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Particle Formation and Aggregation of a Therapeutic Protein in Nanobubble Suspensions

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Abstract

The generation of nanobubbles following reconstitution of lyophilized trehalose formulations has recently been reported.¹ Here, we characterize particle formation and aggregation of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) in reconstituted formulations of lyophilized trehalose. Particle characterization methods including resonant mass measurement and nanoparticle tracking analysis were used to count and size particles generated upon reconstitution of lyophilized trehalose formulations. In addition, accelerated degradation studies were conducted to monitor rhIL-1ra aggregation in solutions containing various concentrations of suspended nanobubbles. Reconstitution of lyophilized trehalose formulations with solutions containing rhIL-1ra reduced nanobubble concentrations and generated negatively buoyant particles attributed to aggregated rhIL-1ra. Furthermore, levels of rhIL-1ra aggregation following incubation in aqueous solution correlated with concentrations of suspended nanobubbles. The results of this study suggest nanobubbles may be a contributor to protein aggregation and particle formation in reconstituted, lyophilized therapeutic protein formulations.

Keywords

protein aggregation; nanobubbles; particle characterization

INTRODUCTION

Sub-visible particles within therapeutic protein products have become the focus of increased regulatory concern due to the association of particles with adverse immune responses in patients.^{2–5} This focus has led to significant efforts within the biopharmaceutical industry to count and characterize populations of sub-visible particles within formulations. Recently, a new source of particles in therapeutic formulations was identified by Zhou et al., who

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showed that large numbers of stable nanobubbles were generated following reconstitution of lyophilized trehalose formulations.¹ Nanobubbles within reconstituted lyophilized formulations will likely contribute to counts of sub-visible particles, but it is not known whether nanobubbles also may promote protein aggregation and particle formation.

Currently, nanobubbles are a growing area of research, with significant focus on understanding the mechanism(s) responsible for the stabilization of the 100–200 nm diameter bubbles^{6–8} in bulk aqueous solutions. Bubbles with diameters on the nanometer scale might be expected to be unstable due to high internal pressures generated by surface tension at the air-water interface.^{9–11} If the surface tension at the nanobubble-water interface were typical of air-water interfaces, the Young-Laplace equation would predict that high pressures within the nanobubbles should result in dissolution and disappearance of the nanobubbles on the order of microseconds.¹² However, studies have reported long-term (meta)stability of nanobubbles, with lifetimes ranging from hours¹³ to several months.⁶ It has been proposed that nanobubble stability may be dependent on the selective adsorption of anions at the nanobubble interface.^{10, 14, 15} At the nanobubble scale, ions adsorbed to the nanobubble interface could be in close enough proximity to result in repulsive forces strong enough to balance the compressive force from surface tension. The balance of these two forces would be expected to result in a nanobubble with a stable diameter where the dissolution of gas from the bubble would be minimized.

Suspensions of nanobubbles have been explored as cleaning agents to remove proteins from surfaces,^{16, 17} but there have been no published examples of research on the effect of nanobubbles on protein aggregation and particle formation. It is well-established that protein molecules have a tendency to interact with and adsorb to various interfaces.^{18, 19} In particular, due to their amphiphilic nature, proteins adsorb readily to air-water interfaces. The tendency of a protein to adsorb to a hydrophobic interface results mainly from the hydrophobic interactions between exposed hydrophobic residues and the air-water interface, although electrostatics may also play a role.^{20, 21} Following adsorption, reorientation of hydrophilic and hydrophobic residues of protein often occurs, resulting in loss of native structure and production of gel-like protein layers.^{22, 23} Conformational changes resulting from adsorption to the air-water interface have been associated with reduced efficacy in protein therapeutics.²⁴ Adsorption of protein particles to nanobubbles could result in protein particles and/or hybrid protein-air bubble nanoparticles, and potentially nucleate protein aggregation in the bulk solution.

In this study, we investigated whether nanobubbles generated following reconstitution of lyophilized disaccharide formulations could induce protein aggregation and particle formation in formulations containing recombinant human interleukin-1 receptor antagonist (rhIL-1ra). Particles generated following reconstitution of lyophilized disaccharide formulations were characterized using a combination of resonance mass measurement (RMM), nanoparticle tracking analysis (NTA) and zeta potential measurements. Lyophilized trehalose and sucrose samples were reconstituted either with a buffer solution alone or with a buffer solution that contained rhIL-1ra, and the number and characteristics of particles present in the resulting solutions were compared. Accelerated degradation studies in aqueous solution were then conducted to determine if the presence of nanobubbles could induce

rhIL-1ra aggregation during extended incubation. In other studies, polysorbate 20 (PS20) and ethylenediaminetetraacetic acid (EDTA) were added to rhIL-1ra formulations that contained nanobubbles to investigate whether these excipients would alter aggregation of rhIL-1ra in nanobubbles suspensions.

MATERIALS AND METHODS

Materials

Recombinant human interleukin-1 receptor antagonist (rhIL-1ra) at a concentration of 100 mg/mL was donated by Amgen (Thousand Oaks, CA). High purity (low endotoxin) grade trehalose was obtained from Pfanstiehl (Waukegan, IL) and EMPROVE® low endotoxin sucrose was purchased from EMD Millipore (Billerica, MA). Urea, sodium chloride and PS20 Surfact-Amps detergent solution were obtained from Fisher Scientific (Hampton, NH), and trisodium citrate dihydrate was acquired from Alfa Aesar (Ward Hill, MA). 2-mercaptoethanol and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). Quick Start™ Bradford 1x dye reagent was obtained from Bio-RAD (Hercules, CA).

Methods

Sample Preparation—All buffer solutions contained 10 mM sodium citrate, and hydrochloric acid was added to adjust to pH values of 4.6, 5.4 and 6.6. Solutions were filtered with a 0.22 µm Millipore filter prior to use (Billerica, MA) unless otherwise noted. A stock solution containing 100 mg/ml rhIL-1ra was dialyzed three times into 10 mM sodium citrate buffer pH 6.6 using 10 kDa molecular-weight cutoff Slide-A-Lyzer cassettes (Pierce, Rockford, IL). Concentrated rhIL-1ra solutions at pH 6.6 were diluted in the respective buffers to obtain 1 mg/mL concentrations of rhIL-1ra at the buffer conditions required for the study.

Lyophilization Procedure—Before use, 20 mL Fiolax glass lyophilization vials purchased from Schott (Lebanon, PA) were soaked overnight in Hellmanex III cleaning solution (Müllheim, Germany). The following day, vials were rinsed and soaked in tap water to remove the remaining detergent. The vials were then thoroughly rinsed with filtered (0.22 µm) deionized ultrapure water and subsequently rinsed with 0.22 µm-filtered 190 proof ethanol. After vials were allowed to dry for at least 12 hours, 3 mL aliquots containing either 10% (w/v) trehalose or 10% (w/v) sucrose dissolved in deionized ultrapure water and filtered with a 20 nm cutoff Anotop 25 inorganic membrane filter (GE Healthcare Life Sciences, Pittsburgh, PA) were added by pipette. The contents of the vials were frozen by submersing the vials in liquid nitrogen for two minutes and stored at –80 °C prior to lyophilization. Frozen samples were loaded into a FTS Lyostar I lyophilizer on shelves precooled to –40 °C and allowed to equilibrate for 300 minutes. After equilibration, primary drying was initiated at a shelf temperature of –20 °C for 1700 minutes with the chamber pressure set to 9.3 Pa. To initiate secondary drying the shelf temperature was raised to 33 °C at a rate of 0.1 °C/min. The shelf temperature was then held at 33 °C for four hours. Upon completion of secondary drying the chamber was back-filled with nitrogen and the vials were sealed.

Reconstitution of Lyophilized Formulations—To reconstitute samples to the original 3 mL volumes to which the vials were filled prior to lyophilization, 2.80 mL of reconstitution solution was pipetted into vials containing lyophilized sucrose or trehalose. The required volume for reconstitution was determined by adding sufficient water so that the sample weight after reconstitution was the same as that prior to lyophilization. Solutions containing 1 mg/mL rhIL-1ra were filtered using a 100 nm Anotop 10 inorganic membrane filter (GE Healthcare Life Sciences, Pittsburgh, PA) prior to reconstituting lyophilized cakes. Particles present in the reconstituted samples were analyzed immediately following reconstitution using the particle characterization methods described below.

Particle Characterization—Particle concentrations and diameters between 50 and 1000 nm were measured using a NanoSight NTA NS300 (Malvern Instruments Ltd, Malvern, UK) instrument. Prior to acquisition, shutter and gain values were optimized for analysis of the nanobubble samples and these settings were held constant for all samples. Sample solutions were continuously injected using a 1 mL silicone oil-free syringe while 30-second videos were recorded in triplicate. Videos were analyzed for particle concentration and diameter using NanoSight NTA 3.0 software, with particle diameter calculated using a solution viscosity of 1.3 centipoise²⁵.

An Archimedes instrument (Malvern Instruments Ltd, Malvern, UK) that relies on the principle of RMM was used to measure particle size distributions for populations of particles differentiated by their respective densities. Concentrations and diameters of negatively and positively buoyant particles were measured for particles with sizes between approximately 0.1 and 5 μm . For calculation of particle diameters, the density of air and rhIL-1ra were provided as 0.00123 g/cm³ and 1.3 g/cm³ respectively. Samples were filtered with a 5 μm filter needle (BD, Franklin Lakes, NJ) prior to loading approximately 150 μL of sample volume. Samples were analyzed for 10 minutes and between samples the sensor was flushed with deionized ultrapure water for 60 seconds.

Zeta potentials were measured using a Zetasizer[®] (Malvern Instruments Ltd, Malvern, UK) using the procedure described by Zhou et al.¹

Accelerated rhIL-1ra Degradation Studies in Nanobubble Suspensions—To determine whether nanobubbles are capable of inducing protein aggregation in formulations containing rhIL-1ra, accelerated degradation studies were conducted using nanobubble suspensions. For degradation studies, nanobubble suspensions were generated immediately before use by reconstituting lyophilized formulations of trehalose with 2.80 mL of 10 mM sodium citrate buffer pH 6.6. For initial studies, nanobubble suspensions generated from reconstitution were used directly or diluted with a filtered (0.22 μm) nanobubble-free control solution consisting of 10% (w/v) trehalose, 10 mM sodium citrate pH 6.6 by factors of 2, 3, 6 and 20. Aliquots (225 μL) of undiluted and diluted nanobubble suspensions as well as the nanobubble-free control solution were combined with 25 μL aliquots of 10 mg/mL rhIL-1ra formulated in 10 mM sodium citrate buffer pH 6.6 to obtain samples containing 1 mg/mL rhIL-1ra and a final volume of 250 μL . Samples were then placed in a 40 °C incubator and after 23 hours rhIL-1ra aggregation was quantified with a Bradford protein assay using the procedure described below. Additional degradation studies utilized the same procedure for

sample preparation described above unless otherwise specified. All subsequent studies were conducted with nanobubble suspensions generated directly from reconstitution of lyophilized formulations without dilution.

To monitor the time-dependent aggregation of rhIL-1ra in nanobubble suspensions, nanobubble suspensions containing 1 mg/mL rhIL-1ra were incubated at a 40 °C and sampled at various time points over a 23 hour period. Following an initial time point, samples were removed from a 40 °C incubator every 3 hours for the first 12 hours and at the completion of the study after 23 hours. Samples were stored at 4 °C until completion of the study and then were analyzed simultaneously. Negligible rhIL-1ra aggregation was found to occur in nanobubble suspensions stored at 4 °C for at least 23 hours.

To evaluate how disaccharide excipients influenced rhIL-1ra aggregation in nanobubble suspensions, rhIL-1ra aggregation was compared in nanobubble suspensions generated by reconstitution of lyophilized sucrose and trehalose. Nanobubbles were generated by reconstituting lyophilized formulations of sucrose using the methods described previously for trehalose, and rhIL-1ra aggregation in the nanobubble-containing reconstituted samples was quantified following 23 hours of incubation at 40 °C.

To determine how other common excipients such as surfactants or chelators might alter rhIL-1ra aggregation in formulations containing suspended nanobubbles, samples containing either PS20 or EDTA were studied. Solutions of 0, 0.05, 0.5, 2.5, 5, 7.5 and 10% (w/v) PS20 were prepared by diluting 10% (w/v) PS-20 solution with 10 mM sodium citrate buffer pH 6.6. Aliquots (5 µL) of these PS20 solutions were added to 225 µL of nanobubble suspensions previously generated by reconstitution of lyophilized trehalose cakes with 10 mM sodium citrate buffer. 20 µL of a 12.5 mg/mL solution of rhIL-1ra were added to these solutions to obtain a final a volume of 250 µL, resulting in a rhIL-1ra concentration of 1 mg/mL and PS20 concentrations of 0, 0.001, 0.01, 0.05, 0.1 0.15 and 0.2 % (w/v). In a separate study, 5 µL of a 250 mM EDTA solution in 10 mM sodium citrate buffer pH 6.6 was added to 225 µL of nanobubble suspension. Nanobubble and EDTA solutions were combined with 20 µL of 12.5 mg/mL of rhIL-1ra, resulting in a final EDTA concentration of 5 mM. For both studies, rhIL-1ra aggregation was quantified using a Bradford assay following 23 hours of incubation at 40 °C.

Quantification of rhIL-1ra Aggregation—Upon completion of the incubation period, 250 µL samples were centrifuged for 20 minutes at 15,000 × g in an Eppendorf 5424R centrifuge that was maintained at 4 °C. Following centrifugation, a pellet of aggregated rhIL-1ra was observable on the bottom of the centrifuge tube. Resulting from the small quantity of aggregated rhIL-1ra, it was found that aggregation could be quantified most accurately by measuring the amount of pelleted rhIL-1ra directly using the Bradford protein assay. For this method, 150 µL of supernatant were carefully removed to avoid disturbing the pellet and the pellet was washed with 400 µL of 10 mM sodium citrate buffer pH 6.6. The washing step was repeated three times. For subsequent washing steps, 400 µL was removed from the supernatant and the volume was replenished with fresh buffer. The samples were centrifuged at 15,000 × g after each washing step. Following the washing steps, 400 µL of supernatant were removed and the pellet was solubilized by adding 100 µL of 8 M Urea,

10% (v/v) 2-mercaptoethanol to the pelleted rhIL-1ra and incubating for 1 hour at room temperature. Aliquots of 150 μ L of the solubilized pellet were then added to a 96 well plate. Standards for rhIL-1ra were prepared in the same plate using a stock solution containing 10 mg/ml rhIL-1ra to obtain rhIL-1ra concentrations between 0.0 to 50 μ g/mL in 10 mM sodium citrate, 4 M urea and 5% (v/v) 2-mercaptoethanol at a volume of 150 μ L. In this concentration range a plot of absorbance versus rhIL-1ra concentration was linear (data not shown) as required by the Bradford protein assay. 150 μ L of Bradford reagent were added to each well and the solutions were mixed thoroughly using a pipette. After a 10 minute incubation period at room temperature the absorbance of each well was measured at 595 nm using a plate reader (Molecular Devices, Sunnyvale California). Using the standard protein solutions, a standard curve of absorbance versus rhIL-1ra concentration was constructed from the absorbance at 595 nm. Using this standard curve, the quantity of rhIL-1ra present in each well could be calculated.

SE-HPLC Analysis of Soluble Aggregates—Size exclusion HPLC (SE-HPLC) was conducted with a Beckman Gold HPLC system (Beckman Coulter, Fullertown California) to detect soluble aggregates of rhIL-1ra. Samples were centrifuged at 15,000 g (4 °C) for 20 minutes prior to injecting 50 μ L of sample into a TSK_{gel} G2000SW_{xl} column (Tosoh Biosciences, Montgomeryville Pennsylvania). Eluent was monitored at 280 nm using a System Gold 168 detector while operating at a flow rate of 0.6 ml/min with a mobile phase consisting of 10 mM sodium citrate with 140 mM sodium chloride (pH 6.5).

Statistics and Reproducibility—For all analyses, at least three independent samples were measured for each condition. The average value of three measurements is reported. Reported errors represent $\pm$ 1 standard deviation.

RESULTS

Characterization of Particles Generated Following Reconstitution

To confirm the previously-reported generation of nanobubbles from lyophilized trehalose cakes, lyophilized formulations consisting of 10% (w/v) trehalose were reconstituted with 10 mM sodium citrate buffer. NanoSight NTA analysis of reconstituted samples detected the generation of a large number of nanoparticles with concentrations on the order of 2×10^9 nanoparticles/mL (Figure 1A). The particle diameters were approximately 100 nm (Figure 1B). Adjusting the pH of the buffer solution used for reconstitution did not appear to significantly alter the size or concentration of particles generated. RMM analysis of the generated particles confirmed that the particles were nanobubbles because large quantities of positively buoyant particles were measured whereas the counts of negatively buoyant particles were negligible (Figure 2). A large difference in the magnitude of nanobubble concentrations can be observed between those measured by RMM and NTA. This discrepancy can be attributed to the reduced sensitivity of RMM, as the lower size limit of detection for this method is typically greater than the mean nanobubble diameter reported by NTA. The particle concentrations measured by both RMM and NTA are consistent with recently published findings of Zhou et al. which reported the generation of nanobubbles in lyophilized formulations of trehalose after reconstitution.¹

To investigate the potential effects of rhIL-1ra on nanobubble formation and nanobubbles on rhIL-1ra particle formation, formulations containing 10% (w/v) trehalose were reconstituted with solutions that contained 1 mg/ml rhIL-1ra in 10 mM sodium citrate at pH values of 4.6, 5.4 and 6.6. Reconstitution of lyophilized cakes with the rhIL-1ra solutions resulted in suspensions with reduced nanobubble concentrations (determined by NTA) as compared to suspensions prepared by reconstitution of lyophilized trehalose cakes with the corresponding rhIL-1ra-free buffer solutions (Figure 1B). In contrast, nanobubble diameters were unchanged by the presence of rhIL-1ra (Figure 1B). Using RMM, negatively buoyant particles that could be attributed to rhIL-1ra particles were detected following reconstitution with buffer containing rhIL-1ra (Figure 2B). The concentration of these negatively buoyant particles was dependent on the solution pH. RhIL-1ra particle formation was minimized (1.7×10^6 particles/mL) at a pH corresponding to rhIL-1ra's isoelectric point of 5.4, and increased at solution pH's above and below the pI of rhIL-1ra (3.4×10^6 and 2.5×10^6 particles/mL at pH 4.6 and 6.6, respectively).

Zeta potential measurements were used to determine the effective charge at the nanobubble surface. At all pH values tested, zeta potentials of nanobubbles suspensions without rhIL-1ra were negative (Table 1). Reconstitution with rhIL-1ra resulted in a significant changes in the magnitude of the zeta potential of the system which were pH dependent. At buffer pH conditions of 4.6 and 5.4 the nanobubble zeta potential closely matched that of the rhIL-1ra alone. At pH 6.6, however, the zeta potential measured in 1 mg/mL rhIL-1ra solutions containing suspended nanobubbles (-20.6 ± 0.6 mV) differed significantly from that of solutions of the rhIL-1ra alone (-3.96 ± 4.7 mV).

Accelerated rhIL-1ra Degradation Studies in Nanobubble Suspensions

To further study the influence of nanobubbles on rhIL-1ra aggregation, accelerated rhIL-1ra degradation studies were conducted wherein rhIL-1ra was added to suspensions of nanobubbles generated by reconstitution of lyophilized trehalose cakes with 10 mM sodium citrate buffer pH 6.6. Incubation of rhIL-1ra in these nanobubble suspensions for 23 hours at 40 °C yielded rhIL-1ra precipitates that were visible following centrifugation. In contrast, no precipitates were observed when rhIL-1ra was incubated in nanobubble-free solutions of 10% (w/v) trehalose. In previous aggregation studies rhIL-1ra has been reported to form soluble irreversible dimers.^{26, 27} Analysis by SE-HPLC of rhIL-1ra samples indicated incubation either with or without nanobubbles did not induce the generation of detectable soluble aggregates (dimers or higher order oligomers, data not shown). This result indicates that quantifying insoluble aggregates reflects the total extent of rhIL-1ra aggregation following incubation at 40 °C for 23 hours.

Quantification of the extent of aggregation indicated that approximately $2.3 \pm 0.2\%$ of the rhIL-1ra formed insoluble aggregates in the presence of nanobubbles (Figure 3A). Diluting the nanobubble suspensions with nanobubble-free, 10% (w/v) trehalose solutions prior to incubation resulted in decreases in rhIL-1ra aggregation that correlated directly with decreasing nanobubble concentrations. During shorter incubation studies, insoluble aggregates could be detected in nanobubble suspensions after 3 hours of incubation (Figure 3B). The quantity of rhIL-1ra aggregates formed in the presence of nanobubbles increased

roughly linearly with time for the first 12 hours of incubation, suggesting an initially constant rate of rhIL-1ra aggregation.

To determine whether nanobubble generation and subsequent associated effects on rhIL-1ra aggregation were a phenomena specific to trehalose, lyophilized cakes consisting of 10% (w/v) sucrose were prepared using the same lyophilization procedure used to prepare lyophilized trehalose samples. To confirm nanobubble generation, lyophilized sucrose formulations were reconstituted with 10 mM sodium citrate buffer pH 6.6 and particle generation was characterized by NTA. 2.5×10^9 nanoparticles/mL were detected, as compared to 2.3×10^9 nanoparticles/mL that had been obtained after reconstitution of the corresponding lyophilized trehalose cakes. Subsequently, accelerated degradation studies were conducted wherein rhIL-1ra was incubated at 40 °C in nanobubble suspensions generated from reconstituted lyophilized sucrose formulations. Similar extents of rhIL-1ra aggregation were observed with the sucrose-generated nanobubble suspensions as had been observed in the trehalose-generated nanobubble suspensions, with $3.0 \pm 0.3\%$ and $3.1 \pm 0.2\%$ of rhIL-1ra forming insoluble aggregates respectively after 23 hours of incubation at 40 °C.

Addition of PS20 to 1 mg/mL solutions of rhIL-1ra containing suspended nanobubbles suspensions resulted in a small but statistically significant ($p=0.007$) increase in the extent of rhIL-1ra aggregation after incubation for 23 hours at 40 °C for all concentrations above the PS20 critical micelle concentration of $\sim 0.006\%$ (w/v) (Figure 4). Without surfactant, slightly less aggregation was observed than in previous studies with $1.9 \pm 0.2\%$ of the rhIL-1ra aggregating. The presence of PS20 resulted in increasing aggregation in the solutions with $3.3 \pm 0.2\%$ of the rhIL-1ra aggregated at a PS20 concentration of 0.2% (w/v).

In nanobubble suspensions in 10% (w/v) trehalose solutions containing 5 mM EDTA, rhIL-1ra aggregation could not be detected either visually or by the Bradford assay following incubation for 23 hours at 40 °C. Particle characterization by NTA confirmed also that the number and size of nanobubbles in the suspension was unaffected by addition of EDTA (data not shown).

DISCUSSION

Reconstitution of lyophilized formulations of the disaccharides trehalose and sucrose generated suspensions of (meta)stable nanobubbles. The concentration and size of nanobubbles described in this work are consistent with the recently published findings of Zhou et al.¹ Reconstituting lyophilized formulations with a solution that contained 1 mg/ml rhIL-1ra resulted in a decrease in nanobubble concentrations, and the generation of negatively buoyant particles attributed to rhIL-1ra aggregates. The reduction in nanobubble concentration may result from the adsorption of rhIL-1ra molecules to the air-water interface. Such adsorption would reduce the surface tension at air-water interfaces, decreasing the rate at which some of the relatively large bubbles present immediately following reconstitution of lyophilized cakes might shrink to nanobubble dimensions.

It has been postulated that nanobubble stability may be dependent on a negative surface charge resulting from adsorbed anions which may generate a repulsive force sufficient to oppose the force of surface tension.^{10, 14, 15} Consistent with previous reports,^{7, 14} our zeta potential results confirmed that nanobubbles are negatively charged. Following reconstitution with rhIL-1ra the magnitude and sign of the zeta potentials in this system were dependent on the net charge on rhIL-1ra molecules, as controlled by altering buffer pH. Zeta potentials measured by electrophoretic light scattering are an average value weighted by the intensity of light scattered by each component. Thus, it is unclear which population of particles would contribute most to this value, because rhIL-1ra monomers, rhIL-1ra aggregates and nanobubbles would all be present following reconstitution of lyophilized disaccharide cakes with a rhIL-1ra solution. In all cases, however, the addition of rhIL-1ra resulted in a reduction in the absolute magnitude of the zeta potential. This decrease in zeta potential could reflect reduced electrostatic repulsion between components, potentially inducing nanobubble destabilization and protein aggregation.

The observed changes in zeta potential of these systems could result from adsorption of rhIL-1ra to the nanobubble interface, resulting in nanobubble zeta potentials similar to those of rhIL-1ra alone at the respective pH. Although proteins are surface active and structural perturbations have been described to occur at interfaces,²⁸ the quantity of rhIL-1ra aggregation observed in this study is not commensurate with the available nanobubble surface area for aggregation to take place. In our accelerated stability studies following incubation in nanobubble suspensions, up to 3% of rhIL-1ra in these solutions formed insoluble aggregates. This extent of rhIL-1ra aggregation is significantly greater than the quantity of rhIL-1ra which could adsorb to the available nanobubble surface area present in our solutions. From a simple approximation assuming monolayer formation and hexagonally closest packing, only 0.0001 mg of rhIL-1ra could adsorb per mL of nanobubbles (2×10^9 nanobubbles/mL). However, the quantity of rhIL-1ra that aggregated in nanobubble suspensions was approximately 300 times greater, suggesting that a direct mechanism wherein surface layers of adsorbed rhIL-1ra molecules form aggregates at the nanobubble-water interface may not be sufficient to explain the extent of aggregation observed.

Furthermore, addition of PS20 at concentrations both above and below the critical micelle concentration did not inhibit aggregation during incubation studies. The presence of nonionic surfactants such as PS20 would be expected to compete with rhIL-1ra molecules for adsorption at the nanobubble interface or to bind to hydrophobic regions of rhIL-1ra reducing surface activity.²⁹ If rhIL-1ra aggregation in the solution was dependent on adsorption to the nanobubble-water interface the addition of surfactant would be expected to reduce or inhibit aggregation. This again suggests that another mechanism may be responsible for generating rhIL-1ra aggregates. Other potential mechanisms may be that nanobubbles serve as nucleation sites for aggregation with further aggregation occurring in the bulk solution or nanobubbles could be capable of catalytically generating structurally perturbed rhIL-1ra species.

At a concentration of 5 mM, EDTA was capable of completely eliminating rhIL-1ra aggregation in nanobubble suspensions. As EDTA is a chelator capable of reducing the thermodynamic activity of a variety of cations, this result suggests contaminants such as

cations leaching from glass container surfaces may be involved in the generation of rhIL-1ra aggregates in our formulations. Whether nanobubbles may facilitate leaching of contaminants from glass surfaces or may interact with contaminants remains unclear. Potentially, cations may be electrostatically attracted to the negatively charged nanobubble interface, resulting in locally higher concentrations at the nanobubble interface. Interactions with contaminants near the nanobubble surface could catalyze perturbations in rhIL-1ra structure resulting in an increased propensity for rhIL-1ra aggregation.

The presence of nanobubbles combined with the generation of rhIL-1ra aggregates following both reconstitution of lyophilized formulations and incubation in reconstituted formulations could be a significant source of sub-visible particles in therapeutic protein formulations. Recent work by both Barnard et al. and Kotarek et al. has identified a correlation between increased concentrations of sub-visible particles and immune responses to marketed therapeutic protein products.^{5, 30} In addition, Rosenberg et al. highlighted the importance of investigating and characterizing sub-visible particles in formulations to develop strategies to mitigate risk and ensure the safety of protein therapeutic products.² As a newly identified source of sub-visible particles likely present upon reconstitution of lyophilized protein products, nanobubbles pose an unknown risk to the safety of protein therapeutic formulations requiring additional research to investigate if nanobubbles could alter the efficacy and immunogenicity of protein therapeutic products.

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Figure 1. NTA particle characterization of particle concentrations (A) and diameters (B) of particles generated following reconstitution of lyophilized trehalose formulations. Samples were reconstituted using 10 mM sodium citrate buffer either without (Black bars) or with (Gray bars) 1 mg/ml rhIL-1ra.

Figure 2. Concentration of positively (A) and negatively (B) buoyant particles measured by resonant mass measurement. Samples were reconstituted using 10 mM sodium citrate buffer either without (Black bars) or with (Gray bars) 1 mg/ml rhIL-1ra.

Figure 3.

(A) Percentage of rhIL-1ra aggregated following incubation for 23 hours at 40 °C with increasing nanobubble concentrations was measured using a Bradford protein assay. (B) The percentage of protein aggregated in nanobubble suspensions was quantified every three hours for twelve hours and then after 23 hours of incubation at 40 °C.

Figure 4. Percentage of rhIL-1ra aggregated in nanobubble suspensions containing 0 to 0.2% PS20 after incubating for 23 hours at 40 °C.

Table 1

Zeta potential values measured in mV using electrophoretic light scattering. Zeta potentials were measured following reconstitution with buffer either with or without 1 mg/ml rhIL-1ra. The zeta potential of 1 mg/ml rhIL-1ra solutions without nanobubbles was also measured.

Solution pH	Nanobubble Suspension	Nanobubbles with 1 mg/ml rhIL-1ra	1 mg/ml rhIL-1ra
4.6	-15.7 ± 1.3 mV	7.0 ± 0.8 mV	6.7 ± 1.8 mV
5.4	-22.3 ± 4.7 mV	-2.2 ± 0.5 mV	-3.1 ± 0.9 mV
6.6	-27.6 ± 4.3 mV	-20.6 ± 0.6 mV	-4.0 ± 4.7 mV