1 **_Original Research Article_**

2 mAb Higher Order Structure Analysis with Protein Conformational Array ELISA

3 **Abstract:**

4 The clinical and biological properties of protein-based therapeutics, or biologics, are closely related to
5 their Higher Order Structures (HOS) which in turn can be altered by many physical and chemical
6 conditions. A novel technology to monitor changes in monoclonal antibody (mAb) HOS is the Protein
7 Conformational Array (PCA) ELISA which uses a bank of more than 30 antibodies to measure protein
8 epitope change on the surface of the mAb. Using this technology, this report provided interesting
9 findings for the first time on the HOS changes in response to the various conditions often encountered
10 during mAb formulation development. Specifically, one IgG1 and four IgG2 native molecules in
11 formulation buffer were compared with the same IgG which had undergone exposure to increased
12 temperature, pH extremes and light exposure. In addition, we also examined the impact of glycation and
13 de-glycosylation on the mAb HOS. This study demonstrated that the PCA ELISA is stability-indicating and
14 can provide detailed HOS information that could be important for the successful development of
15 monoclonal antibodies.

16 **Keywords:**

17 Formulation Development; Monoclonal Antibody; Higher Order Structure; Protein
18 Conformational Array ELISA (PCA ELISA); Conformational Impurity.

19 Introduction

20 Since the introduction to market of the first recombinant biologic, human insulin, in 1982,
21 more than 200 marketed biologics have followed, firmly establishing this class of drugs
22 alongside the earlier small molecule based therapies. Within biologics, monoclonal antibodies

(mAbs) are the fastest growing class of human therapeutics, with more than 40 IgG-based drugs approved(Schellekens H., 2009). Recently an area drawing significant attention in biologics has been the development of biosimilars (Brinks V. et al., 2011; Meager A. et al., 2011; US FDA., 2012). The production of biologics and their generic equivalents, biosimilars, is more complex than the making of generic small-molecule based drugs, in part due to the greater three-dimensional (3-D) structural variations that are possible in a biologic(Beck A. et al., 2014; Berkowitz S A. et al., 2012). Not only does this additional complexity play a role in the quality attributes of the innovator biologics but also in the production of subsequent biosimilars(Berkowitz S A. et al., 2012; Jefferis R., 2009; Schiestl M. et al., 2011). The term 'Higher Order Structure' (HOS) has been used to describe the 3-D structure resulting from the cumulative effects of 1^0, $2^0$and 3^0 structure as well as post translational modifications to the protein. Accordingly, these characteristics of biologics are a function not only of the gene underlying the expression of the protein, but also the cell line in which it is produced. Furthermore the bioprocessing and formulation conditions can impact the HOS of biologics significantly. An additional important factor is that as a biosimilar maker will generally not have access to the same cell line that the innovator used; the resulting biosimilar will generally be at best similar, but not identical to the product that it is attempting to copy. Earlier studies using the PCA ELISA indicated that different mAbs on the market possessed stable and distinctive HOS signatures in their constant regions despite having almost identical amino acid sequences(Wang X et al., 2014; Wang X et al., 2013), suggesting again that the process defines the product. An important question for the regulatory agencies has become: how much and what kind of data are sufficient to establish that the differences between two similar products are not clinically significant. Recently the US Food and Drug Administration (FDA) released draft

46 guidelines on the development of biosimilars(US FDA., 2012). Among the many points covered,
47 the guidelines emphasized the importance of a biologic's 3-D structure for the safety and
48 efficacy of the molecule and also acknowledged that current analytical technologies are still
49 limited in their capability to define the precise 3-D structure of biologics. Therefore, the need for
50 new or improved technologies capable of assessing HOS relative to an approved drug seems
51 clear.

52 The PCA ELISA is a novel technology for mAb conformational analysis that uses a panel of
53 over 30 polyclonal antibodies that were raised against linear and sometimes secondary structure
54 epitopes of the mAb(Wang X et al., 2013) with good specificity. When the protein of interest is
55 in its correctly folded native form, the majority of these linear or secondary structure epitopes
56 will be buried within the 3-D structure and thus will not be recognized by the panel of antibodies.
57 In a typical mAb population, there is a small portion of mAbs that are unfolded or incorrectly
58 folded resulting in normally buried epitope exposure on the surface of the mAb. It is the sum
59 total of all these mAb species that are detected by the panel of antibodies in the PCA ELISA,
60 giving a defined and characteristic signal, or 'fingerprint' for that particular mAb. However, if
61 the protein conformation changes slightly, then the panel of antibodies is primed to recognize the
62 resulting change in epitope exposure. In a previous report, we demonstrated that antibody arrays
63 developed specifically toward several marketed mAbs could detect specific new epitope
64 exposure, caused by temperature induced conformational change with high sensitivity(Wang X
65 et al., 2013). The ability of the PCA ELISA to both interrogate the entire surface of the mAb and
66 also pinpoint to the regions where changes had occurred suggested to us that the antibody array
67 technology could provide a unique measurement of mAb HOS comparability. Recently
68 published work focusing on biosimilar HOS comparability analysis demonstrated that the PCA

69 ELISA can be used to benchmark the innovator mAb and that data was used to compare with
70 several biosimilar candidates(Wang X et al., 2014). Testing with biosimilar mAbs developed in
71 several countries indicated that the HOS comparability can vary greatly. Recently the
72 comparability analysis of the first mAb biosimilar (Remsima) approved by the European
73 Medicines Agency (EMA) was reported(Jung S. et al., 2014). In this study, the PCA ELISA was
74 used as one of the analytical technologies to assess the mAb HOS comparability and the data was
75 consistent with Remsima having high HOS comparability with the reference Remicade molecule.
76 Due to its sensitivity and ease of use, the PCA ELISA has utility in many stages of mAb
77 development, from cell line selection to bioprocess and formulation development. Another
78 benefit of the antibody array technology lies in its ability to quantify small amounts of
79 conformational impurities using an easy-to-operate ELISA format. The sensitivity of the assay
80 can be increased by increasing the concentration of analyzed mAb. As low as 0.1%
81 conformational differences could be detected from all the areas covered by the antibodies, thus
82 providing a very accurate and sensitive measurement of the status of the mAb
83 conformation(Wang X et al., 2013). While we do not yet have extensive data to correlate the
84 impact of this conformational impurity to the safety of the mAb, it is reasonable to postulate that
85 more conformational impurities (epitope exposure) may bring an increased risk in potential
86 immunogenicity(Buttel I et al., 2011; Hermeling S. et al., 2005; Hermeling S. et al., 2004;
87 Jiskoot W. et al., 2009; Maas C. et al., 2007; Rosenberg A., 2006; Schellekens H., 2002, 2005;
88 Sharma B., 2007), and two mAbs on the market, Rituxan and Campath, which showed excessive
89 epitope exposure in the variable region in a previous study (Wang X et al., 2013)were shown to
90 induce cytokine release in clinical studies(Pangalis G A. et al., 2001) and a recent in vivo human
91 whole blood assay(Singulex Poster 2015). Furthermore, exposure of new epitopes will also

92 increase the possibility of the mAb interacting with other regulatory proteins in the body, causing
93 off-target effects. Recent data from PCA ELISA testing on biosimilars has correlated regional
94 structural changes with loss of efficacy for the biosimilar candidate (data not shown).
95 Importantly, some local structural differences that can be elusive to other analytical technologies
96 including bioassays, can be detected with the PCA ELISA, demonstrating complementary value
97 of this epitope-based technology in mAb HOS characterization.

98 In this report we examined the ability of the PCA ELISA to detect HOS changes occurring
99 during novel mAb development. Both IgG1 and IgG2 human mAbs were analyzed for their
100 HOS status under typical bioprocess environment or stressed conditions. The results suggested
101 that the PCA ELISA is stability-indicating and could be of value in the elucidation of the impact
102 of bioprocess and formulation conditions on the HOS of the mAb, providing a sensitive and
103 systematic method for the characterization and improvement of the mAb under development.

104 **Materials and Methods**

105 **Reagents:** All the chemicals were purchased from Sigma-Aldrich (St. Louis, USA). 96-well
106 microplates were purchased from Corning Co. (#9018, Corning, USA. Streptavidin-HRP
107 conjugate (PI-21130) and biotin labeling kits (PI-21425) were obtained from Thermo Fisher
108 (Waltham, USA).

109 **Antibodies and ELISA kits:** All the reagent antibodies and ELISA kits used in this study were
110 products of Array Bridge Inc. (AB000208, St. Louis, USA). Polyclonal antibodies against the
111 antibody peptides were produced in New Zealand White Rabbits. For the sandwich ELISA,
112 antibodies against each region of the mAb molecule were first coated on the 96-well plate; with

113 each antibody coating 6 wells in rows B through G (Row A and H are not used in this analysis to

114 avoid possible edge effects during ELISA assay). In each column of the coated plates, the upper

115 three wells (B, C, and D) were incubated with a reference mAb in triplicate, and the lower three

116 wells (E, F, and G) were incubated with the same mAb after treatment. A biotin-labeled rabbit

117 anti-human IgG antibody was used to detect the mAb-peptide antibody complex, and

118 streptavidin-HRP was used to detect the complex formed by anti-human IgG-mAb-peptide

119 antibody. The signal strength of the sandwich ELISA depends on the relative epitope exposure

120 of the mAb in each region. If there are more epitopes from the mAb that could be recognized

121 by the peptide-derived antibodies, a stronger signal will be produced and vice versa. The

122 sequence assignment of the 31 antibodies used is described below:

123 Constant region sequence assignment is based on Herceptin (trastuzumab) amino acid sequence
124 including Ab13-Ab17 and Ab18-34. For variable regions, Ab1 to Ab6 and Ab7 to Ab12 each is an equal
125 mixture of 8 different antibodies in the same position from 8 marketed mAbs to provide maximum
126 approximation for novel mAbs, the 8 marketed mAbs are: Avastin, Campath, Erbitux, Herceptin, Humira,
127 Remicade, Rituxan and Synagis. Each set of the variable region antibodies were developed separately
128 based on their specific amino acid sequence.

129

	Antibody	Peptide Sequence	Chain Assignment	Sequence Assignment
130	Antibody	Peptide Sequence	Chain Assignment	Sequence Assignment
131	Ab1	DILLTQSPAILSVSP	LC	aa 1-15
132	Ab2	VSPGERVSFSSRASQFVGSSIHWY	LC	aa13-36
133	Ab3	SSIHWYQQRTNGSPRLLIKYASES	LC	aa 31-54
134	Ab4	ASESnleSGIPSRFSGSGSGTDFTLS	LC	aa 51-74
135	Ab5	FTLSINTVESEDIADYYSQQ	LC	aa 71-90
136	Ab6	YSQQSHSWPFTFGSGTNLEVKRTVA	LC	aa 87-111
137	Ab13	IFPPSDEQLKSGTASVVSLLNNFYP	LC	aa 117-141

138	Ab14	NALQSGNSQESVTEQDSKDSTYSL	LC	aa 152-175
139	Ab15	KDSTYSLSSTLTLSKADYEKHKVYASE	LC	aa 168-194
140	Ab16	KVYASEVTHQGLSSPVTKSFNRGES	LC	aa 189-214
141	Ab7	EVKLEESGGGLVQP	HC	aa 1-14
142	Ab8	VQPGGSnleKLSSVASGFIFSNHW	HC	aa 12-33
143	Ab9	NHWnleNWVRQSPEKGLEWVAEIRSKS	HC	aa 31-55
144	Ab10	RSKSINSATHYAESVKGRFTISRDD	HC	aa 52-76
145	Ab11	SRDDSKSAVYLQnleTDLRTEDTGVYY	HC	aa 73-97
146	Ab12	VYYSSRNYYGSTYDYWGQGTTLTVSSA	HC	aa 95-121
147	Ab17	PSVFPLAPSSKSTSGGTAALGSLVK	HC	aa 133-157
148	Ab18	SLVKDYFPEPVTVSWNSGALTSGVHT	HC	aa 154-179
149	Ab19	VHTFPAVLQSSGLYSLSSVVTVPSS	HC	aa 177-201
150	Ab20	VTVPSSSLGTQTYISNVNHKPSNTKV	HC	aa 196-221
151	Ab21	PSNTKVDKKVEPPKSSDKTHTSPPSPA	HC	aa 215-246
152	Ab22	SPPSPAPELLGGPSVFLFPPKPKD	HC	aa 241-264
153	Ab23	SVFLFPPKPKDTL(nle)ISRTPEVT	HC	aa 254-275
154	Ab24	PEVTCVVVDVSHEDPEVKFNWY	HC	aa 272-293
155	Ab25	VKFNWYVDGVEVHNAKTKPREEQYNS	HC	aa 288-313
156	Ab26	KEYKSKVSNKALPAPIEKTISKAKGQP	HC	aa 332-358
157	Ab27	KGQPREPQVYTLPPSRDELTKNQVS	HC	aa 355-379
158	Ab28	KNQVSLTSLVKGFYPSDIAVEWESNG	HC	aa 375-400
159	Ab29	WESNGQPENNYKTTPPVLDSDGSF	HC	aa 396-419
160	Ab30	SDGSFFLYSKLTVDKSRWQQGNVFS	HC	aa 415-439
161	Ab31	NVFSSSV(nle)HEALHNHYTQKSLSLSPGK	HC	aa 436-451

162

163 **Sample Treatments and Analysis:** All the samples were treated at formulated protein
164 concentration under different conditions. For temperature treatment, the samples were
165 incubated at 55°C for 10 days (IgG1) or at 40°C for 14 days (IgG2) and diluted to 5 µg/ml for
166 analysis. For higher pH treatment to generate deamidated mAb, the mAb samples were
167 treated in Tris buffer at pH 8 for 10 days at room temperature before analysis. For acidic pH
168 treatment, the pH was adjusted to pH 3.6 with acetic acid and incubated at room temperature
169 for 10 days before analysis. For light treatment, the mAb sample was treated with white light
170 at 416,000 lux for 52 hours. before the assay. For mAb deglycosylation, the mAb sample was
171 treated with PNGase F at 400:1 enzyme dilution and incubated at 37°C in Tris buffer, 100 mM,
172 pH 8.5 for two hours. For glycation, the mAb sample was treated with 222 mM glucose at 4°C
173 for 1 month. For reporting antibody, a polyclonal anti-human IgG antibody (developed by Array
174 Bridge Inc.) was used which will detect the capturing antibody-mAb complex. The reporting
175 antibody was labeled with biotin which in turn forms a complex with streptavidin-HRP
176 conjugate, TMB (3,3',5,5'-tetramethylbenzidine) was used as substrate for the HRP enzyme
177 activity assay. Following a short development time to allow color formation from the HRP
178 enzymatic activity, an equal volume of 1M sulfuric acid was added to stop the reaction. A
179 spectrophotometer from Molecular Devices (Sunnyvale, USA), the SpectraMax M3 was used to
180 measure the color change at 450 nm.

181 **Results**

182 **Effect of Temperature on mAb HOS (IgG1 vs IgG2)**

183 mAb stability is one of the major quality attributes in the development of the molecule(Arosio P. et al.,
184 2011; Correia I., 2010; Ionescu R. et al., 2008; Ohkuri T. et al., 2010; Vermeer A. and Norde W., 2000). In
185 this study the effect of exposure to increased temperature was examined for both IgG1 and IgG2 mAbs
186 respectively. The IgG1 sample incubated at 55°C for 10 days was compared to a control sample (Fig. 1A),
187 while an IgG2 sample (IgG2-a) stored at 40°C for 14 days was compared to the corresponding control
188 sample (Fig. 1B).

189 **Figure 1. Effect of different temperature on the HOS of IgG1 and IgG2 molecules**

190 1A. Comparison of native IgG1 with IgG1 that had been treated at 55°C for 10 days. 1B.
191 Comparison of native IgG2a with IgG2a that had been treated at 40°C for 14 days. All the
192 samples were tested on the ELISA plate at 5μg/ml.

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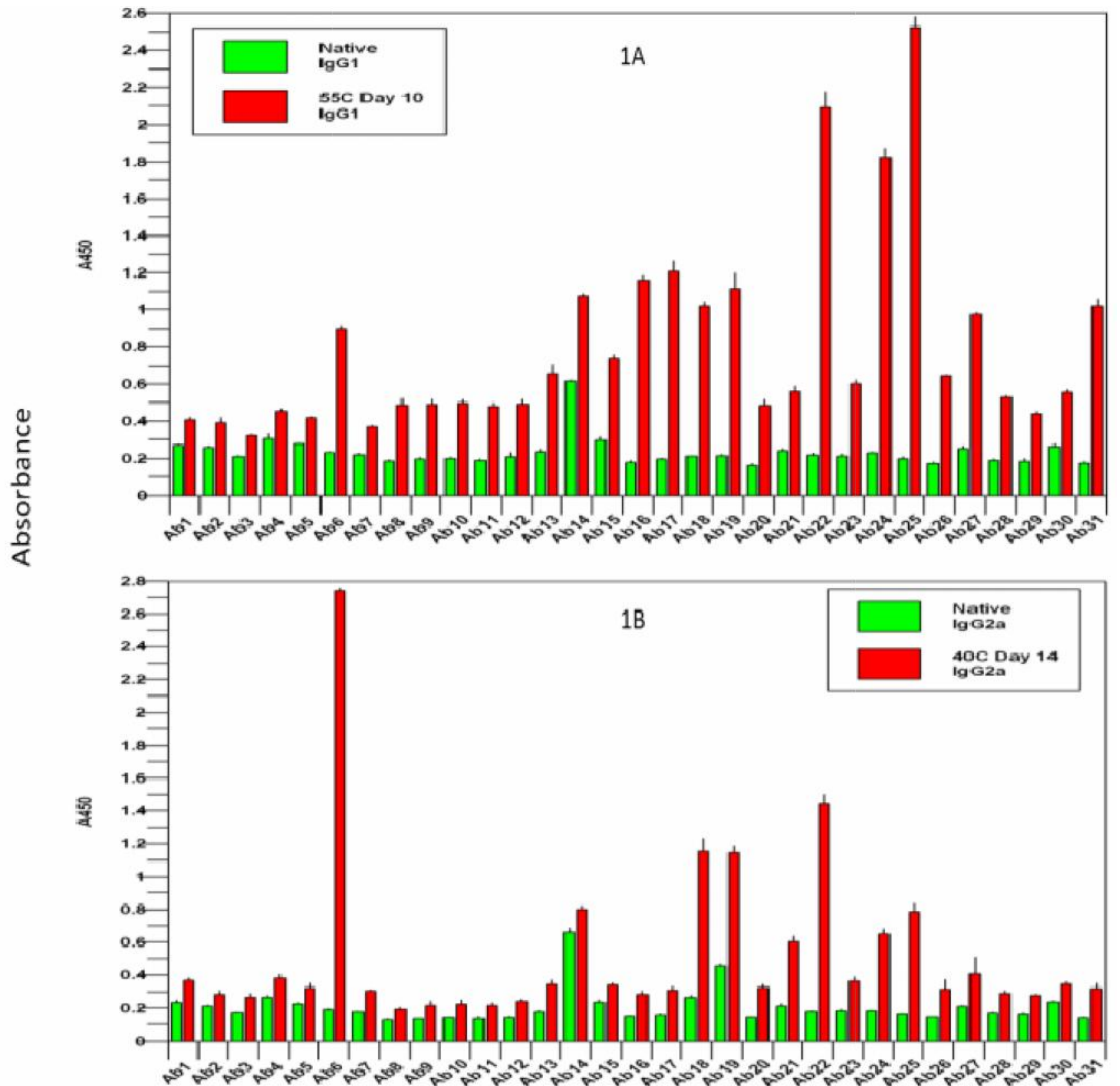


195 In both cases, the PCA ELISA results suggested significant new epitope exposure in both the mAb
196 variable region (covered by pAb1 through pAb12 in the ELISA panel) and constant regions (covered by
197 pAb13 to pAb31) with significant differences between the IgG1 and IgG2 molecules. While both datasets
198 suggested a general partial unfolding of the mAb as indicated by the increased signal across the whole
199 antibody panel, the 'hotspots' of greatest epitope exposure differed between the two mAbs. The IgG1

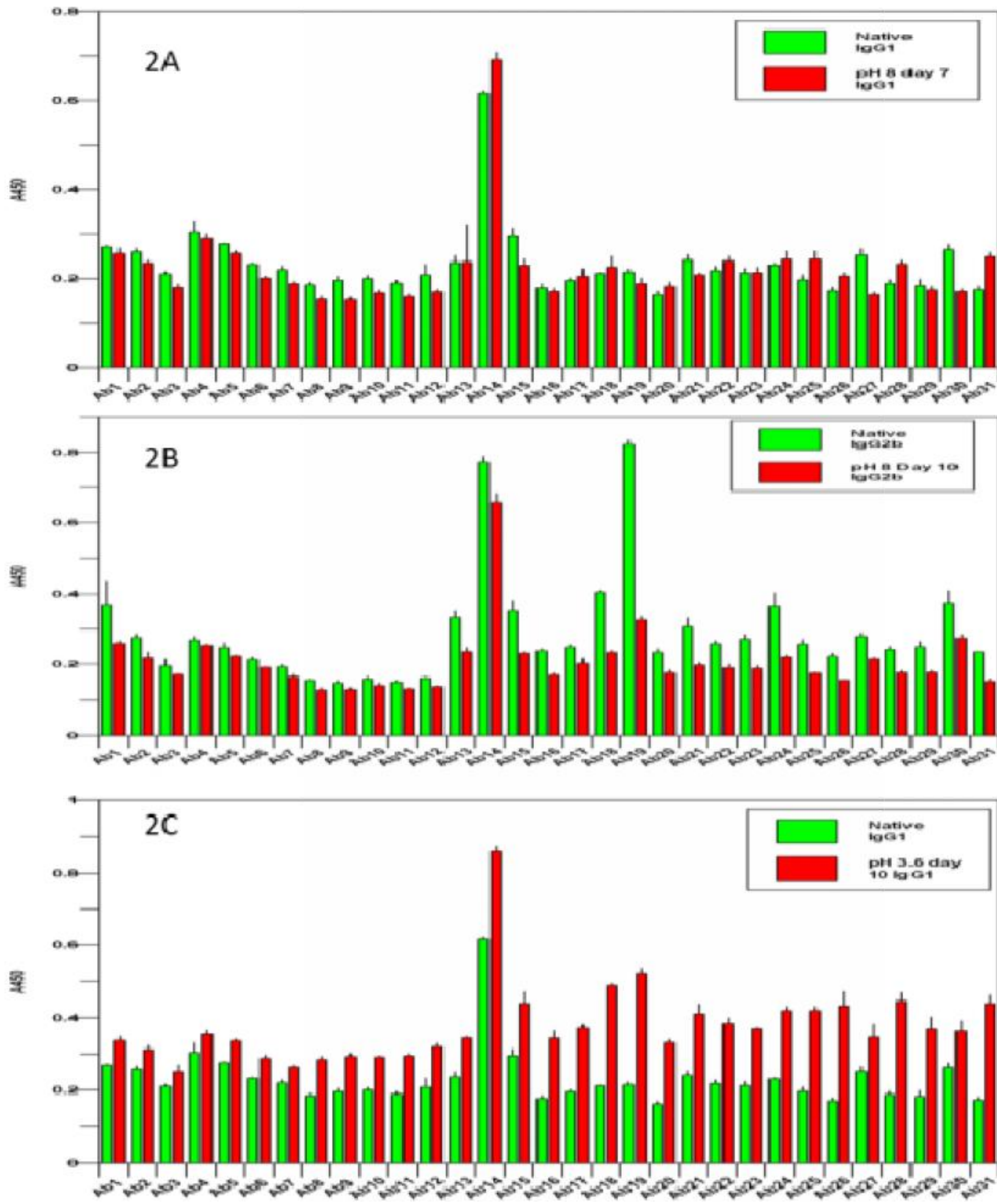
200 result suggested greatest sensitivity to temperature in the light chain at the boundary between the V_L
201 and C_L domains (pAb6), in both chains near the hinge region (pAbs 14-22) and in the heavy chain C_{H2}
202 region (pAbs 22-25). In contrast, the IgG2 result suggested somewhat better stability in the hinge region
203 (pAbs 14-22) but extreme sensitivity in the light chain at the boundary between the V_L and C_L domains
204 (pAb6). The apparent increased stability of the IgG2 mAb in the hinge region could be explained both by
205 the lower temperature incubation and by the additional disulfide bonds present in this region in an IgG2
206 vs IgG1 molecule. In the hinge region, the two heavy chains are linked by 2 disulfide bonds in an IgG1
207 molecule and 4 disulfide bonds in an IgG2 molecule.

208 **Effect of pH on mAb HOS (IgG1 vs IgG2)**

209 It is known that pH has significant impact on the stability of mAb molecules(Mason D. et al., 2012). In
210 this experiment the effect of exposure to basic pH condition on both an IgG1 sample (Fig 2A) and an
211 IgG2 sample (Fig 2B) were examined. In addition, data was collected on the effect of acidic pH condition
212 on sample IgG1 (Fig. 2C).

213 **Figure 2. Effect of different pH on the HOS of IgG1 and IgG2 molecules**

214 2A. Comparison of native IgG1 with IgG1 that had been treated at pH 8.0 for 10 days; 2B.
215 Comparison of native IgG2b with IgG2b that had been treated at pH 8.0 for 10 days. 2C.
216 Comparison of native IgG1 with IgG1 that had been treated at pH 3.6 for 10 days.



217 Absorbance

218

219 For both IgG1 and IgG2, the variable region (pAbs 1-12) appeared relatively stable to 7-10 day
220 exposures to pH 8.0, with slight additional exposures occurring at several epitopes. In contrast, the
221 constant region (pAbs 13-31) of sample IgG2 appeared far more susceptible to pH 8.0 exposures than
222 did the same region of the IgG1 molecule. In contrast to its relative stability to pH 8.0, the IgG1 sample

223 showed significant new epitope exposure across the entire molecule with 10 day incubation to pH 3.6.

224 Low pH (below 4.) has been shown by others(Mason D. et al., 2012) to cause reversible aggregation in

225 IgG molecules, the result here correlated well with previous findings and also demonstrated that PCA

226 ELISA could be used to characterize mAb aggregates induced by lower pH conditions.

227 **HOS Stability to Light Exposure**

228 It is known that light exposure could potentially impact the HOS of biologics(Mason D. et al., 2012).

229 During purification process, mAbs may be exposed to parts of UV-C (200 to 290 nm), UV-B (290 to 320

230 nm) and visible light (400 to 760 nm) under a variety of buffer and pH conditions.

231 **Figure 3. Effect of light on the HOS of IgG1 molecule**

232 Comparison of native IgG1 with IgG1 that had been treated with white light at 416,000 lux for

233 52hrs, both samples was tested on the ELISA plate at 5μg/ml.

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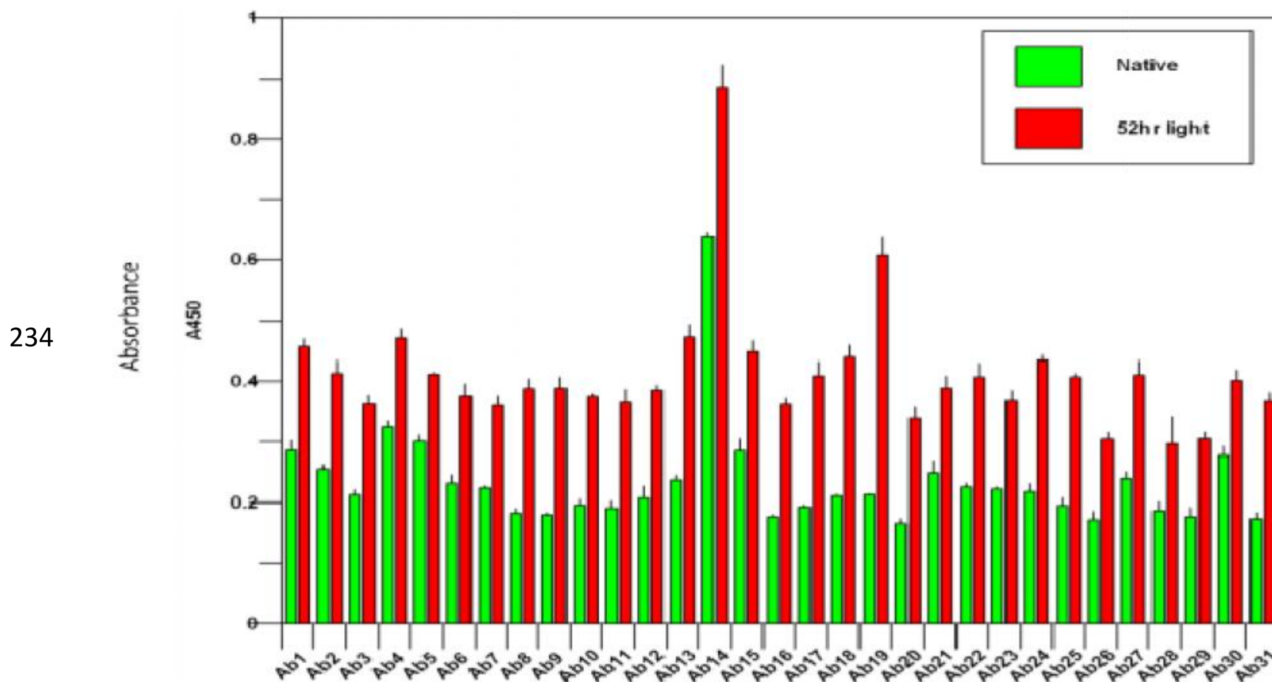

235 The combination of these conditions was known to promote both chemical and physical degradation
236 which may result in conformational changes. In this experiment, a sample of an IgG1 mAb which was
237 irradiated by white light at an intensity of 416,000 lux for 52 hours was compared to a control sample.
238 As can be seen in Fig. 3, this light exposure resulted in a general increase in epitope exposure with an
239 apparent equal distribution of impact (inferred by new epitope exposure) across the entire molecule.
240 The result suggested an unfolding of a small mAb population around 1% as estimated by previous
241 spiking studies. In addition, there is a sub-population of mAbs with specific regional changes around the
242 hinge region (pAb19).

243 **Effect of Deglycosylation on mAb HOS**

244 mAb glycosylation plays important role in its biological function, the mAb glycosylation status could
245 impact the PK/PD of the molecule and also induce immunogenicity(Abes R. and Teillaud J., 2010; Jefferis
246 R., 2007, 2009; Schiestl M. et al., 2011; Sutton B J. and Phillips D C., 1983; Zheng K. et al., 2011),
247 therefore development of mAbs with glycosylation pattern similar to those with clinical success is an
248 important task in mAb development. Previous studies using X-ray crystallography have demonstrated
249 that the mAb carbohydrate chains do not extend into solvent but form a bridge between the two
250 opposing Cγ2 domains(Sutton B J. and Phillips D C., 1983). One of the interesting aspects of
251 glycosylation analysis is to find out the impact of different glycosylation on the Higher Order Structure of
252 the protein. The epitope exposures of deglycosylated and control samples of an IgG2 molecule were
253 compared (Fig 4).

254 **Figure 4. Effect of deglycosylation on the HOS of IgG2 molecule**

255 Comparison of native IgG2c with de-glycosylated IgG2c. Both samples were tested on the ELISA
256 plate at 5μg/ml.

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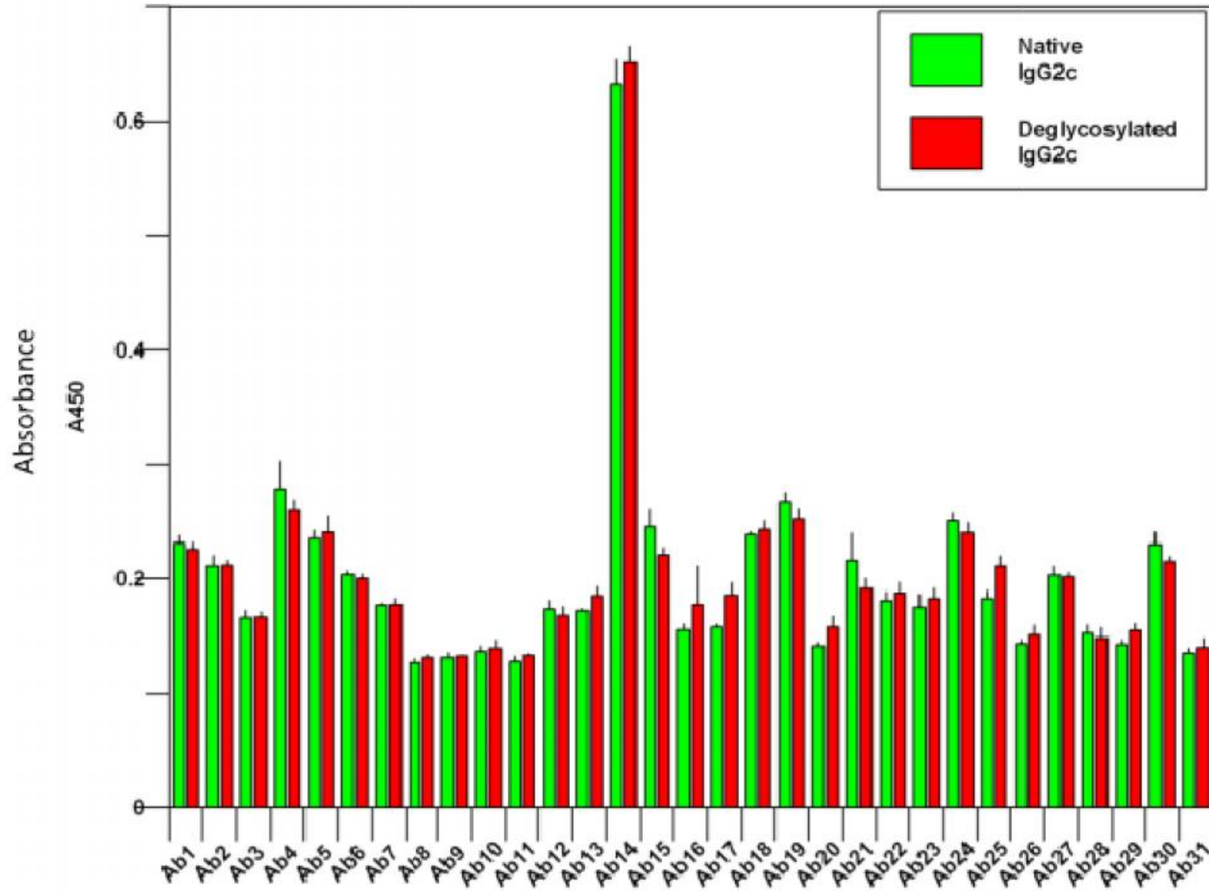

258 N-glycosylation of mAbs occurs within the C_{H2} region at Asn 297(Jefferis R., 2007, 2009) and this
259 epitope is represented in the PCA ELISA by pAb25. In the experimental data (Fig 4), increased epitope
260 exposure at pAb25 was observed. There was also additional epitope exposure at pAb17 which measure
261 epitopes in the hinge region end of the C_L domain. This result indicated that the removal of the glycosyl
262 group caused some additional epitope exposure but not a dramatic conformational change. This is
263 consistent with an earlier study where mAb with and without glycosylation was shown to have similar
264 secondary as well as tertiary structure as analyzed by Fourier Transform Infrared (FTIP) spectroscopy
265 and Intrinsic Fluorescence respectively(Zheng K. et al., 2011).

266 **Effect of Glycation on IgG HOS**

267 mAb glycation is one of the common reactions encountered during the mAb production

268 process(Harris R J., 2005; Harris R J. et al., 2004; Kennedy D M. et al., 1994; Miller A K. et al., 2011; Quan

269 C P. et al., 2008; Zhang B. et al., 2008). To study the impact of mAb glycation on its HOS, a glycated

270 sample of IgG2 was compared to a control sample. No significant HOS changes were observed after

271 glycation (Fig 5), this result was consistent with a previous report indicated that mAb glycation does not

272 impact the bioactivity of the molecule; one of the reasons for not detecting any change in bioactivity

273 could be that the glycation site is not in the CDR regions of this particular mAb.

274 **Figure 5. Effect of glycation on the HOS of IgG2 molecule**

275 Comparison of native IgG2d with glycated IgG2d. Both samples were tested on the ELISA plate

276 at 5μg/ml.

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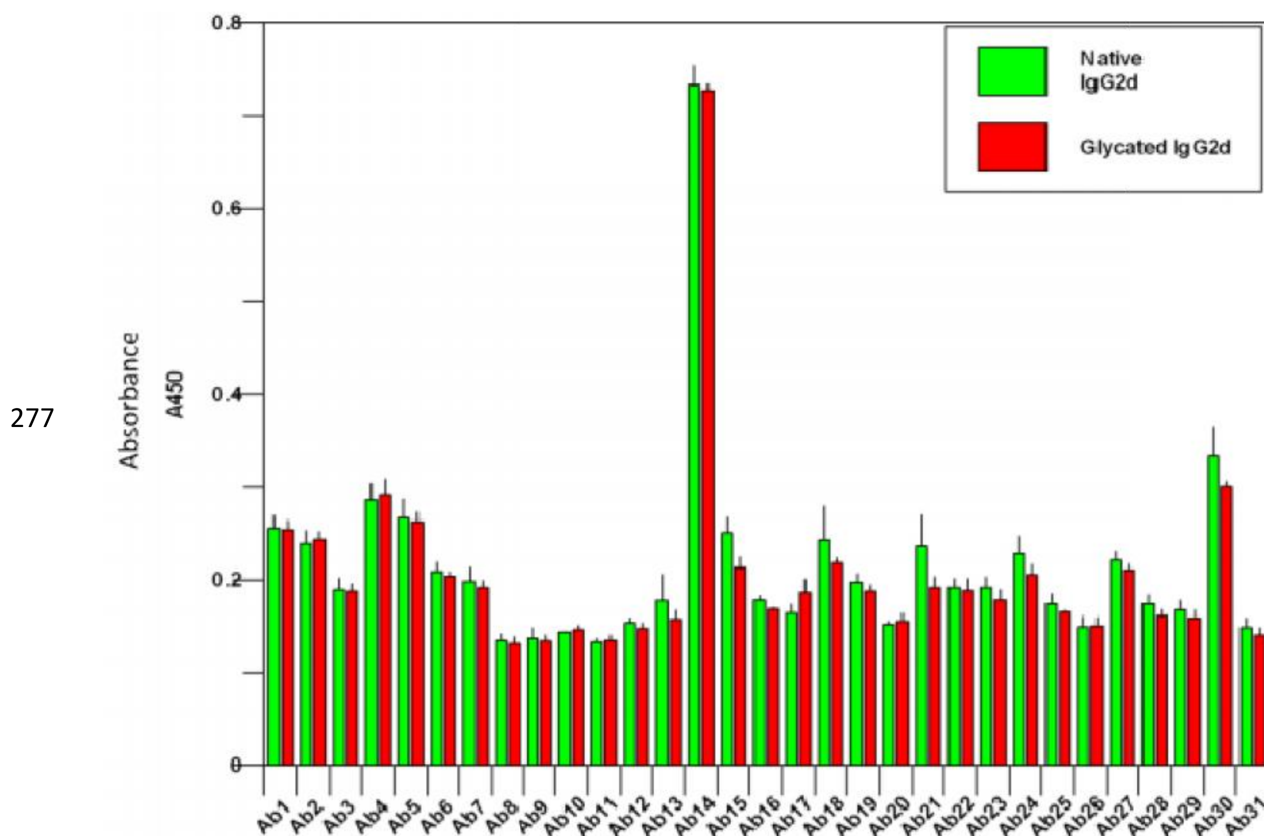
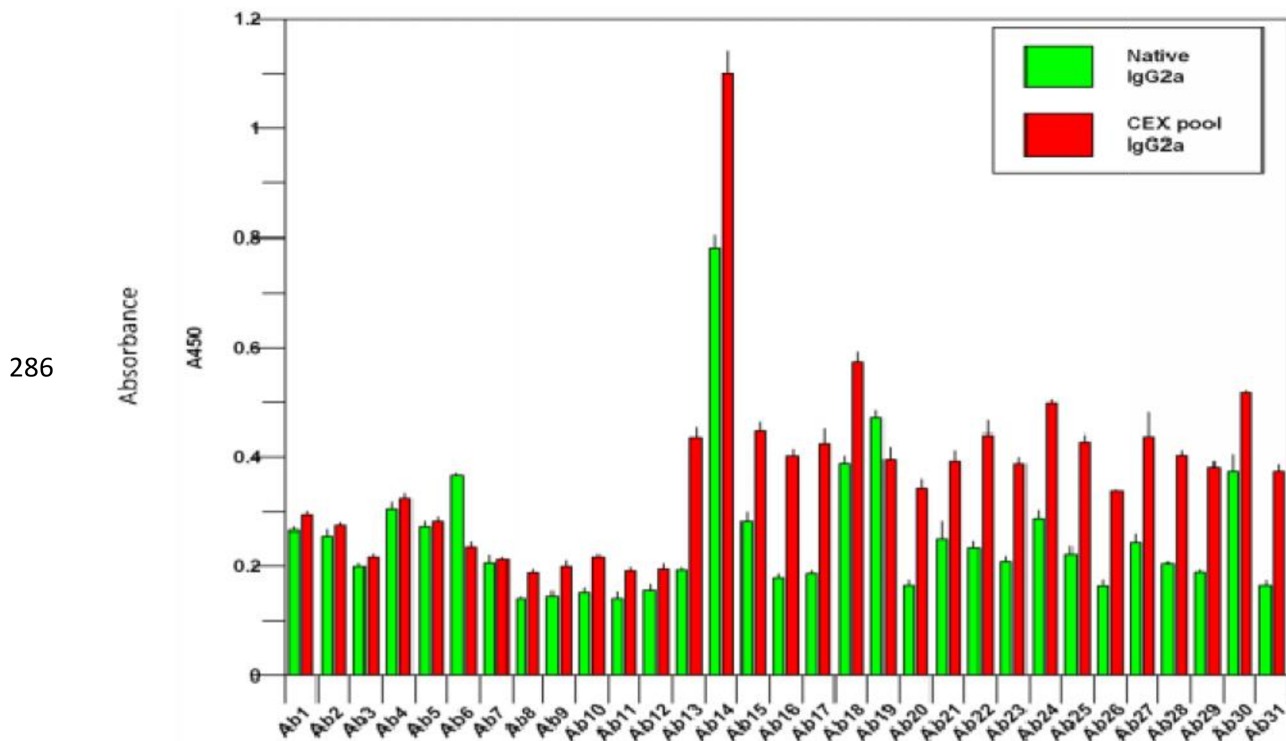


278 **Analysis of Cation Exchange Chromatography (CEX) Variants**

279 Cation exchange chromatography (CEX) is often used to separate charge variants of IgGs. In this study,
280 we examined one particular CEX pool of IgG2 which had several fold higher binding activity in the FcRn
281 assay. The PCA ELISA results (Fig 6) showed significantly higher epitope exposure in the Fc region (pAbs
282 13-31) of this mAb and slightly higher exposure in the variable region (pAbs 1-12).

283 **Figure 6. HOS analysis on an IgG2 molecule with increased FcRn binding**

284 Comparison of native IgG2a with a selected cation exchange chromatography pool of the same
285 IgG2a. Both samples were tested on the ELISA plate at 5μg/ml.

286 

287 Very interestingly, in the region covered by pAb6 which is in the light chain CDR3, there is a decrease
288 of epitope detection, suggesting an inward movement of this region. Another region where a possible
289 inward movement was detected is pAb19 which covers the CH1 domain close to the hinge region. The

290 higher epitope exposure in the Fc region could contribute to the higher binding activity observed in the
291 FcRn assay.

292 **Discussions**

293 While the Protein Conformational Array technology was originally developed to systematically
294 measure the surface epitope exposure and compare conformational status of biosimilar mAb and its
295 corresponding innovator biologics(Wang X et al., 2014; Wang X et al., 2013), it has proven equally useful
296 in measuring conformational impurities produced during degradation studies for novel mAbs. With a
297 relatively large panel of antibodies covering the whole mAb molecule, the surface epitope distribution
298 measured by the PCA ELISA enables biosimilar developers to pinpoint regions of the mAb molecule that
299 are susceptible to process-induced HOS changes and allows the use of the technology to monitor and
300 improve the biosimilar mAb HOS profile compared with that of the innovator reference. This, together
301 with the matching of other critical attributes, will help to produce a biosimilar mAb that is highly similar
302 to the reference mAb. In the current study, different chemical and physical conditions often
303 encountered during process and formulation development were used to study both IgG1 and IgG2
304 molecules for their HOS response. One of the striking findings was the different HOS response between
305 IgG1 and IgG2 under chemical and physical conditions tested. It is known that human IgG1 and IgG2
306 are highly homologous in the constant region with the main difference in the hinge region. However,
307 when treated at higher pH (pH 8), IgG1 molecule is relatively stable with just regional and relatively
308 minor changes (Fig. 2A) whereas in IgG2, significant loss of epitope detection was observed across the
309 whole constant region (Fig. 2B), mAb deamidation data was not available for this two mAbs tested, but
310 it will be interesting to know if the difference was mainly from the differential deamidation of the two
311 molecules or through other mechanisms. Since mAb deamidation results in the loss of the amine group,
312 it is reasonable to expect a decrease of epitope detection using the PCA ELISA. Another interesting

313 observation in the IgG1 vs. IgG2 HOS comparison is the opposite response in the region covered by
314 pAb14. For IgG1, it was an increase of epitope exposure whereas for IgG2, it was the decrease of
315 epitope exposure. When both IgG1 and IgG2 were tested at neutral pH (the reference condition), the
316 HOS status was also dramatically different, suggesting that at least these two IgGs have very different
317 HOS profiles (Fig. 2A, 2B). This is in consistency with a previous study where seven marketed mAbs were
318 analyzed with the PCA ELISA for their HOS status and each showed a unique and different HOS profile,
319 suggesting again that the process defines the product(Wang X et al., 2013). Compared with higher pH,
320 acidic conditions caused more dramatic changes in mAb HOS. As indicated in Fig. 2C, at lower pH, there
321 is a general unfolding of a small portion of the mAb, probably between 0.1% and 0.5% as estimated from
322 a previous study(Wang X et al., 2013). In addition, there were more specific conformational changes in
323 the constant region (pAb14 to pAb31), this is also consistent with a recent study where a purification
324 holding step at lower pH after Protein A affinity column elution resulted in significant new epitope
325 exposure (manuscript in preparation). Compared with different pH conditions, the effect of light seems
326 to cause a general mAb unfolding as indicated in Fig. 3. In addition to a small portion of mAb unfolding
327 (0.1% to 0.5%), there was additional local epitope exposure close to the hinge region as indicated by
328 pAb19; this probably suggests an opening of this area under the conditions tested. Compared to the
329 previous conditions, mAb deglycosylation does not result in significant HOS changes as tested by surface
330 epitope exposure, this is consistent with an earlier study testing the effect of mAb deglycosylation on its
331 secondary and tertiary structure(Zheng K. et al., 2011), however the fact that PCA ELISA can detect a
332 small but quantifiable HOS change around the glycosylation site (Fig. 4, pAb25) demonstrated that this
333 technology is sensitive in the detection of HOS changes resulted from different glycosylation. In an
334 earlier test on a biosimilar mAb, PCA ELISA also detected a minor HOS difference between the biosimilar
335 mAb and the reference molecule and separate glycosylation analysis also indicated a minor difference in
336 the mAb glycosylation pattern (data not shown). mAb glycation is one of the chemical modifications

337 encountered during upstream process; the current study showed that mAb glycation does not result in
338 significant changes in its HOS. This is consistent with the findings from bioassays where no difference
339 was detected between mAbs with and without glycation. However, when a mAb was found to have
340 increased FcRn binding activity, significant HOS difference could be detected (Fig. 6). In this case, a
341 specific fraction of the mAb from a cation exchange column was found to have significant increase in
342 FcRn binding activity, and PCA ELISA analysis showed that major changes in HOS in both the variable
343 region as well as constant region where the FcRn binding supposed to occur. Since there is no increase
344 of epitope exposure across the panel in this test, this result suggests that the mAb is not undergoing
345 general unfolding process. From a regional point of view, there is probably an inward movement in the
346 region corresponding to pAb6 which is located to light chain CDR3. On the other hand, in the constant
347 region, there was an increase of epitope exposure, suggesting an outward movement of mAb structure.
348 It should be pointed out that two possibilities could explain the increased epitope exposure in the
349 constant region: one is that the whole mAb population has a uniform change in their HOS, and the other
350 possibility is that different portions of the mAb changed in different but defined region, and the sum of
351 the changes resulted in the increase in epitope exposure across the whole constant region, further
352 studies are needed to distinguish these two possibilities.

353 In summary, using the Protein Conformational Array ELISA, HOS changes under many chemical and
354 physical conditions encountered during mAb development were observed. More interestingly, it seems
355 that IgG1 and IgG2 may respond to those conditions differently. Furthermore, not all the conditions
356 have similar impact to the mAb HOS; it seems that lower pH, higher temperature and light exposure will
357 result in more changes in the mAb HOS, whereas protein glycosylation, glycation and higher pH have
358 less impact to the mAb HOS status. In a separate study, it is also demonstrated that the upstream and
359 downstream process conditions have major impact to the mAb HOS status (manuscript in preparation).
360 These studies demonstrated that the PCA ELISA could be used in the mAb process development,

especially in purification and formulation development. Another interesting finding from this study was that the PCA ELISA is stability-indicating as demonstrated in the response to stress conditions of different pH and temperature; this suggested that the PCA technology can be a valuable technology for mAb formulation development. Indeed, recent studies indicated that the HOS status of formulated mAb can be assessed by PCA ELISA directly without any treatment; this is different from other analytical analysis where the samples were diluted or treated under conditions that are different from the formulation condition. This study also demonstrated that in addition to its value in providing a molecular level analysis of the mAb HOS and a fingerprint readout of the mAb HOS status for biosimilars, the PCA technology can also be used in novel mAb development, providing sensitive and detailed information for the further improvement of the process to eventually develop a more consistent process and high quality product.

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