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Pharmaceutical Biotechnology

Comparison of Strategies in Development and Manufacturing of Low Viscosity, Ultra-High Concentration Formulation for IgG1 Antibody



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ABSTRACT

Monoclonal antibodies requiring higher doses for exerting therapeutic effect but having lower stability, are administered as dilute infusions, or as two (low concentration) injections both resulting in reduced patient compliance. Present research summarizes impact of manufacturing conditions on ultra-high concentration (≥ 150 mg/mL) IgG1 formulation, which can be administered as one subcutaneous injection. IgG1 was concentrated to ~ 200 mg/mL using tangential flow filtration (TFF). Alternatively, spray dried (SPD) and spray freeze dried (SFD) IgG1, was reconstituted in 30%v/v propylene glycol to form ultra-high concentration (~ 200 mg/mL) injectable formulation. Reconstituted, SPD and SFD IgG1 formulations, increased viscosity beyond an acceptable range for subcutaneous injections (< 20 cP). Formulations developed by reconstitution of SPD IgG1, demonstrated increase in high and low molecular weight impurities, at accelerated and stressed conditions. Whereas, the stability data suggested reconstituted SFD IgG1 was comparable to control IgG1 formulation concentrated by TFF. Also, formulation of IgG1 diafiltered with proline using TFF, reduce viscosity from ~ 21.9 cP to ~ 11 cP at 25 °C and had better stability. Thus, conventional TFF technique stands to be one of the preferred methods for manufacturing of ultra-high concentration IgG1 formulations. Additionally, SFD could be an alternative method for long term storage of IgG1 in a dry powder state.

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Introduction

Due to inherent specificity and potential therapeutic activity, monoclonal antibodies have proven to be one of the most efficient therapeutic agents in treatment of several life threatening disorders.^{1,2} By April 2020, about 84 different antibodies have been approved by European Medical Agency (EMA) and US FDA for various indications. However, majority of the approved antibodies require higher doses (> 100 mg per dose) to demonstrate desired therapeutic effect. Some antibodies at higher concentrations can show limited stability in aqueous solutions, and are manufactured as lyophilized products which are further reconstituted, prior to administration as intravenous infusion (IV).^{3,4} Lyophilization further increases manufacturing cost. At times, antibodies with

larger dose and having poor stability at higher concentration, are injected as two injections at a time (Table 1). All these circumstances together result in reduced patient compliance and adds to the cost of administration.^{5–7}

Recent advances in antibody therapeutics are mainly focused on development of high concentration antibody formulations (> 100 mg/mL concentration) which can administer higher doses in smaller injection volumes. Herceptin SC[®] 600 mg (5 mL injection volume) and Rituxan[®] SC 1600 mg (13.4 mL injection volume), are two such examples of recent developments in high concentration antibody formulations (at ~ 120 mg/mL), and require specialized pumps and auto-devices for subcutaneous delivery, increasing cost of administration. Thus, there is need to develop low viscosity, ultra-high concentration antibody formulations which are stable, cost effective and capable of self-administering larger doses as a single sub-cutaneous injection.⁸

Antibodies approved in past 35 years for various indications like multiple myeloma, metastatic breast cancer, migraine, osteoporosis etc., having doses > 100 mg and concentration ≥ 100 mg/mL, are

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Table 1
Commercialized High Dose Antibody Formulations (>100 mg Dose) Which are Administered as Two Injections for Single Therapeutic Dose.

Therapeutic Protein	Brand Name	Single Therapeutic Dose	Concentration	Injection Volume	Number of Injections for Single Dose
certolizumab-pegol	Cimzia®	400 mg	200 mg/mL	1.0 mL	two
secukinumab	Cosentyx®	300 mg	150 mg/mL	1.0 mL	two
erenumab	Aimovig®	140 mg	70 mg/mL	1.0 mL	two
galcanezumab-gnlm	Emgality®	240 mg	120 mg/mL	1.0 mL	two
romosozumab	Evenity®	210 mg	90 mg/mL	1.17 mL	two

summarized in Fig. 1. These formulations are commercialized as liquid and/or lyophilized presentations. Fig. 1 also includes presentations with large doses, having low active ingredient concentration and are administered as larger volumes by diluting into IV solution. Thus, Fig. 1 highlights potential antibodies which can be developed into ultra-high concentration (>150 mg/mL) formulations.^{3-5,9}

In recent years there has been lot of research on stabilization and viscosity behavior of high concentration antibody formulations.^{10,11} However, there is less research on challenges associated in manufacturing of ultra-high concentration antibody formulations and head-to-head comparative evaluation of their manufacturing methods. Challenges in manufacturing of such antibody formulations are mainly associated with increased viscosity, which exceeds the capabilities of existing manufacturing practices and parenteral delivery systems. Although widely used, tangential flow filtration (TFF) system may have limitation of membrane fouling due to higher viscosity. Hence, alternative membrane geometry and methods to reduce viscosity should be evaluated. Concentration step by TFF also results variation in excipient content (e.g., concentration of polysorbates, buffer and

excipient offset etc.) which may impact the stability of concentrated antibody formulation. Hence, alternate strategies and manufacturing methods for ultra-high concentration should be evaluated. Shire¹² has discussed alternate strategies like lyophilization at high concentration and reconstitution to generate high concentration antibody formulation. High concentration antibody formulations using spray drying technique has been demonstrated by Ginkanga et al.¹³ with stability for 3 months at 40 °C in dry state. However, stability post reconstitution has not been discussed.

Present research is mainly focused on scalable manufacturing strategies to develop ultra-high concentration (>150 mg/mL), low viscosity (<20 cps) antibody formulation suitable for single subcutaneous administration, and provides comparative evaluation of their impact on chemical and structural stability of biosimilar IgG1.^{2,9} Antibody used in the study is a lyophilized biosimilar IgG1 molecule and its commercially available formulation variants are:

- Lyophilized formulation of 440 mg dose at ~22 mg/mL concentration for IV administration.
- Aqueous formulation of 600 mg dose at ~120 mg/mL co-formulated with hyaluronidase and injection volume of ~5 mL

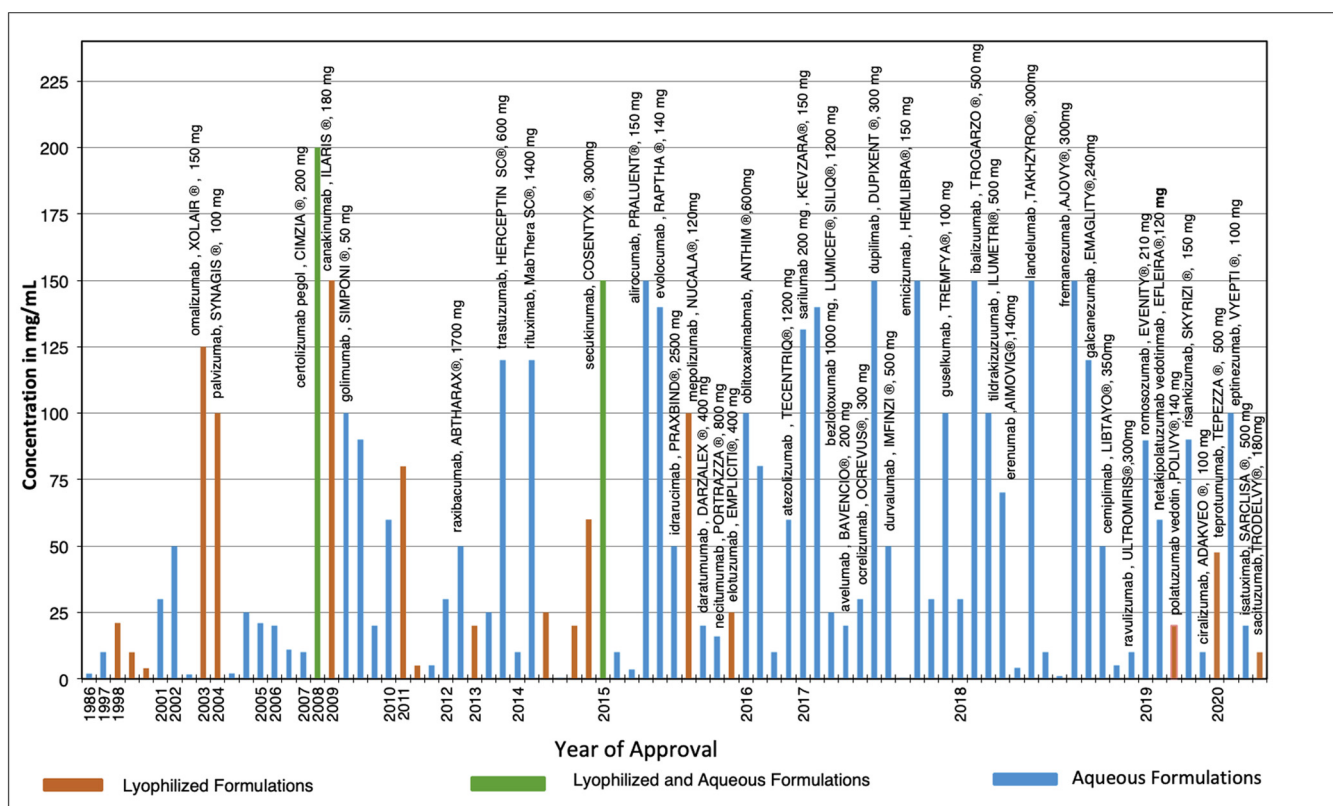


Fig. 1. List of monoclonal antibody formulations with high concentrations (>100 mg/mL) or having higher doses (≥ 100 mg) which can be developed into ultra-high concentration antibody formulation.

for subcutaneous administered using auto device over a period of 5 min.

This paper summarizes research performed in development of ultra-high concentration antibody formulation (≥ 150 mg/mL) having biosimilar IgG1 molecule at ~ 200 mg/mL, resulting in dose of ~ 600 mg in an injection volume of ~ 3.0 mL per injection. Such a formulation could be administered as a single subcutaneous injection instead of using conventional methods of administration. The techniques used for concentration of IgG1 are tangential flow filtration (TFF), spray drying (SPD) and spray freeze drying (SFD).

Material and Methods

All experiments were performed by using a biosimilar IgG1 molecule as a model protein to develop alternative strategies to manufacture ultra-high concentration antibody formulations. IgG1 drug substance (DS) used in this study was manufactured at Lupin Limited (Biotechnology Division), India and is referred as IgG1 hereafter. The IgG1 DS used in the study contains IgG1 at ~ 22 mg/mL concentration as active pharmaceutical ingredient, 10 mM of histidine and histidine HCl as buffering agent at pH 6.0; 19 mg/mL of α , α -trehalose as stabilizer and 0.86% w/v of polysorbate 20 as surfactant. The recommended storage temperature for IgG1 was -20 °C and was thawed to room temperature before further processing. Storage of IgG1 DS at -20 °C, did not result in crystallization of trehalose. This observation was in agreement to earlier research by Jena et al.¹⁴ which demonstrated crystallization of trehalose at -18 °C only after annealing, which is absent during bulk storage of IgG1 DS at -20 °C. Thus, there was no impact of trehalose crystallization on long term storage of IgG1 DS at -20 °C. The excipients used in the study were Ph.Eur. and USP compliant. L-histidine (P/N:1.04352, >99.0% pure) and L-histidine monohydrochloride monohydrate (P/N:1.04354, >99.0% pure), L-arginine hydrochloride (P/N:1.01544, >98.5% pure), ammonium chloride (P/N:1.01142, >99.5% pure), sodium chloride (P/N:1.37017, >99.5% pure), magnesium chloride (P/N:1.02367, >99.0% pure), calcium chloride (P/N:1.37101, >99.0% pure), glycine (P/N:1.03669, >99.0% pure), proline (P/N:1.07430, >99.0% pure) and propylene glycol (P/N:1.07478, $\geq 99.5\%$ pure) were procured from Merck KGaA, Germany. Polysorbate 20 (P/N: JT4116) was procured from JT Baker, USA and α , α -trehalose monohydrate (P/N: T-104-4, >99.0% pure) was procured from Pfanstiehl Inc., USA.

Methods

Concentration of IgG1 by Tangential Flow Filtration (TFF)

Ultracel® 30 kDa membrane with D screen (make: Merck Millipore KGaA, Germany, P/N: P3C030D01), commercially available in 0.11 m² format was used for concentrating IgG1 from ~ 20 mg/mL ($1\times$) to ~ 200 mg/mL ($10\times$) and diafiltration. ~ 6000 mL IgG1 DS, without polysorbate 20 at ~ 20 mg/mL, was ~ 10 fold concentrated at ~ 1.5 bar transmembrane pressure (TMP) during concentration.

Determination of Protein Content

Protein content of IgG1 was determined using UV spectrophotometer (make: Shimadzu, model: UV-1800) at 280 nm. The samples were analyzed by diluting to ~ 0.5 mg/mL, based on the concentration folds. (e.g., initial IgG1 volume of ~ 500 mL is reduced to ~ 250 mL, results in 2 fold concentration. Further concentration will result in reduction of retentate volume to 125 mL in which is 4 fold concentration, and so on).

Histidine Quantification

Histidine quantification was performed using SeQuant® ZIC®-HILIC 5 μ m, 200 Å, 250 mm \times 2.1 mm column (Merck KGaA, Germany; P/N:150458) at 30 °C. A mobile phase of 70% acetonitrile (Merck KGaA, Germany; P/N:100030), 30% 10 mM ammonium acetate (Merck KGaA, Germany; P/N: 101116), pH 5.0 was used in an isocratic mode at 0.5 mL/min flow rate for 15 min at 206 nm. With this method three amino acids can be detected namely: glycine (3.2 min), histidine (5.0 min) and arginine (5.7 min).^{15–17}

Method for Removal of Polysorbate 20 from IgG1

Polysorbate 20 was removed by passing IgG1 through SDR Hyper D resin (Pall Life Sciences, USA; P/N: 20033–023) in flow through mode, using Akta Prime™ FPLC system (GE Life Sciences, USA). ~ 206 mL SDR Hyper D resin was packed in XK50/30 column and IgG1 was loaded at a flowrate of ~ 25 mL/min. This resulted in selective binding of polysorbate 20, and IgG1 without polysorbate 20 was obtained as flow-through.¹⁸ Required amount of polysorbate 20 was later added in final formulation after concentration and diafiltration step.

Viscosity Measurements

Viscosity of the formulations was estimated using a rolling ball and capillary based micro-viscometer (model: Lovis 2000 M/ME, Anton Paar GmbH Austria) having temperature controller. The capillary made of glass with i.d. of 1.62 mm was used for viscosity estimation and the angle of rotation was 20° – 70° .

Glide Force and Break Loose Force Estimation

Glide force estimation of samples was performed using Universal Testing Machine (UTM) (model: UTM LS-1; make: Lloyds, USA) having a 20 N load cell. IgG1 samples were filled into EZ Fill™ 1 mL USP type 1 glass syringes with 27 Gauge, thin wall staked needle having $\frac{1}{2}$ inch length and 3 bevels (make: Nuova Ompi, Italy; P/N: 7600001.7439) and were placed on the stage of UTM testing machine. The friction test was performed in compression mode with preload of 0.5 N at speed of 21 mm/min, followed by test speed or extension rate of 100 mm/min up to length of 26 mm.¹⁹

Spray Drying (SPD) of IgG1 and Reconstitution to Form Ultra-High Concentration Antibody Formulation

A lab scale spray dryer was used for SPD of IgG1. IgG1 DS at 20 mg/mL with polysorbate 20 was spray dried using a spray dryer (model: Spray Mate™; make: JISL, India) having nozzle i.d. of 0.5 mm. The feed flow rate was ~ 4 mL/min at an air pressure of ~ 27 psig. The feed nozzle temperature was at 180 °C and the air flow rate was ~ 10 LPM. Powder of IgG1 from cyclone separator was collected and sealed in air tight vials. Spray dried powder was reconstituted into small volumes of sterile water for injection (WFI) or alternative vehicles to achieve IgG1 concentration of ~ 200 mg/mL.¹³

Spray Freeze Drying (SFD) of IgG1 and Reconstitution to Form Ultra-High Concentration Antibody Formulation

Spray freeze drying (SFD) was performed by spraying of IgG1 into liquid nitrogen and flash freezing followed by bulk freeze drying in a lyophilizer. An aliquot of 200 mL IgG1 at a concentration of 20 mg/mL with polysorbate 20, was sprayed on the surface of liquid nitrogen from a height of 30 cm, using a 0.5 mm spray nozzle pressurized by compressed nitrogen at ~ 30 psig. The spray frozen IgG1 thus obtained was loaded on lyophilizer (model: Lab Scale™; make: LSI, India) having pre cooled shelf at -45 °C. Further, the spray frozen IgG1 was freeze dried to achieve the powder of IgG1 and reconstituted similar to spray dried powder to achieve concentration of IgG1 approximately 200 mg/mL.^{20,21}

Size Exclusion Chromatography (SE HPLC)

The high and low molecular weight impurities, were determined by SE HPLC analysis using a Yarra™ 3 µm SEC-3000 column of 300 mm × 7.8 mm dimensions (make: Phenomenex, USA, P/N: 00H-4513-K0) in an isocratic mode. The column was equilibrated at 0.5 mL/min with mobile phase containing 80 mM sodium phosphate, pH 6.8 with 0.3 M sodium chloride at a column oven temperature of 25 °C. IgG1 samples were diluted to 0.5 mg/mL using mobile phase and detected at 280 nm. The column load for this method was 25 µg of IgG1.

Cation-Exchange Chromatography (CE HPLC) for Charge Variant Analysis

Analysis of acidic and basic charge variants of IgG1 samples was performed using a cation-exchange chromatography using a HPLC system (make: Shimadzu, Japan; model: LC-2010CHT), in a gradient mode. The IgG1 samples were diluted to 1 mg/mL in mobile phase A containing 20 mM of MES (make: Merck KGaA, Germany, P/N: 1.37074) buffer pH 6.8 and a column load of 50 µg was injected on ProPac™ WCX-10 analytical cation-exchange column (4 mm × 250 mm) (make: ThermoScientific, USA; P/N: 054993) at a column temperature of 40 °C. The IgG1 charge variants were eluted using 35% mobile phase B containing 20 mM of MES buffer pH 6.8 and 200 mM sodium chloride at a flow rate of 1 mL/min.

Peptide Mapping for Determination of Oxidation and Deamidation of IgG1

Oxidized and deamidated species of IgG1 were determined by peptide mapping under reduced conditions using quantitative UHPLC-MS technique. IgG1 samples at 2 mg/mL were treated with 0.25% w/w of RapiGest SF™ (make: Waters, USA; P/N: 186002123) at 25 °C for 1 h followed by reduction with 10 mM dithiothreitol (DTT) (make: Sigma Aldrich, USA; P/N: D0632) for 1 h. Samples were alkylated using 20 mM imidoacetamide (make: Sigma Aldrich, USA; P/N: I6125) for 1 h at 25 °C. Further the samples were digested enzymatically with Trypsin (make: ThermoFisher Scientific, USA; P/N: 90058) for 12 h at 37 °C followed by GluC (make: ThermoFisher Scientific, USA; P/N: 90054) for 10 h at 25 °C. The reaction was arrested by addition of 1% v/v of formic acid (Mass grade) and samples were centrifuged at 12500 RPM for 25 min at 25 °C. The reduced supernatant was further diluted to 0.2 mg/mL using acetonitrile with 0.1% (v/v) trifluoroacetic acid of LC/MS grade (make: Merck, P/N: 1.59014) and 10 µL of this sample was analyzed (n = 3) on Acclaim™ VANQUISH™ C18 column, of 2.2 µm particle size and dimensions of 2.1 mm × 250 mm (P/N 074812-V) using Vanquish Flex Binary UHPLC system and further analyzed on Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (all from Thermo Scientific, USA). The data for samples was analyzed by using Xcalibur 4.0 and BioPharma Finder 3.0 software.

Sub-Visible Particle Analysis of IgG1 Samples by Flow Imaging Microscopy

Micron sized sub-visible particles of IgG1 were quantified using Micro-Flow Imaging™ (MFI) (make: Protein Simple, USA; model: 5200). Before every analysis sufficient amount of WFI was primed through the flow path to get a particle free base line. Sample volumes of 0.51 mL were analyzed at a flow rate of 0.17 mL/min and software MFI View Analysis Suite™ version 1.4.0, (Protein Simple, CA, USA) was used for data analysis.

Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) measurements were recorded using CD spectrophotometer (make: Jasco, Japan; model J – 1500). The far-UV CD spectra (195–260 nm) for ultra-high concentration IgG1 from different manufacturing process were collected at 20 °C using

a quartz cell of 0.1 cm path length and protein concentration of 0.2 mg/mL. After accumulation of 3 scans at a scan rate of 1 nm per second, the scans were subsequently corrected by subtracting formulation buffer as blank. Secondary structural components were calculated by CDNN software using molecular mass of 148.4 kDa and total number of 1328 amino acids.

Results and Discussion

Concentration by Tangential Flow Filtration (TFF)

IgG1 was concentrated to ~200 mg/mL using Ultracel® 30 kDa Pellicon® 3 cassettes (Merck Millipore) having 'D screen' geometry. Preliminary experimentation on concentration of IgG1 using Pellicon Biomax® (PES) 30 kDa Membrane ('A screen' membrane) could achieve concentration only up to 100–120 mg/mL (data not included). Ultracel® 'D screen' cassettes was able to achieve higher viscosity and higher concentrations under existing processing limits and conditions conventionally used for Pellicon Biomax® cassettes having 'A screen' geometry. Polysorbates are normally added to final concentrated DS due their propensity to concentrate on TFF membranes. This can pose a risk of aggregate formation due to absence polysorbate during concentration step. Also, preliminary experiments suggested that polysorbate 20 present in the IgG1 was concentrated during the concentration and diafiltration step when Biomax® cassettes 'A screen' geometry was used. This increase in polysorbate 20 concentration would result in inconsistency with respect to TFF process and formulation composition. Hence, polysorbate 20 was removed by passing IgG1 through SDR Hyper D resin in flow through mode (supporting data Fig. 1). IgG1 thus obtained without polysorbate 20 was used for further concentration to ~200 mg/mL using TFF system with D screen cassette. Polysorbate 20 was added to IgG1 after concentration.

Alternatively, to address this challenge of polysorbate 20 concentration, surfactants like sodium deoxycholate can be evaluated while developing ultra-high concentration antibody formulations. According to Malarkani et al.²² and Albani et al.²³ sodium deoxycholate does not concentrate during TFF and helps to prevent any aggregation due to shear during concentration/diafiltration step. Also, sodium deoxycholate is routinely used in pharmaceutical injections and vaccines.^{24–29}

Excipient concentration in final DS differs from initial formulation buffer during concentration and diafiltration of proteins. This is either due to volume of exclusion, preferential hydration or charge dependent Donnan membrane effect. Thus, it is necessary to quantify the excipient concentration after concentration or diafiltration step. IgG1 samples withdrawn at different concentration folds were analyzed for protein content and excipient content during concentration of IgG1. It was observed that trehalose content was unchanged during the concentration process, thus rejecting the volume of exclusion hypothesis. But histidine content significantly reduced as the IgG1 was concentrated to ~200 mg/mL (supporting data Fig. 2).

In this case, reduction of histidine content could be possible due to Donnan membrane effect, wherein the charged proteins are retained by semipermeable membrane and electrostatic interactions result in an unequal distribution of charged solutes across the membrane, resulting in buffer and pH off sets. Miao et al.³⁰ in their work on concentration/diafiltration for antibodies demonstrated low histidine concentration in retentate because of repulsive charge interactions between positively-charged histidine molecules and positively-charged protein molecules. Miranda et al.³¹ demonstrated that IgG1 has an isoelectric point of 8.7. Based on observations of Stoner et al.³² and Teerters et al.³³ it can be concluded that, histidine with isoelectric point of 7.6 will be

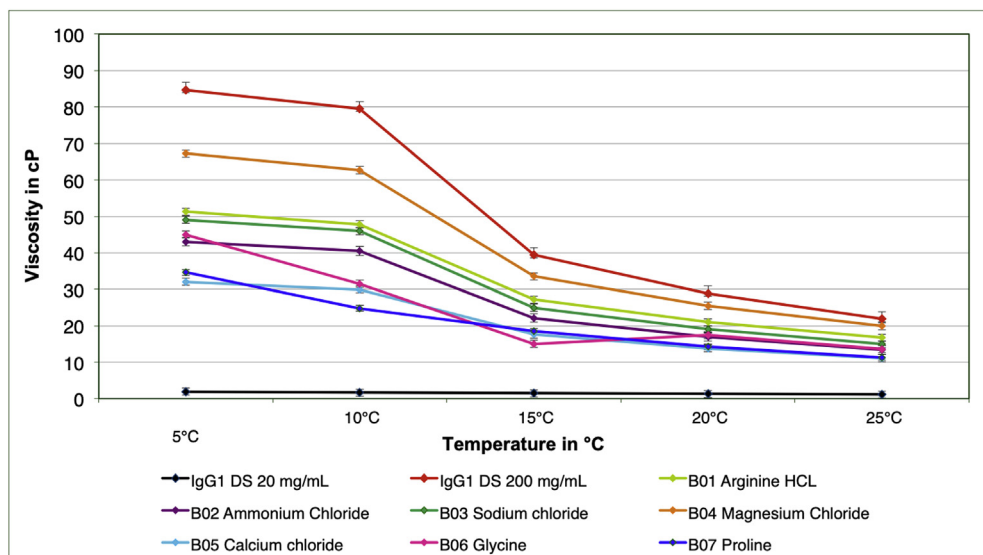


Fig. 2. Impact of various viscosity reducing agents on viscosity of ultra-high Concentration IgG1 DS.

positively charge at pH 6.0. IgG1 with isoelectric point of 8.7 will also have net positive charge at pH 6.0, thus resulting in electrostatic repulsion during diafiltration step. As a result of histidine expulsion in permeate, change in pH of concentrated IgG1 at ~200 mg/mL was observed to increase from 6.0 to 6.5. Thus, diafiltration of IgG1 followed by concentration step results in buffer offset. Hence, strategies like diafiltration with higher buffer strength to compensate for buffer off-set while targeting higher IgG1 concentration, or addition of required histidine, histidine-HCl after diafiltration, or performing concentration step at lower pH ~5.5 (by adding more histidine-HCl in initial stage) followed by diafiltration to achieve target pH of ~6.0 after diafiltration, needs to be evaluated. Alternatively, buffering agents like citrate, phosphate or combinations thereof, which are negatively charged at pH 6.0, should be considered while formulating such antibody formulations. The concentrated IgG1 thus obtained was analyzed for histidine content and required amount of histidine was added to achieve the target concentration. IgG1 DS from above process was further used for developing low viscosity IgG1 formulation at ~200 mg/mL, for subcutaneous administration.

Selection of Viscosity Modifying Agents

Roberts et al.³⁴ reported that salts and amino acids, reduce viscosity of protein formulation based on their ionic strength. Also, they are generally regarded as safe in injectable and were screened to develop low viscosity formulation within acceptable range for subcutaneous injection (i.e. <20 cP). Salts screened as viscosity reducing agents were sodium chloride, ammonium chloride, calcium chloride, magnesium chloride. Whereas, amino acids screened during the study were L-arginine hydrochloride, glycine and proline. The concentration of these viscosity reducing agents was such that the resultant osmolality of solution would be within internal osmolality target range of ~300 ± 20 mOsmols/kg.

About 13 mL IgG1 DS was buffer exchanged with formulation buffer containing viscosity modifiers, using a 10 kDa membrane. Further, these buffer exchanged formulations were filled in EZ Fill™ USP type 1 prefillable syringe (PFS) barrels (make: Nuova Ompi, Italy; P/N:7600001.7439) with Flurotech® coated stopper (make: West, P/N:9000001.6075) and were charged on stability.

The viscosity data clearly indicates that IgG1 formulations containing viscosity modifiers showed lower viscosity at 5 °C and

25 °C (Fig. 2). Calcium chloride showed significant impact on viscosity IgG1 followed by proline and glycine. However, osmolality of formulation containing calcium chloride was too high (~380 mOsmols/kg) than the targeted isotonic range (300 ± 20 mOsmols/kg). As concluded by Roberts et al.³⁴ this could be due to ionic interactions resulting in accumulation of negative charged chloride ions with positively charged protein and expulsion of positive calcium ions at pH 6.0. If the amount of calcium chloride is reduced to match the target osmolality, the viscosity would increase as impact on viscosity is inversely proportional to concentration of viscosity modifier. Hence, calcium chloride was ruled out as potential viscosity modifying agent. The osmolality of IgG1 at 200 mg/mL was marginally lower than the IgG1 at 20 mg/mL concentration and could be attributed to the loss of histidine in permeate during the diafiltration process. The impact of viscosity modifiers on viscosity of IgG1 at ~200 mg/mL concentration is summarized in Table 2.

Impact of Viscosity Modifiers on Stability of Ultra-High Concentration IgG1 at 200 mg/mL

From 6 months stability data, it was observed that all formulations containing viscosity modifiers were stable at real time conditions (5 °C). At accelerated conditions (25 °C ± 2 °C/60% RH), formulations with L-arginine hydrochloride, ammonium chloride, and sodium chloride and magnesium chloride as viscosity modifiers showed significant increase in low molecular weight (LMW) impurities. None of viscosity modifier had any adverse impact on high molecular weight (HMW) impurities.

The formulations containing calcium chloride, glycine and proline did not show significant increase in LMW impurities at accelerated conditions. Formulation containing calcium chloride showed marginal increase in HMW species. Whereas, formulations containing proline and glycine were more stable as compared to the other viscosity modifying agents. The purity of IgG1 with proline and glycine formulations was promising after 6 months at accelerated conditions (25 °C ± 2 °C/60% RH) (Table 2). In order to optimize the formulation composition and to determine their interaction effects, a DoE study with Response Surface Methodology (RSM) considering Central Composite Rotatable Design (CCRD) was performed using Design Expert® Software (supporting data Figs. 4 and 5). Based on observations from DoE study, IgG1 formulation with proline was selected for further studies.

Table 2
Impact of Viscosity Modifying Agents on pH, Osmolality, Viscosity, Injection Forces and Accelerated (25 °C/60% RH) Stability of IgG1 DS Formulation at ~200 mg/mL Concentration up to 6 Months.

Excipients Used as Viscosity Modifiers (and their Concentration in mg/mL)	pH	Osmolality (mOsmols/kg)	Viscosity in cps at 25 °C	Injection Forces (N)		% High Molecular Weight Impurities			% Monomer			% Low Molecular Weight Impurities		
				Glide Force	Break-Loose Force	Day 0	3 Month	6 Month	Day 0	3 Month	6 Month	Day 0	3 Month	6 Month
IgG1 Drug Substance at ~200 mg/mL (NIL)	6.07	70	21.9	9.5	5.5	0.4	1.9	2.0	99.4	96.4	93.8	0.2	1.7	4.2
Arginine HCl (54.7 mg/mL)	6.03	285	16.7	3.8	3.0	0.6	1.0	1.3	99.4	93.3	90.0	0.0	5.7	8.7
Ammonium chloride (13.9 mg/mL)	6.06	300	13.4	4.5	2.3	0.7	1.4	1.4	99.4	92.8	84.8	0.0	5.8	13.7
Sodium chloride (15.2 mg/mL)	6.07	303	15.0	5.4	2.7	0.7	1.1	1.4	99.2	93.5	87.7	0.1	5.4	11.0
Magnesium chloride (25 mg/mL)	6.07	285	19.9	5.3	2.5	1.3	1.1	1.2	98.7	93.7	88.0	0.0	3.9	10.7
Calcium chloride (14.4 mg/mL)	6.14	380	11.1	4.8	2.6	1.7	2.0	2.1	98.2	96.5	94.0	0.1	1.6	3.9
Glycine (9.8 mg/mL)	6.27	291	13.6	4.2	5.0	0.7	1.7	1.4	99.3	96.3	95.1	0.1	2.0	3.5
Proline (15 mg/mL)	6.20	320	11.3	5.7	5.1	0.6	1.3	1.4	99.3	97.0	94.7	0.1	1.8	3.9

Impact of Viscosity Modifiers on Injection Forces and Pain

Dias et al.³⁵ demonstrated that tolerability of high volume subcutaneous injection of ~3.5 mL viscous placebo buffer (like a typical protein formulation), administered over 1 min was associated with more pain than a 1.2 mL bolus injection. The pain was lesser compared to bolus injection when the same viscous placebo buffer was administered over 10 min. Another study evaluating impact of viscosity of monoclonal antibody formulation, injection volume and injection flow rate on SC injection tolerance, Dias et al.³⁵ concluded that injection volume of up to 3 mL having viscosity up to 15–20 cP, are well tolerated without pain, when administered into the abdomen, within 10 s.² Also, for patients with normal dexterity, the limit of viscosity for SC administration is up to 20 cps. Thus, based on conclusions of Bertheau et al.² and Dias et al.³⁵ there is a possibility that ~3.0 mL IgG1 at ~200 mg/mL with proline having viscosity ~11–12 cP could be administered over a period of 5–10 min, with less pain. Prasetyono et al.³⁷ reported that, clinically the moment of pushing the piston sliding inside the syringe plays a crucial role in potential pain resulted by flowing solution inside the tissue. The speed of gliding piston correlates with the flow of the fluid infiltrating the tissue i.e. greater speed results in more pain by stimulating the nerve endings compared to slow flowing injection.

Siew et al.³⁶ and ISO guidance³⁸ describe that injection of solution requires two types of forces as parameters of injectability, i.e. the initial force when piston of syringe is pushed; known as plunger-stopper 'break loose force' and the maintenance force required to keep pushing the piston in a sustained way; known as dynamic 'gliding force'. Both injections forces are affected by the diameter of needle and syringe, as well as viscosity of the solution. However, keeping the container closer system (prefilled syringe and needle) constant, the glide force and break loose force should be impacted by viscosity of the solution. It can be observed that IgG1 at 200 mg/mL having viscosity >20 cP has glide force of ~9.5 N and break loose force is 5.5 N (Table 2). Addition of viscosity modifiers resulted in viscosity below 20 cP with average break loose force of ~4.8 N and an average glide force of ~3.3 N, which can be considered as tolerable injection force with respect to pain perception.

Spray Drying (SPD) and Spray Freeze Drying (SFD) of IgG1

Ginkanga B. et al.¹³ demonstrated that manufacturing of high-concentration antibody formulations by spray drying has no process limitations with respect to concentration step. However, their study mainly focused on bulk storage of spray dried antibody and further formulation to high concentration drug product followed by stability in reconstituted state has not been evaluated. The spray drying (SPD) process involved spraying of IgG1 solution at high pressure through a heated nozzle (180 °C) followed by drying in a chamber with a flow of hot air flow (80 °C). Thus, the quality attributes of the antibody may get impacted during the drying process. An alternative to SPD, spray freeze drying (SFD) process which involves flash freezing of IgG1 in liquid nitrogen followed by bulk freeze drying can be explored. Faghihi et al.³⁹ and Yowa et al.⁴⁰ describe this process as more subtle for proteins and is commercially viable option, but less studied in manufacturing of high-concentration antibody formulations.

The spray dried powder of IgG1 had higher bulk density (was heavier) as compared to spray freeze dried IgG1 powder. Thus, SFD IgG1 may be difficult for handling during dispensing and compounding process, posing the risk of airborne cross contamination. Table 3 summarizes formulation composition of IgG1, total solids per mL of IgG1 and % recovery obtained from SPD and SFD processes. The recovery of IgG1 dry powder was higher (>90%) in case of SPD process as compared to (~85%) SFD process. Lower recovery

Table 3
Formulation Composition for IgG1 and Percentage Recovery From Spray Drying and Spray Freeze Drying.

% Recovery Calculations		
Parameter	Spray Drying (SPD)	Spray Freeze Drying (SFD)
Volume processed in mL	270	275.0
^a Total solids input in mg	5373.5	5472.5
Total solids obtained in mg	4988.2	4660.7
% Recovery	92.8%	85.2%

^a NOTE: Based on total solid of 19.9 mg/mL according to formulation composition of IgG1 DS.

observed in SFD process can be attributed to process loss in an open system as compared to spray drier which had closed system. Impact of SPD and SFD on the morphology of IgG1 is summarized in [supporting data Fig. 3 and Table 4](#).

Reconstitution of Spray Dried and Spray Freeze Dried IgG1 to Form Ultra-High Concentration Antibody Formulation

Wang et al.,⁴¹ Srinivasan et al.⁴³ and Yang et al.⁴² demonstrated the feasibility of preparing crystal forms or amorphous particulate suspension at high-concentration protein formulations was demonstrated. Crystal formation of three different monoclonal antibodies was demonstrated at 150 mg/mL. It was observed that the viscosity of infliximab was highest amongst the three, at 275 cP. However, its equivalent crystalline suspension had a viscosity of less than 40 cP. They also demonstrated that aqueous solution of γ -globulin at 300 mg/mL have viscosity of 370 cP at 25 °C. Suspensions of γ -globulin in a number of organic solvents like ethanol, methanol, isopropanol, 1, 4-butanediol, propylene glycol, benzyl benzoate, PEG200, ethyl acetate, toluene, acetonitrile, etc.) exhibited viscosities up to 38 times lower than those of the corresponding aqueous solutions. This demonstrates a possibility of developing high concentration antibody formulations by suspending SPD and SFD formulation in non-aqueous vehicles.

Propylene Glycol (PG) is one such solvent which is used widely used pharmaceutical injections ([supporting data table 1](#)). Although widely used, there have been concerns for usage of 30% v/v of PG. However, systemic toxicity (resulting in confusion, lactic acidosis and acute kidney injury) due to metabolic acidosis from PG metabolism is rare and is reported only in case of acute ingestion or intravenous intoxications at extremely high doses (>600 mg/dL or >1600 g over 7 days or up to >200 g/day as continuous infusion). As per toxicological assessment by Anderson,⁴⁴ only critically ill patients (having functional impairment of liver and kidney) and premature infants could be sensitive to PG. Rare, idiosyncratic clinical responses have been reported for mild local irritancy. This also has been supported by conclusive reviews from authoritative bodies like US FDA⁴⁵ and EMEA.⁴⁶ As per the US FDA⁴⁵ and EMEA report⁴⁶ the Permitted Daily Exposures (PDE) for PG is 50 mg/kg body weight for adults (considering average weight of adult, male ~75 kg and female ~60 kg) and maximum daily dose of 500 mg/kg body weight is considered safe with no noticeable effects, whatever is the duration and route of administration (except inhalation). Considering 3.0 mL subcutaneous/intramuscular dose of IgG1 in 30% PG, and considering average body weight of 60 kg, the maximum daily dose of PG would be ~15.6 mg/kg body weight. This is well below allowable maximum daily dose. Thus, 30% PG can be considered as alternative vehicle for reconstitution of IgG1 powders obtained from SPD and SFD processes.

Also, the composition of spray dried and spray freeze dried IgG1 powder is identical and, reconstitution of IgG1 powder from SPD and SFD process in WFI would result in IgG1 at ~200 mg/mL

(without any viscosity modifier), but having higher viscosity ([Fig. 2](#)). Thus, with an anticipation to form colloidal suspension of IgG1, 30%v/v of PG was selected as an alternative vehicle for reconstitution of spray dried and spray freeze dried IgG1 for manufacturing of low viscosity, ultra-high concentration IgG1 formulation for intramuscular or subcutaneous administration.

The commercially available formulation variant of IgG1 has a dose of 600 mg for subcutaneous injection. Preliminary experiments suggested that reconstitution of ~2 g of spray dried or spray freeze dried IgG1 powder into ~3.0 mL of WFI, resulted in IgG1 at ~200 mg/mL concentration. Thus, reconstitution of spray dried or spray freeze dried IgG1 powder into 3.0 mL of 30% v/v of PG would result in colloidal suspension at 200 mg/mL of IgG1 with a dose of 600 mg. The reconstitution time of SPD IgG1 in WFI and 30% PG was higher ([supporting data table 2](#)).

Comparative Evaluation of Formulations Manufactured by Different Process

The IgG1 (200 mg/mL) with proline as viscosity modifier (manufactured by TFF) and IgG1 (200 mg/mL) reconstituted in 30% v/v PG in IgG1 formulation buffer (manufactured by SPD and SFD) were filled in 3.5 mL USP type 1 glass syringe having tamper evident OVS® closure (make: Schott Kaisha, India; P/N: SB00303). Spray dried and spray freeze dried IgG1, reconstituted in 30% v/v of PG resulted in a clear but highly viscous solution, instead of a colloidal suspension. The viscosity of SFD sample was ~80 cps and that for SPD samples was ~93 cps when measured at 25 °C. The solution of spray freeze dried IgG1 in 30% v/v of PG was almost colorless, while spray dried IgG1 in 30% v/v of PG had brownish discoloration. These formulations were charged on stability at real time (5 °C), accelerated (25 °C ± 2 °C/60% RH) and stress conditions (40 °C ± 5 °C/75% RH) along with IgG1 at 200 mg/mL (without any viscosity modifier and without 30% PG) as control sample and were compared for impact of manufacturing conditions on stability. [Table 4](#) summarizes impact of processing conditions on accelerated stability of IgG1 up to 6 months and impact of processing conditions and excipient change on other quality attributes like acidic and basic charge variants, oxidized, deamidation impurities, and sub-visible particulate analysis, under real time conditions.

Impact of manufacturing conditions on conformational changes in secondary structure of IgG1 in ultra-high concentration formulations, was evaluated by analyzing formulations from different manufacturing techniques using far UV CD. Lyophilized IgG1 (reconstituted in WFI and having identical composition to SPD and SFD IgG1 powder) was used as additional control sample to compare structural changes due to processing.

The formulation composition of IgG1 used in SPD and SFD process is identical and head-to-head comparison between these two processes can be established after reconstitution in 30% PG. From [Table 4](#), it can be observed that, although the SPD and SFD formulations have identical composition, there is significant increase in % HMWs for formulation obtained from SPD process. Whereas formulation from SFD process is relatively stable and comparable to control sample (without 30% PG). It was observed that acidic and basic variants were not impacted by SPD and SFD conditions nor by adding 30% PG, and were comparable to TFF and control sample. Also, at real time conditions up to 18 months (in presence of 30% PG), there was no significant difference in oxidation of HC-Met 255 and HC-Met 431 and deamidation impurities of IgG1 in comparison with control. This could be because the pH of formulations, IgG1 SPD (pH = 6.2) and IgG1 SFD (pH = 6.1) (containing 30% PG) was within the target range of 6.0 ± 0.3. The sub-visible particle analysis by flow imaging microscopy demonstrated marginally higher particles in IgG1 formulation from SPD

Table 4
Comparative Stability of Formulations from Different Manufacturing Processes (Tangential Flow Filtration, Spray Drying and Spray Freeze Drying) at Accelerated Conditions (25 °C/60% RH) up to 6 Months and Impact of Processing Conditions, Excipient Change on Charge Variants, Oxidation, Deamidation and Sub-Visible Particles at Real Time Conditions up to 18 Months.

Stability Indicating Parameters	Time Point	IgG1 at 200 mg/mL Without Viscosity Modifier (Control)	IgG1 at 200 mg/mL With Proline From Tangential Flow Filtration (TFF)	IgG1 at 200 mg/mL in 30% PG From Spray Freeze Drying (SFD)	IgG1 at 200 mg/mL in 30% PG From Spray Drying (SPD)
Purity by SE HPLC (25 °C/60% RH)					
% High Molecular Weight (HMW) impurities	Day 0	0.7	0.6	0.5	0.5
	6 month	3.6	1.8	3.3	6.8
% Monomer	Day 0	99.0	99.3	98.7	99
	6 month	92.7	94.5	91.9	86.5
% Low Molecular Weight (LMW) impurities	Day 0	0.3	0.1	0.8	0.5
	6 month	3.8	3.7	4.8	6.7
Charge Variants by CEX HPLC (5°C)					
% Acidic variants	18th month	25.6	26.7	24.6	25.8
% Main Form		68.1	66.7	68.6	67.5
% Basic Variants		6.3	6.8	6.9	6.7
^aOxidized species					
LC-MS-MS					
% Abundance (5°C)					
HC Met 255	18th month	2.2 (±0.3)	2.1 (±0.4)	2.2 (±0.4)	2.4 (±0.2)
HC-Met-ox-255					
HC Met 431		1.8 (±0.1)	1.7 (±0.1)	1.7 (±0.3)	1.8 (±0.3)
HC-Met-ox-431					
^aDeamidation species					
LC-MS-MS peptide mapping					
% Abundance (5°C)					
LC-Asn-30: LC-deamid-30	18th month	1.0 (±0.1)	1.0 (±0.1)	1.0 (±0.2)	1.2 (±0.2)
HC-Asn-55: HC-deamid-55		4.6 (±0.3)	4.7 (±0.2)	3.9 (±0.2)	3.7 (±0.5)
HC-Asp-102: HC-isoAsp-102		11.1 (±0.1)	11.3 (±0.4)	10.2 (±0.8)	13.8 (±1.2)
HC-Asp-283: HC-isoAsp-283		1.0 (±0.1)	1.3 (±0.2)	1.1 (±0.3)	1.1 (±0.2)
HC-Asn-387,392,393:HC-deamid-387,392,393		1.1 (±0.1)	1.0 (±0.1)	1.4 (±0.4)	1.0 (±0.3)
^bSub-visible particle analysis by micro flow imaging (MFI) (5°C)					
≥ 2 µm particles per mL	18th month	8569	10,565	10,932	13,588
≥ 5 µm particles per mL		3100	4764	5784	7536
≥ 10 µm particles per mL		707	910	1185	1403
≥ 25 µm particles per mL		86	92	103	137

^a (n = 3, mean = ±S.D.).

^b (n = 3).

process on reconstitution with 30% PG. The particles from SPD process were dark as compared to other strategies and had mixed morphology of both round shaped and elongated particles (supporting data table 4). However, IgG1 formulation from SFD process had relatively lower sub-visible particles and were comparable to control formulation. As both IgG1 SPD and IgG1 SFD have identical composition, the rise in particulates in IgG1 SPD formulation can be attributed to processing conditions and not to the presence of 30% PG. Thus, it can be concluded that differences in quality attributes of SE HPLC and sub visible particulates are due to impact of processing conditions and presence of 30% PG does not have any impact on stability of ultra-high concentration IgG1.

Formulation from TFF additionally has proline, as viscosity reducing agent. It can be seen from the 6 month accelerated (25 °C ± 2 °C/60% RH) stability data (Table 2), that proline does not enhance the stability of IgG1DS. Stability profile of IgG1 without proline (B00) and with proline (B07) is equivalent and comparable

under accelerated conditions up to 6 Months. Hence, proline is just a viscosity reducing agent and not stabilizing agent. Thus, it can be concluded that presence of proline or 30% PG does not have any impact on stability of IgG1 at 200 mg/mL, even under accelerated conditions up to 6 months, and formulations from different manufacturing process having these variations can be compared for studying the impact of manufacturing conditions on stability of ultra-high concentration IgG1 at 200 mg/mL.

From the stability data at accelerated conditions up to 6 months (Table 4) and stressed conditions up to 4 weeks (data not shown) it was observed that, in formulations developed by reconstitution of spray dried IgG1, both HMW and LMW impurities increased (~6.7%) with time. This could be due to severe processing conditions which involved atomization at high nozzle temperature of 180 °C. However, in case of spray freeze dried and reconstituted IgG1, there was significantly less (~3.3%) HMW impurities and were almost comparable to IgG1 control at 200 mg/mL. Thus, SFD could be a

Table 5
Comparison of Wavelength at Zero Intensity, Spectra Minima and Broad Shoulder for IgG1 DS From Different Manufacturing Processes.

Description of Formulation	Wavelength in nm		
	Zero Intensity	Spectra Minima	Broad Shoulder
IgG1 DS at 200 mg/mL (Control)	209.2	217.3	227.2
IgG1 Lyophilized (Control)	209.4	218.2	227.7
IgG1 Spray Freeze Dried	209.6	217.4	227.5
IgG1 Spray Dried	209.8	217.3	228.1
IgG1 from TFF	209.6	215.5	228.1

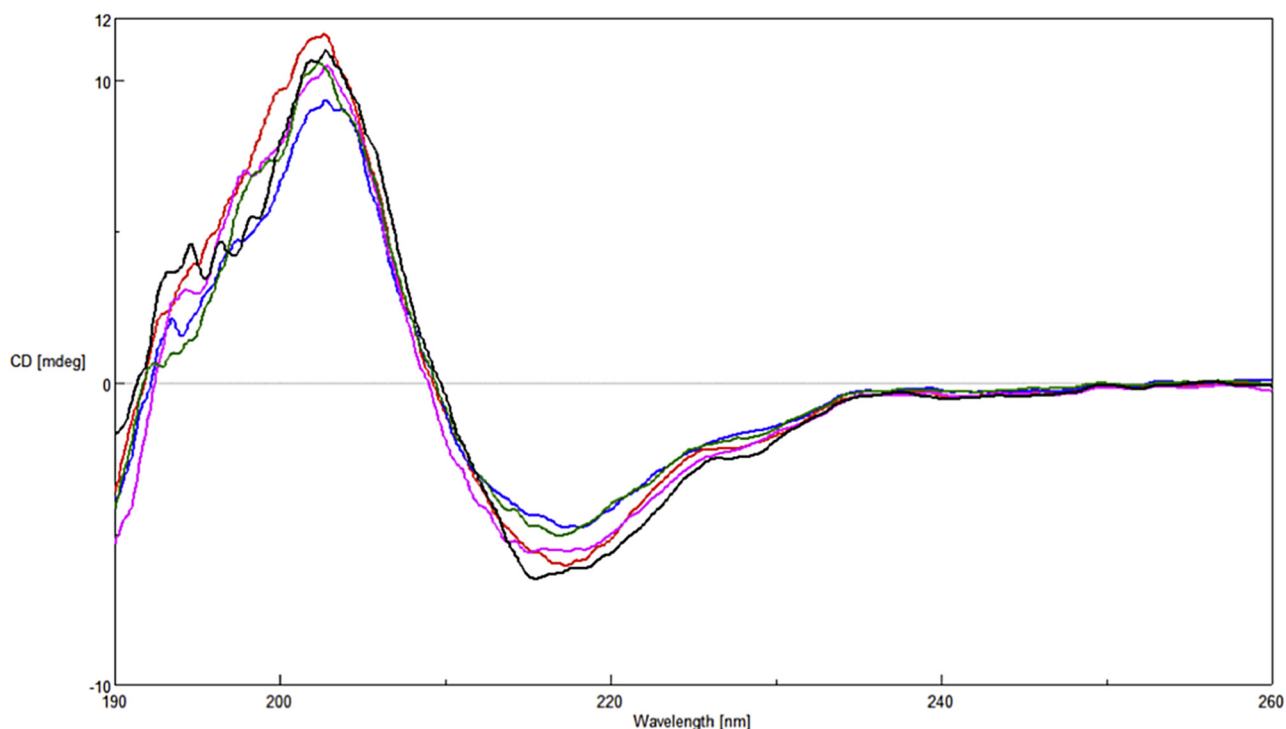


Fig. 3. Circular Dichroism (CD) spectra for ultra-high concentration formulations of IgG1 DS manufactured from different manufacturing techniques like Lyophilization, Spray Drying, Spray Freeze Drying and Tangential Flow Filtration (TFF).

potential method for manufacturing of bulk dried powders of IgG1 formulations with no significant impact on purity. For the IgG1 concentrated by TFF and having proline as viscosity modifying agent, LMW impurities were comparable to control at accelerated conditions but had lesser HMWs (~1.78%).

CD spectra of control sample (IgG1 without proline and without PG) showed zero intensity at 206 nm, minimum intensity at 217 nm and a broad shoulder at ~228 nm which indicates presence of β -sheet as predominant structure (Table 5). These results were consistent with the structure reported for native IgG1 molecule by Pabari et al.⁴⁷ and Lee et al.⁴⁸

IgG1DS from SPD, SFD and TFF, has wavelength at zero intensity, spectra minima and broad shoulder; comparable with IgG1 controls (without proline and without PG and lyophilized IgG1) (Fig. 3 and Table 5). Although there is increase in HMW impurities in SPD IgG1, but there is no co-relation with any change in the secondary structure. The secondary structure analysis summarized in Table 6 confirmed that β -sheet was the predominant structure (48% β -sheet, 5.5% α -helix and 34% was random coil). As demonstrated by Schüle et al.⁴⁹ and Ng et al.⁵⁰ the variation in secondary structure of high concentration IgG1 from different manufacturing conditions was within the error of the measurement technique (i.e. 3–4%)

suggesting that there is no significant impact of difference in excipients and manufacturing conditions on secondary structure of ultra-high concentration IgG1. Therefore, IgG1 concentrated up to 200 mg/mL by different manufacturing techniques and having difference of excipients (proline in formulation from TFF and 30% PG in formulation from SPD and SFD) and manufacturing conditions (e.g., nozzle temperature of 180 °C) does not lead to any change in the secondary structure of IgG1. This confirms that IgG1 remains chemically and conformationally intact when exposed to stresses of above mentioned manufacturing conditions.

Conclusion

A low viscosity ultra-high concentration IgG1 formulation at 200 mg/mL was successfully developed by using TFF process. The limitation of conventionally used 'A' Screen membrane to achieve maximum IgG1 concentration up to ~120 mg/mL was circumvented using 'D' screen membrane (Ultracel® 30 kDa Pellicon® 3). IgG1 was concentrated up to ~200 mg/mL Ultracel® 30 kDa Pellicon® 3 ('D' screen membrane). To overcome concentration of polysorbate 20, using SDR hyper D resin in flow through mode resulted in IgG1 without polysorbate 20. Alternatively, stability of IgG1 with

Table 6
Secondary Structure Analysis of Ultra-High Concentration IgG1 DS Manufactured From Different Manufacturing Techniques Using Circular Dichroism (CD).

Secondary Structural Components	IgG1 Control DS	IgG1 Lyo Reconstituted in Water for Injection	IgG1 SFD Reconstituted in 30% Propylene Glycol	IgG1 SPD Reconstituted in 30% Propylene Glycol	IgG1 TFF With Proline
α -Helix	5.8%	5.3%	5.5%	5.2%	5.9%
β -sheet: Antiparallel	42.2%	42.3%	42.8%	42.7%	42.4%
β -sheet: Parallel	6.1%	5.8%	5.9%	5.9%	5.5%
β turn	14.3%	15.0%	14.5%	14.8%	15.9%
Random Coil	33.5%	33.6%	32.9%	33.9%	34.6%

Accuracy \pm 3%. (n = 3).

surfactants like sodium deoxycholate can be evaluated as they do not concentrate on TFF membranes and can be used as alternative to polysorbates. The challenge of buffer off set observed with buffers like histidine can be addressed calculating the strength of required buffering system to achieve target buffer concentration or by use of excipients with charge opposite to that of IgG1. Viscosity of IgG1 formulation was reduced to ~11cps by adding proline to concentrated IgG1. Considering <20 cps as acceptable viscosity for subcutaneous administration, the maximum tolerable injection force at lower viscosity values was observed to be < 5 N.

Ultra-high concentration formulations manufactured from alternate strategies like SPD and SFD were compared with those manufactured from TFF process. The difference in formulation composition (i.e. presence of proline in TFF based formulation versus presence of 30%PG in formulations reconstituted from SPD and SFD) was not significant from stability data at accelerated conditions up to 6 months and did not have any impact on other quality attributes like oxidation, deamidation and sub visible particles, at real time conditions up to 18 months. This was supported by secondary structural analysis by circular dichroism suggesting that the differences observed in stability and sub-visible particles is only due to difference in processing conditions and not due to difference of excipients. Thus, the formulations from different process can be compared for stability at accelerated conditions.

Reconstitution of IgG1DS powders in 30% v/v of PG did not form any colloidal suspension but formed a clear viscous solution. SFD IgG1 reconstituted in 30% v/v of PG showed comparable stability to control. However, higher viscosity post reconstitution in 30% PG would make it difficult for subcutaneous administration. Thus, further scope of work in this area involves evaluation of alternative solvents for reconstitution of IgG1 powder from SFD technique.

From the comparative evaluation of different methods, TFF stands to be the most preferred method for manufacturing of high concentration antibody formulation. This is followed by SFD which can be a potential method for manufacturing of bulk dried powders of IgG1 with no significant impact on purity.

Declaration of Interests

None.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.xphs.2020.09.014>.

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