

Review

Physical Stability of Proteins in Aqueous Solution: Mechanism and Driving Forces in Nonnative Protein Aggregation

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Irreversible protein aggregation is problematic in the biotechnology industry, where aggregation is encountered throughout the lifetime of a therapeutic protein, including during refolding, purification, sterilization, shipping, and storage processes. The purpose of the current review is to provide a fundamental understanding of the mechanisms by which proteins aggregate and by which varying solution conditions, such as temperature, pH, salt type, salt concentration, cosolutes, preservatives, and surfactants, affect this process.

KEY WORDS: formulation; pharmaceuticals; denaturation; second virial coefficient; conformational stability.

INTRODUCTION

The issue of protein stability was first explained on a fundamental level by Hsien Wu in 1931 (1), when he proposed a theory on protein denaturation after publishing 12 papers on his experimental observations on this topic (2). In 1954, Lumry and Eyring published a seminal paper (3) — “Conformation Changes of Proteins” — that laid the groundwork for what we know today about protein structure, folding, stability, and aggregation.

Protein stability is a particularly relevant issue today in the pharmaceutical field and will continue to gain more importance as the number of therapeutic protein products in development increases. Proteins provide numerous unique and critical treatments for human diseases and conditions (e.g., diabetes, cancer, hemophilia, myocardial infarction). There are already dozens of protein products on the market and hundreds more in preclinical and clinical development (4). However, if a therapeutic protein cannot be stabilized adequately, its benefits to human health will not be realized. The shelf life required for economic viability of a typical protein pharmaceutical product is 18–24 months (5). Achieving

this goal is particularly difficult because proteins are only marginally stable and are highly susceptible to degradation, both chemical and physical (6–9). Chemical degradation refers to modifications involving covalent bonds, such as deamidation, oxidation, and disulfide bond shuffling. Physical degradation includes protein unfolding, undesirable adsorption to surfaces, and aggregation (6,8–10). Nonnative aggregation is particularly problematic because it is encountered routinely during refolding, purification, sterilization, shipping, and storage processes. Aggregation can occur even under solution conditions where the protein native state is highly thermodynamically favored (e.g., neutral pH and 37°C) and in the absence of stresses. This review examines the mechanisms and driving forces in nonnative protein aggregation.

Nonnative protein aggregation (hereafter referred to simply as “aggregation”) describes the assembly from initially native, folded proteins of aggregates containing nonnative protein structures. Aggregation is often irreversible, and aggregates often contain high levels of nonnative, intermolecular β -sheet structures (11). Protein aggregation behaviors, such as onset, aggregation rate, and the final morphology of the aggregated state (i.e., amorphous precipitates or fibrils) have been found to depend strongly on the properties of a protein’s solution environment, such as temperature, pH, salt type, salt concentration, cosolutes, preservatives, and surfactants (10,12–16) as well as the relative intrinsic thermodynamic stability of the native state (17–20).

This review first examines how different solution conditions affect protein stability. Case studies and fundamental insights into how each solution condition affects protein stability are discussed. The second part of this review discusses characteristics, mechanisms, energetics, and driving forces of nonnative protein aggregation. Recent studies on two therapeutic proteins — recombinant human interferon- γ (rhIFN- γ) and recombinant human granulocyte colony-stimulating

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ABBREVIATIONS: rhIFN- γ , recombinant human interferon- γ ; rhGCSF, recombinant human granulocyte colony-stimulating factor; ΔG_{unf} , free energy of unfolding; B_{22} , osmotic second virial coefficient; GdnHCl, guanidine hydrochloride.

factor (rhGCSF) — will be used to provide insight into the mechanistic issues. The present review is not aimed at providing a comprehensive literature review in the area of protein stability and aggregation. Each protein is unique both chemically and physically and therefore will exhibit unique stability behavior. The purpose of the current review is to provide a fundamental understanding of the mechanisms by which proteins aggregate and by which varying solution conditions may affect this process. This insight in turn can be used in the rational design of stable aqueous formulations of therapeutic proteins.

FACTORS INFLUENCING PROTEIN STABILITY

Temperature

Most proteins fold to a specific globular conformation that is essential for their biologic functions. The thermodynamic stability of the native protein conformation is only marginal, about 5–20 kcal/mole in free energy more stable than unfolded, biologically inactive conformations under physiologic conditions (21–25). This thermodynamic stability is much weaker than covalent or ionic bonds (~150 kcal/mole) (26) or the thermal energy of a protein (5–20 kcal/mole is less than one tenth of $k_B T$ per residue, where k_B is Boltzmann's constant and T is the absolute temperature) (21). The small net conformational stability of protein results from a unique balance between large stabilizing and large destabilizing forces. Contributions to the free energy of folding arise from hydrophobic interactions, hydrogen bonding, van der Waals' forces, electrostatic forces (classic charge repulsion or ion pairing), and intrinsic propensities (local peptide interactions) (21). The main force opposing protein folding is the protein's conformational entropy. Both local entropy (e.g., translational, rotational, and vibrational degrees of freedom on the molecular scale) and nonlocal entropy (e.g., excluded volume and chain configurational freedom) are increased on unfolding (21). Because of the small conformational stability of the protein native state, relatively small changes of external variables (e.g., temperature, pH, salt, etc.) in the protein–solvent system can destabilize the structure of the protein, i.e., induce its unfolding.

The thermodynamic stability of the native protein conformation, characterized by the free energy of unfolding (ΔG_{unf}), typically shows a parabolic profile as a function of temperature (27–30). ΔG_{unf} therefore becomes negative at two temperatures, accounting for the unfolding of proteins at both high (e.g., 50–100°C) and low temperatures (e.g., less than 10°C) (21,27,29,30). The molecular origin of the effect of temperature on ΔG_{unf} is complex and is the subject of much ongoing research (21,22,29–31) and is not considered further here.

It has long been known that incubating protein solutions at high temperatures results in physical degradation. Although thermally induced denaturation may be reversible for some proteins, high temperatures usually lead to irreversible denaturation because of aggregation. Examples include the concomitant unfolding and aggregation of recombinant human Flt2 ligand (32), streptokinase (33), recombinant human keratinocyte growth factor (34,35), recombinant consensus interferon (36), rhIFN- γ (37), and ribonuclease A (38,39).

Typically, high temperatures perturb the native protein conformation to a sufficient degree to promote aggregation (13). Importantly, it is usually observed during heating that aggregation starts at temperatures well below the equilibrium melting temperature of the protein (11). This observation suggests that aggregates are not formed from fully unfolded molecules. Rather, as discussed in more detail below, it appears that partially unfolded protein molecules are the reactive species that form aggregates.

Temperature also strongly affects reaction kinetics because rate constants increase exponentially with temperature for activated reactions. Increasing temperature increases the thermal kinetic energy of reactants. As a result, reactant collision frequency, as well as the probability of collisions with enough energy to overcome activation energies, increases with increasing temperature (40). For diffusion-controlled reactions, increasing temperature increases the rate of diffusion of reactant species, thus increasing the rate of reaction. Protein aggregation rates are similarly increased at high temperatures.

Solution pH

pH has a strong influence on aggregation rate. Proteins are often stable against aggregation over narrow pH ranges and may aggregate rapidly in solutions with pH outside these ranges. Examples include recombinant factor VIII SQ (41), low-molecular-weight urokinase (42), relaxin (43), rhGCSF (17,20), deoxyhemoglobin (44), interleukin-1 β (45), ribonuclease A (46), and insulin (47).

Solution pH determines the type (positive or negative) and total charge on the protein, thereby affecting electrostatic interactions. There are two different ways in which electrostatic interactions can affect protein stability. First, classic electrostatic effects are the nonspecific repulsions that arise from charged groups on a protein when it is highly charged, for example, at pH far removed from the isoelectric point (pI) of the protein (21). As the number of charged groups on a protein is increased by increasing the acidity or basicity of the solution, increased charge repulsion within the protein destabilizes the folded protein conformation because the charge density on the folded protein is greater than on the unfolded protein. Thus, pH-induced unfolding leads to a state of lower electrostatic free energy (21). Second, specific charge interactions, such as salt bridges (or ion pairing), can also affect protein conformational stability. In contrast to the nonspecific electrostatic effect, where increasing charges destabilize the folded state, salt bridges stabilize it (21,48,49).

In addition to their effects on protein conformation, charges on protein molecules also give rise to electrostatic interactions between protein molecules. When proteins are highly charged, repulsive interactions between proteins stabilize protein solution colloiddally, making assembly processes such as aggregation energetically unfavorable (20,26,37,38). When proteins possess both positively and negatively charged groups (e.g., at pH values close to the pI), anisotropic charge distribution on the protein surface could give rise to dipoles. In such cases, protein–protein interactions could be highly attractive, making assembly processes such as aggregation energetically favorable (20,50).

Ligands and Cosolutes

The Wyman linkage function and related theories applied by Timasheff *et al.* [see, for example, Timasheff (51)] to protein conformational stability can be used to explain the effects of cosolutes, such as strong binding ligands, excipients, and salts, on protein physical stability. By the Wyman linkage function, differential binding of ligand in a two-state equilibrium will shift the equilibrium toward the state with the greater binding. Thus, for example, binding of polyanions to the native state of acidic fibroblast growth factor (52) or native recombinant keratinocyte growth factor (34) greatly shifts the equilibrium between the native and unfolded states to favor the native state. Likewise, binding of Zn^{2+} to human growth hormone increases the free energy of unfolding (53).

The Wyman linkage function can also be used to explain the effect of weakly interacting ligands (i.e., cosolutes) that affect protein conformational stability and equilibrium solubility at relatively high concentrations. It has been recognized for over a century that high concentrations (≥ 1 M) of certain solutes (e.g., sugars, polyols, and certain salts, such as ammonium sulfate) stabilize the native state of proteins, whereas other solutes act as protein denaturants [e.g., urea and guanidine hydrochloride (GdnHCl)] (51,54,55). These observations can be explained by differences in binding of these weakly interacting solutes to native and unfolded states. Denaturants bind to the unfolded state to a higher degree than to the native state, thus favoring unfolding.

Protein stabilizers such as sucrose and glycerol are preferentially excluded from the surface of a protein molecule, and the degree of exclusion is proportional to its solvent-exposed surface area (51,56,57). These cosolutes are depleted in the domain of the protein, and as a result, water is enriched in that domain. Preferential exclusion can thus be interpreted as negative binding. During unfolding, protein surface area increases, leading to a greater degree of preferential exclusion (e.g., larger negative binding). The net effect of greater negative binding to the unfolded state is to favor the native state.

Another way to state the mechanism by which preferential exclusion stabilizes the native state is to consider that this interaction coincides with an increase in protein chemical potential. By LeChatelier's principle, the system will tend to minimize this unfavorable effect. Thus, protein states with reduced surface area that exhibit lower preferential exclusion are favored over more solvent-exposed states. As a result, the free energy of unfolding is increased in the presence of preferentially excluded solutes. In addition, assembly of monomers into native oligomers, which reduces the specific protein surface area exposed to solvent, is also favored. The same mechanism also explains the decrease in equilibrium solubility of proteins in the presence of preferentially excluded solutes, such as ammonium sulfate (58).

Ligands and cosolutes that alter protein conformational stability also influence the rate of formation of nonnative aggregates. For example, in the presence of polyanions, aggregation of acidic fibroblast growth factor (52) and native recombinant keratinocyte growth factor (34) is greatly inhibited. It has also been shown that the addition of weakly interacting preferentially excluded solutes can reduce the rate of protein aggregation. For example, sucrose has been shown to inhibit aggregation of hemoglobin (59), rhIFN- γ (60,61), keratinocyte growth factor (62), immunoglobulin

light chains (15,19), and rhGCSF (17). In contrast, cosolutes (e.g., GdnHCl) that exhibit greater binding to the denatured state can accelerate aggregation (15,18,19,63). The mechanism for the effects of cosolutes on protein aggregation is discussed below.

Salt Type and Concentration

Electrolytes have complex effects on protein physical stability by modifying conformational stability, equilibrium solubility (e.g., salting-in and salting-out), and rate of formation of nonnative aggregates (38,64–67). For example, Yamasaki *et al.* found that bovine serum albumin could be stabilized against thermal unfolding by kosmotropic salts such as NaSCN and NaClO_4 and destabilized by chaotropic salts at high ionic strength (68). However, low concentrations of chaotropes (10–100 mM) stabilized bovine serum albumin (68). The equilibrium solubility of recombinant human tissue factor pathway inhibitor was decreased in the presence of NaCl (69). The rates of aggregation of recombinant factor VIII SQ (41) and recombinant keratinocyte growth factor (70) were decreased in the presence of NaCl. In contrast, NaCl increased the aggregation rate for rhGCSF (20).

Salts bind to proteins. Ions can interact with unpaired charged side chains on the protein surface. Binding of multivalent ions to these side chains can cross-link charged residues on the protein surface, leading to the stabilization of the protein native state (65). Because the peptide bond has a large dipole moment resulting from a partial positive charge on the amino group and partial negative charge on the carbonyl oxygen, ions can bind to peptide bonds (67), potentially destabilizing the native state. Consistent with the Wyman linkage theory described above, destabilization occurs if ions bind more strongly to nonnative than to native protein states (65).

Electrolytes modulate the strength of electrostatic interactions between the charged groups, both within the protein and between protein molecules. Thus, whereas intramolecular charge–charge interactions affect conformational stability, intermolecular electrostatic interactions affect equilibrium and rate of aggregate formation, as is described in more detail below.

At low concentrations, the predominant effect of ions in solution results from charge shielding, which reduces electrostatic interactions. However, at high concentrations of certain salts, in addition to charge-shielding effects, preferential binding of ions to the protein surface can result in a decrease in thermodynamic stability of the native conformation and an increase in equilibrium solubility (64). Other salts that are preferentially excluded from protein surface show stabilizing or salting-out effects (71).

The net effect of salt on protein stability is thus a balance of the multiple mechanisms by which salt interacts with protein molecules and by which salt affects protein–protein interactions. Because pH determines the type, total, and distribution of charges in a protein, salt-binding effects may be strongly pH dependent.

Preservatives

Antimicrobial preservatives, such as benzyl alcohol and phenol, are often needed in protein liquid formulations to ensure sterility during its shelf life. In particular, multidose

formulations of proteins require effective preservatives to prevent microbial growth after the first dose has been removed from a product vial. Preservatives are also required for certain drug delivery systems, e.g., injection pens that are used for multiple doses, minipumps that are used for continuous injection, and topical applications for wound healing. However, preservatives often induce aggregation of protein in aqueous solution. For example, preservatives (e.g., phenol, *m*-cresol, and benzyl alcohol) have been shown to induce aggregation of human growth hormone (16), recombinant interleukin-1 receptor (72), human insulin-like growth factor I (73), and rhIFN- γ (74).

The mechanism for preservative-induced protein aggregation is not well understood. However, it has been observed that addition of benzyl alcohol perturbed the tertiary structure of rhIFN- γ without affecting its secondary structure, and the rate of rhIFN- γ aggregation increased as the molar ratio of benzyl alcohol to protein increased (74). Also, preservatives reduced the apparent melting temperature of recombinant interleukin-1 receptor (72). These results suggest that preservatives bind to and populate unfolded protein states that are prone to aggregation. However, further research is needed to test this hypothesis and to determine rational strategies to inhibit preservative-induced protein aggregation.

Surfactants

Nonionic surfactants are often added to protein solutions to prevent aggregation and unwanted adsorption (e.g., to filter and container surfaces) during purification, filtration, transportation, freeze-drying, spray-drying, and storage. Surfactants are amphiphilic molecules that tend to orient so that the exposure of the hydrophobic portion to the aqueous solution is minimized. For example, surfactants adsorb at air/water interfaces, forming a surface layer of surfactant molecules oriented so that only their hydrophilic ends are exposed to water. Such orientation and surface adsorption can also occur at solid/water interfaces such as those found in vials, syringes, tubing, and other containers [for a review see Randolph *et al.* (10) and references therein]. Protein molecules are also surface active and adsorb at interfaces. Surface tension forces at interfaces perturb protein structure, often resulting in aggregation. Surfactants inhibit interface-induced aggregation by limiting the extent of protein adsorption (10,75).

As with other cosolutes, differential binding of surfactants to native and unfolded states of protein influences the protein's conformational stability. For some proteins, surfactants bind more strongly to the native state and increase the free energy of denaturation [e.g., human growth hormone (76)]. A more common effect is preferential binding of surfactants to the unfolded state, resulting in a decrease in the native protein state stability (10). Despite that surfactants often cause a reduction in thermodynamic stability of protein conformation, surfactants still can kinetically inhibit proteins aggregation at interfaces. In addition, surfactants have been shown to act as chemical chaperones, increasing rates of protein refolding and thus reducing aggregation (77). The readers are directed to the following reviews, and references therein, for further information: Randolph *et al.* (10), Jones *et al.* (75), and Jones (78).

MECHANISM OF PROTEIN AGGREGATION

Structural Transitions Accompanying Aggregation

Protein aggregation is accompanied by the loss of native protein structure. Such structural transitions have been well documented by Fourier transform infrared spectroscopy (FTIR) studies [for a review, see Dong *et al.* (11) and references therein]. A common feature of protein aggregates — formed in response to thermal, chemical, or physical stresses, or even in the absence of any applied stress — is an increased level of nonnative intermolecular β -sheet structures (11). This structural transition occurs regardless of the initial secondary structural composition of the native protein (11) or the final morphology (amorphous or fibrillar) of the aggregates (14,15,17–19).

Characterization of the Aggregation-Competent Species

Based mostly on studies of thermally induced precipitation, research on protein aggregation first led to the proposal that protein aggregates form from the fully unfolded state [reviewed by Dong *et al.* (11)]. Subsequent research has led to the hypothesis that partially unfolded states aggregate (14,79–86). These partially unfolded states (also called molten globules or acid-denatured “A” states) generally adopt a collapsed conformation that is more compact than the unfolded state and has substantial secondary structure and little tertiary structure (79). They have large patches of contiguous surface hydrophobicity and are much more prone to aggregation than both native and completely unfolded conformations (14).

Recently, several studies have found that even under physiologic solution conditions that are not perturbing of protein tertiary structure and that thermodynamically greatly favor the native state, proteins can form aggregates and precipitate (17–19,87). The protein native conformation is flexible and does not exist as a discrete, single structure (12,63,88,89). Rather, at any instant in time, there exists an ensemble of native substates with a distribution of structural expansion and compaction. Kendrick *et al.* showed that the aggregation of rhIFN- γ proceeds through a transiently expanded conformational species within the native state ensemble (61). Compared to the most compact native species, the expanded species has a 9% increase in surface area (18,87). This conformational expansion is only about 30% of that required for the complete unfolding of rhIFN- γ (87). Furthermore, Webb *et al.* showed that the surface area increase to form the structurally expanded species that precedes rhIFN- γ aggregation is independent of GdnHCl concentration, pressure, or temperature, suggesting a common intermediate for aggregation under these various stresses (87).

Krishnan *et al.* recently showed that under physiologic conditions (neutral pH, 37°C, with no added denaturants), where the native state is greatly favored thermodynamically, rhGCSF aggregated readily (17). The surface area increase needed to form the expanded conformation leading to aggregation was only approximately 15% of that for unfolding (17).

Aggregation Models, Energetics, and Rates

In order to transform protein molecules from natively folded monomers (or higher-order native assemblies, e.g., native dimers) to structurally perturbed, higher-order aggre-



Scheme 1. Lumry-Eyring framework of protein aggregation.

gates, protein molecules in the native state need to undergo both structural changes and assembly processes. The aggregation pathways of many proteins have been analyzed in the well-known Lumry-Eyring framework (3,18,90,91). A representation of this framework, shown in Scheme 1, involves a reversible conformational change of a protein (Scheme 1a) followed by irreversible assembly of the nonnative species to form aggregates (Scheme 1b) (3,90,91).

In Scheme 1, N is the native protein, TS^* represents the transition state preceding the formation of an aggregation intermediate A_I , and A_m and A_{m+I} are aggregates containing m and $m + I$ protein molecules, respectively.

It is generally known that the rate of a reaction is controlled by both thermodynamics and kinetics. The transition state theory used in the model depicted in Scheme 1 can be graphically represented on a reaction coordinate diagram as shown in Fig. 1. The free energies of reactant (N), transition state (TS^*), and products (A_n and A_m) are shown on an arbitrary free energy y -axis. The x -axis represents the course of individual reaction events. A_m is expected to be favored thermodynamically and therefore has the lowest free energy. Each reaction proceeds through energy barriers (curved lines in Fig. 1), which represent energies of the different molecular configurations between reactants and products. The maximum energy configuration is the transition state, and the free energy difference between the transition state and reactant is called activation free energy ($\Delta G^\ddagger$). For a multiple-step reaction, such as protein aggregation, the step that has the highest $\Delta G^\ddagger$ is the rate-limiting step.

Scheme 1 describes a reversible reaction to form a transition state, followed by irreversible reactions. The reaction order for the rate-limiting step determines the apparent order of the aggregation reaction. A number of proteins have been found to follow first order aggregation kinetics (3,18), suggesting that the rate-limiting step is unimolecular (e.g., a conformational change) rather than a bimolecular reaction limited by collision frequency.

In contrast, the aggregation of rhGCSF in pH 7 phos-



Scheme 2. rhGCSF aggregation mechanism with N^* as the transition state species (17).

phate buffer saline (PBS) follows a second-order reaction, suggesting that the rate-limiting step is bimolecular (17). Krishnan *et al.* proposed the mechanism in Scheme 2 for rhGCSF aggregation. Native rhGCSF (N) undergoes a bimolecular, second-order irreversible reaction ($2N \rightarrow A_2$) to form a dimeric aggregation-competent intermediate A_2 . This step proceeds through the formation of a transition state N^* , which is a transiently expanded conformational species within the native state ensemble (17). N^* then irreversibly dimerizes to form A_2 , and this step is rate limiting (17). A_2 then undergoes assembly reactions to form aggregates. N^* could also react irreversibly with an existing aggregate A_n to form a larger aggregate A_{n+1} . The reaction coordinate diagram for Scheme 2 is shown in Fig. 2. Also shown in Fig. 2 is the unfolded state (U), which is thermodynamically unstable with respect to the native state by 9.5 kcal/mole (17,20).

Role of Conformational Stability

It is apparent that the intrinsic conformational stability of the protein native state plays an important role in aggregation. First, aggregation is accompanied by the loss of native protein structures. Second, partially unfolded protein molecules are especially prone to aggregation. Third, the aggregation transition state of some proteins has been identified as a structurally expanded species within the protein native state ensemble (17,18). Hence, aggregation is governed by the conformational stability of the protein native state relative to that of the aggregation transition state.

Kendrick *et al.* showed that the addition of a thermodynamic stabilizer (e.g., sucrose) that increased ΔG_{unf} of

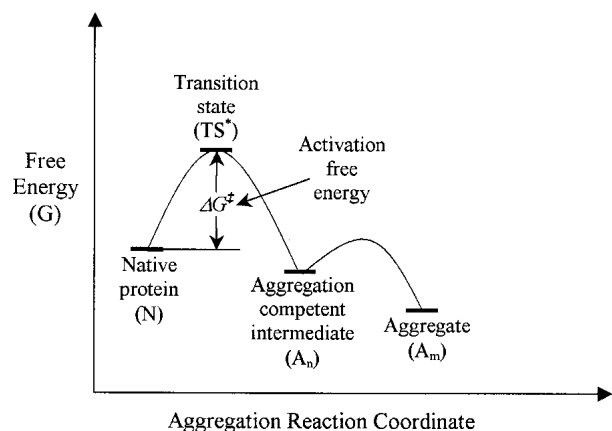


Fig. 1. Schematic reaction coordinate diagram of a protein aggregation (Scheme 1) on an arbitrary free energy y -axis. Curved lines illustrate kinetic energy barriers.

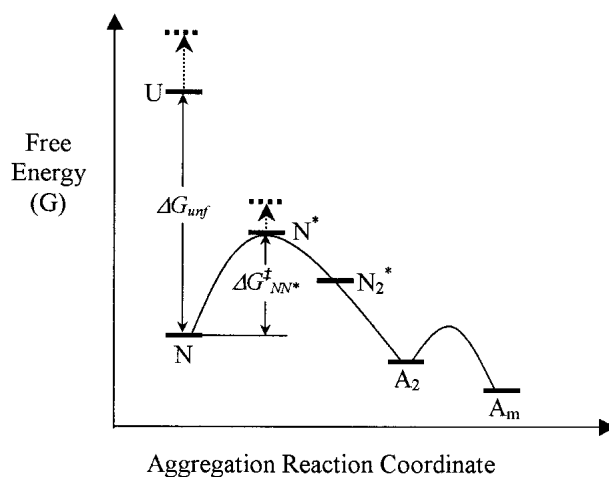


Fig. 2. Schematic reaction coordinate diagram of rhGCSF aggregation in pH 7 PBS. N^* is the transition state species, and $\Delta G_{NN}^\ddagger$ is the activation free energy of aggregation. A_2 is the dimeric aggregation intermediate. Dotted arrows illustrate, relative to protein native state (N), shifts in the free energies of unfolded state (U) and N^* when sucrose is added (20).

rhIFN- γ decreased its aggregation rate (Fig. 3a). The same trend was observed for the aggregation of rhGCSF (Fig. 3b) (17). As discussed earlier, stabilizers such as sucrose increase protein thermodynamic stability because they are preferentially excluded from the surface of protein molecules (92). By the Wyman linkage theory, the addition of sucrose thus drives reactions toward the least solvent-exposed states. The aggregation-prone partially folded states and structurally expanded transition states both have greater surface area than the most compact native state and are therefore expected to be disfavored energetically by the addition of sucrose. Sucrose thus shifts the molecular population toward the most compact species within the native state ensemble. This is verified experimentally by the observation that sucrose greatly reduces the rate of hydrogen-deuterium (H-D) exchange within proteins (17–19,63). A reduction in H-D exchange indicates that the time-averaged conformation of the protein in the presence of sucrose was more compact than in the absence of this stabilizing solute (17–19,63).

The effects of sucrose on ΔG_{unf} and $\Delta G^\ddagger$ (which controls the rate of aggregation) are illustrated by dashed arrows in Figs. 2 and 4. The addition of sucrose increases the free energies, relative to N, of U and N* (upward dashed arrows in Figs. 2 and 4). Increasing the free energy of N* relative to N results in a larger $\Delta G^\ddagger$, thus shifting the equilibrium between

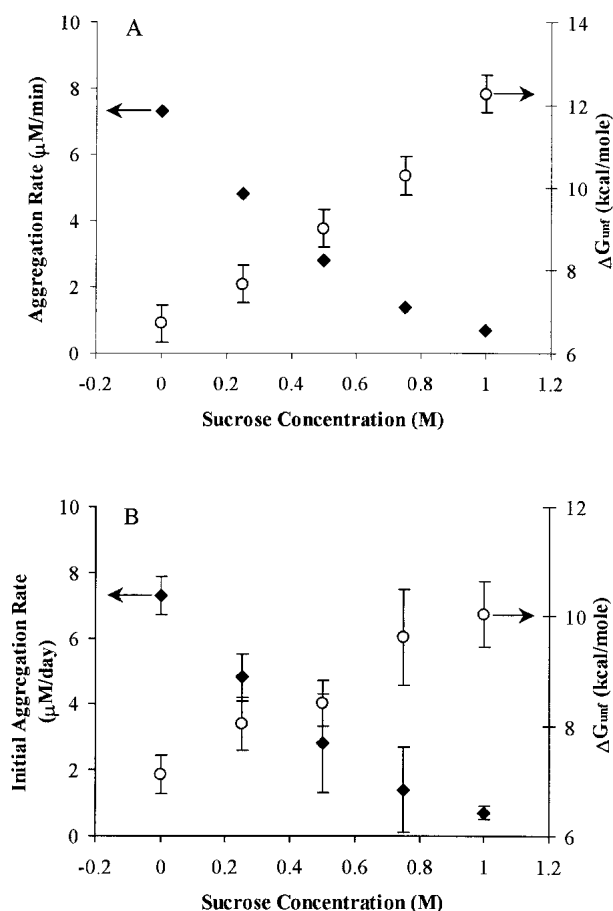


Fig. 3. The effect of conformational stability on the aggregation rates of (A) rhIFN- γ (18,87) and (B) rhGCSF (17). Increasing the free energy of unfolding (ΔG_{unf}) by the addition of sucrose decreased protein aggregation rates.

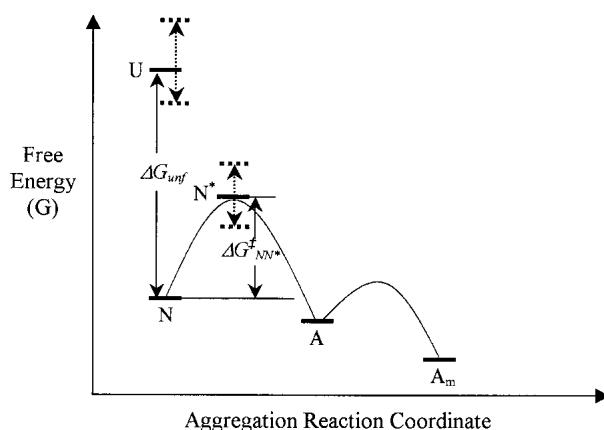


Fig. 4. Schematic reaction energy profile of rhIFN- γ aggregation. A is the monomer aggregation intermediate. Dotted upward and downward arrows illustrate, relative to the protein native state (N), shifts in the free energy of the aggregation transition state (N*) when sucrose or GdnHCl is added, respectively.

N and N* toward N. As a result, the rates of rhIFN- γ and rhGCSF aggregation decrease with the addition of sucrose.

The opposite effect, also illustrated in Fig. 4, of increasing aggregation rate by the addition of chaotropes (e.g., urea and GdnHCl) that destabilize the conformational stability of the native protein, has also been observed (18,19,61). Kim *et al.* found that urea, which decreased the ΔG_{unf} of immunoglobulin light chains (and presumably reduced $\Delta G^\ddagger$), enhanced their fibril formation by both reducing nucleation lag time and increasing fibril growth rate (15). Furthermore, the destabilizing effect of urea could be counteracted by the addition of preferentially excluded cosolutes sorbitol and betaine (15).

Cosolutes that increase the stability of the native conformation have been demonstrated to be effective at reducing protein aggregation. Is conformational stability the only factor controlling the rate of protein aggregation? When denatured by high temperature, ribonuclease A aggregated readily at pH 7.8 at temperatures both above and below the apparent melting temperature, where the protein was mostly unfolded and folded, respectively (38,39). In contrast, ribonuclease A did not aggregate at pH 3 even when exposed to 75°C for 24 h (38,39). The same effect of pH was observed during thermal scanning of rhGCSF. The protein aggregated rapidly at pH 7 and 6.1, but no aggregation was observed at pH 3.5 (20). We discussed earlier that even minor perturbations to the native protein structure can lead to rapid aggregation. However, even when the native conformation of ribonuclease A and rhGCSF became significantly perturbed at high temperatures, these proteins did not aggregate at acidic pH. Thus, protein conformation alone cannot explain the aggregation behaviors of ribonuclease A and rhGCSF observed at different pH during thermally induced denaturation.

Furthermore, as shown in Fig. 3b, ΔG_{unf} values of rhGCSF in pH 7 PBS correlated well with its initial aggregation rate during incubation at 37°C. However, at different solution pHs, very different aggregation behaviors were observed even when the ΔG_{unf} values were comparable (20). ΔG_{unf} values measured for rhGCSF in several different solutions, pH 3.5 HCl, pH 6.1 PBS, pH 7 PBS, were 11.3 ± 0.7 , 11.4 ± 0.6 , and 9.5 ± 0.5 kcal/mole, respectively (20). Aggre-

gation occurred in the pH 6.1 and 7 solutions but did not occur in the pH 3.5 solution (Fig. 5) (20). In addition, aggregation was observed in solution at pH 3.5 in the presence of 150 mM NaCl, although the change in ΔG_{unf} caused by addition of NaCl was statistically insignificant (20). Thus, rhGCSF aggregation behavior in different solutions cannot be explained by its conformational stability alone.

Role of Colloidal Stability

In addition to the structural changes that occur during aggregation, protein molecules also assemble to form higher-order aggregates. Molecular assembly processes occur as a result of attractive intermolecular interactions. Thus, an understanding of protein aggregation also requires information about the nature and magnitude of these interactions. The osmotic second virial coefficient (B_{22}) is a thermodynamic solution parameter that directly quantifies overall protein–protein interactions on the molecular level, which include hard-sphere, electrostatic, van der Waals', and all other short-range interactions. Positive B_{22} values indicate the overall dominance of repulsive forces between protein molecules, where protein–solvent interactions are favored over protein–protein interactions (93) (i.e., proteins are colloidally stable). Negative B_{22} values reflect overall attractive forces between proteins, with protein–protein interactions being favored over protein–solvent interactions (i.e., proteins are colloidally unstable). B_{22} is fundamentally linked to protein phase behavior (94–96) and solubility (97–100), and B_{22} measurements have been used to characterize and predict solution conditions for protein assembly into crystals and for salting out (93,95,98,99,101,102).

The onset of native protein crystallization or precipitation and the morphology of the solid phases formed are predominantly determined by the mechanisms of molecular approach, reorientation, and incorporation of native proteins, which are governed by the strength and range of protein colloidal interactions (103). Assembly of protein molecules into

nonnative aggregates by definition involves the formation of higher-molecular-weight assemblies from initial lower-molecular-weight species. Thus, the same intermolecular interactions that govern protein crystallization and salting out are also expected to be important in the formation of nonnative protein aggregates.

Two major contributions to interactions between protein molecules in aqueous solutions are Coulombic electrostatic interactions and van der Waals' interactions (Fig. 6A). Electrostatic interactions between isocharged surfaces are always repulsive and are described by double-layer interactions whose range and strength are modulated by electrolyte concentration (26). As illustrated in Fig. 6A, when two isocharged particles such as protein molecules approach each other (e.g., starting from a particle surface separation distance marked as point *a* in Fig. 6A), they need to overcome an energy barrier, ΔW_I (located at a separation distance marked as position *b*) to come into physical contact. At distances less than *b*, molecules experience attractive forces, resulting in coagulation. When ΔW_I is high, particles remain kinetically stable as dispersed particles (Fig. 6B, case *i*). When ΔW_I is small (Fig. 6B, case *ii*) or negative (Fig. 6B, case *iii*), particles become colloidally unstable, and coagulation occurs. Energetics of particle assembly processes are thus controlled by the interaction energy (or ΔW_I) between them.

Assembly processes in protein aggregation pathways discussed so far include Scheme 1b and Schemes 2b and 2c. These are the reactions that transform lower-molecular-weight proteins into higher-order aggregates. A more subtle assembly reaction is the formation of the dimeric intermediate A_2 (Scheme 2a) from N in rhGCSF aggregation. Although in pH 7 PBS, N^* is the transition state for the reaction $2N \rightarrow A_2$ (Fig. 2), the reaction also must proceed through a dimeric molecular configuration, N_2^* , to form A_2 (Fig. 2). At pH 3.5, where rhGCSF is highly charged, electrostatic repulsion is stronger than at pH 7, near the isoelectric point. This causes the energy barrier for collisions between protein molecules (ΔW_I) to increase. If ΔW_I is sufficiently high, assembly reac-

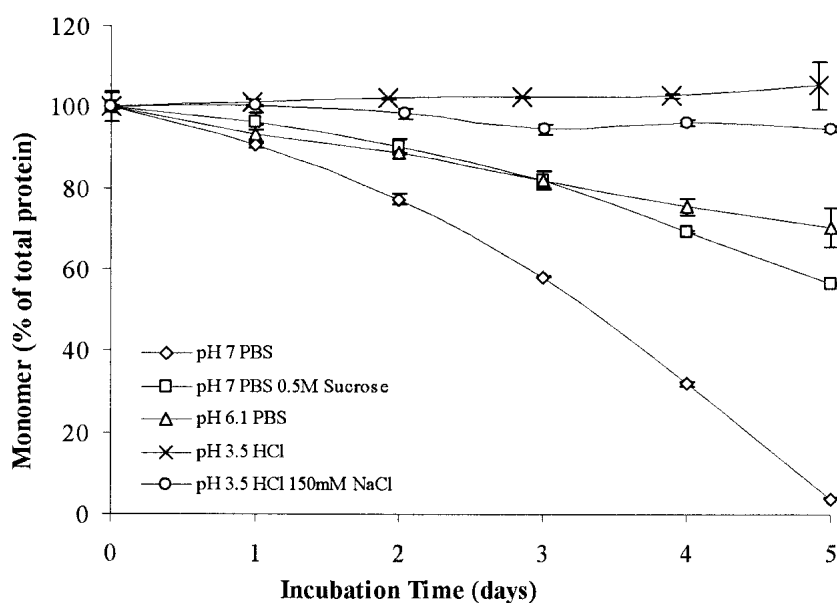


Fig. 5. Aggregation profiles of rhGCSF in different solution conditions during 5 days of incubation at 37°C (17,20).

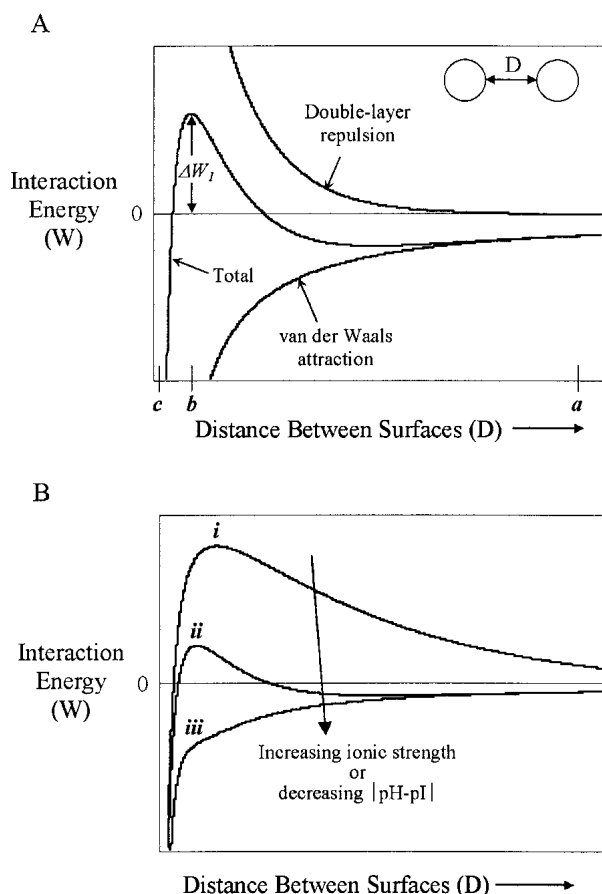


Fig. 6. Schematic interaction energy of two spherical particles interacting at constant and uniform surface potential. A, Total interaction energy is the sum of electric double-layer repulsion and van der Waals' attraction (26). ΔW_I represents the maximum interaction energy barrier. B, Increasing salt concentration screens double-layer repulsion, resulting in a decrease of ΔW_I . Decreases in ΔW_I could also result from a decrease in the absolute value of the difference between solution pH and the isoelectric point ($|\text{pH} - \text{pI}|$) of a protein.

tion becomes the rate-limiting step, and N_2^* is the transition state (20) (Fig. 7).

B_{22} values reflect similar information. As shown in Fig. 8, B_{22} values of rhGCSF in pH 7 and 6.1 PBS are negative, indicating that protein-protein interactions are attractive (Fig. 6B, case iii) (20). As pH is lowered to 3.5, B_{22} value became large and positive, indicating that the overall interactions between rhGCSF molecules changed from attractive to highly repulsive (Fig. 6B, case i) such that no aggregation occurred (20). Solution pH thus changed rhGCSF colloidal stability, from being colloidal unstable at pH 7 and 6.1 to colloidal stable at pH 3.5. This dominant role of colloidal interaction also explains the observation that even when the native state became significantly unfolded during thermal unfolding at pH 3.5, aggregation still did not occur (20).

At pH 3.5, rhGCSF aggregation could be induced by the addition of 150 mM NaCl (Fig. 5) whereas ΔG_{unf} remained relatively unchanged by the addition of NaCl (20). The effect of ionic strength on B_{22} values measured at pH 3.5 is shown in Fig. 9. Salt screens repulsive electrostatic interactions, reducing ΔW_I (or $\Delta G_{NN_2^*}^\ddagger$) sufficiently so that aggregation occurred (Fig. 6B and Fig. 8, case iii). Under these conditions,

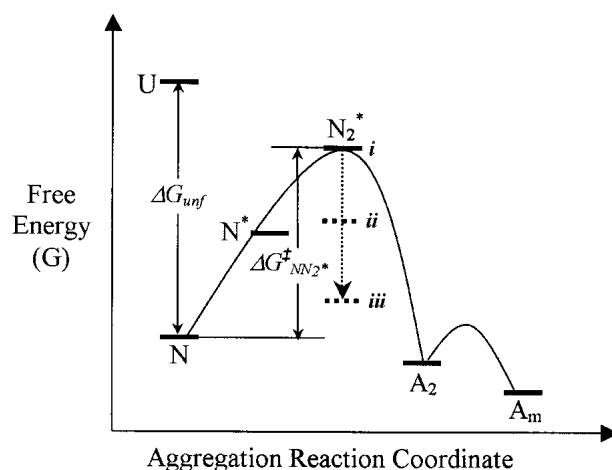


Fig. 7. Schematic reaction energy profile of rhGCSF in pH 3.5 HCl. N_2^* is the transition state species, and $\Delta G_{NN_2^*}^\ddagger$ is the activation free energy of aggregation. Dotted arrows indicate that increases in solution ionic strength (or decrease in $|\text{pH} - \text{pI}|$) decrease $\Delta G_{NN_2^*}^\ddagger$. At low ionic strength (i), ΔW_I is large and positive, resulting in a high $\Delta G_{NN_2^*}^\ddagger$. Increasing ionic strength sufficiently led to a negative ΔW_I (iii), lowering $\Delta G_{NN_2^*}^\ddagger$ enough that N_2^* is no longer the transition state of the aggregation reactions. At high ionic strength, N^* is expected to be the transition state of aggregation (20).

assembly reaction is no longer rate limiting; the transition state is N^* rather than N_2^* . Thus, at pH 3.5 and high ionic strength, conformational stability of the native state again becomes the dominant factor governing rate of rhGCSF aggregation (20).

Nucleation-Dependent Aggregation

Thus far, we have discussed in detail the mechanism and driving forces that are important during the initial stages of aggregation. Following the onset of aggregation, both the number and size of aggregates increase while the native protein population is depleted. Aggregates grow and eventually precipitate out of solution and form visible particles. Aggregation can thus often be detected visually. However, the aggregation of some proteins exhibits a distinct lag phase during which the protein solution remains clear and the loss of native

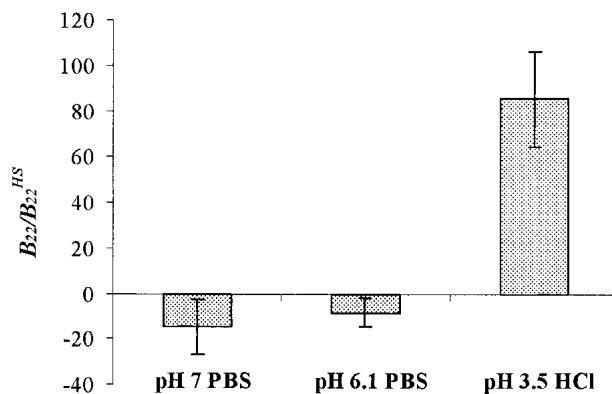


Fig. 8. Osmotic second virial coefficient, normalized to hard sphere contribution, of rhGCSF in different pH solution conditions. Protein-protein interactions changed from strongly repulsive at pH 3.5 to increasingly attractive at pH 6.1 and 7. Error bars are standard errors from the linear regression of light-scattering data (20).

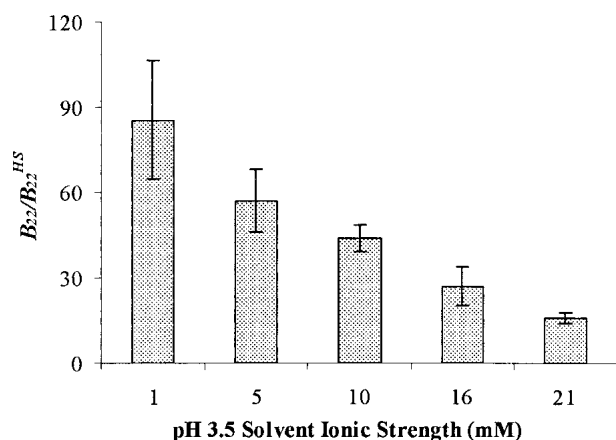


Fig. 9. Osmotic second virial coefficient, normalized to hard sphere contribution, of rhGCSF in pH 3.5 solutions with different ionic strength. Protein-protein charge repulsion becomes increasingly shielded as the ionic strength of the solution is increased. Error bars are standard errors from the linear regression of light-scattering data (20).

protein often is nearly undetectable, followed by rapid, often complete, native protein loss. For example, when α -synuclein was incubated at 37°C, insoluble aggregates were not detected during the first 15 days (17). This lag phase was followed by a rapid loss of monomeric α -synuclein, where after 30 days, essentially all native α -synuclein were lost to aggregation (104). This type of nucleation-dependent aggregation behavior has been observed for a number of other proteins, such as β -lactoglobulin (105), immunoglobulin light chains (15), prion protein (PrP96-111M) (106), and the SH3 domain of the α -subunit of bovine phosphatidylinositol-3'-kinase (107). Furthermore, seeding (e.g., with preformed aggregates) has been found to dramatically reduce lag time and promote aggregation (105,106,108).

Protein aggregation is inherently a nucleation and growth phenomenon where aggregates accumulate, eventually exceeding their solubility and precipitate. The existence of a lag phase in the aggregation of some proteins is caused an energy barrier to assembly (or nucleation). The energy barrier results from the free energy required to create a new solid-liquid interface and depends on aggregate size (109,110). When the size of the new phase is above a critical size where energy barrier is highest, then growth of the nucleus occurs. Nucleation-dependent aggregation behavior is a result of a rate-limiting nucleation step. Furthermore, the barrier to assembly may be orientationally specific. If there is an orientation with a lower free energy to assemble, then growth will occur preferentially in that orientation, resulting in ordered aggregate morphology. For example, fibrils are often associated with nucleation-dependent assembly (105,106,108,111).

For some protein systems undergoing nucleation-dependent assembly, the rate-limiting step is the formation of a prenucleus species (15,104). For example, with α -synuclein the prenucleus was a ditryrosine crosslinked dimer that formed due to protein oxidation (104). When the dimer concentration reached a critical level of about 1–2% of the total protein population, nucleation occurred rapidly. Seeding a sample that contained no dimers with preformed dimers rapidly induce fibril formation, whereas inhibition of dimer for-

mation with methionine (which serves as a radical scavenger) prevented fibril formation.

Similar pathways for therapeutic proteins probably are important for their physical degradation, although there are no published examples in the literature. In these cases, soluble aggregates (which can be noncovalently or covalently crosslinked) can form, for example, during long-term storage in an aqueous formulation, without any assembly into higher order aggregates or precipitates. However, if the soluble aggregate is a prenucleus species and it reaches a critical level (e.g., 1%), it can foster rapid assembly into large aggregates. Thus, a product that appears to have acceptable physical stability for several months, could in the space of a few weeks (or even days) suddenly have unacceptable levels of aggregated protein. Similarly, if the bulk drug product already has soluble aggregates present, the long-term stability of the final formulated product may be compromised.

Thus, it is important for pharmaceutical scientists to pay close attention to the levels of soluble aggregates present in both bulk drug product, as well as the final formulation. For a given therapeutic protein product, it may become essential to have processing steps to reduce the levels of soluble aggregates, in order to obtain acceptable long-term stability in the final formulation.

CONCLUSIONS

Protein aggregation is problematic both *in vivo* and *in vitro*. Because of the low thermodynamic stability of the native protein structure, aggregation often occurs, even under solution conditions where the native protein is both conformationally stable and at concentrations well below its equilibrium solubility. Non-native aggregation of a protein involves at least two steps – conformational changes to the protein native state and assembly of protein molecules into higher order aggregates, and their energetics are controlled by conformational stability, expressed as ΔG_{unf} , and colloidal stability, reflected in the values of B_{22} , respectively. Under solution conditions where conformational stability dominates (i.e., large ΔG_{unf} and negative B_{22}), the first step is rate-limiting. Increasing ΔG_{unf} (e.g., by the addition of sucrose) is effective at decreasing aggregation. In solutions where colloidal stability is high (i.e., large and positive B_{22} values), assembly step is rate-limiting. Solution conditions (e.g., pH and ionic strength) that increases B_{22} are effective at reducing aggregation.

Protein aggregation is therefore controlled by both conformational stability and colloidal stability, and, depending on the solution conditions, either could be rate limiting. To successfully stabilize protein against aggregation, solution conditions need to be chosen not only to stabilize the protein native conformation but also to stabilize protein against attractive intermolecular forces. During development of formulations for therapeutic proteins, the latter goal is often achieved empirically during preformulation studies, where ionic strength, pH, and buffer type are optimized to minimize precipitation and other adverse events (e.g., deamidation). Manipulation of solution conditions during preformulation studies so as to maximize B_{22} may be useful in development of formulations exhibiting long-term storage stability.

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Aggregation of a multidomain protein: A coagulation mechanism governs aggregation of a model IgG1 antibody under weak thermal stress

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Abstract: Using an IgG1 antibody as a model system, we have studied the mechanisms by which multidomain proteins aggregate at physiological pH when incubated at temperatures just below their lowest thermal transition. In this temperature interval, only minor changes to the protein conformation are observed. Light scattering consistently showed two coupled phases: an initial fast phase followed by several hours of exponential growth of the scattered intensity. This is the exact opposite of the lag-time behavior typically observed in protein fibrillation. Dynamic light scattering showed the rapid formation of an aggregate species with a hydrodynamic radius of about 25 nm, which then increased in size throughout the experiment. Theoretical analysis of our light scattering data showed that the aggregate number density goes through a maximum in time providing compelling evidence for a coagulation mechanism in which aggregates fuse together. Both the analysis as well as size-exclusion chromatography of incubated samples showed the actual increase in aggregate mass to be linear and reach saturation long before all molecules had been converted to aggregates. The CH2 domain is the only domain partly unfolded in the temperature interval studied, suggesting a pivotal role of this least stable domain in the aggregation process. Our results show that for multidomain proteins at temperatures below their thermal denaturation, transient unfolding of a single domain can prime the molecule for aggregation, and that the formation of large aggregates is driven by coagulation.

Keywords: Multidomain; protein aggregation; dynamic light scattering; coagulation; Rituximab; monoclonal IgG1 antibody; Tween 80

Abbreviations: CD, circular dichroism; CH2, heavy chain domain 2; DLS, dynamic light scattering; DSC, differential scanning calorimetry; Fab, antigen-binding fragment; Fc, constant fragment; IgG, immunoglobulin gamma; mAb, monoclonal antibody; SEC, size-exclusion chromatography.

Additional Supporting Information may be found in the online version of this article.

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Introduction

Most proteins fold to a specific three-dimensional structure essential for their biological function. The net thermodynamic stability stemming from various stabilizing and destabilizing intramolecular forces is low and may be corrupted by relatively small changes in external variables (pH, temperature, salt, etc.).^{1,2} When the protein structure is destabilized, parts of the interior is exposed and this may lead to irreversible formation of aggregates.^{3–5} The mechanisms leading to aggregates are as diverse as the nature of the aggregates themselves—ranging from oligomers and micelles to amorphous

aggregates and fibrils^{6–9}—and remain a major challenge in protein science.^{10,11}

Due to minute transient perturbations of the molecular structure, heating may induce aggregation even well below the melting temperature of the protein.^{1,12} Furthermore, higher temperature accelerates aggregation because of its impact on the individual rate constants involved in the aggregation kinetics. This is the reason why high temperature is often used to probe the stability of a given protein. Model proteins for folding/misfolding and aggregation are often single-domain proteins (BSA, β -lactoglobulin, lysozyme, insulin, etc.), which exhibit two-state unfolding, with far fewer studies of more complex multidomain proteins despite their acknowledged importance in pharmaceutical sciences.¹³ Low aggregate content is especially crucial in pharmaceutical formulations as aggregates here may elicit an inflammatory immune response to neutralize the aggregate.¹⁴ This would not only diminish the effect of the drug itself but could also lead to more serious adverse effects related to cross-reactivity with autologous proteins.^{15,16}

In this study, we used the monoclonal IgG1 antibody Rituximab¹⁷ as a model system to better understand the mechanisms leading to the formation of large aggregates when incubating a multidomain protein under weak thermal stress. Aggregation of antibodies due to various kinds of stress have been addressed in a number of studies,^{18–24} with a few key observations highlighted later. In this context, we distinguish between the formation of oligomers (dimers, trimers, etc.) and larger aggregates: by size-exclusion chromatography (SEC), oligomers can be separated and characterized individually, while aggregates comprise a broad range of sizes that elute in the void.²⁵ Jøssang and coworkers^{18,19,23} used light scattering under conditions similar to those applied in this work to study aggregate formation of antibodies purified from blood. They found that the antibodies generate large fractal aggregates with no discernable amount of oligomers and that the aggregates were not stabilized by disulfide bonds. In contrast, in a more recent study by Remmele *et al.*,²² the IgG1 formulation Epratuzumab was found to contain dimers, 70% of which were covalently linked. The appearance of large aggregates, if any, was not reported. Finally, Van Buren *et al.*²⁴ incubated an IgG2 monoclonal antibody (mAb) for 15 weeks at 37°C in two different pH regimes, and found the aggregation pathways to be pH dependent: at high pH, dimers had ~75% covalent character, while the formation of large aggregates was largely suppressed; at low pH, on the other hand, both dimers and large aggregates were formed. To generalize these findings, incubation at elevated temperatures may lead to the formation of large aggregates, dimers, or both. The dimers appear to be linked by disulfide bridges, while this appears

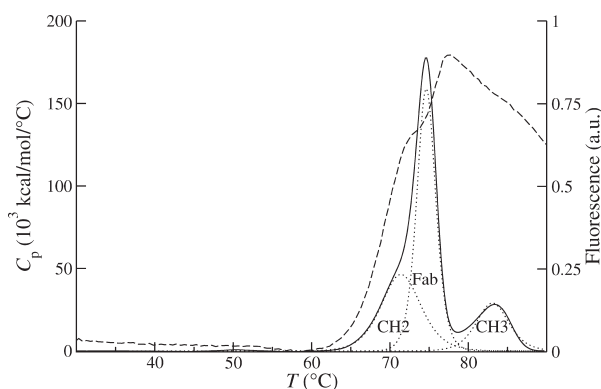


Figure 1. Rituximab unfolds in three steps reflecting the stability of individual domains. The heat-induced unfolding was monitored by Sypro orange fluorescence (dashed line) and DSC (solid line). The scan rate in both experiments was 1°C/min. Each peak in the DSC profile (dotted lines) has been assigned to a specific domain or fragment as devised previously.^{29,30} The activation energy of the first thermal transition corresponding to the CH2 domain was found to be 98.4 kcal/mol (see main text for details).

not to be the case for the aggregates. For certain globular proteins, such as β -lactoglobulin, the oligomers have been shown to be on-pathway to larger aggregates,²⁶ but such a simple link between oligomers and aggregates has yet to be demonstrated for antibodies.

By light scattering, we show that Rituximab aggregation kinetics have a distinct two-phase appearance starting with a rapid doubling of the scattered intensity followed by hours of exponential increase. This observation is in stark contrast to the lag-time behavior, typically ascribed to heterogeneous nucleation,^{27,28} commonly observed in protein fibrillation. Light-scattering analysis demonstrates that the main mechanism for aggregate growth is the continuous coagulation of smaller aggregates into larger ones. The initial rapid generation of aggregates is less clearly resolved. The comparison of light-scattering data with calorimetry data shows that the activation energy of the aggregation process is linked to the stability of the thermally least stable antibody domain.

Results

Thermal denaturation

Figure 1 shows the heat capacity of the protein solution as a function of temperature. The thermal denaturation by differential scanning calorimetry (DSC) resulted in three partially overlapping transitions with T_m values of ~71, 74, and 83°C, respectively. The results are in good agreement with previous results from an IgG1 mAb, in which case the three transitions were assigned to the unfolding of the CH2 domain, the antigen-binding fragment (Fab),

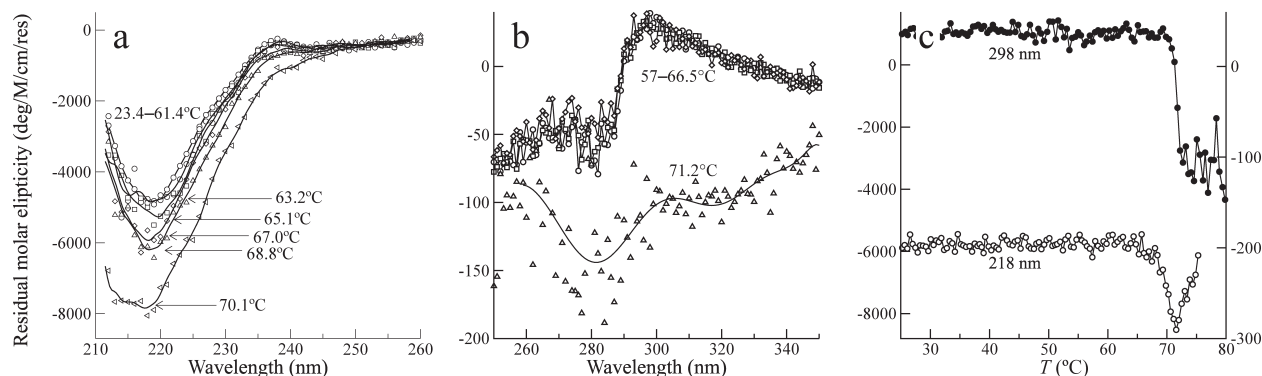


Figure 2. Under the weak thermal stress applied in this study, secondary and tertiary structure elements are essentially conserved. The thermal unfolding was monitored by far and near-UV CD. (a) Far-UV CD temperature scan. The spectra from 23.4 to 61.4°C were similar and have been averaged for clarity. At temperatures above 63°C, the negative signal with a minimum at 218 nm becomes more intense. (b) Near-UV CD spectra with a maximum at 298 nm showing the abrupt loss of tertiary structure. The solid line is a guide to the eye. (c) Thermal scans at 218 and 298 nm demonstrate that the antibody is stable up to ~68°C, at which point the protein denatures.

and the CH3 domain, respectively.^{29–31} CH2 and CH3 comprise the constant fragment (Fc) of the antibody. The denaturation of Rituximab was always irreversible, and the T_m values were also scan rate dependent (results not shown), which precluded full thermodynamic analysis.³² This is a common property of large, complex proteins such as mAbs and has been described earlier.^{30,33} However, the use of the denaturation enthalpy is still permissible according to the first law of thermodynamics, even if the process is irreversible.³⁴ In addition, no distortion, which is sometimes observed when the samples aggregate irreversibly, was seen on the shape of the heat capacity profiles for the Rituximab samples. A calorimetric enthalpy of 320 kcal/mol and a Van't Hoff enthalpy of 144 kcal/mol were obtained for the first thermal transition. From the irreversible two-state model of Sánchez-Ruiz,^{34,35} it is also possible to calculate the activation energy of unfolding given that only the native state (N) and the final irreversible state (F) are significantly populated:

$$N \xrightarrow{k_{ap}} F, \quad (1)$$

where k_{ap} is the first-order rate constant for the irreversible denaturation process. Then the activation energy can be calculated using the following equation:

$$E_a = \frac{eRT_m^2 \Delta C_p^m}{\Delta_N^F H}, \quad (2)$$

where e is the base of the natural logarithm, R is the gas constant, ΔC_p^m is the excess heat capacity measured at T_m , and $\Delta_N^F H$ is the total heat of the process. Using this equation, an activation energy of 98.4 kcal/mol was obtained for the first thermal

transition corresponding to the denaturation of the CH2 domain.

Figure 1 also shows the intensity of the fluorescent probe Sypro orange as a function of temperature. The intensity is essentially unchanged up to 62°C at which point it starts to increase. Two characteristic melting temperatures are observed at 69 and 75°C in accordance with the melting temperatures obtained by DSC at the same temperature rate.

Finally, thermal stability was probed by far- and near-UV CD, which probes secondary and tertiary structure, respectively. Figure 2(a) shows far-UV spectra in the wavelength range from 205 to 260 nm recorded while ramping the sample temperature. The spectra are characterized by a single negative peak with a minimum at 218 nm, which is characteristic of proteins—such as antibodies—rich in β -structure. Below 61.4°C, no change in spectra characteristics was noticed, and they have been averaged for clarity. Above this temperature, the negative signal of the spectra increases and becomes broader. This behavior suggests an amplification on the level of secondary structure before unfolding and has been observed for antibodies previously.^{33,36} Above 70°C, the spectra decreased in intensity as the secondary structure was gradually lost (spectra omitted for clarity). Figure 2(b) shows near-UV spectra in the wavelength range from 250 to 350 nm recorded while ramping the sample temperature. The spectra recorded below 66.5°C all have a maximum at 298 nm. Above that temperature, the characteristic features of the spectra disappear due to the loss of tertiary structure.³³ Figure 2(c) shows the CD signal at 218 and 298 nm as a function of sample temperature from two separate experiments similar to the ones detailed earlier. The figure confirms that the

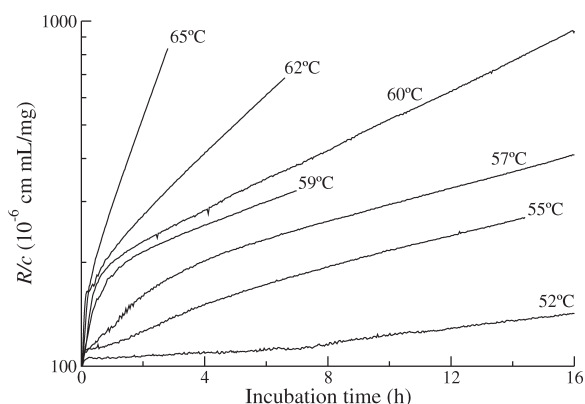


Figure 3. Aggregation kinetics are characterized by two phases: an initial fast phase followed by an exponential phase, and both are temperature dependent. Static light scattering of 1 mg/mL Rituximab samples as a function of incubation time. The kinetics were studied at temperatures ranging from 52 to 65 °C as indicated in the figure.

antibody is remarkably stable in the incubation temperature interval (52–65 °C) applied in this study.

Concentration and temperature dependence of the aggregation kinetics

Figure 3 shows the scattered intensity from 1 mg/mL Rituximab samples incubated at 52–65 °C as a function of incubation time. We observe that the scattered intensity is dominated by two phases: an initial fast increase is followed by several hours of exponential growth. Phenomenologically, we define the initial phase as starting at time zero and lasting until exponential growth takes over, but we also note that the two processes are most likely overlapping. The temperature is seen to affect both the initial phase—which is shortened from about 8 h at 52 °C to being practically instantaneous at 65 °C—as well as the subsequent exponential phase. The two phases of each curve were fit separately with a simple model to extract the rate constants: $I(t) = a(k_{\text{ini}}t / (1 + k_{\text{ini}}t))^2 \exp(k_{\text{exp}}t) + b$, where a is an amplitude, b is a baseline, and k_{ini} and k_{exp} are rate constants of the initial and the exponential phase, respectively. In this phenomenological model, the first phase is fitted by a power law and the second phase by an exponential. Figure 4 shows the natural logarithm of each of these rate constants plotted against the reciprocal temperature in an Arrhenius plot. Both data sets fit a straight line with activation energies of 117 and 71 kcal/mol for the initial and exponential phase, respectively.

Figure 5 shows the scattered intensity of 0.2–10 mg/mL Rituximab samples as a function of incubation time at 60 °C. The concentration dependence of the rate constants of the first phase is approximately linear, $k_{\text{ini}} \propto c$, while the rate constants of the exponential phase have a weak concentration dependence: $k_{\text{exp}} \propto c^{0.2}$ (inset of Fig. 5). The autocorrelation

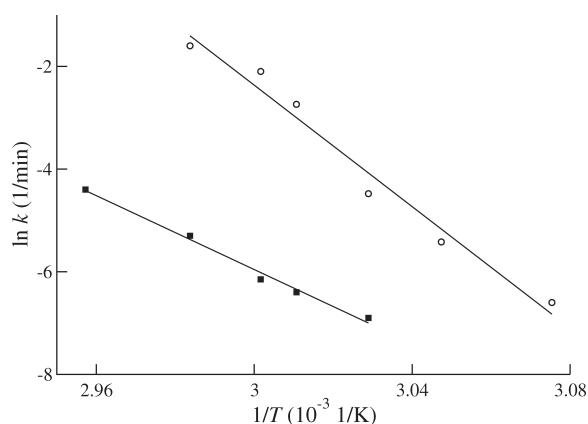


Figure 4. The activation energy of the first phase of the kinetics is similar to the denaturation energy of the CH2 domain. The Arrhenius plot shows the natural logarithm of the rate constants calculated from the kinetics at various incubation temperatures (Fig. 3) plotted against the reciprocal temperatures. The initial phase (open circles) has an activation energy of $E_a = 117$ kcal/mol, whereas the exponential phase (solid squares) has an activation energy of $E_a = 71$ kcal/mol.

functions acquired during each experiment were analyzed using the two-species model in Eq. (9) (Materials and Methods), which assumes a monomer population with a fixed hydrodynamic radius of 5.6 nm and a polydisperse aggregate population. By visual inspection, the model fit every autocorrelation function very well without any constraints on the fitting parameters (not shown). The polydispersity parameter, which is subject to the largest uncertainty especially at low concentration, generally increased during the experiment and reached a maximum value of 0.3 after 16 h of growth. The Tween 80 present in the sample buffer forms micelles with a

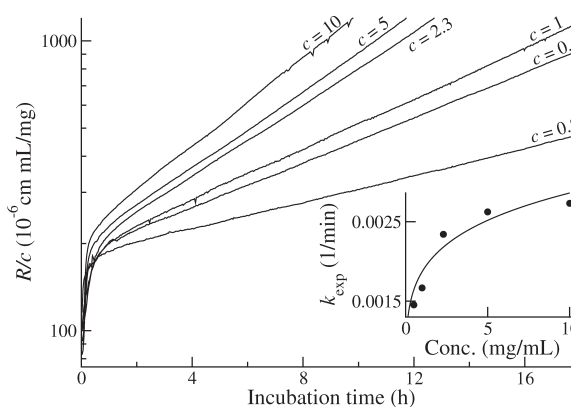


Figure 5. Protein concentration has a surprisingly weak effect on both phases of the aggregation kinetics. Static light scattering of 0.2–10 mg/mL Rituximab samples as a function of incubation time at 60 °C. The kinetics are composed of an initial fast phase followed by an exponential phase. The reaction rates of the exponential phase (inset) have a weak concentration dependence, $k_{\text{exp}} \propto c^{0.2}$ (solid line).

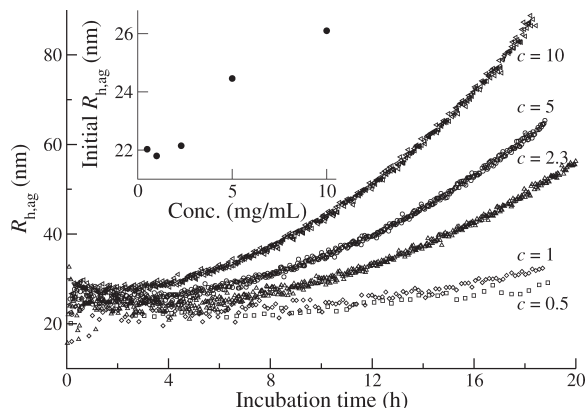


Figure 6. Aggregate sizes increase continuously throughout the incubation period. Aggregate hydrodynamic radius for various concentrations of Rituximab plotted as a function of incubation time at 60°C. The initial hydrodynamic radius, as obtained by extrapolation to time zero, is plotted as a function of the concentration (inset).

hydrodynamic radius similar to that of the protein monomers, but only contributes very little to the total scattered intensity.

Figure 6 shows the increase in the hydrodynamic radius of the aggregate species formed at concentrations of 0.5 to 10 mg/mL as a function of incubation time at 60°C. The hydrodynamic radius of the aggregate population is seen to increase continuously throughout the experiment. The initial hydrodynamic radius of the aggregates, as determined by extrapolation to time zero, increases from 22 to 26 nm with increasing concentration (inset of Fig. 6).

From the light-scattering data, the accumulated aggregate mass and aggregate number density are calculated as follows. First, the intensity due to aggregates is given as a sum of the individual aggregate contributions:

$$I_{\text{ag}}(t) = [1 - \alpha(t)]I(t) = \left[\frac{I_0}{cM_m} \right] \sum_{\text{ag}} c_i(t)M_i(t)P_i(q,t), \quad (3)$$

where $\alpha(t)$ is the monomer fraction defined in Eq. (9), I_0/cM_m represents a normalization of the scattered intensity at time zero with respect to the sample concentration and the molecular weight, and the sum is taken over all aggregate species of concentration $c_i(t)$, mass $M_i(t)$, and form factor $P_i(q,t)$. In the following calculations, we approximate the aggregate species by a single population of aggregates with an average aggregate mass, $\overline{M}_{\text{ag}}(t)$, and an average mass concentration, $\bar{c}_{\text{ag}}(t)$. The above equation is then rewritten as follows:

$$[1 - \alpha(t)]I(t) = \left[\frac{I_0}{cM_m} \right] \bar{c}_{\text{ag}}(t)\overline{M}_{\text{ag}}(t)P_{\text{ag}}(q,t), \quad (4)$$

where $P_{\text{ag}}(q,t)$ is the form factor of the aggregate population. Rearranging Eq. (4), we obtain the fol-

lowing expression for the average aggregate mass concentration:

$$\bar{c}_{\text{ag}}(t) = \frac{cM_m[1 - \alpha(t)]I(t)}{I_0\overline{M}_{\text{ag}}(t)P_{\text{ag}}(q,t)}. \quad (5)$$

We may also obtain an expression for the average aggregate number density, $\overline{N}_{\text{ag}}(t)$, from the expression above:

$$\overline{N}_{\text{ag}}(t) = \frac{\bar{c}_{\text{ag}}(t)}{\overline{M}_{\text{ag}}(t)} = \frac{cM_m[1 - \alpha(t)]I(t)}{I_0\overline{M}_{\text{ag}}^2(t)P_{\text{ag}}(q,t)}. \quad (6)$$

The average mass concentration and average aggregate number density [Eqs. (5) and (6), respectively] are central for understanding the aggregation mechanism. To that end, estimates of the form factor, $P_{\text{ag}}(q,t)$, and the mean mass of an average-size aggregate, $\overline{M}_{\text{ag}}(t)$, are required. The form factor was measured every hour during the kinetics, and a satisfactory fit was obtained using the Fisher-Burford approximation. The Fisher-Burford approximation is an extension of the Guinier law and is originally developed to include mass fractals³⁷:

$$P_{\text{ag}}(q,t) = \left[1 + \frac{2}{3}d^{-1}q^2R_g^2(t) \right]^{-d/2}, \quad (7)$$

where $R_g(t)$ is the radius of gyration, and the fractal dimension, d , concerns the structure of the aggregate. Typical fractal dimensions for protein aggregates are in the range 2.0–2.7.^{38,39} We chose $d = 2.5$, close to the value of 2.56 reported previously for antibodies,¹⁸ but choosing other values in the interval did not qualitatively change the results. The radius of gyration in Eq. (7) was similar to the hydrodynamic radius of the aggregates obtained from the dynamic light-scattering (DLS) analysis, $R_{g,\text{ag}}(t) \sim R_{h,\text{ag}}(t)$, and as the hydrodynamic radius is more accurately determined in the beginning of the experiments, we used this parameter in Eq. (7) when calculating the aggregate mass concentration [Eq. (5)] and the aggregate number density [Eq. (6)]. Some deviation between the hydrodynamic radius and the radius of gyration was observed in the last part of the kinetics in the 10 mg/mL data set. The mean mass of a typical aggregate, $\overline{M}_{\text{ag}}(t)$, is estimated by assuming that the aggregates are fractal objects with an average density equal to the monomer density:

$$\overline{M}_{\text{ag}}(t) = M_m \left[\frac{R_{h,\text{ag}}(t)}{R_{h,m}} \right]^d, \quad (8)$$

Figure 7(a) shows the aggregation number density as a function of incubation time calculated from Eq. (6). For clarity, only the 1 and 2.3 mg/mL data

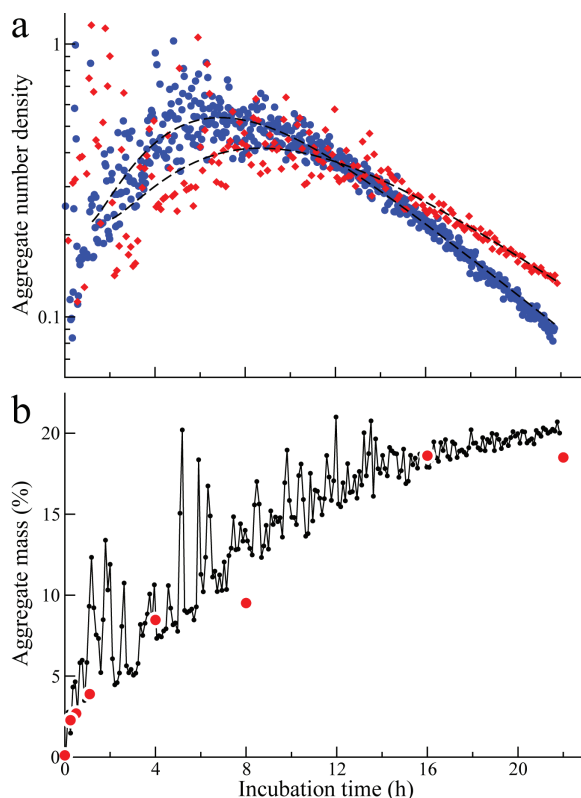


Figure 7. Aggregate number density goes through a maximum in time, and aggregate mass reaches a plateau. These are hallmarks of a coagulation mechanism. (a) Aggregate number density, $\bar{N}_{ag}(t)$, as a function of incubation time. Samples of 1 mg/mL (red diamonds) and 2.3 mg/mL (blue dots) were incubated at 60°C. The dashed lines are guides to the eye. (b) A comparison of aggregate mass percentage, $\bar{c}_{ag}(t)$, of a 1 mg/mL Rituximab sample as a function of incubation time at 60°C as estimated by DLS analysis (black data points) and SEC (red dots). The two approaches measure aggregate mass in fundamentally different ways, but the end result is very much in accordance. The aggregate mass is seen to increase for hours and then reach a plateau. The SEC data point at 22 h was obtained immediately after the DLS experiment shown in the figure.

sets are shown, but the other data sets follow similar behavior. The number density increases for several hours, reaches a maximum, and then decreases in the last part of the kinetics. Figure 7(b) shows the percentage of monomers converted into aggregates as a function of incubation time of a 1 mg/mL solution as calculated from Eq. (5). The aggregate mass percentage increases from 0 to 20% for 16 h, at which point the growth slows down and the aggregate mass reaches a plateau. Figure 7(b) also contains SEC data as discussed later. The increase in aggregate mass is mirrored by a decrease in monomer mass; however, the latter quantity is subjected to larger error in the light-scattering analysis.

The effect of Tween 80 and salts on the Rituximab aggregation kinetics was examined by removing

Tween 80 from the stock solution and then changing the sample buffer into a similar buffer without Tween 80 and also into pure milli-Q water. In Supporting Information Figure 1, the aggregation kinetics of these two samples are compared with the kinetics of a regular 1 mg/mL Rituximab sample and found to have the same distinct two-phase kinetics.

Quantification of aggregate mass by SEC

Aggregate mass may also be measured directly by SEC. In Figure 8, SEC of samples incubated at 60°C for 0, 0.25, 0.5, 1, 4, 8, 16, and 22 h is shown. With increasing incubation time an aggregate peak appears, but as with the light-scattering data, no significant formation of small oligomers is observed. The figure shows eluent absorption at 280 nm as a function of eluent volume. The inset in Figure 8 shows the aggregate mass percentage as a function of incubation time. After 15 min, 2.5% aggregate mass have formed, and in the following hours, the aggregate mass increases until a plateau is reached at ~18% after 16 h. The sum of the monomer and aggregate areas was constant as expected for mass conservation. In Figure 7(b), the same SEC data have been superimposed on the aggregate mass percentage calculated from the light-scattering data, and the agreement is seen to be satisfactory. This supports the main assumption, that the aggregates can be approximated by an average-sized population in deriving Eqs. (5) and (6).

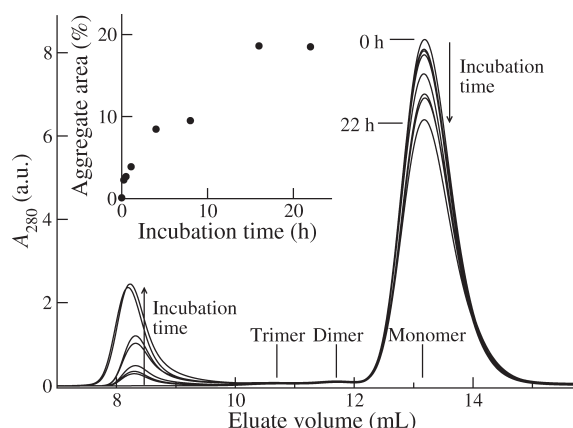


Figure 8. Aggregate mass increases over time and then reaches a plateau. SEC of a fresh 1 mg/mL Rituximab sample is compared with samples incubated at 60°C for 0.25, 0.5, 4, 8, 16, and 22 h. The eluate absorption at 280 nm is plotted as a function of the eluate volume. The monomers elute at 13.2 mL, and the aggregates elute at ~8.2 mL. Only minute amounts of dimers and trimers were present. During incubation, the height of the monomer peak decreases, and the height of the aggregate peak increases as indicated by arrows. The aggregate mass percentage (inset) increases continuously and then reaches a plateau around 18% after 16 h.

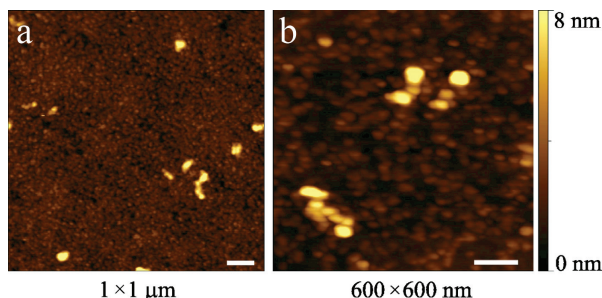


Figure 9. The size of aggregates visualized by AFM is in accordance with aggregate sizes determined by light scattering. (a) A 1 mg/mL Rituximab sample was incubated for 16 h at 60°C. Aggregates with an average radius of 30 nm are embedded on a background of monomers with an average radius of 6 nm. (b) A closer look at aggregates from a similar sample. The scale bars indicate 100 nm.

Aggregate visualization

Aggregated samples were visualized by AFM. Figure 9(a) shows a representative 1 mg/mL sample incubated for 16 h at 60°C with aggregates embedded on the monomer-covered mica. Figure 9(b) shows aggregates from another sample in more detail. The height scale is the same in both pictures. A buffer-only sample showed a clean mica surface and ensures that the buffer preparation does not give rise to any artifacts that could be mistaken for aggregates. A monomeric sample showed the mica surface covered with objects with an average radius of 6 nm. From an analysis of more than 400 aggregates, the average radius was found to be about 30 nm. These observations are in accordance with the DLS and SEC data in Figure 7(b), which show that under these conditions, ~80% of the sample remain monomeric, and also in accordance with the hydrodynamic radius of the aggregates after 16 h of incubation (Fig. 6).

Discussion

In this study, we investigate the aggregation kinetics of an IgG1 antibody when stressed by incubation at temperatures below the lowest thermal transition. In proteins such as antibodies, where several domains unfold separately, the influence of the stability of individual domains on the aggregation propensity is as of yet not clear. Moreover, the exact aggregation mechanisms involved remain largely unresolved when compared with the large number of studies done on single-domain model systems. Untangling the molecular mechanisms leading to aggregates typically involves interpreting a kinetics experiment, which probes the bulk of the aggregating solution. To that end, a large number of models have been proposed, each relying on a specific aggregation mechanism.^{10,11,27} Very often more than one model will fit the data, and this is also the case for the antibody aggregation study at hand. However,

we show how a stringent interpretation of light-scattering data help narrow down the number of plausible mechanisms. In addition, we demonstrate that light-scattering and calorimetry data in concert point to the importance of a specific domain in initiating the aggregation process.

Initial fast formation of aggregates

The principal observation in this study is the fact that the scattered light—under a wide range of concentrations and temperatures—exhibits a distinct two-phase behavior (Figs. 3 and 5). The first aggregation step does not involve the formation of long-lived oligomers, as shown by DLS and SEC data (Figs. 6 and 8, respectively). This phase is puzzling, because it apparently goes to completion and paves the way for the second, slower phase. If the process goes to completion due to a monomer-aggregate equilibrium, we would expect a strong concentration dependence, quite opposite to the actual observations. This kind of fast initial aggregate assembly has been previously observed by other groups. For example, the amyloid β -peptide has been observed to assemble into a micelle-like structure with a hydrodynamic radius of 7 nm at ambient temperature,^{9,40} and β -lactoglobulin has been reported to initially form clusters with a hydrodynamic radius of 15 nm, which later on were incorporated into large fractal structures.⁴¹ In principle, the first process could also rely on a component in the buffer. However, Rituximab, when prepared in milli-Q water and incubated at 60°C, had a similar two-phase behavior (Supporting Information Fig. 1). We hence conclude that Tween 80 and salts have negligible impact on the kinetics presented in this article. The mechanistic details of the initial aggregate formation remain unresolved due to methodological limitations.

Coagulation governs aggregate growth

The second phase is characterized by a remarkable continuous exponential increase in scattered light (Fig. 5). Exponential increase in weight-averaged aggregate mass can be associated with either reaction-limited cluster-cluster aggregation^{42,43} or secondary nucleation mechanisms.^{28,44,45} Secondary nucleation mechanisms are often observed for fibril-forming proteins capable of generating new fibril ends by branching or breakage mechanisms, and reaction-limited cluster-cluster aggregation is a coagulation process where the rate of coalescence of two clusters is not dependent upon cluster size. The fundamental difference between these two mechanisms manifests itself through the conversion of monomers into aggregates: in secondary nucleation, the aggregate mass increases exponentially, while this is not explicit in coagulation. Furthermore, aggregate number density, although not always readily obtainable from light scattering experiments, conveys

important information about the underlying mechanism. We rely on these facts to distinguish between the two mechanisms as detailed in the following.

As mentioned earlier, the exact growth of the aggregate mass provides a litmus test for a coagulation mechanism versus a secondary nucleation mechanism. We calculated this quantity from the DLS data and independently confirmed it by SEC [Fig. 7(b)]. The correlation between DLS and SEC data shows that although not explicit in the light-scattering analysis, mass is conserved. We found that rather than being exponential, the growth in aggregate mass is linear and approaches a plateau. This is clearly inconsistent with a secondary nucleation mechanism. Furthermore, the aggregate number density goes through a maximum in time [Fig. 7(a)], and this is compelling evidence of a coagulation process. The combination of an exponential increase in the scattered light (Figs. 3 and 5), the continuous increase in the aggregate size (Fig. 6), the maximum in aggregate number density [Fig. 7(a)], and the growth in aggregate mass [Fig. 7(b)] leads to the conclusion that a coagulation process, such as reaction-limited cluster-cluster aggregation, governs the growth of aggregates. In the light of the many events involved in the coagulation mechanism, the activation energy of the exponential phase (71 kcal/mol) is not readily associated with a single physical process.

Coagulation processes are essentially second order,⁴⁶ and hence do not readily offer an explanation for the weak concentration dependence observed, $k_{\text{exp}} \propto c^{0.2}$ (inset of Fig. 5). A possible explanation may be that another process with a lower reaction order is effectively controlling the aggregate formation.⁴⁷ For example, concomitant micelle formation in fibrillating amyloid β -peptide solutions has been shown to effectively keep the monomer concentration constant and hence mask the true concentration dependence of the fibrillation process.⁹ In any case, a realistic description of the concentration dependence of the rate constants of the exponential phase should probably take into full account the process governing the initial fast phase.⁴⁸

Conceptually, it is difficult to understand why the majority of the monomers do not convert into aggregates. Although still being investigated in our group, it appears that the plateau in the aggregate mass percentage is not significantly affected by concentration, but rather strongly influenced by temperature (data not shown). Jøssang and Feder¹⁹ made a similar observation for polyclonal antibodies under conditions similar to ours. They showed that when reincubating the heat-stable monomers, they exhibited a much different aggregation kinetics; however, that phenomenon may be ascribed to the inherent heterogeneity of polyclonal antibodies purified from blood. In this study on mAbs, heterogeneity is

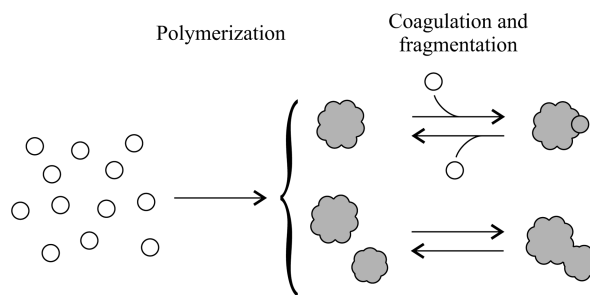


Figure 10. Sketch of the proposed mechanisms involved in Rituximab aggregation. During the first phase, a population of large aggregates is quickly formed by a mechanism not yet fully understood. Then, aggregates increase in size by coagulation, while fragmentation may play a role in preventing the aggregation from going to completion. Coagulation and fragmentation may involve both monomers and aggregates.

unlikely to have a significant impact on the aggregation kinetics. Instead, the partial aggregation could point to the presence of the reverse process of coagulation, namely fragmentation. By fragmentation, aggregates repeatedly break apart into smaller aggregates, and monomers are detached from the aggregates as sketched in Figure 10.

AFM pictures showed structures with an average size of 30 nm embedded on a background of monomers in accordance with our kinetics data. The structures themselves (Fig. 9) are somewhat different from the spherical aggregate structures observed previously by transmission electron microscopy for antibodies⁴⁹ and for a number of other proteins.^{8,26}

Transient unfolding correlates with aggregation

We now turn to the structural events priming molecules for conversion into aggregates. All incubation temperatures were below the denaturation temperature of the CH2 domain ($T_{m,1} = 71^\circ\text{C}$), and no structural changes were detected in the incubation temperature range studied here (Figs. 1 and 2). However, even at temperatures well below the melting temperature, thermal fluctuations are expected to transiently expose the hydrophobic interior to the solvent while keeping structural elements intact, and this may prime aggregation.¹² In fact, even subtle changes to the protein conformation may trigger the prevalence of attractive intermolecular forces.³ Aggregation is hence most likely initiated by minor structural changes to certain parts of the molecule and possibly to just a small fraction of the sample. In the study of IgG2 aggregation mentioned earlier, Van Buren *et al.*²⁴ showed that oligomerization and aggregation were mainly driven by unfolding of the CH2 domain, although no changes in far- and near-UV spectra were observed. The isolated oligomers and aggregates did, however, exhibit minor changes in far-UV CD spectra, demonstrating

that in studies such as ours, where only a small fraction of the monomers aggregate, the signal will be dominated by the most numerous species. A similar conclusion was reached by Kalonia and co-workers⁵⁰ in a study of irreversible aggregation of a mAb under nonstressed conditions. In our study, the CH2 domain is the least stable domain and the overall irreversible aggregation process is most likely determined by the activation barrier for unfolding of this particular domain.⁵¹ We find it intriguing that the activation energy of the initial reaction (117 kcal/mol) is similar to the activation energy of the unfolding of the CH2 domain (98.4 kcal/mol). Thus, a structural change in the CH2 domain is likely to prime the molecule for aggregation.

Conclusions

Temperature-induced aggregation of the model multidomain protein exhibits two phases: the first phase represents the fast assembly of monomers into aggregates with a hydrodynamic radius of about 25 nm by a mechanism not yet fully understood. This phase is followed by a second phase characterized by several hours of exponential growth of the scattered intensity. During this phase, the aggregates continuously increase in size, the aggregate number density goes through a maximum in time, and the aggregate mass percentage approaches a plateau. All of these observations, including the exponential increase in the scattered light itself, are consistent with a coagulation mechanism, likely involving both reaction-limited cluster-cluster aggregation as well as fragmentation (Fig. 10). Therefore, this study gives a rare insight into the complex mechanism controlling the aggregation of multidomain proteins. Transient structural fluctuations are likely to prime the antibody molecules for aggregation, and our data show that the CH2 domain may play an important role to that effect.

Materials and Methods

Protein and reagents

Rituximab (marketed as MabThera by Roche, Basel, Switzerland, and Rituxan by Genentech, San Francisco, CA) 10 mg/mL was in a liquid formulation composed of 7.35 mg/mL NaCitrate·2H₂O pH 6.5 with 9 mg/mL NaCl and 0.7 mg/mL Tween 80.¹³ It was diluted in a similar buffer containing analytical grade tri-NaCitrate · 2H₂O (Merck, Darmstadt, Germany), NaCl (Honeywell Riedel-de Haën, Seelze, Germany), and Tween 80 (Polysorbate 80; Sigma-Aldrich, St. Louis, MO) diluted in milli-Q water (Millipore, Billerica, MA). The buffer was filtered through a Millex-LG sterilizing 0.2-μm filter (Millipore, Billerica, MA). In all light-scattering experiments, the protein samples were filtered through

Anotop 10 filters (Whatman, Maidstone, England) with a pore size of 20 nm. The first three drops were discarded to saturate the filter with protein and Tween 80. Concentration was measured after filtration by absorbance measurement at 280 nm on a Shimadzu UV-2401 PC spectrophotometer (Kyoto, Japan) using the Rituximab extinction coefficient $\epsilon_{280} = 1.7 \text{ mL}/(\text{mg cm})$ available from the U.S. Food and Drug Administration homepage.

Removing Tween 80 and salts from Rituximab

This procedure is described in Section 1.1 of the Supporting Information.

Size-exclusion chromatography

Aggregate mass percentage was measured using a Superdex 200 10/300 GL SEC column (GE Healthcare, Buckinghamshire, UK) connected to a Shimadzu HPLC (Kyoto, Japan). Samples of 1 mg/mL Rituximab were incubated at 60°C for various time intervals in the light-scattering setup described below and transferred directly to a 500 μL loop on the HPLC. The samples were eluted at a flow of 0.8 mL/min in Rituximab sample buffer, and the eluate was monitored by absorption at 280 nm. The percentage of monomers converted to aggregates was estimated from the area of the aggregate peak with respect to the total area of monomers and aggregates. The total integrated area of aggregates and monomers was constant.

Static and dynamic light scattering

The scattered light intensity at 90° and its time autocorrelation function, $g_2(\tau)$, were measured simultaneously, and the structure factor was obtained every hour by measurements at 12 different angles. The Brookhaven light-scattering setup (Brookhaven Instr. Corp., Holtsville, NY) was equipped with a BI-200SM goniometer, a BI-APD avalanche photodiode detector, a BI-9000AT autocorrelator, and a Melles Griot (Carlsbad, CA) Omnichrome Series 43 vertically polarized solid-state laser tuned at a wavelength of either $\lambda_0 = 488 \text{ nm}$ or $\lambda_0 = 514 \text{ nm}$. The temperature was measured with a Fluke 2180A RTD digital thermometer (Everett, WA) in the oil surrounding the circular quartz cuvette and controlled by a Haake F8 circulator (Karlsruhe, Germany) with a tolerance of 0.05°C. Thermal equilibrium in the cuvette was achieved within 5 min.

Absolute values for the scattered intensity (Rayleigh ratio) were obtained by normalization with respect to a toluene measurement performed before each experiment assuming a toluene Rayleigh ratio at 488 and 514 nm of 39.6×10^{-6} and $32.0 \times 10^{-6} \text{ cm}^{-1}$, respectively. Raw intensity data were normalized with respect to the instrument-dependent contrast constant $K = 4\pi^2 n_{\text{buf}}^2 (dn/dc)^2 / \lambda_0^4 N_A$, where n_{buf}

is the refractive index of the buffer, dn/dc is the incremental refractive index, λ_0 is the wavelength, and N_A is the Avogadro constant. Volume and refraction effects were taken into account by multiplying the intensity measurements by $\sin \theta$ and $(n_{\text{buf}}/n_{\text{tol}})^2$, respectively, where θ is the scattering angle and n_{tol} is the refractive index of toluene. The excess Rayleigh ratio was further normalized with respect to the protein concentration, c . All relevant constants are listed in Supporting Information Table I. Autocorrelation functions $g_2(\tau)$ were analyzed by fitting to a double-exponential function assuming two independent populations: a monodisperse monomer population and a polydisperse aggregate population⁵²:

$$g_2(\tau) = \beta \left| \alpha \exp(-D_m q^2 \tau) + (1 - \alpha) \exp(-D_{ag} q^2 \tau) \left(1 + \frac{\mu_2}{2!} \tau^2 \right) \right|^2, \quad (9)$$

where β is the amplitude of the autocorrelation function, α is the relative amplitude of the two populations, D_m and D_{ag} are the diffusion times of the monomer and aggregate population, respectively, μ_2 is the variance about the mean, and $q = 4\pi n_{\text{buf}} \sin(\theta/2)/\lambda_0$ is the scattering vector. Assuming diffusion of spherical particles of radius R_h in a medium of viscosity η , the diffusion times were converted to hydrodynamic radii using the Stokes–Einstein relation $D = k_B T / 6\pi\eta R_h$, where k_B is the Boltzmann constant and T is the temperature. The hydrodynamic radius of the Rituximab monomer was fixed at $R_{h,m} = 5.6$ nm, a value measured in a 1 mg/mL Rituximab solution at ambient temperatures. This hydrodynamic size is in accordance with the value predicted by using the Hydropro software⁵³ on the crystal structure of an intact IgG1 mAb (PDB ID 1hzh).⁵⁴

Refractive index, refractive index increment, and viscosity

For the light-scattering analysis, the refractive index, n , the refractive index increment, dn/dc , and the viscosity, η , must be measured. The refractive index of the Rituximab buffer was measured at 56.0, 60.1, and 66.2°C on an Abbe refractometer (Sun Instr. Corp., Torrance, CA) temperature controlled by a Haake F3 circulator (Karlsruhe, Germany). The refractive index increment was measured on a differential refractometer (BI-DNDC; Brookhaven Instr. Corp., Holtsville, NY). Viscosity of the Rituximab buffer was measured on an AR 1000 rheometer (TA Instr., New Castle, DE) using a 40-mm 4° Ti cone with a truncation length of 26 μm . Viscoelastic spectra were measured at 60°C with the shear stress ramped from 0.15 to 3 Pa in 3 min. The values measured are reported in Supporting Information Table I.

Atomic force microscopy

Aggregates were visualized by AFM. First, samples were diluted 1:1000 in milli-Q water, and then 10 μL sample solution was deposited on a mica surface. After 20 min at room temperature, excessive sample solution was washed off from the mica with 20 drops of milli-Q water. Without any washing, salt and Tween 80 structures easily mistaken for aggregates were observed even when visualizing the buffer alone. The samples were dried gently with a flow of nitrogen. Samples of pure buffer, 1 mg/mL Rituximab, and 1 mg/mL Rituximab aggregated for 16 h at 60°C were visualized on a MultiMode scanning probe microscope (Veeco Instr. Inc., Santa Barbara, CA), equipped with a NanoScope 3D controller and operated in tapping mode. Rigid cantilevers (RFESP; Veeco Instr. Inc.) with resonance frequencies of 81–92 kHz, a spring constant of 3 N/m, and single-crystal Si tips with a nominal radial curvature of 8 nm were used. The typical scan rate was 1.0 Hz. Using Gwyddion 2.10 data analysis software,⁵⁵ the average size of monomers and aggregates was determined by 2D autocorrelation function analysis.

Differential scanning calorimetry

The thermal stability of individual domains were measured by DSC. Measurements were performed on a 10 mg/mL Rituximab solution using a capillary VP-DSC (MicroCal LLC, Northampton, MA) with a cell volume of 0.135 mL. Temperature scans were performed from 25 to 90°C at a scan rate of 1°C/min and an excess pressure of 51 psi. A buffer–buffer reference scan was subtracted from each sample scan prior to concentration normalization. Baselines were created in Origin 7.0 (OriginLab, Northampton, MA) by cubic interpolation of the pretransition and post-transition baselines. Subsequent calculations of thermodynamic parameters were performed using the MicroCal Origin software. Values of calorimetric enthalpy, the Van't Hoff enthalpy, and the midpoint melting temperatures (T_m) were obtained using the non-two-state model in the software.

Circular dichroism

Thermal unfolding of Rituximab was studied by far and near-UV circular dichroism (CD) using a Chirascan CD spectropolarimeter (Applied Photophysics, Surrey, UK). The temperature was ramped linearly from ambient temperature to >75°C at a rate of 1°C/min and spectra acquired with a 1 nm step size, a 1-nm bandwidth, and a 1-nm/sec scan rate. Far-UV CD spectra were recorded from 205 to 260 nm on a 0.15-mg/mL Rituximab sample in a quartz cuvette with a 1-mm path length. The spectra were truncated at 211 nm, at which point the photomultiplier voltage exceeded 650 V. In a similar unfolding experiment, the unfolding was measured at 218 nm,

the global minimum of the far-UV CD spectra. Near-UV CD spectra were measured from 250 to 350 nm on a 0.5-mg/mL Rituximab sample in a quartz cuvette with a path length of 10 mm. In a similar unfolding experiment, the unfolding was measured at 298 nm, the global maximum of the near-UV CD spectra. The signal from a buffer sample was subtracted from each run, and no additional filtering was applied. The CD signal is reported in residual molar ellipticity, and the temperature was recorded directly using a temperature probe placed inside the cuvette.

Sypro orange fluorescence

Sypro orange is a fluorescent probe sensitive to changes in protein hydrophobicity with excitation and emission maxima at 472 nm and 569 nm, respectively.⁵⁶ Thermal unfolding of Rituximab was explored using a high-throughput microarray method,⁵⁷ in which the fluorescence is detected by a MyiQ single-color real-time PCR detection system (Bio-Rad Labs., Hercules, CA). A 480-nm excitation filter with 40-nm bandwidth and a 540-nm emission filter with 50-nm bandwidth were used. Fifty Microliters of 0.2 mg/mL Rituximab with a 2000-fold dilution of Sypro orange (Life Tech., Carlsbad, CA) was transferred to a 96-well PCR plate and sealed with adhesive QPCR tape pads (Porvair Sciences, Leatherhead, UK). Sypro orange fluoresces in the presence of Tween 80, hence the sample was diluted in a buffer made without Tween 80. The Peltier-controlled plate holder was ramped from 25 to 90°C at 1°C/min, and the data were adjusted for the background signal.

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MINIREVIEW

Challenges in the Development of High Protein Concentration Formulations

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ABSTRACT: Development of formulations for protein drugs requiring high dosing (in the order of mg/kg) may become challenging for solubility limited proteins and for the subcutaneous (SC) route with <1.5 mL allowable administration volume that requires >100 mg/mL protein concentrations. Development of high protein concentration formulations also results in several manufacturing, stability, analytical, and delivery challenges. The high concentrations achieved by small scale approaches used in pre-formulation studies would have to be confirmed with manufacturing scale processes and with representative materials because of the lability of protein conformation and the propensity to interact with surfaces and solutes which render protein solubilities that are dependent on the process of concentrating. The concentration dependent degradation route of aggregation is the greatest challenge to developing protein formulations at these higher concentrations. In addition to the potential for nonnative protein aggregation and particulate formation, reversible self-association may occur, which contributes to properties such as viscosity that complicates delivery by injection. Higher viscosity also complicates manufacturing of high protein concentrations by filtration approaches. Chromatographic and electrophoretic assays may not accurately determine the non-covalent higher molecular weight forms because of the dilutions that are usually encountered with these techniques. Hence, techniques must be used that allow for direct measurement in the formulation without substantial dilution of the protein. These challenges are summarized in this review. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 93:1390–1402, 2004

Keywords: protein formulation; high concentration; pharmaceutical; manufacturing; analytical; viscosity

INTRODUCTION

Over the past two decades, recombinant DNA technology has led to the commercialization of many protein therapeutics requiring successful formulations. The most conventional route of

delivery for protein drugs has been intravenous (IV) administration because of poor bioavailability by most other routes, greater control during clinical administration, and faster pharmaceutical development. For products that require frequent and chronic administration, the alternate subcutaneous (SC) route of delivery is more appealing. Particularly when coupled with pre-filled syringe and autoinjector device technology, SC delivery allows for home administration and improved compliance of administration, and may

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result in expanded product markets. Proteins such as monoclonal antibodies are often administered with frequent dosing regimens and at high doses (several mg/kg). Two antibodies, Rituxan[®] and Herceptin[®] that have been approved for the treatment of cancer are intravenously administered in hospitals, but several programs are underway for use of monoclonal antibodies to treat diseases that may require outpatient administration, and hence require the development of SC route of administration. Treatments with high doses, e.g., more than 1 mg/kg or 100 mg per dose, require development of formulations at concentrations exceeding 100 mg/mL because of the small volume (<1.5 mL) that can be given by the SC routes. For some proteins, that have limited solubility, achieving such high concentration formulations may require the use of solubility enhancers. Even for the IV delivery route, where large volumes can be administered, protein concentrations of tens of milligram per milliliter may be needed for high dosing regimens and this may pose solubility challenges for some proteins such as cytokines and enzymes. Development of formulations at high concentrations also poses stability, manufacturing, and delivery challenges related to the propensity of proteins to aggregate at the higher concentrations. These challenges and appropriate analytical techniques for assessing high concentration protein formulations will be discussed.

SOLUBILITY CONSIDERATIONS

A potential challenge to developing a protein formulation that enables the desired dosing is achieving the required protein concentration in solution. The desired protein concentration may be difficult to achieve for some enzymes and cytokines that have limited solubility. The principles governing protein solubility are more complicated than those for small synthetic molecules,^{1,2} and thus overcoming the protein solubility issue takes different strategies. Operationally, solubility for proteins could be described by the maximum amount of protein in the presence of co-solutes whereby the solution remains visibly clear (i.e., does not show protein precipitates, crystals, or gels), or does not sediment at 30,000g centrifugation for 30 min.³ The dependence of protein solubility on ionic strength, salt form, pH, temperature, and certain excipients has been mechanistically explained by changes in

bulk water surface tension and protein binding to water and ions versus self-association.^{1,3-5} Binding of proteins to specific excipients or salts influences solubility through changes in protein conformation or masking of certain amino acids involved in self-interaction. Proteins are also preferentially hydrated (and stabilized as more compact conformations) by certain salts, amino acids, and sugars, leading to their altered solubility.⁵ Protein solubility could also depend on the level of purity of the protein preparation; e.g., fibrinogen solubility was found to be dependent on the initial amount of protein because of the impurities and heterogeneity of the preparation.⁶ Hence, selection of a protein formulation based on preformulation work that uses materials from early process development runs may be a misguided decision and needs to be verified with representative larger scale preparations.

Determining the highest protein concentration achievable remains an empirical exercise because the lability of protein conformation and the propensity to interact with itself, with surfaces, and with specific solutes, all lead to protein solubilities that depend on the process of concentrating. Table 1 lists the various methods for concentrating a protein solution, with their advantages and practical limitations. Early in preformulation studies where material is limited, small-scale methods are needed to achieve high protein concentrations. The most mechanically gentle and rapid methods involve osmotic pressure dependent microdialysis principles.^{7,8} One such method⁷ very rapidly achieves several orders of magnitude increase in protein concentration simultaneous with buffer exchange using only ~0.1 mg protein. Though this approach is a useful tool for rank ordering of solubility in various formulation compositions, for some cytokines, it gave 10-times higher solubilities than those reached by diafiltration/ultrafiltration on a Centricon[®] system that may result in shear induced protein denaturation and lowered apparent solubility (Shahrokh et al., unpublished results). Another approach based on osmotically driven solute concentration involves dialysis against a hygroscopic material such as Sephadex or polyethylene glycols.² This approach requires the use of high purity materials that do not result in protein degradation, and must be done with care to prevent over drying and exposure to very large surface areas since water rapidly leaves the protein solution. In addition, as polyelectrolytes, proteins impact the distribution of diffusible ions across the dialysis membrane to maintain

Table 1. The Commonly Used Methods of Concentrating Proteins

Concentrating Method	Scale	Benefits	Limitations	Used for Solubility Determination
Osmotic driven (dialysis) methods Against solutions	Few μL to few mL	Small-scale; mechanically gentle; rapid Simultaneous buffer exchange	Concentrations achieved may not translate to manufacturing scale	Yes
Against water absorbing materials			Have to buffer exchange later	
Solvent evaporation Vacuum, N ₂ flow	Few μL to few mL	Small-scale	Slow (may lead to degradation); concentrates excipients	Yes, only in solution composition reached at end of process
Lyophilization	mL	Pharmaceutically relevant and scalable	Must protect against drying induced degradation; concentrates excipients	
Precipitation (salt or solvent; SCF)	Few μL to L	Quick and scalable	Solution composition not pharmaceutically relevant	No
Freezing	Few mL to L	Tens of fold concentration is achieved quickly	May lead to irreversible degradation	No
Chromatographic bind and elute	Few μL to many mL	Wide range of scale	Concentrates excipients too Process may damage protein Solution composition not pharmaceutically relevant Conditions may damage protein	No
Filtration Systems Centrifugal (Centricon)	Hundreds of μL to tens of mL	Moderate scale; may include buffer exchange	Adsorption losses; shear induced degradation Findings may not translate to manufacturing scale	Yes, if not material limited
Pressure (Amicon)	Hundreds of mL to L	Manufacturing scale; may include buffer exchange	Large scale impractical for early development phases	
TFF			High viscosity or pore clogging may limit concentrations reached	

electroneutrality as dictated by the Donnan equilibrium.⁹ This could lead to a potentially large change in the ion composition within the protein solution as it concentrates, and could be damaging to the protein. The alternative small-scale methods of solvent evaporation by vacuum or nitrogen flow, and precipitation by salts or solvents,^{10–12} alter the formulation composition and may also result in protein degradation due to the time or materials used in the process. Precipitation and generation of protein powders by supercritical fluids has met some success in maintaining active or structurally native proteins, but this approach remains of limited practical use particularly for small scale studies.^{13,14} Concentration of solutes by freezing¹⁵ is another approach that could achieve a high protein concentration, but alteration in the solute composition, potential damage to the protein during freezing and thawing, and difficulty in retrieving the frozen concentrate all make this an impractical approach. Chromatographic methods that bind the protein and allow elution at lower volumes is another useful tool for concentrating proteins over a large range of scale; these include ion exchange or hydrophobic interaction chromatography, but require buffer exchange after concentration. Filtration is the most common approach for concentrating proteins, and unlike many of the methods discussed is amenable to scale-up for manufacturing. There are numerous filtration systems, each with an extent of adsorption or shear-induced denaturation/aggregation that is dependent on surface area, contact materials, and flow mechanisms. The mechanical and interfacial forces imposed on protein's structure (hence its solubility) by small-scale methods may be different than those by the large-scale processes, and hence, the results obtained by such methods would have to be verified by representative manufacturing processes. The most commonly used methods for large scale manufacturing of high protein concentrations are further discussed in "Manufacturing Considerations."

STABILITY CONSIDERATIONS

Although proteins are more complex than typical small molecule pharmaceuticals, a great deal is now known about the various chemical degradations that occur in proteins, and several reviews are available.^{16–18} Many of the chemical reactions such as deamidation, aspartate isomerization, oxidation, and peptide bond hydrolysis that occur

in proteins are hydrolytically driven requiring the presence of water, and generally the kinetics follows lower order concentration dependency. Aggregation, on the other hand, requiring bi-molecular collisions, and a high concentration dependency is expected to be the primary degradation pathway in high concentration protein formulations. The relationship of concentration to aggregate formation depends on the size of aggregates as well as the mechanism of association.^{12,17} Protein aggregation may result in covalent (e.g., disulfide-linked) or non-covalent (reversible or irreversible) association. Irreversible aggregation by non-covalent association generally occurs via hydrophobic regions exposed by thermal, mechanical, or chemical processes that alter a protein's native conformation.¹⁹ When the protein-unfolding step is rate limiting, the kinetics of protein aggregation often shows pseudo-first order behavior¹⁹ rather than the expected higher order concentration dependency. Further illustration of the importance of the aggregation mechanism on the relationship of concentration to aggregate formation is shown by the inverse concentration dependency of protein aggregation that results from interaction with air–water interfaces.²⁰

Protein aggregation may impact protein activity, pharmacokinetics and safety, e.g., due to immunogenicity.^{18,21,22} Irreversible protein aggregation is recognized as a major degradation product in protein formulations, whereas reversible protein self-association is often overlooked. Reversible protein association is less studied, partly because of poor analytical methodologies (as will be discussed under "Analytical Considerations") and partly because of the perception that aggregates will not be present after typical dilutions are made prior to administration. However, if the rate of dissociation is slow, then reversible aggregation may still impact activity, *in vivo* clearance and safety. Even under conditions where the rate of dissociation is rapid, the equilibrium at high protein concentration may be shifted toward a greater amount of aggregate due to molecular crowding effects.^{23–25} In particular, as the protein concentration increases, the fraction of the total volume occupied by the protein increases. The resulting decrease in the effective volume available to the protein yields a higher apparent protein concentration that in turn favors self-association. This non-ideal solution behavior of increasing the apparent thermodynamic association constant with increasing protein concentration may be shelf-life limiting, especially when the resulting

aggregates are irreversible. In addition, after *in vivo* administration, the protein therapeutic may be placed into a crowded macromolecular environment, which shifts the equilibrium to the associated state even after dilution.

Macromolecular crowding, i.e., excluded volume effects, also can impact protein physical properties such as viscosity which can have major impact on the ability to manufacture high protein concentration formulations (see "Manufacturing Considerations") as well as on the ability to administer the protein drug by injection (Fig. 2A). In general, the viscosity of a macromolecule in solution can be expressed as a virial expansion, where the viscosity, η , can be related to the solvent viscosity, η_0 , and concentration of the protein, c_p , in g/mL by a power series²⁶:

$$\eta = \eta_0 \left(1 + k_1 c_p + k_2 c_p^2 + k_3 c_p^3 + \dots \right) \quad (1)$$

where in equation 1, k_1 is the intrinsic viscosity, which is related to the contribution from individual solute molecules, and k_2 and higher order coefficients are related to effects from interactions of 2, 3, or more protein molecules. This equation can be rewritten in terms of the specific viscosity, $\eta_s = (\eta - \eta_0)/\eta_0$:

$$((\eta - \eta_0)/\eta_0)/c_p = \eta_s/c_p = k_1 + k_2 c_p + k_3 c_p^2 + \dots \quad (2)$$

In the case of significant protein self-association that results in formation of soluble aggregates, the higher order terms will dominate and lead to large increases in viscosity as a function of concentration.

There are two key approaches to mitigating irreversible protein aggregation for improving the shelf life of high concentration protein formulations. One approach uses conditions that stabilize the native conformation of the protein, and the other, reduces molecular collisions that lead to aggregation. For the first approach, molar concentrations of osmolytes, such as sugars, are added that are preferentially excluded from the immediate environment of proteins, and result in preferential hydration.^{27,28} This preferential hydration leads to an increase in the protein's chemical potential since the increased ordering of water in the hydration shell leads to an entropic decrease for the entire thermodynamic system consisting of protein and solvent water. Since a larger surface area such as that found in unfolded proteins requires a greater number of ordered water molecules for preferential hydration, the protein will

assume a more compact folded form to resist the thermodynamically unfavorable decrease in the system's entropy. Thus, the protein will minimize the surface area available for hydration leading to stabilization of the more compact folded form, which then decreases the formation of protein aggregates. However, if the native state of the protein is capable of associating, forming aggregates of native conformers, then the addition of preferentially excluded osmolytes may actually increase the amount of protein aggregate since total surface area will be diminished following protein self association. The potential impact of reversible self-association on product shelf-life or protein physical parameters will depend on the rate of dissociation as well as the possible consequent formation of irreversible aggregates during storage at high concentration. The use of preferentially excluded osmolytes to fix the aggregation problem is limited since addition of high concentrations of excipients may also add to the viscosity and osmolality of the formulation that may render it impractical for use as a parenteral solution. Also, as already noted, the addition of these osmolytes may actually increase the amount of reversible native associated protein.

The second approach to minimize aggregation is to restrict the mobility of proteins in order to reduce the number of collisions. Lyophilization with appropriate excipients may improve protein stability against aggregation by decreasing protein mobility and by restricting conformational flexibility with the added benefit of minimizing hydrolytic reactions consequent to removal of water.²⁹ The addition of appropriate excipients, including lyoprotectants, prevents the formation of aggregates during the lyophilization process as well as during storage of the final product.³⁰⁻³² A key parameter for effective protection is the molar ratio of the lyoprotectant to the protein.³³ Generally molar ratios of 300:1 or greater are required to provide suitable stability, especially for room temperature storage. While this strategy achieves an optimal final formulation at moderate protein concentrations, if the lyophilizer is used to concentrate the protein, additional challenges can result as discussed below.

MANUFACTURING CONSIDERATIONS

Although it may be possible to create a formulation during development that has the desired solubility and stability, the design of processes

that will allow for manufacturing of the formulation at a larger scale is critical for successful implementation of bench top experimental findings. Two commonly used processes will be discussed below.

Tangential Flow Filtration (TFF)

The main technology for buffer exchange and concentrating proteins that has been used at commercial scale is TFF.^{34–36} In this technology, the protein is circulated by pumping through a series of hollow fiber tubes, which allow for the passage of water and small molecules but not large macromolecules. Attainment of high concentration formulations by TFF systems can be difficult because the required membrane flux may dictate higher concentrations at the membrane boundary than the targeted concentration. As an example, when a protein is concentrated to 100 mg/mL, the concentration at the membrane may be as high as 125 mg/mL. Depending on a protein's propensity to interact with and unfold at the surface, this could lead to decreased flux and eventual membrane clogging. Proteins are often shear sensitive, resulting in denaturation,^{37,38} and the continuous circulation through tubings and pulsation of the pumps may generate sufficient shear or cavitation to result in protein unfolding and precipitation. Although it may be possible to filter out the precipitated protein during manufacture of bulk protein, the shear sensitivity of a protein may also lead to formation of aggregates and particulates during filling of the final product that requires some sort of a pumping system. Typical filling systems include piston driven and diaphragm pumps. Piston driven pumps tend to generate more shear than rolling diaphragm pumps and this needs to be considered when filling high concentration protein solutions.

Another parameter that could limit a TFF system's ability to concentrate a protein solution is the solution's high viscosity. The viscosity increase may result in such high back-pressures during the TFF process that it may exceed the capacity of the pumps. In addition, as viscosity increases, it becomes increasingly difficult to remove the final finished product from the TFF unit, leading to economically unacceptable losses. Though these limitations may be handled by appropriate TFF equipment redesign, equipment design can be an expensive proposition. Since the viscosity of a macromolecular solution is dependent on temperature, increased temperatures

would improve the TFF process if the protein stability is not compromised. Alternatively, design of an appropriate formulation to decrease viscosity may help solve manufacturing process limitations. Hence, the formulation may need to be designed to ensure not only acceptable shelf life, but also compatibility with the manufacturing process.

Although TFF is the industry standard for concentrating macromolecules at large scale, there are alternatives. Rotating and vortex systems that enhance mass-transfer have recently been designed.^{36,39} Alternatively, any drying technique with a subsequent reconstitution at lower volumes can be used as will be discussed below.

Drying Techniques for Generating High Concentration Protein Formulations

As discussed earlier in "Solubility Considerations," a high concentration protein formulation can be achieved either by decreasing the solvent content while retaining the protein, e.g., using filtration systems, or by removal of solvent followed by reconstitution with the desired solvent volume. The latter can be achieved either by bulk processing or by dehydrating final product in vials. Bulk drying processes include spray drying,^{40,41} fluid bed drying,^{42,43} vacuum drying,^{44,45} or lyophilization in tray units.⁴⁶ Crystallization has proven to be effective in the development of insulin formulations, and shows great promise for the development of stable monoclonal antibody dosage forms.^{47,48} For parenteral protein products, bulk drying processes require operation under aseptic conditions, and have been used primarily in the aerosol industry. The alternative approach of lyophilizing sterile filtered products in vials is a cost-effective approach for generating high concentration protein formulations.

To use the lyophilizer as a concentrator, a loading volume of protein, V_L , at loading concentration C_L is lyophilized and then reconstituted with a volume of diluent, V_R , where $V_R < V_L$. The final concentration of the drug product, C_F , will then be:

$$C_F = C_L V_L / (V_R + V_S) \quad (3)$$

where V_S is the volume contributed from the remaining solids. To determine the appropriate V_R that gives a desired C_F , one can estimate V_S from the sum of the partial molar volumes of all the excipients. However, the most accurate way to achieve the target protein concentration is to reconstitute with a series of diluent volumes and empirically determine the appropriate reconstitution

volume. In addition to reaching the desired concentration, sufficient volume needs to be used in the reconstitution process to ensure that the required volume for administration can be removed from the vial. Thus, experiments may need to be designed whereby V_L and/or C_L are also varied to define the required overage in the final reconstituted product. These parameters can also have a major impact on the reconstitution properties of the final product. Figure 1 shows the effect of loading concentration on reconstitution times and morphology of the lyophilized solid. Each vial was loaded with the same total mass of protein and formulation excipients (i.e., a lower C_L required a larger V_L to maintain the loading mass). Vials were reconstituted to a final concentration of 125 mg/mL and investigated for solution clarity and disappearance of all solids after 10 min. The right side of Figure 1 shows that as the protein loading concentration is decreased, reconstitution occurs much more readily. Investigation of the morphology of the lyophilized solid by scanning electron microscopy (SEM; left side of Fig. 1) shows a very dense compact structure at 110 mg/

mL compared to a loosely packed layer of flakes at 40 mg/mL. Thus, the ease of wetting of the cake is very likely related to the differences in this morphology. SEM analysis of intermediate concentrations (not shown) demonstrates a gradual transition from a dense solid to the more loosely packed structure.

The process described above allows for designing a formulation with appropriate stability and tonicity. Although isotonicity is not necessarily required for SC administration, it may be desirable for minimizing pain upon administration.⁴⁹ Isotonicity is difficult to achieve because both the protein and the excipients are concentrated during the reconstitution process. As shown in Table 2, excipient:protein molar ratios of 500:1 will result in hypertonic preparations if the final protein concentration is targeted for >100 mg/mL. If the desire is to achieve an isotonic formulation, then a choice of lower molar ratio of excipient:protein will result in a potentially less stable formulation. This was indeed the issue involving a rhuMAb preparation where the hypertonic formulation at 500:1 excipient:protein molar ratio gave a significantly

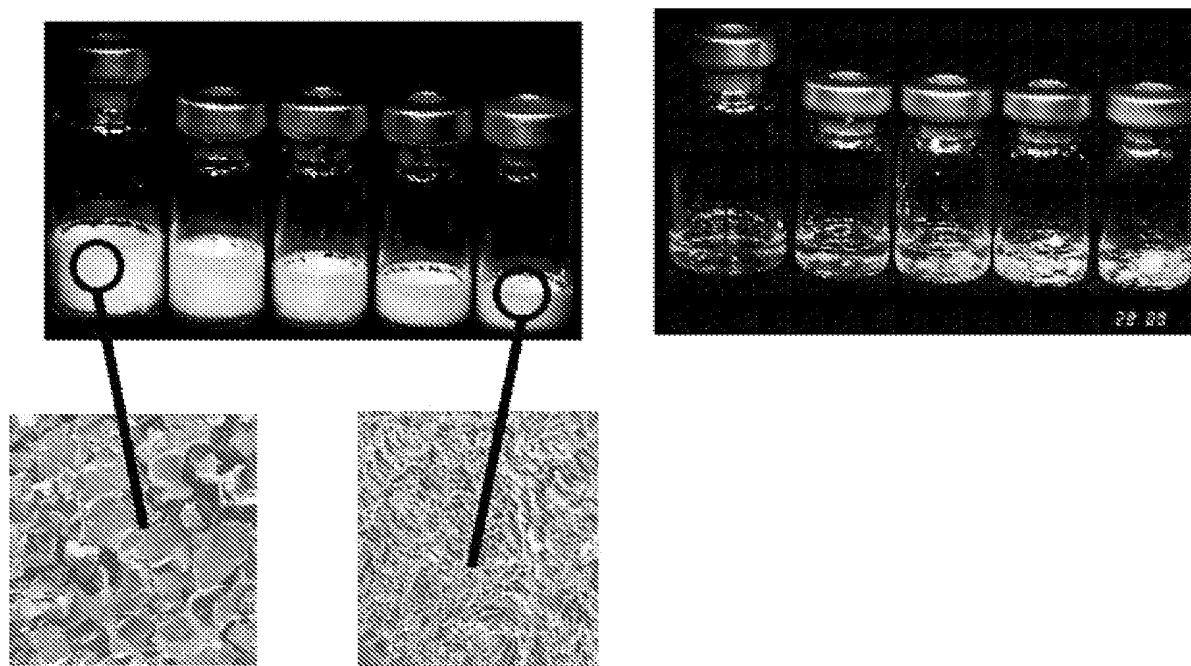


Figure 1. Lyophilization of a monoclonal antibody as a function of loading concentration. Upper left panel: Loading concentration from left to right was 40, 60, 80, 100, and 110 mg/mL, respectively, while maintaining the same total mass of MAb and excipients. Lower left panels: Scanning electron microscopy of lyophilized solid for the 40 and 110 mg/mL MAb loading concentrations. Right panel: 10 min after reconstitution of vials in upper left panel to 125 mg/mL with sterile water for injection.

Table 2. Concentration of Excipient as a Function of Final Reconstituted Protein Concentration and Molar Ratio of Excipient to Protein

Excipient:Protein Molar Ratio	250:1	500:1
C _F (mg/mL)	Excipient concentration (mM)	Excipient concentration (mM)
50	83	167
100	167	333
150	250	500
200	333	666

more stable preparation at controlled room temperature (30°C), but the stabilities at 250 and 500:1 molar ratios were comparable at 2–8°C storage (data not shown). In the case of this particular antibody where the target product profile and frequency of administration allowed for 2–8°C storage, stability was traded off for appropriate tonicity, and the 250:1 molar ratio was selected.

COST OF GOODS AND DELIVERY CONSIDERATIONS

Though high concentration formulations have the cost saving advantage of decreasing bulk storage space or number of product fills, they have undesirable overall cost of goods because of unrecoverable volumes from ultrafiltration units, fill vessels, and product containers. As an example, whereas the required volume overage in vials containing moderate protein concentrations is typically <10% of the fill volume, the overage for a >100 mg/mL protein formulation could be as high as 30% because of greater adherence of the viscous solution to product container surfaces. Unrecoverable product losses in the final dosage form could be minimized by decreasing product contact surface areas, e.g., with the use of smaller or narrower based vials, or by using prefilled syringe configurations where the solution can be pushed off the surfaces with the plunger head.

Protein formulations at high concentrations may also have physical properties that impact the ability to easily deliver the protein drug. For example, higher viscosity preparations may be difficult to administer by injection. Syringes for SC injection are often equipped with 26 or 27 gauge needles. If the viscosity of a high concentration formulation is sufficiently high, it may impact the ability to load and deliver from a syringe, and unless the viscosity can be reduced by appropriate formulation excipients, the high concentration

required for SC delivery may not be attainable. As an example, the protein concentration dependence of viscosity of an antibody is shown in Figure 2A. The time required to load 1 mL of this formulation into a syringe equipped with 27 gauge needle correlates very well with the viscosity of the formulation. Adjustment of the formulation to contain NaCl greatly reduces viscosity (Fig. 2B),

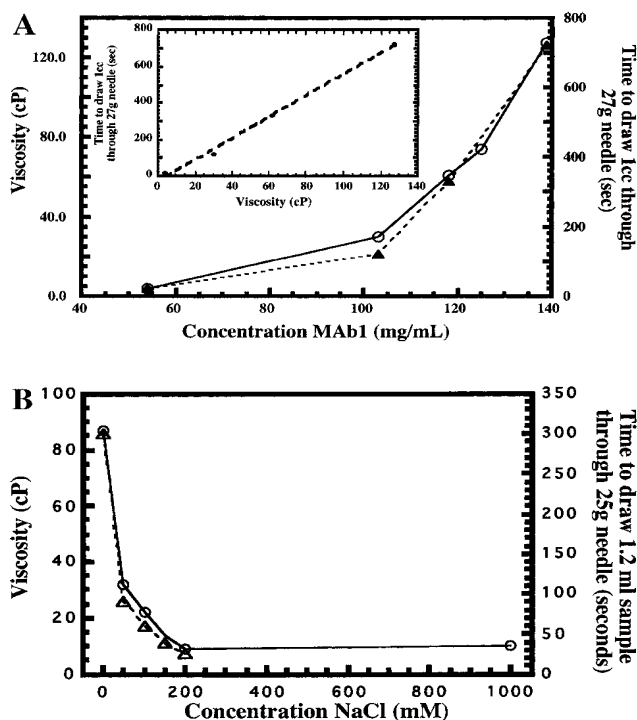


Figure 2. A: Viscosity (solid line, open circles) and syringe (27 gauge) loading time (dashed line, solid triangles) of a monoclonal antibody as a function of concentration. Insert is a linear regression fit of syringe (27 gauge) loading time versus viscosity (loading time = $-25.9 + 5.8 \times \text{viscosity}$, $R^2 = 0.995$). B: Viscosity (solid line, open circles) and syringe (25 gauge) loading time of a monoclonal antibody at 125 mg/mL as a function of NaCl concentration.

and yields reasonable times to draw the formulation into a syringe equipped with a 25 gauge needle. Thus, the alteration of the formulation excipients and the use of a slightly bigger needle yield reasonable times for withdrawal of a high concentration Mab formulation.

ANALYTICAL CONSIDERATIONS

Many of the analytical techniques currently available to explore covalent and conformational modifications in proteins^{50,51} are easily adapted to the study of proteins at high concentration. Moreover, analytical techniques that characterize solid-state protein dosage forms are useful in developing drying methods to produce a high protein concentration subsequent to reconstitution. These techniques include differential scanning calorimetry (DSC),⁵² particularly modulated DSC technology,^{53,54} to determine glass transition temperatures and thermal phase transitions of the solid-state matrix at high protein concentrations, Fourier-Transform Infrared Spectroscopy (FTIR)^{55,56} and Raman spectroscopy to study the protein's secondary structure after drying and during storage, and fluorescence spectroscopy recently used to investigate possible tertiary structural alterations during the drying process.⁵⁷

Generally the analytical technologies that are used to characterize proteins involve dilution to lower concentrations or exposure of the protein to solvent conditions that are very different than the initial formulation composition. This may have considerable impact on the results of the assay since a change in solvent composition or concentration may alter a protein's physical state in a way that is not representative of the initial conditions. This problem is especially important in the analysis of molecular weight/size distribution of proteins. SDS-PAGE, nongel sieving SDS-capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are often used to obtain molecular weight information. These techniques are useful for detecting covalently linked aggregates, or SDS non-dissociable aggregates but can't be used to determine non-covalent protein association states. The often-used gel permeation or size exclusion chromatographic method (SEC) provides such information but has several problems. The protein's interaction with the chromatographic resin and its hydrodynamic volume may alter the elution times; the use of globular protein

standards to estimate the molecular weight may thus be erroneous. When the interactions are ionic in nature due to the charge moieties on the resins, the addition of salts will decrease such interactions, but control experiments are then required to demonstrate that the increased ionic strength of the mobile phase did not perturb the size distribution of the protein. The impact of hydrodynamic volume leading to apparently larger proteins than actual size is especially noted for highly glycosylated proteins that have shapes different from the typical globular protein standards.^{58,59} The use of static light scattering detectors coupled with sizing chromatography (LC/LS)⁶⁰ allows for the absolute determination of molecular mass of a protein and its higher order aggregates and fragments during separation by gel sieving. Since measured elution times are no longer used to estimate molecular size, the problem of protein-resin interaction and molecular conformation are no longer important, provided the interactions do not prevent elution of the protein from the chromatographic column.

The most commonly used analytical technique for quantifying aggregates in pharmaceutical formulations is SEC with UV detection. In high concentration formulations where reversible protein self-association predominates, the determination of aggregate levels by the SEC method may be inconsistent and inaccurate. The SEC procedure involves a high dilution of the protein solution during injection onto the column that may lead to dissociation of aggregates with rapid dissociation rate constants. Moreover, to prevent detector saturation, high concentration protein solutions are generally diluted even prior to injection onto the SEC column. The rapid dissociation of a monoclonal antibody upon dilution⁶¹ yielded varying aggregate levels by SEC, depending on the time and temperature of analysis after sample dilution. To use SEC as an analytical tool for quantification of aggregates and estimation of formulation's shelf life and physical characteristics, it is imperative to have reproducible measurements. For a slower dissociating protein in the example shown in Figure 3, reproducible quantification of aggregates in an 80 mg/mL formulation is achieved by limiting the analysis time to less than 24 h and by controlling the most critical parameters, the protein concentration after dilution and sample temperature (a 5-fold decrease in dissociation rate was observed for every 10°C decrease in temperature of the auto-sampler compartment of the HPLC). The clinically relevant aggregate level for

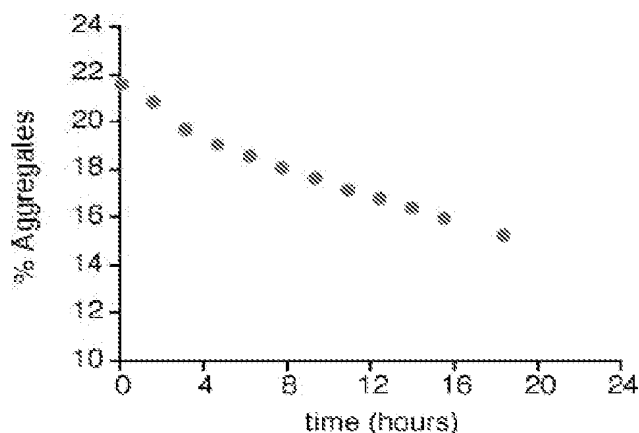


Figure 3. Dissociation of aggregates in an 80 mg/mL protein formulation at 30°C upon dilution to 0.1 mg/mL. At 5°C, the dissociation rate is too small to detect any changes in the initial aggregate level during this time frame.

a protein that undergoes reversible self-association is that which remains after dilution in the IV solution for administration (if applicable) and after initial dilution in blood volume at 37°C. This may have to be determined separately from the routine stability testing of the pharmaceutical product, particularly when dissociation rate is slower than practical for routine analyses.

Technologies such as static and dynamic light scattering^{62–65} and analytical ultracentrifugation^{62–65} provide more representative information about protein self-association states at high protein concentrations because they maintain the concentration during analysis and can also be performed at high concentration. Another technique uses preparative centrifuges coupled with a specially designed microfractionator to obtain sedimentation equilibrium measurements of molecular weight in concentrated as well as multi-component protein systems.⁶⁶ Limitations of the light scattering methods are that they are not quantitative and that they are subject to multiple scattering artifacts at high protein concentrations. Although sedimentation equilibrium centrifugal analysis can be used to characterize an aggregating system, the analysis requires fitting of data to several exponentials and is model dependent.⁶⁷ Sedimentation velocity analysis has been greatly improved and algorithms are now available to determine small amounts of protein aggregates and sedimentation coefficients.^{68,69} The determination of the sedimentation coefficient can provide valuable information regarding overall shape of

the protein when coupled with computations of hydrodynamic bead models.^{70,71} However, correction of apparent molecular weights and sedimentation coefficients due to nonideality at high protein concentrations is difficult to achieve.

SUMMARY AND CONCLUSIONS

Protein properties such as self-association/aggregation, solubility, and viscosity pose challenges to developing pharmaceutically and economically acceptable formulations at high concentration. In addition to maintaining suitable stability, protein properties at high concentration may impact the ability to administer the drug, to manufacture at large scale, and also the yields of the two processes. Analytical methodologies to investigate protein properties at high concentration are also limited and must be considered with caution as they are impacted by the very property under investigation. Very little work has been published on high concentration protein formulation development and this review has touched on the key issues with examples of the potential solutions to the issues. Achieving a suitable formulation requires an integrated approach whereby a stable formulation is developed that can also be successfully administered and economically manufactured.

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REIVINDICACIONES

1. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 210 mM de trehalosa dihidratada, 10 mM de metionina, 0.06% de polisorbato 80, 2,000 U/mL de rHuPH20 a pH 5.5.
2. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 210 mM de trehalosa dihidratada, 10 mM de metionina, 0.06% de polisorbato 80, 2,000 U/mL de rHuPH20 a pH 6.1.
3. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 210 mM de trehalosa dihidratada, 10 mM de metionina, 0.06% de polisorbato 80, 12,000 U/mL de rHuPH20 a pH 5.5.
4. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 210 mM de trehalosa dihidratada, 10 mM de metionina, 0.06% de polisorbato 20, 12,000 U/mL de rHuPH20 a pH 5.5.
5. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 120 mM de cloruro de sodio, 10 mM de metionina, 0.02% de polisorbato 80, 12,000 U/mL de rHuPH20 a pH 5.5.
6. Una formulación farmacéutica del anticuerpo anti-CD20 que comprende 120 mg/mL de Rituximab, 20 mM de L-histidina, 120 mM de cloruro de sodio, 10 mM de metionina, 0.04% de polisorbato 80, 12,000 U/mL de rHuPH20 a pH 6.0.

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Trámite: 11
Evento: 379
Actuación: 444

Re: PI.-F. HOFFMANN-LA ROCHE AG.-Solicitud de Patente de Invención en Fase Nacional PCT para “**COMPOSICIONES LIOFILIZADAS QUE COMPRENDEN RITUXIMAB**”. -Clase **A61K 9/00**.-Expediente número **12-040.141.-**

1. Me refiero al auto dictado por ese Despacho notificado por fijación en línea del 10 de junio del 2019.

2. Con el fin de responder las objeciones expuestas por el Señor Examinador en su Concepto Técnico correspondiente al Oficio N° **4208** en relación con el Título, la claridad del Capítulo Reivindicatorio, la unidad de invención y el nivel inventivo de la presente invención, me permito dirigir su atención a las siguientes modificaciones y argumentos técnicos por medio de los cuales no sólo se superan completamente dichas objeciones, sino que además se establece de manera incontrovertible la patentabilidad de la solicitud en estudio.

3. **PRESENTACIÓN DE SOLICITUD DIVISIONAL.**

En primer lugar, me permito manifestar ante ese Despacho que, con el fin de superar la objeción de Unidad de Invención presentada por el Examinador, es deseo del solicitante fraccionar la materia de la presente solicitud en función de los grupos inventivos señalados en el concepto técnico anteriormente mencionado. Por lo tanto, conforme a lo estipulado en el artículo 36 de la Decisión Andina 486/2000, se ha dividido **la presente solicitud de patente en tres (4) solicitudes**, a saber:

- Una **solicitud parental** con un capítulo reivindicatorio modificado compuesto por **6 reivindicaciones** (que corresponden a las reivindicaciones 1 a 3, 5, 6 y 9 actualmente presentadas), en donde se reclama una formulación farmacéutica del anticuerpo anti-CD20 (Rituximan 120mg/ml), L-histidina, trehalosa, metionina, polisorbatos y rHuPH20;
- Una **primera solicitud divisional** con un capítulo reivindicatorio compuesto por **3 reivindicaciones** (que corresponden a las reivindicaciones 4, 7 y 8 actualmente presentadas en la solicitud parental), en donde se reclama una formulación

farmacéutica del anticuerpo anti-CD20 (Rituximan 120mg/ml), ácido acético o ácido cítrico, trehalosa, metionina, polisorbatos y rHuPH20;

- Una **segunda solicitud divisional** con un capítulo reivindicatorio compuesto por **1 reivindicaciones** (que corresponde a la reivindicación 10 actualmente presentada en la solicitud parental), en donde se reclama una formulación farmacéutica del anticuerpo anti-CD20 (Obinutuzumab 25mg/ml); y
- Una **tercera solicitud divisional** con un capítulo reivindicatorio compuesto por **9 reivindicaciones** (que corresponde a las reivindicaciones 11 a 19 actualmente presentadas en la solicitud parental), en donde se reclama una formulación farmacéutica del anticuerpo anti-CD20 (Ocrelizumab 30mg/ml).

En relación con lo anterior, me permito resaltar que dicho fraccionamiento refleja una división de la materia de acuerdo con los grupos inventivos identificados por el Examinador en el Concepto técnico correspondiente al Oficio N° 4208.

Adicionalmente, es pertinente indicar que los detalles y documentos relacionados con cada una de las solicitudes divisionales se describen en el respectivo memorial de presentación de dichas solicitudes, los cuales han sido allegados el día de hoy.

4. **TÍTULO.**

En relación con el título de la solicitud, me permito manifestar que es deseo del solicitante modificar dicho título de tal forma que refleje la materia reclamada en el capítulo reivindicatorio modificado, el cual se presenta más adelante en este memorial, así las cosas, el nuevo título se lee como sigue:

"COMPOSICIONES LIOFILIZADAS QUE COMPRENDEN RITUXIMAB"

En este sentido, me permito enfatizar que este nuevo título refleja de manera clara el objetivo de la invención, destacando las características técnicas esenciales de la materia reivindicada.

5. **CAPÍTULO REIVINDICATORIO.**

(a) **Modificaciones al Capítulo Reivindicatorio.**

Por otro lado, me permito manifestar que es deseo del solicitante presentar un **capítulo reivindicatorio modificado**, que remplaza al actualmente presentado, el cual es resultado del fraccionamiento de la solicitud en referencia y, que se describe en el

numeral 3 del presente memorial, con el propósito de superar las objeciones presentadas como parte del concepto técnico realizado por ese Despacho.

En conexión con el mencionado **capítulo reivindicatorio modificado**, me permito resaltar que éste no constituye de ninguna manera una ampliación al alcance de la solicitud, tal como fue originalmente presentada, toda vez que define claramente el objeto de la presente invención de acuerdo con las enseñanzas de la descripción. Dichas modificaciones se exponen a continuación:

- Se eliminó la palabra “estable” de la **reivindicación 3**.

De esta forma, **el capítulo reivindicatorio modificado queda constituido por 6 reivindicaciones**, las cuales se refieren de manera más clara y concisa a las composiciones liofilizadas que comprenden rituximab, que son objeto de la presente solicitud, y que cuentan con pleno sustento en las enseñanzas de la descripción.

(b) **Claridad y Concisión del Capítulo Reivindicatorio.**

Con respecto a la claridad y la concisión del capítulo reivindicatorio, el examinador afirma lo siguiente:

- *“El capítulo reivindicatorio contiene un excesivo número de reivindicaciones independientes de la misma categoría que repiten materia, en consecuencia, presentan el mismo alcance. Se sugiere incluir en una sola reivindicación independiente todas las características técnicas esenciales de la invención e incluir en las reivindicaciones dependientes modalidades preferentes.”*

Frente a esta objeción, me permito mencionar que la presente solicitud se ha fraccionado, de acuerdo con la objeción de unidad de invención hecha por el examinador. Razón por la cual el capítulo modificado no contiene un número excesivo de reivindicaciones, si no que este consta ahora de 6 reivindicaciones que comparten las características técnicas esenciales de la invención aquí reclamada.

Con base en lo anterior, amablemente solicito que se retire la objeción a este respecto.

- *“Las reivindicaciones 1-19 no son claras, dado que la estabilidad y el pH se refieren a resultados a alcanzar y no definen a la formulación, puesto que las reivindicaciones incluirían no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características técnicas esenciales, estructurales o funcionales, que contribuyen a la obtención del efecto.”*

A este respecto, primero, me permito aclarar que la palabra estable fue eliminada del capítulo reivindicatorio modificado. Segundo, me permito indicar que el pH, de hecho, si es una característica técnica esencial de la composición reclamada, ya que este permite mantener el anticuerpo inclinado hacia una carga, bien sea positiva o negativa, en donde es más estable, esto teniendo en cuenta el pH al cual se encuentra el punto isoeléctrico nos permite saber dicha característica. Por lo cual, es claro que la persona normalmente versada en la materia fácilmente identificaría el pH como una característica técnica esencial de las composiciones reclamadas.

En este orden de ideas, amablemente solicito que la objeción a este respecto quede totalmente superada.

En relación con los argumentos anteriormente expuestos, me permito señalar que el capítulo reivindicatorio modificado presentado junto con el presente memorial supera completamente las objeciones presentadas por el Señor Examinador. Asimismo, el capítulo reivindicatorio modificado cuenta con la suficiente claridad, concisión y sustento para definir la materia a reclamar, y, por ende, cumple a cabalidad con lo estipulado por el Artículo 30 de la Decisión Andina 486 de 2000.

6. **UNIDAD DE INVENCION.**

Por otro lado, el Señor Examinador señala que "(...) *Derivado de lo anterior, se concluye que no existe un concepto común entre los sistemas farmacéuticos seleccionados para este anticuerpo, es decir, los sistemas utilizados para formular Ocrelizumab, Rituximab y Obintuzumab no resultan equivalentes entre sí.*"

En este sentido, el Señor Examinador sugiere presentar las respectivas solicitudes divisionales para cada uno de los grupos inventivos presentados en esta solicitud, o restringir la solicitud a un solo grupo inventivo.

En respuesta a esta objeción, me permito indicar que, tal como se mencionó en el numeral 3 del presente memorial, es deseo del solicitante presentar tres solicitudes divisionales que comprende la materia de los grupos inventivos II, III y IV señalados por el Señor Examinador. Por su parte, la presente solicitud se refiere únicamente al grupo inventivo I, de acuerdo con las sugerencias del señor examinador.

En consecuencia, el capítulo reivindicatorio modificado se refiere a un único concepto inventivo, el grupo inventivo I, superando así la objeción del Señor Examinador.

7. **NIVEL INVENTIVO.**

Finalmente, el examinador considera que la materia reclamada en las reivindicaciones 1, 2, 3, 5, 6 y 9 (reivindicaciones 1 a 6 del capítulo reivindicatorio modificado) carecería de nivel inventivo con respecto a las enseñanzas de los documentos **D1** (US2007071675) y **D2** (WO2009009407).

En conexión con lo anterior, el examinador inicialmente afirma que el documento D1 es el estado de la técnica más cercano a la invención definida en las reivindicaciones 1 a 6.

En este sentido, el examinador establece que la diferencia entre la invención de las reivindicaciones 1 a 6 y el documento D1 consiste en que la composición reclamada incluye como anticuerpo anti-CD20 al Rituximab. Así, el examinador asegura que no existe evidencia de un efecto técnico asociado a la diferencia mencionada.

No obstante, el examinador que el documento D2 ya menciona el incluir Rituximab en concentración entre 20 a 300mg/ml para obtener composiciones estables.

Por lo tanto, el examinador concluye que la persona del oficio normalmente versada en la materia estaría motivada a incluir el Rituximab del documento D2 en la formulación del documento D1, para así llegar al objeto de las reivindicaciones 1 a 6 de la solicitud de manera obvia. En consecuencia, las reivindicaciones 1 a 6 no tienen nivel inventivo.

En respuesta a tales afirmaciones, me permito indicar que las enseñanzas de los documentos señalados no anticipan ni permiten llegar de forma obvia a la presente invención, con todas y cada una de sus características técnicas esenciales, tal y como se reclaman en el capítulo reivindicatorio modificado de la presente solicitud.

(a) *Problema técnico de la presente invención*

Primeramente, me permito señalar el problema técnico de la invención y como esta lo resuelve, a la luz de la información que se tenía en el momento de la primera presentación de la solicitud bajo estudio.

En este sentido, me permito manifestar que el Rituximab es conocido por ser susceptible a la agregación incluso a bajas concentraciones (0,2 a 10 mg/ mL). Lo anterior fue establecido por Andersen et al. (Protein Science 19: 279-290 (2010) (Anexo I)) quién evaluó la agregación de IgG1 usando Rituximab como un sistema modelo.

De igual manera, se conoce que las composiciones de Rituximab incluyen variantes de carga, tal y como lo demuestra Moorhouse et al. (J. Pharm Biomed Análisis 16: 593-603 (1997), (Anexo II)), y la alteración de la distribución de tales variantes podría potencialmente alterar las actividades biológicas del anticuerpo.

Por otro lado, en septiembre de 2009 se sabía que era un reto la preparación de formulaciones estables que contienen altas concentraciones de una proteína, por ejemplo, aproximadamente 120 mg/mL de Rituximab, tal y como lo explica Shire et al. (J. Pharmaceutical Sciences 93: 1390-1402 (2004), (Anexo III)), quien establece que:

"El desarrollo de formulaciones de alta concentración de proteínas también da lugar a varios problemas de fabricación, de estabilidad, de análisis y de entrega. [...] la degradación dependiente de la concentración de agregación es el mayor desafío para el desarrollo de formulaciones de proteínas en estas concentraciones más altas. Además del potencial para la agregación de proteínas no-nativas y formulaciones particuladas, puede producir auto-asociación reversible, lo que contribuye a las propiedades tales como la viscosidad, que complica la entrega por inyección. Viscosidades mayores, también complican la fabricación de altas concentraciones de proteínas por métodos de filtración". (Ver, Resumen de Shire)

Así entonces, en vista del potencial de Rituximab al agregarse a, incluso, concentraciones relativamente bajas (por ejemplo 0,2 a 10 mg/mL) tal y como se señaló anteriormente, es sorprendente que la preparación de formulaciones de alta concentración de Rituximab (por ejemplo, aproximadamente 120 mg/mL) se mantenga estable durante largo tiempo de almacenamiento.

Por lo tanto, es evidente que esta tarea es aún más difícil, en el caso de que dos proteínas muy diferentes (el anticuerpo IgG1 Rituximab y la proteína rHuPH20) sean formuladas juntas. Tales combinaciones son difíciles de trabajar, así por ejemplo realizar diferentes ensayos con combinaciones de proteínas, como la proteína morfogenética ósea y el colágeno, como lo describen Friess et al. (Pharm. Development & Tech. 4 (3): 387-396 (1999) (Anexo IV)), o como interferón y albúmina, tal como lo enseña Hawe y Friess (J. Pharm. Sci. 96 (11): 2987-2999 (2007) (Anexo V)). Dichos trabajos muestran, precisamente, la dificultad a la hora de realizar experimentación con combinaciones de proteínas diferentes, hecho que no es despreciable para la persona normalmente versada en la materia.

Esto es porque, por lo general, se requiere que los científicos resuelvan una compleja serie de problemas: (i) encontrar un factor determinante para la estabilidad entre la primera y la segunda proteína, respectivamente, (ii) considerar, analizar y minimizar nuevas reacciones químicas, y (iii) analizar, entender y evitar interacciones fisicoquímicas proteína-proteína no deseadas y resultantes de la agregación de proteínas.

Entonces, es relevante que, para la combinación de dos proteínas diferentes en una sola formulación, los inventores de la presente solicitud tuvieron que encontrar un equilibrio entre todos estos parámetros de formulación. Al mismo tiempo, se enfrentaron con nuevos problemas de inestabilidad debido a la co-formulación, ya que al igual que Rituximab, la enzima rHuPH20 también era propensa a problemas significativos de inestabilidad.

Lo anterior, fue reconocido en el año 1952 (Ver por ejemplo la patente de Estados Unidos número 2.795.529, (Anexo VI)). Esta patente informa que la desactivación observada de la hialuronidasa se debe a reacciones complejas, y además se encontró que el EDTA, que se utiliza a una concentración de 0,1 a 1,0 M, junto con NaCl y CaCl₂, ayuda a solventar tales problemas de inestabilidad.

En este punto, también es importante indicar que en el caso de las formulaciones que comprenden dos proteínas activas, deben considerarse las interacciones proteína-proteína, ya que pueden conducir a nuevas vías críticas de agregación o inactivación de proteínas. Las interacciones proteína-proteína se rigen en gran medida por las interacciones carga-carga que fueron dramáticas para la combinación anticuerpo-enzima en la solicitud de patente mencionada.

En particular, el anticuerpo anti-CD20 Rituximab tiene un alto punto isoeléctrico (pI), que es el valor de pH de carga neta cero, de aproximadamente 8,68¹. Por lo tanto, las moléculas del anticuerpo están sustancialmente cargadas positivamente en un agente tampón a un pH de 5,5. Esto conduce a una repulsión neta entre las moléculas que se requieren para la estabilidad del anticuerpo.

Por otro lado, rHuPH20 tiene un pI bajo de aproximadamente 5,73. Esto lleva a una situación en la que casi la mitad de las moléculas de rHuPH20 tienen una carga negativa neta a un pH de 5,5, que es el pH asociado con la estabilidad de Rituximab. En vista de esto, se hubiera esperado que se formaran heteroagregados solubles e insolubles entre Rituximab y rHuPH20, en caso de que el anticuerpo y la enzima se formularan conjuntamente. Esta expectativa se basa en la comprensión básica de como funciona la carga total en las proteínas, según lo determinado por el pI de cada molécula y el pH de la solución, ya que estos pueden afectar las interacciones proteína-proteína en una formulación que las contiene.

Así las cosas, me permito citar a Chi et al. (Pharmaceutical Research 20: 1325-1336 (2003) (Anexo VII)), quienes explican este fenómeno:

¹ <http://www.drugbank.ca/drugs/DB00073>

"[...] las cargas en las moléculas de proteínas también dan lugar a interacciones electrostáticas entre las moléculas de proteínas. [...] Cuando las proteínas poseen grupos de cargas positivas y negativas (por ejemplo, a valores de pH cercanos al pI), la distribución de carga anisotrópica en la superficie de la proteína podría dar lugar a dipolos. En tales casos, las interacciones proteína-proteína podrían ser muy atractivas, haciendo que los procesos de ensamblaje, como la agregación, sean energéticamente favorables [...]"(página 1326, columna derecha, penúltimo párrafo)

Dados los pI opuestos para Rituximab y rHuPH20, y la información conocida en el estado de la técnica antes de la presentación de la solicitud bajo estudio, la persona normalmente versada en la materia se habría preocupado por la interacción entre dos proteínas como resultado de las interacciones proteína-proteína no intencionadas. En consecuencia, **combinar eficazmente Rituximab y rHuPH20 en la misma solución requiere de una gran cantidad de experimentación y esfuerzo del investigador por lograr el objetivo.**

Por lo tanto, por las razones expuestas anteriormente, a la fecha de presentación de la presente solicitud no se esperaba que Rituximab y rHuPH20 pudieran ser co-formulados en una formulación que comprende dicho anticuerpo en una concentración elevada (alrededor de 120 mg/mL) de tal manera que la formulación permaneciera estable en el tiempo.

(b) Diferencias entre la solicitud en cuestión y el documento D1

Así las cosas, me permito mencionar que la solicitud de patente en cuestión está dirigida a proteger una formulación farmacéutica estable para la administración subcutánea de un anticuerpo anti-CD20, específicamente Rituximab, que emplea también hialuronidasa rHuPH20 en la formulación.

Ahora bien, a partir de la atenta lectura de D1 es absolutamente claro que dicha anterioridad no se refiere a una formulación farmacéutica como la reclamada en la presente invención. Contrario a esto, D1 se refiere a proteínas recombinantes de unión multivalentes y multiespecíficas, métodos de fabricación, y específicamente a sus usos en la prevención y/o tratamiento de enfermedades inflamatorias agudas y crónicas y otras enfermedades, tal como se evidencia en el párrafo 2 de dicho documento.

Así mismo, me permito señalar que la reivindicación 1 del documento D1 reclama una proteína de unión (multivalente) que comprende una cadena de polipéptido, donde tal cadena de polipéptido comprende VD1-(X1)_n-VD2-C-(X2)_n, siendo VD1 un primer dominio variable, VD2 un segundo dominio variable, C es un dominio constante, X1 representa un aminoácido o polipéptido, X2 representa una región Fc y n es 0 o 1.

Asimismo, la reivindicación dependiente 2 reclama dominios de cadena pesada VD1 y VD2. Estas son construcciones de dominio de cadena pesada variable, es decir, biespecíficos y multiespecíficos incluso construcciones capaces de unirse dos o más antígenos, dichas construcciones tienen diferentes especificidades de unión de antígeno para VD1 y VD2.

En conexión con lo anterior, vale la pena resaltar que el párrafo 0017 de dicho documento establece que:

*“En otra forma de realización preferida, las proteínas de unión divulgadas anteriormente son capaces de unirse a uno o más objetivos. Preferiblemente, el objetivo se selecciona de entre el grupo formado por citoquinas, proteínas de superficie celular, enzimas y receptores. Preferiblemente, la proteína de unión es capaz de modular una función biológica de uno o más objetivos. Más preferiblemente, la proteína de unión es capaz de neutralizar uno o más objetivos. La proteína de unión de la invención es capaz de unirse a citoquinas seleccionadas del grupo que consiste de linfoquinas, monoquinas, y hormonas de polipéptidos. En una realización específica la proteína de unión es capaz de unir pares de citoquinas seleccionadas del grupo que consiste de IL-1 [alfa] y la IL-1 [beta]; IL- 12 e IL-18, TNF [alfa] e IL-23, TNF [alfa] e IL-13; TNF e IL- 18; TNF e IL-12; TNF e IL-1 beta; TNF y MIF; TNF e IL-17, y TNF e IL-15; TNF y VEGF; VEGFR y EGFR; de IL- 13 e IL- 9; de IL-13 e IL-4 ; de IL-13 e IL-5 ; de IL-13 e IL-25; de IL-13 y TARC; de IL-13 y MDC; IL-13 y MIF; de IL-13 y TGF-[beta]; de IL-13 y LHR agonista; de IL-13 y CL25; de IL-13 y SPRR2a; de IL- 13 y SPRR2b; de IL-13 y ADAM8; y TNF [alfa] y PGE4, IL-13 y PED2, TNF y PEG2. En otra realización, la proteína de unión de la invención es capaz de unir pares de blancos seleccionados del grupo que consiste de **CD138 y CD20**; CD138 y CD40; **CD19 y CD20**; **CD20 y CD3**; CD38 y CD138; **CD38 y CD20**; CD38 y CD40; **CD40 y CD20**; CD-8 e IL-6; CSPG y RGM A; CTLA4 y BTNO2; IGF1 y IGF2; IGF1/2 y Erb2B; de IL- 12 y TWEAK; de IL- 13 e IL - 1[beta]; MAG y RGM; de NgR y RGM A; NogoA y RGM A; OMgp y RGM A; PDL-1 y CTLA4; RGM A y RGM B; Te38 y TNF [alfa]; TNF [alfa] y Blys; TNF [alfa] y de CD-22; TNF[alfa] y CTLA-4; TNF[alfa] y GP130; TNF [alfa] e IL-12p40, y TNF[alfa] y el ligando de RANK.” (Énfasis añadido).*

Entonces, es claro que todas las proteínas de unión mencionadas no se unen a un solo antígeno, sino a más de uno. Por lo tanto, **el número de proteínas de unión divulgadas en este documento es inmenso, además que todas ellas son biespecíficas o multiespecíficas**, en donde entre el inmenso número de proteínas de unión, solo unas pocas se unen a CD20 y otra molécula, tal como CD138, CD19, CD3, CD38 o CD40.

De lo anterior, es claro que el documento D1 está totalmente alejado de la presente invención, pues la molécula de anticuerpo, Rituximab, empleada en la presente solicitud es un anticuerpo monoclonal anti-CD20 conocido en el estado de la técnica. Además, **la**

presente solicitud no busca en ningún momento modificar la estructura del anticuerpo (Rituximab o cualquier otro) para obtener una molécula de unión biespecífica o multiespecífica.

Junto con lo anterior, me permito retomar el problema técnico de la solicitud bajo estudio, el cual se refiere a la dificultad de preparar formulaciones estables que contengan altas concentraciones de dos proteínas. El desarrollo de formulaciones de alta concentración de proteínas resulta en varios desafíos de fabricación, estabilidad, análisis y entrega, tal como se describió en el punto (a) antes mencionado. Además, el Rituximab tiene el potencial de agregarse incluso a concentraciones relativamente bajas (por ejemplo, 0.2 a 10 mg/ml). Al igual que Rituximab, rHuPH20 también era propenso a problemas de inestabilidad significativos.

Por lo tanto, la co-formulación que resulta en una combinación estable de dos proteínas estructural y funcionalmente diferentes: **el anticuerpo monovalente, no biespecífico Rituximab y la enzima hialuronidasa rHuPH20, no puede derivarse de D1 solo relacionando construcciones biespecíficas o multiespecíficas.**

Aunado a lo anterior, me permito citar el párrafo 0038 de D1, el cual se refiere a diferentes modos de administración de dichos constructos biespecíficos y multiespecíficos, es decir, de forma parenteral, subcutánea, intramuscular, intravenosa, intraarticular, intrabronquial, intraabdominal, intracapsular, entre otras. En donde, el párrafo 0184 menciona que las composiciones farmacéuticas de dichos constructos biespecíficos y multiespecíficos son, en general, de administración parenteral.

En relación con todo lo anterior, es importante indicar que, en lo que se refiere a la mención del anticuerpo anti-CD20 en el documento D1, esta solo se da en las reivindicaciones 24 y 25 que cubren cientos de proteínas diana para dichas construcciones biespecíficas y multiespecíficas, desde ABCF1 y terminando con CTLA-4, y en los párrafos 17 y 20 que mencionan distintas modalidades posibles del anticuerpo biespecífico y se indica que puede unirse a CD20 y otro antígeno tal como CD19, CD3, CD40, etc. Por lo tanto, este documento es irrelevante para el campo de formulaciones de anticuerpos monoclonales CD20, y más específicamente para el Rituximab.

En cuanto a la hialuronidasa, el Examinador indica que es mencionada en D1 en una concentración de 100-5000 U/ml, pero me permito aclarar que dicha proteína es nombrada como adyuvante en una composición farmacéutica para administración por vía parenteral, y, adicionalmente, en ninguna parte del documento se menciona una concentración para la misma, contrario a lo que afirma el examinador.

Analizada en detalle la composición farmacéutica de D1 frente a la reclamada en la presente invención, se tiene:

Tabla 1. Comparación de las características técnicas esenciales de la presente invención con las de D1

Características Esenciales	D1 (De acuerdo con el examinador)	Comentarios
Una formulación farmacéutica del anticuerpo anti-CD20 que comprende:	Composición farmacéutica que comprende: (Páginas 30-31, Párrafos [0175 y 0184])	La descripción principal son las moléculas bi y multiespecíficas. Los párrafos [0175 y 0184] solo tienen texto muy general para cualquier composición farmacéutica, por lo que no es relevante para la presente invención.
-Rituximab 120mg/ml	Anticuerpo anti-CD20 0.1-250 mg/ml (página 3, párrafo [0017], página 7, párrafo [0040], página 31, párrafo [0184], página 18, párrafos [0111-0112] y páginas 60-61, Ejemplo 4)	Rituximab no se menciona. La conc. 120 mg/ml no se menciona. Párrafo [0017] menciona moléculas biespecíficas sin relevancia: CD138 y CD20; CD19 y CD20; CD20 y CD3; CD38 y CD20; CD40 y CD20. Párrafo [0040] menciona cientos de ligantes objetivo. <u>Párrafo [0184], menciona 0.1-250 mg/ml para la molécula biespecífica o multiespecífica, pero no rituximab.</u> Página 18, párrafos [0111-0112] menciona cientos de los denominados anticuerpos parentales que no son objeto de la invención de D1. Páginas 60-61, ej. 4) menciona una construcción de DVD-Ig biespecífica CD20-CD3 que es una molécula completamente diferente de rituximab.
-L-Histidina 20mM Cloruro de Sodio 120mM	L-Histidina (1-50mM) Cloruro de Sodio (<300mM) (Página 31, Párrafo [0184])	La formulación D1 es para anticuerpos biespecíficos y multiespecíficos y, por lo tanto, no es adecuada para la co-formulación de dosis altas de rituximab. L-histidina (1-50 mM) y cloruro de sodio (<300 mM) (página 31, párrafo [0184]) son rangos generales. No se menciona específicamente la concentración de 20mM para la L-

		Histidina, ni 120mM para Cloruro de sodio.
- Trehalosa dihidratada 210mM	Trehalosa (240mM) (Página 31, Párrafo [0184])	La trehalosa solo se menciona generalmente, y no se menciona que este en una concentración tal como 210mM, ni en ninguna otra.
-Metionina 10mM	Metionina (1-50mM) (Página 31, Párrafo [0184])	Es un rango general, no muestra una concentración específica o al menos preferida.
-Polisorbato 20/80 0.06%	Polisorbato 20/80 (<0.05%) (Página 31, Párrafo [0184])	Polisorbato 20/80 en un a conc. de 0.06% no se menciona. Este es solo mencionado de forma general.
-rHUPH20 2000 o 12000 U/ml	Hialuronidasa recombinante humana (100-5000 U/ml) (Página 31, Párrafo [0184])	La Hialuronidasa recombinante humana es solo mencionada de forma general, pero jamás se enseña un rango concentración, ni mucho menos una conc. específica, para dicha proteína en D1.

En este punto vale la pena resaltar que en ninguna de las modalidades preferentes o ejemplos de D1 se enseña una composición farmacéutica que contenga como principio activo anticuerpo anti-CD20 únicamente, específicamente Rituximab, presente en alta concentración y que además tenga hialuronidasa en alta concentración. De esta manera, las enseñanzas de D1 no pueden hacer evidente la formulación enseñada en la presente invención.

Por último y en vista de lo anteriormente planteado, es claro entonces que el documento D1 de ninguna manera sugiere una formulación farmacéutica estable del anticuerpo anti-CD20 Rituximab de acuerdo con la presente invención, como una solución técnica al problema abordado en la solicitud de patente en cuestión.

(c) Las enseñanzas de D1 con las enseñanzas de D2 no llegan a la formulación de la presente invención

Teniendo en cuenta lo anteriormente mencionado, atentamente me permito señalar que incluso si la persona medianamente versada en la materia en efecto encontrara alguna razón para partir del documento D1 y se viera inclinado a modificar tales enseñanzas con D2 con el objetivo de llegar a la formulación farmacéutica estable de la presente invención, es claro que dicha anterioridad D2 de ninguna manera enseña o sugiere una composición que comprende un anticuerpo anti-CD20, como Rituximab, que emplea también hialuronidasa rHuPH20 en la formulación.

El documento D2 se refiere a formulaciones muy específicas del ofatumumab monoclonal humano anti-CD20. El ejemplo de la solicitud de patente, así como las figuras, solo se refieren a una formulación de citrato de 17 mg o una formulación de acetato de sodio de 20 mg/ml de ofatumumab (consulte la breve descripción de las figuras en la página 7 y los ejemplos 4.1 y 4.2 en la página 15). También se reivindican formulaciones de acetato de sodio con base libre de arginina y EDTA (véase la reivindicación 1). Por lo tanto, el documento no se refiere a una formulación de dosis altas de rituximab con L-histidina ni a una formulación conjunta con una hialuronidasa para administración subcutánea. Por lo tanto, tampoco D2 es relevante para el nivel inventivo.

En este orden de ideas, ¿Cómo la persona normalmente versada en la materia encontraría motivación alguna en dicho documento D2 para llegar a la formulación reclamada, es más para modificar las enseñanzas del documento D1 con la intención de llegar a la invención bajo estudio?

Adicionalmente, me permito mencionar que la presente invención según se reivindica, son formulaciones altamente concentradas de rituximab que comprenden 120 mg/ml de rituximab formulado con L-histidina 20 mM, y que también comprende metionina 10 mM formulada juntamente con la enzima hialuronidasa altamente concentrada rHuPH20, que sorprendentemente tiene estabilidad a largo plazo.

No es una tarea rutinaria formular dos proteínas diferentes en una solución líquida altamente concentrada, cuya formulación es estable durante un tiempo prolongado en línea con el requisito de una formulación comercial de un producto terapéutico.

En otras palabras, la presente invención proporciona nuevas formulaciones líquidas con dos proteínas diferentes en alta concentración. Tenga en cuenta también que se considera sorprendente lograr una alta estabilidad durante un período prolongado de tiempo, porque de hecho se espera que la alta concentración de hialuronidasa rHuPH20 tenga un impacto negativo en la estabilidad de esas formas de administración subcutánea concentrada.

De esta forma, me permito comparar las características técnicas esenciales del documento D2 con aquellas presentes en la presente solicitud, como sigue:

Tabla 2. Comparación de las características técnicas esenciales con las de D2

Características Esenciales	D2 (De acuerdo con el examinador)	Comentarios
Una formulación farmacéutica del anticuerpo anti-	Formulación que comprende: (Página 20, Reivindicación	Esta solicitud de patente se relaciona principalmente con ofatumumab como anticuerpo anti-CD20

CD20 que comprende:	1)	
Rituximab 120mg/ml	Anticuerpo anti-CD20 (20-300 mg/ml de Rituximab) (página 20 y 23, Reivindicaciones 1-9 y 24)	Si bien el rango del anticuerpo anti-CD20 es de 20-300 mg/ml, no se menciona la concentración muy específica de Rituximab 120mg/ml; además, esta característica no puede derivarse de D2
-L-Histidina 20mM -Cloruro de Sodio 120mM	Arginina 05-5% (Página 20, Reivindicación 1)	No se menciona L-Histidina, ni cloruro de sodio, ni siquiera las concentraciones 20mM o 120 mM, respectivamente
Trehalosa dihidratada 210mM	Acetato de sodio 10-100mM (Página 20, Reclamación 1)	La Trehalosa dihidratada no se menciona, ni mucho menos la concentración de 210mM.
Metionina 10mM	No se menciona	No se menciona
Polisorbato 20/80 0.06%	Polisorbato 80 0.01% - 0.2% (Página 20, Reivindicación 1)	No se menciona la dosis específica de 0.06%
-rHUPH20 2000 o 12000 U/ml	No se menciona	No se menciona

En vista de lo anterior, es claro que el documento D2 no subsana las deficiencias del documento D1, y, además, es importante señalar que **este documento no sugiere la inclusión de hialuronidasa en alta concentración, por ejemplo, de 2.000 U/ml a 12.000 U/ml, ni siquiera menciona la presencia de esta, ni mucho menos que puede ser usada en formulaciones farmacéuticas con alto contenido de anticuerpos anti-CD20 sin causar efectos adversos frente a la disponibilidad del anticuerpo.**

Así las cosas, de todo el análisis previamente hecho, el objeto de la presente invención, es decir, formulaciones muy concentradas del anticuerpo anti-CD20 rituximab, co-formulado con la enzima hialuronidasa altamente concentrada que, sorprendentemente, tiene estabilidad a largo plazo, no se menciona ni pueden considerarse obvias a partir del documento D2, ni mucho menos por la combinación de las enseñanzas de los documentos D1 y D2.

(d) Análisis Hindsight realizado por el Examinador

Con base en lo planteado previamente, me permito señalar que el hecho de citar los documentos D1 y D2 como estado de la técnica más cercano a la presente invención únicamente puede ser el resultado de un análisis de tipo ***hindsight*** por parte del señor Examinador. Esto debido a que dichas anterioridades de ninguna manera mencionan rangos específicos de concentraciones de hialuronidasa tipo rHuPH20, así como tampoco menciona que la formulación obtenida de anticuerpo anti-CD20 sea estable y pueda ser administrada por vía subcutánea.

En este sentido, el hecho de sugerir que la persona medianamente versada en la materia técnica en cuestión enfrentado al problema abordado en la presente invención habría arrancado de las enseñanzas de D1 debido a que dicha anterioridad menciona una composición que combina un anticuerpo y hialuronidasa, implica que dicha persona medianamente versada en la materia ya conocía al menos una parte de la solución al problema técnico abordado en la presente invención, es decir, ya tenía claro que la formulación de anticuerpo altamente concentrado de anti-CD20 con alta concentración de hialuronidasa podía ser efectiva en una formulación como la reclamada en la presente invención.

Lo anterior resulta relevante porque en el estado de la técnica se conocía el efecto negativo de la alta concentración de hialuronidasa sobre la estabilidad de formas concentradas de moléculas de administración subcutánea. Por ello, no resultaba evidente hacer una formulación farmacéutica estable que combina dos proteínas (anticuerpo anti-CD20 como rituximab y hialuronidasa) altamente concentradas.

Visto lo anterior, **me permito manifestar que no resulta adecuado ignorar las diferencias en cuanto a propósito y efecto técnico logrado existentes entre el documento D1 y la presente invención, toda vez que dicho mecanismo de análisis genera una idea errada con respecto a la obviedad de la solución dada en la solicitud en cuestión al problema técnico abordado, desconociendo de paso el esfuerzo requerido para superar los problemas técnicos existentes en el estado de la técnica anterior para obtener una formulación farmacéutica estable para la administración subcutánea de un anticuerpo anti-CD20 Rituximab.**

Por lo tanto, amablemente me permito manifestar ante ese Despacho que la materia del capítulo reivindicatorio modificado presenta un inequívoco carácter inventivo frente a las enseñanzas de los documentos D1 y D2, cumpliendo por lo tanto a cabalidad con los requisitos de patentabilidad establecidos en el Artículo 18 de la Decisión Andina 486/2000.

8. **CONCLUSIÓN.**

Para finalizar, me permito reiterar que el capítulo reivindicatorio modificado presentado junto con este memorial es totalmente claro y conciso al reclamar a composiciones liofilizadas que comprenden rituximab, que están plenamente soportados dentro del texto de la descripción, cumpliendo así con lo exigido por la mencionada Decisión Andina 486 de 2000.

Por otro lado, en virtud de los argumentos discutidos con respecto a los documentos citados, se estableció de manera incontrovertible que las composiciones liofilizadas que comprenden rituximab, que son objeto de la solicitud en estudio de ninguna manera resultarían evidentes para la persona versada en la materia a partir de las enseñanzas de los documentos referenciados, demostrando irrefutablemente la patentabilidad de la presente solicitud.

9. **SOLICITUD DE NUEVO EXAMEN DE PATENTABILIDAD.**

Finalmente, me permito manifestar que es deseo del solicitante requerir a ese Despacho la expedición de un nuevo examen de patentabilidad, lo anterior en caso de que el Señor Examinador no considere suficientes los argumentos y las modificaciones al capítulo reivindicatorio presentados en respuesta al Oficio N° **4208**.

10. Anexo al presente memorial, me permito presentar copia de:

(a) La página **129** que corresponde al **capítulo reivindicatorio modificado**, tal como se discutió en el presente memorial, constituido por **6 reivindicaciones**.

(b) Copia de los **anexos I a VII**, mencionados a lo largo del presente memorial.

11. Adicionalmente, informamos que el pago por concepto del nuevo examen de patentabilidad se efectuó a través de la plataforma SIPI el día de hoy.

12. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,



ALICIA LLOREDA RICAURTE

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Validation of an HPLC method for the analysis of the charge heterogeneity of the recombinant monoclonal antibody IDEC-C2B8 after papain digestion¹

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Abstract

An HPLC procedure was validated for determining the purity with respect to the charge variant distribution of the recombinant monoclonal antibody (MAb) IDEC-C2B8 by high-performance ion-exchange chromatography. Papain was used to fragment the molecule into Fab and Fc fragments prior to chromatographic analysis. Fragmentation allowed the resolution of the variants arising from the cyclization of glutamine to pyroglutamate at the amino-terminus of the light and heavy chains (Fab-pE/Q variants) from the variants resulting from the processing of the carboxy-terminal lysine residues of the heavy chains (Fc-Lys variants). The assay demonstrated good linearity, yielding correlation coefficients of > 0.99 for total protein, Fc-Lys variants and Fab-pE/Q variants. Recovery of total protein from the column was 95.7%. Sample recovery studies demonstrated a mean accuracy of 102% for a Fab fragment over the range 2–10% of the total protein. The limit of detection was 0.2 μg and 0.1 μg for Fc and Fab variants, respectively. The repeatability of the assay and intermediate precision had relative standard deviation (RSD) values of $< 1\%$. Parameters of the papain digest (time, digest stability, reagent stability, pH and papain vendor) and of the chromatography (mobile phase pH, stability, buffer concentration, and column lot and aging) were evaluated for robustness and determined to be acceptable. Data are presented demonstrating the suitability of the assay for determining the product purity of a recombinant MAb. Published by Elsevier Science B.V.

Keywords: Recombinant protein; Monoclonal antibody; Papain; Cation-exchange chromatography; Method validation

1. Introduction

Established methods for the analysis of the charge variants of recombinant proteins include ion-exchange chromatography and isoelectric fo-

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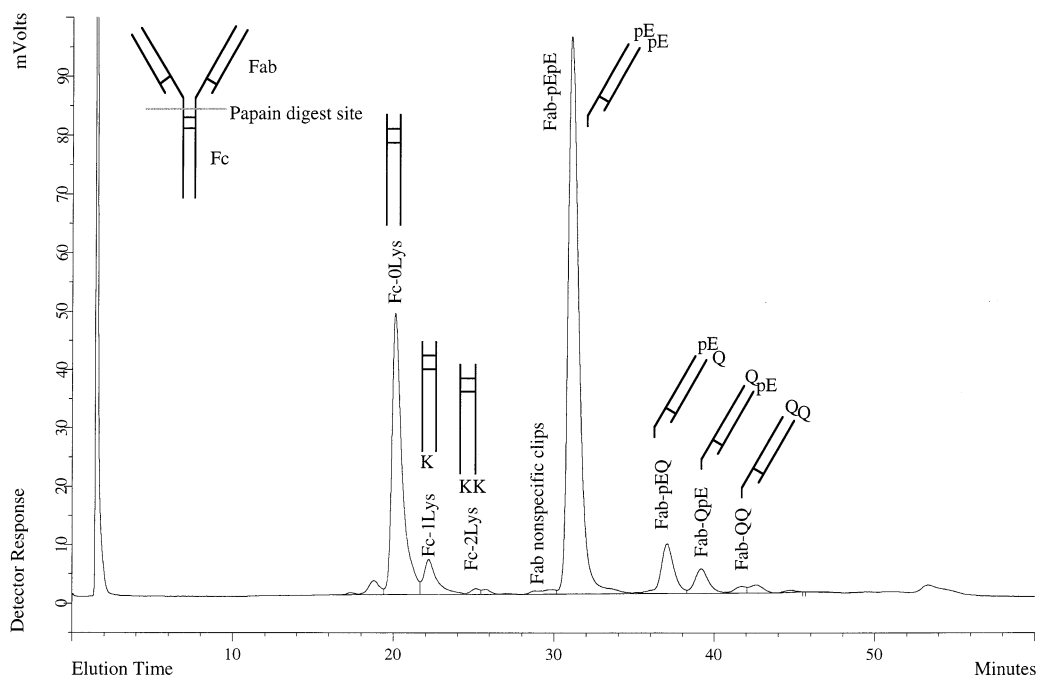


Fig. 1. Typical chromatogram of papain-digested IDEC-C2B8 MAb obtained using a Polymer labs SCX column.

cusing [1]. In ion-exchange chromatography charge variants are separated by differential partitioning on a charged support. Protein charge variants arise from modifications of the amino- and carboxy-termini and amino acid side chains. With small proteins, such as recombinant human growth hormone, resolution of all charge species is possible [2,3]. As molecule size increases the number of possible charge variants increases. As changes in charge from different modifications may be additive or subtractive, ion exchange chromatography becomes more complex while the overall resolution of individual variants may be lost. This is particularly apparent for monoclonal antibodies which are comprised of two light and two heavy chains with overall molecular weights in the region of 150 kDa.

IDEC-C2B8 is a recombinant mouse/human chimeric monoclonal antibody that binds the human CD20 antigen [4]. Inherent charge variants of this MAb arise during cell culture from the enzymatic removal of the C-terminal lysine from one or both heavy chains to reveal a C-terminal

glycine, a common reaction for proteins isolated from cell culture [5]. Additional charge variants arise from the cyclization of the N-terminal glutamine of both the heavy and light chains to produce pyroglutamate, a deamination reaction. These charge variants can occur in random combination at the four N-termini and the two heavy-chain C-termini of the molecule, leading to a possibility of 15 different species [6]. To overcome this complexity, an ion-exchange assay was developed to analyze the charge heterogeneity of the molecule after it has been fragmented by digestion with papain [7].

Papain is a thiol protease that cleaves IgG antibodies at the heavy chain hinge region into three fragments, one Fc and two identical Fab fragments [8,9]. With IDEC-C2B8 papain cleaves the His-Thr bond in the heavy chain hinge sequence -Asp-Lys-Thr-His-Thr-Cys-Pro-Pro-Cys- so that each Fab fragment consists of the light chain and the N-terminal section of the heavy chain. The Fc fragment consists of the disulfide-linked C-terminal section of the heavy chain. As a

Table 1
Linearity and accuracy of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment	Result	
Linearity of response ^a	Fab-pE/Q variants	$R = 1.0$
	Fc-Lys variants	$R = 1.0$
	Total peak area	$R = 1.0$
Column recovery ^b	Total protein	96% Recovery
Sample recovery ^c	2% Variant spike	100% Recovery
	5% Variant spike	103% Recovery
	9% Variant spike	102% Recovery

^a Linearity was calculated from duplicate injections of papain-digested MAb in the range 10–150 µg. The peak area response was plotted against total protein load and the correlation coefficient (R) calculated.

^b A single sample of papain digested IDEC-C2B8 was injected in duplicate with the column in and out of line. %Recovery = [(mean peak area with column in line)/(corrected mean peak area with column out of line)] $\times$ 100%.

^c Mixtures of papain-digested IDEC-C2B8 and purified Fab nonspecific clip were prepared at three different levels and analyzed. The measured values from the analysis for the variant were compared to the theoretical values calculated from the protein concentration and purity. % Recovery = (measured/theoretical) $\times$ 100%.

result of cleavage, the inherent charge heterogeneity associated with the IDEC-C2B8 Fab fragment can be resolved from the heterogeneity associated with the Fc fragment by cation exchange chromatography. Although the use of papain to produce antibody fragments is well known, the application of this approach as part of a method to monitor and release a marketed recombinant

MAb can only occur once the method has undergone a rigorous validation as required by the Food and Drug Administration [10], and other regulatory authorities.

Currently validation studies for recombinant proteins are performed with reference to the guidelines of the International Conference on Harmonization [11,12]. The validation of a method is performed as a series of carefully controlled experiments, each experiment designed to assess a particular aspect of the method, e.g. accuracy, precision (repeatability and intermediate precision), specificity and robustness. The results of each experiment are judged as either acceptable or unacceptable against criteria considered appropriate for the purpose of the method with respect to measuring product purity, potency, efficacy and safety. If the validation results are acceptable the method is considered validated and can be submitted to the appropriate regulatory authority for approval, as part of the product license application. This paper summarizes the validation of the chromatographic analysis of the monoclonal antibody IDEC-C2B8 for charge heterogeneity after digestion with papain.

2. Materials and methods

2.1. Reagents and materials

IDEC-C2B8 was manufactured by IDEC Pharmaceuticals (San Diego, CA). Papain (from *Car-*

Table 2
Precision of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result	
		Fc-Lys variants	Fab-pE/Q variants
Repeatability ^a	Injection	0.9% RSD ($n = 6$)	0.3% RSD ($n = 6$)
	Digestion	0.8% RSD ($n = 6$)	0.3% RSD ($n = 6$)
Intermediate precision	Day-to-day ^b	0.7% RSD ($n = 3$)	0.7% RSD ($n = 3$)
	Analyst-to-analyst ^c	0.5–1.3% Difference ($n = 3$)	0.3–1.5% Difference ($n = 3$)

^a Six replicate injections of a single papain digest and six replicate digestions of IDEC-C2B8 were analyzed.

^b Papain digests of IDEC-C2B8 were prepared and analyzed by a single analyst on three different days.

^c Two analysts analyzed three different lots of IDEC-C2B8 using separate reagents and equipment. The results are the range of differences seen for the three lots.

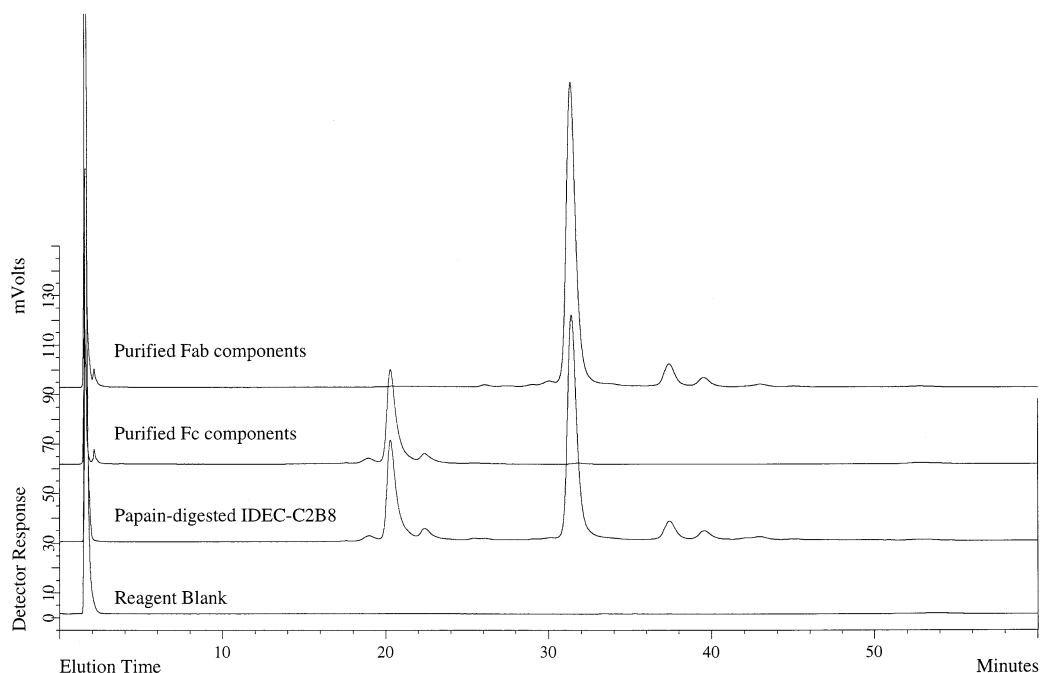


Fig. 2. Specificity of the analysis of papain-digested IDEC-C2B8 MAb.

ica papaya, EC 3.4.22.2) was purchased from Boehringer Mannheim (Indianapolis, IN), Worthington Biochemical (Freehold, NJ) and Serva (Crescent Chemical, Hauppauge, NY). L-Cysteine was from Sigma (St. Louis, MO). 2-(*N*-morpholino)ethanesulfonic acid (MES) was from Research Organic (Cleveland, OH). PD-10 buffer exchange columns were from Pharmacia Biotech (Piscataway, NJ). PL-SCX columns (1000 Å, 8 µm, 150 × 4.6 mm) were from Polymer Laboratories (Amherst, MA), and TSK G3000SW_{XL} columns (7.8 × 300 mm) were from TosoHaas (Montgomeryville, PA). The Poros A/M column (4.6 × 100 mm) was from PerSeptive Biosystems (Framingham, MA). Centrifugal concentrators were from Amicon (Beverly, MA). All other chemicals were of analytical reagent grade.

2.2. Papain digestion

The standard digestion of the recombinant MAb by papain was as follows. IDEC-C2B8 was incubated at a final concentration of 1 mg ml⁻¹ in 0.1 M Tris-HCl, 4 mM EDTA, 1 mM cysteine

(pH 7.4). The digestion was initiated by the addition of papain (diluted to 0.1 mg ml⁻¹ with water) to give a final protein:enzyme ratio of 100:1, the final digestion volume was 1.5 ml. The digestion was carried out for 2–2.5 h at 37°C, then 1 ml of the digested MAb was buffer exchanged into 2 ml of 10 mM MES (pH 6.0) (solvent A) using PD-10 columns.

2.3. Chromatography

Chromatographic analysis by cation exchange chromatography was carried out using a PL-SCX column. 200 µl (approximately 100 µg) of the digested protein was injected onto the column. The gradient for elution was 5 min at 95% solvent A, 5% solvent B (0.2 M NaCl in 10 mM MES pH 6.0) followed by a linear gradient of 5–85% solvent B over 40 min. The flow rate was 1 ml min⁻¹. After elution the column was washed with 100% solvent B for 2 min and re-equilibrated with 95% solvent A. The total run time was 60 min. The column temperature was ambient and elution was monitored at 280 nm (except as noted).

Table 3
Robustness of the papain digestion of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result ^a	
		Fc-Lys variants (% difference)	Fab-pE/Q variants (% difference)
Digest pH	pH 6.8–7.4	1.4	0.8
Time of digest	1–>5 h	1.0 (after 1 h)	0.5 (after 1 h)
Sample stability	Fresh vs. 1 week at 2–8°C	0.3	0.3
Cysteine stability	Fresh vs. 1 day old	0.1	0.2
Papain ^b	Lot 1–3	0.1	0.1

^a Values are the maximum differences seen for the Fc-Lys and Fab-pE/Q values calculated from a single lot of IDEC-C2B8 analyzed according to the conditions indicated.

^b Three different lots from Boehringer Mannheim.

Chromatographic analysis by size exclusion chromatography was carried out using a TSK G3000SW_{XL} column. The mobile phase was 0.2 M potassium phosphate, 0.25 M KCl, (pH 7.0). The flow rate was 1 ml min⁻¹ and the elution was isocratic. The column temperature was ambient and elution was monitored at 280 nm.

2.4. Preparation of Fab and Fc fragments

Fab and Fc fragments of IDEC-C2B8 were prepared from several pooled digests using protein A affinity chromatography. Digested MAb was injected onto a protein A column equilibrated in 10 mM sodium phosphate, 0.15 M NaCl (pH 7.4). The Fab fragments were collected as the flow-through peak and the Fc fragments eluted with 2% (v/v) acetic acid, 0.1 M glycine, the column was washed with 20% (v/v) acetic acid before re-equilibration with buffer and the next injection. Fc and Fab fragment pools were buffer exchanged into solvent A by dialysis with at least three changes of buffer. Finally the protein was concentrated using centrifugal concentrators prior to chromatographic analysis.

Fc-0Lys, Fab-pEpE and a Fab nonspecific clip variant (see Section 3.1 for full terminology) were purified from papain digested MAb by cation exchange chromatography described above with minor modifications. After collection the purified variants were concentrated and buffer exchanged into solvent A using centrifugal concentrators.

3. Results and discussion

3.1. Method characterization

A typical elution profile is shown in Fig. 1. The isoelectric points (*pI*) of the Fc and Fab fragments were estimated from slab gel isoelectric focusing to be pH 7.1 and 9.1, respectively. At pH 6.0 both fragments have a positive charge and adhere to the negatively charged sulfonate groups of the cation ion-exchange matrix and are eluted by a salt gradient in the order of their increasing isoelectric points. The nature of the separated species was determined by mass spectrometry with or without tryptic digestion (manuscript in preparation). Fc variants elute in order of increasing *pI* resulting from the sequential removal of the lysine residues from each carboxy-termini to reveal glycine. Fc peaks are identified using the following terminology. Fc-0Lys (an Fc fragment without lysine residues at either C-terminus), Fc-1Lys (an Fc fragment with a lysine residue at one C-terminus) and Fc-2Lys (an Fc fragment with lysine residues at both C-termini) collectively are termed the Fc-Lys variants.

Fab variants elute in order of increasing *pI* resulting from the sequential cyclization of the basic amino terminal glutamine to the neutral pyroglutamate at each of the four Fab amino-termini. Fab peaks are identified using the following terminology. Fab-pEpE (a Fab fragment with pyroglutamate at both N-termini), Fab-pEQ (a Fab fragment with pyroglutamate at the heavy-chain

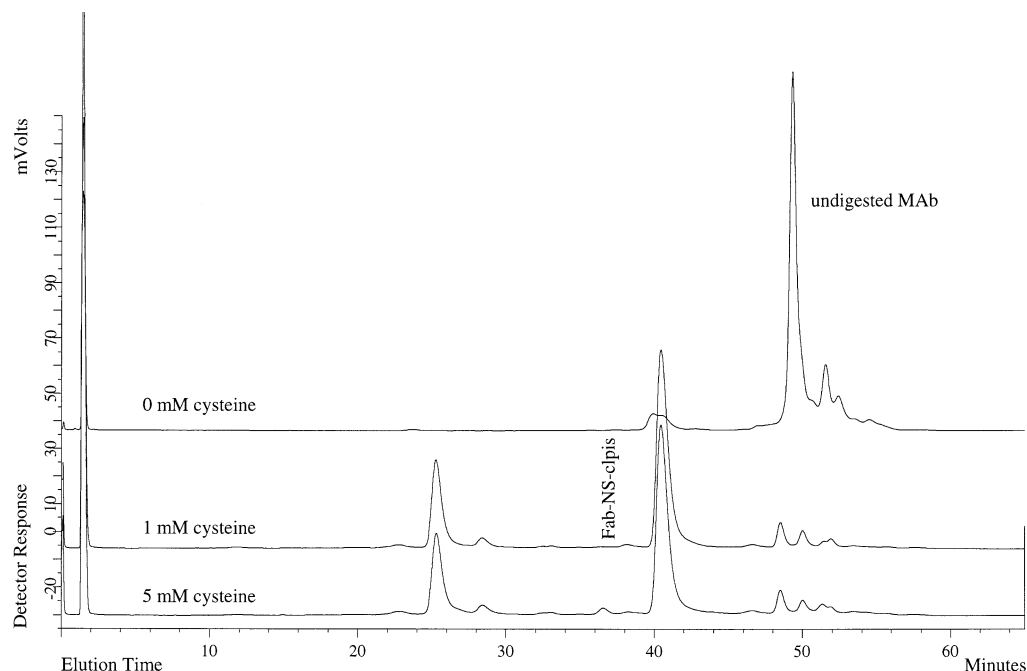


Fig. 3. Effect of cysteine concentration on the papain digestion of IDEC-C2B8 MAb.

N-terminus and glutamine at the light-chain N-terminus) and Fab-QpE (a Fab fragment with glutamine at the heavy-chain N-terminus and pyroglutamate at the light-chain N-terminus) and Fab-QQ (a Fab fragment with glutamine at both N-termini) collectively are termed the Fab-pE/Q variants. In addition to the cleavage producing the Fc and Fab fragments, papain also exhibited a low level of exopeptidase activity resulting in the sequential removal of the histidine and threonine from the new C-terminus of the Fab heavy chain fragment. These further degraded fragments are apparent as peaks eluting just prior to the Fab-pEpE peak and are termed Fab nonspecific clips (Fab-NS-clips). Quantitation was performed using peak area normalization.

3.2. Linearity and accuracy

The results of the linearity and accuracy studies are presented in Table 1. There was a linear response for the analysis of Fc-Lys and the

Fab-pE/Q variants and for the sum of all peak areas as demonstrated by correlation coefficients of >0.99 . Accuracy was determined in three parts by, column recovery, sample recovery and, by the determination of the limit of detection and quantitation. Column recovery was calculated at 95.7% based on a comparison of peak areas determined by making duplicate injections of digested MAb with the column in and out of line. The mean recovery of a purified Fab-NS-clip variant from experimental mixtures of this Fab variant and papain-digested IDEC-C2B8 (corresponding to variant levels of 2–10% of the total protein by mass) was 102%. The detection limit, of serially diluted samples of purified Fc-0Lys and Fab-pEpE variants was 0.2 and 0.1 μg (0.2% and 0.1% based on a 100 μg load), respectively. The limits of quantitation for the same samples were 2 and 0.5 μg , respectively (2% and 0.5% based on a 100 μg load). The limit of quantitation was somewhat less for the Fc variant as these species showed considerably more peak broadening than the Fab variants.

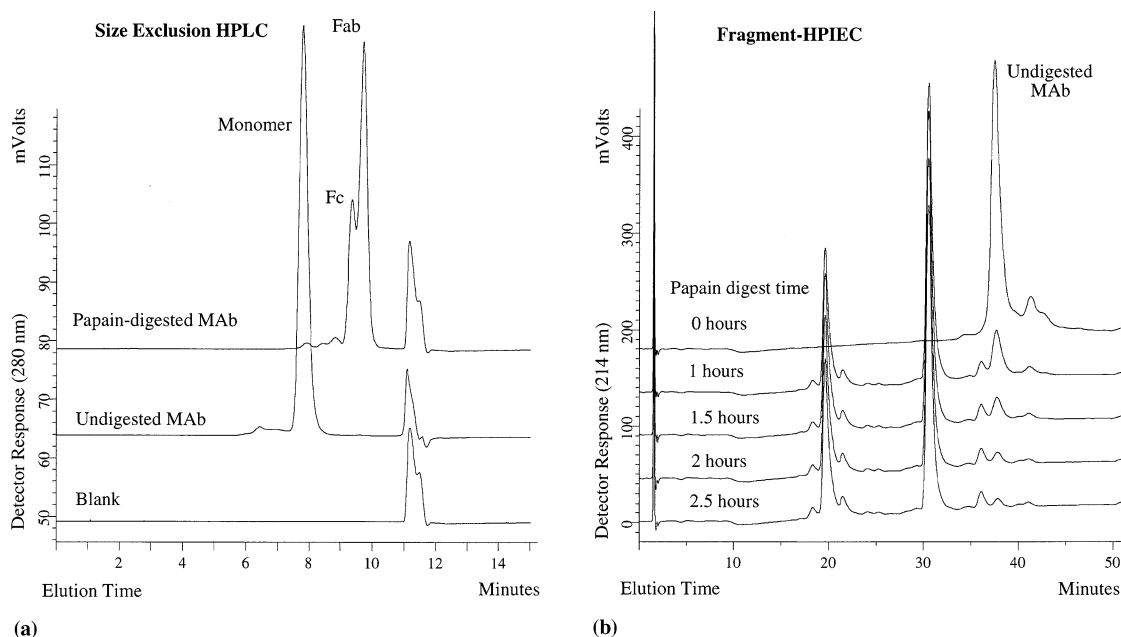


Fig. 4. Completeness of papain digestion of IDEC-C2B8 MAb.

3.3. Precision

Precision was evaluated in two parts, by repeatability and by intermediate precision studies. The results are presented in Table 2. Repeatability studies were performed for the chromatography by evaluating multiple injections and for the digestion by evaluating multiple digests. In both cases for the calculated Fc-Lys and Fab-pE/Q values the RSD values were $< 1\%$. Intermediate precision was determined with three studies. In the first study a second analyst repeated the repeatability studies and again obtained RSD values of $< 1\%$ for both digest and chromatography. In the second study a single analyst prepared and analyzed a single sample of IDEC-C2B8 on 3 separate days. Again RSD values of $< 1\%$ were obtained. In the third study two analysts each analyzed three different lots of IDEC-C2B8. The results of the two analysts showed differences of 0.5–1.3% for the Fc-Lys variants, 0.3–1.5% for the Fab-pE/Q when the three lots were compared. The values represent the highest level of precision testing as the two analysts were using different reagents, columns and instruments. Overall the

results of the precision studies were acceptable for a chromatographic procedure.

Reproducibility by an interlaboratory trial was not assessed for the assay as this aspect of precision is now only considered in the case of the standardization of an analytical procedure, such as for inclusion in a pharmacopoeia [12].

3.4. Specificity

Fab fragments were isolated from Fc fragments by affinity chromatography using protein A. These individually prepared fragments were used to show the specificity of the analysis. The profiles for purified variants and for a blank digest which contained all components except the MAb are shown in Fig. 2. The chromatograms clearly demonstrate that all earlier eluting peaks result from Fc fragments and that they are resolved from the later eluting Fab fragments. Injection of individual components after the PD-10 buffer exchange step indicated that the peak eluting at 2 min resulted from the Tris-EDTA components of the digest buffer.

Table 4
Robustness of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result ^a	
		Fc-Lys variants (% difference)	Fab-pE/Q variants (% difference)
Mobile phase	pH (pH 5.9–6.3)	0.8	1.6
	Molarity (5–20 mM)	0.3	0.1
Column	Column lot 1a–1c ^b	1.5	0.5
	Column lot 1–3 ^c	1.8	1.0

^a Values are the maximum differences seen for the Fc-Lys and Fab-pE/Q values calculated for a single lot of IDEC-C2B8 analyzed according to the conditions indicated.

^b Three different columns from the same column lot.

^c Three different columns from three different column lots.

3.5. Robustness

The robustness of the assay was evaluated to show the reliability of the analysis with respect to deliberate variations in method parameters. This aspect of validation is especially important for assays where an enzyme reaction is included as part of the analysis procedure.

After optimization of the digest for the MAb and papain concentration, for the ease of sample preparation and to give sufficient chromatographic response, the following parameters of the papain digest were studied; cysteine and EDTA concentration, time, digest stability, reagent stability, digest pH, and papain vendor. The results are summarized in Table 3. EDTA was included in the incubation buffer as the presence of divalent cations inhibit papain activity [9], however, in this case EDTA concentrations (0–10 mM) had no effect on the digestion. The cysteine concentration had a significant effect on the digestion as seen by the lack of digestion which occurred at 0 mM cysteine and by the increase in the relative peak area of the Fab nonspecific clips which occurred with increasing concentration (Fig. 3). For this reason the cysteine concentration was optimized to maximize the completeness of digestion while minimizing the production of the Fab nonspecific clips. Cysteine concentrations of 1 mM resulted in nonspecific clip production of less than 1%. The completeness of digestion at 1 mM cysteine was then monitored by the loss of the intact peak which co-migrates with the Fab-QpE

peak by cation exchange chromatography and by size exclusion HPLC by the loss of the monomer peak (Fig. 4a and b). Both studies indicated that at least 96–97% digestion was achieved. At 1 mM cysteine the production of Fab nonspecific clips was still observed within the 2–2.5 h digestion time but overall their levels were so low they did not affect the results. This was confirmed with extended digestion times where even after > 5 h the Fab nonspecific clips represented no more than 1.2% of the total peak area. The stability of the cysteine reagent was assessed as the –SH group in the papain active site must be in the reduced form for activity. The ability of cysteine to activate the papain after storage was evaluated by comparing the results from a sample of IDEC-C2B8 which had been digested with cysteine that (a) had been freshly prepared and (b) stored for 1 day at 2–8°C. The results indicated that any oxidation of cysteine to cystine that occurred within 24 h did not effect the overall activity of the papain.

The pH of the digest was evaluated with no effect seen over the range pH 6.8–7.4. The papain activity and completeness of digestion were probably not affected by pH as the digestion was carried out under conditions of excess enzyme activity. The stability of the digest was evaluated as the papain was not removed by the buffer exchange step. Residual exopeptidase activity was seen on storage by an increase in the area percent of Fab nonspecific clips of digested samples over time. However, storage experiments indicated that

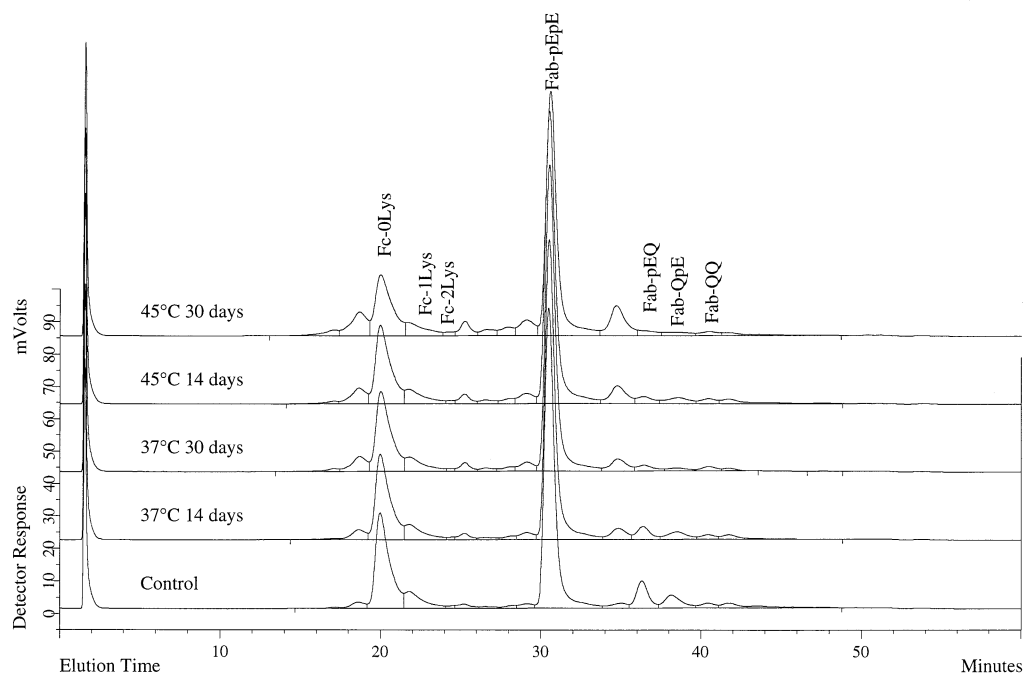


Fig. 5. Thermal degradation of IDEC-C2B8 MAb following storage at elevated temperatures.

a sample could be kept for up to 24 h at ambient or 4 days at 2–8°C before the production of nonspecific clips would affect the results. The source of the papain was evaluated to ensure that if papain was not available from the primary vendor (Boehringer Mannheim), then alternative sources could be used. Papain from Worthington and Serva were evaluated for their ability to digest the MAb on both a weight and activity corrected basis by comparison with the primary vendor. Digestion profiles and data analysis for each vendor were consistent with values for the Fc-Lys and Fab-pE/Q variants within 2% of that seen for the primary vendor.

The robustness of the chromatography section was performed by evaluating changes in mobile phase pH (pH 5.9–6.3), concentration (5–20 mM) and column manufacture (both differences between columns from the same lot and columns from different lots). The results, summarized in Table 4, indicated that none of the parameters studied affected the results.

3.6. Stability indicating properties

Finally, the utility of the assay was assessed by its ability as an indicator of product degradation. Four samples which had been subjected to accelerated stability studies at elevated temperatures of 37 or 45°C for 2 or 4 weeks were analyzed. The chromatographic analysis is presented in Fig. 5; the results are summarized in Table 5. There was both a time- and temperature-dependent decrease in the percent peak areas for both the Fc-Lys and Fab-pE/Q variants. The relative distribution of Fc-0Lys and Fc-1Lys variants did not change, which is consistent with an enzymatic rather than chemical processing of this C-terminal. There was an increase in the area of a more acidic peak eluting prior to Fc-0Lys with both time and temperature. The nature of this peak was not determined but its position is consistent with a degradation reaction associated with an increase in negative charge, such as deamination. The change in peak areas of the individual Fab-pE/Q variants was more complex. N-terminal glutamine

Table 5
Utility of fragment-HPIEC analysis of the recombinant MAb IDEC-C2B8

Conditions ^a	Percent peak area		
	Fc-Lys variants	Fab-pE/Q variants	Fc-Lys + Fab-pE/Q variants
Control, 2–8°C	28.2	63.0	91.2
37°C, 14 days	26.4	60.7	87.1
37°C, 30 days	25.8	59.2	85.0
45°C, 14 days	24.7	57.3	82.0
45°C, 30 days	22.1	50.2	72.2

^a Samples of IDEC-C2B8 were stored as described before analysis.

cyclization appeared to be a temperature-dependent reaction as there was a change in the distribution of the Fab-pE/Q variants consistent with conversion of the partially cyclized species Fab-pEQ and Fab-QpE to the fully cyclized Fab-pEpE at the lower temperature. At the higher temperature there were significant increases in the areas of peaks not related to cyclization. As exposure to elevated temperatures is known to decrease activity for the MAb (data not shown), the changes seen in the chromatographic profile indicate that this method can be used to monitor product quality on a stability basis.

4. Conclusion

A comprehensive validation was performed on an HPLC based assay for the recombinant MAb IDEC-C2B8 which first required the digestion of the protein with papain. The method showed acceptable recovery and appeared rugged and precise with respect to operating conditions. The validation and optimization of the papain-digest step was evaluated and found to be robust.

Cysteine was required in the digest step for papain activity but increasing cysteine concentrations also increased the nonspecific exopeptidase activity of the enzyme. Other parameters including papain and antibody concentrations, digest time, pH and reagent shelf life were chosen such that 96–97% digestion occurred while the production of nonspecific clips was less than 1% of the total peak area. The utility of the assay as both an indicator of product quality and consistency for

lot release and for stability studies was shown by the analysis of thermally degraded samples, which exhibited changes in the chromatographic profile.

The method as validated satisfied all of the rigorous criteria for use as a lot release method and may also be applicable to other recombinant monoclonal antibodies. The success of the method with other antibodies will depend on the relative *pI* values of the Fc and Fab fractions. Replacement of soluble papain with immobilized papain, which would be removed during the buffer exchange step, may increase assay robustness by decreasing the production of nonspecific exopeptidase-related Fab peaks during storage.

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Bone Regeneration with Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Using Absorbable Collagen Sponges (ACS): Influence of Processing on ACS Characteristics and Formulation

Wolfgang Friess, Hasan Uludag, Shannon Foskett & Robert Biron

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RESEARCH ARTICLE

Bone Regeneration with Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Using Absorbable Collagen Sponges (ACS): Influence of Processing on ACS Characteristics and Formulation

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ABSTRACT

The effects of variability in three parameters (mass, cross-linking with CH₂O, and EtO sterilization) of three surgically implantable absorbable collagen sponges (ACS) were studied. Sponges soaked with recombinant human bone morphogenetic protein-2 (rhBMP-2) solution were analyzed for pH, conductivity, and rhBMP-2 precipitation. A method using trinitrobenzenesulfonic acid was developed to quantify the free amino groups of the collagen sponge. With up to 240 min exposure to CH₂O, the amount of free amino groups was reduced to 80%. In comparison, the denaturation temperature as determined by differential scanning calorimetry (DSC) after the sponges were soaked with phosphate-buffered saline, increased from 48 to 55°C, indicating stronger interactions due to cross-linking. Subsequent sterilization with EtO caused a marked decrease in the amount of free amino groups (approximately 33% of nonsterilized controls) independent of previous CH₂O treatment. However, the denaturation temperature was on average 5°C lower in sterilized sponges than in nonsterilized material. In contrast to CH₂O exposure, the strong reaction with EtO appeared to weaken the collagen structure. Resistance of the sponge to collagenase correlated with the degree of collagen cross-linking but was slightly reduced by sterilization. In addition, the pH of ACS soaked with water was substantially increased by sterilization. Protein precipitation was a function of pH and salt concentration

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but there was no effect due to collagen alone. Results indicated that ACS weight has to be limited to avoid rhBMP-2 precipitation.

KEY WORDS: Bone morphogenetic protein; Collagen; Cross-linking; Sterilization.

INTRODUCTION

Bone morphogenetic proteins (BMPs) were first discovered by Urist's group in 1965 (1) and currently constitute a family of more than 20 related proteins that are part of the transforming growth factor- β super family (2). Two recombinant human BMPs, recombinant human bone morphogenetic protein-2 (rhBMP-2) and rhBMP-7, have reached the status of pivotal clinical testing in different bone repair situations and prospective approval is expected within a few years (3–5). Unlike purified human or animal BMP, the recombinant proteins present no disease transmission risk and are available in high purity and large quantities. Regeneration of bone with BMPs will reduce the number of autograft surgeries—more than 250,000 are performed annually in the United States alone (6)—which can cause donor site pain, infection and morbidity, and blood loss, and incur high costs.

Once implanted, BMPs initiate a series of events starting with differentiation of mesenchymal cells into chondroblasts forming a cartilaginous matrix which subsequently calcifies and is replaced by bone (7). To promote osseous regeneration, the osteoinductive rhBMP-2 is combined with osteoconductive, porous biomaterials. This scaffolding supports vascular ingrowth, cellular infiltration and attachment, cartilage formation, and calcified tissue deposition. Purely osteoconductive carriers are preferably composed of collagen and calcium phosphate (hydroxyapatite and/or tricalcium phosphate). In addition, calcium sulfate, polylactide-co-glycolide, and cellulose derivatives have been successfully used (6,8,9). Prerequisites for an ideal carrier substratum are the following: (i) good affinity with BMP and host bone, (ii) no toxicity, immunogenicity, or interference with bone regeneration, (iii) biodegradability, (iv) enhancement of osteogenic activity of BMP with a restrictive release of BMP at an effective dose during a period coincident with the accumulation and proliferation of target cells, (v) amenability to sterilization, and (vi) weight-bearing substrate for attachment of osteoprogenitor cells (10).

The focus of the investigations presented was on absorbable collagen sponges (ACS). These systems are currently under clinical investigation for surgical implantation in combination with rhBMP-2 at sites where bone induction is desired (3,11). Collagen sponges were devel-

oped as wound dressing or hemostyptics and can be loaded with antibiotics for treatment and prophylaxis of soft tissue infections (12,13). Because the sponge exhibits scaffolding properties and positive effects on wound healing and cellular ingrowth, new focus arose for applications in tissue engineering research and in combinations with growth factors (12,14). For bone regeneration in clinical applications, commercially available, approved ACS material is soaked with rhBMP-2 formulation prior to implantation of the wet product. ACS properties such as resorption rate, interactions with rhBMP-2, or in vivo pharmacokinetics could be manipulated and adapted to its application by modifications in the manufacturing process.

The emphasis of this study was on the combined effects of three parameters (sponge mass, cross-linking, and sterilization) on the properties of ACS alone and of the implanted rhBMP-2/carrier formulation. These three characteristics were assumed to directly influence the formulation and protein interactions by chemical reaction with collagen or to induce indirect changes via shifts in pH or ion concentrations. This paper summarizes the results of these investigations.

MATERIALS AND METHODS

rhBMP-2 was produced at Genetics Institute, Inc. (Andover, MA) using a Chinese hamster ovary (CHO) cell expression system (15). Purified protein was formulated at 4 mg/ml in a 5 mM glutamic acid buffer pH 4.5 (glu I; 2.5% glycine, 0.5% sucrose, 0.01% polysorbate 80, 5 mM NaCl, 5 mM glutamic acid). Dilution was performed using a 30 mM glutamic acid buffer pH 4.5 (glu II, 2.5% glycine, 0.5% sucrose, 0.01% polysorbate 80, 30 mM glutamic acid).

ACS Manufacturing

The manufacturing process is shown schematically in Fig. 1. Briefly, purified, fibrillar, bovine collagen dispersion (pH approximately 6.3) was lyophilized to achieve ACS with height corresponding to 2.6, 4.4, or 6.2 mg/cm² weight. The collagen concentration of the dispersion was controlled after hydrolysis with 6 N HCl for 1 hr at 155°C by quantification of the hydroxyproline content



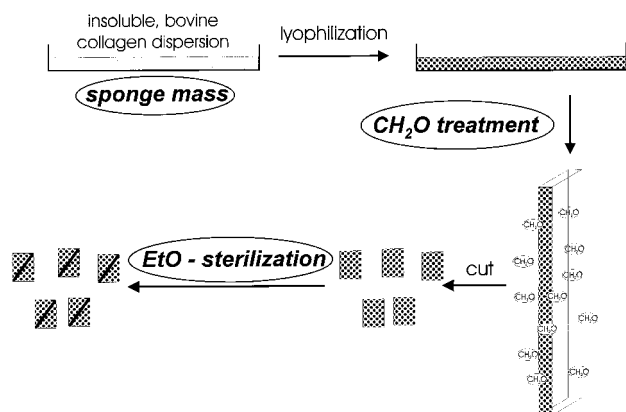


Figure 1. ACS manufacturing process; modified parameters are circled.

(16). Sponges were treated with CH_2O vapor for 30, 90, or 240 min. Cut and packed ACS were sterilized for 4 hr at 45°C with 100% ethylene oxide (EtO).

Transmission Electron Microscopy

The investigations were carried out on a Philips EM 300 (New York, NY). A 0.1% w/w collagen dispersion at pH 6.3 was placed on a formvar-coated copper grid (EMS, Fort Washington, PA), washed with water, and stained for 20 min in 1% phosphotungstic acid pH 6.3. The grid was washed again with water and air-dried.

Scanning Electron Microscopy

The investigations were carried out on an RJ Lee Personal SEM (Traffort, PA). ACS pieces of 6.5 cm^2 were soaked with 650 μl glu I/II mixture (3:5) and rinsed three times with water after 30 min. After dehydration via ethanol gradient, ethanol was exchanged against hexamethyldisilazane (EMS), then samples were air-dried and sputtered with gold.

ACS Degradation

Collagen sponge pieces of 6.5 cm^2 were placed in 5 ml 50 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.4, 0.36 mM CaCl_2) containing 10 μg collagenase type H (Sigma). After 5 hr, 200- μl samples were centrifuged, the supernatant was mixed with bicinchonic acid (BCA) reagent (Pierce, Rockford, IL), and after 2 hr the absorbance at 562 nm was measured and corrected for 25 mg sample weight.

ACS Denaturation Temperature

ACS pieces of approximately 10 mg weight were soaked with 30 μl phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4) pH 7.2 for 2 hr prior to differential scanning calorimetry (DSC) analysis (Seiko DSC 120, Haake, Paramus, NJ). Pieces were heated from 15 to 130°C at $1.25^\circ\text{C}/\text{min}$.

Expressate Analysis

ACS pieces of 6.5 cm^2 were soaked with 650 μl 1.5 mg/ml rhBMP-2 in gluI/II buffer (3:5) pH 4.5. After 30 min, the sponge pieces were squeezed out using a pair of tweezers. The expressed solution was collected; weight, pH, and conductivity (Twin Cond conductivity meter B-173, Horiba, Irvine, CA) were measured; and the expressate was visually inspected for protein precipitation. Anion analysis was performed using a poly(styrene-divinylbenzene) column with trimethylammonium exchanger (PRP-X100, Hamilton, Reno, NV) and 4 mM parahydroxybenzoic acid in 2.5% methanol pH 8.9 (adjusted with LiOH) at 1 ml/min with conductivity detection (model 431, Waters, Milford MA; 0.01 S sensitivity range and 500 μS base sensitivity).

rhBMP-2 Aggregation

NaCl (1, 5, 10, 15, 20, 30, 50 mM), Na_2SO_4 (0.1, 0.5, 1, 1.5, 2, 3 mM) or both ($\text{Na}_2\text{SO}_4/\text{NaCl}$ 0.1/1.5, 0.5/7.5, 1/15, 1.5/22.5, 2/30, 3/45 mM) were added to 1.5 mg/ml rhBMP-2 formulations in gluI/II buffer (3:5) at pH 5.0, 5.5, 6.0, and 6.5. The preparations were gently agitated and visually inspected for precipitation. To confirm visual results, samples were centrifuged and the supernatant was analyzed for rhBMP-2 concentration via UV absorbance at 280 nm.

For analysis in combination with ACS, $7.5 \times 10\text{ cm}$ collagen sponges were rinsed with water (2 liter exchanged four times over 48 hr) and re-lyophilized. First, 325 μl of 3.0 mg/ml rhBMP-2 in gluI/gluII buffer (3:1) was added to 6.5 cm^2 pieces followed by 325 μl of glu II buffer. After 30 min, the expressate was gathered and analyzed as described above.

Determination of Free Amino Groups

To each ACS sample of 1–10 mg, 1 ml 0.1 M borate buffer pH 9.3 and 1 ml 0.1% w/v 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fluka, St. Louis) in 0.1 M



borate buffer pH 9.3 were added. TNBS labels amino groups that lead to trinitrophenyl derivatives (17). The reaction has to be performed at room temperature or slightly elevated temperatures and at a pH between 9 and 10. After the mixture was shaken for 2 hr at 40°C, 2 ml 12 N HCl was added to each sample for hydrolysis and to stop the reaction. Gas development during HCl addition was evaded by the use of borate buffer (18) instead of bicarbonate buffer (19). The collagen material was shaken and hydrolyzed at 80°C for 2 hr. The labeled insoluble collagen material is dissolved and becomes accessible for spectrophotometric quantification. Unlike α -amino group derivatives, ϵ -amino group derivatives are stable to acid hydrolysis (17). A 200- μ l aliquot was diluted to 1 ml with water and absorbance was analyzed at 346 nm.

RESULTS AND DISCUSSION

Electron microscopy demonstrated the presence of insoluble collagen fibers in the starting dispersion [Fig. 2(a)] with diameters of approximately 0.05–0.3 μ m and lengths of approximately 5–250 μ m forming entangled aggregates. After lyophilization, the sponges showed larger pores on the bottom side of up to 300 μ m. The pore diameter decreased toward the top of the sponge, and the structure turned into intertwined individual fibers at the top. During freezing, ice crystals form and squeeze the collagen fibers into the remaining interstices with the pore size distribution depending on the freezing conditions. Placement of a collagen dispersion on frozen shelves leads to formation of large ice crystals started on the bottom side with an increasing number of crystallization points and consequently smaller pores toward the top (20). Freezing the dispersion slowly by reducing the shelf temperature results in a more homogeneous ice crystal formation and consequently even pore size distribution throughout the system (20,21). The wet sponge showed pores reduced in size to approximately 50 μ m. The soak volume, which was approximately one-third of the dry ACS volume, became decisive for the dimensions of the implant [Fig. 2(b)].

Free Amino Groups

Figure 3(a) shows the absorbances for 1–10 mg collagen standard and two nonsterilized ACS materials obtained from free amino group analysis. These initial results indicated two problems: (i) extrapolated curves for different materials did not go through the origin and met

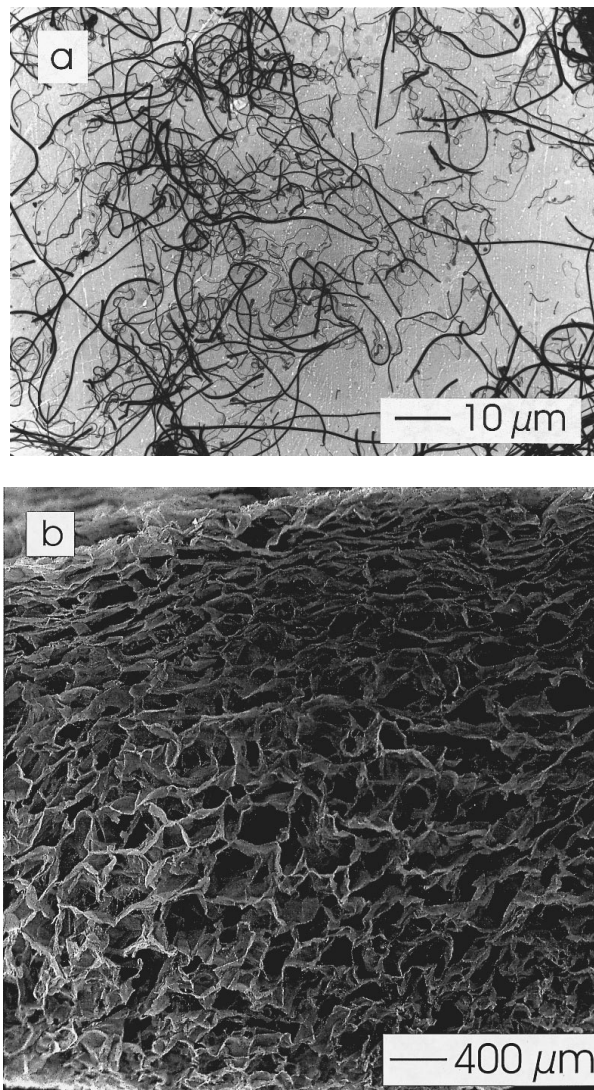


Figure 2. (a) Transmission electron micrograph of collagen dispersion for ACS preparation. (b) Scanning electron micrograph of ACS.

the y-axis at different intercepts, and (ii) the absorbance reached a plateau at higher collagen amounts. Therefore, in order to quantify the amino groups, five specimens for each material in the linear range of the absorbance versus sample weight were analyzed with weights of 1.15–1.35, 1.85–2.05, 2.55–2.75, 3.25–3.45, and 3.95–4.15 mg. The slope of a linear fit through these data points was used in AU per milligram of sample [Fig. 3(b)].

As shown in Fig. 4, the amount of free amino groups decreased significantly (*t*-test) when exposure of ACS to CH₂O was extended by approximately 10% for 90 min



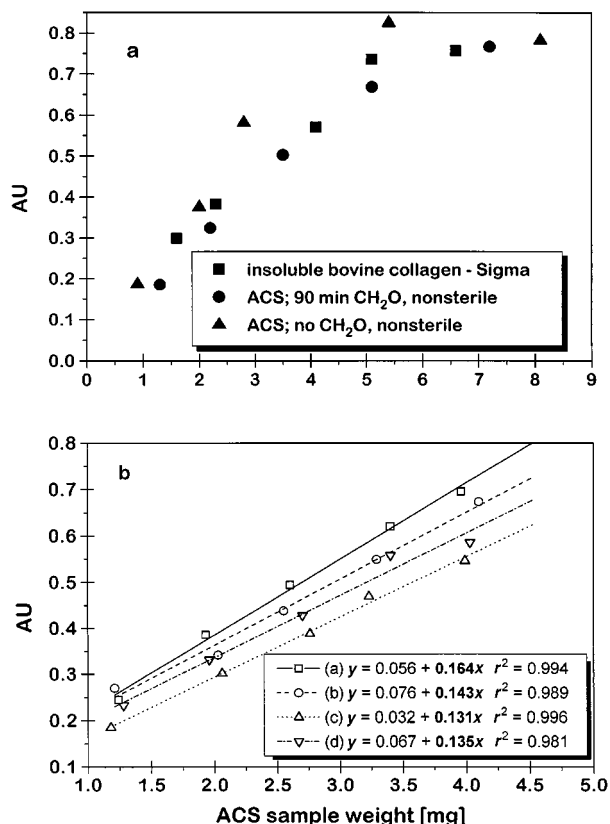


Figure 3. (a) Absorbance from TNBS derivatization. (b) Absorbance from TNBS derivatization for nonsterilized ACS; weights limited between 1 and 5 mg with linear fits. (□) Non-cross-linked, 6.2 mg/cm²; (○) 90 min CH₂O treatment, 6.2 mg/cm²; (△) 240 min CH₂O treatment, 6.2 mg/cm²; (▽) 90 min CH₂O treatment, 2.6 mg/cm².

and by approximately 20% after 240 min treatment, whereas after 30 min a significant change could not be detected. The significantly highest numbers of free amino groups for ACS with 6.2 mg/cm² weight within the 90-min treatment groups indicated that cross-linking may be affected by the ACS weight due to reduced penetration of CH₂O atmosphere into the porous structures. In contrast, there was no significant difference between the 2.6 and 4.4 mg/cm² ACS material, indicating consistency of cross-linking at the lighter ACS weights. The numbers for non-cross-linked material compared well to insoluble bovine tendon collagen control material.

The EtO sterilization procedure induced a decrease in the amount of free amino groups to approximately 35% of the initial number (Fig. 4). A similar degree of derivatization was described by Olde Damink et al. (22). EtO strongly reacts with amino groups at elevated tempera-

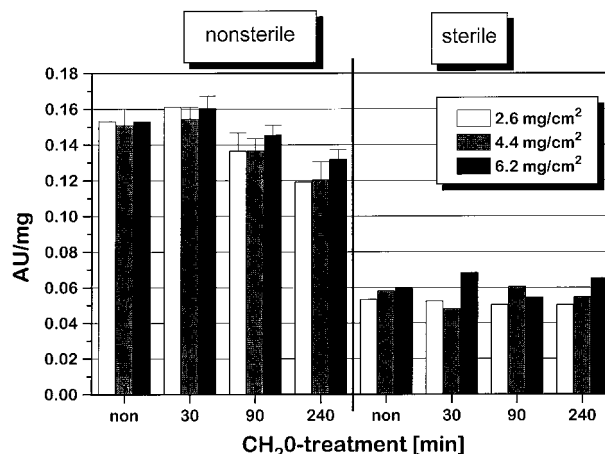


Figure 4. Free amino groups on ACS as a function of sterilization, sponge mass, and cross-linking time.

ture and humidity during 4 hr of exposure, independent of previous CH₂O treatment. The analyzed samples were picked randomly from within the sterilization chamber and amino group modification could be considered consistent throughout the load.

Denaturation Temperature

The reaction of CH₂O and EtO with free amino groups changed the stability of the helical structures, expressed by the denaturation temperature (Fig. 5). The DSC results for nonsterilized ACS followed the same trend as the free

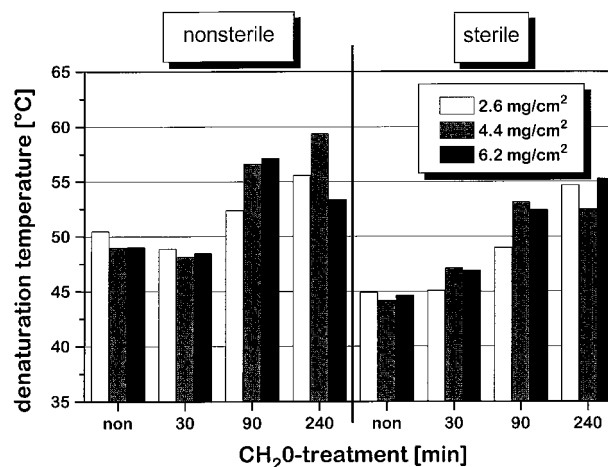


Figure 5. Denaturation temperature of ACS as a function of sterilization, sponge mass, and cross-linking time.



amino group data obtained from TNBS derivatization. After 30 min, a significant change could not be detected, whereas with longer CH_2O treatment the denaturation temperature increased from approximately 48 to 55°C after 90 and 240 min. After cross-linking, more energy and therefore higher temperatures were necessary to break the helical structures. The effect was rather mild compared to increases in shrinkage temperature or denaturation temperature after cross-linking with glutaraldehyde, hexamethylenediisocyanate, or via dehydrothermal treatment (22–24). At longer CH_2O exposure times sample preparation was difficult because of inadequate wettability with PBS, especially for nonsterilized cross-linked material.

Sterilization led to a decrease in denaturation temperature for all groups of ACS by an average of 4°C. EtO bound to the amino groups interferes with the interactions between the collagen structures rather than intensifying the stability by additional cross-linking (22). The sterilized material indicated a steady increase in denaturation temperature with CH_2O exposure time, independent of ACS mass. For a sterilized product, CH_2O treatment cannot be detected by quantification of free amino groups due to strong chemical modification by EtO. However, DSC post-sterilization can be used as an indicator for previous additional chemical cross-linking.

Collagenase Degradation

As can be shown in vitro, chemical modifications due to cross-linking and sterilization alter the ACS degradability by collagenase (Fig. 6). As little as 30 min CH_2O exposure reduced the amount of collagen cleaved within 5 hr. This significant effect of short CH_2O treatment on enzymatic degradation treatment had not been reflected in the amount of free amino groups or the DSC analysis of the denaturation temperature. With longer exposure to CH_2O , the amount of ACS degraded decreased even further. A difference in the effect of 90 or 240 min of CH_2O treatment could be discriminated by the use of a 30-fold higher collagenase concentration, resulting in less degradation for material treated for 240 min compared to 90 min. With sterilization, the enzymatic degradation was enhanced, especially for 30 min CH_2O treatment corresponding to the interference of EtO with the helical structure as seen in the decrease in denaturation temperature.

pH

The final implant was created in situ by combination of an ACS obtained by lyophilization of a collagen dis-

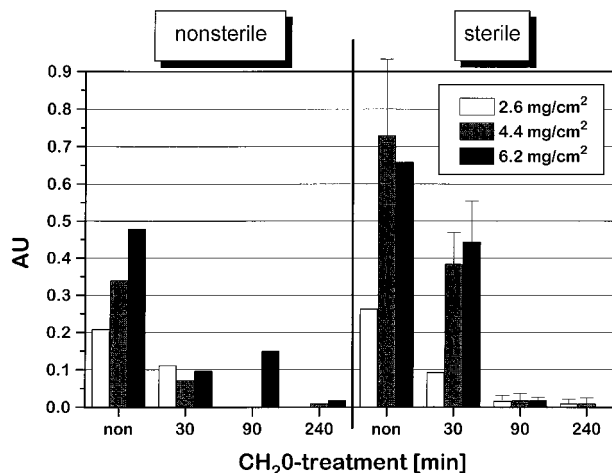


Figure 6. Collagenase degradation of ACS as a function of sterilization, sponge mass, and cross-linking time.

person at pH 6.3 and treated by cross-linking, and sterilization with the liquid rhBMP-2 formulation at pH 4.5 (Fig. 7). This gives rise to interest in the pH of the system because of its effect on the biocompatibility and on the rhBMP-2 structure. BMPs are characterized by low solubility in water and extraction of BMPs from natural sources is performed after removal of soluble proteins by the use of guanidine or urea, preferably in slightly acidic milieu. Furthermore, hypotheses for the physiological role of BMPs in the regulation of bone remodeling are based on the low solubility (25). With rising ACS mass the pH for nonsterilized material increased. Independent of the CH_2O treatment, the formulation pH of 4.5 was increased by approximately 0.25 units for the 2.6 mg/cm² samples, whereas 6.2 mg/cm² ACS material brought the pH up to approximately 5.1 (Fig. 8). The pH of ACS soaked in a volume of water equivalent to the collagen dispersion, which was lyophilized to manufacture the ACS material instead of rhBMP-2 formulation, was ap-

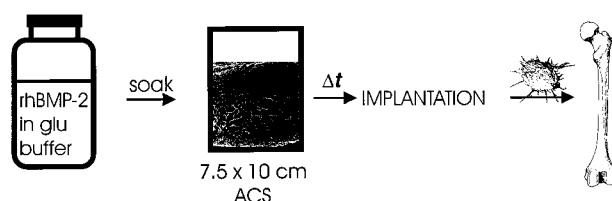


Figure 7. rhBMP-2/ACS combination application; rhBMP-2 is reconstituted and transferred onto ACS; after a waiting period the implant can be used for bone regeneration.



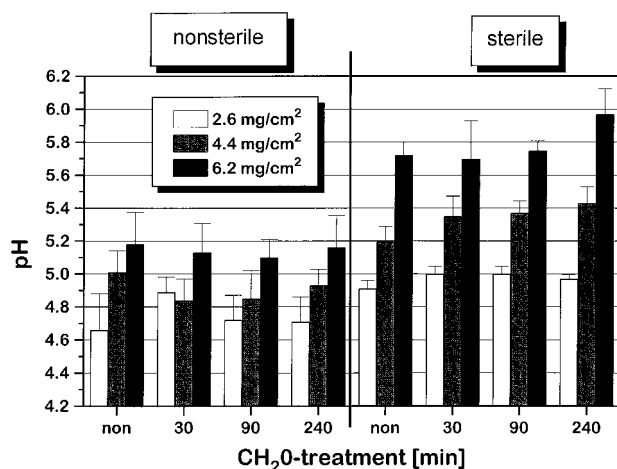


Figure 8. Expressate pH as a function of sterilization, sponge mass, and cross-linking time.

proximately 6.3, identical to the pH of the original dispersion. However, sterilization greatly increased the resulting pH. For the low weight ACS material the pH reached approximately 5.0 and increased to 6.0 for heavy material samples. Again, this effect was independent of the degree of cross-linking. In comparison, incubation of sterilized 4.4 mg/cm² ACS in water as described for non-sterilized material resulted in an expressate pH of approximately 10. In combination with formulation buffer, this pH shift toward higher pH could influence solubility and stability of protein formulations loaded onto the sponge as well as interactions between ACS and protein (26).

Conductivity

An additional factor that could be critical for protein aggregation was the anion concentration in the carrier soaked with rhBMP-2 formulation (27). Ion chromatography results of liquid expressed from sterilized ACS material indicated chloride and sulfate as the two main anions (Table 1). For routine operations conductivity was measured instead of quantification of anions via HPLC. Conductivity, similar to pH, was independent of CH₂O treatment (Fig. 9). With weight, conductivity increased for nonsterilized samples in an almost linear fashion starting at the conductivity for the original buffer formulation of 1.1 mS/cm. In addition, conductivity decreased significantly with sterilization for 6.2 and 4.4 mg/cm² ACS and only a slight trend in reduction was observed for 2.6 mg/cm² ACS. Ion chromatographic investigations

Table 1

Average Chloride and Sulfate Concentrations (mM) in Expressates from Sterilized ACS Material

	Chloride	Sulfate
2.6 mg/cm ²	8.8 ± 2.2	0.25 ± 0.01
4.4 mg/cm ²	14.9 ± 2.7	0.35 ± 0.04
6.2 mg/cm ²	20.2 ± 0.9	0.45 ± 0.02

showed a reduction in chloride concentration in sterilized over nonsterilized implants (data not shown) based on the formation of ethylene chlorohydrin by reaction of inorganic chloride with ethylene oxide (28). For the glu buffer formulation, conductivity remained constant in the pH range from 4.5 to 6.5, indicating that the effect of proton concentration was compensated and the reduction in chloride content by sterilization was the decisive factor for reduction of conductivity. For sterilized 6.2 mg/cm² ACS, both pH and conductivity in the expressate reached their maximum and we decided to evaluate whether the obtained levels would be critical for rhBMP-2 aggregation and interactions with ACS.

rhBMP-2 Precipitation

We investigated the effect of chloride and sulfate on rhBMP-2 solubility both in the liquid formulation itself and when applied to ACS. Concentrations of 15 mM chloride and 1 mM sulfate, previously found in expressates, were used as reference. As can be seen in Fig. 10(a), visible aggregation of rhBMP-2 did occur with in-

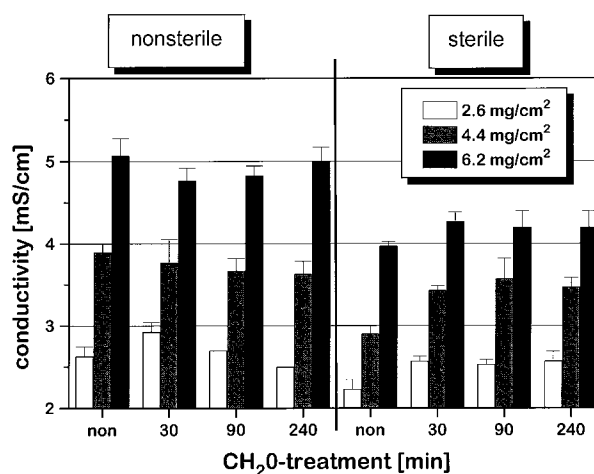


Figure 9. Expressate conductivity of sterilization, sponge mass, and cross-linking time.



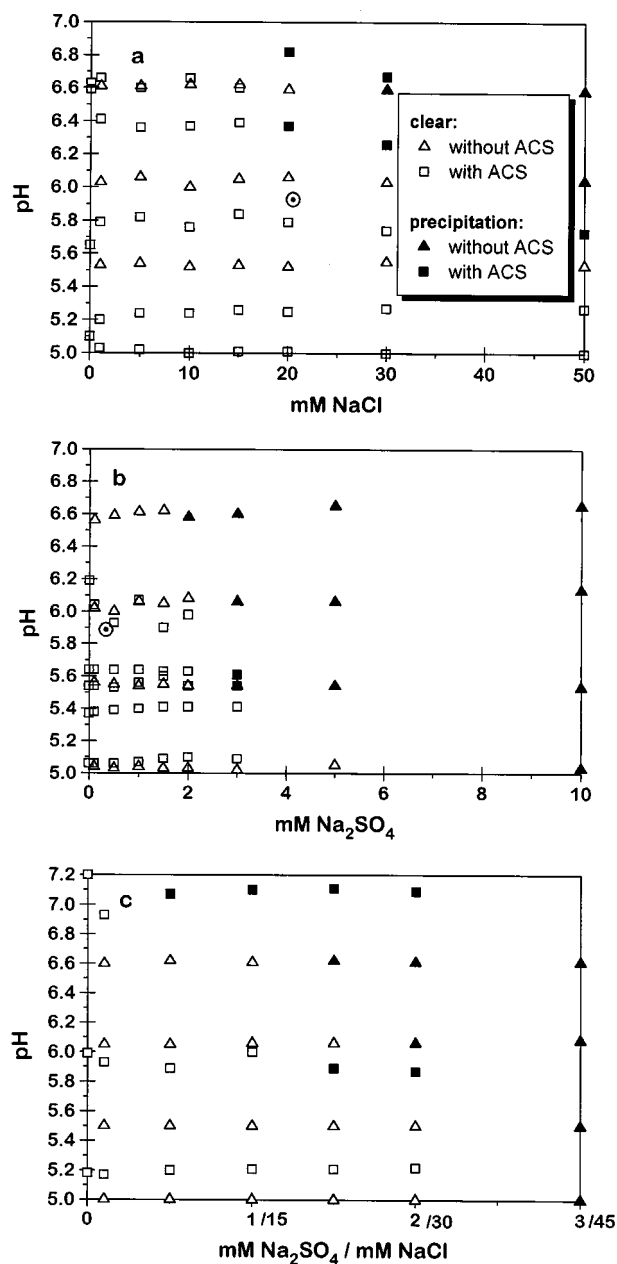


Figure 10. RhBMP-2 precipitation as a function of (a) pH and NaCl, and (b) pH and Na₂SO₄. (c) pH and NaCl + Na₂SO₄; indicates 20.2 mM chloride, 0.45 mM sulfate, and pH 5.9 as found on average for expressates from sterilized 6.2 mg/cm² ACS.

creasing pH (pI of rhBMP-2 ~ 9) and increasing chloride concentrations. However, pH and chloride concentration found in 6.2 mg/cm² sterilized ACS (pH 5.9, 20.2 mM chloride) were well outside the area of precipitation. Precipitation at pH below 6.0 occurred only at 50 mM chloride. The results showed that there was no distinct influence by the presence of ACS, which was freed from salt.

Comparison to the results on the effect of Na₂SO₄ based on the same setup [Fig. 10(b)] demonstrated that rhBMP-2 was more sensitive to the presence of sulfate compared to the presence of chloride. Still, a combination of 0.45 mM sulfate and pH 5.9 as occurred for a 6.2 mg/cm² ACS did not lead to precipitation. More than 3 mM sulfate was necessary at this pH in order to cause visible aggregation of rhBMP-2. Again, the presence of collagen did not induce noticeable differences in the onset of precipitation. In addition, rhBMP-2 precipitation with combinations of chloride and sulfate was studied [Fig. 10(c)]. The chloride-to-sulfate ratio of 15:1 was chosen on the basis of previous expressate analysis (data not shown). The effect of both anions on precipitation was additive and led to precipitation at lower pH, respectively, for lower concentrations of sulfate or chloride than found for each anion separately. Taking the standard deviations and variability of pH and anion concentrations in expressates into account, the results found for sterilized 6.2 mg/cm² ACS come close to the edge of visible aggregation.

Thus, two of the three variables tested, cross-linking and sterilization, led to pronounced chemical modifications in collagen sponges. As a consequence, denaturation temperature and collagenase resistance both increased with CH₂O treatment and decreased with EtO exposure. An important additional factor that had to be considered when utilizing porous collagen systems as protein carriers was the pH shift upon sterilization. This pH increase and the anion content became more critical the higher the ACS mass. ACS weight could not be raised at will in order to modify the implant properties, but was restricted by rhBMP-2 precipitation. Close control of the pH-shift due to sterilization and the anion residuals on the ACS could widen the limits. In addition, ACS mass affected the degree of cross-linking. These variables that were tested cannot be separated, but represent a complex mixture with the factors affecting each other. Consistency in the processing parameters is important. Future studies should focus on the effects of ACS processing parameters on the interactions of the carrier system with rhBMP-2 as well as the importance for in vivo performance (26).



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Stabilization of a Hydrophobic Recombinant Cytokine by Human Serum Albumin

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ABSTRACT: The objective was to evaluate the impact of pH and NaCl content on aggregation, particle formation, and solubility of a hydrophobic recombinant human cytokine in formulations with human serum albumin (HSA) as stabilizing excipient. While cytokine-HSA formulations were stable at physiological pH, a tremendous increase in turbidity at pH 5.0, close to the isoelectric point of HSA was caused by a partially irreversible precipitation. By dynamic light scattering (DLS), disc centrifugation, atomic force microscopy (AFM), and light obscuration it could be shown that the turbidity was mainly caused by particles larger than 120 nm. SDS-PAGE provided evidence that the precipitation at pH 5.0 was mainly caused by the cytokine. The HSA-stabilizers Na-octanoate and Na-N-acetyltryptophanate were less effective in preventing the turbidity increase of unstabilized-HSA compared to NaCl. The interactions between HSA and cytokine were weakened by NaCl, as determined by fluorescence spectroscopy. The positive effect of NaCl on the formulation could be attributed to a direct stabilization of HSA and weaker interactions between HSA and the cytokine, which in consequence provided an overall stabilization of the cytokine. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 96:2987–2999, 2007

Keywords: albumin; fluorescence spectroscopy; light scattering (dynamic); nephelometry; protein aggregation; protein formulation; interaction

INTRODUCTION

The limited solubility of hydrophobic cytokines, which can range below 0.05 mg/mL at physiological pH,¹ associated with a strong aggregation and adsorption tendency are the major challenges during formulation development. One possible approach is the use of the physiologically well tolerated human serum albumin (HSA) as stabilizer for the cytokine. Numerous examples of commercial formulations with HSA as stabilizer

for therapeutic proteins are available, for example, for factor VIII, interferons, urokinase, streptokinase, immunoglobulins, and others.² The stabilizing effect of HSA on a second protein is often explained by the stabilizing properties a polymer can provide.³ There is also evidence that direct interactions between HSA and the active proteins are responsible for the stabilization.⁴ Generally, HSA is extracted from human plasma and therefore a pasteurization process is required to eliminate potential blood born pathogens. According to the US Food and Drug Administration the pasteurization process has to be conducted for 10 h above 60°C.⁵ The FDA requires the addition of 0.16 mM stabilizer per gram HSA, either as combination of 0.08 mM Na-octanoate and 0.08 mM Na-N-acetyltryptophanate or 0.16 mM

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Na-octanoate as single substance.⁵ Na-octanoate and Na-N-acetyltryptophanate increase the denaturation temperature of HSA and thereby, prevent HSA from aggregation and denaturation.^{6,7} In addition, HSA is stabilized by NaCl, which also inhibits the heat-induced aggregation.⁸ These additives brought into the formulations indirectly via HSA may induce stability problems, for example, for proteins which are sensitive to ionic strength. Furthermore, they can impact the physico-chemical properties of other excipients, for example, crystallization behavior or glass transitions which can become critical for lyophilized formulations.⁹ Therefore, it is important to study the impact of these compounds on the formulations.

In the presented study, a hydrophobic cytokine was formulated in combination with stabilized-HSA and mannitol in analogy to commercially available products.² The goal was to elucidate the stabilizing properties of HSA for the hydrophobic cytokine. The physical stability of cytokine-HSA formulations was characterized with special focus on aggregation under different pH and ionic strength conditions. To get comprehensive insight into the aggregation phenomena in cytokine-HSA formulations, turbidimetry, DLS, disc centrifugation, AFM, and light obscuration were performed. We further wanted to measure the interactions between the cytokine and HSA by fluorescence spectroscopy to understand the properties of the cytokine-HSA formulation under different pH and ionic strength conditions.

MATERIALS AND METHODS

Materials

A formulation with 0.25 mg/mL cytokine, 12.5 mg/mL mannitol, and 12.5 mg/mL stabilized-HSA was used. This formulation further contained between 0.08 and 0.1% NaCl as analyzed by ICP-OES, deriving from HSA and pH-adjustment. Unstabilized-HSA (fraction V, 96–99% purity) from Sigma-Chemicals (Steinheim, Germany) was solid and contained no further excipients. Stabilized-HSA from Grifols (Langen, Germany) was used as 20% solution and contained 16 mmol Na-octanoate, 16 mmol Na-N-acetyltryptophanate, and 130–160 mmol/L sodium. As HSA-free cytokine material a bulk with 1.2 mg/mL cytokine in 20 mM glycine at pH 3.0 was used. Sodium chloride, Na-octanoate, and N-acetyl-DL-

tryptophanate were purchased from Sigma. Potassium chloride, lithium chloride, sodium acetate, ammonium chloride, potassium thiocyanate, and potassium iodide were purchased from Merck (Darmstadt, Germany). All salts were of reagent grade and used without further purification.

Turbidity Measurement

Turbidity measurement was performed with a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany). Light at $\lambda = 860$ nm was sent through the samples and the scattered light was detected at a 90° angle. The system was calibrated with formazine as standard and the results were given in formazine nephelometric units (FNU).

Light Obscuration

Particles from 1 to 200 μm were determined by light obscuration measurement using a PAMAS—SVSS-C Sensor HCB-LD-25/25 (Partikelmess- und Analysensysteme GmbH, Rutesheim, Germany). Five aliquots of 0.3 mL were analyzed of each sample.

Zetapotential

The zetapotential was determined with a Zetasizer Nano (Malvern, Herrenberg, Germany). The measurements were performed in the automatic measurement mode using disposable capillary cells (Malvern DTS 1060).

Dynamic Light Scattering (DLS)

DLS performed on a Zetasizer Nano (Malvern, Herrenberg, Germany) was used to characterize protein molecules and particles in the range from 1 to 1500 nm. The Zetasizer Nano is operating with a 4 mW He-Ne-Laser at 633 nm and noninvasive back-scatter technique. The size distribution by intensity and volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software from Malvern (version 4.00).

Disc Centrifugation

The CPS disc centrifugation system (LOT-Oriel GmbH, Darmstadt, Germany) was used to determine

the size distribution of the particles in the range of 20 nm to 2 μ m. Two hundred microliters of the protein solution was applied on the disc, which was rotating with 22000–24000 rpm. A density gradient from 7 to 2% sucrose was built up within the disc. At the edge of the disc the particles were detected with a light source of 470 nm. The time required for the particles to reach the edge of the disc, as well as the absorption signal were transformed to a particle size distribution using Stokes-Law and Mie-theory.

Atomic Force Microscopy (AFM)

AFM was conducted with a Nanoscope VI Dimension Bioscope (Veeco Instruments, Santa Barbara, US). Prior to the measurement a small silica plate was immersed into the sample for about 10 min and subsequently measured in the liquid state. As imaging technique the tapping mode in air was used. Interactions between the sample and the tip were below 300 pN. Type I cantilevers with a nominal spring constant of 36 nN/nm were applied. The scanning speed was adjusted to the respective scanned area and ranged between 0.25 and 2 Hz at a resolution of 512×512 pixels, independent of width of the scanned area. All experiments were performed under atmospheric pressure at 25°C/60% RH.

SDS-PAGE

Nonreducing denaturing SDS-PAGE was used to analyze formulations containing HSA and the cytokine. NuPAGE[®] 10 and 12% Bis-Tris gels 1 mm, 10 wells (Invitrogen, Karlsruhe, Germany) and NuPAGE[®] MOPS running buffer was used for the separation. The electrophoresis was performed at a constant current of 0.03 A per gel. NuPAGE[®] LDS sample buffer was added to the samples, which were denatured for 10 min at 95°C. The gels were stained with SilverXPress[®] Silver Staining Kit (Invitrogen, Karlsruhe, Germany).

Fluorescence Spectroscopy

Fluorescence Spectroscopy was performed using a Varian Cary Eclipse (Darmstadt, Germany). For the single components studies the solutions were measured in 3.0 mL quartz cuvettes at a constant temperature of 20°C. The emission was recorded

from 260 to 450 nm at an emission slit of 5 nm and a scanning rate of 120 nm/s with an excitation wavelength of 280 nm at an excitation slit of 5 nm. The PMT voltage of the detector was set to 400 V. To study protein–protein interactions 300 μ L of the samples were analyzed in 96-well plates at a constant temperature of 20°C. HSA was used at a constant concentration of 0.5 mg/mL, while the cytokine concentration was varied. HSA-cytokine interactions were studied at pH 3.0 and 4.5 with 0.0 and 0.1% NaCl, buffered with 2 mM glycine. The excitation wavelength was 280 nm at an excitation slit of 5 nm and the emission was recorded from 260 to 450 nm at an emission slit of 5 nm and a scanning rate of 30 nm/s. The PMT voltage of the detector was set to 600 V. Fluorescence quenching was monitored to evaluate the degree of HSA-cytokine interaction. Therefore, the fluorescence of the individual components, as well as of the combination was analyzed for the respective conditions. The degree of interaction was determined using Eq. (1):

$$\text{Degree of interaction} = F_0/F \quad (1)$$

with F_0 as calculated sum of the fluorescence intensity of the individual proteins and F measured fluorescence intensity of solution containing both proteins from 290 to 450 nm. A high value for F_0/F can be attributed to stronger interactions between the two proteins.¹⁰

RESULTS AND DISCUSSION

Effect of pH and NaCl on the Turbidity of Cytokine and Cytokine-HSA Formulations

The impact of NaCl and pH on the turbidity of cytokine formulations and cytokine-HSA formulations was evaluated. For 0.25 mg/mL cytokine a significant turbidity increase was monitored above pH 5.5, with maximum values when the formulation pH approached the pI of the cytokine at 9.2¹¹ (Fig. 1a). Upon the addition of NaCl, the precipitation was fostered and the turbidity stepped up already at lower pH values. Huang et al. (2005)¹² demonstrated for human recombinant interferon- α -2a by isothermal titration calorimetry, that the attraction between the protein monomers was increased by the addition of NaCl with the consequence of boosted aggregation induced by hydrophobic interactions and decreasing electro-repulsive forces between the protein molecules. Below pH 5.5 however, no

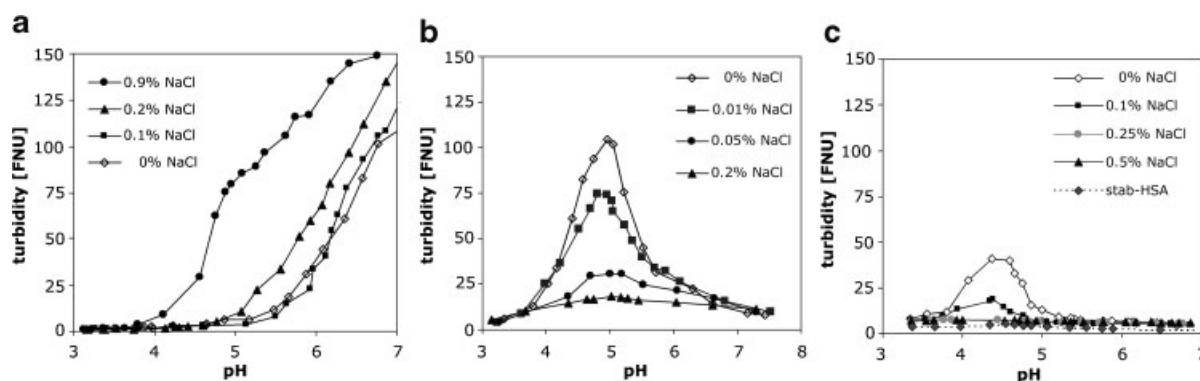


Figure 1. Turbidity of 0.25 mg/mL cytokine (a), 0.25 mg/mL cytokine with 12.5 mg/mL stabilized-HSA (b), and 12.5 mg/mL unstabilized-HSA (solid lines) or stabilized-HAS (dashed line) (c) from pH 3.0 to 7.5 with increasing NaCl concentrations.

turbidity elevation was measured for the cytokine at NaCl concentration up to 0.2%, indicating sufficient solubility of the cytokine under these conditions.

For formulations with 0.25 mg/mL cytokine, 12.5 mg/mL stabilized-HSA and 12.5 mg/mL mannitol a turbidity of 6 FNU was measured at pH 7.4 (Fig. 1b). At physiological pH HSA, which is present in a 50-fold excess compared to the cytokine, exists in its native conformation and can provide sufficient solubilization and stabilization of the cytokine. In several examples HSA is used to stabilize different cytokines at physiological pH^{13,14} as HSA is known to provide a general stabilization and solubilization of a protein and can prevent adsorption.³ When the pH of the formulations was lowered the turbidity increased sharply and reached a maximum of about 100 FNU at pH 5.0 with a similar curve profile as compared to unstabilized-HSA (Fig. 1c). In addition, light obscuration revealed that the number of particles in cytokine-HSA formulations, in particular the particles of 1–2 μm in size increased significantly from 3335 ± 150 particles per mL at pH 7.4 to about 69400 ± 3350 particles per mL when the pH was lowered to 5.0. The apparent correlation between light obscuration and the turbidity data was in agreement with literature.¹⁵

When used as excipient in cytokine-HSA formulations, exclusively HSA stabilized with Na-octanoate, Na-N-acetyltryptophanate, and NaCl is used due to the regulatory requirements. For a solution with 12.5 mg/mL HSA the addition of a total concentration of 2 mM Na-octanoate and Na-N-acetyltryptophanate is required by the FDA.⁵ To determine the impact of these stabilizers on HSA, the turbidity of different HSA solutions was

monitored between pH 3.0 and 7.0. Formulations with 12.5 mg/mL stabilized-HSA exhibited a constant turbidity below 5 FNU from pH 7.0 to 3.0. In contrast 12.5 mg/mL unstabilized-HSA exhibited a significant turbidity increase with a maximum of 50 FNU at pH 4.8 (Fig. 1c). This elevated turbidity is a sign for the presence of larger aggregates and precipitated protein. To elucidate the differences between stabilized and unstabilized-HSA, the impact of the HSA-stabilizers on the turbidity of unstabilized-HSA over the pH-range was analyzed. By adding NaCl to formulations with 12.5 mg/mL unstabilized-HSA the tremendous turbidity increase at pH 4.8 could be significantly reduced to 18 FNU at 0.1% NaCl and to 7.5 FNU at 0.25% NaCl (Fig. 1c). A stabilizing effect of NaCl on HSA and BSA is also described in literature. Saso et al. (1998)⁸ showed a stabilizing effect of NaCl on heat-induced aggregation of HSA which can be attributed to an increase of the denaturation temperature.¹⁶ Yamasaki and Yano (1990, 1991) reported that the denaturation temperature of BSA between pH 4.5 and 9.0 was increased after adding 0.001–1.0 M (0.0058–5.8%) NaCl. They ascribed this stabilizing effect to a screening effect of NaCl on the electrostatic forces and the inhibition of crevice formation within the molecule in the vicinity of the tryptophan residue at position 212.^{17,18} The maximum turbidity for unstabilized-HSA was measured at pH 4.6 close to the pI of HSA at pH 4.8. At its pI, protein–protein interactions are favored due to a reduced interaction energy barrier, which can lead to aggregation.¹⁹ Consequently, Saso et al. (1998)⁸ described, that HSA showed that highest level of heat-induced aggregates in the pH range of 4.5–5.0. The addition of

1–5 mM Na-N-acetyltryptophanate led to a shift of the turbidity maximum to pH 4.5, but the maximum turbidity could be decreased only to values between 30 and 40 FNU (Fig. 2a). Higher concentrations were omitted, due to the poor solubility of the amino acid derivative. When adding N-octanoate the turbidity maximum was shifted to pH 4.5, comparable to Na-N-acetyltryptophanate (Fig. 2b). This comparable effect can be ascribed to the fact that N-octanoate and Na-N-acetyltryptophanate are known to bind to the same high affinity site II located in the subdomain IIIA of HSA.^{20,21} The addition of 1–4 mM Na-octanoate resulted in a less distinct decrease of turbidity compared to Na-N-acetyltryptophanate to values between 30 and 50 FNU. A further

increase in Na-octanoate to concentration between 5 and 10 mM initiated higher turbidities with maximum values above 110 FNU. This emphasized, that Na-N-acetyltryptophanate and Na-octanoate played a minor role in the suppression of the precipitation at the pI of HSA. Apparently, in the concentrations used for stabilizing HSA, NaCl was most effective in the prevention of the turbidity increase at pH 4.8. The addition of Na-octanoate had no apparent impact on the turbidity of the cytokine (data not shown).

The elevated turbidity at pH 5.0 in cytokine-HSA formulations pointed either at a precipitation of HSA or a combination of cytokine and HSA. However, it could also be possible that HSA was no longer capable to provide sufficient stabilization for the formulation, leading to a precipitation of the cytokine. The concept of complex coacervation, characterized as the binding of two oppositely charged macromolecules resulting in a phase separation of formed complex from the solution could as well be considered to explain the precipitation.²² At the turbidity maximum at pH 5.0 HSA still exhibits a negative whereas the cytokine exhibits a positive net charge. Furthermore, it was obvious that the addition of NaCl to the formulations with 0.25 mg/mL cytokine and 12.5 mg/mL stabilized-HSA could significantly reduce the turbidity increase at pH 5.0, indicating a stabilizing effect of NaCl on the formulations (Fig. 1b). An increase in ionic strength is known to dissolve the complexes formed during complex coacervation.²³ For NaCl concentrations between 0.2 and 0.9% the measured turbidity at pH 5.0 reached a plateau phase of about 20 FNU. This wide plateau phase pointed at a rather unspecific stabilizing effect of NaCl, probably induced by electrostatic effects. However, especially above pH 5.0 complex coacervation cannot be used exclusively as explanation for the observed turbidity profile and the stabilizing effect of NaCl. Here a NaCl concentration needs to be selected to keep the balance between the stabilizing effect of NaCl for the formulation and the aggregation inducing effect of NaCl on the cytokine. A suitable NaCl concentration to achieve this balance is found between 0.1 and 0.2% NaCl.

The turbidity maximum of the cytokine-HSA formulations at pH 5.0 was close to the isoelectric point of HSA at pH 4.8.²⁴ Compared to the cytokine-HSA formulation with a turbidity maximum at pH 5.0, the turbidity maximum was located at pH 4.6 for comparable pure HSA

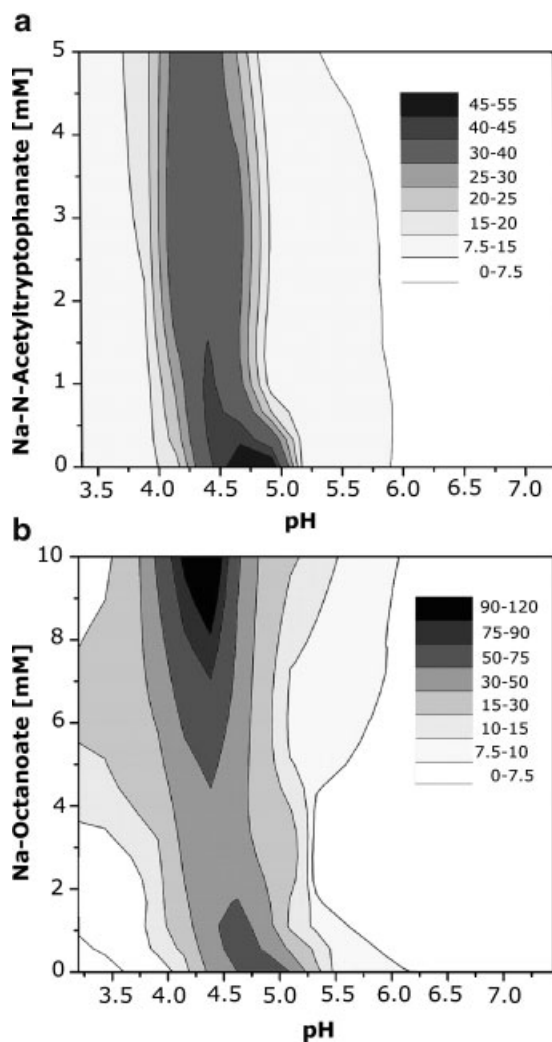


Figure 2. Turbidity in FNU of 12.5 mg/mL unstabilized-HSA with 0 to 5 mM Na-N-acetyltryptophanate (a), and 0 to 10 mM Na-octanoate (b) at pH 3.0–7.0.

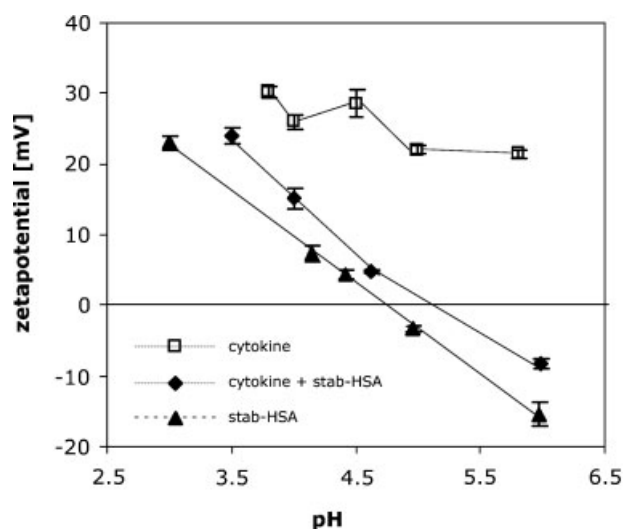


Figure 3. Zetapotential of 0.25 mg/mL cytokine, 12.5 mg/mL HAS, and a combination of 0.25 mg/mL cytokine and 12.5 mg/mL HSA at pH 3.0–6.0.

solutions (Fig. 1 b and c). This shift of the turbidity maximum in cytokine-HSA formulations could be explained by the zetapotential of the formulations (Fig. 3). For stabilized-HSA a zetapotential of 0 mV which marks the isoelectric point was reached at pH 4.7. For cytokine-HSA formulations a zetapotential of 0 mV, was found around pH 5.0. Thus, the pH at which the zetapotential reached 0 mV was shifted to higher pH values due to the addition of the cytokine. Generally, the pI of protein mixtures is found between the pI values of the individual proteins depending on the ratio. This was, for example, shown by Rezwan et al. (2005)²⁵ for bovine serum albumin and lysozyme adsorbed on colloidal particles. Between pH 3.0

and 5.5 the cytokine with its pI of 9 exhibited a positive value for the zetapotential, which was responsible for the shift of the apparent pI in cytokine-HSA formulations. Above pH 5.5 it was not feasible to determine the zetapotential of the cytokine due to the beginning precipitation and its low solubility.

Effect of Salt Type on the Turbidity of Cytokine-HSA Formulations

To evaluate whether the stabilizing effect of NaCl on cytokine-HSA formulations was specific for NaCl different salts were added to the formulation of 0.25 mg/mL cytokine, 12.5 mg/mL HAS, and 12.5 mg/mL mannitol at an ionic strength of $\mu = 0.009$ and 0.034 (Tab. 1). The resulting turbidity maxima were located at pH 5.0 for all studied conditions. The only exception was KSCN at an ionic strength of $\mu = 0.034$ as the maximum turbidity was shifted to pH 4.0. All tested salts led to a decline of the maximum turbidity at pH 5.0 compared to 100 FNU without salt. It is generally difficult to predict the direct effect of salts on protein stability, as it is an interaction of various factors, for example, type of protein, pH, ionic strength, and mechanism of interaction.¹⁹ Ions can be classified by their chaotrope effect or salting in, respectively, cosmotrope or salting out effect in the order of an increasing chaotrope effect for $\text{SO}_4^{2-} < \text{CH}_3\text{COO}^- < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{SCN}^-$ for anions and $\text{NH}_4^+ < \text{K}^+ < \text{Na}^+ < \text{Li}^+ < \text{Mg}^{2+} < \text{Ca}^{2+} < \text{Ba}^{2+}$ for cations.²⁶ A stabilizing or salting out effect is achieved when the addition of salt leads to a preferential hydration of the protein, whereas binding of salts to the protein often yields in a destabilizing, salting in effect.^{27,28}

Table 1. Maximum Turbidity in FNU at pH 5.0 for the Cytokine-HSA Formulation After the Addition of Different Salts at Ionic Strength of $\mu = 0.009$ and $\mu = 0.034$

	$\mu = 0.009$		$\mu = 0.0345$	
	c (%)	Turbidity pH 5.0 (FNU)	c (%)	Turbidity pH 5.0 (FNU)
NaCl	0.05	34.7	0.2	18.9
KCl	0.06	36.4	0.26	19.9
LiCl	0.04	49.3	0.15	19.1
NaCH ₃ COO	0.07	49.0	0.28	21.0
NH ₄ Cl	0.05	54.7	0.18	18.1
KI	0.14	65.4	0.57	37.4
KSCN	0.06	55.8	0.23	35.4 ^a

^aTurbidity maximum at pH 4.0 with 86.2 FNU.

Low concentrations of salts usually increase the solubility of proteins by nonspecific electrostatic interactions. It was evident that the more chaotrope salts KSCN and KI were less effective in preventing the turbidity increase at pH 5.0. When used at the lower ionic strength NaCl and KCl were superior to the other salts in preventing the turbidity increase at pH 5.0. When increasing the ionic strength to $\mu = 0.034$ NaCl, KCl, LiCl, NaCH₃COO, and NH₄Cl all reached comparable values of about 19 to 21 FNU at pH 5.0. This pointed at an unspecific effect of electrostatic interactions which led to the lowering in turbidity at pH 5.0. The stabilizing effect of NaCl is advantageous due to the physiological compatibility of NaCl and the fact that it is brought into the formulations as HSA stabilizer.

Particle Size Analysis in Cytokine-HSA Formulations

So far, only turbiditometry was used to monitor aggregation and particle formation in cytokine-HSA formulations. However, different particle fractions especially particles smaller than 30 nm, as well as larger particles in the micrometer-range may not sufficiently be reflected by turbiditometry. Therefore, various methods complementing each other, namely dynamic light scattering, AFM, disc centrifugation, and light obscuration were used to further characterize the particle formation.

For cytokine-HSA formulations a shift of the first peak in the DLS size distribution by volume from 4.8 nm at pH 7.0 to 7.5 nm at pH 5.0 without NaCl and from 5.6 nm at pH 7.0 to 6.5 nm at pH 5.0 with 0.2% NaCl was observed. For all cytokine-HSA formulations a second peak at 30 to 60 nm with an intensity of approximately 0.1% was present which can be attributed to aggregated protein. For native HSA at neutral pH, a mean diameter of 6–7 nm and for aggregated HSA a broad peak at 30–100 nm was described by Sontum and Christiansen (1997).²⁹ In highly turbid samples without NaCl a third particle class with a maximum at about 500 nm at pH 5.5 and about 1500 nm at pH 5.0 appeared. The increasing turbidity in cytokine-HSA formulations without NaCl was reflected in a rising integrated peak area above 120 nm from 2.7% at pH 7.0 to 67% at pH 5.0 and at the same time a declining integrated peak area between 15 and 120 nm when the pH was lowered from 7.0 to 5.0 (Fig. 4a). In samples with 0.2% NaCl, which

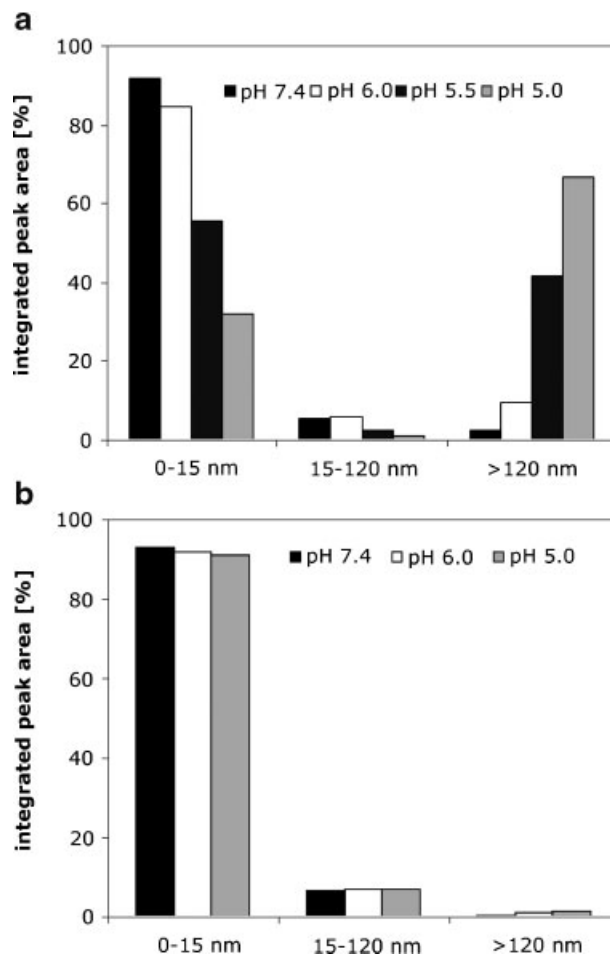


Figure 4. Integrated peak area of the DLS size-distribution by volume in % for cytokine-HSA formulations without NaCl (a) and 0.2% NaCl (b) at pH 7.4–5.0.

exhibited maximally a turbidity of 20 FNU (compare 3.2) less than 1.5% of integrated area of the size distribution by volume could be attributed to the particles larger than 120 nm (Fig. 4b). This strengthened the assumption that particles larger than 120 nm were mainly responsible for the tremendous turbidity increase at pH 5.0 in NaCl free formulations. The results are in agreement with Mahler et al. (2005)¹⁵ who showed for IgG1 formulations that the turbidity determined by UV-absorption at 350 and 550 nm could be attributed to medium sized aggregates.

Disc centrifugation, which is similar to analytical ultracentrifugation, is based on the sedimentation velocity of particles within the density gradient of a disc. The sedimentation velocity depends on particle size and density under the influence of centrifugal forces. With the density

gradient from 7 to 2% sucrose built up within the disc the smallest particles to be detected were 20 nm, as smaller particles would require unfeasible long runtimes. At pH 7.0 about 95% of the intensity could be ascribed to particles smaller than 100 nm without and with 0.2% NaCl (Fig. 5). Thereby, 50% of the cumulative intensity by weight derived from particles smaller than 32 nm without NaCl compared to particles smaller than 40 nm when 0.2% NaCl was added. When lowering the pH of the NaCl-free formulation to 5.0 a significant shift of the size-distribution by weight to larger particles was observed with more the 50% of the intensity by weight attributed to particles larger than 100 nm. For the samples with 0.2% NaCl only 5% of the intensity by weight derived from particles larger than 100 nm.

AFM was used to size and visualize particles in the nanometer-range. Contrary to the other evaluated techniques, AFM studies at pH 5.0 were omitted because of the presence of too large particles in the micrometer-range, which can interfere with the analytics. To size the objects in the samples line-scans were used, which are exemplarily shown for a sizing of 30 nm objects at pH 7.0 (Fig. 6). Thereby, the samples at pH 7.0 exhibited the most homogenous distribution with predominantly small globular objects of 6 to 10 nm next to larger objects with a medium size of 29.4 nm. At pH 6.0, objects with an average size of 31.2 nm and some particles with a diameter of about 80 nm were detected in the sample besides the main fraction at 6 to 10 nm. The incipient precipitation at pH 5.5 was reflected in particles

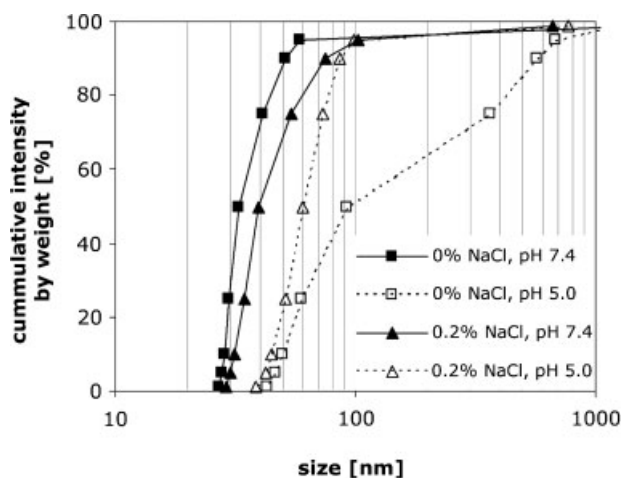


Figure 5. Cumulative particle size distribution by weight of cytokine-HSA with 0.0% NaCl and 0.2% NaCl at pH 7.4 and 5.0 determined with disc centrifugation.

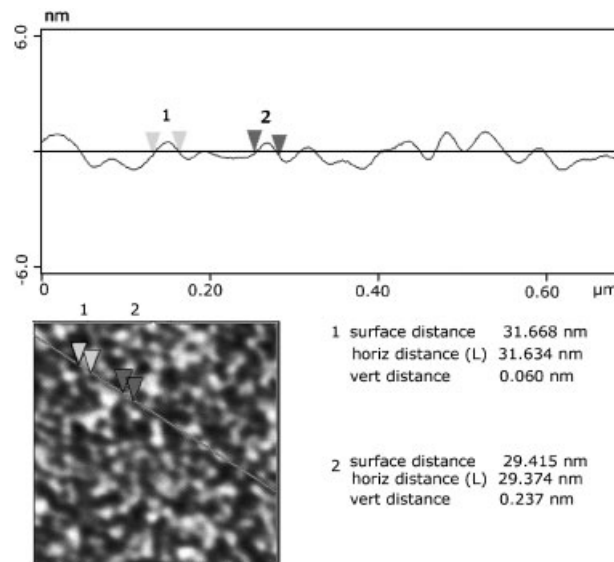


Figure 6. AMF line scan to size particles of 0.25 mg/mL cytokine, 12.5 mg/mL stabilized-HSA at pH 7.4.

with a diameter of 200–400 nm within the sample. Beside these particles, smaller particles of 6–10 nm and 32.4 nm were detected.

The so far described methods focused on characterization of the nanometer-range. Light obscuration was used to determine the number of particles of 1–200 μm . The total number of particles $\geq 1 \mu\text{m}$ at pH 7.0 and 6.0 was slightly higher in the presence of NaCl as compared to the solutions without the addition of NaCl. In the absence of NaCl, an immense increase in particles at pH 5.0, especially in the size range between 1 and 2 μm was observed, due to precipitation of the protein (Fig. 7a). This increase in particles corresponds to the significant turbidity increase for the formulation at pH 5.0. The addition of NaCl was beneficial to inhibit the formation of particles $\geq 1 \mu\text{m}$, as well (Fig. 7b).

A summary of the results obtained by DLS, disc centrifugation, AFM, and light obscuration is displayed in Table 2. In contrast to disc centrifugation and light obscuration, DLS, and AFM were capable to detect particles in the range of 5–10 nm, which represented most of the protein in the samples by number. Particles of a medium size of 30–100 nm were detected by disc centrifugation and DLS for all pH-values. DLS showed that the area of the medium fraction decreased by about 4.5% when the pH was lowered from 7.0 to 5.0 in favor of the larger, turbidity inducing particles. Particles with a size of about 1000–1500 nm were detected by light obscuration, DLS, and disc

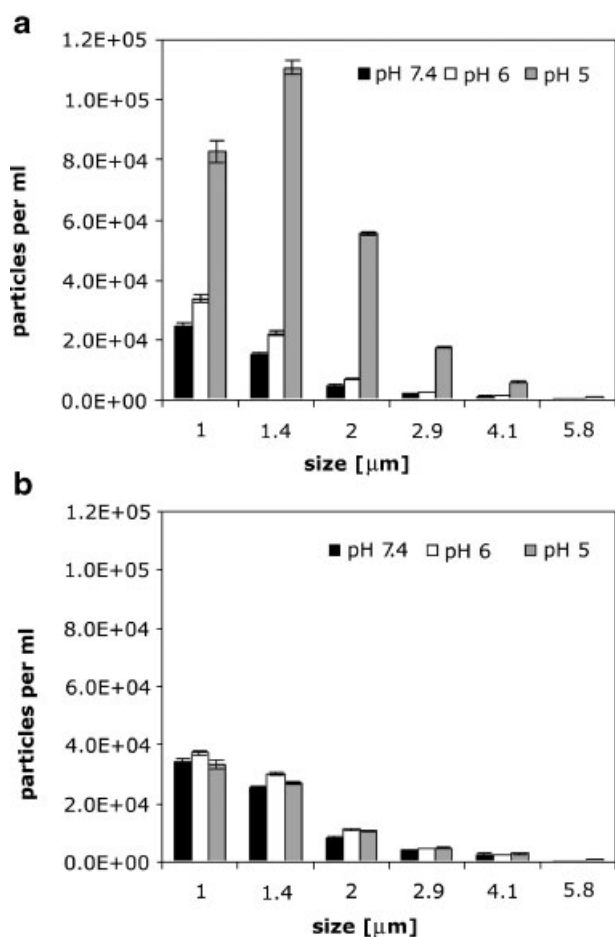


Figure 7. Particles per mL determined by light obscuration for cytokine-HSA formulations with 0.0% NaCl (a), and 0.2% NaCl (b) at pH 7.4, 6.0, and 5.0.

Table 2. Main Particle Sizes of the Cytokine-HSA Formulation Without NaCl Determined With DLS, AFM and Disc Centrifugation

pH	DLS	AFM	Disc Centrifugation	Light Obscuration
7.4	<15 nm (max: 4.8 nm)/91.8% ^a <120 nm (max: 35 nm)/97.3% ^a <1500 nm (no max)/99.9% ^a	6–10 nm 29.4 nm ± 3.2 nm	<50 nm/90% ^b <100 nm/96% ^b <1000 nm/99% ^b	Total number: 44890 1–2 µm/92.5% >2–10 µm/7.5% >10 µm/0.006%
6.0	<15 nm (max: 5.6 nm)/84.5% ^a <120 nm (max: 44 nm) /90.4% ^a <1500 nm (no max)/99.9% ^a	6–10 nm 31.2 nm ± 3.8 nm 80 nm	—	Total number: 62904 1–2 µm/92.7% >2–10 µm/7.2% >10 µm/0.010%
5.5	<15 nm (max: 7.5 nm)/55.7% ^a <120 nm (max: 58 nm)/58.3% ^a <1500 nm (max: 450 nm)/99.2% ^a	6–10 nm 32.4 nm ± 3.5 nm 200–400 nm	—	—
5.0	<15 nm (max: 7.5 nm)/32.1% ^a <120 nm (max: 60 nm)/33.1% ^a <1500 nm (max: > 1000 nm)/99.0% ^a	Not feasible (due to large particles)	<50 nm/10% <100 nm/52% ^b <1000 nm /97% ^b	Total number: 193603 1–2 µm/91.0% >2–10 µm/9.0% >10 µm/0.004%

^aCumulative intensity by volume.

^bCumulative intensity by weight.

centrifugation. Light obscuration showed that especially the number of particles in the size range of 1 to 2 µm increased when the pH was lowered from 7.0 to 5.0. With all methods it could be shown that particle formation was increased when the formulation pH was lowered from 7.0 to 5.0. It was further obvious that NaCl provided a stabilizing effect and could significantly reduce the formation of particles in the formulations.

Characterization of the Precipitation at pH 5.0 in Cytokine-HSA Formulations

Reversibility of the Precipitation at pH 5.0

In order to elucidate the reversibility of the precipitation at pH 5.0 in cytokine-HSA formulations, the pH was lowered to 5.0 and readjusted back to 7.4. Formulations with 0.25 mg/mL cytokine, 12.5 mg/mL stabilized-HSA, and 12.5 mg/mL mannitol, were compared to placebo-formulations containing only 12.5 mg/mL unstabilized-HSA. Unstabilized-HSA was used to eliminate a potential effect of the HSA-stabilizers on turbidity and to show solely the properties of HSA. The turbidity of the cytokine-HSA formulation increased from below 10 FNU at pH 7.4 to about 100 FNU at pH 5.0. When the pH was adjusted back to 7.4 a residual turbidity of 38 FNU remained immediately after the pH-shift and a constant level of 33 FNU was reached after approximately 90 min. This indicated that precipitation, triggered by the

lowering of the pH, was at least partially irreversible, which could become an issue, for example, during pH-adjustment in manufacturing. When 0.2% NaCl was added to the formulations the turbidity increased from 11 FNU at pH 7.4 to 18 FNU at pH 5.0 and remained with 13 FNU about 2 FNU higher than initially when the pH was shifted back to 7.4. For a placebo-formulation with 12.5 mg/mL unstabilized-HSA precipitation and elevated turbidity levels of about 50 FNU were observed around pH 5.0 as well. In contrast to the cytokine-HSA formulation the precipitation of unstabilized-HSA was completely reversible and the turbidity dropped back to the initial value of about 4 FNU when the pH was raised again to 7.4. This indicated that the cytokine or a combination of cytokine-HSA may be responsible for the irreversibility of the precipitation in cytokine-HSA formulations.

SDS-PAGE of the Precipitated Material

To analyze the composition of the precipitated material, SDS-PAGE was performed. For the cytokine-HSA formulation at pH 5.0 the turbid solution was centrifuged and both the supernatant and the precipitated material were analyzed. The HSA-gel showed that only traces of HSA were found in the precipitated fraction (Fig. 8a). In contrast, the cytokine was present to a similar degree in the supernatant and the precipitated fraction (Fig. 8b). This confirmed the assumption that the cytokine was mainly responsible for the irreversible precipitation at pH 5.0.

Fluorescence Spectroscopy of Cytokine-HSA Mixtures

The high turbidity of 100 FNU for the cytokine-HSA formulation compared to 5 FNU for the cytokine and 50 FNU for unstabilized-HSA at pH 5.0 pointed at interaction between the two proteins which leads to the increased precipitation. To gain insight into protein-protein interactions, isothermal titration calorimetry,³⁰ nuclear magnetic resonance spectroscopy,³¹ chromatographic techniques³² or fluorescence spectroscopy¹⁰ would be possible approaches. In the context of this study, fluorescence spectroscopy was used. Both proteins contain tryptophan residues in their amino acid sequences and therefore show an intrinsic fluorescence. HSA contains one Trp residue at position 214 and the cytokine molecule two Trp residues near the surface at position 22 and 143 and one in the hydrophobic core at position 79. Trp residues of a protein can be excited at 280 nm and show characteristic emission spectra with maxima between 310 and 350 nm, depending on the environment.^{33,34} In the emission spectra of the cytokine the emission maximum was located at 340 nm independent of the formulation pH (data not shown). An emission maximum of 340 nm can be attributed to Trp residues on the protein surface, which have contact with bound water and other polar groups.³³ With increasing pH the fluorescence intensity declined, but no shift of the maximum occurred. This revealed that the microenvironment of the Trp residues of the cytokine was not affected by the pH change.³⁵ An increased

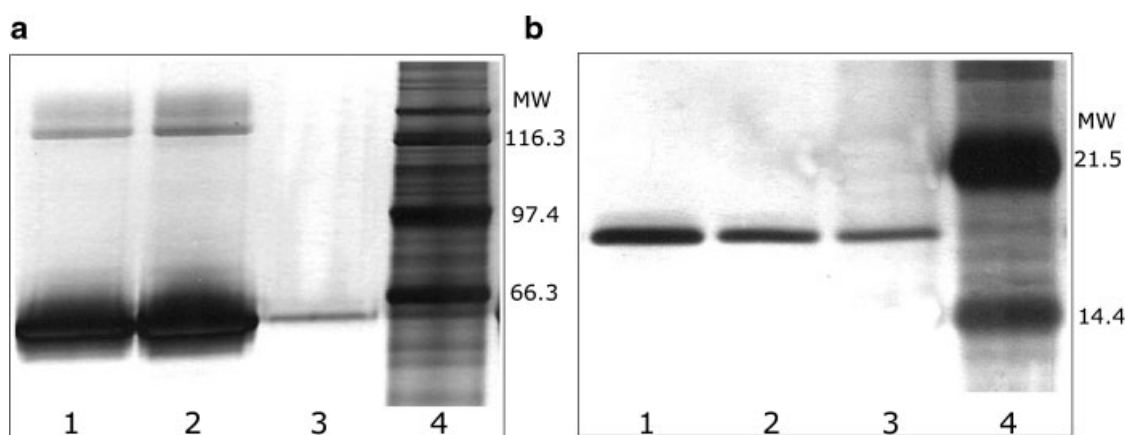


Figure 8. SDS-PAGE of the cytokine-HSA formulation at pH 5.0 showing the turbid solution (1), the supernatant (2), and the precipitated fraction (3), and the marker for HAS (a) and the cytokine (b).

fluorescence intensity which was not accompanied by with a red shift of the maximum was, for example, described for bovine growth hormone upon a pH shift from neutral to acidic.³⁶ For HSA the pH-lowering was accompanied by a shift of the emission maximum from 340 nm between pH 7.8 and 4.8, to 326 nm at pH 3.9 and 321 nm between pH 3.5 and 2.8 (data not shown). The blue shift of the spectrum at lower pH values indicated an exposure of the Trp residues to a less polar environment and a loss of contact to water.

To estimate protein-protein interactions increasing amounts of cytokine were added to a constant amount of 0.5 mg/mL HSA at pH 3.0 and 4.5, with 0 and 0.1% NaCl. It was not feasible to include higher pH values in the study due to the beginning cytokine precipitation above pH 5.0. In Figure 9a the spectra of 0.5 mg/mL HSA, 0.04 mg/mL cytokine as well as the measured and calculated combination of HSA with cytokine are shown exemplarily for pH 3.0. The degree of interactions was determined as F_0/F with a higher value of F_0/F being indicative for stronger protein-protein interactions.¹⁰ For the combination of HSA and the cytokine the interaction parameter F_0/F was increasing at higher cytokine concentrations. At pH 3.0 the interaction parameter F_0/F raised from about 1.1 at 0.02 mg/mL to 1.3 at 0.1 mg/mL cytokine (Fig. 9b). This indicated that interactions occurred to a greater extent at higher cytokine concentrations when HSA was present in excess. The addition of 0.1% NaCl did not impact the strength of the interactions determined at pH 3.0. Significantly stronger interactions between HSA and the cytokine were measured without NaCl at pH 4.5 (Fig. 9c). F_0/F increased from 1.15 to 1.53 when adding between 0.02 and 0.1 mg/mL cytokine to 0.5 mg/mL HSA. At pH 4.5 the addition of 0.1% NaCl weakened the interactions between HSA and cytokine. This became obvious by lower values of F_0/F , which were comparable to the behavior at pH 3.0. Thus, NaCl not only directly stabilized HSA, but additionally reduced the interaction between HSA and the cytokine at pH 4.5. This may offer a further explanation for the beneficial suppression of the turbidity increase in the cytokine-HSA formulation, with the maximum at pH 5.0, by NaCl.

CONCLUSIONS

In cytokine-HSA formulations a significant increase in turbidity and aggregation was observed

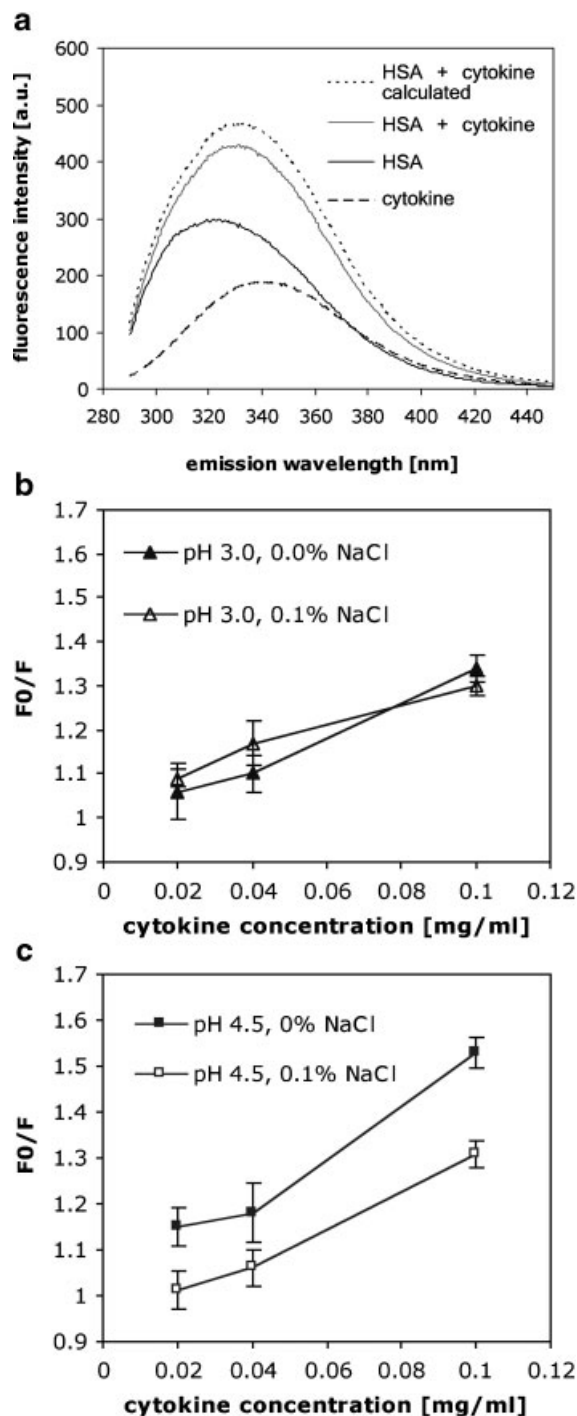


Figure 9. Emission spectra of 0.5 mg/mL HSA with 0.04 mg/mL cytokine in 2 mM glycine at pH 3.0 after excitation at 280 nm (a), and interaction parameter F_0/F for the combination of 0.5 mg/mL HSA with cytokine at pH 3.0 (b), and pH 4.5 (c) with different NaCl concentrations.

below pH 5.5 with a maximum precipitation at pH 5.0. Studies with cytokine formulations and HSA placebo formulation made clear that rather HSA than the cytokine led to the instability at pH 5.0. A significant reduction of the precipitation could be achieved by NaCl, whereas the HSA-stabilizers Na-octanoate and Na-N-acetyltryptophanate only played a minor role. The precipitation at pH 5.0 in cytokine-HSA formulations was partly irreversible and SDS-PAGE revealed that predominantly cytokine precipitated. By characterizing the particle formation process by DLS, disc centrifugation, AFM, and light obscuration it could be shown that the formulations contained mostly monomers with a size of 5–10 nm and a second particle population at 20–60 nm. In highly turbid formulations, for example, when the pH of the cytokine-HSA formulation was lowered to 5.0 a third population at 500–1000 nm emerged. Fluorescence spectroscopy demonstrated that the interactions between HSA and the cytokine were weakened by the addition of NaCl at pH 4.5. As a general conclusion it can be stated that a sufficient stabilization of HSA is a prerequisite to obtain an overall stable formulation of a second protein.

ACKNOWLEDGMENTS

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United States Patent Office

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STABILIZED HYALURONIDASE SOLUTION CONTAINING CALCIUM CHLORIDE

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No Drawing. Application June 17, 1954,
Serial No. 437,594

3 Claims. (Cl. 167—73)

This application is a continuation-in-part of patent application Serial No. 327,175, now abandoned, filed by Harvey E. Alburn and Robert W. Whitley on December 20, 1952 for Stabilized Aqueous Hyaluronidase Solutions.

This invention relates to stabilized aqueous hyaluronidase solutions in which the stabilizing constituent is ethylenediaminetetraacetic acid.

Hyaluronidase is a proteinaceous enzyme having the property of hydrolyzing hyaluronic acid. Hyaluronic acid is a mucopolysaccharide of high molecular weight forming an essential component of ground substance or cement substance of the tissues. Its aqueous solutions have a high viscosity.

Because the high viscosity of aqueous solutions of hyaluronic acid is reduced by the hydrolytic action of hyaluronidase, hyaluronidase has found successful clinical application in promoting the absorption by tissues of therapeutic liquids. The reduced viscosity due to this hydrolysis appears to permit more rapid diffusion of the therapeutic fluid introduced. In one application, for example, by the use of hyaluronidase hypodermaclysis can be substituted in many cases for intravenous injection with attendant advantages to patient and physician. Hyaluronidase can also be used with advantage in local anesthesia. Other uses are known.

Prior to this invention hyaluronidase had been sold in solid form—as a powder in sealed ampules containing lactose as an inert excipient to facilitate handling. Although it would have been more advantageous for medical use to have it available in solution, this was impractical because of the instability of hyaluronidase in aqueous solution in which it becomes denatured at room temperature.

It is an object of this invention to provide an aqueous solution of hyaluronidase suitable for parenteral use and having sufficient stability to enable it to be sold, stored and dispensed as such, e. g. at temperatures from 15° to 30° C.

According to our invention we provide a dilute solution comprising hyaluronidase and ethylenediaminetetraacetic acid ($\text{HO}_2\text{C}\cdot\text{CH}_2)_2\text{N}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{N}(\text{CH}_2\cdot\text{CO}_2\text{H})_2$; the solution also preferably contains a soluble physiologically acceptable salt such as sodium chloride and calcium, added, for example, as calcium chloride. The reaction should be nearly neutral. For this purpose we incorporate a small amount of buffer in the solution.

Ethylenediaminetetraacetic acid, which we abbreviate to EDTA has previously been used to stabilize various chemicals, including pharmaceuticals, because of its metal-ion-"sequestering" power, i. e., its ability to form complexes with heavy metals in which the metal is no longer ionized. Thus it can protect certain products which are sensitive to heavy metal ions.

In the present case the instability of aqueous solutions of hyaluronidase is a more complex affair and is profoundly affected by other conditions than the presence of heavy metal ions, which are not the controlling factor in stability.

The only prior attempt to use EDTA with hyaluronidase

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which we have been able to find is reported in a paper by Meyer and Rapport in J. Biol. Chem., 188 (1951), 485-490. These workers studied the instantaneous deactivation of hyaluronidase by heavy metal ions and its reactivation as well as the prevention of deactivation by several organic and inorganic agents. They envisaged the action as a competition for the heavy metal ion between hyaluronidase and the protective agent. According to these authors, to the extent the protective agent prevails in this competition the hyaluronidase is protected against deterioration due to heavy metals. Among the protective agents tested was "Versene," a commercial form of EDTA. The authors conclude that chelating agents such as oxine and Versene are rather poor competitors of the enzyme for the metal. It is to be noted that these authors investigated only the instantaneous and reversible deactivation of the enzyme and not its long-time stability.

Our investigations have shown that other factors than heavy metals are chiefly responsible for hyaluronidase deactivation and that heavy metals play an insignificant role in its deactivation under practical conditions. For example, the copper-purified enzyme is more stable in aqueous solutions than the alcohol-purified enzyme although the former has a higher content of copper and iron impurities. Alcohol-purified hyaluronidase, however, is preferred from the clinical viewpoint because of its greater purity and from the manufacturing viewpoint because of the greater efficiency of the alcohol-purification process. Surface forces play an important role in deactivating hyaluronidase in aqueous solution. It appears that there is a tendency in the dilute proteinaceous enzyme solution for the protein molecules to concentrate at the air-water interface. Under these conditions the unbalanced surface forces appear to act on some of the weak bonds between the amino acid chains and cause the protein molecules to partially disassociate and lose their enzyme activity. While we do not wish to commit ourselves irrevocably to this theory of inactivation, we have proved by comparative experiments that increasing the air-water interface of dilute aqueous solutions of hyaluronidase greatly increases the rate of deactivation. That this deactivation is not due to oxidation was proved by saturating such solutions with oxygen and finding no increase in deactivation.

EDTA substantially reduces deactivation of hyaluronidase due to surface action.

The addition of certain salts to aqueous hyaluronidase solutions, notably sodium and calcium chloride, improves the stability of such solutions, and if EDTA is also present, increases the stabilizing power of the latter. The presence of calcium chloride has also been found to lessen the pain of injection.

From the above discussion it will be seen that the deactivation of hyaluronidase is a complex reaction in which the presence of heavy metal ions plays an insignificant role. Quite possibly factors as yet undiscovered come into play. It is a remarkable fact, which could not be foreseen from prior knowledge, that EDTA exerts a strong inhibitory effect on all known deactivation reactions of hyaluronidase and its use makes possible the production of a stable solution of hyaluronidase suitable for parenteral injection.

This experimentally determined fact raises the difficult question of the mechanism of the protective action. We have as yet been unable to determine what this is but suggest (without limiting ourselves to this explanation) that the protective action is due in part at least to the formation of a complex including hyaluronidase and EDTA, the complex being more stable than hyaluronidase alone and not interfering with the enzyme action of the latter.

According to our invention we dissolve a small amount

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of hyaluronidase, e. g. 25–1000 TRU/ml., in water, preferably pyrogen-free and containing a small amount of sodium or calcium chloride or both, e. g. 0.12–0.15 M NaCl or 0.00027–0.0027 M CaCl₂ or both, together with 0.1–1.0 mg./ml. EDTA disodium salt dihydrate, and adjust the solution to a reaction ranging from slightly acid to approximately neutral, e. g. pH 4.5–8.0. The ratio of CaCl₂ to EDTA is preferably equimolar with a plus or minus variation of 5%. Since a chelate is apparently formed by calcium chloride and EDTA, this chelate may be prepared in advance as by making a solution of equimolecular amounts of calcium chloride and EDTA and incorporating a suitable amount of this solution in the hyaluronidase solution. The reaction of the hyaluronidase solution may be controlled by adding a suitable buffer such as 0.01 ionic strength phosphate or acetate buffer. A small amount of a preservative agent, such as "Merthiolate" (sodium ethylmercurithiosalicylate) 1:10,000, may also advantageously be present. If phosphate buffer is used, the calcium chloride should be added after the addition of EDTA in order to avoid the precipitation of calcium phosphate. These amounts are intended to be illustrative only and not absolute limitations, as it will be recognized by those skilled in the art that variations may be introduced without departing from the spirit of our invention.

In the average solid hyaluronidase preparation 150 TRU are contained in 0.2 mg. Twenty-five TRU therefore are contained in 0.033 mg. and 1000 TRU in 1.33 mg. For general therapeutic use a solution of 150–200 TRU/ml. is convenient. Where, however, only a small area or volume is to be infiltrated, as for example in dental anesthesia, a dilute solution containing as little as 25–30 TRU/ml. may be advantageous. On the other hand, in some uses, as for example in treating kidney stones, a more concentrated solution is desirable, containing, say, 600 TRU/ml. or more.

We have found further that the addition of a small amount of a simple amino acid, such as glycine and arginine, will in some cases reinforce the protective action of EDTA, but such addition is optional and not essential to our preferred compositions.

In the present disclosure the terms ethylenediaminetetraacetic acid and EDTA are intended to include the simple non-toxic water-soluble salts. EDTA is commercially available in several forms; the dihydrate of the disodium salt having a molecular weight of 372.25 has been found most convenient to use for the purposes of the present invention, but the invention is not limited to the use of this precise salt. The EDTA salt present in the solution is determined in part by the salt added *qua se* and in part by the buffer used to adjust pH or by other added salts. In the examples below, the disodium dihydrate salt of EDTA was used and concentrations are expressed in terms of this salt. The EDTA anion is the important functioning unit. The term TRU indicates "turbidity-reducing units" as used in the turbidimetric assay described by Alburn and Whitley in *J. Biol. Chem.*, 192 (1951), 379–393. Copper-purified hyaluronidase was prepared from fresh-frozen bovine testes by a method based on Hahn, *Biochem. Ztsch.*, 315 (1943), 83–96, and alcohol-purified hyaluronidase was prepared by the method of Tint and Bogash, *J. Biol. Chem.*, 184 (1950), 501.

In the following examples data are furnished on embodiments of our invention and on comparative experiments discussed above in more general terms. These examples are intended to be illustrative only and not to limit our invention, the scope of which is defined in the appended claims.

Assays yielding the results tabulated below were made by the method of Alburn and Whitley (*loc. cit. supra*).

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EXAMPLE 1

Stability of alcohol-purified hyaluronidase in various media at 37° C.

The solutions were made up in 1:10,000 aqueous Merthiolate and contained 192 TRU/ml. Five ml. volumes were placed in 5 ml. vials at 37° C.

Solution	Percent Activity Lost	
	30 days	60 days
Water.....	100	100
0.01 M phosphate buffer, pH 7.0.....	100	100
0.15 M NaCl.....	9	38
0.15 M CaCl ₂	23	39
1 mg./ml. EDTA·Na ₂ ·2H ₂ O.....	75	94
1 mg./ml. EDTA·Na ₂ ·H ₂ O+0.15 M NaCl.....	4	10

EXAMPLE 2

Effect of ratio of air-water interface to volume of solution—without agitation

The solutions of alcohol-purified hyaluronidase were made up in 1:10,000 aqueous Merthiolate and contained 192 TRU/ml. Three ml. or 10 ml. were placed in 10 ml. vials as indicated and stored at 37° C. without shaking.

Solution	Percent Activity Lost, 30 Days	
	3 ml. in vial	Full vial
0.15 M NaCl.....	35	14
1 mg./ml. EDTA·Na ₂ ·2H ₂ O+0.15 M NaCl.....	6	6

EXAMPLE 3

Effect of air-water interface with agitation

Five-tenths ml. of solutions made up as in Example 2 and containing 170 TRU/ml. were placed in 10 ml. vials attached horizontally around the periphery of a ball-mill container rotated at 120 R. P. M. at 37° C.

Solution	Percent Activity Lost, 20 Hours
0.15 M NaCl.....	70
1 mg./ml. EDTA·Na ₂ ·2H ₂ O+0.15 M NaCl.....	19

EXAMPLE 4

Comparison of copper-purified and alcohol-purified hyaluronidase

Solutions of copper-purified hyaluronidase containing 145 TRU/ml., 0.15 M NaCl and 0.01 M phosphate buffer in 1:10,000 aqueous Merthiolate at pH 7.0, with and without EDTA·Na₂, were stored at 37° C. for the indicated times.

Solution	Percent Activity Lost	
	41 days	90 days
Without EDTA.....	19	33
1 mg./ml. EDTA·Na ₂ ·2H ₂ O.....	8	17

Solutions of alcohol-purified hyaluronidase, as above, but containing 192 TRU/ml.

Solution	Percent Activity Lost		
	15 days	32 days	60 days
Without EDTA.....	27	35	37
1 mg./ml. EDTA·Na ₂ ·2H ₂ O.....	3	4	10

Discussion of Examples 1-6

	Metal Impurities in Hyaluronidase in Above Described Tests	
	Cu, percent	Fe, percent
Cu-purified.....	0.2	0.03
Alcohol-purified.....	0.002	0.01

EXAMPLE 5

Long-term stability of hyaluronidase at 37° C.

Solutions of copper-purified hyaluronidase having an activity of 1350 TRU/mg., containing an enzyme concentration of 145 TRU/ml., 0.15 M NaCl and 0.01 ionic strength phosphate buffer, in 1:10,000 aqueous Merthiolate were stored at 37° C. for the indicated times.

Solution	Percent Activity Lost				
	41 days	90 days	136 days	6 months	9 months
Without EDTA.....	19	33	50	55	61
1 mg./ml. EDTA·Na ₂ ·2H ₂ O.....	8	17	32	28	34

EXAMPLE 6

Long-term stability of hyaluronidase solutions containing calcium chloride

The relative stabilities at different temperatures under otherwise comparable conditions of hyaluronidase with and without EDTA and with varying amounts of calcium chloride are shown in the following table for a 6-month period. The solutions were made up by dissolving the ingredients used in the required amounts in 800 ml. pyrogen-free water in the following order, each ingredient being completely dissolved before the next was added: NaCl; EDTA·Na₂ dihydrate; CaCl₂ (from a standardized stock solution); NaH₂PO₄·H₂O; Na₃PO₄·12H₂O; Merthiolate (Lilly). Powdered hyaluronidase was then dissolved to provide about 200 TRU/ml., the solution filtered and diluted to 1 liter. Five ml. volumes were sealed in low-soluble 7 ml. glass vials for the test, the ratio of air-liquid surface in cm.² to volume in cm.³ being about 0.5:1. Each vial contained the ingredients in the concentrations shown in the table, in addition to approximately 8.45 g. NaCl and 0.1 g. Merthiolate per liter and sufficient of the phosphate salts to provide a pH of about 7.0.

Initial Assy, TRU/ml.	EDTA·Na ₂ ·2H ₂ O, g./l.	CaCl ₂ , g./l.	Temp., ° C.	Activity Loss in 6 months, Percent
206.....	1.0	0.268	5	6
			25	12
			37	35
205.....	1.0	0.283	5	1
			25	10
			37	34
205.....	1.0	0.298	5	4
			25	11
			37	34
207.....	1.0	0.313	5	6
			25	15
			37	35
205.....	1.0		5	7
			25	16
			37	35
				After 2 months
179.....			5	68
			25	86
			37	97

Certain conclusions may be drawn from the data assembled above in Examples 1-6:

1. A dilute solution of hyaluronidase in water is extremely unstable, losing all its activity in 15 days at 37° C. Buffering to pH 7 with phosphate buffer does not stabilize it. (Example 1.)
2. Small amounts of NaCl or CaCl₂ improve its stability. (Example 1.)
3. Increasing the air-water interface accelerates its inactivation. (Examples 2 and 3.)
4. The presence of heavy metal ions (Cu and Fe) is not a controlling factor. (Example 4.)
5. The addition of EDTA is effective to inhibit the deactivation rate in the presence of a small amount of sodium or calcium chloride for an extended period of time at an accelerated deactivation temperature (37° C.) under all the conditions investigated. (Examples 1, 2, 3, 4 and 5.)

6. As noted above, the addition of a small amount of a calcium salt was found to eliminate pain on injection of the stabilized hyaluronidase solution. This result is thought due to the formation of a chelate of calcium ion with the EDTA·Na₂. Apparently the free EDTA·Na₂ was responsible for the pain, but the chelate is not painful.

Example 6 shows that the presence of approximately one-quarter to three-tenths gram of calcium chloride per liter has no deleterious effect on the stabilizing action of one gram EDTA per liter, although the latter amount of calcium chloride is sufficient to convert practically all of the EDTA to the chelate form.

These results are surprising. It had hitherto been considered that ethylenediaminetetraacetic acid acted only by deionizing heavy metal impurities, and its use as a protective agent had been limited to such cases. In fact, however, in the only published report on its use with hyaluronidase (Meyer and Rapport, discussed above), it was found not to be an efficient competitor with the enzyme for Cu and Fe ions.

It is to be noted that the tests of Examples 1-6 were accelerated tests, carried out at 37° C. The average range of storage temperatures may be taken as 15°-30° C., the most frequently met being 20°-25° C. Since the rate of loss of activity approximately doubles for a 4° rise in temperature it is quite possible by our invention to provide clinically useful dilute aqueous solutions of hyaluronidase retaining adequate activity for several months.

An example of one embodiment of our invention adapted for clinical use follows.

EXAMPLE 7

A stock solution of analytical grade calcium chloride is prepared in pyrogen-free water which will contain about 30 grams of calcium chloride per liter. The solution is analyzed accurately for calcium concentration by a standard gravimetric procedure.

For each liter of solution to be prepared, to about 800 ml. of pyrogen-free water the following ingredients are added in the order listed. Each ingredient is completely dissolved before the next one is added.

- 8.45 grams of sodium chloride
- 1.000 grams of ethylenediamine tetraacetic acid disodium dihydrate ("Versene" or "Sequestrene" 3 times crystallized)
- 0.298 gram of calcium chloride, added as the stock solution
- 0.557 gram of monosodium phosphate monohydrate (NaH₂PO₄·H₂O)
- 1.919 gram of trisodium phosphate dodecahydrate (Na₃PO₄·12H₂O)
- 0.100 gram of "Merthiolate"

The hyaluronidase is dissolved cold in this solution, filtered through a bacterial filter and sealed aseptically in sterile vials of the desired size. The vials are of low-soluble glass and are of such size as to keep the air-liquid interface at a minimum. They may be, for example, of 1 ml. capacity for single doses containing 150 TRU, or may be multiple dose vials of 5 ml. or more capacity. Multiple dose 5 ml. vials may contain 600-3000 TRU.

EXAMPLE 8

When hyaluronidase is used with organic-base local anesthetics in the form of their salts, such as their hydrochlorides, it is desirable that the solution have a slightly acid reaction, e. g. in the range 4.5-5.5. The following tables give the results of an accelerated aging test of two such solutions, one without and one with EDTA.

The composition of the solutions was the following:

	Solution I	Solution II
NaCl.....	0.15 M.....	0.15 M.....
CaCl ₂		0.298 mg./ml.
Hyaluronidase.....	191 TRU/ml.....	232 TRU/ml.
EDTA·Na ₂ ·2H ₂ O.....		1 mg./ml.
Merthiolate.....		1:10000.
Sodium acetate buffer to.....	pH 5.0.....	pH 5.0.

The solutions were stored at 37° C. and lost activity as follows:

	Percent activity lost				
	1 month	2 months	3 months	4 months	5 months
Solution I.....	32	48	57	61	71
Solution II.....	12	17	22	31	32

A solution such as II may be prepared with an added anesthetic salt, e. g. procaine hydrochloride, and stored and dispensed in that form.

We claim:

1. A stabilized solution for parenteral use comprising hyaluronidase in an amount to furnish 25-1000 TRU per milliliter, an amount of ethylenediaminetetraacetic acid approximately equivalent to 0.1-1.0 m. disodium ethylenediaminetetraacetate dihydrate per milliliter, approximately 0.1-0.2 M NaCl, approximately 0.25-0.3 g. CaCl₂ for each gram of disodium ethylenediaminetetraacetate dihydrate equivalent, and a non-toxic buffer to provide a reaction in the approximate range pH 4.5-8.0.
2. A sterile sealed vial of glass of low water solubility containing a solution as defined in claim 1, the volume of solution with respect to the capacity of the vial being such as to provide a ratio of air-liquid interface to liquid volume not greater than 0.5 expressed as cm.² and cm.³ when the vial is in an upright position.
3. A stabilized sterile hyaluronidase solution in pyrogen-free water for parenteral use having an activity in the range 25-1000 TRU per milliliter and containing in addition to the hyaluronidase approximately 1 mg./ml. disodium ethylenediaminetetraacetate dihydrate, 0.15 M NaCl, 0.3 mg./ml. CaCl₂, and phosphate buffer to provide a reaction of the order of pH 7.3.

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