Selective Sensing of Proteins Using Aptamer Functionalized Nanopore Extended Field-Effect Transistors

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The ability to sense proteins and protein-related interactions at the single-molecule level is becoming of increasing importance to understand biological processes and diseases better. Single-molecule sensors, such as nanopores, have shown substantial promise for the label-free detection of proteins; however, challenges remain due to the lack of selectivity and the need for relatively high analyte concentrations. An aptamer-functionalized nanopore extended field-effect transistor (nexFET) sensor is reported here, where protein transport can be controlled via the gate voltage that in turn improves single-molecule sensitivity and analyte capture rates. Importantly, these sensors allow for selective detection, based on the choice of aptamer chemistry, and can provide a valuable addition to the existing methods for the analysis of proteins and biomarkers in biological fluids.

1. Introduction

Proteins play an essential role in biological function,[1] The accurate recognition and quantification of proteins are of particular significance in biochemical and biomedical applications, including clinical diagnostics and targeted therapeutics.[2,3] Developing tools to detect such targets at low concentrations within a complex mixture has been gaining in importance. Single-molecule techniques have emerged as powerful tools for the observation of individual molecules in real-time, which can provide rich information about molecular structures and interactions with other molecules.[4,5] By monitoring one molecule at a time and statistically analyzing thousands of individual molecules, proteins can be identified by measuring their characteristic signals.[6,7]

Single molecules techniques, such as nanopore sensors are becoming popular due to their label-free operation, ease of fabrication, the ability of active transportation, and in some cases, the ability to tailor the sensor chemistry via surface modification.[8–14] Nanopores have been widely used for the detections of nucleic acids, DNA sequencing,[15] and protein sensing.[16–23] However, detection of proteins remains challenging due to fast analyte transport, low capture rates, and the need for relatively high protein concentration.[24,25] To date, a number of valuable approaches have emerged for nanopore based protein sensing. For example, the use of high salt conditions to slow down the translocation, or the use of high bandwidth instruments to resolve the fast transients.[26,27] However, selectivity is still something that needs to be optimized.

One promising strategy for addressing these challenges is to integrate nanopores with field-effect-transistors (FETs).[28–31] These combined sensors are capable of selectively detecting analytes at the single-molecule level and offer a degree of control over the transport of individual analytes. Our group has also developed nanopore designs coupled with ionic-FETs, where a gate voltage was used to facilitate molecular transport at the single-molecule level.[32,33]

In one of these designs, a nanopore extended FET (nexFET) was functionalized with a polypyrrole (PPy) layer that incorporated protein receptors. By applying the gate voltage our group has demonstrated that molecular transport can be efficiently controlled at the single-molecule level.[34] nexFETs were able to selectively recognize the corresponding antibodies, with improved signal-to-noise and enhanced analyte capture. Despite these improvements, the generality of the detection strategy is somewhat limited as it requires optimization for each antibody–antigen pair.[35] An alternative and perhaps more general approach would be to use aptamers, as they simplify sensor functionalization, and typically can operate in a broader range of environments.[36] Aptamers are commonly selected from a random DNA/RNA sequence library using systematic evolution of ligands by exponential enrichment.
(SELEX), which can be made applicable to a wide range of targets from small molecules to cells and bacteria.\(^\text{[36]}\) Unlike protein ligands/receptors, aptamers have a well-defined charge distribution, that can be used in optimizing electrostatic interactions near the nanopore surface when using gating voltage.\(^\text{[37]}\)

Here, we report on a nexFET sensor based on a double-barrel nanopipette, which was functionalized with aptamers (Figure 1). A method for the fabrication and the functionalization of these sensors is shown, whereby simple aptamer sequence is embedded in a polypyrrole gating layer. A thrombin binding aptamer (TBA) was used for the detection of human α-thrombin, a protein that is generally difficult to detect using more conventional nanopores.\(^\text{[38,39]}\) In contrast, with the aptamer modified nexFET, the gate voltage facilitates single-molecule thrombin detection at low concentrations (pm) with improved signal-to-noise, higher capture rate and lower translocating speed.

2. Result and Discussion

2.1. Fabrication and Characterization of the Aptamer Modified nexFET

The aptamer modified nexFET is fabricated using a double-barrel nanopipette with one hollow barrel terminating with a nanopore, and the other filled with carbon forming a nanoelectrode. The carbon nanoelectrode is further functionalized by a two-step PPy/aptamer electrodeposition with real-time current feedback control.

First, double-barrel quartz capillaries were pulled using a laser-assisted pipette puller to achieve nanopipettes terminating with two 100 ± 20 nm (n = 5, number of repeats) nanopores (Figure 2a(ii); Figure S1, Supporting Information). Carbon was pyrolytically deposited in one barrel and at the end of the nanopore tip, as previously reported.\(^\text{[32,40]}\) (Figure 2b; Figures S3 and S4, Supporting Information). The conductive carbon at the nanopipette tip was electrically connected to carbon inside the barrel, and the electrochemically active carbon area was estimated via linear sweep voltammetry (LSVs) using \(1 \times 10^{-3} \text{ M ferrocenemethanol as the redox mediator (Figure 2f). It should be noted that the uniform carbon coating of the nanopipette tip is essential for the successful fabrication of functional nexFETs. Nonuniform deposition of carbon at the tip and the adjacent nanopore orifice results in an uneven coating of the PPy. This, in turn, would lead to a device that would lack gating control, as the drain–source channel would be dominated by the glass wall rather than the PPy gating layer (Figures S5, S6, and Note S1, Supporting Information).}

Figure 1. Conceptual schematic of an aptamer functionalized nexFET. a) The sensor is based on a double-barrel quartz nanopipette. One of the barrels is hollow and terminates with a nanopore. The inside of the other barrel is filled with pyrolytic carbon, and the tip of the pipette is coated with a thin layer of carbon and a core PPy layer and a shell PPy–aptamer layer. b) A side view schematic of the nanopore, illustrating the sensor operation. When no gate voltage is applied, the inner layer is positively charged, and the outer layer is slightly negatively charged, resulting in limited binding and detection of the target protein. c) Positive gated device (\(V_G = 400 \text{ mV}\)) can catch and sense thrombin selectively, with higher throughput and signal-to-noise. Representative current–time traces showing translocation thrombin (concentration \(50 \times 10^{-12} \text{ m}\)) in \(100 \times 10^{-3} \text{ M KCl, } 1 \times 10^{-3} \text{ M Tris–EDTA, pH 8, under a bias of } V_{DS} = 500 \text{ mV. Scale bar: horizontal: 1 s; Vertical: 20 pA).\)
The carbon nanoelectrode and carbon deposited at the nano-pipette tip served as a site for the electrochemical polymerization and formation of the PPy layers. The electropolymerization process was performed by applying a bias, \( V_G \), to the carbon working electrode to enable the oxidation of pyrrole while the second working electrode was used to monitor the change of the open pore current, \( I_{DS} \), under a small bias across the nanopore, \( V_{DS} \). Precise control of the nanopore dimension can be achieved by the real-time feedback, by monitoring the \( I_{DS} \) (Figure 2c) similar to previous reports.\(^{[32,33]}\) By using this controllable fabrication process, the deposition could be stopped at a defined threshold of the current passing through the nanopore \( I_{DS} \). The process is reproducible and results in PPy-coated nexFET with very similar ionic \( I-V \) characteristics (Figure 2g).

Previously, we added antibody receptors to the PPy precursor prior to electrochemical polymerization.\(^{[32]}\) These nexFET devices had the capability to selectively sense target antibodies as they bind to the receptors embedded in the PPy modified nanopore surface. However, diminished FET performance was observed, due to the excess negative charge of the functional groups embedded in the PPy (Figure S8, Supporting Information). Herein, we improved on the FET performance by developing a two-step electrodeposition protocol: In the first step, a PPy core layer was electropolymerized onto the carbon surface of the nanopipette tip, generating a PPy nanopore (Figure 2c). In the second step, a PPy shell embedded with aptamers was electropolymerized onto the existing PPy layer (Figure 2d). After electrochemical polymerization of the core layer, the ion transport through the nanopore showed a positive ionic current rectification (ICR), indicating the nanopore wall is positively charged due to the protonated amino groups in PPy (Figure 2g). Upon addition of the shell, negative ICR (Note S2, Supporting Information) was observed, confirming the successful imprinting of negatively charged aptamers (Figure 2h). The FET performance of this double-layer system, compared to a single layer system, was improved significantly: the ICR

**Figure 2.** Fabrication and characterization of aptamer modified nexFETs. The aptamer modified nexFET was fabricated using a) double barrel quartz nanopipettes with b) one barrel filled with pyrolytically deposited carbon, followed by c) electropolymerization of a core PPy layer, and d) electrodeposition of a shell PPy layer embedded with aptamers. i) shows the schematic representation and ii) the corresponding fabrication process. e) \( I-V \) characteristics of a double-barrel quartz nanopipette in \( 100 \times 10^{-3} \) M KCl (prior to filling with carbon and electropolymerization). f) Linear sweep voltammograms recorded at the carbon surface area using \( 1 \times 10^{-3} \) M ferrocenemethanol as a redox mediator, see Note S1 in the Supporting Information for additional discussion. g) The \( I-V \)s of multiple devices after the PPy deposition with real-time feedback control. h) The \( I-V \)s for the multiple devices after aptamer/PPy deposition. The insets are the SEM images for the device at each stage. Scale bar: 100 nm. SEM images are shown in Figure S7 in the Supporting Information. In all cases, error bars indicate one standard deviation, \( n = 5 \).
changed from 0.17 to 0.5 within the gate voltage range of −400 to 400 mV in 100 × 10⁻³ M KCl (Figure S9, Supporting Information). Hence, the core PPy layer, which has high conductance, can be used to efficiently control the gate response of the FET, while the shell layer can be used for the selective sensing of target analytes.

Using this two-step PPy functionalization protocol and deposition feedback control, the final PPy coated nanopore diameter was 15.0 ± 0.7 nm (n = 5). This was achieved, by stopping the deposition when the nanopore current reaches a threshold of 2 nA at V_DS = 100 mV. This resulted in an initial PPy coated nanopore ≈40 nm in diameter (Figure 2g; Figure S7, Supporting Information). Following deposition of the shell PPy layer, using a nanopore current threshold of 0.7 nA, the nanopore diameter decreased to ≈15 nm (Figure 2h; Figure S7, Supporting Information). Using this approach, the nanopore I–V characteristics can be easily tuned by changing the set point of the open current to match the size of the target molecules of interest.

2.2. Aptamer Modified nexFET with Different Functionalization Density

Sensor validation was performed using the well-established TBA–thrombin binding system. We used human α-thrombin (37.5 kDa; pI of 7.0–7.4), that is a multifunctional protease in the bloodstream and has a significant role in physiological and pathological processes, such as blood coagulation, thrombosis, and angiogenesis. It is therefore essential to detect thrombin at trace levels (pm–nm range) with high sensitivity. The TBA used in this study, is 15-mer (5‘-GGTTGGTGTGGTTGG-3) ssDNA (Kd between 35–100 nm) and folds into a stable intramolecular G-quadruplex conformation in the presence of K⁺ which is required for thrombin binding.[35,41,42] To ensure that the TBA is sufficiently extended from the PPy shell to facilitate thrombin binding, a 12 base random sequence was added to the aptamer (Figure S11, Supporting Information).

In control experiments, no translocations were observed using plain nanopipettes before PPy polymerization (Figure 3a). With smaller nanopore sizes (15–20 nm), translocation events were rarely observed, even at thrombin concentrations of 1 × 10⁻⁹ M (Figure S10, Supporting Information). This was attributed to several factors including 1) The protein does not easily get transported electrochemically through the nanopore due to its heterogeneous charge of; 2) the translocation signal cannot be resolve from the noise due to the small analyte size and fast transport speed.

Quartz nanopores functionalized with PPy can slow down DNA translocation as a result of the interactions between the DNA and PPy. In this configuration, the translocation of thrombin is slowed by the electrostatic interaction with a more positively charged and smaller nanopore, resulting in sharp blockade events (Figure 3b). However, such a platform is not selective and cannot differentiate between molecules of similar size and charge.

With the addition of the aptamer/PPy shell layer, selectivity is improved, and the target protein can bind and unbind to the corresponding aptamer located on the shell. We studied the effect of TBA concentration, incorporated in the shell, at a constant thrombin concentration of 1 × 10⁻⁹ M. Upon the addition of 100 × 10⁻⁹ M TBA into the PPy precursor prior to electrochemical polymerization, the ICR changed from 1.53 (Figure 3b(ii)) to 0.96 (Figure 3c(ii)). The addition of 1 × 10⁻⁹ M TBA results in a negatively charged nanopore with ICR = 0.38 (Figure 3d(ii)) and 10 × 10⁻⁶ M TBA results in an even more negatively charged nanopore with ICR = 0.15 (Figure 3e(iii)).

At higher TBA concentrations, the number of available binding sites increases, as is evident by the “closed” TBA–thrombin bound state becoming more dominant. The increase in the “closed” fraction is due to the higher capture rate (Figure S12, Supporting Information) and prolonged dwell time (Figure S13, Supporting Information). When the binding sites are more densely packed, the bound molecules can detach, move and reattach to nearby sites.[43,44] On one hand, the aptamers can act as a transport system: the proteins can be accumulated by the aptamers on the whole polymer surface and then attracted by the nanopore under the electrophoretic force, resulting in a higher capture rate (Figure S14a, Supporting Information). On the other hand, the protein can hop along the binding sites located in the orifice and keep occupying the space in the nanopore, leading to prolonged dwell time (Figure S14b, Supporting Information).[29,31,32,44] A common problem when functionalizing nanopores is how to control the number of binding sites; if there are insufficient binding sites, the majority of the proteins will pass through the nanopore undetected (Figure 3c). With too many accessible binding sites (Figure 3e), the nanopore could remain in the blocked state. In this study, by varying the aptamer concentration in the polymerization electrolyte, the number of these functional groups located on the nanopore orifice can be controlled. It was found that a starting TBA concentration of 1 × 10⁻⁹ M was appropriate to ensure a reasonable capture rate (R_C = 1.53 ± 0.25 event s⁻¹, V_DS = 500 mV) while avoiding spontaneous pore blocking (Figure 3d). The V_DS dependence was similar to what would be expected with conventional transport through a nanopore, i.e., at higher bias voltage the capture rate increases, dwell time decreases, and peak amplitude increases (Figure S15, Supporting Information).[29,43,46] To further confirm the sensor is selective only to thrombin, we used an IgG antibody, which cannot bind to TBA, as a control. Compared to thrombin translocation (t_0 = 0.96 ± 0.61 ms, I_peak = 32.32 ± 12.43 pA), the antibody unbound signal is sharp and short (t_0 = 0.40 ± 0.21 ms, I_peak = 58.39 ± 19.09 pA), indicating there are no specific interactions between the TBA and the antibody (Figure S16, Supporting Information).

2.3. Protein Translocation Controlled by Gating

The rate of transport, along with peak amplitude and signal to noise could be tuned by biasing the gate electrode. V_C For instance, a V_C = −400 mV results in only sporadic events (R_C = 0.04 ± 0.01 event s⁻¹, V_DS = 500 mV), Figure 4. A likely explanation is that a negatively charged pore prevents the negatively charged proteins from passing through; the protein passes through without binding to the aptamer, leading to fast translocation.[24] Thrombin has a slight negative charge under the experimental conditions used (pH 8) and was repelled from the nanopore surface under negative gate voltage.
A positive gate voltage helps attract the thrombin towards the TBA and facilitates binding resulting in overall higher capture rate, Figure 4. A $V_G = 400\, \text{mV}$ resulted in a $54$-fold increase in capture rate, $4.1$-fold increase in the mean peak current, and $3.9$-fold increase in the signal-to-noise ratio, compared to $V_G = -400\, \text{mV}$. This was attributed to two contributions 1) the altered pore charge and hence, the increased likeliness of binding between aptamers and proteins, and 2) enhanced capture of the analyte due to electroosmotic flow. When the gate bias is sufficiently positive, the electroosmotic flow has the same direction as the electrophoretic force (from the bath to the inside of the pipette), facilitating analyte transport. Hence, it was possible to detect thrombin at concentrations as low as $50 \times 10^{-12}\, \text{M}$ (Figure S17, Supporting Information). Importantly, it is possible to detect analytes at a concentration well below the dissociation constant and can distinguish between bound and unbound states with single-molecule resolution.

Apart from the capture rate, another key characteristic of the nexFET was an improvement in the signal-to-noise ratio, Figure 4f. This was in large part due to the PPy surface conductivity and its voltage-induced conformational change. Under the experimental buffer conditions, adjacent PPy polymer chains tend to repel each other as they carry a moderately positive charge. As a result, the structure tends to swell and expand, which has also been observed in other elastic polymers such as poly(N-isopropylacrylamide).
Application of a negative gate voltage allows for the polymer to become less positively charged, thus decreasing repulsion between polymer chains and leading to a larger effective nanopore diameter and hence, lower signal-to-noise ratio (Figure S18a, Supporting Information). On the other hand, if a positive gate voltage is applied, then the structure will swell, and the nanopore will have a smaller effective diameter, resulting in a higher signal-to-noise ratio (Figure S18b, Supporting Information). This improvement is particularly useful as many of the proteins are too small to be detected. Interestingly, the dwell time of the protein translocation is not significantly affected by the gate voltage. This is attributed to interactions between the analyte and the nanopore, that do not originate from electrostatic forces but from aptamer–protein specific interactions. Therefore, it is likely that the dwell times (Figure S19, Supporting Information) are dependent on the number of binding sites rather than the gate bias as can be seen the current time traces in Figure 3.

3. Conclusion

In this work, we demonstrate that some of the challenges in nanopore protein sensing, that arise due to fast translocation speeds, low capture efficiency and recognition specificity[50] can be addressed when solid-state nanopores are integrated with a FET and functionalized with DNA aptamers. In particular, we demonstrate that the aptamer modified nexFET can selectively detect thrombin at a low concentration with improved performance. This is due to a combination of factors including nonspecific electrostatic interactions between the nanopore surface and the analyte, specific aptamer–protein interactions, and
potential differences generated at the gating nanoelectrode. The platform has a universal design, and the sensing capability can be expanded further to selectively detect a broad range of analytes for which aptamers are available.

4. Experimental Section

Fabrication and Characterization of Double-Barrel Nanopipettes: The double-barrel capillaries were plasma cleaned followed by careful loading into a laser-based pipette puller (Sutter Instrument, P-2000). Capillaries were heated and pulled to generate a pair of double barrel nanopipettes, as illustrated in Figure 2a(ii). The parameters of the one-step protocol are HEAT 700 FIL 3 VEL 45 DEL 130 PULL 93. It should be noted that the parameters of the pulling protocols are instrument specific and sensitive to both the temperature and humidity of the room.

Fabrication of Carbon-Coated Double-Barrel Nanopipettes with One Carbon Nanoelectrode: The carbon pyrolysis strategy was used, which was previously published.[12,13] Specifically, to deposit carbon only in one barrel, a plug was used to seal off the second barrel. Then, butane/propane was filled in the barrel and heated with a butane torch under a convective argon flow, which prevented the deposited carbon from being oxidized by the air under high temperatures. Within the inert atmosphere and high temperature, butane/propane can be pyrolyzed to form a conductive solid layer made up of carbon. To coat the whole cross-section of the nanopore rather than only one barrel, it is essential to heat location 1 for at least 15 s initially and then gradually move closer to the tip area (locations 2 and 3), as illustrated in Figure 2b(ii). The entire fabrication process took ~60 s.

After fabrication, cyclic voltammetry (CV) was used to characterize the approximate pore size in $100 \times 10^{-3}$ m of KCl (Sigma-Aldrich) before PPy polymerization. The area of the carbon nanoelectrode was examined by SLV using the redox mediator ferrocenemethanol (Sigma-Aldrich) in $100 \times 10^{-3}$ m KCl.

PPy Deposition with Real-Time Feedback Control: Pyrrole (Py, Sigma-Aldrich) was stored under an inert atmosphere, and the quality was always checked before use. The color of the liquid should be transparent.

PPy Predeposition with Real-Time Feedback Control: Pyrrole (Py, Sigma-Aldrich) was stored under an inert atmosphere, and the quality was always checked before use. The color of the liquid should be transparent otherwise, the pyrrole needs to be purified by column chromatography (using Al2O3). PPy was electropolymerized on the conductive surface of the carbon-coated dual-barrel nanopipettes by applying a 600 mV bias (this was determined by the voltage of the oxidation peak on the CV scan from ~800 to 800 mV) on the carbon nanoelectrode using a solution of $500 \times 10^{-3}$ m Py, $200 \times 10^{-3}$ m of lithium perchlorate (LiClO4, Sigma-Aldrich), and $100 \times 10^{-3}$ m of perchloric acid (HClO4, Sigma-Aldrich) in DI water. At the same time, the open barrel was filled with $100 \times 10^{-3}$ m KCl, and a small bias (100 mV) was applied to monitor the open pore current (Figure 2c(ii)). Real-time feedback control is crucial as the conductivity of the nanopore is tightly linked to its size. After PPy polymerization, the nexFET was stabilized in $100 \times 10^{-3}$ m HCl. A CV was performed between ~300 and 300 mV until the current stabilized.

To form the functional layer, a further deposition was conducted using a PPy/aptamer solution. The same feedback control deposition conditions were used as described above.

Ionic Currents and Translocation Measurement: I–V characteristics, FET behavior, and single-molecule sensing were measured using a Multiclamp 700B amplifier and 1530b digitizer (Molecular Devices). Patch A Ag/AgCl electrode was used as the patch electrode to apply the drain-source voltage. The ground/reference electrode was also Ag/AgCl, and it was placed into the bath. A silver wire was incorporated into the carbon and was used to bias the PPy.

All characterization experiments were performed in Tris–EDTA pH-buffered $100 \times 10^{-3}$ m KCl. There are two reasons for choosing this salt concentration: 1) The optimal condition of the nexFET performance was previously validated under different ionic strength conditions. It is clear that at 1 m salt, FET performance is reduced due to the shorter Debye screening length.[12] Furthermore, the intention is to ultimately use such a platform for practical use. One advantage of biosensors based on nanopipette is its geometry that can be used in vivo and living cell detection. Moreover, the highly demanding analytics and diagnosis requires sensors to work well in real samples. Therefore, a condition ($100 \times 10^{-3}$ m KCl, pH 8.0) that was close to the physiological condition was chosen to perform measurements. In the future, using this sensor would be tried to detect targets in real samples and even living cells.

The data was recorded by pClamp software (Molecular Devices) and analyzed using the Nanopore App written by Joshua Edel.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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