Research paper

Microfabricated silicon chip as lipid membrane sample holder for serial protein crystallography

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ARTICLE INFO

Keywords:
Protein crystallography
X-ray free electron laser
Perforated silicon nitride membrane
Microfluidics
Black lipid membrane
Electrochemical impedance spectroscopy

ABSTRACT

Rapid progress in protein crystallography techniques at X-ray free electron lasers (X-FELs) requires the development of new methods for sample delivery. Recording dynamic changes in protein structures is one of the aims. To assess protein dynamics, sample holders for crystallography need to become active devices, providing possibilities to induce in situ changes of the protein conformation during the experiments. We propose an integrated device for crystallographic studies on 2D crystals of membrane proteins. The conceptual device can support physiological conditions and provide a platform for electrophysiological measurements, as well as for electrical stimulation during the diffraction experiments at X-FELs. This allows for triggering conformational changes in membrane proteins, ultimately permitting to take series of crystallographic snapshots of the dynamic behavior of proteins. The device integrates a microfabricated silicon chip between two microfluidic transport layers. We demonstrate the fabrication of the device, describe its key components and show the method of sample loading and lipid bilayer formation with the use of the microfluidic delivery system. Electrochemical impedance spectroscopy (EIS) has been used to characterize the properties of lipid bilayers formed in the microfabricated silicon nitride support. Minimizing the sample consumption, mimicking physiological conditions during crystallographic data collection and an interface for electrochemical characterization and addressing protein 2D crystals are the key properties of the proposed device.

1. Introduction

Membrane proteins account for about 30\% of the proteome in most organisms and perform critical cellular and physiological functions [1]. Nearly 60\% of approved drugs target membrane proteins. High-resolution structure determination of membrane proteins is a prerequisite for understanding of their function, mechanisms of action and for designing new types of drugs. X-ray crystallography is, so far, the most efficient way method of protein structure determination [2]. However, the radiation damage at synchrotron sources coupled with low-expression yield of membrane proteins and crystallization difficulties remain a major obstacle in the field. Furthermore, membrane proteins often crystallize as 2D crystals, which exhibit too low X-ray diffraction yields to be measured at synchrotrons. Another challenge for structural studies is recording the dynamics of proteins, which would only be possible with classical X-ray techniques when using large crystals. All of these challenges are being addressed by diffraction experiments at X-ray free electron lasers (XFELs) [3–6]. These large-scale facilities can produce femtosecond X-ray pulses with much higher intensities compared to synchrotrons, enabling structure determination based on a diffract-before-destroy principle using a method commonly described as serial femtosecond crystallography (SFX). During the data acquisition, samples containing the microcrystals are being moved across the beam path, in a way that the diffraction patterns are taken from a fresh, undamaged sample area with every data collection cycle. Crystals can be delivered to the X-ray beam in a liquid jet or on fixed targets with multiple sample areas [7–9]. The ultrafast nature of the XFEL sources coupled with the fact that the sample can be delivered at room temperature facilitates the pump-probe type experiments in this regime [10–12]. This paves the way for time-resolved crystallography, and dynamic studies of proteins in native-like environment. However, due to the lack of suitable devices time-resolved protein structure determination after electrical stimulation has not been demonstrated yet.

Here, we propose a conceptual integrated device for SFX studies on...
membrane proteins which can support physiological conditions and provide possibilities for electrophysiological measurements and electrical stimulation during the SFX experiments. Coupling SFX with an electrophysiological environment will allow triggering conformational changes in membrane proteins such as voltage-gated ion channels, ultimately leading to taking SFX snapshots of the dynamic behavior of proteins and thus recording movies of molecular gating events.

2. Proposed device

A microfabricated silicon/silicon nitride chip with the primary role to support planar lipid bilayers to be used for incorporation of 2D crystals of membrane proteins is a central part of the device. The chip contains an array of perforated, thin-membrane windows. Silicon nitride is well known for its relatively low background scattering and high X-ray transmission [13-16]. These windows are perforated with hexagonal arrays of circular apertures used as a scaffold for free-standing lipid bilayers. The chip is encased from top and bottom between two additional microfabricated chips of the same size carrying arrays of non-perforated silicon nitride windows positioned over the perforated windows of the central chip. This creates a two-compartment system with cis- and trans-side, a typical architecture used for electrophysiological characterization of planar lipid bilayer systems [17,18]. To obtain a high-quality seal between the three layers of chips, they are separated with tailor-made PDMS layers with microfluidic channels. To electrochemically address the cis- and trans-side of the device, microfabricated Ag/AgCl electrodes are integrated on the covering chips at the top and bottom of the device.

In this report, we present results from a simplified version of the device (Fig. 1). We demonstrate the operation principle, namely the introduction of lipids into the device and establishing routes for electrophysiological characterization of planar lipid bilayers spanning over microfabricated silicon/silicon nitride supports. At this stage of development, it is not absolutely necessary for the device to fulfill the requirements for high X-ray transparency or having integrated micro-electrodes, hence we made the following modifications. Firstly, the central chip, on which lipid membranes are formed, is encased between two pieces of cast poly(dimethyl siloxane) (PDMS) with microfluidic channels instead of silicon/silicon nitride chips. This modification significantly reduces the complexity of the device and enables a visual inspection of the sample area during the experiment to allow for quick localization of problems in the microfluidic part of the device such as clogging of channels or breaking of membranes. A set of Ag/AgCl pellet electrodes, which can be manually positioned in selected microfluidic portholes, is used for electrophysiological characterization of the system.

3. Materials and methods

3.1. Fabrication of the membrane chips

The fabrication scheme of central chip with the perforated silicon nitride membranes is shown in Fig. 2A. Silicon nitride membranes were first created in double-side polished 250 μm thick silicon (100) wafers coated with 250 nm silicon nitride using photolithography for structure definition at the back of the wafer, reactive ion etching to selectively remove the nitride layer, and wet chemical etching of the bulk silicon in aqueous KOH solution at 85 °C, as described in detail by Opara et al. [16].

The whole wafer with nitride windows was then coated with 20 nm Cr thermally deposited in a Balzers BAK 600 evaporator (Evatec AG, Switzerland) and spin-coated with positive-tone resist (PMMA 950 k, 4% solution in ethyl lactate). Patterns of apertures were defined by means of electron-beam lithography (EBL). We have prepared a set of devices with apertures of 12 μm, 6 μm, 1 μm, 500 nm and 200 nm in diameter arranged in hexagonal patterns with periods twice as large as the apertures, resulting in up to 23% of open surface. The e-beam exposure was performed using a Vistec EBPG5000Plus (Raith GmbH, Germany) lithography system operated at 100 kV acceleration voltage. The optimal exposure dose was 950 μC/cm². Proximity effect correction was not required, since the exposure was performed on the membranes yielding very low electron back-scattering. After development in a 1:3 mixture of methyl-isobutyl ketone (MIBK) and isopropanol (IPA), the pattern was transferred into the Cr layer by plasma etching in a Cl₂:CO₂ (50 sccm: 100 sccm) gas mixture. The thin chromium layer served as a hard mask for the subsequent reactive ion etching (RIE) of the silicon nitride membranes in mixture of CHF₃/O₂/Ar. After stripping the PMMA layer, the Cr hard mask was removed using a cerium nitrate-based chromium etchant (Sigma-Aldrich). Single chips were separated.
from the full wafer by manual cleaving along fine lines that were included in the initial pattern on the lithography mask.

3.2. Fabrication of the PDMS microfluidic layers

The mold for the PDMS microchannel system was fabricated using negative-tone SU-8 photopolymer on a Si substrate. A 100 mm silicon wafer was dipped into 2% hydrofluoric acid diluted in DI water to remove the native SiO$_2$ layer on the substrate. SU-82075 (Microchem Corp., USA) was spin-coated on the Si wafer with 1500 rpm for 45 s, to yield a thickness of 100 μm, followed by prebaking at 95 °C for 30 min. After double UV exposure for 30 s through a mask with the designed patterns, the exposed SU-8 was hard-baked at 95 °C for 45 min. Then, the processed SU-8 was developed in propylene glycol monomethyl ether acetate (PGMEA) under continuous agitation.

A mixture of liquid silicone elastomer, PDMS (Sylgard 184, Dow Chemical, USA) with a curing agent (10:1) was casted on the mold to reach a thickness of 5 mm and placed in a leveled oven rack at 80 °C for 4 h. After cooling down, the PDMS was cut to size of the silicon chip and peeled off from the master mold. The portholes (0.7 mm in diameter) were cut with a custom-made cutter. The PDMS layers were treated by flash of oxygen plasma (20 W, 60 s, Oxford Plasmalab 80, Oxford Instruments, UK) to activate the surface and brought into contact with silicon chips. The chips were wetted with a drop of ethanol to allow for ne positioning of the PDMS on the chip and ensure that there were no air bubbles trapped between the bonded layers. The bonding process took a few minutes.

3.3. Preparation of the lipid solution

Ampoules with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, 25 mg/ml, Avanti Lipids) were opened and the chloroform solution was divided between three, 2.5 ml glass vials, pre-rinsed with chloroform. Molecular sieve (4 Å, Fluka, Switzerland) was added to keep the solution water-free and the ampoules with stock solution were stored at −20 °C. When needed, 50 μl of the stock solution was transferred into an empty 2.5 ml vial which was subsequently dried under vacuum for at least one hour. n-decane was added to the dried lipid to a final concentration of 15–20 mg/ml. After mixing, the solution was ready for introduction into the device. Lipids in n-decane were used immediately or stored at 4 °C for up to one day.

3.4. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) was performed using an Autolab PGSTAT30 (EcoChemie, Netherlands) and conducted in the frequency range from 1 MHz to 0.01 Hz at 0 V potential, applying the signal amplitude of 10 mV. All experiments were carried out in a two-electrode system consisting of two pelleted silver/silver chloride (Ag/AgCl) electrodes (0.8 mm in diameter, Science Products, Hofheim, Germany) introduced into the systems through the port holes in the PDMS layer.

4. Measurement setup

The high brightness of XFEL beam allows for registration the diffraction signal even from weakly diffracting 2D protein crystals. Still, thousands of identical copies of the sample need to be probed to provide dataset sufficiently large to be suitable for structural information reconstruction. Multiple active areas on one device save precious exposure time, since reduces the excessive sample holder exchange. For this concept study, we choose to have five measurement areas per device which can be addressed individually through the microfluidic channels. This is sufficient to demonstrate the operation principle and can be scaled up for the final device. Each of the measurement areas consists of a silicon nitride window with an array of microfabricated apertures, creating a perforated, periodic structure as shown in Fig. 1 C. The arrays of apertures in the nanometer range greatly increase the stability of formed lipid membranes compared to larger free-standing lipid layers [19] and allow for higher integration of the devices [20].

Fig. 1 A shows the assembly arrangement of three integral components of the device. Top and bottom PDMS layers with mirrored, microfluidic structures create cis- and trans-side compartments of the device. They enclose the microfabricated silicon chip, where lipid membranes are spanned. The five areas for lipid membrane immobilization marked as black squares are arranged in an X-shape. Microfluidic channels run on both sides of the device over each perforated nitride window, which allow pumping the reagents necessary for lipid membrane formation and electrochemical characterization of the system. The three components of the device are bonded together by exposing the matching elements to oxygen plasma and pressing them together for few minutes. This method reliably produces high quality seals between the PDMS and silicon chip and makes the device watertight. In Fig. 1B, a cross-section through a bonded device is shown. Each of the silicon nitride windows represents an isolated measurement area with two compartments defined by the microfluidic channels. In Fig. 1C a microscope...
image of one of the microstructured windows with an array of apertures for lipid membrane formation is presented. The holes fabricated with EBL are arranged in a hexagonal pattern over an area slightly larger than the window (seen as a brighter square shape in the middle of the image in Fig. 1C) to avoid alignment problems during electron beam lithography. We manufactured a range of chips with aperture sizes from 200 nm to 12 μm. As previously shown, the smaller the size of the apertures, the higher is the expected stability of lipid bilayers inside [19].

4.1. Lipid bilayer formation

Lipid bilayers – also named black lipid membranes (BLM) [21] – are formed in the device, over the perforated silicon nitride windows, by successively pumping the lipid solution in n-decane and buffer in both, the top- and the bottom-channel as indicated in Fig. 3. After the bottom microfluidic channel is filled with an electrolyte (Fig. 3B), a lipid solution is pumped through the top channel which subsequently is also filled with an electrolyte (Fig. 3C). Upon filling the top channel with electrolyte (Fig. 3D), the lipid solution thins down into a bilayer, which spans over all open apertures of the area of the membrane window. If the process does not yield a stable bilayer (determined by the EIS, as described below) the procedure is repeated by first pumping the lipid solution followed by electrolyte through the top channel. Due to the minute thickness of the silicon nitride membrane, and thus of each aperture opening, thinning of the lipid to a bilayer occurs spontaneously [22] and does not require any physical manipulation as commonly applied in the painting method [20]. This method reliably produces lipid bilayers over all tested aperture sizes, with moderate aspect ratios (membrane thickness: aperture diameter) from 1:60 for the 12 μm to 1:1 for the 200 nm apertures in the microstructured silicon nitride windows without the need for manual procedures. Although the lipids were dissolved in decane, which is known to induce swelling of PDMS [23] we were successful in pumping the solvent through the microchannels without apparent damage to the PDMS structures.

4.2. Impedance spectroscopy

To characterize the chips without (empty) and with BLM we successfully used electrochemical impedance spectroscopy (EIS), a powerful technique, for characterization of this type of systems [20]. Nyquist plots were used to analyze the impedance (Z) for the empty chip and for the chip with a lipid bilayer. We used the equivalent electrical circuit models to perform data fitting and extract information regarding the capacitance and resistance of the system, as described by Han et al. [20]. In the case without the lipid membrane, the system consists of three components in series (R_{eq}(R_{mem})(C_{mem})), a resistance assigned to the electrolyte (100 mM NaCl with HEPES pH 7, R_{eq}) and two components composed of a resistance and capacitance in parallel (RC), assigned to the electrodes ((RC)_{el}) and the perforated silicon nitride membrane ((RC)_{mem}). In the case of the chip with the lipid bilayer the system simplifies to R_{eq}(R_{mem}), assigned to the electrolyte (R_{eq}) and the membrane with the lipid bilayer ((RC)_{mem}), because the resistance associated with the electrodes is negligibly small compared to the GΩ resistance expected for the lipid bilayer.

5. Results

Impedance plots in Fig. 4A show that for the empty chip (no lipid bilayer) the extracted impedance values do not exceed 50 kΩ. Fig. 4B shows a typical curve observed after 30 min after bilayer formation. In the low range of frequencies (> 1 Hz) the obtained impedance is in the range of several GΩ and is dominated by the lipid membrane resistance R_{mem}. Such a resistance, often described as a “gigaohm seal”, is a good indication of the present lipid bilayer, covering the whole perforated area of the window. In the higher frequency range, the impedance drops by orders of magnitude and is dominated by an ohmic resistance of the electrolyte.

From fitting of the impedance spectra, the resistance and the capacitance values were extracted for each of the different patterns and sizes of apertures. All the values of R_{mem} and C_{mem} parameters for the chips with- and without the lipid bilayer are presented in Fig. 4C and D. Panel C shows extracted impedance values in relation to the aperture size in the individual chips. The impedance values obtained from the empty chips are at least 5 orders of magnitude smaller than when the lipid bilayers are formed over silicon nitride windows. For empty devices, the measured impedance is in the kΩ range and the extracted capacitance is found to be below the 300 pF. In contrast, fitting analysis of the impedance measurements after bilayer formation on all of the chips yielded resistance values above 1 GΩ. Such a high resistance is a prerequisite for the analysis of membrane proteins with electrophysiological methods.

The determination of the lipid bilayer capacitance poses a challenge because the silicon chip already has a capacitance in the range 10–200 pF. An upper limit of 0.9 μF cm⁻² for the specific capacitance of the empty devices was used to calculate the bilayer capacitances for different chips as presented in Fig. 4C. The obtained specific membrane capacitance values are in agreement with previously reported values (0.5–0.9 μF cm⁻²) [24].

Initially, after pumping the reagents through the microfluidic channels as shown in Fig. 3, lipid bilayers start to form and expand inside the apertures, thus forming the BLM. This thinning process of lipids into bilayers gives rise to an increase in capacitance. Fig. 5 shows capacitance values C_{mem} extracted from the EIS spectra taken every 4 min on a silicon nitride membrane with 200 nm size apertures. In this example, the total capacitance of the system increased over the course of 40 min to stabilize at 15 nF, indicating completion of lipid thinning and the system reaching the stable state. The thinning time varies from chip to chip and lays between 5 and 40 min, without any clear dependence on the aperture size. Factors influencing the speed of BLM formation are mainly the amount and concentration of lipid solution and the speed with which the reagents are delivered to the silicon nitride support in
the chip. Only after the capacitance reached a stable value, we took the measurements to extract the impedance and capacitance values as described above.

6. Discussion and conclusions

The design criteria for free-standing lipid bilayer platform are: good membrane sealing (low leak currents), high membrane stability (with lifetimes > 3 h and high surface yield), the ability of up-scaling (modular design) the ability to reconstitute membrane proteins, general robustness (stability during handling/transport/experimental conditions) and cost-effectiveness [25]. Additional criteria need to be fulfilled for serial crystallography applications. These include in particular a high perforation level of the silicon nitride membranes, high X-ray transparency and arrangement of the membranes in arrays to facilitate a goniometer-based screening.

A high perforation level of the supporting membrane and the fabrication of functional devices were achieved by microstructuring the silicon nitride membrane with electron beam lithography and dry etching. The technique is scalable and allows for much greater integration, necessary for a final device, e.g., in which several membranes will be connected by one microfluidic channel. Apart from EBL, nanoimprint lithography can be used for the fabrication of chips in larger volumes [26] or fs laser ablation of PEEK membranes in fully polymeric devices [18].

We used PDMS components stretching over the whole sample areas, which is not an ideal solution for a crystallography analysis, because of high signal attenuation by the few mm thick PDMS layer [27]. The solution to achieve X-ray compatible microfluidics typically involves decreasing the thickness of the device layers in the beam path and also substituting dense or high-atomic- number materials such as the Si-containing PDMS for lighter, fully organic polymers [28]. It has been demonstrated that the quality of diffraction patterns observed from such devices encased in thin, only a few tens of μm thick PDMS layers, has been sufficient to solve the protein structures [29].

To further reduce X-ray absorption and scattering we propose using silicon nitride membranes in the entire X-ray beam path. In this case, the top and bottom part of the device would also be made from microfabricated silicon chips with membrane windows and via thin PDMS intermediate layers. Those layers would also comprise the microfluidic channels with feedthroughs positioned at the edges, parallel to the device, making the whole setup compact and suited for the X-ray experiments.

Fig. 4. Electrochemical impedance spectra of (A) an empty chip and (B) a chip with formed POPE lipid bilayer. Squares and circles denote impedance and phase shift respectively. The diameter of microfabricated holes on both chips was 12 μm. (C) Collected impedance values and (D) capacitance values obtained from devices with different size but similar total area of the apertures. Open and solid squares denote measurements taken with and without formed lipid bilayers, respectively. Error bars indicate the error resulting from fitting of the model to the measured data.

Fig. 5. Capacitance values extracted from the EIS measurements on the same chip over time.
Another important factor, which is critical for successful experiments in the device, is the stability of the lipid bilayers on the chip. The expected lifetime has to be long enough to allow incorporation of membrane proteins into the device. The stability of BLM systems varies a lot and it is well known that such systems are very susceptible to mechanical vibrations. One way to increase the stability of our system is via careful design of the silicon nitride support. It is known that the lipid bilayer stability can be improved by increasing the ratio of lipid bilayer support contact area to the total lipid bilayer area [20].

The apertures arranged in a hexagonal pattern in membrane windows maximize the available space for lipid bilayer spanning in comparison to orthogonal arrangements. At the same time the space between the apertures is large enough to make the support sufficiently robust for a normal handling. It is known that the stability of lipid bilayers increases with decreasing the size of the apertures [20], but decreasing the size of the apertures too much, so that the aspect ratio of the aperture diameter to the silicon nitride membrane thickness is smaller than 1:1, is not desirable. This will induce problems with aperture clogging with the lipid solution and increase the thinning time. With our samples we started experiencing problems with lipid bilayer formation in case when the nominal aperture diameter was below 500 nm. For larger apertures the lipid bilayer formation was always apparent in the electrochemical measurements.

We believe that by merging the microfluidic approach with an ultra-thin support can lead to a new type of a device in which introduction of 2D crystals of proteins could be performed directly before the diffraction data collection. Moreover, by having a chip which can be accessed via microfluidic port one can envisage a potential for introducing – parallel to crystallographic data collection – electrophysiological-based methods, raising hopes for the further development of time-resolved crystallography.

Acknowledgements

This work was supported by the PSI career return program and the PhD school of the Swiss Nanoscience Institute (Project P1305).

References