

Study of Low Endotoxin Recovery and Its Reversal Strategies

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BRIEF. Low Endotoxin Recovery, a false negative in endotoxin testing, was studied comparatively and for viable solutions.

ABSTRACT. Endotoxins are fragments of bacterial cell walls, lipopolysaccharides (LPS), that can cause dangerous immune reactions if they enter the bloodstream via a parenteral drug or medical device, yet routine laboratory tests can miss them when certain common additives are present. The primary industry method for quantitative detection of endotoxins is the Limulus amoebocyte lysate (LAL) assay. Most recently, within the last fifteen years, it has been discovered that chelant-surfactant-containing formulations exhibit a time- and temperature-dependent “masking” effect on endotoxins named Low endotoxin recovery (LER), essentially a false negative, that could potentially endanger patients. Though it has been widely studied, without some controversy, the phenomenon has yet to be assigned an established mechanism. This study compared two widely used chelators (citrate and EDTA) and evaluated two ways to reverse LER: commercial dispersant and treatments with extreme pH. Evidence showed that both the kinetics and strength of masking are likely closely tied to the strength of the chelator used. The dispersant did not restore endotoxin activity under the tested conditions. Acidic pH shock was observed to marginally increase endotoxin recovery in citrate-containing formulations, while basic pH shock returned complete endotoxin activity with those same formulations. The results of this study support and provide insight into the most current theory on LER: chelators remove divalent cations, weakening LPS aggregates, after which surfactants displace surface-level endotoxin molecules, effectively decreasing endotoxin surface area and activity.

INTRODUCTION.

Endotoxins, or lipopolysaccharides (LPS), are structural components of the outer membrane of Gram-negative bacteria. They are of high concern in parenteral drugs and medical devices because trace amounts of the contaminant can result in significant immune response, and in extreme cases, potentially deadly, anaphylactic shock [1]. For this reason, endotoxins are routinely scanned for in the pharmaceutical industry. Currently, the main methods for doing so rely on Limulus amoebocyte lysate (LAL) or recombinant Factor C (rFC) assays. These methods detect LPS via the Factor C-mediated cascade reaction. Unlike earlier methods, such as rabbit pyrogen testing, these assays can quantify endotoxin concentrations (endotoxins units per milliliter, EU/mL) using control standard endotoxin (CSE) and either the endpoint or kinetic readouts [2]. Formally introduced to the US Pharmacopeia in 1980 (USP <85>), Factor-C-based tests are valuable and widely used due to their sensitivity and relatively quick results, though they are not immune to sample-specific interference [2].

In the last decade, low endotoxin recovery (LER) became a critical issue for the industry to resolve. It was noticed that there is a time-dependent loss of measurable endotoxin reactivity in certain formulations containing a combination of a nonionic surfactant and a chelating agent (most studies use polysorbate and citrate, respectively). The issue was first reported at a 2013 conference by Chen and Vinther [3], causing a significant stir and spurring deeper discussion of the issue in technical reviews and regulatory forums. LER is defined as a drop below the 50% recovery threshold used for quality control during a hold-

time study of spiked (endotoxin addition) sample solutions [3]. Subsequent studies have shown that undetectable (masked) endotoxin in these cases can still retain its pyrogenicity, meaning that the false negatives caused by LER could potentially endanger patients [4]. For this reason, regulatory bodies and the pharmaceutical industry are working to fully understand its mechanism and put in place good practices to contain the problem.

Although the mechanism behind LER isn't fully known, it appears to be a multivariable phenomenon. The chelation of divalent cations by agents such as citrate or EDTA can destabilize supramolecular LPS structures. Previous theories suggested that the surfactant, such as polysorbate or Triton X, later monomerizes the aggregates, since it was known that LPS monomers do not react with Factor C. However, findings have shown that particle size doesn't change after LER occurs, setting up the new theory that the surfactant alters LPS surface presentation and accessibility to Factor C, reducing its reactivity without destroying biological activity [5]. Other factors that affect masking kinetics include chelator identity and concentration, surfactant type, dilution, ionic strength, pH, and temperature [6]. There are some ways of combating LER, including the use of dispersants (e.g. Pyrospense®) or other sample-modifying reagents, changes in pH, and dilution that may prevent or expose masked LPS; these methods' success is largely formulation-dependent and not universal [7].

This study compares the kinetics and reversibility of masking produced by two chelators, commonly used in medical formulations—sodium citrate and disodium EDTA—in the presence of polysorbate. In addition, it evaluates two potential mitigation strategies: a commercial dispersing agent (Pyrospense®) and pH-based treatments (acidic or basic shock). We hypothesize that citrate and EDTA will differ in their LER kinetics and in their responsiveness to Pyrospense® and pH treatments.

MATERIALS AND METHODS.

Chemicals and materials.

Disodium EDTA salt was obtained from ALDOSA. Polysorbate 80 (cosmetic-grade) from commercial distributor Vidumshiki. Sodium citrate was obtained from Shandong Ensign Industry Co. Kinetic chromogenic tachypleus amoebocyte lysate (TAL) endotoxin assay test kit obtained from Xiamen Bioendo; it is an equivalent bacterial endotoxin test (BET) to Kinetic-QCL from Lonza and other LAL-based kits. Endotoxin-free, depyrogenated water; depyrogenated ALPYR test tubes; 1.0 M HCl for BET; and 0.1 M NaOH for BET were obtained from Algimed Techno LLC. Pyrospense® was obtained from Lonza. All reagents were checked for background endotoxin; their levels were negligible relative to endotoxin spikes administered.

Sample preparation.

Methods were adapted from Burgmaier et al. [8]. All samples were prepared to 1.0 mL and incubated at $22 \pm 1^\circ\text{C}$. Unless stated otherwise, working formulations contained 10 mM chelant (≈ 0.0026 g/mL sodium citrate or ≈ 0.0037 g/mL disodium EDTA) and 0.05% (w/v) polysorbate 80 (≈ 47.3 μL per mL). Samples were spiked with endotoxin to a target of 25 EU/mL using 50 EU/mL CSE from *E. coli* O111:B4. After each manipulation (spiking, pH change, dilution) tubes were vortexed ~ 30 s.

LER induction and reversal strategies.

Two methods were used to obtain LER. The first being the short-time (chronological) method [8]. Immediately after spiking, 10 μ L aliquots were removed at predetermined time points (e.g., 0, 10, 30, 60 min), each aliquot was then diluted 1:100 in endotoxin-free water (dilution prevents further endotoxin masking) and stored until assay. The second is the long-time method [8]. Unspiked sample aliquots were incubated for specified durations—time of spiked incubation subtracted from total time (e.g. highest incubation time sample is spiked right away while control is spiked right before assay)—then, each aliquot was spiked with an equal volume of 50 EU/mL CSE, and all spiked aliquots were diluted 1:100 prior to assay. The two approaches account for time-dependent masking and for potential dilution/timing errors.

Pyrospense® was used at 1:200 (v/v) and mixed per manufacturer instructions. In tests for LER reversal, Pyrospense® was added to already masked samples and incubated briefly; for LER prevention evaluations it was added at time 0. For pH shock, masked sample aliquots were treated with acid or base (addition of 100 μ L of 0.1 M HCl or 0.1 M NaOH to 10 μ L aliquots), incubated, then neutralized (addition of 0.1 M NaOH or 0.1 M HCl as needed) and diluted to 1:100 before assay. For pH incubation experiments, 10 μ L aliquots were diluted 1:10 into acid or base, incubated ~1.5 h, neutralized, and diluted to assay conditions before testing (See supplemental Fig. S1 sample preparation chart and supplemental Table S2 with detailed sample information).

Measurement of endotoxin activity.

Endotoxin activity was measured by kinetic chromogenic LAL according to the kit manual. Standards on every plate had a sensitivity of 0.005–5.0 EU/mL; samples and standards were assayed in duplicate. Each sample also had duplicate positive product controls (PPC, 10 μ L of 5 EU/mL into wells). The microplate reader (BioTek ELx808IU) read absorbance at 405 nm and time-to-threshold converted to EU/mL using Toxmaster QC8 software (log-log interpolation). Standard curve acceptance $|r| \geq 0.980$ and PPC recovery acceptance 50–200%. Data reported as mean $\pm$ SD ($n = 2$).

RESULTS.

Producing LER and comparison between Citrate and EDTA-induced LER.

LER was produced experimentally by adding polysorbate 80 and a chelator: citrate (Fig. 1). The dashed line shows the LER threshold of 50% from initial activity, with values beneath the line signifying LER. LER occurred within ten minutes. Further 45-minute kinetics show decreasing endotoxin activity. Prolongated incubation (>1 h) of endotoxin with citrate or EDTA (disodium salt)—of which citrate is the stronger chelator—in the presence of polysorbate 80 results in LER in both samples under conditions employed (supplemental Table S3). The short-term endotoxin activity (<1 h) in the presence of same chelators and surfactant is shown in Fig. 2. After a 10-min incubation, the citrate-treated samples' endotoxin activity declined to 42.4% of the initial concentration, achieving LER, whereas EDTA-treated samples did not show evidence of masking within the 45-minute time frame ($>50\%$ recovery of initial level) (Fig. 2). These findings show that chelator strength may contribute to masking kinetics. Being able to form stronger ionic complexes with divalent cations is likely important for quicker LER kinetics.

Examination of Pyrospense® effectiveness to reverse or prevent chelator-surfactant-induced LER.

Pyrospense® is a metallo-modified polyelectrolyte that is advertised as a dispersing or sample-modifying agent for certain formulations exhibiting LAL inhibition. The reagent was evaluated for reversal (Fig.

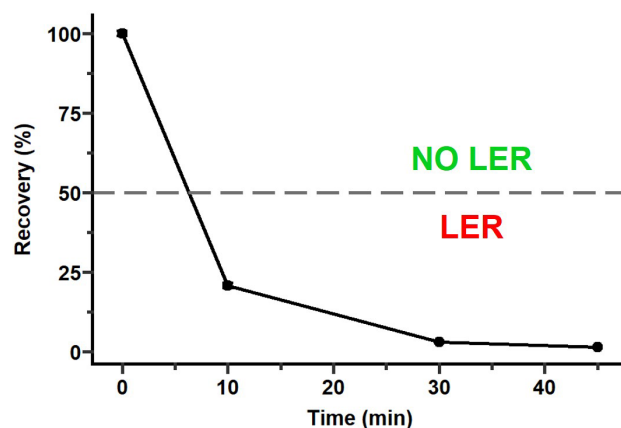


Figure 1. LER in a citrate-polysorbate-containing formulation.

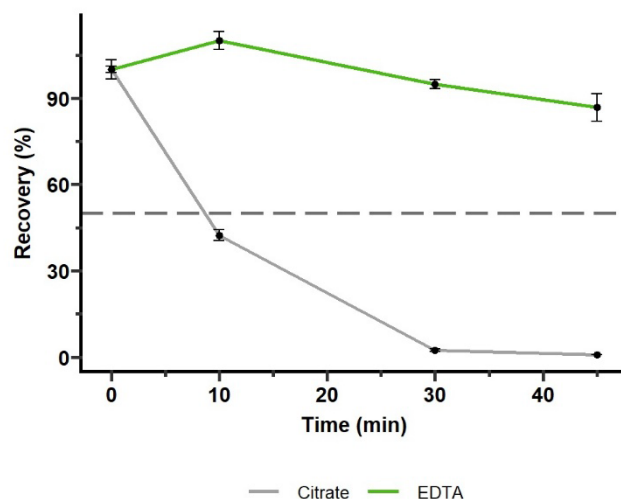


Figure 2. Long-term endotoxin recovery comparison between 10 mM citrate- and 10 mM EDTA-containing polysorbate 80 solutions (0.05% w/v). Starting points were set to 100% and data was normalized for ease of comparison.

3, Panel A) and prevention (Fig. 3, Panel B) of chelant-surfactant-induced LER using the manufacturer's recommendations. Under experimental conditions tested, it can be noticed that initial endotoxin activity levels were greater in solutions with Pyrospense®, suggesting interaction with endotoxin similar to a solubilizing effect (green squared area, Fig. 3, Panel B), yet no decrease in the masking effect was observed (red squared area, Fig. 3, Panels A and B).

Analysis of extreme pH solutions and LER disruption.

This study also explored how pH influences the phenomenon of LER. There are both “controls” and “treatments.” The former are solutions where the matrices are diluted at time 0 to stop endotoxin masking, whether neutral, acidic, or basic. They are used for comparing the effectiveness of “treatments” or pH shocks—the addition of extreme alkaline or acidic solution to a pre-incubated solution (having presumably undergone LER). One observation was the slightly higher recovery of endotoxin reactivity with acidic treatment in citrate-containing solutions than in EDTA-containing solutions (Fig. 4) mirrored in the following assay (Fig. 5).

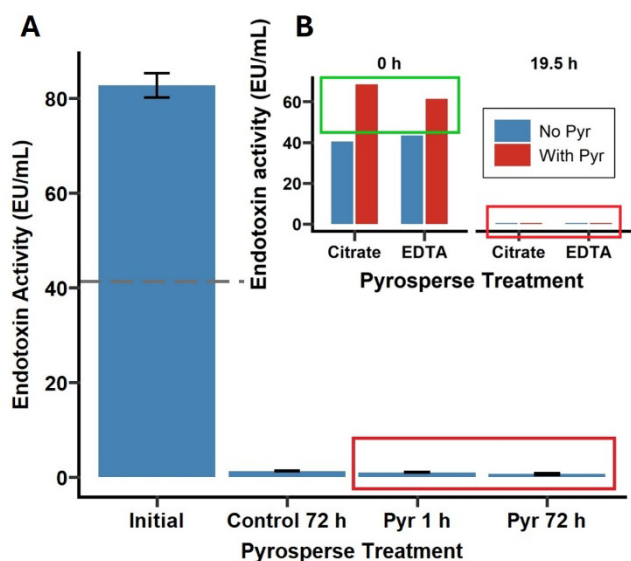


Figure 3. Panel A: Kinetics of LER in the presence of Pyrosperser® in citrate-polysorbate 80 solution. Panel B: Increased endotoxin activity with Pyrosperser® at initial timepoint.

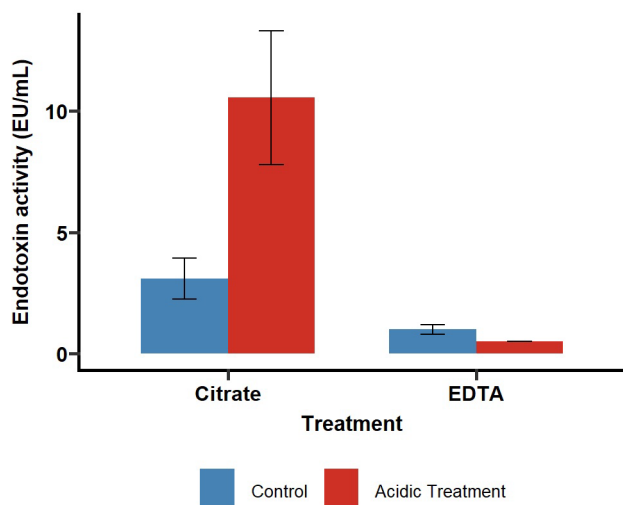


Figure 4. Evaluation of acidic shock in reversing masking. Controls are solutions with LER induced by a 24 h incubation; Acidic Treatment samples were incubated for 23 h to obtain LER, followed by 1 h incubation in 0.1 M HCl. Treatment solutions were neutralized with equimolar amounts of NaOH before measurement.

The most significant result is LER reversal with a basic pH treatment, returning endotoxin activity to around 75 % or between the 50–200% LER threshold (Fig. 5). Most unexpected was the significant drop in recovery in the basic control having EDTA as the chelator to about 24%, meaning LER (Fig. 5).

DISCUSSION.

Chelation Strength and Masking Kinetics.

Comparing citrate and EDTA formulations shows that chelator strength is an important factor of LER kinetics. Citrate-containing solutions showed a rapid sharp decline in recoverable endotoxin activity, while EDTA-containing solutions remained >50% for at least 45 min (Fig. 2). This contrasts with Reich et al. [9], who reported faster kinetics with EDTA than citrate, though their study used 5 mM EDTA of

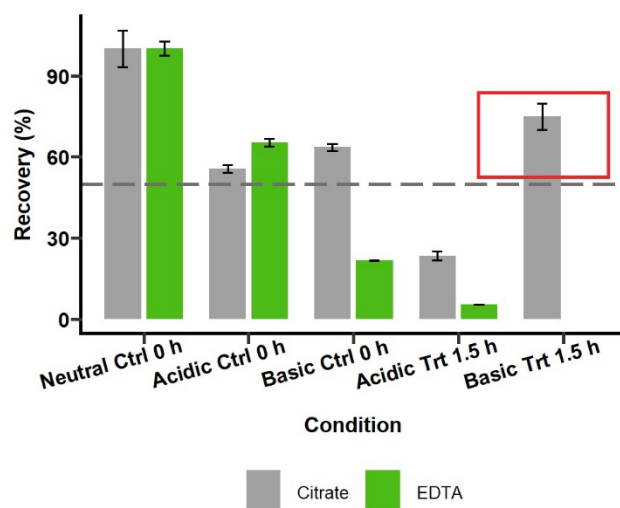


Figure 5. Acidic and basic shock treatment for endotoxin masking reversal. Controls are samples in neutral, acidic (pH~1), and basic (pH~13) media (1:10 diluted to prevent LER) at time 0. Treatment samples were preincubated in neutral conditions for ~1.5 h to achieve LER followed by prompt acid/base (Acidic Trt, Citrate and EDTA) or base/acid (Basic Trt, Citrate only) treatment, respectively. The dashed line is the 50% LER recovery threshold. The red box marks LER reversal.

unspecified salt form, compared to 10 mM disodium EDTA. The difference may be caused by both concentration and speciation, since EDTA is a hexadentate ligand capable of forming highly stable complexes when fully deprotonated, while at neutral pH disodium EDTA exists largely as H_2Y^{2-} or HY^{3-} , reducing its effective chelation strength. Removing stabilizing Ca^{2+} and Mg^{2+} ions from LPS aggregates due to EDTA chelation aids surfactant displacement of endotoxin molecules from micelle surfaces. These findings support the hypothesis that chelator identity and ionic strength are primary drivers of endotoxin masking.

Pyrosperser® Evaluation.

The effect of Pyrosperser® addition was investigated both as a LER prevention and LER reversal strategy. Endotoxin activity levels immediately upon mixing were higher in Pyrosperser-containing solutions (Fig. 3, Panel B), consistent with a solubilizing or stabilizing effect on LPS aggregates. However, the reagent did not prevent the loss of activity over time or recover masked endotoxin following LER (Fig. 3, Panels A, B). Pyrosperser® acts on the endotoxin to increase surface accessibility but may not be able to overcome the chelator-surfactant masking. Since the samples didn't exceed 50% recovery under the tested experimental conditions, it shows that dispersants may not universally combat LER, although they can stabilize endotoxin in formulations. These observations reinforce the importance of studies of the interactions between dispersants and LPS. The observed effect described here may depend upon surfactant type, chelator concentration, ionic composition of the formulation, and other factors.

pH Effects on LER and Mechanistic Implications.

Extreme pH treatments yielded several significant results and insights. Acidic shock (0.1 M HCl, neutralized post-treatment) slightly improved recovery in citrate-containing solutions but failed to overcome masking in EDTA formulations (Figs. 4–5). This implies that acid can't fully disrupt masking interactions in tested conditions. Basic shock (0.1 M NaOH, neutralized post-treatment), on the other hand, restored endotoxin activity to ~75% in citrate-containing formulations exceeding the 50% LER threshold, exhibiting complete reversal (Fig.

5). Among possible explanations there is base-catalyzed hydrolysis of polysorbate 80, which would decrease surfactant displacement of endotoxin and permit reaggregation of LPS micelles. A recent study on LER in blood plasma also found alkaline pH to partially recover masked endotoxin, suggesting electrostatic interference as the mechanism behind the process [10]. Endotoxin itself seems resistant to brief exposure to extreme alkaline conditions, further supporting the viability of this strategy.

EDTA-containing solutions incubated in strong base exhibited severe masking, with recovery dropping to ~24% (Fig. 5, EDTA Basic Ctrl at 0 h). At pH ~13, EDTA exists mostly in its fully deprotonated Y^{4-} form, which has orders of magnitude higher affinity for divalent cations than its protonated species. This increased chelation likely destabilizes LPS micelles more completely, potentially extracting cations from inner layers of aggregates and producing non-reactive monomers. Because the solution was diluted 1:10 intending to prevent LER and partial polysorbate hydrolysis likely took place, surfactant involvement is minimal, suggesting that chelation alone can drive masking under alkaline conditions. This finding indicates that reversal strategies like basic treatment are often chelator-specific.

Model of LER mechanism.

Altogether, this data supports a refined model of LER presented by Tsuchiya [5] (Fig. 6). Chelators destabilize LPS aggregates by extracting stabilizing cations, followed by surfactants displacing surface endotoxin molecules and thereby reducing the accessibility of Factor C. Masking kinetics were influenced by chelator strength, while environmental factors such as pH alter the chemical state of chelator and surfactant drugs. Citrate-driven LER occurs rapidly but is readily reversible under alkaline conditions, while EDTA-driven LER occurs more slowly at neutral pH but is exacerbated under basic conditions, reflecting the high chelation capacity of the Y^{4-} species. These mechanistic distinctions highlight the complexity of LER and the need for tailored mitigation strategies.

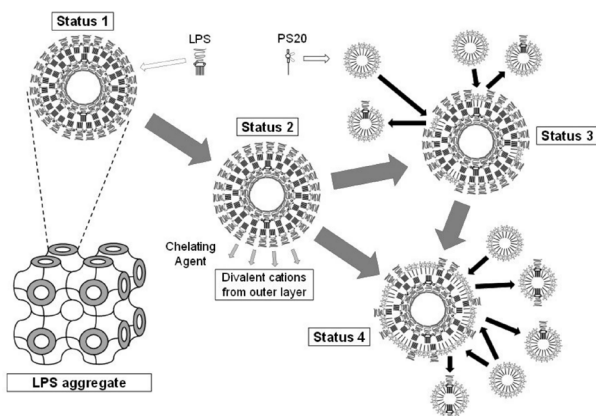


Figure 6. Currently proposed mechanism for chelant-surfactant-induced LER. Reproduced from ref 5. Available under a CC-BY 4.0 license, copyright 2019 Tsuchiya M.

Limitations and Further Exploration.

This study was necessarily limited by small sample size and a single surfactant, polysorbate 80. Additional experiments with other medically relevant surfactants, including Triton X-100 and polysorbate 20, would clarify whether the pH reversal observed here is generally applicable. Further investigation into the hydrolysis of surfactants under

alkaline conditions could validate base treatments as a practical corrective strategy. Expanding to chelants of varying strength and concentration would also help delineate the threshold at which chelation alone destabilizes LPS micelles. Ultimately, understanding chelator-specific masking pathways is critical for developing solutions to LER and determining its mechanism for pharmaceutical quality control.

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SUPPORTING INFORMATION.

Figure S1 – sample prep flow chart; Table S2 – detailed sample information; Table S3 – long-term LER kinetics between citrate and EDTA

REFERENCES.

1. C. Galanos, M. A. Freudenberg, Bacterial endotoxins: biological properties and mechanisms of action. *Mediators Inflamm.* **2**, S11–6 (1993).
2. United States Pharmacopeial Convention. USP–NF, <85> Bacterial Endotoxins Test. Rockville, MD; 2012.
3. J. Chen, A. Vinther. Low endotoxin recovery in common biologics products. Presented at the PDA Annual Meeting, Orlando, FL, 15 to 17 October 2013.
4. H. Schwartz, J. Gornicec, T. Neuper, M. A. Parigiani, M. Wallner, A. Duschl, J. Horejs-Hoeck, Biological activity of masked endotoxin. *Sci. Rep.* **7**, 44750 (2017).
5. M. Tsuchiya, Mechanism of low endotoxin recovery caused by a solution containing a chelating agent and a detergent. *Immunome Research.* **15**, 166 (2019).
6. R. Bu, X. Deng, Y. Cui, J. Jin, B. Mai, K. Meng, X. Liu, J. Chi, Y. Zhang, F. Qiu, Effect of different sample treatment methods on Low Endotoxin Recovery Phenomenon. *J. Microbiological Methods.* **186**, 106241 (2021).
7. M. Tsuchiya, Sample treatments that solve Low Endotoxin Recovery issues. *PDA J. Pharm. Sci. Tech.* **73**, 433–442 (2019).
8. L. Burgmaier, S. Pöhl, M. Avci-Adali, J. Reich, The impact of LPS mutants on endotoxin masking in different detection systems. *Biologicals* **89**, 101808 (2025).
9. J. Reich, P. Lang, H. Gallbert, H. Motschmann, Masking of endotoxin in surfactant samples: Effects on Limulus-based detection systems. *Biologicals* **44**, 417–422 (2016).
10. L. Burgmaier, J. Lifka, M. Avci-Adali, J. Reich, Endotoxin masking in human plasma: The role of protein interactions and lipopolysaccharide structure. *Eur. J. Pharm. Biopharm.* **214**, 114786 (2025).



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