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Sub-dalton Discrimination of Citrullination by a Semi-rationally Designed Aerolysin Nanopore

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Abstract

Citrullination, a post-translational modification (PTM) with a sub-dalton mass shift of only +0.984 Da, plays critical roles in epigenetics and autoimmunity and is a hallmark of several inflammatory diseases, but remains a particularly long-standing challenge to resolve by conventional proteomics. Here we present Physical Interaction Transduction on Nanopores (PIToN), a semi-rational model for relating local pore-analyte interactions to nanopore discrimination. Guided by PIToN, we engineered an aerolysin nanopore carrying the K238E mutation, which together with E258 forms a localized electrostatic constriction that functions as a precision “reading head.” This pore enabled label-free, single-molecule detection of peptide citrullination with 98.3% accuracy and distinguished citrullination from methylation-related histone peptide states. The platform also enabled real-time monitoring of enzymatic citrullination and voltage-dependent selective peptide enrichment with up to 99.7% purity. This work provides a practical route for physico-chemically guided nanopore design and broader applications in PTM analysis and protein characterization.

Introduction

Proteins are not mere participants in biology, but the authors of its dynamic script.¹ As the fundamental architects and executors of cellular life, their functional complexity is shaped by structure formation, enzymatic catalysis, and signaling network orchestration.² The complexity is not fully encoded in the static genome but manifested through the vast, dynamic proteome, where post-translational modifications (PTMs) act as critical “regulatory switches.”³ While the human genome contains roughly 20,000 protein-coding genes, it gives rise to a staggering multitude of over a million distinct protein variants.^{4,5} This dramatic expansion is primarily orchestrated by PTMs, a universe of more than 400 chemical alterations that profoundly reshape protein function, localization, and stability, forming the core of sophisticated cellular regulatory circuits.⁶ For decades, mass spectrometry (MS) has served as the workhorse for detecting these modifications.^{7,8} Yet, its limitations are becoming increasingly apparent, including high costs, labor-intensive workflows, and a critical lack of sensitivity for low-abundance or complex isobaric modifications.⁹ These challenges represent a critical bottleneck,^{10,11} and the journey to fully decipher the proteomic code demands a paradigm shift beyond MS.¹²⁻¹⁴ Developing innovative, scalable strategies that can truly sequence proteins at single-molecule sensitivity is not merely an incremental improvement, but an essential step to illuminate the molecular mechanisms that govern biology and diseases.⁷

This challenge is particularly acute for PTMs that introduce only subtle molecular changes, which conventional proteomics struggles most to decipher. A prime example is protein citrullination (also known as deimination), a Ca^{2+} -dependent modification catalyzed by peptidylarginine deiminases (PADIs).^{15,16} This process converts the positively charged arginine (Arg) into a neutral, non-coded citrulline (Cit) residue, resulting in a minimal mass shift of just 0.984 Da and a slight reduction in hydrogen-bonding capacity.¹⁷ Despite this subtlety, citrullination is a key regulator of fundamental processes, including epigenetics, immunity, and disease (**Fig. 1a**).¹⁷⁻¹⁹ However, MS-based citrullination analysis suffers from critical limitations: an identical mass shift between citrullination and deamidation of asparagine or glutamine, incorrect selection of monoisotopic precursors due to natural isotopic variants,²⁰ and reduced detection sensitivity.²⁰⁻²² Even high-resolution instruments often fail to achieve reliable identification

particularly on histones.²³ Antibody-based detection methods face similar constraints: insufficient specificity (65–75%),²⁴ high cross-reactivity rates (up to 30%),²⁵ and frequent failure to distinguish citrullination from structurally analogous modifications like carbamylation.²⁶⁻²⁸ These inherent shortcomings of conventional tools underscore the pressing need for novel platforms with enhanced sensitivity, specificity, and single-molecule resolution to finally overcome the long-standing bottlenecks plaguing citrullination profiling.

Compounding this detection challenge is the fact that citrullination does not act in isolation; its function is profoundly shaped by intricate crosstalk with other modifications, particularly histone methylation. Citrullination acts as a key “molecular switch” by neutralizing arginine charges to decondense chromatin and promote gene transcription.²⁹⁻³² Yet, its regulatory impact is context-dependent and antagonized by methylation at adjacent residues.³³⁻³⁷ For instance, PADI4-catalyzed citrullination of histone H3R2 facilitates the recruitment of C11orf46, which in turn attracts SETDB1 to catalyze H3K9 methylation, enabling precise control over critical processes like DNA repair and cell cycle progression (**Fig. 1b**). Deciphering this layered epigenetic language therefore requires the simultaneous and site-specific detection of both modifications, a capability that remains elusive with current analytical techniques.^{37,38} Studying these modifications in isolation misses their real-time crosstalk—precisely the dynamic logic that governs cell fate and disease progression. As a notable illustration, analyzing histone citrullination without accounting for adjacent methylation can misjudge transcriptional regulatory states of estrogen receptor-targeted genes, hindering accurate functional interpretation of breast cancer-related pathways.³³

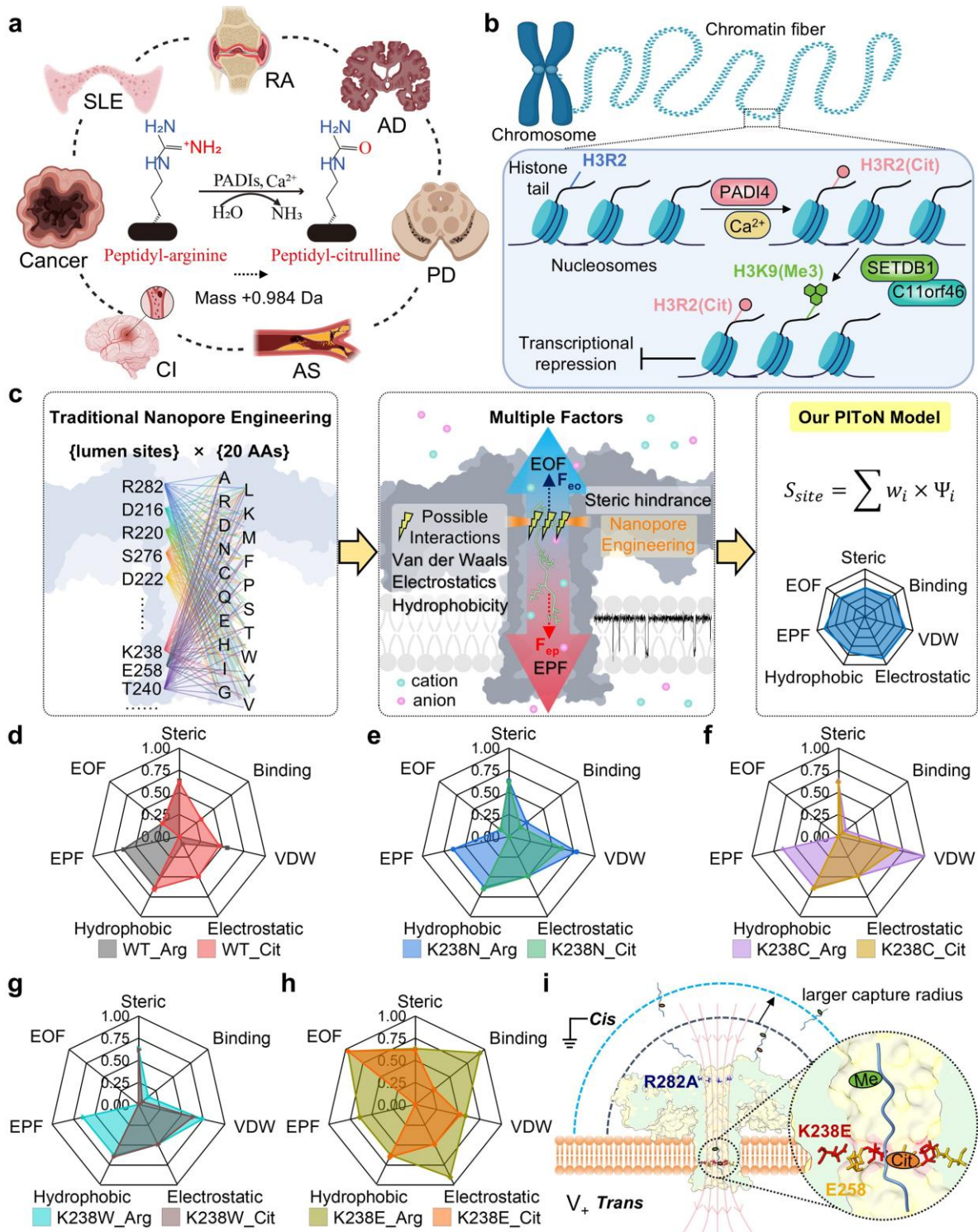


Fig. 1 | Semi-rational design of a nanopore for citrullination discrimination. **a** Schematic illustration of enzymatic conversion of arginine to citrulline by PADIs, resulting in a minimal mass increase of 0.984 Da. This PTM is implicated in a spectrum of human diseases, including autoimmune disorders (e.g.,

systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)), neurodegenerative conditions (e.g., Alzheimer's and Parkinson's diseases (AD and PD)), cancers, cerebral infarction (CI), and ankylosing spondylitis (AS). **b** Schematic of epigenetic crosstalk on histone H3, where citrullination at R2 can recruit machinery that promotes repressive methylation at K9. **c** The left panel illustrates traditional nanopore engineering, including multiple mutation sites and 20 candidate amino acids, which incurs substantial trial-and-error costs. The middle panel demonstrates the complexity of pore-analyte intermolecular interactions, where multiple factors collectively determine the current blockade signals. The right panel presents the PIToN model, which integrates a suite of physical factors. **d–h** Calculated signal response components incorporating seven physical factors for different nanopore mutants including WT (**d**), K238N (**e**), K238C (**f**), K238W(**g**), and K238E (**h**), providing a quantitative basis for the semi-rational selection of pore-lining residues. **i** Experimental nanopore setup, featuring the R282A mutation to enhance peptide event rate and the K238E/E258 constriction that forms a selective “reading head” for discriminating citrullination and its crosstalk with methylation.

Nanopore sensing technique presents a promising route to address the need for precise detection of PTMs. This technique operates by monitoring transient disruptions in ionic current that occur as individual molecules traverse a nanoscale pore.³⁹⁻⁴⁵ The depth and shape of these blockades are influenced by the physical and chemical properties of the translocating molecules.¹⁴ For PTM detection, the prevailing strategy typically focuses on identifying significant steric differences induced by the presence or absence of large modifying groups. Consequently, a range of nanopore mutants have been developed predominately via empirical rather than rational design to detect several PTMs, such as methylation,^{12,46,47} glycosylation,⁴⁸ phosphorylation,^{11,12,49-51} hydroxylation,⁵² sulfation,^{53,54} and others.⁵¹ For PTMs with large steric footprints (e.g., glycosylation or phosphorylation), empirical screening, which often relies on simple physicochemical cues like charge or steric hindrance, is time-consuming and lacks predictable rules, although it can still yield functional nanopores. In sharp contrast, for citrullination, where steric

changes are negligible and physical/chemical interactions dominate the sensing mechanism, this trial-and-error empirical strategy often fails to identify optimal nanopore sensors.

To address these intertwined challenges, including sub-dalton subtlety and complex crosstalk, we first report an integrated theoretical nanopore design model—the Physical Interaction Transduction on Nanopores (PIToN), which provides a semi-quantitative framework for relating physical interactions to measurable ionic current signatures (**Fig. 1c**). Guided by PIToN, we adopted a semi-rational strategy targeting key functional sites (**Fig. 1d–h**) to construct the mutant aerolysin pore R282A/K238E (**Fig. 1i**), where the core modification K238E pairs with the native conserved residue E258 in the pore constriction to form a localized, negatively charged “reading head.” The R282A modification serves as an additional adjustment to boost event frequency. This engineered microenvironment selectively amplifies the subtle chemical differences between arginine and citrulline, enabling single-molecule, label-free discrimination of citrullination with 98.3% accuracy—significantly outperforming high-resolution MS, which achieves $\leq 75\%$ accuracy for citrullination detection,¹⁷ and antibody-based assays, which exhibits 65–75% specificity for distinguishing citrulline from methylation.¹⁸ Molecular dynamics (MD) simulations supported the underlying recognition mechanism and machine-learning-assisted kinetic analysis further enabled time-resolved monitoring of enzymatic activity. Importantly, the sensor maintained high fidelity when confronted with combinatorial citrullination-methylation marks, directly capturing the physiological crosstalk that has long eluded existing tools. Furthermore, we demonstrated a proof-of-concept voltage-dependent selective enrichment mode, enabling enrichment of target peptides with a purity of 99.7%. This work provides a semi-rational design strategy for citrullination-sensitive nanopores and expands the platform from precision analysis toward selective enrichment, highlighting its great potential for PTM proteomics analysis.

Results

Establishment and parameterization of the PIToN model

Conventional nanopore engineering faces two fundamental challenges. First, the combinatorial explosion of pore-lining residue mutations makes systematic optimization impractical—testing even two target positions against the 20 natural amino acids already results in 400 variants (**Fig. 1c**). Second, the sensing signal arises from a complex and coupled interplay of steric, electrostatic, hydrophobic, and electrokinetic effects that are difficult to decouple and predict. Although MD simulations can provide valuable guidance, their routine application to broad mutation screening is computationally demanding, with each full-atom system requiring 2000–3000 core-hours and comprehensive variant screening exceeding tens of thousands of core-hours. Coupled with required specialist expertise, this resource cost limits its practicality as a general prioritization strategy. Consequently, traditional approaches remain largely empirical and nonsystematic, leading to low optimization efficiency and poor generalizability. This limitation becomes particularly pronounced when targeting subtle modifications such as citrullination, where steric changes are minimal and other physical interactions play decisive yet poorly quantified roles.

To address these limitations, we introduced PIToN as a physically motivated, semi-quantitative model that describes how multiple local analyte–pore interactions transduce into measurable ionic current responses. Conceptually, PIToN provides a structured way to relate molecular-scale interaction features to nanopore readouts beyond purely qualitative interpretation.

We proposed that the nanopore sensing response S , typically reflected in blockade-current characteristics and translocation kinetics, can be represented as a weighted linear combination of local interaction descriptors:

$$S = \sum w_i \cdot \Psi_i \quad (1)$$

where Ψ_i denotes the normalized contribution (scaled to 0–1 to eliminate dimensional differences) of the i -th interaction, and w_i represents its corresponding weighting factor. Taken possible interactions into accounts, the formula could be:

$$S = w_1 \cdot \Psi_{Steric} + w_2 \cdot \Psi_{Binding} + w_3 \cdot \Psi_{VDW} + w_4 \cdot \Psi_{Electrostatic} + w_5 \cdot \Psi_{Hydrophobic} + w_6 \cdot \Psi_{Electrophoretic} + w_7 \cdot \Psi_{Electroosmotic} \quad (2)$$

Each term in PIToN is physically motivated and, in principle, can be described by well-defined physical formulations. Given the practical computational constraints, a fully detailed physical description would approach full-scale MD simulations at the peptide or protein level, which is computationally intractable for large-scale screening. We therefore employ reduced descriptors that capture only the most distinctive interaction features differing across systems, while treating shared, common contributions as uniform background. As a first-order approximation, we adopted equal weighting for all terms to avoid over-parameterization and preserve interpretability. Detailed parameter definitions and normalization methods are provided in the **Supplementary Information (SI) Section 1.1**. Within this simplified framework, the steric component is approximated by the change in effective excluded volume, serving as a geometric proxy for local steric compatibility:

$$\Psi_{Steric} = k_s \cdot \Delta V_{eff} \quad (3)$$

where k_s is a lumped geometric scaling factor in the present model, and ΔV_{eff} denotes the change in effective excluded volume of the analyte within the local constricted sensing region. For the present citrullination system, ΔV_{eff} is negligible (<5.4% by hard-sphere or MoloVol estimates,⁵⁵ see **SI Section 1.1**), indicating that steric contribution is not a dominant source of discrimination.

The binding-related term, $\Psi_{Binding}$, was approximated by density functional theory (DFT) calculations of pairwise interaction energies between pore residues and the analyte, thereby providing a relative measure of the strength of local attractive interactions. Because the target citrullination is a site-specific PTM that exclusively alters the side chain of a single arginine residue, while the pore mutation sites are spatially confined to the nanoconstriction region, we deliberately avoided flexible docking of the full peptide. Flexible docking would generate an ensemble of possible conformations, introducing substantial ambiguity in selecting a representative binding pose. Moreover, nonspecific interactions from distal residues could overwhelm the local signal of interest, making it difficult to isolate the contribution of the

modified site and its immediate pore environment. To circumvent these issues, we adopted a reduced host–guest model in which the pore is represented by a single amino acid and the analyte is represented by the side chain of arginine or its citrullinated counterpart, i.e., the only moiety altered by the PTM. This local model captures the key local contacts most directly related to the modification, while maintaining computational tractability and avoiding ambiguity in conformational assignment. DFT was chosen because it provides a first-principles electronic-structure framework for evaluating intermolecular interactions without relying on empirical force-field parameters. All configurations were optimized at the same level of theory, and the lowest-energy conformer was retained for each residue–analyte pair (see **SI Section 1.2** for representative screening using the Arg–Asn pair). A polarizable continuum solvent model was applied to approximate the aqueous environment. Notably, the relative binding tendencies inferred from the DFT-calculated local interaction energies were consistent with the affinity trends obtained from biolayer interferometry (BLI) experiments, where the binding-induced signal variations were measured separately for the intact peptides and the corresponding individual amino acids (**Supplementary Fig. S1**). Although this simplified host–guest model does not fully replicate the complex experimental setting, the consistent affinity trends provide the independent experimental support for the rationale of using DFT-derived interaction energies as local binding descriptors. Accordingly, the resulting interaction energies could be interpreted as a semi-quantitative descriptor for ranking the relative binding tendency across pore mutants, rather than as absolute binding free energies.

The van der Waals (VDW) term, Ψ_{VDW} , is computed from the pairwise Lennard-Jones potential between analyte atoms (i) and pore-lining atoms (j):

$$\Psi_{VDW} = \sum_{i,j} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] \quad (4)$$

where ε_{ij} and σ_{ij} are the Lennard-Jones parameters describing the interaction well strength and equilibrium distance for atom pair (i, j), derived from the CHARMM36 force field using Lorentz-Berthelot combining rules.⁵⁶ r_{ij} is the distance between atoms i and j (see **SI Section 1.1**). For the WT

pore, normalized VDW contributions differed between Arg (0.553) and Cit (0.464), reflecting subtle differences in atomic polarizability and VDW radii.

The electrostatic contribution, $\Psi_{Electrostatic}$, is represented here according to Coulomb's law for pairwise summation:

$$\Psi_{Electrostatic} = \frac{1}{4\pi\epsilon_0\epsilon_r} \sum_{i,j} \frac{q_i Q_j}{r_{ij}} \quad (5)$$

where ϵ_0 and ϵ_r are the vacuum and effective relative permittivities, and q_i and Q_j are partial charges on analyte and pore atoms, respectively. The WT pore exhibited pronounced electrostatic repulsion toward Arg (normalized $\Psi_{Electrostatic} = 0.09$) but neutrality toward Cit (normalized $\Psi_{Electrostatic} = 0.5$), yielding a large contrast $\Delta\Psi_{Electrostatic} = 0.41$; however, this repulsion might shorten Arg dwell time, compromising detection sensitivity.

The hydrophobic contribution is estimated from the change in solvent-accessible surface area (SASA) of hydrophobic moieties upon analyte–pore association, following the established linear relationship between solvation free energy and exposed hydrophobic surface area:

$$\Psi_{Hydrophobic} = \gamma \cdot SASA \quad (6)$$

where γ represents the empirical free energy coefficient per unit area, typically ranging between 0.1–0.2 kJ/mol/Å², while $SASA$ is the solvent-accessible surface area of hydrophobic regions upon formation of the analyte–pore complex. Citrullination slightly increases the hydrophobic contribution in the present descriptor scheme ($\Delta SASA = +3$ Å², normalized $\Psi_{Hydrophobic}(Cit) = 0.660$ vs. $\Psi_{Hydrophobic}(Arg) = 0.638$) for WT nanopore, providing supplementary discrimination capability.

To account for transport-related driving effects that influence translocation kinetics, two phenomenological descriptors were retained: electrophoretic force and electroosmotic force (EPF and EOF). The electrophoretic contribution is expressed as

$$\Psi_{Electrophoretic} = \Psi_{EPF} = q_{eff} \times V_{bias} \quad (7)$$

where q_{eff} is the effective analyte charge (taken as the net charge at pH 7.5), and V_{bias} is the applied voltage. The electroosmotic contribution is expressed as

$$\Psi_{Electroosmotic} = \Psi_{EOF} = \kappa \times |\zeta_p| \times V_{bias} \quad (8)$$

where κ is an effective scaling parameter used to map the assumed pore-surface zeta potential onto an EOF-related descriptor, and ζ_p is the assumed zeta potential at the pore surface. Depending on pore charge distribution and analyte properties, EPF and EOF may cooperate or oppose one another, and the EOF contribution may be attenuated when the analyte occupies the constricted region.⁵⁷ Detailed calculations and parameter derivations are provided in **SI Section 1.1**, including the local simplifications adopted in the present aerolysin-based implementation.

As visualized in the radar plot (**Fig. 1c**), the seven-parameter profile offered an intuitive representation of how each physical interaction term differentially contributed to citrullination recognition, emphasizing the benefit of considering multiple sensing dimensions beyond steric effects alone. Building on previous evidence that K238 is an engineering-relevant position within the constriction region,⁵⁸ PIToN was used here as a semi-rational screening strategy to evaluate candidate mutations. By calculating the multi-parameter response profiles for over 20 diverse amino acid substitutions at this position (**Supplementary Fig. S2**), the model rapidly identified the most promising constructs, a process that would otherwise require months of experimental trial-and-error for protein expression, purification, and characterization. When applied to the panel of engineered aerolysin pores representing key interaction types (**Fig. 1d–h**), PIToN highlighted K238E as the prime candidate for robust citrullination discrimination. Under this equal-weighting approximation, the differential signal response for K238E ($\Delta S = 0.253$; **Fig. 1h**) nearly doubled that of other mutants, including K238C ($\Delta S = 0.144$; **Fig. 1f**), K238N ($\Delta S = 0.128$; **Fig. 1e**), and K238W ($\Delta S = 0.119$; **Fig. 1g**), and significantly surpassed the WT nanopore ($\Delta S = 0$; **Fig. 1d**). This enhanced discrimination capability stemmed from the pronounced specificity of K238E toward Arg ($S = 0.758$), combined with a moderate response to Cit ($S = 0.505$), thereby establishing the largest signal contrast among all tested nanopores and providing a strong basis for highly

sensitive detection (see **SI Section 1.1**). This *in silico* screening helped narrow the experimental search space by focusing validation on a limited subset of chemically distinct candidates.

Detection of citrullination using PIToN-guided engineered nanopores

To translate our PIToN model into an effective sensing platform, we needed an experimental model system capable of rigorously testing the subtlety of citrullination. We selected the *N*-terminal 1–10 peptide of histone H4 (H4₁₋₁₀) for its well-defined citrullination site at arginine 3 (R3) and its direct biological relevance to epigenetic regulation. This peptide pair provided a well-controlled comparison between unmodified H4₁₋₁₀ and its citrullinated variant H4₁₋₁₀R3(Cit) (**Fig. 2a**), for evaluating nanopores engineered at the key constriction residue K238, which our model identified as critical for modulating citrullination-sensitive interactions (**Fig. 2b**).

Peptide translocation through a nanopore is characterized by two primary parameters: the normalized blockade depth I_b/I_o and the dwell time Δt (**Fig. 2c**). To quantify discrimination, we adopted an adapted peak-resolution metric derived from the classical chromatographic definition of resolution for two Gaussian-like distributions. Specifically, the discrimination resolution (R_s) was defined as:

$$R_s = \frac{2 \times |\overline{I_{b1}/I_o} - \overline{I_{b2}/I_o}|}{1.7 \times (w_{1,h/2} + w_{2,h/2})} \quad (9)$$

Where $\overline{I_{b1}/I_o}$ and $\overline{I_{b2}/I_o}$ are the mean I_b/I_o of the two Gaussian fitting peaks of H4₁₋₁₀ and H4₁₋₁₀R3(Cit), and $w_{1,h/2}$ and $w_{2,h/2}$ are their corresponding full width at half maximum (FWHM) values (**Fig. 2d**). The factor 2 originates from the classical peak-resolution definition, and the factor 1.7 arises from the Gaussian approximation relating the peak width at the baseline to the FWHM. A higher R_s indicates better separation and thus more reliable identification.

The WT aerolysin nanopore showed no discernible discrimination between H4₁₋₁₀ and H4₁₋₁₀R3(Cit) (**Fig. 2e**), as reflected in a near-zero experimental resolution ($R_s = 0.01$) and a theoretical response difference ($\Delta S = 0$) consistent with the PIToN prediction. This established a baseline for evaluating

engineered mutants. To validate the PIToN model for citrullination discrimination, we mutated the constriction residue K238 to residues with diverse physicochemical properties, generating K238C, K238N, K238Q, K238Y, and K238W (**Fig. 2b**; see **SI Section 2.1–2.3 and Supplementary Fig. S2–18**). Because exhaustive experimental characterization of all K238 substitutions was impractical, we selected a chemically representative subset of mutants for nanopore fabrication and electrophysiological validation, while retaining the remaining candidates as *in silico* PIToN-screened variants (see **SI Section 1.3**). Neutral polar residue-modified nanopores (K238N and K238Q) yielded moderate improvement in experimental resolution ($R_s = 0.55$ and 0.49 , respectively; **Fig. 2f, g**), which aligned with their modest theoretical discrimination potential ($\Delta S = 0.128$ and 0.127 , respectively; see **SI Section 1.3**). The aromatic mutants (K238Y and K238W) achieved higher experimental resolution ($R_s = 0.62$ and 0.82 ; **Fig. 2i, j**), highlighting the role of enhanced VDW interactions. However, the lower theoretical ΔS values for K238W (0.119) versus K238Y (0.125 ; see **SI Section 1.3**) suggested that, in a simplified equal-weight model, the benefits of increased VDW contributions might be offset by other factors. Notably, K238W induced frequent nonspecific events due to steric hindrance (**Supplementary Fig. S17**), limiting its practical utility. The K238C mutant showed a promising experimental resolution ($R_s = 0.84$; **Fig. 2h**) but exhibited a broad signal distribution ($\text{FWHM} \approx 0.15$), which reduced signal homogeneity and practical separability, thereby limiting its utility as a robust sensing pore.

Strikingly, the K238E mutant achieved the highest experimental resolution ($R_s = 0.90$; **Fig. 2k**; **Supplementary Fig. S19**). This result is in satisfactory agreement with the PIToN model, which predicted the largest differential signal response ($\Delta S = 0.253$) for this mutation. Across the experimentally tested mutant subset, the observed R_s values showed a positive association with the PIToN-predicted ΔS values (Pearson's $r = 0.855$, $p = 0.014$; Spearman's $\rho = 0.679$, $p = 0.094$), indicating that the PIToN-derived signal contrast captures the overall experimental trend in discrimination performance (**Fig. 2l**; see **SI section 1.4**). Notably, the weighting coefficients w_i (equation (1)) were not fitted to the experimental R_s values, making this comparison an a posteriori consistency check rather than a parameter-trained

corresponding citrullinated peptide at the third arginine residue (H4₁₋₁₀R3(Cit)). **b** Schematics of six aerolysin nanopore mutants engineered at the K238 site, alongside the wild-type (WT). **c** Top: a representative single-event trace of H4₁₋₁₀ translocating through the WT nanopore, showing the open-pore current (I_o), blockade current (I_b), and dwell time (Δt). Bottom: the corresponding scatter plot of events. Histograms of I_b/I_o were generated over the range of 0–1 using 100 bins (bin width = 0.01), and the counts were normalized to the total number of detected events to obtain the relative events per bin. The mean I_b/I_o and full width at half maximum (FWHM) were obtained by Gaussian fitting of the histograms. **d** Definition of discrimination resolution (R_s). **e–k, o, q, r** Scatter plots and Gaussian-fitted distributions for the two peptides (20 μ M) traversing the WT (**e**) and engineered nanopores (**f–k, o, q, r**). All experiments were conducted in 4 M KCl, 25 mM HEPES, pH 7.5, at 25 °C, with an applied voltage of –50 mV, unless otherwise specified. **l** Correlations between experimentally determined R_s and PToN-predicted ΔS (black squares), and predicted R_s after correction (blue circles). Red and blue lines represent the corresponding linear fitting curves. **m** Sample current traces from H4₁₋₁₀ peptide (20 μ M) translocations through four nanopores. **n** Event rate comparison for H4₁₋₁₀ (upper panel) and H4₁₋₁₀R3(Cit) (lower panel) across the four nanopores. Three independent nanopore experiments were performed in triplicate, and the error bars represent the standard error. **p** Comparison of electrostatic potential profiles of the WT and R282A/K238E nanopores, calculated by APBS webserver. **s** Binding kinetics of H4₁₋₁₀ peptides with *L*-glutamate-*L*-glutamate (Glu-Glu) immobilized BLI sensor surface at different concentrations. **t** Dissociation constants (K_D) fitting curve for H4₁₋₁₀ (pink), and H4₁₋₁₀R3(Cit) (green) interacted with Glu-Glu.

Although the K238E mutant achieved the superior discrimination, its relatively low event frequency limited detection throughput (**Fig. 2m**). To address this limitation, we targeted the pore-entrance residue R282, which is known to modulate peptide capture.⁵⁹ The single R282A mutation increased the event frequency by approximately threefold compared to the WT pore (**Fig. 2n**). The resulting double mutant, R282A/K238E (EA) retained high citrullination discrimination ($R_s = 0.80$; **Fig. 2o**) while enhancing the

event frequency to about twice that of the K238E single mutant (**Fig. 2n**). Electrostatic potential calculations comparing the WT and the EA nanopore revealed how the two mutations collaboratively reshape the pore's electrostatic landscape (**Fig. 2p**).⁶⁰ The R282A substitution creates a more negatively charged environment at the pore entrance, facilitating the capture of positively charged peptides.⁶¹ Meanwhile, the K238E substitution, in conjunction with the neighboring E258 residue, disrupts intrinsic salt bridges present in the WT pore⁶² and instead forms a contiguous “negative charge well” within the constriction. This reconfigured electrostatic hotspot is precisely positioned to engage and discriminate the subtle charge and structural difference between arginine and citrulline, thereby enabling the high-fidelity sensing observed experimentally.

Systematic perturbations (voltages, solution pH, divalent ions and temperatures) revealed that the robust citrullination discrimination stemmed not from a single dominant interaction, but from a precisely tuned equilibrium with multiple parameters (**Supplementary Fig. S20**). To further validate the general applicability of the nanopore, we tested 8-residue and 16-residue peptides that differ only by a single citrullination site. The nanopore retained clear discrimination towards these peptides of varying lengths (**Supplementary Fig. S21**). Replacing E238 with the shorter aspartate (K238D) drastically reduced the performance ($R_s = 0.33$), illustrating the sensitivity to side chain geometry and local chemistry (**Fig. 2q**). More informatively, replacing the neighboring glutamate with alanine at the position 258 (R282A/K238E/E258A) also impaired the resolution ($R_s = 0.26$ vs. 0.80 for EA) (**Fig. 2r**). This result confirmed that the high-fidelity recognition depended not only on K238E, but also on the synergistic “double glutamate” configuration (E238/E258), which formed a contiguous electrostatic well within the constriction—a structural motif visualized in the electrostatic potential maps that is critical for engaging the subtle charge difference between arginine and citrulline (**Supplementary Fig. S22**). In order to prove this mechanism, binding assays (see **SI Section 2.4; Supplementary Fig. S23**) based on BLI were conducted, which revealed that the Glu-Glu dipeptide exhibits significantly higher affinity for the unmodified H4₁₋₁₀ ($K_D = 63$ nM) than for the citrullinated H4₁₋₁₀R3(Cit) ($K_D = 960$ nM) (**Fig. 2s, t**), corresponding to a ratio of 15.

Collectively, the EA mutant effectively integrates high capture efficiency with excellent discrimination, providing a robust sensing platform for sub-dalton citrullination detection. The overall agreement between PITO-N-guided ranking and experimental performance supports the predictive utility of the model and further illustrates that physico-chemically guided design can effectively address the challenge of subtle molecular recognition.

Molecular mechanism for citrullination discrimination

To elucidate the molecular basis underlying nanopore discrimination of citrullination, we performed all-atom MD simulations of H4₁₋₁₀ and H4₁₋₁₀R3(Cit) peptides translocating through WT and engineered EA aerolysin pores, with the initial peptide configuration shown in **Fig. 3a** (see **SI Video 1–2**). In parallel, ionic currents were estimated from MD-derived pore–peptide conformations using the steric exclusion model (SEM),⁶³ providing a mechanistic framework that is consistent with the experimental observations. Detailed equilibration procedures and analysis protocols are provided in **SI Section 2.5**.

The pore radius profile revealed a slight enlargement at sites 238 and 282 in the EA mutant compared to WT (**Fig. 3b**). The WT aerolysin nanopore has an overall channel length of approximately 10 nm, with two principal constriction regions, each having a radius of approximately 5 Å. Although a larger constriction would typically reduce steric-based resolution, the EA pore maintained high discrimination, indicating that the recognition is not governed solely by volume exclusion, consistent with the multi-parametric PITO-N model. The SMD force profiles plotted against the peptide position along the pore axis (z) differed markedly between the two pores. The WT pore exerted nearly identical time-averaged pulling forces along the representative single trajectories on both peptides (~288 pN, **Fig. 3c**), whereas the EA pore showed a pronounced force disparity, especially near the E238/E258 constriction, where the force exerted on H4₁₋₁₀ was 3.5-fold higher than that on H4₁₋₁₀R3(Cit) (**Fig. 3d**). This force enhancement was consistent with the superior experimental resolution of the EA nanopore.

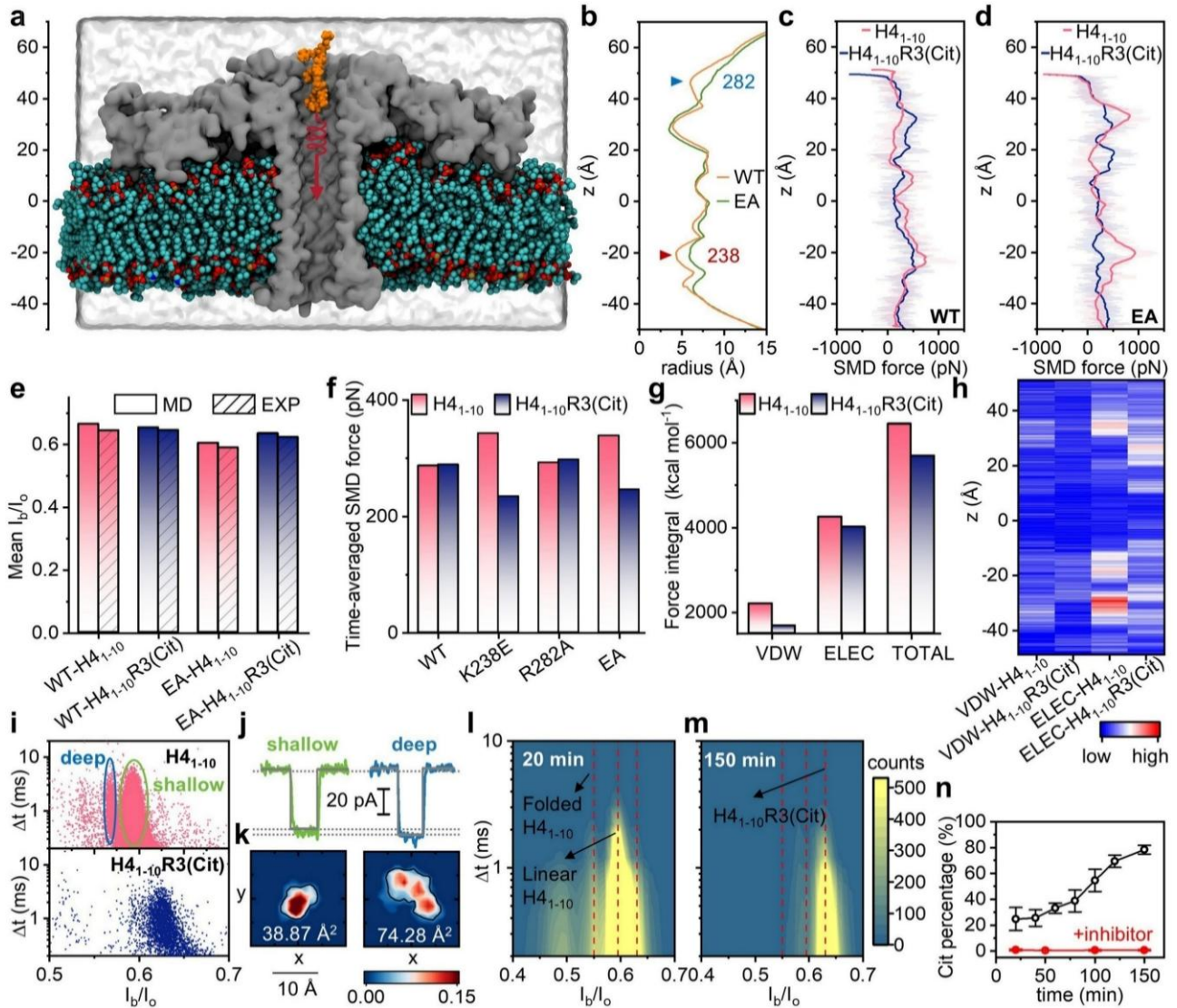


Fig. 3 | Mechanism of citrullination discrimination disclosed by MD simulations. **a** Schematic of the MD simulation setup. **b** Radius profile along the pore axis (z) for WT (orange) and EA (green) nanopores, calculated using the HOLE program, defining the confinement region. The 282 and 238 sites were indicated with blue and red arrows, respectively. **c**, **d** Steered molecular dynamics (SMD) force averaged over 5 Å window for H4₁₋₁₀ (pink) and citrullined H4₁₋₁₀R3(Cit) (blue) peptides translocating through the WT (**c**) and EA (**d**) nanopores. **e** Comparison of the normalized mean I_b/I_o obtained from MD-derived single-trajectory analysis and experimental measurements for the H4₁₋₁₀ and citrullined H4₁₋₁₀R3(Cit) peptides in WT and EA nanopores. **f** Time-averaged SMD forces from representative single SMD trajectories for the peptides across different nanopore variants (WT, K238E, R282A, and EA). **g** Time-

integrated forces for H4₁₋₁₀ (pink) and H4₁₋₁₀R3(Cit) (blue) through the EA nanopore, decomposed into VDW, electrostatic (ELEC), and total contributions. **h** Evolution of VDW and ELEC forces per simulation frame during the peptide transit through the EA nanopore. **(g)** and **(h)** show decomposition results obtained from representative single trajectories. **i** Event distribution analysis with the EA nanopore showed deep and shallow blockade populations for H4₁₋₁₀, and a single population for H4₁₋₁₀R3(Cit). **j** Two typical current traces extracted from the populations in **(i)**. **k** Calculated cross-sectional areas corresponding to the linear and folded peptide conformations. **l, m** Monitoring and quantification of the PAD4-catalyzed citrullination reaction. PAD4-catalyzed citrullination of H4₁₋₁₀ (1.36 mM), was carried out with the addition of 1.36 μ M PAD4, and the reaction buffer containing 200 mM Tris-HCl, 50 mM CaCl₂, incubated at 37 °C. Scatter plots (I_b/I_o vs. Δt) of single-molecule events from the production of at indicated reaction time points: 20 **(l)**, and 150 **(m)** minutes. **n** Predicted reaction kinetics curves. Black curve: time-dependent citrullination percentage; red curve: 0.33 μ M PAD4 with 3.33 mg/mL ethanol extracts of *Terminalia chebula*. All curves generated by the trained neural network model. Three independent nanopore experiments were performed in triplicate, and the error bars represent the standard error.

The difference in pulling forces revealed by steered molecular dynamics (SMD) simulations was mirrored by distinct ionic current signatures. Indeed, the normalized blockade currents (I_b/I_o) predicted from the steric exclusion model (SEM)⁶³ showed good correspondence with experimental measurements ($R^2 = 0.97$, **Fig. 3e**), indicating that the MD–SEM workflow captures the main blockade trend in the present short-peptide system. For descriptive comparison, the time-averaged pulling-force difference between the two peptides, calculated from representative single SMD trajectories, was 1.88 pN in the WT pore and 4.74 pN in R282A, consistent with the relatively weak discrimination observed for these nanopores. In contrast, the corresponding representative differences in K238E and EA were larger (108.52 pN and 92.48 pN, respectively), consistent with the stronger experimental discrimination observed for these pores (**Fig. 3f**). Representative decomposition of non-bonded interactions along the EA pore

suggested that electrostatic contributions were the dominant differentiating component in the trajectories analyzed here: the trajectory-integrated electrostatic force for H4₁₋₁₀ (4249.4 kcal·mol⁻¹) was higher than that for H4₁₋₁₀R3(Cit) (4015.0 kcal·mol⁻¹), whereas the corresponding VDW contributions were comparatively modest (**Fig. 3g**). Spatial mapping localized the strongest electrostatic discrimination to the -30 Å region, near the engineered E238/E258 “reading head” under the present short-peptide conditions, where the electrostatic force on H4₁₋₁₀ exceeded that on H4₁₋₁₀R3(Cit) by a factor of 2.1 (**Fig. 3h**). Whether the same localized reading-head mechanism remains dominant for peptides exceeding the effective pore length remains to be established.

The high resolution of our nanopore platform enabled us to resolve distinct conformational states of the translocating peptides. As shown in **Fig. 3i**, the unmodified H4₁₋₁₀ peptide produces a bimodal distribution of blockade depths when translocating through the EA nanopore, whereas H4₁₋₁₀R3(Cit) yields only a single peak—a reproducible observation (detailed in **Supplementary Fig. S24**). The blockade populations for H4₁₋₁₀ were classified as a major shallow event (~90% of events) and a minor deep event (~10% of events), with representative current traces displayed in **Fig. 3j**. We hypothesized that these two event types originated from different peptide conformations during the translocation (**Supplementary Fig. S25**). Equilibrium MD simulations supported this interpretation, identifying representative linear (cross-section ~38.9 Å²) and folded conformations (cross-section ~74.3 Å²), whose projected cross-sectional areas were consistent with the shallow and deep blockade populations, respectively (**Fig. 3k**; see **SI Video 3–6**). Of note, peptide conformations undergo continuous dynamic fluctuations rather than remaining static; the characteristic conformational populations identified here merely reflect that a fraction of peptides preferentially adopts folded configurations. The nanopore faithfully records this diverse continuum of conformational states, and additional insights into protein conformational landscapes can be further extracted by time-resolved data analysis. In contrast, under the present simulation conditions, the citrullinated peptide was observed predominantly in the linear form, possibly due to reduced intramolecular interactions. Having established the mechanistic basis of discrimination in

the EA pore, we next asked whether this optimized platform could be extended to dynamic enzymatic monitoring.

Time-resolved kinetic profiling of PAD4-catalyzed citrullination

To examine whether the platform could be extended beyond static PTM discrimination, we next applied it to real-time, label-free monitoring of PAD4-catalyzed citrullination (see **SI Section 2.6 and 2.7**). Recording the PAD4-catalyzed citrullination of histone H4 peptide revealed a clear evolution in translocation signatures over time (**Supplementary Fig. S26b–f**). After 20 min of reaction, three populations were resolved: folded H4₁₋₁₀, linear H4₁₋₁₀, and the citrullinated product H4₁₋₁₀R3(Cit) (**Fig. 3l**). As the reaction progressed, the folded peptide signals vanished, the linear arginine population gradually decreased, and the citrullinated product signal grew to dominate the distribution (**Fig. 3m**).

To quantitatively deconvolve these overlapping populations across the full time series, we implemented a machine-learning classifier (see **SI Section 2.8; Supplementary Fig. S26g–i**). A neural network model outperformed all alternative classifiers, achieving 98.3% accuracy in citrullination discrimination, and was subsequently employed to predict the proportion of reaction products. This analysis yielded a precise kinetic profile, showing the citrullinated product fraction progressively increasing to 78.4% at 150 minutes (**Fig. 3n**, black curve). The incomplete conversion likely reflects the intrinsic sequence specificity or thermodynamic equilibrium of PAD4 catalysis. Strikingly, introducing a natural inhibitor candidate, an ethanolic extract of *Terminalia chebula*, strongly suppressed detectable product formation across all measured time points (see **SI section 2.9; Supplementary Fig. S27**), demonstrating the platform's direct utility for real-time inhibitor screening (**Fig. 3n**, red curve).

Voltage-dependent selective event enrichment

Beyond real-time monitoring, we reasoned that the distinct translocation kinetics of the two peptides (**Supplementary Fig. S28–S29**) might be exploited for voltage-controlled selective enrichment. At –50 mV, event rates for both peptides scaled linearly with concentrations (**Supplementary Fig. S28**), yet the

event rate for H4₁₋₁₀ was approximately threefold higher than that for the citrullinated variant under identical conditions. In addition, the distinct voltage thresholds (Supplementary Fig. S29) were observed for H4₁₋₁₀ (−40 mV) and H4₁₋₁₀R3(Cit) (−50 mV), which indicated that the engineered EA nanopore might exhibit voltage-dependent analyte selectivity.

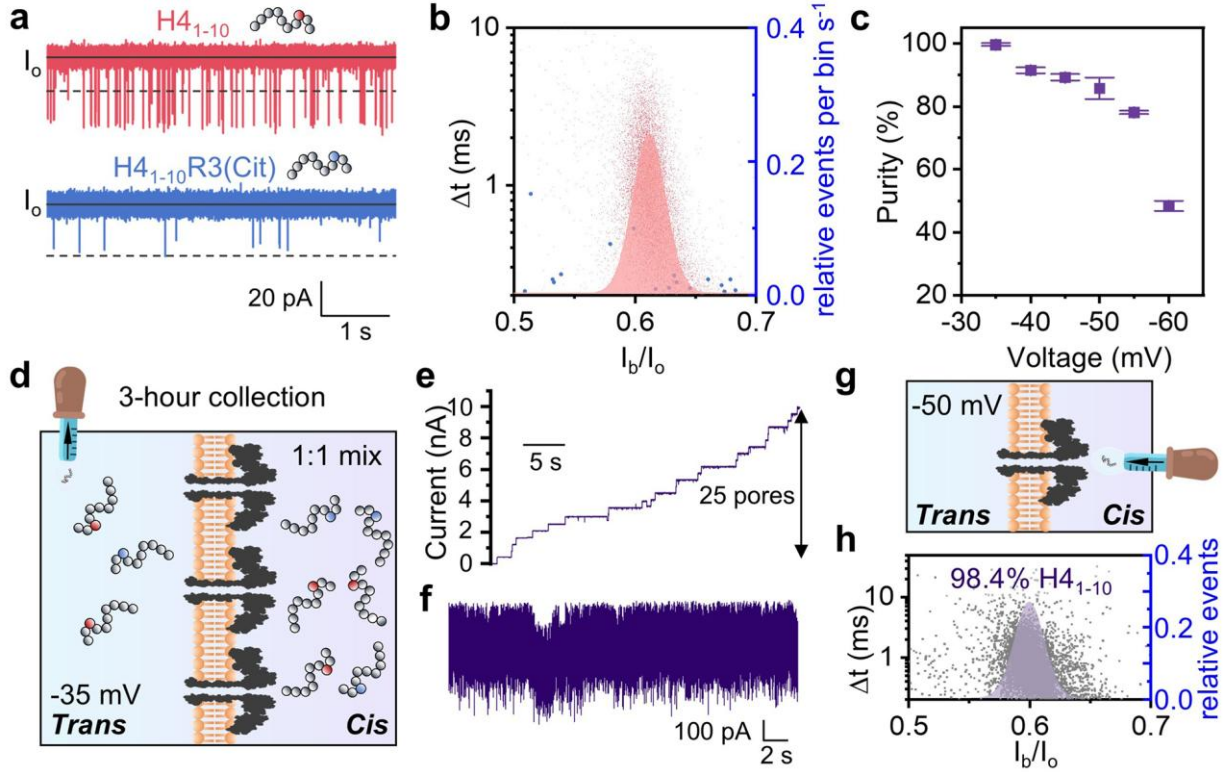


Fig. 4 | Separation of peptides by EA nanopore. **a** Representative current traces of H4₁₋₁₀ (pink) and H4₁₋₁₀R3(Cit) (blue) recorded at −35 mV through EA nanopore. The dashed line indicates the event threshold, defined as $I_o - 12\sigma$, where σ is the baseline noise standard deviation. **b** Scatter plot of events extracted from the recordings in (a). Gaussian fitting of the H4₁₋₁₀ population (pink curve) confirms a single dominant peak. **c** Voltage-dependent purity of H4₁₋₁₀ separation. Data are presented as mean $\pm$ s.e.m. (n = 3 independent experiments). **d** Schematic of the multi-pore separation setup. A 1:1 mixture of H4₁₋₁₀ and H4₁₋₁₀R3(Cit) (final concentration 10 μ M each) was added to the *cis* chamber. Separation was performed at −35 mV. **e** Formation of multiple nanopores in a single lipid bilayer under +200 mV, monitored by stepwise current increases. **f** Continuous current trace after peptide mixture addition, showing frequent translocation spikes. **g** Workflow for evaluating separation efficiency: the

trans-chamber solution from the multi-pore run was transferred to a new single-pore system and recorded at -50 mV. **h** Event scatter plot of the sample from the 3-hour collection, analyzed by **(g)**. The Gaussian-fit peak center is located at $I_b/I_o \approx 0.601$. All experiments were conducted in 4 M KCl, 25 mM HEPES, pH 7.5, at 25°C .

As shown in **Fig. 4a**, at -35 mV the event rate of H4_{1-10} significantly surpasses that of $\text{H4}_{1-10}\text{R3(Cit)}$, when using a current-threshold filter ($I_o - 12\sigma$) to exclude short and incomplete events. Scatter-plot analysis and Gaussian fitting of the events recorded over equal time intervals revealed that only 78 translocation events for the citrullinated peptide, compared with 20,095 events for the unmodified counterpart (**Fig. 4b**). We defined separation purity as the ratio of H4_{1-10} events to the total number of events ($\text{H4}_{1-10} + \text{H4}_{1-10}\text{R3(Cit)}$) within the same recording period. At -35 mV, the purity reached $99.7 \pm 0.4\%$, indicating highly selective enrichment of H4_{1-10} in the *trans* chamber. Further investigation revealed a progressive decrease in purity as the voltage was increased from -35 mV to -60 mV, dropping to $48.4 \pm 1.6\%$ at -60 mV, reflecting the voltage-dependent enhancements in analyte capture and translocation (**Fig. 4c**). This voltage-modulated selectivity is conceptually analogous to chromatographic separation, in which operating conditions influence analyte retention and selectivity. Based on this principle, we interpret these results as evidence that the EA nanopore can function as a nanoscale selective enrichment element under appropriately tuned voltage conditions.

To enhance the practical separation efficiency, we assembled multiple nanopores within a single membrane and introduced a 1:1 mixture of H4_{1-10} and $\text{H4}_{1-10}\text{R3(Cit)}$ into the *cis* chamber (**Fig. 4d**). Following the formation of 25 independent pores (**Fig. 4e**), current traces recorded at the optimal voltage (-35 mV) exhibited frequent translocation spikes (**Fig. 4f**). After collecting the solution from the *trans* chamber over 3 hours, we transferred it to the *cis* side of a new single-pore system (**Fig. 4g**). Using the previously established machine-learning classifier at a detection voltage of -50 mV, 98.4% of the events were categorized as H4_{1-10} (**Fig. 4h**), demonstrating highly efficient enrichment in the EA multi-pore system. Traditionally, nanopores are primarily regarded as analyzers; our results further indicate that,

under suitable pore and voltage conditions, they can also serve as selective enrichment elements. This separation-like capability may prove useful as an upstream enrichment step for downstream analytical workflows, including MS-based identification of PTM peptides.

These findings also have implications for quantitative nanopore analysis.⁶⁴ Although nanopores have been employed for quantification in numerous studies based on the event frequency, a critical factor often overlooked is the inherent capture preference of a given pore for different analytes. While simplified models, such as the capture radius, have been applied to solid-state nanopores, biological pores possess more intricate interior geometries that complicate such approximations. Our results highlight that both analyte concentration and applied voltage govern the event rate, which is essential for reliable quantitative nanopore assays. For quantitative applications, one practical strategy may be to identify operating conditions near the point of balanced capture probability, where analyte-dependent enrichment bias is minimized.

Simultaneous detection of histone citrullination and methylation

Histones are regulated by combinatorial PTMs that synergistically fine-tune transcriptional outcomes. To detect this layered regulatory language, tools must resolve not only individual modifications but also their dynamic interplay. Although engineered nanopores (e.g., the R220S aerolysin mutant) have been reported to distinguish histone methylation states, the observed differences in current blockade remain marginal (~ 0.01 in normalized I_b/I_o),⁴⁶ limiting its capacity to probe subtle PTM crosstalk. Thus, there is a clear need for more sensitive and discriminative sensing interfaces.

To experimentally benchmark the sensor's capability against a set of defined PTMs, we selected the well-studied N-terminal tail of histone H3 (residues 1–10) as a model system. We systematically designed eight peptide variants with different modification states at R2 and K9 (**Supplementary Fig. S30, 31**). As shown in **Fig. 5a**, the variants included the unmodified peptide, single modifications (R2 citrullination H3₁₋₁₀R2(Cit), R2 monomethylation H3₁₋₁₀R2(Me), R2 symmetric dimethylation H3₁₋₁₀R2(Me₂s)), and their combinations with K9 trimethylation (H3₁₋₁₀K9(Me₃)).

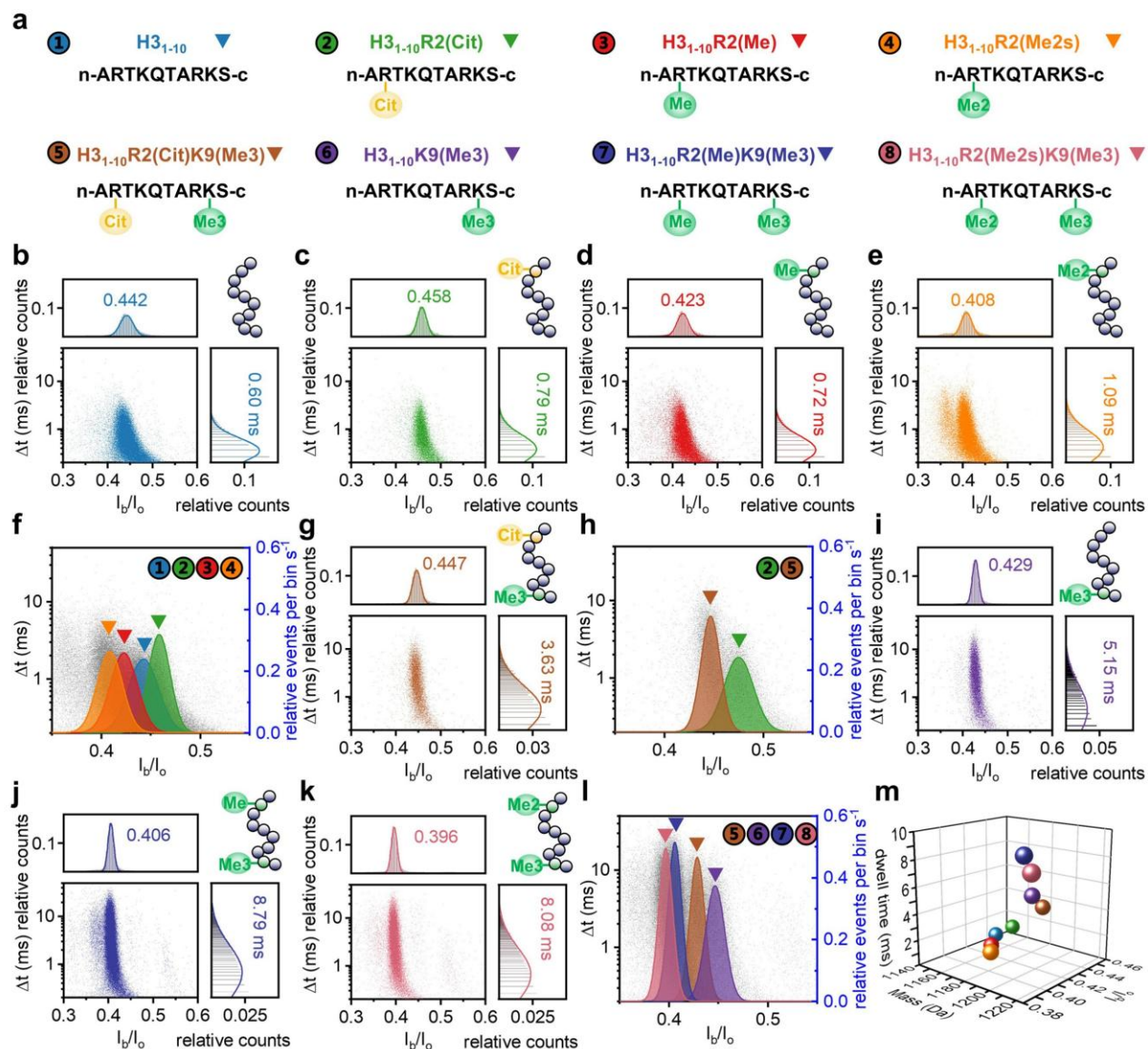


Fig. 5 | Analysis of citrullination and methylation on histone H3 peptides via the EA nanopore. a

Schematics of the eight tested peptides with modifications at R2 and K9, respectively. **b–e, g, i–k** Scatter plots of I_b/I_o versus Δt for individual peptides. I_b/I_o histograms were constructed across a range of 0.3–0.6 with 100 bins, and the counts were normalized to the total detected events to obtain the relative counts. The mean I_b/I_o was determined via Gaussian fitting. Δt histograms were generated with tailored ranges and bin numbers: 0–20 (200 bins) for **b–e**, 0–30 (300 bins) for **g** and **i**, and 0–50 (500 bins) for **j** and **k**. The average Δt was extracted by log-normal fitting of the corresponding histograms. All nanopore experiments were conducted in 4 M KCl, 25 mM HEPES, pH 7.5, at 25 °C, with an applied voltage of –

50 mV, and addition of 20 μ M analytes. **f, h, i** Gaussian-fitted I_b/I_o distributions from the indicated scatter plots, revealing distinct signatures for each modification state and their combinations. **m** Plot of normalized I_b/I_o change and dwell time against mass change for all eight peptides, indicating contributions from non-mass-dependent factors.

The engineered nanopore resolved the different modifications at the R2 site with clear distinction, as reflected in their unique translocation signatures (**Fig. 5b–e**). Citrullination at R2 consistently increased the normalized blockade current, raising the mean I_b/I_o from 0.442 (H3₁₋₁₀) to 0.458 (H3₁₋₁₀R2(Cit)) (**Fig. 5b, c**). In contrast, methylation at R2 decreased the mean I_b/I_o (**Fig. 5d**), with symmetric dimethylation exerting a stronger steric hindrance effect than monomethylation (0.423 for H3₁₋₁₀R2(Me) vs. 0.408 for H3₁₋₁₀R2(Me₂s)) (**Fig. 5e, f**), which could be reasonably attributed to the added steric bulk of the methyl groups. Meanwhile, methylation substantially prolonged peptide dwell times (1.09 ms for H3₁₋₁₀R2(Me₂s), 0.79 ms for H3₁₋₁₀R2(Me), and 0.6 ms for H3₁₋₁₀), citrullination only induced a modest change (0.79 ms for H3₁₋₁₀R2(Cit)).

We next examined the combined effects between R2 modifications and K9 trimethylation. The dual-modified peptide H3₁₋₁₀R2(Cit)K9(Me₃) exhibited an intermediate mean I_b/I_o of 0.447 (**Fig. 5g**), a value between H3₁₋₁₀ and H3₁₋₁₀R2(Cit), reflecting the opposing effects of citrullination (which increases (I_b/I_o)) and methylation (which decreases it). Notably, the dwell time of H3₁₋₁₀R2(Cit)K9(Me₃) increased substantially to ~3.63 ms (**Fig. 5g**), suggesting cooperative modulation of peptide–pore interactions. The methylated variant H3₁₋₁₀R2(Cit)K9(Me₃) could be well identified from H3₁₋₁₀R2(Cit) (**Fig. 5h**). H3₁₋₁₀K9(Me₃) exhibited a mean I_b/I_o of 0.429, corresponding to a deeper current blockade than that of H3₁₋₁₀ (I_b/I_o of 0.442), alongside a prolonged dwell time of 5.43 ms—markedly longer than 0.6 ms observed for H3₁₋₁₀ (**Fig. 5i**). Similarly, peptides containing both R2 methylation and K9(Me₃) modifications showed further reductions in the mean I_b/I_o values: 0.406 for H3₁₋₁₀R2(Me)K9(Me₃), and 0.396 for H3₁₋₁₀R2(Me₂s)K9(Me₃), paired with extended dwell times of 8.79 ms and 8.08 ms, respectively (**Fig. 5j, k**). H3₁₋₁₀R2(Me)K9(Me₃) yielded the lowest mean I_b/I_o (0.396) among all

analytes, due to its largest effective molecular volume (**Fig. 5l**). As summarized in **Fig. 5m**, the EA nanopore unambiguously resolved all eight histone modification states. While the blockade current showed a rough correlation with molecular mass for certain analytes, the overall discrimination stems from a sophisticated interplay of steric volume, charge distribution, and specific non-covalent interactions. This demonstrated that the semi-rationally engineered nanopore served as a versatile molecular interface; it deconvoluted the co-existence and combination between citrullination and methylation, thus advancing the platform from single modification detection to the integrated analysis of combinatorial epigenetic signals.

Deciphering histone citrullination-methylation crosstalk

To decipher the crosstalk between citrullination and methylation on histone H3, we employed the H3₁₋₁₀ peptide as a substrate for PAD4-catalyzed citrullination, given that this peptide contains both a citrullination site (R2/R8) and methylation sites. Both R2 and R8 are possible PAD4 catalytic sites as reported previously, leading to multiple potential end products (**Fig. 6a**). Nanopore analysis of the reacted solution yielded two distinct peaks: a substrate-derived peak of I_b/I_o at 0.447 (occupancy ~39.9%) and a new peak at 0.499 (occupancy ~60.1%), which could be assigned as the product-derived signal (**Fig. 6b**). To verify the product composition, we performed nanopore experiments with synthetic standards H3₁₋₁₀R8(Cit) and H3₁₋₁₀R2(Cit), which exhibited characteristic peaks at 0.491 and 0.458, respectively (**Fig. 6c**). This suggested that H3₁₋₁₀ peptide transformed into H3₁₋₁₀R8(Cit) through the PAD4-catalyzed citrullination with an efficiency of approximately 76.6%.

To further validate this finding, we selected H3₁₋₁₀K9(Me3) as the substrate for PAD4-catalyzed reaction. We hypothesized that trimethylation at K9 would hinder citrullination at the neighboring R8, thereby favoring the formation of H3₁₋₁₀R2(Cit)K9(Me3), as illustrated in **Fig. 6d**. Nanopore analysis of the reaction mixture from **Fig. 6e** showed a prominent peak (occupancy ~86.5%) for the substrate H3₁₋₁₀K9(Me3) at 0.422, demonstrating that trimethylation at K9 indeed impacts citrullination and induces crosstalk. However, the expected product H3₁₋₁₀R2(Cit)K9(Me3) with a predicted peak of I_b/I_o at 0.447

was not detected. Instead, a minor new peak (occupancy ~13.5%) emerged at 0.467, corresponding to the H3₁₋₁₀R8(Cit)K9(Me3) standard, with a characteristic peak at 0.469 (Fig. 6f). This indicated that only about 13.5% of substrate had transformed into H3₁₋₁₀R8(Cit)K9(Me3).

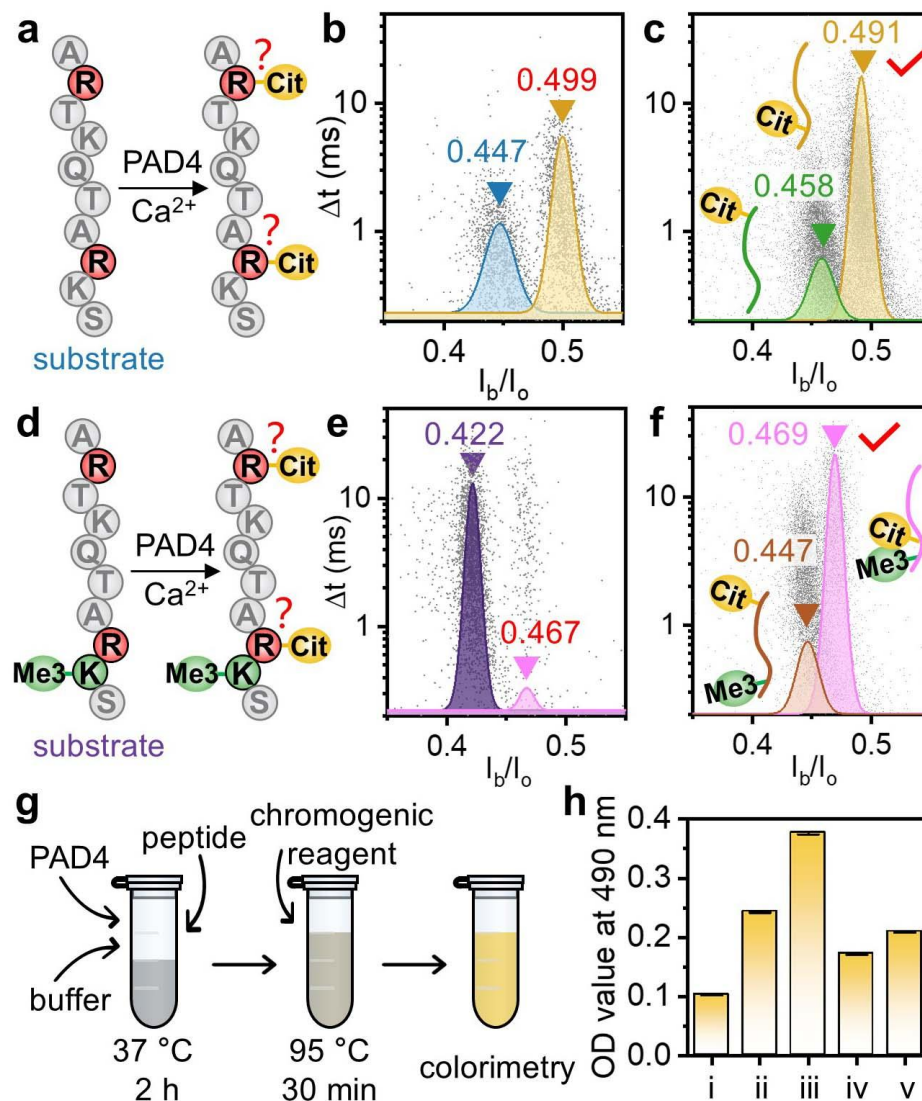


Fig. 6 | PAD4-catalyzed PTM crosstalk between citrullination and methylation on histone H3 peptides via the EA nanopore. a, d PAD4-mediated citrullination of H3₁₋₁₀ (a) and H3₁₋₁₀K9(Me3) (d), with potential citrullination sites highlighted in red. 1.36 mM peptide was added to a reaction buffer solution consisting of 200 mM Tris-HCl (pH 7.6), 50 mM CaCl₂, and 1.36 μM PAD4, the mixture was incubated at 37 °C for 2 h. **b, e** Nanopore analysis of analytes (4 μL) from the 2 h PAD4-catalyzed

586 reaction product of H3₁₋₁₀ (**b**) and H3₁₋₁₀K9(Me3) (**e**). **c, f** Nanopore signals of standard samples (μ M) of
587 H3₁₋₁₀R2(Cit) and H3₁₋₁₀R8(Cit) (**c**), H3₁₋₁₀R2(Cit)K9(Me3) and H3₁₋₁₀R8(Cit)K9(Me3) (**f**). Red arrows
588 denote the corresponding products. All nanopore experiments were conducted in 4 M KCl, 25 mM
589 HEPES, pH 7.5, at 25 °C, with an applied voltage of –50 mV. **g**) Workflow of the colorimetric assay for
590 citrullination detection. 6- or 12-mM peptides, 400 nM PAD4, and the reaction buffer was mixed in one
591 tube. The solution was incubated at 37 °C for 2 h. Then, 100 μ L of freshly prepared color developing
592 solution containing 20 mM diacetylmonoxime (DAMO), 0.5 mM antipyrine, 2.2 M H₃PO₄, 4.5 M H₂SO₄,
593 1.5 mM NH₄Fe(SO₄)₂ was added to chemically modify PAD4-generated citrullinated products, and the
594 derivatization was reacted at 95 °C for 30 min. **h** Optical density (OD) values at 490 nm following PAD4-
595 catalyzed citrullination and subsequent chromogenic reagent reaction for (i) blank (ii) 6 mM and (iii) 12
596 mM H3₁₋₁₀, (iv) 6 mM and (v) 12 mM H3₁₋₁₀K9(Me3). The OD tests were performed three times, and the
597 error bars represent the standard error.

598 To monitor the aforementioned reactions, chromogenic assay tests were conducted (**SI Section 2.10**),
599 where increased optical density (OD) values indicated the formation of citrullinated products (**Fig. 6g**).
600 As shown in **Fig. 6h**, OD values reached 0.243 ± 0.001 and 0.376 ± 0.002 when 6 mM and 12 mM H3₁₋₁₀
601 peptides were introduced to the reaction system, which was significantly higher than the blank control
602 with an OD value of 0.102 ± 0.001 , indicating the efficient PAD4-catalyzed citrullination. In contrast,
603 under the same condition, H3₁₋₁₀K9(Me3) induced slightly OD value increases, proving the inhibitory
604 effect of K9 trimethylation on PAD4 activity. However, this conventional chromogenic assay could not
605 provide the citrullination site information, which is a remarkable drawback. These results indicated that
606 our nanopore system could distinguish positional isomers of equal mass peptidoforms, a capability
607 unattainable with the colorimetric assays. Furthermore, the system effectively validated PAD4-mediated
608 crosstalk between citrullination and methylation on histone H3, offering a versatile tool to decipher
609 complex epigenetic regulatory networks.

Discussion

In summary, we have developed the PIToN model, a semi-rational quantitative model that describes how ionic current signals can arise from diverse physical interactions between analytes and nanopore walls. This model transforms nanopore engineering from empirical screening toward physico-chemically guided design, providing a practical bridge between pore–analyte interactions and high-resolution sensing signals, with potential applicability to various PTMs and molecular discrimination tasks.⁴³

Guided by the PIToN model, we successfully engineered a nanopore sensor featuring a double “reading head” design, achieving label-free, single-molecule detection of citrullination with 98.3% accuracy and sub-dalton resolution, as well as discrimination between citrullination, methylation, and their combinatorial states on histone peptides. In the post-genomic era, high-resolution protein analysis is imperative because PTMs directly define protein function and cellular identity; precise characterization of these modifications is a critical prerequisite for robust nanopore-based protein sequencing. Although nanopore technology holds great promise for single-molecule sequencing, subtle sub-dalton PTMs (e.g., citrullination) have long been a major bottleneck, their misidentification compromises sequencing fidelity and leads to erroneous biological interpretations. Our work helps address this foundational gap, underscoring its significance for advancing nanopore proteomics.

Notably, we expanded the platform’s functionality from analytical detection to voltage-tunable selective enrichment and separation-like behavior at the single-molecule level. Unlike conventional chromatography, which depends on ensemble-averaged interactions and requires micro- to milligram quantities of samples, our approach achieves highly selective enrichment through finely tuned pore–analyte interactions under controlled voltage conditions. This mechanism reduces sample demands, retains a high degree of molecular heterogeneity, and enables target peptide enrichment with 99.7% purity. Although throughput remains a practical limitation for broad application, this can be addressed by parallelizing arrays of nanopores.^{65,66} Scaling such integrated sensing–separation units could transform the platform into a high-throughput technology,⁶⁷ bridging single-molecule precision with the demands of real-world proteomic analysis.

Looking forward, the PIToN model represents a foundational step toward predictive sensor design.⁶⁸ Its performance can be substantially advanced by integration with big-data and artificial-intelligence (AI) workflows. Training on expanded datasets will facilitate iterative refinement of the physical interaction coefficients, and incorporating cooperative multi-site effects will unlock fully predictive nanopore engineering. This advancement will dramatically reduce the time and cost of empirical screening, thereby paving the way for high-throughput profiling of biologically relevant complex PTM landscapes.

Methods

Materials

1,2-Diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, 99%) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). n-Decane (99%) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Trypsin agarose (T1763, from bovine pancreas trypsin) and potassium chloride (for molecular biology) were acquired from Sigma-Aldrich (Shanghai) Trading Co., Ltd. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, reagent grade, 99.5%) was purchased from Sigma-Aldrich. All solutions were prepared with ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}^{-1}$ at 25°C) from a Milli-Q system. The ethanol extract of *Terminalia chebula* was prepared as previously reported¹⁵ and dissolved at $40 \text{ mg} \cdot \text{mL}^{-1}$ in 50% aqueous dimethyl sulfoxide (DMSO). All peptides were purchased from Anhui Guoping Pharmaceutical Co., Ltd. (Anhui, China) with HPLC purity $> 95\%$. N-Benzoyl-L-arginine ethyl ester (BAEE), antipyrine, 2,3-butanedione monoxime, isopropyl β -D-thiogalactoside (IPTG), and glutathione were obtained from Shanghai Yuanye Biochemical Technology Co., Ltd. (Shanghai, China). Dithiothreitol (DTT), ammonium iron sulfate dodecahydrate, and phosphoric acid were purchased from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China).

Nanopore Experiments

Genes encoding mutants (K238C, K238N, K238Q, K238Y, K238W, R282A, R282A/K238E, R282A/K238D, R282A/K238E/K258A) were codon-optimized and synthesized by GenScript, Inc.

(Jiangsu, China). Preparation of recombinant wild-type proaerolysin and mutants followed previously described protocols.^{69,70} Nanopore recordings were performed using a planar lipid bilayer system with two 1-mL *cis/trans* chambers separated by a Delrin perfusion cup ($\approx 150\ \mu\text{m}$ diameter aperture). A DPhPC lipid bilayer was formed across the aperture, and Ag/AgCl electrodes were used for transmembrane potential application and ionic current recording. Peptides were added to the *cis* chamber unless otherwise stated. The recording buffer contained 4 M KCl and 25 mM HEPES (pH 7.5). Recordings were conducted at $-50\ \text{mV}$ (unless specified otherwise), sampled at 250 kHz, and low-pass filtered at 5 kHz using an Axopatch 200B amplifier and 1550B digitizer (Molecular Devices, USA). Ionic current traces were analyzed via the ADEPT 2-state algorithm in MOSAIC software⁷¹ to extract event dwell time (Δt) and relative current blockade depth (I_b/I_o). Only events with $\Delta t \geq 0.2\ \text{ms}$ were retained for quantitative analysis (to avoid unreliable blockade-depth estimation for short events). Scatter plots, histograms, and Gaussian fittings were generated from the filtered event dataset.

Biolayer Interferometry (BLI)

BLI was used to measure the affinity between EE and peptides (H4₁₋₁₀, H4₁₋₁₀R3(Cit)) on an Octet K2 System with amine-reactive 2nd generation (AR2G) biosensors. Glu-Glu ($1\ \text{mg}\cdot\text{mL}^{-1}$) was immobilized onto carboxyl-terminated biosensors via EDC/NHS-mediated coupling between carboxyl groups on the sensor surface and amine residues of Glu-Glu, incubated for 24 h in ultrapure water. Unreacted EE was removed by thorough washing with ultrapure water. EE-modified biosensors were then exposed to peptide solutions (0.005, 0.03, 0.125, 0.25, $1\ \mu\text{M}$) followed by dissociation in ultrapure water. All procedures (sensor preparation and interaction analysis) were performed at $25\ ^\circ\text{C}$. Data were analyzed using ForteBio Data Analysis 11.0 software.

General Molecular Dynamics (MD) Simulations

Model construction followed our previous studies.^{69,72} Briefly, the initial all-atom model of wild-type aerolysin nanopore embedded in a DPhPC membrane, solvated in 1 M KCl with a TIP3P water box (200

Å×200 Å×160 Å), was adopted from prior work. Mutant nanopores (K238E, R282A, R282A/K238E) were constructed using VMD.⁷³ Peptides (H4₁₋₁₀, H4₁₋₁₀R3(Cit)) were built via Avogadro⁷⁴ (H4₁₋₁₀) or modified from H4₁₋₁₀ (H4₁₋₁₀R3(Cit)); citrulline was parameterized with the CHARMM22 force field (SwissSidechain⁷⁵) and other residues with CHARMM36. Peptides were positioned at the *cis* entrance of the pore, aligned along the pore axis.

All MD simulations were performed with NAMD2,^{76,77} and visualization/analysis with VMD. Key simulation parameters: 2 fs time step; particle-mesh Ewald (PME) for long-range electrostatics (1 Å grid); VDW interactions with 12 Å cutoff and 10 Å switching distance; periodic boundary conditions. Systems underwent energy minimization (2000 steps, protein α -carbon restraints), heating to 295 K, and NPT equilibration (α -carbon restraints). A transmembrane bias of –100 mV was applied. Constant-velocity SMD simulations were conducted by pulling peptides (*cis* to *trans*) at 0.5 nm·ns⁻¹ via a virtual spring (69.5 pN/nm), generating 20 ns trajectories. Non-bonded interactions between nanopore and peptides were calculated with the NAMDenergy plugin. Ionic currents (open-pore and peptide-occupied) were estimated via SEM,^{63,78} with current blockade derived from their comparison.

Equilibrium MD simulations (5 ns each) were performed on two snapshots of H4₁₋₁₀ extracted from SMD trajectories (peptide near K238 region). Peptide configuration occupancies were averaged over simulation time using the VMD volmap plugin. Detailed protocols are provided in the Supplementary Information.

PAD4 Expression, Purification, and Activity Assays; Herbal Extract Preparation

Recombinant human GST-PAD4 was expressed in *Escherichia coli* BL21(DE3) and purified by glutathione agarose affinity chromatography as previously reported,^{15,79} with the purified protein stored at –80 °C. Ethanolic extracts of *Terminalia chebula* (purchased from Beijing Tongrentang Chinese Medicine Pharmacy Co., Ltd) were prepared via 75% ethanol reflux extraction, concentration, and freeze-drying, then reconstituted in 50% DMSO to 20 mg/mL and stored at –80 °C after centrifugation. PAD4 activity was measured using a modified colorimetric assay with BAEE or synthetic peptides (H4₁₋₁₀, H3₁₋₁₀, H3₁₋₁₀K9(Me3)) as substrates; reactions were incubated, quenched with a color reagent, heated for color

development, and absorbance was recorded at 490 nm. Detailed experimental procedures are provided in the Supplementary Information.

Machine learning (ML) Analysis

ML classification analyses were carried out using Orange data-mining software (v3.23).⁸⁰ Seven models were evaluated, including AdaBoost, k-nearest neighbors (kNN), logistic regression, naïve Bayes, neural network, random forest, and support vector machine (SVM). The neural network showed the best performance based on AUC and classification accuracy, and was subsequently used to predict the percentage of citrullination products from PAD4-catalyzed reactions.

Data Availability

The source data underlying the figures in this paper are provided. All other relevant data are available from the corresponding author upon reasonable request.

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916 Contributions

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919 assistance from W. Y., M. L., Y. C., W. S., J. W. and J. L.. J. Z. (Juanjuan Zhao) expressed PAD4 enzyme,
920 prepared PAD4 inhibitor and performed OD tests with assistance from Z. Z.. X. Z., D. W. and K. X.
921 performed DFT calculations. Y. B. conceived and supervised the study, discussed the results and helped
922 with the writing. G. Q. conceived and supervised the study, contributed to the writing of the paper,
923 discussed the results and commented on the paper. All authors reviewed and approved the final
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927 **Ethics declarations**

928 Competing interests

929 The authors declare no competing interests.

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