



Kinetic and Functional Studies of Charge and Size Variants in Recombinant Monoclonal Antibodies

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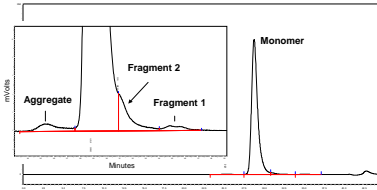
Abstract

Monoclonal antibodies (MoAbs) are characterized by various molecular weight species such as aggregates, monomer and fragments. It is important that such heterogeneity not affect the efficacy of the molecule and we consequently studied the binding characteristics of the different species. Fragments of the MoAb were separated from aggregate and monomer by size exclusion chromatography (SEC). The fragment species that eluted later in the chromatogram were shown by mass spectrometry (MS) to contain heavy and light chain species. The fragment species that partially resolved from the main peak was shown to be the monoclonal antibody with loss of one Fab arm (Fab+Fc). The different molecular species were then characterized by Biacore. The results show that the apparent affinity of the aggregate, monomer and Fab+Fc species were all determined to be similar. Binding of aggregate to its antigen with similar affinity suggest that these aggregates retain their native structure and this was supported by MS analysis which revealed that the aggregates are non-covalently linked. As expected, the Fab+Fc species showed a binding stoichiometry of 1:1 with the antigen. The charge variants of the MoAbs were resolved by weak cation exchange (WCX) chromatography and fractionated into an acidic region, basic region and main species. The individual fractions were then analyzed by Biacore and a cell based bioassay. The results indicate that the affinity and potency of the main species and basic species were comparable to the control. The affinity of the acidic species was about half that of the control and the potency was slightly reduced when compared to the control. Mass spectrometry analysis and analysis of fragments via WCX analysis revealed the presence of fragment species in the acidic region of the ion exchange chromatogram. The reduction in potency and reduced affinity of the acidic species was postulated to be primarily due to fragments. The impact of heterogeneity, either charge or size, on the efficacy of the MoAb was thoroughly evaluated using Biacore (measuring its affinity with antigen).

Objectives

- One of the primary objectives of the study is to obtain the affinity all the charge and size variants through surface plasmon resonance analysis.
- To identify and characterize the size species by mass spectrometry.

Size variants in monoclonal antibodies



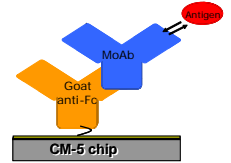
Representative SEC chromatogram for MoAb illustrating the various size variants

- Analysis by SEC indicated the separation of the MoAb into four different species namely the aggregate, monomer, fragment 2 and fragment 1 species.
- The aggregate species or the high molecular weight species.
- The monomer species accounts to about 97% and is the largest peak obtained by SEC analysis.
- The monomer peak has a shoulder peak labeled as Fragment 2.
- The fragment 1 species contains low molecular weight species which elutes after fragment 2.

Methods and Materials

SPR Analysis

- Chip surface was prepared by first immobilizing goat anti-Fc via amine coupling and then capturing MoAb.
- MoAb was captured for 30s at a flow rate of 10 μ l/min.
- Kinetic analysis was done by flowing the antigen the MoAb in concentration series ranging from 25 nM to 0.39 nM.
- Association time was for 5 minutes at a flow rate of 50 μ l/min.
- Dissociation time was for 40 minutes except for runs at 1.56 nM and 6.25 nM where the dissociation time was 2 hours. This is due to slow off rate of the MoAb. The flow rate was 50 μ l/min.



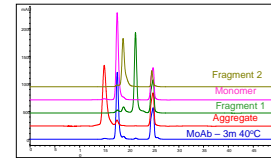
Mass Spectrometry

- Top-down and bottom-up approach was utilized to characterize the charge and size variants.
- All the characterization was done on Agilent Q-TOF LC/MS.

Cell based Bioassay

- Cell based bioassay was done to determine the potency (activity) of the various species.

Fractionation of size species

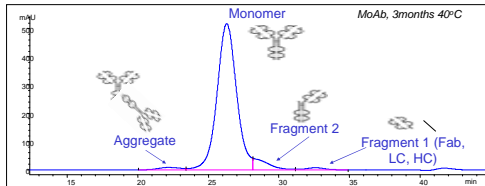


Overlay chromatogram of the fractionated size variants

- Fractionation of the size variants was done using Superdex 200 SEC column on Agilent HPLC system with automated fraction collector

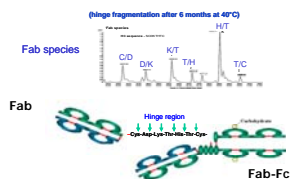
Results

Mass Spectrometry analysis of the size variants



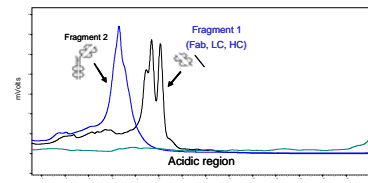
- Aggregate species in MoAb is composed primarily of dimer.
- The monomer is intact MoAb.
- Fragment 2 species is obtained as a result of the loss of single Fab.
- Fragment 1 species which follows the fragment 2 species contains Fab, HC and LC fragments.

Fragment species created by hinge region fragmentation (incubated for 6m at 40°C)



- MS analysis showed that fragmentation in MoAb was in the hinge region of the molecule (kinetic instability of IgG in hinge region)

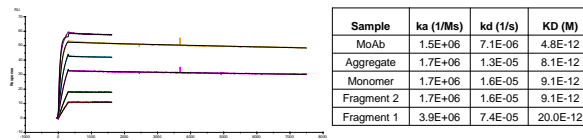
WCX-10 analysis of SEC fractions



WCX-10 profile of purified fragment 1 and 2 species revealed that the fragment species elute in the acidic region of the chromatogram

- Fragment 1 and fragment 2 species of MoAb purified via SEC elute in the acidic region of the WCX-10 profile. (shown in the above overlay)
- Fragmentation is a major cause for increase in acidic species.

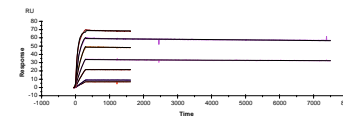
SPR Analysis of the Size Variants



Representative sensogram for the kinetic analysis of the size variants

- The kinetic analysis of the size variants by SPR show that the binding constant for all the species except fragment 1 is comparable to the binding constant of the monomer.
- The binding affinity of the fragment 1 species were significantly reduced. However, the data revealed fragments present in this region were still active.

SPR Analysis of the Charge Variants



Representative sensogram for the kinetic analysis of the charge variants

- Kinetic analysis of the purified charge variants by SPR show that the K_D for all charged species, except the acidic species, were comparable to MoAb.
- K_D for the acidic species was reduced by about half.

Samples	k_a (1/Ms)	k_d (1/s)	KD (M)
MoAb	1.3E+06	5.3E-06	3.9E-12
Acidic species	1.5E+06	2.1E-05	1.5E-12
Main species	1.3E+06	8.6E-06	6.4E-12
Basic Species	1.6E+06	8.8E-06	5.5E-12

Cell Based Bioassay

Sample ID	Biological Activity (%)
MoAb	99
Acidic species	50
Main species	99
Basic Species	87

- With the exception of the acidic region the potency of the various charge species were comparable.
- Reduced potency in AR correlated to reduced affinity as shown by SPR analysis.

Conclusions

- Affinity and potency for all charge variants of the MoAb, except the acidic region, were comparable.
- Aggregate species are dimer of the monomer, fragment 2 is Fab+Fc (loss of Fab) and fragment 1 species consist of Fab, LC & HC fragments.
 - Affinity for all size variants, except fragment 1, were comparable to monomer.
- Fragmentation in MoAb occurs in the hinge region and the fragment species elute in the acidic region.
- Fragmentation of the molecule is one of the predominant cause for the reduced affinity and potency of the acidic species.