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Accurate modeling of a biological nanopore with an extended continuum framework†

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Despite the broad success of biological nanopores as powerful instruments for the analysis of proteins and nucleic acids at the single-molecule level, a fast simulation methodology to accurately model their nanofluidic properties is currently unavailable. This limits the rational engineering of nanopore traits and makes the unambiguous interpretation of experimental results challenging. Here, we present a continuum approach that can faithfully reproduce the experimentally measured ionic conductance of the biological nanopore Cytolysin A (ClyA) over a wide range of ionic strengths and bias potentials. Our model consists of the extended Poisson–Nernst–Planck and Navier–Stokes (ePNP–NS) equations and a computationally efficient 2D-axisymmetric representation for the geometry and charge distribution of the nanopore. Importantly, the ePNP–NS equations achieve this accuracy by self-consistently considering the finite size of the ions and the influence of both the ionic strength and the nanoscopic scale of the pore on the local properties of the electrolyte. These comprise the mobility and diffusivity of the ions, and the density, viscosity and relative permittivity of the solvent. Crucially, by applying our methodology to ClyA, a biological nanopore used for single-molecule enzymology studies, we could directly quantify several nanofluidic characteristics difficult to determine experimentally. These include the ion selectivity, the ion concentration distributions, the electrostatic potential landscape, the magnitude of the electro-osmotic flow field, and the internal pressure distribution. Hence, this work provides a means to obtain fundamental new insights into the nanofluidic properties of biological nanopores and paves the way towards their rational engineering.

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1. Introduction

The transport of ions and molecules through nanoscale geometries is a field of intense study that uses experimental,^{1–8} theoretical^{9–14} and computational methods.^{15–25} A primary driving force behind this research is the development of nanopores as label-free, stochastic sensors at the ultimate analytical limit (*i.e.*, single molecule).^{26–29} These detectors have applications ranging from the analysis of biopolymers such as DNA^{1,2,30–36} or proteins,^{37,38–46} to the detection and quantification of biomarkers,^{47–52} or the fundamental study of

chemical or enzymatic reactions at the single molecular level.^{44,53–59}

Nanopores are typically operated in the resistive-pulse mode, where the changes of their ionic conductance are monitored over time.^{26–28,60} Experimentally, this is achieved by placing the nanopore between two isolated electrolyte compartments and applying a constant DC (or AC) voltage across them. The $\approx 10^8$ difference in ionic resistance of the nanopore (≈ 1 G Ω) compared to the typical electrolyte reservoir (≈ 10 Ω),⁶⁰ causes the full potential change to occur within (and around) the pore. The resulting electric field (10^6 – 10^7 V m^{−1}) electrophoretically drives a given number of ions (*i.e.*, the ‘open-pore’ conductance) and water molecules (*i.e.*, the electro-osmotic flow) through the pore.^{61–64} Similarly, analyte molecules such as DNA and proteins are subject to the same Coulombic (electrophoretic) and hydrodynamic (electro-osmotic) forces.^{10,13,61,65} This guides them towards—and often through—the nanopore, resulting in a temporary disruption of the baseline ionic conductance. Naively speaking, for any given analyte molecule, the rate of these resistive pulses is proportional to its concentration in the reservoir, whereas their

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duration, magnitude and intra-pulse fluctuations contain a wealth of information on its physical properties (*e.g.*, size, shape and charge).^{66–68} Hence, the unique ‘fingerprints’ provided by the (sub-)nanometre-sized sensing volume of a nanopore can reveal information that is typically inaccessible to bulk measurements, such as hidden intermediate states, dynamic noncovalent interactions and the presence of subpopulations.^{67,68} Notably, nanopores have been used to create ultra-long reads of individual DNA strands,³⁶ to determine the shape, volume and dipole moment of proteins,^{39,40} and to investigate the kinetics of single enzymes tethered to^{57,69} or trapped within^{58,59} the pore. Because each current blockade is modulated by the complex interactions between the translocating molecule and the nanopore itself, they will depend on the properties of both. As a result, despite the successful applications mentioned above, the unambiguous interpretation of the ionic current signal remains a notoriously difficult task if a full understanding of the nanofluidic phenomena that underlie them is not available.

The computational approaches most widely used to study nanofluidic transport in ion channels or biological nanopores comprise discrete methods such as molecular dynamics (MD)^{17,24,25,70–76} and Brownian dynamics (BD),^{15,77–82} and mean-field (continuum) methods based on solving the Poisson–Boltzmann (PB) equations^{83,84} and Poisson–Nernst–Planck (PNP) equations.^{85–87} The latter two can be coupled with the Navier–Stokes (NS) equation to include electro-osmotically or pressure driven fluid flow.^{82,88} Due to their explicit atomic or particle nature, MD and BD simulations are considered to yield the most accurate results. However, the large computational cost of simulating a complete biological nanopore system (100 K–1 M atoms) for hundreds of nanoseconds still necessitates the use of supercomputers.^{17,25,73,89} The PNP (–NS) equations, on the other hand, are of particular interest due to their low computational demands and analytical tractability. In a continuum approach, the simulated system is subdivided in several ‘structureless’ domains, the behavior of which is parameterized by material properties such as the relative permittivity, diffusion coefficient, electrophoretic mobility, viscosity or density. Because these properties can only emerge from the collective behavior or interactions between small groups of atoms (*i.e.*, the mean-field approximation), great care must be taken when using them to compute fluxes and fields at the nanoscale, where computational elements may only contain a few molecules.^{90,91} Nevertheless, even though the PNP equations have been used extensively for the qualitative simulation of ion channels,^{15,92,93} biological nanopores^{82,87,94,95} and their solid-state counterparts,^{64,96–98} the extent to which they are quantitatively accurate is often challenged.^{18,20,22,90,91} To remedy the shortcomings of PNP and NS theory, a number of modifications have been proposed over the years. These include, among others, (1) steric ion–ion interactions, (2) the effect of protein–ion/water interactions on their ‘motility’ (*i.e.*, diffusivity and electrophoretic mobility), (3) the concentration dependencies of ion motility, and solvent relative permittivity, viscosity and density.

The steric ion–ion interactions can be accounted for by computing the excess in chemical potential (μ_i^{ex}) resulting from the finite size of the ions,^{85,99,100} or by taking the dielectric-self energy of the ions into account.^{9,101} Gillespie *et al.* combined PNP and density functional theory—where μ_i^{ex} was split up in ideal, hard-sphere and electrostatic components—to successfully predict the selectivity and current of ion channels.⁸⁶ In the Poisson–Nernst–Planck–Fermi model developed by Liu and Eisenberg, steric effects are included by treating water molecules as discrete particles, described using Fermi distributions, and by implementing a mean-field version of the van der Waals potential.^{14,93,102} In another approach, Kilic *et al.* derived a set of modified PNP equations based on the free energy functional of the Borukhov’s modified PB model¹⁰³ and observed significantly more realistic concentrations for high surface potentials compared to the classical PNP equations.¹⁰⁴ To allow for non-identical ion sizes and more than two ion species, this model was later extended by Lu *et al.*, who used it to probe the effect of finite ion size on the rate coefficients of enzymes.¹⁰⁵

The interaction of ions or small molecules such as water with the heavy atoms of proteins or DNA results in a strong reduction of their motility, as observed in MD simulations.^{89,106,107} Since these effects happen only at distances ≤ 1 nm, they can usually be neglected for macroscopic simulations. However, in small nanopores (≤ 10 nm radius), they comprise a significant fraction of the total nanopore radius and hence must be taken into account.^{78,82,87,108} In continuum simulations, this can be achieved with the use of positional-dependent ion diffusion coefficients. An example implementation is the ‘soft-repulsion PNP’ developed by Simakov and Kurnikova,^{87,95} who used it to predict the ionic conductance of the α -hemolysin (α HL) nanopore. Similar reductions in ion diffusion coefficients have been proposed to improve PNP theory’s estimations of the ionic conductance of ion channels.^{81,92,93} The motility of water molecules is expressed by the NS equations as the fluid’s viscosity. Hence, as also observed in MD simulations for water molecules near proteins¹⁰⁷ and confined in hydrophilic nanopores,^{109–111} the water–solid interaction leads to a viscosity several times higher compared to the bulk values. Note that this is valid for hydrophilic interfaces only, as the lack of interaction with hydrophobic interfaces, such as carbon nanotubes, leads to a lower viscosity.¹¹²

It is well known that the self-diffusion coefficient D_i and electrophoretic mobility μ_i of an ion i depends on the local concentrations of all the ions in the electrolyte.¹¹³ Their values typically decrease with increasing salt concentration, and should not be treated as constants. Moreover, even though the Nernst–Einstein (NE) relation ($\mu_i = D_i/k_B T$) is strictly speaking only valid at infinite dilution and a good approximation at low concentrations (< 10 mM), it significantly overestimates the ionic mobility at higher salt concentrations.^{113–116} In an empirical approach, Baldessari and Santiago formulated an ionic-strength dependency of the ionic mobility based on the activity coefficient of the salt⁸⁴ and showed excellent corre-

spondence between the experimental and simulated ionic conductance of long nanochannels over a wide concentration range.¹¹⁷ Alternatively, Burger *et al.* used a microscopic lattice-based model to derive a set of PNP equations with non-linear, ion density-dependent mobilities and diffusion coefficients that provided significantly more realistic results for ion channels.¹¹⁸ Note that other electrolyte properties, such as the viscosity,¹¹⁹ density¹¹⁹ and relative permittivity,¹²⁰ may also significantly affect the ion and water flux. To better compute the charge flux in ion channels, Chen derived a new PNP framework¹²¹ that includes water-ion interactions in the form of a concentration-dependent relative permittivity and an additional ion-water interaction energy term.

To the best of our knowledge, no attempt has been made to consolidate all of the corrections discussed above into a single framework. Hence, we propose an extended set of PNP-NS (ePNP-NS) equations, which improves the predictive power of the PNP-NS equations at the nanoscale and beyond infinite dilution. Our ePNP-NS framework takes into account the finite size of the ions using a size-modified PNP theory,¹⁰⁵ and implements spatial-dependencies for the solvent viscosity,^{107,111} the ion diffusion coefficients and their mobilities.^{78,106} It also includes self-consistent concentration-dependent properties—based on empirical fits to experimental data—for all ions in terms of diffusion coefficients and mobilities,^{84,114} and for the solvent in terms of density, viscosity¹¹⁹ and relative permittivity.¹²⁰ To validate our new framework, we applied it to a 2D-axisymmetric model of Cytolysin A (ClyA),¹²³ a large protein nanopore³⁷ that typically contains 12 or more identical subunits.^{122,126} We choose to model the ClyA-AS type I, a dodecameric variant of the wild type ClyA from *S. Typhii* that was artificially evolved for improved stability.¹²² ClyA-AS has been extensively used in experimental studies of proteins^{8,49,52,59,122,127–131} and DNA,^{7,35,132} and has even been employed as a targeted immunotoxin.¹³³ This allowed us to gauge the qualitative and quantitative performance of the ePNP-NS equations and simultaneously elucidate previously inaccessible details about the environment inside the pore.

The remainder of this paper is organized as follows. In section 2 we describe the equations governing our ePNP-NS framework and detail the construction of the 2D-axisymmetric ClyA model. Next, in section 3, we validate our model by direct comparison of simulated ionic conductance with experimentally measured values. We then proceed to characterize the influence of the bulk ionic strength and the applied bias voltage on cation and anion concentrations inside the pore, the electrostatic potential distribution and magnitude of the electro-osmotic flow. Finally, we touch upon our key findings and their impact in section 4 and describe our protocols in more detail in section 5.

2. Mathematical model

The use of continuum or mean-field representations for both the nanopore and the electrolyte enables us to efficiently

compute the steady-state ion and water fluxes under almost any condition. The dynamic behavior of our complete system is described by the coupled Poisson, Nernst-Planck and Navier-Stokes (PNP-NS) equations, a set of partial differential equations that describe the electrostatic field, the total ionic flux and the fluid flow, respectively.^{85,88,96} The implementation of the full model can be found as a COMSOL report in the ESI.†

2.1. Model geometry

2.1.1. 2D-axisymmetric model of ClyA. ClyA is a relatively large protein nanopore that self-assembles on lipid bilayers to form 14 nm long hydrophilic channels (Fig. 1a). The interior of the pore can be divided into roughly two cylindrical compartments: the *cis lumen* (≈ 6 nm diameter, ≈ 10 nm height), and the *trans* constriction (≈ 3.3 nm diameter, ≈ 4 nm height). Because ClyA consists of 12 identical subunits (Fig. 1b), it exhibits a high degree of radial symmetry, a geometrical feature that can be exploited to obtain meaningful results at a much lower computational cost.^{82,88,96} However, this requires the reduction of the full 3D atomic structure and charge distribution to a realistic 2D-axisymmetric model. To this end, we constructed a full-atom homology model of ClyA-AS type I¹²² and equilibrated it at 298.15 K for 30 ns in an explicit solvent with harmonic constraints on the protein backbone atoms (see section 5 for details). From the final 5 ns of this trajectory we extracted 50 sets of atomic coordinates for ClyA (*i.e.*, every 100 ps). For each of these structures, we computed a 2D atomic density¹³⁴ and charge¹⁷ map (see section 5 for details), which we then averaged to represent the conformational diversity of the side chains. The geometry of the nanopore was then defined as the 25% contour line of the density map, which closely follows the outline of a 30° ‘wedge’ of the full atom structure (Fig. 1c). The equilibrium charge map (Fig. 1d) was loaded directly into our solver as a linear interpolation function (ρ_{pore}^f) and applied across all computational domains.

2.1.2. Global geometry. The complete system (Fig. 1e and f) consists of a large hemispherical electrolyte reservoir ($R = 250$ nm), split through the middle into a *cis* and a *trans* compartment by a lipid bilayer ($h = 2.8$ nm), which contains the nanopore at its center. Both the bilayer and the nanopore are represented by dielectric blocks (see Table 1 for parameters) that are impermeable to ions and water.

2.2. Governing equations

To improve upon the quantitative accuracy of the PNP-NS equations for nanopore simulations, we developed an extended version of these equations (ePNP-NS) and implemented it in the commercial finite element solver COMSOL Multiphysics (v5.4, COMSOL Inc., Burlington, MA, USA). Our ePNP-NS equations self-consistently take into account (1) the finite size of the ions,^{103,105} (2) the reduction of ion and water motility close to the nanopore walls,^{78,82,106,107,110} and (3) the concentration dependency of ion diffusion coefficients and electrophoretic mobilities, as well as electrolyte viscosity, density and relative

Fig. 1 All-atom and 2D-axisymmetric models of ClyA-AS. (a) Axial cross-sectional and (b) top views of the dodecameric nanopore ClyA-AS,¹²² derived through homology modelling from the *E. coli* Cytolysin A crystal structure (PDBID: 2WCD¹²³). Measurements indicate the average 'cylindrical' sizes of the *cis* lumen (6 nm in diameter, 10 nm in height) and the *trans* constriction (3.3 nm in diameter, 4 nm in height). Figures were rendered with VMD.^{124,125} (c) The 2D-axisymmetric geometry was derived directly from the all-atom model using a the radially averaged atomic density (see Methods for details). Hence, it closely follows the outline of a 30° wedge out of the homology model. (d) The fixed space charge density (ρ_{pore}^f) map of ClyA-AS, obtained by Gaussian projection of each atom's partial charge onto a 2D plane (see Methods for details). (e and f) The 2D-axisymmetric simulation geometry of ClyA-AS (grey) embedded in a lipid bilayer (green) and surrounded by a spherical water reservoir (blue). Note that all electrolyte parameters depend on the local average ion concentration $\langle c \rangle = \frac{1}{n} \sum_i^n C_i$ and that some are also influenced by the distance from the nanopore wall d .

permittivity.^{114,119,120} Most of these corrections make use of empirical functions that were fitted to experimental data (Tables 1 and S2 and Fig. S1 to S4†).

2.2.1. Electrostatic field. We make use of Poisson's equation to evaluate the electric potential

$$\nabla \cdot (\epsilon_0 \epsilon_r \nabla \varphi) = -(\rho_{\text{pore}}^f + \rho_{\text{ion}}), \quad (1)$$

with φ the electric potential, ϵ_0 the vacuum permittivity (8.85419×10^{-12} F m⁻¹) and ϵ_r local relative permittivity. The pore's fixed charge distribution, ρ_{pore}^f , was derived directly from the full atom model of ClyA-AS (see eqn (22)). The ionic charge density in the fluid is given by

$$\rho_{\text{ion}} = \mathcal{F} \sum_i z_i c_i, \quad (2)$$

with $\mathcal{F}$ Faraday's constant (96485.33 C mol⁻¹), and c_i the ion concentration and z_i ion charge number of ion i . To account for the concentration dependence of the electrolyte's relative

permittivity, we replaced ϵ_r inside the electrolyte with the expression

$$\epsilon_{r,f}(\langle c \rangle) = \epsilon_{r,f}^0 e^{\epsilon_{r,f}^c(\langle c \rangle)}, \quad (3)$$

with $\langle c \rangle = \frac{1}{n} \sum_i^n c_i$ the average ion concentration, ϵ_r^0 the relative permittivity at infinite dilution and $\epsilon_{r,f}^c$ a concentration dependent empirical function parameterized with experimental data (eqn (S4) and Fig. S3c†).

2.2.2. Ionic flux. The total ionic flux $\mathbf{J}_i$ of each ion i is given by the size-modified Nernst-Planck equation,¹⁰⁵ and can be expressed as the sum of diffusive, electrophoretic, convective and steric fluxes

$$\mathbf{J}_i = -[D_i \nabla c_i + z_i \mu_i c_i \nabla \varphi - \mathbf{u} c_i + D_i \beta_i c_i], \quad (4)$$

where β_i is the steric flux vector

$$\beta_i = \frac{a_i^3 / a_0^3 \sum_j N_A a_j^3 \nabla c_j}{1 - \sum_j N_A a_j^3 c_j}, \quad (5)$$

Table 1 Summary of the parameters and fitting equations used in the ePNP-NS equations

Name	Symbol ^a	Infinite dilution value ^b /function ^c	Ref.
Relative permittivity	$\epsilon_{r,p}$	20	134
	$\epsilon_{r,m}$	3.2	135
	$\epsilon_{r,f}^0$	78.15	120
	$\epsilon_{r,f}^c(\bar{c})$	$1 - \left(1 - \frac{P_1}{P_0}\right)L\left(\frac{3P_2}{P_0 - P_1}\bar{c}\right)$	120
Ion self-diffusion coefficient	$\mathcal{D}_{Na^+}^0$	$1.334 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$	114
	$\mathcal{D}_{Cl^-}^0$	$2.032 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$	114
	$\mathcal{D}_i^c(\bar{c})$	$(1 + P_1\bar{c}^{0.5} + P_2\bar{c} + P_3\bar{c}^{1.5} + P_4\bar{c}^2)^{-1}$	This work
	$\mathcal{D}_i^w(\bar{d})$	$1 - \exp(-P_1(\bar{d} + P_2))$	87 and 106
Ion electrophoretic mobility	$\mu_{Na^+}^0$	$5.192 \times 10^{-4} \text{ m s}^{-1} \text{ V}^{-1}$	136
	$\mu_{Cl^-}^0$	$7.909 \times 10^{-4} \text{ m s}^{-1} \text{ V}^{-1}$	136
	$\mu_i^c(\bar{c})$	$(1 + P_1\bar{c}^{0.5} + P_2\bar{c} + P_3\bar{c}^{1.5} + P_4\bar{c}^2)^{-1}$	This work
	$\mu_i^w(\bar{d})$	$1 - \exp(-P_1(\bar{d} + P_2))$	87 and 106
Ion transport number	$t_{Na^+}^0$	0.396	136
	$t_{Na^+}^c(\bar{c})$	$(1 + P_1\bar{c}^{0.5} + P_2\bar{c} + P_3\bar{c}^{1.5} + P_4\bar{c}^2)^{-1}$	This work
Dynamic viscosity	η^0	$8.904 \times 10^{-4} \text{ Pa s}$	119
	$\eta^c(\bar{c})$	$1 + P_1\bar{c}^{0.5} + P_2\bar{c} + P_3\bar{c}^2 + P_4\bar{c}^{3.5}$	This work
	$\eta^w(\bar{d})$	$1 + \exp(-P_1(\bar{d} - P_2))$	107
Fluid density	ρ^0	997 kg m^{-3}	119
	$\rho^c(\bar{c})$	$1 + P_1\bar{c} + P_2\bar{c}^2$	This work

^a Dependencies on either $\bar{c} = \langle c \rangle / 1 \text{ M}$ (dimensionless average ion concentration) and $\bar{d} = d / 1 \text{ nm}$ (dimensionless distance from the nanopore wall). ^b Values at infinite dilution for a system temperature of 298.15 K. ^c These functions are empirical and hence have no physical meaning. L is the Langevin function ($L(x) = \coth(x) - 1/x$). The values of the fitting parameters P_x of each property can be found in S2 and graphs of the fits in S1 to S4.†

and at steady state

$$\frac{\partial c_i}{\partial t} = -\nabla \cdot \mathbf{J}_i = 0, \quad (6)$$

with $\mathcal{D}_i$ the ion diffusion coefficient, c_i the ion concentration, z_i the ion charge number, μ_i the electrophoretic mobility of ion i . φ is the electrostatic potential, $\mathbf{u}$ the fluid velocity field and N_A Avogadro's constant ($6.022 \times 10^{23} \text{ mol}^{-1}$). a_i and a_0 are steric cubic diameters of respectively ions and water molecules. Because currently there are no experimentally verified values available for a_i and a_0 , we set them to 0.5 nm (max. 13.3 M) and 0.311 nm (max. 55.2 M), respectively.⁹⁹

The reduction of the ionic motility at increasing salt concentrations and in proximity to the nanopore walls was implemented self-consistently by replacing $\mathcal{D}_i$ and μ_i with the expressions

$$\mathcal{D}_i(\langle c \rangle, d) = \mathcal{D}_i^0 \mathcal{D}_i^c(\langle c \rangle) \mathcal{D}_i^w(d) \quad (7)$$

$$\mu_i(\langle c \rangle, d) = \mu_i^0 \mu_i^c(\langle c \rangle) \mu_i^w(d) \quad (8)$$

where $\mathcal{D}_i^0$ and μ_i^0 represent the values at infinite dilution. The concentration dependent factors $\mathcal{D}_i^c(\langle c \rangle)$ and $\mu_i^c(\langle c \rangle)$ are empirical functions fitted to experimental data (between 0 to 5 M NaCl) of respectively the ion self-diffusion coefficients¹¹⁴ (eqn (S1) and Fig. S1†) and the electrophoretic mobilities^{136–139} (eqn (S1) and Fig. S2†). Likewise, the factors $\mathcal{D}_i^w(d)$ and $\mu_i^w(d)$ are empirical functions that introduce a spatial dependency on the distance from the nanopore wall d , and were parameter-

ized by fitting to molecular dynamics data (eqn (S5) and Fig. S4a†).^{78,87,89,106}

Based on the observation that the diffusivity of nanometer- to micrometer-sized particles reduces significantly when confined in pores and slits of comparable dimensions,^{13,140–143} Simakov *et al.*⁸⁷ and Pederson *et al.*⁸² reduced the ion motilities inside the pore as a function of the ratio between the ion and the nanopore radii. We chose not to include this correction into our model, as extrapolating its applicability for ions with a hydrodynamic radii comparable to size of the solvent molecules is questionable.^{141,144}

2.2.3. Fluid flow. As derived by Axelsson *et al.*,¹⁴⁵ the fluid flow and pressure field inside an incompressible fluid with a variable density and variable viscosity is given by the Navier-Stokes equations:

$$\frac{\partial}{\partial t}(\rho \mathbf{u}) + (\mathbf{u} \cdot \nabla)(\rho \mathbf{u}) + \nabla \cdot \boldsymbol{\sigma}_{ij} = \mathbf{F}_{\text{ion}}, \quad (9)$$

where

$$\boldsymbol{\sigma}_{ij} = p\mathbf{I} - \eta[\nabla \mathbf{u} + (\nabla \mathbf{u})^T], \quad (10)$$

together with the continuity equations for the fluid density

$$\frac{\partial \rho}{\partial t} + \mathbf{u} \cdot \nabla \rho = 0, \quad (11)$$

and the divergence constraint for the momentum

$$\nabla \cdot (\rho \mathbf{u}) - \mathbf{u} \cdot \nabla \rho = 0, \quad (12)$$

with $\mathbf{u}$ the fluid velocity, ρ the fluid density, σ_{ij} the hydrodynamic stress tensor, η the viscosity and p the pressure. The external body force density $\mathbf{F}_{\text{ion}}$ that acts on the fluid is given by

$$\mathbf{F}_{\text{ion}} = eN_{\text{A}}\rho_{\text{ion}}\mathbf{E}, \quad (13)$$

with $\mathbf{E} = -\nabla\varphi$ the electric field vector. At steady-state, the partial derivatives w.r.t. time in eqn (9) and (11) become equal to zero:

$$\frac{\partial}{\partial t}(\rho\mathbf{u}) = 0 \quad (14)$$

$$\frac{\partial \rho}{\partial t} = 0. \quad (15)$$

As with the previous equations, we introduced a concentration and wall distance dependency for η and a concentration dependency for the ρ by replacing their constant values by

$$\eta(\langle c \rangle, d) = \eta^0 \eta^c(\langle c \rangle) \eta^w(d) \quad (16)$$

$$\rho(\langle c \rangle) = \rho^0 \rho^c(\langle c \rangle) \quad (17)$$

where η^0 and ρ^0 are the values at infinite dilution (*i.e.*, pure water). The empirical functions $\eta^c(\langle c \rangle)$, $\rho^c(\langle c \rangle)$ and $\eta^w(d)$ were parameterized *via* fitting to experimental¹¹⁹ and molecular dynamics¹⁰⁷ data obtained from literature (eqn (S2), (S3) and (S6) and Fig. S3a, S3b and S4b†).

2.3. Boundary conditions and concentration dependencies

The reservoir boundaries were set up, using Dirichlet conditions, to act as electrodes: the *cis* side was grounded ($\varphi = 0$) and a fixed bias potential was applied along the *trans* edge ($\varphi = V_{\text{b}}$). To simulate the presence of an endless reservoir, the ion concentration at both external boundaries were fixed to the bulk salt concentration ($c_{\text{i}} = c_{\text{s}}$) and the unconstrained flow in and out of the computational domain was enabled by means of a ‘no normal stress’ condition ($\sigma_{ij}\mathbf{n} = 0$). The boundary conditions on the edges of the reservoir shared with the nanopore and bilayer were set to no-flux ($-\mathbf{n}\cdot\mathbf{J}_{\text{i}} = 0$) and no-slip ($\mathbf{u} = 0$), preventing the flux of ions through them and mimicking a sticky hydrophilic surface, respectively. Finally, a Neumann boundary condition was applied at the bilayer’s external boundary ($-\mathbf{n}\cdot(\epsilon_0\epsilon_{\text{T}}\nabla\varphi) = 0$).

All concentration dependent parameters use the local ionic strength rather than their individual ion concentrations. Though valid for electroneutral bulk solutions, this approximation no longer holds inside the electrical double layer (*i.e.*, near charged surfaces or inside small nanopores), where local electroneutrality is violated. The main reasons for making this simplification regardless are the lack of non-bulk experimental data and the absence of a tractable analytical model. Furthermore, we will see that the current implementation of our concentration dependent functions will lead to an excellent agreement with the experimental data in all but the most extreme cases, justifying our choice *a posteriori*.

The ePNP-NS equations revert into the regular PNP-NS equations by disabling the steric flux vector ($\beta = 0$) and all concentration ($e_{\text{r,f}}^{\text{c}} = 1$, $\mathcal{D}_{\text{i}}^{\text{c}} = 1$, $\mu_{\text{i}}^{\text{c}} = 1$, $\eta^{\text{c}} = 1$, $\rho^{\text{c}} = 1$) and wall distance functions ($\mathcal{D}_{\text{i}}^{\text{w}} = 1$, $\mu_{\text{i}}^{\text{w}} = 1$, $\eta^{\text{w}} = 1$).

2.4. On the extension to non-axisymmetric nanopores

The possibility to use a computationally efficient 2D-axisymmetric model is enabled by ClyA’s high degree of rotational symmetry (Fig. 1b). To successfully extend our ePNP-NS framework to non-axisymmetric pores—such as outer membrane porins F (OmpF)¹⁴⁶ or G (OmpG)¹⁴⁷—and very narrow nanopores—such as αHL ,¹⁴⁸ Fragaceatoxin C (FraC),¹⁴⁹ or *Mycobacterium smegmatis* porin A (MspA)¹⁵⁰—these equations should be solved for a full 3D representation. For narrow pores the radial averaging of the geometry might remove features essential for correctly modelling the fluidic properties, such as internal corrugations or opposing charges within the same radial plane. Even though this will increase the computational cost, it would still be significantly faster than either BD or MD approaches (*i.e.*, hours per data point instead of days).

Describing a methodology for setting up a 3D model is beyond of the scope of this manuscript. We expect it to follow along the same lines as the approach outlined above, with the exception that the actual molecular surface and charge distribution of the pore (see the adaptive Poisson-Boltzmann solver (APBS)^{151,152} for a potential approach) could be used instead of a radially averaged one. The ePNP-NS equations themselves would require discretization in Cartesian (x, y, z) rather than cylindrical (r, φ, z) coordinates, but can otherwise remain unchanged.

3. Results and discussion

The current-voltage (I - V) relationships of many nanopores, ClyA included, often deviate significantly from Ohm’s law. This is because the ionic flux arises from a complex interplay between the pore’s geometry (*e.g.*, size, shape and charge distribution), the properties of the surrounding electrolyte (*e.g.*, salt concentration viscosity and relative permittivity) and the externally applied conditions (*e.g.*, bias voltage, temperature and pressure). The ability of a computational model to quantitatively predict the ionic current of a nanopore over a wide range of bias voltages and salt concentrations strongly indicates that it captures the essential physics governing the nanofluidic transport. Hence, to validate our model, we experimentally measured the single channel ionic conductance of ClyA at a wide range of experimentally relevant salt concentrations (c_{s}) and bias voltages (V_{b}). We compared these experimental data with the simulated ionic transport properties in terms of current, conductance, rectification and ion selectivity, of both the classical PNP-NS and the newly developed ePNP-NS equations—finding a quantitative match between the experiment and the simulations.

3.1. Transport of ions through ClyA

3.1.1. Ionic current and conductance. The ability of our model to reproduce the ionic current of a biological nanopore over a wide range of experimentally relevant conditions (between $V_b = -150$ to $+150$ mV and for $c_s = 0.05, 0.15, 0.5, 1, 3$ M NaCl) can be seen in Fig. 2a. Here, we compare IV relationships of ClyA-AS as measured experimentally ('expt.'), simulated using our 2D-axisymmetric model ('PNP-NS' and 'ePNP-NS') and naively analytically estimated ('bulk') using a resistor model of the pore^{122,153} (eqn (S24) and (S25)†). Whereas the classical PNP-NS equations consistently overestimated the ionic current, particularly at high salt concentrations, the predictions of the ePNP-NS equations corresponded closely to the measured values, especially at high ionic strengths ($c_s > 0.5$ M). The inability of the classical PNP-NS equations to correctly estimate the current is expected however, as in this regime the model parameters (*e.g.*, diffusivity, mobility and viscosity, ...) already deviate significantly from their 'infinite dilution' values (see Fig. S1 to S4†). At $c_s < 0.15$ M the ePNP-NS equations tended to minorly overestimate the ionic current, but the discrepancies were much smaller than those observed for PNP-NS. Further, these ionic strengths are not usually tested experimentally. Finally, the bulk model managed to capture the currents surprisingly well at high salt concentrations and positive bias voltages, indicating that, under these conditions, the distribution of ions inside the pore is similar to the bulk electrolyte. In contrast, this simple model faltered in the negative voltage regime, indicating that the resistance of the pore is not only determined by its geometry (*i.e.*, the diameter and length of the 'resistor'), but that it strongly modulated by its electrostatic properties.

The ability of a nanopore to conduct ions can be best expressed by its conductance: $G = I/V_b$. We computed ClyA's conductance with the ePNP-NS equations as a function of bias voltage ($V_b = -200$ to $+200$ mV) and bulk NaCl concentration ($c_s = 0.005$ to 5 M), of which a contour plot can be found in Fig. 2b. The near horizontal contour lines in the upper part of the plot show that, at high ionic strengths ($c_s > 1$ M), ClyA maintains the same conductance regardless of the applied bias voltage. This behavior changes at intermediate concentrations ($0.1 \text{ M} < c_s < 1 \text{ M}$, typical experimental conditions), where maintaining the same conductance level with increasing negative bias amplitudes requires increasing salt concentrations. Finally, at low salt concentrations ($c_s < 0.1$ M), the ionic conductance increases when reducing the negative voltage amplitude but subsequently levels out at positive bias voltages.

The cross-sections of the ionic conductance as a function of concentration at high positive and negative bias voltages (Fig. 2c), serve to demonstrate the differences between these respective regimes. At high positive and negative bias voltages ($V_b = \pm 150$ mV), the slopes of the conductance log-log plots with respect to the bulk salt concentration show linear and bi-linear behavior, respectively. This could be indicative of a different mode of ion conduction of positive and negative bias

voltages, at least for low concentrations ($c_s < 0.15$ M). Even though the bulk model and the PNP-NS equations manage to capture the conductance at respectively high and low ionic strengths, only the ePNP-NS equations perform well over the entire concentration range, particularly at experimentally relevant concentrations (0.1 to 2 M).^{7,8,35,59} Overall, the predictions made using PNP-NS overestimate the conductance over the entire concentration range, but they do converge with those computed with ePNP-NS when approaching infinite dilution ($c_s < 0.01$ M). A more in-depth discussion on the effect of the concentration, wall distance and steric corrections on the ionic conductance can be found in the ESI (Fig. S6a†).

The difference in ionic conduction at opposing bias voltages is also known as ionic current rectification (ICR, α):

$$\alpha(V_b) = G(+V_b)/G(-V_b) \quad (18)$$

with $G(+V_b)$ and $G(-V_b)$ the conductance of the pore at opposing bias voltages of the same magnitude. ICR is a phenomenon often observed in nanopores that are both charged, and contain a degree of geometrical asymmetry along the central axis of the pore.^{21,97,154} As can be seen in Fig. S7,† ClyA exhibits a strong degree of rectification, which is to be expected given its predominantly negatively charged interior and asymmetric *cis* (≈ 6 nm) and *trans* (≈ 3.3 nm) entry diameters (Fig. 1a). Whereas α increases monotonously with the bias voltage magnitude, at least over the investigated range, we found the dependence of α on the ionic strength not to be monotonous, but rather rising rapidly to a peak value at $c_s = 0.15$ M, followed by a gradual decline towards unity at saturating salt concentrations. This concentration is within the transition zone observed in the conductance at negative bias voltages (Fig. 2c) and provides further evidence for a change in the conductive properties of the pore in this regime.

The results and comparisons discussed above indicate that ClyA's conductivity is dominated by the bulk electrolyte conductivity above physiological salt concentrations ($c_s > 0.15$ M). The breakdown of this simple dependency at lower ionic strengths is particularly evident at negative bias voltages and is likely caused by the overlapping of the electrical double layer (EDL) inside the pore (*i.e.*, the Debye length is ≈ 1.4 nm at $c_s = 0.05$ M). This effectively excludes the co-ions (Cl^-) from the interior of the pore and attracts as many counter-ions (Na^+) as needed to screen the fixed charges of the pore. As a result, Cl^- ions do not contribute to ionic current and the conductance is dominated by Na^+ ions attracted by ClyA's 'surface' charges.¹⁵⁵ The presence of only a single ion type inside the pore at low ionic strength may also offer an explanation as to why the ePNP-NS equations are more accurate at higher ionic strengths ($c_s \geq 0.15$ M). Because our ionic mobilities are derived from bulk ionic conductances (*i.e.*, for unconfined ions in a locally electroneutral environment) it is likely that our mobility model begins to break down under conditions where only a single ion type is present.¹⁵⁶ Another cause of the discrepancies could be a slight narrowing of the nanopore at low salt concentrations, which cannot be captured by our simulation due to the static

Fig. 2 Measured and simulated ionic conductance and cation selectivity of single ClyA-AS nanopores. (a) Comparison between the experimentally (expt.) measured, simple resistor pore model (bulk, eqn (S25) $\dagger$) and the simulated (PNP-NS and ePNP-NS) current–voltage (I/V) curves of ClyA-AS at $25 \pm 1^\circ\text{C}$ between $V_b = \pm 200$ mV, and for $c_s = 0.05, 0.15, 0.5, 1$ and 3 M NaCl. The bulk current was calculated by eqn (S24) $\dagger$ by modeling ClyA as two series resistors (eqn (S25) $\dagger$), using the bulk NaCl conductivity at the given concentrations. The grey envelopes represent the experimental errors (standard deviation, $n = 3$). (b) Contour plot of the simulated (ePNP-NS) ionic conductance $G = I/V_b$ as a function of V_b and c_s . (c) log–log plots of G as a function of c_s at $+150$ mV (top) and -150 mV (bottom)—comparing results obtained through experiments, PNP-NS and ePNP-NS simulations, and the simple resistor pore model. The grey envelopes represent the experimental errors (standard deviation, $n = 3$). (d) Contour plot of the Na^+ transport number $t_{\text{Na}^+} = G_{\text{Na}^+}/G$, computed from the individual ionic conductances in the ePNP-NS simulation, as a function of V_b and c_s . The t_{Na^+} expresses the fraction of the ionic current is carried by Na^+ ions (i.e., the cation selectivity). (e) Simulated (PNP-NS and ePNP-NS) values of t_{Na^+} as a function of c_s for $+150$ mV (top) and -150 mV (bottom). Here, the 'bulk' line indicates the bulk NaCl cation transport number, represented by its empirical function $t_{\text{Na}^+}(c_s)$ (see Table 1 and Table S2 $\dagger$). The solid grey line represents $t_{\text{Na}^+} = 0.5$.

nature of its geometry and charge distributions. Nevertheless, our simplified 2D-axisymmetric model, in conjunction with the ePNP-NS equations, is able to accurately predict the ionic current flowing through ClyA for a wide range of experimentally relevant ionic strengths and bias voltages. This suggests that our continuum system can accurately capture the essential physical phenomena that drive the ion and water transport through the nanopore both qualitatively and quantitatively. Hence, we expect the distribution of the resulting properties (e.g., ion concentrations, ion fluxes, electric field and water velocity) to closely correspond to their true values.

3.1.2. Cation selectivity. The ion selectivity of a nanopore determines the preference with which it transports one ion type over the other. Experimentally, it is often determined by placing the pore in a salt gradient (i.e., different salt concentrations in the *cis* and *trans* reservoirs) and measuring the reversal potential (V_r) (i.e., the bias voltage at which the nanopore current is zero).^{7,122} The Goldman-Hodgkin-Katz (GHK) equation can then be used to convert V_r into the permeability ratio $P_{\text{Na}^+} = G_{\text{Na}^+}/G_{\text{Cl}^-}$. Here, we represent the ClyA's ion selectivity (Fig. 2d and e) by the fraction of the total current that is carried by Na^+ ions: the apparent Na^+ transport number

$$t_{\text{Na}^+} = \frac{P_{\text{Na}^+}}{P_{\text{Na}^+} + 1} = \frac{G_{\text{Na}^+}}{G_{\text{Na}^+} + G_{\text{Cl}^-}}, \quad (19)$$

with P_{Na^+} the cation permeability ratio, and G_{Na^+} and G_{Cl^-} the cation and anion contributions to the total conductance. As expected from its negatively charged interior, we found ClyA to be cation selective (i.e., $t_{\text{Na}^+} > 0.5$) for all investigated voltages up to a bulk salt concentration of $c_s \approx 2$ M NaCl (0.5 contour line in Fig. 2d). Above this concentration, t_{Na^+} falls to a minimum of value of 0.45 at $c_s \approx 5$ M, which is still ≈ 1.27 times its bulk electrolyte value of 0.35. This shows that—even at saturating concentrations where the Debye length is < 0.2 nm—ClyA enhances the transport of cations. Below 2 M, the ion selectivity increases logarithmically with decreasing salt concentrations, but it also becomes more sensitive to the direction and magnitude of the electric field: with negative bias voltages yielding higher ion selectivities (Fig. 2e). For example, to reach a selectivity of $t_{\text{Na}^+} \approx 0.9$, the salt concentration must fall to 0.05 M at +150 mV, but only to 0.125 M at −150 mV.

Using the reversal potential method, Franceschini *et al.*⁷ found ClyA's ion selectivity to be $t_{\text{Na}^+} = 0.66$ ($P_{\text{Na}^+} = 1.9$). This corresponds well to the average between the *cis* ($c_s = 1$ M, $t_{\text{Na}^+} = 0.57$, $P_{\text{Na}^+} = 1.3$) and *trans* ($c_s = 0.15$ M, $t_{\text{Na}^+} = 0.84$, $P_{\text{Na}^+} = 5.4$) reservoir concentrations used in their experiment. Therefore, this suggests that although measuring the reversal potential gives valuable insights into the selectivity ion channels and small nanopores, it does not describe the ion selectivity under symmetric conditions. In addition, the GHK equation does not consider the ionic flux due to the electro-osmotic flow and assumes that the Nernst-Einstein relation holds for all used concentrations. These two effects should not be ignored as they contribute significantly to the total conductance of the pore. Furthermore, because the ion selectivity depends

strongly on the ionic strength and often the applied bias voltage, the measured reversal potential will necessarily be influenced by the chosen salt gradient and represents the selectivity at an undetermined intermediate concentration. Tabulated data of the ion selectivity for selected voltages and concentrations can be found in Table S3.†

3.2. Ion concentration distribution

Following the validation of the model in previous section, we now proceed by describing the local ionic concentrations inside ClyA. Detailed knowledge of the ionic environment can be valuable to experimentalists who seek to trap and study single enzymes with ClyA.^{52,127,130} Moreover, it gives insight into the origin of the ion current rectification, ion selectivity and the electro-osmotic flow. Note that the figures below were obtained from a nanoscale continuum steady-state simulation, they represent a time-averaged situation on the order of 10 to 100 ns.¹⁵

3.2.1. Relative cation and anion concentrations. We used the relative ion concentration averaged over the total pore ('PT') volume ($\langle c_i/c_s \rangle_{\text{PT}}$), as a measure for global ionic conditions inside the pore (Fig. 3a). At low ionic strengths ($c_s < 0.05$ M), our simulation predicts a strong enhancement of the Na^+ concentration ($\langle c_{\text{Na}^+}/c_s \rangle_{\text{PT}}$) and a clear depletion of the Cl^- concentration ($\langle c_{\text{Cl}^-}/c_s \rangle_{\text{PT}}$) inside the pore relative to the reservoir concentration, irrespective of the bias voltage. This effect diminishes rapidly with increasing ionic strengths, which can be explained by the electrolytic screening of the negative charges lining the walls of ClyA (i.e., the electrical double layer). At low reservoir concentrations ($c_s < 0.05$ M) the number of ions in the bulk is sparse, leading to the attraction and repulsion of respectively as many Na^+ and Cl^- ions as the chemical potential allows. As the concentration increases, the overall availability of ions improves and the extreme concentration differences between the pore and the bulk are no longer required to offset the fixed charges lining ClyA's interior walls. For example, increasing the reservoir concentration at equilibrium ($V_b = 0$ mV) from 0.005 to 0.05 M causes $\langle c_{\text{Na}^+}/c_s \rangle_{\text{PT}}$ to fall an order of magnitude (34 to 4.4) and $\langle c_{\text{Cl}^-}/c_s \rangle_{\text{PT}}$ to rise an order of magnitude (0.05 to 0.31). Even though at physiological ionic strength ($c_s = 0.15$ M) their concentrations still differ significantly from those in the reservoir ($\langle c_{\text{Na}^+}/c_s \rangle_{\text{PT}} = 2.1$ and $\langle c_{\text{Cl}^-}/c_s \rangle_{\text{PT}} = 0.58$), they do approach bulk-like values ($1.14 \geq \langle c_{\text{Na}^+}/c_s \rangle_{\text{PT}} \geq 1$ and $0.89 \leq \langle c_{\text{Cl}^-}/c_s \rangle_{\text{PT}} \leq 1$) at higher concentrations ($c_s \geq 1$ M).

We also observed a significant difference between their sensitivities to the applied bias voltage, particularly at low salt concentrations (Fig. 3a, left of < 0.15 M line). Whereas the Na^+ concentration shows only a limited response, the Cl^- concentration changes much more dramatically. For example, at $c_s = 0.15$ M and when changing the bias voltage from −150 to +150 mV, $\langle c_{\text{Na}^+}/c_s \rangle_{\text{PT}}$ rises ≈ 1.7 -fold (1.6 to 2.7) and $\langle c_{\text{Cl}^-}/c_s \rangle_{\text{PT}}$ increases ≈ 3.8 -fold (0.28 to 1.06). This difference is clearly visualized by the contour plots of the relative ion concentrations (c_i/c_s) at $c_s = 0.15$ M and for $V_b = -150$ and +150 mV (Fig. 3b). They reveal that the *trans* constriction ($-1.85 < z <$

Fig. 3 Ion concentration distribution inside ClyA-AS. (a) Relative Na^+ and Cl^- concentrations averaged over the entire pore volume ($\langle c_i/c_s \rangle_{\text{PT}}$, eqn (S7)†) as a function of the reservoir salt concentration ($c_s = 0.005$ to 5 M) and bias voltage ($V_b = -200$ to $+200$ mV). (b) Contour plots of the relative ion concentration (c_i/c_s) for both Na^+ and Cl^- for $c_s = 0.15$ M and at $V_b = -150$ and $+150$ mV. (c) The relative Na^+ and Cl^- concentration profiles along the radius of the pore, through the middle of the constriction ($z = -0.3$ nm) and the lumen ($z = 5$ nm), as indicated by the arrows in (b). (d) The average number of ionic charges inside the pore ($\langle Q_{\text{ion}} \rangle_{\text{PT}}$), is distributed between those close to the pore's surface ($\langle Q_{\text{ion}} \rangle_{\text{PS}}$ (i.e., within 0.5 nm of the wall) and those in the 'bulk' of the pore's interior ($\langle Q_{\text{ion}} \rangle_{\text{PB}}$). (e) Cross-section contour plots of the ion space charge density (ρ_{ion}), expressed as number of elementary charges per nm^3 , at $V_b = 0$ mV and for $c_s = 0.05, 0.15, 0.5$ and 5 M. (f) Radial cross-sections of the ρ_{ion} at the center of the constriction ($z = -0.3$ nm) and the lumen ($z = 5$ nm) of ClyA. The vertical line represents the division between ions in the 'bulk' ($d > 0.5$ nm) of the pore and those located near its surface ($d \leq 0.5$ nm).

1.6 nm) remains depleted of Cl^- and enhanced in Na^+ for both $V_b = -150$ mV and $V_b = +150$ mV. This is not the case in the lumen ($1.6 < z < 12.25$ nm), in which the Na^+ concentration is bulk-like for $V_b < 0$ mV and enhanced for $V_b > 0$ mV. Conversely, the number of Cl^- ions becomes more and more depleted in the lumen for increasing negative bias magnitudes, and it is virtually bulk-like at higher positive bias voltages. This is further exemplified by the radial profiles of the ion concentrations (Fig. 3c) through the middle of the constriction (z

$= -0.3$ nm) and the lumen ($z = 5$ nm), which also clearly shows the extent of the electrical double layer.

3.2.2. Ion charge density. The formation of an electrical double layer inside the pore, and the resulting asymmetry in the cation and anion concentrations, gives rise to a net charge density inside the pore (ρ_{ion} , eqn (2)). To investigate the distribution of these charges within ClyA, we divided the total interior volume of the pore into a 'pore surface' (PS) and a 'pore bulk' (PB) region. The PB region encompasses a cylindri-

cal volume at the entry of the pore up until 0.5 nm from the wall ($d \geq 0.5$ nm), the approximate distance from which the wall begins to exert a significant influence on the properties of the electrolyte (Fig. S4†). The PS region includes the remaining volume between the PB domain and the nanopore wall ($d < 0.5$ nm). Integration of ρ_{ion} over the PS and PB regions yields the average number of mobile charges present inside those locations (Fig. 3d): $\langle Q_{\text{ion}} \rangle_{\text{PB}}$ and $\langle Q_{\text{ion}} \rangle_{\text{PS}}$, respectively. Although the total number of mobile charges inside the pore, $\langle Q_{\text{ion}} \rangle_{\text{PT}} = \langle Q_{\text{ion}} \rangle_{\text{PB}} + \langle Q_{\text{ion}} \rangle_{\text{PS}}$, rises appreciatively with increasing reservoir concentrations, the majority of these additional charges are confined to the walls of the pore. Up until a reservoir concentration ≈ 0.15 M, we found $\langle Q_{\text{ion}} \rangle_{\text{PT}}$ to be distributed equally between the surface ($\approx 27 e$) and bulk ($\approx 22 e$) layers. At high salt concentrations ($c_s > 1$ M), the number of charges in the PS region more than doubles (towards $\langle Q_{\text{ion}} \rangle_{\text{PS}} = +58 e$ at 5 M), and those in the PB region diminish (towards $\langle Q_{\text{ion}} \rangle_{\text{PB}} \approx 0 e$ at 5 M). The bias voltage also influences the total number of mobile charges in the pore. As can be seen from our simulation results for three different voltages (Fig. 3d), $\langle Q_{\text{ion}} \rangle_{\text{PT}}$ is approximately +10 to +15 e higher at $V_b = +150$ mV as compared to $V_b = -150$ mV for the full range of ion concentrations. Interestingly, at reservoir concentration > 0.15 M, $\langle Q_{\text{ion}} \rangle_{\text{PB}}$ becomes independent of applied voltage and the changes in $\langle Q_{\text{ion}} \rangle_{\text{PT}}$ can be attributed to $\langle Q_{\text{ion}} \rangle_{\text{PS}}$.

The cross-section contour plots of ρ_{ion} inside ClyA for four different bulk concentrations ($c_s = 0.05, 0.15, 0.5$ and 5 M) reveal the redistribution of the mobile charges with increasing ionic strength in more detail. Up until a bulk concentration of $c_s \leq 0.5$ M, the EDL inside the pore overlaps significantly with itself, as evidenced by the net positive charge density found throughout the interior of the pore (Fig. 3e). Moreover, the absence of Cl^- ions effectively prevents the formation of a negatively charged EDL next to the few positively charged residues lining the pore walls. The situation at high salt concentrations (e.g., 5 M) is very different, with almost no charge density within the PB region of the pore ($d \geq 0.5$ nm), but with pockets of highly charged and alternating positive and negative charge densities close to the nanopore wall (Fig. 3e, right-most panel). This sharp confinement is shown clearly by the radial density profiles (Fig. 3f) drawn through the constriction ($z = -0.3$ nm, purple triangles) and the *lumen* ($z = 5$ nm, green triangles).

It is well known that the activity of an enzyme depends on the composition of the electrolyte that surrounds it.¹⁵⁷ Hence, we expect that the interpretation of kinetic data obtained from enzymes trapped inside the nanopore^{52,130} will benefit from the precise quantification of the ionic conditions inside the pore, including the concentration difference with the reservoir but also the significant imbalance between cations and anions.¹⁵⁸

3.3. Electrostatic potential and energy

The electrostatic potential, or rather the spatial change thereof in the form of an electric field, is one of the primary driving forces within a nanopore. Typically, the potential can be split

into an external, ‘non-equilibrium’ contribution, resulting from the bias voltage applied between the *trans* and the *cis* reservoirs, and an intrinsic, ‘equilibrium’ component, caused by the fixed charge distribution of the pore.⁸ To accurately describe and understand the nanopore transport processes both contributions to the net electric field inside the pore are essential, as their relative magnitudes and directions can significantly influence the transport of ions,^{17,24,73,81} water molecules^{64,159} and biopolymers.^{6,8,13}

3.3.1. A few important charged residues. The interior walls of the ClyA nanopore (Fig. 4a) are riddled with negatively charged amino acids (i.e., aspartate or glutamate), interspaced by a few positively charged residues (i.e., lysine or arginine). When grouping these charges by proximity, we found three clusters with significantly more negative than positive residues: inside the *trans* constriction ($-1.85 < z < 1.6$ nm; E7, E11, K14, E18, D21, D25), in the middle of the *cis lumen* ($4 < z < 6$ nm; E53, E57, D64, K147) and at the top of the pore ($10 < z < 12$ nm; D114, R118, D121, D122). As we shall see, these clusters leave strong negative fingerprints in the global electrostatic potential.

3.3.2. Distribution of the equilibrium electrostatic potential. The electrostatic potential at equilibrium (φ^0 , i.e., at $V_b = 0$ mV) reveals the effect of ClyA’s fixed charges on the potential inside the pore (Fig. 4b). Due to electric screening by the mobile charge carriers in the electrolyte, however, the extent of their influence strongly depends on the bulk ionic strength. The contour plot cross-sections of φ^0 for $c_s = 0.005, 0.05, 0.15, 0.5$ and 5 M (Fig. 4b) and their corresponding radial averages (Fig. 4c) demonstrate this effect aptly. The radial average ($\langle \varphi^0 \rangle_{\text{rad}}$) represents the mean value along the longitudinal axis of the pore and can be computed using

$$\varphi_{\text{rad}} = \frac{1}{\pi R(z)^2} \int_0^{R(z)} \varphi(r, z) 2\pi r dr, \quad (20)$$

where $R(z)$ is taken as the ClyA’s radius inside the pore ($-1.85 \leq z \leq 12.25$ nm), and as fixed values of 4 nm and 2 nm inside the *cis* ($z > 12.25$ nm) and *trans* ($z < -1.85$ nm) reservoirs, respectively. Starting from the *cis* entry ($z \approx 10$ nm), the electrostatic potential is dominated by the acidic residues D114, D121 and D122, resulting in a rapid reduction of $\langle \varphi \rangle_{\text{rad}}^0$ upon entering the pore. Next, $\langle \varphi \rangle_{\text{rad}}^0$ slowly decreases up until the middle of the *lumen* ($z \approx 5$ nm), where the next set of negative residues, namely E53, E57 and D64, lower it even further. After a brief increase, $\langle \varphi \rangle_{\text{rad}}^0$ attains its maximum amplitude inside the *trans* constriction ($z \approx 0$ nm) due to the close proximity of the amino acids E7, E11, E18, D21 and D25, and then quickly falls to 0 inside the *trans* reservoir.

At low ionic strengths ($c_s < 0.05$ M), the lack of sufficient ionic screening results in relatively high negative potentials throughout the entire pore. For example, at low concentrations ($c_s = 0.005$ and 0.05 M), the $\langle \varphi \rangle_{\text{rad}}^0$ inside the constriction ramps up to values of -144 mV ($-5.60 k_B T/e_c$) and -86 mV ($-3.35 k_B T/e_c$), respectively. These values significantly exceed the single ion thermal voltage $k_B T/e_c = 25.7$ mV. Hence, on the

Fig. 4 Equilibrium electrostatic potential inside ClyA-AS. (a) A single subunit of ClyA in which all amino acids with a net charge and whose side chains face the inside of the pore (*i.e.*, those contribute the most to the electrostatic potential) are highlighted. Negatively (Asp + Glu) and positively (Lys + Arg) charged residues are colored in red and blue, respectively. (b) As a result of these fixed charges ClyA exhibits a complex electrostatic potential (φ) landscape at equilibrium (*i.e.*, at $V_b = 0$ mV), and whose values inside the pore we have plotted for several key concentrations ($c_s = 0.005, 0.05, 0.15, 0.5$ and 5 M). Note that even at physiological salt concentrations ($c_s = 0.15$ M), the negative electrostatic potential extends significantly inside the *lumen* ($1.6 < z < 12.25$ nm), and even more so inside the *trans* constriction ($1.85 < z < 1.6$ nm). For the former, localized influential negative 'hotspots' can be found in the middle ($4 < z < 6$ nm) and at the *cis* entry ($10 < z < 12$ nm). (c) Radial average of the equilibrium electrostatic potential along the length of the pore ($\langle\varphi_{\text{rad}}^0\rangle$) for the same concentrations as in (d). Even though the *lumen* of the pore is almost fully screened for $c_s \geq 0.5$ M, the constriction still retains some of its negative influence even at 5 M.

one hand they prohibit anions such as Cl^- from entering the pore, and on the other they attract cations such as Na^+ and trap them inside the pore. For intermediate concentrations ($0.05 \leq c_s < 0.5$ M) the influence of the negative charges becomes increasingly confined to several 'hotspots' near the nanopore walls, most notably at entry of the pore ($10 < z < 12$ nm), in the middle of the *lumen* ($4 < z < 6$ nm), and in the constriction ($-1.85 < z < 1.6$ nm), in accordance with the charge groups discussed in the previous section. Even though the magnitude of $\langle\varphi\rangle_{\text{rad}}^0$ at $c_s = 0.15$ M drops below $1 k_B T/e_c$ inside the *lumen* ($\langle\varphi\rangle_{\text{rad}}^0 \approx -14$ mV), it remains strongly negative inside the constriction ($\langle\varphi\rangle_{\text{rad}}^0 \approx -47$ mV). Finally, at high reservoir concentrations ($c_s \geq 0.5$ M) the potential is close to ≈ 0 mV over the entire *lumen* of the pore, with only a small negative potential remaining inside the constriction. A summary of the most salient $\langle\varphi\rangle_{\text{rad}}^0$ values can be found in Table S4.†

3.3.3. Non-equilibrium electrostatic energy at ± 150 mV. To link back the observed ionic conductance properties to the electrostatic potential, we computed the radially averaged electrostatic energy for a monovalent ion, $\langle U_{E,i} \rangle_{\text{rad}} = z_i e \langle \varphi \rangle_{\text{rad}}$, at $V_b = \pm 150$ mV for the entire range of simulated ionic strengths (Fig. 5a). The resulting plot represents the energy landscape—filled with barriers (hills) or traps (valleys)—that a positive or negative ion must traverse in order to contribute positively to (*i.e.*, increase) the ionic current.

At positive bias voltages, cations traverse the pore from *trans* to *cis* (Fig. 5a, first plot). Upon entering the negatively charged constriction, their electrostatic energy drops dramatically, followed by a relatively flat section with a small barrier for entry in the *lumen* at $z \approx 1.6$ nm. At very low ionic strengths ($c_s < 0.05$ M), the energy at *trans* is significantly lower than the energy of the cation in the *cis* compartment (*e.g.*, $\Delta \langle U_{E,i} \rangle_{\text{rad}} > 2 k_B T$ at 0.005 M), forcing the ions to accumulate inside the pore. At higher concentrations ($c_s > 0.05$ M), the increased screening smooths out the potential drop inside the pore, allowing the cations to migrate unhindered across the entire length of the pore. Anions at $V_b = +150$ mV travel from *cis* to *trans* (Fig. 5a, second plot) and must overcome energy barriers at both sides of the pore. The *cis* barrier prevents anions from entering the pore, but because its magnitude is attenuated strongly with increasing salt concentration (1.7 to $0.5 k_B T$ when increasing the reservoir salt concentration from $c_s = 0.005$ to 0.05 M), it is only relevant at lower ionic strengths ($c_s < 0.05$ M). Once inside the *lumen*, anions can move relatively unencumbered to the *trans* constriction, where they face the second, more significant energy barrier. This prevents them from fully translocating and causes them to accumulate inside the lumen and explains why we observe higher Cl^- concentrations inside the pore at positive bias voltages (Fig. 3b). As with the cations, an increase in the ionic strength significantly reduces these hurdles, resulting in a much smoother landscape for $c_s > 0.15$ M.

Fig. 5 Non-equilibrium electrostatic energy landscape for single ions. (a) Radially averaged non-equilibrium electrostatic energy landscape for single ions, $\langle U_{E,i} \rangle_{\text{rad}} = z_i e \langle \varphi \rangle_{\text{rad}}$, as calculated directly from the radial electrostatic potential at $V_b = +150$ and -150 mV for monovalent cations and anions. The grey arrows indicate the direction in which the ions must travel in order for them to contribute positively to the ionic current. (b) Height of the electrostatic energy barrier ($\Delta E_{B,\text{trans}}$) at the *trans* constriction as a function of the bulk salt concentration. Note that $\Delta E_{B,\text{trans}}$ is much higher for negative voltages and rises logarithmically at lower concentrations. The divergence between $+0$ mV and -0 mV for $c_s < 0.3$ M highlights the difference in barrier height when traversing the pore from *cis* to *trans* or vice versa.

At negative voltages, cations move through the pore from *cis* to *trans*, with a slow and continuous drop of the electrostatic energy throughout the *lumen* of the pore up until the constriction (Fig. 5a, third plot). This results in the efficient removal of cations from the pore *lumen*, and explains the lower Na^+ concentration observed at positive voltages (Fig. 3a). To fully exit from the pore, however, cations must overcome a large energy barrier, which reduces the nanopore's ability to conduct cations compared to positive potentials and hence contributes to the ion current rectification. The situation for anions at negative bias voltages (*i.e.*, travelling from *trans* to *cis*) is very different (Fig. 5a, fourth plot). Their ability to even enter the pore is severely hampered by an energy barrier of a few $k_B T$ at the *trans* constriction. Any anions that do cross this barrier, and those still present in the *lumen* of ClyA, will rapidly move towards the *cis* entry and exit from the pore due to a continuous drop of their electrostatic energy. This effectively depletes the entire *lumen* of anions, which can be observed from the much lower Cl^- concentrations at negative voltages (see Fig. 3a).

3.3.4. Concentration and voltage dependencies of the energy barrier at the constriction. Many biological nanopores contain constrictions that play crucial roles in shaping their ionic conductance properties.^{7,31,50} The reason for this is two-fold, (1) the narrowest part dominates the overall resistance of the pore and (2) confinement of charged residues results in much larger electrostatic energy barriers. With its highly negatively charged *trans* constriction, ClyA's affinity for transport of anions is diminished and that for cations is enhanced compared to bulk, even at high ionic strengths (Fig. 2e).¹²² To further elucidate the significance of the *trans* electrostatic barrier ($\Delta E_{B,i}$), we quantified its height at positive and negative voltages as a function of the salt concentration (Fig. 5b).

Because the application of a bias voltage effectively tilts the energy landscape, it reduces the magnitude of the energy barriers for both positive and negative potentials, as evidenced by the lowering of the curves with increasing bias magnitude (Fig. 5b, light to dark color shading). Likewise, raising the bulk salt concentration results in a continuous decrease of $\Delta E_{B,i}$ due to an increase in the screening of the fixed charges lining the constriction. At moderate to higher reservoir concentrations ($c_s > 0.1$ to 0.5 M, depending on V_b), $\Delta E_{B,i}$ falls below $1 k_B T$ regardless of the bias voltage, and its effect on the ion transport through the pore is significantly reduced.

The ions under the influence of a positive bias voltage (*i.e.*, Na^+ moving from *trans* to *cis* and Cl^- moving from *cis* to *trans*, blue lines in Fig. 5b) experience a $\Delta E_{B,i}$ roughly half that of those under a negative voltage (red lines in Fig. 5b). For example, increasing the salt concentration from 0.005 to 0.15 M, causes $\Delta E_{B,i}$ to drop from 1.8 to $0.73 k_B T$ at $V_b = +150$ mV and from 4.4 to $1.5 k_B T$ at $V_b = -150$ mV. These differences in barrier heights are directly reflected by ClyA's higher degree of ion selectivity at negative compared to positive bias voltages (Fig. 2e).

3.4. Transport of water through ClyA

The charged nature of the inner surface of many nanopores gives rise to a net flux of water through the pore, called the electro-osmotic flow (EOF).^{62,109,160} The EOF not only contributes significantly to the ionic current, but the magnitude of the viscous drag force it exerts on proteins is often of the same order as the Coulombic electrophoretic force (EPF).^{8,161,162} Hence, it strongly influences the capture and translocation of biomolecules including nucleic acids,^{61,72,162} peptides,^{50,163,164} and proteins.^{8,37,49,52,122,127–129} Because the drag exerted by the

EOF depends primarily on the size and shape of the biomolecule of interest and not on its charge,⁸ it can be employed to capture molecules even against the electric field.³⁷ The EOF is a consequence of interaction between the fixed charges on the nanopore walls and mobile charges in the electrolyte and can be described by two closely related mechanisms: (1) the excess transport of the hydration shell water molecules in one direction due to the pore's ion selectivity, and (2) the viscous drag exerted by the unidirectional movement of the electrical double layer inside the pore.^{23,165} The first mechanism likely dominates in pores with a diameter close to that of the

Fig. 6 Concentration and voltage dependency of the electro-osmotic flow inside ClyA-AS. (a) Contour plot of the electro-osmotic flow (EOF) velocity magnitude U at 0.5 M and -100 mV bias voltage. The arrows on the streamlines indicate the direction of the flow. As observed experimentally¹²² and expected from a negatively charged conical nanopore, the EOF follows the direction of the cation (i.e., from *cis* to *trans* under negative bias voltages and vice versa for positive ones). (b) Cross-section profiles of the absolute value of the water velocity $|U_z|$ inside *trans* constriction (at $z = -1$ nm) for various salt concentrations at $V_b = -100$ mV. Notice that at high salt concentrations ($c_s > 1$ M), the velocity profile exhibits two 'lobes' close to the nanopore walls and hence deviates from the parabolic shape observed at lower ionic strengths. (c) Concentration dependency of the electro-osmotic conductance $G_{eo} = Q_{eo}/V_b$, with Q_{eo} the total flow rate through the pore (eqn (25)). In the low concentration regime, G_{eo} increases rapidly between 0.005 and 0.5 M after which it decreases logarithmically for higher concentrations. (d) The rectification of the electro-osmotic conductance ($\alpha_{eo}(V) = G_{eo}(+V)/G_{eo}(-V)$) plotted against the bulk salt concentration. The α_{eo} increases with bias voltage and exhibits an inversion point at $c_s \approx 0.45$ M. (e) Contourmap of the hydrodynamic pressure p at $c_s = 0.15$ M and $V_b = 0$ mV, showing that the large variations in Na^+ concentration along the pore wall result in osmotic pressure 'hotspots' (5 to 30 atm) inside the confined fluid. (f) The axial pressure profile and averaged along the entire radius of the pore at $V_b = 0$ mV.

hydrated ions (≤ 1 nm) such as α HL or FraC,^{50,164} whereas the second is expected to be stronger for larger pores (>1 nm), such as ClyA^{8,37} or most solid-state nanopores.^{62,64} In our simulation, the EOF is generated according to the second mechanism by coupling of the Navier–Stokes and the Poisson–Nernst–Planck equations through a volume force (F_{ion} , eqn (13)). This coupling dictates that the electric field exerts a net force on the fluid if it contains a net ionic charge density—as is the case for the electrical double layer lining the walls of ClyA (Fig. 3d).

3.4.1. Direction, magnitude and distribution of the water velocity. As expected, given ClyA's negatively charged interior surface and the resulting positively charged electrical double layer, the direction of the net water flow inside ClyA follows the electric field: from *cis* to *trans* at negative bias voltages (Fig. 6a). This corresponds to the observations and analysis of single-molecule protein capture¹²² and trapping^{8,127,128} experiments using the ClyA-AS nanopore. Along the longitudinal axis (z) of the pore, the water velocity is governed by the conservation of mass, meaning it is lowest in the wide *cis lumen* and highest in the narrow *trans* constriction (Fig. 6a). For example, at $V_b = -100$ mV and $c_s = 0.5$ M the velocity at the center of the pore is ≈ 0.07 m s⁻¹ in the *lumen* and ≈ 0.21 m s⁻¹ in the constriction.

Along the radial axis (r), u has a parabolic profile with the highest value at the center of the pore and the lowest at the wall due to the no-slip boundary condition (Fig. 6b). Such a parabolic profile contrasts the expected 'plug flow' for an EOF, but follows logically from the overlap of the electrical double layer inside the pore and the resulting uniform volume force, which is analogous to a gravity- or pressure-driven Stokes flow. At concentrations higher than 0.5 M, however, the increasing degree of confinement of the double layer—and its charge—to the nanopore walls (see Fig. 3d, $\langle Q_{\text{ion}} \rangle_s$) results in a flattening of the central maximum and hence a plug flow profile. Interestingly, at very high salt concentrations ($c_s \geq 1$ M) the velocity profile in the constriction exhibits a dimple at the center of the pore (Fig. 6b). This is the result of a self-induced pressure gradient caused by the expansion of the EOF as it exits the pore.¹⁶⁶

3.4.2. Influence of bulk ionic strength and bias voltage on the electro-osmotic conductance. In analogy to the ionic conductance, the amount of water transported by ClyA can be expressed by the electro-osmotic conductance $G_{\text{eo}} = Q_{\text{eo}}/V_b$ (Fig. 6c). Here, Q_{eo} is the net volumetric flow rate of water through the pore and computed by integrating the water velocity across the reservoir boundary (eqn (25)). The strength of the EOF depends strongly and non-monotonically on the bulk ionic strength: G_{eo} rapidly increases with ionic strength until a peak value is reached at $c_s \approx 0.5$ M, followed by a gradual logarithmic decline (Fig. 6c). For example, at $V_b = -150$ mV, G_{eo} first increases from 1.85 nm³ ns⁻¹ V⁻¹ at 0.005 M to 11.3 nm³ ns⁻¹ V⁻¹ at 0.5 M, followed by a gradual decline to 4.00 nm³ ns⁻¹ V⁻¹ at 5 M. As with the ionic conductance, more details on the effect of the concentration, wall distance and steric corrections on the electro-osmotic conductance can be found in the ESI (Fig. S6b†).

The sensitivity of the EOF to the magnitude and sign of the bias voltage is given by the electro-osmotic conductance rectification $\alpha_{\text{eo}}(V) = G_{\text{eo}}(+V)/G_{\text{eo}}(-V)$ (Fig. 6d). For all voltage magnitudes, α_{eo} shows a maximum at $c_s \approx 0.045$ M, after which it falls rapidly to reach unity ($\alpha_{\text{eo}} = 1$) at approximately $c_s \approx 0.45$ M. A minimum is then reached at ≈ 1 M, followed by a gradual approach towards unity at $c_s = 5$ M.

3.4.3. Pressure distribution inside ClyA. The large variations of Na⁺ concentration along the walls of ClyA—up to several orders of magnitude over the course of a few nanometers (see Fig. 3b)—induce regions of high osmotic pressure with peak values up to 30 atm (Fig. 6e). The largest 'hotspots' are located at *cis* entry of the pore ($z = 11$ nm), in the middle of the *lumen* ($z = 4.5$ nm) and inside the entire constriction ($z = -1$ nm) (Fig. 6f), and their influence extends well towards the center of the pore. Up until $c_s \approx 0.5$ M, increasing the reservoir salt concentration does not strongly influence the overall magnitude of the pressure spots. Hence, because such large pressure differences can exert a significant amount of force on particles translocating through nanopores,¹⁶⁷ we expect them to play an important role in the detailed trapping dynamics of proteins inside ClyA.^{8,127}

4. Conclusions

We have developed an extended version of the Poisson–Nernst–Planck–Navier–Stokes (ePNP–NS) equations that is capable of accurately modelling the transport of ions and water through biological nanopores, yielding a wealth of information that is both qualitatively and quantitatively accurate. Our ePNP–NS equations combine many of the improvements to the PNP–NS equations available in literature, in addition to several new corrections. These include the finite size of the ions, self-consistent concentration- and positional-dependent parametrization of the ionic transport coefficients (diffusion coefficient and mobility) and of the electrolyte properties (density, viscosity and relative permittivity).

The use of computationally inexpensive continuum models is pervasive in the solid-state nanopore field, but their application to the structurally more complex biological nanopores has been limited to date. We made use of the radial symmetry of the biological nanopore ClyA to create a 2D-axisymmetric model of the pore which, in conjunction with the ePNP–NS equations, is able to accurately describe the ionic current of ClyA for a wide range of experimentally relevant ionic strengths and bias voltages. Our approach shows that continuum modelling of biological nanopores is not only feasible, but can also be predictive. Our results describe in great detail the properties of ClyA, such as its true ion selectivity, the differences between cation and anion concentrations inside the pore, the distribution and magnitude of the electrostatic potential, the velocity of the electro-osmotic flow and the presence of highly localized 'hotspots' of osmotic pressure.

Finally, the ePNP–NS framework comprises an empirical approach that significantly improves the quantitative accuracy

for continuum simulations of nanoscale transport phenomena. We expect our framework and its principles to be transferable to other biological nanopores and nanoscale transport systems. We believe our model constitutes a powerful and practical tool that can aid with (1) elucidating the link between ionic current observed during a nanopore experiment and the actual physical phenomenon, (2) describing the electrophoretic and electro-osmotic properties of any biological nanopore and (3) guiding the rational design of new variants of existing nanopores. For example, by further expanding and automating our framework, it could be used as a probe to gauge—and tailor—the properties of specific nanopore mutants,^{25,168} or to study the dynamics of translocating proteins by computing the forces that act on model particles.⁸ Both of these applications could play a crucial role in designing the next generation of (biological) nanopore-based biosensors.

5. Materials and methods

5.1. Molecular modelling

5.1.1. ClyA-AS homology model. A full atom model of ClyA-AS¹²² was built and optimized (MODELLER v9.18¹⁶⁹) by introduction of the following point mutations in each of the 12 chains of the wild-type ClyA crystal structure (PDBID: 2WCD¹²³): K8Q, N15S, Q38K, A57G, T67V, C87A, A90V, A95S, L99Q, E103G, K118R, L119I, I124V, T125K, V136T, F166Y, K172R, V185I, K212N, K214R, S217T, T224S, N227A, T244A, E276G, C285S, K290Q. Next, the conformation of all mutated side chains was optimized with a double annealing protocol (heating: 150, 250, 400, 700 and 1000 K, cooling: 1000, 800, 600, 500, 400 and 300 K) where at each temperature the energy was minimized for 200 iterations with a conjugate gradients algorithm (4 fs timestep).¹⁷⁰ The first anneal was performed solely on the mutated residues themselves, and the second run also took the non-bonded interactions with the neighboring atoms into account. The refined nanopore structure was then embedded in the center of an 18 × 18 nm equilibrated DPhPC lipid bilayer patch by manual removal of all overlapping lipids, resulting in 463 lipid molecules. The bilayer was created with the CHARMM-GUI¹⁷¹ membrane builder¹⁷² and equilibrated with NAMD,¹⁷³ as described in detail in ref. 174. The system was then solvated in a box of 18 × 18 × 32 nm by addition of 214640 TIP3 water molecules (VMD's solvate plugin), and the global charge was neutralized by replacing 1276 random water molecules with 674 Na⁺ and 602 Cl[−] ions (VMD's autoionize plugin).¹²⁴

5.1.2. Molecular dynamics simulations. Using molecular dynamics (MD) with NAMD 2.12 (2 fs timestep, CHARMM36 forcefield¹⁷⁵), the final system was minimized for 5 ps, heated from 0 to 298.15 K in 4 ps and equilibrated for 4 ns as NpT ensemble.¹⁷ Finally a 30 ns production run was performed using a NVT ensemble at 298.15 K and the atomic coordinates saved every 5 ps. Note that structural deterioration was prevented by harmonically restraining the protein's Cα atoms to their original positions (spring constant of 695 pN nm^{−1})

during all MD runs.⁷³ A cross-section of the final MD structure, in comparison with the ClyA dodecamer crystal (PDBID: 2WCD¹²³) and cryo-EM (PDBID: 6MRT¹²⁶) structures can be found in Fig. S8.† In addition, an RMSD analysis of the last 10 ns of the molecular dynamics can be found in the ESI (Fig. S9†), where we have compared the per-residue B-factor of the MD trajectory with those given by the 2WCD¹²³ and 6MRT¹²⁶ structures.

5.1.3. Axially symmetric geometry. The 2D-axisymmetric geometry of the ClyA-AS nanopore (Fig. 1c) was derived directly from its full atom model by radially averaging the molecular density. To this end, 50 sets of atomic coordinates were extracted from the final 5 ns of the coordinates of the 30 ns MD production run (*i.e.*, every 100 fs) and aligned by minimizing the RMSD between their backbone atoms (VMD's RMSD tool). Next, we computed and averaged the 3D-dimensional molecular density maps of all 50 structures on a 0.5 Å resolution grid using the Gaussian function¹³⁴

$$\rho_{\text{mol}} = 1 - \prod_i \left[1 - \exp\left(-\frac{d_i^2}{(\sigma R_i)^2}\right) \right], \quad (21)$$

where for each atom i , R_i is its van der Waals radius, $d_i = \sqrt{(x - x_i)^2 + (y - y_i)^2 + (z - z_i)^2}$ is the distance of grid coordinates (x, y, z) from the atom center (x_i, y_i, z_i) and $\sigma = 0.93$ is a width factor. The resulting 3D density map was then radially averaged along the z -axis, relative to the center of the pore to obtain a 2D-axisymmetric density map. The contourline at 25% density was used as the nanopore simulation geometry, after manual removal of overlapping and superfluous vertices to improve the quality of the final computational mesh.

5.1.4. Axially symmetric charge density. The 2D-axially symmetric charge distribution (Fig. 1d) was also derived directly from the 50 sets of aligned nanopore coordinates that were used for the geometry. Inspired by how charges are represented in the particle mesh Ewald (PME) method,¹⁷ we computed the fixed charge distribution of the nanopore $\rho_{\text{pore}}^f(r, z)$ by assuming that an atom i of partial charge δ_i , at the location (x_i, y_i, z_i) in the full 3D atomistic pore model, contributes an amount $\delta_i/(2\pi r_i)$ to the partial charge at a point (r_i, z_i) with $r_i = \sqrt{x_i^2 + y_i^2}$ in the averaged 2D-axisymmetric model. This effectively spreads the charge over all angles to achieve axial symmetry. We assumed a Gaussian distribution of the space charge density of each atom i around its respective 2D-axisymmetric coordinates (r_i, z_i) such that

$$\rho_{\text{pore}}^f(r, z) = \sum_i \frac{e\delta_i}{\pi(\sigma R_i)^2} \exp\left(-\frac{(r - r_i)^2 + (z - z_i)^2}{(\sigma R_i)^2}\right), \quad (22)$$

where R_i is the atom radius, $\sigma = 0.5$ is the sharpness factor and e is the elementary charge. To embed ρ_{pore}^f with sufficient detail, yet efficiently, into a numeric solver, the spatial coordinates were discretized with a grid spacing of 0.005 nm in the domain of ρ_{pore}^f and precomputed values were used during the solver runtime. All partial charges (at pH 7.5) and radii were

taken from the CHARMM36 forcefield¹⁷⁵ and assigned using PROPKA¹⁷⁶ and PBD2PQR.¹⁵² Within COMSOL, the surface charge density $\rho_{\text{pore}}^f(r, z)$ was imported as a 2D linear interpolation function and converted into a pseudo-3D volumetric charge density by normalization over the local circumference of each element (*i.e.*, $2\pi r$). Integration of the resulting charge density over the final mesh yielded a net charge of $-72.9 e$, which is very close to the $-72 e$ of the original atomistic model.

5.1.5. Computing electrophoretic mobilities. To obtain the concentration-dependent ionic mobility μ_i^c from the fitted functions, it must first be derived from the salt's molar conductivity Λ and the ion's transport number t_i before it can be fitted¹¹³

$$\mu_i(c) = \frac{\lambda_i(c)}{z_i \mathcal{F}} \quad \text{with} \quad \lambda_i(c) = \Lambda(c)t_i(c), \quad (23)$$

where $\lambda_i(c)$ is the specific molar conductivity of ion i , and $\mathcal{F}$ the Faraday constant ($96\,485 \text{ C mol}^{-1}$).

5.1.6. Computing the simulated ionic current and electro-osmotic flow rate. The simulated ionic current I_{sim} at steady-state was computed by

$$I_{\text{sim}} = \mathcal{F} \int_S \left(\sum_i z_i \hat{n} \cdot \mathbf{J}_i \right) dS, \quad (24)$$

with z_i the charge number and $\mathbf{J}_i$ the total flux of each ion i across *cis* reservoir boundary S , and $\hat{n}$ the unit vector normal to S . Similarly, the volumetric flow rate (*i.e.*, the volume of water passing through the pore per unit time) is given by

$$Q_{\text{eo}} = \int_S (\hat{n} \cdot \mathbf{u}) dS. \quad (25)$$

5.2. Single-channel nanopore experiments

5.1.1. ClyA expression and purification. ClyA-AS monomers were expressed, purified and oligomerized using methods described in detail elsewhere.^{37,122} Briefly, *E. coli* EXPRESS BL21 (DE3) cells (Lucigen Corporation, Middleton, USA) were transformed with a pT7-SC1 plasmid containing the ClyA-AS gene, followed by overexpression after induction with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG, Carl Roth, Karlsruhe, Germany). The ClyA monomers were purified using Ni^{2+} -NTA affinity chromatography and oligomerized by incubation in 0.2% *n*-dodecyl- β -D-maltopyranoside (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 30 min at 37 °C. Pure ClyA-AS type-I (12-mer) nanopores were obtained using native PAGE on a 4–15% gradient gel (Bio-Rad, Veenendaal, The Netherlands) and subsequent excision of the correct oligomer band.

5.1.2. Recording of single-channel current-voltage curves. Experimental current-voltage curves were measured using single-channel electrophysiology, as detailed elsewhere.^{37,60,122} First, a black lipid bilayer was formed inside a $\approx 100 \mu\text{m}$ diameter aperture in a thin teflon film separating two buffered electrolyte compartments. This was achieved by applying a

droplet of 5% hexane in pentane (Sigma-Aldrich, Zwijndrecht, The Netherlands) over the aperture and leaving it to dry for 1 min at 25 °C. The buffered electrolyte solution was added to both compartments, topped with 10 μL of 6.25 mg mL^{-1} 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids, Alabaster, USA) in pentane. The pentane was left to evaporate for 2 min at 25 °C. A lipid bilayer was formed by lowering and raising the buffer level over the aperture. Minute amounts ($\approx 0.2 \mu\text{L}$) of the purified ClyA-AS type I oligomer were then added to the grounded *cis* reservoir and allowed to insert into the lipid bilayer. Single-channel current-voltage curves were recorded using a custom pulse protocol of the Clampex 10.4 software package connected to AxoPatch 200B patch-clamp amplifier *via* a Digidata 1440A digitizer (all from Molecular Devices, San Jose, USA). Data was acquired at 10 kHz and filtered using a 2 kHz low-pass filter. Measurements at different ionic strengths were performed at $\approx 25 \text{ }^\circ\text{C}$ in aqueous NaCl (Carl Roth, Karlsruhe, Germany) solutions, buffered at pH 7.5 using 10 mM MOPS (Carl Roth, Karlsruhe, Germany).

Conflicts of interest

There are no conflicts to declare.

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