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# Low Viscosity Highly Concentrated Injectable Nonaqueous Suspensions of Lysozyme Microparticles

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## Abstract

Subcutaneous injection of concentrated protein and peptide solutions, in the range of 100–400 mg/mL, is often not possible with a 25- to 27-gauge needle, as the viscosity can be well above 50 cP. Apparent viscosities below this limit are reported for suspensions of milled lysozyme microparticles up to nearly 400 mg/mL in benzyl benzoate or benzyl benzoate mixtures with safflower oils through a syringe with a 25- to 27-gauge needle at room temperature. These apparent viscosities were confirmed using a cone-and-plate rheometer. The intrinsic viscosity regressed from the Kreiger–Dougherty model was only slightly above the Einstein value of 2.5, indicating the increase in viscosity relative to that of the solvent was caused primarily by the excluded volume. Thus, the increases in viscosity from electrical double layer interactions (electroviscous effects), solvation of the particles, or deviations of the particle shape from a spherical geometry were minimal, and much smaller than typically observed for proteins dissolved in aqueous solutions. The small electroviscous effects are expected given the negligible zeta potential and thin double layers in the low dielectric constant organic solvent. The suspensions were resuspendable after a year, with essentially constant particle size after two months as measured by static light scattering. The lower apparent viscosities for highly concentrated protein suspensions relative to protein solutions, coupled with these favorable characteristics upon resuspension, may offer novel opportunities for subcutaneous injection of therapeutic proteins.

## Introduction

Proteins and other polypetides therapeutics have been on the rise in recent years given their lower toxicity and more predictable and selective behavior *in vivo* than for other classes of drugs not naturally found in the body.[1,2] Delivery of high dosages (100–1000 mg) of protein therapeutics has been limited primarily to dilute large volume intravenous injections. The high dilution helps prevent physical and chemical instabilities of proteins that would be encountered at high concentrations.[1–7] Apotentially less invasive method of administration is a subcutaneous injection. Since the injection volume is limited to ~1.5mL, the concentration of the protein therapeutic is often substantially above 100 mg/mL, where loss in stability can become a major issue.[2,3,5–8] In addition, the viscosity of a highly concentrated solution often increases markedly, typically well above 50 cP for proteins with nonspherical shapes, electrical double layer interactions, and hydration of the protein molecule in water, severely limiting the feasibility of subcutaneous injection.[5,7–11] Various electrostatic interactions due to the distortion of the double layer by shear and intramolecular

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Supporting Information Available: Additional information, including the viscosity correlations for the various needle gauges, the entire table of optical density measurements, and the calculation used to estimate the entry distance into the syringe. This material is available free of charge via the Internet at http://pubs.acs.org.

and intermolecular double layer interactions, collectively called the electroviscous effects, can increase the viscosity markedly.[12,13] In some cases, the viscosity increase from electroviscous effects can be mitigated by adding sodium chloride to increase the ionic strength of the solution or by varying the buffer species and pH to reduce the surface charge on the protein.[2,5,7,14] Another limitation is that large excipient concentrations, often up to 20:1 excipient to protein by mass,[15] are often needed to protect against denaturation and aggregation; however, the excipients occupy space in solution otherwise available to the protein.[3,5,8] An alternative to solutions is to form suspensions of an insoluble protein in a nonaqueous solvent. In some cases, smaller excipient levels are needed to stabilize the protein in the solid state relative to in solution and the storage time can be increased to that appropriate for pharmaceutical products.[3,16]

To date, relatively few examples of suspensions of proteins in nonaqueous media have been reported for medicinal purposes as the focus has been on aqueous protein solutions. Highly viscous suspensions of bovine somatotropin, marketed to increase milk production in dairy cows, and a bovine growth hormone releasing factor analogue, used to release somatotropin from the cow's pituitary gland, have been formulated in sesame oil and miglyol oil, respectively.[4,17,18] These viscous suspensions require a large 14- to 16-gauge needle for injection, whereas the preferred needle size for humans is ~25–27 gauge. In addition, a few nonaqueous extended release formulations for the peptide insulin and for very stable proteins such as protein C and a proprietary monoclonal antibody have been produced with the aid of viscosity enhancers and gel forming polymers in the presence of diluents such as benzyl benzoate or benzyl alcohol.[4,19–21] However, injection of these suspensions with a larger 21-gauge needle causes significant pain leading to noncompliance. In addition, the high levels of excipients needed to stabilize the protein particles and form the gel reduce the overall concentration of the protein in the formulation. [19,21–23] Another option is to crystallize the protein or monoclonal antibody and to suspend the crystals in aqueous media. [3,4,24] However, crystallization of high molecular weight proteins can be very difficult due to the high degree of segmental flexibility, and is more feasible for small peptides that have a much lower degree of flexibility.[3,4]

The objective of this study was to produce highly concentrated (up to 400 mg/mL) protein suspensions with viscosities below ~50 cP, the limit for a subcutaneous injection via a 25- to 27-gauge syringe, and to describe theoretically the reasons for the low viscosities. Suspensions of particles smaller than 37 $\mu$m of the model protein, lysozyme, with concentrations from 50 to 370 mg/mL were formulated with benzyl benzoate as the pure nonaqueous solvent or a 50/50 volume mixture of the pharmaceutically acceptable solvents safflower oil and benzyl benzoate. The organic solvents offer various advantages over aqueous solvents including low protein solubility and reduced electrostatic interactions (electroviscous effects) due to the low dielectric constants of the solvent. The experimental apparent viscosities are correlated with the Krieger–Dougherty equation to determine the intrinsic viscosity. The regressed intrinsic viscosity will be shown to be near the Einstein value of 2.5 indicating primarily an excluded volume effect. Thus, the increases in viscosity from the effects of protein shape, solvation, and electroviscous effects are small. In contrast, increases in viscosities from these additional effects can be pronounced for aqueous protein solutions.[5,7] In addition, for successful delivery with concentrated suspensions, the particle size and suspension uniformity must be controlled in order to administer an accurate and uniform dose.[4] The uniform aliquots measured from the suspensions, as well as the slow settling rates, are shown to be sufficient to favor uniform doses. Colloidal stability of the particles is shown by consistent static light scattering measurements of particle size over months.

## Materials and Methods

### Materials

Lysozyme in lyophilized powder form(L6876) was purchased from Sigma Chemical Company (St. Louis, MO). Bovine serum albumin (BSA) in lyophilized powder form (BP671), ACS grade acetonitrile, and USP grade ethanol were used as received from Fisher Chemicals (Fairlawn, NJ). Food grade olive oil and safflower oil were used, while benzyl benzoate was obtained from Acros Organics (New Jersey) and N.F. grade ethyl oleate from Spectrum Chemical Corp. (Gardena, CA).

### Methods

**Suspension Formation—**Lysozyme powder as received was dry milled with a porcelain mortar and pestle for several minutes. The milled powder was then sieved through a number 400 mesh, and particles smaller than 37 $\mu$m were collected. Samples of the particles were then weighed and added to the measured amount of benzyl benzoate or a premixed 50/50 volume mixture of benzyl benzoate and safflower oil to give 50–400 mg/mL concentration of particles in the solvent. Each vial was then shaken by hand to disperse the powder evenly through the suspension without the need for sonication.

**Particle Size Analysis—**Particle size was measured by multi-angle static laser light scattering using a Malvern Mastersizer-S instrument (Malvern Instruments, Ltd., Worcestershire, U.K.). The size of the milled and sieved powder was measured upon suspension in acetonitrile in a large recirculation cell (~500 mL) and also immediately after being added to ethanol in a small batch cell (Malvern, Worcestershire, U.K., ~15 mL). In each case, the obscuration during the measurement was between 10 and 15%. After storing the suspensions for 2 months at room temperature, the particle size was measured again immediately after shaking and diluting the sample in ethanol in the small batch cell.

**Viscosity Measurement—**The viscosity was measured as the time to draw1mL of the sample into a 1 mL BD tuberculin slip tip syringe with a 25 g 5/8″ long or 27 g 1/2″ long needle at room temperature. Each measurement was made at least three times and averaged while maintaining the suction force by holding the end of the plunger at the 1 mL mark each time. Liu and co-workers found this measurement time to be correlated linearly to viscosity.[5–7] Using known viscosities of each pure liquid, benzyl benzoate, ethanol, ethyl oleate, and olive oil, the correlation between the time to draw 1 mL of solution and the viscosity was found for each needle size to give an $r^2$ value greater than 0.999 as expected from the Hagen–Poiseuille equation (eq 1 below). The apparent viscosity of the suspensions in each pure solvent was calculated from these correlations, and the values for the two separate needle sizes were averaged to give a final average apparent viscosity of each sample. Additional samples of the solvent mixture of benzyl benzoate and safflower oil were made from10–90%benzyl benzoate by volume, and the viscosity was calculated as described above.

**Rheology Measurement—**The change in viscosity at various shear stresses was measured using an AR 2000ex cone-and-plate rheometer (TA Instruments, New Castle, DE) for two of the pure benzyl benzoate suspensions (200 and 400 mg/mL particles). Approximately 1 mL of the suspension was placed on the peltier plate, and the 40 mm 2° HAL aluminum cone was lowered to a final truncation gap of 55 $\mu$m. The excess suspension was removed prior to performing a measurement. Twenty-five measurements between the shear stress of 5 and 100 Pa were taken after the equipment equilibrated at room temperature (23 °C). The shear rate was measured up to $10^3$ $s^{-1}$ where inertial and edge effects began to

introduce error. However, the applied torque was within instrument specifications, giving a maximum 2–3% error in the viscosity measurement.

**Average Settling Rate and Maximum Volume Fraction Measurement—**The settling rate of the particles in the solvents was measured by shaking the suspension in a test tube 13 mm in diameter. Pictures were taken with a standard digital camera after 10–1440 min as the height of the sediment–supernatant interface decreased. To measure the final settling volume of the samples, the vials containing the suspensions were left undisturbed for 4 months and images of the settled suspension were taken. All images were analyzed using ImageJ software to determine the distance from the meniscus to the settling front. This approach has been used previously to measure the average settling rate of a concentrated suspension.[25] The maximum volume fraction for the settled suspension was defined by dividing the volume fraction of particles in the overall suspension by the ratio of the volume containing particles after settling for 4 months to the overall volume.

**Quantification of Protein Concentration—**The concentration of lysozyme in an aqueous solution was measured following the protocols for the Micro BCA protein assay (Pierce, Rockford, IL). Each sample was measured in triplicate with relative standard deviations (%RSD) less than 2% in a General Assay 96-well plate. The absorbance was measured at 562 nm in a spectro-photometer ($\mu$Quant model MQX200; Biotek Instruments Inc., Winooski, VT). A standard curve of untreated lysosyme was prepared at concentrations between 2 and 30 $\mu$g/mL.

**Rate of Lysozyme Partitioning to Aqueous Phase—**Partitioning and dissolution of lysozyme from the concentrated suspension was measured in pH 7.4 potassium phosphate buffer. The USP paddle method was used with aVanKelVK6010 dissolution tester with a Vanderkamp VK650A heater/circulator. A total of 900mL of dissolution media was preheated in large 1 L capacity dissolution vessels (Varian Inc., Cary, NC) to 37 °C. A sample of the concentrated suspension giving a total of 18 mg of lysozyme was added. At time increments of 2–240 min, 1 mL samples were taken and analyzed using the Micro BCA protein analysis mentioned earlier.

**Optical Density Measurement—**The lysozyme in suspension was dissolved in water, and the optical density was measured to determine whether large aggregates were present. An amount of 0.1 mL of the concentrated lysozyme suspension was added to a test tube with 4 mL of DI water. This mixture was then gently mixed and left for 3 days for the protein to partition to the water phase. The water phase was then separated, and a sample was diluted and tested for concentration using the Micro BCA protein assay as mentioned above. On the basis of the concentrations measured, the remaining aqueous solution was diluted to 1 mg/mL. This solution was tested for optical density using at least three 200 $\mu$L aliquots in a 96-well plate and analyzed using the $\mu$Quant spectrophotometer at 350 nm. A standard lysozyme aqueous solution was made at 1 mg/mL concentration and exposed to the pure benzyl benzoate solvent and the benzyl benzoate and safflower oil solvent mixture for 3 days and measured as the standard for oil–water interface induced aggregation of the protein. An aqueous solution never exposed to any organic solvent was also measured immediately after it was made and used as a standard for absorbance measurements.

**Suspension Uniformity Measurement—**Three separate 0.1mL aliquots of the resuspended concentrated lysozyme suspensions were added to test tubes with 8 mL of DI water. These mixtures were then gently mixed and left for 1 day for the protein to partition to the water phase. The aqueous phase was then separated and diluted to a theoretical

concentration of 20 $\mu$g/mL if 100% of the protein partitioned. The actual concentration was then analyzed using the Micro BCA protein assay mentioned above.

**Karl Fischer Moisture Analysis—**After being stored for 4 months, a sample of 0.1 mL of the redispersed concentrated suspension was inserted using a 19-gauge needle through the septum of the titration cell of an Aquatest 8 Karl Fischer titrator (Photovolt Instruments, Indianapolis, IN). Each suspension, pure benzyl benzoate, and the benzyl benzoate and safflower oil solvent mixture was measured in triplicate and averaged.

**Polarity Determination—**An aliquot of the suspension was diluted with the solvent until individual particles were visible on a slide through an optical microscope (Bausch & Lomb, 10× magnification). Microelectrophoresis was used to determine if a charge was present on the particles. The diluted particle dispersion was placed between two parallel wire electrodes (0.01-in. diameter stainless steel 304 wire, California Fine Wire) spaced 1 mm apart. The electrodes were secured to a glass microscope slide and observed by optical microscopy. A potential of 10–100 V was applied with the polarity of the electrodes switched every 15–60 s.

## Results

**Formation of Suspensions—**Prior to adding the milled and sieved lysozyme particles to the suspending media, the lysozyme mass percentage was determined to be 91.5% by weight of the particles, consistent with the manufacturer's specifications, according to the micro BCA assay. The average particle size was found to be approximately 20 $\mu$m, according to static light scattering measurements (Figure 1). For the average particle size measurement, a minor secondary submicrometer peak was also visible in all measurements. However, since the 15 mL small batch cell is only calibrated for particle sizes down to 500 nm, this peak was not included in the analysis. Upon adding the suspending media, a uniform suspension was formed by shaking by hand as shown in Figure 2A. Thereafter, the particles settled slowly enough to remain partially suspended even after 24 h (Figure 2B) and took up a significant portion of the volume even after 2 months.

**Apparent Viscosity of Solvent Mixture and Suspensions—**Using the known viscosities of pure solvents, a correlation between the time to draw 1 mL of the sample and viscosity was generated. This type of correlation has been described by Shire and co-workers on the basis of the Hagen–Poiseuille equation, assuming steady, laminar, isothermal flow along the axis of the needle and no slip at the walls[5–7]

$$\langle v\rangle = \frac{R^2}{8\eta}\left(\frac{|\Delta P|}{L}\right) \quad (1)$$

where $v$ is the velocity, $R$ is the inner radius of the needle, $\eta$ is the viscosity, and $\Delta P/L$ is the average pressure drop over the length of the needle. To ensure that the entrance pressure drop was negligible, the entry distance was calculated. The resulting value of 0.0001 cm was significantly less than the 1.27 cm or 1.58 cm length of the needle (see the Supporting Information for calculation). Ensuring that the average pressure drop over the length of the needle remains constant for each sample by maintaining the same suction pressure inside the syringe, the inverse of the velocity of the fluid multiplied by the cross-sectional area gives the time to draw up a specified volume of liquid, in this case 1mL. This time is proportional to the viscosity as shown by the Hagen–Poiseuille equation, and has been very accurately

correlated by Shire and co-workers to the viscosity.[5,7] Using the approximation for shear rate ($\gamma$) in a capillary,

$$\dot{\gamma} = \frac{4Q}{\pi R^3} \tag{2}$$

where $Q$ is the volumetric flow rate and $R$ is the radius of the needle, the shear at the wall over the length of the capillary for both needle sizes was calculated to be on the order of $10^3$ $s^{-1}$. In this shear rate range for two of the suspensions formulated, the apparent viscosity was approximately that measured using a cone-and- plate rheometer at the appropriate shear rate (Figure 5). Because only this high shear rate range is relevant to subcutaneous injection, additional samples were measured with the syringe method alone.

The measured viscosities of the solvent mixtures of benzyl benzoate and safflower oil at room temperature are shown in Figure 3. In this case, since the minimum and maximum values are for the pure solvents, the generalized mixing rule should follow the form

$$f(\eta_m)_L = \sum_i x_i f(\eta_i)_L \tag{3}$$

where $\eta_m$ is the viscosity of the mixture, $i$ is the number of components, $x_i$ is the liquid volume, weight, or mole fraction, and $\eta_i$ is the viscosity of the $i$th component. $f(\eta)_L$ can be $\eta_L$, $\ln(\eta_L)$, $1/\eta_L$, and so on.[26] For our data, the best correlations were obtained with $f(\eta)_L = \ln(\eta_L)$. This theoretical result is shown by the dotted line in Figure 3, assuming no water was present in the system. As will be discussed below, the small amount of residual moisture below 100 $\mu$g/mL in the solvent may be expected to change the viscosity by less than 1%.

The apparent viscosities of the suspensions with increasing concentration were measured for both the pure benzyl benzoate system and the solvent mixture of 50/50 benzyl benzoate and safflower oil. Figure 4 presents the resulting apparent viscosities, averaged from the measurements using two syringe sizes (left $y$-axis), and the average time to draw 1 mL from the 25-gauge syringe (right $y$-axis) as a function of the concentration of lysozyme particles. For subcutaneous delivery, 50 cP is an appropriate maximum viscosity where it will take approximately 20 s for 1 mL of the suspension to be expelled via a 26-gauge syringe.[3] From Figure 4, the highest apparent viscosity measured was approximately 50 cP, where it took approximately 55 s to draw 1mL into a 25-gauge syringe. The disparity in the times measured reflects the smaller suction force that can be applied to draw the volume into the syringe relative to the larger force available to expel the solution from the syringe.

The correlation of the apparent viscosity with the free solvent volume fraction, $1-(\varphi/\varphi_{max})$, was modeled using the Kreiger–Dougherty equation

$$\frac{\eta}{\eta_o} = \left[1 - \left(\frac{\varphi}{\varphi_{max}}\right)\right]^{-[\eta]\varphi_{max}} \tag{4}$$

where $\eta$ is the apparent viscosity of the dispersion, $\eta_o$ is the solution viscosity, $\varphi$ is the volume fraction of particles, $\varphi_{max}$ is the maximum packing fraction, and $[\eta]$ is the intrinsic viscosity (Table 1). $\varphi_{max}$ was approximated experimentally after gravitational settling of the

particles over 4months. It was approximately 0.50 for the pure benzyl benzoate solvent solution and 0.52 for the benzyl benzoate and safflower oil solvent for low shear rates. Using these values, the intrinsic viscosity of the suspension, $[\eta]$, was determined to be 2.7 for the pure benzyl benzoate suspensions and 2.3 for the benzyl benzoate and safflower oil suspensions. Since the exponent $-[\eta]\varphi_{max}$ was used to determine $[\eta]$, uncertainty in the approximation of $\varphi_{max}$ of about 20%can introduce a similar uncertainty in $[\eta]$. However, this uncertainty is small when compared with changes in $[\eta]$ of more than an order of magnitude for proteins with strong interactions in solutions.

**Particle Stability of the Suspension—**The stability of the particles in suspension was measured by using numerous different techniques. First, the average settling rate was measured and compared to the calculated theoretical Stokes settling rate ($U_s$) for the average particle size ($r$)

$$U_s = \frac{2r^2 \Delta\rho g}{9\eta_o} \tag{5}$$

where $\Delta\rho$ is the difference in densities between the solvent and the particles and $g$ is acceleration due to gravity. For a high concentration of particles, the particle crowding will reduce the settling rate to yield

$$U = U_s(1 - \varphi)^{6.55} \tag{6}$$

This modified average Stokes settling rate and the experimentally measured values were found to be within a factor of 2 for most concentrations lower than 300 mg/mL as shown in Table 2. However, for a concentration of 400 mg/mL, the experimental value was 1 order of magnitude lower than the predicted rate (Table 2). At this high concentration, the increase in apparent viscosity at the very low shear rates found in settling ($\sim 10^{-5}$ $s^{-1}$)[27] is much more pronounced, leading to an increased settling time (Figure 5). Shear thinning is expected in concentrated suspensions, as high shear will overcome interparticle forces and rearrange the particles to a more ordered configuration.[27–29]

Three aliquots were taken of an organic suspension to determine the uniformity of the protein within the suspension. The organic suspension was added to an aqueous phase to forma two-phase system as described in the Materials and Methods section. The system was equilibrated for 1 day, to be conservative, as it appeared that the partitioning reached equilibrium in 60 min.

The aqueous phase was then diluted approximately 1000 times, and the concentration of protein was measured. For 8 mL of aqueous phase equilibrated with 0.1 mL of the concentrated nonaqueous suspension, at least 80% of the protein partitions into the aqueous phase in 24 h (Table 3). The %RSD values for the amount of extracted protein were typically below 5%, indicating reasonable uniformity of the protein particles in the redispersed suspension. The %RSD was slightly larger for the highly concentrated 300 mg/mL sample in the mixed solvent.

The particles in suspension were stored for extended time periods to determine whether the particle size changed due to aggregation or other processes including Ostwald ripening. The original particle size was measured via static light scattering immediately after the particles were sieved and resuspended in both acetonitrile, where lysozyme is very insoluble, and

ethanol, where lysozyme is slightly soluble.[30] The uniformity of the two measurements ensures that the time scale of the measurement is much quicker than the time scale of growth of the particles in ethanol (Figure 1). Following 2 months of storage, the samples were diluted in ethanol and immediately tested. The particle size was found to be essentially constant during storage (Figure 1). The visual inspection of one formulated suspension after storage for 1 year and redispersion by shaking confirms that the particles could be redispersed.

The potential effect of electrostatic interactions on the particle stability was tested. However, the lysozyme particles did not display organized movement when the current was reversed for voltages from10 to 100 V for two electrodes spaced 1mmapart in the benzyl benzoate solvent. Thus, the charge on the particles was small as expected given the low dielectric constant of the solvent.

**Protein Stability in the Suspension by Optical Density—**Protein aggregation was investigated by measuring the optical density on sample aliquots that partitioned from the organic to the water phase. The protein was diluted to a concentration of 1 mg/mL, confirmed by dilution of an aliquot to 20 $\mu$g/mL with the micro BCA assay. Additional lysozyme samples in an aqueous solution at the same concentration were exposed to the solvent to measure the effect of the oil–water interface on aggregation. All these solutions were checked for large protein aggregates by comparison with a fresh lysozyme solution at the same concentration. The absorbance at 350 nm of the standard solution, without exposure to the nonaqueous solvent (0.047 ± 0.005), was approximately the same as that for the particles in the pure benzyl benzoate solvent (0.051) and the mixed benzyl benzoate/ safflower oil solvent (0.052). As this is within the error of the experiment, it suggests that the particles did not undergo significant formation of large aggregates over the 4 to 5 month month time period tested. Since lysozyme is a very stable model protein, additional protein denaturation and aggregation studies were not conducted.

Quantification of the moisture content may be used to determine the free and bound water in the suspension. The moisture content was measured for each suspension after being exposed to atmospheric conditions for 2 months. The linear correlations found between the moisture content and suspension concentration indicates that the moisture is directly associated with the protein (Figure 6). The benzyl benzoate solvent contains an average of 20 $\mu$g of water per 0.1mLof solution or approximately 0.02%by weight. The safflower oil and benzyl benzoate mixture contains approximately 74 $\mu$g of water in the same volume sample or approximately 0.074%. The sample with the highest concentration of protein in benzyl benzoate, 400 mg/mL, contained the most moisture, an average of 4450 $\mu$g of water per 0.1 mL of suspension, giving an absolute maximum concentration of 4.5% by weight of the suspension after being stored for 2 months.

## Discussion

### Colloidal Stability of the Protein in Suspension

A key concern for an injectable suspension is the ability to redisperse the particles after settling with gentle agitation.[4] For the various aliquots of the protein in water extracted from the redispersed suspensions, the relatively constant protein concentrations indicate that the suspensions were uniform after gentle shaking. The lack of particle aggregation as measured by static light scattering (Figure 1) and lack of caking of the suspended particles demonstrate significant storage stability. The lack of sintering of the particles is favored by the fact that the hydrophobic poor solvent does not soften the protein chains. The lack of particle growth from Ostwald ripening is not surprising, since the protein is almost completely insoluble in these nonaqueous solvents[30] and will therefore undergo little

diffusion. Furthermore, the lack of particle aggregation is favored by the weak hydrophobic interactions between the protein particles in a low dielectric constant organic solvent.

The average particle size of ~10 $\mu$m is optimal to balance the need for sufficiently small particles to flow through the needle versus large particles to minimize the particle surface area. Milled nanoparticles (~300 nm diameter) were found to produce a highly viscous paste. The high surface area led to stronger interactions between the particles, which increased the viscosity (results not shown).[31] In addition, particles not milled through the 37 $\mu$m sieve caused clogging of the syringe (results not shown). Therefore, an aspect ratio compared to the needle of ~0.1 provided the proper balance of low particle surface area for weak particle interactions (low [$\eta$]) and small enough particles to prevent mechanical clogging of the needle.

The concept of protein particle engineering for the formation of low viscosity suspensions may be generalized beyond the milling technique in this study. For example, micrometer-sized protein particles may be formed by lyophilization or spray drying. In addition, they may be encapsulated in polymer microspheres[18,24] for controlled delivery.

## Effects of Excluded Volume, Shape, Solvation, and Electroviscous Effects on Viscosities of Proteins in Solution versus Protein Suspensions

In the Einstein model for the viscosity of a dilute colloidal dispersion of solid spheres ($\varphi$ < 0.03),[27,32] the slope of the viscosity ratio of the suspension over the solvent versus $\varphi$ is 2.5. This slope signifies the excluded volume increment of viscosity due to the addition of dispersed particles, the intrinsic viscosity, [$\eta$]. The [$\eta$] of concentrated protein colloids and protein suspensions may be regressed from the Kreiger–Doughety equation to give an indication of the effects of excluded volume and other interparticle forces (eq 3).[27,28] The [$\eta$] value can increase from the Einstein value of 2.5 depending on the effects of solvation, particle shape, and electrical double layer forces (electroviscous effects), as well as the shear rate.[1,27] The values near 2.5 for the benzyl benzoate and the benzyl benzoate and safflower oil mixture suspensions in Table 1 indicate that the viscosity increase is governed primarily by the excluded volume effect. The value of 2.3 for the 50/50 benzyl benzoate and safflower oil mixture is slightly below the minimum Einstein 2.5 limit due to experimental uncertainty.

The solvation of protein molecules by a solvent will increase the volume fraction by

$$\frac{\varphi_{\text{solvated}}}{\varphi_{\text{dry}}} = \left[1 + \left(\frac{m_{1,\text{b}}}{m_2}\right)\left(\frac{\rho_2}{\rho_1}\right)\right] \tag{7}$$

where $m_{1,\text{b}}$ is the mass of the bound solvent, $m_2$ is the mass of the particle, $\rho_2$ is the density of the particle, and $\rho_1$ is the density of the solvent.[27] In the case of the Krieger–Dougherty equation, this solvation is manifested as an increase in [$\eta$].[13,27] For the organic solvents in the present study, the increase in $\varphi$ may be expected to be much smaller than that in the case of water, which is a much stronger solvent for solvating proteins.

Since the solvated volume fraction is often unknown, the analysis of protein solutions is typically done using mass concentrations (g/mL) rather than volume fractions, leading to values of the intrinsic viscosity in units of $cm^3/g$ and different higher order relationships derived from the virial expansion.[5,33] For example, a hard quasi-spherical model has been shown to accurately predict viscosities of various protein solutions[5,9,10,34]

$$\frac{\eta}{\eta_o}=\exp\left(\frac{[\eta]c}{(1-\frac{k}{\upsilon}[\eta]c)}\right) \quad (8)$$

where $c$ is the mass protein concentration, $k$ is a crowding factor, and $\upsilon$ is the Simha parameter accounting for the change in shape from a sphere. For various proteins in solution, $[\eta]$ varies from 2.7 for lysozyme to over 200 $cm^3/g$.[33] Even for lysozyme, a protein with a small axial ratio of 1.5,[33] this model shows a dramatic increase in viscosity around a concentration of 300 mg/mL (Figure 4). For glass fibers with axial ratios, 7, 14, and 21, the intrinsic viscosity increases from 3.8 to 5.03 to 6.0, respectively.[28] Our lower values of $[\eta]$ are consistent aspect ratios close to 1, as expected for particles formed by milling.

The electroviscous effect caused by the charge on a protein surface and the associated double layer interactions often produces a marked increase the viscosity of a protein aqueous solution as a function of pH and salinity. The electroviscous effects are as follows: primary, from distortion to the diffuse double layer surrounding the protein molecule, secondary, from interparticle double-layer interactions, and tertiary, from changes in intramolecular double layer interactions that affect the geometry of the system.[12–14,27] The primary electroviscous effect on the intrinsic viscosity for the case of small zeta potentials (<25 mV) and large distortions of the double layer is given by

$$[\eta]=2.5\left\{1+\frac{6(\varepsilon_r\varepsilon_0\zeta)^2}{k\eta_0 R_s^2}\frac{1}{1+Pe^2}\right\} \quad (9)$$

where $\varepsilon_r$ is the relative permittivity, $\varepsilon_0$ is the permittivity of free space, $\zeta$ is the zeta potential, $k$ is the specific conductivity of the continuous phase, $R_s$ is the radius of the colloid, and $Pe$ is the Peclet number.[12,27] For aqueous protein colloids, assuming low ionic strength, an increase in $\zeta$ as the pH moves away from the pI will raise $[\eta]$.[14] For a protein in a lower dielectric solvent, as has been shown for a polymer in methanol,[35] the primary electroviscous effect, as shown in eq 9, has a reduced impact on $[\eta]$. In addition, the $[\eta]$ value has been shown previously to approach the Einstein value of 2.5 upon screening the surface charges on nanoparticles with ligands that adsorb on the charged sites.[36] In our case of nonaqueous protein suspension in low dielectric solvents, the negligible $\zeta$ observed experimentally, as a consequence of the low $\varepsilon$, leads to an insignificant increase in $[\eta]$ from this electroviscous effect. Furthermore, secondary and tertiary electroviscous effects may also be ameliorated by lowering the dielectric constant, thus lowering the surface charge and decreasing the thickness of the double layer.

The negligible elecroviscous effects and increase in $\varphi$ from solvation for organic solvents can be attractive for achieving low $[\eta]$, thereby increasing the achievable volume fraction of particles for a viscosity below 50 cP. These lower apparent viscosities in benzyl benzoate suspensions have also been seen for other milled proteins including bovine serum albumin (Figure 4). Benzyl benzoate and safflower oil are known solvents for parenteral delivery.[37,38] These solvents have been found in formulations including a testosterone propionate solution and a hydroxyprogesterone benzoate preparation for intramuscular delivery, but are much less common than water.[38] Benzyl benzoate has been found to be completely nontoxic in a formulation with 50% or less benzyl benzoate in a fixed (nonvolatile) oil and nonirritating to the skin when injected at 10% benzyl benzoate in oil.[39,40] FDA approval of the use of benzyl benzoate as a pure solvent for subcutaneous delivery awaits further testing.

### Destabilization of Protein Molecules in Suspension

It is challenging to prevent denaturation and aggregation of peptides and proteins at high concentrations, whether in solution or suspension form.[17,41–43] Oxidation due to the oil has been visible for Factor IX in soybean oil, where asmiglyol oil has been used as the solvent to suspend a bovine growth hormone releasing factor analogue for 10 weeks with no reported loss in stability measured by reverse-phase HPLC and FTIR.[17,41] In addition, at high concentrations (up to 300 mg/mL), the stability of bovine somatotropin in sesame oil was confirmed by fluorescence, Raman, and FTIR spectroscopy.[42] In benzyl benzoate, insulin, protein C, and a proprietary monoclonal antibody are stable in sustained release polymer formulations.[19–21]

For the suspensions in this study, insoluble protein aggregates were not visible by optical density for lysozyme at any concentration after 2 months of storage at ambient conditions. Thus, the protein particles redissolved after being in suspension. As the optical density test was conducted in water, this also indicates that the exposure of the protein to the oil–water interface generated between the nonaqueous solvents and an aqueous environment, as will occur upon injection, did not produce insoluble aggregates. Additional characterization of the stability of lysoyzme was not performed, since lysozyme is very stable. Thus, lysozyme would not be a good indicator of the stability of more fragile proteins, with regard to both denaturation and the formation of irreversible protein soluble and insoluble aggregates.[41,42,44–46] However, the moisture content in the suspension was examined, as it is an important factor that influences protein stability. The moisture controls protein hydration in the solid state, which is known to have a large effect on protein stability. For example, the reduced stability of Factor IX in another nonaqueous solvent, methoxyflurane, was attributed to water mediated reaction and conformational changes.[41] On the opposite end, studies of enzymatic activity in organic solvents at low hydration levels have shown increased activity when the protein was only partially hydrated. The increase in activity has been attributed to reduced protein unfolding at low water levels.[47,48] For the suspensions studied, the values measured by Karl Fischer titration indicate that the water incorporated within the suspension tracked the concentration of the protein, and therefore, the slope describes the hydration of the protein in the solvent. In pure benzyl benzoate solvent, slightly less sorption of water was observed (~100 $\mu$g water/mg of protein), versus 140 $\mu$g water/mg of protein for benzyl benzoate/safflower oil after 4 months of storage at room temperature (~23 °C) with no control over the humidity. Both of these values are above that needed for full hydration of the lysozyme protein molecules (~20 $\mu$g water/mg of protein).[47]

To achieve the optimum partially hydrated conditions for increased protein stability using the present suspensions, storage of protein suspensions at lower temperatures and/or low humidities may maximize the stability of a protein in these organic solvents. At these low levels of protein hydration, the sorption of water from organic solvents has been found to be similar to that for solid protein particles exposed to air.[47,49] Since lysozyme and many other proteins have been shown to be stable in the solid phase with the proper excipients and storage conditions, the stability of the protein in the nonaqueous suspension may also be favorable.[44–46]

## Conclusions

The viscosities of concentrated suspensions up to 300–400mg/mL of ~10 $\mu$m milled particles of the model protein, lysozyme, were below 50 cP, the limit for subcutaneous injection through a 25- to 27-gauge needle. The apparent viscosity was correlated with volume fraction at all conditions according to the Krieger– Dougherty equation with an intrinsic viscosity close to 2.5, indicating weak interparticle interactions. The various factors that produce large increases in viscosity for proteins in aqueous solution, including

electroviscous effects from double layer interactions, an increase in $\varphi$ from solvation, and deviations of the particle shape from a spherical geometry, have almost negligible effects for the nonaqueous protein suspensions in this study. The small electroviscous effects are a consequence of the small zeta potential and thin double layers in the low dielectric constant organic solvent. An average particle size of ~10 $\mu$m with an aspect ratio compared to the needle of ~0.1 provided the proper balance of low particle surface area for weak particle interactions (low[$\eta$]) and small enough particles to prevent mechanical clogging of the needle. Static light scattering of the suspensions demonstrated that the protein particle size did not vary for at least 2 months when stored at atmospheric conditions. The low settling rate of the particles (<2 cm/h) contributed to the excellent dose uniformity of 0.1 mL aliquots. The demonstration of injectable low viscosity supsensions with good collidal stability and dose uniformity is an important advancement for the ultimate goal of subcutaneous delivery of therapeutic proteins.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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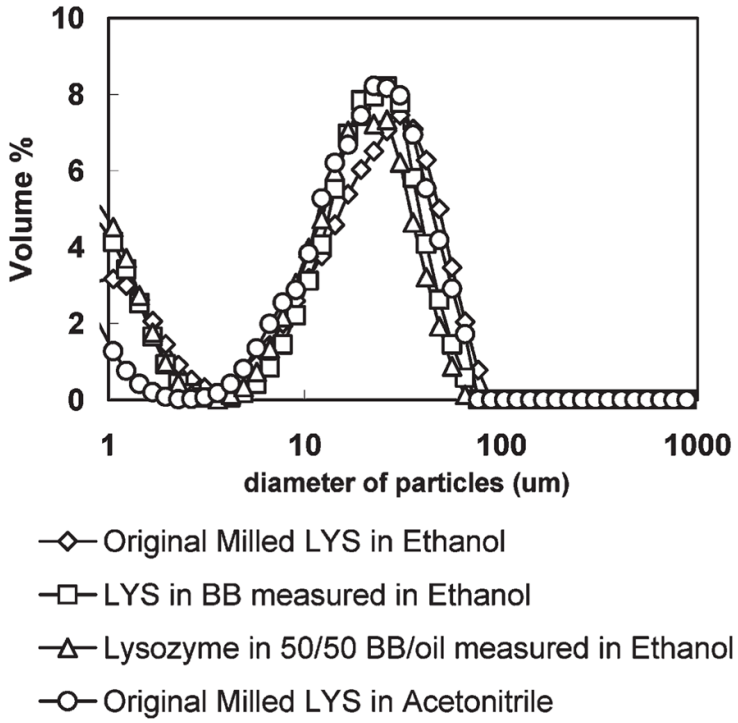


**Figure 1.**
Volume percent of particles versus size measured for the original lysozyme (LYS) milled particles in acetonitrile (○) and ethanol (◇), and after 2 months of storage for the suspensions in pure benzyl benzoate (BB) (□) and a mixture of benzyl benzoate and oil (BB/oil) (△) both measured immediately after being diluted in ethanol to 10–15%obscuration by light scattering.

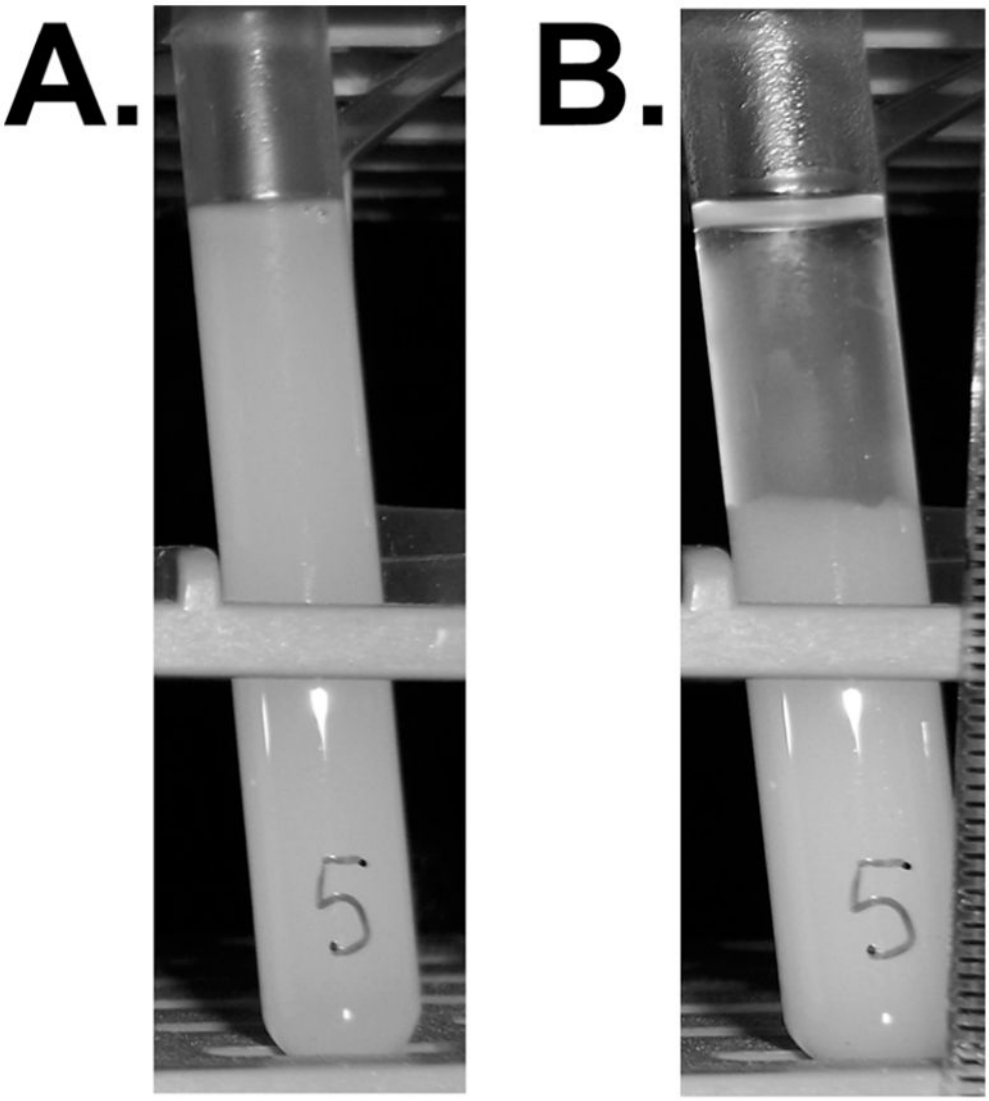


**Figure 2.**
Pictures of the 300mg/mL suspension of lysozyme particles in 50/50 benzyl benzoate and safflower oil (A) after being shaken and resuspended in a test tube (B) after being allowed to settle for 24 h.

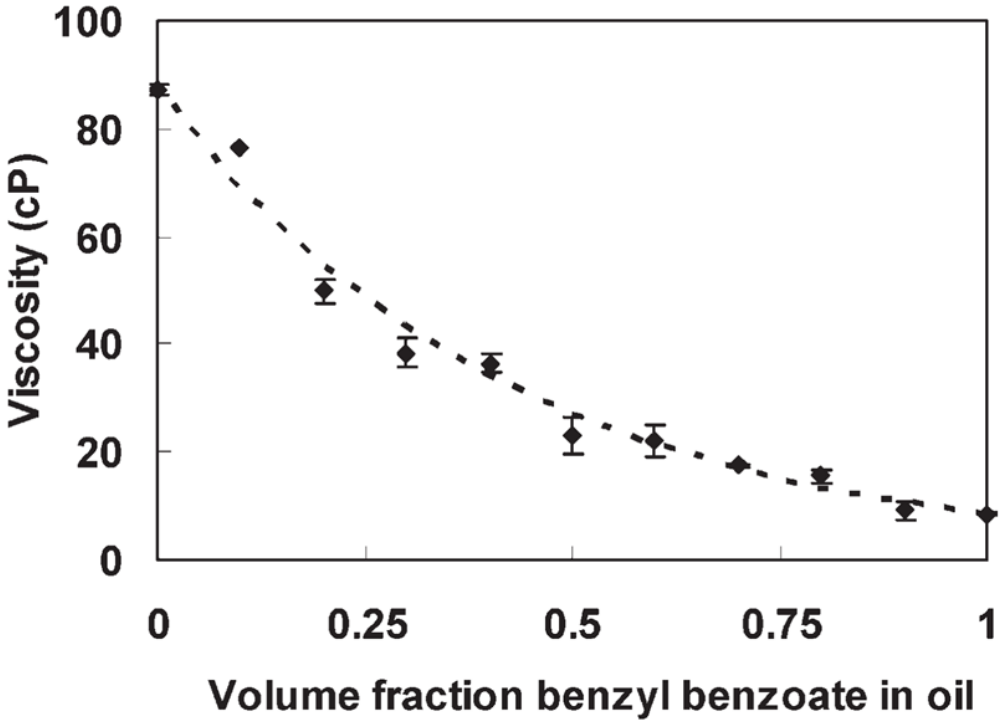


**Figure 3.**
Viscosity of a solution of benzyl benzoate and safflower oil at room temperature at varying concentrations measured experimentally and correlated with eq 3. Error bars indicate the standard deviation of each measurement.

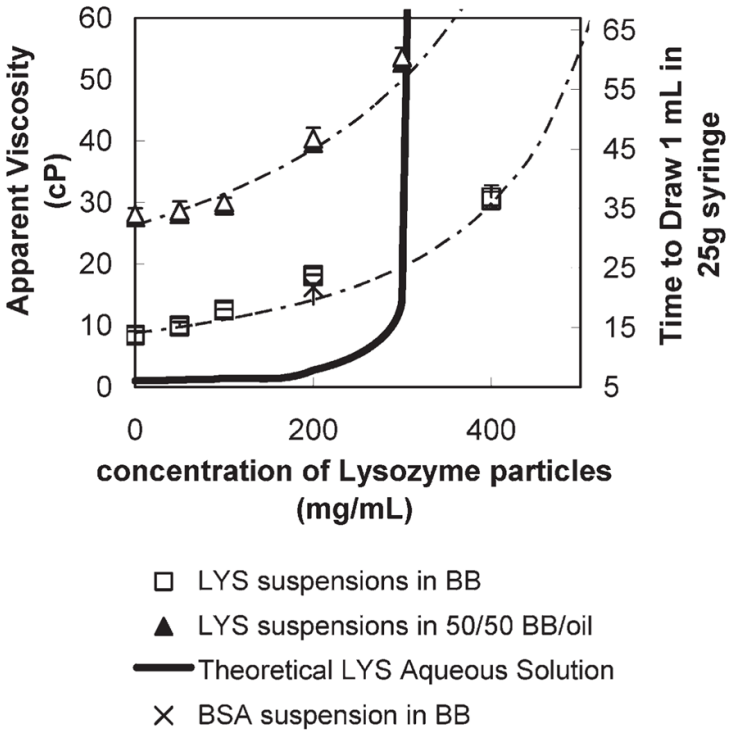


**Figure 4.**
Apparent viscosity as a function of concentration of particle in suspensions of the nonaqueous solvents, benzyl benzoate (BB) (□), and 50/50 benzyl benzoate and safflower oil (BB/oil) (△) with their correlations based on the Kreiger–Dougherty equation (eq 4) and the theoretical viscosity of an aqueous lysozyme (LYS) solution, calculated using the hard quasisphere model (eq 8) (solid line).

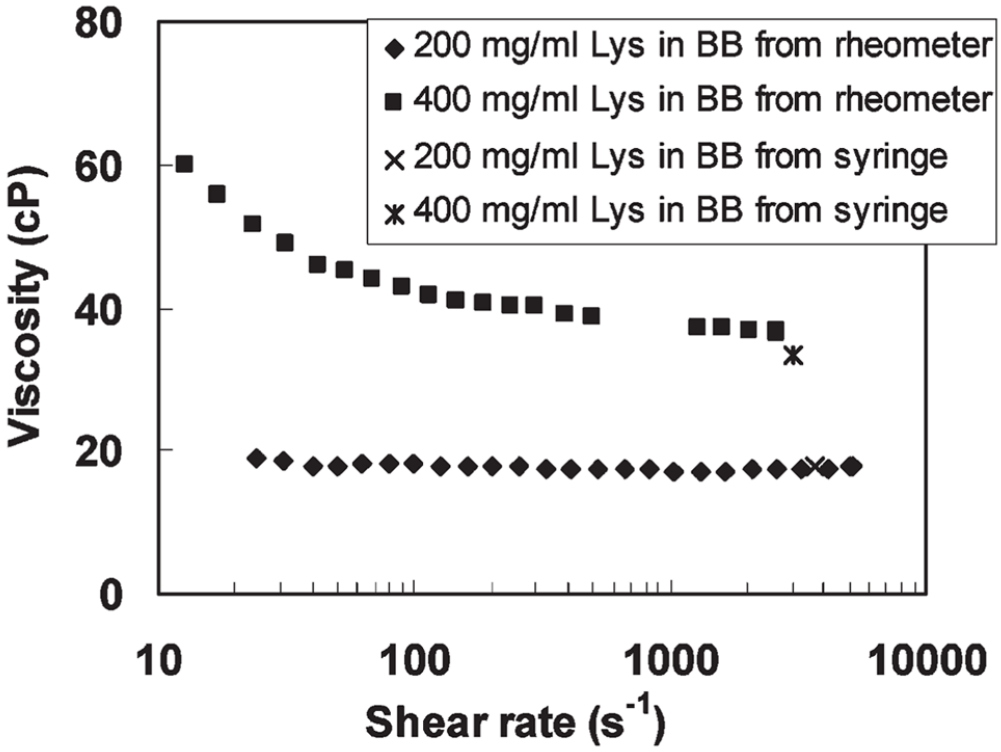


**Figure 5.**
Viscosity confirmation of syringe viscosity measurements using the AR 2000ex rheometer.

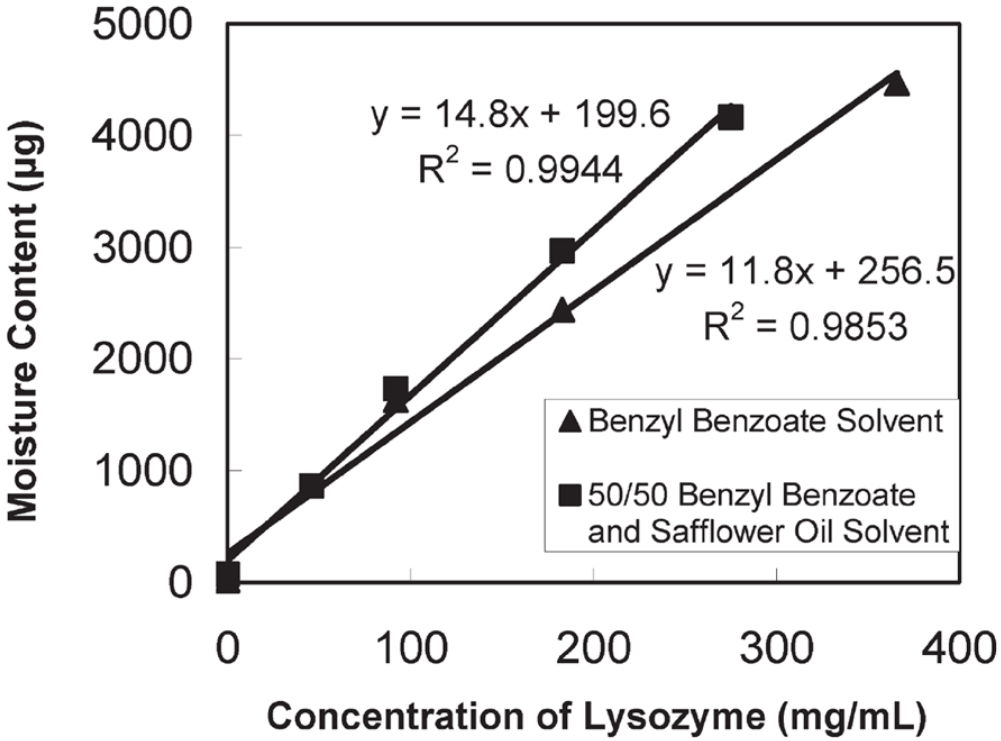


**Figure 6.**
Karl Fisher moisture content analysis of the nonaqueous suspension in benzyl benzoate (▲) and benzyl benzoate and safflower oil (■).

## Table 1

Comparison of the Two Solvent Systems Used for Highly Concentrated Suspensions for the Exponents for the Krieger–Dougherty Equation, the Experimental Maximum Packing Fraction, and Intrinsic Viscosity Average Plus or Minus the Standard Deviation

| solvent system | exponent for Krieger–Dougherty equation ($-[\eta]\varphi_{max}$) | maximum volume packing fraction ($\varphi_{max}$) | intrinsic viscosity ($[\eta]$) |
|---|---|---|---|
| benzyl benzoate suspension | −1.36±0.09 | 0.50 | 2.7±0.18 |
| 50/50 safflower oil and benzyl benzoate suspension | −1.2±0.06 | 0.52 | 2.3±0.12 |

**Table 2**

Comparison of Experimental Settling Rates and Settling Rates Quantified by the Modified Stokes Settling Equation Accounting for Particle Interactions (eqs 5 and 6)

| safflower oil concentration (% of solvent) (remainder is benzyl benzoate) | protein concentration (mg/mL) | volume fraction of particles ($\varphi$) | experimental settling rate (cm/h) | modified Stokes settling rate (cm/h) |
|---|---|---|---|---|
| 50 | 46 | 0.03515 | 1.85 | 0.85 |
| 50 | 92 | 0.0703 | 1.32 | 0.67 |
| 50 | 183 | 0.1406 | 0.32 | 0.40 |
| 50 | 275 | 0.2109 | 0.043 | 0.23 |
| 0 | 46 | 0.03515 | 3.91 | 2.24 |
| 0 | 92 | 0.0703 | 1.64 | 1.76 |
| 0 | 183 | 0.1406 | 0.52 | 1.05 |
| 0 | 366 | 0.2812 | 0.031 | 0.32 |

**Table 3**

Percent of Sample Recovered in Aqueous Phase and Percent Relative Standard Deviation (%RSD) of Three Samples for Suspensions Formed in Benzyl Benzoate with and without Safflower Oil

| safflower oil concentration (% of solvent) (remainder is benzyl benzoate) | particle concentration (mg/mL) | % protein recovered in aqueous | % RSD |
|---|---|---|---|
| 50 | 50 | 84.4% | 2.21% |
| 50 | 100 | 85.8% | 6.61% |
| 50 | 200 | 93.8% | 3.20% |
| 50 | 300 | 83.3% | 9.45% |
| 0 | 50 | 93.1% | 1.52% |
| 0 | 100 | 100% | 3.46% |
| 0 | 200 | 100% | 3.65% |
| 0 | 400 | 88.7% | 4.29% |