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An alternative assay to hydrophobic interaction chromatography for high-throughput characterization of monoclonal antibodies

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The effectiveness of therapeutic monoclonal antibodies (mAbs) is governed not only by their bioactivity, but also by their biophysical properties. Assays for rapidly evaluating the biophysical properties of mAbs are valuable for identifying those most likely to exhibit superior properties such as high solubility, low viscosity and slow serum clearance. Analytical hydrophobic interaction chromatography (HIC), which is performed at high salt concentrations to enhance hydrophobic interactions, is an attractive assay for identifying mAbs with low hydrophobicity. However, this assay is low throughput and thus not amenable to processing the large numbers of mAbs that are commonly generated during antibody discovery. Therefore, we investigated whether an alternative, higher throughput, assay could be developed that is based on evaluating antibody self-association at high salt concentrations using affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS). Our approach is to coat gold nanoparticles with polyclonal anti-human antibodies, use these conjugates to immobilize human mAbs, and evaluate mAb self-interactions by measuring the plasmon wavelengths of the antibody conjugates as a function of ammonium sulfate concentration. We find that hydrophobic mAbs, as identified by HIC, generally show significant self-association at low to moderate ammonium sulfate concentrations, while hydrophilic mAbs typically show self-association only at high ammonium sulfate concentrations. The correlation between AC-SINS and HIC measurements suggests that our assay, which can evaluate tens to hundreds of mAbs in a parallel manner and requires only small (microgram) amounts of antibody, will enable early identification of mAb candidates with low hydrophobicity and improved biophysical properties.

Introduction

The growing interest in using monoclonal antibodies (mAbs) as therapeutics is continuing to fuel efforts to discover mAbs specific for a wide array of targets.[1] Nevertheless, the development of a mAb candidate into a therapeutic drug is a long, expensive and often unsuccessful process. Many mAbs fail in development due to suboptimal drug-like properties, such as poor expressibility and manufacturability, low stability and solubility, high viscosity, and fast serum clearance.[2-6] To minimize downstream risks, several assays have been developed and applied for screening antibody biophysical properties during the early stages of lead selection.[7,8] Some of these assays are designed to detect non-specific interactions between mAbs and various types of biomolecules. For example, cross-interaction chromatography (CIC) probes non-specific interactions between mAbs and immobilized polyclonal antibodies.[9-12] Delayed antibody elution indicates a propensity of mAbs to interact non-specifically with polyclonal antibodies, which is correlated with poor mAb solubility.[10,11] A related ELISA method evaluates non-specific interactions between mAbs and immobilized baculovirus particles (BVPs), and such interactions are in turn correlated with fast clearance in vivo.[3] In addition, a mixture of soluble membrane proteins (SMPs) have been used as a non-specificity reagent, and its relative binding to antibodies is correlated with CIC and BVP results.[13] The SMP assay is particularly useful because it is amenable for use with fluorescence-activated cell sorting (FACS) and enables high-throughput selection of antibodies with low propensity to interact non-specifically.

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Hydrophobic interaction chromatography (HIC), which uses non-biological hydrophobic surfaces to evaluate non-specific interactions, is another common assay for evaluating monoclonal antibodies. The premise of this approach is that increased retention of antibodies on hydrophobic columns at moderate to high salt concentrations is correlated with increased antibody hydrophobicity.[22] The strength of this approach is its ability to provide a more rigorous test of antibody hydrophobicity than other methods that evaluate non-specific antibody interactions. The main weaknesses of this approach are the non-biological nature of the hydrophobic surfaces and the low assay throughput. The latter issue stems from the need for long and sequential chromatography experiments for each mAb variant, and this has limited the use of HIC during early antibody discovery.

We sought to develop an alternative assay to HIC that enables evaluation of mAb interactions at moderate to high salt concentrations while significantly increasing the throughput of such measurements. Our approach builds on a high-throughput method for measuring antibody self-association, namely affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS·).[14,15] This method involves coating gold nanoparticles with polyclonal anti-human capture antibodies and using these conjugates to immobilize mAbs. Antibodies with increased propensity to self-associate, which causes the conjugates to cluster together, are detected via a red-shift of the plasmon wavelength (wavelength of maximum absorbance). AC-SINS measurements of self-association conducted at dilute mAb concentrations (<0.1 mg/mL) have been shown to correlate with interaction and solubility measurements at much higher concentrations (>10 mg/mL).[14,15] We reasoned that this high-throughput approach could be adapted to evaluate antibody properties at high salt concentrations in a manner reflecting the physical basis for the HIC assay. Here we report the characterization of a panel of mAbs using AC-SINS at elevated salt concentrations, and demonstrate how the results from this high-throughput approach are well correlated with those from HIC.

Results

Thirty-two mAbs representing US Food and Drug Administration-approved drugs or candidates in early-to-late stage clinical trials were cloned into mammalian expression vectors using their published sequences, transiently expressed in HEK293 cells, and purified using Protein A for this study. To assess the intrinsic properties of the variable regions, these mAbs were expressed as IgG1 antibodies regardless of their actual isotypes as clinical entities. All mAbs were buffer-exchanged into 1 M ammonium sulfate and normalized to a concentration of 0.5–1 mg/mL prior to loading onto an analytical HIC column. Representative HIC elution traces are shown in **Figure 1A**. Without the buffer exchange step, most of these mAbs in phosphate-buffered saline (PBS) are not retained on the column. On the other hand, when these mAbs were buffer exchanged into high salt (1.8 M ammonium sulfate), some of them precipitated. At our final assay conditions, most of these mAbs eluted between 20–24 min (**Fig. 1B**), corresponding to ammonium sulfate concentrations of 700–500 mM at elution. However, a few mAbs showed late elution times and poor recoveries. Some mAbs even failed to elute from the column, despite the acetonitrile wash.

We also performed AC-SINS for these mAbs in PBS (**Fig. 2A**). Most mAbs show relatively low plasmon wavelengths (< 535 nm) that are similar to control (530 nm). Exceptions include our IgGs having variable region sequences identical to those of ganitumab, ipilimumab, tremelimumab and vesencumab, which all show larger plasmon wavelengths (537–552 nm). Three of the 4 associative mAbs also show significant retention on the HIC column. The exception is ganitumab, which is one of the least hydrophobic antibodies as judged by HIC. Nevertheless, the HIC and AC-SINS data are poorly correlated (**Fig. 2B**), suggesting that they measure different types of interactions.

We reasoned that the correlation between the HIC and AC-SINS measurements could be improved by performing the AC-SINS measurements at similar ammonium sulfate concentrations as used in the HIC experiments. Therefore, we evaluated the plasmon wavelengths of the antibody conjugates (with and without adsorbed mAb) as a function of the ammonium sulfate concentration (**Fig. 3**). The conjugates without adsorbed mAb show little response except at high (>0.8 M) ammonium sulfate concentrations. Interestingly, the highest salt concentrations (0.9-1 M) lead to large plasmon wavelengths (555-560 nm) that are typical of saturating values for AC-SINS.[14-16] We find that other well-behaved antibodies such as the IgG with variable regions sequences identical to adalimumab, polyclonal antibodies or Fc fragment show similar behavior and little response up to 0.8 M ammonium sulfate. In contrast, an antibody with delayed elution on HIC (eculizumab) shows plasmon shifts at lower (0.6 M) salt concentrations. These results suggest that AC-SINS measurements at high salt concentrations may be sampling similar types of interactions as HIC.

To further evaluate the relationship between AC-SINS and HIC measurements in concentrated salt solutions, we analyzed 30 additional mAbs. We find a range of behaviors, examples of which are shown in **Figure 4**. Some mAbs displayed little response and required high salt concentrations (>0.7 M ammonium sulfate) to induce plasmon shifts (**Fig. 4A**). Other mAbs showed plasmon shifts at intermediate (**Fig. 4B**) or low (**Fig. 4C**) ammonium sulfate concentrations. Still others showed complex, intermediate behaviors (**Fig. 4D**).

We investigated whether the plasmon wavelength values could be condensed to a single value for each mAb to facilitate comparison to the HIC data. It was not possible to obtain a midpoint for the plasmon wavelength shifts as a function of ammonium sulfate concentration because of the lack of clear transitions for some mAbs. Instead, we averaged the plasmon wavelengths for each mAb over different ranges of ammonium sulfate concentration. We find little correlation between the HIC retention times and averaged plasmon wavelengths from 300 to 1000 mM ammonium sulfate (**Fig. 5A**). However, there is a strong segregation between mAbs with early- and late-eluting mAbs when averaging the plasmon wavelengths obtained at 700 to 900 mM ammonium sulfate (**Fig. 5B**). Most of the late eluting mAbs show large

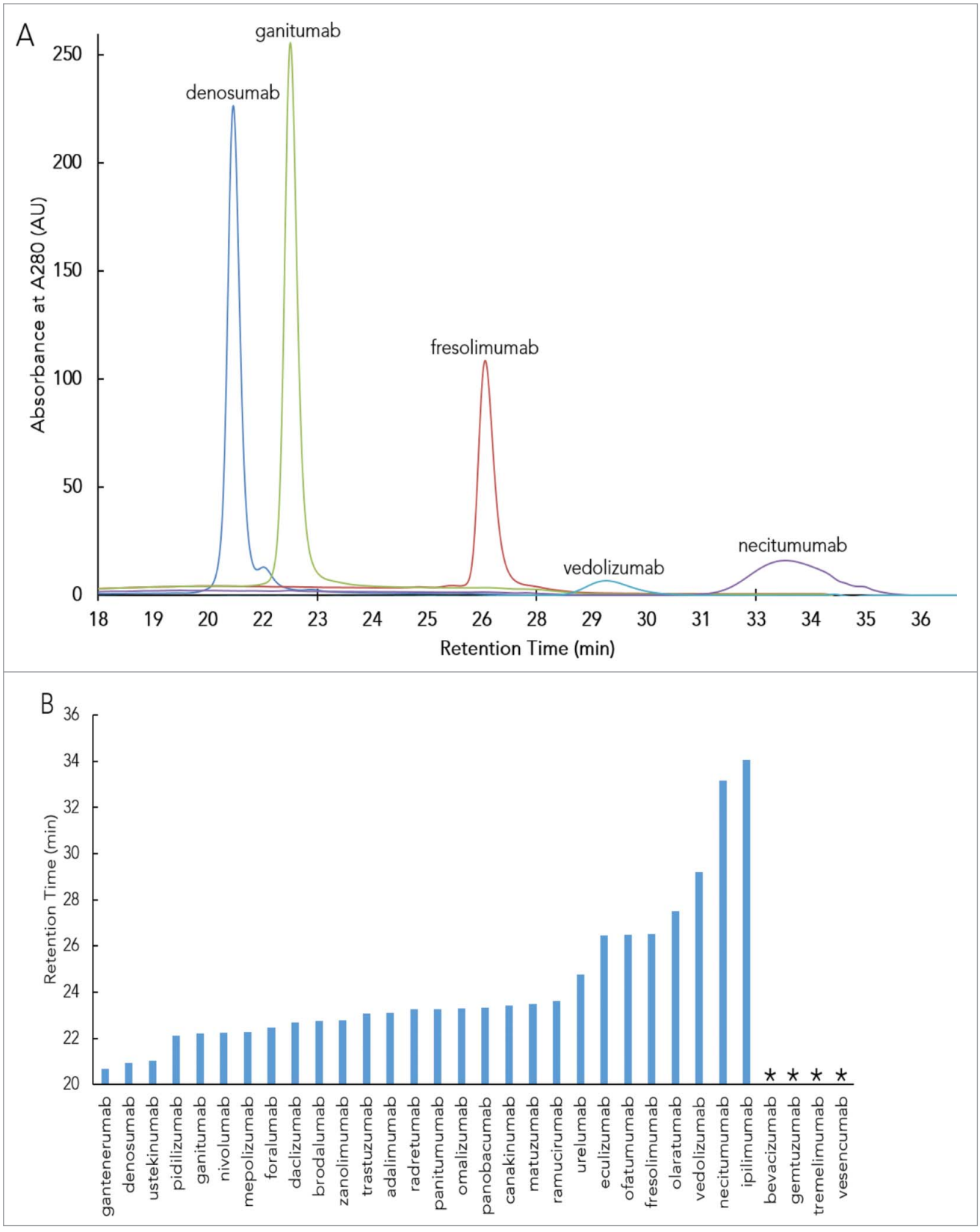

Figure 1. (**A**) Representative chromatograms for 5 mAbs with variable regions identical to clinical-stage molecules on a hydrophobic interaction chromatography (HIC) column. (**B**) HIC retention times for 32 IgGs with variable region sequences identical to those of the indicated clinical-stage mAbs. Four mAbs (*) did not elute.

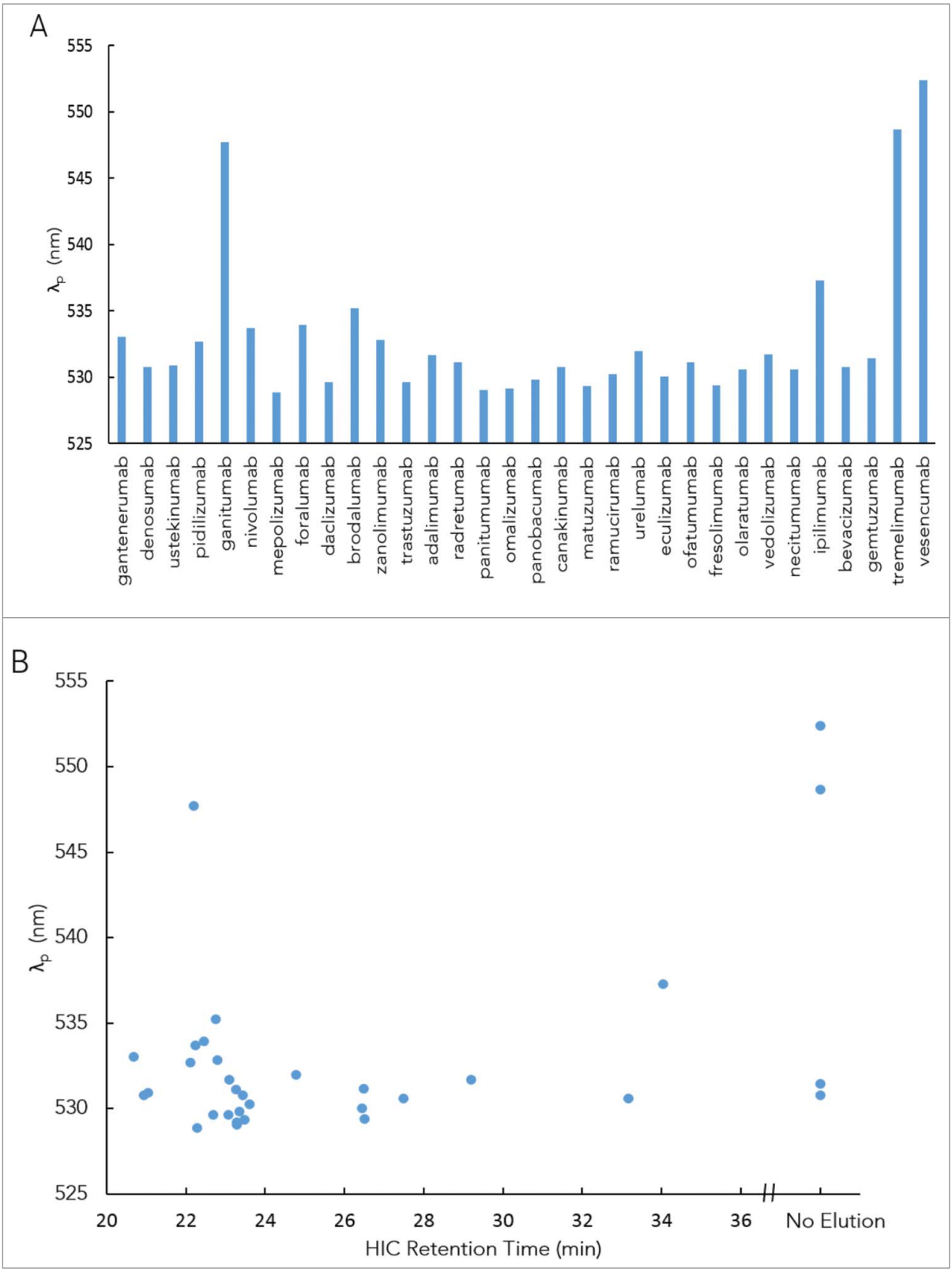

Figure 2. (**A**) Plasmon wavelengths for 32 IgGs with variable region sequences identical to the indicated clinical-stage human mAbs that were obtained using AC-SINS in PBS (pH 7.4). (**B**) Comparison between AC-SINS and HIC results.

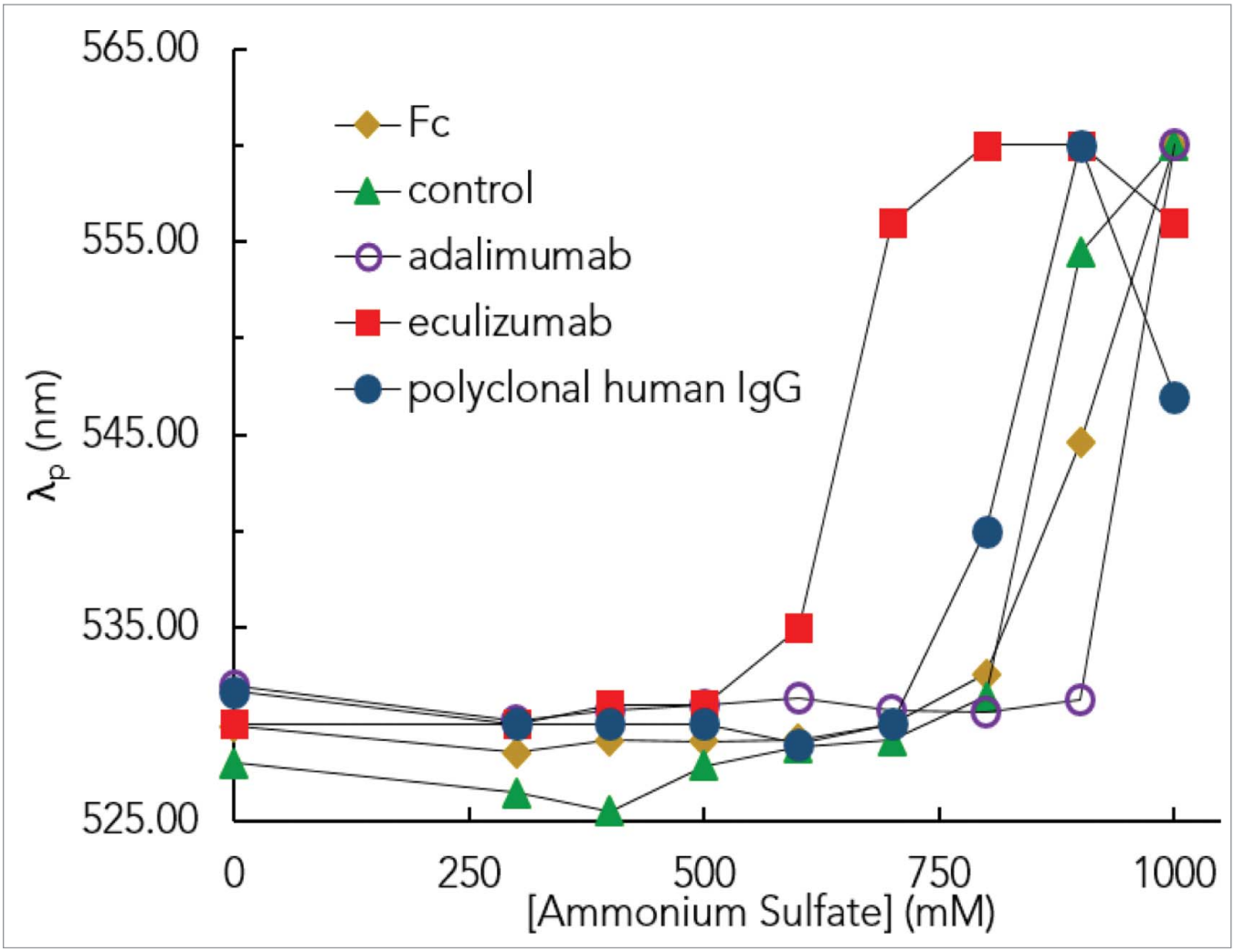

Figure 3. Evaluation of AC-SINS measurements as a function of ammonium sulfate concentration. The control is for gold particles coated only with polyclonal (anti-human Fc) capture antibody and the Fc fragment is from human IgG1 polyclonal antibody.

average plasmon wavelengths between ~–560 nm. On the other hand, mAbs with early elution time show average plasmon wavelengths between ~530–545 nm. Interestingly, a similar segregation effect was observed when comparing the HIC retention times and the plasmon wavelength shifts for only the 800 mM ammonium sulfate concentration (**Fig. 5C**). Rank-order Spearman correlation coefficients between the different sets of measurements were calculated and are presented in the **Table S2**. For example, the HIC retention times and the plasmon wavelength shift measured at 900 mM ammonium sulfate, correlate with a coefficient of 0.72, with a p-value of $1.35*10^{-5}$.

Discussion

The primary aim of this work is to demonstrate how a relatively simple adaptation of our previously described AC-SINS assay[14,15] may, by and large, recapitulate the main features of HIC. How HIC results relate more directly to relevant downstream behavior of antibodies is still a matter of debate and beyond the scope of this manuscript. To facilitate reproducibility of our data by others, we have chosen a set of thirty-two pairs of heavy and light chain variable domains from antibodies with varying degrees of validation in the clinic, with a number of them already approved for therapeutic use (see **Table S1**). For the purposes of this work, we have re-constructed each antibody using a consistent isotype and HEK expression system. This means that the properties of our mAbs relative to those in the clinic are not identical. Therefore, conclusions about how a given sample behaves in our assays cannot be readily extrapolated to what may have been observed in the development of the actual clinical antibodies. We use the USAN-designated nomenclature as short-hand, but it should be understood that our data were not generated with the legitimate clinical material as marketed and as being administered to human patients.

It is critical that mAbs have good biophysical properties to minimize downstream risks during development. Some problems related to these properties, such as solubility and viscosity, can

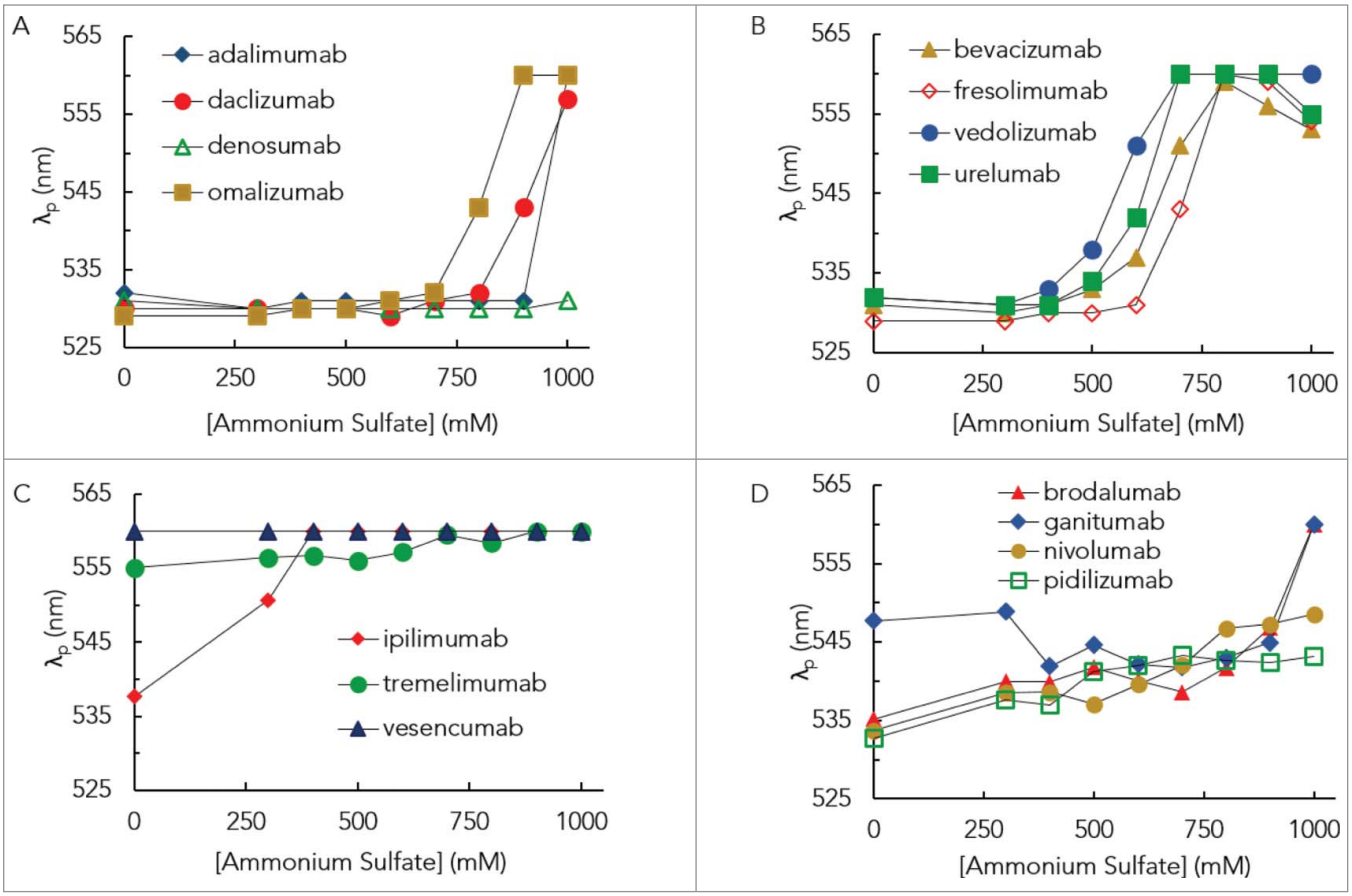

Figure 4. AC-SINS reveals unique sensitivities of human mAbs to ammonium sulfate. Examples of mAbs that display significant plasmon shifts at (**A**) high, (**B**) intermediate and (**C**) low ammonium sulfate concentrations, as well as (**D**) mAbs with more complex and difficult-to-classify behavior.

potentially be addressed via suitable formulations. However, some poor behaviors are related to the antibody sequence and conformation, and cannot be easily fixed. Screening assays that can be applied early in the discovery stage to identify candidates with optimal biophysical properties and stabilities are highly valuable to minimize downstream risks. Among these assays, HIC offers one type of assessment of mAbs for their robustness during manufacturing, long-term storage and delivery.[22] In theory, highly soluble and hydrophilic mAbs that have better tolerance against salt stress are expected to behave robustly during the manufacturing process. On the other hand, hydrophobic mAbs with high sensitivity to salt may display problems such as poor expression, aggregation or precipitation during purification.[18,22]

We constructed a set of 32 antibodies with variable region sequences identical to those of corresponding clinical-stage molecules. We demonstrated that the antibody retention on a HIC column is generally correlated with antibody self-association at high salt measured using AC-SINS. Our results are consistent with previous findings showing that measurements of protein self-association at high salt, as judged by the second osmotic virial coefficient, are generally correlated to HIC retention data.[17] These findings suggest that similar types of interactions mediate both antibody solubility and retention on HIC columns at high salt concentrations. It may be that reduced hydration of antibody surfaces, which occurs most favorably in hydrophobic regions, leads to attractive self-interactions and antibody-surface interactions. Nevertheless, we and others observed some exceptions to the concordance between HIC and self-interaction measurements, which may be due to antibody-ligand interactions in HIC, antibody unfolding on hydrophobic HIC surfaces or other related mechanisms.[17]

It is also notable that antibody solubility measurements in concentrated ammonium sulfate solutions have been reported for evaluating the developability of multiple types of antibody fragments, full-length antibodies and globular proteins.[18-21] Antibodies with high solubility in ammonium sulfate have been shown to be less prone to aggregate at low salt concentrations.[18] This and related findings for globular proteins[19,21] suggest that hydrophobic interactions that are enhanced at high salt concentrations may also contribute to antibody solution behavior at low salt concentrations, including those typically used in antibody formulation. However, it is important to emphasize that non-hydrophobic interactions (such as electrostatic interactions) significantly contribute to antibody solubility, especially at low salt concentrations typical of antibody formulations. Thus, HIC or self-interaction measurements at high salt should be used in concert with other

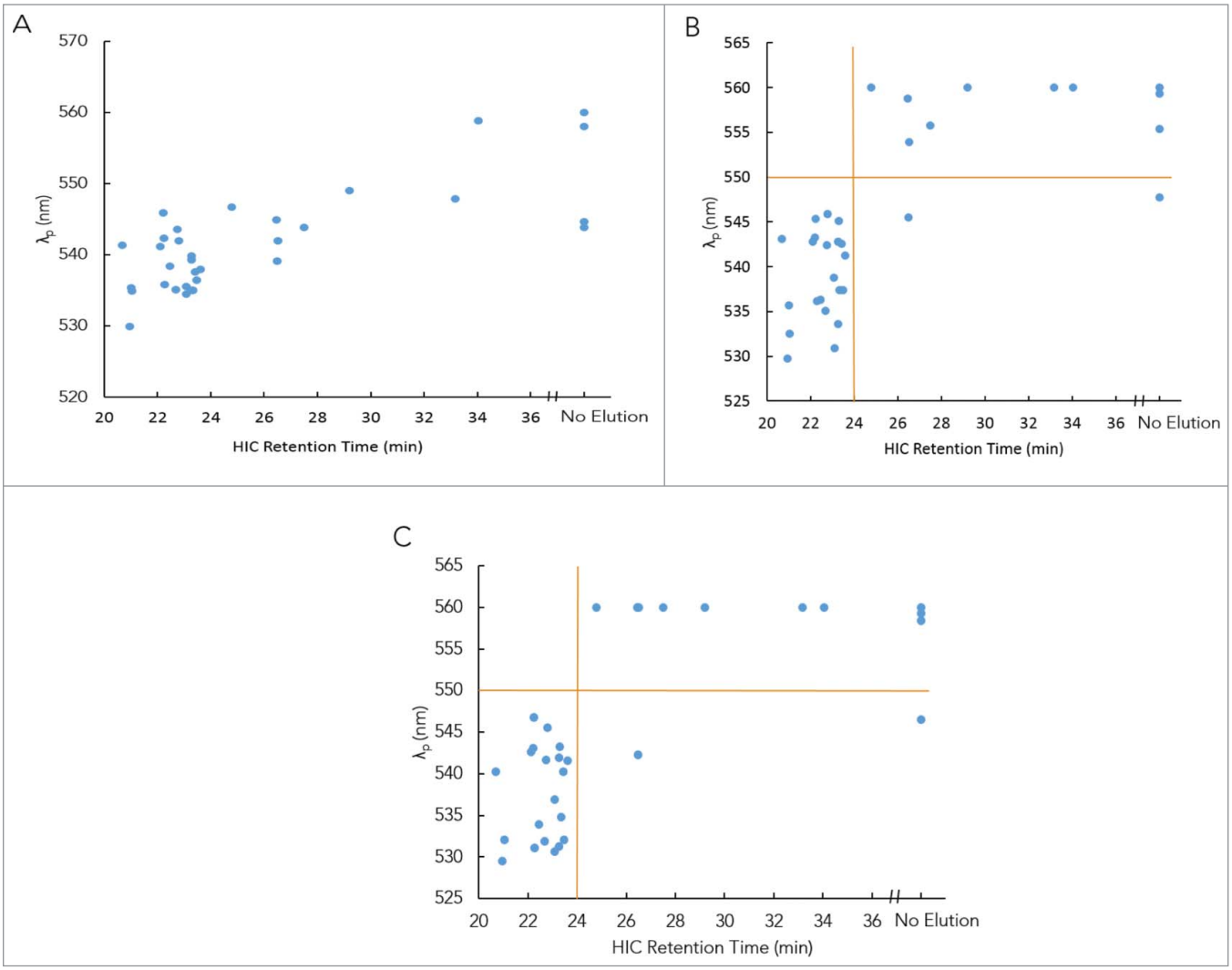

Figure 5. Comparison of AC-SINS and HIC results for 32 clinical stage mAbs. HIC retention times are plotted against (**A**) average plasmon wavelengths for ammonium sulfate concentrations of 300–1000 mM, (**B**) average plasmon wavelengths for ammonium sulfate concentrations of 700–900 mM ammonium sulfate, and (**C**) plasmon wavelengths at 800 mM ammonium sulfate. In **B** and **C**, the cutoff values are 550 nm for AC-SINS and 24 min for HIC.

assays, such as light scattering at reduced salt concentrations, to robustly compare different antibody candidates.

Our approach of using AC-SINS has some advantages and disadvantages relative to HIC and solubility assays reported previously for evaluating antibodies in concentrated ammonium sulfate solutions. The most important advantage of our assay relative to HIC is increased throughput. The ability to evaluate many different antibodies in a parallel manner using AC-SINS enables significant increases in throughput relative to the requirement that each antibody be evaluated in a sequential manner using HIC. Another subtle but important advantage of AC-SINS relative to HIC is that the samples for HIC must be buffer exchanged into high ammonium sulfate concentrations (~1 M) prior to injection to obtain suitable retention, which is not necessary for AC-SINS. A disadvantage of AC-SINS is the need to prepare antibody-gold conjugates, which requires more specialized know-how than HIC. It is also notable that sample size requirements are low in both assays.

The throughput of AC-SINS is theoretically higher than antibody solubility measurements in concentrated ammonium sulfate despite the fact that both assays are conducted in microtiter plates, although direct comparison is required to evaluate such throughput differences. We expect the throughout to be higher for AC-SINS because such measurements do not require a separation step prior to analysis, and measurements of plasmon wavelengths are simpler than measurements of protein concentration. Moreover, AC-SINS requires less protein given that the measurements are conducted at microgram per mL concentrations instead of mg per mL concentrations. However, solubility measurements have a number of advantages over AC-SINS measurements, including that they: (1) are simpler to interpret; (2) are not complicated by presence of non-mAb components such as gold particles and polyclonal antibodies; (3) yield a fundamental property (solubility) directly; and (4) can be conducted for a wide range of types of proteins without the requirement of suitable capture antibodies.

This study focused on the manufacturability/developability assessment of a set of 32 clinical stage mAbs. Twenty mAbs show early elution via our HIC assay, and they may appear to be robust and soluble mAbs. However, 12 mAbs show significantly delayed elution or failed to elute on a HIC column. These mAbs appear to possess increased hydrophobicity, at least upon exposure to moderate to high concentrations of ammonium sulfate, and lead to strong binding to the HIC column. The rate of identification of poorly-behaved clinical stage antibodies by HIC is surprisingly high, which suggests that our HIC (and thus our AC-SINS) assay is overly stringent for prediction of manufacturability. It should be emphasized, of course, that our mAb surrogates for the corresponding clinical antibodies aimed at replicating the variable region sequences only. As illustrated in **Table S1**, the constant regions (heavy chain isotype) can differ substantially and even for the case of IgG1, we used a fixed allotype (IGHG1*01; accession J00228) regardless of the actual allotype of each clinically-developed molecule. Nevertheless, in a few cases (see **Table S1**), evidence may be found suggestive of sub-optimal properties for some IgGs that are closely related to clinical-stage molecules, although these preliminary observations require more investigation.

Materials and Methods

Citrate-stabilized 20 nm gold nanoparticles (15705) were obtained from Ted Pella Inc. (Redding, CA). Poly(ethylene glycol) methyl ether thiol (2000 Da, 729140) was obtained from Sigma-Aldrich (St. Louis, MO). Goat anti-human IgG, Fcγ fragment specific (109-005-098), goat non-specific antibody (005-000-003), goat anti-human IgG F(ab')2 fragment specific (109-005-006) and goat anti-human IgG (H^+L) (109-005-088) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Zeba spin desalting columns (87766, 87768 and 87770) were obtained from Thermo Fisher Scientific (Rockford, IL). 0.22 μm PVDF Millex-GV Filters (SLGVX13NK) were obtained from EMD Millipore (Billerica, MA). Costar 384-Well Polystyrene Plates (12-565-506) were obtained from Fisher Scientific (Pittsburgh, PA). 96-well polypropylene shallow-well plates (5042-1385) were obtained from Agilent Technologies, Inc. (Santa Clara, CA). The clinical antibodies were made recombinantly in HEK293 using published v-region sequence in an IgG1 format. A Spectramax $M2^e$ model plate reader from Molecular Devices was used in this study. Ammonium sulfate was purchased from Sigma-Aldrich (>99%, Cat # 31119-5KG) and 1 M sodium phosphate solution was purchased from TekNova (P2070). A Dionex ProPac HIC-10 column (4.6 × 100 mm, cat # 063655) was also purchased from Thermo-Scientific.

HIC

IgG1 samples were buffer exchanged into 1 M ammonium sulfate and 0.1 M sodium phosphate at pH 6.5 using a Zeba 40 kDa 0.5 mL spin column (Thermo Pierce, cat # 87766). A salt gradient was established on a Dionex ProPac HIC-10 column from 1.8 M ammonium sulfate, 0.1 M sodium phosphate at pH 6.5 to the same condition without ammonium sulfate. The gradient ran for 17 min at a flow rate of 0.75 ml/min. An acetonitrile wash step was added at the end of the run to remove any remaining protein and the column was re-equilibrated over 7 column volumes before the next injection cycle. Peak retention times were monitored at A280 absorbance and concentrations of ammonium sulfate at elution were calculated based on gradient and flow rate.

AC-SINS in the presence of ammonium sulfate

Polyclonal goat anti-human IgG Fc antibodies (capture) and goat non-specific antibodies (non-capture) were buffer exchanged into 20 mM KAc (pH 4.3), followed by adjusted to a concentration of 0.4 mg/mL. A 4:1 volume ratio mix of capture:non-capture IgG solution was prepared to obtain immobilization of 80% capture antibody and 20% non-capture antibody. A 9:1 volume ratio was used to mix gold nanoparticle solution with coating solution. After room temperature incubation for 1 h, thiolated PEG (Sigma Aldrich, 729140, final concentration 0.1 μM) was used to block empty sites on the nanoparticles. These coated and blocked particles were stable in the coating solution at 4°C for up to 2 weeks. The particle solution was then passed through a 0.22 um PVDF membrane (Millex-GV, 13 mm, Millipore). The particles were retained on top of the membrane and the flow-through solution was clear. PBS at 1/10 of the starting volume was used to elute the particles into the collection tube. Ten μL of the concentrated particles were added per well in 96-well plates, and 10 μL of each IgG in PBS was added and allowed to incubate with the particles for 30 min. Then 90 μL of up to 1.22 M ammonium sulfate and 0.1 M sodium phosphate at pH 6.5 was added to the particles, creating ammonium sulfate from 0.3 to 1 M in 0.1 M increments. The particles were allowed to incubate for an additional 90 min. Then 100 μL of the resulting solution was transferred to a polystyrene UV transparent plate, followed by brief centrifugation to bring the solution menisci to the same level. Absorbance data is collected from 510 to 570 nm at increments of 2 nm. Raw absorbance data is exported into an excel file, followed by data processing using a macro. The macro first identifies the wavelength of maximum absorbance in the raw data, then stores the 20 data points around that wavelength in an array. Each point is averaged with the points directly before and after it to reduce error. Using the LINEST function in Microsoft Excel, a second-order polynomial is fit to the data. The coefficients are used to calculate the wavelength at which the slope is equal to zero and the macro then determines whether or not this point is a maximum or minimum. In the case of a maximum, the calculated wavelength is returned, unless it is greater than 560 nm.

AC-SINS

AC-SINS measurements in PBS were performed similarly as above without addition of ammonium sulfate, as described previously.[14]

Disclosure of Potential Conflicts of Interest

Estep P, Caffry I, Yu Y, Sun T, Cao Y, Lynaugh H, Jain T, Vásquez M and Xu Y own company stock in Adimab. Tessier

PM has received consulting fees and/or honorariums related to these and/or related research findings from Adimab, MedImmune, Eli Lilly, Bristol-Myers Squibb, Janssen Biotech, Merck, Genentech, Amgen, Pfizer, Abbott, DuPont, Bayer, Roche and Boehringer Ingelheim.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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