

Low Endotoxin Recovery - Detection of Endotoxins in Common Biopharmaceutical Product Formulations



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...no matter how many instances of white swans we may have observed, this does not justify the conclusion that *all* swans are white.

Karl Popper, 1934, Austrian and British Philosopher

Table of Content

I.	Preface	7
II.	Zusammenfassung	9
III.	Abstract	11
IV.	Abbreviations	12
1	Fundamentals.....	13
1.1	Endotoxin (LPS)	13
1.2	Structure and activity of LPS	15
1.3	Clinical relevance of LPS.....	16
1.4	Need for control of endotoxins and their detection methods	18
1.5	Regulatory aspects	20
1.6	Low Endotoxin Recovery	20
1.7	Purpose of the study	21
2	Material and Methods	23
2.1	Reagents.....	23
2.2	Endotoxins and bacteria.....	23
2.3	Other materials	24
2.4	Preparation of crude endotoxins extracts	24
2.4.1	Preparation 1.....	24
2.4.2	Preparation 2.....	24
2.5	Sample handling.....	25
2.5.1	Addition of endotoxin to a sample.....	25
2.5.2	Sample preparation before endotoxin detection	25
2.5.3	Incubation of endotoxin in a sample.....	25
2.5.4	Preparation of endotoxin recovery kinetics.....	25
2.5.5	Endotoxin recovery and masking controls.....	26
2.5.6	Sample preparation for demasking of endotoxin	26
2.6	<i>Limulus</i> -based endotoxin detection assays	27
2.6.1	Recombinant Factor C assays (rFC)	27
2.6.2	<i>Limulus</i> Amebocyte Lysate assay (LAL)	27
2.7	Silver stained SDS-PAGE	27
2.8	Calculations and plots	28
3	Results and Discussion	29
3.1	Masking of endotoxin in surfactant samples: Effects on <i>Limulus</i> -based detection systems.....	29

3.1.1	Introduction.....	29
3.1.2	Results	31
3.1.3	Discussion	37
3.2	Endotoxin Masking: A kinetically controlled reaction mechanism	41
3.2.1	Introduction.....	41
3.2.2	Results	42
3.2.3	Discussion.....	49
3.3	Heterogeneity of endotoxins and their detectability in common biopharmaceutical formulations.....	54
3.3.1	Introduction.....	54
3.3.2	Results	56
3.3.3	Discussion.....	63
3.4	Demasking of Endotoxin.....	68
3.4.1	Introduction.....	68
3.4.2	Results	71
3.4.3	Discussion.....	79
4	Conclusions.....	86
4.1	Endotoxin demasking – a technical solution.....	86
4.2	Perspectives of sample treatments in BET	87
4.3	Need for structural analysis of endotoxins in complex sample matrices	88
4.4	Clinical relevance of masked endotoxin.....	89
4.5	Key findings	91
5	Appendix.....	93
5.1	List of figures	93
5.2	List of tables	95
5.3	References.....	96
5.4	List of publications.....	107
5.4.1	Selected poster presentations.....	107
5.4.2	Oral presentations.....	107
5.4.3	Published articles.....	108
5.4.4	Intellectual Property.....	108
5.4.5	Declaration	109
5.5	Curriculum Vitae.....	110
5.6	Eidesstattliche Erklärung.....	111
6	Annex.....	113

I. Preface

This work was carried out between October 2012 and June 2016 at the Hyglos GmbH, Research and Development Department, Bernried, Germany in collaboration with the University of Regensburg, Institute of Physical and Theoretical Chemistry, Regensburg, Germany under the supervision of Prof. Dr. Hubert Motschmann.

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II. Zusammenfassung

Bakterien gehören zu den ersten und ältesten Lebewesen auf der Erde und aufgrund ihrer Allgegenwärtigkeit ist der Mensch permanent in direktem Kontakt mit ihnen. In dieser Arbeit wird einem Abbauprodukt der Gram-negativen Bakterien, den sogenannten Endotoxinen, spezielle Aufmerksamkeit gewidmet. Diese sind der Hauptbestandteil der äußeren Membran von Gram-negativen Bakterienzellen und werden während des Wachstums oder beim Absterben und der Lyse freigesetzt. Endotoxine sind für den Menschen generell nicht gesundheitsschädlich, solange sie nicht in den Blutkreislauf eingetragen werden. In diesem Fall können sie jedoch zu schwerwiegenden pathogenen Wirkungen führen (z.B. Sepsis). Ein potentiell Risiko der Übertragung in den Blutkreislauf ist beispielsweise bei der Injektion von Medikamenten gegeben. Um dieses Risiko zu minimieren, sind Hersteller von injizierbaren Medikamenten (Parenteralia) verpflichtet, diese streng auf Endotoxin-Kontaminationen zu überprüfen. Hierzu wird üblicherweise der „*Limulus* Amöbozyten Lysat“ Test verwendet. Dieses Testsystem basiert auf enzymatischen Reaktionen, die der Blutgerinnungskaskade von Pfeilschwanzkrebsen entstammen. Aufgrund der Sensitivität und Anwenderfreundlichkeit stellt diese Methode seit Jahren den „Goldstandard“ zur Bestimmung von Endotoxinen dar. Seit neuestem werden jedoch Unstimmigkeiten während der Testung von biopharmazeutischen Medikamenten beobachtet. In bestimmten Medikamenten konnten die Positivkontrollen nicht innerhalb bestimmter Akzeptanzkriterien detektiert werden. Dieser Effekt wird als „*Low Endotoxin Recovery (LER)*“ bezeichnet und führt zu Bedenken auf Anwender- und Behördenseite, ob diese Testmethode weiterhin zuverlässig und anwendbar ist.

In dieser Arbeit wurde die Detektierbarkeit von Endotoxin in biopharmazeutischen Formulierungen mittels *Limulus*-basierten Testmethoden untersucht, um die Ursachen des LER-Phänomens zu verstehen und die gegenwärtigen Testmethoden zu optimieren. Die präsentierten Daten machen deutlich, dass allgemein verwendete Formulierungsbestandteile der Medikamente den LER-Effekt verursachen können. Als Hauptursache wurde die simultane Anwesenheit von Tensiden und Chelatoren identifiziert. Des Weiteren wird gezeigt, dass LER ein zeitabhängiges Phänomen ist und die Reaktionsgeschwindigkeit hauptsächlich von der Konzentration des Chelators in der Probenmatrix abhängt. Darüber hinaus wurden verschiedene Endotoxine untersucht, die aufgrund ihrer strukturellen Heterogenität unterschiedliche Empfindlichkeiten hinsichtlich der Detektierbarkeit aufweisen. Zusammengefasst deuten die Daten darauf hin, dass sich das Endotoxin in einem zweistufigen Prozess in Tensidmizellen der Probenmatrix einlagert und somit für die Detektion maskiert ist. Mittels dieser Arbeitshypothese wurde eine „Toolbox“, mit verschiedenen amphiphilen und chaotropen Reagenzien zur Demaskierung des Endotoxins entwickelt. Schließlich wird gezeigt, dass durch eine gezielte

Probenvorbereitung der LER-Effekt aufgehoben und das Endotoxin mit herkömmlichen *Limulus*-basierten Testmethoden wieder detektiert werden kann.

III. Abstract

Bacteria are one of the first and oldest living organisms on earth and due to their ubiquity, humans are permanently in close contact to them. This work focuses on special breakdown products of Gram-negative bacteria so-called endotoxins. Endotoxins are the major component of the outer membrane of Gram-negative bacteria. They are released during growth or death and lysis of the bacterial cell. Endotoxins are usually not hazardous to man, as long as they do not enter the circulating blood system. However, if for example endotoxins enter the blood system they can lead to severe pathogenic effects like sepsis. One prominent risk is the accidental injection of contaminated drug products. In order to reduce this risk, manufacturers of parenteral drug products are forced to meticulously control their products before they are released to the market. To this end, *Limulus* Amebocyte Lysate (LAL) assays have been the gold standard for detection of bacterial endotoxins since years. These assays are based on enzymatic reactions derived from the blood coagulation cascade in horseshoe crabs. Most quality control departments in pharmaceutical industry have established such methods to release their drug products, because these assays enable fast and sensitive results. However, in the recent past inconsistencies during testing of biopharmaceutical drug products have been observed. In certain drug products, positive controls of endotoxin were not detectable within given acceptance criteria. This effect is called Low Endotoxin Recovery (LER) and users as well as authorities are concerned about the reliability of the existing test procedures.

In this work, the detectability of endotoxin in biopharmaceutical drug product matrices using *Limulus*-based detection systems was analyzed to understand the phenomenon of LER and to optimize existing test procedures. The results show that common formulation components of biopharmaceutical drug products are capable to induce LER. The minimum prerequisite is the simultaneous presence of surfactants and complex forming agents. It is demonstrated that the occurrence of LER is time-dependent and that the reaction rate of LER is substantially depending on the concentration of the complex forming agents in the sample matrix. Moreover, endotoxins from different sources were studied, because their structural heterogeneity may lead to different masking susceptibilities. Together, these results indicate that LER is caused by masking of endotoxin leading to an alteration of its supramolecular structure, in which endotoxins are embedded in surfactant micelles. The further elucidation of a two-step LER reaction mechanism served as a basis for the development of a toolbox including amphiphilic and chaotropic reagents, which enables the demasking of endotoxin. In conclusion, the dedicated sample treatment using such reagents allows the detection of LER-affected endotoxin by *Limulus*-based detection methods.

IV. Abbreviations

API	Active Pharmaceutical Ingredient
Ara4N	4-Amino-4-Deoxyarabinose
B	BSA
<i>B.cepacia</i>	<i>Burkholderia cepacia</i>
BD	BSA + Dodecanol
BET	Bacterial Endotoxin Testing
BSA	Bovine Serum Albumin
C	Calcium Dichloride
Ci	Citrate
Ca ²⁺	Calcium ²⁺
CBSD	Calcium dichloride+ BSA+ SDS + Dodecanol
CMC	Critical Micelle Concentration
CSE	Control Standard Endotoxin
D	Dodecanol
<i>E.cloacae</i>	<i>Enterobacter cloacae</i>
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic Acid
EU	Endotoxin Unit
FDA	Food and Drug Administration
HLB	Hydrophilic Hydrophobic Balance
LAL	<i>Limulus</i> Amebocyte Lysate
LB	Lysogeny Broth
LER	Low Endotoxin Recovery
LPS	Lipopolysaccharides
MAT	Monocyte Activation Test
NOE	Naturally Occurring Endotoxin
OMV	Outer Membrane Vesicle
<i>P.aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBS	Phosphate-Buffered Saline
<i>P.mirabilis</i>	<i>Proteus mirabilis</i>
PPC	Positive Product Control
<i>R.pickettii</i>	<i>Ralstonia pickettii</i>
rFC	Recombinant Factor C
rpm	Round per Minute
RPT	Rabbit Pyrogen Test
RSE	Reference Standard Endotoxin
RT	Room Temperature
S	SDS
<i>S.maltophilia</i>	<i>Stenotrophomonas maltophilia</i>
<i>S.marcescens</i>	<i>Serratia marcescens</i>
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TRIS	2-Amino-2-(Hydroxymethyl)-1,3-Propanediol

1 Fundamentals

Bacteria are one of the first and oldest living organisms on earth and their diversity is practically unlimited. Since modern analytical techniques enable the determination of genetic codes in bacteria, they can be categorized by their molecular phylogeny[1]. However, for historical and practical reasons bacteria are also classified by structural differences in their cell walls. Based on chemical and physical properties of the cell walls, bacteria can be differentiated between Gram-positive and Gram-negative. This dates back to times in which bacteria were stained (e.g. methyl violet) to enhance the visibility of bacteria [2].

1.1 Endotoxin (LPS)

Endotoxins are a unique group of molecules, which occur naturally in the cell wall of Gram-negative bacteria[3]. They are derived for example from various bacteria like *Neisseria meningitides*, *Vibrio cholera* or *Escherichia coli* (Figure 1A). If administered into the blood stream of mammals, bacteria and their toxic breakdown products can cause severe pathogenic effects including fever and septic shock[4]. Due to their fever inducing capability, endotoxins are also classified as pyrogens.

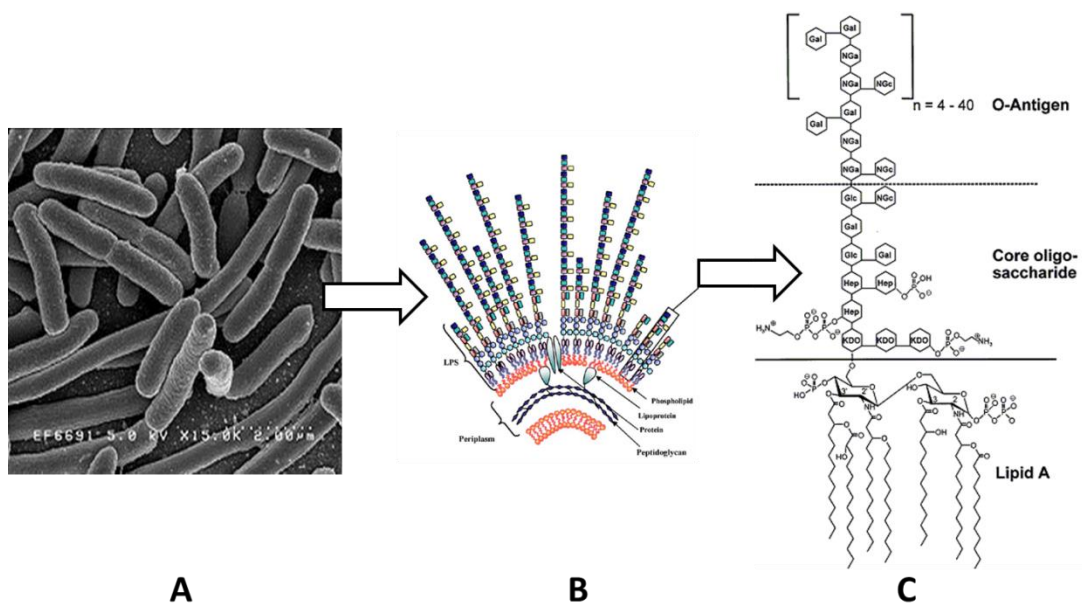


Figure 1 Gram-negative Bacteria

A) Scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip. (Credit: National Institute of Health, National Institute of Allergy and Infectious Diseases, Image Library). **B)** Schematic representation of the Gram-negative membrane. The outer membrane possesses an asymmetric bilayer, in which LPS covers mostly the surface and phospholipids are located in the inner leaflet as counter molecules. In addition, a variety of further components like proteins, lipoproteins, peptidoglycans are present in the cell envelope of Gram-negative bacteria. (Source: [4]) **C)** Chemical structure of LPS from *E. coli* O111:B4 LPS. The particular regions of LPS (lipid A, core region and O-antigen) and the variability of sugar units are indicated. (Source: [5])

The end of the 19th century is of particular importance in the context of endotoxins. Dramatic cholera outbreaks threatened large harbor cities including Hamburg, Germany and St. Petersburg, Russia and have led to a large number of death. During that time, systematic research began and is still ongoing to better understand the hazardous effects of Gram-negative bacteria and endotoxins (Figure 2).[6]

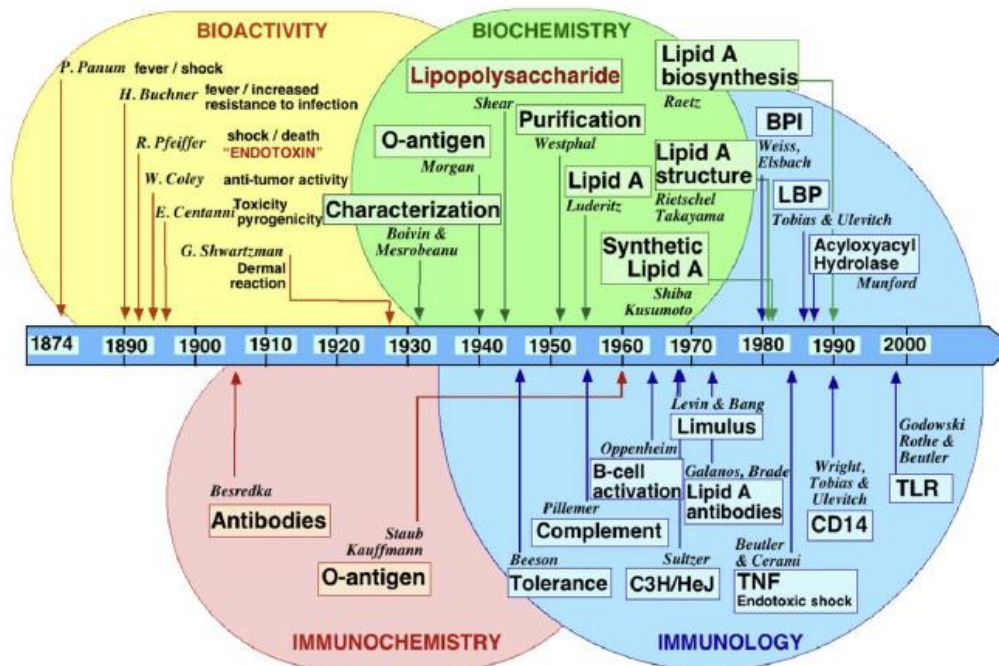


Figure 2 The history of endotoxin research

Research on endotoxins started in the end of 19th century with the description of the deleterious and beneficial bioactivities of endotoxins. A further phase comprised efforts undertaking their biochemical and immunochemical characterization, while the immunological properties of this molecule were mainly defined in the mid-20th century. (Source:[7])

Around 1890, Richard Pfeiffer, a co-worker of Robert Koch, stated:

*“In ganz jungen, aerob gezüchteten Cholerculturen ist ein spezifischer Giftstoff enthalten, welcher ausserordentlich intensive toxische Effecte entfaltet. Dieses primäre Cholergift steht in sehr enger Zusammengehörigkeit zu den Bacterienleibern und ist vielleicht ein integrireder Bestandtheil derselben. Durch Chloroform, Thymol und durch Trocknen können die Cholervibrien abgetödtet werden, ohne dass dieser Giftstoff anscheinend verändert wird.”*¹[8]

¹ In very young aerobically grown *cholera* cultures, a specific toxic substance is contained which exerts extraordinarily intense toxic effects. This primary *cholera* toxin is closely attached to, and probably an integral part of, the bacterial body. By chloroform, thymol, or by drying, the *cholera vibrios* can be killed without any detectable change of the toxin[6].

This was the genesis of the concept of endotoxins. Within the mid of the 20th century, it was figured out that endotoxins are located at the surface of Gram-negative bacteria (Figure 1B) and are liberated when bacteria multiply, die and lyse[9]. During the development of techniques for extracting and preparing endotoxins, lipopolysaccharides (LPS) (Figure 1C) were identified in bearing the toxic principle of endotoxin[3]. To this end, the two terms *endotoxin* and *LPS* are used for the same molecule and thus represent synonyms. However, the term *endotoxin* reflects its biological activity and the term *LPS* its chemical composition[10]. Moreover, it has been shown that LPS are the dominating constituents of the outer membrane of Gram-negative bacteria and LPS play an important role in maintaining the integrity of the membrane architecture and is therefore an essential component for bacterial viability. Noteworthy, LPS are accompanied by certain proteins and lipids, but LPS covers three-quarters of the bacterial surface and one bacterial cell contains approximately 3.5×10^6 LPS molecules.[9]

1.2 Structure and activity of LPS

LPS are a broad and complex group of molecules, which possess a common general architecture[11]. The molecules can be divided in three parts: O-antigen, core region and lipid A (Figure 3)[12]. The latter is based on a phosphorylated diglucosamine which is esterified with fatty acids (e.g. caproic acid (C6), lauric acid (C12), myristic acid (C14), palmitic acid (C16), stearic acid (C18)) and anchors the molecule in the outer membrane of Gram-negative bacteria and is covalently substituted by a saccharide portion. The core region comprises an oligosaccharide containing up to fifteen monosaccharides (e.g. diverse heptoses, glucose, galactose and mannooctulosonic acid) to which a polysaccharide portion of repeating units, the O-specific chain including glucose, galactose, rhamnose and mannose, is linked.[10] Noteworthy, also LPS mutants lacking the O-antigen have been isolated. These LPS forms are denoted as rough LPS, whereas LPS containing O-antigen are called smooth LPS[13]. However, it has been shown that the lipid A part represents the “endotoxic principle” of LPS, and in contrast to the role of the polysaccharides (core region and O-antigen), alterations of the lipid A moiety were found to influence the bioactivity dramatically[14], [15]. Full endotoxic activity is expressed by a molecule containing two hexosamine residues, two phosphoryl groups, and six fatty acids including 3-acyloxyacyl groups with a defined chain length and at a distinct location[9]. As a consequence, not all LPS are toxic, just as not all bacteria are pathogenic[3]. Moreover, due to the hydrophilic (O-antigen and core region) and hydrophobic (lipid A) regions, LPS tend to aggregate in aqueous solutions. Depending on the molecular structure and environmental conditions diverse supramolecular structures either non-lamellar inverted (cubic Q or hexagonal H_{II}) or lamellar can be formed[16]. It has been shown in several studies that the aggregation state of LPS affects its biological activity as well as its detectability.[14], [16]–[19]

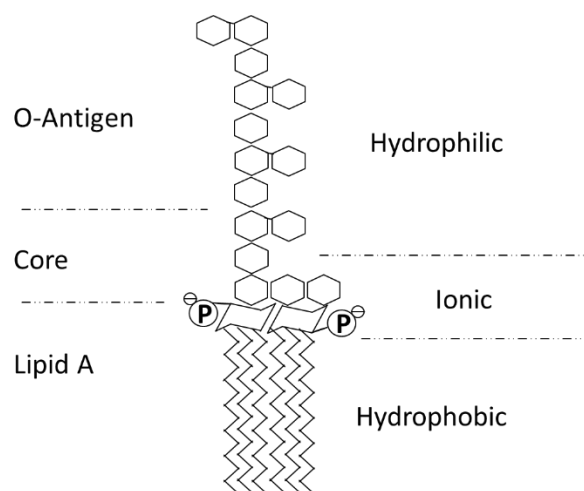


Figure 3 Schematic structure of lipopolysaccharides

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

1.3 Clinical relevance of LPS

Years ago, clinicians have recognized that humans are especially sensitive towards endotoxin. In some cases, intravenous infusions containing bacterial contaminants have led to death and severe pathogenic response in patients.[20]–[22] Thereby, LPS are able to induce a variety of biological effects *in-vivo* (Figure 4). To fight against pathogenicity, bacteria and its LPS are the primary target for interaction with antibacterial drugs and components of the immune system of the host.[17]

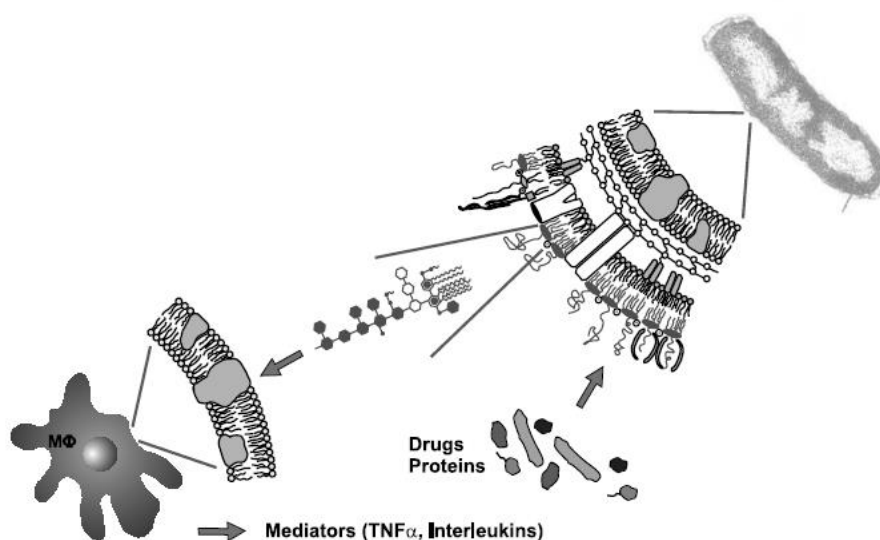


Figure 4 Schematic representation of the activation mechanisms induced by LPS

LPS is released from the bacterial outer membrane by the attack of immune components (drugs, proteins) or simply by cell division. It may interact with serum and membrane proteins, which subsequently leads to an activation of macrophages, which secrete mediators such as tumor necrosis factors alpha and interleukins. (Source: [17])

In order to understand pathogenicity, an important finding has been the identification of the plasma membrane protein Toll-like receptor 4 (TLR4) as the lipid A signaling receptor of animal cells. Activation of TLR4 by lipid A triggers the biosynthesis of diverse mediators of inflammation including tumor necrosis factor (TNF) or interleukins, ultimately resulting in multiple organ failure, septic shock in the case of systemically overproduction.[23] Generally, TLR4 belongs to a family of innate immunity receptors (Figure 5) and besides endotoxins, there are also other pathogens, which are not limited to Gram-negative bacteria (Lipoteichoic acids, peptidoglycans, proteins etc.) and stimulate the innate immune system resulting in pyrogenic reactions. However, endotoxins are considered to be an outstanding alarm marker due to their relatively high pyrogenicity. Endotoxins are active in the picogram range per kilogram bodyweight. Therefore, a little amount of endotoxin can generate a very strong host response.[24] In contrast, peptidoglycans are 50,000 times less pyrogenic than endotoxins[25].

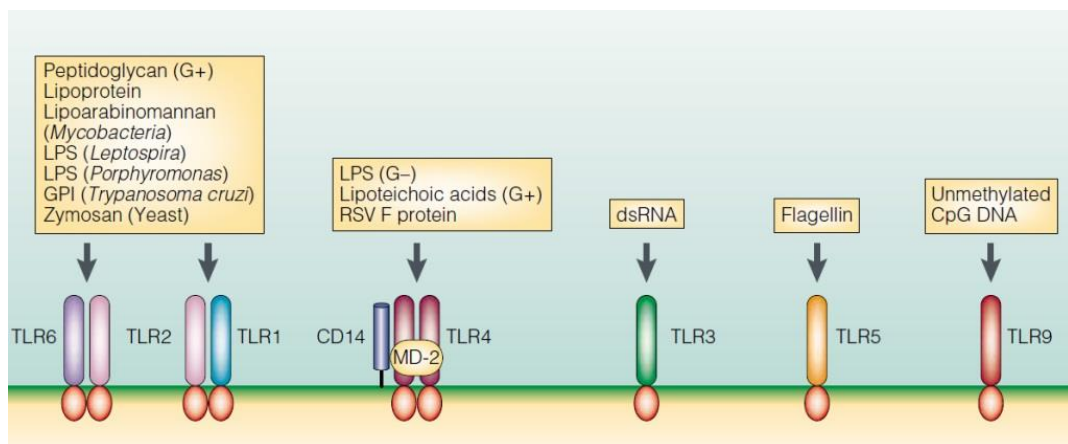


Figure 5 The Toll-Like-Receptor family

Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns. Recognition of LPS by TLR4 is aided by accessory proteins (CD14 and MD-2). TLR2 recognizes a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. TLR3 is involved in recognition of double-stranded RNA. TLR5 is specific for bacterial flagellin, whereas TLR9 is a receptor for unmethylated CpG sequences in DNA. (Source [26])

For further comprehension of the pyrogenicity of endotoxins, dose-febrile response curves for endotoxins have been studied and it was found that man, cat, horse and rabbits have approximately the same threshold to pyrogen simulation by endotoxins. However, larger doses are more pyrogenic and more toxic for man than for rabbit, due to much steeper dose-febrile response curves for man. Dogs and chimpanzee were notably less susceptible to the pyrogenicity of endotoxin than the other species.[27] Moreover, depending on the source of endotoxin different thresholds were found to initiate pyrogenicity. For example approximately 0.1 to 1.0 nanogram per kilogram of bodyweight of endotoxin from *E.coli* is needed, whereas 50 nanogram per kilogram of bodyweight of endotoxin from *P.aeruginosa* are required for pyrogenic response in man[28]. This already gives an indication about the heterogeneity and complexity of

endotoxins. In order to enable a comparison of biological activities, endotoxin units (EU) were introduced, based on the approximated threshold of pyrogenic activity of *E.coli*. 1 EU reflects the biologic activity of 0.1 nanogram of purified endotoxin from *E.coli* in man.[24]

1.4 Need for control of endotoxins and their detection methods

The occurrence of Gram-negative organisms in virtually every environment on earth makes LPS one of the most prevalent complex organic molecules occurring in nature. Gram-negative bacteria have been isolated wherever man has gone: in soil, fresh and salt water, frigid oceans and hot springs. Minimal growth requirements of Gram-negative bacteria allow their growth in the cleanest of water.[24] Therefore, the ubiquity of endotoxins requires routine screening of all fluids and medications prepared for parenteral therapy.[27] Although the pyrogenic dose response curve in man is much steeper than it is in rabbits, the minimum pyrogenic dose on a weight basis in rabbits is in a passable range compared to man[28]. Hence, the Rabbit Pyrogen Test (RPT) was introduced as an *in-vivo* test method for the detection of fever-causing (pyrogenic) contaminations in pharmaceutical products, and has already been manifested in various pharmacopoeias and guidelines around the world in the 1940th[29]. Over the years, alternative *in-vitro* detection methods were discovered and established. One method of pyrogen detection relies on mimicking the human fever reaction, which can be found in the Monocyte Activation Test (MAT). It employs the cytokine response of blood monocytes for the detection of microbiological contaminants.[30] However, handling of appropriate blood or cell lines for running the assay and regulatory issues prolong the universal application of this assay.

Another and more prominent method was discovered by Bang and Levin in the 1960th. They utilized the defense system of an animal with over 450 million years of experience, fighting against microbial attacks. It has been demonstrated that bacterial endotoxins rapidly induce clotting of the blood of horseshoe crabs[31]. The amebocytes in horseshoe crabs' hemolymph contain a coagulation system, which is activated by minute amounts of endotoxin.[32] The principle of this test method is based on an enzymatic reaction cascade. In the presence of LPS, an LPS-sensitive serine protease zymogen Factor C, is autocatalytically activated. The active Factor C* then activates zymogen Factor B to Factor B*, which subsequently activates proclotting enzyme to clotting enzyme. The resulting clotting enzyme converts soluble coagulogen, an invertebrate fibrinogen-like substance, to an insoluble coagulin gel. (Figure 6B)[33]. This cascade system found in the hemocytes allows for an extremely high sensitivity of the lysate to picogram quantities of endotoxins. [34] For production of the so called *Limulus* Amebocyte Lysate (LAL), horseshoe crabs (Figure 6A) are collected when they migrate to shallow coastal waters for reproduction. Once collected, the lively crabs are placed in restraining racks. Sterile needles are

inserted through the muscular hinge between the cephalothorax and abdominal region[24], [32] and up to 150 mL blood of one horseshoe crab can be obtained. If the crabs are handled with care, they normally survive this procedure. Depending on country-specific regulations they are returned into the ocean or are further processed (e.g. fishing bait). However, after collection the blood is centrifuged and the harvested amebocytes are washed. The cells are lysed by addition of distilled water and cellular debris is removed by centrifugation. Finally, the lysate is decanted and can be used for testing.[35] Comparative studies between LAL tests and the RPT on various samples resulted in good agreement and the results achieved by LAL detection methods were more sensitive, when samples were properly handled[36], [37].

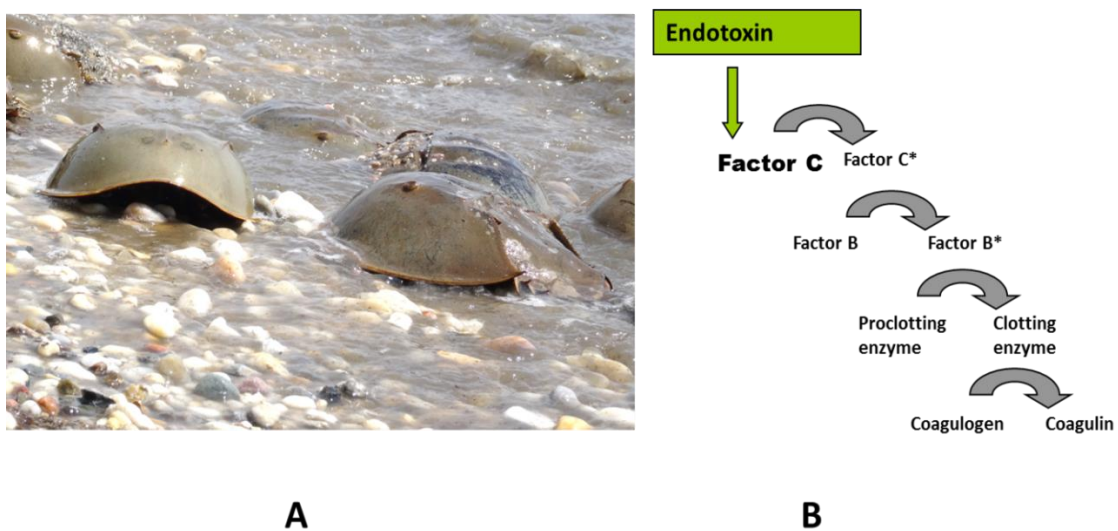


Figure 6 Horseshoe crabs and their endotoxin specific reaction cascade

A) Horseshoe crabs at Pickering beach, Delaware, USA. Horseshoe crabs are found along the northeast coasts in America and southeast coasts in Asia. For reproduction, adult crabs travel during late spring and early summer from deep ocean water to coastal water and females deposit eggs on the beaches.
B) Tentative reaction mechanism for the coagulation cascade of the *Limulus* amebocyte lysate with endotoxin.

Practical experience and technological progress led from simple test techniques, based on the occurrence of gel formation by the reaction of the lysate with endotoxin; to photometric techniques, which are based on the change in lysate turbidity during gel formation; and chromogenic techniques, which are based on the development of color after cleavage of a synthetic peptide-chromogen complex[38]–[40]. Moreover, growing demand and limited resources of horseshoe crabs are leading to the use of recombinant sources of *Limulus*-based enzymes. These novel techniques use, recombinantly produced Factor C (rFC), the first enzyme of the *Limulus* coagulation cascade, and a fluorogenic substrate is generating the signal[41], [42]. In the present work, mainly recombinant test methods were used. Today, these *Limulus*-based methods including LAL and rFC are recognized as the most sensitive *in-vitro* assays available for

bacterial endotoxin testing (BET). These methods are more economical and require a smaller volume of sample for testing than does the RPT and MAT. In addition, a large number of tests can be performed by one individual in a single day.

1.5 Regulatory aspects

Endotoxin is only a concern for man, when it comes into contact with the circulatory blood system. One relevant mechanism for such contact involves medically invasive techniques including injection or infusion of parenteral solutions[24]. Therefore, pharmaceutical regulatory agencies around the world are asking for BET in parenteral drug products. For example, the European Directorate for the Quality of Medicines (EDQM) states that

“bacterial endotoxins are the most common cause of pyrogenicity in pharmaceutical products. Any preparation administered parenterally should be sterile and comply with the test for bacterial endotoxins. Substances to be used in parenteral preparations must comply with the BET, whatever their origin...”[43]

In consequence, manufacturers of parenteral drug products are obliged to perform BET of in-process samples and finished products. Fortunately, the occurrence of contaminations in parenteral drugs, devices, infusions and transfusion solutions has been relatively rare since the introduction of BET.[24] In order to maintain such a high level, the meticulousness in quality control of pharmaceutical products has to be retained and continuously improved.

1.6 Low Endotoxin Recovery

With regard to certain pharmaceutical drug products some inconsistencies were recognized during BET. In 2013, Chen and Vinther from Genentech reported the phenomenon of Low Endotoxin Recovery (LER)[44]. During the establishment of diverse test procedures, to simulate potential contamination events, known amounts of endotoxin were inadequately detectable. Defined amounts of endotoxin were spiked to undiluted drug products and these samples containing endotoxins were incubated for a certain period of time. After incubation, the detection of spiked endotoxin contents resulted in low endotoxin recovery. Interestingly, this phenomenon was mainly observed in biopharmaceutical drug products, in which the Active Pharmaceutical Ingredients (API) are proteins like monoclonal antibodies. For stability reasons, such products are commonly formulated using excipients like phosphate and citrate buffer systems and polysorbates (Table 1). First investigations of affected samples have indicated that excipients of the drug products provoke the phenomenon of LER, but non-harmonized test procedures in industries leading to confounding results. The observation of this phenomenon is meanwhile frequently discussed in many forums, as it may result in an underestimation of hazardous endotoxin contents in injectable drug products. The Food and Drug Administration (FDA) pointed

out that endotoxin might be present in high amounts in a certain drug product and current assays are not detecting it or only detecting “acceptable” levels. Depending on the drug product dose and the potential amount of endotoxin contamination a pyrogenic reaction could occur.[45] In conclusion, to avoid the underestimation of a potential endotoxin contamination, the driving forces of the LER phenomenon have to be understood and current test procedures have to be optimized to ensure entire endotoxin detection in biopharmaceutical drug products.

Table 1 Common formulations of biopharmaceutical drug products

Monoclonal antibodies are prominent active pharmaceutical ingredients (API) in commercial biopharmaceutical drug products. For formulation excipients like phosphate, citrate, sodium chloride and polysorbates are used. (Source: <http://www.rxlist.com>)

Commercial Drug Product:	Active Ingredient:	Formulation components:
Actemra [®]	Tocilizumab	Phosphate, Sucrose, Polysorbate 80
Avastin [®]	Bevacizumab	Phosphate, Trehalose, Polysorbate 20
Erbitux [®]	Cetuximab	Phosphate, Sodium chloride
Humira [®]	Adalimumab	Phosphate, Citrate, Mannitol, Sodium chloride, Polysorbate 80
Lucentis [®]	Ranibizumab	Histidine, Polysorbate 20
Mabthera [®]	Rituximab	Citrate, Sodium chloride, Polysorbate 80
Remicade [®]	Infliximab	Phosphate, Sucrose, Polysorbate 80
Simponi [®]	Golimumab	Histidine, Sorbitol, Polysorbate 80
Soliris [®]	Eculizumab	Phosphate, Sodium chloride, Polysorbate 80
Synagis [®]	Palivizumab	Histidine, Glycine, Mannitol
Tysabri [®]	Natalizumab	Phosphate, Sodium chloride, Polysorbate 80
Xolair [®]	Omalizumab	Histidine, Sucrose, Polysorbate 20

1.7 Purpose of the study

Aim of this work is to improve existing test procedures and to detect endotoxin out of samples, which are affected by the LER phenomenon. First of all, the phenomenon has to be analyzed according to the questioning observations, which were made in pharmaceutical industry. Therefore, endotoxin recovery in common formulation components of drug products including citrate and phosphate buffer systems as well as surfactants like polysorbates has to be investigated. Due to the temporally delayed occurrence of the LER-effect, reaction kinetics has to be examined in order to identify the time limiting parameters of LER and to establish a model system, which enables the simulation of such kinetics. In addition, as endotoxins represent a heterogeneous group of LPS, it has to be examined which influences this variability has on the LER-effect and if it is depending on a certain species of endotoxin. Therefore, endotoxins from different sources have to be tested with regard to their detectability under LER conditions. After analysis of the driving forces, an approach to render the endotoxin detectable has to be figured

out. Due to the regulatory scope in pharmaceutical industry, existing test procedures have to be maintained, but advanced by sample treatments prior to the actual measurement. Thus, purpose of this work is to discover “demasking” agents for sample pre-treatment to detect endotoxin out of LER-affected samples using *Limulus*-based test methods and in turn to reduce the risk of wrong-negative test results during quality control of drug products in pharmaceutical industries.

2 Material and Methods

2.1 Reagents

Polysorbate 20, polysorbate 80, octoxynol 9, ethanol, 1-octanol, 1-decanol, 1-dodecanol, 1-tetradecanol, 1-hexadecanol, 1-octadecanol, sodium chloride, sodium azide, citric acid, trisodium citrate, phosphoric acid, sodium dihydrogenphosphate, potassium dihydrogenphosphate, disodium hydrogen phosphate-heptahydrate, ethylenediaminetetraacetic acid (EDTA), 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), 2-mercaptoethanol, isopropanol, D(+)-glucose, sodium chloride, calcium dichloride and magnesium dichloride were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Ammonium hydroxide, formaldehyde and D(+)-trehalose-dihydrate were obtained from AppliChem GmbH, Darmstadt, Germany. Acetic acid, glycerol, periodic acid, sodium hydroxide, silver nitrate, sodium dodecylsulfate (SDS) and yeast extract (powdered) were obtained from Carl Roth GmbH & Co.KG, Karlsruhe, Germany. Bromophenol blue sodium salt was obtained from Merck Chemicals GmbH, Darmstadt, Germany. Bovine Serum Albumin (BSA) and 20x Tris-tricine/SDS electrophoresis buffer were obtained from Serva Electrophoresis GmbH, Heidelberg, Germany. A bovine polyclonal immunoglobulin G (PAK) and a mouse monoclonal antibody (MAK33) were obtained from Roche Diagnostics Deutschland GmbH, Mannheim, Germany. Tryptone Bacto TM was obtained from Becton Dickinson GmbH, Heidelberg, Germany. Prior to the experiments, all relevant materials have been tested on endotoxin contents and were proven to contain less than 0.05 EU/mL.

2.2 Endotoxins and bacteria

Endotoxin from *E.coli* O55:B5 (gel-filtrate), *P.aeruginosa* and *S.enterica* were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. Phenol-extracted clinical isolate endotoxins from *E.coli*, *K.pneumonia*, *M.morganii*, *Y.enterocolitica*, *N.meningitis*, *P.mirabilis* and *S.marcescens* were a kind gift from Dr. Andreas Wieser, Universitätsklinik München (LMU), München, Germany. Endotoxin from *E.coli* K12 was obtained from InvivoGen, Toulouse, France. Freeze dried bacteria from *E.coli* O55:B5 (DSM 4779), *E.cloacae* (DSM 30054) and *P.aeruginosa* (DSM 500 71) were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Freeze dried bacteria from *E.coli* O113 (Ecor 30) was obtained from Escherichia coli Reference Collection, East Lansing, USA. Freeze dried bacteria from *B.cepacia* (2008 B02-12.20.164) and *S. maltophilia* (DSMZ 50 170) were obtained from Robert Koch-Institute, Wernigerode, Germany. Bacteria from *R.pickettii* (isolate) were a kind gift from Hyglos GmbH, Bernried, Germany.

2.3 Other materials

20% gradient tris-tricin gel was obtained from Anamed Elektrophorese GmbH, Rodau, Germany. 0.2 µm Acrodisc 25 mm Syringe Filters were obtained from Pall GmbH, Dreieich, Germany. Kinetic chromogenic *Limulus* Amebocyte Lysate test was obtained from Lonza Inc., Walkersville, USA. Depyrogenated water, depyrogenated borosilicate glass tubes and recombinant Factor C tests, EndoZyme® and EndoLISA® were obtained from Hyglos GmbH, Bernried, Germany.

2.4 Preparation of crude endotoxins extracts

2.4.1 Preparation 1

For growth of bacteria, 5 mL lysogeny broth (LB) media (10 g/L sodium chloride, 5 g/L yeast extract and 10 g/L tryptone) were inoculated with desired bacterial strain, followed by incubation at 37 °C in a shaking incubator (Platform shaker: Innova 2300, New Brunswick Scientific Co, Enfield, USA; Incubator: Wärmeschrank für Plattformschüttler, Mytron Bio- und Solartechnik GmbH, Heilbad Heiligenstadt, Germany) overnight. Afterwards 200 µL of the preparatory culture were transferred into 500 mL of media (12.8 g/L disodium hydrogenphosphat-heptahydrat, 3 g/L potassium dihydrogenphosphat, 0.5 g/L sodium chlorid, 1 g/L ammonium chloride, 0.01 g/L calcium dichloride and 0.4 wt % glucose) and incubated for 24 hours at 37 °C. Growth of bacteria was tracked by light absorption at 600 nm using a spectro photometer V550 Jasco Germany GmbH, Gross-Umstadt, Germany. Bacterial growth was stopped by temperature reduction to 4 °C, centrifugation at 4500 rpm and sterile filtration (0.2 µm) of the bacterial suspension. For conservation 0.05 (v/v) % sodium azide was added. Required endotoxin concentrations for endotoxin recovery studies were adjusted by dilution with depyrogenated water.

2.4.2 Preparation 2

For growth of bacteria, 5 mL LB media (10 g/L sodium chloride, 5 g/L yeast extract and 10 g/L tryptone) were inoculated with desired bacterial strain, followed by incubation at 37 °C in a shaking incubator (Platform shaker: Innova 2300, New Brunswick Scientific Co, Enfield, USA; Incubator: Wärmeschrank für Plattformschüttler, Mytron Bio- und Solartechnik GmbH, Heilbad Heiligenstadt, Germany) overnight. Afterwards the preparatory culture was transferred into 20 mL of media*¹ and incubated for 18 hours defined temperatures*². Bacterial growth was stopped by temperature reduction to 4 °C and sterile filtration (0.2 µm) of the bacterial suspension. For conservation 0.05 (v/v) % sodium azide was added. Required endotoxin concentrations for endotoxin recovery studies were adjusted by dilution with depyrogenated water.

*Modification of growth conditions:

- 1) LB media:
- a) 100 % media
 - b) 1 % (v/v) media in depyrogenated water
 - c) 100 % media plus 20 mM tris buffer
 - d) 100 % media plus 20 mM EDTA
- 2) Growth temperature:
- i) room temperature (20 – 25 °C)
 - ii) 37 °C

2.5 Sample handling

2.5.1 Addition of endotoxin to a sample

Samples were prepared in 5 mL borosilicate 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 (gel-filtrated) out of a 10,000 EU/mL stock solution. Before adding the endotoxin spikes to the sample, the endotoxin stock solution was shaken at 1400 rpm for 10 minutes using Multi Reax shaker (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany). After spiking, the samples including the endotoxin are shaken again at 1400 rpm for 60 seconds.

2.5.2 Sample preparation before endotoxin detection

To eliminate test interference and ensure validity of endotoxin detection assays, samples were vortexed for 2 minutes at 1400 rpm and diluted in depyrogenated water immediately prior to the measurement. The validity of the measurement was controlled by spiking of defined endotoxin amounts to the diluted samples (Positive Product Control (PPC)). Endotoxin determination in a sample was considered valid, if 50 to 200% of the spiked endotoxin (PPC) were recovered.

2.5.3 Incubation of endotoxin in a sample

For time dependent endotoxin recovery (hold time) studies, endotoxin was incubated in undiluted samples over time. If not otherwise specified, the pH of used buffer systems was adjusted to 7.5. After addition of defined endotoxins spikes to undiluted samples, the resulting solution was vortexed for 60 seconds at 1400 rpm. Samples were subsequently stored without further vortexing at defined temperatures (4° C (2 – 8 °C), RT (20 – 25 °C) and 37°C (35 – 40 °C) for a desired period of time.

2.5.4 Preparation of endotoxin recovery kinetics

Samples for kinetics can be prepared in two opposed sequential arrangements: Online mode (2.5.4.1) vs. reverse mode (2.5.4.2). Both preparations were used. Online mode has the advantage that the endotoxin spike in all samples is exactly the same. Disadvantage is that each time point

needs a new test and standard curve at different days. The advantage and disadvantage performing the reverse preparation are vice versa compared to the online mode.

2.5.4.1 Online mode – kinetics (OM)

Determination of endotoxin masking kinetics was prepared out of one stock solution using the online mode. The start of the kinetics was defined, when at least surfactant, chelator and endotoxin were combined and vortexed. 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. Prior to the measurement, no further dilution was required. Samples were vortexed at least for 2 minutes at 1400 rpm before the individual time points were measured. The online method was used for all preparations of endotoxin masking kinetics, if not otherwise indicated.

2.5.4.2 Reverse mode – kinetics (RM)

Reverse endotoxin masking kinetics was prepared by spiking aliquots of a sample at different time points. The particular spiked and not spiked aliquoted 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. After spiking the time point zero aliquot, all samples were equivalently prepared and measured on the same assay.

2.5.5 Endotoxin recovery and masking controls

To control accuracy of the endotoxin spiked into the undiluted samples, equal amounts of endotoxin were spiked into depyrogenated water (positive control), mixed and identically incubated as the actual sample. For calculation of endotoxin recovery, the determined endotoxin concentrations in the actual sample is compared to the determined endotoxin concentration at time zero in the positive control and stated as percent.

2.5.6 Sample preparation for demasking of endotoxin

2.5.6.1 Preparation of demasking agents (working solution).

For demasking, different molecules (Sodium citrate, calcium dichloride, BSA, SDS, alkyl-alcohols) and mixtures of them were used. Before addition of these molecules to the masked sample a 10-fold concentrated working solutions of the desired component and concentration were prepared. Irrespective of the used alkyl alcohols, the components were dissolved in depyrogenated water. Alkyl alcohols were dissolved in 70% (v/v) ethanol.

2.5.6.2 Sample treatment for demasking of endotoxin.

Endotoxin demasking was performed by the addition of 100 µl of each demasking agent (2.5.6.1 working solution) to the masked sample. The particular agents were sequentially added and 2

minutes vortexed after each addition. After addition of all demasking agents, the samples were incubated for 30 minutes at room temperature without vortexing.

2.6 *Limulus*-based endotoxin detection assays

2.6.1 Recombinant Factor C assays (rFC)

2.6.1.1 EndoZyme®

For detection of endotoxin, a recombinant Factor C test (EndoZyme®), based on a homogenous test format, was used according to manufacturer's instructions. The released amount of fluorescence substrate 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. Standard curves were fit using a four parameter logistic non-linear regression model. The detection limit of the assay was 0.005 EU/mL. EndoZyme was used in all experiments, if not otherwise indicated.

2.6.1.2 EndoLISA®

For detection of endotoxin, a recombinant Factor C test (EndoLISA®), based on a heterogeneous test format, was used according to manufacturer's instructions. The released amount of fluorescence substrate 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. Standard curves were fit using a four parameter logistic non-linear regression model. The detection limit of the assay was 0.005 EU/mL.

2.6.2 *Limulus* Amebocyte Lysate assay (LAL)

For endotoxin detection using *Limulus* Amebocyte Lysate, a kinetic chromogenic LAL assay (Kinetic-QCL™) was used according to manufacturer's instructions. The released amount of chromogenic substrate was measured spectrophotometrically at 405 nm with an Epoch2 absorbance microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). All samples were measured in duplicate and average values were used for further calculations. Standard curves were fit using a linear regression model. Detection limit of the assay was 0.005 EU/mL.

2.7 Silver stained SDS-PAGE

Endotoxin samples (crude extracts, 2.4.1 preparation 1) were vortexed for 30 seconds. 40 µL of the sample were mixed with 10 µL SDS sample loading buffer (1.25 µL tris buffer, 0.5 mg sodium dodecylsulfate, 2.87 µL glycerol, 1.25 µL EDTA, 5 µg bromphenol blue and 0.25 µL mercaptoethanol) and boiled for 10 minutes. 18 µL of each sample were loaded onto a 20%

gradient tris-tricin gel. The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was run in tris-tricine/SDS buffer at 130 V (Electrophoresis power supply: EPS 301, Amersham Pharmacia Biotech, Uppsala, Sweden; Vertical Electrophoresis unit: Mighty small SE260, Hoefer Inc., Holliston, USA) for 90 minutes. For silver staining of the gel the following procedure was used:

- 1) Fixation: Incubation of gel overnight in 150 mL of 25% (v/v) isopropanol and 7% (v/v) acetic acid.
- 2) Oxidation: Incubation of gel for 5 minutes in 75 mL depyrogenated water with 0.5 g of periodic acid and 1 mL of fixation solution (25% (v/v) isopropanol and 7% (v/v) acetic acid solution).
- 3) Washing: Four times 5 minutes wash in depyrogenated water.
- 4) Silver staining: 10 minutes in a solution containing 350 μ L sodium hydroxide (8 M), 1 mL concentrated ammonium hydroxide (28%), 2 mL silver nitrate (20% (w/v) and 75 mL depyrogenated water.
- 5) Washing: Four times 5 minutes wash in depyrogenated water were done.
- 6) Development: 20 min in a solution containing 50 mL depyrogenated water, 50 mg citric acid and 50 μ L formaldehyde (37%). Development was stopped using 10 % (v/v) acetic acid.

2.8 Calculations and plots

For calculation of endotoxin recovery, plotting of graphs and simulation of endotoxin recovery kinetics Microsoft Excel 2010, Version 14.0.7015.1000 was used. Sigmoidal experimental data points were fit using SigmaPlot 2001 for Windows Version 7.0. Calculation of standard curves for determination of endotoxin concentrations, Gen5 Data Analysis Software Version 2.05 from BioTek Instruments GmbH, Bad Friedrichshall, Germany was used.

3 Results and Discussion

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

3.1.1 Introduction

Bacteria and their breakdown products like endotoxins are ubiquitous[46]. The presence of endotoxins in aqueous compositions is an intractable problem, which severely threatens and limits the application of many compositions, in particular if intended for pharmaceutical use. This is especially true for parenteral administered biopharmaceutical drug products. Therefore, there is a risk of endotoxin contamination in the production process of pharmaceutical drug products. To safeguard against potentially hazardous incorporation of endotoxin, measurements must be performed to exclude endotoxin from all steps and products used in the production process of parenteral drug products. For such measurements, a method of choice is the LAL assay. Since decades, these assays are positioned in quality control of pharmaceutical production and have been proven to be a sensitive measure for endotoxins. However, some reports have shown that detection of endotoxins is not always suitable in complex samples[47], [48]. 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[49]. This phenomenon is well known and to identify test interference, positive product controls (PPC) 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 to 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[40]. 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[50], proteins[47] or amphiphilic molecules[51], [52], resulting in a significant change of endotoxin activity. Notably, approaches which eliminate test interference problems are not effective in overcoming such effects[47]. In the 1990's Greaves and co-workers already differentiated between dilution-dependent and dilution-independent interference in environmental samples[53].

In the recent past, inadequate endotoxin detection has been observed in biopharmaceutical drug products[54]. In such cases, the APIs are mainly proteins[55], which are capable of intrinsic binding to endotoxin as previously described by Anspach and co-workers[47]. The inadequate detection of endotoxin might be explained by protein-endotoxin interactions, but furthermore,

therapeutic proteins are usually stabilized by excipients, like nonionic surfactants and certain buffer components[56]. Surprisingly, endotoxin spiking experiments in formulations that lack the API resulted in LER. Such observations of disturbed endotoxin determinations in biopharmaceutical products occurred 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.

Therefore, crucial formulation components of common biopharmaceuticals are extracted from popular biopharmaceutical drug products (table 1) and endotoxin recovery out of such buffer systems containing single and multiple components is investigated. Due to the expected time-dependency of LER, the end-point of the reaction is determined by using different sample incubation temperatures. While multi-parameter interactions between surfactants, complex forming agents and endotoxin are assumed, the particular influence of the buffer components on the detectability of endotoxin is focused. Hence, the impact of pH in a sample, different buffer systems and the effects of different nonionic surfactants are studied. Finally, various endotoxin concentrations are added to a LER causing formulation to evaluate the masking capacity of such a sample.

3.1.2 Results

For analysis of LER, undiluted samples were spiked with defined amounts of endotoxin and incubated over time. With regard to biopharmaceutical drug products, single and mixtures of common formulation components were examined to identify critical components or combinations affecting endotoxin detection. Endotoxin recovery in the presence of different formulation components is shown in table 2.

Table 2 Endotoxin recovery out of single and multiple component samples

Endotoxin recovery over time in presence of single and multiple formulation components is shown. Samples were spiked with an endotoxin amount of 10000 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 up to 1:10000.

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

The recovery was determined after endotoxin spiking of the samples without incubation (T0) and after sample incubation for seven days (T7) at room temperature (RT). Samples 2, 3 and 4, containing only single component additions showed no significant loss of activity over time, according to a validity criteria of 50 to 200% of endotoxin recovery. In contrast, endotoxin could not be detected in samples containing both buffer and surfactant (samples 5 and 6) after an incubation period of seven days. This result shows that endotoxin recovery is substantially affected by the sample incubation period and mixture of formulation components.

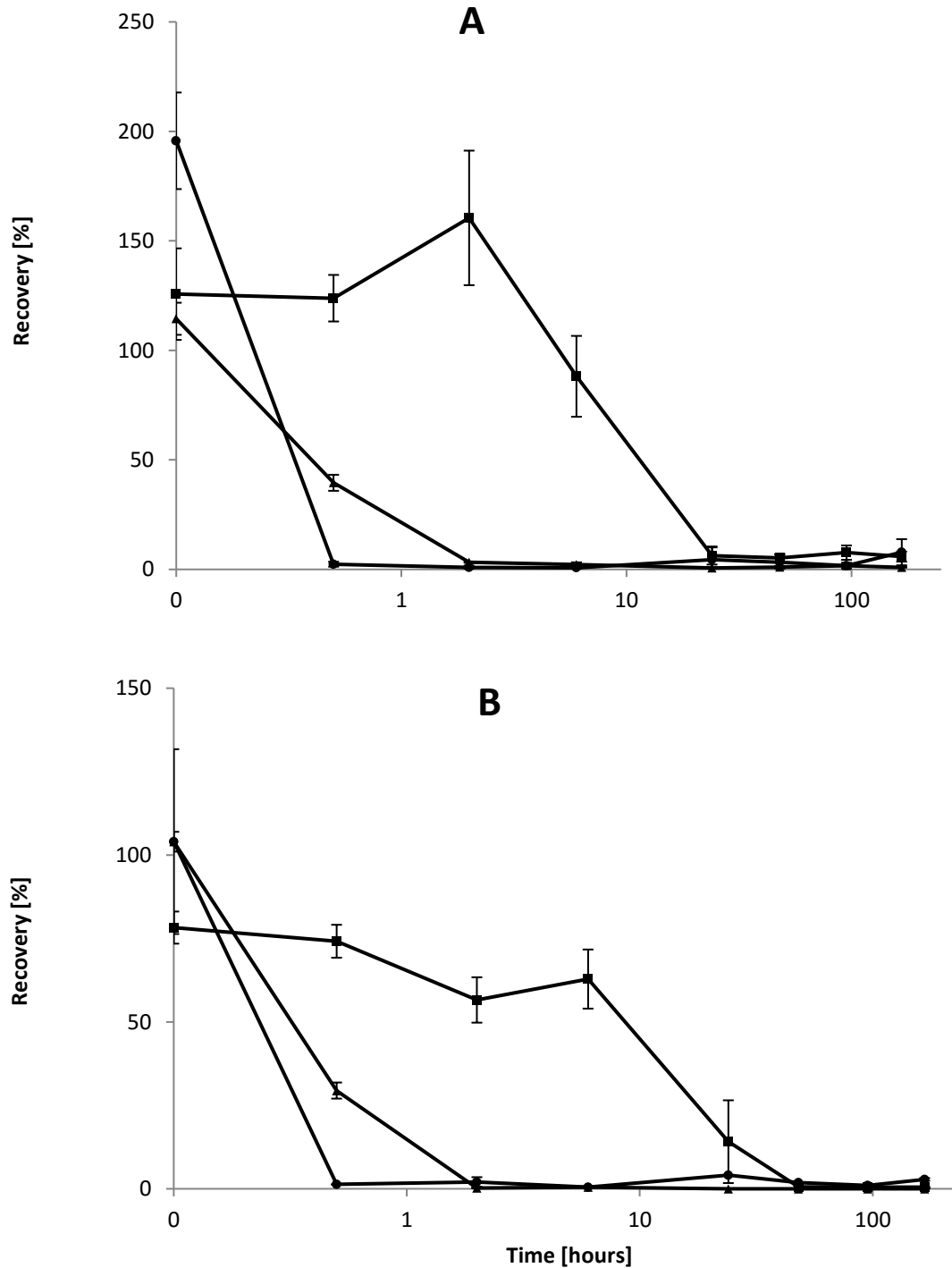
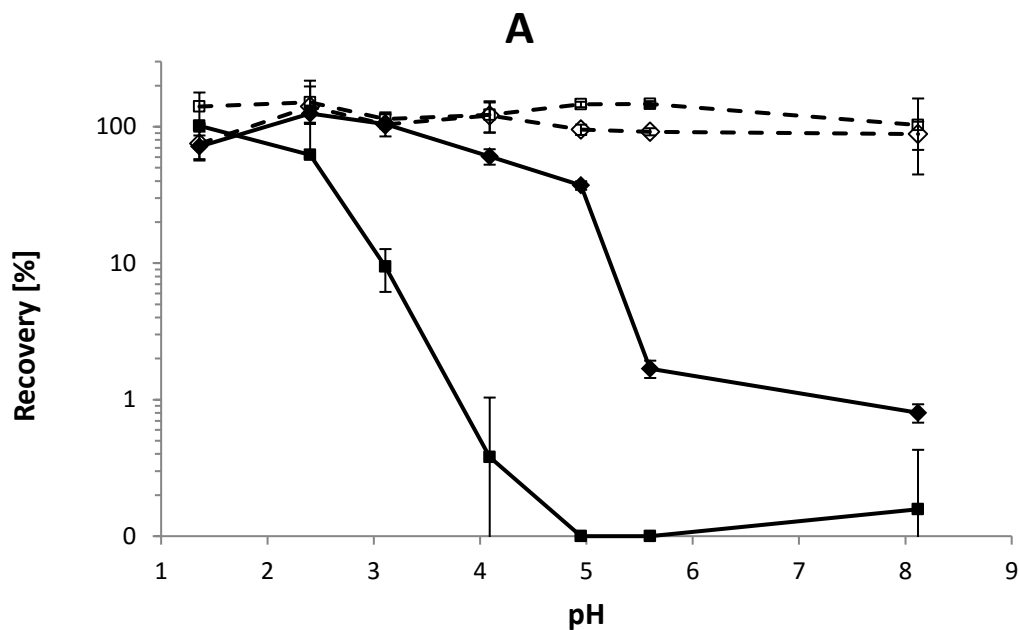


Figure 7 Endotoxin recovery kinetics in citrate-polysorbate formulations

The endotoxin recovery is plotted as a function of the incubation time. The different curves indicate incubation temperatures at 35-40°C (●), 20-25°C (▲) and 2-8°C (■). 100 EU/mL endotoxin were added to samples containing 10 mM sodium citrate and 0.05 % polysorbate 20 and incubated for different time periods (reverse mode, 2.5.3.2). For detection (A) kinetic chromogenic LAL tests and (B) recombinant Factor C tests 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.

To examine the time-dependency of LER more in detail, kinetics of endotoxin recovery in samples containing polysorbate 20 and sodium citrate was analyzed. Figure 7 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 *Limulus* amoebocyte lysate (LAL) test (A) and a recombinant Factor C test (B) for detection. After approximately 24 hours 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.

Together, these experiments show that the combination of a buffer system and a surfactant results in LER over time. Thus, the impact of different buffer systems was studied (Figure 8). In order to investigate pH dependency of endotoxin recovery after sample incubation, defined compositions under different pH conditions were studied (figure 8A). In the absence of surfactants, the variation of pH had no impact on endotoxin detection. This confirmed again the previous results (table 2). 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. The transition to higher pH values hampered endotoxin recovery. In addition, the diverging curve progressions (Figure 8A) indicate an intrinsic effect of each particular buffer system. Hence, endotoxin recovery kinetics using different buffer systems such as ethylenediaminetetraacetic acid (EDTA), citrate and phosphate were studied (Figure 8B).



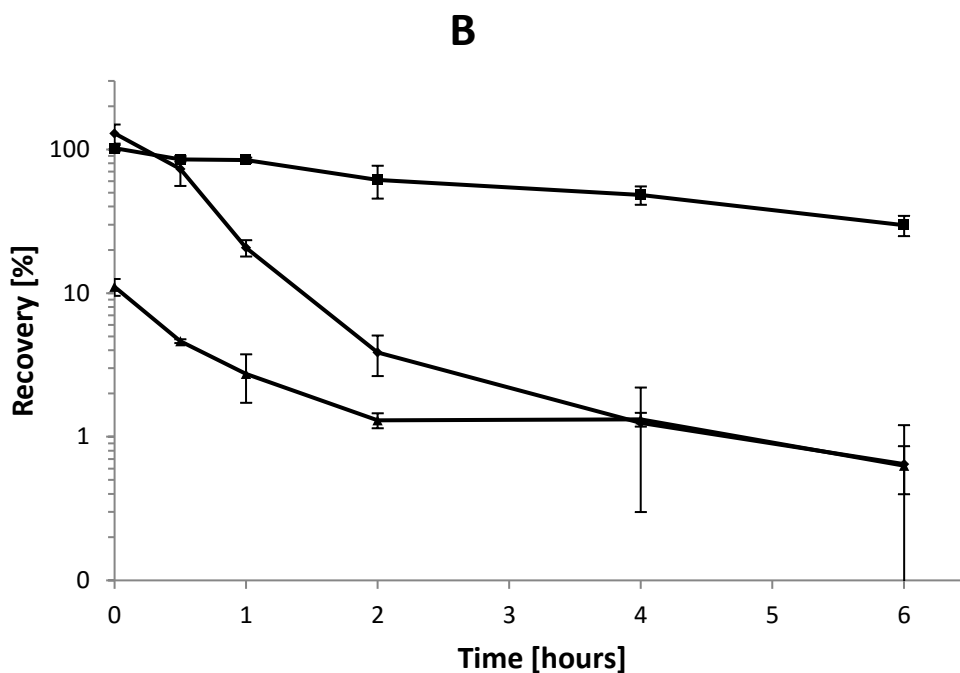


Figure 8 Impact of buffer system on endotoxin recovery

(A) Endotoxin recovery is shown as a function of the pH. The pH varied in a range from 1 to 9 and incubation was at RT for seven days. 100 EU/mL of endotoxin were 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 error bars reflect the standard deviation of three independent replicates (n=3) of the sample. The replicates were measured on the same microtiter plate.

(B): Endotoxin recovery is plotted as function of incubation time using different buffer systems. Sample incubation was at RT. 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 (reverse mode, 2.5.3.2). 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.

The endotoxin recovery in the described buffer systems is plotted as a function of time. The system containing EDTA showed a rapid loss of activity. The loss of activity was slower under citric conditions and slowest under phosphoric conditions. After six hours, recovery was below 30% under 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 figure 9, the endotoxin recovery out of surfactant solutions (polysorbate 20, polysorbate 80 and octoxynol 9) in the presence and absence of citrate are plotted as a function of surfactant concentration. In general, all used surfactants substantially reduced endotoxin detectability in the presence of citrate after seven days of incubation. In absence of citrate, only octoxynol 9 showed low recovery at relatively high concentrations after the incubation period. Without incubation, endotoxins were detectable in all cases (> 50%), except using highest octoxynol 9 concentration including citrate.

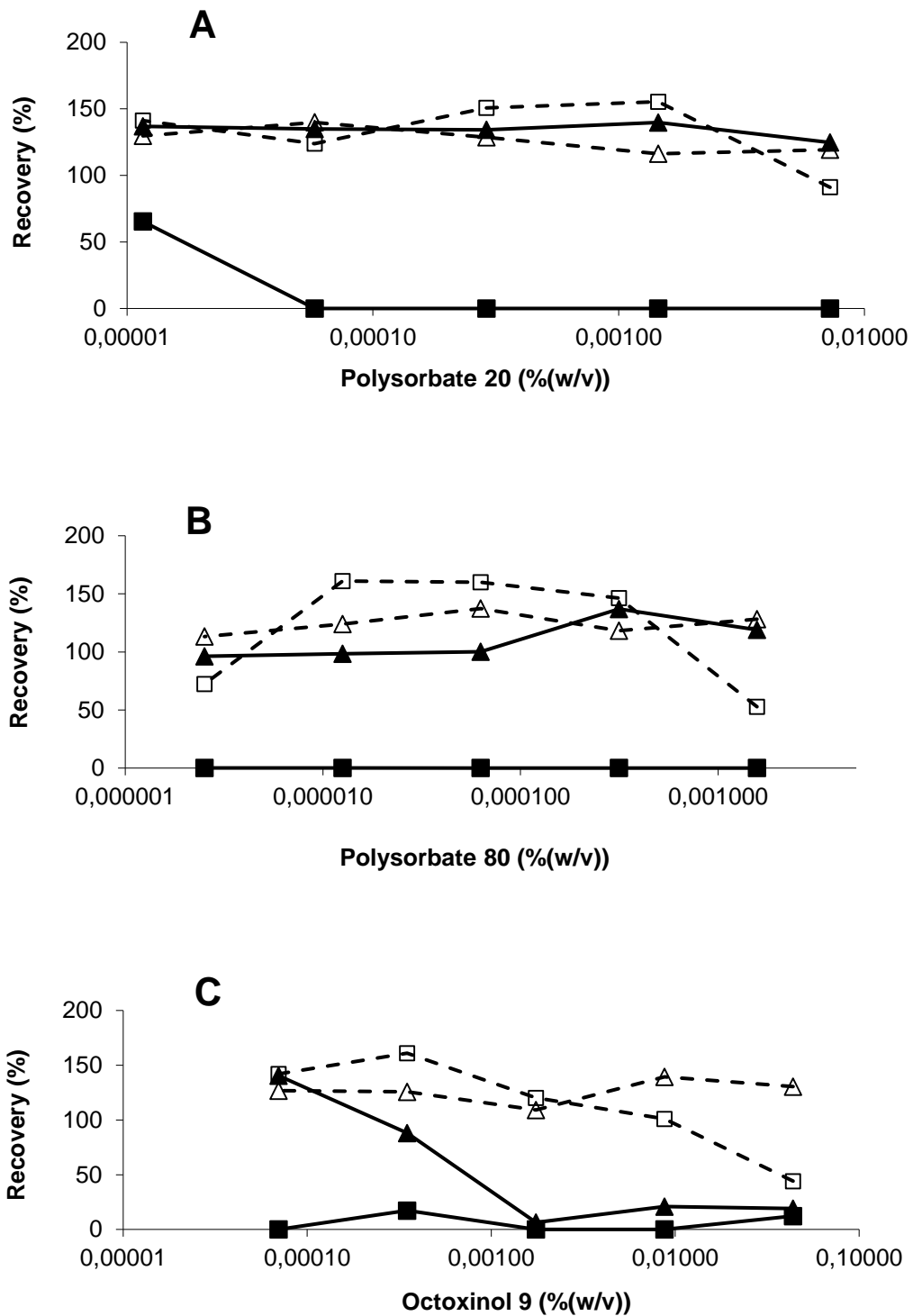


Figure 9 Surfactant dependent endotoxin recovery

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

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 may solely occur after a certain period of time. For a final evaluation, the masking capacity was examined. Endotoxin was titrated into samples containing a citrate buffer system and polysorbate.

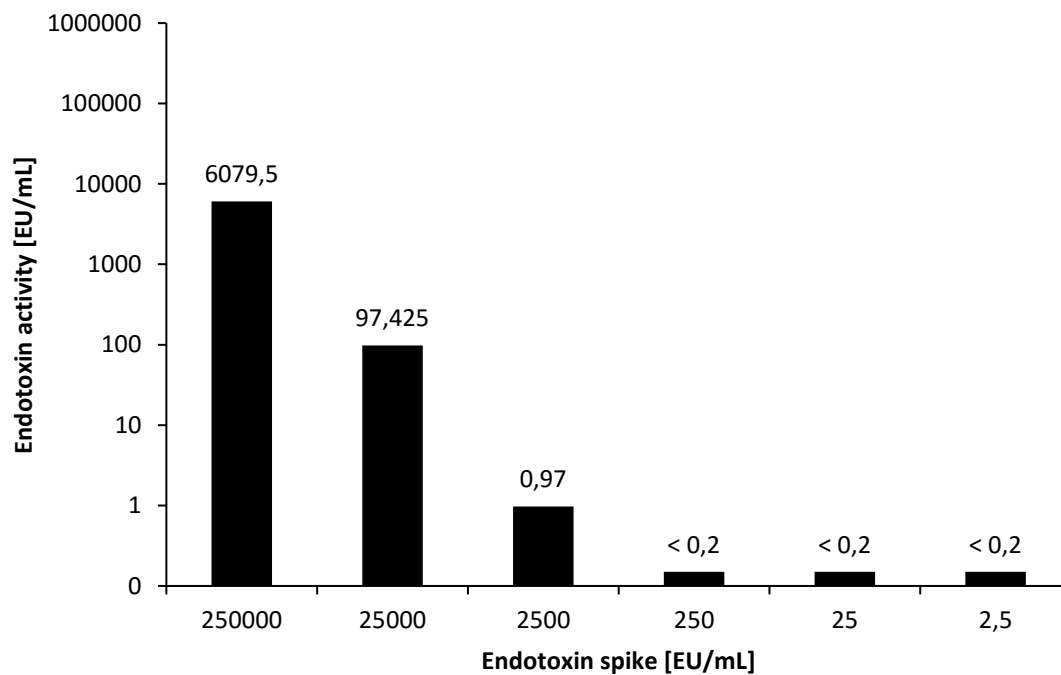


Figure 10 Endotoxin masking capacity of citrate-polysorbate 20 formulation

Detectable endotoxin concentration is shown in relation to the spiked endotoxin concentration. Defined amounts of endotoxin were added to solutions containing 0.05 wt % polysorbate 20 and 10 mM sodium citrate and incubated for seven days at 4 °C. Endotoxin spikes were prepared out of a LPS stock solution containing 10^{-6} EU/mL.

Figure 10 shows the endotoxin masking capacity of such a particular matrix. 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 result illustrates a high masking capacity of common formulation matrices and suggests the need for vigilance in BET under such conditions.

3.1.3 Discussion

BET is a standardized control instrument in pharmaceutical microbiology. To check the absence of test interference and validity of a measurement, PPCs are regularly used according to pharmacopoeial requirements. However, since the observation of inconsistencies during BET, although valid PPC are given, users are partly asked by regulators to establish additional test procedures for storing samples and demonstrating the stability of assayable endotoxin contents[57]. Consequently, there are two different control procedures in BET. The first are PPCs, which are used to identify test interference. Thereby, defined contents of endotoxin are spiked into diluted samples immediately before the measurement is started. The measurement is considered valid, when 50 to 200% of this spike is recovered. The second procedure to investigate LER is the application of “hold time” or “endotoxin recovery” studies. In this case, defined contents of endotoxin are spiked into undiluted samples and the spike is incubated for a certain period of time in the sample before the actual endotoxin measurement. A sample is popularly classified as LER, when less than 50% of the spike is recovered over time.

Basically, test interference directly affects the detection system, but can be overcome by dilution. In the case of LER, sample dilutions up to a factor of 10,000 could not overcome inadequate recovery (Table 2), demonstrating that LER is dilution independent and therefore not caused by test interference. Furthermore, after short incubation periods of endotoxin in the sample, the full endotoxin content could be recovered, showing the functionality of the detection system (Figure 7). These findings confirm a previous observation, namely that under certain conditions the interference in *Limulus*-based detection methods is dilution independent. In this case, it is assumed that the aggregate conformation of LPS is affected and not the detection system itself[53]. Hence, the results above indicate that the phenomenon of LER is also caused by alterations in the aggregate conformation of the endotoxin, meaning the endotoxin is masked. This is also supported by the time-dependent appearance of LER, while test interference appears immediately and is therefore time-independent. This time-dependent behavior of LER 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 range from sub-microseconds to several days, weeks and even month[58]. This time-dependent occurrence of LER might also explain confounding experimental results in pharmaceutical industries (results not published), in which the LER phenomenon was not observed, although masking conditions were present.

3.1.3.1 Effects of complex forming agents

The results 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 surfactants and complex forming components (chelator) suffices to decrease the detectability of endotoxin. The presence of only one of the formulation components does not effectively disturb endotoxin recovery. These findings confirm former assumptions of endotoxin disaggregation [59], [60] and explain the interdependent interaction of surfactant and chelator on endotoxin. Due to the amphoteric and amphiphilic nature of LPS (Figure 3), 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. The rigidity is controlled by the salt form of LPS, which again involves the presence of multivalent cations like Ca^{2+} . [61], [62] 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. In the presence of EDTA the recovery drops faster than in the presence of citrate or phosphate based buffer systems (Figure 8B). Using buffer components with higher metal complex forming capabilities accelerate masking kinetics. Thus, the chelating capability of the buffer component is crucial. The related metal complex formation constants are directly proportional to the denticity of the ligand (rule of thumb [63]). 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 dependent. At low pH values, protons are in competition with cations, which hamper formation of metal complexes [64]. Consequently, LER is affected by the free concentration of protons reflected by 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 (Figure 8A).

3.1.3.2 Effects of surfactants on LER

As shown above, complex forming components alone do not result in inadequate endotoxin detection, additional amphiphilic components like surfactants are necessary. Due to the fact that LPS itself are amphiphilic, these tend to aggregate, driven by the low solubility of hydrophobic fatty acids of lipid A in an aqueous solution [65]. Thus, LPS exhibit certain supramolecular structures, which influence detectability in *Limulus*-based detection systems [66]. 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 [67]. 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. The latter is controlled, to some extent, by ionic interactions as described above. To this end, after the addition of surfactants and chelators to LPS solutions, 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., who have shown that disaggregated LPS molecules (“monomers”) are substantially less active than aggregated LPS in the detection system used[66]. 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[68]. On the other hand, it has been shown that monomeric LPS show a higher potency in activating *LAL* 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.

3.1.3.3 Potential two-step reaction mechanism of LER

In summary, we propose a two-step mechanism of endotoxin masking. Figure 11 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.

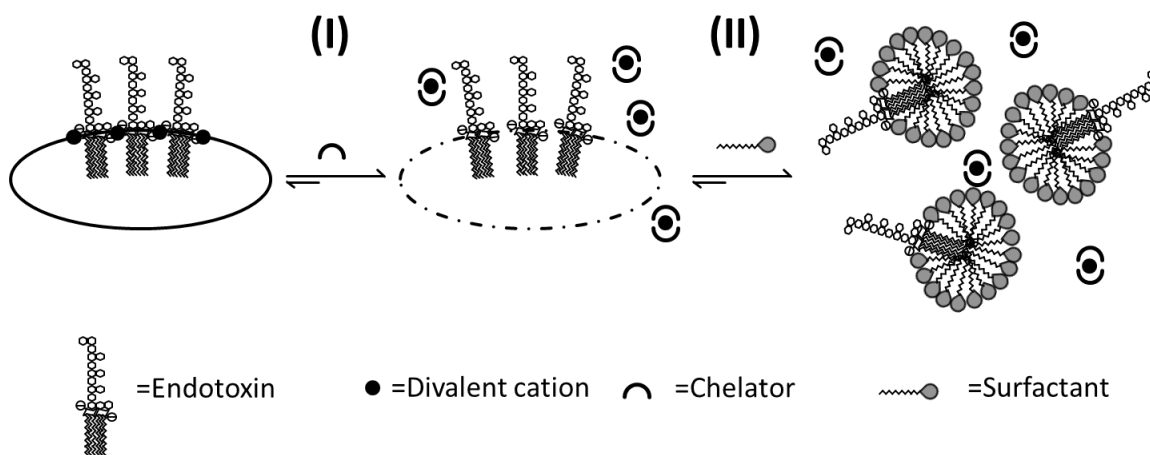


Figure 11 Two-step mechanism of endotoxin masking

Potential reaction mechanism of endotoxin masking, caused by complex forming agents and surfactants is schematically illustrated. In a first step, pure endotoxin aggregates are disturbed by chelators reducing the rigidity of the aggregate. Then, surfactants interact with endotoxin by forming mixed aggregates.

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. Mg^{2+}) and LPS are destabilized, leading to a reduced rigidity of the aggregate. The additional 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 common molar excess of complex forming agent and surfactant (micromolar range) compared to endotoxin content (nanomolar range), mixed surfactant micelles containing monomerized LPS are the most probable resulting supramolecular structure.

Together, this study confirmed the phenomenon of LER in *Limulus*-based detection systems and exemplifies a potential mechanism of endotoxin masking. Due to the time-dependency of 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 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. Capacity experiments have shown that commonly used excipients are capable of masking hazardous amounts of endotoxin. Consequently, the phenomenon of LER has to be especially considered during quality control of drug products including surfactant and complex forming agents. In order to further investigate the time-dependent masking behavior of endotoxin, detailed reaction kinetics is studied in the next chapter (3.2).

3.2 Endotoxin Masking: A kinetically controlled reaction mechanism

3.2.1 Introduction

In the previous chapter (3.1), the phenomenon of LER was studied in common biopharmaceutical product matrices and it is supposed that LER is caused by the interaction of sample matrix and endotoxin, resulting in masking of endotoxin. Basically, LPS aggregate due to their amphiphilic nature and in turn form certain supramolecular structures[70]. Yet, during the transition from detectable to undetectable (masked) endotoxin a change in its supramolecular structure is probable. Moreover, a disaggregation of LPS may occur during masking. With regard to parenteral drug products, regulators consider this phenomenon as a potential safety issue due to the possible underestimation of critical endotoxin levels in a sample[54]. To investigate if a product is affected by LER, an industrial guideline for BET suggests hold time studies[57], which intend to incubate known amounts of endotoxin over time in undiluted products prior to the actual test procedure.

The aim of such hold time studies is to prove assayability of endotoxin in a particular sample over time. In order to provide a better understanding of the LER mechanism and in turn to improve efficiency in planning of such hold time studies 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[56]. Furthermore, such a composition reflects the minimum factors responsible for endotoxin masking and should therefore help to elucidate the driving forces of endotoxin masking. 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[71]. Derivation of a rate law according to the underlying masking reaction enables prediction of the reaction rate depending on a given product formulation and 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 and endotoxin. The variation of concentrations is used to identify whether there are certain reactants controlling the reaction rate. Furthermore, the rate determining step in the proposed two-step reaction mechanism (3.1.3.3) is specified and the derived reaction law is used for the simulation of endotoxin masking kinetics.

3.2.2 Results

Endotoxin masking, caused by the simultaneous presence of surfactants and complex forming agents, has been shown in the previous chapter (3.1). In these studies, mixtures of formulation components influenced the occurrence of masking. To analyze whether the preparation of the samples affects endotoxin masking, kinetics with different order of sample preparations was investigated. Two of the three components (polysorbate, sodium citrate and endotoxin) were pre-incubated overnight and masking kinetics was started by addition of the third component (Figure 12). As expected, all preparations were affected by LER, but diverging kinetics are observed. Endotoxin pre-incubated with sodium citrate shows the fastest masking kinetics and pre-incubation of polysorbate with sodium citrate shows the slowest kinetics. Endotoxin pre-incubated with polysorbate shows likewise slow kinetics. Based on the accelerated reaction kinetics by pre-incubation of endotoxin with sodium citrate, the interaction between endotoxin and sodium citrate appears to be the time limiting reaction step. The large error bars reflect test variables of *Limulus*-based assays, but also the experimental setup. Depending on exact ambient temperatures and hands on time for sample preparation including spiking, mixing as well as vortexing may influence reaction kinetics.

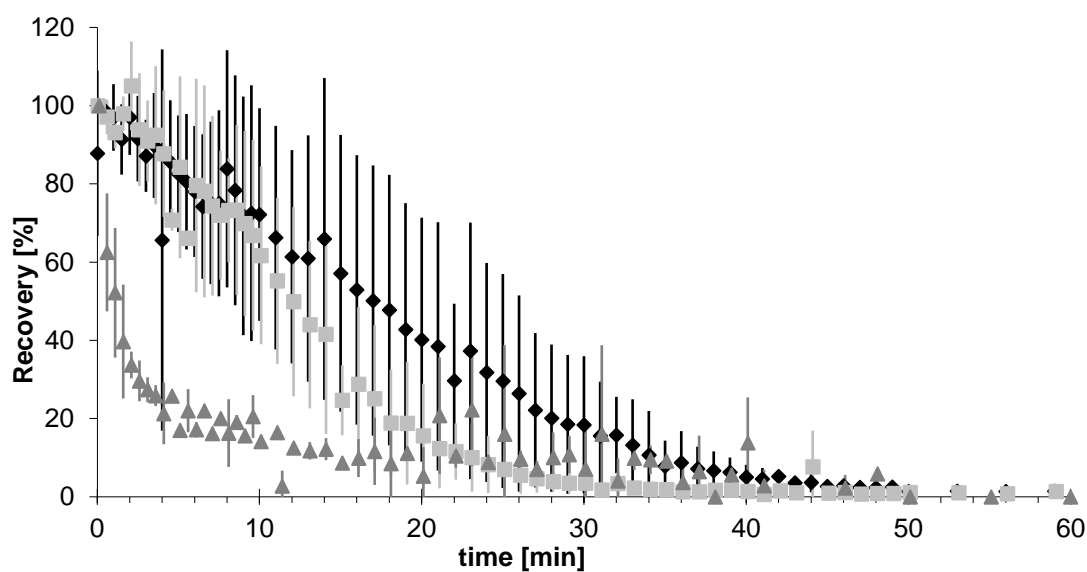


Figure 12 Endotoxin recovery depending on order of matrix component and LPS addition

Endotoxin recovery is plotted as a function of incubation time. 100 EU/mL endotoxin were spiked to solutions containing 0.05 wt % polysorbate 20 and 10 mM sodium citrate. The particular kinetics was generated by different sequential arrangements during sample preparation at RT. In the first kinetics (dark grey triangles (▲)) LPS and sodium citrate were pre-incubated (overnight) and polysorbate was added at time zero (0 min) to start the reaction. In the second kinetics (light grey squares (■)) LPS and polysorbate were pre-incubated (overnight) and sodium citrate was added at time zero (0 min). In the third kinetics (black diamonds (◆)) sodium citrate and polysorbate 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 pre-incubation) and three (polysorbate/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 %.

In order to further analyze the driving forces of this endotoxin masking effect, recovery kinetics using different concentrations of the reactants were analyzed. First of all, recovery of different endotoxin concentrations (50, 500 and 5000 EU/mL) were studied under constant polysorbate/citrate conditions (Figure 13). The recovery over time showed no significant difference using different endotoxin concentrations, indicating that masking is independent of the initial endotoxin concentration. After 10 minutes of incubation, all recoveries are above 50% and after 45 minutes all recoveries are below 7%.

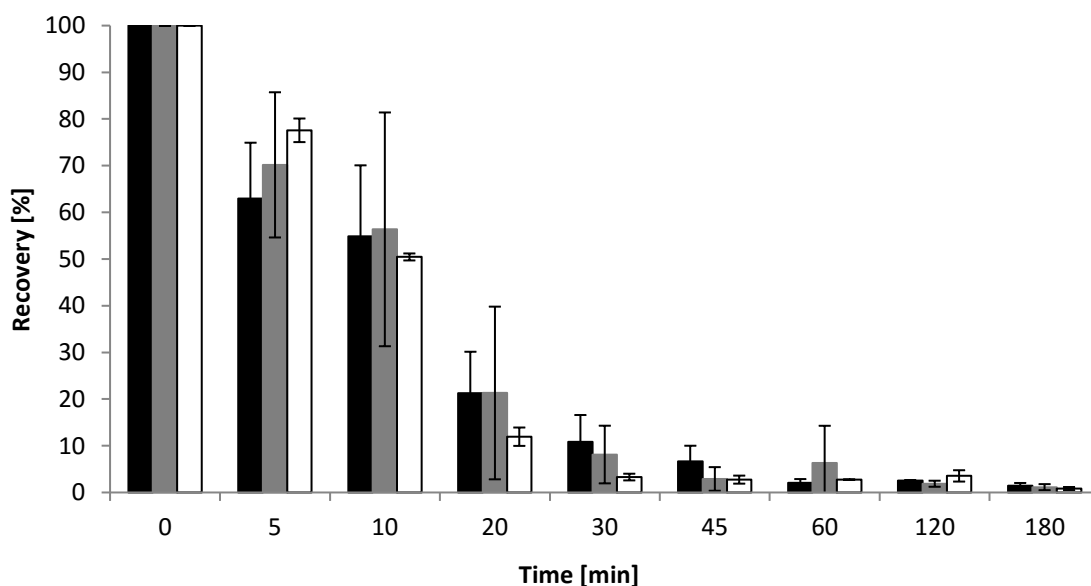


Figure 13 Endotoxin recovery kinetics depending on LPS concentration

Endotoxin recovery is plotted as a function of incubation time. Varying contents of endotoxin were added to samples containing 0.05 wt % polysorbate and 10 mM sodium citrate at RT. The black columns reflect 5000 EU/mL, grey columns 500 EU/ml and white columns 50 EU/mL. For calculation of the data points the mean values of two individual 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 %.

Thereafter, masking of endotoxin was analyzed using reduced concentrations of polysorbate and sodium citrate (Figure 14A). The recovery of endotoxin in a sample containing 0.05 wt % polysorbate and 10 mM sodium citrate was below 1 % after one hour of incubation. Using a sample matrix of 0.0125 wt % polysorbate and 2.50 mM sodium citrate the endotoxin recovery was not reduced after one hour of incubation, but after incubation of 20 hours, the endotoxin recovery was very low, too. Using a sample matrix containing 0.0008 wt % polysorbate and 0.16 mM sodium citrate, no significant decrease in recovery of endotoxin is observed over the analyze period of time (20 hours). Thus, 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 concentration (0.0500, 0.0125 and 0.0008 wt %) was reduced (Figure 14B).

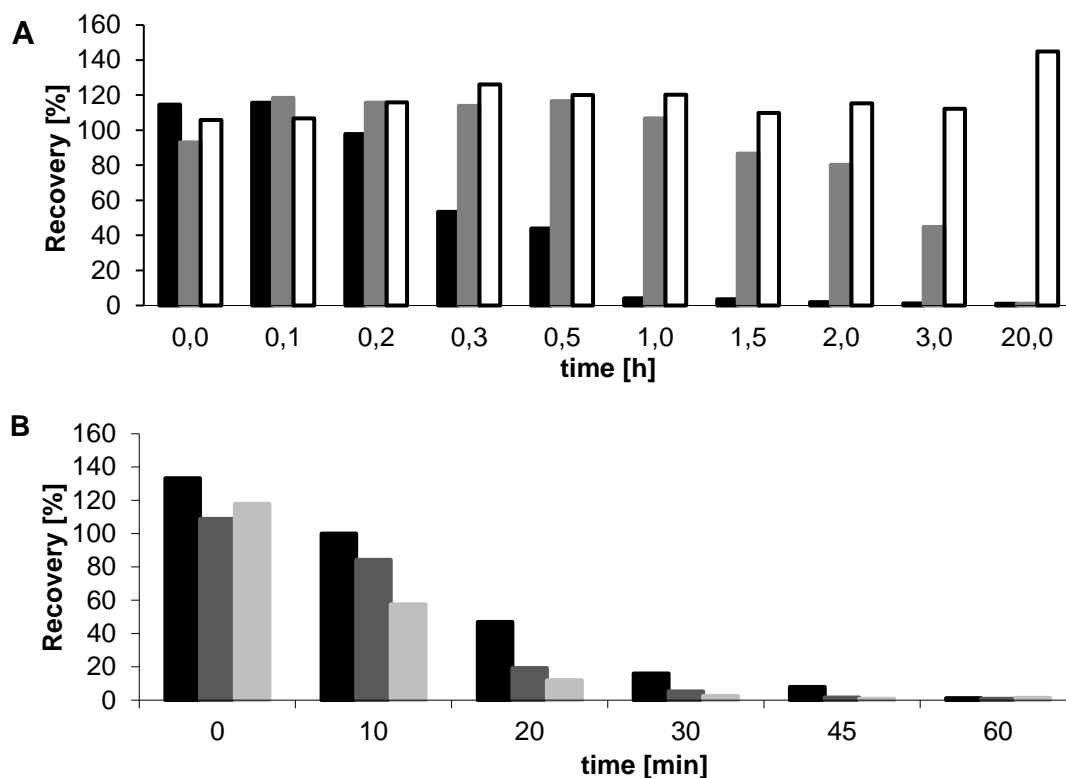


Figure 14 Endotoxin recovery kinetics depending on concentration of matrix components

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 at RT. The three different colored columns reflect different polysorbate and sodium citrate concentrations. The set of black columns correspond to 0.0500 wt % polysorbate and 10.00 mM sodium citrate, the set of grey columns correspond to 0.0125 wt % polysorbate and 2.50 mM sodium citrate and the set of white columns correspond to 0.0008 wt % polysorbate and 0.16 mM sodium citrate.

B) Endotoxin recovery over time in samples containing polysorbate, sodium citrate and LPS is shown. The different colored columns reflect different polysorbate concentrations. The set of black columns correspond to 0.0500 wt % polysorbate, the set of dark grey columns correspond to 0.0125 wt % polysorbate and the set of light grey columns correspond to 0.0008 wt % polysorbate. The concentrations of endotoxin (100 EU/mL) and sodium citrate (10 mM) as well as temperature (RT) were kept constant.

Concentrations of the other components (endotoxin and citrate) were kept constant. In this case, the recovery kinetics is similar within a given measurement uncertainty. The endotoxin recovery is below 2 % independent of the polysorbate concentration after one hour of incubation. This result differs from the previous result, in which the endotoxin was recovered after one hour of incubation (> 100%) at reduced polysorbate and citrate concentrations (Figure 14A). Although the polysorbate concentration was comparably titrated in both cases, the sodium citrate concentrations were different among the two experiments (Figure 14: 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, endotoxin recovery kinetics was examined in the presence of divalent cations (Figure 15A). Here, masking kinetics is delayed in the presence of 1 mM magnesium dichloride compared to the samples without magnesium dichloride. In the presence of 5 mM magnesium dichloride, no

reduced endotoxin recovery is observed within the analyzed time scale. Importantly, the magnesium dichloride was added to the samples prior to endotoxin. In a further experiment, magnesium dichloride was added to the polysorbate/sodium citrate matrix 20 minutes after start of the reaction (figure 15B). 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 dichloride. In comparison, the sample without supplementary addition of divalent cations was masked as expected. Hence, the addition of magnesium dichloride 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 dichloride.

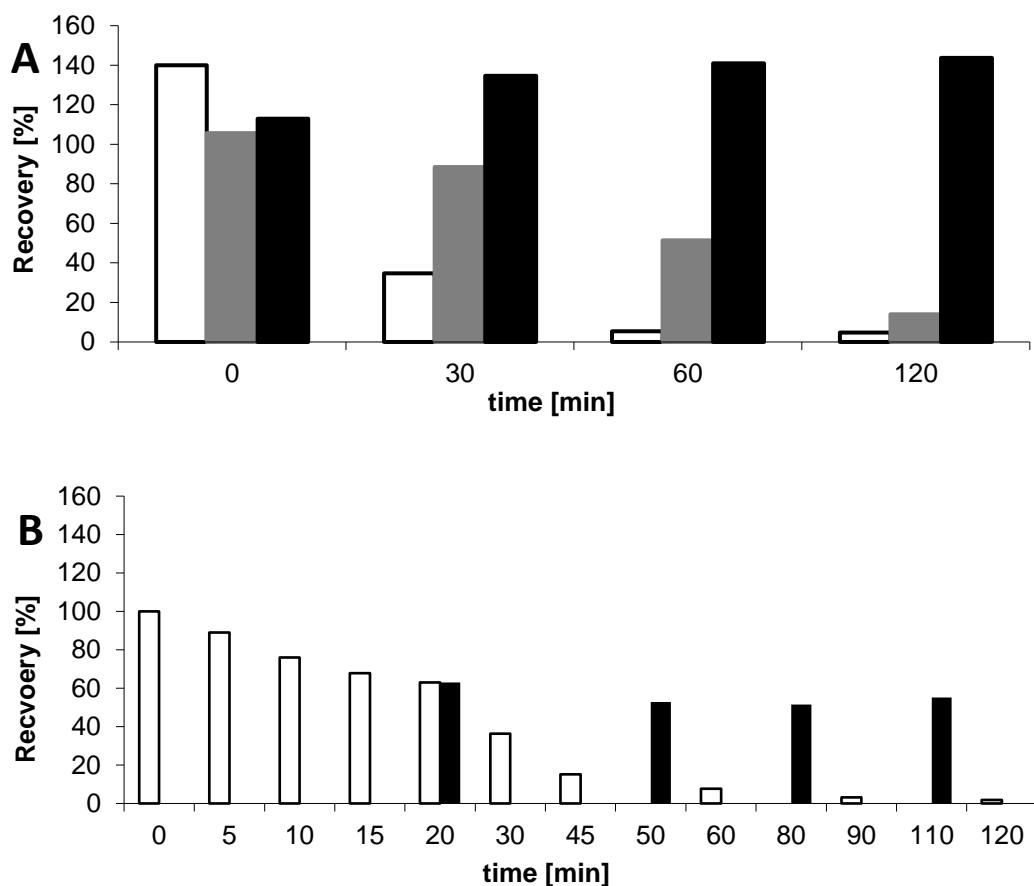


Figure 15 Endotoxin recovery depends on the presence of divalent cations

A) Endotoxin recovery is plotted as a function of time in samples containing polysorbate 20, sodium citrate, endotoxin and magnesium dichloride. The different colored columns reflect different contents of magnesium dichloride (0 mM (white columns), 1 mM (grey columns), 5 mM (black columns)). Concentrations of polysorbate (0.05 wt %), sodium citrate (10 mM) and endotoxin (100 EU/mL) as well as temperature (RT) were kept constant.

B) Endotoxin recovery is plotted as a function of time in a sample containing 0.05 wt % polysorbate, 10 mM sodium citrate and 100 EU/mL endotoxin (white columns). After 20 minutes, the sample was divided into two equivalent aliquots (1 mL each), whereof one aliquot was treated once by the addition of 20 mM (20 μ L of 1 M) magnesium dichloride and the other fraction were continued without treatment. Endotoxin recovery of the treated fraction is expressed by the black columns.

Obviously, there is a relation between masking kinetics and complex formation. For deeper analysis of this effect, masking kinetics using three different citrate concentrations (5, 20, and 80 mM) were recorded (Figure 16). A distinct acceleration of masking by increasing the citrate concentration in the sample from 5 mM to 80 mM is observed. Furthermore, the experimental data points are compared to a model curve using an exponential decay function, in which the endotoxin recovery $[LPS_d]$ is calculated as a function of time t :

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

The parameters of this function were set in analogy to the experimental conditions. Endotoxin recovery at time 0 $[LPS_d]_0$ was set to 100%, time t was given in minutes and k was chosen by chance and set to 7. The sodium citrate concentrations $[Ci]$ were set to 0.005, 0.020 and 0.160 mol/L in the particular curves. The simulated curves are in good agreement with the experimental data, illustrating that the endotoxin masking kinetics is depending on the sodium citrate concentration. Derivation of this model function is discussed in chapter 3.2.3.1 Simulation of LER kinetics.

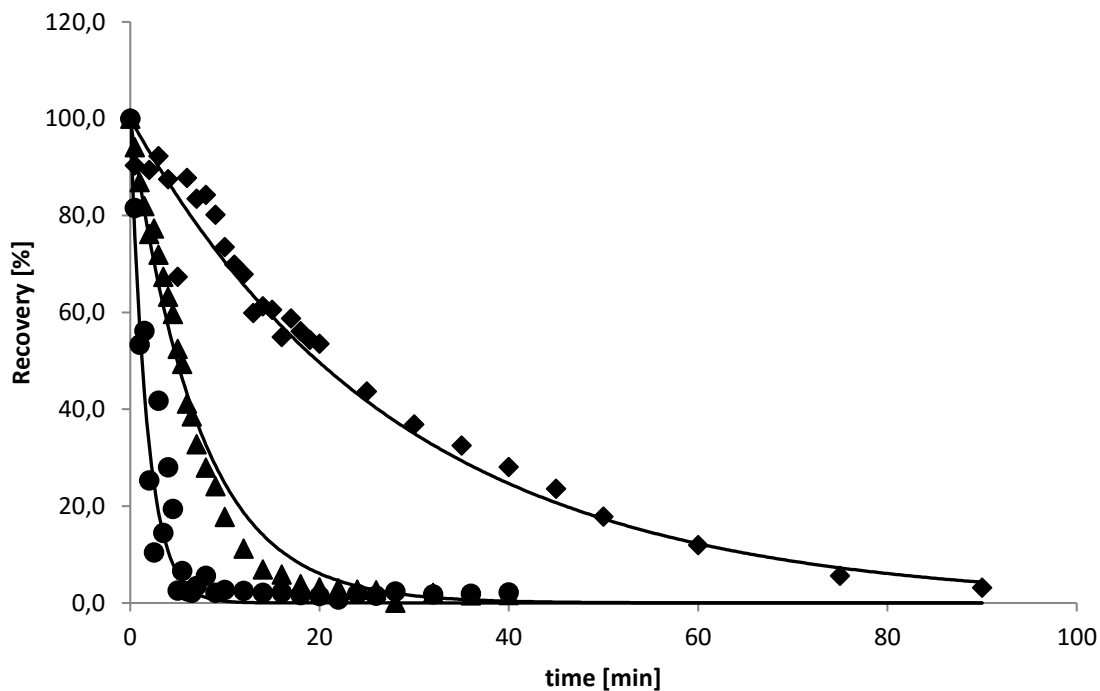


Figure 16 Sodium citrate concentration determines endotoxin recovery

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 (◆), 20 mM (▲), 80 mM (●)). Concentration of polysorbate 20 (0.05 wt %) and temperature (RT) were kept constant. For the corresponding 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 %.

Summarizing the kinetics above, 100 EU/mL endotoxin will be substantially masked within 90 minutes of sample incubation in the presence of at least 5 mM sodium citrate and 0.05 wt % polysorbate 20. Variation of the polysorbate concentration (Figure 14B) showed no significant acceleration or deceleration of the masking kinetics. In contrast, the increase of sodium citrate concentration (Figure 16) resulted in considerably accelerated endotoxin masking kinetics. To determine, whether there is a minimum citrate concentration for endotoxin masking, sodium citrate was titrated under constant polysorbate and endotoxin concentrations and the samples were incubated for seven days prior to endotoxin measurement (Figure 17). According to the previous kinetics, it was assumed that equilibrium of the masking reaction will be established after seven days. Plotting endotoxin recovery as a function of citrate concentration results in a S-shaped data point's 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 facilitate endotoxin masking 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:

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

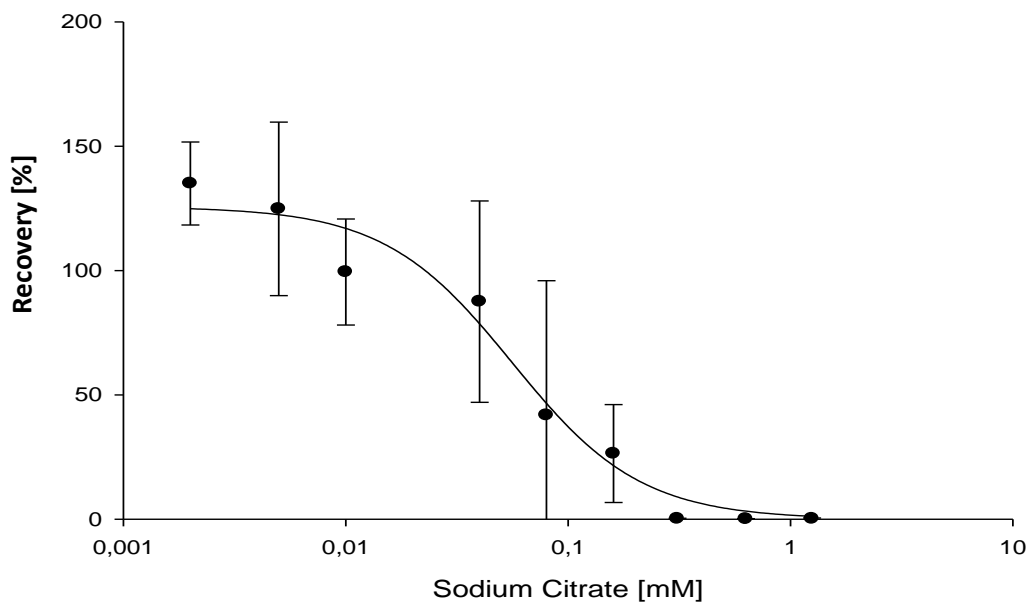


Figure 17 Endotoxin recovery depends on sodium citrate concentration under equilibrium conditions

Endotoxin recovery is plotted as a function of sodium citrate concentration. 50 EU/mL endotoxin and 0.05 wt % polysorbate were incubated with varying sodium citrate concentrations for seven days at RT 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 is used (Equation 2).

A three parameter logistic function (Equation 2) was chosen, because it reflects a sigmoid curve progression and the transition point can be determined easily. The resulting sigmoid curve fit shows endotoxin recovery as a function of sodium citrate concentration. The calculated coefficients “a” equates 125.66, “b” equates 1.50 and “ x_0 ” equates 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 (Figure 17). In consequence, masking of endotoxin does not occur in solutions containing polysorbates and less than 0.06 mM sodium citrate.

3.2.3 Discussion

Endotoxin masking (LER) has been identified as a time-dependent phenomenon and in contrast, test interference occurs instantly[72], [73]. The latter can be therefore clearly distinguished from masking. The results presented here show that endotoxin masking in a sample may not be discovered when the spike is added to the diluted sample, because the original ability of the sample matrix for masking may be weakened or even avoided, when the concentration of a matrix component is reduced. For example, Figure 14A shows a significant delay in time until LER is recognized when endotoxin is spiked in a diluted sample. Due to the fact that test interference is considered since decades, common and compendial BET procedures are trimmed to identify and overcome test interference, but these procedures are not prone to discover and overcome time dependent masking effects. Therefore, it is important to universally include endotoxin spikes into undiluted samples to actual test procedures as well as careful consideration of suitable incubation conditions. In 2012, US FDA has already updated their guidelines for BET[57] and European Pharmacopoeia includes hold time experiments in the coming 9th edition (2017). To this end, it is necessary to generally extend and harmonize worldwide compendial test procedures to thoroughly identify the endotoxin masking capability of a sample.

However, to control the phenomenon of LER, understanding of the masking mechanism is a key factor. There are several examples showing that endotoxins interact with a variety of components, including proteins[47], surfactants[51] or nano particles[74], but the reaction mechanism remains to be elucidated. Most likely, due to the amphiphilic and amphoteric character of LPS[9], hydrophobic and electrostatic interactions are involved. According to previous assumptions the two-step masking mechanism (3.1.3.3) may formally be described as follows:



Chelating buffer components (Ci) destabilize salt bridges formed between divalent cations (M) (e.g. magnesium) and negatively charged substitutes (e.g. phosphates) of LPS (LPS) (Equation 3).



Subsequently, non-ionic surfactants (P) (e. g. polysorbate) 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 depending reaction step is necessary. To this end, the kinetics of endotoxin masking was studied in the presence of citrate and polysorbate. Interestingly, pre-incubation of LPS with citrate and the subsequent addition of polysorbate resulted in very fast masking kinetics. However, pre-

incubation of LPS with polysorbate, and subsequent addition of citrate resulted in somewhat slower kinetics (Figure 12). Furthermore, masking kinetics with increased citrate concentrations and constant polysorbate concentrations (Figure 16) show accelerated reaction rates. In turn, a variation of polysorbate concentration under otherwise identical conditions had no significant impact on the reaction rate (Figure 14B). As a consequence, the first step of the reaction mechanism (Equation 3) seems to control the reaction rate and depends on the citrate concentration.

3.2.3.1 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, because this step is 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:

$$R = d[\text{LPS}_d] / dt \quad (5)$$

In addition, the results implicate that the reaction rate depends on the citrate concentration (Figure 16). Generally, it is supposable that the reaction rate is also depending on the LPS concentration, although the results indicate no concentration dependency of LPS (Figure 13). Thus, the reaction rate R of endotoxin masking is described as depending on detectable LPS [LPS_d] and the citrate concentration [Ci] resulting in the following equation:

$$R = k[\text{LPS}_d][\text{Ci}] \quad (6)$$

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

$$d[\text{LPS}_d] / dt = k[\text{LPS}_d] [\text{Ci}] \quad (7)$$

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[75]:

$$(1/([\text{Ci}]_o - [\text{LPS}_d]_o)) * (\ln([\text{Ci}]/[\text{Ci}]_o) / ([\text{LPS}_d]/[\text{LPS}_d]_o)) = kt \quad (8)$$

Yet, 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 mol/L. In order to convert EU/mL in mol/L it is assumed that 1 EU correlates approximately to 10⁻¹⁰ g (100 pico gram) LPS from *E.coli*[76], [77]. With a molar mass of approximately 10000 g/mol for LPS, 100 EU/mL are equivalent approximately to 10⁻⁹ mol/L[78], [79]. As a consequence, 10⁻³ mol/L citrate is in a substantial molar excess compared to

10^{-9} mol/L of LPS ($[C] \gg [LPS]$). Theoretically, this would already 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, our results (Figure 16) show that the actual citrate concentration has indeed a significant effect on the reaction kinetics. Furthermore, the results indicate that the kinetics seem to be independent of the LPS concentration, which is reasonable, because citrate is in an excess of up to seven orders of magnitude $[C] \gg [LPS]$. A change of the LPS concentration, for example by a factor of 1000, will presumably 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 $[LPS_d]_o$ and leading to the following approximation:

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

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) the 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 function of time, and depending on the citrate concentration:

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

The simulation of masking kinetics with different citrate concentrations, using equation 12, resulted in a good agreement with the experimental data (Figure 16). Consequently, this model reflects very well the observed behavior of the reaction, in which the endotoxin recovery is strongly dependent on the citrate concentration. However, specifications may change, if initial concentrations of the components are substantially changed or if additional components (e.g. salts and proteins) are included in the sample conditions.

3.2.3.2 Minimum citrate concentration

Citrate has been identified to control 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 concentrations was analyzed. The result shows that approximately 0.06 mM of citrate is necessary for masking (Figure 17). This concentration is

orders of magnitude higher than the molar concentration of LPS, assuming that there is no reasonable reaction stoichiometry. Nevertheless, citrate might destabilize LPS aggregates by considering the potential role of citrate during masking. For example, permeabilization (reduction of rigidity) of LPS aggregates occurs when ionic interactions between LPS molecules are disturbed. Primarily, magnesium as well as calcium cations (M^{2+}) stabilize LPS-LPS interactions by the formation of salt bridges [62], [80]–[82]. It has also been shown that complex forming agents can permeabilize such structures [83], [84]. In the presence of citrate, complex formation between divalent cations and chelator occurs. Thus, it is supposed that citrate competes for divalent cations bridging LPS molecules resulting in a permeabilization of LPS aggregates, because of complex formation [85]. Corresponding complex formation constants of magnesium or calcium citrate are in the range around 0.05 mM [86]–[88]. This might explain the required molar excess of citrate compared to LPS for masking, because at lower citrate concentration, the complex formation of calcium or magnesium by citrate is not favored and in consequence the LPS-LPS salt bridges are not destabilized, which prevents intercalation of surfactants and in turn masking of endotoxin.

3.2.3.3 The role of divalent cations

As described above, divalent ions play an important role in the stabilization of supramolecular LPS structures. Aggregates of LPS possess a certain degree of rigidity, maintained by salt bridges between LPS molecules, which in turn affect the susceptibility to masking. To this end, masking rate can be inhibited by the supplementary addition of divalent magnesium ions. This explains former observation showing that under certain circumstances, endotoxin activity in LAL can be maintained through the suppression of aberrant aggregation of endotoxin by saline and buffers [89]. It is most likely that the addition of divalent cations neutralizes the complex formation capability of chelators and favors the stabilized LPS state, because the state of equilibrium is changed, when the sample matrix is changed. Importantly, the retrospective addition of divalent ions to samples in which the endotoxin is masked, enabled no recovery of endotoxin, but the progress of masking can immediately be stopped (Figure 15B). Thus, the effective addition of cations has to take place before endotoxin is already masked. 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 endotoxin masking, the merely addition of divalent ions is not sufficient. For such an approach, also surfactants have to be neutralized or removed. This approach will be discussed in detail in chapter 3.4 demasking endotoxins.

3.2.3.4 Control of reaction rate

In general, to start the reaction of endotoxin masking, a certain energy barrier has to be overcome, which controls the reaction rate. Obviously, the reaction rate can be manipulated by its energy input. For instance, the reaction kinetics can be accelerated chemically by e.g. increasing chelator concentration (3.2) or physically by e.g. increasing incubation temperature of a sample (3.1). Moreover, it is conceivable that the degree of the energy barrier depends on the endotoxin itself. Endotoxin from different sources may have different molecular structures and in turn different masking susceptibilities due to varying stabilization mechanisms of bacteria's outer membrane. In consequence, endotoxins with different stabilizing mechanisms may have different energy barriers and result in manipulated reaction kinetics. To this end, the heterogeneity of endotoxins and their detectability in LER-affected samples is studied in the next chapter (3.3).

3.3 Heterogeneity of endotoxins and their detectability in common biopharmaceutical formulations

3.3.1 Introduction

LPS play an important role in the pathogenesis and manifestation of Gram-negative infections and in particular of septic shock. Due to the fact that the structures of LPS can vary significantly in its O-antigen, core region and lipid A, not all endotoxins possess the same toxicity[17]. However, to control a potential contamination event in drug products by bacterial endotoxins, the sensitive and specific detection of LPS is of great importance. BET has been proven to effectively detect LPS. These tests are based on reactions between the lipid A of LPS and specific enzymes derived from the clotting cascade in horseshoe crabs[33], [90]. For quantitative detection of endotoxin, measures of the unknown samples are compared with standard curves. Such standard curves are prepared by known and defined standard endotoxins. The primary standard in BET is called Reference Standard Endotoxin (RSE), which is endotoxin from *E.coli* O113:H10:K negative. The RSE is purified in several steps including hot phenol-extraction, alcoholic precipitation, enzymatic digestion of nucleic acids and intense dialysis[91]. For a better handling lactose and polyethylene glycol are added. This standard is worldwide accepted and sets the baseline for secondary or tertiary standards. Due to the limited availability of the RSE, vendors of BET systems distribute secondary standards called Control Standard Endotoxins (CSE), which are calibrated against the RSE. The preparations of these secondary standards are inspired by RSE, but the source can differ from *E.coli* O113 and exact production processes and formulations are not published.

After public recognition of the LER phenomenon, many not publicly accessible endotoxin recovery studies in biopharmaceutical drug products and their formulations were performed by endotoxin test providers, contract labs and pharmaceutical companies. The masking effects have been widely confirmed, especially if the tested drug products contain surfactants and chelators. Such hold time experiments are usually performed using standardized endotoxins like CSE and RSE as spike. However, since bacterial endotoxin tests are commercially used, the source and preparations of appropriate standard endotoxins are debated[24]. Especially in the case of LER the source of endotoxin can be discussed, again. It is questionable, whether LER is depending on the endotoxins used in BET. Obviously, depending on the source, preparation and degree of purification, the LPS itself and the accompanying matrix components can vary in a respective preparation[11], [92]. For instance, endotoxins from different bacteria can differ in their molecular structures. There are differences in the lipid A (e.g. acylation), core region (e.g. substitution of sugar units) and O-antigen (e.g. distribution of sugar units). Moreover, depending

on the preparation endotoxin suspensions may vary in their compositions. For example, crude suspension of bacteria, popularly known as Naturally Occurring Endotoxin (NOE), may contain substantial amounts of lipids and proteins and phenol-extracted endotoxin preparations may only contain limited contents of hydrophobic matrix components. Some experiments have supposed that the detectability of selected endotoxins in complex samples might be more robust compared to detectability of commercially available standard endotoxins[93], [94]. Therefore, endotoxins from different bacteria, grown under manipulated conditions, crude and highly purified endotoxins as well as endogenous endotoxins are analyzed with regard to their detectability in a polysorbate/citrate matrix in the present study.

3.3.2 Results

In order to study the masking susceptibility of endotoxins from different bacteria, crude suspensions of bacterial endotoxin were prepared. To this end, bacteria from *E.coli*, *E.cloacae*, *S.marcescens*, *P.aeruginosa*, *B.cepacia*, *S.maltophilia* and *R.pickettii* were grown under equal and defined conditions (2.4.1 preparation 1). After approximately 18 hours of growth, absorbance of all bacterial suspensions were determined (Table 3). Bacteria from *E.coli* and *E.cloacae* showed highest absorbance (> 1.7), whereas *S.maltophilia* and *B.cepacia* showed lowest absorbance (< 0.7). Bacteria from *S.marcescense* showed a medium absorbance of 1.4.

Table 3 Growth of different bacteria and release of endotoxin

Growth of different bacteria and activity of their endotoxins are shown. Bacteria were grown under constant conditions and before harvesting, absorbance (600 nm) of the entire bacterial suspension were determined (2.4.1 preparation 1). Endotoxin activity was determined in the particular sterile-filtrated supernatants of bacterial suspensions.

Source	Absorbance [600 nm]	Activity [EU/mL]
<i>E.coli O55:B5</i>	1.9	146,174
<i>E.coli O113</i>	1.7	402,789
<i>E.cloacae</i>	1.7	189,103
<i>S.marcescens</i>	1.4	116,175
<i>P.aeruginosa</i>	0.9	8,595
<i>B.cepacia</i>	0.7	357
<i>S.maltophilia</i>	0.3	4,557
<i>R.pickettii</i>	0.8	77,815

The absorbance reflects the evolved biomass and in turn is an indicator for growth of the particular bacteria under given conditions. This result clearly indicates varying growth characteristics of different bacteria under given conditions. Endotoxins are usually incorporated in the bacterial cell wall, but during growth, substantial amounts of endotoxin are released into the environment of the bacteria. Therefore, the endotoxin activity in the supernatant is of great interest. Interestingly, the detectable endotoxin activities of the different bacterial supernatants ranged from approximately 400 to 400,000 EU/mL. Between the generated biomass of cells and the detectable activity only a weak correlation is given.

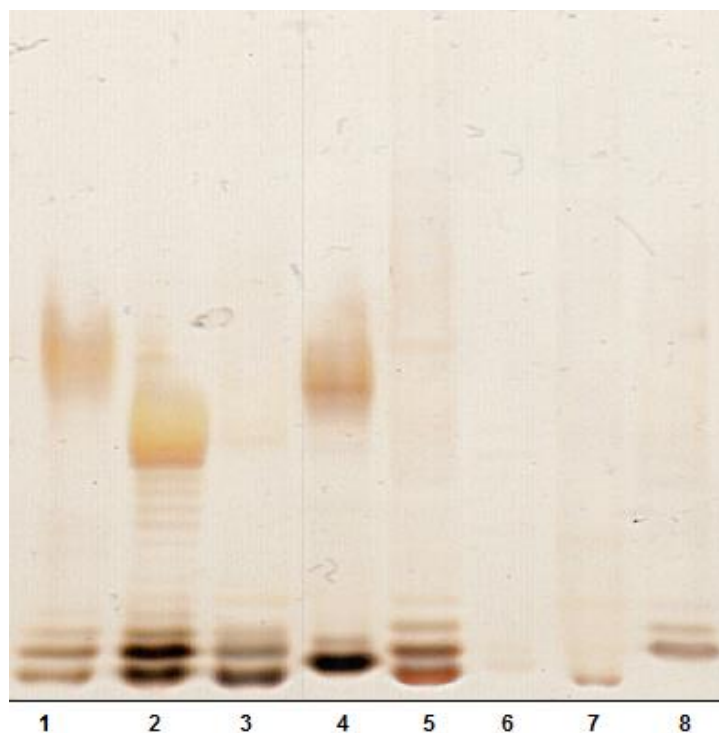


Figure 18 SDS-PAGE of crude bacterial endotoxin preparations from different bacteria

Silver-stained SDS-PAGE of sterile filtrated supernatants of bacterial suspensions (2.4.1 preparation 1) is shown. The lanes reflect endotoxins from *E.coli* O55:B5 (1), *E.coli* O113 (2), *E.cloacae* (3), *S.marcescens* (4), *P. aeruginosa* (5), *B.cepacia* (6), *S. maltophilia* (7) and *R.pickettii* (8).

Furthermore, these bacterial supernatants were applied to SDS-PAGE and silver stained (Figure 18). The typical ladder pattern of LPS can be observed at most lanes and the limited intensities of the bands reflect low concentrations, which are approximately in agreement with the detected activities (Table 3). Upon closer examination, also variations in the arrangement of bands between the different endotoxin samples can be observed, which reflect different molecular structures and proof heterogeneity of endotoxins. To study the masking susceptibilities of crude endotoxin preparations, these preparations were used as endotoxin source for recovery experiments in polysorbate/citrate matrices (Table 4). The crude extracts of endotoxins from *E.coli*, *E.cloacae* and *S.maltophilia* resulted in low recovery already at day 0. Endotoxins from *S.marcescens*, *P.aeruginosa*, and *R.pickettii* showed a gradual loss of recovery over time. The endotoxins from *B.cepacia* could be detected over time and showed no trend in reduced activity. This result clearly indicates that endotoxins from different bacteria, but grown and prepared under equivalent conditions exhibit different masking susceptibilities.

Table 4 Endotoxin recovery of different endotoxins

Recovery of endotoxin from different species over time is shown. Sterile filtrated bacterial supernatants (2.4.1 preparation 1) were diluted to approximately 50 EU/mL in depyrogenated water (positive control). For recovery experiments the endotoxins were spiked with 0.05 wt % polysorbate 20 and 10 mM sodium citrate (reverse mode, 2.5.3.2). Endotoxin recovery was determined after 0, 1, 2, 5 and 7 days of incubation at RT.

Source	positive control	Recovery (%)				
		T0	T1	T2	T5	T7
<i>E.coli O55:B5</i>	40.9	32	7	8	2	0
<i>E.coli O113</i>	64.7	42	0	0	0	0
<i>E.cloacae</i>	50.9	20	4	1	0	0
<i>S.marcescens</i>	60.5	106	46	29	12	8
<i>P.aeruginosa</i>	79.6	135	29	25	23	16
<i>B.cepacia</i>	48.5	248	81	141	92	113
<i>S.maltophilia</i>	37.9	20	1	1	0	0
<i>R.pickettii</i>	68.4	108	73	80	71	55

Furthermore, crude endotoxin extracts from *E.coli O113*, *P.aeruginosa* and *B.cepacia* were prepared under different growth conditions (2.4.2 preparation 2). Thereby, the bacteria were grown under conditions including rich-nutrition media (100 % LB) and elevated temperatures (37 °C) as well as low-nutrition (1 % LB) and ambient temperatures (RT).

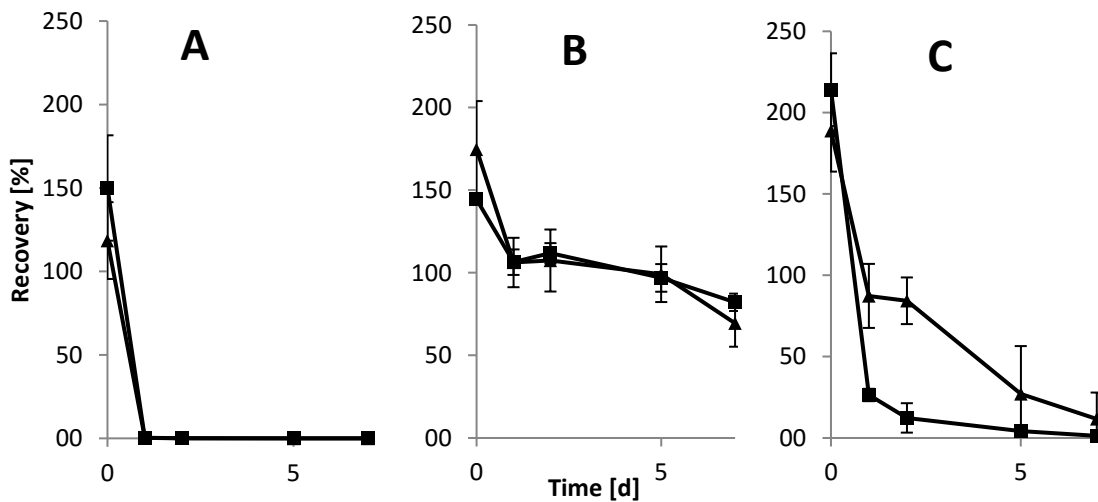


Figure 19 Comparison of crude endotoxin preparations from different bacteria

Endotoxin recovery is plotted as function of incubation time. 100 EU/mL endotoxin from different bacteria ((A) *E.coli O113:H21-*, (B) *B.cepacia* and (C) *P.aeruginosa*) were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20 and incubated at RT (reverse mode, 2.5.3.2). The used endotoxin extracts were derived from two different bacterial growth conditions (2.4.2 preparation 2). Squares reflect recovery of endotoxin from bacteria grown at 37 °C using 100% LB media. Triangles reflect recovery of endotoxin from bacteria grown at room temperature using 1% (v/v) LB media. Each endotoxin was prepared in triplicate. The corresponding endotoxin measurements of three repetitions were analyzed on the same microtiter plate and for calculation of the data points the mean value of the three individual preparations were used. The error bars represent the standard deviation of the three replicates.

Endotoxin from *E.coli* O113 is not recovered independent of the growth conditions of the bacteria (Figure 19A) after one day of incubation. In contrast, the recovery of endotoxin prepared from *B.capecia* shows no significant decline, regardless of the different growth conditions (Figure 19B). Interestingly, recovery of endotoxin from *P.aeruginosa* depends on different growth conditions (Figure 19C). In this case, modified growth conditions resulted in diverging masking kinetics. To this end, it has been described that bacterial growth under limitation of divalent cations may also affect the molecular structures of endotoxin[95]. To further study this effect, bacteria from *E.cloacae* were grown under rich nutrition conditions in the presence (EDTA) and absence (TRIS) of the strong complex forming agent EDTA (2.4.2 preparation 2). The endotoxin recovery kinetics indicates that both endotoxins are affected by masking over time (Figure 20). However, the recovery over time of endotoxin, which was prepared out of EDTA-treated bacterial cells (Figure 20B) is greater than the recovery of endotoxin from bacteria which was not treated with EDTA during bacterial growth (Figure 20A). Comparing the error bars in both experiments, endotoxin recovery from EDTA treated cells tend to be more variable and by chance, a trend to reduced recovery over time is not observed. Although chelators have crucial effects on masking of endotoxin, the presence of chelators during bacterial growth reduces the masking susceptibility of the endotoxin under given conditions.

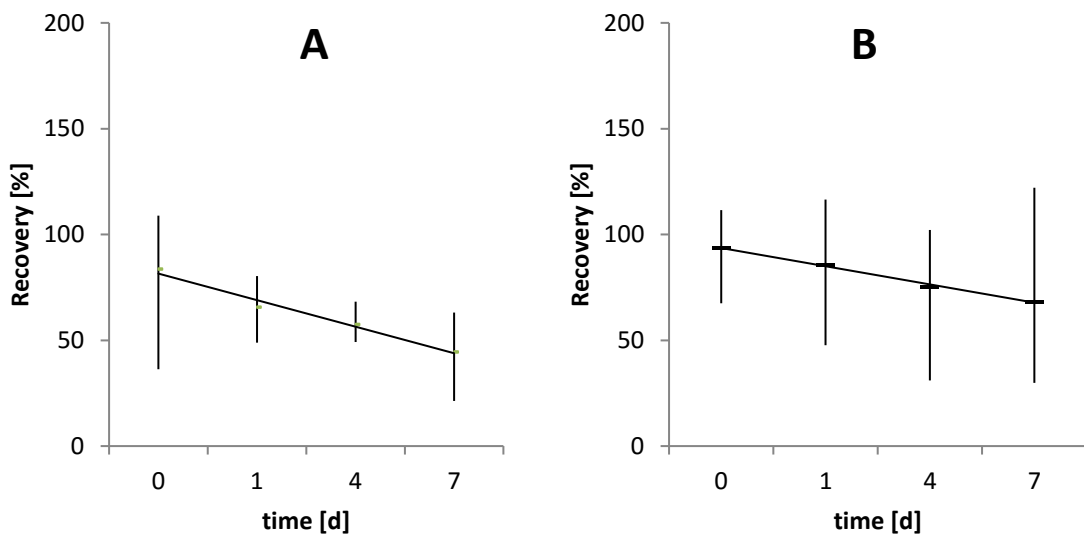


Figure 20 Endotoxin recovery kinetics of endotoxin from *E.cloacae*

Endotoxin recovery is plotted as a function of incubation time. 100 EU/mL endotoxin were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20 and incubated up to seven days at 4°C. The crude endotoxin extracts were derived from *E.cloacae* and the media (100% LB) for bacterial growth at 37 °C was supplemented with (A) tris buffer and (B) EDTA (2.4.2 preparation 2). Each data point represents the mean of three independent measurements. The slope is obtained by linear fit of the mean data points. The error bars represent the lowest and highest determined values at each time point.

However, for investigation of endotoxin masking in quality control of pharmaceutical industries standardized endotoxins are requested by regulatory authorities (US FDA). Such standard endotoxins are prepared by hot-phenol extraction of LPS [91]. Thereby, hydrophobic components like phospholipids and lipoproteins are removed. To analyze, if masking susceptibilities of endotoxin are affected by purification, phenol extracted endotoxins from *E.coli*, *S.marcescens* and *P.mirabilis* were spiked into samples containing a chelator and surfactant and detected over time (Figure 21). In this case, endotoxin from *E.coli* was low (14%) in recovery directly after spiking and no activity of endotoxin was detectable after one day of incubation. The endotoxin from *S.marcescens* was detectable (90%) at day 0, but likewise low (21%) after one day of incubation. Although endotoxin from *P.mirabilis* decreases over time, significant amounts of endotoxin were recovered (>43%) at all time points. These results show different masking susceptibilities of the studied endotoxins, indicating that phenol extraction is not eliminating the heterogeneity of endotoxins from different bacteria.

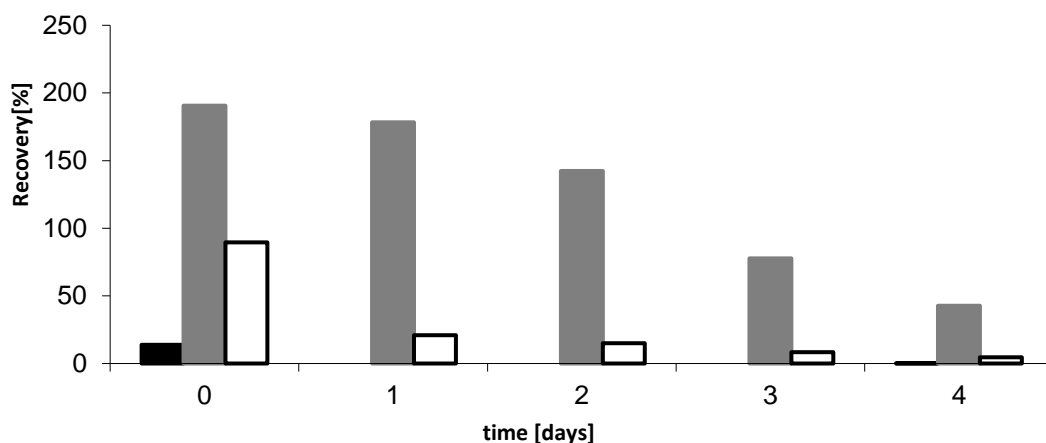


Figure 21 Comparison of phenol-extracted endotoxins from different bacteria

Endotoxin recovery is shown over incubation time. 100 EU/mL of three different endotoxins were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20 and incubated up to 4 days at RT. The used endotoxins were phenol-extracted clinical isolates. The black bars reflect endotoxins from *E.coli*, grey bars reflect *P.mirabilis* and white bars reflect *S.marcescens*.

To further evaluate the impact of endotoxin purification, endotoxins from the same bacterial species (*E.coli* O55:B5), but prepared by different methods were analyzed. Phenol-extracted endotoxin and crude supernatants of bacterial suspension were spiked into a polysorbate/citrate matrix and incubated up to six days at room temperature (Figure 22A). Both preparations show no recovery after one day of incubation, which confirms the pronounced masking susceptibility of endotoxin from *E.coli*. Due to the fast kinetics of masking, the experiment was also performed at decreased incubation temperature (4 °C) (Figure 22B). The reduced incubation temperature was chosen, because endotoxin masking can be decelerated, allowing a better resolution of slight

differences in masking susceptibilities (3.1). After 2 days of incubation 50% and after 14 days 14% of the initial endotoxin content can be detected within the crude extract. In comparison, the recovery of phenol-extracted endotoxin was low after one day of incubation (27%) and no significant content of endotoxin was detectable after three days of incubation. Under these circumstances the detectability of crude endotoxin preparations decreases slower compared to phenol-extracted endotoxin. However, both preparations of endotoxin are affected by masking. In consequence, endotoxin masking kinetics can be affected by the extraction method of endotoxin, but the kinetics rather depends on the source of bacteria.

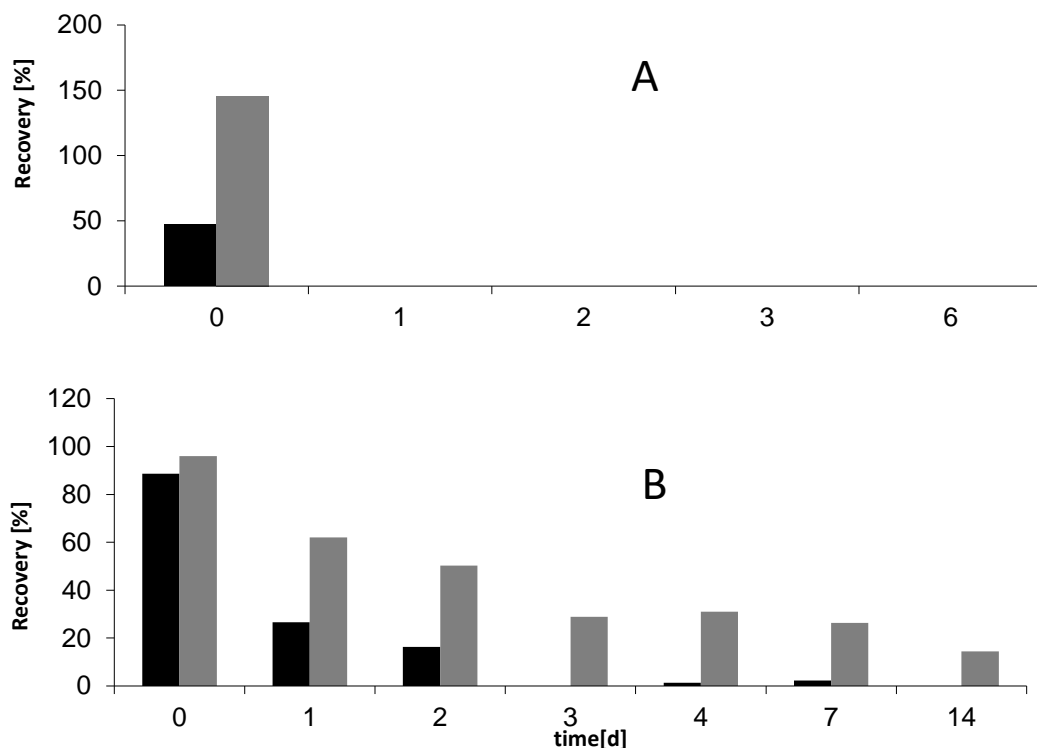


Figure 22 Endotoxin recovery kinetics of different endotoxin preparations

Recovery of endotoxin from *E.coli* O55:B5 is shown over incubation time. 100 EU/mL of gel-filtrated endotoxin (black bars) and sterile filtrated bacterial suspension (grey bars) were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20. The samples were incubated up to 14 days at (A) RT and (B) 4°C.

In all of the examples above, the source of endotoxin was known and the endotoxin was consciously added to samples containing surfactants and chelators. In order to examine a real endotoxin contamination, the detectability of an endogenous contaminated monoclonal antibody was analyzed. Therefore, a lyophilized antibody was solubilized in four different buffer systems and an average endotoxin activity of 135 EU/mL and 114 EU/mL was determined before and after sterile filtration, respectively (Table 5a). The different buffer systems as well as the filtration had no major effects on the detectability of the endogenous endotoxin contamination of the antibody.

Table 5 Detection of an endogenous endotoxin contamination

Activity of an unknown endotoxin contamination under different buffer conditions is shown. 10 mg/mL monoclonal antibody (MAK33) were solubilized in A) 25 mM sodium citrate, pH 6.5; B) 10 mM sodium citrate, pH 7.5; C) 160 mM trehalose, 50 mM sodium phosphate pH 6.2 and D) 10 mM sodium phosphate, pH 7.5). a) Endotoxin content was determined before and after sterile filtration (0.2 µm). For endotoxin detection EndoZyme® was used. b) After filtration 0.07 and 0.05 wt % polysorbate 80 were added to the samples 1.1 and 2.1, respectively. 0.04 and 0.05 wt % polysorbate 20 were added to the samples 3.1 and 4.1, respectively. Endotoxin activity was determined immediately after addition of polysorbate (day 0) and after incubation of three days (day 3) at RT. For endotoxin detection EndoLISA® was used.

a		before filtration	after filtration
#	Sample:	Activity [EU/mL]	Activity [EU/mL]
1.1	MAK + Buffer A	116.9	109.0
2.1	MAK + Buffer B	140.1	118.3
3.1	MAK + Buffer C	128.7	93.5
4.1	MAK + Buffer D	152.8	136.2
	Average	134.6	114.3

b		day 0	day 3
#	Sample:	Activity [EU/mL]	Activity [EU/mL]
1.2	MAK + Buffer A + Polysorbate 80	98.5	2.8
2.2	MAK + Buffer B + Polysorbate 80	95.6	2.1
3.2	MAK + Buffer C + Polysorbate 20	117.9	5.3
4.2	MAK + Buffer D + Polysorbate 20	131.7	6.8

Known from previous studies, the simultaneous presence of polysorbate and a chelator like citrate can mask the endotoxin (3.1 and 3.2). Therefore, polysorbate was added to the antibody solution. Immediately after addition of polysorbate, the endotoxin content was determined and comparable contents of endotoxin were detected. However, when the antibody was incubated for three days at room temperature, the detectable amount of endotoxin significantly decreased in all of the examined samples (Table 5b). This clearly demonstrates that an endogenous endotoxin contamination, which reflects a real naturally occurring endotoxin, may be masked in common formulation matrices.

3.3.3 Discussion

In previous studies (3.1 and 3.2), the phenomenon of LER has been studied, using standardized endotoxins from *E.coli*. For analytical applications, standards are indispensable to determine unknown concentrations and to validate a detection method. However, to investigate if the phenomenon of LER is limited to the use of standardized endotoxins from *E.coli*, detectability of endotoxins from different sources was studied in a typical biopharmaceutical drug product matrix containing polysorbate and sodium citrate. Phenol-extracted endotoxins (Figure 21), crude endotoxin extracts (Table 4, Figure 19 and 20) and endogenous endotoxin (Table 5) were incubated into samples containing polysorbate/citrate. These results show that LER is not limited to standardized endotoxins from *E.coli*. Endotoxins from different sources and alternative preparations of endotoxin can be affected as well as standard endotoxin preparations by LER. Basically, these results clearly demonstrate different masking susceptibilities of different endotoxins. LPS reflect a complex group of molecules, which possess a common general architecture[11] and due to the amphoteric and amphiphilic nature of LPS supramolecular structures are formed[17], [70]. In the case of masking, it is supposed that the presence of complex forming agents destabilize the salt bridges of divalent cations between LPS, leading to a reduced rigidity of the aggregate. The additional presence of a surfactant may then change the initial supramolecular structure and promote the formation of mixed aggregates, thus limiting the detection of endotoxin. Taking this assumption into account, differences in the molecular structure of LPS may explain the diverging masking susceptibilities of endotoxins from different sources.

3.3.3.1 Molecular heterogeneity of LPS

In general, LPS are very heterogeneous molecules. Already the application of a single source LPS on a SDS-PAGE results in a ladder of bands (Figure 18). This reflects the nature and number of sugars within a unit, the nature of the linkages of the sugars as well as the number of repetitive units. O-antigen sugars appear to be most variable, core structures appear to be less variable and in turn Lipid A structures are considered as the most conserved part of LPS within a genus.[9], [96], [97] With regard to the previously described studies on endotoxin masking, it has been demonstrated that destabilization of the LPS aggregates is the crucial step in masking (3.2). Due to the fact that the O-antigen only marginally contributes to the ionic stabilization, the heterogeneity of the O-antigen will be neglected in the following examination. Although lipid A and core region are supposed to be the most conserved part of LPS, diverse molecular structures of LPS have been observed[11], [23], [98]. Relevant modifications are expected within the charged substitutes (e.g. phosphates, amines) as well as acylation (e.g. number, length, saturation) in the core region and lipid A of LPS.

In consequence, LPS from different bacteria may exhibit different molecular structures and therefore, equivalent prepared endotoxins (phenol-extracted) from different bacteria are expected to show different kinetics in endotoxin recovery studies. For instance, recovery of endotoxin from *E.coli* and *S.marcescens* is low (< 25 %) already after one day of incubation (Figure 21). In contrast, the endotoxin from *P.mirabilis* showed substantial detectability over time. Interestingly, the lipid A structures of *E.coli* and *S.marcescens* are assumed to be similar[99], whereas the proposed molecular structure of lipid A from *P.mirabilis* exhibits some differences. Differences are seen for example in the acylation and the substitution of the ester bound phosphate groups linked to the glucomsamine backbone[100]. Thus, modifications in the lipid A of *P.mirabilis* might be a reason for the diverging masking kinetics compared to *E.coli* and *S.marcescens*. A similar behavior was observed when crude preparations of different endotoxins were compared in recovery kinetics (Figure 19, Table 4). The results show endotoxins which are rapidly affected by LER (e.g. *E.coli* O113) and endotoxins which are less susceptible (e.g. *P.aeruginosa*). Endotoxin from *B.cepacia* was not affected by masking within the given conditions. For *B.cepacia*, it is also described that the LPS possess an unusual structure. The bacteria lower the anionic charge of the cell surface by the substitution of 4-amino-4-deoxyarabinose (Ara4N) residues bound to phosphates of the lipid A backbone[101], [102]. Interestingly, endotoxins from *P.mirabilis* and *B.cepacia* are less susceptible to endotoxin masking and both are known for their almost stoichiometric substitution of Ara4N[103]. This might explain the limited masking susceptibility.

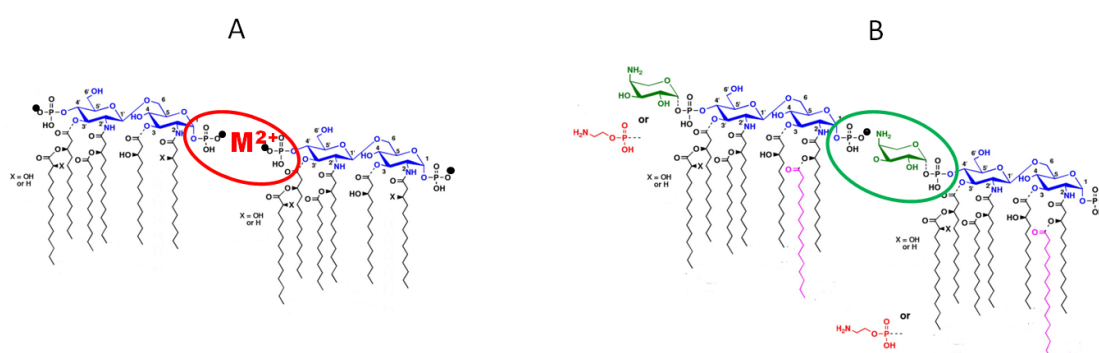


Figure 23 Structural modifications of lipopolysaccharides

Depending on the environmental conditions during bacterial growth phosphorylation or acylation can change the molecular LPS structure. Changed LPS structures can result in different interactions between LPS molecules: Ionic interactions between LPS molecules in the presence (A) and absence (B) of divalent cations. (modified figure from Yan et al.[95])

As described above, chelators destabilize ionic interactions between negative charged LPS molecules and divalent cations. Here, if LPS molecules possess additional positively charged substituents, like Ara4N, LPS molecules can form ionic interactions without divalent cations to

stabilize their supramolecular structures. Under such conditions, LPS structures are independent from divalent cations and chelation of divalent cations has no effects on stability of the supramolecular structures of LPS (Figure 23). Consequently, the intercalation of surfactants into LPS aggregates is limited and the endotoxin is less susceptible to masking under such conditions.

Moreover, different bacteria need different conditions for an optimal growth. Comparing absorbance of bacterial suspensions from different bacteria and the corresponding detectable endotoxin activities, huge differences in growth and endotoxin content are observed (Table 3). However, bacteria are able to adapt themselves to an unfavorable environment to ensure viability. It is known that bacteria are able to modify their primary LPS structure under certain growth conditions, in order to reinforce the external membrane to assure best protection against the environment[95]. Moreover, bacteria possess the ability to alter or regulate their lipid A form under specific environmental conditions[98]. For instance, after growth of bacteria under divalent cation limitation (e.g. in the presence of EDTA), their LPS exhibits raised contents of aminoethanol and Ara4N (Figure 23)[95]. Therefore, using endotoxin from bacteria grown under divalent cation limitation, displays attenuated recovery kinetics (Figure 20), supporting previous assumption. However, it has to be pointed out that not all endotoxins are similarly affected, if growth conditions are modified. Comparing the recovery kinetics of endotoxin from the same source, but grown under different conditions does not automatically result in a change of the masking susceptibility (Figure 19). Thus, due to the unknown source of a potential contamination, it is impossible to predict the species of bacteria, its modifications due to the growth conditions, its molecular structure and consequently its susceptibility to endotoxin masking.

3.3.3.2 Breakdown products of Gram-negative bacteria

In case of a bacterial endotoxin contamination event, LPS might be present in diverse assemblies. For example, if viable bacterial cells are present, LPS are embedded in the outer membrane of the cell to form its outer layer. In addition, LPS can be exposed in so called outer membrane vesicles (OMV), which are segregated by intact cells to improve their protection. Furthermore, during cell division or cell death monomers and multimers of LPS can be released from the bacterial cell. Hence, a set of LPS assemblies (Figure24) can exist in parallel. Noteworthy, the composition of such assemblies can be diverse. A mixture of LPS and accompanying molecules like lipoproteins, phospholipids are not necessarily evenly distributed. For example, OMV frequently contain a high ratio of LPS with extended O-antigens but contain less protein compared to the originated bacteria[104]. Depending on a particular contamination event, the whole set of assemblies can be present in a sample. This would be the case, if there is an acute bacterial contamination event. Otherwise, it is also possible that there are only break down products of the bacteria present in a sample. This can be the case if break down products of the bacteria are

transferred into a sample or parts of the contamination are already eliminated during handling (e.g. chromatography) of the sample.

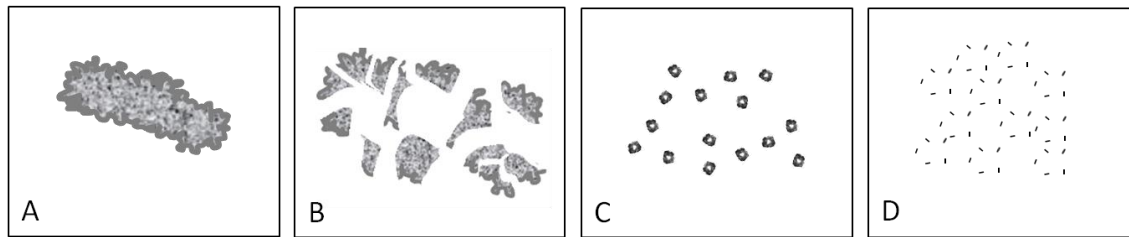


Figure 24 Origin of LPS – Bacterial cells and their breakdown products

(A) LPS are the major building block of the outer membrane of Gram-negative bacteria. Break down products of the bacteria can be (B) fragments of bacteria, (C) OMVs which are segregated by the cell and (D) monomers.

Considering not only the heterogeneity of LPS, but also accompanying bacterial components (e.g. lipids, porins or proteins), a contamination can be very diverse. Keeping this in mind, comparable experiments with regard to LER are only possible using defined endotoxins. Reference or control standard endotoxins meet such requirements, because these are highly purified suspensions. For preparation of such standards, bacterial suspensions from e.g. *E.coli* pass through a set of purification steps in order to meet the ordinary requirements for a qualified standard[4]. During such purification steps, accompanying components like lipids or lipoproteins are removed. The removed components do not directly contribute to the endotoxic potential, as the Lipid A of LPS has been identified responsible for toxicity of Gram-negative bacteria[14], [15]. Moreover, the direct comparison of crude and purified endotoxin preparations gave no indication that the purification process is responsible for the masking susceptibility of an endotoxin (Figure 22). Only at reduced incubation temperatures, which decelerate the process of masking, the crude endotoxin preparations show slower masking kinetics than the highly purified endotoxin. It can be speculated that the supramolecular structures of the less purified endotoxin are partly stabilized by its accompanying membrane molecules, but masking is not prevented. Obviously, crude endotoxin preparations showed also diverse masking susceptibilities (Table 4 and Figure 19) and the recovery of an endogenous contamination was also low over time (Table 5). This proves that endotoxin masking is not driven by a certain preparation of endotoxin. It can be rather supposed that the molecular structure of LPS determines if an endotoxin is susceptible to masking or not. Accompanying molecules other than LPS can modulate the stability against endotoxin masking, but they cannot prevent it.

3.3.3.3 BET and their standard endotoxins

The different behaviors of endotoxins from different sources raise the question, whether the established standard endotoxins from *E.coli* are still adequate in BET? This question was already

discussed in the 1970th, at the time when *E.coli* O113 was determined as source for reference standard endotoxins (RSE)[24]. Finally, endotoxins from *E.coli* were chosen, because they were very well characterized and their toxic effects were studied also in man[28]. The establishment of endotoxin standards from other species was and is possible, but a likewise deep characterization would be necessary. Purification is also necessary in order to standardize and enable comparability of the endotoxin and fulfil the general requirements of a standard. The alternative use of crude endotoxin extracts like the supernatant of a bacterial suspension might reflect in certain cases a potential contamination more realistic, but it is very difficult to standardize such preparations. However, due to the heterogeneity of endotoxins from different sources, it can be supposed that there will be no single standard available, which reflects the diverse nature of bacterial endotoxins. The origin of LPS is inevitable connected to the bacteria and in turn an intrinsic heterogeneity is included. Moreover, it is impossible to predict the source and way of a bacterial endotoxin contamination in a sample. As a consequence, the masking susceptibility of a potential contamination is unknown. In order to ensure reliable detection of endotoxin the masking capability of a sample has to be evaluated. To analyze the masking capability of a sample, endotoxin recovery studies have to be performed with endotoxin spikes, which are susceptible to masking. The results above have shown that standardized endotoxins from *E.coli* exhibit a pronounced susceptibility to endotoxin masking and represent an appropriate source for endotoxin recovery studies. However, an endotoxin spike should reflect the worst case with regard to its masking susceptibility. Although standard endotoxins are susceptible to masking in the investigated cases, it is not proven that standard endotoxins always reflect a worst case situation. With regard to the heterogeneity of endotoxins and the diversity of sample compositions a panel of different endotoxins might be the safest way to determine the masking capability of a sample and to ensure detectability of a potential contamination. Finally, if a sample is identified with the capability of endotoxin masking, a suitable detection method has to be developed in order to detect endotoxin and avoid underestimation of a potential contamination. Such developments are discussed in the following chapter (3.4).

3.4 Demasking of Endotoxin

3.4.1 Introduction

Endotoxin is well detectable in aqueous solutions. However, it becomes significantly less active (i.e. undetectable) by common detection systems, if it is masked by surfactants and chelators (3.1, 3.2 and 3.3). This may have various consequences. The endotoxin can lose its activity, meaning that potential endotoxin contaminations in a drug product are basically not harmful anymore because the endotoxin is masked and pyrogenic reactions are prohibited. For a sustainable suppression of pyrogenic reactions, masking must be irreversible. It has to be ensured that the endotoxin will not be demasked *in-vivo* and becomes pyrogenic again. Noteworthy, modified endotoxins with significantly reduced pyrogenicity are well known and used as an adjuvant to enhance efficacy of vaccination[105]. Due to this fact, it cannot be excluded that the masked endotoxin retains its stimulating effects on the innate immune system, even if the endotoxin is depyrogenated. Last but not least, the detection of endotoxin in a sample, independent of the toxicity of present endotoxins, gives an indication about the quality of the tested sample. Thus, existing test procedures have to be optimized to detect masked endotoxin. Due to the widespread use of *Limulus*-based detection methods, a sample-treatment prior to the use of such conventional test methods is desired. Importantly, successful demasking of endotoxin strongly indicates that endotoxin is not irreversibly deactivated by masking. In consequence, when demasking is possible *in-vitro*, it cannot be excluded that demasking *in-vivo* is also possible.

In order to develop a demasking approach, the nature of endotoxin and the driving forces of masking need to be understood. Due to the amphiphilic nature of LPS, it tends to aggregate in aqueous solutions. The basic cause of aggregation is to lower Gibbs free energy, which is depending on the inner energy and entropy of a system. The latter is predominant in such cases and driving hydrophobic effects.[18] Thereby, a variety of supramolecular structures can be formed, which are obviously depending on the particular conditions (e.g. molecular structure, salinity and polarity). In order to get an idea of a potential supramolecular structure of amphiphilic molecules, the concept of packing parameter is a helpful tool. Israelachvili introduced a dimensionless equation describing the packing parameter S , which in turn is depending on the molecular volume of the hydrophobic moiety, the length of the fully extended hydrophobic moiety and the cross-sectional areas of the hydrophilic and hydrophobic moiety. Depending on the value of such a calculated packing parameter, a particular supramolecular structure of the amphiphilic molecule can be deduced[106].









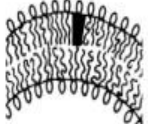
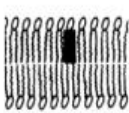


Molecular shape	Cone	Truncated cone	Truncated cone	Cylinder	Inverted truncated cone	Inverted truncated cone
						
Supramolecular structures formed	Spherical micelles	Cylindrical micelles	Flexible bilayers, vesicles	Planar bilayers	Cubic	H ₂
						

Figure 25 Molecular shape of an amphiphilic molecule determines its supramolecular structure

The molecular relationship of amphiphilic molecules is illustrated in relation to their supramolecular structure. Depending on the molecular shape of an amphiphilic molecule (e.g. cone, cylinder and inverted cone) a corresponding supramolecular structure is formed (e.g. micellar, bilayers and inverted). (Source: [18])

Figure 25 gives some examples of supramolecular structures depending on their molecular shape. For example, cone shaped amphiphilic molecules, which often contain only a single hydrophobic tail tend to form spherical micelles (e.g. polysorbates); truncated or cylinder shaped molecules (e.g. phospholipids) often contain two hydrophobic tails and form preferably bilayers; and inverted truncated molecules which contain a pronounced hydrophobic portion tend to form inverted structures (e.g. LPS).[18] This concept does not fully describe the supramolecular behavior of amphiphilic molecules, as further parameters like the fluidity of acyl chains influence likewise the aggregation state. However, the concept of packing parameter helps to understand the formation of supramolecular structures.

Several studies have been performed to explore structure-function relationships of LPS with regard to its biological activity[9], [11], [18], [23], [107]–[109]. The primary lipid A structure of LPS was identified to constitute the endotoxic activity[9]. Moreover, due to the amphiphilic nature of LPS the effects of their supramolecular structures were examined concerning the endotoxic activity[18], [66], [109]. Brandenburg and co-workers proposed to extend the term “endotoxic conformation”, which is used to describe the conformation of a single lipid A molecule required for optimal triggering of biological effects, to “endotoxic supramolecular conformation” which denotes the particular organization of lipid A aggregates in physiological fluids causing biological active LPS [110]. Obviously, there is a relationship between endotoxicity and the supramolecular structure of LPS (Figure 26). Inverted structures possess a higher degree of endotoxicity compared to lamellar structures.[18] This might be comprehensible, as the hydrophobic part of LPS tends to be more accessible in solution. In the case of lamellar structures, the hydrophobic part (lipid A) is

shielded by the sugar units (core region and O-antigen) in solution and is consequently less toxic. Taking this knowledge into consideration, demasking can be achieved by changing the sample environment affecting the aggregation state of endotoxin. Based on this hypothesis sample treatment for demasking are investigated below.

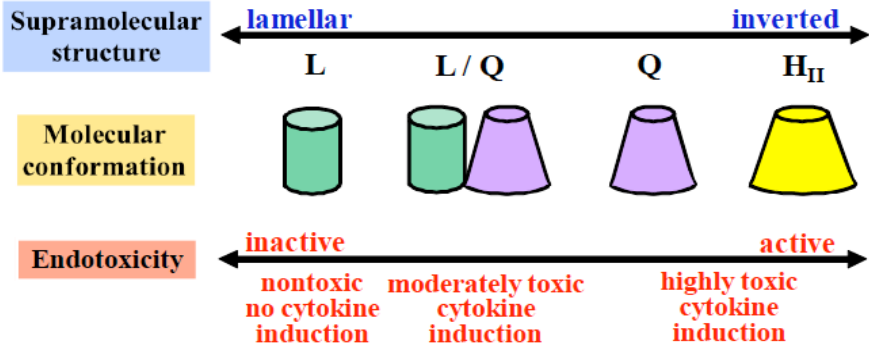


Figure 26 Relationship between supramolecular structures and endotoxicity

Correlation between supramolecular LPS structure and bioactivity is shown. Depending on the molecular structure of LPS and the environmental conditions, LPS form certain supramolecular structures. The latter in turns affects the activity of LPS, in which the lamellar structures possess less endotoxicity compared to inverted structures. (Source: [18])

3.4.2 Results

Various sample compositions are capable to mask endotoxin and render it undetectable (3.1, 3.2 and 3.2). In order to release masked endotoxin out of polysorbate 20 complexes, samples were treated with alkyl alcohols (C8 to C18) prior to endotoxin measurements (Figure 27).

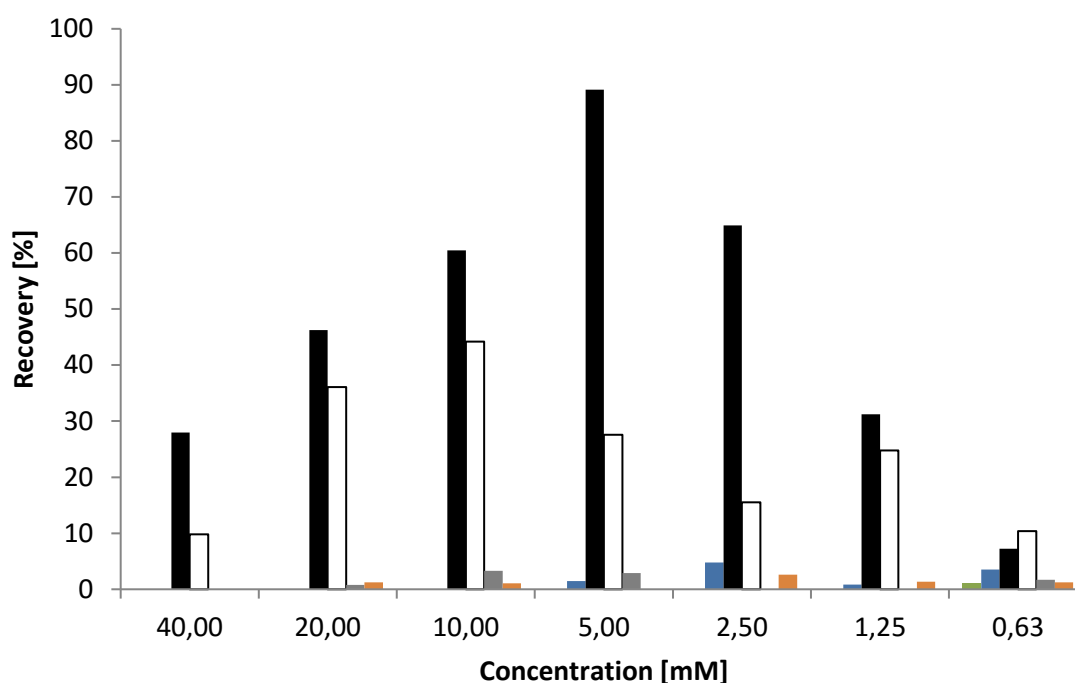


Figure 27 Demasking of endotoxin using co-surfactants

Endotoxin recovery after demasking using different concentrations of alkyl alcohols with various chain lengths is shown. 100 EU/mL of endotoxin were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20 and incubated at least for 24 hours at RT. For demasking, samples were treated using alkyl alcohols with varying chain length from C8 to C18 (1-octanol (green bars), 1-decanol (blue bars), 1-dodecanol (black bars), 1-tetradecanol (white bars), 1-hexadecanol (grey bars), 1-octadecanol (orange bars)). The concentrations of the alcohols ranged from 0.6 to 40.0 mM. For detection of endotoxin EndoLISA® was used.

This result indicates that sample treatment of masked endotoxin using 1-dodecanol and 1-tetradecanol enable substantial recovery. Highest recovery was obtained using concentrations of 5 mM 1-dodecanol and 10 mM 1-tetradecanol, respectively. Using alcohols with alkyl chains lengths below C12 or above C14 resulted in recovery below 10 %. Thus, the alkyl chain length of the alkyl alcohol is crucial for demasking, but it clearly demonstrates that masked endotoxin can be rendered detectable again. There is a small range of 1-dodecanol concentrations which enabled demasking. In order to improve demasking using 1-dodecanol, 10 mg/mL Bovine Serum Albumin (BSA) was added to the particular 1-dodecanol concentration. BSA was chosen due to its capability of binding surfactants (Figure 28). Comparing demasking results in presence and absence of BSA, a consistent increase in the recovery of endotoxin can be observed in the presence of BSA.

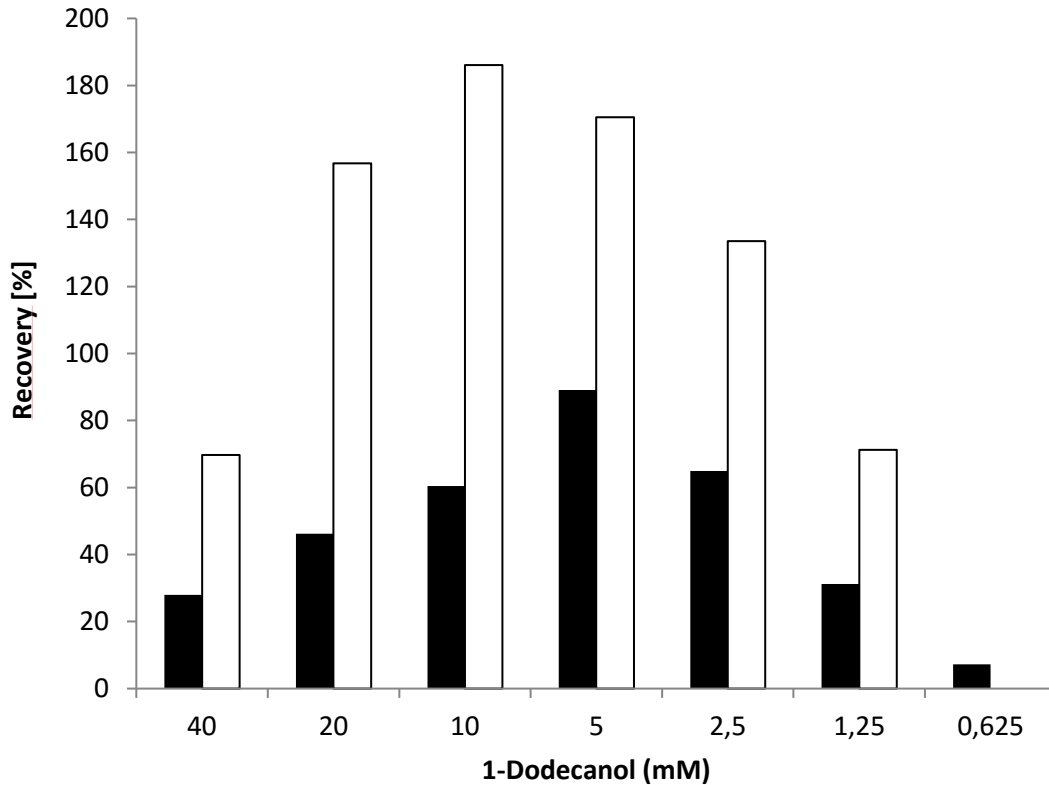


Figure 28 Demasking of endotoxin using dodecanol and BSA

Endotoxin recovery after demasking is shown as function of 1-dodecanol concentration. 100 EU/mL of endotoxin were spiked into samples containing 10 mM sodium citrate and 0.05 wt % polysorbate 20 and incubated for at least 24 hours at RT. Black bars reflect sample treatment using only various concentrations of 1-dodecanol and white bars reflect sample treatment using various concentrations of 1-dodecanol and additional 10 mg/mL BSA. For detection of endotoxin EndoLISA® was used.

To investigate whether demasking is due to similar alkyl chain lengths (C12) of polysorbate 20 and 1-dodecanol, endotoxin was also masked in the presence of polysorbate 80, which possesses C18 alkyl chain and octoxynol 9 which in turn possess a tetramethylbutyl-phenyl group. All of these surfactants induce LER in combination with sodium citrate (3.1). Sample treatment with 1-dodecanol leads to recovery of 29 % out of polysorbate 80. Out of samples containing octoxynol 9, no demasking effects were achieved. The combination of 1-dodecanol and BSA lead to a full recovery out of polysorbate 80, but only to limited recovery out of octoxynol 9 samples (Figure 29).

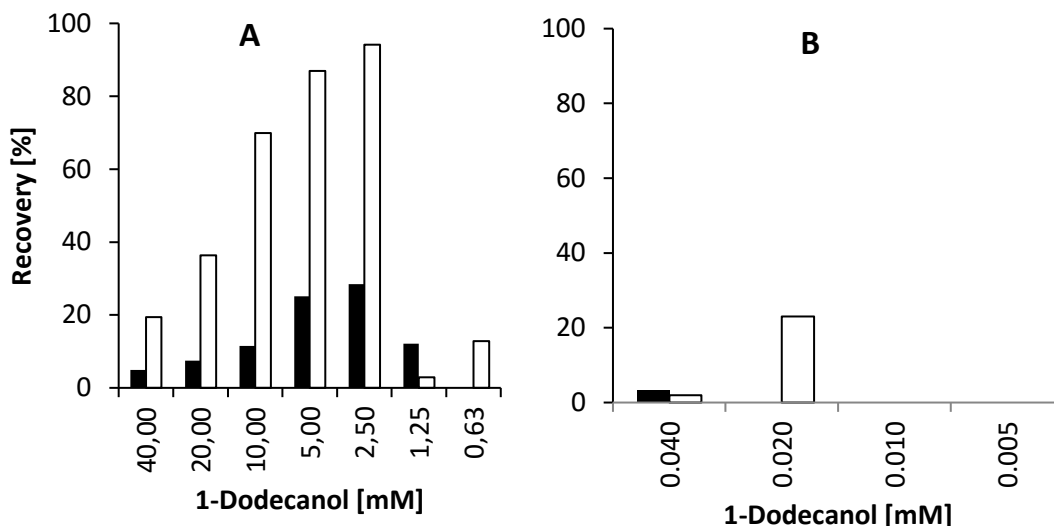


Figure 29 Demasking of endotoxin out of different sample matrices using dodecanol and BSA

Endotoxin recovery is shown after demasking as function of 1-dodecanol concentration. 100 EU/mL of endotoxin were spiked into samples containing 0.05 wt % (A) polysorbate 80 and (B) octoxynol 9. All samples were buffered using 10 mM sodium citrate. The samples were incubated for at least 24 hours at RT. For demasking, samples were treated with 1-dodecanol (black bars) as well as with 1-dodecanol and 10 mg/mL BSA (white bars). For detection of endotoxin EndoLISA® was used.

These results indicate that there is a relationship between the alkyl chains of the masking and demasking components, but the combination of 1-dodecanol and BSA enables demasking out of polysorbate 20 and 80 masking conditions. Demasking out of octoxynol 9 is less effective, indicating that the LPS-octoxynol 9 complex is stabilized under these conditions (Figure 29B). To further enhance demasking, additional agents including sodium dodecylsulfate (SDS) and calcium dichloride were added to the previously used demasking agents.

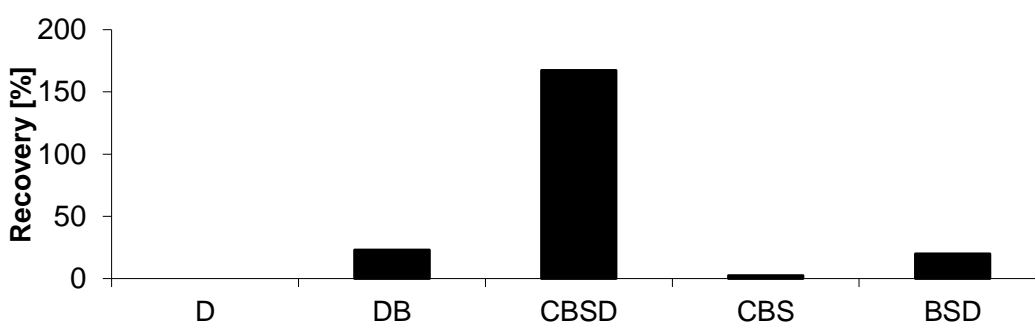


Figure 30 Demasking of endotoxin out of octoxynol 9 matrices

Endotoxin recovery is shown in dependence of various demasking compositions. 100 EU/mL of endotoxin were spiked into samples containing 10 mM sodium citrate and 0.05 wt % octoxynol 9 and incubated for at least 24 hours at RT. Demasking was performed using the following components: 5 mM 1-dodecanol (D), 10 mg/mL BSA (B), 100 mM calcium dichloride (C) and 0.1 wt % sodium dodecylsulfate (S). In different demasking approaches, combinations of the components were used (D, BD, CBSD, CBS and BSD). For detection of Endotoxin EndoLISA® was used.

The combination of calcium dichloride, BSA, SDS and 1-dodecanol resulted in a substantial recovery of endotoxin out of samples containing octoxynol 9 (Figure 30). Table 6 gives an overview of different demasking approaches out of the different surfactant masking conditions. These results indicate that the demasking approach including calcium dichloride, BSA, SDS and 1-dodecanol is suitable for demasking of all examined masking conditions.

Table 6 Comparison of different demasking approaches

Endotoxin recovery after demasking out of different masking surfactants using different approaches is shown. In each case 100 EU/mL of endotoxin were spiked in samples containing 10 mM sodium citrate and 0.05 wt % of the corresponding surfactant (polysorbate 20, polysorbate 80 and octoxynol 9). After incubation of at least 24 hours at RT the samples were treated using 5 mM 1-dodecanol (D), 10 mg/mL BSA (B), 100 mM calcium dichloride (C) and 0.1 wt % sodium dodecylsulfate (S). In different demasking approaches, combinations of the components were used (D, BD and CBSD). For detection of Endotoxin EndoLISA® was used.

	D	BD	CBSD
Masking – Surfactant:	Recovery [%]		
Polysorbate 20	78	170	141
Polysorbate 80	28	94	161
Octoxynol 9	0	23	168

In the experiments shown so far, endotoxin demasking was performed with a commercially available, highly purified endotoxin preparation from *E. coli* 055:B5. According to previous studies (3.3), endotoxin from different sources may have different masking susceptibilities, due to variances in acyl chain length of the lipid A part of LPS, as well as modifications of side chains[3]. Even more, the length of the O-sugar side chains of LPS potentially impacts the demasking approach. It cannot be excluded that highly purified endotoxin and crude endotoxin extracts (often called “NOE”) behave different in demasking mechanism. To address this issue and to exclude the possibility that the demasking approach is specific for the above used LPS from *E. coli* 055:B5, endotoxins from different bacteria with different structures and purities were masked in sample matrixes containing polysorbate 20/citrate (Table 7a), polysorbate 80/citrate (Table 7b) and octoxynol 9/citrate (Table 7c). For subsequent demasking the various approaches using either 1-dodecanol alone, BSA/1-dodecanol or calcium dichloride/BSA/SDS/1-dodecanol were applied. The results clearly show that the ability to successfully demask endotoxin from various masking systems is independent of the source and type of endotoxin used. It shows that demasking is a general technique applicable to various types of endotoxin from various sources, under a variety of masking conditions.

Table 7 Demasking of different endotoxins

Endotoxin recovery before (masking control) and after demasking of endotoxin from different sources and types out of 10 mM sodium citrate and 0.05 wt % (a) polysorbate 20, (b) polysorbate 80 and (c) octoxynol 9 are shown. Approximately 50 EU/mL of the particular endotoxin were spiked into the corresponding sample matrix. The endotoxins were incubated for seven days at RT in the sample matrix. For demasking, the samples were treated using 5 mM 1-dodecanol (D), 10 mg/mL BSA (B), 100 mM calcium dichloride (C) and 0.1 wt % sodium dodecylsulfate (S). In the particular demasking approaches, combinations of the components were used (D, BD and CBSD). For detection of endotoxin EndoLISA® was used.

a) Polysorbate 20 / sodium citrate					
Endotoxin:	Source:	Masking control	Demasking		
			D	BD	CBSD
Recovery [%]					
<i>K.pneumonia</i>	LMU	0	66	128	212
<i>M.morganii</i>	LMU	0	81	110	120
<i>Y.enterocolitica</i>	LMU	0	63	174	243
<i>S.marcescens</i>	LMU	0	128	168	182
<i>N.meningitis</i>	LMU	0	9	23	38
<i>A.baumannii</i>	LMU	0	0	124	655
<i>E.cloacae</i>	Hyglos	0	55	156	187
<i>S.enterica</i>	Sigma	0	42	63	76
<i>E.coli K 12</i>	Invivogen	3	78	80	137
<i>P.aeruginosa</i>	Sigma	0	14	5	179

b) Polysorbate 80 / sodium citrate					
Endotoxin:	Source:	Masking control	Demasking		
			D	BD	CBSD
Recovery [%]					
<i>K.pneumonia</i>	LMU	10	22	12	162
<i>M.morganii</i>	LMU	6	35	23	48
<i>Y.enterocolitica</i>	LMU	0	13	19	236
<i>S.marcescens</i>	LMU	4	28	20	80
<i>N.meningitis</i>	LMU	0	55	14	161
<i>A.baumannii</i>	LMU	8	0	57	918
<i>E.cloacae</i>	Hyglos	0	2	26	85
<i>S.enterica</i>	Sigma	0	1	11	25
<i>E.coli K 12</i>	Invivogen	0	21	12	234
<i>P.aeruginosa</i>	Sigma	0	54	17	78

c) Octoxynol 9 / sodium citrate					
Endotoxin:	Source:	Masking control	Demasking		
			D	BD	CBSD
Recovery [%]					
<i>K.pneumonia</i>	LMU	0	12	173	353
<i>M.morganii</i>	LMU	15	15	39	99
<i>Y.enterocolitica</i>	LMU	7	22	168	309
<i>S.marcescens</i>	LMU	0	105	199	326
<i>N.meningitis</i>	LMU	0	0	11	42
<i>A.baumannii</i>	LMU	0	7	337	511

<i>E.cloacae</i>	Hyglos	24	27	74	183
<i>S.enterica</i>	Sigma	1	1	1	90
<i>E.coli K 12</i>	Invivogen	0	18	10	69
<i>P.aeruginosa</i>	Sigma	2	85	106	176

To this end, demasking has been demonstrated in diverse surfactant/buffer matrices. These matrices were chosen, because pharmaceutical industries often have been using such components for formulation of APIs like proteins (table 1). Further, antibodies constitute frequently formulated pharmaceutical protein products. Hence, the established demasking approaches are applied to systems containing surfactant and an antibody buffered in phosphate and saline. Polysorbate 20 and 80 were chosen as surfactants (Table 8).

Table 8 Demasking of endotoxin out of formulated antibody samples

Endotoxin recovery before and after demasking out of formulated antibody (PAK) samples is shown. 50 EU/ml of endotoxin were spiked into samples containing water, buffer (10 mM sodium phosphate and 50 mM sodium chloride), antibody (10 mg/mL polyclonal antibody) and surfactant (0.05 wt % polysorbate 20 and polysorbate 80). Samples were incubated for three days at RT. For demasking, the sample containing buffer, surfactant and antibody was treated by using calcium dichloride (C), BSA (B), sodium dodecylsulfate (S) and dodecanol (D). For detection of endotoxin EndoLISA® was used.

Masking surfactant:					polysorbate 20	polysorbate 80
Sample:	C [mM]	B [mg/ml]	S [%]	D [mM]	Recovery [%]	
water	-	-	-	-	100	100
buffer	-	-	-	-	102	99
buffer + antibody	-	-	-	-	31	44
buffer + surfactant	-	-	-	-	0	2
buffer + surfactant + antibody	-	-	-	-	0	9
buffer + surfactant + antibody	-	-	-	10.0	17	9
buffer + surfactant + antibody	-	-	-	1.0	20	7
buffer + surfactant + antibody	-	-	-	0.1	0	5
buffer + surfactant + antibody	-	10	-	10.0	41	11
buffer + surfactant + antibody	-	10	-	1.0	3	6
buffer + surfactant + antibody	-	10	-	0.1	2	12
buffer + surfactant + antibody	100	10	0.1	10.0	5	3
buffer + surfactant + antibody	100	10	0.1	1.0	16	23
buffer + surfactant + antibody	100	10	0.1	0.1	67	91

The results show that the buffer solution without polysorbate does not mask the endotoxin. Buffer solutions containing antibody, but no surfactant, resulted in reduced endotoxin recovery suggesting that already the antibody contributes a masking effect. The endotoxin recovery from buffer solutions containing polysorbate and antibody are below 10 % when no demasking

treatments were performed. Thus, not only the surfactants but also the antibody is capable of endotoxin masking. Endotoxin recovery after demasking of such samples, simulating a drug product containing endotoxin, surfactant, buffer and antibody are low using 1-dodecanol alone (< 10%). Using a combination of BSA and 1-dodecanol allows moderate endotoxin recovery (10 to 40 %), but a combination of calcium dichloride, BSA, SDS and 1-dodecanol shows a substantial endotoxin recovery in the presence of Polysorbate 20 and 80 (> 60 %).

To show that demasking is not only possible from solutions containing LPS of a known source, a commercially available mouse monoclonal antibody for diagnostic purpose was used, which contained an “endogenous” LPS contamination from an unknown source (Table 9).

Table 9 Demasking of unknown endotoxin

Endotoxin recovery before and after demasking of endotoxin from an unknown source is shown. A contaminated monoclonal antibody (MAK 33) was dissolved in a buffer containing 25 mM sodium citrate (pH 6.5) and 150 mM sodium chloride. Directly after solubilization of the antibody, an endotoxin content of 11 EU/mg was determined. Endotoxin masking was initiated by addition of 0.07 wt % of polysorbate 80 and incubated for three days at RT. For demasking, the sample containing buffer, polysorbate and antibody was treated by using the indicated concentrations of calcium dichloride (C), BSA (B), sodium dodecylsulfate (S) and dodecanol (D). For detection of endotoxin EndoLISA® was used.

Sample:	C [mM]	B [mg/ml]	S [%]	D [mM]	Recovery [%]
buffer + antibody (0 days)	-	-	-	-	100
buffer + antibody (3 days)	-	-	-	-	57
buffer + polysorbate 80	-	-	-	-	0
buffer + polysorbate 80 + antibody	-	-	-	-	3
buffer + polysorbate 80 + antibody	-	-	-	10	45
buffer + polysorbate 80 + antibody	-	10	-	10	68
buffer + polysorbate 80 + antibody	100	10	0.1	0.1	178

This antibody was dissolved in a buffer composition corresponding to the formulation of the known antibody drug product Rituximab containing sodium citrate, sodium chloride and polysorbate 80 (MabThera®, Rituxan®). The buffer solution containing antibody without polysorbate masks approximately 40 % of the endotoxin contamination within 3 days of incubation at room temperature. Incubation in buffer containing either polysorbate 80 or antibody and polysorbate 80, results in endotoxin recovery below 4%. This shows that an endogenous endotoxin contamination can be masked and that the risk of masking applies not only for purified or crude endotoxin extracts, but also for endogenous endotoxin. Demasking of this endotoxin contamination out of the antibody/surfactant sample resulted in an endotoxin recovery of 45 % using 1-dodecanol, 68 % using a combination of BSA and 1-dodecanol and 179 % using a combination of calcium dichloride, BSA, SDS and 1-dodecanol. This demonstrates that the developed approaches are able to demask endotoxin under conditions of relevance for the

pharmaceutical industry. For detection of demasked endotoxin, the EndoLISA assay was used in all experiments shown above. EndoLISA is the method of choice due to its heterogeneous test format, which reduces test interferences substantially[42]. However, in order to investigate if other test formats and methods are also applicable after demasking, a recombinant Factor C test (homogeneous format) as well as a kinetic chromogenic LAL assay was used for detection of demasked endotoxin. Endotoxin recovery before and after demasking was analyzed out of polysorbate 20 and 80 in phosphate-buffered saline (PBS) (Table 10). The masking controls showed no endotoxin recovery in either sample. However, after demasking substantial contents of endotoxin were recovered in all samples using LAL as well as rFC test methods. This experiment proves that the detection of demasked endotoxin is independent from the detection system used. LAL and rFC are suitable test methods after demasking.

Table 10 Comparison of different detection methods after demasking of endotoxin

Endotoxin recovery out of PBS containing 0.05 wt % polysorbate 20 (P20) and polysorbate 80 (P80) is shown, respectively. For masking, approximately 10 EU/mL endotoxin were spiked into the samples and incubated for three days at room temperature. Afterwards, the samples were treated using 200 mM sodium citrate, 100 mM calcium dichloride, 1 mg/mL BSA, 0.1 wt % sodium dodecylsulfate and 0.1 mM 1-dodecanol. For detection of endotoxin rFC (EndoZyme®) and LAL (Kinetic-QCL™) assays were used.

	Recombinant Factor C		Limulus Amebocyte Lystate	
Sample:	PBS + P80	PBS + P20	PBS + P80	PBS + P20
	[EU/mL]			
Positive control	9	7	12	7
	Recovery [%]			
Before demasking	0	0	3	0
After demasking	65	66	96	47

3.4.3 Discussion

3.4.3.1 Concept of Demasking

Endotoxin masking by surfactants is currently the most prominent masking cause in quality control of biopharmaceutical drug product manufacturing. During masking, the aggregation state of LPS is changed, leading to LPS disaggregation and embedment of LPS in surfactant micelles and in turn the endotoxin becomes undetectable. Considering the molecular shapes of surfactants like polysorbates (cone shape) and LPS (cylindrical / inverted truncated cone shape), mixed aggregates result most likely in micellar structures (Figure 31), given that surfactants are in molar excess. In the case of a potential contamination event, the commonly used surfactants in drug products are in a great molar excess compared to expected LPS concentrations (3.2).

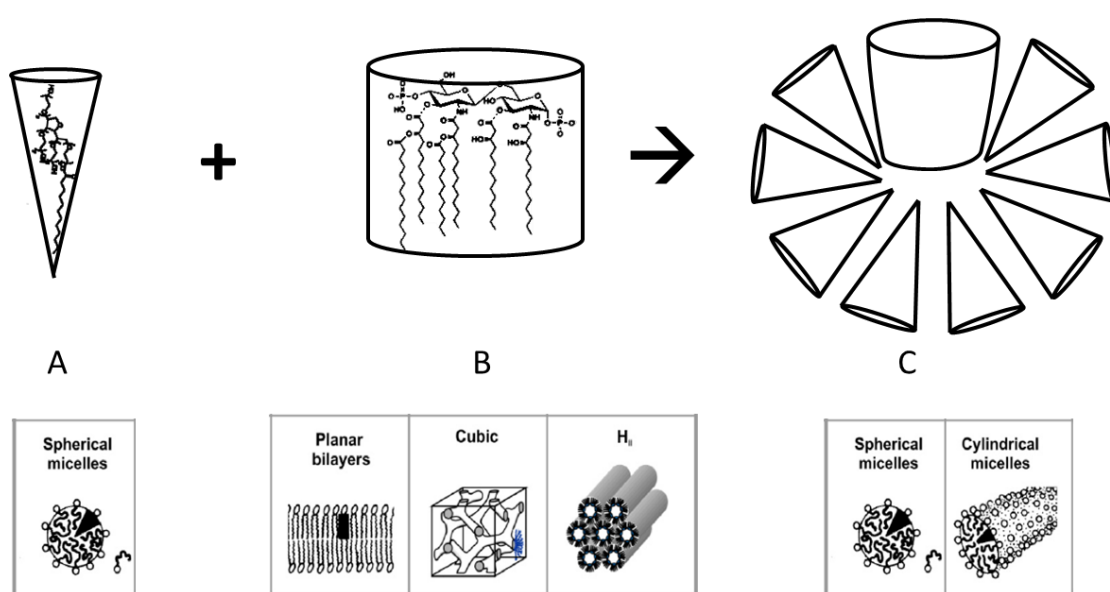


Figure 31 Potential effects on supramolecular structures: Mixing polysorbate and LPS

Formation of mixed polysorbate-LPS aggregates is shown. (A) Polysorbates possess cone shaped structures and form spherical supramolecular structures. (B) LPS possess cylindrical as well inverted truncated cone shaped structures and form bilayers and inverted supramolecular structures. (C) Mixing polysorbates and LPS, the formation of mixed micelles is predicted, under condition that polysorbates are in molar excess.

To detect masked LPS, LPS have to be liberated from their masking complex. Therefore, it was searched for molecules, which are capable to destabilize the LPS-surfactant complex and in turn enable a reassembly of LPS. It is hypothesized that a reorganization of LPS is possible, when surfactants and LPS do not favor the spherical micellar aggregation state. Concurrently, the surfactant LPS complex is unfavored and LPS are released and reassembled. Noteworthy, the pure sample dilution below the critical micelle concentration (CMC) of surfactants was not sufficient (3.1), assuming that mixtures of LPS and surfactants form stable aggregates. Therefore, the application of co-surfactants was considered, because the co-surfactants are capable in affecting the supramolecular arrangement of surfactants (Figure 32).

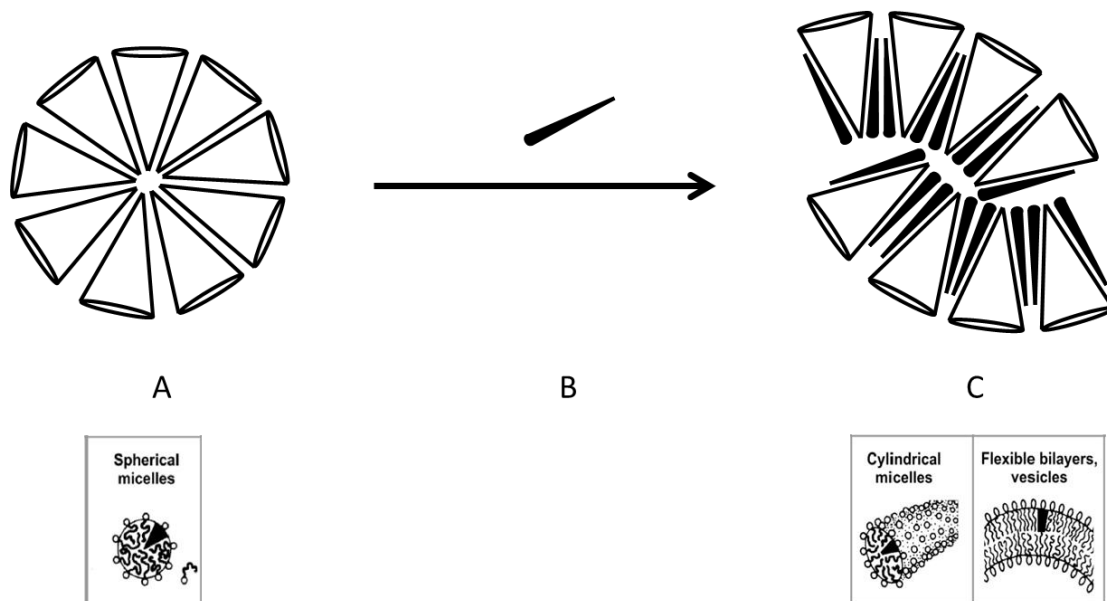


Figure 32 Mixing surfactants and co-surfactants

The change of supramolecular structures is shown when surfactants and co-surfactants are mixed. **(A)** Cone shaped surfactants (e.g. polysorbate) form spherical micelles above critical micelle concentrations (CMC). **(B)** Addition of co-surfactant (e.g. dodecanol) to surfactants. **(C)** Mixing of surfactants and co-surfactants results in alteration of supramolecular structures. The overall hydrophobic portion increases, whereby the hydrophilic portion remains constant. As a result, potentially cylindrical micelles or even bilayers are formed.

Typically, co-surfactants are not able to form micelles because their solubility in water is lower than their critical micelle concentration[111]. But co-surfactants can intercalate into surfactant micelles and swell them[112]. Moreover, co-surfactants like long chained alkyl alcohols can change the overall packing of aggregates and lead to altered aggregation states[113]. In the event of demasking it is expected that co-surfactants intercalate into both surfactant micelles and mixed LPS-surfactant micelles. This disturbs the aggregation states of pure and mixed micelles and new aggregation states are established. It is probable that the surfactants in presence of co-surfactants no longer prefer the formation of spherical micelles, but rather prefer the formation of cylindrical and bilayered aggregates. This reorganization may in turn enable the release of LPS out of masking complex (mixed LPS-surfactant micelles). Moreover, co-surfactants are also capable to interact with the fatty acids of LPS, which can affect the aggregation state of LPS. It is supposed that co-surfactants support the reaggregation of LPS and “catalyze” the formation of inverted truncated cone shaped LPS, which in turn favors the assembly of inverted cubic or hexagonal supramolecular LPS structures (Figure 33). This is the working hypothesis of demasking and the application of this concept will be discussed below (3.4.3.2).

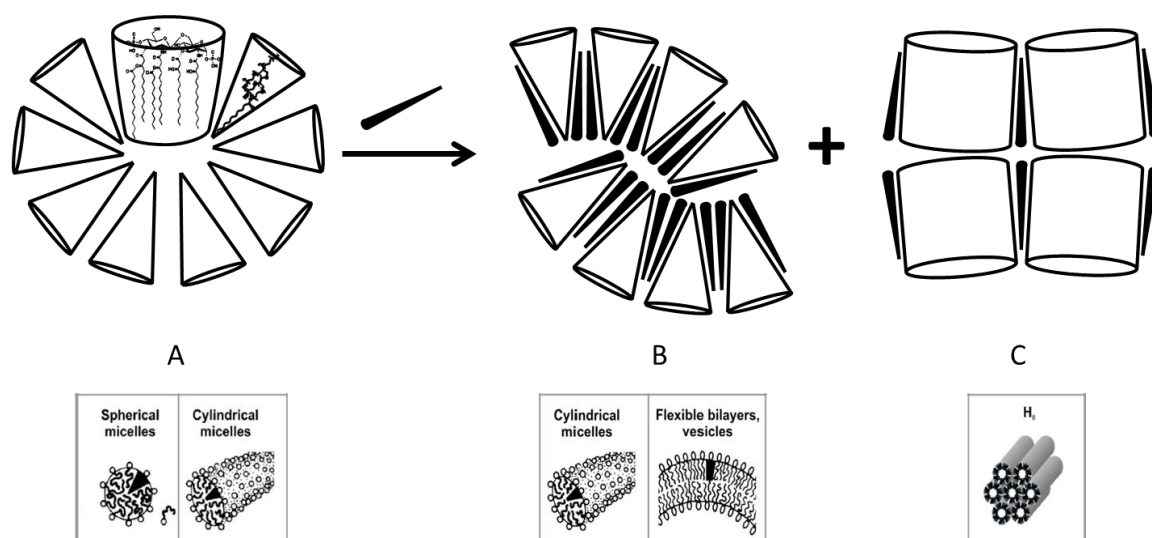


Figure 33 Potential effects on supramolecular structures: Reassembly of LPS

Potential reassembly of LPS is shown. (A) Mixed LPS-polysorbate aggregates form spherical or cylindrical micelles. The addition of a demasking agent (e.g. co-surfactant) changes the overall packing of aggregates and leads to segregation of surfactants and LPS. (B) Surfactants form cylindrical micelles or bilayers, which no longer stabilize monomeric LPS embedded in micelles. (C) LPS forms preferably hexagonal inverted structures which are well detectable.

3.4.3.2 Realization of demasking concept

According to the above described concept (3.4.3.1), co-surfactants were studied in order to demask endotoxin. To this end, long-chained alkyl alcohols were used for endotoxin demasking out of polysorbate 20/citrate samples (Figure 27)[114]. Using alcohols with chain length of C12 resulted in full recovery of endotoxin. The use of C14 alcohols also resulted in a substantial endotoxin recovery, whereas the use of alcohols with longer or shorter alkyl chains was not suitable. The beneficial effects of C12 alkyl alcohol might be explained by its chain length, which fits well to the alkyl chain length of polysorbate 20. Using the C12 alkyl alcohol for demasking out of a matrix, containing polysorbate 80 (Figure 29Aa), the demasking efficiency is lower. In this case, the C12 alkyl chain of the alcohol can interact with the C18 of polysorbate as well, but it possesses a relatively shorter hydrophobic proportion. However, the principle of swelling micelles is still given. The use of alcohols with longer alkyl chains than C12 or C14 would have been beneficial for demasking out of polysorbate 80, but was difficult in handling due to their limited solubility in water. The working solutions of the alkyl alcohols were already solubilized in ethanol to enhance solubility in aqueous solutions. Nevertheless, by adding the working solutions (alkyl chain length > C14) to the particular aqueous samples, the ethanol content is likewise diluted and phase separation occurs. Under such conditions the endotoxin measurement is not necessarily reliable. It is also not possible to increase the ethanol content in the sample to be tested to provide better solubility of the long chained alcohols, because of subsequent interference of ethanol with the enzymatic reaction of the endotoxin assay.

Due to the limited applicability of co-surfactants with alkyl chain lengths above C14, the demasking efficiency out of polysorbate 80 was increased by addition of an ancillary component capable of binding surfactants. It is supposed that the demasking efficiency of 1-dodecanol is extended, by limiting the concentration of “free” surfactants in solution. To this end, BSA was chosen, because it is widely available, well characterized and known to adsorb polysorbates[115], [116]. The use of 1-dodecanol in combination with BSA resulted in significant enhancement of endotoxin demasking out of samples in which polysorbate caused endotoxin masking (Figures 28 and 29A). However, in the case of octoxynol 9 masking, using BSA and 1-dodecanol for demasking was not sufficient. This observation suggests that the masking LPS-octoxynol 9 complex is more stable than the LPS-polysorbate complex and 1-dodecanol is less effective in this case. Comparing polysorbates and octoxynol 9, dissimilarities in their nature are given. Polysorbates comprise of sorbitan substituted with approximately 20 repeat units of polyethylene glycol and an unbranched alkyl chain. Octoxynol 9 comprises of approximately 10 repeat units of polyethylene glycol and phenyl with a branched alkyl chain (Figure 34).

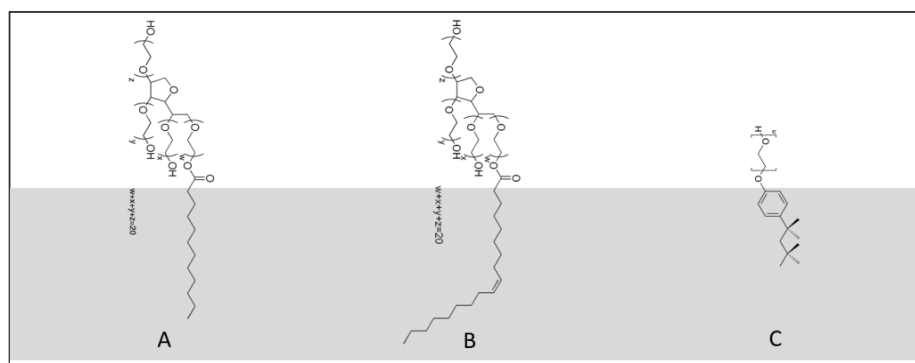


Figure 34 Molecular structures of surfactants

Chemical structures of (A) polysorbate 20, (B) polysorbate 80 and (C) octoxynol 9 are displayed. Polysorbates contain approximately 20 repeat units of ethylenglycol ($=w+x+y+z$), which are distributed across four chains. Polysorbate 20 possess a saturated alkyl chain of 12 C-atoms and polysorbate 80 possess an unsaturated alkyl chain of 18 C-atoms. Octoxynol contains 9-10 repeat units of ethylenglycol ($=n$), which are connected to a tetramethylbutylphenyl group. (source: [117]–[119])

Comparing hydrophilic hydrophobic balances (HLB) of these surfactants, octoxynol 9 (HLB 13.5) is more hydrophobic than polysorbate 80 (HLB 15.0) and polysorbate 20 (HLB 16.7)[120]. Furthermore, octoxynol 9 micelles are described to be more asymmetric than polysorbate micelles and bind less water than those composed of polysorbate[121]. Hence, it is conceivable that octoxynol 9 forms more stable aggregates with LPS compared to polysorbates due to its more pronounced hydrophobic nature. In order to destabilize the LPS-octoxynol 9 complex, it was searched for a well characterized charged surfactant, because these are harsher than non-ionic surfactants[122]. To this end, SDS was chosen, because of its anionic nature, which does not favor ionic interactions with LPS and has the capability to mix very well with octoxynol 9[123].

Moreover, SDS has a high affinity to bind to proteins[124]. This effect may also be beneficial for displacing LPS adsorbed to BSA and further proteins like antibodies. Taking these considerations into account, a combination of SDS, BSA and 1-dodecanol was used for demasking. However, the full content of endotoxin could not be retrieved, so that further optimization was needed. It was supposed that the masking complex is still too rigid under given conditions. It has been described that an increase of ionic strength in octoxynol 9/SDS mixtures can significantly change the aggregation state[125]. Especially in the presence of calcium dichloride, SDS aggregates are swollen and progress from prolate ellipsoids to extended cylinders or rods[126]. Furthermore, divalent cations stabilize LPS aggregates, which may also support the reassembly of LPS and neutralize chelators. Using the combination of calcium dichloride, BSA, SDS and 1-dodecanol (CBSD) for demasking, endotoxin was successfully detected, when it was masked in the presence of octoxynol 9 (Figure 30). These results show that depending on the masking condition different approaches are necessary to demask endotoxin. Interestingly, the most complex approach (CBSD) was also suitable for demasking out of polysorbates and octoxynol.

3.4.3.3 Demasking of endotoxins from different sources

To challenge the described demasking approach, endotoxins from diverse sources were masked using different surfactants and treated with different approaches. Summarizing these results, sample treatment using CBSD enabled demasking of all endotoxins out of all masking conditions. The approach using only 1-dodecanol was expected to be sufficient for demasking out of polysorbate 20 (Table 7a). In fact, most of the endotoxins could be adequately recovered, but a few endotoxins were limited in recovery. This might be explained by the fact that LPS from different bacteria as well as originated from various conditions may possess different molecular structures. There are differences in the length and number as well as modifications in linearity and saturation of the acyl chains in the lipid A part of LPS. Moreover, variations in the composition, decoration and length of the sugar residues in the core region and O-antigen are given[3], [98]. These molecular modifications can have effects on the nature and assembly of LPS. As assumed before, endotoxin masking is driven by hydrophobic effects, and formation of mixed aggregates with additional amphiphilic molecules. Thus, the stability of a LPS masking complex is not necessarily only depending on the masking components, but also on the molecular structure of LPS. Consequently, to demask some of the endotoxins (e.g. *Acinetobacter baumannii*), the more complex and harsh approach (CBSD instead of D) was necessary for full recovery of endotoxin. Moreover, it can be noticed that the detected activity is sometimes partly enhanced compared to the detected activity in pure water of the particular endotoxin (Table 7). Basically, the molecular structure of endotoxin defines the potential activity of endotoxin[11], but the formation of a certain supramolecular structure modulates the detectable activity[18], which is

also depending on the environmental conditions[127]. Thus, inverted structures are more active than lamellar structures[16], [18]. This is comprehensible, because the activity depends on the interaction of a receptor (e.g. Factor C) and lipid A of LPS, which in turn is better accessible by inverted than by regular supramolecular structures. For this reason, it is also possible that LPS in water possess not exactly the same supramolecular structure compared to LPS after demasking, resulting in a diverging detectable activity. However, the overall results in recovery of different endotoxins after demasking are in a passable range, considering variabilities in *Limulus*-based detection methods and the heterogeneity of LPS.

3.4.3.4 Influence of proteins on demasking

In order to simulate the impact of a protein-based API on demasking, samples containing formulated polyclonal and monoclonal antibodies were studied. Also in these cases, the full contents of endotoxin could be recovered after demasking. The applied approaches were similarly effective as in the absence of a protein during masking. Nevertheless, it is observed that a significantly reduced concentration of 1-dodecanol is sufficient for successful demasking in the presence of an antibody (table 8). Further demasking studies have also shown that the required concentrations of demasking components can vary, depending on the concentration and composition of the analyzed sample (data not shown). In consequence, using the discovered components for demasking, the required concentrations of demasking components have to be adjusted individually. Although there is a good perception of the masking and demasking principles, it is difficult to predict the interplay and aggregation state of all sample components while endotoxin masking and demasking. Hence, the described molecules used for sample preparation represent a toolbox of demasking agents. To develop a dedicated sample preparation protocol for demasking endotoxin, broad approaches with various combinations and concentrations of all described demasking components are recommended.

In the case of biopharmaceutical drug products, the API (mainly proteins) is the major component, which predetermines the overall sample conditions. Surfactants are added to such protein based products to saturate hydrophobic interfaces in order to prevent adsorption and aggregation of the API[56]. The results above have been shown that that the phenomenon of LER can be driven by the formulation components lacking the API (3.1 and 3.2), but the API can also contribute to masking of endotoxin and initiate LER (Table 8 and 9). In contrast, BSA has been shown being beneficial during demasking. To this end, depending on the composition and aggregation state of sample compositions, more or less surfactants can be adsorbed by a protein[116] and noteworthy, proteins are also capable in adsorbing LPS[47]. Thus, the presence of a protein can enhance or reduce the endotoxin masking capability of a sample. It is assumed that the “free”

concentration and aggregation state of surfactants in a sample can be affected by the API, which in turn can influence masking and demasking of endotoxins.

3.4.3.5 Endotoxin demasking – rearrangement of endotoxin aggregates

In summary, depending on the particular sample conditions a combination of 1-dodecanol, SDS, BSA and calcium dichloride can render the masked endotoxin detectable again. Noteworthy, 1-dodecanol represents the essential reagent and was present in all demasking approaches. It is supposed that 1-dodecanol provides the major driving force in disturbing endotoxin masking complexes and supporting the rearrangement of LPS. Figure 35 schematically illustrates hypothetical rearrangements of lipid A and polysorbate 20 in the presence of a long-chained alkyl alcohol. The illustration emphasizes the pass through several transition states, in which co-surfactants force swelling of the endotoxin masking complex, followed by forming lamellar structures, which in turn enable a reassembly of detectable LPS.

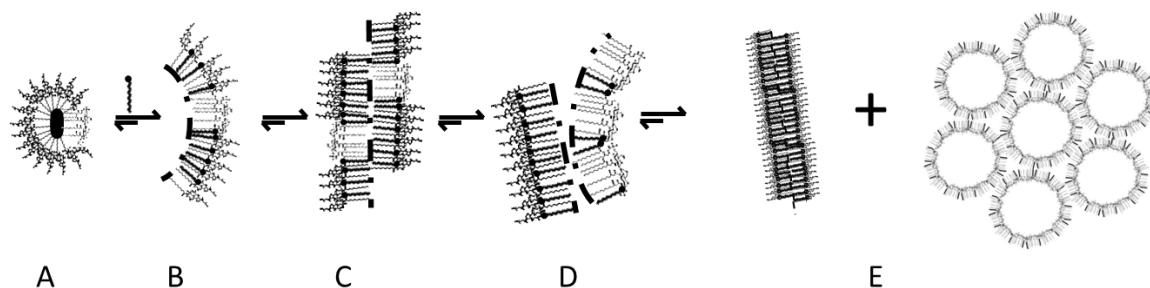


Figure 35 Potential effects on supramolecular structures: Re-arrangements during demasking

Hypothetical rearrangements of endotoxin during demasking are shown. **(A)** The lipid A part of LPS is embedded in the hydrophobic core of a small sized surfactant micelle. **(B)** Co-surfactants intercalate into the mixed surfactant-LPS micelle and swell it. **(C)** Intercalation of co-surfactants rearranges the micellar structures into lamellar and channel structures. **(D)** LPS molecules are free to diffuse. **(E)** Surfactants form bilayers and LPS reassembles into detectable structures (e.g. hexagonal inverted).

In conclusion, the data demonstrate that the detection of endotoxin depends on the particular sample conditions. LPS are reversibly deactivated during masking, because LPS can be detected again after sample treatment. Concomitantly, it cannot be excluded that demasking also takes place *in-vivo*. Thus, masking of endotoxin is most likely driven by alterations in the supramolecular structures of endotoxin, which is controlled by environmental conditions.

4 Conclusions

4.1 Endotoxin demasking – a technical solution

LER has been observed during quality control of biopharmaceutical drug products using *Limulus*-based detection systems[44], [54]. As consequence, the detection of bacterial endotoxins can lead to wrong-negative test results. In this work, the detectability of endotoxins in typical formulation matrices of biopharmaceutical drug products was analyzed in order to understand and overcome LER. The results demonstrate that LER is caused by simultaneous presence of surfactants and complex forming buffer agents. The appearance of LER is time and temperature dependent and complex forming agents were identified in limiting the reaction kinetics. Variation in surfactant and endotoxin concentrations showed no substantial effects on the reaction kinetics, but endotoxins from different sources showed effects on the kinetics. Taken together, the results above lead to the assumption that LER is caused by alteration of the endotoxin aggregation state. Moreover, a two-step masking mechanism is proposed, in which salt bridges between LPS molecules are destabilized and subsequently mixed micelles are formed masking the endotoxin. In order to render endotoxin detectable again, a sample treatment procedure was developed. Thereby, dodecanol was identified very efficient in demasking the endotoxin. The presented results clearly demonstrate that demasking is possible out of various formulation matrices and independent of the endotoxin source. However, the experiments were based on model systems and the conditions of drug products were simulated. The ultimate proof of concept is the application in a real drug product which is affected by LER. The cooperation with a world's leading pharmaceutical company enabled the analysis of endotoxin masking in one of their biopharmaceutical drug products, which is intended for commercial use. The studied product was unequivocally affected by LER (Figure 36A).

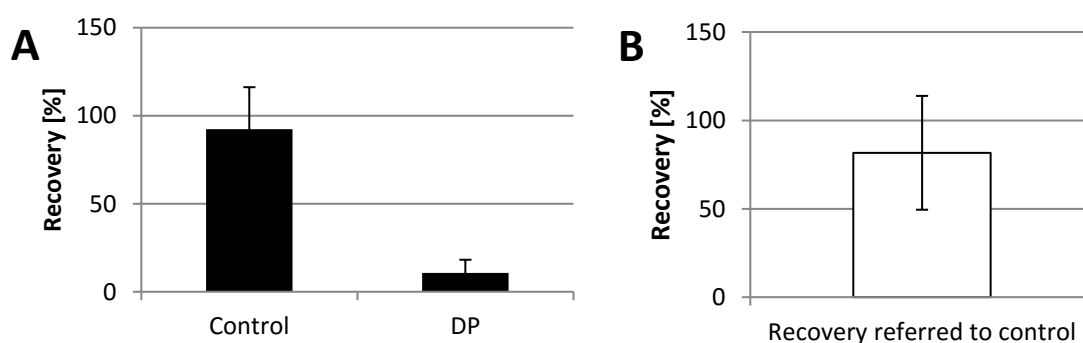


Figure 36 Recovery endotoxin before and after demasking out of a drug product

Endotoxin recovery before and after demasking out of a real life sample is shown. **A)** Recovery of 10 EU/mL endotoxin after incubation for 7 days at 4 °C in depyrogenated water (control) and finished drug product (DP). **B)** Recovery of endotoxin out of the drug product after sample treatment. For detection a LAL assay was used. The error bars reflect the standard deviation of four individual sample preparations (n=4).

For demasking the developed toolbox including ionic and amphiphilic demasking agents was used and a dedicated sample treatment protocol was established to overcome the LER effect in the drug product. Application of the protocol restored the detectability of endotoxin out of the affected drug product (Figure 36B). As expected, not all endotoxins show equivalent masking susceptibilities. For example, endotoxins from *E.coli* O55:B5 are less affected than *E.coli* O113:H21 (RSE) and *E.cloacae* (NOE). However, after sample treatment, all endotoxins were detectable in a range between 50 and 200% recovery. Interestingly, the degree of masking had no impact on demasking efficiency (Figure 37). This approach enabled for the first time an adequate detection of endotoxin over time in this drug product. Consequently, to ensure the detection of potential endotoxin contaminations and to reduce the risk of underestimation of an endotoxin contamination, the sponsor will use this approach in quality control departments in the future to improve patient safety.

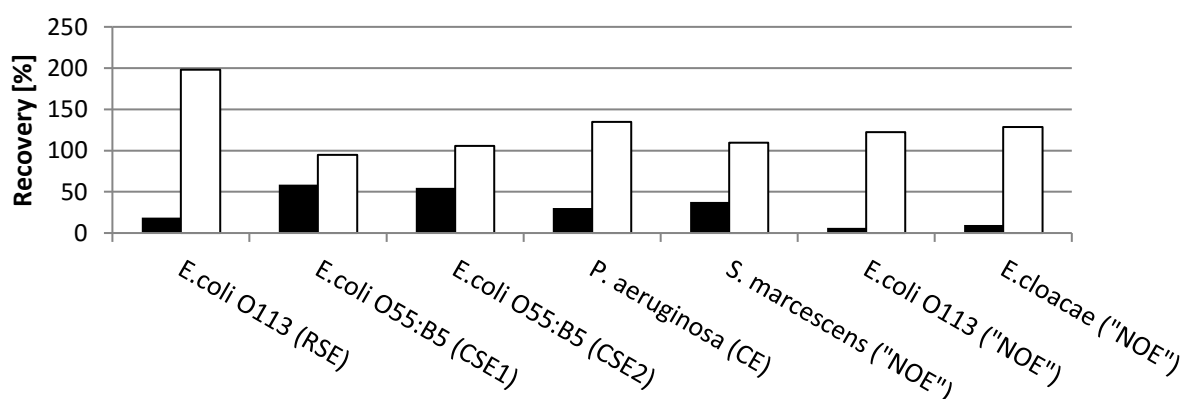


Figure 37 Masking and demasking of different endotoxin out of a drug product

Endotoxin recovery of 2.5 EU/mL of different endotoxins out of a finished drug product after incubation for 15 days at 4°C is shown. Endotoxin was measured before (black bars) and after demasking (white bars). For detection a LAL assay was used.

4.2 Perspectives of sample treatments in BET

The occurrence of LER demonstrates that the requirements for endotoxin testing of modern biological drug products are changing. To this end, snapshot measurements of endotoxin will be extended by time dependent measurements and trends have to be identified. Moreover, the complexity of present and future drug products will not decrease and in consequence, it is expected that individual sample preparations prior to the actual test methods will increase. Moreover, there are further exciting fields of application, which suffer from inadequate endotoxin detection. For example, vaccines can exhibit difficult conditions for BET, because of complex sample formulation including aluminum-based nano particles, which are able to adsorb endotoxin and strongly interfere with the enzyme reaction in *Limulus*-based detection systems. In such a case, the optimization of given detection methods is of great interest. Another challenge

in BET is the group of Advanced Therapy Medical Products, which possess enormously increased sample complexity, because such products often contain living cells. However, the ultimate challenge of endotoxin testing is the analysis of blood samples, because of pronounced masking effects and strong test interference, which substantially reduces sensitivity of the test system. Therefore, fast and sensitive detection of endotoxin in blood samples would be a great achievement in the field of sepsis diagnosis, which in turn would substantially support decisions in the medical treatment of acute infections. Taken together, the presented data contributes to a better understanding of endotoxins and helps to improve detection of bacterial endotoxins in complex sample matrices.

4.3 Need for structural analysis of endotoxins in complex sample matrices

Goal of the present work was to establish a technical solution for endotoxin detection in the case of endotoxin masking (LER) of biopharmaceutical drug products. To this end, a technical solution was developed and a mechanistic model was established, assuming structural rearrangements of LPS during masking and demasking. Yet, to confirm the working hypothesis and to further improve the current methods, detailed structural analysis of endotoxin will be of interest. It might be conceivable to track a change of endotoxin aggregates during masking and demasking with physical methods. For instance, it is most likely that the size of aggregates is changed, which could be determined using scattering methods like Dynamic Light Scattering (DLS) or microscopy methods like Atomic Force Microscopy (AFM). The application of such methods is highly appreciated, but there are a few obstacles that need to be overcome. Due to the heterogeneity of endotoxin, it exists in a broad variety of aggregates with different shapes and sizes. Thus, the analysis of simultaneous alterations of different aggregates will be difficult. A further challenge is the particular endotoxin concentration. Basically, the aggregation state is concentration dependent and relevant LPS concentrations are in the pico- to femtomolar range, which challenges the detection limit of most analytical methods. Further difficulties are given by the molar excess of surfactants compared to LPS. To this end, the detection of structural alterations of LPS might be interfered by surfactant aggregates. Due to these difficulties, it might be reasonable to start experiments using the above studied polysorbate/citrate matrix as sample, but with reduced polysorbate concentrations still allowing for masking, but possibly reducing interference. In addition, using rough mutants lacking the O-antigen as endotoxin source could be beneficial in such experiments, because a reduced heterogeneity of endotoxin can be achieved. Obviously, such conditions do not reflect real conditions in industry, but will support the understanding of endotoxin aggregation. This further elucidation of supramolecular

alterations can be used in diagnostics for improving endotoxin detection methods, but also support fundamental research of stabilities in bacterial membranes.

4.4 Clinical relevance of masked endotoxin

Beyond the presented analytical approach, it is also of great interest to further understand the clinically effects of masked endotoxin. Endotoxin, when masked could be assumed as depyrogenated i.e. the endotoxic activity is neutralized and detection could be assumed as dispensable. At a first glance, this is a solid argument, because *Limulus*-based detection methods are useful for identification of LPS and LPS-like structures in samples like biopharmaceutical drug products. Comparative studies have shown that activities measured with *Limulus*-based detection methods indicate bacterial contaminations very sensitive. However, it has to be kept in mind that *Limulus*-based detection is derived from an invertebrate crab and is not an *in-vivo* measure for endotoxicity in man. Obviously, to study the real pyrogenicity of masked endotoxin, it must be administrated intravenously to man under a variety of conditions (varying concentrations, different endotoxin sources, etc.). Yet, such kinds of studies do not correspond with ethic guidelines. Alternative test procedures are experiments in animals. In Europe it is difficult to perform such studies, because of animal welfare directives of the European Commission. Nevertheless, a few unpublished studies using the RPT have been performed, indicating that rabbits can positively respond to masked endotoxin, but not imperatively. Using another *in-vitro* method which mimics interaction of endotoxin and Toll-like receptors of the human innate immune system might be also beneficial[30]. Interestingly, first results indicate that monocyte activation tests (MAT) are also affected by LER. The interaction of masked endotoxin with Toll-like receptor in the assay seems to be not possible. However, the MAT does not reflect *in-vivo* conditions. It has to be investigated, whether other physiological functions are needed to break up the masked endotoxin complex. Otherwise complex formation of endotoxin by chelator and surfactant might inhibit immune stimulation via Toll-like receptor immune response. In consequence, there is no definite statement available, if masked endotoxin is still hazardous in man. Moreover, it has to be considered that masked endotoxin might activate alternative immune stimulating pathways. In the field of vaccination, depyrogenated endotoxins are actively added to certain drug products to serve as an adjuvant, stimulating the immune system. Such effects are desired and support the development of certain adaptive immunity. In contrast, biopharmaceutical drug products (e.g. therapeutic proteins), which are focus in the present work, can lose their efficacy or lead to life threatening responses due to innate immune response modulating impurities.[128], [129] Hence, in the case of therapeutic monoclonal antibodies, the presence of masked endotoxin could lead to inadvertent side reactions. Consequently,

continuous improvement of endotoxin detection methods is essential to maintain and improve patient safety.

4.5 Key findings

Endotoxin testing is mandatory in quality control of parenteral drug products. Low recovery of known endotoxin contents has led to the presented work and resulted in the following findings:

- The occurrence of the Low Endotoxin Recovery (LER) is time and temperature dependent. LER can be detected after minutes to hours and days of sample incubation depending on the experimental setup. To thoroughly identify if a sample (e.g. drug product) is affected by LER, incubation temperature and periods have to reflect handling and storage procedures of tested samples.
- LER is caused by endotoxin masking. Endotoxin detection assays have been proven functional, but detectability of endotoxin is limited due to alteration of its supramolecular aggregation state, which in turn can be manipulated by the sample matrix. It is supposed that endotoxin is monomerized in its masked state.
- Common formulation components of biopharmaceutical drug products like surfactants and buffer systems as well as proteins can lead to LER. However, only the simultaneously presence of amphiphilic molecules and complex formation agents cause LER. The complex formation capability of a sample matrix strongly determines the reaction rate.
- Endotoxin masking is associated with a two-step reaction mechanism. In a first step, endotoxin aggregates are permeabilized by destabilization of salt bridges between endotoxin molecules. In a second step, amphiphilic molecules like surfactants intercalate between endotoxin molecules and result in masking of endotoxin.
- Endotoxins from different sources possess different susceptibilities to endotoxin masking. Depending on the molecular structure of endotoxin, different stabilization mechanisms are used. Endotoxins with substantial contents of positively charged substituent's (e.g. 4-amino-4-deoxyarabinose) are less susceptible to masking.
- Demasking of endotoxin is possible. Endotoxin can be released from its masking complex and detected again using common detection methods. Sample treatments using dodecanol, calcium dichloride, sodium dodecyl sulfate and bovine serum albumin have been shown to be very effective. It is most likely that endotoxin reagggregates and forms inverted aggregates.
- Sample treatment for demasking is case related. Depending on the sample matrix, above described components and combination thereof as well as concentrations have to be adapted case by case. Co-surfactants like long chain alkyl alcohols have been identified as key components and are necessary in all cases.

5 Appendix

5.1 List of figures

Figure 1 Gram-negative Bacteria	13
Figure 2 The history of endotoxin research	14
Figure 3 Schematic structure of lipopolysaccharides	16
Figure 4 Schematic representation of the activation mechanisms induced by LPS	16
Figure 5 The Toll-Like-Receptor family	17
Figure 6 Horseshoe crabs and their endotoxin specific reaction cascade	19
Figure 7 Endotoxin recovery kinetics in citrate-polysorbate formulations	32
Figure 8 Impact of buffer system on endotoxin recovery.....	34
Figure 9 Surfactant dependent endotoxin recovery	35
Figure 10 Endotoxin masking capacity of citrate-polysorbate 20 formulation.....	36
Figure 11 Two-step mechanism of endotoxin masking	39
Figure 12 Endotoxin recovery depending on order of matrix component and LPS addition	42
Figure 13 Endotoxin recovery kinetics depending on LPS concentration.....	43
Figure 14 Endotoxin recovery kinetics depending on concentration of matrix components	44
Figure 15 Endotoxin recovery depends on the presence of divalent cations.....	45
Figure 16 Sodium citrate concentration determines endotoxin recovery.....	46
Figure 17 Endotoxin recovery depends on sodium citrate concentration under equilibrium conditions.....	47
Figure 18 SDS-PAGE of crude bacterial endotoxin preparations from different bacteria	57
Figure 19 Comparison of crude endotoxin preparations from different bacteria.....	58
Figure 20 Endotoxin recovery kinetics of endotoxin from <i>E.cloacae</i>	59
Figure 21 Comparison of phenol-extracted endotoxins from different bacteria	60
Figure 22 Endotoxin recovery kinetics of different endotoxin preparations.....	61
Figure 23 Structural modifications of lipopolysaccharides	64
Figure 24 Origin of LPS – Bacterial cells and their breakdown products	66
Figure 25 Molecular shape of an amphiphilic molecule determines its supramolecular structure.....	69
Figure 26 Relationship between supramolecular structures and endotoxicity	70
Figure 27 Demasking of endotoxin using co-surfactants.....	71
Figure 28 Demasking of endotoxin using dodecanol and BSA.....	72
Figure 29 Demasking of endotoxin out of different sample matrices using dodecanol and BSA.	73
Figure 30 Demasking of endotoxin out of octoxynol 9 matrices	73
Figure 31 Potential effects on supramolecular structures: Mixing polysorbate and LPS	79

Figure 32 Mixing surfactants and co-surfactants	80
Figure 33 Potential effects on supramolecular structures: Reassembly of LPS	81
Figure 34 Molecular structures of surfactants	82
Figure 35 Potential effects on supramolecular structures: Re-arrangements during demasking	85
Figure 36 Recovery endotoxin before and after demasking out of a drug product	86
Figure 37 Masking and demasking of different endotoxin out of a drug product.....	87

5.2 List of tables

Table 1 Common formulations of biopharmaceutical drug products	21
Table 2 Endotoxin recovery out of single and multiple component samples.....	31
Table 3 Growth of different bacteria and release of endotoxin	56
Table 4 Endotoxin recovery of different endotoxins	58
Table 5 Detection of an endogenous endotoxin contamination	62
Table 6 Comparison of different demasking approaches	74
Table 7 Demasking of different endotoxins.....	75
Table 8 Demasking of endotoxin out of formulated antibody samples	76
Table 9 Demasking of unknown endotoxin.....	77
Table 10 Comparison of different detection methods after demasking of endotoxin	78

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5.4 List of publications

5.4.1 Selected poster presentations

Evaluation of two new recombinant Factor C based assays as alternatives for Limulus blood based endotoxin detection methods, European Society for Alternatives to Animal Testing, Linz, Austria, 2013

Low Endotoxin Recovery in Limulus Based Detection Systems, Dechema Gesellschaft für Chemische Technik und Biotechnologie, Irsee, Germany, 2013

Case study - Low Endotoxin Recovery in bio-pharmaceuticals: Comparison of Natural occurring Endotoxins (NOE) and commercial standards Annual meeting, Parenteral Drug Association, San Antonio, USA, 2014

Endotoxin Contamination in Biopharmaceuticals: False Negative Results Induced by Endotoxin Masking, Bioprocessing Summit, Boston, USA, 2014

Endotoxin Testing – A Gamble on the Test System, Annual meeting, Parenteral Drug Association, Las Vegas, USA, 2015

Challenges of Endotoxin Detection in Biologics Drug Products, Bioprocessing Summit, Boston, USA, 2015

5.4.2 Oral presentations

An advanced Endotoxin Assay: Insights and Strategies for overcoming Low Endotoxin Recovery in complex formulations, Parenteral Drug Association, Tokio, Japan, 2013

New Challenges of Endotoxin Detection in modern Pharmaceuticals, International Endotoxin and Innate Immunity Society, Salt Lake City, USA, 2014

Endotoxin Contamination in BioPharmaceuticals: Overcoming False Negative Results Induced by Endotoxin Masking, Bioprocessing Summit, Boston, USA, 2014

Endotoxin masking & de-masking, Food and Drug Administration, Bethesda, USA, 2014

Heterogeneity of Potential Endotoxin Contaminations in Parenteral Drugs, Parenteral Drug Association, Pharmaceutical Microbiology, Berlin, Germany, 2015

Understanding and Overcoming LER, Lonza Endotoxin Summit, Annapolis, USA, 2015

Understanding the Principles of Endotoxin Masking and Demasking, Bacterial Endotoxin Summit, Iselin, USA, 2015

Endotoxin Masking /Low Endotoxin Recovery Update, Webinar, European Compliance Academy, Heidelberg, Germany, 2015

Overcoming Endotoxin Masking in a Drug Product, PharmaLab, Düsseldorf, Germany, 2015

Masking and Demasking of Endotoxins in Common Biological Product Matrices, Annual Meeting, Parenteral Drug Association, San Antonio, USA, 2016

Detectability of Endotoxin Contaminations in Biologicals, Protein & Antibody Engineering and Development Summit, Shanghai, China, 2016

Understanding and Overcoming LER II, Lonza Endotoxin Summit, Annapolis, USA, 2016

5.4.3 Published articles

J. Reich, K. Heed, H. Grallert, Detection of naturally occurring bacterial endotoxins in water samples, European Pharmaceutical Review magazine, Issue 6, 2014

Z. Hu, T. Murakami, K. Suzuki, H. Tamura, J. Reich, K. Kuwahara-Arai, T. Iba, und I. Nagaoka, „Antimicrobial cathelicidin peptide LL-37 inhibits the pyroptosis of macrophages and improves the survival of polybacterial septic mice“, Int. Immunol., 28, 5, 2016.

L. Wimbish, J. Reich, Better understanding LER and how to detect endotoxins in medicinal products, Pharmaceutical Processing, Vol 31, No. 3, 2016

J. Reich, P. Lang, H. Grallert, H. Motschmann, Masking of Endotoxin in Surfactant Samples: Effects on Limulus-based detection systems, Biologicals, 2016 (in press)

H. Tamura, J. Reich, I. Nagaoka, Bacterial endotoxin assays relevant to host defense peptides, submitted to Juntendo Medical Journal, 62, 2, 2016

5.4.4 Intellectual Property

J. Reich, H. Grallert, Unmasking endotoxins in solution, EP20140172151, 2014

5.4.5 Declaration

The studies presented in chapter 3.1 “Masking of endotoxin in surfactant samples: Effects on Limulus-based detection systems” led to a publication which is was already submitted to the Journal of Biologicals in 2015 ([J. Reich](#), P. Lang, H. Grallert, H. Motschmann, Masking of Endotoxin in Surfactant Samples: Effects on Limulus-based detection systems). The article has been accepted on 26th of April 2016 and is currently in press.

The findings described in chapter 3.4 “Endotoxin demasking” are included in a patent application in 2014 to protect this unique approach. Therefore, parts of this chapter (3.4) were already published in 2015 ([J. Reich](#), H. Grallert, Unmasking endotoxins in solution, EP20140172151, 2014).

5.5 Curriculum Vitae

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Employment History and Experience

09/2016 – **Microcoat Biotechnologie GmbH**, Bernried, Germany
General Manager - endotoxin test service

02/2005 – 12/2008 **Profos AG**, Regensburg, Germany
Product Manager - drugs of abuse (DoA) testing product range
(whilst concluding final elements of University degree)

08/2004 – 01/2005 **Profos AG**, Regensburg, Germany
Marketing Assistant - all technology/product categories.
Industrial year during University degree course.

01/2002 – 08/2002 **Raiffeisen-BayWa Waren GmbH**, Lobsing, Germany
Management Assistant - wholesale outlet

Education and Training

10/2012 – 08/2016 **Universität Regensburg**, Regensburg, Germany
Hyglos GmbH, Bernried, Germany
Doctoral Research Study - Low Endotoxin Recovery

10/2010 – 09/2012 **Universität Regensburg**, Regensburg, Germany
Institute for Separative Chemistry, Marcoule, France
Master of Science (COSOM)

10/2007 – 09/2010 **Universität Regensburg**, Regensburg, Germany
Bachelor of Science (Chemistry)

09/2003 – 07/2007 **University of Applied Sciences**, Regensburg, Germany
Diploma (Business Administration)

09/2002 – 08/2003 **Upper vocational school, BOS**, Regensburg, Germany
Advanced technical college entrance qualification

03/2001 – 12/2001 **Bundeswehr Pionierlehrbatallion**, Ingolstadt, Germany
Military service - Technical training as mobile crane operator

09/1998 – 02/2001 **Raiffeisen-BayWa Waren GmbH**, Lobsing, Germany
Professional training / work experience - Training in commercial aspects of wholesale and export trades.

5.6 Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 05.07.2016

Johannes Reich

6 Annex

Current Topics: Current
Status and Future Perspective
on the Application of Host
Defense Peptides to Medicine

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2016, 62 (2), 132-140



Bacterial Endotoxin Assays Relevant to Host Defense Peptides

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The Limulus amoebocyte lysate (LAL) test is the most sensitive and reliable assay for the detection of trace amounts of bacterial endotoxins (lipopolysaccharides, or LPS), and is an accepted *in vitro* alternative to the rabbit pyrogen test for evaluations of parenteral drugs, biological products, and medical devices. There are three principal LAL tests, which can be categorized as both semi-quantitative and quantitative methods, including gel-clot, turbidimetric, and chromogenic assays. Since the 1970s, these tests have been successfully formulated and commercialized by US and Japanese manufacturers. More recently, in addition to the recombinant factor C-based assay, a novel product containing all of the recombinant coagulation factors from horseshoe crab has been developed, which may lead to the creation of a next generation LAL alternative. Furthermore, there are antimicrobial peptides called "host defense peptides (HDPs)" that play a key role in innate immune responses. The LAL test for HDP-related studies is challenging, because the active site of endotoxin could be masked by the binding with HDPs. Thus, it is very important to properly evaluate the actions of HDPs (human defensins and cathelicidin peptide LL-37) such as the neutralization of LPS, immunostimulatory functions, and anti-endotoxin activity. Moreover, sensitive detection of LPS in cell culture media should be conducted to address the problem of endotoxin contamination in the media. Here, we discuss the progress of LAL-based endotoxin assay technologies, as well as their applications and limitations, with a focus on innovative functional studies of HDPs.

Key words: endotoxin, LPS, LAL test, endotoxin masking, host defense peptides



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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* Amoebocyte 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.

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Antimicrobial cathelicidin peptide LL-37 inhibits the pyroptosis of macrophages and improves the survival of polybacterial septic mice

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Abstract

LL-37 is the only known member of the cathelicidin family of antimicrobial peptides in humans. In addition to its broad spectrum of antimicrobial activities, LL-37 can modulate various inflammatory reactions. We previously revealed that LL-37 suppresses the LPS/ATP-induced pyroptosis of macrophages *in vitro* by both neutralizing the action of LPS and inhibiting the response of P2X₇ (a nucleotide receptor) to ATP. Thus, in this study, we further evaluated the effect of LL-37 on pyroptosis *in vivo* using a cecal ligation and puncture (CLP) sepsis model. As a result, the intravenous administration of LL-37 improved the survival of the CLP septic mice. Interestingly, LL-37 inhibited the CLP-induced caspase-1 activation and pyroptosis of peritoneal macrophages. Moreover, LL-37 modulated the levels of inflammatory cytokines (IL-1 β , IL-6 and TNF- α) in both peritoneal fluids and sera, and suppressed the activation of peritoneal macrophages (as evidenced by the increase in the intracellular levels of IL-1 β , IL-6 and TNF- α). Finally, LL-37 reduced the bacterial burdens in both peritoneal fluids and blood samples. Together, these observations suggest that LL-37 improves the survival of CLP septic mice by possibly suppressing the pyroptosis of macrophages, and inflammatory cytokine production by activated macrophages and bacterial growth. Thus, the present findings imply that LL-37 can be a promising candidate for sepsis because of its many functions, such as the inhibition of pyroptosis, modulation of inflammatory cytokine production and antimicrobial activity.

Keywords: caspase-1, cecal ligation and puncture, cytokines, IL-1 β , sepsis



Facing Up to the Challenge of Low Endotoxin Recovery

Better understanding LER and how to detect endotoxins in medicinal products.

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Endotoxins (also known as lipopolysaccharides) are a major component of the outer membrane of Gram-negative bacteria and elicit a strong immune response in animals. Should they contaminate pharmaceutical products that are administered via injection or medical devices that make contact with the patient's bloodstream, endotoxins can trigger fever, sepsis, or other serious illness. As a result, it is essential that every batch of these pharmaceuticals and medical devices is screened for the presence of such pyrogens.¹

There is a range of tests available for detecting endotoxins including the Rabbit Pyrogen Test (RPT) and tests based on Limulus Amebocyte Lysate (LAL), which is derived from the blood cells of the horseshoe crab. The sensitivity, reliability, and ease-of-use of the LAL test has made it the preferred method in most laboratories. However, an inhibitory phenomenon known as Low Endotoxin Recovery (LER) has raised questions about its use and caused considerable concern in the industry.

Better Understanding LER

LER is the masking of endotoxins in undiluted materials, thought to be attributable to combinations of specific excipients. This differs from the inhibition or interference of endotoxin tests caused by pH, high divalent ion concentrations, chelators, serine proteases, and glucan, which can usually be overcome using a pre-

treatment such as dilution. Since LER was first highlighted by Chen and Vinther in 2013, it has been the subject of much discussion. There has been significant debate about the mechanism behind LER.

It is known that the LPS molecules that make up endotoxins tend to aggregate under certain conditions. For example, in nature they often form a part of bacterial membranes due to their molecular structure, which is composed of both hydrophilic and hydrophobic sections. This is also



Detection of naturally occurring bacterial endotoxins in water samples

A comparison study using two different Kinetic Chromogenic Limulus Amebocyte Lysate (LAL) Assays and three different Endpoint Fluorescent Recombinant Horseshoe Crab Factor C (rFC) Assays

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Endotoxins (chemically lipopolysaccharides) are pyrogenic, heat-stable outer cell membrane components of Gram-negative bacteria¹, such as *Escherichia*, *Klebsiella*, *Enterobacter* and *Orzobacter*, which occur in the environment, e.g. in water². A method comparison study was conducted in order to determine the suitability of different commercially available endotoxin tests to quantify naturally occurring endotoxins in 20 different water samples such as deionised water, tap water and samples from environmental sources like rivers, lakes and springs. The determined endotoxin concentrations ranged from 0.05EU/ml (tap water) to 25.88EU/ml (lake water). The results from all endotoxin methods correlated well with each other (>94.4%), demonstrating that recombinant Factor C (rFC) assays are suitable alternatives to *Limulus* Amebocyte Lysate (LAL) assays for testing naturally occurring endotoxins in water.

Materials & methods

Water Samples: Water samples were collected in sterile and pyrogen-free 50ml tubes from different locations and sources such as rivers, lakes, springs, tap water, mineral water and deionised water. All samples were passed through 0.45µm membrane filters. Subsequently, aliquots were prepared and stored frozen at -20°C until testing.

Kinetic Chromogenic LAL Assays: The two Kinetic Chromogenic LAL assays used in the study were Kinetic-QCL (Lonza Ltd.) and Pyrochrome (Associates of Cape Cod Inc.), both with a labelled sensitivity of 0.005 EU/ml. The horseshoe crab blood coagulation cascade of LAL assays relies on several enzymes. Factor C, the first component in the

cascade, is a protease zymogen which is activated by endotoxin. Factor C in turn activates Factor B. An alternative pathway, via Factor G, can be activated by β-glucan binding. Factor C and Factor G pathways individually process a proclotting enzyme into a clotting enzyme. Chromogenic LAL assays use substrates that are cleaved by the clotting enzyme, resulting in a detectable colour reaction³.

Endpoint Fluorescent rFC Assays: The three Endpoint Fluorescent rFC assays used in the study were EndoZyme, with a labelled sensitivity of 0.005EU/ml (Hyglos GmbH), PyroGene with a labelled sensitivity of 0.01EU/ml (Lonza Ltd.) and EndoLISA, with a labelled sensitivity of 0.05 EU/ml (Hyglos GmbH). These methods use recombinant forms of horseshoe crab Factor C (rFC)⁴, converting a substrate into a detectable fluorescence signal. By using rFC, false-positive β-glucan reactions are eliminated and no horseshoe crabs are exploited. The latter rFC assay EndoLISA differs from the other assays due to its additional sample preparation step consisting of a solid phase pre-coated with an endotoxin-specific phage protein which enables the removal of the sample matrix prior to the detection⁴.

Quantification of endotoxin: The samples were thawed, freshly diluted (1:10) in depyrogenated water and tested in parallel with the five different endotoxin detection assays according to the manufacturer's instructions. The possibility of inhibition or enhancement of the tests due to interfering substances in the samples was determined. This was accomplished by spiking (adding) known amounts of the respective Control Standard Endotoxin (CSE) of each kit to control samples, so called Positive Product Controls (PPCs). The spiked amount was 5EU/ml in all cases except for the PyroGene assay where 1EU/ml spikes were added. Endotoxin concentrations in the samples and PPC recoveries in the control samples were calculated according to the manufacturer's instructions. A test result was considered valid when the corresponding spike was recovered in a range of 50% to 200%.

Table 1: Measured endotoxin values in 1:10 diluted water samples. Valid test results fulfilled the validity criterion of a Positive Product Control (PPC) recovery of 50 to 200%. Highlighted in orange are values which did not fulfill the validity criterion (see Table 2). Endotoxin values below the detection limit of each test are listed as <0.05 (EndoLISA), <0.01 (PyroGene) and <0.005 (EndoZyme, Kinetic-QCL and Pyrochrome).

	Sample #	EndoLISA (EU/ml)	EndoZyme (EU/ml)	PyroGene (EU/ml)	Kinetic-QCL (EU/ml)	Pyrochrome (EU/ml)	MRSA (EU/ml)
Quarry pond	1	4.217	2.443	2.935	11.119	58.108	8.829
Lake	2	0.212	0.492	0.195	0.017	18.898	n.c. ⁵
River	3	0.182	0.268	0.335	0.359	0.342	0.272
River	4	0.169	0.818	1.125	1.229	2.161	0.113
River	5	0.194	0.229	0.887	0.313	12.847	7.326
River	6	0.017	0.691	0.881	0.868	1.284	0.858
River	7	0.083	0.117	1.187	0.188	1.267	0.992
River	8	0.249	0.668	0.217	12.748	28.897	11.284
Spring water	9	0.077	0.097	0.120	0.120	0.034	0.088
Pre-filtered pond	10	1.402	1.092	4.440	2.004	1.691	2.288
Palm forest	11	2.335	7.688	11.024	5.293	12.259	6.713
Tap water	12	<0.05	0.002	0.042	0.045	0.014	0.020
Tap water	13	0.485	0.592	1.815	0.042	0.035	0.829
Tap water	14	<0.05	0.028	0.910	<0.005	0.009	0.028
Tap water	15	<0.05	0.018	0.818	0.048	0.011	0.013
Tap water	16	<0.05	0.158	0.189	0.089	0.108	0.120
Tap water	17	<0.05	<0.005	<0.010	<0.005	0.017	n.c. ⁵
Deionised water	18	<0.05	0.028	0.825	0.055	0.109	0.108
Mineral water	19	<0.05	0.018	<0.010	0.070	0.010	0.017
Mineral water	20	<0.05	0.025	<0.010	0.043	0.007	0.027

⁵ not calculated

Results & discussion

The measured endotoxin concentrations in the 1:10 diluted water samples using the five different endotoxin tests in parallel (Table 1) ranged from 0.005EU/ml (tap water) to 25.88EU/ml (lake water). As expected, the unpurified lake and river samples contained higher endotoxin levels than purified water. However, the water sampled from a natural spring also contained low levels of endotoxin contaminants. No significant deviation in results was observed between the rFC tests and the LAL tests. The results from all tests were in the same order of magnitude. Moderate deviations with regards to the mean values were



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(54) **Unmasking endotoxins in solution**

(57) The invention relates to unmasking endotoxins in compositions so that previously present, but undetectable endotoxins are rendered detectable.

Description

[0001] The present invention relates to unmasking endotoxins in compositions, preferably pharmaceutical compositions, so that present but undetectable endotoxins are rendered detectable. Specifically, the invention relates to a method of unmasking an endotoxin in a composition. The invention further relates to a method of detecting an endotoxin in a composition. The invention further relates to a kit for unmasking an endotoxin in a composition. The invention further relates to the use of a modulator capable of unmasking an endotoxin, e.g. by releasing an endotoxin from a complex between said endotoxin and an endotoxin masker, to unmask an endotoxin in a composition.

Background of the invention

[0002] The presence of endotoxins in aqueous compositions is an intractable problem which severely threatens and/or limits the application of many compositions, in particular if intended for pharmaceutical use. This is especially true of compositions comprising protein products, e.g. recombinant protein products. Naturally occurring endotoxins, especially endotoxins belonging to the class of compounds characterized as lipopolysaccharides (LPS) are molecules produced by certain types of bacteria, for example gram-negative bacteria. Generally, endotoxins such as LPS comprise an extended polysaccharide O-antigen, a core antigen polysaccharide including an outer core component and an inner core component, and a lipid A domain comprising aliphatic amides and aliphatic acid esters. Such endotoxins are found in the outer membrane of gram-negative bacteria, where they contribute to bacterial structural integrity by shielding the organism from chemical attack. Such endotoxins increase the negative charge of the cell membrane of these bacteria, and help to stabilize the overall membrane structure. Such endotoxins elicit strong responses from normal animal, e.g. human, immune systems because normal serum contains lipopolysaccharide (LOS) receptors which normally direct the cytotoxic effects of the immune system against invading bacterial pathogens bearing such endotoxins.

[0003] When present in the human blood in a form disassociated from their source bacteria, endotoxins such as LPS can cause endotoxemia which in severe cases can lead to septic shock. This reaction is due to the endotoxin lipid A component, which can cause uncontrolled activation of the mammalian immune system, in some instances producing inflammatory mediators such as toll-like receptor (TLR) 4, which is responsible for immune system cell activation.

[0004] Bacteria, as well as the endotoxins they produce, are also ubiquitous. For instance, endotoxin contaminants are known to exist in the pipes and hoses of water supply systems, including those of laboratories and facilities for preparing pharmaceutical formulations. The surfaces of containers such as fermentors and glassware used in the process of formulating pharmaceuticals are also commonly contaminated. In addition, as humans carry bacteria and therefore endotoxins on their bodies, so the staff of such facilities in which pharmaceuticals are formulated also represent a possible source of endotoxin contaminants.

[0005] Of course, in addition to the above, gram-negative bacteria themselves find wide use in the production of i.a. recombinant therapeutic proteins, so there is always a danger that endotoxin contamination of aqueous compositions, e.g. pharmaceutical formulations, containing such therapeutic proteins may also arise directly from such bacteria used in the production process.

[0006] To safeguard against potentially hazardous incorporation of endotoxin contaminants, whatever their source, measures must normally be taken to exclude endotoxin from all steps and products used in the production process of such proteins before such solutions may be administered for therapeutic purposes. In fact, the exclusion and/or removal and verifiable absence of all traces of (detectable) endotoxin are among the requirements which must be met when seeking regulatory approval for any new therapeutic, in particular those containing products produced in bacteria, or which have come into contact with bacteria at any point in the production process (see e.g. EMEA, Q6B, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products; 2.1.4 Purity, Impurities and Contaminants; Contaminants; 4.1.3 Purity and impurities; 2) FDA, Q6B, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products; II.A.4. Purity, Impurities and Contaminants; IV.A.3. Purity and Impurities). For instance, all containers holding and/or transferring solutions intended for eventual administration must be rendered endotoxin-free prior to contact with the solution. A depyrogenation oven is used for this purpose, in which temperatures in excess of 200°C are required to break down endotoxins. Based on primary packaging material as syringes or vials, a glass temperature of 250°C and a holding time of 30 minutes is typical to achieve a reduction of endotoxin levels by a factor of 1000. Usually, liquids can not be depyrogenated by heat, therefore different methods are used, such as chromatography (e.g. anion exchange), phase extraction (e.g. Triton X-114), filtration (e.g. ultrafiltration).

[0007] One common assay for detecting the activity of endotoxin is the limulus amoebocyte lysate (LAL) assay, which utilizes blood from the horseshoe crab. Very low levels of endotoxin can cause coagulation by the limulus lysate due to a powerful amplification through an enzyme cascade. However, due to the dwindling population of horseshoe crabs, and the fact that factors exist which interfere with the LAL assay, efforts have been made to develop alternative assays for detecting the presence of endotoxin in solution. The most promising of such methods are enzyme-linked affinity sorbent assays, using a solid phase for endotoxin capturing and subsequent detection by recombinant version of a protein in

EP 2 955 518 A1

the LAL assay, Factor C. The EndoLISA® kit is one such affinity sorbent assay.

- 5 **[0008]** However, even the best available tests for detecting the presence of pyrogens, such as endotoxin, in particular LPS, are often unable to detect LPS in solution. This implies the danger that solutions which are reasonably - in the absence of any detectable endotoxin - thought to be endotoxin-free in fact contain endotoxin which is simply masked so as to be rendered undetectable. Such solutions, e.g. pharmaceutical formulations will not be barred from regulatory approval (at least not due to containing endotoxin), because by all diagnostic appearances, these solutions are endotoxin-free, therefore fulfilling - or at least appearing to fulfill - this regulatory requirement. Clearly, however, administration of such ostensibly endotoxin-free solutions to subjects risks triggering the types of reactions mentioned above. In such instances, one may learn of the presence of masked endotoxin in such solutions too late, after subjects have already developed the types of adverse and potentially life-threatening reactions described above. In addition, from a hygienic standpoint, drug regulatory authorities place great value on positively knowing which substances are contained in pharmaceutical compositions and which are not. This ultimately comes down to the ability to reliably detect all components in a given composition, and one's ability to believe the results obtained in reference to both the presence and absence of all substances tested.
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- 15 **[0009]** There thus exists a strong motivation to provide ways in which all endotoxin present in compositions, including endotoxin which is undetectable because it is being masked by certain other composition components, may be unmasked such that it is rendered detectable. Providing a way to unmask and/or detect hitherto undetectable endotoxin in a composition would greatly assist in promoting patient safety. It is an aim of the present invention to address such needs.