Monovalent IgGs as a Tool for Characterizing Antibody Binding and Function

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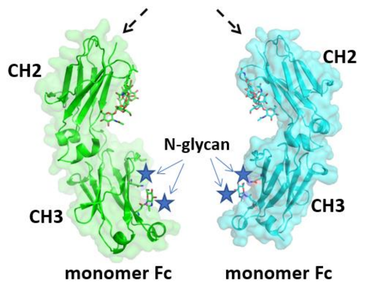
Abstract and Background

Background: Antibodies are a preferred treatment modality, particularly in cancer and autoimmune diseases, with more than 50 approved and more than 500 in various stages of clinical development. As the field advances, antibodies are being engineered for novel specificities and functions. Towards this, additional formats have been designed to engage with multiple targets and create unique functions that are not feasible with the standard bivalent format of an IgG.

Abstract: Assessment of antibody binding affinity is an important aspect of characterization and a pre-requisite for affinity enhancement. The bivalent structure of antibodies can allow co-engagement with two antigen molecules, thereby increasing avidity and limiting assessment of true binding affinity. Many binding assays display immobilized antigen and allow for avid binding, an attribute that may not be representative of binding in situ. Monovalent and monomeric IgGs, containing a single Fab arm joined to a single Fc domain, can serve as a useful tool for characterizing the binding and mode of engagement of therapeutic antibodies. Here, we present a case study comparing the binding and functionality of monovalent and bivalent IgG formats targeting a G-coupled protein receptor (GPCR). Monovalent and bivalent IgGs were recombinantly expressed, purified and characterized in biophysical and biochemical assays. Using a panel of such IgGs we show that bivalent IgGs contributed to increased potency in a cell based in vitro assay, confirming that both arms of the bivalent antibody contribute to binding and functionality.

Design of Monomeric IgG

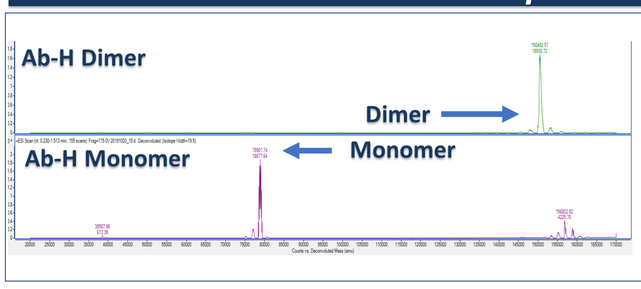


DKTHTSPPSPAPE<u>AA</u>GGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN
AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLP
PSRDELTKNQV**N**LTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFL**N**S<u>T</u>LTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK

The structure of monomeric Fc (PDB 4J12) published by Ishino et al (PMID: 23615911). Engineered glycosylation sites at positions 364 and 407 (EU numbering) block homodimer formation (left). The location of the N-linked glycosylation sites are highlighted with stars in the figure on the left and highlighted in red in the sequence on the right. Underlined are mutations from wild type Fc.

Biophysical Characterization

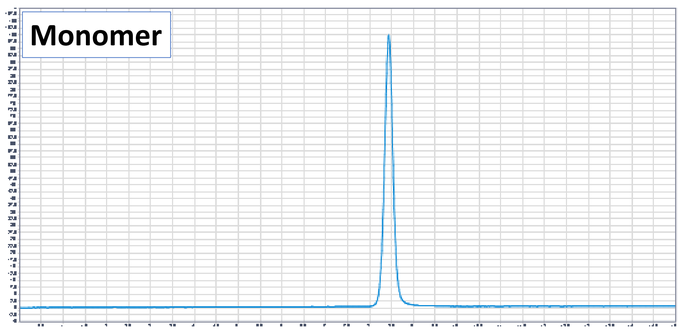
Mass Spectrometry



The mass of monomeric and dimeric IgGs for Ab-H was quantified by Q-tof mass spectrometry under non-reducing conditions. The predominant species for the canonical dimeric IgG was approximately 150 kDa, while the monomeric IgG was approximately 78 kDa. The data confirm that the engineered glycosylation sites prevent dimer formation and favor a soluble, monomeric IgG.

Size Exclusion Chromatography

Antibody	Elution time (minutes)	
	Dimer	Monomer
Ab-A	7.63	8.225
Ab-B	7.65	8.20
Ab-C	7.62	8.19
Ab-D	8.02	8.44
Ab-E	8.06	8.47
Ab-F	9.01	8.92
Ab-G	7.72	8.27



Monomeric and dimeric forms of selected antibodies were separated by size exclusion chromatography on a Yarra™ 3 um SEC-3000 LC Column. The lower molecular weight monomers had longer retention times on the column. Additionally, monomeric antibodies were homogenous with a single predominant elution peak, suggesting the protein is free of aggregate. Ab-F did not differ in the elution time between dimer and monomer; however, the canonical IgG dimer had a longer retention time, suggesting non-specific interaction with the column, presumably caused by the Fab domain. A representative chromatogram for Ab-E monomer is shown, and highlights that the monomer is homogenous and free of high molecular weight aggregates.

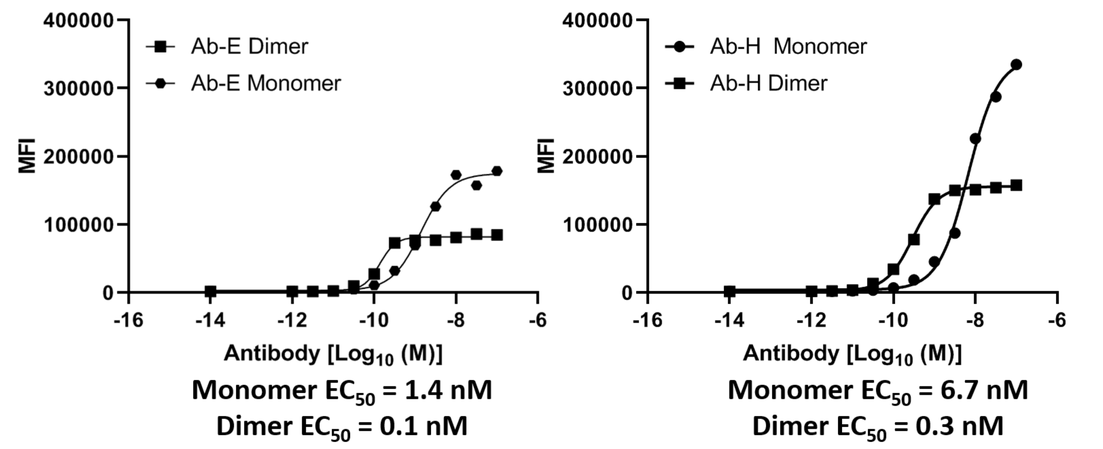
Transient Expression and Purification

Antibody	grams per Liter	
	Dimer	Monomer
Ab-D	0.11	0.39
Ab-H	0.90	1.13
Ab-I	1.18	1.51
Ab-J	1.98	1.39

Monomeric and dimeric IgG were transiently expressed in Expi293 cells. Antibody was purified from cell culture supernatant by protein A affinity chromatography using the AKTA pure system. The amount of purified material obtained for each antibody was comparable for dimeric and monomeric formats, confirming the ability to be expressed and purified by protein A. An example data set from a single round of transfection and purification is highlighted.

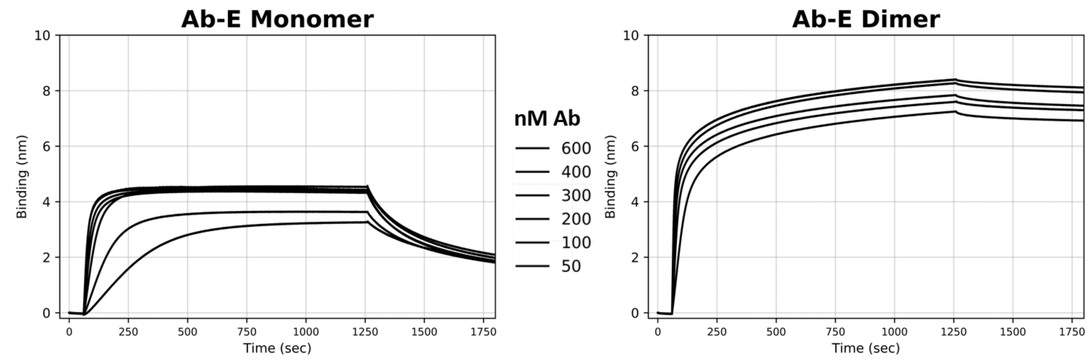
Binding and Functionality

Binding Kinetics Measured by Flow Cytometry and BLI



Cell surface binding of monomeric and dimeric forms of Ab-E and Ab-H as measured by flow cytometry.

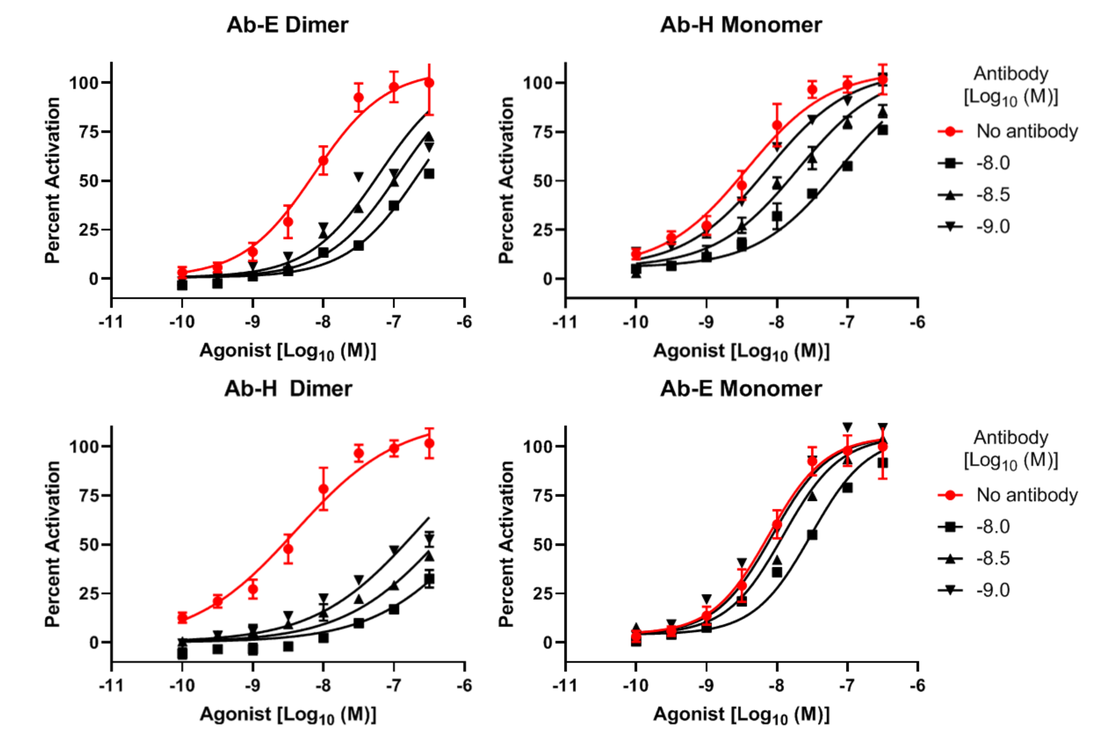
Differences were observed in the binding affinity between monomeric and dimeric forms of Ab-E and Ab-H, with a greater than 1 log shift in EC_{50} between monomer and dimer. Interestingly, a shift in the saturation level was also observed for the monomeric IgG, which would be expected if the stoichiometry of antibody to target ratio shifted from 2:1 to 1:1 for dimer and monomer, respectively. The data suggests that both arms of the bivalent antibody engage with the target GPCR on the cell surface.



Biolayer Interferometry sensorgrams of Ab-E monomer and dimer binding to a linear peptide comprising its core epitope.

A peptide containing the core epitope region of Ab-E was immobilized to a biosensor and binding to Ab-E monomer and dimer was measured at varying concentrations. The monomeric version reached equilibrium allowing for steady state analysis and an accurate quantification of affinity (~28 nM). However, the dimer did not reach steady state and resulted in poor kinetic modeling. The data highlights the utility of monomeric IgG for accurate quantification of binding kinetics.

In Vitro Functional Assay



A cell based reporter assay was used to quantify GPCR agonist dose response with no antagonist (red line) or in the presence of 10^{-8}, $10^{-8.5}$, or 10^{-9} M antagonist (monomeric or dimeric anti-GPCR antibody).

Inhibition of GPCR activation was tested in vitro. The agonist induced GPCR activation with an EC_{50} of approximately 5 nM. The presence of monomeric anti-GPCR antibodies cause a modest shift in agonist EC_{50}; however, the dimeric antibodies produced a much more dramatic shift in agonist EC_{50}, highlighting the contribution of bivalency to functionality.

Conclusions

Antibody valency can impact binding affinity and functionality. The use of monovalent IgG monomers by introduction of N-linked glycosylation sites at the CH3 dimer interface is a valuable tool for assessing the antibody properties. We have presented a case study that details the use of monovalent IgG to assess the contribution of bivalent engagement to anti-GPCR engagement and antagonism. Bivalency increased binding affinity, potency, and stoichiometry of engagement. The monomers can be expressed and purified using the same methods as canonical IgG dimers and are highly pure and soluble. They may also be a useful tool for assessing candidates for selection of Fab domains for incorporation into bispecific antibodies by providing a straightforward method for assessing the true binding affinity of the monovalent Fab domain.

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