton system inside liposomes is to use purified proteins. We designed a recombinant MreB protein, YFP-MreB-18L, where YFP-tagged MreB was fused to a helical amphipathic peptide, 18L,[26] at the C-terminal end (Figure 2A). The recombinant YFP-MreB-18L protein was expressed in *E. coli* and purified using standard procedures (see Methods). Because 18L incorporates spontaneously into phospholipid bilayers owing to its amphipathic nature,[22,24] the protein localized beneath the lipid membrane inside the vesicle. We used a mixture of DOPG/DOPC/DMPC (ratio 0.3:0.2:0.5) to make the liposomes (Figure 2B).[22] This lipid composition was chosen because more than 10% of negatively charged DOPG lipid was necessary for the binding of 18L, and the ratio of DOPC/DMPC was best for making lipid vesicles having typical diameters of 5−20 μm. The liposomes were first prepared in a buffer with no ATP (adenosine triphosphate) and magnesium ions. The pure YFP-MreB-18L protein was uniformly associated with the spherical surface of lipid vesicles but did not polymerize (Figure 2C and Supplementary Figure S1), indicating that YFP-MreB-18L monomers diffused on the membrane with no aggregation.

The polymerization of MreB in bulk solution was induced by the addition of ATP and Mg^{2+} as reported earlier.[17] In the absence of phospholipid, YFP-MreB-18L proteins formed small clusters with a size below the resolution of fluorescent microscopy upon the addition of 1 mM ATP and 5 mM Mg^{2+} (Figure 3A). The polymerization started immediately, and small clusters were detected within 5 min. When encapsulated in a vesicle, the polymerization was faster than the vesicle preparation (which takes 20 min). Consequently, small clusters developed before interacting with the membrane when we mixed ATP and Mg^{2+} in the initial protein solution (data not shown).

To induce the polymerization of YFP-MreB-18L after its membrane localization, we used the pore-forming protein α-hemolysin from *Staphylococcus aureus*. The toxin was added in the external buffered solution to allow the uptake of Mg^{2+} and ATP inside the liposomes. The α-hemolysin protein assembles into heptameric pores of diameter 1.4 nm through which molecules of up to 3 kDa can diffuse. Because YFP-MreB-18L (~64 kDa) is larger than the molecular mass cutoff, YFP-MreB-18L stays localized inside the vesicles.

We induced the polymerization of YFP-MreB-18L in vesicles by adding the pure α-hemolysin protein, ATP, and Mg^{2+} to the external buffered feeding solution only (Figure 3B). ATP and Mg^{2+} diffused slowly inside the vesicle, avoiding the fast clustering of MreB. After 2 h of incubation at 37 °C, YFP-MreB-18L formed cytoskeleton filaments of 4−10 μm inside the vesicles. Characteristic morphologies of YFP-MreB-18L are shown in Figure 3C−F: straight shape (n = 21 out of 45, Figure 3C), buckled φ-like shape (n = 12, Figure 3D), and lemon-like shape (n = 8, Figure 3E). We also found that multiple protrusions of YFP-MreB-18L were generated from single vesicles (n = 4, Figure 3F and F′). The number of samples for this analysis is 45 in this study due to the small yield of liposome preparation. We note that those structures were stable for days.

To distinguish whether the lipid membrane covers the MreB cytoskeleton or is pierced through, we simultaneously observed YFP-MreB-18L and the lipid bilayer with the lipid-specific dye DiI.[27] Two-color visualization revealed that precise co-localization of these two signals (Supplementary Figure S2). The phospholipid vesicles were no longer in a spherical shape but were altered dynamically due to the force exerted by the MreB cytoskeleton.

Because the structure of individual MreB filaments was not visible with the fluorescent microscopy, we used transmission

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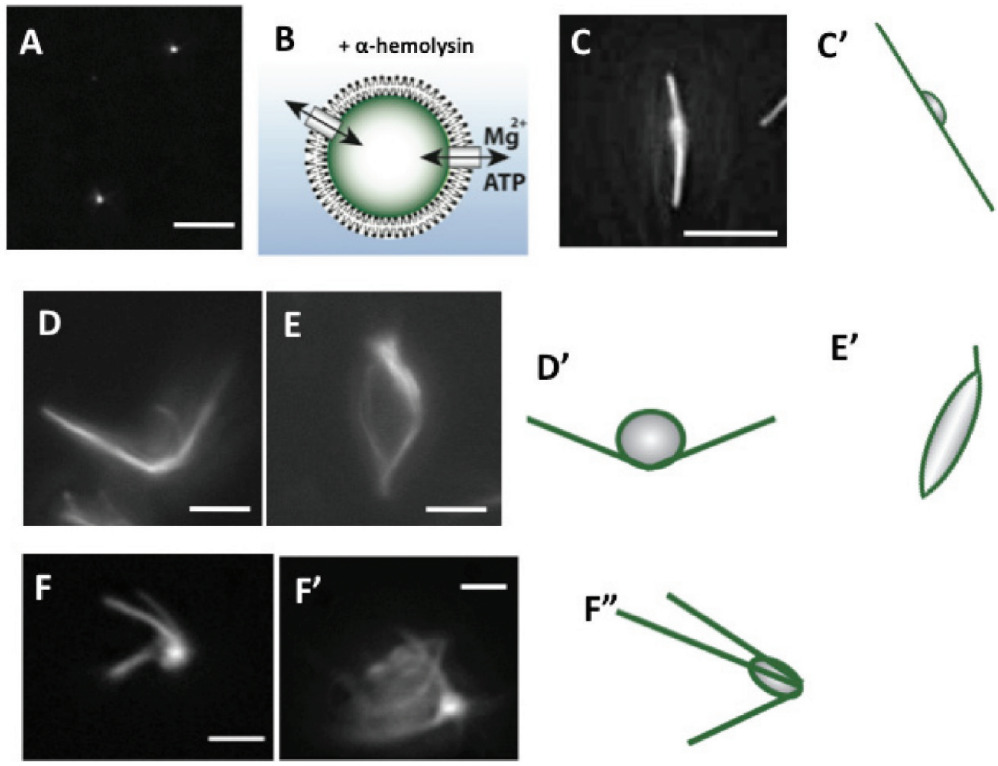

Figure 3. Construction of YFP-MreB-18L cytoskeleton in phospholipid vesicles. (A) Fluorescent microscopy of polymerized YFP-MreB-18L in bulk solution. (B) Scheme of diffusing components through α-hemolysin pore. The polymerization is induced by the diffusion of Mg^{2+} and ATP through α-hemolysin pores. Pore-forming α-hemolysin at 0.5 μM was added to the external feeding solution. (C, D, E, F, and F′) YFP-MreB-18L cytoskeletons in lipid vesicles. Characteristic morphologies are (C) straight, (D) bended, (E) lemon-like, (F and F′) multiple protrusions. (C′, D′, E′, and F″) Illustrations of corresponding morphologies (green represents YFP-MreB-18L, gray lipid vesicle). Scale bar: 3 μm.

electron microscopy (TEM). YFP-MreB-18L was polymerized in bulk solution alone or inside the phospholipid vesicles. Thick and long bundles of YFP-MreB-18L were dominantly observed under the lipid membrane (Figure 4A), while in bulk solution the fusion protein formed thinner and shorter bundle (Figure 4B). Thick and long bundles indicate that the reconstituted YFP-MreB-18L was rigid and much more elongated on a micrometer scale in the presence of lipid membrane. The liposome membrane was not apparent under TEM because the thickness of the membrane is 3−4 nm, greatly smaller than 100−200 nm. However, interestingly small budding membranes were attached on the bundles of MreB (Figure 4A), suggesting a close association between the lipid membrane and YFP-MreB-18L fibers.

We measured the contour length of MreB bundles, which is defined as the total end-to-end distance of the bundle (Figure 4C). The average contour length of YFP-MreB-18L bundles in lipid vesicles is approximately 10 μm (20 μM YFP-MreB-18L), which is 100 times the length of MreB small bundles polymerized in bulk solution. Thus, it is clear that physical contact with the lipid membrane is essential for YFP-MreB-18L to form an elongated rigid bundle.

For bacterial FtsZ of *E. coli* (a tubulin homologue), Osawa et al. have shown that FtsZ formed a ring bundle of slightly curved filaments (125 nm in length) when FtsZ was directly tethered to the surface of tubular vesicles.[16] In the present study we showed more detailed structure analysis with TEM and demonstrated characteristic bundle formation. The membrane-induced assembly of filaments is a common feature in bacterial cytoskeletons; however, our observation suggested structure differences between FtsZ and MreB.

In this work, we demonstrated similar fibrillation and rather loose but fine organization of filaments by using cell-free gene expression of MreB and MreC in phospholipid vesicles. This is the first demonstration of the genetically programmed reconstitution of a filamentous cytoskeleton using a cell-free expression reaction (CFER) in synthetic liposomes, to the best of our knowledge. The series of observations using a CFER system reveals that MreB alone forms small clusters below the micrometer size, while finely organized network structure are observed when MreB and MreC are co-expressed concurrently. The cell-free extract contains the fractions of *E. coli* cytoplasmic proteins, which may help the formation of the MreB cytoskeleton in the presence of MreC. A cell-free reaction also reproduces the conditions of cytoplasm such as molecular crowding, an important biophysical aspect of self-organization in biological systems.[28]

Furthermore, thick and long MreB bundle structures are polymerized inside phospholipid vesicles using a purified YFP-MreB-18L protein. The protein YFP-MreB lacking the 18L peptide cannot form bundled structure even by following the same method of polymerization (Supplementary Figure S3). Membrane-induced bundling of MreB filaments as well as previous study on FtsZ suggests that the formation of the filaments and their assembly take place at the inner membrane.[16] This seems to be a fundamental mechanism and might be a conserved feature among bacterial cytoskeleton systems. In addition, straight and buckled bundles of MreB were observed in our *in vitro* experiment with the purified protein, whereas the

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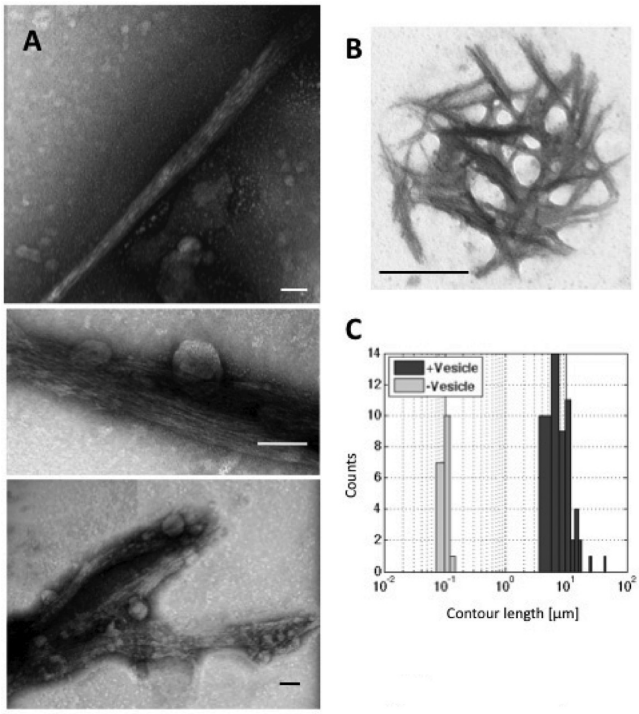

Figure 4. Electron microscopy observation of *in vitro* MreB cytoskeleton. YFP-MreB-18L bundles in the presence (A) and in the absence (B) of lipid vesicles. Cytoskeleton structure of YFP-MreB-18L in the vesicles is thick (100−200 nm) and long (4−10 μm) bundles. In the bulk solution, YFP-MreB-18L polymerizes into short bundles (100−200 nm) and thin bundles (20 nm). Scale bar is 100 nm. (C) The distribution of the contour length of MreB bundles. Horizontal axis is log-scale.

helical and ring-like bundles, which have been observed *in vivo*, were not. According to recent findings that MreB filaments coordinate with cell wall synthesis,[29−31] other protein complexes are necessary for self-organized cytoskeleton systems.

The MreB bundle and network formation in the close proximity of phospholipid membrane would give us an insight for shape regulation machinery in single vesicles. The development of the CFER approach to other cytoskeleton systems stimulates future efforts toward the assembly of artificial vesicle bioreactors capable of self-replication, like living cells.[22,23]

METHODS

Cell-Free Gene Expression. The cell-free gene expression system used in this study has been described previously.[25] *E. coli* core RNA polymerase and the primary sigma factor 70 were present in the extract. The genes used in this study were cloned under a promoter specific to the sigma factor 28. The genes were expressed through a transcriptional activation cascade: $P_{70}\rightarrow\sigma_{28}\rightarrow P_{tar}$-gene. The promoter P_{tar} is specific to sigma 28.

The cell-free expression reaction consists of 33.3% of crude extract (9−10 mg/mL of proteins, final concentration) and 66.6% of reaction buffer and plasmids, with the following final concentrations: 50 mM HEPES pH 8, 1.5 mM ATP, 1.5 mM GTP, 0.9 mM CTP, 0.9 mM UTP, 0.2 mg/mL tRNA, 0.75 mM cAMP, 0.33 mM NAD, 0.26 mM coenzyme A, 1 mM spermidine, 0.068 mM folnic acid, 1 mM DTT, 30 mM 3-phosphoglyceric acid, 1.5 mM amino acids, 3 mM Mg-glutamate, 60 mM K-glutamate, and 2% (w/v) PEG8000. The concentration of plasmids was adjusted depending on the reaction. A concentration of 0.2 mg/mL of Rhodamine BSA was used in the reaction to visualize the phospholipid vesicle. All of the components were purchased from Sigma-Aldrich, except GTP, CTP, UTP (USB Corporation), tRNA (Roche), and amino acids (5Primes).

Plasmids Construction and Protein Purification. Standard molecular cloning procedures were used to construct all plasmids. The plasmid pBESTluc (Promega) was used to construct the plasmids used for cell-free expression. The genes *mreB* and *mreC* were cloned under a P_{tar} promoter:[25,24] pBEST-P_{tar}-UTR1-*mreB* (MreB), pBEST-P_{tar}-UTR1-*yfp-mreB* (YFP-MreB), pBEST-P_{tar}-UTR1-*mreC* (MreC). All of the plasmids have the highly efficient untranslated region containing the T7 *g10* leader sequence, named UTR1 in this work;[32] 0.2 nM plasmid $P_{70}\rightarrow\sigma_{28}$ was used in cell-free expression reactions. A total concentration of 5 nM of P_{tar}-gene plasmids was used with the following proportions: YFP-MreB:MreB:MreC = 2.5:1.25:1.25 nM (Figure 1B and C), YFP-MreB:MreB = 2.5:2.5 nM (Figure 1E and F).

For the work with purified proteins, the genes *yfp-mreB-18L* and *yfp-mreB* (including His6-tag at N-terminal) were cloned into the expression vector pET28a (Novagen).[15] YFP-MreB-18L is a recombinant protein composed of MreB from *E. coli* fused to YFP at the N-terminal end and to the amphipatic peptide 18L at the C-terminal end. YFP-MreB and 18L are separated by a 3 amino acid linker (Ala-Ala-Ala). The sequence coding for 18L was fused to *yfp-mreB* at the Not1/Xho1 sites. Purified YFP-MreB protein without 18L was used as a control in order to observe the effect of membrane localization.

His6-YFP-MreB and His6-YFP-MreB-18L were expressed in *tf2*(DE3)pLysS cells by the addition of 0.5 mM IPTG. We followed Madabhushi et al.'s protocol to obtain crude extract.[17] The soluble supernatant was obtained by ultracentrifugation at 40,000 rpm for 1 h at 4 °C. The proteins were purified by nickel chelate affinity chromatography technique. Purified proteins were eluted in a storage buffer (50 mM Tris/HCl pH 8.0, 100 mM KCl, 0.2 mM ATP, 40% glycerol) and stored at −80 °C.

Liposome Preparation and MreB Polymerization Inside Liposome. The encapsulation of cell-free reactions into liposomes was performed as previously described.[22] The encapsulation of pure proteins into liposomes was performed using the same procedure with the following slight modifications. The phospholipid solution was prepared by dissolving DOPG/DOPC/DMPC (Avanti Polar lipids, ratio of 3:2:5, respectively) in mineral oil (Sigma-Aldrich). One microliter of protein solution was added into 100 μL of phospholipid solution, and this solution was vortexed to create an emulsion. The liposomes were formed into buffer A (50 mM Tris/HCl pH 8.0, 100 mM KCl, 0.2 mM ATP, 10% glycerol) by centrifugation. A final concentration of 0.1 mg/mL of α-hemolysin from *Staphylococcus aureus* (Sigma-Aldrich, H9395) was used in the external buffer A solution to create a permeability of the vesicles to nutrients. The 2X ionic buffer (100 mM potassium glutamate, 2 mM ATP, 5 mM DTT) was mixed with the liposome solution. Magnesium acetate (5 mM final concentration) was added 30 min after incubation to induce the polymerization.

Data Acquisition and Analysis. The fluorescence of YFP was observed with a CCD camera (Retiga 1300, Q-Capture Pro software) mounted on a microscope (Olympus IX-70, epifluorescence) with the proper fluorescence filter sets. Bundles of

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YFP-MreB-18L were observed by transmission electron microscopy (FEI TECNAI G2 Spirit BioTwin, 80 kV, carbon-coated copper grids, negative staining method with 2% uranylacetate). The software ImageJ was used to analyze contour length of MreB bundles.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥These authors equally contributed to this work.

Author Contributions

Y.T.M. and A.L. designed research; Y.T.M. performed research and analyzed data; J.S., Y.T.M., and V.N. performed the cell-free gene expression experiments; Y.T.M. and T.N. purified proteins; Y.T.M. and K.U. took TEM observation; all authors discussed data; and Y.T.M., V.N., and A.L. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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