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Biosimilar mAb in-process Sample Higher Order Structure Analysis with Protein Conformational Array ELISA

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Authors' contributions

This work was carried out in collaboration between all authors. Author XW designed the study and wrote the first draft. Authors MD and GF performed the experiments. Authors GW and JG participated in the design of experiments and critical manuscript review. All authors read and approved the final manuscript.

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ABSTRACT

Biologics higher order structure (HOS) plays an important role in the molecule's biological function and is closely related to its immunogenicity property. A novel technology to study changes in HOS is the Protein Conformational Array (PCA) ELISA which uses a bank of 34 antibodies to measure epitope distribution on the surface of biologics such as mAbs. The objective of this study is to use Protein Conformational Array technology to analyze mAb HOS status during bioprocess development. Under carefully controlled assay conditions, the mAb epitope distribution can be thought of as a 'fingerprint' of the biologics being studied and many physiochemical changes would correlate with changes in HOS. MAbs with additional epitope exposure compared to the reference

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standard or innovator mAb can be considered as conformational impurities. In this study we used the PCA ELISA to follow this epitope 'fingerprint' to study the HOS of two biosimilar mAbs under development; a large number of samples from both upstream and downstream of the process were analyzed. In these two particular cases, an increase in epitope exposure was observed from the two biosimilar mAb cell culture samples in the later stage of the upstream process. During the downstream process, the PCA ELISA indicated that almost all of the mAb conformational impurities were removed, producing a biosimilar candidate with high HOS similarity to the reference standard.

Keywords: Biosimilar mAb; higher order structure; bioprocess development; protein conformational array ELISA; conformational impurity; bioassays.

1. INTRODUCTION

In biologics development, the bioprocess defines the product. Because of the high complexity of biologics in its molecular structure and composition, producing high quality biologics and producing it consistently is the focus in biologics development and production [1]. During bioprocess development, impurity analysis and evaluation is one of the major analytical activities. Bioprocess impurities can be divided into two groups, process-related impurities and product-related impurities. In the process-related impurities, host cell proteins (HCPs) and host cell DNA are the two major molecular classes where analytical methods have been developed at a sensitivity of parts per million (ppm) and parts per billion (ppb) level respectively [2-4], and the methods have provided invaluable information for the successful development of many biologics. On the other hand, the product-related impurities include protein degradation products such as clipped molecules, acidic and basic species, and different disulfide bond formation [5-8], this class also includes different glycosylation isoforms and oxidation species, and different forms of aggregation. Many technologies have been developed over the years and successfully used to characterize and quantify these impurities [6,7]. However, it should also be noted that even with the extensive use of many analytical technologies to characterize and evaluate the biologics molecules, the majority of biologics under development still failed during clinical development. The reasons for the clinical failure can be complex but this outcome suggests that in spite of all the analytical and biological evaluation, there are still many aspects of the biologics molecule which are important to its safety and efficacy that can't be detected or precisely evaluated by existing technologies, hence pointing to the needs to develop novel technologies to provide more insights on the biologics molecule.

It is known that protein HOS is important to the immunogenicity and safety of the molecule, however it is also realized that the precise protein HOS could be difficult to define [8-24]. In our previous studies, it has been shown that the protein conformational array ELISA can detect and quantify the impact of many physical and chemical conditions on the HOS of the molecule which is closely related to its immunogenicity and efficacy [10,22]. Within biologics, monoclonal antibodies (mAbs) are the fastest growing class of human therapeutics, with more than 40 IgG-based drugs approved [25]. Recently an area drawing significant attention in biologics has been the development of biosimilars. 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 [11]. Not only does this additional complexity play a role in the critical quality attributes (CQA) of the innovator biologics but also in the production of subsequent biosimilars [8,26-29]. The term 'higher order structure' (HOS) has been used to describe the 3-D structure resulting from the cumulative effects of 1º, 2º and 3º structure as well as post translational modifications to the protein. The bioprocessing and formulation conditions can impact the 3-D structure of biologics significantly. 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 [21,22], suggesting again that process defines the product. A further investigation on mAb degradation introduced by different pH, temperature, glycosylation, glycation and oxidation suggested that this PCA ELISA is stability-indicating and can precisely determine the impact of product-related degradations on the mAb HOS, which is closely related to the molecule's immunogenicity and efficacy [10].

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 [22]. 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 protein. However, if the protein conformation changes slightly, then the panel of antibodies is primed to recognize the resulting change in epitope exposure. The ability of the PCA ELISA to both interrogate the entire surface of the mAb and also pinpoint the regions where changes had occurred suggested to us that the antibody array technology could provide a unique measurement of biosimilar mAb HOS comparability. Studies 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 [23]. Recently the comparability analysis of the first mAb biosimilar (Remsima) approved by the EMA was reported [15]. In that 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. While it is difficult to correlate the impact of conformational impurity to the safety of the biosimilar mAb, it is known that completely unfolded or even partially unfolded antibody is not part of the normal human immune system thus these species or "conformational impurities" could be considered as "foreign molecules" by the human immune surveillance system and induce immune response. Thus t is reasonable to postulate that more conformational impurities (epitope exposure) may bring an increased risk in potential immunogenicity [19,30-34].

In this report we examined the ability of the PCA ELISA to detect HOS changes during bioprocess development. Two biosimilar mAbs were analyzed for their HOS status in different stages of the bioprocess, including different culture conditions and purification columns. The results suggested that the PCA ELISA could be of value in defining and controlling mAb HOS impurities during the bioprocess development and contribute to the development of high quality biologics consistently.

2. MATERIALS AND METHODS

2.1 Reagents

All the chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri). 96-well micro-plates were purchased from Corning Co. (Corning, New York). Streptavidin-HRP conjugate and biotin labeling kits were obtained from Thermo Fisher Scientific (Rockford, Illinois).

2.2 Antibodies and ELISA Kits

All the antibodies and ELISA kits used in this study were products of Array Bridge Inc. (St. Louis, Missouri, www.arraybridge.com). Polyclonal antibodies against mAb peptides were produced in New Zealand White Rabbits. For the initial (three antibody) testing, peptide antibodies 19, 25 and 30 were coated on separate 96-well microplates, and the in-process samples were diluted to 5 µg/ml to carry out the test. For the full panel ELISA analysis, antibodies against each region of the mAb molecule were first coated on the 96-well plate with each antibody coating one column (6 wells) in rows B through G. In each column of the coated plates, the upper three wells (B, C, and D) were incubated with one mAb (such as the reference material) in triplicate, and the lower three wells (E, F, and G) were incubated with a second mAb (biosimilar) in triplicate. A biotin-labeled rabbit anti-human IgG antibody (developed by Array Bridge Inc.) 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. TMB (3,3', 5,5'-tetramethylbenzidine) was used as substrate for the HRP enzyme activity assay. Following a 15 minute 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 Devise, the SPECTRA max Plus, was used to measure the color change at 450 nm. 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 vice- versa.

3. RESULTS

3.1 Upstream and Downstream Process Sample Analysis

The PCA technology was developed from the amino acid sequence of the mAb and provides a sensitive and systematic measurement of the surface epitope exposure for the mAb of interest [22].

34 different antibodies were raised against overlapping peptides covering the whole mAb molecule, any specific epitope changes of 0.1% or more from that of a reference mAb can be detected and quantified using this technology, Fig. 1 is a diagram on the coverage of the PCA ELISA on the mAb molecule. Since the exact location of each peptide in the 3-D structure is known and the antibodies generated from those defined peptides were shown to have good specificity [22], the PCA ELISA can detect and quantify local conformational changes and help to identify when and where in the bioprocess a conformational change was introduced. It is known that protein conformation is closely related to its efficacy and immunogenicity [9,12,35-38]; the information on the mAb conformation will be valuable in the mAb development. Previous studies have shown that the PCA ELISA can be used for biosimilar mAb HOS comparability analysis [23]; conformational differences were detected from some biosimilar mAbs as compared with the corresponding reference material. It is also interesting to note that the differences detected in the mAb HOS correlated well with other analytical and bioassay results. In this study, two biosimilar mAbs were tested for their HOS status during process development. For biosimilar-1, a total of 30 samples spanning the entire bioprocess were analyzed using the PCA ELISA. Because of the large number of in-process samples, to reduce the complexity of the analysis, for the first step, three PCA ELISA antibodies known to detect conformational changes in some “hot spots” of the mAb molecule were chosen to detect conformational changes. Among the 3 PCA antibodies selected, antibody 19 covers the mAb heavy chain amino acid 154 to 179 (based on Trastuzumab amino acid sequence), at the interface between the Fv and CH1 domain. Antibody 25 covers the Heavy chain amino acid 272 to 293, close to the hinge region and glycosylation site, and finally antibody 30 covers the heavy chain CH3 amino acid 355-379, close to the C-terminal of the heavy chain. The regions covered by the three selected antibodies are shown in Fig. 2.

Ab14 is covering light chain amino acid 135 to 155, Ab16 is covering light chain amino acid 168 to 194 and Ab26 is covering heavy chain CH2, amino acid 288 to 313.

Fig. 3 depicts the results of the PCA test using these three antibodies for biosimilar-1. In the upstream samples, mAbs from different culture conditions (days of culture) were tested directly (prior to any purification steps) for their conformational status. This analysis showed a relatively stable level of conformational impurity (new epitope exposure) up to day 9 and significant increase of conformational impurity at day 10. For downstream process, samples from three purification steps (Protein A, cation exchange and anion exchange columns) were tested. The results indicated that Protein A column eluates had decreased epitope exposure in general compared to the mAbs from the medium with the exception of elute 9 which has significant increase of conformational impurity corresponding to the region of Ab19, suggesting that elution conditions had an impact on the conformational status. In the cation exchange (CEX) column purification, there was a relatively small level of increase in mAb epitope exposure. Following elution of the mAb from the CEX column, there was no significant change in epitope exposure from the anion exchange (AEX) column purification except elute 4 which showed significant increase in epitope exposure in the region covered by Ab19. It will be interesting to know the condition differences between eluate 4 and the other four batches. Finally, the deep filtration retentate and drug substance have similar levels of epitope exposure. When compared with reference standard (last column), the conformational impurity profile seems very similar as measured by the three selected antibodies.

30 samples selected from the biosimilar-1 process were analyzed by three conformational array antibodies. Each sample was diluted to 5 µg/ml and analyzed in triplicates; reference material was used for the assessment of the epitope exposure.

For biosimilar-2 mAb, 14 samples were tested including upstream samples from cell culture harvested from 5 different time points and downstream samples from three purification columns.

18 samples selected from the biosimilar-2 process were analyzed by three conformational array antibodies. Each sample was diluted to 5 µg/ml and analyzed in triplicates.

As shown in Fig. 4, there is a certain degree of epitope exposure as compared with the drug substance, however because no reference standard is available for this candidate, it is difficult to estimate the increases. The mAb from Protein A load had epitope exposure similar to those from the upstream cell culture and after the Protein A column, the majority of the conformational impurity was cleared. On the CEX column, the purification process further decreased conformational impurity as measured by all the three antibodies used. On the anion exchange column (AEX), for an unknown reason, there is a significant epitope exposure in the region covered by Ab19 whereas the other two regions monitored by Ab25 and Ab30 did not show much change. Drug substance has relatively low epitope exposure, however since the reference standard for biosimilar-2 was not available at the time of testing, the HOS status of the drug substance and reference standard can't be compared.

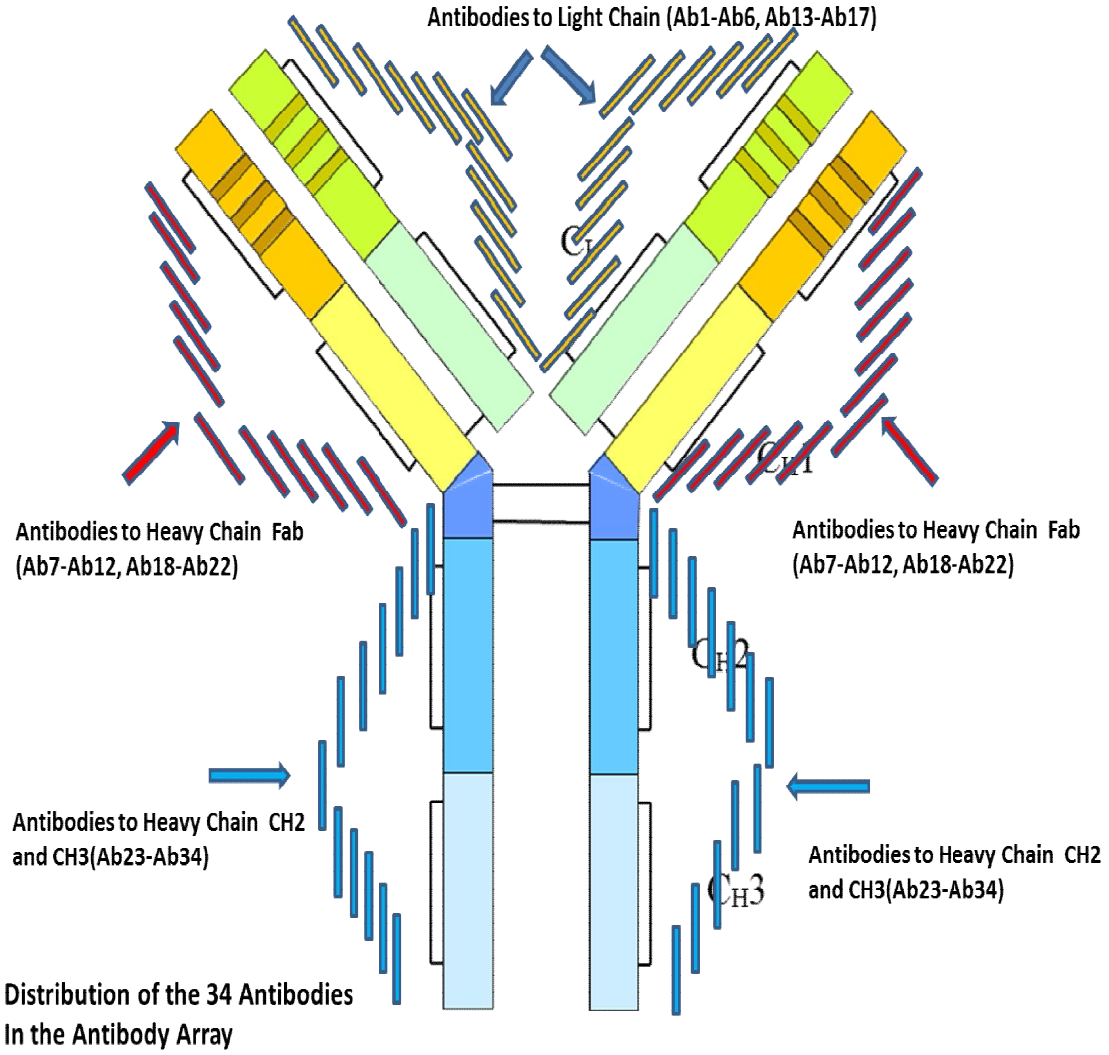

Fig. 1. Diagram of the full panel protein conformational array antibody coverage on monoclonal antibody molecule

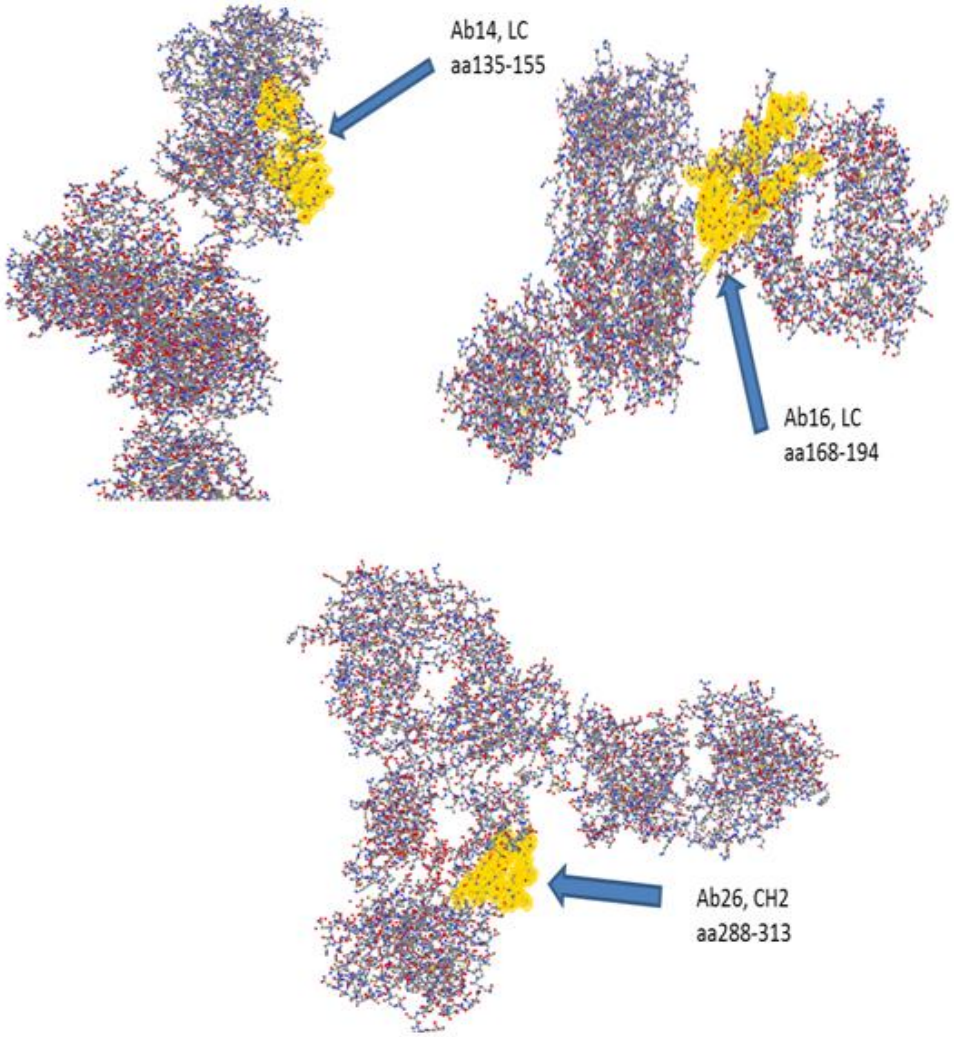

Fig. 2. 3-D diagram of the three antibodies used for the analysis of in-process samples

3.2 Full Panel PCA Elisa Analysis on Drug Substance

After the initial testing of both upstream and downstream process samples by the three selected antibodies above, two samples from Biosimilar-1, cell harvest and drug substance were selected for the analysis with the full antibody panel of 34 antibodies and compared with the reference standard.

Three samples were selected for the full panel conformational array analysis: Protein A Elute 1, Biosimilar-1 drug substance and the reference material.

Fig. 5 showed the testing results covering the mAb variable regions and constant regions of the mAb light and heavy chain respectively. The results indicated that cell harvest had the highest relative epitope exposure among the three samples tested in both the variable region and constant region. Biosimilar-1 drug substance had decreased epitope exposure as compared to cell harvest but similar epitope exposure compared to the reference standard across the full antibody panel. It is interesting to note that for the cell harvest sample, some regions had relatively more epitope exposure than others, such as those regions covered by antibody 2, 3, 8, 18, 24, 25, and to a less extent for the rest of the antibody panel. For biosimilar-2, reference material was not available at the time of testing, therefore only the conformational status of one upstream sample (cell harvest) and drug substance were compared (Fig. 6).

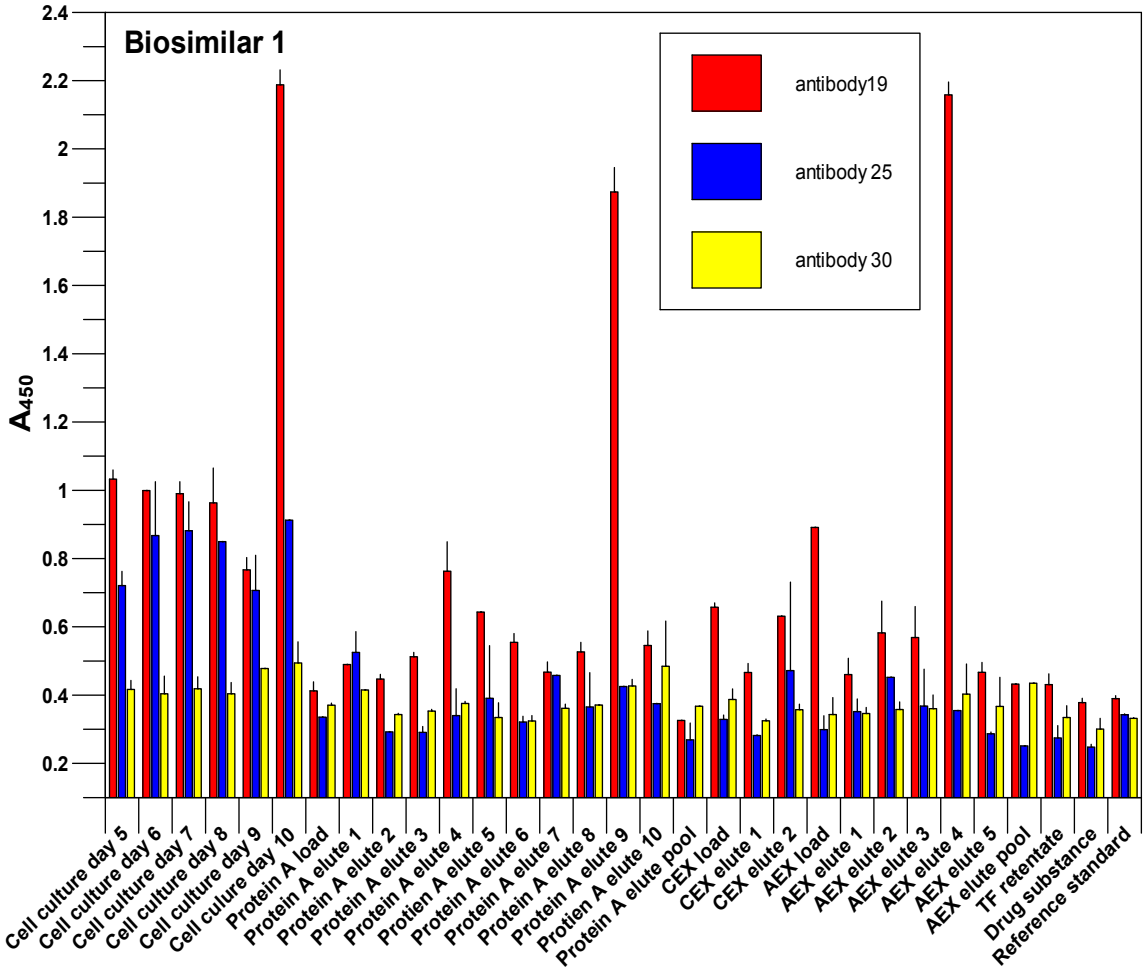

Fig. 3. Biosimilar-1 In-process sample conformational analysis

Two samples were selected for the full panel conformational array analysis: Cell culture day 5 and Drug substance.

As shown in the graph, there was a significant decrease of conformational impurity from cell culture samples to drug substance in the mAb. In specific regions covered by Ab6, 13, 15, 16 and 17, there is significant increase of epitope exposure from the cell harvest sample. It will be interesting to see in future testing how the drug substance conformational impurity compared to that of the reference material.

4. DISCUSSION

The Protein Conformational Array (PCA) ELISA was originally developed to systematically measure the mAb surface epitope exposure and compare conformational status of biosimilar mAbs and their corresponding innovator biologics. With a relatively large panel of antibodies covering the whole mAb molecule, the surface epitope distribution measured by the PCA ELISA enables mAb developers to pinpoint regions of the molecule that are susceptible to process-induced conformational changes or the creation of conformational impurities. In the current study, in-process samples from two biosimilar mAbs were tested for their conformational impurities. For biosimilar-1, the test showed that both the upstream and downstream process can have some impact on the conformational status of the molecule, the full panel analysis also indicated that the increased conformational impurity is not all related to the unfolding of the mAb because different regions of the molecule showed different degree of epitope

exposure, suggesting that the conformational impurity contains mAb species with regional changes. Furthermore, it was shown that almost all the conformational impurities could be removed with the downstream purification process (Fig. 5). It should be noted that in both the upstream (Day 10 cell culture) and downstream (Protein Eluate 9 and AEX Elute 4) steps, there is significant regional epitope exposure, it will be interesting to know how these three samples were handled differently from other samples to induce such conformational changes. Other studies with IgG1 and IgG2 mAbs have shown that many factors could contribute to the increase of conformational impurity including low and high pH, high temperature, oxidation, glycosylation and protein aggregation (data not shown). The data shown here suggested that the protein conformational array is a sensitive method for detecting conformational changes induced by the aforementioned conditions and it can be easily applied to process development. Another salient observation from the biosimilar-1 in-process sample testing is that some regions of the mAb were more sensitive than others to in-process conditions. In the biosimilar-1 case, three antibodies from the panel of 34 were selected for the analysis and it was found that the regions covered by antibody 19 was more sensitive to the in-process conditions that the other two regions looked at (Fig. 3). For biosimilar-2, we found that the culture conditions had a major impact on conformational impurities (Fig. 4). The testing also indicated that the downstream process could remove significant amounts of the conformational impurities. In the full panel analysis of biosimilar-1, it was shown that high similarity were observed in the light chain CDR region (corresponding to Ab2, 3, 6) and heavy chain CDR (Ab11) respectively, and bioassay testing showed that the two mAbs have equivalent bioactivity (data not shown) suggesting that the conformational analysis correlated well with that of the potency assay. In summary, for the full panel conformational analysis, biosimilar-1 has similar epitope exposure across the full panel as compared with the reference material, suggesting that the process is capable of producing mAbs with good HOS similarity. For biosimilar-2, the corresponding reference material was not available at the time of testing, therefore only the relative conformational exposure can be assessed. One of the interesting observations from the biosimilar-2 conformational analysis is that significant new epitope exposure could be introduced during the downstream process as indicated by the AEX elute but eventually this conformational impurity species was either removed or reversed back by the further processing.

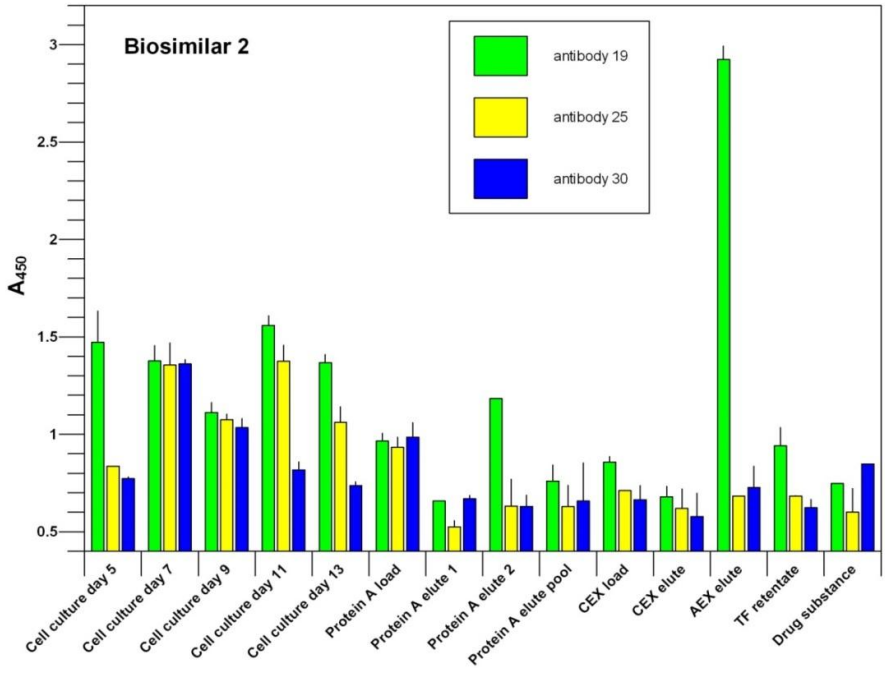

Fig. 4. Biosimilar-2 In-process sample conformational analysis

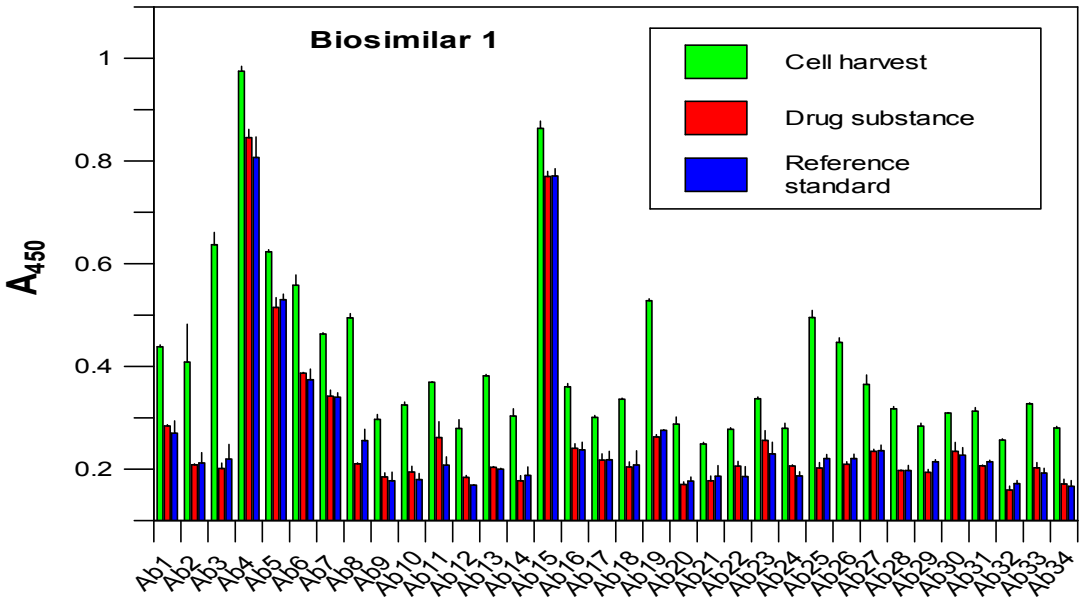

Fig. 5. Biosimilar-1 full panel protein conformational array analysis

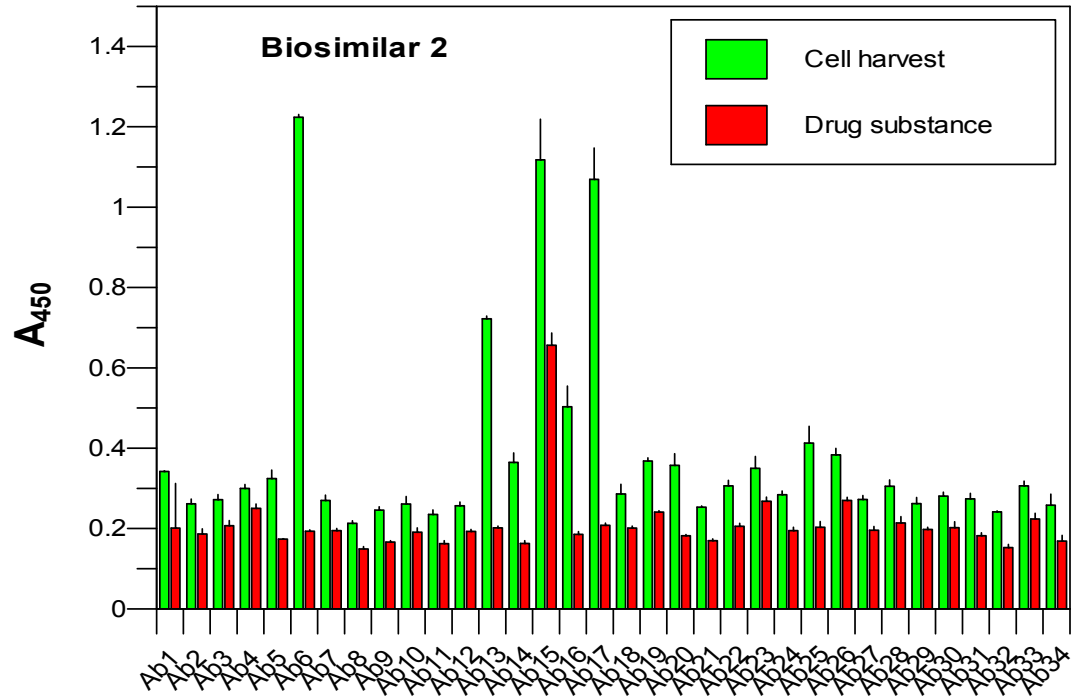

Fig. 6. Biosimilar-2 full panel protein conformational array analysis

5. CONCLUSION

In summary, the analysis of the two in-process biosimilar samples indicated that the protein conformational array ELISA can be used for process development, the assay can be applied to both upstream and downstream samples, and can pinpoint to steps where a conformational impurity profile is changed. One important application for the PCA ELISA is the direct measurement of mAb HOS from the cell culture samples without any prior purification; the affinity of the coating antibodies for only the target of interest means that complex mixtures can be

easily tested without time consuming purification work. In the stage of cell line development, it is convenient to analyze the conformational status of the mAb from each candidate cell line and select the cell line producing the highly similar mAb in their HOS as compared to the reference material. Furthermore, the study presented here also demonstrated that protein conformational array correlated well with that of bioassay in providing mAb conformational characterization systematically and at molecular level.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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