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# Masking of endotoxin in surfactant samples: Effects on *Limulus*-based detection systems

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ABSTRACT

Over the last few decades *Limulus* Amebocyte Lysate (LAL) has been the most sensitive method for the detection of endotoxins (Lipopolysaccharides) and is well accepted in a broad field of applications. Recently, Low Endotoxin Recovery (LER) in biopharmaceutical drug products has been noticed, whereby the detection of potential endotoxin contaminations is not ensured. Notably, most of these drug products contain surfactants, which can have crucial effects on the detectability of endotoxin. In order to analyze the driving forces of LER, endotoxin detection in samples containing nonionic surfactants in various buffer systems was investigated. The results show that the process of LER is kinetically controlled and temperature-dependent. Furthermore, only the simultaneous presence of nonionic surfactants and components capable of forming metal complexes resulted in LER. In addition, capacity experiments show that even hazardous amounts of endotoxin can remain undetectable within such formulation compositions. In conclusion, the LER phenomenon is caused by endotoxin masking and not by test interference. In this process, the supramolecular structure of endotoxin is altered and exhibits only a limited susceptibility in binding to the Factor C of *Limulus*-based detection systems. We propose a two-step mechanism of endotoxin masking by complex forming agents and nonionic surfactants.



## 1. Introduction

Endotoxins are products of Gram-negative bacteria, released during bacterial cell division, lysis and at cell death. Chemically, endotoxins are lipopolysaccharides (LPS). The general structure of LPS comprises three basic units (Fig. 1), O-antigen, Core and Lipid A, wherein the latter is the highly toxic part [1]. Within the mammalian blood circulation, endotoxin can trigger severe physiological reactions (e.g. fever, septic shock) [2,3]. Thus, bacterial endotoxin testing of drug products for parenteral administration is mandatory. However, several applications have shown that detection of endotoxin using widespread *Limulus* Amebocyte Lysate (LAL) based methods are not always feasible in complex protein samples containing endotoxin [4,5].

One reason for inadequate detection of endotoxin is interference of sample constituents with the enzymatic reaction of the *Limulus*-based detection system. In this case, certain components (e.g. heavy metals, protease inhibitors) can directly disturb enzyme activation of the detection system, which is called test interference [6]. This phenomenon is well known and to indicate test interference, positive product controls are performed. To this end, a known amount of endotoxin is added to the sample and immediately measured. A test is considered valid if the spiked endotoxin is recovered in a range of 50–200%. If the validity criterion is not fulfilled, it is recommended to overcome interference by suitable sample treatments such as dilution, filtration, neutralization, dialysis or heating etc. [7]. Another potential reason for inadequate endotoxin detection is the interaction of endotoxin itself with matrix components of the sample. For instance, it has been reported that endotoxin can interact with blood components [8], proteins [4] or amphiphilic molecules [9,10], resulting in a significant change of endotoxin activity. Notably, approaches which eliminate test interference problems are not effective in overcoming such effects [4]. In the 1990's Greaves and coworkers [11] already differentiated between dilution dependent

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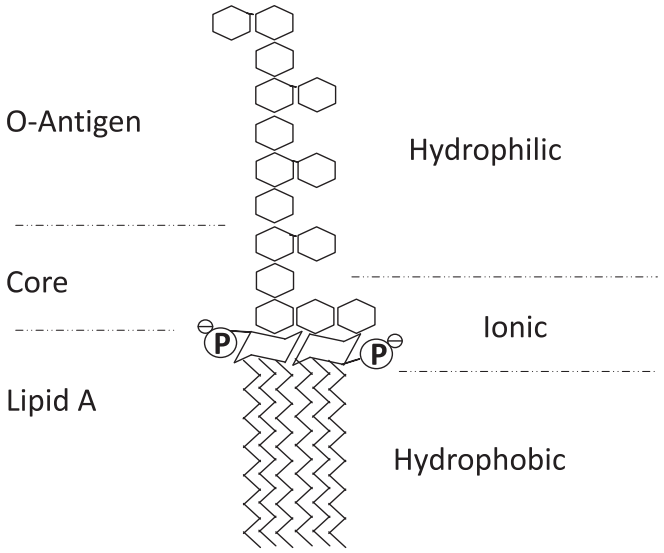


**Fig. 1.** Schematic structure of Lipopolysaccharides (LPS). LPS is an amphiphilic molecule. The fatty acids within the Lipid A are hydrophobic and the polysaccharides in the Core and O-antigen are hydrophilic. In addition, LPS is electrically charged due to substitution (e.g. phosphates) in the core region and on the diglucosamine of Lipid A. With regard to the biological nature, LPS can be divided into the three functional subunits O-antigen, Core and Lipid A. The latter is the toxic fragment of the molecule.

interference and dilution-independent interference in environmental samples. The latter phenomenon is called masking.

In the recent past, inadequate endotoxin recovery over time has been observed in biopharmaceutical drug products [12]. In such cases, the active pharmaceutical ingredients are mostly proteins [13], which are capable of intrinsic binding to endotoxin as previously described by Anspach and co-workers [4]. The inadequate detection of endotoxin might be explained by protein-endotoxin interactions. Nonetheless, therapeutic proteins are usually stabilized by excipients, like nonionic surfactants and certain buffer components [14]. Surprisingly, endotoxin spiking experiments in formulations that lack the active pharmaceutical ingredient (e.g. monoclonal antibody) resulted in endotoxin masking over time. Such observations of disturbed endotoxin determinations in biopharmaceutical products over time and the related risk of undiscovered endotoxin contamination events compelled us to study the impact of common formulation components on the detectability in *Limulus*-based detection systems. The aim of the present study is to understand the mechanism of Low Endotoxin Recovery (LER) in samples containing nonionic surfactants in combination with standard buffer systems.

## 2. Materials & methods

*E.coli* O55:B5 lipopolysaccharide (gel-filtered), polysorbate 20, polysorbate 80, octoxynol 9, citric acid, trisodium citrate, phosphoric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate were obtained from Sigma–Aldrich Chemicals, Steinheim, Germany. Depyrogenated water, depyrogenated borosilicate glass tubes and recombinant Factor C test (EndoZyme®) were obtained from Hyglos GmbH, Bernried, Germany. Kinetic chromogenic *Limulus* Amebocyte Lysate test was obtained from Lonza Inc., Walkersville, USA. Prior to the experiments, all relevant materials were tested for endotoxin content and proven to contain less than 0.005 EU/mL.

*Sample preparation:* Samples were prepared in 5 mL glass tubes with sample volumes of 1 mL per sample. Unless otherwise described, samples were spiked with 10 μL of lipopolysaccharide (LPS) out of a 10,000 EU/mL stock solution. The pH of buffer components was adjusted to 7.5, if not otherwise specified. Before adding the spikes to the sample, the stock solution was shaken at 1400 rpm for 10 min. After spiking, the samples were incubated at defined temperatures and periods of time. Immediately after incubation, the samples were mixed at 1400 rpm for 2 min again, and diluted in depyrogenated water in order to avoid test interference. Necessary dilutions of the particular sample compositions were determined prior to the actual experiment.

*Kinetics:* All samples with different incubation periods were measured on the same microtiter plate, to avoid variation from test to test. Therefore, endotoxin recovery kinetics was performed in a reverse manner. The particular sample was aliquoted and all aliquots were stored under equal conditions over time. The aliquot with the longest endotoxin incubation period was spiked first (e.g. 7 days prior to the measurement). Further aliquots with shorter incubation periods were spiked later in accordance with the respective incubation period. The zero time point aliquot was spiked immediately before measurement. To control accuracy of the spikes at different time points, equal amounts of endotoxin were spiked into depyrogenated water (data not shown).

*Detection of endotoxin:* For detection of endotoxin, a recombinant Factor C test (EndoZyme®) and a kinetic chromogenic *Limulus* Amebocyte Lysate (LAL) test were used according to manufacturer's instructions. The released amount of fluorescence substrate, using the recombinant test, was measured spectrophotometrically at 440 nm with an FLx800 fluorescence microplate reader (BioTek, Bad Friedrichshall, Germany). All samples were measured in duplicate and average values were used for further calculations. Endotoxin concentrations (EU/mL) were calculated using Gen5 Data Analysis Software Version 2.05 (BioTek, Bad Friedrichshall, Germany). Standard curves were fit using a four parameter logistic non-linear regression model. The detection limit of the assay was 0.005 EU/mL. For the LAL test, the released amount of chromogenic substrate was measured spectrophotometrically at 405 nm with an Epoch2 absorbance microplate reader (BioTek, Bad Friedrichshall, Germany). All samples were measured in duplicate and average values were used for further calculations. Endotoxin concentrations (EU/mL) were calculated using Gen5 Data Analysis Software Version 2.05 (BioTek, Bad Friedrichshall, Germany). Standard curves were fit using a linear regression model. Detection limit of the assay was 0.005 EU/mL.

*Calculation of endotoxin recovery [%]:* The determined endotoxin concentrations in the tested samples were compared to the endotoxin concentrations at time zero in positive controls and stated as percent. Positive controls were prepared by spiking LPS into depyrogenated water.

## 3. Results

LER was observed in various samples. In the beginning of the study crucial formulation components of common biopharmaceuticals were examined. Therefore, endotoxin masking of single and multiple components were investigated. The end-point of the reaction was determined by endotoxin recovery kinetics at different temperatures. While multi-parameter interactions between surfactants, complex forming agents and endotoxin were observed, the focus of the investigation was the particular impact of these components on the detectability of endotoxin. Thus, the impact of pH and different buffer systems as well as the effects of different nonionic surfactants were studied. Finally, various endotoxin concentrations were added to a LER causing formulation to evaluate the masking capacity.

Single and mixtures of common formulation components were examined to identify critical components or component

**Table 1**
Endotoxin recovery over time in presence of single and multiple formulation components.

| Sample | Formulation components: | T0 recovery [%] | T7 recovery [%] |
|---|---|---|---|
| 1 | H2O | **100** | **94** |
| 2 | Sodium citrate | **125** | **94** |
| 3 | Sodium phosphate | **95** | **69** |
| 4 | Polysorbate 20 | **91** | **79** |
| 5 | Sodium citrate + polysorbate 20 | **1** | **0** |
| 6 | Sodium phosphate + polysorbate 20 | **52** | **0** |

Samples were spiked with an endotoxin amount of 10,000 EU/mL. Endotoxin was detected after preparation (approx. 45 min., T0) and after sample incubation of seven days (T7) at room temperature. Prior to the measurement samples were diluted (1:1000 and 1:10,000).

combinations affecting endotoxin detection. Endotoxin recovery in the presence of different formulation components are shown in Table 1. The recovery was compared directly after sample preparation (T0) and after sample incubation for seven days (T7) at room temperature. Samples 2, 3 and 4, containing only single component additions showed no significant loss of activity over time, according to the validity criteria of 50%–200% of endotoxin recovery. In contrast, endotoxin could not be measured in samples containing both buffer and surfactant (samples 5 and 6) after an incubation period of seven days. Therefore, the kinetics of endotoxin recovery in samples containing polysorbate 20 and sodium citrate was analyzed. Fig. 2 shows the endotoxin recovery of three identical samples as a function of time at different incubation temperatures (4 °C, RT and 37 °C) using a LAL test (A) and a recombinant Factor C test (B) for detection. After a certain period of incubation, all samples showed low endotoxin recovery in both detection systems. This result clearly indicates that this phenomenon is independent of the test system. Furthermore, the loss of activity was significantly accelerated with increasing incubation temperature.

The latter experiments show that only the combination of a buffer system and a surfactant results in LER. Thus, the impact of different buffer systems was studied and is shown in Fig. 3. In order to investigate pH dependency of endotoxin recovery over time, different pH conditions were studied (Fig. 3A). In the absence of surfactants, the variation of pH had no impact on endotoxin

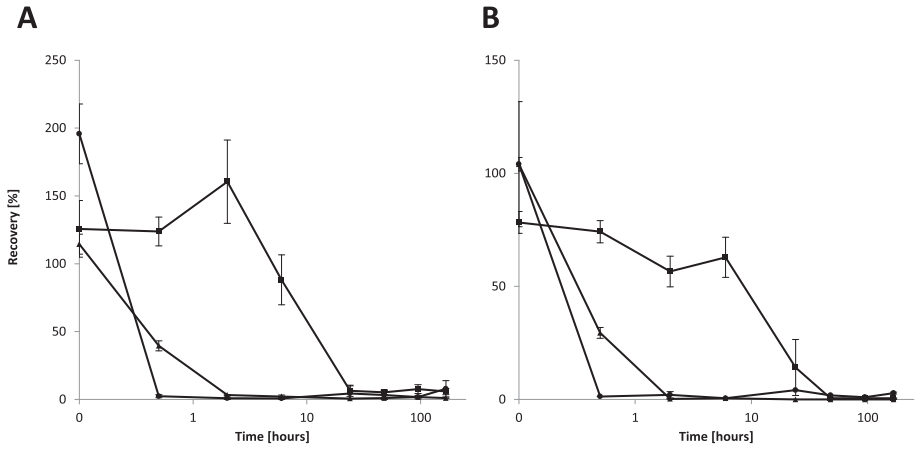


**Fig. 2.** Endotoxin recovery kinetics in citrate-polysorbate formulations. 100 EU/mL endotoxin were added to samples containing 10 mM citrate and 0.05% polysorbate 20 and incubated for different time periods. The endotoxin recovery is plotted as a function of the incubation time. The different curves indicate incubation temperatures at 36–38 °C (●), 21–23 °C (▲) and 2–8 °C (■). For detection kinetic chromogenic LAL test (A) and recombinant Factor C tests (B) were used. The error bars reflect the standard deviation of three independent replicates (n = 3) of the sample. The replicates were measured on the same microtiter plate.

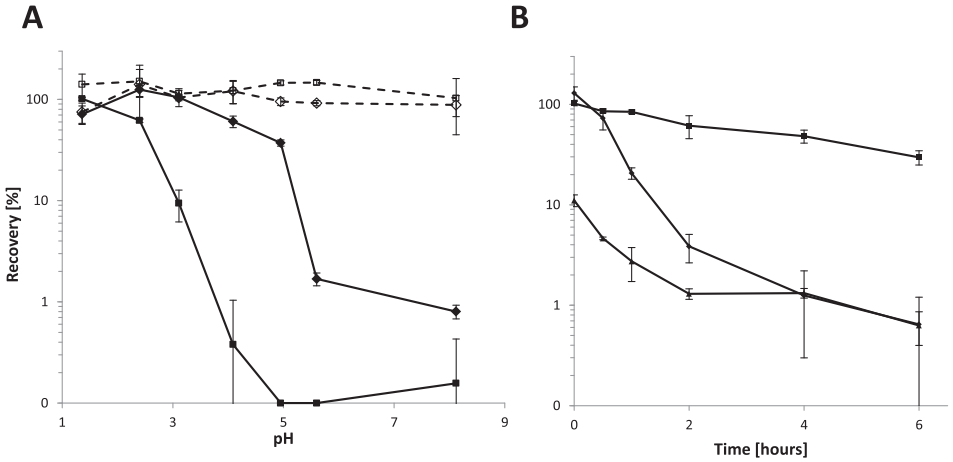


**Fig. 3.** Impact of buffer system on endotoxin recovery. (A): Effect of pH on endotoxin recovery: 100 EU/mL of endotoxin was added to solutions containing 0.05 wt % polysorbate 20 plus 10 mM citrate (■), 0.05 wt % polysorbate 20 plus 10 mM phosphate (◆), citrate only (□) and phosphate only (◇). The pH varied in a range from 1 to 9 and incubation was at room temperature for seven days. The endotoxin recovery is shown as a function of the pH. (B): Effect of buffer systems on kinetics of endotoxin recovery: 100 EU/mL of endotoxin were added to solutions containing a buffer (5 mM EDTA (▲), 10 mM sodium citrate (◆) or 10 mM sodium phosphate (■)) and 0.05 wt % polysorbate 20. Sample incubation was at room temperature. The endotoxin recovery is plotted as function of the incubation time. The error bars reflect the standard deviation of three independent replicates (n = 3) of the sample. The replicates were measured on the same microtiter plate.

detection. However, in the presence of polysorbate the recovery significantly decreased at pH values higher than pH 2 (citrate system) and pH 5 (phosphate system), respectively. Thus, the transition to higher pH values hampered endotoxin recovery. In addition, the diverging curve progressions (Fig. 3A) indicate an intrinsic effect of each particular buffer system. Endotoxin recovery kinetics using different buffer systems such as ethylenediaminetetraacetic acid (EDTA), citrate and phosphate were studied and are shown in Fig. 3B. The endotoxin recovery within the described buffer systems are plotted as a function of time. The system containing EDTA showed the most rapid activity loss. The loss of activity was slower under citric conditions and slowest under phosphoric conditions. After 6 h recovery was below 30% at each condition. As confirmed before, surfactants are significantly involved in reducing the activity of endotoxin in common detection systems. Therefore, the effects of different surfactants at constant buffer and endotoxin conditions were examined. In Fig. 4, the endotoxin recovery out of surfactant solutions (polysorbate 20, polysorbate 80 and octoxynol 9) in presence and absence of citrate are plotted as a function of surfactant concentration. In general, all surfactants significantly reduced endotoxin detectability in the presence of citrate after seven days of incubation. In absence of citrate, only octoxynol 9 showed low recoveries at relatively high concentrations after the incubation period.

Summarizing the results above, nonionic surfactants and complex forming buffer components in combination cause a significant perturbation of endotoxin detection in *Limulus*-based detection systems. The resulting LER is time-dependent and occurs solely after a certain period of time. For a final evaluation, the masking capacity of such formulation matrices was examined. Endotoxin was titrated into samples containing a citrate buffer system and polysorbate. Fig. 5 shows the capacity of such a particular matrix.

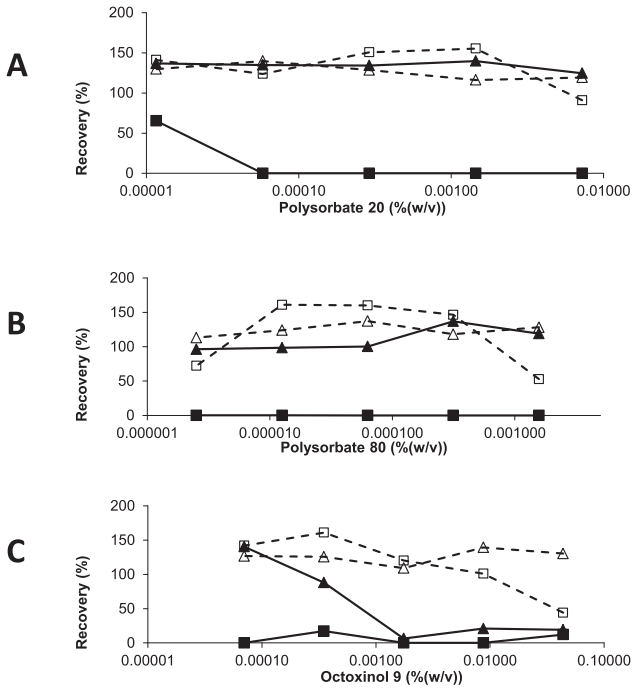


**Fig. 4.** Surfactant dependent endotoxin recovery. 100 EU/mL of endotoxin was added to solutions containing various amounts of polysorbate 20 (A), polysorbate 80 (B) or octoxynol 9 (C). Endotoxin recoveries were determined in the presence of 10 mM citrate immediately after preparation (□) and after incubation for 7 days at room temperature (■). In parallel endotoxin activities were determined in the absence of citrate, without incubation (△) and after incubation (▲). Endotoxin recovery is shown as a function of the particular surfactant concentration.

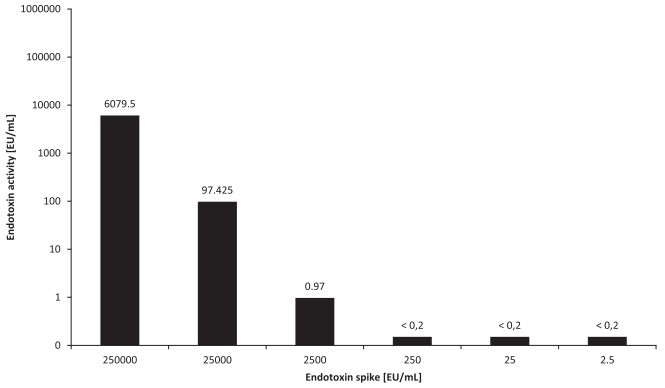


**Fig. 5.** Endotoxin masking capacity of citrate-polysorbate formulation. Defined amounts of endotoxin were added to solutions containing 0.05 wt % polysorbate 20 and 10 mM sodium citrate and incubated for 7 days at 4 °C. Endotoxin spikes were prepared out of a LPS stock solution containing 10E6 EU/mL. After incubation, endotoxin measurements were performed. The detectable endotoxin concentration is shown in relation to the spiked endotoxin concentration.

Spiked endotoxin contents of up to 250 EU/mL resulted in no endotoxin recovery after seven days of incubation. Medium and high-level spikes resulted in very low (<1%) and low endotoxin (<5%) recovery. This illustrates the high masking capacity of common formulation matrices and suggests the need for vigilance in bacterial endotoxin testing under such conditions.

## 4. Discussion

When less than 50% of an endotoxin spike into an undiluted sample is recovered over time, the detection of endotoxin is popularly classified as LER. It is supposed that this phenomenon is caused by endotoxin masking. Therefore it is important to differentiate this phenomenon from test interference. Test interference, which directly affects the detection system, can be excluded because it can be corrected by dilution. Yet, in the case of LER, endotoxin dilutions up to a factor of 10,000 could not overcome inadequate recovery (Table 1). Furthermore, within very short incubation periods with endotoxin in the sample, the full endotoxin content could be recovered, which illustrates the functionality of the detection system. These findings reflect a previous observation, namely that under certain condition the interference in *Limulus*-based detection methods is concentration independent and assumes that the aggregate conformation of LPS is affected and not the detection system itself [11]. The assumption of alterations in the aggregate conformation is supported by the time-dependent appearance of LER. Obviously, the kinetics in Fig. 2 shows a time-dependent phenomenon, while test interference appears immediately and is therefore time-independent. This time-dependent behavior can be illustrated by an alteration of the supramolecular structure of the amphiphilic LPS. In general, the process of aggregation of amphiphilic molecules can be very variable with respect to time-scales for structural changes, which can range from submicroseconds to several days, weeks and even month [15]. This also might explain experimental results, in which the masking phenomenon was not observed, although masking conditions were present [16].

However, the results also demonstrate that endotoxin recovery is affected by the formulation components themselves, even if the active pharmaceutical substance, such as a protein, is absent. The simultaneous presence of a nonionic surfactant and complex forming components (chelator) suffices to decrease the detectability of endotoxin. The presence of only one of the formulation components is not effective in significantly disturbing endotoxin recovery

(Table 1). These findings confirm former assumptions of endotoxin disaggregation [17,18] and explain the interdependent interaction of surfactant and chelator on endotoxin. Due to the ionic and amphiphilic nature of LPS (Fig. 1), complex forming agents disturb the electrostatic interactions and surfactants potentially disturb the hydrophobic interactions in endotoxin aggregates. Certainly, to disturb the supramolecular structure of endotoxin a reduced rigidity is beneficial. This is controlled by the salt form of LPS, which again involves the presence of multivalent cations like $Ca^{2+}$ [19,20]. Consequently, it can be assumed that complex forming agents are in competition with negatively charged patches of the endotoxin. Therefore the salt bridges between LPS molecules are disturbed, which should result in a reduced rigidity of endotoxin aggregates, which in turn facilitates changes in the supramolecular structure. Thus, the chelating capability of the buffer component is crucial. In the presence of EDTA the recovery drops faster than in the presence of citrate or phosphate based buffer systems (Fig. 3b). Using buffer components with higher metal complex forming capabilities accelerate masking kinetics. The related metal complex formation constants are directly proportional to the denticity of the ligand (rule of thumb [21]). A hexadentate ligand like EDTA forms more stable metal complexes than a tridentate ligand like citrate. Furthermore, the equilibrium complex formation ability and the complex stability of a chelator are pH dependant. At low pH values, protons are in competition with cations, which hamper formation of metal complexes [22]. Consequently, masking of endotoxin is affected by the free concentration of protons (pH), which is controlled by the buffer system and its particular acid dissociation constant. This explains the pH dependent endotoxin recovery in different buffer systems (Fig. 2A). However, complex forming components alone do not result in inadequate endotoxin detection, further amphiphilic components like surfactants are necessary. Due to the fact that LPS itself is amphiphilic, it tends to aggregate, because of the low solubility of the hydrophobic fatty acids of Lipid A in an aqueous solution [23]. Thus, LPS exhibits certain supramolecular structures, which influence detectability in *Limulus*-based detection systems [24]. Structural transitions of amphiphilic systems are affected by a large variety of physical and chemical parameters. One major principle to control these structures is the head group repulsions of self-assembling molecules. They can be affected by co-surfactants, electrolytes, and amphiphilic counter ions [25]. If the masking surfactant (e.g.: polysorbate) intercalates between LPS molecules resulting in reduction of head group repulsions, the establishment of a new equilibrium is favored and the supramolecular structure of LPS is altered.

The interaction of nonionic surfactants with LPS aggregates is favored if the LPS aggregates possess a certain degree of rigidity (Fig. 4). The latter is controlled, to some extent, by ionic interactions as described above. Under these circumstances the supramolecular structure of LPS is changed into a structure with a lower affinity to the endotoxin sensitive Factor C of the *Limulus*-based detection system resulting in the measurement of a lower activity. Such a structure could be disaggregated LPS due to a molecular excess of surfactants. This hypothesis fits well to the observation of Mueller et al., which have shown that disaggregated LPS molecules ("monomers") are substantially less active than aggregated LPS in the detection system used [24]. Additionally, Tan et al. proposed a cooperative binding mechanism of LPS to Factor C, which consequently requires more than one LPS molecule in close spatial arrangements [26]. On the other hand, it has been shown that monomeric LPS shows a higher potency in activating *Limulus* Amebocyte Lysate assays than aggregated LPS [27]. Under these circumstances, the inadequate detectability might have steric reasons, in which the activating spots of the LPS (lipid A) are hidden by surfactant molecules and are not accessible for Factor C.

In summary, we propose a two-step mechanism of endotoxin masking. Fig. 6 illustrates the effects of chelating buffer components and nonionic surfactants on endotoxin. In this mechanism the equilibrium LPS structure is shifted to an altered supramolecular structure. In its natural state, LPS monomers tend to aggregate due to the hydrophobic interactions between the Lipid A molecules. Additional ionic interactions, formed by divalent cations and negatively charged substitutes (e.g.: phosphates) of the LPS increase the rigidity of aggregates. By adding a complex forming agent (I), the salt bridges formed by divalent cations (e.g. Mg2+) and LPS are destabilized, leading to a reduced rigidity of the aggregate. The further presence of a surfactant (II) can then change the initial supramolecular structure by formation of mixed aggregates. This structural change leads inevitably to a certain change in detectable activity, as endotoxin activity is dependent on its supramolecular structure. Due to the ordinary molar excess of complex forming agent and surfactant (micro molar range), compared to endotoxin content (nano molar range), mixed surfactant micelles containing monomerized LPS are the most probable resulting supramolecular structure.

Within this study, the phenomenon of LER were confirmed in *Limulus*-based detection systems and exemplifies a potential mechanism of endotoxin masking. The unknown period of endotoxin presence during a potential event of endotoxin contamination in a sample defines the chance of endotoxin recovery. Hence, LER is

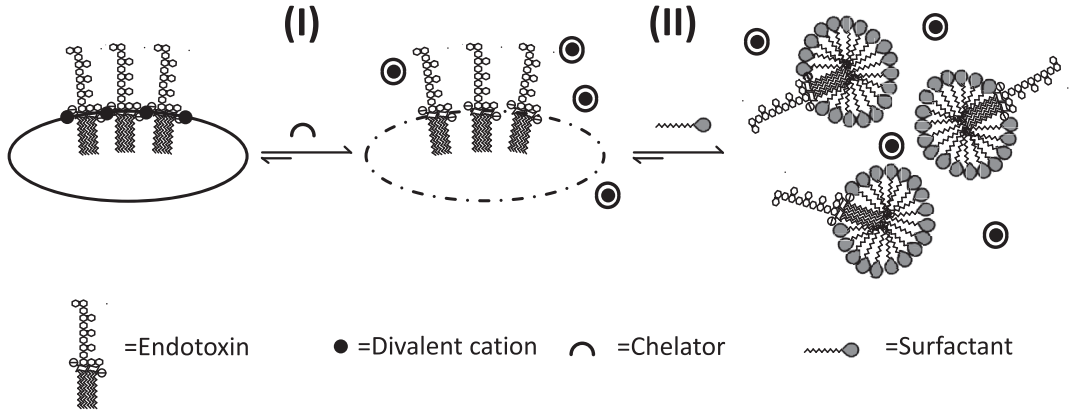


**Fig. 6.** A proposed two-step mechanism of endotoxin masking. Potential equilibration reaction of endotoxin masking, caused by complex forming agents and surfactants, is schematically illustrated. In a first step, pure endotoxin aggregates are disturbed by chelators increasing the permeability of the aggregate. Then, surfactants interact with endotoxin by forming mixed aggregates.

under control of kinetics that governs the formation of mixed endotoxin-surfactant aggregates, which make them less prone to activate the enzymatic reaction of *Limulus*-based detection systems. In conclusion, capacity experiments have shown that commonly used excipients are capable of masking hazardous amounts of endotoxin. Currently, it is not known whether masked endotoxin activates the innate immune system. Bacterial endotoxin testing should be performed with care, especially in the presence of surfactants and chelators.

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