



Refinement of an open-microcavity optical biosensor for bacterial endotoxin test

Nikolay Akimov^a, Jonathan Scudder^{b,c}, Jing Yong Ye^{a,b,*}

^a SAFEbiosense LLC, San Antonio, TX, 78249, USA

^b Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX 78249, USA

^c Texas Biomedical Research Institute, 8715 W Military Dr., San Antonio, TX, 78227, USA

ARTICLE INFO

Keywords:

Limulus amoebocyte lysate
Optical biosensor
Endotoxin
Drug manufacturing quality control

ABSTRACT

The Limulus Amebocyte Lysate (LAL) test is an *in vitro* assay widely used in the pharmaceutical and biotechnology industries to detect bacterial endotoxins. Endotoxin is a structural component of the cell wall of Gram-negative bacteria, which has serious pathogenic effects in the body and may cause dysfunction of multiple organ systems and increased risk of mortality. To address the growing need for LAL assays due to the increased demand from drug and vaccine manufacturers, we have developed a new LAL assay approach. Our detection mechanism is different and improved from those currently used in the industry, leading to increased test sensitivity and reduced assay time. Our study utilizes an open-microcavity photonic-crystal biosensor to quantify endotoxin concentrations. It has demonstrated an improved LAL assay sensitivity by 10 fold compared to the commercial standard methods and reduced the time needed for the assay by more than half. In addition, this approach requires as little as 5 μ L of LAL reagent per test, thereby decreasing costs and conserving horseshoe crabs. The results reported in this paper indicate the possibility of using the photonic-crystal biosensor based approach for significant enhancements of endotoxin testing.

1. Introduction

Quality control is of critical importance in the pharmaceutical and biotechnology industries. If parenteral pharmaceutical products or implantable medical devices are contaminated with endotoxins and come into contact with the bloodstream, they will cause a number of pathophysiological effects. This include a rapid rise in core body temperature, followed by severe shock, thus potentially leading to death, in certain situations, even before a diagnosis is made (Rhee et al., 2019; Thomas, 2019; Ward and Fattahi, 2019). The most common source of endotoxins comes from lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria (Raetz and Whitfield, 2002). As pyrogenic responses and septic shock can be caused by just a segment of a cell membrane rather than live bacteria, the problem is ubiquitous in nature. LPS is a very stable molecule that is difficult to remove from fluids or medications. It cannot be removed by deionizing, destroyed by boiling, autoclaving, nor low pressure plasma sterilization (Moisan et al., 2001). In fact, the only possible ways to remove the pyrogenic effects of LPS are to either denature it by high heat (250 °C for 30 min, 200 °C for 1 h) or treat it with strong acids, bases, or hydrogen peroxide. Note that

sterility, which is much easier to achieve, is not necessarily equal to apyrogenicity. Thus, endotoxins are a pervasive environmental contaminant that is likely present with certain levels in almost all raw materials, laboratory glassware, and final medical products (Gorbett and Sefton, 2005).

Limulus Amebocyte Lysate (LAL) testing is an important part of the pharmaceutical industry quality control toolkit (Bolden et al., 2016; Taylor, 2011), which is approved by the US Pharmacopeia and FDA (FDA, 2014) as a recommended endotoxin testing strategy for oral/injectable medications, USP-grade water at hospitals, and implantable devices (such as hip replacements, artificial hearts, and man-made ligaments) (Taylor, 2011). The LAL test has also been utilized for a range of other applications, including testing endotoxins in food industry (van Duuren-Stuurman et al., 2018; Venter et al., 2006), detecting pyrogens in nanomaterials (Li et al., 2017; Smulders et al., 2012), and verifying safe work environments in ranching and farming (Basinas et al., 2015). The LAL reagent is an aqueous extract of blood cells (amebocytes) from the horseshoe crab (*Limulus polyphemus*), which reacts with bacterial endotoxin and results in a semi-solid mass (coagulation) due to clotting factors contained in the LAL reagent. This reaction forms the basis of the

* Corresponding author. Department of Biomedical Engineering, The University of Texas at San Antonio, San Antonio, TX 78249, USA.

E-mail address: jingyong.ye@utsa.edu (J.Y. Ye).

<https://doi.org/10.1016/j.bios.2021.113436>

Received 29 March 2021; Received in revised form 1 June 2021; Accepted 11 June 2021

Available online 12 June 2021

0956-5663/© 2021 Elsevier B.V. All rights reserved.

three LAL test methods currently used in industry. The most basic test is termed “gel clot” test. This test simply mixes LAL reagents with the analyte solution of interest in a 1:1 ratio at 37 °C. The gel-clot test yields a binary, end-point result 1 h after mixing. A tube is scored as “positive” if the mixed solution gels and the clot withstands 180° inversion without breaking. All other conditions are scored “negative,” even if the clot almost remains intact but then collapses. In addition to the gel-clot test, the other two approaches, turbidimetric and chromogenic LAL assays, are commercially available for endotoxin testing. The turbidimetric assay uses the same enzymatic cascade as the gel clot test, but adds a turbidity scanner to detect the change in scattered light over the reaction process to obtain kinetic analysis, rather than only at the end-point. Chromogenic detection has been developed as another kinetic LAL assay. In the final step of the enzymatic cascade, coagulogen is switched to a chromogenic substrate, which, when cleaved by the activated clotting enzyme, produces a yellow color that can be read with a plate reader.

Despite its important applications, the LAL test frequently faces challenges from interference issues caused by certain chemical or physical factors in samples that affect either the gel clot reaction or detection of the gelation process (Chen and Mozier, 2013). For example, chemical inhibitors cause chelation of divalent cations necessary for the LAL reaction (i.e. EDTA), protein denaturation, or pH disruption (i.e. pH outside 6.0–7.5 range). Physical complicating factors include adsorption of endotoxin, product viscosity, or color of testing samples (FDA, 2014). Turbidimetric and chromogenic methods cannot be used with certain turbid or colored samples (FDA, 2014), as they can affect the reading at the detection wavelength and lead to inaccurate results. The most widely used approach to overcome the interference problems is through serial dilutions of the test sample. This approach relies on the premise that a sample may be diluted to a point at which the interfering factor ceases to affect the test, but at which the endotoxin test method is still sensitive enough to measure the endotoxin limit (threshold pyrogen dose for humans) of the product. The maximum valid dilution (MVD) is defined as the greatest dilution at which the endotoxin limit can be detected. Intrathecally (IT) administered drugs typically have much lower endotoxin limit (McCullough, 2018), thereby rendering it even more difficult to measure when certain interfering factors occur in the LAL assays. Moreover, recent new drug formulations tend to be more and more complex, which require more dilutions, and therefore more sensitive methods, to overcome the interference problem. For example, many new drug products using recombinant techniques have been released recently, which contain chelating agents and detergents, rendering it extremely challenging for accurate LAL assays (Chen and Mozier, 2013). The interference, especially inhibitors, can cause false-negative results that may jeopardize public health and safety, and may lead to huge economic losses to the involved companies (Schwarz et al., 2017).

The need of serial dilutions to minimize the interference problems in the LAL assays puts a strict requirement for high sensitivity of LAL detection methods. To address this challenging issue and the fast-growing demand for LAL assays, this paper reports a new detection mechanism for the LAL assay that is different from the current three LAL techniques. This new approach takes advantage of refractive index (RI) changes in the LAL gel forming process monitored with a novel open-microcavity photonic crystal biosensor. Our last paper (Scudder and Ye, 2018) suggested that there was a sensitivity gain of 200 fold from the use of the photonic crystal biosensor in testing for the presence of pyrogens. After much refinement of the experiments, we have noticed that the previously reported astronomical sensitivity gain was partially due to experimental artifacts. The actual sensitivity gain is, in actuality, ten times greater than the current best commercial sensitivity. This improvement in sensitivity is still a large step further in solving the problem of confounding variables as described above. In addition, this study further demonstrates that the required amount of LAL reagents can be as little as 5 µL for each test, leading to significant cost savings for

the LAL assays. We believe that our biosensor system, due to its higher sensitivity, higher speed and lower operating cost compared to the current commercial systems, is a step forward in providing more data for pharmaceutical and medical device manufacturers to accurately monitor potential endotoxin contaminations of their products and to safeguard public health.

2. Materials and methods

2.1. Biosensor chip preparation

The biosensor used in this work possesses a novel open optical microcavity for highly sensitive bioassays based on a unique detection mechanism that utilizes a photonic-crystal in a total-internal-reflection (PC-TIR) configuration. Different from our previous studies using the PC-TIR sensor for molecular binding assays (Guo et al. 2008, 2010; Zhang et al. 2011, 2013, 2014, 2016), this study is focused on detecting RI changes of LAL analyte solutions caused by endotoxins. The PC-TIR sensor was designed based on our numerical simulations with a transfer matrix approach and fabricated with electron-beam physical vapor deposition, which has five alternating 90.6-nm TiO₂ and 308.8-nm SiO₂ layers coated on a BK-7 glass substrate. Above this periodic structure a cavity layer was formed with 374.6 nm of silica and 13 nm of silicon. The thin silicon layer was designed to give rise to an appropriate level of absorption such that a sharp dip in the reflectance spectrum is introduced at the resonant wavelength of an open optical microcavity formed when the PC structure is used in a TIR configuration. When the RI of analyte solutions on top of the PC-TIR sensor surface changes, the resonant wavelength shifts sensitively for 1490 nm/RIU (Guo et al., 2008). When the wavelength of a probe light is fixed, i.e., a laser beam rather than a broadband white light source, the change in RI can also be quantified by measuring the change in the resonant angle of the sensor. The sensitivity is 48.67°/RIU for the angular interrogation. The smallest detectable RIU change is 2.2×10^{-6} RIU with an imaging chip to monitor the change in the resonant angle in the current system. This detection limit may be improved by increasing the distance between the imaging chip and the sensor. Pyrogen-free silicone wells were purchased from Sigma Aldrich with a dimension of each well measured as 2.8 mm in diameter and 1.85 mm in depth. Three sample wells were sealed on the surface of a PC-TIR sensor. PC-TIR sensor chips were baked at 200 °C for 1 h before usage to remove any possible contaminations.

2.2. Sensor apparatus

A compact sensor system as schematically shown in Fig. 1 was designed and constructed for sensitive detection of the changes in RI of the LAL analyte solutions on the sensor chip surface. A diode laser with an emission wavelength at 635 nm was first collimated and then focused

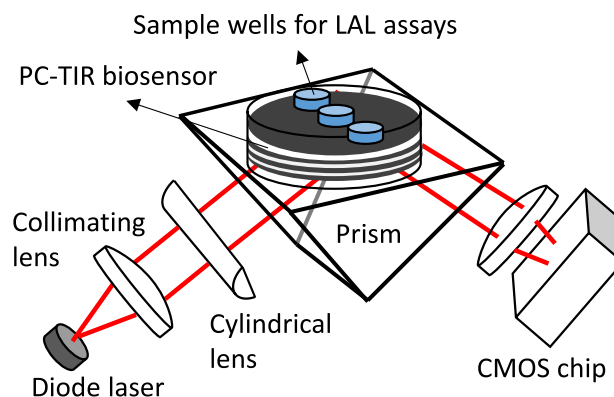


Fig. 1. Schematic drawing of the experimental setup for LAL assays using a PC-TIR biosensor.

into a line across a PC-TIR sensor surface using a cylindrical lens. The PC-TIR sensor was coupled with a BK7 glass prism using refractive-index matching fluid. Three silicone wells were sealed on the sensor surface for LAL reagents and analyte solutions. The output beam reflected from the sensor was collected with a lens and projected onto a CMOS imaging chip. Dark lines that correspond to the resonant angle of the sensor were recorded on the images. The positions of the resonant lines on the images, determined by the RIs of the samples, were analyzed with a MATLAB program with a graphical user interface developed by SAFE-biosense. The software was programed to start data capture processes at a user-defined interval (3–300 s) for any duration as needed. Each captured image was segmented, so that only the resonant lines of the PC-TIR sensor was present in the cropped area. The 2-dimensional array representing each resonant line was averaged along the axis parallel to the resonant line, which resulted a histogram of the pixel intensities across the line. As the resonant line should have a Lorentzian line shape of a Fabry-Pérot cavity (Ismail et al., 2016), the histogram was fitted with a Lorentzian function to precisely determine the resonant line location with a subpixel resolution. Relative changes of the line positions reflect the state of reaction of endotoxins with LAL reagents. The entire system was housed in a chamber with a temperature set at 37 °C.

2.3. *Limulus Amebocyte Lysate (LAL) and standard endotoxin preparation*

The LAL reagent KTA2 (with a marked sensitivity of 0.005 EU/mL) was ordered from Charles River Laboratories. The directions for proper re-hydration and preparation included in the LAL package were followed. Basically, the LAL powder was collected into the bottom of the vial by tapping on a hard surface. The indicated amount of LAL Reagent Water (LRW) was added directly into the vial and mixed gently until the LAL powder dissolved. The control standard endotoxin (CSE) was also purchased from Charles River Laboratories. Included in the CSE package was the Certificate of Analysis, which specifies the potency (pyrogenicity) of the dehydrated endotoxin in EU/mg. The CSE was reconstituted with LAL Reagent Water and vortexed vigorously for 5 min before further dilutions. Serial dilutions were made to prepared the test solutions containing 50, 5, 0.5, 0.05, 0.005, 0.0005 EU/mL endotoxin, respectively.

2.4. Measurement procedure

After the LAL reagents were rehydrated and appropriate dilutions of the CSE were made, a baked biosensor was placed on an equilateral prism coupled with index matching fluid as shown in Fig. 1. Before adding any solutions, a background image of the sensor was first taken. A small amount of LAL reagent (6 μ L) was then mixed with 6- μ L CSE having a concentration ranging from 50 to 0.0005 EU/mL, while another 6- μ L LAL reagent was mixed with 6- μ L LRW for using as a reference. A portion (10- μ L) of the mixture was taken and added into a sample well on the PC-TIR sensor. A coverslip was used to cover the sample wells to prevent evaporation. The probe laser beam focused by a cylindrical lens formed a line across three sample wells on the sensor surface simultaneously. The reflected beam of the probe laser from the sensor containing a range of different angles was projected onto an imaging chip to measure the resonant angle of the sensor. A dark line showed up on the image indicating the resonant angle determined by the RI of the analyte solution in the corresponding sample well. The home-built MATLAB code allowed us to process the images in real time. The background image taken previously was used to subtract the image with the dark lines, resulting in an image with three short sections of bright lines corresponding to the three samples on the sensor surface. The intensity profile of each bright line was obtained from the processed image and fitted with a Lorentzian function to determine the peak position. The images were taken every 3 s and processed to continuously monitor the change of the peak positions. The peak position was observed shifting

with the coagulation process of the sample due to the interactions of the LAL reagent with the endotoxin. After the assay, the sample wells and the sensor chip were cleaned first with acetone, and then with deionized water, dried with compressed air to assure no fluid solution remains on the sensor surface. In order to reuse the biosensor, the cleaned sensor chips were baked at 200 °C on a hot plate or oven for at least 1 h in an aluminum enclosure, to ensure even heat transfer, and to restrict room air contamination.

Measurements of the samples were conducted five times for each CSE concentration to make statistical analysis possible. Standard errors were calculated and plotted as the appropriate error bar in figures showing the measurement results. Standard deviations were also calculated.

3. Results and discussion

In our previous studies we have used PC-TIR sensors for molecular binding assays ranging from well-studied coupling agents to small molecule binding and nucleic acid and cardiac biomarker detection (Guo et al. 2008, 2010; Zhang et al. 2011, 2013, 2014, 2016). In contrast, this study utilizes the PC-TIR sensor to monitor the changes in RI of LAL analyte solutions, which sensitively reflects the amount of

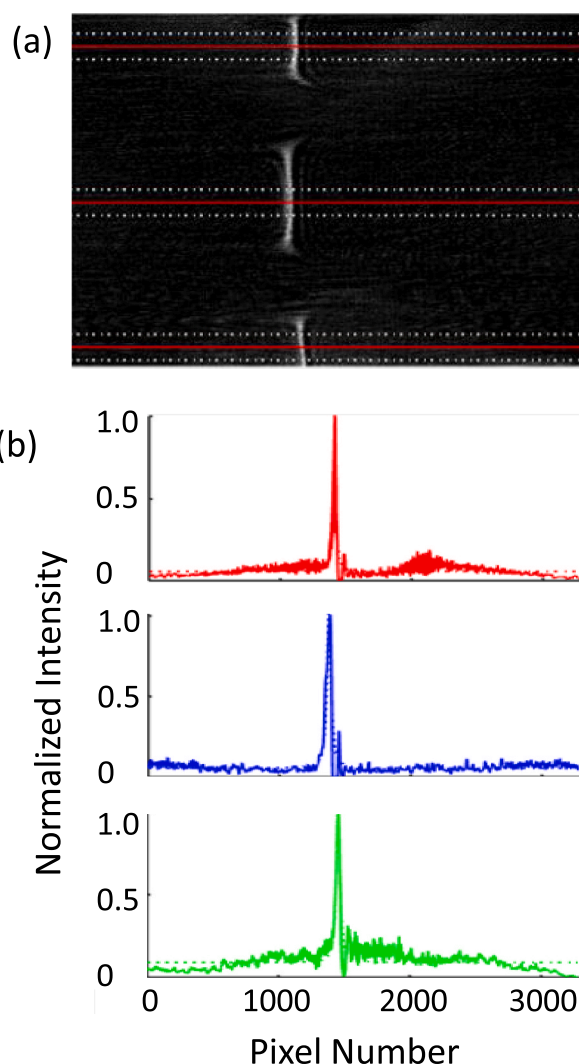


Fig. 2. (a) A representative image of the probe laser beam reflected from a PC-TIR sensor. The position of the three short lines correspond to the resonant angles of the sensor at the three sample wells. (b) Normalized intensity profile across each resonant line. The reflection angle is corresponding to the pixel numbers.

endotoxin in the test samples. Fig. 2(a) shows a representative image of the reflected probe laser beam from a PC-TIR sensor without samples (background image) subtracted by the image after the samples were loaded. Three short bright lines are visible in the image, which correspond to the resonant angles of the sensor for the three sample wells. The dashed lines indicate the selected sections of the resonant lines for data analyses. The normalized intensity profile across each resonant line is plotted in Fig. 2 (b) as a function of the reflection angle (corresponding to the pixel numbers). The sharp resonant peak shifts with time as the LAL reagent responds to the endotoxin.

The peak position of each resonant line was continuously monitored by taking the images every 3 s throughout the LAL assay process for each sample. A Lorentzian function has been utilized to fit each curve in order to precisely determine the peak position, which shifts during the LAL reaction process due to the change of the local refractive index. By fitting the curve rather than simply picking the highest point of the curve, one can obtain a more accurate value of the resonant angle. The sharp resonant condition of the PC-TIR sensor allows for precise determination of the peak position, thus leading to accurate quantification of the RI changes that is related to the endotoxin concentration. The time dependence of the peak positions obtained from fitting the curves for six different concentrations of endotoxin as well as the control sample is shown in Fig. 3. It can be seen that the curve from each sample slowly increases with time initially and at certain time point the curve starts to shift up at a much higher rate before it reaches a plateau. The time point for the rapid increase corresponds to the onset time of the LAL reactions with the endotoxin. Different onset times can be observed clearly for different endotoxin concentrations. Therefore, the onset time can be utilized as a useful parameter to quantify the endotoxin concentration in the test sample.

The data indicate that even the most diluted endotoxin solution used (0.0005 EU/mL) has resulted a curve that is clearly different from the negative control (LAL reagent mixed with water). Based on the average pyrogenicity of the endotoxin, 0.0005 EU/mL equates approximately to 0.00005 ng/mL of endotoxin in the solution, which demonstrates the superior sensitivity of this PC-TIR sensor based approach over the conventional approaches. Compared to the best sensitivity (0.005 EU/mL) obtained in the LAL turbidimetric test by Charles River Laboratories, the sensitivity of the PC-TIR sensor based approach has showed a 10-fold enhancement over the industry standard. In addition, compared to the detection sensitivity of endotoxin at 0.4 ng/mL in a recent study using a plasmonic biosensor in conjunction with antimicrobial polymyxin-B conjugated gold nanoparticles (Manoharan et al., 2019), our results represent a notably higher sensitivity by four orders of magnitudes.

Fig. 3 shows that even the curve for the negative control sample eventually started to shift up at an increased rate in a longer time scale. This shift may be attributed to a possible minute amount of endotoxin in the water used in this measurement. Although the depyrogenated LAL reagent water (LRW) from Charles River was specifically chosen for this experiment to minimize any possible contaminations, it is still possible that an extremely low level (<0.0005 EU/mL) of endotoxin may exist in the sample, as the LRW is only guaranteed to have an endotoxin level less than 0.001 EU/mL. It is therefore likely that the detection limit (0.0005 EU/mL) observed in this experiment is actually due to the limitation of the control sample rather than the detection approach itself. The detection approach may allow distinguishing endotoxins with an even higher sensitivity if the control sample has a more stable curve. The significant improvement of our approach in detection sensitivity can be attributed to the PC-TIR sensor's capability for ultrasensitive measurements of the RI changes in the analyte solutions caused by LAL reactions to the minute amount of endotoxin. As the resonance bandwidth of a PC-TIR sensor is determined by a cavity mode, which is around 1–2 nm, compared to the bandwidth of an SPR sensor (~ 40 nm) (Hoa et al., 2007; Homola, 2003, 2008), the bandwidth of the PC-TIR sensor is 20–40 times narrower than a typical SPR sensor, thus allowing precise measurements of resonant line shifts for enhanced detection sensitivity.

In recent years the increased demand of pyrogen test in the pharmaceutical and biotechnology industries has attracted much effort for the development of new approaches for endotoxin testing, including using quartz sensors (Chalupniak et al., 2014; Liu et al., 2017), electrochemical sensors (Inoue et al., 2012; Su et al., 2012), nanoparticle suspensions (Li et al., 2015; Wang et al., 2014), piezoelectric sensors (Muramatsu et al., 1988), and surface-plasmon-resonance (SPR) based sensors (Abdin et al., 2015; Manoharan et al., 2019). Some of these new approaches still need to further improve their detection sensitivities. For example, the SPR sensor functionalized with molecular imprinted polymers has an LPS detection range of 15.6–500 ng/mL (Abdin et al., 2015), which is still much less sensitive even compared with conventional LAL assays, although this approach may have some potential benefits, including good stability, reasonable cost, easy preparation, and modification (Su and Ding, 2015).

In addition, alternative pyrogen test compounds have been constantly engineered, such as the beta glucan only test, GlucateLL (Obayashi et al., 1995), the monoclonal antibody test (Hartung, 2015; Sander et al., 2008), and recombinant Factor C (Barnett et al., 2012). Some new approaches have showed promising results for improving the endotoxin testing sensitivity. For example, Noda et al. has reported a detection sensitivity of endotoxin as high as 0.0005 EU/mL through

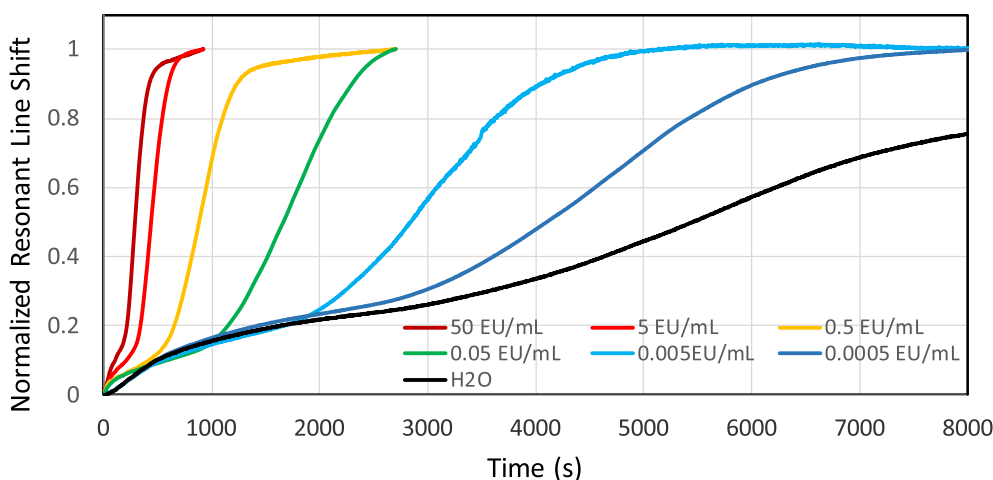


Fig. 3. Representative curves of the time dependence of the resonant line shifts of a PC-TIR sensor for samples with different concentrations of endotoxins; 50, 5, 0.5, 0.05, 0.005 and 0.0005 EU/mL. Different onset times can be observed for different endotoxin concentrations. Even the most diluted endotoxin solution used (0.0005 EU/mL) can be easily distinguished from the negative control.

bioluminescence measurements using mutant firefly luciferase. This approach is an end-point detection rather than kinetic measurements and may have challenges to mass produce enough mutant firefly luciferase to meet the industry demand. Yeo et al. reported another sensitive approach for endotoxin testing in the range of 0.0005–5 EU/mL based on a new electrochemical sensor using a human recombinant toll-like receptor 4 (rhTLR4) and myeloid differentiation-2 (MD-2) complex (Yeo et al., 2011). While the alternative approaches have showed promising potentials, a latest study indicates that correlation between the LAL assays and recombinant alternatives remains unresolved and requires caution, continued development, and testing (Dubczak et al., 2021). In contrast, the PC-TIR sensor based approach utilizes the robust, well-established enzymatic reaction of the LAL reagent, and introduces a fundamentally new detection principle that utilizes the RI changes during the coagulation process, different from the existing turbidimetric and chromogenic methods. While this PC-TIR based approach offers significantly enhanced sensitivity, it keeps the same enzymatic reaction of LAL reagents and does not require substantial modification of the industry standard approaches, thus allowing for easy adoption of this new approach.

In addition to the sensitivity, the time required for LAL assays is also an important consideration in industry applications for higher throughput and cost saving. To illustrate the fast response of the PC-TIR sensor to LAL assays, we took the time derivative of the resonant line shifts to determine the onset time of the coagulation process of the LAL reaction to endotoxins. The time corresponding to the minimum of each derivation curve in Fig. 4 determines the onset time for the endotoxin concentration used for that measurement, as the shift of the resonant line speeds up after that time point due to the onset of the coagulation process, which causes the increase of the local RI. It can be seen in Fig. 4 that the onset time increases with lowering the endotoxin concentration. The average onset time for an endotoxin sample at 50-EU/mL is less than 3 min. The average onset time increases to 32 min for the lowest endotoxin concentration (0.0005 EU/mL) as measured in the current experiment.

A direct comparison between the PC-TIR biosensor measurement results and the turbidimetric measurement results provided by Charles River Laboratories is shown in Fig. 5. The error bars in the figure indicate the standard errors calculated based on 5 measurements with PC-TIR sensor for each endotoxin concentration. The between-run coefficient of variation (CV) is ranging from 9% to 18%, which is smaller than the typical acceptable value (25%) for LAL testing (Sandle, 2014). The results shown in Fig. 5 clearly demonstrates the fast response time, another unique feature of the PC-TIR sensor based LAL assays in addition to its ultrahigh sensitivity. The conventional approaches, including

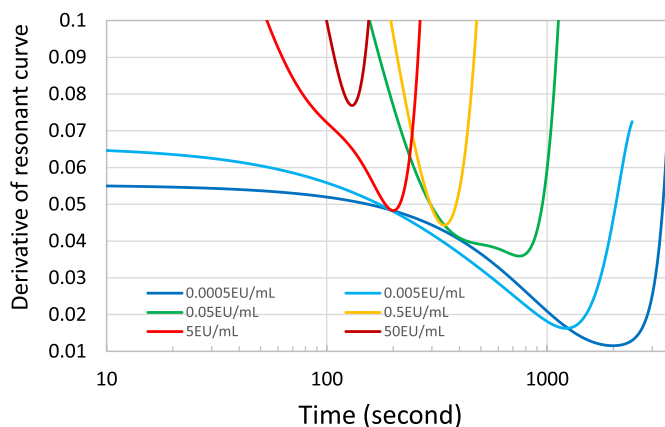


Fig. 4. Time derivatives of the resonant line shifts. The time corresponding to the minimum of each curve determines the onset time of the coagulation process of the LAL reaction to endotoxins, which increases with lowering the endotoxin concentration.

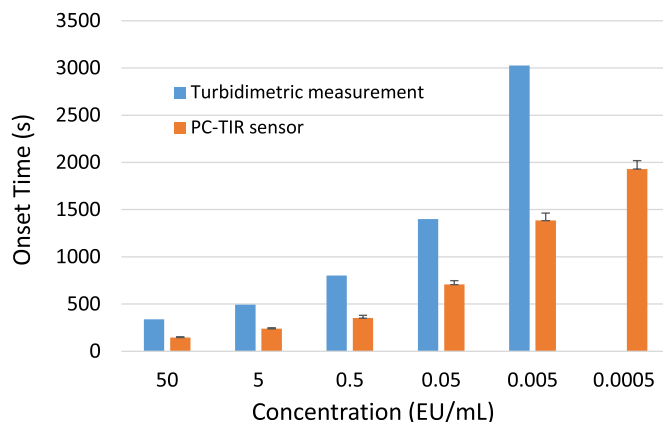


Fig. 5. Comparison of the onset times between the PC-TIR biosensor measurement and the turbidimetric measurement by Charles River Laboratories (CRL). The error bars indicate the standard error for our results, while the error bars are not provided for the results from CRL.

gel clot, turbidimetric, and chromogenic assays, take much longer time to generate assay results, especially for samples with low endotoxin concentrations. It is worth noting that the onset time for the lowest endotoxin concentration (0.0005 EU/mL) measured with the PC-TIR sensor is faster than the onset time for a sample with even 10 times higher endotoxin concentration (0.005 EU/mL) measured with a conventional turbidimetric approach, although both measurements used the same LAL reagents. There is no data available for the 0.0005-EU/mL endotoxin concentration from Charles River Laboratories, as their turbidimetric approach has a detection sensitivity limited to 0.005 EU/mL. The short assay time for the PC-TIR sensor-based detection may be attributed to the fast response of the change in RI due to the onset of the coagulation of the analyte solution. The open microcavity of the PC-TIR sensor has a sharp resonant condition, which is highly sensitive to the local change in the RI of a solution in a proximity of several hundreds of nanometers above the sensing surface. The RI may start to change immediately after the onset of the coagulation process, before a gel clot is formed. The significantly shortened assay time of the PC-TIR approach compared with the other methods is desired for many industry applications, such as with “in-line” quality control of products.

In addition to the ultrahigh sensitivity and fast response time, another unique advantage of the PC-TIR sensor based endotoxin testing is the minimum requirement of the amount of LAL reagents. In the current study, each sample well contains only 5 μ L of LAL reagents, while the typical amount of LAL reagents required for the conventional endotoxin assays ranges from 25 to 100 μ L. Our result has clearly demonstrated that the endotoxin assays based on the PC-TIR sensor can significantly reduce the required amount of LAL reagents. This detection advantage can be attributed to the fact that the PC-TIR sensor is extremely sensitive to the local change of the RI within a range of around 300 nm above the sensor surface, which is in sharp contrast to conventional turbidimetric measurements that require a light path length long enough in the sample to gain necessary sensitivity. The substantial volume reduction of the required LAL reagents with PC-TIR measurements will save on costs of endotoxin assays for the pharmaceutical and biotechnology industries and will also allow the LAL test to be much more sustainable with respect to the horseshoe crab's limited population.

4. Conclusion

This study has shown that our endotoxin test with a PC-TIR sensor provides additional value in comparison to other commercial LAL assays in its enhanced sensitivity, along with expedited discrimination of positive versus negative results. This remarkable improvement can be

attributed to the unique detection principle that utilizes a new physical parameter – the RI of the analyte solution – to monitor the coagulation process during the reaction of the LAL reagent with the analyte solution. In addition to sensitivity and speed, this new approach also allows for a significant reduction in the amount of LAL reagents for the assays, thereby resulting in both a cost savings and conservation of a most valuable resource; the horseshoe crab. The durability, sensitivity, speed, cost effectiveness, resource conservation and practicality of our sensor apparatus versus commercial standards solidifies the PC-TIR sensor as an innovative modality in advancing the evolution of endotoxin testing.

CRedit authorship contribution statement

Nikolay Akimov: Funding acquisition, Investigation, Software, Writing – review & editing. **Jonathan Scudder:** Conceptualization, Writing – original draft, Writing – review & editing. **Jing Yong Ye:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors J. Y. Ye and J. Scudder are inventors for a pending patent (Serial No. 16/162,261), which protects the system described in this paper. The authors N. Akimov and J. Y. Ye are part of the limited liability corporation SAFEbiosense LLC. All the authors have certified that none of the results found within this article were in any way coerced or rewarded by any outside parties.

Acknowledgments

This paper is based upon work supported by the National Science Foundation SBIR Phase I grant (No. 2014915).

References

- Abdin, M.J., Altintas, Z., Tothill, I.E., 2015. In silico designed nanoMIP based optical sensor for endotoxins monitoring. *Biosens. Bioelectron.* 67, 177–183.
- Barnett, M.J., Wadham, J.L., Jackson, M., Cullen, D.C., 2012. In-field implementation of a recombinant factor C assay for the detection of lipopolysaccharide as a biomarker of extant life within glacial environments. *Biosensors* 2 (1), 83–100.
- Basinas, I., Sigsgaard, T., Kromhout, H., Heederik, D., Wouters, I.M., Schläslen, V., 2015. A comprehensive review of levels and determinants of personal exposure to dust and endotoxin in livestock farming. *J. Expo. Sci. Environ. Epidemiol.* 25 (2), 123–137.
- Bolden, J.S., Warburton, R.E., Phelan, R., Murphy, M., Smith, K.R., De Felippis, M.R., Chen, D., 2016. Endotoxin recovery using limulus amoebocyte lysate (LAL) assay. *Biologicals* 44 (5), 434–440.
- Chalupniak, A., Waszczuk, K., Halubek-Głuchowska, K., Piasecki, T., Gotszalk, T., Rybka, J., 2014. Application of quartz tuning forks for detection of endotoxins and Gram-negative bacterial cells by monitoring of Limulus Amoebocyte Lysate coagulation. *Biosens. Bioelectron.* 58, 132–137.
- Chen, L., Mozier, N., 2013. Comparison of Limulus amoebocyte lysate test methods for endotoxin measurement in protein solutions. *J. Pharmaceut. Biomed. Anal.* 80, 180–185.
- Dubczak, J., Reid, N., Tsuchiya, M., 2021. Evaluation of limulus amoebocyte lysate and recombinant endotoxin alternative assays for an assessment of endotoxin detection specificity. *Eur. J. Pharmaceut. Sci.* 159, 105716.
- FDA, 2014. Bacterial endotoxins/pyrogens. <https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/inspection-technical-guides/bacterial-endotoxinspyrogens>.
- Gorbet, M.B., Sefton, M.V., 2005. Endotoxin: the uninvited guest. *Biomaterials* 26 (34), 6811–6817.
- Guo, Y.B., Divin, C., Myc, A., Terry, F.L., Baker, J.R., Norris, T.B., Ye, J.Y., 2008. Sensitive molecular binding assay using a photonic crystal structure in total internal reflection. *Opt Express* 16 (16), 11741–11749.
- Guo, Y., Ye, J.Y., Divin, C., Thomas, T.P., Myc, A., Bersano-Begey, T.F., Baker, J.R., Norris, T.B., 2010. Real-time biomolecular binding detection using a sensitive photonic crystal biosensor. *Anal. Chem.* 82, 5211–5218.
- Hartung, T., 2015. The human whole blood pyrogen test—lessons learned in twenty years. *ALTEX* 32 (2), 79.
- Hoa, X.D., Kirk, A.G., Tabrizian, M., 2007. Towards integrated and sensitive surface plasmon resonance biosensors: a review of recent progress. *Biosens. Bioelectron.* 23 (2), 151–160.
- Homola, J., 2003. Present and future of surface plasmon resonance biosensors. *Anal. Bioanal. Chem.* 377 (3), 528–539.
- Homola, J., 2008. Surface plasmon resonance sensors for detection of chemical and biological species. *Chem. Rev.* 108 (2), 462–493.
- Inoue, K.Y., Takahashi, S., Ino, K., Shiku, H., Matsue, T., 2012. Development of an electrochemical Limulus amoebocyte lysate assay technique for portable and highly sensitive endotoxin sensor. *Innate Immun.* 18 (2), 343–349.
- Ismail, N., Kores, C.C., Gekus, D., Pollnau, M., 2016. Fabry-Perot resonator: spectral line shapes, generic and related Airy distributions, linewidths, finesses, and performance at low or frequency-dependent reflectivity. *Opt Express* 24 (15), 16366–16389.
- Li, Y., Italiani, P., Casals, E., Tran, N., Puentes, V.F., Boraschi, D., 2015. Optimising the use of commercial LAL assays for the analysis of endotoxin contamination in metal colloids and metal oxide nanoparticles. *Nanotoxicology* 9 (4), 462–473.
- Li, Y., Fujita, M., Boraschi, D., 2017. Endotoxin contamination in nanomaterials leads to the misinterpretation of immunosafety results. *Front. Immunol.* 8, 472.
- Liu, T., Zhang, W., Zhou, L., Guo, Z., Tang, Y., Miao, P., 2017. A quartz crystal microbalance sensor for endotoxin assay by monitoring limulus amoebocyte lysate protease reaction. *Anal. Chim. Acta* 961, 106–111.
- Manoharan, H., Kalita, P., Gupta, S., Sai, V.V.R., 2019. Plasmonic biosensors for bacterial endotoxin detection on biomimetic C-18 supported fiber optic probes. *Biosens. Bioelectron.* 129, 79–86.
- McCullough, K.Z., 2018. Calculating Endotoxin Limits for Drug Products. *American Pharmaceutical Review, Endotoxin Supplement*, pp. 14–16.
- Moisan, M., Barbeau, J., Moreau, S., Pelletier, J., Tabrizian, M., Yahia, L.H., 2001. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. *Int. J. Pharm.* 226 (1), 1–21.
- Muramatsu, H., Tamiya, E., Suzuki, M., Karube, I., 1988. Viscosity monitoring with a piezoelectric quartz crystal and its application to determination of endotoxin by gelation of limulus amoebocyte lysate. *Anal. Chim. Acta* 215, 91–98.
- Obayashi, T., Kawai, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Shimada, K., Iwasaki, H., Teshima, H., Kohno, S., 1995. Plasma (1→3)- β -D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* 345 (8941), 17–20.
- Raetz, C.R., Whitfield, C., 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700.
- Rhee, C., Jones, T.M., Hamad, Y., Pande, A., Varon, J., O'Brien, C., Anderson, D.J., Warren, D.K., Dantes, R.B., Epstein, L., Klompas, M., Centers for Disease, C., Prevention Prevention Epicenters, P., 2019. Prevalence, underlying causes, and preventability of sepsis-associated mortality in US acute care hospitals. *JAMA Netw Open* 2 (2), e187571.
- Sander, I., Fleischer, C., Borowitzki, G., Brünig, T., Raulf-Heimsoth, M., 2008. Development of a two-site enzyme immunoassay based on monoclonal antibodies to measure airborne exposure to (1→3)- β -D-glucan. *J. Immunol. Methods* 337 (1), 55–62.
- Sandle, T., 2014. Variability and Test Error with the LAL Assay. *American Pharmaceutical Review*.
- Schwarz, H., Gornicec, J., Neuper, T., Parigiani, M.A., Wallner, M., Duschl, A., Horejs-Hoeck, J., 2017. Biological activity of masked endotoxin. *Sci. Rep.* 7, 44750.
- Scudder, J., Ye, J.Y., 2018. Limulus amoebocyte lysate test via an open-microcavity optical biosensor. *J. Biomed. Opt.* 23 (2), 027001.
- Smulders, S., Kaiser, J.-P., Zuin, S., Van Landuyt, K.L., Golanski, L., Vanoirbeek, J., Wick, P., Hoet, P.H., 2012. Contamination of nanoparticles by endotoxin: evaluation of different test methods. *Part. Fibre Toxicol.* 9 (1), 41–41.
- Su, W., Ding, X., 2015. Methods of endotoxin detection. *J. Lab. Autom.* 20 (4), 354–364.
- Su, W., Lin, M., Lee, H., Cho, M., Choe, W.S., Lee, Y., 2012. Determination of endotoxin through an aptamer-based impedance biosensor. *Biosens. Bioelectron.* 32 (1), 32–36.
- Taylor, N., 2011. The FDA and USP Have Expanded Their Collaboration to Ensure the Quality of Medicines by Modernising Tests and Standards. (Accessed 10 May 2011).
- Thomas, F., 2019. Putting endotoxins to the test. *Pharmaceut. Technol.* 43 (7), 38–42.
- van Duuren-Stuurman, B., Grollers-Mulderij, M., van de Runstraat, A., Duisterwinkel, A., Terwoert, J., Spaan, S., 2018. Identification of sources of endotoxin exposure as input for effective exposure control strategies. *Ann Work Expo Health* 62 (2), 157–166.
- Venter, P., Abraham, M., Lues, J.F., Ivanov, I., 2006. The influence of sanitizers on the lipopolysaccharide composition of *Escherichia coli* O111. *Int. J. Food Microbiol.* 111 (3), 221–227.
- Wang, Y., Zhang, D., Liu, W., Zhang, X., Yu, S., Liu, T., Zhang, W., Zhu, W., Wang, J., 2014. Facile colorimetric method for simple and rapid detection of endotoxin based on counterion-mediated gold nanorods aggregation. *Biosens. Bioelectron.* 55, 242–248.
- Ward, P.A., Fattahi, F., 2019. New strategies for treatment of infectious sepsis. *J. Leukoc. Biol.* 106 (1), 187–192.
- Yeo, T.Y., Choi, J.S., Lee, B.K., Kim, B.S., Yoon, H.I., Lee, H.Y., Cho, Y.W., 2011. Electrochemical endotoxin sensors based on TLR4/MD-2 complexes immobilized on gold electrodes. *Biosens. Bioelectron.* 28 (1), 139–145.
- Zhang, B., Dallo, S., Peterson, R., Hussain, S., Weita, T., Ye, J.Y., 2011. Detection of anthrax lef with DNA-based photonic crystal sensors. *J. Biomed. Opt.* 16 (12), 127006.
- Zhang, B., Tamez-Vela, J., Solis, S., Bustamante, B., Peterson, R., Rahman, S., Morales, A., Tang, L., Ye, J.Y., 2013. Detection of Myoglobin with open-cavity and label-free photonic crystal biosensor. *Journal of Medical Engineering* 808056.
- Zhang, B., Morales, A.W., Peterson, R., Tang, L., Ye, J.Y., 2014. Label-free detection of cardiac troponin I with a photonic crystal biosensor. *Biosens. Bioelectron.* 58, 107–113.
- Zhang, B., Wang, B., Morales, A.W., Scudder, J., Bhattacharyya, M.K., Ye, J.Y., 2016. Study of the interactions of *Fusarium virguliforme* toxin FvTox1 with synthetic

peptides by molecular simulations and a label-free biosensor. *Anal. Chem.* 88 (6), 3024–3030.