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Reversible Cation Response with a Protein-Modified Nanopipette

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The calcium ion response of a quartz nanopipette was enhanced by immobilization of calmodulin to the nanopore surface. Binding to the analyte is rapidly reversible in neutral buffer, and requires no change in media or conditions to regenerate the receptor. The signal remained reproducible over numerous measurements. The modified nanopipette was used to measure binding affinity to calcium ions, with a K_d of $6.3 \pm 0.8 \times 10^{-5}$ M. This affinity is in good agreement with reported values of solution-state protein. The behavior of such reversible nanopore-based sensors can be used to study proteins in a confined environment, and may lead to new devices for continuous monitoring.

Electrical devices that can measure ion current through a nanopore are gaining attention as a new way to design sensors with nanoscale resolution.^{1–2} Receptors immobilized to nanopore-based ion current sensors have included proteins,^{3–5} enzymes,⁶ DNA,⁷ aptamers,⁸ ligands,^{9–10} and small biomolecules,^{11–12} allowing nanoscale measurement of a variety of analytes. Sensing by modulation of ion current in functionalized nanopores is distinct from the technique of resistive-pulse sensing, which is used to characterize macromolecules by translocation through a pore.¹³ To distinguish this mechanism in functionalized nanopipette sensors,¹⁴ we coined the term signal transduction by ion nano-gating (STING), evoking both the role of ion current and the needle-like shape of the nanopipette. Essential to the sensitivity of many solid-state nanopore sensors is selective permeability of electrolytes, or ion current rectification, when a bias is applied across the nanopore. Ion current rectification (ICR) arises from the selective interaction between ions in solution and the surface of a charged, asymmetrically shaped nanochannel, or conical nanopore.¹⁵ Nanomaterials exhibiting ICR and used as sensors include track-etched nanopores in polymer membranes¹⁶ and quartz nanopipettes.¹⁷ In either case, the surface modification of nanopores with appropriate receptors is a key challenge to sensor development.

To date, the reversible binding of analytes with nanopore sensors has proven challenging. However, this is a critical issue if such devices are to be used for applications such as continuous monitoring or repeated measurements with one sensor. Multiple uses for a single sensor will also overcome problems in reproducible nanopore fabrication, which limits quantitative measurements for many sensors reported in the literature. For applications using reversible nanopore sensors, the nanopipette is a promising platform as it can be easily

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SUPPORTING INFORMATION AVAILABLE

Additional figures as noted in text. Experimental details for determination of binding constants.

fabricated at the bench with a range of pore sizes, and receptors can be immobilized using well-established surface chemistry. One key advantage of nanopipettes is they can be precisely and rapidly manipulated between samples, or within a single sample, with nanoscale precision. This allows the sensor to be used as a sub-microscopic imaging tool, as in the case of scanning ion current microscopy (SICM).¹⁸ For either imaging or continuous monitoring applications, a sensor with a stable and reversible signal is required. Functionalized nanopores responsive to pH have shown the best properties in terms of rapidly reversible and selective behavior.^{19–20} A recently reported nanopore-based sensor for peroxide used an immobilized redox enzyme to reversibly modulate the ion current rectification.⁶ Nanopipette sensors functionalized with chelators have been used for metal ion detection, and these can be regenerated by immersion in solution of low pH, reprotonating the ligand.^{9–10}

To achieve reversible cation response with a biological receptor, we prepared nanopipette sensors modified with calmodulin, a calcium binding protein that reversibly chelates calcium ($K_d \sim 10^{-6}$ M) with high selectivity.^{21–22} Intracellularly, interactions between calmodulin and various proteins and enzymes are regulated as free calcium levels fluctuate between 0.1 to 10 μ M. A schematic of reversible binding at the tip of a functionalized nanopipette is shown in Fig. 1. Electrical sensors using immobilized calmodulin have been previously reported for probing both calcium concentration^{23–24} and protein-protein interactions.^{25–26}

Our approach to sensor selection and surface functionalization began with deposition of polyelectrolytes to quartz nanopipettes. Polyelectrolytes have strong electrostatic interactions with the charged pore surface, and the ion current rectification is an excellent indicator of polyelectrolyte binding to the nanopore.^{12, 27–28} Using real-time ion current measurements, the layer-by-layer deposition of polyelectrolytes can be monitored to provide a rectifying nanopore with desired properties such as stability and conductivity. Reasoning that the adsorbed polyelectrolytes may be more stable if protected from liquid during washing and handling, we also investigated silanization of the outside of the nanopipette to confine surface chemistry to the inside of the nanopore.

To immobilize the protein to the nanopipette surface, we leveraged amide bond formation between the amine groups in the protein and carboxylate groups on the outermost polyelectrolyte layer. As reported below, we were able to produce sensors displaying selective and reversible binding of calcium at neutral pH with calmodulin-modified nanopipettes.

EXPERIMENTAL SECTION

Preparation and Characterization of Nanopipette Biosensors

Quartz capillaries with filament (QF100-70-7.5) from Sutter (Novato, CA) were used as received and pulled with a Sutter P-2000 laser puller to give nanopipettes. Puller settings used were heat 620, filament 4, velocity 60, delay 170, pull 180. The settings are variable depending on the puller, and were adjusted as needed to provide nanopipettes showing negative ion current rectification with the desired conductance. The nanopipettes described here showed a resistance of approximately 250 M Ω at a potential of -500 mV, for a calculated pore size of 10 nm (see supporting information for details). The nanopipettes were backfilled with a buffered electrolyte (pH 7 Tris-HCl, 10 mM and KCl, 100 mM) unless otherwise indicated.

The two sensors described are twin nanopipettes from one pulled capillary. Sensor CaM-1 was untreated prior to polyelectrolyte deposition, and CaM-2 was silanized with

trimethylchlorosilane (TMCS) using vapor deposition (see Fig. S1 in supporting information). The nanopipette was placed in a sealed chamber of 0.5 L volume with approximately 0.1 mL of TMCS for 10 minutes. The ion current was similar for silanized and bare nanopipettes, indicating the silanization did not appreciably affect the inner pore. Both nanopipettes were then backfilled with buffered electrolyte and immersed in a bath of the same buffer. The ion current was measured with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) using Ag/AgCl electrode in the nanopipette barrel and a reference Ag/AgCl electrode in the bath. A 500 mV sinusoidal voltage (5 Hz) was applied during subsequent surface treatment. The polyelectrolytes poly-L-lysine (PLL) and polyacrylic acid (PAA) were deposited on the surface of the nanopipette by sequential alternating immersion of the pipette tip into buffered electrolyte containing either PLL or PAA at a concentration of 3 ppm, with immersion in buffer to wash after each polyelectrolyte deposition. A polyelectrolyte layer was determined to be stable if the resulting change in current rectification (positive for PLL, negative for PAA) was maintained during immersion in buffer. Both CaM-1 and CaM-2 were functionalized with four layers: PLL, PAA, PLL, then PAA. The nanopipettes were then immersed in a solution containing 10 mg mL⁻¹ each of NHS and EDC (100 mM pH 6.1 MES buffer, with 50 mM KCl) for one hour. Finally, the tips of the nanopipettes were washed and immersed in a solution of calmodulin (bovine brain, Sigma A-3059), (0.05 mg mL⁻¹ in pH 6.1 MES buffer 100 mM with 50 mM KCl) and incubated for 18 h at 4 °C.

The electrical properties of the sensors and response to metal salts were analyzed using the electrical setup described above. All measurements were carried out in pH 7 buffered electrolyte solution with aliquots of either calcium chloride or magnesium chloride (1 to 10 μ L volumes) directly added to a bath of 0.3 mL buffer. Data was sampled at a rate of 200 Hz using the pClamp software and was processed using OriginPro 8.5. For continuous measurement data, the negative ion current peaks arising from the sinusoidal applied voltage were detected and plotted as a function of time. Line smoothing was done with a 50% percentile filter and a 10-point moving window. Ion current rectification was quantified using the coefficient (r), calculated using the following equation

$$r = \log_{10} \left(\frac{I_{pos}}{I_{neg}} \right)$$

where I_{pos} is the magnitude of the ion current at a potential of 500 mV, and I_{neg} is the magnitude of the ion current at a potential of -500 mV. Errors in ion current reflect the standard deviation between three separate measurements of I_{pos} and I_{neg} , with the same nanopipette after washing in buffer between measurements.

RESULTS AND DISCUSSION

Quartz nanopipettes of pore diameter from 10 to 65 nm were modified by polyelectrolyte deposition followed by protein conjugation. The stability of the surface chemistry was established by monitoring the ion current rectification (ICR) of the nanopipettes in real-time for several steps of functionalization. Twin nanopipettes, pulled from the same capillary, were taken through identical steps of surface treatment (see Fig. 2a and Supplementary Figures S2 and S3). Sensor CaM-1 was used directly after pulling, and CaM-2 was first treated with trimethylchlorosilane (TMCS) vapor to silanize the outer nanopipette tip. The ion current rectification (ICR) was monitored as the nanopipettes were immersed in neutral buffer containing either cationic poly-L-lysine (PLL) or anionic polyacrylic acid (PAA), with addition of subsequent layers only after the rectification remained stable in pure buffer.

The pore diameter of the nanopipettes can be estimated by ion current measurements (see supporting information and Table S1). While the pores sizes calculated by this method were generally in agreement with electron microscopy measurements, we observed that conductance in rectifying nanopipettes is influenced by surface charge as well as pore size. For example, the nanopipette used to make sensor CaM-1 had a resistance of 280 M Ω prior to chemical modification, giving a calculated pore diameter of 10 nm. The resistance increased by 30% after addition of the cationic PLL, indicating the deposited layer decreased the pore diameter by 3 nm. The addition of anionic PAA, however, lowered the resistance to 240 M Ω . This gives the impression that the pore size has increased, which is unlikely. Similarly, the resistance nanopipette used for sensor CaM-2 decreased by 50% after depositing a layer of PLL and PAA on the quartz surface. Because the ion current is stable throughout these measurements, we conclude these discrepancies between layer deposition and decreased resistance are due to changes in surface charge, rather than physical changes to pore geometry. We believe the pore becomes smaller after each deposited layer, but this is overshadowed by the effects on ion conductance of incorporating a highly charged polymer at the nanopore surface. While these factors limit the accuracy of calculating the pore diameter, there is a general trend that increasing surface layers does lead to greater nanopipette resistance. In comparing the resistance for both nanopipettes CaM-1 and CaM-2 after one and two layers of PLL/PAA, respectively, the calculated pore size decreases from 10 nm to 7 nm. At each of these stages, the surface is expected to have a high density of carboxylate ions.

As shown by the I-V curve in Fig. 2b, the bare nanopipette has a negative ICR. After two layers of PLL/PAA, the current is still negatively rectified, but smaller in magnitude indicating a decrease in the pore size after deposition. This behavior continues after immobilization of calmodulin protein, which is also negatively charged at neutral pH (pI $\sim$ 4). The rectification coefficient r reflects the behavior seen in the current voltage curves: $r = -0.27 \pm 0.03$ (bare pipette), -0.71 ± 0.02 (second layer of PLL/PAA), and -0.533 ± 0.014 (CaM). The low error in these measurements demonstrates the stability of the surface at each step. Both the silanized and un-silanized nanopipettes showed similar trends in current rectification throughout the surface modification. The primary difference between the two nanopipettes is that during PLL addition, there was less positive current rectification and a more stable signal in CaM-2, the sensor that was silanized (see Fig. S3). This may be due to effects of the hydrophobic surface on adherence of the polyelectrolytes, but these phenomena require further investigation.

Both the size of the pore and the surface chemistry were observed to influence sensor stability and reversibility. All sensors prepared from nanopipettes, including those with pore sizes of 50 nm (CaM-3) and 65 nm (CaM-4) responded to calcium salts with a decreased current at negative potentials. These sensors with larger pores were both slower to respond and showed a less stable signal. In contrast, CaM-1 and CaM-2, with pore sizes of approximately 10 nm, responded rapidly and gave stable, reversible signals. The stability of the signals from CaM-1 and CaM-2 increased after immobilization of the protein, as measured by repeated measurements with the same sensor. This may be an effect of crosslinking of the carboxylate groups of PAA with amines from PLL, forming a more physically robust surface. In general, we observed an increased signal stability for polyelectrolyte-modified nanopipettes after EDC coupling, including those not responsive to calcium.

At calcium ion concentrations of 0.1 mM, an unmodified quartz nanopipette will show only a 5% change in ion current (see Fig. 3), and requires concentrations of greater than 0.2 mM to show a significant change (see Fig. S4 in supplementary information). In contrast, the calmodulin-modified nanopipette responded strongly at concentrations of 0.1 mM, showing

32% decreased current. This effect appears to be due to specific interactions with the nanopipette surface rather than a change in ionic concentration, as analyte addition to the buffered electrolyte solution represents a change in ionic strength of < 1%. While divalent cations can have distinctive effects on ICR through interaction with anionic surface residues on nanopores,^{29–30} the effects observed here were only seen with calmodulin-functionalized nanopipettes.

The calmodulin-modified nanopipettes responded to calcium ions by a decreased current at negative potentials. No effect was seen at positive potentials, though this may be in part due to the negative current rectification. Thus, the binding of calcium ions can be considered as partially inhibiting the negative current rectification, which is consistent with binding divalent cations to a negatively charged, conical pore surface. The decreased negative rectification did not appear to depend on pore size. For sensors CaM-2, CaM-3, and CaM-4, with pore size ranging from 10 to 65 nm, the response to 0.2 mM calcium salts was a 40% decrease in current, with a 10% relative standard deviation. This leads us to conclude the signal modulation arises from a change in surface charge, rather than a change in protein conformation. On binding calcium ions in solution, the calmodulin protein undergoes an increase in hydrodynamic radius from approximately 2.5 to 3 nm.²¹ Were this conformational change responsible for the signal modulation in the nanopipette sensors, the difference would be expected to be negligible in the larger pores. Instead, sensors with both large and small pores had a response of comparable magnitude.

The binding isotherm was measured for sensor CaM-2 by repeated measurements at calcium concentrations from 0 to 0.2 mM. The K_d value was calculated as $6.3 \pm 0.8 \times 10^{-5}$ M, as compared with reported values on the order of 10^{-6} M.^{21–22} As seen in Fig. 3, the response of the nanopipette is clearly enhanced by immobilization of the calmodulin protein. For calcium detection in biological systems, the most common interfering divalent ion is magnesium. As shown in Fig. 3, nanopipette CaM-2 also shows an enhanced response to magnesium chloride beyond that of an unmodified nanopipette, which is not expected from the native protein. If calmodulin alone is responsible for the calcium response, the selectivity should be roughly 1000-fold for calcium over magnesium. The enhanced response to magnesium ions may be due to chelation of the divalent ions by other groups such as carboxylates from PAA, which shows no calcium/magnesium selectivity.³¹ It is also important to note that the four binding motifs of calmodulin show different cation binding affinities,²² and it is unknown how the immobilization in a nanopore will affect these properties. The selectivity for calcium over magnesium was 3:1 for nanopipette CaM-2 (silanized surface), but only 1.5:1 using nanopipette CaM-1, in which the surface was not silanized. This may be due to passivation of the surface, but needs further investigation. At present, it is unknown how a hydrophobic coating at the surface of a nanopipette tip will affect interactions with ions at the sensitive region inside the pore. For both nanopipettes, the responses to calcium and magnesium were highly reproducible, and the signal returned to the baseline simply by immersing in buffer solution (see Figs. 4b,c and S5).

The response of the calmodulin-modified nanopipettes to calcium ions is both rapid and reversible, requiring no conditions other than an absence of calcium salts to restore the signal to baseline. The response was measured using the signal at a potential of -500 mV. On adding an aliquot of calcium chloride to the bath (final concentration 0.10 mM), nanopipette CaM-1 responded with a decreased current from -700 to -350 pA (Fig. 4a). The response was on the order of seconds, and equilibration to the new condition was rapid. Nanopipette CaM-2, in which the outer surface was passivated with a silane layer, showed a similar current modulation with the same concentration of calcium chloride (change from -1300 to -600 pA). As shown in Fig. 4b, moving the nanopipette from a bath of 0.10 mM calcium chloride to 0.10 mM magnesium chloride resulted in a diminished and completely

reversible response (-1100 pA), showing that the ions are quite labile at the receptor sites. The nanopipette returned to the baseline and continued to detect calcium for several cycles, as shown in Fig. 4c. Both nanopipettes showed a linear response to calcium chloride up to 0.10 mM, though the saturation point was not determined. The detection limit for calcium chloride was 20 μ M using nanopipette CaM-2, which is 3 times the standard deviation from blank measurements.

Nanopipette CaM-1 was used for 20 independent measurements including buffer-only as a control before it stopped responding to calcium chloride, while nanopipette CaM-2 was used for over 40 measurements over a period of two hours before it no longer responded to the analyte. When it ceased sensing, the baseline current for CaM-1 decreased (from -700 to -300 pA). Similarly, the baseline current of nanopipette CaM-2 decreased from -1300 to -900 pA after 20 measurements, after which the response to calcium was diminished. Over the course of roughly 20 more measurements, the baseline signal remained at -900 pA but the calcium response diminished progressively until it became negligible. Because the current did not increase over time, we assume the protein and polyelectrolytes did not detach and become removed from the pore. The decrease in baseline current after repeated use of the nanopipettes could indicate closing of the pore by rearrangement or aggregation of the polyelectrolyte layers.

The issue of selectivity is critical for applications of ion sensing. While the calmodulin modified nanopipettes responded well to micromolar concentrations of calcium salts in buffers containing 100 mM potassium ions, the ion current signal was also sensitive to magnesium ions. Further improvements in protein immobilization may result in selectivity typical of the native solution-state protein. Alternatively, binding events may cause perturbations to current signatures, which may help to differentiate analytes. Unlike the case of detecting discrete ions as they pass through a single, protein-based nanochannel,³² the calmodulin-modified pore contains multiple proteins with multiple binding sites. Thus, current perturbations from individual binding events will be invisible due to averaging. We did observe millisecond-scale perturbations to the ion current in the presence of 0.1 mM calcium ions not observed with magnesium ions at that concentration (Fig. S6). Whether these perturbations are due to conformational changes in the proteins or general constriction of the ion current cannot be determined at this point, but such events may help discriminate analyte binding in future work.

CONCLUSIONS

The calmodulin-modified nanopipette described here shows an enhanced response to calcium ions that is rapidly reversible. The highly stable signal allowed for multiple measurements with a single nanopipette, allowing for determination of binding constants. Improving these methods will be useful for characterizing proteins confined in nanochannels, or for measuring protein-protein interactions using nanopore sensors. Further experiments and theoretical modeling, as well as advanced methods of characterizing nanopore surfaces, will be required to more fully understand proteins immobilized to nanopores. Reversible sensors based on nanopipettes may be used for spatial resolution of ion concentration at the nanoscale (functional mapping) or continuous intracellular measurements of specific analytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of reversible ion binding at the tip of a nanopipette. As calcium ions (yellow spheres) are bound by calmodulin protein (blue), changes to the surface charge at the nanopore will affect the ion current. A decrease in current is expected at a negatively biased pore as a result of divalent cation binding.

Figure 2.

Surface modification and ion sensitivity of the nanopipette biosensor. (a) Schematic of surface functionalization on quartz by deposition of polyelectrolyte layers (red, PLL; blue, PAA) followed by amide bond formation to CaM protein using NHS/EDC coupling. (b) Functionalization of sensor CaM-1. Current-voltage response of the bare pipette (■), after two surface layers of polylysine (PLL) followed by polyacrylic acid (PAA) (●), and after coupling with CaM (▲). Error bars reflect the standard deviation from three measurements with the same sensor, washed in buffer between each measurement.

Figure 3.

Thermodynamic binding curve of calcium and magnesium ions with nanopipette CaM-2. The ion current was measured at each concentration of calcium chloride (■) and magnesium chloride (◆) with a potential of -500 mV. A control nanopipette with an untreated quartz surface is shown with calcium chloride and magnesium chloride (black markers). The nanopipette was washed in buffer between each experiment. The curve shown is a best fit to the binding equation described in the supplementary information to give an apparent dissociation constant. $K_d(\text{Ca}^{2+}) = 6.3 \pm 0.8 \times 10^{-5}$ M. $K_d(\text{Mg}^{2+}) = 3 \pm 1 \times 10^{-4}$ M.

Figure 4.

Response time and reversibility of nanopipettes. (a) Response of CaM-1 when an aliquot of calcium chloride is added (final concentration 0.10 mM). Transient current spikes are due to mixing. (b) Continuous signal obtained for CaM-2 immersed in alternating baths of buffer and calcium chloride (c) Cycles of regenerating baseline signal from CaM-2 after immersing in calcium chloride followed by neutral buffer. Values are the average signal from 30 seconds continuous measurement. Buffer solutions contained pH 7 Tris-HCl (10 mM) and KCl (100 mM). Calcium chloride was added to the buffer to give a concentration of 0.10 mM. Signals shown are the ion current obtained at -500 mV potential.