NOTE

Silicone Oil Induced Aggregation of Proteins

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ABSTRACT: Prior to delivery to the patient, protein pharmaceuticals often come in contact with a variety of surfaces (e.g., syringes and stoppers), which are treated to facilitate processing or to inhibit protein binding. One such coating, silicone oil, has previously been implicated in the induction of protein aggregation. We have investigated the propensity of model proteins to aggregate when silicone oil is present in solution and find significant induction of aggregation in four proteins of various molecular weights and isoelectric points in the presence of 0.5% oil. The ability of silicone oil to induce conformational changes that might be responsible for this aggregation was also examined by a combination of circular dichroism (CD) and derivative UV spectroscopy. Neither method produces evidence of large conformational changes or alterations in thermal stability although in a limited number of cases some small changes suggest the possibility of minor structural alterations. The most probable explanation for silicone oil induced aggregation is that the oil has direct effects on intermolecular interactions responsible for protein association through interaction with protein surfaces or indirectly through effects on the solvent. J Pharm Sci 94:918 927, 2005

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Silicone oil contamination has long been suspected of being responsible in some cases for the aggregation seen in certain protein pharmaceutical preparations. Several publications in the 1980s implicated the release of silicone oil from disposable plastic syringes in the aggregation of insulin.[1–5] The link between insulin aggregation and silicone oil was originally based on the observation that after multiple withdrawals from vials, patients using multi-dose preparations of insulin observed clouding of the solutions. In this regard, Chantelau et al. report a silicone oil contamination of up to 0.25 mg/mL in a 10 mL insulin vial when a standard procedure for filling 1 mL siliconized syringes was performed three times each using 10 syringes.[2] Referencing silicone oil contamination levels reported by a syringe manufacturer,[6] Bernstein calculated that some of his patients who were prescribed low doses of insulin could have vials containing ~4 mg of silicone oil when only 1/3 of the vial had been used.[4] The use of silicone oil is not limited to syringes. It is also used as a coating for porous glass vials to minimize protein adsorption and as a lubricant to prevent the conglomeration of rubber stoppers during filling procedures. In addition, it is the author's experience that questions of silicone oil contamination and its potential role in protein aggregation arise frequently during the pharmaceutical development of proteins generally, although little information about this potential

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problem is available in the scientific literature. Thus, the possibility that silicone oil induces the aggregation of proteins could have important implications for a wide variety of protein formulation and process development related phenomena.

Therefore, the purpose of this investigation was to assess the ability of silicone oil to induce aggregation of a variety of proteins over a range of pH and to investigate whether several biophysical techniques that are sensitive to changes in protein secondary and tertiary structure can detect silicone oil induced conformational changes that might be responsible for aggregation. Four model proteins (ribonuclease A (RNase A), lysozyme, bovine serum albumin (BSA), and concanavalin A (ConA)) with a wide range of different physical characteristics were used (Table 1). The choice of buffer pH was based on both pharmaceutical relevance and the well-characterized pH-dependent oligomerization state of ConA.[7] At the lowest pH examined (4.5), ConA is a dimer. At pH 6.5, it exists in dimeric and tetrameric forms. Above pH 7.0, ConA is primarily a tetramer.

EXPERIMENTAL

Materials

Chicken egg white lysozyme (L7651), bovine serum albumin (A3294), ConA from *Canavalia ensiformis* (C7275), and ribonuclease A from bovine pancreas (R5125) were purchased from Sigma Chemical Company (St. Louis, MO). These proteins can be lyophilized without a high content lyoprotectant. Thus, all proteins were supplied as essentially salt-free lyophilized powders and were used without further purification. Silicone oil (S159–500) was purchased from Fisher Chemical Company (Pittsburgh, PA). All buffer salts (sodium phosphate monobasic, sodium phosphate dibasic, sodium acetate, and sodium chloride) were ACS grade or higher. Solutions were prepared using distilled deionized water.

Methods

Preparation of Stock Solutions

Three buffers (10 mM sodium phosphate, 130 mM NaCl, pH 6.5 and pH 7.2 and 10 mM sodium acetate, 130 mM NaCl, pH 4.5) were used. A stock solution (suspension) of 1% (w/v) silicone oil in buffer was prepared by combining silicone oil and buffer in a 50 mL polypropylene centrifuge tube and sonicating for 10 min in an FS30 (Fisher Scientific) ultrasonicating bath to create a dispersion. All silicone oil suspensions were freshly prepared on the day they were used. Over the period of the experiments, the resulting dispersions were stable as judged by constant optical properties.

Protein solutions were prepared in each buffer by adding buffer to an appropriate amount of lyophilized protein to obtain a protein concentration between 1 and 2 mg/mL. The concentration of the protein was then determined based on its extinction coefficient, and additional buffer was added to adjust the protein concentration to 1 mg/mL. The proteins were kept on ice or refrigerated until used.

Turbidity

Optical density measurements were used to monitor protein aggregation. Equal volumes of protein and silicone oil stock solutions were combined in 1.7 mL microcentrifuge tubes to create concentrations of protein and silicone oil of 0.5 mg/mL and 0.5% (w/v), respectively. The samples were mixed by gentle pipetting and inspected visually to ensure a homogeneous appearance. Control protein samples in each buffer were

Table 1. Model Proteins

Protein	Molecular Weight and (pI)[12]	No. of Various Types of Secondary Structure Units		
		Helices	β-Sheets	Turns
Ribonuclease A (RNase A)	13.7 kDa (8.8)	4	12	13
Lysozyme	14.4 kDa (11.0)	4	5	11
Bovine serum albumin (BSA)	66 kDa (4.9)	60		
Concanavalin A (ConA)[b]	102 kDa (tetramer) (4.5 5.5)	5	26	

[a]Secondary structure content are based on the following structures deposited in the Protein Databank (RNase A: 4RAT; lysozyme: 4LYZ; BSA: 1AO6; ConA: 1APN). http://www.rcsb.org/pdb/

[b]The secondary structure content of ConA is given for a monomer unit.

prepared similarly by substituting the appropriate buffer for the 1% silicone oil suspension. Silicone oil buffer blanks were prepared by combining equal volumes of silicone oil (1%) with the appropriate buffer. The protein samples and their corresponding buffer blanks (all in quadruplet) were then transferred to an untreated 96-well microtiter plate. This plate was placed in a FLUOstar Galaxy (BMG Labtechnologies, Durham, NC) microtiter plate reader that had been preheated to 45°C. The instrument was programmed to record the OD_{360} of the wells every 5 min for 5 h. Immediately before the beginning of a cycle (one round of reading all of the wells in the plate), the plate was gently shaken using a 4 mm orbital displacement for 5 s. The data was transferred to an Excel spreadsheet for analysis. The values recorded for all wells containing protein were corrected for extraneous scattering by subtracting the average of the wells containing the corresponding buffer, with or without silicone oil. Changes in optical density were calculated by subtracting the buffer corrected OD_{360} of the silicone oil-free sample from the sample containing 0.5% silicone oil.

Secondary Structure Changes and T_m Determinations

Circular dichroism (CD) spectroscopy was used to determine the effect of 0.5% silicone oil on the secondary structure and T_m of the model proteins. The samples and buffer blanks were prepared as described above, except a protein concentration of 0.2 mg/mL was employed. A 0.1 cm pathlength cell was used for data collection. Far UV CD spectra of the samples and buffer at 20°C were recorded using a Jasco J-720 CD spectropolarimeter (Easton, MD). The spectra of appropriate buffers were subtracted from the spectra of the protein samples prior to comparison. The T_m of each protein sample was determined by recording the signal at 222 nm as the temperature was increased from 20 to 90°C at a rate of 15°C per hour and analyzing the resulting trace using the Jasco thermal denaturation analysis algorithm.

Second Derivative UV Spectroscopy

The effect of silicone oil (0.5%) on the tertiary structure of the model proteins as a function of temperature was investigated using 2nd derivative analysis of UV spectra. The samples were prepared as described for the OD_{360} studies. The absorbance spectra were collected from 20 to 90°C in 2.5°C intervals using an Agilent 8453 UV-Visible spectrophotometer (Palo Alto, CA). The temperature of the cell holder was controlled using a Peltier device and the sample was equilibrated at each temperature for 5 min prior to data collection. Data were collected at 1 nm intervals with a 25 s averaging time. Spectral analysis was performed using a splining procedure as previously described using ChemStation software (Agilent).[8]

RESULTS AND DISCUSSION

Protein Aggregation

The solution parameters for the aggregation study were selected to permit detection of protein aggregates due to the presence of silicone oil over a relatively short time. This primarily involved selection of an appropriate experimental temperature and silicone oil concentration. Thus, we selected conditions for all proteins such that only small changes in optical density occurred when silicone oil was not present. Although we have attributed the increases in turbidity to protein aggregation, it is possible that the observed increases are caused, at least in part, by the effect of the protein on the silicone oil dispersion itself. Unfortunately, there is no obvious experimental method to easily distinguish between this and turbidity increases due to protein aggregation. Our assumption that protein association is responsible for turbidity is based on the fact that aggregated protein can be separated by centrifugation from the protein/silicone oil emulsions and directly identified in the pelleted material. It is also important to note that by monitoring the OD_{360} of the solutions as a convenient method for rapidly detecting protein aggregation, soluble aggregates may not be detected due to their similar size and reduced refractive indices.

At 45°C, protein aggregation was minimal to undetectable at all three pH values in the absence of silicone oil (Figure 1). By including 0.25% or less silicone oil in the protein samples, there was little to no increase in the protein optical density under these same conditions (data not shown) at short times, indicating that insoluble aggregates were not formed (although the formation of soluble aggregates is not precluded). At a silicone oil concentration of 0.5% and over a period of 5 h, however, the protein solutions exhibited changes in optical density indicative of aggregation

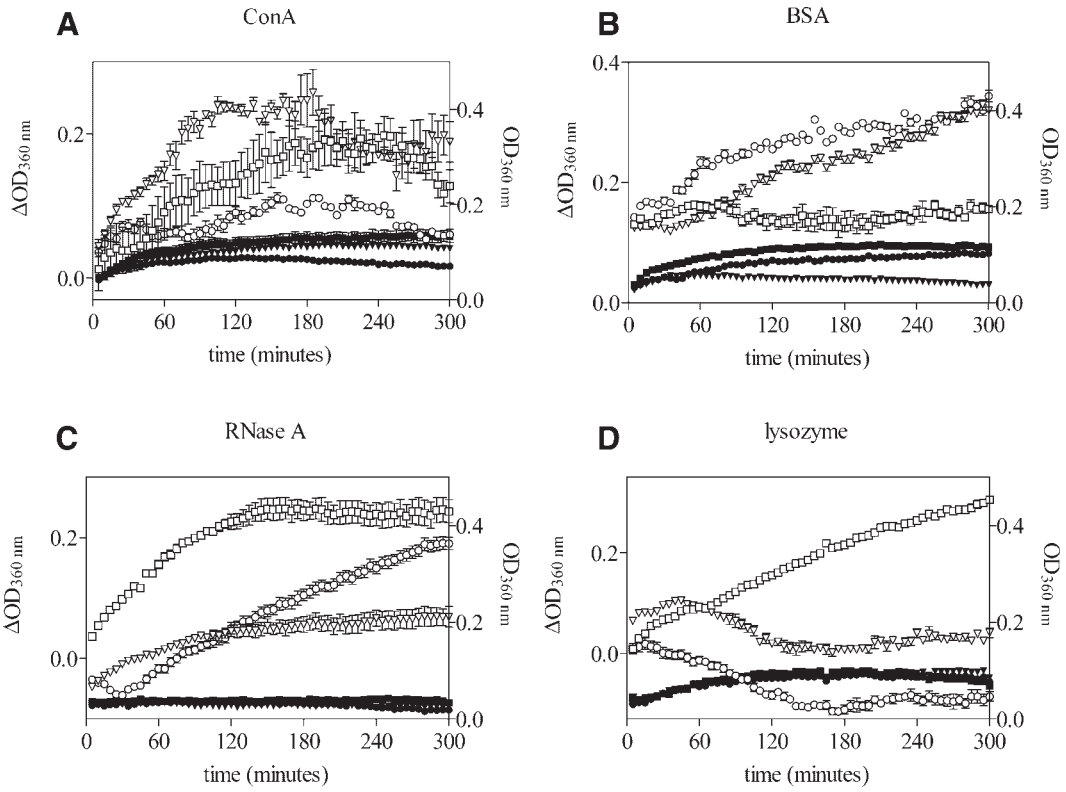

Figure 1. Effect of silicone oil (0.5%) on the optical density of model proteins at 45°C. (A) Con A, (B) BSA, (C) RNase A, (D) lysozyme. The left y-axes refer to the white symbols and the right to the black. ΔOD_{360} is the optical density of the protein sample containing the silicone oil minus the optical density of the corresponding protein sample lacking silicone oil. White squares: ΔOD_{360} at pH 4.5; white triangles: ΔOD_{360} at pH 6.5; white circle: ΔOD_{360} at pH 7.2. The black symbols represent protein samples lacking silicone oil (control samples). Black squares: OD_{360} at pH 4.5; black triangles: OD_{360} at pH 6.5; black circles: OD_{360} at pH 7.2. The error bars represent the SEM of four replicates.

(Figure 1). The extent and rate of the optical density changes are both protein and pH dependent. Although the 0.5% concentration of the oil employed is somewhat higher than that normally detected in protein pharmaceutical preparations, the amount employed can be considered as only a moderately accelerated condition, an approach usually deemed acceptable to replicate the longer times and lower silicone oil concentrations of more immediate interest.

Silicone oil (0.5%) caused the OD_{360} of ConA at pH 4.5 to increase 0.19 U over the course of the first 200 min (Figure 1A). The turbidity remained at this level for another hour, after which there was a slight decrease. At pH 6.5, the initial ConA aggregation occurred more rapidly and induced a greater change in OD_{360} (ΔOD 0.24 U in ~100 min). At ~200 min, the OD_{360} began a slight decline. Silicone oil had the least effect on ConA at pH 7.2, inducing an increase in OD_{360} of only 0.08 U within the first 50 min with the characteristic decline appearing at ~240 min. ConA has previously been described as a hydrophobic protein due to its adsorption to hydrophobic surfaces. The decrease in the effects of silicone oil on ConA at pH 7.2 in comparison to the other pH values may be related to the fact that ConA is a tetramer at pH 7.2, potentially reducing exposure of a subunit interface that could interact with the oil.[9,10] The decline in OD_{360} after an initial increase observed for ConA at all pH values could reflect settling of aggregated material or the creation of particles with a reduced refractive index increment (see below).

The most dramatic silicone oil induced aggregation occurred with BSA, as seen by the significant increase in optical density within the first 5 min at all pH values investigated (Figure 1B). The initial

change in OD_{360} for BSA at each pH was essentially identical (ΔOD_{360} ~0.13 U after 5 min). The change over the course of the experiment was pH dependent. At pH 4.5, the optical density of the BSA/silicone oil suspension increased slightly during the remainder of the incubation (<0.04 U increase in OD_{360} within the first 60 min). The largest increase in the turbidity of BSA was observed at pH 7.2, with a steep increase during the first 60 min followed by a slower rise for the duration of the incubation. The turbidity of the BSA/silicone oil solution at pH 6.5 increased steadily, albeit at a slower rate than the increase observed for the pH 7.2 system, following the initial jump at 5 min. At the end of 5 h, the OD of the pH 6.5 BSA/silicone oil solution was nearly equivalent to that at pH 7.2.

Silicone oil induced aggregation of RNase A was greatest at pH 4.5 (Figure 1C). At this pH, the OD_{360} of the RNase A/silicone oil dispersion increased ~0.2 U during the first 120 min. The pH 6.5 RNase A/silicone oil dispersion displayed a similar trend, but had a much smaller increase in the OD_{360}. At pH 7.2, RNase A aggregation resulted in a decline in the OD_{360} during the first 30 min followed by an increase that only begins to level off during the last 15 min of the 5 h incubation.

The effect of silicone oil on the optical density of lysozyme differed dramatically from the other proteins (Figure 1D). At pH 4.5, silicone oil caused a steady increase in the turbidity over the 5 h, clearly reflecting aggregation. In contrast, the OD_{360} of the lysozyme/silicone oil mixtures at pH 6.5 and 7.2 decreased over extended periods (during 50–140 and 20–165 min, respectively). The pH 6.5 sample containing silicone oil experienced a small increase in OD_{360}, probably due to protein aggregation, prior to the decline. We attribute the decreasing OD to changes in the refractive index increment (dn/dc) of the protein particles as the protein undergoes further aggregation. Note that scattering intensity is proportional to the square of the refractive index increment. Thus, small decreases in the density of scattering particles relative to the monomeric protein itself can in principle lead to significant decreases in scattering.

The results of this silicone oil-induced aggregation study of several proteins reveal only limited information regarding general trends. The most obvious one is that the more hydrophobic proteins, BSA (classified as hydrophobic based on the well known presence of its apolar binding sites[11]) and ConA, had a greater tendency to aggregate than the relatively more hydrophilic ones (lysozyme and RNase A). This result was not unexpected and suggests that the interactions are at least in part apolar in nature. All proteins exhibited a pH-dependence in their tendency to aggregate in the presence of the oil. There was, however, no clear trend (e.g., a protein's isoelectric point and the solution pH at which it experienced the largest (or smallest) change in optical density) to this dependence. For example, although BSA and ConA have similar isoelectric points (4.9 and 4.5–5.5,[12] respectively), ConA had the smallest silicone oil induced change in optical density at pH 7.2 while the pH 4.5 solution exhibited the least change for BSA. Although the increase in optical density was highest at pH 4.5 for both lysozyme and RNase A (two relatively small proteins with basic pIs), the kinetic profiles of the optical density data indicate that they are affected dissimilarly by the silicone oil.

Effects of Silicone Oil on Secondary Structure and Thermal Stability

Why do low concentrations of silicone oil cause proteins to aggregate? One possibility is that the oil structurally alters proteins resulting in aggregation competent states. To test this idea, we used CD to see if silicone oil induced changes in protein secondary structure could be detected. Since absorption flattening is a potential problem in CD studies of proteins at high concentration and in turbid samples, it was necessary to lower the protein concentration to examine the effect of silicone oil on the secondary structure of the proteins. By using short pathlength cells and lower protein concentrations, undistorted CD spectra could be obtained in the presence of the oil. No unusual reduction in CD intensity or red shifts in peaks was observed, arguing that significant flattening was not present. The far UV CD spectra of the four proteins at 20°C in the presence and absence of silicone oil (0.5%) were indistinguishable (data not shown). In most cases, the presence of silicone oil (0.5%) also had no effect on the thermal unfolding temperature (T_m) of the proteins (Figure 2). BSA at pH 4.5 and 7.2 was the only protein for which silicone oil had even a modest statistically significant effect, with a 2–3°C increase and decrease in T_m, respectively. This may at least partially reflect the existence of the well-known apolar binding sites on this protein.

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