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Investigation of the kinetics and mechanism of low endotoxin recovery in a matrix for biopharmaceutical drug products

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ABSTRACT

The inability to detect endotoxin added to undiluted drug samples has been called: Low Endotoxin Recovery (LER). The phenomenon has caused concerns amongst drug manufacturing quality control scientists in that manufactured solutions contaminated with endotoxin could show false-negative results via routine *Limulus*-based tests. The time-dependent appearance of LER has been analyzed in detail to provide a better understanding of the mechanism. The assumption has been that the root-cause of LER involves the interplay of endotoxin with surfactants and results in aggregate structures that are complexed with surfactants. The endotoxin molecules when complexed with surfactants are not accessible for *Limulus*-based detection. The results demonstrate a predominant role of complex-forming agents. It was shown that although the presence of surfactants is a strong prerequisite for masking, it does not determine the kinetics of endotoxin masking. Interestingly, the endotoxin concentration itself had no substantial impact on LER kinetics. By adjusting the ratios of complex-forming constituents, including surfactant, chelator and endotoxin, and by testing the order in which the constituents are added, a new model for simulating masking kinetics has been determined. Our work provides for the first time a model to simulate masking kinetics of endotoxin which lends a better understanding of LER.

1. Introduction

Endotoxins are a unique group of molecules, which occur naturally in the cell wall of Gram-negative bacteria [1]. If administrated into the blood stream of mammals, bacteria and their toxic byproducts can cause severe pathogenic effects including fever and septic shock. It has been shown that lipopolysaccharides (LPS) are the dominating constituents of the outer membrane of Gram-negative bacteria [2]. Moreover, LPS is known as the major factor responsible for toxic manifestations of severe Gram-negative infections including fever, hypotension, shock [3,4]. To this end, the terms LPS and endotoxin are used as synonyms and are therefore interchangeable [3,5–8].

Endotoxin contamination represents a potential safety risk in parenteral drug manufacturing. Therefore, diverse detection methods, including the Rabbit Pyrogen Test (RPT), the Monocyte Activation Test (MAT) and the *Limulus* Amebocyte Lysate (LAL) test [9] have been used. The *Limulus* amebocyte lysate (LAL) test is the most commonly used method for endotoxin detection due to its simplicity and superior sensitivity [10,11]. However, recently inconsistencies during testing of biopharmaceutical drug products have been observed in certain drug product positive controls of endotoxin in undiluted samples that were not recoverable within the allowed acceptance criteria. This effect is called Low Endotoxin Recovery (LER) and users are concerned about the reliability of existing test procedures when testing drugs of specific formulation matrixes. Regulatory authorities consider this phenomenon as a potential safety issue due to the possible underestimation of critical endotoxin levels in a sample during quality control testing of drug products [12].

The LER phenomenon is of particular relevance when surfactants and other ingredients such as salt, urea and other organic substances are present, all molecules representing parameters that strongly influence the critical micelle concentration (CMC) [13,14]. Micelles and other aggregates are assumed to represent the major physico-chemical mechanism of LER by “masking” the endotoxin, thus precluding correct determination in bacterial endotoxin testing [15]. Previously, the LER phenomenon was studied in common biopharmaceutical product matrices and the assumption has been that LER is caused by the interaction of sample matrix and endotoxin, [16,17]. Due to their amphiphilic nature LPS forms specific supramolecular structures [13]. A change in the endotoxin supramolecular structure is likely to occur during the

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transition from detectable to undetectable (masked) forms. Thus, the endotoxin is masked and not accessible by the detection system. Once masking appears, an under-estimation of potential endotoxin contaminations and, therefore, false negative results in the *Limulus*-based test methods may occur. In pharmaceutical quality control units, such false negative results must be strictly avoided. The FDA requires a demonstration of spike recovery in hold time studies to determine whether a particular drug product will cause LER [18]. Therefore, the incubation of known amounts of endotoxin over time in undiluted products prior to the actual *Limulus*-based test procedure are recommended. The aim of such hold time studies is to prove suitability of the endotoxin complex detection in a particular sample over time.

The phenomenon of LER has been recognized by analyzing biopharmaceutical drug products, which normally contain large protein molecules (e.g. monoclonal antibodies) as active pharmaceutical ingredient (API). For stability, APIs are often formulated using citrate or phosphate buffer systems and polysorbates 20 [19]. The LER-effect can be caused by the formulation components only [20] or by a combination thereof with the drug protein. To provide a better understanding of LER and in turn to improve the efficiency of hold time study performance, the time-dependent appearance of LER is analyzed in detail. Therefore, a common formulation matrix containing sodium citrate and polysorbate 20 is used in the present study. This matrix was chosen because it is a common formulation composition for biopharmaceutical drugs products [19]. This composition reflects the minimum requirement for endotoxin masking and should therefore help to elucidate the driving forces of LER. Apart from the temperature dependency of a reaction, the change in concentrations during chemical reactions is often directly proportional to the rate of a reaction [21]. Derivation of a rate law according to the underlying masking reaction enables the prediction of the reaction rate for a corresponding product formulation. Such a prediction would help to plan sample hold-time periods for identification of LER. In order to determine a rate law of the reaction, endotoxin recovery kinetics is recorded using different concentrations of citrate, polysorbate 20 and endotoxin. A variety of concentrations is used to identify whether there are specific reactants controlling the reaction rate. Furthermore, the rate determining step in the proposed two-step reaction mechanism [16] is specified and the derived reaction law is used for the simulation of endotoxin masking kinetics.

2. Material and methods

2.1. Material

Polysorbate 20 (Ph. Eur. Grade), citric acid (Ph. Eur. Grade), trisodium citrate (Ph. Eur. Grade), magnesium chloride (Ph. Eur. Grade) and endotoxin from *E. coli* O55:B5 (Simga-L2637) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Depyrogenated water (EndoGrade®), depyrogenated borosilicate glass tubes 5 mL (EndoGrade®) and *Limulus*-based recombinant Factor C tests (EndoZyme®) were obtained from Hyglos GmbH, Bernried, Germany. Prior to the experiments, all relevant materials had been tested for endotoxin and were proven to contain less than 0.005 EU/mL.

2.2. Sample handling

Samples were prepared in glass tubes with sample volumes of 1 mL per sample. Unless otherwise described, samples were spiked with 10 µL of endotoxin from *E. coli* O55:B5 out of a 10,000 EU/mL stock solution. Before adding the endotoxin spikes to the sample, the endotoxin stock solution was vortexed at 1400 rpm for 10 min using Multi Reax shaker (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany).

For time-dependent endotoxin recovery (hold-time) experiments, endotoxin was incubated in undiluted samples over time. The pH of the solutions was adjusted to 7.5. After addition of defined endotoxin spikes to undiluted samples, the resulting solutions were vortexed for 15 s at 1400 rpm. Samples were subsequently stored without further vortexing at room temperature (19–25 °C) for a specified period of time.

Individual endotoxin masking kinetics was detailed from one stock solution. The start of the kinetics was defined when surfactant, chelator and endotoxin were combined and vortexed together. In order to measure endotoxin at individual points of time, 10 µL of the corresponding sample were transferred to 990 µL of depyrogenated water after desired incubation period (1:100 dilution). Prior to the measurement, no further dilution was required. If needed, diluted samples were stored up to 3 h. Storage at RT for this period of time had no impact on endotoxin recovery of diluted samples. Before measurement, diluted samples were vortexed at least for 2 min at 1400 rpm. The validity of the measurement was controlled by spiking the defined endotoxin amounts into the diluted samples (Positive Product Control (PPC)). Endotoxin determination in a sample was considered valid, if 50%–200% of the spiked endotoxin (PPC) was recovered. To control the accuracy of the endotoxin spiked into the undiluted samples, equal amounts of endotoxin were spiked into depyrogenated water (water control), mixed and identically incubated as the actual sample. The spike into the undiluted sample was considered valid if the water control was in the range of 50%–200% of the theoretical expected spike concentration.

In order to minimize assay to assay variability, time point samples of given kinetics up to 3 h were collected and measured within the same analytical run. To calculate the endotoxin recovery, the resulting endotoxin concentrations for actual sample was compared to the endotoxin concentrations obtained at time zero in the water control and stated as percent.

2.3. Endotoxin detection

For endotoxin detection, a *Limulus*-based test (rFC) was used according to manufacturer's instructions. The amount of fluorescence substrate (amino-methylcoumarin) released was measured fluorometrically at 440 nm (Excitation: 380 nm) with a FLx800 fluorescence microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). All samples were measured in duplicate and average values were used for further calculations, except for the kinetics of Fig. 1, where a single determination was used. Standard curves were fit using a four-parameter logistic non-linear regression model. The detection limit of the assay was 0.005 EU/mL. Microsoft Excel 2010, Version 14.0.7015.1000 was used to calculate endotoxin recovery, plot graphs and to simulate endotoxin recovery kinetics. Sigmoidal experimental data points were fit using SigmaPlot 2001 for Windows Version 7.0. Gen5 Data Analysis Software Version 2.05 from BioTek Instruments GmbH, Bad Friedrichshall, Germany was used to calculate standard curves for determination of endotoxin concentrations.

3. Results

LER has been known to be caused by the simultaneous presence of surfactants and complex forming agents [16]. In this study, we show that various mixtures of formulation components influenced the occurrence of LER. Masking kinetics with different constituent sequence addition of various sample preparations was investigated to analyze whether the preparation of the samples masks endotoxin. Two of the three components (polysorbate 20, sodium citrate and endotoxin) were pre-incubated overnight and the kinetics was started by the addition of the third component (Fig. 1). As expected, LER was observed in all preparations albeit with diverging kinetics. While endotoxin pre-incubated with sodium citrate showed the fastest masking kinetics and pre-incubation of polysorbate 20 with sodium citrate showed the slowest masking kinetics. Endotoxin pre-incubated with polysorbate 20 similarly showed slow masking kinetics. Given the accelerated reaction kinetics by pre-incubation of endotoxin with sodium citrate, the interaction between endotoxin and sodium citrate appears to be the time

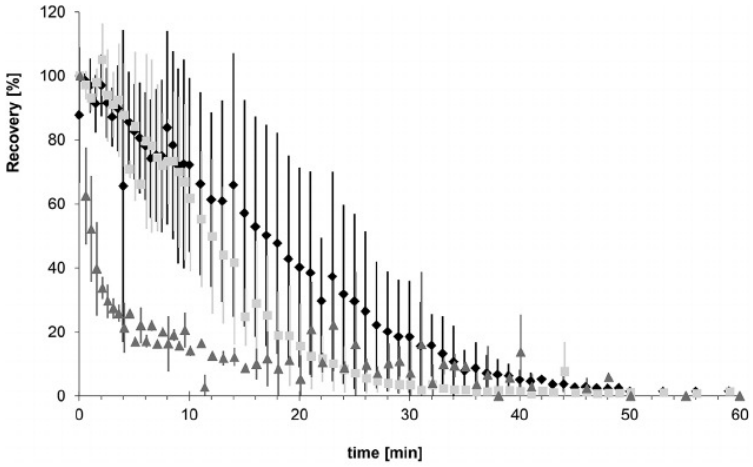

Fig. 1. Endotoxin recovery depending on order of matrix component and LPS addition Endotoxin recovery is plotted as a function of incubation time. 100 EU/mL endotoxin was spiked to solutions containing 0.05 (w/v) % polysorbate 20 and 10 mM sodium citrate. The particular kinetics were generated by different sequential arrangements during sample preparation. In the first kinetic (dark grey triangles; ▲) LPS and sodium citrate were pre-incubated (overnight) and polysorbate 20 was added at time zero (0 min) to start the reaction. In the second kinetic (light grey squares; ■) LPS and polysorbate 20 were pre-incubated (overnight) and sodium citrate was added at time zero (0 min). In the third kinetic (black diamonds; ♦) sodium citrate and polysorbate 20 were pre-incubated (overnight) and LPS was added at time zero (0 min). For calculation of the data points the mean values of two (LPS/sodium citrate and LPS/polysorbate 20 pre-incubation) and three (polysorbate 20/sodium citrate pre-incubation) individually performed repetitions of the kinetics were used and the error bars reflect the corresponding standard deviations. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100%. The mean of the initial measurements of sodium citrate and polysorbate 20 pre-incubation was 143 EU/mL, LPS and polysorbate 20 pre-incubation was 161 EU/mL and LPS and sodium citrate was 10 EU/mL.

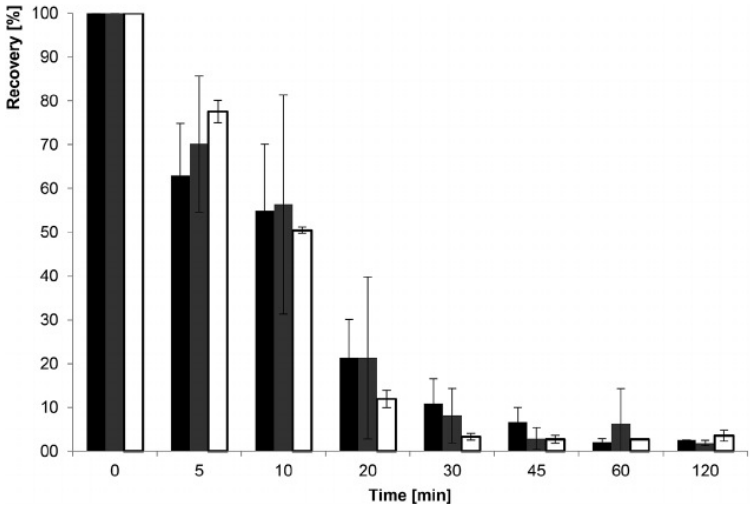

Fig. 2. Endotoxin recovery is independent of LPS concentration Endotoxin recovery is plotted as a function of incubation time. Varying concentrations of endotoxin were added to samples containing 0.05 (w/v) % polysorbate 20 and 10 mM sodium citrate. The black columns reflect 5000 EU/mL, grey columns 500 EU/ml and white columns 50 EU/mL. The particular endotoxin stock solutions for spiking were containing 50,000, 5000 and 500 EU/mL. For calculation of the data points the mean values of two individually prepared kinetics were used and the error bars reflects the corresponding standard deviations. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100%. The mean of the initial measurements of 5000 EU/mL spike was 6993 EU/mL, 500 EU/mL spike was 640 EU/mL and 50 EU/mL spike was 72 EU/mL.

limiting reaction step. The large error bars shown in Fig. 1 reflect test variables of *Limulus*-based assays, but also the experimental setup. Reaction kinetics may be influenced by the exact ambient temperature that occurs and the necessary hands on time for sample preparation including spiking and mixing as well as vortexing. The recovery kinetics using different concentrations of the reactants were tested (Fig. 2) to further analyze the driving forces of endotoxin masking. The recovery of different endotoxin concentrations (50, 500 and 5000 EU/mL) were studied under constant polysorbate 20/citrate conditions. The recovery over time showed no significant difference using various endotoxin concentrations, indicating that masking is independent of the initial endotoxin concentration. After 10 min of incubation, all recoveries were above 50% and after 45 min all recoveries were below 7%.

Next, kinetics was analyzed using reduced concentrations of polysorbate 20 and sodium citrate (Fig. 3A). The recovery of endotoxin in a sample containing 0.05 (w/v) % polysorbate 20 and 10 mM sodium citrate was below 1% after 1 h of incubation. Using a sample matrix of 0.0125 (w/v) % polysorbate 20 and 2.50 mM sodium citrate the endotoxin recovery was not reduced after 1 h of incubation, however, after 20 h of incubation the endotoxin recovery was very low. No LER was observed in matrix containing 0.008 (w/v) % polysorbate 20 and 0.16 mM sodium citrate as observed out to 20 h. Therefore, masking can be delayed or even avoided when the entire sample matrix is diluted before spiking the endotoxin. Furthermore, endotoxin recovery was determined in samples, in which only the polysorbate 20 concentration (0.0500, 0.0125 and 0.0008 (w/v) %) was reduced (Fig. 3B) while the concentrations of the other components (endotoxin and citrate) were kept constant. In this case, the endotoxin recovery is below 2% after 1 h and is independent of the polysorbate 20 concentration after 1 h of incubation. Comparing the individual masking kinetics, there is a tendency to faster masking in the presence of lower polysorbate 20 concentrations. However, this result differs substantially from the previous result where the endotoxin was recovered after 1 h of incubation (> 100%) at reduced polysorbate 20 and citrate concentrations (Fig. 3A). While the polysorbate 20 concentration was comparably titrated in both cases, the sodium citrate concentrations were different in the two experiments (Fig. 3: A vs. B). This again indicates that the role of sodium citrate is crucial within the given experimental conditions. Due to the fact that sodium citrate is capable of forming metal complexes with divalent cations [22], endotoxin recovery kinetics was examined in the presence of divalent cations (Fig. 4A). In this case, the masking kinetics is delayed in the presence of 1 mM magnesium

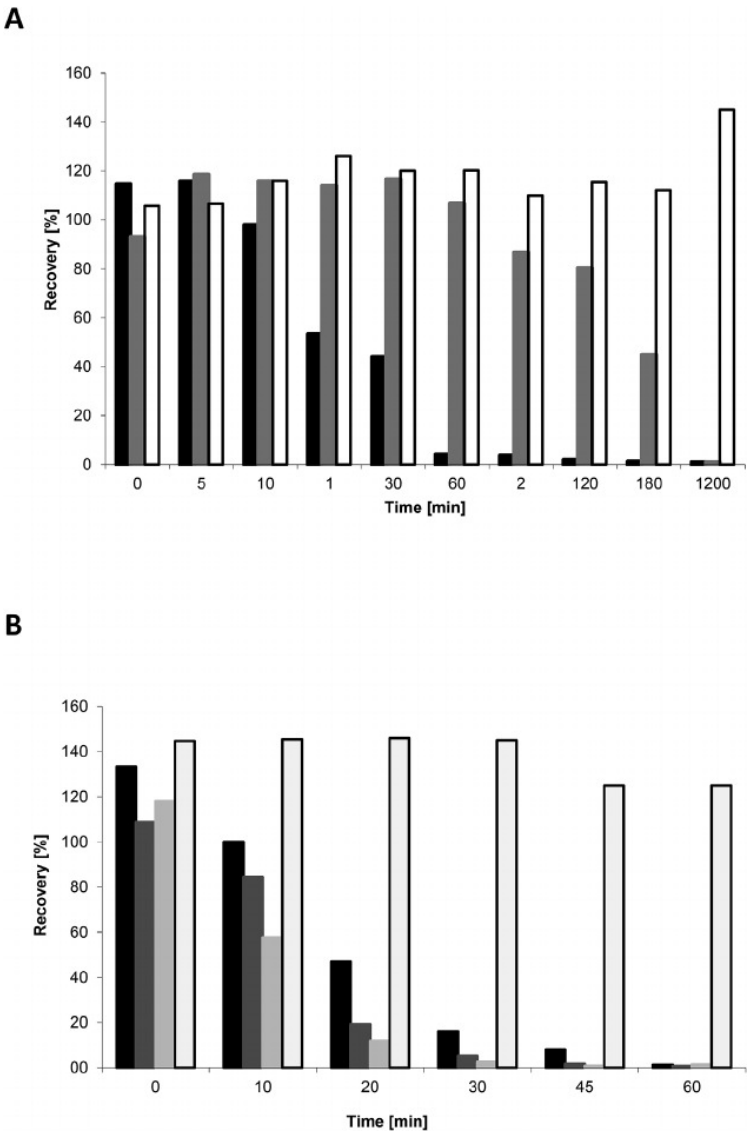

Fig. 3. Endotoxin recovery depending on concentration and matrix components. **A**) Endotoxin recovery is plotted as a function of incubation time. 100 EU/mL of endotoxin were spiked to samples containing polysorbate 20 and sodium citrate. The three different shaded columns reflect different polysorbate 20 and sodium citrate concentrations. The set of black columns correspond to 0.0500 (w/v) % polysorbate 20 and 10.00 mM sodium citrate, the set of grey columns correspond to 0.0125 (w/v) % polysorbate 20 and 2.50 mM sodium citrate and the set of white columns correspond to 0.0008 (w/v) % polysorbate 20 and 0.16 mM sodium citrate. **B**) Endotoxin recovery over time in samples containing polysorbate 20, sodium citrate and LPS is shown. The different shaded columns reflect different polysorbate 20 concentrations. The set of black columns correspond to 0.0500 (w/v) % polysorbate 20, the set of dark grey columns correspond to 0.0125 (w/v) % polysorbate 20, the set of light grey columns correspond to 0.0008 (w/v) % polysorbate 20 and the set of white columns correspond to 0% polysorbate 20. The concentrations of spiked endotoxin (100 EU/mL) and sodium citrate (10 mM) were kept constant.

chloride as compared to the samples without magnesium chloride. In the presence of 5 mM magnesium chloride, no reduced endotoxin recovery is observed within the analyzed time scale. Importantly, the magnesium chloride was added to the samples prior to endotoxin addition. In an additional experiment, 20 mM magnesium chloride was added to the polysorbate 20/sodium citrate matrix 20 min after start of the reaction (Fig. 4B). Magnesium chloride, 20 mM, was chosen, to ensure an excess of cations and to attempt an immediate stop of the masking process. Although the recovery of endotoxin was already reduced at this point of time, no further decrease of endotoxin recovery was observed after addition of magnesium chloride. In comparison, the sample without supplementary addition of divalent cations was masked as expected. Hence, the addition of magnesium chloride can stop endotoxin masking and keep the recovery constant at the actual level. Notably, the original endotoxin activity could not be retrieved after the addition of magnesium chloride. In other words, the supplementary addition of magnesium chloride did not reverse the masking effect that had already occurred.

Obviously, there is a clear relationship between masking kinetics and complex formation. For a deeper analysis of this effect, masking kinetics using four different citrate concentrations (5, 10, 20, and 80 mM) at constant polysorbate 20 concentrations were performed (Fig. 5A–D). A distinct acceleration of masking is observed when the citrate concentration in the sample is increased from 5 mM to 80 mM. Furthermore, the experimental data points were compared to a model kinetic masking curve using an exponential decay function where the endotoxin recovery $[LPS_d]$ is calculated as a function of time t:

$$[LPS_d] = [LPS_d]_0 * \exp(-[Ci]kt) \quad (1)$$

Equation (1) is based on a second order rate law and serves to link the reaction rate with concentrations of the reactants. The parameters of this equation were set analogously to the experimental conditions. Endotoxin recovery at time 0 $[LPS_d]_0$ was set to 100%, time t was given in minutes (min) and the proportionality constant k was set to 4 $(M*min)^{-1}$ as a starting point. The sodium citrate concentrations [Ci] were set to 5, 10, 20 and 80 mM in the specific curves. The simulated

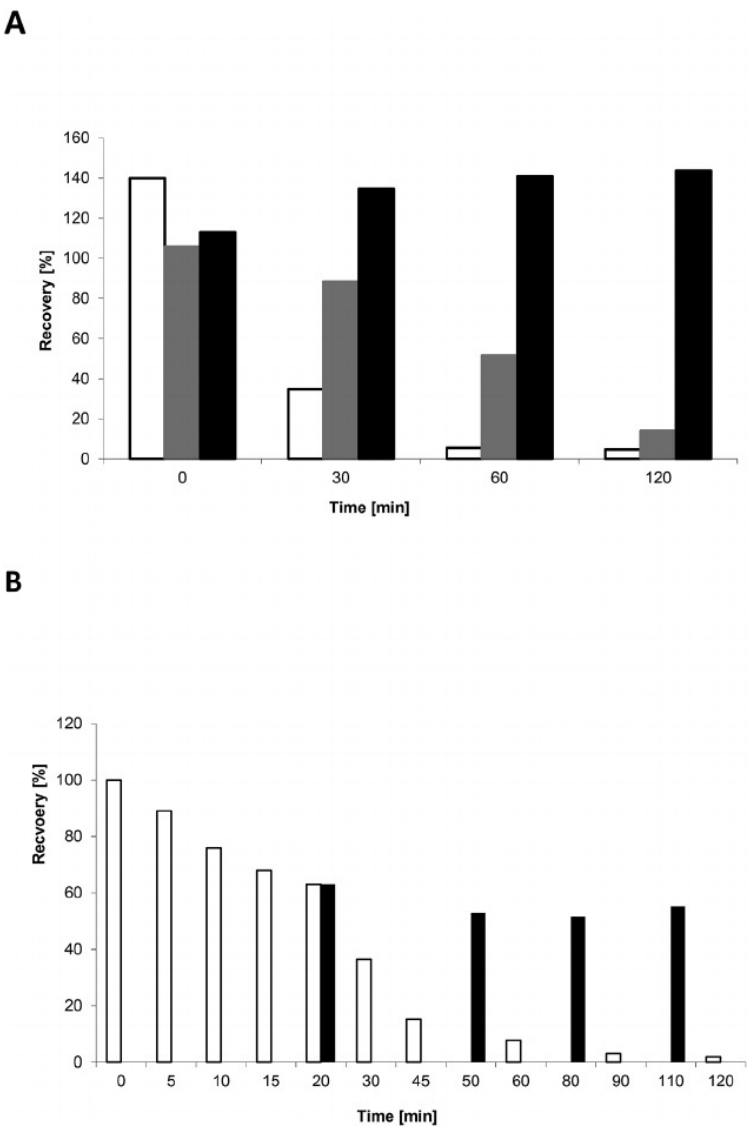

Fig. 4. Endotoxin recovery depends on the presence of divalent cations. **A**) Endotoxin recovery is plotted as a function of time in samples containing polysorbate 20, sodium citrate, endotoxin and magnesium chloride. The different shaded columns reflect different contents of magnesium chloride (0 mM (white columns), 1 mM (grey columns), 5 mM (black columns)). Concentrations of polysorbate 20 (0.05 (w/v) %), sodium citrate (10 mM) and endotoxin (100 EU/mL) were kept constant. **B**) Endotoxin recovery is plotted as a function of time in a sample containing 0.05 (w/v) % polysorbate 20, 10 mM sodium citrate and 100 EU/mL endotoxin (white columns). After 20 min, the sample was divided into two equivalent aliquots (1 mL each), whereby one aliquot was treated once by the addition of 20 mM (20 µL of 1 M) magnesium chloride and measured after 50, 80 and 110 min of total incubation. The other fraction was continued without treatment and measured after 30, 45, 60, 90 and 120 min of total incubation. Endotoxin recovery of the treated fraction is expressed by the black columns.

curves correlate with the experimental data (see Fig. 5), illustrating that the endotoxin masking kinetics depends on the sodium citrate concentration. Derivation of this model function is explained in the discussion section below.

Summarizing the kinetics above, 100 EU/mL endotoxin will be substantially masked within 90 min of sample incubation in the presence of at least 5 mM sodium citrate and 0.05 (w/v) % polysorbate 20. Variation of the polysorbate 20 concentration (Fig. 3B) showed no significant acceleration or deceleration of the masking kinetics. In contrast, the increase of sodium citrate concentration (Fig. 5) resulted in considerable acceleration of the endotoxin masking kinetics. To determine, if there is a minimum citrate concentration for endotoxin masking, sodium citrate was titrated under constant polysorbate 20 and endotoxin concentrations and the samples were incubated for seven days prior to endotoxin measurement (Fig. 6). According to the previous kinetics, it was assumed that an equilibrium of the masking reaction is established after seven days. Plotting endotoxin recovery as a function of citrate concentration results in a S-shaped data point progression, from full recovery at a constant level to no recovery of endotoxin depending on the citrate concentration. This behavior indicates that there is a limiting concentration of citrate to mask endotoxin which can be deduced from the transition point. In order to determine this citrate concentration, the experimental data set was fitted using the following nonlinear fit function:

$$y(x) = a/(1 + (x/x_0)^b) \quad (2)$$

A three-parameter logistic function (Equation (2)) was chosen, because it reflects a sigmoid curve progression, from which the transition point can be determined. The resulting sigmoid curve fit shows endotoxin recovery as a function of sodium citrate concentration. The calculated coefficients “a” equates to 125.66, “b” equates to 1.50 and “x_0“ equates to 0.06. The transition point (x_0) of the curve can be assumed as the limiting citrate concentration and corresponds to a citrate concentration of 0.06 mM under given conditions (Fig. 6). Therefore, LER (recovery < 50%) will not be observed in solutions containing polysorbate 20 and less than 0.06 mM sodium citrate.

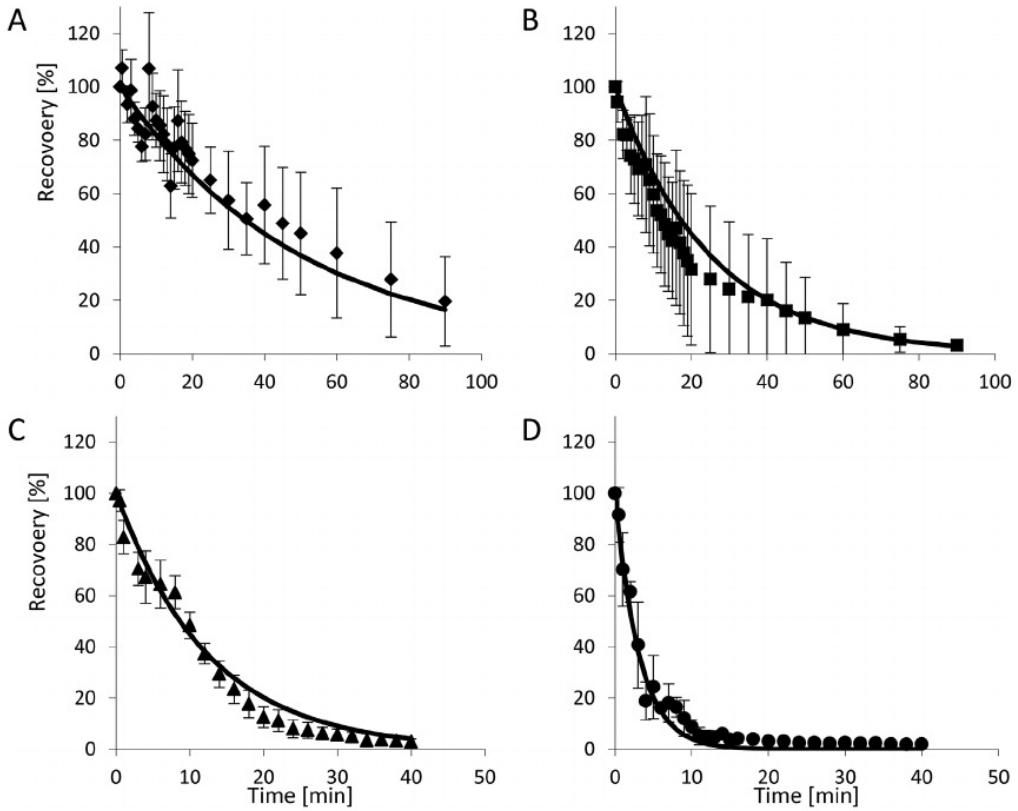

Fig. 5. Sodium citrate concentration determines endotoxin recovery Endotoxin recovery is plotted as a function of incubation time. 100 EU/mL of endotoxin were added to samples containing varied sodium citrate concentrations (5 mM (A), 10 mM (B), 20 mM (C), 80 mM (D). The concentration of polysorbate 20 (0.05 (w/v) %) was kept constant. The data points are mean values of four (A) or three (B, C, and D) individually prepared kinetics and the error bars reflects the corresponding standard deviations. For the simulation (—), an exponential decay function was used (Equation (1)). For a better comparison of independent measurements, the experimental data was normalized and the starting points were set to 100%. Correlation coefficients of experimental and simulated data are 0.95 using 5 mM sodium citrate, 0.98 using 10 mM sodium citrate, 0.99 using 20 mM sodium citrate and 0.99 using 80 mM sodium citrate. The initial measurement of the sample was 131 EU/mL containing 5 mM sodium citrate (A), 140 EU/mL containing 10 mM sodium citrate (B), 141 EU/mL containing 20 mM sodium citrate (C) and 126 EU/mL containing 80 mM sodium citrate (D).

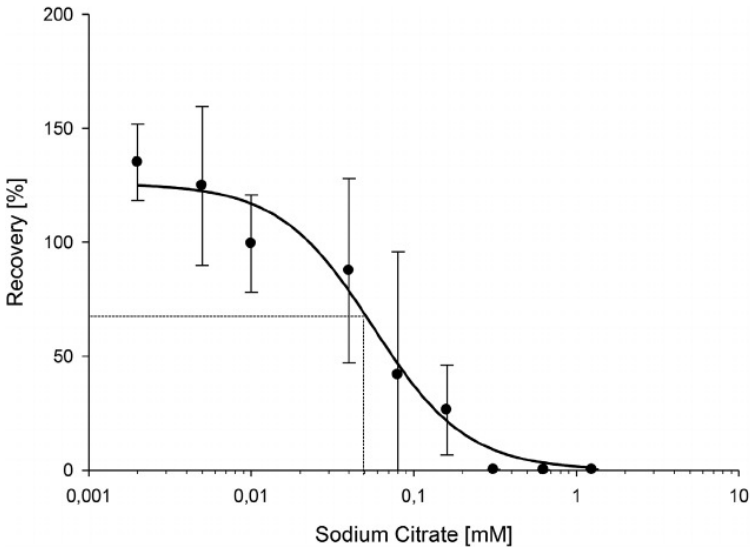

Fig. 6. Endotoxin recovery depends on sodium citrate concentration under equilibrium conditions Endotoxin recovery is plotted as a function of sodium citrate concentration. 50 EU/mL endotoxin and 0.05 (w/v) % polysorbate 20 were incubated with varying sodium citrate concentrations for seven days prior to the measurement. For calculation of the data points the mean values of two individual performed repetitions were used. Error bars reflect standard deviations. For the corresponding fit, a logistic function with three parameters was used (Equation (2)).

4. Discussion

LER has been identified as a time-dependent phenomenon [16,17] whereas, in contrast, test interference occurs instantly [23,24]. The latter can therefore be clearly distinguished from LER. The results presented here show that LER in a sample may not be discovered when the spike is added to the diluted sample. Masking will be not observed or weakened when the concentration of a matrix component is reduced. For example, Fig. 3A shows a significant delay in time until LER is recognized when endotoxin is spiked into a diluted sample. Therefore, it is important to add endotoxin spikes into undiluted samples in order to identify if a sample is affected by LER. Aside from controlling test interference in BET, which is well regulated by international Pharmacopoeias, test procedures for analysis of LER are not regulated, yet. The current FDA guidelines for BET [18] and the 9th edition of European Pharmacopoeia request the performance of hold-time experiments in order to ensure reliable detection of endotoxin over time. However, it is necessary to generally extend and harmonize worldwide compendial test procedures to thoroughly identify the endotoxin masking capability of a sample. Nevertheless, to control the phenomenon of LER, understanding of the masking mechanism is critical. There are several examples showing that endotoxins interact with a variety of components, including proteins [25], surfactants [26] and nano particles [27], but these more complex reaction mechanisms remain to be elucidated. Most likely, due to the amphiphilic and amphoteric character of LPS [28], hydrophobic and electrostatic interactions are involved. Recently, a two-step masking mechanism was described [16], which can be formally described as follows:

$$[\text{M-LPS}] + [\text{Ci}] \leftrightarrows [\text{LPS}] + [\text{M-Ci}] \quad (3)$$

Complex forming agents (Ci) destabilize the salt bridges formed between divalent cations (M) (e.g. magnesium) and negatively charged substitutes (e.g. phosphates) of LPS (LPS) (Equation (3)).

$$[\text{LPS}] + [\text{P}] \leftrightarrows [\text{P-LPS}] \quad (4)$$

Subsequently, non-ionic surfactants (P) (e. g. polysorbate 20) can interact with LPS and result in an altered supramolecular structure of LPS (P-LPS) (Equation (4)), leading to a change in detectable activity. For a deeper understanding of this mechanism, identification of the time dependent reaction step is necessary. To this end, the kinetics of endotoxin masking was studied in the presence of citrate and polysorbate 20. Interestingly, pre-incubation of LPS with citrate and the subsequent addition of polysorbate 20 resulted in very fast masking kinetics, while pre-incubation of LPS with polysorbate 20 and subsequent addition of citrate resulted in somewhat slower kinetics (Fig. 1). Furthermore, masking kinetics with increased citrate concentrations and constant polysorbate 20 concentrations (Fig. 5) show accelerated reaction rates. In turn, a variation of polysorbate 20 concentration under otherwise identical conditions had no substantial impact on the reaction rate (Fig. 3B). Therefore, the first step of the reaction mechanism (Equation (3)) appears to control the reaction rate and is dependent on the citrate concentration.

4.1. Simulation of LER kinetics

To establish a simplified model describing the reaction rate, only the first step of the reaction (Equation (3)) will be considered. The second step of the reaction (Equation (4)) can be neglected, as this step is very fast and does not limit the reaction rate within the given conditions. Basically, the reaction rate [R] is given by the change of detectable endotoxin [LPS_d] as a function of time t and can be expressed as follows:

$$R = d[LPS_d]/dt \quad (5)$$

In addition, the results indicate that the reaction rate depends on the citrate concentration (Fig. 5). Generally, it is assumed that the reaction rate depends on the LPS concentration, although the results here indicate that there is no dependence on LPS concentration (Fig. 2). Thus, the reaction rate R of endotoxin masking depends on the detectable LPS [LPS_d] and the citrate concentration [Ci] resulting in the following equation:

$$R = k[LPS_d][Ci] \quad (6)$$

Equalizing equations (5) and (6) results in a differential function, which is the basis for a second order reaction kinetics:

$$d[LPS_d]/dt = k[LPS_d][Ci] \quad (7)$$

After rearrangement and integration of equation (7), the rate equation of a second order reaction is obtained, provided that the concentrations of LPS [LPS_d] and citrate [Ci] are not equal [29]:

$$(1/([Ci]_o - [LPS_d]_o)) * (\ln([Ci]/[Ci]_o)/([LPS_d]/[LPS_d]_o)) = kt \quad (8)$$

However, the change of detectable [LPS_d] is based on the change of activity, which is usually given in EU/mL, whereas citrate is given in M (mol/L). In order to convert EU/mL in mol/L it is assumed that 1 EU correlates approximately to 10^{-10} g (100 pg) LPS from E. coli [30]. With a molar mass of approximately 10,000 g/mol for LPS, 100 EU/mL are equivalent approximately to 10^{-9} mol/L [31,32]. Therefore, 10^{-3} mol/L citrate is in a substantial molar excess compared to 10^{-9} mol/L of LPS ([Ci]»[LPS]). Theoretically, this would lead to reaction kinetics of pseudo first order, because the concentration of citrate will not change significantly during the reaction and can therefore be neglected. However, results here (Fig. 5) show that the actual citrate concentration has a significant effect on the reaction kinetics. Furthermore, the results indicate that the kinetics are independent of the LPS concentration, which is reasonable, because citrate is in an excess of up to seven orders of magnitude [Ci]»[LPS]. A change of the LPS concentration, for example by a factor of 1,000, will not affect the kinetics, since citrate would still be in excess. Keeping this in mind, the starting molar concentration of citrate $[Ci]_o$ will only be marginally reduced by subtraction of the initial molar concentration of LPS [LPSd] o and leads to the following approximation:

$$[Ci]_o - [LPS]_o = [Ci]_o \quad (9)$$

Concomitantly, the marginal consumption of [Ci] due to the low molar concentration of LPS is also negligible and allows for the following assumption:

$$[Ci]/[Ci]_o = 1 \quad (10)$$

With respect to the equations (9) and (10) equation (8) can be approximated and written as follows:

$$(1/([Ci]_o) * (\ln(1/([LPS_d]/[LPS_d]_o)) = kt \quad (11)$$

Finally, the equation can be rearranged to give the detectable concentration of LPS [LPS_d] as a function of time, and viewed as dependent on the citrate concentration:

$$[LPS_d] = [LPS_d]_o * \exp(-[Ci]kt) \quad (12)$$

Using equation (12), we simulated the kinetics using various citrate concentrations. The simulated curves correlate very well with the experimentally determined data (Fig. 5). Consequently, this model accurately reflects the observed behavior of the reaction, in which the endotoxin recovery is strongly dependent on the citrate concentration. However, specifications may change if the initial concentrations of the components or pH (chelation of the divalent ions is pH dependent) are substantially changed or if additional components (e.g. salts and proteins) are included in the sample conditions.

4.2. Minimum citrate concentration

Citrate has been identified as the key component controlling the reaction kinetics of endotoxin masking. To get a deeper understanding of the role of citrate, the minimal concentration of citrate required to initiate masking at constant polysorbate 20 concentrations was analyzed. Due to the fact that masking of endotoxin is time dependent, the minimal concentration was determined after seven days of sample incubation. The results show that approximately 0.06 mM of citrate is necessary for masking after that incubation period (Fig. 6). Hence, the required minimal concentration is apparently lower, than the commonly used citrate concentrations of approximately 10 mM.

It is important to note that the minimal concentration of citrate is orders of magnitude higher than the molar concentration of LPS, assuming that there is no reasonable reaction stoichiometry. However, citrate concentrations in this range potentially lead to permeabilized and destabilized LPS aggregates. Primarily, magnesium (Mg^{2+}) as well as calcium cations (Ca^{2+}) stabilize LPS-LPS interactions by the formation of salt bridges [14,33–35]. For example, permeabilization (reduction of rigidity) of LPS aggregates occurs when ionic interactions between LPS molecules are disturbed. It has been shown that complex forming agents can permeabilize such structures [36,37]. Thus, it is theorized that citrate competes for divalent cations bridging LPS molecules resulting in a permeabilization of LPS aggregates due to complex formation [38]. The corresponding complex formation constants of magnesium and calcium citrate are in the range around 0.05 mM [22,39,40]. This may explain the molar excess of citrate required for masking as compared to LPS, because at lower citrate concentrations the complex formation of calcium or magnesium by citrate is not favored. Thus, under these conditions the LPS-LPS salt bridges are not destabilized, which prevents the intercalation of surfactants between LPS molecules and thus prevents the masking of endotoxin.

4.3. The role of divalent cations

As described above, divalent ions play an important role in the stabilization of supramolecular LPS structures. LPS aggregates possess a certain degree of rigidity, maintained by salt bridges between LPS molecules, which in turn affect the susceptibility to masking. Thus, the masking rate can be inhibited by the supplementary addition of divalent magnesium ions. This explains a previous observation that showed under certain circumstances the endotoxin activity in LAL can be maintained through the suppression of aberrant aggregation of endotoxin by saline and buffers [41]. It is most likely that the addition of divalent cations neutralizes the complex formation capability of such complex forming agents and favors a stabilized LPS state. Importantly, the retrospective addition of divalent ions to samples in which the endotoxin is masked, enabled no recovery of endotoxin, but showed that the progress of masking can be immediately stopped (Fig. 4B). The effective addition of cations must take place before endotoxin masking occurs. These results demonstrate that the destabilization of LPS (Equation (3)) can be prevented by the addition of divalent ions, but when LPS is already masked, the consecutive reaction step (Equation (4)) is not affected by supplementary addition of divalent ions. To achieve a reversal of masked endotoxin masking (demasking), the mere addition of divalent ions is not sufficient. For such an approach, surfactants like polysorbate would need to be neutralized or removed.

4.4. The role of surfactants

It has been shown that if only a complex forming agent (e.g. sodium citrate) and no surfactant (e.g. polysorbate 20) is present, no masking is expected [16], indicating that surfactants are a prerequisite for the induction of masking. Titration of sodium citrate at constant polysorbate 20 concentrations lead to a substantial deceleration of masking kinetics (Fig. 5). In contrast, the kinetics with reduced polysorbate 20 concentrations showed no trend towards decelerated masking kinetics. Kinetics with reduced polysorbate 20 concentrations showed rather a slight acceleration of masking kinetics. This result is surprising, as an opposite behavior would be expected by reducing the concentration of polysorbate 20. Due to the fact that the presence of polysorbate 20 is indispensable, this result suggests that there is an optimum concentration for masking the endotoxin. This is conceivably, because supramolecular aggregation states are concentration dependent and a certain state might be more effective in masking the endotoxin. However, the change of recovery kinetics depending on polysorbate 20 concentration is minor compared to the change of recovery kinetics that depend on citrate concentration. Moreover, LAL-based test methods are subject to variations and therefore the differences dependent on various polysorbate 20 concentrations should not be over-interpreted.

4.5. Control of reaction rate

To start the reaction of endotoxin masking, a certain energy barrier that controls the reaction rate has to be overcome. The reaction rate can be manipulated by adding energy (e.g. incubation temperatures) to the system or shifting equilibrium states (eg. component concentrations). For instance, the reaction kinetics can be accelerated by e.g. increasing chelator concentration (see Fig. 5) or by e.g. increasing incubation temperature of a sample [16]. Moreover, it is conceivable that the degree of the energy barrier depends on the endotoxin itself. Endotoxin from different sources have different molecular structures [8] and in turn different masking susceptibilities due to varying stabilization mechanisms of various bacterial outer membrane structures. Therefore, endotoxins with different inherent stabilizing mechanisms may have different energy barriers and thus result in manipulated reaction kinetics. To clarify this effect, the heterogeneity of endotoxins as they occur in nature and their detectability in samples affected by LER is currently being studied in this laboratory.

5. Conclusion

The reaction kinetics presented here are consistent with the recently proposed two-step reaction mechanism [16] of endotoxin masking. The first reaction step, chelation of the divalent cations has been identified as the crucial step to mask endotoxin in the presence of surfactants. These results help to understand the time-dependent occurrence of LER. Moreover, confounding observations in similar drug product formulations can be explained, because the singular consideration of the presence of certain excipients is insufficient. The concentrations of components play an important role in the causation of LER. Finally, LER is a complex phenomenon due to the interplay of many sample components (active pharmaceutical ingredient (protein), buffer, surfactants, sugars, salts, etc.), but can be better understood by carefully accounting for all relevant variables. Also note that the occurrence of LPS binding to proteins [25] may occur and does not necessarily require a chelating buffer or polysorbate 20.

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