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Research paper

Rheological and syringeability properties of highly concentrated human polyclonal immunoglobulin solutions

V. Burckbuchler [a,b], G. Mekhloufi [a,*], A. Paillard Giteau [b], J.L. Grossiord [a], S. Huille [b,1], F. Agnely [a,1]

[a] Faculté de Pharmacie, Université Paris-Sud, UMR CNRS 8612, Châtenay – Malabry cedex, France
[b] Laboratoire français du fractionnement et des biotechnologies (LFB Biotechnologies), Les Ulis, France

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ABSTRACT

This study of highly concentrated polyvalent immunoglobulin solutions, IgG, aimed at analyzing the relationships between protein concentration and aggregation on the one hand and viscosity on the other hand. Viscosity variations as a function of IgG concentration showed two well-defined behaviours: a Newtonian behaviour for low-concentrated solutions and a shear-thinning behaviour for highly concentrated ones. The viscosity data fitted very well with the Mooney model, suggesting the absence of intermolecular interactions in the IgG solutions that behaved like a non-interacting suspension of hard particles. The polyclonal nature of IgG seems to prevent intermolecular interaction. The shape factor, determined from Mooney fitting, revealed a non-spherical shape of the polyclonal IgG molecules. The rheological properties were also correlated with the injection force (F) through hypodermic needles by syringeability tests. Here, F was mainly affected by three parameters: the solution viscosity, the injection flow rate, and the needle characteristics. In fact, syringeability tests showed that F increased with IgG concentration and flow rate and decreased with the internal diameter of the needle. A zone for optimal injection conditions was then identified taking into account the different affecting parameters and mainly a maximum force for manual injection, which was fixed at 30 N.

1. Introduction

Human normal immunoglobulins are polyvalent IgG obtained from pools of plasma, collected from many healthy blood donors. They are used as therapeutic agents in two main types of indications [1,2]: namely for substitution in antibody deficiencies and for immunomodulation in a large number of autoimmune and/or systemic inflammatory diseases [3,4]. Due to the limitations in the injection volumes and the large dose requirements of these treatments (hundreds of mg/kg), they are formulated as highly concentrated solutions. The commercially available human normal immunoglobulin preparations are rather concentrated (from 50 to 160 mg/mL) and particularly compared to monoclonal immunoglobulin formulations. Many monoclonal antibodies are marketed at concentrations of 50 mg/mL and above. However, commonly they are found at lower concentration.

Higher concentrations of IgG formulations would thus be of great interest to reduce the infusion volume and even to gain in storage space. The development of protein formulations at high concentrations raises several practical challenges in terms of stability, manufacturing, and delivery, owing to the propensity of proteins to aggregate at higher concentrations [5–8]. Indeed, contrary to chemical reactions which are hydrolytically driven and generally slightly concentration dependent, aggregation that requires bi-molecular collisions is highly concentration dependent. It is likely that aggregation rates increase at higher concentrations simply due to increased encounter frequency. Aggregation is thus expected to be the primary degradation pathway in high-concentration protein formulations. Protein aggregation may have an impact on protein activity, pharmacokinetics, and safety, due to potential immunogenicity [9–11]. It can also be very critical for the manufacturing process. In addition, high-concentration protein formulations may also be difficult to be used, because of their high viscosity, with pre-filled syringes or injection devices proposed for improving administration, compliance, and safety.

Various methods for concentrating a protein solution can be performed, and these present both advantages and practical limitations [6]. Manufacturing of IgG can be accomplished by a multi-step process, including several ultra-/diafiltration steps. Filtration processes are often the last step before formulation. Attainment of very high-concentration formulations by this process can be difficult because of membrane clogging. Moreover, during these steps,

* Corresponding author. UMR CNRS 8612, Université Paris-Sud 11, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay – Malabry cedex, France. Tel.: +33 01 46 83 59 91; fax: +33 01 46 83 58 82.
E-mail address: ghozlene.mekhloufi@u-psud.fr (G. Mekhloufi).
[1] Equal contribution.

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the high local protein concentration, flow-induced shear, shear-induced interfacial interactions (potential exposure of hydrophobic regions of proteins), and changes in ionic strength of the protein solution may induce aggregation [12–14]. Furthermore, the physical properties of protein formulations at high concentration will impact the ability to easily deliver them. In fact, these concentrated solutions often exhibit high viscosities that can prevent them from flowing through syringe needles, mainly 26- or 27-gauge needles. Thus, from a practical point of view, it is of the utmost importance to investigate the relationship between viscosity and protein concentration.

Rheology is a possible approach to study the behaviour of human normal IgG in highly concentrated solution. Many attempts have been made to relate the rheology of a solution to solute characteristics. Einstein first attempted to relate the relative viscosity (η_r) of dilute suspensions of spherical non-deformable, non-interacting particles to the volume fraction (ϕ), they occupy in the system, through the following equation [15,16]:

$$\eta_r = 1 + 2.5\phi \tag{1}$$

where $\eta_r \equiv \frac{\eta}{\eta_0}$. η denotes the effective suspension viscosity and η_0 the viscosity of the suspending medium (pure liquid). This equation is valid in situations where ϕ is lower than 0.05. The Einstein equation was later extended by Mooney [17] for more concentrated suspensions, where crowding effect due to the polydispersity of the particle diameter is considered. In this case, the relative viscosity of the suspension is assumed to be governed by $\eta_r = f(\phi)$, where the function f depends solely upon ϕ. The Mooney equation is written as follows:

$$\eta_r = \exp\left(\frac{S\phi}{1 - K\phi}\right) \tag{2}$$

S denotes the parameter which, in general, depends on the shape of the dissolved particle and on the hydrodynamic interactions of the particles in solution. It is called the shape factor (Simha parameter). K is the crowding factor that corresponds to the inverse of the maximum packing fraction ϕ_{max}. This equation has been modified and applied successfully to many systems, including suspensions and emulsions [18], magnetic particles [19,20], and protein solutions [21–23].

For protein solutions, the rheological behaviour depends on the composition of the system, on the protein concentration, and on the protein solution properties. Most protein solutions exhibit a non-Newtonian behaviour, whereby the rheological properties show stress dependence. This behaviour is due to protein–protein interactions. However, in practice, significantly different flow profiles have been observed for similarly sized proteins [5]. Whilst studies of rheological properties of monoclonal antibodies have already been conducted, no data on polyvalent immunoglobulins are available to date, to our knowledge. As flow characteristics of proteins can markedly differ upon concentration increase due to protein–protein interactions, knowing the rheological behaviour of human normal immunoglobulin would provide valuable information about protein conformation, protein–solvent and protein–protein interactions as reported for monoclonal antibodies [5,23–25].

In the present study, rheological properties of highly concentrated human normal IgG solutions were studied with a cone-plate rheometer, and the Mooney equation was used to predict protein interactions in solution. Furthermore, a syringeability test was specially designed to correlate these rheological properties with the ability of the concentrated solutions to flow through a syringe equipped with a needle. The effects of solution concentration and viscosity as well as the effects of needle diameter and flow rate on the injection force were thus assessed.

2. Materials and methods

2.1. Materials and solution preparation

Human polyclonal immunoglobulin solutions (M_w = 150 kDa) were obtained by the Laboratoire Français de Fractionnement et des Biotechnologies (Les Ulis, France) from pooled human plasma derived from multiple blood donors. The preparation process of the IgG solution consisted in a combination of industrial steps including ethanol fractionation by the Cohn method [26], virus inactivation, a removal step through solvent/detergent treatment, and nanofiltration [27] purification by various chromatographic steps and ultra/diafiltration. The high-concentration IgG solution was produced by concentrating the bulk solution first by a tangential flow filtration (TFF) process using a Millipore Pellicon 2 TFF mini-cassette 0.1 m^2 with a Biomax® 30 kDa membrane and then with a centrifugal ultrafiltration device (Amicon Ultra-15, Millipore). Each 15-mL centrifugal tubes contained a heat-sealed membrane (MWCO of 30 kD and active membrane area of 7.60 cm^2). The centrifugal ultrafiltration was carried out in a centrifuge (Sigma 3K30 Bioblock Scientific) at 7000 rpm and 4 °C. The final IgG concentration varied from 80 g/L to 380 g/L. The final concentration of the IgG solutions was checked by a UV spectrophotometer (UV-160A, Shimadzu Corporation, Japan) at 280 nm, with a 1-cm quartz cell (Hellma). The IgG samples were diluted with MilliQ water so that the absorbance value was in the appropriate range of the spectrophotometer. All chemical reagents were of analytical grade or higher.

2.2. Size exclusion chromatography

Size exclusion chromatography experiments were conducted using a Tricorn Superdex 200 10/300 GL column (GE Healthcare) on an HPLC system (Alliance 2690). After appropriate dilution of samples, the column was loaded with 500 μg of protein and the samples were eluted at room temperature with a pH 7.4 buffer composed of 10 mM phosphate, 138 mM sodium chloride, and 2.7 mM potassium chloride. A flow rate of 0.4 mL/min was used. The elution peaks were monitored at 280 nm with a UV diode array detector.

2.3. Dynamic light scattering measurements

Aggregation was controlled by dynamic light scattering measurements at 632.8 nm wavelength on an ALV/CGS compact goniometer system. Light scattering intensity was monitored at two angles, 30° and 90°, during 3 runs of 30 s. The solutions supplemented with 50 mM sodium chloride were diluted to 5 g/L and filtered (0.22 μm filter). Samples were then allowed to equilibrate at least for 5 min inside the cell chamber prior to analysis. The measurements were performed at 25.0 ± 0.1 °C.

2.4. Immunoglobulin rheological behaviour

The IgG rheological behaviour was studied by means of a controlled-stress rheometer (RheoStress 600, Haake, Thermo Scientific Inc.) equipped with a cone-plate sensor C35/0.5° (with a diameter of 35 mm, a cone angle of 0.5°, and a gap of 0.026 mm). A thermostatic Universal Temperature Control (UTC) was used to maintain the plate temperature at 25.0 ± 0.1 °C. The samples were first incubated at 25.0 ± 0.1 °C for 15 min in a thermostated bath, and then less than 1 mL of samples were immediately placed on the plate for rheological measurements. In each experimental run, the shear stress was increased from 0 to 40 Pa over 3 min and then decreased

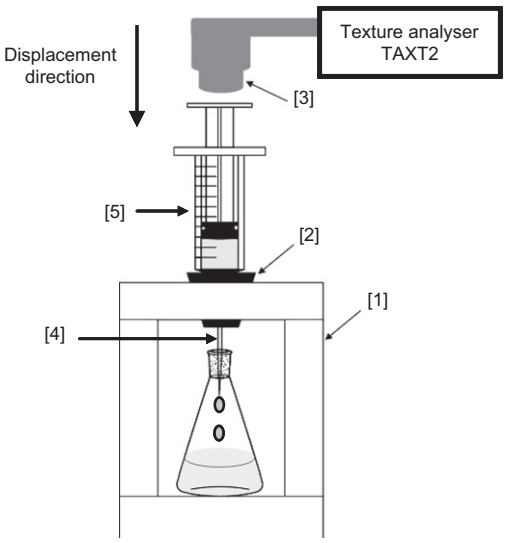

Fig. 1. Experimental set-up for the measurement of the syringeability force developed during IgG injection: [1] metallic support, [2] plastic clamping ring, [3] force transducer, [4] needle, and [5] syringe.

to 0 Pa over 3 min. A solvent trap was used to prevent water evaporation. Three runs were carried out for each sample.

2.5. Syringeability tests

Syringeability of a solution is an important factor to consider for its subsequent intravenous or subcutaneous injection. This is the pressure or the force required for the injection of this solution at a given injection rate via a needle of predetermined gauge and length.

Syringeability of the IgG solutions was studied using an instrument designed for this purpose by the UMR CNRS 8612 laboratory. The force exerted on the plunger of the syringe to inject IgG-concentrated solutions was evaluated by the developed device coupled to a texture analyser (TAXT2, Stable Microsystems, UK) in compression mode. The principle consists in applying a given displacement rate to the plunger of the syringe filled with the IgG solution and in measuring the resulting force (N). The syringeability device is described in Fig. 1.

The IgG-concentrated solutions were filled into 2-mL plastic syringes (Terumo, plunger diameter 9 mm) equipped with 26-, 27-, or 30-G needles. The needles characteristics given by the manufacturers are gathered in Table 1. Each syringe was placed in the metallic support [1] and immobilized with a plastic clamping ring [2]. A force transducer [3] was connected to the texture analyser TAXT2 [4] to measure the force with which the arm of the texture analyser moved and pushed the plunger of the syringe. The displacement rate varied from 0.1 to 1 mm/s, which corresponds to flow rates ranging from 0.4 to 4 mL/min. These flow rate values are comparable to usual injection rates. All experiments were performed at room temperature.

Table 1
Characteristics of hypodermic needle tubing.

Gauge (G)	Length (mm)	Inside diameter (mm)	Outside diameter (mm)
26[a]	12	0.241	0.457
27[a]	20	0.191	0.406
30[b]	13	0.140	0.305

[a] 26G and 27G from Terumo.
[b] 30G from BD Microlance™3.

3. Results and discussion

3.1. Immunoglobulin aggregation

To find out whether soluble aggregates account for viscosity changes of the human polyclonal immunoglobulin in solution, size exclusion chromatography and dynamic light scattering measurements were performed. Solutions prepared with either a high concentration or low concentration showed no significant difference (Table 2 and Fig. 2). It is worth noticing that during these measurements, protein solutions were diluted and that potential reversible aggregates responsible for the increased viscosity of high-concentration protein solution may have been dissociated [23,28]. The decrease in dimers amount in conjunction with an increase in protein

Table 2
Molecular weight distribution of human polyclonal immunoglobulin solutions with increasing concentrations as determined by size exclusion chromatography.

Concentration (g/L)	Monomers (%)	Dimers (%)	Polymers (%)	Fragments (%)
77	89.21	10.15	0.64	–
230	91.56	7.75	0.69	–
300	92.49	6.88	0.63	–

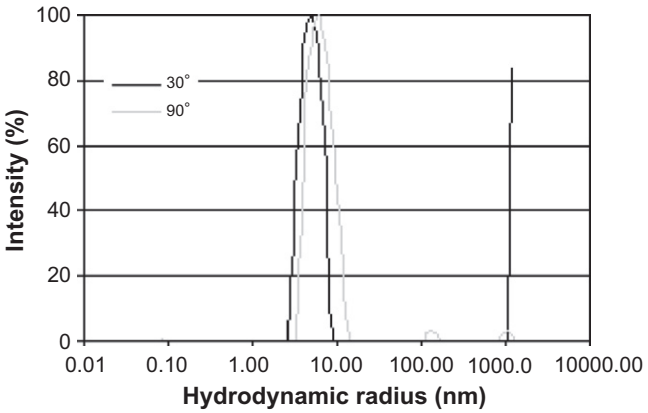

Fig. 2. Size distribution of a 230 g/L solution of human polyclonal IgG (after dilution to 5 g/L) as determined by dynamic light scattering (at angles of 90° and 30°).

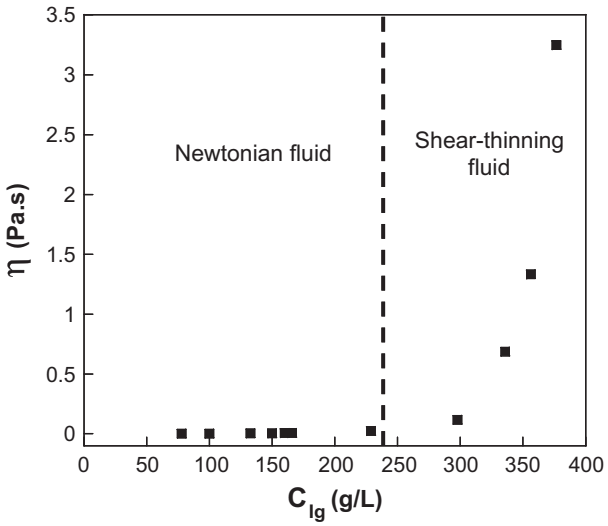

Fig. 3. IgG viscosity variations as a function of IgG concentration.

concentration may also be correlated to this dilution effect (Table 2). The hydrodynamic radius (R_h) of the protein was determined from dynamic light scattering. We obtained R_h = 6.3 nm at 90°.

3.2. Immunoglobulin rheological behaviour

The viscosity of IgG solutions was determined over a concentration range from 80 to 380 g/L at 25 °C. The samples were prepared by diluting the highest IgG concentration (380 g/L) with MilliQ water. Fig. 3 displays the variations of viscosity versus IgG concentration and shows that IgG viscosity is highly dependent on protein concentration. Indeed, IgG viscosity increased with IgG concentration. Rheological measurements exhibited a major transition as a function of IgG concentration between two well-defined behaviours.

For the IgG concentrations ranging from 80 g/L to 229 g/L, the viscosity of the IgG solutions was low and independent of the shear stress, which corresponded to a Newtonian behaviour. Above an IgG concentration of 298 g/L, the viscosity increased drastically. IgG solution at 336 g/L was about 350-fold more viscous than the protein solution at 80 g/L. At these higher IgG concentrations, the protein solutions behaved as shear-thinning non-Newtonian liquids, i.e. the IgG viscosity decreased with a rise in shear stress. It is worth noticing that for shear-thinning solutions, it is possible to define a Newtonian region (called first Newtonian regime) for very low shear stress range [29]. So, for the highly concentrated IgG solutions, the viscosity values were determined in the first Newtonian region (Fig. 4).

These data were analyzed and fitted with a model derived from the Mooney law (Eq. (2)).

In the Mooney original work, the relative viscosity was expressed as a function of the volume fraction of the solvent. As this volume fraction, ϕ, cannot be directly known, it can be expressed as a function of the IgG concentration, c, using the following relation:

$S\phi = [\eta]c$, where $[\eta]$ is the intrinsic viscosity, which is expressed in L/g (or mL/mg) if the unit of the concentration c is g/L (or mg/mL). So, Eq. (2) becomes:

$$\eta_r = \exp\left(\frac{[\eta]c}{1-\frac{K}{S}[\eta]c}\right) \tag{3}$$

The variations of the relative viscosity versus IgG concentration are displayed in Fig. 4. Experimental data are well fitted to Eq. (3) (with R^2 = 0.998), which is outstanding and infrequent.

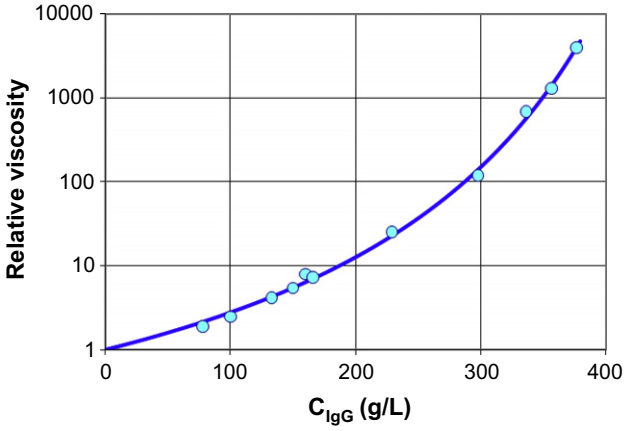

Fig. 4. Variations of the IgG relative viscosity as a function of IgG concentration. The solid line is the result of the Mooney fit.

Interestingly, the very good fit observed suggests that intermolecular interactions are not significant in our system of polyclonal IgG. The solution behaves as a non-interacting hard particles suspension. A previous study on reversible self-association of humanized monoclonal antibodies, MAbs, (with different complementary determining regions) showed that the good fitting of viscosity to Mooney equation depends on the importance of interparticle interactions [25,30,31]. In fact, for MAbs that self-associate at high concentrations (primarily through attractive intermolecular interactions), the fit was not satisfactory, probably due to additional interactions not accounted for by excluded volume effect. The presence of attractive intermolecular interactions results in a bad fit of viscosity data. The fit quality was, however, improved when salt was added (salt decreases association and viscosity) for one kind of MAb: the MAb1 [25]. It is worth noticing that MAb1 displayed a unique behaviour at pH 6 comparing to other studied MAbs [25,31,32]. Extremely strong attractive interactions (through Fab–Fab interactions) were noted at high concentrations (a maximal at ~pH 6.0) [25]. These attractive interactions could be due to the presence of electrostatic charge–dipole, dipole–dipole, and van der Waals forces. The presence of counterions or salt would decrease charge–dipole and dipole–dipole interactions. This particular behaviour seems to be due to the presence of four histidyl groups close to each other in the CDR regions of the Fab fragments of the MAb1 [32]. Near the p*K*a of histidine residue (~6.2), the protein molecule has a small net positive charge. Under crowded conditions (concentration >100 mg/mL), the molecules can approach each other closely (distance of the order of a few angstroms), which results in intermolecular attractive association mainly due to hydrophobic or van der Waals interactions. Addition of salt shields the protein charges and in turn decreases intermolecular interactions. For MAbs which did not appreciably associate at high concentrations (the intermolecular interactions are repulsive) either in the presence or in the absence of salt, viscosity variations were well described by the Mooney equation [25,31]. The addition of salt had little impact on the solution viscosity. The linear fit indicates that the increase in solution viscosity with increasing solute concentration is primarily due to self-crowding or the excluded volume effect. In our case, the polyclonal nature of the IgG seems to prevent intermolecular attractive interaction. This can explain the good fit of viscosity data by Eq. (3). From the Mooney fitting, two adjustable parameters, $[\eta]$ and K/S, were determined. The intrinsic viscosity value was 0.0086 L/g, and K/S value was 0.19. The first parameter can be used to determine the hydrodynamic radius. In fact, if we consider that the proteins can be assimilated to equivalent spheres of hydrodynamic radius R_h, then this parameter is related to the intrinsic viscosity according to the following equation:

$$R_h = \left(\frac{3M[\eta]}{10\pi N_a}\right)^{1/3} \tag{4}$$

With M = 150,000 g/mol and $[\eta]$ = 0.0086 L/g, we obtained R_h = 5.9 nm, which is in good agreement with the value determined from light-scattering experiments. Note that R_h measured by dynamic light scattering is the radius of an equivalent sphere.

In his original work [17], Mooney showed, on the basis of purely geometric considerations, that for rigid spherical particles, values of K should range between 1.35 and 1.91, which corresponds to $\phi_{max,}$ respectively, of 0.74 and 0.52. The maximum packing fraction ϕ_{max} depends on the arrangement of the particles, which in turn is determined by particle shape, size distribution, and shear flow. The maximum packing fraction is the solid volume fraction where solid content is so high that the three dimensional structure makes suspension flow impossible and viscosity tends to infinity. The theoretical value for uniform hard spheres is ϕ_{max} = 0.74, but values

are expected to decrease considerably with increasing particle aspect ratio.

For spherical molecules, the value of S is 2.5. For elongated particles, it is usually found that $S > 2.5$. If $K = 1.35$ and $S = 2.5$, the ratio K/S is 0.54 for a hard sphere particle. So the value of $K/S = 0.19$ suggests that the IgG molecule is not spherical, which is confirmed by literature. In fact, the conformation of IgG molecules has been studied by several techniques which suggested that the IgG structure in solution probably looks like a T or Y letter in shape [33].

Keeping the value $K = 1.35$, a value of $S = 7.1$ is obtained, which confirms the non-spherical shape of IgG molecules. For a prolate ellipsoid, the shape parameter S can be related to the axial ratio $p = a/b$ with a (respectively b) the length of the longer (respectively shorter) semi-axis of the particles. The relation between these parameters can be evaluated by the asymptotic formula previously reported [34,35]:

$$S = 2.5 + 0.4075(p-1)^{1.508} \quad (5)$$

For ellipsoids, the axial ratio value is between 1 and 15.

From this equation, axial ratio is calculated and $p = 6.0$. This p value is in good agreement with the value $p = 5.34$ reported by Monkos and Turczynski for human IgG molecules [21].

3.3. Syringeability tests

The first syringeability experiments were performed under a constant flow rate of $Q = 4$ mL/min to study the effect of the needle diameter on the injection force of IgG solution at different concentrations as shown in Fig. 5. As expected, at fixed needle diameter, the injection force increased with IgG concentration. For example, with a 30-G needle, the injection force at $C_{IgG} = 133$ g/L was 15.5 N and increased to 30.4 N for $C_{IgG} = 180$ g/L. A linear relationship was observed for the variations of the injection force versus IgG viscosity as following: $F = a\eta + f$; where a corresponded to the slope and f to the friction force of the syringe.

At a fixed IgG concentration, the experiments showed a decrease in the injection force with a decrease in the internal diameter of the needle. Indeed, at $C_{IgG} = 166$ g/L, the injection force was 6-fold less significant with a 26-G needle ($F^{26G}_{166g/L} = 3.9$ N) than with a 30-G needle ($F^{30G}_{166g/L} = 24.4$ N). With a manual injection (into air) force limit fixed at $F = 30$ N, these data allow to describe an injection zone as a function of the IgG concentration or IgG viscosity (grey zone in Fig. 5). The limit was delimited by the curve at 30G and 26G. All the IgG preparations in this zone involve reasonable injection forces.

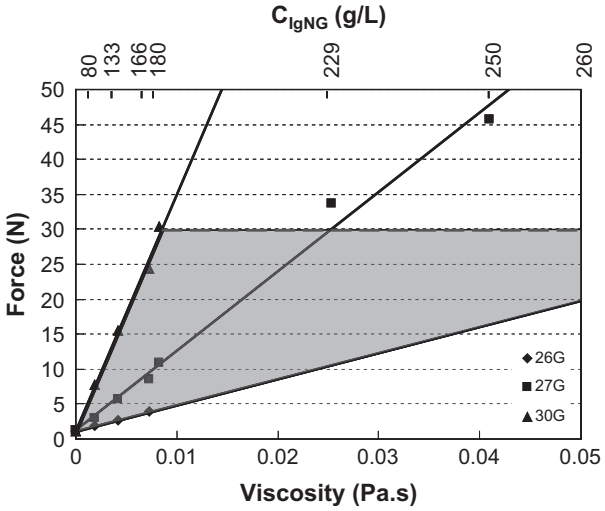

Fig. 5. Variations of the injection force as a function of the viscosity of IgG solutions at different concentrations for three different needle diameters: 26G (diamonds), 27G (squares), and 30G (triangles), for a flow rate $Q = 4$ mL/min. The shaded area corresponds to the manual injection zone.

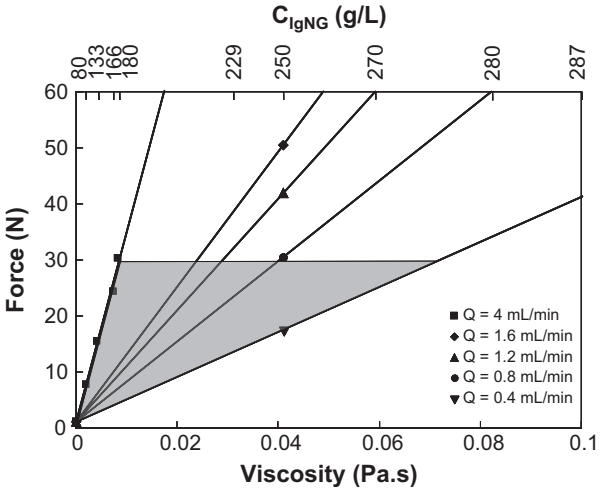

Fig. 6. Variations of the injection force as a function of the viscosity of IgG solutions at different concentrations for different flow rates. A 30-G needle was used for these measurements. The shaded area corresponds to the manual injection zone.

In addition to the needle diameter, flow rates are critical parameters for injection of viscous preparations. The effect of the flow rate on the injection force was thus studied for more or less viscous protein solutions. A needle gauge of 30G was selected for this study. This diameter requires a higher injection force than the other tested needle diameters, but is less deleterious for the skin. The flow rate was varied from 4 mL/min to 0.4 mL/min, which is consistent with classical injection rates. Fig. 6 clearly emphasizes the influence of the flow rate on the injection force. Namely, for a fixed IgG concentration, the injection force increased when the flow rate increased. This result is in agreement with the Poiseuille law (Eq. (6)). The represented injection zone (grey zone in Fig. 6) helps to identify optimal conditions for injection of IgG solutions. For low-concentration protein solutions, a flow rate of 4 mL/min can be used with a 30-G needle. For higher protein concentrations, injection will be possible with a 30-G needle if the flow rate is decreased. This syringeability test could be very useful for the development of highly concentrated protein solutions. It could namely evaluate the ability to inject the preparations, as well as help to define optimal injection conditions. As the results of this test are well correlated to viscosity values, its use could be extended to any viscous preparation.

It is possible to compare the F-values of the syringeability experiments with the theoretical values which can be calculated from Poiseuille law. Indeed, for a Newtonian liquid, the following equation can be derived from Poiseuille law:

$$F = \frac{32D^2 l}{d^4} Q\eta \quad (6)$$

with D, the syringe plunger diameter, l and d, respectively, the needle length and diameter, Q, the flow rate, and η, the solution viscosity.

Fig. 7 provides a schematic illustration of the syringe–plunger–needle association.

The theoretical and experimental values for F are in good agreement. For example, for $C_{IgG} = 250$ g/L (with $Q = 0.8$ m/min, $D = 0.9$ cm, and $\eta = 0.04$ Pa s), the calculated F for 30-G needle was 47 N, whereas the measured one was 30 N. Probably due to the end needle effects and also to the existence of a friction force

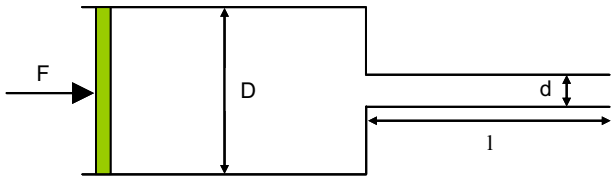

Fig. 7. Schematic representation of the plunger–needle association.

on the syringe walls, their absolute values are not exactly the same, but they have the good order of magnitude and exhibit a deviation of about 50%. From a relative point of view, the variations of the experimental values of F versus Q, η, and d^{-4} might be modelled by Eq. (6) at first approximation. So, this relation can be used for first data screening and to define suitable experimental conditions, before any syringeability experiments.

4. Conclusion

In this study, the solution behaviour of concentrated IgG solutions was investigated. From size exclusion chromatography and dynamic light scattering experiments, it was shown that no irreversible aggregation occurred in the concentration range investigated. Therefore, viscosity and syringeability tests were not affected by any irreversible aggregation which may have occurred upon concentration. This result was confirmed by the excellent fit of the rheological data to the Mooney equation. This study thus demonstrates that polyclonal IgG behaved as non-interacting hard particles in suspension. Syringeability tests allowed us to define the optimal injection conditions, taking into account solution viscosity, flow rate, injection force, and internal diameter of the needle. This preliminary study offers the prospect to formulate more concentrated polyclonal IgG solutions for subcutaneous polyclonal IgG so as to reduce the volume of injection and possibly the frequency of injection for a better patient compliance than the currently marketed ones.

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