Synthesis of Extended, Self-Assembled Biological Membranes containing Membrane Proteins from Gas Phase

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1. Abstract

Membrane proteins carry out a wide variety of biological functions. The reproduction of specific properties that have evolved over millions of years of biological membranes in a technically controlled environment is of significant interest. Here a method is presented that allows the self-assembly of a macroscopically large, freely transportable membrane with Outer membrane porin G from Escherichia Coli. The technique does not use protein specific characteristics and therefore, could represent a method for the generation of extended layers of membranes with arbitrary membrane protein content. Such in-vitro systems are relevant in the study of membrane-protein function and structure and the self-assembly of membrane-based protein complexes. They might become important for the incorporation of the lipid-membranes in technological devices.

2. Introduction

Biological membranes have been fundamental to the evolution of cellular life. They form a selectively permeable boundary partitioning the cell's interior and its organelles and create a homeostatic environment that is the basis for development to higher organisms. As life forms have evolved in complexity, there has been a parallel growth in the functional requirements of membranes. In nature, many physicochemical processes depend on membrane proteins, from photosynthesis over generating electric potentials, to single photon and molecule detection at ambient temperature. The technological reproduction of these properties is of significant interest.

Several techniques can be used to generate large lipid bilayers. The most frequently employed is the Langmuir-Blodgett technique. Pulling a carrier twice through the lipid monolayer formed on an aqueous surface in a controlled way transfers it as a bilayer to the solid support[1]. A second method is the self-assembly of a lipid bilayer on the surface of a flat target by vesicle spreading in an aqueous environment[2].

The incorporation of membrane-proteins into a bilayer can be problematic. Once the bilayer formed adding proteins is can be achieved by vesicular fusion[3]. When lipid monolayers are covalently bound to a solid support, the fusing vesicles that generate the second lipid layer can simultaneously act as the carrier of membrane proteins[4]. In specific cases, membrane proteins can be covalently bound to a surface and assemble a lipid bilayer around themselves[4]. Lipid bilayers, formed non-covalently on a support structure, usually establish only a minimal gap to their underlying surface. This tight spacing can render the successful incorporation of intact membrane proteins impossible[4]. Covalently bound lipid membranes can mitigate this problem by establishing a defined gap between the bilayer and its support via their linker. For this, chemical modification of either the membrane proteins or the lipid molecules is required[4]. An alternative is to use pore-spanning membranes where the carrier is micro-etched with a regular array of approximately 1.2 μm sized depressions. The bilayer formed by vesicular fusing and spreading spans these holes[3]. In all of these cases, the stability of the membrane depends on its carrier, and it is not possible to use the lipid bilayer as such easily in other devices.

One way to form free-floating membranes with proteins is to slowly remove the detergent from a detergent-solubilized membrane protein solution that contains lipids[5]. Detergents can be removed by dialysis, or by using SM-2 Biobeads (Bio-Rad, CA, USA) which act as a trap for the detergent molecules. With the slow removal of the detergent molecules, the proteins either denature or acquire lipid molecules to coat their hydrophobic regions. If the conditions are favorable, the lipid-solubilized membrane proteins can self-order and form two dimensional crystals. However, because there is no flat surface to direct the growth of the lipid membranes, the

process usually does not lead to extended layers. Lipid sheets can roll up and form tubes or multi-layer stacks[5].

Here, a technique is presented that allows the assembly of an extended membrane layer with an arbitrary membrane protein content. Only the dimension of the experimental apparatus limits its size. So far, the focus of the experiments was on the exploration of the basic principles of the membrane layer generation. However, with simple technical modifications of the apparatus, the synthesis of macroscopically large, anisotropic membrane-protein layers might be possible, limited only by the availability of the proteinaceous components.

3. Experimental

3.1. Protein Expression & Purification

Yildiz et al. describe the expression and purification of the outer membrane porin G (ompG)[6]. Briefly, E. coli expresses the amino acids 22 - 301 plus an N-terminal methionine of the OmpG gene having cloning it into a pET26B plasmid vector. Unfolded ompG elutes from an ion exchange column after breaking the cells, centrifuging and solubilizing them in 8 M urea, 1% Triton-X 100 and 25 mM Tris-HCl. The refolding of the protein occurs in a 1% (weight/vol) n-octyl-β-D-glucopyranoside (OG), 3 M urea solution. A final ion-exchange separation succeeds in removing unfolded or partially folded protein from the solution. With ultrafiltration or dialysis using membranes with a 12 kDa weight cutoff against a buffer containing 20% PEG-35000 the protein concentration rises to approx. 50 mg/ml. A buffer containing 10 mM Tris, 1% octylglucoside, 20 pmol/μl phosphatidylcholine with a concentration of 32 pmol/μl ompG serves as the base-solution to prepare ompG layers. The final spraying solution contains an additional 25% glycerol to protect the protein from the high charges.

3.2. Static Secondary Ion Mass Spectrometry

Electrospray prepared targets were investigated using Time-Of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) I and II instruments[7–9]. The TOF-SIMS I instrument had a pulsed Ar^+ ion source. The ions hit a grounded metal target. 8 keV Ar^+ ions were focused onto a 1 mm spot with a primary ion density of 10^{10} Ar^+ ions per (cm^2•second) (static SIMS conditions). Secondary ions were extracted from the grounded target and left to drift into two time-of-flight regions connected by a 163° angular and energy focusing toroidal Poschenrieder condenser lens. A single ion counting device detected analytical ions. They were post-accelerated before being converted to electrons in a channel plate which excited a scintillator. A photomultiplier counted the photon-pulses. For the TOF SIMS II instrument, the Poschenrieder lens was replaced by an energy compensating reflectron which increased the mass resolution to more than 10 000. Significant for this investigation were improvements in the primary Ar ion source. The ion current was more stable than on the TOF-SIMS I instrument, and the diameter of the ion beam on the target was 0.1mm. The, for the time, small focus made spacially resolved scans of the prepared target regions possible.

3.3. Electron Microscopy

Carbon-coated electron microscopic grids were briefly exposed to gas discharge to render them hydrophilic. Lowering a grid carefully onto a liquid meniscus carrying the prepared layers transfers them to the electron microscopy target. Several exchanges of double distilled water washed the specimens extensively. For inspection in the electron microscope, the grids were negatively stained using 1 % wt/vol uranyl acetate. Alternatively, a platinum/carbon beam shot at an angle of 15° covered the surface with a metallic layer of up to 1.5 nm. An FEI Tecnai Spirit BioTWIN transmission electron microscope operating at 120 kV with varying magnifications of up to 150 000x was used for inspection.

3.4. Electrospray based Surface Preparation

3.4.1. Electrospray Apparatus

An electrospray apparatus was built to distribute dissolved material evenly over a flat surface[10] (second generation instrument see

Figure 1). The first instrument had a metal nozzle with an emitter diameter of 250 μm. The balance between the electrostatic pull on the liquid and the flow resistance of the opening regulated the flow rate. The flow resistance was adjustable by varying the height of a precisely fitted metal cylinder within the outlet. Like this, flow rates between 8 and 15 μl/min were achievable. After the initial experiments, the nozzle was replaced by a hand-pulled, gold-coated glass capillary to reduce the stable flow rate to below 500 nanoliters/min[10]. The liquid sample was sprayed from the reservoir under atmospheric conditions onto a flat metal target. By applying a high voltage, the tip of a liquid Taylor Cone, formed at the nozzle, emitted a stream of rapidly spreading, highly charged small droplets. The volatile solvent evaporates in flight, and the dried down residues cover the target region in a circle with a diameter of about 4 mm. The distance between the nozzle and the target is adjustable. For a second generation of the apparatus, the glass capillaries were manufactured by a commercial capillary puller (model P-87 Puller, Sutter Instruments Company, Novato, CA, USA) and mounted in a pressurized container (**Figure 1**). Before spraying, the narrow glass tip was broken to have an orifice of about 1 μm in diameter[11]. With this more mature device, the electrospray operates with stable and constant flow rates between 20 and 100 nanoliters/min.

3.4.2. Surface Preparation

Spraying a rhodamine/acetone solution on an aluminum target generated a thin layer for the investigation with a static Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) instrument.

The target for the ompG protein membrane preparation was the liquid meniscus in a small cylindrical container with a diameter of 3 mm filled with a 10 mM Tris solution, pH 7.5 which contained SM-2 Biobeads (Bio-Rad, CA, USA). The final protocol for the membrane synthesis consists of five steps. The first material sprayed onto the buffer is the equivalent amount of a lipid bilayer of bi-polar lipids dissolved in ethanol: 1 μl 66 pmol/μl lipid with 35% cholesterol, 65% phosphatidylcholine. This preparation is incubated overnight at room temperature. In a second step, a thin glycerol layer is added by spraying a 1:2 solution of glycerol/ethanol onto the surface for about 10 minutes. The third step consists of spraying enough lipid to generate half a bilayer: 1 μl 33 pmol/μl lipid with 35% cholesterol, 65% phosphatidylcholine in ethanol. In the fourth step, the sprayed membrane protein and lipid complete the molecular mix to allow the self-assembly of an intact, the protein containing lipid bilayer. The sprayed solution consisted to 25% of glycerol to protect the protein from the high droplet charges when the water evaporates in flight: 1 μl ~32 pmol/μl ompG, 20 pmol/μl lipid 35% cholesterol, 65% phosphatidylcholine, 1% octylglucoside in 10 mM Tris, 25% glycerol. As the last step, another glycerol layer is added to complete a hydrophilic environment: 1:2 glycerol/ethanol solution for about 10 minutes. The entire assembly is incubated for 6 days at room temperature to allow for the extraction of the octylglucoside detergent by the SM-2 beads from the surface layers.

4. Results & Discussion

4.1. Experimental Foundation

4.1.1. Nano Electrospray Based Surface Preparation

Still unpublished experiments, completed 30 years ago, laid the scientific foundation for the membrane preparation method. The purpose of the project at that time was to build an electrospray apparatus for the homogeneous distribution of a substrate over a metallic surface. A static organic Time-of-Flight - Secondary Ion Mass Spectrometry (TOF-SIMS) instrument measured the homogeneity of the surface[12]. Static organic SIMS is capable of analyzing molecules on surfaces with sub-monolayer sensitivity. Ar^+ ions, hitting a metallic target, transfer their kinetic energy into the surface[12]. Organic molecules lying on the target detach by partially absorbing this kinetic energy. They acquire a charge by ion-association or by their intrinsic ionic state and accelerate away from the surface pulled by strong electric fields. A time-of-flight mass spectrometer measures their mass/charge ratio. The desorption mechanism explains the sub-monolayer sensitivity of static SIMS. If the surface is covered by more than one layer, the uppermost molecules do not absorb sufficient energy from the metal target, cannot desorb, and the static SIMS signal decreases. At the time, before the discovery of the Matrix Assisted Laser Desorption/Ionization (MALDI) and electrospray ionization processes, static SIMS

was one of the most sensitive techniques for the analysis of organic molecules of up to 10 kDa in size.

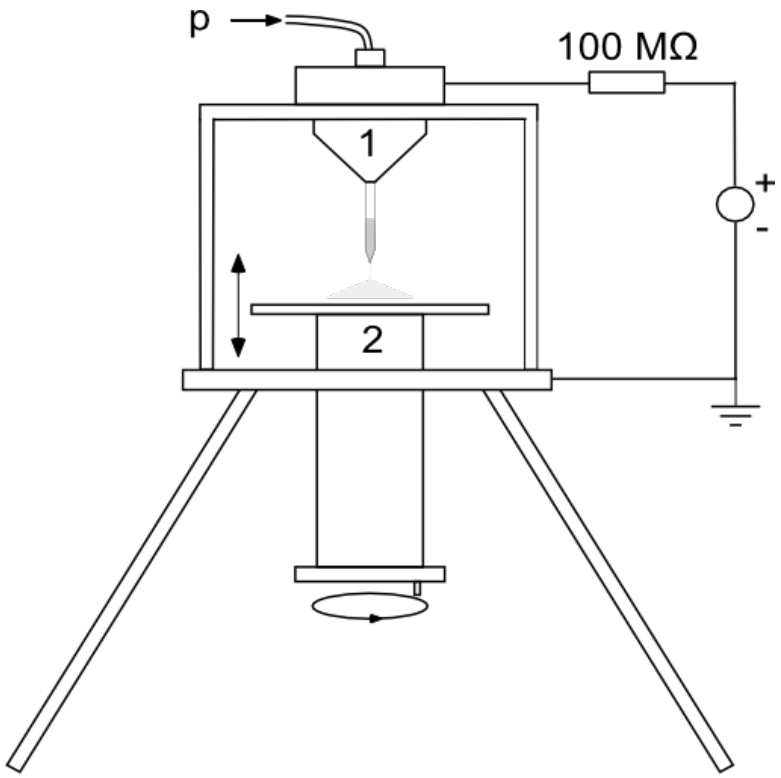

Figure 1

The electrospray apparatus for the preparation of surfaces (2nd generation): a pressurized container holds a gold-coated pulled glass capillary filled with 1 or 2 μl solution (1). An optional small pressure supports the continuous flow of the sample through the glass capillary during operation. The distance between the grounded target (2) and the needle is variable. It was usually 3 cm. The first generation instrument had a 250 μm steel nozzle and after that, a manually pulled glass capillary as an emitter. Its container was not pressurized.

Conventional electrospray generates thin films for many different purposes[13]. In general particles in the μm size range are distributed homogeneously over the prepared surface. The first electrospray apparatus built reproduced this behavior. It operated at conventional flow rates of 10 - 20 μl/min. **Figure 2** panel A shows the scanning electron microscopic picture of a surface prepared with an acetone solution of rhodamine B using this device. After the acetone evaporates in flight rhodamine particles with sizes between 0.2 μm and 1.5 μm cover the aluminum surface. The rhodamine on the surface was visible as a white homogeneous layer. Such a thick preparation is insufficient for a static SIMS investigation which analyses sub-monolayer structures. Theoretical and experimental investigations into the electrospray process itself indicated a way how to improve the homogeneity of the rhodamine on the surface[10]. A stable liquid Taylor cone ejects droplets exclusively from its tip. The radius for the emission of droplets depends on the flow rate. The central equation describing the radius of the emission region from the tip of a Taylor cone is[10]:

$$r_e = \left(\frac{\rho}{4\pi^2 \gamma \tan(\frac{\pi}{2} - \vartheta) \cdot [(\frac{U_a}{U_T})^2 - 1]} \right)^{1/3} \cdot \dot{V}^{2/3} \tag{1}$$

where r_e **the emission radius of droplets at the tip of the Taylor Cone,** ρ **the density of the solution,** γ **the surface tension,**

ϑ **the opening angle of the Taylor Cone (49.3° in the static case),** U_a **the applied and** U_T **the Taylor Cone threshold voltage and** $\dot{V}$ **the flow rate.**

Reducing the flow rate reduces the size of the initially produced droplets (**Figure 2**, panel B). The equation suggests that when spraying an aqueous solution of 1 pmol/μl at a flow rate of 20 nanoliters/min, droplets with the size of the emission radius would contain, on average, only one analyte molecule. By replacing the metallic nozzle with a gold-coated, pulled glass capillary, the flow rate was about 200 nanoliters/min. With this emitter, the metallic aluminum surface did not visibly change when the rhodamine solution was sprayed - even under prolonged preparation of 1 h or more. A raster-electron microscope and a static SIMS mass spectrometer analyzed the obtained targets (**Figure 3**). The raster electron microscopic picture showed no discernible particles even though rhodamine covered the surface of the target (panel A and B).

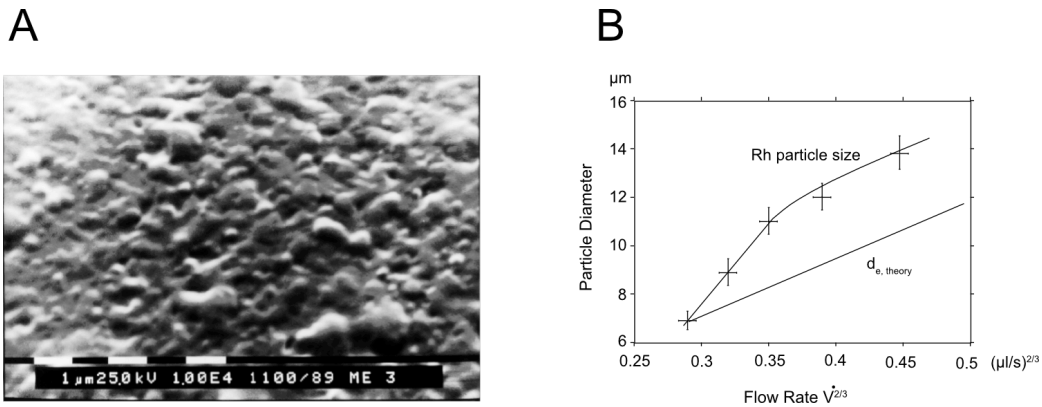

Figure 2

Panel A shows the raster-electron microscopic picture of a surface prepared with an electrospray from the 250 µm nozzle. The surface layer originated from a 30 µM acetone solution sprayed onto a metal target with a flow rate of 8 - 15 µl/min. The image shows a 10 000x magnification of the gold sputtered rhodamine surface. The length of a white bar is 1 µm. The surface is densely covered with 0.2 µm - 1.5 µm sized particles. Panel B shows that the maximal observable particle size on the target depends on the flow rate in agreement with formula 1. When spraying a saturated rhodamine/acetone solution, the particle diameter on the target corresponds roughly to the size of the initially produced droplets.

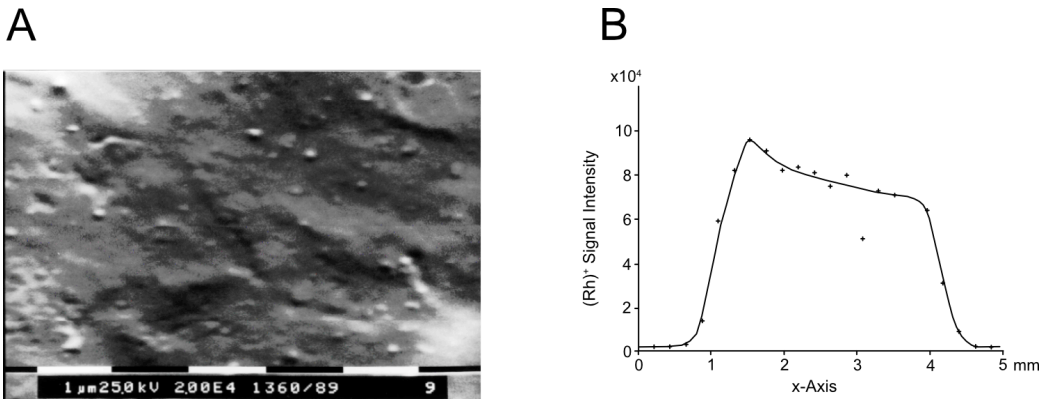

Figure 3

Rhodamine prepared layer using a self-sustained flow rate of approximately 200 nanoliters/min generated with a hand-pulled gold-coated glass capillary: panel A shows the 20 000x magnified surface. While rhodamine covered the entire surface (see panel B), no specific particle sizes could be determined. Panel B shows the static SIMS signal of the same target as in panel A, only before it was sputtered with gold to allow the electron-microscopic investigation. The spatially resolved static SIMS investigation showed that the rhodamine covered an area with a diameter of 4 mm evenly. At the time, this target represented the most homogeneously prepared surface investigated on this machine (TOF-SIMS II [9], https://www.iontof.com/history-ion-tof-sims.html).

Figure 4 panel A shows a slow build up of the static SIMS signal with preparation time and a fall off when the rhodamine coverage becomes too thick. This behavior is similar to a monolayer coverage on clean surfaces in vacuum[12]. The question arose whether the surface is covered by an increasing number of particles, as in panel A of **Figure 2**, or if the coverage is monolayer-like. To answer this question, a series of experiments determined the time required to reach the maximum SIMS response signal $t_{SIMSmax}$ as a function of the rhodamine concentration. If small rhodamine particles cover the target $t_{SIMSmax}$ depends on their cross-section, hence varies with $(c_{Rh})^{-2/3}$. However, if the coverage is monolayer-like, the static SIMS signal responds to the total amount of rhodamine sprayed onto the surface and will depend on $(c_{Rh})^{-1}$. **Figure 4** panel B shows how the preparation time to reach the maximum SIMS signal t is indirectly proportional to the rhodamine concentration. The linearity indicates that the SIMS signal characteristic changes proportionally to the total amount of rhodamine sprayed onto the target. This result is a clear indication that the surfaces generated by the nanoelectrospray preparation behave like molecular monolayers under a static SIMS investigation.

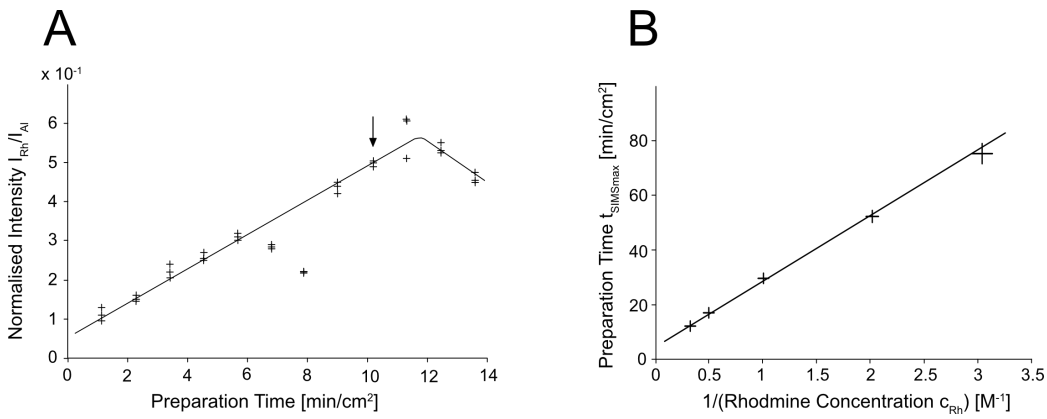

Figure 4

*Static SIMS response characteristic as a function of the amount of rhodamine sprayed onto the surface: panel A shows the increasing Rh^+ signal with preparation time. The sprayed liquid was a 30 μM rhodamine-acetone solution. For each target, three different measurements at different target locations contribute to the analysis. The SIMS signal intensity rises with increasing surface coverage passes through a maximum, and drops off. This characteristic is similar to surfaces which are successively covered by molecular beams in vacuo. During the preparation of targets 6 and 7, the electrospray became temporarily unstable. A more thorough analysis with target 9 using spatially resolved SIMS and electron microscopy revealed more details of the surface topology (**Figure 3**). Panel B shows the relationship between the time taken to reach the maximum SIMS response signal $t_{SIMSmax}$ and the rhodamine concentration in the sprayed solution. This time is indirectly proportional to the rhodamine concentration.*

4.1.2. Molecular Beam Hypothesis

The surface preparation experiments concluded in that when an acetone solution is electro-sprayed from a stable Taylor cone using a metal coated pulled glass capillary with a flow rate in the order of 200 nanoliters/min the compartmentalization of individual analyte molecules into separate droplets is complete or close to complete[14]. Two hypotheses arose from these experiments: firstly that it might be possible to construct an ion source for non-volatile molecules of virtually unlimited mass - that, what electrospray ion sources are today - and secondly that it should be possible to use a nanoelectrospray to synthesize molecularly designed layers of large organic compounds[15].

While these experiments were in its final stage, Prof. Fenn et al. published the first mass spectra of protein ions generated with a conventional electrospray ion source[16,17]. With the realization of the nanoelectrospray ion source at the European Molecular Biology Laboratory (EMBL), the first of these two hypotheses was confirmed[18]. The direct interfacing of the nanoelectrospray ion source with the vacuum system of a mass spectrometer demonstrated that the compartmentalization of analyte molecules is indeed complete. Nanoelectrospray is still the most effective ion source and continues to contribute relevant experimental evidence to discussions about

the mechanism of the electrospray ionization process itself[14].

4.2. Membrane Layer Synthesis

The most complex functional layers involving proteins are cellular membranes. Because purified membrane proteins are only stable when associated with detergent molecules, it is not possible to synthesize such membranes in a layered fashion[6,19]. The question was whether electrosprayed layers could constitute an environment in which they self-assemble. The ability of the nanoelectrospray to generate mono-molecular films was the experimental foundation. The issue to address was: what are the requirements for self-assembly of protein filled lipid bilayers when synthesized in a layered fashion? The course of the experiments suggests the answer: hydrostatic stabilization of the membrane proteins on both sides of the lipid bilayer. Even though a lipid bilayer can form on the surface of a liquid, incorporation of the proteins will only occur when a hydrostatic layer thick enough to enclose the proteins surrounds the lipid layer. This finding corresponds to the observation that membrane proteins in a supported bilayer can denature when the distance between the membrane and the underlying support is too small[4].

The first experimental result was the observation that lipid mono- and bilayers can assemble on a liquid surface when the lipids are sprayed onto them. The substrate selected for the layer generation was the liquid meniscus of a small round container with a diameter of 3 mm corresponding to the size of a standard electron microscopic grid. The surface has a molecular density of 36 $Å^2$ per molecule when distributing 33 pmols phosphatidylcholine evenly over the surface of the meniscus. This value lies between the surface occupancy of 60 Å - 70 Å as measured for phosphatidylcholine molecules in vesicles[20], and approximately 20 Å for a saturated fatty acid molecule in a regular Langmuir-Blodgett film[21]. Considering the linear structure of saturated fatty acids and their dense packing in a regular array, 20 $Å^2$ is certainly too small for the surface occupancy of a phosphatidylcholine molecule in a closed, flat phosphatidylcholine film. When the layer consisted of 66 pmols phosphatidylcholine, the surface viscosity visibly changed, and, after overnight incubation, folds in the surface appeared (see **Figure 7**, supplemental material). The folding did not occur when the layer contained only 33 pmols.

The behavior of outer membrane protein G (ompG) from Escherichia Coli on the surfaces generated by spraying 66 pmols and 33 pmols underlines their different nature. ompG is a relatively resilient 35 kDa monomeric channel protein. It is a membrane crossing protein that denatures in an aqueous buffer if no detergent is present to cover its hydrophobic regions. Its crystal structure shows that it has an outer pore diameter of approximately 2.5 nm in the open conformation with a 2 nm large central channel at its periplasmatic side[6,19]. ompG sprayed onto a surface prepared with 66 pmols phosphatidylcholine denatures upon removal of the detergent. When sprayed onto a surface with only 33 pmols phosphatidylcholine it remains intact.

Figure 5 shows transmission-electron microscopic images of the first successfully assembled membrane layer containing ompG. These images come from an early stage of successful experiments. The preparation protocol was subsequently optimized to generate more extended protein-lipid membranes in a more reproducible way. However, they demonstrate that no support other than the liquid surface is required to allow membrane assembly. The first step for this preparation was to spray 1 μl 22 pmol/μl phosphatidylcholine in ethanol onto the liquid container holding a 10 mM TRIS solution, pH 7.5 and some SM2-Biobeads. Then followed the distribution of 1 μl 16 pmol/μl ompG in 1 w% octylglucoside 10 mM TRIS solution and again 1 μl 22 pmol/μl phosphatidylcholine. The fourth layer consisted of glycerol by spraying approximately 3 μl 1:2:2 solution of glycerol, water, and ethanol. The container was incubated at 37 °C in a closed petri dish in a saturated water vapor atmosphere for seven days to allow the extraction of the octylglucoside detergent by the SM2-Biobeads. Then, the liquid surface was transferred to a carbon-coated copper grid, washed several times with doubly distilled water, negatively stained with 1% uranyl acetate and imaged in an electron microscope.

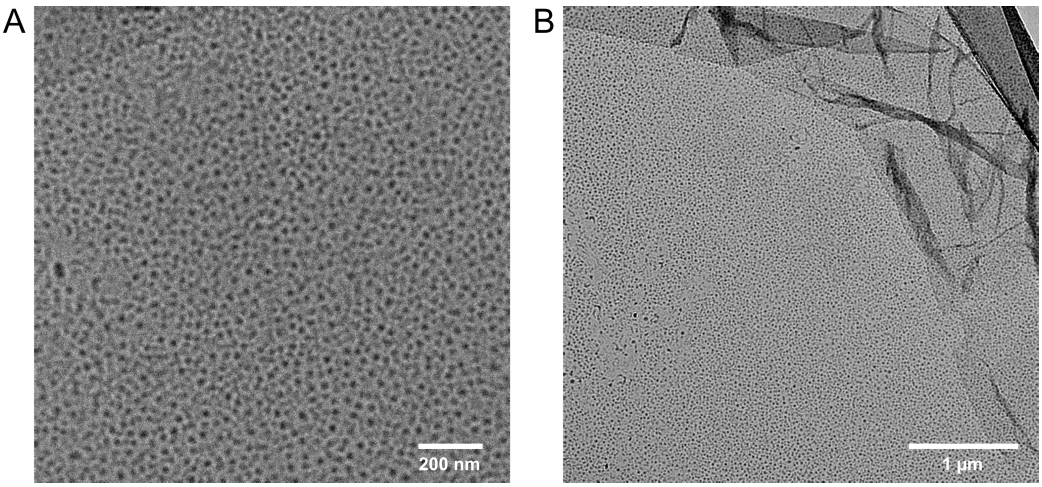

Figure 5

Transmission electron microscopic picture of the first successfully self-assembled ompG containing membrane prepared with nanoelectrospray onto a buffer solution: a negative stain with a 1% uranyl-acetate solution generates the contrast. The length of the white bar is 200 nm for panel A and 1 µm for panel B. The folded structure in panel B is the ruptured and crumbled carbon layer of the EM grid at this location.

One particular feature of the image came as a surprise - the electron dense center of the pores. A possible explanation for this is that the washing steps failed to remove glycerol from the interior of the pores. Without additional measures, liquid water cannot readily permeate pores with a diameter in the low nm range. This aspect is central to Gore-Tex membranes which are permeable to gaseous but not to liquid water. Uranyl-acetate staining, as used for the images in **Figure 5**, tends to enlarge features in EM pictures. To acquire pictures that represent the layer more accurately, we chose platinum shadowing as the staining procedure.

The preparation protocol was further optimized to generate large membrane layers in a more reproducible and robust way (**Figure 6**). The first step consists of spraying 66 pmols of bipolar lipid onto a buffer containing SM-2 biobeads. The container was incubated overnight at room temperature. This procedure generates a layer that retains subsequent molecules in the surface. A thin layer of glycerol laid the foundation for the membrane assembly in a hydrophilic environment by spraying a 1:2 glycerol/ethanol solution. The equivalent of a lipid mono-layer was added (33 pmols) followed by the detergent-solution of ompG and sufficient lipid to form a closed bilayer (20 - 30 pmols). A final glycerol layer completes the hydrophilic environment. The SM-2 beads in the buffer extracted the detergent from the surface in a six days lasting incubation period. After transfer to a carbon coated grid several exchanges of double distilled water removed the glycerol. Exposure to a platinum beam in vacuo generated the contrast for a transmission-electron microscopy investigation. **Figure 6** shows a series of images from one region of a lipid bilayer formed from this assembly. An extended, intact ompG containing layer has formed. Considering that ompG denatures in an aqueous environment when no lipid or detergent molecules are present, the images seem to display a densely packed membrane. The appearance of the structures depends on the addition of ompG to the surface, and the circles are virtually identical in size and shape to a projection of the space-filling model of ompG calculated from crystallization data[19].

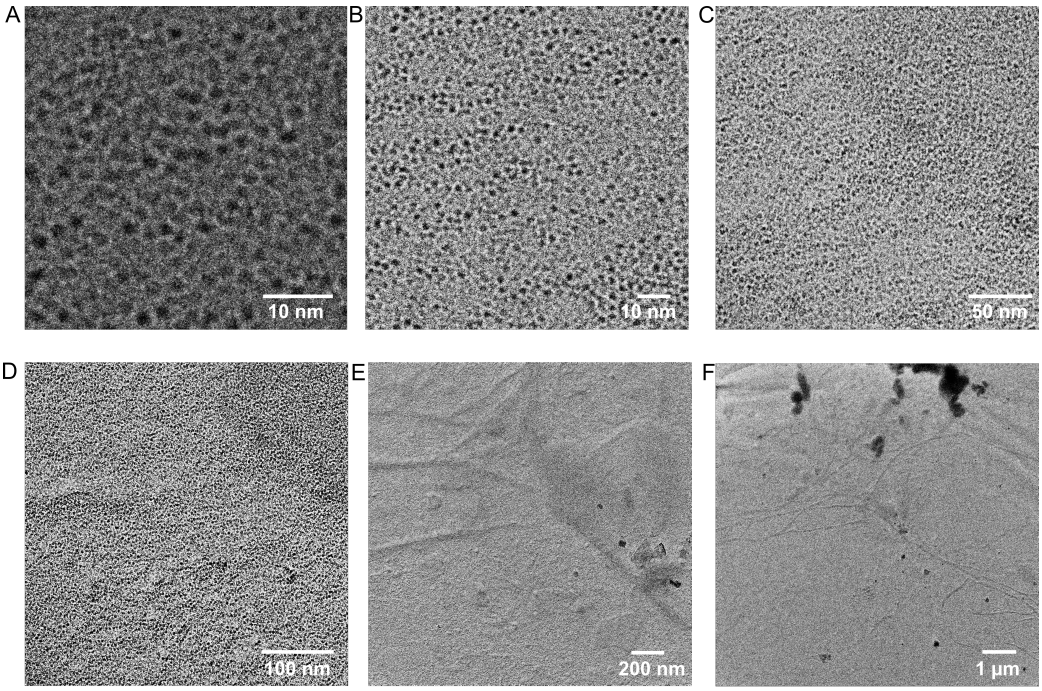

Figure 6

The images show a transmission-electron investigation of an ompG containing membrane prepared with nanoelectrospray and shadowed with a 1.5 nm thick platinum layer at an angle of 15°. The length of the white bar is 10 nm for panel A and B, 50 nm for panel C, 100 nm for panel D, 200 nm for panel E and 1 µm for panel F. A monolayer of the ompG filled membrane seems to cover the entire region.

The interpretation of the observations is controversial. **Table 1** summarises two different models and their respective arguments. For the sake of completeness, the chart reflects the entire discussion. A follow-up manuscript will present the missing experiments already mentioned here[22].

Observation	Homogeneous layer generation as displayed in figure 5 and 6	
	Observations explained by the model	
Model to explain the observation	**OmpG containing lipid bilayer membrane**	**Contaminations and electron microscopic defocussing artefacts**
Homogeneity	✔	The homogeneity is possible but not inherent to a contamination.
The pattern is exclusively observed when adding ompG to the preparation	✔	The contamination is part of the protein purification.
Control samples, prepared in the same way but without the ompG protein, don't show this pattern	✔	The contamination is part of the protein purification.
No denatured ompG protein visible after detergent extraction	✔	-
The structures have the same size as ompG proteins	✔	This is just a coincidence.
The pictures do not look like ompG prepared in solution	The ompG pores are filled with glycerol since the membrane assembled itself in an glycerol layer. A simple washing procedure of the surface fails to remove the glycerol from the pores since water does not enter easily holes that small.	The pictures do not show ompG but a contamination.
The surface is flat - no shadows are visible after having been sputtered with platinum under an 15°angle	The ompG - membrane is flat since the glycerol fills the ompG pores. Additional glycerol is washed away.	- - (Contaminations like dust particles should have shadows.)
Preparations with lysteriolysin O* show structures of the expected size which are ten times larger than the structures of ompG prepared membranes.	✔	- - (It is unclear why the size of the contaminations follows the size of the protein structures.)
Lysteriolysine O pores* display as well an electron-dense center.	Lysteriolysine O pores are ten times larger than ompG pores but they are still too small for water to wash the glycerol out.	This is how solid contaminations with defocussing artefacts look like.
The lysteriolysin O samples* look the same for very different sources of lysteriolyin O (expressed in the laboratory and commercial)	✔	- - (It is possible but not very likely to observe the same contamination in lysteriolysin O preparations of such different origins.)

Table 1

*Two different models to explain the observations displayed in figure 5 and 6. * refers to [22].*

5. Conclusions

The second of the two original hypotheses that nanoelectrospray should allow the composition of surfaces on a molecular level appears to hold. The fact that it was possible to self-assemble a large ompG containing membrane when a lipid monolayer acts as the base but not when it is a lipid bilayer confirms this hypothesis. With this technique, it is possible to generate a flat environment for the self-assembly of an ompG containing membrane upon detergent extraction. When the detergent is removed the ompG molecules either denature or recruit lipid molecules to cover their hydrophobic regions. In this manner, ompG molecules act as nuclei for the formation of a membrane. This process also occurs in a solution. Under those conditions, however, these sheets are generally limited in size. Stacks of membrane-layers or membrane tubes can form[5,23]. The lateral ends of an assembling lipid bilayer are not stable in a hydrophilic environment. Growing sheets tend to role up eliminating their tangling edges. Here, the assembly of a membrane is occurring in a flat milieu. There is not enough space for them to roll up into tubes or form multi-layered sheets.

So far, the experiments did not aim at establishing an anisotropic orientation of the membrane proteins. However, the protein orientation is likely to become oriented when the membrane assembly process proceeds in a static electric field. Will this membrane self-organization process also succeed with other membrane proteins? Proteins, by their very nature, have very individual physicochemical properties. However, considering the general concepts underlying the ompG membrane assembly, the expectation is that the process is genuine. Nanoelectrospray based layer assembly, thus, describes a route towards engineering a new class of materials which might serve as interfaces between microelectronic devices and organisms, become an integral part of biosensors, allow the direct study of small molecule - receptor interaction and the self-assembly of protein-based complexes in membranes or enable the synthesis of complex bio-molecules in membrane-based bio-reactors.

6. Conflict of Interests

The author declares no conflict of interest.

7. Acknowledgments

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9. Supplementary Information

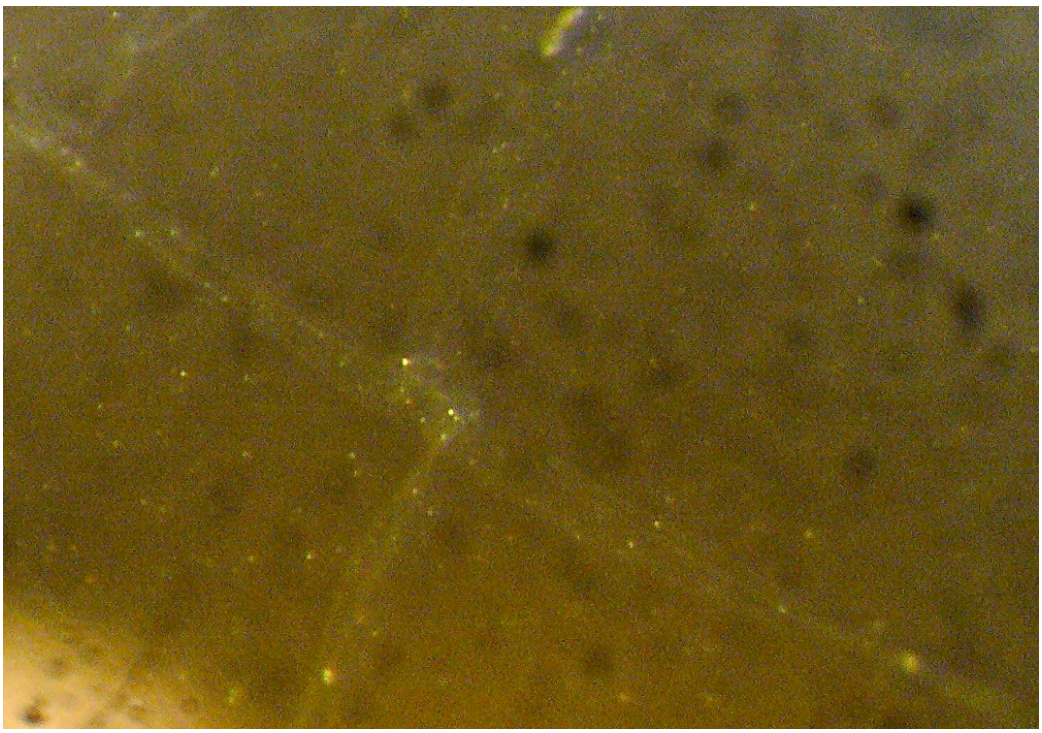

Figure 7

A nanoelectrospray generated lipid bilayer on a buffer solution after overnight incubation at room temperature: some folds of the viscous surface are visible.