



## Assessing site-specific structural changes in monoclonal antibodies utilizing Covalent Labeling Mass Spectrometry (CL-MS)

In recent years, there has been an exponential increase in therapeutic protein candidates trying to make it into the competitive market.

### Introduction

One of the challenges is the risk of degradation of recombinantly produced therapeutic proteins due to the various stress conditions during manufacturing, lyophilization, and storage of the proteins. Thus, detailed assessment of structure-function changes in therapeutic proteins under forced stress and storage conditions are essential quality checks in the development of therapeutic proteins. Herein we present the application of diethylpyrocarbonate (DEPC) based covalent labeling mass spectrometry (CL-MS) as a structural protein analysis method that can be easily adapted in the biopharma industry using the standard LC-MS equipment. DEPC reagent labels side chains of Lys, His, Ser, Thr, Tyr, and Cys with different degrees of reactivity (1).

A model monoclonal antibody (SILuMAb) was used to identify structural changes using DEPC CL-MS in two forced stress scenarios: deglycosylation and long-term storage. Peptide maps of tryptic peptides from DEPC labeled stressed and non-stressed SILuMAb samples were acquired with Bruker impact II QTOF MS and LC separation from Agilent 1260 capillary HPLC. Peptides with DEPC-label at specific amino acids were detected accurately using the fragment ion series generated in MS/MS acquisition. Quantification of DEPC-labeled peptides across different samples (stress conditions) allows for the identification of significant structurally altered sites on the protein.

Keywords:  
Diethylpyrocarbonate, covalent labeling, site-specific, mAbs, labeled peptide intensity

## Material and Methods

### Sample preparation

50  $\mu\text{g}$  SILuMAb (Merck, Darmstadt, Germany) was subjected to two different stress treatments: deglycosylation and long-term storage without stabilizing agents for assessing structural changes compared to non-stressed control samples. Stressed and unstressed SILuMAb samples (3 replicates each) were labeled with 0.04 mM DEPC reagent (Acros Organics, Geel, Belgium) for 5 min at 37°C and later quenched with imidazole. Deglycosylation of SILuMAb was performed with 1  $\mu\text{L}$  of PNGaseF (1000 U) at 37°C for 1h.

### LC-MS/MS analysis

For each sample replicate, 40  $\mu\text{g}$  SILuMAb was denatured in 8 M urea prepared in ammonium bicarbonate buffer. Disulfide bonds in the SILuMAb were reduced with 0.79 mM TCEP at 37°C for 30 min and alkylated with 15 mM IAA for 30 min at RT in the dark. Samples were diluted with 50 mM ammonium bicarbonate to result 1M urea in the samples before starting the digestion. Trypsin (Product no. V5111-Promega, Madison, WI, USA) digestion was conducted for 3 hrs at 37°C. The digested samples were cleaned up with C18 stage tips.

### Chromatography and mass spectrometry

LC-MS/MS analysis of tryptic peptides was performed on an Agilent 1260 capillary HPLC coupled to a Bruker impact II QTOF. The details of the parameters used in LC-MS/MS can be found in Table 1 and Table 2.

RPLC parameters		
Time (min)	%B	Flow rate ( $\mu\text{L}/\text{min}$ )
0	5	12
2	5	12
30	40	12
31	90	12
35	90	12
36	5	12
45	5	12

**Table 1**  
RP-LC parameters used for LC-MS/MS analysis of tryptic peptides generated after DEPC labeling protocol.

ESI source parameters			
Nebulizer	Dry gas	Dry temp	End plate offset
0.7 bar	6L/min	200°C	500 V
Capillary voltage	Scan range	Scan mode	Spectra rate
4500 V	150-2200 $m/z$	Auto MS/MS	2 Hz
MS/MS parameter - Auto MS/MS			
<b>Cycle time</b>	2 sec		
<b>Threshold (Active exclusion)</b>	1970 counts Exclude after 1 spectra Release after 0.1 min Reconsider precursor if current intensity/Previous intensity: 3.0		
<b>MS/MS spectra acquisition (Dynamic)</b>	Max. rate: 8 Hz, Min rate: 2 Hz, Target intensity 2500 counts		

**Table 2**  
MS parameters used for LC-MS/MS analysis of tryptic peptides generated after DEPC labeling protocol.

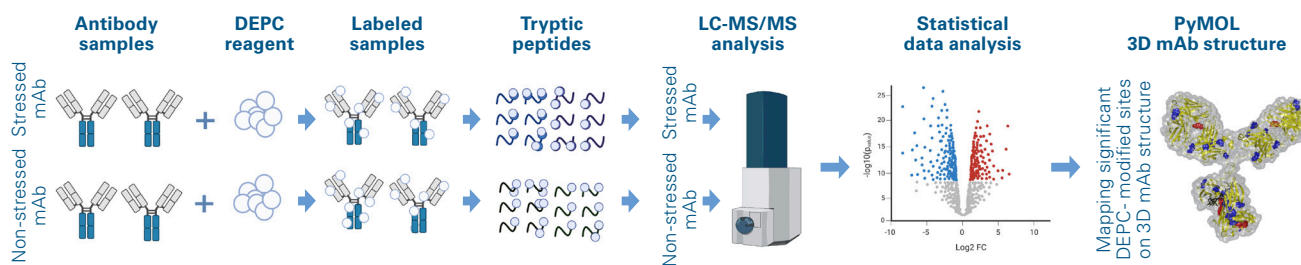
Peptide identification and LfQ quantification were carried out with Byos Software Version v4.5 (Protein Metrics). Statistical data analysis was performed in Perseus while the volcano plots were generated using an in-house R script. The 3D SiLuMAb structure was generated in PyMOL.

## Results and discussion

### 1. Structural changes in deglycosylated SiLuMAb:

The N-glycosylation present on the Fc domain of mAbs plays a major role in the antigen recognition and thereby efficacy of the mAb. The glycan structure is also responsible for the stability of the mAb molecule. The complexity of the N-glycans structure dictates the conformation of the Fc domain. In most cases, the glycans are embedded in the horseshoe structure of the Fc domain (2). A loss/removal of glycans would not lead to an entirely denatured state of a mAb, but it may rather disturb the Fc domain. We aimed to assess the accuracy and sensitivity of our DEPC-based CL-MS protocol for structural changes using the hypothesis that deglycosylation would majorly disrupt the Fc domain.

Bottom-up MS analysis was performed on the labeled sample set of glycosylated and deglycosylated SiLuMAb respectively. The LC-MS/MS-analysis resulted in an HC coverage of 82.67% and an LC coverage of 96.33%, respectively. The efficient fragmentation on Bruker's impact II MS allowed accurate localization of the site of DEPC incorporation either at His, Lys, Thr, Tyr, or Ser residues for a tryptic peptide. 22 sites of DEPC incorporation were detected in each of the sample sets. Statistical data analysis revealed Lys330 position on the HC to have significantly ( $p < 0.05$  and more than a two-fold change in intensity) reduced DEPC labeling in deglycosylated compared to the glycosylated SiLuMAb. Plotting Lys330 onto the 3D structure of SiLuMAb (Figure 2), it was evident that this site is located within the CH2 domain in the Fc portion of SiLuMAb. This site of structural change deduced in our analysis is in the vicinity of the N-glycosylation site (Asn301). This structural change reflects that the hydrophobic patches in the CH2 domain that are supposed to be kept apart by the N-glycans have collapsed after deglycosylation. The significant decrease in the DEPC label is indicative of a local aggregation of the hydrophobic patches around the Lys330 position, indicating a site-specific structural change. Hence, such a CL-MS analysis exhibits the importance of N-glycans in maintaining the structural stability of mAbs.



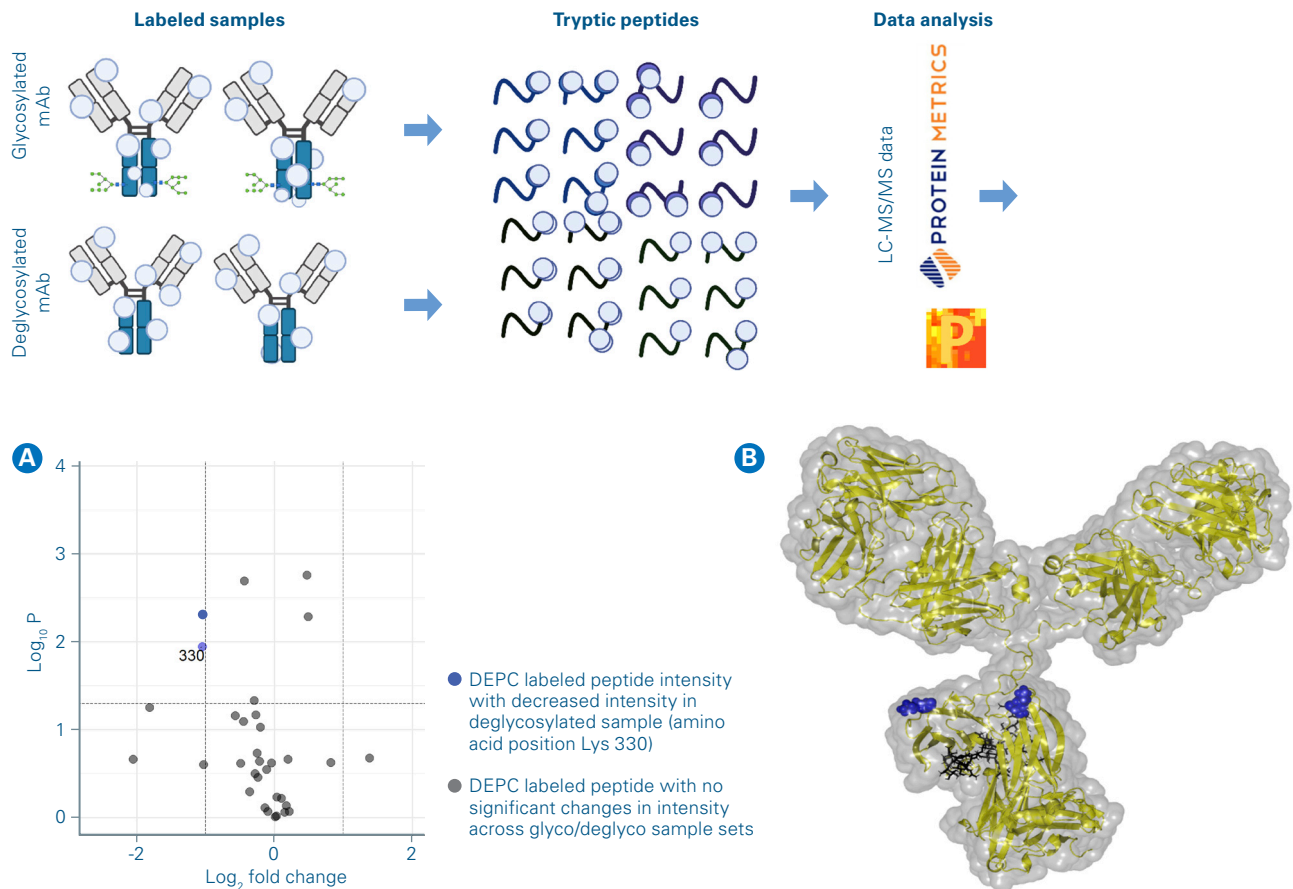
**Figure 1**

**Schematic representation of DEPC labeling protocol for analysis of structural changes in mAb.**

The stressed and unstressed samples were subjected to DEPC reagent for limited time followed by a classical bottom-up protein analysis workflow. The LC-MS/MS data consisting of quantified peptide intensities was statistically sorted for significantly changed labeled peptides using software tools in Perseus. The significantly changed positions named after amino acid position were plotted onto 3D mAb structure to visualize the structurally changed sites.

## 2. Structural changes in long-term stored SILuMAb:

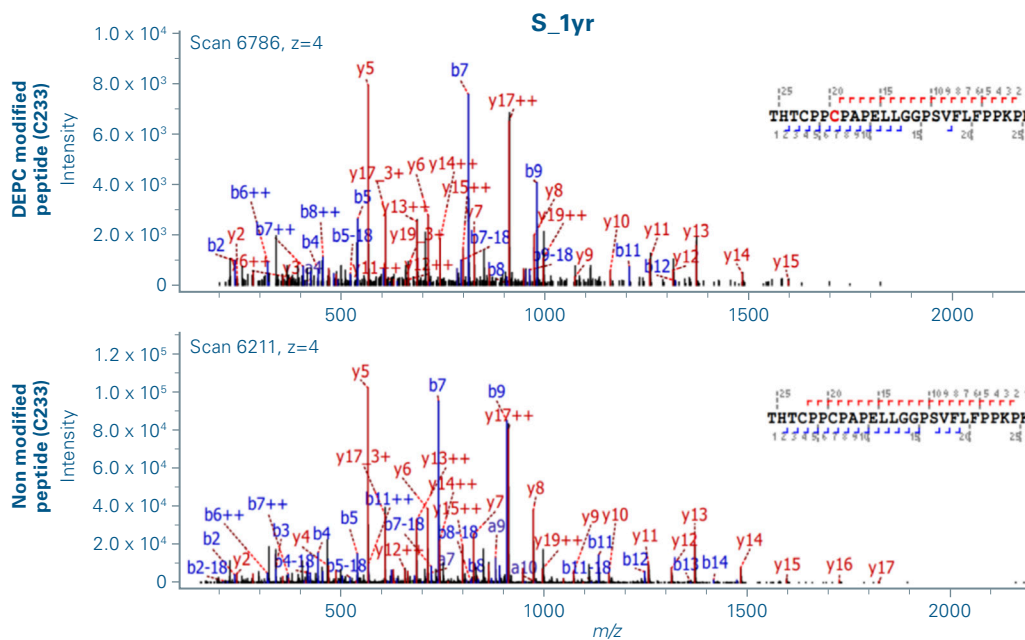
Given the exceptional analytical importance of shelf-life studies in mAbs, this set of experiment used a one-year-old refrigerated (4-8°C) SILuMAb for structural analysis in comparison to a freshly thawed SILuMAb sample. The aim was to assess the intactness of disulfide bonds. Thereby, DEPC label incorporation at cysteines (Cys) was analyzed in detail. A double labeling strategy for Cys was followed. Following the DEPC labeling, samples were reduced and alkylated with IAA. DEPC would only be reactive towards free Cys in the SILuMAb sample. Thereby, all Cys that were not involved in disulfides during the DEPC labeling step will be detected as DEPC labeled Cys while the rest will be detected as carbamidomethylated Cys. Among the 15 Cys in SILuMAb, DEPC label incorporation was accurately detected at 6 of the Cys (a 72 Da mass addition in the fragment ion series) for one-year refrigerated SILuMAb samples. These DEPC labels were however not detected in the freshly thawed SILuMAb samples. An example of one such Cys modified with DEPC is shown in Figure 3.



**Figure 2**

**Structural analysis in deglycosylated SILuMAb vs glycosylated SILuMAb using DEPC CL-MS protocol.**

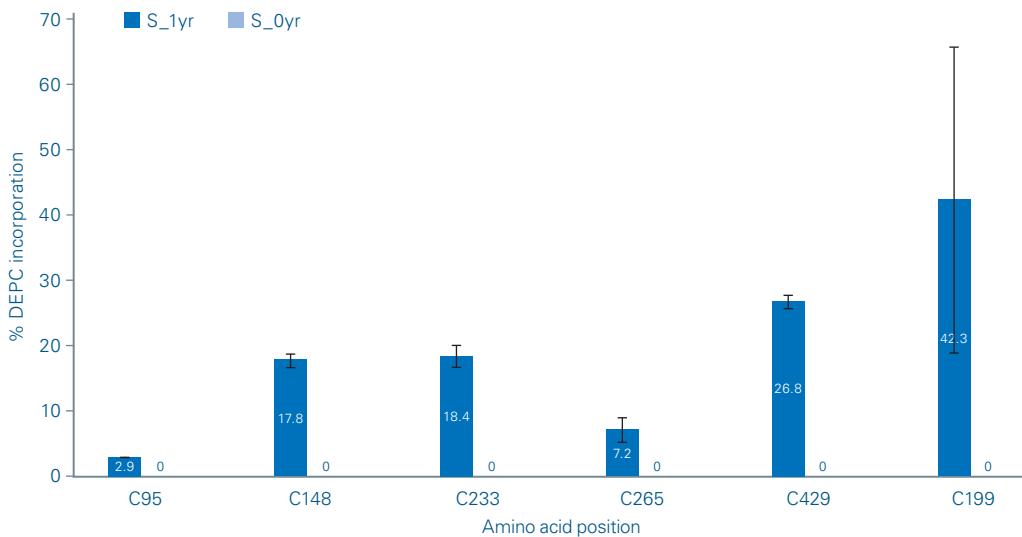
(A) Volcano plot with X-axis of  $\text{Log}_2$  fold change and Y-axis of  $\text{Log}_{10}$  of the p-value, showing the position of amino acid with significantly changed DEPC peptide intensity across the sample sets. (B) 3D model SILuMAb generated in PyMOL referring to the site of structural change (Lys330) in blue spheres and the native position of the N-glycan at Asn 301 in black sticks.



**Figure 3**  
**MS/MS spectra of the peptide containing the DEPC-modified site of Cys233.**  
 The spectra above represent the DEPC-modified Cys highlighted in red color and the spectra below show the same peptide with no modification (b7 ion).

The Cys233 is one of the two interchain Cys, holding the heavy chains of the SILuMAB together by a disulfide bond. DEPC labeling at Cys233 is evidence of a disulfide bond cleavage within the mAb. The DEPC incorporation at Cys residues was also quantified in comparison to the unmodified form thereby giving a % DEPC labeling at a specific site (Figure 4). Cleavage of disulfide bond at multiple Cys in a one-year-old SILuMAB sample indicated that most SILuMAB molecules in the sample are most likely not intact, but exist as truncated forms (3).

This single CL-MS experiment not only indicates the compromised stability of the SILuMAB structure, but also indicates the specific position where the changes could have occurred. These compromised disulfide bonds could change the structure of the SILuMAB in a way to expose the hydrophobic patches of the SILuMAB molecule further leading to aggregation.



**Figure 4**  
**Percentage of DEPC-modified Cys containing peptides in one-year-old and freshly thawed SILuMAB sample.**  
 No DEPC-modified Cys were detected in the freshly thawed sample.

## Conclusion

- The site-specific structural change in deglycosylated mAb in the CH2 domain was detected based on the excellent label-free quantitative accuracy with QTOF technology of the impact II LC-MS system, which compares favorably with previous non-MS studies. This proves the accuracy and superiority of the optimized CL-MS method.
- The MS/MS spectra quality and reproducibility based on impact II fragmentation efficiency was key to site-specific detection of DEPC labels on Cys in the double labeling strategy used in the long-term storage study. Overall, this CL-MS method with double labeling can be used as an efficient initial approach for studying disulfide bonding in proteins.
- For more complex questions about structural protein analysis, this CL-MS method can serve as a preliminary approach to narrow down the hypothesis before diving into methods like HDX-MS, cross-linking MS, and alike.

## References

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