NACE–ESI-MS/MS method for separation and characterization of phosphorylation and acylation isomers of lipid A

Lipid A represents a heterogeneous group of bacterial outer membrane phosphoglycolipids, which play a major role in the pathogenesis of Gram-negative sepsis. The number and position of phosphoryl and acyl groups in lipid A molecules are key structural determinants in their bioactivities. In this study, a NACE–ESI-MS/MS method was developed for the simultaneous analysis of lipid A isomers possessing a different degree of phosphorylation and acylation. Various C4'- and C1-monophosphorylated lipid A isobars, as well as acylation isomers, were baseline separated within 43 min in a separation medium of methanol/dichloromethane/triethylamine/acetic acid 60:40:1.08:0.36 (v/v/v/v). Both normal and reverse CEP polarities could be applied for proper detection of the analytes owing to the combination of a suction effect caused by the nebulizer gas at the outlet end of the capillary and external pressure applied on the inlet vial. The separated lipid A species could be identified unequivocally by their characteristic fragmentation patterns through CID performed in both negative- and positive-ionization modes. The uniqueness of the NACE–ESI-MS/MS method lies in its simplicity and reliability for proving the phosphorylation isomerism (C1 or C4') and acylation pattern of native lipid A species or those designed for therapeutic applications.

Keywords: Acylation isomerism / Lipid A / Nonaqueous CE–MS/MS / Normal and reverse polarity / Phosphorylation isomerism DOI 10.1002/elps.201900251

Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Lipid A is the hydrophobic anchor of lipopolysaccharides (LPSs), also called endotoxins, which cover the surface of the overall majority of Gram-negative bacteria [1]. In severe bloodstream infections, the lipid A moiety of LPS can provoke a strong host immune system activation, which may lead to septic shock [2]. Canonical lipid A, produced by Escherichia coli, is represented by a C1-/C4'-bisphosphorylated β(1→6)linked α-glucosamine disaccharide carrying four 3-hydroxymyristoyl groups at positions C2 and C2' as well as C3 and C3' via amide- or ester linkages, of which two (those at C2' and C3') are further esterified with a lauric and a myristic acid, respectively [3,4]. Such lipid A structure with six acyl chains (restricted to 12–14 carbon in lengths) and two phosphate groups are optimal for the full activation of the LPS receptor [2]. Other naturally occurring lipid A variants may comprise different combinations of phosphorylation and acylation connected to the central diglucosamine backbone. Typically, the structural features that determine the biological potency of these molecules are the number, binding sites, and type of fatty acyl chains, as well as the number and substitution of phosphate groups (e.g., by phosphoethanolamine or glycosyl residues) [5–8]. For instance, the host immune response is weakened in case the lipid A is underacylated (or even overacylated), or it contains only one phosphate group [6]. Interestingly, it has been shown that lipid A with a reducing terminal phosphate (at the C1 position) was more highly inflammatory than lipid A with a nonreducing terminal phosphate (at the C4' position) [9–11]. Some tetra-acylated lipid A could even possess antagonistic properties [12–14]. Of particular note is the nontoxic 3-O-deacyl-4′-monophosphoryl lipid
A derived from Salmonella that has already been approved as a vaccine adjuvant for clinical use [15].

For the structural analysis of individual lipid A species, direct MS/MS using soft ionization methods (such as ESI or MALDI) has become a primary analytical technique providing high selectivity and sensitivity [16]. Most commonly, solvents containing chloroform (or dichloromethane [DCM]) and methanol are used to solubilize the amphiphilic lipid A molecules. However, the complete characterization of native lipid A remains an important analytical challenge, because generally lipid A preparations are highly heterogeneous biological extracts (containing several closely related compounds), even derived from a single bacterial strain [17–20]. This inherent microheterogeneity can result from incomplete biosynthesis as well as from genetic modulation in response to environmental changes to facilitate bacterial survival [21–24]. The exact determination on naturally occurring lipid A variants that have significantly different inflammatory potentials within a bacterial sample has gained growing interest over the last few years. Recognition and identification of minor molecular alterations as well as knowledge about the simultaneous presence of structural isomers (with identical molecular masses) are crucial for our understanding of the overall immune responses to these molecules. For such studies, direct MS/MS is less suitable because of the unavoidable ionization suppression effects and the overlapping of MS signals of isobaric species.

In particular, RP-LC in conjunction with ESI-MS/MS was recently demonstrated as a valuable analytical tool for the profiling of complex lipid A samples based on differences in the lipid A’s hydrophobicity [25–30]. In such a method development, special attention had to be paid to the eluting solvent system in order to provide the best solubility, separation, and MS ionization/fragmentation of the lipid A molecular species. An alternative mode of the analytical approach to LC is CE that is ideally suited for the separation of polar, charged analytes. However, CE online coupled to MS has scarcely been employed to analyze intact lipid A. Until now, only one study has been devoted to the application of CE-MS for the analysis and separation of free lipid A molecular species [31]. In that work, the authors used a mixed aqueous/organic solvent system (isopropanol alcohol, water, triethylamine [TEA], and acetic acid in a ratio of 50:50:0.06:0.02 by volume) as the sample solution, CE electrolyte, and sheath liquid [31]. However, due to the poor detection sensitivity, no MS/MS experiments could be performed during the CE run; thus, the differences between the separated mass isomers of lipid A could not be revealed.

Lipid A compounds are poorly soluble in water. In this sense, NACE represents another promising alternative technique to accomplish lipid A analysis by CE. The main advantages of using neat organic solvents and organic solvent mixtures are improved solubility and better separation selectivity of water-insoluble/barely soluble substances. These are particularly important for applications pertaining to the analysis of complex lipid A, which exhibit a set of closely related molecules of lipophilic character. Moreover, this CE mode offers even better possibilities to be directly applied for MS coupling since the running buffer contains volatile solvents, and there are possibilities of using volatile electrolyte additives, as well.

In this work, a NACE–ESI-MS/MS method was developed for the separation and structural elucidation of a set of lipid A compounds present in a heterogeneous bacterial extract. By means of already well-characterized, E. coli type lipid A species, the advantages of the NACE–ESI-MS/MS system in providing structural information with special attention to lipid A phosphorylation and acylation isomers in the positive- and negative-ionization modes are discussed.

## 2 Materials and methods

### 2.1 Chemicals and samples

Methanol (MeOH) (LC-MS Chromasolv grade), DCM (Chromasolv Plus, for HPLC, ≥99.9%), TEA (eluent additive for LC-MS), acetic acid (AcOH) (eluent additive for LC-MS), sodium hydroxide (NaOH), and ACN were purchased from Sigma-Aldrich (Steinheim, Germany).

### 2.2 Bacterial strains

*Shigella sonnei* phase II strain was cultured at 37°C in a laboratory fermentor on Mueller-Hinton broth at pH 7.2, until it reached the late logarithmic phase (about 10 h), and then collected by centrifugation. The bacterial LPSs were extracted from acetone-dried organisms by the traditional hot phenol/water procedure [32] in a yield of 5% of bacterial cell dry mass and were lyophilized.

### 2.3 Lipid A isolation and sample preparation

Lipid A was released from LPS by mild acid hydrolysis with 1% (v/v) AcOH (pH 3.9) at 100°C for 1 h; then, the solution was centrifuged (8 000 × g, 4°C, 20 min). The sediment containing lipid A was washed four times with distilled water and lyophilized. About 0.1 mg lipid A was dissolved in 200 μL of MeOH:DCM:TEA:AcOH (80:20:0.36:0.12, v/v/v/v) mixture and was centrifuged for 5 min with 21 000 × g.

### 2.4 NACE–ESI-QTOF MS/MS

NACE measurements were carried out with a 7100 CE system (Agilent Technologies, Waldbronn, Germany) controlled by Agilent Chemstation Rev. B.04.03. software. The CE instrument was coupled to a 6530 Q-TOF mass spectrometer (Agilent Technologies, Singapore) equipped with an Agilent Jet Stream ESI interface. The mass spectrometer was controlled by Agilent MassHunter B.04.00 software. The nonaqueous BGE was different mixtures of MeOH, DCM, and ACN, in the presence of TEA and AcOH as ionic additives.
at varying concentrations. The optimal BGE composition was MeOH:DCM:TEA:AcOH (40:60:1.08:0.36, v/v/v/v). Bare fused-silica capillaries (50 μm id, 375 μm od; Polymicro Technologies, Phoenix, AZ, USA) cut to a length of 55 cm were used for the separations. No simultaneous UV-DAD detection was carried out. The samples were injected by the application of 50 mbar pressure for 7 s, followed by the injection of BGE applying 50 mbar pressure for 5 s. Due to evaporation issues of the nonaqueous electrolyte solution, the separation capillary was thermostated to 20°C. The applied voltage was +30 kV in the normal CE polarity mode (anode at the inlet) and −30 kV in the reverse CE polarity mode (cathode at the inlet). Initial voltage ramps were applied, that is, the separation voltages were linearly increased from 0 to +30 kV or −30 kV under 0.2 min. Prior to use, the capillary was preconditioned by rinsing with MeOH for 5 min, followed by 1 M NaOH for 10 min, then by water for 10 min, before rinsing with BGE for 10 min. Between the runs, the capillary was washed with the running solution for 3 min.

The exit tip of the capillary was inserted into the triaxial electrospray interface. The original Agilent stainless steel ESI needle was replaced with the Agilent G7100–60041 platinum needle. To maintain stable electrospray, the capillary tip was positioned, 0.3 mm outside the platinum needle, and about 0.5 cm portion of the polyimide coating was removed. The coaxial sheath liquid consisted of 0.06% (v/v) TEA and 0.02% (v/v) AcOH in MeOH at 5 μL/min flow rate, delivered by an isotropic pump (Agilent Technologies) through a splitter set at 1:100. The electrospray voltage was set at 3.0 kV in both negative- and positive-ion modes. The nitrogen drying gas flow rate was 5.0 L/min at 200°C with a nebulizer nitrogen gas pressure of 15 psi, except for during the sample injection, when the nebulizer nitrogen gas pressure was decreased to 1 psi. The flow rate and temperature of the Agilent Jet Stream curtain gas were 2 L/min and 90°C, respectively. The MS data were collected under full scan mode in the range of m/z 50–2100 at the scan rate of 2 spectra/s. The data sets were screened for specific m/z corresponding to bis-, mono- and nonphosphorylated E. coli type lipid A species with different acylations. Identification of ion peaks was achieved by CID MS/MS experiments in both positive- and negative-ion modes by applying precursor m/z-dependent linear collision energy gradient. The slope of the linear energy gradient was 0.026 in the positive- and 0.044 in the negative-ion mode, and the MS/MS spectra were acquired with auto MS/MS mode in the range of m/z 50–2100 at the scan rate of 2 spectra/s.

3 Results and discussion

3.1 Selection of the nonaqueous BGE compounds

In NACE experiments, methanol is a widely used organic solvent. It is often mixed with ACN because the relatively high viscosity of methanol (0.55 cP) resulting in low apparent mobility of the analytes can be reduced by mixing it with the low-viscosity ACN (0.34 cP) (the two solvents are nearly isodielectric with ɛ higher than 30) [33]. However, in the case of lipid A molecules, the use of ACN proved to be inadequate as it reduced the solubility of the amphiphilic lipid A molecules. To meet the solubility requirements of bacterial phosphoglycolipids, a solvent system based on methanol mixed with DCM was adopted in this study. Although DCM is not a common solvent for NACE experiments due to its low dielectric constant (ɛ = 8.93), it proved to be a good choice because it is an effective solvent for lipid A, besides its relatively low viscosity (0.41 cP) reduces the overall viscosity of the nonaqueous mixture. The influence of the DCM ratio on the separation efficacy of lipid A structural isomers is discussed in a subsequent section.

For the selection of ionic components of the electrolyte, several aspects were considered, such as acid dissociation constant, volatility, and ionization efficiency. The primary consideration for choosing the additives was to perform the electrophoresis in a BGE with a slightly basic pH (so-called apparent pH in nonaqueous solutions) because deprotonation of the phosphate groups in lipid A is promoted at basic (or even neutral) pH values. However, it was taken into account, as well, that the ester bonds could hydrolyze at higher pH levels [34]. Based on a previous report on the CE-MS separation of lipid A in a partially aqueous electrolyte system [31], a combination of triethylamine and acetic acid (in equimolar quantities) was chosen as the electrolyte additives, and the effect of their concentration on the separation of lipid A was explored. The resulting triethylammonium acetate is a volatile salt, which, when mixed with the nonaqueous solvent mixture, maintained an apparent pH at about 8 of the resulting BGE. It should be noted that although triethylamine can cause “memory effect” in LC-MS systems [35], the application of ammonium hydroxide instead of triethylamine was not appropriate, because it led to low signal intensities of lipid A.

3.2 Parameters for NACE–ESI-MS coupling with a triaxial sprayer

Parameters, such as capillary tip position, composition and flow rate of the sheath liquid, CE and ESI voltages, flow rate of the nebulizing gas, pressure and temperature of the drying gas and the Jet stream curtain gas were all optimized to produce a stable spray and the highest signal intensity of each lipid A ion. A good spray formation is favored by the application of low surface tension and easy evaporation of organic solvents [36]. In addition, the ESI spraying is known to be more stable in the presence of a volatile, neutral salt than using only a basic additive [37]. Hence, a sheath liquid setup consisting of pure methanol modified with various (but equimolar) TEA (0.24–0.03%, v/v) and acetic acid (0.08–0.01%, v/v) contents were tested. As the electrospray content of the sheath liquid decreased, the signal intensities increased due to the reduced electrospray signal suppression effect caused by the lower amount of triethylammonium acetate [38]. However, when the lowest percentage of additives in the electrospray was used, it led to current instabilities.
Figure 1. Overlaid extracted ion electropherograms (EIE) obtained by NACE–ESI-QTOF MS analysis of lipid A species present in *S. sonnei* bacterium extract. Capillary dimensions: 55 cm × 50 μm, applied voltage: 30 kV (normal CE polarity, detection at the cathodic end), pressure assistance: 0 min: 0 mbar, 10 min: 30 mbar. BGE: MeOH with 0.36:0.12 (v/v) TEA:AcOH. The left side insert shows the EIE of the tetra-acylated monophosphorylated lipid A isomers at \( m/z \) 1280. The right side insert demonstrates the compound’s migration order. Notations: \( \mu \text{app.} \), apparent mobility; \( \text{bis-P} \), bisphosphorylated lipid A; \( \text{P1} \), C1-monophosphorylated lipid A; \( \text{P4}' \) and \( \text{P4}'^* \), acylation isomers of C4'-monophosphorylated lipid A; \( \text{non-P} \), nonphosphorylated lipid A. The green and red arrows reflect the efficient and apparent mobilities.

Therefore, the next lowest salt concentration (i.e., 0.06% TEA and 0.02% AcOH) was chosen, which provided proper current levels and about a threefold increase in signal intensities (see Supporting Information Fig. S1 for the dependence of peak areas of lipid A components on the salt concentration). Deliverance of this sheath liquid at a flow rate of 5 μL/min was found to be enough to produce high detection sensitivity in both negative- and positive-ion modes of ESI.

It is known that when interfacing CE with MS, a suction effect is generated by the natural aspiration of the nebulizing gas (15 psi was needed to obtain a stable spray) at the separation capillary tip in the ESI ion source [39]. This suction effect is known to have a great influence on the overall hydrodynamic velocity of the fluid inside the separation capillary. In our measurements, a platinum ESI sprayer needle was used instead of the standard stainless steel needle, as recommended for measurements in the reverse CE polarity mode in order to avoid needle corrosion [40]. However, it is noteworthy to mention that using the platinum needle instead of the steel needle, a marked decrease in the flow rate of the solution emerging from the CE capillary was observed. A possible explanation for this could be that the physical dimensions of the platinum needle were (most probably) slightly different compared to the steel needle, which influenced the laminar flow caused by the nebulizer. Nevertheless, during the injection process, it was necessary to drastically reduce the nebulizing gas pressure to avoid the aspiration effect; otherwise, an air segment could enter into the capillary at the inlet side, and the current could be stopped.

### 3.3 NACE–ESI-MS separation of lipid A

First experiments were carried out in order to achieve the separation of nonderivatized bacterial phosphoglycolipids by NACE–ESI-MS. Due to the intrinsic heterogeneity of lipid A, several species were simultaneously present in the sample and detected by high-resolution Q-TOF MS measurements. Such compounds had been previously determined by an HPLC-MS/MS technique developed for the analysis of lipid A heterogeneity in bacteria [25].

Pure methanol with the addition of 0.36:0.12 (v/v) TEA:AcOH (resulting in 14 μA current intensity) was chosen as the primary separation solvent for the analysis of lipid A anions from *S. sonnei*. Figure 1 shows the electropherogram obtained using the normal system polarity. As shown in the figure, the lipid A compounds (carrying a different number of acyl chains) were well resolved according to their level of phosphorylation, that is, non-, mono-, or bisphosphorylated, which exhibited considerable differences in their charge-to-size ratios. The phosphorylated lipid A, being lipophilic, weak acids, possessed a net negative charge under the slightly basic conditions. Therefore, they exhibited anodic mobilities counter EOF and migrated toward the anode by their effective mobilities. However, as a cathodic EOF developed inside the capillary (the silanol groups of the capillary wall start to deprotonate above ca. pH 7 in methanol [33,41]), the flow of the EOF directed the anionic lipid A species toward the cathode (i.e., toward the MS detection in the normal CE polarity setup).

Based on preliminary results, we revealed that the anodic mobilities of the lipid A species and the cathodic mobility of the EOF is in the same order of magnitude, and mostly, that of the EOF is slightly bigger, but the difference between the mobilities is small. As the lipid A zone counter migrates the EOF (except for the nonphosphorylated ones, which move with the EOF), the components with bigger anodic mobilities stay steadily in a given position of the capillary, and hardly reach the detector in the normal polarity mode. Moreover, the EOF velocity in a completely methanolic system is strongly reduced [42]. To maximize the efficiency of the CE-MS sys-
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must be noted that external pressure (30 mbar) was applied
organic solvent is used [43]); thus the run time was almost
produced the EOF (note that the p

addition of DCM to the running electrolyte significantly re-
any charged component was observed.

trolyte additives (0.72:0.24 TEA:AcOH) in the DCM–MeOH
methanol (a protic solvent) using the normal CE polarity
solvent mixtures of 20–60% DCM (an aprotic solvent) in
Whole electropherograms of the complex lipid A sample in
3.4 Separation selectivity and efficiency
Whole electropherograms of the complex lipid A sample in
solvent mixtures of 20–60% DCM (an aprotic solvent) in
methanol (a protic solvent) using the normal CE polarity mode are shown in Fig. 2. The concentrations of the electrolyte additives (0.72:0.24 TEA:AcOH) in the DCM–MeOH mixtures were kept constant to permit a meaningful comparison of the solvent effect. As can be seen in the figure, the addition of DCM to the running electrolyte significantly reduced the EOF (note that the pKa values of the silanol groups on the inner capillary wall can be shifted when a different organic solvent is used [43]); thus the run time was almost 20 min longer as compared to pure methanol. However, it must be noted that external pressure (30 mbar) was applied after 30 min to ensure a reasonable migration time of the sample zone.

Besides the change in migration time, several significant differences in the migration behavior of individual lipid A types could be noticed. For example, the migration zones of the C1 and C4’ monophosphorylation isomers were distinctly resolved above 40% DCM content (Fig. 2B), whereas inversion of the migration order of the C1 mono- and the bis-
phosphorylated peak clusters occurred at the highest DCM percentage applied (Fig. 2C). Most probably, there was a decrease in the dissociation (charge) of the bisphosphoryl compounds in the aprotic DCM. Furthermore, a better resolution was step-by-step observed for the differently acylated, nonphosphorylated lipid A species (moving in the vicinity of the EOF), whereas the best separation for these components was obtained at 40% DCM content. Most probably, the nonionic molecules were transformed into ionic heteroconjugates with the acetate ions present in the BGE [33,43]. The effective mobility of an ionic species is inversely proportional to the size; in this case, the acylation degree of the lipid A. Accordingly, the bigger nonphosphorylated molecules were the slower ones, and they migrated in the close vicinity of the EOF boundary. When the BGE contained no or less than 40% DCM, the affinity of lipid A molecules toward the acetate ions was found to be lower, and consequently, no or poor separation of nonphosphorylated components occurred.

The most important influence of gradual replacement of methanol by DCM was on the separation of the critical pair of tetra-acylation isomers; they were partially resolved at concentrations greater than 40% of DCM in BGE (R = 0.83 at 40% DCM and R = 1.18 at 60% DCM; also see inserts in Fig. 2). Most probably, the two tetra-acyl isomers (possessing different distribution of fatty acids) solvated by DCM had greater differences in their molecular geometry (i.e., solvation shell radii), which in turn means a difference in their charge-to-size ratio. If, however, the polarity of the system was reversed, a substantial improvement in the separation of the two isomers could be obtained. Figure 3 shows the overlaid electropherograms in the reverse CE polarity setup obtained after optimization of the salt concentration in the BGE and by using constant pressure assistance from the inlet [44] (see Supporting Information Fig. S3 for the stacked electropherograms). The application of constant external pressure (3 mbar) was enough to overcome the cathodic mobility of the EOF. The insert in Fig. 3 illustrates that an increase in the electrolyte concentrations (1.08:0.36, TEA:AcOH) allowed the two acylation isomers to fully resolve due to sufficient differences in their electrophoretic mobilities (discussed below). A significant difference between the two runs (i.e., applying either normal or reverse CE separation polarity) was in the migration order of individual lipid A species (which was exactly the opposite), illustrating the altered separation selectivity of the NACE system in the two polarity modes (compare Figs. 2B and 3). Polarity switching is especially useful for the detection of components that migrates at either the very beginning or the rear end of the sample zone, respectively. For example, using normal CE polarity, there was less time for the nonphosphorylated lipid A species to separate from each other as during the reverse CE polarity measurement under the same conditions.

Figure 4 shows the variation in the migration times and plate numbers of five representative lipid A of the different phosphorylation types (including two acylation isomers) as a function of the molar ratio and volume ratio of TEA and AcOH electrolyte additives in the nonaqueous BGE. First,
Figure 2. Overlaid extracted ion electropherograms (EIE) obtained by NACE–ESI-QTOF MS analysis of lipid A species present in S. sonnei bacterium extract. Capillary dimensions: 55 cm x 50 μm, applied voltage: 30 kV (normal CE polarity), pressure assistance: 0 min: 0 mbar, 30 min: 30 mbar. BGE: (A) 20:80 (v/v) DCM:MeOH, (B) 40:60 (v/v) DCM:MeOH, and (C) 60:40 (v/v) DCM:MeOH with additives 0.72:0.24 (v/v) TEA:AcOH in all three cases. The inserts show the EIE of the tetra-acylated monophosphorylated lipid A isomers extracted at m/z 1280. Notations same as in Fig. 1.

The molar ratio of the basic and acidic additives was varied between 1:2, 1:1, and 2:1 ratios besides keeping the total molar number of additives at a constant value (Fig. 4A). The equimolar ratio was selected for further studies because the separation between the acylation isomers using this molar ratio was the best. Then the effect of the total concentration of the additives was studied in both the normal and the reverse CE polarity modes. The results obtained in the reverse polarity mode are depicted in Fig. 4B and C. In reverse mode, the EOF is a counter flow, and the separation was pressure-assisted. An in-
Figure 3. Overlaid extracted ion electropherograms (EIE) obtained by NACE–ESI-QTOFMS analysis of lipid A species present in *S. sonnei*. Capillary dimensions: 55 cm × 50 μm, applied voltage: –30 kV (reverse CE polarity, detection at the anodic end), pressure assistance: 0 min: 3 mbar, 30 min: 30 mbar. BGE: 40:60 (v/v) DCM:MeOH with 0.72:0.24 (v/v) TEA:AcOH. The inserts show the structures and EIEs of the tetra-, penta-, and hexa-acylated, monophosphorylated lipid A isomers extracted at *m/z* 1280, *m/z* 1488, and *m/z* 1716, respectively. Notations same as in Fig. 1.

Figure 4. Influence of the molar ratio of triethylamine and acetic acid electrolyte additives on (A) the migration time in a solvent of 60:40 (v/v) MeOH:DCM, and influence of the salt concentration levels on (B) the migration time and (C) number of theoretical plate height in a solvent of 40:60 (v/v) DCM:MeOH. Capillary dimensions: 55 cm × 50 μm, (A) applied voltage: 30 kV (normal CE polarity), pressure assistance: 0 min: 0 mbar, 20 min: 30 mbar, (B) and (C) applied voltage: –30 kV (reverse CE polarity), pressure assistance: 0 min: 3 mbar, 30 min: 30 mbar. The 1:1 molar ratio of TEA and AcOH is identical with the 0.36:0.12 volume ratio. Notations same as in Fig. 1.

crease of the salt concentration led to suppressed EOF (which could be estimated by the apparent mobility of the nonphosphorylated component in normal mode) and a slight decrease of the electrophoretic mobilities of components. At the lowest salt level, the EOF was bigger than the pressure-assisted flow, and therefore the nonphosphorylated isomer escaped detection. The higher concentration of the electrolyte additives resulted in the focusing of the full sample zone; however, it yielded better resolution of the lipid A ions, due to larger electrophoretic mobility difference between them. Figure 4c shows the increase in peak efficiencies with electrolyte concentrations, which was profound for the P4+ and P4+\* acylation isomers. The corresponding resolutions obtained for the low, medium, and high salt levels were *R* = 1.5, 1.8, 2.1 in reverse polarity mode and *R* = 0.6, 0.8, 1.5 in normal polarity mode. Taken together, the reverse polarity mode using a nonaqueous solvent of 40:60 (v/v) DCM:MeOH containing 1.08:0.36 (v/v) TEA:AcOH seemed to be the best compromise for the efficient separation and MS/MS fragmentation of individual compounds in the complex lipid A sample.

In earlier work, our group has shown the usefulness of a reversed-phase HPLC-ESI-MS/MS technique for the heterogeneity profiling of complex lipid A samples [25]. Several different acylation patterns could be separated, but the resolution of C1- and C4+- phosphorylation isomers could hardly be achieved, as they coeluted. An example of the chromatographic separation result obtained for the same type of lipid A species as those analyzed by NACE–MS is shown in Supporting Information Fig. S4. Given the different separation mechanisms between CE and LC, a change in the
elution order could be seen when comparing the same set of analytes. Namely, by RP-HPLC, the lipid A species were primarily separated by their acylation (i.e., by the total number of hydrophobic acyl chains), followed by the number of polar phosphate groups (i.e., the bisphosphorylated eluted before the monophosphorylated ones). Furthermore, the separation of C1- and C4'-monophosphorylated lipid A species was only partial, or they were unresolved. Meanwhile, the NACE separation enabled baseline separation of the phosphorylation isomers and acceptable separation of the acylation isomers of lipid A. Therefore, NACE–MS and LC–MS, with their different selectivities, should be considered as complementary techniques in lipid A analysis (e.g., NACE–MS can be useful for cross-checking the determination results done by LC–MS).

3.5 Mass spectrometric determination of phosphorylation site and acylation profile

The improved solubility of the lipid A molecules in the BGE containing 40–60% DCM, in turn, led to increased intensity of the detected MS signals and lower detection limits. Thus, it enabled the application of MS/MS fragmentation measurements of the separated compounds, which is needed for accurate structural identification. By applying TEA as an additive to both BGE and sheath liquid, the lipid A components were efficiently ionized in both negative- and positive-ion modes (in two separate runs) as deprotonated molecules [M – H]– or as triethylammonium adduct ions [M + H + TEA]+, respectively. Fragmentation trends under CID conditions of both lipid A ion forms have been reported earlier [25,27,45–47]; thus, the fragmentation profiles obtained for the lipid A isomers could easily be evaluated. In the followings, only the main product ions peaks will be discussed.

Figures 5 and 6 show the positive- and negative-ionization tandem mass spectra obtained by low-energy CID fragmentation of the baseline-separated peak-pairs obtained by NACE. A major advantage in the identification of lipid A structures with a Q-TOF mass analyzer is the detection of dominant first-, second-, and even third-generation product ions. In the positive ion mode tandem mass spectra (Fig. 5), the presence of dominant B-type ions [48] (such as B₁ and B₂) formed by cleavages of the glycosidic bonds at the C1’ and C1 positions of the glucosamine disaccharide) could be observed. These fragment ions can be used as diagnostic ions to detect phosphorylation patterns of lipid A [27,45]. Namely, the B₁ ion indicates the phosphorylation state at the C1 position by the loss of the adduct-forming agent (TEA) along with the C1 substituent (i.e., H₃PO₄ in case of C1-phosphorylation or H₂O in case of C4'-phosphorylation). Furthermore, the B₂ ion gives indirect information on the phosphorylation state at the C4’ position. For instance, the B₂ product appearing at m/z 1006 on the tandem mass spectrum of one of the hexa-acyl precursors indicated that the C4’ position of the diglucosamine backbone was free, whereas the B₁ ion detected at m/z 1086 for the other hexa-acyl isomer confirmed that a phosphate group was attached at C4’.

As mentioned above, BGE with DCM ratio between 40 and 60% (v/v) provides enough resolving power to separate acylation isomers of lipid A. In the mass spectra of compounds (4)P₄’ and (4)P₄’* (Fig. 5) different B₁ ion peaks (m/z 650 and m/z 877) appear beside the identical B₂-type ion peaks at m/z 1264. These fragment ions showed evidence for the presence of two tetra-acylated C4’-monophosphorylated lipid A species with different acylation patterns.

Studies on the negative-ionization mode tandem mass spectra (Fig. 6) are important to gain information mainly on the acylation patterns of lipid A; therefore, it can be used as a complementary technique to positive-ion MS/MS. As described previously [25,27,45], fragmentation of deprotonated monophosphoryl lipid A precursors [M – H]– results in the elimination of only the ester-bound fatty acids (both primary and secondary) and some cross-ring cleavages, whereas there is a lack of dephosphorylated product ions in the tandem mass spectra. Because the fragmentation processes are governed by the position of the single phosphate group, the fatty acyl losses can take place in different manners, either more consecutively (in the case of C4’-phosphorylation) or more competitively (in the case of C1-phosphorylation). Even though there is a difference in the preference of the ester-bond cleavages between the phosphorylation positional isomer structures, highly similar tandem mass spectra are generated, showing several product ions at the same m/z but with different relative intensities. Due to this high similarity, deconvolution of isobaric lipid A structures without prior separation to MS can be a major problem. Thus, a main advantage of the NACE–ESI-MS/MS method is its high selectivity and improved sensitivity, which allow for the exact determination and comparison of CID fragmentation processes of phosphorylation isomers of lipid A.

4 Concluding remarks

For the first time, separation and full structural identification of C1- and C4’-monophosphorylated isomers in bacterial lipid A mixtures was achieved using an optimized NACE method hyphenated with ESI-QTOF MS/MS. Due to the different separation selectivities and analyte migration order obtained by former RP-HPLC experiments (which could hardly separate the phosphate positional isomers), the NACE-MS/MS should be regarded as an excellent orthogonal technique to LC-MS/MS. A unique feature of this pressure-assisted NACE-MS separation is that the migration order of the compounds can be changed by switching the CE voltage polarity, providing increased sensitivity for the detection of minor components.

It is well known that lipid A consisting of either C1- or C4’-phosphorylated lipid A structures differentially activate the innate immune system; however, little is known about the extent and biological significance of the natural structural variations in the lipid A occurring within the same bacterial strain. As shown by our NACE results, significant variation occurs in both phosphorylation and acylation of
C1-monophosphorylated lipid A

![CID mass spectra for C1-monophosphorylated lipid A](image)

- Compound (6)P1
  - CID energy: 50 eV

- Compound (6)P1
  - CID energy: 40 eV

- Compound (6)P1
  - CID energy: 30 eV

C4'-monophosphorylated lipid A

![CID mass spectra for C4'-monophosphorylated lipid A](image)

- Compound (6)P4'
  - CID energy: 50 eV

- Compound (6)P4'
  - CID energy: 40 eV

- Compound (6)P4'
  - CID energy: 30 eV

Figure 5. CID mass spectra obtained by positive-ion ESI-QTOF MS/MS of the C1 and C4' phosphorylation isomers and two C4' tetra-acylated isomers separated by NACE. The numbers in brackets show the number of fatty acyl chains in the lipid A molecule. P1, C1-monophosphorylated lipid A; P4' and P4'*, acylation isomers of C4'-monophosphorylated lipid A; non-P, nonphosphorylated lipid A.

lipid A within a single strain of S. sonnei. The thorough characterization of the structural heterogeneity by different analytical procedures is critical to understanding the structure–function relationships of lipid A. Such investigations are at the forefront of medical research. Thus, we believe that NACE–ESI-MS/MS can be a good screening procedure of phosphorylation isomers in different bacterial samples or lipid A-based therapeutics.

The research was supported by the grants ÚNKP-18-3-III New National Excellence Program of the Ministry of Human Capacities, NKFIH FK-129038, and NKFIH K-125275.

The authors have declared no conflict of interest.
Figure 6. CID mass spectra obtained by negative-ion mode ESI-QTOF MS/MS of the C1 and C4’ phosphorylation isomers and two C4’ tetra-acylated isomers separated by NACE. The numbers in brackets show the number of fatty acyl chains in the lipid A molecule. P1, C1-monophosphorylated lipid A; P4’ and P4’*, acylation isomers of C4’-monophosphorylated lipid A; non-P, non-phosphorylated lipid A.
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