Transparent Nanopore Cavity Arrays Enable Highly Parallelized Optical Studies of Single Membrane Proteins on Chip

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ABSTRACT: Membrane proteins involved in transport processes are key targets for pharmaceutical research and industry. Despite continuous improvements and new developments in the field of electrical readouts for the analysis of transport kinetics, a well-suited methodology for high-throughput characterization of single transporters with nonionic substrates and slow turnover rates is still lacking. Here, we report on a novel architecture of silicon chips with embedded nanopore microcavities, based on a silicon-on-insulator technology for high-throughput optical readouts. Arrays containing more than 14 000 inverted-pyramidal cavities of 50 femtoliter volumes and 80 nm circular pore openings were constructed via high-resolution electron-beam lithography in combination with reactive ion etching and anisotropic wet etching. These cavities feature both, an optically transparent bottom and top cap. Atomic force microscopy analysis reveals an overall extremely smooth chip surface, particularly in the vicinity of the nanopores, which exhibits well-defined edges. Our unprecedented transparent chip design provides parallel and independent fluorescent readout of both cavities and buffer reservoir for unbiased single-transporter recordings. Spreading of large unilamellar vesicles with efficiencies up to 96% created nanopore-supported lipid bilayers, which are stable for more than 1 day. A high lipid mobility in the supported membrane was determined by fluorescent recovery after photobleaching. Flux kinetics of α-hemolysin were characterized at single-pore resolution with a rate constant of 0.96 ± 0.06 × 10−7 s−1. Here, we deliver an ideal chip platform for pharmaceutical research, which features high parallelism and throughput, synergistically combined with single-transporter resolution.

KEYWORDS: Silicon-on-insulator chips, transport kinetics, membrane proteins, optical readout, supported lipid bilayers, nanopores, microcavities

To date, membrane proteins (MPs) are targeted by more than half of all developed drugs,1 underlining their outstanding importance for pharmaceutical industry and basic research. They are involved in a multitude of essential cellular processes including bioenergetics, transport, and communication. Especially, their role in transport processes including bioenergetics, transport, and communication.

High-throughput screening applications are confined by the complexity of interfaces between electronics and ionic systems, except for some advanced techniques using whole-cell or automated patch-clamp recordings.8−11 For parallel analysis of electrogenic transporter activities, an alternative electrophysiological method has been based on solid-supported membranes.12,13 Yet, this technique is unable to achieve the single-transporter level. While these electrode-based approaches are capable of measuring ionic fluxes or the translocation of ionic substances they still lack the efficiency or ability to characterize transporters with nonelectrogenic solutes. Other tools for the analysis of MPs have been utilized, including tethered bilayers,14,15 native vesicle arrays,16−21 and micro-black-lipid membranes,22−24 which are compatible with nonionic cargos.

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So far, these applications are unsuitable for highly parallelized kinetic studies of MPs. In the past decade, supported lipid bilayers (SLBs) have gained attention due to their long-term stability and their ability to mimic native membranes. However, the incorporation of transmembrane proteins is inherently limited because of their close proximity to the solid support. To overcome this drawback, silicon-based microcavities were spanned with membranes creating a platform of supported and free-standing lipid bilayers with techniques such as spreading of giant unilamellar vesicles (GUVs), large unilamellar vesicles (LUVs), or shear-driven SLB formation. In order to transfer the methodology from laboratory research to industrial screening applications, it is required to have in vitro systems (i) revealing highly automated channel recordings for high-throughput applications, (ii) minimizing the sample consumption, and (iii) possessing long-term stability to ensure repeatability without suffering from degradation of sensor interfaces. Recent advances in semiconductor fabrication techniques of micro- and nanostructures with well-defined dimensions as well as the development in the field of solid supported membranes provide a promising direction for studying MP mediated transport processes.

Silicon-based chips containing arrays of nanopores with microcavities combined with fluorescent readout are in the focus of particular interest, as they enable highly parallel protein recordings across lipid membranes. Nonelectrogenic membrane transport proteins can be incorporated via self-spreading of liposomes and their kinetics can be investigated, which is, however, not feasible with electrical setups. Only a few examples for multiplexed analysis using silicon arrays were published, including the characterization of the mechanosensitive channel of large conductance (MscL), the F$_{0}$/F$_{1}$-ATP synthase, and α-hemolysin. Recently, SLBs were produced with the aid of organic solvent across femtoliter- or attoliter-sized cavities, suffering from the fact that organic solvents may be harmful to MPs. Kleefen and colleagues used nanopores with femtoliter compartments, fabricated by electron-beam lithography (EBL), reactive dry, and anisotropic wet etching of a Si$_{3}$N$_{4}$ covered silicon chip. In particular, the bulk bottom handle wafer substrate was fully opaque for visible light, hence not accessible for common backside fluorescent microscopy but limited to upward oriented microscopes. Because of this limitation and owing to the large buffer background fluorescence intensity, it was not possible to separate fluorophores in cavities and buffer reservoir for unbiased single-transporter recordings. Nevertheless, fluxes of fluorescent substances through α-hemolysin and saponin were characterized at single-protein resolution. Urban and colleagues utilized silicon-on-insulator (SOI) substrates, which were processed via reactive ion etching (RIE) creating cylindrical cavities. Chemical vapor deposition of silicon dioxide (SiO$_{2}$) was applied to reduce the cylindrical micrometer openings to nanometer scales. Thereby, rough surfaces with intrusions of SiO$_{2}$ were obtained, leading to diminished spreading abilities as well as limited diffusion properties for encapsulated substances. Nevertheless, an analysis of MP kinetics was presented for MscL and α-hemolysin.

Here, we present a novel architecture of silicon chips containing macroscopic-scale arrays of optically transparent nanopore microcavities that feature a superior homogeneity in shape and size, constituting well-defined femtoliter compartments in SOI substrates. Our unprecedented chip architecture combines the advantages of both previously reported chip structures: (i) extremely smooth, low-strain surface and pore surroundings with well-defined and tapered pore edges, and (ii) top and bottom chip transparency, allowing for full optical readout access for inverted microscopy. Hence automated and multiplexed readout as well as screening become possible. Cavity volume, nanopore diameter, and the patterning of cavity arrays (size and shape) can be precisely controlled on wafer scales via high-resolution EBL in combination with RIE and...
anisotropic wet etching. Our chips contain more than 14,000 inverted-pyramidal cavities arranged in a rectangular pattern with pore distances of 10 μm and openings of 80 nm. As validated by atomic force microscopy (AFM) analysis, the surfaces are extremely smooth on pore surroundings with well-defined and tapered pore edges, hence providing optimal spreading conditions for lipid bilayers. The material properties of the employed SiO2 and Si3N4 layers allow an optically transparent chip design for parallel fluorescence readout of both cavities and buffer reservoir for unbiased single-transporter recordings in a highly parallel fashion. We show that as anticipated, our chip platform indeed features high spreading efficiencies of large unilamellar vesicles (LUV), high lipid mobility, and long-term stability of the free-standing, nanopore-supported lipid bilayer. As proof of concept, kinetics of the model α-hemolysin toxin were analyzed down to single-pore resolution in high-throughput.

Experimental section. Silicon-on-Insulator Substrate. Si3N4 coated, prime-quality 4-in. diameter SOI wafers (Si-Mat, Kaufering, Germany) were composed of a top 3.0 ± 0.5 μm thick undoped silicon (100) device layer, which was separated from the 380 ± 15 μm undoped bulk silicon (100) substrate by a 100 ± 10 nm buried oxide layer (SiO2, “BOX”). Stoichiometric Si3N4 (thickness ~50 nm) was then deposited on both sides of the substrate by low-pressure chemical vapor deposition (LPCVD). The wafers were sawed into 13 × 13 mm² pieces for the following nanopore cavity structure fabrication.

Fabrication and Characterization of Nanopore Cavity Arrays with Transparent Bottoms. The fabrication process of nanopore cavity arrays is schematically depicted in Figure 1. First, a large pyramidal pit was structured on the backside of the chip by a combination of optical lithography, RIE, and aqueous potassium hydroxide (KOH (aq)) wet etching. For this step, Shipley S1818 photoresist was spun onto the wafer at a speed of 6000 rpm for 60 s followed by a soft-bake at 115 °C for 3 min. We then exposed the resist to UV light (wavelength 365 nm, power density ~6.2 mW/cm²) for 15 s through a square mask (1.8 × 1.8 mm²) at the center of the wafer using a mask aligner (SUSS MicroTec, Garching, Germany). Subsequently, the chip was developed in DEV 351 solution (diluted 1:5 vol % in DI water) for 30 s, followed by a RIE (Oxford Instruments, Oxfordshire, U.K.) step using a C4F8/O2 gas mixture for 85 s to remove the exposed Si3N4 layer. After rinsing with acetone, isopropanol, and drying with nitrogen flow, the chips were anisotropically chemically etched in 20 wt % KOH (aq) solution at 80 °C to remove bulk silicon from the backside, resulting in a large pyramidal etch pit. The etching was stopped after 3 h when the etch pit reached the BOX layer below, which served as an etch stop.

For the fabrication of cavity arrays, the chips were extensively rinsed with water to remove KOH residues prior to the following EBL process. For EBL, an e-beam resist (AR-P 6200, Allresist, Strausberg, Germany) was spun on the chip front side at 3000 rpm for 60 s and prebaked at 150 °C on a hot plate for 1 min, resulting in a resist thickness of ~100 nm. Arrays of nanopores (120 × 120 pores) with diameter of 80 nm and pore distance of 10 μm were patterned by an eLiNe system equipped with the Elphy Quantum pattern generator (Raith, Dortmund, Germany) at an acceleration voltage of 30 kV and with beam currents of ~30 pA. The development of the patterns was done in developer AR 600-546 (Allresist, Strausberg, Germany) for 1 min. Subsequently, the chips were rinsed with isopropanol and dried under nitrogen flow. After a postbaking at 130 °C for 1 min, the nanopores patterns were transferred to the Si3N4 layer by RIE, as described above. The residual resist was then removed by immersion in acetone and isopropanol for 10 min each, followed by drying with nitrogen. Finally, arrays of femtoliter cavities with inverted pyramidal shape were formed by an anisotropic wet etching step of the Si device layer in 15 wt % KOH (aq) at 50 °C, using the Si3N4 nanopore as an etch mask. Under these conditions, the etching of mainly Si(111) planes proceeds at a very low rate due to the high etching anisotropy (0.9 ± 0.1 μm h⁻¹). The final cavity volume is adjusted by the etching time. For a 50 femtoliter cavity volume, the etching takes about 3.5 h, resulting in cavities with free-standing Si3N4 membranes of ~36 μm² in size, forming the cavity tops, and transparent cavity bottom areas of ~3 μm².

The nanopore cavity arrays were characterized by scanning electron microscopy (SEM) using the eLiNE system (Raith, Dortmund, Germany) at an acceleration voltage of 5 kV. The surface topography of the Si3N4 membranes on top of the cavities, including the single nanopores, was imaged by high-resolution AFM, utilizing a Veeco Dimension V system (Veeco Instruments Inc., New York, United States) equipped with a Veeco Nanoscope V controller in Tapping Mode. For all topographic measurements, Si cantilever probes (model: SSS-NCLR (super sharp), Nanosensor, Neuchâtel, Switzerland) were used, having nominal force constant of 48 N/m and resonance frequency of 190 kHz. These probes feature a tip radius of about 2 nm, which helps to minimize tip/sample convolution effects. The surface roughness was determined as root-mean-square (rms) value.

Liposome Preparation. LUVs composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids Inc. (Hamburg, Germany) and mixed in molar ratios of 4:3:3 to a concentration of 5 mg/mL and subsequently resolved in HEPES buffer (20 mM HEPES/NaOH, 150 mM NaCl, pH 7.4). LUV formation was conducted as described. Liposomes were extruded 21 times through 100 nm polycarbonate membranes at the LiposoFast-Basic extruder (AVESTIN, Mannheim, Germany). Vesicle sizes were analyzed using the nanoparticle tracking system NanoSight LM10 (Malvern, Herrenberg, Germany).

Chip Preparation. The SOI chips were glued on 8-well sticky-slides (ibidi, Planegg/Martinsried, Germany), followed by oxygen plasma cleaning for 2 min at 0.3 mbar and at 80% power settings with a plasma cleaner (Diener Electronics, Ebhausen, Germany). After immersion in 300 μL of ethanol for 5 min, successive exchange of ethanol was conducted by dilution with HEPES buffer including 5 mM CaCl₂ (total volume 500 μL). This step was repeated 15 times to completely dilute ethanol and ensure wetting of the microcavities. Different dyes were encapsulated via spreading (1 h incubation) of LUVs (1 mg/mL), followed by buffer exchange to remove lipid and fluorophore excess. Optical readout was performed with the confocal laser scanning microscope (CLSM) LSM 880 (AxioObserver from Zeiss, Jena, Germany) equipped with a Plan-Apochromat 20x/0.8 M27 air objective or with the automated NyONE epifluorescence microscope (SYNENTEC GmbH, Elmshorn, Germany) equipped with a 20× magnification air objective (Olympus UPlanSApo 20x/0.75). Images were recorded at different time intervals dependent on the
experiment. Each assay was stopped by adding 25 μL Triton-X 100 10% (v/v) to induce SLB rupture that induce rapid efflux of fluorescent molecules and represented additional proof for the absence of unspecific binding.

**Spreading Efficiency, Long-Term Stability, and Lipid Fluidity of the SLB.** The spreading of LUVs and the resulting SLB were visualized via fluorophore encapsulation. Ten micromolar of ATTO655 (ATTO-TEC, Siegen, Germany) and 5 μM of Oregon Green (OG) dextran (10 kDa) (ThermoFisher Scientific, Darmstadt, Germany) were added, followed by liposome spreading and buffer exchange. Sealing efficiencies were calculated as ratio of dye encapsulated cavities regarding total cavities per field of view. Pearson correlation coefficients were determined for the location of both fluorophores in the individual cavities with ImageJ 1.51o. The long-term stability was monitored at different time points (1, 3, and 24 h) after vesicle spreading with the NyONE microscope. For this purpose, 5 μM of ATTO655 was encapsulated inside the cavities.

FRAP experiments were conducted at the CLSM with LUVs composed of POPC/POPE/POPG as described and additionally doped with 3,3′-dioctadecyl-oxacarbocyanine perchlorate (DiO 0.5 mol %) (ThermoFisher Scientific, Darmstadt, Germany). After spreading, small circular areas of 8 to 15 μm² diameter were bleached within 4 s with high laser intensities. Fluorescence recovery was monitored for ~1 min in ~100 ms intervals dependent on the bleached area. The normalized fluorescence intensity \(I_t\) was calculated according eq 1

\[
I_t = \left[ I_0 + I_{inf} \left( \frac{t}{\tau_{1/2}} \right) \right] \left[ \left( 1 + \frac{t}{\tau_{1/2}} \right) \right]^{-1}
\]

in which \(I_0\) is the initial fluorescence, \(I_{inf}\) is the fluorescence intensity after recovery, and \(\tau_{1/2}\) is the time at half-maximal fluorescence intensity. The lateral lipid diffusion coefficient \(D\) was determined regarding eq 2

\[
D = \frac{r_o^2 \gamma}{4 \tau_{1/2}}
\]

with \(r_o\) as the radius of the excitation spot and \(\gamma\) as the bleaching parameter.47

**Single-Transporter Recordings.** Ten micromolar of ATTO655 (0.6 kDa) and 5 μM of OG dextran (10 kDa) were encapsulated via liposome spreading. After buffer exchange, α-hemolysin was added to final concentrations of 500, 100, or 50 nM. Data were collected in 11 s intervals for 3–4 h with the CLSM. Unspecific binding of enclosed dyes was foreclosed by addition of 25 μL Triton-X 100 10% (v/v) after the experiment. The resulting time traces were processed via the Zen 2.1 black software from Zeiss and the corresponding mean gray values were calculated via ImageJ, followed by plotting and fitting of the translocation kinetics with the Nanocal software (Nanospot GmbH). Two different types of exponential decays were identified: (i) monoexponential decays and (ii) more sigmoidal decreasing curves abruptly converting to exponential decays. Both types of curves were fitted with monoexponential equations, whereas the inflection point of curve type (ii) was used as lower fit boundary (see SI Figure 6).

First-order rate constants \((k_{el})\) were further processed with Origin 9.1 Pro (OriginLab), including descriptive statistics to count traces in individual intervals, peak analysis (Origin 9.1 Pro, peak analyzer), and Gaussian fitting.48 Rate constant errors were obtained for both the fitting error of the single exponentials and the Gaussian distribution fit of the histograms. To provide an upper bound estimate for the total error, we list the larger derived value of both in each case, respectively.

**Figure 2.** Silicon nanopore chip design for multiplexed parallel recordings. Eighty nanometer pores in a Si₃N₄ layer were fabricated via EBL and RIE, followed by wet etching with KOH (aq) to create more than 14,000 inverted-pyramidal cavities per chip. (A) SEM micrograph of a portion of the whole homogeneous array with nanopore cavities appearing as dark gray squares. (B,C) SEM images presenting a single cavity, disclosing a single nanopore in the center of the Si₃N₄ membrane with diameter of ~80 nm. (D) Front side view of a single cavity after removal of most of the Si₃N₄ membrane, disclosing the truncated, inverted pyramidal shape of the cavity with transparent bottom (black square). (E–G) Images of the chip backside after the final KOH etch step showing an array of homogeneous but smaller squares compared to the front side. These squares correspond to the truncated, inverted pyramid tops, hence to the transparent cavity bottoms (cf. dark square in panel D). (H) AFM topography showing a 3D rendered image of a nanopore with highly rounded and smooth edges of the pore opening.
Results and Discussion. Device Fabrication and Characterization. Nanopore cavity arrays with transparent cavity bottoms were realized by a combination of different processes that included optical lithography, EBL, RIE, and KOH (aq) wet etching. The fabrication flowchart is schematically shown in Figure 1 and detailed in Experimental Section. A SOI wafer with 3 μm silicon (100) device layer and 100 nm buried oxide (BOX) was used as a starting substrate, which was then coated with 50 nm Si₃N₄ by LPCVD on both sides. A square window in the Si₃N₄ layer on the backside of the wafer was processed by optical lithography and RIE. Subsequently, an anisotropic wet etch in KOH solution was performed to remove the thick bulk Si through this window, resulting in a large etch pit of pyramidal shape truncated at the BOX layer (etch stop layer; Figure 1A). Here, the shape of a large pyramidal cavity is formed because the etch rate of Si perpendicular to the (100) crystal planes is much higher than perpendicular to the (111) planes.²⁹ In 20% (wt/v) aqueous KOH solution at 80 °C, we observed that the ratio of etch rates (100)/(111) ranged from 45:1 to 50:1.

In the second step, a square array of nanopores (120 × 120 pores) with a pore diameter of 80 nm and pore distance of 10 μm was patterned in the Si₃N₄ top layer by high-resolution EBL and RIE (Figure 1B,C). Finally, the wafer was etched again in KOH solution to fabricate a homogeneous nanopore cavity array (Figure 1D). The size of the cavities was well controlled by adjusting the etching time (see Experimental Section).

The nanopore cavity array was characterized by SEM. The cavities form a well-ordered array with highly homogeneous cavity sizes (Figure 2A). The Si₃N₄ layers on top of the cavities are intact and appear as dark gray squares as they are almost transparent for the electron beam. An image of a single cavity reveals a single pore in the very center of the Si₃N₄ membrane with a diameter of ~80 nm (Figure 2B,C). The magnification of one cavity with the Si₃N₄ top layer almost removed confirms the inverted pyramidal shape of the cavity (Figure 2D). The pyramid tip, visible as a small black square, is truncated at the cavity bottom composed of a SiO₂ layer.

An SEM investigation from the chip backside verifies that the cavities are homogeneous in size (Figure 2E–G). The cavity array was well aligned with the BOX layer window exposed from the backside, both in the center of the chip. The dimensions of the cavity top and bottom squares can be accurately measured by SEM. By further knowing that the angle between the cavity top (Si (100) plane) and the cavity sidewalls (Si (111) plane) is 54.74° and that the height of the cavities is 3.0 ± 0.5 μm (Si device layer thickness), the volume of the cavities can be calculated. In this study, chips with cavity volumes of 50 ± 3 femtoliters were used.

The topography of the nanopores was investigated by high-resolution AFM in tapping mode using a Si cantilever probe featuring a tip with radius ~2 nm, thereby minimizing tip/sample convolution effects. Figure 2H shows a 3D rendered AFM image of a nanopore, revealing that the nanopore opening has well curved and smooth edges. The topography of the Si₃N₄ layer in the area of a certain nanopore cavity was also detailed. SI Figure S1A shows a single cavity with the areas in a single cavity opening and in an area where no cavities were structured.
SLB: Spreading Efficiency, Long-Term-Stability, and Mobility. The formation of pore-spanning lipid bilayers occurs via self-spread of liposomes. Vesicles with an average diameter of ~150 ± 27 nm (SI Figure S2) were used for SLB formation induced by adding CaCl₂ (5 mM CLSM). The latter are mainly in the underlying cavities. These findings are consistent with perfect colocalization of both dye molecules in the microcavities. Colocalization deviations, lacking either green or red fluorescence, can be caused by variations of the encapsulation efficiencies. The latter are mainly influenced by the physical properties of the fluorophores, for example, size and hydrophobicity, especially of the dextran conjugate. We like to note that regions of interest without a defined colocalization were neglected in further analysis. The homogeneity of fluorophore encapsulation, an optimal cavity filling, and the high signal-to-background ratio of our novel device are illustrated as magnifications of sections of Figure 3A, cf. Figure 3B below.

Optimal conditions for optical single-transporter translocation assays were provided by perfect wetting abilities and low unspecific binding of fluorescent dyes (SI Figure S3). Furthermore, compartmentalization of fluorophores in cavities and buffer reservoir can be achieved and imaged due to the transparency of the chip design. For this purpose, ATTO655 was trapped via vesicle spreading, followed by buffer exchange and OG dextran addition (SI Figure 4, merge channel). The focus area of the CLSM was adjusted horizontally to the cavities to visualize solely the chip cavities. The rounded edge shape and the surface of the pore surroundings with nanometer roughness revealed perfect conditions for liposome spreading with 80–96% sealing efficiency.

As illustrated above, advanced applications will be the investigations of slow, nonionic transporters. Therefore, long observation periods with suspended lipid bilayers stable for multiple hours are required. The long-term stability was analyzed for 24 h and visualized via selectively entrapped ATTO655 molecules. Previous studies revealed high SLB stabilities for nanopores in the range of 100 nm. Our SLBs are stable for 3 h without any leakage of fluorophores (see Figure 4A). Even after 24 h, more than 67% of the cavities are still sealed. Notably, fluorophore bleaching as well as membrane permeability can be neglected due to millisecond illumination times and the low membrane interaction factor of ATTO655. Unspecific fluorophore occlusion was ruled out by adding Triton-X 100 10% (v/v) after 24 h to solubilize the SLB. Continuous homogeneous coverage as well as the dynamic properties of the SLB, which are important to preserve the physiological environment of MPs, were examined via FRAP on the SLBs doped with DIO (0.5 mol %). Fluorescence recovery of 97.1 ± 0.3% and a lateral diffusion coefficient of 0.85 ± 0.20 μm² s⁻¹ are consistent with an intact homogeneous, fluid suspended lipid bilayer determined in nine independent measurements. These values are in agreement with the lateral...
diffusion coefficient of lipids measured on glass or silicon based surfaces, which range from 1 to 3 μm² s⁻¹ (Figure 4B).53−55 In summary, all results reveal a successful spreading process, resulting in a stable impermeable and extremely fluidic suspended lipid bilayer, which is able to spatially and temporally compartmentalize molecules in cavities and buffer reservoir. The barrier was used to investigate the transport processes via optical readout and ensures a nature-like environment for integral proteins.

**Single-Transporter Recording of the Membrane Protein Complex α-Hemolysin.** α-Hemolysin was chosen as model membrane protein complex to investigate MPs at single-transporter resolution and in high-throughput. This bacterial pore-forming toxin binds to the choline groups of phospholipids, followed by oligomerization and spontaneous insertion into lipid bilayers.41 The crystal structure of this heptameric protein revealed a pore with its narrowest opening of 1.4 nm.56−59 Nevertheless, apertures up to 2.9 nm were reported, dependent on lipid composition and buffer/salt conditions, allowing the diffusion of molecules up to 2 kDa in size.56 Prior to SLB formation, 10 μM of ATTO655 (0.6 kDa) and 5 μM of OG dextran (10 kDa) were compartmentalized inside the cavities, followed by buffer exchange and imaging via CLSM. The kₑff values were determined for single and double incorporations of α-hemolysin after separation in 35 classes for each histogram. (A) Schematic illustration of the transport experiments with possible scenarios including α-hemolysin-mediated transport, rupture, and cavities without fluorescence changes. (B) Exemplary efflux traces of ATTO655 at different pores at the same chip, mediated by α-hemolysin. (C–E) Histograms of the ATTO655 efflux rates for 500, 100, and 50 nM of α-hemolysin, respectively.

**Figure 5.** Flux kinetics of single α-hemolysin pores. Ten micromolar of ATTO655 (0.6 kDa) and 5 μM of OG dextran (10 kDa) were compartmentalized inside the cavities, followed by buffer exchange and imaging via CLSM. The kₑff values were determined for single and double incorporations of α-hemolysin after separation in 35 classes for each histogram. (A) Schematic illustration of the transport experiments with possible scenarios including α-hemolysin-mediated transport, rupture, and cavities without fluorescence changes. (B) Exemplary efflux traces of ATTO655 at different pores at the same chip, mediated by α-hemolysin. (C–E) Histograms of the ATTO655 efflux rates for 500, 100, and 50 nM of α-hemolysin, respectively.
Here, ATTO655 acts as a flux reporter whereas the dextran-coupled fluorophore serves as membrane impermeable control. Hence, SLB rupture is indicated by a rapid efflux of both fluorophores, while α-hemolysin-mediated translocation events are classified by an exponential decline of the ATTO655 signal and a constant OG signal (Figure 5A). In total, four different situations were observed: (i) α-hemolysin-mediated transport, (ii) membrane rupture, (iii) complex kinetics, and (iv) undefined events. The latter mainly comprised events without control signals of the OG channel, whereas complex kinetics include processes with two declining steps or spontaneous signal increases in one of the two channels. Only traces displaying single-exponential decays in the ATTO655 channel (red) together with a constant signal in the OG channel (green) were accepted for final analysis. Exemplary efflux traces of ATTO655 are shown in Figure 5B. On the basis of the spontaneous insertion and oligomerization of the toxin, the single-exponential decline occurs at different time points, which is similar to previous studies.38

Single-transporter recordings were successively investigated by decreasing the protein concentration. α-Hemolysin coalesces to heptameric pores before those insert into the membrane.60 The likelihood for multiple protein insertion per silicon nitride nanopore drastically diminishes with declining protein concentration. We started by applying α-hemolysin (500 nM final), which has been reported to address the range of single-transporter recordings.36,41 The efflux rates (k_{eff}) of 275 α-hemolysin traces are summarized in a histogram composed of 35 classes. A broad Gaussian distribution was observed with an average efflux value of 3.29 ± 0.23 × 10^{-3} s^{-1} (Figure 5C). Contrary to our observation, single-translocation processes are expected to consist of narrow distributions, as shown for the protein MscL.36 To identify single-transporter events the concentration of α-hemolysin was further decreased successively down to 100 and 50 nM (Figure 5D,E, respectively).

Two hundred and four traces of α-hemolysin-mediated translocation events were analyzed after adding α-hemolysin (100 nM final). The classification as well as the intervals were chosen as described (Figure 5D). Two populations were identified by peak analysis and fitted with Gaussian distributions. The average efflux values of the first Gaussian peak (red) at 1.23 ± 0.25 × 10^{-3} s^{-1} clearly suggest that at 500 nM of α-hemolysin (see Figure 5C) single-transporter recordings were superimposed by the elevated statistics of multiple protein insertions per silicon nanopore. This statement is corroborated by similar average efflux values of 3.25 ± 0.44 × 10^{-3} s^{-1} at 100 nM of α-hemolysin (Figure 5D, blue curve) in respect to the average efflux value of 3.29 ± 0.23 × 10^{-3} s^{-1} at 500 nM of α-hemolysin (Figure 5C).

The single-transporter regime was reached by finally decreasing the concentration of α-hemolysin down to 50 nM. Here, 117 efflux traces were analyzed and distributed as described above, and two distinct Gaussian peaks were identified. The first population (red) raises at the expense of the second one (blue) (Figure 5E). The 10-fold lower concentration than previously reported for single α-hemolysin recordings,38,41 in combination with only 4.8% of sealed cavities showing monoexponential decays, indicates that the single-transporter regime has been reached because the likelihood of protein insertion per silicon nitride nanopore has decreased drastically. We interpreted our findings as incorporation of one or two α-hemolysin molecules inside a single silicon nitride nanopore. Thereby, we identified an average efflux value of 0.96 ± 0.06 × 10^{-3} s^{-1} for single- and an average efflux constant of 2.89 ± 0.12 × 10^{-3} s^{-1} for double-protein recordings (Figure 5E). By decreasing the α-hemolysin concentration, the main peak shifted from two protein insertions per nanopore in panel C to one protein per nanopore in panel E.

Slight shifts in the exact average efflux values at all three concentrations can be explained by statistic variations, especially for the final concentration of 50 nM α-hemolysin. However, all corresponding values are comparable in the range of error. Nevertheless, the concentration series of α-hemolysin clearly demonstrates that our silicon chips are suitable for single-transporter recordings. Additionally, the efflux values for single α-hemolysin recordings are in the range of previous data for MscL.36 Efflux rates of α-hemolysin in the order of 10^{-5} s^{-1} were not reproduced.41 However, a later study showed single-protein recordings of α-hemolysin with rates of 5.5 × 10^{-4} s^{-1}.37 Their results are in good agreement with our findings and small deviations occur based on different fluorophores, lipids, buffer/salt conditions, and variations in chip architecture and volumes. These parameters make it difficult to compare the exact α-hemolysin efflux kinetics. Nevertheless, our values are in the range of reported α-hemolysin efflux rates, which demonstrates that our novel chip design is suitable for single-protein recordings.37

We finally tested if the efflux kinetics are altered by encapsulation of two dyes in one cavity. Therefore, 10 μM of ATTO655 was encapsulated inside the cavity and 5 μM of OG dextran (10 kDa) was applied after SLB formation (SI Figure S4). α-Hemolysin was added (500 nM final) and 186 traces were analyzed resulting in an average efflux value of 3.40 ± 0.13 × 10^{-3} s^{-1} (SI Figures S5A and S6), consistent with the results above (Figure 5C). Furthermore, rupture events of the SLB were analyzed revealing more than 10-fold faster efflux rates for the diffusion of 10 μM ATTO655, indicating that our single-protein recordings are not biased by lipid rupture processes (SI Figure S5B). In short, single-pore efflux kinetics of α-hemolysin at different concentrations were presented that demonstrate the viability of the transparent nanopore-chips for high-throughput single-transporter studies.

**Conclusion.** We presented a novel SOI-based nanopore cavity platform for highly parallelized single-transporter studies using optical readout. The fabrication of the chips, which comprise large, homogeneous arrays of cavities with both top and bottom optical transparency, were controlled precisely on wafer-scale, delivering ideal conditions for screening approaches. A homogeneous coverage of the chip surface with an SLB was verified by fluorescent dye encapsulation experiments. On the basis of the transparent nature of SiO2 and Si3N4 it is possible to track influx as well as efflux processes of fluorescent solutes with high contrast. Single-protein resolution was clearly demonstrated with the bacterial toxin α-hemolysin. The self-spreading process allows the use of proteoliposomes. Thereby, reconstitution of MPs in liposomes can be used to make the presented chip design suitable for medically relevant transporters, like the transporter associated with antigen processing (TAP)61 or Thermus thermophilus multidrug resistance proteins A and B (TmrAB), a functional homologue of human TAP.62 The high lipid mobility as well as the long-term stability of the SLB create a perfect platform for the characterization of nonionic transporters in single-protein resolution, which we intend to elaborate on in the future. By reducing the cavity volume and increasing the
number of microcapacities per field of view, higher parallelism can be accomplished, based on thinner top silicon layer architectures and smaller pore-to-pore distances. Further, vector-based cluster analysis may become a future alternative approach to data analysis for extracting pore populations with transporters.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.8b01252.

Figures S1–S6 (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(18) Christensen, A. L.; Lohr, C.; Christensen, S. M.; Stamou, D. Lab Chip 2013, 13, 3613–3625.


(61) Mayerhofer, P. U.; Tampé, R. J. Mol. Biol. 2015, 427, 1102–1118.
