Supplemental Information for

Covalent Labeling/Mass Spectrometry of Monoclonal Antibodies with Diethylpyrocarbonate: Reaction Kinetics for Ensuring Protein Structural Integrity

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CONTENT

SUPPLEMENTAL EXPERIMENTAL SECTION

A. DEPC Labeling Reaction and Dose-Response Plot

DEPC can modify His, Lys, Ser, Thr, Tyr, and N-termini, and the labeling results in a single type of modification product with a mass addition of 72.02 Da. Under the conditions used in this study, the bimolecular reaction between DEPC and a specific site in the protein should follow second order kinetics.[1]

Nucleophilic side chain (P) + DEPC (X) → Carbethoxylated P + CO_2 + CH_3CH_2OH

If the reaction follows second order kinetics, the rate is defined by:

$$Rate = -\frac{d[P]}{dt} = k[P][X] \quad \text{(S-1)}$$

where [P] is the concentration of unmodified mAb at time t, [X] is the DEPC concentration at time t, and k is the second-order rate coefficient.

Let $a = reaction\ progress = [P]_0 - [P] = [X]_0 - [X]$ (S-2)

where $[P]_0$ is the initial concentration of unmodified mAb, $[X]_0$ is the initial concentration of DEPC

$$-\frac{d[P]}{dt} = \frac{da}{dt} = k([P]_0 - a)([X]_0 - a) \quad \text{(S-3)}$$

$$\frac{da}{([P]_0 - a)([X]_0 - a)} = k\,dt \quad \text{(S-4)}$$

$$\int_0^a \frac{da}{([P]_0 - a)([X]_0 - a)} = k \int_0^t dt \quad \text{(S-5)}$$

$$\frac{1}{[X]_0 - [P]_0}\left[\ln\frac{[P]_0}{[P]_0 - a} - \ln\frac{[X]_0}{[X]_0 - a}\right] = kt \quad \text{(S-6)}$$

$$\frac{1}{[X]_0 - [P]_0}\left[\ln\frac{[P]_0[X]}{[P][X]_0}\right] = kt \quad \text{(S-7)}$$

$$\ln\left(\frac{[X]_0[P]}{[X][P]_0}\right) = -kt[X]_0 + kt[P]_0 \quad \text{(S-8)}$$

For each specific labeling site, the plot between $\ln\left(\frac{[X]_0[P]}{[X][P]_0}\right)$ and $[X]_0$ was produced for a given peptide from LC-MS/MS data of that peptide.

B. LC-MS Analysis of DEPC-Labeled Light and Heavy Chains of the mAbs

Aliquots of rituximab and the NISTmAb (10 mg/mL, 70 μM) were reacted with DEPC. Information about sample preparation and DEPC labeling reaction conditions can be found in the main text. After labeling and quenching, TCEP was added to the DEPC-labeled samples at a 500:1 TCEP:mAb molar ratio to reduce the disulfide bonds of the mAb and yield heavy and light chains. The resulting mixtures were diluted in 50 mM phosphate buffer at pH 7.4 to a final concentration of 0.15 mg/mL.

Online LC-MS analyses were performed on the TCEP-reduced samples. A sample containing approximately 2 μg protein was loaded on a Thermo Scientific Ultimate 3000 HPLC system (Waltham, MA). The separation was performed on a Waters Acquity UPLC Protein BEH C4 column (50 mm x 2.1 mm, 1.7 μm particle size, 300 Å pore size; Milford, MA) with a flow rate of 200 μL/min. LC/MS-grade water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as mobile phases. Desalting was performed at 5% B during the first 4 min after sample injection. A linear gradient of solvent B was increased from 5% B to 100% B over 12 min. The gradient was held at 95% B for additional 3 min to flush a column.

Mass spectra were acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer (Waltham, MA). The electrospray needle voltage was kept at ~4 kV (positive mode), and the ion transfer tube temperature was set to 330 °C. In-source activation energy was applied at 35 V to help remove water and other adducts from protein ions. Mass spectra were acquired on an Orbitrap analyzer, with a resolution of 15,000. The AGC target and maximum injection time were set to 1×10^6 ions and 100 msec, respectively. Measurements were conducted in the high mass range mode with 3 microscans per spectrum.

SUPPLEMENTAL FIGURES

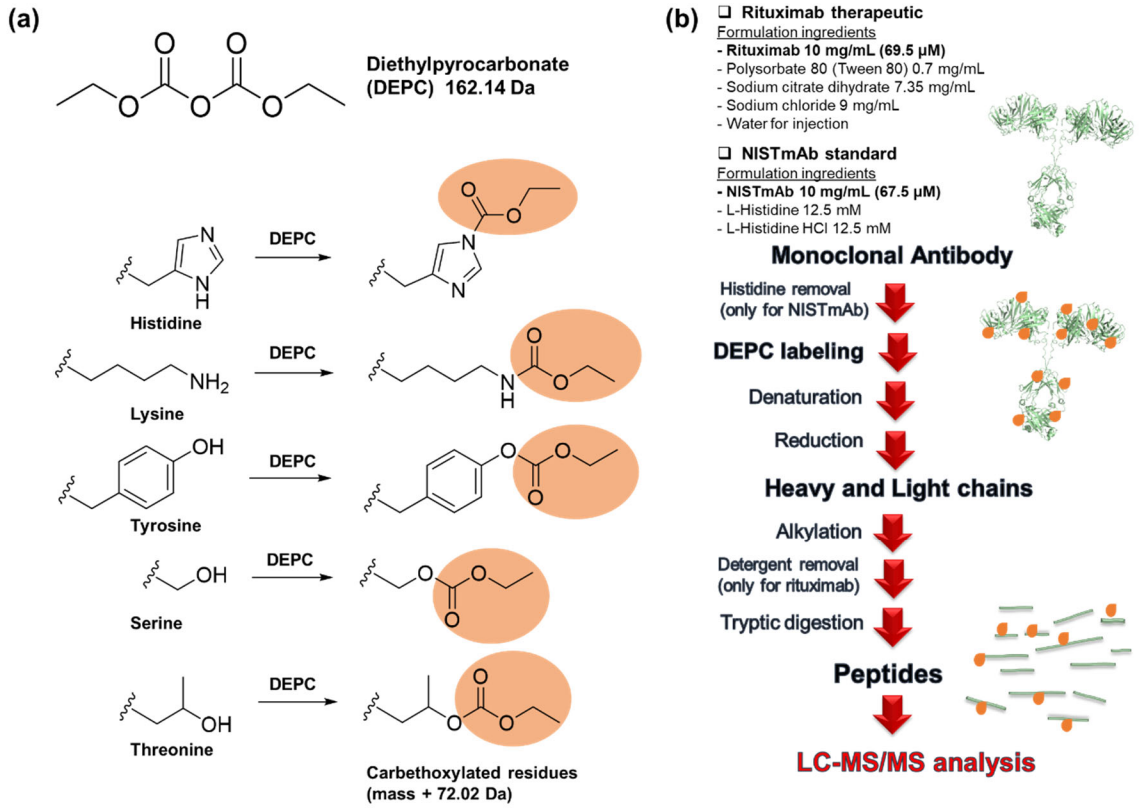


Figure S1. An antibody's structural properties are encoded into the mass of antibody via DEPC covalent labeling. (a) Chemical structure of DEPC and labeling reaction products for amino acid residues that are modified by DEPC. (b) Workflow for DEPC covalent labeling combined with bottom-up MS analysis via proteolytic digestion and LC-MS/MS to identify labeling sites and determine label levels at each modified residue in the mAbs.

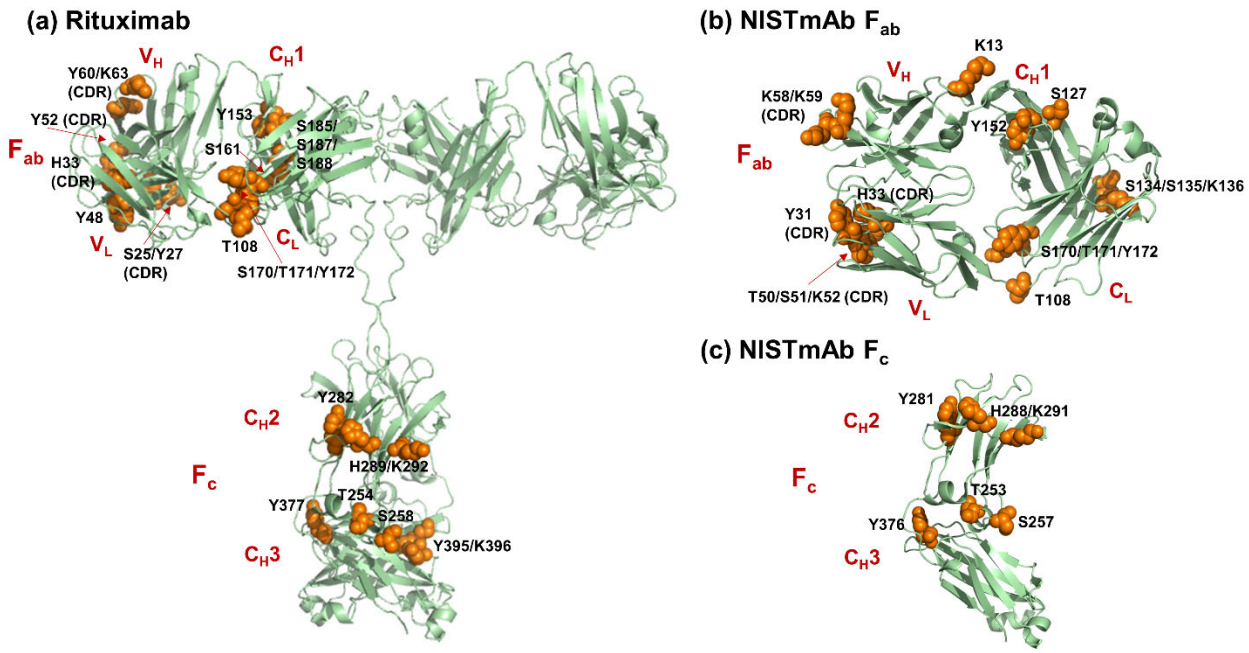
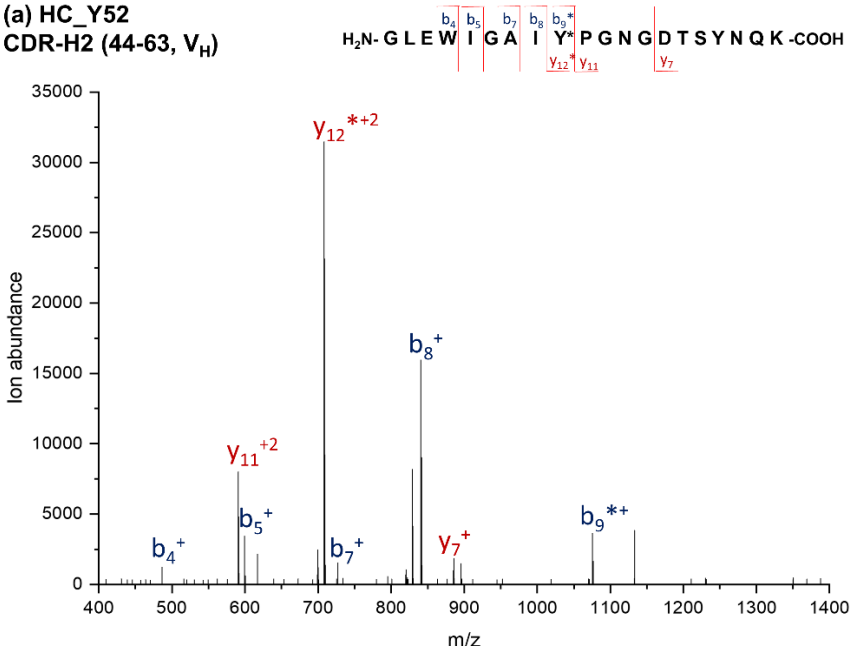
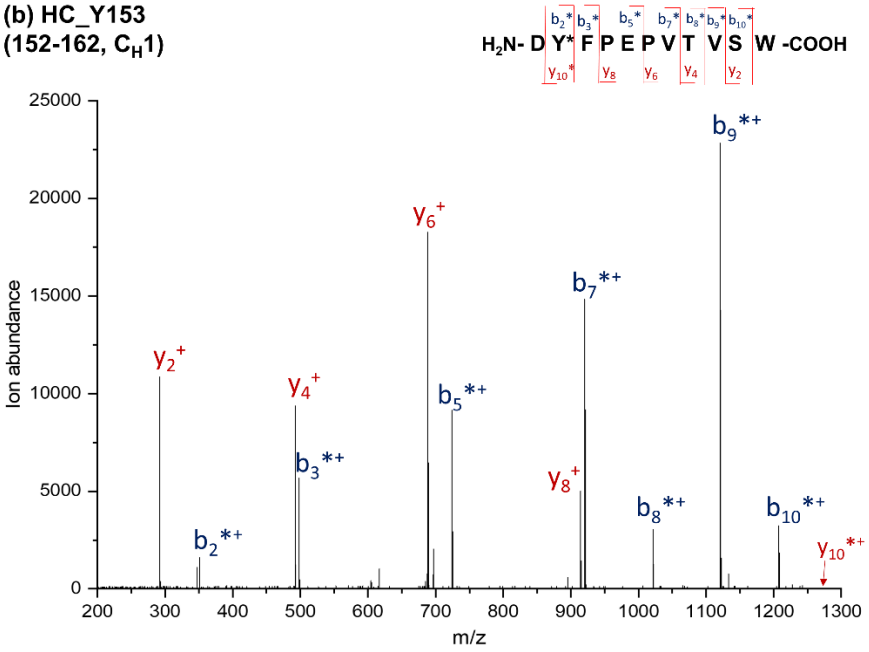


Figure S2. Labeling sites (orange spheres) from a set of representative peptides that were selected from different domains throughout the antibody structures. (a) Rituximab - F_{ab} (PDB 4KAQ) and F_c (PDB 4W4N) structures of rituximab are aligned to the human IgG1 model, using the molecular visualization system PyMOL.[2, 3] Note that, for clarity, only one symmetric unit of the rituximab structure is labeled in this figure. (b) NISTmAb F_{ab} (PDB 5K8A) and (c) NISTmAb F_c (PDB 4W4N)

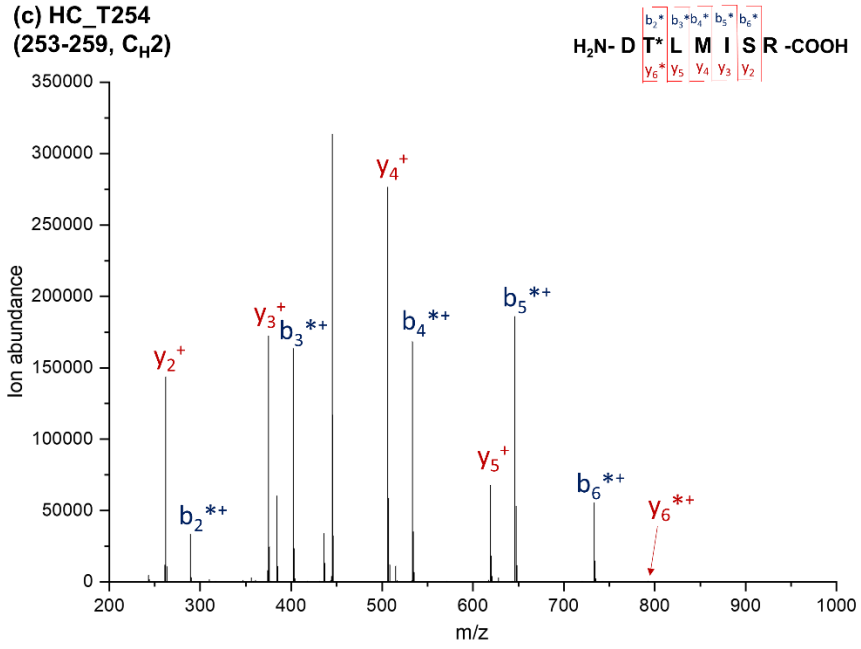
(a) HC_Y52
CDR-H2 (44-63, V_H)



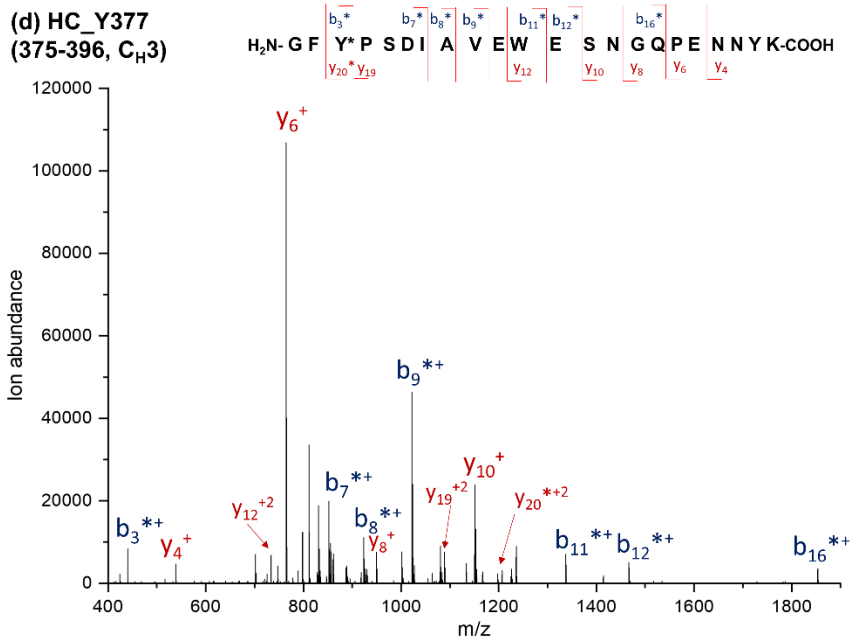
(b) HC_Y153
(152-162, C_H1)



(c) HC_T254
(253-259, C_H2)



(d) HC_Y377
(375-396, C_H3)



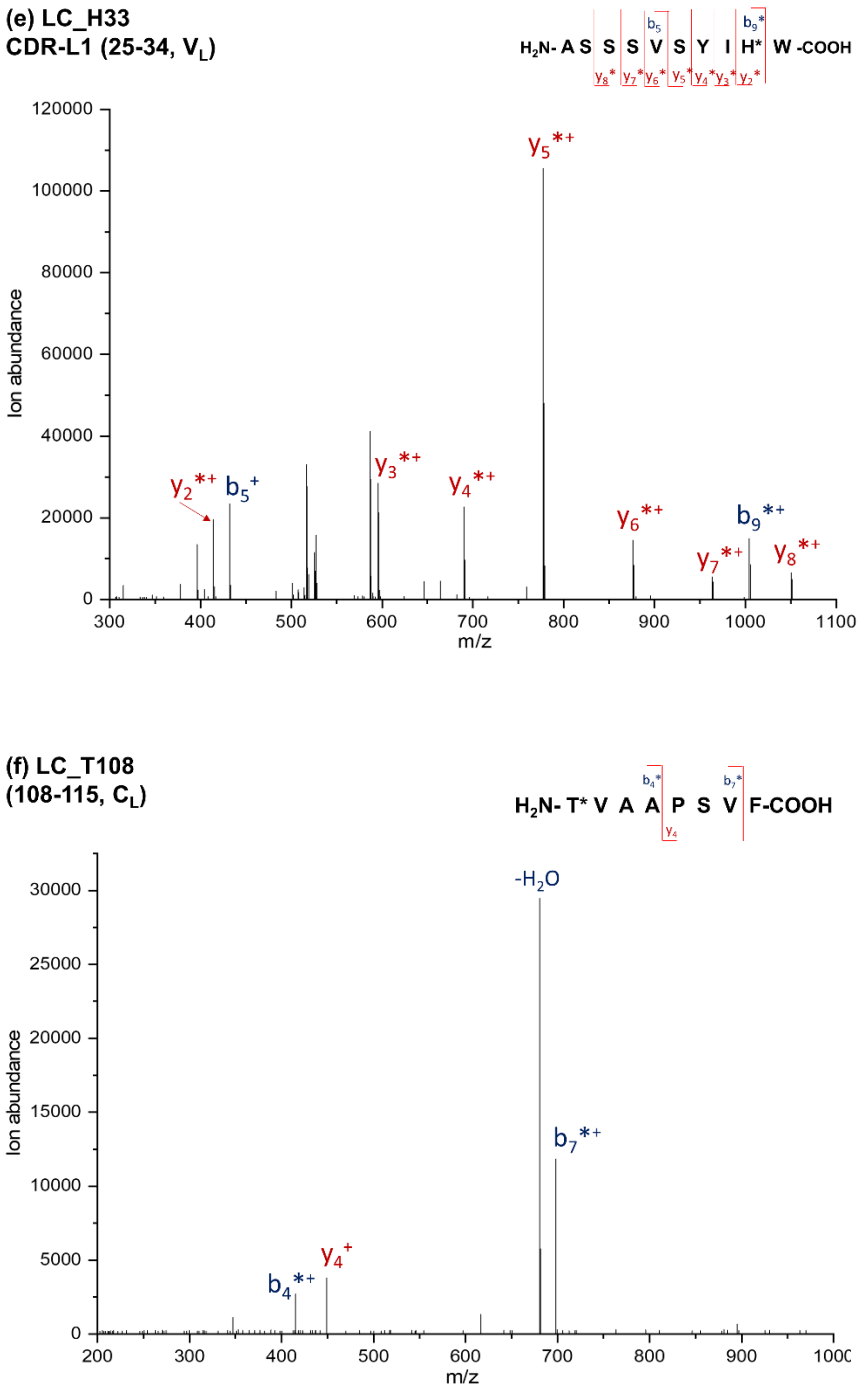
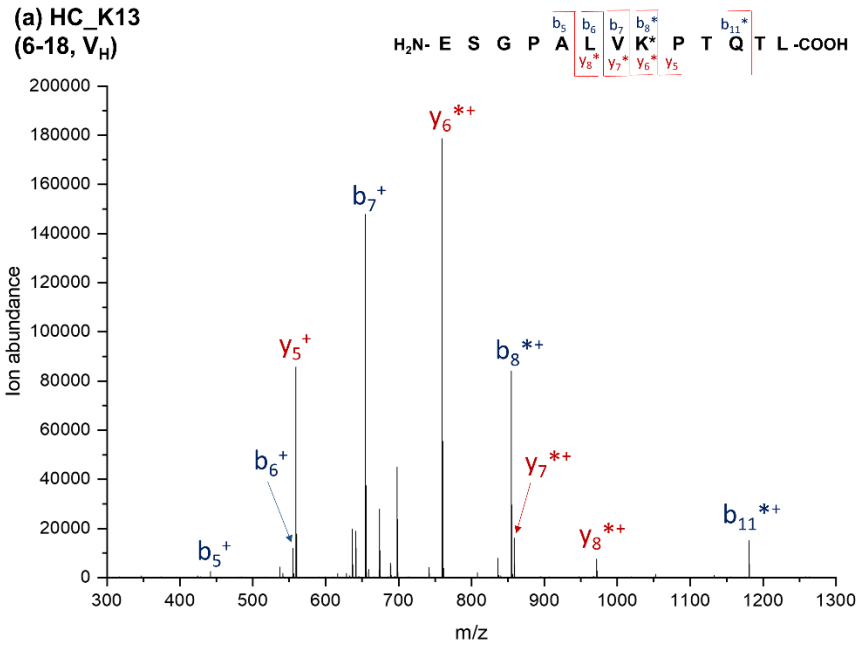
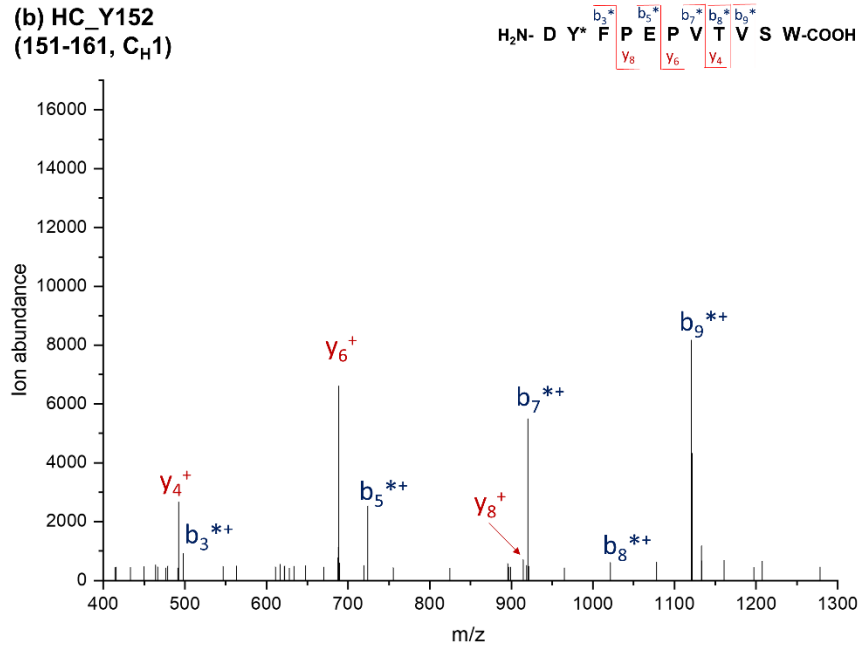
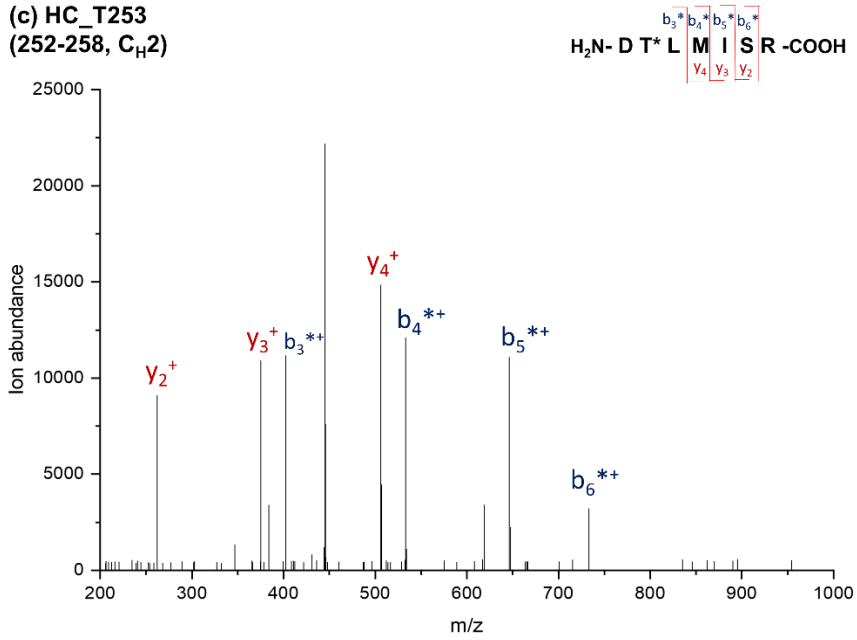


Figure S3. MS/MS assignments for DEPC-labeled peptides from each of the six antibody domains in heavy chain (HC) and light chain (LC) of rituximab, (a) V_H, (b) C_H1, (c) C_H2, (d) C_H3, (e) V_L, and (f) C_L

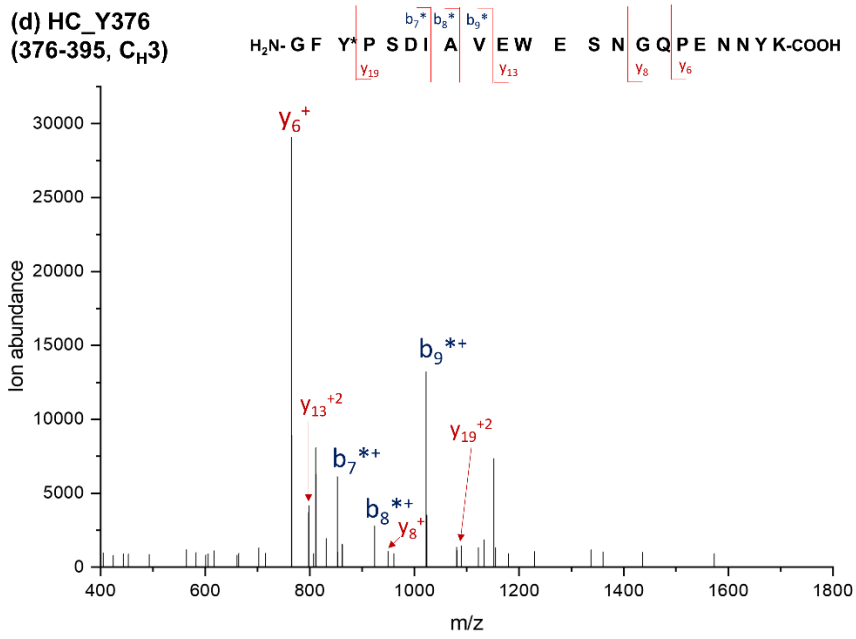

(a) HC_K13
(6-18, V_H)
H_2N- E S G P A L V K* P T Q T L -COOH
Ion abundance
m/z
y_6^{*+}
b_7^+
y_5^+
b_8^{*+}
b_6^+
y_7^{*+}
b_{11}^{*+}
y_8^{*+}
b_5^+


(b) HC_Y152
(151-161, C_H1)
H_2N- D Y* F P E P V T V S W-COOH
Ion abundance
m/z
b_9^{*+}
y_6^+
b_7^{*+}
y_4^+
b_5^{*+}
y_8^+
b_3^{*+}
b_8^{*+}

(c) HC_T253
(252-258, C_H2)



(d) HC_Y376
(376-395, C_H3)



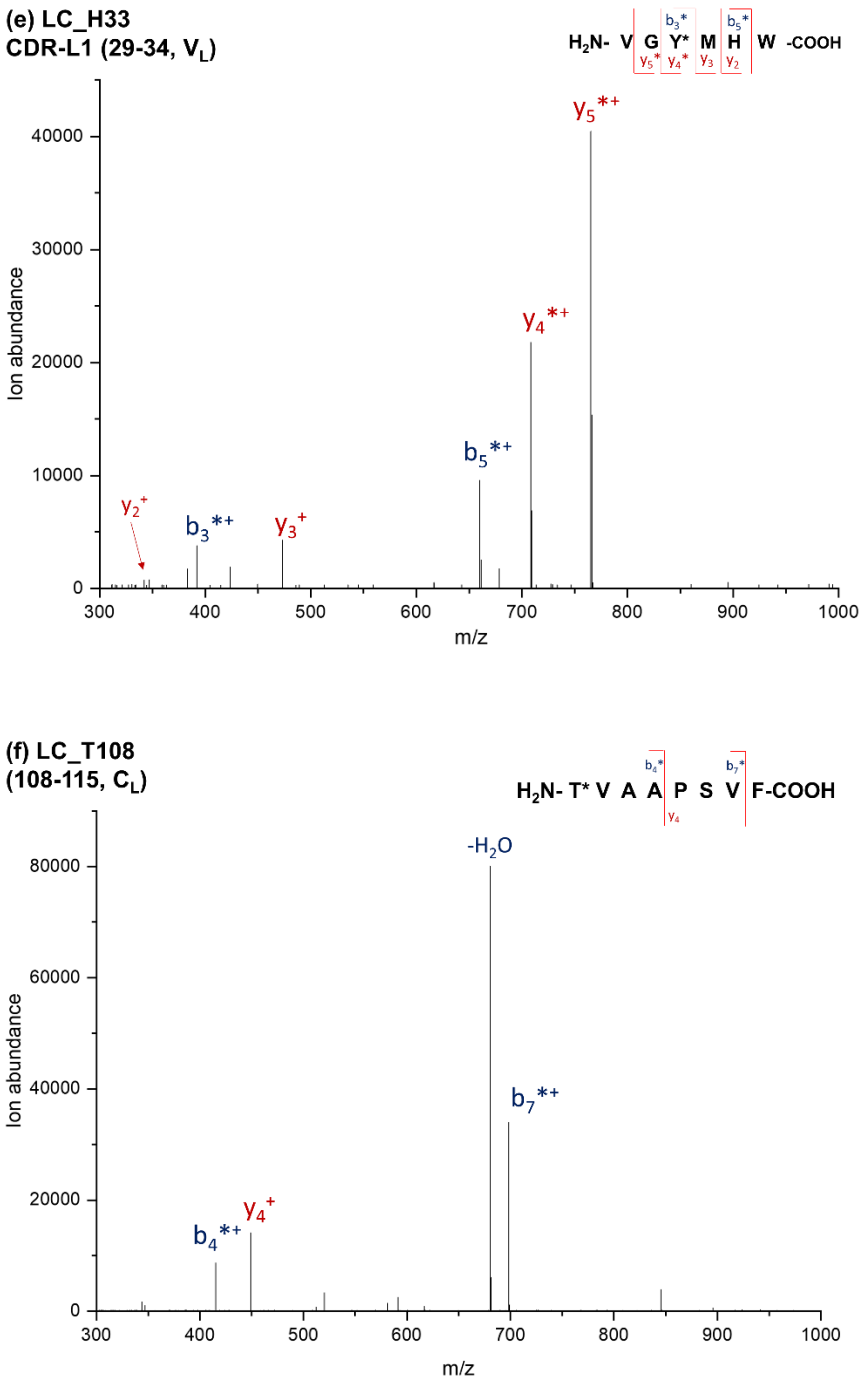
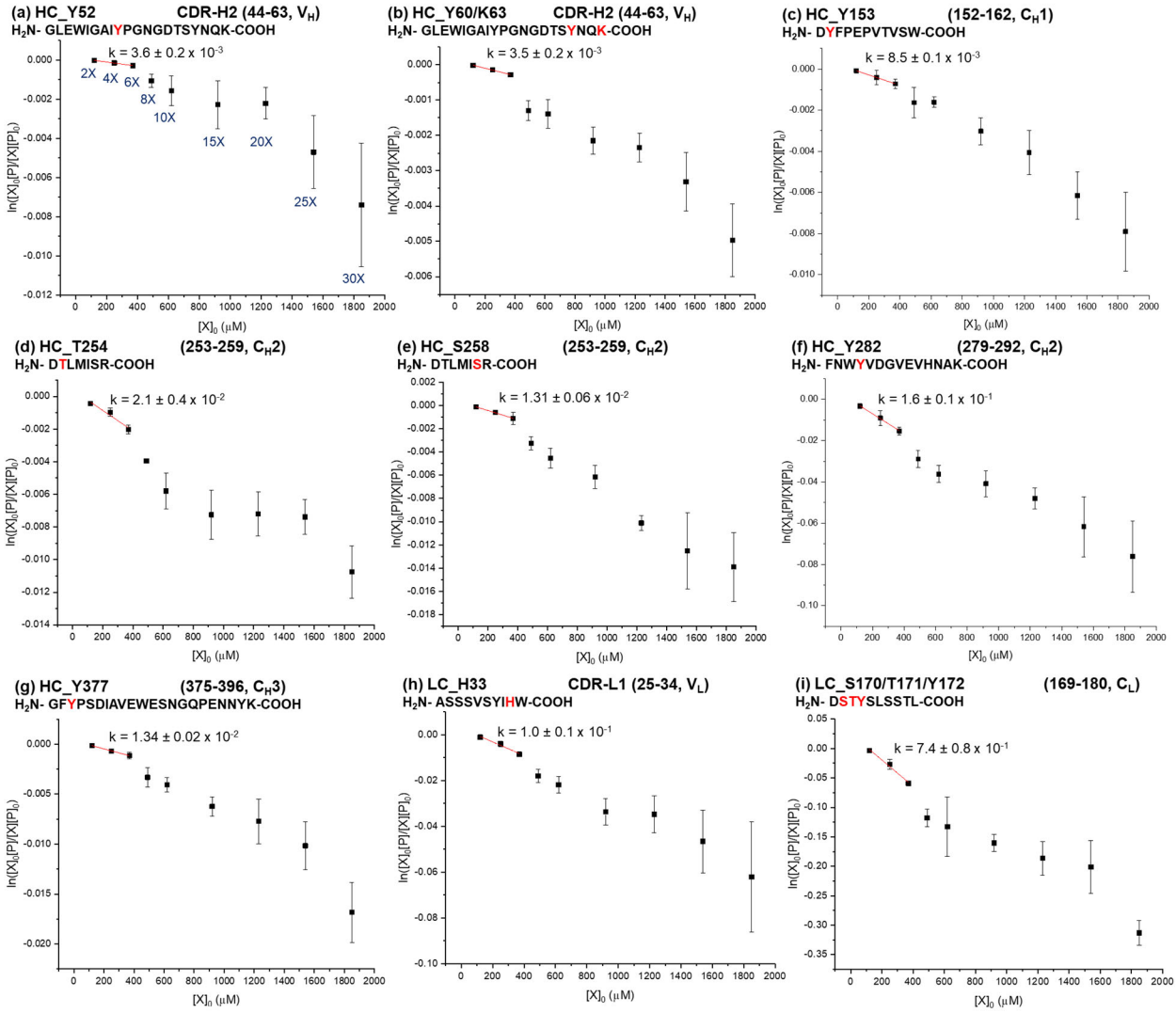


Figure S4. MS/MS assignments for DEPC-labeled peptides from each of the six antibody domains in heavy chain (HC) and light chain (LC) of NISTmAb, (a) V_H, (b) C_H1, (c) C_H2, (d) C_H3, (e) V_L, and (f) C_L

❑ **Linearity up to 6X**



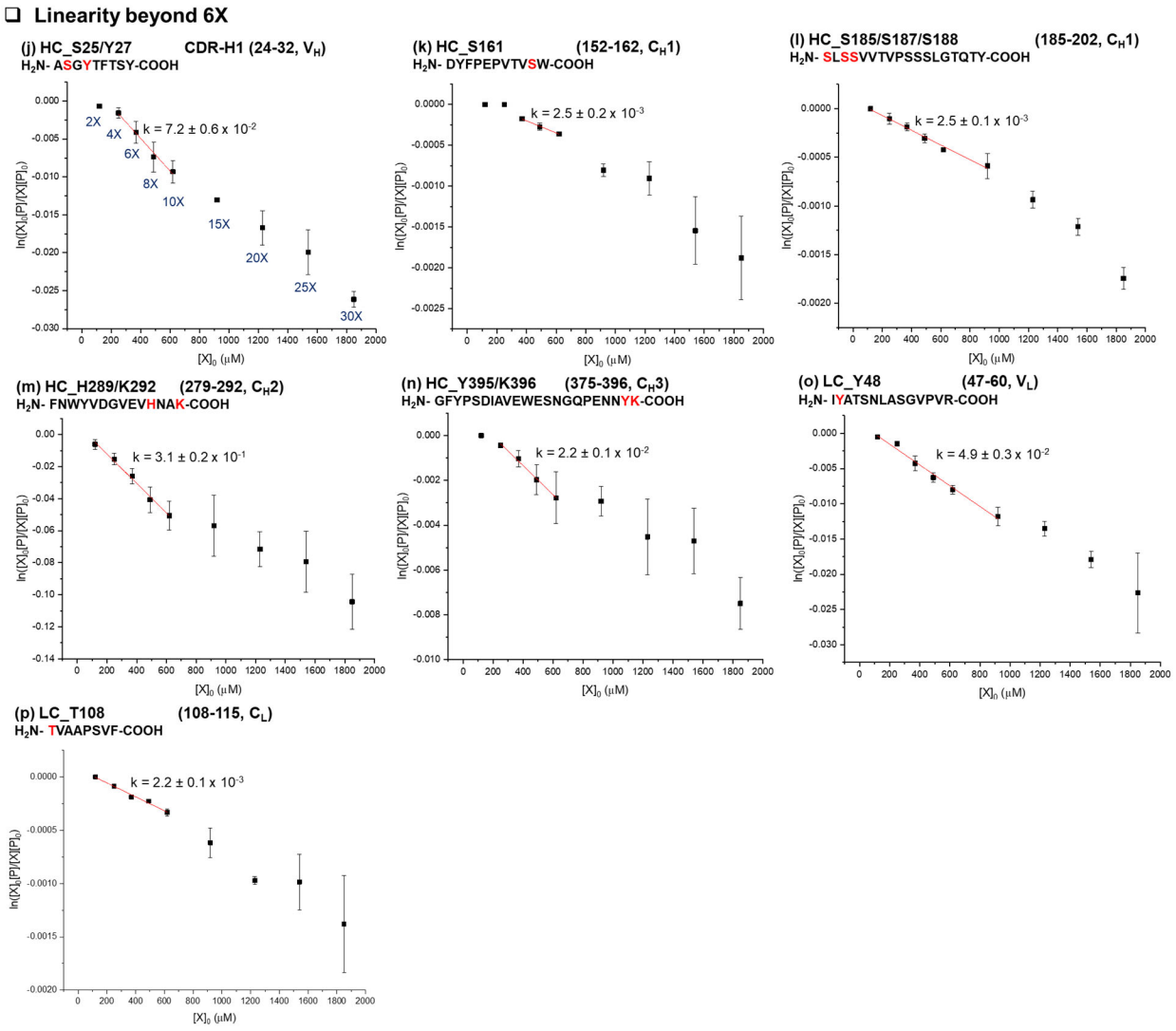
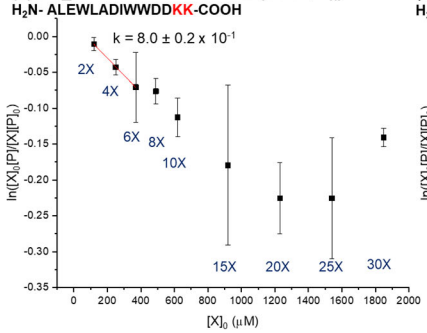


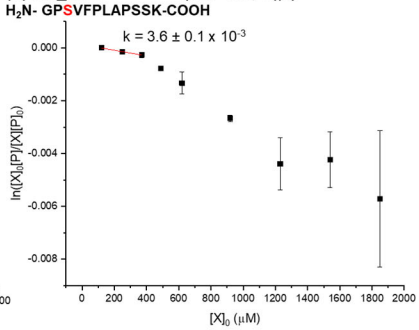
Figure S5. Dose-response plots for selected proteolytic fragments of rituximab after labeling with DEPC at different concentrations varying from 2- to 30-fold DEPC to protein molar ratio (2X to 30X). The plots of reactive residues from different antibody domains (V_H, C_H1, C_H2, C_H3, V_L, and C_L) in heavy chain (HC) and light chain (LC) are shown here. From all of the representative peptides, linear relationships are observed between the unmodified fraction $\ln(\frac{[X]_0[P]}{[X][P]_0})$ and the DEPC concentrations at low reagent concentrations up to 6X [plots (a) to (i)] or beyond 6X [plots (j) to (p)]. The rate coefficient (k) values in M^{-1} s^{-1} are obtained by dividing the measured slopes by the reaction time and the error bars are calculated from standard error of slope.

❑ **Linearity up to 6X**

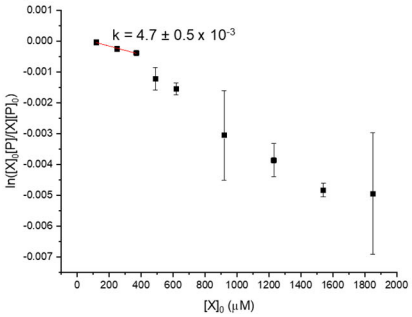
(a) HC_K58/K59 CDR-H2 (46-59, V_H)
H_2N- ALEWLADIWWDDKK-COOH



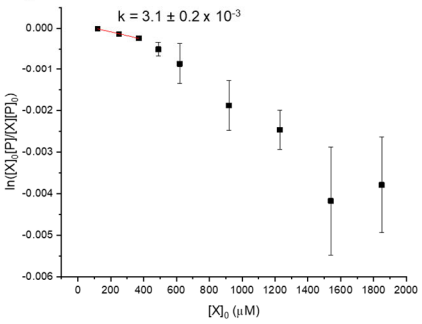
(b) HC_S127 (125-136, C_H1)
H_2N- GPSVFPLAPSSK-COOH



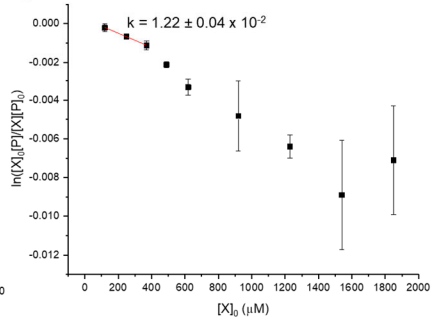
(c) HC_T253 (252-258, C_H2)
H_2N- DTLMISR-COOH



(d) HC_S257 (252-258, C_H2)
H_2N- DTLMISR-COOH

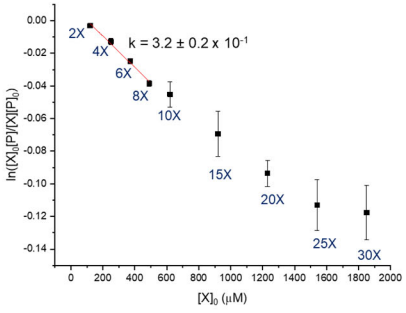


(e) LC_T50/S51/K52 CDR-L2 (45-52, V_L)
H_2N- LLIYDTSK-COOH

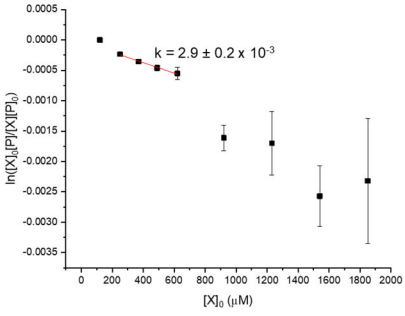


❑ **Linearity beyond 6X**

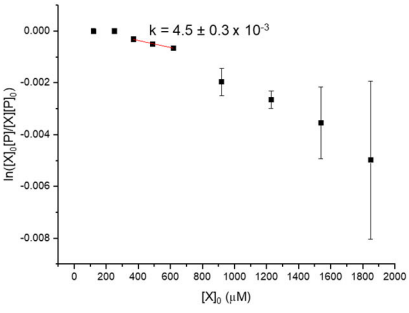
(f) HC_K13 (6-18, V_H)
H_2N-ESGPALVKPTQTL-COOH



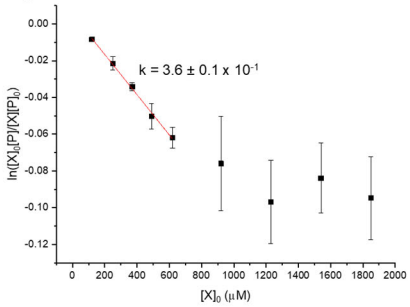
(g) HC_S134/S135/K136 (125-136, C_H1)
H_2N- GPSVFPLAPSSK-COOH



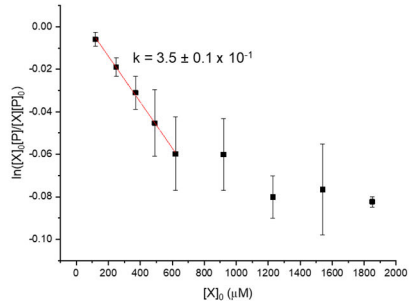
(h) HC_Y152 (151-161, C_H1)
H_2N- DYFPEPVTVSW-COOH



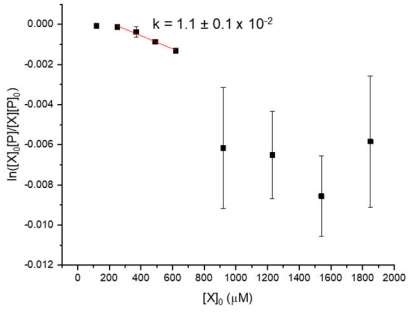
(i) HC_Y281 (279-292, C_H2)
H_2N- FNWYVDGVEVHNAK-COOH



(j) HC_H288/K291 (279-292, C_H2)
H_2N- FNWYVDGVEVHNAK-COOH



(k) HC_Y376 (374-395, C_H3)
H_2N- GFYPSDIAVEWESNGQPENNYK-COOH



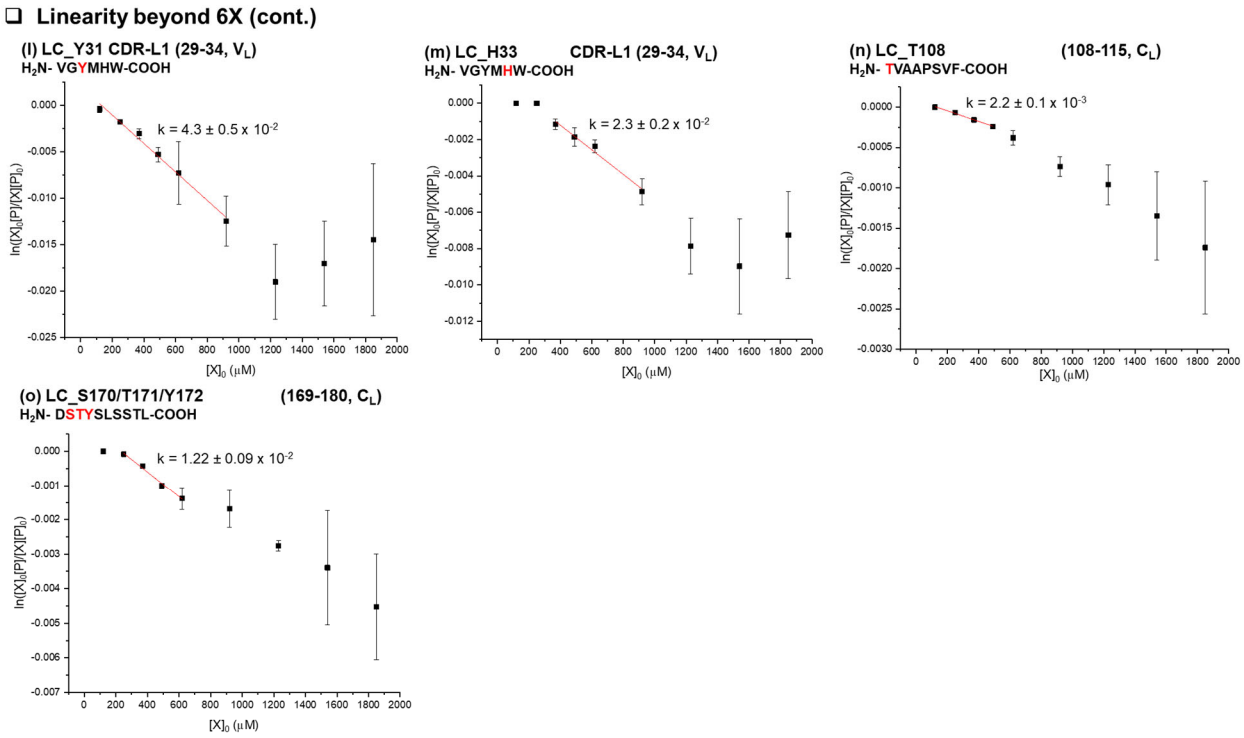


Figure S6. Dose-response plots for selected proteolytic fragments of NISTmAb after labeling with DEPC at different concentrations varying from 2- to 30-fold DEPC to protein molar ratio (2X to 30X). The plots of reactive residues from different antibody domains (V_H, C_H1, C_H2, C_H3, V_L, and C_L) in heavy chain (HC) and light chain (LC) are shown here. From all of the representative peptides, linear relationships are observed between the unmodified fraction $\ln(\frac{[X]_0[P]}{[X][P]_0})$ and the DEPC concentrations at low reagent concentrations up to 6X [plots (a) to (e)] or beyond 6X [plots (f) to (o)]. The rate coefficient (k) values in M^{-1} s^{-1} are obtained by dividing the measured slopes by the reaction time and the error bars are calculated from standard error of slope.

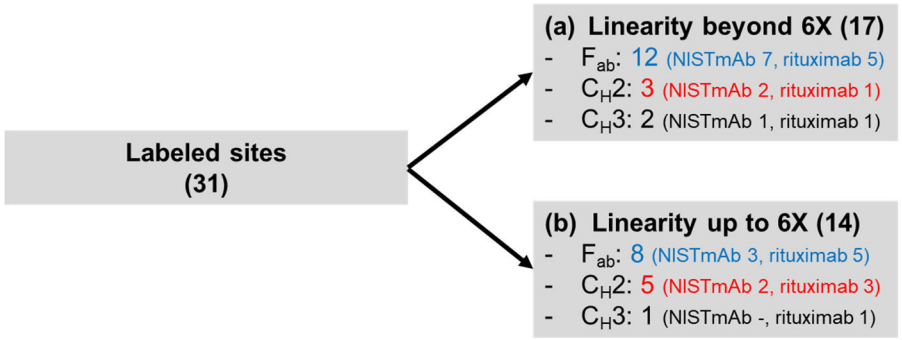


Figure S7. Trends of structural perturbations with regard to different antibody domains. The F_{ab} has a greater percentage of sites whose linearity goes beyond 6X (60%, 12 out of 20 sites) whereas C_H2 has a smaller percentage of sites whose linearity goes beyond 6X (38%, 3 out of 8 sites). Considering the number of F_{ab} sites in each antibody, NISTmAb has higher number of sites whose linearity is beyond 6X (70%, 7 out of 10 sites) while rituximab has lower number (50%, 5 out of 10 sites).

SUPPLEMENTAL REFERENCES

1. Mendoza, V. L.; Vachet, R. W., Protein Surface Mapping Using Diethylpyrocarbonate with Mass Spectrometric Detection. *Anal. Chem.* **2008,** *80* (8), 2895-2904.
2. Limpikirati, P.; Hale, J. E.; Hazelbaker, M.; Huang, Y.; Jia, Z.; Yazdani, M.; Graban, E. M.; Vaughan, R. C.; Vachet, R. W., Covalent labeling and mass spectrometry reveal subtle higher order structural changes for antibody therapeutics. *mAbs* **2019,** *11* (3), 463-476.
3. Schrodinger, LLC *The PyMOL Molecular Graphics System, Version 1.8*, 2015.