



HAL
open science

Self-Assembly of Solid-Supported Membranes Using a Triggered Fusion of Phospholipid-Enriched Proteoliposomes Prepared from the Inner Mitochondrial Membrane

Celine Elie-Caille, Ophelie Fliniaux, Jacques Pantigny, Jean-Claude Maziere, Christian Bourdillon

► **To cite this version:**

Celine Elie-Caille, Ophelie Fliniaux, Jacques Pantigny, Jean-Claude Maziere, Christian Bourdillon. Self-Assembly of Solid-Supported Membranes Using a Triggered Fusion of Phospholipid-Enriched Proteoliposomes Prepared from the Inner Mitochondrial Membrane. *Langmuir*, 2005, 21(10), pp.4661-4668. 10.1021/la066973k . hal-00096251

HAL Id: hal-00096251

<https://hal.science/hal-00096251v1>

Submitted on 19 Sep 2006

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Re-submitted as article to *Langmuir*

(N° LA 048212 N)

**Self-Assembly of Solid-Supported Membranes Using a Triggered Fusion
of Phospholipid-Enriched Proteoliposomes Purified from
the Mitochondrial Respiratory Chain.[†]**

Céline Elie-Caille,[†] Ophélie Fliniaux,[†] Jacques Pantigny,[†] Jean-Claude Mazière[‡] and Christian Bourdillon^{†*}

[†] *CNRS UMR 6022, Université de Technologie de Compiègne, B.P. 20529, 60205 Compiègne Cedex, France.*

[‡] *EA 2087 Université de Picardie Jules Verne, CHU d'Amiens, Place Victor Pauchet, 80054, Amiens Cedex, France.*

Corresponding author: * Christian Bourdillon
UMR 6022
Université de Technologie de Compiègne, B.P. 20529
60205 Compiègne Cedex, France.
e-mail: Christian.Bourdillon@utc.fr
Tel. (33) 3 44 23 44 05
Fax: (33) 3 44 20 39 10

Abstract

A general procedure for the formation of solid-supported artificial membranes containing transmembrane proteins is reported. The main objective was to directly use the pool of proteins of the native biomembrane (here the inner membrane from mitochondria of human carcinogenic hepatic cells) and to avoid purification steps with detergent. Proteoliposomes of phospholipids-enriched inner membranes from mitochondria were tethered and fused onto a tailored surface via a streptavidin link.

The failure of some preliminary experiments on membrane formation was attributed to strong non-specific interactions between the solid surface and the protuberant hydrophilic parts of the transmembrane complexes. The correct loading of uniform membranes was performed after optimization of a tailored surface, covered with a grafted short-chain polyethylene glycol, so that non-specific interactions are reduced.

Step by step assembly of the structure and triggered fusion of the immobilized proteoliposomes were monitored by surface plasmon resonance and fluorescence photobleaching recovery, respectively. The long range lateral diffusion coefficient (at 22°C) for a fluorescent lipid varies from $2.5 \cdot 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for tethered lipid bilayer without protein to $10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for a tethered membrane containing the transmembrane proteins of the respiratory chain at a protein area fraction of about 15%. The decrease in the diffusion coefficient in the tethered membrane with increase in protein area fraction was too pronounced to be fully explained by the theoretical models of obstructed lateral diffusion. Covalent tethering links with the solid are certainly involved in the decrease of the overall lateral mobility of the components in the supported membrane at the highest protein to lipid ratios.

Introduction

Solid-supported phospholipids bilayers are currently very attractive for the development of new models of the biological membrane.^{ii,iii,iv,v,vi} Recent successful approaches for the incorporation of proteins in supported bilayers have demonstrated the importance of a water-filled space between the solid template and the bilayer.^{vii,viii,ix,x,xi} The current best molecular architectures at the solid interface consist of a tethering layer such as a polymer spacer covered by a lipid bilayer.

Without proteins, the two-dimensional structure of tethered phospholipid bilayers is generally obtained in two steps by combination of several molecular assembly techniques: Langmuir-Blodgett transfer,^{vii,xii} Langmuir-Schaefer transfer,^{xiii} self-assembly of various thiolate spacers on gold,^{xiv,xv,xvi,xvii,xviii} adsorption or grafting of polymers,^{xix,xv} or ligand-receptor recognition.^{xx,xxi,xxii} These first steps are followed by the final spreading out of the phospholipid bilayer through a spontaneous vesicle fusion process.

Compared to these numerous methodologies, the choice of strategies for the incorporation of integral proteins in the supported membrane is still limited. Since the first demonstration in 1984 by Brian and McConnell,^{xxiii} the literature describes two main possibilities, always performed after the isolation of the membrane protein of interest with the help of a convenient detergent:

- i) direct incorporation into the supported bilayers. The detergent-solubilized protein solution is put in the presence of either a supported monolayer,^{xi,xxiv,xviii,xvi} or a bilayer.^{xxv,xxvi} and the detergent is removed.
- ii) preliminary reconstitution of the purified protein in proteoliposomes and spontaneous fusion of the proteoliposomes on a appropriate interface such as: a tethered peptide,^{xxvii,xvi} a hydrophobic first monolayer,^{xii,vii,ix,xviii} a polymer layer,^{xxviii,xxix} naked gold,^{xxx} or gold in the presence of thiolipids.^{xxxi}

As the mechanism of supported bilayer formation is complex and not yet completely understood, both strategies appear to be controlled by several interdependent parameters such as: the hydrophilic/hydrophobic nature of the tethered spacer, proteoliposome-surface interactions, proteoliposome stability related to the nature of the integral protein, interactions between the surface and the protuberant parts of the proteins, heterogeneity of the protein contents related to the vesicle size. This is clearly not an exhaustive list. It is highly unlikely that the optimal conditions for the formation of supported membranes are identical from one transmembrane protein to another. These problems were discussed in a recent paper of Graneli et al.^{xxxii} For example, the authors have demonstrated that the hydrophilic domains of an integral protein can severely hamper the fusion process. The higher the (protuberant protein)/(lipid) ratio in the proteoliposomes, the greater the effect.

Within this general context, we are interested in the reconstitution of a complete functional membrane assembly as a working electron transfer chain. Our mid-term goal is to achieve electrocatalytic coupling between the reconstituted electron transfer chain and an electrochemical interface via the lateral mobility of the quinone pool.^{xxxiii} We previously demonstrated the feasibility of this coupling in two dimensions in the simpler case of a peripheral enzyme: pyruvate oxidase coupled to ubiquinone.^{xxxiv}

The reconstitution of the electron transfer chain on a convenient template is a more complex task than the incorporation of only one purified protein and, in view of the discussion above, there is a need to innovative reconstitution strategies.

Firstly, to produce the proteoliposomes solution, it seems unrealistic, on the purification point of view, to reconstitute the electron transfer machinery after individual isolation of each enzyme complex. We decided therefore to start with the purified inner membrane of mitochondria and to use the concept of "lipid-enriched membranes" developed for other purposes, twenty five years

ago, in the Hackenbrock group.^{xxxv,xxxvi} The proteoliposomes are then produced by fusion of native membranes with exogenous lipid vesicles in such a way that the lipid to protein ratio can be controlled.

Secondly, the driving force controlling the approach and docking of the proteoliposomes on the surface has to be as specific as possible. In this case, the surface can be independently adapted at the molecular level to control unspecific binding of both proteins and lipids. Such specific fixation of vesicles was previously described, using for example, biotin/avidin affinity,^{xxii,xxxvii} or metal-chelator affinity.^{xxi}

Thirdly, an important constraint in most of the reconstitution methodologies is the need for a **spontaneous** fusion and assembly of the protein/lipid material on the tethering structure. The hydrophilic/hydrophobic balance of every molecular component has to be carefully adjusted for this purpose. Moreover, even if the interaction of vesicles with the surface is generally fast, the lateral fusion and connection between the membranes is often slow (several hours) and poorly controlled. Sometimes there is no spontaneous fusion at all,^{xxxviii,xxxvii} and the promotion of fusion by an osmotic stress has been proposed.^{xxxix} In our previous papers on the formation of supported phospholipid bilayers without protein, we have demonstrated the possibility of a clear separation in time between a preliminary step of vesicles fixation followed by a deliberate triggered and fast fusion of the immobilized lipid material.^{xi,xli,xlii} In this work, we exploit the same principle using proteoliposomes.

The present study was made on flat surfaces using surface plasmon resonance (SPR), fluorescence microscopy and fluorescence photobleaching recovery (FRAP) as the main characterization techniques. However our interest is in the development of an assembly process also convenient for the high surface areas encountered in our microporous templates.^{xli} With this

idea in mind, optimization was performed without any step requiring Langmuir transfer methodologies.

Materials and methods:

Materials:

Diphosphatidylglycerol (cardiolipin), from bovine heart, L- α -phosphatidyl-ethanolamine-dioleoyl (DOPE) synthetic, L- α -phosphatidyl-ethanolamine-dipalmitoyl-N-NBD (NBD-DPPE) synthetic, L- α -phosphatidylcholine (egg-PC) type XVI-E from egg yolk, biotin-amidocaproic acid 3-sulfo-N-hydroxysuccinimideester (NHS-Ic-biotin), streptavidin, n-octyl β -D-glucopyranoside (OG) were purchased from Sigma (St Quentin Fallavier, France). Hydroxy ethyl piperazine ethane sulfonic acid (HEPES), ethylene diamine tetraacetic acid (EDTA), ethylene glycol bis-aminoethylether tetraacetic acid (EGTA), dithiothreitol (DTT), phenyl methane sulfonyl fluoride (PMSF), digitonine, bovine serum albumin (BSA) fraction V, cytochrome c from horse heart, polyethylene glycol (PEG-8000), average molecular weight: 8000 g mol⁻¹ were also from Sigma. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl (biotinylated DPPE) was from Avanti polar-lipid. NHS-PEG-333 was from Quanta bioDesign (Powell, OH). The monoclonal antibody labeled with Alexa fluor 488 was a mouse IgG anti-human cytochrome oxidase from Molecular Probes (Eugene, OR). Aminopropyl-dimethylethoxysilane (ADMS), aminoethanethiol and buffers were from (Aldrich, Strasbourg, France). Organic solvents were HPLC grade. Water with a typical resistivity of 18 M Ω was produced from a Milli-Q purification system (Millipore, Les Ulis, France).

HepG2 cells culture:

The HepG2 cell line was provided from the American Type Culture Collection (HB 8065, Rockville, USA). HepG2 cells were grown in 75 cm² tissue culture flasks, containing MEM medium supplemented with 10% (v/v) foetal calf serum, 100units/mL penicillin, 100µg/mL streptomycin and 2mM glutamine. Cells were maintained in a humidified atmosphere in 5% CO₂ at 37°C.

Preparation of mitochondria from HepG2 cells:

The protocol was based on methods described by Rickwood et al.^{xliii} After 10 days of culture, cells were harvested and rinsed twice with PBS. A centrifugation at 395g for 10 minutes was used to pellet HepG2 cells, which were then resuspended into a hypoosmotic buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, pH 7.4). Cells were disrupted with a glass homogeneizer, and the solution was centrifuged at 900g for 5 minutes. The supernatant was then centrifuged at 12000g for 10 minutes. The pellet of HepG2 mitochondria was resuspended in a storage buffer (0.65 M sorbitol, 10 mM Tris, 1 mM EDTA, 0.1% (m/V) BSA, pH 7.5) and stored at -80°C.

Preparation of submitochondrial particles from mitochondria

Mitoplasts were prepared as previously described.^{xliv} Briefly, mitochondria were treated with digitonin (0.4 mg/mg mitochondrial protein) for 1 minute and centrifuged twice at 12000g for 10 minutes. The protocol of submitochondrial particles preparation was adapted from Schatz and Racker.^{xlv} The pellet (mitoplasts) was resuspended in the storage buffer (see above) diluted three times and sonicated four times for 15 seconds at 15W with a Branson Sonifier 500 (Danbury, CT). A centrifugation at 27000g for 15 minutes was performed to separate submitochondrial particles. The supernatant was stored at -80°C.

Preparation of inner mitochondrial membrane fragments:

KCl (0.15 mM) was added to the supernatant described above. Gel filtration chromatography was then performed using Sephacryl S300 HR packed in a small column (12 cm³). The column was eluted at a flow rate of 0.25 mL/min with a buffer containing 10 mM Tris, and 0.1 M KCl at pH 7.5 and fractions were collected at 90 seconds intervals. Inner mitochondrial membrane fragments were eluted first.

Sub-cellular fractionation experiments were performed at 4°C and controlled by protein assay (Biorad) and complex III activity measurement via the reduction of cytochrome c followed at 550 nm (adapted from the procedure of Rieske).^{xlvi}

Substrates and PEG/streptavidin sublayer:

Microscope cover-slides (Corning) were first cleaned in chromic acid at 60°C and thoroughly rinsed in Milli-Q water. They were stored in water until use. Gold surfaces used for SPR measurements were prepared on glass by vapor deposition of a thin layer of chromium (2 ± 0.1 nm) followed by 48 nm of gold (Edwards model E306A deposition apparatus, working at a pressure below 2 10⁻⁶ mbar). A first monolayer of short-chain alkylamine was created on the glass surface by silanization with a freshly prepared ADMS solution (1%, v/v) in acetone/water (95/5 v/v) for 2 hours, then thoroughly rinsed with acetone and dried. Similarly, a monolayer of short-chain alkylamine was assembled on gold by overnight dipping in an ethanol/water solution (80/20 v/v) of cysteamine 10mM and rinsing. The following steps were then identical on the two substrates:

First the slides were dipped for 30 minutes in a 2 mM NHS-biotin (or 10 mM NHS-PEG-333 or mixed NHS-biotin/NHS-PEG-333) solutions in a 50 mM pH 8 phosphate buffer. After thorough rinsing, the slide was dipped for 15 minutes in a streptavidin solution at a convenient concentration (typically $4 \mu\text{g cm}^{-3}$) in PBS buffer (0.01 M, pH 7.4 phosphate buffer + 0.15 M NaCl). After rinsing in a detergent twice for 5 minutes in 50mM OG, and several rinsing with the appropriate buffer in at least three different baths, the substrates were ready for experiments directed at vesicle fusion.

Small unilamellar vesicles (SUV):

Lipids, fluorescent probe and biotin-DPPE were mixed in the required ratio (typically: 45% egg-PC; 35.5% DOPE; 18% cardiolipin; 0.5% biotinylated DPPE; 1% NBD-DPPE) from stock solutions in chloroform, then dried under nitrogen flow and desiccated under vacuum for 1 hour. The dried film was resuspended from the walls of the glass tube by vigorous vortexing in 5 mL of buffer (typically, 1 mM lipids in 10 mM, pH 7.5 Tris/HCl buffer + 0.1 M KCl). This solution was sonicated four times, for 3 minutes each at 15 W, to clarity, the temperature being maintained at about 30 °C. The SUV solutions were cleared of titanium particles by centrifugation at 3000 g for 5 minutes and then diluted at the appropriate concentrations in the Tris buffer.

Phospholipid-enriched inner membranes (proteoliposomes):

Purified inner membrane fragments from liver mitochondria and small unilamellar vesicles were mixed in various proportions and sonicated 16 times 15 seconds at 15 W. Proteoliposomes were then centrifuged at 3000 g for 5 minutes to remove titanium particles.

Tethered membrane formation on the PEG/streptavidin sublayer:

In the reference method, the streptavidin sublayer was loaded with the biotinylated vesicles for 1 hour in a solution at 0.1 mM of lipids. Extensive rinsing was then performed by exchanging the solutions several times to eliminate the non-specifically adsorbed vesicles but with care not to allow the air/water meniscus to reach the substrate level.

The fusion of the immobilized vesicles was triggered by replacing the buffer solution with a 30% (w/v) PEG-8000 solution. After 5 minutes contact, the concentrated PEG solution was rinsed by gentle stirring with the buffer solution at least 8 times. The tethered bilayers were found to be stable for at least 2 days.

Surface plasmon resonance analysis:

The SPR instrument was a Biacore X (Biacore, Sweden) used with sensor chips of unmodified gold surface. After rinsing the surface by a 15 minutes injection of a 50 mM OG solution at a flow rate of 20 $\mu\text{L}/\text{min}$, the different solutions of reactants were introduced at a flow rate of 5 $\mu\text{L}/\text{min}$.

Fluorescence microscopy and FRAP on a confocal scanning light microscope:

A commercial confocal scanning light microscope (CSLM) (LSM 410 from Zeiss, Germany) was used for both fluorescence and fluorescence photo-bleaching recovery measurements (FRAP). An inverted Zeiss microscope (Axiovert 135) equipped with a 40x oil-immersion objective, NA 1.3 and with an argon ion laser (488 nm, 15 mW) was controlled by the LSM4 software from Zeiss.

The glass slide was mounted, the supported bilayer up, in an open cell allowing the introduction of about 1 mL of solution. The uniformity of the emitted fluorescence from the supported bilayer labeled with NBD- DPPE (1 mol%) was imaged at 525 nm at a scan speed of 4 seconds for the

production of 512x512 pixel images. The lateral diffusion coefficient of the fluorescent probe was measured by FRAP after calibration under the conditions detailed in a previous paper.^{xlii}

Results and discussions:*Phospholipids-enriched proteoliposomes from inner membranes of HepG2 mitochondria.*

As explained in the Introduction, our goal is the reconstitution of a respiratory chain in a supported bilayer. The starting material was a culture of HepG2 cells, a human hepato-cellular carcinoma cell line which is especially rich in mitochondria.^{xlvii} After purification of the mitochondria, the inner membranes were extracted and purified according to previously described methods.^{xliii,xliv}

Due to the very high protein content of the inner membranes of mitochondria, there is little hope of fusing purified membrane fragments (submitochondrial particles) directly onto any surface. As the phospholipid surface area in the inner membrane represents only 50% of the total area,^{xlviii} the physicochemical behaviors of these particles can clearly not be predicted by reference to pure lipid vesicles. Thus we propose to prepare phospholipid-enriched proteoliposomes by fusion of small unilamellar vesicles (SUV) with the submitochondrial fragments in order to dilute the membrane proteins. In this way, the complete range of lipid/protein ratios from pure SUV to intact inner membranes (typically 0.5 μmol of lipid / mg of protein) can be obtained. Moreover, from the experimental point of view, the dilution step allows the introduction of functionalized lipids (biotin-DPPE) or probes (NBD-DPPE, Q₁₀), useful for the formation or the characterization of the tethered membrane.

Since the pioneering work of the Hackenbrock group,^{xxxv} several fusion techniques have been described to dilute membrane proteins.^{xlix,1} Here, purified inner membrane fragments from liver mitochondria and SUV (see Methods) were mixed in various proportions and fusion was provoked by sonication. We preferred this technique (and not for example fusion by a freeze-thaw technique)^{xlix} as the subsequent process of supported bilayer formation is expected to be more efficient when small proteoliposomes are used.

Finally, for a rapid evaluation of enzyme functionality we have chosen to follow the activity of one of the membrane complexes. The activity of cytochrome bc_1 was routinely monitored (through the decyl-ubiquinone / cytochrome c assay) at each step of the process.

The lipid composition for the vesicles used for lipid enrichment was chosen to be similar to that of the inner membrane of mammalian mitochondria. As phosphatidylcholine (PC = 40 mol%), phosphatidylethanolamine (PE = 34 mol%) and cardiolipin (18 mol%) are the major species,^{li} we used egg-PC complemented with DOPE and cardiolipin. Moreover, in our case, the adjustment of PE in the vesicles to more than 30% increased the sensitivity to PEG fusion, as it has been demonstrated for SUV in solution that the fusogen efficiency of PEG is dependent on the PE concentration in vesicles.^{lii}

Table I gives a summary of purification-dilution results on a typical batch of HepG2 cells, re-suspended from tissue culture flasks after 10 days of culture. At each step, the process was optimized for purity and not for yield. Exclusion chromatography at the last step of the purification produced pure inner membrane fragments without soluble proteins as attested by the high lipid/protein ratio of this fraction (0.5 to 0.6 $\mu\text{mol}/\text{mg}$).^{xxxvi}

Molecular design of the functionalized template.

In the first instance, we tested our procedure previously developed for the assembly of a tethered bilayer from pure lipid vesicles.^{xl,xlii} Biotinylated proteoliposomes of various lipid/protein ratios were immobilized on a simple streptavidin sublayer on silanized glass surfaces and, after rinsing, fusion was triggered by a concentrated solution of polyethylene glycol (PEG-8000). The mobility of a fluorescent probe (NBD-DPPE) before and after the PEG treatment was measured by FRAP as previously described.^{xliii} The results were disappointing, in that the overall process of formation of the tethered bilayer was severely hindered by the transmembrane proteins incorporated with the proteoliposomes. Controls without proteins demonstrated, as expected, that the PEG-triggered

fusion of pure SUV produced routinely a fluid tethered bilayer (diffusion coefficient of the fluorescent probe typically $2 \cdot 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ at ambient temperatures).^{xlii} But in the presence of transmembrane proteins, even at the highest lipid/protein ratio of $60 \mu\text{mol/mg}$, the bilayer structure was neither fluid nor continuous as established by FRAP experiments where the fluorescent probe mobility was nil.

We have interpreted these negative results in the same manner than Graneli et al.^{xxxii} The water exposed domains of the membrane proteins, protuberant from the membrane surface, hamper the formation of the supported bilayer. The non-specific-binding (NSB) phenomena occurring between the protuberant parts and the support unfortunately dominate the assembly process. The PEG treatment cannot laterally fuse immobilized proteoliposomes as the lipid and protein materials are probably only pasted onto the solid surface in a disorganized fashion. Therefore, it was necessary to reconsider the structure of the solid interface in order to drastically reduce NSB. The layer-by-layer assembly of the functionalized template on the solid surface was restructured in the following manner (Fig. 1):

- The first step for grafting a monolayer of short-chain aliphatic amine onto the surfaces was unchanged. It consisted in either a silanization by ADMS on glass or a chemisorption of aminoethanethiol on gold. This step is very convenient as it allows use of the same assembly processes on the amine sub-layers, whatever the support. Thus comparisons between different characterization methods, (FRAP on glass surfaces and SPR on gold surfaces) are possible.
- The major modification was to decrease as much as possible NSB between protuberant proteins present in the proteoliposomes and the template surface. The ability of grafted PEGs to repel proteins is well known,^{liii} and we have tested the grafting efficiency of a short-chain NHS-PEG (MW = 333) for this purpose. NHS-PEG-333 is a methoxy-terminated very short oligo-PEG of

only three monomers. We have chosen a short polymer chain to avoid if possible the interactions between the brush of PEG and the internal face of the bilayer (as assumed on Fig 1).

- The strategy of streptavidin immobilization was also revisited. We tried to put enough streptavidin molecules per cm^2 in such a way that the surface can be covered by affinity with closed-packed immobilized biotinylated proteoliposomes, but avoiding an excess which would lead to non-specific binding between streptavidin and proteins. The projection of a proteoliposome covers a significantly larger surface area than a streptavidin itself larger than the space required for the primary grafted biotin. If it is assumed that the proteoliposomes are 50 nm (in diameter), a rough calculation gives a required low grafting coverage of about $10^{-13} \text{ mol cm}^{-2}$ of biotin. NHS-*lc*-biotin was then diluted with another NHS reactant such as a NHS-PEG for the grafting step on the amine sub-layer (similar strategies for gold functionalization using mixed alkylthiolates have been already described in the framework of diagnostic technologies, see for example Nelson et al.^{liv} for a review).

The experimental conditions for assembly, especially concerning reactants concentrations, were carefully optimized with the help of SPR measurements.

Firstly, the controlled grafting of biotin and the specific recognition by streptavidin were studied in relation to the NHS-*lc*-biotin /NHS-PEG molar ratio. The SPR experiments started on the amine monolayer with the injection of, for example, mixed NHS-*lc*-biotin and NHS-PEG-333 solution into the flow cell. Figure 2 presents an example of SPR shifts for a mixed NHS reactant at 0.1 mol% of NHS-biotin. After rinsing, the response unit (RU) shift of the Biacore base line was 320 RU. This value is expected in view of the data (not shown) obtained with the pure NHS-*lc*-biotin solution (500 ± 20 RU) and for a pure NHS-PEG-333 solution (310 ± 20 RU). According to the calibration of Stenberg et al.,^{lv} 100 RU corresponds to a coverage of about 10 ng cm^{-2} . Coverages of 50 ng cm^{-2} ($\sim 10^{-10} \text{ mol cm}^{-2}$) for a monolayer of pure NHS-*lc*-biotin (MW = 557)

and 31 ng cm^{-2} ($\sim 10^{-10} \text{ mol cm}^{-2}$) for a monolayer of NHS-PEG-333 are of the order of magnitude expected. However, the true characterization of the mixed layer was performed in the next step of streptavidin recognition of immobilized biotins. For 0.1 mol% of NHS-biotin, we found (Figure 2) 95 RU that is to say $1.5 \cdot 10^{-13} \text{ mol cm}^{-2}$ of streptavidin which is of the expected order of magnitude. The last step in Figure 2 demonstrated the functionality of the immobilized streptavidins. A biotinylated cytochrome c was specifically recognized at the right level on the modified surface.

In parallel, the reliability of the streptavidin loading onto the mixed PEG/biotin surfaces was checked by successive injections of streptavidin solutions at 0.4 to 40 $\mu\text{g/mL}$. This allowed for the determination of the saturation plateaus, given the surface concentration of the immobilized protein as a function of biotin/PEG ratio (Figure 3). For a 100% biotin monolayer, we obtained a maximum streptavidin coverage of 1600-1700 RU. The surface coverage is thus about 160 ng cm^{-2} or $2.7 \cdot 10^{-12} \text{ mol cm}^{-2}$. A closed-packed (crystalline), two-dimensional monolayer of streptavidin of $5.8 \cdot 10^{-12} \text{ mol cm}^{-2}$ has been observed by atomic force microscopy.^{lvi} Our result for a maximum streptavidin coverage of 46 % of full coverage is thus consistent with the random sequential covalent immobilization of rigid particles (spheres) which cannot theoretically exceed 50.3 % of the solid surface area.^{lvii}

Finally, as our goal was to produce a reliable streptavidin coverage at the low level of 1 to $2 \cdot 10^{-13} \text{ mol cm}^{-2}$ (about 60 to 120 RU), another important optimization step was the control of non-specific binding of streptavidin (Table II). As expected, NSB are considerably decreased on oligo-PEG surfaces even at the high concentration of 40 $\mu\text{g/mL}$. For a given contact time of the streptavidin solution with the modified surfaces, the level of NSB increases with the concentration. This knowledge facilitates the choice of streptavidin concentrations convenient for

a saturation loading of surfaces at low biotin/PEG ratios and low NSB loading. For example, at the lowest biotin/PEG of 0.1 mol%, injection of a solution at 4 $\mu\text{g}/\text{mL}$ for 15 minutes saturated the biotins and routinely gave 110 ± 20 RU of streptavidin.

Non-specific protein adsorption on the functionalized PEG/streptavidin surface.

The present experiments were performed on the monolayer of PEG-333 containing 0.1 mol% of biotin and loaded with about $1.5 \cdot 10^{-13}$ mol cm^{-2} of streptavidin. For evaluation of the NSB, we chose two proteins of opposite net charges at pH 7.4, cytochrome c (MW = 14000 $\text{g}\cdot\text{mol}^{-1}$; $\text{pH}_i = 10.2$) and bovine serum albumin (BSA, MW = 67000 $\text{g}\cdot\text{mol}^{-1}$; $\text{pH}_i = 4.8$). The results (Table III), obtained with protein solutions at concentrations higher than apparent membrane protein concentrations in the proteoliposomes used in the next chapter, demonstrate a significant reduction of NSB compared to the blanks on cysteamine surfaces.

Triggered fusion of immobilized proteoliposomes on the functionalized PEG/streptavidin surface.

As the RPS technique is only sensitive to the overall amount of immobilized materials, without regard to its organization, we used FRAP measurement of the long-range lateral mobility of lipids to establish that a continuous supported membrane was formed. The lipid-enriched membranes at different lipid/protein ratios from 3 $\mu\text{mol}/\text{mg}$ to 60 $\mu\text{mol}/\text{mg}$ (as described in Table I) were loaded onto the PEG/streptavidin structure on glass surfaces according to Figure 1:

- i) 50 μL of biotinylated proteoliposomes were deposited on the modified functionalized surface (typically $1.5 \cdot 10^{-13}$ mol of streptavidin per cm^2). After one hour of contact at ambient temperature, the surface was gently rinsed, any contact with an air interface being avoided.
- ii) The sample under the buffer solution was mounted on the inverted confocal microscope where a first measurement of the fluorescent probe mobility was performed.

iii) The surface was then treated with a concentrated solution of PEG (30% w/v of PEG-8000 in Tris-buffer) for 5 minutes to trigger fusion. After careful rinsing with buffer, the second mobility measurement was performed.

The typical fluorescence photobleaching experiment summarized in Figure 4 demonstrated that triggered fusion of phospholipid-enriched membranes on the new PEG/streptavidin surface was successful. Before PEG treatment, the absence of recovery in the sequence of fluorescence images shows both that the vesicles are correctly immobilized on the tailored surface and that long-range diffusion is negligible. After triggered fusion, the long-range diffusion coefficient (D) and the mobile fraction (M) of the fluorescent lipid were measured from the recovery curves according to the theoretical framework originally developed by Axelrod et al.^{lviii} (see experimental details and calibration in a previous paper).^{xlii}

For the phospholipids-enriched proteoliposomes at lipid/protein ratios from 3 to 60 $\mu\text{mol/mg}$, we found that M , the mobile fraction, was always at least $95 \pm 5\%$, but that the diffusion coefficient was severely affected by the presence of transmembrane proteins (Figure 5). This datum consistently reveals a large decrease in the lateral mobility of lipids when the protein area fraction increases. Such a behavior has been previously described theoretically, for example, within the archipelago model,^{lix} or experimentally in biophysical studies on membranes models like proteoliposomes,^{lx, lxi} or oriented planar lipid multilayers.^{lxii}

As in the paper of Blackwell and Whitmarsh,^{lxi} for ready comparison with previously published results, we have converted the lipid/protein ratio into protein area fraction (C_P) using the following calculation:

By definition: $C_P = A_P/(A_P + A_L)$ with A_P = the apparent area of embedded protein and A_L = the apparent area of lipid. A_L is easily calculated from the lipid content if the cross-sectional area per lipid molecule is known (0.64 nm^2 for egg-PC) thus: $A_L = 0.64 N n_L/2$ for one mg of protein with

n_L the lipid to protein ratio ($\mu\text{mol}/\text{mg}$). In contrast, A_P is difficult to evaluate in our case as we did not incorporate purified membrane proteins of known cross-sectional area as was done in most of the previous studies. Our protein pool included all the different proteins of the mitochondrial inner membrane. For this reason we have based our calculation on the result of Sowers and Hackenbrock,^{lxiii} assuming a C_P of about 0.5 for the inner membrane of mammal mitochondria. As our purified fraction of the inner membrane (Table I) contains typically $0.55 \mu\text{mol}/\text{mg}$, $A_L = 1060 \text{ cm}^2$ for $0.55 \mu\text{mol}$ of lipid, 1 mg of protein represents a surface area $A_P = 1060 \text{ cm}^2$ and finally: $C_P = 1060 / (1060 + 1930 n_L)$ [1]

The mobility results are presented in the form of a relative diffusion rate: $D^* = D/D_0$ where D is measured at a given protein area fraction and D_0 is the diffusion coefficient for the tethered bilayer of pure lipid (here: $2.5 \cdot 10^{-8} \text{ cm}^2 \text{ s}^{-1}$). A comparison of the present results with values of D^* from FRAP measurement on hydrated PC multilayers (line A) and from pyrene fluorescence-quenching technique on proteoliposomes (line B) is presented in Figure 6. In both cases, purified membranes proteins were incorporated in the models, bacteriorhodopsin (MW = 260000) and cytochrome bf (MW = 130000), respectively. Apparently, in the present study, the proteins have a greater effect on D^* , at high protein area fraction, than do bacteriorhodopsin or cytochrome bf. This may reflect the fact that the protein pool obtained by dilution of the mitochondria inner membrane is quite different from the homogenous fractions used in the other experiments (here, the molecular weights of the inner membrane proteins are highly heterogeneous, roughly from 25000 for small porins to 550000 for ATP synthase and even 900000 for complex I). The theoretical models of obstructed lateral diffusion of lipids in a membrane (see Saxton for example)^{lix} predict a mobility in general slightly higher than the present experiments indicate,

even if similar dependence of D^* on the area fraction of proteins can be qualitatively found in the case of partial aggregation of the proteins.^{lix}

Another explanation of the low mobility in the tethering membrane would be that covalent links or interactions are established between the solid template and any part of the supported bilayer. Evidently, a fraction of the lipids is immobilized through the pre-organized tethering chain: DPPE-biotin-streptavidin-biotin-support (Figure 1). Several papers have recently explored the effect of tethering density on the lateral mobility of lipids,^{vii,lxiv} or peripheral proteins.^{vii,lxv} In these studies, different variants of lipopolymers were grafted as tethering moieties. At the low density used in the present study (less than 1 mol% of the lipid in the first monolayer is implicated in the tethering process) the different authors observed unrestricted motion within the bilayer. However, here, the situation is more complex as several membrane complexes of the electron chain exhibit a water-soluble domain, protuberant from the bilayer on both sides and on the same scale (at least 5 nm) as the size of the streptavidin, itself protuberant (about 5 nm) from the thin oligo-PEG sublayer (see Figure 1). If the diffusion space in two dimensions of these large proteins is reduced by an excluded volume phenomenon with the streptavidin cushions, the overall membrane mobility could be affected.

To explore this hypothesis we have studied the lateral mobility of one typical complex of the electron chain: cytochrome oxidase. A fluorescent monoclonal antibody against human cytochrome oxidase was incubated (at 1 $\mu\text{g}/\text{mL}$ for 30 minutes) on the surface of tethered membranes prepared at a lipid dilution of 14 or 30 $\mu\text{mol}/\text{mg}$. In parallel, reference samples were prepared by incubation of the same antibody solution on pure phospholipid-tethered bilayers. The fluorescent images made on the CSLM (not shown) demonstrate the specific recognition of the oxidase on the supported membranes by the labeled antibody (negligible fluorescence on blanks).

The fluorescence was quite uniform at the observed resolution (some μm). Bleached areas were produced under the same conditions as Figure 4 but the contrasts were understandably weaker as the dye concentrations were lower. Fluorescence recovery was negligible which indicated no lateral mobility of the probe. We therefore conclude that the mobility of the different elements of the diluted membrane, lipids and proteins, are certainly hindered by the tethering process.

Conclusion

In the present paper, we propose a strategy for the reconstitution by self-assembly of a tethered membrane incorporating transmembrane proteins. We have optimized the procedure in such a way as to facilitate the formation of the supported bilayer without purification of the membrane proteins. Two main problems have been overcome:

- i) Non-specific binding interactions between the water-exposed domain of the membrane proteins and the template were reduced with the help of a protective layer (oligo-PEG) grafted onto the solid surface.
- ii) As the fusion of immobilized proteoliposomes on this surface is unlikely to be spontaneous, we triggered the formation of the continuous membrane by a fusogen agent.

Long-range continuity of the membranes (hundred of microns) was affirmed by FRAP experiments after the fusion process, demonstrating a mobile fraction of the fluorescent lipid close to 100% and a lateral mobility directly related to the protein content. As the supported membrane lies on a bed of streptavidin cushions which also tether the bilayer, the overall mobility of the membrane components seems to be more affected by the protein fraction than in non-tethered model like proteoliposomes or multilayers.

Acknowledgments:

The supports of this research by the CNRS through an ACI grant N° A2N062 and by La Région Picardie through a grant from the "pôle IBFBio" are both gratefully acknowledged.

Table I : Lipid enrichment of inner membrane of liver mitochondria: typical purification batch starting from the equivalent of $\sim 300 \text{ cm}^2$ of culture of HepG2 cells in flasks.

Purification step	protein amount	lipid/protein ratio	cytochrome bc ₁ activity
		□ $\mu\text{mol lipid/ mg protein}$)	□ $\mu\text{mol cyt c / min / mg}$)
HepG2 cells	15 to 25 mg	–	–
purified mitochondria	1.5 to 2.5 mg	0.2 to 0.25	0.6
purified inner membranes	0.12 to 0.16 mg	0.5 to 0.6	1.2
lipid-enriched proteoliposomes	$\sim 50 \mu\text{g}$	3 to 60	1.0

Table II : RPS evaluation of specific (on biotin) and non-specific binding of streptavidin on various modified surfaces. In all cases, the assemblies started from a monolayer of cysteamine on gold. PEG-333 is a methoxy-terminated very short oligo-PEG of only three monomers.

functionalized surfaces	ratio biotin/PEG (mol %)	streptavidin concentration ($\mu\text{g/mL}$)	SPR shift (RU)
unmodified cysteamine	-	0.4	210 ^a
		4	950 ^a
		40	1600 ^a
NHS-PEG-333	0 %	0.4	~ 30 ^a
		4	50 ^a
		40	180 ^a
NHS-PEG-333 + NHS-1c-biotin	0.1 %	4	120 ^b
	0.6 %	4	310 ^b
	2.1 %	40	820 ^b
	8.5 %	40	1410 ^b
	100 %	40	1600 ^b

^a after injection of $80 \mu\text{L}$ of the streptavidin solution at a flow rate of $5 \mu\text{L min}^{-1}$.

^b at the saturation plateau

Table III : RPS evaluation of non-specific binding of proteins on PEG/streptavidin functionalized surfaces. NHS-Ic-biotin / NHS-PEG-333 at 0.1 mol%. Loaded streptavidin : 90 RU or $1.5 \cdot 10^{-13} \text{ mol cm}^{-2}$.

Protein	non-specific binding on cysteamine surface	non-specific binding on PEG/streptavidin surface
cytochrome c at 100 $\mu\text{g/mL}$	1100 RU	50 RU
BSA at 200 $\mu\text{g/mL}$	2300 RU	210 RU

Figure captions:

Figure 1: Schematic view of the step-by-step assembly for the supported membrane. Whatever the solid support, glass or gold, the process starts from the same amine monolayer.

Note that the formation of the continuous bilayer is triggered by a concentrated solution of polyethylene glycol (PEG, MW = 8000) at the last step.

Figure 2: Experimental trace of the SPR response given by the Biacore apparatus on a gold surface modified with cysteamine. First step: grafting of NHS-Ic-biotin (0.1 mol% in NHS-PEG-333). After exchange with a PBS buffer, loading of streptavidin (0.5 $\mu\text{g/mL}$ in PBS buffer) at a final level of 95 RU ($\sim 1.5 \cdot 10^{-13} \text{ mol cm}^{-2}$). The last step was to check the functionality of the immobilized streptavidin with a biotinylated cytochrome c. NSB of native cytochrome c on the same surface at the same concentration gave 20 RU (not shown) . Thus $45 - 20 = 25$ RU of specifically immobilized cytochrome c correspond to about $1.7 \cdot 10^{-13} \text{ mol cm}^{-2}$, the same order of magnitude as the streptavidin coverage.

Figure 3: Streptavidin saturation of the biotinylated surfaces at different NHS-Ic-biotin/NHS-PEG-333 ratios. Streptavidin solutions vary from 0.4 to 40 $\mu\text{g/mL}$ in PBS buffer pH 7.4. The biotin/PEG ratio indicated is the molar fraction of the solutions of mixed NHS reactants used for grafting. The SPR data are not corrected for NSB.

Figure 4: Fluorescence photobleaching experiments demonstrating the triggered fusion of immobilized proteoliposomes in the formation of a supported membrane.

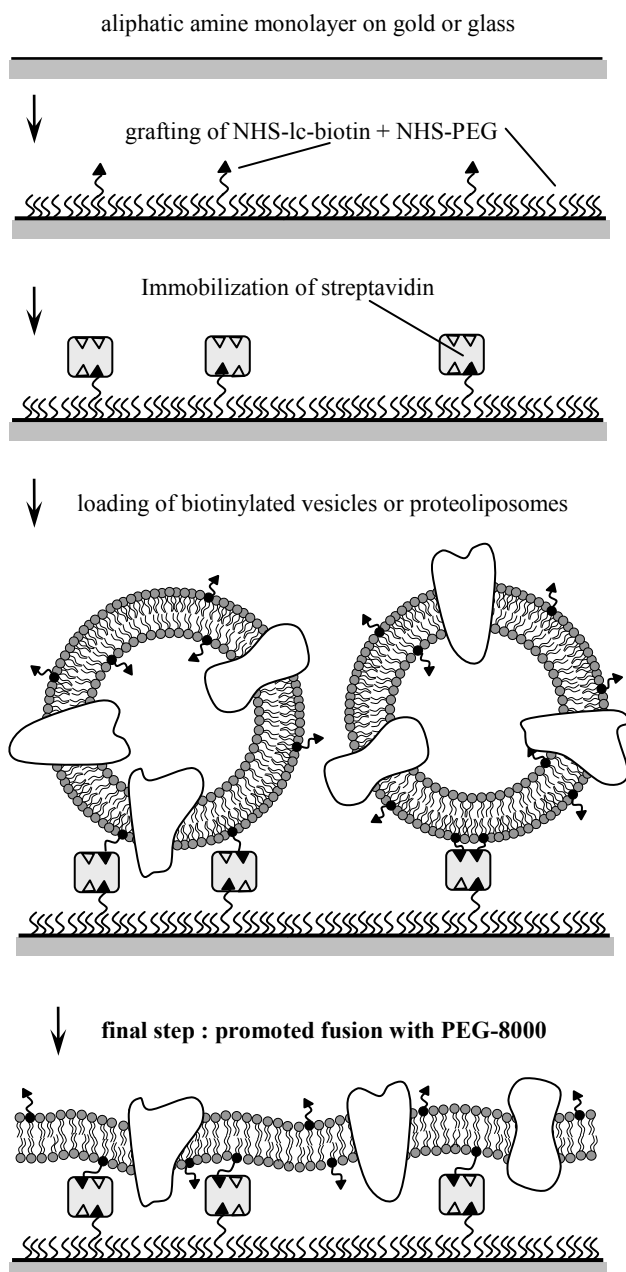
A: sequence of scanning confocal images recorded on the PEG-streptavidin layer loaded with proteoliposomes (0.1 mM of lipid) at the dilution of 14 $\mu\text{mol lipid/mg protein}$.

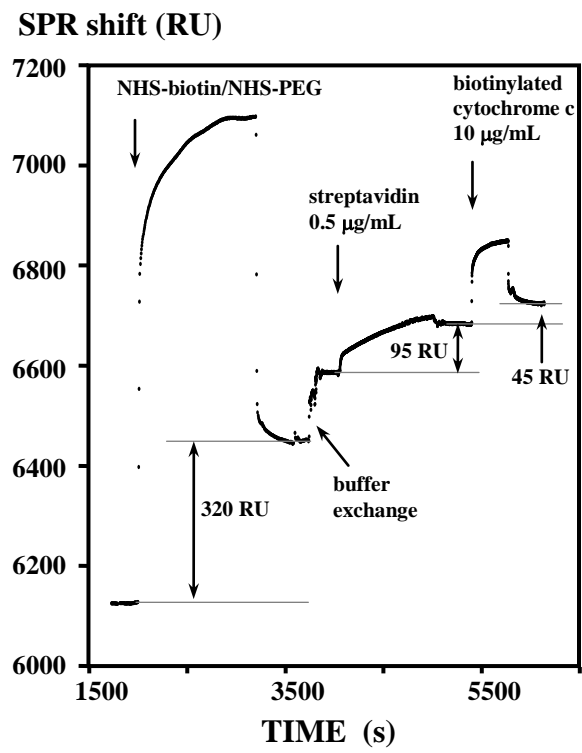
B: the same FRAP experiment after triggered fusion by a PEG treatment (same result with a freeze-thaw step). $D = 1.7 \pm 0.3 \cdot 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ and $M = 95 \pm 5 \%$ at 22 $^{\circ}\text{C}$.

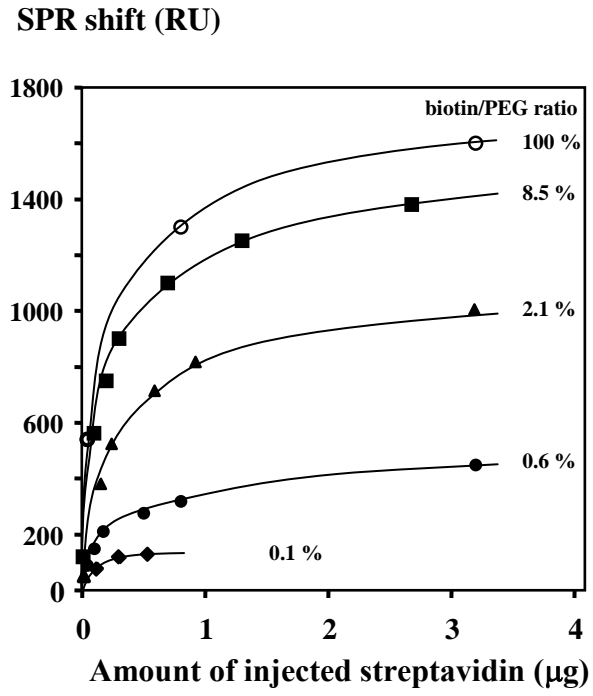
The fluorescent probe was NBD-DPPE (1 mol%). Images: 310x310 μm .

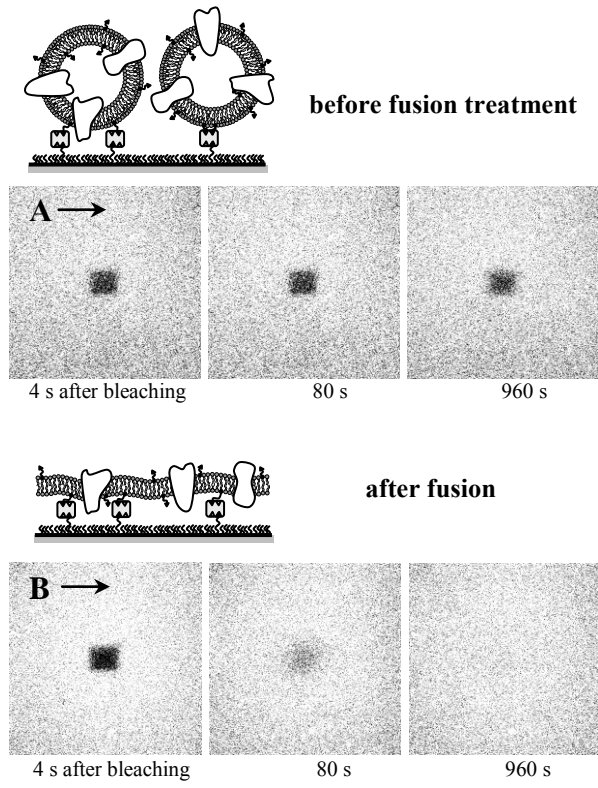
Figure 5: Tethered bilayers made from phospholipid-enriched inner membrane of mitochondria by the multi-step process of Figure 1: diffusion coefficient of NBD-DPPE (1 mol%) in the bilayer at various lipid/protein ratio. D from fitting of the fluorescence recovery curves and the lipid mobile fraction was at least 95 %. Proteoliposomes from three cell culture batches. FRAP measurements at ambient temperature : $22 \pm 1^{\circ}\text{C}$. Relative error on D : $\pm 15\%$.

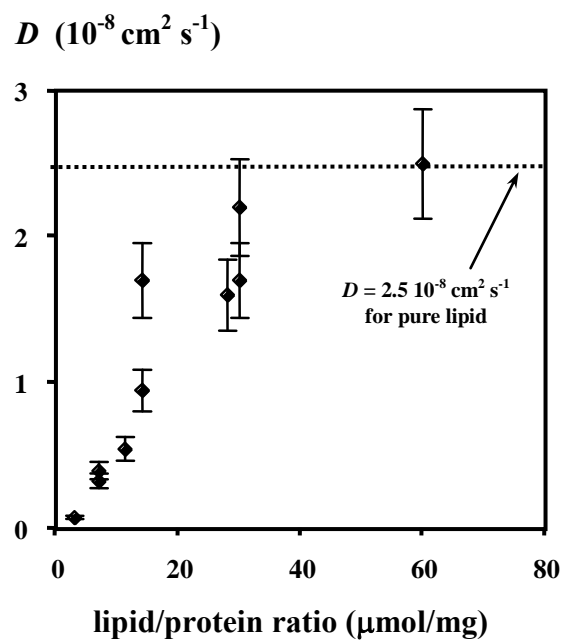
Figure 6: Relative diffusion rate from data of Figure 5 (\blacklozenge) presented as a function of the protein area fraction (C_P) in the tethered membrane. C_P was calculated from equation [1]. Line A is the best fit for the experimental data from Schram et al.^{lxii} on bacteriorhodopsin in egg-PC multilayers. Line B from the experimental data of Blackwell and Whitmarsh,^{lxi} for cytochrome bf incorporated in soybean-PC liposomes.

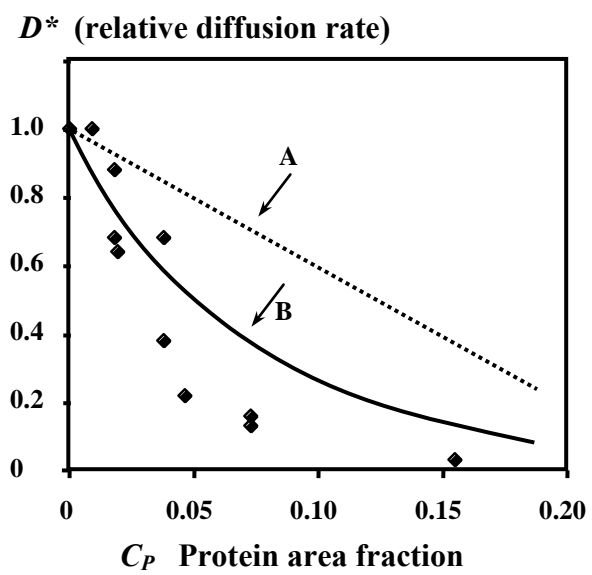
Elie-Caille *et al.* Figure 1

Elie-Caille *et al.* Figure 2

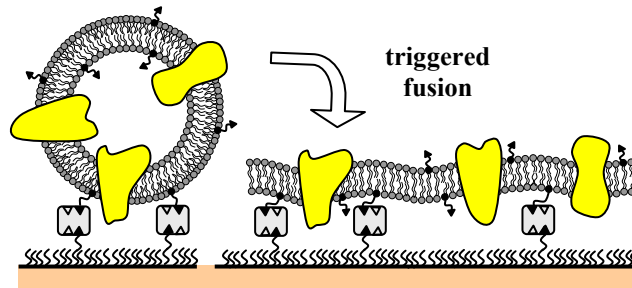
Elie-Caille *et al.* Figure 3

Elie-Caille *et al.* Figure 4

Elie-Caille *et al.* Figure 5

Elie-Caille *et al.* Figure 6

Elie-Caille *et al.* TOC Image



References:

- (i) This paper is dedicated to the memory of Professors Jacques Moiroux and Jean-Marc Laval, without whose skills and enthusiasm this project would never have got off the ground.
- (ii) Tamm, L.K.; McConnell, H.M. *Biophys. J.* **1985**, *47*, 105-113.
- (iii) Sackmann, E. *Science*, **1996**, *271*, 43-48.
- (iv) Boxer, S.G. *Curr. Op. Chem. Biol.* **2000**, *4*, 704-709.
- (v) Sinner, E-K.; Knoll, W. *Curr. Op. Chem. Biol.* **2001**, *5*, 705-711.
- (vi) Richter, R.P.; Lai Kee Him, J.; Brisson, A. *Materials today*, **2003**, *6*, 32-37.
- (7) Wagner, M.L.; Tamm, L.K. *Biophys. J.* **2000**, *79*, 1400-1414.
- (viii) Zhao, J.; Tamm, L.K. *Langmuir*, **2003**, *19*, 1838-1846.
- (ix) Kiessling, V.; Tamm, L.K. *Biophys. J.* **2003**, *84*, 408-418.
- (x) Sackmann, E.; Tanaka, M. *TIBTECH*, **2000**, *18*, 58-64.
- (xi) Heyse, S.; Ernst, O.P.; Dienes, Z.; Hofmann, K.P.; Vogel, H. *Biochemistry*, **1998**, *37*, 507-522.
- (xii) Puu, G.; Gustafson, I.; Artursson, E.; Ohlsson, P.A. *Biosens. Bioelectr.* **1995**, *10*, 463-476.
- (xiii) Morigaki, K.; Baumgart, T.; Jonas, U.; Offenhausser, A.; Knoll, W. *Langmuir*, **2002**, *18*, 4082-4089.
- (xiv) Spinke, J.; Yang, J.; Wolf, H.; Liley, M.; Ringsdorf, H.; Knoll, W. *Biophys. J.* **1992**, *63*, 1667-1671.
- (xv) Lang, H.; Duschl, C.; Vogel, H. *Langmuir*, **1994**, *10*, 197-210.

-
- (xvi) Naumann, R.; Schmidt, E.K.; Jonczyk, A.; Fendler, K.; Kadenbach, B.; Liebermann, T.; Offenhausser, A.; Knoll, W. *Biosens. Bioelectr.* **1999**, *14*, 651-662.
- (xvii) Cornell, B.A.; Braach-Maksvytis, V.L.B.; King, L.G.; Osman, P.D.J.; Raguse, B.; Wieczorek, L.; Pace, R.J. *Nature*, **1997**, *387*, 580-583.
- (xviii) Terrettaz, S.; Mayer, M.; Vogel, H. *Langmuir*, **2003**, *19*, 5567-5569.
- (xix) Wong, J.Y.; Majewski, J.; Seitz, M.; Park, C.K.; Israelachvili, J.N.; Smith, G.S. *Biophys. J.* **1999**, *77*, 1445-1457.
- (xx) Bieri, C.; Ernst, O.P.; Heyse, S.; Hofmann, K.P.; Vogel, H. *Nature Biotechnol.* **1999**, *17*, 1105-1108.
- (xxi) Radler, U.; Mack, J.; Persike, N.; Jung, G.; Tampé, R. *Biophys. J.* **2000**, *79*, 3144-3152.
- (xxii) Fisher, M.I.; Tjarnhage, T. *Biosensors Bioelec.* **2000**, *15*, 463-471.
- (xxiii) Brian, A.A.; McConnell, H.M. *Proc. Natl. Acad. Sci. USA.* **1984**, *81*, 6159-6163.
- (xxiv) Burgess, J.D.; Rhoten, M.C.; Hawkridge, F.M. *Langmuir*, **1998**, *14*, 2467-2475.
- (xxv) Salamon, Z.; Hazzard, J.T.; Tollin, G. *Proc. Natl. Acad. Sci. USA.* **1993**, *90*, 6420-6423.
- (xxvi) Salamon, Z.; Wang, Y.; Soulages, J.L.; Brown, M.F.; Tollin, G. *Biophys. J.* **1996**, *71*, 283-294.
- (xxvii) Naumann, R.; Jonczyk, A.; Kopp, R.; Van Esch, J.; Ringsdorf, H.; Knoll, W.; Graber, P. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2056-2058.
- (xxviii) Lindholm-Sethson, B.; Gonzalez, J.C.; Puu, G. *Langmuir*, **1998**, *14*, 6705-6708.
- (xxix) Goennenwein, S.; Tanaka, M.; Hu, B.; Moroder, L.; Sackmann, E. *Biophys. J.* **2003**, *85*, 646-655.
- (xxx) Salafsky, J.; Groves, J.T.; Boxer, S.G. *Biochemistry*, **1996**, *35*, 14773-14781.

-
- (xxxii) Sevin-Landais, A.; Rigler, P.; Tzartos, S.; Hucho, F.; Hovius, R.; Vogel, H. *Biophys. Chem.* **2000**, *85*, 141-152.
- (xxxiii) Graneli, A.; Rydstrom, J.; Kasemo, B.; Hook, F. *Langmuir*, **2003**, *19*, 842-850.
- (xxxiiii) Marchal, D.; Boireau, W.; Laval, J-M.; Moiroux, J.; Bourdillon, C. *Biophys. J.* **1998**, *74*, 1937-1948.
- (xxxv) Marchal, D.; Pantigny, J.; Laval, J-M.; Moiroux, J.; Bourdillon, C. *Biochemistry*, **2001**, *40*, 1248-1256.
- (xxxvi) Schneider, H.; Lemasters, J.J.; Hochli, M.; Hackenbrock, C.R. *Proc. Natl. Acad. Sci. USA.* **1980**, *77*, 442-446.
- (xxxvii) Schneider, H.; Lemasters, J.J.; Hochli, M.; Hackenbrock, C.R. *J. Biol. Chem.* **1980**, *255*, 3748-3756.
- (xxxviii) Jung, L.S.; Shumaker-Parry, J.S.; Campbell, C.T.; Yee, S.S.; Gelb, M.H. *J. Am. Chem. Soc.* **2000**, *122*, 4177-4184.
- (xxxix) Keller, C.A.; Kasemo, B. *Biophys. J.* **1998**, *75*, 1397-1402.
- (xl) Seitz, M.; Ter-Ovanesyan, E.; Hausch, M.; Park, C.K.; Zasadzinski, J.A.; Zentel, R.; Israelachvili, J.N. *Langmuir*, **2000**, *16*, 6067-6070.
- (xli) Proux-Delrouyre, V.; Laval, J-M.; Bourdillon, C. *J. Am. Chem. Soc.* **2001**, *123*, 9176-9177.
- (xlii) Proux-Delrouyre, V.; Elie, C.; Laval, J-M.; Moiroux, J.; Bourdillon, C. *Langmuir*, **2002**, *18*, 3263-3272.
- (xliii) Berquand, A.; Mazeran, P-E.; Pantigny, J.; Proux-Delrouyre, V.; Laval, J-M.; Bourdillon, C. *Langmuir*, **2003**, *19*, 1700-1707.
- (xliv) Rickwood, D.; Wilson, T.M.; Darley-Usmar, V.M. *In Mitochondria: a practical approach*, V.M. Darley-Usmar, D. Rickwood and T.M. Wilson (Eds), IRL Press, Oxford, 1986, p6.

-
- (xlv) Velours, J.; Guerin, B.; Duvert, M. *Arch. Biochem. Biophys.* **1977**, *182*, 295-304.
- (xlv) Schatz, G.; Racker, E. *Biochem. Biophys. Res. Comm.* **1966**, *22*, 579-584.
- (xlvi) Rieske, J.S. in *Methods in Enzymology*, Vol X, R.W. Eastbrook, M.E. Pullman (Eds) Academic Press, New York, 1967, p239.
- (xlvii) Aden, D.P.; Fogel, A.; Plotkin, S.; Damjanov, I.; Knowles, B.B. *Nature*, **1979**, *282*, 615-616.
- (xlviii) Gennis, R.B. *Biomembranes, Molecular Structure and Function*; Springer-Verlag: New-York, 1989.
- (xlix) Casadio, R.; Venturoli, G.; Di Gioia, A.; Castellani, P.; Leonardi, L.; Lelandri, B.A. *J. Biol. Chem.* **1984**, *259*, 9149-9157.
- (l) Millner, P.A.; Grouzis, J.P.; Chapman, D.J.; Barber, J. *Biochim. Biophys. Acta*, **1983**, *722*, 331-340.
- (li) Daum, G.; Vance, J.E. *Prog. Lipid Res.* **1997**, *36*, 103-130.
- (lii) Haque, M.E.; McIntosh, T.J.; Lentz, B.R. *Biochemistry*, **2001**, *40*, 4340-4348.
- (liii) For example: Prime, K.L.; Whitesides, G.M. *J. Am. Chem. Soc.* **1993**, *115*, 10714-10721.
- (liv) Nelson, K.E.; Gamble, L.; Jung, L.S.; Boeckl, M.S.; Naeemi, E.; Golledge, S.L.; Sasaki, T.; Castner, D.G.; Campbell, C.T.; Stayton, P.S. *Langmuir*, **2001**, *17*, 2807-2816.
- (lv) Stenberg, E.; Persson, B.; Roos, H.; Urbaniczky, C. *J. Colloid Interface Sci.* **1991**, *143*, 513-526.
- (lvi) Reviakine, I.; Brisson, A. *Langmuir*, **2001**, *17*, 8293-8299.
- (lvii) Finegold, L.; Donnell, J.T. *Nature*, **1979**, *278*, 443-445.
- (lviii) Axelrod, D.; Koppel, D.E.; Schlessinger, J.; Elson, E.; Webb, W.W. *Biophys. J.* **1976**, *16*, 1055-1069

-
- (lix) For example: Saxton, M.J. *Biophys. J.* **1993**, *64*, 1053-1062.
- (lx) Chazotte, B.; Wu, E.S.; Hackenbrock, C.R. *Biochem. Soc. Trans.* **1984**, *12*, 463-464.
- (lxi) Blackwell, M.F.; Whitmarsh, J. *Biophys. J.* **1990**, *58*, 1259-1271.
- (lxii) Schram, V.; Tocanne, J-F.; Lopez, A. *Eur. Biophys. J.* **1994**, *23*, 337-348.
- (lxiii) Sowers, A.E.; Hackenbrock, C.R. *Proc. Natl. Acad. Sci. USA.* **1981**, *78*, 6246-6250.
- (lxiv) Naumann, C.A.; Prucker, O.; Lehmann, T.; Ruhe, J.; Knoll, W.; Frank, C.W.
Biomacromolecules, **2002**, *3*, 27-35.
- (lxv) Purrucker, O.; Fortig, A.; Jordan, R.; Tanaka, M. *Chem.Phys.Chem.* **2004**, *5*, 327-335.