1 **Ligand-bound forced degradation as a strategy to generate functionally relevant**
2 **analytical challenge materials for assessment of CQAs**

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Abstract

Therapeutic monoclonal antibodies (mAbs) contain a variety of amino acids that are susceptible to enzymatic, chemical, and physical modifications. These modifications can happen throughout production, purification, formulation, and storage and many are known to affect the biological activity of a mAb. Methods that are able to characterize and evaluate these attributes are critical in order to understand how they might alter biological activity. Methods capable of site-specific monitoring of these critical quality attributes are extremely valuable to biopharmaceutical research but also require well-defined materials with site-specific attribute modifications. Here, we describe the development and application of a strategy to generate functionally relevant analytical challenge materials that have unique site-specific attributes. This method involves the use of a ligand that is bound to the mAb during oxidative stress resulting in unique oxidation patterns with some methionine residues protected while others are exposed to oxidation. These unique materials were used to develop a rapid surface plasmon resonance (SPR) assay that could rapidly detect methionine oxidation in both the Fab and Fc regions using specific molecular probes. The addition of uniquely oxidized materials to our data set enabled us to determine specific methionine residues vital to binding. Further analysis showed that antibody oxidation could also be rapidly detected in multiple domains from qualitative thermal melting using intrinsic tryptophan fluorescence. Methionine oxidation of an antibody was explored in this study, but we envision this method could be useful to explore structure function relationships of a variety of antibody modifications and modifications to other biologically relevant protein drugs.

1. Introduction

Therapeutic monoclonal antibodies (mAbs) are an important class of biological therapeutics widely used for the treatment of cancer, autoimmune diseases, and various infectious diseases. mAbs are susceptible to a variety of enzymatic, chemical, and physical modifications throughout production, purification, formulation, and storage that are known to alter their biological properties. The result is a complicated mixture of product variants that can pose a challenge to current analytical measurements and complicates interpretation of a modification's impact on stability and/or function. The potential impact of product variants are evaluated during mAb development by imparting intentional stressors in excess to induce artificial changes in product quality, a process called forced degradation. Later in development, stability testing is performed on the formulated drug substance or drug product, wherein stressors model more real-world intrusions (Li et al. 2015).

Post-translational modifications (PTMs) are changes in the polypeptide occurring after translation due to enzymatic processing, although chemically induced and/or exacerbated changes are typically also included in a broader definition (Li et al. 2015). One of the most common and pervasive PTMs that affects mAbs is oxidation, which can be caused by peroxides, metal ions, and light. Methionine is the amino acid most susceptible to oxidation in mAbs, while oxidation of several other amino acids like tryptophan, cysteine, lysine, and histidine have also been reported (Chen et al. 2019). Methionine oxidation in the Fc region has been widely studied and shown to cause reduced Fc mediated activity by decreasing interactions with the neonatal Fc receptor (FcRn) and other Fc receptors (Bertolotti-Ciarlet et al. 2009, Pan et al. 2009, Gao et al.

66 2015, Cymer et al. 2017). Specifically, oxidation of Met 255 and Met 431 (actual
67 sequence number varies owing to differing complementarity-determining region (CDR)
68 lengths and often is referred to as Met 252 and Met 428) have been demonstrated to
69 cause a decrease in binding of FcRn. Oxidation of amino acids in the CDR has also
70 been implicated in decreased antigen binding (Dashivets et al. 2016). Others have
71 shown that Met oxidation can interfere with immunoglobulin G (IgG) oligomerization,
72 which is needed for C1q binding and complement dependent cytotoxicity (Mo et al.
73 2016). Investigation of the biological impact of antibody methionine oxidation is
74 important for many aspects of antibody function, and higher resolution information on
75 the effects of a specific oxidized methionines are of great interest.

76 Because of the global nature of stress protocols, all susceptible methionine
77 residues are oxidized, albeit at varying rates. The rate of oxidation depends on a variety
78 of factors, with solvent accessible surface area being the major contributing factor (Pan
79 et al. 2009, Sokolowska et al. 2017). Although many studies have demonstrated
80 correlations between the biological impact of IgG and methionine oxidation, direct
81 assessment of a specific methionine residue can be difficult to evaluate because it is
82 challenging to generate materials with oxidation at a specific methionine residue.
83 Genetic manipulation of specific methionine residues has been used; methionine to
84 leucine to block oxidation at a site or methionine to glutamine to mimic an oxidized
85 methionine. These genetic modifications allowed for the expression of mAbs with site
86 selective oxidation mimics useful in deciphering structure-function relationships of IgG
87 binding to FcRn (Gao et al. 2015). However these sequence-engineered materials have
88 altered primary amino acid sequence and do not directly represent the same product.

89 Another approach attempted to enrich Met-oxidized species using affinity
90 chromatography with ligands that can distinguish methionine oxidation at certain
91 residues (Stracke et al. 2014). This approach can enrich a certain population of Met-
92 oxidized antibodies, but is rather cost prohibitive at scale and still cannot provide
93 sufficiently pure material.

94 As an alternative, we envisioned a method that allows for a binding partner
95 known to interact with specific methionine residues to mask oxidation at that site when
96 chemical oxidation was performed in the presence of that ligand. By decreasing the
97 solvent exposure, the kinetic rate of oxidation should decrease drastically. The publicly
98 available IgG1κ monoclonal antibody Reference Material 8671, NISTmAb, was chosen
99 as an example material due to its usefulness in evaluation and development of
100 emerging analytical measurement technologies (Schiel, Davis and Borisov 2015). Here
101 we present a method to generate functionally relevant, selectively oxidized materials
102 that are useful as analytical challenge materials. We chose to generate a library of
103 these selectively oxidized mAbs in combination with traditionally oxidized samples and
104 characterized each sample with mass spectrometry to access the quantity of residue-
105 specific modifications. Furthermore, we use these unique challenge materials to
106 demonstrate how a rapid surface plasmon resonance-based assay and a thermal
107 unfolding assay can differentiate the impact of oxidation at different regions of the mAb,
108 providing highly valuable information that could be useful in the assessment of critical
109 quality attributes of antibody therapeutics.

110 2. Materials and methods

111 2.1 Preparation of the stressed samples

112 *2.1.1 NISTmAb alone vs NISTmAb with protein A in solution*

113 Two sets of oxidized samples were generated, one with NISTmAb alone (Nox 1, Nox 3,
114 and Nox 6) and the other with a mixture of NISTmAb and protein A (PAox 1, PAox 3,
115 PAox 6). Nox samples were generated as follows: 100 µL of NISTmAb at 10 mg/mL
116 was added to each vial followed by the addition of 22.6 µL of phosphate-buffered saline
117 (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4) (PBS) pH 7.4 and 4.2 µL of
118 30 % (w/w) H_2O_2 to give a final concentration of H_2O_2 of 1 % and a final concentration of
119 NISTmAb of 7.88 mg/mL. Samples were incubated at 25 °C, protected from light.
120 Samples were centrifuged for 5 min at 10,000 rpm and reactions were stopped by buffer
121 exchanging back into formulation buffer (pH 6.0, 25 mmol/L L-Histidine) using zeba spin
122 desalting columns 7K MWCO (Thermo Scientific) at the following time points: 1 h, 3 h,
123 and 6 h to generate $^{1\%}Nox_{1hr}$, $^{1\%}Nox_{3hr}$, and $^{1\%}Nox_{6hr}$. PAox samples were generated
124 as follows: 100 µL of NISTmAb at 10 mg/mL was added to each vial followed by the
125 addition of 22.6 µL of protein A at 25 mg/mL in PBS and 4.2 µL of 30 % (w/w) H_2O_2 to
126 give a final concentration of H_2O_2 of 1 % and a final concentration of NISTmAb of 7.88
127 mg/mL. Samples were incubated at 25 °C, protected from light. Samples were
128 centrifuged for 5 min at 10,000 rpm and reactions were stopped by buffer exchanging
129 back into formulation buffer (pH 6.0, 25 mmol/L L-Histidine) using zeba spin desalting
130 columns 7K MWCO (Thermo Scientific) at the following time points: 1 h, 3 h, and 6 h to
131 generate $^{1\%}PAox_{1hr}$, $^{1\%}PAox_{3hr}$, and $^{1\%}PAox_{6hr}$.

132 *2.1.2 Standard in solution oxidation time course*

133 To achieve forced oxidation, 8.01 µL of 30 % (w/w) H_2O_2 was added directly to a 800 µL
134 vial (8 mg at 10 mg/mL) of NISTmAb RM 8671 samples to obtain a final concentration

135 of 0.3 % H_2O_2 and 9.9 mg/mL of NISTmAb. Samples were incubated at 25 °C, protected
136 from light. Samples were centrifuged for 5 min at 10,000 rpm and reactions were
137 stopped by buffer exchanging back into formulation buffer (pH 6.0, 25 mmol/L L-
138 Histidine) using zeba spin desalting columns 7K MWCO (Thermo Scientific) at the
139 following time points: 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 16 h, 32 h. A NISTmAb Bex sample
140 was generated by buffer exchanging a NISTmAb RM 8671 vial into formulation buffer
141 using the same method as oxidized samples. A NISTmAb control sample was also
142 generated by incubating a NISTmAb vial without the addition of H_2O_2, at 25 °C and
143 protected from light for 32 h and then buffer exchanged into formulation buffer using the
144 same method as oxidized samples. All buffer exchanged samples were measured for a
145 concentration (all samples were between 7 mg/mL to 8 mg/mL) using a NanoDrop
146 1000C, aliquoted to 50 µL, and stored at -80 °C .

147 *2.1.3 Oxidation while bound to protein A column*

148 NISTmAb (8 mg) was loaded onto a 1 mL MabSelect SuRe protein A column (GE
149 Lifesciences) and the column was washed with PBS pH 7.4 for 5 column volumes. To
150 achieve forced oxidation a solution of H_2O_2 in PBS was run over the column at a flow
151 rate of 0.5 mL/min for a specific amount of time. Five CPA samples were generated
152 using a different amount of H_2O_2 and different exposure times: $^{0.3\,\%}CPA_{2hr}$, $^{0.3\,\%}CPA_{4hr}$,
153 $^{0.3\,\%}CPA_{6hr}$, $^{0.0375\,\%}CPA_{16hr}$, and $^{3\,\%}CPA_{1hr}$ (Where the superscript denoted H_2O_2 % and
154 subscript denotes time exposed to H_2O_2 on the protein A column). After the oxidation,
155 the column was washed with PBS pH 7.4 for 5 column volumes to get rid of any excess
156 H_2O_2. Bound IgG was eluted using a citric acid solution (100 mmol/L pH 3.0) and quickly
157 neutralized with 1.5 mol/L tris buffer pH 8.8. Fractions containing eluted NISTmAb were

158 combined and concentrated using amicon ultra centrifugal filter unit 10 KDa (Millipore).
159 Samples were then centrifuged for 5 min at 10,000 rpm and buffered exchanged back
160 into formulation buffer (pH 6.0, 25 mmol/L L-Histidine) using zebra spin desalting
161 columns 7K MWCO (Thermo Scientific) and aliquots were stored at -80 °C. The
162 concentrations of all samples were measured using a Nanodrop 2000 C system and
163 ranged from 7 mg/mL to 8 mg/mL.

164 **2.2 LC-MS analysis**

165 *2.2.1 IdeS Subunit Analysis*

166 The antibody samples were diluted to 3 mg/mL into 0.25 mol/L Tris-HCl, pH 7.5
167 and one unit of IdeS protease was added for every 1 µg of antibody. The samples were
168 digested by incubating the solution at 37 °C for 30 minutes. The digested samples were
169 then diluted 1:10 into a denaturing buffer comprised of 6 mol/L guanidine HCl in 0.25
170 mol/L Tris-HCl, pH 7.5. The mAbs were then reduced by adding dithiothreitol (DTT) to a
171 final concentration of 25 mmol/L for 60 min at 45 °C. The samples were then buffer
172 exchanged into 0.1 % formic acid (FA) and 10 % acetonitrile in LC-MS grade water
173 using a zebra spin filter column. 2.5 µg of each peptide digest was injected onto a
174 Waters UPLC Protein Ethylene Bridged Hybrid C4 column (150 mm x 2.1 mm i.d. 1.7
175 µm BEH particles, 300 Å) set to 60 °C and analyzed by liquid chromatography-
176 electrospray ionization-mass spectrometry (LC-ESI-MS/MS) using an Agilent 1200
177 Infinity II series LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF. The
178 chromatographic method was initiated with 80 % Mobile Phase A (0.1 % FA in water)
179 and 20 % Mobile Phase B (0.1 % FA in acetonitrile) with a flow rate of 0.4 mL/min. The
180 separation was achieved over 20 min starting with a 5 min isocractic hold at 20 % B

181 followed by a gradient to 45 % B in 15 min. The column was then washed by ramping
182 up to 95 % B in 1 min followed by a 4 min hold at 95 % B. The column was equilibrated
183 by returning the flow to 20 % B followed by a 2 min hold at 20 % B. The MS instrument
184 was operating in positive ion mode with the following source settings: gas temp 350 °C,
185 drying gas 8 l/min, nebulizer 2.4 x 10^5 Pa, sheath gas temp 275 °C, sheath gas flow 11
186 l/min, VCap 5000 V, nozzle voltage 1000 V, fragmentor 250 V, skimmer 65 V, Oct 1 RF
187 Vpp 750 V. The mass range for MS1 was 300 – 3200 m/z and data was acquired at 1
188 spectra/s.

189 *2.2.2 Peptide mapping analysis*

190 The antibody samples were prepared and digested following a previous
191 publication (Mouchahoir and Schiel 2018). Briefly, the samples were denatured in a
192 buffer comprised of 6 mol/L guanidine HCl, 1 mmol/L ethylenediaminetetraacetic acid
193 (EDTA) in 0.1 mol/L Tris-HCl, pH 7.8. The mAbs were then reduced with a final
194 concentration of 5 mmol/L dithiothreitol (DTT) for 60 min at 4 °C and alkylated with a
195 final concentration of 10 mmol/L of iodoacetamide (IAM) for 60 min at 4 °C. The
196 samples were then buffer exchanged into 1 mol/L urea in 0.1 mol/L Tris, pH 7.8 and
197 trypsin was added at a 1:18 (enzyme: sample) mass ratio. The digestion was incubated
198 for 4 h at room temperature. The reaction was stopped by adding 0.1 % formic acid (FA)
199 in LC-MS grade water was added at a 1:1 volume ratio and the digests were stored at -
200 80 °C until analysis. 2.5 µg of each peptide digest was injected onto a Agilent Zorbax
201 RRHD StableBond C18 column (150 mm x 2.1 mm i.d. 1.8 µm BEH particles, 300 Å)
202 set to 40 °C and analyzed by LC-ESI-MS/MS using an Agilent 1200 Infinity II series LC
203 system coupled to an Agilent 6545XT AdvanceBio LC/Q-Tof. The chromatographic

204 method was initiated with 99 % Mobile Phase A (0.1 % FA in water) and 1 % Mobile
205 Phase B (0.1 % FA in acetonitrile) with a flow rate of 0.25 mL/min. The separation was
206 achieved over 72 min starting with a 5 min isocractic hold at 1 % B followed by a steep
207 gradient to 10 % B in 1 min ending with a gradient to 35 % B in 64 min. The column was
208 then washed by ramping up to 90 % B in 2 min followed by a 5 min hold at 90 % B and
209 then back down to 1 % B in 2 min followed by an isocratic at 1 % B for 2 min. The
210 gradient was then raised to 10 % B over 2.5 min, then to 45 % B in 8 min, and lastly to
211 90 % B in 1.5 min. A final isocratic hold at 90 % for 6 min was performed and the
212 column was equilibrated by returning to 1 % B for 14 min before the next sample. The
213 MS instrument was operating in positive ion mode with the following source settings:
214 gas temp 325 °C, drying gas 13 l/min, nebulizer 2.4 x 10^5 Pa, sheath gas temp 275 °C,
215 sheath gas flow 12 l/min, VCap 4000 V, nozzle voltage 500 V, fragmentor 175 V,
216 skimmer 65 V, Oct 1 RF Vpp 750 V. The mass range for MS1 was 100 m/z to 2400 m/z
217 and 50 m/z to 2400 m/z in MS2. Ions were selected for MS/MS with a narrow 1.3 m/z
218 window then fragmented by collision induced dissociation using the formula, collision
219 energy= 3.6*(m/z)/100 + 4.8. The MS2 conditions were as follows: Top 10, 3000 counts
220 abs threshold/ 0.001 % rel threshold, active exclusion enabled with exclusion after 3
221 spectra and released after 0.2 min. The MS data was analyzed using Genedata
222 Expressionist software. Briefly, raw data was imported into Genedata Expressionist and
223 a workflow was run that includes background subtraction followed by retention time
224 alignment, MS peak detection, charge assignment, MS/MS consolidation and peak
225 detection. The MS1 mass tolerance was set at 10 ppm and MS2 mass tolerance was 50
226 ppm for peptide identification. The oxidation percentage of each Met residue was

227 calculated by dividing the peak area of the oxidized peptide by the sum of the peak
228 areas of both oxidized and nonoxidized peptide.

229 **2.3 Size-exclusion chromatography (SEC) analysis**

230 SEC analysis was performed according to a previously developed method
231 (Turner et al. 2018). Briefly, all samples were analyzed on an Agilent high pressure
232 liquid chromatography system using isocratic elution (100 mmol/L sodium phosphate
233 supplemented with 250 mmol/L sodium chloride, pH 6.8) at 0.30 mL/min and monitored
234 at 280 nm. 60 µg of antibody sample was injected onto a Waters Acquity UPLC Protein
235 BEH SEC column (1.7 µm particle size, 200 Å pore size, 4.6 x 150 mm length).

236 **2.4 SPR analysis**

237 SPR experiments were performed using a Biacore T200 system (GE Healthcare)
238 with analysis temperature set to 25 °C and sample compartment temperature set to 15
239 °C. Series S Sensor Chip CAP, PBS-P+ Buffer 10x [0.2 mol/L phosphate buffer with
240 27 mmol/L KCl, 1.37 mol/L NaCl and 0.5 % v/v Surfactant P20 (Tween 20)], and Biotin
241 CAPture Kit were all obtained from GE Healthcare. A peptidic epitope of the NISTmAb
242 with the sequence NSELLSLINDMPITNDQKKLMSNN and N-terminal acetylation, C-
243 terminal amidation, and a C-terminal biotinylated lysine residue was synthesized by
244 Genscipt. Recombinant biotinylated protein A (29989) and protein L (21189) were
245 purchased from Thermo Fisher.

246 Measurements were conducted using a double-capture method via the
247 oligonucleotide-immobilized CAP sensor chip, Biotin CAPture reagent (streptavidin
248 bound to an oligonucleotide complementary to the strand on the CAP chip), and the

249 various biotinylated ligands (protein A, F peptide, and protein L). Biotin capture reagent
250 was injected for 300 s at a flow rate of 2 μL/min to capture approximately 3000 relative
251 units (RU) followed by a 60 s injection at 5 μL/min into Fc2 of biotinylated protein A
252 (0.02 μg/μL) to give a capture level of 275 RU to 305 RU, another 60 s injection at 5
253 μL/min into Fc3 of biotinylated F peptide (5 μg/μL) to give a capture level of 295 RU to
254 305 RU, and a final 60 s injection at 5 μL/min into Fc4 of biotinylated protein L (0.02
255 μg/μL) to give a capture level of 220 RU to 230 RU. Samples were diluted in running
256 buffer (PBS-P+ pH 7.4) to a concentration of 200 nmol/L and flowed over each flow
257 channel at a flow rate of 50 μL/min. Each complex was allowed to associate and
258 dissociate for 100 s and 300 s, respectively. Following the association and dissociation
259 phases of the experiment, the chip was regenerated with an injection of regeneration
260 buffer 1 for 120 s (6 mol/L guanidine-HCL, 0.25 mol/L NaOH) and injection of
261 regeneration buffer 2 for 120 s (30 % acetonitrile in 0.25 mol/L NaOH).

262 A binding affinity response point was taken at the maximum binding level at the
263 end of the association phase for each sample. The RU value was normalized for each
264 ligand by dividing each data point by the maximum value of NISTmAb Bex for that
265 ligand in each experiment. The relative binding level for each sample to each ligand was
266 calculated by averaging sample replicates over three independent experiments and the
267 standard deviation along with %CV were also calculated.

268 **2.5 Thermal Unfolding analysis**

269 Thermal unfolding experiments were performed using a Tycho NT 6.0 system
270 (NanoTemper). All samples were diluted to 1 mg/mL in formulation buffer (pH 6.0, 25

271 mmol/L L-Histidine) before analysis. Samples were heated from 35 °C to 95 °C over 3
272 min, and intrinsic fluorescence at 350 nm and 300 nm were monitored, providing a
273 relative thermal stability for each sample. Unfolding profiles were generated by plotting
274 the fluorescence ratio of 350nm /330 nm. NanoTemper software then calculated the
275 inflection temperatures by taking the first derivative of this ratio where max and min
276 peaks correlate to inflection temperatures. The average value and standard deviation of
277 inflection temperatures and initial ratio was calculated using three sample replicates.

278 **2.6 Statistical Analysis**

279 Graphpad Prism version 9.1.2 was used for all statistical analysis. The unpaired t-test
280 was performed with the following settings: Assume Gaussian distribution and two-tailed
281 p value calculation. The nonlinear regression analysis was performed using either a
282 straight line model for linear data or One phase decay model for nonlinear data using
283 standard settings. The standard error of regression (Sy.x) was calculated and the 90%
284 prediction bands (the area that 90% of future data points are expected) were also
285 plotted. Replicates were accounted for by using the number of samples (N) and the
286 standard deviation.

287 **3. Results and Discussion**

288 **3.1 *Method to change methionine oxidation kinetics at a specific site: Solution***
289 ***phase protein A protection proof of principle***

290 Oxidative stress of NISTmAb in the presence of protein A in solution was used to
291 initially assess the ability of protein A to mask, and thereby protect, Fc methionine
292 residues from oxidation. The levels of oxidation of NISTmAb alone and NISTmAb with

293 protein A in solution (1:2 molar ratio) were compared after exposure to a strong
294 accelerated oxidative stress condition, 1 % hydrogen peroxide (H_2O_2) solution, for 1 h, 3
295 h, and 6 h. The global oxidation levels of both sets of oxidized samples, NISTmAb alone
296 ($^{1\%}Nox_{1hr}$, $^{1\%}Nox_{3hr}$, $^{1\%}Nox_{6hr}$) and NISTmAb with protein A ($^{1\%}PAox_{1hr}$, $^{1\%}PAox_{3hr}$,
297 $^{1\%}PAox_{6hr}$) were monitored by liquid chromatography mass spectrometry (LC-MS) and
298 compared to the unstressed NISTmAb RM 8671. A rapid subunit mass analysis was
299 employed which entailed enzymatic digestion with IdeS to specifically cleave IgG in the
300 hinge region, resulting in three subunits after reduction of disulfide bonds: Fc/2, Fd', and
301 LC. A representative spectrum of all three subunits of NISTmAb RM 8671 is shown in
302 Fig. S1**.** The analytical method was capable of identifying all previously reported
303 proteoforms of the NISTmAb and deemed suitable for preliminary oxidation screening.
304 A full list of identified masses, including observed and theoretical masses for all
305 proteoforms, can be found in Table S1.

306 Exposure to H_2O_2 resulted in up to six methionine oxidation events observed on
307 the NISTmAb: three events in the Fc region, one in the LC, and two in the Fd subunit
308 (Figs. 1 to 3). Each glycoform of the Fc was observed to oxidize at the same rate,
309 therefore the mass range was zoomed in to focus on the G0F glycoform for easier
310 visualization in Fig. 1. As the Fc methionine residues oxidize over time in the Nox
311 samples, four distinct species can be detected corresponding to 0, 1, 2, and 3 oxidized
312 methionine residues with each oxidation event adding +16 da (Figs. 1 A through C).
313 Longer time courses resulted in a larger relative abundance of scFc containing more
314 oxidation events. The oxidation profile of the scFc of PAox samples showed
315 substantially less oxidation than Nox at all time points (Figs. 1 D through F).

316 The oxidation profiles of the LC and Fd subunits of Nox and PAox samples, however,
317 were quite similar and showed nearly the same oxidation levels at each timepoint (Figs.
318 2 and 3, A through C vs. D through F). The LC subunit shows two distinct species
319 corresponding to 0 and 1 oxidized methionine while the Fd subunit shows three distinct
320 species corresponding to 0, 1, and 2 oxidized methionine residues (Figs. 2 and 3, A
321 through C). The IdeS subunit analysis of these samples indicate that methionine
322 oxidation kinetics of the Fc region of NISTmAb due to H_2O_2 exposure can be
323 significantly slowed when protein A is added to the solution. Presumably the protein A
324 remains bound to NISTmAb Fc to a sufficient degree, blocking putative oxidation sites,
325 while the oxidation rates in the Fab region remain relatively unchanged.

326 While our in solution oxidation results were promising, the solution phase
327 protection method had a few limitations leading to a final product that was not ideal for
328 further downstream studies:1) protein A was still in solution and while bound to
329 NISTmAb cannot easily be removed 2) protein A is a multidomain protein that has five
330 different domains that all bind IgG Fc with varying degrees (Ljungberg et al. 1993) and
331 3) some protein A domains have also been shown to bind to the Fab region. To address
332 these issues, another strategy was developed that takes advantage of a commercially
333 available resin, MabSelect SuRe, which contains an engineered protein A covalently
334 conjugated to agarose matrix. The MabSelect SuRe resin contains a tetramer of a Z
335 domain, an alkali tolerant mutant of the B domain of protein A, which has multiple
336 properties that address previously mentioned drawbacks of the free in solution oxidation
337 method: 1) the protein A is attached covalently to the resin and has low ligand leaching
338 (Healthcare 2005) 2) the use of only the Z domain has less affinity and selectivity

339 variation vs. the multi-domain protein A and 3) the Z domain has been shown to have
340 little to no Fab binding (Jansson, Uhlén and Nygren 1998). On column oxidation was
341 therefore pursued for the generation of samples with unique oxidation profiles when
342 compared to traditional solution phase oxidation without protein A.

343 **3.2 Generation of selectively oxidized NISTmAb samples and comparison to**
344 **standard forced oxidized time course samples using LC-MS/MS analysis**

345 After the in solution proof of principle studies and the rationale for a more
346 optimized approach, a larger scale study was performed to generate both standard
347 forced oxidized samples along with a set of uniquely oxidized samples generated by
348 oxidation on a protein A column for comparison purposes. Traditional accelerated
349 forced oxidized samples (no protein A) with a wide range of total oxidation were
350 generated using conditions known to produce materials with low levels of oxidation all
351 the way to near complete oxidation of all susceptible methionine residues. A lower H_2O_2
352 % was used, 0.3 %, so that functionally relevant materials with very low levels of
353 oxidation could be generated. Samples were stressed for a range of time points from 30
354 minutes to 32 h at 25 °C focusing on early time points in the linear oxidation range and
355 named according to the H_2O_2 % and amount of time oxidized ($^{0.3\,\%}Nox_{0.5hr}$, $^{0.3\,\%}Nox_{1hr}$,
356 etc). A NISTmAb buffer exchange reference (NISTmAb Bex) was made without the
357 addition of H_2O_2 but following a similar buffer exchange workup and NISTmAb control
358 (NISTmAb Ctrl) was made without the addition of H_2O_2 but a 32 h incubation at 25 °C to
359 control for any changes that might occur over time. The exact conditions are detailed in
360 the materials and methods. Another set of selectively oxidized samples was also
361 generated by performing the forced oxidation while NISTmAb was bound to a protein A

362 column. Specifically, three samples were generated using the same 0.3 % H_2O_2 to allow
363 for a direct comparison to time course stressed samples: 2 h, 4 h, and 6 h and named
364 accordingly $^{0.3\,\%}CPA_{2hr}$,$^{0.3\,\%}CPA_{4hr}$, $^{0.3\,\%}CPA_{6hr}$. Two other selectively oxidized samples
365 were generated to evaluate on/off equilibrium effects: one with gentle stress over a long
366 time period (0.0375 % H_2O_2 for 16 h), $^{0.0375\,\%}CPA_{16hr}$ and another with heavy stress for a
367 short time period (3 % H_2O_2) for 1 h, $^{3\,\%}CPA_{1hr}$. The exact conditions of all on column
368 oxidized samples are detailed in the materials and methods.

369 To demonstrate site specific changes in the oxidation profile of on column
370 stressed material, tryptic peptide mapping combined with liquid chromatography mass
371 spectrometry (LC-MS) was performed. Following peptide identification by tandem mass
372 spectrometry, the extent of quantifiable methionine oxidation was determined by
373 quantitative evaluation of the modified tryptic peptides relative to their respective
374 unmodified parent peptides as described in methods section. All peptides containing a
375 given Met residue were collated to provide the residue-specific quantification results.
376 Among the eight methionine residues, only six changed significantly under our $^{0.3\,\%}Nox$
377 accelerated stress conditions: HC M34, M101, M255, M361, M431 and LC M4 as
378 summarized in Fig. 4A. The levels of other typical modifications (deamidation,
379 isomerization, glycation) were monitored and found that no other significant
380 modifications occurred in any of the $^{0.3\,\%}Nox$ samples. The $^{0.3\,\%}Nox$ samples showed an
381 increase in oxidation over time (Fig. 4A). Typically, methionine residues in the Fc are
382 known to be the most susceptible to oxidation while methionine residues in the variable
383 regions of the Fab vary in their susceptibility to oxidize depending on a variety of factors
384 including solvent-accessibility of the methionine residue, expression host, and process

385 conditions (Yang et al. 2017). Specifically, the oxidation rates of susceptible methionine
386 residues in NISTmAb ordered as the following: M255>M101>M431>M361>LC M4>M34.
387 The oxidation profile of $^{0.3\,\%}$Nox samples can be generalized with three tiers of oxidation
388 with the first and most susceptible being M255 and M101, followed by M431 and M361
389 second, and third and least susceptible being M34 and LC M4.

390 M255 was oxidized at much lower rate in the $^{0.3\,\%}$CPA samples when identical
391 timepoints were considered (indicated by black dashes in Fig. 4B), implying that when
392 NISTmAb is bound to protein A, M255 is protected from oxidation. In addition to the
393 dramatic protection observed for M255 oxidation, a slight decrease (and hence minor
394 protection) in oxidation at M431 was also shown in the $^{0.3\,\%}$CPA$_{2hr}$ sample but not seen
395 in the 4 h and 6 h samples. While a direct comparison in the $^{0.3\,\%}$CPA$_{4hr}$ and $^{0.3\,\%}$CPA$_{6hr}$
396 samples did not show lower oxidation at M431 it was clear that oxidation was lower at
397 M431 than M361 in CPA samples but in Nox samples the opposite was true so
398 oxidation in the presence of protein A did seem to have a slight protective effect on
399 M431 as well. This phenomenon is in agreement with the known relative solvent
400 exposure of protein A-bound IgG1; protein A is known to be in close contact with M255
401 while M431 is also nearby (Deis et al. 2015). Interestingly, a higher rate of oxidation at
402 M101 (and to a lesser degree M361 and LC M4) was observed when compared to other
403 residues. This could indicate a conformational change of the Fab when IgG is bound to
404 protein A that makes M101 more accessible and/or an orientational effect while ligand-
405 bound. Specifically, the oxidation rates of susceptible methionine residues in CPA
406 samples ordered as the following: M101>M361>M431>LC M4>M255>M34. The
407 oxidation profile of CPA samples can be generalized with three tiers of oxidation with

the first and most susceptible being M101, followed by M361, M431, and LC M4 second, and third and least susceptible being M34 and M255.

A similar phenomenon was seen in the $^{0.0375\ \%}CPA_{16hr}$ and $^{3\ \%}CPA_{1hr}$ samples with M255 showing a much slower oxidation rate relative to the other methionine residues when compared to standard forced oxidized $^{0.3\ \%}Nox$ samples; M255 displayed the fastest oxidation rate in Nox samples but in CPA samples it was much slower, second to last. $^{0.0375\ \%}CPA_{16hr}$ was shown to be a very interesting material with a high level of M101 oxidation (52 %) while having only small amounts (<15 %) of oxidation at all other sites. This material could be of interest to elucidate specific effects M101 oxidation might have on NISTmAb while minimizing the influence of other oxidation effects. $^{3\ \%}CPA_{1hr}$ was shown to be the most oxidized CPA sample, and the strategy of heavy stress for a short period did not seem to provide the same level of protection at M255 when compared to the milder conditions. Taken together, these measurements indeed confirm the ability of a ligand to mask a specific epitope from oxidation and the ability to generate uniquely oxidized challenge materials.

3.3 Surface Plasmon Resonance (SPR): Assessment of NISTmAb using domain specific molecular probes

Previous studies have shown that oxidation in both the Fab and Fc regions can affect binding to both antigen and Fc receptor targets. In order to characterize both domains and therefore potentially unique bioactivity of the novel challenge materials created herein, a surface plasmon resonance (SPR) assay was designed that could assess binding at distinct and separate locations using a set of NISTmAb binding proteins as molecular probes. Specifically, ligands were selected that are known to bind

431 in both the Fab and Fc regions so functionality of both domains could be assessed. Two
432 common bacterial proteins were selected that are known to have a high affinity for
433 NISTmAb and have known binding sites: protein A which binds to the Fc and protein L
434 which binds to the variable region of the LC Fab without interfering with antigen binding
435 site. A peptidic epitope, F peptide, known to bind with high affinity to the NISTmAb CDR
436 of the Fab was also included. A schematic diagram showing the three molecular probes
437 and the relative location they bind to NISTmAb is shown in Fig. 5. A reversible biotin
438 capture system was used for each of the ligands that has been shown to be an efficient
439 SPR assay platform that allows for a generic regeneration condition and repeatable
440 capture (Karlsson, Fridh and Frostell 2018). The assay depends on a special sensor
441 chip CAP which has a pre-immobilized oligonucleotide on the surface. Sequential steps
442 of the assay include: 1) A biotin capture reagent (streptavidin modified with the
443 complementary oligonucleotide) is hybridized to the CAP chip, 2) The biotinylated ligand
444 (protein A, protein L, or F-peptide) is captured by streptavidin, 3) the
445 adsorption/desorption of the analyte is observed, and 4) finally the sensor chip surface
446 is completely regenerated back to the bare oligonucleotide (Fig. S2).

447

448 A unique target of the current assay was to demonstrate that reliable and
449 quantitative differentiation of the binding could be achieved with a single replicate of
450 material and sole observation of the maximum response level as opposed to detailed
451 concentration-dependent equilibrium and/or kinetic fitting models. Repeatability of this
452 platform (additional details in materials and methods) was demonstrated using 20
453 consecutive cycles of ligand capture and NISTmAb Bex as the analyte. Response levels

454 for each of the biotinylated ligands were repeatable with coefficient of variations (CV) of
455 less than 3.4 % (Table S2). Very repeatable ligand capture levels are vital for assay
456 performance and were achieved by the addition of 0.1 % bovine serum albumin to both
457 protein A and protein L solutions but not needed in the F peptide solution. The binding
458 response for each NISTmAb Bex replicate was also confirmed to be repeatable with
459 CVs of less than 3.3 % (Table S3). These results indeed confirmed that the assay was
460 repeatable and that this assay could provide reliable data on our oxidized sample set.
461 The final experimental design for future use therefore consisted of 20 cycles with 3
462 startup cycles to prep the surface, 3 NISTmAb Bex samples run at the beginning,
463 middle, and end, and 14 samples run once in a randomized order. Samples were all
464 diluted to 200 nmol/L concentration and run over once per experiment. Each experiment
465 was repeated 3 times providing 3 measurements for each oxidized sample and 9
466 measurements of NISTmAb Bex. After analysis of all samples, the repeatability and
467 reproducibility of the assay was also confirmed by measuring the response level in
468 response units (RU) for each of the biotinylated ligands after each cycle. All three
469 ligands also showed intra assay CV less than 2 % and inter assay CV of less than 5 %
470 (Tables S4 through S6). All oxidized samples were characterized by size exclusion
471 chromatography (SEC) to ensure that samples did not have any substantial changes in
472 high molecular weight (HMW) or low molecular weight (LMW) species that would have
473 interfered with SPR and thermal unfolding studies. The HMW, monomer, and LMW
474 species were determined for each sample and summarized in Table S7. Overall, all the
475 oxidized samples except ${}^{0.0375\,\%}CPAox_{16hr}$ showed only minor differences when

476 compared to NISTmAb Bex and the small difference in that one sample was noted but
477 was not considered an issue for further biophysical studies.

478 3.4 Surface Plasmon Resonance (SPR): Effects of methionine oxidation on 479 NISTmAb binding to domain specific molecular probes

480 *3.4.1 General Trends of Nox Samples*

481 The relative binding affinity of oxidized NISTmAb samples binding to rotein A can
482 be seen in Fig. 6A and the representative sensorgrams in Fig S3 A and B. A significant
483 decrease in relative binding affinity correlating to increasing oxidation levels can be
484 quickly identified when looking at $^{0.3\,\%}$Nox time course samples. The least oxidized
485 sample, $^{0.3\,\%}Nox_{0.5hr}$, showed relatively small changes (94.7 %) in binding to protein A
486 relative to NISTmAb Bex, whereas the $^{0.3\,\%}Nox_{32hr}$ sample, which is the most oxidized
487 sample with near complete oxidation of the six methionine residues shown to oxidize
488 under our conditions, showed a drastic decrease in the relative binding affinity to protein
489 A (38.5 %). The F peptide binding results showed a similar trend of decreasing relative
490 binding affinity with increasing global oxidation as shown in Fig. 6B and the
491 representative sensorgrams in Fig S3 C and D. The least oxidized sample, $^{0.3\,\%}Nox_{0.5hr}$,
492 showed relatively small changes (94.6 %) in binding to F peptide relative to NISTmAb
493 Bex. On the other side of the time course scale, the $^{0.3\,\%}Nox_{32hr}$ sample, which is the
494 most oxidized sample with near complete global oxidation, showed large changes to the
495 relative binding to F peptide (53.7 %). While the protein A and F peptide binding data
496 showed major changes due to oxidation, the Protein L binding data showed very little
497 changes as oxidation increased (Fig. 6C) (Fig. S3 E and F). The least oxidized sample,
498 $^{0.3\,\%}Nox_{0.5hr}$, showed no real changes (99.3 %) in binding to Protein L relative to

499 NISTmAb Bex. Even the near completely oxidized $^{0.3\,\%}Nox_{32hr}$ sample showed only a
500 small change in relative binding to Protein L (92.6 %).

501 *3.4.2 General Trends of CPA samples*

502 The relative binding affinity of $^{0.3\,\%}$CPA samples binding to Protein A, F peptide,
503 and Protein L can also be seen in Fig. 6A, Fig. 6B, and Fig. 6C, respectively**.** The
504 binding level of identical time points of $^{0.3\,\%}$Nox samples is shown with a solid line in
505 each figure. The relative binding affinity of $^{0.3\,\%}$CPA samples to Protein A was higher
506 when compared to identical time points of $^{0.3\,\%}$Nox samples, implying that protection
507 from oxidation due to the Protein A masking did indeed help maintain rebinding to a
508 Protein A ligand. This phenomenon, while interesting, was expected as M255 has been
509 shown to be vital for high affinity Protein A binding and was shown to be protected from
510 oxidation in the 0.3 %CPA samples.

511 Protein A masking had little to no effect on Protein L binding as indicated in Fig.
512 6C. This may be the expected result considering Protein A and Protein L binding
513 regions are spatially distributed as demonstrated in Fig. 5, and no methionine residues
514 of IgG are known to be involved in Protein L binding, a supposition supported by our
515 observation of little to no change in Nox sample binding to Protein L. Protein A masking
516 did, however, have an effect on F peptide binding (Fig. 6B). The relative binding affinity
517 of $^{0.3\,\%}$CPA samples to F peptide was lower when compared to identical time points of
518 $^{0.3\,\%}$Nox samples, implying that the changes in oxidation rates due to the protein A
519 masking did affect F peptide binding. Qualitative comparison of Fig. 4B reveals that
520 M101 had the largest increase in oxidation vs. the equivalent Nox time points. A
521 correlation would make sense considering M101 is located in the CDR and therefore

522 probable to have some effect on antigen binding. On the other hand, M361 and LC M4
523 are also consistently more oxidized in the 0.3 %CPA samples, making a conclusive
524 correlation subjective at best without epitope mapping and of course neglecting the
525 possibility of allosteric effects.

526 *3.4.3 Site-Specific Correlation: % Met oxidized vs Relative Binding (RU)*

527 Peptide mapping analysis was used to evaluate oxidation at specific methionine
528 residues and combined with relative binding affinity by SPR to further explore site-
529 specific structure/function correlations. Fig. 7 A through D shows a consistent trend for
530 Met residues in the $^{0.3\,\%}$Nox samples (in black), an increase in oxidation at each site
531 appears to correlate with a decrease in protein A relative binding. Based on these
532 samples alone, however, no confident site-specific correlations can be made because
533 oxidation at all methionine residues is increasing and roughly correlating with a
534 decrease in protein A binding. Inclusion of the CPA samples (in pink), however, allow
535 for a more selective and confident site-specific relationship because oxidation rates at
536 some residues remain unaltered, while others change as a result of ligand protection.
537 CPA samples for M101 and M361, for example, show a higher relative binding at the
538 same % oxidation (Figs. 7 A and C) and these data stay widely from the $^{0.3\,\%}$Nox
539 regression line and fall completely out of 90% prediction bands. These data indicate
540 oxidation at these sites is not the dominant driver of protein A binding reduction.
541 However, CPA samples for M255 and M431 trend more closely to the $^{0.3\,\%}$Nox
542 regression line (Figs. 7 B and D), indicating decreased protein A binding is more closely
543 associated with oxidation at these sites. Previous reports have indeed demonstrated
544 that oxidation in the Fc region disrupts protein A binding as it binds to a region where

545 M255 and M431 are located and where the C_H2 and C_H3 domains interact (Deis et al.
546 2015) (Gallagher, Galvin and Karageorgos 2018). In the above example, it is our
547 position that if a specific residue is a causal factor in reducing binding, then alteration of
548 the kinetics of that oxidation (e.g. slowed via ligand-bound protection) will not alter the
549 trend of relative binding vs. site-specific %Met oxidation. Specifically, CPA data points
550 will deviate farther from the Nox regression model when residues are not involved in
551 binding while CPA data points will trend closer to the Nox regression model when they
552 are involved in binding. To quantitate this distance, the standard error of regression
553 (Sy.x) of CPA data points was calculated from the Nox regression model. The Sy.x
554 values are shown in Table S8 and show significantly larger values for M101 and M361,
555 more intermediate value for M431, and lowest for M255. In summary, oxidation at M255
556 correlates strongly to decreased protein A binding while oxidation at M431 might have a
557 minor secondary effect and oxidation at M101 and M361 seem to play no direct role in
558 decreased protein A binding.

559 A similar trend can be seen in Fig. S4 A through D for Met residues in the $^{0.3}$
560 $^{\%}$Nox samples, an increase in oxidation at each site appears to correlate with a
561 decrease in F peptide relative binding. More selective and confident correlations are
562 achieved when the CPA samples are added to these plots. CPA samples for M34 and
563 M255 show a lower relative binding to F peptide at the same % oxidation and these
564 data stay widely from the $^{0.3\,\%}$Nox regression line and fall completely out of 90%
565 prediction bands (Figs. S4 C and D). CPA samples for LC M4 and M101 , on the other
566 hand, trend more closely to the Nox regression line (Fig. S4 A and B). Again the Sy.x
567 was calculated and values are shown in Table S9. The values are significantly higher

568 for M34 and M255, more intermediate for LC M4, and lowest for M101. In summary,
569 oxidation at M101 correlates strongly to decreased F peptide binding while oxidation at
570 LC M4 might have a minor secondary effect and oxidation at M34 and M255 seem to
571 play no direct role in decreased F peptide binding. Previous reports have shown that the
572 oxidation of a residue in the CDR of an antibody can disrupt antigen binding (Haberger
573 et al. 2014), and the current F peptide binding data indicates that methionine oxidation
574 can indeed cause a significant decrease in the ability of NISTmAb to bind to a peptide
575 antigen.

576 **3.5 Surface Plasmon Resonance (SPR): Sensitivity to detect changes in Oxidation**

577 To determine what samples had statistical differences in the binding affinity when
578 compared to the unstressed NISTmAb Bex sample, an unpaired t-test was used to
579 calculate the differences between the $^{0.3\,\%}$Nox samples and the NISTmAb Bex sample
580 (Table S10). First, any differences due to storage at room temperature for an extended
581 period of time were ruled out by demonstrating that the binding of NISTmAb Bex and
582 NISTmAb Ctrl were not statistically different. Looking next at the least oxidized sample,
583 $^{0.3\,\%}\text{Nox}_{0.5\text{hr}}$, it did indeed show significant differences in binding to Protein A when
584 compared to NISTmAb Bex with a corresponding p value of 0.0028. All other samples
585 were also statistically different than NISTmAb Bex with p-values of less than 0.0001. An
586 unpaired t-test was again used to determine the statistical difference in relative binding
587 affinity to F peptide. The binding of NISTmAb Bex and NISTmAb Ctrl were not
588 statistically different when binding to F peptide which confirms that no modifications due
589 to storage at 25 °C for 32 h led to any detectable changes. Again the least oxidized Nox
590 sample, $^{0.3\,\%}\text{Nox}_{0.5\text{hr}}$, could be differentiated from NISTmAb Bex with F peptide binding

591 as the relative binding affinity was statistically different with a p value of 0.0008. All
592 other samples were also statistically different than NISTmAb Bex with p-values of less
593 than 0.0001. A summary of p values for all samples can be found in Table S8. Taken
594 together both Protein A and F peptide binding are good indicator of oxidation and even
595 small amounts of oxidation could be detected using this approach.

596 The sensitivity of detection of specific oxidized methionine residues in NISTmAb
597 could be estimated by using the experimental data from our least oxidized sample, $^{0.3\,\%}Nox_{0.5hr}$. Protein A binding was indicative of methionine oxidation at M255 and the
599 sensitivity of detection was estimated to be 10 % of M255 oxidation from experimental
600 data of sample $^{0.3\,\%}Nox_{0.5hr}$. F peptide binding was indicative of methionine oxidation at
601 M101 and the sensitivity of detection was estimated to be 5 % of M101 oxidation from
602 experimental data of sample $^{0.3\,\%}Nox_{0.5hr}$.

603 Overall the trends for the traditional Nox samples were as expected – higher
604 global oxidation, indicated increase in oxidation at all susceptible Met residues (Fig. 4A),
605 resulted in decreased binding for oxidation-affected interactions. In general, oxidation
606 affects NISTmAb binding to Protein A > F peptide and only the smallest amount for
607 Protein L. The general trend also held for the CPA samples, oxidized samples
608 demonstrated lower binding affinity compared to NISTmAb Bex. Both protein A and F
609 peptide make excellent molecular probes to distinguish methionine oxidation in
610 NISTmAb as they can detect only minor changes in oxidation but Protein L was unable
611 to detect these minor changes. This SPR assay has been demonstrated to be a rapid
612 method to detect oxidation at both the Fc and Fab regions in the same analytical run.

613 ***3.6 Assessment of NISTmAb stability with thermal unfolding assay***

614 In the discussion above we utilized the uniquely oxidized CPA samples to
615 demonstrate that localized changes in oxidation would inevitably affect ligand binding in
616 that region. The SPR assay developed was therefore inferred to be sensitive to
617 oxidation of specific Met residues. A logical question to then ask is whether other
618 biophysical assays indicative of stability may also be localized using similar techniques.
619 Qualitative thermal melting studies with intrinsic tryptophan fluorescence were therefore
620 performed to identify correlations between methionine oxidation and domain stability.
621 Oxidation of IgG is known to destabilize the Fc domain of IgG resulting in changes in
622 the melting temperature (Chumsae et al. 2007, Gao et al. 2015). Previous differential
623 scanning calorimetry (DSC) measurements of NISTmAb have determined that there are
624 three distinct melting temperatures, 69.2 °C, 83.1 °C, and 93.4 °C, corresponding to
625 C_H2, C_H3, and Fab domains (Gokarn et al. 2015). To examine the thermal stability of
626 stressed samples in the current study, a thermal unfolding assay was performed that
627 measures the intrinsic fluorescence from aromatic amino acid residues. Changes in the
628 fluorescence ratio 350 nm / 330 nm were measured with increasing temperature, and
629 the midpoint unfolding inflection temperatures (Ti) were calculated along with the initial
630 350 nm / 330 nm ratio.

631 Representative unfolding profiles of NISTmAb Bex, a lightly oxidized sample ($^{0.3\,\%}Nox_{2hr}$),
632 and a heavily oxidized sample ($^{0.3\,\%}Nox16_{hr}$) are shown in Fig. 8. These
633 unfolding profiles showed two clear changes that relate to the level of oxidation. First,
634 the initial ratio of detected fluorescence signal decreases while oxidation increases
635 indicated by the downward shift of the profile at 35 °C (the beginning of the assay
636 measurement). This decrease signifies a change in the amount of solvent exposed

637 tryptophan or tyrosine residues. Second, the thermal stability of oxidized samples
638 decreased as methionine oxidation increased indicated by the leftward shift (decreasing
639 value) in both Ti_1 and Ti_2. Three distinct Ti's could be determined for NISTmAb Bex:
640 72.5 °C (Ti_1), 80.0 °C (Ti_2), and 89.1 °C (Ti_3). The software was unable to determine Ti_3
641 for all samples, however manual inspection of the first derivative of the raw data shows
642 Ti_3 was similar for all oxidized samples. The complete results of the unfolding
643 measurements including the initial ratio and unfolding temperatures for all samples can
644 be found in Fig. 9.

645 *3.6.1 General Trends of Nox samples*

646 When looking over the thermal unfolding data it is evident that as oxidation
647 increases the initial ratio and both unfolding temperatures decrease significantly. A
648 small decrease in the initial ratio can be seen in Fig. 9A correlating to increasing
649 oxidation levels when looking at $^{0.3\,\%}$Nox time course samples. The decrease of the
650 initial ratio is small in value, 0.003, for the least oxidized sample, $^{0.3\,\%}Nox_{0.5hr}$, and 0.021
651 for the most oxidized sample. While these values are indeed small, the measurement of
652 the initial ratio was shown to be extremely precise with the average coefficient of
653 variation of 0.09 % indicating that even small differences could be reliably measured.

654 A large decrease in the Ti_1 can be seen in Fig. 9B correlating to increasing
655 oxidation levels when looking at $^{0.3\,\%}$Nox time course samples. The least oxidized
656 sample, $^{0.3\,\%}Nox_{0.5hr}$, showed a small decrease in Ti_1 of 0.3 °C relative to NISTmAb Bex.
657 Whereas, the $^{0.3\,\%}Nox_{32hr}$ sample, the most oxidized sample, showed a drastic decrease
658 in Ti_1 of 8.45 °C. Similarly, a large decrease in the measured Ti_2 can be seen in Fig. 9C
659 correlating to increasing oxidation levels when looking at $^{0.3\,\%}$Nox time course samples.

660 The least oxidized sample, $^{0.3\,\%}Nox_{0.5hr}$, showed a small decrease in Ti_2 of 0.15 °C
661 relative to NISTmAb Bex. Whereas, the $^{0.3\,\%}Nox_{32hr}$ sample, the most oxidized sample,
662 showed a substantial decrease in Ti_1 of 5.95 °C.

663 *3.6.2 General Trends of CPA samples*

664 The initial ratio and thermal unfolding temperatures of $^{0.3\,\%}CPA$ samples can also
665 be seen in Fig. 9A, Fig. 9 B, and Fig. 9C, respectively**.** The measured initial
666 ratio/unfolding temperature of identical time points of $^{0.3\,\%}Nox$ samples is shown with a
667 solid line in each figure for comparison purposes. The initial ratio of $^{0.3\,\%}CPA$ samples
668 was much lower when compared to identical time points of $^{0.3\,\%}Nox$ samples, implying
669 that changes in the methionine oxidation pattern due to the Protein A masking did
670 decrease the initial ratio. This significant decrease in initial ratio was seen in heavily
671 oxidized Nox samples suggesting that oxidation of M101 is likely to be the cause of the
672 change as it is heavily oxidized in all CPA samples. This phenomenon is confirmed
673 when comparing the other CPA samples especially $^{0.0375\,\%}CPA_{16hr}$ which has very low
674 levels of oxidation at all sites except M101. $^{0.0375\,\%}CPA_{16hr}$ has a similar amount of
675 oxidation at M101 as $^{0.3\,\%}Nox_{6hr}$ and these samples indeed have similar initial ratio
676 values, 1.002 and 1.001 respectively. Taken together oxidation at M101 seems to have
677 a strong effect on the initial ratio and oxidation at this site seems to induce a structural
678 change that exposes a tyrosine or tryptophan residue likely in the Fab region.

679 Protein A masking also had an effect on the measured Ti_1 (Fig. 9B). The Ti_1 of
680 $^{0.3\,\%}CPA$ samples was much higher at time points 4 h and 6 h but essentially the same
681 at 2 h when compared to identical time points of $^{0.3\,\%}Nox$ samples. This implies that
682 changes in the oxidation rates due to Protein A masking did increase the stability of the

683 C_H2 domain by blocking oxidation. Presumably, this effect is mostly due to the much
684 lower amount of oxidation at M255 in CPA as it is the most protected residue and also is
685 located in the C_H2 domain. While the initial ratio and Ti1 values were significantly
686 affected by Protein A masking oxidation, the value of Ti_2 of $^{0.3\,\%}$CPA samples was
687 slightly higher at 2 h and slightly lower at 4 h and 6 h compared to $^{0.3\,\%}$Nox samples
688 (Fig. 9C). The changes in oxidation rates due to the protein A masking had a smaller
689 effect on the stability of the C_H3 domain of NISTmAb. This effect is most likely due to
690 the higher amounts of oxidation at M361 and M431 in the CPA samples both of which
691 are located in the C_H3 domain of NISTmAb.

692 *3.6.3 Site-Specific Correlation: % Met oxidized vs Initial ratio/unfolding temperatures*

693 Peptide mapping analysis was used to evaluate oxidation at specific methionine
694 residues and combined with thermal unfolding analysis to further explore site-specific
695 structure/function correlations. Plots of the initial ratio of the $^{0.3\,\%}$Nox samples against
696 the % oxidized of methionine at all sites were generated and nonlinear regression
697 analysis was used to model Nox data sets (Figs. S5 A through F). A consistent trend
698 can be seen for all Met residues in the Nox data, an increase in oxidation at each site
699 correlates with a decrease in the value of the initial ratio. More selective and confident
700 correlations are achieved when the CPA samples are added to these plots. CPA
701 samples for M34, M255, and M431 stay widely from the $^{0.3\,\%}$Nox regression model and
702 fall completely out of 90% prediction bands (Figs. S5 B, D, and F). On the other hand,
703 CPA samples for LC M4, M101, and M361 trend more closely to the Nox regression
704 model (Fig. S5 A, C, and E). The Sy.x of CPA data was calculated and the values are
705 shown in Table S11. The Sy.x was significantly higher for M34, M255, and M431, more

706 intermediate for LC M4 and M361, and lowest for M101. In summary, oxidation at M101
707 correlates strongly with the decrease in initial ratio, while oxidation at LC M4 and M361
708 could play a more secondary role. Methionine oxidation at these sites seem to induce
709 structural change that causes a tyrosine or tryptophan residue to be less exposed,
710 resulting in a shift of the fluorescence toward lower wavelengths.

711 Strong correlating trends can be seen when Ti_1 is plotted vs % of methionine
712 oxidation in the Nox samples (Figs. S6 A through F). Again, CPA samples were added
713 to these plots to get a more precise idea of which oxidized methionine residues play a
714 stronger role in the decrease of thermal stability of NISTmAb. CPA samples for LC M4,
715 M101, and M361 stay widely from the $^{0.3\,\%}$Nox regression model and fall completely out
716 of 90% prediction bands (Figs. S6 A, C, and E). On the other hand, CPA samples for
717 M34, M255, and M431 trend more closely to the Nox regression model (Fig. S6 B, D,
718 and F). The Sy.x of the CPA data was calculated and the values are shown in Table
719 S12. The Sy.x was significantly higher for LC M4, M101, and M361, more intermediate
720 for M34 and M431, and lowest for M255. These data indicate that oxidation at M255
721 correlates strongly with the decrease in thermal stability of the CH2 domain, while
722 oxidation at M34 and M431 could play more secondary roles.

723 Similar strong correlations are observed after plotting Ti_2 vs % of methionine
724 oxidation in the Nox samples (Figs. S7 A through F). CPA samples for M101 stay widely
725 from the Nox regression model whereas all others trend closely to their respective
726 model. The Sy.x of the CPA data was calculated and the values are shown in Table
727 S13. The Sy.x was higher for M101 while all other methionine residues show relatively

728 low values. These data indicate that global methionine oxidation at multiple residues
729 contribute to the decrease in thermal stability of the CH3 domain.
730

731 **4. Perspectives**

732 In order to evaluate methods that are capable of attribute specific monitoring,
733 well-defined materials with site specific attribute modifications are needed. We explored
734 a strategy that could generate selectively oxidized antibody materials with specific
735 attribute changes that differ from global chemical oxidation. This ligand masking method
736 takes advantage of the ability of a specific binding partner to bind and mask its binding
737 region on an antibody. Once bound, an accelerated stress procedure was performed to
738 generate a product with a unique methionine oxidation pattern. This approach
739 generated materials that showed protection from oxidative stress at a specific
740 methionine residue, M255, while other methionine residues were exposed to varying
741 levels of oxidation. While this method still cannot provide perfect control over oxidation,
742 unique materials with specific attribute modifications were generated that could not be
743 made using a traditional approach. Other strategies can achieve similar unique
744 materials; however, this method has some distinct advantages that make it an
745 interesting approach. First, no genetic manipulations are needed that can mimic or
746 block oxidation which can be arduous and require the expression and purification of
747 new mAbs. Also these oxidation mimicking mutants (*e.g.*, glutamine to mimic oxidized
748 methionine) do share similarities in chemical structure and hydrophobicity. They are
749 indeed different in structure, and the exact contribution still must be demonstrated.
750 Another typical method relies on tedious chromatographic separations in combination

751 with fractionation that can be difficult on a larger scale. In some cases oxidation variants
752 can be almost impossible to separate without a very specialized affinity column.

753 Assigning criticality to a specific amino acid residue can be a challenging task
754 due to the global nature of typical stress studies. While certain residues are typically
755 modified at higher rates than others, multiple residues are most often modified, making
756 correlations between a site-specific modification and biological activity difficult. Often
757 multiple residues in both the Fab and Fc regions are susceptible to modifications, but
758 the effect of each are difficult to differentiate. The ligand masking approach allowed
759 generation of materials with unique modification patterns. The effect of this oxidation
760 was explored by measuring changes in relative binding affinity and the thermal stability
761 of these modified materials when compared to NISTmAb Bex. With the help of these
762 uniquely stressed materials, specific attribute changes could be correlated to specific
763 changes in these analytical assays; a pre-requisite to assigning attribute criticality with
764 heightened specificity.

765 In this study, we explored the effect methionine oxidation has on the ability of
766 NISTmAb to bind to three IgG binding proteins including protein A, an antigen mimic F
767 peptide, and protein L. A SPR assay was developed that demonstrated the ability of
768 these proteins to serve as molecular probes to rapidly reveal stress induced oxidation in
769 NISTmAb samples. High throughput analytical screening technologies are important to
770 quickly identify critical process parameters and to monitor critical product quality
771 attributes. Both protein A and F peptide could easily distinguish samples with low levels
772 of oxidation whereas protein L was not a good ligand for detecting oxidation. Peptide
773 mapping analysis combined with relative binding affinity by SPR analysis allowed for

774 correlations to be made between increases in oxidation at specific methionine residues
775 and decreases in binding to a specific molecular probe. The SPR assay in this study
776 serves as an interesting approach to rapid analysis of oxidation at multiple sites that
777 uses very little material and can be run without any sample prep. One could envision an
778 SPR readout that can accurately estimate the % oxidation at a variety of specific sites if
779 the right molecular probes were used. In addition, other targeted assays could be
780 developed using different molecular probes that are able to detect other stress induced
781 modifications such as deamidation or aggregation. This sort of label-free binding assay
782 could provide rapid results about the integrity of antibody samples and detect a variety
783 of higher order structural changes.

784 In addition to usefulness in assessing attribute criticality, such selectively
785 oxidized samples also serve as useful and interesting materials for various analytical
786 challenges. Analytical challenge materials are a vital part of demonstrating and
787 determining method capability. Traditional accelerated degradation studies can indeed
788 produce useful materials that can contain a variety of induced amino acid modifications
789 that can challenge various analytical methods. In some cases, the ability of a method to
790 detect and to quantitate a site specific modification might be required, so a more
791 selectively modified challenge material would be of great advantage. These materials
792 with selective modifications can serve as important resources to determine if a method
793 can distinguish site specific modifications versus global changes. For example, the
794 ability of the different LC-MS methods used to characterize oxidized materials in this
795 study is clearly distinguished when comparing IdeS subunit monitoring versus peptide
796 mapping. IdeS subunits can monitor oxidation levels of each subunit (i.e. scFc, Fd, and

797 LC), but peptide mapping is able to determine oxidation at the peptide levels giving us
798 residue specific information. A similar phenomenon can be seen when comparing the
799 SPR and thermal unfolding analysis as the SPR method can give us information about
800 the oxidation levels at specific epitopes while the thermal unfolding studies provide a
801 correlation between oxidation and domain stability. The selectively oxidized CPA
802 samples provided useful and interesting challenge materials for these assays and
803 helped push the limits of each method to detect domain and residue specific oxidation.

804 **5. Conclusions**

805 In summary, we developed a strategy to generate uniquely stressed antibody
806 materials by performing the stress in the presence of a bound ligand Protein A. These
807 materials were characterized with mass spectrometry to quantitate site specific
808 methionine oxidation. Substantial changes in the oxidation rate and level of multiple
809 methionine residues were shown when compared to materials stressed in solution
810 without bound Protein A. With these uniquely oxidized materials in hand, we developed
811 a rapid SPR assay that could detect methionine oxidation in both the Fab and Fc
812 regions using specific molecular probes. The addition of our uniquely oxidized materials
813 to our data set allowed us to hone in on specific methionine residues vital to binding.
814 Further analysis showed that antibody oxidation could also be rapidly detected using
815 thermal unfolding analysis as the stability decreases in multiple domains. The industry
816 relevant stress of accelerated oxidation was used, but other industry relevant stress
817 conditions could be chosen and a similar ligand masking approach could protect
818 specific residues from modification. In the future more studies may reveal other proteins
819 or peptides that can provide site specific protection and use these masking agents to

820 elucidate specific structure function attributes related to a variety of antibody
821 modifications. In addition to monoclonal antibodies, the use of other modalities such as
822 other protein drugs or viral vectors could be of great interest especially when knowledge
823 of structure function relationships is not as developed as mAbs.

824 **Declaration of competing interest.**

825 The authors declare that there are no conflicts of interest. The authors would like to
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829 **Disclaimer**

830 Certain commercial equipment, instruments, and materials are identified in this paper in
831 order to specify the experimental procedure. Such identification does not imply
832 recommendation or endorsement by the National Institute of Standards and
833 Technology, nor does it imply that the material or equipment identified is necessarily
834 the best available for the purpose.

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908 **Figure captions.**

909 **Fig. 1 Deconvoluted LC-MS Spectrum of Fc subunit zoomed in to G0F A) $^{1\%}Nox_{1hr}$ B) $^{1\%}Nox_{3hr}$ C) ^{1}**
910 **$^{\%}Nox_{6hr}$ D) $^{1\%}PAox_{1hr}$ E) $^{1\%}PAox_{3hr}$ F) $^{1\%}PAox_{6hr}$ *Denotes adduct peaks that do not correspond to actual**
911 **oxidized antibody peaks**

912 **Fig. 2 Deconvoluted LC-MS Spectrum of LC subunit A) $^{1\%}Nox_{1hr}$ B) $^{1\%}Nox_{3hr}$ C) $^{1\%}Nox_{6hr}$ D) $^{1\%}PAox_{1hr}$ E) ^{1}**
913 **$^{\%}PAox_{3hr}$ F) $^{1\%}PAox_{6hr}$ *Denotes adduct peaks that do not correspond to actual oxidized antibody**
914 **peaks**

915 **Fig. 3 Deconvoluted LC-MS Spectrum of Fd subunit A) $^{1\%}Nox_{1hr}$ B) $^{1\%}Nox_{3hr}$ C) $^{1\%}Nox_{6hr}$ D) $^{1\%}PAox_{1hr}$ E) ^{1}**
916 **$^{\%}PAox_{3hr}$ F) $^{1\%}PAox_{6hr}$ *Denotes adduct peaks that do not correspond to actual oxidized antibody**
917 **peaks**

918 **Fig. 4. Relative abudance of oxidized methionine residues in NISTmAb samples A) NISTmAb Bex and**
919 **Ctrl with Nox Samples B) CPA samples. Black dashes (−) are used in the 0.3 % CPA samples to denote**
920 **the relative abundance of each methionine residue from equaivlanet Nox sample for comparison**
921 **purposes.**

922 **Fig. 5 Diagram of molecular probes used in SPR assay and the relative location they bind.**

923 **Fig. 6. Relative binding affinity of NISTmAb samples: A) Protein A binding affinity (RU) B) F peptide**
924 **binding affinity (RU) C) Protein L binding affinity (RU). The standard deviation of each measurement is**
925 **noted with error bars. Black dashes (−) are used in the 0.3 % CPA samples to denote the relative**
926 **binding affinity from equivlent timed Nox sample for comparison purposes.**

927 **Fig. 7. Correlation between Prot A binding and Met ox ($^{0.3\%}Nox$ samples in black and CPA samples in**
928 **pink) and linear regression analysis of Nox samples (solid black line) with 90% prediction bands**
929 **(dotted black line) A) M101 B) M255 C) M361 D) M431**

930 **Fig. 8. Unfolding profiles of NISTmAb Bex, a lightly oxidized sample ($^{0.3\%}Nox_{2hr}$), and a heavily oxdized**
931 **sample ($^{0.3\%}Nox_{16hr}$) with calculated inflection temperatures for the C_H2 (Ti_1) and C_H3 (Ti_2) domains(**

932 **Fig. 9. Thermal unfolding analysis using Tycho NT 6.0 A) Initial 350nm / 330nm ratio B) Inflection**
933 **Temperature 1 C) Inflection Temperature 2. The standard deviation of each measurement is noted**
934 **with error bars. Black dashes (−) are used in the 0.3 % CPA samples to denote the relative binding**
935 **affinity from equivlent timed Nox sample for comparison purposes.**

936