FEBS Letters 555 (2003) 160–169 FEBS 27739

# Membrane protein reconstitution and crystallization by controlled dilution

H.-W. Rémigy[a], D. Caujolle-Bert[b], K. Suda[a], A. Schenk[a], M. Chami[a], A. Engel[a,*]

[a] *M.E. Müller Institute for Microscopy, Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland*
[b] *1 Rue Henri Coeur, 38420 Domène, France*

Received 25 August 2003; accepted 1 September 2003

First published online 8 October 2003

Edited by Gunnar von Heijne, Jan Rydström and Peter Brzezinski

**Abstract Efficient reconstitution of membrane proteins for functional analyses can be achieved by dilution of a ternary mixture containing proteins, lipids and detergents. Once the dilution reaches the point where the free detergent concentration would become lower than the critical micellar concentration, detergent is recruited from the bound detergent pool, and association of proteins and lipids is initiated. Here we show that dilution is also suitable for the assembly of two-dimensional crystals. A device has been designed that allows controlled dilution of a protein–lipid–detergent mixture to induce formation of densely packed or crystalline proteoliposomes. Turbidity is used to monitor the progress of reconstitution on-line, while dilution is achieved by computer-controlled addition of buffer solution in sub-microliter steps. This system has mainly been tested with porin OmpF, a typical β-barrel protein, and aquaporin-1, a typical α-helical protein. The results demonstrate that large, highly ordered two-dimensional crystals can be produced by the dilution method.**
**© 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.**

*Key words:* Two-dimensional crystallization; Lipid–detergent interaction; Membrane protein solubilization; Reconstitution

## 1. Introduction

A major challenge in the structural analysis of membrane proteins is their crystallization. Three-dimensional (3D) crystallization requires the membrane protein to be solubilized and essentially devoid of lipids. To assemble two-dimensional (2D) crystals, the isolated, detergent-solubilized membrane protein is reconstituted in the presence of lipids, which restores its native environment. Besides the fact that 2D crystals can be prepared from small amounts of proteins, their major advantage is the functional reconstitution of the membrane protein: both structure and function can be assessed within the same protein–lipid assembly [1]. The principle of reconstitution, i.e. the detergent removal-driven assembly of lipids and proteins, is simple, yet the specific conditions working for one membrane protein cannot usually be applied to another. In addition, reconstitution performed at a low lipid-to-protein ratio (LPR) to enforce regular packing of the protein enhances protein aggregation. The prospect of improving 2D crystallization in the presence of lipids provides a strong motivation to further investigate the reconstitution process. The characterization of the micelle–vesicle transition [2–5] has led to an increased understanding of the binary detergent–lipid systems and has allowed relevant experimental variables for protein reconstitution to be defined [6–8]. A similar effort is now required for ternary mixtures of lipid, detergent and protein.

For detergent-mediated reconstitution, the detergent-solubilized protein is mixed with lipids also solubilized with detergent to form a homogeneous solution of mixed protein–detergent and lipid–detergent micelles. Detergent removal then results in the formation of protein aggregates in the worst case, and in the progressive formation of proteoliposomes with large 2D crystalline regions in the best case. Dynamic light scattering allows this process to be monitored [9].

The reconstitution events are dictated by the respective affinities between the components of the ternary mixture. If the detergent binds more strongly to the protein than to the lipids, pure lipid vesicles assemble upon detergent removal, while the protein remains in its soluble form. Further detergent removal will precipitate the protein unless it is able to integrate into preformed vesicles. The protein will also aggregate if it releases the detergent more readily than the lipid does. Ideally, a starting condition should be established where mixed detergent–protein and mixed detergent–lipid micelles have exchanged constituents to the extent that the mixture consists mainly of ternary detergent–protein–lipid micelles. Assuming that the protein remains in its native, properly folded state during the solubilization and isolation steps, this ideal situation is likely to foster perfect reconstitution and possibly 2D crystallization.

The pertinent interactions depend on the shape and surface charges of the components. For a given protein, the lipid–detergent mixture, pH, counterions and temperature must all be optimized. In addition, the concentration, the ratio of the components and the detergent removal rate are critical. This gives a multidimensional parameter space that needs to be experimentally sampled, a similar task to that carried out

*Corresponding author. Fax: (41)-61-2672109.
*E-mail address:* andreas.engel@unibas.ch (A. Engel).

*Abbreviations:* $C_{12}E_8$, *n*-dodecyl-octaethyleneglycol ether; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio] propanesulfonic acid; CMC, critical micellar concentration; DM, *n*-decyl-β-D-maltopyranoside; DDM, *n*-dodecyl-β-D-maltopyranoside; DMPC, dimyristoyl-glycerophosphatidylcholine; DOPE, dioleoyl-glycerophosphatidyl-ethanolamine; DOPG, dioleoyl-glycerophosphoglycerol; LPR, lipid-to-protein ratio; octyl-POE, octyl-polyoxyethylene; OG, *n*-octyl-β-D-glucopyranoside; OTG, *n*-octyl-β-D-thioglucopyranoside; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TX100, Triton X-100

0014-5793/03/$22.00 © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/S0014-5793(03)01105-0

in 3D crystallization screens. The difficulty of such experiments is the management of the screens and the assessment of results. With 2D crystallization the latter is particularly cumbersome because 2D crystals cannot be detected by light microscopy and screening by electron microscopy is time-consuming.

Previous work on the reconstitution process has mainly concentrated on the lipid–detergent interaction. Membrane solubilization has been described as a two-step process involving bilayer destabilization and lipid solubilization [2,6]. Conversely, membrane reconstitution involves the transition from mixed micelles to an intermediate state characterized by strongly light scattering filamentous structures that exist as a 'gel-like' network, and the subsequent transition into vesicles [5,6,10]. Turbidimetry [11,12], dynamic light scattering [3,10], neutron scattering [5] and cryo-electron microscopy [4,13] have been used to characterize this process. An elegant study has classified several detergents commonly used for protein crystallization according to their membrane disruption potency using isothermal titration calorimetry [14]. Although a wealth of experimental data on 2D crystallization is available and has been summarized in pertinent reviews [6–8,15–17], neither the transition of ternary micelles into proteoliposomes or regular structures during detergent removal nor the spontaneous integration of solubilized membrane proteins into lipid bilayers is well understood.

Generally used methods to bring the detergent concentration below the critical micellar concentration (CMC) include dialysis [10], adsorption of the detergent to Biobeads [18], and dilution of the ternary mixture [11,19]. Most crystallization attempts have been carried out in bulk solution [6–8,17], but some involve interfaces [20–24]. Among the latter type the monolayer crystallization technique is the most successful [23,24]. Nevertheless, the crystals suitable for atomic resolution structure determination have so far been produced in bulk solution by the dialysis method [25,26].

The difficulties in controlling detergent removal by dialysis or adsorption to Biobeads with sufficient accuracy have led to preliminary experiments aimed at inducing 2D crystallization by dilution [9]. To facilitate such dilution experiments and achieve better reproducibility, we built a dilution machine, which allows the transition of solubilized components into aggregates during controlled dilution to be monitored by turbidimetry. Since reproducible starting conditions can only be achieved when the concentrations of all constituents in the dilution mixture are known, we measured the detergent concentration by shape analysis of sitting drops. Here we present the features of the dilution machine, experimental results on lipid solubilization that can easily be compared with published data, and a set of 2D crystallization experiments.

## 2. Materials and methods

### 2.1. The dilution cell with optical detector

The cuvette is made of Teflon and has a volume of 450 μl (Fig. 1a). Two transparent plastic coverslips (CrystalClene, Molecular Dimensions, Florida, USA) are used to close this V-shaped cell, allowing a 670 nm laser beam to traverse the solution (Imatronic, Berkshire, UK). The chamber is temperature-regulated by a sensor and a Peltier element (Peltron, Fürth, Germany) working in a feedback loop controlled by a microcontroller linked to a computer (see below). The dilution buffer flow is set by the opening rate of a micro-valve (Lee, Westbrook, USA) combined with a home-built syringe pump controlled by a computer (see below). Each opening/closing cycle of the solenoid can last from 40 ms to several seconds. The buffer volume injected during each opening of the solenoid ranges from 0.05 to 1.5 μl, depending on the syringe. The minimum volume for an experiment is 15 μl. This is required to enable the laser beam to illuminate the reaction volume without interference with the liquid surface. Also it permits a paddle to stir the mixture. The light sensor is a photodiode (Centronic, Croydon, UK) equipped with a small axial tube mounted at the center of the diode to absorb the unscattered light. The collected scattered light is a direct measure of the structures assembled.

### 2.2. Control unit

The eight channels of the dilution machine are controlled by a Motorola 68000 microcontroller. The state of the sensors is recorded every 20 μs, and integrated by the embedded software of the microcontroller. From time to time, the computer retrieves these data and also sends the commands and parameters to the microcontroller that ensures the temperature changes or dilutions to be made at the required rates and final volume. A Linux-based ix86 PC is used to hold the Graphical User Interface (GUI, Fig. 1b), and to print the final dilution/scattering curves. All the software modules needed to run the dilution machine were assembled into a package called GISK, available at www.sourceforge.net. GISK is under the GNU general public license and is entirely written in C. This interface allows all parameters needed to manage a dilution experiment to be set and the system to be calibrated. A flow calibration procedure is required for each channel. After calibration, the initial sample volume, the final dilution, the pause between valve openings and the temperature are set for each channel. Two optional fields can be activated. They change the rate of dilution or the temperature according to a defined scattering threshold or a preset dilution value. These parameters are entered for each of the eight channels. At the bottom of the GUI, eight buttons activate or inactivate each channel. The period over which the computer has to integrate the signal, i.e. the degree of smoothing, can be set (typically 30 s). Extra features permit the settings of the communication port (in case more than eight channels are used), the rate of the data flow, the printers, the debugging level and the automatic resetting of the microcontroller to be defined. The software is multithreaded so that each channel is controlled separately in order to have not only experimental flexibility but also the most accurate dilution rate and reproducibility. The data, additional comments and the settings are saved in a default (based on the current date) or a defined file. This file can be automatically printed, showing the final dilution curves. During the experiment a table shows the current light scattering values and the remaining time.

### 2.3. Electron microscopy

Reconstituted proteoliposomes were absorbed to carbon films rendered hydrophilic by glow discharge in air. The grids were washed twice with distilled water and stained with saturated uranyl formate. Micrographs were taken on a Hitachi H-7000 transmission electron microscope operated at 100 kV and a magnification of 50 000× or 5000×. Well-ordered crystals were adsorbed to carbon film, quick-frozen in a thin buffer layer or embedded in trehalose [27] and recorded at liquid nitrogen temperature and low dose in a Philips CM200-FEG transmission electron microscope operated at 200 kV. Suitable high-resolution micrographs were scanned at 50 or 100 lines/mm (corresponding to a pixel size of 0.4 or 0.2 nm) using a Primescan 7100 (Heidelberg Druckmaschinen, Heidelberg, Germany).

### 2.4. DMPC solubilization

To evaluate the lipid solubilization efficiency of several common detergents, a 10 mg/ml DMPC solution was diluted by OG, DM, DDM, TX100, OTG or CHAPS (see Abbreviations) solutions. DMPC was purchased from Avanti Polar Lipids (Alabama, USA). OG, OTG, DM and DDM were purchased from Anatrace (Ohio, USA), TX100 from Sigma-Aldrich (Missouri, USA) and CHAPS from Dojindo Molecular Technology (Maryland, USA). Detergent solutions were made using a 20 mM Tris–HCl (pH 7.5) buffer, with a final detergent concentration of 10 mg/ml for OG, OTG, DM and CHAPS and 5 mg/ml for TX100 and DDM. DMPC in chloroform was first dried using an argon or nitrogen stream and kept in a desiccator overnight to eliminate residual chloroform. The dried lipids were weighed and diluted with buffer to a final concentration of 10 mg/ml (typical volume 3 ml). To obtain homogeneous small unilamellar vesicles the lipid solution was sonicated using a Branson sonifier 250 tip for 3 min at 20% of the maximum output power. Initial lipid

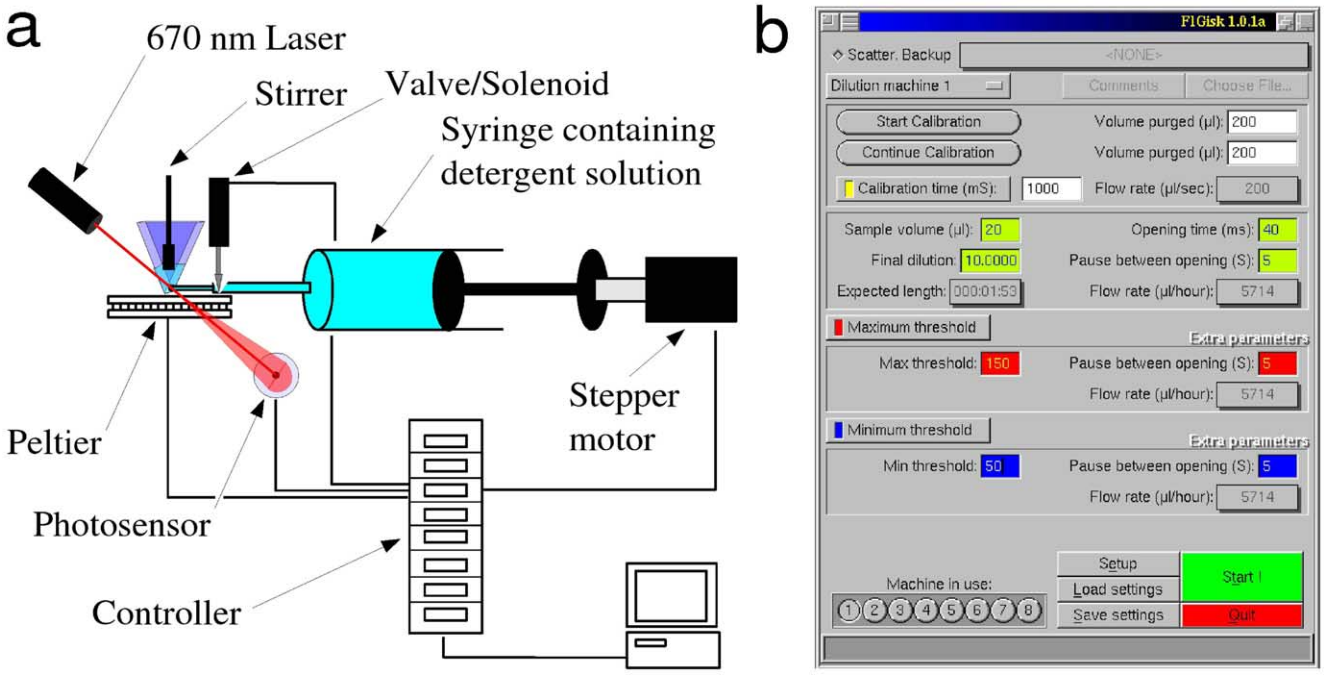


Fig. 1. Dilution machine. a: The dilution buffer (with or without detergent) is supplied to the triangular cuvette by a microsyringe that is driven by a screw mounted on the stepper motor. The valve prevents diffusion between cuvette and reservoir. Proteoliposome assembly is monitored using a photodiode that collects the scattered laser light. A Peltier element controls the temperature of the cuvette. b: A graphical user interface allows all pertinent parameters of the eight channels to be individually set and monitored during a dilution run. Final results are automatically stored in a protocol and printed.

volumes loaded to the cuvettes were between 20 and 60 µl, and lipid concentrations started at 1, 2, 3, 4, 5, 6, 7 and 8 mg/ml. Experiments were made at room temperature and lasted from 30 min to 5 h. During dilution, a constant mixing was applied (about 300 rpm). The rate of the volume increase was constant and the dilutions were varied between 1.5- and 5-fold, depending on detergent solubilization capabilities. To obtain statistically relevant data, dilution series with the same detergent but different lipid concentrations were carried out in parallel and multiple times using the eight channels of the dilution machine.

### 2.5. OmpF reconstitution

*2.5.1. OmpF in octyl-POE.* OmpF was produced with the *Escherichia coli* strain BZB1110/pMY222 and purified as reported previously [28]. DMPC was prepared as described above, and entirely solubilized (10 mg/ml DMPC, 10 mg/ml octyl-POE). The protein was also solubilized in 10 mg/ml octyl-POE. In the ternary protein–lipid–detergent mixture, the initial OmpF concentration ranged from 1 to 4 mg/ml, LPR from 0.2 to 5 (0.2, 0.5, 1, 1.5, 2, 3, 4 and 5) and the octyl-POE concentration was 10 mg/ml at the beginning of the dilution experiment. The buffer used for the dilution contained 20 mM HEPES–NaOH (pH 7), 100 mM NaCl, 10 mM $MgCl_2$, 3 mM $NaN_3$. An initial volume of 20 µl was diluted 12–15-fold at room temperature, varying the total dilution time from 15 min to 24 h.

*2.5.2. OmpF in DDM.* To test the dilution machine's ability to handle 2D crystallization with low-CMC detergents, DDM was added to OmpF solubilized in 10 mg/ml octyl-POE. The solution was then dialyzed for 20 h against the same buffer previously used for dilution (20 mM HEPES–NaOH (pH 7), 100 mM NaCl, 10 mM $MgCl_2$, 3 mM $NaN_3$) to eliminate octyl-POE. Analysis of the shape and dimensions of a sitting drop showed the DDM concentration to be 0.35 mg/ml after dialysis. DMPC solubilized in 10 mg/ml octyl-POE was then added to reach an LPR of 2. 20 µl of this ternary mixture was diluted 13-fold at room temperature over 2 h.

Experiments were carried out three times to ensure reproducibility. The resulting crystals were first inspected by negative stain electron microscopy and eventually subjected to phospholipase A2 (Sigma) treatment [29].

### 2.6. Photosystem II reconstitution

Photosystem II (PSII) was purified from spinach membranes as described [30] and solubilized in 5 mg/ml OTG. Because the preparation contained endogenous lipids, no extra lipids were added. The dilution buffer was 40 mM MES–NaOH (pH 6.5), 20 mM NaCl, 1 mM $CaCl_2$, 1 mM Zn acetate, 1 mM Na ascorbate, 3 mM $NaN_3$. A 20 µl aliquot of a 1.3 mg/ml PSII solution was diluted 15-fold over different time ranges (10, 20, 40, 60 and 120 min, and 6, 12 and 24 h). Experiments were performed at room temperature with constant stirring (around 300 rpm). Negatively stained samples were prepared at the end of each experiment to characterize the resulting structures by electron microscopy.

### 2.7. Reconstitution of E. coli translocase

SecYEG was prepared according to [31], using 0.3 mg/ml DDM to keep the purified protein in solution at a concentration of 1 mg/ml. Different lipid mixtures were explored, which were prepared at a concentration of 10 mg/ml as described above. The lipids were solubilized in 10, 20 or 40 mg/ml OG. Protein and lipid solutions were mixed to reach an LPR between 0.2 and 1. A 13-fold dilution was achieved at room temperature and over a time of 2 h. Dilution was made using a 50 mM Tris–HCl (pH 8) buffer containing 50 mM KCl and 3 mM $NaN_3$. Reconstitution experiments were also performed under similar conditions using dialysis to remove the detergent over time periods of weeks to several months. In addition, Biobeads were used to absorb DDM from identical mixtures.

### 2.8. Aquaporin-1 (AQP1) reconstitution

AQP1 was extracted from human erythrocytes using OG and purified as described previously [32]. *E. coli* lipids were prepared as described above, and entirely solubilized in OG (10 mg/ml *E. coli* lipids, 10 mg/ml OG). In the ternary protein–lipid–detergent mixture, the initial AQP1 concentration was 1 or 2 mg/ml, the LPR was varied from 0.3 to 2 (0.3, 0.5, 0.6, 1, 1.5, 2) and the OG concentration was 10 mg/ml at the beginning of the dilution experiment. The buffer used for the dilution contained 20 mM MES–NaOH (pH 6), 200 mM NaCl, 2 mM dithiothreitol, 3 mM $NaN_3$ and various amounts of $MgCl_2$. An initial volume of 20 µl was diluted 12-fold at 25°C, at 40°C, or alter-

nating between 25°C and 40°C, varying the total dilution time from 2 to 24 h. AQP1 reconstitutions were also performed in DDM under similar conditions. Lipids were also solubilized in DDM as described above, and the DDM concentration of the ternary mixture was 0.1 mg/ml before dilution (measured by drop shape analysis). The resulting crystals were negatively stained for conventional electron microscopy, or either quick-frozen or embedded in trehalose for low-dose cryo-electron microscopy.

## 3. Results

### 3.1. DMPC solubilization

The light scattering signal during a complete run, where DMPC is diluted with a DM solution, is shown in Fig. 2a. The saturation and solubilization points can be directly recognized (Fig. 2a, arrows and arrowheads, respectively). The saturation point is characterized by a light scattering maximum (λ-shaped curve, see [10,12]), which results from the network of detergent-saturated lipids (this 'gel-like' phase point was also described by [13]). By adding slightly more detergent, the turbidity falls rapidly to a point where no scattered light is detected anymore (Fig. 2a, arrowheads). These points indicate a linear relation between the lipid and the detergent concentrations (Fig. 2b,c). Linear regression yielded the parameters to calculate the minimum amount of detergent required to completely solubilize a given lipid with a given detergent (Fig. 2, caption). Similar results were also recorded with the detergents OG, DDM, TX100, OTG and CHAPS. Interestingly, attempts to monitor turbidity during DMPC solubilization with cholate, $C_{12}E_8$, and octyl-POE failed to produce the 'gel-like' phase. This is contrary to previous experiments [10], and probably a result of the continuous stirring employed.

### 3.2. 2D crystallization of porin OmpF

On dilution of a ternary mixture containing OmpF (2 mg/ml), DMPC (4 mg/ml) and octyl-POE (10 mg/ml), the light scattering intensity showed a λ-shaped curve (Fig. 3a [12]). Such a maximum occurs when a significant population of

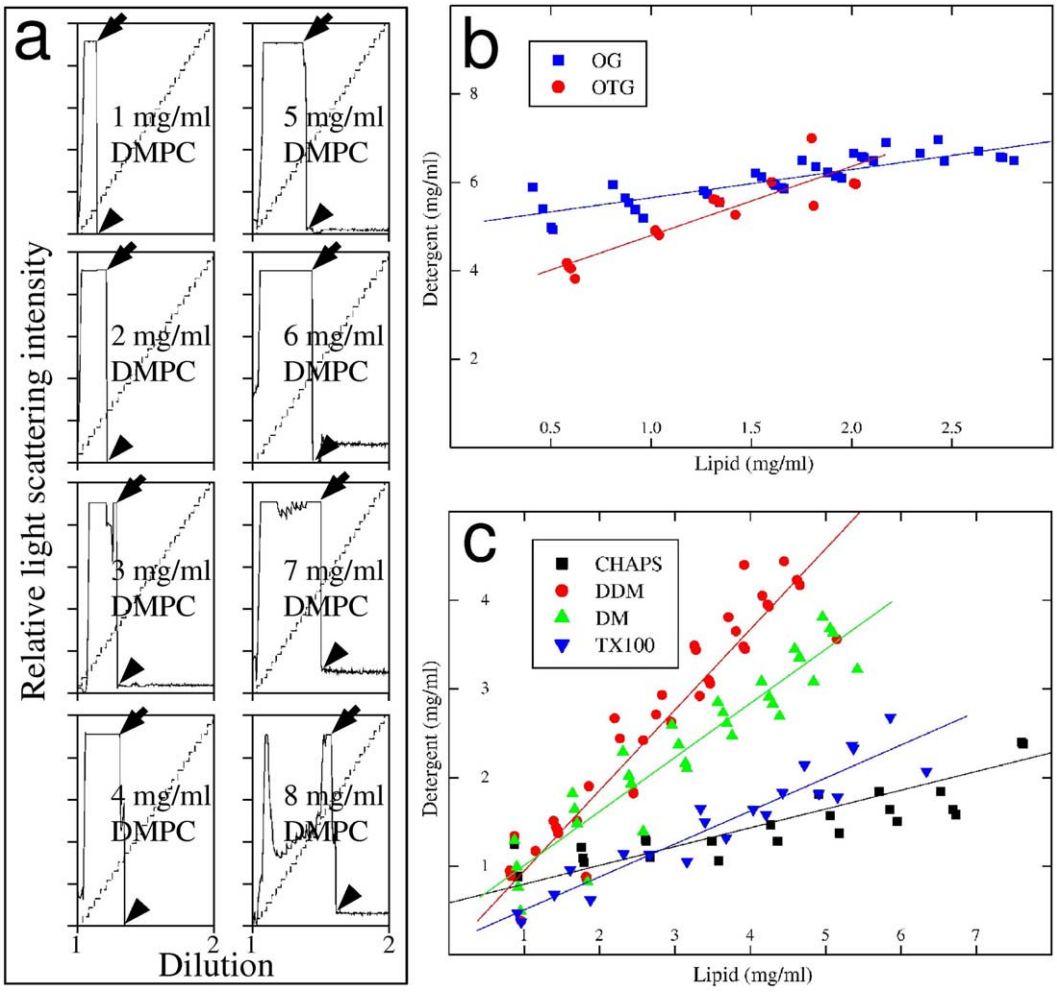


Fig. 2. Solubilization capacity of different detergents. DMPC solutions (1–8 mg/ml) were solubilized by adding a 20 mM Tris–HCl (pH 7.5) buffer containing OG, DM, DDM, TX100, OTG or CHAPS at a fixed rate, and monitoring the light scattering signal. Experiments lasted from 30 min to 5 h and dilutions were between 1.5- and 5-fold, depending on the solubilization capacity of the detergent. a: Parallel registration of the dilution profiles acquired for different concentrations of DMPC with DM (10 mg/ml). Arrows mark the point where the 'gel-like' phase occurred (saturation point), and the arrowheads indicate the solubilization point. The dotted line represents the dilution rate during the experiment. b: DMPC concentration plotted against OG and OTG concentration at the solubilization point (arrowheads in a). c: DMPC solubilization by DM, DDM, TX100 or CHAPS. Linear regression yields the parameters ($a$, $b$) to calculate the detergent concentration $d = a \times l + b$ required for a specific DMPC concentration $l$ (OG: $a = 0.64$, $b = 5.01$; DM: $a = 0.61$, $b = 0.40$; DDM: $a = 0.91$, $b = 0.04$; TX100: $a = 0.37$, $b = 0.13$; OTG: $a = 1.56$, $b = 3.24$; CHAPS: $a = 0.21$, $b = 0.58$). The parameters indicate the solution capacity of the respective detergent.

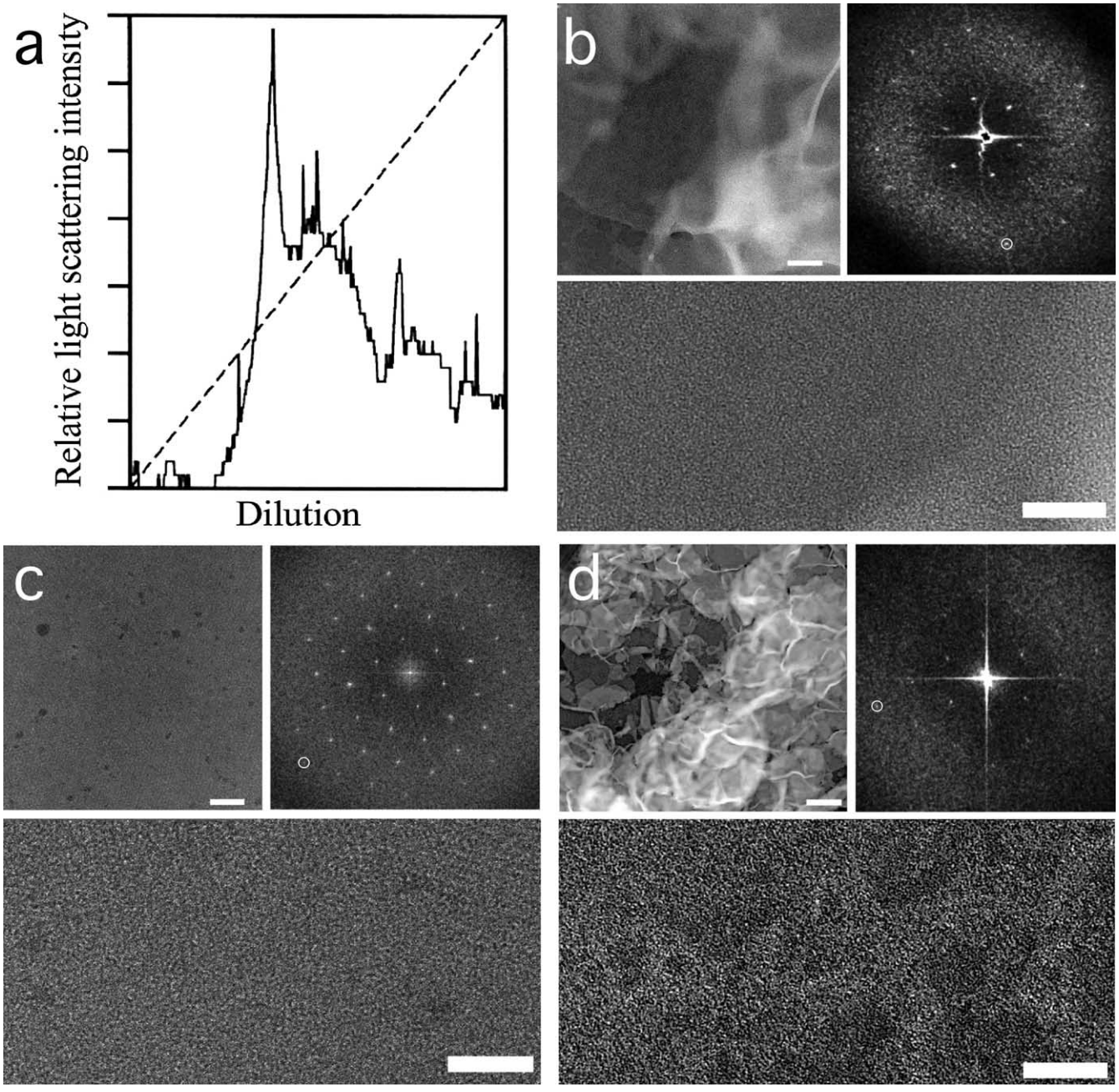


Fig. 3. OmpF reconstitution. a: Typical λ-shaped curve of the scattered light during dilution of the ternary mixture OmpF/DMPC/octyl-POE. The sharp onset indicates the formation of large structures upon reaching the CMC of octyl-POE. Upon further dilution, the light scattering decays. Negatively stained samples were observed by electron microscopy. b: OmpF crystals obtained after 2 h and a 10-fold dilution (top left, overview; top right, diffraction pattern with the marked spot at $(3.1\ \text{nm})^{-1}$; bottom, high magnification). c: Same crystals as in b, but after phospholipase A2 treatment (top left, overview; top right, diffraction pattern with the marked spot at $(1.8\ \text{nm})^{-1}$; bottom, high magnification). d: Crystals of lesser quality were obtained when OmpF was solubilized in DDM (top left, overview; top right, diffraction pattern with the marked spot at $(3.7\ \text{nm})^{-1}$; bottom, high magnification). In all panels the scale bars represent 120 nm (top left) or 90 nm (bottom).

large structures or a large population of smaller structures appears. In agreement with previous results, electron microscopy characterization excluded the latter case [10]. Upon dilution of such a detergent/lipid/protein mixture, the population of large aggregates, proteoliposomes or 2D crystals starts to increase when the detergent concentration is too low to keep both protein and lipid solubilized; this is concomitant with a distinct increase of the light scattering signal. Beyond a certain point, the contribution of each structure to the total light scattering remains unchanged and the intensity recorded diminishes as a result of progressing dilution.

In the experiments made the dilution rate had a critical influence on the assembly products, as suggested by a previous experiment [9]. Fast (15 min) and slow (1, 2, 3, 6 and 24 h) dilution experiments were performed. Fast dilution led to small proteoliposomes having a diameter ranging from 40 to 100 nm and a minority having diameters ranging from 200 to 400 nm. No regular packing of the protein was observed. In

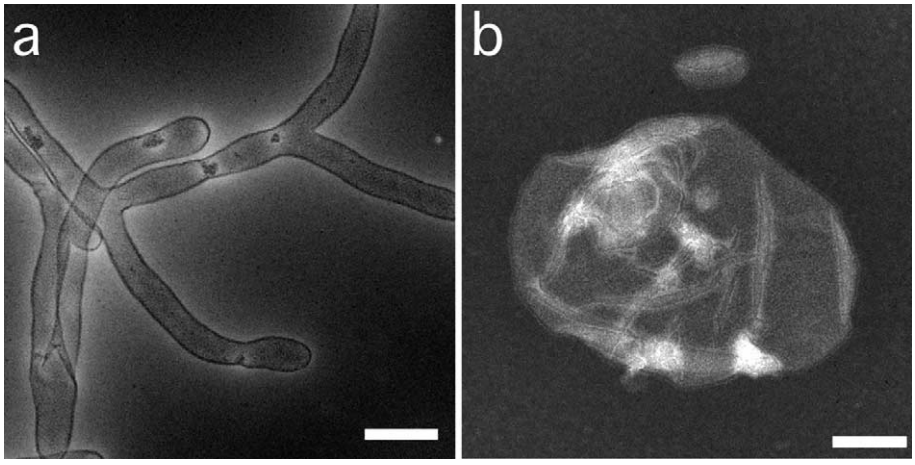


Fig. 4. Reconstitution of PSII. a: Tubular crystals were obtained by dilution over 20–120 min. b: Densely packed vesicles assembled when the dilution time was 12 h. In both cases the initial sample was diluted 15-fold at room temperature. Scale bars represent 320 nm.

contrast, when the dilution period was longer than 1 h, periodic structures were observed, in spite of continuous stirring at 300 rpm. The best crystals were obtained at an LPR of 1 and a dilution time of 2 h. The crystalline vesicles had an average diameter of 1.5 μm. Micrographs of such negatively stained sheets revealed trigonal lattices ($a = b = 9.5 \pm 0.2$ nm; $\gamma = 60°$), which diffracted up to 3 nm resolution (Fig. 3b). Crystallinity was significantly improved by phospholipase A2 treatment [10,29]: these lattices diffracted up to 1.8 nm resolution ($a = b = 7.3 \pm 0.2$ nm; $\gamma = 60°$, Fig. 3c).

DDM-solubilized OmpF also reconstituted into ordered arrays when mixed with DMPC solubilized in octyl-POE at an LPR of 1, yielding trigonal lattices with unit cell dimensions $a = b = 9.5 \pm 0.2$ nm (Fig. 3d). In general, the quality of these lattices was inferior to those obtained with octyl-POE-solubilized OmpF.

### 3.3. 2D crystallization of PSII

The dilution runs made with ternary mixtures containing 1.3 mg/ml PSII and an unknown amount of native lipids, all solubilized in 5 mg/ml OTG, were carried out at room temperature over different time periods (10 min to 24 h), to a final dilution of 15-fold. When dilution was performed over 20–120 min, examination of negatively stained samples in the electron microscope showed tubular crystals as reported previously (Fig. 4a [30]). No particles or aggregates were visible in the background confirming that the reconstitution was complete. In contrast, when the dilution was performed over a longer period (6–24 h) densely packed sheets formed and only a few crystalline tubes were observed (Fig. 4b). Before dilution, sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed the solubilized PSII core complex to comprise the CP47, CP43, D1, D2, CP29 and CP26 polypeptides, the three OEC proteins (17, 23 and 33 kDa), and several smaller proteins having a mass below 10 kDa. However, the reconstituted PSII complex did not contain the 17 and 23 kDa polypeptides (data not shown).

### 3.4. E. coli *translocase reconstitution*

When a ternary mixture containing various amounts of different DOPE/DOPG mixtures (7:3 and 3:7, w/w; 0.3–1.3 mg/ml) and SecYEG at 1.3 mg/ml was diluted over a time period of 2 h at room temperature, complete reconstitution of the translocase was reproducibly achieved, yielding small vesicles and no aggregates. All other lipid mixtures explored failed to integrate the translocase. Electron microscopy of negatively stained samples revealed that SecYEG solubilized in 0.1 mg/ml DDM is properly integrated in the bilayer even at a low LPR. Sometimes, regular rows of round particles with a stained central pit were found that exhibited a hexagonal diffraction pattern (Fig. 5). According to the row spacing the particle diameter is approximately 7.3 nm. However, no large crystals suitable for electron diffraction were obtained during these experiments.

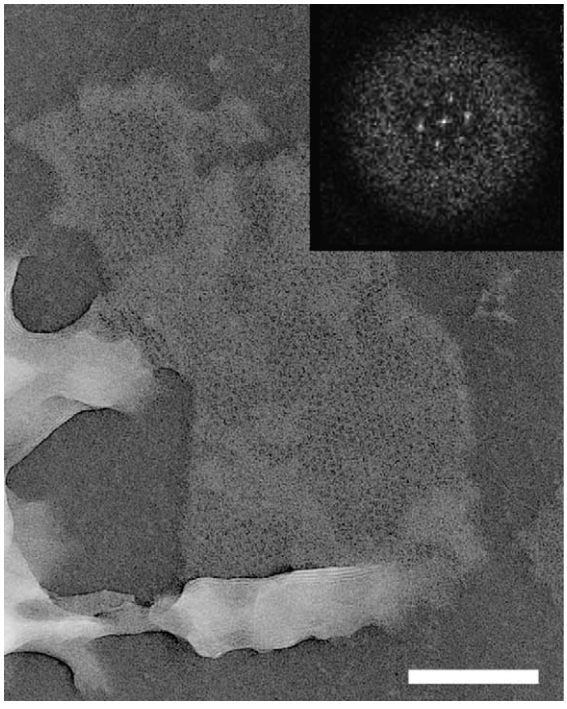

Fig. 5. *E. coli* SecYEG translocase reconstituted by dilution. A DOPE/DOPG mixture solubilized in OG was added to the translocase solubilized in DDM. The 15-fold dilution was performed at room temperature and lasted 24 h. Occasional regular arrays of round particles suggest feasibility of 2D crystallization. The scale bar represent 120 nm and the first diffraction order is at $(7.3\ \text{nm})^{-1}$.

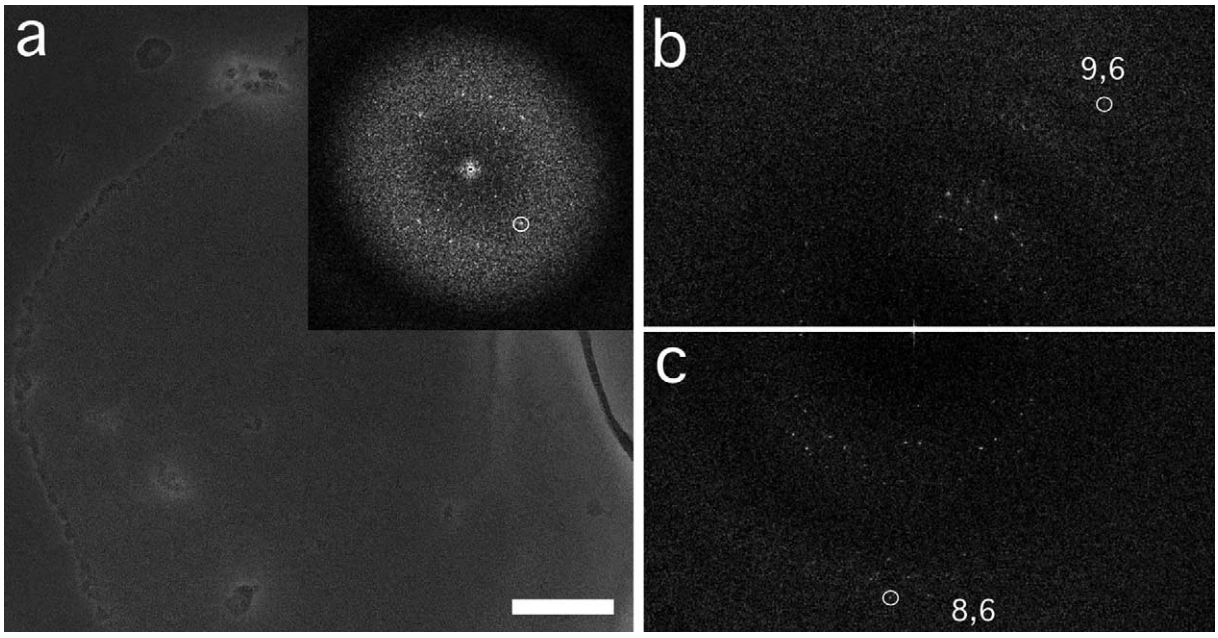


Fig. 6. Reconstitution of AQP1. a: Negatively stained samples demonstrate the existence of large regular lattices as documented by the diffraction pattern (inset). Diffraction patterns calculated from large unstained crystals recorded at low temperature and low dose indicate that the AQP1 crystals produced by dilution are well-ordered. The diffraction pattern in b is calculated from a crystal prepared directly after the dilution run and reveals orders up to a resolution of $(0.8\ \text{nm})^{-1}$. The diffraction pattern in c is from a crystal produced by dilution, which was subjected to 36 h of dialysis and exhibits spots to a resolution of $(0.9\ \text{nm})^{-1}$. The scale bar represents 320 nm.

### 3.5. 2D crystallization of AQP1

The experiments carried out with AQP1 were designed to determine the influence of $Mg^{2+}$, the LPR, the temperature, the dilution rate and the stirring. In addition, an attempt was made to reconstitute AQP1 that had been solubilized in DDM instead of OG. Standard conditions were derived from previous experiments: *E. coli* lipids were used exclusively, and the buffer contained 20 mM MES–NaOH (pH 6), 200 mM NaCl, 2 mM dithiothreitol, 3 mM $NaN_3$ with different amounts of $MgCl_2$ [33,34]. Interestingly, none of the conditions explored with a mixture of DDM solubilized AQP1 (1 mg/ml) and DDM-solubilized *E. coli* lipids yielded crystalline arrays. Small, probably empty vesicles and protein aggregates emerged independently of the $MgCl_2$ concentration, the dilution rate and the LPR.

In contrast, large, well-ordered tetragonal arrays ($a = b = 9.6 \pm 0.2$ nm; $\gamma = 90°$) were obtained from ternary mixtures containing 1 mg/ml AQP1, 10–50 mM $MgCl_2$ and various amounts of *E. coli* lipids (LPR 0.5–1), all solubilized in OG (10 mg/ml, final concentration), provided that the dilution took approximately 2 h and stirring was omitted (Fig. 6a). When the mixture was stirred during dilution, only small vesicles were found interspersed with protein–lipid aggregates, in stark contrast to the results from porin crystallization runs (see above). Important prerequisites for the formation of large 2D AQP1 crystals by the dilution method are (i) the presence of $Mg^{2+}$, (ii) an LPR of 0.5–1, and (iii) a dilution time of at least 2 h. The temperature (room temperature or 40°C) did not markedly change the quality of the crystals. The best crystals were produced at an LPR of 1, 10 mM $MgCl_2$, and either 25°C or 40°C. They were subsequently subjected to a 36 h dialysis against the same buffer. The sheets became larger, but the crystallinity did not improve as judged by the diffraction patterns calculated from micrographs of unstained, quick-frozen crystals recorded at liquid nitrogen temperature, which revealed spots out to $(0.8\ \text{nm})^{-1}$ (Fig. 6b,c).

## 4. Discussion

### 4.1. Starting conditions

Favorable starting conditions for all types of reconstitutions may be chosen from a rich literature on 2D crystallization experiments driven by detergent dialysis or adsorption to Biobeads (see [7,8,16–18]). In general the LPR should be in the range of 0.2–2, and the protein concentration should not be less than 1 mg/ml. The latter requirement is rather stringent because commonly used purification protocols yield protein concentrations of only 0.1–2 mg/ml. Therefore, proteins are concentrated using Centricon filtration or similar techniques. However, retention of detergent micelles in the Centricon tube leads to a much increased detergent concentration, which must be reduced.

The choice of the detergent is critical, since it must both preserve the protein's native state during solubilization and purification, and possess the correctly balanced binding affinities to the protein and the lipid. Low-CMC non-ionic detergents have been shown to stabilize many fragile proteins, but they can only be efficiently removed using Biobeads. For both dialysis- and dilution-driven reconstitution runs, an unnecessary excess of detergent is definitely an unsuitable starting condition.

Not only is it critical to adjust the starting detergent concentration, but excess detergent may also destabilize membrane protein complexes. Over time, complexes may thus disassemble, recruiting detergents from the excess pool. The next stage may be the loss of the ternary structure, individual α-helices becoming embedded in detergent micelles, or ultimately, the complete loss of the secondary structure. Provided

that enough detergent is available, the devastating problem cannot be seen: the protein solution remains clear. We suggest that the amount of detergent in the protein mixture should just suffice to keep the native protein in solution. Therefore, the measurement of the detergent concentration prior to the dilution series is one prerequisite for reproducibility. To optimize the starting concentration we have monitored Biobeads-driven detergent removal by analyzing the shape and dimensions of sitting drops.

Likewise, the lipid solution should not contain excess detergent. Microcalorimetry has been used to determine the POPC solubilization capacity of detergents with a wide range of properties [14], yielding a rough guideline for preparing the lipid solution. As demonstrated by the examples presented in Fig. 2, the large lipid solubilization capacity of CHAPS [14] is also observed for DMPC, whereas DDM and DM appear to solubilize DMPC better than POPC. Our experiments gave similar results as those reported by Lambert et al. [13], who studied the solubilization of egg lecithin with DDM. However, there are small but significant differences. Therefore, the solubilization point of a specific detergent–lipid combination needs to be determined under the conditions of the reconstitution experiment to ensure that the required reproducibility is obtained.

Of particular interest is a situation that facilitates the formation of ternary mixed micelles upon mixing solubilized proteins and lipids. To this end, a concentrated solubilized lipid solution is diluted into a less concentrated protein solution to achieve the appropriate LPR. The detergent concentration in the lipid solution is adjusted so that it will be pushed below its CMC in this step, forcing the protein–detergent micelles to accommodate lipids. It may also be advantageous to select different detergents to solubilize the protein and the lipid, thereby adjusting the respective interactions. For instance OTG (initially used to grow 2D crystals of PSI [35]) has been shown to promote the formation of extended crystalline sheets when added in sub-CMC amounts to the reconstitution mixtures [36]. Likewise it has been possible to produce 2D crystals of lactose permease solubilized in DDM by adding OG-solubilized lipids [37].

### 4.2. Dilution machine

The need for an automated dilution device emerged from published results and our own experiments. Here we describe the machine we built to fulfill this requirement: the system provides a flow rate calibration protocol, yielding excellent reproducibility. The experiment to determine DMPC solubilization capacity of different detergents (Fig. 2) documents the potential of the system. It is also easy to use. Changing dilution conditions is fast and operator attention is not required during experiment. All light scattering intensity vs. dilution curves are printed on the same sheet automatically at the end of the run, facilitating comparison of results. In addition, it is possible to withdraw some sample during a dilution run to monitor the reconstitution process. Because the detergent concentration just depends on the dilution rate, controlled reconstitution is also achieved with ternary complexes containing a mixture of different detergents.

Membrane protein complexes are often solubilized by mild detergents to preserve their native structure. In fact, many membrane proteins are destabilized upon extraction from native membranes, especially when short-chain (high-CMC), harsh detergents are used. Because of the high dilution achievable with the dilution machine (the final volume can be up to 450 μl and the minimum sample volume is 15 μl, making the maximum dilution 30-fold), reconstitution using low-CMC detergents such as DDM, TX100, or $C_{12}E_8$ is possible. In contrast, dialysis of micelles containing such detergents take weeks at room temperature, and months at 4°C. The most successful way to remove low-CMC detergents from a solution is by using the Biobeads method. However, mastering the rate of detergent removal using this approach is not trivial especially with small volumes, and most of the detergent is usually removed within 30 min (see [38]). Therefore, dilution of ternary systems containing low-CMC detergents appears to be an appropriate alternative way to warrant the controlled removal of the detergent bound to proteins and lipids during the micelle to vesicle transition. It can be performed at any desired speed and the temperature does not affect the rate of detergent removal (unlike dialysis or Biobeads experiments). The dilution rate can be reproducibly and finely tuned at different stages of the dilution, and light scattering can be used to monitor the aggregation of the components. In addition, detergent mixtures can easily be adjusted to reach the respective CMCs at the same dilution. Because the temperature profile has been important to optimize the growth of 2D crystals by dialysis (e.g. for cyanobacterial PSI [35], light-harvesting complex II [25], AQP1 [39], GlpF [40] and lactose permease [37]), a Peltier device combined with a temperature feedback control was implemented in the present dilution machine.

### 4.3. Reconstitution and crystallization

*4.3.1. OmpF.* Porin OmpF from *E. coli* is an ideal test protein. Its particular β-barrel fold and trimeric configuration make it resistant to even the harshest detergents and it exhibits a pronounced propensity to form a range of crystals forms with different lipids at LPRs ranging from 0.2 to 2 [10]. The purpose of the dilution series with OmpF was to establish typical light scattering diagrams for different conditions and to correlate them with the resulting structures observed by negative stain electron microscopy as is illustrated in Fig. 3. A lower limit to the dilution time required for the formation of 2D crystals is around 1 h. This corroborates the findings of an initial set of dilution experiments where samples rapidly diluted below CMC revealed unstructured proteoliposomes [9]. The observation is also consistent with reports discussing Biobeads-driven 2D crystallization experiments, which indicate that a too high detergent removal rate impedes the formation of 2D crystals [18]. A dilemma thus emerges: on the one hand, every attempt should be made to integrate a delicate membrane protein rapidly in the bilayer to preserve its native structure, but on the other hand, time must be provided for the system to crystallize.

The 2D crystallization of OmpF was achieved reproducibly by dilution and yielded crystal lattices similar to those previously reported [9,10]. The best OmpF crystals were obtained when a 10-fold dilution was performed over 1–2 h with protein and lipid all solubilized in octyl-POE. It is noteworthy that crystal growth is not hindered when the ternary system is stirred during the dilution process, which is consistent with the unusual propensity of this protein to crystallize. Consistent with previous experiments, octyl-POE-solubilized OmpF yielded better crystals than DDM-solubilized OmpF [10]. This

is in contrast to the situation with lactose permease, which required the presence of DDM for successful crystallization [37].

*4.3.2. PSII.* The experiments with PSII represent an attempt to improve the quality of tubes previously reported [30] by increasing the dilution time period. The observation that upon slow dialysis aggregates form relates to the dilemma between fast integration of the protein in the bilayer and slow crystallization. If the protein is lost in aggregates, the concentration of the soluble protein will be too low for crystallization to occur. In the case of PSII the choice of OTG is apparently not ideal, as also suggested by the loss of the 17 and 23 kDa subunits.

The heat from the absorbed laser light could reduce a protein's stability and cause aggregation during long dilution runs ($>2$ h). If this had been an inhibiting factor for PSII, turbidity should always have been detected after the same exposure time during the different dilution experiments (12 and 24 h). This was not the case, in our experiments the scattering signal reproducibly increased at the same dilution point but not at the same dilution time. Furthermore, by adding different initial concentrations of OTG (2, 5, 10 and 15 mg/ml), we confirmed that when the amount of detergent was higher, a longer time was required at a given dilution rate to reach the aggregation point. In addition, the preparation had the same green color after dilution as at the beginning of the experiment. These observations suggest that the laser light did not significantly affect the PSII crystallization trials.

*4.3.3. Translocase.* Many attempts to crystallize or even to reconstitute SecYEG from *E. coli* at high packing density failed in our hands. When solubilized in OG, the enzyme lost its biological activity within hours at 4°C, and also lost its ability to integrate in a bilayer, no matter which lipid mixture was employed. Protein stability was improved when DM was used, but DDM preserved full activity of the solubilized enzyme over several days at room temperature. Therefore, dialysis and Biobeads experiments were carried out with DDM, but unfortunately with no success. In contrast, several dilution runs reproducibly yielded reconstitutions devoid of large aggregates, revealing densely packed proteoliposomes and small 2D crystals. Interestingly, the best conditions (SecYEG solubilized in DDM, lipids solubilized in OG) were close to those leading to 2D crystals of lactose permease [37]. The experiments were not continued because crystals suitable for electron crystallography were produced and the structure solved to 0.8 nm in-plane resolution [41].

*4.3.4. AQP1.* As a result of its propensity to form 2D and even 3D crystals, the atomic structure of AQP1 has been determined by electron [26] and X-ray crystallography [42]. We selected this protein to test the dilution method because it is an excellent representative of α-helical membrane proteins, and because we have extensive experience in handling this protein.

Unexpectedly, AQP1 could not be reconstituted by dilution when solubilized in a low-CMC detergent such as DDM. This is in contrast to the intuition that DDM would enhance the stability of the protein and hence foster its integration in the bilayer. Because dilution-driven 2D crystallization worked reproducibly when the protein and lipids were solubilized in OG, we conclude that AQP1 and *E. coli* lipids have different binding affinities to DDM. This hypothesis will be explored further.

Large coherent 2D AQP1 crystals were obtained under conditions similar to those previously reported for dialysis [34]. Using mainly negative stain electron microscopy, we could not observe a critical influence of the LPR as long as it was in the range of 0.5–1. Since excessive lipid can be removed using lipase treatment [29,43], we did not further explore this point. However, magnesium ions play an important role, the minimum $Mg^{2+}$ concentration was found to be approximately 10 mM. It is interesting to note that the optimum conditions for dialysis experiments were LPR = 0.5–0.75, $MgCl_2 > 25$ mM, and a temperature of 37°C [33]. Because dilution does not remove the detergent, we also checked whether subsequent dialysis would change the 2D crystals formed during a dilution run. With the best 2D AQP1 crystals, we did not detect a significant change in samples negatively stained or quick-frozen after 36 h of dialysis, but a conclusive answer will only be obtained from electron diffraction experiments.

### 4.4. Conclusion

The experiments presented demonstrate that dilution, which is commonly used for the reconstitution of proteoliposomes for functional assays, can also promote the 2D crystallization of membrane proteins. Because crystallization experiments are generally done at the highest possible protein concentration, these findings are quite counter-intuitive and may suggest that the major step in 2D crystallization is the reconstitution event. At this point, the membrane protein is highly concentrated by incorporation in the bilayer and may well crystallize, even though its overall concentration is reduced during the dilution. Since both dilution rate and temperature are precisely controlled with the dilution machine described, reproducible reconstitution conditions are easy to achieve thus providing the ideal experimental environment to systematically investigate the reconstitution process. In contrast to dialysis or Biobeads adsorption, dilution allows the controlled removal of the detergent bound to proteins and lipids. This opens the possibility of slowing down the process at the onset of crystal growth. Our results suggest that dilution is a promising technique for 2D crystallization of membrane proteins. After reconstitution under controlled conditions, there is room to improve the crystals. This may be achieved by complete removal of the detergent using dialysis or Biobeads, as well as by elimination of excessive lipids by lipase treatment, which all need to be further explored.

*Acknowledgements:* The authors thank Prof. Demetrios Ghanotakis and Dr. Aspasia Spyridaki for the kind gift of photosystem II core complex. The authors are also grateful to Drs. Shirley Müller and Dimitrios Fotiadis for inspiring discussions. Drs. Arnold Driessen and Chris van der Does have kindly provided SecYEG membranes, and Gilbert Parat has been helpful in building the electronics. This work was supported by the M.E. Müller-Foundation of Switzerland, the Swiss National Foundation (Grant NF 31-59415.99), the European Union-Quality of Life and Management of Living Resources Project (Grants QLRT-2000-00778 and QLRT-2000/00504), and the Swiss National Center of Competence in Research (NCCR) 'Structural Biology'.

## References

[1] Walz, T., Smith, B.L., Zeidel, M.L., Engel, A. and Agre, P. (1994) J. Biol. Chem. 269, 1583–1586.
[2] Lichtenberg, D. (1985) Biochim. Biophys. Acta 821, 470–478.
[3] Schurtenberger, P., Mazer, N. and Känzig, W. (1985) J. Phys. Chem. 89, 1042–1049.

[4] Walter, A., Vinson, P., Kaplun, A. and Talmon, Y. (1991) Biophys. J. 60, 1315–1325.
[5] Hjelm, R.P., Thiyagarajan, P. and Alkan-Onyuksel, H. (1992) J. Phys. Chem. 96, 8653–8661.
[6] Rigaud, J.-L., Pitard, B. and Levy, D. (1995) Biochim. Biophys. Acta 1231, 223–246.
[7] Ringler, P., Heymann, B.J. and Engel, A. (2000) in: Membrane Transport (Baldwin, S.A., Ed.), pp. 229–268, Oxford University Press, New York.
[8] Rigaud, J., Chami, M., Lambert, O., Levy, D. and Ranck, J. (2000) Biochim. Biophys. Acta 1508, 112–128.
[9] Dolder, M., Engel, A. and Zulauf, M. (1996) FEBS Lett. 382, 203–208.
[10] Engel, A., Hoenger, A., Hefti, A., Henn, C., Ford, R.C., Kistler, J. and Zulauf, M. (1992) J. Struct. Biol. 109, 219–234.
[11] Almog, S., Kushnir, T., Nir, S. and Lichtenberg, D. (1986) Biochemistry 25, 2597–2605.
[12] Ollivon, M., Eidelman, O., Blumenthal, R. and Walter, A. (1988) Biochemistry 27, 1695–1703.
[13] Lambert, O., Levy, D., Ranck, J.L., Leblanc, G. and Rigaud, J.L. (1998) Biophys. J. 74, 918–930.
[14] Heerklotz, H. and Seelig, J. (2000) Biophys. J. 78, 2435–2440.
[15] Fujiyoshi, Y. (1992) Tanpakushitsu Kakusan Koso 37, 560–566.
[16] Jap, B.K., Zulauf, M., Scheybani, T., Hefti, A., Baumeister, W., Aebi, U. and Engel, A. (1992) Ultramicroscopy 46, 45–84.
[17] Kühlbrandt, W. (1992) Q. Rev. Biophys. 25, 1–49.
[18] Rigaud, J.L., Mosser, G., Lacapere, J.J., Olofsson, A., Levy, D. and Ranck, J.L. (1997) J. Struct. Biol. 118, 226–235.
[19] Racker, E., Chien, T.F. and Kandrach, A. (1975) FEBS Lett. 57, 14–18.
[20] Aoyama, K., Ogawa, K., Kimura, Y. and Fujiyoshi, Y. (1995) Ultramicroscopy 57, 345–354.
[21] Cyrklaff, M., Auer, M., Kühlbrandt, W. and Scarborough, G.A. (1995) EMBO J. 14, 1854–1857.
[22] Auer, M., Scarborough, G.A. and Kühlbrandt, W. (1999) J. Mol. Biol. 287, 961–968.
[23] Lebeau, L., Lach, F., Venien-Bryan, C., Renault, A., Dietrich, J., Jahn, T., Palmgren, M.G., Kühlbrandt, W. and Mioskowski, C. (2001) J. Mol. Biol. 308, 639–647.
[24] Levy, D., Chami, M. and Rigaud, J.L. (2001) FEBS Lett. 504, 187–193.
[25] Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Nature 367, 614–621.
[26] Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A. and Fujiyoshi, Y. (2000) Nature 407, 599–605.
[27] Fujiyoshi, Y. (1998) Adv. Biophys. 35, 25–80.
[28] Holzenburg, A., Engel, A., Kessler, R., Manz, H.J. and Aebi, U. (1989) Biochemistry 28, 4187–4193.
[29] Mannella, C.A. (1984) Science 224, 165–166.
[30] Tsiotis, G., Walz, T., Spyridaki, A., Lustig, A., Engel, A. and Ghanotakis, D. (1996) J. Mol. Biol. 259, 241–248.
[31] Manting, E.H., van Der Does, C., Remigy, H., Engel, A. and Driessen, A.J. (2000) EMBO J. 19, 852–861.
[32] Denker, B.M., Smith, B.L., Kuhajda, F.P. and Agre, P. (1988) J. Biol. Chem. 263, 15634–15642.
[33] Fotiadis, D., Suda, K., Tittmann, P., Jeno, P., Philippsen, A., Muller, D.J., Gross, H. and Engel, A. (2002) J. Mol. Biol. 318, 1381–1394.
[34] Walz, T., Smith, B., Zeidel, M., Engel, A. and Agre, P. (1994) J. Biol. Chem. 269, 1583–1586.
[35] Ford, R.C., Hefti, A. and Engel, A. (1990) EMBO J. 9, 3067–3075.
[36] Chami, M., Pehau-Arnaudet, G., Lambert, O., Ranck, J.L., Levy, D. and Rigaud, J.L. (2001) J. Struct. Biol. 133, 64–74.
[37] Zhuang, J., Prive, G.G., Werner, G.E., Ringler, P., Kaback, H.R. and Engel, A. (1999) J. Struct. Biol. 125, 63–75.
[38] Levy, D., Bluzat, A., Seigneuret, M. and Rigaud, J.L. (1990) Biochim. Biophys. Acta 1025, 179–190.
[39] Walz, T., Smith, B.L., Agre, P. and Engel, A. (1994) EMBO J. 13, 2985–2993.
[40] Braun, T., Philippsen, A., Wirtz, S., Borgnia, M.J., Agre, P., Kühlbrandt, W., Engel, A. and Stahlberg, H. (2000) EMBO Rep. 1, 183–189.
[41] Breyton, C., Haase, W., Rapoport, T.A., Kühlbrandt, W. and Collinson, I. (2002) Nature 418, 662–665.
[42] Sui, H., Han, B.G., Lee, J.K., Walian, P. and Jap, B.K. (2001) Nature 414, 872–878.
[43] Jap, B.K. and Li, H. (1995) J. Mol. Biol. 251, 413–420.