Determination of *Caulobacter crescentus* glycan strand length distribution by LC-UV-MS

Ludmila Alexandrova[1], Leigh K. Harris[2], Julie A. Theriot[2], and Allis Chien[1], Stanford University

[1] Vincent Coates Foundation Mass Spectrometry Laboratory, [2] Biophysics Program, Department of Biochemistry, and Howard Hughes Medical Institute

Overview

The shape and mechanical stability of most bacteria are maintained by the murein (peptidoglycan) sacculus. Murein is a polymeric macromolecule made of glycan strands cross-linked by short peptides, and is the main structural component of the cell wall. Glycan strands consist of (β1-4)-linked N-acetylglucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) dimers up to 30 and more disaccharide units.

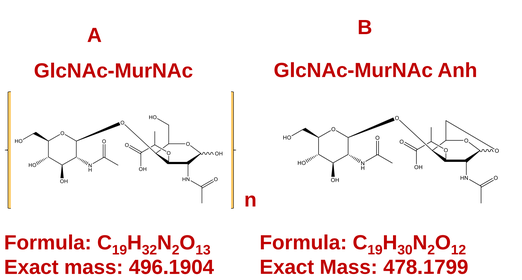
The length distribution of the glycan strands in amidase-treated peptidoglycans from *Caulobacter crescentus* has been investigated by a LC-UV-MS method. Mass measurement enables individual glycan length confirmation, and UV detection enables quantification. The ultimate goal is to understand what role glycan strand length plays in determining the overall shape and mechanical stability of bacterial cells under different physiological and drug perturbations.

Introduction

Murein (peptidoglycan) is a hetero-polymer made of linear glycan strands of alternating, β1-4-linked GlcNAc and MurNAc residues, that are cross-linked by short peptides (1,3,4,5). The peptides are attached by an amide linkage to the lactyl group of MurNAc and contain rare D-amino acids. The terminal residues of the glycan strands are GlcNAc and 1,6-anhydroMurNAc, which is MurNAc with an intramolecular ether linkage from C-1 to C-6 (MurNAc Anh).

The goal of the study was to determine the length distribution of glycan strands isolated from *Caulobacter crescentus* cells grown in different media and drug conditions. After glycan strand length distribution pattern is established and confirmed by accurate mass, the method can be run routinely on a low-resolution mass spectrometer. An HPLC-UV-MS method combining reversed-phase HPLC with ultraviolet and mass spectrometry detection for the separation, detection, identification and quantification of glycan strands up to 12 disaccharide units was established to further investigate glycan strand degree of polymerization under different treatment conditions.

SCHEME 1. Structures of glycan strand disaccharide units. A: non-reduced form (GlcNAc-MurNAc) and B: terminal residue as a reduced form (GlcNAc-MurNAc Anh)



Methods

Sample preparation: Murein sacculi were isolated from *Caulobacter crescentus* and digested with the amidase AmiD from *Escherichia coli* (2). Briefly, cells were harvested and boiled with SDS. Sacculi were harvested by ultracentrifugation and washed with water. Enzymatic peptidoglycan cleavage was performed overnight at 37°C followed by heat inactivation. Samples of purified glycan strands were diluted 2-fold with water, and 10-60 µL aliquots were injected for LC-UV-MS analysis.

LC-MS: For determination of the individual glycan lengths, high resolution mass spectrometry (HRMS) data were acquired on an Acquity UPLC (Waters) connected to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific), using ultra high resolution at 100,000 FWHM. For subsequent routine quantitative analysis, the method was transferred to a low resolution system consisting of an 1100 series HPLC-UV (Agilent Technologies) integrated with an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific).

Glycan strands were separated according to polymer length on an Agilent C18 column (4.6x50mm); the mobile phase was A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) with gradient elution from 5% to 11% B for 30 minutes at a flow rate of 0.5 mL/min. For quantitation, 60 µL of high concentration sample was injected onto the column, followed by post-UV 1:1 flow split. UV detection of glycans was achieved at 202 nm. Mass spectrometry utilized heated electrospray ionization (HESI) with positive/negative ion mode switching. Negative ionization mode was highly selective for detecting glycan strands, and positive ionization mode was useful for confirming both glycan strand and residual muropeptide components within the UV peak.

Quantitative comparison: Quantitative assessment was based on UV peak area ratio calculations. The percentage of each component was calculated as a percent ratio of peak area to the sum of peak areas of glycan strands consisting of 1 to 12 subunits.

Results

Determination of glycan length distribution was carried out by a newly developed LC-UV-MS method. This established method enables chromatographic separation of glycan strands and confirmation of their degree of polymerization up to approximately 20 disaccharide subunits. The individual length of the glycan strands was determined using LC-HRMS. During this experiment, characteristic MS features representing glycan oligomers were established and confirmed by accurate mass measurements within 5 ppm mass accuracy. Quantification of glycan strand length distribution was based on UV detection calculated as a peak area percent ratio. The predominant quantifiable lengths of the glycan strands isolated from *Caulobacter crescentus* cells were up to 12 disaccharide units. Preliminary results for the determination of glycan length distribution following treatment with fosfomycin showed changes in the degree of polymerization for glycan strands: In drug-treated samples, long chain components (4 to 12 disaccharide units) decreased while short chain components (1 to 3 subunits) increased.

TABLE 1. Glycan strand data by number of subunits: theoretical and experimental values. Data acquired in negative mode (HESI-) from murein sacculi isolated from *Caulobacter crescentus* using the LC-HRMS method.

Glycan Strand (Number of GlcNAc-MurNAc subunits)	Retention time (min)	Molecular formula	Exact Mass (Da)	Exact m/z (Da) Calculated (charge state) HESI-	Exact m/z (Da) Measured (charge state) HESI-	Mass accuracy (ppm)
1	3.5	C19H30O12N2	478.1799	477.1721 (z=1)	477.1727 (z=1)	1.32
2	8.7	C38H58O24N4	956.3598	955.3520 (z=1)	955.3528 (z=1)	0.87
3	13.8	C57H90O36N6	1434.5397	716.2621 (z=2)	716.2649 (z=2)	4.02
4	16.7	C76H120O48N8	1912.7196	955.3520 (z=2)	955.3549 (z=2)	3.07
5	18.7	C95H150O60N10	2390.8995	1194.4420 (z=2)	1194.4444 (z=2)	2.08
6	20.3	C114H180O72N12	2869.0794	1433.5319 (z=2)	1433.5356 (z=2)	2.60
7	21.9	C133H210O84N14	3347.2593	1114.7453 (z=3)	1114.7482 (z=3)	2.63
8	23.1	C152H240O96N16	3825.4392	1274.1386 (z=3)	1274.1408 (z=3)	1.75
9	24.0	C171H270O108N18	4303.6191	1433.5319 (z=3)	1433.5353 (z=3)	2.39
10	24.1	C190H300O120N20	4781.7990	1592.9252 (z=3)	1592.9275 (z=3)	1.46
11	25.6	C209H330O132N22	5259.9789	1752.3185 (z=3)	1752.3214 (z=3)	1.67
12	26.3	C228H360O144N24	5738.1588	1433.5319 (z=4)	1433.5353 (z=4)	2.39

Figure 1. Representative LC-MS chromatograms obtained on LC-HRMS platform with polarity switching. Under these reversed phase conditions, the glycans elute as a series of chromatographic peaks according to their degree of polymerization. A: Negative mode. B: Positive mode. Extent of polymerization is specified by the number of disaccharide subunits (n).

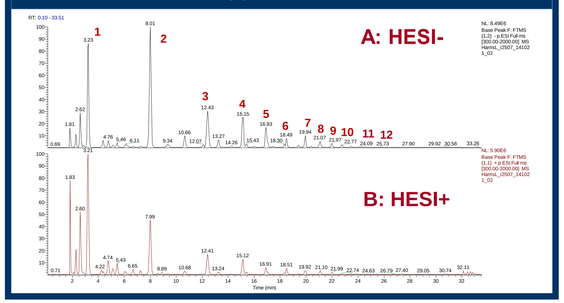


FIGURE 2. Representative high resolution MS of a glycan strand consisting of 7 disaccharides (retention time 19.9 min. in Fig. 1). A: Unzoomed spectrum, showing triply and doubly charged ions. B: Zoomed view of same spectrum, showing the triply charged ion cluster.

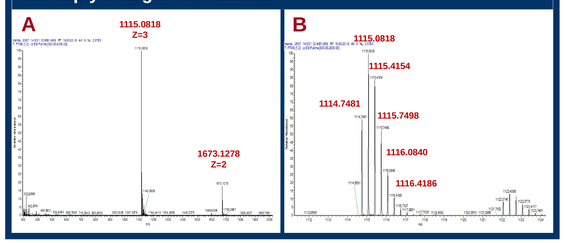


Figure 3. Representative chromatograms from LC-UV-low resolution MS. Chromatographic peaks are labeled by number of disaccharide units. A: Base peak MS chromatogram. B: UV chromatogram (202nm). C: Low resolution MS of glycan strand consisting of 7 disaccharides.

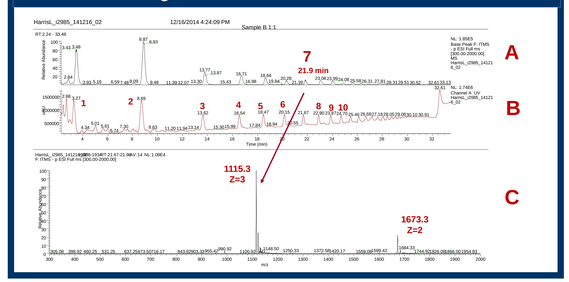
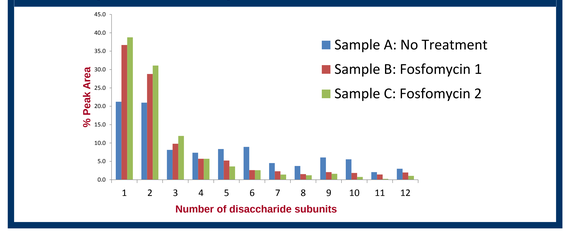


FIGURE 5. Comparison of glycan strand length distribution in samples isolated from *Caulobacter crescentus* cells grown under different drug conditions. UV response is independent of the ionization efficiency of multiply charged ions, providing an accurate quantitative assessment of each component in the glycan strand mixture. Cells grown with fosfomycin show a consistent trend toward shorter glycan strands (1 to 3 subunits).



Conclusions

- Determination of glycan length distribution was carried out by a newly developed LC-UV-MS method.
- This established method enables chromatographic separation of the glycan strands and confirmation of their degree of polymerization up to approximately 20 subunits.
- The predominant quantifiable lengths of the observed glycan strands were up to 12 disaccharide units.
- Drug (fosfomycin) treatment did affect glycan length distribution: highly polymerized components (4-12 subunits) decreased, and short chain components (1-3 subunits) increased.

References

1) Vollmer W. et.al., FEMS Microbiol Rev. 32; 2008; 259-286
2) Hayhurst E. et.al.,PNAS; 2008; vol. 105, No.38; 14603-14608
3) Takacs C.N. et.al.; PLOS ONE; 2013; vol. 8, issue 2; e57579
4) Bui N.K. et.al.;J. Bacteriol., 2009; vol. 191, No. 2; 494-505
5) Morlot C. et.al.; Genes & Development; 2010; vol. 24; 411-422

Acknowledgements

Thanks to the Vincent and Stella Coates Foundation