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RESEARCH PAPER

Non-Aqueous Suspensions of Antibodies are Much Less Viscous Than Equally Concentrated Aqueous Solutions

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ABSTRACT

Purpose The aim of this study was to markedly lower the viscosities of highly concentrated protein, in particular antibody, formulations. An effective approach elaborated herein for γ-globulin and a monoclonal antibody is to replace aqueous solutions with equimolar suspensions in neat organic solvents.

Methods Viscosities of aqueous solutions and non-aqueous suspensions of the model protein bovine γ-globulin and a murine monoclonal antibody were examined under a variety of experimental conditions. In addition, protein particle sizes were measured using dynamic light scattering and light microscopy.

Results Concentrated suspensions of amorphous γ-globulin powders (up to 300 mg/mL, composed of multi-micron-sized particles) in absolute ethanol and a number of other organic solvents were found to have viscosities up to 38 times lower than the corresponding aqueous solutions. Monoclonal antibody follows the same general trend. Additionally, the higher the protein concentration and lower the temperature, the greater the viscosity benefit of a suspension over a solution.

Conclusions The viscosities of concentrated γ-globulin and monoclonal antibody suspensions in organic solvents are drastically reduced compared to the corresponding aqueous solutions; the magnitude of this reduction depends on the solvent, particularly its hydrogen-bonding properties.

KEY WORDS amorphous · formulation · proteins · suspension · viscosity

ABBREVIATIONS

MAb Monoclonal antibody

INTRODUCTION

Monoclonal antibody therapeutics are increasingly common in clinical practice (1–3). A major impediment to their even wider use is the need for highly concentrated formulations because sufficient therapeutic potencies often require protein doses of hundreds of milligrams (4). The U.S. Food and Drug Administration (FDA) does not permit subcutaneous injections of volumes over ~1.5 mL with a viscosity exceeding ~50 centipoise (cP) (5). Such a requirement is often onerous since multi-hundred-mg/mL protein solutions are typically very viscous. This thick consistency with a strong resistance to flow makes concentrated protein formulations challenging to handle and administer to patients. Hence lowering their viscosity is critical to harness the benefits of protein-based pharmaceuticals.

Recently, new strategies toward the aforementioned goal, such as the addition of hydrophobic salts (6,7) and of molecular crowding agents (8,9) to concentrated protein solutions, have afforded much reduced viscosities. An alternative promising approach is to replace aqueous protein solutions with crystalline suspensions (4,10). However, crystallizing antibodies is unpredictable and time-consuming due to their high molecular weight, abundant glycosylation, and structural flexibility (3,4). In the present study, we overcome this drawback and demonstrate that viscosities of suspensions in neat ethanol and

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numerous other organic solvents, made from amorphous powder of γ-globulin (a model protein for monoclonal antibodies) and a murine monoclonal antibody, are drastically lower than those of equally concentrated aqueous solutions.

MATERIALS AND METHODS

Monoclonal antibody (designated herein as MAb) was kindly provided by Sanofi-Aventis Company (Frankfurt, Germany). The 147-kDa murine MAb with an isoelectric point of 6.6 was supplied at 8.6 mg/mL in 10 mM citrate buffer (pH5.5). Bovine γ-globulin (product #G5009), other reagents, and most solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO); absolute ethanol was from VWR International (Radnor, PA).

Viscosities were measured using a Brookfield DV-II Pro viscometer equipped with a cone-and-plate geometry, a CPE 40 or CPE 52 spindle, and a temperature-controlling water bath. The viscometer was pre-calibrated using a CAP0L standard (supplied by Brookfield) and water. Measurements were performed with 500-μL samples in the "external" mode, where increasing shear rates equivalent to10%–100% of instrument torque (from 3.0 to 53 s^{-1} and 23 to 200 s^{-1}) were used, depending on viscosity. The viscosity values reported herein are an average of at least duplicate measurements taken at 25°C unless stated otherwise. Plots of viscosity *vs.* shear rate were typically non-linear, characteristic of non-Newtonian pseudoplastic solutions and suspensions (11); however, such solvents as ethanol and ethyl acetate, when measured alone as controls, exhibited ideal Newtonian behavior. All protein formulations exhibited a non-Newtonian dependence, as expected for concentrated solutions and colloidal suspensions (12,13). The resultant plots were extrapolated to zero-shear values from the highest three shear rates (7,11). Apparent viscosities calculated from these linear extrapolations were accepted when the R^2 exceeded 0.95 (11).

Particle diameters of γ-globulin nanosuspensions were measured using a DynaPro NanoStar Light Scattering instrument from Wyatt Technology (Santa Barbara, CA) in the dynamic light scattering mode. Measurements were performed in duplicate according to manufacturer's instructions in a quartz cuvette at a laser wavelength of 658 nm.

Particle diameters of micron-sized γ-globulin suspensions were measured using a Zeiss Axioplan II upright microscope with a halogen transmitted light source. Images were collected with a Qimging color camera at 10- and 25-fold magnifications. A protein suspension in 2 μL of ethanol was smeared across a glass microscope slide, quickly covered with a No. 1 glass cover slip and immediately sealed with nail polish to prevent evaporation of ethanol. ImageJ software was used to determine the average diameter of ~200 protein particles per sample.

γ-Globulin aqueous solutions were prepared at the desired concentration by adding water or aqueous buffer to protein powder, followed by centrifugation at 1,300×*g* for 60 min using an Eppendorf 5810R centrifuge equipped with an A-4-81 rotor. Volume was adjusted as necessary: for example, only 3.5 mL of water was added to 1.3 g of γ-globulin to prepare 5 mL of protein solution at 260 mg/mL. A stir bar was added, and the resulting mixture was gently shaken overnight at room temperature to achieve complete dissolution of the protein. The pH of this solution was 6.0 unless otherwise indicated. Subsequently, the γ-globulin solution was centrifuged at 500×*g* for 10 min to remove air bubbles prior to viscosity measurements.

To make protein nanosuspensions using such precipitating agents as salts and 4,000-Da poly(ethylene glycol) (PEG4000), a solution of γ-globulin was prepared with a final volume below 5 mL. A precipitant was then added to the desired concentration, and the mixture at room temperature was gently either shaken overnight to dissolve the salt or stirred for 1 h to dissolve the PEG4000, and centrifuged at 500×*g* for 10 min to remove air bubbles. In both cases, the volume was then adjusted as necessary.

To prepare protein nanosuspensions with PEG and another added excipient, a nanosuspension of γ-globulin with PEG was formed first as described in the preceding paragraph. A surfactant solution or a solid salt was then added to the desired concentration, and the mixture was gently stirred for 3 h (overnight for the salts). The mixture was centrifuged at 500×*g* for 10 min, and the volume was subsequently adjusted as necessary.

To prepare nanosuspensions with ethanol as a precipitating agent, a 50 mg/mL γ-globulin solution prepared as described above was added drop-wise to aqueous ethanol with stirring on an IKA (Wilmington, NC) RET stirring hot plate to reach a final protein concentration of 15 mg/mL and a final ethanol concentration of 50% (v/v). The resulting nanosuspension was directly used for viscosity measurements. An analogous protocol was used to make aqueous suspensions with ethanol: a concentrated γ-globulin solution prepared as described above was mixed with neat ethanol to give a final ethanol concentration in the range of 80–95% (v/v) and a final γ-globulin concentration of 260 mg/mL.

To prepare γ-globulin suspensions in neat organic solvents, the latter was added to protein powder to the desired concentration (*e.g.*, 5 mL to 1.3 g). The mixture was centrifuged at 1,300×*g* for 10 min. A stir bar was introduced, and the suspension was tapped to release the thick protein pellet and stir bar. The resultant suspension was gently shaken for 3 h at room temperature and centrifuged at 500×*g* for 10 min. The volume was adjusted as necessary, and the suspension was stirred for 5 min at room temperature to re-suspend protein particles

before viscosity measurements. The same viscosities were observed whether the suspension was formed by adding solvent to γ-globulin or *vice versa*.

Ethanol precipitation of γ-globulin was performed in a cold room from a 260 mg/mL protein solution prepared as described above with both liquids chilled to 4°C. The final ratio of absolute ethanol to protein solution was 4:1 (v/v). The first portion of cold ethanol was added drop-wise to a protein solution with vigorous stirring. The remaining three portions were added in a slow stream. The resultant mixture was stirred for 10 min and filtered through a Corning Disposable Sterile Filter System with a 0.22-μm-pore polyethersulfone membrane. After 10 min, the collected solid (which should be slightly sticky but not wet) was disturbed and left on the filter to dry. After another 5 min, the solid was transferred from the filter to a weigh paper and dried for 1 h. During this drying step, the solid was repeatedly flattened and then scraped up in order to help release trapped ethanol to result in a white, very fine, free-flowing powder.

Solid MAb was prepared from the buffered aqueous solution in which it was received from Sanofi-Aventis by concentration, lyophilization, and liquid-nitrogen milling. First, it was spin-concentrated to approximately 200 mg/mL in a 50-kDa MWCO Amicon Ultra-15 device (EMD Millipore) with a regenerated cellulose membrane. The concentrated solution was flash frozen and lyophilized for up to 48 h until dryness. A mortar and pestle were placed in a crystallization dish and pre-cooled in liquid nitrogen. Solid MAb was placed in the mortar and frozen for 10 min. Next, the protein was milled for 10 min; liquid nitrogen was added to the mortar and dish as needed to prevent crystallization of water within the mortar and on the end of the pestle. After milling, the suspended protein was poured into a glass scintillation vial and covered with a Kimwipe™ to allow the liquid nitrogen to evaporate. The solid MAb was then stored at 4°C for short-term use.

Aqueous solutions were prepared by incrementally adding solid MAb powder to an appropriate volume of water or aqueous buffer in a 1.5-mL Eppendorf tube (solvent was chosen to give the same citrate concentration in all samples). The mixture was centrifuged in a VWR Galaxy 7 Microcentrifuge at 1,500 rpm for 1 min and then gently shaken at room temperature until near-complete dissolution occurred (typically 30–60 min). Additional powder was added and the process repeated until the final concentration of protein – as determined by absorbance at 280 nm – was achieved.

To prepare MAb suspensions in neat organic solvent, protein powder was added to a 5-fold (w/v) excess of solvent in a 15-mL Falcon tube. The mixture was centrifuged at 1,000×*g* for 10 min using an Eppendorf 5810R centrifuge equipped with an A-4-81 rotor and then gently shaken for 1 h at room temperature. The protein was pelleted at 2,500×*g* for up to 10 min and the solvent aspirated. Fresh solvent was added to the desired concentration (as determined by the total volume of the suspension: the sum of the volumes of liquid and the pelleted solid). The powder was resuspended by gentle shaking for 5 min immediately prior to viscosity measurements.

RESULTS AND DISCUSSION

Murine, chimeric, humanized, and human IgG monoclonal antibodies comprise the majority of clinically validated therapeutics and experimental proteins (1,2). Due to their relatively low specific activities, highly concentrated solutions of these antibodies are often necessary for subcutaneous injections; unfortunately, they are typically very viscous making injections difficult. Herein we have explored this phenomenon and a way of circumventing it by employing concentrated suspensions of amorphous protein powders, rather than conventional aqueous solutions. Bovine γ-globulin has been used as a model antibody for most of this study.

First, we investigated concentration dependence of the viscosity of aqueous solutions of γ-globulin. As seen in Fig. 1, the expected (7,14,15) exponential (as opposed to linear) increase in solution viscosity was observed at high protein concentrations. At 300 mg/mL, for example, the γ-globulin solution viscosity at 25°C is 370±20 cP, far exceeding that permitted for subcutaneous injections (6). Using dynamic light scattering, we determined that the average effective diameter of γ-globulin molecules in this solution is 37 nm (Table I). Simple calculations reveal that protein molecules in this solution must be in close proximity to each other, with an average intermolecular distance of less than one

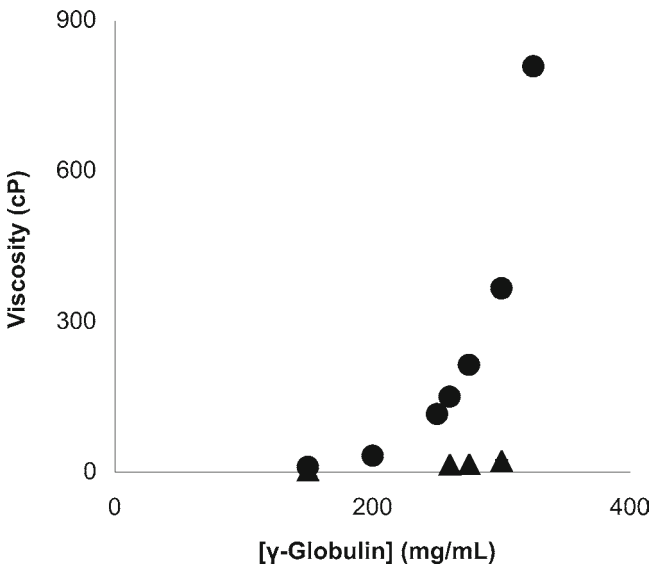

Fig. 1 Concentration dependence of viscosities of solutions (*circles*) and suspensions (*triangles*) of γ-globulin at 25°C. Solutions were prepared in de-ionized water to give a final pH of 6.0 and suspensions using neat ethanol. The data points incorporate the standard deviations which, however, are often smaller than the symbols.

Table I Viscosities at 25°C and Protein Particle Sizes of 300 mg/mL γ-Globulin Formulations in 20 mM Aqueous Histidine-HCl Buffer (Final pH of 5.2)

Formulation	Viscosity (cP)	Particle diameter (nm)
Solution	370 ± 17	37 ± 6.1
Suspension formed by 1% w/v PEG4000	530 ± 12	110 ± 2.3
Dissolved suspension	n.d.	36 ± 1.7

third of a molecular diameter (based on a spherical-shape approximation with a Stokes diameter of 10.5 nm for γ-globulin (16)). The resultant intimate intermolecular contacts and abundant hydrophobic and electrostatic interactions (7) create a strong resistance to flow, *i.e.*, high viscosity. This high γ-globulin concentration was selected for most of the subsequent study.

The essence of our approach to lowering viscosity was to form a γ-globulin suspension (as opposed to a solution), in which interior protein molecules within a suspended particle are "hidden". To this end, the commonest protein precipitating agent, ammonium sulfate (17), was added to a 300 mg/mL γ-globulin solution. At salt concentrations less than 1 M (pH 6.0, 25°C), however, no visible suspension was observed; indeed the particle diameter measured by light scattering was the same 37 nm as in buffered solution. At ammonium sulfate concentrations exceeding 1 M, a protein suspension did form but was too viscous to measure.

Next, another precipitating agent, 4,000-Da poly(ethylene glycol) (PEG4000) was investigated. PEG, typically used in relatively low concentrations, has been examined as a macromolecular crowder (4, 8, 9, 18). Addition of 1% PEG4000 to a 300 mg/mL γ-globulin solution produced a translucent mixture suggesting a nanosuspension (*i.e.*, having a particle diameter comparable to a half of the wavelength of visible light). Indeed, particle size analysis thereof by dynamic light scattering yielded a diameter of 110 nm (Table I). Henceforth, translucency was used as a tentative visual indicator of nanosuspension formation.

The viscosity of this nanosuspension was measured to be 530 cP, which is 1.4-fold above that of a γ-globulin solution of the same concentration (Table I). We found that this increase in viscosity was not due to irreversible denaturation or aggregation of the protein upon precipitation with PEG; when the nanosuspension was diluted, thereby re-dissolving the protein, the average particle diameter dropped to 36 nm, *i.e.*, the same as for γ-globulin in the original solution (Table I).

We previously ascertained that hydrophobic intermolecular interactions are largely responsible for high viscosities of concentrated solutions of monoclonal antibodies (7). To test this possibility for γ-globulin, we added various surfactants to its PEG-induced nanosuspension to disrupt hydrophobic contacts. Cationic, anionic, non-ionic, and zwitter-ionic surfactants indeed all slightly lowered the viscosities of PEG nanosuspensions of γ-globulin, with the most potent (non-ionic Pluronic F68) affording a 30% drop (Table II). As seen in the table, however, a similar viscosity-lowering effect was observed for γ-globulin solutions. Though marginal in magnitude and certainly falling short of our ultimate objective, these effects suggest that hydrophobic interactions play a role in the high viscosities of concentrated solutions and nanosuspensions of γ-globulin.

To test whether another factor – electrostatic interactions – contributes to viscosity increases in nanosuspensions, we added 1 M salts to weaken these ionic interactions. However, both increasing and decreasing viscosities were observed in PEG-induced nanosuspensions of γ-globulin depending on the salt (Table III). Interestingly, the addition of NaCl, recently reported to greatly decrease the viscosity of monoclonal antibody solutions (14), actually raised the viscosity of the suspension some 2.5 fold. The effects of salt excipients on solution viscosity thus seem to be both protein-dependent and not applicable to suspensions.

Since arginine salts are common additives in pharmaceutical formulations (19), we explored them next. Arginine-HCl was the most potent salt among those tested, decreasing the viscosity of a 300 mg/mL γ-globulin nanosuspension 6-

Table II Viscosities at 25°C of 300 mg/mL γ-Globulin Solutions and 1% (w/v) PEG-induced Nanosuspensions in the Presence of Surfactants in 20 mM Aqueous Histidine-HCl Buffer (Final pH of 5.2)

Surfactant		Viscosity (cP)[a]	
Compound (% w/v)	Classification	Solution	Nanosuspension
None		370 ± 17	530 ± 12
BKC[b] (0.15)	Cationic	300 ± 6.8	400 ± 3.2
BKC[b] (0.04)	Cationic	310 ± 3.6	390 ± 2.0
SDS[c] (0.1)	Anionic	320 ± 1.7	400 ± 2.4
Tween 80 (0.1)	Non-ionic	340 ± 3.4	480 ± 3.1
Tween 80 (0.001)	Non-ionic	340 ± 17	470 ± 7.6
Pluronic F68 (0.06)	Non-ionic	240 ± 1.0	400 ± 4.9
Pluronic F68 (0.01)	Non-ionic	n.d.	380 ± 12
CHAPS[d] (1.2)	Zwitter-ionic	320 ± 3.8	410 ± 6.1
CHAPS[d] (0.3)	Zwitter-ionic	n.d.	440 ± 11

n.d. not determined

[a] The surfactants themselves, even at the highest concentrations used, made negligible contributions to observed viscosities. The *p*-values reflecting the viscosity differences between solutions and the corresponding nanosuspensions were in all cases no greater than 0.01. The same applies to the viscosity differences between the nanosuspension without surfactant and those with surfactants, except that in this case no *p*-value exceeded 0.03

[b] Benzalkonium chloride

[c] Sodium dodecyl sulfate

[d] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

Table III Viscosities at 25°C of 300 mg/mL γ-Globulin Solutions and 1% (w/v) PEG-induced Nanosuspensions in the Presence of Various 1 M Salts in 20 mM Aqueous Histidine-HCl Buffer (Final pH of 5.2)

Salt	Viscosity (cP)	
	Nanosuspension	Solution
None	530 ± 12	366 ± 17
Arginine-HCl	89 ± 1.6	89 ± 0.30
Tris–HCl	130 ± 2.1	110 ± 5.3
$H_2N(CH_2)_2OH$-HCl	140 ± 4.5	150 ± 17
Guanidine-HCl	280 ± 3.1	n.d.
NH_4Cl	390 ± 35	n.d.
$NaNO_3$	540 ± 6.0	n.d.
KCl	770 ± 24	n.d.
L-Lysine-HCl	780 ± 32	n.d.
D-Lysine-HCl	880 ± 23	n.d.
NaCl	1300 ± 60	n.d.
CH_3COONa	1500 ± 230	n.d.

n.d. not determined

fold (Table III). As seen in the table, ethanolamine-HCl and Tris–HCl were also quite potent, reducing viscosity 3.8- and 4.1-fold, respectively. However, although all these hydrochlorides substantially decreased γ-globulin nanosuspension viscosities, their effect on a 300 mg/mL protein solution was equally beneficial (Table III), thus making formation of nanosuspensions superfluous.

Analogs of arginine-HCl were then used to investigate any effects on viscosity resulting from the nature of the salt's anion. As seen in Table IV, arginine acetate was the most effective, reducing nanosuspension viscosity 6.6-fold. Moreover, it is one of only two salts affording PEG-induced γ-globulin nanosuspensions that are slightly less viscous than the corresponding solution (Table IV). The general pattern, though, remained unchanged; even when modification of PEG-induced aqueous nanosuspensions with surfactants or salts lowered viscosity, the effect was still comparable to that achieved by adding the same excipients to a protein solution.

Table IV Viscosities at 25°C of 300 mg/mL γ-Globulin Solutions and 1% (w/v) PEG-induced Nanosuspensions in the Presence of Various 1 M Arginine Salts in 20 mM Histidine-HCl Buffer (Final pH of 5.2)

Anion	Viscosity (cP)	
	Nanosuspension	Solution
None	530 ± 12	366 ± 17
CH_3COO^-	80 ± 9.5	121 ± 13
Cl^-	89 ± 1.6	89 ± 0.30
NO_3^-	89 ± 4.2	94 ± 3.2
Succinate	100 ± 2.6	130 ± 4.3
Fumarate	105 ± 5.2	100 ± 4.9
$HCOO^-$	110 ± 11	n.d.
CF_3COO^-	110 ± 7.9	n.d.
Camphorsulfonate	140 ± 1.0	n.d.
$CH_3CH_2COO^-$	150 ± 11	n.d.
SO_4^{2-}	160 ± 0.13	n.d.
Citrate	180 ± 3.7	n.d.
Glutamate	210 ± 17	n.d.

n.d. not determined

We therefore explored still additional precipitating agents. Sodium citrate (18) at a 0.43 M concentration formed a translucent nanosuspension of γ-globulin with a viscosity of 770 cP and an average particle diameter of 120 nm (Table V). However, this viscosity was higher than that of the corresponding solution. Ethanol is also a common precipitant for proteins, including γ-globulins (17, 20). Indeed, in 50% (v/v) aqueous ethanol, γ-globulin formed a translucent nanosuspension with a particle diameter of 110 ± 9.9 nm. Curiously, ethanol-induced nanosuspensions formed only at low protein concentrations. We measured the viscosity of a dilute 15 mg/mL γ-globulin nanosuspension to be 20 cP, *i.e.*, 5-fold higher than that of the corresponding protein solution (Table VI). Thus, as with sodium citrate, a nanosuspension is again more viscous than a solution.

Protein nanoparticles comprise a bimodal population: those protein molecules buried within the particle and those on the surface that are directly exposed to other particles. One would expect that decreasing the fraction of the latter protein molecules should reduce viscosity. When formed with sodium citrate, PEG, or ethanol, nanoparticles have a mean diameter of 113 nm (Tables I, V, and VI); a straightforward calculation (for an idealized spherical nanoparticle with a diameter of 113 nm composed of idealized spherical protein particles with a diameter of 37 nm, all but one protein molecule will be on the surface: 113 nm–2 × 37 nm = 39 nm; in other words, the nanoparticle contains essentially three protein molecules across) reveals that the vast majority of the protein molecules in particles of this size are on the surface. Hence such nanoparticles fail to effectively shield a significant fraction of γ-globulin molecules. In

Table V Viscosities at 25°C and Protein Particle Sizes of 300 mg/mL γ-Globulin Solutions and Nanosuspensions (Induced by 0.43 M Na Citrate) in 20 mM Aqueous Histidine-HCl Buffer (Final pH of 5.2)

Formulation	Additive	Viscosity (cP)	Particle diameter (nm)
Solution	None	370 ± 17	37 ± 6.1
Nanosuspension	None	770 ± 33	120 ± 3.7
Solution	1 M Arginine acetate	120 ± 13	n.d.
Nanosuspension	1 M Arginine acetate	110 ± 3.7	n.d.

n.d. not determined

Table VI Viscosities at 25°C and Protein Particle Sizes of a 15 mg/mL γ-Globulin Solution and Ethanol-induced Nanosuspension in 20 mM Aqueous Histidine-HCl Buffer (Final pH of 5.2)

Formulation	Viscosity (cP)	Particle diameter (nm)
Solution	4.0 ± 0.14	21 ± 0.45
Nanosuspension	20 ± 0.10	110 ± 9.9

n.d. not determined

contrast, simple geometrical considerations predict that forming larger protein particles should (i) increase the fraction of interior (hidden) protein molecules and (ii) substantially diminish the number of protein particles in solution, thereby increasing the free space available to each.

We observed that high concentrations of γ-globulin in 80–100% (v/v) ethanol formed dense and opaque suspensions, as opposed to translucent nanosuspensions. We further analyzed this type of a suspension, necessarily comprised of particles much larger than nanoparticles. In particular, we examined the dependence of suspension viscosity on ethanol concentration. At 260 mg/mL, a γ-globulin suspension in neat (*i.e.*, 100%) ethanol had the lowest viscosity: 15 ± 0.81 cP (Fig. 2). Importantly, this is 9-fold lower than the viscosity of the corresponding aqueous solution of γ-globulin!

Examination of this γ-globulin suspension in neat ethanol by light microscopy revealed a wide distribution of particle sizes in the micron range, with a mean particle diameter of 8.9 μm (Table VII). In contrast to their much smaller counterparts discussed above, particles of this size should display only a small fraction of their component protein molecules on the surface, with the rest being hidden in the interior; in addition, such suspensions should have much larger inter-particle distances compared to nanosuspensions, let alone solutions, of γ-globulin.

It is well established that enzymic proteins are remarkably stable when suspended in neat organic solvents (21).

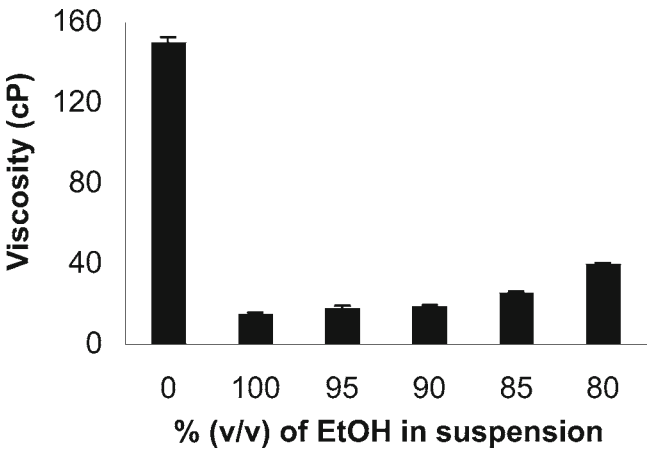

Fig. 2 Viscosities at 25°C of a 260 mg/mL aqueous solution and aqueous-ethanol suspensions of γ-globulin. Solutions were prepared in 20 mM histidine-HCl buffer (final pH of 5.2) and suspensions using neat ethanol.

We confirmed this principle for γ-globulin suspended in absolute ethanol by removing the solvent under vacuum, dissolving the dried protein powder in water, and measuring the resultant solution viscosity. The original and post-suspension solution viscosities were similar, suggesting that under these conditions the protein suffers no irreversible solvent-induced unfolding and/or aggregation.

Figure 1 depicts the dependence of viscosity of γ-globulin suspensions in neat ethanol on protein concentration. One can see that the greatest drop in viscosity compared to the corresponding aqueous solution, 16-fold, is achieved at the highest protein concentration of 300 mg/mL. Therefore, while the viscosity of γ-globulin suspensions is lower than that of the solution at all protein concentrations, the higher the concentration the greater the advantage (Fig. 1).

We further found that as the temperature was lowered from 25° to 10°C, viscosities of a 260 mg/mL solution and ethanol suspension increased 3.0 and 1.7 fold, respectively (Fig. 3). This pattern also holds true for 300 mg/mL γ-globulin formulations, where solution and suspension viscosities at 25 C were 370 ± 17 cP and 23 ± 2.3 cP, respectively. At 17°C, the solution viscosity rose 1.8 fold, while that of the suspension only 1.3 fold (Fig. 3). Therefore, the lower the temperature and higher the concentration of the protein, the greater the viscosity benefit of an ethanol suspension of γ-globulin compared to the corresponding solution.

Thus far, all γ-globulin formulations were prepared from the solid protein as it was obtained from the manufacturer. Ideally, however, we would prepare γ-globulin powders ourselves to control the process and ensure its reproducibility. To this end, we employed cold-ethanol precipitation, which is a rapid isolation method widely used for antibody purification (17, 20), to prepare γ-globulin powder from the commercial one. As seen in Table VII, the resultant protein possessed nearly the same aqueous solution and ethanol suspension viscosities as the commercial γ-globulin powder; hence cold-ethanol precipitation appears to cause no permanent damage to the protein. In agreement with this conclusion, the particle size in the resultant suspension (Table VII) was similar to that seen when a suspension was prepared from commercial γ-globulin.

Table VII Viscosities at 25°C and Protein Particle Sizes of a 260 mg/mL Aqueous Solution and Ethanol Suspension of Cold-ethanol-precipitated γ-Globulin

Protein preparation	Viscosity (cP)		Particle diameter (μm)[a]	
	Solution	Suspension	Mean	Median
Commercial	150 ± 2.5	15 ± 0.81	8.9 ± 6.1	7.06
Ethanol-precipitated	140 ± 10	16 ± 1.3	9.6 ± 6.7	8.06

[a] Upon a 10-fold dilution, the distribution of particle sizes did not change (data not shown)

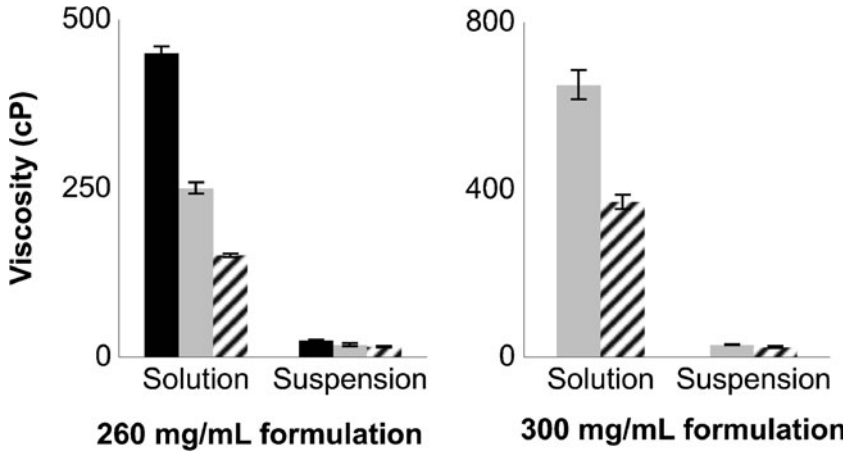

Fig. 3 Temperature dependence of viscosities of γ-globulin suspensions of two different concentrations in neat ethanol measured at 10°C (*black bars*), 17°C (*gray bars*), and 25°C (*hatched bars*).

Next, using the aforementioned cold-ethanol-precipitated γ-globulin, we investigated the generality of our findings. Suspensions of lysozyme in organic/oil mixtures had been previously shown to produce low-viscosity formulations (8, 22), although their viscosities were not directly compared to those of aqueous protein solutions. Here we selected over a dozen relatively simple neat organic solvents (Table IV), and the viscosities of a 260 mg/mL suspension of γ-globulin in each of them (as well as the viscosities of the solvents themselves) were measured. As seen in Table VIII, most of these protein suspensions were much less viscous than a γ-globulin solution in water. In fact, the lowest viscosity overall was observed with the tetrahydrofuran suspension: only 3.6 cP, constituting a 38-fold reduction compared to protein solution (Table VIII, Fig. 4). While tetrahydrofuran is not FDA-approved as a component of drug formulations, isopropanol is (23). A 260 mg/mL suspension of γ-globulin in this solvent also has a low viscosity of 7.7 cP, that is, 18 times reduced over the corresponding protein solution.

It is tempting to speculate why some of the γ-globulin suspensions (Table VIII) are more viscous than others. Inspection of the data suggests that the viscosity roughly relates to the hydrogen-bonding ability of the solvent. Those solvents that are unable to donate a hydrogen atom for hydrogen-bonding (*i.e.*, ethyl acetate, *N*-methylpyrrolidone, and toluene) or that possess only one such hydrogen (isopropanol, ethanol, and methanol) as a group result in the lowest protein suspension viscosities. In contrast, solvents that contain two hydrogen atoms available for H-bond donation (PEG200, propylene glycol, 1,4-butanediol) have viscosities over 6-fold higher. Thus it appears that those solvent molecules that are "sticky", *i.e.*, form the most extensive hydrogen bonding with protein particles, result in the greatest suspension viscosity; as seen in Table VII, this rationale applies to γ-globulin's aqueous solution as well. It is worth noting that a recent publication (24) reported an opposite conclusion for three monoclonal antibodies suspended in miglyol, benzyl benzoate, and/or ethyl lactate (even aside from obtaining no viscosity reduction benefits from suspending monoclonal antibodies in them). This difference is likely due to the fact that all three solvents tested were esters, thus providing little chemical diversity. The same conclusion applies to another 2012 publication (25).

Finally, using murine monoclonal antibody (MAb) we explored the generality of the findings presented in Table VIII that the viscosity of γ-globulin suspensions in neat organic solvents is drastically lower than in the

Table VIII Viscosities at 25°C of Suspensions of Ethanol-precipitated γ-Globulin at 260 mg/mL in Various Neat Organic Solvents

Number of hydrogen atoms available for H-bonding	Solvent[a]	Viscosity (cP)	
		EtOH-precipitated protein powder suspended in solvent	Solvent itself
≤ 1	**1**	3.6 ± 0.003	0.32 ± 0.01
≤ 1	**2**	5.8 ± 0.50	0.46 ± 0.10
≤ 1	**3**	6.3 ± 0.70	0.49 ± 0
≤ 1	**4**	7.5 ± 0.24	0.33 ± 0.004
≤ 1	**5**	7.7 ± 1.6	2.2 ± 0.11
≤ 1	**6**	8.1 ± 1.3	1.9 ± 0.050
≤ 1	**7**	12 ± 1.0	0.40 ± 0.010
≤ 1	**8**	14 ± 1.6	1.72 ± 0.03
≤ 1	**9**	15 ± 0.89	0.87 ± 0.010
≤ 1	**10**	16 ± 1.3	1.1 ± 0.12
≤ 1	**11**	25 ± 2.0	0.61 ± 0
≤ 1	**12**	26 ± 1.0	7.8 ± 0.36
2	Water	140 ± 10	0.90 ± 0.010
2	**13**	160 ± 6.5	48 ± 0.23
2	**14**	200 ± 1.0	43 ± 0.10
2	**15**	240 ± 23	55 ± 1.5

The data for aqueous solution are included for comparison

[a] It is worth noting that the solvents **2**, **5**, **6**, **10**, **12**–**14**, and, of course, water are FDA-approved for use in IM/IV pharmaceutical formulations; the remaining solvents are not (Ref. 23)

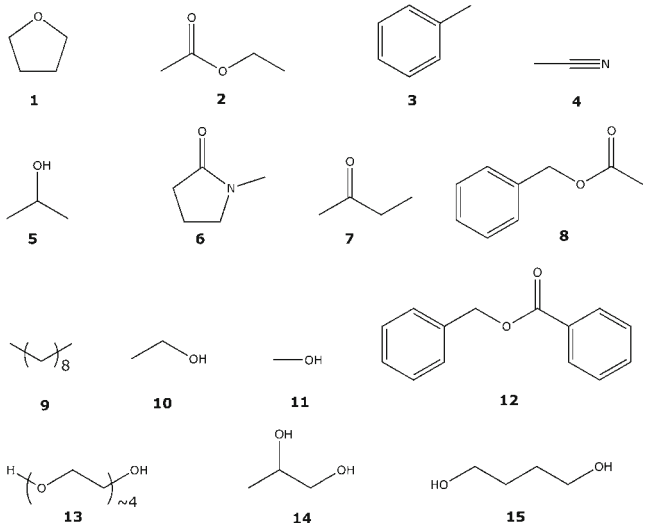

Fig. 4 Chemical structures of organic solvents in which antibody powders were suspended. Tetrahydrofuran (**1**), ethyl acetate (**2**), toluene (**3**), acetonitrile (**4**), isopropanol (**5**), *N*-methylpyrrolidone (**6**), methyl ethyl ketone (**7**), benzyl acetate (**8**), decane (**9**), ethanol (**10**), methanol (**11**), benzyl benzoate (**12**), PEG 200 (**13**), propylene glycol (**14**), and 1,4-butanediol (**15**).

corresponding aqueous solution. To this aim, we obtained a MAb solution made at the pharmaceutical company Sanofi-Aventis. To prepare a powder, the solution was concentrated approximately 20-fold and lyophilized to yield a dense solid. This solid was then milled in liquid nitrogen to minimize putative protein damage resulting from heat and/or moisture. Using this milled powder we prepared an aqueous solution at 200 mg/mL. Its viscosity, 14±0.75 cP (Table IX), is very close to the 12±0.14 cP for a solution of the non-milled powder (*i.e.*, a MAb solid obtained from direct lyophilization of the original 8.6 mg/mL solution), pointing to no damage/aggregation of MAb imparted by liquid nitrogen milling. When we suspended this milled MAb powder in toluene – a solvent that afforded one of the lowest suspension viscosities in γ-globulin studies (Table VIII) – at the same 200 mg/mL concentration, its viscosity was 4.9±0.68 cP (Table IX), *i.e.*, a 2.5-fold reduction compared to that of the solution.

Table IX Viscosities at 25°C of 200 mg/mL[a] Aqueous Solution (Final pH of 5.2), as well as a Suspension in Toluene, of Murine MAb

Protein preparation	Viscosity (cP)
Solution	12±0.14
N_2-Milled MAb solution[b,c]	14±0.75
N_2-Milled[b] MAb toluene suspension	4.9±0.68

[a] It was not possible to examine more concentrated preparations because solid MAb at 200 mg/mL comprised almost the entire volume of the suspension in organic solvents

[b] "N_2-milled" refers to solid MAb obtained by milling in liquid nitrogen

[c] A minor portion of the MAb was insoluble in water, presumably due to irreversible aggregation. Protein concentration determined by measuring absorbance at 280 nm

CONCLUSION

In this work, we successfully endeavored to lower the viscosities of concentrated antibody formulations by switching from solutions to suspensions of amorphous powders. Methods to prepare low-viscosity suspensions of the model protein γ-globulin and a full-length murine MAb in organic solvents were elaborated and validated. Protein-protein and protein-solvent hydrophobic, electrostatic, and hydrogen-bonding interactions were identified as critical in the observed viscosity reductions. By suspending protein powders in organic solvents possessing one or no hydrogen atoms available for H-bonding, we prepared highly concentrated but still non-viscous antibody formulations.

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