



Ultrasensitive mycotoxin detection by STING sensors

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ABSTRACT

Signal transduction by ion nano-gating (STING) technology is a label-free biosensor capable of identifying DNA and proteins. Based on a functionalized quartz nanopipette, the STING sensor includes specific recognition elements for analyte discrimination based on size, shape and charge density. A key feature of this technology is that it does not require any nanofabrication facility; each nanopipette can be easily, reproducibly, and inexpensively fabricated and tailored at the bench, thus reducing the cost and the turnaround time. Here, we show that STING sensors are capable of the ultrasensitive detection of HT-2 toxin with a detection limit of 100 fg/ml and compare the STING capabilities with respect to conventional sandwich assay techniques.

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1. Introduction

Mycotoxins are small (MW ~700 g/mol), toxic chemical products formed as secondary metabolites by a few fungal species that readily contaminate crops in the field or after harvest. These compounds pose a potential threat to human and animal health through the ingestion of food products prepared from contaminated commodities.

At this time, the great diversity of toxins represents a challenge; detection methods are currently specific for individual toxins or groups of similar toxins. Because each toxin requires a different method, standardization of techniques to detect all mycotoxins remains elusive. Likewise, practical requirements for high-sensitivity detection and the need for a specialized laboratory setting create challenges for routine analysis. Therefore, depending on the physical and chemical properties of the toxins, procedures have been developed around existing analytical techniques, which offer flexible and broad-based methods of detecting compounds (Turner et al., 2009).

Traditionally, thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) have been employed for toxin detection. However, the tedious sample preparation and cleanup often lead to inconsistent results and poor sensitivity (Daly et al., 2000). Various research groups have employed surface plasmon resonance (SPR) - based sensors for applications such

as inhibition immunoassays (Stubenrauch et al., 2009) and antibody affinity analysis (Reid et al., 2007). SPR analysis changes in the interfacial optical properties of modified electrodes induced by the binding of biomolecules on the surface. Although the SPR platform is capable of label-free, real time monitoring of molecules as small as 200 Da, this requires highly sophisticated and expensive equipment (Skottrup et al., 2008).

In their 2002 study, Schnerr et al. (2002) developed an inhibition immunoassay for the rapid quantification of the trichothecene mycotoxin deoxynivalenol using the SPR-based Biacore system. Despite its versatility, the complexity and the cost of the Biacore instrumentation remain very high (Mullett et al., 1998). The low molecular weight of mycotoxins is often not enough to induce significant change upon binding to the sensor surface. Consequently, an alternative assay strategy is required for mycotoxin detection using SPR. One of the most established laboratory-based biochemical assays for pathogen detection is ELISA, which is based on the detection of pathogen-specific surface epitopes using antibodies (Cunningham, 2000). With its very high specificity and exceptional sensitivity, ELISA is often referred to as the gold standard of toxin detection. Nevertheless, current assays typically involve reporter molecules or labels conjugated to enzymes or fluorescent markers, which makes ELISA restricted to advanced laboratory settings with specialized read-out equipment (Skottrup et al., 2008). Accurate and rapid read-out on site would provide vital efficiency in toxin detection, reducing potential risks of further unnecessary food-borne pathogen contamination. However, implementing ELISA into a point-of-use test remains challenging due to the sheer complexity of the instrumentation involved. In 2009, Valdés et al. (2009)

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reviewed the application of nanotechnology-based platforms for the detection of mycotoxins.

More recently, our lab employed a magnetic nanotag (MNT) detection platform for multiplexed mycotoxin detection (Mak et al., 2010). Real-time measurements were conducted upon the addition of MNTs onto the spin-valve sensor surface immobilized with capture antibodies for mycotoxins (aflatoxin-B1, zearalenone and HT-2). The MNT technology demonstrated detection limits for mycotoxins in the pg/ml level.

Here we describe a new technique, signal transduction by ion nano-gating (STING), which uses a functionalized quartz nanopipette as an electrochemical biosensor. A key feature of this technology is that it does not require any nanofabrication facility; each nanopipette can be easily, reproducibly, and inexpensively fabricated and tailored at the bench, thus reducing the cost and the turnaround time. The electrochemical sensitivity of the device is maximized at the nanopipette tip, essentially an elongated cone, making the dimension and geometry of the tip orifice crucial for biosensor performance (Umehara et al., 2009). Permanent blockade, or gating, from binding events at the nanoscale-sized tip opening cause distinctive changes to the nanopipette electrical signature. The electrical changes are then detected with simple electrochemical measurements in real time without any need for labeling. For a more detailed explanation of the ion nano gating mechanism and the electrochemical characteristic of nanopipette electrodes, see our recent review, (Actis et al., 2010). The selectivity of the nanopipette sensor can be customized for many different targets by introducing highly specific bio-recognition agents such as antibodies (Umehara et al., 2009), DNA (Fu et al., 2009), and aptamers (Ding et al., 2009). The quartz pipettes also provide an ideal interface to append such bio-receptors using established surface-modification chemistry. Nanopipette-based platforms have been used to 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). We have recently demonstrated that STING technology can selectively detect interactions such as biotin–streptavidin and protein–protein binding (Umehara et al., 2009).

In this paper, we discuss application of the STING platform for the ultrasensitive detection of a mycotoxin belonging to the species *Fusarium*, HT-2 toxin. The detection of HT-2 toxins presents unique challenges due to their low molecular weight (<500 Da) and their insolubility. We examine the sensor's limit of detection and linear range and compare the STING capabilities with respect to conventional sandwich assay techniques.

2. Experimental

2.1. Reagents

Poly-L-lysine (PLL; 19320-A) was purchased from Electron Microscopy Sciences (Hatfield, PA). Sulfo-SMCC was purchased from G biosciences (Maryland Heights, MO). Monoclonal antibody for HT-2 (anti-HT-2, clone C6B4) was purchased from Advanced Immuno-Chemical Inc. (Long Beach, CA). HT-2 toxin was acquired from Sigma–Aldrich (T4138). Polyclonal antibody HPV16 E6 (C-19) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used as a control. PBS solutions at pH 7.4 were prepared using standard method. Ridascreen® ELISA kits for zearalenone was purchased from RBiopharm AG (Darmstadt, Germany). *N,N*-Dimethylformamide (99% purity) was purchased from Sigma–Aldrich and used without further purification. Aqueous reagents were prepared using nanopure water with >18 M Ω cm^{−1} resistance.

2.2. STING sensors fabrication

Nanopipettes were fabricated from quartz capillaries with filaments, with an outer diameter of 1.0 mm and an inner diameter of 0.70 mm (QF100-70-5; Sutter Instrument Co.). Prior to pulling, glass capillaries were cleaned with sulfuric acid/hydrogen peroxide (piranha solution) (Tabard-Cossa et al., 2007).

Caution: The piranha solution is a highly energetic oxidizer. It reacts very violently with organic materials.

The capillary was then pulled using a P-2000 laser puller (Sutter Instrument Co.) preprogrammed to fabricate nanopipettes with an inner diameter of 50 nm. Parameters used were: Heat 700, Fil 4, Vel 60, Del 150, and Pul 192. The resulting nanopipette tips had inner diameters ranging from 37 to 82 nm, with the mean diameter of 56 nm (Karhanek et al., 2005).

2.3. Surface functionalization: antibody immobilization

Antibodies were immobilized through the following steps. First, nanopipettes were internally coated by filling with a 0.01% solution of poly-L-lysine in water, followed by centrifugation at 4600 rpm for 3 min. The centrifugation step helps to get the solution to the very tip of the nanopipette. After the removal of excess PLL solution, the nanopipettes were baked at 120 °C for 1 h to stabilize the PLL coating (Umehara et al., 2006). The nanopipette was then filled with a sulfo-SMCC solution (2 mg/ml, 10 mM EDTA and 50 mM PBS), centrifuged at 4600 rpm for 3 min and then incubated at room temperature for 1 h. Nanopipettes were then filled with 0.01 M PBS and centrifuged for at least 3 times to remove any unreacted sulfo-SMCC molecules. Sulfo-SMCC contains an amine-reactive *N*-hydroxysuccinimide (NHS ester) that reacts with the PLL amino groups, leaving a maleimide group available for the antibodies cross link through a thioether bond. The nanopipettes were then incubated with antibody solution (10 μ g/ml IgG, PBS, 1 h, 37 °C). Antibody-functionalized nanopipettes were then filled at least 3 times with PBS and centrifuged, rinsed two more times with a PBS (0.01 M)/DMF (80/20) solution to remove any unbound antibody and to provide a smooth electrolyte filling throughout the tip.

2.4. Measurement setup

Since the current flowing through the nanopipette is too small to polarize a reference electrode (Wei et al., 1997), a two electrode setup was used. A typical setup is shown in Fig. 1. The STING sensor, acting as the working electrode, is backfilled with the working buffer, and a Ag/AgCl electrode is inserted. Another Ag/AgCl electrode is placed in bulk solution acting as auxiliary/reference electrode. Both electrodes are connected to the Axopatch 700B amplifier with the DigiData 1322A digitizer (Molecular Devices), and a PC equipped with pClamp 10 software (Molecular Devices). Since nanobubbles are the dominant source of noise in solid state nanopores (Smeets et al., 2006), every solution was degassed prior use. The system remained unstirred for the duration of the measurement, which was conducted at room temperature.

3. Results and discussion

Fig. 1 schematically illustrates the operation of the STING platform. The electrochemical sensitivity of the device is maximized at the nanopipette tip, essentially an elongated cone, making the dimension and geometry of the tip orifice crucial for biosensor performance (Umehara et al., 2009). The STING sensor is based on nanopipettes fabricated using a P-2000 laser puller where the protocol was optimized to get reproducible pore opening within the range 30–70 nm. We extensively tested the reproducibility of the STING sensor fabrication as well as the surface chemistry and these

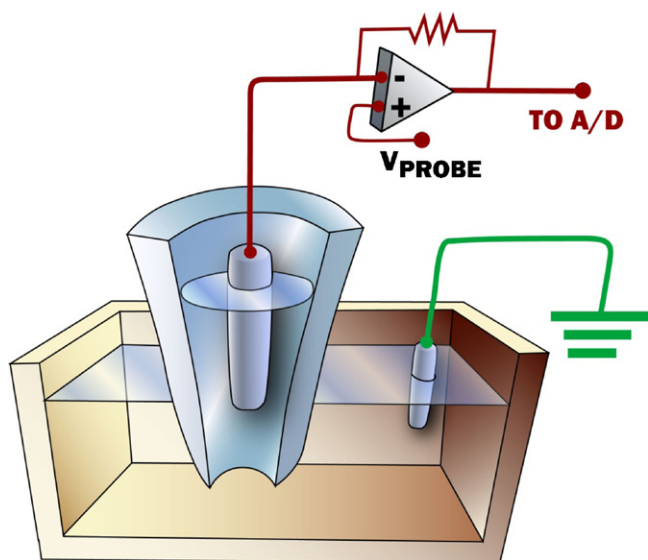


Fig. 1. STING platform: measurement setup. The STING sensor, acting as the working electrode, is backfilled with the working buffer, and an Ag/AgCl electrode is inserted. Another Ag/AgCl electrode is placed in bulk solution acting as auxiliary/reference electrode.

have been described previously (Umehara et al., 2009). Briefly, we established that measured ionic current correlates with pore size, and this allows pipettes to be screened for a desirable ion current range. To date we have obtained a 25% success rate in achieving the target pore size. Thus nanopipette fabrication remains the highest source of inefficiency, but with careful quality control we have been able to limit the impact on experimental results. We are currently establishing a fabrication protocol based on chemical etching rather than thermal pulling that will allow a better control of the sensor fabrication. We investigated as well the optimal storage conditions for our sensors.

To introduce selectivity, the nanopipette internal wall is functionalized with poly-L-lysine (Umehara et al., 2006), to which antibodies are immobilized through the sulfo-SMCC cross-linking

chemistry (Raj et al., 2009) (Fig. 2). After extensive rinsing, nanopipettes are backfilled with the working buffer (PBS/DMF, 80/20, v/v) and an Ag/AgCl wire is inserted to comprise a STING electrode module. It is important to point out that the sensitivity of the STING sensor depends on conditions of the functionalized surface at the very tip, where the nanoscale pore limits ion flow. Theoretically, the limit of detection could be determined by the number of molecules that generate a detectable signal at the 50-nm nanopipette tip. In practice, however, consumption of target molecules on areas of no sensitivity, such as the outer nanopipette sidewalls, could prevent devices from achieving the theoretical detection limit. Therefore, we functionalized only the inner walls of the nanopipette, in order to reduce any analyte consumption far from the sensing region. Furthermore, it has been recently shown that molecules can be trapped (Clarke et al., 2005) or concentrated (Calander, 2009) at the tip of a nanopipette by applying a DC voltage. The applied voltage increases the probability of a binding event inside the sensing region, where the antibodies are attached. First, the STING sensor was immersed into the working buffer and a constant voltage of -400 mV was applied between the electrode placed inside the nanopipette and the external one placed into working buffer. A blank measurement was then recorded and only sensors showing a baseline variation lower than 5% over a 15 min run were employed for the HT-2 toxin detection. Due to their extremely low solubility in water, the mycotoxins were first dissolved in pure DMF separately. The final sample consists of the HT-2 toxin diluted in the working buffer. Once a signal baseline was established, a defined amount of HT-2 toxin was added to the reservoir; the variation of the measured current was recorded over time and compared to the baseline previously recorded. We have tested the shelf-life of the sensors at different stages of functionalization. Poly-L-lysine coated STING sensors can be stored up to 2 weeks at room temperature while STING sensors functionalized with antibodies were stored up to 2 days in a humidified chamber at 4°C without any noticeable loss of performance.

3.1. HT-2 toxin detection

Fig. 3 shows that the added mycotoxins instantly reduce the current amplitude, through stepwise blockades, indicating that

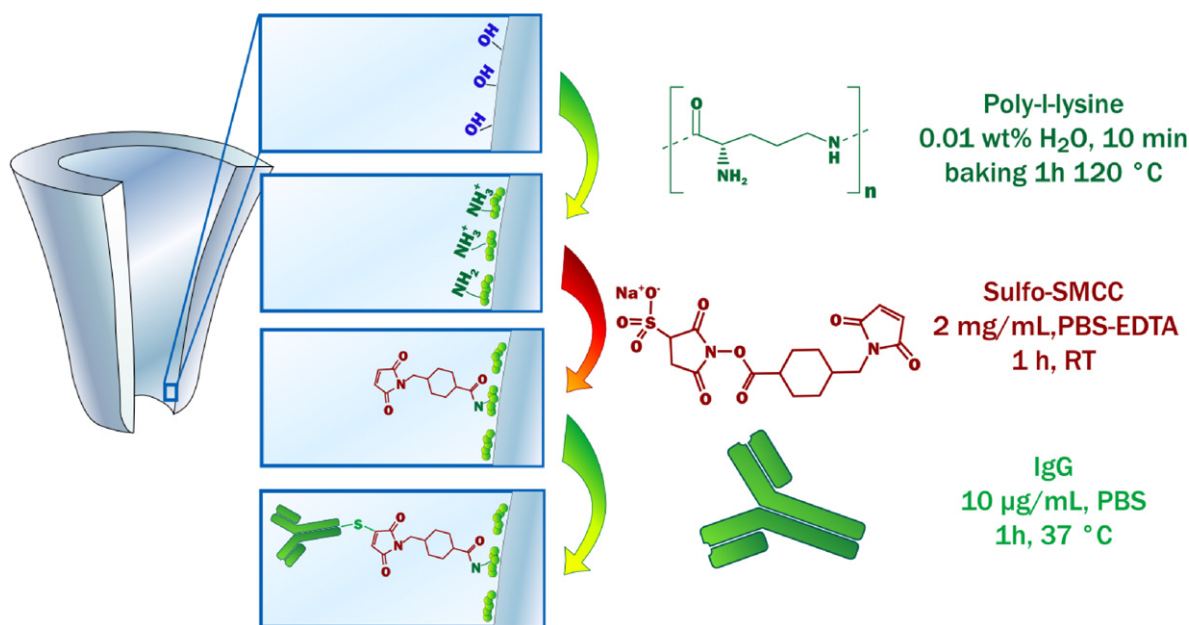


Fig. 2. Schematic illustration of the surface chemistry for immobilization of probe antibody to the nanopipette. First, PLL is physisorbed onto the negatively charged quartz surface. Sulfo-SMCC is then employed to cross-link amino groups from the PLL and the cysteine residues of the antibody.

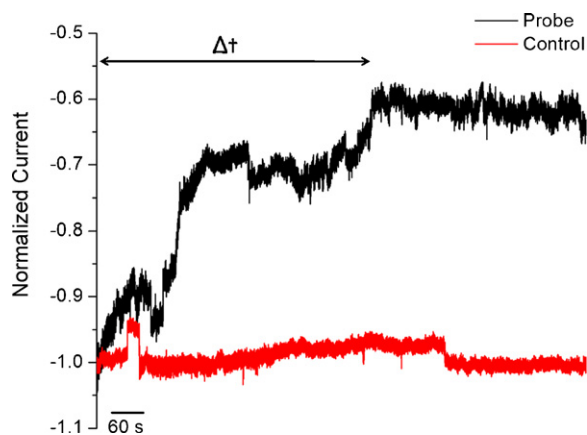


Fig. 3. Selective HT-2 toxin detection by STING sensor. After the addition of HT-2 toxin molecules at a final concentration of 1 ng/ml, stepwise blockades were recorded by the probe nanopipette (black line), functionalized with anti HT-2 toxin, whereas essentially no change was observed with the control nanopipette (red line). Applied voltage = −400 mV vs Ag/AgCl. Solution: PBS (0.01 M)/DMF (80/20, v/v). Control nanopipette functionalized with anti-HPV16E6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a binding event took place on the STING sensor. Their sequential binding at the close proximity of the STING sensor opening alters the local impedance, thus inducing stepwise changes in the recorded ionic current. A similar behavior was observed on STING sensors backfilled with the working buffer containing the HT-2 toxin (see Supporting information Fig. S3) rather than having them added in the reservoir. However, due to the conical shape of the nanopipette tip, different blockade patterns are identified depending of the particular location where the binding takes place. These permanent blockades are also distinguishable from those produced by molecular translocation, as protein molecules traversing the nanopipette generate shorter and temporary blocks with a duration ranging in microseconds (Ding et al., 2009). To corroborate this assumption we are designing and developing models which help us to better interpret or explain phenomena underlying the obtained current signals.

3.2. Specificity

We verified the specificity of the STING sensor by functionalizing a nanopipette with control antibodies, anti-HPV16E6 IgG, that do not bind HT-2 toxin. No stepwise blockade was observed when HT-2 toxin was added in the reservoir (Fig. 3) or inside the nanopipette (see Supporting information Fig. S3), thus confirming the specific detection of the mycotoxin by the STING sensor and a surprisingly negligible signal from non-specific adsorption.

We systematically investigated the source of the signal, showing that the signal reduction was generated by a specific antibody–antigen interaction rather than accumulation of molecules at the tip of the STING sensor. No permanent blockade was observed in nanopipettes backfilled with the probe antibody and immersed in a solution containing the toxin (see Supporting information Fig. S2). This indicates that the STING sensor is highly sensitive to binding events at its inner surface rather than in solution.

3.3. Limits of detection

Next, we studied the signal dependence on toxin concentration in order to determine the detection limit and the dynamic range of the STING platform. The conical shape of the sensing region generates different binding signatures of target molecules depending on

Table 1

Average Δt for HT-2 toxin detection. Errors are calculated from three independent measurements.

HT-2 toxin concentration (pg/ml)	Δt (s)
10000	440 ± 35
1000	463 ± 25
100	636 ± 50
10	1050 ± 280
1	1140 ± 216
0.1	1760 ± 320

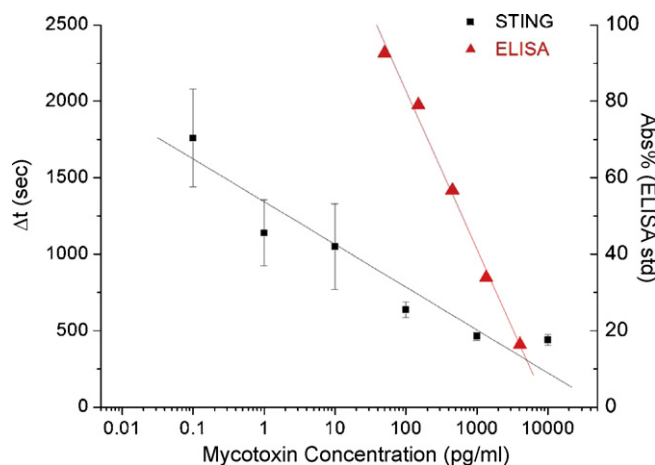


Fig. 4. Comparison between standard curves from ELISA kit (red triangles, linear fit, $R=0.970$) and the STING detection (black squares, linear fit, $R=0.997$) for HT-2 toxin detection. Results show a dynamic range for the STING platform of at least 2 orders of magnitude with a detection limit 3 orders of magnitude lower than ELISA. Error bars are calculated from three independent measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the specific location where the binding occurs. Hence, the quantification of mycotoxin in solution is problematic if the decrease in the ionic current is taken as an indicator. On the other hand, we noticed that the time to reach a saturation level, Δt , of the HT-2 toxin binding curve can be used for a direct correlation of analyte concentration. The moment when HT-2 toxin solution is added to the reaction reservoir is defined as $t=0$. The binding event to the immobilized antibodies took place immediately upon contact and was recorded in real time until a saturation level was reached. The Δt for different HT-2 toxin concentrations were investigated and results are summarized in Table 1. Δt increased from 440 ± 35 s for a mycotoxin concentration of 10 ng/ml to 1760 ± 320 s for 0.1 pg/ml. We have also tested the detection at concentrations above 10 ng/ml. For higher concentrations (data not shown), the sensors did not show significant decrease in Δt , indicating that the all available binding sites were already saturated. Error bars reflect the sensor-to-sensor variation that is mainly attributable to differences in pore size. The results, summarized in Table 1, demonstrate that our STING platform can achieve a dynamic range of 5 orders of magnitude, exceeding the dynamic range (3 orders of magnitude) of the commercial ELISA kits we have tested. Furthermore, we have compared the limit of detection (LOD) and the dynamic range of our system to commercially available ELISA kits for mycotoxin from the genus *Fusarium*, demonstrating LOD three orders of magnitude lower than that obtained with ELISA. The standard curves for the ELISA kit and the STING platform are shown in Fig. 4 and show the superior performance of STING sensors.

4. Conclusion

In conclusion, we demonstrated here the potential of STING sensors as an analytical method for the ultrasensitive detection of mycotoxins, with a detection limit as low as 100 fg/ml and linear range of 3 logs. The STING sensor performance compared favorably with commercially available ELISA kits for mycotoxins, whose linear range is 2 logs and lower limit of detection is generally around 100 pg/ml.

STING paves the way for a new class of label-free, real time biosensors with unmatched sensitivity. The fully electrical read-out as well as the ease and low cost fabrication are unique features that give this technology enormous potential. Moreover, STING technology can be easily multiplexed using multiple sensors, with the nanopipette geometry preventing electrode cross-talk. Unlike other biosensing platforms, nanopipettes can be precisely manipulated with submicron accuracy and can be used to study single cell dynamics. We are currently investigating other applications of STING sensors, such as the response of oncoproteins both *in vitro* and inside living cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.016.

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