

Development of NISTmAb-derived Antibody-drug Conjugate (ADC) Standards

Shanhua Lin¹, Terry Zhang², Brian Agnew³, Trina Mouchahoir⁴, and John Schiel⁴, 1: Thermo Fisher Scientific, Sunnyvale, CA, USA; 2: Thermo Fisher Scientific, San Jose, CA, USA; 3: Thermo Fisher Scientific, Eugene, OR, USA; 4: National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA

ABSTRACT

Purpose: Develop NISTmAb-derived antibody-drug conjugate (ADC) standards.

Methods: Antibody-drug conjugates (ADCs) were made using SiteClick™ enzyme-based N-glycan labeling of NISTmAb with DIBO-angiotensin II, DIBO-Biotin and DIBO-AF488. Intact ADCs as well as IdeS generated F(ab)₂ and scFc fragments were analyzed using a Thermo Scientific™ MABPac™ RP column coupled with a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer.

Results: Enzymatic pre-treatment, or “trimming” of the antibody glycans with GlycINATOR® endoglycosidase S2, prior to azide activation, results in highly-reproducible drug-to-antibody ratios (DARs) of 2.0. MS analysis demonstrate the successful synthesis of NISTmAb-angiotensin II (NISTmAb-ATII), NISTmAb-Biotin, and NISTmAb-AF488.

INTRODUCTION

We propose the development of the first-ever NISTmAb derived antibody-drug conjugate standard. Antibody-drug conjugates (ADCs) have become a promising class of cancer therapeutics since the approval of Adcetris in 2013 and Kadcyra in 2014. And currently, there are 40 plus ADCs in the clinical pipeline. ADCs are created by coupling an anticancer drug (e.g. a cell toxin or cytotoxin) to an antibody that specifically targets a tumor marker. Quantitative pharmacokinetic (PK) studies are regularly conducted as part of pre-clinical data collection, clinical evaluation, and are required for regulatory approval. However, the innate heterogeneity of antibodies and ADCs, due to variation in glycan structure and drug-to-antibody ratios (DARs), greatly complicates these studies. As such, a stable, well-characterized homogeneous ADC standard would be of great help in these analyses. There are currently no commercially available homogeneous ADC standards on the market today to support the multitude of analytical analyses required for ADC characterization. Most recently, the National Institute of Standards and Technology (NIST) has developed and released a monoclonal antibody (NISTmAb standard¹) that is intended for use in evaluating the performance of methods for physicochemical and biochemical analyses of monoclonal antibodies. It also provides a standard test antibody for development of novel technology for therapeutic protein characterization. Here we propose the use of SiteClick™ site-specific antibody labeling technology^{2,3} Thermo Fisher Scientific™ in combination with the NISTmAb to develop the first non-toxic, NISTmAb-derived, ADC standard (NISTmAb-ADC). We chose three different payloads as model drugs with distinctly different properties and functionalities that could be used in a broad spectrum of analytical applications down the road.

MATERIALS AND METHODS

Enzyme Labeling

In a one-pot activation reaction, NISTmAb (NIST, RM8671) was incubated at 37 °C with GlycINATOR enzyme (Genovis) for 1 hour at 37 °C, then UDP-GalNAz substrate, β-Gal-T1(Y289L) enzyme, and MnCl₂ were added, and the mixture was incubated overnight (16 hrs) in histidine buffer, pH 6.0 (NISTmAb storage buffer used throughout the complete labeling process) at 30 °C. Excess UDP-GalNAz and β-Gal-T1(Y289L) were removed using 50 kD MW cut-off ULTRA spin filters Amicon. The purified azide-activated antibodies were labeled with DIBO-angiotensin II, DIBO-biotin and DIBO-AF488 at 20 °C overnight (16 hrs) and then purified using 50 kD cut-off filters as above. (Scheme 1).

Generation of scFc and F(ab)₂ Fragments

Antibodies were cleaved using IdeS enzyme (Genovis) to generate scFc and F(ab)₂ fragments.

LC/UV and LC/MS Analysis

Intact and fragment samples were separated and analyzed on Thermo Scientific™ Vanquish™ UHPLC system, using a MAbPac RP analytical column, 4.0 μm, 2.1 × 50 mm column (p/n 088648) at 80 °C. When coupling UHPLC with a UV detector, H₂O/TFA/acetonitrile mobile phase was used. When coupling UHPLC with Orbitrap Fusion Lumos Tribrid Mass Spectrometer instrument, H₂O/FA/acetonitrile mobile phase was used. The MS acquisition method was set with a full scan at both 15,000 (FWHM, at m/z 200) and 120,000 resolution in positive mode. The method parameters were: AGC 2e5, IT 200 ms, in-source CID 0 ev and 35 ev, scan range: 800-3000, 1000-3500 m/z, spray voltage 3.8 kv, sheath gas 60, aux gas 20, capillary temperature 350 °C, s-lens 30, probe heater temperature 150 °C.

Data Analysis

Mass of intact molecules and fragments from unmodified antibody, azide-tagged intermediate, and ADCs were analyzed using Thermo Scientific™ Protein Deconvolution 4.0 using the Xtract algorithm for isotopically resolved MS spectra and theReSpect algorithm for isotopically unresolved MS spectra.

RESULTS

NISTmAb was used to synthesize ADCs with DIBO-Angiotensin II, DIBO-Biotin, and DIBO-AF488. There are 2 N-glycans per antibody Fc domain, one on each heavy chain. After “trimming” of the antibody glycans with GlycINATOR enzyme, there is one labeling site available per heavy chain. Therefore, up to 2 payload molecules (angiotensin, biotin, or AF488) can be conjugated to 1 mAb molecule. HPLC analyses of intact (Figure 1) and scFc fragments (Figure 2) before and after GlycINATOR-based glycan cleavage and SiteClick labeling shows complete conversion of labeled scFc domains.

HPLC profile of the scFc fragment (Figure 2 left panel) showed that there are “twin peaks” for each ADC, regardless of the payload molecules (angiotensin II, biotin, or AF488). High resolution LC/MS analysis demonstrated that these “twin peaks” have identical masses, indicating that they are structural isomers. NMR studies have shown that DIBO enantiomers were formed after the conjugation.

MS spectra of scFc fragments were acquired using 120K resolution and therefore the deconvolution was carried out using the Xtract algorithm which is designed for isotopically resolved peaks. The deconvoluted spectra of scFc and its derivatives at each modification step (treated with GlycINATOR enzyme, labeled with azide, and conjugated to Angiotensin II, Biotin, or AF488) are shown in Figure 3, right panel. The theoretical mass and observed mass of scFc are listed in Table 1. The mass accuracy is within 2 ppm.

Scheme 1. SiteClick™ site-specific labeling of antibodies, conjugation to the chitobiose core

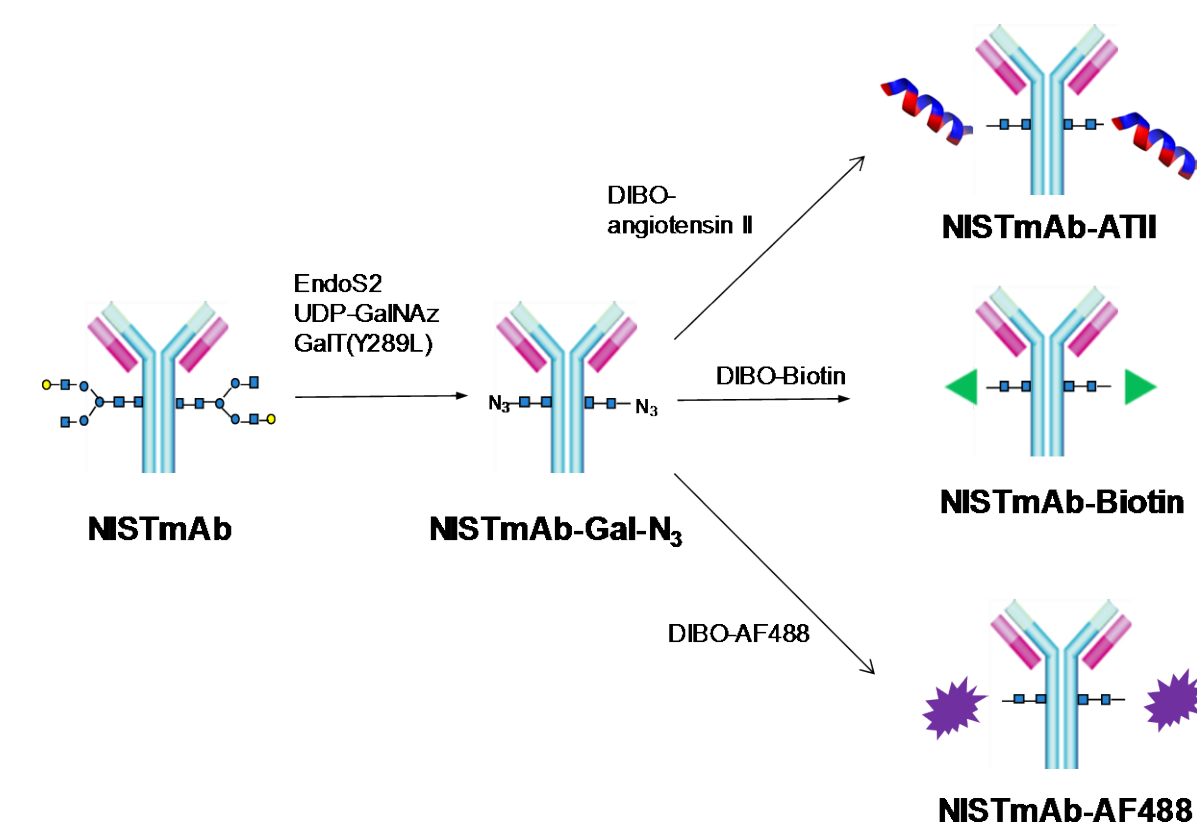


Table 1. Accurate mass assignments of scFc Fragments (Monoisotopic) for the NISTmAb and ADCs

IdeS Fragments	Theoretical Mass (Da)	Observed Mass (Da)	Mass Accuracy (ppm)
scFc-G0F	25216.431	25216.477	1.8
scFc-Gal-N ₃	24365.142	24365.182	1.6
scFc-ATII	26068.896	26068.933	1.4
scFc-Biotin	25222.475	25222.515	1.6
scFc-AF488	25512.391	25512.428	1.5

Figure 1. LC/MS analyses of intact NISTmAb, azide-activated NISTmAb, NISTmAb-ATII, NISTmAb-Biotin, and, NISTmAb-AF488. Left panels show UV spectra and right panels show mass spectra. The full scan spectra are collected on a Orbitrap Fusion Lumos Tribrid MS instrument at a resolution of 15K (FWHM, at m/z 200)

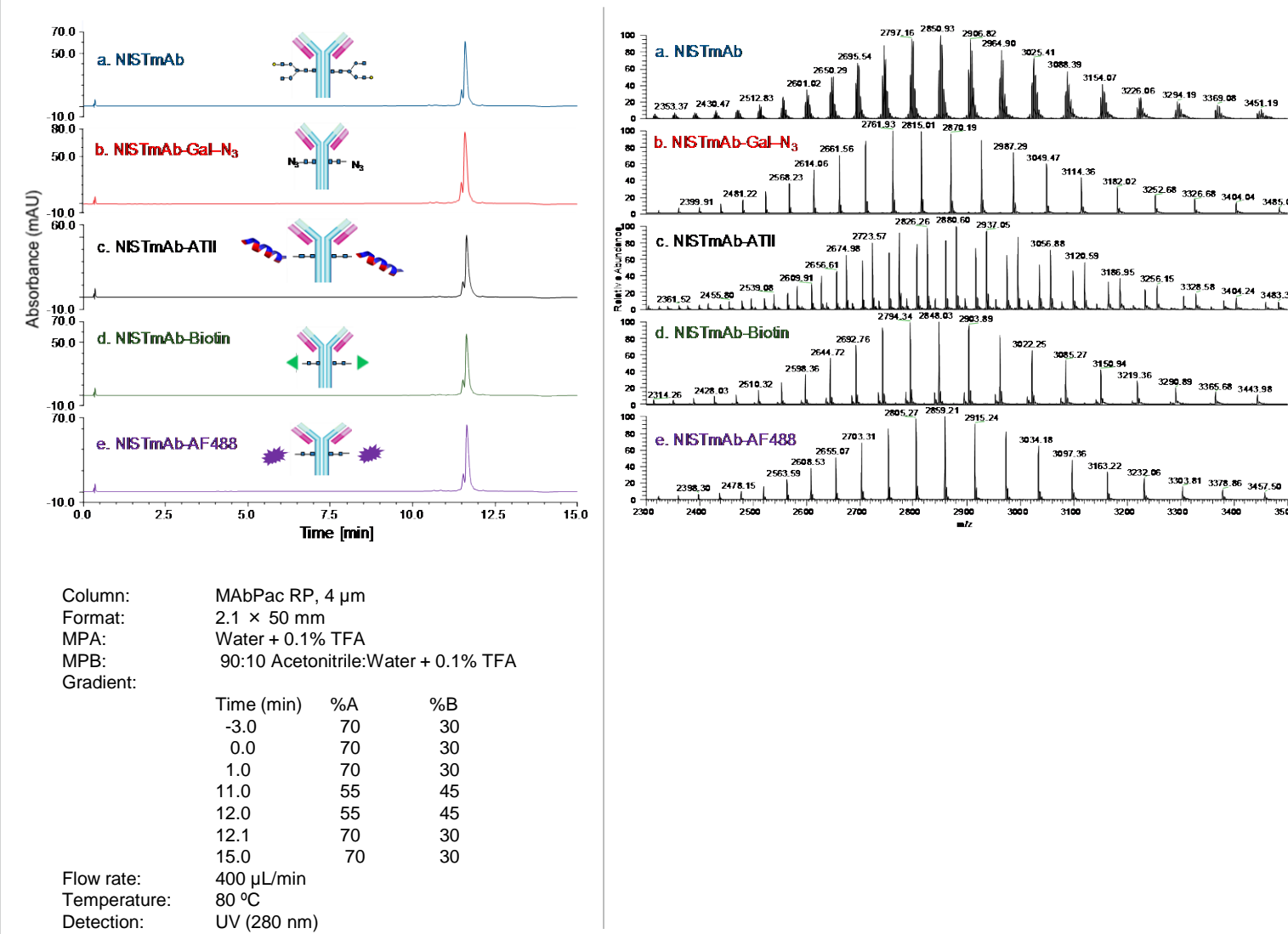


Figure 2. LC/MS analyses of scFc fragments from NISTmAb, NISTmAb-Gal-N3, NISTmAb-ATII, NISTmAb-Biotin, and, NISTmAb-AF488. Left panels show UV spectra and right panels show mass spectra. The full scan spectra are collected on a Orbitrap Fusion Lumos Tribrid MS instrument at a resolution of 120K (FWHM, at m/z 200)

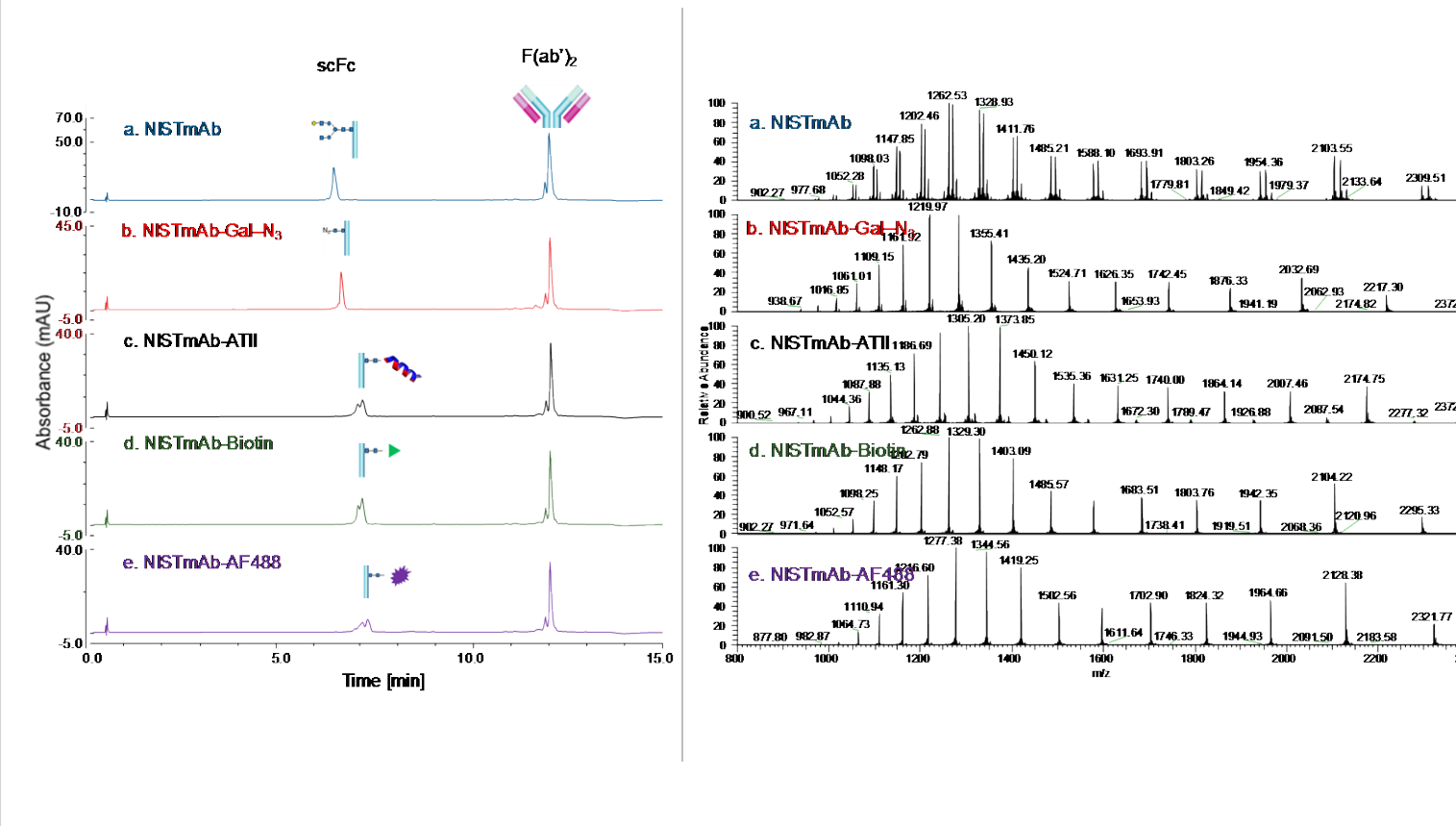
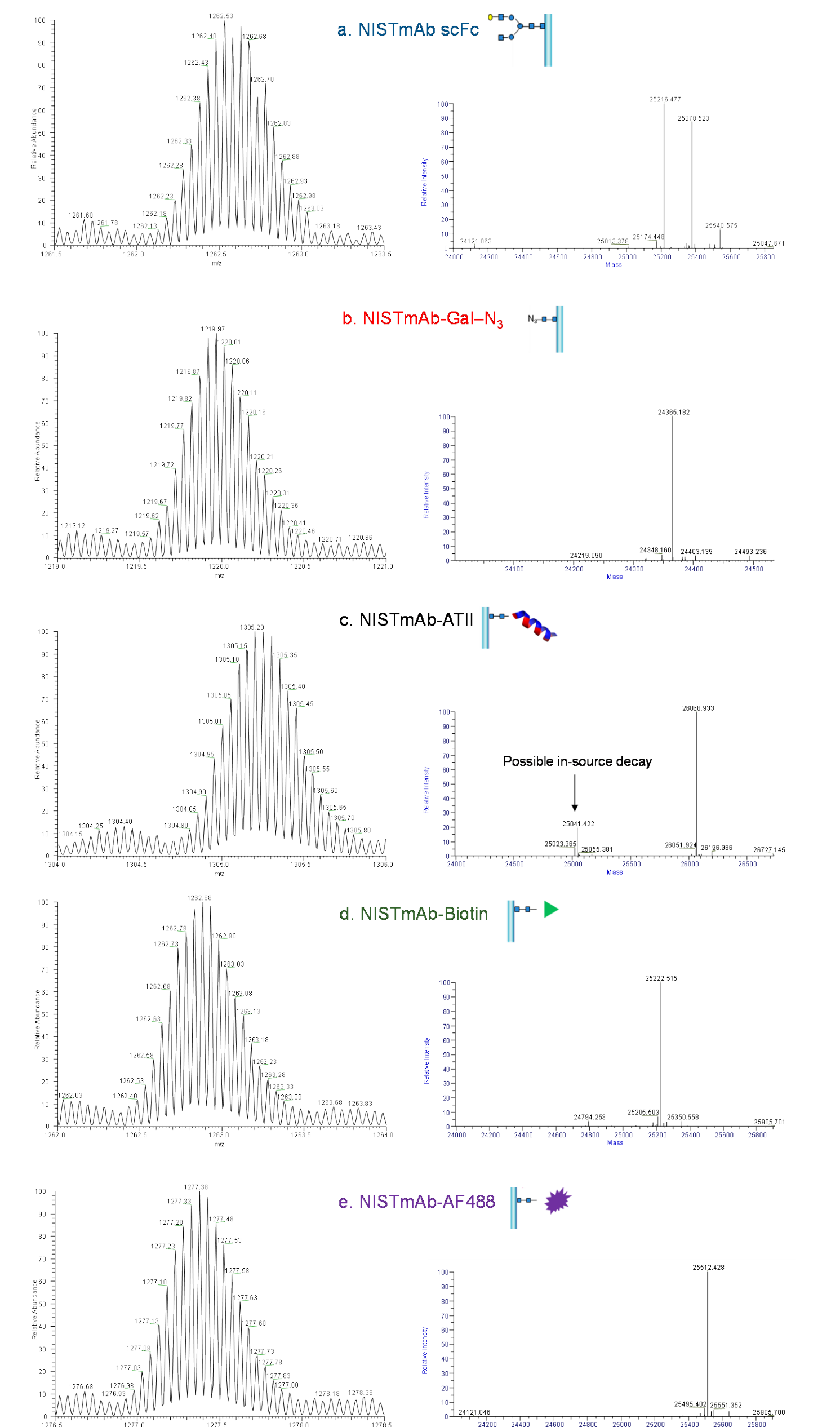


Figure 3. Monoisotopic spectra of scFc fragments from NISTmAb, NISTmAb-Gal-N₃, NISTmAb-ATII, NISTmAb-Biotin, and, NISTmAb-AF488. Left panels show monoisotopic spectra at charge state +20 and right panels show deconvoluted spectra



CONCLUSIONS

- NISTmAb ADC conjugates (NISTmAb-ATII, NISTmAb-Biotin, and NISTmAb-AF488) with a drug-to-antibody ratio (DAR) of 2.0 have been successfully synthesized and purified.
- Complete primary LC-MS evaluation of intact antibody conjugates and fragments has confirmed DARs, labeling specificity to heavy chain N-linked glycosylation sites, and expected molecular weights.
- Complete comprehensive evaluation and characterization of ADC standards including peptide mapping, characterization of posttranslational modifications, and stability studies on final ADC standards is underway.

REFERENCES

- State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study. John E. Schiel, Darryl L. Davis, Oleg V. Borisov. 2015 Oct 15., American Chemical Society. ISBN13: 9780841230293.
- Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huigen cycloadditions. Ning X, Guo J, Wolfert MA, Boons GJ. Angew Chem Int Ed Engl. 2008;47(12):2253-5.
- Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. Ramakrishnan B, Qasba PK. J Biol Chem. 2002 Jun 7;277(23):20833-9

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Collaborative Research Contract (CRC) Grant Program for funding this project. We would like to personally thank CRC Program Leader Dr. David Odelson for facilitating the CRADA discussions and execution with NIST.

TRADEMARKS/LICENSEING

© 2018 Thermo Fisher Scientific Inc. All rights reserved. GlycINATOR is a trademark of Genovis AB. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.