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Single domain antibodies for the detection of ricin using silicon photonic microring resonator arrays

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Abstract

Ricin is a lethal protein toxin derived from the castor bean plant. Given its notorious history as a biowarfare agent and homicidal weapon, ricin has been classified as a category B bioterrorism agent. Current ricin detection methods based on immunoassays lack the required sensitivity and specificity for many homeland security surveillance applications. Importantly, many conventional antibody-based methodologies are unable to distinguish ricin from RCA 120, a non-toxic protein also found in the castor bean plant. Single domain antibodies (sdAbs), which are recombinantly derived from immunized llamas, are known to have high affinities for ricin A or B chains, and low cross-reactivity with RCA 120. Herein, we demonstrate the use of silicon photonic microring resonators for antibody affinity profiling and one-step ricin detection at concentrations down to 300 pM using a 15 minute, label-free assay format. These sdAbs were also simultaneously compared with a commercial anti-RCA IgG antibody in a multi-capture agent, single target immunoassay using arrays of microrings, which allowed direct comparison of sensitivity and specificity. Given the rapidity, scalability, and multiplexing capability of this silicon-based technology, this work represents a step toward using microring resonator arrays for the sensitive and specific detection of biowarfare agents.

INTRODUCTION

Since ancient times, biological agents have been used as weapons by both militaries and terrorist organizations.[1] The use of ricin was considered by both the US and British militaries in both the First and Second World Wars, and was also employed in the infamous 1978 poisoned umbrella assassination of Bulgarian dissident Georgi Markov.[1, 2] More recently, the anthrax-containing letters sent to media outlets and two U.S. Senators in 2001 in the aftermath of 9/11 attacks, and similar attacks in 2003 and 2004, brought bioterrorism surveillance to the forefront of homeland security efforts.[1] Accordingly, there are pressing needs to develop robust analytical tools for the detection of ricin, and other potential biowarfare agents.

Ricin is a ~60 kDa proteinaceous toxin derived from the seeds of the castor bean plant, *Ricinus communis*.[3] The castor bean plant is grown worldwide and is the main raw material for production of castor oil, which has a broad range of industrial and medical applications.[2] As a byproduct of oil production, ricin is easily obtainable in large quantities,[2, 4] fueling fears that this agent could easily fall into the hands of terrorist organizations. A type 2 ribosome inactivating protein (RIP), ricin's structure consists of an A chain and B chain linked by disulfide bonds.[3, 5, 6] The B chain is a lectin that binds to the galactose residues of glycoproteins and glycolipids on the cell surface, which facilitates ricin entry into the

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cytosol.[6] The chains are cleaved apart, and the A chain depurinates an adenine residue from the 28S rRNA of ribosomes at a rate of ~1500 ribosomes/min, which leads to inhibition of protein synthesis and eventually causes cell death.[3]

The lethal dose of ricin varies dramatically depending upon the route of exposure, but inhalation represents the most dangerous mode, with a median lethal dose (LD_{50}) of 3-5 μg/kg for inhalation versus 20 mg/kg via ingestion. This high lethality, ease of extraction, and high accessibility of ricin led to its classification as a category B bioterrorism agent by the Centers of Diseases Control and Prevention (CDC).[7]

At present, common approaches for ricin detection includes polymerase chain reaction (PCR),[8-10] assays measuring the catalytic activity of ricin,[11-13] , and immunoassays.[14-20] Both PCR and catalytic activity assays are indirect methods for detecting ricin. PCR only detects nucleic materials from the plant origin of the toxin, and therefore is not applicable to detect purified ricin,[21, 22] while catalytic activity assays lack specificity towards ricin, since the catalytic activity of all RIPs are similar.[21, 23] Because of these limitations, most studies in the literature have utilized immunoassays for ricin detection. Immunoassays generally rely upon antibody recognition elements and can be used in a variety of formats, including radioimmunoassays,[14] enzyme-linked immunosorbent assays (ELISA),[15] electroluminescence,[16] fluorescent-based flow cytometry,[17] optical waveguide sensors,[18] surface plasmon resonance (SPR)[19] and colorimetric hand-held assays,[20] Importantly, the broad reliance upon immunoaffinity methods has generated strong interest in developing stable and robust antibodies that are specific for ricin.[23-25]

One limitation to ricin immunoassay development is that polyclonal immunoglobulin G (IgG) antibodies often do not have high specificity for ricin, and although monoclonal antibodies have improved specificity, they also have limited stability.[26] An alternative to conventional IgG antibodies is a class of recombinant antibodies known as single-domain antibodies (sdAb). sdAbs are derived from a special class of heavy-chain antibodies, which are found in animals of the *Camelidae* family, and also in sharks.[27-31] Unlike IgG antibodies, which consist of two heavy chains and two light chains linked by disulfide bonds, sdAbs do not have light chains, thus only a variable domain (VHH) on the heavy chain is responsible for antigen binding.[28, 29] This VHH region can be cloned and expressed as a recombinant sdAb,[27] with ten times lower molecular weight (~15 kDa),[27, 30] as compared to a standard IgG. Importantly, sdAbs are robust to heat and chemical treatment as they can refold to maintain their antigen affinity after denaturation.[29, 32] These properties make sdAbs attractive capture agents for immunoassays of various formats. Anderson *et al.*[27] have recently developed a series of anti-ricin sdAbs and demonstrated their high affinity, specificity, and robustness in ELISA and bead-based immunoassay formats. These sdAbs were selected from a phage display library constructed by extraction of the mRNA of heavy chain antibodies in lymphocytes of immunized llamas, followed by PCR amplification to clone resulting sdAb genes into a phage display vector, and transformed to *E. coli* cells for antibody production.

In this paper, we demonstrate the applicability of anti-ricin sdAbs for agent detection on a label-free microring array detection platform. Silicon photonic microring resonators are an emerging class of chip-integrated sensors that have been used to detect a range of biomolecular targets: including protein[33-36] and nucleic acid[37-40] biomarkers, and viruses.[41] Microring resonators are refractive index-based sensors that are sensitive to the local environment near the microring surface. When the surface is modified with capture agents, such as antibodies, the binding of the target antigen is readily detected as a shift in the resonance wavelength supported by the microcavity. These changes are monitored as a function of time and used to quantify the amount of analyte in solution, or alternatively used

to interrogate the kinetics of binding interactions. In addition to the high surface sensitivity and analytical versatility, advantages of this silicon photonic sensing technology come from its genesis in well-established semiconductor fabrication methodologies, which make the sensors highly scalable, inherently multiplexable, and cost-effective. Herein, we demonstrate the applicability of this technology for the relatively rapid and quantitative detection of ricin using sdAbs down to a concentration of 300 pM in a label-free assay format. Furthermore, we verify that the sdAbs are significantly more specific than a standard IgG antibody when challenged with the molecularly similar, but non-toxic ricin analogue RCA 120. Importantly, we feel this work establishes this silicon photonic as a useful platform for detection biowarfare agents, since the multiplexing capability and cost effective nature of the technology would lend itself well to network surveillance efforts in which large numbers of sensor arrays could be distributed as a network for autonomous environmental monitoring.

EXPERIMENTAL

Materials

Unless specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. 3-N-((6-(N'-isopropylidenehydrazino))nicotinamide)propyltriethoxysilane (HyNic Silane) and succinimidyl 4-formyl benzoate (S-4FB) were purchased from Solulink (San Diego, CA). *Ricinus communis* agglutinin II (ricin), *Ricinus communis* agglutinin I (RCA 120) and a polyclonal goat anti-RCA antibody were purchased from Vector Laboratories, Inc. (Burlingame, CA). Polyclonal Chicken anti-saporin was purchased from Advanced Targeting Systems (San Diego, CA). Single domain antibodies (sdAb) C8 and B4 used in the experiments were a generous donation from Drs. George Anderson and Ellen Goldman at the Naval Research Laboratory. Aniline and glycine were purchased from ACROS Organics (Geel, Belgium). Zeba spin desalting columns were purchased from Thermo Fisher Scientific (Rockford, IL).

All buffers were made from purified water (ELGA PURELAB filtration system; Lane End, UK) and the pH was adjusted with 1 M HCl or 1M NaOH. Phosphate buffered saline (PBS) was reconstituted from Dulbecco's phosphate buffered saline packets purchased from Sigma-Aldrich (St. Louis, MO). The low pH glycine buffer consisted of 10 mM glycine and 160 mM NaCl adjusted below pH 3.0. PBST-BSA buffer consisted of 0.1mg/ml bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 in 10 mM PBS at pH 7.4. The sensor chip blocking buffer consisted of 2% (w/v) BSA and 0.01% (w/v) sodium azide in 10 mM PBS at pH 7.4.

Sensor chip layout and instrumentation

The microring resonator instrument and sensor chips were acquired from Genalyte, Inc. (San Diego, CA). Instrumentation and sensor chips designs have been previously described in detail.[33, 42] Briefly, the sensor chips are 6 mm×6 mm in size, and fabricated from silicon-on-insulator wafers. Each chip consists of 32 microrings adjacent to linear waveguides. The entire chip is spin-coated with a perfluoropolymer cladding, with annular openings etched to expose 24 rings to be used as sensors exposed to solution, while the remaining 8 rings are left under the cladding to serve as thermal control rings to correct for temperature drift. Light from a tunable external cavity laser in the instrument (wavelength centered at 1560 nm) interrogates each individual microring via grating couplers placed at the edge of the chip. The scan speed of the system is ~250 ms/ring with the entire array interrogated every ~9 seconds.

Antibody immobilization on sensor chip surface

Sensor chips were batch-functionalized by the following procedures: The chips are first cleaned for 30 s in piranha solution (3:1 ratio of concentrated sulfuric acid to 30% hydrogen peroxide), then rinsed with copious amount of distilled water and dried under a stream of nitrogen (Caution! Piranha solutions are extremely dangerous, reacting explosively with trace quantities of organics.). A 20 μL drop of a 1 mg/ml HyNic silane solution in 95% ethanol and 5% N,N-dimethylformamide (DMF) was spotted on the surface of each sensor chip for 30 min, after which the chips were rinsed in 100% ethanol and dried under nitrogen to remove the excess HyNic Silane.

Separately, antibodies were conjugated with S-4FB molecules by first buffer exchanging the antibodies into 100 mM pH 6.0 PBS using Zeba spin desalting columns. The resulting concentration of antibodies in PBS was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). After the concentration was determined, a five-fold molar excess of S-4FB (0.1 mg/ml in DMF) was added and allowed to incubate at room temperature for 2 hrs. Unreacted S-4FB was then removed by buffer exchanging antibodies into 100 mM pH 7.4 PBS using the Zeba spin columns. The final concentration of the S-4FB-modified antibodies was again determined by the NanoDrop spectrophotometer and adjusted to 50 μg/ml.

Immediately before attachment to sensor chip surface, 4FB-modified antibodies were diluted to 25 μg/ml in PBS containing 100 mM aniline.[43] Approximately 1 μl aliquots of 4FB-modified antibodies were deposited on specific microrings on the sensor chip surface with the aid of a stereo microscope to direct spotting positions, while a selected set of rings were blocked with 2% w/v BSA (unexposed to any antibodies) to serve as control rings. The antibody solution-coated chips were then placed in a saturated humidity chamber overnight at room temperature. Afterwards, the sensor chips are immersed in chip blocking buffer overnight to block the chips surfaces prior to performing binding or detection experiments.

Assay Procedure

Ricin, RCA 120, and saporin standard solutions were made via serial dilution of stock solutions in PBST-BSA. For each assay, a chip was placed in a holder with a two-channel microfluidic set-up defined by a Mylar gasket sandwiched between the holder and a Teflon lid. A syringe pump was used to control solution flow across 12 active sensor rings in each of two flow channels, the schematic of which was described in a previous publication.[33] Assays were conducted at 30 μl/min flow rate. Before each assay run, glycine buffer was flowed across the chip surface for 2 min to remove excess blocking BSA, before establishing a stable baseline by flowing PBST-BSA running buffer for at least 4 min. The analyte solution is then flowed across the chip for 10 min, followed by a 5 min PBST-BSA rinse. Each sensor chip is used only once without regeneration.

Data Analysis

All microring responses were corrected for baseline thermal drift using the microrings occluded by the cladding layer as references. Each active microring signal was also corrected by setting the response of one blank control ring unmodified with antibodies as the “zero” reference to the response of antibody-modified rings. The initial slopes for all the sensograms of ricin standards and unknown samples were determined by a linear regression fit of the first five minutes upon binding of ricin to the sdAb immobilized on the microrings, after which the resulting slopes were averaged among the replicated measurements of each sdAb-modified ring exposed to the same sample. OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA) was used to fit a linear regression plot to correlate the initial slopes with concentration of the ricin standards in the calibration curve, while the concentration of the

unknown sample was quantified by interpolating its corresponding initial slope on the calibration curve.

RESULTS and DISCUSSION

As specificity towards ricin is of great importance when developing assays for this target our initial efforts focused on evaluating the reactivity and cross-reactivity of sdAbs towards ricin and RCA 120, respectively. The molecular weight of ricin is ~60 kDa and it sometimes referred to as RCA 60. Meanwhile, as its name suggests, RCA 120 is twice the mass of ricin, having a tetrameric structure that is >80% homologous to ricin, yet is much less toxic.[44] A commercially available goat anti-RCA IgG was arrayed next to the sdABs B4 and C8, as well as the BSA blocked control rings. All of the microrings were then simultaneously exposed to 10 nM RCA 120. In a separate experiment, an identically arrayed sensor chip was exposed to 10 nM ricin. Figure 1 shows the responses of the arrays to both RCA 120 and ricin. All of the ricin and RCA-specific capture agents show strong responses to ricin; however, the anti-RCA IgG shows a much larger response to RCA 120 as compared to the sdAbs, verifying the enhanced specificity of the sdAb capture agents.

In addition to specificity for ricin over RCA 120, we also investigated the cross-reactivity of the C8 sdAb against saporin, another naturally obtained RIP. Initially, we tested saporin against an anti-saporin antibody to confirm binding affinity of the molecule (Figure S-1, Supporting Information). Using a sensor chip functionalized with C8 sdAbs, we consecutively exposed the rings to 30 nM saporin follow by 30 nM ricin. As shown in Figure 2, Responses from the C8 immobilized microrings further demonstrate the specificity of this sdAb towards ricin over another RIP.

These cross-reactivity and detection results further support previous reports by Anderson *et al.,* which show that C8 has the highest binding response to ricin while B4 have the lowest non-specific binding to RCA 120.[27, 30] Having established that sdAb C8 offered good specificity and sensitivity, we sought to demonstrate the quantitative detection capabilities for ricin on our sensor platform. We flowed a set of standard ricin solutions, prepared in PBST-BSA to concentrations of 10 nM, 3 nM, 1 nM, 0.3 nM and 0 nM, across an array of microrings functionalized with sdAb C8. The binding responses to each concentration of ricin interacting with eight microrings per sensor chip were then recorded and corrected using the BSA-blocked rings, as shown in Figure 2. Responses for four representative microrings are shown in the figure for the sake of clarity; however, the average initial slopes and standard deviations for all eight sensors are provided in the Supporting Information.

We previously showed the ability to perform rapid, label-free quantitation based upon the initial slope of binding response upon introduction of the antigen-containing solution.[33] Using the data from Figure 3, but including fits to all eight binding curves recorded at each concentration of ricin, we created a calibration plot that could be used for determination of an unknown. Figure 3 shows the resulting calibration curve. We then utilized this calibration curve to determine the concentration of a solution having an unknown ricin concentration prepared in PBST-BSA. Comparison of the sensor response with the calibration curve allowed us to determine the unknown concentration to be 4.2 ± 0.4 nM. This value and error, determined as the 95% confidence interval for n=8 measurements, was in good agreement with the “as prepared” value of 4.5 nM.

Finally, we determined the limit of detection for label-free ricin detection by analyzing the noise present in the measurement. Specifically, we determined assay “slope noise” (σ) of the running buffer baseline, which is a measure of how precisely we can determine the initial slope of the sensor binding response. Using the determined value of 0.09 pm/min for this

system, we then determine the limit of detection as 3σ (~0.27 pm/min). Evaluation of this noise level against the ricin standard binding curve points to an overall limit of detection of 200 pM. Furthermore, it is worth noting that we have previously shown that assay sensitivity, and specificity, can be further increased by using a secondary capture agent and tertiary binding events.[34-36]

For applications in biodefense, a rapid, real-time ricin detection system is needed to ensure a prompt and efficient response capacity. Herein we demonstrate a label-free detection methodology that achieves a relevant limit of detection within a rapid (<15 min) assay format. Admittedly, the matrix described in this manuscript is quite proteinaceous, but relatively well-controlled compared to that encountered in many analytical matrices. However, the detection of airborne agents is a rather unique potential application area, as samples collected using air filtration are typically resuspended in a convenient buffer of choice. As mentioned above, ricin and many other biowarfare agents pose very high inhalation threats, and therefore air and surface sampling, both of which often involve suspension in a neat buffer solution, are commonly utilized for these agents.[8, 45, 46] This practical operation procedure adds support to the utility of this rapid and label-free, buffer-based assay for ricin and its potential for future deployment as sensor networks for biowarfare agent surveillance.

CONCLUSION

In this work, we demonstrated that silicon photonic microring resonator arrays are a powerful and promising tool for detecting biowarfare agents such as ricin. Our evaluation of anti-ricin sdAbs on the microring arrays platform is consistent with the previous reports [27, 30, 47] that show sdAbs to have selective affinity towards ricin, yet minimal cross-reactivity with the non-toxic analogue RCA 120. Using the sdAb C8 as a capture agent, we also illustrated a rapid, real-time, one-step quantitative approach of ricin detection, detecting a concentration of 300 pM in a 15 minute, label-free assay format. Future efforts will focus on further improving assay performance and the creation of multiplexed detection panels towards the goal of surveillance for multiple agents within environmental matrices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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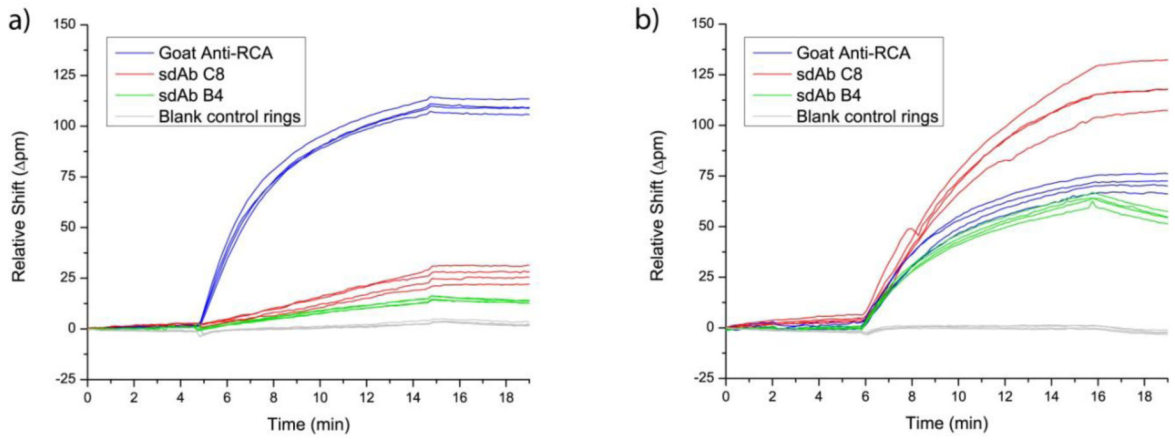

Figure 1.

Responses of a 3-capture agent sensor array exposed to 10 nM of a) RCA 120 and b) ricin. sdAb clones C8 and B4 both show greater selectivity for ricin compared to the goat anti-RCA IgG, which shows the largest response to RCA 120. Both sdAb clones show a significantly reduced response to RCA 120 while displaying good binding responses to ricin. In both sensing experiments, blank control rings show insignificant levels of non-specific binding.

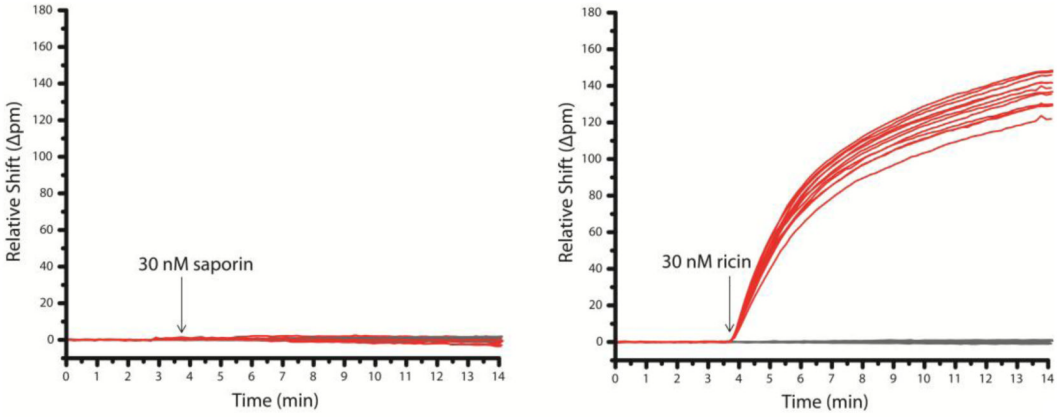

Figure 2.

Response of sdAb C8-modified microrings upon addition of 30 nM saporin, followed by addition of 30 nM ricin (red lines). Sensors were initially in PBST-BSA buffer and arrows indicate the times when analyte solutions were introduced. Dark gray lines indicate responses of thermal control rings.

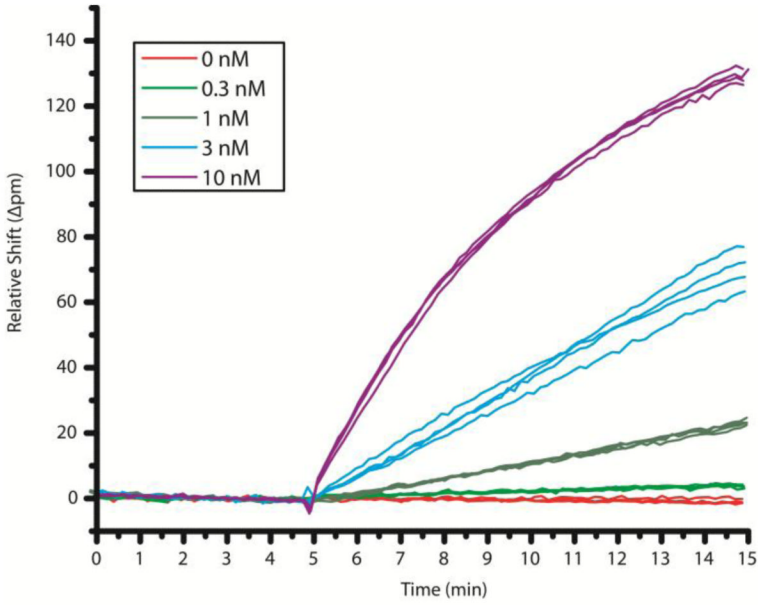

Figure 3.

Concentration-dependent binding response of ricin as a function of target concentration. Each measurement was made eight times redundantly on the same sensor chip, functionalized identically with sdAb C8. For the sake of clarity, data from four rings is presented. Following the establishment of an initial baseline by equilibrating with PBST-BSA running buffer, ricin-containing solutions were flowed across the array (staring at t = 5 min) and persisting for a total of 10 minutes.

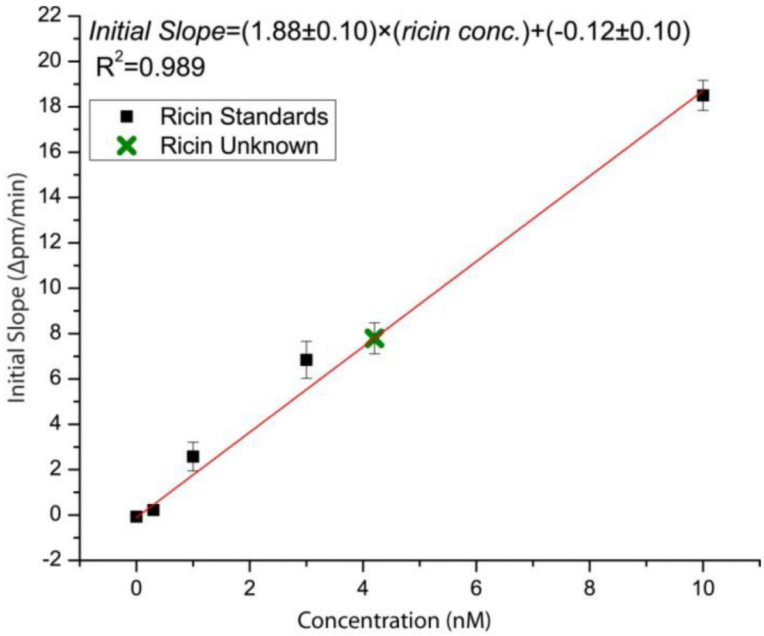

Figure 4.

Calibration curve illustrating the concentration-dependent response of sdAb C8 functionalized microrings to solutions of various concentrations. Real-time binding curves were obtained (as in Figure 3) for samples prior to the analysis of a prepared solution containing an unknown amount of ricin. The sensor response for the unknown solution was then compared against the standard calibration curve, allowing for quantitative detection. Error bars represent the 95% confidence interval from n=8 measurements.