# EVALUATION OF TWO DIFFERENT METHODS FOR ENDOTOXIN DETECTION IN NANOPARTICLE SUSPENSIONS

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**Abstract**

Nowadays nanomaterials are widely used in industry, medicine, cosmetics, remediation of persistent pollutants, and therefore it is important to test their possible harmful effects on both human health and environment. However, nanoparticles can be often contaminated by bacterial endotoxins (lipopolysaccharides, pyrogens) that can cause false positive results of the (eco)toxicological tests. For this reason, nanoparticle samples should always be screened for endotoxin presence before performing any toxicological studies. Some types of nanoparticles interfere with traditional methods determining the level of endotoxin contamination. The aim of this study was to evaluate two different methods for endotoxin detection and to compare their performance. The first commonly used method, chromogenic LAL assay, is fast, and relatively cheap, but it is known that the test is susceptible to inconclusive results due to nanomaterial interactions. The second one, EndoLISA, is a new, ELISA-based assay in which nanoparticle interference is minimized due to specific binding of endotoxin onto a surface. Our results demonstrate that EndoLISA assay can be less sensitive than chromogenic LAL assay in low endotoxin concentrations, but can be used at higher endotoxin levels in which LAL test cannot be applied.

**Keywords:** Chromogenic LAL assay, EndoLISA, endotoxin, LPS, nanoparticles

## 1. INTRODUCTION

### 1.1. Endotoxins

Endotoxins, or lipopolysaccharides (LPS), are essential biologically active structural components of the cell wall of all Gram-negative bacteria [1]. Endotoxins are very potent stimulators of the immune system even at very low concentrations. Their interaction with immune cells (monocytes, macrophages and dendritic cells) results in the production of the broad range of secondary messenger molecules (cytokines, chemokines, etc.) [2]. Production of cytokines and other mediators is responsible for many pathophysiological reactions, such a fever or hypotension [3]. Exposure to high doses of endotoxins can induce strong immunostimulation leading to septic shock, tissue damage, multiple organ failure, and disseminated intravascular coagulation, all of which can potentially cause death.

Nanoparticles are increasingly being used in biology and medicine. Endotoxin contamination can cause false-positive results of toxicity screens [4], and should be, therefore, carefully assessed before performing these tests. Nanoparticles are often synthesized in the laboratories that have not been approved for production of nanomaterials for clinical use. Moreover, nanoparticles are characterized by large surface area and high reactivity that both, along with the previously mentioned fact, increase the risk of endotoxin contamination [5].

### 1.2. Detection of LPS in nanoparticle suspensions

Endotoxins can be detected by different methods: *in vivo* performed rabbit pyrogen test (RPT), *Lymulus amebocyte* lysate test (existing in three formats: gel clot, turbidimetric and chromogenic), recombinant factor C assay, monocyte activation-cytokine assay or EndoLISA endotoxin detection (**Figure 1**). LAL assay has established itself recently as one of the most widely used methods for endotoxin quantification in nanomaterials due to its convenient use and relatively low price. The LAL assay is based on clottable proteins present in the blood cells (amebocytes) of the horseshoe crab (*Limulus polyphemus*) [6]. However, it has been shown that many nanoparticles interfere with this assay [7]-[9]. Recently, EndoLISA assay has been launched on the market as a new product quantifying endotoxins. It is an ELISA-based assay promising no interference of nanoparticles with the assay due to specific binding of endotoxin onto a surface.

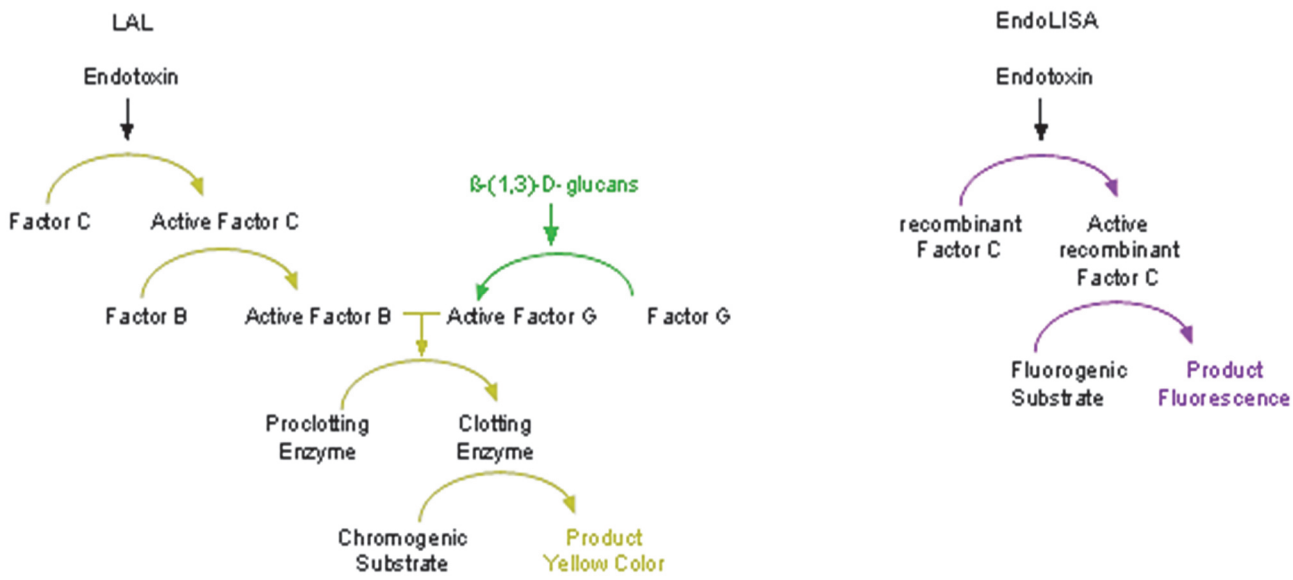


**Figure 1** Principle of LAL test (A) and EndoLISA assay (B)

The aim of this study is to compare the performance of LAL chromogenic end-point assay and EndoLISA test in nanoparticle suspensions. Specifically, we focus on the detection limits of the assays being important factors influencing the choice of the proper method for endotoxin detection.

## 2. MATERIALS AND METHODS

### 2.1. Nanoparticle synthesis and characterization

$SiO_2$ endotoxin-free nanoparticles with a hydrodynamic diameter of 100nm (CBNI NPs) were synthetized at Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin. The particle dispersion was characterized using dynamic light scattering (DLS) and differential centrifugal sedimentation (DCS).

### 2.2. Chromogenic LAL assay

Endpoint chromogenic QCL-1000W LAL assay was purchased from Lonza (Walkersville, MD, USA). The LAL assay was performed according to the manufacturer's instructions.

Calibration curves were prepared by spiking known amounts (0.1, 0.25, 0.5 and 1 EU/ml) of endotoxin provided by manufacturer into endotoxin-free water. $SiO_2$ nanoparticles were diluted to final concentration (100 µg/ml) by mixing at 1000 rpm for 30 seconds. Samples of nanoparticles spiked with *E. coli* endotoxin (0.1, 0.5, 1, 1.5 and 2.5 EU/ml) were incubated for 30 minutes without shaking. All samples were prepared in duplicates. The absorbance signal of the nanoparticle suspensions was detected by Synergy HTX (BioTek) under wave length 410 nm and once again after the nanoparticles were removed by centrifugation for 10 minutes at 4600 rpm.

Standard curve was plotted using linear regression model and the endotoxin concentration of the spiked nanoparticle suspensions was determined (**Table 1**).

### 2.3. EndoLISA

The commercial EndoLISA® test (Endpoint Fluorescent Endotoxin Detection Assay) was purchased from Hyglos (Regensburg, Germany). All solutions were prepared with endotoxin-free water provided in the kit. While performing the test we followed manufacturer's instructions.

Standard solutions were prepared by serial dilutions of *E. coli* endotoxin provided by the manufacturer to obtain following concentrations: 0 (blank), 0.05, 0.5, 5, 50, 100, and 500 EU/ml and vortexed at 1400 rpm for 20 minutes. $SiO_2$ nanoparticles were diluted to final concentration (100 µg/ml) by mixing (1000 rpm) for 30 seconds. Samples of nanoparticles spiked with *E. coli* endotoxin (0.1, 0.5, 1, 1.5, 2.5, 5, 25, 50 and 250 EU/ml) were incubated for 30 minutes without shaking. Afterwards, nanoparticle samples and standard dilutions (100 µl) were added into the wells of the microplate. 20 µl of Binding Buffer was added into all wells. The wells were sealed with cover foil and incubated at 37 °C for 90 minutes with continuous mixing at 450 rpm.

After incubation, the liquid was poured out by inverting the plate and dashing the liquid into a basin and 150 µl of Wash Buffer was added into each well using a repetitive pipette. This was repeated twice. Then the Assay Reagent (100 µl) was added to each well and the fluorescence signal was detected by Synergy HTX (BioTek) at 37 °C under excitation (400/30) and emission (460/40) filters at time zero and after 90 minutes.

Zero minutes values were subtracted from 90 minutes values. The standard curve was plotted using 4-parameter logistic regression model and the endotoxin concentration of the spiked nanoparticle suspensions was determined (**Table 1**).

## 3. RESULTS AND DISCUSSION

### 3.1. Nanoparticle characterization

The hydrodynamic diameter of the $SiO_2$ nanoparticles determined using DLS was found to be 100 nm while the size determined by DCS was 83 nm. This observed difference in size is always present in similar samples and can be explained by a myriad of phenomena. Two important ones being that hydrodynamic diameter includes the adsorbed water and salt shell on the particles and the propensity of DLS to overestimate the large particle fraction. In all cases studied, both peaks were monomodal with a PdI <0.2 (DLS).

### 3.2. Chromogenic LAL assay and EndoLISA results comparison

First important step was to plot standard curve from known endotoxin concentrations. The coefficients of correlation (r values) for standard curves were 0.991 (**Figure 2a**) for LAL assay and 0.993 for EndoLISA (**Figure 2b**).

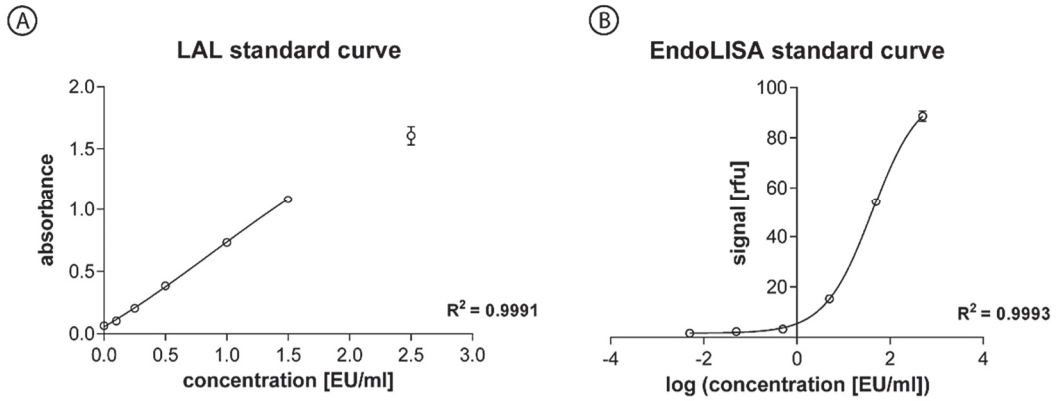


**Figure 2** A) LAL calibration curve (linear model);
B) EndoLISA standard (calibration) curve (4-Parameter Logistic Regression Model)

The results of the spiking experiments of the chromogenic LAL assay and EndoLISA are summarized in **Table 1**. Endotoxin values of spiked samples estimated by LAL assay well corresponded to the endotoxin concentration the samples were spiked with. The highest deviation of the spike endotoxin concentration was 16.6 % in case of 2.5 EU/ml, however value 2.5 EU/ml exceeds the sensitivity range of this assay (0.1 - 1 EU/ml). We did not detect any interference (inhibition or enhancement) of the $SiO_2$ nanoparticles with the assay. This commonly reported phenomenon can invalidate results of the LAL assay [5], [8]-[10] because it causes overestimation or underestimation of the endotoxin concentration in the sample and represents the main reason why alternative methods for endotoxin detection are needed.

**Table 1** Comparison of results obtained with LAL assay and EndoLISA

| | LPS detection method | | | | | |
|---|---|---|---|---|---|---|
| | **LAL chromogenic assay** | | | **EndoLISA** | | |
| Spike concentration [EU/ml] | Detected endotoxin concentration [EU/ml] | Standard. deviation | Deviation of the spike endotoxin concentration (%) | Detected endotoxin concentration [EU/ml] | Standard deviation | Deviation of the spike endotoxin concentration (%) |
| **0** | **0.031** | 0.0010 | - | **0.058** | 0.0000 | - |
| **0.1** | **0.089** | 0.0010 | **10.9** | **0.289** | 0.0000 | **-189.1** |
| **0.5** | **0.560** | 0.0123 | **-6.9** | **0.379** | 0.1564 | **24.1** |
| **1** | **1.050** | 0.0327 | **-5.0** | **0.659** | 0.1720 | **34.1** |
| **1.5** | **1.523** | 0.0205 | **-1.5** | **1.178** | 0.0000 | **21.4** |
| **2.5** | **2.084** | 0.0082 | **16.6** | **2.258** | 0.0000 | **9.7** |
| **5** | - | - | - | **5.245** | 0.2923 | **-4.9** |
| **50** | - | - | - | **49.229** | 3.5723 | **1.5** |
| **250** | - | - | - | **184.986** | 19.2943 | **26.0** |

EndoLISA was not as accurate as LAL assay at low endotoxin concentrations although the sensitivity of the assay should be higher (from 0.05 EU/ml to 500 EU/ml) compared to chromogenic LAL assay. The accuracy of the endotoxin concentration estimation improved at higher endotoxin concentrations with the exception of the sample spiked with 250 EU/ml in which deviation of the spike endotoxin concentration was 26%. EndoLISA

is, hence, an appropriate tool when higher endotoxin concentrations are expected or in the case when the interference of nanoparticles with LAL assays is observed, but when none of these is true, LAL assay represents more affordable and convenient way to detect endotoxins in nanoparticle suspensions. However, EndoLISA is a relatively new assay that will be probably thoroughly studied in the future and its accuracy can be optimized for determining low endotoxin levels.

## 4. CONCLUSION

The performance of LAL chromogenic assay and EndoLISA for endotoxin quantification in nanoparticle suspension was compared. We detected no interference of $SiO_2$ nanoparticles with LAL assay. Chromogenic LAL assay was shown to be more accurate at low endotoxin concentrations (up to 1.5 EU/ml) but EndoLISA was suitable to reliably quantify higher endotoxins levels than LAL assay. Further studies are needed to determine the limits of both assays for endotoxin quantification in nanoparticle suspensions.

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