Monoclonal antibody higher order structure analysis by high throughput protein conformational array

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ABSTRACT
The elucidation of antibody higher order structure (HOS) is critical in therapeutic antibody development. Since HOS determines the protein bioactivity and chemo-physical properties, this knowledge can help to ensure that the safety and efficacy attributes are not compromised. Protein conformational array (PCA) is a novel method for determining the HOS of monoclonal antibodies. Previously, we successfully utilized an enzyme-linked immunosorbent assay (ELISA)-based PCA along with other bioanalytical tools to elucidate the structures of antibody aggregates. In this study, applying a new multiplex-based PCA with 48-fold higher throughput than the ELISA-based one we revealed structural differences between different antibody molecules and antibody structure changes affected by various processing conditions. The PCA analysis of antibody molecules clearly demonstrated significant differences between IgG1 and IgG4 subclasses in epitope exposure and folding status. Furthermore, we applied small angle X-ray scattering to decipher mechanistic insights of PCA technology and validate structural information obtained using PCA. These findings enhance our fundamental understanding of mAbs’ HOS in general. The PCA analysis of antibody samples from various processing conditions also revealed that antibody aggregation caused significantly higher exposure of antibody epitopes, which potentially led to a “foreign” molecule that could cause immunogenicity. The PCA data correlated well with protein stability results from traditional methods such as size-exclusion chromatography and protein thermal shift assay. Our study demonstrated that high throughput PCA is a suitable method for HOS analysis in the discovery and development of therapeutic antibodies.

Introduction
As a group, protein therapeutics comprising monoclonal antibodies (mAbs) have grown substantially over the past twenty years.1,2 It is widely known that an antibody’s three-dimensional (3D) conformation affects its biological functions.3,4 Understanding structure-activity relationships and the role of higher order structure (HOS) in the mechanism of action of therapeutic proteins is essential to define and control the critical product quality attributes.5 Regulatory agencies have increasing expectations on HOS characterization of biologics for quality control, and set an especially high bar for biosimilar products.6,7 Determining or monitoring the conformational changes of mAbs in development offers in-depth understanding of the impact of process conditions on protein quality and may lead to further improvement of product and process performance.

Common structural characterization methods such as nuclear magnetic resonance (NMR), X-ray crystallography, circular dichroism (CD), and mass spectrometry (MS) have various limitations when applied to biologics development. NMR and X-ray can provide structural information at atomic level resolution, but need special sample treatments or preparations such as isotope labeling or crystallization to obtain suitable structural information.8,9,10 Lengthy data acquisition periods and instrument availability further limit their application in the industry environment. CD can provide secondary or tertiary structural information depending on the wavelength range used.11 Complicated buffer matrices in samples generated along the development process may have strong absorbance at the near or far UV range, which limits the application of CD in biologics development. In addition, the CD signal is the sum of the whole mAb, and thus it is difficult to determine which part of the protein contributes to the CD signal changes. MS, especially hydrogen-deuterium exchange MS (HDX-MS), can specifically pinpoint where the conformation changes occur. However, MS is expensive, low throughput, and time-consuming and thus is not suitable for application in biologics development.12,13,14 A protein conformational array (PCA) method was recently developed to gain HOS information of mAbs in therapeutic antibody development.15,16 It was utilized to analyze conformational variants and determine structural similarity between bio-similar and its reference product,15,16 but it can also be used in development of novel therapeutic antibodies. In the PCA method, a set of 34 capture antibodies (pAb1-pAb34) were developed to bind 34 IgG fragments, respectively, from a marketed antibody. For a generic set of capture antibodies used for novel mAb development, the capture antibodies for the constant region are based on the amino acid sequence of trastuzumab (Herceptin17), including pAb13-pAb34. Each of pAb1-
pAb12 target variable regions, and they are used as an equal mixture of 7 different capture antibodies targeting the same position from 7 marketed mAbs. This pooled antibody strategy has demonstrated good sensitivity and coverage toward novel mAbs under development. The 34 IgG fragments used as antigens for capture antibody generation are sequential, with slight overlap to cover the whole sequence of the IgG antibody. When the IgG antibody of interest is well folded, these 34 fragments have limited or no accessibility for the 34 corresponding capture antibodies because of their relative position and orientation in the mAb, and thus have very weak or no interaction with those capture antibodies. Incomplete folding or partially unfolding of IgG provides more accessibility of IgG fragments and stronger interaction with capture antibodies. Therefore, the strength of the interaction between capture antibodies and IgG antibody of interest becomes an indicator of IgG folding status and the local accessibility of each IgG fragment. The full set of accessibility data for 34 fragments, along with the IgG of interest, provides the HOS information of the IgG.

A sandwich enzyme-linked immunosorbent assay (ELISA) was developed by immobilizing the 34 capture antibodies on 34 separate plate wells to capture each IgG fragment on the mAb of interest.\(^{16,17}\) Both the degree and the location of the conformational change can be detected by this assay. The results correlated very well with the results from HDX MS in our recent study.\(^{13}\) However, this method is labor intensive since at least 34 wells are needed for one IgG sample. In the commercial kit, three 96-well plates can only run three samples in duplicate with one control. A new high throughput method was recently developed by combining these 34 capture antibodies with Lumimex’s multiplexing technology. Instead of immobilizing 34 capture antibodies in 34 separate wells, these antibodies are immobilized on 34 different magnetic beads that are color coded and can be mixed together to capture 34 IgG fragments in a single well. Compared to the ELISA method, this multiplexing method significantly increases the throughput by 48 fold since one 96-well plate can test 48 samples in duplicate, which is more applicable to support HOS analysis of large volume of samples in biologics development.

In this work, we demonstrated the application of this new high throughput PCA assay to support novel mAb development. We deciphered the mechanistic insight of mAb structure revealed by PCA using small angle X-ray scattering (SAXS) and correlated the structure information obtained using PCA to protein stability information acquired using size-exclusion chromatography (SEC) and protein thermal shift (PTS) assay. Our study demonstrated that the high throughput PCA is highly efficient for: 1) comparison of the structural difference between different IgG molecules; 2) distinguishing mAb stability in different buffers and monitoring structure changes along the process; and 3) evaluating the effects on structures by different conditions. Therefore, this method can be used to screen mAbs in early discovery for developability and manufacturability and study structural comparability during cell line or process changes that occur in different clinical phases. Furthermore, this PCA can provide specific regional structure information in a high throughput fashion.\(^{16}\) The structural data obtained by PCA provides in-depth product and process understanding to support mAb development for both biosimilars and novel mAbs. To our knowledge, this is the first report of antibody HOS analysis using multiplex-based high throughput PCA.

### Results

**IgG1 and IgG4 differences revealed by PCA and SAXS**

**PCA analysis of different mAbs in IgG1 and IgG4 subclasses**

PCA analysis was applied to six different mAbs, including two IgG1 (mAb1 and mAb2) and four IgG4 (mAb3-mAb6). The results for the six mAbs are shown in Fig. 1. The fluorescence signals, which represents epitope exposure from PCA analysis, of IgG1 mAbs are generally 60% lower than those of IgG4 mAbs, clearly demonstrating the more compact structure of IgG1 compared to IgG4. In the IgG1 group, mAb1 has slightly higher signals on a few regions captured by antibodies 20, 23, 25, 26 and 28 (most in CH2 domain), while mAb2 has slightly higher signal than mAb1 on regions captured by antibodies 6, 10, 13, 17, 31, and 33 (most in CL and CH3 domain). In the IgG4 group, the signal patterns of four molecules are similar with the exception of mAb4, which has a slightly higher signal than those of the other three IgG4.

**Structural characterization of different mAbs using small angle X-ray scattering**

mAb1-5 were subjected to structural characterization using SAXS (Fig. 2). The SAXS curves (Fig. 2A) show that the scattering intensities of IgG4 (mAb3-5) are stronger than those of IgG1 (mAb1-2) at small angles, which results in the larger gyration radii of IgG4 than those of IgG1 (Table S-1). In Kratky plots (Fig. 2B), peaks at low angles (Q around 0.04) are clearly resolved between IgG1 (mAb1-2) and IgG4 (mAb3-5), which suggests that IgG4 are larger scatterers. Pair distance distribution curves (Fig. 2C) show that all mAbs have bimodal distance distributions and mAb3-5 have larger maximum diameters (Dmax) than those of mAb1-2. Further analysis of 1D data leads to the 3D molecular envelope of mAbs. Our data show that the molecular volumes of IgG4 are larger than those of IgG1 (Table S-1). Homology models of mAbs were fitted to their 3D molecular envelopes (Fig. 2D). The accessibility of each of the 34 fragments was calculated using fitted homology models. Our results show that the fragments in IgG4 are easier to access, which explains why the domain volumes of IgG4 are larger and PCA signals of IgG4 are higher.

**Study of mAb5 stability in different buffers using PCA, SEC, and PTS**

**PCA analysis of mAb5 in different buffers**

A set of 20 buffers with different salt type, concentration, and pH (Table S-2) were screened to optimize the mAb5 stability in the downstream process development. The first group of buffers contains one of three salts at 25 mM (glycine succinic acid, sodium acetate, and citrate phosphate) with or without 200 mM sodium chloride at pH 3.5 or 6.0. The second group of buffers contains...
one of two salts at 25 mM (Tris-HCl and sodium phosphate) with or without 200 mM sodium chloride at pH 6.0, 7.0, or 8.0. mAb5 samples in these 20 different buffers were analyzed using PCA. Only results of the first 12 samples are shown in Fig. 3A due to the space limitation. Clearly, the signal of mAb5 samples in buffer 2, 6, and 10 are 3–5 times higher than those of other samples. This suggests that buffer 2, 6, and 10 containing high salt (200 mM NaCl) at low pH (3.5) may have caused antibody unfolding and aggregation. The signals of regions captured by antibodies 19, 20, and 31 in buffers 1, 5, and 9 (no NaCl at low pH) are lower than those in buffers 2, 6 and 10 (high NaCl at low pH), but higher than those in other buffers.

**High molecular weight species analysis of mAb5 in different buffers**

HMW species were assessed using SEC to determine the stability of mAb5 in the above mentioned buffer conditions. As shown in Fig. 3B, mAbs in buffers 2, 6 and 10 have significantly

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**Figure 1.** PCA data of 6 different mAbs (mAb1: black, mAb2: red, mAb3: green, mAb4: blue, mAb5: cyan, and mAb6: magenta). mAb1-2 are IgG1 molecules and mAb3-6 are IgG4 molecules. The error bar is the standard deviation from two repeats. The regions of IgG of interest recognized by these capture antibodies are labeled underneath the X-axis.

**Figure 2.** (A) Comparison of the SAXS curves of different mAbs. The scattering intensity was plotted as the function of the square of the momentum transfer $Q$ ($Q = 4\pi \sin(\theta)/\lambda$, where $\lambda$ is the wavelength of the X-ray). (B) Kratky plots based on the SAXS data of different mAbs. (C) The pair distance distribution $P(r)$ of different mAbs from the indirect Fourier transformation of the scattering intensity curves. (D) Homology models were fitted to average ab initio envelopes of representative IgG1 (left) and representative IgG4 (right). (mAb1: black, mAb2: red, mAb3: green, mAb4: blue, and mAb5: cyan).
higher percentages of HMW (55%, 15%, and 24%, respectively), which correlates with their higher signal intensity by PCA (Fig. 3A). Since the increase in the PCA signal is an indication of increased epitope exposure, these results suggest that the formation of mAb HMW including aggregates resulted in more epitopes exposed. The lower HMW percentage in other buffers (around 3%) also correlates well with their lower PCA signals. These three buffers contain high salt concentration (200 mM NaCl) at low pH (pH 3.5), but with different buffer species. All other buffers have no NaCl at pH 3.5 or have higher pH (pH 6–8) with or without 200 mM NaCl.

**Thermal stability of mAb5 in different buffers**

PTS assay was conducted to investigate the thermal stability of mAb5 in these 20 different buffers. The fluorescence intensity of the PCA analysis was plotted as the function of the temperature from 25 to 95°C. The first derivative of the fluorescence intensity – temperature function was also plotted to show the state transition clearly. PTS data of Buffer 2, 6, and 10 are shown in Fig. 4 with Buffer 1 as their control. The thermal PTS data show the mAb in buffers 2, 6, and 10 has less thermal stability. The derivative plot shows two peaks, the first peak around 65°C and the second one around 76°C. Before the first domain starts unfolding (around 55°C), the fluorescence signal of Buffer 2, 6, and 10 is higher than their control Buffer 1. At the first transition (around 65°C), the ΔF/ΔT in buffers 2, 6, and 10 is only one fourth or less of that in Buffer 1. At the second transition (around 76°C), the ΔF/ΔT in Buffer 2, 6, and 10 is about half of that in Buffer 1.

**PCA analysis of mAb5 in process development**

**PCA analysis of upstream process development samples**

In this experiment, the conformational changes of mAb5 during the cell culture process were studied by PCA. Cell
culture samples were collected every 2 days from Day H-6 to Day H during the cell culture process. The mAbs in these samples were directly analyzed by PCA without Protein A chromatography. The results of these cell culture samples in different culture days are shown in Fig. 5A. All antibody regions except those captured by antibody 19 and 20 are similar. The signal of the regions captured by antibody 19 and 20 reaches the maximum in Day-4 and then decreases with increasing cell culture days.

PCA analysis of downstream process development samples

The mAb5 purification was conducted using a platform downstream process. Samples collected include Protein A Elution (PAE), virus inactivation (VI), cation exchange elution (CEX), and anion exchange flow-through (AEX). The PCA results of these samples are shown in Fig. 5B. The signal of VI sample is generally higher than those of other samples, indicating more exposure of antibody regions due to low pH treatment-induced structure change. Signal of PAE sample is marginally higher in certain regions than the signal of CEX and AEX samples, although the PAE sample has a much lower pH (about 4.5).

Discussion

IgG1 and IgG4 structural differences

To date, IgG1 and IgG4 are the predominant IgG subclasses of therapeutic mAbs approved in the US and European Union (68 out of 77). Although IgG1 and IgG4 have high sequence similarity, they have substantially different properties, which could be attributed to structural differences. Our study demonstrates that PCA is a sensitive and robust method that can clearly identify the conformational differences between IgG1 and IgG4. We further utilized an orthogonal method, small angle X-ray scattering to characterize IgG1 and IgG4 structural differences, which built strong correlation with PCA results.

In the IgG subclasses, IgG1 and IgG4 are highly conserved, but are different in their hinge regions and upper CH2 domains. These regions are involved in binding to both Fcγ receptors (FcγR) and C1q. The difference in these regions between IgG1 and IgG4 leads to different half-life and effector functions, such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, and activating complement. These functional differences are attributed to structural differences. PCA signal strength reflects the accessibility of 34 IgG fragments captured by the corresponding generic set of 34 mAbs. The less folded IgGs will expose the epitopes more to the capture antibodies and produce stronger signal and vice versa. Therefore, the PCA signal implies the folding status of the IgG of interest. The significant PCA signal difference between IgG1 and IgG4 suggests that IgG1 mAbs fold tighter than IgG4 mAbs, which leads to less exposure of regions in IgG1. The total PCA signal of studied mAbs is directly proportional to the solvent-accessible surface area (SASA) calculated using the SAXS fitted homology models (Fig. 6A). The higher SASA due to less folding, the higher PCA signal for that IgG molecule. The trend of PCA signal also correlates with gyration radius, Dmax, and molecular volume of these IgG molecules (Table S-1), which further confirms the folding status. These results suggest that PCA can be used to effectively monitor and compare the general folding status of different IgG molecules.

The more flexible structure (Fab and Fc) of IgG4 than IgG1 is fundamentally supported from two perspectives. First, IgG4 has a shorter hinge region, but two more Pro residues in the hinge region, than IgG1. This leads to a more rigid hinge region for IgG4, which renders the other parts of the molecule (Fab...
and Fc) more flexible during Brownian motion. Secondly, the inter heavy-light chain disulfide bond linkage in IgG4 makes the Fab more flexible. In IgG4, the last residue Cys in the kappa light chain forms an inter heavy-light chain disulfide bond with the Cys residue in the middle of CH1 domain, which leaves one sheet of the CH1 sandwich unbundled. In IgG1, the last residue Cys in the kappa light chain forms an inter heavy-light chain disulfide bond with the Cys residue in the end of CH1 domain, which leaves the CH1 domain stapled together with the CL domain.

**Correlation of mAb stability information between PCA, SEC and PTS**

Process development for mAb purification and formulation requires various buffers with different pH, salinity type and concentration, which can have detrimental effects on protein stability and other quality attributes. In this study, PCA was successfully applied to determine mAb HOS in different buffering conditions. The accessibility of 34 mAb regions determined by PCA affects the mAb stability, which were well correlated with the stability information obtained by SEC and protein thermal shift.

It has been demonstrated that protein aggregation is a major factor for biologics immunogenicity.\(^{26,27}\) The sensitive and systematic measurement of mAb HMW species including aggregates should provide valuable information during process development. The high HMW percentage shows that the mAb5 is less stable in buffers 2, 6, and 10 (Fig. 3B). These three buffers all have low pH (3.5) and high salt concentration (200 mM NaCl). SEC is a standard method for HMW characterization of therapeutic proteins. Although SEC is automated by UPLC, the throughput is not as high as other methods such as PCA and PTS. PCA and PTS can also provide additional structural information as discussed in the following sections.

Our PCA data for the same set of samples suggest that mAb5 in the buffers 2, 6 and 10 has higher signal and less stability, which matches the finding from SEC analysis. Furthermore, we can tell that mAb5 has lower signal in buffer 1, 5, and 9. These three buffers have low pH (3.5) without NaCl. This suggests the unusual high HMW by SEC and high PCA signal in buffers 2, 6, and 10 is attributed to the combination effect of both low pH (3.5) and high salt concentration (200 mM). In addition, PCA results suggest that the more affected regions such as 19, 20, 26, 28, 30, and 31 are close to or within the Fc region of mAb5. This might be caused by the dissociation of two salt bridges between two CH2-CH3 chains due to low pH-induced protonation of glutamic acid in the salt bridge.\(^{28,29}\)

However, our study shows low pH itself did not cause much aggregation. Rather, the salt addition at low pH might promote the hydrophobic interactions between dissociated Fc from different monomers and cause high aggregation. Protein aggregation is known as a major product-related impurity causing immunogenicity; however, the molecular basis of the protein aggregates causing immunogenicity has not been elucidated. In this study, it was demonstrated that mAb aggregation led to a significant increase in epitope exposure as measured by PCA (Fig. 3). It is thus possible that this increased new epitope exposure from the aggregate formation may transform the mAb from a “self” molecule to a “foreign” molecule for the human immune system, thus eliciting the immune response. Since PCA is a more sensitive and systematic analysis of mAb HOS status, this result also suggested that the PCA assay might be used to predict the immunogenicity potential for mAbs with detectable aggregates. Cytokine release testing using human whole blood to examine the possible correlation between mAb HOS and immunogenicity is ongoing.

PTS, also referred to as differential scanning fluorimetry (DSF), has been a routine method to screen protein stability in different buffer conditions.\(^{31}\) As shown in Fig. 4, before the protein starts melting, the fluorescence signal from PCA analysis (reflecting epitope exposure) in buffer 2, 6, and 10 are slightly higher than that in buffer 1. This higher fluorescence signal indicated partial unfolding in aggregates of mAb5 due to low pH and high salt (Table S-2). During the transition stage, the height of the transition peak is reversely proportional to the percentage of aggregate, indicating the unfolding of monomer. Our study of full length mAb5, mAb5 Fab, and mAb5 Fc (Figure S-1) shows that the first peak is attributed to the melting of mAb5 Fc domain. Therefore, PTS data suggest that the mAb5 Fc domain is more sensitive to the low pH and high salt than mAb5 Fab. The indication well matches PCA results that pAb1-18 signals for Fab are generally lower than pAb19-34 signals for Fc (Fig. 3A).

**Monitoring mAb conformational change in bioprocessing**

Recombinant mAbs undergo various dramatic environmental changes along the process from upstream cell culture to downstream purification.\(^{15,22,33}\) mAbs may change their conformation in response to these environmental changes. We applied PCA to determine mAb conformational changes along the
Different buffer matrices. In this study, mAb5 is subjected to beyond the scope of this study. Information of this region requires further investigation, and is beyond the scope of this study.

In our downstream process, mAb5 encountered affinity-based bind-elution in Protein A chromatography, low pH virus inactivation steps and neutral pH with higher salt concentration in the CEX and AEX polishing steps. Our data in Fig. 5B shows that the signal of the sample from the viral inactivation step is higher than those of samples from other steps. This suggests that low pH treatment (pH 3.5 for 60 min) in the viral inactivation leads to partial mAb denaturation, exposing buried regions captured by assay antibodies. mAb5 is stable at pH 4.5 (PAE) in the original sample concentration varied from 10 mg/mL to 50 mg/mL. All samples were adjusted to a concentration of 100 μg/mL using the dilution buffer before they were used in PCA.

During the cell culture of mAb5 in a 5 L bioreactor, 15 mL of cell culture samples were collected on Day H-6, H-4, H-2 and H (Day of Harvest). The cell culture samples were centrifuged to remove cells and filtered using a 0.2 μm filter. The titers of Day H-6, H-4, H-2 and H samples were 1.2, 1.8, 2.3, and 2.9 mg/mL, respectively. All samples were diluted to 100 μg/mL using the dilution buffer before PCA.

Downstream process samples of mAb5 were collected after PAE, VI, CEX, and AEX. The concentrations of PAE, VI, CEX, and AEX samples are 16.5, 16.2, 12.0, and 3.4 mg/mL, respectively. All samples were diluted to 100 μg/mL using the dilution buffer before PCA. To investigate the conformational change and stability of mAb5 in different buffers, a matrix of buffers with different buffer salt types (Tris, phosphate, citrate, acetate), salt concentrations (with and without 200 mM sodium chloride), and pH (3.5-8.0) were prepared using a TECAN liquid handler. The mAb5 was reconstituted in these different buffers by doing buffer exchange using Sephadex G-25 desalting columns. The buffer exchanged mAb5 samples were incubated at room temperature for 24 hours before samples were further analyzed using PCA, SEC and PTS.

Protein conformational array

PCA was conducted using MILLIPLEX InnoPlex Beta kit (MilliporeSigma). In the kit, each of 34 antibodies was conjugated to an individual color-coded magnetic bead. The mixture of color-coded and antibody-conjugated magnetic bead was suspended and aliquoted into 96-well plate provided with the different mAbs or mAb structure changes, PCA analysis may lead to specific actions such as re-engineering of susceptible region(s) or optimization of process and formulation conditions to minimize HOS changes that might cause mAb immunogenicity or affects its efficacy. Monitoring the antibody structure changes during process development is essential to safety and efficacy of the final product. The high throughput format PCA ensures its applicability in supporting antibody development. Our work has contributed to the development of a novel, high throughput method for characterizing higher order structure of mAbs that provides in-depth product and process understanding to facilitate mAb discovery and development and ensure final product quality.

Materials and methods

Chemicals

All chemicals used in this study were from Sigma-Aldrich unless specified.

Sample preparation for protein conformational array

All recombinant therapeutically mAbs (mAb1-6) were produced at Bristol-Myers Squibb using a mammalian cell expression system. The original sample concentration varied from 10 mg/mL to 50 mg/mL. All samples were adjusted to a concentration of 100 μg/mL using the dilution buffer before they were used in PCA.

In this study, we demonstrated broad applications of a novel high throughput format PCA, including comparing HOS of different mAb molecules, analyzing structure associated stability in different buffers, and monitoring HOS change in antibody processing. The PCA analysis led to important findings, including significant IgG1 and IgG4 difference in folding and epitope exposure, and molecular-level understanding of antibody aggregation, which could potentially cause immunogenicity. For fundamental understanding and verification of this method, we revealed the mechanistic insight of IgG1 and IgG4 conformational differences distinguished by PCA with structural characterization using SAXS. We correlated the HOS information obtained from PCA to IgG stability analyzed by traditional methods of SEC and PTS. One of the advantages of PCA compared with other technologies for HOS analysis is its high sensitivity and ability to address specific regional changes. Besides providing a general structure fingerprint to compare
kit. After the incubation at 4°C with shaking, the supernatant was discarded and the magnetic beads were retained in the ELISA plate using a magnet. All protein samples were diluted to 100 μg/mL using the dilution buffer before use. 100 μL of each protein sample were added to the ELISA plate and incubated with the antibody-conjugated magnetic beads at 4°C with shaking. Uncaptured proteins were discarded and the captured proteins on beads were retained in the ELISA plate using the magnet. After the reporting antibody reaction, color development, and stopping solution addition, the samples were sipped to a channel using a Luminex MAGPIX system controlled by Luminex xPonent. The magnetic beads were identified using two beams of decoding light and the captured protein on the magnetic beads was detected using fluorescence. The data were presented with the identity of these 34 antibodies as the x-axis and the fluorescence intensity indicating the amount of captured protein of interest as the y-axis.

**Size-exclusion chromatography**

SEC assay was conducted in Waters Acquity UPLC system using a BHE SEC 200 column. In each injection, 10 μg of sample was injected to the column and the mobile phase of 20 mM potassium phosphate, 200 mM sodium chloride, pH 6.8 was used in equilibration and elution. The data acquisition and analysis were done in Empower 3.

**Protein thermal shift**

Protein Thermal Shift Starter Kit (Thermo Fisher Scientific) was used to study the thermal stability of IgG molecules (mAb1-5) and the mAb5 stability in different buffers. Sample preparation was done by following the vendor’s user manual with minimal modification. Briefly, the dye was pre-diluted to 16X dye from 5000X dye using the Protein Thermal Shift Buffer. All protein samples were diluted using the Protein Thermal Shift Buffer to a 100 μL stock solution pre-diluted to 16X dye. Protein Thermal Shift Buffer (5.0 μL), protein stock solution (12.5 μL) and pre-diluted 16X dye (2.5 μL) were mixed in a 96-well PCR plate with each protein sample having four repeats. After spinning down the plate in a benchtop centrifuge to ensure no air bubbles in the PCR plate, the melting curve of samples were collected in a Vii A 7 QPCR instrument with excitation filter of 580 ± 10 nm and emission filter of 623 ± 10 nm and a temperature ramp rate of 1°C/min from 25°C to 99°C. The melting curves were analyzed in Protein Thermal Shift™ software (V1.2).

**SAXS experiments and 3D model analysis**

All mAb1-5 were buffer exchanged to phosphate buffer containing 20 mM sodium phosphate, 150 mM sodium chloride, and pH 7.2 using Amicon filters with molecular weight cut-off of 30 kDa following manufacturer’s instruction. The synchrotron SAXS data were collected on beam line 16ID (LIX) in National Synchrotron Light Source II at the Brookhaven National Laboratory (Upton, New York). Samples were loaded using the homemade automated sampler. Scattering from the IgG protein solutions with the concentration of 1 mg/mL was measured at 4°C in the momentum transfer up to 2.0 Å⁻¹. Momentum transfer is defined as Q = 4π sin(θ)/λ, where θ is the scattering angle and λ is the X-ray wavelength (λ = 1.5 Å). Data analysis was performed using the software suite ATSAS (V2.6). PRIMUS35 was used to generate Guinier plot and Karyt plot. GNOM36 was used to generate pair distance distribution and DAMMIN37 was used to generate 3D models. SUPALM38 was used to fit the homology models of IgGs to their SAXS models. Get Area was used to analyze the solvent accessibility of fragments in Pymol (V1.7, Schrödinger, LLC). JMP (V10) was used to analyze the correlation between solvent accessibility and PCA signal.

**Abbreviations**

- AEX anion exchange flow-through
- CD circular dichroism
- CEX cation exchange elution
- ELISA enzyme-linked immunosorbent assay
- Fab antigen-binding fragment
- Fc crystallizable fragment
- HC heavy chain
- HDX hydrogen/deuterium exchange
- HMW high molecular weight species
- HOS higher order structure
- LC light chain
- mAb monoclonal antibody
- MW molecular weight
- MS mass spectrometry
- NMR nuclear magnetic resonance
- PAE Protein A Elution
- PCA protein conformational array
- PTS protein thermal shift
- SAXS small angle X-ray scattering
- SEC size-exclusion chromatography
- VI virus inactivation

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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