PARTICLES IN THERAPEUTIC PROTEIN FORMULATIONS – RELEVANCE TO
AIR-WATER AND SILICONE OIL-WATER INTERFACES

BY

PINAKI BASU

B. Pharm., Jadavpur University, 2002

M.S., University of Toledo, 2005

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This thesis for the Doctor of Philosophy degree by

Pinaki Basu

has been approved for the

Pharmaceutical Sciences Program

by

Michael F Wempe, Chair
John F. Carpenter, Advisor
LaToya Jones Braun
Krishna Mallela
Theodore Randolph

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ABSTRACT

Therapeutic protein drug products are used in the treatment and mitigation of human diseases for which no or very few alternative therapies are available e.g. Factor VIII, insulin, erythropoietin, interferon alpha, bevacizumab, etanercept etc. Siliconized prefilled syringes are often utilized as a primary container for therapeutic protein drug products. Despite efforts to maintain high product quality, the formation of unwanted particles is occasionally observed in therapeutic protein formulations in siliconized prefilled syringes. During the last few years, interest in sub-visible particles in therapeutic protein drug products has significantly increased due to its potential of eliciting an adverse immunogenic response. Consequently, evaluating the source and level of sub-visible particles in therapeutic protein drug products is important. In the studies that comprise my thesis, it was found that agitation of therapeutic protein formulations in the presence of siliconized beads accelerated the formation of sub-visible particles. Perturbation of the tertiary structure of therapeutic protein resulted from adsorption at the silicone oil-water interface. The addition of excipients, such as sucrose and sodium chloride, reduced tertiary structural changes. Lastly we found that rupturing the therapeutic protein layer at the air-water or silicone oil-water interface(s) accelerated the
formation of sub-visible particles in therapeutic protein formulations. Overall, the work described in this thesis demonstrates that assessment of sub-visible particles in therapeutic protein formulations advances our understanding of therapeutic protein stability in the presence of silicone oil droplets/siliconized interfaces.

The form and content of this abstract are approved. I recommend its publication.

Approved: John F. Carpenter
I dedicate this work to my fiancé and parents
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TABLE OF CONTENTS

CHAPTER

I.  INTRODUCTION ........................................................................................................... 1

II.  IgG₁ AGGREGATION AND PARTICLE FORMATION INDUCED BY SILICONE-WATER INTERFACES ON SILICONIZED BOROSILICATE GLASS BEADS: A MODEL FOR SILICONIZED PRIMARY CONTAINERS ................................................. 5

Introduction ....................................................................................................................... 5

Materials and Methods ...................................................................................................... 7

Materials ............................................................................................................................ 7

Methods ............................................................................................................................ 8

Glass Bead Siliconization .................................................................................................... 8

IgG₁ Sample Preparation ................................................................................................. 9

Agitation Studies ............................................................................................................... 9

Effect of Transient Exposure of IgG₁ Formulations to Silicone-Water Interface ................................................................. 11

Size Exclusion Chromatography .................................................................................... 12

Measurement of Sub-Visible Particles .......................................................................... 12

Results .............................................................................................................................. 13

Effect of Siliconized Beads on IgG₁ Aggregation during Orbital Agitation in pH 5.0 Buffer ........................................................................................................... 13

Buffered (Excipient-Free) Formulation .......................................................................... 13

Formulation with 0.01% Polysorbate 20. ....................................................................... 14

Formulation with 0.01% Polysorbate 20 and 0.5 M Sucrose. .................................. 14

Formulation with 150 mM Sodium Chloride ................................................................ 16

Formulation with 0.5 M Sucrose. .................................................................................. 18

Influence of pH on Aggregation Induced by Siliconized Beads ................................... 18
Effect of End-Over-End Rotation in pH 5.0 Buffer ......................... 20
Exposure of IgG₁ Formulations to Silicone-Water Interfaces in the Absence of Air-Water Interfaces ........................................... 25
Discussion .................................................................................. 25
Relevance of Studying Therapeutic Protein Interactions with the Interface between Water and Immobilized Silicone .................................. 25
Effects of Siliconized Beads and Agitation on IgG₁ Aggregation and Particle Formation ......................................................... 27
Effects of pH and Excipients on IgG₁ Aggregation and Particle Formation in the Presence of Silicone-Water Interfaces .................. 29
Utility of Siliconized Glass Beads as a Model to Study Protein Particle Formation due to the Silicone-Water Interface ...................... 30

III. ALBINTERFERON α₂b ADSORPTION TO SILICONE OIL-WATER INTERFACES: EFFECTS ON PROTEIN CONFORMATION, AGGREGATION AND SUB-VISIBLE PARTICLE FORMATION .................................. 32
Introduction .................................................................................. 32
Materials and Methods ................................................................. 34
Materials ..................................................................................... 34
Methods ...................................................................................... 35
Albinterferon α₂b Sample Preparation ........................................... 35
Silicone Oil-in-Water Emulsions .................................................... 36
Estimation of Total Interfacial Area of Silicone Oil Microdroplets in Aqueous Emulsion .......................................................... 36
Measurement of Albinterferon α₂b Adsorption onto Silicone Oil Microdroplets ................................................................. 37
Fluorescence Quenching ............................................................... 39
Light Microscopy ........................................................................ 40
Zeta Potential .............................................................................. 41
Siliconization of Glass Beads ....................................................... 41
Flow Microscopy. ................................................................. 65

IgG\textsubscript{1} Adsorption at the Air-Water Interface by Pendant Drop Tensiometry. ................................................................. 66

IgG\textsubscript{1} Gelation at the Air-Water Interface. ......................... 67

IgG\textsubscript{1} Adsorption on Silicone Oil Droplet. ......................... 68

IgG\textsubscript{1} Adsorption on Immobilized Silicone Interface. ............... 68

Electrostatic Potential of IgG\textsubscript{1}. ......................................... 70

Results ........................................................................................................... 70

Interface Rupture Experiment .......................................................... 70

Air-Water Interface .................................................................................... 70

Silicone Oil-Water Interface ...................................................................... 71

IgG\textsubscript{1} Adsorption at Air-Water Interface .................................... 74

IgG\textsubscript{1} Gelation at the Air-Water Interface .................................. 75

IgG\textsubscript{1} Surface Charge ................................................................. 77

IgG\textsubscript{1} Adsorption at Silicone Oil-Water and Immobilized Silicone-Water Interfaces ................................................................. 77

Discussion ................................................................................................. 78

Conclusions ............................................................................................... 83

V. CONCLUSIONS AND RECOMENDATIONS ............................................. 85

REFERENCES .......................................................................................... 88
LIST OF TABLES

Table

II.1 Particle concentrations of orbitally agitated and stationary IgG samples in 10 mM sodium acetate buffer pH 5.0, in the presence and absence of siliconized beads. .......... 17

II.2 Particle concentrations of orbitally agitated and stationary IgG samples in 10 mM sodium acetate pH 5.0, in the presence or absence of siliconized beads. ....................... 19

II.3 Particle concentrations of orbitally agitated and stationary IgG samples in 10 mM sodium phosphate buffer pH 7.4, in the presence and absence of siliconized beads. ....... 21

II.4 Particle Concentrations of End-on-End Agitated IgG Samples in 10 mM Sodium Acetate Buffer (pH 5.0), in the Presence or Absence of Siliconized Beads and the Presence ........................................................................................................................................ 24

II.5 Particle concentrations of end-on-end agitated IgG samples in 10 mM sodium acetate buffer pH 5.0, in the presence or absence of siliconized beads and the presence or absence of headspace (partially filled). ........................................................................................................ 26

III.1 Adsorption footprint of albinterferon α2b on silicone oil in 10 mM sodium phosphate pH 7.2 buffer in different formulations. ............................................................ 46

III.2 Zeta potential (δ) of silicone oil droplets, native albinterferon α2b and silicone oil adsorbed albinterferon α2b in different formulations. .......................................................... 49

IV.1 Gelation time and elastic modulus of IgG layer at the air-water interface. ......... 75

IV.2 Adsorption loading and footprint of IgG at silicone oil-water interface............ 78
LIST OF FIGURES

Figure

II.1 Levels of IgG₁ monomer, dimer and soluble aggregates in 10 mM sodium acetate pH 5.0 buffer during orbital agitation at 4°C................................................................. 15

II.2 Levels of IgG₁ monomer and dimer in 10 mM sodium phosphate pH 7.4 buffer during orbital agitation at 4°C. ................................................................. 22

II.3 Levels of IgG₁ monomer, dimer and soluble aggregates present in 10 mM sodium acetate pH 5.0 buffer during end-on-end rotation at room temperature. .................. 23

III.1 Panel A shows the adsorption isotherm of albinterferon α₂b on silicone oil and Panel B shows the adsorption footprint of the same.................................................. 45

III.2 Acrylamide quenching of albinterferon α₂b in the presence and absence of silicone oil droplets in different formulations in 10 mM sodium phosphate pH 7.2 buffer........ 47

III.3 Stern-Volmer quenching constant (K_{SV}) of native (dotted bar) and silicone oil containing (checkered bar) albinterferon α₂b samples in different formulations........... 48

III.4 Optical microscopy showing silicone oil droplet coalescence in different formulations of 3 mg/mL albinterferon α₂b solution......................................................... 50

III.5 Size exclusion chromatography results showing soluble monomer and dimer percent of albinterferon α₂b upon agitation in the presence and absence of 200 µg silicone oil. ........................................................................................................ 53

III.6 Size exclusion chromatography results showing monomer and dimer levels of albinterferon α₂b in vials rotated end-over-end in the presence and absence of 50 mg siliconized glass beads. ......................................................... 55

III.7 Binned sub-visible particle concentration of albinterferon α₂b samples agitated in the presence and absence of siliconized beads for seven days. ....................... 56

IV.1 Schematic diagram of the experimental set-up for interface rupture. .............. 64

IV.2 Illustrative figure showing the real-time acquisition of protein particles and silicone oil droplets in IgG₁ samples by resonant mass measurement.......................... 66

IV.3 Concentration of particles in IgG₁ samples at pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations........................................................................................................ 72

IV.4 Concentration of particles in IgG₁ samples at pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations........................................................................................................ 73
IV.5 Percent monomer remaining in IgG₁ samples from air-water interface rupture (Panel A) and silicone oil-water interface rupture (Panel B) experiments. ............................ 74

IV.6 Interfacial tension of IgG₁ samples at the air-water interface at pH 5.0 (green line) and pH 7.4 (red line) formulations. ............................................................................. 76

IV.7 Representative plot of elastic (G’ in red) and viscous (G” in blue) modulus of IgG₁ at air-water interface in pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations. ............ 76

IV.8 Surface charge distribution of IgG₁ at pH 5.0 (Panel A) and pH 7.4 (Panel B) formulations. .............................................................................................................. 77

IV.9 Adsorption footprint of IgG₁ on silicone oil droplets in pH 5.0 (open square) and pH 7.4 (open triangle) formulations................................................................. 79

IV.10 Adsorption breakthrough curve IgG₁ in pH 5.0 (green) and pH 7.4 (red) formulations (Panel A). ........................................................................................................... 80
CHAPTER I

INTRODUCTION

Therapeutic protein drug products are used in the treatment and mitigation of human diseases. The primary containers utilized for therapeutic protein formulations are many e.g. vial, prefilled syringes, dual-chambered syringes, cartridges, auto-injector pens, etc. The primary containers consist of components that are often made of different materials. Consequently, a therapeutic protein formulation can be in contact with different surfaces of the primary container.

During the last few years, particles in therapeutic protein drug products have been subjected to increased scrutiny by regulatory agencies. An important reason for increased scrutiny can be attributed to concerns regarding the potential of particles in therapeutic protein products to elicit an adverse immunogenic response. Typically, the particles in therapeutic protein formulations are from the primary container (e.g. glass particles, silicone oil droplets) or proteinaceous particles. Studies assessing the source, level and nature of particles in therapeutic protein formulations are few in literature.

Particle formation and protein aggregation in siliconized prefilled syringes is of particular interest. The glass barrel interior of siliconized prefilled syringes contains a layer of immobilized silicone oil that facilitates plunger movement. Occasionally, particle levels in therapeutic protein formulations contained in siliconized prefilled syringes are high. The increase in particle level is thought to be due to sloughing of silicone oil droplets from the syringe barrel into therapeutic protein formulations.

A therapeutic protein formulation in siliconized prefilled syringe remains in contact with the immobilized silicone layer of the syringe barrel. The silicone oil
interface in a prefilled syringe can be the immobilized silicone oil on syringe barrel or silicone oil droplets that sloughs into protein formulations. Agitation of therapeutic protein formulations with silicone oil droplets has been reported to accelerate aggregation. But the effect of agitation on particle formation and aggregation due to immobilized silicone interface is not known. In Chapter II, siliconized glass beads were utilized to mimic immobilized silicone interface in therapeutic IgG₁ formulations. Formulation screening was performed to evaluate the effect of excipients on sub-visible particle formation in IgG₁ samples containing siliconized beads. It was found that sub-visible particle counts in IgG₁ samples increased due to protein aggregation in agitated samples containing siliconized beads. Formulation excipients significantly affected IgG₁ aggregation and particle formation. Compared to pH 5.0, IgG₁ aggregation and particle formation was accelerated at pH 7.4. Monitoring sub-visible particles was found to be a sensitive method to evaluate aggregates in IgG₁ formulations.

Silicone oil layer on the barrel of a prefilled syringe may slough into a therapeutic protein formulation and form silicone oil droplets. Consequently counts of sub-visible particles in a therapeutic protein formulation may increase in a prefilled syringe. Agitation of therapeutic protein formulations in the presence of silicone oil droplets has been reported to accelerate aggregation. Because the use of siliconized beads to study protein aggregation in a pharmaceutically relevant application was not known, comparison of therapeutic protein aggregation during agitation of protein samples in the presence of either immobilized silicone interface or silicone oil droplets are not known.

In Chapter III, aggregation of a therapeutic fusion protein, albinterferon α₂b, was separately evaluated in samples containing siliconized beads or silicone oil droplets.
Albinterferon $\alpha_{2b}$ aggregation in buffer-only sample was accelerated upon agitation in the presence of silicone oil droplets. Levels of sub-visible particles were also higher in an identical formulation upon agitation with siliconized beads. Aggregate levels between albinterferon $\alpha_{2b}$ samples agitated with silicone oil droplets or immobilized silicone interfaces were found to be significantly different. Inhibition of albinterferon $\alpha_{2b}$ adsorption on silicone oil droplets and an increase in conformational stability of albinterferon $\alpha_{2b}$ coincided with reduced aggregation in 0.01% (w/v) polysorbate 80 and 300 M sodium chloride formulations respectively. This study adds to the growing body of evidence that agitation of therapeutic protein formulations in the presence of silicone oil interface accelerates aggregation and particle formation.

The study described in Chapter IV expands our understanding of silicone oil-water interface in accelerating particle formation in therapeutic protein formulations. In this study, rupture of IgG$_1$ layer at the air-water or silicone oil-water interfaces accelerated particle formation in therapeutic IgG$_1$ samples. The importance of assessing the rate of IgG$_1$ adsorption and gelation at the air-water interface in context to particle formation in IgG$_1$ samples was demonstrated in this study. Like previous observations, results obtained from Chapter IV showed that assessment of sub-visible particles in therapeutic protein samples is a sensitive measure of detecting aggregates.

From the results of Chapter IV, we found that sample pH significantly affected particle formation in IgG$_1$ samples. During rupture of air-water interface, particle formation was relatively faster at pH 7.4 than at pH 5.0. The difference in the relative particle levels formed in pH 5.0 and pH 7.4 samples during interface rupture was attributed to relative difference in the electrostatic surface potential of IgG$_1$. 
The utility of adsorption breakthrough curves in assessing IgG\textsubscript{1} the rate and extent of IgG\textsubscript{1} adsorption at immobilized silicone-water interface was introduced in Chapter IV. The shape of the adsorption breakthrough curve of IgG\textsubscript{1} were found to be significantly different at pH 5.0 and pH 7.4 samples. The shape of the adsorption breakthrough curve was used to assess IgG\textsubscript{1} interaction at the immobilized silicone–water interface. The result suggested that interaction of IgG\textsubscript{1} with the immobilized silicone-water interface was significantly different in pH 5.0 and pH 7.4 samples.

The work described in this thesis demonstrated that understanding therapeutic protein interaction at the air-water or silicone oil-water interfaces is important in context to particle formation in therapeutic protein formulations. These interfaces are pharmaceutically relevant interfaces that can potentially interact with the therapeutic protein formulations during transport and storage. The work described in this thesis suggests that therapeutic protein adsorption at air-water or silicone oil-water interfaces and protein association rates in the adsorbed layer affect sub-visible particle formation in therapeutic protein formulations.
CHAPTER II

IgG₁ AGGREGATION AND PARTICLE FORMATION INDUCED BY SILICONE-WATER INTERFACES ON SILICONIZED BOROSILICATE GLASS BEADS: A MODEL FOR SILICONIZED PRIMARY CONTAINERS¹

Introduction

Particle formation in therapeutic protein products is a significant concern for product quality. ³⁻⁵ In prefilled syringes, formation of particles in protein formulations has been linked to the presence of silicone oil that is used as a lubricant on barrels and stoppers.⁶,⁷ Silicone oil sloughs off into the solution,⁸ and protein may adsorb to the resulting silicone oil droplets.⁹ As a consequence of adsorption to silicone oil-water interface, proteins may aggregate.⁶,¹⁰,¹¹ Additionally it has been reported that the neutralization of surface charge on silicone oil microdroplets upon protein adsorption can lead to agglomeration of the droplets.⁹

Silicone oil-induced protein aggregation can vary depending on the protein’s properties, formulation conditions, and the presence of additional stresses such as agitation.⁶,⁹,¹¹ In order to develop formulations that optimize the stability of a given therapeutic protein in prefilled syringes during transport and storage, relevant accelerated stability studies are needed.

Previously, robustness of the formulation to silicone oil was tested by spiking protein formulations with silicone oil emulsions and monitoring protein aggregation.¹⁰,¹¹ However, detection and quantification of subvisible proteinaceous particles in the

presence of silicone oil droplets are challenging because it is difficult to distinguish silicone oil microdroplets from protein particles using particle counting instrumentation. Attempts to distinguish between proteinaceous particles and silicone oil droplets have been made with microflow imaging data using optical filters, but the approach is not effective for particles less than about 5 µm in diameter.\textsuperscript{12}

Furthermore, in addition to the impact of silicone oil droplets, the effects of the silicone oil-water interface on protein stability must take into account the surface of the silicone layer remaining on the glass surface of the syringe. Syringes are siliconized by either spraying pure liquid silicone oil on the interior surfaces of the syringe barrel, or, alternatively, by baking on a layer of oil that is sprayed as an emulsion of oil in water. The latter treatment provides a silicone layer that adheres to the glass surface and is far less prone to sloughing off into the solution\textsuperscript{13}. However, the protein will still be exposed to the baked-on silicone-water interface. In the current study, we evaluated the propensity of an IgG\textsubscript{1} to aggregate and to form subvisible particles in the presence of an immobile silicone-water interface. As a model for this interface, we used covalently siliconized borosilicate glass beads to provide an immobilized silicone-water interface of known surface area.\textsuperscript{14} Because the silicone is covalently attached to the glass surface, this system avoids the potential interference caused by silicone oil droplets during subvisible particle counting.

In this study, we examined the effect of immobilized silicone-water interfaces under static and conditions of agitation on IgG\textsubscript{1} aggregation and subvisible particle formation. In addition, the effects of key formulation variables such as solution pH, the presence of a nonionic surfactant and the addition of sucrose were investigated.
Aggregation and loss of monomeric protein were quantified by size exclusion chromatography (SEC), and particles were sized and counted by microflow imaging (MFI).

**Materials and Methods**

**Materials**

Highly purified antistreptavidin IgG was provided by Amgen Inc. (Thousand Oaks, CA) in a stock formulation containing 30 mg/ml protein, 5% sorbitol and 10 mM sodium acetate, pH 5. Non-porous borosilicate glass beads (1 mm diameter, catalog # Z273619; ≤ 106 µm diameter, catalog # G8893) were purchased from Sigma Aldrich (St. Louis, MO). Siliconizing agent Surfasil® and Pierce Slidealyzer® (product# 66012) with 20kDa molecular weight cutoff were purchased from Thermo Scientific (Rockford, IL). Buffer salts were procured from Fisher Scientific (Fair Lawn, NJ). Buffer solutions were prepared in deionized MilliQ® water. Polysorbate 20 was purchased from Croda Inc. (Edison, NJ). Endotoxin-free sucrose was from Ferro Pfanstiehl (Waukegan, IL). Presterilized 2 and 3 cc Type 1 borosilicate glass vials and silicone-free Westar® stoppers were purchased from West Pharmaceutical Services (Lionville, PA). Barrier tips (1 mL) were from Neptune (catalog# BT1250, San Diego, California) and were used to pipette samples for subvisible particle analysis.
Methods

**Glass Bead Siliconization.** Glass beads were siliconized using a modified version of previously published protocols.\textsuperscript{14,15} For larger sized glass beads, the beads were incubated in 4N hydrochloric acid for one hour to remove surface contaminants, rinsed thoroughly with doubly-distilled water and dried overnight at 50°C. Surfasil® (1 mL) was diluted in 10 mL of HPLC-grade acetone and added to 10 g of dry glass beads contained in a 50 mL Pyrex® beaker. The sample was stirred continuously with a glass stir rod during the addition of the siliconizing fluid. The beads were kept in the Surfasil®-acetone solution overnight at room temperature. The solution was decanted, and the beads were rinsed five times with 10 mL of acetone with a gentle swirling motion to remove unreacted Surfasil®. Then the siliconized beads were washed five times with 10 mL of HPLC-grade methanol and dried at 50°C for 30 minutes. After drying, the siliconized beads were washed three times by swirling with 10 mL MilliQ® water, after which they were again dried overnight at 50°C. The siliconized beads were stored at 4°C in a sealed container until use.

The smaller-sized glass beads (≤106 µm) were siliconized in a similar fashion as described above. The siliconization step was performed by the addition of a 20 mL solution (1 part Surfasil® in 3 parts acetone) into 20 g beads that were incubated overnight at room temperature. The excess solution was decanted, and the siliconized beads were rinsed with excess of acetone and methanol respectively followed by overnight drying at 50°C. The siliconized beads were stored at 4°C in a sealed container until use.
**IgG₁ Sample Preparation.** Stock IgG₁ solution was dialyzed overnight at 4°C against formulation buffer (either 10 mM sodium acetate, pH 5, or 10 mM sodium phosphate, pH 7.4) in Pierce® 20 kDa molecular weight cutoff dialysis cassettes. Dialysis of stock IgG₁ solution was also carried out in either pH 5 or 7.4 buffer in the presence of 0.5 M sucrose or 150 mM NaCl. The concentration of IgG₁ in the solution following dialysis was determined by measuring the UV absorbance using an Agilent® 8540 spectrophotometer (Santa Clara, CA) and an extinction coefficient of 1.54 mL/mg-cm at 280 nm. The solutions were then diluted to an IgG₁ concentration of 1 mg/mL using appropriate aliquots of filtered (0.22 µm) buffers. For the preparation of formulations containing polysorbate 20, an aliquot of freshly prepared stock of 10% (w/v) polysorbate 20 was added to the dialyzed IgG₁ solutions to achieve a final surfactant concentration of 0.01% (w/v). This solution was diluted to an IgG₁ concentration of 1 mg/mL using appropriate buffer containing 0.01% (w/v) polysorbate 20.

Using a spatula, siliconized beads (50 mg of 1 mm diameter in size) were added to 3 cc vials (for orbital agitation) or 2 cc vials (for end-over-end rotation). Control samples were prepared in a similar fashion with the omission of glass beads. In all cases, filling and closure (Westar® 13 mm stoppers) of vials were performed in a laminar flow hood to minimize exogenous particle contamination. Vial stoppers were crimped with aluminum seals.

**Agitation Studies.** IgG₁ aggregation and particle formation after agitation in vials was monitored in samples prepared with and without siliconized beads. Two different
agitation methods were employed: orbital shaking and end-over-end rotation. An orbital shaker (VWR® Model 3600 ADV Orbital shaker, catalog # 89032-096, Radnor, PA) was used for orbital agitation studies whereas a rotator (American Equipment Exchange®, Rotmix Model No: RKVSD, Haverhill, MA) was used for the end-over-end rotation studies.

For orbital agitation, 1 ml samples were placed in 3 cc glass vials in the presence or absence of 50 mg of siliconized beads. Vials were placed upright in secure-fitting 7x7 box dividers within cardboard boxes, which in turn were taped to an orbital shaker. Samples were agitated at 4°C and 350 rpm. We chose this agitation speed based on a previous study that reported 350 rpm was sufficient to cause accelerated IgG \(^1\) degradation in samples containing silicone oil droplets\(^{11}\). Three vials were removed on each of days 1, 3, 5, 7, 14, 21 or 28, and samples were analyzed using size exclusion chromatography (see below) for levels of monomer, dimer and soluble aggregates. Samples were analyzed for subvisible particle concentrations by microflow imaging (see below).

For end-over-end rotation, 1 mL aliquots of IgG \(^1\) formulations were placed in 2 cc vials with or without 50 mg of siliconized beads. With this volume of solution in the vials there was headspace and an associated air-water interface. To examine the effects of the absence of an air-water interface, another set of vials were completely filled with IgG \(^1\) formulations so that there was no headspace. Vials were agitated end-over-end at a speed of 20 rpm on the rotator at room temperature. Three vials were removed on each of days 1, 3, 5 and 7, and samples were analyzed for protein monomer and aggregates with size exclusion chromatography (SEC) and for particles with microflow imaging.
**Effect of Transient Exposure of IgG₁ Formulations to Silicone-Water Interface.** Approximately 1.6 g of siliconized beads (≤ 106 µm) in acetone was packed into an empty TSK guard column (part# 13075, Tosoh Bioscience LLC, Montgomeryville, PA). The packed column was washed with HPLC-grade water at a flow rate of 1 mL/min for ten minutes using an Agilent® 1100 HPLC pump (Santa Clara, CA). After washing the column with water, a Masterflex® peristaltic pump (Cole Parmer®, Illinois) with platinum-cured silicone tubing (catalog# 96420-13) was used to pump sodium acetate or sodium phosphate buffer through the column at 0.1 mL/min for fifteen minutes. PEEK tubing and fittings were used to connect peristaltic pump tubing to the siliconized bead column. For each sample, the column was freshly packed with siliconized beads and new pump tubing was used.

Following equilibration of the column with buffer solution, an IgG₁ sample (0.5 mg/mL, in either pH 5 or pH 7.4 buffer, with and without 0.01% polysorbate 20) was passed through the column at a flow rate of 0.1 mL/min for six minutes at room temperature. The residence time of the column was approximately 3.5 minutes.

Eluate from the column was collected in individually-wrapped polypropylene Falcon tubes (Fisher Scientific ® Fair Lawn, NJ catalog #352196). The samples were analyzed for subvisible particles by MFI (see below). As a control, buffer solution (no IgG₁) was passed through the siliconized bead column and analyzed for subvisible particles. IgG₁ formulations were also pumped through an identical set-up in the absence of the siliconized beads column and analyzed for subvisible particles.
**Size Exclusion Chromatography.** SEC was used to determine the relative levels of IgG\(_1\) monomer, dimer and soluble aggregates. Guidelines of terminologies to describe therapeutic protein aggregates has been reported in literature.\(^{16}\) In the current study, high molecular weight IgG\(_1\) aggregate (≤ 0.2 µm) detected by SEC was termed as soluble aggregate. SEC analysis was performed on an Agilent® 1090 HPLC system using a TSK-GEL G3000SWXL (Tosoh Bioscience LLC, Montgomeryville, PA) column and a 0.2 micron prefilter. Prior to SEC analysis, samples were centrifuged in 500 µL polypropylene centrifuge tubes at 12,000 g for fifteen minutes using an Eppendorf® bench top centrifuge (Centrifuge Mini Spin®, ColeParmer®). Ten µL of the supernatant was injected onto the SEC column. The IgG\(_1\) was eluted using 100 mM sodium phosphate, 300 mM NaCl, pH 7 buffer as the mobile phase, which was pumped at a flow rate of 0.6 ml/min. The UV absorbance of the eluting IgG\(_1\) was detected at 215 nm. The percentages of IgG\(_1\) monomer, dimer and soluble aggregates were determined from peak areas of the SEC chromatograms relative to those for untreated IgG\(_1\) solution.

**Measurement of Sub-Visible Particles.** Insoluble protein aggregates detected by MFI were termed as sub-visible particles. The levels of subvisible particles (≥1 µm) in IgG\(_1\) formulations were quantified using microflow imaging (MFI, Model # DPA 4100, Protein Simple®, Santa Clara, CA) with MFI View application software (version 6.9.8). The instrument was configured in the set point 3 mode (low magnification and open aperture settings with 100 µm flow cell, part number 4002-002-001). The background illumination of the MFI instrument for a given sample was optimized using a corresponding IgG\(_1\)-free buffer solution. For sample loading into the MFI instrument,
samples (500 µl) were pipetted out of the vial using a Neptune barrier pipette tip. A volume of 150 µL was allowed to flow through the MFI flow cell before particle counts were obtained. Total protein mass in particles was estimated based on particle counts and equivalent circular diameters, as previously described.\textsuperscript{17}

\section*{Results}

\textbf{Effect of Siliconized Beads on IgG\textsubscript{1} Aggregation during Orbital Agitation in pH 5.0 Buffer}

\textbf{Buffered (Excipient-Free) Formulation.} Orbital agitation of the IgG\textsubscript{1} formulation with siliconized beads caused extensive protein aggregation, with 80\% of the monomer converted to soluble and insoluble aggregates (Figure II.1, Panels A1-A3). In contrast, agitation of samples in the absence of siliconized beads, or incubation of unagitated samples with siliconized beads resulted in minimal conversion of IgG\textsubscript{1} monomer into aggregates.

Increasing levels of particles were observed during agitation of samples with siliconized beads (Table II.1). Following five days of orbital agitation, the average level of particles ≥1 µm diameter reached 870,000 particles/mL, which surpassed the maximum limit of the MFI instrument. In contrast, protein-free control solutions of 10 mM sodium acetate pH 5 buffer agitated for 7 days with siliconized beads did not show a substantial increase in subvisible particles (Table II.1). Orbital agitation of IgG\textsubscript{1} formulation in the absence of siliconized beads did not lead to significant increase in the subvisible particle concentrations, even after 28 days. Similarly, incubation of IgG\textsubscript{1} with
siliconized beads in the absence of agitation did not lead to observable increase in subvisible particle counts.

**Formulation with 0.01% Polysorbate 20.** In agitated samples containing siliconized beads, less IgG₁ aggregation was observed in the formulation containing 0.01% polysorbate 20 compared to IgG₁ aggregation in excipient-free buffered formulation. Initially, agitation of samples for 7 days with siliconized bead did not cause a loss of IgG₁ monomer (Figure II.1, Panel B1). After 28 days of agitation, 34% monomer was converted to aggregates. Agitation of samples in the absence of siliconized beads or stationary incubation of samples with siliconized beads did not cause IgG₁ aggregation.

Compared to the results for the formulation without excipients, the formation of particles during agitation with siliconized beads was significantly inhibited in formulations containing polysorbate 20. The concentrations of particles of size ≥1 µm in samples agitated with siliconized beads increased from 6000 particles/mL at Day 0 to 550,000 particles/mL at Day 7 and 320,000 particles/mL at Day 14 (Table II.1). Agitation for 28 days increased the particle concentration past the upper quantification limit of the MFI instrument. In contrast, samples agitated without siliconized beads for up to 28 days showed no increase in counts of subvisible particles (Table II.1).

**Formulation with 0.01% Polysorbate 20 and 0.5 M Sucrose.** Agitation with siliconized beads in sucrose and polysorbate resulted in a comparable loss of IgG₁ monomer to that observed in 0.01% polysorbate 20 formulations.
Figure II.1 Levels of IgG₁ monomer, dimer and soluble aggregates in 10 mM sodium acetate pH 5.0 buffer during orbital agitation at 4°C.

Treatments were: IgG₁ samples agitated without beads (open circle); IgG₁ stationary samples containing 50 mg siliconized beads (open triangle); and IgG₁ samples agitated with 50 mg siliconized beads (open square). The formulations conditions represented in the different panels are: Panel A, Buffered (Excipient-free); Panel B, 0.01% PS 20; Panel C, 0.01% PS 20 + 0.5 M sucrose; Panel D, 150 mM NaCl; and Panel E, 0.5 M sucrose. Values presented are mean ± SD for three independent replicate vials. Labels 1, 2 and 3 denote percentages of IgG₁ monomer, dimer and soluble aggregates in IgG₁ samples.
Following 28 days of agitation, approximately 36% of monomer was converted to aggregates (Figure II.1, Panel C). Significant IgG₁ aggregation was not observed during agitation without siliconized beads or stationary incubation with siliconized beads (Figure II.1, Panel C1).

The formulation containing 0.01% polysorbate 20 and 0.5 M sucrose was the only condition in which subvisible particle concentration in samples agitated with siliconized beads could be measured through 28 days without exceeding the maximum limit of the MFI instrument. Following 28 days of agitation with siliconized beads, the average concentration of subvisible particles of size ≥1 µm was 130,000 particles/mL (Table II.1). There was not a significant increase in the counts of subvisible particles in the stationary samples that were incubated with siliconized beads or in samples agitated without siliconized beads.

**Formulation with 150 mM Sodium Chloride.** The loss of IgG₁ monomer in formulations containing 150 mM NaCl during agitation with siliconized beads was accelerated compared to that in formulations without excipients (Figure II.1, Panel D1). After 7 days of agitation, the protein was 100% aggregated. In contrast, aggregation was not observed after 28 days of agitation in the absence of siliconized beads or during stationary incubation with siliconized beads.

In the samples agitated in 150 mM NaCl with siliconized beads, particles formation was accelerated in comparison to that observed in other formulations. The particle concentration surpassed the upper quantification limit of the MFI instrument by the first day (Table II.2).
Table II.1 Particle concentrations of orbitally agitated and stationary IgG₁ samples in 10 mM sodium acetate buffer pH 5.0, in the presence and absence of siliconized beads.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days</th>
<th>Particles/mL</th>
<th>Cumulative Mass (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥1 µm</td>
<td>≥5 µm</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4,000±400</td>
<td>1000±400</td>
</tr>
<tr>
<td>Agitated IgG₁ (No Beads)</td>
<td>28</td>
<td>7,000±9,000</td>
<td>200±30</td>
</tr>
<tr>
<td>Stationary IgG₁ (with Beads)</td>
<td>28</td>
<td>11,000±700</td>
<td>1,000±200</td>
</tr>
<tr>
<td>Agitated IgG₁ (with Beads)</td>
<td>5</td>
<td>870,000±900,000</td>
<td>30,000±10,000</td>
</tr>
<tr>
<td>Agitated Buffer (No IgG₁, with Beads)</td>
<td>28*</td>
<td>&gt;800,000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7,000±3,000</td>
<td>100±70</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>6,000±2,000</td>
<td>100±300</td>
</tr>
<tr>
<td>Agitated IgG₁ (No Beads)</td>
<td>28</td>
<td>7,500±3,000</td>
<td>200±40</td>
</tr>
<tr>
<td>Stationary IgG₁ (with Beads)</td>
<td>28</td>
<td>4,000±100</td>
<td>400±100</td>
</tr>
<tr>
<td>Agitated IgG₁ (with Beads)</td>
<td>7</td>
<td>550,000±50,000</td>
<td>14,000±19,000</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>320,000±340,000</td>
<td>130,000±90,000</td>
</tr>
<tr>
<td></td>
<td>28*</td>
<td>&gt;800,000</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,000±400</td>
<td>400±130</td>
</tr>
<tr>
<td>Agitated IgG₁ (No Beads)</td>
<td>28</td>
<td>4,000±300</td>
<td>600±30</td>
</tr>
<tr>
<td>Stationary IgG₁ (with Beads)</td>
<td>28</td>
<td>8,000±10,000</td>
<td>1,100±1,200</td>
</tr>
<tr>
<td>Agitated IgG₁ (with Beads)</td>
<td>14</td>
<td>60,000±40,000</td>
<td>5,000±2,000</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>130,000±100,000</td>
<td>6,000±2,000</td>
</tr>
</tbody>
</table>

“Control” denotes non-agitated ‘Day 0’ IgG₁ sample containing siliconized beads. “ND” denotes not determined.
Also, the particle concentrations in samples agitated in the absence of siliconized beads also increased (Table II.2). However, in the samples incubated with siliconized beads without agitation, there was not an increase in counts of particles.

**Formulation with 0.5 M Sucrose.** Agitation of samples with siliconized beads in 0.5 M sucrose accelerated aggregation compared to the results in the excipient-free buffered formulation (Figure II.1, Panel E1). By day 7 of incubation, 20% soluble aggregates were formed in the supernatant fraction. No monomer loss was observed in samples agitated without siliconized beads or kept stationary with siliconized beads for 28 days.

After Day 1, particle concentrations exceeded the limit of the MFI instrument (Figure II.1, Panel B3). Agitation of the samples in the absence of siliconized beads increased subvisible particle concentrations (Table II.2). Small increases in particle concentrations were also observed in stationary samples incubated with siliconized beads for 28 days (Table II.2).

**Influence of pH on Aggregation Induced by Siliconized Beads**

In a previous study, it was reported that the colloidal stability of the same antistreptavidin IgG1 was reduced in 10 mM sodium phosphate buffer, pH 7.4, compared to that in 10 mM sodium acetate buffer, pH 5. This effect was attributed to the proximity of the alkaline pH to the isoelectric point of the protein (pI = 8.7). As a result, protein aggregation during agitation in the presence of silicone oil microdroplets was greatly accelerated at pH 7.4 compared to that observed at pH 5.9
Table II.2  Particle concentrations of orbitally agitated and stationary IgG\textsubscript{1} samples in 10 mM sodium acetate pH 5.0, in the presence or absence of siliconized beads.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days</th>
<th>Particles/mL</th>
<th>Cumulative Mass (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥1 µm</td>
<td>≥5 µm</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>12,000±1,000</td>
<td>3,000±200</td>
</tr>
<tr>
<td>Agitated IgG\textsubscript{1} (No Beads)</td>
<td>28</td>
<td>25,000±6,000</td>
<td>10,000±3,000</td>
</tr>
<tr>
<td>Stationary IgG\textsubscript{1} (with Beads)</td>
<td>28</td>
<td>5,000±600</td>
<td>1000±300</td>
</tr>
<tr>
<td>Agitated IgG\textsubscript{1} (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,000±300</td>
<td>200±100</td>
</tr>
<tr>
<td>Agitated IgG\textsubscript{1} (No Beads)</td>
<td>28</td>
<td>500,000±80,000</td>
<td>10,000±5,000</td>
</tr>
<tr>
<td>Stationary IgG\textsubscript{1} (with Beads)</td>
<td>28</td>
<td>18,000±1,000</td>
<td>2,000±100</td>
</tr>
<tr>
<td>Agitated IgG\textsubscript{1} (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
</tr>
</tbody>
</table>

*Samples where the particle concentration exceeded 800,000 particle/mL in ≥1 micron diameter size range. “ND” denotes not determined.

Therefore, we investigated the effect of reduced colloidal stability in pH 7.4 phosphate buffer on IgG\textsubscript{1} aggregation and subvisible particle formation during agitation in the presence of siliconized beads.

Agitation with siliconized beads at pH 7.4 accelerated aggregation relative to results obtained at pH 5 (Figure II.2, Panel A1). After 14 days agitation at pH 7.4, monomer was not detected, and the presence of 0.01% polysorbate 20 did not inhibit aggregation (Figure II.2, Panel A1 and B1). In either 0.5 M sucrose or 150 mM NaCl formulations, aggregation was accelerated compared to results obtained in pH 7.4 buffer alone, with complete loss of monomer within 7 day (Figure II.2, Panel D1 and E1).

Agitation of samples in the absence of siliconized beads did not cause a significant loss of the monomer in any of pH 7.4 formulations except 0.5 M sucrose formulation, where 8% loss of monomer was observed following 28 days of agitation...
Stationary incubation with siliconized beads did not cause significant decrease in monomer in any of the pH 7.4 formulations.

Following one day of agitation with siliconized beads, counts of particles exceeded the limit of the MFI instrument in all pH 7.4 formulations (Table II.3). In the absence of siliconized beads, particle formation during agitation in pH 7.4 buffer was only observed in 150 mM NaCl and 0.5 M sucrose formulations. The particle levels in stationary samples incubated with siliconized beads did not increase substantially during 28 days of incubation (Table II.3).

**Effect of End-Over-End Rotation in pH 5.0 Buffer**

End-over-end rotation of IgG\textsubscript{1} formulations was performed in an attempt to facilitate the movement of protein solution over the silicone-water interface under mild agitation conditions, in the presence and absence of air in the headspace. Air-bubbles were not entrained in the solutions during end-over-end rotation, unlike the bubble entrainment observed during orbital agitation. Also, as the vial rotated end-over-end, it was observed that the siliconized beads only occasionally underwent tumbling in the solution but mostly remained attached to the vial wall.

End-over-end rotation of samples in pH 5 buffer with siliconized beads in partially-filled vials (i.e., with an air-filled head space) resulted in aggregation. In these samples, approximately 80% monomer was lost and 12% soluble aggregates were formed in 7 days (Figure II.3).
Table II.3  Particle concentrations of orbitally agitated and stationary IgG<sub>1</sub> samples in 10 mM sodium phosphate buffer pH 7.4, in the presence and absence of siliconized beads.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days</th>
<th>≥1 µm (Particles/mL)</th>
<th>≥5 µm (Particles/mL)</th>
<th>≥10 µm (Particles/mL)</th>
<th>≥20 µm (Particles/mL)</th>
<th>≥50 µm (Particles/mL)</th>
<th>Cumulative Mass (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffered (Excipient-free)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4,000±400</td>
<td>400±300</td>
<td>100±20</td>
<td>3±5</td>
<td>0±0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (No Beads)</td>
<td>28</td>
<td>7,000±7,000</td>
<td>700±100</td>
<td>400±300</td>
<td>80±30</td>
<td>0±0</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>28</td>
<td>4,000±900</td>
<td>1,000±100</td>
<td>200±100</td>
<td>70±40</td>
<td>0±0</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td><strong>PS 20</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,000±2000</td>
<td>300±100</td>
<td>100±20</td>
<td>3±5</td>
<td>0±0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (No Beads)</td>
<td>28</td>
<td>30,000±25,000</td>
<td>140±1,000</td>
<td>400±300</td>
<td>100±100</td>
<td>0±0</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>28</td>
<td>7,000±2,000</td>
<td>1,000±400</td>
<td>300±20</td>
<td>60±10</td>
<td>0±0</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td><strong>PS 20+ Sucrose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,000±500</td>
<td>200±100</td>
<td>60±60</td>
<td>20±30</td>
<td>0±0</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (No Beads)</td>
<td>28</td>
<td>4,000±400</td>
<td>1,000±600</td>
<td>400±300</td>
<td>100±100</td>
<td>0±0</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>28</td>
<td>1,500±1,000</td>
<td>200±200</td>
<td>50±40</td>
<td>0±0</td>
<td>0±0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td><strong>150 mM NaCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,300±400</td>
<td>300±100</td>
<td>100±10</td>
<td>4±5</td>
<td>0±0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (No Beads)</td>
<td>28</td>
<td>11,000±2,000</td>
<td>1,100±200</td>
<td>300±100</td>
<td>100±20</td>
<td>0±0</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>28</td>
<td>2,200±500</td>
<td>600±300</td>
<td>230±170</td>
<td>100±70</td>
<td>0±0</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td><strong>0.5 M Sucrose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3,000±400</td>
<td>130±100</td>
<td>20±10</td>
<td>3±4</td>
<td>0±0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (No Beads)</td>
<td>28</td>
<td>200,000±85,000</td>
<td>30,000±20,000</td>
<td>9,000±7,000</td>
<td>300±1,000</td>
<td>20±10</td>
<td>33.5 ± 23.2</td>
</tr>
<tr>
<td>Agitated IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary IgG&lt;sub&gt;1&lt;/sub&gt; (with Beads)</td>
<td>28</td>
<td>6,000±300</td>
<td>2,000±300</td>
<td>1,000±100</td>
<td>150±40</td>
<td>0±0</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

*Samples where the particle concentration exceeded 800,000 particle/mL in ≥1 micron diameter size range. “ND” denotes not determined.
Figure II.2 Levels of IgG₁ monomer and dimer in 10 mM sodium phosphate pH 7.4 buffer during orbital agitation at 4°C.

Treatments were: IgG₁ samples agitated without beads (open circle); IgG₁ stationary samples containing 50 mg siliconized beads (open triangle); and IgG₁ samples agitated with 50 mg siliconized beads (open square). The formulations conditions represented in the different panels are: Panel A, Buffered (Excipient-free); Panel B, 0.01% PS 20; Panel C, 0.01% PS 20 + 0.5 M sucrose; Panel D, 150 mM NaCl; and Panel E, 0.5 M sucrose. Values presented are mean ± SD for three independent replicate vials. Labels 1, 2 and 3 denote percentages of IgG₁ monomer, dimer and soluble aggregates in IgG₁ samples.
The loss of monomer was inhibited in the presence of 0.01% polysorbate 20 (Figure II.3, Panel D). In the absence of siliconized beads end-over-end rotation of partially filled vials did not cause measurable loss of monomer, either in buffer alone or in 0.01% polysorbate 20 (Figure II.3).

**Figure II.3** Levels of IgG1 monomer, dimer and soluble aggregates present in 10 mM sodium acetate pH 5.0 buffer during end-on-end rotation at room temperature.

Treatments were: Partially filled vials without siliconized beads (open triangle); partially filled vials with siliconized beads (open circles); and completely filled vials with siliconized beads (open squares). Panels A, B & C show percentages of monomer, dimer and soluble aggregates for Buffered (Excipient-free) IgG1 samples. Panels D, E & F show percentages of monomer, dimer and soluble aggregates for IgG1 samples containing 0.01% polysorbate 20. Values presented are mean ± SD for three independent replicate vials.

In contrast to results observed in vials with headspace, in completely filled vials containing siliconized beads, end-over-end rotation did not result in measurable aggregation (Figure II.3). This was expected because end-over-end rotation of fully filled vials in the absence of movement of the siliconized beads did not cause fluid mixing and was analogous to quiescent incubation.
Table II.4 Particle Concentrations of End-on-End Agitated IgG1 Samples in 10 mM Sodium Acetate Buffer (pH 5.0), in the Presence or Absence of Siliconized Beads and the Presence

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days</th>
<th>Particles/mL</th>
<th>Mass (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥1 µm</td>
<td>≥5 µm</td>
</tr>
<tr>
<td>IgG1, Buffered (Excipient-free)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5,000±1,000</td>
<td>500±300</td>
</tr>
<tr>
<td>Partially filled (no Beads)</td>
<td>7</td>
<td>30,000±10,000</td>
<td>700±200</td>
</tr>
<tr>
<td>Completely filled (no Beads)</td>
<td>7</td>
<td>10,000±1,000</td>
<td>2,000±1,40</td>
</tr>
<tr>
<td>Partially Filled (Siliconized beads)</td>
<td>1*</td>
<td>&gt;800,000</td>
<td>-</td>
</tr>
<tr>
<td>Completely Filled (Siliconized Beads)</td>
<td>7</td>
<td>25,000±9,000</td>
<td>2,000±1,200</td>
</tr>
<tr>
<td>IgG1, 0.01% PS 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5,000±1,000</td>
<td>300±40</td>
</tr>
<tr>
<td>Partially filled (no Beads)</td>
<td>7</td>
<td>3,000±1,000</td>
<td>500±130</td>
</tr>
<tr>
<td>Completely filled (no Beads)</td>
<td>7</td>
<td>3,000±1,000</td>
<td>400±100</td>
</tr>
<tr>
<td>Partially Filled (Siliconized beads)</td>
<td>3</td>
<td>30,000±6,000</td>
<td>1,000±100</td>
</tr>
<tr>
<td>Completely Filled (Siliconized Beads)</td>
<td>7</td>
<td>70,000±60,000</td>
<td>3,000±2,000</td>
</tr>
<tr>
<td>Buffer (No IgG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially Filled (Siliconized beads)</td>
<td>0</td>
<td>4,000±1,000</td>
<td>200±10</td>
</tr>
<tr>
<td>Completely Filled (Siliconized Beads)</td>
<td>7</td>
<td>5,000±1,000</td>
<td>300±200</td>
</tr>
<tr>
<td>Buffer (No IgG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially Filled (Siliconized beads)</td>
<td>0</td>
<td>7,000±4,000</td>
<td>300±200</td>
</tr>
<tr>
<td>Completely Filled (Siliconized Beads)</td>
<td>7</td>
<td>7,000±3,000</td>
<td>20±10</td>
</tr>
</tbody>
</table>

* samples where the particle concentration exceeded 800,000 particle/mL in ≥1 micron diameter size range. "ND" denotes not determined.

In partially filled vials with siliconized beads, end-over-end rotation of IgG1 in buffer led to the rapid formation of subvisible particles, with particle counts in excess of the limit of the MFI instrument by Day 1 (Table II.4). Particle formation in samples was inhibited in the presence of 0.01% polysorbate 20. In the absence of siliconized beads, agitation of IgG1 in buffer resulted in only modest increase in particles counts after 7
days, which was substantially inhibited in the presence of 0.01% polysorbate 20 (Table II.4). In fully filled vials, end-over-end rotation in samples containing IgG\textsubscript{1} let to minimal particle formation. In partially- or fully-filled vials containing buffer without protein, end-over-end rotation for 7 days did not result in significant increase in particle counts.

**Exposure of IgG\textsubscript{1} Formulations to Silicone-Water Interfaces in the Absence of Air-Water Interfaces**

There was a small increase in particle counts when IgG\textsubscript{1} formulations were flowed through a column packed with siliconized beads (Table II.5). Samples formulated at pH 7.4 formed a larger number of particles compared to samples formulated at pH 5. A control experiment wherein buffer without IgG\textsubscript{1} was passed through the siliconized bead column did not result in an increase in concentrations of subvisible particles. Other control experiments showed that particle formation in IgG\textsubscript{1} formulations did not result from contact with tubing surfaces attached to the column (data not shown). Incorporating 0.01% polysorbate 20 in IgG\textsubscript{1} samples that were passed through the siliconized bead column resulted in an increase in the concentration of particles of diameter $\geq 1 \mu$m and a decrease in the concentration in $\geq 5 \mu$m and larger sized particles (Table II.5).

**Discussion**

**Relevance of Studying Therapeutic Protein Interactions with the Interface between Water and Immobilized Silicone**

Therapeutic proteins packaged in prefilled syringes may interact with both the silicone oil coatings on the walls of the syringe barrels and the silicone oil droplets that slough off the silicone layer.
Table II.5 Particle concentrations of end-on-end agitated IgG1 samples in 10 mM sodium acetate buffer pH 5.0, in the presence or absence of siliconized beads and the presence or absence of headspace (partially filled).

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH 5</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control IgG1</td>
<td>Control IgG1</td>
</tr>
<tr>
<td></td>
<td>IgG1 pumped (no column)</td>
<td>IgG1 pumped (no column)</td>
</tr>
<tr>
<td></td>
<td>IgG1 pumped through column</td>
<td>IgG1 pumped through column</td>
</tr>
<tr>
<td></td>
<td>Buffer pumped through column (No IgG1)</td>
<td>Buffer pumped through column (No IgG1)</td>
</tr>
<tr>
<td></td>
<td>≥1 µm Mass (µg)</td>
<td>≥1 µm Mass (µg)</td>
</tr>
<tr>
<td></td>
<td>2,000±300</td>
<td>1,000±100</td>
</tr>
<tr>
<td></td>
<td>100±30</td>
<td>240±100</td>
</tr>
<tr>
<td></td>
<td>30±10</td>
<td>90±70</td>
</tr>
<tr>
<td></td>
<td>4±7</td>
<td>10±10</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2000±100</td>
<td>700±100</td>
</tr>
<tr>
<td></td>
<td>20±10</td>
<td>10±20</td>
</tr>
<tr>
<td></td>
<td>3±6</td>
<td>4±6</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>0.1 ± 0</td>
<td>0.2 ± 0</td>
</tr>
<tr>
<td></td>
<td>3,000±600</td>
<td>4,000±1,000</td>
</tr>
<tr>
<td></td>
<td>200±200</td>
<td>1,000±100</td>
</tr>
<tr>
<td></td>
<td>50±60</td>
<td>400±50</td>
</tr>
<tr>
<td></td>
<td>10±20</td>
<td>200±10</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>10±1</td>
</tr>
<tr>
<td></td>
<td>0.1 ± 0.1</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10,000±1,000</td>
<td>8,000±200</td>
</tr>
<tr>
<td></td>
<td>20±30</td>
<td>10±10</td>
</tr>
<tr>
<td></td>
<td>6±10</td>
<td>5±10</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>3±5</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td></td>
<td>200±50</td>
<td>200±20</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

“ND” denotes not determined.

With baked-on silicone, the propensity to emulsify oil droplets from the wall is reduced, but the silicone-water interface at the syringe wall could still be a source of instability to proteins. There have been at least a few published studies on the effects of silicone oil droplets on therapeutic protein aggregation. However, the impact of the layer of silicone or other lubricants on syringe barrels on aggregation have not been received as much attention in the literature. In one study, it appears that the main effect of baked-on silicone was to reduce the amount of subvisible particles appearing in an antibody formulation compared to that noted in syringes with sprayed-on silicone oil; but there were no data differentiating particles due to protein and those due to silicone oil microdroplets. In another study, syringes coated with a proprietary lubricant did not shed...
lubricant droplets into protein formulations but aggregation and particle formation were observed during storage in the syringes for formulations of recombinant protective antigen, abatacept and anti-staphylococcal enterotoxin B antibody.\textsuperscript{6} To gain more insight into the effects of immobilized silicone-water interfaces on protein stability, we studied protein aggregation and particle formation in the presence of siliconized glass beads.

A theoretical calculation of the surface area of 50 mg of 1 mm outer diameter borosilicate glass beads gives a total surface area of 1.2 cm\textsuperscript{2}, assuming the density of borosilicate glass beads as 2.46 gm/cc\textsuperscript{19}. Total surface area of glass beads =

\[
\frac{0.05}{\left(\frac{4}{3}\pi r^3\right)\rho} = \frac{3\times 0.05}{r}\rho \quad \text{Equation (1)}
\]

where \(r\) is the radius of glass bead and \(\rho\) is density of borosilicate glass beads. In agitation study, the surface area of siliconized bead (1.2 cm\textsuperscript{2}) per milliliter of IgG\textsubscript{1} solution was relevant to the silicone surface area of a 1 mL prefilled syringe having an approximate value of 10 cm\textsuperscript{2}/mL.\textsuperscript{9} The total surface area of siliconized beads (\(\leq 106 \mu m\) diameter) packed in the column was approximately 0.3 m\textsuperscript{2} (unpublished data) which is greater than the immobilized silicone surface area of a 1 mL prefilled syringe.

**Effects of Siliconized Beads and Agitation on IgG\textsubscript{1} Aggregation and Particle Formation**

During transport, protein formulations packaged in prefilled syringes are subjected to agitation. Generally, current filling techniques do not allow syringes to be filled completely, resulting in an air bubble in the syringe that contributes to agitation-
induced stresses during shipping. To mimic shipping-induced agitation stresses in syringes and vials, several methods have been used including orbital shaking, end-over-end rotation and vortexing.\textsuperscript{20-23} In the current study orbital agitation and end-over-end rotation were used to apply agitation stresses. During both types of agitation, it was observed that the siliconized beads remained stationary, with the IgG\textsubscript{1} solution passing over them. With both agitation methods, there was a synergistic effect of siliconized beads and agitation on protein aggregation and particle formation.

Similar observations have been reported in other studies in which protein aggregation was enhanced by agitation in the presence solid hydrophobic beads. Aggregation of insulin in the presence hydrophobic polytetrafluoroethylene (PTFE) beads\textsuperscript{24} or siliconized glass beads\textsuperscript{25} occurred only during end-over-end agitation. Similarly, \(\alpha\)-synuclein was shown to aggregate during end-over-end agitation in the presence of PTFE beads.\textsuperscript{26}

The mechanism by which agitation and a water-silicone interface act synergistically to cause the formation of protein aggregates and subvisible particles is not understood. In our study, when the IgG\textsubscript{1} formulation was flowed through a column of siliconized glass beads, in the absence of an air-water interface, substantial numbers of protein particles were formed. Thus, we speculate that the synergism between agitation and the silicone-water interface may be explained, at least in part, by the enhanced transport of native protein to the silicone-water interface and/or perturbed aggregation-prone protein molecules away from the interface by agitation. Practically, if syringes could be filled with no or minimal air bubbles, this transport would be minimized and protein stability could be improved.
Effects of pH and Excipients on IgG\textsubscript{1} Aggregation and Particle Formation in the Presence of Silicone-Water Interfaces

Silicone oil-water interfaces have been reported to have negative charge\textsuperscript{9,27} At both pH’s tested in the present study (5.0 and 7.4), the IgG\textsubscript{1} would be expected to be positively charged because its estimated pI is 8.7. However, the greater positive charge of the protein at pH 5 might have been expected to enhance the rate of adsorption of the IgG\textsubscript{1} to the silicone-water interface and, hence, the rates of protein aggregation and particle formation. However, the opposite was observed. The increased particle counts and protein aggregation observed at pH 7.4 compared to those noted at pH 5.0 are likely due to the lower colloidal stability of the IgG\textsubscript{1} at the alkaline pH\textsuperscript{11}. Similar pH effects were seen for the same protein during agitation in the presence of emulsified silicone oil microdroplets\textsuperscript{9}. Furthermore, the presence of 150 mM NaCl should weaken charge-charge interactions between the IgG\textsubscript{1} and the silicone-water interface, and thus might be expected to result in decreased aggregation. Again the opposite effect was observed in the current study, and the explanation appears to be that the effects of NaCl on the protein’s colloidal stability are dominant. It has been shown previously that the presence of 150 mM NaCl reduced the colloidal stability of the IgG\textsubscript{1}, resulting in enhanced aggregation during agitation in the presence of emulsions of silicone microdroplets\textsuperscript{11}.

In addition to colloidal stability, the conformational stability of protein molecules has been shown to affect protein aggregation rates in bulk solution\textsuperscript{28}. However, for the IgG\textsubscript{1} used in the current study, the conformational stability - as inferred from thermal stability studies - was comparable at pH 5.0 and 7.4, and was insensitive to the presence of 150 mM NaCl\textsuperscript{9}. Furthermore, in the current study, the addition of 0.5 M sucrose,
which increases protein conformational stability in bulk solution, actually accelerated IgG₁ aggregation and particle formation. This provides further support that colloidal rather conformational stability controls the aggregation and particle formation of the IgG₁ during agitation in the presence of silicone-water interface.

Sucrose may increase aggregation of the IgG₁ during this stress because it can increase the adsorption of protein molecules to the silicone-oil interface⁹ and may decrease stability of protein molecules adsorbed to interfaces.²⁹ In contrast, polysorbate 20 minimizes interfacial adsorption of proteins,³⁰-³² which may account for the reduction in protein aggregation in the present study observed at pH 5.0. However, at pH 7.4 the surfactant was not effective at inhibiting aggregation during agitation in the presence of siliconized beads. Presumably the protein was so colloidal unstable at pH 7.4 that polysorbate-induced reduction in protein adsorption to interfaces was not sufficient to overcome aggregation propensity. Alternatively, degradation of the polysorbate 20 during the 28-day incubation may have reduced its capacity to inhibit protein adsorption.³³,³⁴

Utility of Siliconized Glass Beads as a Model to Study Protein Particle Formation due to the Silicone-Water Interface

It has recently been shown that particle counts can be an extremely sensitive measurement of protein aggregation, allowing quantitation of protein damage at a resolution greatly exceeding that obtained with other methods such as size exclusion chromatography.¹⁷ However, because of the significant interference from silicone oil droplets, levels of protein particles are difficult to evaluate in prefilled syringes lubricated with sprayed-on silicone oil. Also, protein particle counts are difficult to quantify in
formulation screening studies employing emulsions of silicone oil. In contrast, the covalently attached silicone layer used in the present study does not interfere with particle counts, allowing for a highly sensitive measure of protein particle formation and a useful system for formulation screening.
CHAPTER III

ALBINTERFERON α2b ADSORPTION TO SILICONE OIL-WATER INTERFACES: EFFECTS ON PROTEIN CONFORMATION, AGGREGATION AND SUB-VISIBLE PARTICLE FORMATION

Introduction

Therapeutic proteins are increasingly formulated in prefilled syringes, largely for patient convenience and dosing compliance. Silicone oil is used to lubricate the barrel-plunger assembly and is thought to contribute to the widely-observed protein aggregation in pre-filled syringes. In several studies, the combination of silicone oil and agitation in the presence of air-water interfaces led to synergistic increases in protein aggregation. In contrast, in the same studies and others, incubation of proteins in the presence of silicone oil without agitation caused minimal aggregation.

It seems likely that adsorption of the protein to the silicone oil-water interface initiates the process by which silicone oil induces protein aggregation. The levels of adsorption for other proteins to this interface have been reported to be between 1.5 – 6.5 mg/m². A few studies have shown that non-ionic surfactants inhibit protein adsorption on silicone oil. In one study with an IgG₁, diminished adsorption correlated with lower levels of protein aggregation during agitation in the presence of silicone oil. However, it is not known if inhibition of adsorption of protein to the silicone oil-water interface is generally effective at inhibiting aggregation during agitation.

Submitted to the Journal of Pharmaceutical Sciences for Publication
In two recent studies, both focusing on monoclonal antibodies, adsorption to silicone oil-water interfaces was accompanied by perturbations in the tertiary structure of the proteins. Britt et al. showed that three different humanized antibodies showed essentially the same degree of tertiary structure perturbation after adsorption to silicone oil-water interfaces as that observed after incubation in 8M urea. In contrast, somewhat smaller perturbations of tertiary structure were observed for another antibody upon adsorption to silicone oil microdroplets, and the perturbations were diminished when the ionic strength of the formulation was increased. Interestingly, agitation-induced aggregation of the antibody in the presence of silicone oil was also reduced at high ionic strength.

The connections between protein adsorption on silicone oil microdroplets, accompanying protein tertiary structural changes and aggregation during agitation have only been evaluated in a single study of a monoclonal antibody that did not investigate the effects of addition of surfactants. We hypothesize that addition of nonionic surfactants to formulations will not only inhibit protein adsorption to the silicone oil-water interface and associated protein structural perturbations, but will also inhibit protein aggregation during agitation. Furthermore, we hypothesize that in surfactant-free formulations the use of solution conditions that reduce the degree of structural perturbation of protein adsorbed to the silicone oil-water interface will also reduce aggregation during agitation.

In the present study, we used albinterferon α2b as a model protein. Albinterferon α2b is a fusion protein composed of human serum albumin (HSA) and interferon α2b (Ifn-α). Four albinterferon α2b formulations were evaluated in the current study. These
formulations contained either buffer (10 mM sodium phosphate, pH 7.2), buffer with 300 mM sodium chloride, buffer with 300 mM sucrose or buffer with 0.01% (w/v) polysorbate 80. In these formulations, we measured the adsorption of albinterferon α2b to silicone oil microdroplets and assessed accompanying tertiary structural changes. In addition, we evaluated the effect of agitation on the aggregation of albinterferon α2b in the presence and absence of low concentrations of silicone oil microdroplets (0.2 mg/mL). This concentration of silicone oil was used to approximate typical silicone oil concentrations found in commercially available prefilled syringes.7

Furthermore, in luer-lock prefilled syringes or prefilled cartridges, silicone oil may be present as an immobilized layer that is “baked-on” the interior glass surfaces of syringe barrels. In a previous study, we showed the utility of using siliconized glass beads as a model for baked-on silicone interfaces to assess their effects on protein aggregation and sub-visible protein particles formation.38 In the current study, we also use siliconized beads to assess the influence of baked-on silicone water interfaces and agitation on the formation of sub-visible particles and aggregates in albinterferon-α2b formulations.

Materials and Methods

Materials

Purified albinterferon α2b was provided by GlaxoSmithKline (Rockville, MD) as a stock solution containing 5 mg/ml albinterferon α2b, 36 mg/mL mannitol and 23 mg/mL trehalose in 10 mM sodium phosphate buffer, pH 7.2. Reagent grade buffer salts were
purchased from Fisher Scientific (Fair Lawn, NJ). Polysorbate 80 (Product # 4117-04) and endotoxin-free sucrose (Product# 4005-04) were obtained from J.T. Baker (Avantor Performance Materials Inc., Phillipsburg, New Jersey). Medical grade silicone oil (Dow Corning 360\textsuperscript{TM}, 1000 cSt) was purchased from Dow Corning Corporation\textregistered (Midland, Michigan). Hexane was purchased from Acros Organics\textsuperscript{TM} (New Jersey, USA). 20 kDa molecular weight cutoff Pierce Slidealyzer\textsuperscript{®} dialysis cassettes (product# 66012) were purchased from Thermo Scientific\textsuperscript{®} (Rockford, Illinois). Sterile 3 cc Type-I glass vials were purchased from SCHOTT\textsuperscript{®} (Elmsford, NY), and sterile vial stoppers (West 4432/50 13 mm serum stopper, USP Type I) were obtained from West\textsuperscript{®} Pharmaceutical Services (Lionville, PA). Barrier tips (1 mL, catalog# BT1250) were purchased from Neptune\textsuperscript{®} (San Diego, California). Non-porous (1 mm) borosilicate glass beads (catalog # Z273619) were obtained from Sigma-Aldrich (St. Louis, MO). The siliconizing agent Surfasil\textsuperscript{®} was purchased from Thermo Scientific (Rockford, IL).

**Methods**

**Albinterferon α\textsubscript{2b} Sample Preparation.** The buffer (10 mM sodium phosphate, pH 7.2) used throughout the study was prepared from deionized water, filtered through a 0.22 μm nitrocellulose membrane and used for dialysis of the stock protein solution. To prepare excipient-free formulations, albinterferon α\textsubscript{2b} was dialyzed for twelve hours (4°C) against buffer using dialysis cassettes. For formulations with excipients, albinterferon α\textsubscript{2b} was dialyzed against buffer solutions containing either 300 mM sucrose or 300 mM sodium chloride. For the preparation of albinterferon α\textsubscript{2b} formulations containing polysorbate 80, an aliquot of freshly prepared stock of 1% (w/v) polysorbate 80 in buffer
was added to the dialyzed protein sample to achieve a final polysorbate 80 concentration of 0.01% (w/v). Albinterferon \( \alpha_{2b} \) concentrations were determined from UV absorbance at 280 nm (Agilent® 8540 spectrophotometer, Santa Clara, CA), using an extinction coefficient of 0.621 AU*(mL/mg)*cm (provided by GlaxoSmithKline). The final albinterferon \( \alpha_{2b} \) concentration was adjusted to 3 mg/mL by dilution of the dialyzed protein solution with the appropriate formulation.

Silicone Oil-in-Water Emulsions. Surfactant-free, silicone oil-in-water emulsions were prepared following a previously published method. The resulting emulsion was collected in a clean glass bottle and stored at room temperature until use. Stock solutions of the excipients (2 M sodium chloride, 1 M sucrose or 1% polysorbate 80) were prepared separately in buffer and added to an appropriate volume of the emulsion. The final concentrations of these excipients in the silicone oil emulsion were 300 mM sodium chloride, 300 mM sucrose or 0.01% (w/v) polysorbate 80. Following the addition of excipients, the emulsions were swirled gently and allowed to stand for ten minutes at room temperature before use.

The silicone oil concentration in the emulsion was determined following a previously published method wherein the silicone was first extracted into hexane and then analyzed by infrared spectroscopy at 1220-1300 cm\(^{-1}\).

Estimation of Total Interfacial Area of Silicone Oil Microdroplets in Aqueous Emulsion. The size distribution of the silicone oil microdroplets in the aqueous emulsion was determined with a Beckman Coulter LS230 (Fullerton, CA), using a
The silicone oil microdroplet number distribution in the buffer-only formulation along with the concentration of silicone oil in the emulsion were used to estimate the total interfacial area of silicone oil microdroplets. Consistent with observations reported by Ludwig et. al., the addition of either 300 mM sucrose or 300 mM sodium chloride to the buffer-only silicone oil emulsion increased microdroplet diameter (data not reported). The droplet diameter in the 0.01% polysorbate 80 formulation was comparable to that in buffer-only formulation (data not reported).

Measurement of Albinterferon α2b Adsorption onto Silicone Oil Microdroplets. A centrifugation method was used to measure albinterferon α2b adsorption on silicone oil microdroplets in aqueous solutions. First, 980 µL samples containing various concentrations of silicone oil emulsion were prepared by pipetting increasing volumes of stock silicone oil emulsion into a buffer solution in polypropylene Eppendorf tubes (Fisher Scientific Catalog # 05-402-25). Then, 20 µL of 0.25 mg/mL albinterferon α2b was added to achieve an albinterferon α2b concentration of 5 µg/mL in the final sample. This method was used to prepare samples in buffer-only, 300 mM sucrose, and 300 mM sodium chloride. For studies with polysorbate 80, in order to prevent pre-adsorption of the surfactant on silicone oil microdroplets prior to protein addition, samples for 0.01% (w/v) polysorbate 80 formulation containing 5 µg/mL albinterferon α2b and silicone oil emulsion were prepared by a slight modification of the above method. First, 470 µL of buffer was pipetted into Eppendorf tubes. Then 10 µL of 1% (w/v) polysorbate 80 in buffer-only solution was added followed by the addition of
20 µL of a 0.25 mg/mL albinterferon α2b solution in buffer. Increasing volumes of stock silicone oil emulsion were then added to albinterferon α2b samples containing polysorbate 80, and the final sample volume was adjusted to 1 mL with buffer.

The samples were incubated at room temperature for one hour, and then centrifuged in an Eppendorf Minispin® centrifuge (Hamburg, Germany, 7000*g) for fifteen minutes. Centrifugation resulted in the creaming of silicone oil microdroplets to the top of aqueous solution, leaving a clear aqueous solution below. Approximately 200 µL of this subnatant layer were removed with a micropipette, taking care to minimize disturbance of the creamed silicone layer. The subnatant sample was placed in an autosampler vial, and 90 µL were analyzed by high performance size exclusion chromatography (HP-SEC) (described below). The adsorbed mass of albinterferon α2b was calculated using equation 2.9,40

\[ M_0*(A_0 - A)/ A_0 \]  
... Equation (2)

where the initial mass of albinterferon α2b in the sample (M0) is 5 µg, (A) is the integrated total peak area of the HP-SEC chromatogram for the subnatant and (A0) the peak area for the silicone oil-free albinterferon α2b control sample. Three replicates of each sample type were separately incubated, centrifuged and analyzed by HP-SEC. The average adsorbed protein mass with standard deviation was plotted against the silicone oil-water interfacial area.

The data from adsorption measurements were also used to estimate the adsorption footprint of albinterferon α2b on silicone oil microdroplets.44 The non-adsorbed fraction of the albinterferon α2b was plotted against the silicone oil microdroplet interfacial area.
per milligram of adsorbed albinterferon α2b, and the adsorption footprint determined from the x-intercept of a linear regression fit to the data.44,45

**Fluorescence Quenching.** Tryptophan fluorescence of albinterferon α2b was quenched with acrylamide. Acrylamide stock solutions (9M) were freshly prepared in buffer prior to each experiment. Native albinterferon α2b at 0.1 mg/mL was prepared in various formulations and used for acrylamide quenching of the native control. Solutions containing unfolded albinterferon α2b were prepared by overnight incubation of 0.1 mg/mL albinterferon α2b in 8M guanidine hydrochloride (GdHCl). To measure fluorescence, 3 mL of the sample were placed in a 1-cm square quartz cuvette. Aliquots of the acrylamide solution were added to the albinterferon α2b solution using a 20 µL Hamilton® glass syringe. Following each addition of acrylamide solution into the protein sample, the samples were gently mixed with a polypropylene transfer pipette. The fluorescence spectra were recorded at 25°C in a SLM-Aminco® spectrofluorometer using standard 90° optics (Urbana, Illinois) at a scan rate of 0.95 nm/s. The slit widths of excitation and emission monochromators were both set at 4 nm. Tryptophan was excited at 295 nm and the emission spectrum between 300-400 nm was recorded. The fluorescence intensities for the native and silicone oil-adsorbed albinterferon α2b samples were monitored at 328 nm, whereas intensities for unfolded protein were monitored at 346 nm. The fluorescence spectrum of an identical blank solution (protein free) was subtracted from that of the protein sample to obtain the final fluorescence spectrum.

To study the effects of adsorption of albinterferon α2b on fluorescence quenching, sufficient silicone oil microdroplets were added so that essentially 100% of the protein
was adsorbed. Acrylamide quenching of albinterferon α2b adsorbed to silicone oil samples was measured using front face optics. The incidence angle of the UV light in the front face mode was kept at 53° to minimize scattering interferences due to the silicone oil microdroplets. To correct for any scattering effects, the fluorescence spectrum of a blank solution containing silicone oil microdroplets was collected and subtracted from that of albinterferon α2b samples.

The Stern-Volmer equation (equation 3) was used to calculate the quenching constant (K_{SV}) for the control, unfolded and silicone oil-containing albinterferon α2b samples.

\[
\frac{F_0}{F} = 1 + K_{SV}[Q] \ldots \text{Equation (3)}
\]

where \(F_0\) and \(F\) are the fluorescence intensity at \(\lambda_{\text{max}}\) in the absence and presence of acrylamide, respectively, and [Q] is the molar concentration of acrylamide in the sample. Fluorescence quenching for each acrylamide concentration was determined from three samples which were incubated separately. Each formulation was analyzed three separate times. The average \(K_{SV} \pm \) standard deviation is reported.

**Light Microscopy.** Agglomeration of silicone oil microdroplets in albinterferon α2b formulations during stationary incubation was examined by light microscopy. Samples for light microscopy were prepared by incubating 1 mL albinterferon α2b solution (3 mg/mL) containing 200 µg/mL of silicone oil emulsion for one day in a covered glass petri dish at room temperature. A light microscope (Nikon Eclipse TE 300, Melville, NY) equipped with NIS Elements BR (Version 3.2) software was used for
image acquisition at 40x magnification. The incubations were performed with triplicate samples for each formulation, and representative images are shown.

**Zeta Potential.** The zeta potentials of silicone oil droplets with and without adsorbed albinterferon α_{2b} were measured by Malvern Nanosizer® (Malvern Instruments Ltd., Malvern, UK). Samples with essentially 100% of the albinterferon α_{2b} adsorbed on silicone oil droplets were prepared in the different formulations. Approximately 1 mL of this sample was pipetted into the disposable capillary cell (catalog # DTS 1061) and used for measurement. The zeta potential of 1 mg/mL albinterferon α_{2b} in solution without silicone oil was also measured. All measurements were performed at 25°C in triplicate samples for each formulation, with averages and standard deviations reported.

**Siliconization of Glass Beads.** Siliconization of glass beads was performed following a previously published method explained in detail elsewhere.\textsuperscript{14,15}

**Agitation Study.** Albinterferon α_{2b} samples were prepared in the different formulations: buffer-only, 0.01% polysorbate 80, 300 mM sodium chloride and 300 mM sucrose. These samples were pipetted into sterile 3 cc Type-I glass vials. Then an aliquot of the silicone oil emulsion in the appropriate formulation was added such that the final sample volume was 1 mL. The final concentration of silicone oil in each vial was 200 µg/mL, and that of albinterferon α_{2b} was 3 mg/mL.

For the albinterferon α_{2b} samples containing siliconized glass beads, 50 mg siliconized beads were first weighed in 3 mL vials using an analytical balance. Then a 1
mL aliquot of the 3 mg/mL albinterferon α2b formulation was pipetted into these vials. To minimize contamination by exogenous particles, this procedure was carried out in a laminar flow hood (Nuaire Biological Safety Cabinet, Class II, Type A2, Model number Nu-437-600 Series 2). Albinterferon α2b samples (1mL) without silicone oil emulsion or siliconized glass beads were prepared by pipetting 1 mL of 3 mg/mL protein solution into vials.

All the vials were stoppered with sterile vial stoppers (West 4432/50 13 mm serum stopper, USP Type I) and crimped with aluminum seals. The sealed vials were fit snugly into a cardboard box (Fisher Scientific catalog # 12-009-31) having a 7x7 box divider. The box was covered with a lid, and the base of the box was secured on a rotator (Rotmix Model No: RKVSD, Haverhill, MA) by metal screws. The box containing vials was rotated at 20 rpm at room temperature. This mode of agitation facilitated end-over-end rotation of albinterferon α2b formulations in the glass vials. Identical sample vials containing albinterferon α2b solution with and without silicone oil or siliconized glass beads were kept stationary at room temperature. Triplicate vials of each sample were removed for analysis on Days 0, 1, 3, 5, and 7.

**High Performance Size Exclusion Chromatography.** HP-SEC analysis of albinterferon α2b samples was performed using an Agilent® 1100 HPLC system (Santa Clara, CA). Samples from adsorption (90 µL) and agitation studies (10 µL) were injected onto a size exclusion column (Tosoh Haas G3000) having a 0.2 µm pre-filter. Albinterferon α2b was eluted from the column using a mobile phase (50 mM sodium phosphate and 600 mM sodium sulfate at pH 6.7) pumped at a flow rate of 0.6 mL/min.
The albinterferon α₂b eluted from the column was detected by UV absorbance at 215 nm. For samples from the agitation incubation study, the integrated areas of monomer and dimer were determined separately and compared to the values for an unstressed albinterferon α₂b control sample.

**Sub-Visible Particle Measurement.** The concentration and size distribution of sub-visible particles was measured by optical flow microscopy (Micro flow imaging, model # DPA 4100, (Protein Simple®, Santa Clara, CA) using MFI View® application software (version 6.9.8). The instrument was configured in the set point 3 mode (low magnification, open aperture), with a 100 µm flow cell (part number 4002-002-001) that allowed the detection of particles 1-70 µm in diameter. The illumination was optimized with buffer solution prior to the analysis of samples. To perform particle analysis, 500 µL of each of the sample were pipetted gently into the instrument using a barrier tip. Prior to data acquisition 150 µL of sample was allowed to flow through the cell, followed by collection of data for the next 350 µL of sample.

**Result and Discussion**

**Effect of Excipients on the Adsorption of Albinterferon α₂b to the Silicone Oil-Water Interface**

In all of the formulations tested, except for that containing polysorbate 80, albinterferon α₂b adsorbed onto the silicone oil droplets (Figure III.1, Panel A). The adsorbed mass of albinterferon α₂b ranged between 1.8 - 2.0 mg/m² silicone-water interface (Table III.1). Compared to the buffer-only formulation, the presence of 300 mM
sucrose or 300 mM sodium chloride did not alter the adsorbed mass of albinterferon $\alpha_{2b}$ significantly. The adsorption of albinterferon $\alpha_{2b}$ to silicone oil microdroplets was comparable to values for other proteins reported in the literature.$^{9,36,39,40}$ For example, Gerhardt et al. determined that the adsorption of a therapeutic monoclonal antibody onto the silicone oil-water interface was $2.7 \pm 0.6 \text{ mg/m}^2$. $^{36}$ Britt et al., reported values for three different monoclonal antibodies ranging from about 0.6 to 1.8 mg/m$^2$. $^{40}$ Ludwig et al. found that values for four proteins (lysozyme, bovine serum albumin, abatacept and trastuzumab) varied between about 0.8 to 1.5 mg/m$^2$. $^{9}$ Thus, it appears that adsorption of albinterferon $\alpha_{2b}$ at the silicone oil-water interface is similar to that for other proteins.

Furthermore, as has been shown previously with other proteins,$^{9,36,39,40}$ adsorption of albinterferon $\alpha_{2b}$ to the silicone oil-water interface was not observed in the presence of polysorbate 80. Presumably surfactant adsorption to the interface interferes with adsorption of protein molecules and/or the surfactant displaces adsorbed protein molecules.$^{9,47-50}$

**Adsorption Footprint of Albinterferon $\alpha_{2b}$ at Silicone-Water Interface**

The experimentally determined adsorption footprint for albinterferon $\alpha_{2b}$ at the silicone oil-water interface was about 0.5 m$^2$/mg (Table III.1). The exact molecular dimensions of albinterferon $\alpha_{2b}$ are unknown, but a conservative theoretical estimate for the adsorption footprint of a monolayer of albinterferon $\alpha_{2b}$ can be made by assuming that the surface area occupied per albinterferon $\alpha_{2b}$ molecule is the cross-sectional area of a sphere of equivalent volume.
Figure III.1  Panel A shows the adsorption isotherm of albinterferon α2b on silicone oil and Panel B shows the adsorption footprint of the same. Panel B shows predicted adsorption footprint of fusion protein for end on (open circle) and side on (closed star) modes of adsorption and measured albinterferon α2b adsorption footprint on silicone oil. Formulations are denoted as following- Buffer-only (open square), 300 mM sucrose (open triangle, inverted), 300 mM sodium chloride (open triangle) and 0.01 % polysorbate 80 (open diamonds). Values presented are mean + SD for three independent replicate samples.

Using the molecular weight of albinterferon α2b and an estimated specific density of 1.33 g/ml, the resulting theoretical footprint for monolayer coverage is 0.2 m²/mg. This estimate assumes perfect surface packing of albinterferon α2b. Other packing models such as the random sequential adsorption model would yield higher estimates for the footprint of albinterferon α2b at monolayer coverage. In contrast, lower footprints would be expected if multilayer adsorption occurred. Thus, the experimentally observed footprint value is consistent with monolayer coverage of albinterferon α2b at the silicone oil-water interface. Similar observations have been reported in literature where surface coverages of proteins adsorbed on silicone oil microdroplets were estimated to be monolayer.9,36,40
Table III.1 Adsorption footprint of albinterferon α2b on silicone oil in 10 mM sodium phosphate pH 7.2 buffer in different formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Footprint (m²/mg)</th>
<th>Loading (mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer-only</td>
<td>0.5 ± 0.0</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>300 mM sucrose</td>
<td>0.5 ± 0.0</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>300 M sodium chloride</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Tertiary Structure of Albinterferon α2b Adsorbed on Silicone Oil

Albinterferon α2b contains three tryptophan residues; one tryptophan resides in the HSA domain and two in the Ifn- α domain (determined from albinterferon α2b sequence provided by GlaxoSmithKline). The $K_{SV}$ values reported in this study represent the overall quenching of the tryptophan residues in the fusion protein and do not differentiate between the quenching of individual tryptophan residues in the HSA and Ifn- α domains. A change in $K_{SV}$ is indicative of tertiary structural perturbations in proteins.44,52

$K_{SV}$ values for albinterferon α2b in silicone oil-free samples were similar between buffer-only and 300 mM sucrose formulations (Figure III.3). However, $K_{SV}$ value for silicone oil-free samples was significantly decreased (p < 0.05) in the 300 mM sodium chloride formulation relative to the buffer-only formulation. Sodium chloride increases the conformational stability of albinterferon α2b, stabilizing a compact native conformation of the protein.53 In the buffer only and the 300mM sodium chloride formulations, $K_{SV}$ values for albinterferon α2b adsorbed on silicone oil microdroplets were significantly higher relative to the silicone oil-free samples. However, in 300 mM
sucrose, $K_{SV}$ values for albinterferon $\alpha_{2b}$ for oil-free and silicone oil-containing samples were indistinguishable ($p > 0.05$). Significant adsorption of albinterferon $\alpha_{2b}$ on silicone oil microdroplets did not occur in formulations that contained 0.01% (w/v) polysorbate 80. Hence $K_{SV}$ for albinterferon $\alpha_{2b}$ was not measured in silicone oil-containing samples in formulations that contained polysorbate 80.

Figure III.2 Acrylamide quenching of albinterferon $\alpha_{2b}$ in the presence and absence of silicone oil droplets in different formulations in 10 mM sodium phosphate pH 7.2 buffer. Native albinterferon $\alpha_{2b}$ (open triangle), Unfolded albinterferon $\alpha_{2b}$ (open triangle, inverted), Buffer-only (open circle), 300 mM sodium chloride (open square) and 300 mM sucrose (open diamonds). Values presented are mean ± SD for three independent replicate samples.

$K_{SV}$ values for other therapeutic proteins adsorbed at silicone oil–water interface have been reported to increase relative to those for native protein, suggesting perturbation of the tertiary structure in protein molecules adsorbed to silicone oil oil.$^{36,40}$ Gerhardt et al. reported that adsorption of therapeutic IgG$_1$ on silicone oil microdroplets was accompanied by a tertiary structural change indicated by an increased $K_{SV}$ value. The
increase in the $K_{SV}$ for the adsorbed IgG₁ was significant, and $K_{SV}$ for adsorbed IgG₁ was comparable to the $K_{SV}$ for unfolded IgG₁.³⁶

In the current study, a small increase in the $K_{sv}$ of albinterferon α₂b adsorbed at the silicone oil-water interface was observed in the buffer-only and 300 mM sodium chloride formulations. This result suggests that the tertiary structure of albinterferon α₂b is slightly perturbed when adsorbed to the silicone-oil interface in these formulations. Interestingly, in the presence of 300 mM sucrose, there was no change in $K_{SV}$ upon adsorption to silicone oil. Thus, it appears that sucrose stabilizes albinterferon α₂b against tertiary structural perturbation due to adsorption.

**Figure III.3** Stern-Volmer quenching constant ($K_{SV}$) of native (dotted bar) and silicone oil containing (checkered bar) albinterferon α₂b samples in different formulations. Double stars denotes $p < 0.05$, single star denotes $p < 0.01$ and ‘NS’ indicates not significant. Values presented are mean ± SD for three independent replicate samples.
Albinterferon $\alpha_{2b}$ Adsorption on Silicone Oil Microdroplets and its Effect on Zeta-Potential

The zeta-potential of silicone oil microdroplets in protein-free samples ranged between -19 to -27 mV and that of albinterferon $\alpha_{2b}$ in silicone oil free samples ranged between -10 to -22 mV (Table III.2). The negative zeta potential of silicone oil microdroplets in aqueous samples has been attributed to adsorption of hydroxyl ions to silicone oil as well as to orientation of the hydroxyl/hydronium ions at the silicone oil-water interface.

Adsorption of the negatively charged albinterferon $\alpha_{2b}$ on silicone oil microdroplets decreased the average zeta-potential by 15 mV in the buffer-only formulation and 6 mV in the 300 mM sucrose formulation (Table III.2).

Table III.2 Zeta potential ($\delta$) of silicone oil droplets, native albinterferon $\alpha_{2b}$ and silicone oil adsorbed albinterferon $\alpha_{2b}$ in different formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>SOE* (No protein) $\delta$(mV)</th>
<th>Albinterferon $\alpha_{2b}$ $\delta$(mV)</th>
<th>Albinterferon $\alpha_{2b}$ +SOE* $\delta$(mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>-54±3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Buffer-only</td>
<td>-27±1</td>
<td>-18±1</td>
<td>-45±2</td>
</tr>
<tr>
<td>0.01% PS 80</td>
<td>-19±1</td>
<td>-10±4</td>
<td>-</td>
</tr>
<tr>
<td>300 mM sodium chloride</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300 mM Sucrose</td>
<td>-23±1</td>
<td>-22±2</td>
<td>-29±1</td>
</tr>
</tbody>
</table>

* SOE denotes silicone oil emulsion

In protein-free samples, the zeta-potential of silicone oil microdroplets was lower in the polysorbate 80 formulation in comparison to that in the buffer-only formulation. Polyoxyethylene groups of non-ionic surfactants have been postulated to shift the shearing plane of zeta-potential in oil in water emulsions. It is possible that a similar phenomenon lowered the zeta-potential of silicone oil microdroplets in formulations.
containing the non-ionic, polyoxyethylene-containing surfactant polysorbate 80.\textsuperscript{34} Since albinterferon $\alpha_{2b}$ did not adsorb onto silicone oil microdroplets in formulations containing polysorbate 80, zeta-potential in this formulation was only measured in the absence of protein. Zeta potentials could not be measured in 300 mM sodium chloride formulation due to the high ionic strength of the solution.

**Figure III.4** Optical microscopy showing silicone oil droplet coalescence in different formulations of 3 mg/mL albinterferon $\alpha_{2b}$ solution.

Panel A: Buffer only, Panel B: 0.01% polysorbate 80, Panel C: 300 mM sodium chloride, Panel D: 300 mM sucrose.

**Agglomeration of Silicone Oil Microdroplets in Albinterferon $\alpha_{2b}$ Formulations**

Silicone oil microdroplets can agglomerate in therapeutic protein formulations.\textsuperscript{9,40} Agglomeration of silicone oil microdroplets has the potential to form visible particles,
which can affect the acceptability of protein therapeutics in prefilled syringes. In the current study, agglomeration of silicone oil microdroplets was observed in 300 mM sodium chloride and 300 mM sucrose formulations of the protein, but not in the buffer-only and 0.01% (w/v) polysorbate 80 formulations (Figure III.4).

Typically, particles with zeta potentials of absolute values greater than 30 mV are considered to be colloidally stable. In the buffer-only formulation, due to a relatively large negative zeta-potential, the silicone oil microdroplets containing an adsorbed layer of albinterferon $\alpha_{2b}$, were colloidally stable and did not agglomerate. The zeta potential of silicone oil microdroplets containing an adsorbed albinterferon $\alpha_{2b}$ layer was -29mV in the 300 mM sucrose formulation and only small agglomerates of oil microdroplets were observed.

The addition of sodium chloride increased the ionic strength of the albinterferon $\alpha_{2b}$ formulation, which decreased the electrostatic charge barrier between the albinterferon $\alpha_{2b}$ coated silicone oil microdroplets. As a result, the silicone oil microdroplets agglomerated in 300 mM sodium chloride formulation of albinterferon $\alpha_{2b}$.\textsuperscript{40}

Agglomeration of silicone oil microdroplets was not observed in the polysorbate 80 formulation. Although the microdroplets had zeta potentials that would suggest colloidal instability, the bulky polyoxyethylene chains of adsorbed polysorbate 80\textsuperscript{34} may sterically hinder agglomeration of silicone oil microdroplets.\textsuperscript{58}
Effect of Excipients on Albinterferon $\alpha_{2b}$ Aggregation during Agitation in the Presence of Silicone Oil Microdroplets

Aggregation of albinterferon $\alpha_{2b}$ was measured in vials that were agitated by end-over-end rotation. Agitation of albinterferon $\alpha_{2b}$ samples in the absence of silicone oil microdroplets did not result in aggregation in any of the formulations (Figure III.5). Also, quiescent incubation of albinterferon $\alpha_{2b}$ formulations in the presence of silicone oil microdroplets did not induce aggregation (Figure III.5); nor did quiescent incubation in the absence of silicone oil (data not shown).

In the buffer-only formulations, agitation of albinterferon $\alpha_{2b}$ in the presence of silicone oil microdroplets resulted in relatively rapid loss of monomer to insoluble aggregates (Figure III.5 - Panel A1). Agitation-induced aggregation of albinterferon $\alpha_{2b}$ in suspensions of silicone oil microdroplets was reduced in the presence of 300 mM sucrose. (Figure III.5 - Panel D1), and albinterferon $\alpha_{2b}$ aggregation was inhibited in 300 mM sodium chloride and 0.01% polysorbate 80 formulations (Figure III.5 – Panel B1 & C1).

The combination of agitation and silicone oil interfaces has been shown to act synergistically to accelerate protein aggregation. A similar synergism was observed with albinterferon $\alpha_{2b}$. Structurally perturbed albinterferon $\alpha_{2b}$ molecules adsorbed at the silicone oil-water interface may self-associate to form aggregates. It is possible that agitation facilitated sloughing of aggregated albinterferon $\alpha_{2b}$ from the silicone oil interface.

In formulations containing 300 mM sodium chloride, perturbation of albinterferon $\alpha_{2b}$ tertiary structure in the silicone oil adsorbed layer was minimized. As a result
aggregation was inhibited during agitation of samples containing silicone oil microdroplets in 300 mM sodium chloride formulation. In addition, sodium chloride minimizes aggregation by increasing the conformational stability of albinterferon α2b in solution.\(^{53}\) In combination, we postulate that these protective mechanisms inhibited albinterferon α2b aggregation in 300 mM sodium chloride formulation.

Albinterferon α2b adsorbed to silicone oil microdroplets retained more native-like tertiary structure in formulations containing 300 mM sucrose compared to the buffer-only formulation. As expected, in these sucrose-containing samples also exhibited reduced aggregation compared to that observed in the buffer-only formulation.

**Figure III.5** Size exclusion chromatography results showing soluble monomer and dimer percent of albinterferon α2b upon agitation in the presence and absence of 200 µg silicone oil.

The different panel shows the agitation results for different formulation conditions which are as follows: Panel A – buffer only, Panel B - 0.01% polysorbate 80, Panel C – 300 mM sodium chloride and Panel D – 300 mM sucrose. Open circle denotes samples agitated in the absence of silicone oil, Open square denotes agitated samples in the presence of silicone oil & open diamonds denotes stationary samples of fusion protein incubated with silicone oil. Values presented are mean + SD for three independent replicate vials.
In formulations containing polysorbate 80, two non-exclusive potential mechanisms may play a role in inhibiting aggregation. First, specific binding of polysorbate 80 to native albinterferon α2b might increase the conformational stability of albinterferon α2b and result in lower rates of aggregation. Second, polysorbate 80 might act to reduce aggregation by inhibiting of adsorption of albinterferon α2b to the silicone-water interface, along with the associated perturbations in albinterferon α2b tertiary structure.

**Effect of Excipients on Albinterferon α2b Aggregation during Agitation of Formulations with Siliconized Beads**

Albinterferon α2b aggregation was not observed in samples agitated in the absence of siliconized beads (Figure III.6). During agitation in the presence of siliconized beads, albinterferon α2b monomer was lost to insoluble aggregates in the 300 mM sucrose formulation (Figure III.6). Albinterferon α2b did not aggregate in buffer-only, 0.01% polysorbate 80 and 300 mM sodium chloride formulations during agitation of samples with siliconized beads.

The counts of sub-visible particles in silicone-free agitated samples for albinterferon α2b in formulations containing buffer only, 300 mM sodium chloride or 0.01% (w/v) polysorbate 80 (Figure III.7) were comparable to that for control samples. But with the silicone oil-free formulation containing 300 mM sucrose, significant increases in both 1 – 5 and 5 – 10 µm particle counts were caused by agitation. Also, the counts of sub-visible particles in quiescent samples did not increase during incubation of albinterferon α2b samples in the presence or absence of siliconized beads. Albinterferon
agitated with siliconized beads exhibited an increase in 1 – 5 and 5 – 10 µm size ranges of particles (Figure III.7). Increases in counts of sub-visible aggregates in 20 – 50 µm and ≥ 50 µm diameter size bins were significantly higher in buffer-only and 300 mM sucrose formulations but not in 0.01% polysorbate 80 and 300 mM sodium chloride formulations.

The possibility of particles forming in agitated samples due to sloughing of the silicone layer (e.g. silicone oil microdroplets) from siliconized beads was eliminated from a control experiment. Agitation of buffer solution (protein free) with 50 mg siliconized beads did not increase the counts of sub-visible particles in comparison to silicone-free buffer samples (data not shown).

**Figure III.6** Size exclusion chromatography results showing monomer and dimer levels of albinterferon α2b in vials rotated end-over-end in the presence and absence of 50 mg siliconized glass beads.

The different panels shows results for the various formulation conditions: Panel A – buffer-only, Panel B - 0.01% polysorbate 80, Panel C – 300 mM sodium chloride and Panel D – 300 mM sucrose. Open circle denotes samples agitated in the absence of beads, open square denotes agitated samples in the presence of 50 mg siliconized beads and open diamond denotes stationary samples of fusion protein incubated with 50 mg siliconized beads. Values presented are mean + SD for three independent replicate vials.
As proposed for experiments with silicone oil droplets, with siliconized glass beads tertiary structural change of albinterferon $\alpha_{2b}$ adsorbed to the silicone-water interface possibly led to albinterferon $\alpha_{2b}$ aggregation and subsequent sub-visible particle formation during agitation of albinterferon $\alpha_{2b}$. However, during agitation of albinterferon $\alpha_{2b}$ in the presence of silicone oil microdroplets, aggregation was higher in buffer-only formulation compared to that in the 300 mM sucrose formulation. Conversely, during agitation of albinterferon $\alpha_{2b}$ in the presence of siliconized beads, aggregation was higher in 300 mM sucrose formulation. Perhaps differences in the silicone oil interfacial area, density and fluidity of silicone-water interface between silicone oil microdroplets and siliconized beads led to the differences that were observed in albinterferon $\alpha_{2b}$ aggregation.

**Figure III.7** Binned sub-visible particle concentration of albinterferon $\alpha_{2b}$ samples agitated in the presence and absence of siliconized beads for seven days.

- Buffer only
- 0.01% polysorbate 80
- 300 mM sodium chloride and
- 300 mM sucrose

“Agitated” denotes albinterferon $\alpha_{2b}$ samples agitated without siliconized beads, “STA Beads” denotes albinterferon $\alpha_{2b}$ samples incubated with siliconized beads without agitation and “AG Beads” denotes albinterferon $\alpha_{2b}$ samples agitated with siliconized beads. Values presented are mean $\pm$ SD for three independent replicate vials.
Prefilled syringes are siliconized by spraying a surfactant-free silicone oil emulsion on the inside wall of syringe and cartridge barrels. With cartridges or luer-lock syringes, the lubricated barrels are often then passed through a heating tunnel. This process is commonly known as the “baked-on” technique. This technique vaporizes the low molecular weight siloxanes in silicone oil and immobilizes the silicone oil on the syringe barrel.¹³ The “baked on” technique is thought to minimize sloughing of the immobilized silicone oil layer thereby reducing the interaction between therapeutic protein and silicone oil microdroplets. But as shown recently with a model monoclonal antibody and in the current study, the silicone-water interface of silicone coated glass can also operated synergistically with agitation to promote formation of protein particles and aggregates.

**Conclusion**

Therapeutic protein aggregation in siliconized prefilled syringes is problematic. Therapeutic proteins have the potential to adsorb on silicone oil microdroplets and undergo tertiary-structural changes. As a result proteins can aggregate and form particles, especially during agitation. Excipients such as sucrose may inhibit tertiary structural change of proteins adsorbed to the silicone-water interface, resulting in reduced aggregation. Also, incorporation of a surfactant to inhibit protein adsorption at the silicone oil–water interface should reduce protein aggregation in prefilled syringes and cartridges. Overall, the results show that excipients can affect both adsorption of a protein to a silicone oil-water interface and the resulting alteration in protein conformation, and
that the combination of these effects can modulate the degree of interface-induced protein aggregation and particle formation.
CHAPTER IV

PARTICLE FORMATION IN IgG₁ FORMULATIONS DURING RUPTURE OF
AIR-WATER AND SILICONE OIL-WATER INTERFACES IN IgG₁ SAMPLES³

Introduction

Proteins may adsorb to interfaces. As a result, the native conformation of the protein can be perturbed and the protein may aggregate.⁵⁹ For example, adsorption of a monoclonal antibody on glass particles has been reported to perturb the native conformation of the protein in the adsorbed layer.⁴⁵ Aggregation of insulin in agitated samples containing Teflon spheres has been linked to insulin unfolding at the Teflon-water interface.²⁴ In another study, adsorption of recombinant human platelet-activating factor acetylhydrolase on silica nanoparticles caused aggregation by a heterogeneous nucleation-like mechanism.⁶⁰

Compatibility of a therapeutic protein with its primary container-closure is an important issue in product development. The complexity of primary containers has significantly increased (prefilled syringes, dual cartridge syringes, auto-injector devices, etc.) and contains different number and nature of interfaces that contacts therapeutic proteins formulations. These interfaces include glass, plastic, stoppers and silicone-oil.² Among these interfaces, silicone oil used in prefilled syringes has been linked to protein aggregation.⁶,¹¹,³⁶ Several studies have reported that silicone oil and agitation together accelerates aggregation.⁶,¹¹,³⁶ However the mechanism of this apparent synergism has not been definitively elucidated.

³ Manuscript in Preparation
During transportation, protein formulations may undergo agitation. Agitation introduces air-microbubbles in protein samples and may accelerate aggregation.\(^{29,61,62}\) As such, agitation is a stress that is frequently assessed during development of therapeutic protein formulations. Aggregation of therapeutic proteins in the presence of air-water and silicone oil-water interfaces has been typically assessed during accelerated agitation (e.g. orbital agitation, rotation or vortexing). In the absence of agitation, adsorption of protein at silicone oil-water\(^{40}\) and glass-water\(^{45}\) interfaces can potentially cause perturbation of protein tertiary structure in the adsorbed layer.

Several studies have reported that agitation increases the number of particles in therapeutic protein formulations containing silicone oil-water interfaces. In one study, perturbation of the native conformation of IgG\(_1\) in the adsorbed layer at the silicone oil-water interface was linked to aggregation in agitated formulations.\(^{36}\) Another study postulated that agitation may slough adsorbed IgG\(_1\) from the silicone oil-water interface, accelerating aggregation.\(^{38}\) But the mechanism of aggregation or particle formation is not well understood.

Protein aggregates at air-water interface has been linked to particles in IgG\(_1\) samples. For example periodic compression/relaxation of IgG\(_1\) samples has been reported to increase the surface pressure in IgG\(_1\) layer at air-water interface that released aggregates into the bulk sample above a critical compression ratio. As a result, particle concentration in the IgG\(_1\) samples increased. In a different study, IgG\(_1\) aggregation was reported to accelerate during mechanical rupture of the adsorbed IgG layer at the air-water interface.\(^{63}\) But the level of sub-visible particles in protein samples was not assessed.
It is possible that rupture of the adsorbed protein layer at the silicone oil-water interface increases particle counts in therapeutic protein formulations. This may explain, at least in part, the link between agitation and silicone oil in accelerating particles in therapeutic protein samples. But studies evaluating the effect of silicone oil-water or air-water interface rupture on particle formation in IgG₁ samples are not known. We hypothesize that rupture of the air-water or silicone oil-water interface increases the counts of protein particles in therapeutic protein formulations. A model IgG₁, antistreptavidin, was used to test our hypothesis.

In a previous study we reported during agitation of IgG₁ (antistreptavidin) samples particle formation was accelerated at pH 7.4 compared pH 5.0 samples. In the same study it was speculated that low colloidal stability of an identical IgG₁ at pH 7.4 accelerated aggregation. Charge distribution on IgG₁ molecule is expected to be different at pH 7.4 than at pH 5.0. Charges on protein molecules affect protein association at interfaces, a phenomenon that may affect particle formation in IgG₁ samples during interface rupture. So we chose pH 5.0 and pH 7.4 to evaluate the effect of interface rupture on particle formation in IgG₁ samples.

Materials and Methods

Materials

IgG₁, antistreptavidin, was provided by Amgen, Inc. (Thousand Oaks, CA) in 10 mM sodium acetate pH 5.0 buffer containing 5% sorbitol. Buffer salts were purchased from Fisher Scientific (Fair Lawn, NJ). Medical grade silicone oil was purchased from Dow Corning (Dow Corning 360™, 1000 centi stokes, Midland, MI). Glass beads, ≤ 106
µm (catalog # G8893) were purchased from Sigma-Aldrich (St. Louis, MO). Guard column was from TOSOH Biosciences LLC (Part# 13075, Montgomeryville, PA). Pierce Slidealyzer® (product# 66012) having 20kDa molecular weight cutoff and siliconizing agent Surfasil® were purchased from Thermo Scientific (Rockford, IL). Hexane and HPLC grade acetonitrile were purchased from Acros Organics™ (New Jersey, USA). UV-flow cell (Catalog # 583.1F-Q-1/Z15) was from Strana Cell® (Atascadero, CA). Presterilized 3 cc Type-I glass vials were from SCHOTT® (Elmsford, NY), and silicone-free Westar® stoppers were purchased from West Pharmaceutical Services (Lionville, PA).

Methods

**IgG₁ Sample Preparation.** Buffer solutions, 10 mM sodium acetate pH 5.0 and 10 mM sodium phosphate pH 7.4 buffers, were prepared using MilliQ® water. Antistreptavidin was dialyzed overnight at 4°C separately against 10 mM sodium acetate pH 5 and 10 mM sodium phosphate pH 7.4 buffers. Following dialysis, IgG₁ concentration was measured using an Agilent® 8540 spectrophotometer (Santa Clara, CA). An extinction coefficient of 1.54 mL/mg-cm at 280 nm (extinction co-efficient provided by Amgen, Inc.) was used to estimate IgG₁ concentration.

**Air-Water Interface Rupture Experiment.** Buffer solutions, Transient rupture of the air-water interface in IgG₁ solution was performed by an experimental set-up similar to that described by Ruduiik et.al. First, 1 mL of 0.3 mg/mL IgG₁ solution in pH 5.0 or pH 7.4 formulation was transferred to a glass vial. Vials were filled with IgG₁ sample under a laminar air flow hood (Nuaire Biological Safety Cabinet, Class II, Type
A2, Model number Nu-437-600 Series 2). The vials containing IgG₁ solution were stoppered and crimped with aluminum seals. The stopper of IgG₁ containing vial was punctured vertically by an 18” gauge needle (BD, ready slip™) at an off-centered position (Figure IV.1, Panel A). The height of the needle was adjusted such that approximately 0.5 cm of the needle remained submerged in IgG₁ solution. The vials were clamped on a rotator (Rotomix Model No: RKVSD, Haverhill, MA) with the vertical axis of the vial making an angle of 30° to the horizontal beam of the rotator. The base of the rotator was maintained at 30°C to the horizontal. The vials were rotated at 25 rpm at room temperature. During rotation of vials under the above conditions, the needle tip periodically dipped in IgG₁ solution.

Two set of control experiments were performed. In the first control experiment, IgG₁ samples were rotated without a needle in an identical manner as described above. For the second control experiment, the vial stopper containing IgG₁ sample was punctured vertically at the center by a needle. The needle tip was adjusted to contact the vial bottom. The vials were secured on the rotator, upside down (Figure IV.1, Panel B). The rotator was kept inclined at 50° to the horizontal and rotated at 25 rpm at room temperature. During rotation, the needle always remained submerged in IgG₁ solution.

**Silicone Oil-Water Interface Rupture Experiment.** Transient rupture of the silicone oil-water interface in IgG₁ samples was performed as follows. First, glass vials were filled with 1 mL of 0.3 mg/mL IgG₁ solution. Then 100 µL of silicone oil was gently added using a positive displacement pipette tip.
Due to its lower density, silicone oil phase separated from the aqueous solution. The vials were stoppered, crimped and punctured with a needle either at a centered or an off-centered position in an identical manner as described above. IgG₁ samples containing silicone oil were rotated in the presence or absence of needle in an identical manner as described for the air-water interface experiments.

**Size Exclusion Chromatography.** An Agilent 1100 high performance liquid chromatography (HPLC) system (Santa Clara, CA) with a TOSOH G300 column (TOSOH Biosciences, PA) was used for size exclusion chromatography. The HPLC system was equilibrated with mobile phase buffer (300 mM sodium phosphate pH 7.0) at 0.6 mL/min. Fifty microliters of IgG₁ sample was injected and the eluting peak was detected at 280 nm by UV absorbance. The monomer peak was integrated by the Chemstation software provided by Agilent.
**Resonant Mass Measurement.** Sub-visible particles (≥ 0.3 µm) in IgG₁ samples was measured by Archimedes Particle Metrology System (Affinity Biosensors LLC, Santa Barbara, CA). Particle Lab software (version 1.8.1180) provided with the equipment was used for data collection and analysis. A density value of 1.33 g/mL was utilized for protein particles. For silicone oil containing samples, 0.97 g/mL was used as the density in the detection of silicone oil droplets. A resonant microfluidic channel, Micro-A788, was used for particle analysis. The instrument bins the silicone oil droplets as a positively buoyant peak and the protein particles as a negatively buoyant peak. The total concentration of protein particles in IgG₁ samples was obtained from the negatively buoyant peaks.

During sample measurement, approximately 500 µL of IgG₁ solution was transferred into an empty polypropylene eppendorf tube (Fisher Scientific catalog number 02-681-271). The sample was analyzed for either 10 minutes or until 200 particles were detected by the particle counter. Particle concentration in IgG₁ samples was analyzed from three separate vials. All particle size distributions were truncated at 0.3 µm. The average ± standard deviation of total concentration of negatively buoyant particles (≥ 0.3 µm) is reported.

**Flow Microscopy.** Cumulative counts of particles in IgG₁ samples having an equivalent circular diameter of ≥ 1 µm were estimated by microflow imaging (model #DPA 4100, Protein Simple®, Santa Clara, California). MFI view application software (version 6.9.8) provided with the equipment was utilized for data acquisition. During sample analysis, 500 µL of IgG₁ sample was gently pipetted into a barrier tip (Neptune
catalog# BT1000) and placed on the inlet port of the instrument. Prior to sample analysis, the background illumination of the MFI instrument was optimized with the sample buffer. Total particle concentration in IgG₁ samples was measured from three separate vials. Average ± standard deviation of total particle concentration is reported. Mass of sub-visible particles was estimated following a previous method.¹⁷

![Figure IV.2 Illustrative figure showing the real-time acquisition of protein particles and silicone oil droplets in IgG₁ samples by resonant mass measurement.](image)

IgG₁ Adsorption at the Air-Water Interface by Pendant Drop Tensiometry. Adsorption kinetics of IgG₁ at the air-water interface was assessed by pendant drop tensiometry. Pendant drop tensiometer measures air-water interfacial tension by axisymmetric drop shape analysis.⁶⁴ We used a custom-built pendant drop tensiometer equipped with an Artcam-130MI black and white CMOS camera (Artray, Tokyo, Japan) mounted on a VZM 450 microscope (Edmunds optics, Barrington, NJ). The image of outer diameter of the steel capillary used to form air bubble was used to calibrate the camera for interfacial tension measurement. FTA32 software (First ten angstroms, Portsmouth, VA) was used for image acquisition and analysis. Approximately 2 mL of
IgG₁ solution (0.1 mg/mL) was introduced inside the bubble chamber using a 5 mL plastic syringe (Cat. No. S7510-10, National Scientific, Rockwood, TN). Then an air-bubble was created on the tip of steel capillary by injecting air with a 100 µL Hamilton glass syringe. After the pendant shaped air-bubble was formed in IgG₁ solution, image acquisition was performed. Initially the camera was set to capture fifty frames in the first ten seconds. This was followed by the acquisition of two thousand images.

**IgG₁ Gelation at the Air-Water Interface.** IgG₁ gelation at air-water interface was assessed with the aid of an interfacial shear rheometer. The details of the construction and the working principle of the interfacial shear rheometer has been described by Sahin et.al. The procedure described in a previous study was followed in the current experiment to assess gelation time of IgG₁ at the air-water interface. Before each experimental run, the glass channel was cleaned with piranha solution (70% H₂SO₄+30% H₂O₂). During an experiment, a calibration run was first performed with 40 mL buffer solution in the glass channel. Following calibration, 30 mL of buffer solution was removed and replaced with an equal volume of IgG₁ solution so that IgG₁ concentration in the final solution was 5 µg/mL. Gelation measurement for IgG₁ samples was performed overnight. The elastic (G’) and viscous (G’´) modulus was determined from the experiment. Data analysis was performed using Igor Pro (version 4.0.6.1). G’ and G’´ were plotted versus time and the point where G’´ intersected G’ was reported as the gelation time.
**IgG₁ Adsorption on Silicone Oil Droplet.** Silicone oil in water emulsion (o/w) was prepared and characterized following a method described in detail by Ludwig *et al.* Surfactant-free, silicone oil-in-water emulsions were prepared and characterized in 10 mM sodium acetate pH 5.0 and 10 mM sodium phosphate pH 7.4 buffers. Adsorbed amount and footprint of IgG₁ on silicone oil droplets were measured by a centrifugation method described in previous in Chapter III. IgG₁ levels in silicone oil containing samples were determined from size exclusion chromatography. Area integration of SEC chromatograms was used to estimate IgG₁ adsorption onto silicone oil droplets.

**IgG₁ Adsorption on Immobilized Silicone Interface.** We utilized the method described by Klose *et al.* to assess IgG₁ adsorption at an immobilized silicone-water interface from adsorption breakthrough curves. Siliconized beads were prepared by siliconization of 20 g non-porous glass beads (≤106 µm diameter) following a previous published method. Then, 1.6 g of siliconized beads were packed in an empty TSK gel column (Part# 13075, Tosoh Bioscience LLC, Montgomeryville, PA). The column inlet was connected to an Agilent® HPLC pump and flushed with MilliQ water for 10 minutes at 1 ml/min. After flushing with water, the column was used for adsorption breakthrough curve measurement.

A Masterflex® peristaltic pump (Cole Parmer®, Illinois) with platinum cured tubing (catalog# 96420-13, Cole Parmer®, Illinois) was used to pump samples. The pump tubing was connected with the inlet of the siliconized bead column with PEEK tubing. The column outlet was connected to an UV flow cell (Starna Cells, Inc.™, Atascadero, CA, Catalog# 583.1F-Q-1/Z15) with PEEK tubing. Prior to an experiment,
the column was equilibrated with buffer at 0.2 mL/min for 10 minutes. IgG₁ solution (1 mg/ml) was pumped at 0.2 mL/min for five minutes. The UV absorbance at 280 nm was collected by an Agilent® 8543 spectrophotometer using the kinetic mode of the Chemstation software. The ratio of actual (Cᵢ) and final (C₀) absorbance of IgG₁ sample at 280 nm was plotted against time (seconds) to obtain the adsorption breakthrough curve. Uracil is a non-interacting compound that was used to determine t₀.²²,²³ The inflection time of the breakthrough curve from the x-axis was reported as the breakthrough time. IgG₁ (mg) adsorbed on siliconized beads was determined using equation (3).

\[ m_{\text{protein}} = \dot{v} c_p (t_c - t_0) - \dot{v} \int_{t_0}^{t_c} c(t) dt \]  

…Equation (4)

where \( m_{\text{protein}} \) is the mass (mg) of IgG₁ adsorbed, \( \dot{v} \) is the volume flow rate (0.2mL/min), \( t_c \) and \( t_0 \) are the breakthrough times for IgG₁ and a non-interacting compound (uracil) respectively. Using GRAMS/AI® 7.0 (Thermo Scientific, Rockford, Illinois), area integration was performed to determine \( \int_{t_0}^{t_c} c(t) dt \).

Specific surface area of siliconized beads was determined by Brunauer–Emmett–Teller (BET) isotherm analysis. The experiment was conducted using Gemini surface area analyzer (Micromeritics®, Norcross, GA). Nitrogen was used as the adsorbing gas. The mass of IgG₁ adsorbed being known, the adsorption loading of IgG₁ at silicone interface was determined as follows:
\[ \Gamma = \frac{m_{\text{protein}}}{A_{\text{spez}} \times m_{\text{adsorbent}}} \] … Equation (5)

where \( \Gamma \) is \( \text{IgG}_1 \) adsorption loading (mg/m\(^2\)), \( A_{\text{spez}} \) is the specific surface area of beads determined from BET and \( m_{\text{adsorbent}} \) is the mass of siliconized beads in the column.

**Electrostatic Potential of \( \text{IgG}_1 \).** Because the crystal structure of antistreptavidin is not known, the structure of related protein human \( \text{IgG}_1\)-b12 (Protein data bank entry 1HZH) was used as a template structure. A method described in detail by Yadav et al. was followed to assess the electrostatic potential of \( \text{IgG}_1 \).\(^{67}\) Briefly, the PDB file 1HZH was first converted to a PQR file in PDB2PQR converter\(^{68}\) using an AMBER force field. Then PROPKA was utilized to separately determine the protonation state of ions at a pH 5.0 and 7.4. Two separate PQR files were generated for \( \text{IgG}_1 \) at pH 5.0 and pH 7.4. The PQR files were used to generate the electrostatic potential by Adaptive Poisson Boltzman Solver (version 1.3) using the linearized Poisson-Boltzman equation. The calculation of surface charge was performed at a temperature of 298K, water dielectric constant of 78 and 2.0 Angstrom ionic radius. The electrostatic potential of \( \text{IgG}_1 \) was visualized using Pymol molecular graphic software (Schrodinger LLC, San Diego, CA).\(^{69}\)

**Results**

**Interface Rupture Experiment**

**Air-Water Interface.** Particle concentration was significantly higher in \( \text{IgG}_1 \) samples (pH 5.0 and pH 5.0) where the air-water interface was periodically ruptured (Figure IV.3, Panels A1 and B1). Control experiment showed that particle concentration
in IgG₁ samples did not increase significantly in the absence of interface rupture. In contrast, total concentration of particles in the interfacially ruptured IgG₁ samples increased significantly. Upon sixty hours of air-water interface rupture, the total concentration of protein particles in IgG₁ samples were $1.5 \times 10^7 \pm 0.4 \times 10^7$ particles/mL and $1.2 \times 10^7 \pm 0.6 \times 10^7$ particles/mL in pH 5.0 and pH 7.4 formulations respectively.

A significant increase in total particle concentration was also observed in IgG₁ samples that underwent rupture of the air-water interface (Figure IV.3, Panels A2 and B2). Upon sixty hours of air-water interface rupture, the average concentration of particles in IgG₁ samples were $0.1 \times 10^6 \pm 0.0 \times 10^6$ particles/mL at pH 5.0. In contrast, the particle concentration for the sixty hour ruptured pH 7.4 IgG₁ samples was $1.9 \times 10^6 \pm 1.4 \times 10^6$ particles/mL that was significantly higher than pH 5.0. Particle counts in IgG₁ samples rotated in the absence of needle did not increase significantly (data not reported).

Loss of IgG₁ monomer was not significant in pH 5.0 samples the air-water interface of which was ruptured (Figure IV.5, Panel A2). For pH 7.4 samples, although IgG₁ monomer decreased slightly in the ruptured samples, the loss was not significant upon 60 hours of interface rupture. Loss of IgG₁ monomer was not significant in samples rotated without interface rupture.

**Silicone Oil-Water Interface.** Periodic rupture of the silicone oil-water interface in IgG₁ samples significantly increased particle concentration (Figure IV.4, Panels A1 and B1). Control experiment showed that particle concentration in IgG₁ samples did not increase significantly without interface rupture. Upon sixty hours of interface rupture, the
total concentration of particles in IgG₁ samples were $1.5 \times 10^7 \pm 0.6 \times 10^7$ particles/mL and $0.8 \times 10^7 \pm 0.3 \times 10^7$ particles/mL in pH 7.4 and pH 5.0 formulations respectively.

**Figure IV.3** Concentration of particles in IgG₁ samples at pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations.

Open circles denotes IgG₁ samples in which the air-water interface was ruptured, Open squares denotes IgG₁ samples in which the air-water interface was not ruptured. Panels containing the numbers “1” and “2” represent particle concentration measured by resonant mass measurement and flow microscopy respectively. The average ± standard deviation of particle concentration from triplicate samples is reported.

Particle concentration also increased significantly in IgG₁ samples that underwent rupture of the silicone oil-water interface (Figure IV.4, Panels A2 and B2). Upon sixty hours of air-water interface rupture, the average concentration of particles in IgG₁ samples were $5.2 \times 10^6 \pm 2.7 \times 10^6$ particles/mL and $0.8 \times 10^6 \pm 0.5 \times 10^6$ particles/mL in pH 7.4 and pH 5.0 formulations respectively. Particle concentration did not increase
significantly in IgG₁ samples rotated in the absence of interface rupture. Similarly, particle counts in IgG₁ samples containing a layer of silicone oil and rotated in the absence of needle, did not increase significantly (data not reported). Silicone oil droplets in IgG₁ samples can potentially increase particle concentration by flow microscopy. But increase in particle concentration due to silicone oil droplets was not significant in the current study.

**Figure IV.4** Concentration of particles in IgG₁ samples at pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations.

Open circles denote IgG₁ samples in which the silicone oil-water interface was ruptured. Open squares denotes IgG₁ samples in which the silicone oil-water interface was not ruptured. Panels containing the numbers “1” and “2” represent particle concentration measured by resonant mass measurement and flow microscopy respectively. The average ± standard deviation of particle concentration from triplicate samples is reported.
IgG₁ monomer loss did not occur in the majority of IgG₁ samples. A significant loss of IgG₁ monomer was only observed in the 60 hour pH 7.4 IgG₁ samples where the silicone oil-water interface was ruptured (Figure IV.5, Panel B2).

**Figure IV.5** Percent monomer remaining in IgG₁ samples from air-water interface rupture (Panel A) and silicone oil-water interface rupture (Panel B) experiments.

Panels containing the numbers “1” and “2” represents pH 5.0 and pH 7.4 samples respectively. Open circles denote IgG₁ samples in which interface was ruptured. Open squares denote IgG₁ samples in which the interface was not ruptured. The average ± standard deviation of % IgG₁ monomer from triplicate samples is reported.

**IgG₁ Adsorption at Air-Water Interface**

Adsorption of IgG₁ at the air-water interface lowered the interfacial tension in pH 5.0 and pH 7.4 samples (Figure IV.6). Compared to pH 7.4 formulation, lowering of the interfacial tension was significantly drastic in pH 5.0 samples of IgG₁. In pH 5.0
formulation, the interfacial tension decreased from 72 mN/m to 55 mN/m in two hours. But in two hours, the interfacial tension only decreased from 72 mN/m to 69 mN/m in pH 7.4 samples.

**IgG₁ Gelation at the Air-Water Interface**

We assessed IgG₁ gelation at air-water interface to gain insights into the mechanism of particle formation during needle rupture of the air-water interface in IgG₁ samples. Representative figures of the interfacial shear modulii of IgG₁ in pH 5.0 and pH 7.4 formulations with time are shown in Figure IV.7. During IgG₁ gelation, the elastic (G’) and viscous (G’”) modulii gradually increased, eventually intersecting one another. An increase in either G’ or G’” was not observed in protein-free buffer solution (data not shown).

Two significant differences in gelation data were observed between pH 5.0 and pH 7.4 formulations of IgG₁. First, IgG₁ gelation time was significantly shorter at pH 7.4 than pH 5.0 (Table IV.1). Secondly, the increase in the elastic modulus (G’) with aging time was significantly faster at pH 7.4 than pH 5.0. For example, after one hour the G’ of IgG₁ was 0.37 ± 0.01 mN/m in pH 7.4 and 0.14 ± 0.03 mN/m in pH 5.0 samples.

**Table IV.1** Gelation time and elastic modulus of IgG₁ layer at the air-water interface.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>pH 5.0</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelation Time (hrs)</td>
<td>0.80 ± 0.10</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td>Elastic Modulus (G’) at 1 hr</td>
<td>0.14 ± 0.03</td>
<td>0.37 ± 0.01</td>
</tr>
</tbody>
</table>
Figure IV.6 Interfacial tension of IgG\textsubscript{1} samples at the air-water interface at pH 5.0 (green line) and pH 7.4 (red line) formulations. The average ± standard deviation of interfacial tension from triplicate samples is reported.

Figure IV.7 Representative plot of elastic (G' in red) and viscous (G'' in blue) modulus of IgG\textsubscript{1} at air-water interface in pH 7.4 (Panel A) and pH 5.0 (Panel B) formulations.
IgG₁ Surface Charge

The electrostatic potentials of IgG₁ at pH 5.0 and pH 7.4 are shown in Figure IV.8. It was observed that the surface charge distribution of IgG₁ in pH 5.0 formulation was predominantly positive. Neutral or negatively charged patches were not detected. Unlike pH 5.0 formulation, the pH 7.4 formulation contained neutral uncharged and negatively charged patches as well as positive charges.

![Figure IV.8](image)

**Figure IV.8** Surface charge distribution of IgG₁ at pH 5.0 (Panel A) and pH 7.4 (Panel B) formulations.

IgG₁ Adsorption at Silicone Oil-Water and Immobilized Silicone-Water Interfaces

Adsorbed amounts of IgG₁ on silicone oil droplets in pH 5.0 and pH 7.4 formulations were comparable and in agreement with reported values²⁻⁴⁰ (Figure IV.9, Table IV.2). The adsorption footprint of IgG₁ at monolayer coverage has been reported to range between 0.26-1.04 m²/mg.⁴¹ The experimentally determined adsorption footprint of IgG₁ at the silicone oil-water interface were comparable to estimated values of IgG₁ footprint for monolayer coverage.
Table IV.2 Adsorption loading and footprint of IgG₁ at silicone oil-water interface.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Silicone Oil</th>
<th>Siliconized Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adsorption Footprint (m²/mg)</td>
<td>Adsorbed Mass (mg/m²)</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>0.46 ± 0.02</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>0.40 ± 0.01</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

The relative retention times of the IgG₁ adsorption breakthrough curves were 243 ± 4 seconds in the pH 5.0 formulation and 257 ± 5 seconds in the pH 7.4 formulation (Figure IV.10). From BET measurements, the estimated specific surface area of the siliconized beads was estimated to be 0.18 ± 0.01 m²/gm. The surface area of immobilized silicone packed in the column was estimated to be 0.3 m² using values of specific surface area and average weight of siliconized beads (1.6 g). Finally, using these values, IgG₁ adsorbed at the immobilized silicone-water interface (Γ) was estimated to be 3.0 ± 0.1 mg/m² in the pH 5.0 formulation and 2.4 ± 0.2 mg/m² in the pH 7.4 formulation (Table IV.2).

Discussion

Rupturing the adsorbed IgG₁ layer at the air-water interface accelerated particle formation in IgG₁ samples. Adsorption of protein at the air-water interface is often accompanied with gelation of the adsorbed protein layer. During gelation, condensed layers of protein films are formed at the air-water interface. Interfacially adsorbed gelled protein layers are viscoelastic and may undergo deformation upon application of a mechanical stress. The gelled IgG₁ layer at the air-water interface was presumably
punctured during interface rupture, leading to increased counts of particles. A few studies have linked interfacially adsorbed protein layers to aggregation during dynamic processes e.g. agitation, shearing. In one such study, shearing of the IgG₁ layer adsorbed at the steel-water interface was reported to form aggregates in IgG₁ formulations. In another study, rupturing the air-water interface in IgG samples was reported to accelerate aggregation.\textsuperscript{63}

Interfacial shear rheometry was performed to assess interaction between IgG₁ molecules adsorbed at the air-water interface. It was observed that compared to pH 5.0, IgG₁ gelation at the air-water interface was significantly faster at pH 7.4. The iso-electric pH (pI) of IgG₁ was 8.6 (value provided by Amgen Inc.).

![Figure IV.9](image.png)

**Figure IV.9** Adsorption footprint of IgG₁ on silicone oil droplets in pH 5.0 (open square) and pH 7.4 (open triangle) formulations.

The average ± standard deviation of adsorption footprint from triplicate samples is reported. Error bars may be smaller than the symbol.
Figure IV.10 Adsorption breakthrough curve IgG₁ in pH 5.0 (green) and pH 7.4 (red) formulations (Panel A).

The average ± standard deviation from triplicate samples is reported. Example first derivative of the adsorption breakthrough curve (Panel B).

Hence charges on IgG₁ molecule was expected to be predominantly positive at pH 5.0 than at pH 7.4. An assessment of the surface electrostatic potential of IgG₁ molecules at pH 5.0 and pH 7.4 suggested that charge distribution on IgG₁ molecules was significantly different in these two pHs that in turn may affect rate of IgG₁ gelation. IgG₁ molecule at pH 7.4 contained neutral, positive and few negative patches; at pH 5.0 charges on IgG₁ was predominantly positive (Figure IV.8). Consequently, at pH 7.4, charge repulsion between IgG₁ molecules was reduced which presumably accelerated protein association and gelation of the IgG₁ layer at the air-water interface. Similar observations have been reported in literature where gelation of interfacially adsorbed protein at the air-water interface was accelerated at the iso-electric pH.⁷²,⁷³

In the current study, increase in the elastic modulus of the IgG₁ layer adsorbed at the air-water interface was significantly faster at pH 7.4, compared to pH 5.0 (Table IV.1). Interestingly, elastic modulus of the IgG₁ layer adsorbed at the air-water interface correlated with particle formation. Compared to the pH 5.0, increase in particle counts in
IgG₁ samples was significantly accelerated at pH 7.4 where the elastic modulus of the adsorbed IgG₁ film was also higher. The elastic modulus or G’ of the interfacial protein gel at the air-water interface is indicative of the recoverable energy stored in the gelled protein layer. In literature, larger value of elastic modulus of adsorbed protein film has been associated with fracture of interfacially adsorbed protein film. Since gelation of IgG₁ at air-water interface was relatively faster in pH 7.4 samples, G’ of the IgG₁ film at the air-water interface increased rapidly that possibly accelerated particle formation.

Interface rupture and particle formation in IgG₁ samples appears to be a dynamic process. Upon interface rupture, the protein layer at the air-water interface is displaced, prompting IgG₁ adsorption at the freshly exposed interface. Results from the pendant drop tensiometry suggested that lowering of the interfacial tension during IgG₁ adsorption at the air-water interface occurred relatively faster at pH 5.0 than at pH 7.4. Possibly, IgG₁ at the air-water interface interacted strongly and rapidly gelled that decreased the rate of lowering of the interfacial tension in pH 7.4 samples. A similar observation has been reported in literature where reduction in the rate of lowering of interfacial tension at the air-water interface was postulated to be a result of strong interaction between protein molecules in the adsorbed layer. The rate of lowering of interfacial tension is expected to be important in relation to particle formation during interface rupture experiment. If air-water interfacial tension is lowered rapidly, energy released during interface rupture will be smaller, a factor that is postulated to affect particle formation in IgG₁ samples.

The effect of silicone oil-water interface rupture on particle formation in IgG₁ samples was also assessed in the current study. Like the air-water interface results,
particle counts significantly increased in IgG$_1$ samples during rupture of the silicone oil-water interface. Particle concentration in the interfacially ruptured IgG$_1$ samples was also significantly higher at pH 7.4 than pH 5.0. Perhaps a phenomenon similar to that described for the air-water interface increased the levels of particles in silicone oil samples. A monolayer of IgG$_1$ adsorbed at the silicone oil-water interface,$^{79,80}$ gelled, which when ruptured formed particles in IgG$_1$ samples. Several studies have reported an increase in the G’ of the adsorbed protein layer at the oil-water interface.$^{81-83}$

IgG$_1$ adsorption at the immobilized silicone-water interface in pH 5.0 and pH 7.4 samples were also assessed from adsorption breakthrough curves. Apart from determining adsorbed amounts of IgG$_1$, the shape of the adsorption breakthrough curves has been utilized to assess protein interactions at solid-liquid interfaces.$^{84}$ The adsorbed mass of IgG$_1$ measured from the breakthrough curves was comparable to monolayer coverage (Table IV.2), an observation that was similar to IgG$_1$ adsorption on silicone oil droplets. However, the shapes of the adsorption breakthrough curves of IgG$_1$ at the immobilized silicone-water interface were significantly different between pH 7.4 and pH 5.0 samples. At pH 7.4, the adsorption breakthrough curve was predominantly steep. In contrast, the adsorption breakthrough curve in pH 5.0 samples significantly broadened prior to reaching a plateau (Figure IV.10, Panel A). Broadening of the adsorption breakthrough curve has been suggested to be reminiscent of protein conformational changes,$^{85}$ molecular rearrangements$^{86}$ in the interfacially adsorbed layer and reversible binding sites for adsorption.$^{84}$ A first derivative of the adsorption breakthrough curve demonstrates the significant differences in curve shape between pH 5.0 and pH 7.4 samples. The right shoulder of the first derivative curve illustrates significant tailing at
pH 5.0 in comparison to pH 7.4 samples (Figure IV.10, Panel B). It is possible that broadening of the adsorption breakthrough curve at pH 5.0 occurred due to molecular rearrangement of adsorbed IgG\textsubscript{1} molecules or reversible binding at the immobilized silicone-water interface the origin of which can be attributed, at least in part, to the positive charges on IgG\textsubscript{1}. In contrast, electrically neutral IgG\textsubscript{1} adsorbed at the immobilized silicone-water interface presumably interacted strongly at pH 7.4, minimizing subsequent IgG\textsubscript{1} adsorption; as a result, the breakthrough curve was steep and symmetric. A similar observation has been reported in literature. Broadening of the adsorption breakthrough curve of lysozyme on a non-porous column matrix (silica, glass) was due to “secondary equilibrium effects” caused by molecular rearrangement of the pre-adsorbed protein. The use of breakthrough curves is important in understanding IgG\textsubscript{1} interactions at an immobilized silicone-water interface, and provides us with a picture of molecular interactions occurring in the adsorbed protein layer.

**Conclusions**

Particles formed in therapeutic protein formulations due to agitation are problematic. Therapeutic proteins may undergo adsorption and gel at the air-water or silicone oil-water interface. Dynamic processes like agitation during shipping have the potential to rupture the interfacially adsorbed IgG\textsubscript{1} layer. Consequently, particles can be formed in therapeutic protein formulations. Interfacial adsorption and gelation of protein appears to be a key component in particle formation during dynamic processes causing interfacial rupture. The pH of the formulation appears to significantly affect interfacial
adsorption and viscoelasticity of adsorbed protein films that in turn influence particle formation.
CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Therapeutic proteins are an important class of biotherapeutic that is of significant interest in the biopharmaceutical field. The primary containers used for therapeutic proteins are different in variety and complexity. Primary containers for therapeutic protein drug products encompass vials, prefilled syringes, dual-chambered syringes, cartridges and auto-injector pens. Consequently, therapeutic protein formulation comes in contact with the interfaces of the primary container. Advantages like ease of self administration and reduced cost makes prefilled syringes an attractive choice as a primary container for drug products. Therapeutic proteins are often developed in siliconized prefilled syringes e.g. Humira, Enbrel. Occasionally, visible particles occasionally are formed in therapeutic protein formulations in prefilled syringes have been linked to silicone oil. Also, the level of sub-visible particles in therapeutic protein formulations in siliconized prefilled syringes is often very high.

Silicone oil in prefilled syringes has been often linked to particles in therapeutic protein formulations. Prior to the work described in this thesis, a detailed understanding of sub-visible particles in therapeutic protein formulation due to silicone oil interface was not known. Relevant approaches to assess sub-visible aggregates in therapeutic protein formulation containing silicone oil interface were very limited. Additionally, the mechanism behind the apparent synergism between agitation and silicone oil that has been reported to accelerate therapeutic protein aggregation remained unclear. The work described in this thesis advances our understanding on protein adsorption and aggregation at silicone oil-water interface. The work described in this thesis put forwards approaches
that can be utilized in the evaluation of particles in therapeutic protein formulations that are intended to be developed for siliconized prefilled syringes. Assessment of aggregates, sub-visible particles and protein adsorption at the silicone oil-water and air-water interfaces has led to the following conclusions.

1. Formation of protein particles is accelerated in therapeutic protein samples during agitation with immobilized silicone interface – Mass of protein particles represents a tiny fraction of total protein in the sample.

2. Tertiary structure of therapeutic protein adsorbed at the silicone oil-water interface can be potentially perturbed – Perturbation of tertiary structure in the adsorbed layer can be minimized by incorporating excipients like sucrose and sodium chloride in the formulation.

3. Surfactants in therapeutic protein formulations can minimize aggregation and particle formation in agitated samples containing silicone oil droplets or immobilized silicone interface.

4. Rupture of therapeutic protein layer at air-water or silicone oil-water interfaces accelerate the formation of sub-visible particles in protein samples.

5. The rate of gelation of the adsorbed protein layer at the air-water interface affects particle formation during interface rupture – Charge distribution of protein appears to affect protein gelation at the air-water interface.

6. Adsorption breakthrough curves can be utilized to assess therapeutic protein adsorption at immobilized silicone-water interface.
More work is warranted to understand particle formation in therapeutic protein formulations at silicone oil-water and air-water interfaces. In this work, two pHs (pH 5.0 and pH 7.4) were utilized to evaluate particle formation in IgG1 samples. A similar experimental approach can be utilized to evaluate particle formation in different antibody formulations during interface rupture. Gelation of therapeutic protein at silicone oil-water interface was not assessed in the current study. Additional studies are required to assess therapeutic protein gelation at silicone oil-water interface.
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