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Supporting Information

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Ultrasensitive and Selective Protein Recognition with Nanobody-Functionalized Synthetic Nanopores

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Experimental Procedures

Determination of Dimensions of Single Nanopores

The dimensions of single nanopores were calculated according to previously established protocols (Apel P *et al*. Nucl. Instrum. Methods Phys. Res. B, **2001**, 184, 337–346). Briefly, the base opening *D* was calculated according to **Equation S1** as a function of the etching time *t*.

Equation S1 $$D = 2.13\ \frac{nm}{min} \cdot 2 \cdot t$$

To this end, membranes were fixed between two custom-made conductivitiy cells. The *cis* side was filled with etching solution (9 M NaOH) while the *trans* side was filled with a stopping solution (1 M KCl, 1 M formic acid). A gold electrode was then placed into each cell and a voltage of -1 V was applied. Initially, no current was measured across the membrane, but approximately after 2-3 h the etchant breaks through the membrane. Consequently, a substantial increase in the current is detected across the membrane which defines the breakthrough point and demonstrates the successful formation of a nanopore. The etching was generally stopped after the current reaches a value of a few nA. The progress of an exemplary etching procedure is shown in **Figure S1** while the experimentally determined etching times *t* and the calculated base opening *D* are summarised in **Table S2**.

The tip opening *d* was experimentally determined in separate *I-V* measurements. To this end, the etched nanopore was fixed between two custom-made conductivitiy cells that were filled with an electrolyte (1 M KCl, pH 3.0). A pair of Ag/AgCl-electrodes was then placed into each cell and connected to the picoammeter/voltage source (Keithley 6487, Keithley

instruments, Ohio). A voltage was subsequently ramped between -0.5 V and 0.5 V for 3 periods, and the resulting current was measured. The tip opening *d* was subsequently derived from the slope value of the experimentally determined *I-V* curve according to **Equation S2**.

Equation S2

$$I = \underbrace{\frac{D\,\pi\kappa}{4L} \cdot d}_{slope\ value} \cdot U$$

Here, *L* refers to the channel length corresponding to the foil thickness (12 µm), *I* refers to the measured current, *U* refers to the applied voltage and *κ* refers the specific conductivity of the 1 M KCl solution (10.48 S/m at 25 °C, pH 3.0), which was experimentally measured using a pH/mV Meter (Mettler Toledo^{TM} FiveEasy^{TM} F20). An exemplary *I-V* measurement is shown (**Figure S2**) while the experimentally determined slope values and the calculated tip opening *d* are summarised in **Table S3**

Recombinant Protein Expression

mCherry, sfGFP, Nb^{GFP}-$(GGS)_2$-SpyCatcher002, Nb^{mCh}-$(GGS)_2$-SpyCatcher002 and $Nb^{\alpha Am}$-$(GGS)_2$-SpyCatcher002 were produced in *E. coli* BL21(DE3) transformed with the corresponding plasmid pACYCT2-sfGFP-His_6, pFLinkC-XE-T7-mCherry-Strp-LPETGG, pFLinkC-XE-T7-Nb^{GFP}-$(GGS)_2$-SpyCatcher002-GASPAG-Strp, pFLinkC-XE-T7-$NB^{mCherry}$-$(GGS)_2$-SpyCatcher002-GASPAG-Strp and pFLinkC-XE-T7-$NB^{\alpha Am}$-$(GGS)_2$-SpyCatcher002-GASPAG-Strp, respectively. In all cases, a preculture was used to inoculate 800 mL of LB medium (supplemented with 90 µg/mL ampicillin in all cases exept for pACYCT2-sfGFP-His_6 where 25 µg/mL chloramphenicol was used) in a 3 L shaking flask. Protein expression was induced with 0.5 µM IPTG when cells reached an OD_{600} 0.6 and left to express for 4 h at 25 °C. Cultures

were harvested by centrifugation at 8,000 x g for 20 min at 4 °C and washed once with Buffer W (150 mM NaCl, 100 mM Tris-HCl pH 8.0) before being stored at -20 °C until further use.

Purification of Strep-tagged Proteins

On the day of purification, cells expressing Strep-tagged proteins were resuspended and thawed in 40 mL Buffer W (150 mM NaCl, 100 mM Tris-HCl, pH 8.0) and crushed *via* four passes through an Emulsiflex C3 (Avestin). After centrifugation at 25,000 × g for 1 h at 4 °C, the sample was filtered through a 0.45 µm syringe filter. Strep-tagged proteins were purified by affinity chromatography on 1 mL StrepTrap HP colums using an automated ÄKTA Pure L protein purification system according to manufacturer's instructions (GE Healthcare). Proteins were generally eluted with Buffer E (150 mM NaCl, 100 mM Tris-HCl, 2.5 mM desthiobiotin pH 8.0) and the concentration estimated by UV absorption at 220 nm. Finally, proteins were transferred into storage buffer (100 mM KCl and 10 mM Tris-HCl, pH 8.0) by gravity flow size exclusion chromatography on PD10 columns before aliquots were flash frozen in liquid nitrogen and stored at -80 °C until further use.

Purification of sfGFP

The His_6-tagged protein sfGFP was purified in an equivalent fashion by affinity chromatography on 1 mL Ni^{2+}-NTA colums using an automated ÄKTA Pure L protein purification system according to manufacturer's instructions (GE Healthcare). Here, Buffer W additionally contained 20 mM imidazole while Buffer E for elution contained 250 mM imidazole instead of 2.5 mM desthiobiotin.

Purification of MBP-SpyTag-Tether

The SpyTag-tether was generally produced as an MBP-fusion protein in *E. coli* BL21(DE3) transformed with the plasmid pET24-MBP-SpyTag-tether-LPETGG. A preculture was used to inoculate 2 L LB medium supplemented with 50 µg/mL kanamycin in a 5 L shaking flask. Protein expression was induced with 0.5 µM IPTG when cells reached an OD_{600} 0.5 and left to express for 4.5 h at 30 °C. Cultures were harvested via centrifugation and stored at -20 °C overnight. The following day, cells were resuspended in 70 mL Buffer W (150 mM NaCl, 100 mM Tris-HCl pH 8.0) and crushed *via* four passes through an Emulsiflex C3 (Avestin). After centrifugation at 25,000 × g for 1 h at 4 °C, the supernatant was sonicated for 30 s on ice to destroy any residual chromosomal DNA before being filtered through a 0.45 µm syringe filter. Proteins were purified by affinity chromatography on 1 mL StrepTrap HP colums using an automated ÄKTA Pure L protein purification system according to manufacturer's instructions (GE Healthcare). The protein was eluted in Buffer E (150 mM NaCl, 100 mM Tris-HCl, 2.5 mM desthiobiotin, pH 8.0) and the concentration estimated by UV absorption at 220 nm. Protein aliquots were flash frozen in liquid nitrogen and stored at -80 °C until further use.

Azp-coupling to the MBP-SpyTag-Tether

Purified proteins were thawed on ice. Azp-coupling reactions were performed in 100 µL reactions in 1.5 mL reaction tubes in 100 mM NH_4HCO_3 pH 7.8. Azp was added from a 1 M stock (stored in 1 M acetic acid) to a final concentration of 100 mM. Sortase A5M was included to a final concentration of 10 µM and the MBP-SpyTag-Tether was included at 60 µM. The Azp-coupling reaction was performed for 3 h at 37 °C according to established protocols [24]. Subsequently, buffer was exchanged into 50 mM Tris-HCl, 2 mM DTT, pH 8.0

by ultrafiltration through a Vivaspin 20 centrifugal concentration with a 10 kDa molecular weight cut off at 10,000 × g in a table top centrifuge.

Proteolytic Removal of MBP

Prior to application to the polymer foils, the SpyTag-Tether was cleaved off MBP using 30 µg/mL TEV protease in 50 mM Tris-HCl pH 8.0, 2 mM DTT. TEV cleavage was performed at 18 °C overnight at 15 rpm on a 3D rocker. On the following day, the SpyTag-tether was separated from the larger proteins (i.e. TEV protease, Sortase A5M, and residual MBP cleavage product) in the reaction mix by ultrafiltration through a Vivaspin 500 centrifugal concentrator with a 10 kDa molecular weight cut off at 10,000 × g in a table top centrifuge.

Summary of Protein Sequences

Nb^{GFP}-SpyCatcher002

Blue: Nanobody-GFP. Yellow: SpyCatcher002, Red: Strep-Tag

MGQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRSSY
EDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGQGTQVTVSSGGSGGSG
VTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTWISDGH
VKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHTGGASPAGGWSH
PQFEKGIA*

Nb^{mCherry}-SpyCatcher002

Pink: Nanobody-mCherry. Yellow: SpyCatcher002, Red: Strep-Tag

MGQVQLVESGGSLVQPGGSLRLSCAASGRFAESSSMGWFRQAPGKEREFVAAISWSGGATNY
ADSAKGRFTLSRDNTKNTVYLQMNSLKPDDTAVYYCAANLGNYISSNQRLYGYWGQGTQVTV
SSGGSGGSGVTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKT
ISTWISDGHVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHTGG
ASPAGGWSHPQFEKGIA*

Nb$^{\alpha AM}$-SpyCatcher002

Grey: Nanobody-α-amylase, Yellow: SpyCatcher002, Red: Strep-Tag

MGQVQLVESGGGSVQAGGSLSLSCAASTYTDTVGWFRQAPGKEREGVAAIYRRTGYTYSADS

VKGRFTLSQDNNKNTVYLQMNSLKPEDTGIYYCATGNSVRLASWEGYFYWGQGTQVTVSSGG

SGGSGVTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTW

ISDGHVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHTGGASPA

GGWSHPQFEKGIA*

MBP-SpyTag-Tether

Yellow: MBP; Green: TEV-site; Pink: SpyTag002; Dark Green: TVMV-site. Red: Purification tag. Grey: Sortase recognition motif

MGDRGPEFMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAAT

GDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSL

IYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYD

IKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSK

VNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGA

VALKSYEEELVKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKD

AQTNSSSENLYFQSGPPPPLPPKRRRGVPTIVMVDAYKRYKGGSGGSETVRFQSGSTSGSGK

PGSGEGSTKGWSHPQFEKGLPETGG

Immobilized Azp-functionalised SpyTag-tether:

SGPPPPLPPKRRRGVPTIVMVDAYKRYKGGSGGSETVRFQSGSTSGSGKPGSGEGSTKGWSH

PQFEKGLPET-Azidopropanamin(Azp)

GFP

Green: sfGFP. Red: His_6-tag.

MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVT

TLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIE

LKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNT

PIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGTSGGHHHHH

H*

mCherry

Pink: mCherry. Red: Strep-Tag

MGVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPF

AWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEF

IYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTT

YKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGGASPAGGWSH

PQFEKGLPETGG*

Porcine Pancreatic α-Amylase (Protein Sequence Derived of Uniprot without Signal Sequence)

QYAPQTQSGRTSIVHLFEWRWVDIALECERYLGPKGFGGVQVSPPNENIVVTNPSRPWWERY

QPVSYKLCTRSGNENEFRDMVTRCNNVGVRIYVDAVINHMCGSGAAAGTGTTCGSYCNPGNR

EFPAVPYSAWDFNDGKCKTASGGIESYNDPYQVRDCQLVGLLDLALEKDYVRSMIADYLNKL

IDIGVAGFRIDASKHMWPGDIKAVLDKLHNLNTNWFPAGSRPFIFQEVIDLGGEAIQSSEYF

GNGRVTEFKYGAKLGTVVRKWSGEKMSYLKNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGA

SILTFWDARLYKVAVGFMLAHPYGFTRVMSSYRWARNFVNGQDVNDWIGPPNNNGVIKEVTI

NADTTCGNDWVCEHRWRQIRNMVWFRNVVDGQPFANWWANGSNQVAFGRGNRGFIVFNNDDW

QLSSTLQTGLPGGTYCDVISGDKVGNSCTGIKVYVSSDGTAQFSISNSAEDPFIAIHAESKL

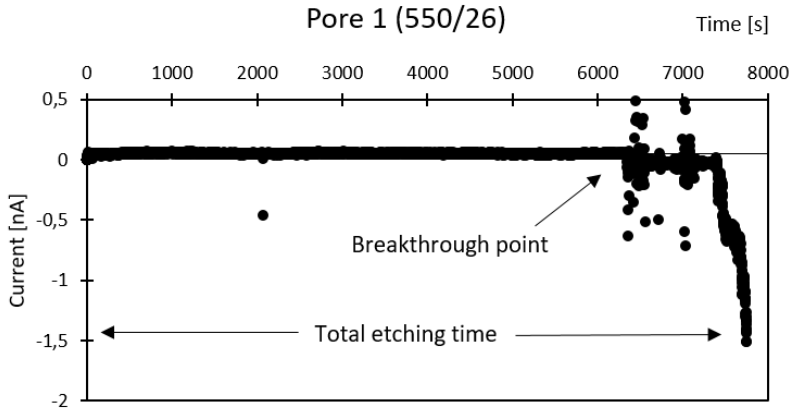

Figure S1: Exemplary experimental data used to calculate the diameter of the base of single pore membranes. The dimensions of the base opening of single pore membranes were calculated according to **Equation S1** based on the total etching *t*. Pore 1 corresponds to the membrane used in **Fig. 2A** in the main manuscript.

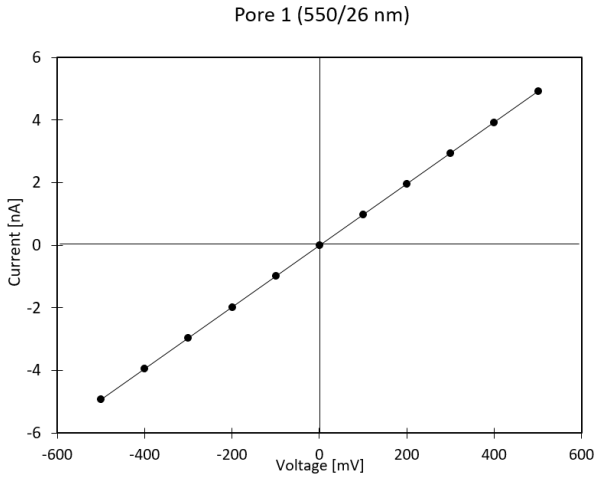

Figure S2: Exemplary experimental data used to calculate the diameter of the tip of single pore membranes. The dimensions of the tip opening *d* were calculated according to **Equation S2** based on the slope value of the measured *I-V* curve. Error bars were in the range of several pA and do not protrude beyond the actual data points. Pore 1 correspond to the membrane used in **Fig. 2A** in the main manuscript.

Table S1: Summary of nanopore membranes used in this study.

Nanopore ID	Density	Tip and Base Diameter	Receptor	Ligand	Recording
Fig. 2A	Single Pore	Tip: 26 nm; Base: 550 nm	Nb^{GFP}	GFP	Electrical
Fig. 2B	Single Pore	Tip: 45 nm; Base: 650 nm	$Nb^{mCherry}$	mCherry	Electrical
Fig. 2C	Single Pore	Tip: 38 nm; Base: 580 nm	$Nb^{\alpha\text{-}Amy}$	α-Amy	Electrical
Fig. 3A	Single Pore	Tip: 51 nm; Base: 680 nm	Nb^{GFP}	1. α-Amy 2. mCherry 3. GFP	Electrical
Fig. 3B	Single Pore	Tip: 42 nm ; Base: 510 nm	$Nb^{mCherry}$	1. GFP 2. α-Amy 3. mCherry	Electrical
Fig. 4A	10^7 per cm^2	Cylindrical: 200 nm	Nb^{GFP}	mCherry	CLSM
Fig. 4B	10^7 per cm^2	Cylindrical: 200 nm	Nb^{GFP}	GFP	CLSM
Fig. 4C	10^7 per cm^2	Cylindrical: 200 nm	Nb^{mCh}	GFP	CLSM
Fig. 4D	10^7 per cm^2	Cylindrical: 200 nm	Nb^{mCh}	mCherry	CLSM
Fig. 5A	10^7 per cm^2	Tip: 33 nm; Base: 490 nm	Nb^{GFP}	GFP	CLSM
Fig. 5B	10^7 per	Tip: 28 nm ; Base: 425 nm	$Nb^{mCherry}$	GFP	CLSM

	cm^2				

Table S2: Summary of etching times *t* used to calculate the diameter of the base opening

Pore ID	Total Etching time [min]	Base Opening (D) [nm]
Fig. 2A	129.214	550.45
Fig. 2B	152.586	650.02
Fig. 2C	136.209	580.25
Fig. 3A	159.683	680.248
Fig. 3B	119.715	509.984
Fig. 5A	115.104	490.341
Fig. 5B	99.845	425.341

Table S3 Summary of slope values used to calculate the diameter of the tip opening

Pore ID	Slope Value [pA mV^{-1}]	Tip Diameter [nm]
Fig. 2A	9.8356	26
Fig. 2B	20.096	45
Fig. 2C	15.191	38

Fig. 3A	23.735	51
Fig. 3B	14.789	42
Fig. 5A	11.154	33
Fig. 5B	8.1916	28

Table S4: Properties of individual proteins used in this study

Protein / Peptide	**Size in kDa**	**Theoretical pI**	**Estimated charge in PBS at pH 7.4 (according to Prot pi)**
SpyTag-Tether	7.5 (72 AA)	10.2	+5.2
Nb^{GFP}- SpyCatcher002	27.3 (256 AA)	5.0	-9.8
Nb^{mCh}- SpyCatcher002	27.9 (265 AA)	5.1	-7.8
$Nb^{\alpha Am}$-Spycatcher002	27.6 (261 AA)	5.0	-9.8
sfGFP-His_6	27.8 (248 AA)	6.3	-6.1
mCherry-Strep	29.0 (260 AA)	5.6	-7.5
α-Amylase (porcine pancreas), glycosylated	51-54 (475 AA)	6.4/7.5	-4.9 w/o considering glycosylation