Original Research Article

mAb Higher Order Structure Analysis with Protein Conformational Array ELISA

Abstract:

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

Keywords:

Formulation Development; Monoclonal Antibody; mAbs; Higher Order Structure; HOS; Protein Conformational Array ELISA; Conformational Impurity.

Introduction

Since the introduction to market of the first recombinant biologics, human insulin, in 1982, more than 200 marketed biologics have followed, firmly establishing this class of drugs 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 biologics(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 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

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

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

demonstrated that the PCA ELISA can be used to benchmark the innovator mAb and that data was used to compare with several biosimilar candidates(Wang X et al., 2014). Testing with biosimilar mAbs developed in several countries indicated that the HOS comparability can vary greatly. Recently the comparability analysis of the first mAb biosimilar (Remsima) approved by the EMA was reported(Jung S. et al., 2014). In this study, the PCA ELISA was used as one of the analytical technologies to assess the mAb HOS comparability and the data was consistent with Remsima having high HOS comparability with the reference Remicade molecule. Due to its sensitivity and ease of use, the PCA ELISA has utility in many stages of mAb development, from cell line selection to bioprocess and formulation development. Another benefit of the antibody array technology lies in its ability to quantify small amounts of conformational impurities using an easy-to-operate ELISA format. The sensitivity of the assay can be increased by increasing the concentration of analyzed mAb. As low as 0.1% conformational differences could be detected from all the areas covered by the antibodies, thus providing a very accurate and sensitive measurement of the status of the mAb conformation(Wang X et al., 2013). While we do not yet have data to correlate the impact of this conformational impurity to the safety of the mAb, it is reasonable to postulate that more conformational impurities (epitope exposure) may bring an increased risk in potential immunogenicity(Buttel I et al., 2011; Hermeling S. et al., 2005; Hermeling S. et al., 2004; Jiskoot W. et al., 2009; Rosenberg A., 2006; Schellekens H., 2002, 2005; Sharma B., 2007). Furthermore, exposure of new epitopes will also increase the possibility of the mAb interacting with other regulatory proteins in the body, causing off-target effects. Recent data from PCA ELISA testing on biosimilars has correlated regional structural changes with loss of efficacy for the biosimilar candidate (data not shown). Importantly, some local structural differences that can be elusive to other analytical technologies including

bioassays, can be detected with the PCA ELISA, demonstrating complementary value of this epitope-based technology in mAb HOS characterization.

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

Materials and Methods

Reagents: All the chemicals were purchased from Sigma-Aldrich. 96-well microplates were purchased from Corning Co. (#9018). Streptavidin-HRP conjugate (PI-21130) and biotin labeling kits (PI-21425)were obtained from Thermo Fisher.

Antibodies and ELISA kits: All the reagent antibodies and ELISA kits used in this study were products of Array Bridge Inc. (AB000208). Polyclonal antibodies against the peptides were produced in New Zealand White Rabbits. For the sandwich ELISA, antibodies against each region of the mAb molecule were first coated on the 96-well plate; with each antibody coating 6 wells in row B through G. In each column of the coated plates, the upper three wells (B, C, and D) were incubated with a reference mAb in triplicate, and the lower three wells (E, F, and G) were incubated with the same mAb after treatment. A biotin-labeled rabbit anti-human IgG antibody was used to detect the mAb-peptide antibody complex, and streptavidin-HRP was used to detect the complex formed by anti-human IgG-mAb-peptide antibody. The signal

strength of the sandwich ELISA depends on the relative epitope exposure of the mAb in each region. If there are more epitopes from the mAb that could be recognized by the peptide-derived antibody, a stronger signal will be produced and vies versa.

Sample Treatment and Analysis: All the samples were treated at formulated protein concentration under different conditions. For temperature treatment, the samples were incubated at the indicated temperature for either 10 days (IgG1) or 14 days (IgG2) and diluted to 5 µg/ml for analysis. For higher pH treatment to generate deamidated mAb, the mAb samples were treated in Tris buffer at pH 8 for 10 days at room temperature before analysis. For acidic pH treatment, the pH was adjusted to pH 3.6 with acetic acid and incubated at room temperature for 10 days before analysis. For light treatment, the mAb sample was treated with white light at 416,000 lux for 52 hrs. before the assay. For mAb deglycosylation, the mAb sample was treated with PNGase F at 400:1 enzyme dilution and incubated at 37°C in Tris buffer, 100 mM, pH 8.5. For glycation, the mAb sample was treated with 222 mM glucose at 4°C for 1 month. For the sandwich ELISA, six rows of anti-peptide antibodies were coated in 96-well plate from row B to G. For comparability studies, the first three rows (B, C, D) were used for reference mAb, while rows E, F and G were used for the analysis of the mAb under various treatment. For reporting antibody, a polyclonal anti-human IgG antibody (developed by Array Bridge Inc.) was used which will detect the capturing antibody-mAb complex. The reporting antibody was labeled with biotin which in turn forms a complex with streptavidin-HRP conjugate, TMB (3,3',5,5'-tetramethylbenzidine) was used as substrate for the HRP enzyme activity assay. Following a short development time to allow color formation from the HRP enzymatic activity, an equal volume of 1M sulfuric acid was added to stop the reaction. A

spectrophotometer from Molecular Devices, the SpectraMax M3 was used to measure the color change at 450 nm.

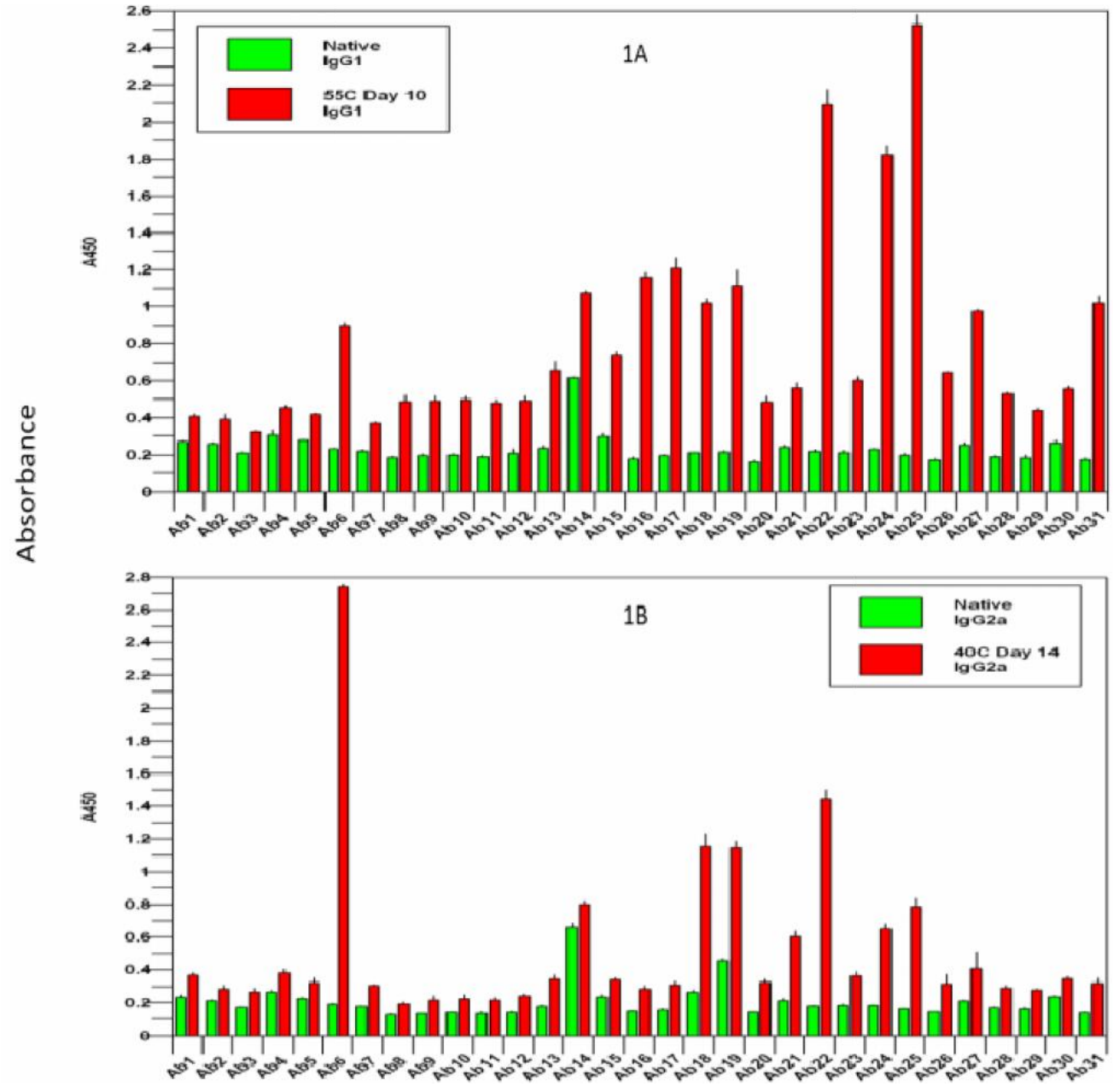
Results

Effect of Temperature on mAb HOS (IgG1 vs IgG2)

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

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

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



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

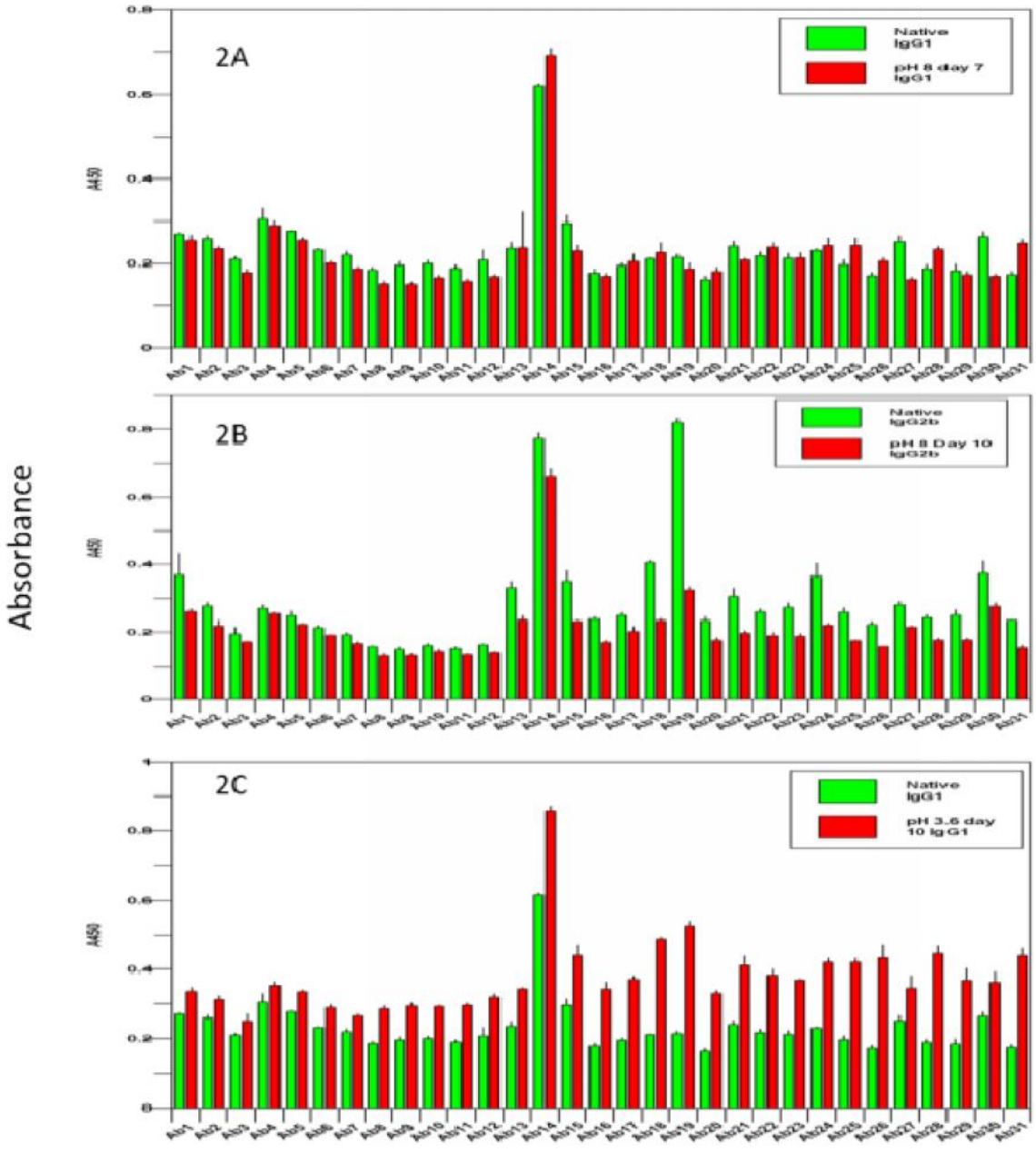
result suggested greatest sensitivity to temperature in the light chain at the boundary between the V_L and C_L domains (pAb6), in both chains near the hinge region (pAbs 14-22) and in the heavy chain C_{H2} region (pAbs 22-25). In contrast, the IgG2 result suggested somewhat better stability in the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

Effect of pH on mAb HOS (IgG1 vs IgG2)

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

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

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



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

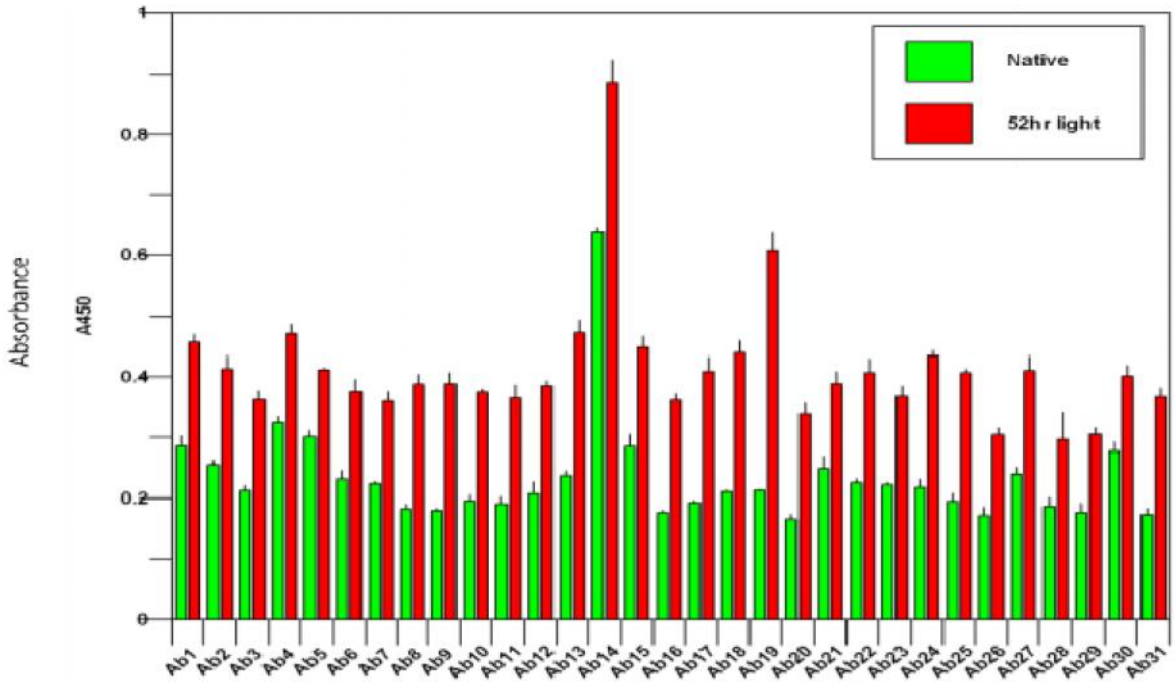
showed significant new epitope exposure across the entire molecule with 10 day incubation to pH 3.6. Low pH (below 4.) has been shown by others(Mason D. et al., 2012) to cause reversible aggregation in IgG molecules, the result here correlated well with previous findings and also demonstrated that PCA ELISA could be used to characterize mAb aggregates induced by lower pH conditions.

HOS Stability to Light Exposure

It is known that light exposure could potentially impact the HOS of biologics(Mason D. et al., 2012). During purification process, mAbs may be exposed to parts of UV-C (200 to 290 nm), UV-B (290 to 320 nm) and visible light (400 to 760 nm) under a variety of buffer and pH conditions.

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

Comparison of native IgG1 with IgG1 that had been treated with white light at 416,000 lux for 52hrs, both samples was tested on the ELISA plate at 5μg/ml.



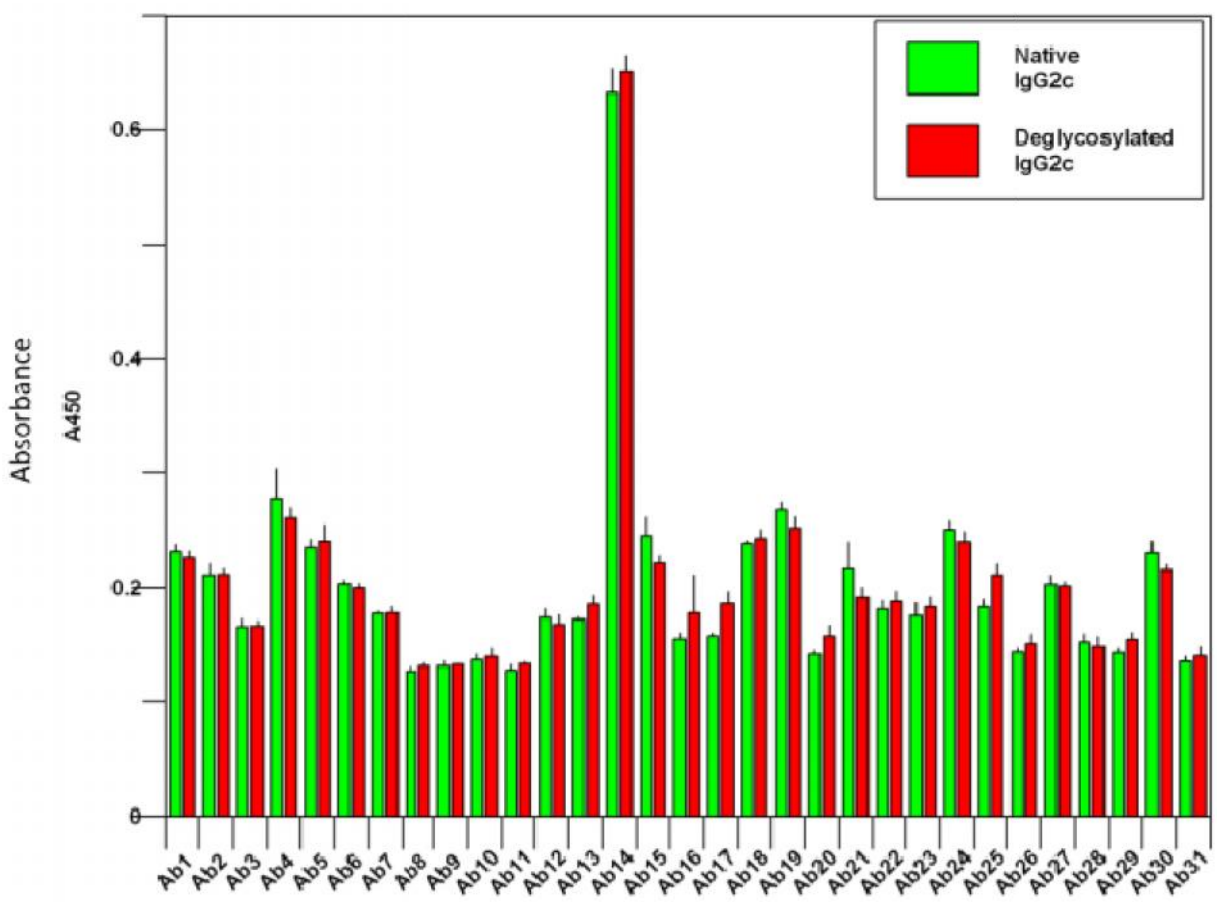
The combination of these conditions was known to promote both chemical and physical degradation which may result in conformational changes. In this experiment, a sample of an IgG1 mAb which was irradiated by white light at an intensity of 416,000 lux for 52 hours was compared to a control sample. As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

Effect of Deglycosylation on mAb HOS

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

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

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



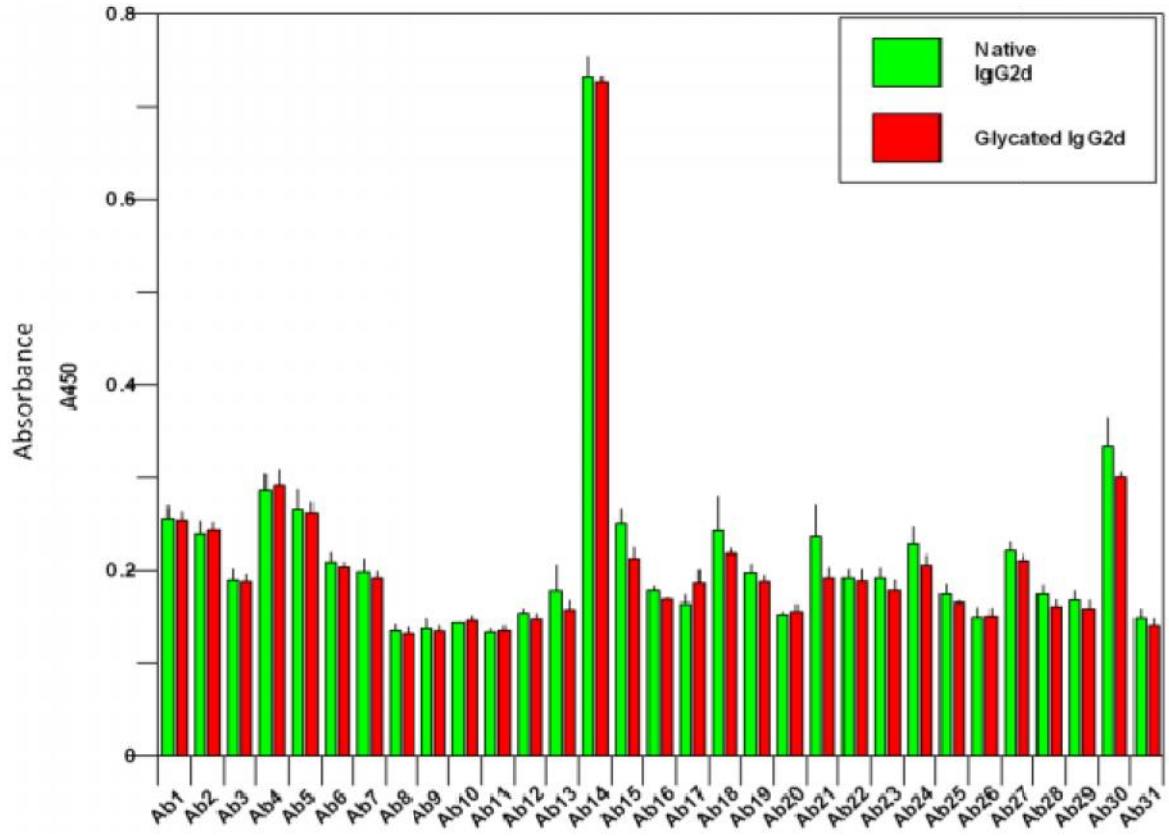
N-glycosylation of mAbs occurs within the C_{H2} region at Asn 297(Jefferis R., 2007, 2009) and this epitope is represented in the PCA ELISA by pAb25. In the experimental data (Fig 4), increased epitope exposure at pAb25 was observed. There was also additional epitope exposure at pAb17 which measure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

Effect of Glycation on IgG HOS

mAb glycation is one of the common reactions encountered during the mAb production process. To study the impact of mAb glycation on its HOS, a glycated sample of IgG2 was compared to a control sample. No significant HOS changes were observed after glycation (Fig 5), this result was consistent with a previous report indicated that mAb glycation does not impact the bioactivity of the molecule.

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

Comparison of native IgG2d with glycated IgG2d. Both samples were tested on the ELISA plate at 5μg/ml.

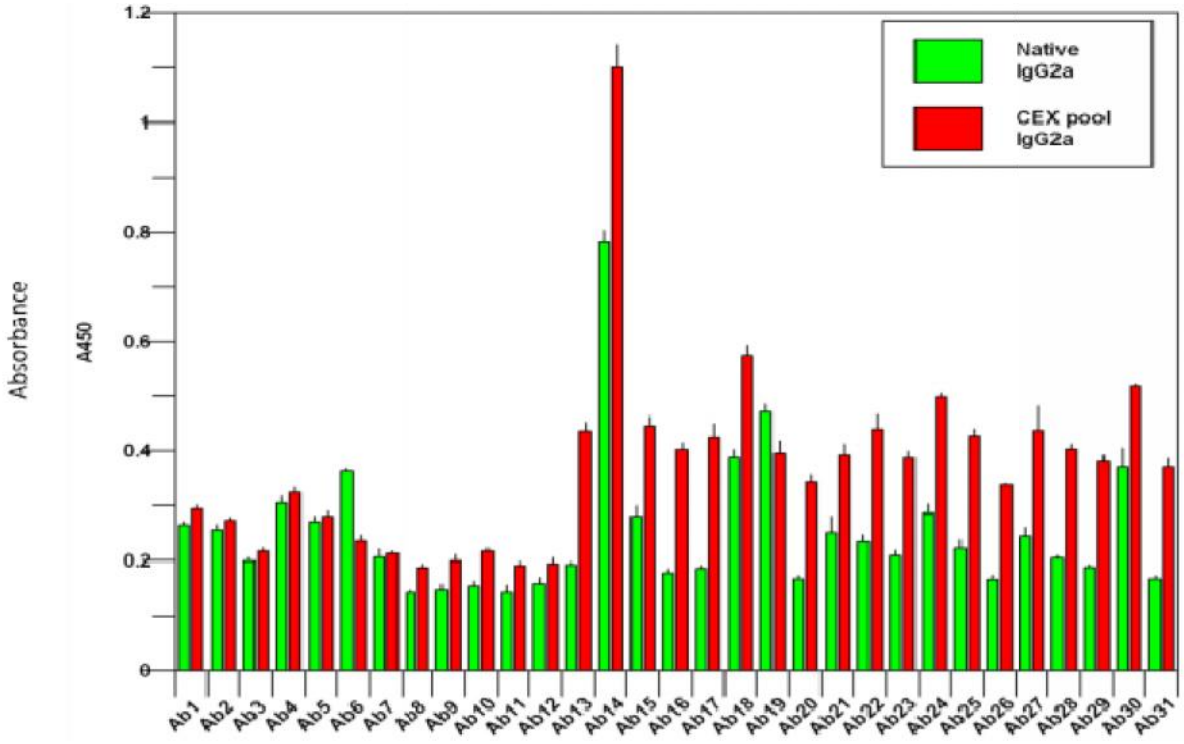


Analysis of Cation Exchange Chromatography (CEX) Variants

Cation exchange chromatography (CEX) is often used to separate charge variants of IgGs. In this study, we examined one particular CEX pool of IgG2 which had several fold higher binding activity in the FcRn assay. The PCA ELISA results (Fig 6) showed significantly higher epitope exposure in the Fc region (pAbs 13-32) of this mAb and slightly higher exposure in the variable region (pAbs 1-12).

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

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



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

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

Discussions

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

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

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

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

especially in purification and formulation development. Furthermore, this study demonstrated that the PCA technology can help to quantify regional changes in the mAb and more importantly providing valuable inputs for the further improvement of the process to make more consistent and high quality products. 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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