Introduction

Functional protein and polymer-based networks often characterized as gel-like are responsible for a wide range of biological, chemical and pharmaceutical processes. For example, actin filaments are responsible for extended inter and intracellular movements, among many other functions, while the viscoelastic and structural properties have been extensively studied. In addition, some pathogenic disease states exhibit physicochemical characteristics that resemble gel-like behavior. Synthetic gels and polymers are widely used in industry; in particular, pharmaceutical gels are manipulated for delivery applications. The use of high concentration monoclonal antibody (mAb) formulations in the biotechnology industry has renewed interest in diverse disciplines ranging from immunoglobulin solubility, disease states to fundamental soft condensed matter physics.

In this work, we demonstrate formation of an antibody gel and characterize the physicochemical properties of this filamentous bundle. Citrate is shown to trigger filament formation which is of potential broad ranging significance. In vivo, ions have been implicated in a variety of pathogenic disease states that result from cross-linking and formation of protein networks. Although the viscoelastic properties of these protein networks may not always be known, several studies suggest that the cellular environment is gel-like and many of the filamentous networks are mechanically characterized as a solid with structural disorder and high volume fractions of solvent.

Several neurodegenerative diseases result from formation of protein aggregates that have been correlated with anionic compounds in vivo. While protein aggregation is vital for processes where microtubule and actin assemble to enable chromosome segregation and formation of the contractile ring (e.g., in cell division), it could lead to disorders ranging from Parkinson disease to cryoglobulinemia. At the forefront of neurodegenerative disease research is a crusade to elucidate protein aggregation, which leads to deposits of naturally-occurring proteins in the brain. For example, in Creutzfeldt-Jakob disease (CJD), prion proteins (PrP) form deposits in the brain and transforming growth factor β-induced proteins (TGFBIp) aggregate into amyloid or non-amyloid fibril deposits, resulting in corneal dystrophies and other related diseases. Purified immunoglobulin proteins from cryoglobulinemia patients assemble into crystalline structures at reduced temperatures (< 5°C). Scientists have established links between missense mutations and protein deposits, although the mechanism of protein self-association remains elusive. Furthermore, in vivo protein–anion interactions are thought to be responsible for protein storage in the extracellular matrix, chaperone activity, macromolecular transport, and cellular communication. Although the direct role of these interactions is not known for antibodies in vivo, it is nonetheless likely that they occur.
Citrate binds numerous macromolecules and has been shown to induce antibody self-association. Previous reports have shown that in the presence of multica-rboxylates such as citrate, a mAb can form ordered filament assemblies that crosslink and bundle into networks. Little is known about the thermodynamic or structural requirements for filament assembly or the interaction between citrate and antibodies. In this work, isothermal titration calorimetry (ITC) was used to measure the heat associated with substrate–ligand interactions. TEM, FTIR and Raman spectrosocpy, and mechanical rheology were used to investigate the structural changes within mAbs, and to quantify the mechanical strength of the protein gel under varying assembly conditions.

The heat given off upon titrating a stock solution of citrate into a mAb solution was measured (Fig. 1A). The differential heat released reduces over time possibly due to the saturation of substrate–ligand interaction sites (Fig. 1A). We hypothesize that the substrate contains positively charged amino acid residues that directly interact with the negatively charged citrate ions. The observed enthalpies are consistent with an interaction between citrate and an amino acid residue. The area under the curve for the differential heat given off was used to generate a titration curve (Fig. 1B) and fit to Equation 1 (See Materials and Methods). This equation fit the data well and no unique higher order analysis was considered. A titration curve of citrate titrated into buffer without protein was also obtained and subtracted from the titration with protein (Fig. 1B) to eliminate heats of dilution of citrate salts. To minimize contributions from polymerization of monomeric units of mAb, experiments were conducted only up to a threshold assembly ratio of 64:1 (moles of citrate: moles of mAb) previously determined.

Temperature and pH significantly affects K and ΔH of mAb–citrate interaction. Isothermal titration calorimetry was used to investigate the effect of pH on the interaction thermodynamics (Fig. 2). For pH 4, 5, 6 and 7, the respective K values were 567 M⁻¹, 801 M⁻¹, 854 M⁻¹, and 184 M⁻¹ (Fig. 2A and Table 1). The association constants suggest that the binding/interaction affinity between citrate and mAb exhibits a maximum at pH 5–6 and is 5-fold lower at pH 7 (Fig. 2A). As the pH increases, the theoretical net positive charge of the protein decreases from +42 (pH 5) to +2.5 (pH 7), while the amount of fully ionized citrate ions increases from 4% (pH 5) to 89% (pH 7) (Fig. 2B). As pH values increase past pH 6, the net mAb charge decreases (+2.5 at pH 7 and +0.6 at pH 8) and there is also a marked decreased in association constant between mAb and citrate ions, implying a possible role for histidine ions which are deprotonated over the same pH range. A similar trend is observed with the measured apparent enthalpy of interaction, including a maximum enthalpy magnitude at pH 5 (Fig. 2C; Table 1). Enthalpies at pH 4, 5, 6 and 7 are ~53.3 kcal/mol, ~73.5 kcal/mol, ~25.3 kcal/mol and ~16.1 kcal/mol, respectively, implying an exothermic reaction/interaction.

The enthalpy of interaction remained relatively unchanged when experimental temperatures were varied between 10°C and 30°C (Fig. 2D and Table 2). At a constant pH 6, the enthalpies of interaction were ~26.0 kcal/mol, ~26.9 kcal/mol, ~25.3 kcal/mol, and ~26.1 kcal/mol, for 10°C, 17.5°C, 25°C,
and 30°C, respectively. At 40°C, ΔH reduced slightly to –22.4 kcal/mole, possibly due to thermal perturbations or conformational change of the mAb (Fig. 2D; Table 2). The association constant, on the other hand, decreased with increasing temperature with association constants of 1427 M⁻¹, 1089 M⁻¹, 854 M⁻¹, 651 M⁻¹ and 542 M⁻¹ for 10°C, 17.5°C, 25°C, 30°C and 40°C, respectively (Fig. 2E; Table 2). These association constants represent a weak interaction between citrate and mAb (For example, an association constant of 1427 M⁻¹ is equivalent to a dissociation constant of ~700 μM). These weak interactions are expected because the assembly of mAb monomers into a filamentous network is reversible. There is also a downward shift in the pKₐ of histidine residue as temperature is increased from 10°C and 40°C; this shift in pKₐ could contribute to the changes in observed association constants by decreasing the ionization of the histidine.

The free energy of interaction between mAb and citrate was calculated to be between 3 and 4 kcal/mole and the free energy increased slightly with increasing pH (constant temperature of 25°C) and temperature (constant pH 6) (Tables 1 and 2).

The secondary structure and amino acid environment is altered after gelation. Given the observed pH effect on mAb–citrate interactions and its possible structural implications, the pH-dependent conformational heterogeneity of mAb was evaluated using FTIR and Raman spectroscopy (Fig. 3). Subtle alterations in the secondary structure and environment surrounding ionizable and aromatic amino acid residues were observed as a function of pH. In general, the pH 6 mAb spectrum is characterized by intermediate intermolecular β-sheet and disordered structure based on relative height, width and position of bands at 1612–1620 and 1645 cm⁻¹ (Fig. 3A). The spectra for pH 4 and 8 (5 and 7) solutions exhibit the greatest (least) amount of intermolecular β-sheet and disordered structure (Fig. 3A). Interestingly, pH 6 spectra appear to have unique structural characteristics compared with all other solution conditions. The pH 6 spectra are closest to

![Figure 2](image)

**Figure 2.** Effect of temperature and pH thermodynamic parameters of mAb–citrate interaction. (A) Observed association constant, kₐ, is maximum between pH 5–6, at a constant temperature of 25°C. (B) Plot showing that as solution pH increases, the net positive charge of mAb decreases and percentage of fully ionized state of citrate increases. (C) Observed enthalpy of interaction, ΔH, as a function of solution pH. At constant pH 6, both enthalpy of interaction, (D), and association constant, (E), decrease with increasing temperature. The slope indicates a constant heat capacity change ΔCₚ of interaction of ~117 cal/mole.K

| pH  | Kₐ M⁻¹  | ΔH kcal/mol | ΔG kcal/mol |
|-----|---------|-------------|-------------|
| 4.0 | 566.5 ± 22.2 | -53.3 ± 1.1 | -3.7 ± 0.1  |
| 5.0 | 800.5 ± 38.4 | -73.5 ± 1.8 | -3.9 ± 0.2  |
| 6.0 | 853.8 ± 35.8 | -25.3 ± 0.5 | -3.9 ± 0.2  |
| 7.0 | 184.1 ± 18.4 | -1.6 ± 0.1  | -3.0 ± 0.4  |

![Table 1](table)

**Table 1.** Thermodynamic parameters at constant temperature of 25°C

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pH 5 and 7 with regard to distinct characteristics, which is not surprising. Specific changes are observed in regions characteristic of histidine protonation state, including bands at 1095, 1440 and 1592 cm$^{-1}$ (Fig. 3A and B). Although concrete assignment cannot be made because of overlapping absorbance in this region, the changes are most likely due to histidine considering the residues previous vibrational assignments, residue pK$_a$s, and the pH where spectral changes are observed.

Upon gelation, subtle changes in the β-sheet structure and surrounding environment of Glu and Lys residues at pH 4, 5, 7 and 8 are observed near 1555 and 1530 cm$^{-1}$, which is not surprising considering the pK$_a$'s and strong absorption coefficients for these residues (Fig. 3C). As previously mentioned, changes are also observed in regions that may be the result of histidine protonation state.

In addition, the aromatic residues appear to play an important role in both the pH dependent structural heterogeneity and gelation behavior based on changes in peaks at 1000, 1070, 1205, 1340 and 1515 cm$^{-1}$. The spectral changes observed upon gelation at pH 6 are dominated primarily by shifts in peak positions that may be assigned to Trp, Tyr, and Ser residues. Interestingly, the change in peak position at 1243 and 1282 cm$^{-1}$ suggests modulation of the H-bonding environment surrounding Trp and Tyr residues (Fig. 3C).

Raman spectroscopy was used to further investigate the significance and participation of aromatic amino acid residues in the gelation process (Fig. 3D and E). The intensity ratio ($I_{1500}/I_{1350}$) and band frequency red shift near 880 cm$^{-1}$ have been shown to correlate with hydrogen bonding of Tyr and Trp residues, respectively (Fig. 3E). Increased hydrogen bonding to the indole ring is observed as a function of increasing gelation time based on the 880 cm$^{-1}$ peak position shifts, which is consistent with the FTIR results. In addition, the $I_{1500}/I_{1350}$ ratio suggests a change in Tyr environment from that of both a donor and acceptor of strong hydrogen bonds before gelation to that of increasing mobility of mAb filaments (Fig. 3E). This suggests that the elasticity increased to ~2000 Pa over the same time duration (Fig. 4A).

Solutions that were between pH 5 and 6 were also more solid-like as measured by their phase angle (Fig. 4B). At pH 4, the phase angle was 90° suggesting that there was negligible elasticity, which was probably due to fewer and non-overlapping filaments. At pH 5, 6, and 7, the phase angle had dropped to 14°, 30° and 40°, respectively (Fig. 4B). After gelation for 90 min, these gels were subject to an increasing frequency with a constant shear deformation of 0.5% (Fig. 4C) to study the tendency of mAb filaments to move in solution. The extent of increase is proportional to the ability of mAb filaments to move in solution. Between 1 rad/s and 100 rad/s, mAb solution elasticity increased slightly from ~2500 Pa to ~3000 Pa for pH 5 and ~2600 Pa to 4200 Pa for pH 6, while at pH 4 and pH 7, solution elasticity significantly increased ~2000× and 4000×, respectively (Fig. 4C).

Table 2. Thermodynamic parameters at solution pH 6

| Temperature °C | K$_M$ M$^{-1}$ | ΔH Kcal/mol | ΔG Kcal/mol |
|---------------|---------------|--------------|-------------|
| 10.0          | $1427 \pm 49.1$| $-26.0 \pm 0.4$| $-4.0 \pm 0.1$|
| 17.5          | $1089 \pm 42.8$| $-26.9 \pm 0.5$| $-4.0 \pm 0.2$|
| 25.0          | $853.8 \pm 34.8$| $-25.3 \pm 0.5$| $-3.9 \pm 0.2$|
| 30.0          | $651.4 \pm 29.1$| $-26.1 \pm 0.6$| $-3.9 \pm 0.2$|
| 40.0          | $541.7 \pm 18.0$| $-22.4 \pm 0.4$| $-3.9 \pm 0.1$|

Table 2. Thermodynamic parameters at solution pH 6

The environment of the ensemble average of all 12 Trp residues, however, may be considered hydrophilic.

Structural information obtained from FTIR and Raman spectroscopy was complemented with electron micrographs (Fig. 3F), which showed filament bundles that appeared rigid. These structures of 50 mg/ml mAb assembled in 50 mM citrate were indistinguishable over the range of pHs tested (pH 4 to pH 7), although the number of filament bundles appeared more in pH 5–6 compared with either pH 4 or pH 7 (not shown).

Similar to previous studies, these filament bundles showed larger bundles containing smaller filament bundles. These larger bundles varied widely in length up to 5 μm and had diameters as large as 150 nm (Fig. 3F).

mAb gel elasticity is highly dependent on solution pH and temperature. Gelation of mAb solutions, triggered by the addition of millimolar concentrations of sodium citrate, was measured by the onset of elasticity on a mechanical rheometer (Fig. 4). The network elasticity of 100 g/L mAb was dependent on solution pH when assembly was triggered with 50 mM sodium citrate at 25°C (Fig. 4A). Solutions at pH 4 and 7 did not show any significant increase in elasticity even after 90 min, while at pH 5 and pH 6 the elasticity increased to ~2000 Pa over the same time duration (Fig. 4A).

We further tested the gelation properties of mAb filaments at a fixed pH of 6 and temperatures between 10°C and 30°C (Fig. 4D–F). The elastic modulus increased with temperature in the presence of 50 mM sodium citrate. For the time range monitored, at 10°C the network elastic modulus was relatively low with a steady-state value of 14 Pa; the steady-state elastic modulus increased rapidly with temperature to 750 Pa at 20°C and to 1200 Pa at 30°C. In addition to increased elasticity modulus at temperatures of 20°C and 30°C, the solutions were also more solid-like as assessed by their phase angle. After ~30 min of gelation, the elastic modulus of gels at 30°C decreased, probably due to interfilament interactions or steric hindrances.
to filament reorganization in solution (Fig. 4D). After gelation, these gels were similarly subjected to an increasing frequency with a constant shear deformation of 0.5% (Fig. 4E) to study their tendency to move in solution. As temperature increased, the extent of mobility in solution decreased, suggesting more solid network structures (Fig. 4E). This was confirmed by the phase angle, which showed that solutions assembled at 10°C was 60°, while the phase angle was reduced to ~30° for solutions assembled at ≥ 20°C indicative of more solid structures (Fig. 4E, inset). Depending on the type of interfilament interaction, network elastic modulus may increase/stiffen or decrease/soften under increasing shear. We subjected mAb filaments to shear deformations of increasing amplitude (Fig. 4F) to assess the mechanical response. The modulus of mAb gels decreased rapidly for gels formed at 10°C in agreement with a more liquid-like character exhibited by its phase angle, whereas at higher temperatures, mAb gels were independent of the applied deformation amplitude at low shear deformations (Fig. 4F). Low shear deformation/strain typically define the linear rheological regime for which applied deformation and induced stress are proportional, and an elastic modulus declines steeply past a threshold deformation value more commonly referred to as a yield strain. mAb gels softened at a yield strain amplitude of 0.6 and 1% for gels assembled at 20°C and 30°C, respectively (Fig. 4F).

**Figure 3.** Structure of mAb is altered by pH and state of gelation. Plot showing second derivative FTIR spectra of 100 mg/mL mAb at varying solution pH from pH 4–8 from (A) 1380 cm⁻¹ to 1700 cm⁻¹ and (B) 1000 cm⁻¹ to 1500 cm⁻¹. (C) Second derivative FTIR spectra of 100 mg/mL mAb before and 30 min after addition of 100 mM citrate at pH 6. Each spectrum was subtracted with a spectrum of corresponding buffers without proteins. (D) Raman spectra of mAb gelation over time at constant pH 6 from 700 cm⁻¹ to 1100 cm⁻¹ (E) Raman spectra showing ratio of intensities at 830 cm⁻¹ and 850 cm⁻¹ over time. Inset, showing full Raman spectrum of mAb gelation over time at constant pH 6 from 300 cm⁻¹ to 1700 cm⁻¹. (F) Electron micrographs of 50 mg/mL mAb after the addition of citrate. Analysis of micrographs showed big bundles containing smaller filament bundles. Individual straight filaments were of varying lengths and had an average diameter of 4 nm. Scale bar is 0.2 μm.
used. We hypothesize here that these carboxylate ions may exhibit competitive binding. Since acetate ions do not trigger gelation of mAb proteins, we added sodium acetate at varying concentrations to mAb proteins prior to the addition of 50 mM sodium citrate (Fig. 5). We monitored the elastic modulus of mAb gels

**Figure 4.** Mechanical properties of mAb. (A) Profile shows increase in elastic modulus, G', of mAb gels at various pH upon addition of citrate. (B) The phase angle of mAb gels after 1.5 h of gelation as a function of pH. The phase angle is 90° for a viscous liquid like water or glycerol, which has no elasticity, and 0° for a highly elastic material like a crosslinked polyacrylamide gel. (C) Frequency dependent elastic modulus of 100 mg/mL mAb solution. Measurements were taken immediate after the citrate showing prolonged lag phase. Concentration of all gels are 100 mg/mL. Symbols in (A and C) are pH 4 (asterisk), pH 5 (open circles), pH 6 (closed squares), pH 7 (closed circles). Effect of temperature on the time-dependent (D), frequency-dependent (E), strain-dependent (F), elastic modulus of mAb gels. Symbols in (D), (E and F) are 10°C (open circles), 20°C (closed diamonds), and 30°C (closed squares). (E, Inset) Profile showing the phase angle of mAb gels at steady-state as a function of temperature. The amplitude of shear deformations in all experiments was 1%.

Monovalent carboxylates may inhibit mAb gelation due to competition. Previous reports show that gelation of mAb filaments is dependent on multivalent carboxylates (such as citrate), not monovalent carboxylates (such as acetate), and the extent of gelation is dependent on the concentration of multi-carboxylate

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over time and observed that the elastic modulus of these gels was significantly reduced in the presence of acetate (Fig. 5A). In this concentration range, the reduction did not appear to be correlated with the quantity of acetate added. To determine if this was an ionic strength effect and not specific to a competition between binding sites of citrate and acetate, we repeated the same experiment using sodium chloride and found that sodium chloride also reduced the elastic modulus of mAb gels, but not to the same extent as acetate (Fig. 5B). Upon further examination, unlike sodium acetate, the concentration of sodium chloride in solution correlated strongly with the extent of gelation (Fig. 5B). While there is an ionic strength effect, sodium acetate appears to compete for binding/interaction with citrate and inhibit assembly even at much lower concentrations.

To supplement these rheological findings, we assessed the thermodynamics of interaction between citrate and mAb and found that it changes significantly in the presence of acetate ions in solution. Sodium acetate was added to mAb solutions, at a fixed temperature of 25°C and pH 6, and incubated prior to adding sodium citrate (Fig. 5C). Association constant, $K_a$, decreased steeply with increasing amounts of acetate present, indicating that the interaction between citrate and mAb was decreasing. This result supports the rheology data, which shows reduced elasticity of mAb gels when acetate was present in solution. Similar to citrate, since the interaction between acetate and mAb is not covalent, the process can be regarded as an ion-exchange process as explained by the theory of polyelectrolyte solutions. In this theory, because a stoichiometric amount of citrate interacting with mAb exists in equilibrium, there is a corresponding and equivalent release of acetate from mAb. On a log-log plot, the observed association constant between citrate and mAb increases linearly with acetate concentration (Fig. 5D) and can be described as

$$\log k_d = Z \log [acetate] + \text{constant}$$

![Figure 5. Competition of Carboxylates during mAb gelation. (A) Elastic modulus of mAb gels is affected by the concentration of acetate presence in solution prior to initiating gelation. Symbols are 0 mM (closed circles), 10 mM (open circles), 100 mM (closed squares), and 250 mM (open squares). (B) Profile showing the steady-state elasticity of mAb gels containing varying concentrations of sodium acetate (hatch bars) and sodium chloride (black bars). The amplitude of shear deformations in all experiments was 1%. (C) Citrate is titrated into protein solutions containing fixed concentrations of acetate. Heat is released after each addition of citrate into the solution over time and used to calculate the thermodynamic parameters (see Fig. 1). Plots show association constant between mAb and citrate as a function of acetate concentration in solution. Titration was performed at pH 6.0 and 25°C. (D) Log-log plot of dissociation constant and acetate concentration in solution. The slope of the plot indicates that ~1.3 acetate ions were released from mAb in the presence of a citrate ion.](image-url)
where Z represents the number of ionic interactions between the mAb and citrate ions, which results in the release of Z-bound acetate ions from mAb; and the constant represents contributions from nonionic interactions, including interacting mAb molecules in solution, that are otherwise not accounted for. The slope of the plot indicates that ~1.3 acetate ions were released from mAb in the presence of a citrate ion57 (Fig. 5).

**Discussion**

Proteins have been long shown to interact with ions through direct binding (e.g., calcium-binding protein, calmodulin) and through other electrostatic interactions.15,64 Ionic interactions can trigger functional or structural changes in many proteins that are relevant in vivo. For example, multiple highly negatively charged anions have been shown to bind diverse proteins with functional consequence.65-67 In addition, the cellular environment is highly polyanionic and has been proposed to function as a molecular highway for intracellular communication.18 In vivo, antibodies can associate to form structures as large as pentamers, although the role and importance of ions in the formation of these structures is still not clear. In vitro, antibodies can self-associate either reversibly or irreversibly and can associate into organized filament bundles that assemble into a network. Proteins aggregate in the presence of anions according to the Hofmeister series and these anions may interact with water molecules or directly with amino acid residues on a protein to increase aggregation.68 Pesinmeyer et al. also show that the presence of anions, and not cations, in solution results in lower melting temperatures and conformational stability of antibodies.68

Antibodies self-associate under specific solution conditions and in the presence of multivalent carboxylates to form well ordered filaments.15 The exact nature of the interaction is unknown although it is probably due to one or a combination of electrostatic, short-range, and possibly dispersion forces. Rheology, calorimetry, microscopy, and spectroscopy analysis provide better insight into this interesting phenomenon that a mAb, like many cytoskeletal proteins, can form gels composed of ordered filamentous networks.

**Optimal pH required for gelation suggests critical role for histidine residue.** The highest interaction between citrate and mAb occurs between pH 4 and 6, in agreement with gelation where the gels feature highest elastic modulus in the same pH range. MAbs are composed of multiple amino acids that are ionized depending on their pK, and solution pH. At pH slightly below 5, histidine, lysine and arginine residues are positively charged and can potentially interact with negatively charged citrate ions. As the solution pH is raised above pH 6, however, citrate ions are fully ionized and only lysines and arginines remain protonated, while histidine becomes deprotonated. The deprotonation of histidine residue correlates with the inability of mAb to form gels, suggesting significant protein–ion interaction mediated by histidine. The side chains of lysines and arginines are more flexible and as such may be entropically challenging, while histidine is composed of a more rigid ring structure that may be favorable to such interactions.

In addition to the formation of filaments, as assessed by electron microscopy, the elastic modulus association constants also increased within a similar pH range. Network elasticity is directly proportional to citrate concentration present in solution, implying that citrate acts as a crosslinking agent. At pH 4, there is virtually no elastic modulus, which can be explained by the protonated state of citrate in solution. Elasticity is present, but it is significantly diminished at pH 7 when histidine residues are relatively uncharged. The phase angle of mAb solutions at pH 4 is ~90°. A phase angle of 90° implies the rheological behavior of a liquid such as glycerol, whereas a phase angle close to 0° implies an elastic solid, such as a stiff rubber, hence there are little to no overlapping filaments formed at pH 4. mAb gels at pH 5 have a phase angle of ~20° that increases with pH, which results in a solid-like property due to interfilament interactions. Solid-like behavior is also observed when mAb gels are exposed to increasing shear deformations. Rapidly applied deformations do not allow sufficient time for filaments within the gel to relax, while slowly applied deformations allow the time for filaments within the gel to rearrange and relax the stress. The elasticity of the extreme pH 4 and pH 7 solutions display a frequency-dependent spectrum indicative of loosely overlapping or uncrosslinked filaments; at pH 5 and pH 6, mAb gels feature frequency-independent elasticity that is similar to cross-linked or bundled polymers.69

To determine thermodynamic parameters due mainly to ion–protein interactions, mAb concentrations below the threshold for gelation were used for isothermal calorimetry studies; however, there were undoubtedly still some protein–protein interactions. Nonetheless, the observed association constants were maximal between pH 5 and pH 6 while the enthalpies of interaction were highest at pH 5, which further supports results from other assays indicating an optimum pH range for gelation of mAb. Since the ionic state of histidine residue changes in this pH range, it is likely that histidine plays a role in the interaction of mAb with citrate. Histidine is implicated in FcRn-mediated transport and can be used to regulate the pharmacokinetic half-life of therapeutics as evidenced by FcRn-albumin and FcRn-IgG complexes, which are strongest around pH 6.70-72 Furthermore, the saturation of the binding isotherm for the interaction of mAb with citrate at citrate:protein ratio of 20–30 correlates to a total of number of 22 histidines within each mAb molecule.

**Carboxylate ion interaction is specific.** Isothermal calorimetry results show that an enthalpy of interaction is present when citrate is mixed with mAb. Higher association and affinities are accompanied by higher exothermic enthalpies of interaction at an optimum pH between 5 and 6. Taken together, these results suggest greater interaction, which is also confirmed by increased gelation over the same pH range. The exothermic enthalpy remains relatively unchanged over a temperature range between 10°C and 30°C probably due to a stable mAb structure; however, at 40°C there is a slightly lower exothermic enthalpy, suggesting that the interaction is thermodynamically and not kinetically limited. At lower temperatures, where there is less kinetic energy in the system, interaction is purely thermodynamically driven and there is a corresponding increase in affinity and association
constant. This stronger association at lower temperature does not result in higher filament network elasticity. Although citrate associates stronger with mAb protein subunits at lower temperatures, mAb filament networks exhibit lower mechanical integrity at lower temperatures.

While ionic strength affects mAb gelation in a concentration-dependent manner, monocarboxylates (such as acetate), which by themselves do not trigger gel formation, significantly affect interaction between citrate ions and the mAb protein. In the presence of acetate, the association constants between citrate and mAb decreases, which suggests competition between citrate and acetate for the same binding/interaction region, consequently the elasticity of gels significantly reduces when acetate is present. If the presence of citrate in protein formulations results in self-association in other proteins, then compounds similar to acetate could be useful in preventing protein interactions. Formation of filamentous structures in drug formulation requires optimal conditions that could vary significantly for different proteins. These conditions include the type of protein, protein concentration, citrate concentration, number of histidine residues and ion accessibility of histidine residues within a protein. Together, these results denote a specific and competitive interaction between carboxylates and mAb.

Secondary structure of mAb is affected by gelation. Spectroscopic analysis reveals that, upon gelation, the mAb exhibited subtle changes in secondary structure mainly around aromatic and hydrophobic residues with an increase in β sheet structure. As suggested by the spectroscopic results, it is likely that filament formation results in hydrophobic aromatic residues becoming less solvent exposed with additional alteration of the H-bonding environment. These changes are likely to be correlated with the secondary structure alterations observed. In addition, confirmation of the role of histidine was provided by FTIR and Raman spectroscopy; however, the changes observed cannot be definitely assigned to histidine alone because of overlapping bands. At pH 4 and 8, the mAb has increased disordered structure compared with pH 5 and 7, which is not surprising. Increased disordered structure is expected at pH 4 because of partial unfolding and disruption of salt bridges, which has previously been observed with other antibodies. In fact, partial unfolding may aid in reducing the rigidity of structures at low and high pH due to increased conformational fluctuations. Changes in intermolecular β-sheet were observed with spectra at pH 4 and 8, which were different from the pH 5, 6 and 7 spectra. These changes indicate differences in hydrogen bonding and transition dipole coupling (TDC) and may be due to increased self-association. TDC is a resonant interaction between neighboring oscillating dipoles that render the Amide I band sensitive to secondary structure. This coupling interaction is dependent on the relative orientation and distance of these dipoles. These changes have previously been observed for mAbs as a function of pH, but the effects of citrate-induced gelation has not been studied. In fact, Kamerzell et al. correlate changes in disordered and intermolecular β sheet structure with increased solution viscosity and self-association of some mAbs at ultrahigh concentrations. Similar spectroscopic changes are observed at pH 5 and 6 in this study, which correlates with the TEM and rheology results.

Possible mechanism and applications. Like many proteins, antibodies can self-associate reversibly at high concentrations in vitro and form large structures. Immunoglobulins purified from the plasma of patients in certain disease states have been shown to form insoluble aggregates upon lowering their solution temperature. In this report, we investigated the thermodynamic interactions between citrate and mAb, and postulate that positively charged histidine may be crucial to initiate interaction, followed in importance by other aromatic and hydrophobic residues that may assemble and stabilize structures. Interestingly, histidine protonation has been linked to many different physiological processes. For example, protonated histidine modulates ion channels, required for viral fusion protein activation and invasion of host cells driven by specific surface proteins, and participates in hundreds of highly specific and functional metal coordinated interactions. Formation of an IgG-FcRn complex is dependent on conserved histidine amino acid in the Fc-region, while a recent study suggests that histidine plays a role in the FcRn-albumin complex. While there is no direct physiological evidence of histidine interacting with citrate, we postulate that polycarboxylates such as citrate could act as a bridge between histidine molecules, protein assembly may be possible after some conformational changes, whereby it may become favorable for one molecule to dock with the other. The Yukawa model of interacting proteins describes a phenomenon of an interaction potential that could finally result in bundling of individual filaments through a counterion effect in which filament surface charges are neutralized, followed by filament-alignment by Van der Waals interactions. Since the two Yukawa potential has typically been used in low ionic strength and small self-association systems, it may not be predictive in a strongly associating system.

In vivo, antibodies are known to form dimers (IgA) and pentamers (IgM); these larger structures enable them to bind antigens with greater avidity to form larger complexes and possibly increased function to direct immune responses in living organisms. In medical care, the administration of high concentration antibodies has advantages for chronic indications, subcutaneous delivery, and possibly higher bioavailability, but the increased viscoelasticity could limit its use. Temperature, pH, and ionic strength are useful tools in controlling the type and degree of protein assemblies and they can be exploited for extended release matrixes or as more stable biomaterials. Depending on its specificity, crosslinkers or epitopes can be engineered to crosslink filament bundles to form more rigid biomaterials for a wider variety of applications.

Materials and Methods

Sample preparation. A humanized mAb was constructed from an IgG1 human framework with light chains. The antibody was expressed in Chinese hamster ovary (CHO) cell lines and purified using protein A affinity chromatography and ion exchange chromatography. Buffer exchanges during protein isolation and
the final formulation steps were performed using a tangential flow filtration (TFF) system. The antibody (MW ~150 kDa; pI = 7.8) was stored in a range of pH conditions: 30 mM pyridine hydrochloride buffer at pH’s 4, 5 and 6, and 30 mM imidazole hydrochloride buffer at pH’s 6, 7 and 8. All chemical reagents were analytical grade or higher.

**mAb concentration determination.** The concentration of mAb solutions were assessed using a Hewlett Packard 8453 diode array spectrophotometer with a 1-cm quartz cuvette. Concentration was calculated using an absorbivity of 1.60 cm⁻¹ (mg/mL)⁻¹ for mAb, as determined by quantitative amino acid analysis.

**Rheological studies.** Studies were conducted using a temperature-controlled cone-and-plate Modular Compact Rheometer 300 (Anton Paar). The apparatus is equipped with a solvent trap to prevent solvent evaporation. mAb solutions at 100 g/L were first added onto the plate, followed by the addition of millimolar concentrations of citrate. Experiments were performed under various pH and temperature conditions. The rheometer’s mechanism of action is as follows: the cone is attached to a motor, which applies an oscillatory shear deformation with controlled frequency and amplitude, and shear deformation of the mAb solutions is measured by a torque transducer attached to the cone. Rheological parameters are presented as a function of the elastic (storage) modulus, G’ (ω), and the viscous (loss) modulus, G” (ω). The viscoelastic properties of mAb gelation were measured in two intervals: (1) 90 min at 0.5% strain (γ) and 1 rad/s of oscillatory frequency (ω), as supplied by the cone; (2) 8.5 min at 0.5% γ and variable ω ranging from 0.01 to 100 rad/s. Steady-state data were extrapolated from the elastic and viscous moduli generated from variations in oscillatory frequency and from applied strain.

**Raman spectroscopy.** Spectra were generated using a Horiba Jobin Yvon Raman spectrometer with LabSpec software. The excitation laser beam was 532 nm and the real time exposure of sample to laser (RTD) was 5 sec. A 50 x objective scanned each sample for 10 sec from 1800 cm⁻¹ to 600 cm⁻¹. Spectra of 100 g/L mAb solutions at pH 4 through 8 were taken before and after the addition of millimolar concentrations of citrate, at room temperature. Time-dependent gelation studies were conducted at 15 min intervals following citrate addition. Spectra were corrected by subtracting buffer background.

**Fourier-transform infrared spectroscopy** (FTIR). Spectra were generated using a Nicolet 6700 FT-IR spectrometer (OMNIC software) equipped with a zinc selenide attenuated total reflection (ATR) accessory and a KBr beamsplitter. Data were collected using 128 scans per sample with a 4 cm⁻¹ resolution. Spectra of 100 g/L mAb solutions, at pH 4 through 8, were taken before and after the addition of millimolar concentrations of citrate, at room temperature. Time-dependent gelation studies were conducted at 15 min intervals following citrate addition. Spectra were corrected for by buffer subtraction.

**Isothermal titration calorimetry (ITC).** Experiments were conducted in a VP-ITC MicroCalorimeter (MicroCal, Inc.), which was electrically calibrated prior to experiments. Samples were degassed using a ThermoVac accessory. Titrant-to-buffer experiments were performed prior to protein experiments to facilitate data calibration. The reference power was set to 10 μcal/s to optimize the baseline. Protein samples (60–70 μM) were loaded into the calorimeter cell (~1.8 mL) and titrations were performed using 40 injections of mM concentrations of citrate at 5 μL/injections with 2 min intervals with stirring at 307 rpm as suggested by the manufacturer. Titrations were performed under different experimental conditions for temperature-dependent (10°C, 25°C and 30°C) and pH-dependent (pH 4–7) studies. The instrument was cleaned with ~0.5 L 5% Decon and ~1.0 L Millipore water using a ThermoVac and the data were analyzed using the Microcal software.

Binding curves were fit using a nonlinear model of “one set of sites,” which assumes that the binding sites on the macro-molecule are homogenous and that each ligand binds a single substrate.33 For simplification, we assume that all binding sites are homogeneous and independent. The model fit the data well and no further higher order analysis was required. **Equation 1** (Microcal Software) shown below was to calculate the thermodynamic properties of the interaction.

\[
Q = \frac{N \Delta H V}{2} \left[1 + \frac{L_0}{N M_t} + \frac{1}{N K M_t} \left(1 + \frac{L_0}{N M_t} + \frac{1}{N K M_t} \right)^2 \frac{4 L_0}{N M_t} \right]^{1/2}
\]

In this equation, Kᵣ is the association constant, N is the number of binding sites, ΔH is the enthalpy of interaction, Mᵣ is the substrate concentration, Lᵢ is the ligand concentration, and V is the volume of the solution. The mAb has multiple positively charged amino acids that could potentially interact with negatively charged citrate ions.

**Transmission electron microscopy (TEM).** Micrographs were generated using a Phillips 410 transmission electron microscope with a magnification between 50,000x and 105,000x. Ten μl of assembled mAb solutions at pH 4 through 8 were placed on electron microscope grids, followed by crosslinking with 2% glutaraldehyde. The grids were then washed with water and stained with 2% uranyl acetate solution. After blotting with filter paper and drying, micrographs were taken at various magnifications.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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