Current noise of a protein-selective biological nanopore

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Abstract

1/f current noise is ubiquitous in protein pores, porins, and channels. We have previously shown that a protein-selective biological nanopore with an external protein receptor can function as a 1/f noise generator when a high-affinity protein ligand is reversibly captured by the receptor. Here, we demonstrate that the binding affinity and concentration of the ligand are key determinants for the nature of current noise. For example, 1/f was absent when a protein ligand was reversibly captured at a much lower concentration than its equilibrium dissociation constant against the receptor. Furthermore, we also analyzed the composite current noise that resulted from mixtures of low-affinity and high-affinity ligands against the same receptor. This study highlights the significance of protein recognition events in the current noise fluctuations across biological membranes.

Keywords

PhuA, ion channel, membrane protein engineering, protein detection, protein dynamics, single-molecule electrophysiology

1 | INTRODUCTION

Nanopores are powerful tools for single-molecule protein identification, detection, and analysis [1–11], suggesting realistic prospects in quantitative proteomics [12–21]. The detection mechanism employs early developments in single-channel electrical recordings [22]. If a single nanopore is located within an insulating membrane and exposed to an electrolytic fluid, then the ionic current traverses the nanopore whenever a voltage bias is applied. Partitioning of either a peptide or a protein into the nanopore occludes the permeation pathway, so that changes in the current signature are noted in the form of transient current blockades. The current amplitude of these blockades, as well as their duration and frequency are at the heart of standard event analysis in single-molecule nanopore detection of proteins. This is the time-domain analysis of single-channel current recordings. An alternative way to analyze these electrical measurements is to inspect the frequency domain using a fast Fourier transformation method. In this manner, the power spectral density (PSD) can be determined, illustrating the distribution and density of statistical current fluctuations acquired at various frequencies.

The nature and amplitude of current noise of both biological [23–38] and synthetic [39–50] nanopores have been extensively examined. Early studies of bacterial porins have revealed the presence of 1/f noise in the low-frequency domain with a range of 0–100 Hz [27]. In a logarithmic representation of a PSD diagram, the low-frequency 1/f noise denotes a linear increase in the amplitude of current noise by a decrease in the frequency. In general, it is accepted that 1/f noise is produced by infrequent and significant fluctuations in the number and local mobility of diffusing charges [51–53]. Moreover, the low-frequency 1/f noise is present in many transmembrane pores [33,37,38,50], porins [29,30,32,54–57], and channels [39,58].
some β-barrel protein pores [23,24,36,59] and porins [31,34] exhibit frequency-independent white noise in the low-frequency regime. We have recently shown that a protein nanopore fused to an external protein receptor exhibits 1/f noise whenever reversible captures of a high-affinity protein ligand occurred at the tip of the nanopore [60]. It is worth mentioning that the ligand-captured and ligand-released current substrates only showed white noise (e.g., flat noise or frequency independent current noise). Therefore, we concluded that the low-frequency 1/f noise was generated during reversible switches between the two current substrates of the nanopore.

In this study, we inquired whether the low-frequency 1/f current noise of a protein-selective nanopore also depends on the binding affinity of the ligand. Here, the stem of the biological nanopore, simply called t-FhuA (Figure 1A) [61], is the 22-stranded β barrel of ferric hydroxamate uptake component A (FhuA) of Escherichia coli [62,63]. We then fused a small 110-residue RNase barnase (Bn) [64], an external receptor domain, to the N terminus of truncated ferric hydroxamate uptake protein A (t-FhuA) via a Gly/Ser-rich flexible hexapeptide tether. Further, we also covalently attached an unstructured dodecapeptide adaptor (O) to the N terminus of Bn to serve as an external sensing element that changes the single-channel electrical signature of the nanopore upon its transient binding to a protein ligand. This extensive protein engineering facilitated the design and creation of a protein-selective biological nanopore [61], here named Bn-t-FhuA, for the real-time monitoring of protein detection. In a recent study [60], barstar (Bs) [65], a high-affinity 89-residue ligand [66], produced reversible bindings with the tethered Bn receptor, creating the low-affinity and high-affinity protein ligands. Finally, we examined the composite effect of a binary mixture of these ligands with different binding affinities.

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### Statement of significance

Protein and synthetic nanopores represent key elements used in protein detection, identification, and characterization. Electrical recordings of single nanopores have traditionally employed the analysis of time-resolved current fluctuations resulting from their interactions with various protein analytes. An alternative approach of this data processing is inspecting the frequency domain of statistical current fluctuations. Here, we show that a protein-selective biological nanopore exhibits either low-amplitude Lorentzian current noise or high-amplitude 1/f current noise. Moreover, we show that a binary mixture of low-affinity and high-affinity protein ligands produces 1/f current noise. This outcome is likely determined by the dominant role of the high-affinity protein ligand in creating significant fluctuations in the diffusion of charges through the nanopore.

### 2 MATERIALS AND METHODS

#### 2.1 Cloning and mutagenesis of the protein nanopore and protein ligands

Cloning was performed employing the pPR-IBA1 expression vector [62]. The obn(ggs)2-t-fhua gene encoded the Bn-t-FhuA protein nanopore. This gene encompassed DNA sequences for an extensively t-FhuA [61], a Gly/Serine-rich hexapeptide arm (GGSGGS), a small RNase Bn [64], a dodecapeptide adaptor (MGDRGPEFELGT) [67], as well as KpnI sites at both ends [61,68]. Fusion of these polypeptides was conducted at the N terminus of t-FhuA. The overall gene of this modular protein nanopore was created using individual genes of Bn and t-FhuA, respectively, and assembly PCR reactions. The RNase activity of Bn was suppressed using an H102A mutant of Bn. In this way, Bn-t-FhuA exhibited no toxic activity within the expression host [64,69]. The bs gene of the Bs, a high-affinity protein inhibitor of Bn, included a double-alanine mutant, C40A/C82A [70]. The gene that encoded, D39A Bs, a low-affinity protein ligand against Bn, was developed using inverse PCR [68].

#### 2.2 Protein expression, solubilization, purification, and refolding

Specific methods and protocols for protein expression and purification Bn-t-FhuA were previously described [61,71]. Solubilization of Bn-t-FhuA was performed using 200 mM KCl, 8 M urea, 50 mM Tris-HCl, pH 8 to a concentration of ∼15 μM. This solubilization process was conducted for several hours at room temperature. Denatured Bn-t-FhuA samples were refolded by adding n-dodecyl-β-D-maltopyranoside (DDM) to a final concentration of 1.5% (w/v) [72], then slowly dialyzed...
Reversible protein ligand captures by a single protein-selective biological nanopore. (A) The composition of a protein-selective biological nanopore, whose scaffold comprises a heavily truncated ferric hydroxamate uptake protein A of Escherichia coli (t-FhuA) [61,68]. This outer membrane β-barrel protein pore, which serves as the permeation pathway for ions’ transit, was fused to a barnase (Bn) protein receptor via a flexible hexapeptide tether. An unstructured dodecapeptide adaptor (O) was engineered at the N terminus of Bn. Barstar (Bs), a protein ligand, is transiently captured at the tip of this nanopore (Bn-t-FhuA). (B) In the absence of Bs, the open-state current, \( I_{on} \), was \(-50.0 \pm 0.1\) pA. The single-channel electrical trace shows upward current spikes, whose residual current, \( I_{c} \), was \(-47.5 \pm 0.5\) pA. The transmembrane potential was \(-40\) mV. Single-channel electrical traces were low-pass filtered at 1 kHz using an 8-pole Butterworth filter. For (B) and (C), all-points histograms of current amplitudes of individual peaks were illustrated on the right side. These histograms were developed using 10 s-duration single-channel electrical traces.
against 200 mM KCl, 20 mM Tris-HCl, pH 8, at 4°C for at least 3 days. For single-channel electrical recordings, protein samples were 20-fold diluted in 200 mM KCl, 20 mM Tris-HCl, pH 8, 0.5% DDM. Protein expression and purification of Bs protein ligands, both wild-type and mutants, were previously reported [61,68].

2.3 | Single-channel electrical recordings using planar lipid bilayers

Single-channel electrical traces were recorded using planar lipid membranes [73,74]. The experimental solution contained 300 mM KCl, 10 mM Tris-HCl, pH 8. The sample of protein nanopore was added to the cis side of the chamber (Figure 1A), which was grounded. The final concentration of Bnt-FhuA was in the range of 0.3–1 ng µL⁻¹. Single-channel electrical currents were recorded employing an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) and digitized using a Digidata 1440A acquisition system (Axon Instruments). The applied transmembrane potential was −40 mV. All single-channel electrical traces were sampled at a frequency of 50 kHz and low-pass filtered at 10 kHz using an 8-pole Butterworth filter, model 900B (Frequency Devices, Ottawa, IL, USA). The acquisition and analysis of single-channel electrical recordings were accomplished using pClamp 10.5 software package (Axon Instruments) and Origin 8.5 (OriginLab, Northampton, MA, USA). All electrical traces were acquired at room temperature (23 ± 1°C).

2.4 | Noise analysis of single-channel current recordings

ClampFit 10.5 (pClamp, Axon Instruments) was used to produce individual PSD plots. This process was achieved by employing a built-in power spectrum function, which was applied to single-channel electrical traces. In general, 10 s-duration single-channel electrical traces were used for creating PSD plots. Averaged PSDs were obtained using 15 distinct PSDs. These PSDs were acquired using n = 3 independently reconstituted nanopores with five PSDs of each nanopore experiment. For noise analysis, all traces were low-pass filtered at a frequency of 10 kHz using an 8-pole Butterworth filter. They were used to obtain average values of low-frequency limit of current noise, \( S(0) \) [37,75], \( S(0) \) was acquired using the lowest experimental frequency of the PSD [60]. Average single-channel currents of the ligand-released and ligand-captured substrates were determined using Gaussian fits of all-points current amplitude histograms. Such average currents were used to determine the average absolute current differences between the two substrates of the nanopore (ΔI). These values were employed to compute the model-dependent \( S(0) \) values. For fits of the white noise in the low-frequency domain of the PSD, a Lorentzian function was used:

\[
S(f) = \frac{S(0)}{1 + \left( \frac{f}{f_c} \right)^2}
\]

where \( f_c \) is the corner frequency. For fits of 1/f noise, a linear function was employed in a log-log representation. These latter fits were executed in the frequency range of \( f_{\text{min}}, f_{\text{cut}} \). Here, \( f_{\text{min}} \), and \( f_{\text{cut}} \) denote the minimum experimental frequency of the PSD and the cut-off frequency of the 1/f current noise, respectively. \( f_{\text{cut}} \) shows to the onset of the low-frequency 1/f noise.

3 | RESULTS AND DISCUSSION

3.1 | Low-affinity ligand does not produce 1/f noise at concentrations much lower than the equilibrium dissociation constant

In this work, we examined the nature of current noise produced by D39A Bs, a low-affinity Bs ligand. The low-affinity D39A Bs ligand interacts with Bnt-FhuA with the rate constant of association, \( k_{\text{on}} = 0.12 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \), and the rate constant of dissociation, \( k_{\text{off}} = 281 \text{ s}^{-1} \), which give the \( K_D \) of ~146 µM [61]. The electrical signature of Bnt-FhuA in the presence of the low-affinity D39A Bs comprises ligand-captured (O_{oc}) and ligand-released (O_{or}) events that undergo closely similar transitions between identical current substrates for the high-affinity Bs ligand (Figures 1B, C, and 2; Figure S1).

However, the Bs-captured time, \( \tau_{\text{oc}} \), for the high-affinity Bs ligand and low-affinity D39A Bs ligand with the tethered Bn measured ~1.16 s and ~3.5 ms, respectively [61]. The low-affinity D39A Bs ligand enabled us to test whether the low-frequency 1/f current noise is still detectable at ligand concentrations much lower than its corresponding \( K_D \). At a concentration of 181.7 nM D39A Bs, reversible switches between O_{or} and O_{off} were noted (Figure 2A), representing the interactions of the tethered Bn receptor with the low-affinity D39A Bs ligand. As expected, their frequency increased at elevated ligand concentrations of 362.4 nM (Figure 2B) and 723.4 nM (Figure 2C). The supplementary single-channel electrical trace, which was presented at the bottom of Figure 2, shows three such examples of very short-lived current transitions produced by the low-affinity D39A Bs ligand. Moreover, the all-points histograms of current amplitudes on the right side of Figure 2 showed an additional peak, whose substrate was named O_{c}. The current transitions between O_{or} to O_{c} were likely produced by flickering fluctuations of the O peptide adaptor, while the nanopore dwelled on the D39A Bs-released O_{or} blocked substrate. Although the amplitude of this O_{c} peak fluctuated from trace to trace due to the stochasticity of this process, the amplitude of the O_{or} peak was amplified at increasing D39A Bs concentrations. We have never observed current transitions between the D39A Bs-captured O_{oc} open substrate and O_{c} substrate. In a previous study [60], we have documented that in the O_{or} substrate, which included transitions to and from O_{c} substrate, the nanopore exhibits a high-frequency current noise, but no low-frequency 1/f current noise.

Next, we employed noise analysis to generate the PSD (Materials and Methods) of Bnt-FhuA in the presence of the low-affinity D39A Bs ligand at various concentrations. Surprisingly, we found that the low-frequency 1/f current noise is absent under these conditions.
FIGURE 2  Detection of a weakly binding protein ligand by a single protein-selective biological nanopore. The weakly-interacting D39A Bs ligand was added to the cis compartment. $O_{on}$ represents the Bs-released open substate, whereas $O_{off}$ indicates the Bs-captured open substate. (A) At 181.7 nM D39A Bs, $I_{on}$, $I_{off}$, and $I_{c}$ were $-50.0 \pm 0.1$ pA, $-59.4 \pm 0.7$ pA, and $-46.8 \pm 1.5$ pA, respectively. (B) At 362.4 nM D39A Bs, $I_{on}$, $I_{off}$, and $I_{c}$ were $-48.7 \pm 0.2$ pA, $-59.0 \pm 0.7$ pA, and $-44.9 \pm 4.0$ pA, respectively. (C) At 723.4 nM D39A Bs, $I_{on}$, $I_{off}$, and $I_{c}$ were $-50.4 \pm 0.1$ pA, $-58.9 \pm 0.5$ pA, and $-46.3 \pm 0.5$ pA, respectively. Current amplitudes of the peaks were provided as mean ± SEM of individual Gaussian fits. The additional trace in (C) shows examples of brief D39A Bs capture events. The transmembrane potential was $-40$ mV. The single-channel electrical traces were low-pass filtered at 1 kHz using an 8-pole Butterworth filter. All-points histograms of current amplitudes of individual peaks were illustrated on the right side of the figure. These histograms were developed using 10 s-duration single-channel electrical traces.

(Figure 3). Therefore, reversible switches no longer generated $1/f$ current noise despite their high probability (Figure 2). In this article, the low-frequency limit of current noise, $S(0)$, is the current noise amplitude that corresponds to the lowest experimentally measured frequency of the PSD. We determined the background noise of Bnt-t-FhuA at a zero transmembrane potential and in the absence of D39A Bs. This value was $\sim 10^{-29}$ A² Hz⁻¹, which is in a good accord with the calculated total background noise. This zero-voltage and ligand-free background noise includes both the Johnson-Nyquest thermal noise $[29,49,76–80]$ of $2.0 \times 10^{-29}$ A² Hz⁻¹ and the shot noise $[29,49,78–80]$ of $1.6 \times 10^{-29}$ A² Hz⁻¹. Our calculations were achieved using a single-channel conductance of $\sim 1.26$ nS for Bnt-t-FhuA $[60]$. We noted a three-order of magnitude increase in $S(0)$, to a value of $\sim 10^{-26}$ A² Hz⁻¹, when a transmembrane potential of $-40$ mV was applied.
Figure 3  Power spectral density (PSD) of single-channel current noise of Bn-t-FhuA recorded at different D39A Bs concentrations. Representative PSDs of single-channel current noise were recorded at different D39A Bs concentrations, as follows: (A) 181.4, (B) 362.4, and (C) 723.4 nM. PSDs were obtained using 15 distinct PSDs. These PSDs were acquired using $n = 3$ independently reconstituted nanopores with five PSDs of each nanopore experiment. Corresponding Lorentzian spectral fits (Materials and Methods) were displayed on the left side. Here, the minimum analyzed frequency, $f_{\text{min}}$, was 0.19 Hz. The corner frequency, $f_c$, for the three D39A concentrations were the following: (A) 35.0 ± 1.9 Hz, (B) 49.7 ± 2.0 Hz, and (C) 22.3 ± 1.2 Hz. These values were provided as mean ± SEM from individual Lorentzian fits (Materials and Methods). The lower dashed line is the background noise ($\sim 2.06 \times 10^{-29}$ A$^2$ Hz$^{-1}$), which was computed using Johnson-Nyquist noise function [76,77].

At a concentration of 181.7 nM D39A Bs, we noted a flat Lorentzian noise at frequencies in the range of 0–100 Hz, a low-frequency domain that is characteristic to 1/f noise [50] (Figure 3A). A flat noise was also observed at higher D39A Bs concentrations of 362.4 nM (Figure 3B) and 723.4 nM (Figure 3C). In addition, we represented the hypothetical 1/f noise lines on the right panels of Figure 3 to highlight the absence of such a noise signature in the low-frequency domain. Recently, we have shown that the amplification in low-frequency 1/f noise by a protein ligand of a protein-selective biological nanopore is determined at least in part by reversible switches between ligand-captured and ligand-released states [60]. Here, our experiments with the low-affinity D39A Bs ligand suggest that the reversible switches are not sufficient for the generation of this noise signature.

3.2 Quantitative analysis of the white noise produced by the low-affinity protein ligand

A detailed analysis of the noise amplitude in the low-frequency regime revealed that $S(0)$ increases by increasing the concentration of the D39A Bs ligand (Figure S2–S4). This outcome was acquired even if we normalized the PSDs to the squared single-channel currents.
corresponding to the \( O_{\text{on}} \) substate, \( I_{\text{on}}^2 \) (Figure S5). Our finding is not surprising, given that the theoretical prediction of the low-frequency limit of current noise, \( S(0) \) [23,81,82], obeys Machlup’s equation [81]:

\[
S(0) = 4(\Delta f)^2 \tau_{\text{off}} \left( \frac{L}{K_D} \right)^2 \left( 1 + \frac{L}{K_D} \right)^3
\]

(2)

The function (2) reaches the maximum value at a ligand concentration, \([L]\), equal to \( K_D/2 \). Here, \( \Delta f \) is the absolute single-channel current difference between the \( O_{\text{on}} \) and \( O_{\text{off}} \) substates. \( S(0) \) is a biphasic function with respect to its variable, \([L]\). This means that the function (2) increases when \([L]\) increases up to its corresponding \( K_D/2 \), yet it decreases when \([L]\) increases beyond \( K_D/2 \) (Table S1, Figure S6). Using the kinetic rate constants of binding interactions of the low-affinity D39A Bs ligand with Bn-t-FhuA [61], the maximum \( S(0) \) value, \( S(0)_{\text{max}} \), would be \( 210.8 \times 10^{-27} \, \text{A}^2 \text{Hz}^{-1} \) at a ligand concentration of 73 \( \mu \text{M} \) D39A Bs.

However, our experimental \( S(0) \) values for inspected submicromolar D39A Bs concentrations were much smaller than \( S(0)_{\text{max}} \). For instance, \( S(0) \), in terms of mean \( \pm \) SD (\( n = 3 \) distinct nanopores), was \((2.7 \pm 1.1) \times 10^{-27} \, \text{A}^2 \text{Hz}^{-1}, (6.4 \pm 1.2) \times 10^{-27} \, \text{A}^2 \text{Hz}^{-1}, (11.6 \pm 3.5) \times 10^{-27} \, \text{A}^2 \text{Hz}^{-1}, \) and \((17.3 \pm 3.0) \times 10^{-27} \, \text{A}^2 \text{Hz}^{-1} \) at D39A Bs concentrations of 90.7 nM, 181.4 nM, 362.4 nM, and 723.4 nM, respectively (Figures S4 and S6, Table S2). This outcome is likely because these concentrations of the low-affinity D39A Bs ligand are at least two orders of magnitude lower than the \( K_D/2 \). Furthermore, no 1/\( f \) noise was noted within this concentration regime despite the presence of numerous reversible switches between the D39A Bs-captured and D39A Bs-released substates. Here, in light of the outcomes of our previous publication [60], we tentatively interpret that these ligand-induced reversible current switches are required, but not sufficient, to generate the low-frequency 1/\( f \) noise of a protein-selective biological nanopore (Figure 1A).

To test this interpretation, we next examined the ligand-induced current noise of E76A Bs, a medium-affinity ligand for Bn, whose measured \( K_D \) was \( \sim 1.06 \, \mu \text{M} \) (Figure S7, Table S3). In this case, we added 100 nM E76A Bs to the cis side of the chamber. This ligand concentration was a five-fold lower concentration than its corresponding \( K_D/2 \). Therefore, \([L]\) was in the same order of magnitude with the \( K_D/2 \). In accord with our interpretation, Bn-t-FhuA showed a signature of low-frequency 1/\( f \) noise in these conditions (Figure S8, Table S4). In other words, the fractional occupancy of the Bn receptor by the medium-affinity E76A Bs ligand, \( f_o \), was within the same order of magnitude with the expected fractional occupancy reached in the presence of \( K_D/2 \) concentration of the ligand. For example, if \([L]\) = \( K_D/2 \), then the ligand-released time is \( \tau_{\text{on}} = 2\tau_{\text{off}} \), because \( K_D = ([L]\tau_{\text{on}}/\tau_{\text{off}}) \). This means that the fractional occupancy, which is defined by \( f_o = \tau_{\text{off}}/\tau_{\text{on}} + \tau_{\text{off}} \), is 33.3%. At \([L]\) values of a 5-fold and a 100-fold lower concentration than the \( K_D/2, f_o \) is \( \sim 9.1\% \) and \( \sim 0.5\% \), respectively. Our interpretation is also consistent with a prior study in which high concentrations, in the low-micromolar range, of the high-affinity Bs produced Lorentzian noise in the low-frequency domain of the PSD [60]. In this case, \([L]\) was much higher than its corresponding \( K_D/2 \).

3.3 Comparison of current noise produced by the low-affinity and high-affinity protein ligands

Next, we quantitatively compared and contrasted the noise signatures of Bn-t-FhuA under closely similar conditions, but either in the presence of high-affinity or low-affinity protein ligands. Figure 4A shows the averaged PSDs of the background and baseline current noise, which were recorded at zero transmembrane potential and at \(-40 \, \text{mV} \), respectively, both of which in the absence of the protein ligand. Remarkably, at ligand concentrations near to each other, 181.7 nM D39A Bs and 201.6 nM Bs produced signatures of low- and high-affinity white current noise and high-amplitude 1/\( f \) noise, respectively. This distinction in the signature and amplitude of current noise appeared despite frequent ligand-induced reversible switches between the ligand-captured and ligand-released substates in the presence of 181.7 nM D39A Bs (Figure 2A). These concentrations of the low-affinity D39A Bs and high-affinity Bs ligands were 787-fold lower and 3.2-fold higher than their \( K_D \) values, respectively. On the other hand, at 723.4 nM D39A Bs, the event frequency, \( f_e \), was \( \sim 1.4 \, \text{s}^{-1} \). Yet, at 201.6 nM Bs, \( f_e \) was \( \sim 1.6 \, \text{s}^{-1} \). Although \( f_e \) values were almost...
FIGURE 5  The low-frequency domain of current noise determined for binary mixtures comprising high- and low-affinity protein ligands. Representative low-frequency domain PSDs of single-channel current noise recorded in the presence of different binary mixtures, as follows: (A) 1440 nM D39A Bs and 25 nM Bs, (B) 1439 nM D39A Bs and 100 nM Bs, and (C) 1435 nM D39A Bs and 399 nM Bs. The lower dashed line is the baseline current noise acquired at a transmembrane potential of $-40$ mV. Corresponding linear fits were displayed on the left side for data points in the range of $0.19-10.07$ Hz. At a Bs concentration of 25, 100, and 399 nM, the linear slope, $c$, was $1.64 \pm 0.05$, $1.52 \pm 0.06$, and $1.10 \pm 0.04$, respectively. These values were provided as mean $\pm$ SEM of linear fits of $S(f)$ in a logarithmic representation.

identical in these cases, the low-affinity D39A Bs ligand produced low-amplitude white noise (Figure 3C), whereas the high-affinity Bs ligand created high-amplitude $1/f$ noise (Figure 4A) in the low-frequency domain. These quantitative comparisons confirm that $f_c$ of the Bn receptor by the protein ligand is an additional key determinant in the amplification of the low-frequency $1/f$ noise.

$S(0)$ of Bn-t-FhuA in the presence of 201.6 nM Bs, in terms of mean $\pm$ SD ($n = 3$), was $(31.8 \pm 5.5) \times 10^{-24}$ A$^2$ Hz$^{-1}$. This noise amplification is over three orders of magnitude greater than $S(0)$ acquired at 181.7 nM D39A Bs ($6.4 \pm 1.2) \times 10^{-27}$ A$^2$ Hz$^{-1}$, $n = 3$) (Table S2). This $S(0)$ value for Bs is in accordance with the model-dependent $S(0)$ of $\sim 20.5 \times 10^{-24}$ A$^2$ Hz$^{-1}$ (Equation 2), which was calculated using the kinetic rate constants of the Bn-Bs binding interactions under these experimental conditions [61]. Here, the Bs-amplified $1/f$ noise exhibited a cut-off frequency, $f_{cut}$, of 50.54 Hz, indicating that this noise signature occurred in the range of 0–100 Hz. Yet, the dependence of $S(f)$ on the experimental frequency was sharper than $1/f$. This suggests that the low-frequency domain of $S(f)$ undergoes a $1/f^c$ dependence [39], with a slope, $c$, in terms of mean $\pm$ SEM, of $1.49 \pm 0.02$ in a logarithmic representation of the PSD.

Figure 4B presents the dependence of $S(0)$ on the ligand concentration. It should be noted that $S(0)$ is strongly dependent on the high-affinity Bs concentration in a submicromolar range. In contrast, $S(0)$ is weakly dependent on the low-affinity D39A Bs concentration under
CONCLUSIONS

Representative PSDs acquired at different Bs ligand concentrations and superimposed on each other. (B) S(0) was acquired at different Bs ligand concentrations. S(0) was the following: (i) (61.8 ± 1.5) × 10⁻²⁴ A² Hz⁻¹ at 1440 nM D39A Bs and 25 nM Bs, (ii) (37.5 ± 6.0) × 10⁻²⁴ A² Hz⁻¹ at 1439 nM D39A Bs and 100 nM Bs, and (iii) (19.6 ± 2.7) × 10⁻²⁴ A² Hz⁻¹ at 1435 nM D39A Bs and 399 nM Bs. S(0) values were provided as mean ± SD using n = 3 independently reconstituted nanopores. Similar conditions. Again, the magnitude of S(0) for the low-affinity D39A Bs ligand was between two and four orders of magnitude smaller than that value corresponding to the high-affinity Bs ligand.

3.4 Composite noise of binary mixtures containing the low-affinity and high-affinity ligands

Based on the results obtained above, we questioned the effect of the concurrent presence of the low-affinity and high-affinity Bs ligands on the amplitude and signature of current noise. Therefore, the PSDs of Bn-t-FhuA were examined in the presence of binary mixtures of weakly-interacting D39A Bs and strongly-interacting Bs. In these binary mixtures, the concentration of the low-affinity D39A Bs was maintained at a value of 1.4 µM, whereas the concentration of the high-affinity Bs was changed in a range of 0–400 nM. In this way, we mixed ligands that either produce low-amplitude white current noise or high-amplitude 1/f current noise. Under these experimental conditions, we noted the coexistence of the D39A Bs-induced short-lived and Bs-induced long-lived current transitions (Figure S9). Notably, the coexistence of the low-affinity and high-affinity ligands in solution produced low-frequency 1/f noise, suggesting the dominant role of the strongly binding protein in the magnitude and signature of current noise (Figures 5 and 6A).

In addition, S(0) depended on the concentration of the high-affinity Bs ligand as one would expect for a strongly interacting protein ligand, according to Machlup’s equation (Equation 2) (Figure 6B; Figure S10). For example, the highest S(0), in terms of mean ± SD (n = 3), of (61.8 ± 1.5) × 10⁻²⁴ A² Hz⁻¹ was obtained at 25 nM Bs, a ligand concentration slightly lower than the corresponding Kd/2 of 32 nM [61]. At 100 nM Bs and 399 nM Bs, the corresponding S(0) values, shown as mean ± SD (n = 3), decreased to (37.5 ± 6.0) × 10⁻²⁴ A² Hz⁻¹ and (19.6 ± 2.7) × 10⁻²⁴ A² Hz⁻¹, respectively (Table S5). This outcome was not changed even if we normalized the PSDs to the squared single-channel currents corresponding to the Oon substate, f(on)² (Figure S11). In addition, these S(0) values compared well with the corresponding values acquired in the presence of the same Bs concentrations, yet in the absence of the low-affinity D39A Bs ligand [60]. Moreover, the cut-off frequency, fcut, namely the onset frequency at which 1/f current noise occurs in the low-frequency regime, also depended on the high-affinity Bs concentration. For instance, fcut values at 25, 100, and 399 nM Bs, in terms of mean ± SD (n = 3), were 14.3 ± 2.0 Hz, 27.7 ± 3.9 Hz, and 48.5 ± 3.1 Hz, respectively.

The slope of the low-frequency 1/f noise also depended on the concentration of the high-affinity Bs ligand. The generalized formula of the low-frequency 1/f current noise is the following [27,83–85]:

\[
S(f) = \frac{\alpha f^\beta}{N f^c}
\]

Here, \( \alpha \) is Hooge’s phenomenological parameter, \( f \), \( N \), and \( c \) denote the experimental frequency, number of channels within a membrane, and slope of the low-frequency PSD fit in a logarithmic representation, respectively. We also noted that \( c \) was over 1 for all examined high-affinity Bs concentrations (Table S5). This is in contrast to other outer membrane proteins, which exhibit intrinsic 1/f current noise with a slope, \( c \) of ~1 [27,29]. However, a slope different than 1 was noted in other instances [39,41,86]. Furthermore, our \( \alpha \) values were in a range close to those previously determined for other \( \beta \)-barrel porins [27].

4 CONCLUSIONS

In summary, we show that a single protein-selective biological nanopore with an externally engineered protein receptor can either generate low-amplitude white current noise or high-amplitude 1/f current noise in the low-frequency domain. This significant distinction in both the magnitude and signature of current noise strongly depends on the fractional occupancy, \( f_{on} \), of the protein receptor by the protein ligand. At a very low \( f_{on} \), which corresponds to ligand concentrations much lower than the Kd/2, transient ligand-receptor interactions cannot elevate the amplitude of current noise beyond what one would expect for white current noise in the low-frequency domain. However, at a significantly increased \( f_{on} \), which corresponds to ligand concentrations in the same order of magnitude with the corresponding value of Kd/2, reversible switches between the ligand-captured and ligand-released substates produce a significant amplification in the low-frequency 1/f current noise. The drawback of time-domain analysis in the form of time-resolved events is the relatively long sampling time, especially in the case of high-affinity protein ligands, which produce long-lived binding events. In the case of low-affinity protein ligands, it is advantageous to determine the concentration of a given sample. The PSDs of 10 s-duration electrical traces can be obtained and analyzed in a matter of minutes, providing a much shorter processing time than one would expect for the analysis of time-resolved events. Finally, we show that competitive binding between the low-affinity and high-affinity ligands against the same Bn receptor produced 1/f current noise in a concentration-dependent fashion of the strongly
binding ligand. Our findings agree well with theoretical predictions for the analyte-induced amplification in current noise of protein channels and pores [23,87,88]. With further developments, future explorations of single-molecule protein detection using nanopores may couple the time-domain and frequency-domain analyses of current recordings [30,54].

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Jiaxin Sun, Avinash Kumar Thakur, and Liviu Movileanu designed research. Jiaxin Sun and Avinash Kumar Thakur performed research and analyzed data. Jiaxin Sun, Avinash Kumar Thakur, and Liviu Movileanu wrote the paper.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online https://doi.org/10.1002/pmc.202100077 in the Supporting Information section at the end of the article.