Investigating the Factors Affecting Endotoxin Masking in Human Blood Plasma: Internal and External Contributions

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"Every failure is a step to success. Every detection of what is false directs us towards what is true: every trial exhausts some tempting from error.”

– William Whewell

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List of abbreviations

AMPs	Antimicrobial peptides
APCs	Antigen-presenting cells
API	Active pharmaceutical ingredient
BDG	(1→3)-β-D-glucan
BET	Bacterial endotoxin testing
DCs	Dendritic cells
DIC	Disseminated intravascular coagulation
E. coli	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicines Agency
EU	Endotoxin units
FDA	Food and Drug Administration
Gal	Galactose
Hep	L-glycero-D-manno-heptose
HSA	Human serum albumin
ICU	Intensive care units
IFN-γ	Interferon-gamma
IL-1beta	Interleukin-1 beta
IL-6	Interleukin-6
KDO	3-deoxy-2-oxo-D-manno-octonic acid
LAL	Limulus Amebocyte Lysate
LER	Low endotoxin recovery
LPS	Lipopolysaccharide
MAT	Monocyte Activation Test
NOE	Naturally occurring endotoxin
PAMP	Pathogen-associated molecular pattern
PPC	Positive Product Controls
PRRs	pattern recognition receptors
rFC	Recombinant Factor C
RT	Room temperature
S. minnesota	*Salmonella Minnesota*

SEC	Size exclusion chromatography
Th cells	T-helper cells
TLR	Toll-like receptor
TNF-alpha	tumour necrosis factor alpha
Tregs	Regulatory T-cells

Abstract (EN)

Endotoxin detection is essential for pharmaceutical safety and clinical diagnostics, but remains challenging due to interference with formulation components and biological matrices. This thesis investigates factors affecting detection, focusing on storage conditions, the effect of lipopolysaccharide (LPS) structural variants on low endotoxin recovery (LER), and masking mechanisms in human plasma.

First, the influence of container materials on (1→3)-β-D-glucan (BDG) recovery was assessed, as BDG interferes with endotoxin measurements by falsely activating the Limulus amebocyte lysate (LAL) cascade via factor G. While short-term storage had no effect, long-term storage at -20°C reduced recoveries, especially in polyethylene containers. This highlights the importance of storage material selection for reliable BDG detection.

Next, the effect of LPS structural diversity on endotoxin masking was analysed using rough LPS mutants (Ra – Re) from *S. minnesota* and *E. coli*. Polysaccharide length influenced masking susceptibility: rough mutants, with a lower hydrophilic/hydrophobic ratio and stronger negative charge, showed greater resistance to masking due to enhanced cation binding and stabilised supramolecular structures, resulting in higher recovery rates. Masking kinetics varied between detection systems, with LAL and recombinant Factor C (rFC) assays showing similar masking behaviour, while the Monocyte Activation Test (MAT) showed improved recovery.

Finally, endotoxin detection in human blood was investigated, where plasma proteins contribute to masking. While previous research has emphasised electrostatic interactions, this study found that highly charged LPS structures, such as Re LPS, are less susceptible to masking, suggesting additional contributing factors.

Overall, this dissertation advances the understanding of endotoxin masking, structural influences on detection and external factors affecting test reliability. These findings will help to optimise endotoxin testing, reduce false negatives and improve pharmaceutical and clinical safety.

Zusammenfassung (DE)

Der Nachweis von Endotoxin ist essenziell für die pharmazeutische Sicherheit und klinische Diagnostik, wird jedoch durch Wechselwirkungen mit Formulierungsbestandteilen und biologischen Matrices erschwert. Diese Dissertation untersucht Einflussfaktoren, insbesondere Lagerbedingungen den Einfluss struktureller LPS-Varianten auf Low Endotoxin Recovery (LER) und Maskierungsmechanismen im menschlichen Blut.

Zunächst wurde der Einfluss verschiedener Behältermaterialien auf die (1→3)-β-D-glucan (BDG) -Rückgewinnung analysiert, da BDG die Bestimmung von Endotoxin stören kann. Während kurzfristige Lagerung keine Effekte zeigte, führte langfristige Lagerung bei −20 °C zu verringerten Glucan-Rückgewinnungen, insbesondere in Behälter aus Polyethylen. Dies unterstreicht die Bedeutung der Behälterwahl für verlässliche BDG-Analysen.

Anschließend wurde untersucht, wie die strukturelle Diversität von LPS die Maskierung beeinflusst. Hierfür wurden raue LPS-Mutanten von *S. minnesota* und *E. coli* verwendet, die keine vollständigen O-Antigen-Ketten besitzen. Die Studie zeigte, dass die Polysaccharidlänge die Maskierungsempfindlichkeit beeinflusst. Raue Mutanten waren resistenter, da ihr geringeres hydrophiles/hydrophobes Verhältnis und ihre stärkere negative Ladung die Kationenbindung und Stabilisierung supramolekularer Strukturen begünstigen. Maskierungsmechanismen variierten je nach Nachweissystem: Während der Limulus-Amöbozyten-Lysat-Test (LAL) und der rekombinante Faktor C-Test (rFC) eine vergleichbare Maskierung zeigten, erzielte der Monozyten-Aktivierungstest (MAT) eine höhere Endotoxin-Rückgewinnung.

Schließlich wurde der Nachweis von Endotoxin im Blut untersucht, wo Plasmaproteine zur Maskierung beitragen. Während frühere Studien elektrostatische Wechselwirkungen betonten, zeigte diese Arbeit, dass hochgeladene LPS-Strukturen wie Re-LPS weniger maskierungsanfällig sind, was auf weitere Einflussfaktoren hindeutet.

Diese Dissertation trägt zum Verständnis der Endotoxin-Maskierung, struktureller Einflüsse auf die Detektion und externer Faktoren bei. Die gewonnenen Erkenntnisse helfen, Testverfahren zu optimieren, falsch-negative Ergebnisse zu reduzieren und die pharmazeutische und klinische Sicherheit zu verbessern.

1. Publications

1.1 Publications and manuscripts embedded in this thesis

Publication 1: **Burgmaier L**, Illes B, Leiss M, Avci-Adali M, Reich J.
Effects of Different Container Types on (1→3)-β-D-glucan Recovery.
Molecules. 2023 Oct 4;28(19):6931. doi: 10.3390/molecules28196931

Publication 2: **Burgmaier L**, Pölt S, Avci-Adali M, Reich J.
The impact of LPS mutants on endotoxin masking in different detection systems.
Biologicals. 2024 Nov 24;89:101808. doi: 10.1016/j.biologicals.2024.

Publication 3: **Burgmaier L**, Lifka J, Avci-Adali M, Reich J.
Endotoxin Masking in Human Plasma: The Role of Protein Interactions and Lipopolysaccharide Structure.
Eur J Pharm Biopharm. 2025 Jun 13:114786. doi: 10.1016/j.ejpb.2025.

1.2 Personal contribution

Publication 1:

Luisa Burgmaier:	Experimental performance, Data analysis and evaluation, Conceptualization and study design, Manuscript writing
Bernhard Illes:	Conceptualization and study design, Data analysis and evaluation, Manuscript review and editing
Michael Leiss:	Conceptualization and study design, Manuscript review and editing
Meltem Avci-Adali:	Manuscript review and editing
Johannes Reich:	Manuscript review and editing, Supervision, Conceptualization and study design

Publication 2:

Luisa Burgmaier:	Experimental performance, Data analysis and evaluation, Conceptualization and study design, Manuscript writing
Stefan Pölt:	Experimental performance, Data analysis and evaluation, Manuscript review and editing
Meltem Avci-Adali:	Manuscript review and editing
Johannes Reich:	Manuscript review and editing, Supervision, Conceptualization and study design

Publication 3:

Luisa Burgmaier:	Experimental performance, Data analysis and evaluation, Conceptualization and study design, Manuscript writing
Johanna Lifka:	Experimental performance, Data analysis and evaluation
Meltem Avci-Adali:	Manuscript review and editing
Johannes Reich:	Manuscript review and editing, Supervision, Conceptualization and study design

2. Introduction

2.1 Endotoxins and their structures

Endotoxins, chemically known as lipopolysaccharides (LPS), are large molecules embedded in the outer membrane of Gram-negative bacteria [1]. These molecules are critical for bacterial survival in harsh environments, providing structural stability and acting as a permeability barrier to toxic substances. While essential for bacterial physiology, endotoxins pose a major threat to human health when introduced into the bloodstream or sterile body sites, as they can trigger a cascade of immune responses [2].

From a structural point of view, LPS can be divided into three distinct regions: lipid A, core oligosaccharide, containing outer and inner core regions and O-specific polysaccharide (Figure 1).

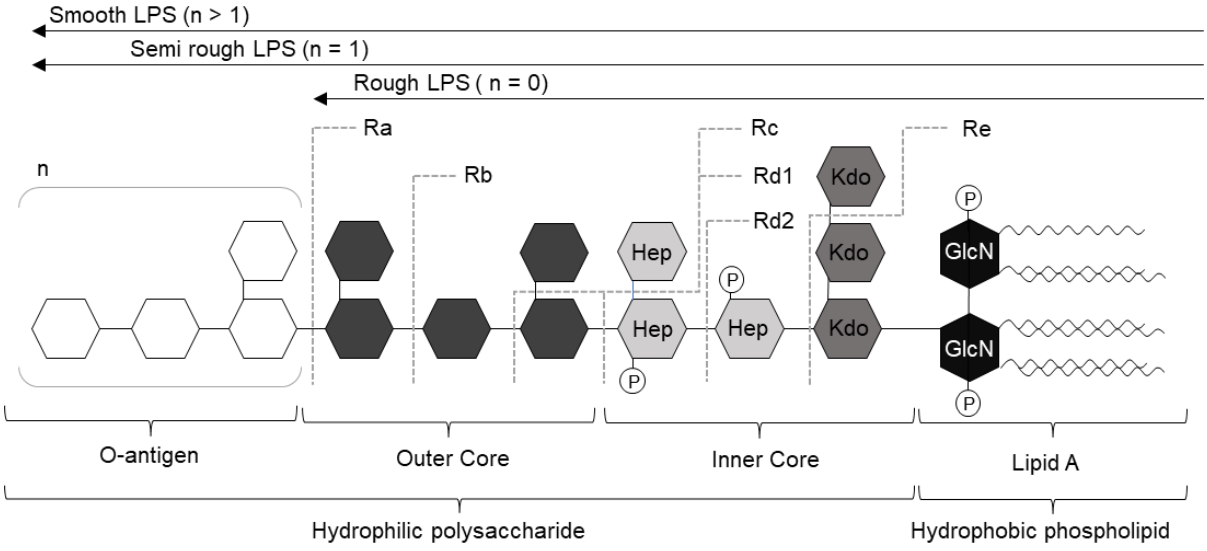

***Figure 1: Schematic representation of LPS**. Lipid A consists of a di-phosphorylated disaccharide GlcN (glucosamine) backbone. The core is divided into inner and outer core. The inner core consists of Kdo (3-deoxy-D-manno-octulosonic acid) and heptose (L-glycero-D-manno) residues and is phosphorylated with various groups such as phosphate (labelled P). The O antigen is composed of 1 to 50 repeating sugar subunits (n = 1-50). Different LPS mutants are labelled with Ra - Re. (Modified from [3])*

Lipid A: Lipid A occupies the proximal position of LPS. It is hydrophobic and consists of a bisphosphorylated disaccharide of glucosamine esterified with fatty acids including caproic acid (C6), lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18) [4]. Lipid A anchors LPS molecules to the outer membrane of the bacterial cell wall through hydrophobic interactions with the acyl chains of phospholipids, forming a lipid bilayer. The stability of this layer is enhanced by salt bridges between

divalent cations and the phosphate groups of neighbouring lipid A molecules [1]. In particular, the lipid A region represents the endotoxic principle of LPS, as changes in its structure significantly affect the bioactivity of the molecule [5,6]. Full endotoxic activity is observed in molecules containing two hexosamine residues, two phosphoryl groups and six fatty acids, including specific 3-acyloxyacyl groups with defined chain lengths at different positions [7]. However, not all LPS molecules are toxic, just as not all Gram-negative bacteria are pathogenic [8]. For example, certain *E. coli* strains are harmless commensals in the human gut.

Core oligosaccharide: Attached to lipid A, the core oligosaccharide forms the second part of LPS and can be further subdivided into an inner and outer core. The inner core, which is more conserved, contains unique sugars such as 3-deoxy-2-oxo-D-manno-octonic acid (KDO) and L-glycero-D-manno-heptose (Hep), with carboxyl and phosphate groups contributing to its negative charge. This negative charge facilitates the binding of divalent ions such as Mg^{2+} and Ca^{2+}, which is critical for maintaining the structure and function of the bacterial outer membrane [9]. In contrast, the outer core typically consists of hexoses such as glucose, galactose and N-acetylglucosamine, which show more variability between bacterial strains [7]. The core region contains up to fifteen monosaccharides and links to the polysaccharide portion to form the O-specific chain [10].

O-specific polysaccharide (O-Antigen): The outermost region of LPS, the O-specific polysaccharide, consists of repeating oligosaccharide units. This polysaccharide varies significantly between bacterial strains in terms of sugar type, sequence and substitution patterns, resulting in different serotypes. Common sugars found in this region include glucose, galactose, rhamnose and mannose. The presence or absence of the O-specific polysaccharide determines whether bacteria are classified as smooth (S-type) or rough (R-type), with smooth types having a complete O antigen and rough types lacking it. The naming of these classes is based on the colony morphology observed on agar plates, where smooth colonies have a glossy appearance and rough colonies have a matte appearance [11]. Functionally, the O-specific polysaccharide plays a key role in bacterial survival, particularly in the host environment. In pathogens such as *Salmonella* species, it helps to evade host immune defences by providing a barrier to the membrane attack complex of the complement system. As it is the most exposed part of Gram-negative bacteria, the O-specific polysaccharide is often

targeted by the host immune system, including antibody binding, and is therefore referred to as the O-antigen [12].

The absence of O-antigen can occur naturally or as a result of genetic mutation. While all rough mutants lack O-antigen, the length of the oligosaccharide core varies depending on the gene loci involved. Rough mutants without O-antigen but with a complete inner and outer core structure are called Ra-LPS. In contrast, rough mutants with a significantly reduced core are termed Re-LPS or "deep rough" LPS [13]. Re-LPS represents the smallest LPS structure that supports bacterial membrane integrity, while Ra-LPS has the longest core configuration among rough LPS. Mutations at different loci result in core lengths between Ra and Re-LPS [8]. In wild-type organisms, which predominantly express smooth LPS, there is significant size heterogeneity among LPS molecules. Approximately 40-50% of these molecules are semi-rough, characterised by the absence of the O-antigen repeating units [11].

Due to its amphiphilic nature, with hydrophilic (O-antigen and core region) and hydrophobic (lipid A) regions, LPS tends to aggregate in aqueous solutions. Depending on the molecular structure and environmental conditions, LPS can form various supramolecular assemblies, including non-lamellar, inverted cubic or hexagonal phases, as well as lamellar structures. Several studies have shown that the aggregation state of LPS influences both its biological activity and detectability [5,14–16].

2.2 Clinical relevance of endotoxins

Endotoxins can induce an adverse reaction in the picogram range per kilogram of host body weight, meaning that even minimal amounts can lead to significant immune responses. The intensity and threshold to induce pyrogenicity depends on the source of the endotoxin. Endotoxin units (EU) have been introduced to allow comparison of the biological activities of different LPS. The units are based on the approximate threshold of pyrogenic activity of *E. coli* LPS, where 1 EU corresponds to the biological activity of 0.1 nanogram of purified *E. coli* endotoxin in humans [17].

Due to their potent immunostimulatory effects, endotoxins play an important role in clinical settings. When endotoxins enter the human bloodstream, they can trigger a systemic inflammatory response known as endotoxemia [18]. This condition is particularly critical in sepsis, a life-threatening organ dysfunction caused by a

dysregulated immune response to infection. Sepsis remains a major cause of morbidity and mortality in intensive care units (ICUs) worldwide [19]. The lipid A component of LPS is primarily responsible for the endotoxic effects as it activates the innate immune system by binding to Toll-like receptor 4 (TLR4) on host cells. This interaction initiates a signalling cascade leading to the production of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-alpha), interleukin-1 beta (IL-1beta) and interleukin-6 (IL-6). In vivo, these cytokines contribute to fever, hypotension, endothelial activation, and increased vascular permeability [20].

The severity of endotoxin-induced sepsis highlights the importance of early diagnosis and prompt treatment. As sepsis progresses, organ dysfunction and systemic complications make effective therapy increasingly difficult, emphasizing the need for early intervention. Current therapeutic strategies for sepsis include fluid resuscitation, administration of broad-spectrum antibiotics, and the use of vasopressors. However, despite advances in medical care, sepsis remains associated with high mortality. This underscores the urgent need for novel therapeutic approaches that target endotoxin activity and, more importantly, for reliable methods to detect endotoxin at an early stage to enable timely and effective treatment. [21,22]

In addition to sepsis, endotoxins have been implicated in several non-infectious inflammatory diseases [23]. Chronic exposure to low levels of endotoxins, often referred to as metabolic endotoxemia, has been linked to metabolic disorders such as insulin resistance, obesity and atherosclerosis. In these conditions, endotoxins derived from the gut microbiota can enter the bloodstream through a compromised intestinal barrier, triggering low-grade systemic inflammation [24–26].

The clinical relevance of endotoxins extends beyond human health to the pharmaceutical and biomedical industries. Endotoxin contamination in parenteral drugs, intravenous fluids and medical devices can pose serious risks to patients, making endotoxin detection and removal a critical aspect of product development and quality control [27,28]. Overall, understanding the clinical implications of endotoxins is essential to improve patient outcomes in both infectious and non-infectious diseases. Continued research into the mechanisms of endotoxin action, detection technologies and therapeutic interventions holds promise for reducing the global burden of endotoxin-related disease.

2.3 Detection of endotoxins

Given that Gram-negative organisms inhabit nearly every environment on Earth, LPS is among the most abundant complex organic molecules in nature.[29]. Gram-negative bacteria thrive in diverse habitats, from soil and freshwater to the extremes of icy oceans and scalding hot springs. Gram-negative bacteria can grow in the cleanest water due to their minimal growth requirements. The ubiquity of endotoxins therefore requires routine and reliable screening of all fluids and drugs prepared for parenteral therapy. [1]

Over the years, several assays have been developed to detect endotoxins, with the Limulus amebocyte lysate (LAL) assay being the most widely used. To address some of the limitations of LAL, alternative methods such as the Recombinant Factor C (rFC) assay and the Monocyte Activation Test (MAT) have been introduced. Each method uses a different mechanism with unique advantages and challenges.

Introduced in the 1960s, the LAL test remains the gold standard due to its high sensitivity and specificity. It is derived from the blood of horseshoe crabs (*Limulus polyphemus* or *Tachypleus tridentatus*) and uses a natural coagulation cascade triggered by endotoxin binding. The occurrence of LPS activates an LPS-sensitive serine protease zymogen Factor C. The active form of the enzyme Factor C* then activates zymogen Factor B to Factor B*, following a forming formation of the clotting enzyme from proclotting enzyme (Figure 2). The clotting enzyme converts coagulogen, an invertebrate fibrinogen-like substance, to coagulin, resulting in a gel [30]. The whole enzymatic cascade is present in the Limulus amoebocyte lysate and allows for an extremely high sensitivity of the lysate to nanogram quantities of endotoxins [31,32]. The gel-clot method (Figure 2 A) relies on the occurrence of gel forming and is closest to the coagulation reaction happening in vivo [33]. Chromogenic and turbidimetric methods are referred to as photometric techniques. They are based on development of colour after cleavage of a synthetic chromogenic substrate by an enzyme activated by the endotoxin or a turbidity change after gel formation in lysate, respectively (Figure 2 B, C) [34,35]. The rates of absorbance change in the chromogenic method and the turbidimetric method are proportional to the endotoxin concentration. Despite its effectiveness, the LAL assay has several limitations, including reliance on animal resources and susceptibility to interference from certain pharmaceutical formulations, resulting in issues such as low endotoxin recovery (LER) [36].

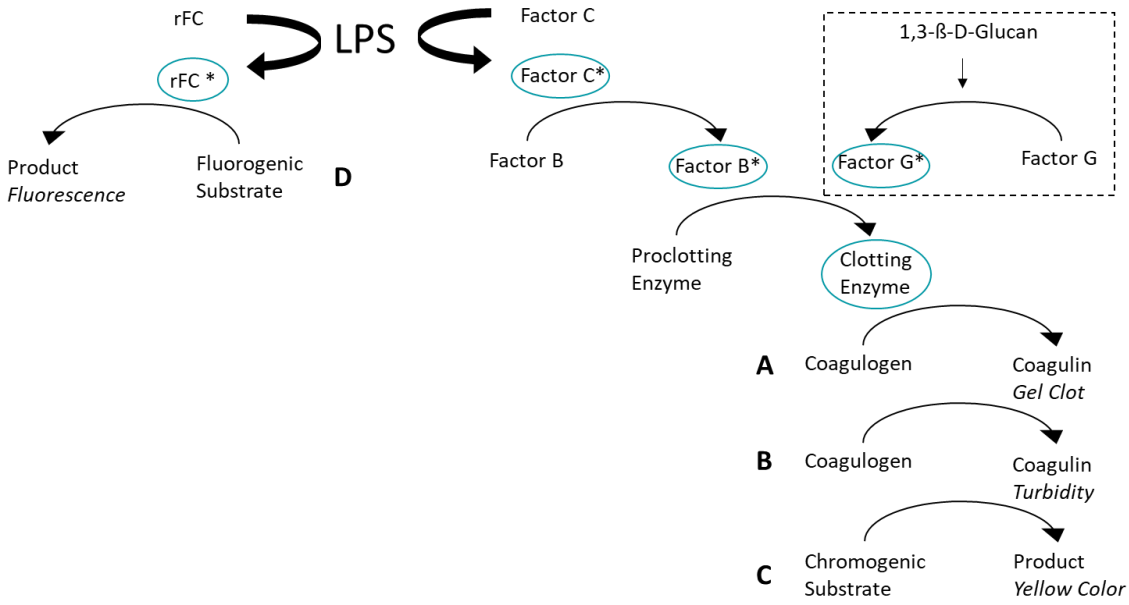

Figure 2 Reaction mechanism of the Limulus coagulation cascade system and its usage as endotoxin test. *Activation of Factor C through LPS results in the formation of coagulin gel. Unspecific activation of the cascade by factor G through glucans is also possible. Substrates and Products A) used for gel clot assay; B) used for turbidimetric assay; C) used for chromogenic assay are shown. D) Activation of recombinant Factor C (rFC) by LPS and fluorogenic substrate and product used for recombinant assay.*

The rFC assay offers a synthetic alternative that addresses the ethical and sustainability concerns associated with horseshoe crab harvesting. It uses recombinant technology to produce Factor C, which specifically binds to endotoxins and elicits a measurable response (Figure 2 D). This assay is recognised as being ethical, sustainable and less prone to interference, with performance comparable to or better than LAL [37–39]. Regulatory bodies, including the Food and Drug Administration (FDA) and European Medicines Agency (EMA), have begun to recognise the rFC assay as a valid alternative for endotoxin testing [40,41].

Unlike LAL and rFC, which specifically detect endotoxins, MAT can identify a wider range of pyrogens than endotoxins, known as non-endotoxin pyrogens (NEPs). Examples of NEPs include lipoteichoic acid from Gram-positive bacteria and peptidoglycan from fungi. The MAT uses human monocytes, monocytic cell lines or whole blood to measure the biological response to pyrogens. When pyrogens are present, they bind to Toll-like receptors on monocytes, triggering the release of cytokines such as IL-1 beta, which are then quantified by ELISA or similar techniques. MAT offers the advantages of detection of endotoxins and NEPs, closer simulation of human response and ethical compliance as it is entirely in vitro. However, it is more complex, costly and less standardised than LAL or rFC [42].

To summarise the key differences, LAL and rFC assays are highly sensitive and specific for endotoxins, whereas MAT provides broader pyrogen detection for endotoxins and NEPs. LAL is animal-dependent, whereas rFC and MAT are animal-free, making them more sustainable options.

2.4 Limitations in the detection of endotoxins

2.4.1 Test Interference

Assay interferences are a significant limitation in endotoxin detection. Various factors present in the sample matrix can lead to false-positive or false-negative results by interfering with the assay reaction. For LAL assays, common interferents include certain proteins, detergents or salts that can either inhibit the clotting reaction or cause non-specific reactions, making accurate quantification difficult. These interferences can result from the physicochemical properties of the sample, such as pH, ionic strength or the presence of chelating agents, which affect the assay's ability to reliably detect endotoxins. The LAL reaction is optimised for pH values between 6.4 and 8.0; deviations from this range will suppress reactivity. Divalent cations such as calcium and magnesium are essential for LAL activity as they help to disperse endotoxin aggregates. Chelating agents such as citrate and heparin can inhibit LAL by binding calcium, reducing sensitivity. [43]

Another specific challenge for LAL assays is the presence of (1→3)-β-D-glucans (BDG), which can activate the LAL pathway independently of endotoxins. By activating factor G, it leads to the formation of the clotting enzyme (Figure 2), leading to false-positive results [30]. BDGs are polysaccharides composed of glucose monomers that can trigger an inflammatory response, similar to endotoxins. They are produced by most fungi as a cell wall component, but they are also found in a wide range of other eukaryotic and prokaryotic organisms, like yeast and algae [44]. Unlike LAL, rFC based assays are not sensitive to glucans, making them preferable in certain applications. Overcoming test interferences often involves sample preparation steps such as dilution, pH adjustment or the use of endotoxin-free materials, but these can introduce additional variability [45].

2.4.2 Sample Interference – Masking of Endotoxin

Sample interference occurs when an endotoxin spike cannot be recovered over time, even though valid Positive Product Controls (PPCs) indicate that the enzymatic reaction is functional and no direct test interference is present. This phenomenon is

known as LER and is characterised by a time-dependent reduction in detectable endotoxin levels, typically observed in undiluted samples containing specific formulation components (e.g. chelators or buffers).

In 2013, Chen and Vinther first reported LER during bacterial endotoxin testing (BET) of biopharmaceuticals. They observed that spiked endotoxins were insufficiently detectable after incubation in certain formulations. [46] LER primarily affects biopharmaceutical products where the active pharmaceutical ingredients (APIs) are large proteins, such as monoclonal antibodies. These products are often formulated with excipients such as phosphate and citrate buffers and polysorbates to improve stability.

Reich et al. [36] proposed a two-step mechanism for endotoxin masking. First (Figure 3, A), chelating agents disrupt the salt bridges between endotoxins and divalent cations such as magnesium, reducing the structural stability of endotoxin aggregates. Second (Figure 3, B), surfactants solubilize the loosened aggregates by forming mixed micelles with monomeric endotoxin, thereby altering its structure and reducing detectability. Ionic surfactants, which combine chelating and hydrophobic properties, can induce masking directly by breaking up endotoxin aggregates and forming undetectable complexes.

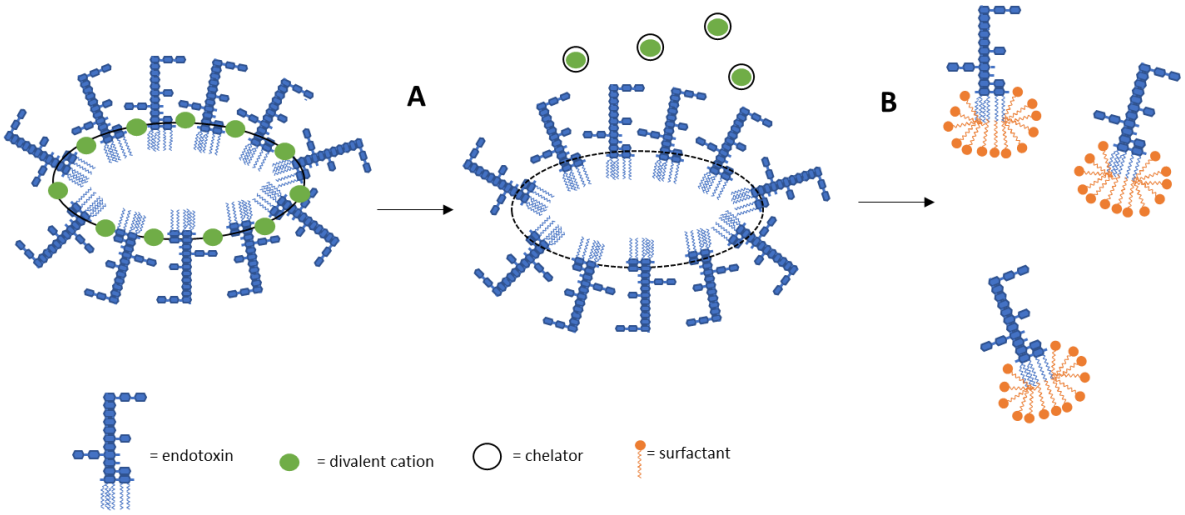

Figure 3: Two-step mechanism of endotoxin masking *Reich et al. proposed a potential mechanism of endotoxin masking, caused by a chelator and a surfactant. A) Chelators disturb pure endotoxin aggregates and reduce the rigidity of the aggregates. B) Surfactants interact with endotoxin and form mixed aggregates which masked the endotoxin for enzymatic detection. (adapted from [36])*

However, LER is not limited to formulations containing both chelators and surfactants. Williams highlighted that proteins in biopharmaceutical formulations can independently induce LER. Proteins can bind endotoxins via ionic or hydrophobic interactions, altering their aggregation state and rendering them undetectable. [1]

Blood is a particularly challenging matrix for endotoxin detection due to its complex composition of proteins, lipids and cells. These components can interact with endotoxins and either inhibit or enhance detection, depending on the specific interactions. There is currently no universally reliable method for detecting endotoxin in blood, which complicates clinical diagnosis and safety assessment. [47–49] This limitation is critical in scenarios such as sepsis diagnosis, where rapid and accurate detection of endotoxins could significantly improve patient outcomes.

The FDA has expressed concern that LER may lead to underestimation of endotoxin levels in drugs, resulting in a potential risk of pyrogenic reactions. To mitigate this risk, regulatory authorities recommend thorough validation of endotoxin detection methods for each specific product matrix. Understanding the mechanisms underlying LER and ensuring accurate endotoxin detection remains critical for safeguarding patient health and maintaining product quality in pharmaceutical and medical device manufacturing [40].

3. Aim of work

The primary objective of this thesis is to improve the reliability of endotoxin detection in complex biological matrices, in particular human plasma, by systematically investigating the factors that contribute to endotoxin masking. The study will follow a structured approach to analyse the mechanisms behind masking, identify key influencing factors and develop strategies to improve detection accuracy.

External factors affecting endotoxin detection, including storage conditions and potential contaminants in the samples, will first be investigated. This will be combined with an investigation of how (1→3)-β-D-glucan detection with the LAL-based assay is affected by storage conditions.

The focus of the research will then be on internal factors, with an emphasis on the structural diversity of endotoxins. Variations in LPS structure, including differences in O-antigen chains and core oligosaccharides will be evaluated under masking conditions to determine their effect on endotoxin detectability.

Finally, the study will investigate endotoxin masking in human plasma by assessing the interactions between endotoxins and plasma components, in particular proteins. Various strategies to overcome masking will be investigated, including salt-based treatments, pH adjustments and the use of polyanionic substances such as heparin. The effectiveness of these methods in restoring endotoxin detectability will be systematically evaluated.

Ultimately, the vision of the research is to improve endotoxin detection methods to increase the reliability of endotoxin testing in clinical diagnostics and pharmaceutical quality control. By overcoming the limitations of endotoxin masking, the study aims to contribute to more accurate safety assessments of blood products and injectable drugs, thereby reducing the risk of false-negative results and improving patient safety. This research provides a comprehensive framework by sequentially addressing external factors, internal structural diversity, and endotoxin masking, ensuring a systematic approach to enhancing endotoxin detection strategies.

4. Results

4.1 Publication I

Effects of Different Container Types on (1→3)-β-D-glucan Recovery

Burgmaier Luisa, Illes Bernhard, Leiss Michael, Avci-Adali Meltem, Reich Johannes

Molecules/ published 2023, October 4

Reliable detection of (1→3)-β-D-glucan is critical in pharmaceutical quality control, as this contaminant can trigger inflammatory responses and interfere with endotoxin measurements. To ensure accurate detection, it is essential to understand how storage conditions and container materials affect glucan recovery.

This study investigated the effect of different container types, borosilicate glass, polypropylene and polyethylene, on BDG recovery under different conditions, including room temperature (RT) and long-term freezing at -20 °C. During short-term storage (up to 3 hours) at RT, all containers showed glucan recoveries above 80%. However, after 24 to 48 hours, polyethylene containers showed significantly lower recoveries (57% and 46%), while borosilicate and polypropylene containers maintained consistent results.

In case of long-term storage at -20 °C, glucan recovery decreased with time for all types of containers. Borosilicate and polypropylene containers maintained higher recoveries (76% to 80%), whereas polyethylene containers had lower and more variable recoveries (68%). Freeze/thaw cycles in polyethylene containers, reduced recoveries to 66%, while the recovery rate in borosilicate and polypropylene containers remained stable at close to 100%.

The study also tested the effect of surface area to volume ratio using two borosilicate containers of different dimensions. After 28 days of storage at 4 °C, both showed similar recoveries of around 89%, indicating no significant effect of this factor in this study.

In conclusion, container material can play a critical role in BDG recovery. Borosilicate glass and polypropylene are the most reliable options, whereas polyethylene containers run the risk of underestimating contamination, particularly during long-term storage or freeze/thaw processes. These findings highlight the importance of selecting appropriate containers to ensure accurate glucan detection in pharmaceutical quality control.

4.2 Publication II

The impact of LPS mutants on endotoxin masking in different detection systems

Burgmaier Luisa, Pölt Stefan, Avci-Adali Meltem, Reich Johannes

Biologicals/ published 2024, November 24

Endotoxin masking poses a potential risk to patient safety by rendering endotoxins in pharmaceutical products undetectable. This study investigates how structural variations in LPS from *Salmonella minnesota* and *Escherichia coli* affect masking and how this can be detected using different assays: the LAL test, the rFC test, and the MAT. It builds on previous work on (1→3)-β-D-glucan detection, which showed that container materials and storage conditions influence recovery. These findings highlight how technical and biological factors affect reliable pyrogen detection in pharmaceutical products.

The susceptibility to masking strongly depends on polysaccharide chain length. Rough LPS mutants with shorter chains show slower masking than smooth LPS with longer chains. This is attributed to a lower hydrophilic/hydrophobic ratio in rough mutants, reducing masking, and their stronger negative charge, which stabilises supramolecular structures by increasing affinity for divalent cations.

Masking kinetics varied between bacterial strains. *E. coli* mutants masked faster than *S. minnesota*, likely due to the higher hydrophobicity of *S. minnesota* lipid A. In both strains, mutants with longer polysaccharide chains showed faster masking.

LAL and rFC assays showed similar masking behaviour and masked smooth LPS and rough mutants with longer chains (Ra, Rb) within 45 minutes. In contrast, MAT showed higher endotoxin recovery for rough mutants, suggesting it can detect masked endotoxins for longer. However, MAT showed lower activity for short-chain mutants due to their reduced potency in immune activation.

Short-term studies (up to 45 minutes) showed rapid masking for smooth LPS and longer-chain mutants, while rougher mutants masked only after longer incubation of up to 72 h. Masking was induced by a matrix containing sodium citrate and polysorbate 20. The study concludes that LPS structure, particularly polysaccharide length and charge, significantly influences masking behaviour.

4.3 Publication III

Beyond Electrostatic Interactions: The Key to Reliable Endotoxin Analysis in Human Plasma

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Accurate detection of endotoxin in human blood is critical for sepsis diagnosis and safe drug administration. However, masking by blood components complicates detection using the LAL assay. This study investigates masking mechanisms and shows that electrostatic interactions alone cannot fully explain the effects, with LPS structure and additional binding forces playing key roles. The findings complement previous studies showing that both technical factors such as container materials and biological properties like LPS structure influence pyrogen detection and can lead to underestimation of contamination.

Endotoxins interact with plasma proteins such as human serum albumin (HSA) and lysozyme. While HSA showed limited masking at physiological levels, lysozyme, even at low concentrations (~6 µg/ml), contributed significantly via electrostatic interactions. Denatured proteins masked less effectively, and their reduced hydrophobic interfaces suggest a role for hydrophobic interactions.

High salt solutions disrupted masking in lysozyme samples but were ineffective in HSA and plasma, indicating additional binding mechanisms. pH adjustment to 11 or 12 improved recoveries by reducing electrostatic interactions, but full recovery remained elusive. Smooth LPS (S-type) with complete O-antigen chains masked more rapidly due to hydrophilic interactions, whereas rough mutants retained higher recoveries.

Size exclusion chromatography (SEC) of plasma fractions showed that masking depended on specific protein interactions rather than total protein concentration. The results suggest masking results from a combination of electrostatic, hydrophilic, and structural interactions.

In conclusion, endotoxin masking in plasma involves complex mechanisms beyond electrostatics, including specific protein interactions and LPS structural diversity. Effective detection requires strategies addressing these diverse mechanisms to ensure reliable diagnostics and patient safety.

5. Discussion

Endotoxin detection in human blood plays a critical role in clinical diagnostics and contamination control of blood-derived products in pharmaceutical quality assurance. Although several studies have reported variable levels of endotoxin in humans, methodological limitations make these values difficult to interpret with certainty. One study found endotoxin levels in the blood of 116 healthy donors ranging from 0.01 to 0.5 EU/mL, with a mean of about 0.1 EU/mL [50]. Another published study documented similar results, with endotoxin levels generally below 1 EU/mL [51]. Some research has indicated that endotoxin levels in the blood of healthy individuals can be even lower, typically below 0.05 EU/mL [52].

Both the U.S. Food and Drug Administration (FDA) and the European Pharmacopoeia set a limit of 5 EU/kg body weight per hour for endotoxin exposure in injectable drugs and medical devices [40,53]. The threshold is a standardized constant derived from studies that assessed the endotoxin levels required to induce fever in rabbits [54,55]. For an average adult weighing 70 kg, this translates to a total endotoxin load of 350 EU, which, distributed across an approximate blood volume of 5 L, results in a concentration of about 0.07 EU/mL. This limit is established to protect individuals from even the smallest amounts of endotoxins, which could trigger adverse immune responses when introduced through injectable drugs or other parenteral products. In clinical settings, however, endotoxin levels in the blood can be significantly higher, often serving as a marker for the severity of the condition. These elevated endotoxin levels observed in sepsis patients, as highlighted by studies such as those by Opal *et al.,* ranging from 0.5 to several EU/mL, with severe cases reaching up to 8 EU/mL indicate a fundamentally different clinical scenario compared to the controlled conditions of pharmaceutical manufacturing. The high endotoxin levels observed in sepsis are linked to systemic inflammation and poor clinical outcomes, underscoring the critical role of LPS in the pathophysiology of sepsis [51]. Therefore, while the 0.07 EU/mL limit is crucial for safeguarding against external endotoxin exposure, the higher endotoxin levels observed in sepsis patients represent a separate and more complex issue in clinical practice.

This difficulty raises concerns about the reliability of current endotoxin assays, particularly in detecting the full spectrum of LPS concentrations in clinical settings. The variability in reported endotoxin levels across different studies suggests that

measurement techniques might not consistently capture true LPS levels, potentially leading to underestimation or misinterpretation, and impacting clinical decisions and patient management [56]. In addition to sepsis, LPS levels also show significant variability in other disease contexts. For example, in chronic liver diseases, research by Duan *et al.* has demonstrated that circulating LPS levels vary significantly among patients with different stages of liver fibrosis. This variability suggests differential responses to LPS-induced hepatic inflammation and fibrogenesis [57]. Similarly, studies investigating neuroinflammatory conditions have found varying levels of LPS in cerebrospinal fluid and brain tissues of patients with neurodegenerative diseases. Banks et al. emphasized the complexities involved in measuring LPS-induced neuroinflammation and the necessity for standardized protocols to accurately assess LPS levels across different biological matrices [58].

Given the critical role of endotoxin in sepsis and other inflammatory conditions, reliable detection methods are essential for timely diagnosis and effective treatment. Sepsis remains a leading cause of mortality, thus delays or inaccuracies in endotoxin measurement can significantly impact patient outcomes. Advancing detection methods and understanding endotoxin interactions will improve sepsis management and therapeutic interventions.

The studies presented here examine various aspects of the complex issue of endotoxin detection in human blood, addressing both external and internal influencing factors. External factors, defined as environmental or procedural conditions influencing the sample from the outside, include storage conditions and potential accompanying contaminants. Internal factors, referring to properties inherent to the sample itself, include the structural diversity of endotoxins and their masking mechanisms, as well as the limitations of current detection systems in complex biological matrices like human blood. By addressing these challenges, the combined results offer valuable insights into endotoxin behaviour, the reliability of detection methods, and the influence of interfering factors.

5.1 External factors influencing endotoxin detection

The chromogenic variant of the LAL assay employed for BDG detection relies on the coagulation cascade of horseshoe crab amoebocytes. While native lysates contain both Factor C and Factor G, activated by endotoxin and BDG respectively, specificity for BDG is achieved by eliminating Factor C. As a result, the assay becomes

exclusively responsive to BDG [59]. Despite this specificity, both endotoxin and BDG assays remain highly susceptible to external influences, particularly container materials and handling conditions, which can significantly impact analytical accuracy.

Our study demonstrated that polyethylene containers resulted in significantly lower BDG recoveries during long-term storage and freeze/thaw cycles, whereas borosilicate glass and polypropylene containers provided more stable recoveries. These findings suggest that adsorption or degradation over time may reduce BDG detectability, potentially leading to false-negative results.

The differences in adsorption behaviour among the tested materials can be attributed to their distinct surface properties. Polyethylene, composed of nonpolar -CH_2 groups, presents a highly hydrophobic surface that favours van-der-Waals forces and hydrophobic interactions with the large and flexible but overall hydrophilic BDG molecules [60]. These interactions often lead to irreversible or strongly retained binding, thereby reducing the analyte's availability for detection. In contrast, borosilicate glass offers a hydrophilic, negatively charged surface with polar silanol groups capable of reversible hydrogen bonding with BDG [61]. This interaction supports better dispersion and availability of BDG in solution. Polypropylene, although nonpolar, exhibits higher crystallinity and reduced surface flexibility, resulting in fewer interactions with BDG compared to polyethylene, which explains its good performance [62].

These observations align well with previous research emphasizing the impact of surface chemistry on biomolecule adsorption. The comparable BDG recoveries in our study observed in different sized borosilicate containers suggest that surface chemistry plays a more significant role than surface area in this context, providing practical guidance that container volume within typical ranges may not substantially affect glucan detection.

Similar material-dependent effects are well-documented in endotoxin analysis. Novitsky *et al*. demonstrated that different container surfaces influence endotoxin recovery, primarily due to their adsorptive properties [63]. While borosilicate glass and polystyrene containers exhibited high endotoxin recoveries, polypropylene containers showed significantly lower recoveries, confirming the adsorptive nature of certain surfaces. The amphipathic nature of LPS leads to complex interactions with container surfaces. Hydrophilic, negatively charged materials like borosilicate glass promote

stable dispersion of LPS aggregates, whereas hydrophobic surfaces such as polyethylene and polypropylene facilitate aggregation and physical losses [64]. Comparable mechanisms govern endotoxin behaviour, although the amphipathic nature of lipopolysaccharides introduces additional complexity. LPS molecules consist of both hydrophilic polysaccharide chains and a hydrophobic lipid A moiety, and the ratio between these regions can vary significantly among bacterial species or LPS mutants [65]. This structural variability directly affects how LPS interacts with container surfaces and other sample components.

Moreover, sample matrix composition, especially protein content and charge, substantially influences endotoxin adsorption. Yokota *et al.* investigated the impact of cationic proteins on endotoxin adsorption to glass surfaces [66]. While glass generally minimizes endotoxin loss in water-based solutions, the presence of cationic proteins led to significantly lower endotoxin recoveries. This suggests that charge-mediated adsorption plays a major role in endotoxin loss in glass containers. In contrast, polystyrene tubes exhibited better recoveries in the presence of cationic protein but performed worse when non-cationic proteins were present. Cationic proteins can mediate adsorption by bridging negatively charged glass surfaces, while polystyrene's interaction varies depending on protein characteristics [66].

These findings highlight the importance of matrix effects in endotoxin analysis, which may also be relevant for BDG quantification. Our study focused on BDG recovery in water samples, and therefore does not account for the potential influence of more complex matrices such as those containing proteins or other charged components. The parallels to endotoxin behaviour suggest that matrix-dependent effects could significantly impact BDG detectability as well. Given the growing scientific interest in glucans due to their immunomodulatory properties, future research should investigate the influence of realistic pharmaceutical formulations on BDG detection [67]. While endotoxins are strictly regulated because of their significant impact on patient safety, BDG are not currently subject to regulatory control, although their biological activity may justify closer examination [68].

Our findings, along with results from the other studies, underscore the necessity to experimentally validate the choice of storage materials in the context of specific sample matrices to avoid assay artifacts and ensure accurate quantification. The observed differences in recovery depending on protein content and container surface emphasize

the importance of container selection, particularly when analysing protein-rich samples, as such interactions can significantly influence assay performance for both endotoxins and BDG. This highlights the need for follow-up studies using more complex and pharmaceutically relevant matrices to fully understand and control potential interferences.

Matrix effects are not limited to storage conditions but also extend to the pre-analytical phase, particularly in blood-based samples. The choice of anticoagulant is a critical factor when analysing endotoxins in blood. Heparinized blood samples often yield more reliable endotoxin results compared to EDTA- or citrate-treated samples. This difference underscores the importance of optimizing blood collection methods to minimize interferences in LAL assays, for example by standardizing protocols, preferring heparin, and ensuring prompt, pyrogen-free handling. [69] In our studies, we consistently used heparin as the anticoagulant to minimize such interferences and ensure comparability of results across experiments.

Moreover, the blood sample itself can also cause interference. Several serine proteases, including trypsin, factor X, and α-thrombin, can hydrolyse the chromogenic substrates used in the LAL assay, leading to false-positive results. Conversely, serine protease inhibitors, such as trypsin inhibitor and antithrombin III, may cause false-negative results by inhibiting LAL clotting enzymes[70]. While these interferences are typically addressed by heat treatment (75 °C, 15 min) and sample dilution, our findings show that these measures are not consistently effective. Heat treatment alone led to highly variable results, with residual interference still observed in several plasma samples (data not shown). We found that a minimum dilution of 1:500 was required to reliably suppress plasma-associated interferences in the chromogenic LAL assay. However, this level of dilution significantly compromises assay sensitivity, making the detection of low endotoxin levels challenging or impossible.

While the LAL assay is FDA-approved for BDG detection in human serum, its clinical application faces challenges due to limited sensitivity and specificity. The sensitivity of BDG assays typically averages in the mid-70% range, meaning that negative results do not reliably exclude invasive fungal infections. Furthermore, specificity is compromised by false-positive results caused by albumin, intravenous immunoglobulin, and other components. [59] These limitations complicate clinical decision-making and suggest the presence of additional, unidentified factors

influencing BDG detection. Despite these challenges, BDG assays remain valuable for early fungal infection detection, offering a faster alternative to traditional blood cultures, which require 2-3 days of incubation. Optimizing assay reliability through improved sample handling and a better understanding of external influences is crucial for enhancing diagnostic accuracy.

These findings highlight the critical role of external factors, particularly storage container material and sample handling, in the detection of both endotoxins and BDG using the LAL assay. While endotoxin detection is further complicated by masking effects and structural variability, BDG assays primarily require stringent control of external variables. Addressing these factors through the use of pyrogen-free, low-binding containers, standardized sample preparation protocols (e.g., use of heparinized blood and rapid processing), and targeted assay optimization (such as heat treatment and validated dilution steps) is essential for ensuring reliable endotoxin and BDG detection, ultimately improving pharmaceutical safety and clinical diagnostics.

5.2 Internal factors influencing endotoxin detection

While external factors affecting endotoxin detection and LER, such as container effects and matrix components like buffers, APIs and surfactants, have been extensively studied, internal factors such as the structural diversity of LPS remained unexplored. Evaluations using defined endotoxins are often recommended to assess assay performance. However, naturally occurring contamination is more complex due to the enormous structural diversity of LPS resulting from different bacterial growth conditions and mutations. Different LPS variants exhibit different masking susceptibilities, often for reasons that are not fully understood. Although some research has focused on the effects of naturally occurring endotoxin (NOE), these studies primarily examine whether purified LPS variants, which do not occur naturally in their isolated form, introduce artefacts. However, they did not investigate the structural influence of LPS on masking, but rather focused on the effects of purified endotoxins. A better understanding of the link with the structure of LPS is therefore essential to support the development of more reliable detection methods for endotoxins [8,71–73].

In this thesis the effect of LPS mutants on the LER phenomenon and its detection in different test systems was evaluated. The aim was to investigate how structural variations in LPS affect masking processes and ultimately endotoxin detectability.

Despite its importance, this phenomenon is still not fully understood. Therefore, this study analyzed the role of LPS mutants in LER within pharmaceutical formulation matrices and human plasma, assessing their detectability using the most commonly employed endotoxin assays.

To begin with, the endotoxin activities per mol of each mutant were determined, revealing a wide range of assay responses across the different LPS structures. This variability is partly explained by the strong tendency of LPS molecules to form aggregates, which complicates the accurate determination of their molecular mass and, consequently, the calculation of molar activity [8]. As molarities were estimated in this study, slight variations in activity per mol may occur, particularly in *E. coli* where different core types with different polysaccharides complicate accurate measurements. For *S. minnesota* mutants, activity decreased progressively from smooth LPS to mutants with shorter sugar chains, except for the Ra mutant. A similar trend was observed for *E. coli* mutants, where activity decreased from mutant Rc to lipid A.

Interestingly, for both strains, lipid A exhibited the lowest activity, which was unexpected as lipid A is generally considered to be the most toxic component of LPS. Lipid A is a hydrophobic anchor embedded in the bacterial membrane and is recognized by the host immune system as a pathogen-associated molecular pattern (PAMP) [20,74]. Lipid A is the most conserved component of LPS but still exhibits significant structural variability. This variability is primarily due to differences in the number, length, and saturation of acyl chains, as well as the degree of phosphorylation. For example, *E. coli* lipid A typically contains six acyl chains, while lipid A *from Pseudomonas aeruginosa* and *Helicobacter pylori* contains fewer acyl chains, which affects their endotoxic activity and recognition by the immune system [75,76]. The reduced activity could be attributed to the shorter sugar chains, which may hinder the mutants' ability to form the supramolecular structures required for detection by Factor C. This finding is consistent with previous observations that the size and state of LPS aggregate correlate with activity in LAL assays [74,77]. Mueller *et al.* also reported that LPS monomers showed no biological activity in either MAT or LAL assays [78]. Furthermore, while endotoxin aggregation is necessary for stimulation, other studies have shown that the structure of the aggregates also plays a critical role [79].

In addition, changes in solubility caused by the shortened sugar chains and increased hydrophobicity may affect the conformations of LPS, thereby influencing its

detectability. Nevertheless, further investigation of different lipid A structures is warranted as these variations have a significant impact on endotoxin activity. As highlighted by Dardelle *et al.*, differences in lipid A composition, such as the number and length of fatty acid chains, the presence and position of phosphate groups and potential modifications, lead to different LPS behaviours [80].

While LAL and rFC can only detect endotoxic activity, the MAT can also indicate biological activity. The MAT is based on the activation of immune cells, e.g. by LPS. The immune cells then express and release cytokines and chemokines [81]. It is noteworthy that the MAT in our study tends to detect lower levels of endotoxin activity, particularly in mutants with shorter sugar chains. It is known that mutants consisting of shorter sugar chains and lacking O-antigen are less potent than those with O-antigen [82]. The O-antigen is the most variable part of LPS, with differences in the composition and arrangement of sugar residues contributing to a wide range of serotypes. This diversity is a key factor in the ability of bacteria to evade the host immune system and is a major determinant of the antigenic specificity of LPS. O-antigen variation allows bacteria to adapt to different environments and hosts, making it a critical factor in pathogenicity and immune evasion [83]. While lipid A is crucial for TLR4 activation, the core oligosaccharide and O-antigen can modulate the immune response by influencing the accessibility and presentation of lipid A. Variations in the core region can affect the interaction of LPS with LPS-binding proteins and receptors, thereby modulating the downstream signalling pathways [12]. The O-antigen play a crucial role in adhesion processes and shield bacteria from hostile environments, such as host-derived antibacterial agents, thereby enhancing virulence. Bacteria that lack O-antigen or possess negatively charged cores are typically non-virulent and unable to survive within animal or plant hosts [83–85]. This could explain the lower endotoxin levels for mutants with missing O-antigen and shortened core saccharide when measured with MAT.

5.3 Impact of LPS structural variability on masking kinetics in pharmaceutical buffers

The primary objective of this study was to investigate the susceptibility of different LPS mutants to LER. In *S. minnesota*, a clear correlation between polysaccharide chain length and masking kinetics was observed: smooth LPS exhibited the fastest masking, followed by Ra and Rb. Although Ra and Rb retained recoveries above 50% after 45

minutes (LAL and MAT), a trend toward lower recoveries was evident. Mutants with truncated chains (Rc, Rd1, Rd2, Re, lipid A) showed no masking in short-term experiments but demonstrated progressive masking during longer incubation (5 days). Consistently, the Rc mutant, which has the fourth highest polysaccharide content, exhibited the fourth fastest masking in long-term kinetics in both measurement methods. These results suggest that while shortened LPS structures delay masking, they are not resistant to it. The masking rate strongly correlates with the saccharide content, which determines the hydrophilic/hydrophobic balance of the amphiphilic LPS molecule. As lipid A remains constant across mutants, rougher variants display greater hydrophobicity [3]. Consequently, rougher mutants exhibit greater hydrophobicity. Changing this ratio in amphiphilic molecules alters the physicochemical properties of the resulting aggregates. This shift alters aggregate structure, as seen in the micelle sizes, 24 nm for smooth LPS and 91 nm for the Re mutant [65].

In addition to the reduced hydrophilic/hydrophobic ratio, the shortening of the polysaccharide chain indirectly increased the charge density in the LPS mutants. This is because the negatively charged groups in LPS are predominantly located in the inner core region [86]. In mutants lacking an outer core, such as the Rd1 mutant, some negative charges remain through lipid A, but the absence of the typically neutral outer core polysaccharides prevents the distribution of these charges over a wider structure.

Reich *et al.* identified the removal of divalent cations as the rate-limiting step in the masking process [36]. Only after chelation of the divalent cations, detergents can be incorporated into the supramolecular structure and disrupt it. Unpublished work by us showed a clear relationship between the rate of masking and the amount of chelator for LPS mutants. A titration of the chelator in the masking matrix was performed. After 7 days we were able to show that smooth LPS was masked by the lowest chelator concentration (0.0625 mM), whereas the short mutants were only masked by the highest chelator concentration (10 mM) and lipid A was not masked at all at the chelator concentration tested. This indicates that negative charges contribute to the stabilization of the aggregates. The importance of negative charges is further highlighted by Rice *et al.* study in 2018, which examined the role of Ca^{2+} bridges between the HEP5 and HEP3 phosphate groups in the Rd1 mutant of *S. Minnesota* [87]. In contrast, the Rd2 mutant lacks the HEP5 sugar, preventing similar salt bridge formation. This difference is likely to explain the significant reduction in recovery observed in the Rd2 mutant after

24 h in the rFC assay and after 48 h in the MAT, while the recovery of the Rd1 mutant remains stable. Sali *et al.* [65] showed that rough mutants typically have higher negative charge densities, which enhance electrostatic interactions by increasing the affinity for divalent cations.

While in *S. minnesota* mutants the masking rate increased consistently with the hydrophobic/hydrophilic ratio, from rough to smooth LPS, this trend was not observed in the *E. coli* mutants. Despite its higher hydrophilic character, smooth *E. coli* LPS did not exhibit the fastest masking. This indicates that, unlike in *S. minnesota*, the hydrophobic/hydrophilic ratio alone cannot explain the masking behavior of *E. coli* LPS variants. Unlike *S. minnesota*, which has only one core polysaccharide type, *E. coli* has five different core polysaccharide types [74]. This diversity of core types is likely to contribute to the remarkably low endotoxin activity of the Ra mutant. In contrast to other mutants, the Ra mutant shows 10 to 100 times lower reactivity in the endotoxin assay. Classified as core type R2, the Ra mutant contains a neutral galactose (Gal) residue in its inner core [88]. Previous studies have shown that variations in charged moieties, such as inner core sugars and their functional groups, can significantly affect reactivity; for example, removal of a negative charge has been reported to result in a 100-fold reduction in activation of the LAL cascade [7].

Smooth LPS corresponds to the *E. coli* core type R1, which is characterised by the presence of glucosamine in the inner core region [89]. This saccharide contains a positively charged amino group that facilitates interactions between LPS molecules even in the absence of divalent cations. Studies have shown that the incorporation of aminoarabinose, another sugar with an amino group can increase the number of inter-lipid hydrogen bonds between LPS molecules [87]. This increased stabilisation reduces the reliance on salt bridges formed by divalent cations. Although the presence of glucosamine in smooth *E. coli* LPS reduces the overall negative charge density and affinity for Ca^{2+}, it may contribute to a slower masking process due to increased aggregate stability [3].

Despite species-specific differences, both *S. minnesota* and *E. coli* mutants showed increased masking susceptibility with longer saccharide chains. However, this trend was more pronounced in *S. minnesota*. Interestingly, *E. coli* mutants tended to mask faster overall in our study, suggesting additional structural factors such as lipid A composition contribute to the observed differences.

Lipid A from *E. coli* is predominantly hexa-acylated (~ 90%), with a smaller proportion (~ 10%) being penta-acylated [90]. Conversely, in *S. minnesota*, lipid A is hepta-acylated in 20% to 70% of cases, depending on growth conditions [91]. For mutants with identical saccharide chain lengths, this results in greater hydrophobicity in *S. minnesota* mutants compared to *E. coli* mutants, leading to a slower masking process. In addition, under certain growth conditions, *S. minnesota* LPS can incorporate aminoarabinose sugars, further stabilising aggregates through hydrogen bonding as previously described [92]. This finding is consistent with the observations of Reich *et al.*, who tested several NOEs for their masking behaviour. Among these, the NOE from *Burkholderia cepacia* showed prolonged resistance to masking compared to other endotoxins. [73] In particular, LPS from the *Burkholderia* genus often contains multiple aminoarabinose substitutions on both lipid A and the core region, contributing to increased aggregate stability [93]. These findings highlight how species-specific features like acylation degree and core composition influence aggregate stability and baseline masking susceptibility.

However, masking in pharmaceutical formulations is not solely driven by intrinsic LPS properties. It also depends on formulation components, particularly chelators and surfactants. The interplay of formulation components, in particular complexing agents (chelators) and non-ionic surfactants, has been identified as a key factor influencing endotoxin recovery [36]. Studies indicate that neither chelators nor surfactants alone are sufficient to cause masking; rather, their simultaneous presence is necessary to significantly reduce detectability of LPS [36,94,95]. Chelators such as EDTA, citrate and phosphate disrupt the electrostatic interactions within endotoxin aggregates by competing with divalent cations (e.g. Ca^{2+}, Mg^{2+}), thereby reducing the rigidity of the aggregates [96]. The degree of masking is directly proportional to the chelating strength of the buffer component, with EDTA having the strongest effect. In addition, the pH of the buffer system modulates chelation efficiency, which further influences endotoxin recovery. A lower pH reduces the availability of cations for complex formation, thereby altering the extent of masking [77].

Non-ionic surfactants, such as polysorbates, disrupt the hydrophobic interactions that stabilise endotoxin aggregates. These surfactants intercalate between LPS molecules, modifying their supramolecular structure. As a result, endotoxin aggregates become mixed micelles with surfactants, altering their affinity for the Factor C activation

mechanism in Limulus-based detection systems. The presence of both chelators and surfactants shifts the equilibrium towards less detectable LPS structures, resulting in reduced endotoxin recovery [77,96].

The underlying mechanism of masking appears to follow a two-step process: (I) chelators disrupt salt bridges within LPS aggregates, reducing rigidity; (II) surfactants subsequently integrate into the disrupted structures, forming mixed micelles that further reduce detectability [36]. Given the time-dependent nature of this phenomenon, the duration of endotoxin presence in a pharmaceutical sample may influence the degree of masking. A novel one-step mechanism for zwitterionic surfactant-induced endotoxin masking is proposed by Ørving *et al*. [97]. The principle is similar to a previously proposed two-step mechanism for endotoxin masking, but in the case of zwitterionic surfactants, these two steps are combined: disruption of the salt bridges and hydrophobic interactions with LPS in one molecule. These molecular interactions occur rapidly when both endotoxin and the zwitterionic surfactant are present in the same matrix. The amphiphilic nature of the zwitterionic surfactant allows it to disrupt both electrostatic and hydrophobic interactions simultaneously, resulting in an immediate and stable masking effect [97].

These results demonstrate that masking is the product of both intrinsic LPS architecture and extrinsic matrix effects. A full understanding requires consideration of molecular structure, environmental context, and the dynamic behaviour of LPS aggregates in solution.

5.4 Impact of LPS structural variability on masking kinetics in human plasma

The study demonstrates that the structural diversity of lipopolysaccharides (LPS), ranging from smooth (S-type) LPS with complete O-antigen chains to rough (Ra, Rc) and deep-rough (Re) mutants, significantly influences masking dynamics in human plasma. We observed that the masking patterns of these LPS chemotypes in plasma closely resemble those seen in pharmaceutical matrices containing chelators and surfactants. In such matrices, masking is typically attributed to LPS disaggregation by agents such as EDTA or polysorbates, which reduces LAL reactivity. However, the precise mechanism in plasma remains elusive and likely involves additional endogenous factors such as proteins or lipoproteins. Despite this complexity, the parallels suggest that similar interactions occur in both systems, modulated by blood-

specific components. While demasking strategies can often reverse masking in pharmaceutical products, reversibility in plasma is uncertain, raising both methodological concerns and physiological implications.

Among the tested LPS types, S-type LPS displayed the fastest and most pronounced masking. This may be attributed to its full-length O-antigen chain, which facilitates hydrophilic interactions and hydrogen bonding with plasma proteins, accelerating and amplifying the masking effect. In contrast, rough and deep-rough mutants, as well as lipid A, exhibited slower masking kinetics and higher endotoxin recovery rates. The truncated polysaccharide chains of these variants provide a smaller hydrophilic surface area, reducing their ability to form extensive interactions and lowering their masking potential [3].

Additionally, the molecular conformation of LPS appears to contribute to masking efficiency. The extended O-antigen in S-type LPS supports the formation of supramolecular structures that may enhance accessibility to masking components. In contrast, rough mutants, lacking complete polysaccharide chains, adopt different conformations that may hinder such interactions. Although this structural difference helps explain the slower masking of rough LPS, it does not entirely account for the significantly higher recovery rates observed. The limited hydrophilic surface area of rough LPS likely restricts further interaction with masking agents, thereby improving residual detectability [98,99].

The supramolecular organisation of LPS is critical for both its detection in LAL assays and its biological activity. LPS molecules naturally form aggregates through hydrophobic interactions between the lipid A acyl chains, which are further stabilised by divalent cations such as Mg^{2+} and Ca^{2+} [94]. These aggregates are essential for the activation of Factor C in the LAL assay, which specifically detects LPS in its aggregated state [77]. Dissociation of LPS into smaller units or monomers significantly reduces its ability to trigger Factor C, thereby reducing the sensitivity of the assay.

In biological contexts, LPS aggregation is also associated with toxicity, as disaggregated LPS is less pyrogenic. Detergents or plasma proteins can induce dissociation, leading to reduced toxicity, although removal of these agents can restore both aggregation and biological activity. This suggests that a minimum aggregate size is required for full biological potency [100–102]. However, it remains unclear whether LPS that evades detection in LAL assays, retains its biological activity. Studies suggest

that masked LPS in LER solutions (i.e. chelating agent and surfactant) can still stimulate the release of pro-inflammatory cytokines, and thereby induce an immune response [103]. Previous studies have shown that monomeric Re LPS from E. coli is more active than aggregates in the LAL assay. In contrast, Mueller *et al.* reported that E. coli Re LPS and Lipid A are exclusively active in their multimeric form, both in the LAL assay and in stimulating cytokine production from human mononuclear cells [78,104].

Given the strong correlation between LPS aggregation and its biological activity, it is important to determine whether the same LER effects that inhibit LPS binding to Factor C also prevent recognition by the immune system through the TLR4-MD-2 complex. Tan *et al.* concluded that the LPS binding domain of Factor C interacts cooperatively with multiple lipid A molecules, suggesting that more than one LPS molecule is required for Factor C binding and that monomeric LPS alone may be insufficient to activate Factor C [105]. In contrast, monomeric LPS must be presented to the TLR4-MD-2 complex to initiate the signalling cascade. The accessory proteins lipopolysaccharide binding protein (LBP) and CD14 enhance LPS recognition by extracting LPS from aggregates and converting it into monomers [106]. However, it has been suggested that LBP binds efficiently to LPS aggregates, which may explain the link between aggregates and biological activity. This is likely due to LBP´s varying affinities for different aggregate forms and its inability to bind monomeric LPS. Consequently, LBP cannot deliver monomeric LPS to CD14, which is necessary for its presentation to the TLR4-MD-2 complex [107].

Understanding how LPS aggregation influences Factor C activation and TLR4 recognition is crucial, as the innate immune system relies on pattern recognition receptors (PRRs) such as TLR4 to detect microbial components like LPS. Structural differences in LPS can lead to differential activation of these receptors, resulting in variable cytokine profiles. For example, the acylation pattern of lipid A determines the strength of TLR4-mediated signalling and the subsequent production of cytokines like TNF-α, IL-6, and IL-1β [107].

Beyond innate immunity, LPS also shapes adaptive immune responses by modulating antigen-presenting cells (APCs) and T-cell activation. When LPS interacts with APCs such as dendritic cells (DCs), it triggers their maturation and enhances their ability to present antigens to T-cells, thus initiating the adaptive immune response. The

structural diversity in LPS plays a crucial role in shaping the type and magnitude of this immune response. Different LPS structures can influence the maturation process and the pattern of cytokine production by DCs, which subsequently affects the differentiation of T-helper cells (Th cells) into various subtypes, including Th1, Th2, or regulatory T-cells (Tregs) [108]. For example, LPS from *E. coli* is known to be a strong inducer of DC maturation, leading to a robust production of pro-inflammatory cytokines that promote a Th1-biased immune response. This type of response is characterized by the production of interferon-gamma (IFN-γ), which is crucial for combating intracellular pathogens. In contrast, LPS from other bacterial species may induce different cytokine profiles in DCs, potentially favouring Th2 responses, which are associated with antibody production and the defence against extracellular pathogens, or promoting the development of Tregs, which play a role in immune tolerance and preventing autoimmunity [13]. The structural elements of LPS, including variations in lipid A, the core oligosaccharide and the O-antigen, determine its interaction with TLRs on DCs, shaping not only the immediate innate immune response but also the long-term adaptive immune response.

Integrating knowledge of LER effects on both Factor C and TLR4-MD-2 interactions with insights into in vivo aggregation and immune activation could clarify the biological relevance of masked LPS. Notably, early and accurate detection of endotoxin in blood could be a valuable biomarker for sepsis, enabling timely intervention. However, the application of LAL-based assays in sepsis diagnostics remains limited [70]. Numerous plasma components, including proteins, lipoproteins, bile salts and amphiphilic peptides, interfere with LPS detection by altering its structural and chemical properties [48]. These interactions can inactivate LPS or prevent Factor C activation, contributing to false-negative results [109–112].

Furthermore, the immune response in sepsis is highly dynamic, characterised by alternating periods of hyperactivation and immunosuppression. These fluctuations can result in varying levels of detectable endotoxin and different thresholds of immune activation throughout the course of the disease. The body's endotoxin clearance mechanisms, such as hepatic clearance and immune neutralisation, further complicate detection by significantly reducing circulating endotoxin levels [113,114].

This thesis investigates why previous methods have struggled to produce consistent results and aims to identify the factors and binding mechanisms that interfere with endotoxin detection in human blood.

The SEC experiment identified 20 fractions without masking and 47 with strong masking effects. Masking correlated with later-eluting proteins, suggesting an association with specific protein properties rather than a random effect. While protein concentration influenced endotoxin recovery, similar concentrations in masking and non-masking fractions gave different results, suggesting a protein-specific effect. Some proteins, such as HSA, were present in almost all fractions without a direct correlation with masking and require further investigation.

To study the effects of individual plasma proteins and to verify previous findings on the role of electrostatic interactions, we selected model plasma proteins with specific properties, i.e. different net charges. Antimicrobial peptides (AMPs) neutralise LPS through a combination of electrostatic and hydrophobic interactions. Positively charged residues bind to the negatively charged phosphate groups of LPS, while hydrophobic residues incorporate into the lipophilic core of LPS. This dual mechanism disrupts the bacterial outer membrane and neutralises endotoxins [115–117]. Lysozyme stands out as a key antimicrobial enzyme and an essential component of innate immunity. While it primarily targets Gram-positive bacteria by hydrolysing peptidoglycan in their cell walls, conventional lysozymes also exhibit peptidoglycan-independent antimicrobial activity due to their strong cationic properties [118]. The results showed that lysozyme effectively masks endotoxins even at low concentrations (~ 6 µg/ml). However, the masking could be reversed by adding high salt solution to the lysozyme sample, suggesting a sole electrostatic mechanism behind LPS and endotoxin interaction. This is also in line with earlier findings by Brandenburg *et al.* [119].They were also able to show that this interaction alters the structure of LPS aggregates, shifting them from cubic (highly endotoxic) to lamellar (less endotoxic) forms.

In contrast, HSA, the most abundant plasma protein with concentrations of 35 to 50 mg/ml, showed limited masking capacity under physiological conditions and resulted in endotoxin recovery rates of over 50%. Albumin is known to bind and solubilise LPS, including its lipid A component, and can partially mask endotoxin activity. However, this interaction primarily involves non-electrostatic forces and induces only minor changes in endotoxin structure and charge, reflecting its limited masking potential.

[120,121] Although albumin contributes to endotoxin binding, it cannot explain the extent of masking observed in human plasma and should not be considered as primary masking agent, demonstrating that masking is influenced by the specific binding characteristics of individual proteins rather than their overall abundance.

The interaction between endotoxins and individual blood proteins has been the subject of numerous recent studies [122–125], focusing primarily on binding mechanisms and their impact on the immune system. However, these studies rarely address the impact of such interactions on endotoxin detection using standard LAL assays. While it is well established that plasma proteins can bind to endotoxins, there is no definitive evidence that these interactions directly cause masking effects. It remains possible that despite binding, endotoxins can still be detected under certain conditions. Therefore, further research is needed to clarify whether specific protein-endotoxin interactions contribute to masking and how they affect assay performance.

Although we showed that electrostatic interactions between the negatively charged phosphate groups of LPS and the positively charged amino acids, such as lysine and arginine, of plasma proteins possibly play an important role in endotoxin masking, the results also suggest that electrostatic forces alone do not fully explain the phenomenon [69]. This conclusion is reinforced by the partial effectiveness of strategies targeting electrostatic interactions alone.

High concentrations of salts, such as NaCl, disrupt electrostatic interactions by neutralising molecular charges, a common approach in protein purification to minimise non-specific binding [126]. The Na^+ and Cl^- ions shield opposing charges by surrounding them, thereby weakening or eliminating the attractive forces between molecules bound primarily by electrostatic interactions [127]. If endotoxin-protein interactions remain intact, as shown by masking, despite high salt concentrations, this suggests that other types of forces are involved in maintaining the interaction. These could include hydrophobic interactions, which are not affected by ionic strength, or more stable covalent bonds. Adjusting the pH of plasma samples, which reduces the positive net charge on plasma proteins, resulted in improved endotoxin recovery, with average recovery rates exceeding 50% when endotoxin was added after adjustment. However, when pH modification was applied to samples where masking had already occurred, recovery remained incomplete, suggesting that masking in these cases involves mechanisms beyond simple electrostatic binding. This highlights the need for

further research into the nature of the underlying interactions. Recent work by Harm *et al.* [128] explored the mitigation of electrostatic interactions by the addition of the polyanionic substance heparin alongside divalent cations. However, their use of a heparin injection solution with additional components, like sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, may have influenced the results. In our experience, heparin alone, even in combination with divalent cations, has shown limited success in completely resolving endotoxin masking.

6. Conclusion and outlook

The detection of endotoxins in complex biological matrices such as human plasma remains a significant challenge due to various interfering factors that can lead to false-negative results. This study systematically addressed these challenges by investigating external effects, the impact of endotoxin structural diversity and endotoxin masking mechanisms, to contribute to the development of more reliable detection strategies.

First, external factors were investigated, which revealed that sample storage conditions and container material significantly influence the detection of endotoxin and BDG. These findings highlight the importance of optimised handling procedures to prevent loss of detectability due to adsorption or degradation of the BDG.

In the second study, the influence of the structural diversity of LPS on detectability was analysed. Different LPS chemotypes were shown to have different susceptibilities to masking, with smooth LPS showing the fastest masking kinetics due to its increased hydrophilic interactions, whereas rough mutants and lipid A were more resistant. These results highlight the need to consider endotoxin structure when assessing detection reliability.

Finally, endotoxin masking in human plasma was systematically investigated and key interactions between endotoxins and plasma proteins were identified as the primary contributors to masking. While electrostatic interactions play an important role, additional hydrophobic and structural factors were found to influence detectability. Various approaches, including pH modifications, salt-based treatments and polyanionic substances, have been tested to restore endotoxin recovery. However, no universal demasking strategy could be identified, highlighting the need for tailored solutions depending on the specific sample matrix.

While this study provides important insights into endotoxin masking and its impact on detection reliability, several aspects remain to be explored in future research.

First, the investigation of endotoxin-protein interactions was limited to two model plasma proteins. Extending this research to a wider range of plasma proteins is essential to better understand the complexity of endotoxin masking in biological

matrices. Future studies should not only focus on the binding interactions, but also simultaneously evaluate the impact on endotoxin detectability to ensure a comprehensive assessment of masking effects.

In addition, further research is needed to investigate how structural modifications of endotoxins occur in masking matrices such as blood and how these changes affect detectability. Combining structural analysis of endotoxins in a masked state with detection assays could provide valuable insights into the mechanisms that contribute to loss of detectability in biological samples.

This study did not provide a definitive solution for reversing endotoxin masking in blood. However, the findings contribute to future work in this area and emphasise that efforts should not focus solely on electrostatic interactions. Given the complexity of masking mechanisms, future research should explore additional molecular interactions that may play a role in endotoxin sequestration and develop targeted strategies to counteract them.

Another important aspect that remains unclear is the relationship between endotoxin masking and the immune response. It is not yet known whether masked LPS can induce an immune response, which would have important implications for clinical diagnosis and therapeutic intervention. Methods such as the MAT can provide initial insights, but remain limited due to their in vitro nature. Understanding whether masking represents a physiological immune mechanism could reshape our approach to endotoxin detection and risk assessment.

Finally, it is important to investigate how structural differences in LPS affect not only detectability but also recognition by the immune system. Variations in LPS structure could lead to differences in pyrogenicity, which could influence the severity of immune responses. A deeper understanding of these relationships could help to refine detection assays and improve safety assessments for pharmaceutical and clinical applications.

In conclusion, while this study has provided a strong foundation for understanding endotoxin masking in human plasma, further research is needed to explore protein interactions, structural modifications of masked endotoxins, immune recognition and potential demasking strategies. Addressing these issues will be critical to advancing

endotoxin detection methods and ensuring more reliable safety assessments in pharmaceutical and clinical settings.

7. References

1. K. L. WILLIAMS, *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems*, Springer International Publishing AG, Cham (2019).
2. N. OHNO and D. C. MORRISON. “Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity,” *European journal of biochemistry*, **186,** *3*, 629 (1989).
3. A. GORMAN and A. P. GOLOVANOV. “Lipopolysaccharide Structure and the Phenomenon of Low Endotoxin Recovery,” *European journal of pharmaceutics and biopharmaceutics official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V*, **180**, 289 (2022).
4. B. W. SIMPSON and M. S. TRENT. “Pushing the envelope: LPS modifications and their consequences,” *Nat Rev Microbiol*, **17,** *7*, 403 (2019).
5. A. B. SCHROMM et al. “Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion,” *European journal of biochemistry*, **267,** *7*, 2008 (2000).
6. C. GALANOS et al. “Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities,” *European journal of biochemistry*, **148,** *1*, 1 (1985).
7. E. T. RIETSCHEL et al. “Bacterial endotoxin: molecular relationships of structure to activity and function,” *FASEB journal official publication of the Federation of American Societies for Experimental Biology*, **8,** *2*, 217 (1994).
8. M. CAROFF and D. KARIBIAN. “Structure of bacterial lipopolysaccharides,” *Carbohydrate research*, **338,** *23*, 2431 (2003).
9. O. LÜDERITZ et al. “Endotoxins of Gram-negative bacteria,” *Pharmacology & therapeutics*, **15,** *3*, 383 (1981).
10. O. HOLST. “Biochemistry and cell biology of bacterial endotoxins,” *FEMS Immunology and Medical Microbiology*, **16,** *2*, 83 (1996).
11. P. J. HITCHCOCK et al. “Lipopolysaccharide nomenclature--past, present, and future,” *Journal of Bacteriology*, **166,** *3*, 699 (1986).
12. C. ERRIDGE et al. “Structure and function of lipopolysaccharides,” *Microbes and infection*, **4,** *8*, 837 (2002).
13. S. AGRAWAL et al. “Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos,” *Journal of immunology (Baltimore, Md. 1950)*, **171,** *10*, 4984 (2003).
14. K. BRANDENBURG et al. “Influence of the supramolecular structure of free lipid A on its biological activity,” *European journal of biochemistry*, **218,** *2*, 555 (1993).
15. K. BRANDENBURG et al. “Physicochemical properties of bacterial glycopolymers in relation to bioactivity,” *Carbohydrate research*, **338,** *23*, 2477 (2003).
16. K. TAKAYAMA et al. “Physicochemical properties of the lipopolysaccharide unit that activates B lymphocytes,” *The Journal of biological chemistry*, **265,** *23*, 14023 (1990).
17. K. L. WILLIAMS, *Endotoxins*: *Pyrogens, LAL Testing and Depyrogenation,* 3rd ed., Taylor and Francis, Hoboken (2013).
18. R. S. MUNFORD. “Endotoxemia-menace, marker, or mistake?,” *Journal of Leukocyte Biology*, **100,** *4*, 687 (2016).
19. Y. SAKR et al. “Sepsis in Intensive Care Unit Patients: Worldwide Data From the Intensive Care over Nations Audit,” *Open Forum Infectious Diseases*, **5,** *12*, ofy313 (2018).
20. C. R. H. RAETZ and C. WHITFIELD. “Lipopolysaccharide endotoxins,” *Annual review of biochemistry*, **71**, 635 (2002).
21. E. SANTACROCE et al. “Advances and Challenges in Sepsis Management: Modern Tools and Future Directions,” *Cells*, **13,** *5* (2024).
22. C. M. COOPERSMITH et al. “Surviving sepsis campaign: research priorities for sepsis and septic shock,” *Intensive Care Medicine*, **44,** *9*, 1400 (2018).

23. F. F. ANHÊ et al. “Metabolic endotoxemia is dictated by the type of lipopolysaccharide,” *Cell Reports*, **36,** *11*, 109691 (2021).
24. S. MOHAMMAD and C. THIEMERMANN. “Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions,” *Frontiers in Immunology*, **11**, 594150 (2020).
25. P. D. CANI et al. “Metabolic endotoxemia initiates obesity and insulin resistance,” *Diabetes*, **56,** *7*, 1761 (2007).
26. C. J. WIEDERMANN et al. “Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the Bruneck Study,” *Journal of the American College of Cardiology*, **34,** *7*, 1975 (1999).
27. J. COHEN. “The immunopathogenesis of sepsis,” *Nature*, **420,** *6917*, 885 (2002).
28. “Clinical sepsis and death in a newborn nursery associated with contaminated parenteral medications--Brazil, 1996,” *MMWR. Morbidity and mortality weekly report*, **47,** *29*, 610 (1998).
29. W. R. KEENE et al. “Observations on the pyrogenic response and its application to the bioassay of endotoxin,” *J. Clin. Invest.*, **40,** *2*, 295 (1961).
30. S. IWANAGA. “Biochemical principle of Limulus test for detecting bacterial endotoxins,” *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, **83,** *4*, 110 (2007).
31. J. LEVIN and F. B. BANG. “A DESCRIPTION OF CELLULAR COAGULATION IN THE LIMULUS,” *Bulletin of the Johns Hopkins Hospital*, **115**, 337 (1964).
32. E. H. MÜRER et al. “Isolation and studies of the granules of the amebocytes of Limulus polyphemus, the horseshoe crab,” *Journal of cellular physiology*, **86,** *3 Pt 1*, 533 (1975).
33. H. SAKAI et al. “Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant,” *Journal of Pharmaceutical Sciences*, **93,** *2*, 310 (2004).
34. *Comparison of endotoxin testing methods for pharmaceutical products* (2002).
35. P. DOLEJŠ and K. VAŇOUSOVÁ. “A collection of horseshoe crabs (Chelicerata: Xiphosura) in the National Museum, Prague (Czech Republic) and a review of their immunological importance,” *aramit*, **49**, 1 (2015).
36. J. REICH et al. “Masking of endotoxin in surfactant samples: Effects on Limulus-based detection systems,” *Biologicals*, **44,** *5*, 417 (2016).
37. M. SCHNEIER et al. “Current technologies to endotoxin detection and removal for biopharmaceutical purification,” *Biotechnology and bioengineering*, **117,** *8*, 2588 (2020).
38. P. LI et al., *Biotechnology Efforts to Conserve Horseshoe Crabs Through the Development of Recombinant Factor C-Based Endotoxin Test*, in: R.H. Carmichael et al. (Eds.), *Changing global perspectives on horseshoe crab biology, conservation and management*, 501, Springer, Cham (2016).
39. J. L. DING and B. HO. “Endotoxin detection--from limulus amebocyte lysate to recombinant factor C,” *Sub-cellular biochemistry*, **53**, 187 (2010).
40. *FDA (*Mon, 15 July 2024).
41. Deutscher Apotheker-Verlag Doktor Roland Schmiedel, *European Pharmacopoeia, 11th Ed., English: 11.0 - 11.2 Subscription to Main volume + Supplement 1 + Supplement 2,* 11. Auflage, Deutscher Apotheker Verlag, Stuttgart (2022).
42. T. HARTUNG. “Pyrogen testing revisited on occasion of the 25th anniversary of the whole blood monocyte activation test,” *ALTEX*, **38,** *1*, 3 (2021).
43. J. F. COOPER. “Resolving LAL Test interferences,” *Journal of parenteral science and technology a publication of the Parenteral Drug Association*, **44,** *1*, 13 (1990).
44. A. MUELLER et al. “The influence of glucan polymer structure and solution conformation on binding to (1--3)-beta-D-glucan receptors in a human monocyte-like cell line,” *Glycobiology*, **10,** *4*, 339 (2000).

45. T. SANDLE. “Bacterial Endotoxin Test using LAL methodology: overcoming interfering factors,” *European Pharmaceutical Review*, **26,** *4*, 20 (2021).
46. *Low endotoxin recovery in common biologics products* (2013).
47. A. GNAUCK et al. “The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects,” *Journal of Immunological Methods*, **416**, 146 (2015).
48. J. LEVIN et al. “Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria,” *The New England journal of medicine*, **283,** *24*, 1313 (1970).
49. *Detection of endotoxin in human blood and demonstration of an inhibitor* (1970).
50. Z. NÁDHÁZI et al. “Plasma endotoxin level of healthy donors,” *Acta Microbiologica et Immunologica Hungarica*, **49,** *1*, 151 (2002).
51. S. M. OPAL et al. “Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock,” *The Journal of infectious diseases*, **180,** *5*, 1584 (1999).
52. S. J. DAS et al. “Role of serum endotoxin, FGF19, TLR2, TNF-α, IL-12 and IL-10 in NAFLD-associated T2DM pathogenesis: Insights into Th1 bias and protective mechanisms,” *Indian J Gastroenterol*, 1 (2024).
53. “Bacterial Endotoxins | USP,” https://www.usp.org/harmonization-standards/pdg/general-methods/bacterial-endotoxins (current as of Jan. 17, 2025).
54. R. DABBAH et al. “Pyrogenicity of E. coli 055:b5 endotoxin by the USP rabbit test--a HIMA collaborative study,” *Journal of the Parenteral Drug Association*, **34,** *3*, 212 (1980).
55. R. E. WACHTEL and K. TSUJI. “Comparison of limulus amebocyte lysates and correlation with the United States Pharmacopeial pyrogen test,” *Applied and environmental microbiology*, **33,** *6*, 1265 (1977).
56. J.-L. VINCENT et al. “Sepsis definitions: time for change,” *Lancet (London, England)*, **381,** *9868*, 774 (2013).
57. Y. DUAN et al. “Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease,” *Nature*, **575,** *7783*, 505 (2019).
58. W. A. BANKS et al. “Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit,” *J Neuroinflammation*, **12,** *1*, 223 (2015).
59. J. W. PICKERING et al. “Evaluation of a (1-3)-beta-D-glucan assay for diagnosis of invasive fungal infections,” *Journal of clinical microbiology*, **43,** *12*, 5957 (2005).
60. J. BARBERI and S. SPRIANO. “Titanium and Protein Adsorption: An Overview of Mechanisms and Effects of Surface Features,” *Materials (Basel, Switzerland)*, **14,** *7*, 1590 (2021).
61. Y. YU et al. “The hydrophilic-to-hydrophobic transition in glassy silica is driven by the atomic topology of its surface,” *J. Chem. Phys.*, **148,** *7*, 74503 (2018).
62. F. PONCIN-EPAILLARD et al. “Surface treatment of polymeric materials controlling the adhesion of biomolecules,” *Journal of Functional Biomaterials*, **3,** *3*, 528 (2012).
63. T. J. NOVITSKY et al. “Factors affecting recovery of endotoxin adsorbed to container surfaces,” *Journal of parenteral science and technology a publication of the Parenteral Drug Association*, **40,** *6*, 284 (1986).
64. F. ELTABONI et al. “Fluorescence Spectroscopy Analysis of the Bacteria–Mineral Interface: Adsorption of Lipopolysaccharides to Silica and Alumina,” *Langmuir* (2020).
65. W. SALI et al. “Polysaccharide Chain Length of Lipopolysaccharides From Salmonella Minnesota Is a Determinant of Aggregate Stability, Plasma Residence Time and Proinflammatory Propensity in vivo,” *Frontiers in microbiology*, **10**, 1774 (2019).
66. H. YOKOTA et al. “Adsorption of endotoxins on glass in the presence of cationic proteins,” *Journal of pharmaceutical and biomedical analysis*, **25,** *5-6*, 1001 (2001).
67. E. J. MURPHY et al. “β-Glucan Metabolic and Immunomodulatory Properties and Potential for Clinical Application,” *Journal of fungi (Basel, Switzerland)*, **6,** *4* (2020).

68. M. GORGONE et al. “Circulating 1,3-Beta-D-Glucan is Associated with Lung Function, Respiratory Symptoms, and Mediators of Matrix Degradation in Chronic Obstructive Pulmonary Disease,” *Chronic obstructive pulmonary diseases (Miami, Fla.)*, **9,** *3*, 325 (2022).
69. S. HARM et al. “An in vitro study on factors affecting endotoxin neutralization in human plasma using the Limulus amebocyte lysate test,” *Sci Rep*, **11,** *1*, 4192 (2021).
70. H. TAMURA et al. “Outstanding Contributions of LAL Technology to Pharmaceutical and Medical Science: Review of Methods, Progress, Challenges, and Future Perspectives in Early Detection and Management of Bacterial Infections and Invasive Fungal Diseases,” *Biomedicines*, **9,** *5*, 536 (2021).
71. “Case Studies of Microbial Contamination in Biologic Product Manufacturing,” https://www.americanpharmaceuticalreview.com/Featured-Articles/36755-Case-Studies-of-Microbial-Contamination-in-Biologic-Product-Manufacturing/ (current as of Jan. 08, 2025).
72. “Low Lipopolysaccharide Recovery versus Low Endotoxin Recovery in Common Biological Product Matrices,” https://www.americanpharmaceuticalreview.com/featured-articles/167451-low-lipopolysaccharide-recovery-versus-low-endotoxin-recovery-in-common-biological-product-matrices/ (current as of Jan. 08, 2025).
73. J. REICH et al. “Low Endotoxin Recovery-Masking of Naturally Occurring Endotoxin,” *International Journal of Molecular Sciences*, **20,** *4*, 838 (2019).
74. T. GUTSMANN et al. “Structural prerequisites for endotoxic activity in the Limulus test as compared to cytokine production in mononuclear cells,” *Innate immunity*, **16,** *1*, 39 (2010).
75. C. R. H. RAETZ et al. “Lipid A modification systems in gram-negative bacteria,” *Annual review of biochemistry*, **76**, 295 (2007).
76. R. S. MUNFORD and A. W. VARLEY. “Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria,” *PLoS pathogens*, **2,** *6*, e67 (2006).
77. C. WANG et al. “Understanding lipopolysaccharide aggregation and its influence on activation of Factor C,” *Journal of Colloid and Interface Science*, **552**, 540 (2019).
78. M. MUELLER et al. “Aggregates are the biologically active units of endotoxin,” *The Journal of biological chemistry*, **279,** *25*, 26307 (2004).
79. K. BRANDENBURG et al. “The expression of endotoxic activity in the Limulus test as compared to cytokine production in immune cells,” *Current medicinal chemistry*, **16,** *21*, 2653 (2009).
80. F. DARDELLE et al. “Diversity, Complexity, and Specificity of Bacterial Lipopolysaccharide (LPS) Structures Impacting Their Detection and Quantification,” *International Journal of Molecular Sciences*, **25,** *7*, 3927 (2024).
81. Y. JEON et al. “Monocyte activation test (MAT) as an ethical alternative to animal testing,” *BMB Reports*, **58,** *3*, 105 (2025).
82. A. NEGWER. “Low Endotoxin Recovery,” Universitäts- und Landesbibliothek Bonn.
83. C. WHITFIELD and M. S. TRENT. “Biosynthesis and export of bacterial lipopolysaccharides,” *Annual review of biochemistry*, **83**, 99 (2014).
84. C. ALEXANDER and E. T. RIETSCHEL. “Bacterial lipopolysaccharides and innate immunity,” *Journal of endotoxin research*, **7,** *3*, 167 (2001).
85. A. SILIPO et al. “Glyco-conjugates as elicitors or suppressors of plant innate immunity,” *Glycobiology*, **20,** *4*, 406 (2010).
86. S. G. WILKINSON. “Bacterial lipopolysaccharides--themes and variations,” *Progress in Lipid Research*, **35,** *3*, 283 (1996).
87. A. RICE and J. WERESZCZYNSKI. “Atomistic Scale Effects of Lipopolysaccharide Modifications on Bacterial Outer Membrane Defenses,” *Biophysical Journal*, **114,** *6*, 1389 (2018).
88. G. HÄMMERLING et al. “Structural investigations on the core polysaccharide of Escherichia coli 0100,” *European journal of biochemistry*, **22,** *3*, 331 (1971).

89. I. ORSKOV et al. “Serology, chemistry, and genetics of O and K antigens of Escherichia coli,” *Bacteriological Reviews*, **41,** *3*, 667 (1977).
90. N. QURESHI et al. “Complete structural determination of lipopolysaccharide obtained from deep rough mutant of Escherichia coli. Purification by high performance liquid chromatography and direct analysis by plasma desorption mass spectrometry,” *The Journal of biological chemistry*, **263,** *24*, 11971 (1988).
91. C. GALANOS et al. “Biological activity of synthetic heptaacyl lipid A representing a component of Salmonella minnesota R595 lipid A,” *European journal of biochemistry*, **160,** *1*, 55 (1986).
92. E. GARCÍA VÉSCOVI et al. “Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence,” *Cell*, **84,** *1*, 165 (1996).
93. A. de SOYZA et al. “Chemical and biological features of Burkholderia cepacia complex lipopolysaccharides,” *Innate immunity*, **14,** *3*, 127 (2008).
94. J. REICH et al. “Investigation of the kinetics and mechanism of low endotoxin recovery in a matrix for biopharmaceutical drug products,” *Biologicals journal of the International Association of Biological Standardization*, **53**, 1 (2018).
95. M. TSUCHIYA. “Mechanism of Low Endotoxin Recovery Caused by a Solution Containing a Chelating Agent and a Detergent,” *Immunome Res*, **15,** *1*, 1 (2019).
96. L. A. CLIFTON et al. “Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models,” *Langmuir the ACS journal of surfaces and colloids*, **31,** *1*, 404 (2015).
97. R. BECH ØRVING et al. “Bacterial Endotoxin Testing-Fast Endotoxin Masking Kinetics in the Presence of Lauryldimethylamine Oxide,” *Microorganisms*, **8,** *11* (2020).
98. U. SEYDEL et al. “The generalized endotoxic principle,” *European journal of immunology*, **33,** *6*, 1586 (2003).
99. W. RICHTER et al. “Morphology, size distribution, and aggregate structure of lipopolysaccharide and lipid A dispersions from enterobacterial origin,” *Innate immunity*, **17,** *5*, 427 (2011).
100. J. A. RUDBACH and A. G. JOHNSON. “RESTORATION OF ENDOTOXIN ACTIVITY FOLLOWING ALTERATION BY PLASMA,” *Nature*, **202**, 811 (1964).
101. S. I. OROSZLAN et al. “REVERSIBLE INACTIVATION OF AN ENDOTOXIN BY INTRACELLULAR PROTEIN,” *Biochemical Pharmacology*, **12**, 1131 (1963).
102. E. RIBI et al. “Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate,” *Journal of Bacteriology*, **92,** *5*, 1493 (1966).
103. H. SCHWARZ et al. “Biological Activity of Masked Endotoxin,” *Sci Rep*, **7,** *1*, 44750 (2017).
104. K. TAKAYAMA et al. “Monomeric Re lipopolysaccharide from Escherichia coli is more active than the aggregated form in the Limulus amebocyte lysate assay and in inducing Egr-1 mRNA in murine peritoneal macrophages,” *Journal of Biological Chemistry*, **269,** *3*, 2241 (1994).
105. N. S. TAN et al. “Definition of endotoxin binding sites in horseshoe crab factor C recombinant sushi proteins and neutralization of endotoxin by sushi peptides,” *FASEB journal official publication of the Federation of American Societies for Experimental Biology*, **14,** *12*, 1801 (2000).
106. L. MAZGAEEN and P. GURUNG. “Recent Advances in Lipopolysaccharide Recognition Systems,” *International Journal of Molecular Sciences*, **21,** *2* (2020).
107. B. S. PARK and J.-O. LEE. “Recognition of lipopolysaccharide pattern by TLR4 complexes,” *Experimental & molecular medicine*, **45,** *12*, e66 (2013).
108. B. PULENDRAN et al. “Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo,” *Journal of immunology (Baltimore, Md. 1950)*, **167,** *9*, 5067 (2001).

109. D. S. S. M. UPPU and J. HALDAR. “Lipopolysaccharide Neutralization by Cationic-Amphiphilic Polymers through Pseudoaggregate Formation,” *Biomacromolecules*, **17,** *3*, 862 (2016).
110. A. GRIES et al. “Biophysical analysis of the interaction of the serum protein human β2GPI with bacterial lipopolysaccharide,” *FEBS Open Bio*, **4**, 432 (2014).
111. K. BRANDENBURG et al. “Biophysical characterization of the interaction of high-density lipoprotein (HDL) with endotoxins,” *European journal of biochemistry*, **269,** *23*, 5972 (2002).
112. Y. KACONIS et al. “Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides,” *Biophysical Journal*, **100,** *11*, 2652 (2011).
113. K. BRANDENBURG et al. “Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin,” *Biological chemistry*, **382,** *8*, 1215 (2001).
114. D. PETSCH and F. B. ANSPACH. “Endotoxin removal from protein solutions,” *Journal of biotechnology*, **76,** *2-3*, 97 (2000).
115. M. GOUGH et al. (1996-12-11996-12-1T00:011996-12-1T00:00).
116. A. GIACOMETTI et al. “Potential therapeutic role of cationic peptides in three experimental models of septic shock,” *Antimicrobial agents and chemotherapy*, **46,** *7*, 2132 (2002).
117. A. IWAGAKI et al. “Influence of synthetic antiendotoxin peptides on lipopolysaccharide (LPS) recognition and LPS-induced proinflammatory cytokine responses by cells expressing membrane-bound CD14,” *Infection and immunity*, **68,** *3*, 1655 (2000).
118. S. A. RAGLAND and A. K. CRISS. “From bacterial killing to immune modulation: Recent insights into the functions of lysozyme,” *PLoS pathogens*, **13,** *9*, e1006512 (2017).
119. K. BRANDENBURG et al. “Biophysical characterisation of lysozyme binding to LPS Re and lipid A,” *European journal of biochemistry*, **258,** *2*, 686 (1998).
120. G. JÜRGENS et al. “Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A,” *Journal of endotoxin research*, **8,** *2*, 115 (2002).
121. SUNIL A. DAVID, *The Interaction of Lipid A and Lipopolysaccharide with Human Serum Albumin*, in: H. Brade (Ed.), *Endotoxin in health and disease*, 413, CRC Press, Boca Raton (2020).
122. K. BRANDENBURG et al. “Temperature dependence of the binding of endotoxins to the polycationic peptides polymyxin B and its nonapeptide,” *Biophysical Journal*, **88,** *3*, 1845 (2005).
123. M. C. FALK et al. “Aggregation of serum proteins with lipopolysaccharide (LPS): characterization of the precipitable LPS-protein complex,” *Journal of endotoxin research*, **3,** *2*, 129 (1996).
124. J. ANDRÄ et al. “Interactions between lipid A and serum proteins,” *Advances in experimental medicine and biology*, **667**, 39 (2010).
125. M. TRIANTAFILOU et al. “Serum proteins modulate lipopolysaccharide and lipoteichoic acid-induced activation and contribute to the clinical outcome of sepsis,” *Virulence*, **3,** *2*, 136 (2012).
126. N. WENGER, *TriAltus Bioscience (*6 January 2020).
127. X. XIAO et al. “Salt Destabilization of Cationic Colistin Complexation within Polyanionic Microgels,” *Macromolecules*, **55,** *5*, 1736 (2022).
128. S. HARM et al. “Heparin enables the reliable detection of endotoxin in human serum samples using the Limulus amebocyte lysate assay,” *Sci Rep*, **14,** *1*, 2410 (2024).

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9. Appendix

9.1 Publications in Original

9.1.1 Publication I

molecules

MDPI

Article

Effects of Different Container Types on (1→3)-β-D-glucan Recovery

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Abstract: It has long been known that containers for sample analysis or storage can play a role in endotoxin recovery and have to be taken into account when determining endotoxin concentrations. However, there is little data on the effects of containers regarding (1→3)-β-D-glucan, which plays a role as a contaminant in endotoxin measurements. To determine the effect of the container on (1→3)-β-D-glucan measurements, four different types of containers were investigated at different temperatures and stored for up to 28 days. For short-term storage for 3 h at room temperature, no effect of the container on the (1→3)-β-D-glucan recovery could be observed, but for storage at −20 °C, the results indicate that the storage time and temperature influences (1→3)-β-D-glucan detection. All containers showed a trend of lower recoveries over time, but the polyethylene container showed a significantly lower recovery compared to the other containers. We also showed that freeze/thaw cycles had a strong influence on the recovery of (1→3)-β-D-glucan in polyethylene containers. Our study showed that the container can affect not only the detection of endotoxins but also the detection of (1→3)-β-D-glucans.

Keywords: (1→3)-β-D-glucan; container effect; sample hold time; SHT; freeze/thaw; spike recovery

check for updates

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1. Introduction

The contamination of pharmaceutical products with glucans can occur within a number of upstream and downstream processes, such as filtration during purification. The potential origins are cellulose-based materials (e.g., cellulose filters) or fungal contamination of raw materials (e.g., sucrose-containing buffers) [1,2]. (1→3)-β-D-glucans are polysaccharides composed of glucose monomers that can trigger an inflammatory response, similar to endotoxins. They are produced by most fungi as a cell wall component, but they are also found in a wide range of other eukaryotic and prokaryotic organisms, like yeast and algae. They are also a component of plant tissue. They possess high structural diversity, e.g., differences in polymer length. This also results in different properties regarding biological activity and solubility. The least water-soluble particulate forms are associated with more potent immunological activity [3,4].

The detection of (1→3)-β-D-glucans employs a similar enzymatic cascade to the detection of endotoxins. Both substances trigger the Limulus Amoebocyte Lysate (LAL) reaction, utilizing blood cells (amoebocytes) sourced from horseshoe crabs. In the cascade mediated by LPS, three distinct serine protease zymogens are involved: factor C, factor B, and proclotting enzyme, in addition to coagulogen, a protein possessing the capacity to form gel-like structures. This cascade commences with LPS catalyzing the conversion of zymogen factor C into the active factor C_a. Subsequently, factor C_a activates factor B, leading to the conversion of proclotting enzyme into clotting enzyme. The clotting enzyme then cleaves two peptide bonds in coagulogen, a molecule resembling fibrinogen,

to form insoluble coagulin gel. The above-described cascade can also be initiated by (1→3)-β-D-glucan. Initially, factor G, a zymogen of serine protease, undergoes activation and subsequently triggers the activation of the proclotting enzyme [5]. As a consequence, employing the LAL assay for endotoxin measurement may yield false-positive results in the presence of (1→3)-β-D-glucans [6]. Fortunately, solutions are available to address this interference issue. To mitigate such interference, the test can be altered to include glucan-inhibiting substances [7], or an alternative approach involving the use of a recombinant factor C assay can be used. In the recombinant factor C (rFC) assay, factor G is absent, preventing the cascade's activation by this protein in response to (1→3)-β-D-glucans. rFC-based testing methods have proven to be reliable and, notably, exhibit greater specificity than LAL tests [8]. For glucan detection, factor C is removed from the lysate, allowing the remaining factor G to initiate the cascade upon encountering (1→3)-β-D-glucans [9].

In comparison with bacterial endotoxins, (1→3)-β-D-glucans exhibit milder immunological activity but retain immunomodulatory characteristics. They are recognized for their ability to bind to and stimulate various cell types, such as monocytes and macrophages [10–12]. Furthermore, it has been observed that (1→3)-β-D-glucans can augment the detrimental effects of endotoxin-induced toxicity, thereby impacting the immune system adversely [13]. Unlike bacterial endotoxins, there is no universally accepted standard for detecting (1→3)-β-D-glucans. Nevertheless, there is an emerging pattern in the industry and among global regulatory bodies to recognize and quantify (1→3)-β-D-glucan impurities and to comprehend the factors that affect their detection [1,14,15].

One often-overlooked aspect of contaminant detection is the effect of the container. It has long been established that, besides the low endotoxin recovery phenomenon [16,17], the container (e.g., adsorption) can also influence endotoxin determination. Different types of plastic lead to differences in endotoxin recovery and even the initial endotoxin concentration or source of the endotoxin can lead to differences in recovery [18]. Another factor that can influence endotoxin recovery is the storage time of the samples in a specific container, with containers made of certain materials like polycarbonate showing decreasing recoveries over time. This effect of endotoxin adsorption to the surface of containers is more strongly pronounced in plastic containers than in those made of borosilicate [19]. However, this effect can also be influenced by the samples, leading to differences in adsorption in the presence of different materials or the charge of the samples [20]. Taking all of these factors into account, borosilicate containers are the preferred containers for performing endotoxin testing and studies.

However, little is known about how differences in container properties affect the recovery of (1→3)-β-D-glucan. As (1→3)-β-D-glucan contamination is often accompanied by endotoxin contamination, it is important that accurate measurements can also be performed for (1→3)-β-D-glucan, regardless of the container type, to obtain an accurate picture of the total contamination.

This study investigated the recovery of (1→3)-β-D-glucan from containers composed of borosilicate, polypropylene, or polyethylene for short-term storage at room temperature (RT; 18–25 °C) and long-term storage at −20 °C (−30–−10 °C). This study aimed to investigate the temperature, time, and container dependencies of (1→3)-β-D-glucan recovery and to provide recommendations for the best practice for obtaining the most accurate results.

2. Results and Discussion

In the following, we present our findings regarding the contamination of different containers with (1→3)-β-D-glucan and the influence of storage under different conditions. First, a short-term storage at RT was performed, which mimicked the processing time between sampling and possible analyses, such as immediate in-process controls. Subsequently, a sample-hold time study was performed at −20 °C to mimic the storage of, e.g., retain samples. In addition, the influence of freezing and thawing was investigated.

2.1. Initial Contamination of Containers

First, before the investigation of the effects of the container on the recovery of (1→3)-β-D-glucan, the contamination of different container types was determined. For this, 10 containers of each type were filled with 1 mL reagent-grade water (RGW) heated to 37 °C and vortexed for 15 s. The containers were incubated for 60 min at RT, and after 30 min or 60 min, the test containers were vortexed again for 15 s. The (1→3)-β-D-glucan content was measured without additional dilution.

The results of these investigations are summarized in Table 1. All 10 individual containers tested per material showed no initial contamination above the limit of detection (6.26 pg/mL) with (1→3)-β-D-glucan. The positive product controls (PPC), which ensured that no test interference occurred, were valid for all samples.

Table 1. Initial contamination of containers used in this study.

Container	Average (1→3)-β-D-glucan Concentration [pg/mL]	Average PPC	Number of Valid Measurements
Borosilicate 1	<6.26	109	10
Borosilicate 2	<6.26	134	9 (1 CV > 30%)
Polyethylene	<6.26	113	10
Polypropylene	<6.26	96	10

Labware is often labelled as sterile, RNAse-free, DNAse-free, or pyrogen-free. Sterile defines a product as absent of any living organisms; however, nonviable biologic contaminations may not be removed via standard sterilization processes. RNAse- and DNAse-free defines, as stated, a product that is free of RNAse or DNAse. Endotoxin-based contaminations could be excluded under the pyrogen-free label [21]. None of these labels guarantee the exclusion of glucan contaminations. It is, therefore, important to exclude possible contaminations prior to sampling. Our data showed glucan contamination below the detection limit for all tested containers, so the investigated containers were suitable for glucan sample collection.

2.2. Short-Term Storage at Room Temperature

For the investigation of the effects of short-term storage on the recovery of (1→3)-β-D-glucan, four different containers were filled with 1 mL (1→3)-β-D-glucan-spiked RGW to imitate a glucan contamination. The initial spike concentration was set at 25 pg/mL. All samples were measured directly after adding the spiked sample and after an incubation time of 3 h or 24 h at RT. As a confirmation of the spike concentration, a water control was prepared using the same spike solution.

The results of the short-time storage study are shown in Figure 1. For the measurement of the samples at time point (Tmp) 0 h, no significant differences between the different containers are observed. All containers reach a recovery above 80% calculated to the water control at Tmp 0 h (borosilicate 1: 92%, borosilicate 2: 88%, polyethylene: 107%, polypropylene: 95%). The measurement after storage of the samples for 3 h at RT also showed valid recovery of the glucan spike (85%, 84%, 88%, 87%). Even after 24 h and 48 h of storage, all samples show stable (1→3)-β-D-glucan recoveries around 100%, except for the sample in the polyethylene container. The recovery in this sample is only 57% after 24 h and even sinks below the 50% limit after 48 h (46%). Thus, it shows a significant decrease compared to the water control at Tmp 0 h.

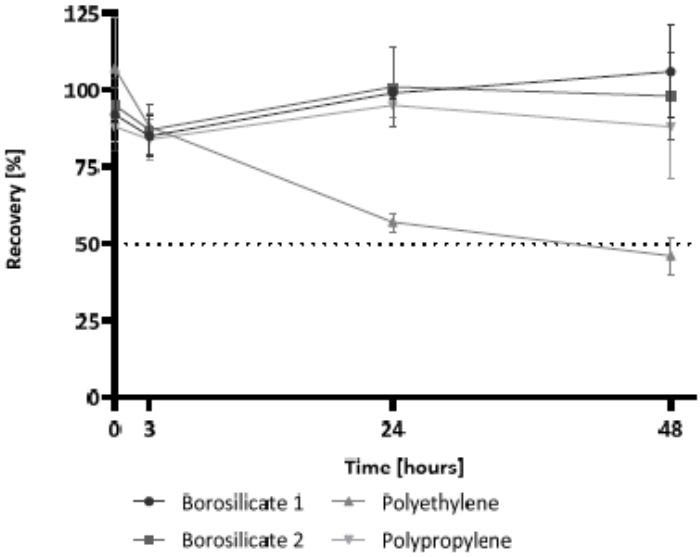

Figure 1. (1→3)-β-D-glucan recovery after the short-term storage of spiked reagent-grade water in four types of containers at room temperature for up to 48 h. (1→3)-β-D-glucan recovery for each time point is shown. Time point zero (0 h) of the water control with the 25 pg/mL (1→3)-β-D-glucan spike was used as the reference value and set to 100% for calculations. The recovery is shown relative to this reference value. Each point shows the mean recovery of three replicates. The error bars reflect the standard deviation of replicates (n = 3). The dotted line marks 50% recovery.

Regardless of the differences in material or surface-area-to-volume ratios, all containers appeared to be well suited for glucan detection after short-term storage for 3 h. If samples are stored for longer than 3 h, effects appear with the use of polyethylene containers. The use of polyethylene containers poses the risk of underestimating potential contamination. These results are in contrast to the container effects observed for endotoxin analysis. Meadows et al. showed that, up to 24 h, there was no significant difference in endotoxin recovery in different containers of different materials and surface-area-to-volume ratios (polystyrene tube, polypropylene tube, glass tube) [19].

2.3. *Long-Term Storage at −20 °C*

As the short-term storage of samples in the containers did, in most cases, not correspond to the storage conditions used in industry, longer storage times at −20 °C were also investigated. It was also hoped that freezing the samples might reduce or prevent the observed container effects. Sample preparation was identical to that of the short-term storage experiments. Samples were stored at −20 °C and thawed for measuring after specific storage times (0 h, 1 d, 7 d, 14 d, 28 d). The recoveries at each time point for each container type are shown in Figure 2.

For time point 0 h, all container types showed comparable recoveries. This was in common with the results for time point Tmp 0 h of the short-term storage study. After 1 d of storage and thawing, all of the containers showed valid recoveries in a similar range (borosilicate 1: 89%, borosilicate 2: 86%, polyethylene: 88%, polypropylene: 78%). The mean recoveries over all time points were: for borosilicate 1, 76% with a coefficient of variation (CV) of 10%; for polypropylene, 77% (CV 10%); for polyethylene, 68% (CV 21%); and for borosilicate 2, 80% (CV 11%). It was notable that for the polyethylene container, the mean recovery was the lowest and also showed the highest variance.

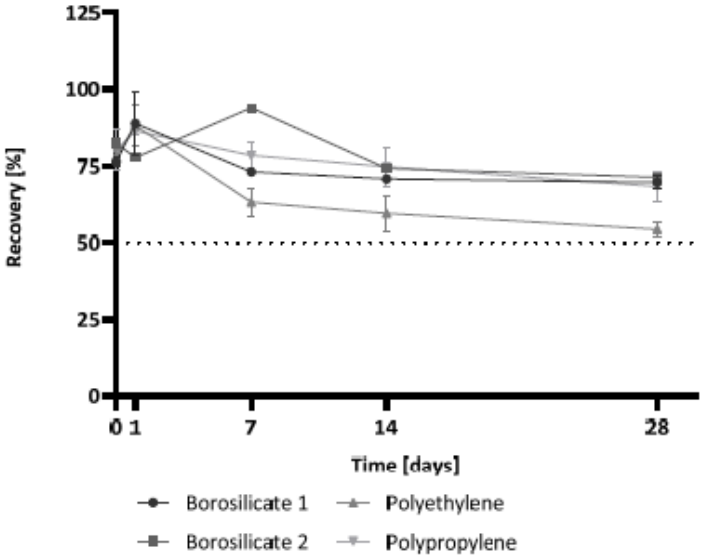

Figure 2. (1→3)-β-D-glucan recovery after the long-term storage of spiked reagent-grade water in four types of containers at −20 °C for up to 28 days. (1→3)-β-D-glucan recovery for each time point is shown. Time point zero (0 h) of the water control with the 25 pg/mL (1→3)-β-D-glucan spike was used as the reference value and set to 100% for calculations. The recovery is shown relative to this reference value. Each point shows the mean recovery of three replicates. The error bars reflect the standard deviation of replicates (n = 3). The dotted line marks 50% recovery.

These results indicate that the storage time influences glucan detection regardless of the container type. However, the container material also appears to have an effect. Although all containers show a trend of lower recoveries after storage at −20 °C and all recoveries remain above 50% of the water control at Tmp 0 h for all time points, the use of polyethylene containers again has the risk of underestimating a potential contamination. However, the data show that the container effects of polyethylene occur much later when stored at −20 °C than at RT. This could, therefore, indicate an energy-dependent process.

2.4. Freeze and Thaw Comparison

To investigate whether a freeze-and-thaw process also has an influence on recovery depending on the container type, a further experiment was performed. Water spiked with (1→3)-β-D-glucan was used to fill each of the containers and subjected to a freeze/thaw cycle with 15 min thawing time before measuring. As a reference, water spiked with (1→3)-β-D-glucan was used to fill each container and was not subjected to the freeze/thaw cycle. As can be seen in Figure 3, all samples in the different containers reached recoveries over 50% of the control. In the borosilicate 1, borosilicate 2, and polypropylene containers, recoveries of 100%, 134%, and 95%, respectively, were determined. Only for the polyethylene container was the recovery lower (66%). This indicates that the freeze/thaw cycle also influences the recovery of (1→3)-β-D-glucan in polyethylene containers.

The results indicate that the freeze/thaw cycle generally has no effect on the recovery of beta-glucan. For three of the four containers, the recovery rate was close to 100% of the respective control without freezing. For borosilicate 1, borosilicate 2, and polypropylene, storage time and storage temperature appear to have a greater impact on glucan detection than freeze/thaw cycles.

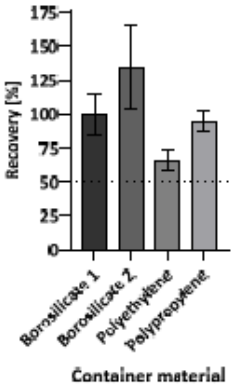

Figure 3. Mean (1→3)-β-D-glucan recovery after one freeze/thaw cycle of spiked reagent-grade water in four types of containers. A water control with a 25 pg/mL (1→3)-β-D-glucan spike that was not subjected to the freeze/thaw cycle was used as the reference value and set to 100% for calculations. The recovery is shown relative to this reference value. Each bar shows the mean recovery of three replicates. The error bars reflect the standard deviation of replicates (n = 3). The dotted line marks 50% recovery.

To further investigate the effect of thawing times and possible container dependencies, the recovery of (1→3)-β-D-glucan was investigated after 5, 20, and 30 min of thawing. The overall process was handled as before where a 15 min thawing time was used before measuring. The only difference was the time the samples were allowed to thaw and acclimate before measuring. After thawing times of 5 min and 20 min, the recoveries for all containers aside from borosilicate 1 were below 75% compared to the control, which was not subjected to the freeze/thaw cycle. While the recoveries for the samples in the borosilicate 2 and polypropylene containers increased with a longer thawing time (30 min), the polyethylene container showed an even lower recovery. This was only slightly above 50% (52%) (Figure 4).

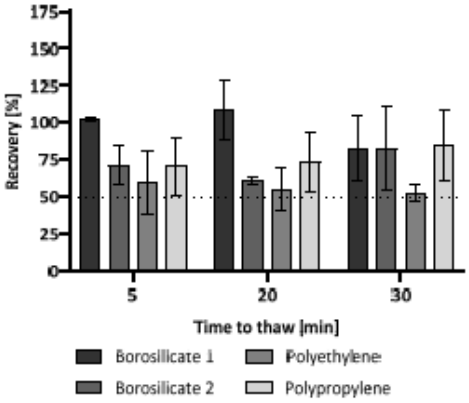

Figure 4. (1→3)-β-D-glucan recovery after one freeze/thaw cycle with different thawing times in four container types. (1→3)-β-D-glucan recovery for each thawing time is shown. A water control with a 25 pg/mL (1→3)-β-D-glucan spike that was not subjected to the freeze/thaw cycle was used as the reference value and set to 100% for calculations. The recovery is shown relative to this reference value. Each bar shows the mean recovery of two replicates. The error bars reflect the standard deviation of replicates (n = 2). The dotted line marks 50% recovery.

2.5. Dependence of Surface-Area-to-Volume Ratio

In a further experiment, the influence of the surface-area-to-volume ratio was investigated. For this study, only the two borosilicate containers were compared, as they differed only in their surface-area-to-volume ratio and were, therefore, best suited for comparison. For this purpose, samples were kept in the containers at 4 °C for 28 days. In order to avoid freeze/thaw effects, storage at 4 °C was chosen. This is also in common with the storage of samples in industry when a sample cannot be immediately analysed. Sample preparation was performed according to the −20 °C long-time storage experiments. Samples were stored at 4 °C and measured directly after adding the spiked sample and after a specific storage time (0 h, 1 d, 7 d, 14 d, 28 d). The recoveries at each time point for the two borosilicate containers are shown in Figure 5.

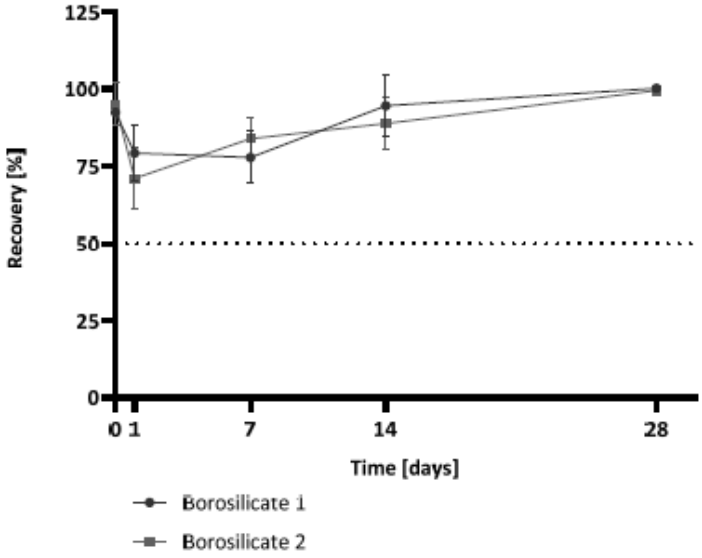

Figure 5. (1→3)-β-D-glucan recovery after the long-term storage of spiked reagent-grade water in two types of borosilicate containers at 4 °C for up to 28 days. (1→3)-β-D-glucan recovery for each time point is shown. Time point zero (0 h) of the water control with the 25 pg/mL (1→3)-β-D-glucan spike was used as the reference value and set to 100% for calculations. The recovery is shown relative to this reference value. Each bar shows the mean recovery of three replicates. The error bars reflect the standard deviation of replicates (n = 3). The dotted line marks 50% recovery.

Both containers show similar behaviours over the tested time interval. For time point 0 h, both containers showed recoveries near 100% (92% for borosilicate 1, 95% for borosilicate 2). After 1 d of storage, the recoveries are slightly lower for both sample containers compared to Tmp 0 h (79% for borosilicate 1, 71% for borosilicate 2). Nevertheless, both container types revealed recoveries of 100% after storage for 28 d. The mean recoveries over all time points were: for borosilicate 1, 89% (CV 11%), and for borosilicate 2, 88% (CV 13%). Although both containers have different surface-area-to-volume ratios, the recoveries are almost identical.

The surface-area-to-volume ratio plays an important role in many biological processes and influences, for example, pharmacokinetic and pharmacodynamic parameters [22]. Also, in assay development, the surface/volume ratio is a considering factor. In solid phase assays like ELISA (enzyme-linked immunosorbent assay), a larger surface-area-to-volume ratio implies a faster adsorption of molecules from the liquid phase because the liquid is exposed to more surface [23]. Therefore, it is conceivable that a container with a

larger surface-area-to-volume ratio has a greater impact on (1→3)-β-D-glucan recovery than a container with a smaller surface-area-to-volume ratio. The sample and possible (1→3)-β-D-glucan contamination are more exposed to the material. However, our data indicate that borosilicate as a container material does not influence the recovery when stored at 4 °C over 28 d and, therefore, the surface-area-to-volume ratio does not influence recovery.

3. Materials and Methods

3.1. Materials and Reagents

(1→3)-β-D-glucan standard, lyophilized Glucatell reagent, and RGW were purchased from Associates of Cape Cod (East Falmouth, MA, USA). The used container types and their suppliers are summarized in Table 2.

Table 2. Container types used in this study.

Container	Material	Volume	Supplier
PyroControl	Borosilicate glass (1)	10 mL	ACILA AG (Mörfelden-Walldorf, German)
EndoGrade Glass Test Tubes	Borosilicate glass (2)	6 mL	BioMerieux (Marcy-l'Étoile, France)
Nalgene Cryoware	Polyethylene	2 mL	Thermo Fisher Scientific (Waltham, MA, USA)
CELLSTAR Tubes	Polypropylene	15 mL	Greiner bio-one GmbH (Frickenhausen, Germany)

3.2. Beta-Glucan Assay

The (1→3)-β-D-glucan concentrations were determined using the Glucatell assay kit following the manufacturer's instructions (Associates of Cape Cod). The detection is based on a modification of the Limulus Amoebocyte Lysate (LAL) pathway and therefore specific for (1→3)-β-D-glucan. As procedure, the kinetic mode using onset O.D. was chosen, with a log—log plot of the onset times versus the standard concentrations. The glucan standard was reconstituted with RGW to provide a solution of 100 pg glucan/mL. The standard curve was prepared via serial dilution of the stock solution (100 pg/mL–3.125 pg/mL). A total of 25 µL of the standard or sample was added to each well of the microtiter plate. The Glucatell reagent was reconstituted with RGW and Pyrosol buffer, and then 100 µL of the reagent was added to each well of the plate. The Glucatell lysate/sample mixture was incubated at 37 °C ± 1 °C and measured using an ELx808 Absorbance Microplate Reader (BioTek, Bad Friedrichshall, Germany) equipped with a 405 nm filter. Analysis was executed using the Gen5 Secure software, version 3.11 (BioTek, Bad Friedrichshall, Germany). All samples were tested in duplicate. The glucan concentration was determined from the mean of the readings interpolated from the kinetic standard curve. Samples were accepted as valid if the CV of the duplicates was less than 30%.

3.3. Calculation of Recovery Rate

The determined (1→3)-β-D-glucan content in the tested samples was determined relative to the total content at time point zero in water controls and stated as a percent. Water controls were prepared by spiking (1→3)-β-D-glucan in RGW using a 25 pg/mL (1→3)-β-D-glucan spike solution.

For the freezing/thawing experiments, the content of (1→3)-β-D-glucan in the tested sample was determined and the recovery rate was determined relative to the content in the water spiked with a 25 pg/mL (1→3)-β-D-glucan spike solution that was used to fill each container that was not subjected to the freeze/thaw cycle.

The obtained results were rated as valid when the recovery of the (1→3)-β-D-glucan content was between 50% and 200%.

The calculation of the mean and standard deviation as well as the creation of figures were performed using Graph Pad Prism, version 10.0.0.

4. Conclusions and Future Directions

The growing scientific evidence of the immunomodulatory effects of beta-glucans results in an increasing need for its reliable quantification. While there are established detection methods, little is known about the potential influences on beta-glucan analysis and more work needs to be performed to validate these methods and to establish compendial procedures for their application [24–27].

It is known that endotoxin recovery depends on various parameters, including but not limited to the plastic resin manufacturer, sample container manufacturer, and endotoxin species. However, less is known about the influence of these parameters on (1→3)-β-D-glucan.

Our study shows that the container can also have an influence on the detection of (1→3)-β-D-glucans. The material and storage time as well as storage temperature play important roles. In this study, four different container types for storage at different temperatures and time intervals were analysed. The data showed that the polypropylene and borosilicate containers had the most reliable results over time. The standard deviation of the average of glucan recovery across all time points in the long-term study was 7% for borosilicate 1, 6% for borosilicate 2, and 8% for polypropylene compared to 13% for the polyethylene container. Consequently, sampling containers should be selected with care. In general, all tested containers were suitable for short-term storage, as no initial contamination of the containers could be detected. When the measurement is performed within 3 h of the sampling event, all containers can be recommended. If samples for the detection of (1→3)-β-D-glucans are subjected to storage for more than 3 h in the container, independent of storage temperature, polyethylene is not recommended as a container material. Borosilicate glass as well as polypropylene containers appear to be equally suitable for the detection of (1→3)-β-D-glucans. Although polypropylene is chemically quite similar to polyethylene, it is much harder, stronger, and more thermally resilient. These differences could influence the adsorption properties of the materials [28].

The long-term storage study was conducted at only one temperature (−20 °C), as this is the most common storage temperature for samples for long-term storage in industry. However, it would be interesting to observe how other temperatures (4 °C, −80 °C) affect (1→3)-β-D-glucan detection in all four containers used in this study. This will be the subject of further studies.

Another point that should not be overlooked is the surface-area-to-volume ratios of the containers. It is possible that this also plays a role in the observed effects on the recoveries as the polyethylene container had the lowest volume of the investigated containers. The comparison of both borosilicate containers, which possess different surface-area-to-volume ratios, has shown that during long-term storage at 4 °C the surface/volume ratio has no influence on the recovery of (1→3)-β-D-glucan, although a dependence on the surface/volume ratio cannot be completely excluded with the investigations performed. The effects of surface-area-to-volume ratios of other container materials should be further investigated in follow-up studies. It should be noted that in this initial investigation, only water spiked with a standard (1→3)-β-D-glucan was used. Thus, it would be interesting to know if the sample matrix or glucan species also contribute to this container effect. This should be part of further research.

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References

1. Barton, C.; Vigor, K.; Scott, R.; Jones, P.; Lentfer, H.; Bax, H.J.; Josephs, D.H.; Karagiannis, S.N.; Spicer, J.F. Beta-glucan contamination of pharmaceutical products: How much should we accept? *Cancer Immunol. Immunother.* **2016**, *65*, 1289. [CrossRef] [PubMed]
2. Legentil, L.; Paris, F.; Ballet, C.; Trouvelot, S.; Daire, X.; Vetvicka, V.; Ferrières, V. Molecular Interactions of β-(1→3)-Glucans with Their Receptors. *Molecules* **2015**, *20*, 9745. [CrossRef] [PubMed]
3. Mueller, A.; Raptis, J.; Rice, P.J.; Kalbfleisch, J.H.; Stout, R.D.; Ensley, H.E.; Browder, W.; Williams, D.L. The influence of glucan polymer structure and solution conformation on binding to (1→3)-beta-D-glucan receptors in a human monocyte-like cell line. *Glycobiology* **2000**, *10*, 339. [CrossRef] [PubMed]
4. Kim, Y.T.; Kim, E.H.; Cheong, C.; Williams, D.L.; Kim, C.W.; Lim, S.T. Structural characterization of beta-D-(1 → 3, 1 → 6)-linked glucans using NMR spectroscopy. *Carbohydr. Res.* **2000**, *328*, 331. [CrossRef] [PubMed]
5. Jun, A.; Shigenori, T.; Hiroshi, T.; Yuko, S.; Hazime, S. Activation of limulus coagulation factor G by several (1→3)-beta-D-glucans: Comparison of the potency of glucans with identical degree of polymerization but different conformations. *J. Biochem.* **1993**, *113*, 683.
6. Roslansky, P.F.; Novitsky, T.J. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J. Clin. Microbiol.* **1991**, *29*, 2477. [CrossRef] [PubMed]
7. Associates of Cape Cod Glucashield: Glucan Inhibiting Buffer. Available online: https://www.acciusa.com/pdfs/accProduct/inserts/Glucashield_13DGlucan_Inhibiting_Buffer.pdf (accessed on 11 July 2023).
8. Piehler, M.; Roeder, R.; Blessing, S.; Reich, J. Comparison of LAL and rFC Assays-Participation in a Proficiency Test Program between 2014 and 2019. *Microorganisms* **2020**, *8*, 418. [CrossRef] [PubMed]
9. Ostrosky-Zeichner, L.; Alexander, B.D.; Kett, D.H.; Vazquez, J.; Pappas, P.G.; Saeki, F.; Ketchum, P.A.; Wingard, J.; Schiff, R.; Tamura, H.; et al. Multicenter clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **2005**, *41*, 654. [CrossRef]
10. Emurphy, E.J.; Rezoagli, E.; Major, I.; Rowan, N.J.; Laffey, J.G. β-Glucan Metabolic and Immunomodulatory Properties and Potential for Clinical Application. *J. Fungi* **2020**, *6*, 356. [CrossRef]
11. MNovak, M.; Vetvicka, V. *β*-Glucans, History, and the Present: Immunomodulatory Aspects and Mechanisms of Action. *J. Immunotoxicol.* **2008**, *5*, 47–57.
12. Chan, G.C.; Chan, W.K.; Sze, D.M. The effects of beta-glucan on human immune and cancer cells. *J. Hematol. Oncol.* **2009**, *2*, 25. [CrossRef] [PubMed]
13. Neun, B.W.; Cedrone, E.; Potter, T.M.; Crist, R.M.; Dobrovolskaia, M.A. Detection of Beta-Glucan Contamination in Nanotechnology-Based Formulations. *Molecules* **2020**, *25*, 3367. [CrossRef] [PubMed]
14. Liss, B.; Cornely, O.A.; Hoffmann, D.; Dimitriou, V.; Wisplinghoff, H. 1,3-β-D-Glucan contamination of common antimicrobials. *J. Antimicrob. Chemother.* **2016**, *71*, 913. [CrossRef] [PubMed]
15. Vigor, K.; Emerson, J.; Scott, R.; Cheek, J.; Barton, C.; Bax, H.J.; Josephs, D.H.; Karagiannis, S.N.; Spicer, J.F.; Lentfer, H. Development of downstream processing to minimize beta-glucan impurities in GMP-manufactured therapeutic antibodies. *Biotechnol. Prog.* **2016**, *32*, 1494. [CrossRef] [PubMed]
16. Reich, J.; Tamura, H.; Nagaoka, I.; Motschmann, H. Investigation of the kinetics and mechanism of low endotoxin recovery in a matrix for biopharmaceutical drug products. *Biol. J. Int. Assoc. Biol. Stand.* **2018**, *53*, 1–9. [CrossRef] [PubMed]
17. Reich, J.; Lang, P.; Grallert, H.; Motschmann, H. Masking of endotoxin in surfactant samples: Effects on Limulus-based detection systems. *Biol. J. Int. Assoc. Biol. Stand.* **2016**, *44*, 417. [CrossRef] [PubMed]
18. Novitsky, T.J.; Schmidt-Gengenbach, J.; Remillard, J.F. Factors Affecting Recovery of Endotoxin Adsorbed to Container Surfaces. *PDA J. Pharm. Sci. Technol.* **1986**, *40*, 284.
19. Meadows, C. Aseptic Sampling Best Practices: Endotoxin Binding Affinity. Available online: https://www.sartorius.com/download/21658/technical-note-endotoxin-binding-affinity-sp-4006-e-data.pdf (accessed on 27 September 2023).
20. Yokota, H.; Kiyonaga, H.; Kaniwa, H.; Shibanuma, T. Adsorption of endotoxins on glass in the presence of cationic proteins. *J. Pharm. Biomed. Anal.* **2001**, *25*, 1001. [CrossRef]
21. Eppendorf AG. Eppendorf Purity Grades Selection Guide. Available online: https://www.eppendorf.com/de-de/service-support/quality-certificates/purity-grades/ (accessed on 27 September 2023).
22. Munn, R.; Whittem, T.; Woodward, A.P. The Surface Area to Volume Ratio Changes the Pharmacokinetic and Pharmacodynamic Parameters in the Subcutaneous Tissue Cage Model: As Illustrated by Carprofen in Sheep. *Front. Vet. Sci.* **2022**, *9*, 905797. [CrossRef]

23. Esser, P. The Surface/Volume Ratio in Solid Phase Assays: Application Note. 2014. Available online: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwin_KiWi9qBAxV-gP0HHWZqB70QFnoECBwQAQ&url=https%3A%2F%2Fassets.fishersci.com%2FTFS-Assets%2FLCD%2FProduct-Bulletins%2FD19566.pdf&usg=AOvVaw23-bzcrqu2vHu-n1kKHThE&opi=89978449 (accessed on 27 September 2023).
24. Hong, F.; Hansen, R.D.; Yan, J.; Allendorf, D.J.; Baran, J.T.; Ostroff, G.R.; Ross, G.D. β-Glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumouricidal granulocytes as killers cells. *Cancer Res.* **2003**, *63*, 9023.
25. Li, B.; Allendorf, D.J.; Hansen, R.; Marroquin, J.; Ding, C.; Cramer, D.E.; Yan, J. Yeast beta-glucan amplifies phagocyte killing of iC3b-opsonized tumor cells via complement receptor 3-Syk-phosphatidylinositol 3-kinase pathway. *J. Immunol.* **2006**, *177*, 1661. [CrossRef]
26. Vannucci, L.; Krizan, J.; Sima, P.; Stakheev, D.; Caja, F.; Rajsiglova, L.; Horak, V.; Saieh, M. Immunostimulatory properties and antitumor activities of glucans (Review). *Int. J. Oncol.* **2013**, *43*, 357. [CrossRef]
27. Whitehead, A.; Beck, E.J.; Tosh, S.; Wolever, T.M. Cholesterol-lowering effects of oat β-glucan: A meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2014**, *100*, 1413. [CrossRef]
28. Baur, E.; Osswald, T.; Rudolph, N. *Saechtling Kunststoff Taschenbuch*, 31st ed.; Carl Hanser Verlag GmbH & Co. KG: München, Germany, 2013.

9.1.2 Publication II

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The impact of LPS mutants on endotoxin masking in different detection systems

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ABSTRACT

Endotoxin masking poses a potential risk to patient safety by rendering endotoxin undetectable. While research often focuses on international endotoxin standards (RSE), the effects of LPS mutants on Low Endotoxin Recovery (LER) are poorly understood. Our study investigated *S. minnesota* and *E. coli* mutants with incomplete O-antigen chains (rough LPS) using Limulus amebocyte lysate (LAL), recombinant Factor C (rFC) and the monocyte activation test (MAT). All tested methods detected the mutants, with variations in activity observed. Measurements over time in a common drug formulation (10 mM sodium citrate and 0.05 % (w/v) polysorbate 20) showed different masking kinetics for the mutants using different methods. We were able to show that LAL and rFC have comparable kinetics, whereas MAT showed improved recovery of masked endotoxin. The study showed that the mutation of LPS have an effect on masking, independent of the assay system. We propose that polysaccharide length affects masking susceptibility, with lower hydrophilic/hydrophobic ratios caused by the shortened polysaccharide chain (rough LPS) reducing masking. In addition, the stronger negative charge of the rough mutants increases cation affinity and is suggested to contribute to the stabilization of supramolecular structures, making the rough mutants less susceptible to masking than the smooth mutants.

1. Introduction

The sources of microbial contamination in pharmaceutical manufacturing can be diverse. Contamination comes from many sources: water, personnel, raw materials and equipment, to name but a few. This shows that microbial contamination is diverse and endotoxins can come from a variety of sources [1]. Endotoxins, chemically known as lipopolysaccharides (LPS), are part of the outer membrane of Gram-negative bacteria. Multiple studies showed that endotoxins cause inflammatory responses when entering the human bloodstream. This can lead to severe physiological reactions, from fever to septic shock and death [2–4]. The toxicity of these molecules derives from the interaction with mammalian host cells, which leads to the production of potent bioactive mediators, like cytokines [5]. LPS, mainly lipid A is recognized as the main contributor to toxicity linked with Gram-negative bacteria.

However, endotoxin is not a homogeneous molecule and its structure can vary significantly depending on the bacterial species producing the LPS, the contamination status, i.e. intact bacteria, outer membrane vesicles (OMV) or degradation products caused by lysis or cell division. Growth conditions also affect the structure, resulting in different substitutions in lipid A, core and o-antigen (e.g. amino arabinose, phosphates, amino acids), the number of fatty acids in lipid A and polysaccharides in the core, and the number of repeats in the o-antigen [6].

Despite the highly ordered structures of LPS in all Gram-negative bacterial strains, which correspond to a fundamental three-unit composition – O-antigen, core, and lipid A – variations in these constituents lead to diverse overall structures, resulting in different LPS classifications (Fig. 1).

The classification of LPS as high molecular weight (smooth) or low molecular weight (rough) depends on the presence or absence of O-antigen in its structure [8]. The absence of O-antigen can occur naturally or due to genetic mutation. While all rough mutants lack O-antigen, the length of the oligosaccharide core varies depending on the affected gene loci. Rough mutants without O-antigen but with a complete inner and outer core structure are termed Ra-LPS. In contrast, rough mutants with a significantly reduced core, are termed Re-LPS or "deep rough" LPS [9, 10]. Re-LPS represents the smallest LPS structure supporting bacterial

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membrane integrity, while Ra-LPS has the longest core configuration among rough LPS. Mutations at different loci yield core lengths ranging between Ra and Re-LPS [8]. In wild-type organisms, which predominantly express smooth LPS, there is significant size heterogeneity among the LPS molecules. Approximately 40–50 % of these molecules are semi-rough, characterized by the absence of the O-antigen [8].

Endotoxin contamination poses a potential safety concern in the context of parenteral drug manufacturing [11,12]. Various detection methods were therefore established, including the rabbit pyrogen test (RPT), the monocyte activation test (MAT), and the Limulus amebocyte lysate (LAL) test. The LAL test, renowned for its simplicity and high sensitivity, stands as the preeminent method for endotoxin detection in scientific applications [13–18]. In recent years, the recombinant Factor C (rFC) test has become increasingly widespread, indicating a trend towards replacing the LAL test [19].

A controversial topic of debate in the context of evaluating current testing methods results is the issue of "Low Endotoxin Recovery" (LER) [20]. The term LER describes the inability to recover more than 50 % of activity when endotoxin is added to an undiluted product, which calls into question the reliability of current endotoxin detection methods [21]. The presence of LER has been extensively confirmed [22–27]. However, the clinical relevance of this phenomenon remains under discussion. Schwarz et al. [26] demonstrated in their study that masked endotoxins still trigger immune reactions. Furthermore, a human whole blood assay has been shown to resolve LER and detect the pyrogenic activity of endotoxin, while demonstrating reduced activation of the LAL cascade and activation of the TLR receptor system in MAT [28]. Additionally, modern drugs are often associated with side effects such as cough, fever, and inflammation [29,30]. These symptoms make it difficult to distinguish whether the reactions are caused by the treatment itself or by potential undetected contamination. This lack of differentiation poses a significant challenge for regulatory agencies, as they struggle to accurately assess the safety profile of these drugs. Until this issue is fully understood, endotoxin masking must be considered a potential risk and further study of the LER phenomenon is essential.

Standardized endotoxins like Reference Standard Endotoxin (RSE) are often used as spikes in hold time experiments [31]. LER is particularly relevant when surfactants and other components like salt, urea, and various organic substances – which are common in drug formulations – are present. Due to its amphiphilic nature, LPS can be modified by these components, leading to the spontaneous formation of LPS aggregates, which cannot be reliably detected. The transition from a detectable to an undetectable (masked) form is probably accomanied by a change in the supramolecular structures of the endotoxin. As a result, the endotoxin is masked and inaccessible for detection [24,26,32].

Despite the significant variability in LPS molecules, studies investigating LER have focused on structurally defined RSE. Several studies have shown varying susceptibility to the LER phenomenon. For example, some crude LPS preparations (i.e. NOEs (naturally occurring endotoxins)) have been found to be less susceptible to this phenomenon [33]. Considering that masking is associated with a structural change, it is surprising that different LPS structures, such as mutants, have not been extensively explored in this context. Due to the inherent heterogeneity of LPS, naturally occurring contaminations are likely to consist of both smooth and rough LPS variants. This heterogeneity results from genetic mutations and variations in growth conditions, leading to considerable structural variability in LPS. As the exact nature of the contamination is unknown, the specific LPS structures involved remain undefined, making it difficult to predict their composition and behaviour.

Understanding the connection between LPS supramolecular arrangements and their detection in LAL assays by Factor C, as well as LPS recognition via the TLR4-MD2 cascade in MAT, is crucial for unraveling the mechanism of LER. In this study, the causes of masking were investigated in more detail by examining the influence of endotoxin structures and their susceptibility to the LER phenomenon.

2. Materials and methods

2.1. Chemicals and material

Polysorbate 20 and trisodium citrate were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Endotoxin-free water and borosilicate glass tubes were obtained from Microcoat Biotechnologie GmbH, Bernried, Germany. Kinetic chromogenic Limulus amebocyte lysate test (Kinetic-QCL™) was obtained from Lonza Inc., Walkersville, USA. The recombinant Factor C test (EndoLISA®) was obtained from Biomerieux, Marcy-l'Étoile, France, and the monocyte activation test (HaemoMAT®) from Haemochrom Diagnostica GmbH, Essen, Germany. Prior to the experiments, all relevant materials were tested for endotoxin content and proven to contain less than 0.005 EU/mL.

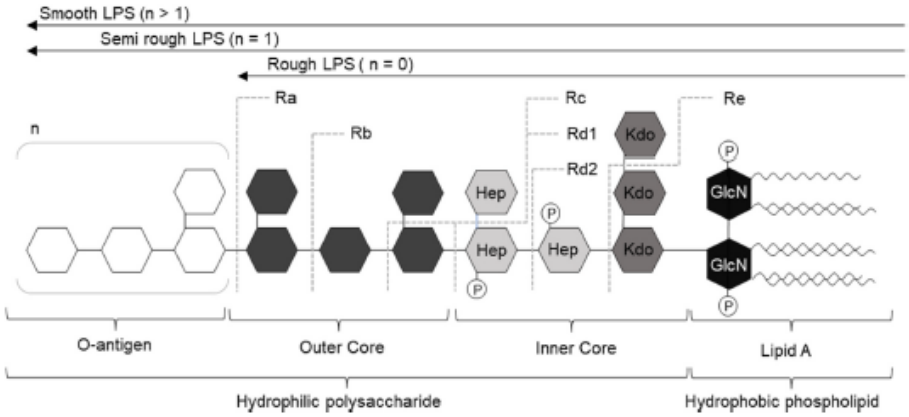

Fig. 1. Schematic representation of smooth LPS and different LPS mutants (labelled Ra - Re). Lipid A consists of a di-phosphorylated disaccharide GlcN (glucosamine) backbone. The core is divided into inner and outer core. The inner core consists of Kdo (3-deoxy-D-manno-octulosonic acid) and heptose (L-glycero-D-manno) residues and is phosphorylated with various groups such as phosphate (labelled P). The O antigen is composed of 1–50 repeating sugar subunits (n = 1–50) (Modified from Ref. [7]).

2.2. Lipopolysaccharides

LPS mutants from *Escherichia coli* O8:K27 (S-form), EH 100 (Ra), J5 (Rc), R515 (Re) and LPS mutants from *Salmonella minnesota* (S-form), R60 (Ra) R345 (Rb), R5 (Rc), R7 (Rd1), R3 (Rd2), R595 (Re) as well as the Lipid A from *E. coli* R515 and *S. minnesota* R565 were obtained from BIOMOL GmbH, Hamburg, Germany. The provided concentration was 1 mg/mL for each solution.

2.3. Preparation stock solutions

To determine the endotoxin activity, samples with a concentration of 1 mg/mL of the respective mutant were measured using a kinetic chromogenic assay (Kinetic-QCL™). The detected activities are listed in Table 2 and used to prepare 10,000 EU/mL solutions of each of the LPS mutants.

2.4. Preparation of samples and measurement of masking kinetics

Samples were prepared in 5 mL glass tubes with sample volumes of 1 mL per sample. Unless otherwise described, samples were spiked with 10 µL lipopolysaccharide (LPS mutant) out of a 10.000 EU/mL stock solution. Stock solutions were shaken at 1400 rpm for 30 min before adding the spikes to the samples. After spiking, the samples were incubated at room temperature and for different periods.

Samples with different incubation periods were measured on the same microtiter plate, to avoid variation from test to test. For the short-time incubation (up to 45 min), the endotoxin recovery kinetics were measured in an online manner. Therefore, the samples were spiked with the respective LPS mutant. After a specific time interval, a 10 µL aliquot was taken from each sample and diluted 1:100 in endotoxin-free water. This was necessary to stop masking effects and to avoid test interferences.

For the long-time incubation, the endotoxin recovery kinetics were measured in a reverse manner. Samples were aliquoted and stored for the time of the study. The aliquots with the longest incubation time were spiked first with the respective mutant. Further aliquots with shorter incubation periods were spiked later in accordance with the respective incubation period. The timepoint 0 aliquots were measured immediately after adding the LPS spikes. All the samples were diluted 1:100 in endotoxin-free water for measurement.

2.5. Detection of endotoxin

The kinetic chromogenic assay (Kinetic-QCL™), the recombinant Factor C assay (ENDOLISA®) and the monocyte activation test (HaemoMAT®) were used. All assays were performed following the supplier's instructions.

For both the LAL-based and the rFC-based assay, 100 µL of samples, positive product controls (PPC) and the prepared standard curve dilutions were added in duplicate to a 96-well microtiter plate. A 10 µL endotoxin spike was added to the PPC wells. Lysate (containing the LAL enzyme cascade) or reaction mixture (containing the rFC) was then added.

For the LAL-based method the amount of released chromogenic substrate was measured at 405 nm with a BioTek ELX808 absorbance microplate reader (BioTek Instruments, Inc. Winooski,VT) coupled with WinKQCL™ software version 5.3.3 (Lonza Walkersville, MD) at 37 ± 1 °C. The mean of the duplicates per sample was used to determine endotoxin concentrations (EU/mL) using a standard curve fitted with a linear regression model. The detection limit of the assay was 0.005 EU/mL (Kinetic-QCL™).

For the recombinant Factor C assay, the amount of released fluorescence substrate was measured spectrophotometrically at 440 nm with an FLx800 fluorescence microplate reader (BioTek, Bad Friedrichshall, Germany) at 37 ± 1 °C coupled with Gen5 Secure software (version 3.11, BioTek). The mean of the duplicates per sample was used to determine endotoxin concentrations (EU/mL) using a standard curve fitted with a four-parameter logistic non-linear regression model. The detection limit of the assay was 0.005 EU/mL (ENDOLISA®).

For MAT, 20 µL of the samples, PPCs and the prepared standard curve dilutions were added in triplicates to a 96-well microtiter plate. A 20 µL endotoxin spike was added to the PPC wells and 20 µL cell culture medium was added to all other wells. Thawed PBMCs were then diluted in pre-warmed cell culture medium and 160 µL of cell suspension (PBMC) was added per well of the 96-well plate. The plate was incubated at 37 °C ± 2 °C and 5 % ± 0.5 % CO_2 for 17–19 h. After incubation, the supernatant was collected. The amount of IL-6 released was measured using the IL-6 ELISA (part of the HaemoMAT®) at 450–630 nm with a BioTek ELX808 absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT) coupled with Gen5 Secure software (version 3.11, BioTek). The mean of the triplicates per sample was used to determine endotoxin concentrations (EU/mL) using a standard curve fitted with a four-parameter logistic non-linear regression model. The detection limit of the assay was 0.125 EU/mL (HaemoMAT®).

2.6. Calculation of endotoxin recovery for the measurement of kinetics and statistics

The endotoxin concentrations determined in the samples were compared with the endotoxin concentrations at timepoint 0 and expressed as a percentage.

Calculations of means, standard deviations (SD) and all other statistical tests were performed using Graph Pad Prism, version 10.1.1. For comparison between groups (e.g. smooth LPS detected by LAL vs. smooth LPS detected by rFC), ANOVA was performed with Tukey's multiple comparison test. p-values less than 0.05 were considered statistically significant. Significance is indicated in the manuscript as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.7. Estimation of molecular mass of the different LPS mutants

The estimation of the molecular mass of the mutants was based on the generic polysaccharide shown in Fig. 1. For the molecular mass of the individual monosaccharides, an average rounded value of 200 g/mol was determined (Kdo = 238 g/mol; Hep = 210 g/mol; Gal = 180 g/mol; Glc = 180 g/mol). The molecular mass for Lipid A and smooth LPS was estimated based on literature values, with an average of 1800 g/mol for Lipid A (noting that its mass can vary significantly) and approximately 10.000 g/mol for smooth LPS [34–37]. It should be noted that the molecular mass of smooth LPS can also vary depending on the number of sugar repeats in the O-antigen.

The primary focus of this analysis is the relative relationship between the estimated values of different mutants, which have been presented with as much precision as possible. Due to the naturally occurring variety of bacterial strains, absolute magnitudes can vary. All estimated values are summarized in Table 1.

Of note, the same molecular mass was estimated per mutant for both strains, which does not reflect reality.

3. Results

3.1. Activity of different LPS mutants

Overall, thirteen LPS mutants with different structures were tested. Eight mutants, including smooth LPS and the lipid A derived from *S. minnesota* and five mutants, including smooth LPS and lipid A from *E. coli* (see Materials for further strain information). In an initial approach, the activity of the LPS mutants was determined. For this purpose, solutions of 1 mg/mL of each mutant were measured with a kinetic chromogenic assay (KCA). To account for differences in the molarities of the LPS mutants, the activities are presented as activity per

Table 1
Estimation of molecular mass of the different mutants.

Mutant	Estimate number of monosaccharides	Molecular mass of polysaccharide [g/mol]	Estimate molecular mass [g/mol]
LPS smooth	>11	>2200	10000
LPS Ra	11	2200	4000
LPS Rb	9	1800	3600
LPS Rc	6	1200	3000
LPS Rd1	5	1000	2800
LPS Rd2	4	800	2600
LPS Re	2	400	2200
Lipid A	0	0	1800

mol. The molarities of each mutant were estimated based on the molecular mass of their monosaccharides and relevant literature (Details can be found in the Methods section). The molarities and corresponding activities of the mutants are summarized in Table 1. Activities were for *S. minnesota* LPS smooth 90,900,000 EU/mol, Ra 54,000,000 EU/mol, Rb 72,000,000 EU/mol, Rc 66,900,000 EU/mol, Rd1 67,200,000 EU/mol, Rd2 66,300,000 EU/mol, Re 34,100,000 EU/mol and lipid A 10,206,000 EU/mol. The activity per mol tends to decrease with decreasing polysaccharide chain. However, for the Re mutant, the mutant with the shortest sugar chain and for lipid A alone, the activity per mg decreases. Mutants of *E. coli*, showed the following activities: LPS smooth 93,800,000 EU/mol, Ra 3,764,000 EU/mol, Rc 109,200,000 EU/mol, Re 91,960,000 EU/mol and lipid A 26,460,000 EU/mol (Table 2). The activities for smooth LPS from *S. minnesota* and *E. coli* were similar. Overall, *E.coli* mutants exhibited a higher activity per mol. Interestingly, the Ra mutant of *E. coli*, showed very low activity compared to all other mutants.

3.2. Comparison of assay systems for the detection of LPS mutants

To assess whether commercially available assay systems are able to detect heterogeneous LPS, we performed tests with different LPS mutants on different assay systems. This effort also aimed to determine whether rFC-based assays could replace the LAL assay. The endotoxin activities of selected LPS mutants obtained by KCA measurements (Table 2) were used to prepare 10,000 EU/mL solutions for each respective mutant. To compare the capabilities of different assay systems in detecting LPS mutants of S. minnesota and *E. coli*, the KCA assay, the rFC-based assay, and the MAT were applied. The KCA assay is designed to detect endotoxin activities in the range of 0.005–50 EU/mL, the rFC-based assay in the range of 0.05–500 EU/mL, and the MAT used in this study can detect activities in the range of 0.125–1 EU/mL.

For mutants of S. minnesota, all three assay systems successfully detected the structural diversity of LPS, as illustrated in Fig. 2. No statistically differences were observed for smooth LPS and mutants Rb, Rc, Rd1, and Re. However, for the Ra and Rd2 mutants, a slight significance ($p < 0.05$) was observed when comparing the assays. For the Ra mutant, the MAT detected lower endotoxin activities compared to the rFC-based assay. For the Rd2 mutant, the MAT detected lower endotoxin activities compared to the KCA. Significant differences ($p < 0.001$) were found between the three test systems, especially when analysing lipid A from S. minnesota. However, these results should be interpreted with caution due to the relatively large standard deviations and the limited number of tests conducted. The rFC assay showed the highest endotoxin activities compared to KCA and MAT. Lower endotoxin activities were detected with the MAT than with the KCA and rFC assays. Thus, higher endotoxin activities were detected with the KCA than with the MAT, but lower activities than with the rFC. It is noteworthy that for lipid A, the within-test variability observed for KCA and rFC also increased, as indicated by the standard deviation (SD).

Similar to observations made with the *S. minnesota* mutants, all test systems were able to detect the mutants of *E. coli*. However, when *E. coli* mutants were examined, they showed more differences between the assays compared to *S. minnesota*. Fig. 3 illustrates the activities of these mutants, measured through KCA, rFC, and MAT assays. Notably, all tested *E. coli* mutants exhibited significant differences in endotoxin activity across various assay systems, with particular emphasis on the Ra mutant, as well as mutants Re and lipid A. For smooth LPS, we observed lower endotoxin activities when measured with the MAT compared to the KCA. For the Ra mutant, the KCA detected lower endotoxin activities compared to the rFC and the MAT. The MAT also detected lower activities for the Ra mutant compared to the rFC. For both the Rc and Re mutants, we found that the KCA detected higher endotoxin activities compared to the rFC and the MAT. The MAT detected lower endotoxin activities compared to the KCA and the rFC for lipid A. In most cases, the results indicate that the activity observed in the MAT is comparatively

Table 2
Endotoxin activities of the mutants (measured with the kinetic chromogenic assay (K-QCL)). The mean and the coefficient of variation (CV [%]) of three independent experiments (conducted in duplicate) is shown (n = 3).

Strain	Mutant	Estimated molecular mass [g/mol]	Activity [EU/mol]	CV [%]
S. minnesota	LPS smooth	10000	90,900,000	1
	LPS Ra	4000	54,000,000	4
	LPS Rb	3600	72,000,000	16
	LPS Rc	3000	66,900,000	8
	LPS Rd1	2800	67,200,000	11
	LPS Rd2	2600	66,300,000	10
	LPS Re	2200	34,100,000	2
	Lipid A	1800	10,206,000	24
E. coli	LPS smooth	10000	93,800,000	1
	LPS Ra	4000	3,764,000	12
	LPS Rc	3000	109,200,000	15
	LPS Re	2200	91,960,000	4
	Lipid A	1800	26,460,000	11

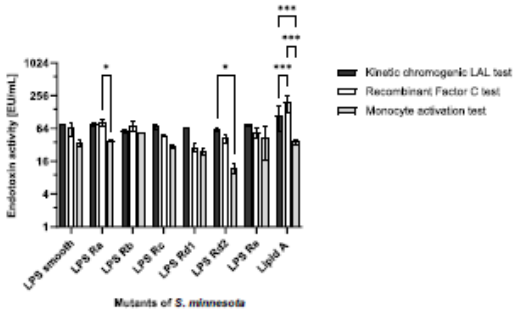

Fig. 2. Comparison of the endotoxin activity of the different mutants from *S. minnesota* in various assay systems (LAL, rFC, MAT). The mean ± standard deviation of the mean (SD) of three independent experiments (conducted in duplicates) is shown (n = 3) and plotted on log2 scale. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

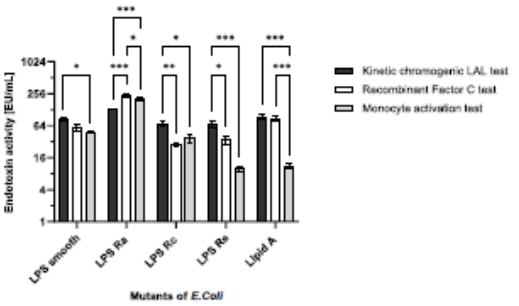

Fig. 3. Comparison of the endotoxin activity of the different mutants from *E. coli* in various assay systems (LAL, rFC, MAT). The mean ± standard deviation of the mean (SD) of three independent experiments (conducted in duplicates) is shown (n = 3) and plotted on log2 scale. *p < 0.05; **p < 0.01; ***p < 0.001.

lower than in other assay systems (for mutant Ra, Re and lipid A). This suggests that the measured LPS preparations may not possess the same level of potency in activating the immune system compared to their ability to activate Factor C. The most significant differences are determined between the LAL and the MAT assay. These results highlight the importance of considering assay specific variations when evaluating endotoxin activity.

3.3. Short-time analysis of the masking kinetics of the S. minnesota mutants

After proving that the used test systems are able to detect various LPS structures of the LPS mutants, we focused on investigating their masking behaviour. We employed a known masking matrix comprising 10 mM sodium citrate and 0.05 % (w/v) polysorbate 20 and recorded the masking kinetics using the same assay systems as above (LAL, rFC, and MAT) for 45 min. In accordance with the definition of masking, samples with a recovery of less than 50 % were classified as masked. Samples were also considered invalid if the recovery was greater than 200 %.

Distinct masking behaviours among the LPS mutants were observed in the LAL-based assay kinetics (Fig. 4 (a)). Smooth LPS demonstrated the fastest masking, similar to the smooth RSE. Within 20 min, the recovery of smooth LPS fell below 50 %. Similarly, after 30 min the recovery of RSE fell below 50 %. Notably, mutants Ra and Rb, possessing shorter polysaccharide chains than the smooth LPS, exhibited recoveries of around 50 % after 45 min. In contrast, all other mutants, characterized by shorter polysaccharide chains (including lipid A), showed no masking after 45 min, with recoveries ranging from 70 % to 121 %.

In the masking kinetics measured with the rFC assay (Fig. 4 (b)), the RSE as well as the smooth LPS and Ra mutant, exhibited masking after 20 min. Mutant Rb showed recovery below 50 % after 30 min. Conversely, all the other mutants displayed recoveries above 50 %. For mutant Rd2, Re and lipid A recoveries were 95 %, 88 % and 92 %, respectively after 45 min. Noteworthy are the recovery patterns of mutants Rc and Rd1. For the Rc mutant, the recovery increased until 20 min to a recovery of 161 %, after which it gradually declined and showed recovery of 121 % after 45 min. In the case of Rd1, the recovery demonstrated a continuous increase over the 45-min duration and showed recovery of 177 % after 45 min. However, it is important to note that both mutants remained below the 200 % threshold throughout – indicating a valid recovery.

The MAT (Fig. 4 (c)) revealed that the RSE as well as the smooth LPS, exhibited masking after 30 min, consistent with the observations from the other two detection systems. All other mutants showed no indications of a LER within the given time frame. Notably, the Rd1 mutant,

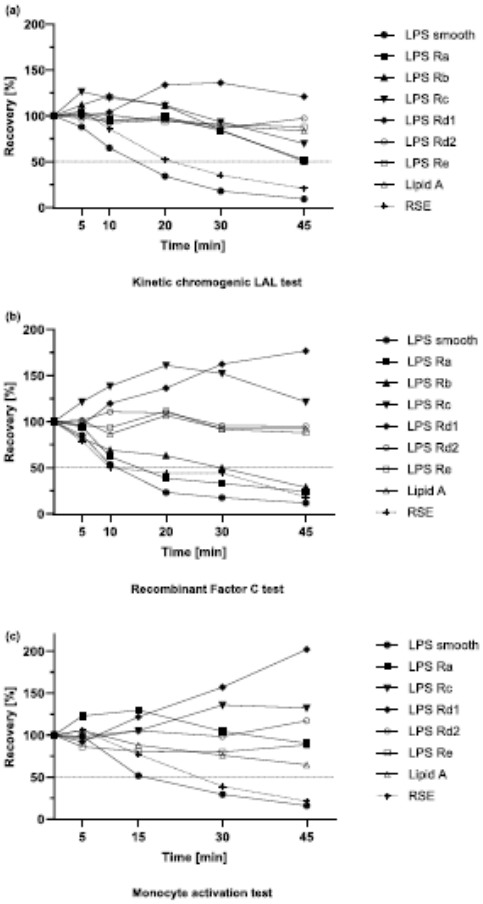

Fig. 4. Masking susceptibility of different mutants from *S. minnesota*. Endotoxin recovery was plotted as a function of incubation time. 100 EU/mL endotoxin from different mutants were spiked into samples containing 10 mM sodium citrate and 0.05 % (w/v) polysorbate 20 and incubated at room temperature (RT) for up to 45 min. For detection, (a) kinetic chromogenic LAL test, (b) recombinant Factor C test and (c) monocyte activation test were used. For calculation of the data points the mean values of three individually performed repetitions (n = 3) of the kinetics were used. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100 %.

which had shown increased endotoxin activity in both the LAL and rFC assays, also showed increased activity in the MAT. This activity exceeded 200 % after 45 min. It is important to note that no data is available for the Rb mutant in MAT as all measurements performed exceeded the detection limit.

3.4. Short-time analysis of the masking kinetics of the E. coli mutants

In the LAL-based assay conducted on *E. coli* mutants (Fig. 5 (a)), the fastest masking was observed for mutant Ra, with recoveries falling below 50 % after just 5 min. Smooth LPS, mutant Rc and RSE also showed masking, but at a slightly slower rate, with recoveries falling

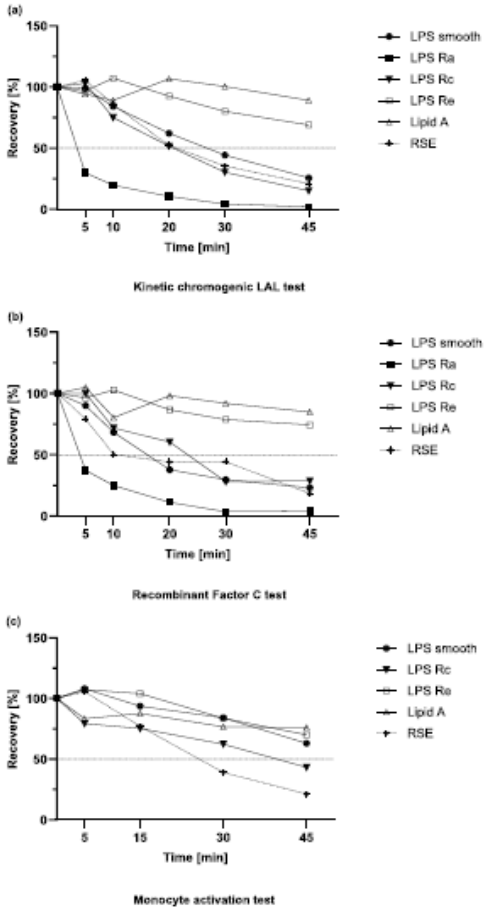

Fig. 5. Masking susceptibility of different mutants from *E. coli*. Endotoxin recovery was plotted as a function of incubation time. 100 EU/mL endotoxin from different mutants were spiked into samples containing 10 mM sodium citrate and 0.05 % (w/v) polysorbate 20 and incubated at room temperature (RT) for up to 45 min. For detection, (a) kinetic chromogenic LAL test, (b) recombinant Factor C test and (c) monocyte activation test were used. For calculation of the data points the mean values of three individually performed repetitions (n = 3) of the kinetics were used. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100 %.

below 50 % after 30 min. Similar to the mutants of S. minnesota, those with longer sugar chains (LPS smooth, Ra, Rc) displayed faster masking kinetics. Mutant Re and lipid A showed no masking after 45 min. The recovery was 69 % for Re and 89 % for lipid A.

The rFC assay (Fig. 5 (b)) showed similar recovery patterns to the LAL assay. Mutant Ra showed the fastest masking kinetics with recoveries below 50 % after 5 min, followed by RSE, smooth LPS and mutant Rc. RSE and smooth LPS had recoveries below 50 % after 20 min and mutant Rc after 30 min. Re and lipid A showed no masking behaviour within the time interval studied, with recoveries of 74 % and 85 % respectively.

In the MAT assay (Fig. 5 (c)), only the recovery of mutant Rc (as well as the RSE) fell below 50 % within 45 min. The smooth LPS, Re, and lipid A exhibited a trend toward lower recoveries over time, although they consistently remained above the 50 % threshold. Smooth LPS showed a recovery of 63 %, Re of 70 % and lipid A 76 % after 45 min. No data were available for mutant Ra in the MAT, since all measurements exceeded the limit of detection. While this mutant showed the fastest masking in the other assays, it is important to note that it also showed significantly increased activity in these assays compared to the water control (data not shown). However, this activity decreased rapidly over time. It is therefore surprising that this trend for mutant Ra is not reflected in the MAT measurements.

In summary, both the LAL and rFC assay showed comparable masking kinetics for the two bacterial strains. Specifically, S. Minnesota mutants showed recoveries below 50 % for smooth LPS to Rb mutants, whereas shorter mutants (Rc to lipid A) showed recoveries above 50 % for both. For MAT, the recovery rate was below 50 % only for smooth LPS in S. Minnesota. In *E. coli*, smooth LPS to Rb mutants showed recoveries below 50 % after 45 min in LAL and rFC assay, while recoveries were above 50 % for mutant Re and lipid A. The MAT showed recoveries below 50 % only for Rc mutant. The results showed that endotoxin was detectable for longer in MAT compared to LAL and rFC. In addition, S. Minnesota mutants showed a slower masking compared to *E. coli* mutants.

3.5. Long-time analysis of the masking kinetics of the S. minnesota mutants

After observing the masking kinetics within a 45 min interval and recognising the variability in masking behaviour among LPS mutants, we extended our investigations to include long-term kinetics at specific time points, including 24, 48, 72 and 96 h. Given the comparable kinetic patterns obtained from the LAL and rFC assays, the focus for further investigation was narrowed to the rFC assay and MAT.

For S. minnesota strains, including the Rc, Rd1, Rd2, Re, and lipid A mutants, no masking behaviour was evident within the first 45 min measured with the rFC assay (Fig. 4 (b)). The Rc mutant showed the first masking with recovery rates below 50 % after 48 h, while the remaining mutants showed recovery rates less than 50 % after 72 h (Fig. 6 (a)).

When S. minnesota mutants were subjected to MAT, only the smooth LPS mutant showed recovery rates below 50 % within 45 min (Fig. 4 (c)). Next, all mutants from Ra to lipid A were examined for their long-term kinetics (Fig. 6 (b)). After 24 h, both the Ra and Rb mutants and, surprisingly, lipid A showed recoveries below 50 %. Consistent with the results of the rFC assay (Fig. 6 (a)), all mutants showed masking behaviour after 72 h.

3.6. Long-time analysis of the masking kinetics of the E. coli mutants

In contrast to S. minnesota mutants, only the Re and lipid A mutants of the *E. coli* strain showed no masking behaviour within the first 45 min as measured by the rFC assay (Fig. 5 (b)). Thus, long-term kinetic analysis in the rFC assay was performed for these two mutants (Fig. 7 (a)). Mutant Re showed recoveries below 50 % after the 24 h, while lipid A showed similar results after 72 h, reflecting the kinetics observed for S. minnesota lipid A.

For measurements with MAT, in addition to mutant Re and lipid A, smooth LPS showed recoveries above 50 % at the 45 min time point (Fig. 5 (c)). Shown in Fig. 7 (b), both smooth LPS and mutant Re fell below 50 % recovery after 24 h, indicating masking. Lipid A showed a recovery below 50 % after 72 h, consistent with all other mutants from S. minnesota and *E. coli*.

Although none of the mutants tested were resistant to masking behaviour and showed recoveries below 50 % after 72 h at the latest, they exhibited different kinetics. Some mutants showed masking behaviour as early as 45 min, while others did not show it until 72 h.

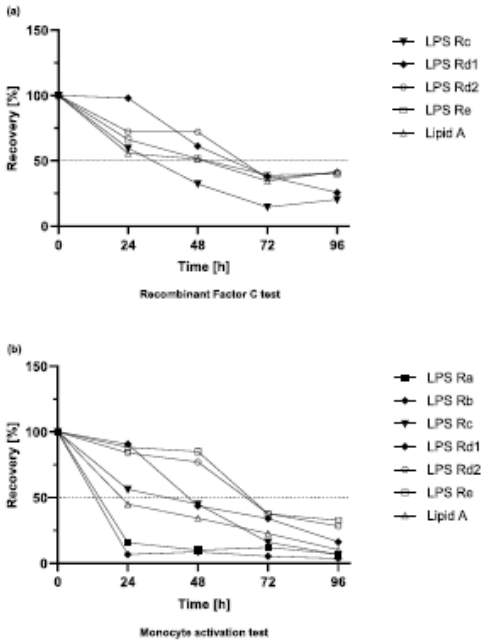

Fig. 6. Masking susceptibility of different mutants from *S. minnesota*. Endotoxin recovery was plotted as a function of incubation time. 100 EU/mL endotoxin from different mutants were spiked into samples containing 10 mM sodium citrate and 0.05 % (w/v) polysorbate 20 and incubated at room temperature (RT) for up to 96 h. For detection, (a) recombinant Factor C test and (b) monocyte activation test were used. For calculation of the data points the mean values of three individually performed repetitions (n = 3) of the kinetics were used. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100 %.

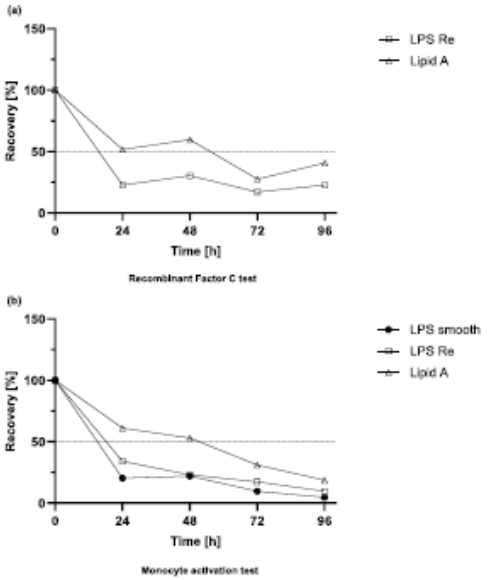

Fig. 7. Masking susceptibility of different mutants from *E. coli*. Endotoxin recovery was plotted as a function of incubation time. 100 EU/mL endotoxin from different mutants were spiked into samples containing 10 mM sodium citrate and 0.05 % (w/v) polysorbate 20 and incubated at room temperature (RT) for up to 96 h. For detection, (a) recombinant Factor C test and (b) monocyte activation test were used. For calculation of the data points the mean values of three individually performed repetitions (n = 3) of the kinetics were used. For a better comparison of independent measurements, the data was normalized and the starting points were set to 100 %.

4. Discussion

The detection of endotoxin levels is of paramount importance, particularly in the pharmaceutical and biomedical industries. Endotoxins represent one of the most prevalent and potent pyrogens, posing a significant risk of contamination in pharmaceutical products. Contaminations resulting from endotoxins can lead to fever and even progress to septic shock, underscoring the critical need for effective detection methods. Despite the availability of established detection methods, the challenge presented by LER phenomenon is noteworthy. Studies using defined structural endotoxin are recommended to assess the effectiveness of detection methods. However, natural contaminations are far more complex, given the infinite diversity of LPS arising from various growth conditions and mutations. Different masking susceptibilities are known, often with unknown underlying reasons [1,33,38,39]. It was aimed to investigate the effect of LPS mutants on the LER phenomenon and their detection using different test systems. The structure of LPS influences masking processes, which in turn has a profound effect on detectability. Interestingly, this phenomenon has not been extensively studied. This study therefore elucidates the effect of LPS mutants on LER and its detectability in the most commonly used endotoxin assays.

4.1. Activity of different LPS mutants

First, the endotoxin activities per mol of each mutant were determined, revealing a wide range of assay activities within the LPS structures. The activity of smooth LPS towards mutants with shorter sugar chains decreased for *S. minnesota* mutants, with the exception of the Ra mutant. A similar decrease in activity was observed for *E. coli* mutants, starting from mutant Rc to lipid A. Due to the strong tendency of LPS molecules to form aggregates, it is difficult to determine their exact molecular mass [39]. Since the molarities were estimated, there may be slight deviations in the activities per mol, especially for *E. coli* due to the presence of different core types with different polysaccharides, which will be discussed later. However, for both strains, lipid A showed the lowest activity, which was unexpected as lipid A is usually considered to be the toxic part of LPS [40,41]. The observed reduced activity could be associated with their shortened sugar chains, which consequently reduces the mutant's ability to form supramolecular structures required for detection by Factor C. This would be consistent with previous observations that the size and aggregation state of LPS aggregates correlate with activity in LAL [12,41]. Mueller et al. [42] found no biological activity of LPS monomers in either MAT or LAL. In addition to these observations the endotoxin must be aggregated for stimulation, other studys have shown that the structure of the aggregate aslo plays an important role [43,44]. In addition, changes in solubility due to the shorter sugar chain and greater hydrophobicity may also play a role by affecting conformations. Nevertheless, different lipid A structures should also be investigated in a follow-up study, as these have a major influence on the activity of the endotoxins. As demonstrated in Dardelle et al.'s [45] study, LPS exhibit varied reactions primarily due to differences in their lipid A composition, including variations in the number and length of fatty acid chains, the presence and positioning of phosphate groups, and any potential modifications or substitutions.

4.2. Comparison of assay systems for the detection of LPS mutants

In the first part of this study, we investigated the capability of different test systems to detect various LPS structures. All test methods, independent of the detection mechanism, were suitable for the detection of LPS mutants. Regarding naturallly occurring contaminations in water or pharmaceutical samples, it is crucial to ensure that a reliable test method is used. Natural occurring contaminations may consist of different LPS molecules from different strains comprising different chain lengths [6].

While significant differences between LAL, rFC, and MAT in *S. minnesota* mutants were mainly restricted to lipid A, for *E. coli* differences were detected within all mutants. Looking at the significant differences, almost all are within a factor of 2 for LAL and rFC measurements, which is not unusual for endotoxin measurements. Additionally, caution is advised when interpreting these statistically significant differences, as they are based on only three tests with relatively large standard deviations. Compared to MAT, LAL and rFC are based on the same mechanistic principle. Consistently, the results suggest that rFC is equivalent to LAL and can therefore be used to replace LAL. The significant differences between MAT and LAL assay are strikingly higher than within LAL/rFC. It is noteworthy that particularly for mutants possessing shorter sugar chains, the MAT tends to detect lower amounts of endotoxin activity. The MAT is based on the activation of immune cells, e.g. by LPS. Subsequently, immune cells express and release cytokines and chemokines. Mutants consisting of shorter sugar chains and lacking O-antigen are known to be less potent than those with O-antigen. This could be an explanation for the lower recovery of the MAT for mutants with shorter sugar chains [39,46].

It would be very interesting to investigate further mutants from different strains and determine the ability to detect these different LPS structures in common test systems. In this study, we focused on mutants of *E. coli* and *S. minnesota*. *E. coli*, chosen as a strain representing all international reference standards, served as a comparison and also provided insight into the variability in masking behaviour within a species. *Salmonella*, a common and abundant pathogenic strain, was used as an additional example of contamination, as these are the best characterised LPS mutants.

4.3. Masking kinetics of the mutants

To compare whether one of the assays has an advantage in terms of sensitivity, reliability and accuracy in detecting endotoxin in a masking matrix, a kinetic study was performed. The LAL and rFC assays showed similar kinetics in both mutant strains. Both assays showed masking for *S. minnesota* smooth LPS and the mutants Ra, Rb and Rc in the 45 min time interval, but no masking for the mutants Rc, Rd1, Rd2, Re and lipid A. For the *E. coli* mutants, both assays showed masking for smooth LPS, Ra and Rc, and no masking for Re and lipid A. The MAT initially appears to detect the selected LPS mutants in masking matrix better than the LAL and rFC assays, with most mutants (except smooth LPS from *S. minnesota* and LPS Rc from *E. coli* and the RSE) still showing over 50 % recovery in this assay after 45 min. In a study by Schwarz et al. [26], masked endotoxin was reported to be biologically active in inducing the release of proinflammatory cytokines in human monocytes. This could explain the higher residual recovery in MAT after 45 min, as Factor C may be less activated by masked LPS compared to the TLR-4 receptor. Negwer et al. [47] also demonstrate a significantly reduced but still present residual activity of masked LPS in MAT compared to LAL assays.

Recoveries between LAL and rFC were nearly identical for almost all mutants, considering masking criteria (50 %-200 % recovery). This suggests that the complete LAL cascade, compared to an assay exclusively containing Factor C, does not fundamentally differ in capturing masking kinetics. In addition, the possibility of false positives due to glucans is excluded. These would have activated the LAL cascade via Factor G, which is present in LAL but not in the recombinant Factor C assay [47].

However, it is important to note that LPS reacts very sensitive to the masking solution used in this study, which consists of citrate and polysorbate 20. Therefore, the kinetics show some variability. This becomes evident when examining the standard deviations of individual measurements (Supplement 1). Even replicates of RSE in the same matrix showed very high fluctuations. This is most likely attributed to the concentration of polysorbate 20; minimal changes here lead to highly varied reactions of the LPS. Of note, it has been demonstrated (data not shown) that a lower concentration of polysorbate 20 initially increases masking capability (while reducing masking time), but then a reduction in the concentration of polysorbate 20 reduces the masking capability again.

Masking kinetics are strongly influenced by the sample matrix. Polysorbate 20, combined with citrate buffer, is a potent inducer of masking, whereas polysorbate 80 with citrate buffer shows slower masking kinetics [24,48]. Recent studies conducted masking kinetics in a polysorbate 80-citrate matrix at and concluded that there was no masking of NOEs [49]. However, the conditions were not completely conclusive. The masking kinetics were only performed at 4 °C, which does not reflect the real scenario. It is known that masking is temperature dependent, with stronger masking at higher temperatures [24]. Other studies using polysorbate 20 demonstrated that NOEs also exhibit masking behavior, albeit with varying susceptibilities [26,33]. This highlights how significantly masking kinetics are influenced by the sample matrix as well as the molecular structure of the LPS.

The smooth LPS from *S. minnesota* consisting of the highest polysaccharide content, is masked most rapidly, followed by the Ra and Rb mutant with the second and third highest polysaccharide content. Although the Ra and Rb mutants exhibited recoveries above 50 % after 45 min in two out of three assays, they showed a tendency to lower recoveries, demonstrating a similar dependence of masking speed on polysaccharide content. For the five mutants with shorter polysaccharide chains (Rc, Rd1, Rd2, Re, and lipid A), there no masking was observed after 45 min. They neither exhibit lower recoveries nor a discernible trend towards decreasing recoveries. Only hold time studies of 5 days showed masking for these mutants. Consistently, the mutant with the fourth highest polysaccharide content (Rc) was the fourth fastest to be masked, considering both measurement methods for the long-time kinetics. The long-time masking kinetics suggest that the masking process is slowed down and that these mutants are not completely resistant to the masking phenomenon.

The LPS mutants of *S. minnesota* suggested that the rate of masking is closely linked to the proportion of polysaccharides. This proportion determines the ratio of hydrophilicity to hydrophobicity, as lipid A remains constant within a species [7]. Therefore, rougher mutants exhibit higher hydrophobicity. Altering this ratio in amphiphilic molecules results in a modification of the physicochemical properties of aggregates. Smooth LPS from *S. minnesota* forms micelles with a diameter of approximately 24 nm, while the Re mutant of *S. minnesota* forms micelles with a diameter of about 91 nm [36]. In addition to a reduction in the hydrophilic-to-hydrophobic ratio, the shortening of the polysaccharide chain of LPS indirectly resulted in an increased charge density in LPS mutants. This is because the negatively charged groups of LPS are primarily located in the inner core region [50]. When mutants lack an outer core, as seen in the Rd1 mutant up to lipid A, some of the negatively charged groups are still present. However, the outer core polysaccharides (typically neutral), onto which the negative charge can be distributed, are absent.

Reich et al. [24] reported that the rate-determining step in the demasking process is the removal of divalent cations. Only after these cations are removed by a chelator, the detergent can integrate and alter the supramolecular structure. The impact of these negative charges is also evident in the examination of Rice's work in 2018 [51]. In a Rd1 mutant of *S. minnesota*, bridges are formed between the HEP5 and HEP3 phosphate groups through Ca^{2+}. However, the Rd2 mutant lacks the

HEPS sugar, preventing the salt bridge formation in a similar manner. This could explain why a significant decrease in recovery is observed in the Rd2 mutant after 24 h (for the rFC assay) and after 48 h (for the MAT), while the recovery of the Rd1 mutant remains unchanged.

Wahib Sali [36] demonstrated that rough mutants tend to exhibit larger negative charge densities. These charge densities enhance electrostatic interactions, triggered by higher affinities to divalent cations. The magnitude of this influence could be investigated by further studies, in which mutants are exposed to different concentrations of the chelator at a constant detergent concentration, similar to the studies of Reich et al. [24].

Smooth LPS is not the fastest masked mutant for *E. coli*. The increase in masking speed cannot be solely explained by the hydrophobic-to-hydrophilic ratio. It is noteworthy that, unlike *S. minnesota* mutants, which have only one core polysaccharide type, *E. coli* has five distinct core polysaccharide types [41]. These diverse core polysaccharides may also influence the remarkably low endotoxin activity of the Ra mutant. In contrast to other mutants, the reactivity of the Ra mutant is 10–100 times lower. The Ra mutant, classified as core type R2, has a neutral Gal in the inner core [52]. Other studies have shown that differences in charged molecules, such as inner core sugars and associated functional groups, can also affect reactivity. For example, it has been shown that the removal of a negative charge results in a 100-fold reduction in activation [53].

Smooth LPS corresponds to an *E. coli* core type R1 [54]. This core type differs from others by having glucosamine in the inner core region. This saccharide has a positively charged amino group, which allows LPS molecules to interact with each other even in the absence of divalent cations. There are studies describing that the incorporation of aminoarabinose, also a sugar with an amino group, leads to a higher number of inter-lipid hydrogen bonds among LPS molecules [51]. This additional stabilisation reduces the significance of salt bridges involving divalent cations. Even though glucosamine in the inner core of smooth LPS from *E. coli* lowers the negative charge density and thereby reduces the affinity for Ca^{2+}, it might enable slowed masking through this additional stabilisation [7].

The mutants of both strains exhibited a comparable dependence of the masking susceptibility on polysaccharide chain length, although the influence was more pronounced in *S. minnesota*. For *E. coli* mutants, an additional dependence on different core types was assumed. When comparing the respective mutants of both species, it was found that *E. coli* mutants tended to mask more rapidly.

The main differences between the mutants lie in their lipid A. For *E. coli*, lipid A is hexa-acylated in about 90 % of cases and penta-acylated in about 10 % of cases [55]. In *S. minnesota*, lipid A is 20 %–70 % hepta-acylated, depending on growth conditions [56]. For the same LPS mutants (i.e., with the same saccharide chain length), this results in higher hydrophobicity in *S. minnesota* mutants compared to *E.coli* mutants, leading to a slowed masking process. Additionally, *S. minnesota* LPS can substitute aminoarabinose sugars depending on growth conditions, contributing to additional aggregate stabilisation through hydrogen bonding, as described above [57]. This observation aligns with findings by Reich et al. [33], where several naturally occurring endotoxins (NOE) were examined for masking. The NOE from Burkholderia Cepacia did not mask over an extended period compared to others. LPS from the Burkholderia genus often carries multiple aminoarabinoses, which are substituted on both lipid A and the core region [58].

5. Conclusions

Our research shows that the structural properties of LPS play a critical role in influencing both its potency and its detection. One key factor is the length of the polysaccharide chain, which significantly modulates LPS activity. For example, rough LPS mutants exhibit greater variability in detection compared to their smooth counterparts, highlighting the importance of structural differences. Furthermore, the masking kinetics of LPS are determined by its molecular structure, with rough LPS mutants exhibiting slower masking kinetics than smooth LPS. This suggests that different LPS structures interact uniquely with surrounding substances in a given sample. The hydrophilic to hydrophobic ratio plays a critical role in masking susceptibility. A lower ratio, as seen in mutants with shorter polysaccharide chains, corresponds to a lower masking rate. In addition, rough LPS mutants tend to carry an increased negative charge, indicating a greater affinity for divalent cations, which may stabilize supramolecular structures and reduce susceptibility to masking.

The use of a model matrix for masking in this study has improved our understanding of masking mechanisms. However, further studies are needed to explore the influence of LPS structure in other masking matrices. In addition to molecular structure, the sample matrix itself has a significant influence on masking kinetics. Components such as polysorbate 20 vs. polysorbate 80, buffers such as citrate, phosphate, or histidine, and protein structure strongly influence the extent of masking. Thus, the factor of structural variability investigated in this study is only one of several influencing factors.

Contamination can be highly heterogeneous and unpredictable. Some studies suggest that using NOEs as reference materials in LER studies could provide a more realistic assessment of masking behavior compared to RSE. However, this approach has limitations. Because the structure of environmental endotoxin contamination is usually unknown and different forms of LPS have different susceptibilities to masking, using NOEs as reference materials may not be practical or predictive for all contamination scenarios. It is likely that real-world contamination consists of a mixture of rough and smooth LPS, which adds complexity to detection and recovery. Our results show that smooth LPS is particularly susceptible to masking, making it a "worst-case" scenario for endotoxin detection. Consequently, RSE composed of smooth LPS provides a suitable spike material for LER studies due to its high susceptibility to masking, also in accordance with PDA Technical Report 82 [59].

CRediT authorship contribution statement

Luisa Burgmaier: Writing – original draft, Visualization, Formal analysis, Conceptualization. **Stefan Pölt:** Writing – review & editing, Formal analysis, Conceptualization. **Meltem Avci-Adali:** Writing – review & editing. **Johannes Reich:** Writing – review & editing, Supervision, Conceptualization.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2024.101808.

Appendix

References

[1] Case Studies of Microbial Contamination in Biologic Product Manufacturing," https://www.americanpharmaceuticalreview.com/Featured-Articles/36755-Case-Studies-of-Microbial-Contamination-in-Biologic-Product-Manufacturing/(currentasofJan. 13, 2024).
[2] Esmaeilli D, et al. Bioactivity and immunological evaluation of LPS from different serotypes of Helicobacter pylori. Iran J Microbiol 2013;5(2):142.
[3] Martich GD, et al. Response of man to endotoxin. Immunobiology 1993;187(3–5):403.
[4] Cohen J. The immunopathogenesis of sepsis. Nature 2002;420(6917):885.
[5] Ciesielska A, et al. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cellular and molecular life sciences CMLS 2021;78(4):1233.
[6] Simpson BW, Trent MS. Pushing the envelope: LPS modifications and their consequences. Nat Rev Microbiol 2019;17(7):403.
[7] Gorman A, Golovanov AP. Lipopolysaccharide structure and the phenomenon of low endotoxin recovery. European J Pharmaceutics Biopharmaceut J Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V 2022;180:289.
[8] Hitchcock PJ, et al. Lipopolysaccharide nomenclature–past, present, and future. J Bacteriol 1986;166(3):699.
[9] Complete structural determination of lipopolysaccharide obtained from deep rough mutant of Escherichia coli. Purification by high performance liquid chromatography. 1988.
[10] Agrawal PK, et al. 1H and13C NMR assignments of a lipopolysaccharide obtained from the deep rough mutant ofEscherichia coliD31m4. Magn Reson Chem 1998;36(1):1.
[11] Li Y, et al. Endotoxin contamination in nanomaterials leads to the misinterpretation of immunosafety results. Front Immunol 2017;8:472.
[12] Wang C, et al. Understanding lipopolysaccharide aggregation and its influence on activation of Factor C. J Colloid Interface Sci 2019;552:540.
[13] Cirefice G, et al. The future of pyrogenicity testing: phasing out the rabbit pyrogen test. A meeting report. Biol J Int Associat Biol Standard 2023;84:101702.
[14] Vipond C, et al. Limitations of the rabbit pyrogen test for assessing meningococcal OMV based vaccines. ALTEX 2016;33(1):47.
[15] Spoladore J, et al. Standardized pyrogen testing of medical products with the bacterial endotoxin test (BET) as a substitute for rabbit Pyrogen testing (RPT): a scoping review. Technol In Vitro Int J 2021;74:105160. *published in association with BIBRA.*
[16] Schneier M, et al. Current technologies to endotoxin detection and removal for biopharmaceutical purification. Biotechnol Bioeng 2020;117(8):2588.
[17] Brown J, et al. Using the monocyte activation test as a stand-alone release test for medical devices. ALTEX 2021;38(1):151.
[18] Park C-Y, et al. Comparison of the rabbit pyrogen test and Limulus amoebocyte lysate (LAL) assay for endotoxin in hepatitis B vaccines and the effect of aluminum hydroxide. Biol J Int Associat Biol Standard 2005;33(3):145.
[19] Piehler M, et al. Comparison of LAL and rFC assays-participation in a proficiency test program between 2014 and 2019. Microorganisms 2020;8:3.
[20] Chen J VA. Low endotoxin recovery in common biologics products. 2013. Orlando, FL.
[21] Cao Y, et al. Low endotoxin recovery and its impact on endotoxin detection. Biopolymers 2021;112(11):e23470.
[22] Reich J, et al. Investigation of the kinetics and mechanism of low endotoxin recovery in a matrix for biopharmaceutical drug products. Biol J Int Associat Biol Standard 2018;53:1.
[23] Tsuchiya M. Factors affecting reduction of reference endotoxin standard activity caused by chelating agent/detergent matrices: kinetic analysis of low endotoxin recovery. PDA J Pharm Sci Technol 2017;71(6):478.
[24] Reich J, et al. Masking of endotoxin in surfactant samples: effects on Limulus-based detection systems. Biol J Int Associat Biol Standard 2016;44(5):417.
[25] Bolden J, et al. Results of a harmonized endotoxin recovery study protocol evaluation by 14 BioPhorum Operations Group (BPOG) member companies. Biol J Int Associat Biol Standard 2017;48:74.
[26] Schwarz H, et al. Biological activity of masked endotoxin. Sci Rep 2017;7:44750.
[27] Bech Ørving R, et al. Bacterial endotoxin testing-fast endotoxin masking kinetics in the presence of lauryldimethylamine oxide. Microorganisms 2020;8:11.
[28] Schromm AB, et al. Supramolecular assembly of micellar aggregates is the basis of low endotoxin recovery (LER) in a drug formulation that can be resolved by a whole blood assay. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2024;173:116286.
[29] Kroschinsky F, et al. New drugs, new toxicities: severe side effects of modern targeted and immunotherapy of cancer and their management. Crit Care 2017;21(1):89.
[30] DRUGS.COM. "Muromonab-cd3 Side Effects: Common, Severe, Long Term," https://www.drugs.com/sfx/muromonab-cd3-side-effects.html (current as of Oct. 16, 2024).
[31] *FDA* (Sat, 9 February 2019).
[32] Tsuchiya M. Sample treatments that solve low endotoxin recovery issues. PDA J Pharm Sci Technol 2019;73(5):433.
[33] Reich J, et al. Low endotoxin recovery-masking of naturally occurring endotoxin. Int J Mol Sci 2019;20:4.
[34] Rimler RB. Comparisons of Pasteurella multocida lipopolysaccharides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine relationship between group B and E hemorrhagic septicemia strains and serologically related group A strains. J Clin Microbiol 1990;28(4):654.
[35] Black IM, et al. Structure of lipopolysaccharide from liberibacter crescens is low molecular weight and offers insight into candidatus liberibacter biology. LIMS 2021;22(20):11240.
[36] Sali W, et al. Polysaccharide chain length of lipopolysaccharides from Salmonella Minnesota is a determinant of aggregate stability, plasma residence time and proinflammatory propensity in vivo. Front Microbiol 2019;10:1774.
[37] Pupo E, et al. Intact rough- and smooth-form lipopolysaccharides from Escherichia coli separated by preparative gel electrophoresis exhibit differential biologic activity in human macrophages. FEBS J 2013;280(4):1095.
[38] Low Lipopolysaccharide Recovery versus Low Endotoxin Recovery in Common Biological Product Matrices," https://www.americanpharmaceuticalreview.com/Featured-Articles/167451-Low-Lipopolysaccharide-Recovery-versus-Low-Endotoxin-Recovery-in-Common-Biological-Product-Matrices/(currentasofJan.13, 2024).
[39] Caroff M, Karibian D. Structure of bacterial lipopolysaccharides. Carbohydr Res 2003;338(23):2431.
[40] Raetz CRH, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem 2002;71:635.
[41] Gutsmann T, et al. Structural prerequisites for endotoxic activity in the Limulus test as compared to cytokine production in mononuclear cells. Innate Immun 2010;16(1):39.
[42] Mueller M, et al. Aggregates are the biologically active units of endotoxin. J Biol Chem 2004;279(25):26307.
[43] Brandenburg K, et al. The expression of endotoxic activity in the Limulus test as compared to cytokine production in immune cells. Curr Med Chem 2009;16(21):2653.
[44] Dardelle F, et al. Diversity, complexity, and specificity of bacterial lipopolysaccharide (LPS) structures impacting their detection and quantification. Int J Mol Sci 2024;25:7.
[45] Jerala R. Structural biology of the LPS recognition. Int J Med Microbiol IJMM 2007;297(5):353.
[46] Negwer A. Low Endotoxin Recovery Wiederfindung und pyrogene Aktivität von Endotoxinen. 2020.
[47] Roslansky PF, Novitsky TJ. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. J Clin Microbiol 1991;29(11):2477.
[48] Correa W, et al. Biophysical analysis of lipopolysaccharide formulations for an understanding of the low endotoxin recovery (LER) phenomenon. LIMS 2017;18(12):2737.
[49] Bolden J, et al. It is time to reconsider the use of naturally-occurring endotoxins in endotoxin recovery studies: Part 2 of a BioPhorum harmonized endotoxin recovery study. Biol J Int Associat Biol Standard 2024;87:101779.
[50] Wilkinson SG. Bacterial lipopolysaccharides–themes and variations. Prog Lipid Res 1996;35(3):283.
[51] Rice A, Wereszczynski J. Atomistic scale effects of lipopolysaccharide modifications on bacterial outer membrane defenses. Biophys J 2018;114(6):1389.
[52] Hämmerling G, et al. Structural investigations on the core polysaccharide of Escherichia coli 0100. Eur J Biochem 1971;22(3):331.
[53] Rietschel ET, et al. The chemical structure of bacterial endotoxin in relation to bioactivity. Immunobiology 1993;187(3–5):169.
[54] Orskov I, et al. Serology, chemistry, and genetics of O and K antigens of Escherichia coli. Bacteriol Rev 1977;41(3):667.
[55] Qureshi N, et al. Complete structural determination of lipopolysaccharide obtained from deep rough mutant of Escherichia coli. Purification by high performance liquid chromatography and direct analysis by plasma desorption mass spectrometry. J Biol Chem 1988;263(24):11971.
[56] Galanos C, et al. Biological activity of synthetic heptaacyl lipid A representing a component of Salmonella Minnesota R595 lipid A. Eur J Biochem 1986;160(1):55.
[57] García Véscovi E, et al. Mg2+ as an extracellular signal: environmental regulation of Salmonella virulence. Cell 1996;84(1):165.
[58] de Soyza A, et al. Chemical and biological features of Burkholderia cepacia complex lipopolysaccharides. Innate Immun 2008;14(3):127.
[59] *Low endotoxin recovery*, No. 82. Bethesda: Parenteral Drug Association; 2019.

9.1.3 Publication III

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Endotoxin masking in human plasma: The role of protein interactions and lipopolysaccharide structure

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ABSTRACT

Reliable endotoxin detection in human plasma is compromised by masking effects, which interfere with Limulus Amebocyte Lysate (LAL)-based assays. While electrostatic interactions have been considered a major cause of masking, our study demonstrates that they alone cannot fully explain the phenomenon. We show that masking occurs rapidly in plasma, with endotoxin recoveries dropping below 50% within minutes. Although increasing the pH to 12 partially restores detection, high-salt treatments fail to disrupt endotoxin-protein complexes, indicating additional stabilizing forces. Plasma fractionation experiments revealed that specific proteins, particularly lysozyme, contribute significantly to masking, while human serum albumin plays only a minor role at physiological concentrations. Furthermore, structural differences between lipopolysaccharides (LPS) influence masking behavior: smooth LPS variants are masked more rapidly than rough mutants, suggesting that hydrophilic interactions and molecular conformation play a crucial role. Our findings highlight that endotoxin masking is not solely driven by electrostatic interactions but results from a complex interplay of structural and biochemical factors. Recognizing these mechanisms is essential for developing reliable detection strategies, ensuring the accuracy of endotoxin testing in clinical and pharmaceutical applications.

1. Introduction

Endotoxins, also known as lipopolysaccharides (LPS), are essential components of the outer membrane of gram-negative bacteria [1,2]. Their structural complexity and biological activity play a critical role in bacterial infections, triggering robust immune responses. When endotoxins enter the bloodstream, they are recognised by the immune system, primarily through binding to Toll-like receptor 4 (TLR4). This interaction activates the innate immune response, resulting in the release of pro-inflammatory cytokines [3]. While this response is essential to eliminate invading pathogens, excessive activation can lead to severe inflammatory conditions such as sepsis and septic shock, highlighting endotoxins as key players in disease pathology [4]. Because of their pathogenic potential, strict monitoring of endotoxin contamination is essential, particularly in intravenous drugs and biologicals. Regulatory standards, as outlined in various pharmacopoeias, set strict limits for endotoxin levels (5 endotoxin units per kg body weight per hour for intravenous applications) [5,6]. Adherence to these guidelines is essential to ensure patient safety, particularly in clinical settings where immunocompromised individuals are at high risk.

Sepsis, a life-threatening condition characterised by an extreme immune response to infection, remains a major clinical challenge. It can be caused by gram-negative or gram-positive bacteria and even fungal components [7,8]. Current diagnostic methods rely on clinical assessment and biomarkers such as procalcitonin and C-reactive protein, which indicate systemic inflammation but do not directly identify the underlying pathogens [9]. Early and accurate detection of endotoxins in blood could serve as a critical marker for sepsis diagnosis, allowing for prompt and targeted treatment. Integrating endotoxin assays into diagnostic protocols could improve specificity, allowing clinicians to differentiate between bacterial and non-bacterial causes of inflammation. Despite the promise of endotoxin detection in the diagnosis of sepsis, there is still no reliable detection method. [10]. Blood cultures, the gold standard for identifying bacterial infections, are time-consuming and often have low sensitivity, especially after antibiotic administration [11,12]. Non-culture-based methods, such as the Limulus Amebocyte Lysate (LAL) assay and the recombinant Factor C assay, offer a faster alternative for the detection of endotoxins [13,14].

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However, their clinical utility has been limited by inconsistencies between LAL-detected endotoxin levels and patient clinical status or disease severity [10,15–17].

One of the major challenges in applying the LAL assay to human blood is the phenomenon of endotoxin masking, which can lead to false-negative results [18–20]. This phenomenon, also known as Low Endotoxin Recovery (LER), complicates the detection process by making endotoxins undetectable by current methods despite their presence. In the complex matrix of human plasma, various substances may interfere with the detection process [21,22]. Plasma components, including proteins, lipoproteins, and bile salts, contribute to LER by interacting with endotoxins and altering their structural and chemical properties. Amphiphilic and cationic polypeptides, as well as various plasma proteins, chelating agents, and surfactants, can bind to LPS, inactivating it and preventing it from triggering Factor C in the LAL assay [23–26].

Accurate endotoxin detection is critical not only for the treatment and prevention of sepsis but also for ensuring the safety of blood products and pharmaceuticals. Undetected endotoxin contamination poses a serious risk to patient safety, particularly in vulnerable populations relying on intravenous therapies [27]. Therefore, understanding the mechanisms behind endotoxin masking and developing strategies to counteract these effects is critical. Improving the performance of the LAL-based test methods, which remains the gold standard for endotoxin detection in industrial and pharmaceutical settings, requires addressing these inhibitory factors and optimising sample preparation methods. Various approaches have been proposed to mitigate the inhibitory effects of plasma components on LAL assays, such as sample dilution and heating [28–30]. However, these methods have limitations and a standardised, reliable method for the quantification of endotoxin in human plasma has yet to be established. For example, while sample dilution can reduce the concentration of inhibitors, it also dilutes the endotoxin, reducing detection sensitivity. Heat treatment can denature proteins and reduce their binding to LPS, but it can also affect the structure and reactivity of endotoxin.

Understanding and addressing the challenges presented by the complex matrix of human blood are essential for enhancing the reliability of LAL assays in clinical diagnostics and pharmaceutical quality control. This study aims to identify the factors and binding mechanisms that interfere with endotoxin detection in human blood, explore why previous methods have failed to achieve consistent results and elucidate the underlying mechanisms. Overcoming these challenges will improve diagnostic accuracy, enhance treatment outcomes for sepsis patients, and ensure the safety of therapeutic products.

2. Materials and methods

2.1. Collection of human blood plasma

The study was conducted in accordance with the guidelines of the Declaration of the World Medical Association of Helsinki, and the plasma used in the following experiments was obtained by internal blood collection. Blood samples were collected from 10 donors by trained personnel using the Vacuette R safety blood collection set to obtain whole human blood. Lithium heparin tubes (BD VACUETTE® TUBE 9 ml LH Lithium Heparin; Becton Dickinson (BD) GmbH, Heidelberg, Germany) were used, which were then centrifuged at 2000 g for 10 min. The separated plasma was collected, pooled, and frozen in cryotubes at −80 °C. These samples were thawed and used for all experiments in this paper. Written consent to participate and approval for publication was obtained from each volunteer.

2.2. Sample spiking with endotoxin

Unless otherwise specified samples were prepared in 5 mL glass tubes (EndoGrade Glass Test Tubes from BioMerieux, Marcy-l'Étoile, France) and spiked with reference standard endotoxin (RSE; *E.coli* O113:*H*10:K; EDQM Council of Europe, Strasbourg, France) from a 10,000 EU/mL stock solution. The stock solution was shaken at 1400 rpm for 30 min before the spikes were added to the samples. All samples were spiked to a theoretical endotoxin value of 100 EU/mL.

2.3. Endotoxin masking kinetics of human whole blood and plasma

Fresh human heparinised blood and human plasma samples were prepared in 5 mL glass tubes with a volume of 500 µL per sample. Samples were spiked and incubated at room temperature. After 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 4 h, and 24 h, a 5 µL aliquot was taken from each sample and diluted 1:500 in LRW (LAL reagent water; EndoGrade Water from BioMerieux, Marcy-l'Étoile, France). Prior to the experiments, the samples were tested for initial endotoxin content. For both whole blood and plasma, endotoxin levels were below the limit of detection (LOD < 2.5 EU/mL).

2.4. pH shift of the plasma samples

Human plasma samples were prepared in 5 mL glass tubes with a volume of 500 µL per sample. A 2 M NaOH solution was used to adjust the pH to 11 and 12, pH was tested with pH test strips (Merck KGaA, Darmstadt, Germany). Samples were spiked and incubated for 1 h at room temperature and then 5 µL aliquot was taken from each sample and diluted 1:500 in LRW.

2.5. Plasma separation by size exclusion chromatography (SEC)

A 10 mL sample of human plasma was applied to a self-packed Sephacryl™ S-300 HR column (column dimensions: 50 mm × 480 mm; bed volume: 940 mL; resin from Cytiva, Marlborough, MA, USA). The separation range for globular proteins of 10—1500 kDa. 1x PBS (Dulbecco's Balanced Salt Solution (DPBS) without Ca, Mg, pH: 7.0 – 7.3; Gibco™) was used as the running buffer. The flow rate was adjusted to 1 mL/min. A volume of 5 ml per fraction was collected, resulting in 67 fractions.

To characterise the fractions, an SDS gel of the fraction groups was performed and the protein concentration of the samples was determined according to Bradford (as described below). For the analysis of the masking behaviour of the individual fractions, samples were prepared in 5 mL glass tubes with a sample volume of 1 mL per sample. The samples were spiked and incubated at room temperature for 24 h. A 5 µL aliquot was taken from each sample and diluted 1:500 in LRW.

2.6. Masking behaviour of plasma proteins

For human serum albumin (Cat. No. 1850–0028, SeraCare, Milford, MA, USA), solutions ranging from 5 mg/mL to 200 mg/mL were prepared according to physiological conditions. For lysozyme (Cat. No. L1667, Sigma-Aldrich, St. Louis, MO, USA) solutions ranging from 1 µg/mL to 1000 µg/mL were prepared. Stock solutions were tested for endotoxin content and found to contain < 2.5 EU/mL at the highest concentrations tested (200 mg/mL for HSA and 1000 µg/mL for lysozyme). Samples were prepared in 5 mL glass tubes with a sample volume of 500 µL per sample. Samples were spiked and incubated for 1 h at room temperature and then a 5 µL aliquot was taken from each sample and diluted 1:500 in LRW.

2.7. Approaches for overcoming masking

Human plasma samples were prepared in 5 mL glass tubes with a sample volume of 500 µL per sample. The samples were spiked and incubated for 1 h to ensure masking.

A high-salt approach using varying molarities of sodium chloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was employed. Therefore, 3 M NaCl stock solution in TRIS buffer pH 8 (Sigma-Aldrich

Chemie GmbH, Steinheim, Germany) was prepared, and 100 µL of this solution was added to 500 µL of the masked sample. In a second experimental setup, 6 M NaCl was used and combined with the masked sample at different ratios (20:1, 10:1, 5:1, 4:1, 2:1). Afterwards samples were diluted 1:500 in LRW.

A pH shift approach was also used. The pH was shifted using 2 M NaOH. Appropriate volumes were added to the masked sample and the pH was tested using pH test strips (Merck KGaA, Darmstadt, Germany). In the first experiment, the samples were diluted at a ratio of 1:500 in LRW water, and in the second experiment at a ratio of 1:500 in dilution buffers. Calcium chloride and magnesium chloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solutions were prepared (concentrations of $CaCl_2$ solution: 0.1, 0.5, and 1 mM, concentrations for $MgCl_2$ solution: 1, 5, and 10 mM.

2.8. Behaviour of different LPS structures

LPS mutants from *Salmonella minnesota* (S-form), R60 (Ra), R5 (Rc), R595 (Re) as well as the Lipid A from *S. minnesota* R595 were obtained from BIOMOL GmbH, Hamburg, Germany. The provided concentration was 1 mg/mL for each solution. To determine the endotoxin activity, solution of the respective mutant were measured using a kinetic chromogenic assay (Kinetic-QCL™). The detected activities were used to prepare 10,000 EU/mL solutions of each of the LPS mutants. Human plasma samples were prepared in 5 mL glass tubes with a sample volume of 500 µL per sample. Samples were spiked with 10 µL lipopolysaccharide (LPS mutant) from a 10,000 EU/mL stock solution. The stock solution was shaken at 1400 rpm for 30 min before the spikes were added to the samples. After spiking, samples were incubated at room temperature. After 10 min, 30 min, 60 min, 120 min, and 24 h, a 5 µL aliquot was taken from each sample and diluted 1:500 in LRW.

2.9. Detection of endotoxin

The kinetic chromogenic assay (Kinetic-QCL™; Lonza, Walkersville, MD) was used to detect endotoxin activities. All samples were diluted 1:500 with LRW or equivalent buffer to avoid plasma interference with the LAL assay. The validity of the measurements was confirmed by spiking defined amounts of endotoxin into the diluted samples, referred to as the Positive Product Control (PPC). 100 µL of each standard, sample, and PPC were transferred in duplicate to a 96-well microplate. For the PPC wells, 10 µL of a 5 EU/mL standard endotoxin solution was added, resulting in a final spike concentration of 0.5 EU/mL. The plate was then incubated for at least 10 min at 37 °C in a microplate reader. Then 100 µL of reconstituted LAL reagent was rapidly added. The amount of chromogenic substrate released was measured at 405 nm using a BioTek ELX808 absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT) coupled to WinKQCL™ software version 5.3.3 (Lonza Walkersville, MD) at 37 ± 1 °C. The mean of the duplicates per sample was used to determine endotoxin concentrations (EU/mL) using a standard curve fitted with a linear regression model. The sensitivity of the assay was 0.005 to 50 EU/mL.

2.10. Gel electrophoreses

Plasma fractions were separated by 4 – 12 % SDS-PAGE and protein bands were visualised by Coomassie Blue staining. A ServaGel Neutral HSE gel (SERVA Electrophoresis GmbH, Heidelberg, Germany) was used to monitor proteins from 6.5 to 200 kDa. Running buffer, protein marker and staining solution were purchased from Thermo Fisher Scientific Inc, Waltham, MA.

2.11. Determination of protein concentration

Protein concentrations were determined using the Bio-Rad (Hercules, CA) Protein Assay based on the Bradford method. The standard procedure for microtiter plates was used. A BSA standard curve was used as the protein standard. The standard and samples were assayed in duplicate. Absorbance was measured at 595 nm using an absorbance microplate reader (Microplate Reader 3550, Bio-Rad, Hercules, CA).

2.12. Calculation of endotoxin recovery and statistics

The endotoxin concentrations determined in the samples were compared with the theoretical endotoxin concentrations and expressed as a percentage.

Calculations of means, standard deviations (SD), and all other statistical tests were performed using Graph Pad Prism, version 10.1.1. p-values less than 0.05 were considered statistically significant. Significance is indicated in the manuscript as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Endotoxin masking kinetics of human whole blood and plasma

To determine the masking behaviour of human whole blood and plasma, endotoxin recovery was recorded over time (Fig. 1). At time 0, the recoveries of whole blood and plasma were very distinct. While whole blood showed a recovery of 120 % ± 13 %, plasma had a diminished recovery of only 58 % ± 6 %. The recovery in whole blood remained above 50 % for the first 60 min, ranging from 113 % ± 30 % to 66 % ± 12 %. After 4 h, the recovery was only 30 % ± 6 %, with no further decrease after 24 h. For plasma, recovery drops below 50 % after the first 5 min to 29 % ± 7 %. Recovery further decreased to 16 % ± 5 % at 30 min and remained there at 4 h (17 % ± 5 %). After 24 h, the recovery rate had increased slightly to 26 % ± 12 %. After 24 h, the recovery rates in plasma and whole blood were similar, but the results showed that masking kinetics were faster in plasma than in whole blood. This suggests that plasma lacking cellular components is more susceptible to masking. Human plasma was therefore chosen for further analysis.

3.2. Influence of alkaline plasma on endotoxin masking

Based on the previous results, we hypothesized that endotoxin masking in human plasma is primarily driven by electrostatic interactions rather than hydrophobic effects [22,31]. To investigate the potential role of electrostatic interactions, endotoxin recovery was tested in plasma samples with different pH levels.

Samples at pH 8 (initial pH of plasma sample) were compared with samples adjusted to pH 11 and pH 12. At pH 8, endotoxin recovery was 15.5 % ± 11.5 %. At pH 11 there was a slight improvement in recovery to 20.5 % ± 4.4 %. However, at pH 12 the recovery increased significantly to 57 % ± 11.9 % (Fig. 2).

These results suggest that electrostatic interactions could play a key role in endotoxin masking, as an elevated pH level of 12 led to increased endotoxin recovery. Of note, the assay criteria remained valid independent of the pH, confirming that the reduced recovery at lower pH levels is not due to assay limitations but rather to specific sample-dependent effects.

3.3. Plasma separation by size exclusion chromatography (SEC) reveals specific protein groups involved in endotoxin masking

To further investigate the proteins involved in the masking process, plasma samples were separated by size using SEC. A total of 67 fractions were collected and analysed for protein concentration and endotoxin masking behaviour. The protein concentration and endotoxin recovery of each fraction are shown in Fig. 3 (a). Protein concentrations of the fractions varied between 0.1 mg/mL and 8.9 mg/mL. The protein concentrations exhibit minimal values for fractions 1 through 21, with a

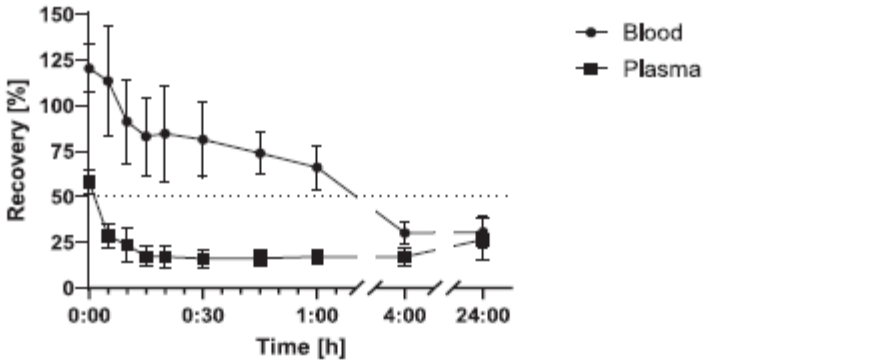

Fig. 1. Masking kinetics in human heparinized whole blood and heparin plasma. Endotoxin recovery was determined by kinetic chromogenic LAL test and plotted as a function of incubation time. 100 EU/mL endotoxin was spiked into samples and incubated at room temperature (RT) for up to 24 h. Data points were calculated using mean kinetics from five healthy donors (n = 5) and error bars reflect the standard deviation. The dotted line represents 50 % recovery.

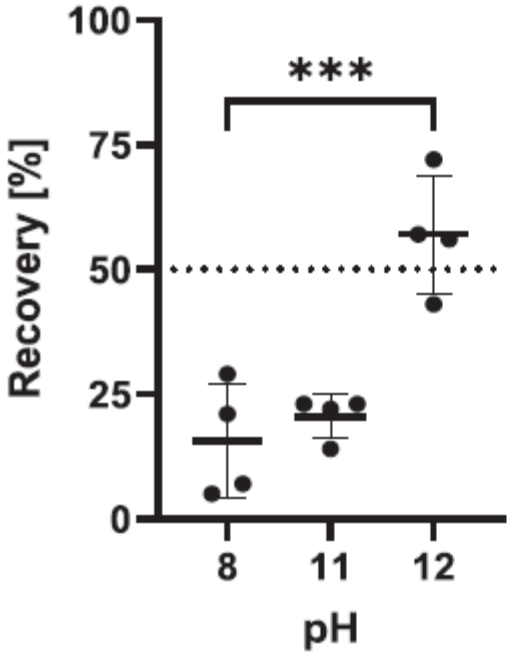

Fig. 2. Endotoxin recovery at different pH levels. Plasma samples were adjusted to pH 11 and 12. 100 EU/mL endotoxin was spiked into samples and incubated at room temperature (RT) for 30 min. For detection kinetic chromogenic LAL test was used. Data points represent individual measurements (n = 4) and error bars reflect the standard deviation. The dotted line represents 50 % recovery of spiked endotoxin. Data were analyzed by Kruskal-Wallis test (non-parametric one-way ANOVA) with Dunn's multiple comparison test: *** $p < 0.001$.

range of 0.8 mg/mL to 1.9 mg/mL. Subsequently, a concentration gradient was observed, culminating in a maximum at fraction 51, where the concentration reached 8.9 mg/mL ± 0.8 mg/mL. Following the peak, a continuous decrease in concentration was observed from

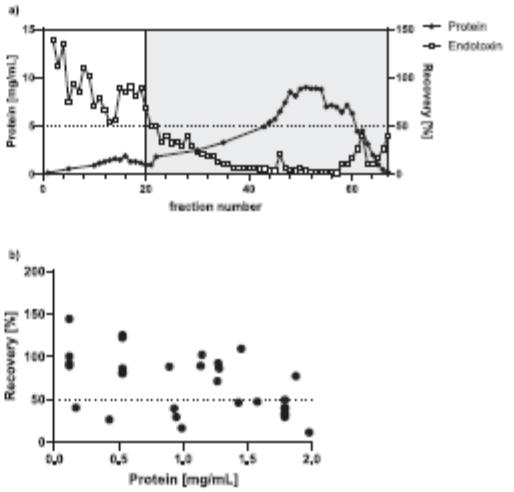

Fig. 3. Endotoxin recovery and protein concentration for individual plasma fractions after size exclusion chromatography. 100 EU/mL endotoxin was spiked into each fraction and incubated at room temperature (RT) for 24 h. For endotoxin detection kinetic chromogenic LAL test was used. Protein concentrations were determined after Bradford. Data points represent individual measurements (n = 3). The dotted line represents 50 % recovery. (a) Protein concentration (mg/mL) and endotoxin recovery (%) across different fractions. The shaded area indicates fractions with masking behaviour (Endotoxin recovery < 50 %). (b) Scatter plot of endotoxin recovery (%) as a function of protein concentration (mg/mL).

fraction 52 (8.8 mg/mL ± 1.1 mg/mL) to fraction 64 (2.0 mg/mL ± 0.1 mg/mL). The terminal fractions (65–67) exhibited concentrations below 1 mg/mL. This concentration profile demonstrates a distinct elution pattern, with a gradual but slow increase, followed by a sharp increase and a gradual decline in protein concentration across the collected fractions.

Determination of endotoxin recovery for individual fractions revealed that the first 20 fractions exhibited no masking within the first 24 h. Endotoxin recoveries ranged from 133 % ± 11 % for fraction 4 to 54 % ± 9 % for fraction 12. From fraction 21 onwards a strong masking

behaviour was observed. First with a slight decrease in endotoxin recovery ranging between 20 % and 30 % (fractions 21 to 31) followed by a further decrease with residual recovery of 2 % – 3 % for fractions 49 to 57. After fraction 57, the recovery increased slightly again, but did not reach 50 %. For example, fraction 67 showed a recovery of 40 %. This recovery profile showed a gradual decrease followed by a slight increase in endotoxin recovery across the collected fractions.

SDS-PAGE analysis revealed that the early eluted fractions contained proteins larger than 116 kDa as well as proteins with approximately 25 kDa. Interestingly, the further fractions, which were expected to contain smaller proteins, showed an additional band around 66 kDa. This band was present in all fractions except the first, suggesting that this protein could be human serum albumin (HSA), given its abundance and molecular weight (Appendix A1).

Initially, it appeared that protein concentration within the fractions was crucial for the masking behaviour. However, by plotting endotoxin recovery against the protein concentration of the fractions (Fig. 3 (b)), we determined a masking behaviour despite low protein concentrations. These results suggest that specific protein groups, rather than protein concentration alone, could be the main factors in masking.

3.4. Individual protein contributions to endotoxin masking: the role of HSA and lysozyme

Since the SEC analysis and the literature [32] suggested that HSA might contribute to endotoxin masking, we tested its masking behaviour in isolation. Other previous experiments suggested that masking is mediated by additional proteins that are smaller than HSA and bind to endotoxins via electrostatic interactions. These proteins are expected to have a positive charge and an isoelectric point (IEP) above the plasma pH of 8. Therefore, in addition to HSA, lysozyme was chosen as a model protein for potential masking because of its smaller size (15 kDa) and high IEP (11.8) [33].

Human plasma typically contains HSA at approximately 40 mg/mL, accounting for 60 % of the total protein content (70 mg/mL). Masking effects were observed at HSA concentrations exceeding the physiological range (Fig. 4 (a)). At concentrations of 100 mg/mL, 80 mg/mL, and 60 mg/mL, recoveries were 38 % ± 11 %, 48 % ± 3 %, and 51 % ± 9 %, respectively. Concentrations at the physiological level (40 mg/mL) led to increased recoveries (67 % ± 12 %). At lower concentrations, recoveries ranged between 100 % and 77 % (Fig. 4 (a)). These results indicate that while HSA contributes to masking, it requires considerably higher concentrations that are below physiological HSA levels.

To evaluate whether the masking effects observed at 100 EU/mL endotoxin also occur at lower, more clinically relevant concentrations, we conducted an additional experiment using 10 EU/mL endotoxin and varying concentrations of HSA (Appendix A2). In the water control (0 mg/mL HSA), recovery was 74 %, suggesting a lower baseline recovery. At physiological HSA levels (40 mg/mL), recovery was slightly above 50 %. These results indicate a somewhat stronger masking effect at lower endotoxin concentrations. However, the overall pattern remained consistent with that observed at 100 EU/mL, suggesting that the qualitative behavior of HSA is maintained across different endotoxin concentrations.

Lysozyme, present in human plasma at approximately 5–15 µg/mL, was tested at concentrations ranging from 1 µg/mL to 1000 µg/mL. No masking was observed at concentrations between 1 µg/mL and 5 µg/mL, with endotoxin recoveries ranging from 118 % to 67 %. However, at 6 µg/mL the recovery decreased to 47 %. A further decline in recovery was observed with subsequent increases in lysozyme concentration. At 15 µg/mL the residual recovery was 13 % and at 1000 µg/mL it dropped to 2.5 % (Fig. 4 (b)).

These results demonstrate a clear protein concentration dependence of lysozyme for endotoxin masking. This suggests that even small amounts of lysozyme can contribute significantly to endotoxin masking possibly through electrostatic interactions.

3.5. Disrupting electrostatic interactions in endotoxin masking

Given the previous findings suggesting the involvement of electrostatic interactions in endotoxin masking, the next step was to investigate how these interactions could be disrupted. To overcome electrostatic interactions in biochemical processes, two common approaches are the use of high-salt solutions and pH adjustments. High-salt solutions increase the ionic strength of the medium, effectively shielding charged groups on molecules like proteins. This reduces the active forces between oppositely charged sites, breaking electrostatic bonds. Similarly, pH adjustment alters the ionization state of these molecules: raising the pH deprotonates proteins, reducing their positive charge, and weakening their interaction with negatively charged molecules.

3.5.1. Limited effect of high salt solutions on plasma endotoxin recovery

To overcome electrostatic interactions, the masked sample was exposed to a high salt concentration by first preparing a 3 M NaCl solution. We compared the behaviour of the masked endotoxin plasma sample, the masked endotoxin HSA sample (200 mg/mL) as an example of a cationic plasma protein, and the masked endotoxin lysozyme sample (15 µg/mL) as an example of a cationic plasma protein. The results are shown in Fig. 5 (a). For plasma, endotoxin recovery was 11 % ± 0.5 % when diluted in the saline buffer and 10 % when diluted in water, indicating that high salt concentrations do not enhance recovery. Similar behaviour was observed with the HSA sample, where the recovery was 29 % ± 2 % in saline buffer and 19 % in water. In contrast, for the masked endotoxin in the lysozyme sample, the recovery increased to 97 % ± 15 % when diluted in saline buffer, compared to only 33 % when diluted in water.

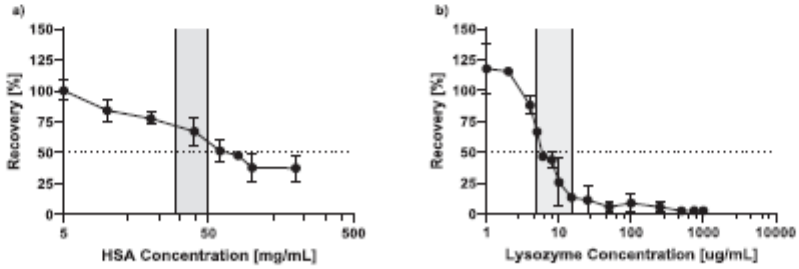

Fig. 4. Endotoxin recovery as a function of protein concentration (a) human serum albumin (b) lysozyme. 100 EU/mL endotoxin was added to each sample and incubated for 30 min at room temperature (RT). A kinetic chromogenic LAL test was used for endotoxin detection. Data points represent the mean of individual measurements (n = 3) and error bars reflect the standard deviation. The dotted line represents 50 % recovery of spiked endotoxin. The shaded area indicates the physiological concentration range of the respective protein in human plasma.

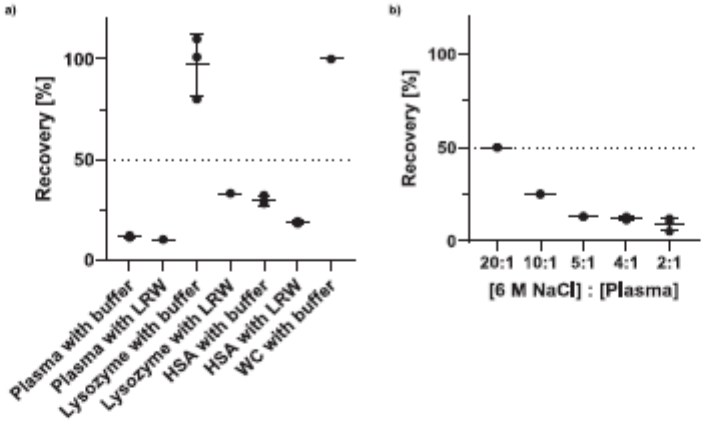

Fig. 5. Endotoxin recovery under high-salt conditions. 100 EU/mL endotoxin was added to each sample (Plasma, Lysozyme, HSA, Water (WC)) and incubated for 30 min at room temperature (RT). Samples were treated with (a) 3 M NaCl-containing buffer or LRW, and (b) varying ratios of 6 M sodium chloride (NaCl) to plasma. For detection, a kinetic chromogenic LAL test was used. Data points represent individual measurements (n = 3) and error bars reflect the standard deviation. Cross dots indicate measurements below the limit of detection (LOD). The dotted line indicates 50 % recovery of the spiked endotoxin.

The results showed that for the lysozyme sample, the high salt buffer is sufficient to recover LPS from the lysozyme-LPS complex, but this is not the case for the HSA or plasma samples. To investigate whether a higher salt concentration is necessary, we mixed our plasma sample with a 6 M NaCl buffer at different ratios. The recoveries are shown in Fig. 5 (b). For mixtures with increased NaCl concentration, the recoveries were below the LOD due to the high dilutions required. All endotoxin recoveries were below 50 % (20:1), 25 % (10:1), and 13 % (5:1), respectively. For the 4:1 and 2:1 mixtures (salt:plasma), endotoxin was measured, but the recoveries were below 50 %, with 12 % and 9 %, respectively. We are aware that the high salt-to-plasma ratios introduced an additional dilution of the samples, which was necessary to ensure valid measurement conditions, including a valid positive product control. As a consequence, recovery values below 50 %, as observed for the 20:1 mixture, fell below the LOD. However, this approach was required to investigate the effect of very high salt concentrations. Any substantial improvement in recovery, particularly above 50 % would have remained detectable despite the dilution. The absence of such an effect suggests that high ionic strength alone does not release masked endotoxin from plasma. Instead, high salt concentrations appear effective only in disrupting electrostatic interactions, such as those between endotoxin and cationic proteins like lysozyme. These findings indicate that additional masking mechanisms are likely present in plasma that are not resolved by salt treatment alone.

3.5.2. *pH adjustment alone insufficient to improve endotoxin recovery in plasma samples*

The second approach to mitigate electrostatic interactions between plasma components and endotoxins was to adjust the pH of plasma samples to more basic levels. Since the net charge of proteins is influenced by the surrounding pH, most proteins become negatively charged in a basic environment when their IEP is lower than the sample pH. Thus, plasma samples were tested at pH 11 and 12, using physiological plasma pH 8 as a control. In addition, this pH adjustment was combined with dilution in different buffers containing divalent cations (e.g., $MgCl_2$ or $CaCl_2$), which are known to support the assembly of supramolecular structures [34,35]. Results are shown in Fig. 6. The control sample at pH 8, diluted in LRW, showed an endotoxin recovery of 15 % ± 11 %. In contrast, recoveries increased significantly at higher pH levels, reaching 32 % ± 7 % at pH 11 and 48 % ± 3 % at pH 12 when diluted in LRW.

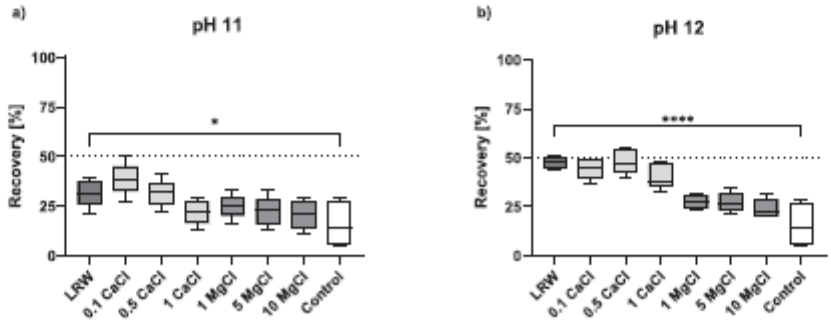

Fig. 6. Endotoxin recovery at different pH levels. 100 EU/mL endotoxin was added to each sample and incubated for 30 min at room temperature (RT). Plasma samples were adjusted to (a) pH 11 and (b) pH 12, and diluted in different buffers. Kinetic chromogenic LAL test was used for the detection. Data points represent the mean of individual measurements (n = 5) and error bars reflect the standard deviation. Dotted line represents 50 % recovery of spiked endotoxin. Data were analyzed by Kruskal-Wallis test (non-parametric one-way ANOVA) with Dunn's multiple comparison test: **** $p < 0.0001$, * $p < 0.05$.

Although recoveries did not exceed 50 %, these results show a clear trend towards improved endotoxin recovery with increasing pH.

The addition of divalent cation buffers resulted in diverse results. At pH 11, a slight but not significant improvement was observed with 0.1 mM $CaCl_2$, resulting in a recovery of 39 % ± 8 % compared to 32 % ± 7 % when diluted in LRW. However, $MgCl_2$ buffers did not improve recovery and in some cases even worsened it. The highest recovery with $MgCl_2$ at pH 11 was 25 % ± 6 % using 1 mM $MgCl_2$. At pH 12, no improvement compared to LRW dilution was observed, with the best condition being 0.5 mM $CaCl_2$ (48 % ± 6 %). $MgCl_2$ buffers at pH 12 again showed impaired recoveries, with a maximum of 28 % ± 3 % at 1 mM $MgCl_2$.

These results suggest that increasing the sample pH could reduce electrostatic interactions between endotoxins and plasma proteins, leading to improved endotoxin detection. However, the variability in recovery rates and the inability to consistently exceed 50 % suggest that factors other than electrostatic interactions contribute additionally to endotoxin masking in human plasma.

3.6. Behaviour of different LPS structures in human plasma

It was hypothesised that electrostatic interactions alone may not be responsible for masking in human plasma, as previous results suggested the potential involvement of other factors. To further investigate the driving forces behind the masking behaviour of human plasma, we tested different LPS preparations with different polysaccharide chain lengths and therefore different net charges of molecules. These included S-type LPS from *Salmonella minnesota* and its mutants: semi-rough LPS Ra, rough LPS Rc, deep-rough LPS Re, and the lipid A.

The experiments revealed different masking behaviours among the LPS variants (Fig. 7). S-type LPS showed the most pronounced and rapid masking, with a residual endotoxin recovery of only 5 % ± 1 % after 10 min. Similarly, LPS Ra, LPS Rc, and RSE showed substantial masking within the same time frame, with recoveries of 28 % ± 10 %, 26 % ± 2 %, and 13 % ± 5 %, respectively. In contrast, LPS Re and lipid A showed less masking initially, with recoveries of more than 50 % after 10 min (66 % ± 11 % and 60 % ± 11 %, respectively). However, the masking effects became more pronounced with time. For lipid A, recovery dropped below 50 % within 30 min (47 % ± 7 %). LPS Re followed a slower trajectory, maintaining recoveries above 50 % until 2 h, when it dropped to 37 % ± 4 %.

Notably, after 2 h, no further significant decrease in recovery was observed for any of the LPS variants. At 2 h, recoveries for each LPS type were as follows: S-type LPS at 15 % ± 2 %, RSE at 12 % ± 1 % and LPS Re at 37 % ± 4 %. After 24 h, recoveries showed minimal changes: S-type LPS increased slightly to 10 % ± 1 %, RSE to 12 % ± 1 %, and LPS Re to 43 % ± 2 %.

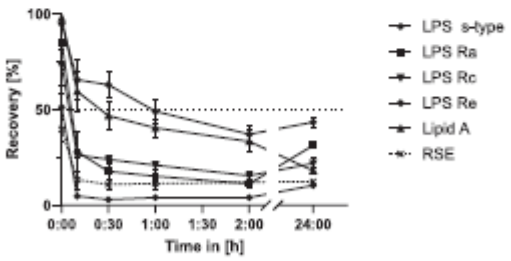

Fig. 7. Masking susceptibility of different mutants from *S. minnesota* in human plasma (LPS s-type, R60 (Ra), R5 (Rc), R595 (Re), Lipid A from *S. minnesota* R595). Endotoxin recovery was plotted as a function of incubation time. 100 EU/mL endotoxin from different mutants was spiked into plasma sample and incubated at room temperature (RT) for up to 24 h. For detection, kinetic chromogenic LAL test was used. Data points were calculated using mean kinetics from three healthy donors (n = 3) and error bars reflect the standard deviation. For a better comparison of independent measurements, the data was normalized to the respective water controls. The dotted line represents 50 % recovery of spiked endotoxin.

These results highlight significant differences in masking kinetics and behaviour between smooth and rough LPS variants. The rapid and sustained masking observed not only for S-type LPS but also for rough mutants possessing an almost complete core oligosaccharide contrasts sharply with the slower and less pronounced effects seen for LPS Re and lipid A. This pattern suggests that structural differences, particularly within the core oligosaccharides, play a critical role in endotoxin masking kinetics in human plasma.

4. Discussion

The detection of endotoxins in human blood is challenging due to the complex interactions between endotoxins and various blood components, including plasma proteins. Whole blood, which contains erythrocytes, leukocytes, and platelets, showed weaker masking effects than plasma, which mainly consists of water, proteins, and nutrients [36]. Water and nutrients have no known masking effect. This suggests that the more concentrated non-cellular components in plasma mainly contribute to the masking of LPS, whereas cellular components appear to play a minor role in endotoxin masking.

These interactions of plasma components can result in binding to endotoxins or altering the structure of endotoxins, making them less detectable by assays such as the LAL test, leading to false negative results. The required recovery rate according to regulatory guidelines (50 % – 200 %) of spiked endotoxin is not achieved using the standard protocol, as our data showed, which is probably why LAL assays have not yet found their way into medical diagnostics.

It is important to note that the endotoxin concentration used in our study was 100 EU/mL, which is considerably higher than levels typically found under physiological or clinical conditions. Circulating endotoxin concentrations in human plasma, even during pathological states such as sepsis, are usually in the low EU/mL range [37]. However, the reliability of reported endotoxin levels at such low concentrations remains questionable, given the limitations of current detection methods [38].

The use of a high LPS concentration in our experiments was chosen to ensure sufficient assay sensitivity and to enable robust comparative analyses across different conditions and treatments. At lower concentrations, such as 10 EU/mL, the required dilution factor of 1:500 to obtain valid measurements would have caused recoveries below 25 % to fall below the limit of detection. Nonetheless, it must be acknowledged that some masking phenomena observed at higher concentrations may not directly translate to physiological conditions. High LPS levels can promote aggregate formation or exhibit interaction kinetics that differ from those occurring at lower, more clinically relevant concentrations. To address this limitation, we performed an additional experiment using 10 EU/mL endotoxin and varying HSA concentrations of (Appendix A2). At physiological HSA levels (~40 mg/mL), recovery remained just above 50 %, while pronounced masking was only observed at higher, non-physiological concnetrations.

Therefore, while our findings provide important mechanistic insights, future studies should investigate masking behavior at lower endotoxin concentrations to confirm their relevance under in vivo conditions.

Previous studies have investigated dilution and heat treatment, but these methods primarily address assay interferences rather than the underlying mechanisms of endotoxin masking [39]. Dilution reduces the overall concentration of interfering components, while heat treatment denatures proteins such as serine proteases that interfere with the assay. However, these approaches do not provide a comprehensive solution to overcome masking. Due to the inherent structure of endotoxin, several interactions are possible. Hydrophobic interactions at the lipid A region, hydrophilic interactions at the sugar residues, and electrostatic

interactions at the negatively charged phosphate groups.[40].

Our study confirms previous findings that one of the major causes of masking is the electrostatic interaction between the negatively charged phosphate groups of endotoxins and the positively charged amino acids (e.g., lysine, arginine) of plasma proteins [22]. Our study provides critical insights into the factors influencing endotoxin masking in human blood, with particular emphasis on the role of plasma proteins and the structural diversity of LPS.

Our SEC experiment successfully identified fractions with and without apparent endotoxin masking. While 20 fractions showed recoveries above 50 %, which would typically suggest no strong masking, this threshold does not necessarily rule out partial masking or transient interactions. Overall, 47 fractions exhibited reduced recovery (< 50 %), indicating more pronounced masking effects. Fewer masking fractions had initially been expected, which would have facilitated the identification of specific masking proteins. However, a clear trend emerged: early-eluting fractions showed higher recovery, while later-eluting protein-rich fractions were associated with stronger masking. This indicates that masking is not a random phenomenon but is likely linked to specific protein properties. While a general correlation between protein concentration and endotoxin recovery was observed, similar concentrations in masking and non-masking fractions resulted in different recoveries, further supporting the idea that masking is protein-specific and not merely concentration-dependent. SDS-PAGE analysis revealed incomplete separation due to protein dimerisation and aggregation. Some proteins were present in almost all fractions, making the identification of masking agents difficult. For example, HSA was detected in almost all fractions, but did not correlate with masking and requires further investigation. Although in some cases similar protein concentrations were measured in the non-masking and masking fractions, a difference in recovery could be seen. This suggests that the effect is not only concentration dependent but also influenced by the specific type of protein present. An outstanding protein in this context is lysozyme, which belongs to the class of antimicrobial enzymes/peptides (AMP) and is a critical component of innate immunity. Lysozyme typically targets gram-positive bacteria by hydrolysing the peptidoglycan in their cell walls. However, conventional-type lysozymes also exhibit peptidoglycan-independent antimicrobial activity due to their highly cationic nature [41]. Our findings show that lysozyme, even at low concentrations (~6 µg/mL), effectively masks endotoxins. However, lysozyme differs from many AMPs in that its interaction with LPS appears to be driven primarily by electrostatic forces. This is supported by our observation that endotoxin masking by lysozyme can be easily reversed by increasing the ionic strength, i.e. by adding salt. In contrast, recovery of masked endotoxins in plasma remains a challenge, suggesting that additional factors, possibly involving hydrophobic or hydrophilic interactions contribute to endotoxin masking in this complex biological matrix.

In contrast, HSA, the most abundant human plasma protein (35–50 mg/ml), showed limited masking potential under physiological conditions, resulting in endotoxin recovery rates above 50 %. Albumin is known to bind and solubilise LPS, including the lipid A component, and can mask endotoxin activity to some extent. However, this binding is primarily through non-electrostatic interactions and results in only minor changes in endotoxin structure and charge, indicating a limited masking potential [32,42]. The fact that endotoxin recovery from HSA solutions was not possible by increasing ionic strength suggests that hydrophobic interactions play a major role in this binding. Given that HSA possesses lipid transport properties, it is plausible that its interaction with endotoxin involves hydrophobic domains, contributing to LPS solubilization rather than strong masking. Therefore, although albumin binds endotoxin, it does not account for the extent of masking observed in human plasma and cannot be considered a major masking agent. This highlights that masking is not solely determined by protein abundance but rather by the specific binding properties of individual proteins.

Rauchhaus *et al.* [44] introduced the HDL-LPS hypothesis, which proposes that high-density lipoprotein (HDL) neutralises LPS by binding to it and facilitating its clearance by the liver [43]. HDL has both positively charged apolipoproteins and a hydrophobic phospholipid layer, allowing it to interact with LPS through electrostatic and hydrophobic forces [44]. This interaction reduces the bioactivity of LPS by preventing its recognition by macrophages, thereby attenuating inflammatory responses and reducing the risk of sepsis [45]. In addition, other studies have shown that endotoxins can also be neutralised by plasma protein fractions free of lipoproteins and that LAL activity is not affected by the binding of human lipoproteins to endotoxin [22]. While our results suggest that electrostatic interactions between negatively charged phosphate groups of LPS and positively charged amino acid residues of plasma proteins contribute to masking, they are unlikely to represent the sole mechanism.

High concentrations of salt, such as NaCl, disrupt electrostatic interactions by shielding the charges on molecules. This method is also used in protein purification to reduce non-specific binding between proteins and impurities between them [46]. The Na^+ and Cl^- ions from the salt can surround and neutralise the charges, thereby weakening or eliminating the attractive forces between oppositely charged molecules. This process can lead to the dissociation of molecules held together primarily by electrostatic forces [47]. If endotoxin-protein interactions remain intact, as shown by masking, despite high salt concentrations, this suggests that other types of forces are involved in maintaining the interaction. These could include hydrophobic interactions, which are not affected by ionic strength, or more stable covalent bonds. Increasing the pH of plasma samples to pH 11 and 12, which reduces the positive charge of plasma proteins, improved recovery of endotoxin in average over 50 % when endotoxin was subsequently added and prevented partial masking. However, once masking had already occurred, pH adjustments failed to reverse masking. This suggests that endotoxin masking is likely to be mediated by mechanisms beyond simple electrostatic binding, and that further investigation into the nature of these interactions is required to fully understand the masking phenomena. We also note that pH values of 11 and 12 may not only affect the net surface charge of plasma proteins and thereby their interactions with other molecules such as endotoxin, but also alter intramolecular interactions within the proteins themselves. Changes in the ionization state of amino acid side chains can disrupt ionic bonds, hydrogen bonds, or other stabilizing interactions critical for maintaining native protein conformation [48,49]. Consequently, structural rearrangements or even partial denaturation may occur, further complicating the interpretation of observed effects. This limitation underscores the need for additional structural analyses to distinguish between effects related to altered protein charge and those caused by conformational changes. In future studies, it would therefore be valuable to investigate individual masking-relevant proteins such as lysozyme under defined pH conditions, assessing both the masking effects and the protein structure in parallel. This approach may provide more detailed insights into the underlying mechanisms of endotoxin masking. Recent studies by Harm *et al.* [50] also focused on resolving electrostatic interactions through the addition of the polyanionic substance heparin and the supplementation of divalent cations. However, their approach utilized a heparin injection solution containing additional components, which could influence the results. In our experience, heparin alone, even when combined with divalent cations, has shown limited effectiveness in fully resolving endotoxin masking (data not shown).

The masking behaviour observed with different LPS structures supports this conclusion. One finding of our study is the influence of LPS structure on masking behaviour in human plasma samples. Different LPS chemotypes, ranging from S-type LPS with complete O-antigen chains to rough (Ra, Rc) and deep-rough (Re) mutants, showed different masking dynamics. Sali *et al.* [51] showed that rough mutants tend to have higher negative charge densities, which would typically enhance electrostatic interactions. Therefore, one would expect rough and deep-rough mutants to mask faster than S-type LPS in plasma samples. However, our

investigation showed different results. We could show that S-type LPS exhibited the fastest and most extensive masking. The presence of the full-length O-antigen increases hydrophilic interactions and hydrogen bonding with plasma proteins. These additional interactions are likely to increase the efficiency and speed of masking, making S-type LPS more susceptible. In contrast, rough and deep-rough mutants (Ra, Rc, Re), as well as lipid A, showed slower masking and higher endotoxin recovery rates. The truncated polysaccharide chains reduce the hydrophilic surface available for interactions, thereby limiting the masking potential. Additional factors, such as molecular conformation may also play a role in masking dynamics. The presence of the O-antigen in S-type LPS allows the formation of distinct supramolecular structures that may increase accessibility for interactions with masking components. In contrast, rough (Ra, Rc) and deep-rough (Re) mutants, which lack parts of the polysaccharide chain, form different conformations that may limit their accessibility for binding. This structural difference likely contributes to the slower masking observed with rough LPS. However, while molecular conformation influences the rate of masking, it does not fully explain the magnitude of the differences in endotoxin recovery between S-type and rough LPS. The reduced hydrophilic surface area of rough mutants reduces their ability to engage in additional interactions, which possibly contributes to their higher residual recoveries [52,53]. Interestingly, the observed behaviour is consistent with a previous study of ours, in which we demonstrated that in a common masking matrix of chelator and surfactant, rough LPS masked more slowly than smooth LPS [54].

The supramolecular structure of LPS plays a critical role not only for its detection in LAL assays, but also for its biological activity. LPS molecules naturally form aggregates due to hydrophobic interactions between lipid A acyl chains and electrostatic stabilisation by divalent cations such as Mg^{2+} and Ca^{2+} [18]. These aggregates are essential for the activation of Factor C in the LAL assay, which relies on the detection of LPS in its aggregated form [55]. When LPS dissociates into smaller subunits or monomers, its ability to activate Factor C andconsequently the LAL assay is significantly reduced. Biologically, the aggregated state of LPS is also required for optimal toxicity. Studies have shown that disaggregated LPS is less pyrogenic than aggregated LPS. Similary, when detergents or plasma proteins induce LPS dissociation, toxicity is reduced. However, upon removal of these agents, LPS can regain its supramolecular structure and biological activity. This suggests that a minimum aggregate size is required to elicit full biological potency [56–58].

This raises the question of whether endotoxins are being degraded rather than neutralised or masked. Studies by Oroszlan *et al.* showed that the binding of LPS to a cationic liver protein resulted in a loss of biological activity, which was reversible upon the addition of polyglucose sulphate [56]. Similarly, Rudbach *et al.* [58] found that plasma proteins reduced LPS pyrogenicity, but this effect was reversed when the proteins were dissociated from LPS, restoring its toxicity [58]. These results suggest that the reduction in toxicity is due to masking or neutralisation rather than irreversible degradation. Further research by Ribi *et al.* using sodium deoxycholate revealed that LPS dissociates into inactive subunits, reducing pyrogenicity by 100–200 fold. However, when the bile salt was removed, LPS reaggregated, and pyrogenicity was restored. Notably, the addition of plasma proteins prevented this reaggregation, reinforcing the hypothesis that masking, rather than degradation, is responsible for the loss of biological activity [57]. Thus, the reversible nature of these interactions suggests that LPS toxicity can be suppressed through dissociation or binding to proteins, but the potential for reaggregation implies that the endotoxin remains intact and capable of regaining full biological potency once the masking agents are removed.

It remains unclear whether LPS that evades detection in LAL assays, retains its biological activity. Studies suggest that masked LPS in LER solutions (i.e. chelating agent and surfactant) can still stimulate the release of pro-inflammatory cytokines, and thereby induce an immune response [59]. Schromm *et al.* [60] provide evidence that the aggregation state of LPS impacts biological and analytical detection methods alike [60]. Previous studies have shown that monomeric Re LPS from *E. coli* is more active than aggregates in the LAL assay [61]. In contrast, Mueller *et al.* [62] reported that E. coli Re LPS and Lipid A are exclusively active in their multimeric form, both in the LAL assay and in stimulating cytokine production from human mononuclear cells [62]. Given the strong correlation between LPS aggregation and its biological activity, it is important to determine whether the same LER effects that inhibit LPS binding to Factor C also prevent recognition by the immune system through the TLR4-MD-2 complex. Tan *et al.* concluded that the LPS binding domain of Factor C interacts cooperatively with multiple lipid A molecules, suggesting that more than one LPS molecule is required for Factor C binding and that monomeric LPS alone may be insufficient to activate Factor C [63]. In contrast, monomeric LPS must be presented to the TLR4-MD-2 complex to initiate the signalling cascade. The accessory proteins lipopolysaccharide binding protein (LBP) and CD14 enhance LPS recognition by extracting LPS from aggregates and converting it into monomers [64]. However, it has been suggested that LBP binds efficiently to LPS aggregates, which may explain the link between aggregates and biological activity. This is likely due to LBPs varying affinities for different aggregate forms and its inability to bind monomeric LPS. Consequently, LBP cannot deliver monomeric LPS to CD14, which is necessary for its presentation to the TLR4-MD-2 complex [65]. To further refine our understanding, combining findings from studies of the effects on LER effects on Factor C activation and TLR4-MD2 recognition with research on how in vivo conditions influence the supramolecular structure and biological toxicity of LPS could greatly enhance our understanding of the biological activity of masked LPS in human plasma.

5. Conclusions

Overcoming endotoxin masking in blood remains a major challenge. In conclusion, our study underscores the complexity of this phenomenon and highlights that while electrostatic interactions play an important role, they are not the sole factor. Electrostatic shielding or neutralisation approaches, such as high salt treatments, have been tested with limited success. It is known that high salt concentrations or a pH shift can reduce electrostatic interactions by changing the ionic strength, but in complex biological environments like blood, additional forces such as hydrophilic interactions, molecular conformation, and specific protein binding contribute to inconsistent results.

The resolution of endotoxin masking is further complicated by structural variations in endotoxin structures themselves, such as different LPS mutants. Rough LPS variants, for example, have different charge densities and structural configurations compared to S-type LPS. Interestingly, rough and deep-rough mutants tend to mask more slowly. At the same time, they have an increased negative charge density. This suggests that the affinity of endotoxins for plasma proteins and their subsequent masking is influenced by more than just electrostatic forces. Furthermore, a key question in endotoxin masking is whether masked endotoxins can still induce an immune response. Given that LPS is one of the most potent pyrogens, it is reasonable to assume that it retains immunogenic potential even when undetectable by the LAL assay. Therefore, caution is needed when using the term "endotoxin neutralisation" in plasma, as the absence of detection does not necessarily equate to a loss of its pyrogenic potential or biological activity. This concern is supported by studies showing that masked endotoxins can still induce immune responses. The structural diversity of LPS molecules must also be considered, as some LPS structures are less susceptible to masking or mask more slowly. This variability is more representative of naturally occurring conditions, where different forms of LPS circulate simultaneously during infections. Consequently, endotoxins that remain unmasked or are masked more slowly may continue to activate the immune response. Understanding which structural forms of LPS are primarily responsible for immune activation and how masking affects this activation is crucial.

Future research should focus on developing detection methods that account for the structural diversity of LPS molecules as well as the various masking mechanisms mediated by blood components. Our study represents an important step toward understanding these complex interactions by systematically exploring how different physicochemical interventions, such as pH shifts and changes in ionic strength, affect endotoxin recovery. At the same time, future studies will benefit from combining endotoxin masking experiments with complementary protein structural analyses. This will help to disentangle whether observed effects are primarily due to changes in surface charge or to conformational alterations of plasma proteins. Extreme conditions, such as high pH values, are likely to affect both aspects simultaneously. Therefore, integrating structural biology approaches with functional endotoxin assays could further strengthen mechanistic understanding and support the interpretation of results obtained in complex biological matrices like blood.

Taken together, these findings lay the groundwork for more reliable diagnostics, improved sepsis management, and a deeper understanding of endotoxin-driven immune responses. Expanding on this foundation through interdisciplinary approaches may also enable the development of therapeutic strategies targeting masked or unmasked endotoxins in inflammatory diseases.

CRediT authorship contribution statement

Luisa Burgmaier: Writing – original draft, Visualization, Formal analysis, Conceptualization. **Johanna Lifka:** Writing – review & editing. **Meltem Avci-Adali:** Writing – review & editing. **Johannes Reich:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix

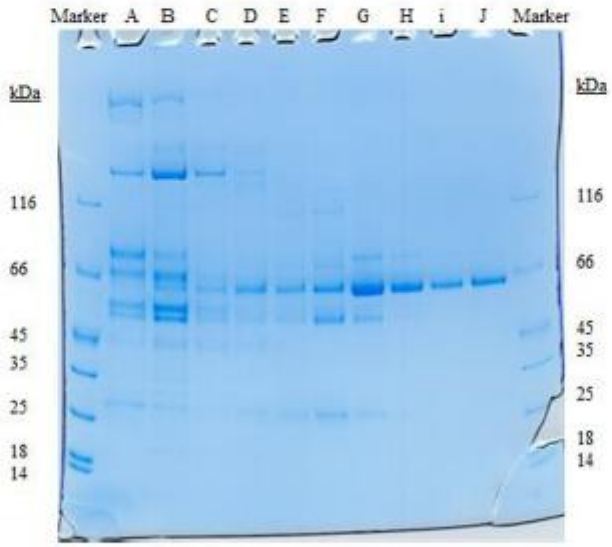

Fig. A1. SDS-PAGE of the SEC fractions, fractions were summarised in groups A – J (A: 1 – 4, B: 5 – 9, C: 1017, D: 18 – 21, E: 22 – 29, F: 30 – 34, G: 35 – 42, H: 43 – 51, i: 55 – 62, J: 63 – 67).

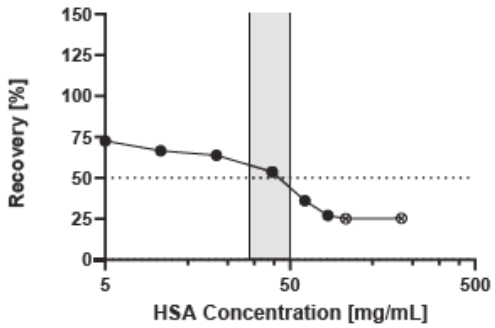

Fig. A2. Endotoxin recovery as a function of protein concentration human serum albumin. 10 EU/mL endotoxin was added to each sample and incubated for 30 min at room temperature (RT). A kinetic chromogenic LAL test was used for endotoxin detection. Data points represent the mean of individual measurements (n = 2). Cross dots indicate measurements below the limit of detection (LOD). The dotted line represents 50 % recovery of spiked endotoxin. The shaded area indicates the physiological concentration range of the protein in human plasma.

Data availability

Data will be made available on request.

References

[1] E.T. Rietschel, H. Brade, Scientif. Am. (1992).
[2] A. Farhana, Y.S. Khan, *StatPearls: Biochemistry*, Lipopolysaccharide, Treasure Island (FL), 2024.
[3] T.H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses, Clin. Microbiol. Rev. 22 (2) (2009) 240–273. Table of Contents.
[4] M.J. Delano, P.A. Ward, The immune system's role in sepsis progression, resolution, and long-term outcome, Immunol. Rev. 274 (1) (2016) 330.
[5] *FDA (Sat, 3 November 2018).*
[6] P. Malyala, M. Singh, Endotoxin limits in formulations for preclinical research, J. Pharm. Sci. 97 (6) (2008) 2041.
[7] G. Martin, et al., The international PROGRESS registry of patients with severe sepsis: drotrecogin alfa (activated) use and patient outcomes, Crit. Care 13 (3) (2009) R103.
[8] A.E. Jones, et al., Procalcitonin test in the diagnosis of bacteremia: a meta-analysis, Ann. Emerg. Med. 50 (1) (2007) 34.
[9] Z. Bradley, N. Bhalla, Point-of-care diagnostics for sepsis using clinical biomarkers and microfluidic technology, Biosens. Bioelectron. 227 (2023) 115181.
[10] H. Tamura, et al., Outstanding contributions of LAL technology to pharmaceutical and medical science: review of methods, progress, challenges, and future perspectives in early detection and management of bacterial infections and invasive fungal diseases, Biomedicines 9 (5) (2021) 536.
[11] K. Wildi, et al., Factors associated with positive blood cultures in outpatients with suspected bacteremia, Eur. J. Clin. Microbiol. Infect. Dis. 30 (12) (2011) 1615.
[12] J. Morrison, et al., Retrospective analysis of clinical data associated with patients enrolled in a molecular diagnostic feasibility study highlights the potential utility for rapid detection of bloodstream infection, Am. J. Emerg. Med. 32 (6) (2014) 511.
[13] J. Levin, The role of endotoxin in the extracellular coagulation of Limulus blood, Bull. Johns Hopkins Hosp. 115 (1964) 265.
[14] M. Piehler, et al., Comparison of LAL and rFC assays-participation in a proficiency test program between 2014 and 2019, Microorganisms 8 (2020) 3.
[15] R.J. Elin, et al., Lack of clinical usefulness of the limulus test in the diagnosis of endotoxemia, N. Engl. J. Med. 293 (11) (1975) 521.
[16] A. Gnauck, et al., The Limulus Amebocyte Lysate assay may be unsuitable for detecting endotoxin in blood of healthy female subjects, J. Immunol. Methods 416 (2015) 146.
[17] A. Gnauck, et al., Chasing a ghost?–Issues with the determination of circulating levels of endotoxin in human blood, Crit. Rev. Clin. Lab. Sci. 53 (3) (2016) 197.
[18] J. Reich, et al., Investigation of the kinetics and mechanism of low endotoxin recovery in a matrix for biopharmaceutical drug products, Biologicals 53 (2018) 1.
[19] J. Reich, et al., Masking of endotoxin in surfactant samples: effects on Limulus-based detection systems, Biologicals 44 (5) (2016) 417.
[20] J. Reich, et al., Low endotoxin recovery-masking of naturally occurring endotoxin, Int. J. Mol. Sci. 20 (2019) 4.
[21] J. Levin, et al., Detection of endotoxin in human blood and demonstration of an inhibitor, J. Lab. Clin. Med. 75 (6) (1970) 903.
[22] S. Harm, et al., An in vitro study on factors affecting endotoxin neutralization in human plasma using the Limulus amebocyte lysate test, Sci. Rep. 11 (1) (2021) 4192.
[23] D.S. Uppu, J. Haldar, Lipopolysaccharide neutralization by cationic-amphiphilic polymers through pseudoaggregate formation, Biomacromolecules 17 (3) (2016) 862.
[24] A. Gries, et al., Biophysical analysis of the interaction of the serum protein human β2GP1 with bacterial lipopolysaccharide, FEBS Open Bio 4 (2014) 432.
[25] K. Brandenburg, et al., Biophysical characterization of the interaction of high-density lipoprotein (HDL) with endotoxins, Eur. J. Biochem. 269 (23) (2002) 5972.
[26] Y. Kaconis, et al., Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides, Biophys. J. 100 (11) (2011) 2652.
[27] E.C. Tidswell, A nontrivial analysis of patient safety risk from parenteral drug- and medical device-borne endotoxin, Drugs R&D 23 (1) (2023) 65.
[28] D.A. Dubose, et al., Comparison of plasma extraction techniques in preparation of samples for endotoxin testing by the Limulus amoebocyte lysate test, J. Clin. Microbiol. 11 (1) (1980) 68.
[29] T.J. Novitsky, Limulus amebocyte lysate (LAL) detection of endotoxin in human blood, J. Endotoxin Res. 1 (4) (1994) 253.
[30] T. Obayashi, Addition of perchloric acid to blood samples for colorimetric limulus test using chromogenic substrate: comparison with conventional procedures and clinical applications, J. Lab. Clin. Med. 104 (3) (1984) 321.
[31] D. Petsch, et al., Proteinase K digestion of proteins improves detection of bacterial endotoxins by the Limulus amebocyte lysate assay: application for endotoxin removal from cationic proteins, Anal. Biochem. 259 (1) (1998) 42.
[32] G. Jürgens, et al., Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A, J. Endotoxin Res. 8 (2) (2002) 115.
[33] K. Brandenburg, et al., Biophysical characterisation of lysozyme binding to LPS Re and lipid A, Eur. J. Biochem. 258 (2) (1998) 686.
[34] P. Garidel, et al., Divalent cations affect chain mobility and aggregate structure of lipopolysaccharide from Salmonella minnesota reflected in a decrease of its biological activity, Biochimica et Biophysica Acta (BBA)-Biomembranes 1715 (2) (2005) 122.
[35] S. Snyder, et al., Lipopolysaccharide bilayer structure: effect of chemotype, core mutations, divalent cations, and temperature, Biochemistry 38 (33) (1999) 10758.
[36] G. Müller-Newen and P. E. Petrides, *Blut – Bestandteile und Blutplasma*, in: P.C. Heinrich et al. (Eds.), *Löffler/Petrides Biochemie und Pathobiochemie*, 10th ed., 1133, Springer, Berlin, Heidelberg (2022).
[37] S.M. Opal, et al., Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock, J Infect Dis 180 (5) (1999) 1584.
[38] J.-L. Vincent, et al., Sepsis definitions: time for change, Lancet 381 (9868) (2013) 774.
[39] R.I. Roth, et al., Optimization of detection of bacterial endotoxin in plasma with the Limulus test, J. Lab. Clin. Med. 116 (2) (1990) 153.
[40] C. Erridge, et al., Structure and function of lipopolysaccharides, Microbes Infect. 4 (8) (2002) 837.
[41] S.A. Ragland, A.K. Criss, From bacterial killing to immune modulation: recent insights into the functions of lysozyme, PLoS Pathog. 13 (9) (2017) e1006512.
[42] A. Sunil, DAVID, *the Interaction of Lipid a and Lipopolysaccharide with Human Serum Albumin*, in: H. Brade (Ed.), *Endotoxin in Health and Disease*, 413, CRC Press, Boca Raton, 2020.
[43] M. Rauchhaus, et al., The endotoxin-lipoprotein hypothesis, Lancet (london, England) 356 (9233) (2000) 930.
[44] K. Emancipator, et al., In vitro inactivation of bacterial endotoxin by human lipoproteins and apolipoproteins, Infect. Immun. 60 (2) (1992) 596.
[45] J.M. Cavaillon, et al., Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide, Infect. Immun. 58 (7) (1990) 2375.
[46] N. Wenger, TriAltus Biosci. (2020).

[47] X. Xiao, et al., Salt destabilization of cationic colistin complexation within polyanionic microgels, Macromolecules 55 (5) (2022) 1736.
[48] Z. Gao, et al., Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate, Food Res. Int. (Ottawa, Ont.) 131 (2020) 109045.
[49] H.G. Kristinsson, H.O. Hultin, Changes in trout hemoglobin conformations and solubility after exposure to acid and alkali pH, J. Agric. Food Chem. 52 (11) (2004) 3633.
[50] S. Harm, et al., Heparin enables the reliable detection of endotoxin in human serum samples using the Limulus amebocyte lysate assay, Sci. Rep. 14 (1) (2024) 2410.
[51] W. Sali, et al., Polysaccharide chain length of lipopolysaccharides from salmonella minnesota is a determinant of aggregate stability, plasma residence time and proinflammatory propensity in vivo, Front. Microbiol. 10 (2019) 1774.
[52] W. Richter, et al., Morphology, size distribution, and aggregate structure of lipopolysaccharide and lipid a dispersions from enterobacterial origin, Innate Immun. 17 (5) (2011) 427.
[53] U. Seydel, et al., The generalized endotoxic principle, Eur. J. Immunol. 33 (6) (2003) 1586.
[54] L. Burgmaier, et al., The impact of LPS mutants on endotoxin masking in different detection systems, Biologicals 89 (2025) 101808.
[55] C. Wang, et al., Understanding lipopolysaccharide aggregation and its influence on activation of factor C, J. Colloid Interface Sci. 552 (2019) 540.
[56] S.I. Oroszlan, et al., Reversible inactivation of an endotoxin by intracellular protein, Biochem. Pharmacol. 12 (10) (1963) 1131.
[57] E. Ribi, et al., Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate, J. Bacteriol. 92 (5) (1966) 1493.
[58] J.A. Rudbach, A.G. Johnson, Restoration of endotoxin activity following alteration by plasma, Nature 202 (4934) (1964) 811.
[59] H. Schwarz, et al., Biological activity of masked endotoxin, Sci. Rep. 7 (1) (2017) 44750.
[60] A.B. Schromm, et al., Supramolecular assembly of micellar aggregates is the basis of low endotoxin recovery (LER) in a drug formulation that can be resolved by a whole blood assay, Biomed. Pharmacotherapy = Biomed. Pharmacotherapie 173 (2024) 116286.
[61] K. Takayama, et al., Monomeric Re lipopolysaccharide from Escherichia coli is more active than the aggregated form in the Limulus amebocyte lysate assay and in inducing Egr-1 mRNA in murine peritoneal macrophages, J. Biol. Chem. 269 (3) (1994) 2241.
[62] M. Mueller, et al., Aggregates are the biologically active units of endotoxin, J. Biol. Chem. 279 (25) (2004) 26307.
[63] N.S. Tan, et al., Definition of endotoxin binding sites in horseshoe crab factor C recombinant sushi proteins and neutralization of endotoxin by sushi peptides, FASEB J. Off. Publ. Federation Am. Soc. Exp. Bio. 14 (12) (2000) 1801.
[64] L. Mazgaeen, P. Gurung, Recent advances in lipopolysaccharide recognition systems, Int. J. Mol. Sci. 21 (2) (2020) 379.
[65] B.S. Park, J.-O. Lee, Recognition of lipopolysaccharide pattern by TLR4 complexes, Exp. Mol. Med. 45 (12) (2013) e66.