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Improving monoclonal antibody selection and engineering using measurements of colloidal protein interactions

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Abstract

A limitation of using monoclonal antibodies as therapeutic molecules is their propensity to associate with themselves and/or with other molecules via non-affinity (colloidal) interactions. This can lead to a variety of problems ranging from low solubility and high viscosity to off-target binding and fast antibody clearance. Measuring such colloidal interactions is challenging given that they are weak and potentially involve diverse target molecules. Nevertheless, assessing these weak interactions – especially during early antibody discovery and lead candidate optimization – is critical to preventing problems that can arise later in the development process. Here we review advances in developing and implementing sensitive methods for measuring antibody colloidal interactions as well as using these measurements for guiding antibody selection and engineering. These systematic efforts to minimize non-affinity interactions are expected to yield more effective and stable monoclonal antibodies for diverse therapeutic applications.

Introduction

There are a daunting number of factors that influence the effectiveness and success of therapeutic monoclonal antibodies (mAbs). The most important issues relate to the specific biological pathways being targeted. For example, the optimal binding affinity and epitope on a target antigen (mediated by the antibody variable domains) as well as the optimal type and level of effector function (mediated by the antibody constant domains) are dependent on the specific therapeutic target. The pharmacokinetics and biodistribution of therapeutic mAbs, which are influenced by the target antigen and recycling Fc receptors, also significantly impact their effectiveness. Thus, specific (affinity) interactions involving the variable and constant domains of mAbs are key determinants of their therapeutic activity.

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Conflicts of interest

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Nevertheless, the same variable and constant regions of mAbs that mediate affinity interactions can also participate in non-affinity interactions with either themselves (self-interactions) or with other molecules (polyspecific interactions). The potential negative ramifications of these colloidal interactions are significant and are also important determinants of the success of therapeutic mAbs. Attractive self-interactions between antibodies (either in their native or non-native conformations) can lead to aggregation, abnormally high viscosity, liquid-liquid phase separation and opalescence.¹⁻⁶ Aggregation is particularly concerning because of the suspected immunogenicity of antibody aggregates,⁷⁻¹⁰ while high viscosity is problematic for subcutaneous delivery applications.^{11,12} Polyspecific antibody interactions are also concerning because they can lead to off-target effects as well as fast antibody clearance.^{13,14}

Therefore, it is critical to evaluate non-affinity antibody interactions early in therapeutic discovery and lead candidate optimization to minimize problems that can occur later in development. However, these interactions are difficult to measure because they are relatively weak and can involve a large number of potential molecules. This is particularly challenging during early antibody discovery because of the large number of candidate mAbs (tens to thousands), as well as their low concentrations (<100 µg/mL) and purities (unpurified cell supernatants). Here we review important recent progress in characterizing antibody colloidal interactions using biophysical methods during early antibody discovery and discuss how these measurements are being used to improve antibody selection and engineering.

Antibody self-interactions

Antibody self-association is the most fundamental and widely studied type of non-affinity antibody interaction. It is logical that mAbs can self-associate based on their multidomain architecture, symmetry, and non-uniform distribution of solvent-exposed hydrophobic and charged residues (Fig. 1). The variable heavy (V_H) and light (V_L) domains each display three solvent-exposed peptide loops (complementarity determining regions or CDRs) that commonly contain hydrophobic and charged residues to mediate high-affinity binding. Several studies have confirmed that hydrophobic and electrostatic interactions involving CDRs can mediate antibody self-association and aggregation.¹⁵⁻²³ More generally, attractive electrostatic interactions involving the Fab²³⁻²⁵ and Fc²⁶ regions of some antibodies have been shown to mediate self-association. The Fc regions of antibodies contain solvent-exposed hydrophobic residues involved in binding to Fc receptors and can also influence antibody self-association.^{22,26-31} The bivalency of mAbs naturally amplifies these self-interactions.^{16,32}

There are several valuable methods for measuring antibody self-association, including static³³⁻³⁶ and dynamic light scattering,^{34,37-41} neutron^{42,43} and X-ray⁴⁴⁻⁴⁷ scattering, analytical ultracentrifugation,^{6,38,48,49} membrane osmometry,^{3,50} self-interaction chromatography,⁵¹⁻⁵⁷ self-interaction nanoparticle spectroscopy,⁵⁸⁻⁶³ and bilayer interferometry.⁶⁴ The most powerful and insightful methods such as neutron scattering, membrane osmometry, and analytical ultracentrifugation are generally the lowest throughput

and most difficult to use during early antibody candidate selection. These methods are outside the scope of this review and have been reviewed previously.^{48,65,66}

Methods such as dynamic light scattering, self-interaction nanoparticle spectroscopy and biolayer interferometry provide lower resolution information but afford increased throughput and flexibility for characterizing mAb candidates during early antibody discovery. The most widely used method is dynamic light scattering, which can be performed in a microplate format enabling tens to hundreds of samples to be evaluated.^{37,39–41} As a measure of antibody self-association, mutual diffusion coefficients (D) are evaluated as a function of antibody concentration (c). This enables evaluation of the diffusion interaction parameter (k_D):

$$D=D_0(1+k_Dc)$$

where D_0 is the self-diffusion coefficient. k_D is not a direct measure of antibody self-association because it has both thermodynamic and hydrodynamic contributions. Therefore, it must be interpreted carefully, as discussed elsewhere.³⁸ Nevertheless, multiple studies have confirmed that k_D is generally well correlated with the second virial coefficient,^{37,39,40} a thermodynamic measure of pairwise protein interactions. Measurements of k_D are typically conducted at antibody concentrations of 1–20 mg/mL, which typically requires approximately one mg of purified mAb per k_D measurement. Although it is undesirable from the prospective of early antibody discovery that dynamic light scattering requires purified and concentrated mAbs, advances in high-throughput expression and purification methods are reducing this bottleneck.⁶⁷

Several studies have demonstrated the power of dynamic light scattering at improving mAb candidate selection and characterization.^{23,37–40,68–72} One of the most comprehensive studies to date measured k_D values for 29 different mAbs at several different solution conditions and compared these measurements conducted at 1–20 mg/mL to viscosity measurements conducted at much higher concentrations (up to 175 mg/mL).³⁷ Impressively, the k_D measurements are strongly correlated with viscosity measurements (Fig. 2), and demonstrate the utility of dynamic light scattering for efficiently assessing properties of concentrated solutions at modest antibody concentrations. Other studies have also demonstrated that k_D measurements are typically correlated with antibody solubility and aggregation rates.^{38,40,59,73}

To improve throughput of antibody self-interaction measurements during early antibody discovery, it is important to reduce the need for purifying and/or concentrating mAb candidates. This is challenging because typical mAb concentrations in unpurified cell culture supernatants are <0.1 mg/mL using small scale, transient transfections. Components in the media can also interfere with measurements of antibody self-association. One logical approach for characterizing unpurified mAb samples is to use a method that involves an affinity capture step in which antibodies are selectively adsorbed on a solid support and assayed. Although surface plasmon resonance methods such as Biacore have been used for this application,^{26,74} these methods are limited in their ability to assay weak interactions due to the low surface area for interaction.

A related approach – namely bio-layer interferometry⁷⁵ – has been reported recently for characterizing antibody self-association that holds potential for assaying unpurified mAb samples.⁶⁴ This method measures the interference pattern of light reflected from a surface with immobilized protein relative to a control surface to evaluate the wavelength shift, which is proportional to the thickness of the protein coating. By first coating the biosensor surface with an affinity capture antibody (e.g., goat anti-human antibody), dilute mAbs (~0.1 mg/mL) are adsorbed and the propensity of non-adsorbed mAbs to self-associate with immobilized mAbs is assayed. This approach has been shown to differentiate between highly and poorly soluble mAbs,⁶⁴ and it shows good correlation with lower throughput methods for measuring self-association such as self-interaction chromatography (which has been used for optimizing antibody solubility).^{54–56} Bio-layer interferometry experiments have been conducted in the presence of a non-specific protein (BSA), suggesting that it may be compatible with unpurified mAb samples that are common during early antibody discovery. This approach has good throughput because it can be conducted in 384-well plates. Minor limitations include low sensitivity at highly dilute mAb concentrations (~0.01 mg/mL) and the need for specialized equipment. Nevertheless, this powerful method holds significant potential for use during early antibody candidate selection.

Another promising approach for characterizing unpurified mAbs during early antibody discovery is affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS).^{59,63} This method involves coating gold nanoparticles with different ratios of capture (goat anti-human) to non-capture (goat non-specific) antibodies, and then using these conjugates to selectively immobilize mAbs from dilute antibody solutions (0.001–0.05 mg/mL; Fig. 3). The colloidal stability of the mAb-gold conjugates is governed by the self-association propensities of the immobilized mAbs. Associative mAbs lead to reduced separation distances between mAb-gold conjugates and red-shifting of the wavelength of maximum absorbance (plasmon wavelength, λ_p). Evaluation of the plasmon wavelength as a function of the capture to non-capture antibody ratio enables sensitive discrimination between different mAbs.

AC-SINS has been used to discriminate between high and low solubility mAbs that differ only by single mutations in their CDRs (Fig. 3).⁵⁹ Interestingly, the AC-SINS measurements are weakly impacted by cell culture media, suggesting AC-SINS may be compatible with unpurified cell culture supernatants. Moreover, the AC-SINS findings obtained at dilute mAb concentrations (0.05 mg/mL) are strongly correlated with dynamic light scattering and solubility experiments conducted at orders of magnitude higher concentrations (1–50 mg/mL). In addition, AC-SINS has good throughput because it is typically conducted in 384-well plates and requires only a standard absorbance plate reader. Weaknesses of AC-SINS include the possible impact of antibody immobilization on mAb self-association, and the potential interference of additives and contaminants that interact strongly with gold. Nevertheless, this approach appears promising for guiding initial mAb candidate selection.

Antibody cross-interactions (polyspecificity)

The potential for antibodies to interact non-specifically with multiple targets has long been recognized.⁷⁶ For example, antibodies generated from immature B-cells are typically

polyspecific, while those from mature B-cells are generally much more specific.⁷⁷ Quantifying polyspecificity is challenging given the large number of possible target molecules and the wide range of possible affinities.^{77,78} Polyspecificity is especially concerning for antibody therapeutics because of potential off-target effects and the negative impact that such cross-interactions may have on antibody pharmacokinetics.^{13,14}

Therefore, measurements of antibody polyspecificity are increasingly being incorporated into the analysis of therapeutic mAb candidates.^{13,14,79} A particularly exciting study reported the characterization of a large panel of mAbs that displayed a wide range of antibody clearance rates in monkeys and humans.¹⁴ Importantly, the differences in clearance rates were unrelated to their target antigens or interactions with recycling Fc receptors. Therefore, the investigators reasoned that non-specific interactions between mAbs and off-target molecules may lead to fast clearance. To test this hypothesis, they evaluated the binding of mAbs to baculovirus particles that present a complex mixture of phospholipids, carbohydrates, glycoproteins, nucleic acids and other types of molecules. Interestingly, the relative binding of mAbs to immobilized baculovirus particles in an ELISA assay was positively correlated with the rate of mAb clearance both in monkeys and humans (Fig. 4). Similar results for a subset of the mAbs were obtained using mammalian cells, but the use of cells as a non-specific binding reagent is problematic because some of the antigens are endogenous cell-surface proteins. Mutations that reduced mAb binding to baculovirus particles also reduced the rate of antibody clearance. Other physical properties of antibodies – such as net charge, isoelectric point and hydrophobicity – showed little correlation with the rate of antibody clearance. Strengths of this approach include good throughput and the potential to evaluate unpurified mAbs early in antibody discovery due to the ELISA format. Future work will need to evaluate if the correlation with antibody clearance rates can be further improved using other types of non-specific reagents alone or in combination with baculovirus particles. Nevertheless, these findings highlight the value of evaluating non-specific interactions for guiding the selection of mAb candidates with desirable (long) circulation times.

Another approach for incorporating measurements of polyspecificity into the early discovery process was reported using an alternative polyspecificity reagent.¹³ Instead of using baculovirus particles, the investigators generated a soluble membrane protein reagent from homogenized mammalian cells prepared with a mild surfactant. The binding of biotinylated membrane proteins to antibody libraries displayed on the surface of yeast was evaluated with fluorescence-activated cell sorting (FACS) to select antibody variants with low non-specific binding relative to high specific binding to the target antigen. Antibody binding to the membrane protein reagent was correlated with binding to baculovirus particles, which suggests the FACS measurements may also be correlated with antibody clearance rates. The correlation between different assays also suggests that similar types of non-specific interactions are detected using different polyspecificity reagents. The strengths of this approach include the excellent throughput of FACS and the ability to identify antibodies with low polyspecificity at an unusually early stage in the antibody discovery process. Future work will need to evaluate if other polyspecificity reagents (including baculovirus particles) further improve the detection of non-specific antibody interactions either alone or in combination with soluble membrane proteins.

A simpler, complementary method for evaluating antibody polyspecificity is to evaluate the interaction of mAbs with polyclonal antibodies.⁷⁹ This approach has been elegantly demonstrated in the form of cross-interaction chromatography in which human polyclonal antibodies are immobilized on chromatography particles and the retention times of human mAbs are evaluated from a polyclonal antibody column (Fig. 5).^{13,16,63,64,79} Longer retention times correspond to increased non-specific interactions between mAbs and polyclonal antibodies. There is some correlation between the propensity of human mAbs to interact with polyclonal antibodies and the propensity of mAbs to interact with baculovirus particles and soluble membrane proteins,¹³ suggesting that the three assays measure similar types of non-affinity interactions. Nevertheless, the baculovirus particle and membrane protein assays are more strongly correlated than are the cross-interaction chromatography and soluble membrane protein assays, which is consistent with the less complex nature of polyclonal antibodies relative to the other reagents. Strengths of cross-interaction chromatography include its simplicity, moderate throughput using a liquid chromatography system with an autosampler, relatively low mAb consumption (<10 µg per measurement), and the potential to evaluate unpurified mAbs if such samples can be modestly concentrated to ~100 µg/mL. Future work will need to further improve the throughput of the assay, reduce the required mAb concentrations for use in early antibody discovery, and evaluate the use of other immobilized molecules in addition to human polyclonal antibodies to improve detection of non-specific antibody interactions.

An interesting question is whether antibodies that are prone to self-associate (and which are poorly soluble and/or viscous) are also prone to interact promiscuously with other molecules. Indeed, cross-interaction chromatography was originally developed for identifying mAbs with low solubility.⁷⁹ A closely related method (self-interaction chromatography^{52,56,57}) could be used in which self-interactions between immobilized and non-immobilized mAbs are measured. However, the throughput of this method is low for screening different mAbs because a separate column must be prepared for each antibody variant. Nevertheless, cross-interaction chromatography measurements are correlated with mAb solubility (which is governed by antibody self-association).^{16,20,79,80} This suggests that interactions leading to non-specific antibody cross-association are similar to those that mediate antibody self-association. It is likely that the basis for the success of using cross-interaction chromatography measurements as a surrogate for self-interaction measurements is the similarity in the size, structure and composition of human mAbs and human polyclonal antibodies. Interestingly, a poorly soluble human mAb that interacts strongly with human polyclonal antibodies is less interactive with murine polyclonal antibodies,⁸⁰ suggesting that the extreme similarity between human mAbs and human polyclonal antibodies is important for detection of non-affinity antibody interactions (at least for those that govern antibody solubility). Nevertheless, exceptions between mAb self-interactions and cross-interactions with human polyclonal antibodies are expected and have been reported.⁶³ These findings caution against over-interpreting cross-interaction measurements without evaluating antibody self-association, and highlight the need for additional research to understand similarities and differences between antibody self- and cross-interactions.

A related intriguing question is whether selection and/or evolution of high-affinity antibodies generally increase the risk for non-specific antibody interactions. This seems

logical for antibodies that use hydrophobic residues in their CDRs to mediate high-affinity binding. Indeed, the hydrophobic CDRs of multiple high-affinity antibodies obtained from phage libraries have been shown to also mediate non-specific antibody interactions.^{16,18,20,32} Elegant work has shown how cross-interaction chromatography can be used to detect these colloidal interactions and how directed evolution methods can be used to reduce these interactions without compromising binding affinity.²⁰ Conventional wisdom suggests that high-affinity antibodies obtained from animals and/or eukaryotic cell display methods have a lower risk of non-specific antibody interactions due to the increased quality control mechanisms during evolution and selection. Indeed, a recent survey of >90 murine hybridoma antibodies revealed that they all display low non-specific interactions via cross-interaction chromatography.⁸⁰ Nevertheless, several antibodies derived from animals and/or eukaryotic cell display methods have been reported to be associative and/or display undesirable solution properties at high antibody concentrations required for subcutaneous delivery.^{13,14,63,64} Additional work is needed to better define the relative risks of obtaining associative antibodies using different antibody discovery methods. Nevertheless, these findings suggest that diverse antibody discovery methods can be used to successfully identify antibodies with both high affinity and low propensity to interact non-specifically if effective assays of colloidal interactions can be incorporated into the early discovery process.

Conclusions and Future Directions

The intense interest in developing antibodies as therapeutics has led to many innovative approaches for detecting weak protein interactions that can negatively impact antibody developability and therapeutic activity. It will be important to refine these methods to be compatible with the extreme requirements of early antibody discovery during which large numbers of dilute and unpurified mAbs must be characterized. Important next steps include defining the accuracy and precision of these methods, as well as comparing several of them at the same time to better evaluate their similarities and differences. It will also be critical to better define how different types of colloidal interactions influence properties such as antibody solubility, viscosity and antibody clearance rate. Finally, it will be important to evaluate whether measurements of antibody colloidal interactions prior to purification and characterization of mAbs can improve the discovery and development of new antibody candidates with desirable biophysical and pharmacokinetic properties.

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Figure 1.

Monoclonal antibodies are complex multidomain proteins that can associate with themselves or other molecules via non-affinity (colloidal) interactions. The Fab crystal structure of a poorly behaved antibody (PDB 3G6A) is highlighted with the heavy chain complementarity-determining regions (CDRs) in green and the light chain CDRs in blue. For this antibody, three consecutive aromatic residues (red) in heavy chain CDR3 mediate attractive colloidal interactions and poor antibody solubility.¹⁸

Figure 2.

Measurements of diffusion coefficients as a function of antibody concentration are correlated with viscosities of concentrated antibody solutions. Mutual diffusion coefficients are measured using dynamic light scattering for moderately concentrated antibody solutions (typically 1–20 mg/mL). Measurements of diffusion coefficients as a function of antibody concentration are used to calculate the diffusion interaction parameter (k_D). The exponential viscosity coefficients are obtained from viscosity measurements as a function of antibody concentration (50–175 mg/mL). The k_D and viscosity measurements are reproduced from a previous report.³⁷

Figure 3.

Identification of mutations that reduce antibody self-association and increase solubility using affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS). (A) Gold nanoparticles are coated with mixtures of non-capture (gray) and capture (red) polyclonal antibodies, the latter of which is specific for human mAbs (blue). After capturing human mAbs, attractive mAb self-interactions are detected due to reduced interparticle separation distances and increased plasmon wavelengths. (B) Plasmon wavelength measurements as a function of the ratio of non-capture to capture antibody reveal that two mAbs with single mutations in their complementarity determining regions have reduced self-association. The inset images show that the mAbs that display reduced self-association at dilute concentrations (1–50 $\mu\text{g/mL}$) also do not phase separate when concentrated orders of magnitude (50 mg/mL). The red boxes highlight the phase separation for the three mAbs with elevated self-association. The plasmon wavelengths and images of antibody solutions are reproduced from a previous report.⁵⁹

Figure 4.

Non-affinity interactions between monoclonal antibodies and baculovirus particles are correlated with the rate of antibody clearance in humans. Baculovirus particles are immobilized in microtiter plates, and the relative binding of antibody variants is evaluated. Increased antibody binding is correlated with increased rate of antibody clearance. The data is reproduced from a previous report.¹⁴

Figure 5.

Detection of monoclonal antibodies that interact non-specifically with polyclonal antibodies using cross-interaction chromatography. Polyclonal antibody is immobilized on chromatography particles, and the relative retention of different monoclonal antibodies is evaluated. Increased retention signifies attractive antibody interactions. The chromatograms are reproduced from a previous report.⁷⁹