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CALCIUM ION BINDING STUDY IN HUMAN Cx46
CONNEXON

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DEDICATORY

I dedicate this doctoral thesis to my fiancé Esteban Silva, who not only accompanied and supported me throughout this long and arduous process but also served as a constant source of encouragement and motivation. His unwavering belief in my abilities and his unconditional love were the driving forces behind the completion of this journey. I am profoundly grateful for his presence and dedication, without which this achievement would not have been possible.

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Resumen

Los iones de calcio extracelular (Ca^{2+}) son un regulador crítico de los canales de conexina desacoplados (conexones) humanos; sin embargo, los mecanismos moleculares precisos que gobiernan esta interacción aún no se comprenden a cabalidad. En esta tesis, se investigan las dinámicas de unión de Ca^{2+} y la modulación funcional del conexón de Conexina 46 humana (C-hCx46) utilizando extensas simulaciones de dinámica molecular (MD). A partir de una estructura molde ovina de alta resolución, se construyó un modelo 3D totalmente atomístico de C-hCx46. Nuestras simulaciones revelan un modo de interacción de Ca^{2+} novedoso y altamente dinámico, caracterizado por una región de confinamiento toroidal delimitada por residuos cargados negativamente y dispuestos simétricamente (E42, D43, D47 y E48). A diferencia de C-hCx26 —que emplea una trampa electrostática rígida debido en gran parte a la sustitución E42K—, C-hCx46 acomoda múltiples iones de Ca^{2+} que transitan libremente entre subzonas.

Los análisis termodinámicos y estructurales, que incluyen el uso de modelos de iones multi-sitio (CAL y CAM) y “sistemas de juguete” (toy systems) reducidos, demuestran que esta carga dinámica depende sinérgicamente tanto de la simetría de carga local como de la arquitectura estéricamente restringida del poro intacto. Este volumen de unión expandido proporciona una significativa ganancia entrópica, aumentando enormemente los microestados accesibles y explicando la mayor capacidad de carga y afinidad de C-hCx46. Funcionalmente, la unión de Ca^{2+} no ocluye completamente el canal, sino que actúa como un modulador cinético. Disminuye parcialmente la permeación de K^+ (hasta un 42%) mediante el apantallamiento electrostático del potencial negativo nativo del poro, e induce una profunda reducción de más de dos veces en la permeabilidad del agua (aproximadamente un 57% de caída). Esta severa restricción en el flujo hídrico no se debe simplemente a un menor co-transporte iónico, sino que es impulsada por un mecanismo sinérgico: la alta energía libre electrostática de solvación de Born del Ca^{2+} estructura fuertemente el agua para formar un “tapón de hidratación”, mientras que el rápido movimiento transversal de estos iones dentro de la zona de confinamiento crea una barrera cinética mediante un efecto de “barrido dinámico” que interrumpe continuamente las redes de puentes de hidrógeno del agua.

En última instancia, este trabajo establece un nuevo paradigma para la regulación por cationes divalentes en conexones —pasando de sitios de

unión rígidos a un confinamiento dinámico impulsado por entropía— y proporciona un marco biofísico integral para continuar explorando la plasticidad estructural y la selectividad de la familia de las conexinas humanas.

Abstract

Extracellular calcium ions (Ca^{2+}) are a critical regulator of human uncoupled connexin channels (connexons), yet the precise molecular mechanisms governing this interaction remain poorly understood. In this thesis, we investigate the Ca^{2+} binding dynamics and functional modulation of the human Connexin 46 connexon (C-hCx46) utilizing extensive molecular dynamics (MD) simulations. Leveraging a high-resolution ovine template, we constructed a fully atomistic 3D model of C-hCx46. Our simulations reveal a novel, highly dynamic Ca^{2+} interaction mode characterized by a toroidal confinement region outlined by symmetrically arranged, negatively charged residues (E42, D43, D47, and E48). In contrast to the closely related C-hCx26—which employs a rigid electrostatic trap largely due to an E42K substitution—C-hCx46 accommodates multiple Ca^{2+} ions that freely transit between subzones.

Thermodynamic and structural analyses, including the use of multi-site ion models (CAL and CAM) and reduced “toy systems”, demonstrate that this dynamic loading relies synergistically on both the local charge symmetry and the sterically restricted architecture of the intact pore. This expanded binding volume provides a significant entropic gain, vastly increasing the accessible microstates and explaining the higher loading capacity and affinity of C-hCx46. Functionally, Ca^{2+} binding does not fully occlude the channel but acts as a kinetic modulator. It partially decreases K^+ permeation (up to 42%) via electrostatic screening of the pore’s native negative potential, while inducing a profound, more than two-fold reduction in water permeation (an approximate 57% decrease). This severe restriction in water flux is not merely due to reduced ion co-transport, but is driven by a synergistic mechanism: the deep Born electrostatic free energy of solvation of Ca^{2+} highly structures surrounding water molecules to form a “hydration plug”, while the rapid transversal movement of these ions within the confinement zone creates a kinetic barrier via a dynamic “sweeping” effect that continuously disrupts water hydrogen-bond networks.

Ultimately, this work establishes a new paradigm for divalent cation regulation in connexons—shifting from rigid binding sites to entropically driven dynamic confinement—and provides a comprehensive biophysical framework to further explore the structural plasticity and selectivity of the human connexin family.

Publication

The work carried out for this thesis was involved directly in the production of the following publication:

- **Stepke** et al. (2026). A Symmetric region Produces a Novel Calcium Binding Mode in the Human Cx46 Connexon. *Protein Science*, PRO-25-0382.R1 [http:DOI](http://DOI)

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Chapter 1

Introduction

“I believe that the more diversified the descendants from any one species become in structure, constitution, and habits, by so much will they be better enabled to seize on many and widely diversified places in the polity of nature”

Charles Darwin
On the Origin of Species

1.1 The connexon formed by human Connexin 46

This work focuses on the study of connexons formed by human connexin 46 (C-hCx46) and their interaction with calcium ions (Ca^{2+}), addressing the dynamics of this interaction, as well as some effects resulting from it. Therefore, each of the concepts necessary to understand the approach, methodology, and findings of this work will be explained below.

Connexins: Building Blocks of the Puzzle

Generally, connexin genes are organized in a consistent way. They are composed of two exons with a variable-length intron in between (Willecke et al., 2002). Analysis of these gene sequences across the chordate phylum shows that connexins are exclusive to this group and demonstrate a significant degree of evolutionary stability, meaning they have remained largely unchanged (Cruciani and Mikalsen, 2007). While vertebrates have connexins, invertebrates use functionally analogous proteins called innexins, with a markedly different amino acid sequence (Phelan, 2005). Connexins are differentially expressed, displaying spatial and temporal specificity modulated by a variety of transcription factors, such as Sp transcription factors (Sp1 and Sp3), some members of the Jak / STAT pathway, and activator protein 1 (AP-1) (Miyoshi et al., 2001). In addition, specific cells connexin expression are regulated by Nkx2, HNF-1, Mist1, and NFkB (Oyamada et al., 2005). Furthermore, connexin expression is regulated by epigenetic mechanisms, including histone modification, DNA methylation, and miRNA activity (Vinken et al., 2009). The connexins topology (See Fig. 1.1) involves intracellular carboxy-terminal and amino-terminal domains, as well as four trans-membrane segments connected with two extracellular and one intracellular loop (Saez and Leybaert, 2014). There are 21 human connexins (hCxs) with a wide expression in virtually all human tissues (Oyamada et al., 2005). Connexins are named based on their predicted molecular weight, and one of these being human connexin 46 (hCx46), predicted to have a molecular weight of 46 kDa, who is predominantly expressed in mature fiber cells (Beyer and Berthoud, 2014, Berthoud and Beyer, 2009, Puk et al., 2008), and have been associated to lens malfunction and cataract (Beyer and Berthoud, 2014, Retamal et al., 2015), and

will be studied here.

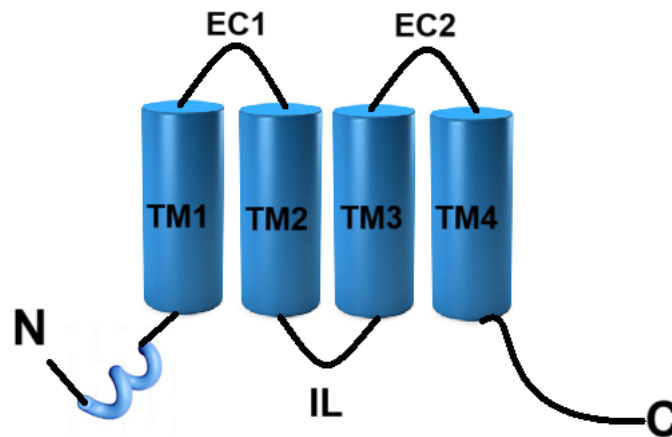


Figure 1.1: connexins topology. Transmembrane regions (TM1, TM2, TM3, TM4) are represented with blue cylinders, Extracellular loops (EL1 and EL2), Intracellular Loop (IL), amino-terminal (N) and carboxy-terminal (C) domains are represented with black lines, a relevant alpha helix of N is represented by a blue spiral

Connexon: when the name matters

Connexons are formed of six connexin proteins (Saez and Leybaert, 2014), which assemble with either identical connexin subunits (homomeric) or different connexins (heteromeric). Initially, the most studied structure was the gap junction (Kumar and Gilula, 1986), which consists of two connexons anchored to each other and allowing intercellular communication (See Fig. 1.2). However, over time, connexons were found in the cell membrane that were not anchored to any other connexon. Because of this, they were initially called "hemichannels," as they were considered half of a gap junction. As time went on, it was reported that these hemichannels are completely functional on their own, and their function, associated with communication with the extracellular space, is just as important as the function of gap junctions (Goodenough and Paul, 2003). This is why, although "hemichannel" is still a widely used name in the literature, I prefer to call them connexons, and it is probably only a matter of time before this name completely replaces "hemichannel".

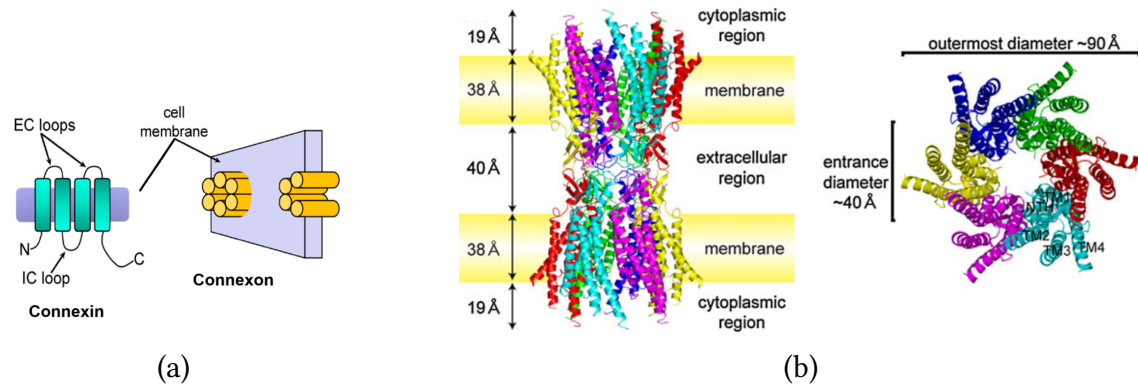


Figure 1.2: Connexon: the functional channel formed by connexins. (A) connexin hexamerization into a connexon (Modified from Fiori et al., 2017) (B) two connexons coupled forming a gap junction, the sizes are useful to study uncoupled connexons too (Modified from Maeda and Tsukihara, 2011)

Although neither the voltage sensor domain nor the gating mechanism of connexon has been determined, they can be described with a three-state electrophysiological model (See Fig. 1.3), involving two gates (González et al., 2007): The first gate is an extracellular one, described as a “slow gating” (Bargiello et al., 2018), controlling the transition between the closed configuration at hyperpolarizing potential and the full-open configuration with a maximum conductance close to zero millivolt (González et al., 2007). The second one is an intracellular “fast gating” (Bargiello et al., 2018), controlling the transition between the full-open configuration and the semiconductive configuration, decreasing the conductance from its maximum to approximately a quarter of its peak value. The voltage range involved in this process depends on each of the hCxs forming the connexon, but it’s generally greater than zero millivolts (González et al., 2007)

Connexon aqueous pores enable the bidirectional flux of small molecules and water between the intracellular and extracellular environments. When open, the connexon pore provides a continuous conduit through the membrane, whose permeability and selectivity are modulated by structural and electrochemical factors.

In the case of **Cx46**, the pore architecture allows osmotic water movement across the membrane. The hydrophilic nature of the pore, along with its substantial inner diameter, facilitates passive water flow driven by osmotic gradients (Beahm and Hall, 2002; Retamal and Altenberg, 2022).

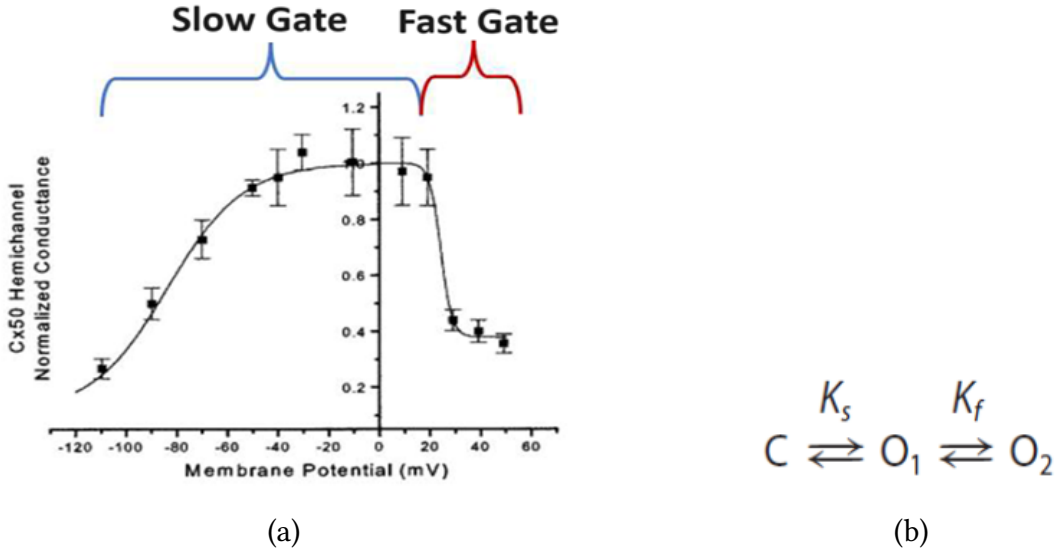


Figure 1.3: Connexon Electrophysiology. (A) Example graph of normalized conductance Vs Membrane potential in an connexon (Modified from Beahm and Hall, 2002) (B) three state model scheme for C-Cx: closed state (C), a full-open state (O_1) and a semiconductive state (O_2).

Structural modeling of hCx46 reveal a funnel-shaped geometry in the pore, with a central constriction that may serve as a selective gate (Jagielnicki et al., 2024). This region, rich in polar residues, is thought to coordinate water molecules and act as a bottleneck that modulates permeability depending on physiological context. Under pathological or mutational changes, altered pore dynamics can lead to excessive water influx, cell swelling, and compromised lens function (Woo et al., 2021).

1.2 C-hCx46 modulation by Ca^{2+}

Calcium ions (Ca^{2+}) are among the most versatile and universal regulators in biological systems. They act as second messengers in a wide variety of cellular processes, including muscle contraction, exocytosis, synaptic plasticity, and the modulation of ion channels. Their ability to either activate or inhibit protein targets depends on spatial and temporal concentration gradients, as well as the identity and structural features of their binding partners (Zhou et al., 2016). In many systems, Ca^{2+} modulates ion channel activity either by promoting channel opening or by blocking conduction via direct interactions or electrostatic effects.

Connexons represent an important model of Ca^{2+} regulation. These channels enable the diffusion of ions and small molecules either to the extracellular space or between adjacent cells when forming gap junctions. Multiple studies have demonstrated that extracellular Ca^{2+} can inhibit connexon conductance, suggesting a functional block that does not require large conformational rearrangements. Functionally, the connexons are defined as large-diameter channels for ions and small molecules, with exclusion limits on the order of 1 kDa mass or about 1.2 nm diameter, these small molecules includes ATP, amino acids, reduced glutathione, NAD^+ , IP_3 , prostaglandin E_2 and cyclic nucleotides (Cherian et al., 2005, Stridh et al., 2008, Kang et al., 2008, Gossman and Zhao, 2008, Jiang et al., 2011, Valiunas, 2013 and Bruzzone et al., 2001). Given the large conductance and modest selectivity of the aqueous pore of connexon (Saez and Leybaert, 2014), its opening must be highly regulated to maintain cell viability.

C-hCx46 is a cation-selective channel that shows high voltage sensitivity and is strongly regulated by divalent cations, such as calcium (Pinto et al., 2017; Trexler et al., 2000). The regulation of C-hCx46 by extracellular calcium ion binding in particular has been reported (Pinto et al., 2017), the IC_{50} for Ca^{2+} reported was 0.17mM, and a hill coefficient of 2 suggest cooperativity between individual interaction sites, also this publication suggest that the stabilization of C-hCx46 in its closed configuration is the probable molecular mechanism of regulation, likely involving a reorganization of specific interactions within the connexon that lead to a closed channel (Lopez et al., 2016). To date, calcium ion binding sites have not been reported for C-hCx46, and the specific effects of calcium ion binding are still unclear. This work aims to close that knowledge gap by using molecular dynamics simulations to study the behavior of calcium ions with the extracellular zone of the connexon.

Connexin 26 (Cx26) has been extensively studied through high-resolution structural approaches, providing insight into its calcium-dependent regulation. The crystal structure **PDB 5ER7** (Bennett et al., 2016), resolved at 3.6 Å by X-ray diffraction under calcium-bound conditions, represents the gap junctional form of Cx26 with extracellular Ca^{2+} present. In contrast, the cryo-EM structure **PDB 7QER** (Brotherton et al., 2022), resolved at 3.4 Å, was obtained under calcium-free conditions. Surprisingly, both structures show nearly identical global conformations, ruling out pore occlusion or large-scale closure as the

mechanism of inhibition. Instead, in the Ca^{2+} -bound structure (5ER7), calcium coordination sites were identified at the subunit interfaces near the extracellular vestibule. These sites, primarily formed by negatively charged residues such as Asp50, Glu42, Glu47, and Glu49, enable Ca^{2+} chelation. Computational electrostatic analyses demonstrated that Ca^{2+} binding generates a local positive electrostatic barrier that inhibits cation permeation without physical pore occlusion (Bennett et al., 2016).

Although the calcium-free structure (7QER) does not explicitly represent an “open” state, it serves as a reference for the unblocked configuration, reinforcing the idea that calcium does not induce a large structural transition, but may be modulates conduction electrostatically (García et al., 2019).

In the case of Connexin 46 (Cx46), no crystal structure is yet available. Nonetheless, electrophysiological and mutagenesis studies suggest a regulatory mechanism similar to that of Cx26, in fact, site-directed mutations at these positions result in reduced calcium sensitivity, supporting the hypothesis of a conserved electrostatic slow gating mechanism among connexins (Pfahnl and Dahl, 1999; Verselis et al., 2011).



Figure 1.4: Sequence alignment of Ca^{2+} binding zone. From top to bottom are the sequences of the hCx46 and hCx26. The black selection shows the amino acids deemed as possible interaction sites with divalent cations based in detected coordinate for Ca^{2+} of hCx26 crystal (PDB 5ER7)

When comparing the sequence of the interaction region reported in the crystal structure of hCx26 with that of hCx46 (See Fig. 1.4), we can observe that it is highly conserved, with the most notable difference being the substitution of E42 with K. As a bit of a spoiler—and as will be shown later—this region is highly conserved across the entire human connexin family. It is particularly interesting to note that the E42K mutation introduces a change in local electrostatic properties, as it replaces an acidic polar amino acid with a basic one. This leads to the central question of this

study: does this difference affect the dynamics of interaction with Ca^{2+} , and how significant is the impact of this substitution?

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Chapter 2

Methodology

“The old that is strong does not wither,
Deep roots are not reached by the frost.”

J.R.R. Tolkien
The Fellowship of the Ring

2.1 Building Models of protein systems

The generation of three-dimensional protein models is primarily addressed through two computational strategies: homology modeling and artificial intelligence-based prediction. Homology modeling, exemplified by the Modeller software (Webb and Sali, 2017), leverages the known structures of related proteins to build a model of the target protein, based on sequence similarity. The process begins with the alignment of the protein sequence of interest to known 3D structures, referred to as templates. From this alignment, the program calculates spatial restraints, such as distances and angles, based on statistical probabilities derived from extensive databases of protein alignments. These restraints, combined with energy functions that ensure the fidelity of protein chemistry, constitute an objective function. The optimization of this function, using sophisticated algorithms, results in the generation of the final 3D model. A crucial component in the evaluation and selection of models generated by MODELLER is the DOPE (Discrete Optimized Protein Energy) scoring function. DOPE employs a statistical potential derived from known protein structures to assess the probability of a model being correct, by analyzing atomic interactions and distances present in the structure. In this way, DOPE allows for the discernment of the highest quality and most reliable models. Furthermore, MODELLER includes functions to refine loop regions, which further improves the accuracy of the final model

In contrast, AI-based prediction, with AlphaFold (Jumper et al., 2021a) as its most prominent exponent, employs deep neural networks to predict the structure from numerous small fragments of sequences from various proteins.

The human connexin C-hCx46 has been the subject of computational modeling to understand its three-dimensional structure. Previous models of C-hCx46 relied on hCx26 as a template, despite a not particularly high sequence identity of 49.12%. However, the recent publication of the sheep connexin 46 crystal structure, which exhibits a significantly higher sequence identity (70.59% in the whole sequence and up to 94.87% in the extracellular domains), offers an opportunity to generate more accurate models. Although the artificial intelligence tool AlphaFold was considered due to its recognized capabilities, it was opted not to use it in this study. This decision was based on two main factors: the high quality of the new

experimental template, which allows for convergence of models with traditional methods, and AlphaFold's current limitations in differentiating between distinct conformational states of the protein. This work focuses on the construction of a refined model of C-hCx46, leveraging the structural information of the sheep template and exploring comparative modeling methodologies to accurately represent its three-dimensional conformation.

2.2 Computer simulations of biological systems

This thesis presents a comprehensive study conducted exclusively through molecular dynamics (MD) simulations. Key aspects of this research include the construction of a human Cx46 model, given the absence of an crystal structure, the establishment of initial simulation conditions informed by bioinformatics analyses, and the execution of simulations using NAMD with the charmm36m force field.

Molecular Dynamics

Molecular dynamics (MD) is a computational simulation method used to study the physical movements of atoms and molecules. By solving Newton's equations of motion and employing force fields to model interatomic interactions, MD provides insights into the time-dependent behavior of molecular systems, revealing details about their structure and dynamic properties (Karplus and Petsko, 1990). In MD, the Hamiltonian plays a fundamental role by defining the total energy of the system (Jolicard, 1995). This operator, which is the sum of the kinetic and potential energies of all particles, guides the temporal evolution of the system through Hamilton's equations. The conservation of the Hamiltonian during the simulation is a crucial indicator of the accuracy and stability of the calculation, ensuring that the system behaves according to the principles of classical mechanics. Deviations from Hamiltonian conservation can signal errors in the simulation setup, the choice of force field, or the time integration algorithm. The Hamiltonian is decomposed into kinetic energy, which reflects atomic motion and relates to temperature, and potential energy, which describes interatomic interactions and determines the structure. Since all atoms have

a fixed charge and mass, the energy of the system is defined by

$$\mathcal{H}(\mathbf{p}, \mathbf{q}) = \mathcal{K}(\mathbf{p}) + \mathcal{V}(\mathbf{q}) \quad (2.1)$$

where $\mathbf{p}$ is the momentum vector of the particles in the system, and $\mathbf{q}$ is the position vector of the system. For a 3D system containing N particles, the momentum ($\mathbf{p}$) and position ($\mathbf{q}$) are represented by vectors with $3N$ elements. Given that potential energy is solely a function of particle positions and kinetic energy depends only on velocities, we can compute their respective partial derivatives.

$$\frac{\partial \mathcal{H}}{\partial \mathbf{q}} = -\frac{d\mathbf{p}}{dt} = -\mathbf{m} \frac{d\mathbf{v}}{dt} = -\mathbf{m} \frac{d^2 \mathbf{r}}{dt^2} \quad (2.2)$$

$$\frac{\partial \mathcal{H}}{\partial \mathbf{p}} = \frac{d\mathbf{q}}{dt} \quad (2.3)$$

Eq. (2.2) provides forces, and Eq. (2.3) velocities. Numerical integration of these yields particle positions and velocities at $t + \delta t$. Leapfrog and (Velocity) Verlet are common integration schemes.

Potential Energy Decomposition

The potential energy component, $\mathcal{V}$, is divided into bonded ($\mathcal{V}^b$) and non-bonded ($\mathcal{V}^{nb}$) interactions (Su et al., 2012), each with specific sub-components. Bonded interactions, describing covalent connections, are typically modeled using harmonic potentials:

$$\mathcal{V}^b = \mathcal{V}^{bond} + \mathcal{V}^{angle} + \mathcal{V}^{dih} + \mathcal{V}^{improp} \quad (2.4)$$

where $\mathcal{V}^{bond}$ represents bond stretching, $\mathcal{V}^{angle}$ bond angle bending, $\mathcal{V}^{dih}$ dihedral angle torsion, and $\mathcal{V}^{improp}$ improper dihedral angles, ensuring planarity.

Non-bonded interactions, encompassing longer-range forces, are composed of Lennard-Jones ($\mathcal{V}^{LJ}$) and Coulombic ($\mathcal{V}^E$) potentials:

$$\mathcal{V}^{nb}(\mathbf{q}) = \mathcal{V}^{LJ}(\mathbf{q}) + \mathcal{V}^E(\mathbf{q}) \quad (2.5)$$

The Lennard-Jones potential, modeling van der Waals interactions, is short-ranged and often truncated for computational efficiency, typically represented by:

$$\mathcal{V}^{LJ} = 4\epsilon \left(\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^6 \right) \quad (2.6)$$

where σ is the distance at which the potential is zero, and ε is the depth of the potential well.

The Coulomb potential, describing electrostatic interactions, is long-ranged and computationally demanding. For a system of N particles:

$$\mathcal{V}^E = \sum_{i=0}^N \sum_{j \neq i}^N \frac{1}{4\pi\epsilon_0} \cdot \frac{q_i q_j}{r_{ij}} \quad (2.7)$$

where q represents the particle charge, r_{ij} the inter-particle distance, and ϵ_0 the vacuum permittivity. Due to its long-range nature, direct computation of the Coulomb potential is inefficient for large systems, necessitating approximation methods like Particle Mesh Ewald.

Efficient Electrostatics: Periodic Boundaries and Particle-Mesh Ewald

Calculating electrostatic interactions via the Coulomb formula, Eq. (2.7), scales as $\mathcal{O}(N^2)$, rendering it impractical for large systems. To address this, Particle-Mesh Ewald (PME), an accelerated Ewald summation method, is employed, leveraging periodic boundary conditions (PBC).

Periodic Boundary Conditions (PBC)

PBC simulate an infinite system by replicating a simulation box in all directions (White et al., 2008). When a particle exits one side, it re-enters the opposite side, effectively eliminating boundary effects. Mathematically, for the i -th coordinate:

$$x_i(t + \delta t) = x_i(t) + L_{x_i} \quad x_i(t) < L_{x_i}/2 \quad (2.8)$$

$$x_i(t + \delta t) = x_i(t) - L_{x_i} \quad x_i(t) > L_{x_i}/2 \quad (2.9)$$

where L_{x_i} is the box dimension along that axis.

Ewald Summation

Ewald summation divides the Coulomb potential into short-range (real space) and long-range (reciprocal space) components (Deserno and Holm,

1998). For a cubic system with PBC:

$$E = \frac{1}{2} \sum_{i,j=1}^N \sum'_{\mathbf{n} \in \mathbb{Z}^3} \frac{q_i q_j}{|\mathbf{r}_{ij} + \mathbf{n}L|} \quad (2.10)$$

where $\mathbf{n}L$ accounts for periodic images. By splitting the $1/r$ term, the Ewald formula becomes:

$$E = E^{(r)} + E^{(k)} + E^{(s)} + E^{(d)} \quad (2.11)$$

with terms representing real space, reciprocal space, self-energy, and dipole corrections:

$$E^{(r)} = \frac{1}{2} \sum_{i,j}^N \sum'_{\mathbf{m} \in \mathbb{Z}^3} q_i q_j \frac{\text{erfc}(\alpha|\mathbf{r}_{ij} + \mathbf{m}L|)}{|\mathbf{r}_{ij} + \mathbf{m}L|} \quad (2.12)$$

$$E^{(k)} = \frac{1}{2L^3} \sum_{\mathbf{k} \neq 0} \frac{4\pi}{k^2} e^{-k^2/4\alpha^2} |\tilde{\rho}(\mathbf{k})|^2 \quad (2.13)$$

$$E^{(s)} = -\frac{\alpha}{\sqrt{\pi}} \sum_i^N q_i^2 \quad (2.14)$$

$$E^{(d)} = \frac{2\pi}{(1 + 2\epsilon')L^3} \left(\sum_i^N q_i \mathbf{r}_i \right)^2 \quad (2.15)$$

where $\tilde{\rho}(\mathbf{k}) = \sum_{j=1}^N q_j e^{-i\mathbf{k} \cdot \mathbf{r}_j}$ is the Fourier transformed charge density. Despite improvements, Ewald summation still scales as $\mathcal{O}(N^{3/2})$.

Particle-Mesh Ewald (PME)

Particle-Mesh Ewald is a more efficient variant of the Ewald Summation technique. It leverages the Fast Fourier Transform (FFT) to accelerate calculations. This acceleration is made possible by transferring particle charges onto a grid using an 'assignment function', W_p^P . This function dictates how the charge of each particle is distributed across the grid's nodes. In a simplified one-dimensional case, this translates to spreading a charge's influence across neighboring grid points.

$$W_p^{(P)} = W^{(P)}(x - x_p) \quad (2.16)$$

where function W is usually set up to be a spline. This formulation requires that the charge density (ρ) be redefined in terms of the mesh points, which is simply

$$\rho_M(x) = \frac{1}{h} \int_0^L W^{(P)}(x - x_p) \rho(x) dx \quad (2.17)$$

for a grid of step size h and total length L . An example for the charge distribution onto a 1-dimensional mesh with 3 points is given in Appendix E in Deserno and Holm, 1998.

The charges, now distributed on the mesh points, allow us to compute the reciprocal space contribution of the Ewald sum using Fast Fourier Transforms (FFT). The main advantage of this approach lies in the reduction of computational complexity from $\mathcal{O}(N^2)$ to $\mathcal{O}(N \log N)$. This is achieved by expressing the reciprocal space energy as a convolution that can be efficiently computed using FFTs.

The reciprocal space energy in PME is Eq. (2.13) with our modified charge distribution onto a mesh

$$E^{(k)} = \frac{1}{2L^3} \sum_{\mathbf{k} \neq 0} \frac{4\pi}{k^2} e^{-k^2/4\alpha^2} |\tilde{\rho}_M(\mathbf{k})|^2 \quad (2.18)$$

where $\tilde{\rho}_M(\mathbf{k})$ is the Fourier transform of the mesh charge density. The computation can then be summarized in the following three steps:

- 1) Forward transform of the mesh charges:

$$\tilde{\rho}_M(\mathbf{k}) = \text{FFT}[\rho_M(\mathbf{r})] \quad (2.19)$$

- 2) Multiplication in Fourier space:

$$\tilde{\phi}(\mathbf{k}) = \tilde{\rho}_M(\mathbf{k}) \frac{4\pi}{k^2} e^{-k^2/4\alpha^2} \quad (2.20)$$

- 3) Inverse transform to obtain the potential:

$$\phi_M(\mathbf{r}) = \text{FFT}^{-1}[\tilde{\phi}(\mathbf{k})] \quad (2.21)$$

The forces on the particles are then computed by interpolating the mesh-based potential gradient back to the particle positions using the same assignment function $W^{(P)}$. This ensures that the forces are continuous as particles move through the system, which is essential for energy conservation in molecular dynamics simulations.

Calculation of Average Electrostatic Potential Maps

To perform this type of analysis, it is highly recommended to create a new system with the following considerations: utilizing the lowest possible ionic concentration to minimize noise from moving ions, which can disrupt potential calculations. Additionally, simulating the molecule with constraints, especially restricting the backbone in proteins, reduces perturbations and ensures more accurate potential assessments by maintaining structural stability. To obtain a distribution of the electrostatic potential in our simulations (Aksimentiev and Schulten, 2005), we evaluate long-range electrostatic forces via PME, every point charge is approximated by a spherical Gaussian normalized to give the original charge upon integration.

$$\rho(\mathbf{r}_i) = q_i \left(\frac{\beta}{\sqrt{\pi}} \right)^3 e^{-\beta^2 |\mathbf{r} - \mathbf{r}_i|^2} \quad (2.22)$$

The positive parameter β determines the width of the Gaussian. The electrostatic potential is obtained by solving the Poisson equation

$$\nabla^2 \phi(\mathbf{r}) = -4\pi \sum \rho_i(\mathbf{r}) \quad (2.23)$$

To determine the mean electrostatic potential, instantaneous electrostatic potentials $\phi(\mathbf{r})$ were averaged over the entire MD trajectory

Calculation of permeation events

The system was spatially segmented along the z -axis into five distinct regions. The lower and upper bulk regions correspond to coordinates $z < Z_{\text{inf}}$ and $z > Z_{\text{sup}}$, respectively. Between these boundaries, two cylindrical zones were defined at the entry and exit of the pore, each of height δ and radius r , spanning the intervals $Z_{\text{inf}} < z < Z_{\text{inf}} + \delta$ (lower cylinder) and $Z_{\text{sup}} - \delta < z < Z_{\text{sup}}$ (upper cylinder). The intermediate region of the pore was defined by $Z_{\text{inf}} + \delta < z < Z_{\text{sup}} - \delta$.

A complete permeation event was identified when an atom moved continuously across all five regions in sequence. In the case of permeation in the positive z direction, this corresponds to the following ordered transition:

$$\begin{aligned}
 z &< Z_{\text{inf}} \\
 &\rightarrow Z_{\text{inf}} < z < Z_{\text{inf}} + \delta \\
 &\rightarrow Z_{\text{inf}} + \delta < z < Z_{\text{sup}} - \delta \\
 &\rightarrow Z_{\text{sup}} - \delta < z < Z_{\text{sup}} \\
 &\rightarrow z > Z_{\text{sup}}
 \end{aligned}$$

The reverse sequence was used to identify permeation in the negative direction. To ensure reliable detection, the parameter δ was chosen considering both the temporal resolution of the trajectory and the geometrical length of the pore. The number of permeation events in each direction was recorded cumulatively across all frames, allowing quantification of directionality and temporal dynamics.

Calculation of osmotic permeability coefficient (p_f)

The osmotic permeability coefficient (p_f) was determined following the method described by Zhu (Zhu et al., 2004). A non-dimensional variable $n(t)$ is defined in its differential form dn :

$$dn = \sum_{i \in S(t)} \frac{dz_i}{L(t)}, \quad (2)$$

where $S(t)$ represents the water molecules within the pore, $L(t)$ is the pore length, and:

$$dz_i = z_i(t) - z_i(t - \delta t), \quad \delta t = 1 \text{ ps}. \quad (3)$$

Integrating over time yields:

$$n(t) = \int_0^t \frac{dt}{L(t)} \sum_{i \in S(t)} dz_i. \quad (4)$$

The average-squared displacement (MSD) of $n(t)$ is calculated to derive the diffusion coefficient D_n using Einstein's relations:

$$\langle n^2(t) \rangle / t = \frac{1}{M} \sum_m n^2(t) \Big|_{n(t=t_0)=0, \delta t=1 \text{ ps}}, \quad (5)$$

$$D_n = \frac{\langle n^2(t) \rangle}{2t}. \quad (6)$$

with D_n , p_f is computed as:

$$p_f = v_w D_n, \quad (7)$$

where $v_w = \frac{V}{N_A}$ is the average water molecule volume, with $V = 18 \text{ cm}^3 \text{ mol}^{-1}$ and N_A being Avogadro's number. The (p_g) was computed in bins along the z -axis (Caviglia et al., 2024; Hashido et al., 2007).

Determination of binding sites

A three-dimensional probability density map for each calcium ion was calculated using a 1 Å resolution grid and probabilities were integrated along the XY planes (on the Z axis) and radially (XY plane integration) for analysis.

The probabilities were then integrated along the Z plane and a toroidal region was defined around the center of mass of residues 42, 43, 47, and 48 of the neighboring chains, with a radius of 10 Å. Six valid sub-zones were defined, taking the fractions of the toroidal region limited by the neighboring monomers. The movement of calcium ions between sub-zones within this toroid was analyzed over the course of the simulation. The rate of site transitions (in μs^{-1}) for each Ca^{2+} was calculated using the following expression:

$$Rt_{\text{Ca}^{2+}} = \frac{Tr}{Fvs} \cdot 100,000$$

where, Tr and Fvs are the transitions between valid sites and the frames where the Ca^{2+} was in a valid sub-zone, respectively. The Weighted Ratio (Wr) combines the rates of all calcium ions, weighted by the proportion of frames with different numbers of valid calcium ions (n).

$$Wr = \sum_{n=1}^6 \left(\frac{\sum Rt_{\text{Ca}^{2+}} \cdot w(n)}{n} \right)$$

where $w(n)$ is the fraction of frames with exactly n ions. A minimum time of 0.5 ns was the criteria for staying in sub-zone to avoid over counts if the

ion was within a sub-zone limit, and a maximum time of 5 ns the largest observed time of a transition between not adjacent sub-zones. Wr allows to compare the transition rate for the 3 and 6 ion models.

2.3 Protocols

Modelling

C-hCx46 and C-hCx26 models

The amino acid sequence of hCx46 (accession number NP_068773 . 2) was retrieved from the NCBI database and aligned with the sequence of the sheep Cx46 Cryo-EM structure (PDB: 7JKC; (Flores et al., 2020), which shares 70.59% sequence identity and has a 1.9Å resolution. The amino acid sequence of hCx26 (accession number AAL87696 . 1) was retrieved from the NCBI database and aligned with the sequence of hCx26 crystal structure (PDB:7QER; (Brotherton et al., 2022). Monomeric models were built using the comparative modeling by satisfaction of spatial restraints method via the *Modeller* software (Martí-Renom et al., 2000). The modelled sequence was from residues 1 to 225, as the C-terminal of the template structure was not resolved. For hCx26, this procedure was performed to model missing residues of the original structure (13, 102-128, and 218-230); 500 structural models were generated for each monomer; loop modeling was carried out via a simulated annealing procedure for regions without a structural template (for hCx46, these were residues 1 and 110-139) with default parameters; the optimal models were selected by their DOPE score (Martí-Renom et al., 2000). In Fig. 2.1, we show the DOPE score of the best model and a comparison with a hCx46 model derived from AlphaFold2 (AF2) (Jumper et al., 2021b). One could note that the main difference arises in the intracellular region, specifically between residues 110 to 139 while the extracellular half maintains an almost identical overlap, being this the region of interest in this study; quite interestingly, the structure of the 110-139 region was not resolved in the original CryoM structure, which suggests that this region is unstructured, as the Modeller model produces; on the other hand, the AF2 model tends to form alpha-helical structures on both extremes of this loop (see dashed circle in Fig. 2.1). The DOPE scores for both models are below 1 for every residue, which is a good score.

Consequently, the Modeller model seems to better reflect the experimental evidence, specifically the 110-139 region.

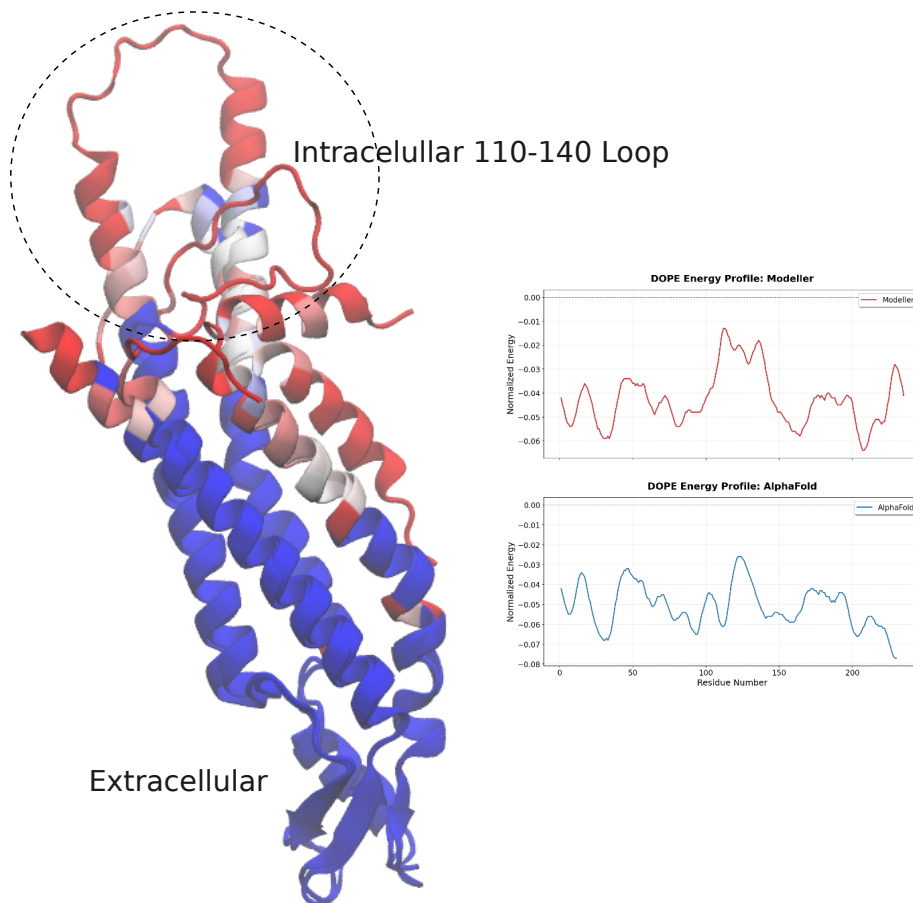


Figure 2.1: Modeller vs AF2 model for Cx46. On the left, structural alignment colored by RMSD between both models. Main differences arise in the 110-139 loop, which was not resolved in the template structure. AF2 model tends to form helical structures in regions that were not resolved in the template, which suggests that are flexible and unstructured. On the right, DOPE score for each model. As expected, the main differentia in the DOPE score arise in 110-139 region.

The hexameric hCx46 and hCx26 connexons models (C-hCx46 and C-hCx26) were constructed by replicating and arranging monomers based on the coordinates of the membrane domains in the 7JKC (Flores et al., 2020) and 7QER (Brotherton et al., 2022) structures. The hexamers were then embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer membrane containing 440 POPC molecules using the *CHARMM-GUI* membrane builder (Jo et al., 2008). The reason to choose POPC is

that it is the most abundant lipid in most eukaryotic cells, thus it serves as a model membrane. As we are studying two different proteins that are expressed in different cell types, this is a reasonable choice for this exploratory study. The system were solvated with TIP3P (Jorgensen et al., 1983) water molecules and neutralized with KCl at a concentration of 0.15 M. The final systems consisted of approximately 300K atoms and after relaxation the final dimensions of the systems were of $140 \times 140 \times 140 \text{ \AA}^3$, and the membrane thickness was of around 40 $\text{\AA}$.

Calcium ions (CAL model) were placed manually at six locations according to the crystallographic structure binding zones (PDB: 5ER7 (Bennett et al., 2016)). These were defined by the center of mass of the amino acid pairs E42-D43 and D47-E48. For C-hCx46, two models were constructed: one with 3 calcium ions (using interspersed sites) and the other with 6 (filling all the sites). For C-hCx26, only the 6 ion system was built. The CHARMM36m force field, with the CHARMM-WYF modifications for Lennard-Jones parameters, was used for proteins, lipids and calcium ions (Huang et al., 2016; Khan et al., 2016; Klauda et al., 2010). Lennard-Jones specific interactions for chloride, potassium and calcium were also employed (Han et al., 2018; Savelyev and MacKerell, 2015). Additionally, systems were prepared using a multi-site Ca^{2+} (CAM model. (A. Zhang et al., 2020)). In this case, only one model with 6 ions was prepared for both C-hCx26 and C-hCx46 systems. A representative depiction of the simulated systems is shown in Fig. 2.2.

Molecular dynamics simulations

C-hCx46 and C-hCx26 simulations

All C-hCx46 and C-hCx26 molecular dynamics (MD) simulations were carried out using NAMDv3.0 software (Phillips et al., 2020) with the CHARMM36m parameter set (Huang et al., 2016). Pressure and temperature were maintained at around 1 atmosphere and 310 K, respectively, using Langevin dynamics (Grest and Kremer, 1986) and the Nosé Hoover method (Martyna et al., 1994). A 12 $\text{\AA}$ cut-off was applied for Lennard-Jones and real space electrostatics, with a smoothing function applied between 10 and 12 $\text{\AA}$. Long-range electrostatics were estimated using the Particle Mesh Ewald (PME) method (Darden et al., 1993). All bonds involving hydrogen were fixed to their reference values using the SHAKE algorithm

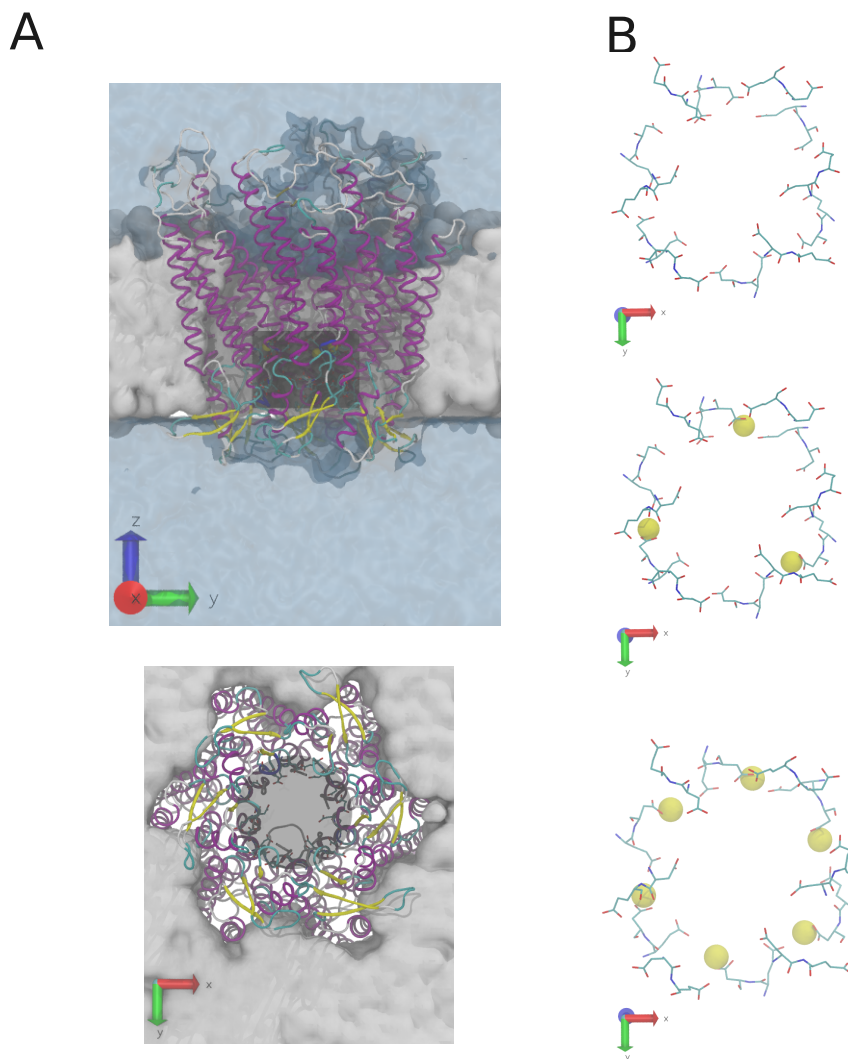


Figure 2.2: Example of C-hCx46 simulated systems.(A) Side and bottom view of a C-hCx46 embedded in POPC membrane and solvated with TIP3P water. The dark shaded area depicts the Ca^{2+} binding region. The protein is depicted in a cartoon representation and colored by its secondary structure; the white surface and the transparent light blue region represents the membrane and the solvent molecules, respectively (B) From top to bottom, simulated systems with 0, 3 and 6 Ca^{2+} bound. Only D42, D43, D47 and E48 are depicted in a licorice representation, while the Ca^{2+} are presented as yellow spheres. For C-hCx26, only the 0 and 6 Ca^{2+} system were simulated.

(Ryckaert et al., 1977). Hydrogen Mass Repartitioning (HMR) was applied to improve simulation efficiency, allowing the use of a timestep of 4 fs for real space interactions (Hopkins et al., 2015) while reciprocal space interactions were computed every 8 fs. The systems were energetically minimized with steepest descent and conjugate gradient methods for 10,000 cycles, keeping protein and bilayer atoms fixed. Three subsequent minimizations (20,000 cycles each) were performed removing restraints on the bilayer first and then on the protein. Subsequently, a thermalization MD was carried by rescaling velocities at constant volume until reaching the target temperature of 310K, this procedure was done for 1 ps. For the systems employing the Ca^{2+} CAM model (A. Zhang et al., 2020) a similar MD protocol was employed only differing in the timestep, which in this case was of 2 fs for real space interactions and 4 fs for reciprocal interactions. After 100 ns of equilibration in NPT dynamics, three independent MD simulations of 500 ns were performed for each model. The total simulation time for all models was of around 8 μs . For all simulations configuration were saved every 10 ps.

To explore the kinetic and structural effects of the use of the HMR procedure with a 4fs timestep on the cation, we carried out MD simulations of a single Ca^{+2} in a water box of $3\times 3\times 3$ nm for 1 μs @2fs or @4fs timestep. To evaluate the effects on the dynamics and structuring of the employed timestep, we computed the radial distribution function ($g(r)$) between the Ca^{+2} and water oxygen and diffusion coefficients (D) for the cation and water oxygen employing the method of Bullerjahn et al (Bullerjahn et al., 2020). For completeness, we also carried out an MD simulation of the multi-site CAM calcium model (A. Zhang et al., 2020) in water. This model is not stable @4fs (due to the use of strong harmonic restraints), thus only @2fs results are presented. As shown in Fig. 2.3 the $g(r)$ @2fs and @4fs are identical, while the CAM model exhibits 2 peaks with a lower intensity; the integration of the first solvation shell leads to an energy difference of around 1.1 kJ/mol , which is below $K_b T$. The peaks are also slightly displaced towards higher values (around 0.5 Å) while sigma values for the Ca^{+2} -oxygen interaction are above 2 Å (Huang et al., 2016; Khan et al., 2016; A. Zhang et al., 2020). For Ca^{+2} and water, we observe a slight increase in D (of around 7% compared to CAL@2fs) upon an increment in the timestep (see Table 2.1 and Fig. 2.4); the CAM model exhibits a small reduction in D (around 14% with respect to CAL@4fs).

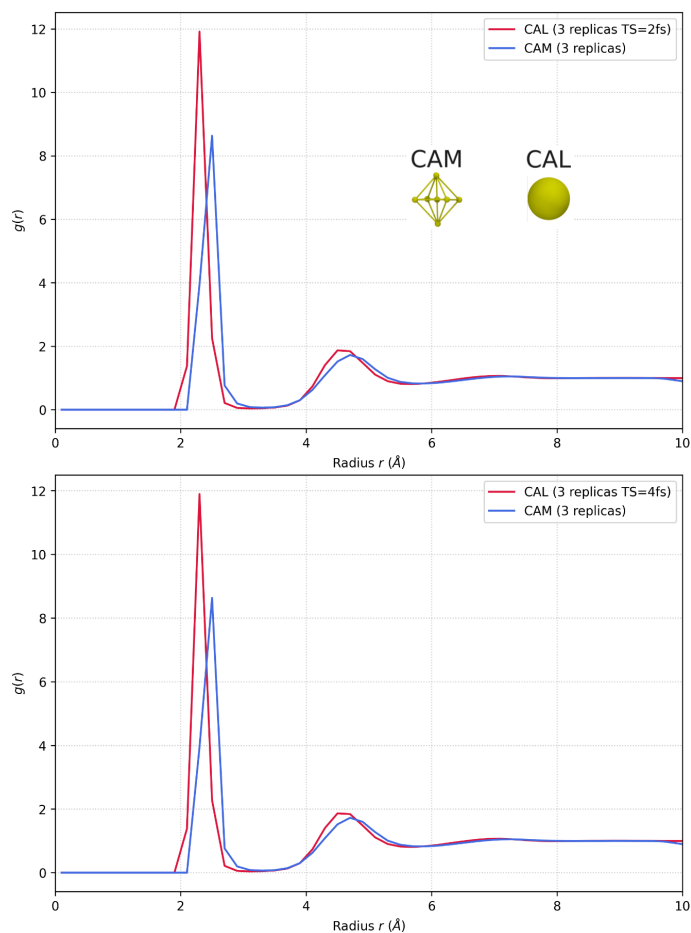


Figure 2.3: Radial Distribution functions ($g(r)$) of Ca^{2+} -O pair for a system of a single ion in water box of 3 nm.

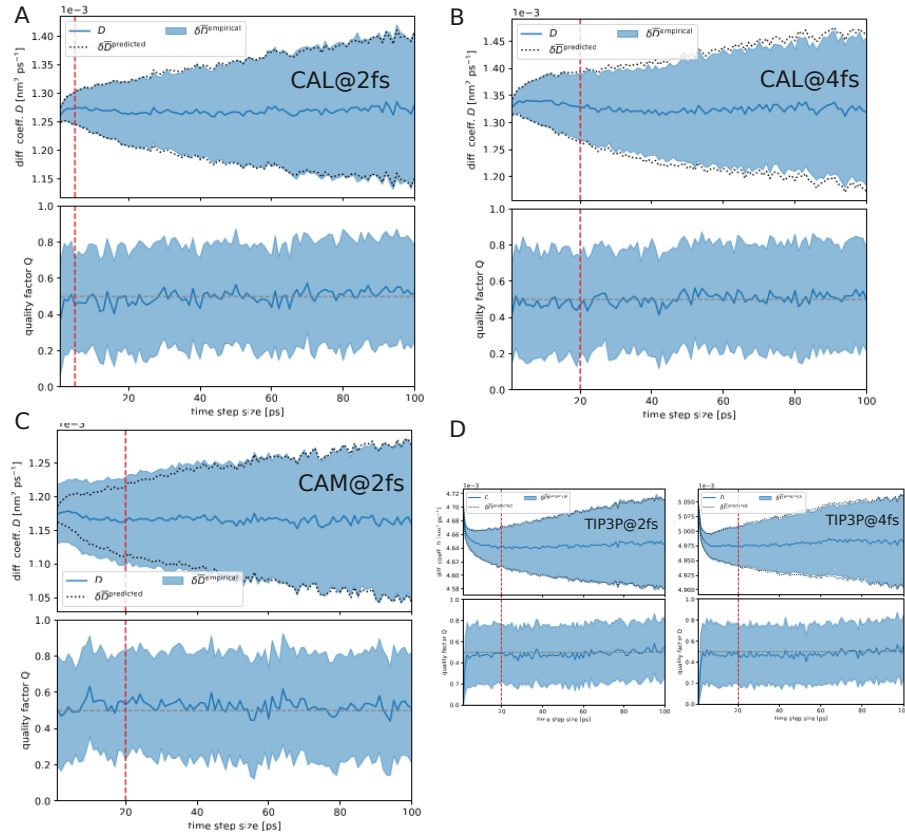


Figure 2.4: Diffusion coefficient (D) and quality factor as function of different recording-time steps lengths for CAL@2fs (A), CAL@4fs (B), CAM@2fs (C), TIP3P@2fs (D, left) and TIP3P@4fs (D, right). D was computed from $3 \times 3 \times 3$ nm cubic box of single ion (CAM or CAL) in water, which was simulated for $1 \mu\text{s}$. The shaded light-blue regions depict the estimated error. The reported values in Table S1 are at a recording time-steps of 20 ps. For more details on the calculation of D , refer to the work of Bullerjahn et al. Bullerjahn et al., 2020.

Table 2.1: Diffusion coefficients (D) for CAL, CAM and TIP3P models at 298 K

System	D ($10^{-3} \text{ nm}^2/\text{s}$)
CAL@2fs	1.27 ± 0.03
TIP3P@2fs	4.65 ± 0.02
CAL@4fs	1.33 ± 0.07
TIP3P@4fs	4.98 ± 0.03
CAM@2fs	1.16 ± 0.05

Overall, these results allowed us to conclude that a 4 fs time-step does not significantly affect the Ca^{2+} intrinsic dynamics or structuring in water. The CAM model exhibits a lower diffusivity and similar RDF in water.

Determination of binding sites

The three-dimensional probability density map for each calcium ion was calculated using a 1 Å resolution grid with the *VolMap* plugin in *VMD* (Ak-simentiev et al., 2008) and the data obtained were processed as previously described.

Water and and ions content in pore analysis

The amount of K^+/Cl^- ions and water molecules inside the pore during simulations was analyzed using the MDAnalysis Python library (Gowers et al., 2016). The pore of C-hCx46, modeled as a cylinder centered at the origin with a length of 60 Å and a radius of 20 Å, was divided in bins of 1 Å resolution and the amount of water molecules, K^+ and Cl^- ions was determined in each bin for all frames and normalized by the bulk density of each species.

Water and ions permeation events

The MD trajectories were processed as previously described using the ProDy 2.0 Python library (S. Zhang et al., 2021) for Water and K^+/Cl^- . The pore of C-hCx46, modeled as a cylinder centered at the origin with a length of 60 Å and a radius of 20 Å.

Electrostatic potentials calculation

For this analysis, two new systems were constructed: one with three calcium ions and another without calcium ions. Both systems contain the minimum number of potassium or chloride ions necessary for system neutralization to minimize the irrelevant noise in the electrostatic potential calculations. The conexon and membrane movements were limited to also reduce noise by applying positional harmonic restraints to all heavy atoms of the protein. The systems were equilibrated for 100 ns at 310 K using

NPT dynamics without any constraints, followed by 50 ns of the restrained simulations. An electrostatic potential map resulting from an average over the trajectory was obtained using by solving Poisson equations in a grid via the PMEpot plug-in of VMD (Aksimentiev et al., 2008). Afterward, a cylinder of 20 Å radius and 120 Å height was defined, centered on the pore's midpoint, and the radial average along the Z axis was computed.

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Chapter 3

Results

“The things that really change the world, according to Chaos theory, are the tiny things. A butterfly flaps its wings in the Amazonian jungle, and subsequently a storm ravages half of Europe.”

Neil Gaiman y Terry Pratchett
Good Omens

3.1 Ca^{2+} Binding Analysis

Initial Ca^{2+} location validation

Based on similarities with hCx26, six putative Ca^{2+} sites were defined in hCx46 using the geometric center of E42-D43 and D47-E48 pairs. Two models were created: one with 3 Ca^{2+} ions (interspersed) and one with 6 (full occupancy). Analysis of Z-axis probability revealed a well-defined region between these amino acid pairs, supporting the proposed initial positions (Fig. 2.2 and Fig. 3.1). A minor interaction region 24 Å below the center was also detected, but not included in interaction analyses.

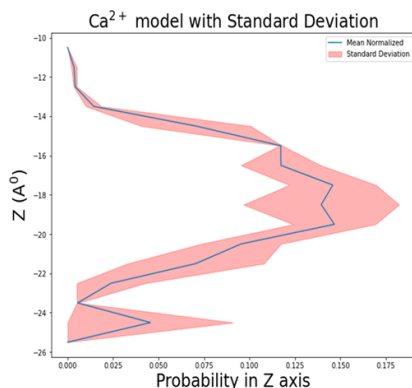


Figure 3.1: Integration of XY planes along Z-axis of the Ca^{2+} probability density grid.

A symmetric cavity in C-hCx46 increases Ca^{2+} transitions between binding sites

Before analyzing the interaction between Ca^{2+} and C-hCx46, we must first consider the interaction between Ca^{2+} and C-hCx26, as described in the literature Bennett et al., 2016. Understanding this context will allow for a more precise evaluation of the significance of the findings presented in this study. As depicted in Fig. 3.2A, the most significant structural differences between C-hCx26 and C-hCx46 are primarily located in the intracellular half of the pore. In this region, several structural rearrangements are observed, particularly in charge distribution. Furthermore, some of the alpha helices in C-hCx46 extend further to the intracellular side compared to those in

C-hCx26. In contrast, the extracellular half of the pore remains structurally conserved despite sequence differences between the two proteins. Both the secondary structure elements and the overall charge distribution appear largely unchanged. A particularly notable example are residues near the confinement region (See Fig. 3.2A). Here, residues D43, D47 and E48, highlighted in red, are conserved in both C-hCx26 and C-hCx46. These residues are positioned near the confinement site and maintain a similar arrangement in both connexins. However, a key difference is observed in the same region: Residue E42 in C-hCx46 is replaced by a K in C-hCx26 (highlighted in blue). This substitution alters the local charge distribution, disrupting the symmetrical charge arrangement seen in C-hCx46 and we will see that it changes the ion kinetics within its binding zone.

Given the well-defined Ca²⁺ sites identified for hCx26 and the structural similarities shown in Fig. 3.2 we set out to find similar sites in our hCx46 simulations. In detail, the ions were placed manually in six locations based on their positions in the structure of hCx26 crystallized with Ca²⁺ (PDB: 5ER7 Bennett et al., 2016), in which the ions interact with the D47-E48 amino acid pair of hCx26. However, due to the existence of another amino acid pair in hCx46, E42-D43, (see Fig. 3.2B), the geometric center of the four amino acids (E42-D43 and D47-E48) was chosen as the initial site. Two models with the CAM models were constructed: one with 3 Ca²⁺ (using interspersed sites) and the other with 6 (filling all sites). A radius of 10 Å was established for the toroid based on the stable positioning of Ca²⁺ (see Fig. 3.3A). Quite interestingly, no well-defined binding site was identified. Instead, the ions move through a toroidal confinement zone near the sites reported for hCx26 and eventually traverse the pore center in a transient and rapid manner (≤ 3 ns). A 6 Ca²⁺ model employing the CAM model exhibits a similar behavior. Representative Ca²⁺ trajectory within the confinement zone for the CAL and CAM models are depicted in Fig. 3.3A and Fig. 3.3C, respectively. On the other hand, in C-hCx26, it is observed that Ca²⁺ tends to remain in its initial binding zone throughout the simulation for both Ca²⁺, as shown in Fig. 3.3B and Fig. 3.3D.

For C-hCx46 and the CAL models, two initial calcium occupancies were tested: one with three Ca²⁺ ions and another with six initial Ca²⁺ ions, each positioned equidistantly at the centers of mass of the previously defined sub-zones. In each case, we monitored the retention of the ions in any subzone during the simulations. The occupancy fraction time of Ca²⁺ in

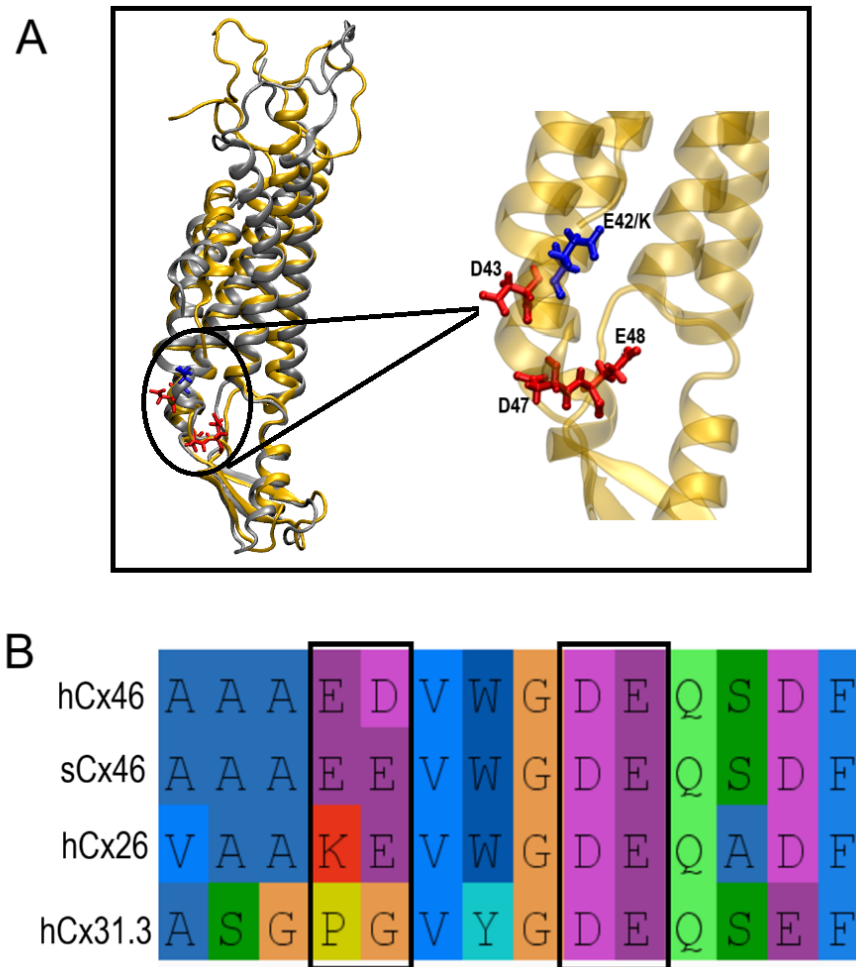


Figure 3.2: Ca^{2+} binding Site Comparison among different connexins. (A) Structural alignment between hCx46 (in yellow) and hCx26 (in gray). E or D residues near the confinement region, which are conserved in both connexins, are highlighted in red. In contrast, a D in hCx46 is replaced by a K in C-hCx26 and is highlighted in blue. (B) Sequence alignment of Ca^{2+} binding zone. From top to bottom are the sequences of the hCx46 model used here, sheep Cx46 used as template, hCx26 from the crystal structure with bound Ca^{2+} , and hCx31.3 used as non-interaction site control Lee et al., 2020. The black selection shows the amino acids deemed as possible interaction sites with divalent cations

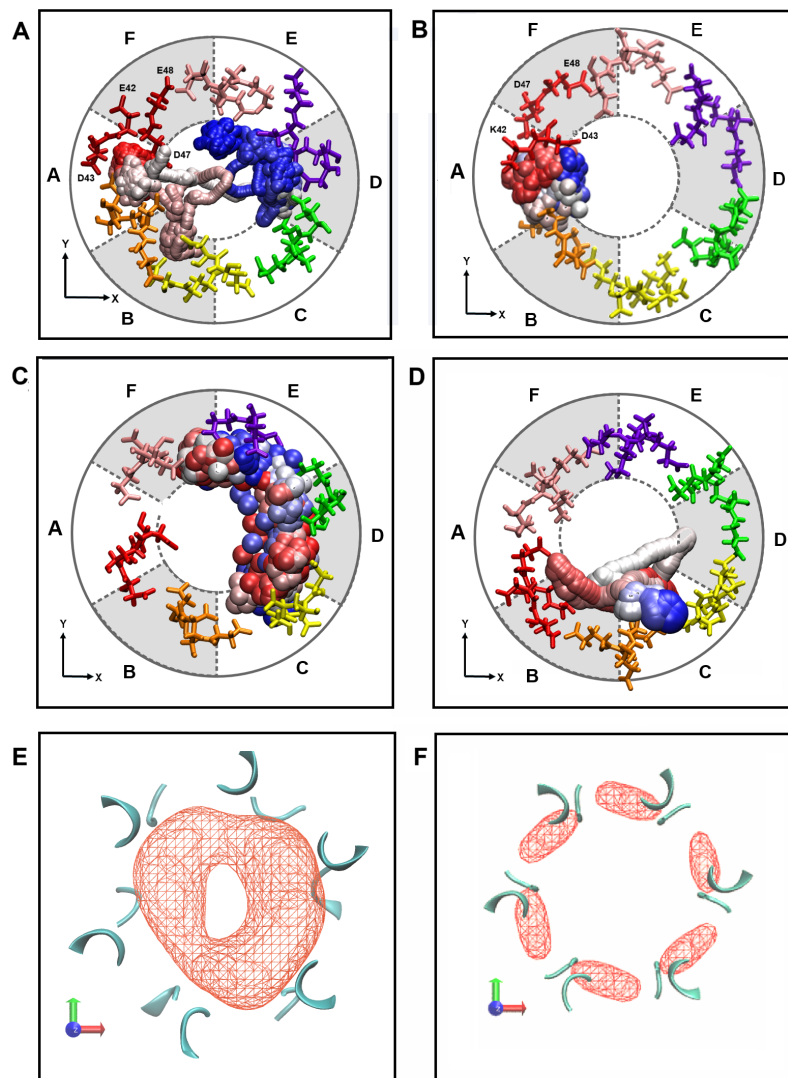


Figure 3.3: Ca^{2+} Visualization of a representative Ca^{2+} ion trajectory, colored from red (at start of simulation) to blue (at end of simulation). (A) Representative Ca^{2+} ion (CAL model) trajectory in C-hCx46 (B) Representative Ca^{2+} ion (CAL model) trajectory in C-hCx26 (C) Representative Ca^{2+} ion (CAM model) trajectory in C-hCx46. (D) Representative Ca^{2+} ion (CAM model) trajectory in C-hCx26. (E) Identification of C-hCx46 region of isopotential of approximately -0.8 Volts based on the electrostatic surface potential maps along the pore in Fig. 3.7, which coincides with the toroidal confinement zone proposed in this study. (F) Identification of six C-hCx26 isopotential regions of similar intensity using the same methodology, which are smaller in size and located closer to the extracellular exit of the pore.

the confinement zone is detailed in Table 3.1, this value corresponds to the fraction of the total simulation time (500 ns) during which a determined number of Ca^{2+} ions are present in the confinement zone, regardless of the specific subzone they occupy. Our results show that the six confinement zones were never fully occupied. Furthermore, we observed a significant number of exchanges among subzones, as depicted by the dynamics of one calcium ion shown in Fig. 3.3A, a behavior that was observed for all Ca^{2+} ions in all replicas of both models. The latter was quantified in the form of average occupancies and the average ratio of transition events between sub-zones, as presented in Tables 3.1 & 3.2, respectively (results for each replica are presented in Tables S2 to S6). For the 3 ion model, the occupancy oscillates between 3 and 2 while for the 6 ion model the occupancy is between 3 and 5 ions. Thus, on average, 4 ions can bind to C-hCx46. We observe that for both models the transitions exhibit no statistical differences with an average of around 80 per microsecond. The 6 Ca^{2+} system employing the CAM model is in qualitative agreement with similar average occupancies (around 4) but a low number of transitions (around 40 per microsecond), as presented in Tables 3.1 & 3.2. The latter implies that these transitions seem to be the result of the symmetrical nature of the binding region. This spatial symmetry is confirmed when the average electrostatic potential is computed in the absence of Ca^{2+} (see methods). As presented in Fig. 3.3E, a toroidal isopotential surface of -0.8 volts is generated by this symmetric cavity which explains the free movement of the ions, as this region exhibits a smooth energy surface. When the same calculation was performed for C-hCx26, regardless of the Ca^{2+} ion model, Table 3.1 shows that average occupancies are around 1 and 2 with almost no transitions between sites. For C-hCx46 and the CAL models, two initial calcium occupancies were tested: one with three Ca^{2+} ions and another with six initial Ca^{2+} ions, each positioned equidistantly at the centers of mass of the previously defined sub-zones. In each case, we monitored the retention of the ions in any subzone during the simulations. The occupancy fraction time of Ca^{2+} in the confinement zone is detailed in Table 3.1, this value corresponds to the fraction of the total simulation time (500 ns) during which a determined number of Ca^{2+} ions are present in the confinement zone, regardless of the specific subzone they occupy. Our results show that the six confinement zones were never fully occupied. Furthermore, we observed a significant number of exchanges among subzones, as depicted

by the dynamics of one calcium ion shown in Fig. 3.3A, a behavior that was observed for all Ca²⁺ ions in all replicas of both models. The latter was quantified in the form of average occupancies and the average ratio of transition events between sub-zones, as presented in Tables 3.1 & 3.2, respectively (results for each replica are presented in Tables S2 to S6). For the 3 ion model, the occupancy oscillates between 3 and 2 while for the 6 ion model the occupancy is between 3 and 5 ions. Thus, on average, 4 ions can bind to C-hCx46. We observe that for both models the transitions exhibit no statistical differences with an average of around 80 per microsecond. The 6 Ca²⁺ system employing the CAM model is in qualitative agreement with similar average occupancies (around 4) but a low number of transitions (around 40 per microsecond), as presented in Tables 3.1 & 3.2. The latter implies that these transitions seem to be the result of the symmetrical nature of the binding region. This spatial symmetry is confirmed when the average electrostatic potential is computed in the absence of Ca²⁺ (see methods). As presented in Fig. 3.3E, a toroidal isopotential surface of -0.8 volts is generated by this symmetric cavity which explains the free movement of the ions, as this region exhibits a smooth energy surface. When the same calculation was performed for C-hCx26, regardless of the Ca²⁺ ion model, Table 3.1 shows that average occupancies are around 1 and 2 with almost no transitions between sites. In Fig. 3.3F, six discontinuous isopotential regions are observed in accordance with the six binding sites and the restricted Ca²⁺ dynamics observed for C-hCx26. In Fig. 3.3F, it was observed that during the whole MD simulation, for a system with six ions initially placed in each of these six regions, there were no exchanges between sites and all ions remained in their site. The occupancies timeseries, shown in Fig. 3.4, further suggest that the calcium binding modes in C-hCx46 and C-hCx26 are indeed different.

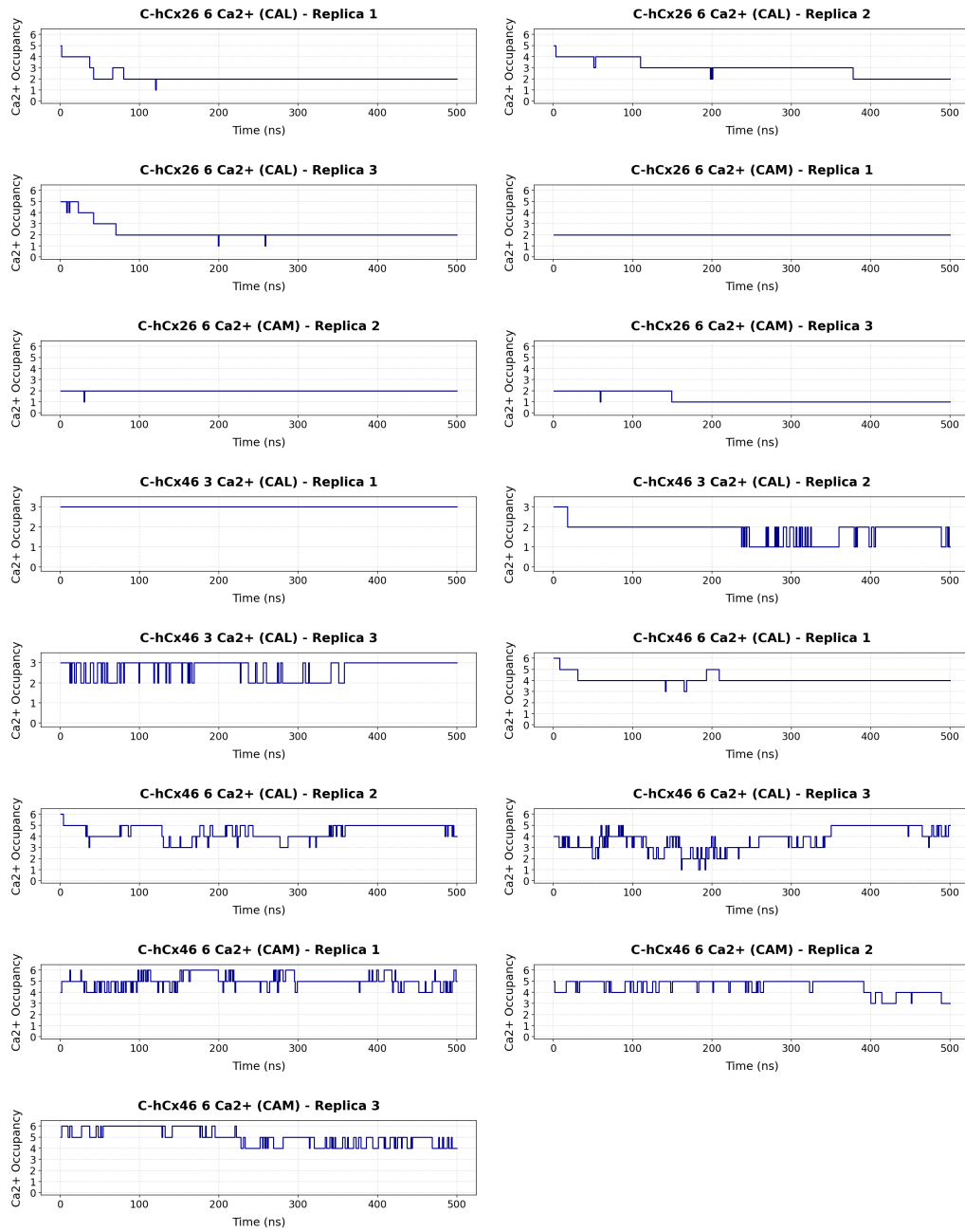


Figure 3.4: Calcium occupancies as function of time for all simulated systems.

Table 3.1: Average Ca²⁺ Occupancy Fraction Time and SEM

Ions	C-hCx26		C-hCx46		
	6 Ca ²⁺ CAL	6 Ca ²⁺ CAM	3 Ca ²⁺ CAL	6 Ca ²⁺ CAL	6 Ca ²⁺ CAM
1	0.00 ± 0.00	0.24 ± 0.24	— — —	0.00 ± 0.00	0.00 ± 0.00
2	0.67 ± 0.21	0.76 ± 0.24	0.36 ± 0.22	0.04 ± 0.02	0.00 ± 0.00
3	0.21 ± 0.16	0.00 ± 0.00	0.64 ± 0.22	0.11 ± 0.07	0.02 ± 0.02
4	0.11 ± 0.05	0.00 ± 0.00	— — —	0.53 ± 0.13	0.25 ± 0.04
5	0.02 ± 0.01	0.00 ± 0.00	— — —	0.28 ± 0.09	0.56 ± 0.07
6	0.00 ± 0.00	0.00 ± 0.00	— — —	0.03 ± 0.02	0.16 ± 0.09

Table 3.2: Average weighted rate of Ca²⁺ transitions (*events/μs*) and SEM

C-hCx26		C-hCx46		
6 Ca ²⁺ Model		3 Ca ²⁺ Model	6 Ca ²⁺ Model	
CAL	CAM	CAL	CAL	CAM
6.15 ± 1.85	7.99 ± 0.84	67.33 ± 32.08	89.67 ± 10.97	41.00 ± 7.87

3.2 Ca²⁺ binding effect

Ca²⁺ binding decreases the kinetics and occupancy of water and K⁺ at the extracellular side in C-hCx46

To explore the effects of calcium binding on the conductance and transport properties of the C-hCx46 and C-hCx26 connexons, we measured the average relative densities of water, K⁺ and Cl[−] along the pore in our simulations. These results are shown in Figs. 3.5 and 3.6. The results for each replica are presented in Fig. S4 to S9. For water and C-hCx46, we observed a slight decrease in the average density in the extracellular half of the channel in the models with Ca²⁺. This effect is more pronounced in the model with six initial Ca²⁺ ions, as shown in Fig. 3.5A. No differences are observed when employing the CAM model. For C-hCx26, as shown in Fig. 3.5B we observe lower water densities with no apparent effects

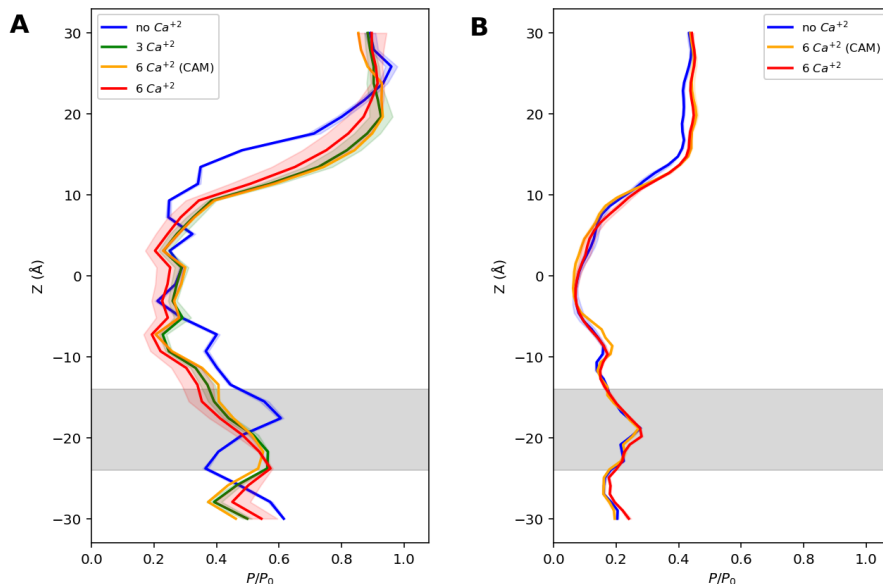


Figure 3.5: Average relative water number density with respect to the bulk (P/P_0) along the pore, the Ca^{2+} confinement zone is shaded in gray. The errors represent the SEM of the average and are shaded in the same color as the average for each model: (A) C-hCx46 models (B) C-hCx26 models

of Ca^{2+} binding on this descriptor. Regarding K^+ densities in C-hCx46, a decrease was observed in the Ca^{2+} confinement zone, which was again accentuated in the model with six initial Ca^{2+} ions for both CAL and CAM models, as shown in Fig. 3.6A. A similar effect is observed for C-hCx26, however in this case K^+ densities above the binding region are close to zero, as shown in Fig. 3.6B. Regarding Cl^- densities profiles, in C-hCx46 they are close to zero (see Fig. 3.6C) which is to be expected since C-hCx46 is a highly cation-selective channel. For C-hCx26, the Cl^- densities profiles are higher, specially at the intracellular side. Calcium binding increases these profiles, but we observe differences in the densities peaks between the CAL and CAM models, which are displaced by around 15 Å. In general, we consistently observe that Ca^{2+} leads to a reduction in water and K^+ densities. Remarkably, this effect is propagated along the pore, specially in regions just above the binding pocket.

In addition to occupancy, we also measured water, K^+ and Cl^- permeation events from our simulations. Table 3.3 shows the average count of permeation events obtained from the three replicas of each system. When analyzing potassium permeation events in C-hCx46, a decrease of approx-

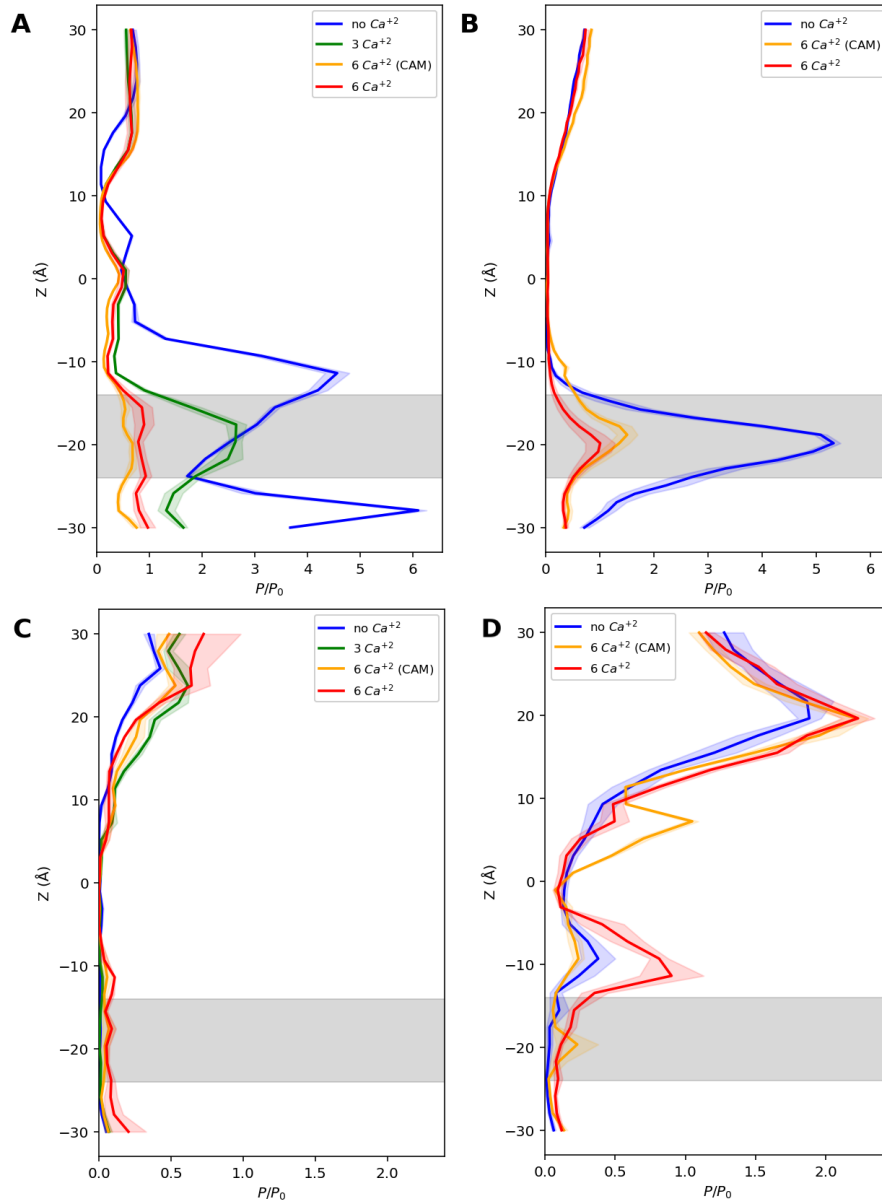


Figure 3.6: Average relative ion number density with respect to the bulk (P_0) along the pore, the Ca^{2+} confinement zone is shaded in gray. The errors represent the SEM of the average and are shaded in the same color as the average for each model: (A) K^+ in C-hCx46 models (B) K^+ in C-hCx26 models (C) Cl^- in C-hCx46 models (D) Cl^- in C-hCx26 models

imately 30% was observed in the models with Ca^{2+} , and no statistically significant difference was found between the two initial Ca^{2+} occupancies or the calcium model. For C-hCx26, regardless of ion occupancy or calcium model, no permeation is observed. Regarding Cl^- permeation, only in the C-hCx26 model we observe some permeation events. Calcium binding seems to increase permeability for the CAL model, while the CAM model exhibits no effects with similar permeation events compared to the systems without calcium. The latter could be attributed to the slightly lower occupancies observed for the CAM model (see Table 3.1). These results are in accordance with the densities shown in Figure 3.6. In the case of water and in the absence of Ca^{2+} , C-hCx46 exhibits around a 30% increase in permeation events compared to C-hCx26, which is in line with the water densities shown in Fig. 3.5. Overall, Ca^{2+} leads to a reduction in the number of permeation events for both connexons; however, there is quantitative disagreement and opposing effects when the CAL and CAM models are compared. For C-hCx26, the CAM model greatly reduces water permeation; on the other hand, in C-hCx46, the reduction in water permeation is less pronounced when using the CAM model. To further inspect the nature of the observed decrease in water, we calculated the osmotic permeability (p_f) along the pore (see Fig. S1). Interestingly, a decrease in p_f around and above the Ca^{2+} confinement zone is observed, which became more evident in the model with six initial Ca^{2+} ions. This is in line with the reduction in water density. For C-hCx46, no significant differences were observed between the models with three or six initial Ca^{2+} ions in the osmotic permeability (p_f). However, a more than two-fold decrease (approximately 57%) was observed with respect to the model without Ca^{2+} .

Table 3.3: Ions and Water Permeation Events

Connexin	Model	K ⁺ (events/ μ s)	Cl ⁻ (events/ μ s)	Water (events/ μ s)
C-hCx26	no Ca ²⁺	—	23 $\pm$ 4	75986 $\pm$ 2808
	6 Ca ²⁺ CAL	—	53 $\pm$ 6	72378 $\pm$ 789
	6 Ca ²⁺ CAM	—	17 $\pm$ 5	46576 $\pm$ 8533
C-hCx46	no Ca ²⁺	289 $\pm$ 11	—	110719 $\pm$ 1195
	3 Ca ²⁺ CAL	206 $\pm$ 40	—	49142 $\pm$ 1152
	6 Ca ²⁺ CAL	167 $\pm$ 66	—	47052 $\pm$ 2272
	6 Ca ²⁺ CAM	171 $\pm$ 16	—	77340 $\pm$ 1631

The data in Table 3.1 implies that not only a structural effect (reduction in densities) is produced by Ca²⁺ binding but also a reduction in the water and K⁺ permeability.

To gain a deeper insight into the Ca²⁺ interaction with C-hCx46 and C-hCx26, we also computed the mean potential along the pore in a specialized simulation (See Fig. 3.7).

A lighter color scheme was used to illustrate the simplification of the system: the average surface electrostatic potential in the simplified model without Ca²⁺ is shown in light blue, and the average in the simplified model with three Ca²⁺ ions is shown in orange. The standard deviation of the averages is represented by shading in the same color associated with each model. Fig. 3.7B shows a slice in the xz plane of the volumetric map of the surface electrostatic potential, cutting through the center of the pore, to illustrate the effect quantified in Fig. 3.7A. The left panel of Fig. 3.7B corresponds to the simplified model without Ca²⁺, and the right panel to the simplified model with three Ca²⁺.

For C-hCx46, a considerably negative surface electrostatic potential (depicted in red) is observed near the extracellular end of the pore, surrounded by a positive surface electrostatic potential (depicted in blue). This asymmetric electrostatic pathway (more electronegative toward the extracellular end) is screened by the presence of Ca²⁺. This is clearly observed by comparing the reddish tone of the left slice with the more orange tone of the right slice in Figure 3.7B, and is further supported by the quantitative results presented in Fig. 3.7A. For C-hCx26, as shown in Figures 3.7C and

D, the electrostatic profiles are more asymmetrical, being positive towards the extracellular docking side and negative towards the intracellular side. This more positive value at the intracellular side is partially due to the E15K substitution in the N-terminal helix of C-hCx26. Calcium binding shifts the overall potential towards positive values, but its effects are less pronounced compared to C-hCx46.

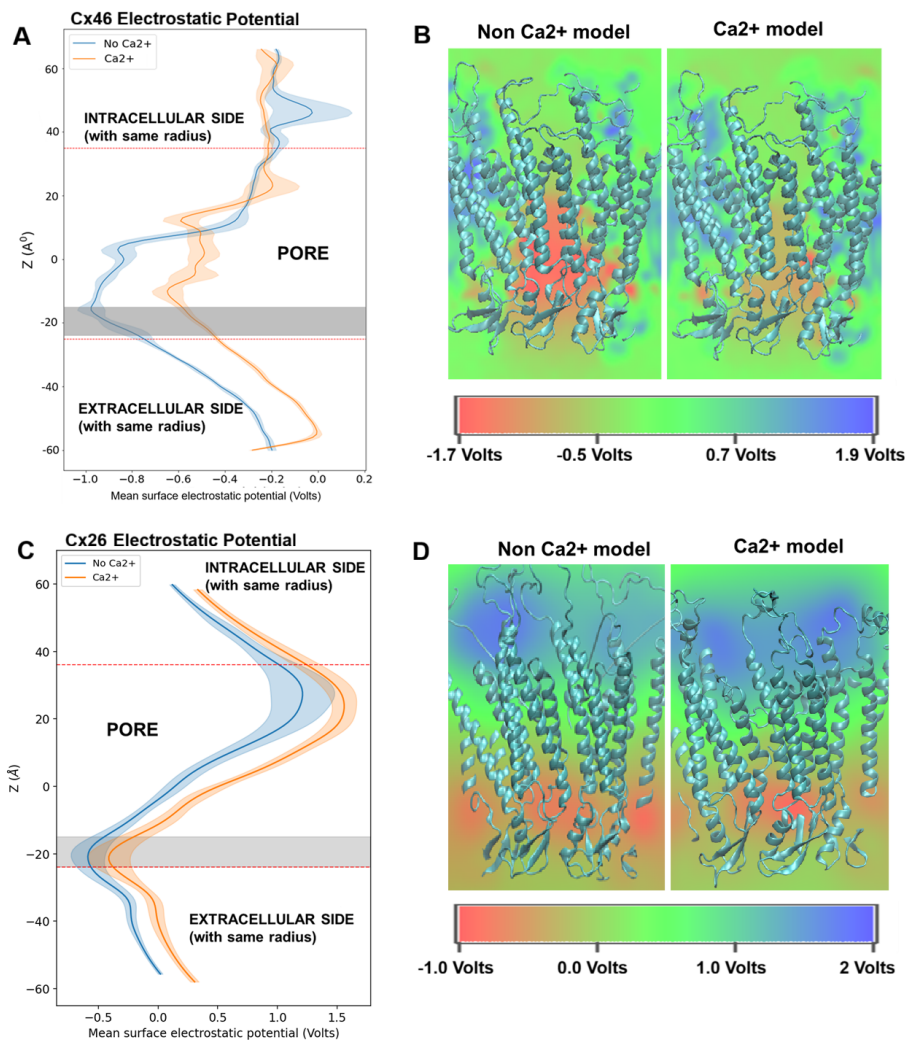


Figure 3.7: Mean surface electrostatic potential along the pore. The errors represent the SD of the average and are shaded in the same color as the average for each model (A) average surface electrostatic potential along the pore in Ca²⁺ model vs no Ca²⁺ model for C-hCx46 (B) 2D average surface electrostatic potential taken from an x-z plane located at the center of the pore for the Ca²⁺ and no Ca²⁺ models of C-hCx46. (C) average surface electrostatic potential along the pore in Ca²⁺ model vs no Ca²⁺ model of C-hCx26 (D) 2D average surface electrostatic potential taken from an x-z plane located at the center of the pore for the Ca²⁺ and no Ca²⁺ models for C-hCx26.

Assessing the Impact of the E42K Substitution on Ca^{2+} Interaction Geometries Using Toy Systems of C-hCx46 and C-hCx26

To assess the impact of the E42 to K substitution (a major alteration involving the replacement of a negative charge with a positive one) a reduced model (toy system) was constructed. This system incorporated the E42-D43 and D47-E48 pairs to mimic the charged boundaries of the C-hCx46 interaction zone, and the K41-D42 and D46-E47 pairs to represent the corresponding region in C-hCx26. The results deviated from our expectations; both systems displayed a similar frequency of inter-site transitions, an event that theoretically should not occur in C-hCx26 (Table 3.4). Additionally, the geometry of the interaction region in both models shifted from a toroid to a cylinder, a structural change that is also clearly discernible in the electrostatic isopotential surface of the C-hCx46 toy system (Fig.3.9).

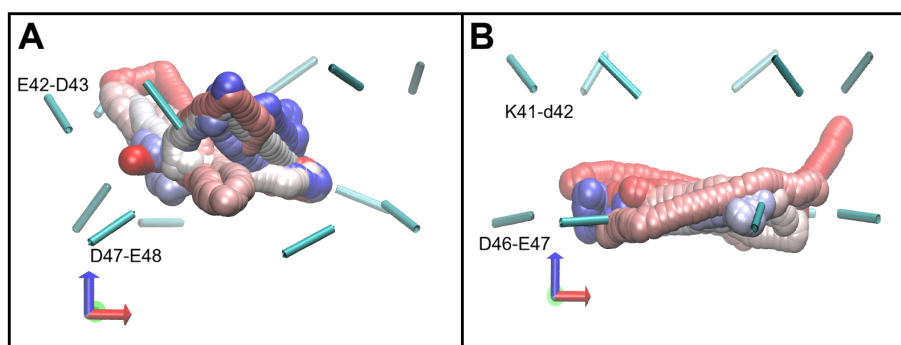


Figure 3.8: Ca^{2+} Visualization of a representative Ca^{2+} ion trajectory, colored from red (at start of simulation) to blue (at end of simulation). (A) Representative Ca^{2+} ion (CAL model) trajectory in toy system of C-hCx46 (B) Representative Ca^{2+} ion (CAL model) trajectory in in toy system of C-hCx26

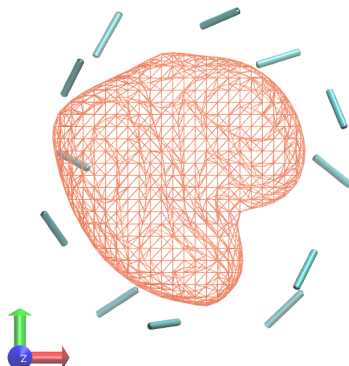


Figure 3.9: Isopotential Zones in toy system of C-hCx46

Table 3.4: Single Ca²⁺ Transition Rates for Toy systems

System	Transition Rate (events/ μ s)
C-hCx26@CAL	49.63 ± 5.71
C-hCx26@CAM	27.45 ± 2.92
C-hCx46@CAL	45.33 ± 6.67
C-hCx46@CAM	27.33 ± 2.67

Despite these initially discouraging observations, a compelling effect emerged: the Ca²⁺ interaction zone within the C-hCx26 toy system aligns with the lower half of the interaction zone observed in the C-hCx46 toy system (specifically, the region where the negatively charged D46-E47 pair is conserved, Fig.3.8). Consequently, it can be concluded that while the charged boundaries of the confinement zone play a crucial role, additional structural factors are necessary to fully modulate the electrostatic potential of the pore.

3.3 Supplementary Material

Table S1: Fraction of simulated time with 3-10 helix

Model	Time Fraction
No Ca^{2+}	0.07 ± 0.07
3 Ca^{2+}	0.54 ± 0.018
6 Ca^{2+}	0.72 ± 0.09

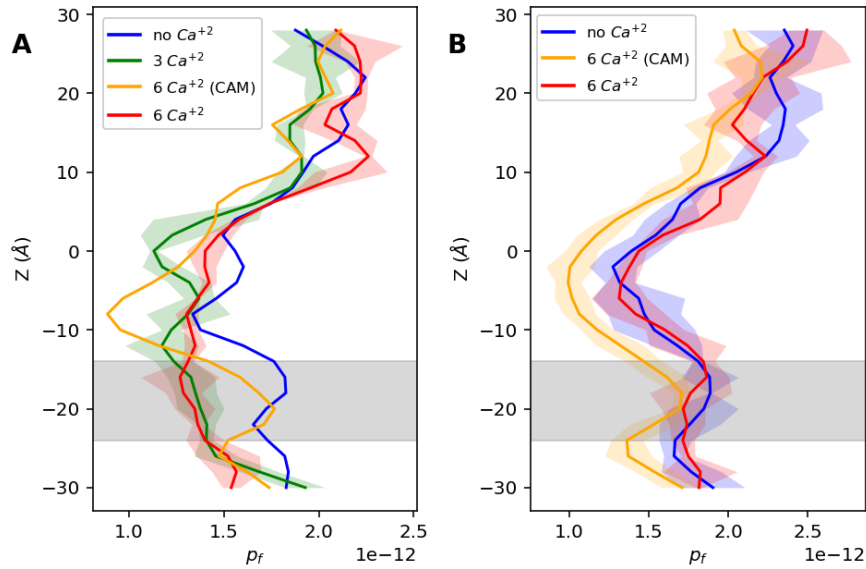


Figure S1: Average unitary permeability osmotic coefficient. The Ca^{2+} confinement zone is shaded in gray. The errors represent the SEM of the average p_f and are shaded in the same color as the average for each model: (A) C-hCx46 models (B) C-hCx26 models.

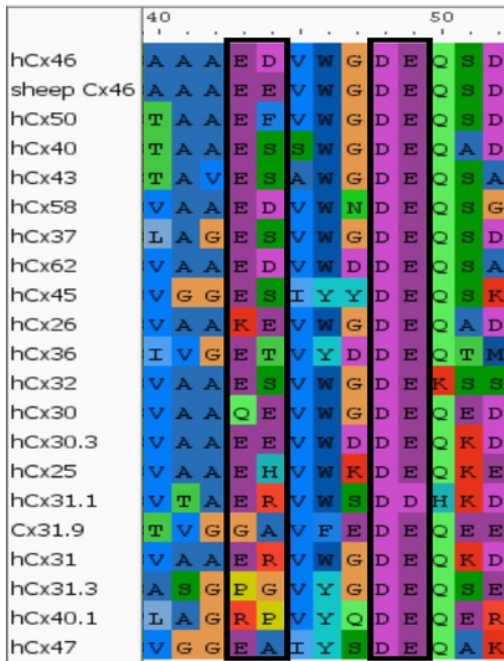


Figure S2: Sequence alignment of Ca^{2+} binding zone of human connexins sequences.

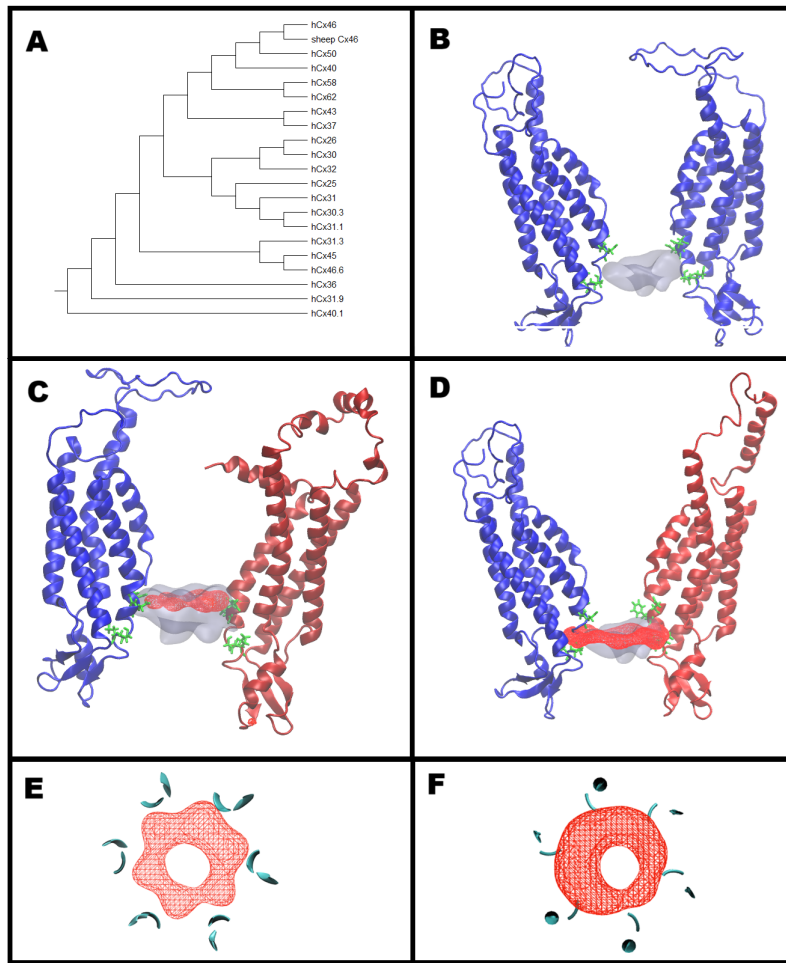


Figure S3: Isopotential Zones in hCx43 and hCx50. (A) A dendrogram based on multiple sequence alignment is shown to illustrate similarities among members of the human connexin family. (B) Two non-contiguous monomers of C-hCx46 are displayed in blue, with the isopotential zone corresponding to the Ca^{2+} interaction region highlighted in translucent blue. (C) Structural alignment between C-hCx46 and C-hCx43: one monomer of C-hCx46 is shown in blue and one monomer of C-hCx43 in red. The isopotential region overlapping with the Ca^{2+} interaction site is shown in translucent blue, and a region with similar characteristics in Cx43 is marked with a red mesh. (D) Structural alignment between C-hCx46 and C-hCx50: one monomer of C-hCx46 is shown in blue and one monomer of C-hCx50 in red. The isopotential region overlapping with the Ca^{2+} interaction site is shown in translucent blue, and a region with similar characteristics in Cx50 is marked with a red mesh. (E) Isopotential Zone of C-hCx43 (F) Isopotential Zone of C-hCx50,

Table S2: Calcium Ion Occupancy in the confinement zone for each C-hCx46 MD

Ions	3 Ca²⁺ (CAL) model			6 Ca²⁺ (CAL) model			6 Ca²⁺ (CAM) model		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
2 Ca²⁺	0.00	0.75	0.32	0.01	0.01	0.10	0.00	0.00	0.00
3 Ca²⁺	1.00	0.25	0.68	0.01	0.02	0.30	0.00	0.07	0.00
4 Ca²⁺	—	—	—	0.89	0.39	0.32	0.17	0.31	0.28
5 Ca²⁺	—	—	—	0.08	0.50	0.28	0.63	0.62	0.43
6 Ca²⁺	—	—	—	0.01	0.08	0.00	0.20	0.00	0.29

Table S3: Calcium Ion Occupancy in the confinement zone for each C-hCx26 MD

Ions	6 Ca²⁺ CAL			6 Ca²⁺ CAM		
	R1	R2	R3	R1	R2	R3
1 Ca²⁺	0.00	0.00	0.00	0.00	0.00	0.71
2 Ca²⁺	0.89	0.25	0.86	1.00	1.00	0.29
3 Ca²⁺	0.04	0.54	0.06	0.00	0.00	0.00
4 Ca²⁺	0.07	0.21	0.04	0.00	0.00	0.00
5 Ca²⁺	0.00	0.00	0.04	0.00	0.00	0.00
6 Ca²⁺	0.00	0.00	0.00	0.00	0.00	0.00

Table S4: Weighted rate of calcium transitions (*events/μs*) for each C-hCx26 MD

Replica	Weighted rate (<i>events/μs</i>)	
	6 Ca ²⁺ (CAL)	6 Ca ²⁺ (CAM)
Replica 1	9.76	6.33
Replica 2	5.00	8.68
Replica 3	3.68	8.97

Table S5: Weighted rate of calcium transitions (*events/μs*) for each C-hCx46 MD

Replica	Weighted rate (<i>events/μs</i>)		
	3 Ca ²⁺ (CAL)	6 Ca ²⁺ (CAL)	6 Ca ²⁺ (CAM)
Replica 1	37.71	92.10	56.70
Replica 2	131.43	61.67	32.09
Replica 3	32.85	115.24	34.21

Table S6: Permeation events (*events/μs*) of K⁺ and water for each C-hCx46 model

Replica	K ⁺ permeation (<i>events/μs</i>)		
	No Ca ²⁺	3 Ca ²⁺	6 Ca ²⁺
Replica 1	278	167	69
Replica 2	289	206	167
Replica 3	301	245	266
Replica	Water permeation (<i>events/μs</i>)		
	No Ca ²⁺	3 Ca ²⁺	6 Ca ²⁺
Replica 1	108650	47650	45700
Replica 2	110719	49142	47052
Replica 3	112788	50634	48404

Table S7: Permeation events (*events/μs*) of Cl⁻ and water for each C-hCx26 model

Replica	Cl ⁻ permeation (<i>events/μs</i>)		
	No Ca ²⁺	6 Ca ²⁺ (CAL)	6 Ca ²⁺ (CAM)
Replica 1	24	42	8
Replica 2	28	64	18
Replica 3	16	52	24
Replica	Water permeation (<i>events/μs</i>)		
	No Ca ²⁺	6 Ca ²⁺ (CAL)	6 Ca ²⁺ (CAM)
Replica 1	73693	73956	30423
Replica 2	81573	71596	49885
Replica 3	72693	71581	59421

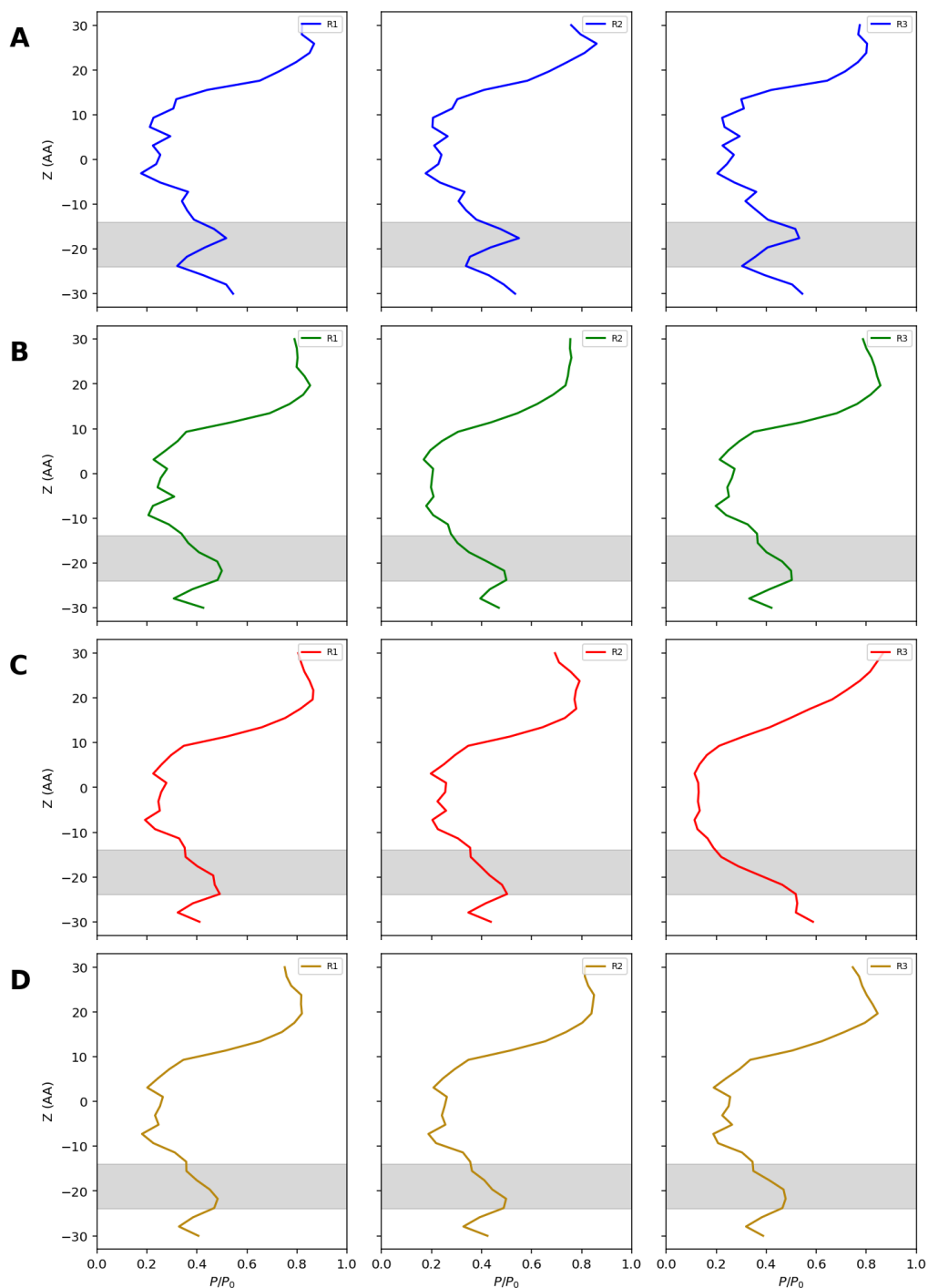


Figure S4: Relative water number density with respect to the bulk (P/P_0) along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas.

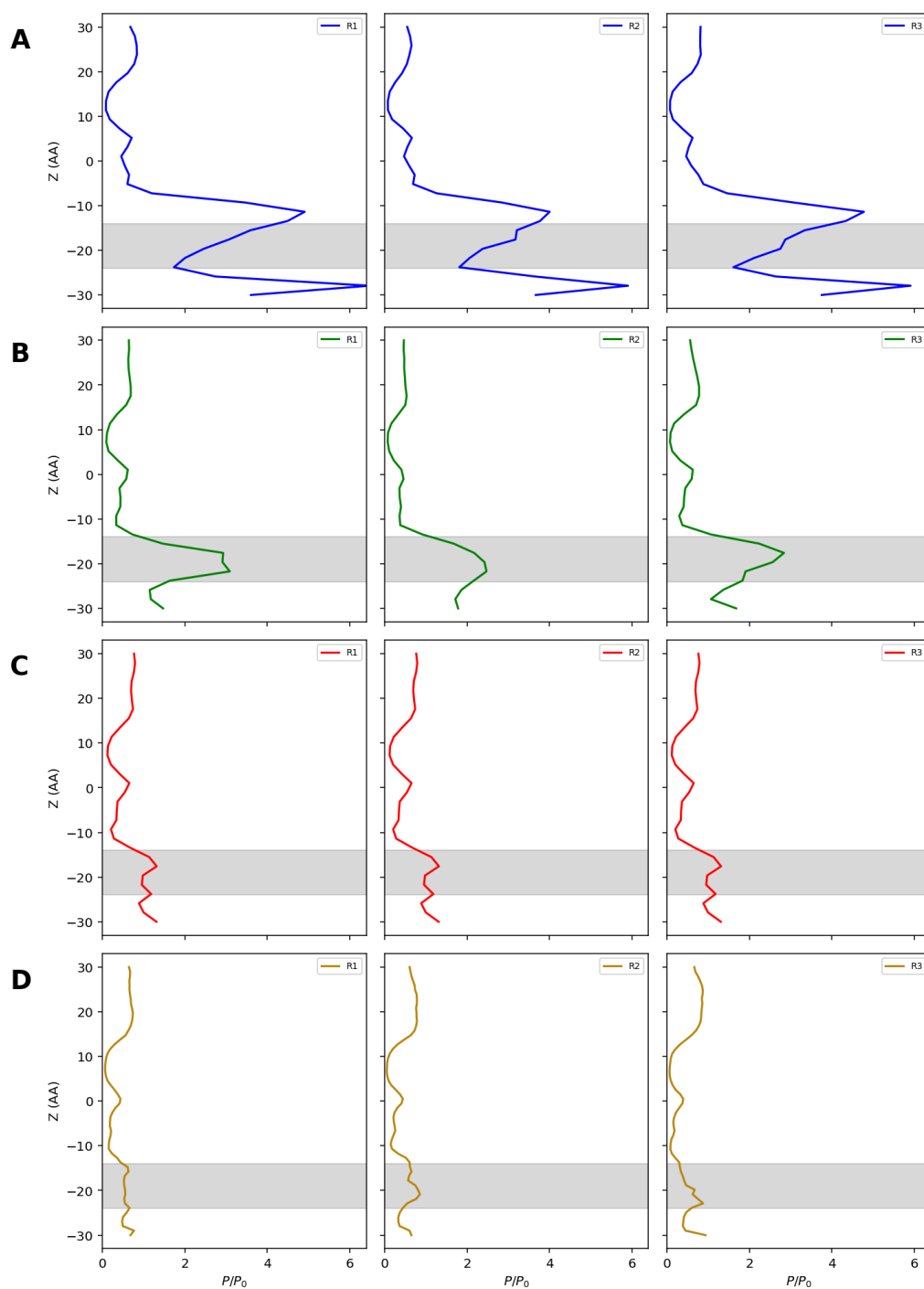


Figure S5: Relative K^+ number density with respect to the bulk (P/P_0) along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas.

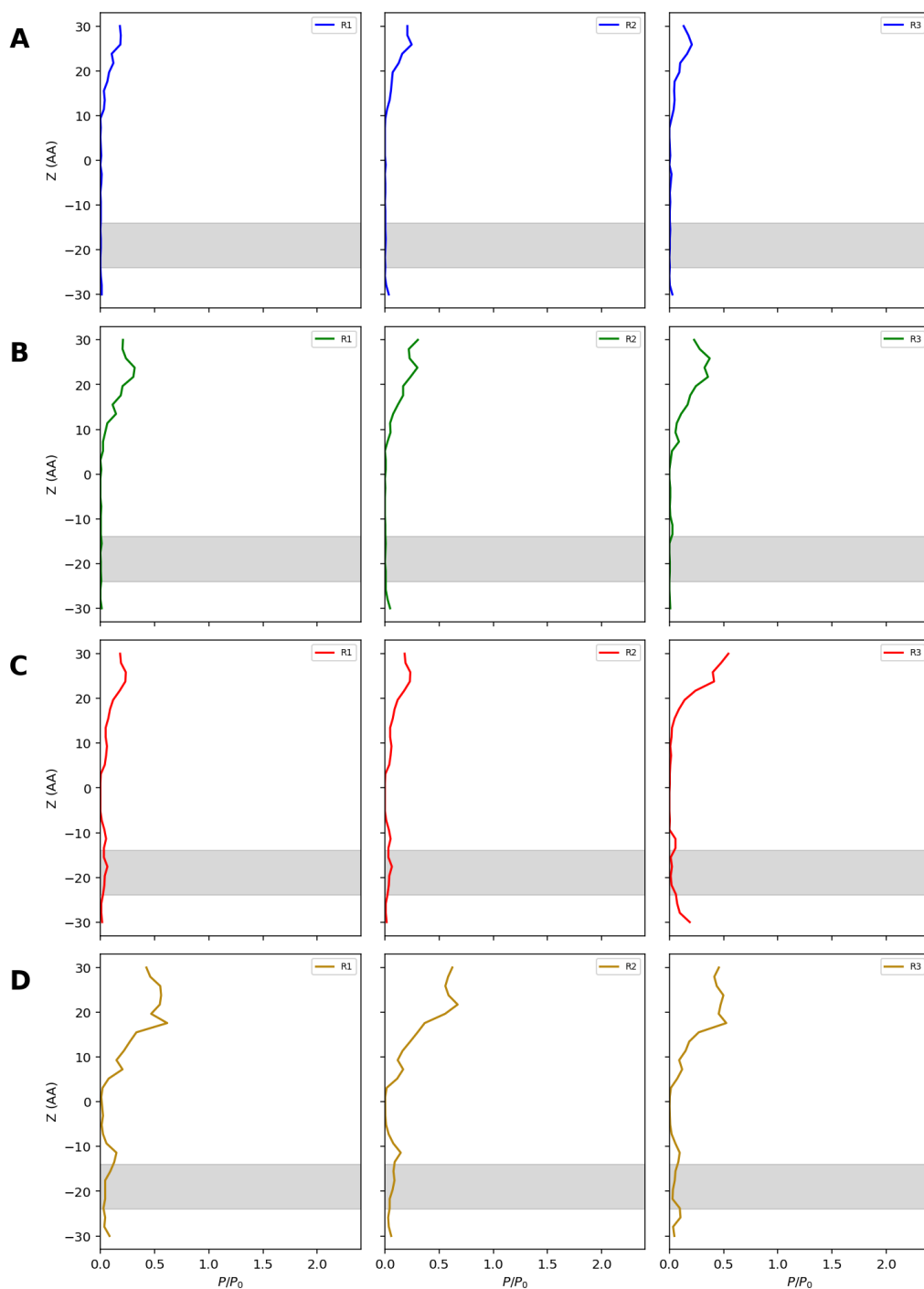


Figure S6: relative Cl^- number density with respect to the bulk (P/P_0) along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas

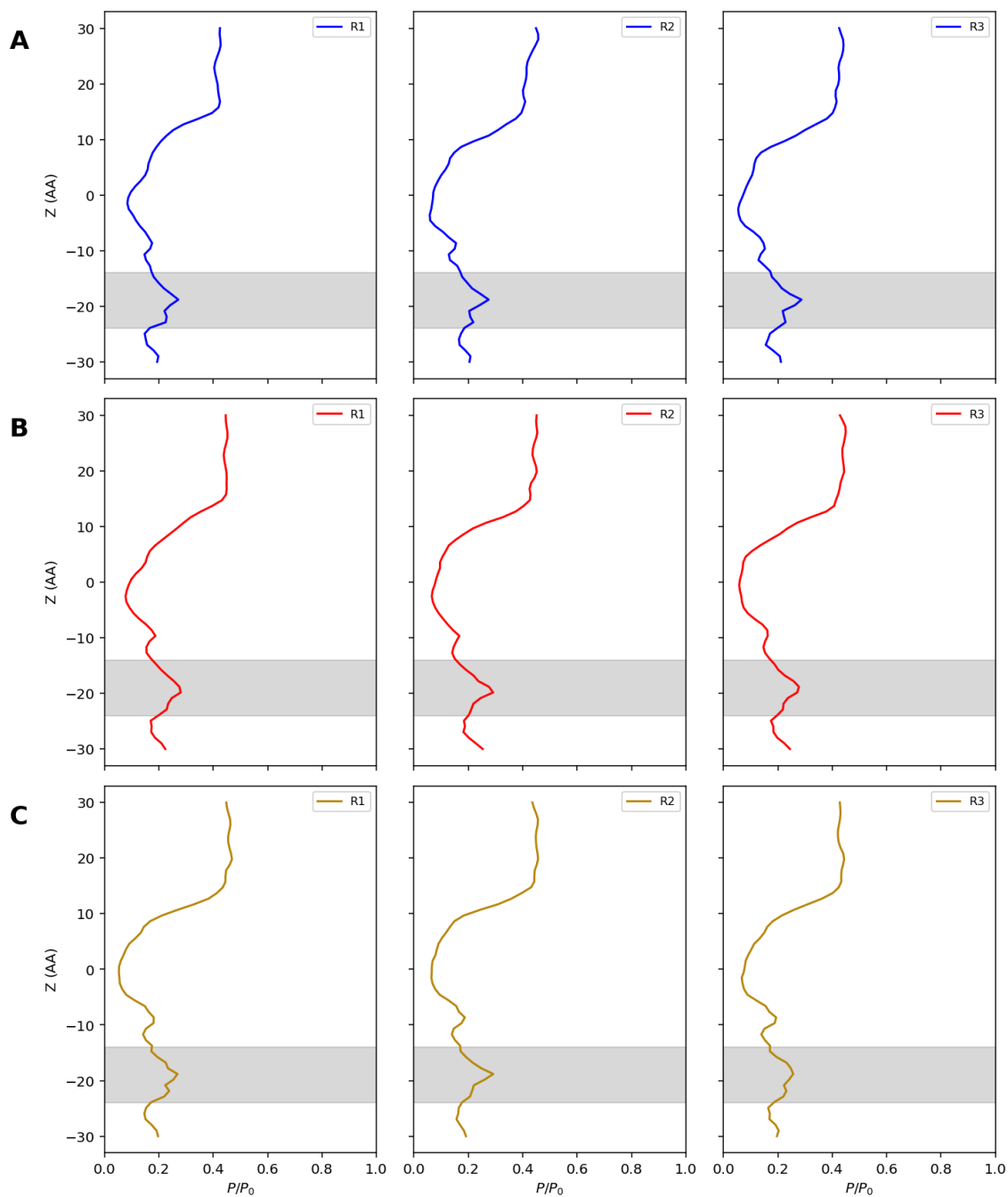


Figure S7: Relative water number density with respect to the bulk (P/P_0) along the pore for Each C-hCx26 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 6 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAM) model replicas.

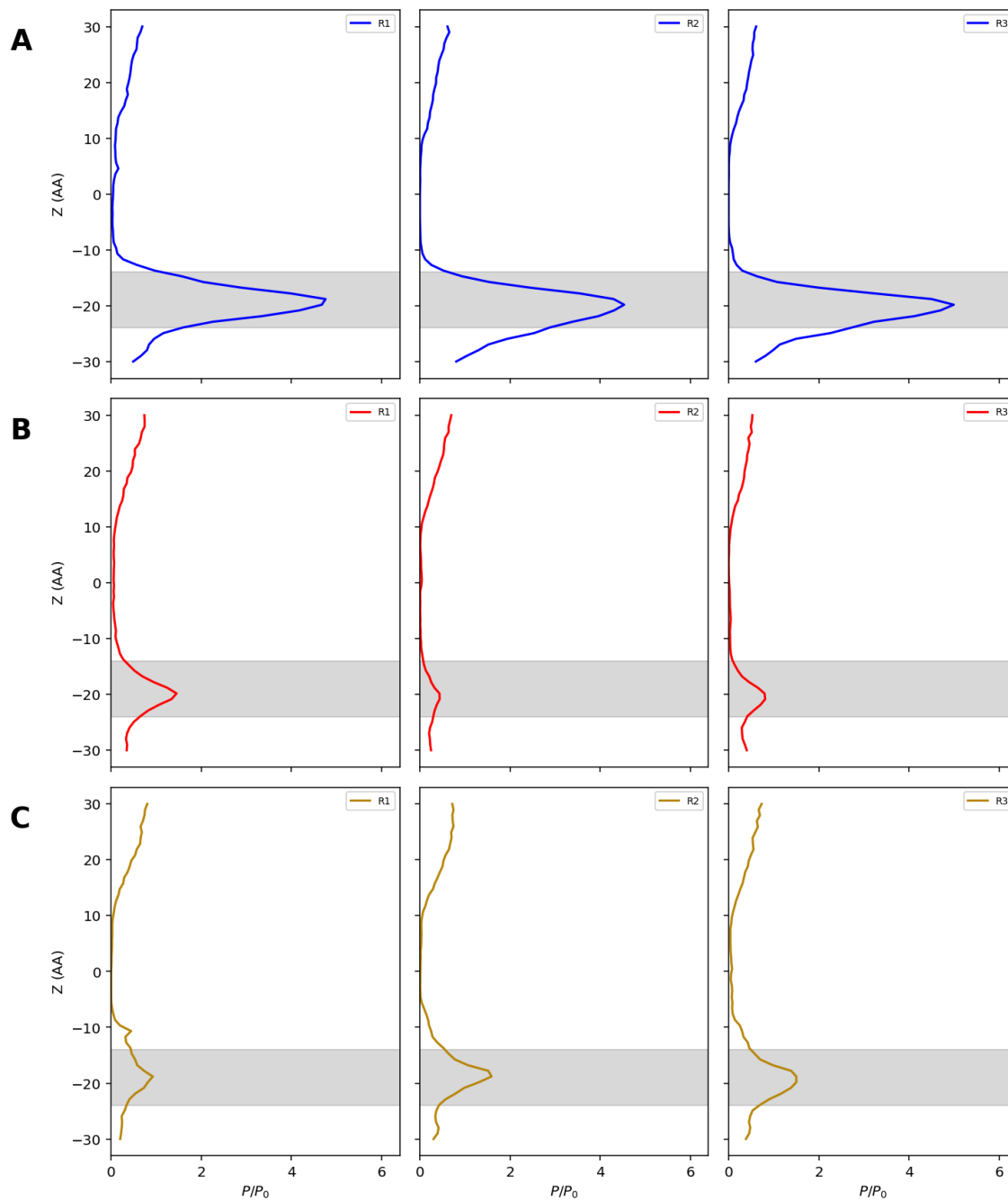


Figure S8: Relative K^+ number density with respect to the bulk (P/P_0) along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas.

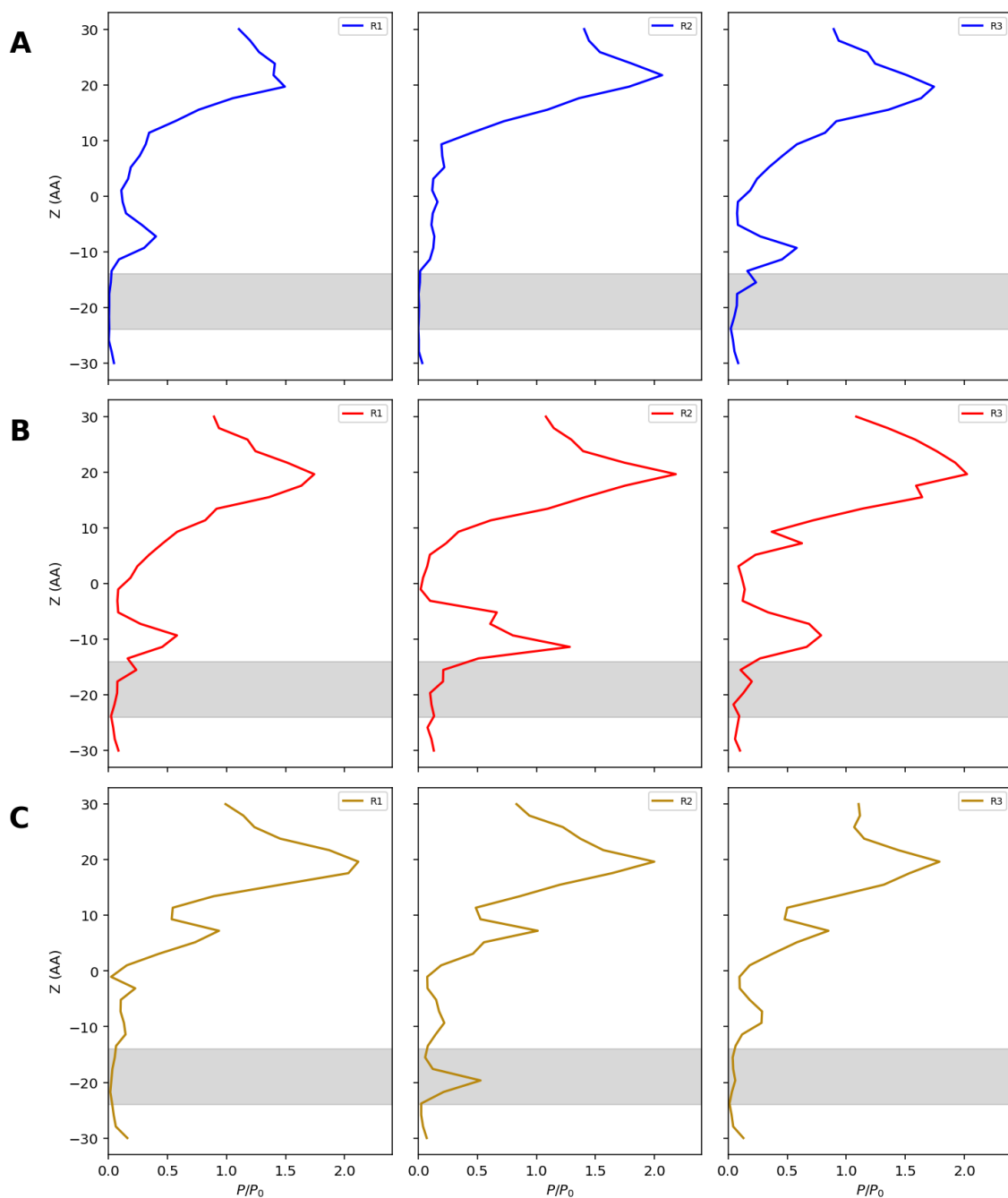


Figure S9: Relative Cl^- number density with respect to the bulk (P/P_0) along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas.

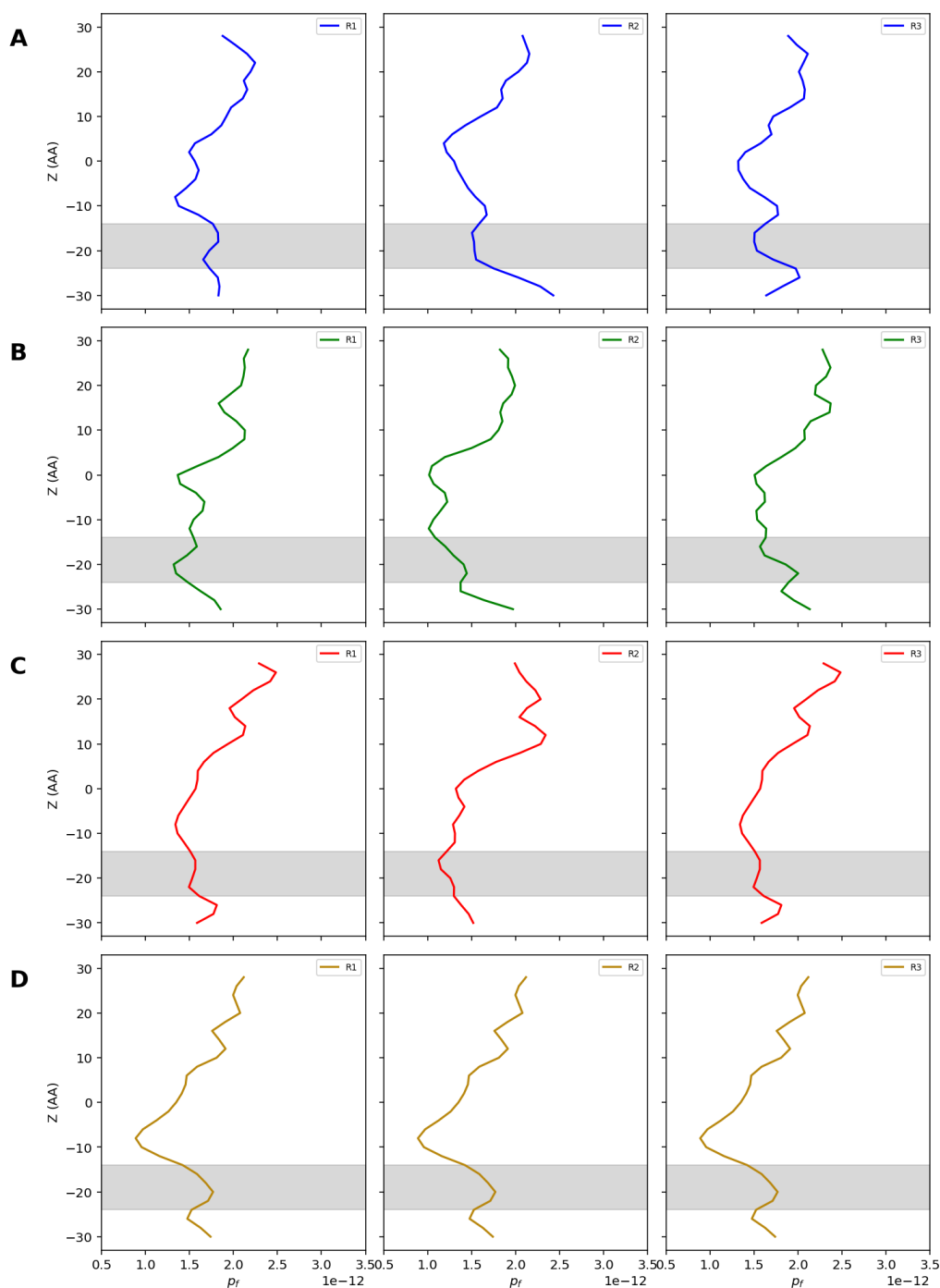


Figure S10: Unitary permeability osmotic coefficient along the pore for Each C-hCx46 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 3 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAL) model replicas. (D) 6 Ca^{2+} (CAM) model replicas.

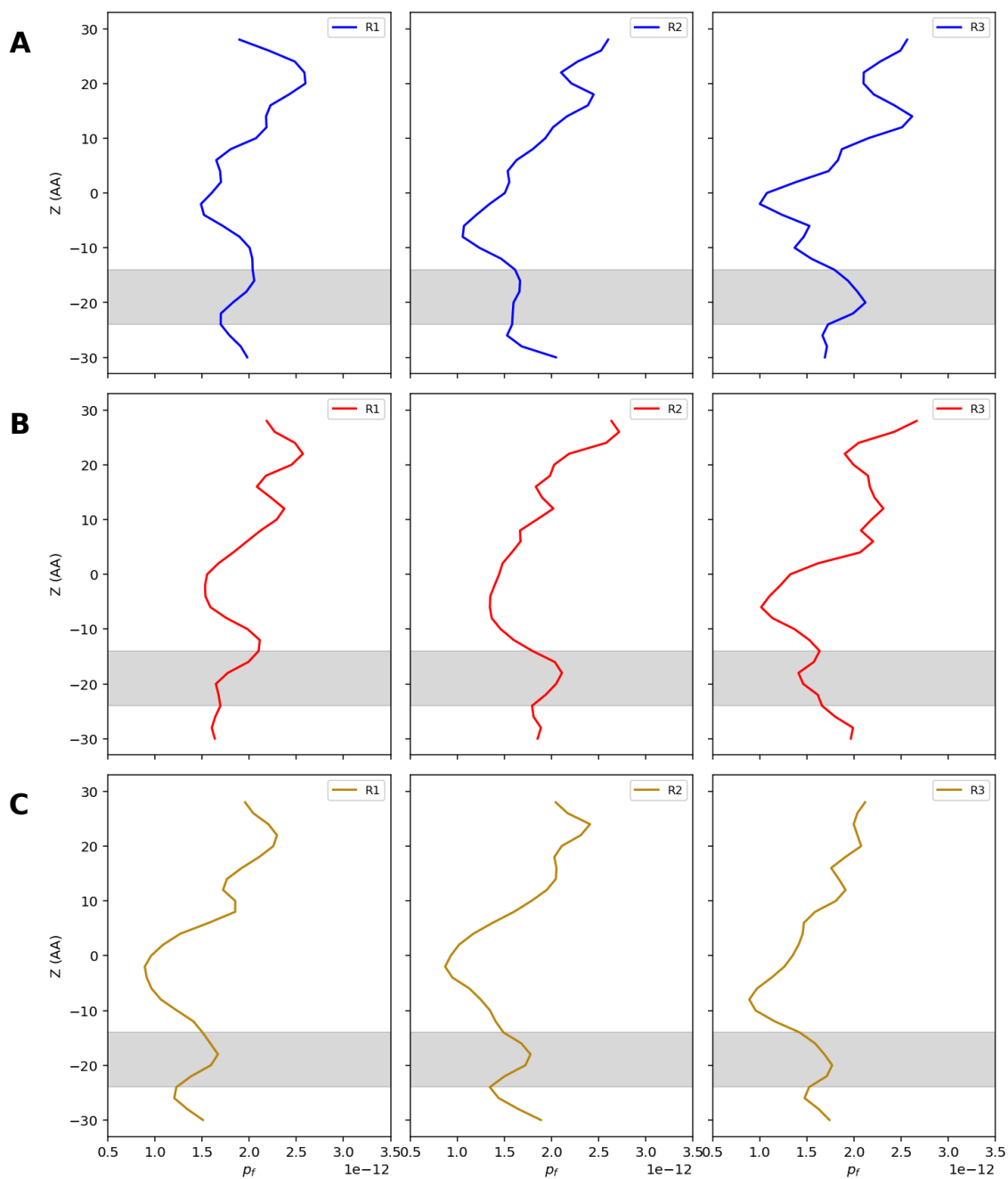


Figure S11: Unitary permeability osmotic coefficient along the pore for Each C-hCx26 MD, the Ca^{2+} confinement zone is shaded in gray. (A) No Ca^{2+} model replicas. (B) 6 Ca^{2+} (CAL) model replicas. (C) 6 Ca^{2+} (CAM) model replicas.

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Chapter 4

Discussion

“The idea that the future is unpredictable is undermined every day by the ease with which the past is explained.”

Daniel Kahneman
Thinking, Fast and Slow

4.1 Ca^{2+} Confinement Zone, Charge Symmetry, and Entropic Gain

C-hCx46 exhibits a unique calcium interaction behavior that is fundamentally distinct from the classical binding pockets observed in C-hCx26. Initially, this divergence might appear to be solely driven by the symmetry of the negatively charged residues (E42-D43 and D47-E48) that outline the boundaries of a toroidal confinement region. However, as our reduced “toy system” experiments demonstrate (Section 4.4), this local symmetric charge distribution is a necessary but not sufficient condition. The symmetric residues must operate synergistically with the sterically restricted architecture and the specific dielectric environment of the intact extracellular pore. Together, the protein scaffold and the symmetrical charges produce a smooth, quasi-equipotential electrostatic landscape, allowing for a dynamic equilibrium where multiple ions can be accommodated and delocalized simultaneously.

Unlike C-hCx26, where the substitution of E42 for a lysine (K) breaks this symmetry and—when placed within the full pore architecture—creates a localized potential trap closer to the extracellular exit, the C-hCx46 pore facilitates constant ion movement between subzones. Our results suggest that the rigid binding behavior observed in C-hCx26, which rapidly reaches a plateau with virtually zero re-entry events, is a direct consequence of this broken symmetry interacting with the confined pore environment.

Crucially, the capacity of C-hCx46 to consistently bind a higher number of Ca^{2+} ions can be explained thermodynamically by an entropic gain. In statistical mechanics, the entropy S of a system is related to the number of accessible microstates Ω by the Boltzmann equation:

$$S = k_B \ln \Omega \quad (4.1)$$

Because the toroidal isopotential region in C-hCx46 provides a significantly larger accessible volume compared to the restricted pockets in C-hCx26, the number of positional microstates (Ω) favorable for Ca^{2+} interaction is vastly increased. This volumetric expansion renders a highly favorable entropic contribution ($\Delta S > 0$) to the binding free energy ($\Delta G = \Delta H - T\Delta S$). This entropic gain effectively offsets the electrostatic repulsion between the multiple calcium ions, allowing higher occupancy. Despite this repulsion, transient proximity between Ca^{2+} ions occurs in C-hCx46, highlighting a

finely tuned electrostatic confinement mechanism that maintains dynamic loading Trexler et al., 2000.

4.2 Dynamics and Stability of the Ion Models

To assess the robustness of these interaction dynamics, we evaluated the confinement zone using both CAL and CAM multi-site ion models. While the CAM model—parametrized to yield lower interaction energies—showed lower transition frequencies due to its reduced diffusivity in hydrated environments, these transitions remained significantly higher than those in C-hCx26. Given that the ions move along an isopotential surface in C-hCx46, diffusivity rather than binding enthalpy appears to govern this dynamic structuring. Both models qualitatively confirm that the delocalized, entropically favorable loading capacity is a robust and unique feature of Cx46, triggered by its specific amino acid composition at the extracellular mouth.

4.3 Channel Selectivity and the Electrostatic Bottleneck

Impact on Water and Potassium Permeation

A significant finding of this study is that Ca^{2+} binding does not fully occlude the channel, but rather kinetically modulates ion and water flow. For K^+ , permeation events decreased by 29% and 42% in the 3-ion and 6-ion models, respectively. However, the impact on water is far more profound, with osmotic permeability dropping more than two-fold (an approximate 57% decrease).

This striking reduction in water permeation raises the question of whether the decrease in water flux is merely a secondary effect of reduced ion co-transport (electro-osmotic drag) or a direct consequence of Ca^{2+} binding. Analyzing the permeation events in C-hCx46 (Table 3.3), the reduction of K^+ permeation by approximately 122 events/ μs (in the 6-ion CAL model) correlates with a massive drop of over 63,000 water permeation events/ μs . If this water reduction were exclusively due to the loss of K^+ -driven co-transport, each potassium ion would need to drag an un-

physical number of water molecules (≈ 500). Furthermore, in the C-hCx26 model, Ca^{2+} binding (CAL model) actually increases Cl^- permeation while simultaneously decreasing water permeation, effectively decoupling water flux from ion permeation.

Therefore, the severe restriction in osmotic permeability must be a direct result of the calcium ions themselves, acting through a synergistic combination of thermodynamic and kinetic barriers. Thermodynamically, this can be quantitatively understood through the Born model of ion solvation, which approximates the electrostatic free energy of solvation (ΔG_{solv}) as:

$$\Delta G_{\text{solv}} = -\frac{N_A z^2 e^2}{8\pi \epsilon_0 r} \left(1 - \frac{1}{\epsilon_r} \right) \quad (4.2)$$

where N_A is Avogadro's number, z is the ion valence, e is the elementary charge, r is the effective ionic radius, and ϵ_r is the relative permittivity of the solvent. As shown in Equation 4.2, ΔG_{solv} scales with the square of the ion's valence (z^2). Consequently, the estimated solvation energy is $\Delta G_{\text{solv}} \approx -22 \text{ kcal/mol}$ for K^+ and deepens significantly to -122 kcal/mol for Ca^{2+} . The extremely strong solvation of Ca^{2+} causes water molecules to become highly structured and tightly bound to the resident calcium ions, creating a functional "hydration plug".

Crucially, these highly hydrated Ca^{2+} ions are not static. Kinetically, they transit rapidly within the toroidal confinement zone ($\approx 90 \text{ events}/\mu\text{s}$ for the CAL model). This constant transversal movement effectively acts as a dynamic "sweeping" mechanism, continuously disrupting the hydrogen-bond networks required for continuous water flux. This kinetic barrier is elegantly supported by our multi-site (CAM) model simulations. In C-hCx46, the CAM model diffuses more slowly (transitions drop to $\approx 41 \text{ events}/\mu\text{s}$), which reduces the frequency of this dynamic sweeping and, consequently, allows the water permeation to partially recover compared to the CAL model. Conversely, in C-hCx26, where the Ca^{2+} ions are rigidly trapped (almost zero transitions), the static CAL ion presents a much smaller steric obstacle, allowing continuous water wires to form and bypass the ion almost entirely.

Thus, the inhibition of water permeation in C-hCx46 is driven by a powerful synergy: the reduction of K^+ co-transport, the thermodynamic formation of a Ca^{2+} "hydration plug", and the kinetic disruption caused by its high-speed dynamic sweeping across the pore.

4.4 Assessing the Impact of the E42K Substitution

To isolate the specific impact of the E42K substitution, a reduced "toy system" comprising only the charged boundary residues was constructed. Surprisingly, the isolated residues displayed equalized inter-site transition rates for both C-hCx26 and C-hCx46. This demonstrates that the local electrostatics of the amino acid pairs alone cannot account for the rigid Ca^{2+} trapping observed in C-hCx26. Instead, the full protein scaffold is required to spatially restrict water mobility and lower the local dielectric constant. The E42K substitution effectively acts as an electrostatic trap only when placed inside the specific, sterically restricted architecture of the intact connexon pore.

4.5 Emergence of 3-10 Helices and Long-Range Communication

Interestingly, the presence of extracellular Ca^{2+} correlates with the emergence of 3-10 helices in the intracellular loop (ICL) (Table S1). Although these domains are spatially distant, this transient structural transition suggests a mechanism of subtle allosteric and electrostatic communication across the protein scaffold. These motifs may reflect the channel's inherent structural plasticity and could serve as precursors to the larger conformational rearrangements required for channel gating.

4.6 Relation to Fast Gate Closure and the Semiconducting State

Recent structural findings Flores et al., 2025 link intracellular Ca^{2+} to fast gate closure via N-terminal helix (NTH) rearrangement. Our data align with this paradigm, demonstrating extracellular calcium binding while the channel remains in the open state. The ability of Cx46 to bind calcium at both intra- and extracellular sites—unlike channels such as Cx31.3, which bind calcium exclusively intracellularly Lee et al., 2020—suggests a complex cross-regulatory mechanism. We speculate that extracellular binding,

by altering the electrostatic potential across the pore, may modulate the affinity of the intracellular sites, acting as a prerequisite for transitioning to the semiconducting or fully closed states.

4.7 Prospects for Other Connexins: Exploratory Comparative Analysis

To explore the evolutionary conservation of this toroidal confinement mechanism, we performed sequence alignments and electrostatic profiling across the human connexin family (Fig. S2, S3A). Regions with similar equipotential profiles to C-hCx46 were clearly identified in C-hCx43 and C-hCx50 (Figs. S3C–F). These candidates share high sequence similarity in the critical E42–D48 regions and present promising avenues for future studies. This exploratory screening provides a strategic roadmap for subsequent simulations, identifying which connexins likely utilize this "dynamic loading" mechanism versus those that employ a rigid localization trap.

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Chapter 5

Conclusions & Perspectives

There is nothing like looking, if you want to find something.
You certainly usually find something, if you look,
but it is not always quite the something you were after.

The Hobbit
J.R.R. TOLKIEN

In this research, the dynamics of calcium ion (Ca^{2+}) interaction with the human connexon 46 (C-hCx46) were thoroughly investigated utilizing robust molecular dynamics (MD) simulations. The primary aim was to elucidate the molecular and biophysical bases of its calcium-mediated regulatory mechanism, clearly distinguishing C-hCx46 from closely related hemichannels such as C-hCx26.

Initially, leveraging the high-resolution ovine Cx46 (sheep Cx46) template, we successfully constructed a highly refined, full-atomistic 3D model of C-hCx46. A combined approach of comparative modeling methods and artificial intelligence evaluations verified robust structural convergence, providing a highly reliable representation of the extracellular vestibule critical for subsequent functional studies.

Our findings elucidate a novel, highly dynamic Ca^{2+} binding mode. We characterized a toroidal confinement region outlined by a symmetrical arrangement of negatively charged residues (E42, D43, D47, and E48). Crucially, through the evaluation of reduced “toy systems” and multiple multi-site ion models (CAL and CAM), we demonstrated that this local charge symmetry is a necessary but not sufficient condition; it must operate synergistically with the sterically restricted architecture of the intact protein scaffold. Together, they create a quasi-equipotential electrostatic landscape that allows Ca^{2+} to transit freely among subzones at high rates (≈ 80 events/ μs), rather than being locked into rigid binding pockets.

Thermodynamically, this symmetric and expanded binding volume provides a significant entropic gain ($\Delta S > 0$), vastly increasing the number of accessible microstates for the interacting ions. This offers a compelling molecular explanation for the higher Ca^{2+} binding affinities and larger loading capacities reported for C-hCx46 compared to C-hCx26, where the E42K substitution creates a rigid, localized electrostatic trap that severely limits dynamic occupancy.

Functionally, our simulations reveal that extracellular Ca^{2+} binding modulates, rather than fully occludes, the channel’s permeability. We observed a partial reduction in K^+ permeation (up to 42%) driven by the electrostatic screening of the pore’s negative surface. More strikingly, water permeation experienced a severe, more than two-fold reduction (an approximate 57% decrease). We demonstrate that this phenomenon goes beyond a simple reduction in ion co-transport (electro-osmotic drag). Instead, it is driven by a powerful synergy of thermodynamic and kinetic

barriers. As described by the Born electrostatic free energy of solvation, the highly charged Ca^{2+} ions strongly structure surrounding water molecules, effectively forming a “hydration plug”. Concurrently, the rapid transversal movement of these solvated ions within the confinement zone establishes a kinetic barrier via a dynamic “sweeping” mechanism, continuously disrupting the hydrogen-bond networks required for water permeation.

Furthermore, our comparative analyses highlighted intrinsic differences in pore selectivity. We demonstrated that the native K15E substitution in C-hCx46 facilitates its characteristic cation selectivity, contrasting with the unmodified C-hCx26 scaffold which repels cations due to its positive extracellular rim. Finally, the transient emergence of 3-10 helices in the distant intracellular loop upon extracellular Ca^{2+} binding suggests a mechanism of indirect allosteric communication across domains. This indicates that extracellular binding may act as a vital precursor to the larger conformational rearrangements required for fast gate closure or transitioning to the semiconducting state.

All in all, this study establishes a new paradigm for divalent cation regulation in connexons, shifting the focus from rigid binding sites to dynamic, entropically driven confinement zones. Preliminary sequence and electrostatic screenings suggest that this “dynamic loading” mechanism is conserved in other human connexins, such as C-hCx43 and C-hCx50. These candidates present promising avenues for future biophysical explorations, providing a strategic roadmap to further decode the structural plasticity and regulatory complexity of the human connexin family.

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