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Nanotube−Vesicle Networks with Functionalized Membranes and Interiors

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Abstract: We describe nanotube−vesicle networks with reconstituted membrane protein from cells and with interior activity defined by an injection of microparticles or molecular probes. The functionality of a membrane protein after reconstitution was verified by single-channel ion conductance measurements in excised inside-out patches from the vesicle membranes. The distribution of protein, determined by fluorescence detection, in the network membrane was homogeneous and could diffuse via a nanotube connecting two vesicles. We also show how injecting small unilamellar protein-containing vesicles can differentiate the contents of individual containers in a network. The combination of membrane activity and interior activity was demonstrated by ionophore-assisted accumulation, and internal Calcium Green-mediated detection, of Ca^{2+} within a single network container. This system can model a variety of biological functions and complex biological multicompartment structures and might serve as a platform for constructing complex sensor and computational devices.

Introduction

In biological systems, such as single cells, the parallel handling of small numbers of molecules is inherent. A differentiated eucaryotic cell can perform some 10^3-10^4 different chemical operations simultaneously, depending on the protein content, at an overall burning rate of roughly 10^6 molecules/s of ATP (i.e., $\sim 10^6$ chemical or physical operations/s). All this is performed in a volume element of roughly a picoliter. How is this chemical multiprocessing capability in extremely small volumes possible? We can in part find a solution to this problem by looking at four main features:[1] (1) compartmentalization, that is, usage of specialized reaction containers (organelles, $10^{-15}-10^{-21}$ L) with controlled input/output properties; (2) molecular recognition, that is, highly specific interactions between reacting molecules or binding interactions in a sorting/counting step; (3) a combination of small scale and complex function, which is true both on the single-molecule level (i.e., a single protein can function as an enzyme, an ion channel, a transporter, a light detector, etc) and on the level of a single organelle; and (4) targeted transport and controlled mixing of components, for example, by the use of vesicles as cargo carriers between organelles.

We wish to mimic these four properties of a single cell in order to design extremely small-scale devices for a variety of applications in biosensors and microfluidics. In the majority of systems using molecules as signaling entities, sequential chemical operations have to be performed on the molecules, requiring selective interactions and well-defined reaction steps. Since this is not easily accomplished in solid-state microfabricated materials, we have focused on the development of soft microfabrication technologies that can be implemented in artificial biological cell and cell-network design. In particular, we have explored the possibility of building new lipid membrane-based networks (mainly from phospholipid bilayer materials in their liquid crystalline phase) consisting of micrometer-sized containers ($10^{-12}-10^{-15}$ L) linked by nanotubes with diameters ranging from 100 to 300 nm.[2]

To allow communication between the network interior and exterior, selective mediation of a chemical signal through the membrane is of great importance. Membrane translocation of small molecules into individual containers can be created by transient membrane pore formation using highly spatially focused microelectroporation with carbon microelectrodes.[3,4] Microelectroporation has the advantage that single containers can be addressed and that the timing as well as relative loading efficiency can be precisely controlled. A drawback in microelectroporation is not being able to select exactly which compound is to be translocated. In contrast, membrane proteins, that is, transporters and channels, are highly selective signal mediators because of inherent molecular recognition and permeability properties. Additionally, the gating function of many membrane proteins can be controlled by membrane potential or specific ligands. In this work, we show that nanotube−vesicle

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networks can be made with an active protein incorporated in their membranes and that artificial organelles can be enclosed within the interior of individual network vesicles. Also, we demonstrate the concept of transmembrane signal translocation and detection within an individual vesicle in a network.

These systems provide a new paradigm in microsystems bioengineering, as they are based on biomimetic components and biological principles of operation. They can be programmed to perform a complex task, or a set of tasks, at the expenditure of minute amounts of energy in extremely small-scale and compact structures. Complex chemical reactions possibly involving many steps, such as enzyme-catalyzed reactions, hybridizations, and derivatizations, are performed rapidly because of fast diffusive mixing with minimal dilution. These membrane-based devices might therefore find applications in novel analytical, biosensor, and computational devices, where a series of manipulations have to be performed on an initially extremely small volume containing a signaling molecule or an analyte. Basically, these devices are inspired by liquid and material transport in biological systems, that is, packaging and transport in vesicular and tubular structures, controlled fusion of transport vesicles to target containers, and chemical transformations in ultrasmall-scale membrane-enclosed containers, as well as the usage of multicompartment structures with catalysis and signal transduction across membrane barriers.

Material and Methods

Chemicals. Eosin-5-maleimide (EMA) and FM1-43 were from Molecular Probes (Leiden, Netherlands). Polar extracts of soybean lecithin (SBL, average $M_w = 800$) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). A23187, phenylmethylsulfonylfluoride (PMSF), and Triton X-100 were from Sigma (St. Louis, MO, USA). Biobeads SM-2 were obtained from Biorad (Hercules, USA). Outdated human red blood cells were obtained from Sahlgrenska University Hospital, Sweden. All other chemicals were of highest obtainable purity. Water was purified with a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Reconstitution of Labeled Erythrocyte Membrane Protein into Liposomes. All procedures were performed at 0 °C unless indicated. Eosin-5-maleimide-labeled erythrocyte ghosts were prepared as previously described.[5,6] EMA binds preferentially to Lys-430 in the first extracellular loop of anion exchanger 1 (AE1).[7] The labeled membrane (1 mg/mL protein) was solubilized with 5 mM Triton X-100 in 0.12 M KCl, 0.5 mM EDTA, 1 mM $MgSO_4$, and 1% glycerol, at pH 7.4 (1 h). Cell debris was removed by centrifugation (27 000 × *g*, 1 h). The solubilized protein was mixed with an equal volume of detergent−lipid solution, which contained 5 mM SBL and 10 mM Triton X-100 in the same buffer. The protein−lipid−detergent mixture was incubated for 30 min. Reconstitution by detergent removal was done with Biobeads SM-2 at 20 °C.[8] The resulting proteoliposome solution was diluted 1:10 (v/v) into 1 mg/mL SBL in 120 mM KP_i, 5 mM Tris-HCl, 1 mM $MgSO_4$, and 0.5 mM EDTA, at pH 7.4. To homogenize the solution, it was repeatedly passed through two stacked polycarbonate filters with a pore size of 200 nm. The homogenized proteoliposomes were used to form giant unilamellar vesicles.

Characterization of Protein Labeling and Reconstitution. Protein concentration was determined using the NanoOrange kit (Molecular Probes, Leiden, Netherlands) in the presence of 0.01% SDS and with BSA as standard. Determination of protein was done by SDS-PAGE (10% separating gel) with silver staining according to Merril and Pratt.[9] Proteins were identified by apparent molecular weight, with reference to widely published data. The protein pattern was characteristic of erythrocyte membranes and contained most membrane-associated proteins including α- and β-spectrin, the anion exchanger AE1, glycophorin A, and others. To determine the target specificity of protein labeling by EMA, unstained protein gels were scanned with laser light at 488 nm. EMA was predominantly coupled to a protein with an apparent molecular weight of ∼105 (±5) kDa, corresponding to AE1.[6]

Proteoliposomes were precipitated by centrifugation (45 min, 105 000 × *g*). The supernatant and the pellet were separated and dissolved in 0.15 M NaCl and 5 mM Tris-HCl, at pH 7.4, containing 0.2% SDS. Fluorescence emission from the respective fractions was measured using a spectrofluorometer ($\lambda_{exc} = 488$ nm, $\lambda_{em} = 550$ nm). Virtually all detectable (>99.9) fluorescence was associated with the proteoliposomes, that is, reconstituted AE1. Background fluorescence, and light scattering contributions, was obtained from a parallel experiment using plain SBL liposomes.

Preparation of Giant Vesicles. Giant vesicles were formed as described except that soybean lipid (SBL) from Avanti was used.[10] In short, 1−5 μL of proteoliposomes in 120 mM KP_i, 5 mM Tris-SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA, and 1% (v/v) glycerol, at pH 7.8, were placed on a borosilicate coverslip and dehydrated under reduced pressure for 30−40 min at 20 °C. The approximate lipid concentration was 1 mg/mL. When the lipid film was completely dry, it was carefully rehydrated with 120 mM KP_i, 5 mM Tris, 1 mM $MgSO_4$, and 0.5 mM EDTA, at pH 7.8, unless otherwise stated in the text.

To produce protein-containing giant vesicle networks, we used the Karlsson method for network construction.[13,17] Briefly, a pulled borosilicate-glass micropipet with an outer-tip diameter of 0.5−1 μm, back-filled with aqueous medium and mounted onto an electroinjection system, was pressed into the unilamellar blister of a surface-immobilized GUV−MLV containing reconstituted membrane proteins. With one or several transient anodic rectangular DC-voltage pulses of field strengths between 10 and 40 V/cm and a duration of 1−4 ms applied over the micropipet, the membrane of the unilamellar blister was penetrated. The micropipet was then pulled out and away from the mother liposome, forming a lipid nanotube connection between the mother vesicle and the pipet tip. When the nanotube had reached the desired length (10−20 μm), the aqueous medium was slowly (∼50 × 10^{-15} L s^{-1}) injected into the nanotube using a pressurized-air-driven microinjector, forming a small vesicle at the outlet of the micropipet tip. Once this satellite vesicle reached a desired diameter, typically 5−15 μm, it was immobilized at a targeted location by gently pressing the vesicle to the surface, thereby allowing it to adhere to the surface. After the pipet tip was withdrawn, it was disconnected from the lipid membrane by applying one or several cathodic DC-voltage pulses over the micropipet, typically using field strengths of 60−100 V/cm.

Patch-Clamp Recording of Ion Channels Reconstituted in GUV Membranes. Single-channel recordings were obtained by using the patch-clamp technique.[11] Patch pipets filled with 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, and 10 mM Hepes, at pH 7.2,

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were used. The same buffer was used for vesicle formation. After seal formation, the pipet was carefully removed from the vesicle surface, resulting in an excised patch (inside-out configuration). An Axopatch 200A amplifier (Axon Inc.) was used at a gain of 20 mV/pA. The signal was digitized at 10 kHz and filtered with an 8-pole Bessel filter set at 1 kHz. The pipet electrode was set to command voltage, and the bath electrode was held at virtual ground. For all single-channel recordings, the applied membrane voltage is given as the pipet potential relative to the grounded bath electrode. All experiments were performed at room temperature (20° ± 2°). Data analysis was performed in Clampfit 8.1 (Axon Inc, USA), using a digital 8-pole Bessel filter set at 200 Hz. Protein-free GUV membranes, acting as a control, were electrically silent.

Microscopy and Micromanipulation. All experiments were performed on an inverted microscope (Leica DM IRB, Wetzlar, Germany) equipped with Leica PL Fluotar 40x and 100x objectives and a water hydraulic micromanipulation system (high graduation manipulator, Narishige MWH-3, Tokyo; coarse manipulator, Narishige MC-35A, Tokyo). No. 1 rectangular cover slip glasses were placed directly on the microscope stage.

Fluorescence imaging was achieved by sending the output of an Ar^+ laser (Spectra-Physics 2025-05, 488 nm) through a 488-nm line interference filter followed by a spinning disk to break the coherence and scatter the laser light. The laser light was collected by a lens and was sent through a fluorescein filter (Leica I-3) into the objective to excite the fluorescent dyes. The fluorescence was collected by the objective, detected by a three-chip color CCD camera (Hamamatsu, Kista, Sweden), and recorded on VHS (Panasonic S-VHS AG-5700). Digital images were acquired with an Argus-20 system (Hamamatsu, Kista, Sweden) and further processed with Adobe Photoshop graphic software.

Electroinjection and network formation were performed using a microinjection system (Eppendorf Femtojet, Hamburg, Germany) in combination with a pulse generator (Digitimer Stimulator DS9A, Welwyn Garden City, U.K.). Carbon fiber microelectrodes (5-μm diameter, Dagan Corp., Minneapolis, MN, USA) were used as counter electrodes.

Results and Discussion

Reconstitution of Membrane Protein into Vesicles. Reconstitution of membrane proteins into giant vesicles is a common method to study protein activity in a simplified environment.[12] We have previously demonstrated that networks of giant vesicles (GUVs) conjugated by nanotubes can be formed from a single GUV using an electroinjection technique.[13] Here, we advance the possibilities of the technique by introducing protein into the membrane of the vesicles and bioactive components into their interiors. As a source of membrane protein, we chose human erythrocytes. Verification of protein incorporation into the GUV membrane was provided by two separate approaches. Laser-induced fluorescence (LIF) detection of labeled membrane protein was used to optically determine the distribution of protein within the membrane of GUVs, and ion-channel current measurements were used to verify retained protein activity.

Plasma membrane protein was prepared from erythrocytes of which the anion exchanger AE1 had been covalently labeled with eosin-5-maleimide (EMA). Membrane protein was then extracted from cells with the detergent Triton X-100 and reconstituted into small proteoliposomes. The proteoliposomes were subjected to a dehydration−rehydration process to form giant vesicles. Rehydration resulted in multilamellar structures with uni- or oligolamellar blisters (GUV−MLV). Examination by LIF microscopy showed that the EMA-labeled AE1 protein was distributed homogeneously in the unilamellar membrane blisters from which networks are formed (Figure 1).

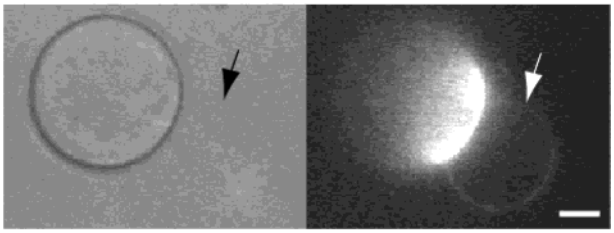

Figure 1. Nomarski (left) and fluorescence (right) images of EMA-labeled AE1 distribution in a giant vesicle, with the arrow indicating a unilamellar membrane blister used to form networks. Bar = 5 μm.

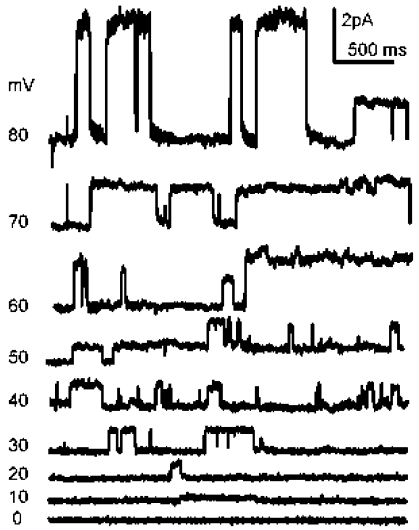

Figure 2. Single-channel recordings at different holding potentials (0−80 mV) from a unilamellar membrane patch excised from a vesicle membrane blister containing erythrocyte membrane protein.

After investigating the distribution of membrane protein, we confirmed that the protein remained active after the dehydration−rehydration procedure. For this purpose, we excised inside-out membrane patches from the membrane blisters. We identified channel activity as rapid jumps between discrete current levels, representing the opening and closing of conducting channels. As a representative example, current traces from a single membrane patch, recorded at various positive membrane potentials, are reproduced in Figure 2. The membrane patch in question appeared to contain up to three channels. Assuming that the channels were identical, we estimated the conductance to be ~5 pS. The identity of the channel was not further investigated, although we presume it to be a nonvoltage-gated chloride channel. From this result, we drew the conclusion that membrane proteins reconstituted into GUVs by the chosen procedure were functionally active and that they would remain active during formation of vesicle networks.

Formation of Vesicle Networks with Membrane Protein. Complex networks consisting of several containers could be produced from single-protein-containing vesicles. An example is displayed in Figure 3, which shows an eight-container network of genus=3 topology. We found that the lifetime of a protein-containing network after full assembly (30−60 min) was shorter than those for networks made from pure soybean lipid. The precise reason for this effect has not been investigated in depth.

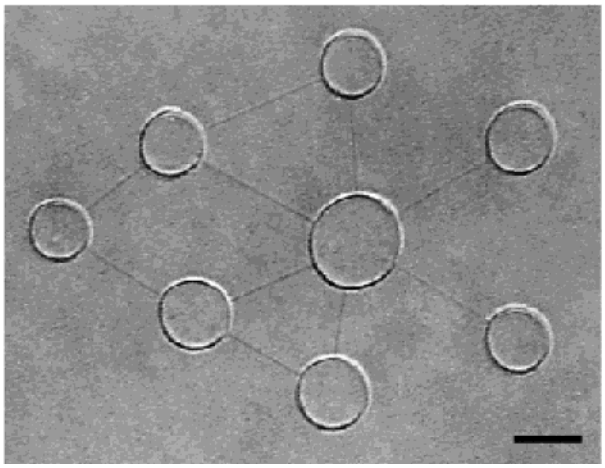

Figure 3. A nanotube−vesicle network derived from a giant vesicle containing erythrocyte membrane protein. Bar = 5 μm.

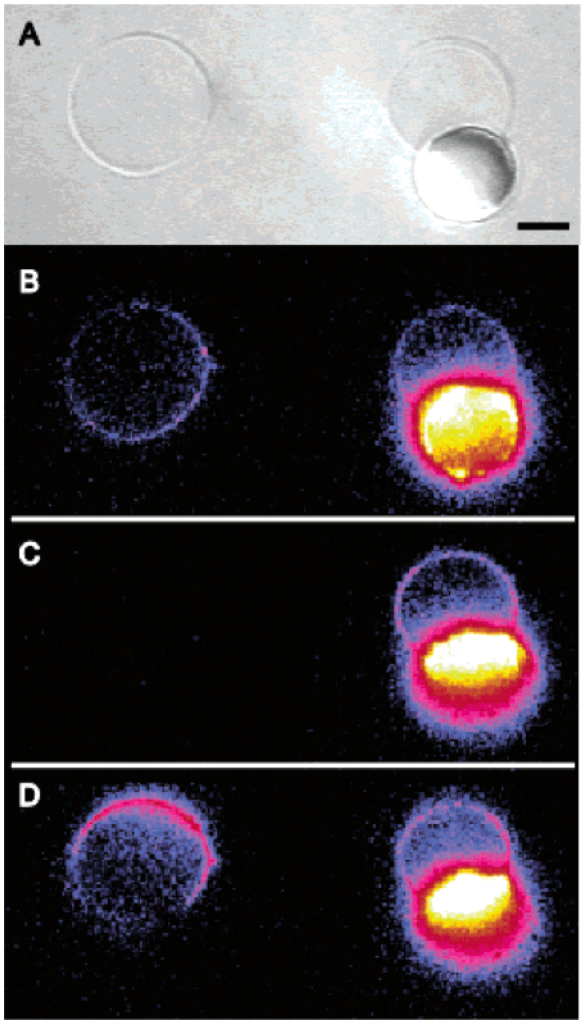

Figure 4. Membrane protein in a vesicle network diffuses freely between containers connected by a lipid nanotube. (A) Brightfield image of the network. (B) EMA-labeled AE1 distribution after the formation of the network was homogeneous. (C) AE1-associated EMA was bleached in the left vesicle and was recovered (D) after diffusion of EMA-labeled AE1 from the right vesicle via the nanotube. The fluorescence images were intensity-indexed for clarity. Bar = 5 μm.

Since the vesicles in a network are continuous with the interconnecting nanotubes, we assumed that diffusion of membrane protein between containers could occur. We investigated

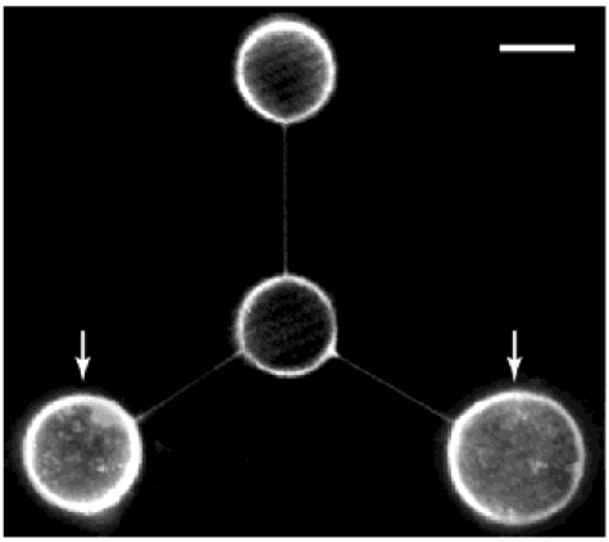

Figure 5. Differentiation of network container interiors. The containers indicated by arrows contain small membrane protein-containing vesicles (seen as fluorescent spots), whereas the top and middle containers are empty. Membranes were stained with FM1-43 for enhanced fluorescence microscopy. Bar = 5 μm.

nanotube-mediated protein diffusion in fluorescence recovery after a photo bleaching experiment. A two-vesicle network containing an EMA-labeled AE1 protein was constructed (Figure 4A). As seen in the LIF micrograph in Figure 4B, AE1 was distributed in both the satellite vesicle (left) and mother vesicle (right).

The satellite vesicle protein-associated fluorescence was bleached by laser irradiation to a level indistinguishable from the background (5 min), leaving the mother vesicle unaffected (Figure 4C). The system was then allowed to rest in the dark for an hour. After this time, membrane fluorescence had accumulated in the satellite vesicle (Figure 4D). The only possible supply route was the nanotube, with the mother vesicle being the protein reservoir. We believe that in an unperturbed two-vesicle network of this design the lipid flow between containers is negligible, suggesting that the protein translocation we have observed occurred by diffusion only.[14]

Differentiation of Container Contents and Introduction of Artificial Organelles. If membrane-bound ion channels or transporters are combined with various enzyme activities confined within specific containers in a network, it is possible to create signaling pathways that mimic, for example, biological feedback loops or self-sustaining networks. Ideally, each interior reaction system, or component responsible for a desired operation, should be unable to diffuse through a nanotube to a neighboring container. Spatially confined activity can be obtained by binding the activity to particles, in the form of lipid-membrane-based artificial organelles or colloidal particles having diameters larger than the nanotube diameter (100−300 nm in our networks).[14] In Figure 5, we demonstrate how a nanotube−vesicle network can be differentiated with respect to internal contents. In this example, a four-vesicle network is shown.

During formation of the network, two of the four vesicles were filled with proteoliposomes (~200 nm diameter) containing reconstituted erythrocyte membrane protein. With respect to morphology, the proteoliposomes can be seen to represent intracellular organelles or an emulsion of microsomes prepared

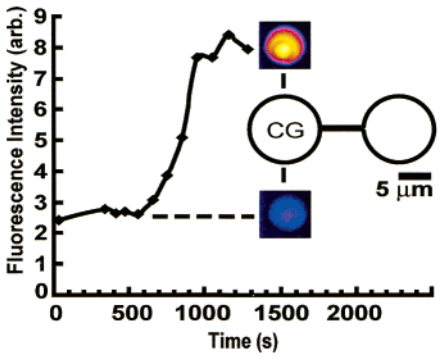

Figure 6. Individual vesicles in a network can be given a specific sensor function. The satellite vesicle (left container in the scheme) contained 5 μM Calcium Green (CG). After the addition of $CaCl_2$ to the external solution, A23187-mediated influx of Ca^{2+} into the network was seen as an increase in fluorescence in the satellite vesicle only. The lower and upper fluorescence images of the satellite vesicle represent interior fluorescence at zero and saturating Ca^{2+} levels, respectively. The fluorescence images were intensity-indexed for clarity.

from intracellular membrane. Since the activity of the organelle or colloidal particle can be designed to a high specificity, each network vesicle functionalized in this manner can be considered as a separate body with exclusive biomimetic machinery.

To demonstrate differentiation of interior activity, we formed a simple two-vesicle network in which the satellite vesicle interior, but not the mother vesicle, contained Calcium Green (Figure 6). The solution in which the network was formed (100 mM NaCl, 0.5 mM EGTA, and 10 mM Tris-HCl, at pH 7.4) contained 100 nM ionophore A23187 to allow selective entry of Ca^{2+} into the network. Application of $CaCl_2$ (2 mM) to the external medium resulted in an increase in fluorescence in the satellite vesicle, while no increase was observed in the mother vesicle during the time of the experiment. This demonstrates how an individual vesicle in a network can be defined, by the combination of the membrane component and the internal medium, to perform a specific sensor function.

Although we did not detect diffusion of the calcium probe from the satellite vesicle to the mother vesicle during this experiment, this problem has to be taken into consideration. To minimize diffusion of a probe between containers, it can be linked to a high-molecular weight species, such as dextran, or to colloidal particles.

Conclusion and Outlook

We have developed techniques for producing microscopic networks of containers and nanotubes using lipid bilayer membranes and proteins as the major building blocks. The material properties of the liquid crystalline bilayer phases not only allow for the construction of topologically complex structures but also provide a housing environment for the membrane proteins. Proteins can be introduced to the networks either in their outer membranes or as integrated parts of organelles and/or catalytically active particles, which can be confined in individual containers. The experimental data provided here describe the methodology for the construction of such a system and are to be considered as proof of a concept. The major obstacle remaining concerns segregation of individual types of membrane protein to specific regions of the outer membrane. If the spatial distribution of proteins can be controlled, it would be feasible to construct more complex systems. Ion channels or transporters reconstituted in the network would then be able to sense concentration gradients of substances in the network environment, signal their presence, and allow chosen ions and compounds to enter different compartments where they can trigger chemical reactions. Hypothetically, a construction of this type could be used to study multicomponent reaction systems or to study complex signaling systems, for example, G-protein-coupled receptor signaling,[15] cellular circuits, for example, gap-junction-mediated cell-to-cell signaling,[16] or to design complex chemical sensors.

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