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Diffusion of Soluble Aggregates of THIOMABs and Bispecific Antibodies in Serum

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Abstract

Submicrometer aggregates are frequently present at low levels in antibody-based therapeutics. Although intuition suggests that the fraction of the aggregate or the size of the aggregate present might correlate with deleterious clinical properties or formulation difficulties, it has been challenging to demonstrate which aggregate states, if any, trigger specific biological effects. One source of uncertainty about the putative linkage between aggregation and safety or efficacy lies in the likelihood that noncovalent aggregation differs in ideal buffers versus in serum and biological tissues; self-association or association with other proteins may vary widely with environment. Therefore, methods for monitoring aggregation and aggregate behavior in biologically relevant matrices could provide a tool for better predicting aggregate-dependent clinical outcomes and provide a basis for antibody engineering prior to clinical studies. Here, we generate models for soluble aggregates of THIOMABs and a bispecific antibody (bsAb) of defined size and exploit fluorescence correlation spectroscopy to monitor their diffusion properties in serum and viscosity-matched buffers. The monomers, dimers, and trimers of both THIOMABs and a bsAb reveal a modest increase in diffusion time in serum greater than expected for an increase in viscosity alone. A mixture of larger aggregates containing mostly bsAb pentamers exhibits a marked increase in diffusion time in serum and much greater intrasample variability, consistent with significant aggregation or interactions with serum components. The results indicate that small aggregates of several IgG platforms are not likely to aggregate with serum components, but nanometer-scale aggregates larger than trimers can interact with the serum in an Ab-dependent manner.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

*Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.6b01097. SPR data for binding of capped and uncapped THIOMAB binding to Protein A, demonstrating the labeling process has no effect on binding function, and hydrophobic chromatography results with the bsABs, demonstrating complete conversion of parental mAbs to the bsAb (PDF)

Therapeutic proteins, including monoclonal antibodies (mAbs), Fc fusion proteins, bispecific antibodies (bsAbs), and other proteins that are based on the immunoglobulin (Ig) framework, dominate the pool of biologics currently in commercial development.^{1–23} Collectively, these drugs have the potential to treat many diseases that have been intractable to treatment with small molecule drugs.¹ Antibody-based therapeutics can be challenging and expensive to characterize and manufacture, and regulatory criteria concerning their production are still being revised. Aggregation status is a critical property often determined in standard dilute solutions, but difficult to measure in biological tissue or serum.

Part of the concern over protein aggregates is their potential for increasing immunogenicity, which can negatively impact the safety and efficacy of a drug.^{4–6} The mechanism by which aggregates activate the immune system likely involves the introduction of novel epitopes from partial unfolding and the presence of regularly repeated epitopes.⁷ Binding of C1q as the first step of the complement pathway is highly dependent on oligomeric state, with IgG hexamers causing higher levels of complement activation.^{8,9} Similarly, Ab multimers have been shown to have affinities for Fc receptors higher than those of the monomeric molecules, and this trend may contribute to the immune response.¹⁰ The generation of antidrug Abs may result in anaphylaxis, cytokine release syndrome, and other potentially fatal infusion reactions in addition to increased clearance and a resulting loss of efficacy.^{11,12}

Many methods for monitoring the aggregation of biologics in formulation buffers are available. Size-exclusion chromatography (SEC) and dynamic light scattering (DLS) are commonly used to determine size distributions. However, it is not possible to assess the aggregation state in biological media at therapeutic concentrations using these and other optical techniques. Because the aggregation status can change with the environment, the behavior of a protein in buffer is not necessarily predictive of its properties in serum.^{13,14} In addition, distinct aggregation states of antibody-based drugs could interact with serum or tissue proteins differently and thereby impact their functional and therapeutic properties. Methods that directly monitor the aggregation in serum could facilitate our ability to better predict aggregation behavior or to better understand clinical properties of biologics. While there has been success using surface plasmon resonance and Ab-coated nanoparticles to achieve these ends, the majority of strategies involve the use of surface-based binding to monitor protein interactions within complex mixtures.^{15,16}

Fluorescence correlation spectroscopy (FCS) exploits confocal microscopy to monitor the diffusion of highly dilute solutions of a fluorescently labeled molecule. The autocorrelation

function of the fluorescence signal provides the translational diffusion time (τ_D) of the molecules, which is linearly proportional to the hydrodynamic (or Stokes) radius and therefore can be used to report on the aggregation state.^{17,18} Crucially, FCS allows the measurement of diffusive properties in optically dense and nonideal samples such as tissue or serum that cannot be analyzed by standard optical or light scattering methods. Although FCS has been applied in biological media, its utility for monitoring the aggregation status of therapeutic proteins in the serum has not been documented. Here we utilize fluorescently labeled THIOMABs and a bispecific IgG with defined, irreversible aggregation states as models to study their tendency to further aggregate in serum, or to interact with serum proteins. The results indicate a modest, viscosity-independent, increase in τ_D values for dimers or trimers compared to monomers in serum versus buffer, and a significant increase in τ_D for aggregates of bsAbs with molecular weights corresponding to pentamers when they are placed in serum. This highlighted the important effects that even relatively small changes in the nanometer-scale aggregation profile of a biologic may have on its aggregation state in serum and therefore potentially on its efficacy and disposition.

MATERIALS AND METHODS

Materials

Ab residues were identified using Eu numbering. THIOMAB (trastuzumab, anti-Her2 S400C in human IgG1) was provided by Genentech, Inc. The parental Abs used to create bsAb, antirespiratory syncytial virus F405L and anti-gp120 K409R (both in human IgG1), were expressed in Expi293 cells (Thermo Fisher, A14635) according to the manufacturer's protocol by cotransfecting heavy and light chain plasmids at a 1:3 molar ratio. Purification was performed by equilibrating a protein A column in 1× phosphate-buffered saline (PBS), loading the filtered supernatant, washing with PBS, and eluting Abs with 100 mM sodium citrate (pH 3.5), followed by immediate dialysis into fresh PBS. OneShot FBS was purchased from Thermo Fisher (A3160401), and chemicals were purchased from Sigma.

Disulfide Cross-Linked THIOMAB

Three milligrams of THIOMAB capped with cysteine and/or glutathione was first uncapped as described previously by being incubated with a 20 molar equivalents of dithiothreitol for 16 h at room temperature.¹⁹ Following reduction, the Ab was purified using a 1 mL cation exchange column. The column was equilibrated in 20 mM succinate (pH 5.0), and the sample was diluted in the same buffer prior to loading to allow binding to the column. Following a 10 mL wash at pH 5.0, the Ab was eluted using Tris buffer [50 mM Tris and 150 mM NaCl (pH 7.5)].

After preparation of reduced thiols in THIOMAB, a 6× equivalent (40 μ M) of CuSO₄ was added to catalyze reoxidation of cysteines, including the creation of inter-Ab disulfides. This reaction mixture was incubated for 2 h at room temperature and resulted in a small amount of visible precipitate. Immediately following oxidation, a 6× equivalent of Alexa 488 maleimide (Thermo Fisher, A10254) was added to the sample from a 5 mg/mL stock in dimethyl sulfoxide (DMSO) and incubated for 3 h at room temperature. An additional buffer exchange was required after SEC to remove free dye using a 0.5 mL desalting column with a

7K molecular weight cutoff. Afterward, the ratio of dye to Ab was 0.2 for monomer and 0.1 for dimer as determined by absorbance at 280 and 493 nm.

Glutaraldehyde Cross-Linked THIOMAB

Three milligrams of THIOMAB was uncapped as described above, but using PBS [20 mM NaP_i and 150 mM NaCl (pH 7.2)] to elute from the cation exchange column rather than Tris buffer. A 6× equivalent of Alexa 488 maleimide was added immediately after uncapping from a 5 mg/mL stock in DMSO and incubated for 2 h at room temperature. The sample was concentrated to 400 μ L, and then 2 μ L of 12% (w/v) glutaraldehyde in water was added. After 10 min at room temperature, 30 μ L of 1 M Tris (pH 8.0) was added to quench the reaction. The insoluble aggregate was removed by spinning the sample for 10 min at 16000g. Following SEC, the labeling efficiency was ~2 fluorophores per Ab for each THIOMAB oligomer.

Glutaraldehyde Cross-Linked bsAb

bsAb was generated using cFAE as described previously.^{20,24} Briefly, 2.5 mg of anti-RSV F405L and 2.5 mg of anti-gp120 K409R in human IgG1 were co-incubated with 75 mM mercaptoethylamine for 5 h at 31°C, followed by dialysis into PBS. After formation of bsAb, 1 M NaHCO₃ was added to equal $1/10$ the reaction volume, and a 6× molar equivalent of Alexa 488 NHS ester (Thermo Fisher, A20000) was also added from a 5 mg/mL stock in DMSO. Labeling was performed for 2 h at room temperature. The sample was then glutaraldehyde cross-linked as with THIO-MAB described above. Ratios of dye to Ab ranged from 0.5 to 2, with a lower level of dye incorporation for the larger oligomers.

Size-Exclusion Chromatography

Purification of cross-linked oligomers was performed with a Superdex 200 10/300 GL (GE, 17517501) column with PBS as the running buffer at 0.5 mL/min and collection of 0.3 mL fractions. To estimate the size of larger oligomers, a standard curve of molecular weight versus retention time was generated using a gel filtration standard (Bio-Rad, 151–1901). Although vitamin B₁₂ is included in the mix of standards, it was excluded from the calibration curve as it deviated from the trend of the other standards (likely because of its small mass of 1350 Da and extended shape relative to proteins).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Purified oligomers were run on a nonreducing SDS–PAGE gel to visualize their composition. Gels containing 4–15% (w/v) acrylamide were used with a Tris-glycine running buffer containing 0.1% (w/v) SDS and a Laemmli sample buffer lacking reductant. Lanes contained either the starting monomer sample, the cross-linked sample, or 5–25 μ L of an SEC-purified fraction. For determination of aggregation reversibility, the loading buffer contained 50 mM DTT for reduced samples. The percentage of each species in a sample was calculated by dividing the density of its band by the total density of bands in its lane.

Viscometry

The dynamic viscosities of Tris buffer, Tris buffer with 9.90% (v/v) glycerol, and FBS were determined at 20 °C using a Lovis 2000 M/ME rolling ball viscometer at 70° relative to horizontal. Values in the text are the average and standard deviation of 10 replicates.

Fluorescence Correlation Spectroscopy

Experiments were performed on an Axio Observer D1 microscope (Zeiss) equipped with an LDH-D-C-485 ps pulsed diode 485 nm laser, a Tau-SPAD single-photon counting module, and HydraHarp 400 for autocorrelation (PicoQuant). Time traces and autocorrelations were collected using SymPhoTime 64 software (PicoQuant).

Free the dye and tagged Ab, samples were analyzed at 1.5 $\mu\text{g/mL}$ regardless of oligomer size or 10 nM fluorophore in the case of the free dye. In some cases, Ab samples had to be concentrated after SEC so that they could be diluted 1:100 in the appropriate medium. All buffers were filtered through a 0.22 μm filter prior to sample dilution. Sample aliquots of 50 μL were placed on 22 $\times$ 22-1 cover glass. With a laser power of 140 μW , ten 60 s traces (or five 120 s traces) were obtained for each sample and contained 200 sampling points and a maximal lag time of 10 s. Traces were fit individually using Igor Pro over the range of 0.01–1000 ms, initially using the following one-component three-dimensional diffusion model:^{17,18}

$$G_{1\text{comp}}(\tau) = \frac{1}{N} \left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \left(\frac{1}{1 + \frac{\tau}{s^2 \tau_D}} \right)^{0.5}$$

where $G(\tau)$ is the autocorrelation at a given lag time τ , N is the average number of particles in the focal volume, τ_D is the translational diffusion time, and s is the ratio of long to short axes of the focal volume. The value of s was fixed at 10 for all samples based on its value for the free dye in Tris buffer. For larger oligomer samples in particular, autocorrelations did not fit well to a one-component model and were instead fit to include two components. When both τ_D values were allowed to float, one τ_D was similar to that of the free dye. Therefore, final fits for all samples include two components with one τ_D fixed at the τ_D obtained for the chemically passivated free dye in the corresponding medium. Additional parameters ρ_{dye} and ρ_{Ab} represent the fraction of each species, and $\rho_{\text{dye}} + \rho_{\text{Ab}} = 1$.

$$G_{2\text{comp}}(\tau) = \frac{1}{N} \left[\rho_{\text{dye}} \left(\frac{1}{1 + \frac{\tau}{\tau_{D,\text{dye}}}} \right) \left(\frac{1}{1 + \frac{\tau}{s^2 \tau_{D,\text{dye}}}} \right)^{0.5} + \rho_{\text{Ab}} \left(\frac{1}{1 + \frac{\tau}{\tau_{D,\text{Ab}}}} \right) \left(\frac{1}{1 + \frac{\tau}{s^2 \tau_{D,\text{Ab}}}} \right)^{0.5} \right]$$

The Ab τ_D , which was allowed to float, is the τ_D plotted in Figure 6. For the majority of samples, the τ_D of the Ab contributed more than 85% of the total signal with the remainder

from the free dye component. The dye component did not exceed 50% of the overall signal for any sample.

Statistical Analysis

Because diffusion times were not normally distributed for all data sets, the Mann–Whitney test was used for comparison of τ_D values in glycerol buffer and FBS. Significant differences ($P < 0.05$) are listed in Table 1.

RESULTS

Generation of Oligomeric Standards

To determine whether Ab oligomers have different propensities for self-association in serum or association with serum components, we prepared three distinct types of Ab oligomers using covalent cross-linking strategies. First, THIOMABs containing engineered cysteine residues at a solvent-exposed region of the C_H3 region (S400C) were labeled and site-specifically cross-linked.²¹ The engineered thiol groups (initially capped with cysteine or glutathione) were reduced prior to addition of a small amount of CuSO₄ to yield dimers with inter-Ab disulfide bonds (Figure 1A). As a result of disulfide bond formation at the site of the engineered Cys, this method creates Ab dimers that are uniform in size as well as shape. After oxidation to a dimer, the sample was labeled at the remaining reduced cysteines using Alexa 488 maleimide. Importantly, there are two free thiols per Ab molecule regardless of oligomeric state, because each disulfide bond involves only one cysteine of each Ab. Therefore, labeling was performed after oxidation to allow for maximal dimerization. Monomeric THIOMAB that was reduced and labeled retained function as assessed by binding to protein A, indicating proper disulfide formation in the hinge region (Figure S1).

Second, as an alternative to well-defined disulfide linkages, glutaraldehyde was used to randomly tether nucleophilic residues of different Ab molecules via two reactive aldehydes separated by five carbon atoms (Figure 1B). The abundance of lysine residues in IgG, coupled with several possible mechanisms of glutaraldehyde cross-linking, leads to a heterogeneous mixture of Ab oligomers.^{22,23} Therefore, even dimers of the same mass may have significant differences in shape or exposed surfaces because of distinct sites of cross-linking. Prior to amine-targeted cross-linking, the solvent-exposed cysteine residues of the THIOMAB were fluorescently labeled using a maleimide derivative of Alexa 488.

As a third model system, a bsAb was created using controlled Fab-arm exchange (cFAE), which involves the co-incubation under reducing conditions of parental Abs harboring complementary mutations that drive bsAb formation.²⁴ Full formation of bsAb from parental Abs was verified by hydrophobic interaction chromatography, where a single peak corresponding to bsAb was observed between the peaks for parental Abs (Figure S2). Because the Abs used for creation of bsAb did not contain engineered cysteine residues, they were instead labeled at primary amine groups using Alexa 488 *N*-hydroxysuccinimide (NHS) ester. After labeling, glutaraldehyde was added to cross-link molecules of bsAb at unreacted amines (Figure 1C). As with glutaraldehyde cross-linked THIOMAB, this sample

is expected to have significant heterogeneity in the site of cross-linking as well as oligomer shape.

The type of linkage for cross-linked samples was verified by nonreducing and reducing SDS-PAGE (Figure 2). Any covalent oligomer that can be reduced to heavy and light chains with DTT was likely oligomerized via inter-Ab disulfide bonds. Thus, the copper-oxidized sample was expected to be fully dissociated in the presence of 50 mM dithiothreitol (DTT) because of reduction of intra- and inter-Ab disulfide bonds. Conversely, the glutaraldehyde cross-linked samples contain nonreducible linkages between Ab heavy and light chains. Indeed, addition of DTT converted CuSO₄-oxidized THIOMAB to free heavy chain (50 kDa) and light chain (25 kDa), while the oligomers formed with glutaraldehyde still contained larger species corresponding to irreversibly linked Ab domains.

After cross-linking and labeling, different oligomeric states of Ab were purified using size-exclusion chromatography (SEC). The purified fractions were subjected to nonreducing SDS-PAGE to visualize the purity of oligomeric standards (Figure 3). For each preparation of cross-linked Ab, a distribution of oligomeric states was obtained with small oligomers being the most abundant. While the disulfide-linked sample contained mostly monomeric and dimeric Ab, the glutaraldehyde cross-linked samples also contained larger oligomeric species. Thus, monomer and dimer molecules were isolated from the CuSO₄-treated mixture, while monomer, dimer, trimer, and a mixture of larger oligomers were purified from glutaraldehyde-treated Abs. Densitometry of electrophoretic gels revealed the proportion of each oligomer in the purified fractions (Table 2). Monomer and dimer fractions were >75% pure, while larger oligomers were significantly more heterogeneous. It is important to note that monomers purified from the cross-linking reactions were used for FCS experiments, rather than using the unreacted monomer. In the case of the chemically cross-linked samples, both the monomer and the oligomers may be coupled to molecules of glutaraldehyde that did not react with other molecules of Ab but that may have reacted with the Tris used to quench the reaction. In addition to separating the different oligomers, SEC was also useful for removing unreacted molecules of the free dye and cross-linking reagents.

Gel filtration was used to estimate the size of species in the larger oligomer fractions (Figure 4). On the basis of their known masses and measured retention times, fractions of monomer, dimer, and trimer from glutaraldehyde cross-linked THIOMAB and monomer and dimer from cross-linked bsAb fell close to a calibration curve generated using gel filtration standards. The retention time of larger oligomer fractions was then used to estimate the size of these species. For glutaraldehyde cross-linked THIOMAB, the larger oligomers were roughly consistent with a hexameric species, having a calculated mass of 840 kDa and an estimated aggregation state of 5.60. Large oligomers of bsAb were found to correspond roughly to pentamers based on a calculated mass of 728 kDa, which is 4.85 times that of the monomer.

Benchmarks of Viscosity

The diffusion of a labeled particle is dependent not only on the size of the particle but also on the in situ viscosity.^{25,26} Therefore, transferring Ab from buffer to serum results in an increased τ_D simply because of the higher viscosity of the serum. To control for the

viscosity of the medium, a buffer was prepared to have the same viscosity as fetal bovine serum (FBS). Via comparison of the τ_D of an Ab in FBS with that in the viscosity-matched buffer, it is possible to determine whether any changes in hydrodynamic properties have occurred, in particular, whether Abs have formed interactions with serum components or other molecules of Ab.

The dynamic viscosity, which is independent of density, was determined for Tris buffer and FBS at 20 °C. As expected, the Tris buffer had a viscosity significantly lower than that of FBS (1.0294 ± 0.0006 and 1.3657 ± 0.0010 cP, respectively). To account for the effect of viscosity on diffusion properties, a solution containing 9.90% (v/v) glycerol in Tris buffer was prepared that matched the viscosity of FBS but lacked protein. The glycerol-containing buffer had a viscosity of 1.3658 ± 0.0014 cP, which is well within experimental error of the measured viscosity of serum.

Fluorescence Correlation Spectroscopy

FCS is a sensitive single-molecule, small-ensemble technique that reports on the hydrodynamic properties of fluorescent particles based on how rapidly they diffuse through the ~1 fL observation volume of a confocal microscope. An increase in a particle's diffusion time (τ_D) may result from an increase in complex size (as occurs during binding or aggregation) or a decrease in compactness (as occurs during unfolding). Independent of the diffusion time, FCS also reports on the brightness per particle, an orthogonal measure of aggregation state. Thus, FCS is well-suited for determining whether Ab oligomers have altered hydrodynamic properties in serum compared to viscosity-matched buffer. Any increase in τ_D above that seen in the matched buffer would suggest that labeled Abs are associating with proteins present in the serum sample or undergoing self-association that is facilitated by the crowded environment of the serum.

The τ_D of labeled Ab oligomers was determined after dilution into simple Tris buffer, high-viscosity glycerol buffer, and FBS. Oligomers were diluted for FCS analysis so that the final solution contained >99% of the appropriate medium. FCS autocorrelation curves of glutaraldehyde cross-linked THIO-MAB (Figure 5) demonstrate that Abs diffuse more slowly in high-viscosity glycerol buffer than in simple Tris buffer based on a shift of the curve to longer lag times. Interestingly, the autocorrelation curves of monomeric, dimeric, and trimeric THIO-MAB (Figure 5B–D, respectively) appeared to be very similar between glycerol buffer and FBS, suggesting that small oligomers do not associate significantly with the serum. However, larger THIO-MAB oligomers (Figure 5F) showed an increase in τ_D in FBS compared to those in buffers based on the shift in autocorrelation to longer lag times. The clearest example of serum associations was seen with the reactive Alexa 488 maleimide (Figure 5A), which exhibited a modest increase in τ_D from Tris buffer to glycerol buffer and a large increase in τ_D upon dilution into FBS. The significant right shift of the curve in FBS indicates that molecules of the free dye interact covalently with serum proteins through reaction to cysteine residues. When the reactive maleimide was first chemically passivated with 2-mercaptoethylamine, the autocorrelation curve in FBS was similar to that in glycerol buffer.

To calculate τ_D values from autocorrelation curves, a three-dimensional diffusion equation was fit to the data. Fits were significantly improved by using a two-component model, suggesting that some samples contained residual molecules of the free dye (Figure 5E, F). Thus, one τ_D was fixed at the value of the chemically passivated free dye in the appropriate medium, and the other τ_D was a fitted parameter. Diffusion times for Ab oligomers in Tris buffer, glycerol buffer, and FBS are plotted in Figure 6 with a data point shown for each individual replicate. All dye and Ab samples experienced slightly slower diffusion in glycerol buffer than in Tris buffer because of the viscosity effect. As previously noted, both maleimide and NHS esters of free Alexa 488 had significantly longer τ_D values in FBS than in either buffer as a result of covalent reaction with serum proteins. Dyes chemically passivated with 2-mercaptoethylamine or Tris did not show this increase in τ_D , demonstrating that significant noncovalent interactions did not occur.

For disulfide-linked THIOMAB, there was a small increase in τ_D from monomer to dimer in each of the media examined. These THIOMAB monomers and dimers showed similar τ_D values in glycerol buffer and FBS, indicating that long-lived serum interactions did not occur. Diffusion times of glutaraldehyde cross-linked THIOMAB oligomers were similar to those of copper-oxidized THIOMAB oligomers and did not vary greatly with environment. Although larger oligomers appeared to have longer τ_D values, the differences were within experimental error.

A similar pattern was observed with glutaraldehyde cross-linked bsAb for which the τ_D again increased with the size of the oligomer and with the viscosity of the medium. There was a large variability in the τ_D when large oligomers were placed in FBS, which likely results from the tendency of large oligomers to interact with serum components. The crude mixture of cross-linked bsAb oligomers was analyzed and was similar to the sample of the monomer. Because the mixture contained mostly monomer and small amounts of larger oligomers, this result is not unexpected.

The FCS results are summarized quantitatively in Table 1. In accordance with the autocorrelation data, the τ_D values were longer in FBS and glycerol buffer than they were in Tris buffer. To determine whether different oligomers interacted with the serum, the τ_D values of each oligomer in glycerol buffer and FBS were compared. Because the viscosities of these two media were the same, any differences in τ_D indicate serum-induced interactions. Interestingly, glutaraldehyde cross-linked THIO-MAB dimer and hexamer and glutaraldehyde cross-linked bsAb dimer and trimer appeared significantly larger ($P < 0.05$) in FBS than in glycerol buffer. This result suggests that glutaraldehyde-generated oligomers in general are more likely to interact with serum than the more homogeneous disulfide-linked oligomers. Furthermore, the pentamer fraction of glutaraldehyde cross-linked bsAb was quite different ($P < 0.001$) in these two media. Such a difference in τ_D indicates that these large oligomers behave distinctly in the serum environment than in simple buffers. The implication is that oligomers larger than trimers may experience serum interactions, but that aggregation is dependent upon the Ab framework.

DISCUSSION

Small amounts of subvisible or submicrometer aggregates are present in many preparations of therapeutic antibodies and related platforms.²⁷ However, the behavior of small oligomers in serum or tissue is not well characterized, and their interactions with serum components, or with each other, could impact their clearance, their immunogenicity, and their efficacy. To assess the tendency of small aggregates to further oligomerize in serum, or to interact with serum components, we prepared fluorescently labeled standards of defined oligomer composition and applied FCS to monitor changes in apparent size in serum versus viscosity-matched buffers

The use of a two-component model was required because a single component could not adequately describe the data in all cases. In particular for the larger oligomers, there was a significant contribution to the autocorrelation from a free dye component. Although all samples were purified by SEC prior to analysis, it is likely that the hydrophobic dye molecules were selectively trapped in larger oligomer fractions. After dilution of Abs for FCS analysis, the noncovalently bound dye could dissociate from the protein component. A measurable contribution from free dye is not uncommon in FCS experiments and is routinely dealt with using two-component fitting as employed here.^{28,29} Although some samples likely contained the free dye, monomeric Ab, and large aggregate, it was not possible to use a three-component model without overfitting.

On the basis of the measured τ_D , the following equations can be used to calculate the hydrodynamic radius (R_H) of a particle:

$$R_H = \frac{k_B T}{6\pi\eta D} \quad D = \frac{r^2}{4\tau_D}$$

where k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, D is the translational diffusion coefficient, and r is the short axis of the cross section of the focal volume. Using the measured τ_D of 0.24 ms for Alexa 488 NHS ester along with its diffusion coefficient from the literature allows for calculation of the r parameter for this experimental setup.³⁰ Then, the average τ_D of 2.52 ms for all Ab monomer samples yields an R_H of 54 Å, which is in good agreement with other reported values of 55–56 Å obtained by FCS.^{31,32} The increase in τ_D from Ab monomers to dimers was subtle but within the precision of the method, averaging just a 1.14-fold increase in apparent size. This similarity in diffusion properties for distinct oligomeric states is expected, because a given change in mass will cause a much smaller change in diffusion. For globular proteins, the τ_D is proportional to the cube root of the particle's mass, meaning that a doubling of mass from monomer to dimer would cause an only 1.26-fold increase in τ_D .¹⁷ Because antibodies are not perfectly spherical, it is reasonable to expect an even smaller change in diffusion if dimerization results in a more compact shape. Studies of unstressed Abs by analytical ultracentrifugation have yielded R_H values of 54 and 62 Å for the monomer and compact dimer, respectively, which match the values of 54 and 61 Å, respectively, obtained here by FCS.³³ Thus, the modest differences in diffusion properties between oligomeric fractions are fully consistent with changes in their apparent sizes.

Abs experienced slower diffusion in serum or glycerol-containing buffer than in Tris buffer, consistent with the diffusion rate change in a more viscous environment. In fact, τ_D values increased overall 1.31-fold from Tris buffer to glycerol buffer, consistent with the 1.33-fold difference in viscosity. Across the oligomers tested, diffusion was slightly slower in FBS than in glycerol buffer. This trend may represent weak or transient interactions between labeled Abs and proteins in the serum. Significant differences ($P < 0.05$) between these two media were only seen, however, for certain samples of glutaraldehyde cross-linked Ab. Because glutaraldehyde modifies the Ab surface in addition to cross-linking, it was not entirely unexpected that Abs incubated with the agent would interact more with the serum. For instance, single molecules or polymers of glutaraldehyde could react with nucleophilic Ab side chains to introduce strands of glutaraldehyde or Tris-capped glutaraldehyde, in addition to the inter- and intra-Ab cross-links. These surface modifications add more area to the Abs that may interact with molecules in the serum. Consistent with this hypothesis, the τ_D of the THIOMAB monomer that was reacted with CuSO_4 (an unmodified Ab monomer) was lower than that of the THIOMAB monomer or bsAb monomer that was reacted with glutaraldehyde. Thus, while all Ab oligomers showed serum-induced differences in diffusion, these differences were significant only when the Ab contained surface modifications from the glutaraldehyde cross-linking step. Alternatively, the higher rate of incorporation of dye for the glutaraldehyde cross-linked samples could have increased the level of serum interactions by the additive effects of several weak dye-serum interactions. In either case, the result has implications for antibody-drug conjugates prepared via lysine coupling, which frequently contain more than two drug-linker moieties that presumably provide a source of nonspecific interactions with serum proteins analogous to those proposed here for glutaraldehyde.³⁴

However, the difference between Ab diffusion in glycerol and FBS was far more significant ($P < 0.001$) for the large oligomer fraction of bsAb despite a large increase in variability for this sample. This substantial increase in τ_D suggests interactions with serum beyond those caused by surface decoration with glutaraldehyde adducts. Larger Ab oligomers may form stronger or longer-term interactions with serum components simply because of their increased surface area. The level of partial unfolding may be increased in certain large oligomers to accommodate inter-Ab linkages, and this could introduce the opportunity for hydrophobic oligomer-serum interactions. Likewise, bridging flocculation from the domains present in the larger oligomers could result in serum component interactions.³⁵ Besides interacting directly with components of FBS, serum might facilitate the self-association of large Ab oligomers via the excluded volume effect.³⁶ The possibility that multiple weakly interacting dye molecules could cause serum association through an avidity effect was considered and would be possible for the THIOMAB hexamers that contained ~12 dye molecules per molecule of hexamer. However, for bsAb samples, the pentamer fraction contained fewer dye molecules (~3) than the dimer fraction (~4), while the pentamer diffused more slowly in serum than the dimer did. Thus, the serum interactions observed for this sample were protein- rather than dye-mediated.

It is notable that large oligomers of the bsAb showed slower diffusion in FBS than in buffer, while this shift did not occur for similarly sized oligomers of THIOMABs generated by the same cross-linking method. A likely explanation is that serum interactions are dependent

upon the variable region of the Ab. It is known that differences in Fab sequence can cause substantial changes in melting temperature that are related to stability.³⁷ Thus, if either Fab from the bsAb is inherently more prone to unfolding or aggregation than the Fab from the TH10MAB, the bsAb may have slower diffusion. This effect would be accentuated at large oligomer states in which the molecules become multivalent for each Fab. An alternative explanation is that the framework plays a role, and that bsAbs in general experience more serum interactions than mAbs do. However, because bsAbs generated by cFAE contain only two point mutations that minimally affect biophysical properties and stability, the variable region is perhaps the more feasible cause for the difference in diffusion.³⁸

These studies highlight the utility of FCS as a means of assessing protein aggregation and other association events in biological media such as the serum. Although FCS is useful for monitoring a specific protein within complex mixtures, there is concern that the hydrophobic dye molecule could accelerate aggregation or otherwise alter Ab properties. In fact, the Ab–dye conjugates used here are in many ways similar to antibody–drug conjugates, which are known to have altered properties relative to those of naked Abs.³⁴ To minimize dye-induced changes, the average incorporation of dye was kept below two molecules for each molecule of Ab. Previous studies have shown no significant differences between naked and Alexa 488-labeled Abs with a dye:Ab ratio of 2 by SEC, nanoparticle tracking analysis, and light obscuration.¹⁵ This is consistent with the FCS result showing that free dye interacts with serum only when it contains a reactive moiety for covalent modification. If dye molecules did significantly alter the properties of the Abs, then higher dye:Ab ratios would be expected to yield larger τ_D values by FCS. However, dye incorporation was slightly lower for higher-order oligomers (possibly because of the competition of cross-linking and labeling chemistries), and it was these oligomers that showed the slowest diffusion.

Taken together, these experiments demonstrate that a range of soluble Ab oligomers generated with distinct cross-linking strategies experience slower diffusion in the serum than would be expected on the basis of viscosity. This finding suggests that Abs in general interact transiently with components of the serum. On the basis of increases in average τ_D and variability, nanometer-scale soluble oligomers estimated as bsAb pentamer aggregate in serum to a greater extent than bsAb monomers, dimers, and trimers. Although this aggregation phenomenon was shown to be Ab-dependent, it implies that oligomerization results in significant hydrodynamic changes only after a certain size threshold is exceeded. Thus, the time- and cost-intensive practice of removing all traces of dimer may be unnecessary in terms of preventing adverse reactions. On the other hand, there are important pharmacokinetic and immunological responses that depend on the size and conformation of administered proteins that have not been investigated here. For example, submicroneter Ab aggregates (0.1–1 μm) may have an altered distribution compared to that of monomers and micrometer oligomers, and small native-like oligomers (10–40 nm) have been shown to be more immunogenic than monomer or micrometer-sized aggregates.^{39,40} As the large bsAb oligomers generated here exhibited altered diffusion in FBS (forming 6–50 nm aggregates), these findings may suggest a correlation between serum interactions and immunogenicity. While techniques like FCS are useful for detecting serum-induced changes in protein behavior, more work is needed to elucidate the precise causes and mechanisms of aggregate-induced immunogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ab	antibody
bsAb	bispecific antibody
cFAE	controlled Fab-arm exchange
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
Fab	antigen-binding fragment
Fc	crystallizable fragment
FBS	fetal bovine serum
FCS	fluorescence correlation spectroscopy
IgG	immunoglobulin G
mAb	monoclonal antibody
NHS	<i>N</i> -hydroxysuccinimide
PBS	phosphate-buffered saline
R_H	hydrodynamic radius
SEC	size-exclusion chromatography
τ_D	translational diffusion time

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Figure 1.

Preparation of covalent Ab oligomers. (A) THIOMAB was oxidized with CuSO_4 to induce inter-Ab disulfide bonds and then labeled at its remaining reduced cysteines using Alexa 488 maleimide. (B) THIOMAB was labeled with Alexa 488 maleimide and subsequently cross-linked with glutaraldehyde to create larger oligomers. (C) A sample of bsAb was labeled with Alexa 488 NHS ester and then cross-linked with glutaraldehyde. Green stars represent molecules of Alexa 488, and dashed lines denote cross-linking through undefined sites on the Abs.

Figure 2.

SDS-PAGE of Ab oligomers reveals the chemical nature of cross-links. THIAMAB oligomers induced by oxidation with CuSO₄ were dissociated to heavy and light chains upon addition of 50 mM DTT to the sample loading buffer. Glutaraldehyde cross-linked samples retain heavier species in the presence of DTT, indicative of nonreducible linkages.

Figure 3.

Size-exclusion chromatograms plotted as normalized absorbance at 280 nm (left) and nonreducing SDS-PAGE gels (right) showing the purification of (A) disulfide-linked THIOMAB, (B) glutaraldehyde cross-linked THIOMAB, and (C) glutaraldehyde cross-linked bsAb oligomers. Monomer (1) and dimer (2) fractions were purified for all samples, while trimer (3) and larger oligomer (4) fractions were also obtained for glutaraldehyde cross-linked Abs. Gels include lanes for unreacted Ab (0) and the cross-linked reaction mixture (X). For the large oligomer fractions, contrast was increased compared to those of other lanes to resolve faint bands.

Figure 4.

SEC calibration curve generated from gel filtration standards (○) used to estimate the size of larger oligomers (■) as roughly hexamer (THIOMAB) and pentamer (bsAb). Known Ab oligomers are shown for comparison (gray triangles) and include glutaraldehyde cross-linked THIOMAB monomer, dimer, and trimer and glutaraldehyde cross-linked bsAb monomer and dimer. The monomer and dimer points overlap for the different Ab samples. Standards include bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), and horse myoglobin (17 kDa).

Figure 5.

Normalized FCS autocorrelations of (A) free Alexa 488 maleimide and glutaraldehyde-reacted THIOMAB (B) monomer, (C) dimer, and (D) trimer in Tris buffer (blue), glycerol buffer (red), and FBS (black). The free dye in FBS is shown as Alexa 488 maleimide that was chemically passivated with 2-mercaptoethylamine (black solid lines) and as the reactive maleimide (black dotted lines). The autocorrelations of the larger oligomer fraction are plotted along with (E) one-component and (F) two-component fits; bottom panels show fit residuals. Ten replicates are overlaid for each oligomer–medium combination.

Figure 6.

Diffusion times resulting from fits of FCS autocorrelation to a two-component model (with one τ_D fixed to that of the chemically passivated free dye in the appropriate medium). Purified oligomers of disulfide-linked THIOMAB, glutaraldehyde cross-linked THIOMAB, and glutaraldehyde cross-linked bsAb were analyzed in a simple Tris buffer (blue circles), glycerol-containing buffer (red squares), and FBS (black triangles). Free Alexa 488 was tested as the reactive maleimide and NHS ester derivatives and after quenching with 2-mercaptoethylamine or Tris.

Table 1Average Diffusion Times (milliseconds) $\pm$ Standard Deviation of Ab Oligomers Reported by FCS

sample	fraction	Tris	glycerol	FBS
disulfide THIOMAB	monomer	2.02 $\pm$ 0.12	2.78 $\pm$ 0.24	2.85 $\pm$ 0.08
	dimer	2.37 $\pm$ 0.08	3.68 $\pm$ 0.38	3.50 $\pm$ 0.62
glutaraldehyde THIOMAB	monomer	3.02 $\pm$ 1.14	3.41 $\pm$ 0.95	3.57 $\pm$ 0.55
	dimer	3.56 $\pm$ 1.58	3.39 $\pm$ 0.42	4.07 $\pm$ 0.43 ^a
	trimer	2.62 $\pm$ 0.18	4.03 $\pm$ 0.19	4.14 $\pm$ 0.41
	hexamer	3.04 $\pm$ 0.69	4.26 $\pm$ 0.92	5.08 $\pm$ 0.64 ^a
glutaraldehyde bsAb	monomer	2.52 $\pm$ 0.59	2.93 $\pm$ 0.22	3.17 $\pm$ 0.35
	dimer	2.69 $\pm$ 0.23	4.17 $\pm$ 0.60	5.15 $\pm$ 1.41 ^a
	trimer	2.66 $\pm$ 0.44	4.03 $\pm$ 0.39	4.87 $\pm$ 0.92 ^a
	pentamer	3.31 $\pm$ 1.56	3.71 $\pm$ 0.96	11.6 $\pm$ 9.1 ^b

^a $P < 0.05$ between glycerol buffer and FBS.^b $P < 0.001$ between glycerol buffer and FBS.

Table 2

Purities of Oligomer Fractions Assessed by SDS–PAGE Densitometry

sample	fraction	% monomer	% dimer	% trimer	% tetramer
disulfide THIOMAB	monomer	99	1	–	–
	dimer	13	83	4	–
glutaraldehyde THIOMAB	monomer	97	3	–	–
	dimer	16	83	1	–
	trimer	11	20	67	3
glutaraldehyde bsAb	hexamer	6	5	3	86
	monomer	99	1	–	–
	dimer	22	77	1	–
	trimer	24	40	33	3
	pentamer	16	17	13	55