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The Role of Protein-Protein Interactions in Inducing Interfacial Aggregation

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The role of protein-protein interactions in inducing interfacial aggregation

by

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B.S., University of Texas at Austin, 2013
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A thesis submitted to the
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This thesis entitled:
The role of protein-protein interactions in inducing interfacial aggregation
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has been approved for the Department of Chemical and Biological Engineering

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
The synergic exposure to silicone oil-water interfaces and to agitation has been shown to promote the aggregation of therapeutic proteins. Silicone oil is typically used as a lubricant for manufacturing surfaces and pharmaceutical containers such as glass prefilled syringes. This is a problem as protein aggregates formed at the silicone oil-water interface may shed into bulk solutions, leading to product recalls or adverse immunogenicity upon injections into patients.

Protein aggregation in solution occurs as a result of attractive protein-protein interactions (PPI). PPI can be characterized using techniques such as dynamic light scattering which measures the dependence of molecular diffusivity on protein concentration. However, numerous factors unrelated to intermolecular forces can also impact protein diffusion. We therefore investigated the influence of multicomponent diffusion in a ternary protein-salt-water system on protein diffusion. This analysis demonstrated that large changes in protein diffusivity with protein concentration could result even in the absence of PPI. Therefore, careful interpretation is needed if protein diffusivity is used to measure PPI and colloidal stability of protein formulations.

PPI between proteins adsorbed at interfaces currently cannot be measured directly and the forces driving interface-induced aggregation are poorly understood. Therefore, we investigated the effects of PPI measured in solution on aggregation of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) at silicone oil–water interfaces. Attractive PPI measured in solution correlated with stronger interfacial aggregate gels while when PPI were decreased, weaker interfacial aggregate gels were formed. Upon mechanical perturbation in siliconized syringes, the strongest interfacial gels correlated with the most monomeric loss.
In addition, we suggested that interfacial aggregation could be reduced by decreasing attractive electrostatic PPI through the covalent addition of poly(ethylene)glycol (PEG) to rhIL-1ra. Monomeric loss in siliconized syringes was reduced upon PEGylation although PEGylation decreased protein’s conformational stability. PEGylation reduced rhIL-1ra’s net attractive PPI, causing the formation of weaker aggregate gels at the silicone oil-water interface. Therefore, attractive PPI measured in solution dominated the aggregation mechanism at the silicone oil-water interface for rhIL-1ra and those forces could be decreased upon PEGylation.
Dedication

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Chapter 1

Introduction

1.1 Protein-based therapeutics

The use of peptide and protein based pharmaceutical products to treat an extensive variety of diseases has been growing exponentially over the past twenty years. Subsequently, there are currently more than 200 different proteins or peptides-based therapeutics approved for clinical use by the US Food and Drug Administration (FDA), and many more are in the development phase (1). These drugs target a range of medical conditions that include hormone deficiencies such as diabetes, haemostasis, metabolic enzyme deficiencies (i.e. Gaucher disease, Hunter syndrome), cancer, and immunoregulation (i.e. rheumatoid arthritis) (2). Protein-based pharmaceuticals offer several advantages over their small-molecule therapeutic counterpart as they are highly specific and serve complex functions that cannot be mimicked by simple chemical compounds. In addition, their high specificity decreases the potential for interfering with normal biological processes that could cause adverse effects. All of these benefits have led to tremendous growth in protein and peptide-based biotechnology products with sales increasing from $36 billion in 2001 to $163 billion in 2012 (1).

Proteins are large macromolecules made up of amino acids and each protein is characterized by a unique three-dimensional structure that corresponds to its biologically active state. A protein’s native structure is a balance among various interactions including covalent linkages, hydrophobic interactions, electrostatic interactions, hydrogen bonding, van der Waals forces, and protein-solvent interactions. These forces play an important role in maintaining protein structural integrity and function. However, proteins are only marginally stable (\( \Delta G_{\text{unf}} \approx 5-40 \text{ kcal mol}^{-1} \)) and are susceptible to degradation (3, 4). Degradation pathways can be divided into two main categories: chemical degradation and physical degradation. Chemical degradation arises due to modification of covalent bonds or to chemical changes to the protein sequence such as disulfide scrambling, proteolysis, oxidation, and deamidation (5, 6). Physical degradation includes undesirable adsorption to interfaces, changes in protein structural integrity, and molecular...
assembly, resulting in protein aggregation, or precipitation (7–9). Aggregation involves the assembly of monomeric proteins or of larger order protein oligomers usually due to attractive intermolecular interactions (10–16). Protein aggregation is a complex phenomenon where multiple steps can be necessary for aggregation to occur, such as a change in protein conformation upon adsorption to an interface which could destabilize it and result in molecular assembly (13, 17–20). Degradation leading to aggregation is problematic as it can be induced by stresses occurring regularly throughout a protein’s life cycle, compromising the integrity of the drug product (21).

1.2 Protein aggregation impact on product quality and safety

Proteins must be folded correctly to function effectively as drug molecules, so degradation that results in aggregation can be detrimental. As a result, pharmaceutical products are held to strict standards of chemical and physical purity by regulatory agencies throughout the manufacturing process and the shelf-life of the product (22). Therefore, preventing protein aggregation is a pressing issue for the pharmaceutical industry. In many cases, aggregation is irreversible, impacting both product quality and efficacy (13). Protein aggregation can lead to an increase in formulation viscosity which can negatively impact intramuscular or subcutaneous delivery (23). More importantly, aggregation raises safety concerns as aggregates may elicit immune responses after injection into patients (24, 25). A study on human growth hormone (hGH) showed a link between aggregated hGH and the development of persistent antibodies to hGH (26). Insulin aggregates caused cutaneous allergy in patients and antigenicity correlated with levels of aggregates (27). If an immune response occurs, patients can become immune to the drug or worse, develop an autoimmune disease (26). For instance, neutralizing anti-interferon-α antibodies developed in 61% of patients, resulting in loss of responsiveness towards therapy in some cases (28). In addition, protein aggregates can become large enough to form subvisible and visible particles which may impact product quality, efficacy, and safety, making the product unacceptable for clinical use (29, 30). These proteinaceous particles, along with non-proteinaceous particles arising from leachables (i.e., stainless steel, silicone oil, glass) have received increasing attention from regulatory agencies, such as the United States Pharmacopeia
(USP) which defines the number of particles ≥ 25 µm and ≥ 10 µm that are acceptable in an injectable drug, although smaller sizes are also a concern (22, 29).

1.3 Protein aggregation

Controlling and mitigating protein aggregation is necessary to ensure the safe delivery of protein therapeutics and to maintain their efficacy after injection into patients. One strategy to achieve this objective is to develop robust formulations to keep biopharmaceutical proteins stable during the manufacturing steps and the storage process. This requires a thorough understanding of protein aggregation pathways and of the effect of formulation solution conditions (pH, salt concentration, salt type, addition of excipients) on protein stability. The forces involved in the formation of protein aggregates in solution are well understood, where aggregate assembly occurs as a result of attractive intermolecular interactions between proteins (10–15). In addition, the intrinsic conformational stability of the protein native state also plays an important role in its propensity to aggregate where intermediates that are structurally altered compared to the native state have been found to precede aggregation (13, 31, 32). The resulting protein aggregates are typically accompanied by large quaternary structural changes, with increased intermolecular β-sheet content (33–35).

1.4 Formation of aggregates due to interfaces

Even under solution conditions where protein stability appears to be optimized to maximize protein stability, aggregates and particles may form (36–38). This is because proteins ubiquitously adsorb to interfaces, and upon adsorption, their stability, previously optimized in solution, may be compromised upon adsorption to an interface (39). Due to interfacial stresses, those aggregates can detach and leach back into bulk solution, thereby forming particles and possibly impacting product quality and safety. This scenario is problematic as protein-based products encounter interfaces throughout their life cycle such as with process equipment, container-closure systems, and delivery devices. The types of interfaces encountered include liquid-liquid interfaces such as silicone oil-water interfaces from filling pumps, rubber stopper, and prefilled syringes, solid-liquid interfaces from container walls, ice-water interfaces from freeze-thaw
cycles, and air-water interfaces from air bubbles present during mixing, filling, and storage in containers (40–43).

Furthermore, leachables that can shed from product contact surfaces can provide additional sites that can lead to adsorption and subsequent aggregation. Such surfaces include stainless steel from pumps and fittings, silicone from pharmaceutical containers, pump fittings and tubing, cellulose from filters, and silica from glass containers. Adsorption to interfaces has been found to lead to the formation of aggregates or to the loss of activity of the pharmaceutical product. Teflon® caused aggregation of insulin (44), leachates from tungsten caused protein precipitation (45, 46), stainless steel particles caused aggregation of a mAb (47, 48), and clinical lots of recombinant human platelet-activating factor acetylhydrolase (rhPAF-AH) showed visible particles upon incubation with nano-sized silica particles (36). In some of those cases, such as with rhPAF-AH, aggregation proceeded through a heterogeneous nucleation-controlled mechanism where the exogenous particles present in solution were seeds on which the protein adsorbed, nucleate, and grow into large aggregates (36). However, in some other studies, such as with adsorption to cellulose and silica, no additional monomer loss was observed after the initial adsorption phenomenon (48).

Protein adsorption to interfaces displays three distinct regimes: 1) protein diffusion to the interface and initial adsorption, 2) adsorbed protein conformational changes, continued protein adsorption, and protein interfacial rearrangement, 3) surface saturation, increased protein intermolecular binding and gel layer formation. The initial adsorption phenomenon is relatively rapid while interfacial structural changes and interfacial gelation can occur over a time course of multiple hours (49–51). Once adsorbed to an interface, protein molecules may form covalent or non-covalent bonds between each other, through disulfide bridging or hydrogen bonding, until an extensive 3D elastic network is formed (51–57). The adsorbed protein layer can contain extensive intermolecular β-sheet structures as a result of both hydrophobic and electrostatic interactions between protein molecules that can be measured by dye binding assays using extrinsic fluorescence (58).

1.5 Interfacial mechanical stresses
In addition to the damaging effect of interfaces on protein stability, stresses applied to interfacially adsorbed protein layers have been observed to cause increasing protein aggregation as these interfacial aggregates are brought into solution upon stress, causing surface renewal for the adsorption of a new protein layer (59–61). Ruduiik et al. demonstrated that IgG aggregates formed at the air-water interface and were transported into solution by transient stresses applied on the water surface (62). Therefore, the rupture of interfacial films was an important source of aggregates in solution. In addition, Bee et al. demonstrated that compression-dilation cycles at the air-water interface caused a model monoclonal antibody to form particles and that when the compressions were halted, the particles did not nucleate additional particles and the existing particles did not grow larger. These observations demonstrated that aggregates were formed at the air-water interface during periods of high surface stress and were stable after their detachment from the surface and into bulk solution. At the silicone oil-water interface, similar observations were made (63). Anti-streptavidin IgG 1 adsorbed to a silicone oil-water interface did not stimulate aggregation during incubation unless samples were also agitated (37). In addition, Mehta et al. showed that the rupture of interfacial gel layer formed at silicone oil-water interfaces led to increased particle formation (56). Furthermore, agitation of therapeutic fusion protein albinterferon-α2b in the presence of a silicone oil-water interface caused aggregation and subvisible particle formation (64). Although some of these interfacial stresses were amplified compared to what therapeutic proteins typically experience during their life cycle, over the shelf life of a protein (approximately 2 years), external stresses periodically encountered can be detrimental to protein stability and cause interface-induced aggregation (37, 60, 65–68).

1.6 Importance of silicone oil-water interface in pharmaceuticals

Many packaging components for parenteral products require the use of some form of lubrication to ensure good functionality. The low surface tension of silicone oil (20-25 mJ m$^{-2}$) provides it with good wetting properties, allowing it to spread readily on most solid surfaces. As such, silicone oil is used as a lubricant or coating in devices such as syringes, needles, and pharmaceutical containers. Such devices include pre-filled syringes in which the barrel is usually coated with silicone to ensure that the piston easily
moves within the syringe. The use of pre-filled syringes as storage and delivery devices is increasing in the health care industry as pre-filled syringes greatly enhance ease of use and dose accuracy, increase patient compliance, reduce contamination risk, and require little to no overfill volume (69, 70). Multiple commercial products are currently available in pre-filled syringes such as Humira®, Actemra®, Kineret®, and Copaxone® and more are expected to enter the market in the near future (69).

Upon storage in pre-filled syringes, the protein product is in contact with the silicone oil-water interface throughout its shelf life. This contact is problematic as proteins adsorb readily to the hydrophobic interface and may form interfacial aggregates that can contaminate the formulation. Earlier studies demonstrated the contamination of insulin syringes with silicone oil lead to “cloudy formulation” and elevated aggregate levels (71–73). More recently, interfacial stresses at the silicone oil-water interface on adsorbed protein layers were seen to increase protein aggregation (see previous section). In syringes, similar observations were made: upon incubation of lysozyme and a monoclonal antibody in siliconized pre-filled syringes, particle formation greatly increased when mechanical interfacial stress was applied (61). Silicone oil-induced protein aggregation is thus a problem in the pharmaceutical industry so gaining a better understanding of the forces responsible for protein interfacial aggregation is paramount towards keeping protein drug products safe and efficacious for patients.

1.7 Role of protein-protein intermolecular interactions on protein stability

To effectively prevent protein loss due to aggregation at the silicone oil-water interface, a mechanistic understanding of the forces governing aggregation is necessary. In solution, protein aggregation is the collective result of at least two processes—an assembly process dominated by intermolecular forces and protein structural changes. In principle, either of these two processes could be rate limiting (13). The energetic barrier for protein association is a balance of electrostatic repulsive forces and attractive interactions between protein molecules. The magnitude and sign of the interaction energy between two protein monomers can be measured with the osmotic second virial coefficient, $B_{22}$ shown in Eq. 1.1 below:
\[ B_{22} = \frac{2\pi}{M} \int_{0}^{\infty} r^2 \left( e^{-\frac{u(r)}{kT}} - 1 \right) dr \]

where \( M \) is protein molecular weight, \( r \) is intermolecular separation distance, \( u(r) \) is the interaction potential, \( k \) is the Boltzmann constant, \( T \) is absolute temperature, and the subscripts 2 in \( B_{22} \) follow Scatchard notation and stand for the protein monomer (15, 74–77). \( B_{22} \) encompasses all interactions between two protein monomers, including the hard-sphere potential, repulsive electrostatic interactions, van der Waals attractive forces, and other interactions such as charge-charge, charge-dipole, dipole-dipole, charge-induced dipole, and dipole-induced dipole (15, 74, 75). Changes in formulation pH and excipient concentration/identity can affect the magnitude and sign of protein intermolecular interactions (13, 78–80). Therefore, measuring this parameter is a useful screening tool in early drug development to optimize protein stability in solution.

In the context of classic electrostatic effects where proteins are modeled as uniformly charged spheres, nonspecific repulsion arises from overall protein charge and increasing ion concentration results in charge shielding of electrostatic repulsion forces between protein molecules (80, 81). Saluja et al. observed that increasing the ionic strength of hen-egg white lysozyme and of a monoclonal antibody in solution decreased the colloidal stability of each protein (reduced positive \( B_{22} \) values) and led to an increase in protein aggregation (80). In addition, Chi et al. demonstrated that decreasing the net charge of granulocyte colony-stimulating factor (rhGCSF) by decreasing the difference between formulation pH and its isoelectric point (pI) resulted in a lower \( B_{22} \) value (less repulsive intermolecular interactions) and an increase in monomer loss (13). However, \( B_{22} \) can be dominated by relatively few protein-protein configurations in which opposing surfaces arising from charge or structural anisotropy contribute disproportionately strongly to protein intermolecular interactions (74). For example, bovine chymotrypsinogen showed an increase in \( B_{22} \) values (less negative \( B_{22} \) values) with increasing ionic strength, indicating that attractive electrostatics interactions dominated despite protein’s finite positive charge (74, 82). This behavior is contrary to classic electrostatic effects of uniformly charged sphere where increasing ionic strength should increase screening of repulsive protein intermolecular interactions and lead
to the decrease of a positive $B_{22}$ value. Neal et al. ascribed this increase in $B_{22}$ instead to few, highly attractive configurations arising from charge anisotropy gathered from crystallographic data of the protein molecules (74).

The extent of interfacial aggregation may be controlled by intermolecular protein interactions of adsorbed proteins at the silicone oil-water interface where the same intermolecular forces are expected to be present, albeit at different magnitudes relative to forces in bulk solution. For instance, the properties of protein gels at air-water and oil-water interfaces are influenced by formulation conditions, such as pH or ionic strength and such properties also impact protein intermolecular interactions (56, 83, 84). For example, Tang et al. suggested that weaker repulsive interactions due to charge shielding could explain their observation that stronger gels of silk fibroin were formed at silicone oil-water interfaces (83). In addition, Ruhs et al. observed that the strongest interfacial gels of $\beta$-lactoglobulin were formed at pH closest to the protein’s pI, where the protein’s net charge was minimal (84). Although in both of these examples pH and ion concentration had an effect on interfacial viscoelastic protein behavior, the roles they played on protein intermolecular interactions at the interface were not explicitly studied.

### 1.8 Surfactants to protect against protein interfacial aggregation

A common strategy used by the biopharmaceutical industry to decrease protein interfacial aggregation is to add nonionic surfactants to therapeutic protein formulations (85–87). However, adding these surfactants to reduce protein adsorption and protect against aggregation is not always effective (85–90). The most commonly used surfactants in protein formulation are polysorbate 20 and polysorbate 80 (87, 90). However, polysorbates are known to degrade via oxidation and hydrolysis and cause oxidative damage to the protein via production of reactive oxygen species (85–87, 90). Additionally, surfactants can adsorb strongly to materials contacted during processing, such as filters and tubing, thus complicating the manufacturing process (91). Furthermore, at high protein concentrations, surfactants may not be successful in displacing all proteins adsorbed to interfaces, thereby not protecting fully against interfacial aggregation (89). Finally, increased aggregation has been reported during quiescent incubation with polysorbate for
recombinant Factor IX and PEG granulocyte colony stimulating factor (88, 92). Therefore, the addition of surfactants is not always the most suitable strategy to prevent protein aggregation and alternatives should be explored.

1.9 Measuring protein intermolecular interactions

Prior to investigating the role of intermolecular forces on protein aggregation, a better understanding of the strengths and the limitations of current techniques to measure protein intermolecular interactions is necessary. $B_{22}$ values can be measured by membrane osmometry, however, this method is not experimentally viable for the high-throughput stability screenings that are needed for numerous therapeutic protein candidates as it requires extensive amounts of product (79). In addition, membrane osmometry is time intensive as osmotic equilibrium needs to be reached for each sample. As a result, new developments in experimental methods have related the osmotic virial coefficient to Rayleigh light scattering, leading to the widespread use of static light scattering to measure $B_{22}$ (76, 93). Nevertheless, even with advances in the measurement of $B_{22}$, relatively large quantities of proteins are still needed and sample preparation and analysis are quite long as extensive filtration is required to limit scattering from contaminant particles. More recently, the use of the interaction parameter $k_D$ has gained favor in the pharmaceutical industry as a more high-throughput means to quantify protein-protein interactions in solution. $k_D$, measured by dynamic light scattering (DLS), is increasingly used due to advances in technology leading to high throughput DLS instruments such as plate readers requiring as little as 4 µL sample volume. $k_D$ is related to $B_{22}$ through Eq. 1.2:

$$k_D = 2MB_{22} - (\xi_1 + \bar{v}_2)$$

where $\xi_1$ denotes the first-order concentration coefficient in the virial expansion of the friction coefficient and $\bar{v}_2$ is the protein’s partial excluded volume (94). Therefore, $k_D$ reflects both thermodynamic protein-protein interactions from $B_{22}$ but also hydrodynamic interactions. Experimentally, the link between $B_{22}$ and $k_D$ is not always apparent as the hydrodynamic component under dilute conditions is sometimes neglected.
or its importance is minimized (95). This could be problematic as the hydrodynamic component could dominate under semi-dilute or concentrated regime (96, 97).

\[ k_D \text{ can be measured by DLS using Eq. 1.3:} \]

\[ D_{m,2} = (k_D c + 1)D_{s,2} \]  \hspace{1cm} (1.3)

where \( D_{m,2} \) is the protein’s mutual diffusion coefficient and \( D_{s,2} \) is protein intrinsic diffusivity. As opposed to \( B_{22} \) which is obtained by measuring the weighted protein scattering intensity averaged over time, \( k_D \) is calculated through measurements of protein diffusivity by probing relaxation times of microscopic concentration fluctuations in solutions (98). Proteins in solution are macroions whose Brownian diffusion can be perturbed by the presence of other ions. For example, salt concentration gradients with tunable amplitude have been used to strongly amplify protein migration (99–101). To the best of the author’s knowledge, the effect of co-solute on protein diffusivity was never studied in the context of protein stability measurements. This could be problematic as protein/co-ion gradients may alter protein diffusivity, resulting in \( k_D \) values that are not representative of protein intermolecular interactions.

1.10 Sterically preventing intermolecular interactions to mitigate interface-induced aggregation

We proposed earlier that similar forces as the ones controlling aggregation in bulk solution could be responsible for the formation of interfacial aggregates. Therefore, optimizing formulation conditions to reduce attractive intermolecular protein interactions or to maximize repulsive interactions could prevent the formation of interfacial aggregates. In addition, we hypothesize that sterically preventing intermolecular interactions of protein adsorbed to the interface could limit interfacial aggregation. Proteins with covalently added linear or branched polymers represent an emerging class of biopharmaceuticals. The most prevalent of these conjugates are poly(ethylene glycol)-grafted, or “PEGylated,” proteins (102–104). PEGylated proteins have multiple potential therapeutic advantages over unmodified proteins, including longer in vivo circulating half-lives, which decreases dosing frequency, decreases proteolysis, and reduce the risk of immunogenicity (24).
The covalent addition of PEG to protein molecules may prevent close contact between the protein moieties, mitigating intermolecular bond formation required for aggregation. In bulk solution, PEGylation of lysozyme led to a change from attractive to repulsive protein-protein interactions (105). This decrease in net attractive forces between protein molecules may have contributed to the reduction of particles formed when stresses such as freeze-thaw and shaking of siliconized vials were applied (105). Multiple studies also demonstrated that protein PEGylation sterically limited protein adsorption to interfaces as well as decreased interfacial aggregation. Daly et al. observed that upon PEGylation of lysozyme, protein adsorption to a silica surface was reduced due to increased excluded volume from the PEG polymer (106). In addition, Pai et al. demonstrated that interfacial lysozyme aggregation could be reduced upon its adsorption to a hydrophobic interface though PEGylation and that a higher degree of PEGylation resulted in a larger reduction in interfacial aggregates (107).

1.11 Thesis Objectives

As the amount of protein-based therapeutic products continues to rise, it becomes increasingly urgent to mitigate protein aggregation. Although the forces controlling aggregation are well understood in solution, fundamental knowledge is lacking on the role these forces play in governing interface-induced aggregation. This is problematic as therapeutic proteins encounter interfaces throughout their life cycle which can potentially damage the therapeutic product. Upon adsorption to interfaces, a protein’s structure can be compromised and interfacial aggregates can form and leach back into bulk formulation solution upon interfacial stress. These aggregates can increase the risk of unwanted immune responses upon injection in the patient, raising both efficacy and safety issues for the drug product. One interface of interest is the silicone oil-water interface, where silicone oil is used as a coating material or lubricant for pharmaceutical containers, rubber stoppers, and pre-filled syringes. These syringes are increasingly used as storage and delivery devices for protein therapeutics, exposing the protein product to the silicone oil-water interface throughout its shelf life. Therefore, gaining a better understanding on the effect of protein
intermolecular interactions on protein interfacial aggregation should be an important step towards the development of aggregation control strategies.

The first objective of this project was to gain a better understanding on the limitation of the current techniques to measure protein intermolecular interactions. We used model proteins lysozyme and a mAb to investigate the role protein-ion charge separation could play on protein diffusivity and how that could influence the interaction parameter $k_D$. We experimentally measured protein diffusivity as a function of concentration using DLS. Then, we compared the results to a model of protein diffusivity obtained using Nernst-Planck multicomponent diffusion equation which only considers cosolvent and protein charge, molecular weight, and concentration. We hypothesized that protein diffusivity could be strongly dependent on local electric potential gradients generated from protein-ion charge separation in solution, yielding $k_D$ values not truly representative of protein-protein interactions.

The second objective of this thesis was to understand the role of intermolecular interactions on interfacial protein aggregation. We used model protein interleukin-1 receptor antagonist (rhIL-1ra), a 17 kDa cytokine inhibitor, marketed in silicone oil-lubricated syringes (108). We hypothesized that similar forces responsible for aggregation in solution would cause aggregation at the silicone oil-water interface. More specifically, we studied the role of specific intermolecular interactions, arising from structural or charge anisotropy, on the interfacial behavior of rhIL-1ra. We first measured the effects of protein-protein interaction strength on interfacial viscoelastic properties and aggregation of rhIL-1ra. In particular, we correlated interfacial viscoelastic properties determined using interfacial shear rheometry with bulk protein-protein interactions ($B_{22}$) that were measured by static light scattering. To investigate quaternary protein structural changes associated with interfacial gel formation, thioflavin-T (ThT) was used as a marker of extensively ordered β-sheet structures. Finally, rhIL-1ra solutions in siliconized syringes were agitated to create interfacial mechanical stress, and monomer loss was measured by size exclusion high performance liquid chromatography (SE-HPLC) to determine the relationship between interfacial gel formation and aggregation in solution.
The last objective of this thesis was to decrease attractive intermolecular protein interactions at the silicone oil-water interface through the grafting of a PEG polymer to rhIL-1ra. We hypothesized that the covalent addition of a 20 kDa branched PEG to rhIL-1ra should sterically reduce protein intermolecular interactions, thereby decreasing interface-induced aggregation. We analyzed monomer loss caused by interfacial stress in siliconized and unsiliconized syringes. Additionally, we explored the potential driving forces for the different behavior of the PEGylated compared to the non-PEGylated rhIL-1ra such as adsorption properties and interfacial structural changes. We first focused on notable secondary and tertiary conformational changes upon adsorption to the silicone oil-water interface. Then, we analyzed the effect of PEGylation on intermolecular protein-protein interaction with $B_{22}$ measurements. Interfacial viscoelastic properties and interfacial aggregation were then measured and correlated to $B_{22}$ to determine whether PEG chains could sterically hinder interfacial intermolecular protein-protein interactions.
Chapter 2

Challenges in predicting protein-protein interactions from measurements of molecular diffusivity


2.1 Introduction

Weak protein-protein interactions are a major determinant of protein stability and aggregation (80, 109, 110), and are therefore of great importance in the pharmaceutical industry. Since protein aggregation can reduce efficacy or worse, elicit an immune response (24, 25, 111, 112), studies of weak protein-protein interactions can provide useful screening tools in early drug development. One measure of protein-protein interactions is the osmotic second virial coefficient $B_{22}$, which describes the non-ideal solution behavior resulting from two-body interactions between solutes (denoted by the subscript 2) (76, 113). $B_{22}$ is often normalized against its hard sphere value $B_{2}^{HS}$. Values of $B_{22}/B_{2}^{HS} > 1$ indicate net repulsive interactions between solutes, whereas values below unity indicate net attractive conditions relative to purely steric repulsion (15, 114, 115). Techniques such as static light scattering and membrane osmometry have been developed to measure $B_{22}$ (113, 114, 116–118). However, $B_{22}/B_{2}^{HS}$ is difficult to measure with high throughput techniques and has limited applicability to protein-protein interactions under pharmaceutically relevant conditions because it is a dilute solution property (119).

Recently, the use of the interaction parameter $k_D$ has gained favor as a more high-throughput means to quantify protein-protein interactions (98). The interaction parameter can be obtained directly with such techniques as dynamic light scattering (DLS) (94, 98) or Taylor dispersion analysis (TDA) (120) by measuring the dependence of protein diffusivity $D_{m,2}$ on protein concentration $c$:

$$k_D = \left( \frac{\partial D_{m,2}}{\partial c} \frac{1}{D_{2,2}} \right)_{T,P,\mu_1,\mu_2}$$  \hspace{1cm} (2.1)
where $D_{s,2}$, the single-particle diffusion coefficient, is a constant obtained at infinite dilution, and $\mu$ is the chemical potential. Subscripts follow Scatchard notation where water is denoted as subscript 1, protein as subscript 2, and co-solute as subscript 3 (77). Under dilute conditions where higher-order interactions can be neglected, $k_D$ is related to $B_{22}$ through:

$$k_D = 2MB_{22} - (\xi_1 + \overline{\nu}_2)$$

(2.2)

where $\xi_1$ is the first-order concentration coefficient in the virial expansion of the frictional coefficient, $M$ is the protein’s molecular weight, and $\overline{\nu}_2$ is the protein’s partial specific volume (94). Thus, $k_D$ values reflect both thermodynamic as well as hydrodynamic interactions.

High-throughput DLS instruments have been developed to facilitate rapid determination of $k_D$ for colloidal stability screening during early drug development (121–123). However, multiple factors may affect interpretation of DLS data, some of which have not been explicitly investigated in the context of protein stability screening. In particular, proteins in solution are macroions whose Brownian diffusion can be perturbed by the presence of other ions. Indeed, multiple studies (99–101) have shown that when charged proteins undergo mutual diffusion with small, mobile counter-ions such as Cl$^-$, charge separation is prevented because diffusion-induced local electric fields slow the motions of the small counter-ions and speed those of the protein macroions. Thus, salt concentration gradients with tunable amplitude have been used to achieve strongly amplified particle migrations not representative of specific colloidal forces between proteins (99–101, 124). This effect is heightened at low ionic strengths where chemical potential gradients of ionic species are consequential.

DLS measures diffusivity by probing relaxation times of microscopic concentration fluctuations in solution, whereas the mutual diffusion coefficient is best measured using classical techniques based on macroscopic concentration gradients (125). In general this is not a practical issue, as demonstrated in past work (stemming from the Onsager regression hypothesis (126, 127)) that microscopic fluctuations give the same transport coefficients as the ones observed macroscopically (128, 129).
$k_D$, as described in Eq. 2.2, can be separated into two components: a thermodynamic contribution and a hydrodynamic contribution. Because it includes hydrodynamic forces, $k_D$ has been used to probe solution behavior at high protein concentration, such as when measuring protein crystallization propensity (130), making it a useful tool for protein-based pharmaceuticals whose concentrations often exceed 100 g L$^{-1}$ due to small volume requirements (131). However, caution should be taken when making conclusions regarding protein-protein interactions based on $k_D$ measurements under these conditions. At high concentration, where thermodynamic non-idealities, crowding effects and higher order interactions (e.g., $B_{222}$) can alter net interactions (80, 132), collective behavior can be dramatically different from that observed under dilute conditions.

This study aims to address some of the limitations of using $k_D$ to measure protein-protein interactions. First, we highlight the dependence of protein diffusion on electrolyte concentrations and local electric potential gradients by using Nernst-Planck equation to predict the effects of electrolytes on protein mutual diffusion coefficients. We then compare these predictions to experimental diffusivity results. We also show that changes in $D_{s2}$ obtained from DLS at low ionic strength need not imply changes in protein conformation. Finally, a theoretical hard sphere model is used to determine the effect of excluded volume on $k_D$ as a function of protein concentration. The results demonstrate the utility of comparing $k_D$ to its hard sphere value to estimate the contribution of hydrodynamic interactions to $k_D$.

2.2 Theory

2.2.1 Enumeration of components

For the protein – salt – water systems that we consider here, there are three ionic and one neutral species, which are the proteins lysozyme or mAb (P$^+$), counter-ions (Cl$^-$), co-ions (K$^+$), and the neutral solvent species, water. We can neglect water dissociation, whereas KCl completely dissociates into K$^+$ and Cl$^-$. We assume that only Cl$^-$ counter-ions interact with the positively charged protein while K$^+$ diffuses freely. Although additional specific and non-specific protein-solute interactions are possible, such as ligand binding to specific sites and salt exclusion effects from the protein surface, we neglect them in the scope of this study (133, 134).
2.2.2 Modeling protein diffusion due to a chemical potential gradient

We model the multicomponent diffusion system composed of proteins, Cl\(^{-}\) and K\(^{+}\), using Nernst-Planck equation which describes the flux of charged species \((N_{i})\) under the influence of composition gradients and of an electric field (135, 136). The Nernst-Planck equation can be simplified by assuming steady-state, the absence of an external electric potential gradient coupled with a net charge balance of zero, \[ \sum_{i=1}^{n} z_{i} N_{i} = 0 \], and neglecting the effects of non-ideality. Flux is then a function of only composition and local electric potential gradients. For a dilute solution comprising of three ionizable components \((n = 3)\) with well-defined net charges, the equation combined with a multicomponent Fickian diffusion equation becomes (137):

\[
\begin{bmatrix}
D_{m,p} \\
D_{m,CI} \\
D_{m,K^+}
\end{bmatrix} = \begin{bmatrix}
1 - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,CI} & - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,K^+} & - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,p} \\
- \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,CI} & 1 - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,K^+} & - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,p} \\
- \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,CI} & - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,K^+} & 1 - \sum_{i=1}^{n} z_{i}^2 x_{i} D_{s,p}
\end{bmatrix} \tag{2.3}
\]

where \(x_{i}\) are the mole fractions of the three ionic species, \(z_{i}\) are the net charges of each species, and \(D_{s,i}\) are the single-particle diffusion coefficients (for derivation, see Appendix). Eq. 2.3 enables us to calculate the mutual diffusion coefficients of each species, \(D_{m,i}\), based solely on coupled transport due to chemical potential gradients. DLS measures the eigenvalues of the diffusion coefficient, \(D_{m,p}\) matrix and the smallest eigenvalue is close to the protein mutual diffusion coefficient (138). Constants used in the model are \(D_{s,i}\) and \(z_{i}\). \(D_{s,CI}\) and \(D_{s,K^+}\) are 1.97 \times 10^{-5} \text{cm}^2 \text{s}^{-1} and 1.96 \times 10^{-5} \text{cm}^2 \text{s}^{-1}, respectively. They are obtained from electrical conductivity measurements in water at infinite dilution and were corrected for temperature and viscosity (139, 140). \(D_{s,p}\) values obtained from DLS at high salt concentrations (500 mM) are used for lysozyme (1.20 \times 10^{-6} \text{cm}^2 \text{s}^{-1}) and the mAb (4.40 \times 10^{-7} \text{cm}^2 \text{s}^{-1}). The charge of K\(^{+}\) and Cl\(^{-}\) ions are +1 and -1, respectively. Protein charge is determined from literature, because calculations of net charge based on amino acid sequences often overestimate the magnitude of protein’s overall charge (141, 142).
The variables in Eq. 2.3 are the mole fractions \( x_i \) of each mobile species. To determine the appropriate values of \( x_i \) corresponding to each experimental condition, a short explanation of sample preparation is necessary. Each protein was dialyzed at 23 mg mL\(^{-1}\) against the desired salt solution. During dialysis, unequal partitioning of ions occurred to neutralize the protein’s charge across the membrane due to the Donnan effect (143–145). The total concentration of ions on the retentate side (\( \text{ret} \)) of the membrane after dialysis is represented quantitatively in the Donnan equilibrium equations below (for derivation, see Appendix):

\[
[K^{+,\text{ret}}] = [Cl^{-,\text{perm}}] \left( 1 - \frac{z_{\text{op}} [P^+]}{[Cl^{-,\text{ret}}]} \right)^{0.5}
\]

\[
[Cl^{-,\text{ret}}] = [Cl^{-,\text{perm}}] \left( 1 + \frac{z_{\text{op}} [P^+]}{[Cl^{-,\text{ret}}]} \right)^{0.5}
\]

where \([P^+]\) is protein concentration on the retentate side, \( \text{perm} \) indicates the permeate side during dialysis, and the amount of bound counter-ion on the retentate side is \( z_{\text{op}} [P^+] \). Under conditions of low total mobile ion concentration, unequal partitioning is relatively large, whereas it diminishes at higher mobile ion concentrations. In addition, electro-neutrality dictates an excess of counter-ions and a shortage of co-ion on the retentate side of the dialysis membrane relative to permeate concentrations (146). If, after dialysis, the protein is diluted to lower concentrations, the final concentrations of co-ion \( K^+ \) will increase with dilution while the counter-ion \( Cl^- \) concentrations will decrease.

### 2.2.3 Calculation of excluded volume contributions to \( k_D \)

High protein concentrations impose a limitation on the use of Eq. 2.2 as a measure of protein-protein interactions. This follows because for most solutions it is difficult to separate the contributions to \( k_D \) from higher-order protein-protein interactions from those due to hydrodynamic effects. Attempts have been made to draw empirical relations between \( k_D \) and \( B_{22} \) in order to extract friction coefficients, but in general these relations are not universal as \( \xi_1 \) is affected by molecular symmetry, shape, and type (78, 121, 147–151).

Solution viscosities are strongly dependent on protein concentration, and protein diffusivities have been empirically found to be proportional to the inverse of the solution viscosity (152). Thus, we postulate...
that at high protein concentration, viscous effects account for a large portion of $k_D$, leading to negative $k_D$ values that are not truly representative of protein-protein attractive interactions. Although interactions other than volume exclusion may affect protein diffusion in concentrated solutions (97, 153, 154), past work (e.g., scaled particle theory (155, 156)) has shown that protein diffusion coefficients can be predicted at high protein concentration based on simple hard particle models without interactions beyond excluded volume contributions.

As the particle concentration in a hard sphere suspension increases, the crowding reduces diffusional mobility – a hydrodynamic rather than thermodynamic consequence that impacts solution viscosity (157). The concentration-dependence of the viscosity ($\eta$) of a solution containing a hard sphere (HS) solute can be described by the generalized form of the Mooney equation (158):

$$\eta = \eta_0 \exp \left( \frac{S\phi}{1 - k\phi} \right)$$

(2.5)

where $\eta_0$ is the viscosity of the solvent, $\phi$ is the protein volume fraction in the solution, $k$ is the self-crowding factor, and $S$ is a parameter that depends on the shape of the protein, its hydrodynamic interactions, and temperature. The volume fraction can be expressed as $\phi = c\overline{v}_2$, where $\overline{v}_2 = 0.7 \text{ mL g}^{-1}$ (151, 159). For a hard sphere, $S = 2.5$ and $1.35 < k < 1.91$, where $k$ depends on the packing density (158). Although the Mooney model offers reasonable prediction of viscosity using only excluded volume, in some cases, structural details can become important (160). For example, lysozyme and the mAb can be more accurately treated as ellipsoids of revolution rather than as HSs (161). When modeled in this fashion, values for $S$ and $k$ for lysozyme at 296 K have been determined experimentally by Monkos and are 3.01 and 2.91, respectively (162). For the mAb, $S$ is 2.6; this value was calculated using dimensions $a = 14$ and $b = 10$ nm, following Eq. 13 in Monkos (162). The self-crowding factor, $k$, can be calculated by $k = 1/\langle OSP \rangle$, where $\langle OSP \rangle$ is the average occluded surface packing. Using data from Fleming and Richards, $k$ for the mAb was estimated to be 2.3 (163).
To provide an estimate how protein diffusion depends on solution viscosity, we used the Stokes-Einstein equation for the diffusion of a spherical particle (140, 164):

\[ D = \frac{k_B T}{6\pi \eta R} \]  

(2.6)

where \( R \) is protein hydrodynamic radius, \( k_B \) is Boltzmann’s constant, and \( T \) is temperature. Eqs. 2.5 and 2.6 may be combined after substituting \( \phi = e \bar{v}_2 \) to yield:

\[ D = \frac{k_B T}{6\pi R \eta_0 \exp\left(\frac{2.5 c \bar{v}_2}{1-kc \bar{v}_2}\right)} \]  

(2.7)

Following Eq. 2.1, and using the approximations that for a HS \( D = D_{m,2} \) and that \( D_{s,2} \) is the diffusion coefficient calculated from Eq. 2.6 at infinite protein dilution, the derivative of Eq. 2.7 is taken as a function of protein concentration and is divided by \( D_{s,2} \) to estimate the contribution of viscosity to \( k_D^{HS} \):

\[ k_D^{HS} = -\frac{2.5 \bar{v}_2}{(kc \bar{v}_2 - 1)^2} \exp\left(\frac{2.5 c \bar{v}_2}{1-kc \bar{v}_2}\right) \]  

(2.8)

We note that this model for \( k_D^{HS} \) incorporates only hard sphere interactions between proteins with no attractive and/or electrostatic repulsive forces as long as the distance between their respective centers exceeds the surface contact distance. A similar equation modeling the concentration dependence of the mutual diffusion of hard spheres has been derived previously in Phillies (165). \( k_D^{HS} \) thus differs from \( k_D \) because it omits possible electro-viscous effects due to protein-protein charge interactions as well as other protein-protein interactions. \( k_D^{HS} \) is analogous to the thermodynamic normalization factor \( B_{Z2}^{HS} \) and it can be used to normalize \( k_D \) to facilitate comparisons between measurements of different protein types and/or at different protein concentrations.

2.3 Materials and methods

2.3.1 Proteins and Reagents

Chicken egg-white lysozyme (molecular weight 14.3 kDa, \( r_H = 1.89 \) nm, with > 90% purity) was obtained from Sigma - Aldrich (St. Louis, MO) as a lyophilized powder and was reconstituted in 0.02 μm filtered deionized Milli-Q water to form a stock solution at a concentration of 30 mg mL\(^{-1}\). Purified mAb
(molecular weight 145 kDa, $r_H = 5.1$ nm) was donated by Medimmune, Inc. (Gaithersburg, MD) at a stock concentration of 23 mg mL$^{-1}$ in 10 mM NaAc. Analysis by size exclusion chromatography showed that the mAb and the lysozyme were pure and free of soluble aggregates. Potassium chloride salt was obtained from Sigma-Aldrich ($\geq 99.0\%$ purity). All stock solutions were prepared with deionized Milli-Q water filtered with 0.22 μm Millipore filter (Billerica, MA).

**2.3.2 Protein Sample Preparation**

Proteins were exchanged into the final formulation using a bench-scale equilibrium dialysis kit by placing protein samples in 3.5 kDa MWCO dialysis cassettes (Pierce, Rockford, IL). They were dialyzed overnight against excess KCl solution, adjusted to pH 6.0 with HCl. After dialysis, protein samples were diluted with 0.02 μm filtered KCl solution to desired protein concentrations. Protein concentrations were determined using a ThermoScientific NanoDrop2000 Spectrophotometer (Waltham, WA) at 280 nm with an extinction coefficient of 2.52 mL mg$^{-1}$ cm$^{-1}$ for lysozyme (166) and 1.45 mL mg$^{-1}$ cm$^{-1}$ for the mAb (17). Protein concentrations were measured in triplicate after each dialysis and/or dilution step. Finally, prior to light scattering experiments, samples were filtered with 0.1 μm (Whatman, Maidstone, UK) filters to minimize light scattering due to impurities such as dust. All solutions were used within a week of preparation and their pH was checked the day of sample preparation.

**2.3.3 Dynamic Light Scattering (DLS) to obtain protein diffusion coefficients**

DLS studies were conducted at 296 K using a DynaPro™ Dynamic Light Scattering Instrument from Wyatt Technology (Goleta, CA) at a 90° scattering angle using a 830 nm diode laser. Protein samples were inserted into a 12 μL quartz cuvette (Wyatt Technology) and the cuvette was placed into the MicroSampler unit of the DLS instrument. Each sample was equilibrated for two minutes before measurements were recorded. Due to instrument limitations, protein concentration was varied from 2 mg mL$^{-1}$ to 25 mg mL$^{-1}$ for lysozyme and from 0.7 mg mL$^{-1}$ to 23 mg mL$^{-1}$ for the mAb. At least 8 scans of 10 seconds each, with polydispersity less than 30% and sum of squares less than 2 were accumulated for each sample. The laser power was varied from 10 - 50% to achieve constant amplitudes of the autocorrelation function for all measurements. All samples were measured in triplicate. Temporal fluctuations of light
intensity scattered at 90° were processed by a correlator, yielding the intensity autocorrelation function. The samples were monodisperse so the method of cumulants was used to obtain the mutual diffusion coefficient, $D_{m,2}$ (167) from the autocorrelation function. For a more detailed description of the theory behind the DLS and method of cumulants, see detailed review (98). Finally, $D_{m,2}$ was plotted against protein concentration to find $k_D$.

2.3.4 Circular Dichroism (CD) spectroscopy to measure protein secondary structure

Far-UV CD spectra were collected to monitor protein secondary structure. Samples were placed in a 0.5 mm path length quartz cuvette and spectra recorded from 190 nm to 260 nm for solutions containing 2 mM and 10 mM KCl, at pH 6.0. Samples with 90 mM KCl were monitored from 195 nm to 260 nm to keep the high-tension voltage below 600 V (168). Triplicate samples were analyzed and ten scans per sample were measured using a Chirascan-plus spectrometer (Applied Photophysics, Leatherhead, UK). After subtraction of a blank solution spectrum from the raw average signal, the spectra were normalized by calculating the mean residue ellipticity:

$$\theta = \frac{[\theta]_{obs} M}{\text{residues} \times cL}$$

(2.9)

Where $[\theta]_{obs}$ is the observed ellipticity, residues is the number of amino acid residues per protein molecule, and $L$ is the cell path length.

2.3.5 Static Light Scattering (SLS) to measure protein apparent molecular weight

A Brookhaven light scattering system (Brookhaven Instruments Corporation, Holtsville, NY) was used to measure apparent molecular weights. Protein samples were prepared at concentrations ranging from 0.5 mg mL$^{-1}$ to 5.0 mg mL$^{-1}$ in formulations with defined ionic strengths at pH 6.0. The refractive index increment of the protein/solvent pair was assumed to be constant at 0.185 mL g$^{-1}$ for the range of ionic strength studied (169). The scattering intensity was measured at 90° using a 2 mm pinhole size for lysozyme and a 1 mm pinhole size for the mAb at 296 K using a mini L-30 compact diode 637 nm laser. The absolute Rayleigh ratio of the samples were obtained using pure benzene as a calibration standard. Triplicate samples were prepared for each formulation condition and scattering intensity was acquired for
each sample replicate. Disturbances due to dust were minimized by using a built-in statistical dust rejection function.

2.4 Results

2.4.1 Nernst-Planck theory for non-interacting protein molecules accurately predicts experimentally-determined diffusivities.

In Fig. 2.1 a and b, experimental DLS data for both lysozyme and mAb are compared to predictions of the multicomponent diffusion model described by Eq. 2.3. The predictions of the model, $D_{m,2}$ versus protein concentration, are shown as lines in both figures. Concentrations of ionizable species, which reflect experimental sample preparation, are input parameters in the model. The concentrations of K$^+$ and Cl$^-$ ions present in solution, after dialysis, are calculated using Eq. 2.4 a and b, respectively. For example, after dialyzing a 23 mg mL$^{-1}$ solution of lysozyme against 2.3 mM KCl, [K$^{+}\text{ret}$] is 0.5 mM whereas [Cl$^{-}\text{ret}$] is 10 mM. In contrast, when this concentrated protein sample is diluted to a protein concentration of 1.5 mg mL$^{-1}$ by adding 2.3 mM KCl, the concentrations of [K$^{+}\text{ret}$] and [Cl$^{-}\text{ret}$] are 2 mM and 2.7 mM, respectively. These resulting electrolytes and protein concentrations are used with Eq. 2.3 to calculate diffusivities.

![Figure 2.1](image-url) DLS data for lysozyme (A) and mAb (B) in 2 mM KCl (circles), 5 mM KCl (diamonds), 10 mM KCl (triangles), and 90 mM KCl (inverted triangles) at pH 6.0. Error bars represent the standard deviation of triplicate measurements. Most error bars are smaller than symbols. Lines show the fit of the model $D_{m,2}$ versus $c$, calculated using Eq. 2.3 for 2 mM KCl (solid line), 5 mM KCl (dashed line), 10 mM KCl (dash-dotted line), and 90 mM KCl (dotted line) at pH 6.0. The inset in (B) shows a zoomed-out version of the model to demonstrate curvature.
Both experimental and theoretical results demonstrate that at low ionic strength and low protein concentration, protein diffusivity increases with increasing protein concentration. At higher ionic strength however, this trend becomes weaker, and above an ionic strength of ca. 90 mM the diffusivity is essentially independent of protein concentration. The model fits DLS data for both proteins well at low protein concentrations (up to ca. 10-20 mg mL\(^{-1}\)), but at higher concentrations of mAb, where protein-protein interactions neglected in the Nernst-Planck model likely become more important, the agreement is only qualitative, with experimental data showing a more sharply curved profile than the calculated model data (see insert of Fig. 2.1 b). The agreement between the model – which does not incorporate protein-protein interactions – and DLS data for both proteins demonstrates that local electric field gradients at low ionic strength account for the majority of protein mutual diffusion behavior (137). Tables 1.1 and 1.2 show the apparent \(k_D\) values for lysozyme and the mAb, respectively, calculated from the slopes of experimental diffusivity vs. concentration data in Fig. 2.1 a and b using Eq. 2.10:

\[
D_{m,2} = D_{i,2}(1+k_Dc)
\]  

(2.10)

We chose popular protein concentration ranges commonly employed in the literature to calculate \(k_D\) from DLS data obtained at ca. 2 – 12 mg mL\(^{-1}\) and ca. 2 – 25 mg mL\(^{-1}\) protein concentrations (95, 138, 149, 170, 171), and noticed large discrepancies between \(k_D\) values calculated over the two concentration ranges. This effect is due to the non-linearity of the curves at low ionic strength and it highlights a potential problem that could arise when using diffusivity as a function of protein concentration to measure the strength of protein-protein interactions.

**Table 2.1.** Apparent \(k_D\) values obtained from lysozyme diffusivity values by DLS, calculated using Eq. 2.10. At low ionic strengths, \(k_D\) values are obtained at different lysozyme concentration range due to the non-linearity of the experimental data. Error bars represent fitting error.

<table>
<thead>
<tr>
<th>Ionic strength (mM KCl)</th>
<th>Lysozyme concentration range (mg mL(^{-1}))</th>
<th>Apparent (k_D) (mL g(^{-1})) from DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2-12</td>
<td>119.1 ± 18.7</td>
</tr>
<tr>
<td>2</td>
<td>2-25</td>
<td>45.6 ± 9.3</td>
</tr>
<tr>
<td>5</td>
<td>2-12</td>
<td>75.6 ± 4.8</td>
</tr>
<tr>
<td>5</td>
<td>2-25</td>
<td>59.0 ± 3.5</td>
</tr>
<tr>
<td>10</td>
<td>2-25</td>
<td>51.0 ± 2.8</td>
</tr>
<tr>
<td>90</td>
<td>2-12</td>
<td>-1.5 ± 1.7</td>
</tr>
</tbody>
</table>
Table 2.2. Apparent $k_D$ values obtained from the mAb diffusivity values by DLS, calculated using Eq. 2.10. At low ionic strengths, $k_D$ values are obtained at different mAb concentration range due to the non-linearity of the experimental data. Error bars come from fitting error.

<table>
<thead>
<tr>
<th>Ionic strength (mM KCl)</th>
<th>mAb concentration range (mg mL$^{-1}$)</th>
<th>Apparent $k_D$ (mL g$^{-1}$) from DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2-12</td>
<td>85.0 ± 4.2</td>
</tr>
<tr>
<td>2</td>
<td>2-25</td>
<td>21.5 ± 5.2</td>
</tr>
<tr>
<td>5</td>
<td>2-12</td>
<td>29.9 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>2-12</td>
<td>27.9 ± 1.0</td>
</tr>
<tr>
<td>90</td>
<td>2-12</td>
<td>-7.8 ± 2.4</td>
</tr>
</tbody>
</table>

Effect of net protein charge on protein diffusion

Both Donnan equilibrium equations and the multicomponent diffusion model are sensitive to net protein charge so the accuracy of any predicted protein diffusivity is dependent on assumed protein charge. This sensitivity could explain some discrepancy between the models and experimental data as the estimation of the net charge on the proteins is challenging (141, 142). Although protein charge as a function of solution pH is frequently estimated using the protein’s primary amino acid sequence and assumed pK$_a$ values for ionizable residues, this approach often overestimates the actual protein’s absolute net charge (172–174). Actual charges are likely to be different from theoretical values due to specific protein-salt interactions and the effects of ionic strength on the pK$_a$ values of amino acid residues (142, 172). Indeed, Lehermayr et al. showed that the estimated net charge of a series of eight mAb’s differed by up to 70% from their measured net charge at pH 6.0 (173). Extrapolating from the data of Lehermeyer et al., the mAb used here would be predicted to have a net charge between +8 and +10 at the experimental pH of 6.0, in contrast to its theoretical value of +30.1 (based on sequence and assumed pK$_a$ values). We found that the “best fit” charge of the model for all DLS results is +8.7, consistent with the extrapolation from the data of Lehermayr et al. At pH 6.0, in 0.1 M KCl, the net charge of lysozyme from titration curves is +7.5 (172), whereas under our low ionic strength conditions we found the lysozyme “best fit” effective charge to be +4.8. Again, it is likely that the two estimates vary due to the effects of protein conformation and ionic strength on the pK$_a$ values of amino acid residues.
2.4.2 Under low ionic strength conditions, conformational changes should not be inferred from DLS data

Protein conformational changes as a function of solution conditions have been inferred from measurements of protein diffusivity (e.g. Sarangapani et al. (171)) by extrapolating the infinite dilution single-particle diffusion constant, \( D_{s,2} \), from DLS data using the linear relationship between \( D_{m,2} \) and \( D_{s,2} \) (Eq. 2.10) (144). Protein size (i.e. hydrodynamic radius) is then calculated from \( D_{s,2} \) using the Stokes-Einstein equation for a spherical particle (Eq. 2.6) and assuming \( D = D_{s,2} \). However, because the mutual diffusivity of a protein depends not only on its concentration, but also on the ratio of its concentration to that of other ionic species, \( k_D \) is not constant as a function of protein concentration. Thus, experimentally, the choice of protein concentrations used in the extrapolation of Eq. 2.10 as well as the ionic strength will affect the apparent value of \( D_{s,2} \). If Eq. 2.6 is then used to calculate the hydrodynamic radius of the protein, this in turn will result in artefactual implications of ionic strength or protein concentration dependent changes in protein conformation.

DLS experimental data from Fig. 2.1 a and b were used to calculate \( D_{s,2} \) by linear extrapolation to zero protein concentration. In solutions containing 2 mM KCl, only concentrations up to 12 mg mL\(^{-1}\) for lysozyme and 15 mg mL\(^{-1}\) for mAb were analyzed to maximize \( R^2 \) of a linear fit. \( D_{s,2} \) values at the lowest ionic strength were 50% higher for lysozyme and 10% higher for mAb compared to their respective \( D_{s,2} \) values at higher ionic strengths (see Fig. 2.2 a and b). If interpreted in the context of the Stokes-Einstein

![Figure 2.2](image)

Figure 2.2. The self-diffusion coefficient, \( D_{s,2} \), as a function of ionic strength calculated by linear extrapolation of DLS data for lysozyme (A) and mAb (B). Results are obtained by finding the intercept of the linear portion of DLS data in Fig. 2.1. Error bars represent the standard deviation of triplicate measurements and extrapolation to zero protein concentration.
equation, these dramatic changes in diffusivity as a function of ionic strength would yield the improbable implication that lysozyme is tightly folded at low ionic strengths but unfolds at moderate ionic strengths. In fact, when fully unfolded (e.g. in high concentrations of chaotrope), the diffusivity of lysozyme decreases by 55% (175), a similar drop to what occurs in Fig. 2.2a.

To confirm that ionic strength did not affect protein conformation, each protein’s secondary structure was examined by far-UV CD. The spectra for both lysozyme and mAb are shown in Fig. 2.3a and b after subtraction of the blank solution spectrum and conversion to molar ellipticity. The spectra at all three ionic strengths (2 mM, 10 mM, 90 mM) overlap each other, indicating no change in protein secondary structure as a function of solution ionic strength for either protein. Finally, static light scattering was used to determine protein apparent molecular weight at dilute protein concentrations to check for potential monomer-dimer associations or aggregation across the range of ionic strength studied (2-90 mM). The apparent molecular weights for both proteins agreed with their respective theoretical molecular weights within 95% confidence intervals, consistent with a lack of dimerization or other oligomerization under these solution conditions (data shown in Fig. S2 and Fig. S3 in Supporting Material).

Figure 2.3. Normalized far-UV CD spectra in KCl at pH 6.0 for lysozyme (A) and the mAb (B) at 2 mM KCl (solid black line), 10 mM KCl (dashed gray line), and 90 mM KCl (dashed black line), at pH 6.0. Each curve represents the average of triplicates of 10 scans each. Error bars are not shown, for clarity.
2.4.3 The excluded volume contribution to $k_D$ is always negative and becomes increasingly large at high protein concentration

The influence of excluded volume on $k_D$ for proteins modeled as hard-spheres is calculated using Eq. 2.8, where $k = 1.35$ for densely packed spheres (158, 176), such as in a face-centered cubic lattice (thin black line of Fig. 2.4). $S$ and $k$ parameters depend on protein shape and on packing habits, respectively. $S$ and $k$ for lysozyme were determined experimentally by Monkos, by treating lysozyme as a prolate ellipsoid of revolution and he found that $S = 3.01$ and $k = 2.91$ (162). Those values were used to calculate excluded volume contributions to $k_D$ for lysozyme as function of protein concentration (thick line of Fig. 2.4). Excluded volume contributions to $k_D$ were also calculated for the mAb by treating the protein as an ellipsoid of revolution with $S = 2.6$ and $k = 2.3$ (dashed line). At zero protein concentration, $k_D^{HS}$ is -1.75 mL g$^{-1}$, whereas the excluded volume contribution to $k_D$ for lysozyme and the mAb are -2.1 mL g$^{-1}$ and -1.8 mL g$^{-1}$, respectively. Interestingly, those values are negative although they do not take into account any protein-protein interactions beyond simple steric effects. In addition, excluded volume increases with protein concentration, due to increasing solution viscosity based solely on volume fraction, making $k_D$ more negative in the process.

2.5 Discussion

DLS is a high-throughput, automated technique that can measure protein colloidal stability, assess aggregation, and screen high-concentration protein solutions. These features make DLS a useful tool during protein screening in the formulation process. However, in formulations of low ionic strength, the protein concentration dependence of protein mutual diffusivities (e.g., as determined from DLS measurements) is

Figure 2.4. Excluded-volume contribution to $k_D$ calculated using Eq. 2.8 for an HS with $S = 2.5$ and $k = 1.35$ (thin black line), for lysozyme modeled as a prolate ellipsoid with $S = 3.01$ and $k = 2.91$ (thick line), and for the mAb with $S = 2.6$ and $k = 2.3$ (dashed line).
dominated by effects resulting from co-solute chemical potential gradients, and at high volume fractions of protein, diffusivities are strongly affected by excluded volume effects on solution viscosity. Thus, under either of these conditions, $k_D$ values do not quantitatively reflect protein-protein interactions.

### 2.5.1 Protein diffusion is influenced by the concentration of electrolytes

Protein diffusion is mediated by electrostatic interactions, hydrodynamic interactions, thermodynamic interactions, and intrinsic excluded volume effects. The interaction parameter $k_D$ takes all of these interactions into account, so care should be taken when interpreting $k_D$ values in light of only one of the effects (e.g., protein-protein interactions (148, 177) or protein hydrodynamic size (171, 178)).

We used a Nernst-Planck multicomponent diffusion model to estimate the effect of electrostatics on $k_D$. Electrostatic interactions are governed by ionic strength, counter-ion valence, protein concentration, and charge density. Our protein – salt – water model includes three ionic species: a positively charge protein and a monovalent salt which dissociates into co- and counter-ion $K^+$ and $Cl^-$. Many protein-based pharmaceuticals are formulated in low ionic strength buffers or prepared at high protein concentrations (131), where the molar concentration of co-ions and counter-ions is similar to that of the protein. In these regimes, electrochemical-potential gradients of the ionic species play an important role in driving electrolyte diffusion.

Comparing experimental DLS data to the multicomponent diffusion model demonstrates that the concentration dependence of protein diffusion for both lysozyme and the mAb is largely accounted for by electrochemical potential gradients. Even in the absence of protein-protein interactions, large and positive $k_D$ values are predicted to arise in solutions of low ionic strengths. We believe that this is an important discovery as $k_D$ is often interpreted as a measure of protein-protein intermolecular interactions to assess protein colloidal stability.

At lower ionic strengths, there is pronounced curvature in plots of diffusivity vs. protein concentration obtained from both DLS experimental data as well as from the model. This phenomenon has been observed for $\alpha$-chymotrypsinogen under low ionic strength conditions and it was explained in terms of strongly repulsive protein-protein interactions (179). However, the curvature, which is readily apparent
in the diffusivities calculated from the Nernst-Planck equation, is rather an indication of a decrease in the molar ratio of counter-ions to protein at higher protein concentration that results in reducing electrostatically-driven migration. In principle, this curvature could also be associated with non-ideal behavior, reflecting monomer-dimer or monomer-trimer interactions. However, we can discount higher-order interactions as the cause of this curvature, which is seen under conditions where both proteins volume fractions are well below 2% and peaks from DLS are monodisperse. This curvature can cause inaccuracy when using DLS to measure protein colloidal stability since apparent $k_D$ values depend on the experimental range of protein concentrations chosen, as highlighted in Table 2.1 and Table 2.2. This curvature is not limited to DLS experiments, indeed, measurements of lysozyme diffusion coefficients at low ionic strength by Taylor dispersion analysis (TDA), a microcapillary flow technique, also yield diffusivity vs. concentration plots with a curvature similar to those observed by DLS (see Supporting Material, Fig. S6).

For both proteins, the model $a priori$ fits the data well. Discrepancies can be explained by the simplicity of the model and some major underlying assumptions. The model is sensitive to the protein’s net charge, which is treated as a constant but realistically expected to depend on solution ionic strength. We also neglected protein-salt specific interactions and mAb glycosylation, which could lead to asymmetric charge distributions on the protein’s surface and impact diffusion induced by a local electric field.

We also demonstrated that electrostatic interactions can impact not only the protein’s mutual diffusion coefficient but the infinite dilution single-particle diffusion constant, $D_{s,2}$, as well. Clearly protein-protein interactions are negligible at infinite dilutions of protein molecules, but protein-cosolute interactions still cannot be neglected because, as is evident from Nernst-Planck theory, where the presence of counter-ions induces dramatic effects on protein diffusion. Using experimental DLS data, we measured changes in $D_{s,2}$ without detecting any conformational changes by CD spectroscopy or monomer-dimer association by SLS. We postulate that Stokes-Einstein equation is not applicable for DLS data at low ionic strength as this equation neglects electrostatic interactions present in a ternary protein – salt – water system even at infinitesimal protein concentrations. This issue does not apply solely to light scattering experiments, however, as large deviations in $D_{s,2}$ for lysozyme at low ionic strength have been seen in diffusivity
measurements from pulsed-gradient spin-echo NMR studies (180). This is an important observation since changes in protein diffusivity are often used in conjunction with the Stokes–Einstein equation to infer protein conformational changes such as unfolding. For example, Sarangapani et al. (171) concluded that BSA increased in size by 20% from pH 7.2 to 5.0 and by 40% from pH 7.2 to 3.0 based on fluctuations in $D_{s,2}$ values measured by DLS in 20 mM ionic strength buffers. Those findings are surprising considering that other studies did not observe such significant changes in the size of BSA as a function of pH (pH > 2) under similar buffer conditions (181, 182). Furthermore, Tanford & Buzzell demonstrated that apparent size changes in BSA as a function of pH are due to electroviscous effects alone and not to the deformation of the BSA molecule (183).

2.5.2 $k_D$ can be strongly positive or negative with no attractive or repulsive forces present

Thus far, we have discussed factors affecting $k_D$ measured at low protein concentrations. However, many protein-based pharmaceuticals are prepared in highly concentrated (100-200 mg mL$^{-1}$) solutions (131, 184). At these high concentrations, electrostatic forces can become negligible compared to hydrodynamic forces from excluded volume (96, 97). We showed, using Eq. 2.8, that at high protein concentration, the excluded volume contribution to $k_D$ becomes relatively large and negative. This effect is purely steric and not determined by underlying microscopic colloidal forces such as VDW interactions. This effect is even more dramatic when lysozyme and the mAb are modeled as prolate ellipsoids as opposed to spheres. To obtain a complete picture of $k_D$ for lysozyme as a function of protein concentration, with no repulsive or attractive forces present aside for steric forces, the multicomponent diffusion model was added.

Figure 2.5. Theoretical $k_D$ values as a function of lysozyme concentration calculated by contributions from excluded volume and electrostatics using Eqs. 2.3 and 2.8, with 2 mM KCl (solid line), 5 mM KCl (dashed line), 10 mM KCl (dash-dotted line), and 90 mM KCl (dotted line) at pH 6.0.
to Fig. 2.4 (see Fig. 2.5). The electrophoretic impact on diffusion is evident at low protein concentration whereas excluded volume contributions dominate as protein concentration increase. This figure highlights the possibility of obtaining a wide range of apparent $k_D$ values, both positive and negative, even in the complete absence of actual attractive or repulsive forces.

2.6 Conclusions and recommendations

$k_D$ is a parameter that can be measured with high throughput and is a promising tool for understanding solution behavior of protein formulations. However, because it reflects both hydrodynamic and thermodynamic effect on protein solutions, careful interpretation is needed if it is used to measure protein-protein interactions and colloidal stability of protein formulations. Especially at low ionic strength, $k_D$ values are dominated by electrophoretic effects rather than by protein-protein interactions. Likewise, at high protein concentrations, viscous effects arising from excluded volume may largely determine the value of $k_D$. We recommend comparing experimentally-determined $k_D$ values against calculated contributions to $k_D$ excluded volume effects.

2.7 Author contributions

All authors contributed extensively to this work. L.L.S and M.A.D conducted DLS experiments. L.L.S created the model and analyzed output data with contributions from T.W.R and D.K.S. L.L.S performed the remaining of the experiments (CD and SLS) and wrote the manuscript. T.W.R and D.K.S supervised and edited the paper.

2.8 Appendix

2.8.1 Derivation of Eq. 2.3

Diffusion of charged species is affected by composition and by electric potential gradients generated by the transport of ionizable species in solution. A charged protein in a solution of small and fast salt ions will create minute charge separations between the protein and oppositely charged ions. This scenario generates local electric potential gradients that accelerate the protein and slows down the oppositely charged ion. As more salt is added however, the diffusion potential diminishes and trace amounts of the charged protein have a negligible influence on this phenomenon. For a quantitative understanding of the
composition effects on protein diffusivity, it is necessary to start with the generalized Maxwell-Stefan
equation (185, 186).

\[ u_i \nabla c_i + \nabla F_i = 0 \]  \hspace{1cm} (2.A1)

where \( F_i \) is flux density of species \( i \), and \( u_i \) is mobility. Mobility can be expressed by diffusivity alone. Of all possible forces, only electric and composition gradients are included; this neglects effects of non-ideality (activity coefficients are assumed to be unity) and pressure gradients. If only the friction between the solute and water is considered, which is applicable for dilute solutions, the above expression reduces to the Nernst-Planck equation:

\[ u_i \nabla c_i + \nabla \left( D_{s,j} \nabla c_i + \frac{D_{s,j}}{RT} c_i z_j e \nabla \phi \right) = 0 \]  \hspace{1cm} (2.A2)

Where \( \phi \) is the electric potential, \( e \) is Faraday’s constant, \( R \) is the ideal gas constant, and \( T \) is temperature. For simplicity, it is assumed that the gradient takes place only in the z-direction. With a net charge of zero, without external electric potential gradient \( \sum_{i=1}^{N} z_i N_i = 0 \) (where, in the current example \( n = 3 \)), \( d\phi / dz \) can be determined as a function of the gradient in diffusion and can be substituted into the Nernst-Planck equation:

\[ N_j = -c_j D_{s,j} \left( -x_i z_i \sum_{j=1}^{n} z_j D_{s,j} \frac{dx_j}{dz} + \sum_{j=1}^{n} z_j^2 D_{s,j} x_j \right) \]  \hspace{1cm} (2.A3)

Putting this in matrix form with three species in the structure of \(-N_i = D_{m,i} c_f (dx_i / dz)\) yields Eq. 2.3 (137).

We note that Eq. 2.3 takes into account the fluxes of all three charged species (protein, co-ion, and counter-ion) but because species diffusivities are coupled and conditions of electroneutrality need to be maintained, Eq. 2.3 could have been reduced to a 2x2 Fickian diffusion matrix as the counter-ion contribution is not needed (101).

**2.8.2 Derivation of Eqs. 2.4 a and b**

Assuming the solution is ideal, the chemical potential of the salt is:
\[ \mu_{\text{KCl}} = \mu_{\text{KCl}}^0 + RT \ln[K^+][Cl^-] \]  

(2.4)

During dialysis, at equilibrium, the standard state chemical potential must be the same in both phases:

\[ [K^{+,\text{ret}}][Cl^{-,\text{ret}}] = [K^{+,\text{perm}}][Cl^{-,\text{perm}}] \]  

(2.5)

The net charge of each phase must equal zero, a condition known as electro-neutrality, which is expressed mathematically as:

\[ [K^{+,\text{perm}}] = [Cl^{-,\text{perm}}] \]  

(2.6)

\[ z_{(P^{+})}[P^{+}] + [K^{+,\text{ret}}] = [Cl^{-,\text{ret}}] \]  

(2.7)

Substitution of 2.5 into 2.7 yields:

\[ [K^{+,\text{ret}}] = [Cl^{-,\text{ret}}] \left( 1 - \frac{z_{(P^{+})}[P^{+}]}{[Cl^{-,\text{ret}}]} \right) \]  

(2.8)

Eq. 2.5 and 2.6 are substituted into the [Cl^{-,\text{ret}}] term of Eq. 2.8 which gives Eq. 2.4.a. Similar steps starting with 2.7 can be taken to obtain Eq. 2.4.b.

2.9 Supporting Material

**Figure S2.1.** Apparent molecular weight (M_{app}) of lysozyme (A) and the mAb (B) as a function of salt concentration calculated by linear extrapolation to zero protein concentration of the static light scattering data. Error bars represent 95% confidence intervals of the fitted parameters.
**Figure S2.2.** Static light scattering data for lysozyme (A) and the mAb (B) at pH 6.0 at 2 mM KCl (circle), 5 mM KCl (diamond), 10 mM KCl (square), and 90 mM KCl (triangle) used to generate Fig. S1. Error bars represent the standard deviation of triplicate measurements and are smaller than most data points.

**Figure S2.3.** Near-UV CD for lysozyme in 5 mM KCl pH 6.0. Black line indicate spectra at 1.5 mg mL\(^{-1}\) and gray line at 15 mg mL\(^{-1}\) protein concentration. Error bars not shown for clarity. Each curve represents the average of triplicates of ten scans each. Data indicates no conformational changes of lysozyme as a function of protein concentration.

**Figure S2.4.** Far-UV CD mAb in 2 mM KCl (dotted gray line), 10 mM KCl (long dash gray line), 10 mM NaAc (dashed gray line), and 90 mM KCl (black line), all at pH 6.0. Error bars not shown for clarity. Each curve represents the average of triplicates of ten scans each.
Chapter 3

Protein-protein interactions controlling interfacial aggregation of rhIL-1ra are not described by simple colloid models


3.1 Introduction

Since proteins are only marginally stable, adsorption to interfaces can destabilize them, leading to protein aggregation, both in vitro and in vivo (10, 19, 21, 187–190). Protein aggregation in vivo has been linked to many human disorders (e.g., Alzheimer’s, Parkinson’s, type II diabetes mellitus, prion disorders) (191–193). Moreover, in therapeutic applications of proteins and peptides, aggregation can lead to reduced product efficacy or adverse immune responses in patients (25, 194).

Aggregate assembly occurs as a result of attractive intermolecular interactions between proteins (10–15). Thus protein aggregation in solution can often be mitigated by adjusting formulation conditions to control protein-protein interactions (PPI), the magnitude of which can be characterized using techniques such as static and dynamic light scattering (13, 98, 114, 147). On the other hand, PPI between protein molecules adsorbed at interfaces currently cannot be measured directly. Moreover, many studies fail to isolate interface-induced aggregate formation from aggregation processes occurring in bulk solution, potentially obscuring the mechanisms for interface-induced aggregation (37, 38). Here, we specifically examine aggregation due to interfacial protein adsorption, independently from aggregation in the vicinal bulk solution.

Protein solutions are often described as colloidal dispersions whose kinetic stability results primarily from repulsive long-range electrical double-layer forces that shield attractive van der Waals forces according to the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of interparticle interactions.
(13, 81). However, a more sophisticated view that accounts for the structural and functional anisotropy of protein molecules suggests that PPI can be dominated by contributions from a relatively small number of specific protein-protein configurations (e.g., due to charge heterogeneity or structural anisotropy) (74). Here, we study the interfacial adsorption and gelation behavior of a model protein whose PPI are dominated by such specific interactions. Importantly, this protein exhibits specific PPI that have the opposite dependence on ionic strength compared to predictions based on uniformly charged sphere models, allowing us to distinguish the role of specific PPI from electrostatic double-layer repulsive forces in the context of interface-mediated aggregation.

Protein molecules may unfold, aggregate, and form viscoelastic interfacial gel layers when they adsorb to interfaces (195). These interfacial gels may be stabilized through protein-protein intermolecular interactions such as disulfide bridging or hydrogen bonding (57). The properties of protein gels at air-water and oil-water interfaces can be influenced by formulation conditions, such as pH or ionic strength (42, 56, 83, 84). The pH and ionic strength dependence of these interfacial viscoelastic properties are often rationalized in the context of models describing uniformly charged spheres. For example, Tang et al. suggested that weaker repulsive interactions due to charge shielding could explain their observation that stronger gels of silk fibroin were formed at silicone oil-water interfaces (83). However, it can be difficult to differentiate interactions arising from double-layer repulsive electrostatic forces from specific protein interactions such as dipole-dipole, π-interactions, and salt bridges (50, 83, 84).

When a protein gel layer at an interface is mechanically disrupted (e.g., during shipping and handling of pharmaceutical formulations), pieces of the interfacial gel may detach and be transported into the bulk solution as particles (53, 62, 196). Liu et al. found a link between the formation of strong interfacial gels and increased interface-induced aggregation for keratinocyte growth factor-2 at air-water interfaces (42). However, the underlying causes for these connections are not completely understood. We hypothesize that particle formation arising from interface-induced aggregation can be reduced by modulating PPI to weaken the viscoelastic properties of interfacial gel layers.
Silicone oil-water interfaces, typically found in glass prefilled syringes (PFS), are a pharmaceutically-relevant environment where proteins adsorb and aggregate. The inner walls of syringe barrels are typically coated with a layer of silicone oil (linear polydimethylsiloxane) to ensure smooth movement of the syringe plunger (69, 70). PFS serve as both delivery devices and storage containers, exposing proteins to silicone oil-water interfaces within the syringes for the entire 18 – 24 month shelf-lives of therapeutic proteins. This is a problem as protein adsorption to silicone oil-water interfaces contributes to the formation of particles that may be shed from interfaces into bulk solutions, leading to product recalls or adverse immunogenicity if particles are injected into patients (25, 61, 194, 197).

The model protein used in this study, recombinant human interleukin-1 receptor antagonist (rhIL-1ra), a 17 kDa cytokine inhibitor, is marketed in silicone oil-lubricated PFS (108). PPI for rhIL-1ra become less attractive as the ionic strength of the bulk solution increases, even under conditions where the net protein charge is expected, based on electrical double-layer theory, to yield repulsive interactions that weaken at higher ionic strengths. Thus, both the sign and magnitude of PPI for rhIL-1ra are dominated by orientation-specific attractive electrostatic forces, which have been suggested to include cation-π interactions (198, 199). Here, we exploited the dominance of these PPI over electrostatic double-layer repulsive forces to determine how PPI interactions affect rhIL-1ra viscoelastic properties at the silicone oil-water interface. In particular, we correlated interfacial viscoelastic properties determined using interfacial shear rheometry with bulk PPI interactions which were measured by static light scattering. To investigate quaternary protein structural changes associated with interfacial gel formation, thioflavin-T (ThT) was used as a marker of extensively ordered β-sheet structures. Finally, rhIL-1ra solutions in PFS were agitated to create interfacial mechanical stress, and monomer loss was measured by size exclusion high performance liquid chromatography (SE-HPLC) to determine the relationship between interfacial gel formation and aggregation in solution.

3.2 Results
3.2.1 Protein-protein interactions in bulk solution

rhIL-1ra PPI in bulk solution as a function of NaCl concentration were determined using static light scattering. Debye plots (Fig. 3.1) of scattering as a function of protein concentration showed decreasing slopes as NaCl concentration increased. Osmotic second virial coefficient values \( B_2 \) were calculated using Eq. 3.1, as described in the method section, and were normalized by the hard-sphere second virial coefficient, \( B_{HS} \). The quantity \( B_2/B_{HS} - 1 \) increased systematically with ionic strength from \(-3.0 \pm 0.1\) in 60 mM NaCl, to \(-2.2 \pm 0.3\) in 150 mM NaCl, to \(-1.0 \pm 0.1\) in 500 mM NaCl, and finally to \(-0.2 \pm 0.2\) in 800 mM NaCl. All \( B_2/B_{HS} - 1 \) values were negative, indicating that net attractive protein-protein interactions dominate in bulk solution. \( B_2/B_{HS} - 1 \) values increased with increasing ionic strength, until at 800 mM NaCl, they approached 0, the value expected for hard-sphere repulsion.

3.2.2 rhIL-1ra adsorption to silicone oil-water interfaces

To determine whether ionic strength affected protein-silicone oil interactions, the values of protein surface coverage at the silicone oil-water interface were measured and used to estimate the free energy change of adsorption (\( \Delta G_{\text{ads}} \)). Figure 3.2 shows the surface coverage of rhIL-1ra at the silicone oil-water interface as a function of protein concentration remaining in solution at the lowest and highest ionic strengths employed. The surface coverage increased rapidly as bulk protein concentration increased from 0 to 0.2 mg mL\(^{-1}\), and plateaued when the protein concentration in solution reached approximately 1 mg mL\(^{-1}\), indicating surface saturation. This behavior is conventionally parametrized using a Langmuir
isotherm, although not all the assumptions of this simplistic model are met due to the complexity of protein adsorption to silicone oil-water interfaces (19). Nevertheless, data were successfully described using the two parameters for the Langmuir model, namely the monolayer surface coverage, $\theta$, and the apparent equilibrium constant, $K_{eq}$ (200). The saturated coverage, $\theta$, for both ionic strengths was $3.0 \pm 0.3$ mg m$^{-2}$. This value was consistent with the theoretical monolayer coverage of 2.9 mg m$^{-2}$, calculated using hexagonal packing for disks (packing efficiency $= 0.91$) and a characteristic radius $r_H = 1.7$ nm (48). Values for the apparent free energy change of adsorption were calculated using the relationship $\Delta G_{ads} = -RT\ln(K_{eq})$ and were $-20.1 \pm 0.8$ kJ mol$^{-1}$ and $-20.2 \pm 1.1$ kJ mol$^{-1}$ for 60 and 800 mM NaCl solutions, respectively. Interestingly, the values of $\Delta G_{ads}$ and of surface coverage between the lowest and highest ionic strength conditions were within experimental error, suggesting that interfacial protein-silicone oil interactions were essentially independent of ionic strength. This experiment was repeated at longer incubation times (9 h and 24 h) to test whether the surface coverage changed as a function of time. No changes in rhIL-1ra surface coverage were observed after 9 h and 24 h of incubation in a silicone oil emulsion (data shown in Figure S3.6 of the supplementary materials), suggesting that no additional protein layers formed at the silicone oil-water interface during the incubation times used in this study.
3.2.3. Interfacial shear rheology

PPI for rhIL-1ra in bulk solution were sensitive to buffer ionic strength, but the interfacial protein coverage was insensitive to ionic strength. This allowed us to explore the effects of PPI on the properties of the interfacial protein layer at constant surface coverage. Interfacial shear moduli at various ionic strengths are shown in Figure 3.3 as a function of aging times. Initially, the storage modulus $G'$ was small, and the loss component $G''$ dominated, consistent with the predominantly viscous nature of the interface. However, after approximately 3–4 h of aging, $G'$ increased (indicating a viscoelastic interface) and eventually surpassed $G''$ after approximately 5 h in all four buffer conditions tested. This crossover point, where $G' > G''$, represents the so-called interfacial gel transition, and the time at which it occurs is called the ‘gelation time’, as indicated by dashed vertical lines in Figure 3.3 (201). The values of the gelation times are shown in Table 3.1. The gelation time in 800 mM NaCl was lower than at the other three NaCl concentrations. However, this value was associated with a large uncertainty due to the fact that $G'$ and $G''$ remained equal, within experimental uncertainty, for a relatively long time interval, making the precise gelation point difficult to distinguish for this particular salt concentration. After surpassing the loss modulus, the storage modulus continued to increase and eventually plateaued after approximately 9 h. The mechanical strength of the interfacial gels,
summarized in Table 3.1, was obtained by averaging G' values for last 3 h of each experiment, from 11 – 14 h. As shown in Table 3.1, as ionic strength of the solution was decreased, stronger interfacial gels were formed.

Table 3.1. Summary of gelation times and elastic and viscous moduli at each NaCl concentration in 10 mM MES, pH 6.5 for 1 mg mL⁻¹ rhIL-1ra.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Gel time (h)</th>
<th>G' (mN m⁻¹)</th>
<th>G'' (mN m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhIL-1ra in 60 mM NaCl</td>
<td>5.4 ± 0.8</td>
<td>5.5 ± 0.6</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>rhIL-1ra in 150 mM NaCl</td>
<td>5.3 ± 1.2</td>
<td>4.2 ± 0.5</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>rhIL-1ra in 500 mM NaCl</td>
<td>5.2 ± 0.7</td>
<td>2.4 ± 0.9</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>rhIL1-ra in 800 mM NaCl</td>
<td>1.3 ± 1.4</td>
<td>1.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

3.2.4 ThT fluorescence of incubated rhIL-1ra

The addition of tris(2-carboxyethyl)phosphine (TCEP) had no effect on gelation time or strengths (data not shown), indicating that gel formation was not due to the formation of disulfide bonds between rhIL-1ra monomers. Instead, we speculated that a physical gel was formed through noncovalent interactions (57). Because intermolecular β-sheet formation has been linked to protein aggregation behavior at various interfaces, we probed for the possible formation of intermolecular β-sheet during gel formation using ThT (58, 107). ThT is a commonly used dye for detecting the presence of extensively ordered β-sheet structures. A representative emission spectrum for rhIL-1ra incubated with silicone oil for 24 h (Fig. 3.4) shows a marked increase in fluorescence emission intensity at 480 nm upon the addition of ThT, indicating the formation of ordered intermolecular β-sheets upon incubation. Figure 3.4 also shows that in the absence of silicone oil–water interfaces, no fluorescence

Figure 3.4. Example of emission fluorescence spectrum for 1 mg mL⁻¹ rhIL-1-ra incubated for 24 h. Solid black line shows the spectrum before the addition of 120 μM ThT in 60 mM NaCl 10 mM MES pH 6.5 in the presence of silicone oil, and dashed black line after 120 μM ThT has been added. Dotted black line shows the spectrum after addition of 120 μM ThT in 60 mM NaCl 10 mM MES pH 6.5 without any silicone oil present, and dotted grey line shows the spectrum after addition of 120 μM ThT in 800 mM NaCl 10 mM MES pH 6.5, without any silicone oil. Error bars from three independent experiments for each spectrum are not shown for clarity.
increase was detected upon the addition of ThT, indicating the absence of aggregates containing ordered β-sheet structures when silicone oil-water interfaces were absent.

After incubating rhIL-1ra for 1 h with a silicone emulsion (SOE), a slight increase of approximately 15% in ThT fluorescence intensity occurred at all four ionic strengths (Fig. 3.5). After 4 h of incubation of rhIL-1ra solutions with a SOE, samples that contained 60 mM NaCl showed a 90% increase in ThT emission fluorescence, but increases were insignificant for all other salt concentration tested. After 12 h of incubation, ThT emission fluorescence prominently increased for rhIL-1ra incubated in both 60 and 150 mM NaCl. ThT fluorescence increased slightly for samples incubated in 500 mM NaCl whereas no significant increase in ThT emission fluorescence was observed for samples incubated in 800 mM NaCl. Finally, after 24 h, only rhIL-1ra incubated in 60 mM NaCl showed a significant increase in ThT emission fluorescence compared to fluorescence measured after 12 h of incubation. The results of this ThT fluorescence study indicated that relatively little ordered intermolecular β-sheet structures formed when rhIL-1ra was incubated with a SOE on time scales consistent with the formation of gels (ca. 5.3 h) and that adding ThT to rhIL-1ra incubated at the lowest ionic strength for 24 h had the greatest emission fluorescence increase.

3.2.5 rhIL-1ra incubation in syringes

Losses of rhIL-1ra monomer from solution were observed when syringes were rotated end-over-end in the presence of a silicone oil-water interface (Fig. 3.6). The greatest amount of rhIL-1ra monomer loss occurred in solutions at the lowest NaCl concentration, 60 mM NaCl, in rotated siliconized syringes, where up to 15% monomer loss occurred after 21 days (Fig. 3.6A). As ionic strength increased, protein
monomer loss decreased, and at the highest ionic strength, 800 mM NaCl, less than 4% monomer was lost. Moreover, monomer loss in siliconized syringes increased steadily over time, where the greatest monomer loss occurred after three weeks of incubation. Incubation studies in un-siliconized syringes were performed as a control to isolate the effect of silicone oil on rhIL-1ra stability (Fig. 3.6B). Monomer loss increased slightly over time in syringes which were rotated with up to 3% monomer loss after 21 days of incubation in 60 mM, 150 mM, and 500 mM NaCl. No monomer loss was seen for rhIL-1ra incubated in solutions containing 800 mM NaCl. Without mechanical interfacial perturbation, the amount of monomer loss remained below 4% and 2.5% in siliconized and un-siliconized syringes, respectively (Fig. S3.1 in supplementary materials).

To determine whether loss of monomeric rhIL-1ra was a result of an aggregation phenomenon occurring in the bulk solution or at the interface, siliconized syringes containing rhIL-1ra were rotated for 14 days. They were then removed from the rotation apparatus and incubated quiescently at room temperature for an additional 7 days. Monomer loss did not continue to increase after syringes were removed from the rotation apparatus (Fig. 3.7). This result implies that protein aggregates were formed solely at the interface and that aggregates, once transported to the bulk, did not induce further monomer loss in bulk solution.

![Figure 3.6. Percent monomer loss from a solution containing 1 mg mL\(^{-1}\) rhIL-1ra incubated for 21 days in rotating (0.05 Hz) syringes, in 10 mM MES pH 6.5 and 60 mM NaCl (black diamonds), 150 mM NaCl (dark grey diamonds), 500 mM NaCl (light grey diamonds), and 800 mM NaCl (white diamonds) in A) siliconized syringes B) un-siliconized syringes.](image-url)
3.2.6 Microflow imaging of subvisible particles

Although we observed as much as 15% loss of monomeric rhIL-1ra after mechanical perturbation of silicone oil-water interfaces, no soluble aggregates were detected by SE-HPLC. In contrast, flow microscopy, which reliably detects particles ≥ 2 µm in diameter, showed large numbers of aggregate particles. Representative images of particles formed after 21 days of incubation in 500 mM NaCl, 10 mM MES, pH 6.5 are shown in Figure 3.8. Images of silicone oil droplets have a spherical shape due to their liquid nature and immiscibility with aqueous buffer. Larger droplets also have a characteristic bright spot in the center due to the refraction of light (202). In contrast, protein particles are irregular in shape and many have a translucent center.

*Figure 3.7.* Loss of soluble monomer from a solution of 1 mg mL⁻¹ rhIL-1ra incubated in siliconized syringes in 10 mM MES pH 6.5 and 60 mM NaCl (black), 150 mM NaCl (dark grey), 500 mM NaCl (light grey), and 800 mM NaCl (white). Diamonds represent samples that were incubated with rotation at 0.05 Hz. Rotation was stopped after 14 days (dotted line) and syringes were left to incubate quiescently, where squares represent measurements taken from quiescently incubated samples. Error bars are standard deviations of triplicate samples.

*Figure 3.8.* Example of images recorded using microflow imaging for solutions containing 1 mg mL⁻¹ rhIL-1ra incubated in rotating syringes (0.05 Hz) in 10 mM MES and 500 mM NaCl, at pH 6.5 in A) siliconized syringes B) un-siliconized syringes. Sample images were randomly selected out of A) 47494 images B) 2790 images.
3.3 Discussion

DLVO theory predicts that electrostatic double-layer forces between charged protein molecules, modeled as colloids, make repulsive contributions to $B_{22}$. These repulsive contributions diminish with increasing ionic strength due to screening effects. Other interactions not described by this simple charged-colloidal model, such as cation-$\pi$ interactions, can also play a role in modulating PPI in bulk solution (198, 203). For example, attractive intermolecular forces, such as cation-$\pi$ interactions, have been suggested as a factor for rhIL-1ra aggregation in bulk solution at increased temperatures (132, 198). In contrast to screening effects on repulsive electrostatic interactions described in DLVO theory, screening causes cation-$\pi$ interactions to become less attractive at higher ionic strengths. Thus, increasing anion concentrations in bulk solution decreases rhIL-1ra’s tendency for self-association and resulted in reduced rate of aggregation in bulk aqueous solution upon incubation at increased temperatures (198, 204). $B_{22}$ values for rhIL-1ra measured as a function of ionic strength confirm this behavior, where interactions at lower ionic strength are attractive and decrease in magnitude at higher ionic strength. The ionic strength effect on $B_{22}$ values for rhIL-1ra can thus be explained in terms of these cation-$\pi$ interactions, although other attractive forces, of electrostatic nature, could also be relevant.

PPI in bulk solutions have been well-characterized, resulting in effective formulation strategies to mitigate solution-phase aggregation. In contrast, strategies to prevent aggregation at interfaces are lacking, in part due to difficulties in measuring interfacial PPI. We hypothesized that the extent of interfacial aggregation may be controlled by intermolecular interfacial PPI, and that the same types of interactions are present at interfaces as in bulk solution, albeit at different relative magnitudes. At the interface, adsorbed proteins, at sufficiently high concentrations to reach a monolayer coverage, are tightly packed and their orientational freedom is reduced (74, 121). Therefore, orientation-specific interactions of rhIL-1ra, such as cation-$\pi$ interactions, may be enhanced in adsorbed protein layers, dominating over long-range electrical double-layer repulsive interactions at the interface.
In previous work, the extent of interfacial aggregation was correlated to interfacial viscoelastic properties, where stronger gels led to increased protein aggregation (42). However, the underlying reasons were not completely clear. In addition, the interfacial viscoelastic properties of adsorbed proteins have been measured as a function of pH, ionic strength, and interfacial packing, but were often explained in terms of simple colloid models described by electrical double-layer theory which could not be separated from other types of protein interactions which arose from structural or charge anisotropy (42, 83, 84). Due to the unique nature of PPI for rhIL-1ra in bulk solution, we proposed to distinguish the role of specific PPI from electrostatic repulsive double-layer interactions in the context of interface-mediated aggregation.

3.3.1 Attractive electrostatic PPI at the silicone oil-water interface contribute to interfacial gel strength

As shown in Fig. 3.9, the viscoelastic properties of rhIL-1ra at the silicone oil-water interface (e.g. interfacial gel strength) were strongly correlated with the magnitude of PPI in solution (e.g. \( B_{22}/B_{HS} - 1 \) values), with the most attractive intermolecular interactions correlating with the strongest interfacial gels. Importantly, we demonstrated that this was not associated with changes in rhIL-1ra surface affinity or coverage, since these properties were independent of ionic strength. This strong empirical correlation between bulk interaction strength and interfacial gel strength suggested that the same types of PPI measured in the bulk solution (e.g., cation-\( \pi \) interactions (198, 199)) may also drive increased interactions between adsorbed proteins, leading to stronger interfacial gels. The trend observed between interfacial gel strength and ionic strength was quite distinct from observations for other

**Figure 3.9.** Relationship between the strength of gels formed at the silicone oil-water interface and the amount of monomer loss in siliconized syringes, rotated at 0.05 Hz for 21 days. This relationship is plotted as a function of \( B_{22}/B_{HS} - 1 \). Interfacial elastic modulus values for rhIL-1ra are shown in black on the left y-axis and monomer loss values are shown on the right y-axis. Lines connecting the points are added to guide the eyes.
proteins, in which stronger gels were formed at higher ionic strengths and were explained through the screening of double-layer repulsive forces, although PPI were not measured (56, 83, 84). Electrostatic charge screening arises from the addition of ions in solution which shield double-layer repulsive forces described by DLVO theory (81). These forces exponentially decay with the intermolecular distance between two charged particles in solution. They are relatively long-range compared to other intermolecular forces and are expected to dominate in bulk solution at pH away from the pI, at low ionic strength. For an interfacial gel protein layer however, these double-layer repulsive forces might not dominate PPI because the separation between proteins is smaller than characteristic Debye screening lengths. Instead, other interactions, potentially masked in bulk solution by long-range electrostatic double-layer forces, may dominate in interfacial layers. Indeed, interfacial gels of rhIL-1ra were strongest at lower ionic strengths where more attractive electrostatic interactions were measured in bulk solution.

The formation of interfacial gels requires an interconnected network of protein molecules, connected at multiple points, that spans macroscopic distances. For our magnetic rod interfacial rheometer, this distance must be at least the characteristic channel half-width (0.5 cm). Thus, the creation of interfacial gels cannot be ascribed to simple monomer-monomer interactions between protein molecules. β-sheet structures in proteins adsorbed to interfaces often correlate with the extent of interfacial aggregation and ultimately may lead to the formation of viscoelastic films (58, 107). The relative amount of extensively ordered β-sheet structures formed was probed by ThT fluorescence and was highly correlated with interfacial gel strength, with samples that exhibited higher intermolecular β-sheet content yielding stronger gels. This observation implied that increasing protein cross-linking, through the formation of ordered intermolecular β-sheet structures, contributed to the strength of interfacial gels. These non-covalent bonds also presumably stabilized interfacial aggregates (even upon removal via mechanical stress). More attractive protein interactions at low ionic strength likely favored more cross-linking by ordered intermolecular β-sheet and further interfacial aggregation. Because of close proximity and restricted rotation at the interface, localized attractive interactions could have thus become particularly important and helped favor intermolecular β-sheet structures.
Gelation times (on the order of 5 hours) were much greater than times needed for equilibrium adsorption to be achieved (i.e., a few minutes (55)) and increased gel strength could not be attributed to continued adsorption of rhIL-1ra to the interface, since only monolayer coverages of rhIL-1ra were observed. Instead, we surmise that rate-limiting structural or orientational rearrangements within the protein layer are necessary for organized β-sheet structures to supply the multi-point attachments needed to form macroscopic gels, and that more attractive intermolecular forces favor the formation of stronger interfacial gels with more β-sheet content (205). Indeed, the slow gelation times of approximately 5 h were reflected in the β-sheet formation kinetics of rhIL-1ra in the presence of a silicone oil emulsion, where very little organized β-sheet content was observed prior to 5 h incubation times.

3.3.2 rhIL-1ra monomer loss in syringes

Proteins that are formulated in silicone oil-lubricated syringes are exposed not only to silicone oil-water interfaces but also to air-water interfaces as a result of air bubbles being introduced during syringe filling and stoppering processes. The synergistic effects of silicone oil-water interfaces, air-water interfaces, and agitation (which causes interfacial motion) generate protein particles that remove gelled protein aggregates from silicone oil-water interfaces and transport them into the bulk (61). In the experiments reported here, we mimicked the disruption of the gelled protein layer that occurs without introducing an air-water interface (i.e., an air bubble), which would have introduced more complexity to the system. To do so, we added two siliconized glass beads that mechanically disrupted protein gels at the silicone oil-water interface as they moved up and down the syringe during rotation. This method has been shown previously to increase the amount of particles generated in syringes, while isolating the effect of the silicone oil-water interface from the air-water interface (61).

Up to 15% of the rhIL-1ra monomers were lost from the bulk solution after three weeks of end-over end rotation in siliconized syringes. Protein loss could not be ascribed solely to adsorption to interfaces as only approximately 0.3% of the total amount of rhIL-1ra in syringes was absorbed at any given time. Nor could losses be attributed to aggregation caused by bulk shear forces associated with the movement of the beads along the walls of the syringe, as only marginal differences in monomer loss were observed.
between rhIL-1ra formulations in un-siliconized syringes that were rotated and those that were incubated quiescently. Instead, our combined results show that monomer loss occurred due to aggregation at the silicone oil-water interface, and a greater loss ensued during agitation because this interface was periodically renewed during interfacial mechanical stress caused by the movement of siliconized glass beads. This claim was further supported by the absence of soluble aggregates detected by SE-HPLC, which implied that aggregate formation by nucleation then growth through monomer addition in the bulk phase was unlikely. Additionally, images recorded with microflow imaging microscopy revealed agglomerates of aggregated protein and silicone oil droplets, consistent with a mechanism of interfacial aggregates being sheared off the interface.

Aggregates formed at the interface and transported into the bulk solution are not always colloidally stable, so aggregates released into the bulk solution could potentially undergo further aggregation. On the other hand, aggregates could form solely at the interface and upon interfacial mechanical stress (i.e., as applied by the moving glass beads) leach into the bulk without further destabilizing monomers in solution (37, 53, 61, 62). To determine which scenario occurred in this study, we stopped the rotation of siliconized syringes containing rhIL-1ra after 14 days of incubation. We observed no further protein loss for the following 7 days of quiescent incubation, consistent with the conjecture that monomer loss was solely due to aggregates forming at the interface. It is interesting that monomeric rhIL-1ra does not aggregate noticeably in the bulk liquid, even under conditions where $B_{22}$ values show that monomer-monomer interactions are attractive. This could be due to several factors. Chi et al. showed that aggregation kinetics are influenced by both PPI and by the protein’s conformational stability (13). rhIL-1ra is conformationally stable in solution ($\Delta G_{\text{unf}} = 5.4 \text{ kcal/mol}$ and is not a function of ionic strength (206)), so even though negative $B_{22}$ values would be expected to correlate with both increased frequency and increased lifetimes of monomer-monomer collisions, the free energy of unfolding provides an energy barrier that over our experimental timescales prevents these collisions from resulting in significant irreversible aggregation. Such an energetic barrier might also explain the negligible growth of aggregates by monomer addition in the bulk solution. In contrast to rhIL-1ra’s stability against aggregation in the bulk solution, for protein
adsorbed at the interface, the very high local concentrations of protein lead to a proximity effect that, combined with conformational destabilization, result in the observed surface aggregation.

### 3.3.3 Greater rhIL-1ra monomer loss in siliconized syringes correlates with stronger interfacial gels

Interface-induced rhIL-1ra aggregation in PFS that were agitated via the movement of internal glass beads correlated with stronger interfacial viscoelastic protein gels (Fig. 3.9). We suggest that shear forces caused by the movement of beads in siliconized PFS resulted in the detachment of interfacial gel fragments into the bulk solution, where they were detected as aggregates. Even though beads went up and down rapidly with respect to gel formation time, the contact area between a passing bead and cylinder walls was small, and only removed a fraction of the surface gel per pass – providing sufficient time for the renewal of interfacial gels prior to the next exposure to mechanical stress from a passing bead. Stronger gels had a higher degree of ordered intermolecular β-sheet cross-linking. Therefore, molecules within the pieces of these stronger gels were more likely to remain aggregated after the shear forces stripped the gels from the silicone oil-water interface. In solutions with higher solution ionic strength, fewer multi-point attachments were created, so interfacial viscoelastic protein gels were weaker and led to lower levels of irreversibly aggregated protein molecules.

### 3.4 Conclusions

Earlier analyses of colloidal stability of proteins focused on electrostatic protein-protein interactions based on DLVO theory, where increasing ionic strength is expected to increase the screening of protein charges, thereby decreasing the strength of repulsive electrostatic interactions (13). However, other electrostatic forces such as orientation-specific electrostatic attractive interactions can play an important role in contributing to interface-induced protein aggregation. Optimal stability against protein aggregation may therefore not occur in formulation buffers at the lowest ionic strengths. Consequently, taking into account the nature of PPI at interfaces should be an important step towards the development of aggregation control strategies.

### 3.5 Materials and methods
3.5.1 Materials
rhIL-1ra (pI = 5.3) was manufactured and purified at Amgen Inc. The stock formulation contained 150 mg mL\(^{-1}\) protein in citrate buffered saline (CSE): 10 mM sodium citrate buffer pH 6.5, 140 mM sodium chloride, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). rhIL-1ra was stored at -80°C until needed. Nano-strip® solution was obtained from VWR (Radnor, PA) (caution: Nano-strip® should be handled with extreme caution; do not store in a closed container). Low viscosity polydimethylsiloxane (PDMS) (5 cSt at 25°C) used for interfacial rheology studies was purchased from Sigma-Aldrich (St. Louis, MO). Pierce bicinchoninic acid (BCA) assay kit, used to measure protein concentration, was purchased from Sigma-Aldrich. PDMS (Dow Corning 360, 1000 cSt) used for making silicone oil in water emulsions, as well as for the siliconization of the syringes was of medical grade and purchased from Nexeo Solutions (Denver, CO). The syringes used in the incubation studies were BD Hypak SCF 1 mL long 27G1/2 (BD Medical-Pharmaceutical Systems, Franklin Lakes, NJ). Solutions of 60 mM – 800 mM NaCl were buffered at pH 6.5 with 10 mM MES and contained an additional 0.05 g L\(^{-1}\) sodium azide to prevent bacterial growth. All buffer salts were purchased from Sigma or Fisher Scientific and were of reagent grade or higher quality. Buffers were prepared using ultrapure water (Elga PURELAB flex, Woodridge, IL) and filtered with 0.2 μm nitrocellulose membrane filters.

3.5.2 Dialysis and sample preparation
Dialysis for buffer exchange from the stock formulation was performed against excess solution overnight using Pierce 3500 MWCO Slide-A-Lyzer Dialysis Cassettes (ThermoFisher Scientific, Waltham, MA). The dialyzed protein was then filtered with 0.1 μm Anotop10 Whatman inorganic syringe filters (General Electric, Boston, MA) before dilution. After dialysis, the protein was diluted to the desired concentrations using the appropriate buffer. Protein concentrations were measured using a Nanodrop 2000 (ThermoFisher Scientific) with an extinction coefficient of 0.77 L g\(^{-1}\) cm\(^{-1}\) (206). The purity of rhIL-1ra stock was verified (≥ 98% monomer) using SE-HPLC after dialysis. Near-ultraviolet circular dichroism (UV-CD) was used to verify the structural integrity of rhIL-1ra as a function of ionic strength. Near UV-
CD method and results are shown in Fig. S3.3 in supplementary materials as no changes in protein tertiary structure were observed as a function of ionic strength.

### 3.5.3 Static light scattering

A Brookhaven light-scattering system (Brookhaven Instruments, Holtsville, NY) was used to measure the osmotic second virial coefficient, $B_{22}$. Dialyzed and 0.1 µm filtered stock of rhIL-1ra samples were diluted to protein concentrations ranging from 1 mg mL$^{-1}$ to 7.0 mg mL$^{-1}$ using 0.02 µm filtered buffer at designated ionic strengths. The protein concentrations were checked by UV-vis at 280 nm. The refractive index increment of the protein-solvent pair (0.185 mL g$^{-1}$) was measured for rhIL-1ra at 60 mM and assumed to be constant for the range of ionic strengths studied (169). The scattering intensity from a mini L-30 compact diode 637 nm laser was measured at 90° with a 2 mm pinhole at 296 K for triplicate samples at each protein concentration. Disturbances due to dust were minimized by using a built-in statistical dust rejection function. The scattering intensity of the buffer was also measured and subtracted from that of the samples. The excess Rayleigh ratios ($R_{90°}$) of the samples were then obtained using pure benzene as a calibration standard. $B_{22}$ values at each ionic strength were calculated using the relationship:

$$\frac{Kc}{R_{90°}} = \frac{1}{M} + B_{22}c$$

where $K$ is the optical constant, $M$ is protein molecular weight, and $c$ is rhIL-1ra concentration obtained by UV-vis (113, 116). $B_{22}$ values were normalized by the theoretical value of the hard-sphere second virial coefficient $B_{HS}$, which is given by:

$$B_{HS} = \frac{16\pi r^2 N_A}{3M^2}$$

where $r$ is rhIL-1ra radius ($r_{H} = 1.7$ nm measured using dynamic light scattering(132)) and $N_A$ is Avogadro’s number (207). $B_{HS} = 1.7 \times 10^{-10}$ mL mol$^{-2}$ can be used to normalize $B_{22}$ where $B_{22}/B_{HS} - 1 < 0$ indicates net attractive forces between protein monomers, $B_{22}/B_{HS} - 1 = 0$ indicates net repulsive forces due to steric forces only, and $B_{22}/B_{HS} - 1 > 0$ indicates net repulsive forces between protein monomers (15, 114).
3.5.4 Preparation and characterization of silicone oil emulsions

Silicone oil emulsions (SOE) were prepared in water as previously described using a Emulsiflex C5 high-pressure homogenizer (Avestin, Inc., Ottawa, Ontario, Canada) (208). During the emulsification process, the majority of the silicone oil collected on the walls of the emulsifier leaving a final emulsion which contained approximately 1% w/v silicone oil. The SOE was stored for a minimum of 4 days at 4 °C prior to use and was assumed to be stable for 3 weeks, as per previous observations in our lab (208).

Liquid-liquid extraction and subsequent Fourier Transform Infrared Spectroscopy (FTIR) analysis were used to determine the concentration of silicone oil present in the SOE. This technique was previously described in Ludwig et al. (208). The area under the curve of known silicone oil concentrations standards measured by ATR-FTIR (Thermo Nicolet 6700 FTIR, Waltham, MA) were used to determine the concentration of silicone oil in the SOE (Fig. S3.4 in supplementary materials). The final concentration of silicone oil was 8.1 ± 0.6 mg mL⁻¹. Silicone oil droplet size distributions were then measured using a Beckman Coulter LS230 (Fullerton, CA) using the value of 1.4046 for the refractive index of the silicone oil (209). The silicone oil surface area in the SOE was calculated using both the silicone oil droplet size distribution (Fig. S5) and the silicone oil concentration, measured by FTIR. The surface area of silicone oil per volume of emulsion was 433 ± 40 cm² mL⁻¹.

3.5.5 Determination of protein surface coverage

The amount of rhIL-1ra adsorbed to the silicone oil-water interface was determined using a modification of the bulk depletion method described in Gerhardt et al. where a BCA assay was used instead of a Bradford assay for measuring protein concentration (17). Briefly, aliquots of dialyzed rhIL-1ra were mixed with a SOE at 60 and 800 mM NaCl, in 10 mM MES at pH 6.5. The samples were incubated for 1 h and were then centrifuged to separate the silicone oil phase from the aqueous phase. No pellets were observed and the subnatant was collected for analysis. To determine the protein concentration remaining in bulk solution after protein adsorption to the silicone oil-water interface, a BCA assay was performed on the extracted subnatant. Following the manufacturer’s instructions, standard curves were generated using rhIL-1ra at known concentrations. Equal volume of BCA reagent was added to rhIL-1ra samples prior to
incubation at 60 °C for 1 h. The absorbance was measured at room temperature at 562 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Protein loss due to adsorption to the silicone oil-water interface was determined using mass balance by subtracting the amount of protein remaining in the bulk from the original amount added. Surface coverage was determined by dividing the concentration of protein adsorbed by the surface area per volume of silicone oil emulsion.

To determine whether adsorption increased over time, rhIL-1ra was also incubated for 5 h and 24 h following the same procedure as described above. Small pellets were observed for both incubation times after centrifugation and were re-suspended in the subnatant prior to protein concentration analysis by BCA assay.

### 3.5.6 Interfacial shear rheology

To study gelation of rhIL-1ra at the silicone oil-water interface, a custom-built interfacial shear rheometer was used as previously described (55, 56, 210). 40 mL of buffer solution was placed in a rectangular glass container (14.5 x 3 cm). A magnetic rod (diameter x length = 0.06 x 2.54 cm²), with anodized black and white stripes was inserted in the middle of a 2.6 cm polytetrafluoroethylene (PTFE) tube and then sealed with paraffin wax. The magnetic rod assembly was aligned in the middle of a glass channel (width of 1 cm) and a 5 mL aliquot of 5 cSt silicone oil was layered on top to cover the entire area of the solution in the glass channel.

Sinusoidal oscillatory forces were applied on the rod by electromagnetic coils placed on both ends of the glass channel. The movement of the magnetic rod due to the applied forces was tracked by a CCD camera to determine the resulting strain. The stress ($\sigma$) and strain waves ($\gamma$) were then used to calculate the complex dynamic interfacial shear modulus ($G^*$), represented as a combination of an elastic (storage) modulus ($G'$) and a viscous (loss) modulus ($G''$), where $G^* = G' + iG''$. The respective moduli were calculated using the following:

$$G' = \frac{|\sigma|}{\gamma} \cos(\theta) \quad (3.3)$$

$$G'' = \frac{|\sigma|}{\gamma} \sin(\theta) \quad (3.4)$$
where $\theta$ is the phase angle between the stress and strain curves (210).

The bulk solution contribution to the complex dynamic interfacial shear was first measured in the absence of protein by varying the frequency of the sinusoidal current applied to the coils (from 0.011 to 4 Hz) as described in Bantchev & Schwartz (55). After calibration, 400 $\mu$L of protein solution was added to the aqueous phase to obtain a final rhIL-1ra concentration of 1 mg mL$^{-1}$. The rheology measurement was initiated immediately at 0.125 Hz and was carried out for 14 h. By this time, $G'$ and $G''$ values reached an apparent asymptotic value, changing by <15% from the start of the plateau which occurred after 11 h of measurements. Final viscoelastic properties were therefore calculated by averaging $G'$ and $G''$ values between 11 – 14 h.

Before each measurement, the glass channel and rectangular container were cleaned by first rinsing with 2% Micro-90 (International Products Corporation, Burlington, NJ), ultrapure water, ethanol, and hexane. They were then soaked in Nano-strip® solution for 2 h at 60 °C to remove any surface contaminants. The rod assembly was cleaned with 2% Micro-90 and rinsed with water. It was then placed in a magnetic coil (0.06 Tesla) for 1 h to magnetize the rod.

To determine any effect from intermolecular disulfide bonding on gels formed at the silicone oil-water interface, the reducing agent TCEP, was also added to a final concentration of 6 mM in a separate set of experiments to prevent intermolecular disulfide bond formation. The pH of the solution was adjusted to pH 6.5 with concentrated NaOH. This solution was then added to the glass channel prior to starting the calibration step and the same procedure as described above was followed for interfacial shear rheology measurements.

### 3.5.7 Thioflavin-T fluorescence to probe for rhIL-1ra intermolecular β-sheet formation

To monitor the formation of rhIL-1ra gels at the silicone oil-water interface, ThT was used as a marker of intermolecular β-sheet formation (35, 58). ThT detects mature fibrils with extensively ordered structures. A stock solution of ThT at a concentration of 2.5 mM was prepared in water and protected from light. The solution was passed through a 0.2 $\mu$m filter and was stored at 4 °C. rhIL-1ra was added to 12.5 mL SOE to obtain a final concentration of 1.2 mg mL$^{-1}$ in 10 mM MES pH 6.5 at the desired NaCl
concentration. The volume was standardized to 15 mL with the appropriate buffer to achieve a final rhIL-1ra concentration of 1 mg mL\(^{-1}\) at a constant surface area of silicone oil. Samples were prepared in triplicate and incubated at room temperature. To prevent silicone oil from creaming out of suspension during long incubation times, the samples were placed on a gentle rocking platform (20 tilts/min at 20° angle, Model 200, VWR, Radnor, PA). 3 mL aliquots from each triplicate vials were removed at each time point of incubation. Using a SLM Aminco Fluorimeter (SLM Instruments, Urbana, IL), samples were excited at 405 nm and emission spectra were recorded in triplicate from 460 to 530 nm with a bandpass filter of 10 nm. Fluorescence intensities were averaged from 475 – 485 nm to minimize variations due to instrumental noise at 480 nm. Fluorescence measurements were conducted using a front-face cell orientation to minimize effects due to SOE turbidity. Emission spectra were recorded prior to the addition of ThT to establish a baseline. Each triplicate sample was then doped with ThT to achieve a final concentration of 120 µM ThT. Measurements were conducted at room temperature, and samples were equilibrated for approximately three minutes prior to readings.

To ensure that the addition of silicone oil and salt did not affect the excitation or emission spectra of ThT, controls containing 60 mM and 800 mM NaCl, in 10 mM MES pH 6.5, with and without SOE were incubated for 1 h and 24 h on a rocking platform (20 tilts min\(^{-1}\)). A slight but consistent increase in fluorescence emission intensity was recorded for all samples once 120 µM ThT was added. This increase in fluorescence emission intensity due to the presence of ThT was subtracted from the data. As expected, ionic strength and the presence of SOE did not affect ThT excitation or emission intensity (211). In addition, controls showing the emission spectra of rhIL-1ra after incubation in 60 and 800 mM NaCl, 10 mM MES pH 6.5 for 24 h, on a rocking platform (20 tilts min\(^{-1}\)), without silicone oil were also measured.

3.5.8 Incubation of protein formulations in PFS

Siliconized glass syringes were cleaned to remove their original silicone oil coating before application of a new silicone oil layer to the bare glass surface to ensure uniform coverage between syringes used in incubation studies. Some syringes used for controls were not re-siliconized. To remove the silicone oil coating, syringes were cleaned according to the following procedure. A 2% Micro-90 solution was
pipetted in and out of the syringe six times. This was followed by multiple rinses with ultrapure water and then with ethanol. Next, n-hexane (ACS grade; ThermoFisher Scientific) was pipetted in and out of the syringes six times and the syringes were left to air dry. Syringes were then submerged in 60°C Nano-strip® solution for 2 h. The syringe needles stayed out of solution. Syringes were then rinsed with ultrapure water, dried with ethanol and left to air dry. Some syringes were then re-siliconized using the “liquid silicone oil” method described in Gerhardt et al. (61). 4 mm diameter borosilicate glass beads (Sigma-Aldrich, St. Louis, MO) were cleaned, and half of those were siliconized using the same method as the siliconization of the syringes. Two beads, appropriately siliconized or un-siliconized, were added to each syringe prior to filing with protein solution.

Dialyzed rhIL-1ra was diluted to 1 mg mL⁻¹ using the appropriate buffer. 1.35 mL of protein formulation at each ionic strength was then pipetted into the syringe containing two glass beads, and the syringe was stoppered, making sure no air bubbles were present. Triplicate syringes were prepared for each incubation condition at each time point. Half of the siliconized and un-siliconized syringes were rotated end-over-end at 0.05 Hz (3.2 rpm) while the other half was laid horizontally for quiescent incubation at room temperature.

At each time point, syringes were un-stoppered and the protein solution was retrieved using a thin tip pipette so as not to disturb any protein adsorbed onto the sides of the syringe. 0.8 mL of that protein solution was centrifuged at 20,000g for 1 h to separate the silicone oil and any insoluble protein aggregates from the aqueous phase while the rest was used for microflow imaging measurements. Insoluble protein aggregates formed a pellet at the bottom of the sample tube while silicone oil and any adsorbed rhIL-1ra formed a thin white layer at the top of the sample after centrifugation. Approximately 300 μL of the subnatant was removed from each micro-centrifuge tube for analysis by HPLC.

SE-HPLC was used to detect levels of monomers and soluble aggregates. The mobile phase consisted of 0.1M sodium sulfate, 0.1M sodium phosphate dibasic, and 0.1 g L⁻¹ sodium azide at pH 6.8. 50 μL of each sample was injected in triplicate at a flow rate of 0.5 mL min⁻¹ for 40 min on a TSK-GEL G3000SWxl column with a guard column (Tosoh Biosciences, Montgomeryville, PA). The absorbance
was monitored at 280 nm for the duration of the experiment. Agilent software was then used to calculate the area under each peak.

**3.5.9 Flow Imaging Microscopy**

A flow imaging microscopy instrument (Benchtop FlowCAM; Fluid Imaging Technologies Yarmouth, ME) was used to visualize particles ≥ 2 µm formed due to incubation in siliconized and un-siliconized syringes. 0.2 mL sample volume taken from the incubation study in syringes was injected at a 0.08 mL/min flow rate through a FC100 flow cell mounted with a 10x objective. The flash duration was set such that the average intensity mean of the image was between 155 – 160. Images of the observed particles were recorded.

**3.5.10 Statistical Analysis**

JMP statistical software (SAS Institute Inc., Cary, North Carolina) was used to fit a Langmuir isotherm to the surface coverage data recorded. The software minimized the mean squared error of a non-linear model with a two parameter fit.

**3.6 Acknowledgements**

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**3.7 Supplementary materials**

*Methods: near-ultraviolet (UV) circular dichroism (CD)*

Near-UV spectra were collected using a Chirascan™-plus CD spectrometer (Applied Photophysics, Beverly, MA) to examine the tertiary structure of rhIL-1ra in bulk solution as a function of NaCl concentration. A 10 mm path length quartz cuvette (Hellma, Plainview, NY) was used to collect spectra in a wavelength range from 240-320 nm using a step of 0.5 nm, a bandwidth of 1 nm, and a scan speed of 0.5 seconds per point. 10 replicates of each sample were collected in triplicate measurements. All spectra were corrected for the contribution of the buffer solution and normalized using the mean residue ellipticity.
**Figure S3.1.** Percent monomer loss from a solution containing 1 mg mL\(^{-1}\) rhIL-1ra incubated for 21 days at room temperature in syringes quiescently, in 10 mM MES pH 6.5 and 60 mM NaCl (black diamonds), 150 mM NaCl (dark grey diamonds), 500 mM NaCl (light grey diamonds), and 800 mM NaCl (white diamonds). The incubation conditions are as follows: A) siliconized syringes with two 4 mm siliconized beads B) un-siliconized syringes with two 4 mm un-siliconized beads. Error bars are standard deviations of triplicate samples.

**Figure S3.2.** Fluorescence emission spectra for rhIL-1ra incubated overnight at room temperature in 10 mM MES pH 6.5 and A) 60 mM NaCl B) 800 mM NaCl. Dotted lines represent sample without ThT added and solid lines are with 120 μM ThT. Contribution from free ThT in buffer has been subtracted.
Figure S3.3. Near-UV CD spectra of rhIL-1ra in 10 mM MES, pH 6.5 with 60 mM NaCl (solid) and 800 mM NaCl (dashed). Buffer spectra was subtracted for both ionic strength condition prior to calculating molar ellipticity. Each spectrum is an average of triplicate measurement of 10 scans each.
Figure S3.4. Silicone oil concentration standard curve. Each square point represents the area under the curve between 1280 and 1240 cm\(^{-1}\) of an infrared absorbance spectrum of silicone oil in hexane standard solution. The solid line represents the linear least square fit of the data. Error bars represent standard deviations from replicate measurements.

Figure S3.5. Particle size distribution of 1\% w/v silicone oil in water emulsion. Particle size was calculated assuming Mie Scattering from spherical particles using the value of 1.4046 for the refractive index of the silicone oil.
Figure S3.6. rhIL-1ra surface coverage on silicone oil as a function of incubation time in 60 mM NaCl 10 mM MES pH 6.5, for 1 h (black), 5 h (grey), 24 h (white). Each data point is an average of three independent measurement. Only concentrations above saturation were measured, ranging from 0.5 mg mL$^{-1}$ to 0.9 mg mL$^{-1}$ total rhIL-1ra concentration.
Chapter 4

Steric repulsion forces contributed by PEGylation of interleukin-1 receptor antagonist (rhIL-1ra) reduce gelation and aggregation at the silicone oil-water interface

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4.1 Introduction

Adsorption of proteins to interfaces may cause structural changes that can lead to interfacial aggregation and gel formation (54–56, 84, 212). Interfacial protein gel layers are extensive 3D elastic networks that are stabilized by protein-protein intermolecular interactions, such as hydrogen bonding, that can result in the formation of non-native intermolecular β-sheet structures (57, 195). Mechanical disturbances at interfaces (e.g., generated by fluid shear exerted when protein solutions are pumped or expelled from syringes, interfacial contraction and dilation caused by agitation of interfaces, or by the motion of air bubbles within containers and pre-filled syringes) may cause gelled protein aggregates to detach and be transported into bulk solutions (42, 53, 61, 62, 68, 212–215). Such protein aggregates may elicit harmful immune responses after injection into patients (24, 25, 194). Furthermore, protein aggregates can become large enough to form insoluble particles which may impact product quality and efficacy, making the product unacceptable for clinical use (29, 47, 216).

Reducing interface-induced aggregation and gelation would improve the quality and safety of protein drug products. Studies by Pai et al. (107) demonstrated that the conjugation of poly(ethylene)glycol (PEG) to lysozyme reduced interfacial aggregation at a hydrophobic interface. Furthermore, Holm et al. (105) observed that the PEGylation of lysozyme decreased its conformational stability in solution, but particle formation resulting from thermal and interfacial stresses was still reduced for PEG lysozyme relative to that for the native lysozyme protein, an observation that was ascribed in part to the steric role of PEGylation in decreasing attractive protein-protein interactions measured in solution. However, the
influence of PEGylation on the propensity of lysozyme to form interfacial aggregate gels was not investigated in either case. Moreover, the forces responsible for forming interfacial aggregates are still incompletely understood.

We hypothesize that intermolecular forces related to those occurring between protein molecules in bulk solution may govern the aggregation of proteins at interfaces. Formulation conditions for therapeutic proteins typically are carefully optimized to minimize protein aggregation in the bulk solution. Aggregation in solution is believed to occur due to molecular protein assembly resulting from attractive intermolecular forces (13, 31, 217). These forces can be measured using the osmotic second virial coefficient ($B_{22}$), which accounts for all interaction energies between two protein monomers (the subscript “2” denotes monomeric protein) including hard-sphere potential, van der Waals, and electrostatic interactions (15, 75). We propose that reducing protein attractive intermolecular interactions through protein PEGylation – quantified in solution by $B_{22}$ measurements – will also reduce intermolecular interactions and aggregation of protein at interfaces. PEGylation may affect a protein’s structural stability, which may also play a role in the protein’s propensity to aggregate (217–219). Moreover, structural changes, including the formation of non-native intermolecular $\beta$-sheet structures, are often observed in protein aggregates (220, 221).

Preventing the formation of interfacial aggregates is a challenge, since a comprehensive knowledge is lacking regarding the forces governing protein stability at interfaces, in contrast with the situation for proteins in solution. This is in part due to the absence of analytical techniques capable of measuring protein interfacial interactions and stability, as the analytical techniques employed to characterize proteins and their interactions in solution are often not amenable to the characterization of proteins adsorbed at interfaces. We propose that intermolecular forces similar to those occurring in bulk solution may govern the aggregation of proteins at interfaces, suggesting that optimizing colloidal and conformational protein stability in solution could also reduce interfacial aggregation.

The silicone oil-water interface is of particular interest in the pharmaceutical industry. Silicone oil is used to facilitate the insertion of rubber stoppers during filling procedures and is also used as a coating
and lubricant in pre-filled syringes (69, 70, 222). Pre-filled syringes are used as both storage and delivery devices, so therapeutic proteins may be in contact with silicone oil-water interfaces for extended periods, increasing the possibility of the formation of gelled interfacial aggregates. Gaining a better understanding of the forces responsible for the formation of gelled protein aggregates at the silicone oil-water interface would therefore help the development of aggregation-control strategies.

We showed previously that protein aggregation at the silicone oil-water interface could be decreased by reducing attractive electrostatic forces between recombinant human interleukin-1 receptor antagonist (rhIL-1ra) molecules (212). Here, we hypothesize that rhIL-1ra interfacial aggregation can also be reduced by introducing repulsive steric interactions between protein molecules through the covalent addition of a 20 kDa PEG polymer to rhIL-1ra. Since PEGylation may affect rhIL-1ra structural integrity, we also examined the role of conformational stability in the interfacial aggregation of rhIL-1ra and PEG rhIL-1ra.

4.2 Materials and methods

4.2.1 Materials
rhIL-1ra (17.3 kDa) and PEG rhIL-1ra (37.3 kDa) were manufactured and purified by Amgen Inc. The rhIL-1ra stock formulation contained 150 mg mL\(^{-1}\) protein in 10 mM sodium citrate buffer pH 6.5, 140 mM sodium chloride, and 0.5 mM ethylenediaminetetraacetic acid. The PEG rhIL-1ra stock contained 1 mg mL\(^{-1}\) protein. Both proteins were stored at -80°C until used in experiments. MALDI-TOF analysis was done on both proteins to confirm the covalent addition of a 20 kDa PEG polymer to rhIL-1ra.

The syringes used were BD Hypak SCF 27G1/2 (BD Medical-Pharmaceutical Systems, Franklin Lakes, NJ). The silicone oil (Polydimethylsiloxane, Dow Corning 360, 1000 cSt) used for making emulsions, as well as for the siliconization of the syringes was of medical grade and purchased from Nexeo Solutions (Denver, CO). Nano-strip® solution was obtained from VWR (Radnor, PA) (caution: Nano-strip® should be handled with extreme caution; do not store in a closed container). The Pierce bicinchoninic acid (BCA) assay kit, low viscosity polydimethylsiloxane (5 cSt at 25°C), thioflavin-T (ThT), and
acrylamide were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate monobasic and phosphate dibasic reagents were purchased from ThermoFisher Scientific (Waltham, MA). Phosphate buffer included 0.05 g L⁻¹ sodium azide to prevent bacterial growth. Buffers were prepared using ultrapure water (Elga PURELAB flex, Woodridge, IL) and filtered with 0.2 μm nitrocellulose membrane filters. All chemicals used were of reagent grade or higher quality. All experiments and measurements, unless noted otherwise, were conducted at room temperature.

4.2.2 Dialysis and sample preparation

The stock proteins were dialyzed against excess 10 mM phosphate buffer pH 6.5, overnight using Pierce 3500 MWCO Slide-A-Lyzer Dialysis Cassettes (ThermoFisher Scientific). The dialyzed proteins were then filtered with 0.1 μm Anotop10 Whatman inorganic syringe filters (General Electric, Boston, MA). Protein concentrations were measured using a Nanodrop 2000 at 280 nm (ThermoFisher Scientific) with extinction coefficients of 0.77 mg mL cm⁻¹ for rhIL-1ra and 0.35 mg mL cm⁻¹ for PEG rhIL-1ra (206). The purity of each stock was verified by SEC-HPLC. rhIL-1ra was > 98% pure and PEG rhIL-1ra contained approximately 2% rhIL-1ra monomer and 2% higher molecular weight species.

4.2.3 Protein incubation in syringes

Siliconized glass syringes were cleaned to remove their original silicone oil coating before the application of a new, more uniform silicone oil layer to the bare glass surface. Some syringes used as controls were not re-siliconized. The procedures for the removal of the silicone oil coating as well as for the re-siliconization steps were previously described in Sorret et al. (212). 4 mm diameter borosilicate glass beads (Sigma-Aldrich) were also cleaned, and half of those were siliconized using the same method as the siliconization of the syringes. Two beads, appropriately siliconized or un-siliconized, were added to each siliconized or un-siliconized syringe, respectively, prior to filing with protein solution to provide the capability for mixing in the syringes without introducing confounding air-water interfaces.

Dialyzed rhIL-1ra and PEG rhIL-1ra were diluted to equimolar concentrations, 30 μM, using 10 mM phosphate buffer. 1.35 mL of protein formulation was then pipetted into the syringe containing two glass beads, and the syringe was stoppered, making sure no air bubbles were present. Triplicate syringes
were prepared for each incubation condition. Half of the syringes were rotated end-over-end at 3.5 rpm while the other half was laid horizontally for quiescent incubation. At each time point, syringes were unstoppered and the protein solution was sampled using a thin-tip pipette so as not to disturb any protein adsorbed onto the sides of the syringe. The protein solution sample was then centrifuged at 20,000g for 1 h to separate the silicone oil and any insoluble protein aggregates from the aqueous phase. Insoluble protein aggregates formed a pellet at the bottom of the sample tube while silicone oil and any adsorbed protein formed a thin white layer at the top of the sample after centrifugation. Approximately 500 μL of the subnatant was removed from each tube for analysis by SE-HPLC.

SE-HPLC was used to detect levels of monomers and soluble aggregates. The mobile phase consisted of 20 mM phosphate 200 mM NaCl pH 7.0 and 7% (v/v) acetonitrile at a flow rate of 0.5 mL min⁻¹ on a TSK-GEL G3000SWxl column with a guard column (Tosoh Biosciences, Montgomeryville, PA). The absorbance was monitored at 280 nm and Agilent software was used to calculate areas under each peak.

4.2.4 Preparation and characterization of silicone oil emulsions

1% w/v silicone oil emulsions (SOE) were prepared in water as previously described (208, 212). To decrease the silicone oil droplet size, the pressure of the emulsifier was gradually increased to 7,000 psi for a total of 7 passes at that final pressure. The SOE was then stored for a minimum of 4 days at 4 °C prior to use and was assumed to be stable for 3 weeks, as determined previously (208). Liquid-liquid extraction and subsequent Fourier Transform Infrared Spectroscopy (FTIR) analysis were used to determine the concentration of silicone oil present in the SOE (212). Silicone oil droplet size distributions were then measured using a Beckman Coulter LS230 (Fullerton, CA) (208, 212). The specific surface area contributed by silicone oil droplets in the SOE was calculated using both the silicone oil droplet size distribution and the silicone oil concentration and was on average approximately 600 cm² mL⁻¹. Size distribution and FTIR data can be found in the Supplemental Results.
4.2.5 Determination of interfacial protein coverage
The amount of protein adsorbed to the silicone oil-water interface was determined using the bulk
depletion method described in Sorret et al. (212) and Gerhard et al. (61). Briefly, aliquots of each dialyzed
protein were mixed with a SOE in 10 mM phosphate buffer at pH 6.5 to achieve protein concentrations
from 0 mg mL\(^{-1}\) to 1 mg mL\(^{-1}\). The samples were incubated for 1 h and were then centrifuged (20,000 g)
to separate the silicone oil phase from the aqueous phase. No pellets were observed and the supernatant was
collected for analysis. To determine the protein concentration remaining in bulk solution after protein
adsorption to the interface, a BCA assay was performed on the extracted supernatant, following
manufacturer’s instructions. Standard curves were generated using rhIL-1ra and PEG rhIL-1ra at known
concentrations. The BCA reagent was added to the samples prior to incubation at 60˚C for 1 h, and
absorbance at 562 nm was measured at 25˚C using a SpectraMax M2 microplate reader (Molecular Devices,
Sunnyvale, CA). Surface coverage was determined by dividing the concentration of protein adsorbed by
the surface area per volume of the emulsion.

Surface coverage as a function of protein concentration remaining in bulk solution was
parametrized using a Langmuir model that included two parameters: the monolayer surface coverage, \(\theta\),
and the apparent equilibrium constant, \(K_{eq}\) (19, 200). The fitting of the data was achieved using JMP
statistical software (SAS Institute Inc., Cary, North Carolina) which minimized the mean squared error for
this two-parameter model.

4.2.6 Fluorescence quenching of intrinsic tryptophan residues
Fluorescence quenching measurements were conducted to measure the effect of adsorption to
silicone oil-water interfaces on the tertiary structure of both proteins. As a control, unfolded rhIL-1ra and
PEG rhIL-1ra were prepared by incubating each protein overnight in 8 M urea. Protein samples in urea and
in phosphate buffer were prepared at 0.1 mg mL\(^{-1}\) while for samples containing the silicone oil emulsion,
the protein concentration was increased to 0.3 mg mL\(^{-1}\) for rhIL-1ra and 0.2 mg mL\(^{-1}\) for PEG rhIL-1ra so
that essentially all protein was adsorbed on the silicone oil surface, as determined from the measured
adsorption isotherm. The samples containing the emulsion were incubated for 1 h on a rocking platform
(20 tilts min\(^{-1}\), 20° angle, Model 200, VWR, Radnor, PA) to ensure complete adsorption. To test if conformational changes occurred over time, selected samples were tested after 45 h on the rocking platform. No differences were detected between samples incubated for 1 h or 45 h (see Supplemental Results). Native tryptophan residues were excited at 295 nm and fluorescence emission spectra were collected from 305 nm to 380 nm. A front-face cell orientation was used for all samples to minimize scattering effects due to the emulsion turbidity. 7.6 M acrylamide, used as a fluorescence quencher, was prepared fresh in 10 mM phosphate buffer, at pH 6.5. Aliquots of the acrylamide stock were added to the samples to progressively quench tryptophan residues until a concentration of 0.15 M acrylamide was reached. Fluorescence quenching experiments were performed in triplicate for each condition using a SLM Aminco Fluorometer (SLM Instruments, Urbana, Illinois). To determine the extent of fluorescence quenching, the data were plotted on a Stern-Volmer plot according to the equation:

\[
\frac{F}{F_0} = K_{SV}[Q] + 1
\]

where [Q] is the acrylamide quencher concentration, F is the fluorescence intensity at the emission maximum in the presence of the acrylamide quencher, \(F_0\) is the fluorescence intensity at the emission maximum in the absence of the quencher, and \(K_{SV}\) is the Stern-Volmer constant (223).

### 4.2.7 Protein conformational stability in solution

Chemically-induced protein denaturation experiments were performed using fresh stocks of approximately 9 M urea prepared in 10 mM phosphate buffer, adjusted to pH 6.5. The concentration of each urea stock solution was determined by refractive index measurements on an Abbemak II digital refractometer (Reichert Technologies, Buffalo NY) (224, 225). Both dialyzed stocks of rhIL-1ra and PEG rhIL-1ra were diluted using buffer and/or urea to achieve the desired final urea concentrations between 0 M and 8 M at a fixed protein concentration of 1 mg mL\(^{-1}\). Protein concentrations were determined by UV-vis at 280 nm. Samples were prepared in triplicate and were incubated overnight. Urea-induced unfolding was monitored using a Chirascan Circular Dichroism spectrometer (Applied Photophysics, Leatherhead UK). Full spectra from 250 nm to 310 nm in 0 M and 8 M urea were obtained to verify essentially complete unfolding of both proteins. Wavelengths to monitor protein unfolding during kinetic measurements were
chosen based on the largest spectral differences in 0 M and 8 M urea for both proteins. Consequently, the mean residue ellipticities for rhIL-1ra and for PEG rhIL-1ra were measured at 282 nm and at 293 nm, respectively. A 10 mm path-length quartz cuvette was used to take triplicate measurements of each sample for 40 s intervals (400 points) with a bandwidth of 1.0 nm. A two-state approximation was used to analyze the transition between the native and the denatured states of the protein. The data in the transition region were analyzed using a linear extrapolation model to obtain the free energy of unfolding, \(\Delta G_{\text{unf}}\).

**4.2.8 Bulk solution protein-protein interactions measurements**

A Litesizer™ 500 Particle Analyzer (Anton Paar, Ashland, VA) was used to measure the osmotic second virial coefficient, \(B_{22}\). Protein samples were diluted using 0.02 µm filtered 10 mM phosphate buffer and sample concentrations were checked by UV-vis at 280 nm. The refractive index increments (dn/dc) of the protein-solvent pairs were measured using a digital refractometer. dn/dc values in phosphate buffer were 0.189 ± 0.007 for rhIL-1ra and 0.162 ± 0.011 for PEG rhIL-1ra. The scattering intensity was measured at 90° for each triplicate sample at each protein concentration. The scattering intensity of the buffer was also measured and subtracted from that of the samples. The excess Rayleigh ratios \(R_{90°}\) of the samples were then obtained using pure toluene as a calibration standard. \(B_{22}\) values for each protein were calculated using the relationship:

\[
\frac{Kc}{R_{90°}} = \frac{1}{M} + B_{22}c
\]  

(4.2)

where \(K\) is the optical constant, \(M\) is protein molecular mass, and \(c\) is protein concentration (113, 116). \(B_{22}\) values were normalized by the theoretical value of the hard-sphere second virial coefficient \(B_{HS}\),

\[
B_{HS} = \frac{16\pi r^3 N_A}{3M^2}
\]  

(4.3)

where \(r\) is the protein hydrodynamic radius measured by dynamic light scattering (\(r_{\text{H}}= 1.7\) nm and 4.37 nm for rhIL-1ra and PEG rhIL-1ra, respectively) and \(N_A\) is Avogadro’s number (74, 207). Calculated \(B_{HS}\) values of 1.7 x 10^{-4} mL mol g^{-2} and 2.6 x 10^{-4} mL mol g^{-2} for rhIL-1ra and PEG rhIL-1ra, respectively, were used to normalize \(B_{22}\), where \(B_{22}/B_{HS} – 1 < 0\) indicates net attractive energies between protein monomers and \(B_{22}/B_{HS} – 1 > 0\) indicates net repulsive energies between protein monomers (15, 114). When \(B_{22}/B_{HS} – 1\)
= 0, the net energies between protein molecules are consistent with those arising from the steric repulsive contribution of non-interacting hard spheres (74, 207).

### 4.2.9 Interfacial shear rheology measurements

Rheology experiments at the silicone oil-water interface were performed using a custom-built interfacial shear rheometer, as described previously (55, 212). Before each measurement, the glass channel and a rectangular container were cleaned by rinsing with 2% Micro-90 (International Products Corporation, Burlington, NJ), ultrapure water, ethanol and hexane. The channel and container were then soaked in Nano-strip® solution for 2 h at 60°C to remove any surface contaminants. They were then rinsed with ultrapure water, ethanol, and allowed to air dry. A ferromagnetic rod (diameter x length = 0.06 x 2.54 cm²), anodized with black and white stripes, was cleaned with 2% Micro-90 and rinsed with ultrapure water. The rod was then placed in the middle of a clean polytetrafluoroethylene tube, sealed with paraffin wax, and magnetized in a magnetic coil (0.06 Tesla) for 1 h. 40 mL of 10 mM phosphate buffer was placed in the clean glass container containing the glass channel. The magnetized rod assembly was then aligned in the middle of a glass channel and a 5 mL aliquot of 5 cSt silicone oil was layered on top to cover the entire area of the solution in the glass container.

Time-dependent sinusoidal oscillatory forces were applied to the striped magnetic rod by electromagnetic coils placed on both ends of the glass channel. The movement of the rod due to these applied forces was tracked by a CCD camera. The stress (σ) and strain waves (γ) were then used to calculate the elastic (storage) modulus (G') and a viscous (loss) modulus (G'') using the following:

\[
G' = \frac{|\sigma|}{\gamma} \cos(\theta) \quad (4.4)
\]

\[
G'' = \frac{|\sigma|}{\gamma} \sin(\theta) \quad (4.5)
\]

where \(\theta\) is the phase angle between the stress and strain curves (210).

The contribution from the bulk solution to the complex dynamic interfacial shear was first measured in the absence of protein by varying the frequency of the sinusoidal current applied to the coils.
as described in Bantchev & Schwartz (55). After calibration, an aliquot from a stock protein solution was added to the aqueous phase to obtain a final protein concentration of 0.05 mg mL\(^{-1}\) and the rheology measurement was initiated immediately at 0.125 Hz.

4.2.10 Thioflavin-T fluorescence to probe for the formation of protein intermolecular β-sheet structures

ThT was used to monitor the formation of extensively ordered intermolecular β-sheet during protein gelation at the silicone oil-water interface (35, 107, 211, 212). A stock solution of ThT at a concentration of 2.5 mM was prepared in water. The solution was passed through a 0.2 µm filter and was stored protected from light at 4 °C for up to 4 weeks. Aliquots from dialyzed stocks of rhIL-1ra or PEG rhIL-1ra were added to an emulsion in 10 mM phosphate buffer to obtain final concentrations of 0.3 mg mL\(^{-1}\) rhIL-1ra and 0.12 mg mL\(^{-1}\) PEG rhIL-1ra so that essentially all the protein added was adsorbed onto the interface. To prevent silicone oil from creaming out of suspension during long incubation times, each triplicate sample was placed on a gentle rocking platform (20 tilts min\(^{-1}\) at a 20° angle). 3 mL aliquots from each triplicate vials were removed after 48 h of incubation. Fluorescence measurements were conducted using a front-face cell orientation to minimize scattering effects due to SOE turbidity. Samples were excited at 405 nm and emission spectra were recorded from 460 nm to 700 nm with a bandpass excitation and emission filter of 4 nm. Emission spectra were recorded prior to the addition of ThT to establish a baseline. Each triplicate sample was then doped with ThT to achieve a final concentration of 120 µM ThT and were then equilibrated for approximately 3 min prior to readings.

The small fluorescence emission intensity increase associated with the fluorescence of free ThT in solution was subtracted from all fluorescence emission data. In addition, to ensure that the addition of silicone oil did not affect the excitation or emission intensity of ThT, controls containing the emulsion in 10 mM phosphate buffer were prepared. No changes in fluorescence emission intensity was recorded once ThT was added to these buffered emulsion controls.

4.2.11 Structural analysis by Far-UV CD

Structural changes in protein molecules incubated with a silicone oil emulsion were monitored by measuring changes in molar ellipticity using Far-UV CD. Wallace et al., as well as others, documented
signal flattening when using CD to characterize membrane proteins due to the heterogeneous distribution of chromophores adsorbed to large (~1 μm) particles (226–229). In our case, droplets of silicone oil in the emulsion had an average diameter of approximately 0.1 μm, and the CD spectrum of PEG rhIL-1ra was undistorted in the presence of SOE, suggesting that any flattening effects were minimal. This agrees with other works in which reliable CD data were obtained for proteins bound to vesicles with the same average diameters as the silicone oil droplets used in this study (227, 230).

Samples were prepared in triplicate by diluting proteins with either phosphate buffer or with a silicone oil emulsion phosphate buffer to a final protein concentration of 0.05 mg mL$^{-1}$ for rhIL-1ra and 0.08 mg mL$^{-1}$ for PEG rhIL-1ra. Protein concentrations were determined using UV-vis for samples in phosphate buffer. Concentrations could not be measured for samples containing the emulsion due to strong scattering at 280 nm, so concentrations in samples containing the emulsion were assumed to be identical to those in buffer alone. Approximately 70% and 25% of the total amount of rhIL-1ra and PEG rhIL-1ra in each sample were adsorbed to the interface, respectively. It was not possible to further reduce protein concentrations so that a larger fraction of the total protein was adsorbed to the interface due to low signal to noise ratio. A 4 mm path-length quartz cuvette was used to collect spectra from 205-260 nm in 0.5 nm steps, with a 2 nm bandwidth, and a scan rate speed of 0.5 s per point. Ten scans of each sample were collected. The lower end of the wavelength range was truncated to 205 nm so that the high-tension voltage did not exceed 900 V. Spectra were corrected for the contribution from either phosphate buffer or from the emulsion in phosphate buffer. Each spectrum was converted to the mean residue molar ellipticity, $[\Theta]_\lambda$, by:

$$[\Theta]_\lambda = \frac{M_o \Theta_\lambda}{10cL}$$

where $M_o$ is the mean residue molecular mass, $\Theta_\lambda$ is the measured ellipticity (degrees), $c$ is the protein concentration (g mL$^{-1}$), and $L$ is the path-length (cm) (168). Data were smoothed with a built-in smoothing algorithm from the Chirascan CD spectrometer software.

4.3 Results
4.3.1 Incubation in syringes

Fig. 4.1A shows rhIL-1ra and PEG rhIL-1ra monomer loss as a result of incubation in rotating syringes (i.e., with mechanically-applied interfacial perturbation) and in quiescent syringes (Fig. 4.1B). The greatest amount of monomer loss occurred for rhIL-1ra after 21 days of incubation in rotating siliconized syringes, where up to 8% monomer was lost. This represented twice the monomer loss measured for PEG rhIL-1ra incubated under equivalent conditions. For rhIL-1ra, monomer loss was only 2% during quiescent incubation and in the absence of a silicone oil-water interface. For PEG rhIL-1ra, however, the presence of a silicone oil-water interface did not affect the loss of monomeric protein in rotating or quiescently incubated syringes.

4.3.2 Protein adsorption to the silicone oil-water interface

Fig. 4.2 shows the interfacial coverage of rhIL-1ra and PEG rhIL-1ra at the silicone oil-water interface as a function of protein concentration remaining in solution. For rhIL-1ra, the interfacial coverage increased rapidly as bulk protein concentration increased from 0 mg mL$^{-1}$ to 0.2 mg mL$^{-1}$, and then plateaued when the protein concentration in solution reached approximately 1 mg mL$^{-1}$, indicating surface

\begin{center}
\includegraphics[width=0.5\textwidth]{Figure_4.1.png}
\end{center}

\textbf{Figure 4.1.} Soluble protein loss monitored by SE-HPLC after the incubation of rhIL-1ra and PEG rhIL-1ra in 10 mM phosphate buffer at pH 6.5, with rotation (0.06 Hz) (A) and without rotation (B) at room temperature. Gray columns denote rhIL-1ra samples and white columns denote PEG rhIL-1ra samples. Solid and hash patterns represent samples that were incubated in siliconized syringes and un-siliconized syringes, respectively.
For PEG rhIL-1ra, the interfacial coverage increased gradually as bulk protein concentration increased and the coverage plateaued when the concentration in solution reached 1 mg mL\(^{-1}\). The saturation coverages for both proteins, parametrized using the Langmuir isotherm model, were 3.1 ± 0.2 mg m\(^{-2}\) for rhIL-1ra and 1.2 ± 0.3 mg m\(^{-2}\) for PEG rhIL-1ra. This threefold decrease in interfacial saturation coverage reflected a less concentrated molecular packing of PEGylated rhIL-1ra molecules at the silicone oil-water interface which could be explained by the lower molecular density of PEG rhIL-1ra compared to that of rhIL-1ra.

For rhIL-1ra, the saturated coverage value measured was consistent with theoretical monolayer coverage of 2.9 mg m\(^{-2}\), calculated using an adsorption model based on hexagonal close packing of disks (packing efficiency = 0.91) (48). The theoretical monolayer coverage of PEG rhIL-1ra was calculated using the hydrodynamic radius was measured by DLS (\(r_\text{H} = 4.4\) nm) and a similar packing density as for rhIL-1ra, although a different adsorption geometry might be occurring due to the attachment of a branched PEG polymer to rhIL-1ra (106, 231, 232). The theoretical disk coverage for PEG rhIL-1ra was 0.9 mg m\(^{-2}\), consistent with the measured saturated coverage of 1.2 ± 0.3 mg m\(^{-2}\). Furthermore, values for the apparent free energy change of adsorption were calculated from the interfacial adsorption data using the relationship \(\Delta G_{\text{ads}} = -RT\ln(K_{eq})\), where R is the ideal gas constant, T is temperature, and \(K_{eq}\) is the equilibrium constant (unitless) (233). \(\Delta G_{\text{ads}}\) values were \(-20.2 ± 0.6\) kJ mol\(^{-1}\) and \(-19.2 ± 1.4\) kJ mol\(^{-1}\) for rhIL-1ra.
and PEG rhIL-1ra solutions, respectively. Overall, these observations suggested that the presence of PEG did not fundamentally influence the energetics of rhIL-1ra interactions with silicone oil-water interfaces.

### 4.3.3 Protein tertiary structure at the silicone oil-water interface

The Stern-Volmer plots for proteins in buffer, proteins adsorbed to silicone oil, and urea-unfolded proteins are shown in Figure 4.3. The calculated $K_{sv}$ values were $6.4 \pm 0.2 \text{ M}^{-1}$ and $6.2 \pm 0.2 \text{ M}^{-1}$ for native rhIL-1ra and PEG rhIL-1ra, respectively, indicating a similar tertiary structure in the context of the proteins’ two tryptophan environments. $K_{sv}$ values for both proteins unfolded in the presence of 8M urea were also similar, $8.2 \pm 0.1 \text{ M}^{-1}$ for rhIL-1ra and $8.0 \pm 0.2 \text{ M}^{-1}$ for PEG rhIL-1ra. These values were higher than for proteins in buffer, indicating an increase in accessibility of the tryptophan residues to the quencher in the unfolded protein molecules. A sharp decrease in the $K_{sv}$ value was observed for rhIL-1ra adsorbed to the silicone oil surface ($K_{sv} = 4.2 \pm 0.1 \text{ M}^{-1}$), indicating that the accessibility of the tryptophan residues to the quencher was more restricted than for proteins in buffer and urea-unfolded samples. When PEG rhIL-1ra was adsorbed to the silicone oil surface, the $K_{sv}$ value was $8.3 \pm 0.2 \text{ M}^{-1}$, indicating that tryptophan residues in adsorbed PEG rhIL-1ra were more accessible to the quencher than in the bulk solution, and were comparable in quencher-accessibility to the residues for urea-unfolded protein.

![Figure 4.3. Stern-Volmer plots for A) rhIL-1ra and B) PEG rhIL-1ra in 10 mM phosphate buffer, pH 6.5 (white diamonds), urea-unfolded (black diamonds), and adsorbed protein to silicone oil emulsion (gray diamonds). $K_{sv}$ values are determined from the slope of the inverse relative fluorescence versus acrylamide concentration. Each data point represents the average of three samples with error bars representing the standard deviation from the average.](image-url)
4.3.4 Conformational stability of rhIL-1ra and PEG rhIL-1ra in solution

The free energies of unfolding ($\Delta G_{\text{unf}}$) for rhIL-1ra and PEG rhIL-1ra in solution were determined by urea-induced unfolding (Fig. 4.4). The fraction of protein denatured as a function of urea concentration showed an earlier onset of unfolding for PEG rhIL-1ra than for rhIL-1ra. This observation was reflected in the $\Delta G_{\text{unf}}$ values, which were $0.9 \pm 0.1$ kJ mol$^{-1}$ for PEG rhIL-1ra and $2.2 \pm 0.2$ kJ mol$^{-1}$ for rhIL-1ra. These results indicated that PEGylation decreased the conformational stability of rhIL-1ra in bulk solution.

4.3.5 Protein-protein interaction in bulk solution

The nature and magnitude of protein monomeric intermolecular interactions were characterized in bulk solution for both rhIL-1ra and PEG rhIL-1ra. The Debye plots (Fig. 4.5) of scattering intensity as a function of protein concentration had a negative slope for rhIL-1ra and a positive slope for PEG rhIL-1ra. As expected, the intercept for PEG rhIL-1ra was lower than for rhIL-1ra, indicating a larger molecular mass for PEG rhIL-1ra. Osmotic second virial coefficient values were calculated using Eq. 4.2 and were normalized by the hard-
sphere second virial coefficient of each protein (Eq. 4.3). The quantity $B_{22}/B_{HS} - 1$ was $-5.1 \pm 0.1$ for rhIL-1ra, indicating that net attractive protein-protein interactions dominated in bulk solution. For PEG rhIL-1ra, $B_{22}/B_{HS} - 1$ was $0.1 \pm 0.1$, consistent with the apparent interaction energy associated with non-interacting hard-sphere particles.

### 4.3.6 Interfacial shear rheology

In solution, protein-protein interactions were more attractive for rhIL-1ra than for PEG rhIL-1ra, and the molecular packing of rhIL-1ra molecules at the silicone oil-water interface was denser than that for PEG rhIL-1ra. To test whether the sparser molecular packing and the reduction of attractive protein-protein interactions affected the strength of interfacial protein gels, we measured interfacial shear moduli as a function of aging time (Fig. 4.6).

Initially, for both proteins, the storage moduli $G'$ were small, and the loss component $G''$ dominated, consistent with the predominantly viscous nature of the interface. However, after approximately 6 h of aging, $G'$ increased for rhIL-1ra and eventually surpassed $G''$ after approximately 8 h. This crossover point, where $G' > G''$, represents the so-called interfacial gel transition and for PEG rhIL-1ra, it occurred much later, after approximately 27 h of aging. After surpassing the loss modulus, the storage modulus continued to increase for the entire duration of the experiment for both proteins, however, this increase was much steeper for rhIL-1ra than for PEG rhIL-1ra, resulting in stronger interfacial gels for rhIL-1ra relative to PEG rhIL-1ra. Each rheology experiment was stopped 10 h after the proteins’ respective gelation time for uniform comparison of
interfacial gel strengths. The mechanical strength of rhIL-1ra interfacial gel at this point was over five times stronger ($4.7 \pm 1.0 \text{ mN m}^{-1}$) than that of PEG rhIL-1ra ($0.9 \pm 0.2 \text{ mN m}^{-1}$).

4.3.7 Intermolecular β-sheet formation in the presence of a silicone oil-water interface

Stronger gels have been correlated with increased intermolecular β-sheet formation and these structures have been linked to protein aggregation behavior at various interfaces (58, 187, 212). We therefore probed for the possible formation of intermolecular β-sheet structures during gel formation using ThT (35, 211). Figure 4.7 shows ThT relative fluorescence emission of rhIL-1ra and PEG rhIL-1ra incubated in the presence and absence of a silicone oil emulsion for 48 h. No ThT fluorescence emission increases were measured for either protein after 48 hours of incubation in buffer only. When rhIL-1ra was incubated for 48 h in the presence of silicone oil-water interfaces, a strong increase in ThT emission fluorescence at 480 nm was observed, indicating the presence of extensively ordered β-sheet structures. In contrast, no emission fluorescence increase was detected for adsorbed PEG rhIL-1ra after 48 h.

4.3.8 Protein structural analysis by far-UV CD

Interfacial aggregation upon adsorption to the silicone oil-water interface was monitored based on changes in molar ellipticity as protein aggregates are often associated with high intermolecular β-sheet content (187, 190, 221, 234, 235). Both proteins were mixed with a silicone oil emulsion in phosphate buffer. Based on the measured interfacial saturation coverage (Fig. 4.2), approximately 70% and 25% of the total amount of rhIL-1ra and PEG rhIL-1ra protein in each sample were adsorbed to the interface,
respectively. It was not possible to further lower protein concentrations so that more of the total protein amount were adsorbed to the interface due to low signal to noise ratio. Both PEG rhIL-1ra and rhIL-1ra had similar spectra in solution (Fig. 4.8), indicating that PEGylation did not strongly affect the rhIL-1ra secondary structure. After adsorption to the silicone oil-water interface however, a large structural change was observed for rhIL-1ra relative to its native structure in solution. In contrast, when PEG rhIL-1ra adsorbed to the silicone oil-water interface, only a slight decrease in molar ellipticity occurred, and the shape of the spectrum was retained.

4.4 Discussion

4.4.1 Mechanism of aggregation in syringes

Therapeutic proteins formulated in siliconized pre-filled syringes are exposed not only to silicone oil-water interfaces but also to air-water interfaces due to air bubbles being introduced during syringe filling and stoppering processes. Previously, accelerated protein particle formation was observed due to the air bubble movement within syringes (61). Capillary forces at the three-phase (silicone oil–water–air) contact line removed silicone oil and gelled protein aggregates from the interface and transported them into bulk solution (61). The bare interface was then repopulated by proteins, leading to the renewal of a gelled layer and further aggregation. In the incubation study reported here, we mimicked the disruption of the gelled protein layer that occurs during agitation without introducing an air-water interface (i.e., an air bubble). To do so, siliconized glass beads were inserted into each syringe. Those beads mechanically disrupted protein gels at the silicone oil-water interface as they moved up and down the syringe during rotation, sliding along the syringe walls. This method has been shown previously to increase the amount of particles generated in
syringes due to interfacial protein adsorption, while isolating the effect of the silicone oil-water interface from the air-water interface (61).

8% of the initial rhIL-1ra monomer content was lost after 21 days of incubation in rotating siliconized syringes. This loss could not be attributed to aggregation caused by bulk shear forces associated with the movement of the beads along the walls of the syringe because monomer losses observed for rhIL-1ra in un-siliconized syringes were similar in syringes that were rotated and in those that were incubated quiescently. Instead, monomer loss occurred due to aggregation at the silicone oil-water interface during agitation because this interface was periodically renewed during interfacial mechanical stress caused by the movement of siliconized glass beads. In addition, after stopping the rotation of siliconized syringes, no further monomer loss occurred (data not shown). This result implies that adsorbed proteins formed aggregates at the silicone oil-water interface which were then transported into bulk solution after the movement of the beads down the syringe mechanically perturbed the aggregated layer (56, 61, 62). In the absence of a silicone oil-water interface, rhIL-1ra did not aggregate appreciably, despite the net attractive protein-protein interactions measured in solution. This could be because the energy barrier for irreversible protein aggregation prevented protein-protein collisions from resulting in significant irreversible aggregation over the experimental timescales in this study (236).

4.4.2 Effect of rhIL-1ra PEGylation on monomer loss in siliconized syringes

Monomer loss from solutions of PEGylated rhIL-1ra incubated in siliconized syringes agitated by rotation for 21 days was two-fold lower than monomer loss in comparable rhIL-1ra samples. This difference in monomer loss could potentially be ascribed to differences in monolayer packing at the interface. Based on the fitted Langmuir isotherm, PEGylation of rhIL-1ra reduced interfacial adsorption by a factor of 6, presumably due to the steric hindrance of the PEG polymer. The sparser packing of rhIL-1ra within PEG rhIL-1ra molecules could thus have prevented interactions that were necessary between rhIL-1ra molecules for interfacial aggregation to occur, resulting in the detachment of fewer PEG rhIL-1ra aggregates after mechanical interfacial disturbance. In addition, no significant increase in monomer loss was observed for PEG rhIL-1ra samples in siliconized and un-siliconized syringes that were agitated
compared to those that were incubated quiescently. These results suggest that these losses, unlike the losses from rhIL-1ra solutions, were most likely not due to interfacial aggregation, but instead occurred in bulk solution.

### 4.4.3 Forces behind the formation of protein aggregates at the silicone oil-interface

Sorret et al. previously demonstrated that attractive forces measured in solution correlated with stronger gels and increased protein aggregate formation at the silicone oil-water interface, suggesting that optimizing protein stability in solution could also reduce interfacial aggregation (212). In this study, we compared the nature of protein-protein interactions and protein conformational stability in bulk solution to gelation, aggregation, and conformational changes at the interface. These forces measured in solution could be present at different magnitudes in adsorbed protein layers however, the conformational changes measured upon adsorption could expose previously buried sites. In addition, orientation-specific interactions may be enhanced in adsorbed protein layers due to mobility restriction upon adsorption.

**PEGylation destabilizes rhIL-1ra conformation**

PEGylation destabilized rhIL-1ra by decreasing ΔG$_{unf}$ and modifying the protein’s tertiary structure in solution (Fig. S4.4 in Supplemental Results). Upon adsorption to the interface, PEG rhIL-1ra became more unfolded, resembling its urea-unfolded conformational state. Partially unfolded proteins are generally more prone to aggregation due to greater exposure of their otherwise buried hydrophobic sites, so unfolded proteins could have led to increased monomer loss during incubation (221, 237, 238). However, this was not observed, suggesting that PEG rhIL-1ra unfolding upon adsorption did not lead to increased aggregation. Instead, it is likely that PEGylation sterically prevented intermolecular protein interactions between rhIL-1ra molecules at the interface, thereby mitigating interfacial aggregation and monomer loss.

**Attractive protein-protein interactions are needed to form interfacial protein aggregates**

Attractive molecular interactions that favor protein-protein connections may result in aggregation. In solution, net attractive interactions were measured between rhIL-1ra monomers, whereas these interactions were shielded sufficiently in PEG rhIL-1ra so that protein-protein interactions resembled those of non-interacting hard-spheres. At the interface, the very high local concentration of protein led to a proximity effect that potentially amplified the role of protein-protein interactions (212). The formation of
ordered intermolecular β-sheet structures upon incubation with a silicone oil-water emulsion were much greater for rhIL-1ra than for PEG rhIL-1ra. This increase could be a result of attractive interactions between adsorbed rhIL-1ra proteins, similar to those measured in solution. However, because the conformation was altered for protein molecules adsorbed to the interface, other forces, not measured in solution, could also exist. Analogous to the hard-sphere behavior observed in solution, the PEGylation of rhIL-1ra potentially sterically limited the formation of intermolecular bonds at the interface, thereby preventing ordered intermolecular β-sheet structures from forming.

4.4.4 Structural characterization of interfacial aggregates

The secondary structure of rhIL-1ra adsorbed to silicone oil-water interfaces was drastically different from that of the native protein in solution; however, this difference was not observed for PEG rhIL-1ra adsorbed to silicone oil. We hypothesized that rhIL-1ra structural changes were due to intermolecular β-sheet structures that formed when rhIL-1ra adsorbed to the interface, based on analogous changes observed in previous studies. For example, Tanaka et al. (239) observed a similar spectrum change between myoglobin and aggregated myoglobin comprised of intermolecular antiparallel β-pleated sheet, and Yu et al. (240) also observed a strong increase in ellipticity for degraded rhIL-1ra incubated at 25°C for 60 days and concluded that it was due to intermolecular β-sheet structures from aggregated species. The similarity between the spectrum for PEG rhIL-1ra in solution and in the presence of a silicone oil-water interface revealed that no secondary structural changes occurred in PEG rhIL-1ra adsorbed to silicone oil, consistent with the absence of intermolecular β-sheet structures observed by ThT fluorescence.

Data from the fluorescence quenching of rhIL-1ra tryptophan residues further supported the presence of intermolecular β-sheet structures at the silicone oil-water interface. The decrease in $K_{SV}$ for rhIL-1ra adsorbed to silicone oil-water interfaces relative to $K_{SV}$ values in solution suggests that rhIL-1ra’s tryptophan residues became less accessible to the quencher after adsorption to the interface, potentially due to the burial of the tryptophan residue inside the intermolecular network during the formation of aggregate structures. Dusa et al. (241) noted a similar occurrence during aggregation of α-synuclein where a decrease in $K_{SV}$ was associated with the formation of fibrils due to the burial of tryptophan residues inside the fibrillar
network. In contrast, no secondary structural changes occurred for PEG rhIL-1ra, consistent with the absence of intermolecular β-sheet structures formed by PEG rhIL-1ra molecules adsorbed to silicone oil-water interfaces.

4.4.5 Stronger gels contribute to greater monomer loss upon mechanical interfacial stress

Sorret et al. previously showed that interface-induced protein aggregation in agitated siliconized syringes correlated with stronger interfacial viscoelastic protein gels (212). These stronger gels also had higher degrees of ordered intermolecular β-sheet cross-linking. In this study, we observed a similar effect, where rhIL-1ra formed gels that were roughly five times stronger than gels formed by PEG rhIL-1ra molecules. rhIL-1ra gels contained ordered intermolecular β-sheet structures whereas no ordered intermolecular β-sheet structures were observed for PEG rhIL-1ra gels, which provided an explanation for their relatively low strengths. Presumably, these weak gels formed as a result of other protein intermolecular interactions such as intermolecular hydrogen bonds or less-ordered intermolecular β-sheets that did not cause ThT fluorescence (57, 238, 242). This result was consistent with the hypothesis that PEGylation sterically interfered with rhIL-1ra’s capacity to form the multi-point attachments necessary to form extensive strong elastic gel networks. The weaker gels formed by PEGylated rhIL-1ra may have been less irreversibly aggregated, explaining the lower levels of aggregated protein molecules observed when these gels were mechanically perturbed by agitation. In contrast, the pieces of stronger gels formed by rhIL-1ra were more likely to remain aggregated after the shear forces in the agitated syringes stripped the gels from the interface.

Another (non-exclusive) explanation for the lower levels of aggregates seen after mechanical perturbation of PEGylated rh-IL1ra gels compared to rhIL-1ra gels is the time required for gel formation. Gelation was slower for PEG rhIL-1ra than for rhIL-1ra. For both proteins, mechanical perturbation of the interface resulted in removal of gels from the interface into the bulk, but the slower rate of gel formation for the PEGylated protein may have caused the characteristic time required for gels to re-form to be longer
than the characteristic time for agitation-induced surface renewal, leading to incomplete gelation and low aggregation levels.

4.5 Conclusions
This study demonstrated that the intermolecular forces measured in solution through $B_{22}$ measurements were predictive of interfacial aggregation behavior for rhIL-1ra and PEG rhIL-1ra. rhIL-1ra interfacial aggregation was driven by attractive intermolecular forces and PEGylation provided a steric barrier to aggregation despite PEG rhIL-1ra having a lower conformational stability and a disturbed tertiary structure at silicone-oil-water interfaces. rhIL-1ra attractive intermolecular interactions led to increased intermolecular $\beta$-sheet structures formed at the silicone oil-water interface and resulted in stronger intermolecular gels. When the interface was mechanically perturbed, these gels detached from the interface, resulting in increased monomer loss in solution. In contrast, PEGylation reduced the potential for PEG rhIL-1ra to form intermolecular bonds, resulting in only weak interfacial gels and lower levels of agitation-induced aggregation. These findings helped gain a better understanding on the forces responsible for the formation of interfacial gelled protein aggregates, therefore helping the advancement of aggregation mitigation strategies at interfaces.

4.6 Acknowledgements
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4.7 Supplemental Results

**Figure S4.1.** Silicone oil concentration standard curve. Each square point represents the area under the curve between 1280 and 1240 cm\(^{-1}\) of an infrared absorbance spectrum of silicone oil in hexane standard solution. The solid line represents the linear least square fit of the data. Error bars represent standard deviations from replicate measurements.

**Figure S4.2.** Particle size distribution of the silicone oil in water emulsion. Particle size was calculated assuming Mie Scattering from spherical particles using the value of 1.4046 for the refractive index of the silicone oil.
Figure S4.3. Stern-Volmer plots after incubation in 10 mM phosphate buffer, pH 6.5 for A) rhIL-1ra for 0 h (gray) and 24 h (black) and for B) PEG rhIL-1ra for 0 h (gray) and 45 h (black).
Figure S4.4. Tertiary structure comparison by near-UV CD spectra of rhIL-1ra (solid) and PEG rhIL-1ra (dashed) in 10 mM phosphate buffer, pH 6.5. Buffer spectra was subtracted for both prior to calculating molar ellipticity. Protein concentration was obtained from UV-vis spectrometer at 280 nm.
Chapter 5

Conclusions and future recommendations

5.1 Conclusions

Silicone oil, which is used as a lubricant or coating in pharmaceutical containers, has been implicated in the aggregation of therapeutic proteins. Upon adsorption to silicone oil-water interfaces, proteins can aggregate to form interfacial gels which upon mechanical disturbances can be transported into solution, thereby compromising the safety and efficacy of the protein-drug product. In bulk solution, protein aggregation occurs in part due to attractive protein-protein interactions (PPI). Numerous efforts are spent optimizing physical properties to minimize protein aggregation in solution (11, 13, 31, 217). As a result, multiple methods have been developed to measure the magnitude and nature of protein intermolecular interactions in solution. However, the analytical techniques employed to characterize PPI in solution are often not amenable to the characterization of proteins adsorbed onto interfaces. Therefore, knowledge is lacking regarding the forces governing protein stability adsorbed to interfaces, limiting the development of interfacial aggregation reduction strategies. In this work, we examined the role PPI measured in solution played on protein interfacial gelation and aggregation.

First, we investigated some of the limitations of using the interaction parameter \( k_D \) to measure PPI in solution. This parameter has gained favor as a more high-throughput means to quantify PPI over the more traditional osmotic second virial coefficient, \( B_{22} \). \( k_D \) can be obtained directly by dynamic light scattering (DLS) by measuring the dependence of molecular diffusivity on protein concentration. This parameter is often used to infer whether protein-protein interactions are repulsive or attractive, resulting in solutions that are colloidally stable or unstable, respectively. We investigated the influence of multicomponent diffusion in a ternary protein-salt-water system on protein diffusion and \( k_D \) in the context of Nernst-Planck theory. This analysis demonstrated that large changes in protein diffusivity with protein concentration resulted even for hard-sphere systems in the absence of protein-protein interactions. We demonstrated that, especially at low ionic strength, \( k_D \) values were dominated by electrophoretic effects.
rather than by PPI. Because $k_D$ reflects both hydrodynamic and thermodynamic effects on protein solutions, careful interpretation is needed if it is used to measure PPI and colloidal stability of protein formulations.

We then examined the role specific PPI played on protein aggregation at silicone oil-water interfaces. The model therapeutic protein used in this study was recombinant human interleukin-1 receptor antagonist (rhIL-1ra), a cytokine inhibitor, marketed in silicone oil-lubricated syringes. Orientation-specific attractive electrostatic forces dominated both the sign and magnitude of PPI for rhIL-1ra in solution, where attractive PPI dominated at low ionic strengths and their magnitude decreased with increasing ionic strength. This behavior contrasts with repulsive interactions that would be expected based on uniformly charged sphere models commonly applied to proteins in solution. We exploited the dominance of these PPI to determine how they affected rhIL-1ra viscoelastic properties and aggregation at the silicone oil–water interface. We showed that more attractive PPI correlated with stronger interfacial gels and that stronger gels correlated with increased intermolecular $\beta$-sheet content. In addition, the loss of monomeric rhIL-1ra in response to mechanical perturbation in siliconized syringes was highest under the conditions where the strongest gels were observed. Aggregation was thus highest where specific attractive interactions dominated, under low ionic strength conditions. These results demonstrate that specific electrostatic forces measured in solution can play an important role in contributing to interface-induced protein aggregation.

After showing that interfacial aggregation could be decreased by reducing attractive electrostatic forces between rhIL-1ra protein molecules, we demonstrated that interfacial aggregation could also be reduced by introducing repulsive steric interactions between proteins through the covalent addition of a 20 kDa polyethylene glycol (PEG) polymer to rhIL-1ra. PEGylation affected rhIL-1ra structural integrity, so as a result, we also examined the role of conformational stability on the interfacial aggregation propensity of rhIL-1ra and PEG rhIL-1ra. Mechanical interfacial perturbation in siliconized syringes containing rhIL-1ra or PEG rhIL-1ra resulted in the loss of soluble monomeric protein. However, the loss of rhIL-1ra was twice that for PEG rhIL-1ra, even though in solution, PEG rhIL-1ra had a lower Gibbs’ free energy of unfolding, and at the silicone oil-water interface it exhibited a more perturbed tertiary structure. Net PPI
for rhIL-1ra were attractive, but increased steric repulsion from PEGylation led to net PPI that were repulsive for PEG rhIL-1ra. Attractive intermolecular interactions for rhIL-1ra were associated with increase intermolecular β-sheet content for layers of adsorbed proteins at the silicone oil-water interface, whereas no intermolecular β-sheet structures were observed for adsorbed PEG rhIL-1ra. In addition, rhIL-1ra formed gels that were five times stronger than those formed by PEG rhIL-1ra. Thus, the steric repulsion introduced by the PEGylation of rhIL-1ra resulted in decreased interfacial gelation and in the reduction of agitation-induced aggregation, in spite of the destabilizing effects of PEGylation on the protein’s conformational stability. This study demonstrated that interfacial aggregation of rhIL-1ra was driven by attractive intermolecular forces and that PEGylation provided a steric barrier to aggregation at silicone-oil-water interfaces.

5.2 Future recommendations

The work presented here showed that interfacial aggregation could be decreased during interfacial mechanical disturbances by reducing attractive electrostatic interactions. This work was limited to studying the silicone oil-water interface, but additional work should be done in the future to continue to explore the effects of PPI measured in solution on interfacial aggregation as other liquid-liquid interfaces, such as phthalate-water interfaces have been shown to cause aggregation. It would be interesting to determine whether attractive intermolecular protein interactions govern gel formation and aggregation at that pharmaceutically-relevant interface as well. During formulation optimization, we therefore recommend taking into account the strength of interfacial gels as we demonstrated in the work that upon the disruption of gelled layers, aggregates can be transported back into solution. In particular, the availability of commercial interfacial shear rheometers make it convenient to study the properties of interfacial viscoelastic gels.

The role of PPI on inducing aggregation at solid surfaces, such as glass, cellulose, steel, and cyclic olefin copolymer (COP), should be explored as well to obtain a more comprehensive understanding of interfacial aggregation. Although gel strength at solid-water interfaces cannot be measured directly,
Thioflavin-T fluorescence assay could be used to determine levels of interfacial aggregation upon adsorption to these solid surfaces. In addition, front-face fluorescence should be used to measure tertiary structures associated with aggregated protein adsorbed by fluorescence measurements of intrinsic tryptophan residues.

We demonstrated that attractive PPI could be decreased by the covalent addition of a PEG polymer. Other post-translational modifications that increase protein stability in solution should also be explored. For example, protein glycosylation has been used to improve protein stability in vivo and in vitro (243). Yet, the role of glycosylation on protein interfacial aggregation has not been explored. Multiple sources have reported that increasing the degree of glycosylation increases protein colloidal stability by sterically inhibiting PPI (244–246). Therefore, these glycosylation events, similar to PEGylation, could provide steric barrier to aggregation.

Finally, it would be interesting to look at different site-directed mutations of rhIL-1ra to control the nature and magnitude of PPI without changing our protein model. Direct control of PPI through mutagenesis could help understand whether PPI modified in solution are also affected upon adsorption to interfaces. For rhIL-1ra, the replacement of lysine-93 with alanine by site-directed mutagenesis resulted in dramatically suppressed rhIL-1ra aggregation, potentially by eliminating rhIL-1ra single anion-binding site (198). This mutation would be a good starting point to determine whether a unique amino acid replacement that affected bulk solution protein intermolecular interactions could affect interfacial aggregation as well.
Bibliography


2053–2065.

Appendix

A.1 Dynamic light scattering parameters and sample data

Procedure
Follow Dr. Annette Erbse protocol.

Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample filtration</td>
<td>0.1 μm</td>
</tr>
<tr>
<td>Buffer filtration</td>
<td>0.02 μm</td>
</tr>
<tr>
<td>Concentration measurement</td>
<td>280 nm</td>
</tr>
<tr>
<td>Temperature</td>
<td>23°C</td>
</tr>
<tr>
<td>SOS</td>
<td>&lt; 3 for lysozyme, &lt; 2 for 3M</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Number of acquisition</td>
<td>≥ 8 per sample</td>
</tr>
<tr>
<td>Model</td>
<td>Globular protein (dn/dc 0.187)</td>
</tr>
<tr>
<td>Water properties</td>
<td>1.333 RI at 20°C; 1cP</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Measured</td>
</tr>
<tr>
<td>Laser power</td>
<td>Adjusted to keep counts around 300,000 cts/s</td>
</tr>
</tbody>
</table>

Example DLS data

Sample: 7 mg mL⁻¹ 3M protein in 5 mM KCl.

![Correlation curve](Image)
<table>
<thead>
<tr>
<th>Item</th>
<th>Time (s)</th>
<th>Intensity (Cnt/s)</th>
<th>R (nm)</th>
<th>Diam (nm)</th>
<th>%Pd</th>
<th>SOS</th>
<th>D (cm²/s)</th>
<th>Pd Index</th>
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<tbody>
<tr>
<td>Acq 1</td>
<td>12765.3</td>
<td>351864</td>
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<td>18</td>
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<td>Acq 5</td>
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<td>4.4415</td>
<td>8.88299</td>
<td>11.1</td>
<td>0.43</td>
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<tr>
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<td>8.86423</td>
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<tr>
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<td>8.91757</td>
<td>12.3</td>
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<td>2.30E-05</td>
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<tr>
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<th>%Mass</th>
<th>D (cm²/s)</th>
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<td>100</td>
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A.2 SEC-HPLC parameters and data analysis

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<td>0.5 mL/min</td>
</tr>
<tr>
<td>Run Time</td>
<td>30 min</td>
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<tr>
<td>Autosampler Temperature</td>
<td>4 °C</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>20 °C</td>
</tr>
<tr>
<td>Detection</td>
<td>280 nm</td>
</tr>
<tr>
<td>Injection Load</td>
<td>As described in methods</td>
</tr>
<tr>
<td>Injection Speed</td>
<td>200 µL/min</td>
</tr>
<tr>
<td>Draw position</td>
<td>3 mm</td>
</tr>
<tr>
<td>Needle Wash</td>
<td>water</td>
</tr>
<tr>
<td>Seal wash</td>
<td>Every 99 min for 1 min</td>
</tr>
<tr>
<td>Pressure limit</td>
<td>75 min</td>
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<tr>
<td>Flow</td>
<td>Isocratic</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>As described in methods</td>
</tr>
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Chromatogram examples

rhIL-1ra 1 mg mL\(^{-1}\) in 10 mM phosphate buffer, pH 6.5 (mobile phase: 0.1 M Na\(_2\)HPO\(_4\), 0.1 M Na\(_2\)SO\(_4\), pH 6.8)

Zoom-in and integration:

rhIL-1ra 1 mg mL\(^{-1}\) in 10 mM phosphate buffer pH 6.5 (mobile phase: 20 mM phosphate, 200 mM NaCl, pH 7.0 + 7% (v/v) acetonitrile)
Both integrated chromatograms show 1% dimer impurity in rhIL-1ra stock samples.

PEG rhIL-1ra 1 mg mL$^{-1}$ in 10 mM phosphate buffer pH 6.5 (mobile phase: 20 mM phosphate, 200 mM NaCl, pH 7.0 + 7% (v/v) acetonitrile)
Zoom-in and integration:

Comparison to rhIL-1ra (red) shows that peaks between 21-26 min are from unPEGylated rhIL-1ra:
A.3 Rheology set-up and calibration

Cleaning

1. Clean glass channel and trough with 2% micro90, water, ethanol, hexanes. Let air dry
2. Put glass channel and trough in hot nanostrip® solution under the hood (caution: read MSDS for proper handling of nanostrip®) for 2 hours
3. Rinse with ultrapure water then ethanol and let air dry
4. Clean and paraffin the magnetic rod
   a. Assure that the magnetic rod (inserted into a PTFE tube) has distinct banding prior to use
   b. Gently clean the magnetic rod with ethanol and wipe
   c. Heat paraffin and coat the ends of the magnetic rod in paraffin to seal the PTFE tube
   d. Measure and record magnetic rod parameters (weight and length)
   e. Magnetize the needle
      i. Place the magnetic rod inside the glass rod and place it within the colloid
      ii. Make sure the rod is centered in the channel otherwise channel might not be clean enough
      iii. Unplug the cords from power supply 0. Connect the red cord from the large coil to + on power supply and black on – on power supply.
      iv. Turn on power supply
      v. Magnetize the rod for 1h using labview virtual instrument “Magnetize for the rod”

Instrument calibration

1. Fill the channel with 40mL of buffer
2. Place the two combs so that the plate in the center stays up (not necessary)
3. Add appropriate amount of 5 cSt silicone oil to cover surface (~2.5 mL)
4. Float and center the magnetic rod on the oil-water interface between the two glass plates.
5. Set the power supplies to low (“LO”)
6. Run the labview virtual instrument Found under Lea-> methods-> buffer or found under: “00 Main Panel 0217”

7. Focus the camera on the magnetic needle. (see picture below) Determine the camera output by pressing continue and reviewing the signals.

8. Enter the rod length, and wavelength, also enter the channel diameter (width between glass plates 1 cm). Enter the appropriate wait time (2 min for calibration) on the Rod P screen and press start.

9. Select the oscillation experiment type (Osc), number 0, and press OK.

10. Check “show Pos?” so you can see the tracking of the rod live.

11. After approximately one hour, change the power supply from low (“LO”) to high (“HI”) power while the instrument is in wait mode and run for another hour.

12. Review the acquired files by running the virtual instrument “FileOpenerB” and importing the calibration data. Data should look like this:

   ![Calibration from Igor Pro software](image)
Experimental run

1. Run the labview virtual instrument “00 Main Panel 02F17” found under Lea -> methods -> protein.

2. Change the array for the imaging: In this file, click window -> show diagram-> third page click on make acq -> click window show diagram-> to see code -> change left most array from 0 to 6, to change it to 10 Volts.

3. Maintain the camera focus at the position established during calibration. Press the continue button followed by next to advance to the rod screen.

4. Enter the rod weight, length, and wavelength. Also enter the channel diameter (width between glass plates) then continue by pressing next.

5. Confirm the LS-15 power supplies are both configured to high (“HI”) power.
6. Enter the appropriate wait time on the Rod P screen and press start. 3 min if you are running experiment overnight since each file takes up a lot of space.

7. Select the oscillation experiment type (Osc) and number 1 and press OK.

8. Record the start time in your notebook.

9. Await conclusion of the first set of oscillations and then create a file to save the acquired data.

10. Allow rheometer to acquire data at least double the time required for gelation to occur

11. Data analysis:

   i. Review the files associated with both the calibration and experimental runs using the virtual instrument “SimplifiedFileOpenerB”

   ii. Press -> and select the .CO1 file

   iii. Sequentially view each file by advancing through a series using the forward button in the lower right corner of the screen.

   iv. Evaluate each file as “good” or “bad”. Should have clear sinusoidal pattern as shown below:
A.4 Intrinsic fluorescence of tryptophan sample data

Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument</td>
<td>SLM Aminco (JD-490, FP-099)</td>
</tr>
<tr>
<td>Software</td>
<td>Model 8100 spectrofluorometer</td>
</tr>
<tr>
<td>Configuration</td>
<td>Front-face</td>
</tr>
<tr>
<td>Monochromators</td>
<td>Excitation: 405 nm 4 nm bandpass</td>
</tr>
<tr>
<td>Emission scan</td>
<td>450-700 nm, 0.95 nm/sec, 1 sec integration</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>EmL, HV. See notebook for parameters but keep voltage &amp; gain constant between samples/run to be consistent</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioflavin T final concentration</td>
<td>120 μM</td>
</tr>
<tr>
<td>Sample volume</td>
<td>3 mL</td>
</tr>
<tr>
<td>Equilibration time</td>
<td>3 min</td>
</tr>
</tbody>
</table>

0.1 mg/mL rhIL-1ra in 10 mM phosphate buffer, pH 6.5, quenched by acrylamide 0 M – 0.3 M:
A.5 Making and characterizing SOE

Making silicone oil emulsion

1. Clean emulsifier with 2% micro 90 in water for two full chambers (tank pressure 100 psi)
2. Rinse with ultrapure water at least 3 full chambers (tank pressure 100 psi)
3. Add 138 mL ultrapure water + 12 mL 1000 cSt silicone oil (medical grade)
4. Shear for 15 min on dial 40 to mix oil and water well
5. Turn on tank to 120 psi
6. Do two passes from 1000-3000 psi reading from emulsifier large pressure gauge
7. Increase tank pressure to 130 psi
8. Do seven passes from 3000-8000 psi reading from emulsifier large pressure gauge
9. Let emulsion collected cool back down to room temperature and store at 4°C for up to 4 weeks

Determination of silicone oil concentration in emulsion

1. Dilute your emulsion 10x in hexane and vortex for 1 minute so you can see phase separation
2. Centrifuge for 1 h at 1200xg at room temperature and then vortex for another minute
3. Prepare standards:
   a. Make 50 mg/mL stock: 205 μL 1000 cSt silicone oil silicone oil + 3.759 mg/mL hexane
      i. **Caution:** Good dilution is very important, add hexane first making sure it does not drip and cap right away.
   b. Make 3.12 mg/mL and do serial dilutions until 0.099 mg/mL using hexane
4. Use FTIR in the Medlin lab, cell Harrick VariGATR
5. Purge FTIR with nitrogen for 30 min
6. Install ATR cell GAT_V N18
7. Use Omnic Software to collect data:
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td># scans</td>
<td>32</td>
</tr>
<tr>
<td>Resolution</td>
<td>4</td>
</tr>
<tr>
<td>Final format</td>
<td>Absorbance</td>
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<tr>
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</tr>
<tr>
<td>File handling</td>
<td>Check save interferograms</td>
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<td>4000-750</td>
</tr>
<tr>
<td>Atmospheric suppression</td>
<td>automatic</td>
</tr>
<tr>
<td>Collect background</td>
<td>ON</td>
</tr>
</tbody>
</table>

8. Measure background with 9 full drops of hexane on clean cell. Make sure sample has evaporated off the cell prior to take a measurement and that sample did not drip outside the cell
   a. Make sure background is at zero where silicone oil adsorbs (around 1260 cm\(^{-1}\))

9. Saturate cell with high concentration sample 3.12 mg/mL, making sure to shake sample prior to using

10. Can now collect adsorption data for standards and samples

*Example of analysis silicone oil standard on GRAMS software*

0.39 mg/mL silicone oil in hexane standard analysis on GRAM software

Zap from 1220 to 1300 and integrate from 1240-1280 cm\(^{-1}\) using multipoint leveled and zero baseline:
Silicone oil concentration standard curve

Each square point represents the area under the curve between 1280 and 1240 cm\(^{-1}\) of an infrared absorbance spectrum of silicone oil in hexane standard solution. The solid line represents the linear least square fit of the data. Error bars represent standard deviations from replicate measurements.

Particle size distribution using LS230 Laser Diffraction Particle Size Analyzer

1. Fill chamber of LS230 Beckman Coulter Laser Diffraction Particle Size Analyzer with ultrapure water
2. Add 0.5 mL micro90 to clean (fill then drain)
3. Repeat one more time with micro90 then rinse with ultrapure water three times
4. Set up computer for run:
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Details</th>
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<td>Measure offsets</td>
<td>Only at start of experiment</td>
</tr>
<tr>
<td>Align</td>
<td>Do for each sample</td>
</tr>
<tr>
<td>Measure background</td>
<td>Do for each sample</td>
</tr>
<tr>
<td>Measure loading</td>
<td>Do for each sample</td>
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<tr>
<td>Enter sample info</td>
<td>Do for each sample</td>
</tr>
<tr>
<td>Enter run settings</td>
<td>Do for each sample</td>
</tr>
<tr>
<td>Include PIDS data</td>
<td>Do for each sample</td>
</tr>
<tr>
<td>Measure background</td>
<td>90 seconds</td>
</tr>
<tr>
<td>Run length</td>
<td>90 sec</td>
</tr>
<tr>
<td>Optimal model</td>
<td>For silicone oil, select silicone.rf780d</td>
</tr>
<tr>
<td></td>
<td>where RI should be 1.405 and fluid 1.333</td>
</tr>
</tbody>
</table>

5. Measure background and record peak maximum in lab notebook (should be around \(10^5\))

6. Add sample (~400 μL) or until PIDS level is between 45-55%.
   a. Make sure obscuration level is below 2% before adding sample and that it stays below 6% at the end of run

7. Press DONE and copy over data

8. Drain and clean instrument

*Particle size distribution from LS230 example*
### Number statistics (Arithmetic) example

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<tr>
<td>Mean:</td>
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</tr>
<tr>
<td>Median:</td>
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</tr>
<tr>
<td>Q(3,2):</td>
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<tr>
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<td>S.D.:</td>
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<td>Variance:</td>
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<tr>
<td>Kurtosis:</td>
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</table>
A.6 Protein full mass analysis by mass spectrometry

*rhIL-1ra analysis*

Molecular weight spectrum for rhIL-1ra from MS ESI:

![Molecular weight spectrum for rhIL-1ra from MS ESI](image)

*PEG rhIL-1ra analysis*

The plot below demonstrates some dimers are present in the rhIL-1ra sample (as confirmed by SEC-HPLC).

![MaxEnt-derived MW spectrum](image)

It was not possible to do MS-ESI on PEG rhIL-1ra as shown below:
Zoom-in on rhIL-1ra peak in PEG rhIL-1ra sample demonstrates some unPEGylated rhIL-1ra in PEG rhIL-1ra sample (as confirmed by SEC HPLC).

Zoom-in on expected area where PEG rhIL-1ra should appear:
Results for PEG rhIL-1ra using MALDI are shown below. Results demonstrate the attachment of a 20kDa PEG to rhIL-1ra.

Zoom-in:
A.7 Peptide mapping using LTQ mass spectrometry

*rhIL-1ra theoretical sequence*

sp|P18510|IL1RA_HUMAN Interleukin-1 receptor antagonist protein OS=Homo sapiens GN=IL1RN PE=1 SV=1 (Uniprot database)

MRPSGRKSSKMQAIFRIWDVNQKTFYLRNNQLVAGYLQGPVNLEEEKIDVPIEPHALFLGIHGGKMCLSCVKGDETRLQEAVNITDLENRKQDKRFAIRSDSGPTTSFESAACPGLCTAMEADQPVSLTMMPDEGMVTKFYQED

Amgen product has an additional methionine at C-terminal.

*Sample preparation*

1. rhIL-1ra and PEG rhIL-1ra were reduced and unfolded overnight in 8M urea and 5 mM TCEP in 0.1M Tris pH 8.0 buffer at 0.5 mg mL⁻¹.

2. 100 µL aliquots of each sample were taken for alkylation and digestion.

3. The samples were diluted to 2M urea by adding 400 µL of 0.1 M Tris pH 8.0 to each sample to reduce urea concentration to 2M for optimal trypsin digestion.

4. 7.5 µL of 0.2 M iodoacetamide in water was added to each sample. The samples were incubated at 29 °C for 30 min while protected from light to allow the alkylation reaction to proceed to completion.

5. 20 µL of 0.1 mg/mL trypsin solution was added to each sample (1:25 trypsin:protein ratio) and incubated at 37 °C for 1 hr while protected from light to allow the digestion to proceed to completion.

6. A blank solution was simultaneously prepared with the samples by aliquoting 100 µL of the 8 M urea, 0.1 M Tris, 5 mM TCEP, pH 8.0 buffer and adding 400 µL of 0.1 M Tris pH 8.0. 7.5 µL of 0.2 M IAM was added and incubated at 29 °C for 30 min. 20 µL of 0.1 mg/ml trypsin was added and incubated at 37 °C for 1 hr.

7. Samples were immediately placed onto the HPLC autosampler set to 4 °C for injection onto LC-MS.
Methods

Instruments (Elion labs): LTQ-1 (Thermo LTQ XL MS, Data Analysis Software: Thermo XCalibur 2.2 SP1.48) EL-A8, HPLC-6 (Agilent 1290 binary pump, Data Analysis Software: ChemStation OpenLAB CDS Rev. C.01.07 SR3 [465]) EL-A27

Mobile phases: Mobile Phase A: 0.1% TFA in MilliQ water, Mobile Phase B: 0.1% TFA in ACN

Column: Waters Acquity BEH C-18, 2.1x 150 mm, 1.7 µm, 300 Å, Part: 186003687, Serial Number: EA990

Method Parameters

Method Name: 20180408_MAD_BEH_TrypMap_35C_IL1ra

<table>
<thead>
<tr>
<th>UPLC parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>0.2 mL/min</td>
</tr>
<tr>
<td>Run Time</td>
<td>45 min</td>
</tr>
<tr>
<td>Autosampler Temperature</td>
<td>5 °C</td>
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<tr>
<td>Column Temperature</td>
<td>35 °C</td>
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<tr>
<td>UV Detection</td>
<td>220 nm</td>
</tr>
<tr>
<td></td>
<td>280 nm</td>
</tr>
<tr>
<td>FLD Detection</td>
<td>Excitation: 292 nm</td>
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<tr>
<td></td>
<td>Emission: 350 nm</td>
</tr>
<tr>
<td>Injection Load</td>
<td>20 µL</td>
</tr>
<tr>
<td>Injection Speed</td>
<td>100 µL/min</td>
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<tr>
<td>Needle Wash</td>
<td>water</td>
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<table>
<thead>
<tr>
<th>Gradient</th>
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<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
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<td>35.0</td>
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<td>5</td>
</tr>
<tr>
<td>45.0</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

| Total Run Time | 45 min |
| Divert Valve   | 0 min - 5 min: to waste |
|                | 5 min – 32 min: to detector |
|                | 32 min – 45 min: to waste |

Results from UPLC

220nm detection:

All detections:
PEG IL-1ra FLD (ex 292 nm, em 350 nm)

PEG IL-1ra 220 nm

PEG IL-1ra 280 nm
Observations: There is no interference from blank spectra. Similar peaks appear for both rhIL-1ra and PEG rhIL-1ra, inferring they have similar degradation patterns. The fraction containing PEG seems to elute around 24 min (does not appear at 280 nm nor in fluorescence, but does show with absorbance at 220 nm). This peak is not present for rhIL-1ra. There is a peak at approximately four minutes for rhIL-1ra at 220 nm that is not appearing for PEG rhIL-1ra. A large peak appears between 19-24 min for both rhIL-1ra and PEG rhIL-1ra. This is potentially undigested peptides.
**Analysis**

Calculated experimental monoisotopic masses of observed peaks from UPLC for rhIL-1ra:

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Sample Name</th>
<th>Observed m/z (monoisotopic)</th>
<th>z</th>
<th>Experimental MW (monoisotopic)</th>
<th>Theoretical MW (monoisotopic) from GPMAW</th>
<th>Peptide Num from GPMAW</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>IL-ra Unfolded</td>
<td>703.32</td>
<td>1</td>
<td>702.31</td>
<td>702.36</td>
<td>1</td>
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<td>9.2</td>
<td>IL-ra Unfolded</td>
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<td>651.29</td>
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<td>9.9</td>
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<td>896.39</td>
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<td>10.2</td>
<td>IL-ra Unfolded</td>
<td>453.36</td>
<td>1</td>
<td>452.35</td>
<td>N/A</td>
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<tr>
<td>10.2</td>
<td>IL-ra Unfolded</td>
<td>498.16</td>
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<td>497.15</td>
<td>N/A</td>
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<tr>
<td>10.4</td>
<td>IL-ra Unfolded</td>
<td>902.44</td>
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<td>901.47</td>
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<tr>
<td>10.8</td>
<td>IL-ra Unfolded</td>
<td>781.30</td>
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<td>780.29</td>
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<td>11.9</td>
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<td>12.2</td>
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<td>977.30</td>
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<tr>
<td>12.5</td>
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<td>13.4</td>
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<td>14.0</td>
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<td>922.00</td>
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<td>2011.10</td>
<td>2011.13</td>
<td>8</td>
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</tbody>
</table>

Theoretical digested segments of rhIL-1ra using Trypsin (from GPMAW software)

Segments number 2, 12, 14, 3, and 13 m/z are too small to appear on the LTQ (range > 400 was used). The range should be decreased to 200 m/z in the future to encompass number 3 and 13. Segment 16 is too large to appear m/z > 4000. A different digestion method will be used to analyze the degradation of this segment. Further analysis on biopharma could not resolve the large peak observed between 19 min and 25 min (see boxed in data below). Trypsin only cleaves a peptide chain after lysine or arginine residues, except if followed by a proline residue. A different digestion method will be added to trypsin digestion
Theoretical rhIL-1ra peptide sequence and masses after treatment with Chymotrypsin enzyme (C-term to F/Y/W, not before P) when all cysteines have been treated with IAM (source: ExPASy). This enzyme will successfully cleave segments that could not be analyzed after Trypsin digestion alone (104-146 residue). It should be used alone, or in tandem with Trypsin to further analyze rhIL-1ra and PEG rhIL-1ra degradation.
<table>
<thead>
<tr>
<th>mass</th>
<th>position</th>
<th>#MC</th>
<th>artif.modification(s)</th>
<th>modifications</th>
<th>peptide sequence</th>
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<tbody>
<tr>
<td>4589.3384</td>
<td>59-99</td>
<td>0</td>
<td>Cys_CAM: 67, 70</td>
<td>4703.3814</td>
<td>LGIHGGKMCLSCVKSGDTRLQLEVNNLDSLSENFKQDKRF</td>
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<td>2627.3020</td>
<td>122-147</td>
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<td>Cys_CAM: 123</td>
<td>2864.3235</td>
<td>LCTAMEADQPVSITMPDEGLWVTKF</td>
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<td>2571.3816</td>
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<td>1167.5640</td>
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<td>820.3254</td>
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<td>166.0862</td>
<td>149-149</td>
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<td>F</td>
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</tbody>
</table>
A.8 Structural analysis by Circular Dichroism

Lysozyme

Tertiary structure for lysozyme in 5 mM KCl pH 6.0 as a function of lysozyme concentration. Black line indicate spectra at 1.5 mg mL\(^{-1}\) and gray line at 15 mg mL\(^{-1}\) protein concentration. Each curve represents the average of triplicates of ten scans each.

<table>
<thead>
<tr>
<th>Near-UV CD Parameters for lysozyme</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration determination</td>
<td>UV-vis 280nm</td>
</tr>
<tr>
<td>Background subtracted</td>
<td>Yes</td>
</tr>
<tr>
<td>Wavelengths</td>
<td>240-340nm</td>
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<tr>
<td>Time (s) per point</td>
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<tr>
<td>Step (nm)</td>
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</tr>
<tr>
<td>Voltage</td>
<td>&lt; 300 for 1mg/ml &lt; 700 15mg/ml</td>
</tr>
<tr>
<td>Repeats</td>
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<tr>
<td>Temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Smoothing</td>
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<tr>
<td>MW g/mol</td>
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<tr>
<td>MRW</td>
<td>111.773</td>
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<tr>
<td>Pathlength (cm)</td>
<td>0.05</td>
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<tr>
<td>Residues total</td>
<td>129</td>
</tr>
</tbody>
</table>

![Graph showing molar ellipticity vs. wavelength](image-url)
Monoclonal antibody:

Far-UV CD for the mAb in 2 mM KCl (dotted gray line), 10 mM KCl (long dash gray line), 10 mM NaAc (dashed gray line), and 90 mM KCl (black line), all at pH 6.0. Error bars not shown for clarity. Each curve represents the average of triplicates of ten scans each.

<table>
<thead>
<tr>
<th>Far-UV CD Parameters for mAb</th>
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<tbody>
<tr>
<td>Concentration determination</td>
<td>UV-vis 280nm</td>
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<td>Background subtracted</td>
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<tr>
<td>Wavelengths</td>
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<tr>
<td>Time (s) per point</td>
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<tr>
<td>Step (nm)</td>
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<tr>
<td>Voltage</td>
<td>&lt; 500</td>
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<tr>
<td>Repeats</td>
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</tr>
<tr>
<td>Temperature</td>
<td>Room temperature</td>
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<tr>
<td>Smoothing</td>
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<tr>
<td>Residues total</td>
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</table>
rhIL-1ra

Near-UV CD spectra of rhIL-1ra in 10 mM MES, pH 6.5 with 60 mM NaCl (solid) and 800 mM NaCl (dashed). Buffer spectra was subtracted for both ionic strength condition prior to calculating molar ellipticity. Each spectrum is an average of triplicate measurement of 10 scans each.

<table>
<thead>
<tr>
<th>Near-UV CD Parameters for rhIL-1ra</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration determination</strong></td>
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<tr>
<td>Background subtracted</td>
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<tr>
<td><strong>Wavelengths</strong></td>
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<tr>
<td>Time (s) per point</td>
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<tr>
<td>Step (nm)</td>
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<tr>
<td>MRW</td>
</tr>
<tr>
<td>Pathlength (cm)</td>
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<tr>
<td>Residues total</td>
</tr>
</tbody>
</table>

![Near-UV CD Spectra](image-url)
Tertiary structure comparison for rhIL-1ra and PEG rhIL-1ra

Near-UV CD spectra of rhIL-1ra (solid) and PEG rhIL-1ra (dashed) in 10 mM phosphate buffer, pH 6.5. Buffer spectra was subtracted for both prior to calculating molar ellipticity.