WHAT (ELSE) TRAVELS SILENTLY THROUGH BIOLOGICAL NANOPORES?

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TEST AND MEASUREMENT STRATEGIES FOR THE DEVELOPMENT OF BIOLOGICAL NANOVALVES

Daniel L. Burden, Joshua J. Meyer, Richard D. Michael, Sophie C. Anderson, Sophia M. Peña, Kristin Joy Leong-Fern, Lily Anne E. Van Ye, Elizabeth C. Meyer, Lisa M. Keranen-Burden

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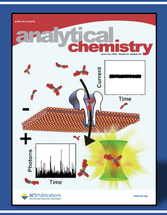

ABSTRACT

Most everything known about the luminal passage of materials through ion channels and nanopores arises from sensitive electrical measurements. Whether nanopores are biological, solid-state, synthetic, hybrid, glass capillary-based, or protein ion channels in cells and tissues, characteristic signatures embedded in the flow of ionic current are foundational to understanding functional behavior. In contrast, this work describes passage through a nanopore that occurs without producing an electrical signature. We refer to the phenomenon as "silent translocation." By definition, silent translocations are invisible to the standard tools of electrophysiology and fundamentally require an ancillary measurement technique for positive identification. As a result, the phenomenon has been virtually unexplored in the literature. Here, we uncover the existence of silent translocations using a derivative of Cyanine 5 (sCy5a) that passes through the alpha hemolysin (αHL) nanopore. Simultaneously acquired single-molecule fluorescence and single-channel electrical recordings from bilayers formed over a closed microcavity demonstrate that translocation does, indeed, take place, albeit infrequently. We report observations of silent translocation as a function of time, dye concentration, and nanopore population in the bilayer. Lastly, measurement of the translocation rate as a function of applied potential permits estimation of an effective energy barrier for transport through the pore, as well as the effective charge on the dye, all in the absence of an information-containing electrical signature. The findings initiate a search for additional compounds that translocate silently but have yet to be discovered.

BACKGROUND

Alpha Hemolysin Oligomer

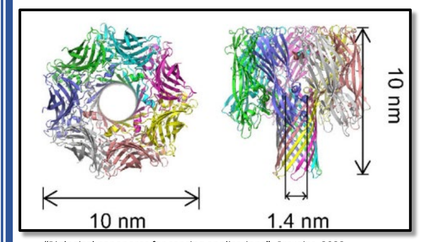

Figure 1: Side and top view of an oligomerized heptameric αHL nanopore, secreted from *Staphylococcus aureus*.[1]

"Biological nanopores for sensing applications", *Proteins,* **2022.**

Electrical Measurement of Translocation

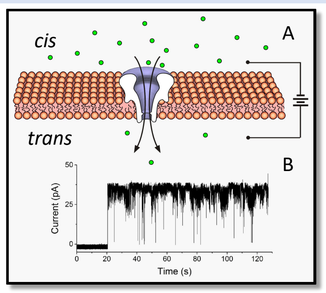

Figure 2: Current fluctuations can be generated with an αHL nanopore embedded in a lipid membrane by applying a potential via two electrodes on opposite sides of the bilayer (A). Analytes (green) that traverse or interact with the pore momentarily block ion flow (B).[2]

- Single-molecule electrical events have been used to characterize numerous translocating analytes.[3]

Optical Measurement of Translocation

- A few publications have documented fluorescent dyes that translocate αHL due to diffusion.[4,5]
- However, only simultaneous dual modality measurements can confirm electrical silence:
  - Optically determined translocation energetics have not yet been published.
  - The existence of analytes that do not produce electrical signals as they traverse single nanopores (i.e., silent translocations) have not yet been confirmed.

MATERIALS AND METHODS

Electrical & Optical Apparatus

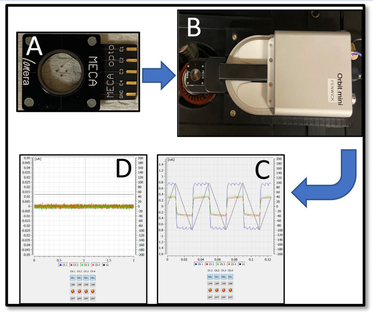

Figure 3: (A) MECAopto chip inserted into an (B) Orbit Mini (Nanion) allows application of an oscillating triangle wave (C) to confirm membrane formation. An applied DC voltage (D) drives analyte through the pore, tracks the number of αHL insertions, and produces fluctuation in current levels that reveal translocation events.

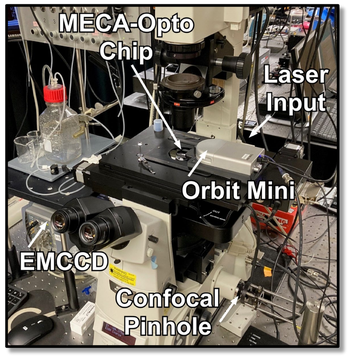

Figure 4: Single-molecule confocal fluorescence microscope.

Relative Molecular Scale & Orientation

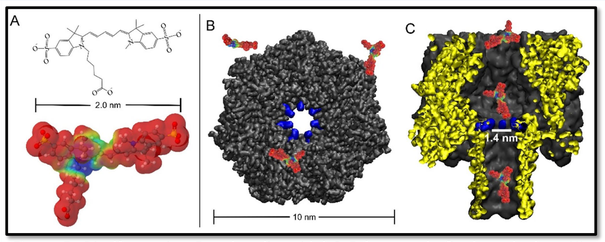

Figure 5: Disulfo-cyanine 5 carboxylic acid (sCy5a) can only fit through the αHL nanopore with a limited range of orientations.

RESULTS

Electrical Silence

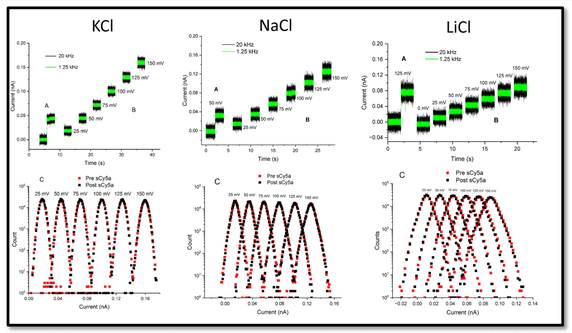

Figure 6: Current recordings are electrically silent in the presence of sCy5a (i.e., without statistically distinguishable current fluctuations) in multiple electrolytes (1 M) at all applied potentials.

RESULTS (cont.)

Optical Confirmation of Translocation

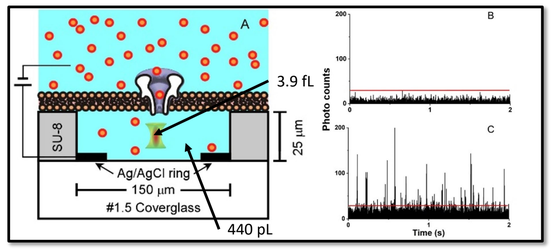

Figure 7: Confirming dye translocation via single-molecule fluorescence. Confocal optical detection in a MECAopto microcavity (A). The bilayer-capped microcavity is first photobleached to eliminate background counts (B). Application of +100 mV drives sCy5a through the nanopore, causing single-molecule photon bursts from the confocal detection volume positioned below the membrane (C).

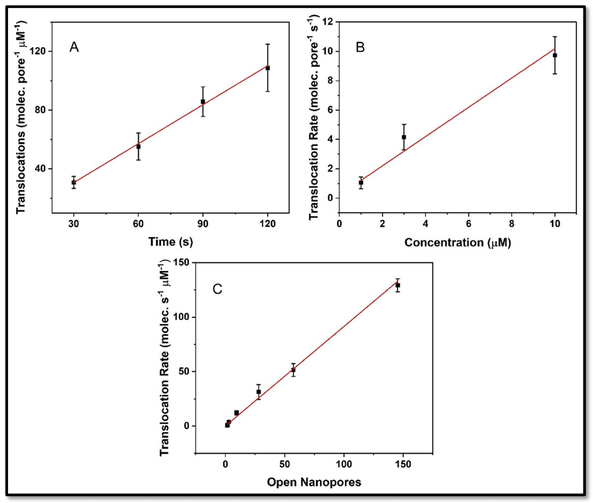

Figure 8. The number of translocated sCy5a molecules increases linearly as a function of accumulation time (slope ~0.90), sCy5a dye concentration on the *cis*-side of the membrane (slope ~1.0), and number of open αHL nanopores in the membrane (slope ~0.91).

Simulation and Analysis

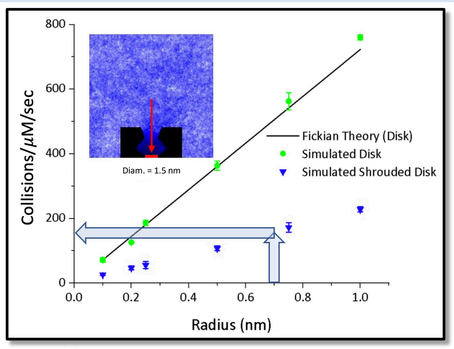

Figure 9: Fickian diffusion to a disk absorber is compared to digital simulations for estimating the collision frequency (ν) of sCy5a with the theoretical barrier. Inset: absorbing disk (red) shrouded by αHL contour (black). Surrounding color intensity corresponds to time-averaged molecule-location probability.

Main Finding: $\nu \cong$ **150 collisions/μM/nanopore/second**

RESULTS (cont.)

Voltage-Dependent Translocation

Figure 10: Transportation velocities through αHL at positive and negative applied potentials. Electroosmotic flow ($\overrightarrow{v_{eo}}$) and electrophoretic ($\overrightarrow{v_e}$) velocities change direction with potential. Concentration-driven diffusion ($\frac{dx_d}{dt}$) is independent of potential.

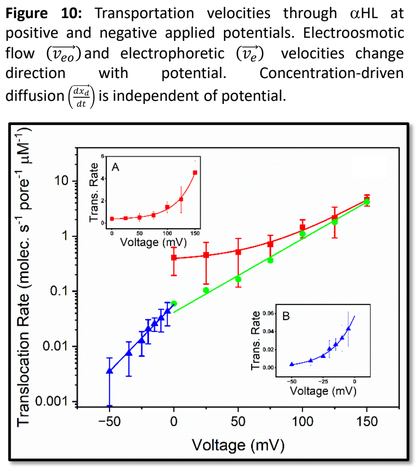

Figure 11: The normalized measured translocation rate ($R_{\vec{m}}^{\pm}$) of sCy5a follows an Arrhenius-like potential dependence[6] at both positive (red) and negative (blue) applied potentials. Positive potentials involve two subpopulations of sCy5a translocation to account for a potential independent transportation rate offset (R_i). This offset is not present at negative potentials. A discontinuity in $R_{\vec{m}}^{\pm}$ occurs at 0 mV due to the reversal of electrophoretic and electroosmotic flow, which either assists, or opposes, diffusion.

$$R_0^{\pm} = e^{-U_{eff}^{\pm}/kT}$$
$$R_m^{+} = R_i + R_0^{+} * e^{(|z_{eff}^{+}|eV)/kT}$$
$$R_{\bar{m}}^{-} = R_0^{-} * e^{(|z_{eff}^{-}|eV)/kT}$$

Figure 12: Arrhenius-like transition-state relation describes the measured $R_{\vec{m}}^{\pm}$. Least-squares analysis of the data yield $U_{\bar{eff}} \approx U_{eff}^{+} = (7.9 - 8.1)kT$, $|z_{eff}^{+}| = 0.78$, and $|z_{eff}^{-}| = 1.4$.

CONCLUSIONS

- For the first time, single-molecule optical measurements reveal translocations of molecules (sCy5a) that produce no electrical signal while traversing a nanopore (i.e., silent translocations).[7]
- For the first time, an apparatus capable of acquiring simultaneous single-molecule fluorescence and current recordings was used to determine the activation energy for translocation through the αHL nanopore (~8kT) and the effective charge of the translocator ($|z_{eff}^{+}| = 0.78$, $|z_{eff}^{-}| = 1.4$).[7]
- Confirmation of the first silent translocator in αHL initiates a search for additional compounds that might also undergo translocation silently in numerous different nanopores, especially those of physiological relevance.

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