NanoComposix's Guide to Endotoxin Measurement and Analysis

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Note to the Reader: *We at nanoComposix have published this document for public use in order to educate and encourage best practices within the nanomaterials community. The content is based on our experience with the topics addressed herein, and is accurate to the best of our knowledge. We eagerly welcome any feedback the reader may have so that we can improve the content in future versions.*

Please contact us at info@nanocomposix.com or 858-565-4227 with any questions or suggestions.

Introduction

Endotoxin is a toxin that is maintained within the cell walls of gram-negative bacteria and is only introduced into the environment after destruction of the bacterial cell wall. The term is synonymously used to denote lipopolysaccharides, which as the name suggests, consists of a sugar chain connected to a lipid moiety (Figure 1). Lipopolysaccharides are a major constituent of the outer cell wall of gram negative bacteria. Upon introduction to the bloodstream, endotoxins interact with specific immune cell receptors, disrupting function. Bacterial endotoxins are also known to be pyrogens (agents that induce fever). For these reasons, endotoxin concentration is of great interest to biological researches, pharmaceutical scientists, toxicologists and anyone interested in the properties of materials *in vitro*.

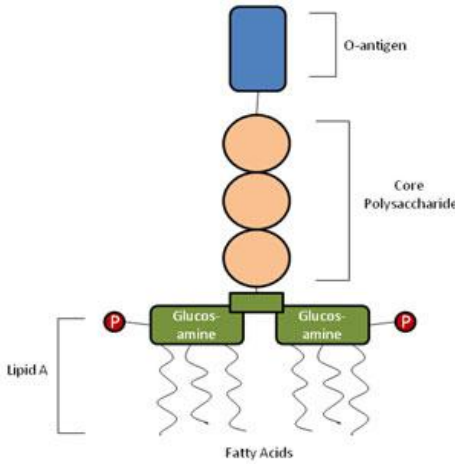

Figure 1: Example of an endotoxin (Lipopolysaccharide) structure

Blood from the Horseshoe Crab (Limulus Polyphemus) contains proteins that coagulate upon exposure to endotoxins (Figure 2). This property is utilized by biological and pharmaceutical researchers in a Limulus Amebocyte Lysate test (LAL test) to determine endotoxin levels in aqueous samples. Endotoxin levels are measured in EU/mL where "EU" stands for Endotoxin Units. One EU is equivalent to approximately 100 pg of E. Coli lipopolysaccharide—the amount present in about 10^5 bacteria. Results are expressed in EU/mL rather than a mass metric since the potency (biological activity) of a given mass of endotoxin varies wildly. By reporting the values in EU, it allows comparison of a variety of different endotoxin sources by correlating to an optical density from the test, not a mass based metric.

Figure 2: Limulus Polyphemus

At nanoComposix, we utilize a popular variation of the LAL test called the Kinetic Turbidity Assay. The Kinetic Turbidity Assay is based upon the reaction between endotoxin and a Limulus Polyphemus Lysate. If endotoxin is present, the lysate reagent causes a clotting reaction which increases the solution optical density. The measurement is performed on an Associates of Cape Cod Pyros Kinetix® Flex 64-well spectrophotometer tube reader that monitors the increase in optical density at 660 nm as a function of time. The time that it takes to reach a threshold optical density (20 mAbs) is known as the onset time, which is correlated to an endotoxin concentration from a standard curve created by dilution from a known endotoxin standard (Figure 3).

Sample Preparation

Samples are analyzed as received in water or, if the sample is a powder, after re-dispersion in endotoxin free water. A minimum volume of 150 uL is required to perform a test; however a larger volume is preferred if multiple dilutions are required to determine the appropriate measurement dilution factor.

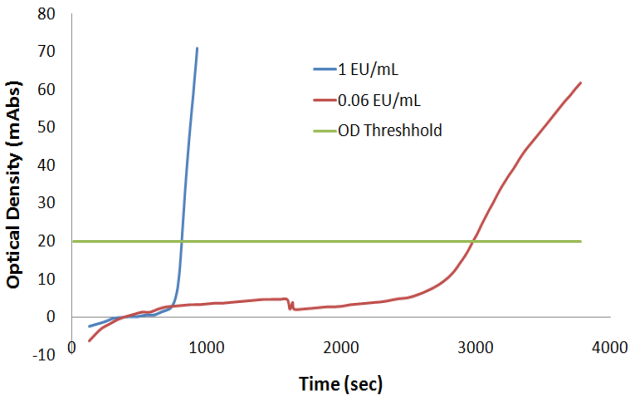

Figure 3: OD vs. Time data

Sample Analysis

At nanoComposix, we measure endotoxin levels of nanoparticle suspensions following a modified version of the National Characterization Laboratory (NCL) Method STE-1.2 that is available online.

A volume of 100 µL of sample is added to a clean, depyrogenated reaction tube. A suspension of LAL reagent is prepared, and 100 µL of this LAL reagent suspension is added to 100 µL of sample in the same tube to initiate the LAL/Endotoxin clotting cascade. The reaction tube is then vigorously vortexed for ~5 seconds and immediately inserted into the Pyros Kinetix® Flex 64-well spectrophotometer tube reader to monitor the increasing optical density that accompanies the LAL/Endotoxin reaction.

Under ideal conditions, the assay has a limit of detection of ~0.001 EU/mL, but in practice a number of factors can prevent the assay from reaching this level of sensitivity:

High Absorbance at 660nm: Many aqueous samples may have high absorbance at 660nm and this absorbance can interfere with the LAL test readout. For example, larger silver nanoparticle formulations of significant extinction at 660nm (Figure 4) and if the background OD at 660nm is relatively high (e.g. > 0.1 or 0.2), then this can reduce the dynamic range of the assay.

> *Solution for High Absorbance Issues:* For samples with high OD at 660 nm, the sample must be diluted to reduce the baseline OD.

Aggregated Nanoparticles: While any aqueous sample can be measured, colloidal dispersions can be challenging to measure due to possible onset of aggregation before or

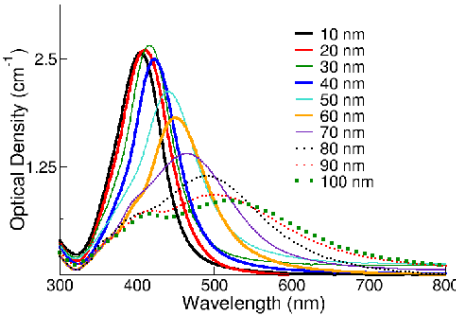

Figure 4: UV-Vis spectrum of Ag nanoparticles

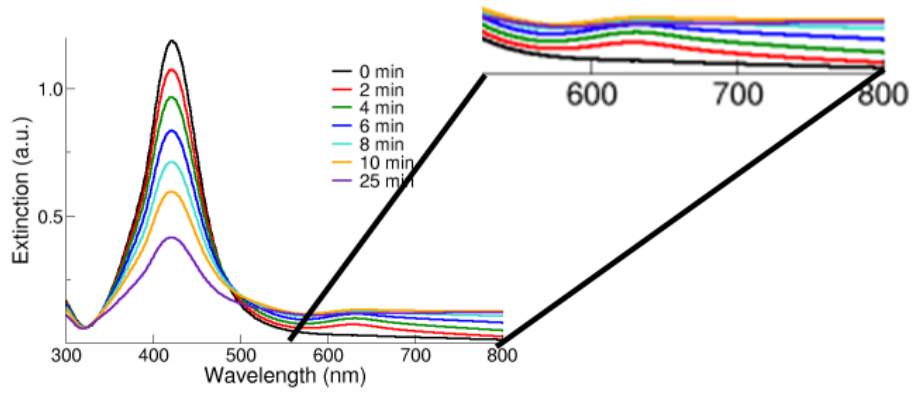

Figure 5: Agglomerating Silver Nanoparticles

during the measurement. Many nanoparticles have unusual size, shape, and agglomeration dependent optical properties that interfere with standard endotoxin tests. For example, de-stabilization (and subsequent aggregation) of silver and gold nanoparticles reduce the magnitude of the primary plasmon peak (~300-800nm depending on the particles size) and introduces a new absorbance peak between 600 and 800nm or an elevated baseline, which is in the range of the laser used for endotoxin analysis. It is important that you understand the stability of your colloidal dispersions and optical properties before, during and after potential aggregation. This knowledge will help in obtaining quality data. See Figure 5 for a time-dependant plot of aggregation of Ag nanoparticles in the presence of 0.15M NaCl.

> *Solution for Aggregation Issues:* The initial nanoparticle solution is diluted to a level where the optical absorbance of the solution at 660 nm is less than 0.05 OD (initially and during any subsequent agglomeration process). While this reduces the ultimate sensitivity of the assay to a 0.001 EU/mL dilution level, it produces reproducible and reliable results.

Strongly acid or basic samples: The pH of the sample needs to be between 6.0 and 8.0 in order for the serine protease enzymatic sequence of the LAL reaction to take place. The LAL reagent has a natural buffering capacity and for most samples this is not an issue, however if the sample is known to have a very high or low pH it should be neutralized before analysis.

> *Solution for Very Acid or Basic Samples*: If the solution pH is outside of the acceptable range, the sample should either be diluted or pH adjusted with an appropriate buffer made with LAL water.

False Positives: Certain classes of materials can induce a "result enhancement" or "false positive" reading by interacting with the LAL reagent and inducing coagulation. This is known to occur with (1->3)-B-D-glucan, trypsin and tannic acid. There are additional controls that can be run to determine if false positives are influencing your result and if you suspect that your

solution may contain anything that may induce a false positive, please make sure to identify the material on the sample submission sheet.

Solution for Detection of False Positives: If there is a suspected false positive, the sample can be re-run with an addition of a known amount of endotoxin added to the solution. If the EU/mL does not increase linearly with the amount of endotoxin, then this is a sign that there is a false positive interference. Additional control tests can be formed to measure the presence and concentration of B-D-glucans.

Interpreting Your Results

Endotoxin data from you sample(s) will be straight-forward and easy to interpret. Your results will be reported as EU/mL (endotoxin unit/mL) value which will be calculated from the standard calibration curve based on the onset time. As previously mentioned, the Pyros Kinetix® tube reader measures the amount of time it takes to for your sample to reach a threshold optical density. The EU/mL value is calculated from this onset time by calculation from a standard curve of onset times from a known EU/mL endotoxin standard (Figure 6). The concentration range of standards is from 1.0 to 0.001 EU/mL at log intervals and only standard curves that have a correlation coefficient greater than 0.980 are utilized for measurements.

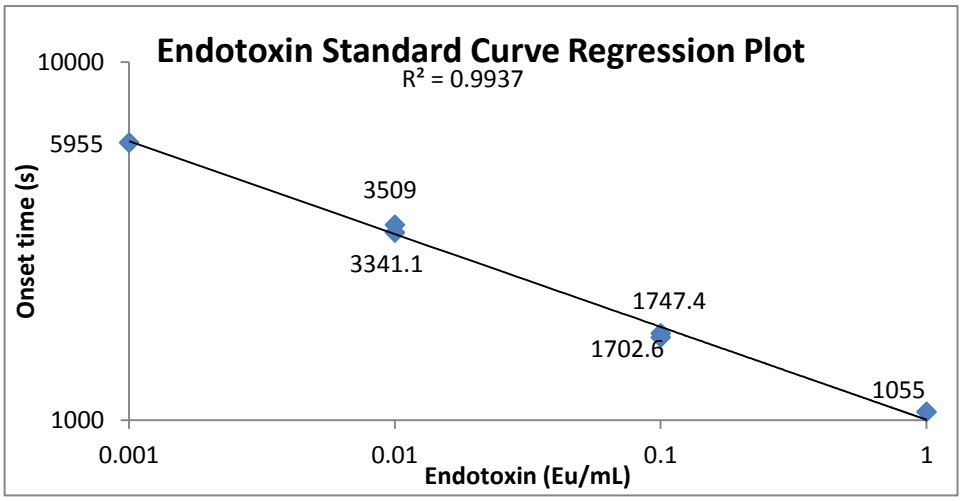

Figure 6: Endotoxin Standard Curve Regression Plot

The results will be reported in a nanoComposix generated .pdf file containing all of the pertinent information of your sample: The sample name (lot number), product description, measured endotoxin concentration, dilution factor, and dilution-corrected concentrations. See Figure 7 for an example of tabulated endotoxin data.

Lot Number	Product	Measured Concentration (EU/mL)	Dilution Factor	Dilution Corrected Endotoxin Conc. (EU/mL)
8092154	Calf Serum	0.213	1:100	21.3
8092154	Calf Serum	0.229	1:100	22.9
8092154	**average value**	**0.221**	**1:100**	**22.1**
1076961	20nm Au colloid*	1.55	1:500	775*
1076961	20nm Au colloid*	1.52	1:500	760*
1076961	**average value**	**1.535**	**1:100**	767.5

*****Measured endotoxin concentration is outside the range of standards. Result obtained by extrapolating from the standard curve

Figure 7: Tabulated Endotoxin Data