Distinguishing Carbohydrate Isomers with Rapid Hydrogen/Deuterium Exchange – Mass Spectrometry

Journal:	*Journal of the American Society for Mass Spectrometry*
Manuscript ID	js-2020-00314f.R1
Manuscript Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	Liyanage, O.Tara; Baylor University Department of Chemistry and Biochemistry, Department of Chemistry and Biochemistry Quintero, Ana; Baylor University, Chemistry and Biochemistry Hatvany, Jacob; Baylor University Department of Chemistry and Biochemistry, Gallagher, Elyssia; Baylor University Department of Chemistry and Biochemistry,

Distinguishing Carbohydrate Isomers with Rapid Hydrogen/Deuterium Exchange – Mass Spectrometry

O. Tara Liyanage, Ana V. Quintero, Jacob B. Hatvany, Elyssia S. Gallagher*

Department of Chemistry & Biochemistry

Baylor University

One Bear Place #97348

Waco, TX 76798

*Address reprint requests to:

Elyssia S. Gallagher

One Bear Place #97348, Waco, TX 76798

Telephone: (254) 710-2783

Email: elyssia_gallagher@baylor.edu

Abstract

Carbohydrates play key roles in facilitating cellular functions, yet characterizing their structures is analytically challenging due to the presence of epimers, regioisomers, and stereoisomers. In-electrospray – hydrogen/deuterium exchange – mass spectrometry (in-ESI HDX-MS) is a rapid HDX method that samples solvated carbohydrates with minimal instrument modification. When applied to proteins, HDX is often measured after multiple timepoints to sample the dynamics of structures. Herein, we alter the HDX reaction time by modifying the spray-solvent conductivity, which changes the initial size of ESI droplets, and thus, the droplet lifetimes. We show that this change in droplet lifetime alters the magnitude of HDX for carbohydrate-metal adducts. Furthermore, we illustrate how monitoring HDX at multiple timepoints enables three trisaccharide isomers (melezitose, maltotriose, and isomaltotriose) to be distinguished. This work illustrates the feasibility of this method for characterizing solvated carbohydrates, including isomeric species which differ only by linkage.

Introduction

Carbohydrates are ubiquitous in nature and play roles in many biological processes such as energy storage, structural support, and cellular recognition [1]. Carbohydrates form complex polymers due to the presence of epimers, regioisomers, and stereoisomers, making their structural characterization challenging.

NMR spectroscopy has been used to characterize carbohydrates; however, the requirement for large amounts of purified sample limit the utility of this method [2]. Alternatively, mass spectrometry (MS), has seen an increased prominence in carbohydrate and glycan analyses. Ion mobility-MS has been used to separate metal-adducted carbohydrates, including some isomers [3, 4]. Yet, the choice of the metal ion, size of the carbohydrate, and resolving power of the instrument limit the separation resolution [2, 5]. Tandem mass spectrometry (MS/MS) coupled to liquid chromatography has been instrumental in characterizing monosaccharide sequences in glycans [6]. Yet, unambiguous assignment of carbohydrate linkages has been inhibited by minimal cross-ring cleavage of glycans, particularly when utilizing common fragmentation methods, such as collision induced dissociation [7]. Furthermore, much of the MS/MS data is interpreted with the knowledge of the enzymes that are present in the organism that produced the glycan, minimizing the possible isomeric features that could be present and thus represented in the data [2]. Recently, hyphenated techniques coupling MS/MS with IR spectroscopy have been used to accurately assign carbohydrate isomers. However, these techniques require sophisticated instrumentation or modifications that limit their widespread use.

In-electrospray (ESI) – hydrogen/deuterium exchange (HDX)-MS has been applied to rapidly characterize carbohydrate conformations [8-10]. This technique requires minimal instrument modification, making it compatible with widespread use. HDX-MS methods are

regularly used to compare differences in the structures of two protein states, *eg*. apo- versus holo- [11]. Yet, the rapid rate of HDX for hydroxyls has limited these methods in being applied to carbohydrates. Furthermore, when comparing the structures of two proteins, HDX is often measured over multiple timepoints to ensure that differences in the dynamics of the structures are observed [11, 12]. We hypothesize that being able to sample small differences in carbohydrate structures, such as the difference between regioisomers, will require monitoring HDX at multiple, rapid exchange times. Previously, we showed that in-ESI HDX samples solvated carbohydrates since metal-carbohydrate adducts do not exchange in the gas phase [9]. Thus, the HDX reaction is quenched upon complete desolvation of the analyte. Based on this, we have demonstrated that the HDX reaction time can be altered by changing the initial ESI droplet size [10]. Herein, we modify the spray-solvent conductivity to alter the initial ESI droplet size, and thus the lifetimes of the droplets and time for HDX. We show that when sampled at multiple HDX timepoints, in-ESI HDX can be used to differentiate trisaccharide isomers.

Experimental

Maltotriose and maltoheptaose were purchased from Cayman Chemical (Ann Arbor, MI). All other materials were from Sigma-Aldrich (St. Louis, MO). Nanopure water was acquired from a Purelab Flex 3 water purification system (Elga, Veolia Environment S. A., Paris, France).

In-ESI HDX experiments used a published method [9]. Briefly, the ESI source was saturated with D_2O vapor by placing a D_2O droplet (200 µl) on a metal plate. Experiments utilized an Ion Max source coupled to an LTQ Orbitrap Discovery (Thermo Fisher, MA). Data was averaged between 4.0 min and 4.2 min after placing the D_2O droplet on the plate in the source since the $D_2O_{(g)}$ content was found to be consistent at this time. ESI parameters were spray voltage:

3.5 kV, sheath gas: 12 arb units, auxiliary gas: 0 arb units, and capillary temperature 300 °C. Between runs, the source was kept open for 2 min to prevent solvent vapor build-up [13]. Undeuterated spectra were collected with neither the metal plate nor D_2O droplet inside the ESI source.

For experiments studying the effect of conductivity on in-ESI HDX, the melezitose concentration was varied from 100-750 μM. To the melezitose samples, we added either NaCl (50 μM-8 mM) or Girard's T reagent (86 μM-13 mM). Spray solvents were 99:1 (v/v) methanol: water or 100 % nanopure water, as stated. Conductivities were measured using a Thermo Scientific Orion® 013005MD DuraProbe™ 4-Electrode Conductivity Cell Probe coupled to an Orion Star A215 conductivity meter (Waltham, MA).

For experiments distinguishing carbohydrates isomers using in-ESI HDX, separate solutions of each carbohydrate (melezitose, maltotriose, and isomaltotriose) were prepared in solvents of 99:1 (v/v) methanol: water. The carbohydrate concentration was kept constant at 500 μM, while the conductivity was varied by changing the concentration of NaCl in solution, resulting in conductivities ranging from 10-100 μS/cm. An internal standard was prepared by derivatizing maltoheptaose with Girard's T reagent [14]. Briefly, maltoheptaose was reacted with an excess of Girard's T reagent for 3 hrs at 75 °C in a solvent of 8.5: 1.5 (v/v) methanol: acetic acid. The reaction mixture was concentrated by drying under a stream of nitrogen. Excess Girard's T reagent was then removed by passing the reaction mixture through PD Minitrap™ G-10 columns (GE Healthcare, Chicago, IL). Eluted fractions containing derivatized maltoheptaose were collected, lyophilized, and reconstituted in methanol. Isomer samples were spiked with internal standard (50 μl). Derivatized maltoheptaose from the same reaction was used as the internal standard for all analyses.

Using experimental mass-to-charge values (*m/z*) and intensities (*I*), the weighted average mass (*M*) of undeuterated and deuterated metal-adducted carbohydrates was calculated.

$$M = \left[\frac{\sum(m/z) \cdot I}{\sum I} \right] z \qquad (1)$$

The number of HDX (*#D*) was calculated by subtracting the weighted average mass of the undeuterated analyte from that of the deuterated analyte.

$$\#\mathrm{D} = M_{\mathrm{Deuterated}} - M_{\mathrm{Undeuterated}} \qquad (2)$$

The relative HDX describes the ratio of the number of HDX (#D) for the carbohydrate to that for the internal standard.

$$Relative\ HDX = \frac{\#D_{carbohydrate}}{\#D_{internal\ standard}} \qquad (3)$$

All measurements were performed on a minimum of four replicates. The number of HDX and relative HDX are presented as the average ± standard deviation. For figures, each data point represents the average with error bars indicating standard deviation. Statistical analyses used Students' *t*-test at the 95% confidence interval.

Results and Discussion

When HDX is performed during ESI, the magnitude of exchange is dependent on both the conformation of the analyte and the lifetime of the ESI droplets. Herein, we altered the lifetime of the ESI droplets to sample the structural dynamics of carbohydrate isomers. During ESI, a Taylor cone emits positively charged droplets, which undergo evaporation and fission until they reach nm-sized droplets from which metal-adducted clusters of melezitose are released [15]. These clusters then undergo coulombic repulsion, primarily forming adducts with a single carbohydrate and metal ion. Since metal-adducted carbohydrates do not exchange in the gas-phase [9], the HDX

reaction is quenched upon complete desolvation of the ESI droplets. Thus, the main factor governing the lifetime of ESI droplets is the evaporative rate, which depends on the solvent vapor pressure and the square of the initial droplet diameter [16].

de Juan and de la Mora proposed a scaling law (Equation 4) that describes the initial diameter of ESI droplets (D), which is dependent on the flow rate of the spray solvent (Q), electrical conductivity (κ), dielectric constant (ϵ), and permittivity of free space (ϵ_0). G (ϵ,Π_μ) is a slowly varying function of the dielectric constant (ϵ) and the viscosity variable (Π_μ) [17].

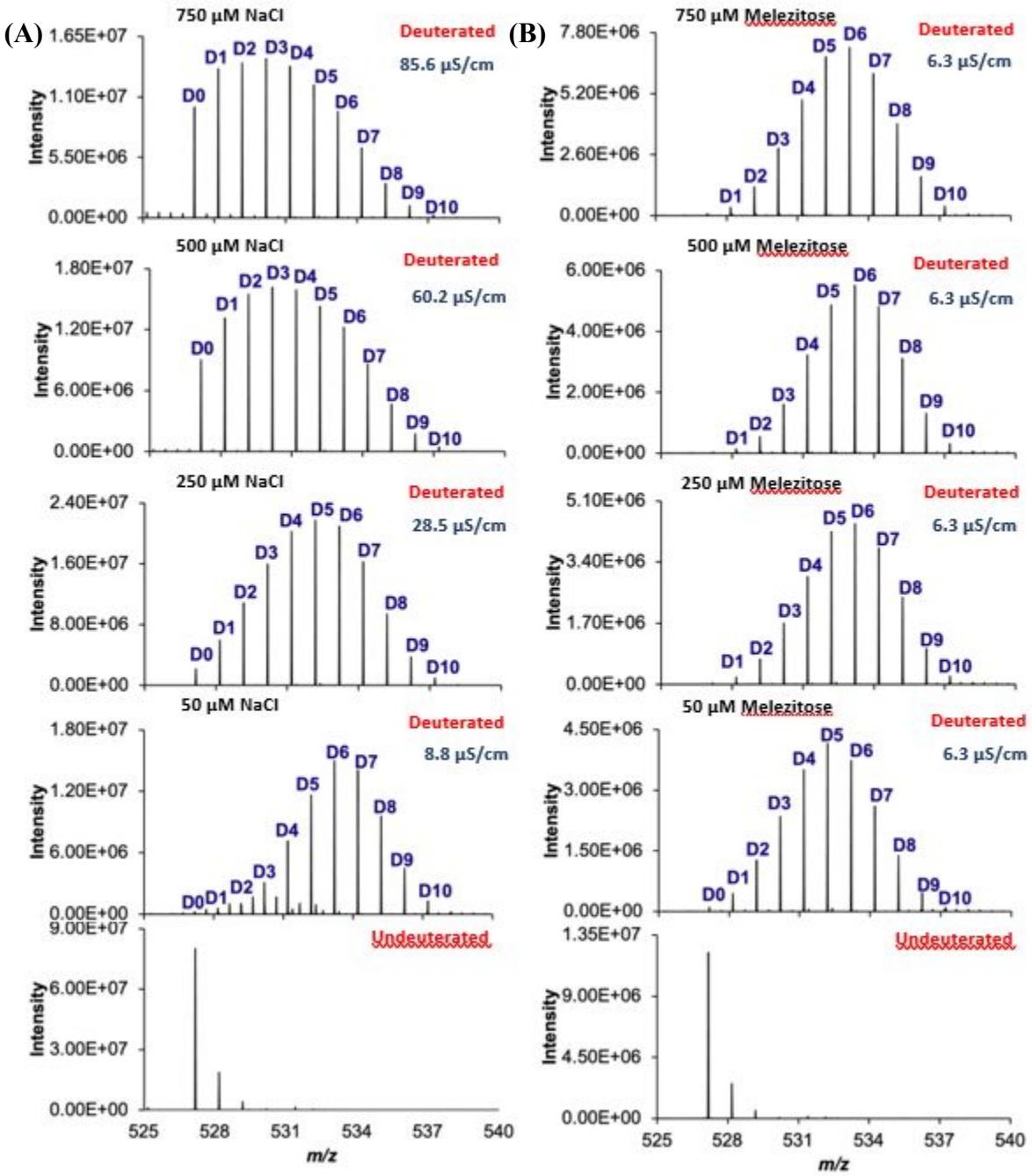
$$D = G(\boldsymbol{\epsilon},\Pi_\mu)\left[\frac{\epsilon\epsilon_0 Q}{\kappa}\right]^{1/3} \qquad (4)$$

According to the scaling law, spray-solvent conductivity and ESI droplet diameter are inversely proportional. Olumee *et al.* used phase anemometry experiments to measure decreasing ESI droplet diameters with increasing spray-solvent conductivities [18].

Previously, we reported that the concentrations of metal ions and melezitose affect the magnitude of HDX observed during in-ESI HDX [9]. These experiments varied both the metal ion and melezitose concentrations while maintaining a constant molar ratio. Herein, we studied the isolated effects of the metal ion and melezitose concentrations. When the NaCl concentration was varied from 50 μM to 750 μM, with a fixed melezitose concentration (500 μM), the distribution of peaks in the mass spectra shifted to lower *m/z* values (Figure 1A). This correlates with the number of HDX decreasing from 5.8 ± 0.3 D to 3.2 ± 0.1 D (Figure 2A). During in-ESI HDX, spray solutions with increasing concentrations of metal ions correlate to increasing solution conductivities. Hence, the increase in conductivity (8.8 μS/cm to 85.6 μS/cm) resulted in smaller ESI droplets, decreasing the time available for exchange, and yielding a decrease in the magnitude of HDX. The magnitude of HDX also decreased when the spray-solvent conductivity was

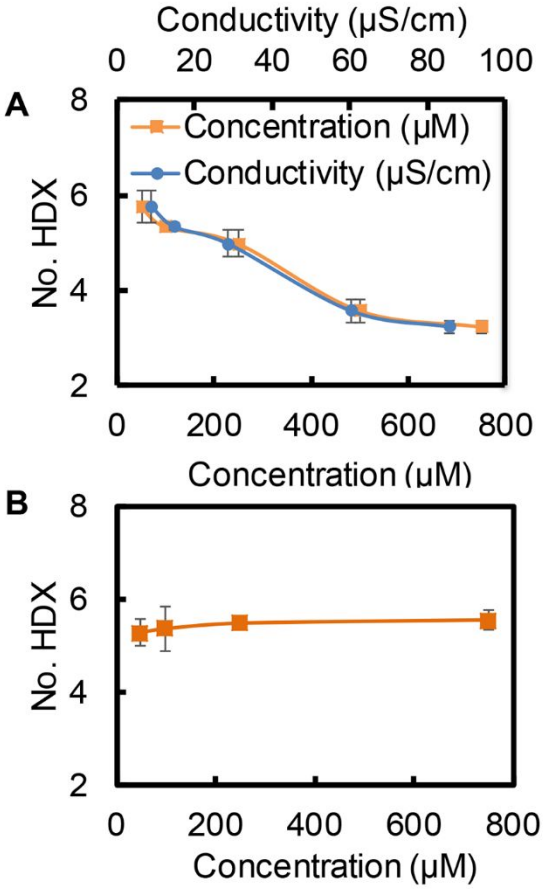
increased for spray solvents of 100 % water containing either NaCl or charged, organic molecules (Figure S1).

Figure 1. Representative mass spectra of [Melezitose + Na]$^+$ comparing the effect of either the (A) metal ion concentration (with 500 µM melezitose) or (B) melezitose concentration (with 50 µM Na$^+$). The spray solvent was 99:1 (v/v) methanol: water.



When the concentration of melezitose was varied from 50 μM to 750 μM with a fixed NaCl concentration (50 μM), the number of HDX remained consistent since the conductivity did not change (Figure 2B). In the mass spectra, the peak distributions were comparable with the D5 or D6 peak being the most intense (Figure 1B). The analyte concentration has been reported to result in clustering during ESI [19], which could decrease the number of exposed sites and decrease the number of HDX. However, changes in the melezitose concentration, which would reflect the extent of clustering, did not affect the magnitude of HDX (Figure 1B and 2B). Recent studies by our group, have shown that metal-adducted melezitose is released into the gas phase by the charged residue model and clustering was observed when the amount of solvent was low [15]. However, due to the presence of Na^+, coulombic repulsion resulted in disassembly of most of the clusters. Thus, clustering is observed at late stages of ESI, just before melezitose is released into the gas phase, at which point exchange no longer occurs for metal-adducted carbohydrates [9, 15]. Since we did not observe a difference in the number of HDX in relation to the melezitose concentration, we hypothesize that the clusters of melezitose do not exist long enough to alter the observed number of HDX.

Figure 2. Plots illustrate the number of HDX (No. HDX) for [Melezitose + Na]$^+$ upon changing the concentration of (A) Na^+ with a constant melezitose concentration (500 µM) or (B) melezitose with a constant Na^+ concentration (50 µM). (A) shows that the number of HDX is consistent for samples with increasing NaCl concentrations or solution conductivities. Error bars (n = 5) are present for each data point but are not visible when smaller than figure symbols.

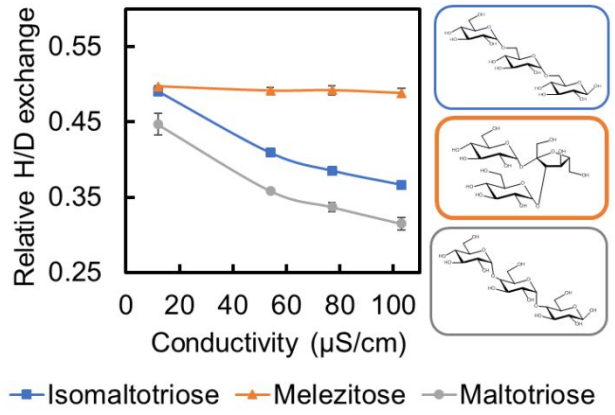


Previously, we showed that using an internal standard reduces day-to-day and run-to-run variability that result from changes in humidity and residual solvent vapor inside the ESI source [14]. Further, we established that using the internal standard effectively reduced the variability of in-ESI HDX measurements for carbohydrates with different sizes. Here, the internal standard was added to each carbohydrate-isomer sample. When analyzed at the same conductivity, we expect the internal standard in each sample to ionize in the same way and have the same time for HDX.

Thus, the internal standard is expected to behave similarly in each isomer sample at constant conductivity; therefore, differences in the relative HDX for the isomers are due to differences in the isomer structures.

To evaluate the utility of in-ESI HDX to distinguish conformations of metal-adducted isomers, we compared the relative HDX values at a conductivity of 12 μS/cm for three trisaccharides: melezitose, maltotriose, and isomaltotriose. The relative HDX value for maltotriose (0.447 ± 0.014) differed from that for isomaltotriose (0.491 ± 0.004) and melezitose (0.497 ± 0.001), but melezitose and isomaltotriose could not be distinguished. To distinguish melezitose from isomaltotriose it was necessary to sample multiple time points of HDX. We controlled the HDX reaction time by altering the spray-solvent conductivity. We analyzed three additional spray-solvent conductivities – 54 μS/cm, 77 μS/cm, and 103 μS/cm, or HDX reaction times. The relative HDX values for melezitose, isomaltotriose, and maltotriose were distinguishable from each other at all three of these conductivities, or HDX reaction times (Table S1). For each of these conductivities, larger differences in the relative HDX values were observed between melezitose and isomaltotriose compared to isomaltotriose and maltotriose. Melezitose differs from isomaltotriose and maltotriose by both the constituent monosaccharides and their linkages. Alternatively, maltotriose and isomaltotriose both contain three glucose units, but differ only by linkage. These results demonstrate that conformational differences between carbohydrates can be detected by in-ESI HDX. Furthermore, in agreement with our hypothesis, rapidly sampling the dynamics of carbohydrates at multiple HDX timepoints enabled the differentiation of isomers, including regioisomers. Representative mass spectra for all three isomers at all conductivities are provided in the Supplemental Information (Figures S2, S3, and S4).

Figure 3. Relative HDX of trisaccharide isomers detected as [Trisaccharide + Na]$^+$. Error bars (n = 4) are present for each data point but are not visible when smaller than figure symbols. The structures are shown for isomaltotriose (blue squares), melezitose (orange triangles), and maltotriose (grey circles).



Conclusions

This work shows, for the first time, that carbohydrate isomers can be distinguished by comparing in-ESI HDX for two or more structures. Furthermore, we illustrate that slight variations in structure, including differences in regioisomers, can be distinguished when we sample HDX for multiple exchange times. Here, we controlled the time for HDX by altering the spray-solvent conductivity, which changes the initial size of the ESI droplets and thus the desolvation time. While we have demonstrated the importance of varying HDX reaction time to distinguish isomers using conductivity, alternative methods of changing the size of ESI droplets and thus desolvation time would be expected to yield similar results. Since carbohydrate-metal adducts do not exchange in the gas phase, this method samples solvated carbohydrates. Thus, this method provides an opportunity to sample these solvated, potentially biologically relevant carbohydrate structures, which cannot be readily analyzed by other analytical techniques. In our ongoing work, we are applying in-ESI HDX to analyze other carbohydrate isomers of varying size and with different

isomeric features, including branching. Finally, we show that in-ESI HDX can be used to compare differences in the structures and dynamics of carbohydrates, a class of biomolecules that have historically been challenging to analyze.

Acknowledgements

OTL was supported by the Welch foundation (AA-1899). AVQ and JBH were supported by the National Science Foundation (CHE-1945078). We thank the Baylor University Mass Spectrometry Center.

Supplemental Information

The supplemental information includes representative mass spectra and data illustrating the effects of conductivity on HDX when using aqueous spray solvents.

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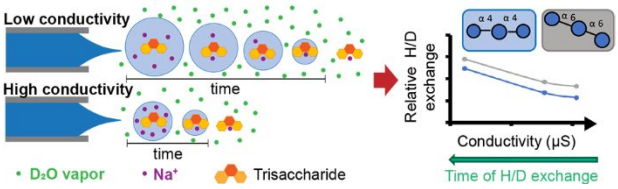

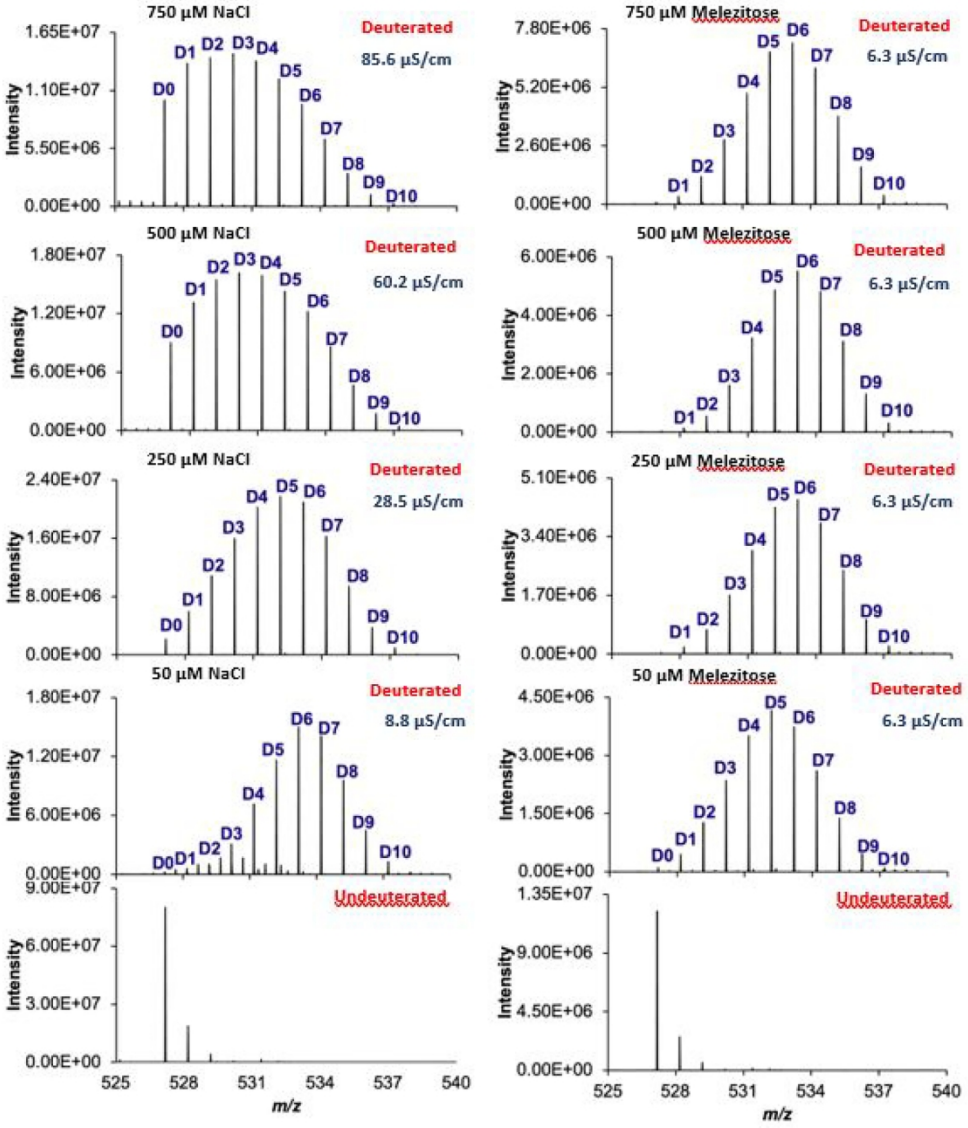

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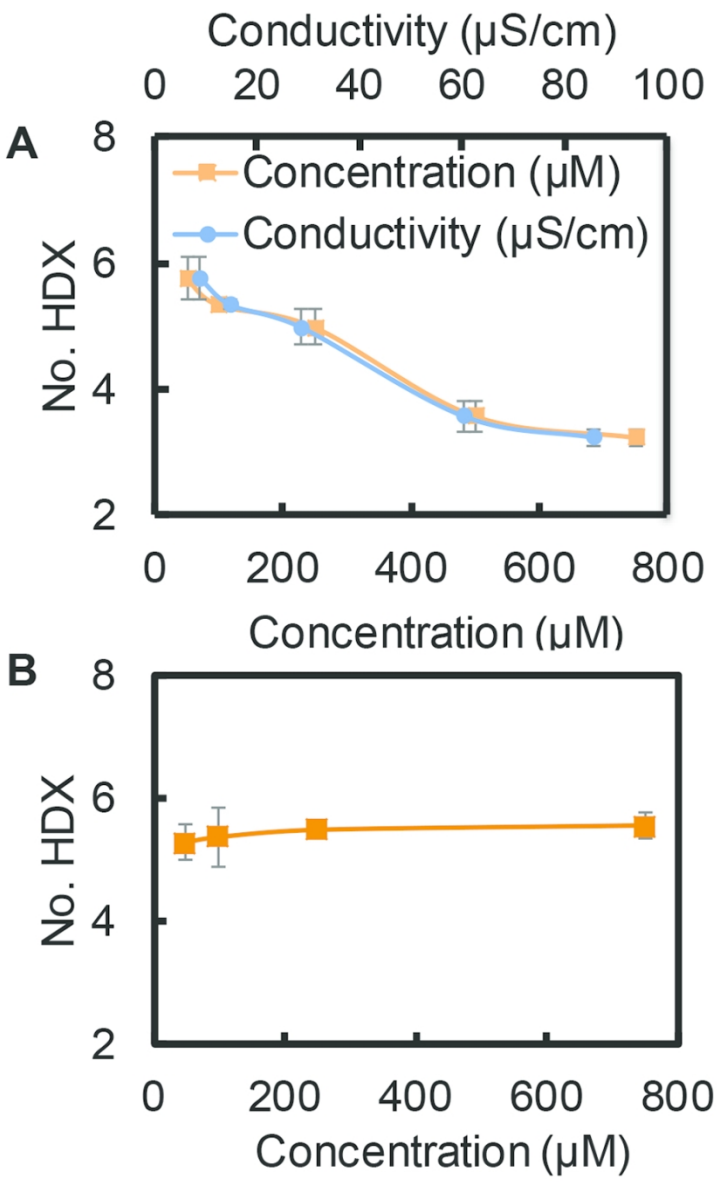

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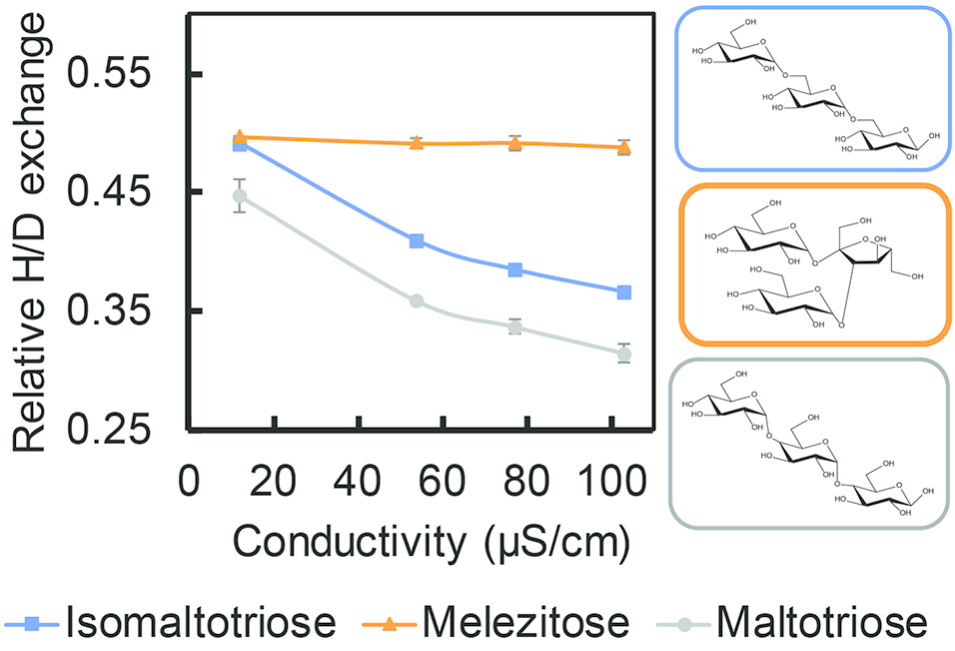

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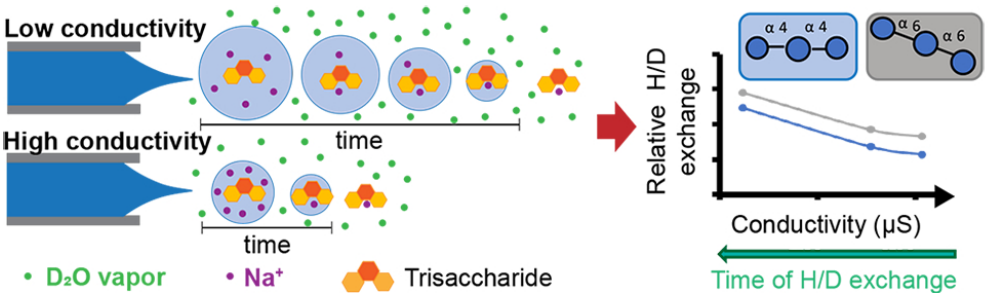

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