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Holographic Characterization of Protein Aggregates in the Presence of Silicone Oil and Surfactants

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ABSTRACT

Characterizing protein aggregates in the presence of silicone oil is a long standing challenge for the pharmaceutical industry. Silicone oil is often used as a lubricant in devices that deliver and store therapeutic protein products and has been linked to protein aggregation, which can compromise a drug's effectiveness or cause autoimmune responses in patients. Most traditional technologies cannot quantitatively distinguish protein aggregates and silicone oil in their native formulations for sizes less than 5 μm. We use holographic video microscopy to study protein aggregation to demonstrate its capability to quantitatively distinguish protein aggregates and silicone oil in the presence of varying amounts of the surfactants SDS and polysorbate 80 in the size range of 0.5-10 μm without the need for dilution or special sample preparation. We show that SDS denatures proteins and stabilizes silicone oil. We also show that polysorbate 80 may limit protein aggregate formation if it is added to an IgG solution before introducing silicone oil.

Introduction

Therapeutic protein products are becoming increasingly important in the treatment of diseases such as cancer, autoimmune diseases, and metabolic disorders.[1] Purification processes to stabilize protein formulations and minimize aggregation[2] have not proven sufficient to definitively prevent aggregate formation during production, transport, storage, and handling. Controlling protein aggregation in protein-based pharmaceuticals, from formulation to delivery, is critical to maintain effectiveness and safety of the product.[3,4]

Protein aggregate detection is particularly challenging in the presence of silicone oil. Silicone oil is a common lubricant in many products used in handling protein products such as prefilled syringes and vial stoppers, which can result in trace levels of silicone oil contamination in protein formulations.[5] Most conventional particle characterization technologies cannot distinguish quantitatively between silicone oil and protein aggregates for sizes less than 5 μm, making it difficult to unambiguously confirm the presence of protein aggregates. In addition, previous studies have linked the presence of silicone oil to the formation of protein aggregation in some cases.[5,6]

The use of nonionic surfactants, such as polysorbate 80 (PS80), has been proposed as an additive to prevent aggregation in protein formulation.[7] Nonionic surfactants can prevent protein aggregation by protecting the protein from interfacial stresses and interface-induced protein aggregation.[7,8] In some cases, nonionic surfactants can reduce protein adsorption to oil/water interfaces when introduced before or with the protein.[9] One proposed mechanism is that surfactants compete with proteins for adsorption at the interface, which inhibits protein adsorption and denaturation.[10] An alternative mechanism proposes that the decrease in aggregation is a result of an increase in free energy of unfolding the protein from its native state in the presence of surfactant.[11]

There is a need for simultaneous measurement of both protein aggregates and silicone oil droplets in their native solution. There are a variety of technologies for characterizing protein aggregates[12] but many such as dynamic light scattering and light obscuration are unable to distinguish proteins aggregates from other contaminants. To understand protein aggregation induced by silicone oil, previous studies have measured antibody concentration spectroscopically. In these studies, aggregation was measured indirectly, by filtration of the samples.[6] Microflow imaging captures images of particles and is limited to distinguishing different particle species by image shape parameters.[13] Resonant mass measurement can be used to distinguish protein aggregates from silicone oil droplets by measuring the buoyant mass of each particle. However, it is limited to smaller

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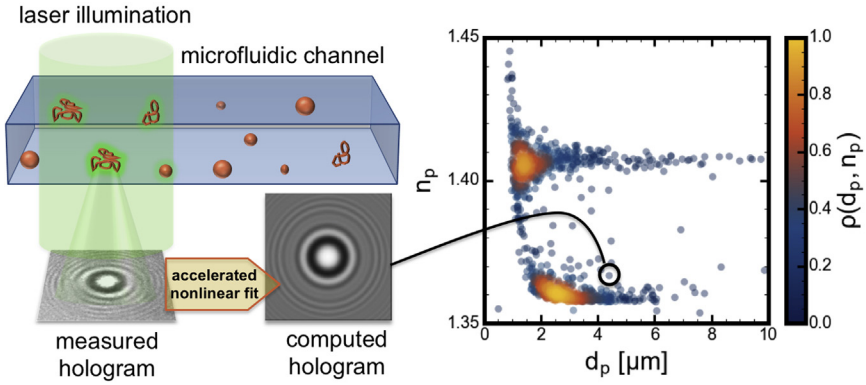

Figure 1. Particles flow through a microfluidic channel and are illuminated by a laser beam. The scattered light from a particle interferes with the incident light to form a hologram. The holograms are captured with a camera. The measured holograms are fit to Lorenz-Mie scattering theory with an accelerated fitting algorithm to obtain the particle's diameter, dp, and refractive index, np. Each point in the scatter plot represents the diameter and refractive index measurements of a single particle. The color indicates the point density in the diameter-refractive index space.

particles and small sample volumes, typically on the order of 100 nL.[14,15]

We use holographic video microscopy (HVM) to directly measure the size distributions of protein aggregates and silicone oil present in the native sample. HVM is a particle-resolved technology that is able to distinguish multiple different particle species in a single sample even when they are the same size.[16] HVM uses quantitatively analyzed holograms to measure concentration and characterize particles from 0.5 μm to 10 μm and has been shown in previous studies to reliably detect and characterize protein aggregates in this size range.[17] Other technologies may require dilution, with potential of changing the composition of the sample or be unable to distinguish protein aggregates from other contaminants for the size of less than 5 μm or to provide quantitative measurements of the concentrations of multiple species.[17] A unique capability of HVM is the ability to distinguish particles such as silicone oil droplets from protein aggregates by measuring each particle's refractive index. These experiments provide quantitative concentrations of multiple species without dilution of the sample.

To demonstrate the ability of HVM to measure protein aggregates and silicone oil in native solutions, we study several IgG and silicone oil mixtures, with the surfactants SDS and PS80. SDS, an ionic surfactant, is known to denature proteins while stabilizing silicone oil. We perform a comparative study of protein and silicone oil mixtures with and without SDS to confirm these effects. A second study compares protein aggregation in different formulations of IgG and silicone oil mixtures with added PS80 to investigate the role of the surfactant in protein aggregation induced by silicone oil. Both studies offer examples of how HVM can directly measure the concentrations and size distributions of silicone oil and protein aggregates when both are present.

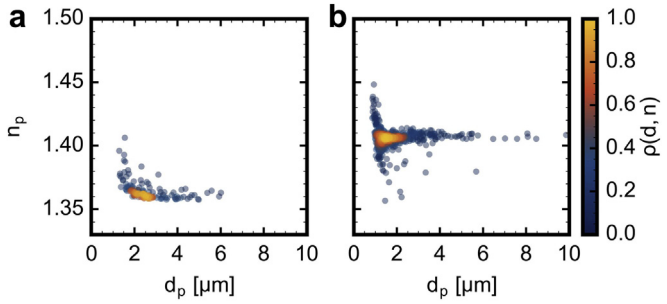

Figure 2. HVM measurements of (a) 0.5 mg/mL IgG in Tris-HCl buffer and (b) 0.6% silicone oil in Tris-HCl buffer emulsion. The concentrations measured in (a) and (b) are 4.5E + 4 ± 3E + 3 particles/mL and 1.76E + 5 ± 4E + 3 particles/mL, respectively. Each sample was measured once. The errors in concentration represent the standard deviation of the mean of the concentrations of each sample after the data have been broken up into 5 segments.

Materials and Methods

Formulation With Ionic Surfactant SDS

We prepared a 2 mg/mL bulk solution of IgG by dissolving IgG (HU-GF-ED-120916DG; Molecular Innovations) in Tris-HCl buffer (UltraPure 1M Tris-HCl pH 7.5) and gently inverted the sample ten times. A stock silicone oil emulsion was prepared by mixing silicone oil (SYLGARD 184, DOW CORNING) with Tris-HCl buffer and vortexing for 1 min to obtain a 2.5% (w/v) silicone oil emulsion. A 2% (w/v) SDS stock solution was prepared by dissolving SDS (Fisher BioReagents, BP8200-100) in Tris-HCl, vortexing for 20 s, letting stand for 10 min and filtering with a 0.22 μm syringe filter (MILLEX GP Filter Unit SLGP033RS).

A 0.5 mg/mL IgG and 0.6% (w/v) silicone oil mixture was prepared by mixing the stock 2 mg/mL IgG solution and 2.5% (w/v) silicone oil emulsion and subsequently diluting with Tris-HCl. A 0.5 mg/mL IgG and 0.6% (w/v) silicone oil mixture with SDS was prepared from a mixture of IgG with SDS and a mixture of silicone oil with SDS. A 1 mg/mL IgG, 1% (w/v) SDS solution was prepared by mixing the 2 mg/mL IgG bulk solution with 2% (w/v) SDS solution. Then, a 1.2% (w/v) silicone oil, 1% (w/v) SDS emulsion was prepared

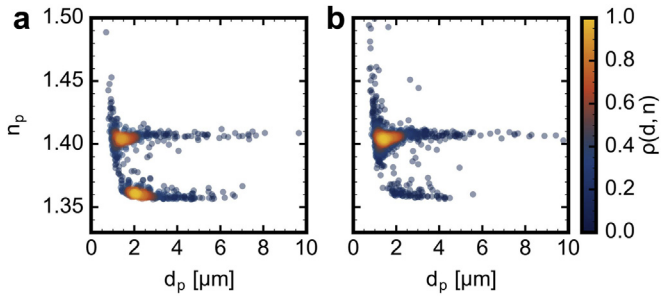

Figure 3. Size and refractive index measurements of IgG/silicone oil mixtures without SDS and with SDS. (a) IgG 0.5 mg/mL mixed with silicone oil and no SDS. (b) The same sample with 1 mg/mL of SDS, showing very few protein aggregates and an increased concentration of silicone oil droplets.

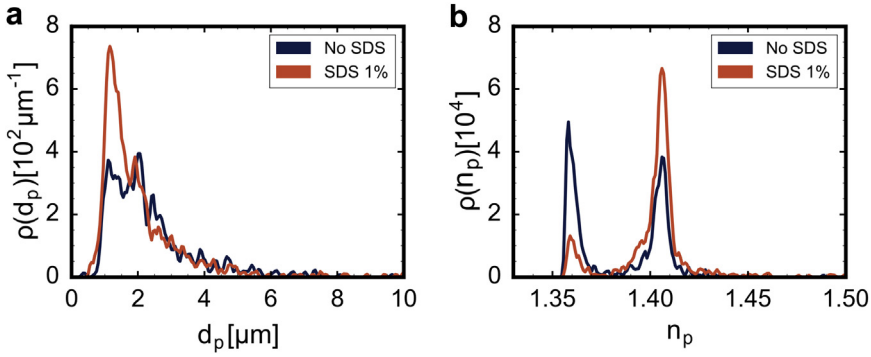

Figure 4. Size and refractive index density distributions of the IgG/silicone oil mixtures. The area under each curve represents the total number of particles measured. (a) The density distribution of the IgG and silicone oil as a function of particle diameter dp. (b) The density distribution of IgG and silicone oil as a function of particle refractive index np.

by mixing the 2.5% (w/v) silicone oil bulk emulsion with 2% (w/v) SDS bulk solution. Finally, a 0.5 mg/mL, 0.6% (w/v) silicone oil and 1% (w/v) SDS solution was prepared by gently mixing the 1 mg/mL IgG, 1% (w/v) SDS solution and the 1.2% (w/v) silicone emulsion, 1% (w/v) SDS solution.

Formulations With Nonionic Surfactant Polysorbate 80

Samples for PS80 experiments were made from stock solutions of IgG, silicone oil, and PS80 all in a Tris-HCl buffer. Human IgG (HUGF-ED-120916DG; Molecular Innovations) was dissolved in Tris-HCl buffer (UltraPure 1M Tris-HCl pH 7.5) and gently inverted 10 times to obtain a 2 mg/mL IgG stock solution. Silicone oil (SYLGARD 184; Dow Corning) was mixed with Tris-HCl and vortexed for 1 min to obtain a 2.5% (w/v) silicone oil emulsion. PS80 (59924-100G-F; Sigma-Aldrich) was mixed in Tris-HCl buffer, vortexed for 10 s, let stand for 5 min, and vortexed again for 10 s to obtain a 0.1% (w/v) stock solution. The 0.1% (w/v) PS80 solution was then filtered with a 0.22 μm syringe filter (MILLEX GP Filter Unit SLGP033RS).

Control

Control samples were prepared of the IgG solution and the silicone oil emulsion in their final concentrations without mixing the 2 samples. We prepared a 0.5 mg/mL IgG solution by diluting the 2 mg/mL IgG stock solution with Tris-HCl buffer and gently inverting 10 times. The 0.6% (w/v) silicone oil emulsion was prepared through a serial dilution of the 2.5% (w/v) silicone oil stock emulsion with Tris-HCl buffer.

Formulation A: Polysorbate 80 Added Before Mixing

As an intermediate step, a 1 mg/mL IgG and 0.05% (w/v) PS80 solution was prepared by mixing the 2 mg/mL IgG stock solution and 0.1% (w/v) PS80 stock solution and gently inverting 10 times. In addition, as an intermediate step, a 1.25% (w/v) silicone oil, 0.05% (w/v) PS80 solution was prepared by mixing the 2.5% (w/v) silicone oil stock solution and 0.1% (w/v) PS80 stock solution and inverting the solution 10 times. The 1 mg/mL IgG, 0.05% (w/v) PS80 solution was mixed with the 1.25% (w/v) silicone oil, 0.05% (w/v) PS80 solution, and inverted gently 10 times to obtain a final solution of 0.5 mg/mL IgG, 0.6% (w/v) silicone oil, and 0.05% (w/v) PS80.

Formulation B: Polysorbate 80 Added After Mixing

The 2 mg/mL IgG stock solution was mixed with the 2.5% (w/v) silicone oil stock emulsion and gently inverted 10 times to obtain a 1 mg/mL IgG and 1.25% (w/v) silicone oil solution. The 1 mg/mL IgG and 1.25% (w/v) silicone oil solution were mixed with the stock 0.1% (w/v) PS80 solution and gently inverted to obtain a final mixture of 0.5 mg/mL IgG, 0.6% (w/v) silicone oil, and 0.05% (w/v) PS80 solution.

Holographic Video Microscopy

HVM measures the size and refractive index of subvisible colloidal particles.[16,18-20] Examples of the results derived using HVM are shown in Figure 1. In HVM, particles flowing through a microfluidic channel are illuminated with a collimated laser beam. The light scattered by the colloidal particle interferes with the

Table 1
Average Concentration of Particles Binned According to Their Respective Sizes for Samples With PS80 Added Before and After Mixing

Size [μm]	PS80 Added before Mixing		PS80 Added after Mixing	
	Average Concentration [1/mL]	Error [1/mL]	Average Concentration [1/mL]	Error [1/mL]
<1	1.31E + 05	1.99E + 04	1.99E + 05	1.78E + 04
1-2	5.42E + 04	1.06E + 04	1.90E + 05	1.41E + 04
2-3	1.48E + 04	7.08E + 03	4.11E + 04	1.08E + 04
3-4	5.91E + 03	3.53E + 03	5.22E + 03	3.64E + 03
4-5	1.64E + 03	2.20E + 03	2.94E + 03	2.71E + 03
5-6	3.28E + 02	9.85E + 02	6.53E + 02	1.31E + 03
6-7	0.00E + 00	0.00E + 00	0.00E + 00	0.00E + 00
7-8	0.00E + 00	0.00E + 00	0.00E + 00	0.00E + 00
8-9	0.00E + 00	0.00E + 00	0.00E + 00	0.00E + 00
9-10	0.00E + 00	0.00E + 00	0.00E + 00	0.00E + 00
>10	0.00E + 00	0.00E + 00	0.00E + 00	0.00E + 00
Total	2.08E + 05	1.50E + 04	4.39E + 05	2.57E + 04

Each sample presented in the table was measured once.
The errors in concentration represent the standard deviation of the mean of the concentrations of each sample after the data have been broken up into 5 segments.

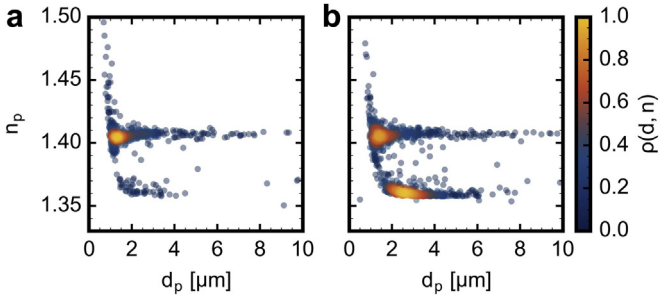

Figure 5. Size and refractive index measurements of IgG/silicone oil mixtures with polysorbate 80 added before and after mixing. (a) Adding polysorbate 80 before mixing the IgG and silicone oil resulted in little protein aggregation. (b) Adding the polysorbate 80 after mixing the IgG and silicone oil resulted in a high concentration of protein aggregates.

incident beam to produce an interference pattern, which is a hologram of the particle. The holograms are recorded with a camera and are analyzed with Lorenz-Mie theory. A parametric fit of the data is performed comparing computed holograms to the experimental data, to obtain the particle diameter d_p and refractive index n_p.[16,21] Although the theory assumes spherical particles, HVM provides meaningful results by finding the effective sphere that best approximates the particle. This approach has been shown to provide accurate results even with significant departures from sphericity.[20] HVM has previously been used in the study of protein aggregates and suspensions including chemical-mechanical planarization slurries and waste water.[17,22,23]

xSight Setup

HVM measurements were made using the commercially available Total Holographic Characterization® instrument, xSight (Spheryx Inc., New York, NY). The measurements were made at 25.0°C. An aliquot of 150 µL was injected into the reservoir of xCell50 (Spheryx Inc.) with 50 µm optical path length. For each measurement, a 3 µL volume of the sample was selected to be analyzed by xSight. xSight measures particles from 0.5 to 10 µm in diameter. xSight has been demonstrated to measure accurately concentrations of 10^3-10^7 particles/mL.[23] To demonstrate sample to sample repeatability, a sample of IgG (0.5 mg/mL in Tris-HCl buffer) was prepared. Five aliquots were measured using xSight and the resulting concentration measurements had a coefficient of variation of 12%.

Results and Discussion

Controls

Measurements of samples of IgG alone (0.5 mg/mL in Tris-HCl buffer) and silicone oil emulsion alone (0.6% [w/v] silicone oil in Tris-HCl buffer) are shown below. These results are control measurements for the behavior of each of these components in the absence of the other and in the absence of any added surfactant. In Figure 2, each particle is represented by a single point plotted as a function of the particle's index of refraction versus the size of the particle. In both control samples, the size distributions are similar with the largest concentration of particles occurring in the range of 1-3 µm. The index of refraction of the protein aggregates (n = 1.35-1.38), however, is different from that of the silicone oil (peak at n = 1.41). This difference in index of refraction distinguishes the two different species when they are both present in a sample.

SDS Experiment

HVM measurements of mixtures of IgG and silicone oil with and without SDS are shown in Figure 3 in scatter plots of index of refraction versus size. Figure 3a is a mixture of IgG and silicone oil without the addition of SDS. The features in Figure 3a are a combination of the features present in Figure 2a, the IgG control, and Figure 2b, the silicone oil control. The silicone oil appears as a narrow distribution centered at a refractive index n = 1.41, consistent with the refractive index of silicone oil. The sample contains protein aggregates, reflected by the high density of the distribution at a refractive index n = 1.36. Previous studies that used similar preparations with various model proteins have confirmed the presence of protein aggregates with a combination of UV spectroscopy and turbidity measurements.[5] The IgG protein aggregate population is spread over a range between the buffer's refractive index n = 1.335 and the refractive index of IgG,[24] similar to what is observed in the IgG control in Figure 2a. The broader range of index of refraction of the protein aggregate distribution is explained by the effective sphere model.[17,18] HVM measures the effective refractive index of the combination of the lower index buffer and higher index protein encapsulated by a hypothetical colloidal sphere. Previous studies have confirmed that the measured refractive index lies between the respective refractive indexes of the media and that of the pure protein. The variation of the measured index depends on the aggregates' porosity, with larger aggregates typically containing more buffer in a more porous structure, thereby leading to a lower refractive index. In addition, previous studies have also shown that the refractive index measurement can offer insight into the morphology of the protein aggregate correlated to the amount of buffer that is able to penetrate into the aggregate's structure.[17,18] In the effective sphere model, particles that are complexes of silicone oil and protein aggregates should have refractive indices between pure silicone oil and pure protein refractive index. In our experiments, we do not see many particles in this intermediate refractive index range, so we do not consider such particles in this work.

The addition of SDS significantly changes the distribution of aggregates as seen in changes in the scatter plots, shown in Figure 3b. SDS is known to denature proteins, and therefore should decrease the amount of protein aggregates in the sample.[25] In addition, SDS stabilizes silicone oil by inhibiting coalescence.[26] This

Table 2
Average Concentration of Particles Binned According to Their Respective Indexes of Refraction for Samples With PS80 Added Before and After Mixing

Refractive Index Range	PS80 Added Before Mixing		PS80 Added After Mixing	
	Average Concentration [1/mL]	Error [1/mL]	Average Concentration [1/mL]	Error [1/mL]
< 1.38	1.90E + 04	9.14E + 03	1.91E + 05	2.29E + 04
> 1.38	1.89E + 05	2.08E + 04	2.48E + 05	1.92E + 04
Total	2.08E + 05	1.50E + 04	4.39E + 05	2.57E + 04

Protein aggregates are expected at n < 1.38, and silicone oil droplets are expected at n > 1.38.

Table 3
The Concentrations of Silicone Oil Emulsion Droplets That Are Eluted From Various Syringes and Needles

Refractive Index Range	Control [mL^{-1}]	BD Needle Ref 305270 [mL^{-1}]	Nipro Needle Ref 22-11 [mL^{-1}]	BD Needle Ref 305270 with PS20 [mL^{-1}]
>1.38	9.9E + 2	8.9E + 3	6.1E + 4	4.0E + 4

All samples were deionized water except the last, which also contains 0.5 mg/mL of PS20.
The control sample was prepared with a standard Eppendorf pipette.

stabilization leads to a higher concentration of silicone oil emulsion droplets in the sample. As expected, when SDS is added, the feature characteristic of the IgG aggregates decreases and the feature characteristic of the silicone oil increases as can be seen in Figure 3b, where 1% (w/v) SDS has been added to the sample. SDS denatures proteins and binds to most proteins with a ratio of 1.4 mg SDS per mg of protein.[27] Figure 3b suggests that most of the protein aggregates have dissociated. Fully denaturing the proteins usually requires a temperature cycling step, which has been excluded from our procedures.

HVM measures particle refractive index in addition to particle size, to determine the effect of SDS on the IgG aggregate distribution and the silicone oil distribution simultaneously. Figure 4a shows the data from these experiments, where probability density is plotted as a function of the particle size. In this plot, it is not possible to distinguish the size distribution and concentration of protein aggregates from the size distribution and concentration of silicone oil droplets. In Figure 4b, the probability density is plotted as a function of particle index of refraction. In this plot, protein aggregates are distinguishable from silicone oil by their indexes of refraction. The peak at low index of refraction (1.35-1.38) corresponds to protein aggregates, and the peak at high index of refraction (>1.38) corresponds to silicone oil emulsion droplets. Without any SDS present, the lower index protein aggregate peak at $n = 1.36$ is larger than the higher index silicone oil peak at $n = 1.41$. Adding 1% (w/v), SDS increases the silicone oil peak in the refractive index distribution, while the protein aggregate peak decreases dramatically.

Polysorbate 80 Experiment

HVM measurements show a significant difference in protein aggregate concentration depending on whether the surfactant, PS80, is added before or after the IgG is mixed with the silicone oil. The concentrations binned by size are presented in Table 1. Each sample was measured once, and the errors in concentration represent the standard deviation of the mean of the concentrations of each sample after the data have been broken up into 5 segments. Adding PS80 before mixing (Fig. 5a) resulted in 10 times fewer protein aggregates on average compared to the case where PS80 was added after mixing the IgG and silicone oil, see Table 2. The concentrations of protein aggregates and silicone oil are measured by binning the particles according to the characteristic refractive index ranges for each species (Table 2). In the scatter plot, this variation is reflected by the changes in the feature characteristic of protein aggregates and the feature characteristic of silicone oil. For comparison, Table 3 presents the typical concentrations of silicone oil observed when measuring deionized water (or deionized water with 0.5 mg/mL polysorbate 20) ejected from standard syringes and needles.

We perform a similar analysis by viewing the particle density distributions as a function of the particle size (Fig. 6a) and as a function of the particle refractive index (Fig. 6b). The area under each curve represents the total number of particles measured. The refractive index plots show that adding the PS80 after mixing results in a larger peak at the refractive index of protein aggregates.

Additional information about the size distributions of protein aggregates and silicone oil droplets can be determined from the HVM experiments by incorporating the refractive index measurements. Figure 7a shows the size distributions of particles detected in the same sample as in Figure 6a, from the experiments where the PS80 was added before mixing (orange line). The data were binned according to refractive index as either lower index protein aggregate particles ($n < 1.38$) or higher index silicone oil particles ($n > 1.38$). Figure 7a shows the plots of the separated size distributions of protein aggregates (purple curve) and silicone oil (yellow curve) that result when PS80 is added before mixing. In Figure 7a, the sample contains predominantly silicone oil and few protein aggregates. Similar results are shown in Figure 7b for the separated size distributions of protein aggregates and silicone oil for the experiments when PS80 is added after mixing. In this case, there are more protein aggregates present.

Figure 8 presents the concentrations of protein aggregates and silicone oil for both experiments. The particles are again grouped by refractive indexes $n < 1.38$ and $n > 1.38$, reflecting the refractive index ranges of the protein aggregates and silicone oil, respectively. The concentrations of silicone oil ($n > 1.38$) are comparable for both formulations. However, the concentrations of protein aggregates

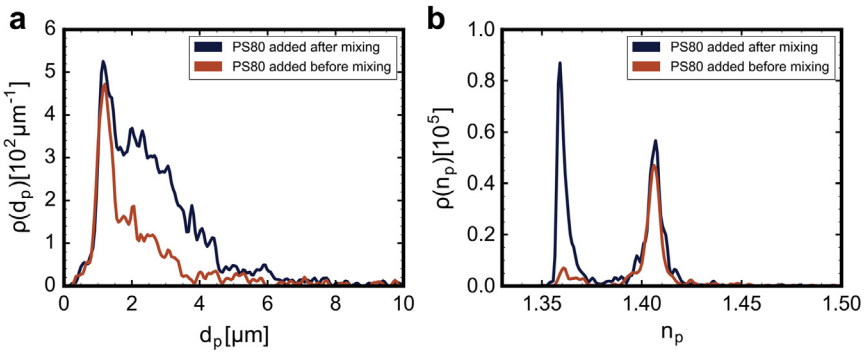

Figure 6. Size and refractive index density distributions of the IgG/silicone oil mixtures. The area under each curve represents the total number of particles measured. (a) Size density distributions of IgG/silicone oil mixture with polysorbate 80 added before and after mixing. (b) Refractive index density distribution of IgG/silicone oil mixture with polysorbate 80 added before and after mixing.

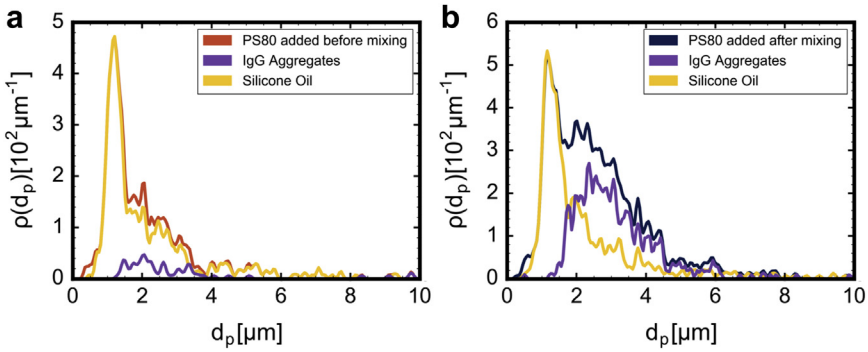

Figure 7. Size density distributions of the IgG and silicone oil mixtures in which polysorbate 80 has been added either before or after mixing. The area under the curves represents the total number of particles measured. Partial distributions are plotted for particles with np < 1.38, labeled "IgG aggregates" and for particles with np > 1.38, labeled "silicone oil." The labels correspond to the refractive index ranges of the distributions of IgG aggregates and of silicone oil droplets in the control measurements (Fig. 2). (a) Polysorbate 80 added before mixing the IgG and silicone oil (orange curve). The sample consists primarily of silicone oil (yellow curve) and few IgG aggregates (purple curve). (b) Polysorbate 80 added after mixing the IgG and silicone oil (dark blue curve). Both the IgG aggregates (purple curve) and silicone oil (yellow curve) are substantial, with the average size of the IgG aggregates larger than the average size of the silicone oil.

differ significantly depending on whether the PS80 was added before or after mixing the IgG and silicone oil. The figure shows a significantly lower concentration of protein aggregates when PS80 was added before mixing. This result agrees with previous studies that PS80 can prevent protein aggregation and suggests that PS80 can protect proteins from silicone oil–induced aggregation. The ability to identify each particle by the refractive index is an effective method to quantitatively distinguish the concentration of each species under different experimental conditions.

Conclusion

HVM was used to quantitatively distinguish species in heterogeneous mixtures of IgG aggregates and silicone oil. Each particle was identified based on the measurement of its index of refraction and its size. A series of experiments with different surfactants has demonstrated that subtle changes in protein aggregate concentrations and size distributions can be quantitatively measured with HVM. The behavior of each species was studied simultaneously, in the same experiment based on the differences in the index of refraction. With HVM, we were able to isolate the effects of the surfactants on the proteins and silicone oil in a series of experiments. The HVM measurements show that adding the ionic surfactant SDS to an IgG/silicone oil mixture stabilizes the silicone oil and denatures the IgG, in agreement with previous studies. In a separate set of experiments, adding PS80 before mixing the IgG and silicone oil resulted in fewer protein aggregates than when adding the PS80 after mixing, or not adding polysorbate at all. These results are consistent with previous studies that suggest surfactants can prevent protein aggregation by changing the interaction between silicone oil and proteins.[5-7] These results demonstrate how HVM can be used to provide a new window into the effects of additives on the propensity of proteins to form aggregates.

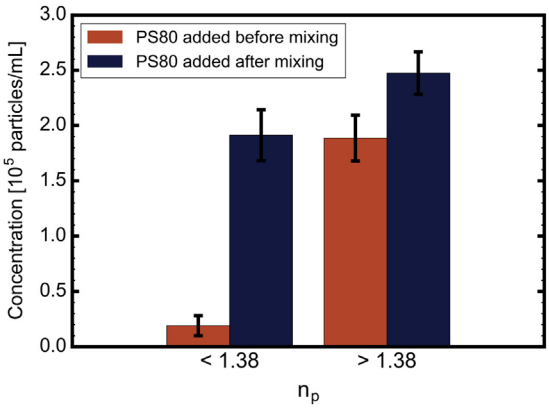

Figure 8. Concentrations of IgG/silicone oil mixtures with polysorbate 80 added before and after mixing. The particles are identified as either IgG aggregate or silicone oil by the refractive index, where np < 1.38 are IgG aggregates and np > 1.38 are silicone oil. Each sample was measured once. The errors in concentration represent the standard deviation of the mean of the concentrations of each sample after the data have been broken up into 5 segments.

Acknowledgments

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