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Direct Reconstitution of Plasma Membrane Lipids and Proteins in Nanotube−Vesicle Networks

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We demonstrate here that nanotube−vesicle networks can be constructed directly from plasma membranes of cultured cells. We used a combination of dithiothreitol (DTT) and formaldehyde to produce micron-sized plasma membrane vesicles that were subsequently shaped into networks using micromanipulation methods previously used on purely synthetic systems. Only a single cell is required to derive material sufficient to build a small network. This protocol covers the advantages of reconstitution in vesicles, such as full control over the solution environment, while keeping the proteins in their original surroundings with the proper orientation. Furthermore, control of membrane protein and lipid content in the networks is achievable by employing different cell types, for example, by overexpression of a desired protein or the use of specialized cell-types as sources for rare proteins and lipids. In general, the method provides simple accessibility for functional studies of plasma membrane constituents. Specifically, it provides a direct means to functionalize nanotube−vesicle networks with desired proteins and lipids for studies of transport activity both across membranes (protein-mediated) and across nanotubes (diffusion), and substrate conversion down to the single-molecule limit. Nanotube−vesicle networks can adopt different geometries and topologies and undergo shape changes at will, providing a flexible system for changing the physical and chemical environment around, for example, a membrane protein. Furthermore, the method offers unique possibilities for extracting membrane and protein material for nanotechnological sensor and analytical devices based on lipid membrane networks.

Introduction

We have previously demonstrated techniques for the formation of lipid nanotube−vesicle networks (NVNs), consisting of surface-immobilized vesicle containers interconnected with lipid nanotubes.[1,2] Networks of surface-immobilized unilamellar vesicles (∼5−25 μm in diameter) conjugated with nanotubes typically 50−150 nm in radius can be made with complex geometries and topologies.[2−5] The membrane composition and container contents can be controlled allowing chemical programming of networks in studies of enzyme kinetics,[6] reaction−diffusion phenomena,[7] and single-biomolecule detection.[8] Materials contained in the networks can be routed using diffusion, Marangoni flows, and electrophoresis.[4,7−9] Thus, networks of nanotubes and vesicles serve as a platform to build nanofluidic devices operating with single molecules and particles and offer new opportunities to study chemistry in confined biomimetic compartments. The networks can furthermore be used to build nanoscale chemical laboratories for applications in analytical devices as well as to construct computational and complex sensor systems that can also be integrated to living cells. Nanotube-conjugated cells also exist in nature, and evidence has been found that cells can exchange materials through such channels.[10,11]

An important challenge in constructing NVNs has been to obtain membrane proteins and lipids from natural sources in high yields and with maintained functionality. A wide range of methods have been developed for reconstitution of membrane proteins in artificial SUVs[12,13] and subsequently GUVs, that can be used to build networks.[14] Techniques for reconstitution in GUVs include fusion of proteoliposomes generated by detergent-mediated reconstitution or insertion of proteins into preformed GUVs via peptide-induced fusion.[14−17] It is a practical way to study a membrane protein of choice in a simplified environment, but this approach has several limitations. For one, the proteins have to be removed from their natural environment, usually by using detergents, and it is not always possible to ensure that all the proteins are reconstituted uniformly in their desired orientation. Moreover, as the isolation procedures can be quite harsh, protein activity may be significantly reduced, and also experimental work can be tedious and time-consuming, as suitable reconstitution protocols for each desired protein have to be developed. The most elegant approach would be to form the NVNs directly from a native cell membrane, but such an extraction of membrane from a cell is complicated as it is firmly attached to the

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cytoskeleton with microscopic folds and invaginations as a discriminating feature. However, upon chemical or mechanical stress,[18−22] cytokinesis,[23,24] or cell movement,[25,26] as well as during apoptosis,[27] cells can form unilamellar protrusions, also known as membrane blebs. Such structures would serve as perfect precursors for NVN formation as they would be compatible with the micromanipulation tools used for synthetic vesicles.[2] During bleb formation, the membrane detaches from the underlying cytoskeleton and acquires a hemispherical shape,[28] which is caused by internal fluid flow into the protrusion, while maintaining the overall cell volume. Bleb formation can be triggered in various ways, and depending on the applied method blebs can be structurally different,[29−31] but a majority lack organelles and cytoskeletal structures. Consequently, the cell presumably still maintains its full functionality, while adapting to external stress factors by continuously modifying cytoskeletal adhesion to the plasma membrane. Little is known about the function of membrane blebs and how they affect the further development of the cell, but it appears that alterations of the physical properties of the cell are vital to compensate for environmental changes. This indicates a close relationship between the physical parameters of the plasma membrane and the biochemical functions of the cell.

In this work we apply a combination of dithiothreitol (DTT) and formaldehyde which has been used for isolating micron-sized membrane blebs that have pinched off from the plasma membrane.[30,31] Previous research has shown that the formaldehyde concentration used causes no detectable aggregation of surface components,[32] and other studies have characterized blebs in terms of their ultrastructure and their protein and lipid composition.[33] DTT is frequently used for reducing protein disulfide bonds, which might affect the activity and surface structure of membrane proteins. It cannot access buried disulfide bonds, so its effect is probably limited to the membrane surface area. Naturally formed blebs, e.g., those caused by cell damage, usually heal with the assembly of actin on the cytoplasmic surface, restoring support of the membrane. DTT as a reducing agent might prevent this retraction together with formaldehyde, which is a known blocking agent for sulfhydryl groups. A bleb can be perceived as the more cell-like analogue to vesicles created from lipid mixtures, as it does not possess the same complexity as the native cell membrane and has a comparatively similar size. We demonstrate that membrane blebs can be used to form surface-adhered networks of plasma membrane vesicles, with typical vesicle diameters of 5−10 μm and tube lengths up to tens of microns using the microelectroinjection technique developed earlier.[2] This method provides high yields, and composition, orientation, as well as function are conserved. Furthermore, given that only about 250 μm^2 of membrane is needed to build a three-vesicle network (given a 5 μm vesicle diameter and 40 μm total tube length), such a network can be built from a single cell. Thus, cells can be cherry-picked from a tissue or a culture to build a NVN with a desired membrane makeup.

Materials and Methods

Chemicals. WGA Alexa 488 was obtained from Invitrogen (Molecular Probes). D-Glucose, HEPES, and $CaCl_2$ were from Merck, and $MgCl_2$, KCl, and NaCl were purchased from Sigma. DTT (dithiothreitol) was from Fluka, and DMEM and fetal calf serum were obtained from PAA Laboratories.

Cell Culturing. NG-108 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine modified to contain 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 1 mM sodium pyruvate and supplemented with 10% fetal calf serum (GTF), penicillin, streptomycin, and HT medium supplement.

Formation of Membrane Blebs. NG-108 cells were cultured for 2−4 days in glass bottom dishes (Willco Wells). The media was removed, and the cells were washed twice with HEPES buffer, containing 140 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES at pH 7.4. They were then submerged in 1 mL blebbing solution, containing 25 mM formaldehyde, 20 mM DTT, 2 mM $CaCl_2$, 10 mM HEPES, and 0.15 M NaCl at pH 7.4. The cells were incubated for 30 min at 37°. The solution was removed, and the cells were rinsed twice with HEPES buffer. Afterwards the cells were covered with 2 mL HEPES buffer, and the dish was placed on an inverted microscope stage.

Labeling. To verify the presence of membrane proteins in the plasma membrane vesicles, Wheat Germ Agglutinin Alexa Fluor 488 conjugate (Invitrogen-Molecular Probes) was used. It was dissolved in 0.1 M $NaHCO_3$, pH 8.3, at a stock concentration of 1 mg/mL. Labeling was performed after bleb formation for 15 min at room temperature at a final concentration of 5 μg/mL.

Confocal Imaging. A confocal laser scanning microscopy system (Leica TCS SP2 RS, Wetzlar, Germany), with a PL APO CS 63×/1.2 W CORR objective, was used for acquisition of confocal fluorescence images. The 488-nm line of the Ar laser was used for excitation of the dye, and emission was collected by a photomultiplier tube with a set sensitivity in the 500−600-nm spectrum. During acquisition, images were stacked (2−4 frames). Simultaneously, DIC-images from the reflected light of the scanning lasers were collected.

Formation of Plasma Membrane Networks. The procedure is based on the microelectroinjection technique described elsewhere.[2,34] The electroinjections were controlled by a microinjection system (Eppendorf Femtojet, Hamburg, Germany) and a pulse generator (Digitimer Stimulator DS9A, Welwyn Garden City, U.K.). The injection tips used for formation of the networks were prepared from borosilicate capillaries (GC100TF-10, Clark Electromedical Instruments, Reading, U.K.). A CO_2-laser-puller (model P-2000, Sutter Instrument Co. Novato, CA) was used to create the injection tips. Silver wire or carbon fiber electrodes were used as a counter electrode.

Results and Discussion

Membrane blebs are essentially hemispherical and unilamellar, and they have a similar size as GUVs. Consequently, they can be manipulated in the same way with the microelectroinjection technique developed for formation of NVNs from synthetic vesicles.[1,2,35] However, when using synthetic vesicles, the unilamellar bleb is connected to a multilamellar vesicle that serves as a lipid reservoir enabling formation of fairly large networks.[36] Similarily, cells which basically have surface-tension-

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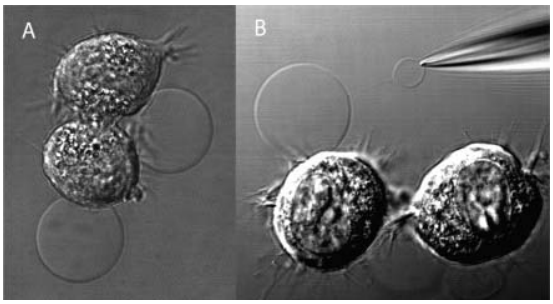

Figure 1. (A) DIC image of adherent NG108-15 cells, displaying membrane blebs induced by a combination of DTT and formaldehyde. (B) Subsequently, one bleb was electroinjected with a buffer-filled micropipet. Following translation of the micropipet, a nanotube connection is formed. Buffer injection leads to the formation of a daughter vesicle at the micropipet tip.

less shells also have a large plasma membrane reserve, but the size of this excess area is cell-type-dependent. Studies on fibroblasts indicate only about 1% membrane reservoir,[37] whereas neuronal cells can swell ~5-fold corresponding to an area increase of about 300% to its original value.[38] As cells originally derived from neuronal tissue are being used in this work, this amount of material is sufficient to build networks with 2−10 nodes depending on their diameter.

For experiments, adherent NG108 cells were grown on untreated glass-bottom dishes, and the formation of large membrane blebs (mother blebs) was induced chemically using a combination of formaldehyde and dithiothreitol (DTT) (Figure 1A). Whereas it appears likely that most cell-membrane components can be extracted into a bleb, the subsequent morphological shape change and the preceding cellular processes may cause a different composition of the bleb membrane compared to the native membrane.[39] For instance, membrane proteins that are interacting with the cytoskeleton may not move over to the protruding bleb, and a change in lateral diffusion of proteins may cause an altered membrane protein distribution.[32] The presence of lipid rafts on plasma membrane vesicles has also been investigated.[40] A sufficiently large bleb is electroinjected with a buffer-filled micropipet. An open connection between the bleb and the cell interior is present as fluorescent dyes injected into the bleb rapidly diffuse into the cell interior (data not shown). The micropipet is then pulled away from the bleb having the membrane sealed around the tip, thus forming a nanotube connection between the two containers. Buffer solution is then injected into the nanotube orifice, with a flow rate of 10−20 fL/s, forming a daughter vesicle which is placed on the glass surface. Figure 1B displays a DIC image of an adherent cell with a protruding bleb, connected via a nanotube to the daughter vesicle which is still attached to the micropipet. After immobilization of the daughter vesicle, the pipet is detached by one or several short dc-voltage pulses (10−40 V/cm), after which a new daughter vesicle from the same mother bleb can be formed. The new vesicle will be connected to the immobilized one by a membrane nanotube. In Figure 2, we show a schematic representation of the method. Organelles and cytoskeletal structures remain in the adherent cell, while the bleb most likely encloses low-molecular weight cytosolic components. The bleb and the subsequently formed daughter vesicle have membrane proteins embedded in the membrane. However, larger structures

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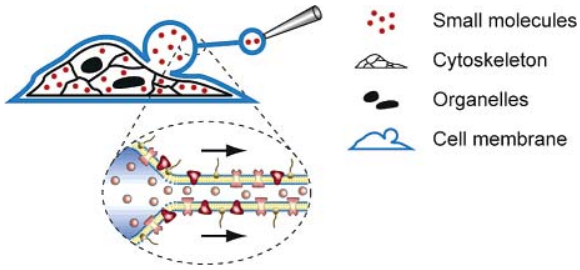

Figure 2. Schematic representation showing the formation of a daughter vesicle from a cell membrane bleb. Organelles and cytoskeletal structures remain in the cell, while the bleb most likely encloses low molecular weight cytosolic molecules. Both the bleb and the daughter vesicle have membrane proteins embedded in the membrane.

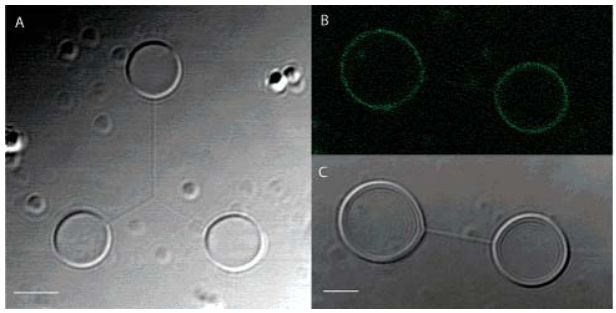

Figure 3. Plasma membrane vesicle networks. (A) A DIC image of a network of three vesicles, interconnected by a nanotube three-way junction. Note that the vesicle at the lower right displays a connecting nanotube to the mother bleb. (B) Contrast-enhanced fluorescence image of a network of two vesicles, connected by a nanotube. The membrane glycoproteins were labeled with the fluorescent dye WGA Alexa 488. (C) The corresponding DIC image of the two-vesicle network (scale bars: 5 μm).

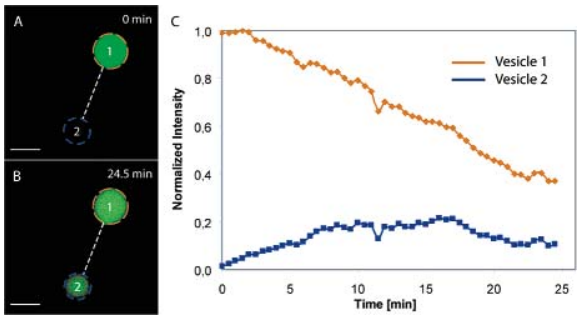

Figure 4. Diffusion of a fluorescent dye in a two-vesicle network. (A, B) Fluorescent images of the network, where the vesicles are filled with the fluorescent dye Alexa 488 hydrazide. Images show the recovery of dye at 0 and 24.5 min after bleaching of vesicle 2. The boundary of the vesicles and the connecting nanotube are marked with dashed lines. The decrease of fluorescence in vesicle 1 in panel B is not seen due to contrast enhancement. (C) Normalized fluorescence intensity of the measurement plotted versus time. The colors of each curve correspond to the color of the dashed line around the vesicles in panels A and B. The decrease of fluorescence in vesicle 1 and the weak increase in vesicle 2 are due to dissipation effects and focus drift during the measurement. Scale bars represent 5 μm.

such as organelles, if present in the mother bleb, cannot pass through the nanotubes because of size-constraints.

By using this method, we were able to construct networks of different connectivity (Figure 3). Panel A of Figure 3 shows a DIC image of a network of three vesicles, linked to each other by a three-way nanotube junction. In panel B a fluorescence micrograph of a simple network of two vesicles connected by

a nanotube is shown, where the presence of membrane glycoproteins was verified using a selective dye (WGA-Alexa 488), which binds to sialic acid and *N*-acetylglucosaminyl residues of glycoproteins. Panel C displays the corresponding DIC image. The manipulation of the cell membrane was as simple as that with synthetic vesicles, and networks were easily formed. However, the amount of membrane material that can be obtained from a cell limits the size range for networks. Furthermore, diffusion of a fluorescent dye (Alexa 488 hydrazide) between network compartments through a nanotube was confirmed by FRAP (fluorescence recovery after photobleaching) measurements, as shown in Figure 4. Images in panels A and B show the fluorescent images of the network at 0 and 24.5 min, respectively, after bleaching of vesicle 2. The measured normalized intensities plotted against time are displayed in panel C. Apart from diffusional relaxation, fluorescence signal is lost by bleaching and leakage of dye which can be treated collectively in a dissipation term.[7] However, the experiment shows that materials can be exchanged between vesicles through nanotube conjugation.

Concluding Remarks

We present a technique for the formation of nanotube−vesicle networks originating from unilamellar cell membrane protrusions obtained from single cells. The presented technique provides full control over the solution environment inside and outside the vesicles, and vesicles with different interior solution composition can be created. We verified the presence of membrane proteins in the vesicles by labeling the sugar residues of membrane glycoproteins. Finally, we demonstrated diffusion of fluorescent molecules between two vesicles via a connecting nanotube. A surface-immobilized membrane vesicle network which naturally incorporates native membranes as well as membrane proteins provides a powerful platform to study, e.g., receptor activity, reaction and diffusion behaviors as a function of geometry, topology, and size. Such systems can provide reductionistic models of cell- or organelle networks, such as the Golgi-endoplasmatic reticulum, or they can provide new platforms for biohybrid nanotechnological devices.

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