Electronic Supplementary Material (ESI) for Analyst.

Supporting information

Analyzing glycans cleaved from a biotherapeutic protein using ultrahigh-resolution ion mobility spectrometry together with cryogenic ion spectroscopy

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1. Clean up procedure

We used C18 cartridges to separate N-linked glycans from O-glycopeptides, proteins and other contaminants. The procedure was performed as follows:

1. C18 cartridge was conditioned with 1 ml of methanol (x3) and then with 1 ml of 5% acetic acid (AcOH) (x3).
2. The glycan sample was loaded onto the C18 column; the sample container was rinsed with 100 µL 5% acetic acid 2 times to make sure all the sample was transferred.
3. N-glycans were eluted with 1 ml 5% AcOH (x3)
4. The resulting N-glycan sample was dried using speed vacuum (SpeedVac, Eppendorf).

In order to remove salts and detergents, we used porous graphitic carbon (PGC) cartridges.

1. The PGC cartridge was conditioned with 1 ml ACN (x3), followed by 1 ml of 60% ACN (x3).
2. It was then equilibrated with 1 ml of water (x3).
3. The glycan mixture was loaded onto the PGC cartridge, either directly after deglycosylation with PNGase F or after cleanup with C18 cartridges.
4. The glycan mixture was washed twice with 1 ml water, discarding the filtrate.
5. Glycans were eluted with 100 µl of 40% ACN/60% 100 mM ammonium formate pH 4.5.
6. Samples were dried using a SpeedVac.

2. Fraction collection

The digested sample was separated on an XBridge Glycan BEH Amide Column (3X150 mm, 2.5 µm) (Waters) at 0.4 mL/min at 60°C. The glycans were eluted by following linear gradients: 22% mobile phase A (ammonium formate buffer, 100mM) and 78% mobile phase B (ACN) for 38.5 min, then by increasing mobile phase A from 22% to 44% in 1 min, then from 44% to 100% in 1 min. In the final wash step, mobile phase A was held at 100% for 5 min. Prior to analysis, the column was equilibrated by running 22% mobile phase A and 78% mobile phase B for 2 min. The auto-sampler was kept at 8°C.

A typical chromatogram for the studied glycans is shown in Fig. S1. The fractions for each glycan were collected for 1 min, and were further analyzed with ion mobility and cryogenic spectroscopy.

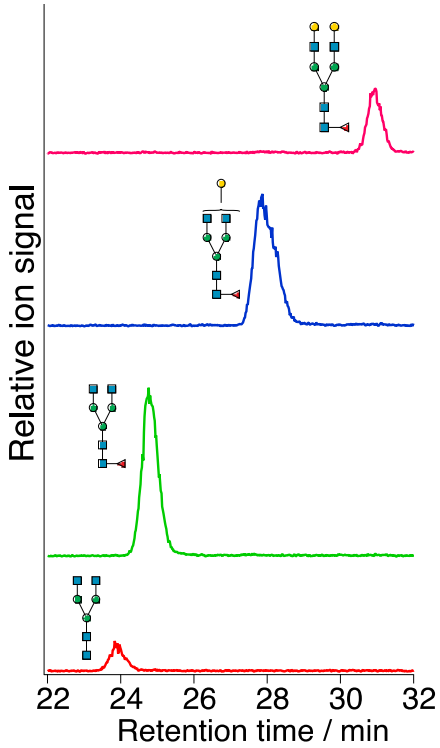

Figure S1. Typical chromatogram of fractions of the eluted glycans : G0, G0F, G1F and G2F.

3. Ion mobility spectrometry settings

The optimal TW amplitude and speed in SLIM-IMS region were found experimentally for each glycan. Typically, the IMS parameters used for the experiments were: RF frequency 885 kHz, RF amplitude 150 Vpp, TW amplitude 20 V, TW speed 500 m/s, drift gas pressure 3 mbar (He).

The signal enrichment process for the ions of interest is performed as follows. The ion packets (130 µs wide) are released from the ion funnel trap (IFT) at a repetition rate of 5 Hz to 10 Hz, depending upon the time window that was needed to mobility-separate the ions. After 1.2 m of separation, the ions interest are selected and introduced to the SLIM on-board trap 1 (Fig. S2). [1] This is done by briefly applying TW potential to the trap entrance electrodes and a 50 V bias to the blocking electrodes on the main track, which guides these ions into the trap. After this, they can be stored for a defined period of time before they are released for additional cycles of mobility separation, or to the cryogenic trap for IR spectroscopy. Releasing ions from the trap is performed by applying TW potential to the trap exit electrode. For signal enrichment purposes, ions from the next IFT pulse were also separated on the 1.2 m drift path, selected, and stored in the same on-board trap, which typically resulted in doubling of the amount of trapped, mobility selected ions. Although the on-board trap started to overfill after 3 and more IFT packets were used for signal enrichment, an increase in signal was still observed when using up to 6 IFT ion packets.

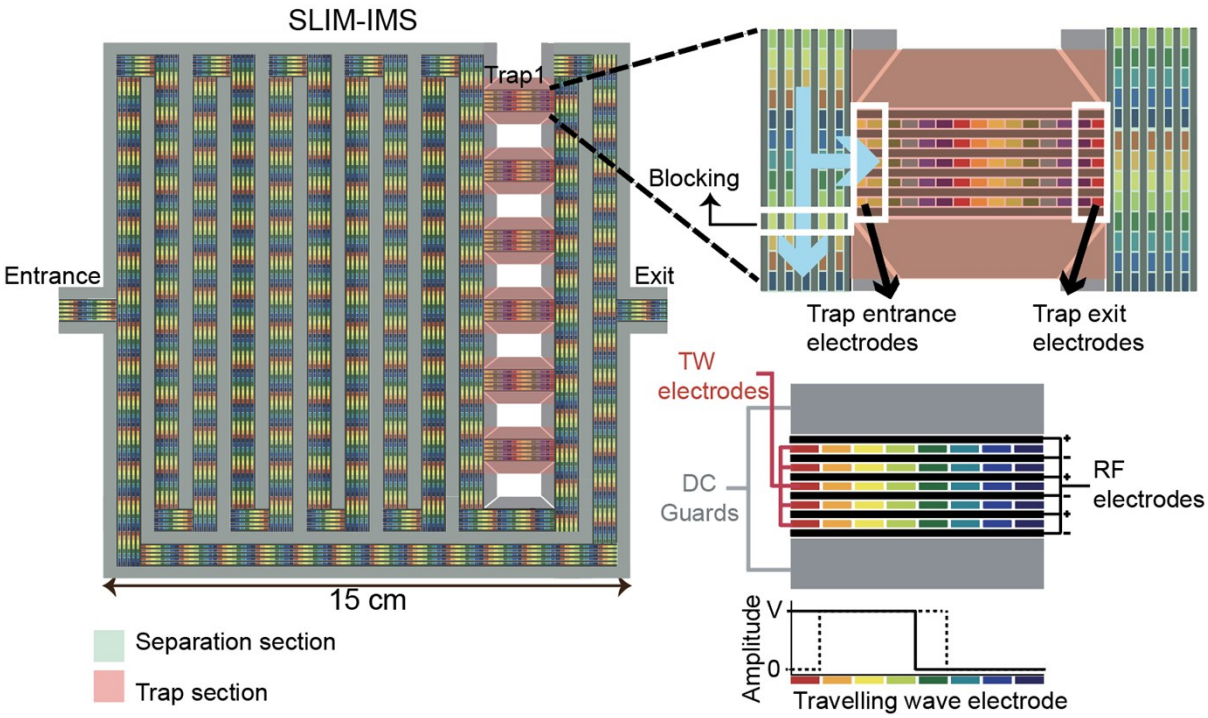

Figure S2. Layout of the SLIM-IMS device employed in this work. Left: scheme of the SLIM board. Right: details of on-board traps used for enrichment. Adapted from Ref. 1.

4. Quantification of spectral comparison with standards

To quantify the similarity between IR spectra of reference and cleaved glycans, we determined the correlation coefficient which provides a measure of the degree of similarity between two data vectors.

The correlation coefficient (r) between two vectors x and y is defined as

$$r = \frac{\sqrt{cov(x,y)}}{\sqrt{var(x)} \cdot \sqrt{var(y)}}$$

where

$$cov(x,y) = \sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y});$$

$$var(x) = \sum_{i=1}^{n} (x_i - \bar{x})^2;$$

$$var(y) = \sum_{i=1}^{n} (y_i - \bar{y})^2;$$

and:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i;$$

$$\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i;$$

It can be seen that the vectors x and y have to be normalized on the total number of points of the vector.

Table 1 presents the correlation coefficients that demonstrate the similarity between the spectra of the doubly protonated standard and the cleaved sugars.

Table. 1 The results of the correlation coefficient method applied to the spectra of each corresponding pair between the standard and the cleaved sugars.

	G0(cleaved)	G0F(cleaved)	G1F(cleaved)	G2F(cleaved)
G0(standard)	0.9599478	0.91782683	0.8777069	0.86271922
G0F(standard)	0.89727567	0.97722808	0.8992873	0.90079167
G1F(standard)	0.91029232	0.92792514	0.98836676	0.9602757
G2F(standard)	0.86496819	0.8818684	0.96427554	0.98312956

The similarity between the standard and the cleaved sugars, as measured by the correlation coefficient, are the following: 95.9 % for G0, 97.7 % for G0F, 98.8 % for G1F, and 98.3 % for G2F.

The correlation coefficient between the spectra of G1F and G2F is quite high (96%) due to similarity between their overall spectra that do not contain distinctive features in the weakly hydrogen-bonded OH stretch region (3450-3550 cm^{-1}). However, if we use only the free OH stretch region (3580-3700 cm^{-1}) for comparison, then the resulting correlation coefficient between G1F and G2f is 91.2%.

It is important to note that the arrival-time distribution (ATD) of G1F and G2F is distinctly different, and thus in cases where the spectral correlation between two species is high, the ATD can also be used to distinguish them. Moreover, the spectra of Figure 7 were measured without selecting any part of the ATD. Doing so produces spectra that have considerably more structure, as shown in Figure 5. This needs to be done only in cases where the non-mobility-resolved spectra are highly correlated.

5. Determination of detection limits

We used Etanercept having a mass of ~128 kDa, of which, 12-13 kDa can be attributed to N-glycans.[2] Thus, in the 600 μg sample of Etanercept that we used, ~60 μg corresponds to N-glycans. To determine an upper bound for our limit of detection, we assume that we lose none of the sample during preparation and clean-up.

The glycan G0 (~1316 Da) has ~1 % abundance among N-glycans attached to Etanercept.[3] This represents ~0.73% by mass, which would mean that we would have ~0.44 µg of this particular glycan. We typically had ~ 1 ml of the combined fractions together for G0, which gives us ~0.33 µM

of the cleaved sugar G0. The measurement of one IR spectrum takes in average 3 mins for the range from 3400 cm^{-1} to 3750 cm^{-1}, and using an upper limit to the nESI flow rate of 100 nl/min, this means we use 300 nL of solution. From this, we can estimate that a total amount of sample upon which we made our measurement is ~100 fmol. If we repeat the same calculation for G0F, which is the most abundant N-glycan from Etanercept (21.8%), this means that our measurement was made on ~2 pmol.

References

1. P. Bansal, V. Yatsyna, A. H. AbiKhodr, S. Warnke, A. Ben Faleh, N. Yalovenko, V. H. Wysocki and T. R. Rizzo, *Anal. Chem.*, 2020, in press (doi:10.1021/acs.analchem.0c01265).
2. T. Wohlschlager, K. Scheffler, I. C. Forstenlehner, W. Skala, S. Senn, E. Damoc, J. Holzmann and C. G. Huber, *Nature Comm.*, 2018, **9**, 1713.
3. S. Houel, M. Hilliard, Y. Q. Yu, N. McLoughlin, S. M. Martin, P. M. Rudd, J. P. Williams and W. Chen, *Anal. Chem.*, 2014, **86**, 576-584.