Tracking free-toxin distribution in xenografts after *in-vivo* ADC dosing by MALDI Imaging

MALDI-MRMS imaging of free released toxin in xenografts after ADC *in-vivo* dosing.

Abstract

Antibody-drug conjugates (ADC), a fast-growing class of anticancer drugs, typically comprise a cytotoxic payload that is chemically linked to a monoclonal antibody. The antibody enables selective targeting of the toxin to tissues that express the cognate antigen on cell surfaces. Drug safety and efficacy of ADCs depend to a large extent on the virtually exclusive release of the toxic payload in target tissues. MALDI magnetic resonance mass spectrometry (MRMS) imaging offers ultra-high resolving power and mass accuracy needed for label-free, highly specific, and spatially-resolved assessment of toxin (or toxin still conjugated to remnants of the chemical linker) distribution during ADC *in-vivo* pharmacology studies. Here, we show free toxin distribution in tumor xenograft-bearing mice after administration of a single dose of an ADC using MALDI-MRMS Imaging and SCiLS™ Lab software for visualization.

Keywords: solariX XR 7T, SCiLS™ Lab 2021, mass spectrometry imaging, ADC, BioPharma, Toxin, MRMS

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Introduction

First generation ADCs are already used in cancer therapy and future generations of ADCs with innovative payloads are in clinical trials. Linker technologies and protein scaffolds are an active research focus for many pharmaceutical companies. Therefore, there is high demand for new analytical methods that aid in understanding their *in-vivo* distribution ("drug metabolism and pharmacokinetics"; DMPK) and efficacy.

Preclinical characterization of ADCs includes the evaluation of the distribution of the drug and its metabolites in rodents. Techniques like autoradiography (ARG) and LC-MS/MS are commonly utilized to gain information about drug distributions and possible off-target effects. ARG is sensitive, but requires radioactive test compounds, which are typically not commercially available and connected to safety hazards. Additionally, this technique is unable to distinguish between the parent drug and its radioactive metabolites. LC-MS/MS analysis allows for the determination of total drug content in organs and xenograft samples, but the tissue is homogenized during sample preparation, and critical spatial information is lost. MALDI mass spectrometry imaging (MALDI-MSI) generates mass spectra from tissue sections. As a label-free technology, it enables visualization of the spatial distribution of molecules of interest and clear differentiation between the drug and its metabolites. Combining MALDI-MSI with ultra-high mass resolving power and mass accuracy allows for identification of compounds using their exact mass as well as isotopic fine structure (IFS).

Experimental

Animal study design and drug administration

Animal tissue was obtained from Heidelberg-Pharma.

CB17-Scid mice were injected with human multiple myeloma cell line NCI-H929. After sufficient xenograft growth, volume varied between 140 mm^3 to 270 mm^3, each mouse was injected intravenously with a single dose of 15 mg/Kg ADC. The control group did not receive any treatment. After 48 hours, the xenografts were harvested and snap-frozen on dry ice. Samples were stored in cryo tubes at -80°C.

Tissue sectioning

Frozen xenograft samples were allowed to reach cutting temperature for 15 min before sectioning at 10 µm thickness in a Leica CM1950 Cryostat (Leica Biosystems, Nussloch, Germany). Chamber and head temperatures were set to -15°C and -13°C, respectively. Sections were thaw-mounted onto Indium-Tin-Oxide (ITO) coated glass slides and stored at -80°C in a small plastic slide-holder (5-Slide-Mailer) sealed with parafilm. Storage time was less than 2 weeks.

Sample preparation

Stored slides were taken from the ultralow freezer and acclimatized to room temperature in a desiccator below atmospheric pressure for 15 minutes. Afterwards, the parafilm sealing of the 5-Slide-Mailer was

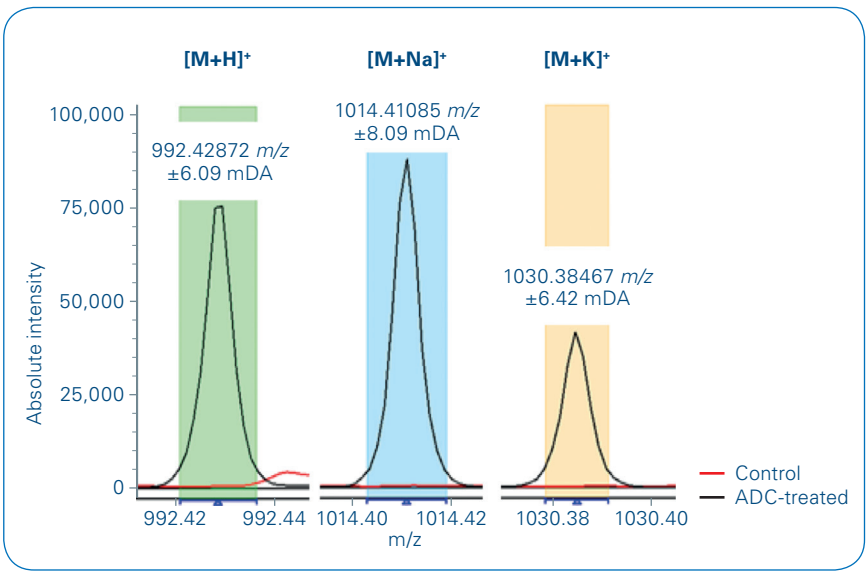

Figure 1. Average spectra of the xenograft groups. Average spectrum of the ADC dosed group (black) vs the control group (red). The signals corresponding to the toxin are only found in the ADC treated group. The signal window used for further evaluation is indicated as background color and above the respective peak.

Table 1: Matrix deposition settings for the HTX M5 Sprayer

Matrix	2,5-DHB
Solvent	50% Acetonitrile/ Water with 0.1% TFA
Concentration	35 mg/mL
Flowrate	120 µL/min
Layers	8
Line distance	3 mm
Nozzle temp.	75°C
Tray temp.	25°C
Speed	1200 mm/min
N_2-Pressure	10 psi

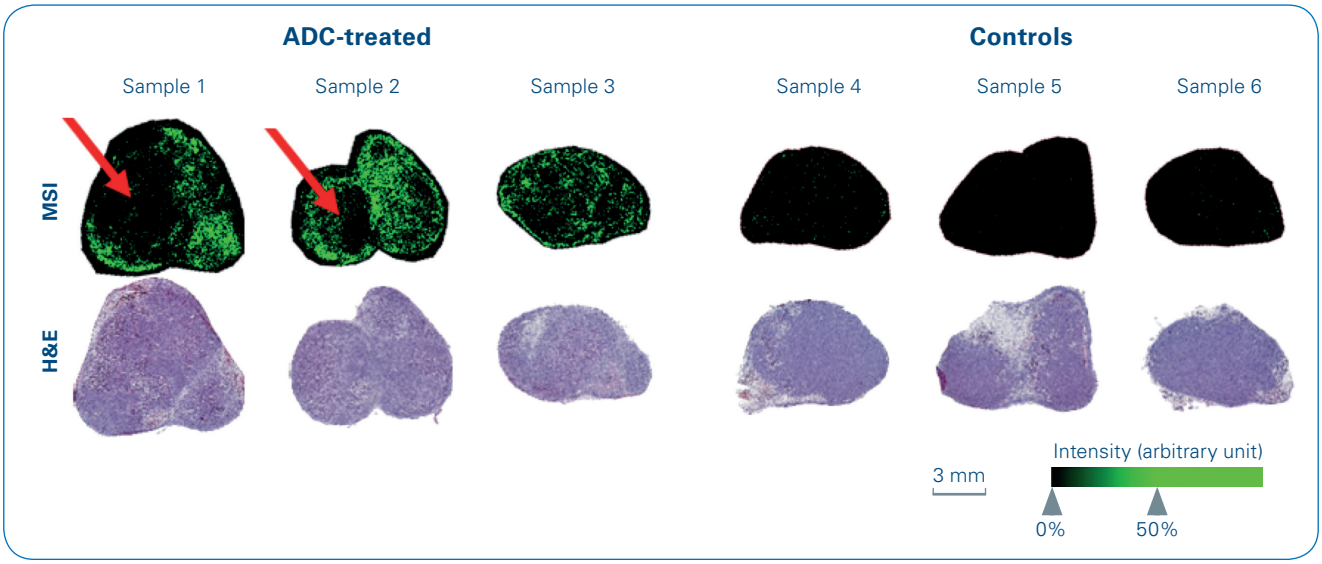

Figure 2. Comparison of the toxin distribution in ADC-treated (sample 1-3) and controls (sample 4-6). The blended ion images of the $[M+H]^+$-, $[M+Na]^+$-, and $[M+K]^+$-species are shown as green color code (0-50%) as shown in the right lower corner. H&E stained sections within 50 µm distance of the measured sections are displayed below the respective xenograft.

Table 2: Instrument settings

Mode	Absorption
Size	4 M
Polarity	Positive
Mass low	100.33
Mass high	3000
Frequency	1000 Hz
Laser Shots	150
Laser focus	Small
Data reduction factor	99%
Isolate	Yes
Q1 mass	950 *m/z*
Isolation window	200 *m/z*
Time of flight	1.4 ms
RF Amplitude	250 Vpp
Frequency	4 MHz
Sweep excitation power	22%

removed and the slides were prepared for measurement. Total Brain Lipid (TBL) standard (1 mg/mL in 10:10:1 MeOH/$CHCl_3$/H_2O) was deposited on the slides, and sections were scanned before matrix application using an Aperio CS2 slide scanner at 20x magnification. Resulting images were reduced to 10% image size and transformed to the TIF LZW format to reduce the scan size (Imagescope software, Leica Biosystems) for teaching.

Matrix Deposition

The MALDI matrix 2,5-Dihydroxybenzoic acid (DHB) was deposited using an HTX M5 Sprayer equipped with a heated tray (HTX Technologies, Chapel Hill, NC, USA). DHB solution consisted of 35 mg/mL DHB in 50/50 Acetonitrile/Water containing a total of 0.1% Trifluoroacetic acid (TFA). The matrix deposition settings are summarized in Table 1.

Table 3: Theoretical and observed m/z *and the calculated mass error in parts per million (ppm).*

	m/z (Theoretical)	*m/z* (Observed)	error (ppm)
$[M+H]^+$	992.4295	992.4287	-0.806
$[M+Na]^+$	1014.4114	1014.4109	-0.493
$[M+K]^+$	1030.3854	1030.3847	-0.679

MALDI MS Measurement

Ultra-high resolution MALDI MSI positive ion mode measurements were performed on a solariX XR 7T Magnetic Resonance Mass Spectrometer (MRMS) equipped with a 7.0-T actively shielded cryomagnet and an ESI/MALDI dual ion source utilizing smartbeam-II laser technology (wavelength 355 nm). The data point transient of the acquired spectra was 4 M resulting in an estimated resolving power of 260,000 at *m/z* 400. Sweep excitation power was set to 22% and time-of-flight to 1.4 ms.

For measurement setup, ftmsControl and flexImaging software were used. Prior to measurement, the instrument was externally calibrated in the mass range between 700 to 1500 Da using TBL standard. After calibration, an isolation window was set to 950 ± 100 Da and MS imaging data was recorded using the following settings: For Experiments 1 and 2 (Rep. 1, Rep.2): raster width 100 µm, 300 laser shots per pixel, 1 ms ToF; for Experiment 3: raster width 75 µm,

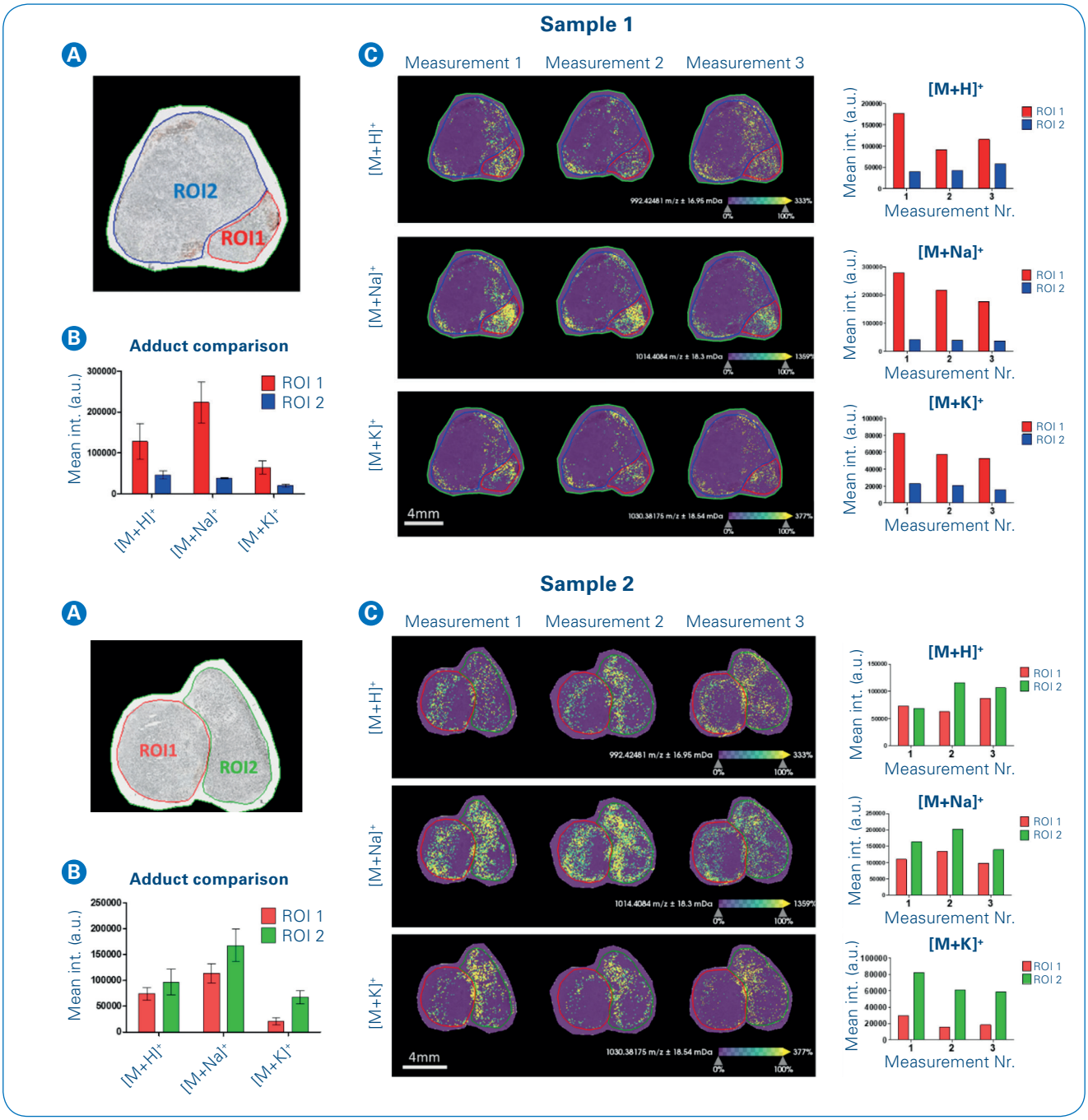

Figure 3. Comparison of ion distribution and intensities for sample 1 and 2. A scan of the respective tissue section and the division into two distinct regions of interest is shown (A). ROI mean intensities over the 3 measurements were averaged and displayed as bar graph. The analyte is primarily detected as sodium adduct (B). Ion images of the analyte and its adducts compared in the different measurements (25% transparency setting). Similar ion distribution patterns can be observed for the different adducts. Bar graphs of the mean intensities in the ROIs for each measurement are displayed (C). The comparability between measurements could be further improved by the use of an internal standard [1,2].

150 laser shots per pixel and a ToF of 1.4 ms. The specified instrument settings are summarized in Table 2.

For visualization, data sets were opened with SCiLS™ Lab software Version 2021a as raw data and then TIC-normalized.

Results and discussion

Free toxin signals formed by protonation or addition of sodium or potassium were identified using their calculated theoretical mass (Table 3). The average spectrum of the treated-versus control tissues group is shown in Figure 1.

All adducts of the analyte were unambiguously detected in xenograft sections of the ADC-treated mice based on the high mass accuracy of the MRMS platform. The resulting mass error was calculated to be below 1 ppm. The theoretical and observed *m/z* are shown in Table 3.

The distribution of the protonated analyte and its alkali adducts were blended using the blend image mode in SCiLS™ Lab, and the blended image is shown as green color gradient in Figure 2.

Toxin distribution was non-homogenous and some inner parts of the tumors displayed no toxin signal. This was particularly true for samples 1 and 2 (red arrows). Presence of necrotic tissue or increased distance to blood vessels are possible explanations, but the precise nature of this non-homogeneous distribution would require in-depth histopathology analysis or immunostaining. A combination with immunostaining for markers of hypoxia, blood vessels or the ADC antibody itself would be plausible.

To evaluate the reproducibility of the approach, a total of three sections for sample 1 and 2 ADC-dosed mice were MALDI imaged. Visually distinct regions were selected as regions of interest and their mean intensities were compared. The distribution of the protonated payload compound and its sodium and potassium adduct in the sections is shown in Figure 3. Additionally, the mean intensities in the selected ROIs are visualized as bar graphs.

Similar distribution patterns were observed for each sample demonstrating the reproducibility of the method.

Conclusion

We successfully mapped the distribution free-toxin/payload, presumably released from ADCs in cancer xenografts after *in-vivo* dosing. This was enabled by the ultra-high mass resolution, sub-ppm mass measurement accuracy of the analyte and the isolation capability of the solariX XR 7T. In conclusion, the solariX MRMS facilitated investigation of toxin distributions after ADC dosage. By providing insight into the distribution of ADC-delivered toxins in xenografts, further options arise to analyze, optimize, and compare therapeutic ADCs.

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