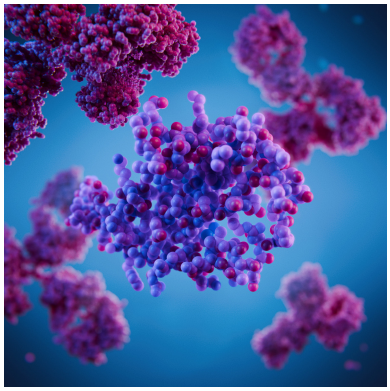
Application Note

Biopharma/Pharma

Hybrid UV-Vis/MS Assay for Free Cysteine Determination in Monoclonal Antibodies

Using the Agilent Cary 3500 UV-Vis and Agilent 6545XT AdvanceBio LC/Q-TOF MS

Suitable for Agilent
1290 Infinity III LC



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Abstract

Cysteine residues in monoclonal antibodies (mAbs) play key structural roles through disulfide bond formation. Free cysteines can arise from incorrect pairing or reduction during processing, affecting product quality. In this application note, a hybrid method is used that quantifies reactive cysteine residues using the Agilent Cary 3500 UV-Vis spectrophotometer at 412 nm by measuring the 2-nitro-5-thiobenzoic acid (TNB) chromophore formed with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent). High-resolution LC/QTOF mass spectrometry (MS) is then used to identify the specific protein chains labeled with TNB. The results correlate the overall thiol-toprotein ratio from UV-Vis data with the site-specific TNB labeling patterns observed by MS, providing both quantitative and structural insight into protein thiol reactivity.

Introduction

MAbs are Y-shaped immunoglobulin molecules composed of two heavy chains (HC) and two light chains (LC). A typical human IgG1 antibody contains 32 cysteine residues, many of which form interchain and intrachain disulfide bonds essential for structural stability and antigen binding.[1] However, some mAbs also include additional cysteines within their hypervariable regions that do not form disulfide bonds. These free or unpaired cysteines can compromise molecular stability, increase aggregation propensity, and diminish biological activity.[2]

Because cysteine residues, and the disulfide bonds they form, play a central role in maintaining proper folding and function, the presence of free thiols arising from mispairing, partial reduction, or bioprocess variability can affect product quality.[3] Free thiol quantification is therefore a critical analytical parameter, supporting assessment of thiol-to-protein ratios in both native and reduced mAbs. This information is particularly important for quality control, comparability studies, and the design of antibody-drug conjugates (ADCs), where controlled cysteine reactivity influences conjugation efficiency and drug-to-antibody ratio (DAR).[4]

This application note describes a hybrid analytical workflow that combines (Figure 1):

- UV-Vis spectroscopy using the Agilent Cary 3500 Multicell UV-Vis spectrophotometer
- High-resolution MS using the Agilent 6545XT AdvanceBio LC/Q-TOF

This hybrid UV-Vis and LC/Q-TOF MS method offers accurate determination of free thiol content in mAbs and structural confirmation of cysteine modification sites.

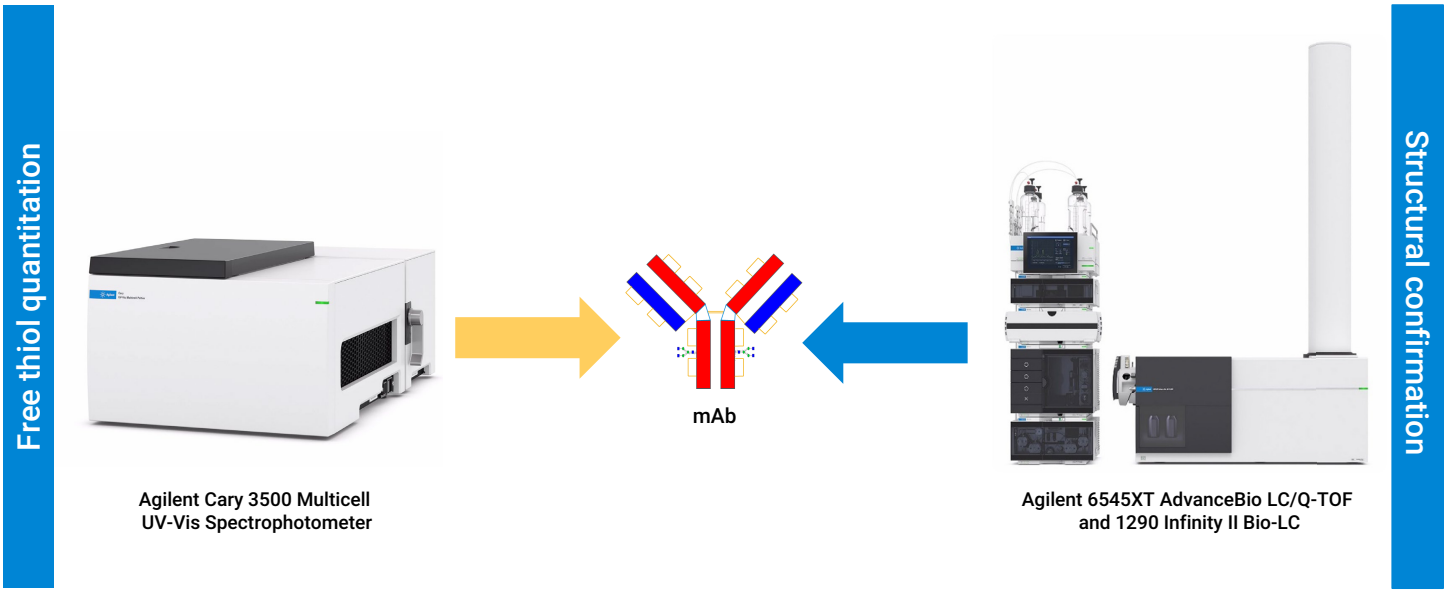

Figure 1. Workflow of the hybrid spectroscopy-mass spectrometry assay for free cysteine analysis in monoclonal antibodies.

Experimental

Reagents and chemicals

Biosimilar and innovator samples of rituximab were purchased from a local distributor in Singapore. 5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB), l-cysteine (Cys), tris(2-carboxyethyl) phosphine (TCEP), ethylenediaminetetraacetic acid (EDTA), formic acid, acetonitrile, sodium dihydrogen phosphate, and sodium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vivaspin 500 centrifugal concentrator spin columns (10 kDa MWCO) were from Sartorius. Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA, USA). All mobile phase components were LC/MS grade.

Ellman's assay

Table 1. Quantification of sulfhydryls using cysteine standards by Cary 3500 UV-Vis.

Standard	Volume of Reaction Buffer	Amount of Cysteine (M.W. = 121)	Final Concentration
A	1,000	181.5 mg	1.5 mM
B	50	250 µL of A	1.25 mM
C	100	200 µL of A	1.0 mM
D	150	150 µL of A	0.75 mM
E	200	100 µL of A	0.5 mM
F	250	50 µL of A	0.25 mM
Blank	300	0	0

Stock solutions

- **Reaction buffer:** 0.1 M sodium phosphate buffer (pH 8.0) + 1 mM EDTA
- **DTND:** 4 mg/mL in reaction buffer (~10 mM)

Procedure

Preparation of cysteine standards:

Dissolve L-cysteine (M.W. = 121) in reaction buffer to prepare the following standard solutions as indicated in the previous table.

Assay setup:

In separate microcentrifuge tubes, add:

- 5 µL of Ellman's reagent solution
- 250 µL of reaction buffer
- 25 µL of Cys STD

Incubation:

Mix thoroughly and incubate at room temperature for 15 minutes.

Measurement:

Measure the absorbance at 412 nm using a Cary 3500 UV-Vis with multiple cuvettes at the same time.

Data analysis:

- Plot the absorbance values of the standards to generate a standard curve automatically from Agilent Cary UV Workstation software.
- This curve was used to determine the concentration of sulfhydryl groups in the unknown samples. A linear calibration curve with $R^2 \geq 0.95$ is set for assay acceptance.

Antibody reduction protocol

MAb samples (10 mg/mL stock) were first diluted to 5 mg/mL with phosphate buffer at pH 8.0. For reduction: TCEP was added to mAb solution for a final TCEP concentration of 10 mmol/L. The samples were then incubated for 4 hours at room temperature. Both innovator and biosimilar samples were spin filtered using Vivaspin 500 centrifugal concentrator spin columns (10 kDa MWCO) with phosphate buffer at pH 8.0. The samples were centrifuged for 5 minutes five times, and the concentrated upper reservoir solution was used for further analysis. A similar method was used for mAbs without TCEP treatment, which acted as controls. After spin filtering, samples were stored at –20 °C until analysis.

Analytical equipment

- An Agilent 1290 Infinity II bio LC system included the following modules:
  - Agilent 1290 Infinity II bio high-speed pump (G7120A)
  - Agilent 1290 Infinity II bio multisampler (G7137A)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)
- Agilent Cary 3500 UV-Vis Multicell Spectrophotometer

Software and data processing

- Agilent MassHunter data acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1
- Agilent MassHunter Qualitative Analysis version 12.0
- Agilent Cary UV Workstation software version 1.6

LC/MS analysis

The LC separation was performed on an Agilent PLRP-S column, 2.1 × 50 mm, 5 µm (part number PL1912-1502) and the raw data were acquired with MassHunter data acquisition software (Tables 2 and 3). The data analysis was performed with MassHunter BioConfirm.

Table 2. Liquid chromatography parameters.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent PLRP-S, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)
Thermostat	10 °C
Solvent A	Water with 0.1% formic acid
Solvent B	Acetonitrile with 0.1% formic acid
Flow Rate	0.5 mL/min
Gradient Program (Reduced/Subunit)	Time (min) %B 0 25.00 1.00 25.00 6.50 60.00 7.50 60.00 7.60 25.00 8.50 25.00
Injection Volume	1 µL
Column Temperature	55 °C

Table 3. MS data acquisition parameters.

Parameter	Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)
Mode	Positive
Ion Source	Dual AJS ESI
Gas Temperature	350 °C
Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer1	65 V
OctopoleRF Peak	750 V
Min Range	800 *m/z*
Max Range	5,000 *m/z*
Scan Rate	1.00 spectra/sec
Reference Masses	922.0097, 1821.9523 *m/z*

A Cary 3500 Multicell UV-Vis spectrophotometer was used in this study. Data acquisition was performed using Cary UV Workstation software (version 1.6) using the parameters shown in Tables 4 and 5. Ultra-microvolume rectangular cells with a UV pathlength of 10 mm and 70 µL fill volume (part number 5062-2496) were used. A sample volume of 50 µL was used for each cell. The Cary 3500 allows the measurement of such small sample volumes due to its highly collimated, uniform beam, which is less than 1.5 mm wide, and the permanent optical alignment of its stationary cell holders.

Table 4. Scan parameters.

Parameter	Value
X Mode	nm
Y Mode	Absorbance
Averaging Time	0.2 s
Spectral Bandwidth	2.00 nm
Data Interval	1.00 nm
Scan Range Start	500.00 nm
Scan Range Stop	250.00 nm
Multiple Experiments	1 zone

Table 5. Concentration setup parameters.

Parameter	Value
X Mode	nm
Y Mode	Absorbance
Averaging Time	0.100 s
Spectral Bandwidth	2.00 nm
Fit Type	Linear
Minimum R^2	0.9500
Wavelengths	412.00 nm

Results and discussion

Quantitative analysis using the Cary 3500 UV-Vis system produced a highly linear cysteine calibration curve across the tested concentration range, supporting accurate determination of thiol content. The calibration curve was generated by simultaneously measuring standards in a multicell configuration, enabling increased throughput. The calibration curve shows good linearity from 0.25 to 1.5 mM cysteine concentration, with an R^2 value of 0.9990 (Figure 2B). Thiol-toprotein ratios varied substantially between native and reduced states and among innovator and biosimilar products. Native mAbs exhibited very low free thiol levels (0.06–0.14), consistent with predominantly disulfide-bonded structures. In contrast, reduced samples demonstrated elevated thiol availability, with thiol-toprotein ratios ranging from 6.6 to 10.3, reflecting exposure of cysteine residues.

Similar results are reported in the literature for partially reduced mAbs.[4,5] The molar ratio of free sulfhydryl groups to mAbs (free-SH:protein ratio) of each sample was calculated based on the absorbance. The free-SH:protein ratio was calculated by Equation 1.

The standard curve also serves as a useful indicator of assay performance. By examining the full absorbance spectra for each standard solution, the isosbestic point can be identified to verify that the molar ratio between Ellman's reagent and the test sample remains consistent across all concentrations. For Ellman's assay, this isosbestic point occurs at approximately 357 nm (Figure 2A). In addition, a well-defined peak at 412 nm confirms that the sample falls within the assay's optimal working range.

Equation 1.

$$\text{Free sulfhydryl (SH):protein ratio (mol/mol)} = \frac{\text{Molar number of the sulfhydryl groups in mAb (mol)}}{\text{molar number of mAb (mol)}}$$

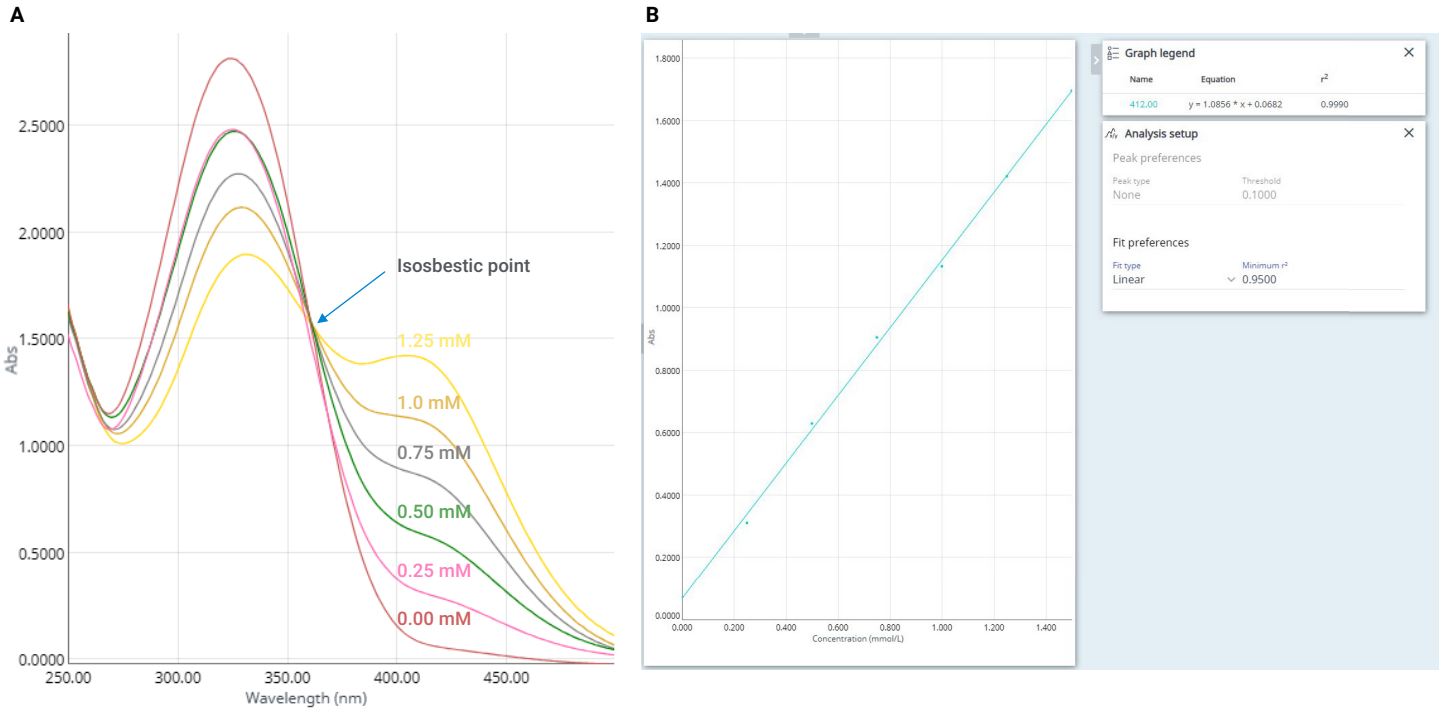

Figure 2. (A) Absorbance spectra of cysteine standards with DTNB. (B) Calibration curves of Cys using the DTNB reagent.

Table 6. Thiol-to-mAb ratio as determined by the calibration curve.

Monoclonal Antibody	Thiol-to-mAb Ratio
Innovator native	0.14
Innovator reduced	6.9
Biosimilar 1 native	0.06
Biosimilar 1 reduced	10.3
Biosimilar 2 native	0.13
Biosimilar 2 reduced	8.4

MS analysis was performed on DTNB-reacted, reduced monoclonal antibody samples using LC/Q-TOF instrumentation, enabling detailed characterization of TNB labeling across heavy and light chains. Deconvoluted spectra consistently revealed distinct TNB-modified species and glycoforms, including G0F, G1F, and G2F with TNB attached.

For the light chain, MS data confirmed the presence of free thiols, a +1 TNB-conjugated species, and retention of the native intrachain disulfide bond (Figure 3). The heavy chain exhibited four expected intrachain disulfide bonds and multiple TNB conjugation states, with clear resolution of glycoform heterogeneity (Figure 4).

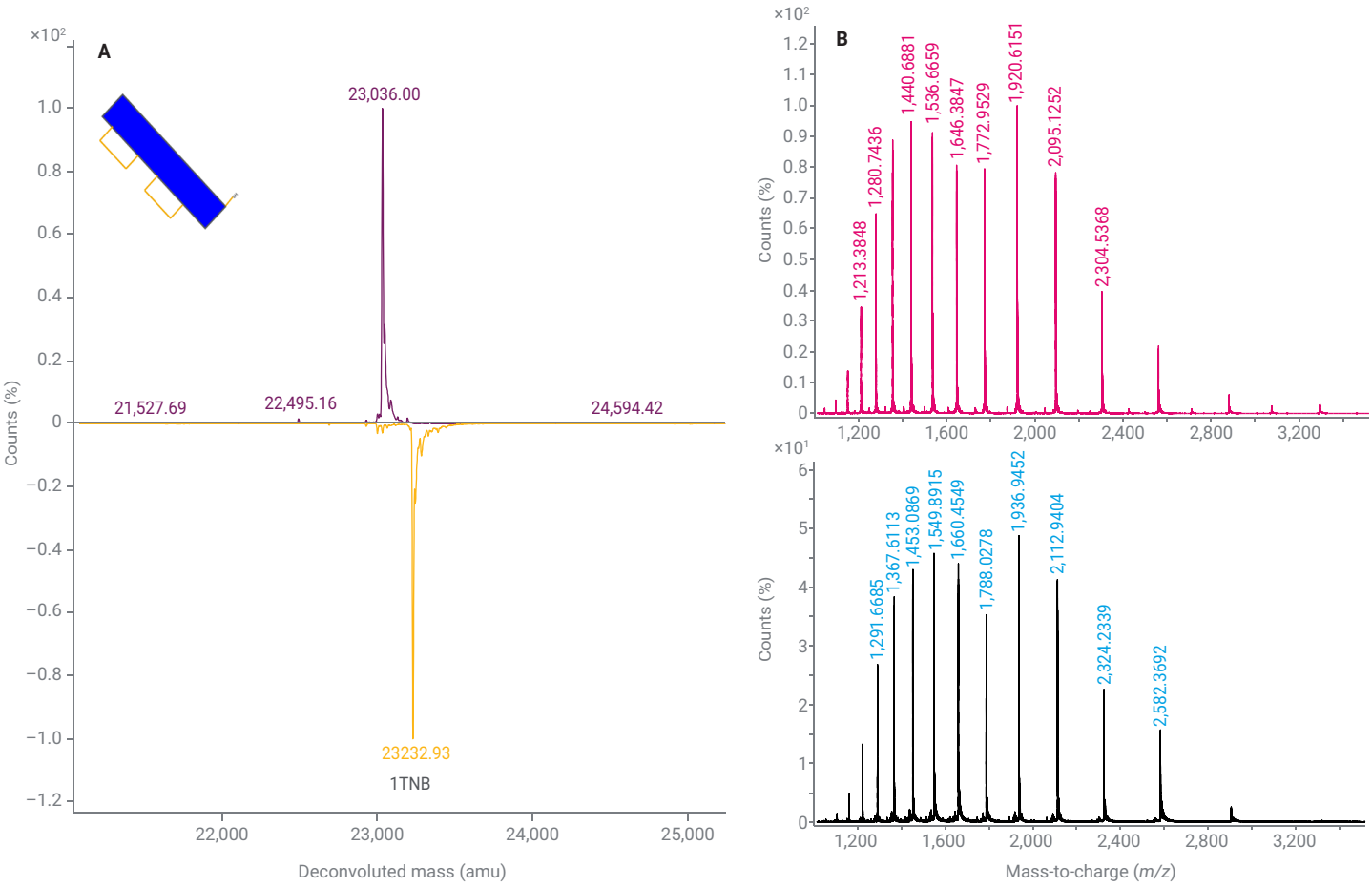

Figure 3. (A) Deconvoluted light chain mass spectrum of DTNB-conjugated mAb (bottom) compared to unconjugated antibody (top). (B) Mass spectrum of DTNB-conjugated mAb and unconjugated antibody.

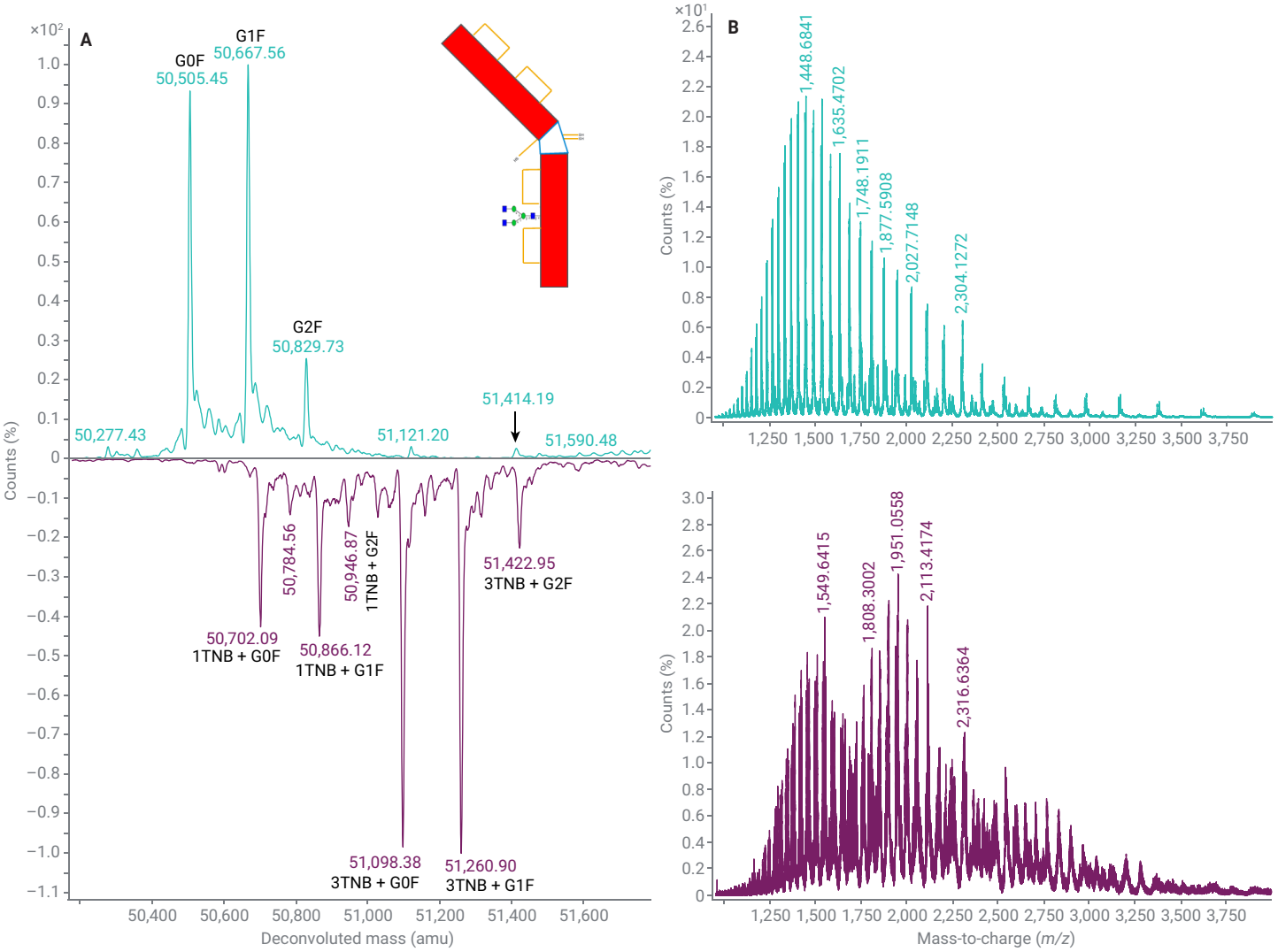

Figure 4. (A) Deconvoluted heavy chain mass spectrum of DTNB-conjugated mAb (bottom) compared to unconjugated antibody (top). TNB-modified species and glycoforms are annotated including G0F, G1F, and G2F. (B) Mass spectrum of DTNB-conjugated mAb and unconjugated antibody.

Across both innovator and biosimilar antibodies, MS reproducibly identified one TNB label on the light chain and three on the heavy chain, consistent with four reactive cysteine residues under reducing conditions. For intact mAbs, this corresponds to a total of eight reactive cysteines, aligning well with results from the DTNB assay. Figure 5 provides a consolidated overview of the partial reduction behavior of the monoclonal antibodies with TCEP and highlights the specific cysteine sites that become available for reaction with DTNB.

The schematic illustrates how controlled reduction selectively disrupts targeted interchain disulfide bonds while preserving key intrachain linkages, thereby exposing a defined set of free thiol groups on both the heavy and light chains. These newly generated thiols correspond directly to the reactive sites detected by MS and quantified through the DTNB assay. Together, the figure summarizes the structural basis for the observed TNB labeling pattern and validates the number and location of cysteine residues that participate in the reaction under the applied reduction conditions.

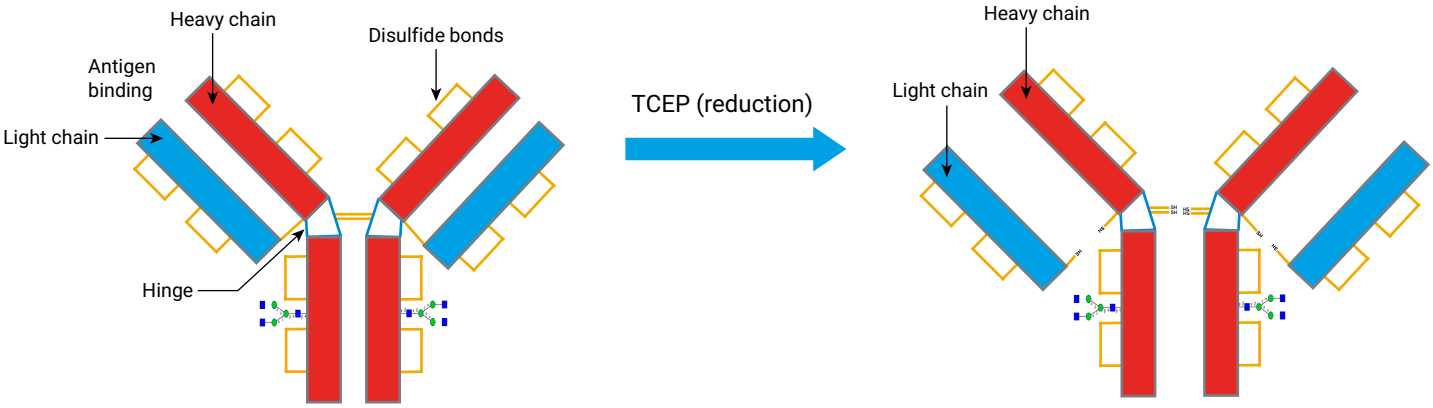

Figure 5. Schematic representation of the partial reduction reaction and sites that are available for DTNB reaction.

Conclusion

The hybrid analytical approach combining UV-Vis quantification with MS confirmation proved highly complementary. The Agilent Cary 3500 Multicell UV-Vis spectrophotometer provided rapid, precise measurements of free thiols, supported by its multicell configuration that improves throughput and repeatability. Mass spectrometry subsequently verified the number and location of TNB labels, enabling structural confirmation and enhancing confidence in the spectroscopic data. Together, these tools enabled comprehensive evaluation of cysteine reactivity, applicable to both innovator and biosimilar mAbs. The workflow is readily extendable to other cysteine containing biologics, offering a robust platform for quality control, comparability assessments, and applications such as antibody-drug conjugate development where cysteine accessibility is critical.

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