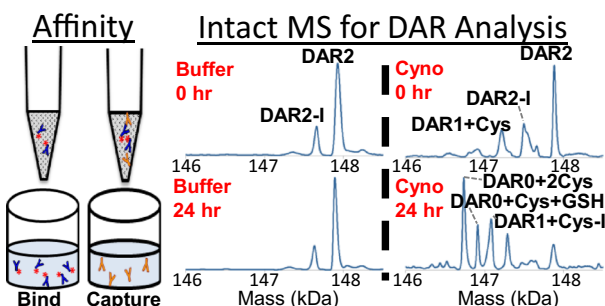CrossMark

RESEARCH ARTICLE

Automated On-tip Affinity Capture Coupled with Mass Spectrometry to Characterize Intact Antibody-Drug Conjugates from Blood

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Abstract. Antibody-drug conjugates (ADCs) present unique challenges for ligand-binding assays primarily due to the dynamic changes of the drug-to-antibody ratio (DAR) distribution in vivo and in vitro. Here, an automated on-tip affinity capture platform with subsequent mass spectrometry analysis was developed to accurately characterize the DAR distribution of ADCs from biological matrices. A variety of elution buffers were tested to offer optimal recovery, with trastuzumab serving as a surrogate to the ADCs. High assay repeatability (CV 3%) was achieved for trastuzumab antibody when captured below the maximal binding capacity of 7.5 μg. Efficient on-tip deglycosylation was also demonstrated in 1 h followed by affinity capture. Moreover, this tip-based platform affords higher throughput for DAR characterization when compared with a well-characterized bead-based method.

Keywords: Affinity capture, Antibody-drug conjugate, Drug antibody ratio

Introduction

Antibody-drug conjugates (ADCs) are designed to deliver a highly potent cytotoxic agent to the targeted tumor cells [1, 2]. To improve efficacy without compromising safety, ADCs must retain the conjugated drug in circulation prior to delivering and releasing the cytotoxic payload in the tumor cells. Therefore, it is essential to characterize the stability of intact ADCs with inherent structural information, which allows for direct measurement of the drug antibody ratio (DAR) distribution. This relative quantitation of DAR distribution can provide insights for identifying factors for rational drug design of antibody conjugation sites, and the chemistry of

linkers and payloads that can potentially affect ADC stability [3, 4].

Previously, chromatographic methods such as hydrophobic interaction chromatography (HIC) [5, 6], size exclusion chromatography (SEC) [7–9], and ion exchange chromatography (IEX) coupled with UV detection have been employed to separate and quantify ADCs with different DAR [8]. However, these methods are not suitable for unambiguous identification of ADC species resulting from partial drug loss in complex biological matrices (e.g., blood, plasma, serum, cell lysate). Alternatively, immunoassays such as enzyme-linked immunosorbent assay (ELISA) [5, 10, 11] and affinity capture liquid chromatography mass spectrometry (LC-MS) [9, 12, 13] have been employed to characterize the DAR distribution of ADCs in biological matrices. Although ELISA allows extraction and identification of therapeutic antibodies with and without the drug molecules in a fairly high throughput manner, MS can provide more precise identification of multiple ADC species in addition to degradations such as deconjugation and payload metabolism in a single LC-MS injection. Due to sample

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complexity, purification using affinity capture such as with streptavidin immobilized on magnetic beads is required for complex biological matrices prior to MS analysis [9, 12]. However, the conventional bead-based affinity capture requires extensive sample handling procedures compared to ELISA. Moreover, the time-consuming sample handling process during affinity capture can potentially introduce artificial modifications to ADCs, such as deamidation [14].

Here, we describe an automated affinity capture approach based on streptavidin-incorporated pipette tips that addresses these challenges. After binding, targeted ADCs were deglycosylated on-tip followed by an enriched elution for increased sensitivity for MS analysis. The robustness of the tip-based affinity capture was validated by comparing the experimental outcomes with a bead-based method. While there are prior studies using such approaches for analyzing intact antibodies [15], to the best of our knowledge, comparing affinity tips versus conventional magnetic bead capture for ADC stability analyses has not been reported.

Experimental

Materials

MSIA Streptavidin D.A.R.T.'S tips were from Molecular BioProducts (San Diego, CA, USA). HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20) was from GE Healthcare (Piscataway, NJ, USA). Anti-HER2 monoclonal antibody (trastuzumab) [16], target antigens and ADC molecules were generated in-house. Peptide N-glycosidase F (PNGase F) was from New England Biolabs (Ipswich, MA, USA). Endoglycosidase S (IgGZERO) was from Genovis (Cambridge, CA, USA).

Affinity Capture, On-tip Deglycosylation, and Elution

All reagents used for affinity capture were contained in ABgene 96-well PCR plates. Streptavidin was covalently conjugated to the monolithic resin at the distal end of the pipette tip by the manufacturer. Repetitive mixing of the solutions with automated aspirating and dispensing were implemented by Dynamic Devices Oasis LM600. For biotinylation, 0.5-ml aliquot of the target antigen at a concentration of approximately 4 mg/ml was mixed with appropriate molar amounts of 20 mM EZ-Link NHS-PEG4-Biotin (Thermo Fisher Scientific, Rockford, IL, USA) in 1× phosphate-buffered saline (PBS), to achieve an optimized mole challenge ratio of 1:10. The reaction mixture was incubated in darkness at room temperature for 0.5 h. A NAP-5 column was equilibrated with 10 ml of 1× PBS, loaded with the biotinylated target antigen, and eluted by 1 ml of PBS buffer. Biotinylated target antigen was then ready for immobilization onto proper solid supports. Prior to affinity capture, the ADCs of interest was spiked into buffer (PBS pH 7.4, 0.5% BSA, 10 ppm Proclin) or blood at a concentration of 100 μg/ml followed by 0 or 24 h incubation at 37 °C.

A schematic of the protocol on the tip-based affinity capture is shown in Fig. 1. Volumes of the reagents and the pipetting iterations and cycle times in each step are listed in Table 1. Initially, the MSIA tips were washed and equilibrated with HBS-EP. Biotinylated antigen ECD (10 μg) was then loaded onto the tip (Fig. 1a) followed by a brief wash with HBS-EP. For capture of the target ADCs, 10 μL buffer or blood with ADCs was added to the plate and diluted to a final volume of 160 μL with HBS-EP. Binding of the ADCs onto the antigen was then performed through pipetting the analytical sample (Fig. 1b). The tip was then extensively washed 3× with HBS-EP to remove any matrix residue remaining on the tip (Fig. 1c). Deglycosylation of the ADCs was performed on tip at room temperature. Sixty microliters HBS-EP containing 1 μL PNGase F or 1.5 μL lgGZERO was pipetted through the tip briefly. After mixing, the deglycosylation solution was held and retained inside the tip to completely cover the resin to minimize drying (Fig. 1d). ADCs can either be deglycosylated for 1 h with lgGZERO or overnight with PNGase F. After deglycosylation, the sample was dispensed into the plate followed by several tip washes with HBS-EP, water, and finally 10% acetonitrile (Fig. 1e). ADCs were eluted off the tip using 30% acetonitrile and 2% formic acid as the elution buffer. For comparisons with on-tip affinity capture, magnetic bead affinity capture was also performed in parallel as described previously [12]. Briefly, streptavidin-coated paramagnetic beads were incubated in the biotinylated ECD solution for 2 h. Rinsed beads were incubated with the ADC sample for 2 h. Bound ADCs was then deglycosylated overnight by incubating the beads in PNGase F solution. The beads were then washed to remove any impurities and incubated with the elution buffer for 15 min to release the ADCs into solution.

LC-MS

Ten microliters ADC samples were injected and loaded onto a Thermo Scientific PepSwift RP monolithic column (500 μm × 5 cm) maintained at 65 °C. The ADCs were then separated on the column using a Waters Acquity UPLC system at a flow rate of 20 μL/min with the following gradient: 20% B (95% acetonitrile + 0.1% formic acid) at 0–2 min; 35% B at 2.5 min; 65% B at 5 min; 95% B at 5.5 min; 5% B at 6 min. The column was directly coupled for online detection with a Waters Synapt G2-S Q-ToF mass spectrometer operated in positive ESI with an acquisition mass range from m/z 500 to 5000.

Data Analysis

Deconvolution of the raw spectrum within a selected ADC elution time window was implemented with Waters BiopharmaLynx 1.3.3. The drug loss or adducts formation were identified according to the corresponding mass shifts from the starting ADC material. Relative abundance of each ADC species in the analytical sample was represented by its MS signal intensity. The relative ratios of ADCs with different DARs were calculated by dividing the intensity of the specific ADC species with the intensity from the total ADC species.
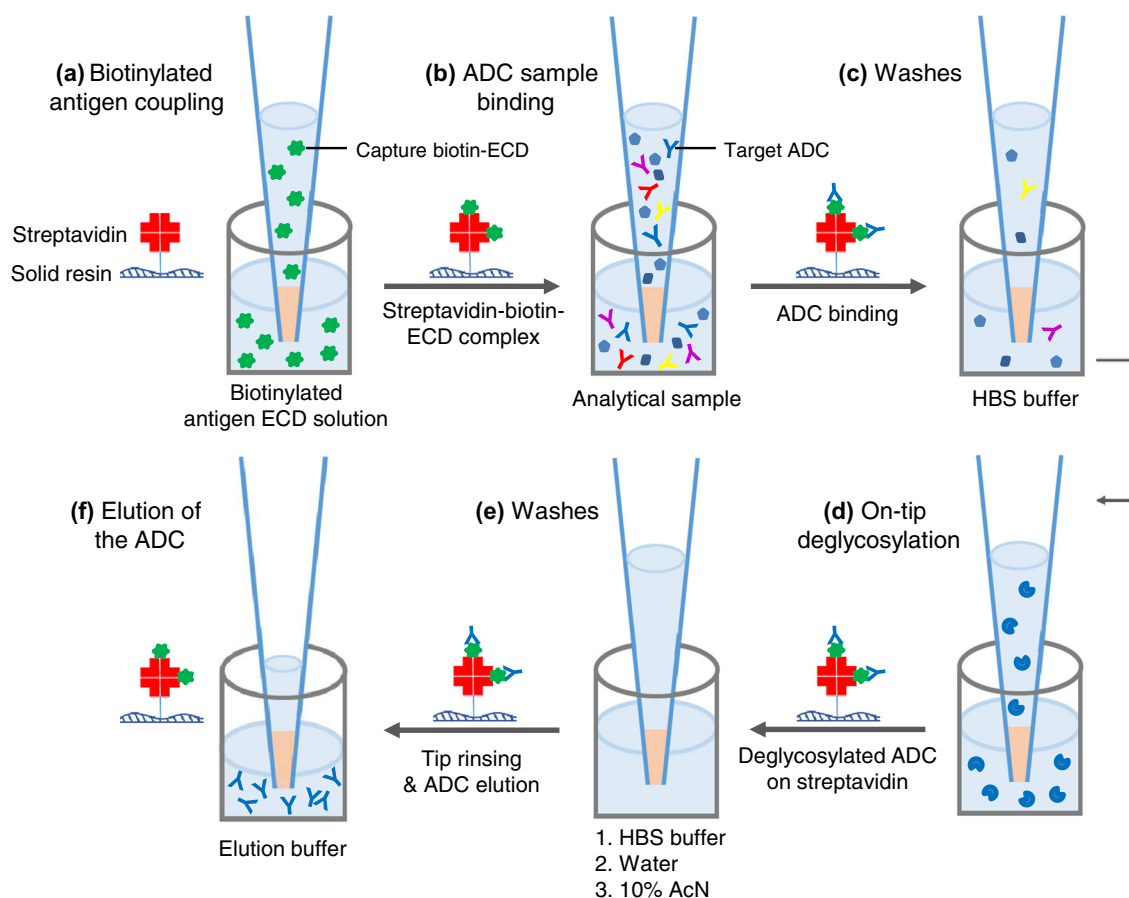
Figure 1. Schematic of the tip-based affinity capture process. Through mixing, (**a**) biotinylated antigen probe and (**b**) target ADCs are bound onto streptavidin and the immobilized antigen, respectively. After binding, (**c**) impurities from the biological sample are washed away from the tip followed by (**d**) ADC deglycosylation. Finally, (**e**) ADCs are purified by extensive washes followed by (**f**) elution off the tip with elution buffer

Results and Discussion

Optimizing the Elution Conditions for On-tip Affinity Capture

In order to improve the elution efficiency for on-tip affinity capture, various formulations of elution buffer, including 30% acetonitrile or 10% methanol with formic acid or trifluoroacetic acid concentrations at 1, 2, and 5%, were examined. Meanwhile, elution of trastuzumab with 20, 200, 450, 700, and 1000 pipetting cycles were also tested. The optimal elution conditions were determined to be 30% acetonitrile + 2% formic acid with 700 pipetting cycles, yielding $37 \pm 5\%$ trastuzumab recovery. To confirm the stability of ADCs under the relatively acidic conditions, ADCs with acid labile linker drugs were spiked into the optimal elution buffer and incubated overnight at room temperature. Compared to elution in 30% acetonitrile with 0.1% formic acid, the optimal elution buffer yielded similar MS profiles suggesting high stability (Supplementary Fig. 1).

Tip Binding Capacity Using Antigen ECD to Capture ADCs

Tips for affinity capture have limited binding sites for capturing ADCs given the specific amount of streptavidin incorporated. Overloading the tip may potentially render a bias when capturing ADCs with different DAR values. To determine the binding capacity, we spiked in trastuzumab amounts ranging from 0.03–12 µg in triplicate. The recovery binding curve in Fig. 2 indicated the tip was saturated at 7.5-µg trastuzumab loading. A linear correlation ($R^2 = 0.9977$) with high repeatability (CV 3%) was observed at loadings below 2 µg. In contrast, the variation increases to 7% at saturation point, presumably attributed to manufacturing variations in the tip binding capacity.

Table 1. Details of the Liquid Handling for Each Step in Affinity Capture

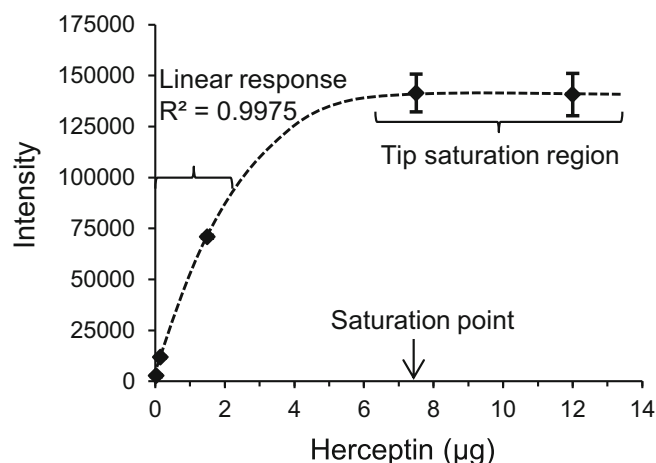| Steps	Well volume (µL)	Pipette volume (µL)	Pipette iterations	Time (min)
Wash	200	175	10	1
ECD binding	120	100	500	30
Wash	200	175	10	1
ADC binding	160	100	500	30
3× wash	200	175	10	1
Deglycosylation	60	30	30	60/overnight
3× wash	200	175	10	1
Elution	40	30	700	25

Figure 2. Calibration curve of the MSIA tip used in affinity capture with HER-2 ECD as the capture probe for trastuzumab. At ranges with low trastuzumab amount (< 2 µg), spiked in recovery is linear. Decreased repeatability was observed when the tip was saturated at 7.5 µg

Thus, keeping the loading amount below the tip binding capacity may be crucial for high analytical reproducibility if absolute quantitation is desired.

On-tip Deglycosylation of the ADCs

Deglycosylation of ADCs at room temperature was implemented with on-tip affinity capture prior to elution. Overnight deglycosylation using an increased amount of PNGase F was applied to the tip-based method and completion was confirmed with the loss of the polysaccharides seen through MS analysis (Supplementary Fig. 2). Alternatively, lgGZERO was capable of completely deglycosylating ADCs in 1 h, which further decreased the overall time of the entire affinity capture procedure (from reagent preparations to obtaining the MS-ready ADC samples) to 3 h without compromising the signal intensity (Fig. 3).

Comparisons of ADC Characterization Using Tip-Based and Beads-Based Affinity Capture

With the established optimized protocol, the tip-based affinity capture was applied to measure DAR distributions for in-vitro whole blood stability analysis for two engineered THIOMAB™ ADCs [6], namely, ADC 1 and ADC 2. The cysteine engineered ADCs contains two sites for drug conjugation with a maximum of DAR 2. Since the drugs are covalently attached to the antibody, denatured intact protein MS as opposed to native MS can be used for more sensitive intact protein characterization.

To assess for affinity capture bias, the DAR difference was compared for samples with and without affinity capture. Each of the two ADCs was spiked at equal ratios of DAR 0 and DAR 2 in buffer and serum, which will result in a theoretical average DAR 1. The buffer samples were injected directly (no affinity capture) for LC-MS analysis, whereas the serum samples underwent bead-based affinity capture followed by LC-MS. Subtracting the DAR for the buffer samples with the serum samples led to a DAR difference of 0.14 and 0.08 for ADC 1 and ADC 2, respectively.
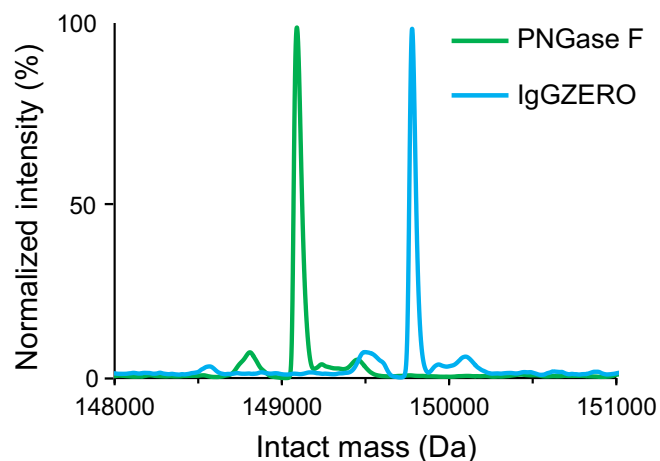

Figure 3. Deconvoluted spectra (normalized intensity) of on-tip deglycosylated ADCs using PNGase F (green) or IgGZERO (blue) show primarily a single peak indicating the cleavage of the polysaccharides from the ADCs. The observed 689 Da mass difference is attributed to different cleavage sites on the sugar chain (IgGZERO leaves a GlcNAc and Fuc on the antibody upon cleavage)

For characterizing ADC stability, the two ADCs were spiked into the HBS-EP buffer, primate blood and rodent blood followed by incubation for 0 and 24 h prior to affinity capture. To assess the accuracy of the tip-based affinity capture approach, the same batch of ADC samples underwent bead-based affinity capture and analyzed using the same LC-MS method. For these two ADCs, the starting materials were predominantly DAR 2. Figure 4a–c and g–i shows the deconvoluted mass spectra of ADC 1 and ADC 2 after 24-h incubation in different matrices using the tip-based affinity capture approach. For comparisons, the bead-based affinity capture results are also displayed (Fig. 4d–f, j–l). The accurate MS detection of ADC species in various matrices showed minor differences between samples captured by the tip-based vs bead-based method. The small variations between the two assays could be attributed to either binding format or affinity capture time differences since the sample binding step in the bead-based affinity capture required an extra 1.5 h, thus exposing the conjugates to matrix proteins longer. As expected, the variation was most apparent when monitoring the shorter incubation time (Supplementary Fig. 3). We did however observe broader mass peaks from the affinity tips that may be attributed to unidentified adducts. Further detailed comparison between 0 and 24 h incubation shows comparable degradation profiles suggesting that both affinity capture methods are complementary (Supplementary Fig. 3).

The peak intensities and mass shifts reveal very different levels of degradation for the two ADCs after 24 h catabolism. Further, degradation differences were also highly dependent on the blood species being used for ADC incubation. Figure 5 shows the comparisons of DAR profiles between buffer and cynomolgus blood using tip-based affinity capture. The relatively stable profiles in buffer contrasts the highly unstable profiles from cynomolgus blood samples for ADC 1.
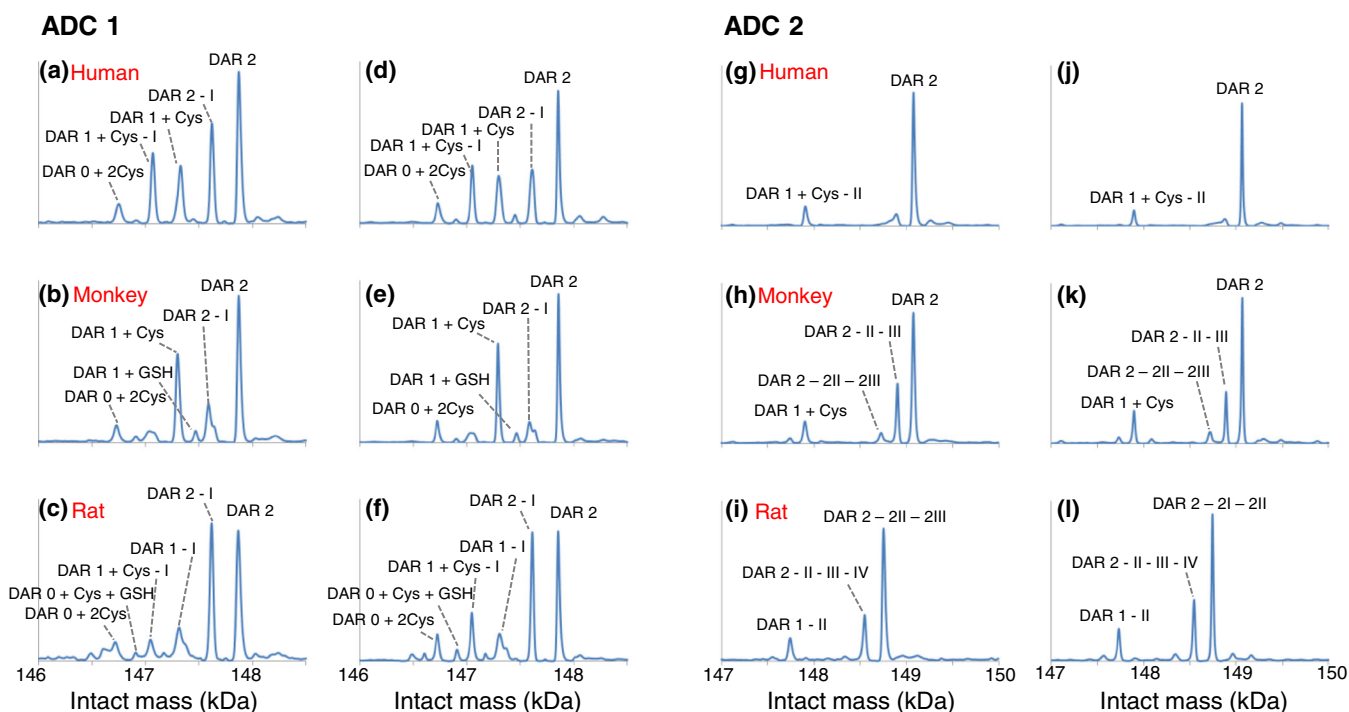
ADC 1

ADC 2

Figure 4. Deconvoluted mass spectra of **a–c** ADC 1 and **g–i** ADC 2 after 24 h incubation in human, monkey, and rat blood using tip-based affinity capture. For comparison, **d–f** and **g–l** are the same corresponding ADC samples analyzed using conventional bead-based affinity capture. Cys = cysteine, GSH = glutathione, I, II, III, and IV refers to different proprietary chemical moieties cleaved from the linked ADC drug

Conclusions

Here, we demonstrated an automated affinity capture platform based on streptavidin-incorporated tips that effectively identified various degradation products of ADCs in biological matrices. Beside whole blood, the method can be easily implemented on other biological matrices including cell/tissue lysate, plasma, and serum. This tip-based affinity capture platform is amenable to automation integration thus capable of achieving high throughput

workflow. With the low volume resin, concentration enrichment by eluting into smaller volumes is possible. The platform can be further coupled with downstream sample handling such as enzymatic digestion of the captured ADCs to gain insights on degradations at the amino acid level of the antibody [14].

Acknowledgements

Intact MS for DAR Analysis

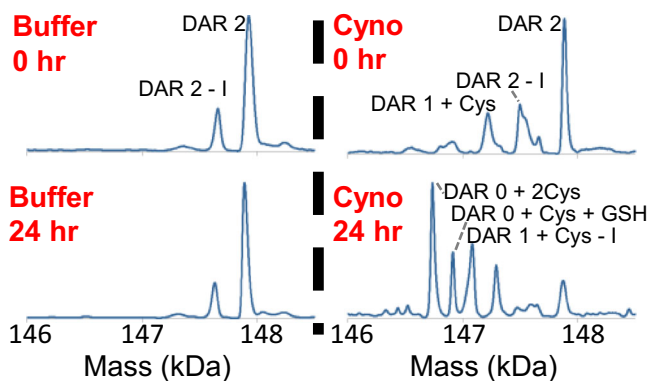**Figure 5.** Deconvoluted mass spectra for ADC 1 after 0 and 24 h incubation in buffer and cynomolgus blood from tip-based affinity capture

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