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Cell-Free Preparation of Functional and Triggerable Giant Proteoliposomes

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Giant liposomes—1 to 100 μm spherical compartments defined by a lipid bilayer membrane— are extremely useful as models of cells and cell membranes.[1,2] Various methods for giant liposome preparation have been developed to achieve well-defined membrane properties and specific composition of the inner core.[3–8] The essential role of membrane proteins in many biological functions of membranes has led to growing interest in working with giant liposomes with giant proteoliposomes, that is, giant liposomes with membrane proteins embedded in the lipid membrane. These are especially useful for in vitro biological, biophysical, and biochemical investigations of membrane proteins in a well-defined lipid environment.[9–16] Although a finely tuned lipid composition of liposome membranes is easily achieved by simply adjusting the stoichiometry and nature of the lipids used, functionalization of liposomes with membrane proteins remains highly challenging. The most widely used functionalization method is based on the incubation of purified proteins with previously prepared giant liposomes. Insertion of proteins into membranes is achieved either by use of surfactants or with small vesicles (diameter < 1 μm) that are then fused to the membrane.[9,11,17–19] Obtaining suitable protein samples can prove a major limitation to this widely used method, as is the difficulty to exert genuine control over the insertion process. This conventional process can also be time-consuming and give low yields of functional, reconstituted membrane protein.[20] To overcome these problems, it is possible to combine the preparation of liposomes with in vitro cell-free membrane protein synthesis, a rapidly expanding methodology.[21] Membrane proteins, such as stearoyl-CoA desaturase,[22] bacteriorhodopsin,[23] voltage-dependent anion channel (VDAC), and Bak proapoptotic protein,[24] have been successfully synthesized by using *Escherichia coli* or wheat germ extract (WGE) cell-free expression systems, and inserted into small unilamellar vesicles (SUVs).

To the best of our knowledge, in vitro synthesis and insertion of membrane proteins in their functional state into giant liposomes has only been accomplished in a few cases. Cell-free extracts (*E. coli* extract, WGE, or rabbit reticulocyte lysate) have been used to synthesize α-hemolysin pore proteins from *Staphylococcus aureus*,[25] apo cytochrome b_5,[26] as well as connexins Cx32 and Cx43,[27] thereby leading to preparation of the corresponding functionalized giant proteoliposomes. Kaneda et al. even showed direct cytosolic delivery with connexin-functionalized liposomes.[27] A reconstituted cell-free gene expression medium was also successfully used to obtain giant liposomes defined by membranes functionalized with *sn*-glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase.[28] Successful examples of protein insertion into membranes are usually performed with giant liposomes prepared by natural swelling. Although this preparation method is simple, it has some severe limitations, such as low yield and uncertain liposome properties, due to significant multilamellarity and size dispersion. Also, as proteins are expressed both inside and outside liposomes, control over protein orientation is not possible. Cell-free expression of membrane-related cytoskeleton proteins was recently achieved from inside polymersomes generated by a microfluidic flow-focusing device,[29] and in liposomes obtained by centrifugation,[30] thus providing possible control over directionality of protein insertion; however, confinement of the gene expression medium to the inside of the polymeric or lipidic vesicles might limit the yield of expression.

Here we describe a robust, one-pot preparation method to generate functional giant proteoliposomes at a high yield within a few hours. We expressed a membrane protein with a cell-free, reconstituted expression system outside well-defined giant liposomes that were obtained by spontaneous transfer of sucrose-in-oil droplets into a water phase through an oil/water interface. We used a DNA plasmid coding for connexin-43 (Cx43), a mammalian membrane protein able to form either pores (dephosphorylated state) or gap junctions (phosphorylated state).[31–34] We analyzed the insertion of Cx43 into the membrane, the role of the membrane in this process, and the functionality of the inserted protein. We also explored the possibility of triggering functionalization of these giant proteoliposomes with a straightforward temperature stimulus.

Figure 1 shows our experimental procedure. Sucrose microdroplets were transferred by gravity from a phospholipid-containing oil into a water phase to form giant liposomes. The membrane protein was rat-heart connexin fused to an enhanced green fluorescent protein (EGFP) reporter (Cx43-EGFP); it was expressed from a synthetic plasmid in a commercial, cell-free, reconstituted gene expression medium (PURExpress).[35,36] To promote droplet transfer through an interface,

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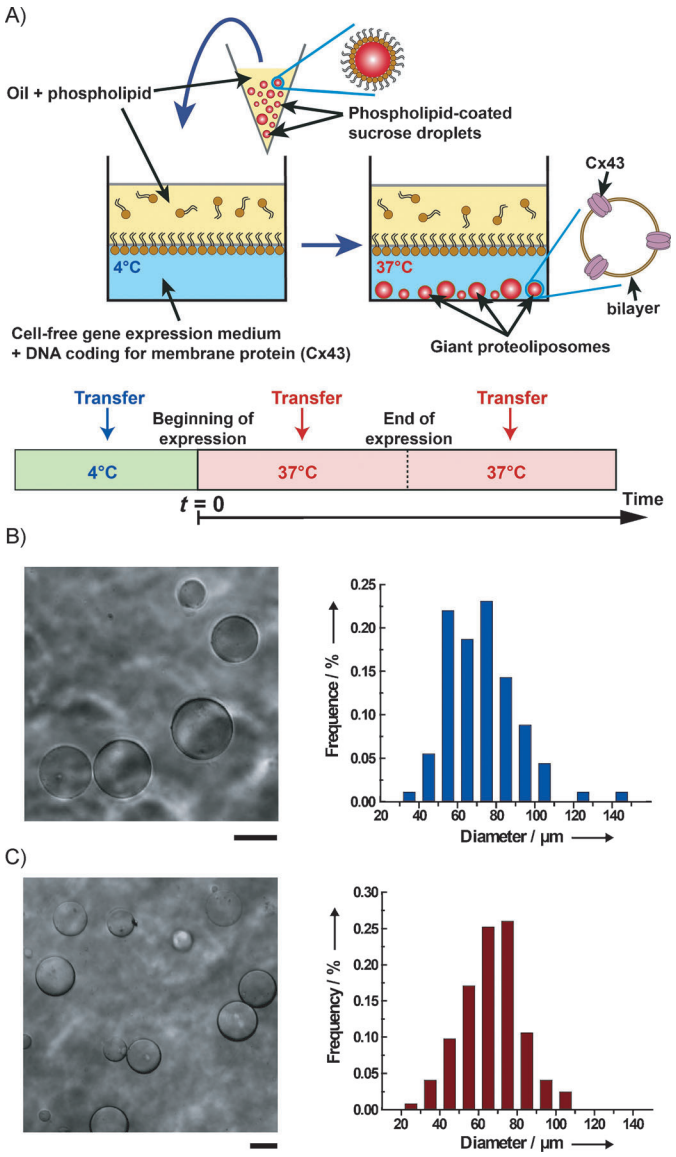

Figure 1. Preparation and characterization of giant proteoliposomes. A) Sucrose droplets initially dispersed in a phospholipid-containing oil (0.5 mM Egg PC in mineral oil) are gravity-transferred to generate giant liposomes in a cell-free gene expression medium (PURExpress) containing DNA (10 ng μL^{-1}) encoding Cx43-EGFP, a connexin-43 membrane protein fused to EGFP fluorescent reporter protein. The transfer can be done before (at 4 °C), during (at 37 °C), or after (at 37 °C) gene expression. B) and C) Phase contrast image and diameter distributions of giant liposomes obtained by transfer B) before and C) after the beginning of expression at 37 °C. Scale bars: 50 µm.

external forces are frequently applied, for instance by centrifugation.[25,37–39] Here, droplets spontaneously transferred through the interface by gravity.[40] The high osmolarity of the gene expression medium (around 990 mOsm under our experimental conditions) meant the sucrose concentration in the microdroplets needed to be adjusted to guarantee isotonic conditions. To evaluate the impact of the liposome membrane on expression, droplets were added above the interface either at 4 °C followed by a rapid rise to 37 °C to start gene expression ($t = 0$), or at 37 °C (during or after gene expression; Figure 1 A). In all cases, 100 to 200 spherical liposomes (20–120 µm in diameter) were obtained within 1–2 min at the bottom of each observation chamber (Figure 1 B and C). This proves that our method allows easy and rapid preparation of well-defined giant liposomes in high osmolarity environments. Western blot analysis showed that Cx43-EGFP was successfully expressed in the outer medium (i.e., in the aqueous phase surrounding the liposomes) after incubation at 37 °C, regardless of the presence or absence of liposomes (Figure S1 in the Supporting Information).

Confocal microscopy was then used to characterize the spatiotemporal distribution of Cx43-EGFP in the samples and to assess protein insertion in liposome membranes. The EGFP fluorescence in the bulk, outside liposomes, systematically increased with incubation time but, interestingly, the fluorescence intensity of the membrane was strongly dependent on the time of transfer (Figure 2 A). When droplets were transferred prior to protein expression, a bright fluorescent membrane was observed for all liposomes. In contrast, when they were added after expression had started, a much lower fluorescence signal was observed at the membrane. To quantify this phenomenon, the radial profile of fluorescence intensity was measured for several tens of liposomes, thus enabling us to establish an average radial profile of fluorescence intensity (Figure 2 B). Analysis of liposomes transferred before expression shows that the fluorescence intensity outside the liposomes increased with incubation time, in agreement with Cx43-EGFP expression in the outer medium. Interestingly, we saw a fluorescence peak at the membrane position; this also increased with time, thus showing efficient accumulation of Cx43-EGFP in the membrane during membrane protein expression (Figure 2 C). Liposomes transferred during expression showed a marked difference in radial profile. For a fixed incubation time (180 min), the fluorescence peak at the membrane increased with increasing contact time with the liposomes (Figure 2 D). The presence of the liposomes during protein expression thus seems to be critical for efficient protein insertion in membranes. To further analyze this effect, we dramatically increased the timescale: samples were incubated overnight before transferring the droplets. All membrane proteins were therefore expressed before the transfer. In this case, even though the outside fluorescence was comparable to that observed with the previous protocols, we did not observe any fluorescence peak at the membrane, despite the long contact time (up to 120 min) between liposomes and the protein-containing aqueous phase (Figure 2 E). These results show that insertion of Cx43-EGFP into the membrane is achieved only if the expression is carried out in the presence of liposomes. The importance of cotranslational insertion of membrane proteins has been shown in the presence of SUVs;[41,42] however, to the best of our knowledge, this is the first time that it has been demonstrated with giant liposomes. Expressing a membrane protein from a DNA plasmid in the presence of a membrane is therefore an easy and efficient strategy to prepare giant proteoliposomes with strong accumulation of a membrane protein at the liposome membrane.

Under our expression conditions, Cx43-EGFP was produced in a dephosphorylated state, in which the pore within a func-

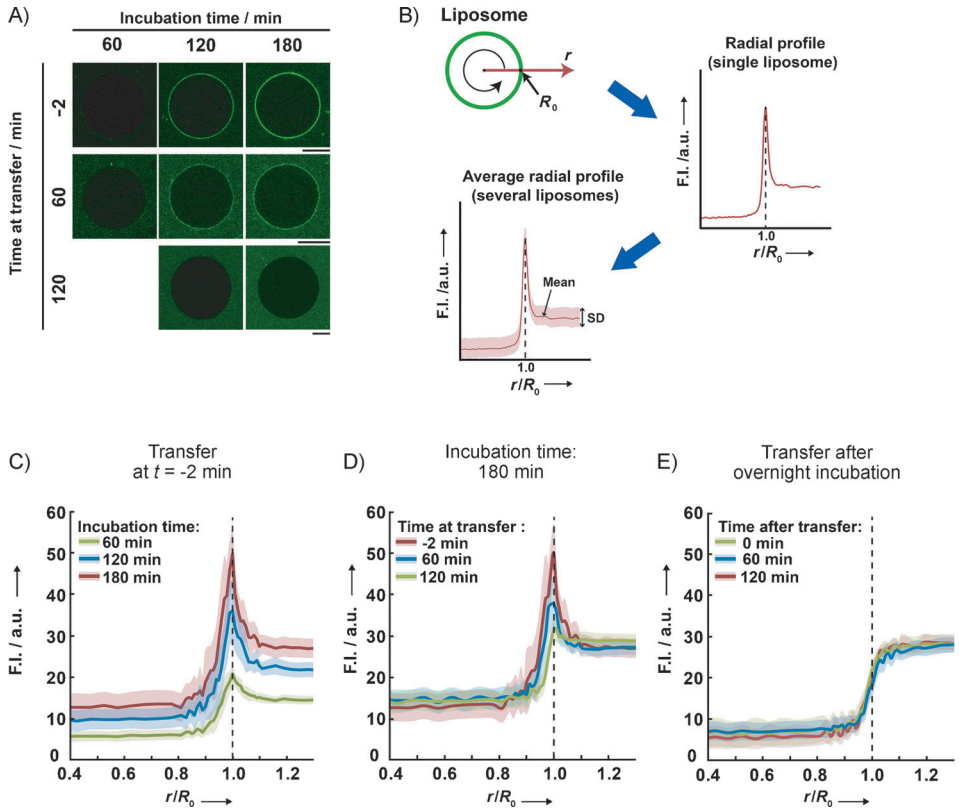

Figure 2. Efficient protein insertion into the membrane is achieved when liposomes are present during Cx43-EGFP expression. A) Representative time-lapse confocal microscopy images (EGFP fluorescence) of giant liposomes transferred before (top) or after (middle and bottom) starting incubation ($t=0$) at 37 °C, as a function of incubation time. Scale bars: 20 μm. B) Normalized radial profile of fluorescene intensity (F.I.) is established for each liposome prior to averaging for several tens of liposomes. C) Average radial profile as a function of incubation time at 37 °C for liposomes transferred before incubation. D) Average radial profile as a function of the time at which the transfer was performed. The same incubation (37 °C, 180 min) was used for all samples. E) Average radial profile for liposomes transferred after overnight incubation at 37 °C as a function of time after transfer.

tional connexin is permanently open.[31,32] Thus, the functionality of the giant proteoliposomes prepared by our method was characterized by leakage experiments. By adding propidium iodide (PI) fluorescent dye to the sucrose solution used to prepare droplets, we made giant proteoliposomes encapsulating PI. In the absence of the DNA plasmid, the intensity of the encapsulated solution decreased very slightly, probably due to photobleaching (Figures 3 A, panel 1). This shows that in the absence of protein expression, most of PI remained encapsulated in the liposomes. In contrast, when membrane proteins were expressed after liposome transfer (Figure 3 A, panel 2), a significant decrease in PI fluorescence inside liposomes was observed, attributed to leakage of liposomal content. Although PI fluorescence is known to increase in the presence of DNA, no significant increase in fluorescence was observed outside liposomes; this is attributed to dye dilution upon leakage to the outside medium. By monitoring simultaneously the fluorescence of PI inside liposomes and that of the EGFP tag on the connexin proteins, we observed a strong correlation between leakage and insertion of Cx43-EGFP into the membrane (Figure S2). When droplets were transferred after overnight incubation of the sample, the PI fluorescence of the encapsulated solution remained stable, while Cx43-EGFP was only detected in the bulk outside the liposomes and not in the membrane (Figures 3 A, panel 3). Analysis of the average PI fluorescent profile of the liposome population revealed a similar trend (Figure 3 B). This confirmed that only expression of Cx43-EGFP in the presence of liposomes led to a significant decrease in PI fluorescence. Specific leakage of PI in the presence of Cx43-EGFP at the membrane was thus a first indication that the expressed protein was in a functional form (i.e., able to induce membrane permeability). To further assess the functionality of the proteoliposomes, we repeated the leakage experiment with water-soluble dyes of different molecular weights (Figure 3 C). In the absence of DNA, when proteins were not expressed, all dyes remained inside liposomes. In contrast, when Cx43-EGFP was expressed from the plasmid in the presence of liposomes, leakage was strongly dependent on the molecular weight of the dye. Although significant leakage was observed for small molecules such as PI ($M_w = 668\ g\,mol^{-1}$), fluorescently labeled dextrans ($M_w > 4400\ g\,mol^{-1}$) displayed reduced leakage behavior. The leakage strongly decreased as the molecular weight of the dyes increased. As all dextran molecules have the same hydrophilic/hydrophobic balance, the molecular weight dependence can be attributed to a size effect, rather than to passive diffusion through the membrane. To the best of our knowledge, the cutoff value of dephosphorylated Cx43 has not been reported. Note that the molecular weight cutoff of the phosphorylated form of Cx43 is 1000–1800 Da when Cx43 establishes gap junctions.[33,34] The molecular weight dependence we observed with dephosphorylated Cx43 was in the same order of magnitude. Our results are thus in agreement with expression of a functional connexin-43 creating pores in the membrane that specifically allow small molecules to flow out of the liposomes.

Our method relies on the expression of membrane proteins from a DNA plasmid to achieve functionalization of liposome membranes. Thus it was possible to harness the temperature dependence of the expression step to achieve membrane functionality triggerable by temperature. As protein expression is inhibited at 4 °C yet activated at 37 °C, we implemented a temperature trigger, by keeping the sample at low tempera-

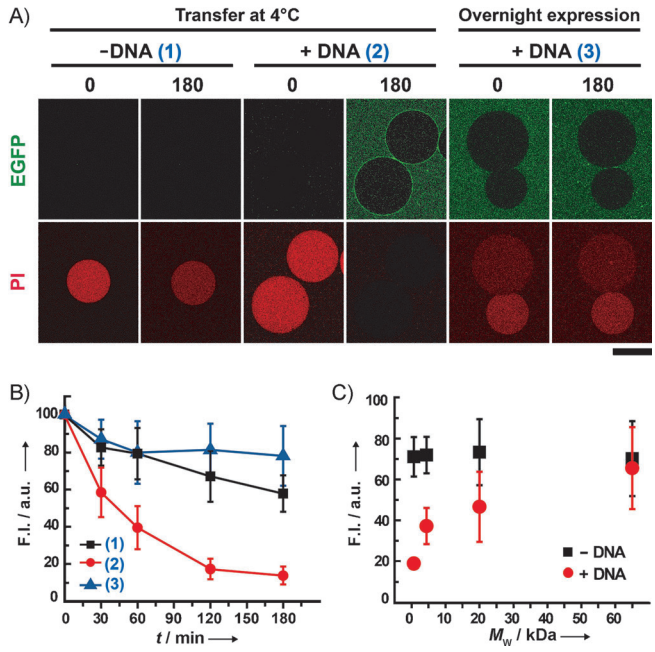

Figure 3. Functionality assays. A) Representative time-lapse confocal microscopy images of giant liposomes encapsulating 40 μg mL^{-1} propidium iodide (PI) fluorescent dye (top: EGFP fluorescence, bottom: PI fluorescence), in the absence (−DNA) or presence (+DNA) of 10 ng μL^{-1} DNA coding for Cx43-EGFP, and obtained by transfer at 4 °C before incubation at 37 °C (panels 1 and 2), or by transfer after overnight incubation at 37 °C (panel 3). For each condition, the same liposomes are shown at $t=0$ and 180 min. Scale bar: 50 μm. B) Normalized PI fluorescence intensity (F.I.) as a function time, for the three conditions shown in A). C) Normalized fluorescence intensity (F.I.) inside giant liposomes obtained by transfer at 4 °C before incubation at 37 °C for 120 min, in the absence (−DNA, black squares) or presence (+DNA, red disks) of 10 ng μL^{-1} DNA encoding Cx43-EGFP, as a function of the molecular weight of the encapsulated dye. Dyes were PI (40 μg mL^{-1}, $M_w=$ 668 g mol^{-1}) and TRITC-labeled dextrans (200 μg mL^{-1}, various molecular weights). In B) and C), the intensity was normalized to 100 at $t=0$ for each liposome. Data are shown as mean ± SD.

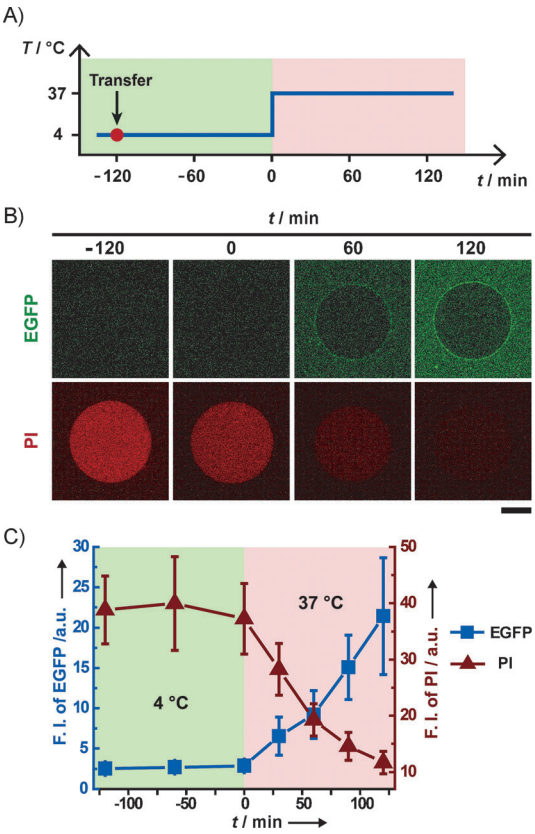

Figure 4. Temperature triggers membrane permeability. A) Giant liposomes encapsulating 40 μg mL^{-1} PI are transferred in the presence of 10 ng μL^{-1} DNA encoding Cx43-EGFP and maintained at 4 °C for 120 min prior to starting the incubation at 37 °C ($t=0$). B) Representative time-lapse confocal microscopy images of giant liposomes (top: EGFP fluorescence, bottom: PI fluorescence). Scale bar: 20 μm. C) EGFP and PI fluorescence intensities (F.I.) as a function of time. Data are shown as mean ± SD.

ture (4 °C) for 2 h before incubating it at 37 °C. Liposomes were therefore first generated at 4 °C from a sucrose solution containing PI and kept at this temperature for 2 h (Figure 4A). As expected, no protein expression was observed, and, interestingly, the PI dye remained encapsulated in the inner compartment of the liposomes (Figure 4B and C). Upon a quick increase in temperature to 37 °C, expression and insertion of Cx43-EGFP was observed, accompanied by strong leakage of PI to the outer medium (Figure 4B and C). These results show that temperature can be used to trigger the functionality (here, membrane permeability) of giant proteoliposomes produced by our method.

We have described a one-pot, fully synthetic method to prepare giant proteoliposomes within a few hours, without any preliminary protein manipulation or purification. The method is easily implementable in any laboratory as it only relies on commercially available systems (standard chemicals, a gene expression medium, and a synthetic gene) and it does not require any specialized equipment. Demonstrated here for a specific membrane protein, this method is readily adaptable to other proteins by simply encoding the target protein in the synthetic gene. An important finding is that the presence of the liposome membrane is necessary during expression for efficient protein insertion into the liposome membrane. This emphasizes the cooperativity between the synthetic expression of the protein, its folding, and its insertion into the liposome membrane, which appears to have a chaperone-like function. Further studies could help decipher the mechanisms interconnecting these processes. Another advantage of our method is that liposomes are directly formed in the gene expression medium, thus avoiding any dilution or mixing step, which could decrease the yield of expression. Moreover, proper orientation of proteins in biological membranes is crucial for protein function. In most in vitro systems, this parameter is poorly controlled, as most synthetic membranes are symmetric, and protein insertion relies on a passive mechanism. With our method, we express the membrane proteins on a specific side of the membrane, and we are currently analyzing whether this affects the orientation of the protein upon its insertion into the membrane. This will provide the foundation for both better understanding and improved control of the protein reconstitution step. For all these reasons, we believe that our method—a robust technique to generate giant functional proteoliposomes—will be useful for biologists and biophysicists who

want to study proteins in a well-defined membrane environment and avoid the tedious steps of protein purification and reconstitution. Our method can also be useful from a chemical perspective. Indeed, giant liposomes are also usually considered as microscale (bio)chemical reactors defined by their lipid membrane.[43–46] However, this membrane is passive in most cases. Here, we showed that we can program a particular membrane function (e.g., permeability) by controlling the expression of a membrane protein from the liposome environment. We demonstrated that temperature can trigger this functionalization, by simply controlling the expression of the protein. Other functions could be explored with other membrane proteins, such as receptors involved in signal transduction to trigger reactions inside liposomes from an outer chemical or biological stimulus. Other classical triggers of in vitro gene expression, such as light,[47–51] could also be used to control such functionalities.

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