Catalytic site-selective substrate processing within a tubular nanoreactor

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Supplementary Information

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1. Identifying the covalent adducts by current patterns

To confirm the identity of adducts formed upon thiol-disulfide interchange, we probed their behaviour in response to voltage steps (Fig. S1).

For an αHL-oligo adduct (Fig. S1a), the negative charges on the oligonucleotide within the barrel re-orientated the chain upon switching the applied potential between high positive and negative values (e.g. ±150 mV). At low potentials (e.g. +50 mV), the pulling force was insufficient to overcome the energy barrier for re-orientation. Therefore, due to condensation of the oligonucleotide, the current level recorded at a low potential (e.g. +50 mV) was lower if the immediately previous applied potential was of opposite polarity. The ability of an αHL-oligo adduct to move back and forth between two cysteines has been further developed to construct a molecular "hopper" that can advance up and down a multi-cysteine track. In this case, the re-orientation of the αHL-oligo adduct by switching the applied potential enabled alternating sequences of hops in upward and downward directions. For example, a high negative potential (e.g. -150 mV) applied after a downward sequence re-oriented the αHL-oligo adduct upward to initiate an upward sequence of hops along the multi-cysteine track.

For an αHL-peptide adduct (Fig. S1b), the peptide fragment was locked inside the upper vestibule of the αHL pore with traptavidin acting as a stopper. In this case, the current pattern at a low applied potential (e.g. -50 mV) was the same following high potentials of opposite polarity (e.g. ±150 mV), which indicates that the conformation was unaffected.

Supplementary Fig. 1 Current changes caused by αHL-polymer adducts in response to voltage steps. a, An αHL-oligo adduct was formed after thiol-disulfide interchange between POC2 and nanoreactor 119. A step to +50 mV produced a different level of current blockade depending on whether the step was made from -150 mV or from +150 mV (purple arrows), indicating that a switch in oligonucleotide orientation occurs at high applied potentials. b, An αHL-peptide adduct was formed after thiol-disulfide interchange between POC2 and nanoreactor 113. After a step to -50 mV, the current levels of the three sub-conductance levels were the same whether the step came from +150 mV or from -150 mV (green arrows), in keeping with the presence of a covalent adduct locked inside the vestibule of the αHL pore by the traptavidin stopper. Traces were filtered at 200 Hz. Conditions: 2 M KCl, 20 mM HEPBS, 20 μM EDTA, 5 mM DTT (trans), pH 8.5, 20 ± 1 °C.
2. Site-selectivity and regioselectivity of thiol-disulfide interchanges between nanoreactor 115/143 and POC1

Supplementary Fig. 2 Thiol-disulfide interchanges between nanoreactor 115/143 and POC1. a, An αHL-oligo adduct was formed at Cys-115 following threading. The oligonucleotide transferred between Cys-115 and Cys-143 by Sγ attack (orange: transfer from 115 to 143; brown: transfer from 143 to 115) until Sγ was attacked to form the cross-strand disulfide (purple). Here, we show an unusual example in which six transfers were observed, the most we recorded. An αHL-DTT adduct was subsequently observed (blue) as DTT reduced the disulfide to regenerate the two free cysteine residues. b, An αHL-oligo adduct was formed at Cys-143 following threading. The oligonucleotide transferred to Cys-115 (brown) from which release took place through Sγ attack by Cys-143 (purple). Traces were filtered at 50 Hz. Insets showing αHL-DTT adducts were filtered at 1000 Hz. Conditions: 2 M KCl, 20 mM HEPBS, 20 μM EDTA, 5 mM DTT (trans), pH 8.5, 20 ± 1 °C. c, The kinetic model and the derived rate constants for the thiol-disulfide interchanges observed with nanoreactor 115/143 and POC1. Dwell time analysis and rate constant estimations were performed by using the maximum interval likelihood algorithm of QuB.
3. Regioselectivity of thiol-disulfide interchange between DTT and αHL adducts

Previously, a transient intermediate was detected upon DTT reduction of a mixed-disulfide formed between an αHL pore with a single Cys-117 and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)².

In the present work, DTT was used to release covalent disulfide adducts—either αHL-oligo adducts or αHL-peptide adducts—from nanoreactors. The DTT thiolate can attack either the nanoreactor cysteine sulfur (Sγ) or the sulfur atom donated by the polymer: an oligonucleotide or a peptide (Sδ) (Fig. S3a). When Sδ is attacked, an αHL-DTT adduct is formed, which has a distinctive breakdown signal (Fig. 5). We investigated the regioselectivity of release of αHL-polymer adducts by DTT, by monitoring the formation of the αHL-DTT adduct (Table S1, Fig. S3a). The Sγ regioselectivity is defined as the number of αHL-DTT adducts detected against the overall number of events:

\[
\text{regioselectivity (\%) } = \frac{n(S\gamma)}{n(S\gamma) + n(S\delta)} \cdot 100
\]

In general, DTT attacked Sδ more frequently than Sγ (Table S1). Complete Sδ regioselectivity was seen when the mixed disulfide was formed between nanoreactor 113 or nanoreactor 143 and the polymer (an oligonucleotide or a peptide). In contrast, complete Sγ regioselectivity was seen when a mixed disulfide was formed between nanoreactor 113 and DTNB (Table S1, Fig. S3b), and over 80% reaction at Sγ was seen when the mixed disulfide was formed between nanoreactor 143 and DTNB. This ruled out the possibility that the αHL-DTT adducts were too short-lived to be detected.

For the thiol-disulfide interchange between DTT and covalent adducts, the constituent sulfurs are not chemically and sterically equivalent and the DTT is freely diffusing in solution. Therefore, no spatial control was exerted on the attacking thiolate and innate regioselectivity was observed (Table S1).

The lifetime of an αHL-DTT adduct (<ταHL-DTT>)—the mean waiting times before cyclization (Table S1)—varied between the nanoreactors. This implies that the nanoreactor cysteine thiols, which are the leaving groups upon DTT thiolate attack, have different pKa values³.

Supplementary Fig. 3 Regioselectivity of thiol-disulfide interchange between DTT and αHL adducts. A mixed-disulfide is formed (a) with a macromolecular substrate to give either an αHL-oligo adduct or an αHL-peptide adduct, or (b) with DTNB. When the DTT attacks the cysteine sulfur (Sγ), the transient intermediate can be detected by single-channel electrical recording.
Supplementary Table 1. Regioselectivity of thiol-disulfide interchange between DTT and αHL adducts, and the lifetimes of the αHL-DTT adducts

|                  | DTT/αHL adducts (αHL-oligo adducts and αHL-peptide adducts combined) | DTT/DTNB |
|------------------|------------------------------------------------------------------------|----------|
|                  | Regioselectivity n(Sγ) / n(Sγ) + n(Sδ)                                 | Regioselectivity n(Sγ) / n(Sγ) + n(Sδ) |
| 113              | 0/137 (0%)                                                            | 47/47 (100%)             | 85 ± 8 |
| 115              | 41/143 (29%)                                                          | 220 ± 30                 | 85/85 (100%) | 150 ± 20 |
| 143              | 0/133 (0%)                                                            | /                       | 82/82 (100%)             | 18 ± 2 |
| 117              | 59/220 (27%)                                                          | 73 ± 6                   | 63/63 (100%)             | 66 ± 6 |
| 119              | 3/198 (1.5%)                                                          | 34 ± 30c                 | 0/145d                   | / |
| 121              | 3/23 (13%)                                                            | 110 ± 80c                | 0/60d                    | / |
| 115/143          | 59/59 (100%)                                                          | 9.4 ± 1.7                | Not tested               |

a. Dwell-time analysis was performed by using the maximum interval likelihood algorithm of QuB unless stated otherwise.
b. /, not measurable
c. <τ_DTT-αHL> was calculated as the arithmetic mean due to insufficient events with nanoreactor 119 or 121.
d. We speculate that the current levels of the adducts formed upon DTNB reaction with nanoreactor 119 or 121 cannot be distinguished from the current level of the αHL-DTT adduct. Future experiments with alternative reducing reagents (e.g. dihydrolipoic acid) might give identifiable current steps for the transient intermediates.
4. Current levels for each substrate/nanoreactor combination

Supplementary Table 2. Residual current levels (I$_{\text{res}}$%) for individual substrate/nanoreactor combination

|   | +150 mV$^a$ |   |   |
|---|-------------|---|---|
|   | Threading$^b$ | $\alpha$HL-Oligo$^b$ | $\alpha$HL-Peptide$^c$ |
| POC1 | 113 | 33% | 39% | 43%-51% |
|     | 115 | 31% | 45% | / |
|     | 117 | 34% | 47% | / |
|     | 143 | 31% | 44% | / |
|     | 115/117 | 30% | 45%, 47% | / |
|     | 115/143 | 30% | 45% | / |
| POC2 | 113 | 33% | 39% | 39%-56% |
|     | 115 | 32% | 45% | 27%-50% |
|     | 117 | 33% | 47% | 42%-49% |
|     | 119 | 34% | 53% | / |
|     | 143 | 32% | 44% | 27%-54% |
| POC3 | 115 | 32%, 33%, 34%$^d$ | 45% | 24%-52% |
|     | 117 | 32%, 33%, 34%$^d$ | 47% | 29%-56% |
|     | 119 | 32%, 33%, 34%$^d$ | 53% | 28%-48% |
|     | 121 | 32%, 33%, 34%$^d$ | / | / |
| POC4 | 113 | 33%, 34%, 35%$^d$ | 39% | 28%-56% |
|     | 117 | 33%, 34%, 35%$^d$ | 47% | 29%-54% |
|     | 119 | 33%, 34%, 35%$^d$ | 53% | 33%-54% |
|     | 121 | 33%, 34%, 35%$^d$ | / | / |
|     | 123 | 34%, 35%, 36%$^d$ | / | / |

$^a$. All the measurements in the Table were carried out at +150 mV.

$^b$. The standard deviations of I$_{\text{res}}$% are less than 0.5% (derived from n > 3 separate experiments for each substrate/nanoreactor combination).

$^c$. Three discrete interchanging levels were recorded for each adduct. However, adduct to adduct variation in I$_{\text{res}}$% was seen. The observed range of I$_{\text{res}}$% for all experiments is reported here.

$^d$. Three discrete interchanging levels were recorded at the threading stage.
5. Conformational flexibility of macromolecular substrates at the threaded stage

Interchanging current levels were observed during the substrate-threaded stage, and were more prominent with substrates containing a longer peptide segment (i.e. POC3 and POC4) (Fig. S5).

Conformational switching during the threaded stage was correlated with fewer phosphodiester units remaining within the electric field for POC3 and POC4. Consequently, less pulling force was imparted to the peptide, resulting in greater conformational freedom.

Supplementary Fig. 5 Interchanging current levels recorded with POC3 threaded into nanoreactor 119. Three interchanging current levels were observed. Neither the αHL-oligo adduct nor the αHL-peptide adduct was formed specifically from one of the three levels. Traces were filtered at 200 Hz. Conditions: 2 M KCl, 20 mM HEPBS, 20 μM EDTA, 5 mM DTT (trans), pH 8.5, 20 ± 1 °C.
6. Synthesis and characterization of macromolecular substrates

6.1. Chemicals
Acetic anhydride (Ac₂O), acetonitrile (HPLC grade), 5-azidopentanoic acid, N,N-diisopropylethylamine (DIPEA), diethyl ether, dimethylformamide (DMF), hexafluoro-2-propanol (HFIP), N,N-hydroxybenzotriazole (HOBt), 1-methyl-2-pyrrolidinone (NMP), Nα-Fmoc-Nε-biotinyl-L-lysine, piperidine, triethylamine (TEA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. HBTU, Fmoc-Phe-OH, and Rink Amide MBHA resin LL (100-200 mesh) were from Novabiochem. Fmoc-NH-polymethylene glycol (PEG)(12)-COOH and Fmoc-Gly-Gly-OH were purchased from Iris Biotech GmbH.

6.2. Peptide segment preparation using solid-phase peptide synthesis
The peptide segments for POC1 and POC2 (Fig. S6a, S7a) were synthesized by using manual solid-phase peptide synthesis, adapted from previously reported protocols. Peptide segments for POC3 and POC4 were purchased from Peptide Protein Research Ltd. Peptide elongation was carried out on 0.25 mmol Rink Amide MBHA resin LL (0.37 mmol g⁻¹) by the Fmoc method with HBTU and DIPEA as coupling reagents. The resin was washed and swollen overnight in DMF and Fmoc was removed by treatment with 20% (v/v) piperidine in NMP at room temperature. The peptide segments were assembled through standard chain elongation by using the reactants Nα-Fmoc-Nε-biotinyl-L-lysine, Fmoc-Phe-OH, Fmoc-NH-PEG(12)-COOH, Fmoc-Gly-Gly-OH (for POC2 peptide segment) and 5-azidopentanoic acid. For each of the coupling reactions, a solution of 1.0 mmol reactant, 0.95 mmol HBTU, and 2.0 mmol DIPEA in 1.9 mL DMF was added to the resin, which was then shaken for 12 min. After each coupling, the resin was washed with NMP and capped with 1.5 mmol Ac₂O, 0.044 mmol HOBt, and 0.39 mmol DIPEA in 3 mL DMF for 2 min. The capped resin was then washed with NMP again before the next coupling. After the completion of chain assembly, the resin was washed sequentially with DMF and ethanol and dried in vacuo. Peptides were cleaved from the resin by treatment with TFA for 2 h. The TFA was evaporated in a stream of nitrogen and the peptide was precipitated with cold diethyl ether, followed by centrifugation, and several trituration treatments with diethyl ether.

6.3. Peptide segment purification and characterization
The crude peptide segment for POC1 or POC2 was dissolved in DMF and purified by preparative RP-HPLC (Dionex UltiMate 3000; Vydac C18 column: 250 × 22 mm, 10-15 μm (particle size); linear gradient: 5-95% eluant B in eluant A over 45 min, flow rate: 15 mL min⁻¹; eluant A: 0.1% TFA in water; eluant B: 0.1% TFA in acetonitrile). The identity and purity of the fractions were determined by analytical LC-MS (Waters LCT accurate-mass time-of-flight instrument (ESI-positive); Chromolith RP-18e column: 50 mm × 2 mm; linear gradient: 5-100% eluant B in eluant A over 8 min, flow rate: 1 mL min⁻¹; eluant A: 0.1% formic acid in water; eluant B: 0.1% formic acid in acetonitrile). The fractions with the correct mass were combined, lyophilized, and characterized by LC-MS and analytical RP-HPLC (Agilent 1260 Infinity HPLC; Polaris C18 column: 150 × 4.6 mm, 5 μm (particle size); linear gradient: 5-95% eluant B in eluant A over 10 min, flow rate: 1 mL min⁻¹; eluant A: 0.1% TFA in water; eluant B: 0.1% TFA in acetonitrile) (Fig. S6b, S6c, S7b, S7c).
Supplementary Fig. 6. LC-MS and HPLC characterization of the POC1 peptide segment. (a) Structure of the peptide segment. (b) The mass of the peptide segment ([M+H]+) was calculated to be 1243.7 g mol\(^{-1}\) and found to be 1244.6 g mol\(^{-1}\). (c) The purity of peptide segment was confirmed by analytical HPLC.

Supplementary Fig. 7. LC-MS and HPLC characterization of the POC2 peptide segment. (a) Structure of the peptide segment. (b) The mass of the peptide segment ([M+H]+) was calculated to be 1358.8 g mol\(^{-1}\) and found to be 1358.8 g mol\(^{-1}\). (c) The purity of the peptide segment was confirmed by analytical HPLC.
6.4. Generation and characterization of macromolecular substrates

Oligonucleotides modified with dibenzocyclooctyne-disulfide (DBCO-SS) at the 5' terminus were purchased from biomers.net.

![5' DBCO-SS modification](image)

5' DBCO-SS-A40 oligo (10 μL, 1 mM in MilliQ water) was added to each peptide segment (1 μL, 10 mM in MilliQ water). The mixtures were left at room temperature for 30 min to form the peptide-oligo conjugates (POC1-4) by a copper-free click reaction (Fig. S8).

The conjugates were purified by semi-preparative HPLC (Agilent 1260 Infinity; Supelco Discovery BIO Wide Pore C18 column: 250 x 10mm, 10 μm; linear gradient: 10-90% eluant B in eluant A over 30 min, flow rate: 4.5 mL min⁻¹; eluant A: 0.1% TFA in water; eluant B: 0.1% TFA in acetonitrile) and identified by LC-MS (UPLC-MS Waters XEVO G2-QTOF (ESI-negative); ACQUITY UPLC Oligonucleotide BEH C18 Column: 2.1 mm X 50 mm, 1.7 μm (particle size); linear gradient: 0-70% eluant B in eluant A over 8 min, flow rate: 0.2 mL min⁻¹; eluant A: 8.6 mM TEA, 200 mM HFIP in 5% methanol/water (v/v); eluant B: 20% eluant A in methanol).
Supplementary Fig. 8. LC-MS characterization of macromolecular substrates POC1-4. The two peaks in each chromatogram have the same mass, corresponding to the two regioisomeric conjugates formed by the copper-free click chemistry between the peptide segment (blue) and the oligonucleotide (green). The mass of POC1 was calculated to be 14327 g mol\(^{-1}\) and found to be 14327 g mol\(^{-1}\). The mass of POC2 was calculated to be 14440 g mol\(^{-1}\) and found to be 14441 g mol\(^{-1}\). The mass of POC3 was calculated to be 14615 g mol\(^{-1}\) and found to be 14616 g. The mass of POC4 was calculated to be 14729 g mol\(^{-1}\) and found to be 14730 g mol\(^{-1}\).
7. Preparation of cysteine-containing αHL monomers

The construction of single-cysteine nanoreactors 113, 115, 117, 119, 121 and the double-cysteine nanoreactor 115/117 based on pT7-αHL-D8H6, which encodes the wild-type αHL with an octa-aspartate tail and a His-tag at the C terminus, have been previously reported5.

Plasmid pT7-αHL-143C-D8H6 encodes an αHL mutant containing a cysteine at position 143 as well as an octa-aspartate tail and a His-tag at the C terminus. It was generated from pT7-αHL-D8H6 by in vivo homologous recombination. Two sets of PCR reactions were carried out. For the first reaction, the template was first linearized by NdeI (New England Biolabs) before PCR with the forward mutagenic primer: 5′-CAAATGTTTTTGATTTGTCATACACTGAAATATGTTC-3′ and the reverse nonmutagenic primer (SC47): 5′-CAGAAGTGGTGCTGCAACTTTAT-3′. For the second reaction, the template was linearised by HindIII (New England Biolabs) before PCR with the reverse mutagenic primer: 5′-GAACATATTTTGATGACAAATCGAAACATTTG-3′ and the forward nonmutagenic primer (SC46): 5′-ATAAAGTTGCAGGACCACTTCT-3′. The linearized templates and primers were mixed with 1XPhusion Flash HF Mastermix (New England Biolabs) and put through the following cycling program: 94°C for 5 min, then 30 cycles of 94°C (30 s), 50°C (30 s), 72°C (30 s), and then 50°C for 5 min. After PCR, 5 μL of each reaction were mixed and transformed into E. coli XL10-Gold cells (Agilent). The transformed cells were allowed to grow at 37°C overnight on LB (Luria Broth)-carbenicillin plates. Plasmid DNA was isolated from colonies by using the QIAprep Spin Miniprep Kit (QIAGEN). Successful mutagenesis was confirmed by DNA sequencing.

Similarly, pT7-αHL-115C143C-D8H6 was made from pT7-αHL-115C-D8H6 with the same two mutagenic primers. pT7-αHL-123C-D8H6 was made from pT7-αHL-WT-D8H6 with the two mutagenic primers: 5′-CGGATTCAACGGTTGTGTTACTGTTGTGATGATACAGG-3′ (Forward); 5′-CCTGTATCATCACCAGTAACAAACCGTTGAATCCG-3′ (Reverse).
8. Preparation of αHL heptamers containing cysteine(s) on one of the seven subunits

Engineered αHL polypeptides were expressed by using a commercial *in vitro* transcription-translation (IVTT) kit: *E. coli* T7 S30 Extract System for Circular DNA (Promega). To suppress transcription by *E. coli* RNA polymerase, the T7 S30 extract provided in the kit was treated with rifampicin prior to use (1 μg mL⁻¹, final concentration). A standard reaction comprised: DNA template (3.2 μg), amino acid mix minus methionine (supplied with the kit, 5 μL), S30 premix without amino acids (supplied with the kit, 20 μL), [³⁵S]methionine (2 μL, 1,200 Ci mmol⁻¹, 15 mCi mL⁻¹, MP Biomedicals), T7 S30 extract (supplied with the kit, 15 μL), and nuclease-free water to a final volume of 50 μL. To make heteroheptamers, a mixture of plasmids encoding the WT αHL and the mutant monomer were mixed in a ratio 6:1 (WT: mutant). The IVTT mixture was incubated at 37°C for 1 h.

Heptamerization was carried out by the addition of rabbit erythrocyte membranes (3 μL, ~1 mg protein mL⁻¹) to the IVTT reaction mixture (50 μL), followed by incubation at 37°C for another 1 h. The mixture was then centrifuged for 10 min at 25,000 × g. The supernatant was removed and the pellet resuspended in MBSA buffer (200 μL, 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 150 mM NaCl, 1 mg mL⁻¹ bovine serum albumin, pH 7.4). The wash with MBSA was repeated before the pellet was resuspended in 2X Laemmli sample buffer (50 μL) and electrophoresed in a 5% SDS polyacrylamide gel at 70 V for 15 h.

The αHL heteroheptamers containing different numbers of mutant subunits were separated in the gel based on their different electrophoretic mobilities, which were determined by the number of octa-aspartate (D8) tails. The top and bottom bands corresponded to WT₇ and (mutant-D8H6)₇, respectively. The second band from the top was the desired heteroheptamer containing a single mutant subunit.

To extract heptameric pores, the gel was first dried without fixation on Whatman 3M filter paper under vacuum for 5 h at room temperature. After visualization by autoradiography with Kodak BioMax MR film, the desired bands were cut from the gel with a scalpel. Each excised band was rehydrated in TE buffer (300 μL; 10 mM Tris·HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) for 1 h at room temperature. The filter paper was then removed, and the rehydrated gel was macerated with a pestle. The resulting slurry was filtered through a 0.2 μm hydrophilic membrane filter (Proteus Mini Clarification Spin Column, Generon). The filtrate was stored in 10 μL aliquots at -80°C.
9. Single Channel Recording

9.1. General
1,2-Diphytanoyl-sn-glycerol-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids. Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich.

Planar bilayer recordings were performed following the method established by Montal and Mueller. Two Delrin compartments were separated by a 25 μm-thick Teflon film containing an aperture (60 μm in diameter). The aperture was pre-treated with 1% (v/v) hexadecane in pentane. Each compartment was then filled with buffer (500 μL, 2 M KCl, 20 mM N-(2-hydroxyethyl)piperazine-N’-(4-butanesulfonic acid) (HEPBS), 20 μM EDTA, pH 8.5). In addition, the trans compartment contained 5 mM DTT. Drops of DPhPC in pentane (5 mg mL⁻¹) were added to both compartments. Repetitive up-and-down pipetting of the buffer solution resulted in the formation of a lipid bilayer across the aperture. A trans-bilayer potential was applied with two Ag/AgCl electrodes, each contained within a salt bridge formed from 3 M KCl in 3% (w/v) low-melt agarose.

Supplementary Fig. 9. Recording chamber (adapted from ref. 8)

Ionic currents were recorded by using a patch clamp amplifier (Axopatch 200B, Axon Instruments) with a 4-pole low-pass Bessel filter (80 dB/decade) at room temperature (20 ± 1°C). Signals were digitized with a Digidata 1320A digitizer (Molecular Devices), connected to a computer running the pCLAMP 10.3 software suite (Molecular Devices). Unless stated otherwise, the signal was filtered with a corner frequency of 5 kHz and sampled at 25 kHz.

9.2. Monitoring turnovers
The substrate (400 μM in 1 μL MilliQ water) was added to a solution of traptavidin (Kerafast) (40 μM in 10 μL phosphate-buffered saline, pH 7.4) and incubated at room temperature (20 ± 1°C) for 15 min to form the traptavidin-tagged substrate. A planar bilayer was first formed across the aperture in the recording chamber by using the method established by Montal and Mueller (described above). The cis compartment of the recording chamber containing αHL nanoreactor heptamers was stirred until a single pore inserted into the bilayer, as seen on the current readout. The traptavidin-tagged substrate (3 μL, 36 μM) was then added to the cis compartment, which contained 500 μL of recording buffer (2 M KCl, 20 mM HEPBS, 20 μM EDTA, pH 8.5). In addition, the trans compartment contained 5 mM DTT. To drive the macromolecular substrate into the nanopore, a potential of +150 mV was applied to the trans side. The threaded substrate reduced the current to an Iₐₜₘₜ of ~33% (see Table S2 for each substrate-
nanoreactor combination). After the thiol-disulfide interchange between the substrate and the nanoreactor, the current increased as one segment of the substrate departed the nanoreactor ($I_{eq}$ of 39%-53% for αHL-oligo adducts and ~24-56% for αHL-peptide adducts, see section 3, Table S2). DTT later released the adduct and returned the nanoreactor to its initial state ready for another round of thiol-disulfide interchange.

9.3. Single-channel data analysis

Current traces were idealized by using Clampfit 10.3 (Molecular Devices). The idealized data were analyzed with QuB 2.0 software (www.qub.buffalo.edu). Dwell time analysis and rate constant estimations were performed by using the maximum interval likelihood (MIL) algorithm of QuB.
10. References

1. Qing, Y., Ionescu, S. A., Pulcu, G. S. & Bayley, H. Directional control of a processive molecular hopper. *Science* **361**, 908–912 (2018).

2. Luchian, T., Shin, S.-H. & Bayley, H. Single-molecule covalent chemistry with spatially separated reactants. *Angew. Chem. Int. Ed.* **42**, 3766–71 (2003).

3. Singh, R. & Whitesides, G. M. Thiol-disulfide interchange. In *Sulphur-Containing Functional Groups* 633–658 (John Wiley & Sons, Inc., 1993).

4. Lee, J. *et al.* Semisynthetic nanoreactor for reversible single-molecule covalent chemistry. *ACS Nano* **10**, 8843–8850 (2016).

5. Pulcu, G. S., Mikhailova, E., Choi, L.-S. & Bayley, H. Continuous observation of the stochastic motion of an individual small-molecule walker. *Nat. Nanotechnol.* **10**, 76–83 (2015).

6. Miles, G., Bayley, H. & Cheley, S. Properties of *Bacillus cereus* hemolysin II: A heptameric transmembrane pore. *Protein Sci.* **11**, 1813–1824 (2009).

7. Montal, M. & Mueller, P. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. U. S. A.* **69**, 3561–3566 (1972).

8. Lu, S., Li, W.-W., Rotem, D., Mikhailova, E. & Bayley, H. A primary hydrogen–deuterium isotope effect observed at the single-molecule level. *Nat. Chem.* **2**, 921–928 (2010).

9. Nicolai, C. & Sachs, F. Solving ion channel kinetics with the QuB software. *Biophys. Rev. Lett.* **08**, 191–211 (2013).

10. Qin, F., Auerbach, A. & Sachs, F. Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys. J.* **70**, 264–280 (1996).