Resolving self-association of a therapeutic antibody by formulation optimization and molecular approaches

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Abbreviations: CDR, complementarity-determining regions; PDB, Protein Data Bank

A common challenge encountered during development of high concentration monoclonal antibody formulations is preventing self-association. Depending on the antibody and its formulation, self-association can be seen as aggregation, precipitation, opalescence or phase separation. Here we report on an unusual manifestation of self-association, formation of a semi-solid gel or “gelation.” Therapeutic monoclonal antibody C4 was isolated from human B cells based on its strong potency in neutralizing bacterial toxin in animal models. The purified antibody possessed the unusual property of forming a firm, opaque white gel when it was formulated at concentrations >30 mg/mL and the temperature was <6°C. Gel formation was reversible with temperature. Gelation was affected by salt concentration or pH, suggesting an electrostatic interaction between IgG monomers. A comparison of the C4 amino acid sequences to consensus germline sequences revealed differences in framework regions. A C4 variant in which the framework sequence was restored to the consensus germline sequence did not gel at 100 mg/mL at temperatures as low as 1°C. Additional genetic analysis was used to predict the key residue(s) involved in the gelation. Strikingly, a single substitution in the native antibody, replacing heavy chain glutamate 23 with lysine (E23K), was sufficient to prevent gelation. These results indicate that the framework region is involved in intermolecular interactions. The temperature dependence of gelation may be related to conformational changes near glutamate 23 or the regions it interacts with. Molecular engineering of the framework can be an effective approach to resolve the solubility issues of therapeutic antibodies.

Introduction

Monoclonal antibodies are large, complicated molecules that can challenge formulation efforts. Solution properties such as pH, ionic strength, temperature and excipients are selected to maintain physical and chemical stability and to ensure long product shelf life.1 Information about stability gathered in early formulation studies is also important to ensure that the manufacturing process is compatible with the product. Biochemical properties of the antibody such as net charge, charge distribution and surface exposed hydrophobic patches are some of the important factors influencing physical stability in dilute solutions.2,3 Short-range interactions, such as dipole interactions, excluded volume effects and short range attraction, become important as protein concentration increases.4

Formulation of therapeutic antibodies at high concentrations is advantageous in circumstances where subcutaneous administration is desired.5 In addition, high-concentration formulations are also ideal for storage and shipment of a drug during new disease outbreaks. As with other therapeutic proteins, antibodies formulated at high concentrations have been found to exhibit a range of phenomenon related to protein-protein interactions. Aggregation, precipitation, crystallization, liquid-liquid phase separation, opalescence and high viscosity are all manifestations of self-interactions that can be promoted by high antibody concentration.1,5-11 The different outcomes are due to the nature of the forces dominating the interactions (electrostatic, hydrophobic, short range attraction) and specific solution conditions.4 These phenomena differ fundamentally from rapid heat- or chemically-induced aggregation or precipitation in that the intermolecular interactions can be reversed by changing the solution conditions such as pH, ionic strength, temperature or excipients, suggesting that a native or near-native protein conformation is maintained.3

While formulation approaches are often effective at minimizing aggregation and other degradation pathways, protein engineering methods provide an alternative approach to stabilization.

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Guided by the analysis of amino acid sequences and by 3-dimensional structural models, predictions can be made about the propensity of a protein to aggregate, sequences that cause instability and alterations in sequence that can improve solubility. Equipped with this information, precise amino acid substitutions can be designed that reduce the undesirable properties while maintaining high affinity for the target antigen.

C4, an antibody that neutralizes diphtheria toxin, was identified in a screen of antibody secreting cells isolated from a volunteer immunized with Td vaccine. This human antibody has the potential to be used as a therapeutic antibody due to its high affinity for diphtheria toxin and strong neutralizing potency in animal models. When formulated at >30 mg/mL, purified C4 exhibited a tendency to form a white semi-solid gel at low temperatures, a property that was not acceptable for a product intended to be stored at 2–8°C. Two approaches, formulation and protein engineering, were employed to resolve this problem.

During formulation screening, the antibody was concentrated to ~100 mg/mL using centrifugal ultrafiltration devices. Storage of the formulated antibody at 4°C resulted in the formation of a firm white gel. A photograph of the gelled antibody at different temperatures is shown in Figure 1. The gelation was completely reversible upon warming to room temperature. Interestingly, repeated cycles of gelation and liquefaction did not create visible particles at room temperature or increase the dimer content as assessed by size-exclusion chromatography-high-performance liquid chromatography. The biological function of the antibody was also not affected during multiple cycles of gelation as tested by an antigen binding ELISA and a toxin neutralization assay (data not shown).

To further characterize the conditions that led to gel formation, a series of samples at different antibody concentrations (10–100 mg/mL) were prepared and incubated at 1–10°C. A white gel formed in samples with concentrations as low as 40 mg/mL and at temperatures as high as 9°C (Table 1). The gel became less firm and less opaque as the antibody concentration decreased or as temperature increased. The transition from homogenous gel to liquid occurred over a temperature range of 1–2°C, and was rapid, reaching completion in less than 5 min. These properties demonstrate that the protein-protein interactions leading to gel formation are favored by higher antibody concentrations and lower temperatures.

The effect of formulation on antibody C4 gelation

To address the effect of formulation buffer on gelation, antibody C4 was formulated at 100 mg/mL in solutions with pHs ranging from 3.8 to 7.8. The different buffers used to provide pH buffering over this range are listed in Table 2. The samples were incubated overnight at 1°C and then the temperature was raised in 1°C increments to 8°C. The temperatures at which the solutions changed from partially cloudy to clear are shown in Table 2. No gel formed when C4 was formulated in acetate, succinate or histidine buffers at pH ≤5.5. At >200 mg/mL in acetate...
Antibody C4 was formulated at 100 mg/mL in 20 mM sodium citrate pH 6.0 and incubated at temperatures from 1–10 °C. The temperature was increased in 1 °C increments. (+) Gel formation, (−) no gel formation, (+/−) unclear solution but no gel formation.

Table 1. Effect of antibody concentration and temperature on gel formation

| Temperature (°C) | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|-----------------|----|----|----|----|----|----|----|----|----|----|
| mg/mL           | 100| 90 | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 |
| +/−             | +/−| +/−| +/−| +/−| +/−| +/−| +/−| +/−| +/−| +/−|
| −               | −   | − | − | − | − | − | − | − | − | − |

Effect of antibody concentration and temperature on gel formation. Some gelation was observed at pH 5.5, but not at pH 4.6 or 5.0 (not shown). These results indicate that both pH and buffer type affect gelation. The effect of pH on gelation suggests that electrostatic interactions are needed for a stable interaction. There is precedence for the buffer substance, citrate in particular, to be involved in gelation of a monoclonal antibody. In that instance, however, it was speculated that the trivalent nature of the citrate anion played a role in promoting gelation. While there is some evidence in the data presented here that at pH 5.5 citrate promotes gelation (compare with the results using acetate buffer in Table 2), evidence to the contrary comes from the observation of gelation in histidine buffer, which is a buffer based on a secondary amine rather than a carboxylic acid.

The effect of sodium chloride was also tested in 2 formulations–sodium citrate pH 6.0 and sodium phosphate pH 7.2.

Addition of 100 mM NaCl to either buffer did not affect the melting temperature (Table 3). Addition of 200 mM NaCl lowered the melting temperature of the citrate-buffered sample by 2 °C, but increased the melting temperature of the phosphate-buffered sample by 1 °C. Addition of 400 mM NaCl was sufficient to completely block gelation in both buffers. These data indicate that ionic interactions between antibody molecules, or a conformational change that is sensitive to pH and ionic strength, are important for gel formation.

Framework change resolves the gelation of C4

Since gelation did not occur at pH less than 5.5, the relative affinity of C4 for its target antigen at this pH was measured. The K<sub>D</sub> at pH 5.5 was 4.7 nM, a significant reduction from the K<sub>D</sub> at pH 7 (0.3 nM). An alternative approach to resolve an antibody solubility issue is through protein engineering. Antibody framework regions, which maintain the structure of the variable region, can sometimes be modified to improve protein solubility without affecting the antigen recognition activity of the complementarity-determining regions (CDRs). Here, we investigated whether changes to the framework amino acid sequence of C4 might reduce gelation. The C4 heavy and light chain variable regions were aligned with human germline sequences from V-Base. Several amino acid sequence differences were noted in the framework regions flanking the CDRs. The C4 heavy and light chain constant region sequences are identical to the IgG1 germline because only the variable region of the original C4 antibody was cloned into a standardized expression vector that carries human germline IgG1 constant regions. Several derivatives of C4 were generated by gene synthesis in which the variable region framework sequences were restored to the germline consensus sequence. Germline C4-G-IgG1 is a derivative of C4 in which the framework regions in both the heavy and light chain variable regions have been restored to the consensus germline sequence. Two chimeric C4 derivatives were created by swapping native and germline subunits between native C4 and germline C4-G-IgG1: one with a native heavy chain and a germline light chain (H<sub>G</sub> L<sub>C</sub>), the second with a germline heavy chain and a native light chain (H<sub>C</sub> L<sub>G</sub>.

All derivatives of C4 were formulated at 50 mg/mL in sodium citrate pH 6.0 in a manner similar to the native C4 described above. The complete germline variant, C4-G-IgG1, and the H<sub>G</sub> L<sub>C</sub> chimera did not form gels when incubated at 1 °C (Table 4). Subsequent analysis showed that C4-G-IgG1at

Table 2. Effect of pH and formulation on gelation

| Buffer   | pH  | Gel Melting Temperature |
|----------|-----|------------------------|
| Succinate| 3.8 | no gel                 |
|          | 4.5 | no gel                 |
|          | 4.8 | no gel                 |
| Acetate  | 4.6 | no gel                 |
|          | 5   | no gel                 |
|          | 5.5 | no gel                 |
| Histidine| 5.5 | no gel                 |
|          | 6   | 2 °C                   |
|          | 6.5 | 5 °C                   |
| Citrate  | 5.5 | 7 °C                   |
|          | 6   | 7 °C                   |
|          | 6.5 | 7 °C                   |
| Phosphate| 6.8 | 6 °C                   |
|          | 7.2 | 6 °C                   |
|          | 7.6 | 6 °C                   |
| Imidazole| 7.1 | 7 °C                   |
|          | 7.7 | 7 °C                   |
|          | 7.9 | 7 °C                   |

Table 3. Effect of NaCl on gelation

| NaCl Concentration (mM) | Sodium Citrate pH 6.0 | Sodium Phosphate pH 7.2 |
|-------------------------|-----------------------|-------------------------|
| 0                       | 9 °C                  | 6 °C                    |
| 100                     | 9 °C                  | 6 °C                    |
| 200                     | 7 °C                  | 7 °C                    |
| 400                     | 7 °C                  | 7 °C                    |

Antibody C4 was dialyzed against water and then added to 10x concentrated buffers. Solutions were equilibrated at 1 °C and then the temperature was increased in 1 °C increments over time until all solutions were clear.
Antibody C4 was formulated at 50 mg/mL in 20 mM sodium citrate pH 6.0 and incubated at temperatures from 1–12°C. The antigen specific affinity was measured by Fortebio Octet.

100 mg/mL did not form a gel at 1°C. In contrast, the HN LG chimera formed a gel at lower concentrations and at higher temperatures than did the native antibody. These results suggest that a main determinant of gelation is located in the heavy chain variable region. The native light chain variable region appears to modulate the intermolecular interactions that lead to gelation.

In addition to changing the framework in variable region, the possibility of switching the antibody isotype to IgG2 by changing the constant region was explored. The native C4 variable region sequence was cloned into an expression vector containing the human germline IgG2 constant region sequence. IgG2 isotype antibodies, like IgG1, have a relatively long half-life, which is required for in vivo efficacy of C4 as a preventative treatment. C4-IgG2 did not form a gel when concentrated at 50 mg/ml in sodium citrate pH 6.0 (Table 4), suggesting that an interaction between the variable and constant regions may also have an effect on the gel formation.

While the hypervariable loops in the variable region confer much of the specificity and affinity toward a target epitope, residues in framework regions can interact directly with the target, or indirectly by influencing the structure and position of the hypervariable loops. The antigen-specific affinities of C4-IgG1, C4-G-IgG1 and C4-IgG2 were therefore compared in kinetic assays. The K_D value of C4-G-IgG1 is very similar to the native C4-IgG1; whereas C4-IgG2 has a slightly lower affinity than its IgG1 counterparts (Table 4). C4-G-IgG1 was also subsequently assayed with an in vivo toxin neutralization assay. C4-G-IgG1 possessed efficacy as high as the native C4-IgG1 in protection against diphtheria lethal toxin challenge (data not shown). Thus, C4-G-IgG1 has been selected for large batch antibody production for clinical development.

**Table 4. Prevention of gelation by framework change and isotype switch**

| Antibody Variant | Gel Melting Temperature | Relative Affinity K_D (nM) |
|------------------|------------------------|---------------------------|
| Native C4-IgG1   | 7°C                    | 0.51                      |
| Germline C4-G-IgG1 | No gel                | 0.33                      |
| Germline Heavy C4-IgG1/ Native Light C4-G-IgG1 | No gel | 0.79 |
| Native Heavy C4-G-IgG1/ Germline Light C4-IgG1 | 12°C | 0.87 |
| Native C4-IgG2   | No gel                 | 1.56                      |

Antibody C4 was formulated at 50 mg/mL in 20 mM sodium citrate pH 6.0 and incubated at temperatures from 1–12°C. The antigen specific affinity was measured by Fortebio Octet.

**Table 5. C4 framework amino acid sequences differ from consensus germline sequence**

| Position | Native a.a. | Type          | RSA   | Germline a.a. | Type         |
|----------|-------------|---------------|-------|---------------|--------------|
| 23       | Glutamic acid | Negative charge | surface | Lysine       | Positive charge |
| 45       | Proline     | Hydrophobic   | core   | Leucine      | Hydrophobic  |
| 74       | Proline     | Hydrophobic   | surface | Threonine    | Polar uncharged |
| 76       | Phenylalanine | Aromatic      | surface | Isoleucine   | Hydrophobic  |
| 77       | Threonine   | Polar uncharged | core   | Serine       | Polar uncharged |
| 79       | Valine      | Hydrophobic   | core   | Alanine      | Hydrophobic  |
| 82       | Aspartic acid | Negative charge | surface | Glutamic acid | Negative charge |
| 85       | Serine      | Polar uncharged | surface | Arginine     | Positive charge |
| 87       | Threonine   | Polar uncharged | surface | Arginine     | Positive charge |

Comparison of amino acid sequences of antibody C4 and germline variable regions revealed 9 residue differences. Program NACCESS was used to calculate relative solvent accessibilities (RSA) for all residues.

**Discussion**

The dramatic effect of pH on gelation suggests that electrostatic interactions involving heavy chain residues are important for C4 gelation. To investigate the molecular basis of this effect, we generated a homology model of the C4 monoclonal antibody structure. Differences between C4 and germline framework sequences that involved surface-exposed charged residues in the heavy chain were examined in detail (Table 5). From this analysis, residues 23, 85, and 87 were identified as having significant changes involving charged residues between the native and germlines sequences.

The properties of a single mutation at residue 23 and a double mutation at residues 85 and 87 were examined. Individual mutations were introduced to the native heavy chain sequence by site-directed mutagenesis. Expressed antibodies were formulated at 50 mg/mL in 20 mM sodium citrate pH 6.0 and incubated at temperatures from 1–12°C. Strikingly, the single residue change of glutamate at residue 23 to lysine resulted in a completely soluble antibody with no observable gel formation under these conditions (Table 6). In contrast, mutation of serine 85 and threonine 87 to arginine lead to gel formation at higher temperature than the native antibody, similar to the phenotype of the HN LG chimeric antibody. These results suggest that a glutamate at position 23 plays a key role in determining whether C4 will form a gel at low temperatures and at high concentrations. The mutation of serine 85 and threonine 87 to arginine creates an additional positively-charged patch on the antibody, which could provide an additional site for interaction with any negatively-charged patches.
Native C4-IgG1/C14 background. Each variant was formulated at 50 mg/mL in sodium citrate, manipulated to minimize the protein self-interactions.1,7-11,13

For a long enough time, to promote the stable intermolecular contacts that cause self-association. Solution conditions can often be manipulated to minimize the protein self-interactions.1,7-11,13

The data reported here show that formulation pH and ionic strength had a large effect on gelation. No gelation was seen at pH below 5.5 and all samples formulated at pH greater than or equal to 6.0 gelled. At pH 5.5, gelation was evident if citrate or histidine buffers were used, but not acetate buffer. Addition of 400 mM sodium chloride prevented gelation at pH 6.0 and 7.2. The pH dependence of gelation and its inhibition by sodium chloride suggest that electrostatic interactions are important for gelation.

If electrostatic interactions are a driving force for gelation, then it would be expected that repulsion would predominate interactions where the molecules are of like charge.4 However, while the net charge of C4 in the buffers used here is positive (the pI is 8.4), there are likely patches of negative charge that could interact stably with positively-charged portions of another C4 monomer, analogous to the hydrophobic residues of an anti-LINGO-1 antibody that were thought to form part of an inter-antibody interface and reduce solubility in that context.13 A lower solution pH will decrease the charge density of negatively-charged patches on the antibody surface by increasing protonation of glutamate and aspartate residues. Reducing the negative charge density should weaken the electrostatic interactions that are involved in self-association. Lower pH will also increase the positive charge of histidine residues, leading to increased repulsion. The finding that the tendency to gel changes over the pH range 5–6 suggests that histidine is involved in self-association because the pKa of the imidazole side chain is approximately 6. Yadav et al. reported that specific glutamate, aspartate and histidine residues in the light and heavy chain CDRs affected the viscosity and intermolecular interactions of an IgG1 antibody.1,17

That being said, pH can also affect other properties, such as conformation, that could contribute to gelation, but are unrelated to histidine or acidic amino acid residues.

Three molecular approaches (framework change, targeted mutagenesis and isotype switch) were used to address the gelation problem. All 3 approaches were able to improve the solubility of C4 and prevent gelation at high concentration. Comparison of amino acid sequences of C4 and germline variable regions indicated that there were several residue differences in framework regions. Conversion of framework in the variable region to germline (C4-G-IgG1) appeared to resolve the gelation while maintaining the affinity and potency of native C4. Targeted mutagenesis revealed the critical residues in the native framework region that contribute to gelation. Strikingly, a single residue change E23K in the heavy chain appeared to be sufficient to resolve the solubility issue. The pKa of the glutamate side chain is approximately 4.2. According to the Henderson-Hasselbalch equation, at pH of 5 it is 86% ionized and at pH 6 it is 98% ionized. A region of negative charge around glutamate would lead to a greater tendency to self-associate because of the overall positive surface charge of the antibody. The substitution of a negatively-charged glutamate residue with a positively-charged lysine residue will disrupt the negatively-charged patch on the protein surface around residue 23, reducing electrostatic interactions between molecules.

The electrostatic surface potential surrounding heavy chain residue 23 becomes more positive when the pH is reduced from 7.0 to 5.0 or when the E23K mutant is introduced at pH 7.0. The higher positive charge would decrease the interactions between molecules and prevent association. Alternatively, the replacement of glutamate with lysine could alter the conformation or stability of framework region 1 in a manner that prevents self-association.

Similar gelation phenomenon has been reported previously with immunoglobulins from patients with pathological elevation of plasma immunoglobulin levels. Patients with this disorder can develop cryoglobulinemia, which is characterized by crystallization, aggregation or gelation of immunoglobulins at low temperatures. The gelation is reversible upon changing temperature and concentration.6,18 The gel formed by one particular cryoglobulin antibody was proposed to be a kinetically arrested state that forms in a supersaturated solution at low temperature. If this model is correct for C4, variations in concentration, formulation pH, or rate of temperature change might result in aggregation or crystallization. These states were not observed in our experiments.

Isolation of antigen-specific antibodies from human B cells and engineering into a recombinant IgG form has been considered as an efficient way to discover human therapeutic antibodies.19 One advantage of this approach over other screening methods such as phage display is that the resulting antibodies have been naturally selected for affinity and solubility under physiological conditions. However, for some drug delivery routes, or for delivery of high doses, antibodies have to be formulated at high concentration, which can increase self-association interactions. In the case of diphtheria treatment, patients with severe cases of systemic infection may require large systemic doses of C4. The gelation phenomenon we observed here suggests that caution should be taken when selecting lead antibody candidates with proven biological activities. Formulation optimization and intra-ER crystal body observation can be applied along with other biological assays at the early stage of the selection.20

The biochemical and molecular approaches we developed in this study can be readily applied to resolve similar solubility issues of other therapeutic antibodies.

**Table 6. Effect of heavy chain mutations on gelation and antigen affinity**

| Heavy Chain Variant | Gel Melting Temperature | Relative Affinity Kd (nM) |
|---------------------|-------------------------|--------------------------|
| Native C4-IgG1      | 5°C                     | 0.51                     |
| Germline C4-G-IgG1  | No gel                  | 0.33                     |
| Native C4-IgG1 (E23K)| No gel                  | 0.80                     |
| Native C4-IgG1 (S85R/T87R) | 12°C                  | 0.63                     |

The indicated point mutations were introduced into the native antibody C4 background. Each variant was formulated at 50 mg/mL in sodium citrate pH 6.0 and incubated at 1–12°C.

10°C, with the highest concentration of antibody resulting in the highest transition temperature. Lower temperatures and higher concentrations would increase the likelihood of 2 or more antibody molecules coming together in the necessary orientation, and for a long enough time, to promote the stable intermolecular contacts that cause self-association. Solution conditions can often be manipulated to minimize the protein self-interactions.1,7-11,13

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Materials and Methods

Production of human IgG1 antibody C4 and derivatives

The human IgG1 monoclonal antibody C4 was isolated from antibody-secreting B cells of an immunized human volunteer and selected as a therapeutic candidate having shown protective activity in animal studies. The genes encoding C4 variable regions were obtained from single cell RT-PCR and cloned into a mammalian expression vector in-frame with the human IgG1 heavy chain constant region or human kappa light chain constant region contained in the vector. For antibody framework change, C4 antibody heavy and light chain gene sequences were aligned with the consensus germline sequences in V base (http://www2.mrc-lmb.cam.ac.uk/vbase/vbase-intro2.php). The derivative C4-G-IgG1 was designed by replacing native framework sequences with the consensus germline framework sequence in the variable region of heavy and light chains. The designed antibody gene was generated by gene synthesis and cloned into expression vectors for antibody production. To generate mutant C4 derivatives, residues in the framework region were mutated to their counterparts in the chosen germline gene by site-directed mutagenesis. For class switch to IgG2, the variable region of C4 was subcloned into expression vectors in frame with the human IgG2 constant region in the vector.

To produce material for formulation studies, stable CHO cell lines were generated for antibody C4 and each derivative. Vectors containing heavy chain and light chain antibody genes were combined into a polycistronic vector. Chimeric variants were created by mixing germline and native heavy and light chain expressing vectors into one polycistronic vector. Vectors were electroporated into CHO cells and stable transfecant cell lines were selected and expanded for large scale antibody purification. Antibodies were purified by protein A chromatography with pH adjusted to 6 with 2M Tris base. After purification the material was buffer exchanged into 20 mM sodium citrate pH 6, using tangential flow filtration and formulated initially at 5–10 mg/mL.

Gelation assays

Ultrafiltration concentrators with 10 kDa cutoff were purchased from Millipore (Centriprep and Microcon). Antibody concentration was determined by measuring absorbance at 280 nm in a DU530 spectrophotometer (Beckman) with a 1 cm quartz cuvette or a Nanodrop spectrophotometer (Thermo Fisher). Samples were diluted to <1 mg/mL for measurements with the spectrophotometer or <10 mg/mL for measurements with the Nanodrop. Low temperature incubations were performed in a NESLAB RTE140 circulating water bath. Chemicals for formulation were purchased from Sigma-Aldrich and were ACS or USP grade.

Purified native antibody was concentrated from an initial concentration of 7–10 mg/mL to 115 mg/mL using ultrafiltration concentrators. Centrifugation was performed at 20°C to prevent gelation during concentration. The concentrated samples were diluted in 20 mM sodium citrate pH 6 to concentrations ranging from 10–100 mg/mL. Samples were dispensed into thin-walled PCR tube strips and incubated overnight at 1°C. The temperature was increased in 1°C increments. After 30 min at each temperature, the visual appearance of each tube was noted. Analysis of chimeric and IgG2 class switch mutants were done in the same manner except that samples were initially concentrated to 60 mg/mL and then diluted to 10–50 mg/mL final concentration.

To study the effect of buffer and pH, native antibody was dialyzed against water, then concentrated to 60 mg/mL. Twenty-seven microliters of concentrated antibody were mixed with 3 µL of 10x concentrated formulation buffers listed in Table 2 a in thin wall PCR tube strips. The pH of the diluted formulation buffers was determined prior to use. The strips were incubated overnight at 1°C and the appearance of the tubes was recorded. The temperature was increased in 1°C increments until no gelling was visible. The appearance of each tube was noted at each temperature. For experiments where sodium chloride concentration was varied, the antibody was dialyzed against water and concentrated to 120 mg/mL. Aliquots of concentrated antibody were then dialyzed against the test formulation buffers overnight at room temperature. Concentrations of the dialyzed antibody were adjusted to 100 mg/mL with dialysis buffers and the samples were incubated in the manner described above.

Assays for C4 affinity

The relative affinity of C4 and C4 derivatives for the target antigen was measured using Fortebio Octet technology. Antibodies were immobilized on the biosensor to avoid potential bivalent effect from antigen immobilizations. Anti-human IgG biosensors were used to capture C4 and its derivatives (all at 10 µg/ml) in phosphate-buffered saline followed by association with the target antigen at 3 concentrations: 12.5 µg/mL, 25 µg/mL and 50 µg/mL. Association (Kon) and dissociation (Koff) rate constants were calculated for the target antigen and used to derive the dissociation constant (Kd). All calculations were made using software supplied by Fortebio. The signal from a no-analyte reference control (buffer only) was subtracted from test results for calculating kinetic constants. X² and R² values were evaluated to confirm the suitability of a 1:1 binding model in all analysis.

Molecular modeling

A structural model for the C4 Fv region was generated by the Modeller program (PMID 18428767), using structural templates selected from the Protein Data Bank (PDB) (PMID 10592235) based on similarity to the C4 heavy and light chain sequences and CDR loop lengths: an anti-polylactide-butyrate antibody (PDB code 2D7T), and the heavy chain from an anti-angiopoietin 2 antibody (PDB code 4IMK). Five models were generated by Modeller, of which the top model was selected based on DOPE score, and refined by Rosetta version 3.5 (PMID 21187238) using the “relax” protocol with heavy atom constraints. The program NACCESS (www.bioinf.manchester.ac.uk/naccess/) was used to calculate relative solvent accessibilities (RSA) for all residues, which were used to distinguish surface (RSA > 35%) versus core (RSA < 35%) residues on the antibody structure.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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