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Characterization of Antigenic Oligosaccharides from Gram-Negative Bacteria via Activated Electron Photodetachment Mass Spectrometry

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Abstract

Lipooligosaccharides (LPS), composed of hydrophilic oligosaccharides and hydrophilic lipid A domains, are found on the outer membranes of Gram-negative bacteria. Here we report the characterization of core oligosaccharides of LPS by activated-electron photodetachment mass spectrometry. Collision induced dissociation (CID) of these phosphorylated oligosaccharides produces simple MS/MS spectra with most fragment ions arising from cleavages near the reducing end of the molecule where the phosphate groups are located. In contrast, 193 nm ultraviolet photodissociation (UVPD) generates a wide array of product ions throughout the oligosaccharide including cross-ring fragments that illuminate the branching patterns. However, there are also product ions that are redundant or uninformative, resulting in more congested spectra that complicate interpretation. In this work, a hybrid UVPD-CID approach known as activated-electron photodetachment (a-EPD) affords less congested spectra than UVPD alone and richer fragmentation patterns than CID alone. a-EPD combines UVPD of negatively charged oligosaccharides to yield abundant charge-reduced radical ions which are subsequently interrogated by collisional activation. CID of the charge-reduced precursors results in extensive fragmentation throughout the backbone of the oligosaccharide. This hybridized a-EPD approach was employed to characterize the structure and branching pattern of core oligosaccharides from lipopolysaccharides of *E. coli*.

Graphical Abstract

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Supporting Information Available

Figures show the structure of *E. coli* R1 dodecasaccharide, fragmentation nomenclature and graphical representation, monoisotopic masses, expansions of regions of the UVPD spectra, charge state dependence of UVPD, various a-EPD spectra, fragmentation maps, and tables summarizing all identified fragmentations from UVPD and a-EPD.

Introduction

Gram-negative bacteria are responsible for some of the most deadly global pandemics (e.g. bubonic plague, cholera).^{1–3} Many of the challenges related to combating infections caused by Gram-negative bacteria lie in the membrane structure of the bacteria.^{4–6} The surface of Gram-negative bacteria protects from normal modes of attack by antibiotics and detergents, as it is decorated with a complex system of lipopolysaccharides (LPS). LPS consist of lipid A, a conserved core oligosaccharide (OS) portion, and an outermost highly repetitive O-antigen glycan. The lipid A portion is anchored into the bacterial membrane via hydrophobic fatty acid acyl chains connected to a diglucosamine backbone and is responsible for a significant portion of the toxicity related to Gram-negative bacteria.^{7,8} Structural modifications of lipid A, presenting as variations in acyl chain length as well as the presence of covalent modifications of the diglucosamine backbone, determine the immunogenicity of lipid A.^{9–12} The mechanism of action of LPS revolves around the way in which lipid A is recognized by Toll-like receptor 4 (TLR-4) after extraction of LPS from the bacterial membrane, which is one reason why lipid A has been studied more extensively than the structural component of the oligosaccharide portion.⁵ The core OS region mediates pathogenesis as well as antibiotic and antimicrobial resistance and evasion of host defense systems.^{13,14} The incorporation of additional sugar molecules and phospho-modifications modulate the properties of the core OS region.^{5,14} The outer O-antigen region can also undergo a number of modifications, such as phospho-modifications, acetylation, and glycosylation which contribute to increased bacterial survival.^{5,15,16} In the LPS of *E. coli*, there are five unique core OS structures: K-12 and R1 – R4.¹⁷ NMR and mass spectrometry have revealed the saccharide composition and connectivity of each of these core types of LPS.^{18,19}

An array of MS/MS approaches^{20,21} have been used to analyze the lipid A portion (the most widely studied of all the domains),^{22–27} rough type LOS (LPS molecules without the highly repetitive O-antigen portion),^{28–42} smooth type LPS (LPS molecules containing the O-antigen portion),^{20,43–46} and to a lesser extent the oligosaccharide core.^{32–34,47,48} Characterization of intact LPS and LOS are the most demanding endeavors because of their amphiphilic structures, and the most effective strategies have combined multiple ion

activation methods and multi-stage MSⁿ strategies to maximize the information obtained from the complex structures.^{28–46} Conventional CID typically allows localization of glycylation, phosphorylation, and acylation of the lipid A portion of LPS and LOS, but has been less successful for oligosaccharide connectivity information owing to insufficient production of X- and A-type ions, cross-ring cleavage products typically used for characterizing branching patterns of oligosaccharides.⁴² Top-down methods have gained attention owing to the compelling goal of characterizing the lipopolysaccharides as intact species,²⁸ thus alleviating the need for hydrolysis and deacylation methods that simplify the molecules at the expense of degradation of important structural features. New ion activation methods have also provided richer fragmentation patterns or afforded complementary information compared to collisional activation.^{26,33} For example, low energy collisional activation of LOS predominantly results in cleavage of the lipid A portion from the oligosaccharide portion, thus allowing each portion to be characterized individually in subsequent stages of activation (MS³).²⁸ Using UVPD for the MS³ step provides saccharide connectivity through the production of cross-ring cleavages, whereas using HCD offers saccharide branching information through glycosidic fragmentation.²⁸ An integrated hybrid strategy combining ultraviolet photodissociation (UVPD) and high-energy collision-induced dissociation (HCD) has been developed to characterize LOS in a top-down manner.³⁵ UVPD causes more extensive fragmentation throughout the entire molecule, including the lipid A portion and cross-ring cleavages of sugars, whereas HCD is more efficient for glycosidic fragmentation, a factor which ultimately has limited the scope of HCD for complete molecular characterization.³⁵

With respect to the oligosaccharide portions of LPS, the oligosaccharides are composed of common monosaccharides such as glucose, galactose, glucosamine, galactosamine, and heptose, among others, which are linked to the lipid A portion of LPS via 3-deoxy-D-*manno*-octulosonic acid (Kdo). Numerous MS/MS methods have been used to characterize oligosaccharides in the context of glycans from glycoproteins,⁴⁹ but characterization of the oligosaccharide domains of LPS represents an especially great challenge owing to their decoration with covalent modifications such as phosphorylation and phosphoethanolamine groups which add an extra degree of structural complexity.⁴¹ Collisional activation of oligosaccharides from LPS typically produces glycosidic cleavage fragment ions centered about charged sites (such as phosphates), but not cross-ring cleavages which are key for identification of branching patterns.^{42,47,48} For example, Corsaro *et al.* explored the LOS from cold-adapted *Psychromonas arctica* by using FT-ICR with in-source fragmentation to generate B/Y ions which elucidated the oligosaccharide portions of the molecules.³² Kelly *et al.* characterized oligosaccharides of *Moraxella catarrhalis* by capillary electrophoresis coupled to ESI-MS,³³ reporting that collisional activation of the O-deacylated LOS resulted in production of complementary B/Y ions and oxonium ions.³³

In the present study, collision induced dissociation (CID and HCD), UVPD, and a-EPD are used to interrogate the structures of *E. coli* oligosaccharides. a-EPD is a hybrid activation method that utilizes UVPD to yield charge-reduced precursors via electron detachment, followed by collisional activation of the charge-reduced precursors to generate diagnostic fragment ions. a-EPD typically produces a broader array of fragment ions than collisional activation alone, and it is a promising option for characterization of negatively charged ions,

as previously demonstrated for acidic peptides,^{50,51} nucleic acids,^{52–56} lipid A,^{57,58} and heparin oligosaccharides.⁵⁹ As shown here, the collision-based activation methods generate abundant glycosidic fragment ions localized around the charged phosphate residues but do not result in diagnostic fragment ions throughout the rest of the oligosaccharide. UVPD produces fragment ions that span the entirety of the oligosaccharide structures, including informative cross-ring fragmentation that can alleviate uncertainty regarding branching patterns. However, often redundant or repetitive fragment ions are generated by UVPD which may complicate or clutter the spectra. a-EPD is employed as an alternative approach to retain simultaneously the benefits of both collisional activation and photodissociation: fewer redundant fragment ions are produced with greater sensitivity (a trait of collisional activation) while fragmentation is observed throughout the entire molecule (a trait of ultraviolet photodissociation), allowing complete characterization of the oligosaccharides.

Experimental

Oligosaccharides from LPS of *E. coli* (both individual oligosaccharides and purified mixtures of *E. coli* R1, R2, and R4) were obtained from Glycobiotech (Kükels, Schleswig-Holstein, Germany) and used without further purification. The oligosaccharides were generated from intact lipopolysaccharides by de-O-acylation using hydrazinolysis, de-N-acylation by strong base, desalting, and anion-exchange chromatography. The structure of one oligosaccharide, *E. coli* R1 dodecasaccharide, is shown as both a ChemDraw version and a graphical representation in Figure S1. The graphical representations are used throughout the rest of the study. Fragmentation nomenclature is illustrated in Figure S2, showing the diagnostic cleavages that lead to A/X cross-ring cleavage species and B/Y and C/Z ions. The structures for all of the oligosaccharides are shown in Figure S3.

Oligosaccharides were dissolved in 50:50 water:methanol to a concentration of approximately 10 μ M. All samples were analyzed using a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer (San Jose, CA, USA) equipped with a 193 nm Coherent Excistar excimer laser (Santa Clara, CA, USA) for photodissociation in the high pressure linear ion trap as previously described.²⁸ A borosilicate glass emitter (1.2 mm o.d.) was pulled to a fine tip (less than 1 μ m o.d.) using a Sutter Instrument P-2000 laser puller (Novato, CA, USA) and coated in an Au/Pd mixture for static electrospray. Each emitter was loaded with approximately 10 μ L of sample. An applied voltage ranging from 900 – 1200 V was utilized for stable ESI in the negative ion mode. MS¹ mass spectra were collected using an AGC (automatic gain control) target of 1×10^5 . All spectra consisted of 2 μ scans and were acquired using a maximum injection time of 100 ms and a resolution of 120K at m/z 400. Multiple methods of ion activation were used, all following isolation using a quadrupole with the selection width set to 5 m/z . CID and UVPD were performed in the high pressure linear ion trap, and HCD was performed in the ion routing multipole (IRM). a-EPD MS³ experiments were performed with UVPD (typically 5 pulses at 4 mJ per pulse) for MS² and collisional activation (NCE 15) of the charge-reduced precursor ion for MS³. Fragment ion interpretation was completed manually (using Domon and Costello nomenclature)⁶⁰ with the aid of the GlycoWorkbench software.⁶¹

Results and Discussion

Electrospray ionization (ESI) of oligosaccharides from deacylated LPS of *Escherichia coli* results in the production of deprotonated species ranging from the 2- to 5- charge states, as exemplified by the spectrum for *E. coli* R1 dodecasaccharide in Figure 1a. The structure for the R1 dodecasaccharide is given in Figure S1. CID, HCD, UVPD and a-EPD were employed for the characterization of the oligosaccharide anions. Examples of the CID and HCD mass spectra of the R1 dodecasaccharide (4-) are shown in Figure 1b and 1c. Both CID and HCD produced an array of highly abundant and diagnostic fragment ions originating from glycosidic cleavages, as mapped on the structure in Figure 1d. Among the fragment ions produced, the most prominent were the ($B_{7\alpha} + Y_{2\beta}$), $B_{7\alpha}$, $Y_{2\beta}$, $Y_{2\alpha}$, $B_{5\alpha}$, and $Y_{4\beta}$ ions, as well as neutral losses of CO_2 and phosphate associated with the identified fragment ions. The fragment ions generally contained the reducing end of the molecule where the majority of the phosphate moieties are localized. The production of B- and Y-type fragment ions near the reducing end allows characterization of the general composition of the oligosaccharide, but provides no insight about the branching pattern at the non-reducing end of the molecule. For the remainder of this study, CID was used as the activation method for the MS^3 modes owing to the lower abundances of neutral losses from B/Y products compared to HCD, as seen in Figures 1b/1c.

Figure 2 shows the mass spectra obtained for the same *E. coli* R1 dodecasaccharide (4-) using UVPD in MS^2 (Figure 2a) and MS^3 (Figure 2b) strategies. Identified fragment ions are listed in Table S1 and Table S2, respectively. For the latter MS^3 mode, 193 nm UVPD was followed by subsequent collisional activation of the charge-reduced precursor; this net process is known as a-EPD. Both spectra in Figure 2 display a diverse array of diagnostic fragment ions, with UVPD producing many B-, X-, Y-, and Z-type ions, as well as charge-reduced precursor ions in the 3- and 2- charge states. Expansions of the most congested regions of the UVPD spectrum are shown in Figure S4 to illustrate the rich fragmentation patterns. A map of the fragments from UVPD (Figure 2c) indicates comprehensive characterization of the oligosaccharide, including $^{1,5}X_7$ and $^{0,2}X_7$ cross-ring cleavage products that allow identification of the branching pattern of the oligosaccharide. There are, however, a number of other product ions that do not contribute new information, such as multiple cross-ring cleavages of the same saccharide (e.g. $^{1,5}X_1$ and $^{0,2}X_1$ ions originating from the second glucosamine residue at the reducing end of R1 dodecasaccharide) and redundant cleavages between neighboring residues (e.g. Y_8 and Z_8 fragment ions generated by cleavages between the two galactose residues at the non-reducing end). While UVPD typically yields a wide array of assignable fragment ions, they may cause spectral congestion.⁶²

The charge-reduced precursor ions observed in Figure 2a are a common hallmark of UVPD of negatively charged ions and can be subsequently mass-isolated, collisionally activated and converted to other informative ions via a hybrid process termed a-EPD. Collisional activation of the charge-reduced radical species in the 3- charge state yielded primarily B- and Y-type product ions that span the entire length of *E. coli* R1 dodecasaccharide (Figure 2b), allowing complete characterization of both the non-reducing and reducing ends of the oligosaccharide, rather than the more limited fragmentation of the glycosidic bonds near the

reducing end for CID only (Figure 1b). Figure 2d illustrates the fragmentation map obtained through this hybrid a-EPD approach. In particular, a series of Y-ions are produced from the non-reducing end of R1 dodecasaccharide (e.g. Y₈, Y₇, Y_{6β}, and Y₅), and a series of B- and Y-ions are generated from the reducing end (e.g. Y_{4α}, B_{5α}, Y_{2α}, and B_{7α}, among others). Additionally, an abundant ^{1,5}X₇ ion (*m/z* 1092.24 in the 2- charge state) is produced by a-EPD which reveals the branching pattern at the non-reducing end.

For all charge states, the production of charge-reduced precursor ions is among the dominant pathways upon UVPD, and the efficiency of charge reduction decreases with decreasing charge state. The charge state dependence of UVPD was evaluated for the 4-, 3-, and 2- precursors of *E. coli* R1 dodecasaccharide (Figure S5). UVPD produces informative fragment ions for all of the charge states; however, the number of fragment ions produced for the 4- charge state was greater and afforded the most complete coverage of the oligosaccharide while minimizing the number of uninformative neutral losses. a-EPD of the 2- charge-reduced precursor originating from the 3- charge state of *E. coli* R1 dodecasaccharide revealed diagnostic B- and Y-type fragment ions (Figure S6, Table S3). However, the number and abundances of fragment ions were lower than obtained by a-EPD of the 3- charge-reduced precursor originating from the 4- charge state (Figure 2b). The production of less informative MS³ spectra was consistently observed for lower charge states of other oligosaccharides, and thus for the remainder of the study the highest charge state of greatest abundance was targeted for all subsequent MS³ analysis.

The UV photoabsorption cross-sections of oligosaccharides are relatively low, hence requiring the use of UVPD parameters (5 pulses, 4 mJ per pulse) that exceed those used for much larger molecules like proteins (1 pulse, 1 mJ per pulse) which contain amide chromophores. Activation using 5 pulses reduces the abundance of the precursor ion by ~75%, and the resulting fragment ions are typically 10X less abundant than the charge-reduced precursor. The performance of a-EPD diminishes for very lowest abundance oligosaccharides, although the exceptional signal-to-noise ratio of product ions measured in an Orbitrap mass analyzer substantially extends the feasibility of the a-EPD method and allows confident distinction of fragment ions from noise.

Another dodecasaccharide (*E. coli* R2 dodecasaccharide, see structure in Figure S3b) was analyzed to evaluate the ability of a-EPD to provide unique fragmentation patterns that confirm saccharide branching. The R2 dodecasaccharide (MS¹ spectrum in Figure S7) has a similar composition to the R1 dodecasaccharide, differing only by one glucosamine versus one galactose residue (approximately 1 Da less for the glucosamine-containing oligosaccharide), but with notable variations in the branching pattern at the non-reducing end of the molecule. Figure 3a shows the a-EPD spectrum obtained for the R2 dodecasaccharide upon activation of the 4- charge state (*m/z* 620.39) for UVPD and subsequent collisional activation of the charge-reduced 3- precursor (*m/z* 827.19). The identified fragment ions are summarized in Table S4. The a-EPD spectra for the two dodecasaccharides (Figure 2b and Figure 3a) have a number of similarities, but there are unique diagnostic fragment ions that facilitate characterization of the reducing ends of each of the molecules. The R1 dodecasaccharide produced a highly abundant Y₇ ion of *m/z* 719.81, a product that was not observed for the R2 dodecasaccharide. The R2

dodecasaccharide, however, produced a unique product resulting from cross-ring cleavage $^{1,5}X_7$, resulting in the ion of m/z 1092.77 (2-). Furthermore, the B-type product ions identified for the R2 dodecasaccharide were shifted in mass by ~1 Da, confirming the presence of the glucosamine moiety at the reducing end of the molecule instead of a glucose residue. The same type of mass offset was noted for the B₈, B_{5a} and B_{7a} ions, but was not observed for the Y-type ions at the non-reducing end of the molecule (e.g. Y₅ was observed at m/z 837.93 (2-) for both dodecasaccharides). Figure 3b displays the detailed fragmentation map of the R2 dodecasaccharide that confirmed its structure.

Another pair of structurally similar oligosaccharides, *E. coli* R1 triadecasaccharide and R3 triadecasaccharide, was characterized via the a-EPD approach as summarized in Figure 4 (Table S5 and Table S6). The branching and arrangement of the sugars is the same for the R1 and R3 triadecasaccharides but the composition is different, resulting in a net mass difference between R1 (Figure S3c) and R3 (Figure S3d) of 1 Da. The MS¹ spectra of the R1 (Figure S8a) and R3 triadecasaccharides (Figure S8b) show that the 3- charge state (m/z 855.70 and 855.36, respectively) is dominant, reflecting the presence of only three phosphate moieties on the oligosaccharide structures. a-EPD of the R1 triadecasaccharide (Figure 4a) yields diagnostic B- and Y-type ions that completely map the structure (Figure 4b). The a-EPD spectrum of the R3 triadecasaccharide is shown in Figure 4c, exhibiting comprehensive B- and Y-type fragment ions, as summarized in Figure 4d. These two oligosaccharides are readily differentiated based on the 1 Da mass shift for the B-type ion series, for which the B ions of the R1 triadecasaccharide were 1 Da heavier than the series of B-ions from the R3 triadecasaccharide.

One smaller oligosaccharide (*E. coli* J-5 nonasaccharide, Figure S3f) with a similar phosphorylation pattern and glucosamine modifications as the *E. coli* R1 triadecasaccharide and *E. coli* R3 triadecasaccharide was also characterized (Figure S9, Table S7). This oligosaccharide has an identical reducing end of the molecule as the other oligosaccharides, but lacks the branched region at the non-reducing end. Formation of the 3- charge state (m/z 638.82) is dominant for the J-5 nonasaccharide (Figure S9a) upon ESI, reflecting the presence of only three phosphates. a-EPD of J-5 (Figure S9b) reveals diagnostic fragment ions which afford complete structural characterization, as mapped on the structure in Figure S9c. The truncated structure of J5 is revealed by the simplified fragmentation spectrum (as compared to longer oligosaccharides analyzed) and the comprehensive array of Y-type fragment ions generated throughout the molecule.

To demonstrate the utility of the a-EPD strategy for characterization of more complex samples, several mixtures of oligosaccharides isolated from the lipopolysaccharides of *E. coli* comprised of distinct cores were analyzed via a shotgun approach. The variation in cores is thought to influence the hydrophobicity, permeability and biofilm formation properties of the bacteria. The mixtures are derived from R1, R2 and R4 cores. The MS¹ spectrum of the *E. coli* R1 core oligosaccharide mixture is shown in Figure S10a. The charge state distribution of the mixture of oligosaccharides extends from 2- to 5- and mirrors the one observed for the *E. coli* R1 dodecasaccharide, with greater complexity in the pattern of polysaccharides. An expansion of the region from m/z 550 to 675 which encompasses the 4- charge state (Figure S10b) shows a number of unique species that were further

interrogated using the hybrid a-EPD approach. As the dominant ion present in the spectrum is similar to the one observed for *E. coli* R1 dodecasaccharide, this structure was used as a guide for building the structures of the other unknown oligosaccharides in the mixture.

Five species from the *E. coli* R1 oligosaccharide mixture (ions of m/z 565.62, 600.65, 620.64, 640.63, and 660.90, all in the 4- charge state) were selected for further analysis, and the resulting a-EPD mass spectra are displayed in Figure 5. As shown in Figure 5a, the ion of m/z 565.62 (4-) exhibited a unique and simpler fragmentation pattern than the others. The mass difference between this species and *E. coli* R1 dodecasaccharide ($m = 220.08$ Da) suggests it is missing one sugar, consistent with the absence of one Kdo residue near the reducing end of the molecule. Further analysis of the product ions confirms this hypothesis, as the $B_{7\alpha}$ and B_8 ions are shifted by the mass of a single Kdo residue, whereas the mass of the $B_{5\alpha}$ product is identical to the one in Figure 2b. The Z_8 ion (3-) of m/z 694.14 substantiates the mass of the entire structure having one fewer Kdo residue, and the Y_1 and $Y_{2\alpha}$ ions (m/z 258.04 and 499.07, respectively), confirm that the reducing end of the molecule is unchanged from the previous structures studied.

Figure 5b highlights the a-EPD spectrum of the ion of m/z 600.65 ($z = 4-$). The fact that this ion is 79.96 Da lower in mass than the most abundant species in the mixture is suggestive of one less phosphate. In the higher m/z range of the a-EPD spectrum, the presence of a $C_{5\alpha}$ ion (m/z 1291.36, $z = 1-$) confirmed that the loss of the phosphate moiety occurred at either one of the terminal glucosamine residues or the LD-Hep residue at the sixth position from the non-reducing end of the oligosaccharide. The consistent presence of the Y_1 and $Y_{2\alpha}$ product ions eliminated the possibility of phosphate loss from the terminal glucosamines, leaving only the LD-Hep residue as the site of the missing phosphate group. The product ions $Y_{4\alpha}$, $B_{7\alpha}$ and ($B_{7\alpha} + Y_{2\beta}$), all which contain the LD-Hep at the sixth position, also lacked the phosphate group, thus confirming its position for this oligosaccharide. The Y_7 and $Y_8/Y_{6\beta}$ ions observed in the 3- charge state confirm both the absence of one of the phosphate residues throughout the structure and the branching pattern of the saccharides at the non-reducing end of the molecule.

Figure 5c shows the a-EPD spectrum of m/z 620.64, the most abundant species in the mixture, and the fragmentation pattern is nearly identical to the known *E. coli* R1 dodecasaccharide described earlier (Figure 2). The m/z values of the identified product ions were similar to those of *E. coli* R1 dodecasaccharide, suggesting that the structures are the same.

The a-EPD spectrum of the ion of m/z 640.63 (4-) is shown in Figure 5d. The mass difference between this species and the oligosaccharide in Figure 5c is 79.96 Da, consistent with an oligosaccharide structure containing one additional phosphate group. The Y_1 , Y_2 , and $Y_{4\alpha}$ ions confirm that the reducing end of the oligosaccharide is unchanged relative to the oligosaccharide in Figure 5c, eliminating this region as a site of modification. The branching pattern of the oligosaccharide at the non-reducing end was revealed by the presence of the $Y_8/Y_{6\beta}$ ions, which also confirmed that the mass addition must be localized between the Y_7 position and the Y_4 position. The Y_5 ion (m/z 877.13, $z=2-$) suggests that the mass modification is localized on the LD-Hep branch, and the $Y_{4\beta}$ ion confirms the

additional phosphate residue is connected to the branched LD-Hep moiety, as depicted in the structure shown to the right in Figure 5d.

The a-EPD spectrum of the ion of m/z 660.90 is displayed in Figure 5e. Owing to the mass addition of 159.92 Da relative to the base dodecasaccharide (Figure 5c), this oligosaccharide is expected to contain one extra glucosamine. The diagnostic $B_{5\alpha}$ ion that was previously used to confirm the structure of the non-reducing end of the oligosaccharide was not present, suggesting that the extra glucosamine moiety might be located either in the middle of the saccharide backbone or the non-reducing end. The presence of the Y_1 , $Y_{2\alpha}$ and $Y_{4\alpha}$ product ions, also observed for the other oligosaccharides in this mixture, suggested a similar structure for the reducing end of the molecule as the one observed in Figure 5c. The product ion of m/z 1143.23 (2-) further corroborates this glucosamine assignment and is identified as a $Y_{4\beta}$ ion which originates from the simultaneous loss of both a LD-*glycero*-D-*manno*-heptose and a glucosamine moiety. Thus, the structure is identical to the oligosaccharide of m/z 620.64 (Figure 5c) with an additional glucosamine on LD-Hep3.

The oligosaccharides from the *E. coli* R2 and R4 cores were analyzed, and the shotgun MS¹ profiles are shown in Figure 6b,c along with that of the *E. coli* R1 core oligosaccharide mixture (Figure 6a) for comparison. The specific composition and distribution of oligosaccharides varied for each mixture, as summarized in Figure 6d. Mass differences in the observed ions reflected variations in the glucose/galactose/glucosamine compositions of the oligosaccharides. For example, the R1 and R4 core mixtures both contained oligosaccharides with masses 1 Da greater than the oligosaccharides in the R2 mixture, suggesting the difference between the two sets of structures lies in the presence of a glucose/galactose versus a glucosamine residue. The difference between R1 and R4 is a glucose (R1) versus a galactose (R4)¹⁷ at the non-reducing end of the molecule, shown graphically in Figure S3a and Figure S3e, respectively. There is no mass difference between the two oligosaccharides. The distributions in Figure 6d confirm the variation in oligosaccharides from the cores found in the LPS of *E. coli*. The profiles of these core types exhibit a number of similarities with respect to the general abundances and structural compositions of the oligosaccharides. The most abundant species in each mixture were dodecasaccharides containing four phosphates, varying only in the glucose, galactose, or glucosamine patterns at the non-reducing ends of the molecules. Furthermore, varying amounts of triadecasaccharides were also observed for the mixtures, suggesting another degree of variability between the cores.

Conclusions

UVPD followed by collisional activation in a MS³ approach offered a versatile strategy for comprehensive characterization of oligosaccharides from LPS. This hybrid a-EPD process reduced the spectral congestion and improved S/N compared to UVPD alone and yielded cross-ring cleavage products not observed for CID alone. CID of the oligosaccharides primarily generated fragment ions arising from cleavages near the charged phosphate sites, leaving the distal end of the oligosaccharides largely uncharacterized. UVPD did a remarkable job of fragmenting the entire oligosaccharide, independent of charge sites and charge state, but generated redundant fragment ions that complicated interpretation of the

spectra. The a-EPD strategy combined the attributes and mitigated the deficiencies of each activation method. This approach was applied in a shotgun manner to profile a number of oligosaccharides derived from the lipopolysaccharides of *E. coli* possessing different cores. This method offers promise for examination of profiles of oligosaccharides extracted from LPS of Gram-negative bacteria, particularly in the context of mapping modifications of the phosphate groups that are correlated with resistance to antibiotics.^{14,57,63,64}

The shotgun method used in the present study affords unsurpassed throughput for fast screening of mixtures at the expense of deeper analysis of low abundance components and possible concerns about overlapping isomeric/isobaric species. Even greater spectral decongestion and signal enhancement could be obtained by implementing the a-EPD method as part of a chromatographic workflow. A liquid chromatography (LC) or ion mobility (IM) separation method coupled with this a-EPD-MS approach would facilitate sequential isolation and characterization of each oligosaccharide individually and reduce the chances for production of chimeric MS/MS spectra caused by simultaneous activation of isomeric/isobaric species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

(a) MS¹ spectrum of *E. coli* R1 dodecasaccharide (monoisotopic mass 2486.56 Da). (b) CID (NCE 20) and (c) HCD (NCE 10)) of *m/z* 620.64 (4⁻) of R1. The precursor is labeled with a star. A red asterisk denotes neutral phosphate loss from the charge-reduced dodecasaccharide. (d) The identified product ions are mapped onto the structure of R1. Fragment ions resulting from the cleavage of two or more backbone bonds are labeled as sums (e.g. $(B_{7\alpha} + Y_{2\beta})$).

Figure 2:

(a) UVPD (4 mJ per pulse and 5 pulses) of m/z 620.64 (4-) of *E. coli* R1 dodecasaccharide and (b) a-EPD (UVPD (4 mJ/5 pulses) of m/z 620.64 (4-) followed by CID (15 NCE) of m/z 827.51 (3-)) of *E. coli* R1 dodecasaccharide (monoisotopic mass 2486.56 Da) reveals diagnostic product ions differentiating the branching pattern and substitution of a glucosamine residue for a glucose or galactose. The precursor is labelled with a star. A red asterisk denotes neutral phosphate loss from the precursor ion or charge-reduced dodecasaccharide. (c,d) The identified UVPD and a-EPD product ions are mapped onto the structure of the R1 dodecasaccharide, respectively. Fragment ions resulting from the cleavage of two or more backbone bonds are labeled as sums (e.g. $(B_{7\alpha} + Y_{2\beta})$). Summaries of the identified fragment ions are shown in Table S1 and Table S2.

Figure 3.

(a) a-EPD (UVPD (4 mJ per pulse and 5 pulses) of m/z 620.39 (4-) followed by CID (15 NCE) of m/z 827.19 (3-) of *E. coli* R2 dodecasaccharide (monoisotopic mass 2485.56 Da). The precursor is labelled with a star. A red asterisk denotes neutral phosphate loss from the precursor ion or charge-reduced dodecasaccharide. (b) The identified product ions are mapped onto the structure of R2. Fragment ions resulting from the cleavage of two or more backbone bonds are labeled as sums (e.g. $(B_{7\alpha} + Y_{2\beta})$). The identified fragment ions are summarized in Table S4.

Figure 4.

(a) a-EPD (UVPD (4 mJ per pulse and 5 pulses) of m/z 855.70 (3-) followed by CID (15 NCE) of m/z 1283.53 (2-)) of *E. coli* R1 triadecasaccharide. The precursor is labeled with a star. The red asterisk denotes neutral phosphate loss from the precursor ion. a-EPD reveals diagnostic product ions differentiating the branching pattern and substitution of a glucosamine residue for a glucose or galactose. (b) The identified product ions are mapped onto the structure of *E. coli* R1 triadecasaccharide. (c) a-EPD (UVPD (4 mJ per pulse and 5 pulses) of m/z 855.36 (3-) followed by CID (15 NCE) of m/z 1283.04 (2-)) of *E. coli* R3 triadecasaccharide. (d) The identified product ions are mapped onto the structure of *E. coli* R3 triadecasaccharide. The identified fragment ions for these oligosaccharides are shown in Table S5 and Table S6.

Figure 5:

(a-e) a-EPD (UVPD (4 mJ per pulse and 5 pulses) followed by CID (15 NCE)) of selected precursor ions (4- charge state) from the complex mixture *E. coli* R1 oligosaccharide mixture. A red asterisk denotes neutral phosphate loss from the precursor ion or charge-reduced oligosaccharide. Labels that contain a slash mark (such as Y_8/Y_6) indicate the resulting products are isomeric and can't be differentiated. The identified oligosaccharide structures are shown to the right of each spectrum.

Figure 6:

Expansions of the 4- and 3- charge state regions of the MS¹ spectra of three *E. coli* oligosaccharide core mixtures (a) R1, (b) R2, and (c) R4. (d) Distribution of the identified oligosaccharides for each of the core mixtures categorized based on charge state, z = 4- or 3-, and features of the oligosaccharides relative to the basic core structure (+glucosamine, +phosphate group, -phosphate group, -3-deoxy-D-manno-2-octulosonic acid (Kdo)). All of the oligosaccharides in the R2 mixture were 1 Da lighter than the oligosaccharides in the R1 and R4 mixtures, indicating the presence of a glucosamine residue rather than a glucose or galactose residue.