Published in final edited form as:

Biosens Bioelectron. 2011 July 15; 26(11): 4503–4507. doi:10.1016/j.bios.2011.05.010.

Reversible thrombin detection by aptamer functionalized STING sensors

Paolo Actis^{1,2,3}, Adam Rogers¹, Jeff Nivala¹, Boaz Vilozny^{1,3}, R. Adam Seger^{1,3}, Olufisayo Jejelowo², and Nader Pourmand^{1,3}

¹Department of Biomolecular Engineering, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA

²Department of Biology, Texas Southern University, 3100 Cleburne Street, Houston, TX 77004, USA

³Advanced Studies Laboratories, UC Santa Cruz and NASA Ames Research Center, Moffett Field, California 94035

Abstract

Signal Transduction by Ion NanoGating (STING) is a label-free technology based on functionalized quartz nanopipettes. The nanopipette pore can be decorated with a variety of recognition elements and the molecular interaction is transduced via a simple electrochemical system. A STING sensor can be easily and reproducibly fabricated and tailored at the bench starting from inexpensive quartz capillaries. The analytical application of this new biosensing platform, however, was limited due to the difficult correlation between the measured ionic current and the analyte concentration in solution. Here we show that STING sensors functionalized with aptamers allow the quantitative detection of thrombin. The binding of thrombin generates a signal that can be directly correlated to its concentration in the bulk solution.

1. Introduction

Nanopore-based sensing platforms hold great promise not only in basic research but also for medical diagnostics (Dekker 2007). The vast majority of nanopore-based sensing platforms monitor temporary ion current blockades as molecules translocate through the nanopore (Martin and Siwy 2007). Notably, however, permanent blockage arising from affinity-based binding between probes tethered to the nanopore surface and their target in solution can be more informative, as specific interactions can be easily detected (Actis et al. 2011; Umehara et al. 2009).

Signal Transduction by Ion NanoGating (STING) is a recently developed sensing technology based on a functionalized quartz nanopipette. STING relies on a simple electrochemical readout that can transduce binding events at the tip of the nanopipette without the need for secondary probes or labels. One of the major advantages of this technology is that STING sensors can be easily and reproducibly tailored at the bench without the need of a nanofabrication facility. The high impedance of the nanopipette tip constrains the sensitivity of the device, making the dimension and geometry of the tip orifice

© 2011 Elsevier B.V. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

crucial for the sensor performance (Actis et al. 2010b). Signal transduction occurs when charged molecules interact with the nanopipette surface and/or when affinity-based events cause a physical occlusion of the nanopipette orifice. The conical geometry, the nanometer size pore, and surface charges generate a peculiar electrochemical behavior referred to as current rectification, in which a nanopipette responds to a symmetric input voltage with an asymmetric current output (Wei et al. 1997).

Current rectification arises from the diffuse electrical double layer (ddl) thickness that is comparable to the diameter of the nanopipette. The electrostatic interaction between ionic species and fixed charges on the nanopipette surface affect its permselectivity, thus causing current rectification. By monitoring current rectification, STING sensors can accurately report on specific interactions occurring at the nanopipette tip.

Furthermore, STING technology can be easily integrated with piezoactuators to generate a sensor with high spatial resolution. As a nanopipette approaches a surface, the ionic current through the pipette will decrease due to “current squeezing,” a well known effect, exploited to great benefit in scanning ion conductance microscopy (SICM) (Hansma et al. 1989). Besides sensing, nanopipette based platforms have been used to control chemical reactions at the nanoscale (Vilozny et al. 2011), investigate single-molecule biophysics (Clarke et al. 2005), for the controlled delivery of molecules inside a single cell (Laforge et al. 2007), and to image cells at the nanoscale (Klennerman and Korchev 2006).

Two limitations exist for this otherwise useful nanopipette sensor technology. We recently reported that antibodies tethered to the nanopipette surface can be employed as molecular recognition elements for the sensitive and specific detection of proteins and environmental toxins. However, antibody-based detection schemes have some drawbacks, including production, cost, limited target analytes, and limited shelf lives. Another limitation of STING technology is the difficulty in correlating a variation of the measured ionic current with the analyte concentration in solution. Several papers have been published showing the potential of functionalized nanopipettes for biosensing (Ding et al. 2009; Fu et al. 2009; Umehara et al. 2009); none of these, however, used the variation of the ionic current as a binding indicator. In a previous paper, we employed the time to reach a saturation level of the antigen binding curve for a direct correlation of analyte concentration (Actis et al. 2010a). This indicator, however, is heavily influenced by experimental conditions and it would be extremely difficult to standardize as minor changes in the experimental setup can largely affect the measured signal.

To address the first of these limitations, we explore here the use of aptamers instead of antibodies. Aptamers are single-stranded oligonucleotides, designed through an *in vitro* selection process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (Ellington and Szostak 1990; Tuerk and Gold 1990). Aptamers have similar affinity and selectivity for targets as antibodies, but they can be chemically synthesized, stored in ambient conditions, and easily regenerated. In addition, aptamers can be engineered to undergo a large-scale conformational response to specific molecules, largely affecting the ion transport through the nanopipette (Abelow et al. 2010).

Here we show, the reversible thrombin detection on aptamer functionalized STING sensors. We selected thrombin and its specific aptamer because this bioaffinity pair is well studied (Bock et al. 1992; Mir et al. 2006; Willner and Zayats 2007), and thrombin is the last enzyme in the clotting cascade functioning to cleave fibrinogen to fibrin which forms the fibrin gel of a hemostatic plug or a pathologic thrombus (Ishihara et al. 1997). We demonstrate that thrombin binding causes a variation in the output ionic current, due to the

partial occlusion of the nanopipette pore that can be directly correlated with the concentration of the analyte in solution.

2. Materials

2.1 Reagents

Poly-L-lysine and Polyacrylic acid were purchased from Electron Microscopy Sciences (Hatfield, PA) and Aldrich (Saint Louis, MO), respectively. N-hydroxysuccinamide (NHS) and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL). The 5' amino-modified thrombin aptamer with a six carbon spacer (5'-AmM-C6-GGTTGGTGTGGTTGG-3') was obtained from Integrated DNA Technologies. Thrombin, BSA and human serum were acquired from Sigma (Saint Louis, MO). Ultrapure water was used to make all aqueous solution reagents.

2.2 STING sensor fabrication

STING sensors can be inexpensively fabricated from quartz capillaries using a commercially available laser puller. The precise control of the dimensions and geometry of the nanopipette is crucial, as the sensitive region of the sensor is the nanopipette tip (Umehara et al. 2009). Nanopipettes were constructed out of filament-containing quartz capillaries of 1.0 mm outer diameter and 0.70 mm inner diameter (QF100-70-5; Sutter Instrument Co., Novato, CA). The capillary was pulled using a P-2000 laser puller (Sutter Instrument Co., Novato CA) preprogrammed to fabricate nanopipettes with an inner diameter of ~ 50 nm (Fig. S1 and S2) (Karhanek et al. 2005). Parameters used were: Heat 625, Fil 4, Vel 60, Del 150, and Pul 192.

2.3 Aptamer Immobilization

The thrombin aptamer was immobilized on the inside of the nanopipette tip through a four step coating process: Nanopipettes were first filled with a 0.01% poly-L-lysine aqueous solution and allowed to rest ~5 minutes to allow the solution to saturate the tip. The nanopipettes were then drained and baked at 120 °C for 1 hr. After baking and rinsing of the nanopipette with water, this process was repeated with a 0.01% polyacrylic acid aqueous solution. After baking of the PAA layer, the nanopipettes were filled with a fresh EDC/NHS solution (10 mg/mL in MES Buffer), and allowed to rest at room temperature for 1hr. The EDC/NHS solution was then removed and the nanopipettes were rinsed three times with water. Finally, the nanopipettes were filled with a 2 μ M aptamer solution in PBS and allowed to rest at room temperature overnight. Any remaining aptamer solution was rinsed out and nanopipettes were washed three times with PBS solution prior to experimental use. After thrombin detection, STING sensors were regenerated by immersion in fresh PBS buffer (Fig. 1).

2.4 Measurement Setup

The experimental setup has been described previously (Actis et al., 2010). Briefly, a two Ag/AgCl electrode setup was used, in which the working electrode was inserted inside the STING sensor (aptamer-functionalized nanopipette backfilled with a PBS solution at pH 7.4) and a reference electrode was placed in the bulk PBS solution. The electrodes were controlled by a Multiclamp 700B amplifier with a DigiData 1440A digitizer (Molecular Devices), interfaced with pClamp 10 software on a PC (Molecular Devices). For all experiments, a 500 mV, 5Hz sine wave was applied to induce ion current flow. One sinusoidal cycle produced a pair of positive and negative peak amplitude values. For biosensing experiments only the negative peaks were computed, as the binding of thrombin caused a larger effect than on the positive ones. All experiments were performed at room

temperature. Additional data analysis and workup were performed using Origin 8.5 software (Origin Labs).

3. Results and Discussion

3.1 Thrombin Detection

The rectification coefficient is a useful indicator of the rectifying properties of a nanopipette and therefore of the fixed charges on the sensor surface.

The rectification coefficient, r , is defined as the logarithm of the ratio between the current measured at particular positive voltage and the current measured at the same voltage but with the opposed polarity.

$$r = \log_{10} \frac{I_+}{I_-}$$

A bare quartz nanopipette shows a negative current rectification, since its surface is negatively charged. One of the key advantages of STING technology is that every functionalization step can be monitored in real time, allowing quality control for every STING sensor fabricated (Fig. 2). The physisorption of a positively charged polyelectrolyte, such as poly-L-lysine (PLL) on the negatively charge nanopipette affects its permselectivity by introducing positively charged amino groups (Umehara et al. 2006). The rectification coefficient increased from -0.1 for the bare sensor to $+0.25$ after the physisorption of PLL. Similarly, the physisorption of polyacrylic acid (PAA) on a PLL modified nanopipette introduces a negatively charged carboxylic group on the sensor surface and inverts and magnifies the rectification coefficient to -1.4 . The reaction of the PAA-modified nanopipette with NHS/EDC cancels the current rectification as EDC reacts with carboxyl groups to form neutral, amine-reactive O-acylisourea intermediates. The subsequent overnight coupling with amino modified thrombin aptamer introduces a negative rectification ($r = -0.6$), as negatively charged oligonucleotides are tethered to the nanopipette. The specific recognition of thrombin causes an increase in the rectification coefficient ($r = -0.4$) as the aptamer negative charge is shielded by neutral thrombin molecules.

Due to the conical shape of the nanopipette, the impedance of the sensor rapidly decreases far away from the very tip. This effect confines the sensitivity to an area that is a few micrometers from the nanopipette orifice. The impedance changes due to molecular recognition on the sensor surface, however, depend on the specific location where the binding occurs.

The interaction of thrombin with its specific aptamer tethered on the STING sensor causes a partial occlusion of the nanopipette pore. The aptamer is in equilibrium between a random structure and a folded state, an equilibrium which is shifted towards the four-stranded structure by binding to thrombin (Mascini 2009). The normalized current decreases by 13% in less than a minute. STING sensors reproducibly respond to a fixed thrombin concentration ($200 \mu\text{g/mL}$) with a decrease in the normalized current of $(10 \pm 3)\%$ for 5 STING sensors used for the study. As a control, we performed the same experiment using bovine serum albumin (BSA) instead of thrombin. The aptamer has no affinity for BSA, and therefore any signal change must be attributed to non-specific interaction with the STING sensor. No change was detected on the normalized current under incubation with BSA, indicative of the selectivity of thrombin binding and detection in this system.

Most of the STING sensors employed in this study showed a noise level $\leq 3\%$ of the measured current. In some cases, however, the noise level was higher, as Figure 3 shows. In this case the change in the output current caused by the binding of was fitted to a sigmoid to mitigate the noise. The variation of the measured ionic current upon thrombin binding is a direct indication of the concentration of thrombin in the bulk solution (Fig. 4). STING sensors are responsive to thrombin in the range 10–200 $\mu\text{g/mL}$, which is in agreement with values reported in the literature (Bini et al. 2007; Wang and Liu 2009).

Several groups developed more sensitive platform for the detection of thrombin. Wang et al. developed an electrochemical assay based on the aptamer labeling with 6-(Ferrocenyl)hexanethiol loaded silica nanocapsules allowing detection of thrombin within the range 0.1–5 nmol/L (Wang et al. 2011). Dai and Kool recently reviewed the application of fluorescent sensors that make use of DNA structures to monitor enzymatic activities. They discussed in detail new strategies to increase the detection limit of fluorescent aptasensors for the detection of thrombin (Dai and Kool 2011). Change et al. developed a highly sensitive and specific fluorescence resonance energy transfer (FRET) aptasensor for thrombin based on the dye labeled aptamer assembled graphene that detects thrombin in the low pM range. All these approaches allow the ultrasensitive detection of thrombin, bypassing the inherent K_d (around 100nM) of the thrombin-aptamer couple (Bock et al. 1992) by introducing some sort of labeling or post-processing. STING technology transduces the biomolecular interaction between thrombin and its aptamer in real-time without any need for labeling. These unique features will allow the continuous monitoring of thrombin not constraining the measurements to a single time point. We believe that by optimizing the aptamer immobilization at the nanopore, we will be able to reduce analyte consumption and increase the sensitivity of STING sensors. Furthermore, Calander demonstrated that the applied voltage can be used to concentrate molecules at the tip of a nanopipette (Calander 2009). This property can be further exploited to enhance the limit of detection of STING sensors by concentrating thrombin at the tip of the nanopipette.

3.2 Reversible Thrombin binding

One of the major advantages of aptamers over antibodies is their ability to reversibly bind their target analytes. We found that a simple immersion of the STING sensor into a fresh PBS solution causes the release of the bound thrombin molecules. STING sensors were regenerated up to 4 times without a noticeable loss of performance (Fig. 5). These findings are in contrast with those described in the literature showing that either heat, salt concentration, pH of the medium, or chelating agents (Jayasena 1999) are necessary to regenerate the sensor. We believe that the confinement of the aptamer into a nanopore affects its binding affinity with thrombin, thus causing the release of thrombin by simple immersion in a PBS buffer.

3.3 Response in Serum

We investigated the response of the STING sensor to thrombin in various serum concentrations. The immersion of a STING sensor in a diluted serum solution causes an instantaneous decrease in the output current caused by adsorption of serum proteins on the sensor surface which is dependent on the serum concentration. The performance of the sensor is slightly affected by concentrations of serum up to 20%. Higher concentrations of serum caused the immediate clogging of the STING sensor which can, however, be completely recovered by immersing it into a fresh PBS solution. Interestingly, we observed that, while in pure PBS, the binding of thrombin caused a reduction in the measured current, in a serum solution the current increased upon the addition of thrombin. We speculate that this behavior is caused by the displacement of weakly adsorbed serum proteins induced by selective thrombin recognition (Figure 6).

4. Conclusion

We have demonstrated the selective and reversible thrombin detection using aptamer-functionalized STING sensors. We showed a direct correlation between the signal change and the analyte concentration in the bulk solution. The reversible binding of thrombin on aptamer-functionalized STING sensors was confirmed both in pure buffer solution and in diluted serum. The reversible binding allows individual sensor calibration, neglecting the variability caused by the fabrication process. Reversible STING sensors will allow the study of biomolecular interactions requiring a continuous analyte monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants from the National Aeronautics and Space Administration Cooperative Agreements [NNX08B47A and NNX10AQ16A], and the National Institutes of Health [P01-HG000205]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Aeronautics and Space Administration or the National Institutes of Health. The authors want to acknowledge Robert Hoelle for technical assistance with the SEM imaging.

References

- Abelow AE, Schepelina O, White RJ, Vallee-Belisle A, Plaxco KW, Zharov I. Biomimetic glass nanopores employing aptamer gates responsive to a small molecule. *Chemical Communications*. 2010; 46(42):7984–7986. [PubMed: 20865192]
- Actis P, Jejelowo O, Pourmand N. Ultrasensitive mycotoxin detection by STING sensors. *Biosensors and Bioelectronics*. 2010a; 26(2):333–337. [PubMed: 20829024]
- Actis P, Mak A, Pourmand N. Functionalized nanopipettes: toward label-free, single cell biosensors. *Bioanalytical Reviews*. 2010b; 1(2):177–185. [PubMed: 20730113]
- Actis P, Vilozy B, Seger RA, Li X, Jejelowo O, Rinaudo M, Pourmand N. Voltage-Controlled Metal Binding on Polyelectrolyte-Functionalized Nanopores. *Langmuir*, null-null. 2011
- Bini A, Minunni M, Tombelli S, Centi S, Mascini M. Analytical Performances of Aptamer-Based Sensing for Thrombin Detection. *Analytical Chemistry*. 2007; 79(7):3016–3019. [PubMed: 17338547]
- Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature*. 1992; 355(6360):564–566. [PubMed: 1741036]
- Calander N. Analyte Concentration at the Tip of a Nanopipette. *Analytical Chemistry*. 2009; 81(20): 8347–8353. [PubMed: 19751050]
- Clarke RW, White SS, Zhou D, Ying L, Klenerman D. Trapping of proteins under physiological conditions in a nanopipette. *Angew Chem Int Ed Engl*. 2005; 44(24):3747–3750. [PubMed: 15883978]
- Dai N, Kool ET. Fluorescent DNA-based enzyme sensors. *Chemical Society Reviews*. 2011
- Dekker C. Solid-state nanopores. *Nat Nano*. 2007; 2(4):209–215.
- Ding S, Gao C, Gu L-Q. Capturing Single Molecules of Immunoglobulin and Ricin with an Aptamer-Encoded Glass Nanopore. *Analytical Chemistry*. 2009; 81(16):6649–6655. [PubMed: 19627120]
- Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature*. 1990; 346(6287):818–822. [PubMed: 1697402]
- Fu Y, Tokuhisa H, Baker LA. Nanopore DNA sensors based on dendrimer-modified nanopipettes. *Chem Commun (Camb)*. 2009; (32):4877–4879. [PubMed: 19652811]
- Hansma P, Drake B, Marti O, Gould S, Prater C. The scanning ion-conductance microscope. *Science*. 1989; 243(4891):641–643. [PubMed: 2464851]

- Ishihara H, Connolly AJ, Zeng D, Kahn ML, Wu Zheng Y, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*. 1997; 386(6624): 502–506. [PubMed: 9087410]
- Jayasena SD. Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. *Clin Chem*. 1999; 45(9):1628–1650. [PubMed: 10471678]
- Karhanek M, Kemp JT, Pourmand N, Davis RW, Webb CD. Single DNA Molecule Detection Using Nanopipettes and Nanoparticles. *Nano Letters*. 2005; 5(2):403–407. [PubMed: 15794633]
- Klenerman D, Korchev Y. Potential biomedical applications of the scanned nanopipette. *Nanomedicine (Lond)*. 2006; 1(1):107–114. [PubMed: 17716213]
- Laforge FO, Carpino J, Rotenberg SA, Mirkin MV. Electrochemical attosyringe. *Proceedings of the National Academy of Sciences*. 2007; 104(29):11895–11900.
- Martin CR, Siwy ZS. Learning Nature's Way: Biosensing with Synthetic Nanopores. *Science*. 2007; 317(5836):331–332. [PubMed: 17641190]
- Mascini, M., editor. *Aptamers in Bioanalysis*. New Jersey: Wiley; 2009.
- Mir M, Vreeke M, Katakis I. Different strategies to develop an electrochemical thrombin aptasensor. *Electrochemistry Communications*. 2006; 8(3):505–511.
- Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*. 1990; 249(4968):505–510. [PubMed: 2200121]
- Umehara S, Karhanek M, Davis RW, Pourmand N. Label-free biosensing with functionalized nanopipette probes. *Proceedings of the National Academy of Sciences*. 2009; 106(12):4611–4616.
- Umehara S, Pourmand N, Webb CD, Davis RW, Yasuda K, Karhanek M. Current Rectification with Poly-L-Lysine-Coated Quartz Nanopipettes. *Nano Letters*. 2006; 6(11):2486–2492. [PubMed: 17090078]
- Vilozny B, Actis P, Seger RA, Pourmand N. Dynamic Control of Nanoprecipitation in a Nanopipette. *ACS Nano*, null-null. 2011
- Wang Y, He X, Wang K, Ni X, Su J, Chen Z. Electrochemical detection of thrombin based on aptamer and ferrocenylhexanethiol loaded silica nanocapsules. *Biosensors and Bioelectronics*. 2011; 26(8): 3536–3541. [PubMed: 21376563]
- Wang Y, Liu B. Conjugated Polyelectrolyte-Sensitized Fluorescent Detection of Thrombin in Blood Serum Using Aptamer-Immobilized Silica Nanoparticles as the Platform. *Langmuir*. 2009; 25(21): 12787–12793. [PubMed: 19785411]
- Wei C, Bard AJ, Feldberg SW. Current Rectification at Quartz Nanopipet Electrodes. *Analytical Chemistry*. 1997; 69(22):4627–4633.
- Willner I, Zayats M. *Electronic Aptamer-Based Sensors*. *Angewandte Chemie International Edition*. 2007; 46(34):6408–6418.

Figure 1. Schematic representation of the measurement setup and thrombin binding on the aptamer functionalized STING sensor.

Figure 2. Monitoring of the functionalization of the STING sensor through electrochemical measurements. All measurements were carried out in a PBS solution (pH 7.4). Rectification coefficient calculated at $\pm 500\text{mV}$.

Figure 3. Variation of the normalized current upon thrombin and BSA interaction with the STING sensor. Thrombin and BSA concentration ($150\mu\text{g/mL}$). Solution: PBS (pH 7.4).

Figure 4. Response of the STING sensor to increasing concentration of thrombin. The red line shows the linear fit of the linear part of the curve ($R=0.95$). Solution: PBS (pH 7.4).

Figure 5. Regeneration of the STING sensors after binding of thrombin. Sensors were regenerated by simple immersion into a fresh PBS solution. Thrombin Concentration: 400 $\mu\text{g/mL}$.

Figure 6.

Response of STING sensors to thrombin in different concentrations of serum in PBS. Thrombin Concentration: 400 $\mu\text{g/mL}$. In pure PBS buffer, the binding of thrombin induces a reduction in the measured current. By contrast, in a serum solution the current increases upon addition of thrombin. The STING sensor is still responsive to thrombin in PBS, after detection in serum.