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A biolayer interferometry-based assay for rapid and highly sensitive detection of biowarfare agents

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ABSTRACT

Biolayer interferometry (BLI) is an optical technique that uses fiber-optic biosensors for label-free real-time monitoring of protein–protein interactions. In this study, we coupled the advantages of the Octet Red BLI system (automation, fluidics-free, and on-line monitoring) with a signal enhancement step and developed a rapid and sensitive immunological-based method for detection of biowarfare agents. As a proof of concept, we chose to demonstrate the efficacy of this novel assay for the detection of agents representing two classes of biothreats, proteinaceous toxins, and bacterial pathogens: ricin, a lethal plant toxin, and the gram-negative bacterium *Francisella tularensis*, the causative agent of tularemia. The assay setup consisted of biotinylated antibodies immobilized to the biosensor coupled with alkaline phosphatase-labeled antibodies as the detection moiety to create nonsoluble substrate crystals that precipitate on the sensor surface, thereby inducing a significant wavelength interference. It was found that this BLI-based assay enables sensitive detection of these pathogens (detection limits of 10 pg/ml and 1×10^4 pfu/ml ricin and *F. tularensis*, respectively) within a very short time frame (17 min). Owing to its simplicity, this assay can be easily adapted to detect other analytes in general, and biowarfare agents in particular, in a rapid and sensitive manner.

Biolayer interferometry (BLI) is an optical technique that uses fiber-optic biosensors for label-free real-time monitoring of protein–protein, protein–nucleic acid, and protein–small molecule interactions [1]. In this system, the interaction of an analyte with an immobilized ligand on a biosensor surface forms a molecular layer that in turn creates a proportional shift in the interference pattern of a reflected light. BLI-based analytical systems (e.g., Octet) use a semi-automated dip-and-read format, thereby eliminating the need for microfluidics. Yet, in cases where the analyte molecule is small or present at low concentrations, the interference signal is close to or below the detection limit. To overcome this shortcoming, a signal enhancement step was recently introduced using horseradish peroxidase (HRP)-labeled secondary antibody that induces precipitation of nonsoluble crystals directly on the sensor, hence greatly amplifying the signal [2]. Indeed, the coupling of BLI with enzyme-linked immunosorbent assay (ELISA, also termed BLI–ELISA) was shown to enable sensitive and rapid (10–20 min) detection of antibodies elicited against norovirus [3]. Therefore, we hypothesized that the obvious advantages of the BLI system (automation, fluidics-free, and on-line monitoring) coupled with a signal enhancement step would be highly suitable for rapid and sensitive detection of biowarfare agents. As a proof of concept, we chose to demonstrate the efficacy of this novel assay on two distinct types of pathogenic agents: ricin and *Francisella tularensis*.

Ricin, derived from the plant *Ricinus communis*, is one of the most lethal toxins known. It consists of two covalently linked subunits; the A-subunit (RTA) is an *N*-glycosidase that irreversibly inactivates the 28S rRNA of the mammalian 60S ribosome subunit, and the B-subunit (RTB) is a galactose-specific lectin that mediates the binding of the toxin to cell membranes [4]. Due to its high toxicity, availability, and ease of production and dissemination, ricin is considered a potential bioterror agent and is classified as a category B select agent by the Centers for Disease Control and Prevention (CDC). Currently, there is no available antidote against

Abbreviations used: BLI, biolayer interferometry; ELISA, enzyme-linked immunosorbent assay; CDC, Centers for Disease Control and Prevention; UEA, *Ulex europaeus*; AP, alkaline phosphatase; HRP, horseradish peroxidase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; LOD, limit of detection; LOQ, limit of quantification; CV, coefficient of variation.

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ricin exposure, underlining the need to develop effective countermeasures. Toward this end, some studies identified small molecules, aptamers, or sugar analogs that inhibit ricin toxicity [5], yet none of these was found to be effective in vivo. To date, the most promising anti-ricin therapy is based on neutralizing antibodies passively administered [6,7]. Due to the rapid progression of the clinical symptoms after ricin poisoning (acute respiratory failure within several hours after pulmonary exposure) [8], treatment should ensue as soon as possible in order to be effective. Therefore, a rapid, sensitive, and specific detection assay for ricin is imperative. Over the years, various analytical methods for ricin detection were reported, including an immunological-based assay [9], mass spectroscopy [10], and an in vitro cell-based assay [11]. Although some of these assays exhibit satisfactory sensitivity, they involve multiple steps and are time-consuming (1 h and up to several hours).

The second select agent addressed in this study is the gram-negative bacterium *F. tularensis*. This pathogen is the causative agent of tularemia, a highly infectious and fatal disease that manifests severe clinical symptoms, including skin and gastrointestinal lesions as well as pneumonia [12]. Due to its high infectivity, ease of dissemination, and the potential to cause high morbidity and mortality in humans, *F. tularensis* is listed as a category A biothreat agent by the CDC. Because early and sensitive detection of *F. tularensis* is of high importance in order to initiate prompt lifesaving antibiotic medical treatment, several assays were introduced over the last decade aiming for sensitive and specific detection of this agent [13–15]. Yet, as is the case with ricin, these assays are lengthy and require multiple steps and/or complicated procedures.

Here, we report the development of an automated, BLI-based detection assay that enables ultra-sensitive and specific identification of ricin and *F. tularensis* in clinical samples within a very short time.

Materials and methods

Reagents

Appropriate safety procedures were applied to ensure the handling of biological samples according to the statutory requirements. Pure ricin was prepared as described previously [6]. *F. tularensis* subsp. *holarctica* strain LVS (ATCC 29684) was inactivated by exposure of 5×10^9 cfu/ml to three doses of 75,000 μJ/cm^3 ultraviolet (UV) radiation. *Yersinia pestis* Kimberley53 [16], *Salmonella typhimurium* SL3261, and *Escherichia coli* TG1 (Lucigen) were inactivated using formaldehyde. Anti-ricin chimeric monoclonal antibodies were purified from FreeStyle Max 293 cells (Life Technologies, USA) supernatant as described previously [11]. Anti-*F. tularensis* polyclonal IgG fraction was obtained by Protein G chromatography of hyper-immune rabbit serum immunized by LVS (six repeated doses of 10^8–10^9 cfu). Abrin was prepared as described previously [17]. *Ulex europaeus* (UEA) was obtained from Sigma–Aldrich. Purified antibodies were biotinylated using an EZ-Link Sulfo-NHS-Biotin kit (Pierce, USA). Alkaline phosphatase (AP) labeling of the antibodies was carried out using the Lightning-Link Alkaline Phosphatase Conjugation kit (Innova Biosciences, UK). Of note, each new batch of conjugated antibody is tested and compared with the previous batch to ensure consistent formation of substrate crystals.

Biolayer interferometry

Binding studies were carried out using the Octet Red system (Forte Bio). All steps were performed at 30 °C with shaking at 1500 rpm in a 96-well plate containing 200 μl of solution in each well. PBS buffer (pH 7.4) containing 10 mg/ml bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 was used throughout this study for antibody and analyte dilution and for washing the sensors. Streptavidin-coated biosensors were loaded with biotinylated antibodies (5 μg/ml) for 300 s (until the biosensor was fully saturated), followed by a 60-s wash. The sensors were then reacted for 300 s with increasing concentrations of antigen, washed again for 60 s, and submerged in wells containing AP-labeled antibodies for another 300 s. The sensors were washed again and immersed in BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) substrate solution for 300 s. The maximum change in light interference in the last step was determined for each antigen concentration (using the previous wash step as the baseline value), and a standard curve was plotted using nonlinear regression (Prism software, GraphPad Software). The limit of detection (LOD) and limit of quantification (LOQ) for each antigen were determined as the average of the light interference value of each antibody pair measured in the absence of antigen plus 3 times and 10 times the standard deviation for LOD and LOQ, respectively (in accordance with the ICH guidelines for validation of analytical procedures). At the end of the assay, insoluble crystals are formed on the surface of the sensors, and the sensors cannot be regenerated.

Results and discussion

The use of a nonsoluble substrate to enhance the binding signal of antibodies to the BLI biosensor bound antigen was demonstrated before [3]. Here, we modified this methodology to enable sensitive detection of biowarfare antigens. As outlined in Fig. 1, in the first step of the assay, capture antibody-labeled biosensors are incubated with antigen-containing samples for 5 min, followed by a quick 1-min wash step. The sensor is then submerged for another 5 min in a well containing AP-labeled antibody, washed, and submerged in BCIP/NBT substrate solution, resulting in the formation of nonsoluble substrate crystals that precipitate on the sensor surface, thereby inducing a significant wavelength interference. It should be noted that although BLI enables real-time, label-free detection of protein binding to the sensors, it is effective only if the antigen is present at high concentrations. However, in this study, where the antigen levels are very low, they usually do not induce significant interference shift, thereby requiring the addition of another step to enhance the signal.

Assay setup for detection of ricin

One of the key features needed for sensitive “sandwich” assay is a set of high-affinity antibodies that target different epitopes and can be used as a pair. We previously isolated a panel of anti-ricin antibodies from antibody phage display libraries derived from the lymphatic organs of ricin-immunized macaques [18]. Here, we chose two antibodies: MH1, which targets the A-subunit of ricin, and MH75, which targets the B-subunit of ricin. These two antibodies were found to be highly potent in ricin neutralization in vitro [11] and to possess extremely high affinity toward the toxin ($K_D < 1$ pM) [18]. To test whether these two antibodies can be used as a capture/detection antibody set, MH1 was biotinylated and immobilized on the streptavidin-coated sensor, forming a highly stable complex. The sensor was then submerged in a ricin-containing well, resulting in a wavelength shift (Fig. 2). The addition of MH75 to this complex resulted in an additional shift, indicating that these two antibodies indeed bind different non-overlapping epitopes on ricin.

Next, we optimized the signal level in the last step of the assay, namely the wavelength interference shift induced by the alkaline phosphatase activity that results in the formation of the insoluble

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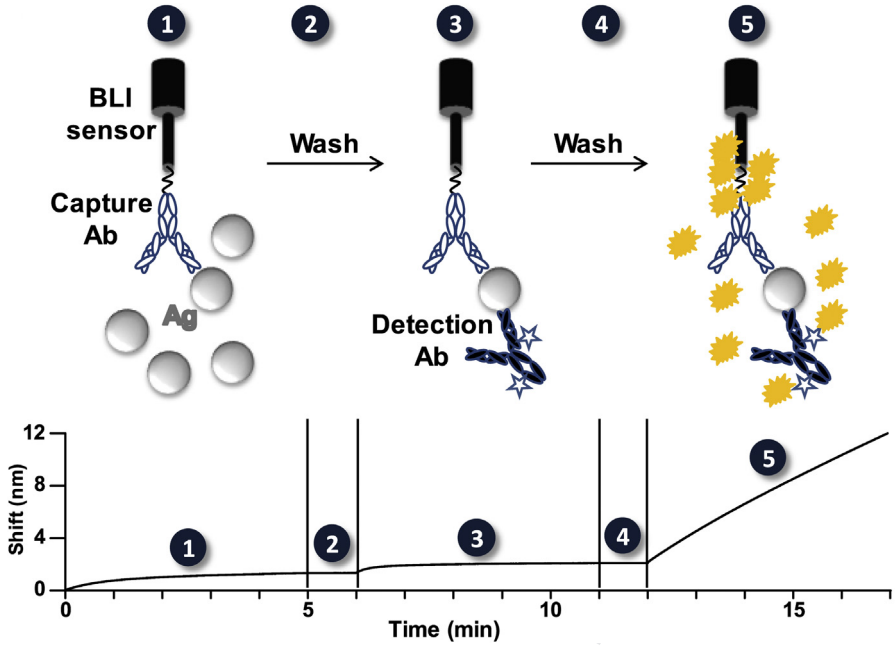

Fig.1. Schematic representation of the BLI-based detection system steps. The streptavidin-coated sensor containing immobilized capture biotinylated antibody (Ab) is incubated with the antigen (Ag) (1) for 5 min. The complex sensor–Ab–Ag is then washed for 1 min (2), followed by another 5 min of incubation with AP-labeled detection Ab (3). Following another short wash (4), the sensor is incubated for 5 min with the AP substrate BCIP/NBT, thereby forming nonsoluble crystals that precipitate on the sensor surface (5), inducing a significant enhancement of the signal (wavelength shift). The lower panel exemplifies the sensogram corresponding to each step of the assay. It should be noted that at low antigen concentrations the shift in the wavelength at steps 1 to 4 is below the detection limit.

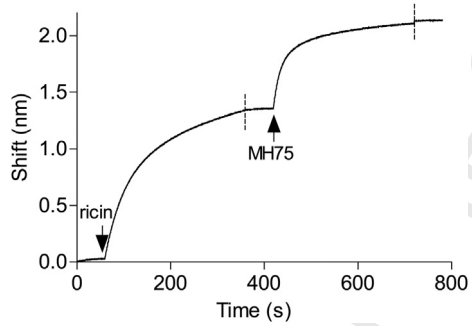

Fig.2. Pairwise mapping analyses of antibodies binding to ricin. Real-time binding of antibodies to ricin was measured using Octet BLI. The streptavidin biosensor coated with biotinylated MH1 antibody was submerged in a ricin-containing well (indicated by an arrow), and the wavelength interference was recorded. Following a short wash (indicated by a dashed line), the sensor was immersed with MH75 antibody, followed by another wash step.

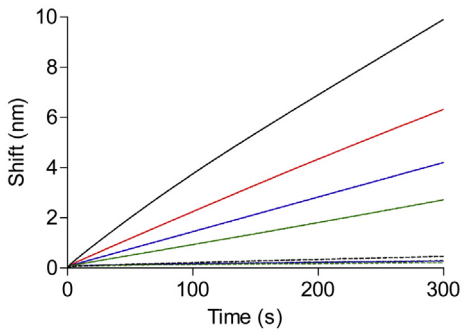

Fig.3. Signal enhancement by AP substrate as a function of AP–MH75 concentration. MH1-coated biosensors were submerged in ricin-containing wells (1 ng/ml; solid lines) or in buffer (dashed lines). The sensors were then immersed with different concentrations of AP–MH75 and incubated with BCIP/NBT for 300 s, and the wavelength interference for each sensor was recorded. AP–MH75 concentrations: 0.5 μg/ml (green lines); 1 μg/ml (blue lines); 2 μg/ml (red lines); 10 μg/ml (black lines). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

crystal precipitate on the sensor surface. To this end, MH1-loaded sensors were incubated with ricin (1 ng/ml), followed by incubation with various concentrations of AP-labeled MH75 (AP–MH75) antibody. As a control, measurements were performed in the absence of ricin to determine the background value for each concentration of labeled MH75 antibody. Indeed, a clear dose response was found between the concentrations of AP–MH75 and the interference levels (Fig. 3). In contrast, insignificant changes of the background levels were monitored as the AP–MH75 concentrations increased, thereby providing an excellent signal-to-noise ratio. Based on these results, it was decided to use AP–MH75 at 10 μg/ml (signal-to-noise ratio of 22 for the maximal wavelength shift at 300 s) throughout the rest of this study. It should be noted that the use of AP–MH75 at higher concentrations may lead to a wavelength shift above 30 nm, a value that is beyond the sensor's limits.

Previous studies incorporated horseradish peroxidase to produce the nonsoluble crystals rather than AP [2,3]. However, by comparing the signal induced by the HRP-labeled MH75 antibody with that induced by the AP-labeled antibody (performed under similar conditions), we found that the use of AP resulted in a superior signal-to-noise ratio (data not shown). Moreover, the maximal BLI shift at the end of the enhancement step induced by AP was significantly higher than that induced by HRP, hence improving the assay sensitivity.

Calibration curve for ricin

To generate a standard curve, samples containing ricin in the range of 30–2000 pg/ml were tested and the curves of wavelength

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interference at the last step of the assay were recorded (Fig. 4A). The maximal wavelength shift ranged between 1.6 nm (coefficient of variation [CV] = 24%) and 25 nm (CV = 5%) for the lowest and highest ricin concentrations tested, respectively, with an average background level of 0.6 nm (CV = 16%). These values (from three independent sets of experiments) were fitted (R^2 = 0.99) using nonlinear regression to produce a standard curve for the detection of ricin (Fig. 4B). The theoretical LOD for ricin was set as the average plus 3 times the standard deviation of the background level and was calculated to be 10 pg/ml (inset of Fig. 4B). The LOQ was set as the average plus 10 times the standard deviation of the background level and was calculated to be 30 pg/ml.

The use of high-affinity antibodies to capture the antigen and maintain a stable complex throughout the next steps is crucial. To this end, ultra-high-affinity anti-ricin antibodies (K_D < 1 pM) were incorporated, so that at the assay's time frame (and in fact even up to several hours) virtually no measurable dissociation was observed [18]. If antibodies with low affinity values (>nM) are to be incorporated into the BLI-based system, then the capture step should be extended and the next steps should be shortened in order to maintain high sensitivity.

Sensitivity and specificity of ricin detection assay

The high reproducibility observed between independent repetitions of the standard curve calibration for ricin prompted us to determine whether the calibration curve can be used "off-line" for unknown samples, thereby bypassing the need for a standard curve in each measurement. To that end, ricin was spiked into buffer at three different concentrations and the samples were analyzed in the BLI system. As expected, by using the preset calibration curve, ricin concentrations could be accurately determined (Table 1).

Next, we sought to investigate the possibility of detecting ricin is serum samples. In several reports, it was shown that the presence of serum components may result in high background signals and, therefore, that clinical samples should be diluted 1:100 before quantification by various detection systems [3,9,15]. Here, we found that control serum samples diluted 1:10 did not induce signals above the background level (of buffer). Consequently, serum was spiked with 5 ng/ml ricin and the sample was analyzed to determine the toxin concentration using the preset calibration curve. Under these conditions, the recorded concentration of ricin was lower than expected (~900 pg/ml; Table 1). Similar results were obtained when ricin was spiked directly into a buffer containing 10% control serum, suggesting that ricin is adsorbed to an unknown component (probably a glycoprotein) in the serum. These findings are in good agreement with the findings of Koja and coworkers [19], who demonstrated that, on spiking serum or cerebrospinal fluid (CSF) samples with ricin, the fraction of free ricin dropped dramatically and was not available for immunological-based detection. It is also likely that the percentage of ricin available for immunological-based detection may change as a function of the toxin level in the serum. Thus, although the current assay can be implemented for ricin detection in clinical serum samples, the above observation should be taken into consideration when calculating the exposure dose by extrapolation of the values measured in plasma.

An important aspect that should be addressed when developing a sensitive assay for ricin detection is to minimize false-positive identification due to closely related antigens. Abrin is a highly toxic plant toxin derived from rosary peas (*Abrus precatorius*) that belongs to the RIP-2 family [20]. Although it is not phylogenetically related to ricin, the two toxins share 50% homology in their amino acid sequence and possess identical catalytic activity. Yet, abrin-neutralizing antibodies are not effective against ricin, emphasizing the need to eliminate false-positive responses in a ricin detection system. Accordingly, samples containing abrin at a concentration of 1000 pg/ml (100 times the assay LOD) were analyzed in the BLI-based system, yet they did not produce a response above the background level (Table 1). As another control for the assay specificity, we analyzed samples containing the plant lectin *U. europaeus* [21]. UEA does not share protein sequence homology with ricin, but it exhibits a similar pattern of sugar moieties. We

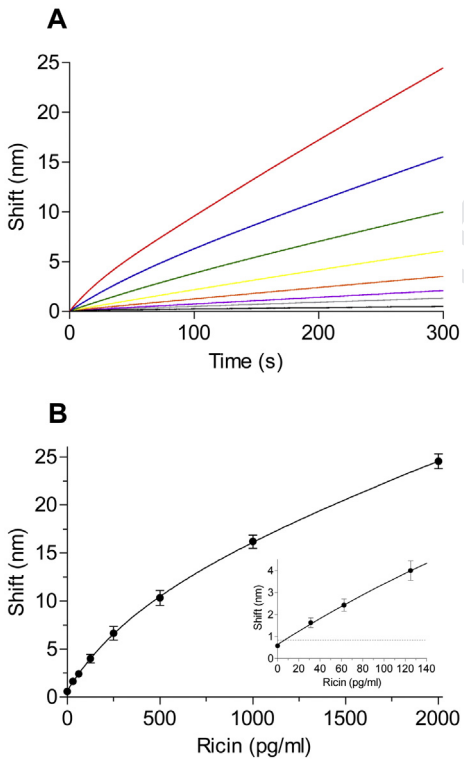

Fig.4. Calibration curve for ricin. (A) MH1-coated biosensors were submerged in buffer (black line) or with serially increasing concentrations of ricin (30–2000 pg/ml, indicated by the respective gray to red lines). The sensors were then immersed in AP–MH75 (10 μg/ml) and incubated with BCIP/NBT for 300 s. The wavelength interference for each sensor is presented. (B) The endpoint value of each sensogram is plotted as a function of ricin concentration. Points are averages ± standard errors of the mean of three independent sets of measurements, and data were fitted by nonlinear regression. Inset: dashed line indicates limit of detection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Antigen quantification in spiked buffer and serum samples and cross-reactivity with various biowarfare agents.

	Ricin (pg/ml)		*F. tularensis* (10^4 cfu/ml)	
	Spiked	Detected	Spiked	Detected
Buffer	250	230	10	15
	500	500	100	120
	1000	950		
Serum	5000	890	100	130
			1000	1800
Abrin	1000	<LOD[a]		
U. europaeus	1000	<LOD[a]		
E. coli			100	<LOD[a]
S. typhimurium			100	<LOD[a]
Y. pestis			100	<LOD[a]

[a] Refers to the shift induced by these agents in the standard curve of each respective antigen.

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have previously documented that vaccination with ricin elicits a polyclonal antibody response that is directed in part toward the sugar moieties [22]. Therefore, it was important to determine that the antibodies employed in this system will not cross-react with other plant lectins. Indeed, samples that contained 1000 pg/ml UEA did not generate signals that were above the background level, indicating that this novel detection system is both highly sensitive and highly specific to ricin.

Over the years, attempts were made to develop a sensitive immuno-based assay for the detection of ricin, and it was shown that it is possible to detect toxin levels below the ng/ml level [23,24]. A direct comparison between these assay formats is difficult because several parameters such as LOD values, assay length, number of steps, and reagent cost should be taken into consideration. For example, by combining digital and analog calibration curves using the SIMOA technology, the LOD could be lowered from 64 pg/ml to 10 fg/ml [9], yet this method involves several steps and the assay length is approximately 60 min. In another recent example of an ELISA microarray assay, the LOD value for ricin was found to be 8 pg/ml for a 16-h assay, whereas shortening the time to 2 h resulted in an LOD of 130 pg/ml [25]. Thus, the novel assay introduced in this study provides a highly sensitive ricin detection assay within a very short time period by a simple, automated, fluidics-free and on-line monitoring technology. It should also be noted that by prolonging the incubation time of the capture antibody with the antigen (step 1), the amount of immobilized ricin will increase, thereby producing a higher wavelength shift in the last step, which in turn may improve assay sensitivity.

Sensitive detection of F. tularensis

The promising results detailed above prompted us to ask whether the novel detection system is suitable for bacterial detection in biological samples. To address this issue, we chose *F. tularensis* as a model. Because the bacterial surface exhibits a diverse protein repertoire as well as repetitive antigens (e.g., lipopolysaccharide [LPS]), we hypothesized that hyper-immune sera of rabbits that were immunized with live *F. tularensis* bacteria contain antibodies directed against these antigens. Hence, the anti-*F. tularensis* antibodies could be used as both the capture and detection moieties. To test this possibility, the IgG fraction of anti-*F. tularensis* hyper-immune rabbit sera (termed T5) was purified, biotinylated (b-T5), and immobilized on a sensor. The sensors were then submerged in a buffer containing inactivated 1×10^8 cfu/ml *F. tularensis* LVS strain, resulting in a significant wavelength shift (Fig. 5A). After a short wash, the complex was submerged in a well that contained nonbiotinylated T5 ensuing another shift, thereby indicating that the same antibody fraction can be used to create the "sandwich" layers needed to capture and detect *F. tularensis*. Therefore, we conjugated AP to T5 (AP–T5) and determined the optimal concentration (8 μg/ml) that generated the highest signal-to-noise ratio (a value of 30).

Next, a standard curve for the detection of *F. tularensis* was generated using samples that contained bacteria in the range of $4–250 \times 10^4$ cfu/ml (Fig. 5B). The maximal wavelength shift ranged between 1.5 and 25 nm (CV = 20%) for the lowest and highest LVS concentrations tested, respectively, with an average background level of 0.4 nm (CV = 3%). These values were fitted ($R^2 = 0.91$) using nonlinear regression, and the LOD and LOQ values were calculated to be 1×10^4 and 2×10^4 cfu/ml, respectively (inset of Fig. 5B).

Sensitive immuno-based detection of *F. tularensis* (<10^3 cfu/ml) was shown recently using a combination of immunosorbent assay and gold-nanoparticles-linked oligonucleotide [15]. Yet, this assay is long (>2 h), involves many steps, and (therefore) is less suitable for rapid detection of this pathogen. In contrast, in a recent comparison of

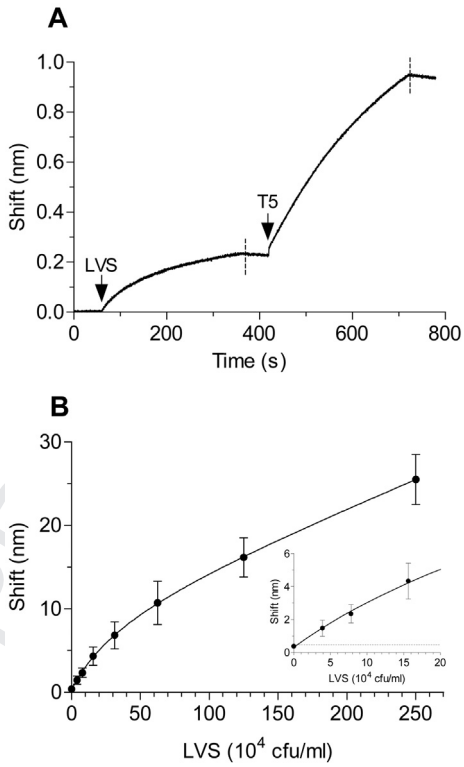

Fig.5. BLI-based detection of *F. tularensis*. (A) The streptavidin biosensor coated with biotinylated polyclonal anti-*F. tularensis* IgG fraction (T5) was submerged in a well containing inactivated *F. tularensis* LVS strain (indicated by an arrow), and the wavelength interference was recorded. Following a short wash (indicated by a dashed line), the sensor was immersed with nonlabeled T5, followed by another wash step. (B) T5-coated biosensors were submerged in samples containing increasing concentrations of inactivated *F. tularensis* LVS strain. The sensors were then immersed with AP–T5 and incubated with BCIP/NBT for 300 s. The endpoint value of each sensogram is plotted as a function of *F. tularensis* concentration. Points are averages ± standard errors of the mean of three independent sets of measurements, and data were fitted by nonlinear regression. Inset: dashed line indicates limit of detection.

11 commercially available rapid tests for the detection of *F. tularensis* (results are given within 15–40 min), the LOD values ranged between 10^5 and 10^7 cfu/ml [26], highlighting our finding that the BLI system provides high sensitivity within a very short time frame.

Assay specificity and F. tularensis detection in serum

As was demonstrated earlier for ricin, we found that the standard curve for *F. tularensis* can also be used as an "off-line" calibration for accurate determination of *F. tularensis* concentrations in buffered solutions (Table 1). The presence of serum had no effect on the background levels of the assay, and the concentration of *F. tularensis* in spiked serum samples at clinically relevant concentrations could be determined (Table 1), indicating the suitability of this assay for early detection of *F. tularensis* in clinical samples.

To evaluate the specificity of this assay, samples were spiked with other gram-negative bacteria: *E. coli*, *S. typhimurium*, and *Y. pestis*. It was found that, even at high concentration of the bacteria (1×10^6 cfu/ml), their presence did not induce a signal that was above the background level (Table 1), indicating that the assay is highly specific for *F. tularensis* detection.

Conclusions

In this study, we have shown that the use of a BLI-based biosensor can be expanded and applied for rapid, sensitive, and

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specific detection of ricin and *F. tularensis*, which represent two classes of biothreats, proteinaceous toxins, and bacterial pathogens. The low detection limits of these agents, demonstrated by the novel assay (10 pg/ml and 1×10^4 pfu/ml for ricin and *F. tularensis*, respectively), meet the need for their rapid identification to initiate prompt lifesaving medical treatment. To use this novel system to its full extent, specific and high-affinity antibodies should be applied. Although in this work we demonstrated the capability of the BLI-based assay to detect two specific pathogens, the methodology can be easily adapted for the detection of any other analytes in general, and biowarfare agents in particular, in a rapid and sensitive manner.

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