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Short Communication

Application of hanging drop technique to optimize human IgG formulations

Guohua Li, Purna C. Kasha, Sameer Late and Ajay K. Banga

Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Mercer University, Atlanta, GA, USA

Abstract

Objectives The purpose of this work is to assess the hanging drop technique in screening excipients to develop optimal formulations for human immunoglobulin G (IgG).
Methods A microdrop of human IgG and test solution hanging from a cover slide and undergoing vapour diffusion was monitored by a stereomicroscope. Aqueous solutions of IgG in the presence of different pH, salt concentrations and excipients were prepared and characterized.
Key findings Low concentration of either sodium/potassium phosphate or McIlvaine buffer favoured the solubility of IgG. Addition of sucrose favoured the stability of this antibody while addition of NaCl caused more aggregation. Antimicrobial preservatives were also screened and a complex effect at different buffer conditions was observed. Dynamic light scattering, differential scanning calorimetry and size exclusion chromatography studies were performed to further validate the results.
Conclusions In conclusion, hanging drop is a very easy and effective approach to screen protein formulations in the early stage of formulation development.
Keywords dynamic light scattering; hanging drop; human IgG; protein formulation; size exclusion chromatography

Introduction

Proteins are unstable when exposed to various chemical and physical processes. Physical instability of proteins, such as aggregation in solutions, can cause protein denaturation. The aggregation of proteins is an important concern in formulation and process development in the pharmaceutical industry. Since all protein-based drug molecules pose unique challenges with respect to stability, and hence efficacy, they need special techniques to characterize formulations to develop a robust and efficacious therapeutic product.[1]

Hanging drop vapour diffusion is a method that has been proposed for screening crystallization conditions and protein solubility.[2–5] This technique is often used in the nuclear magnetic resonance (NMR) field because protein solubility is the main obstacle for liquid protein NMR. In this method, a very small volume of protein solution is mixed with different buffer conditions. Changes in the external buffer environment of the protein can lead to structural changes, which in turn may lead to protein aggregation. Aggregation can result in visible precipitation or particulate formation. The precipitation, which appears as a cloud in the solution, is then monitored by optical microscopy. The protein content and aggregation extent is varied when exposed to different formulations; hence, the aggregation can be scored comparatively under a microscope based on the size and distribution of aggregates in the solution.[3]

To increase the solubility and stability of proteins in different formulations, suitable buffer systems, excipients and additives need to be screened carefully for selection. The buffer components can dramatically affect solubility, such as the incremental solubility of tissue plasminogen activator (tPA) in imidazole, phosphate, histidine, succinate, EDTA and citrate, with a 50-fold difference in solubility from imidazole to citrate.[6] In addition, the pH of the vehicle and excipients can also affect the solubility of a protein.

Several stabilizers can prevent aggregation of proteins or stabilize against hydrolysis. Sugars, such as sucrose, are good stabilizers in solutions and dry state. Sucrose has been shown to protect haemoglobin from oxidation during lyophilization and storage.[7] Peptides and proteins, being biological products, are susceptible to microbial contamination.

Correspondence: Prof Ajay K. Banga, Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Mercer University, Atlanta, GA 30341, USA.
E-mail: banga_ak@mercer.edu

Therefore, the preservative is a critical component in liquid formulation, especially for multi-dose formulations. However, the effect of preservatives on solubility and stability is complex because several preservatives may cause precipitation in the reconstituted solutions. It was demonstrated that only benzyl alcohol was able to meet the regulatory requirements after screening six preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol and *m*-Cresol) for a humanized monoclonal antibody in the development of a multi-dose intravenous formulation.[8]

Monoclonal antibodies are used as therapeutic agents for inflammatory and immune diseases as well as cancer therapy. At high dose, intravenous IgG is used as an anti-inflammatory agent for the treatment of autoimmune disorders.[9] However, good solubility and chemical and physical stability of antibody in a favoured formulation is essential to achieve the desired efficacy and shelf life. Therefore, in this study, the effects of different formulation parameters on physicochemical stability and solubility of human IgG were investigated. Human IgG is a well-studied immunoglobulin with an average molecular mass of 150 kDa and it represents an important class of therapeutic proteins. It is commercially available as a parenteral solution or lyophilized powder.

The main focus of this study was to investigate the feasibility of hanging drop vapour diffusion in the screening of IgG formulations with different pH, salt concentration, buffer species and excipients. The selected screened formulations with zero or low score were further characterized using dynamic light scattering (DLS), differential scanning calorimetry (DSC) and size exclusion chromatography (SEC) assays.

Materials and Methods

Materials

Lyophilized human immunoglobulin G was obtained from Sigma Aldrich (St Louis, MO, USA). Quik Optimize kit containing 4.0 M monobasic sodium phosphate and 4.0 M dibasic sodium phosphate was purchased from Hampton Research (Laguna Niguel, CA, USA). Reagent concentrations in the range 0.2–4.0 M and pH values in the range 5.0–8.2 can be easily formulated with Quik Optimize. The McIlvaine buffer system is composed of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate, which can be formulated for a pH range of 2.2–8. At each pH value, the mixture of these two components was set as 1× and the dilution of McIlvaine buffer was based on this initial 1× buffer. 1× McIlvaine buffer at pH 4, 5, 6, 7 and 8 has an approximate concentration of 140, 150, 160, 180 and 200 mM, respectively. Linbro plates (24 wells), 22 mm glass cover slides and vacuum grease were obtained from Hampton Research (CA, USA). All other chemical reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA). All materials were reagent grade or better.

Hanging drop screening

In the hanging drop screening, 1 ml of different buffer solutions was pipetted into each well of a Linbro plate. Then, a drop (1 μl) of protein solution containing 20 mg/ml IgG in filtered HPLC water was pipetted onto a glass cover slide. To this drop, 1 μl of buffer was added. The protein solution was then mixed with buffer by gently drawing and expelling the solutions in and out of the pipette tip. The glass cover slide was then inverted and sealed onto the wells by using vacuum grease. The plate was allowed to rest undisturbed at room temperature for vapour diffusion to take place. The plates were observed under a stereomicroscope (LEICA MZ6; Houston, TX, USA) once every day for the first week, and then once a week thereafter. The drops were carefully examined under the stereomicroscope with 6.3×~40× magnification. Observations were recorded to indicate whether the drop was clear or contained precipitated particles. The precipitate appeared as a white spot against the black background when a condition of proper contrast of light and black was used to observe the drops. Aggregation was scored based on the fraction of the drop covered by the precipitate. An arbitrary scale of 0–4 was used, with 0 for no aggregates and 4 for aggregates completely covering the whole drop.[3]

Size-exclusion chromatography

Soluble aggregates were determined by size-exclusion chromatography (SEC). SEC-HPLC was performed using a TSK-GEL G3000SW$_{XL}$ 7.8 × 300 mm column (TOSOH Bioscience, Montgomeryville, PA, USA) and Alliance HPLC system with Empower software (Milford, PA, USA). A mobile phase consisting of 20 mM sodium phosphate and 100 mM NaCl at a final pH of 6.8 was used at a flow rate of 0.8 ml/min. The test buffers containing 10 mg/ml of human IgG dissolved were prepared and filtered through a 0.22-μm filter before injection. A sample volume of 20 μl was injected for analysis. Elutes were detected at 214 nm and peak integration was performed for quantification.

Dynamic light scattering measurements

DLS studies were performed in a PDDLS/Batch detector system (Franklin, MA, USA) at a fixed angle of 90° to investigate the formation of soluble aggregates in the formulations. Data was analysed in the precision Deconvolve/Deconview software (Franklin) and the soluble aggregates were determined by a change in average hydrodynamic radius (Rh) (n = 3). Each sample of 1 ml (1 mg/ml) was loaded in the test cuvette for measurement.

Thermal analytical studies

A decrease in the denaturation temperature reflects a destabilizing effect of formulation on IgG stability. The denaturation temperature was measured using a TA DSC Q100 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) at a scan rate of 2°C/min. Twenty microlitres of 10 mg/ml IgG formulation was sealed in an aluminium hermetic pan and scanned between 30 and 100°C under nitrogen purge with a flow rate of 50 ml/min.

Statistical analysis

Differences between various treatments were statistically identified using the Kruskal–Wallis test, in conjunction with Dunn's post-hoc test ($P \leq 0.05$ denoting significance).

Results and Discussion

Hanging drop study

A stereomicroscope was used to observe aggregation associated with IgG and the corresponding scores for different levels of aggregates were assigned according to the scoring criterion as described previously (Figure 1). The results indicate that different solution conditions promote the formation of aggregates *in vitro*. These stable protein aggregates are caused by the formation of amyloid fibrils, a process involving a change from the protein's native soluble form to insoluble aggregates.[10]

Effect of pH and buffer concentration

The stability of proteins is highly pH dependent and can be affected significantly by the presence of high concentration of salts.[11] Protein aggregation can be significantly affected by the pH.[12] Therefore, a minor change in pH can lead to problems.[1] Peptides and proteins are generally least soluble at a pH range near to their pI value.[13] Also, the ionic and salt concentration of a solution can affect the solubility of a protein. The effect of salt on protein stability is complex. The salt concentration for maximum solubility of proteins falls within a very narrow range. A 50 mM change in salt concentration results in a 20-fold difference in solubility of T7 RNA polymerase.[14] Sodium and potassium phosphate buffers with concentration of 200, 100, 50, 25 and 12.5 mM and a pH between 5 and 8.2 were screened for IgG solubility (Figure 2). The extent of insoluble aggregate was very low at 25 mM and 50 mM buffer concentration. In addition, the visible aggregation was low at pH closer to the pI (pI of human IgG = 7–8) for these buffer concentrations (Figure 2).[15] For all the examined buffer concentrations (except 200 mM), no visible aggregation in sodium and potassium phosphate buffer at pH 5.0–6.5 appeared. However, when the pH of the buffer was close to the pI range, there was visible aggregation (Figure 2). The aggregation profiles of all tested buffers follow the trend of increased insoluble aggregation when the pH is close to the pI of the protein. These results were in line with the study of an analogue of human IgG1, in which the monomer decreased with increasing pH of the solution.[16] It has also been reported that the optimum pH range for IgG storage is 5.0–6.0, and these results were in accordance with those of Szneczi *et al*.[17] Figure 3 shows the effect of solution pH and concentration of McIlvaine buffer on IgG aggregation is comparable with that of sodium and potassium phosphate buffer. It shows that McIlvaine buffer at pH 5 and 7 displayed least impact on the IgG stability, though IgG undergoes aggregation or precipitation when exposed to 1× (150 mM) McIlvaine at pH 5 and 0.25× (45 mM) at pH 7. The effect of buffer concentration on IgG aggregation is either an ionic strength effect or a specific ion effect as the effect of buffer pH is almost identical for sodium/potassium phosphate buffer and McIlvaine buffer. It is also known that aggregation of polyclonal IgG decreases at lower pH due to moving away from the isoelectric range, while the tendency of denaturation increases. The denatured or unfolded protein will adsorb to the air–water interface or surfaces of the container and is susceptible to further inactivation by aggregation with neighbouring molecules.[18] From the theory of Sanchez-Ruiz *et al*.,[19] the irreversible denaturation process can be written as:

$$N \leftrightarrow^{ku}{}_{kf} U \rightarrow^{ki} I \quad (1)$$

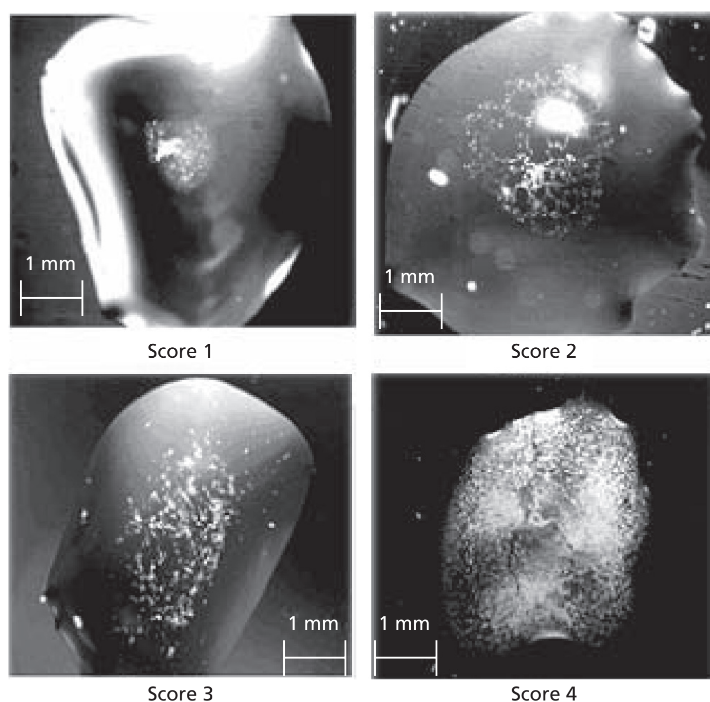

Figure 1 Aggregation scales under a stereomicroscope

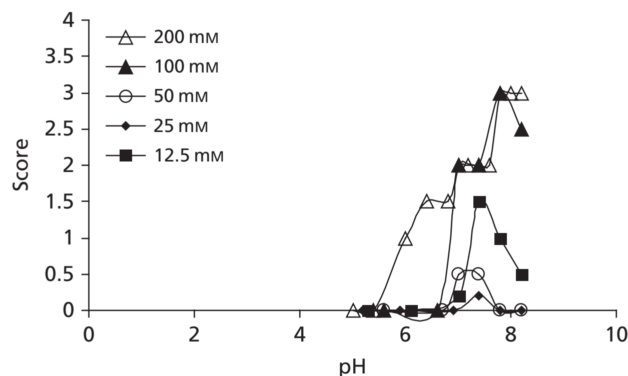

Figure 2 The aggregation profile of human IgG exposed to different pH and concentration of Na/K phosphate buffers. Coefficient of variation < 3%.

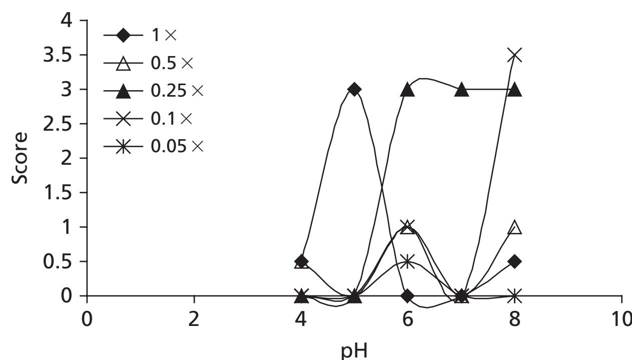

Figure 3 The effect of pH and dilution of McIlvaine buffer on the aggregation of human IgG. The concentration of 1×, 0.5×, 0.25×, 0.1× and 0.05× at pH 4 is 140, 70, 35, 14 and 7 mM, respectively; 150, 75, 37.5, 15 and 7.5 mM at pH 5; 160, 80, 40, 16 and 8 mM at pH 6; 180, 90, 45, 18 and 9 mM at pH 7; 200, 100, 50, 20 and 10 mM at pH 8. Coefficient of variation < 2%.

where *N* is native state, *U* is unfolded state (reversible state) and *I* represents the final irreversible denatured state which often forms aggregates. When ki >> kf, unfolded molecules will be converted to aggregates instead of going back to the native state, N. Therefore by comparing the stability at pH 5, 6 and 7, it is possible that for 1× McIlvaine buffer at pH 5 the denaturation tendency is stronger, which finally leads to aggregates occurring in this buffer environment. Using these results, we conclude that adjusting the pH in the optimal range is essential for the solubility and stability of IgG in solution at lower buffer concentration. These results are in agreement with data reported by Sarciaux *et al.*,[20] where higher concentration of buffer favoured the formation of dimers.

Effect of excipients

Excipients typically used in formulation of protein include salts, sugars, buffers and amino acids. Sugars have been reported to stabilize proteins against a variety of stress situations. Sucrose and sodium chloride are frequently used in formulation to stabilize proteins. Sucrose can stabilize proteins because the unfolded state of the protein is not favoured thermodynamically in the presence of sucrose.[6] Salts are excluded from the protein–solvent interface because of increased surface tension of water, and therefore they can also stabilize proteins.[6] In addition, the presence of ions, such as Cl^-, Na^+ and Ca^{2+}, presumably hinder hydrophobic association, which is involved in the interaction between protein molecules.[12] The use of sucrose as an excipient should be approached with caution for IgG formulation due to reported acute renal toxicity when high concentration (5–10%) is used.[17,20] Therefore, a very low concentration (0.1%) of sucrose was introduced to formulations for studying its effect on the stability of IgG in this study (Table 1). Among the screened formulations, the findings suggested that IgG was more stable for some formulations in the presence of sucrose (the representatives of these formulations are listed in Table 1). However, sodium chloride showed some precipitation in some samples at a concentration of 0.58% (0.1 M). A similar effect of sodium chloride on whey protein aggregation was reported previously. At higher ionic strengths (> 0.1 M NaCl) protein aggregation was promoted.[12] In one of the studies, it has been proved that addition of sodium chloride to a constant concentration of Na vs K phosphate buffer results in more aggregates relative to buffer alone.[18] The salt concentration is very critical for protein stability because higher concentration of sodium chloride may allow stronger intermolecular attractions that can lead to aggregation.[21] In general, the stabilizing effect of some salts is dependent on the nature of protein, pH, charge and concentration of salt used.

Some pharmaceutical formulations must contain antimicrobial agents to protect them from microbial contamination. Preservatives are also required for certain drug delivery systems. However, preservatives often induce aggregation of protein in aqueous solution. For example, preservatives of phenol, *m*-Cresol and benzyl alcohol have been shown to induce aggregation of human growth hormone.[22] In addition, the effectiveness of preservative is influenced by pH and buffer components. It was reported that benzyl alcohol is effective only in the pH range of 4–7 and Tweens inactivate paraben and phenolic preservatives.[8] Therefore, the effect of preservatives on protein stability is a major concern. In this study, formulations were prepared by adding three preservatives, namely 0.5% benzyl alcohol, 0.02% propylparaben and 0.1% *m*-Cresol. The selected formulations with zero or low score were selected to evaluate the effect of preservatives on IgG. The results of this study showed that aggregate formation is not only influenced by different preservatives, but also dependent on the combination effect

Table 1 Effect of sucrose and sodium chloride on IgG aggregation

Buffer, pH	Hanging drop score		
	No excipient	0.1% Sucrose	0.58% NaCl
0.1 × Mc (20 mM), 8	0.5	0.0	1.0
0.25 × Mc (37.5 mM), 5	2.5	1.5	2.5
1 × Mc (150 mM), 5	0.0	0.0	2.0

Mc, McIlvaine buffer. Coefficient of variation < 2%.

of pH and buffer concentration. IgG was more stable in 25 mM Na/K phosphate buffer at pH 5 after addition of these three test preservatives (Figure 4). When the pH of the buffer was increased to 6, buffer concentrations of 200 mM and 25 mM favoured the stability of human IgG (Figure 5). Comparatively, preservatives had a complex effect on aggregation when exposed to 0.05× McIlvaine buffer at a pH range of 4–6 (Figure 6). It was shown that 0.05× (8 mM) McIlvaine buffer with pH 6 had least effect on the aggregation. Therefore, it was clear that the aggregation not only depended on the selected preservative, but also correlated with the pH and concentration of buffer. Overall, all these profiles indicate that human IgG is more stable in the presence of 0.5% benzyl alcohol when compared with the other two preservatives. However, previous studies concluded that a humanized monoclonal antibody was more stable when exposed to formulations containing propylparaben.[8] Though the mechanism for preservative-induced protein aggregation is not completely understood, it has been shown that addition of benzyl alcohol might perturb the tertiary structure of rhINF-γ without affecting its secondary structure.[23]

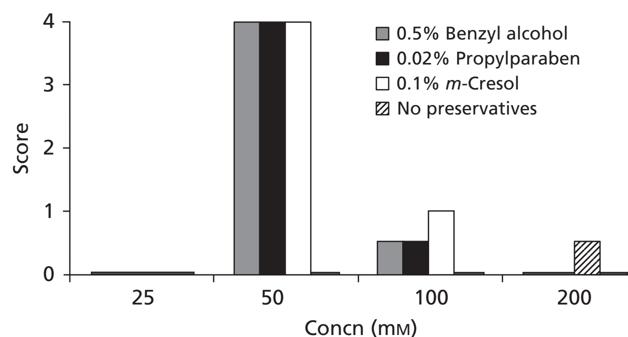

Figure 4 Effect of Na/K phosphate buffer (pH 5) with addition of preservatives on IgG aggregation. Coefficient of variation < 3%.

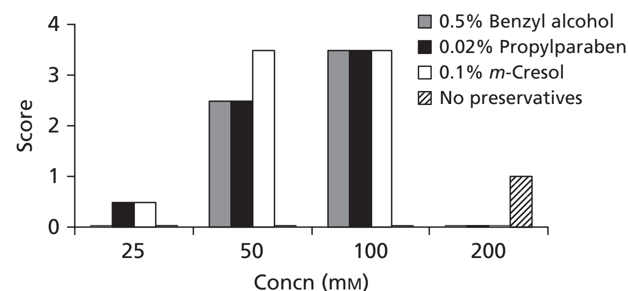

Figure 5 Effect of Na/K phosphate buffer (pH 6) with addition of preservatives on IgG aggregation. Coefficient of variation < 3%.

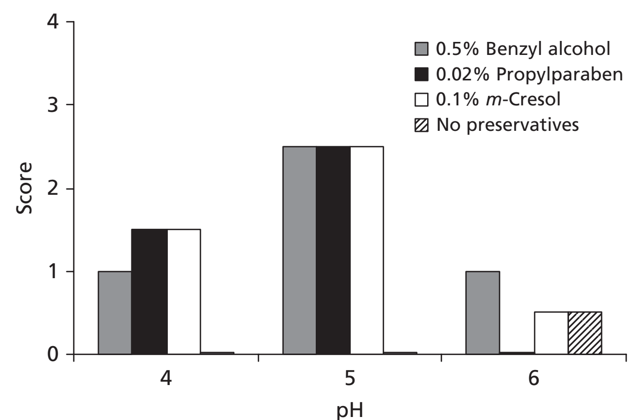

Figure 6 Effect of 0.05× McIlvaine buffer (pH 4–6) with addition of preservatives on IgG aggregation. The concentration of 0.05× at pH 4, 5, 6 is 7, 7.5 and 8 mM, respectively. Coefficient of variation < 3%.

Dynamic light scattering analysis

A primary application of DLS is to study protein aggregation. Association and dissociation of monomeric and polymeric forms of a protein are of interest because of its biological activity, which can be studied with DLS.[16] DLS is a powerful technique to measure hydrodynamic radius and the presence of aggregates in protein samples. The hydrodynamic radius is defined as the radius of a hard sphere. An increase in Rh or broadening of the distribution peak can suggest an increase in protein size and can be seen as an indicator of soluble aggregates.[24] The hydrodynamic radius of monodisperse human IgG is 5 ~ 8 nm.[24] The average hydrodynamic radius of IgG as a function of pH/buffer is summarized in Table 2. The analysis showed that pH change from 4 to 6 had not induced significant change in the average hydrodynamic radius in 0.05× McIlvaine buffer. The size distribution of IgG increased from 9.7 nm to 26 nm with pH change from 5 to 4 in 0.1× McIlvaine buffer (Table 2). Similarly, an increase in Rh from 11.7 nm at pH 5 to 32 nm at pH 4 in 0.25× McIlvaine buffer was observed. Thus, IgG forms larger soluble aggregates in 0.1× McIlvaine or 0.25× McIlvaine buffer with pH 4 in terms of Rh value. At pH 5, IgG does not have any aggregation compared with formulations at pH 4. Comparatively, 0.05× McIlvaine buffer had least effect on soluble aggregation as the Rh value obtained at this condition was close to the hydrodynamic radius range of monomeric IgG. The results shown in Table 2 suggest only monomeric IgG was detected and no protein aggregates were present for 25 mM sodium and potassium phosphate buffer at pH 6 suggesting the protein was in monodisperse form. However, when the pH changed to 5, there was significant increase in the average Rh value (42 nm), suggesting a large portion of soluble aggregates was formed.

Combining the results from hanging drop screening and DLS analysis, it was concluded that 0.05× McIlvaine buffer with a pH range of 4–6 and 25 mM Na vs K phosphate buffer with pH 6 resulted in the most stable human IgG formulation.

Differential scanning calorimetry study

Thermal denaturation of IgG was studied by differential scanning calorimetry. High melting temperature (T_m) of protein unfolding transition determined by DSC is indicative of high protein stability, and thereby an important parameter when screening different protein formulations. The comparison of thermograms recorded at varying pH and buffer concentration was carried out and results of melting temperatures (T_m) are shown in Table 2. For McIlvaine buffers, IgG

Table 2 Formulation optimization using size-exclusion chromatography, dynamic light scattering and differential scanning calorimetry

Formulation	pH	Score (by hanging drop)	Rh (nm) (by DLS)	% Monomer (by SEC)	T_m (°C) (by DSC)
0.05× Mc (7 mM)	4	0	12.0	94.54	46.1
0.05× Mc (7.5 mM)	5	0	12.0	92.69	56.6
0.05× Mc (8 mM)	6	0.5	9.7	91.47	64.5
0.1× Mc (14 mM)	4	0	26.0	94.76	70.0
0.1× Mc (15 mM)	5	0	9.7	89.80	74.3
0.25× Mc (35 mM)	4	0	32.0	92.18	63.1
0.25× Mc (37.5 mM)	5	0	11.7	92.71	64.2
25 mM Na/K PB	5	0	42.0	92.43	57.1
25 mM Na/K PB	6	0	7.8	91.52	66.9

DSC, differential scanning calorimetry; DLS, dynamic light scattering; Mc, McIlvaine buffer; PB, phosphate buffer; Rh, average hydrodynamic radius; SEC, size-exclusion chromatography; T_m, melting temperature. Coefficient of variation < 2%

Table 3 Effect of protein concentration on IgG aggregation

Formulation	Area percentage calculated from UV signal at 214 nm		
	% Aggregates	% Dimers	% Monomers
0.1× Mc (20 mg/ml, pH 5)	7.36	4.81	87.83
0.1× Mc (10 mg/ml, pH 5)	6.52	3.89	89.59
0.1× Mc (5 mg/ml, pH 5)	6.16	4.00	89.85

Mc, McIlvaine buffer; 0.1 × McIlvaine buffer at pH 5 has a concentration of 15 mM. Coefficient of variation < 3%

shows the greatest stability at pH 5 in 0.1× (15 mM) solution (T_m, 74.3°C). IgG at pH 6 in 25 mM sodium and potassium phosphate buffer is more stable than at pH 5 (T_m: 67 and 57°C, respectively). The decrease in T_m values with decreasing pH for the same buffer was observed (Table 2). These results are in agreement with Szenczi *et al.*[17]

Size-exclusion chromatography

SEC-HPLC is a technique for quantitative assessment of soluble aggregation or polymers from protein aggregation. A concentration of 5 mg/ml IgG was injected into the SEC column for all cases. The percentage of monomeric IgG under variable formulation conditions is summarized in Table 2. The aggregation peak from 0.05× McIlvaine buffer with pH 6 was approximately 8.5%, but when the pH changed to 4 the aggregate percentage was decreased to 5.5%. In 0.1× McIlvaine buffer, the aggregate percentage was changed from 10.2% to 5.24% when the pH changed from 5 to 4. SEC data was compared with DSC and DLS data to assess product stability over time. Surprisingly, SEC results were incomparable with DLS and DSC data. This is probably because SEC could not give rise to a correct molecular mass determination due to the difference of retention and interaction of proteins with the column material in SEC. DLS can detect small traces of aggregates and is very sensitive to changes in the aggregate composition. Therefore it has been suggested to combine these techniques together for protein study.[25] Interestingly, for the same buffer species, the IgG monomer content in SEC analysis decreased with increasing pH of the solution. This tendency is the same as reported previously.[16] As expected, SEC data shows that IgG aggregation increased with higher protein concentration tested by using 0.1× McIlvaine buffer (pH 5) (Table 3).

Conclusions

It is demonstrated that selection of pH and buffer concentration in the optimal range is essential for solubility and stability of IgG in solution. The hanging drop method requires a very small quantity of protein. The ease of use and the versatility of testing stability of protein at different conditions make this method a very effective tool for optimizing the IgG liquid formulation in the early stages of formulation development. In conclusion, the hanging drop technique addressed in this study can be a cost effective and rapid method to explore and select new excipients listed as Generally Recognized as Safe (GRAS) for formulation of proteins prior to detailed characterization of the formulation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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