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High-Throughput Assay for Measuring Monoclonal Antibody Self-Association and Aggregation in Serum

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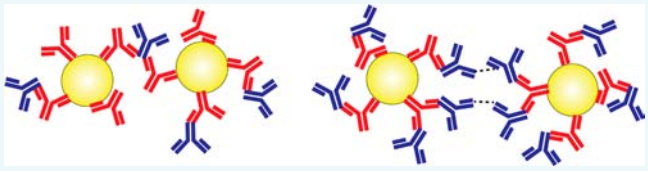
Supporting Information

ABSTRACT: Subcutaneous delivery is one of the preferred administration routes for therapeutic monoclonal antibodies (mAbs). High antibody dosing requirements and small injection volumes necessitate formulation and delivery of highly concentrated mAb solutions. Such elevated antibody concentrations can lead to undesirable solution behaviors such as mAb self-association and aggregation, which are relatively straightforward to detect using various biophysical methods because of the high purity and concentration of antibody formulations. However, the biophysical properties of mAbs in serum can also impact antibody activity, but these properties are less well understood because of the difficulty characterizing mAbs in such a complex environment. Here we report a high-throughput assay for directly evaluating mAb self-association and aggregation in serum. Our approach involves immobilizing polyclonal antibodies specific for human mAbs on gold nanoparticles, and then using these conjugates to capture human antibodies at a range of subsaturating to saturating mAb concentrations in serum. Antibody aggregation is detected at subsaturating mAb concentrations via blue-shifted plasmon wavelengths due to the reduced efficiency of capturing mAb aggregates relative to monomers, which reduces affinity cross-capture of mAbs by multiple conjugates. In contrast, antibody self-association is detected at saturating mAb concentrations via red-shifted plasmon wavelengths due to attractive interparticle interactions between immobilized mAbs. The high-throughput nature of this assay along with its compatibility with unusually dilute mAb solutions (0.1−10 μg per mL) should make it useful for identifying antibody candidates with high serum stability during early antibody discovery.



INTRODUCTION

Monoclonal antibodies (mAbs) are one of the most successful protein therapeutics to date given their remarkable biological and biophysical properties.[1,2] In terms of bioactivity, antibodies are the workhorses of the humoral immune system. They are natural adaptor molecules that bind to foreign molecules via their Fab fragments and immune cells via their Fc fragments. These multiple functionalities give mAbs potent therapeutic activities that are difficult to achieve with other types of biomolecules or small molecule drugs. The attractive biophysical properties of mAbs are also key to their success as therapeutics, as mAbs typically have excellent conformational and colloidal stabilities.[3−5]

Nevertheless, antibodies differ significantly in the sequence of their complementarity-determining regions (CDRs), and can also differ in the sequence of their variable and constant regions depending on the IgG subtype. These differences give rise to a surprisingly wide range of solution behaviors, including undesirable ones such as low solubility, opalescence, high viscosity, and formation of different types of aggregates and particles.[6−10] Much effort is being focused on early assessment of the biophysical properties of mAbs during antibody discovery to avoid problems that arise later in development when antibodies are purified and formulated at high concentrations.[5,11−16] Likewise, many innovative protein engineering strategies have been reported for improving antibody solubility and other biophysical properties.[3,17−21]

Another important yet poorly understood attribute of mAbs is their biophysical properties in serum. While conventional wisdom suggests that mAbs are highly specific and only interact with their target antigens and Fc receptors, several studies have shown that mAbs can display a range of propensities to interact nonspecifically with themselves and diverse molecules, including those in serum.[12,22,23] These promiscuous interactions are particularly concerning because they can potentially lead to undesirable behaviors including antibody aggregation, off-target effects, and fast antibody clearance.[22,24]

Therefore, it is important to evaluate the biophysical properties of antibodies in serum as part of the antibody discovery and development process, yet such measurements are challenging given the complex and concentrated nature of serum. One general approach that avoids direct analysis of

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mAbs in serum is to evaluate the propensity of antibodies to interact with serum components (such as polyclonal antibodies) or complex mixtures of cellular or viral proteins.[12,22−24] These approaches have proven to be surprisingly useful, and multiple reports have demonstrated that the propensity of mAbs to interact nonspecifically with such molecules is correlated with low antibody solubility and/or fast antibody clearance.[12,22,23]

Nevertheless, it is critical to assess the biophysical properties of mAbs in serum to more fully and accurately predict their serum stability. Multiple approaches have been reported to address this important challenge.[24,25] For example, a recent study demonstrated several promising assays for characterizing antibodies in serum.[24] These include light microscopy, dynamic light scattering, and surface plasmon resonance. These studies demonstrate that antibodies such as trastuzumab (Herceptin) or bevacizumab (Avastin) prepared in dextrose solutions form particles when mixed with human plasma, while other antibodies such as infliximab (Remicade) do not. Moreover, the same antibodies formulated in saline do not form particles, revealing complex mAb behavior in human plasma that is at least partially controllable via the composition of antibody formulations.

Despite this excellent progress in developing methods for evaluating antibody properties in serum, there is still a need for simple and high-throughput assays capable of evaluating mAb stability in serum for the large numbers (tens to thousands) of mAb candidates that are common during early stages of antibody discovery. The goal of this work is to develop an assay for evaluating mAb self-association and aggregation in serum. Our approach is based on our recent development of a nanoparticle-based assay (affinity-capture self-interaction nanoparticle spectroscopy, AC-SINS) for measuring mAb self-association.[16] The premise of this method is that gold nanoparticles are coated with polyclonal antibodies specific for human mAbs, and the polyclonal antibody-gold conjugates are used to capture human mAbs from either purified or unpurified samples. Attractive self-interactions between immobilized mAbs cause the conjugates to cluster together, which can be detected via a change in color of the gold suspension and a red shift in the plasmon wavelength (wavelength corresponding to the maximum absorbance). The plasmon shifts obtained by AC-SINS at dilute antibody concentrations (1−50 μg/mL) are correlated with light scattering measurements at several orders of magnitude higher antibody concentrations.[16] Attractive attributes of this approach include its compatibility with dilute and unpurified samples, and the use of common lab equipment (absorbance plate reader).

We reasoned that the ability of AC-SINS to capture and characterize mAbs from unpurified solutions may enable direct characterization of mAb self-association and aggregation in serum. Although AC-SINS has not been reported for detecting antibody aggregation, we posited that such aggregation would lead to reduced plasmon wavelengths due to reduced binding efficiency of the aggregates to the capture conjugates and/or increased thickness of the adsorbed mAb coating. We also posited that the sensitivity of AC-SINS measurements could be enhanced by evaluating plasmon wavelengths from subsaturating to saturating concentrations of adsorbed mAb. Here we report that mAb self-association and aggregation can be evaluated in the same assay, and we use this approach to discriminate between mAbs with dissimilar propensities to self-associate and aggregate in the absence and presence of serum.

RESULTS

AC-SINS Measurements as a Function of the Amount of Immobilized Human Antibody. We selected two mAbs (both IgG1 molecules) for our initial studies, one that is known to be poorly soluble and self-associative (mAb A), and a second one that is significantly more soluble and less associative (mAb B). Previous studies have demonstrated that mAb A (CNTO607) is poorly soluble and displays significant interaction with polyclonal antibodies (as evaluated using cross-interaction chromatography with retention factor values >0.5 for mAb A and <0.05 for mAb B).[23,26,27] Using these mAbs and a human polyclonal antibody (pAb) mixture as a control, we first adsorbed the human antibodies on gold nanoparticles coated with goat anti-human Fc antibodies at low (0.1 μg/mL), moderate (1 μg/mL), and high (10 μg/mL) antibody concentrations. We quantified the number of human antibodies adsorbed per particle, and found 0.2−0.6 (0.1 μg/mL), 2−5 (1 μg/mL), and 7−15 (10 μg/mL) antibodies bound per particle (Table 1). The immobilization density at 10 μg/mL is similar to the theoretical prediction for a monolayer mAb coverage (8−21 mAbs per particle) on polyclonal antibody-coated particles.[28] This estimate is based on the predicted density of capture antibodies (2−5.5 mg/m^2), the fact that each capture antibody can bind two human antibodies, and the assumption that half of the randomly immobilized capture antibodies are oriented suitably for binding human antibodies.

Table 1. Measurements of the Number of Adsorbed Human Antibodies Per Anti-Fc Conjugate as a Function of the Initial Antibody Concentration in PBS[a]

antibody	initial antibody concentration (μg/mL)	immobilized antibodies per particle
mAb A	10	7.8 ± 1.4
	1	2.0 ± 0.5
	0.1	0.23 ± 0.04
mAb B	10	11 ± 1
	1	4.6 ± 0.2
	0.1	0.50 ± 0.08
pAb	10	15 ± 1
	1	4.7 ± 1.5
	0.1	0.55 ± 0.2

[a]The data are averages of three repeats and the errors are standard deviations.

Next, we evaluated the absorbance spectra of the antibody conjugates (Figure 1). The plasmon wavelength for the control conjugates without mAb is 534 nm. At 0.1 μg/mL mAb, the plasmon wavelengths of mAb A (539 nm) and mAb B (537 nm) are similar to that of the control (534 nm), revealing that immobilization of less than one mAb per particle is insufficient to mediate significant cross-capture of mAbs by immobilized capture antibodies. At an antibody concentration of 1 μg/mL, we observe significant plasmon shifts for both mAb A (551 nm) and mAb B (549 nm). The similar plasmon shifts for the two significantly different mAbs is consistent with the fact that affinity cross-capture is mediated by the Fc fragment and weakly impacted by the unique Fab fragments of the two different antibodies. However, the behavior of the mAbs is significantly different at higher antibody concentrations (10 μg/mL), as mAb A has an even larger plasmon wavelength (554 nm), while mAb B has a similar plasmon wavelength (538 nm) as the control (534 nm). This is consistent with that fact that

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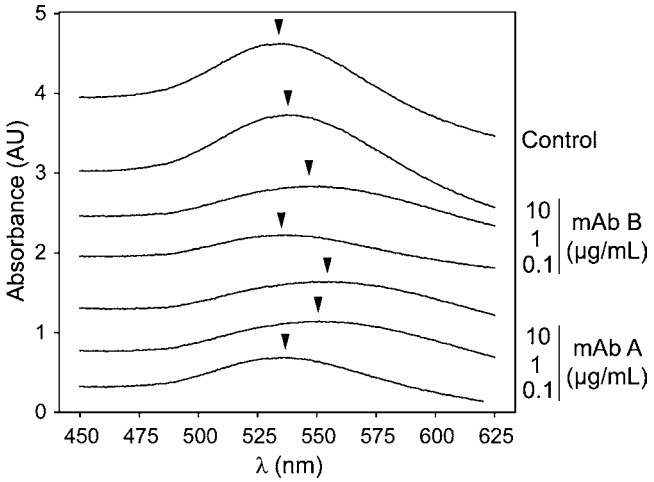

Figure 1. Absorbance spectra of antibody−gold conjugates as a function of mAb concentration. The spectra are for gold nanoparticles (20 nm) coated with goat anti-human Fc antibody in the absence or presence of mAbs in PBS (10 mM sodium phosphate, 1.8 mM potassium phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4). The measurements were obtained after 2 h of coincubation of human antibodies and capture conjugates. The samples were supplemented with goat nonspecific antibody (144 μg/mL final concentration) to maximize specificity. The arrowheads indicate the plasmon wavelengths. The spectra are shifted vertically in an arbitrary manner for clarity.

saturating levels of immobilized mAb enable evaluation of antibody self-association.

To evaluate this behavior in more detail, we measured the plasmon wavelengths for mAb A and B over a larger range of concentrations (0.001−10 μg/mL) in the presence of a constant amount of goat nonspecific polyclonal antibody (144 μg/mL) to maximize specificity (Figure 2). To define antibody concentrations that lead to cross-capture, we also evaluated the plasmon response for human pAb as a control. The human pAb shows little response except at 1 μg/mL (Figure 2), further suggesting that this concentration leads to affinity cross-capture. The response of mAbs A and B are

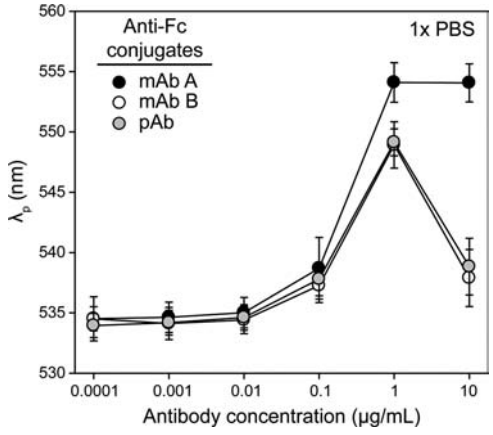

Figure 2. Plasmon wavelengths for goat anti-human Fc conjugates as a function of human antibody concentration. The plasmon wavelength for the control conjugates (without human antibody) was 534 nm. The assay conditions were the same as those described in Figure 1. The data are averages of three repeats, and the error bars are standard deviations.

similar at this intermediate concentration. At the highest concentration (10 μg/mL), mAb B shows similar plasmon wavelengths as human pAb, which is consistent with the high solubility of mAb B. We confirmed that these results are weakly dependent on the incubation time of the mAb with the conjugates (2 and 24 h; Supporting Information Figure S1), and that they are strongly dependent on the use of an affinity capture antibody (Figure S2).

We also investigated whether the orientation of the captured mAbs has an impact on the plasmon measurements by changing the capture antibody from goat anti-human Fc to goat anti-human Fab. Previous studies have shown that mAb A contains a hydrophobic triad of aromatic residues within heavy chain CDR3.[27] These hydrophobic residues are responsible for the poor solubility of mAb A, and mutating them to alanine residues greatly increases solubility.[26,27] We hypothesized that immobilizing mAb A via its Fab fragment would preferentially display its Fc fragment, thereby reducing exposure of this hydrophobic patch and leading to reduced interaction. Indeed, mAb A shows reduced plasmon wavelengths at all antibody concentrations for anti-Fab conjugates (Figure 3) relative to

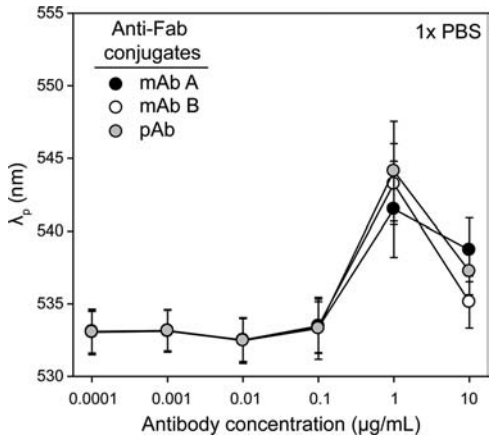

Figure 3. Effect of changing the orientation of human antibody immobilization on the plasmon wavelength measurements as a function of human antibody concentration. Gold nanoparticles were coated with goat anti-human Fab antibody and evaluated using the same conditions as described in Figure 1. The plasmon wavelength for the control (without human mAb) was 532 nm. The data are averages of five repeats, and the error bars are standard deviations.

anti-Fc conjugates (Figure 2). mAb A self-interactions are significantly reduced (as detected at 10 μg/mL) by immobilizing this antibody via its Fab fragment, and they are only modestly higher (539 nm) than those for mAb B (535 nm) and pAb (537 nm; Figure 3). Our results suggest that the Fab fragment is more interactive than the Fc fragment for mAb A, and that the Fab fragment is particularly important in mediating mAb A self-association.

We suspected that our measurements would be influenced by changes in solution conditions such as reduced ionic strength, as observed previously in studies of antibody self-association.[29−37] Thus, we investigated the impact of reducing the salt concentration from 140 to 10 mM on our plasmon measurements (Figure 4). Notably, the plasmon wavelength of the human pAb increases significantly at 10 μg/mL, while it is weakly impacted for mAbs A and B. This is consistent with the high solubility of mAb B, as it appears less self-associative than the polyclonal antibody mixture. The plasmon wavelengths at 1

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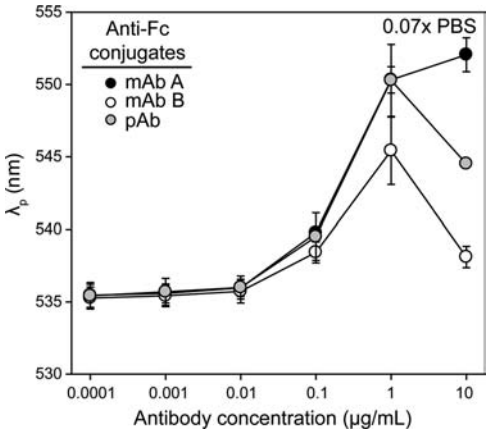

Figure 4. Effect of reducing the salt concentration on the plasmon wavelength measurements as a function of human antibody concentration. The plasmon wavelength measurements were obtained at low salt (10 mM sodium phosphate, 1.8 mM potassium phosphate, 9.8 mM NaCl, 0.2 mM KCl; pH 7.4) using gold nanoparticles coated with goat anti-human Fc antibody. The assay conditions (except for the salt concentration) were the same as those described in Figure 1. The plasmon wavelength for the control (without human mAb) was 535 nm. The data are averages of three repeats, and the error bars are standard deviations.

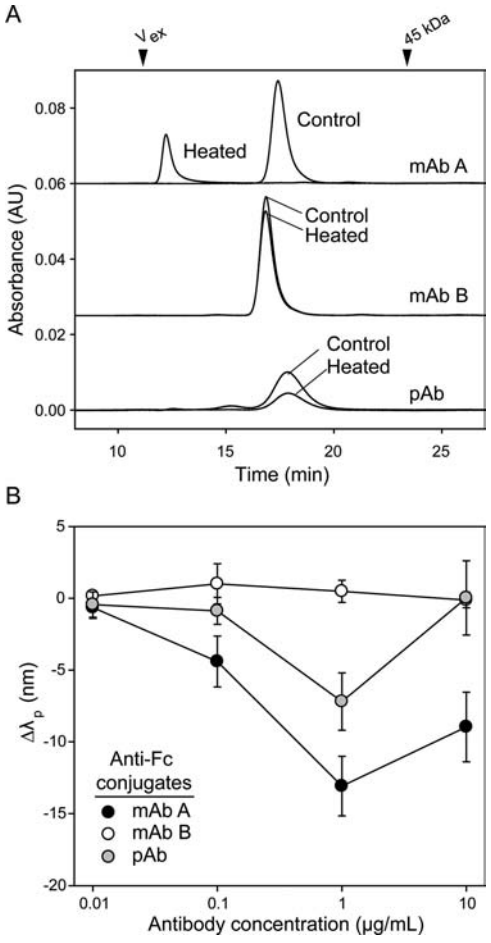

Figure 5. Impact of transiently heating human antibodies on the plasmon wavelength measurements. Human antibodies (100 μg/mL) were heated at 65 °C for 1 h in PBS (pH 7.4). (A) Size-exclusion chromatography of the human antibodies before and after heat stress in PBS. (B) Plasmon shifts for human antibodies after heat stress relative to before heat stress in PBS for anti-Fc conjugates. The plasmon wavelength for the control (without human antibody) was 534 nm. The data are averages of three repeats, and the error bars are standard deviations.

μg/mL are generally similar for the three antibodies at low (Figure 4) and moderate (Figure 2) salt concentrations, which is consistent with the fact that affinity cross-capture is expected to be weakly impacted by the salt concentration.

Detection of Antibody Aggregation Using AC-SINS. We next asked whether our assay could be used to detect antibody aggregation. We posited its sensitivity to antibody aggregation would be maximal at the cross capture condition of 1 μg/mL. This is because this condition leads to significant plasmon shifts that are strongly dependent on the antibody concentration and the ability to efficiently immobilize human antibodies. To test this hypothesis, we stressed the human antibodies at 65 °C for 1 h, sedimented any insoluble aggregates that formed, and evaluated the soluble fraction via size-exclusion chromatography (Figure 5A). We find that mAb A shows significant aggregation, while mAb B does not. The human pAb shows intermediate behavior, as its concentration appears modestly reduced.

We next quantified the concentrations of the three antibodies after heating, diluted them to the proper final concentrations, and evaluated their plasmon shifts relative to unheated samples as a function of human antibody concentration for anti-Fc conjugates (Figure 5B). Notably, mAb A shows significant negative plasmon shifts relative to the unheated control at the cross capture condition (1 μg/mL), while mAb B does not. The human pAb shows intermediate behavior, consistent with the size-exclusion chromatography results (Figure 5A). Importantly, the reduced plasmon wavelengths for mAb A and human pAb are not due to the presence of insoluble antibody aggregates because we obtained similar results with and without sedimentation after heating (Figure S3). Moreover, the reduced signals are also not due to reduced antibody concentration, as we evaluated and approximately adjusted the antibody concentrations after heating. Instead, we find that the reduced plasmon wavelengths are due to reduced antibody immobilization, as the number of immobilized mAb A molecules per particle is reduced from 2.0 ± 0.5 (control) to 0.32 ± 0.2 (heated) at 1 μg/mL, and from 7.8 ± 1.4 (control) to 1.4 ± 0.6 (heated) at 10 μg/mL. Finally, we quantified the sensitivity of the assay for detecting aggregation by mixing different ratios of heated and unheated mAb A (Figure S4). We find samples with >50% heated mAb A lead to reductions in the plasmon wavelength of at least 2 nm and can be differentiated from samples without heat-induced aggregates.

Evaluation of mAb Self-Association and Aggregation in Serum. We also asked whether AC-SINS could be used to evaluate mAb self-association and aggregation in serum. We used mouse serum to avoid interference from human polyclonal antibodies in human serum. To evaluate whether mouse serum interferes with immobilization of human antibodies, we measured the number of immobilized antibodies per conjugate as a function of serum concentration (Table 2). In general, serum had a modest impact on the number of immobilized antibodies over a wide range of serum concentrations (1–50%).

Therefore, we evaluated plasmon wavelengths for the antibody conjugates in serum as a function of the human antibody concentration (Figure 6). Importantly, the plasmon

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Table 2. Measurements of the Number of Adsorbed Human Antibodies Per Anti-Fc Conjugate as a Function of the Initial Antibody Concentration in Different Amounts of Mouse Serum[a]

antibody	% serum	initial antibody concentration (μg/mL)	immobilized antibodies per particle
mAb A	1	10	10 ± 2
		1	2.1 ± 0.3
		0.1	0.25 ± 0.04
	10	10	8.6 ± 2.0
		1	2.2 ± 0.4
		0.1	0.20 ± 0.03
	50	10	8.8 ± 1.9
		1	2.4 ± 0.7
		0.1	0.16 ± 0.01
mAb B	1	10	11 ± 1
		1	4.3 ± 0.2
		0.1	0.51 ± 0.05
	10	10	8.0 ± 2.3
		1	3.3 ± 0.3
		0.1	0.34 ± 0.05
	50	10	13 ± 1
		1	2.7 ± 0.4
		0.1	0.31 ± 0.07
pAb	1	10	15 ± 2
		1	4.5 ± 1.0
		0.1	0.56 ± 0.02
	10	10	16 ± 1
		1	3.8 ± 0.3
		0.1	0.45 ± 0.02
	50	10	13 ± 2
		1	2.7 ± 0.6
		0.1	0.21 ± 0.11

[a]The data are averages of three repeats and the errors are standard deviations.

wavelength measurements discriminate between mAb A and the other human antibodies in 1 and 10% serum (Figure 6). The presence of serum does not interfere significantly with the spectra of the gold conjugates (Figure S5). The plasmon wavelengths are generally reduced as the serum concentration is increased (Figure 6). Moreover, we confirmed that these results are similar for different incubation times (2 and 24 h; Figure S6) and temperatures (25 and 37 °C; Figure S7).

We also investigated whether it was possible to detect antibody aggregates in serum (Figure 7). The human antibodies were transiently heat stressed and their concentrations were adjusted to obtain the correct final concentrations. The heat stressed antibody samples were then mixed with different amounts of serum and combined with anti-Fc conjugates. We observed significant reductions in the plasmon wavelengths ($\Delta\lambda_p < -5$ nm) for mAb A samples at 1 and 10 μg/mL (Figure 7), which is consistent with our results in the absence of serum (Figure 5). We also evaluated the sensitivity for detecting mAb A aggregates in serum when mixing unheated and heated mAb samples, and found that samples with ≥50% heated mAb A led to changes in plasmon wavelengths of at least 2 nm (Figure S8), as observed for samples without serum (Figure S4). These results demonstrate that our assay can detect antibody aggregates in serum with modest interference from serum.

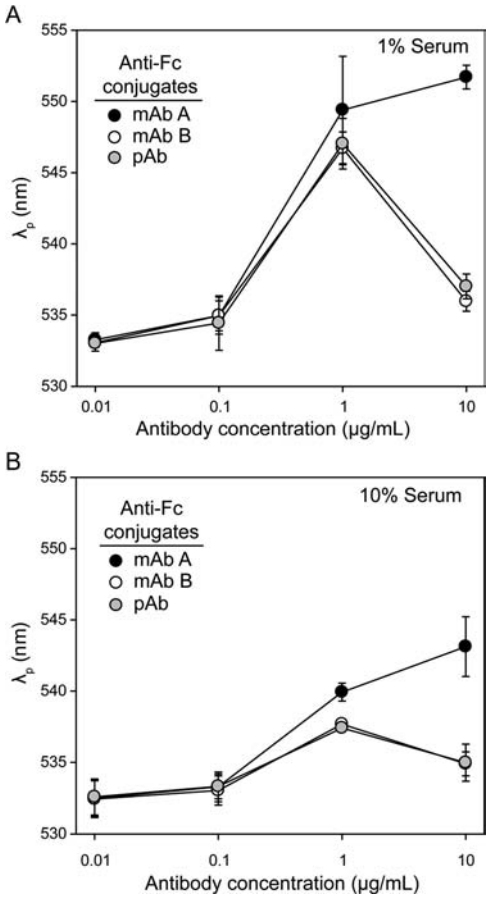

Figure 6. Plasmon wavelength measurements for human antibodies in the presence of serum. Human antibodies were immobilized on gold nanoparticles coated with goat anti-human Fc antibody in the presence of different amounts of serum, and directly assayed in mouse serum. The plasmon wavelengths for the control conjugates (without human antibody) were 533 nm (1% serum) and 532 nm (10% serum). The assay conditions were the same (except for the addition of serum) as those described in Figure 1. The data are averages of three repeats, and the error bars are standard deviations.

DISCUSSION

The main goal of this work was to evaluate the biophysical properties of mAbs directly in serum. Our approach using nanoparticle probes was inspired by previous work that used fluorescent labels for selectively analyzing the properties of mAbs in serum via an analytical ultracentrifuge equipped with a fluorescence detector.[25,38,39] A strength of this approach relative to our assay is the much smaller size of the probe (which is less likely to influence the properties of antibodies), while disadvantages are the low throughput and the need for specialized equipment.

While our approach is similar in some ways to our previous reports related to AC-SINS,[14,16,40−43] there are several key differences that deserve further consideration. In this work, we varied the amount of human antibody from low subsaturating levels to saturating ones, while our previous work typically used saturating levels of human antibody. We find similar cross-capture signals for human mAbs and pAb at subsaturating levels of adsorbed antibody (2−5 human antibodies per particle) regardless of their biophysical properties. This is logical because the cross-capture signal should be strongly dependent on affinity interactions involving Fc or Fab fragments, and weakly

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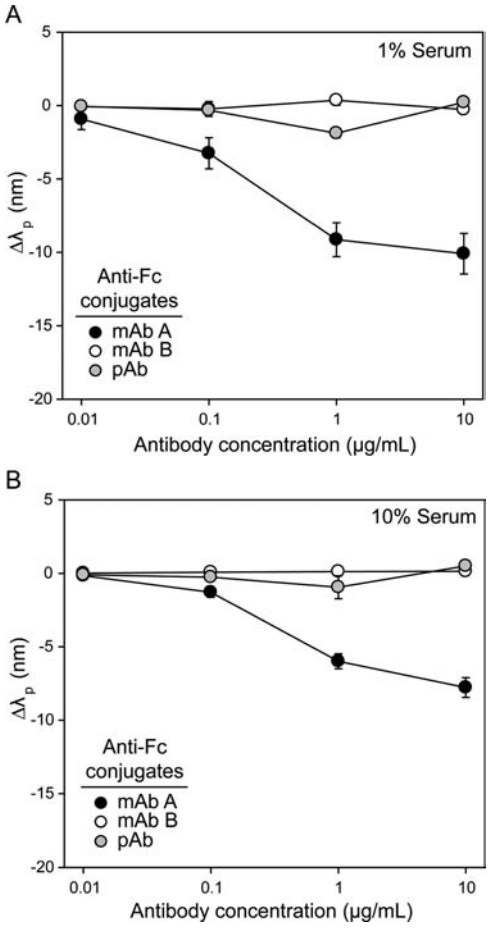

Figure 7. Effect of transiently heating human antibodies on the plasmon wavelength measurements in the presence of serum. Human antibodies (100 μg/mL) were heated at 65 °C for 1 h in PBS (pH 7.4) in the absence of serum, and adsorbed on anti-Fc conjugates in the presence of (A) 1% serum and (B) 10% serum. The plasmon shifts are for human antibodies after heat stress relative to before heat stress. The antibody concentrations were measured and adjusted after heat stress. The plasmon wavelengths for the control conjugates (without human antibody) were 535 nm (1% serum) and 534 nm (10% serum). The data are averages of four repeats, and the error bars are standard deviations.

influenced by the self-association behavior of the human mAbs. This cross-capture signal is a valuable control for our assay to confirm that well-behaved mAbs with low propensity to self-associate, which normally give low signals above background, are properly immobilized.

Another valuable aspect of evaluating affinity cross-capture of antibodies at subsaturating mAb concentrations is the ability to detect antibody aggregation. Prior to this work, we did not know whether antibody aggregates would increase or decrease the measured plasmon wavelengths. For heat stressed antibody samples with aggregates detectable by size-exclusion chromatography (mAb A), we find that plasmon wavelengths are reduced relative to nonaggregated controls even after accounting for antibody concentration changes due to aggregation. We confirmed that this is due to reduced immobilization of aggregated mAb A, which results in reduced affinity cross-capture. Future studies are required to determine whether different types of antibody aggregates (including those formed due to other types of stresses) will also display reduced immobilization relative to monomers.

Interestingly, we also observe reduced plasmon wavelengths for heat stressed polyclonal antibody samples that do not contain detectable aggregates (despite also accounting for concentration changes after heat stress). Notably, these changes are only detectable at subsaturating (1 μg/mL) antibody concentrations corresponding to affinity cross-capture. This result highlights the importance of using subsaturating antibody concentrations for detecting antibody aggregation. More work is needed to understand whether the reduced plasmon wavelengths for heat stressed pAb samples are due to reduced efficiency of antibody immobilization and/or reduced interaction between immobilized antibodies.

We evaluated the sensitivity of our assay to several parameters to identify optimal conditions and assay limitations. One factor that significantly impacted the sensitivity of our assay is the type of capture (anti-human) antibody that was used to coat the gold particles. The anti-Fc antibody conjugates yielded significant differences between mAb A and B at saturating antibody concentrations (10 μg/mL), consistent with the large differences in self-association for these two antibodies.[26,27] Surprisingly, we obtained poor discrimination between them using anti-Fab conjugates at any mAb concentrations. As noted earlier, mAb A (CNTO607) is known to have a hydrophobic patch within HCDR3,[26,27] and immobilizing this antibody via its Fab fragments may partially shield this CDR. Our limited findings suggest that anti-Fc polyclonal antibody is optimal for maximizing sensitivity of our assay, which is also consistent with our previous results for different mAbs.[16]

Another important factor that impacted the sensitivity of our assay is the solution environment. Most of our experiments were conducted in PBS (pH 7.4), which is a relevant physiological solution condition and a robust one for our assay because the background plasmon wavelengths without mAb are low. Interestingly, we were unable to differentiate between mAb B and human polyclonal antibody in PBS (Figures 2 and 3), but we could detect that mAb B displays lower self-association than human polyclonal antibody in a lower ionic strength solution condition (pH 7.4, 10 mM phosphate, 9.8 mM NaCl, 0.2 mM KCl, Figure 4). This simple example highlights the sensitivity of our assay to changes in solution conditions and demonstrates the opportunity to improve sensitivity by changing these conditions. However, we have previously reported that some solution conditions, such as those with histidine buffers at low salt, result in high background for a closely related form of this assay and can complicate interpretation of the measurements.[40]

Our analysis of the sensitivity of AC-SINS for detecting antibody aggregation demonstrates a limitation of our work. While we were able to distinguish heat stressed samples for mAb A and human polyclonal antibody from control samples, the sensitivity of these measurements was modest. We find that mixtures containing at least 50% aggregated mAb A are necessary to robustly differentiate such mixtures from their unstressed controls both in the absence and presence of serum. Further developments are necessary to improve the sensitivity to the point that antibody aggregation is detectable at levels (e.g., <10%) that are of broad interest during antibody development and formulation.

The addition of serum also impacts the sensitivity of our assay. We generally find that increased concentrations of serum correspond to reduced plasmon wavelengths. One potential reason for this may be that components in serum reduce or

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displace adsorbed mAbs. However, our findings suggest that this is not the case because the amount of adsorbed human antibody is weakly dependent on the presence of serum, despite that the mAbs and human polyclonal antibody were adsorbed in the presence of serum (Table 2). We suspect that components of serum interact with immobilized human antibodies and reduce their propensity to self-associate. Nevertheless, it is significant that we could detect mAb A self-association in solutions containing 10% serum (Figure 6), confirming that serum does not significantly mask mAb self-association. This is consistent with the ability of mAbs to self-associate and aggregate in serum despite the high concentration of non-mAb components.[24]

■ CONCLUSIONS

We have developed a simple and widely accessible assay for evaluating antibody self-association and aggregation in serum. The strengths of AC-SINS include the ease of use for analyzing many (tens to hundreds) mAbs in parallel, its use of dilute (μg/mL) antibody solutions, and its ability to detect mAb self-association and aggregation in serum. Future studies should focus on improving the sensitivity of AC-SINS for detecting antibody aggregation. It will also be important to evaluate this assay using a larger panel of mAbs than we did in this study, especially those with well-characterized and dissimilar biophysical properties in serum. Nevertheless, we expect that our assay will be useful during early antibody discovery for guiding the selection of mAbs with low propensity to self-associate and aggregate in both standard antibody formulations as well as in serum.

■ MATERIALS AND METHODS

Materials. Citrate-stabilized 20 nm gold nanoparticles (15705) were obtained from Ted Pella Inc. (Redding, CA). The mAbs (IgG1s) were prepared as described earlier.[26] Polyclonal goat anti-human antibodies (Fc region, 109-005-098; $F(ab')_2$ region, 109-005-006) and a polyclonal goat nonspecific antibody (005-000-003) were obtained from Jackson ImmunoResearch (West Grove, PA). Glacial acetic acid (ACS grade, AC12404-0010), potassium acetate (ACS grade, P171), and sodium chloride (ACS grade, S271) were obtained from Thermo Fisher Scientific (Waltham, MA). Polyclonal IgG from human serum (reagent grade, I4506), potassium phosphate monobasic (ACS grade, P0662), sodium phosphate dibasic dihydrate (reagent grade, 30435), and potassium chloride (molecular biology grade, P9541) were obtained from Sigma-Aldrich (St. Louis, MO). Mouse serum (C57BL/6) was obtained from BioreclamationIVT (Westbury, NY). Alexa Fluor 488 NHS Ester (succinimidyl ester, A-20000) was obtained from Life Technologies (Carlsbad, CA). Polysorbate 80 (PS-80; Tween 80, BP338-500) was obtained from Thermo Fisher Scientific (Waltham, MA).

Methods. *Capture Antibody Immobilization.* Anti-Fc and anti-$F(ab')_2$ capture antibodies were buffer exchanged using Zeba desalting columns (PI-89882, Thermo Fisher Scientific) into 20 mM acetate (pH 4.3). UV absorbance measurements were performed at 280 nm, and the antibody concentrations were calculated using extinction coefficients of 1.927 mL/(mg·cm) for the anti-Fc antibody and 1.29 mL/(mg·cm) for the anti-$F(ab')_2$ antibody. Each capture antibody was diluted to 0.4 mg/mL in the acetate buffer. A volume of 100 μL of each capture antibody was mixed with 900 μL of citrate-stabilized gold nanoparticles (0.77 nM or 4.67×10^{12} particles/mL) and incubated overnight at room temperature. The conjugates were then centrifuged for 5 min at 15 000 rpm, 95% of supernatant was removed, and the pelleted conjugates were resuspended in the remaining 5% of the original solution.

mAb Immobilization and Plasmon Analysis of Nanoparticle Conjugates. Nonspecific goat antibody (10 mg/mL), mAb (100 μg/mL), and human polyclonal antibody (100 μg/mL) solutions were prepared in a solution of either PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, 1.8 mM potassium phosphate; pH 7.4) or a low salt phosphate buffer (9.8 mM NaCl, 0.2 mM KCl, 10 mM sodium phosphate, 1.8 mM potassium phosphate; pH 7.4), both with 550 ng/mL PS-80. The mAbs and human pAb were then serially diluted into the nonspecific goat antibody to arrive at 0.00011−11 μg/mL mAb and 160 μg/mL nonspecific goat antibody. Next, one part of capture antibody−gold conjugates was mixed with nine parts by volume of the mAb solution containing nonspecific goat antibody in a 384-well transparent polystyrene plate (12565506, Fisherbrand; final volume of 50 μL per well) to obtain final concentrations of 0.0001−10 μg/mL mAb, 144 μg/mL nonspecific goat antibody, and 495 ng/mL PS-80. The antibody concentrations were determined via UV absorbance measurements at 280 nm using an extinction coefficient of 1.4 mL/(mg·cm) for the human antibodies and 1.28 mL/(mg·cm) for the nonspecific goat antibody. The human antibody concentrations obtained via UV measurements were also checked for consistency using the BCA assay (Pierce; Rockland, IL), and the values were found to be similar to each other (<5% difference) and modestly higher (1.25−1.3 fold) for BCA measurements compared to the UV measurements.

To determine the number of immobilized human antibodies per conjugate, human antibodies were labeled with a fluorescent dye (Alexa Fluor 488) following the manufacturer's instructions. Labeled antibodies were then purified and the degree of labeling was calculated. Typical labeling ratios were 12−18 dye molecules per antibody. Labeled antibodies were then mixed with capture antibody−gold conjugates, as described above except this was done in microcentrifuge tubes (polypropylene, 87003-294, VWR; 100 μL per tube) instead of well plates. After 2 h, samples were spun for 5 min at 15 000 rpm, and 50 μL of the supernatant was transferred to 384-well black polystyrene plates (nontreated plate; 262260, Thermo Fisher Scientific). The fluorescence intensity was measured using a Tecan Safire^2 plate reader at 483 nm (excitation) and 525 nm (emission). The amount of immobilized antibody was calculated using a standard curve relating fluorescence intensity to labeled antibody concentration.

The absorbance spectra (400−650 nm) of the antibody−gold conjugates were measured using a Tecan Safire^2 plate reader (after 2 h of mAb adsorption unless otherwise stated). The plasmon wavelength was calculated using an Excel macro that fit 40 data points around the maximum absorbance wavelength to a second order polynomial function and then set the first derivative to zero.

mAb Aggregation and Characterization. Human antibodies were prepared in PBS at 100 μg/mL in PCR tubes (20170-004, VWR) and were heated at 65 °C for 1 h. After cooling to room temperature, the antibody concentration was evaluated using the micro BCA assay (23235, Thermo Fisher Scientific) and adjusted appropriately. The heated human antibodies were then serially diluted into the nonspecific goat antibody to arrive

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at 0.011−11 μg/mL mAb and 160 μg/mL goat nonspecific antibody. Next, one part of capture antibody−gold conjugates was mixed with nine parts of the mAb solution in a 384-well transparent polystyrene plate (final volume of 50 μL per well) to arrive at final concentrations of 0.01−10 μg/mL mAb and 144 μg/mL nonspecific goat antibody. To characterize the heated human antibodies, samples were centrifuged after heating and cooling at 15 000 rpm for 30 min, and the supernatant was analyzed using size-exclusion chromatography (TSK Gel G3000SWxl column, 0.78 × 30 cm, Tosoh Bioscience) without further modification or dilution.

AC-SINS Analysis in Serum. Nonspecific goat antibody (180 μg/mL) and human antibody (100 μg/mL) solutions were prepared in a solution of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 1.8 mM potassium phosphate; pH 7.4) with 550 ng/mL PS-80 and different amounts of mouse serum (1, 10 or 50% vol/vol). The human antibodies were then diluted and used for AC-SINS as described above. However, the spectra of the conjugates in serum were subtracted from a control serum spectrum without conjugates, and the resulting difference spectra were analyzed to obtain the plasmon wavelength for each sample.

■ ASSOCIATED CONTENT

Supporting Information

Analysis of the impact of incubation time and temperature, type of capture antibody, sedimentation of aggregated samples, and fraction of aggregates in mAb samples on the plasmon wavelength measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): P.M.T. has received consulting fees and/or honorariums for presentations of this and/or related research findings at MedImmune, Eli Lilly, Bristol-Myers Squibb, Janssen, Merck, Genentech, Amgen, Pfizer, Adimab, Abbott, DuPont, Bayer, Abbvie, Roche and Boehringer Ingelheim.

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