Supporting Information

Semisynthetic Nanoreactor for Reversible Single-Molecule Covalent Chemistry

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SUPPORTING EXPERIMENTAL PROCEDURES

Preparation of NTF plasmids

1) Preparation of pTXB3-NTF126: The DNA encoding residues 1-126 of αHL was obtained by PCR amplification from a plasmid (pT7-SC1) containing the WT αHL gene. The primers (5'-TATGGATTCAACGGTAATGTTACTGGTTGCATCACGGGAGATGCACTAGT-3' (forward) and 5'-GTAACATTACCGTTGAATCCATAAGTTAAAGTACTCATATACTCTTTTGT-3' (reverse)) encode flanking restriction enzyme sites (NcoI and SapI, NEB). PCR reaction contained 25 μL of 2X Phusion high-fidelity PCR master mix (NEB), 22 μL of water, 1 μL of 100 μM forward and reverse primers, and 1 μL of pTXB3 (100 ng μL^{-1}). The thermocycling conditions were: 3 min of initial heating at 95°C followed by 30 cycles of 95°C for 5 s, 60°C for 10 s, 72°C for 1 min and a final elongation step at 72°C for 10 min. The PCR product was electrophoresed through a 1 % agarose gel. The product band was excised from the gel and purified with a QIAquick gel extraction kit (Qiagen). The gel-purified DNA was mixed with pTXB3 and digested by the two restriction enzymes [Digestion components: 2 μL of 10X Tango™ buffer (NEB), 1 μL of NcoI, 1μL of SapI, 1 μL of the gel-purified DNA (28 ng μL^{-1}), and 15 μL of nuclease-free water]. The digested DNAs were purified again using a QIAquick PCR purification kit (Qiagen) to remove the restriction enzymes. The purified DNA was ligated into cut pTXB3 by T4 DNA ligase (NEB) at 16°C overnight [Ligation components: 2 μL of 10X T4 DNA ligase buffer, 1 μL of T4 DNA ligase, 1 μL of pTXB3, 1 μL of insert DNA, and 15 μL of nuclease-free water].

2) Preparation of pTXB3-NTF113M: pTXB3-NTF113M plasmid was prepared by the same cloning procedure as previously described[1]. The primers used to PCR-amplify DNA encoding 1-113 αHL residue are 5'-CGCGCGCCATGGCAGATTCTGATATTAATATTAAA-3' (forward) and 5'-ATTGATACAAAAGAGTATATGTGCGGAAGAGCCAAGGG-3' (reverse).

3) Preparation of pTXB3-NTF113F: pTXB3-NTF113F plasmid for NTF_{113F} expression was obtained by homologous recombination, which resulted in the removal of DNA encoding residues 114-126. pTXB3-NTF126 was digested by one of two enzymes (NcoI or SpeI) and each of the two linearized plasmids was

used as a PCR template. Two sets of two primers were used to produce a plasmid containing a gene encoding residues 1-113F: 5'-CGATTGATACAAAAGAGTATTTCTGCATCACGGGAGATGCACTAGT-3' (forward, B_fwd) and 5'- CAGAAGTGGTCCTGCAACTTTAT-3' (reverse, A_rev) were mixed with the plasmid template linearized by NcoI and 5'- ATAAAGTTGCAGGACCACTTCTG-3' (forward, A_fwd) and 5'-GAAATACTCTTTTGTATCAATCGAAT-3' (reverse, B_rev) were mixed with the plasmid template linearized by SpeI (**Figure S2C**). The underlined sequences in the primers represent the codon for M113F. The thermocycling conditions were: 3 min of initial heating at 95°C followed by 30 cycles of 95°C for 5 s, 60°C for 10 s, 72°C for 4 min and a final elongation step at 72°C for 10 min. The size of the products was checked in a 1% (w/v) agarose gel. The two PCR fragments (10 µL each) were mixed together with *E. coli* XL-10 Gold Ultracompetent cells (100 µL, Agilent) and incubated on ice for 30 min. The cells were plated onto ampicillin plates (100 µg mL^{-1}), which were incubated overnight at 37°C. A colony was picked and grown overnight again in 10 mL LB medium containing the same concentration of ampicillin. Plasmid pTXB3-NTF113F was extracted with a QIAprep miniprep kit (Qiagen) and the sequence of the insert was verified by Sanger sequencing with sequencing primers provided from Source Bioscience (T7F and T7R).

Preparation of CTF plasmids

pT7-SC1-CDH plasmid encoding CTF_{127} was prepared by the same method as previously described[1]. pT7-$CTF_{\Delta114}$-DH was obtained by two successive homologous recombinations beginning with pT7-TBMΔ6, which resulted in the removal of αHL codons 1-113 and the addition of DNA encoding a D_8H_6 tag (**Figure S5**). The homologous recombination procedure carried out to produce pT7-$CTF_{\Delta114}$-DH was the same as described in the preparation of pTXB3-NTF113F section except that different restriction enzymes and primers were used. 1) Removal of αHL codons 1-113 from pT7-TBMΔ6: NdeI and HindIII were used to digest pT7-TBMΔ6. Two sets of primers were required to remove the codons 1-113 and replace codon 114 with a Cys codon. 5'-GTTTAACTTTAAGAAGGAGATATACATATGTGTACTTTAACTTATGGAGGTGATG -3' (forward,

C_fwd) / 5'-CAGAAGTGGTCCTGCAACTTTAT-3' (reverse, A_rev) and 5'-ATAAAGTTGCAGGACCACTTCTG-3' (forward, A_fwd) / 5'-ATGTATATCTCCTTCTTAAAGTTAAAC-3' (reverse, C_rev). 2) Addition of D_8H_6 tag codons to the 3'-end of the CTF gene in pT7-TBMΔ6: Two additional sets of primers were required to form the D_8H_6 tag: 5' –CGACGATGATCACCACCATCACCATCATTGATAAGCTTGGATCCGGCTGC-3' (forward, D_fwd) / 5'-CAGAAGTGGTCCTGCAACTTTAT-3' (reverse, A_rev) and 5'-ATAAAGTTGCAGGACCACTTCTG-3' (forward, A_fwd) / 5'-GGTGATCATCGTCGTCATCATCGTCATCATTTGTCATTTCTTCTTTTTC-3' (reverse, D_rev). 3) Preparation and purification of NTFs: The sequence-verified NTF plasmids (pTXB3-NTF126, pTXB3-NTF113M, and pTXB3-NTF113F) were transformed separately into T7 Express Competent *E. coli* cells (NEB). The cells were plated onto ampicillin plates (100 μg mL^{-1}), which were incubated overnight at 37°C. In order to culture a starter, a colony was transferred into LB medium (5 mL) containing the same concentration of ampicillin and grown overnight. The starter was transferred into 100 mL of LB medium and the culture monitored at 37°C until the OD_{600} reached ~0.6. IPTG was added (100 μM final concentration) to induce expression and the culture was continuously grown for 4 h under the same conditions. The cells were centrifuged at 4,000 g for 20 min at 4°C. To lyse the cells, 5 mL of BugBuster® (Merck Millipore) was added to the cell pellets and shaken at 20°C for 30 min. The lysates were centrifuged again at 4,000 g for 30 min at 4°C. 1 μL of the supernatants and pellets were examined by SDS/PAGE to verify the overexpressed NTFs were mainly in the cell pellet (inclusion bodies). The cell pellets were resuspended in a denaturing buffer (5 mL of 8 M urea, 50 mM NaH_2PO_4 (pH 7.0), 250 mM NaCl, 5 mM TCEP). The concentration of urea in the resuspended cell pellets was diluted to 1 M by mixing with 35 mL of 50 mM NaH_2PO_4 (pH 7.0), 250 mM NaCl, 5 mM TCEP for the chitin-binding domain (CBD) to refold and bind to the chitin column. The resuspended pellets were loaded onto a chitin column (bed volume: 5 mL) and washed with 100 mL of 1 M urea, 50 mM NaH_2PO_4 (pH 7.0), 250 mM NaCl, 5 mM TCEP. NTF-$^{\alpha}$thioesters were released by intein-mediated thiol-cleavage of the fusion protein

on the column with MESNa (sodium 2-sulfanylethanesulfonate). Each fraction was collected at each step and analyzed by SDS/PAGE. The eluted NTF-$^{\alpha}$thioesters were characterized by LC-MS.

Preparation and purification of CTFs

pT7-SC1-CDH and pT7-$CTF_{\Delta 114}$-DH plasmids were separately transformed into *E. coli* BL21 (DE3, pLysS, Promega). The cells were plated onto ampicillin (100 μg mL^{-1}) and incubated overnight at 37°C. A colony was picked and inoculated in LB medium (5 mL) containing the same concentration of ampicillin. The medium was grown at 37°C overnight for the starter culture. The starter was again transferred to 100 mL of LB medium containing the same concentration of ampicillin. The culture was incubated at 37°C until the OD_{600} reached ~0.6. IPTG was added (100 μM final concentration) to induce expression and the culture was further grown for 4 h. The cells were centrifuged at 4,000 g for 20 min at 4°C. To lyse the cells, 5 mL of BugBuster® (Merck Millipore) was added to the cell pellets and shaken at 20°C for 30 min. The lysates were centrifuged again at 4,000 g for 30 min at 4°C. CTFs were found in the cell pellet and a denaturing buffer (5 mL of 6 M Gu·HCl, 50 mM NaH_2PO_4 (pH 7.5), 250 mM NaCl, 5 mM TCEP) was added to dissolve the CTFs in the inclusion bodies. For separation of CTFs, the resuspended cell pellets were filtered by a syringe filter (Millipore, 0.22 μm) to remove cell debris before purification.

Purification of CTFs containing the His_6-tag was carried out with an automated chromatography system *(ÄKTA* purifier, GE Healthcare Life Sciences) at a flow rate of 0.5 mL min-1 by using a Ni-NTA column (HisTrap, 1mL). Prior to injection, the Ni-NTA column was washed with 10 column volumes (CV) of distilled water and subsequently equilibrated with 5 CV of eluant A (6 M Gu·HCl, 50 mM phosphate (pH 7.5), 250 mM NaCl, and 5 mM TCEP). The cell pellet dissolved in eluant A was directly injected onto the column and the column was washed with 5-10 CV of buffer A to elute unbound protein. The separation was performed by applying a gradient from 0 to 50 % of eluant B in eluant A (eluant B: 6 M Gu·HCl, 50 mM phosphate (pH 7.5), 250 mM NaCl, 5 mM TCEP, and 500 mM imidazole). The separation was monitored at 280 nm. The purified CTFs were used directly without any further step to remove imidazole.

Synthesis of Fmoc-N^6-(3-oxobutanoyl)lysine (Fmoc-Ket-OH)

Fmoc-Lys-OH, dichloromethane (DCM), and glacial acetic acid were obtained from Novabiochem and N-hydroxysuccinimidyl acetoacetate (NHA) was from Sigma-Aldrich. 1.0 mmol NHA was dissolved in 20 mL DCM and added to a 50 mL DCM suspension containing 1.1 mmol Fmoc-Lys-OH and 1.1 mmol *N*,*N*-diisopropylethylamine (DIEA). The reaction mixture was stirred for 6 h. The reaction was monitored by silica gel thin layer chromatography (TLC) using 10 % (v/v) MeOH in DCM containing a drop of acetic acid. The crude product was purified by flash column chromatography (silica gel) using 7 % (v/v) MeOH in DCM. The product was washed with 5 % Na_2CO_3 (3 × 50 mL) and water (2 × 50 mL) and subsequently dried over Na_2SO_4. The final product was concentrated by a rotary evaporator (Buchi, R-200) giving a yield of 48 %. A ^{1}H NMR spectrum of Fmoc-Ket-OH was obtained using a Bruker AVIII HD 400. ^{1}H NMR (400 MHz, DMSO-d_6); δ in ppm: 1.28-1.36 (m, Lys γ -CH_2-, 2H), 1.36-1.43 (m, Lys δ -CH_2-, 2H), 1.51-1.77 (m, Lys β -CH_2-, 2H), 2.15 (s, Lys κ -CH_3-, 3H), 3.05 (m, Lys ε -CH_2-, 2H), 3.28 (s, Lys θ -CH_2-, 2H), 3.84 (m, Lys α -CH-, 1H), 4.18-4.32 (m, Fmoc -CH-CH_2-, 3H), 7.30, 7.32, 7.35 (t, Fmoc (2) -CH-, J = 8 Hz, 2H), 7.39, 7.41, 7.42 (t, Fmoc (3) -CH-, J = 8 Hz, 2H), 7.71, 7.72 (d, Fmoc (1) -CH-, J = 7 Hz, 2H), 7.89, 7.91 (d, Fmoc (4) -CH-, J = 8 Hz, 2H), 8.04, 8.05, 8.06 (t, Lys ζ NH-, J = 5 Hz, 1H). ESI-MS: Calculated mass for $C_{25}H_{28}N_2O_6$: $[M+H]^+$ = 453.51, observed mass: 453.18.

Preparation of CSP-Nbz

Dawson Dbz AM resin, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N^{α}-Fmoc protected amino acids were from Novabiochem. *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), *N*,*N*'-diisopropylcarbodiimide (DIC), hydroxybenzotriazole (HOBt), dichloromethane (DCM, anhydrous), diethyl ether, acetonitrile (HPLC-grade), guanidine hydrochloride (Gu·HCl), trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were from Sigma-Aldrich. Boc-Thz-OH was from Bachem. 500 mg of Dawson resin (0.5 meq/g, reaction scale: 0.25 mmole) was subjected to manual peptide elongation by the Fmoc method using HBTU and DIPEA as a coupling reagent to obtain CSP-Nbz. After Fmoc removal from the resin by treatment with 20% (v/v) piperidine in DMF at room temperature, standard chain elongation was performed. For each coupling reaction, 0.5 M

HBTU, 1.1 M DIPEA, and 0.4 M of Fmoc-Xaa-OH in DMF were added to the resin. For coupling of Fmoc-Ket-OH, 0.6 M DIC and 0.6 M HOBt in DMF were used, and the reaction vessel was vortexed overnight at room temperature. After each coupling, the resin was washed with DMF and dried *in vacuo*. Side-chain protection was as follows: Fmoc-Tyr(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH and Fmoc-Asn(Trt)-OH. The N-terminal residue was incorporated as Boc-Thz-OH. CSP[Thz^{114}-Gly^{126}]-Nbz was prepared by using a previously reported protocol.[2] After the completion of chain assembly, the resin-bound protected peptide was washed with anhydrous DCM. 100 mM *p*-Nitrophenyl chloroformate in anhydrous DCM (10 mL) was added to the resin with N_2 purging. The resin was washed with DCM after 1 h and treated with two portions of 0.5 M DIPEA in DMF (2 X 10 mL) for 15 min each. The resin was then washed with DMF, followed by DCM, and dried *in vacuo*. Peptides were cleaved from the resin and side-chain and N-terminal protecting groups were removed by treatment with TFA containing 5% (v/v) DCM, and 5% (v/v) TIS. After 2 h, the mixture was concentrated by evaporating the solvent. The CSP[Thz^{114}-Gly^{126}]-Nbz was precipitated with cold diethyl ether and isolated by centrifugation with several diethyl ether washes.

Purification of peptides

Preparative peptide purification was carried out with a Dionex UltiMate 3000 HPLC. Peptides were dissolved at 20 mg mL^{-1} in DMF prior to injection and separation was performed by using a Vydac C_{18} column (250 × 22 mm, 10-15 μm) at a flow rate of 15 mL min^{-1} with a gradient from 5 to 95% of eluant B in eluant A, over 45 min (eluant A: 0.1% TFA in water; eluant B: 0.1% TFA in acetonitrile). The separation was monitored at 220 and 280 nm. The purified peptides were lyophilized and characterized by LC-MS.

Analytical LC-MS

NTF, CTF, and CSP-Nbz were analyzed on a Waters LCT accurate-mass time-of-flight instrument (ESI-TOF MS) by using the positive ion mode. LC-MS employed a Chromolith RP-18e 50 mm × 2 mm column with a linear gradient of 5-100% eluant B in eluant A, over 8 min, with a flow rate of 1 mL min^{-1} (eluant A: 0.1% formic acid in water; eluant B: 0.1% formic acid in acetonitrile). The mass spectra were

generated with MassLynx software (version 4.1, Waters) by combining mass spectra obtained over the major peak in the total ion chromatogram.

Analytical HPLC

The purified synthetic peptide (SP-Nbz) was further examined on an analytical reverse-phase column (Polaris C_{18} 5u, 100 mm × 4.6 mm) on an Agilent 1260 Infinity HPLC at a flow rate of 1 mL min^{-1} with a gradient from 5 to 95% of eluant B in eluant A, over 13 min (eluant A: 0.1% FA in water; eluant B: 0.1% FA in acetonitrile). The separation was monitored at 280 nm.

Purification of SM

The final ligation product (synthetic monomer, SM) was exchanged into denaturing purification buffer (8 M urea, 50 mM NaH_2PO_4 (pH 7.0), 5 mM TCEP) by successive concentration, dilution and re-concentration with a centrifugal filter (MWCO, 3k). The concentrated material (< 100 μL) was injected onto a gel filtration column (Superdex™ 200 10/300 GL), which was equilibrated with the same buffer and eluted at 0.4 mL min^{-1}. The separation was monitored at 280 nm.

Folding of purified SM

The denaturing buffer (8 M urea, 50 mM NaH_2PO_4 (pH 7.0), 5 mM TCEP) used for the purification of SM was exchanged for the folding buffer (10 mM Tris·HCl, pH 7.4, containing 1 mM EDTA) with a centrifugal filter (Amicon, MWCO 3k). SM in 8 M urea (500 μL) was centrifuged at 14,000 g for 20 min (4°C) to a volume of 100 μL. 400 μL of flow-through was discarded. The folding buffer (400 μL) was added to the filter and mixed with the concentrated SM by pipetting to dilute the urea 5-fold. The urea concentration was further reduced to ~60 mM by repeating the dilution and concentration.

Hemolysis assays

1) Preparation of rabbit red blood cells - Before hemolysis was performed, the rabbit red blood cells obtained from Envigo were washed with MBSA buffer (10 mM 3-[N-morpholino]propane sulphonic acid (MOPS), 150 mM NaCl, pH 7.4, containing 1% bovine serum albumin). 450 μL of rabbit red blood cells was transferred to a 1.5 mL microcentrifuge tube and mixed with 900 μL of MBSA buffer. The rabbit red

blood cells were centrifuged at 1,000 rpm for 2 min. The supernatant was discarded and the pellet was diluted to 10 mL with MBSA buffer.

2) Hemolysis was performed on a transparent 96-well microplate. αHL monomers were serially two-fold diluted with MBSA such that each well contained a volume of 50 μL. For example, SM_f (1 μL) was mixed with MBSA (99 μL) in the first well. Half of the first well (50 μL) was transferred to the next well containing MBSA (50 μL) and mixed. In this way, serial dilution was continued to the twelfth well. Rabbit red blood cells (50 μL) prepared by a protocol described above were then added to each well, starting with the well that contained the most dilute protein (*i.e.* twelfth well). Lysis was recorded by monitoring the decrease in light scattered at 595 nm with a microplate reader (Bio-Rad).

Ionic current recordings and single-molecule chemistry

Ionic current measurements were performed with a planar bilayer apparatus at room temperature. Data were analyzed with pClamp (Ver. 10.1, Molecular Devices). OriginPro9.1 was used for plotting IV curves and statistical analyses. A bilayer of 1,2-diphytanoly-sn-glycero-3-phophatidylcholine (DPhPC, Avanti Polar Lipids) was performed across an aperture of 100 μm diameter in a Teflon film (Goodfellow) separating the *cis* and *trans* compartments. Gel-purified αHL heptamers were added to the grounded *cis* compartment. After the insertion of a single pore into a bilayer, the cis compartment was manually perfused to prevent further insertions. Currents were measured by using Ag/AgCl electrodes connected to a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Signals were sampled by a Digidata 1322A digitizer (Axon Instruments). βCD (β-cyclodextrin) kinetics was determined by adding aliquots of βCD sequentially added to the *trans* compartment to produce concentrations of 25, 50, and 75 μM for all the αHL pores (WT_7, WT_6SM_1, $(SM_{\Delta6\text{-}113M})_7$, $(SM_{\Delta6\text{-}113F})_7$, and WT_6SM_{ket1}. For am_7βCD kinetics, an aliquot of am_7βCD was added to the *trans* compartment of $(SM_{\Delta6\text{-}113F})_7$ pore to give a final concentration of 10 μM. For the determination of each set of kinetic constants, three separate experiments were performed and data acquired from 10 min (>1,000 events) were analyzed. Events were collected by Single-Channel searches with the Clampfit software. Very short events (<0.1 ms) were excluded. Dwell times were obtained by fitting unbinned cumulative histograms to single exponentials. For single-molecule thiolate

chemistry, 5 μL of 10 mM methyl-PEG-OPSS (MPO) (5 kDa, Nanocs) dissolved in 1 M KCl, 10 mM Tris (pH 8.5) was added to the *trans* compartment, which contained 500 μL of the same buffer. For single-molecule ketone chemistry, 50 μL of 20 mM methyl-PEG-amine (MPA) (1.1 kDa, Thermo Scientific) dissolved in 1M KCl, 50 mM sodium acetate (pH 3.4) was added to the *trans* compartment containing 500 μL of the same buffer.

SUPPORTING FIGURES

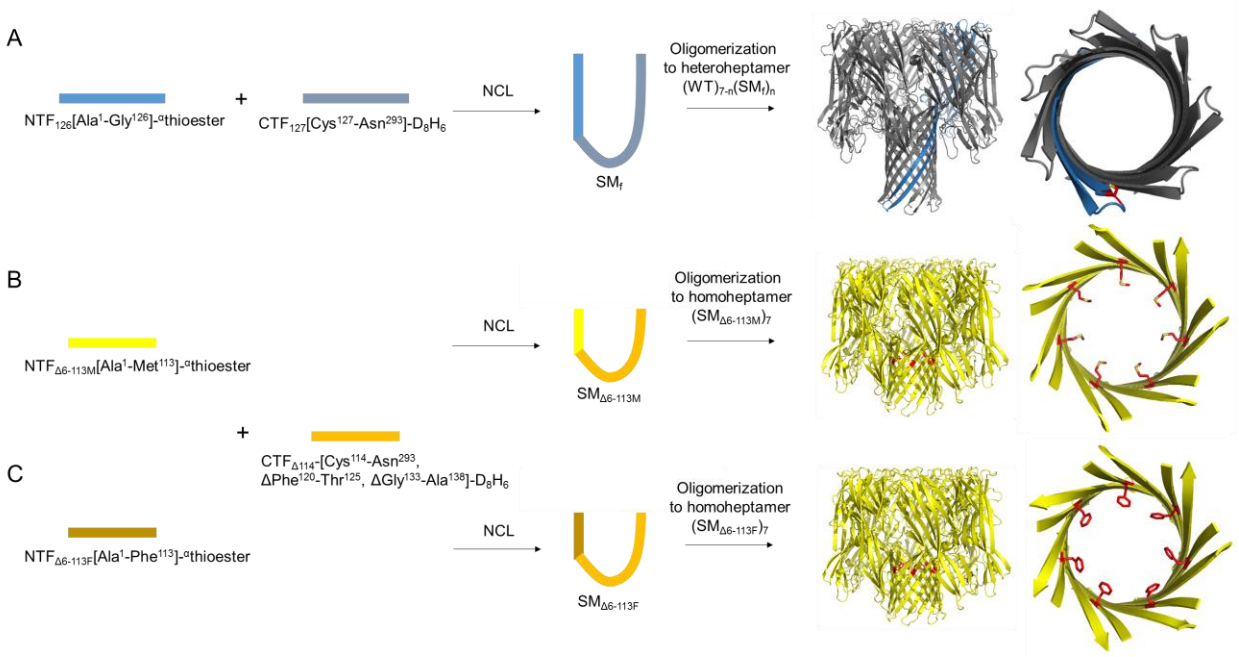

Figure S1. Schematic representation of three αHL pores built by native chemical ligation from two recombinant polypeptides. NTF and CTF produced from *E. coli* were coupled to form αHL monomers. By using three different NTFs and two different CTFs, we synthetized three different αHL monomers (SM_f (**A**), $SM_{\Delta6\text{-}113M}$ (**B**), and $SM_{\Delta6\text{-}113F}$ (**C**)). We folded the synthetic monomers and oligomerized them to form heptameric pores. WT_6SM_{fl}, $(SM_{\Delta6\text{-}113M})_7$, and $(SM_{\Delta6\text{-}113F})_7$ were used as nanoreactors. The residues depicted in red in a), b), and c) are Cys-127, Met-113, and Phe-113, respectively. The images of the αHL pores were prepared using Modeller and then reconstituted in Pymol. Note that the WT_6SM_{fl} pore is a bottom view, while $(SM_{\Delta6\text{-}113M})_7$, and $(SM_{\Delta6\text{-}113F})_7$ are top views.

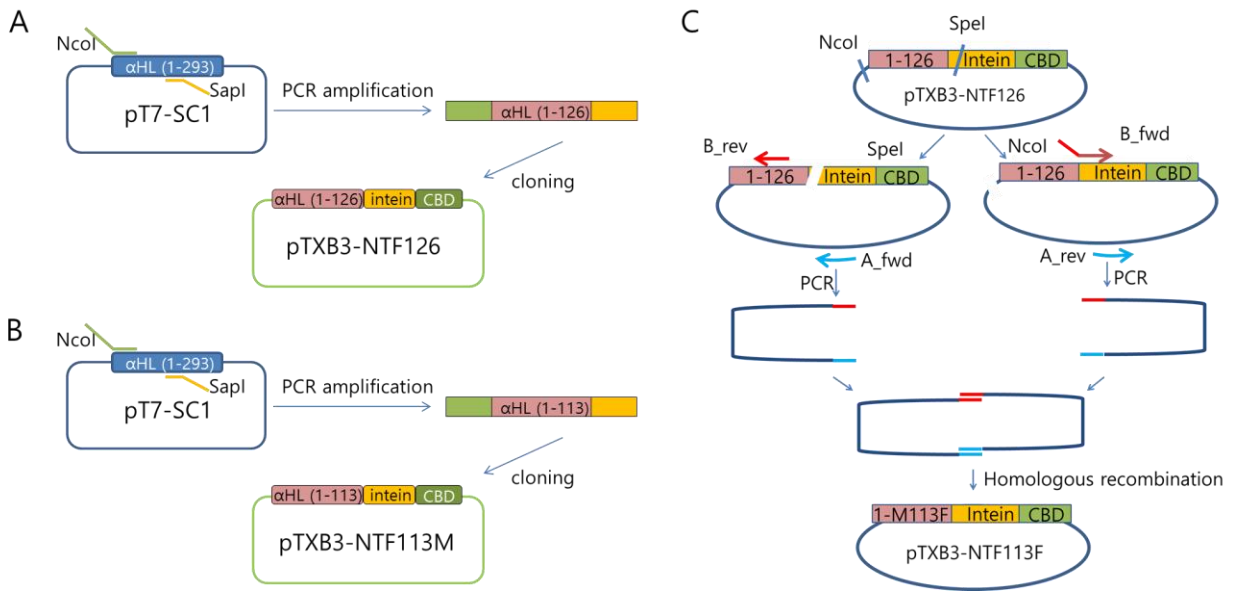

Figure S2. Preparation of the NTF plasmids. The target gene encoding αHL residue 1-126 (**A**) and 1-113 (**B**) were PCR-amplified from the plasmid (pT7-SC1) bearing a full-length αHL gene. The DNAs were separately cloned into pTXB3 upstream sequence of an intein and a chitin-binding domain (CBD). C**)** The B_rev and B_fwd primers were used with A_fwd and A_rev to produce pTXB3-NTF113F by using exactly same method described in **Figure S2B**.

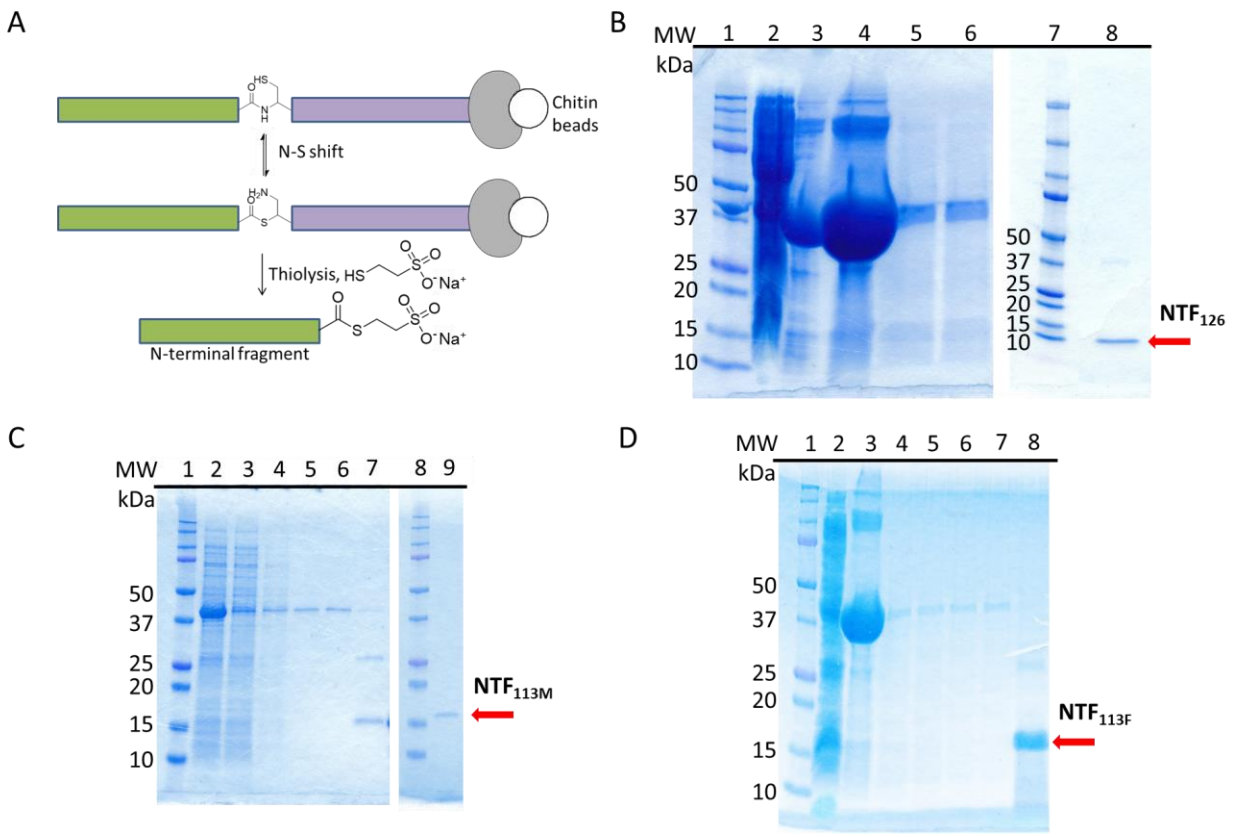

Figure S3. Preparation of NTFs. A) Schematic representation of intein-mediated cleavage by a thiol to prepare an NTF-$^{\alpha}$thioester. A linear thioester intermediate at the N-terminus of the intein is formed by an N-S acyl rearrangement at the Cys. (purple: *Mxe GyrA* intein, grey: chitin binding domain, white circle: chitin beads). An NTF-$^{\alpha}$thioester was obtained by on-column cleavage from a chitin affinity column with MESNa. The preparation was monitored by using SDS/PAGE. **B)** Purification of NTF_{126}. Lane 1 and 7: molecular mass markers (MM); lane 2: cell lysate debris before separation of supernatant and pellet; lane 3: cell extract supernatant (CS); lane 4: cell extract pellet (CP); lane 5: flow-through (FT) from the chitin affinity column; lane 6: wash; lane 8: NTF_{126} (red arrow, 14.1 kDa) eluted from the column under gravity flow. **C)** Preparation of NTF_{113M}. Lane 1 and 9: MM; lane 2: CP; lane 3: FT; lane 4-6: washes; lane7: NTF_{113M} (red arrow) was eluted together with unreacted fusion protein and the chimera (~41 and ~28 kDa); lane 9: The eluted proteins at lane 7 were added to a fresh chitin bead column and re-eluted. **D)** Preparation of NTF_{113F}. Lane 1: MM; lane 2: CS; lane 3: CP; lane 4: FT; lane 5-7: washes; lane 8: NTF_{113F} (red arrow) eluted from the column.

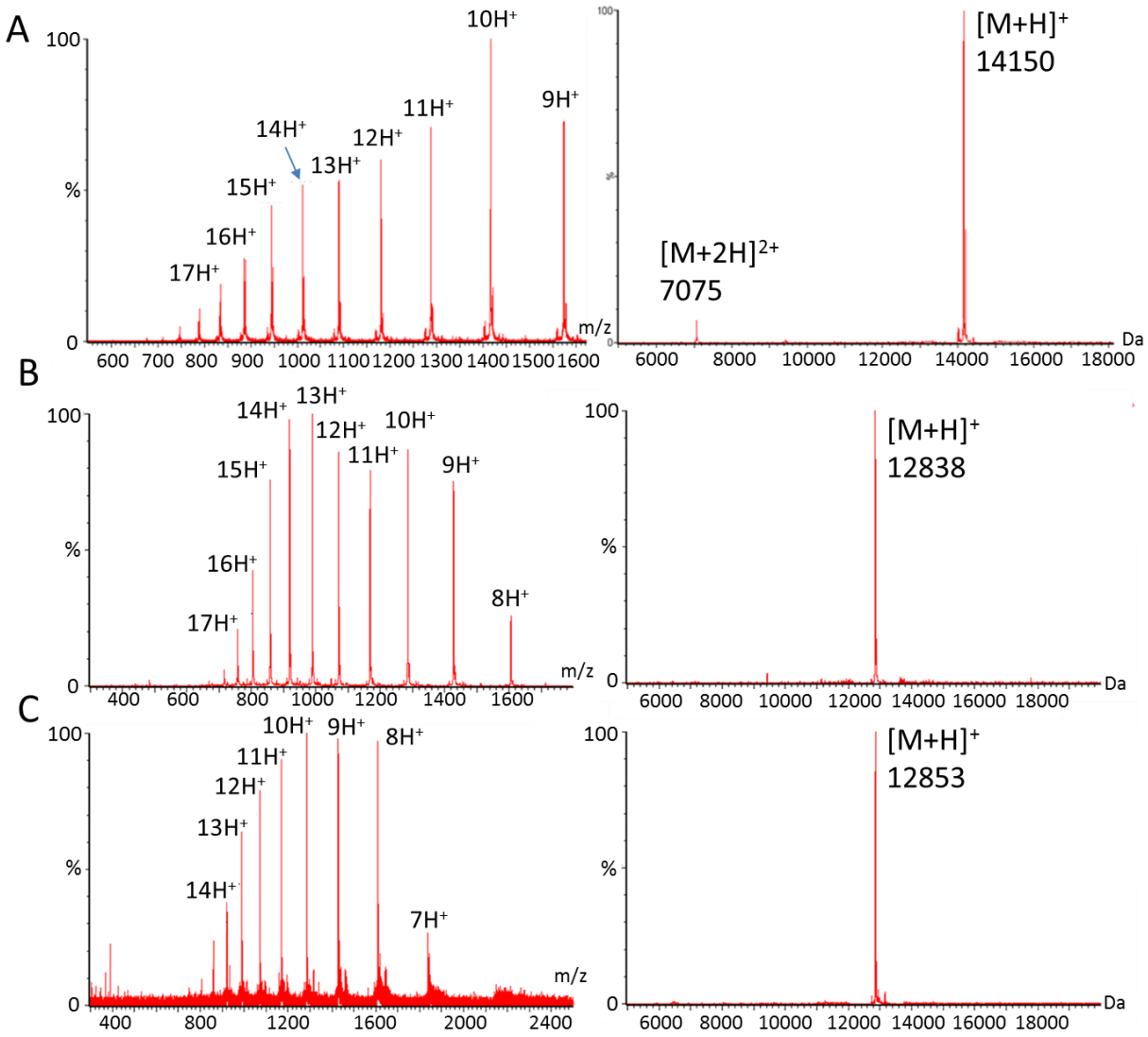

Figure S4. Characterization of NTFs. ESI-MS (left) and of deconvoluted mass spectrum (right) of the NTF_{126} (**A**), NTF_{113M} (**B**), and NTF_{113F} (**C**). NTF_{126}: $[M+H]^+$ = 14,150 (obs), 14,150 Da (calcd); NTF_{113M}: $[M+H]^+$ = 12,838 (obs), 12,838 Da (calcd); NTF_{113F}: $[M+H]^+$ = 12,853 (obs), 12,854 Da (calcd).

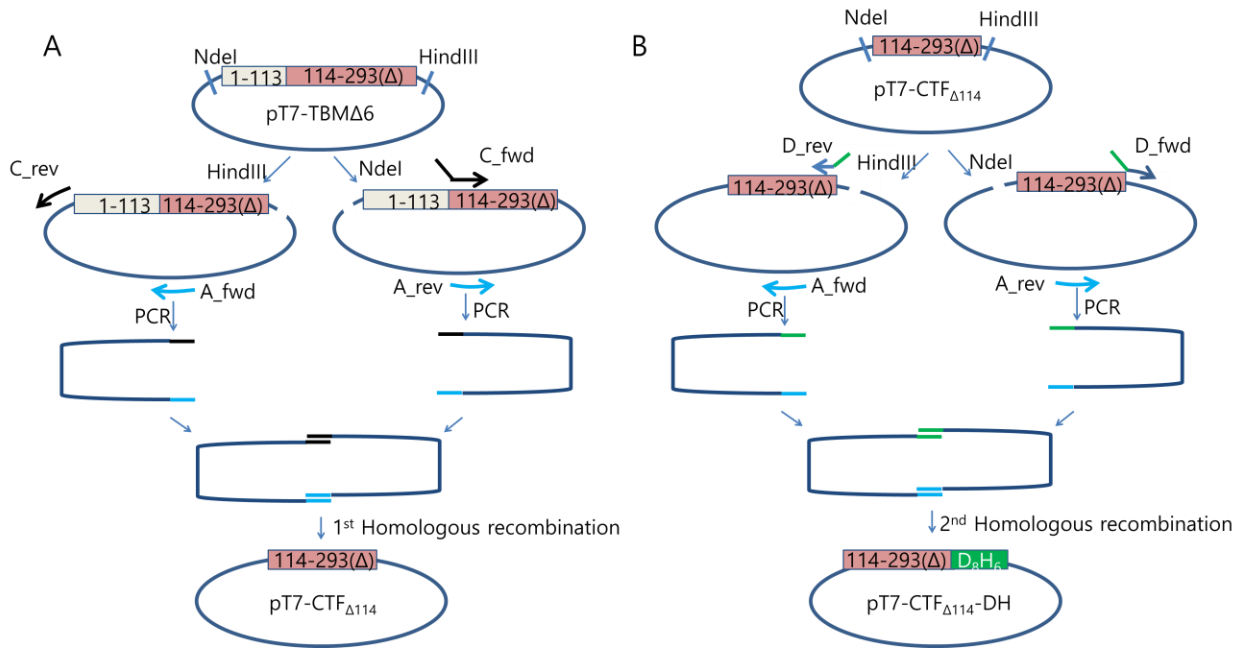

Figure S5. Preparation of the pT7-CTF$_{\Delta 114}$ plasmid. The plasmid containing genes for CTF114Δ were produced by two successive homologous recombinations. **A)** The pT7-TBMΔ6 plasmid containing the gene for the TBMΔ6 monomer was separately treated with two restriction enzymes (NdeI and HindIII). The A_fwd/C_rev primers were used to obtain the left-hand fragment by PCR template of the HindIII-digested plasmid, while C_fwd/A_rev were used to obtain the right-hand fragment from the NdeI-digested plasmid. The two fragments, which have homologous ends, were mixed and recombined *in vivo* to generate a circular plasmid (pT7-CTF$_{\Delta 114}$) replacing the residues (1-113) upstream of the CTF by a gene initiated with Met-Cys codons. **B)** A second homologous recombination with A_fwd/D_rev and D_fwd/A_rev primers yielded the plasmid pT7-CTF$_{\Delta 114}$-DH containing the D_8H_6 tails at the C-terminus.

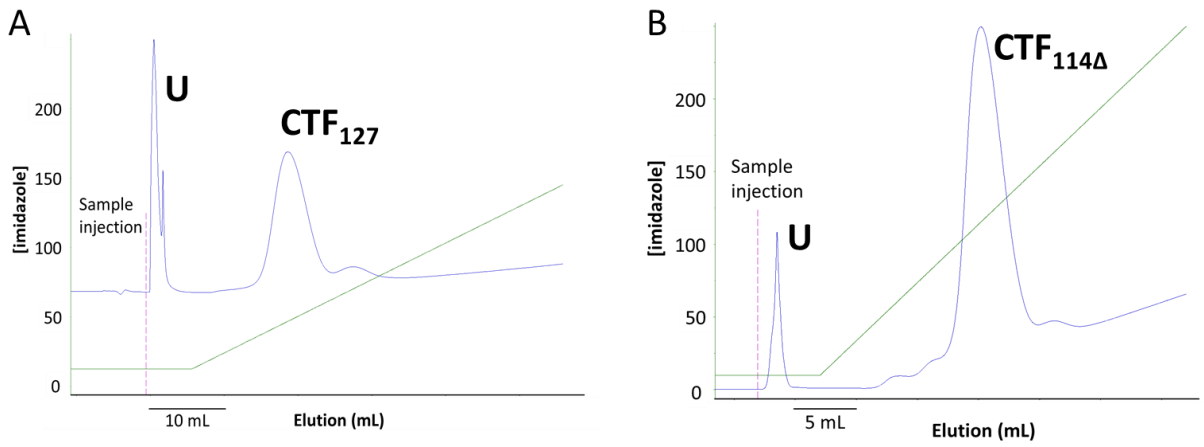

Figure S6. Purification of CTFs on a Ni-NTA affinity column. The cell lysate was loaded onto the column and the column was washed to remove unbound proteins (U) before increasing the concentration of imidazole in the buffer. The buffer containing 500 mM imidazole increased in a slow gradient (green line, gradient change: 1% min^{-1}) and the CTFs (CTF_{127} and $CTF_{114\Delta}$ for (**A**) and (**B**), respectively) containing the His_6-tag at the C-terminus were eluted when the eluant contained 50-100 mM imidazole. The fractions of the CTF peak were pooled and directly used for characterization and the pyruvate removal step. The separation was monitored at 280 nm.

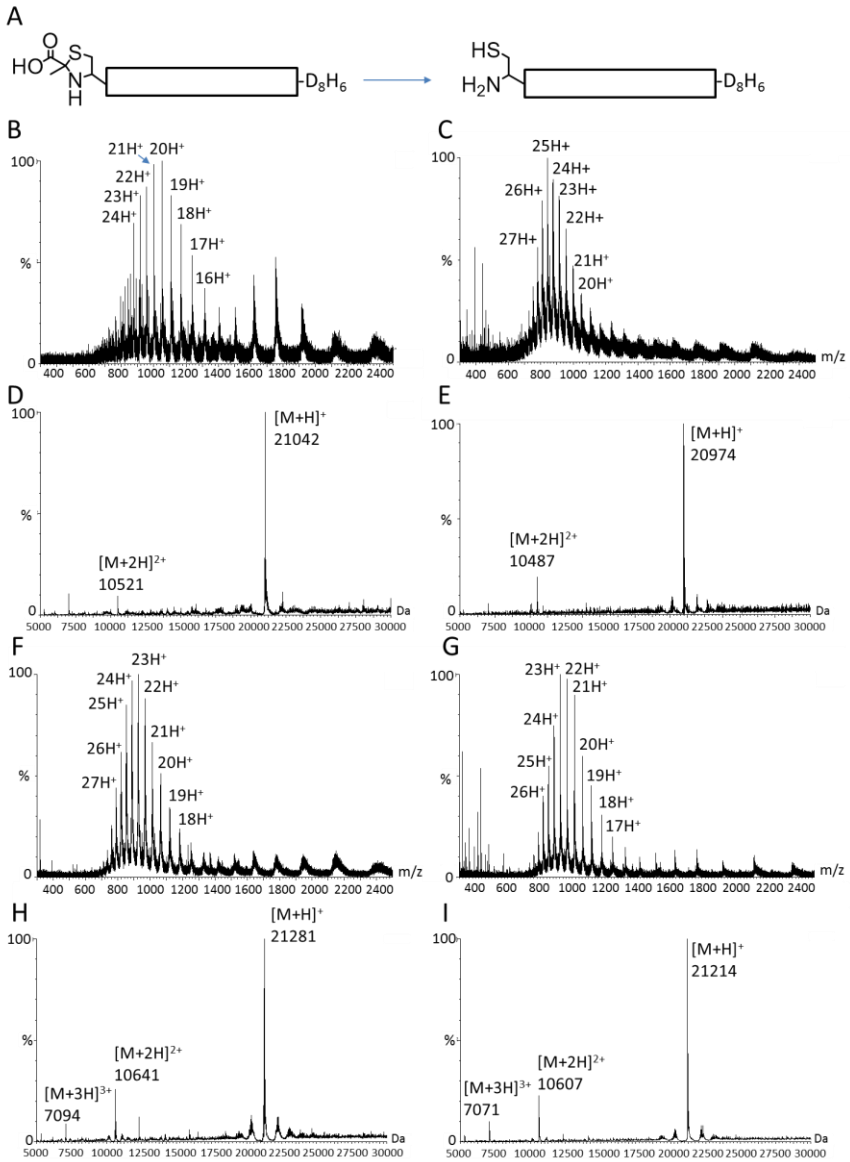

Figure S7. Characterization of CTFs by LC-MS. A) Schematic representation of pyruvate removal on N-terminal cysteine, which was masked by reaction with pyruvate after the initiator Met was cleaved in the host. An N-Cys was generated by removing pyruvate with 0.4 M $HONH_2 \cdot HCl$ at pH 4.0. **B)** and **C)** ESI-MS spectrum of the CTF_{127} and $HONH_2$-treated CTF_{127}, respectively. **D)** and **E)** Deconvoluted mass spectrum of the CTF_{127} and $HONH_2$-treated CTF_{127}, respectively. CTF_{127}: $[M+H]^+$ = 21,042 (obs), 21,044 Da (calcd); $HONH_2$-treated CTF_{127}: $[M+H]^+$ = 20,974 (obs), 20,974 Da (calcd). **F)** and **G)** ESI-MS spectrum of the $CTF_{\Delta 114}$ and $HONH_2$-treated $CTF_{\Delta 114}$, respectively. **H)** and **I)** Deconvoluted mass spectrum of the $CTF_{\Delta 114}$ and $HONH_2$-treated $CTF_{\Delta 114}$, respectively. CTF_{127}: $[M+H]^+$ = 21,281 (obs), 21,282 Da (calcd); $HONH_2$-treated CTF_{127}: $[M+H]^+$ = 21,214 (obs), 21,212 Da (calcd).

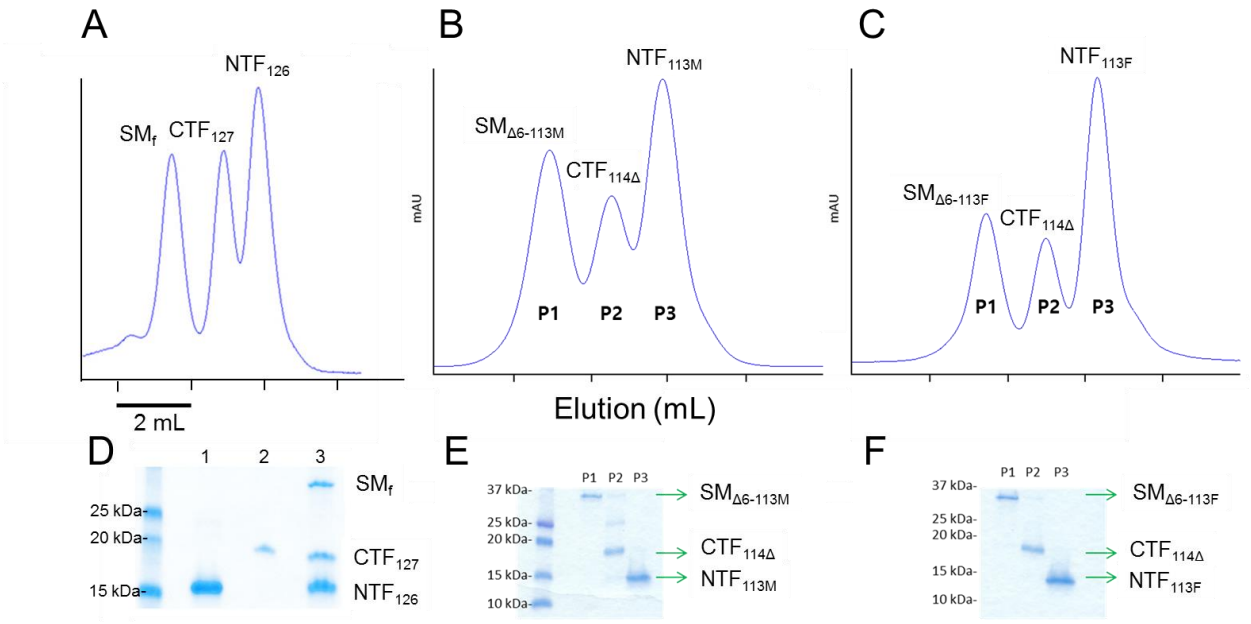

Figure S8. Purification of the synthetic monomers produced by two-fragment coupling. A, B, and **C)** The crude ligation products were purified by gel filtration (Superdex^{TM} 200 10/300 GL column) under denaturing conditions (8 M urea, 50 mM NaH_2PO_4 (pH 7.0), 5 mM TCEP). The separation was monitored at 280 nm. The fractions eluted in the first peaks were collected to obtain SMs. **D)** The fragments (NTF_{126} and CTF_{127}; lane 1 and 2, respectively) used for the synthesis of SM_f and the crude NCL products (lane 3) were examined by SDS/PAGE prior to purification. **E)** and **F)** The fractions collected at each of peaks (P1, P2, and P3) were loaded onto SDS-gels to examine the purity of the synthetic monomers.

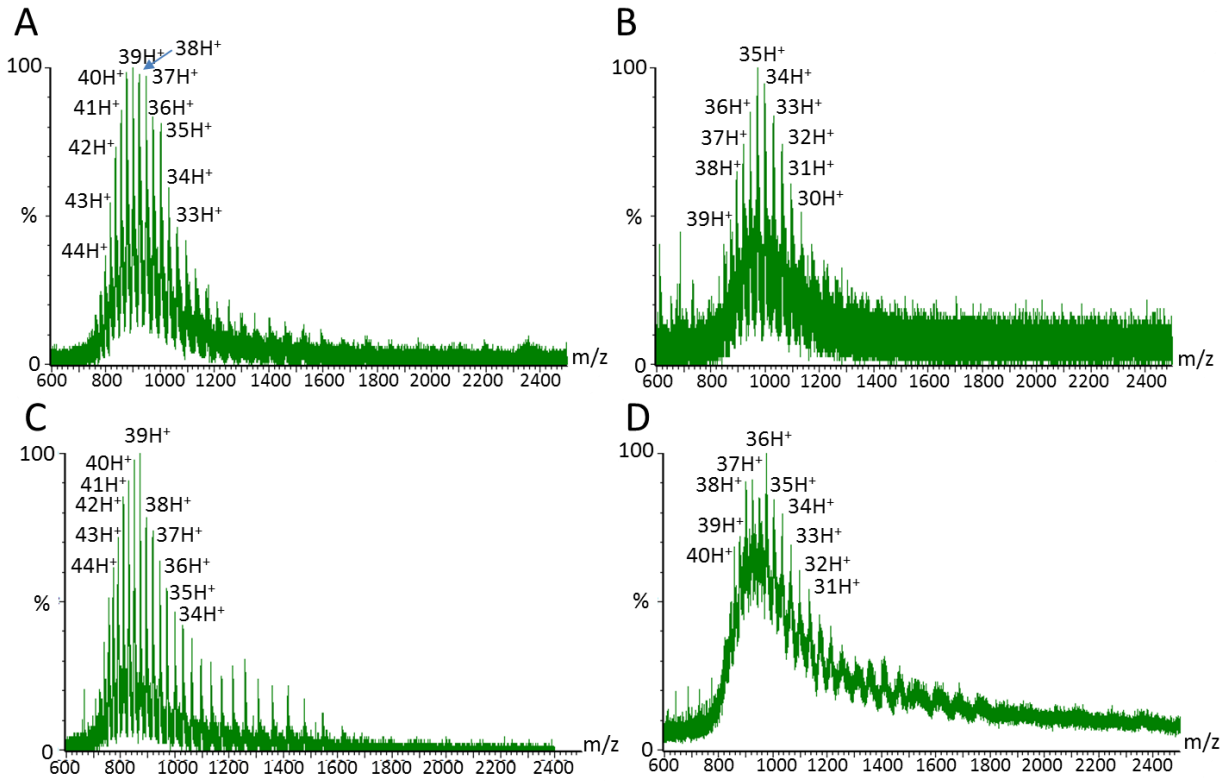

Figure S9. Characterization of SMs by LC-MS. ESI-MS spectrum of SM_f (**A**), $SM_{\Delta 6\text{-}113M}$ (**B**), $SM_{\Delta 6\text{-}113F}$ (**C**), and SM_{ket} (**D**) used to obtain deconvoluted mass spectrum in **Figure 1B-D** and **Figure 3B**.

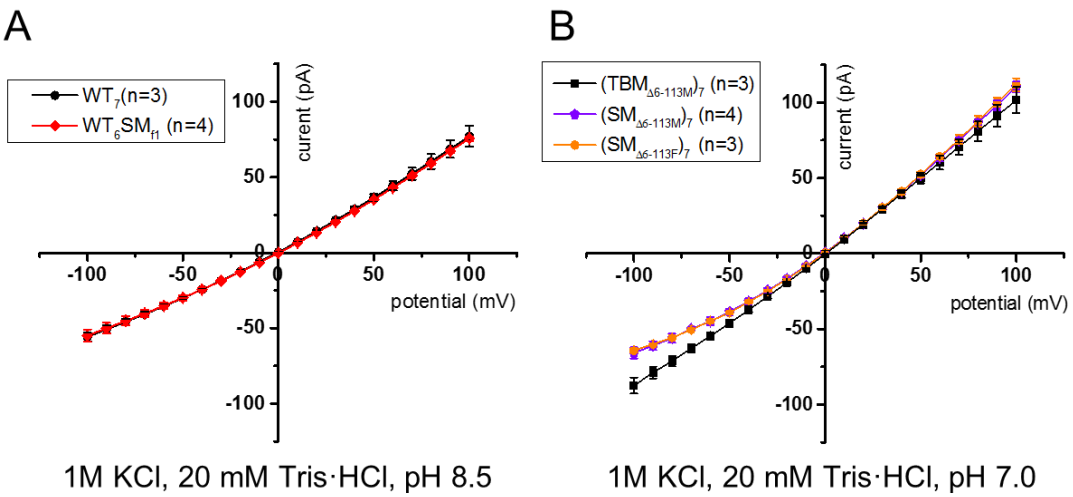

Figure S10. Comparison of current-voltage characteristics of αHL pores containing synthetic monomers with αHL pores only containing subunits prepared by IVTT. A) I-V curve of WT_7 (black) and WT_6SM_{f1} (red). **B)** I-V curve of $(TBM_{\Delta6\text{-}113M})_7$ (black), $(SM_{\Delta6\text{-}113M})_7$ (purple), and $(SM_{\Delta6\text{-}113F})_7$ (orange). $(TBM_{\Delta6\text{-}113M})_7$ containing E111N/K147N (NN) mutation loses the rectification properties of WT, which shows larger conductance at the negative potential[3, 4].

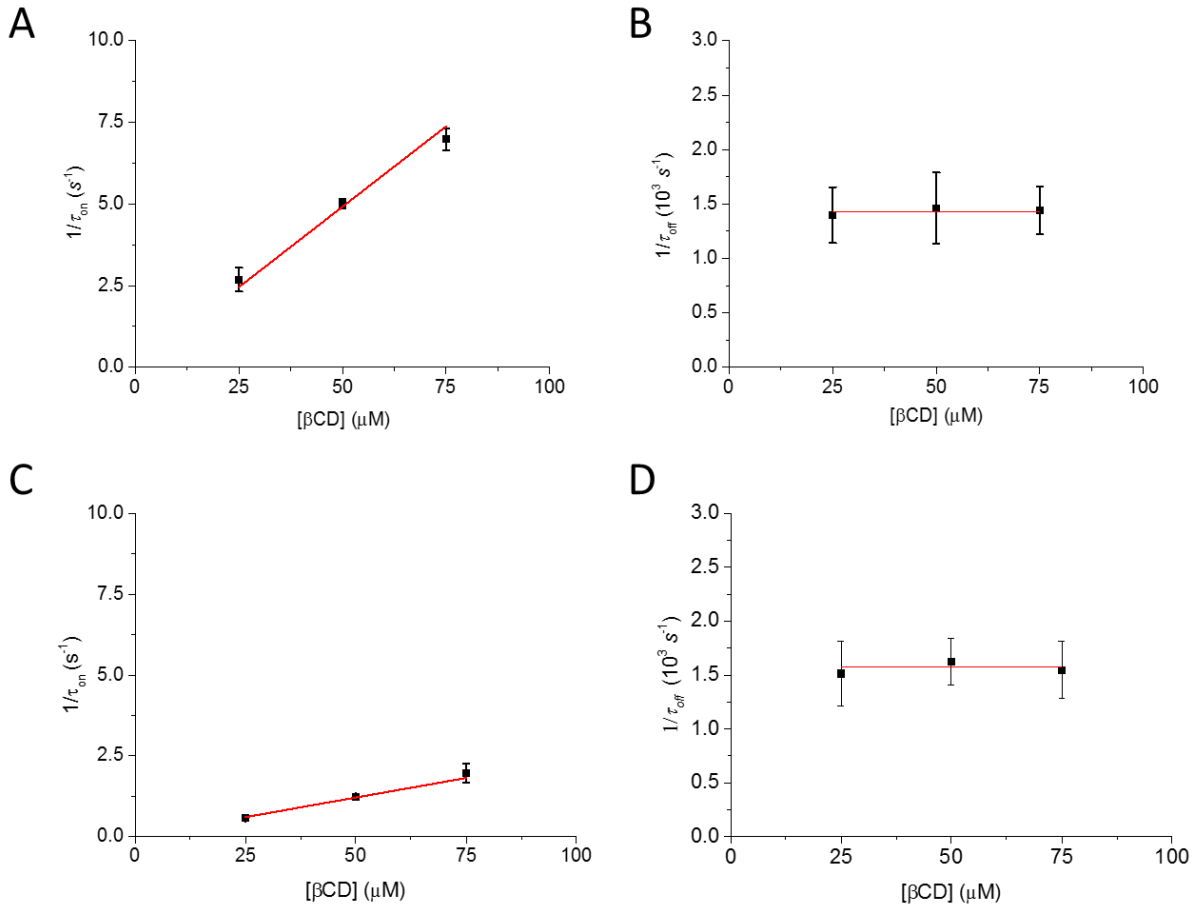

Figure S11. Kinetic analysis of βCD binding to synthetic αHL pores. The association (k_{on}) and dissociation rate constants (k_{off}) were determined from plots of $1/\tau_{on}$ and $1/\tau_{off}$ *versus* [βCD] by using linear fit tools in Origin 9.1. Dissociation constants (K_D) were obtained from the rate constants ($K_D = k_{off}/k_{on}$). WT_6SM_{fl}: $K_D = 14.5 \pm 0.4 \times 10^{-3}$ M (n = 3); $k_{on} = 10.0 \pm 0.2 \times 10^4$ $M^{-1} \cdot s^{-1}$ (**A**), $k_{off} = 14.5 \pm 0.2 \times 10^2$ s^{-1} (**B**). $(SM_{\Delta 6\text{-}113M})_7$: $K_D = 6.5 \pm 0.2 \times 10^{-2}$ M (n = 3); $k_{on} = 24.2 \pm 0.7 \times 10^3$ $M^{-1} \cdot s^{-1}$ (**C**), $k_{off} = 15.7 \pm 0.3 \times 10^2$ s^{-1} (**D**). The measurements for the two different αHL pores were carried out in 1 M KCl, 20 mM Tris·HCl, at +50 mV, but in different pH (WT_6SM_{fl}: pH 8.5, $(SM_{\Delta 6\text{-}113M})_7$: pH 7.0).

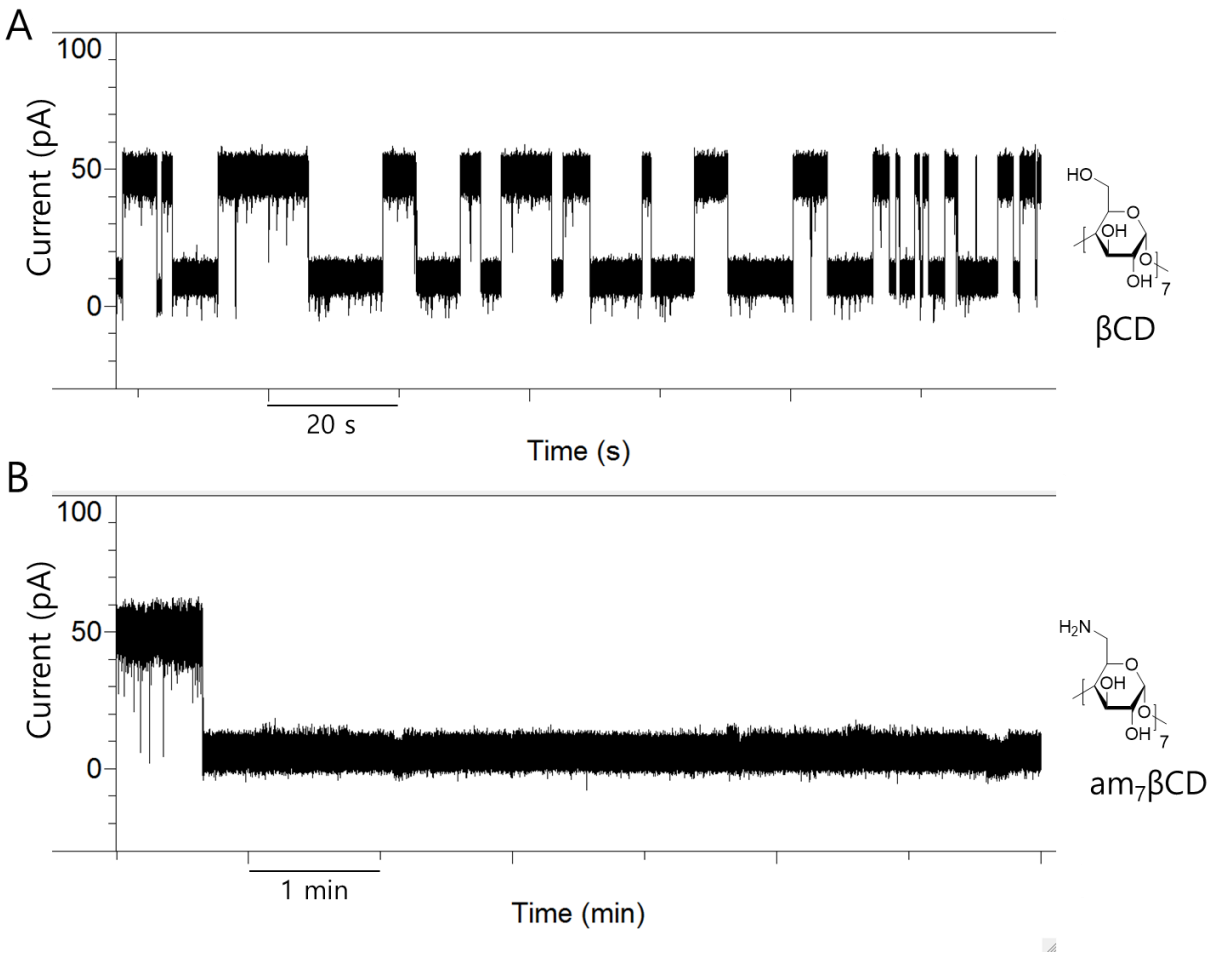

Figure S12. Representative current trace of cyclodextrin bindings to a single homoheptameric $(SM_{\Delta 6\text{-}113F})_7$ pore. (A) Binding of βCD (k_{on} = 7.4 ± 0.5 × 10^4 $M^{-1}s^{-1}$, k_{off} = 4.5 ± 0.8 s^{-1}, and K_D = 6.1 ± 0.2 × 10^{-5} M^{-1}) **(B)** Permanent binding of am_7βCD to the $(SM_{\Delta 6\text{-}113F})_7$ pore. The measurement was carried out in 1 M KCl, 20 mM Tris·HCl (pH 7.0), at +50 mV. The current was filtered at 5 kHz and sampled at 25 kHz. Further digital filtering was carried out at 2 kHz (8-pole lowpass Bessel filter) for display.

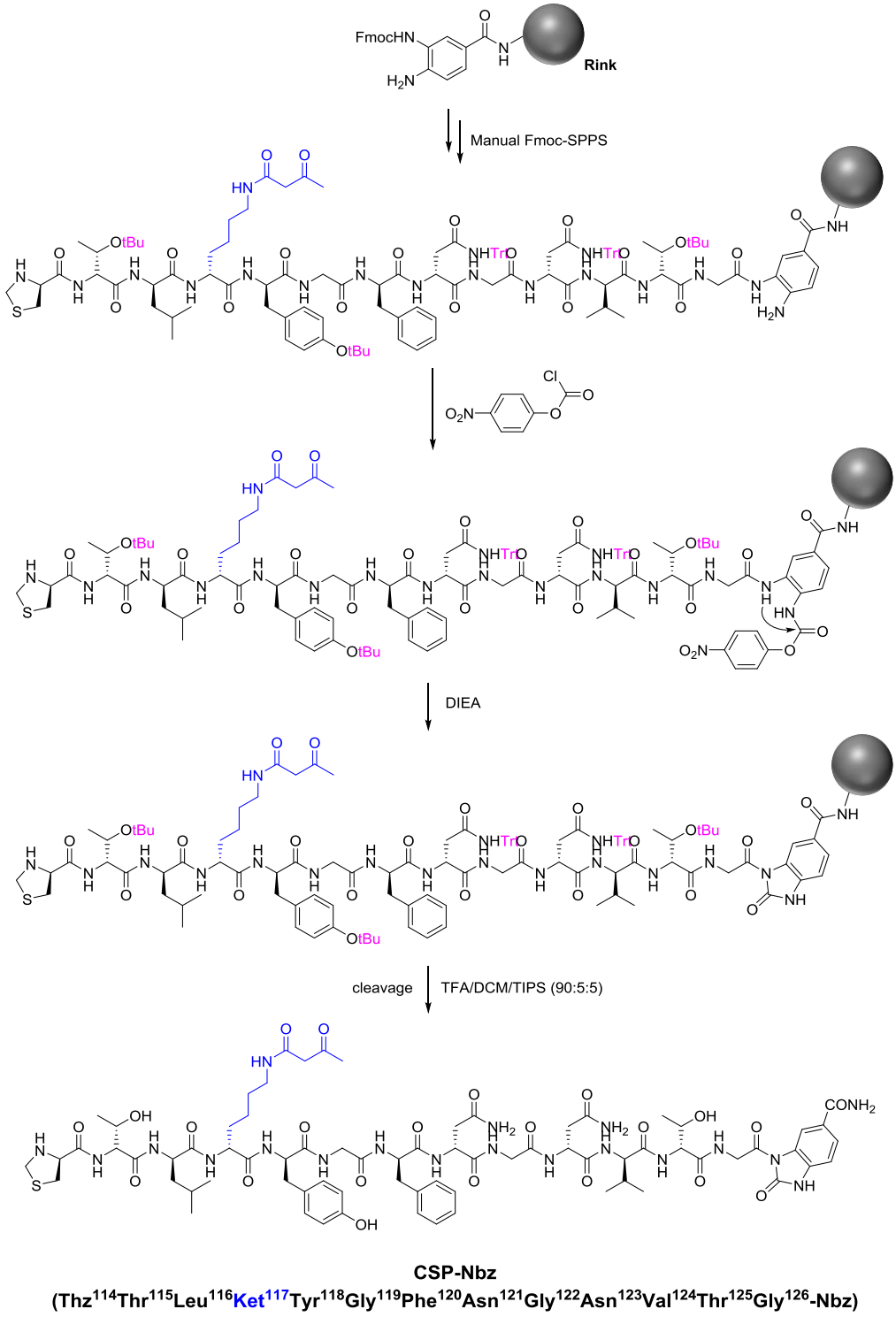

Figure S13. Synthesis of CSP-Nbz. Synthetic scheme for CSP-Nbz bearing a ketone group

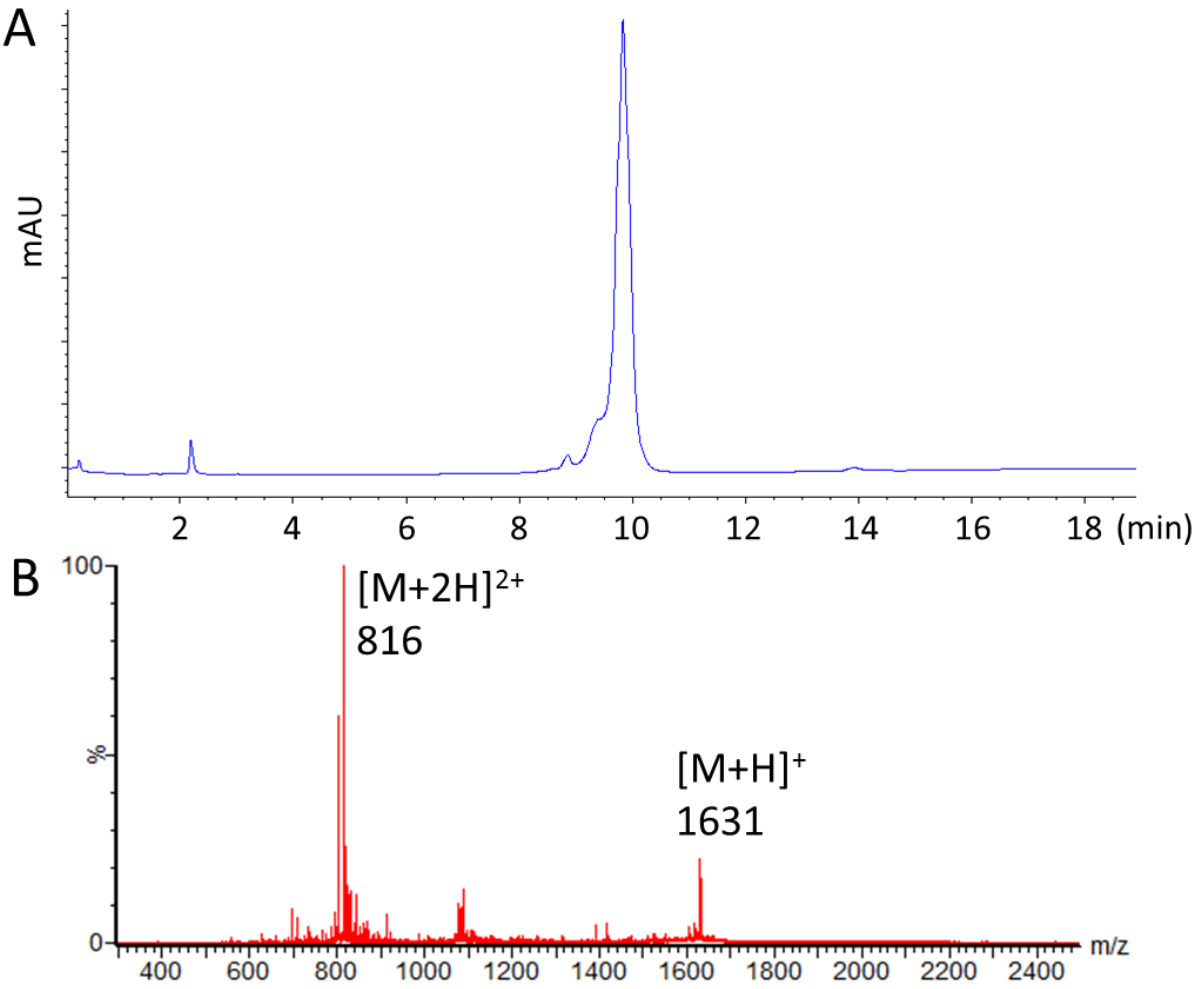

Figure S14. Characterization of CSP-Nbz. A) The peptide purified by HPLC with a preparative C_{18} reverse-phase column (Vydac 218TP) was further characterized by analytical LC using a C_{18} analytical column (Polaris C_{18} A 5u). **B)** ESI-MS spectrum for CSP-Nbz ($[M+H]^+$ = 1,631 (obs) 1,629 (calcd))

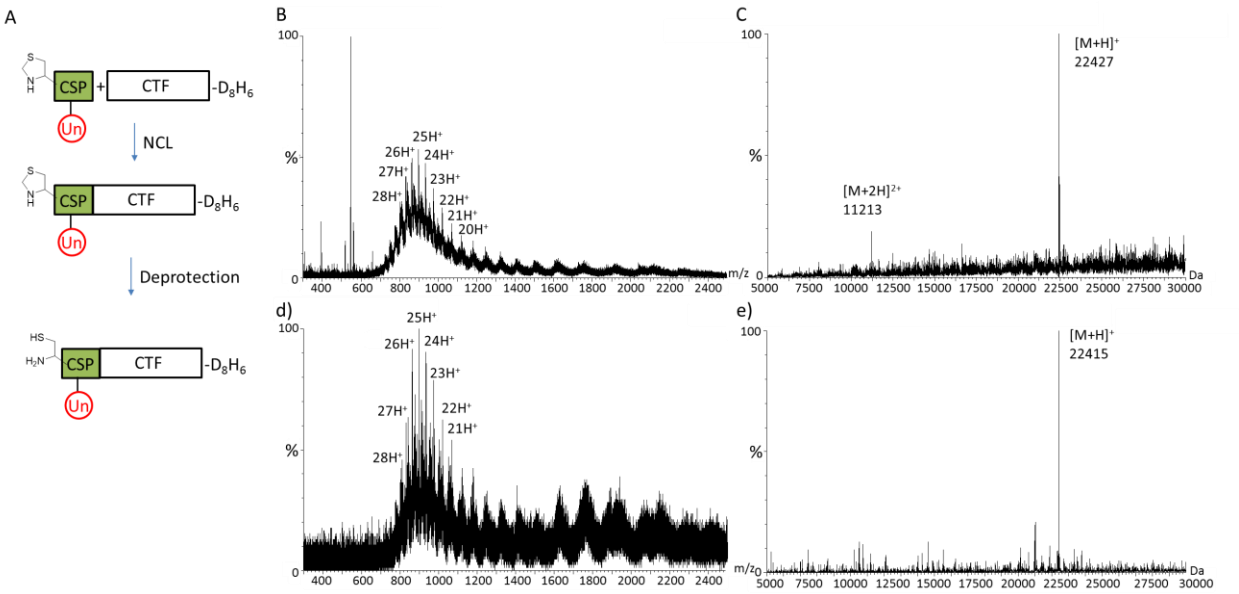

Figure S15. Characterizations of the first NCL product and Thz-deprotected product for SM_{ket}. A) Schematic representation of the first NCL and deprotection to regenerate N-Cys. ESI-MS and deconvoluted mass spectrum of the first ligation product (CSP-CTF) (**B** and **C**, respectively): [M+H]+ = 22,427 (obs), 22,426 (calcd). ESI-MS and deconvoluted mass spectrum of the Thz-deprotected first ligation product (**D** and **E**, respectively): [M+H]+ = 22,415 (obs), 22,414 (calcd).

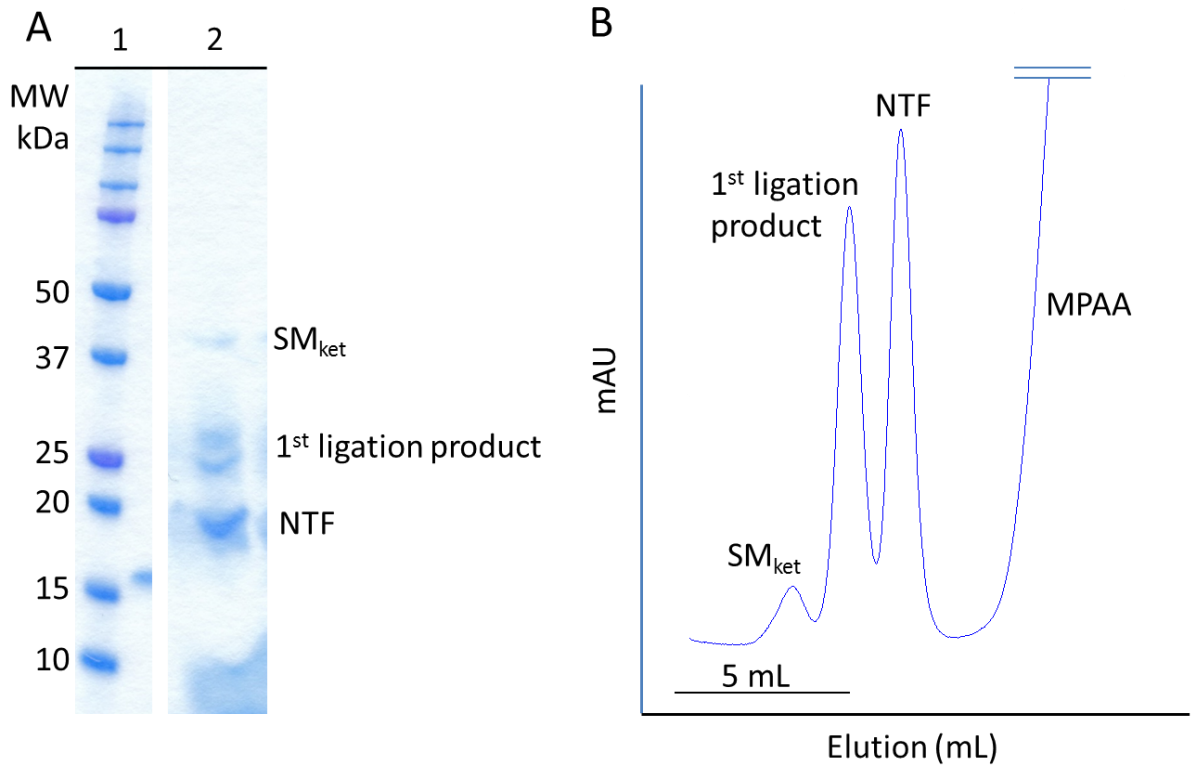

Figure S16. The second native chemical ligation step and purification of SM_{ket}. A) The crude product was examined by SDS/PAGE. Lane 1: molecular mass markers; lane 2: crude second ligation product. The intensity of the polypeptide bands in the gel by using ImageJ (NIH) gave a yield of ~19% for the second ligation reaction. **B)** The crude second ligation product was purified by gel filtration under denaturing conditions (8 M urea, 50 mM NaH_2PO_4 (pH 7.0), 5 mM TCEP). The fraction eluted in the first peak (SM_{ket}) of the column (Superdex^{TM} 200 10/300 GL column) was collected. The separation was monitored at 280 nm.

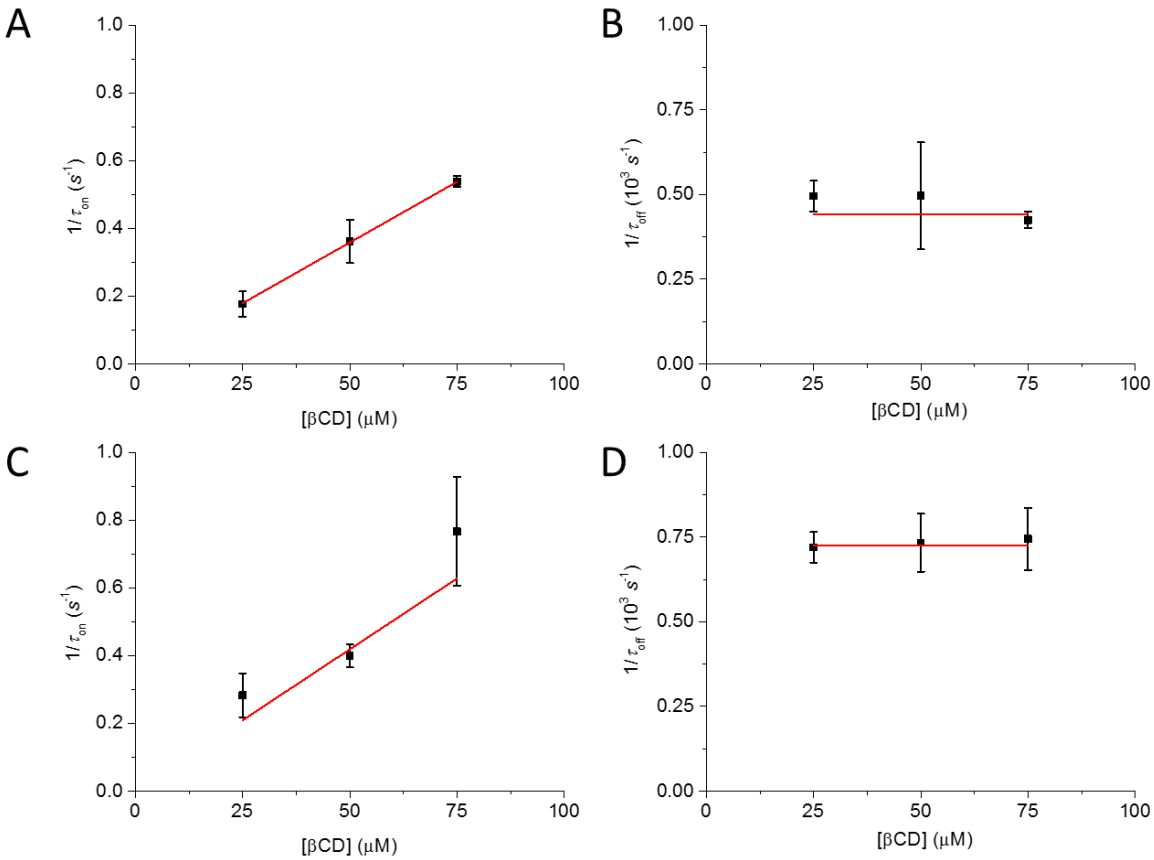

Figure S17. Comparison of the βCD binding kinetics for the WT_7 and WT_6SM_{ket1} pores. Kinetic constants were calculated by using $k_{on} = 1/\tau_{on}$ [βCD] and $k_{off} = 1/\tau_{off}$, where [βCD] is the concentration of βCD. k_{on} was obtained from the slope of the linear fit of $1/\tau_{on}$ *versus* [βCD]. k_{off} was independent of [βCD] and was obtained from the average of the $1/\tau_{off}$ values. WT_7: $K_D = 6.1 \pm 0.2 \times 10^{-2}$ M (n = 3); $k_{on} = 71.7 \pm 0.1 \times 10^2$ $M^{-1} \cdot s^{-1}$ (**A**), $k_{off} = 4.4 \pm 0.2 \times 10^2$ s^{-1} (**B**). WT_6SM_{ket1}: $K_D = 8.6 \pm 0.7 \times 10^{-2}$ M (n = 3); $k_{on} = 83.7 \pm 6.8 \times 10^2$ $M^{-1} \cdot s^{-1}$ (**C**), $k_{off} = 7.2 \pm 0.1 \times 10^2$ s^{-1} (**D**). The measurement was carried out in 1 M KCl, 50 mM Na acetate buffer (pH 3.4), at +50 mV.

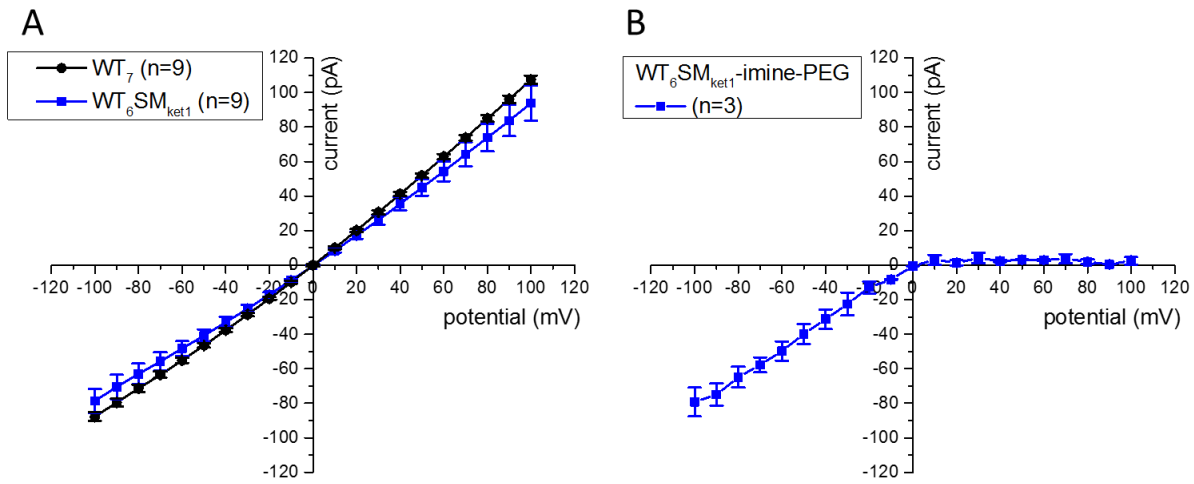

Figure S18. Current-voltage characteristics of WT_6SM_{ket1}. A) The IV curves were obtained from mean values measured with WT_6SM_{ket1} (blue) and WT_7 (black). The latter were made from WT subunits prepared by IVTT. **B)** The IV curve shows almost no current at all applied positive potentials after the reaction of the ketone with MPA. The measurements were carried out in 1 M KCl, 50 mM Na acetate buffer (pH 3.4).

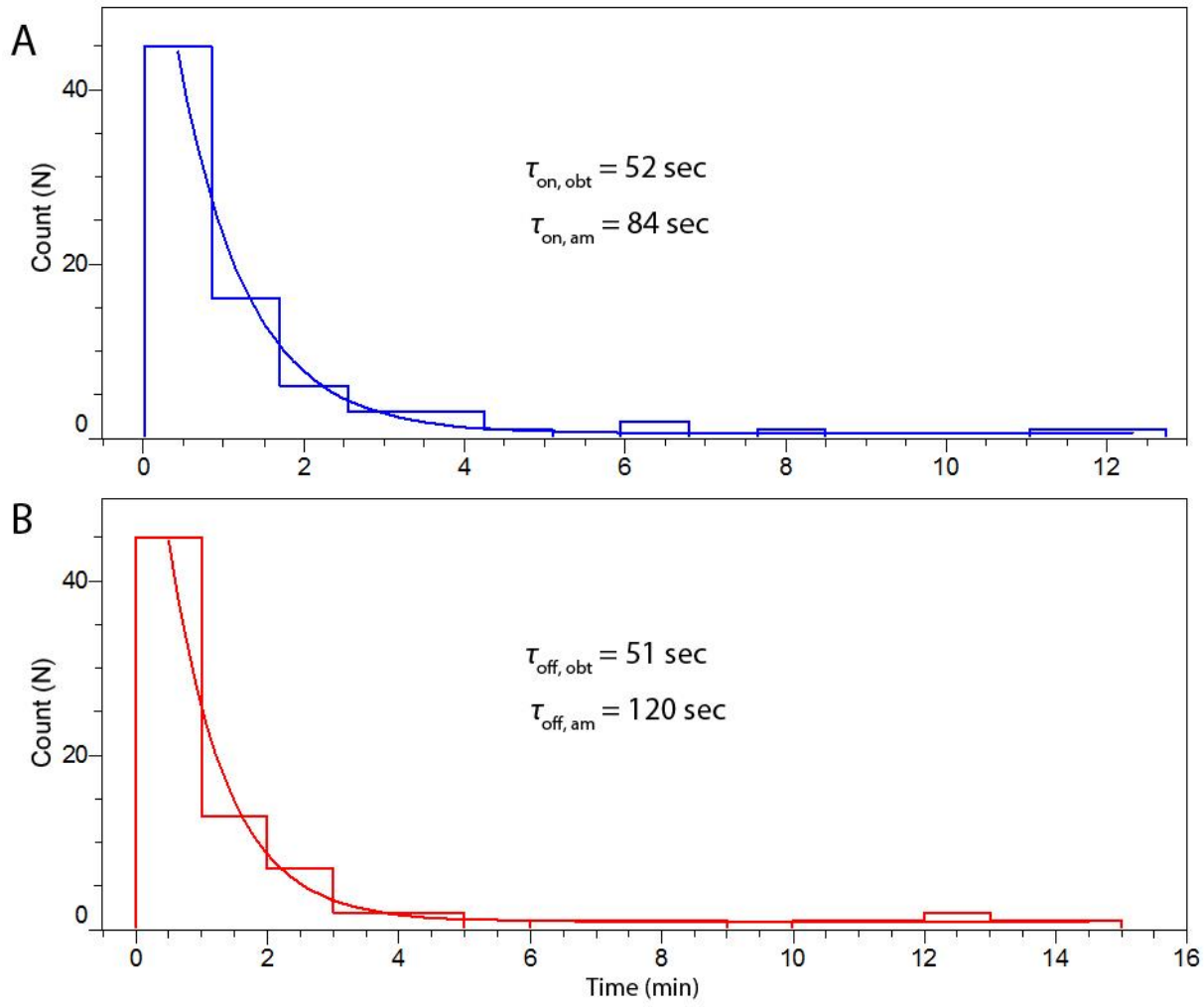

Figure S19. The mean lifetime of the oxime (A) and O-alkyloxime (B). Individual lifetimes (τ_{on} and τ_{off} for the mean lifetime of oxime and O-alkyloxime) from all recordings (number of measurements (n) = 79 and 78 for τ_{on} and τ_{off}) were plotted and fitted to an exponential function to yield a mean lifetime of (A) τ_{on} = 52 ± 2 sec (correlation coefficient, r = 0.94) and (B) τ_{on} = 51 ± 2 sec (r = 0.93). The lifetimes obtained from the fitted exponential curves (obt) are in the same range of the arithmetic means of the individual lifetimes (am).

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