SUPPORTING MATERIAL FOR

Covalent Labeling and Mass Spectrometry Reveal Subtle Higher Order Structural Changes for Antibody Therapeutics

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CONTENT

SUPPLEMENTAL METHODS

A) Biophysical Characterization

Intrinsic Fluorescence Spectroscopy. Tryptophan fluorescence measurements were performed on a Photon Technology International Quantamaster-4SE spectrofluorometer. Rituximab samples were diluted to 1 μM in 50 mM phosphate buffer at pH 7.4 prior to analysis. Heated samples were then cooled to 37 °C prior to a measurement. A 200-μL solution of 1 μM rituximab sample was transferred to a quartz cuvette. Fluorescence spectra were acquired at room temperature using an excitation wavelength of 285 nm with a slid width of 1 nm and an emission scan range of 310 – 440 nm with a slit width of 0.2 nm.

Circular Dichroism (CD) Spectroscopy. Far-UV CD analyses were performed on a Jasco J-1500 spectropolarimeter. CD spectra were recorded at room temperature over a scan range of 250 to 195 nm. Rituximab samples were diluted to 1 μM in 50 mM phosphate buffer at pH 7.4 prior to analysis. Heated samples were cooled to 37 °C prior to a measurement. A 200-μL solution of 1 μM rituximab sample was then transferred to a quartz cuvette. The CD spectrometric parameters were set as follows: a scan resolution (data pitch) of 0.5 nm, a scan rate of 20 nm/min, a band width of 2 nm, and a digital integration time of 1 sec. Triplicate measurements were performed for each sample at room temperature. After background subtraction, raw CD outputs (θ, degree) were converted into mean residue ellipticity using **Eq. S-1**.[1]

$$[\theta]^{MR}\ (degree\ cm^2 dmol^{-1}) = \frac{100 \times \theta\ (millidegree)}{C\ (mol\ L^{-1}) \times N \times l\ (cm)} \quad \text{(S-1)}$$

where $[\theta]^{MR}$ is mean residue ellipticity in deg cm^2 $dmol^{-1}$, θ is raw signal output in mdeg, C is rituximab concentration in molar, N is the number of amino acid residues in a protein, and l is path length of a cuvette in cm.

Dynamic Light Scattering (DLS). Hydrodynamic radii of native and thermally-stressed rituximab were measured at room temperature using a Malvern Zetasizer ZSP – DLS system. Rituximab samples were diluted to 1 μM in 50 mM phosphate buffer at pH 7.4 prior to analysis. Heated samples were cooled to 37 °C prior to a measurement. A 1-mL solution of 1 μM rituximab sample was transferred to a plastic cuvette. Back scattering was detected at a measurement angle of 173^{O}, and volume particle size distribution of the sample was recorded. Five replicate

measurements were performed for each sample at room temperature. Measurement duration was set according to the preset levels (automatic mode).

Size-Exclusion Chromatography (SEC). SEC separation of rituximab after heating was performed at room temperature on an Agilent 1260 Infinity HPLC system using a TSKgel SuperSW3000 column (30 cm x 7.8 mm ID, 5 μm particle size; Tosoh Bioscience LLC). Rituximab samples were diluted to 1 mg/mL in 50 mM phosphate buffer at pH 8.0 prior to analysis. Heated samples were cooled to 37 °C prior to a chromatographic run. A sample containing approximately 50 μg rituximab was loaded on an SEC column. The mobile phase (pH 6.5) comprises 50 mM sodium phosphate, 400 mM sodium perchlorate, and 10% isopropanol, and an isocratic flow rate of 0.5 mL/min was used. A variable wavelength UV detector set at 280 nm was used for detection.

B) Determination of the labeling limits of quantitation (LOQ)

Peptide Spiking Experiments The model peptide H_2N-VVSVLTVLHQDWLNGK, which comes from the rituximab sequence (HC peptide with amino acids 306-321), was custom synthesized (AnaSpec Inc.). A variety of DEPC-modified species (with different modification sites) were generated from covalent labeling of the model peptide (10 μM) at a DEPC to protein molar ratio of 10 to 1 at 37°C for 5 min, which resulted in labels at N-terminus, H314, and K321. Labeled sites were identified and modification percentages were determined by LC-MS/MS. A mixture of DEPC-labeled peptides were spiked into a digest of unlabeled rituximab (2.57 μM matrix) at varying concentrations, and the resulting solutions were subsequently analyzed by LC-MS/MS. Signals of the spiked peptide, as measured by LC-MS, were used to estimate the the LOQ based on signal-to-noise ratio of response.

C) Activity Assays

Alamar Blue Assay A range of stress temperatures were used in this study. Rituximab samples were incubated at 37°C (control), 50°C, 60°C, and 68°C prior to an assay. The conditions used for this assay were derived from Zhang *et al*[2]. Briefly, Raji cells (ATCC CLL-86) were grown in a T75 flask using RPMI media supplemented with fetal bovine serum (FBS). Once confluent, cells were washed with phosphate-buffered saline (PBS) and diluted to 1 x 10^6 cells/mL in RPMI media. 90 μL of cells were delivered into a 96 well plate, and 10 μL of control or heat-treated

rituximab diluted in RPMI media was added at the concentrations indicated (4 ng/mL rituximab). Plates were then incubated for 30 min at 37 °C, then each well was supplemented with 10% Invitrogen™ normal human serum (#31876, Thermo Fisher Scientific). Plates were returned to 37 °C for 4 h, then 11 µL of Invitrogen™ Alamar Blue reagent (#DAL1025, Thermo Fisher Scientific) was added per manufacturer's specifications. After 1 hour, fluorescence signal was read using an excitation wavelength of 560 nm and emission of 590 nm. Data was generated on a Synergy H1 microplate reader (BioTek) and results were exported to Microsoft Excel for analysis. Each sample was generated in triplicate, and independently generated to confirm trends were the same.

Rituximab bridging ELISA Custom rituximab ELISA plates were generated and used as previously described in Cragg *et al*[3] and Hampson *et al*[4] with some modifications. Briefly, anti-human capture antibody (clone SB2H2 recognizing the F_c region of human antibody, #MCA2531, Biorad) was diluted 1 to 1000 in coating buffer (15 mM sodium carbonate, 28.5 mM sodium bicarbonate, pH 9.6), and 100 µL was added to each well of a 96 well NUNC MaxiSorp flat-bottom plate (#44-2404-21, Thermo Fisher Scientific). Plates were then incubated overnight at 4 °C, then blocked with 200 µL of 1% bovine serum albumin (BSA) in PBS for 2 h. Plates were washed three times with PBST prior to use. For the rituximab quantitation and standard curve generation, samples were diluted in phosphate-buffered saline with Tween® 20 (PBST) at the concentrations indicated (1 to 500 ng/mL rituximab). For the binding assay, 10 ng/mL rituximab was used in the experiment. Rituximab samples were incubated at 37°C (control), 45°C, 55°C, and 65°C prior to an assay. 100 µL of sample was added to each well and incubated for 1 h at room temperature. Wells were then washed five times with PBST, and the horseradish peroxidase (HRP) – labeled anti-rituximab detection antibody (clone MB2A4 anti-idiotypic antibody, #MCA2260P, Bio-Rad) was added at a 1: 60,000 dilution in blocking buffer. Samples were incubated for 90 minutes, then washed five times with PBST. Plates were then developed using 100 µL of HRP substrate for 45 min and stopped with the addition of 50 µL of 3 M sulfuric acid. Data was generated on a Synergy H1 microplate reader (BioTex) measuring absorbance at 450 nm and normalized against the absorbance at 630 nm to clear up any background signal, and the results were exported to Microsoft Excel for analysis. Each sample was generated independently in triplicate.

Raji Cell Pull-Down Assay A range of stress temperatures were used in this study. Rituximab samples were preheated at 37°C (control), 50°C, 60°C, and 68°C. Control and heat-

treated rituximab was diluted to 200 ng/mL in PBS and mixed 1:1 with either Raji cells (1 x 10^6 cells/mL) in PBS, or PBS alone was used as a control. The total concentration of rituximab in the experiment was 100 ng/mL. Free rituximab was then quantified using the ELISA plates, and normalized against the control wells. Absolute quantitation was performed using standard curves generated from each heat-treated sample independently to confirm quantitation was in the linear part of the standard curve.

SUPPLEMENTAL FIGURES AND TABLES

DEPC labeling with MS detection as a tool for HOS analysis of rituximab

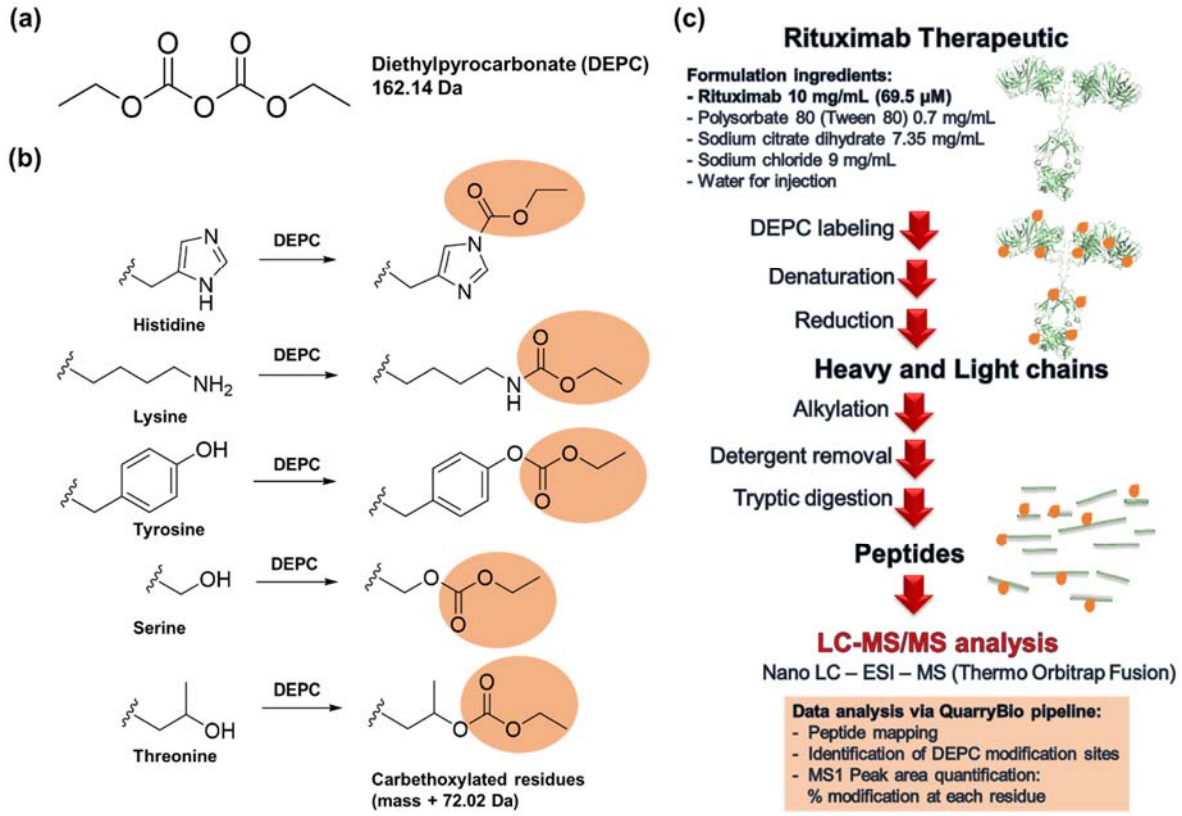

Figure S1 DEPC CL-MS as a tool for structural analysis of rituximab. In an MS-based approach for structural analysis, a protein's structural properties are encoded into the mass of protein via DEPC covalent labeling. This figure shows (a) chemical structure of DEPC, (b) reactions of amino acid residues that are modified in covalent labeling with DEPC, and (c) workflow for DEPC covalent labeling combined with bottom-up MS analysis via proteolytic digestion and LC-MS/MS to identify labeled sites and determine label levels at each modified residue. Peptide identification and peak area quantification were performed using a custom software pipeline developed by QuarryBio Inc.[5]

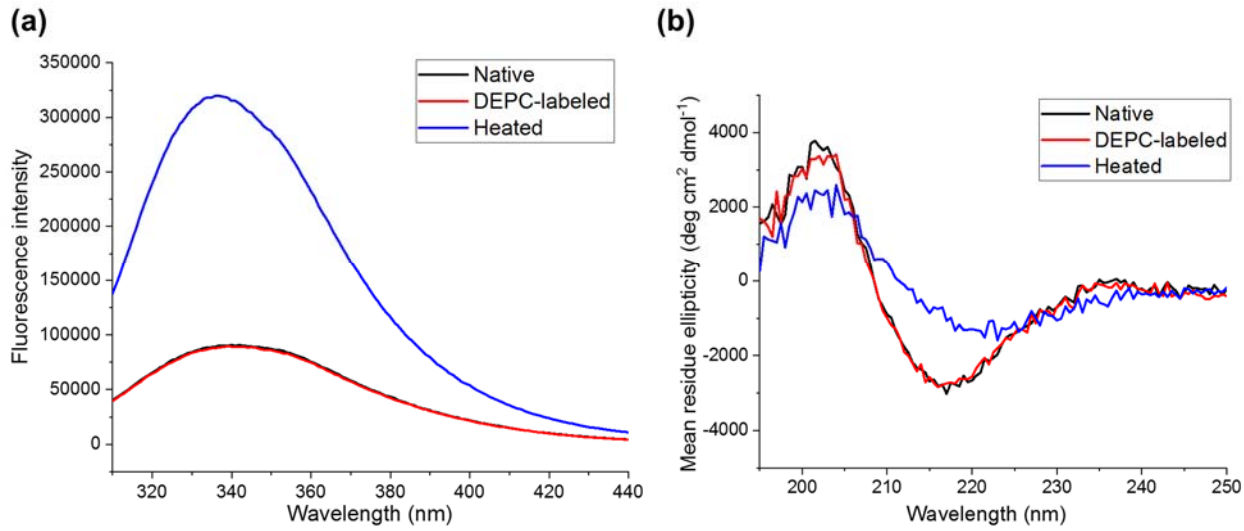

Figure S2 Controlling DEPC to rituximab molar ratio to minimize structural perturbations of rituximab during the labeling reaction. The DEPC to rituximab molar ratio is limited at 4 to 1 in order to minimize structural perturbations of rituximab during the labeling reaction. Previous studies on a variety of proteins indicate that a 4 to 1 ratio is appropriate to minimize labeling-induced structural changes.[5-7] The structural integrity of rituximab after covalent labeling can be confirmed via: (a) tryptophan fluorescence spectra and (b) circular dichroism spectra obtained from rituximab samples under native (black), DEPC-labeled (red), and heated at 70 °C for 15 min (blue) conditions. Heated rituximab was used to indicate spectral changes due to the structural change. The essentially identical overlap between the spectra of the native and DEPC-labeled rituximab samples indicates that covalent labeling has only little or no effect on the structural perturbation of rituximab.

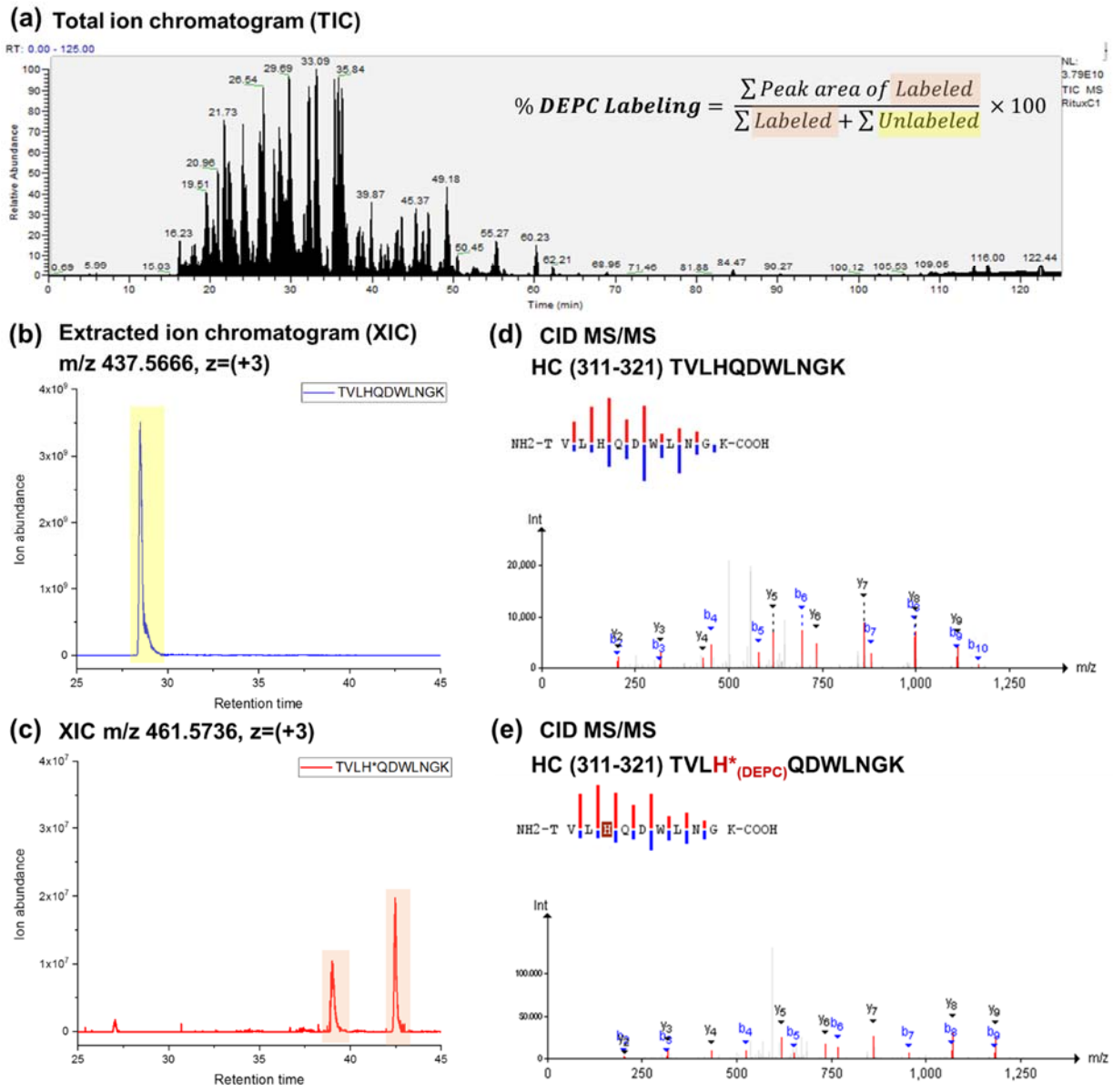

Figure S3 Illustration of how the DEPC modification levels are calculated. After DEPC labeling and proteolytic digestion, (a) LC-MS analysis of the digested rituximab is performed. During LC-MS, peptides are subjected to CID MS/MS in a linear quadrupole ion trap for identication, while peptide ion abundances measured by the Orbitrap are used for peak area quantification. Peak areas of (b) unlabeled and (c) labeled peptides in a chromatogram are used to calculate the labeling percent (see **Eq. 3** in a main text). CID tandem spectra of (d) unlabeled and (e) labeled peptides obtained at specific retention time are used for peptide sequencing and identification of DEPC labeled site. Two LC peaks for the labeled peptide in (c) are observed because the His side chain has two nitrogens that are separately labeled to produce isomers that can be separated by LC.

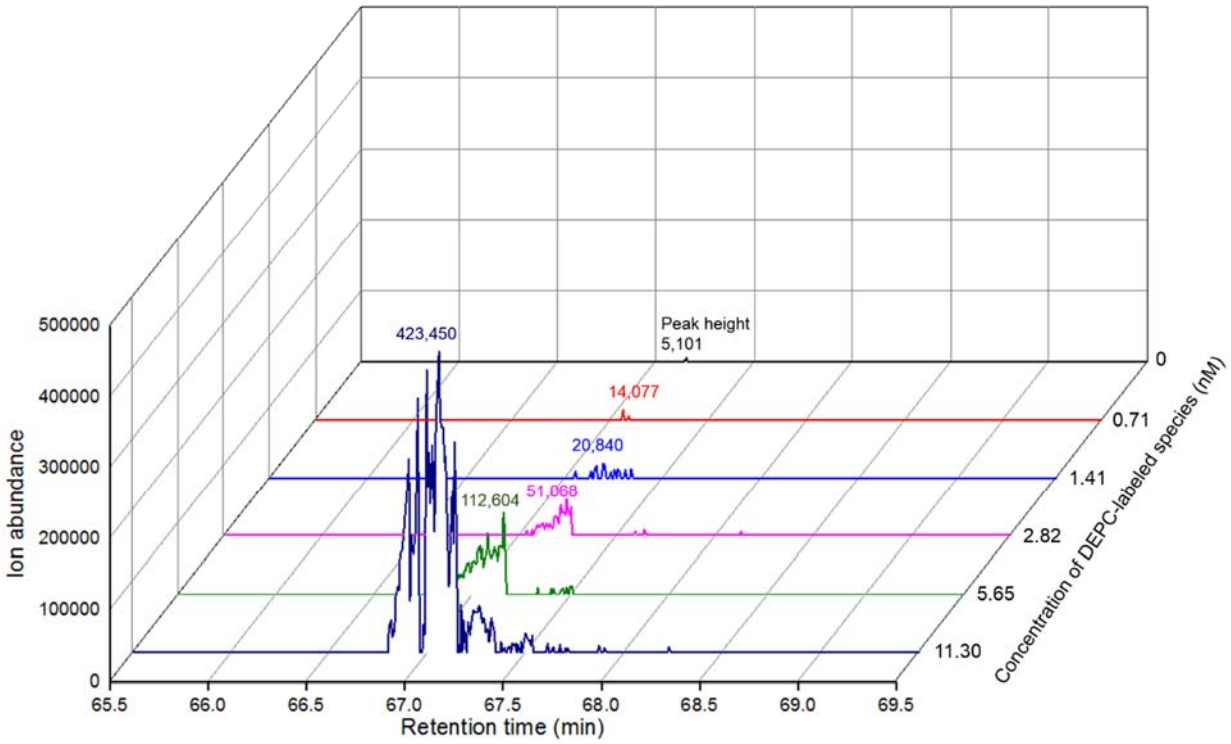

Figure S4 Representative extracted ion chromatograms (XICs) used to estimate the labeling LOQ for a DEPC-labeled model peptide (H_2N-VVSVLTVLHQDWLNGK*). This peptide, which comes from the rituximab sequence, was synthesized and DEPC labeled before spiking into a digest of unlabeled rituximab at varying concentrations. Shown above are the XICs for peptide labeled at the Lys residue. Peak heights of the spiked peptide, as measured by LC-MS, are used to estimate the the LOQ based on signal-to-noise ratio of response. When the blank analysis gives a result with a nonzero signal-to-noise ratio, the LOQ is defined as the analyte concentration corresponding to a signal-to-noise ratio that is 10 times the blank signal-to-noise ratio (see **Eq. S-2** below).[8, 9] From the data in this figure, we find that the LOQ is 2.82 nM, which is equivalent to a labeling level of 0.001%. These experiments were repeated three times.

Calculation of LOQ for the K labeled peptide (H_2N-VVSVLTVLHQDWLNGK*)

- **Blank measurement**

Peak height $H_{bl} = 5{,}101$ (noise)

- **LOQ determination for H_2N-VVSVLTVLHQDWLNGK***

LOQ is defined as the analyte concentration corresponding to the signal-to-noise ratio of 10.

Peak height $H_{LOQ} = 10H_{bl}$ (S-2)

$H_{LOQ} = (10 \times 5{,}101) = 51{,}010$

The closest data point that yield signal above H_{LOQ} from our surrogate peptide experiment is at

$X_{LOQ} = 2.82\ nM$

Peak area of this DEPC-modified peptide at 2.82 nM spiked = 20,250.23

Total peak area of unmodified and modified peptides at 2.82 nM spiked = 1,640,538,555.33

∴ The LOQ is at a labeling level = $\frac{20{,}250.23}{1{,}640{,}538{,}555.33} \times 100 = 0.001\%$

Calculation of LOQ for the N-terminally labeled peptide (H_2N*-VVSVLTVLHQDWLNGK)

(Raw data not shown here)

- **Blank measurement**

Peak height $H_{bl} = 56{,}723$ (noise)

- **LOQ determination for H_2N*-VVSVLTVLHQDWLNGK**

LOQ is defined as the analyte concentration corresponding to the signal-to-noise ratio of 10.

Peak height $H_{LOQ} = 10H_{bl}$ (S-2)

$H_{LOQ} = (10 \times 56{,}723) = 567{,}230$

The closest data point that yield signal above H_{LOQ} from our surrogate peptide experiment is at

$X_{LOQ} = 183\ nM$

Peak area of this DEPC-modified peptide at 183 nM spiked = 433,880.64

Total peak area of unmodified and modified peptides at 183 nM spiked = 1,644,219,196.06

∴ The LOQ is at a labeling level = $\frac{433{,}880.64}{1{,}644{,}219{,}196.06} \times 100 = 0.03\%$

DEPC CL-MS for probing subtle structural changes of rituximab

Table S1 DEPC modification percentages for individual residues of rituximab under native conditions and after heating to 45 °C for 4 h. A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n = 4). Error bars shown in a table are standard deviations. Color coding of the cells (introduced below) indicates the extent of covalent labeling (CL) change which falls within low (L), medium (M), and high (H) bins.

	High	Medium	Low	Low	Medium	High
Absolute difference (His, Lys)	≥ 20%	5% to 20%	≤ 5%	≥ -5%	-5% to -20%	≤ -20%
Ratio difference (Ser, Thr, Tyr)	≥ 70%	40% to 70%	≤ 40%	≥ -40%	-40% to -70%	≤ -70%
	Increased labeling				Decreased labeling	

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
Light chain - V_L domain					
S 5	0.03% ± 0.02%	0.03% ± 0.01%	No		
S 7					
S 12	0.07% ± 0.02%	0.05% ± 0.03%	No		
S 14	1.5% ± 0.7%	0.63% ± 0.30%	No		
K18	0.17% ± 0.11%	0.09% ± 0.09%	No		
T 20					
T 22					
S 26					
S 27	0.19% ± 0.10%	0.24% ± 0.29%	No		
S 28	0.04% ± 0.04%	0.10% ± 0.10%	No		
S 30					
Y 31	0.22% ± 0.13%	0.31% ± 0.32%	No		
H 33	0.51% ± 0.42%	0.09% ± 0.10%	No		
K 38	0.10% ± 0.06%	0.15% ± 0.08%	No		
S 41	0.01% ± 0.02%	0.01% ± 0.01%	No		
S 42	0.03% ± 0.02%	0.01% ± 0.01%	No		
K 44	0.13% ± 0.06%	0.13% ± 0.05%	No		
Y 48	0.05% ± 0.03%	0.07% ± 0.04%	No		
T 50	0.11% ± 0.05%	0.09% ± 0.07%	No		
S 51	0.05% ± 0.03%	0.03% ± 0.02%	No		
S 55	0.02% ± 0.02%	0.01% ± 0.00%	No		
S 62	3.6% ± 2.6%	1.8% ± 1.4%	No		
S 64	2.1% ± 1.8%	1.2% ± 1.0%	No		
S 66	1.3% ± 1.2%	0.79% ± 0.32%	No		
T 68	1.3% ± 1.2%	0.79% ± 0.32%	No		
S 69	0.02% ± 0.02%	0.02% ± 0.01%	No		
Y 70	0.02% ± 0.02%	0.02% ± 0.01%	No		
S 71	50% ± 7%	30% ± 19%	No		
T 73	7.5% ± 4.8%	5.3% ± 4.4%	No		
S 75	7.6% ± 4.9%	5.5% ± 4.6%	No		

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
T 84	0.08% ± 0.04%	0.07% ± 0.04%	No		
Y 85					
Y 86					
T 91					
S 92	1.7% ± 0.8%	3.7% ± 3.7%	No		
T 96	1.9% ± 0.8%	3.9% ± 3.7%	No		
T 101					
K 102	0.00% ± 0.00%	0.00% ± 0.00%	No		
K 106					
Light chain - C_L domain					
T 108					
S 113	0.02% ± 0.01%	0.14% ± 0.11%	No		
S 120					
K 125					
S 126	0.06% ± 0.10%	0.02% ± 0.01%	No		
T 128					
S 130					
Y 139					
K 144					
K 148	1.3% ± 1.5%	1.3% ± 0.8%	No		
S 155	4.5% ± 1.5%	9.3% ± 3.6%	Yes	+4.9% ± 3.9%	+109% ± 95%
S 158	4.5% ± 1.5%	9.3% ± 3.6%	Yes	+4.9% ± 3.9%	+109% ± 95%
S 161	4.5% ± 1.5%	9.3% ± 3.6%	Yes	+4.9% ± 3.9%	+109% ± 95%
T 163	0.61% ± 0.55%	0.89% ± 0.51%	No		
S 167	0.64% ± 0.58%	0.99% ± 0.56%	No		
K 168					
S 170					
T 171					
Y 172					
S 173					
S 175					
S 176					
T 177					
T 179	0.53% ± 0.96%	0.06% ± 0.06%	No		
S 181	0.97% ± 0.52%	0.31% ± 0.06%	Yes	-0.67% ± 0.53%	-68% ± 65%
K 182	16% ± 3%	8.0% ± 5.3%	No		
Y 185					
K 187	90% ± 4%	92% ± 4%	No		
H 188	78% ± 9%	87% ± 10%	No		
K 189	77% ± 9%	86% ± 9%	No		
Y 191	3.4% ± 1.3%	6.1% ± 4.8%	No		
T 196	3.4% ± 1.5%	4.9% ± 1.0%	No		
H 197	3.7% ± 1.6%	5.3% ± 0.9%	No		
S 201					
S 202					
T 205					
K 206	0.92% ± 0.39%	1.3% ± 0.6%	No		
S 207					
Heavy chain – V_H domain					
K 13	2.5% ± 1.7%	2.7% ± 1.2%	No		

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
S 17	2.4% ± 1.6%	2.6% ± 1.2%	No		
K 19	0.20% ± 0.06%	0.22% ± 0.07%	No		
S 21					
K 23	0.82% ± 0.70%	2.5% ± 2.8%	No		
S 25					
Y 27	43% ± 1%	44% ± 4%	No		
T 28	41% ± 2%	44% ± 3%	No		
T 30	21% ± 8%	17% ± 8%	No		
S 31	0.30% ± 0.35%	0.52% ± 0.34%	No		
Y 32	0.30% ± 0.35%	0.52% ± 0.34%	No		
H 35	90% ± 5%	84% ± 7%	No		
K 38	56% ± 17%	57% ± 13%	No		
T 40					
Y 52	0.35% ± 0.11%	0.26% ± 0.17%	No		
T 58					
S 59					
Y 60					
K 63	1.64% ± 0.64%	1.11% ± 0.58%	No		
K 65					
K 67	80% ± 11%	82% ± 15%	No		
T 69	4.0% ± 2.2%	25% ± 18%	No		
T 71	26% ± 5%	39% ± 21%	No		
K 74	32% ± 5%	36% ± 17%	No		
S 75					
S 76	26% ± 7%	6.5% ± 4.2%	Yes	-20% ± 8%	-76% ± 38%
S 77	14% ± 4%	2.3% ± 1.6%	Yes	-12% ± 4%	-84% ± 38%
T 78	0.55% ± 0.54%	0.45% ± 0.42%	No		
Y 80	1.1% ± 1.0%	1.4% ± 0.6%	No		
S 84	2.5% ± 0.4%	3.4% ± 5.8%	No		
S 85					
T 87					
S 88	3.6% ± 0.8%	4.4% ± 1.6%	No		
S 91					
Y 94	0.13% ± 0.08%	0.22% ± 0.12%	No		
Y 95	2.8% ± 1.7%	3.4% ± 1.7%	No		
S 99					
T 100					
Y 101					
Y 102					
Y 107					
T 115	0.11% ± 0.17%	0.57% ± 0.91%	No		
T 116	0.11% ± 0.17%	0.57% ± 0.91%	No		
T 118					
S 120	7.9% ± 4.4%	2.7% ± 0.9%	No		
Heavy chain – C_H1 domain					
S 123	9.5% ± 2.0%	5.6% ± 5.1%	No		
T 124	14% ± 4%	7.5% ± 6.0%	No		
K 125	42% ± 12%	25% ± 15%	No		
S 128	0.01% ± 0.01%	0.05% ± 0.03%	No		

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
S 135	0.05% ± 0.02%	0.25% ± 0.17%	No		
S 136	0.04% ± 0.02%	0.03% ± 0.02%	No		
K 137	0.05% ± 0.02%	0.22% ± 0.12%	Yes	+0.17% ± 0.13	
S 138	1.4% ± 0.9%	1.3% ± 0.7%	No		
T 139	0.03% ± 0.02%	0.02% ± 0.02%	No		
S 140	0.33% ± 0.19%	0.06% ± 0.03%	Yes	-0.26% ± 0.20%	-81% ± 77%
T 143	0.30% ± 0.18%	0.07% ± 0.04%	Yes	-0.24% ± 0.19%	-78% ± 78%
K 151					
Y 153	0.01% ± 0.00%	0.01% ± 0.01%	No		
T 159	4.6% ± 2.7%	2.8% ± 1.2%	No		
S 161	1.8% ± 0.5%	0.7% ± 0.8%	No		
S 164	14% ± 4%	8.2% ± 4.6%	No		
T 168	7.9% ± 3.8%	4.9% ± 3.2%	No		
S 169	0.38% ± 0.59%	0.53% ± 0.31%	No		
H 172	35% ± 4%	35% ± 8%	No		
T 173					
S 180	7.5% ± 1.6%	9.1% ± 10.6%	No		
S 181	7.3% ± 1.7%	8.9% ± 10.6%	No		
Y 184					
S 185					
S 187	0.86% ± 0.93%	1.2% ± 1.2%	No		
S 188	1.4% ± 1.1%	1.4% ± 1.3%	No		
T 191	1.5% ± 0.8%	0.39% ± 0.52%	No		
S 194	0.04% ± 0.02%	0.17% ± 0.10%	Yes	+0.13% ± 0.10%	+313% ± 281%
S 195	1.4% ± 0.7%	0.8% ± 0.5%	No		
S 196	1.6% ± 1.1%	0.95% ± 0.47%	No		
T 199					
T 201	0.55% ± 0.36%	2.4% ± 3.7%	No		
Y 202	0.55% ± 0.36%	2.4% ± 3.7%	No		
H 208	1.5% ± 0.2%	1.0% ± 0.7%	No		
K 209	7.8% ± 3.1%	6.4% ± 2.9%	No		
S 211	0.00% ± 0.00%	0.00% ± 0.00%	No		
T 213					
K 214	0.30% ± 0.26%	0.20% ± 0.18%	No		
K 217					
K 218					
K 222	100% ± 0%	100% ± 0%	No		
S 223	0.80% ± 0.56%	1.3% ± 0.8%	No		
Heavy chain – Hinge region and C_H2 domain					
K 226	39% ± 12%	73% ± 20%	Yes	+34% ± 23%	
T 227	0.07% ± 0.07%	0.14% ± 0.12%	No		
H 228	16% ± 8%	14% ± 3%	No		
T 229	16% ± 8%	14% ± 3%	No		
S 243	0.06% ± 0.05%	0.04% ± 0.03%	No		
K 250					
K 252	100% ± 0%	100% ± 0%	No		
T 254					
S 258					
T 260					

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
T 264	0.04% ± 0.03%	0.03% ± 0.02%	No		
S 271	5.1% ± 2.5%	4.2% ± 1.9%	No		
H 272	5.1% ± 2.4%	4.2% ± 1.9%	No		
K 278	0.13% ± 0.07%	0.16% ± 0.07%	No		
Y 282	0.08% ± 0.07%	0.06% ± 0.02%	No		
H 289	0.06% ± 0.05%	0.04% ± 0.01%	No		
K 292	0.01% ± 0.01%	0.01% ± 0.01%	No		
T 293	7.4% ± 2.8%	23% ± 12%	Yes	+16% ± 12%	+220% ± 188%
K 294	7.4% ± 2.8%	23% ± 12%	Yes	+16% ± 12%	
Y 300					
S 302	83% ± 4%	75% ± 17%	No		
T 303	83% ± 4%	75% ± 17%	No		
Y 304					
S 308					
T 311	1.6% ± 0.5%	1.2% ± 0.7%	No		
H 314	0.29% ± 0.10%	0.25% ± 0.20%	No		
K 321	4.7% ± 1.6%	3.3% ± 1.7%	No		
Y 323					
K 324	94% ± 5%	92% ± 3%	No		
K 326					
S 328					
K 330	100% ± 0%	100% ± 0%	No		
K 338	0.53% ± 0.14%	0.48% ± 0.28%	No		
T 339					
S 341	27% ± 3%	22% ± 8%	No		
K 342					
K 344					
Heavy chain – C_H3 domain					
Y 353	27% ± 16%	39% ± 25%	No		
T 354	0.01% ± 0.00%	0.01% ± 0.01%	No		
S 358	0.11% ± 0.05%	0.17% ± 0.08%	No		
T 363	0.12% ± 0.06%	0.19% ± 0.08%	No		
K 364	0.11% ± 0.05%	0.17% ± 0.09%	No		
S 368	0.03% ± 0.03%	0.04% ± 0.03%	No		
T 370	7.5% ± 1.3%	14% ± 4%	Yes	+6.5% ± 3.9%	+87% ± 54%
K 374	7.5% ± 1.3%	14% ± 4%	Yes	+6.5% ± 3.9%	
Y 377	0.63% ± 0.33%	0.65% ± 0.22%	No		
S 379					
S 387					
Y 395					
K 396	0.20% ± 0.10%	0.05% ± 0.02%	Yes	-0.15% ± 0.10%	
T 397					
T 398	9.6% ± 4.0%	6.4% ± 2.8%	No		
S 404	9.0% ± 4.0%	5.7% ± 2.9%	No		
S 407	1.6% ± 0.4%	0.9% ± 1.1%	No		
Y 411					
S 412					
K 413					
T 415					
K 418					

Residues	Native	Stressed 45 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
S 419					
S 428	0.13% ± 0.06%	0.14% ± 0.10%	No		
S 430	6.0% ± 0.8%	5.2% ± 1.0%	No		
H 433	13% ± 3%	15% ± 2%	No		
H 437	14% ± 2%	11% ± 2%	No		
H 439	1.4% ± 0.6%	1.4% ± 0.3%	No		
Y 440	0.00% ± 0.00%	0.05% ± 0.04%	No		
T 441					
K 443	1.1% ± 0.6%	1.3% ± 0.7%	No		
S 444					
S 446					
S 448	17% ± 2%	26% ± 4%	Yes	+9.1% ± 5.0%	+54% ± 31%
K 451					

Table S2 DEPC modification percentages for individual residues of rituximab under native conditions and after heating to 55 °C for 4 h. A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n = 3). Error bars shown in a table are standard deviations. Color coding of the cells (introduced below) indicates the extent of covalent labeling (CL) change which falls within low (L), medium (M), and high (H) bins.

	High	Medium	Low	Low	Medium	High
Absolute difference (His, Lys)	≥ 20%	5% to 20%	≤ 5%	≥ -5%	-5% to -20%	≤ -20%
Ratio difference (Ser, Thr, Tyr)	≥ 70%	40% to 70%	≤ 40%	≥ -40%	-40% to -70%	≤ -70%
	Increased labeling					Decreased labeling

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
Light chain - V_L domain					
S 5	0.03% ± 0.01%	0.04% ± 0.00%	No		
S 7					
S 12	0.03% ± 0.01%	0.02% ± 0.01%	No		
S 14					
K18	0.56% ± 0.06%	0.66% ± 0.22%	No		
T 20	17% ± 4%	8% ± 2%	Yes	-10% ± 4%	-56% ± 26%
T 22					
S 26					
S 27					
S 28					
S 30					
Y 31					
H 33	0.08% ± 0.02%	0.21% ± 0.02%	Yes	+0.14% ± 0.03%	
K 38	0.15% ± 0.05%	0.24% ± 0.02%	No		
S 41	0.04% ± 0.01%	0.02% ± 0.02%	No		
S 42					
K 44	0.06% ± 0.02%	0.15% ± 0.01%	Yes	+0.08% ± 0.02%	
Y 48	0.09% ± 0.04%	0.13% ± 0.01%	No		
T 50	0.03% ± 0.03%	0.05% ± 0.01%	No		
S 51					
S 55					
S 62					
S 64					
S 66	18% ± 4%	15% ± 2%	No		
T 68	18% ± 4%	15% ± 2%	No		
S 69					
Y 70	14% ± 5%	11% ± 4%	No		
S 71					
T 73	53% ± 14%	86% ± 3%	Yes	+32% ± 15%	+61% ± 32%
S 75	53% ± 14%	86% ± 3%	Yes	+32% ± 15%	+61% ± 32%
T 84	1.3% ± 0.2%	2.6% ± 0.5%	Yes	+1.3% ± 0.6%	+103% ± 46%
Y 85	1.3% ± 0.2%	2.6% ± 0.5%	Yes	+1.3% ± 0.5%	+100% ± 43%

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
Y 86					
T 91	0.03% ± 0.02%	0.10% ± 0.01%	Yes	+0.07% ± 0.02%	+225% ± 196%
S 92	1.8% ± 0.6%	2.1% ± 0.6%	No		
T 96	2.0% ± 0.6%	2.2% ± 0.6%	No		
T 101	0.18% ± 0.01%	0.14% ± 0.02%	No		
K 102	0.08% ± 0.02%	0.10% ± 0.01%	No		
K 106					
Light chain - C_L domain					
T 108	1.3% ± 0.4%	1.1% ± 0.3%	No		
S 113	1.0% ± 0.3%	0.8% ± 0.1%	No		
S 120					
K 125					
S 126					
T 128	5.4% ± 1.0%	3.1% ± 0.3%	Yes	-2.3% ± 1.1%	-42.4% ± 21.7%
S 130	5.4% ± 1.0%	3.1% ± 0.3%	Yes	-2.3% ± 1.1%	-42.4% ± 21.7%
Y 139					
K 144					
K 148	7.2% ± 1.7%	9.7% ± 0.8%	No		
S 155					
S 158					
S 161					
T 163	0.00% ± 0.00%	0.01% ± 0.01%	No		
S 167					
K 168	0.48% ± 0.19%	0.47% ± 0.16%	No		
S 170					
T 171					
Y 172					
S 173					
S 175					
S 176					
T 177					
T 179					
S 181					
K 182	5.0% ± 2.1%	19% ± 10%	No		
Y 185	23.5% ± 0.3%	27% ± 2%	Yes	+4% ± 2%	+16% ± 8%
K 187	85% ± 3%	86% ± 4%	No		
H 188	55% ± 2%	64% ± 3%	Yes	+10% ± 4%	
K 189	60% ± 3%	66% ± 3%	No		
Y 191	0.74% ± 0.09%	0.98% ± 0.33%	No		
T 196					
H 197	0.11% ± 0.03%	0.16% ± 0.03%	No		
S 201					
S 202					
T 205					
K 206	0.37% ± 0.13%	0.38% ± 0.10%	No		
S 207	4.2% ± 2.3%	2.8% ± 2.0%	No		
Heavy chain – V_H domain					
K 13	2.7% ± 0.5%	3.7% ± 0.3%	Yes	+1.0% ± 0.6%	
S 17					
K 19	3.5% ± 0.7%	4.5% ± 0.4%	No		

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
S 21	24% ± 4%	11% ± 4%	Yes	-13% ± 6%	-55% ± 27%
K 23	86% ± 5%	92% ± 4%	No		
S 25					
Y 27					
T 28					
T 30					
S 31					
Y 32	0.05% ± 0.03%	0.40% ± 0.26%	No		
H 35	23% ± 3%	4% ± 1%	Yes	-19% ± 3%	
K 38	30% ± 2%	22% ± 3%	Yes	-8% ± 3%	
T 40					
Y 52	1.1% ± 0.2%	1.3% ± 0.2%	No		
T 58	0.12% ± 0.01%	0.15% ± 0.02%	No		
S 59	0.09% ± 0.01%	0.09% ± 0.03%	No		
Y 60	0.09% ± 0.01%	0.09% ± 0.03%	No		
K 63					
K 65					
K 67	23% ± 7%	33% ± 2%	No		
T 69					
T 71					
K 74	79% ± 4%	87% ± 1%	Yes	+8% ± 4%	
S 75					
S 76	2.4% ± 0.2%	1.5% ± 0.2%	Yes	-0.8% ± 0.3%	-35% ± 13%
S 77	0.01% ± 0.01%	0.01% ± 0.01%	No		
T 78	0.28% ± 0.10%	0.26% ± 0.23%	No		
Y 80					
S 84	1.8% ± 0.7%	1.4% ± 0.6%	No		
S 85	0.32% ± 0.06%	0.28% ± 0.14%	No		
T 87					
S 88	0.02% ± 0.01%	0.01% ± 0.01%	No		
S 91	0.25% ± 0.04%	0.23% ± 0.09%	No		
Y 94	0.25% ± 0.04%	0.23% ± 0.09%	No		
Y 95					
S 99					
T 100					
Y 101					
Y 102					
Y 107					
T 115	14% ± 4%	12% ± 1%	No		
T 116					
T 118	71% ± 3%	59% ± 7%	Yes	-12% ± 7%	-17% ± 11%
S 120	84% ± 2%	70% ± 7%	Yes	-14% ± 7%	-17% ± 9%
Heavy chain – C_H1 domain					
S 123	1.2% ± 0.3%	1.6% ± 0.2%	No		
T 124					
K 125					
S 128	0.92% ± 0.44%	1.4% ± 1.0%	No		
S 135	1.1% ± 0.5%	1.3% ± 1.1%	No		
S 136					
K 137					

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
S 138					
T 139	0.13% ± 0.07%	0.12% ± 0.04%	No		
S 140	0.03% ± 0.02%	0.02% ± 0.01%	No		
T 143	0.01% ± 0.01%	0.01% ± 0.01%	No		
K 151					
Y 153	0.02% ± 0.02%	0.07% ± 0.05%	No		
T 159					
S 161	1.8% ± 0.2%	2.2% ± 0.4%	No		
S 164	0.002% ± 0.001%	0.016% ± 0.005%	Yes	+0.014% ± 0.005%	+890% ± 849%
T 168	1.3% ± 0.4%	2.4% ± 1.2%	No		
S 169	0.23% ± 0.13%	0.35% ± 0.14%	No		
H 172	0.23% ± 0.13%	0.35% ± 0.14%	No		
T 173	1.1% ±0.2%	2.2% ± 0.7%	No		
S 180					
S 181					
Y 184					
S 185					
S 187	10% ± 3%	11% ± 2%	No		
S 188	10% ± 3%	10% ± 2%	No		
T 191	4.6% ± 1.1%	4.3% ± 0.7%	No		
S 194					
S 195					
S 196	0.01% ± 0.00%	0.05% ± 0.03%	No		
T 199	0.01% ± 0.00%	0.05% ± 0.03%	No		
T 201					
Y 202					
H 208	0.00% ± 0.00%	0.00% ± 0.00%	No		
K 209	6.4% ± 2.0%	8.0% ± 0.9%	No		
S 211	0.00% ± 0.00%	0.00% ± 0.00%	No		
T 213					
K 214	0.50% ± 0.38%	0.40% ± 0.12%	No		
K 217					
K 218					
K 222	99% ± 1%	100% ± 0%	No		
S 223	0.06% ± 0.04%	0.04% ± 0.00%	No		
Heavy chain – Hinge region and C_H2 domain					
K 226	33% ± 7%	72% ± 5%	Yes	+39% ± 8%	
T 227	1.4% ± 0.8%	1.6% ± 1.2%	No		
H 228	36% ± 7%	43% ± 15%	No		
T 229	36% ± 7%	42% ± 14%	No		
S 243					
K 250					
K 252	100% ± 0%	100% ± 0%	No		
T 254					
S 258	5.2% ± 1.8%	3.1% ± 0.8%	No		
T 260	0.70% ± 0.58%	0.81% ± 0.33%	No		
T 264	11% ± 2%	16% ± 11%	No		
S 271	10% ± 1%	15% ± 11%	No		
H 272	0.26% ± 0.08%	0.24% ± 0.06%	No		

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
K 278	0.16% ± 0.10%	0.28% ± 0.07%	No		
Y 282	0.39% ± 0.02%	0.33% ± 0.04%	No		
H 289	0.49% ± 0.13%	0.41% ± 0.03%	No		
K 292					
T 293	38% ± 6%	85% ± 8%	Yes	+46% ± 10%	+122% ± 32%
K 294					
Y 300	50% ± 1%	56% ± 7%	No		
S 302	38% ± 3%	16% ± 6%	Yes	-22% ± 7%	-58% ± 18%
T 303					
Y 304					
S 308					
T 311					
H 314	0.24% ± 0.08%	0.23% ± 0.06%	No		
K 321	0.29% ± 0.04%	0.28% ± 0.01%	No		
Y 323					
K 324					
K 326					
S 328					
K 330	100% ± 0%	100% ± 0%	No		
K 338	2.4% ± 1.7%	4.4% ± 0.5%	No		
T 339					
S 341					
K 342					
K 344					
Heavy chain – C_H3 domain					
Y 353	0.44% ± 0.62%	14% ± 2%	Yes	+14% ± 2%	+3118% ± 4418%
T 354					
S 358					
T 363	0.006% ± 0.002%	0.012% ± 0.003%	Yes	+0.006% ± 0.003%	+111% ± 67%
K 364	0.15% ± 0.07%	0.29% ± 0.21%	No		
S 368	0.02% ± 0.02%	0.18% ± 0.01%	Yes	+0.16% ± 0.03%	+1018% ± 1537%
T 370					
K 374	4.1% ± 1.6%	7.8% ± 2.0%	No		
Y 377	11% ± 2%	12% ± 7%	No		
S 379	13% ± 2%	13% ± 7%	No		
S 387	11% ± 2%	12% ± 7%	No		
Y 395	2.2% ± 0.8%	2.4% ± 1.0%	No		
K 396	0.82% ± 0.22%	0.58% ± 0.27%	No		
T 397	0.87% ± 0.29%	1.0% ± 0.2%	No		
T 398	12% ± 1%	17% ± 3%	No		
S 404	5.2% ± 1.3%	4.8% ± 1.8%	No		
S 407	15% ± 4%	30% ± 6%	Yes	+15% ± 7%	+101% ± 52%
Y 411	7.5% ± 5.1%	20% ± 4%	Yes	+12% ± 6%	+163% ± 138%
S 412					
K 413					
T 415					
K 418	29% ± 24%	46% ± 4%	No		
S 419	19% ± 15%	33% ± 2%	No		
S 428	0.86% ± 0.08%	1.2% ± 0.4%	No		
S 430	6.8% ± 2.2%	12% ± 2%	No		

Residues	Native	Stressed 55 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
H 433	15% ± 2%	13% ± 4%	No		
H 437	5.8% ± 2.9%	7.4% ± 0.9%	No		
H 439	5.4% ± 0.9%	4.2% ± 3.2%	No		
Y 440	0.02% ± 0.01%	0.02% ± 0.01%	No		
T 441					
K 443	0.47% ± 0.24%	0.35% ± 0.07%	No		
S 444					
S 446					
S 448	71% ± 12%	92% ± 4%	Yes	+22% ± 13%	+30% ± 18%
K 451	41% ± 16%	79% ± 7%	Yes	+37% ± 18%	

Validation of DEPC CL-MS data using rituximab activity assays

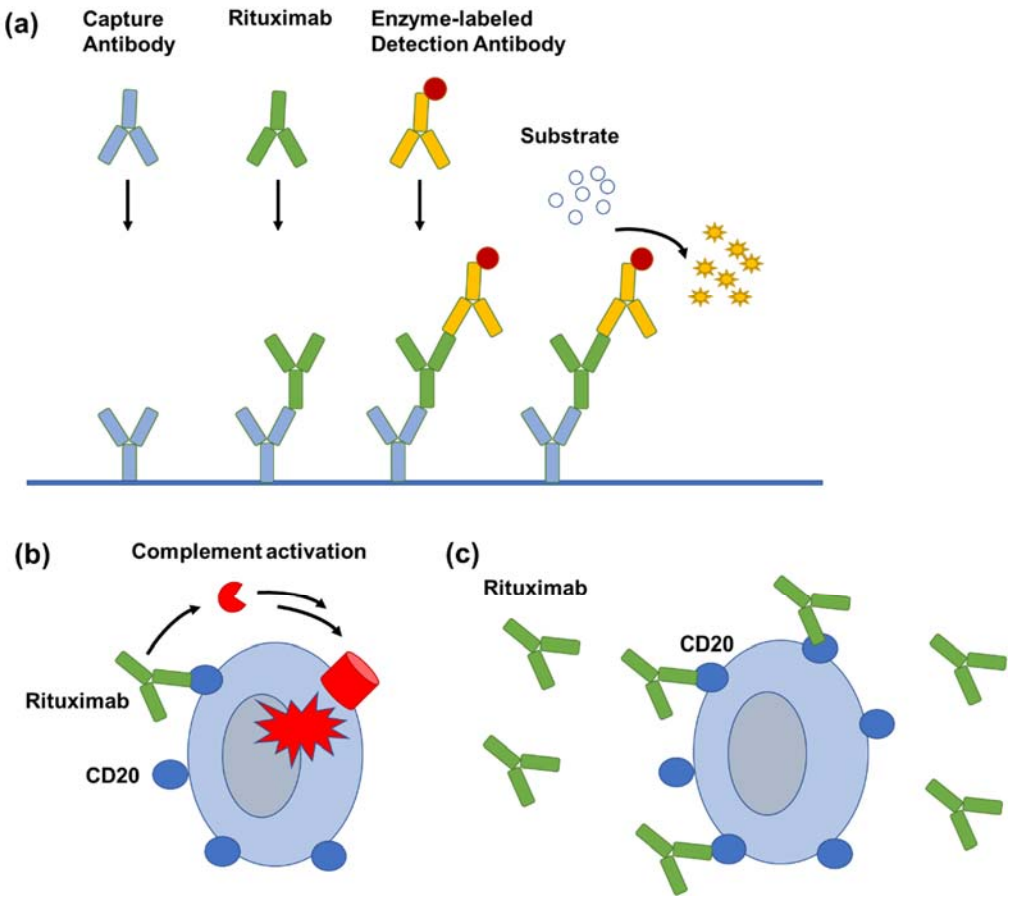

Figure S5 Schematics of three types of activity assays used in this study. (a) The pharmacokinetic bridging format of the ELISA was used to evaluate binding of the F_c region to a capture antibody. The F_c of rituximab was first bound to an anti-human capture antibody, and the F_{ab} was then bound to an anti-rituximab detection antibody. (b) Complement-dependent cytotoxicity (CDC) activity of the F_c region was assessed using an Alamar blue assay. In this assay, Raji B cells were incubated with control or stressed rituximab. Rituximab binds to CD20 antigen expressed of cell surface, and cytotoxicity can be exerted by F_c after a normal human serum complement is added. CDC activity was then measured via a cell viability assay, allowing it to act as a functional indicator of the HOS of the F_c region. (c) The functional activity of F_{ab} binding to its antigen CD20 was measured using a Raji cell pull-down assay. A study has shown that rituximab is bound to the surface of CD20 cells *in vitro*.[3] In this experiment, CD20-positive Raji B cells were treated with control or stressed rituximab without human serum added, and the unbound rituximab was then quantified using ELISA This assay could measure binding activity of the F_{ab} region to its CD20 antigen on the cell surface.

Investigation of conformation changes upon higher heat stress using DEPC CL-MS

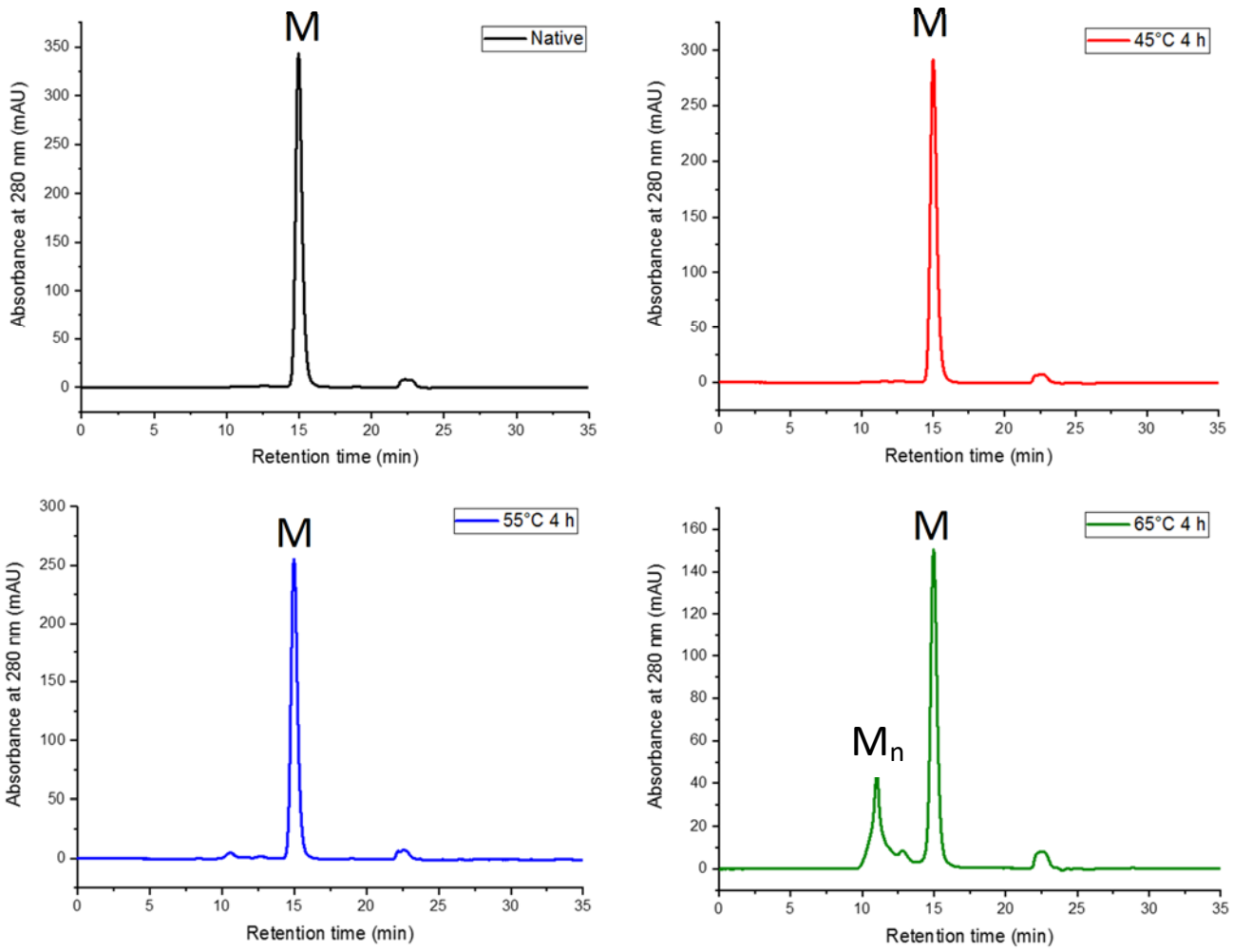

Figure S6 Size-exclusion chromatography (SEC) of rituximab at 37 °C (native, black) and thermally-stressed conditions, after incubation of the rituximab formulation at 45 °C for 4 h (red), 55 °C for 4 h (blue), and 65 °C for 4 h (green). After heating at 65 °C, the chromatogram of the stressed rituximab indicates the significant formation of high molecular weight species (i.e. M_n), whereas the other temperatures show almost exclusively monomeric rituximab (i.e. M).

Table S3 DEPC modification percentages for individual residues of rituximab under native conditions and after heating to 65 °C for 4 h. A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n = 3). Error bars shown in a table are standard deviations. Color coding of the cells (introduced below) indicates the extent of covalent labeling (CL) change which falls within low (L), medium (M), and high (H) bins.

	High	Medium	Low	Low	Medium	High
Absolute difference (His, Lys)	≥ 20%	5% to 20%	≤ 5%	≥ -5%	-5% to -20%	≤ -20%
Ratio difference (Ser, Thr, Tyr)	≥ 70%	40% to 70%	≤ 40%	≥ -40%	-40% to -70%	≤ -70%
	Increased labeling					Decreased labeling

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
Light chain - V_L domain					
S 5	0.03% ± 0.01%	0.01% ± 0.01%	Yes	-0.02% ± 0.01%	-62% ± 40%
S 7					
S 12	0.03% ± 0.01%	0.01% ± 0.01%	No		
S 14					
K18	0.56% ± 0.06%	0.33% ± 0.27%	No		
T 20	17% ± 4%	13% ± 8%	No		
T 22					
S 26					
S 27					
S 28					
S 30					
Y 31					
H 33	0.08% ± 0.02%	0.61% ± 0.49%	No		
K 38	0.15% ± 0.05%	0.22% ± 0.06%	No		
S 41	0.04% ± 0.01%	0.12% ± 0.04%	No		
S 42					
K 44	0.06% ± 0.02%	0.09% ± 0.04%	No		
Y 48	0.09% ± 0.04%	0.06% ± 0.01%	No		
T 50	0.03% ± 0.03%	0.00% ± 0.00%	No		
S 51					
S 55					
S 62					
S 64					
S 66	18% ± 4%	12% ± 4%	No		
T 68	18% ± 4%	12% ± 4%	No		
S 69					
Y 70	14% ± 5%	1.8% ± 2.3%	Yes	-12% ± 5%	-87% ± 48%
S 71					
T 73	53% ± 14%	73% ± 13%	No		
S 75	53% ± 14%	73% ± 13%	No		
T 84	1.3% ± 0.2%	2.9% ± 1.3%	No		
Y 85	1.3% ± 0.2%	2.9% ± 1.3%	No		
Y 86					

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
T 91	0.03% ± 0.02%	0.01% ± 0.01%	No		
S 92	1.8% ± 0.6%	4.3% ± 2.2%	No		
T 96	2.0% ± 0.6%	4.3% ± 2.1%	No		
T 101	0.18% ± 0.01%	0.07% ± 0.05%	Yes	-0.11% ± 0.05	-60% ± 30%
K 102	0.08% ± 0.02%	0.04% ± 0.02%	No		
K 106					
Light chain - C_L domain					
T 108	1.3% ± 0.4%	0.66% ± 0.32%	No		
S 113	1.0% ± 0.3%	0.52% ± 0.26%	No		
S 120					
K 125					
S 126					
T 128	5.4% ± 1.0%	3.2% ± 1.9%	No		
S 130	5.4% ± 1.0%	3.2% ± 1.9%	No		
Y 139					
K 144					
K 148	7.2% ± 1.7%	4.9% ± 1.0%	No		
S 155					
S 158					
S 161					
T 163	0.00% ± 0.00%	0.00% ± 0.00%	No		
S 167					
K 168	0.48% ± 0.19%	0.40% ± 0.21%	No		
S 170					
T 171					
Y 172					
S 173					
S 175					
S 176					
T 177					
T 179					
S 181					
K 182	5.0% ± 2.1%	13% ± 8%	No		
Y 185	23.5% ± 0.3%	25% ± 4%	No		
K 187	85% ± 3%	78% ± 4%	No		
H 188	55% ± 2%	62% ± 8%	No		
K 189	60% ± 3%	65% ± 6%	No		
Y 191	0.74% ± 0.09%	0.98% ± 0.24%	No		
T 196					
H 197	0.11% ± 0.03%	0.57% ± 0.22%	Yes	+0.46% ± 0.23%	
S 201					
S 202					
T 205					
K 206	0.37% ± 0.13%	0.61% ± 0.22%	No		
S 207	4.2% ± 2.3%	2.7% ± 0.6%	No		
Heavy chain – V_H domain					
K 13	2.7% ± 0.5%	3.5% ± 1.4%	No		
S 17					
K 19	3.5% ± 0.7%	3.4% ± 1.5%	No		
S 21	24% ± 4%	12% ± 8%	No		

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
K 23	86% ± 5%	96% ± 5%	No		
S 25					
Y 27					
T 28					
T 30					
S 31					
Y 32	0.05% ± 0.03%	0.27% ± 0.20%	No		
H 35	23% ± 3%	11% ± 3%	Yes	-12% ± 4%	
K 38	30% ± 2%	38% ± 10%	No		
T 40					
Y 52	1.1% ± 0.2%	1.4% ± 0.2%	No		
T 58	0.12% ± 0.01%	0.21% ± 0.15%	No		
S 59	0.09% ± 0.01%	0.19% ± 0.16%	No		
Y 60	0.09% ± 0.01%	0.19% ± 0.16%	No		
K 63					
K 65					
K 67	23% ± 7%	70% ± 17%	Yes	+48% ± 19%	
T 69					
T 71					
K 74	79% ± 4%	52% ± 8%	Yes	-27% ± 9%	
S 75					
S 76	2.4% ± 0.2%	4.0% ± 1.2%	No		
S 77	0.01% ± 0.01%	0.03% ± 0.01%	Yes	+0.02% ± 0.01%	+165% ± 127%
T 78	0.28% ± 0.10%	0.16% ± 0.09%	No		
Y 80					
S 84	1.8% ± 0.7%	1.3% ± 0.5%	No		
S 85	0.32% ± 0.06%	0.26% ± 0.09%	No		
T 87					
S 88	0.02% ± 0.01%	0.03% ± 0.01%	No		
S 91	0.25% ± 0.04%	0.21% ± 0.06%	No		
Y 94	0.25% ± 0.04%	0.21% ± 0.06%	No		
Y 95					
S 99					
T 100					
Y 101					
Y 102					
Y 107					
T 115	14% ± 4%	14% ± 5%	No		
T 116					
T 118	71% ± 3%	55% ± 5%	Yes	-16% ± 6%	-22% ± 9%
S 120	84% ± 2%	69% ± 4%	Yes	-15% ± 4%	-18% ± 5%
Heavy chain – C_H1 domain					
S 123	1.2% ± 0.3%	1.2% ± 0.2%	No		
T 124					
K 125					
S 128	0.92% ± 0.44%	0.30% ± 0.16%	No		
S 135	1.1% ± 0.5%	0.35% ± 0.34%	No		
S 136					
K 137					
S 138					

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
T 139	0.13% ± 0.07%	0.23% ± 0.10%	No		
S 140	0.03% ± 0.02%	0.05% ± 0.01%	No		
T 143	0.01% ± 0.01%	0.02% ± 0.01%	No		
K 151					
Y 153	0.02% ± 0.02%	0.01% ± 0.01%	No		
T 159					
S 161	1.8% ± 0.2%	1.2% ± 0.2%	Yes	-0.66% ± 0.31%	-36% ± 18%
S 164	0.00% ± 0.00%	0.02% ± 0.02%	No		
T 168	1.3% ± 0.4%	2.2% ± 1.5%	No		
S 169	0.23% ± 0.13%	0.23% ± 0.18%	No		
H 172	0.23% ± 0.13%	0.23% ± 0.18%	No		
T 173	1.1% ±0.2%	1.9% ± 0.9%	No		
S 180					
S 181					
Y 184					
S 185					
S 187	10% ± 3%	11% ± 2%	No		
S 188	10% ± 3%	11% ± 2%	No		
T 191	4.6% ± 1.1%	4.4% ± 0.1%	No		
S 194					
S 195					
S 196	0.01% ± 0.00%	0.08% ± 0.02%	Yes	+0.07% ± 0.02%	+728% ± 276%
T 199	0.01% ± 0.00%	0.05% ± 0.02%	Yes	+0.05% ± 0.02%	+689% ± 396%
T 201					
Y 202					
H 208	0.00% ± 0.00%	0.04% ± 0.01%	Yes	+0.04% ± 0.01%	
K 209	6.4% ± 2.0%	4.0% ± 1.0%	No		
S 211	0.00% ± 0.00%	0.21% ± 0.02%	Yes	+0.21% ± 0.02%	+6074% ± 5773%
T 213					
K 214	0.50% ± 0.38%	0.19% ± 0.12%	No		
K 217					
K 218					
K 222	99% ± 1%	98% ± 2%	No		
S 223	0.06% ± 0.04%	0.05% ± 0.01%	No		
Heavy chain – Hinge region and C_H2 domain					
K 226	33% ± 7%	39% ± 17%	No		
T 227	1.4% ± 0.8%	1.2% ± 0.8%	No		
H 228	36% ± 7%	40% ± 12%	No		
T 229	36% ± 7%	40% ± 12%	No		
S 243					
K 250					
K 252	100% ± 0%	100% ± 0%	No		
T 254					
S 258	5.2% ± 1.8%	1.2% ± 1.5%	Yes	-4.0% ± 2.3%	-77% ± 52%
T 260	0.70% ± 0.58%	0.16% ± 0.10%	No		
T 264	11% ± 2%	9.6% ± 2.3%	No		
S 271	10% ± 1%	9.3% ± 2.2%	No		
H 272	0.26% ± 0.08%	0.19% ± 0.10	No		
K 278	0.16% ± 0.10%	0.24% ± 0.03%	No		

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
Y 282	0.39% ± 0.02%	0.39% ± 0.08%	No		
H 289	0.49% ± 0.13%	0.34% ± 0.23%	No		
K 292					
T 293	38% ± 6%	82% ± 27%	No		
K 294					
Y 300	50% ± 1%	30% ± 13%	No		
S 302	38% ± 3%	10% ± 16%	Yes	-28% ± 16%	-74% ± 43%
T 303					
Y 304					
S 308					
T 311					
H 314	0.24% ± 0.08%	0.20% ± 0.03%	No		
K 321	0.29% ± 0.04%	0.37% ± 0.06%	No		
Y 323					
K 324					
K 326					
S 328					
K 330	100% ± 0%	100% ± 0%	No		
K 338	2.4% ± 1.7%	1.5% ± 0.4%	No		
T 339					
S 341					
K 342					
K 344					
Heavy chain – C_H3 domain					
Y 353	0.44% ± 0.62%	0.00% ± 0.00%	No		
T 354					
S 358					
T 363	0.006% ± 0.002%	0.002% ± 0.001%	Yes	-0.004 ± 0.002	-66% ± 40%
K 364	0.15% ± 0.07%	0.10% ± 0.08%	No		
S 368	0.02% ± 0.02%	0.00% ± 0.00%	No		
T 370					
K 374	4.1% ± 1.6%	3.5% ± 2.4%	No		
Y 377	11% ± 2%	8.5% ± 4.9%	No		
S 379	13% ± 2%	10% ± 5%	No		
S 387	11% ± 2%	8.5% ± 4.9%	No		
Y 395	2.2% ± 0.8%	2.3% ± 0.3%	No		
K 396	0.82% ± 0.22%	0.73% ± 0.19%	No		
T 397	0.87% ± 0.29%	1.0% ± 0.2%	No		
T 398	12% ± 1%	16% ± 10%	No		
S 404	5.2% ± 1.3%	4.9% ± 2.7%	No		
S 407	15% ± 4%	23% ± 12%	No		
Y 411	7.5% ± 5.1%	13% ± 6%	No		
S 412					
K 413					
T 415					
K 418	29% ± 24%	36% ± 18%	No		
S 419	19% ± 15%	25% ± 15%	No		
S 428	0.86% ± 0.08%	0.41% ± 0.23%	Yes	-0.45% ± 0.24%	-52% ± 29%
S 430	6.8% ± 2.2%	8.1% ± 4.6%	No		
H 433	15% ± 2%	16% ± 3%	No		

Residues	Native	Stressed 65 °C 4 h	Significant?	Absolute difference (H, K)	Ratio difference (S, T, Y)
H 437	5.8% ± 2.9%	5.2% ± 0.9%	No		
H 439	5.4% ± 0.9%	2.5% ± 0.3%	Yes	-2.9% ± 0.9%	
Y 440	0.02% ± 0.01%	0.01% ± 0.01%	Yes	-0.01% ± 0.01%	-60% ± 42%
T 441					
K 443	0.47% ± 0.24%	0.25% ± 0.15%	No		
S 444					
S 446					
S 448	71% ± 12%	47% ± 9%	No		
K 451	41% ± 16%	5.1% ± 5.4%	Yes	-36% ± 17%	

Discussion: Subtle structural changes of rituximab as revealed by DEPC CL-MS

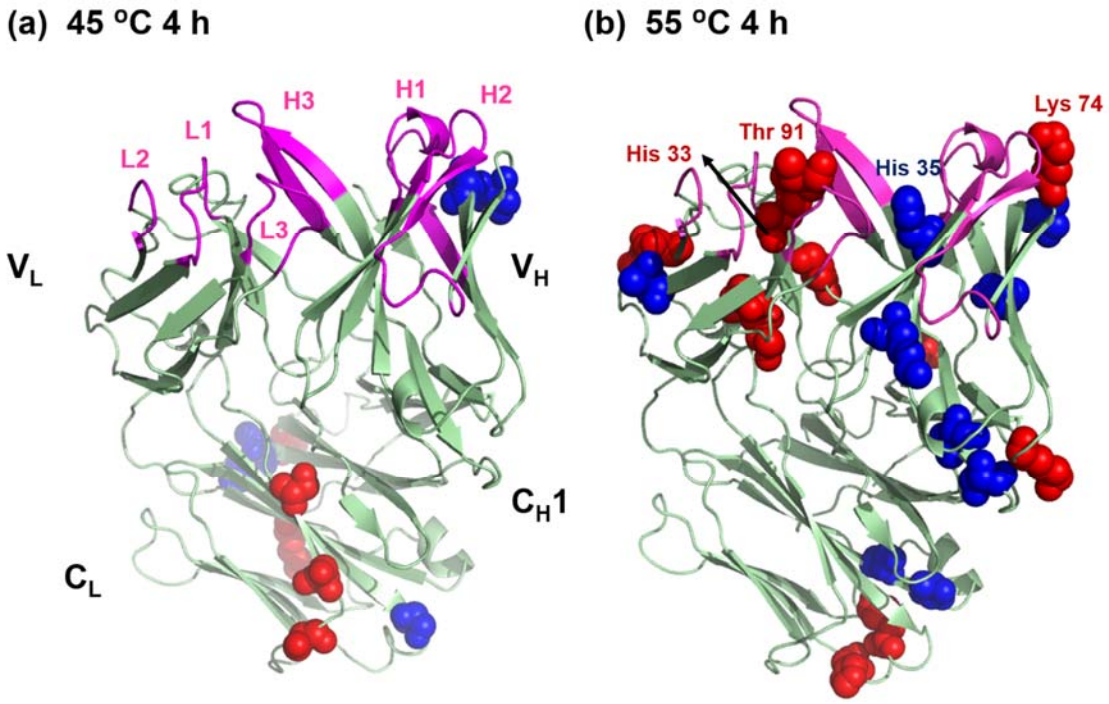

Figure S7 Illustration of rituximab's F_{ab} domain highlighting structural changes that may affect CDRs after heat stress at (a) 45 °C for 4 h and (b) 55 °C for 4 h. Red represents the increase in label level while blue represents the decrease in label level. Six CDRs of rituximab (H1-3 in heavy chain and L1-3 in light chain) have been previously reported in Du *et al.*,[10] and they are highlighted in magenta. Thr91 (light chain), His33 (light chain), His35 and Lys74 (heavy chain) which sit in or nearby CDRs are found to undergo significant changes in labeling after heat stress at 55 °C. (PDB accession code 4KAQ)

Discussion: HOS changes and aggregation of rituximab as revealed by DEPC CL-MS

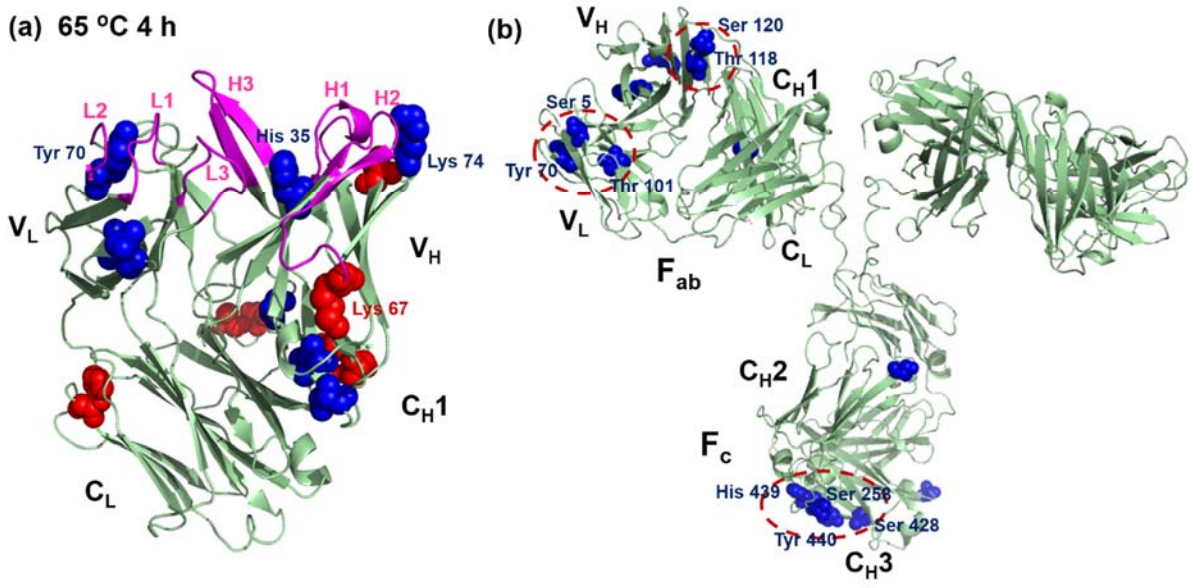

Figure S8 Illustration of rituximab structure highlighting structural changes that may affect CDRs and the possible aggregation sites after heat stress at 65 °C for 4 h. (a) Cartoon representation of the F_{ab} domain after the heat stress. Red represents the increase in label level while blue represents the decrease in label level. Six CDRs of rituximab (H1-3 in heavy chain and L1-3 in light chain) have been previously reported in Du *et al.*,[10] and they are highlighted in magenta. Tyr70 (light chain), His35, Lys67, and Lys74 (heavy chain), which sit nearby CDRs, are found to undergo significant changes in labeling after heat stress at 65 °C. (PDB accession code 4KAQ) (b) Cartoon representation of possible aggregation sites in the F_{ab} and F_c regions. Only decreases in labeling are shown in this figure. Those residues cluster to each other with a distance less than 12 Å. Note that only one asymmetric unit of rituximab structure is labeled in this figure. (See **Fig. 3** for more details about rituximab's molecular model.)

SUPPLEMENTAL REFERENCES

1. Myers JK, Pace CN, Scholtz JM. Helix Propensities Are Identical in Proteins and Peptides. Biochemistry 1997; 36:10923-9.
2. Zhang H, Song L, Ye H, Hu L, Liang W, Liu D. Characterization of a Novel Humanized Anti-CD20 Antibody with Potent Anti-Tumor Activity against Non-Hodgkin's Lymphoma. Cellular Physiology and Biochemistry 2013; 32:645-54.
3. Cragg MS, Bayne MB, Tutt AL, French RR, Beers S, Glennie MJ, et al. A new anti-idiotype antibody capable of binding rituximab on the surface of lymphoma cells. Blood 2004; 104:2540.
4. Hampson G, Ward TH, Cummings J, Bayne M, Tutt AL, Cragg MS, et al. Validation of an ELISA for the determination of rituximab pharmacokinetics in clinical trials subjects. Journal of Immunological Methods 2010; 360:30-8.
5. Borotto NB, Zhou Y, Hollingsworth SR, Hale JE, Graban EM, Vaughan RC, et al. Investigating Therapeutic Protein Structure with Diethylpyrocarbonate Labeling and Mass Spectrometry. Analytical Chemistry 2015; 87:10627-34.
6. Mendoza VL, Vachet RW. Protein Surface Mapping Using Diethylpyrocarbonate with Mass Spectrometric Detection. Analytical Chemistry 2008; 80:2895-904.
7. Liu T, Marcinko TM, Kiefer PA, Vachet RW. Using Covalent Labeling and Mass Spectrometry To Study Protein Binding Sites of Amyloid Inhibiting Molecules. Analytical Chemistry 2017; 89:11583-91.
8. Miller JN, Miller JC. Statistics and chemometrics for analytical chemistry. Harlow, England, United Kingdom: Pearson/Prentice Hall, 2005.
9. Shrivastava A, Gupta V. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of Young Scientists 2011; 2:21-5.
10. Du J, Wang H, Zhong C, Peng B, Zhang M, Li B, et al. Structural Basis for Recognition of CD20 by Therapeutic Antibody Rituximab. Journal of Biological Chemistry 2007; 282:15073-80.