Analytical Biochemistry 506 (2016) 22–27

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

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

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ARTICLE INFO

Article history:
Received 14 March 2016
Received in revised form
19 April 2016
Accepted 21 April 2016
Available online 5 May 2016

Keywords:
Biolayer interferometry
Ricin
Tularemia
Octet
Biowarfare agents

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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http://dx.doi.org/10.1016/j.ab.2016.04.018
0003-2697/

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