Supporting Information

Screening of aggregation properties of cyclic peptides by protein nanopore

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Section S1 Materials and Instruments

P1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, powder, ≥99%) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). n-Decane (99%) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Trypsin agarose (T1763, buffered aqueous suspension, from bovine pancreas trypsin) and potassium chloride (for molecular biology, ≥99.0%) were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Tris(hydroxymethyl) aminomethane (Tris, 99%) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, ≥99.0%), 1-naphthylamine (99%), 1-aminopyrene (97%), D-lactose monohydrate (98%), D-(+)-maltose monohydrate (98%), 4,4-diaminodiphenyl ether (98%, Cas No. 101-80-4), and sodium cyanoborohydride ($NaBH_3CN$, 95%) were purchased from Beijing Innochem Science & Technology Co., Ltd. (Beijing, China). 3'-p-Anisidine(99%), 1-(4-aminophenyl)-1,2,2-triphenylethene (97%), D-(+)-cellobiose (98%), and 1-amylamine (98%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). 3,4-(4-Methoxyphenoxy) aniline (CAS No. 31465-36-8, 97%) was purchased from Ark Pharm, Inc. (Arlington Heights, IL, USA). 2-mercaptoethanol (99%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). CDA (H-Cys[1]-Asp-Gly-Arg-Pro-Asp-Arg-Ala-Cys[1]-OH), CTY (H-Cys[1]-Thr-Pro-Arg-Ser-Ala-Asn-Tyr-Cys[1]-OH), CPT (H-Cys[1]-Pro-Glu-Lys-Trp-Leu-Gly-Thr-Cys[1]-OH), CMT (H-Cys[1]-Met-Thr-Pro-Asn-Pro-Thr-Thr-Cys[1]-OH), CPM (H-Cys[1]-Pro-Trp-His-Asn-Leu-Ile-Met-Cys[1]-OH), CST(H-Cys[1]-Ser-Arg-Ser-Met-Asp-Ser-Thr-Cys[1]-OH), CKT (H-Cys[1]-Lys-Pro-Thr-Ile-Ala-Asp-Thr-Cys[1]-OH) were purchased from ChinaPeptides Co., Ltd. (Shanghai, China). A series of mono- and multi-phosphorylated peptides (PPs) are purchased from ChinaPeptides Co., Ltd. (Shanghai, China).

Coomassie Brilliant Blue was purchased from Sangon Biotech, and the Bicinchoninic Acid (BCA) Protein Assay Kit was obtained from Beyotime Biotechnology (China). All solutions were prepared using ultrapure water from a Milli-Q system (resistivity of 18.2 MΩ·cm at 25 °C). Other general reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ag/AgCl electrode was prepared by electroplating from a silver wire (0.5 mm in diameter) as the anode (a platinum wire served as the cathode) in 0.1 M HCl under a constant 1 mA current for 10 minutes.

NMR spectra were obtained using a Bruker Avance III 400 MHz spectrometer. UV-Vis spectra were obtained using a PerkinElmer LAMBDA 365 UV-Vis spectrophotometer. Mass spectrometry (MS) data were acquired on a Waters ESI-Q-TOF mass spectrometer (Manchester, UK). EPIC detection assays were performed on a Tag-free Corning® Epic® system (New York, USA). Bio-Layer Interferometry (BLI) assays were conducted using an Octet K2 System (Molecular Devices LLC, USA). Current-voltage (I-V) characteristics were obtained using a Keithley 6487 picoammeter (Tektronix Inc., USA).

Section S2 Preparation of Recombinant Proaerolysin

The gene encoding proaerolysin was amplified from the reference sequence (GenBank accession number P09167). [1]The open reading frame (ORF) was flanked by EcoRI and HindIII restriction sites and subcloned into a pET22b(+) vector containing a C-terminal polyhistidine (His) tag. The recombinant plasmid was transformed into E. coli Rosetta Blue competent cells. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM) at 16 °C for 24 h. The cells were harvested and disrupted by sonication. The soluble supernatant containing the denatured protein was collected and purified using a Ni-NTA His-Bind resin column. The purified proaerolysin was subsequently refolded, and its concentration was determined using the BCA Protein Assay Kit. The purity of the protein was verified by 15% SDS-PAGE with Coomassie Blue staining. The purified protein was stored at −80 °C until further use.

Section S3 Single-Channel Recording

Experimental Setup. The single-channel recording apparatus consisted of a custom-designed double-chamber perfusion cell separated by a phenolic resin partition containing a circular aperture (diameter: 150 μm). To minimize external noise, the apparatus was enclosed in a copper box serving as a Faraday cage.

Lipid Bilayer Formation. A lipid solution was prepared by dissolving DPhPC (3 mg) in n-decane (100 μL). [2]Prior to assembly, the area surrounding the aperture on both sides of the cup was pre-painted with the lipid solution and allowed to dry for approximately 10 min to evaporate the solvent. The cup was then mounted in the chamber, and both the cis and trans compartments were filled with 1 mL of the recording electrolyte (10 mM Tris-HCl, 1 M KCl, 1 mM EDTA, pH 8.0, unless otherwise stated).

Specifically, a high ionic strength of 1 M KCl was employed to provide a highly conductive environment, which maximizes the spatial resolution and signal-to-noise ratio required to resolve the subtle current blockades of extremely small cyclic peptides. The pH was maintained at 8.0 to ensure both the structural stability of the aerolysin pore within the lipid bilayer and a consistent charge state for the peptide analytes. Furthermore, 1 mM EDTA was introduced to chelate trace divalent metal ions, preventing potential non-specific peptide precipitation or lipid membrane destabilization.

A pair of Ag/AgCl electrodes was immersed in the electrolyte solutions of the cis and trans chambers, respectively. The lipid bilayer was formed using the painting/air-bubble technique: a small amount of lipid solution was applied to the aperture in the cis chamber, followed by raising and lowering the liquid level across the aperture using a syringe until a stable bilayer was formed. The formation of the bilayer was confirmed by the observation of zero current between the electrodes.

Pore Insertion. Upon the formation of a stable lipid bilayer, the aerolysin protein solution was added to the cis chamber. A positive potential of +200 mV was applied to facilitate pore insertion. Once a single pore insertion was detected (indicated by a step-change in current), the applied potential was immediately reduced to +50 mV. The solution in the cis chamber was then gently perfused or agitated to remove unbound protein and prevent multiple pore insertions.

Section S4 Identification of peptide ligands targeting the C-terminus of SUMO-1 remnant by phage display

The C-terminus of SUMO-1 remnants “DVIEVYQEQTTGG” (DV12) and “QTGG” were selected as targets of the peptide ligands. For DV12, dodecapeptide (Ph.D.-12) library was used to screen against DV12 through a complete biopanning procedure. Firstly, to minimize nonspecific binding, an initial negative screening with BSA was performed. Briefly, 100 μL of 0.5% BSA solution was incubated overnight at 4°C in 96well plates, followed by six washes with 0.01% TBST. Subsequently, the plate was exposed to 100 μL of the original phage library (~1×1012 plaque-forming units, PFUs). After 30 minutes of shaking at room temperature, the supernatant was collected and used to infect E. coli ER2738 cells for amplification as per the manufacturer's instructions. Then, four rounds of affinity screening against DV12 were conducted. 100 μL of DV12 (200 μg/mL) was added to a 96-well plate (100 μL/well), followed by overnight incubation at 4°C. The plate was washed three times with 0.01%

TBST solution and blocked with 0.05% BSA solution at 4°C for 1 h. After blocking, 100 μL of phage broth (from the previous BSA negative screening step, titer approximately 1010 pfu/mL) was added to the plate and incubated for 30 min (25°C, 100 rpm). After a quick wash with TBST, 100 μL of 0.2 M glycine-HCl buffer (pH 2.2) was added and shaken for 10 min (25 °C, 200 rpm). The eluate phages were collected, neutralized with 1 M Tris-HCl (pH 9.1), and stored at –20°C with glycerol for further use. The titer was then measured and expanded to the desired titer for subsequent screening rounds. Besides, in the third and fourth rounds, washing with 0.05% TBST was performed to enhance stringency and improve biopanning affinity.

For QTGG, a loop constrained heptapeptide (Ph.D.-C7C) library was used to screen against QTGG. The biopanning procedures were the same as described above. One round of negative screening with BSA and three rounds of affinity screening against QTGG was performed. After completing all biopanning procedures, 16 well-isolated phage clones from the last round of DV12 screening, as well as 10 well-isolated phages from the thirds round of QTGG screening, were randomly selected for DNA sequencing. Phage DNA was extracted by using M13 singlestranded DNA extraction kit (BioTeke, China) and DNA sequencing was entrusted to Shanghai Sangon Biotech Corporation (Shanghai, P. R. China).

Section S5 Ionic Current Data Analysis

Single-channel ionic current recordings were analyzed using the open-source MOSAIC software developed by Balijepelli et al.[3] This software facilitates the characterization of physical interactions between macromolecules (e.g., DNA, proteins, or synthetic polymers) and the nanopore, which typically manifest as multiple discrete states within the current signal. In contrast to conventional analysis methods, the algorithms implemented in MOSAIC enable the identification of distinct discrete current states within transient signals, allowing for the estimation of short-lived states that were previously uncharacterizable. Unless otherwise specified, the experimental data in this work were processed using the "2-state" and "ADAPT" algorithms integrated within the MOSAIC package, and ionic current traces were recorded for 5-minute windows due to the limit of the algorithms.

Section S6 EPIC test assay

The aggregation assay was conducted to study the assembly behavior of cyclic peptides on the surface of a biosensor using the Label-free Corning® Epic® system. An Epic 384-Well Uncoated Cell Assay Microplate was used for detecting aggregation formation. Prior to measurements, peptides were dissolved into water to prepare a series of solutions with different concentrations from 100 nM to 1 mM, respectively. A microplate was first soaked in 30 μL water (soaking buffer) and temperature-equilibrated in the Epic reader for at least 1 hour. After a baseline measurement was taken for 2 min, a series of peptides solution with a volume of 10 μL were added into microplates with 30 μL water (1:4 dilutions). Following the mixture addition, the 384-well microplate was returned immediately to the Epic reader and signal changes were recorded as continuous time traces for at least 30 min. Each of the assays was performed at least four times with satisfactory reproducibility.

Section S7 Inter-pore and Day-to-day Reproducibility Test

To validate the reproducibility of our measurements, we conducted inter-pore and day-to-day reproducibility tests. Experiments were repeated on three separate days, during which nanopores were reconstituted following the previously described insertion procedure. The CPM cyclic peptide (1.2 μM) was dissolved in 1 mL of recording electrolyte (10 mM Tris-HCl, 1 M KCl, 1 mM EDTA, pH 8.0), with solutions prepared fresh daily prior to measurements, and ionic current traces were recorded for 5-minute windows. The results are shown in Fig. S3.

Section S8 Bio-Layer Interferometry (BLI) Assays

The binding affinity and kinetic interactions between the cyclic peptides CTY and CPM were characterized using a Fortebio Octet K2 System (Molecular Devices, LLC, USA), which utilizes Bio-Layer Interferometry (BLI) technology. Amine Reactive 2nd Generation (AR2G) biosensors were employed for the assays. The ligand peptide, CTY, was covalently immobilized onto the sensor surface via standard amine coupling chemistry. Specifically, the carboxyl groups on the AR2G sensors were activated using a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Subsequently, the activated sensors were immersed in a solution of CTY (0.1 mM in 10 mM sodium acetate, pH 5.0) and incubated overnight to facilitate the formation of amide bonds. Following immobilization, unreacted reagents were quenched with 1 M ethanolamine

(pH 8.5) for 5 min, and the functionalized sensors were thoroughly washed with Milli-Q water to remove any non-specifically bound species. For kinetic analysis, the CTY-loaded sensors were dipped into wells containing the analyte, CPM, at various gradient concentrations, and the association and dissociation responses were recorded in real-time. The resulting sensor grams were globally fitted to a 1:1 binding model using the Fortebio Data Analysis software to determine the equilibrium dissociation constant. The data represents a single titration series.

Section S9 Disulfide Reduction and Ring-Opening Kinetics

To investigate the kinetic dynamics of the co-assembly process, a disulfide reduction assay was performed. The addition of 2-mercaptoethanol was utilized to cleave the intramolecular disulfide bonds, thereby converting the cyclic peptides into their linear counterparts. In a typical experiment, both cyclic peptides (e.g., CTY and CPM, 1.0 μM each) were introduced into the electrolyte solution. Upon the establishment of a stable current baseline or assembly state, 2-mercaptoethanol was added to the chamber to reach a final concentration of 0.1 mM. The ionic current was recorded continuously throughout the process to monitor the structural evolution of the co-assemblies before and after the addition of the reducing agent.

Section S10 Molecular Docking Simulations

To elucidate the binding mode and interaction mechanism between the two cyclic peptides, molecular docking studies were performed using AutoDock software.[4] The structural models of the cyclic peptides were first pre-processed, during which polar hydrogen atoms were added to the structures. In the docking setup, the cyclic peptide CPM was defined as the receptor, while CTY was designated as the ligand. The grid box was centered and sized to encompass the entire structure of the CPM receptor, serving as the active binding search space. The Lamarckian Genetic Algorithm (LGA) was employed as the global search method. The simulation parameters were configured to generate a maximum of 20 distinct docking conformations (models). The optimal binding mode was selected based on the lowest binding energy and the scoring function ranking. Finally, the molecular interactions of the best-scored conformation were analyzed and visualized using the PyMOL molecular graphics system. The results are shown in Figure S5.

Section S11 Supplementary Figures

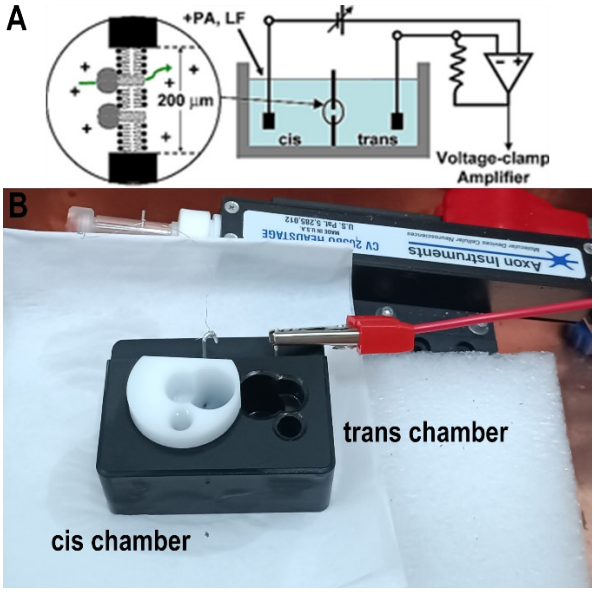

Fig. S1 Schematic illustration (a) and photograph image (b) of the experimental instruments used in biological nanopore tests.

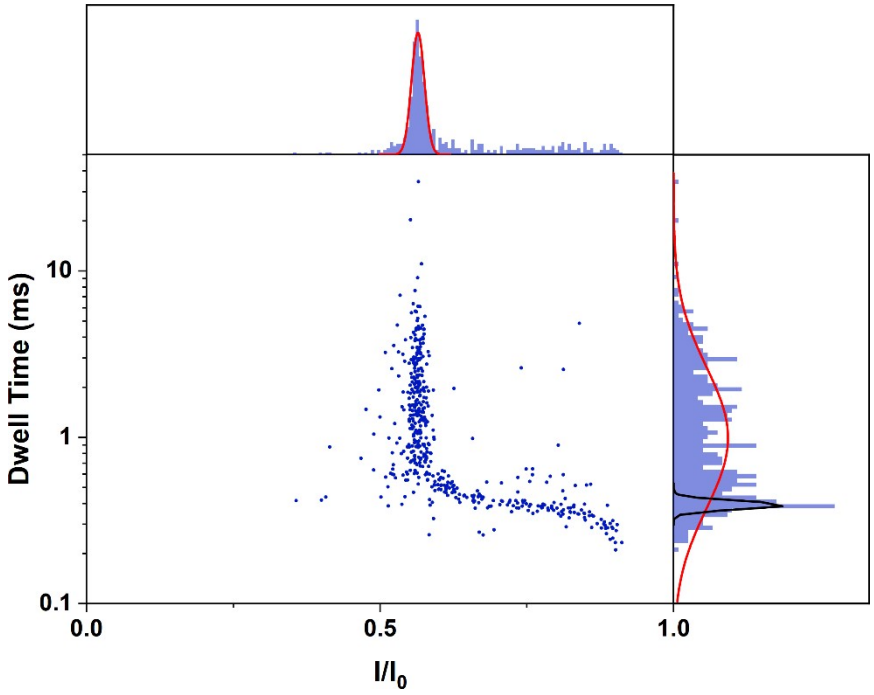

Fig. S2. Schematic illustration of the event selection criteria based on scatter plot distribution. The scatter plots display the relationship between event duration (dwell time) and blockage current amplitude. The marginal histograms positioned at the top and right represent the event frequency distributions along the respective axes. The superimposed red and black curves denote Gaussian fits to the histograms, utilized to extract the mean values and standard deviations of the event populations.

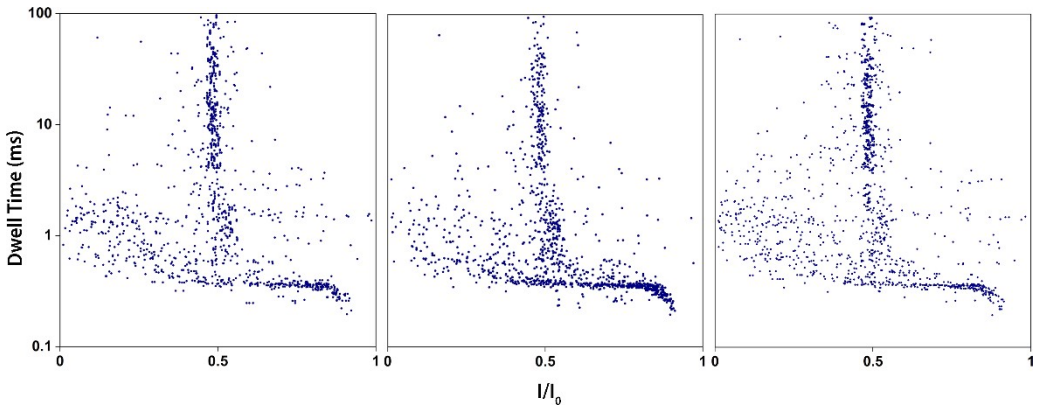

Fig. S3 Inter-pore and day-to-day reproducibility of the nanopore experiment. The relative standard deviation of the capture event frequency across the three days was calculated to be 3.7%, and the relative current ratio kept consistent (I/I_0=0.48) in all three experiments.

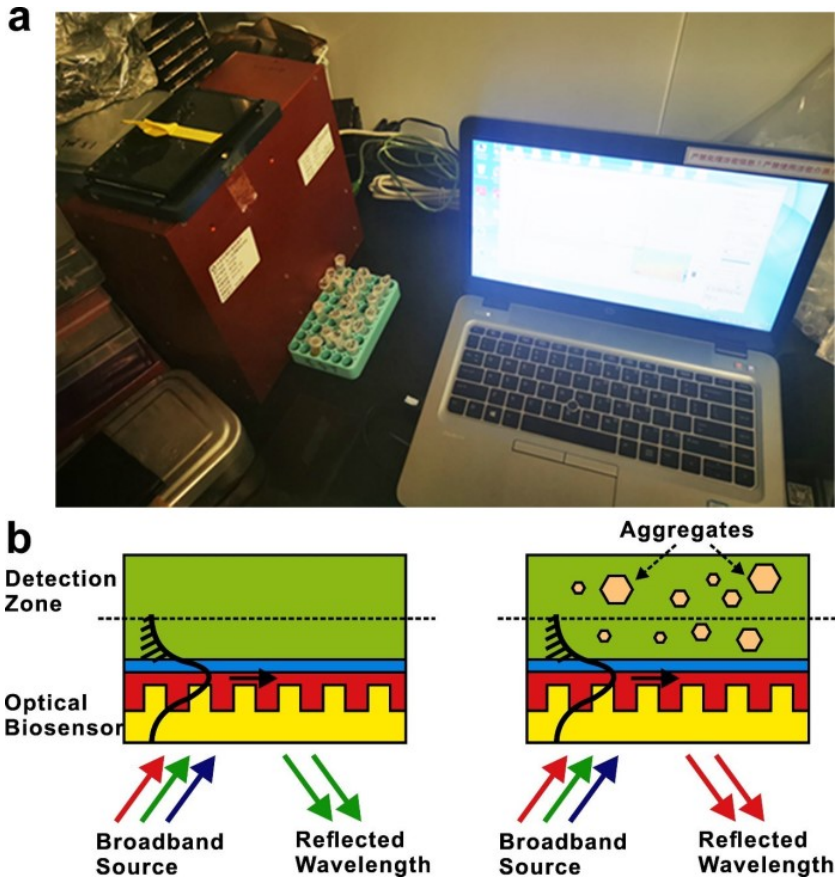

Fig. S4 (a) Photo of the label-free Corning® Epic® system and its detection mechanism. (b) Compound aggregation can be detected using a simple add and read assay format. The compound aggregation formation at the surface of the biosensor is monitored by measuring the wavelength shift of reflected resonant light. This information and the schematic diagrams are obtained from the Corning Epic operation manual.

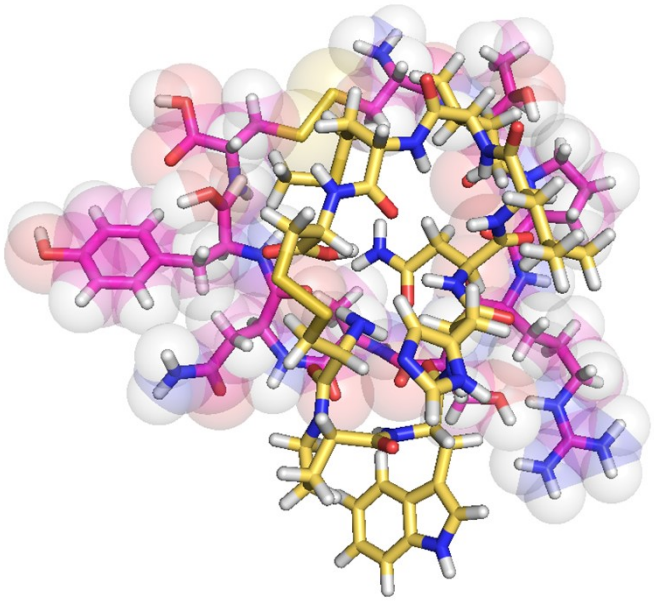

Fig. S5 Theoretical calculation results characterizing the interaction between CTY and CPM. The optimal binding conformation was obtained from molecular docking simulations.

Section S12 References

(1) Cressiot, B. et al. ACS Sens., 2019, 4, 530–548.

(2) Cao, C., Liao, D.-F., Yu, J., Tian, H. & Long, Y.-T. Nat. Protoc., 2017, 12, 1901–1911.

(3) Forester, J. H. et al. Anal. Chem. 2016, 23, 11900–11907

(4) Morris, G. M., et al. J. Comput. Chem. 2009, 30, 2785–2791.