Supporting Information

Proteins on supported lipid bilayers diffusing around proteins fixed on acrylate anchors

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MATERIALS AND METHODS

Lithography. Multiphoton polymerization is achieved using a 780 nm femtosecond pulsed laser (FemtoRay 780, 50 MHz repetition rate, 100 fs pulse duration, Menlo Systems GmbH, Germany). An oil immersion objective lens (Zeiss α-plan Apochromat, 100x, numerical aperture (NA) = 1.46, Carl Zeiss AG, Germany) is used for focusing the beam into the photoresist. Power adjustment was carried out with an acousto-optic modulator (Q1133, Isomet Ltd., UK). The point spread function was monitored by detection of the backscattered excitation beam with an avalanche photodiode (APD-SPCM-AQRH, PerkinElmer Optoelectronic Inc., USA). Sample movement was carried out by a three axes piezo stage (P-562.3CD, Physik Instrumente PI, Germany) with a bidirectional positioning accuracy of 2 nm / 2 nm / 4 nm (x/y/z) and 200 µm travel range in each direction. This stage is mounted on top of a coarse xy-motor-stage (M-686.D64, Physik Instrumente PI, Germany) with a travel range of 25 mm x 25 mm. The two stages are driven in closed loop using two controllers (E710.3CD and C-867.260, Physik Instrumente PI, Germany). A LabView program (LabView, National Instruments Corp., USA) is used for control of the lithography and imaging processes. A sketch of the system can be found in Ref. [1]

Fluorescence microscope. Fluorescence images were taken with an Olympus IX81 inverted microscope (Olympus Austria GmbH, Austria). For excitation, lasers with wavelengths of 642 nm (PhoxX 642, Omicron-Laserage Laserprodukte GmbH, Germany), 532 nm (Cobolt Samba, Cobolt, Sweden) and 491 nm (Cobolt Calypso, Cobolt, Sweden) were used. The beams were focused by an oil immersion objective lens (Olympus UApoN, 100x, NA = 1.49). The emitted fluorescence light was detected by an EMCCD-camera (Andor iXon3 897 (back illuminated), Andor Technology Ltd., UK) with 512 x 512 active pixels and single photon sensitivity. A three axes piezo stage (TAO 3-axis sample scanning module, JPK Instruments, Germany) with 100 µm x 100 µm x 10 µm travel range in x, y and z direction was used for sample positioning. Illumination time of 5 ms is controlled by acousto-optic modulators (1205C-2, Isomet Corp., USA). The delay between two consecutive images was 100 ms. The system is controlled by a custom-made program (LabView, National Instruments Corp., USA).

Materials. Carboxy-functional polymer structures were fabricated using a photoresist consisting of two different acrylates and a photoinitiator. The monomers are pentaerythritol triacrylate (PETA, Sigma Aldrich Co., USA) containing 300 ppm-400 ppm monomethyl ether hydroquinone (MEHQ) as inhibitor, and 20 wt% 2-carboxyethyl acrylate (CEA, Sigma Aldrich Co., USA) containing 900 ppm-1100 ppm MEHQ. 1 wt% Irgacure® 819 (IC 819, BASF Schweiz AG, Switzerland) was added as photoinitiator. Structures without carboxy-functionality were fabricated using pure PETA with 1 wt% of IC 819. The chemical structures of the photoresist ingredients can be found in Figure S1.

Sample preparation. All polymer structures were fabricated on glass slides cleaned with acetic acid (#1.5 Menzel Gläser, Germany). To fabricate the polymer structures, 0.3 µl of photoresist was drop cast on the glass substrate. Polymerization is achieved by focusing a femtosecond laser beam into the photoresist. No additional pre-preparation methods (like for e.g. spin-coating) are required. The polymer anchors for immobilization of proteins were fabricated using 4.5 mW of 780 nm femtosecond pulses and an illumination time of 20 ms per anchor. The polymer grids were fabricated as follows[2]: First, a pure PETA grid was fabricated using an excitation power of 5.5 mW. The remaining pure PETA photoresist was diluted (100x amount) with a CEA containing photoresist (PETA with 20 wt% CEA and 1 wt% IC 819). The carboxy functional grids were written using an excitation power of 5 mW. All laser powers were measured directly before entering the objective lens. After polymerization, the structures were developed by rinsing with acetone (Merck KGaA, Germany) and drying with a nitrogen stream. Samples were stored in air at room temperature.

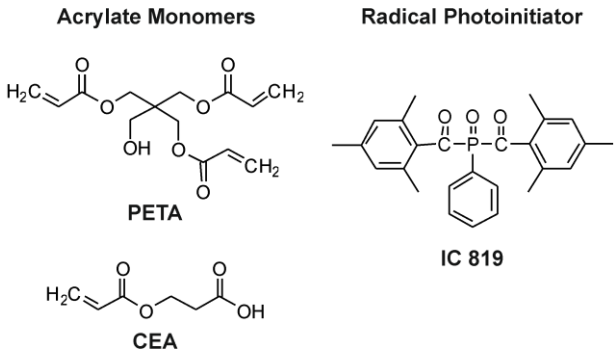

Figure S1: Chemical structures of the monomers and the radical photoinitiator. The carboxy functional polymer structures were fabricated using 20 wt% of 2-carboxyethyl acrylate (CEA) within pentaerytritol triacrylate (PETA) and 1 wt% of Irgacure® 819 (IC 819) as photoinitiator. The unfunctionalized acrylate structures were written using PETA with 1 wt% IC 819.

Protein expression and purification. The DNA fragment encoding photoswitchable monomeric protein orange (PSmOrange)[3] was synthesized and cloned into a pET-30(a) vector (Merck KGaA, Germany) using NdeI and EagI restriction sites (Eurofins Genomics GmbH, Germany). For purification and functional binding, a poly-histidine tag (protein amino acid sequence – EFGGGSGGGSGGGGTGGTSHHHHHHHHHHHH) was added to the C-terminus of PSmOrange. Emerald GFP (EmGFP) was cloned into the pRSET plasmid (Thermo Fisher Scientific Inc., USA).

Competent E. coli cells (One Shot BL21 Star (DE3), Invitrogen for psmOrange, One Shot BL21 Star (DE3) pLysS for EmGFP) were transformed with plasmid DNA using the heat shock method, and the cells were plated onto selective LB agar plates supplemented with kanamycin or ampicillin, respectively. Single colonies were picked and grown in LB-Miller medium. Protein expression was induced by addition of 0.1 mM Isopropyl-b-D-Thiogalactopyranoside.

Bacteria were incubated at 22° C for 72 h with shaking at 120 rpm. Cells from six 1l cultures were pelleted by centrifugation, resuspended in lysis buffer (50mM Tris, 300mM NaCl, 20mM imidazole, 1% Triton-X-100, 1mg/ml lysozyme, pH 7.8) supplemented with protease inhibitor (Protease Inihibitor Cocktail Tablet, AppliChem GmbH, Germany), and lysed by several freeze-thaw cycles using liquid nitrogen. Proteins were purified by affinity chromatography using Ni-NTA resin (Qiagen GmbH, Germany) and size exclusion chromatography using a 26/60 Superdex 200 column (GE Healthcare GmbH, Germany). Protein concentration and maturation rate were determined photometrically. Sample purity was analyzed by SDS-PAGE. Proteins were concentrated (Amicon, 10,000 MWCO) to ~0.2 mM and stored in 50% glycerol stocks at −20 °C.

PSmOrange sequence:

MVSKGEENNMAIIKEFMRFKVRMEGTVNGHEFEIEGEGEGRPYEGFQTAKLKVTKGGPLPFAWDILSPLFTY
GSKAYVKHPADIPDYFKLSFPEGFKWERVMNYEDGGVVTVTQDSSLQDGEFIYKVKMRGTNFPSDGPVMQK
KTMGWEASSERMYPEDGALKGEIRMRLKLKDGGHYTSEVKTTYKAKKSVQLPGAYIVGIKLDITSHNEDYTI
VEQYERAEGRHSTGGMDELYKEFGGGSGGGSGGGGTGGTSHHHHHHHHHHH

Buffer solutions. HEPES buffer (50 mM, pH 7.5) was prepared by dissolving 0.6 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Carl Roth GmbH & Co KG, Germany) in 50 ml ddH_2O. EDTA buffer (50 mM, pH 7.4) was prepared by dissolving 931 mg ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Carl Roth GmbH & Co KG, Germany) in 50 ml ddH_2O. Carbonate buffer (50 mM, pH 9.6) was prepared as follows: 0.21 g $NaHCO_3$ (Merck KGaA, Germany) and 0.265 g of Na_2CO_3 (Merck KGaA, Germany) were mixed with 50 ml ddH_2O. Additionally, HEPES buffer (500 mM, pH 9.6) using 1.19 g of HEPES mixed with 10 ml ddH_2O water was prepared. Buffers were stored at 4°C. Phosphate buffered saline (pH 7.4) was used as received (Merck KGaA, Germany).

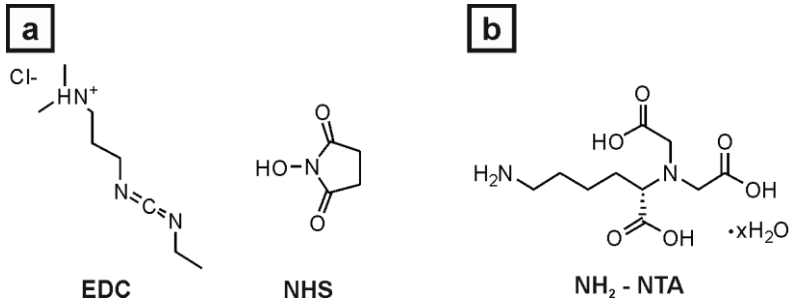

Figure S2: Chemical structures of the molecules used for functionalization of carboxy-functional polymer structures. a) EDC and NHS are used for conversion of the carboxy groups to NHS-esters. **b)** After conversion of carboxy groups to NHS esters, NH_2-NTA was bound to the polymer structures to enable his-tag protein binding.

Hybrid protein platform. A homemade reaction chamber consisting of two component resin (Twinsil®, Picodent GmbH, Germany) was glued on the glass slide followed by rinsing with ethanol for cleaning and incubation with EDTA buffer for 5 minutes. Then, EDTA buffer was exchanged with HEPES buffer (50 mM, pH 7.5). 30 µl of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine vesicle solution (POPC, Avanti Polar Lipids Inc., USA) with 4 wt% of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N (cap biotinyl) (sodium salt) (16:0 Biotinyl Cap PE, Avanti Polar Lipids Inc., USA) in phosphate buffered saline was added and incubated for 20 minutes. This lead to a lipid bilayer spiked with biotinylated lipids, leaving the carboxy-functional polymer structures uncovered[4]. After washing with HEPES buffer (50 mM, pH 7.5), the carboxy-groups on the surface of the polymer structures were converted to NHS esters. Conversion was achieved by using the heterobifunctional cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma Aldrich Co., USA) in combination with N-hydroxysuccinimide (NHS, Sigma Aldrich Co., USA) (Figure S2a). We incubated with 0.1 M NHS and 0.4 M EDC dissolved in ddH_2O for 30 minutes, followed by a washing step using HEPES buffer (50 mM, pH 7.5). In order to functionalize the polymer structures with nitrilotriacetic acid (NTA), 4 mg of N_α-N_α-Bis(carboxymethyl)-L-lysine hydrate (NH_2-NTA, Sigma Aldrich Co., USA) (Figure S2b) was dissolved in a mixture of different buffers (500 µl HEPES pH 7.5, 25 µl HEPES pH 9.6 and 20 µl EDTA) similar to Ref.[5] added and incubated for 30 minutes, followed by another washing step with HEPES buffer (50 mM, pH 7.5). Then, 10 µl of a 10 mM aqueous solution of nickel (II) chloride was incubated for 10 minutes. After washing with HEPES buffer (50 mM, pH 7.5) blocking of the remaining carboxy groups and NHS esters is achieved by adding 10 µl of 0.1 wt% ovalbumin (albumin from chicken egg white, Sigma Aldrich Co., USA) and incubating for 10 minutes. Subsequently, his-tagged fluorescent proteins, i.e. his-tagged EmGFP or his-tagged PSmOrange, were added and immobilized to the nickel-NTA binding sites on the polymer structures. After an additional washing step with HEPES buffer, 1 µl streptavidin labeled with Alexa Fluor® 555 with a concentration of 1 µg/ml (Life Technologies, USA) was attached to the biotin molecules within the lipid bilayer.

CONTROL EXPERIMENTS

Unspecific binding of proteins to structures.

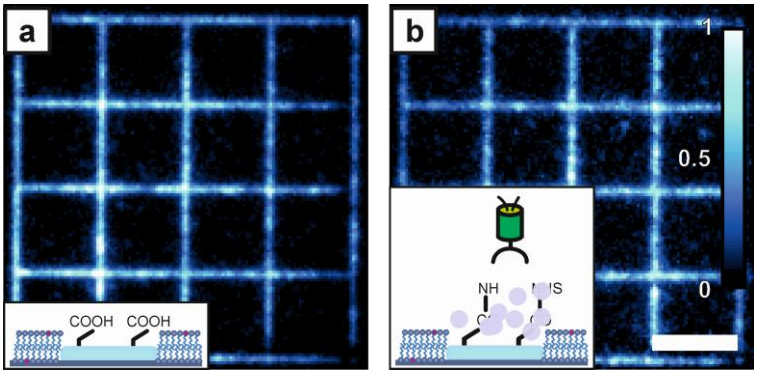

Figure S3: Passivation efficiency of albumin in absence of nickel ions and NTA. a) Auto-fluorescence (excitation wavelength 491 nm) of a carboxy acrylate structure in HEPES buffer (pH 7.5). **b)** Carboxy groups were activated with NHS esters and passivated with albumin. NTA and Ni are omitted. After incubation with his-tagged EmGFP, the average intensity is comparable; EmGFP is not binding to the polymer structures. The scale bar of 5 µm in (b) and the calibration bar in (b) are valid for (a) and (b).

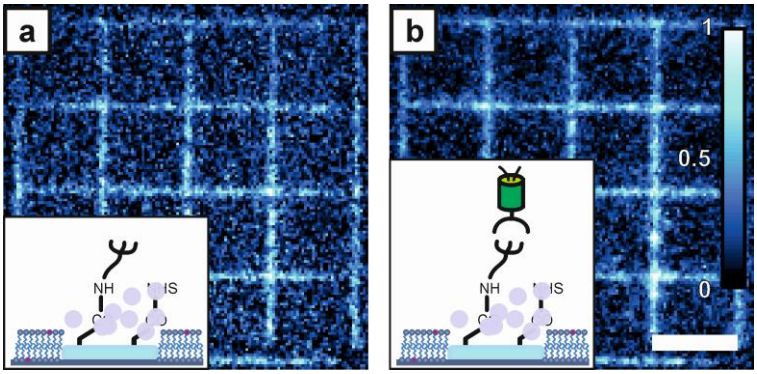

Figure S4: Unspecific binding of his-tagged EmGFP to NTA modified carboxy-acrylate structures in the absence of nickel ions. a) Auto-fluorescence signal (excitation wavelength 491 nm) of the carboxy functional structure before incubation with fluorescent proteins and in the absence of nickel ions. The structure was passivated using 0.1% albumin. **b)** Fluorescence image of the carboxy structure after incubation with his-tagged EmGFP in the absence of nickel ions. The fluorescence signal did not increase significantly in comparison to a). The scale bar of 5 µm in (b) and the calibration bar in (b) are valid for (a) and (b).

The average number of GFP molecules for the experiment (see Figure 2 in the manuscript) was ~ 0.18 molecules/pixel (PETA + 20 wt% CEA with 1 wt% IC 819) and for the negative control (PETA with 1 wt% IC 819) the signal is ~ 0.02 molecules/pixel (pixelsize 160 nm, 5 ms illumination time, excitation intensity ~ 3.3 kW/cm^2). For the negative control shown in Figure S3b, the signal is 0.009 GFP molecules/pixel, for the negative control shown in Figure S4b

the signal is 0.02 GFP molecules/pixel. For determination of the number of molecules, we calculated the ratio of the average signal of the structure and single GFP signal determined by a gaussian fitting.[6] The unspecific binding is about 7 times lower than the specific binding.

Bilayer Quality. We determined the diffusion constants on glass slides with different cleaning procedures: on the one hand on glass slides cleaned with acetic acid (as used in the original manuscript) and on the other hand on glass slides cleaned with peroxymonosulfuric acid. In order to quantify the influence of the remaining unpolymerized photoresist left over after washing, we also placed a droplet of photoresist on the glass slides (cleaned with acetic acid or peroxymonosulfuric acid) and washed it away with acetone before spreading the lipid vesicles.

The diffusion constants were determined by manual single particle tracking (as described in 7). In order to evaluate a possible influence of crosslinking of biotin via streptavidin, we not only carried out the experiments with streptavidin-Alexa Fluor® 555 bound to biotinylated lipids, but also with the lipophilic fluorescent tracer DiD (1 mg dissolved in 1 ml ethanol, Invitrogen/Thermo Scientific, USA).

Table 1. Dependence of diffusion constants of DiD and streptavidin-Alexa Fluor® 555 within supported lipid bilayers on the treatment of the glass substrates. Glass slides were either cleaned using peroxymonosulfuric acid or with acetic acid. On some of the cleaned glass slides, the photoresist PETA with 20 wt% CEA and 1 wt% IC 819 was applied and subsequently removed by washing with acetone prior to the spreading of the lipid bilayer.

Substrate	**D_{DiD}** / $\mu m^2/s$	**D_{SA}** / $\mu m^2/s$
Peroxymonosulfuric acid cleaned glass slide	0.30 ± 0.03	0.27 ± 0.06
Peroxymonosulfuric cleaned glass slide with photoresist applied and washed	0.29 ± 0.03	0.25 ± 0.06
Acetic acid cleaned glass slide	0.15 ± 0.02	0.13 ± 0.02
Acetic acid cleaned glass slide with photoresist applied and washed	0.16 ± 0.02	0.12 ± 0.05

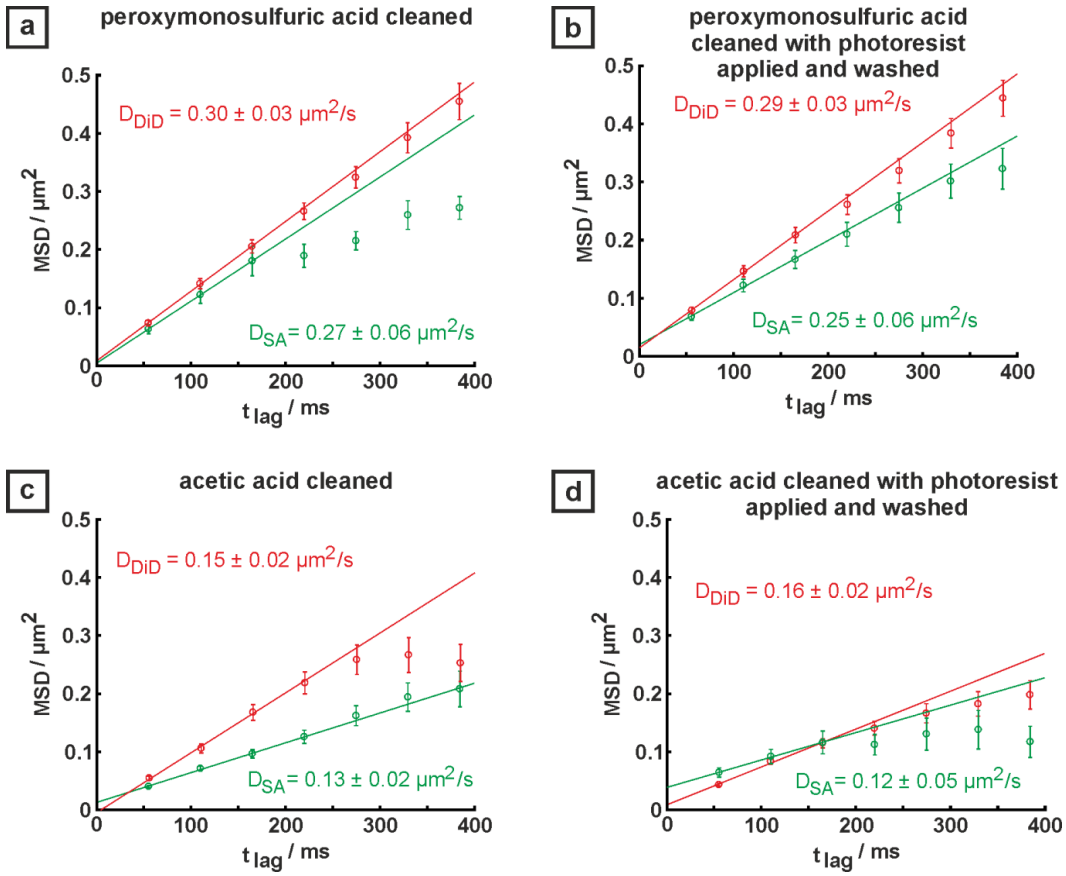

Figure S5. Diffusion constant of different fluorescent probes within the lipid bilayer on differently treated substrates. Red depicts the diffusion constant for DiD incubated lipid bilayers and green the diffusion constant of the streptavidin-Alexa Fluor® bound to the biotinylated lipids within the lipid bilayer.

Overall, we observed that the cleaning procedure strongly influences the diffusion properties of the lipid bilayer. Best results are yielded on peroxymonosulfuric acid treated glass coverslips. In this case, the diffusion constant (D) is in the range between 0.2 to 0.4 $\mu m^2/s$ (see Figure S5a and b) which is consistent with published data. The application of the photoresist and washing has some, but a minor influence. The diffusion constant is decreased by less than 10%.

For with acetic acid cleaned slides, D is in the range of 0.1 to 0.2 $\mu m^2/s$ (see Figure S5c and d). In general, the diffusion constant of streptavidin is lower than the diffusion constant of DiD.

Polymer lines as barrier for lipids. Equidistant horizontal polymer lines (height ca. 20 nm, width ca. 100 nm) have been structured and incubated with POPC vesicles. For bilayer visualization, the lipid bilayer has been incubated with DiI (see Figure S6). Figure S6a shows a DiI signal originating from a lipid bilayer which has bleached (500 ms bleach pulse, I = 3.3 kW/cm^2) in the center. It is clear that diffusion is stopped by the upper and lower horizontal line but DiI starts to diffuse in from the left and the right. Figure S6b depicts the same area after ~ 500 ms recovery time.

The DiI signal recovery occurs only from the left and the right side, not from the top or bottom. This indicates that the polymer lines act as barriers, hindering lipid exchange between the bleached and non-bleached area.

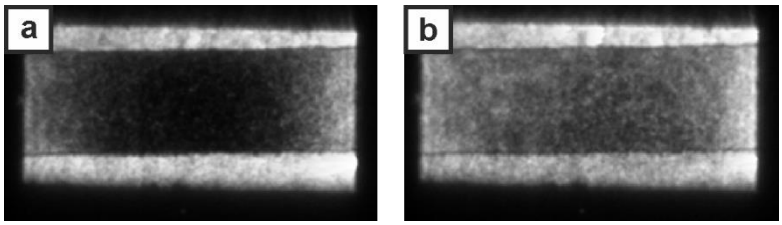

Figure S6: Polymer lines as barriers for the supported lipid bilayer. a) Bleached image of a lipid bilayer labeled with DiI. Two polymer lines with a height of about 20 nm act as barriers. b) Recovery image after 500 ms recovery time. The DiI labeled lipids diffuse from left and right into the bleached region, but not from above or below the polymer barriers. Excitation wavelength: 491 nm

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