Osmosis-Driven Motion-Type Modulation of Biological Nanopores for Parallel Optical Nucleic Acid Sensing

Yuqin Wang,†,§ Shuanghong Yan,†,§ Panke Zhang,†,§,∥ Zidao Zeng,§,¶ Di Zhao,†,§ Jiaxin Wang,§ Hongyuan Chen,‡,¶,§,∥ and Shuo Huang,†,‡,§

† State Key Laboratory of Analytical Chemistry for Life Sciences, ‡ Collaborative Innovation Centre of Chemistry for Life Sciences, and § School of Chemistry and Chemical Engineering, Nanjing University, 210023 Nanjing, China
§ Department of Chemistry, University of Pittsburgh, 219 Parkman Avenue, Pittsburgh, Pennsylvania 15260, United States

ABSTRACT: Recent developments in nanopore sequencing have inspired new concepts in precision medicine but limited in throughput. By optically encoding calcium flux from an array of nanopores, parallel measurements from hundreds of nanopores were reported, while lateral drifts of biological nanopores set obstacles for signal processing. In this paper, optical single-channel recording (oSCR) serves to track nanopores with high precision and a general principle of nanopore motion kinetics is quantitatively investigated. By finely adjusting the osmosis-oriented interactions between the lipid/substrate interfaces, motions of nanopores could be controllably restricted. Improved signal-to-noise ratio is observed from motion-restricted nanopores, which is experimentally demonstrated. To systematically evaluate oSCR with asymmetric salt concentrations, a finite element method simulation is established. oSCR with an array of immobilized nanopores suggests new strategies for sequencing DNA by microscopic imaging in high throughput and is widely applicable to the investigation of other transmembrane proteins.

KEYWORDS: nanopore, single molecule, transmembrane protein, gene sequencing, lipid membrane, high throughput, single-particle tracking

1. INTRODUCTION

The success of nanopore sequencing has inspired new concepts in precision medicine and point-of-care diagnosis. A commercialized nanopore sequencer (the MinION, Oxford Nanopore Technologies) now sequences DNA with particularly long read-length (6–60 kb) and single-molecule resolution in a USB-stick-sized device. This portability of MinION has enabled successful pathogenic sequencing of Ebola virus in Africa and even the first ever space sequencing scenario of trajectory crossing over between pores (Figure S1) or pores escaping out of the FOV. A pore in motion always reduces the signal-to-noise ratio (SNR) in the extracted fluorescence traces because of uneven laser excitation along its lateral drift trajectory. For practical need of parallel biosensing by oSCR, biological nanopores should be functioning but immobilized, better in a highly ordered array for addressability.

Recent developments in nanopore arrays and calcium imaging-based optical single-channel recordings (oSCRs) have suggested an alternative way to monitor nanopore translocation events in parallel via fluorescence imaging. In our previous work, translocation events from hundreds of nanopores are simultaneously detected within the same field of view (FOV). Clear sequence-specific recognition is demonstrated for homopolymeric DNA and miRNA samples. In principle, parallel detections with up to $10^7$ pores mm$^{-2}$ density are achievable with negligible cost for consumables, which inspires an innovative nanopore sequencing platform to possess millions of sensors in a fingertip size as a disposable diagnosis device for routine clinical uses.

However, unlike solid-state nanopores, the thermal drift of biological nanopores sets obstacles for image acquisition and data extraction during oSCR. Image analysis by spot tracking uses tremendous computation power and gets complicated in the scenario of trajectory crossing over between pores (Figure S1) or pores escaping out of the FOV. A pore in motion always reduces the signal-to-noise ratio (SNR) in the extracted fluorescence traces because of uneven laser excitation along its lateral drift trajectory. For practical need of parallel biosensing by oSCR, biological nanopores should be functioning but immobilized, better in a highly ordered array for addressability. However, protein immobilization by chemical modification or tag protein introduction may generate unpredictable consequences such as decreased protein preparation yield, protein denaturation, failure of oligomerization, and induced noises.

Received: December 3, 2017
Accepted: February 12, 2018
Published: February 12, 2018
In this paper, we present a universal and tag-free strategy to motion-modulate and immobilize biological nanopores. In brief, oSCR is performed over a droplet interface bilayer (DIB) immediately above a layer of spin-coated hydrogel as reported before. DIB, self-assembled by 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), establishes a semipermeable diffusion barrier for water molecules but not ions and macromolecules. Asymmetric salt concentrations in different sides of the DIB drive slow net movement of water molecules across the bilayer (Figure S2), which modulates the separation between the hydrogel substrate and the DIB. Hydrophilic attractions between the hydrogel and the lipid head group inhibit thermal diffusion of the lipid molecules but conversely retard the motion of the inserted ion channels as well.

To investigate the lateral motion kinetics of biological nanopores in different asymmetric buffer conditions across a lipid bilayer, oSCR is utilized to track individual nanopores in the DIB. Long been used as biosensors, limited studies about the dynamics of transmembrane proteins have been reported. Some pioneering works have investigated the lateral motions of protein nanopores using single-particle tracking of labeled fluorescent dyes and chelating reagents. To mimic a patch clamp instrument, oSCR can sometimes replace traditional electrophysiology measurements when the time resolution of acquisition is not critical. However, the physical origin of Ca$^{2+}$-induced fluorescence involves complicated processes such as diffusion of ions, electrical migration of charged species, and dynamic equilibrium between fluorescent dyes and chelating reagents. To better understand the oSCR system with asymmetric salt concentrations, a finite element method (FEM) model coupling all contributing terms listed above is established and evaluated.

2. EXPERIMENTAL SECTION

2.1. Materials. Hexadecane, silicone oil AR30, pentane, ethylenediaminetetraacetic acid (EDTA), Triton X-100, and Genapol X-80 are from Sigma-Aldrich. DPhPC is from Avanti Polar Lipids. Fluo-8H′sodium salt (Fluo-8) is from AAT Bioquest. 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) is from Sigma-Aldrich. Low melting point agarose is from Takara. Ethanol and acetone are from Sinopharm. Potassium chloride, sodium chloride, sodium hydrogen phosphate, and sodium dihydrogen phosphate are from Aladdin. (HEPES) is from Shanghai Yuanye Bio-Technology. E. coli strain BL21(DE3) pLysS and BL21(DE3) are from BioMed. Dioxide-free isopropyl-β-D-thiogalactopyranoside, ampicillin sodium, kanamycin sulfate, and imidazole are from Solarbio. LB broth and LB agar are from Hopebio. All the items listed above are used as received.

The potassium chloride buffer (0.3–2.5 M KCl, 10 mM HEPES, pH 7.0) and the calcium chloride buffer (0.75 M CaCl$_2$, 10 mM

Figure 1. Imaging and tracking of α-HL nanopores in a DIB. (a) Schematic diagram of the DIB setup. A DIB is supported on a spin-coated agarose layer over the coverslip. A 60X TIRF objective is used to both illuminate and image the area. A constant voltage (+100 mV) is applied using Ag/AgCl electrodes present in the droplet and the agarose substrate. Simultaneously, the cationic Ca$^{2+}$ ions flow from the agarose (trans) into the droplet (cis) through each individual nanopore establishing a stable gradient of Ca$^{2+}$ ions in the vicinity area above each pore. This gradient of Ca$^{2+}$ generates a sharp image contrast when bound with the calcium-sensitive dye Fluo-8 evenly distributed in the droplet. Thus, the center of each pore could be visually tracked. Dashed box: motion tracks of α-HL nanopores in a DIB. (b) Top: a serial snapshot of α-HL nanopores (cis: 0.5 M KCl, trans: 0.75 M CaCl$_2$). Pores appear as bright moving spots in a DIB in different frames. Bottom: corresponding motion tracks of each pore in different frames. The circles indicate the pore position in the current frame, while the tracks depict the trajectories of drifting nanopores up to this time. The pore tracking analysis was performed using the ImageJ plugin: MosaicSuite. Scale bar: 20 μm. (c) Immobilizing α-HL nanopores by varying the KCl concentration in the droplet. The lateral motions of α-HL nanopores are gradually suppressed when the KCl concentration in cis is increased from 0.5 to 1.5 M. Scale bar: 20 μm.
conductivity of the protein nanopore. The purification of the protein activities is performed as previously published.\textsuperscript{15} A DIB of [KCl] in cis (the droplet in Figure 1a). Pore motions are gradually suppressed with increasing [KCl] in cis, while the salt concentration in trans is kept the same. No significant drifting is further observed when [KCl] reaches 1.5 M. (b) MSD of representative trajectories of \(\alpha\)-HL nanopores in different [KCl] in the droplet. The MSD is proportional to the power exponent \(\alpha\), where anomalous exponent (\(\alpha\)) is linked with the motion forms. When [KCl] = 0.3 or 0.5 M, the pores follow superdiffusion (\(\alpha > 1\)). When [KCl] = 1 M, the pores follow Brownian diffusion (\(\alpha \approx 1\)). When [KCl] = 1.5–3 M, the pores follow subdiffusion (\(\alpha < 1\)). Here, the MSD curves are almost overlapped, the details of which are supplemented in the box on the left. Representative tracks are present for [KCl] = 0.5 M (red), 1 M (blue), and 2.5 M (purple). The purple dotted trajectory in the dashed box is scaled up ten times for demonstration. (c) Mean values of anomalous exponent as a function of [KCl] in the droplet for both \(\alpha\)-HL and MspA pores. (d) Mean values of generalized diffusion coefficients as a function of [KCl] in the droplet for both \(\alpha\)-HL and MspA pores. The two coefficients are calculated according to the logarithmic MSD curve for each track. Thirty tracks were recorded for each condition in (c,d).

HEPES, pH 7.0) are prepared prior to use. For simplicity, 0.3–2.5 M KCl buffer stands for 0.3–2.5 M KCl, 10 mM HEPES, pH 7.0, if not otherwise stated. Particularly, the KCl buffer is treated with Chelex 100 resins (Bio-Rad) overnight before use to get rid of potential divalent ion contaminations. High-performance liquid chromatography (HPLC)-purified DNA (Table S1) is dissolved in DNase-/RNase-free water prior to use. To form hairpin DNA structures, the HPLC-purified DNA is dissolved in electrolyte buffer (1.5 M KCl, 10 mM HEPES, pH 7.0), heated up to 95 °C, and gradually cooled down (−5 °C/min) to room temperature (25 °C) on a PCR thermal cycler (ABI 2720).

2.2. Methods. 2.2.1. Preparation of Biological Nanopores. WT \(\alpha\)-HL and MspA NNNRRKK nanopores are expressed with E. coli BL21(DE3) and purified with nickel affinity chromatography (Supporting Methods 1 and 2; Figures S3 and S4). The purified nanopores are further characterized (Figure S5) with a patch clamp amplifier (Axon 200B, Molecular Devices) to verify the ionic conductivity of the protein nanopore. The purified protein is directly used in oSCR.

2.2.2. Imaging Biological Nanopores with oSCR. oSCR of nanopore activities is performed as previously published.\textsuperscript{15} A DIB of DPhPC is formed between an aqueous droplet and a thin layer of the hydrogel over a coverslip immersed in lipid/oil solution (Supporting Methods 3–6). Nanopores dissolved in the droplet spontaneously insert into the DIB and form ion pathways across the bilayer. A pair of custom Ag/AgCl microelectrodes are inserted into the droplet and the hydrogel, respectively, to apply the potential. At positive potential, Ca\(^{2+}\) ions flow from the hydrogel into the droplet and a gradient of Ca\(^{2+}\) is established in the vicinity of each nanopore. The established Ca\(^{2+}\) gradient generates a stable image contrast when bound with calcium-sensitive dye Fluo-8 in the droplet. A 60X total internal reflection fluorescence (TIRF) objective (N.A. = 1.49, Nikon) is used for illuminating and imaging the DIB area. A 100 mW 473 nm fiber-coupled laser (Changchun New Industries Optoelectronics Technology) illumination is used to excite the fluorescence. An electron-multiplying charge-coupled device (EM-CCD) (iXon 897, Andor) is used to acquire the image results in stacks (Supporting Methods 7).

2.2.3. Pore Tracking and Mean Square Displacement (MSD) Analysis. MosaicSuite in ImageJ is used to track fluorescence traces could be extracted continuously from different frames, fluorescence traces could be extracted continuously by linking values in different frames in series. The intensity is then normalized so that the open pore fluorescence equals to 0.8 and the lowest blockage fluorescence equals to 0.

2.2.5. FEM Simulation of oSCR. FEM simulations are performed in a COMSOL 5.3 program in a personal computer (Intel i5 (3.3 GHz), 32 Gb RAM).

3. RESULTS AND DISCUSSION

3.1. Pore Motion Kinetics with Asymmetric Salt Concentrations. Experimentally, we image \(\alpha\)-HL nanopores in a DIB setup (Figure 1a). By optically probing Ca\(^{2+}\) flux through a pore using indicator dye Fluo-8, a nanopore appears as an extremely bright spot sized 3–5 μm in diameter. During pore tracking by oSCR, a constant bias \(V = +100\) mV is applied, which drives a sustained Ca\(^{2+}\) flow into the droplet and thus motion of individual nanopores. The two coefficients are calculated according to the logarithmic MSD curve for each track. Thirty tracks were recorded for each condition in (c,d).
µm × 136 µm) with an EM-CCD (1 ms exposure, iXon 897, Andor). Assisted by the MosaicSuite plugin in ImageJ, coordinates of each pore in different frames are extracted by performing super localization for each spot. A series of coordinates form a trajectory. Representative lateral movements and the corresponding trajectories of six α-HL pores are demonstrated in Figure 1b with 1 ms temporal resolution. Intuitively, ion channel proteins inserted in a self-assembled lipid bilayer should be free to move laterally as long as the membrane is freely standing. The motion type of inserted nanopores is thus expected to be Brownian. However, it is discovered that the motion type of nanopores in such a DIB is highly dependent on how hydrated the hydrogel layer immediately below the DIB is. Yet, no systematic investigation of this phenomenon has been reported before.

As reported, Brownian motion of lipid molecules is more restricted when the lipid membrane is in direct physical contact with a glass surface in a supported membrane ($D_{\text{app}} = 4.6 \, \mu m^2 \, s^{-1}$) than in a free-standing configuration ($D_{\text{lum}} = 20.6 \, \mu m^2 \, s^{-1}$).\(^1\) Whereas in another report,\(^2\) the mobility of heptameric α-HL nanopores in a DIB over hydrogel ($D_{\text{o-HL}} = 42.7 \, \mu m^2 \, s^{-1}$) is shown comparable to that of a lipid molecule in the same DIB ($D_{\text{lum}} = 90.0 \, \mu m^2 \, s^{-1}$), indicating the presence of a rich water layer in the agarose substrate immediately below the DIB. We hypothesize that this water-rich layer of the hydrogel serves to separate the interface between the membrane and the glass, which frees lipid molecules from motion restrictions because of nonspecific adhesion.

Being a highly porous and biocompatible hydrogel material, the layer of agarose on the trans side of the DIB (Figure 1a) plays a critical role in osmotic-driven movement of hydrophilic CaCl\(_2\)-containing medium as a sustained Ca\(^{2+}\) source for induced fluorescence. It may also serve as a polymer cushion to modulate lipid motion or motion of transmembrane proteins inserted in a DIB.\(^2\) Thus, by introducing a suitable unbalanced osmolarity across the DIB,\(^3\,\(^4\)\) an adjustable physical contact between the bilayer and the agarose substrate could be established and the pore motion is controllably modulated with a wide dynamic range. To test this, by tentatively varying the KCl concentrations in the droplet (0.5–1.5 M), drifts of α-HL nanopores are observed to be statistically suppressed (Figure 1c). When the KCl concentration achieves 1.5 M, pores are immobilized within a restricted area (<1 µm) (Figure 1c). Note that the chemical content is kept at 0.75 M CaCl\(_2\), 10 mM HEPES, pH = 7.0 all through the paper if not otherwise stated. This phenomenon of pore immobilization around the isotonic condition (cis: 1.5 M KCl, trans: 0.75 M CaCl\(_2\)) across the DIB thus suggests that motion of nanopores may be finely switched to be diffusive or confined by establishing a salt concentration difference across the bilayer. Although nanopore measurements with a salt gradient have been intensively investigated before,\(^5\)–\(^10\) osmosis-driven motion-type modulation of the nanopore itself has not been reported to the best of our knowledge.

To fully validate the dependence of pore motion with varying osmotic pressures on the DIB, a wide range of KCl concentrations (0.5–2.5 M) in the droplet are tested, while keeping the CaCl\(_2\) concentration in the agarose unchanged (0.75 M). Motion trajectories of α-HL within 5 s are shown in Figure 2a. For 0.3–1 M KCl in the droplet, pores appear to follow a series of stochastic motions (Video S1). By increasing the KCl concentration in the droplet, the stochastic motion is systematically retarded (Video S2), which supports our hypothesis that an increased DIB–agarose interaction is critical to the motion modulation of the pore.

To quantitatively investigate the pore motion kinetics in different KCl concentrations, MSBD analysis is performed to classify the motion type and to obtain quantitative kinetics parameters similar to reported.\(^11\) MSBD describes the average of the squared distances between a spot's start and end position as a function of the time lag $t_{\text{lag}}$ and KCl concentration. For a single trajectory, the MSBD is calculated as

$$\langle \text{MSD}(t_{\text{lag}} = m\Delta t) \rangle = \frac{1}{N - m} \sum_{i=1}^{N-m} |\vec{r}(t_i + m\Delta t) - \vec{r}(t_i)|^2$$

where $\vec{r}(t)$ represents the position of the particle at time $t$ (the subscript being the frame index), $N$ represents the total number of frames in the observed time period, $\Delta t$ represents time separations between adjacent frames, and $m$ is the defined index number to calculate $t_{\text{lag}} = m\Delta t$. Generally, for a k-dimensional diffusion process, the MSBD follows a power law with $t_{\text{lag}}$ according to eq 2

$$\langle \text{MSD}(t_{\text{lag}}) \rangle = 2k\Delta t^\alpha$$

where $k$ and $\alpha$ represent the generalized diffusion coefficient and the anomalous exponent, respectively.\(^12\)

The value of $\alpha$ classifies the motion type of the nanowire (Figure S6). For $\alpha = 1$, the motion type is Brownian. Otherwise, it is classified as subdiffusive ($\alpha < 1$) or superdiffusive ($\alpha > 1$). By fitting the logarithmic of the MSBD curve, the anomalous exponent and the diffusion coefficient can be derived from the slope and the intercept of the fitting as demonstrated in eq 3.

$$\log(\langle \text{MSD}(t_{\text{lag}}) \rangle) = \log(2kD) + \alpha \log(t_{\text{lag}})$$

Representative MSBD curves in each salt concentration are shown in Figure 2b. MSBD analysis for 30 independent tracks in each KCl concentration is performed to establish the statistics. In 0.3 and 0.5 M KCl, pores follow superdiffusive type of motion, as judged from the anomalous exponents in Figure 2c ($\alpha = 1.205 \pm 0.234$ in 0.3 M KCl, $\alpha = 1.123 \pm 0.347$ in 0.5 M KCl), indicating the existence of anisotropy for pore motion. As observed, when the salt concentration in the droplet (0.3–0.5 M KCl) is far lower than that in the agarose substrate (0.75 M CaCl\(_2\)), the whole DIB is lifted up because of osmosis. This DIB is more lifted up on the edge than in the center because of the surface tension of the aqueous droplet, generating a curved bilayer (Figure S7) with inhomogeneous interactions with the hydrogel layer, causing Brownian motion of nanopores with a slight bias toward the edge of the DIB. Still, a mixture of many factors (interaction with DIB boundary, new pore insertions, inhomogeneous hydrogel coating, etc.) in DIB contributes to the motion modulation of the pore.
hydrophilic interaction between the DIB and the agarose substrate (Table S2).

If this phenomenon of pore motion modulation results from the retardation of the lipid molecules, any transmembrane protein should be compatible with this method as long as it inserts into DIB and transports Ca$$^{2+}$$. To test the universal compatibility of our immobilization strategy, another widely used biological nanopore (MspA) is introduced (Supporting Methods 2, Figures S4 and S5). MspA is a conical-shaped protein nanopore ideal for DNA sequencing applications because of its sharp recognition site. The incorporation of the MspA nanopore on the oSCR platform is promising to break through the bottleneck of throughput in the nanopore sequencing industry, where pore immobilization during oSCR-based nanopore sequencing is urgently needed. As demonstrated in Figure 2c,d, the MspA nanopore shows similar motion rules in dependence of KCl concentrations in the droplet (0.3–2.5 M) while the CaCl$_2$ concentration in the hydrogel is kept unchanged (0.75 M) (Table S3, Figure S6). The motion-type transition also occurs around the isotonic condition (cis: 1.5 M KCl, trans: 0.75 M CaCl$_2$), which supports our hypothesis that osmosis-driven pore motion-type modulation is universally applicable to different types of transmembrane proteins.

3.2. Probing Lateral Mobility of Lipid Molecules with Asymmetric Salt Concentration Across a DIB. If osmosis-driven pore motion modulation is independent of the type of the inserted nanopores, it implies that the phenomenon of pore motion modulation originates from lipid motion modulation. To directly probe the diffusion characteristics of lipid molecules in this DIB setup, a fluorescence recovery after photobleaching (FRAP) assay is carried out (Supporting Methods 9, Figure S8) for various KCl concentrations (0.5–2.5 M) in the aqueous droplet, while keeping the CaCl$_2$ concentration (0.75 M) unchanged in the hydrogel substrate.

Experimentally, the DIB area is first imaged with a 488 nm laser in the TIRF mode (Figure S8aI). The DIB is fluorescently bright, indicating its successful labeling with DiO. Bright scattering spots, which may be aggregates of dye molecules, are not affecting the following FRAP assay. A 405 nm laser spot (5 mW) is focused on the DIB, and a circular area of 20 $\mu$m$^2$ is scanned for 15 s to photobleach DiO within the scanned area. A dark spot is observed immediately after switching off the 405 nm laser (Figure S8aII), indicating successful photobleaching within the scanned area. This dark spot gradually recovers because of lateral motion of the lipid molecules. The extracted FRAP curve shows an exponential relationship against time (Figure S8b). The recovery time constant is derived according to the fitting result of the curve based on eq 4, where $F_{pre}$ stands for the fluorescence intensity before the photobleaching and $b$ stands for the fraction of fluorophores that are photobleached.

$$F(t) = F_{pre} (1 - b e^{-t/\tau})$$  \hspace{1cm} (4)

$t_{1/2}$ is defined as the half recovery time constant, which could be derived from $t_{1/2} = \tau \ln 2$. The diffusion coefficient of the fluorophore is calculated from eq 5 adapted from the literature, where $w$ stands for the radius of the photobleached area. For a uniform circular spot, $\gamma$ has a constant value of 0.88.

$$D = \frac{\gamma}{\pi w^2}$$  \hspace{1cm} (5)

According to the derived diffusion coefficient values, it is evident that the diffusion of the fluorophore is significantly slowed when the KCl concentration is raised (Figure S8c), which consequently increase the interaction between the DIB and the hydrogel modulated by osmosis. This FRAP assay confirms again that osmosis-driven motion-type modulation of nanopores in oSCR is a consequence of controlled lipid motion retardation within a DIB.

3.3. SNR Evaluation from Drifting Nanopores. To resolve minute signal discrimination between DNA sequences in an oSCR setup for DNA sequencing purposes, the SNR of the recorded fluorescence traces needs to be fully optimized. Various sources of noises could contribute to the fluctuation of the recorded fluorescent traces. These sources include the power fluctuation of the illumination laser, photobleaching of the Ca$$^{2+}$ indicator dye, misfolded protein nanopores, mechanical vibration of the system, or photon noises captured from the measurement environment. These noise sources could...
be eliminated or minimized with improved engineering designs at the expense of a higher instrumentation cost.

Another critical noise source is from the inhomogeneous laser illumination in the whole DIB, which could be disastrous when thermal motion of nanopores is significant. Osmosis-driven pore immobilization thus effectively improves the SNR with no extra engineering efforts. To quantitatively compare how the SNR of oSCR is improved for immobilized pores, oSCR with a fixed applied potential (+100 mV) for individual α-HL nanopores is recorded with different KCl concentrations in the aqueous droplet (0.3−3.0 M), while keeping the CaCl₂ concentration (0.75 M) in the hydrogel unchanged. For each salt concentration, 15 independent traces are included for statistics. Each trace is scale-normalized so that the mean of the whole trace (12 s duration) equals to 1. The standard deviation (STD) of the trace is taken as a quantitative measure of the noise while the signal is kept equal to 1. Representative traces (Figure 3a) clearly demonstrate how the SNR is improved for aqueous droplets with high KCl concentrations. Distinct fluctuations in oSCR traces with low salt concentrations in the droplet (0.3−1.0 M KCl) result from the thermal drift of nanopores (Figure S9). According to the statistics from 15 independent recordings of each condition, the noise level (STD of the whole trace) is systematically reduced for more than 20-fold (Figure 3b, Table S4) with no particular investment from the instrumentation.

3.4. Validation of Biosensing Capabilities of Immobilized Nanopores. To verify that the native structure of the immobilized nanopores is preserved, translocation events of ssDNA are monitored during oSCR for both α-HL and MspA. A 3’-protruding hairpin DNA (Table S1) is designed as a model analyte, which mimics a transient step during nanopore sequencing. The 3’ overhang facilitates pore capture, whereas the terminal hairpin enables prolonged duration to fit the temporal resolution of the EM-CCD (1 ms). After thermal annealing (Materials), the hairpin DNA is dissolved in an α-HL-containing droplet, which forms the cis side of a DIB. At a fixed potential (+100 mV), fluorescent spots blink spontaneously corresponding to successive translocation events. The measurements are done with both 1.5 M (Figure 4b) and 1 M KCl (Figure 4c) in the cis side and 0.75 M CaCl₂ in the trans side for comparison. Consistent with patch clamp recording results, oSCR readout from hairpin translocation through α-HL includes two characteristic blockade levels (Figure 4a). The unzipping processes driven by the applied voltage generate long duration plateaus (II, Figure 4a), whereas translocations of unzipped DNA show short durations with deeper blockade amplitudes (III, Figure 4a). Because of the uneven excitation intensity in different positions within the FOV, oSCR from...
immobilized α-HL nanopore gives rise to a stable baseline and enhanced SNR (Figure 4b) compared to that of a drifting pore (Figure 4c).

The same experiment is performed with MspA. Typical translocation events through an MspA nanopore include only one characteristic blockade level (Figure 4d), which is consistent with that obtained by Gundlach et al. using patch clamp recording.44 Similarly, an increased SNR is also observed when the MspA nanopore is immobilized (cis: 1.5 M KCl, trans: 0.75 M CaCl2) (Figure 4e,f). However, when the KCl concentration reaches 2 M, immobilized MspA pores occasionally appear permanently blocked by the hairpin DNA, which may result from a gating phenomenon or difficulties of analyte unzipping in such a condition. The nature of this phenomenon needs to be further investigated, while α-HL does not show any similar gating activities, likely due to a more rigid β-barrel structure it possesses. Still, the optimum KCl concentration for nanopore sensing should be around or slightly higher than the isotonic concentration, when the nanopore is restricted while its native structure is still retained.

3.5. FEM Simulation of oSCR with Asymmetric Salt Concentrations. Different from patch clamp-based electrophysiology, which has been intensively studied both theoretically and experimentally, oSCR for nanopore biosensing is an emerging field of study with new complexities (such as coupling among features from indicator dyes, measurement geometries, diffusion features of ions, etc.) yet to be discovered.

To better understand the mechanism of oSCR under asymmetric salt concentrations particularly for our DIB geometry, FEM simulation is performed to model the distribution of [Ca2+/Fluo-8] (bound form of Ca2+ and Fluo-8, the only fluorescent chemical specie in our model) in the immediate vicinity of a pore. Following previous studies,29 Nernst–Planck equations (eqs 6–12) are applied to estimate the motion of ions around a nanometer-sized pore:

\[ \frac{\partial}{\partial t} [\text{Ca}^{2+}] = D_{\text{Ca}} \nabla^2 [\text{Ca}^{2+}] + \frac{D_{\text{Ca}} z_{\text{Ca}} F}{k_B T} \cdot \nabla \cdot ([\text{Ca}^{2+}] \nabla V) \]

\[ + \beta_{\text{Fluo}} [\text{FluoCa}] + \beta_{\text{EDTACa}} [\text{EDTACa}] - \alpha_{\text{Fluo}} [\text{Ca}^{2+}] - \alpha_{\text{EDTACa}} [\text{EDTA}] [\text{Ca}^{2+}] \]

\[ \frac{\partial}{\partial t} [\text{K}^+] = D_{\text{K}} \nabla^2 [\text{K}^+] + \frac{D_{\text{K}} z_{\text{K}} F}{k_B T} \cdot \nabla \cdot ([\text{K}^+] \nabla V) \]

\[ \frac{\partial}{\partial t} [\text{Cl}^{-}] = D_{\text{Cl}} \nabla^2 [\text{Cl}^{-}] + \frac{D_{\text{Cl}} z_{\text{Cl}} F}{k_B T} \cdot \nabla \cdot ([\text{Cl}^{-}] \nabla V) \]

\[ \frac{\partial}{\partial t} [\text{Fluo}] = D_{\text{Fluo}} \nabla^2 [\text{Fluo}] + \frac{D_{\text{Fluo}} z_{\text{Fluo}} F}{k_B T} \cdot \nabla \cdot ([\text{Fluo}] \nabla V) \]

\[ + \beta_{\text{Fluo}} [\text{FluoCa}] - \alpha_{\text{Fluo}} [\text{Ca}^{2+}] \]

\[ \frac{\partial}{\partial t} [\text{FluoCa}] = D_{\text{FluoCa}} \nabla^2 [\text{FluoCa}] + \frac{D_{\text{FluoCa}} z_{\text{FluoCa}} F}{k_B T} \cdot \nabla \cdot ([\text{FluoCa}] \nabla V) \]

\[ - \beta_{\text{Fluo}} [\text{FluoCa}] + \alpha_{\text{Fluo}} [\text{Fluo}] [\text{Ca}^{2+}] \]

\[ \frac{\partial}{\partial t} [\text{EDTACa}] = D_{\text{EDTACa}} \nabla^2 [\text{EDTACa}] + \frac{D_{\text{EDTACa}} z_{\text{EDTACa}} F}{k_B T} \cdot \nabla \cdot ([\text{EDTACa}] \nabla V) \]

\[ - \beta_{\text{EDTACa}} [\text{EDTACa}] + \alpha_{\text{EDTACa}} [\text{EDTA}] [\text{Ca}^{2+}] \]
\[
\frac{\partial}{\partial t} [\text{EDTACa}] = D_{\text{EDTACa}} \nabla^2 [\text{EDTACa}]
+ \frac{D_{\text{EDTACa}} z^2 \text{EDTACa} F}{k_B T} V \cdot \nabla ([\text{EDTACa}] V V)
- \beta_{\text{K}} [\text{EDTACa}] + \alpha_{\text{K}} [\text{EDTA}] [\text{Ca}^{2+}]
\]  

(12)

While the electrostatic potential is governed by Poisson's equation (eq 13)

\[
\nabla^2 V = -\frac{F}{\varepsilon} (z_{\text{Ca}} [\text{Ca}^{2+}] + z_K [K^+] + z_{\text{Cl}} [\text{Cl}^-])
\]  

(13)

Here, \(D\) is the diffusion constant (\(D_{\text{Fluo}} = D_{\text{Fluochrome}} = 15 \mu m^2 s^{-1}\)), \(D_{\text{Ca}} = D_{\text{Cl}} = D_{\text{EDTA}} = D_{\text{EDTACa}} = 200 \mu m^2 s^{-1}\). \(z\) is the charge number (\(z_{\text{Ca}} = +2\), \(z_K = +1\), \(z_{\text{Cl}} = -1\)). \(F\) is the Faraday constant. \(k_B\) is the Boltzmann constant. \(T\) is the temperature (300 K). \(V\) is the electric potential. \(\alpha\) is the forward binding rate (\(\alpha_{\text{K}} = 5 \mu M^{-1} s^{-1}\), \(\alpha_{\text{Ca}} = 150 s^{-1}\)). \(\beta\) is the backward binding rate (\(\beta_{\text{K}} = 0.75 \mu M^{-1} s^{-1}\), \(\beta_{\text{Ca}} = 450 s^{-1}\)). \(\varepsilon\) is the dielectric constant permittivity of water. The boundary condition on the cis side is set to 0 mV and varying KCl concentrations (0.5 M–2.5 M), whereas the boundary condition on the trans side is kept constant when varying the KCl concentration in the droplet, the size variation of the fluorescence spot is almost negligible from the results in Figure 5d. This result is consistent with our experiment, when the appearance of tracked spots is not significantly changed during immobilization by osmosis.

According to the full width at half-maximum of the PSF in 1.5 M KCl, the minimum pore-to-pore separation for independent optical recording is theoretically estimated to be 5 \(\mu m\), which is on the same order of magnitude compared with our experimental results (~3 \(\mu m\)) before.13 This size corresponds to an effective measurement density of 4.6 \(\times\) 10^4 pore mm^{-2} when the FOV is fully occupied by nanopores in a hexagonal array. The size of a fluorescence spot depends on parameters such as the geometry of the nanopore, the salt concentrations on both sides of the bilayer, the transmembrane potential, and so forth. This numerical simulation thus helps us to understand the restrictions among the parameters. For different applications, some parameters of interest could be strengthened while making the others less important and vice versa.

4. CONCLUSIONS

In summary, we have quantitatively investigated the lateral motion of biological nanopores within a DIB using oSCR. oSCR-based ion channel tracking not only provides a novel tool for the study of motion kinetics of any transmembrane proteins, but it may also serve to investigate a wide range of protein–protein interactions or protein–lipid interactions with high resolution and wide FOV. By modulating asymmetric salt concentrations across a DIB, we have developed a simple but universal approach to immobilize nanopores for high-quality oSCR in high throughput. Although immobilized, the nanopores are kept in their natural forms, when DNA analytes could still translocate through the pores. Pore immobilization also facilitates fluorescence trace extraction and may further improve the temporal resolution of oSCR to ns regime when equipped with a confocal illumination and a photodiode acquisition device.12 Pore immobilization in a highly ordered array will be the next step toward practical and addressable high-throughput DNA sequencing on a microscopic platform. According to our refined FEM model and experimental evaluations, a nanopore array with a density of ~10^7 pores mm^{-2} is feasible, which suggests an extremely portable sequencing device while still maintaining its high-throughput power. Considering the simple assembly, high SNR, and the negligible cost of consumables of oSCR, we foresee its potential as a prototype for disposable point-of-care diagnosis with single-molecule resolution.13,45
Preparation of α-hemolysin, MspA, lipid/oil solution, and droplet solution; coverslip pretreatment; DIB formation; TIRF microscopy and optical recording; electrical voltage protocol generation; FRAP assay; trajectory overlap of two drifting α-HL nanopores; osmotic pressure-induced water migration across a DIB; purification and characterization of α-HL and MspA with nickel affinity chromatography; SCRs of α-HL and MspA; diffusion coefficient histogram of α-HL and MspA with different [KCl] in the droplet; DIB conformations in different KCl concentrations; lateral mobility of a DIB determined using FRAP measurements; poor SNR caused by stochastic pore drifts with inhomogeneous excitation; model geometry of the FEM simulation; nucleic acid abbreviations and sequences; MSD analysis results of diffusion coefficients and abnormal exponents for α-HL; MSD analysis results of diffusion coefficients and abnormal exponents for α-HL; and STD analysis of fluorescence curves with different KCl concentrations (PDF)

Videos S1–S3: Tracking of α-HL nanopores, immobilization of α-HL nanopores, and simulated oSCR of a virtual nanopore with different applied biases (ZIP)

**ACKNOWLEDGMENTS**

The authors would like to thank Prof. Hagan Bayley (University of Oxford) and Prof. Mark Wallace (King’s College of London) for useful discussions; Dr. Matthew Cheetham (King’s College of London) for discussions on the FEM modeling; and Prof. Wei Wang (Nanjing University), Prof. Yin Ding (Nanjing University), and Dr. Hua Su (Nanjing University) for assistance with fluorescence recovery after photobleaching measurements. This work is supported by the National Natural Science Foundation of China (grant nos. 21327902, 21675083, and 91753108), Fundamental Research Funds for the Central Universities (grant nos. 020514380078 and 020514380120), and 1000 Plan Youth Program of China.

**REFERENCES**