View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Kent Academic Repository

Kent Academic Repository

Full text document (pdf)

Citation for published version

Gourbatsi, Evdoxia and Povey, Jane F. and Smales, Christopher Mark (2018) The effect of formulation variables on protein stability and integrity of a model IgG4 monoclonal antibody and translation to formulation of a model ScFv. Biotechnology Letters, 40 (1). pp. 33-46. ISSN 0141-5492.

DOI

https://doi.org/10.1007/s10529-017-2443-x

Link to record in KAR

http://kar.kent.ac.uk/63854/

Document Version

Author's Accepted Manuscript

Copyright & reuse

Content in the Kent Academic Repository is made available for research purposes. Unless otherwise stated all content is protected by copyright and in the absence of an open licence (eg Creative Commons), permissions for further reuse of content should be sought from the publisher, author or other copyright holder.

Versions of research

The version in the Kent Academic Repository may differ from the final published version.
Users are advised to check **http://kar.kent.ac.uk** for the status of the paper. **Users should always cite the published version of record.**

Enquiries

For any further enquiries regarding the licence status of this document, please contact:
researchsupport@kent.ac.uk

If you believe this document infringes copyright then please contact the KAR admin team with the take-down information provided at **http://kar.kent.ac.uk/contact.html**

KAR Kent Academic Repository

University of Kent

The effect of formulation variables on protein stability and integrity of a model IgG4 monoclonal antibody and translation to formulation of a model ScFv

Evdoxia Gourbatsi, Jane F Povey, and C Mark Smales

School of Biosciences and Industrial Biotechnology Centre, University of Kent, Canterbury CT2 7NJ, UK

Address correspondence to: Mark Smales. Ph (+44) 01227 823746; email c.m.smales@kent.ac.uk

ABSTRACT

Objectives: There are a number of blockbuster monoclonal antibodies on the market used for the treatment of a variety of diseases. Although the formulation of many antibodies is achieved in 'platform' formulations, some are difficult to formulate that can result in an inability to develop a finished drug product. Further, a large number of antibody inspired or based molecules are now being developed and assessed for biotherapeutic purposes and less is understood around the required active protein drug concentrations, excipients and additives required in final product formulations.

Results: We investigated the effect of formulation variables (pH, buffer composition, glycine and NaCl concentration, time and temperature of accelerated stability studies) on antibody solubility/aggregation and activity using a Plackett-Burman Experimental Design approach. We then used the findings from this study and applied these to the formulation of a single chain variable fragment (ScFv) molecule. Our data shows that prediction of ScFc stability from a model monoclonal antibody could be achieved although further formulation optimization was required. Mass spectrometry analysis confirmed changes to the mass and hence authenticity of both the model antibody and ScFv under formulation conditions that did not provide appropriate conditions for protection of the molecules.

Conclusions: We report and discuss on the role of the different formulation conditions on maintaining protein integrity and show using mass spectrometry that protein integrity is compromised under particular conditions. The implications for predicting successful formulations for protein molecules is discussed and how antibody formulations could be used to predict formulation components for novel antibody based molecules.

Keywords: Formulation, monoclonal antibody, ScFc, stability, aggregation, recombinant biotherapeutic protein

INTRODUCTION

Monoclonal antibody (mAb) based drugs have been a huge success in the clinic and there are many more in development for the treatment of diseases including cancer and rheumatoid arthritis (Ivanov et al 2009; Ford et al ; Kurata et al; Wang et al 2007; Goi et al). Although recombinant proteins can be successfully formulated and stabilised by lyophilisation (Smales et al 2002; Povey et al 2009), most of the mAb products currently in the clinic are administered by intravenous infusion (Zhu et al 2014). Due to the injection volume that is best for administration (≤ 1.5 ml), and the large dose requirements for most antibody treatments (≥100-400 mg per treatment), this generally means that antibodies must be formulated as high concentration solutions (Dani et al 2007). The development of such high concentration formulations can be challenging due to the increased potential for inter-molecular interactions and macromolecular 'crowding' in solution, and concentration-dependent aggregation (Dani et al 2007). Such deleterious effects can lead to issues with stability, activity, pharmacokinetics, potentially patient safety, and practical issues in delivery (Harn et al 2007).

The stability of a mAb may be compromised throughout all stages of production and manufacturing by factors including shear, presence of deleterious host cell proteins, pH, buffer composition and temperature. The most common issue associated with instability is aggregation, which is more prominent in high protein concentration solutions (Dani et al 2007; Demeule et al 2007a; Demeule et al 2007b; Harn et al 2007). Beyond the practical issues of drug delivery, aggregation can also reduce the efficacy of the protein (Cudd et al 1995) and result in toxic effects and potentially increased immunogenicity (Hermeling et al 2004). Aggregates are usually formed after storage at extreme temperatures and pH values that induce protein unfolding and increase the intermolecular interactions of hydrophobic regions which lead to an increase in aggregation events (Carpenter et al 1999; Liu et al 2008), however at high protein concentrations such events can be observed in the absence of extreme temperature or pH when formulation conditions are unfavourable.

Although many mAb based drugs have been produced and used as delivery vehicles for other therapeutic molecules, and the formulation of many IgGs is undertaken in platform formulations, there remain cases where it is difficult to successfully formulate particular mAb molecules resulting in an inability to develop a finished drug product. Further, molecules that are constituted of domains or sections of antibody have now also been developed and require different active protein drug concentrations, excipients and additives. Formulation development

of recombinant proteins is usually undertaken using a screening approach whereby excipients and additives are changed in order to find a combination that provides the desired stability profile (Almeida et al 2017; Gourbatsi et al 2016). Here, we report on the investigation of the effect of different formulations variables on protein integrity as measured by aggregation, activity, structural and chemical stability using a model mAb IgG4 molecule. We then ask the question as to whether the mAb information could be applied to the design of a formulation of a model single chain Fv (ScFv) molecule. From the findings, we discuss how an understanding of the model mAb and ScFv formulation requirements can be extrapolated to aid in further formulation design of ScFv's.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma-Aldrich (Dorset, UK) and were of analytical grade or higher. The model mAb was an IgG4 lambda molecule that had been produced in Chinese hamster ovary (CHO) cells, purified and formulated in a storage buffer of 50 mM sodium acetate (pH 5.5).

Plackett-Burman Design of Experiments. The effect of formulation variables (pH, buffer composition, glycine and NaCl concentration, time and temperature of accelerated stability studies) on antibody solubility/aggregation and activity were investigated. In order to investigate the effect of these variables on protein aggregation and stability whilst limiting the potential number of conditions to investigate, a Plackett-Burman Experimental Design was implemented (Supplementary Tables 1 and 2) in a manner to that previously described (Gourbatsi et al 2016). Triplicate samples were investigated for each formulation and the mean of all analyses calculated. A two-tailed t-test was used to compare sample means i.e. the low and high values of each variable.

Dialysis, freeze-drying and resuspension of samples in test formulations. In order to remove any existing buffer salts present, protein samples were initially dialysed against the appropriate formulation buffer. The concentration of antibody in solution was monitored before and after storage by measuring the absorbance at 280 nm using a NanoDrop ND-1000 spectrophotometer. After storage under the appropriate conditions, samples were centrifuged at 200 X g for 4 min in a benchtop 5702 centrifuge (Eppendorf UK). The pellets were carefully separated from the supernatants and resolubilized in 100 µl 8 M urea, 0.25 M Tris-HCl (pH 8.75), 1 mM EDTA. The samples were shaken for several minutes and centrifuged for a further 4 min and then the

concentration determined by A_{280} measurement. A visual record of samples before and after storage was also recorded using a Nikon D70 camera.

SDS-PAGE analysis. Standard SDS-PAGE was undertaken under non-reducing conditions using a 7.5% acrylamide resolving gel and for reduced samples using a 12% acrylamide resolving gel. Samples were adjusted to 1 mg/ml for analysis. The relative quantification of bands was undertaken using the freeware ImageJ software (https://imagej.nih.gov/ij/).

Equilibrium denaturation studies. The stability of the model mAb in high concentration formulations was studied by denaturation analysis using guanidine hydrochloride as the denaturant using the method previously described by Williamson *et al.* (1994).

Fluorescence spectroscopy. A Cary Eclipse fluorescence spectophotometer (Varian Ltd, Oxford, U.K.) was used for the collection of fluorescence data. Suspensions of 1 mg/ml (low protein concentration) and 90 mg/ml (high protein concentrations) were examined. The excitation wavelength was 295 nm and the emission was measured between 300 to 400 nm at 900 V with 10 nm slits. The excitation filter was set to auto and the detector voltage was 900 V. Measurements were taken from an average of four scan passes. For data analysis, the graphs were plotted in Excel and the λ_{max} determined. The data was normalized by plotting the ratio of the intensity over maximum wavelength intensity.

Size-exclusion chromatography-high performance liquid chromatography (SEC-HPLC). The model mAb was analysed on a Waters 600 series HPLC associated with a Waters 486 tunable absorbance detector and absorbance monitored at 220 nm. Samples were diluted to 1 mg/ml and 20 µl injected onto a pre-equilibrated TSK-Gel 3000SWxl column with 0.2 M sodium phosphate buffer pH 7.5. For generation of a standard curve, dilutions of known concentrations of mAb were prepared in 0.2 M sodium phosphate buffer pH 7.5. A constant flow rate (1.0 ml/min) was used for all runs.

Data analysis. All data was analysed using the Sequential Design of Expert tool (EasyStats, DX7, Version 7.1.6, Stat-Ease® Inc., Minneapolis, U.S.A.) to investigate and correlate the effect of individual variables and predict the best formulation conditions for long term storage at 4°C and 25°C. The criteria used for selection of the best formulation conditions were to optimize pH, buffer composition, glycine and NaCl concentrations to maximise the concentration of mAb in solution, and the visual appearance to be minimized (0 was scored for a clear solution and 1 was scored for a sample that precipitated), and the λ_{max} (fluorescence) to be closest to the standard/control sample.

Heavy and light chain analysis of the model mAb by mass spectrometry. Mass spectrometry analysis was undertaken as previously described (Gourbatsi et al 2016).

Analysis of a model single chain fragment variable region (ScFv). A single chain fragment variable region (ScFv) was provided by MedImmune in PBS (pH 7.3). The fragment was dialysed against dH_2O, freeze-dried and then 0.61 mM samples prepared in appropriate formulations based upon date collected from the model mAb analysis. Samples were analysed as described for the model mAb.

RESULTS

Determination of model mAb solubility with varying formulation. Following standard industrial procedure, an initial impression as to how different formulations affected mAb stability was undertaken visually against an intense light background. Visual analysis indicated that formulations 1, 3, 8 and 12 (Supplementary Table S2) resulted in the majority of mAb precipitating out to form a gel like aggregate (data not shown). The effect of formulation variables on initial protein solubility and any further effect during accelerated storage studies at raised temperature were then investigated by measuring the absorbance at 280 nm immediately after formulation and then again after the relevant storage time (Supplementary Table S1). Changes in the concentration as determined by A_{280} were calculated and the results and statistical analysis are shown in Supplementary Tables S3 and S4 respectively. From the A_{280} analysis and two tailed t-test statistical testing there was not a single factor identified as being the statistically significant factor impacting upon concentration remaining in solution upon formulation. However, after storage the most significant variable factors were, not surprisingly, protein concentration and the temperature of storage. Of the other variables, although none of these was above the 80% significance level, these generally had more of an effect initially upon formulation than after storage (Supplementary Table S4). The data did show, along with the visual data, that several formulations were particularly unsuitable (1, 3, 8, 12) and although all of these were subjected to the high temperature condition, other high temperature formulations were not adversely affected. Of the high protein concentration samples, formulations 4, 9 and 10 did not precipitate compared to high concentration samples 1, 8 and 12. However, formulation 4 was at low temperature for the maximum time investigated, formulation 9 and 10 at low temperature whilst formulations 1 and 12 were stored for the maximum time investigated at maximum temperature and formulation 8 at

maximum temperature for 2 h. This suggests that storage temperature was the key variable in the initial study.

SDS-PAGE analysis of MAb samples in the different formulations. SDS-PAGE analysis of all supernatants and pellets of the mAb samples was undertaken in order to investigate changes in protein aggregation (precipitate versus supernatant) (Figure 1). Where it was necessary to resolubilized pellets this was undertaken in 8 M urea solution (formulations 1, 3, 8, and 12) and for these samples supernatant analysis was not possible. For the mAb sample in formulation 12, not all protein was initially in solution and thus the precipitate observed after storage was not due to the temperature of storage. Differences in the banding pattern in the non-reduced SDS PAGE gel after storage of formulations 3R (where R stands for resolubilized pellet), 6 and 7 compared to the standard indicates that these formulations and conditions had an impact upon the mAb sample that was not evident from the A_{280} measurements alone (Figure 1A). Further, upon reduced SDS-PAGE analysis, the samples in formulations 6 and 11 showed a different banding profile from the standard sample (Figure 1B).

Determination of soluble aggregates in mAb formulations by size exclusion chromatography (HPLC-SEC). Quantitative analysis of the amount of soluble aggregate in the different formulated samples was undertaken by size exclusion chromatography and the area under the different peaks representing aggregates of different size determined. Across the samples four major peaks/areas were observed (Figure 2). These are labelled as peaks I, II, III and IV in figure 2E where peak III corresponds to the fully assembled and intact mAb monomer. Two peaks eluted earlier than the standard corresponding to higher molecular weight aggregates (I and II at 5-6 mins and 6-7 mins respectively, Figure 2) and one later eluting peak (IV 8-9 mins, Figure 2) that was smaller than the monomer and is likely due to disassembled mAb. A summary of the integration of the area under each peak as a percentage of the total area for each sample is depicted in Figure 3. The analysis of samples in formulation 2 showed clear evidence of higher molecular weight aggregates as well as disassembled antibody material despite the fact that other methods of analysis had shown no effect of this formulation on stability. Resolubilised material from formulation 8 also revealed the presence of higher order aggregate (Figure 2). The amount of each peak in the HPLC-SEC analysis as a percentage of the total peak area is reported in Figure 2. In all cases more aggregate or disassembed material was present in the formulated samples after storage than in the standard

samples. High molecular weight aggregate was observed after storage of mAb in low protein concentrations and high protein concentration formulations.

mAb stability in different formulations as determined by fluorescence and guanidine hydrochloride denaturation studies. In order to determine whether any large structural changes were observed in the different mAb high protein concentration formulations, fluorescence studies were undertaken. Figure 4 shows the λ_{max} of the mAb samples in the different formulations, an indication of how accessible tryptophan residues are to the solvent. The fluorescence curves show that there was a shift in the observed λ_{max} after storage in formulation 12 and smaller shifts after storage in formulations 1, 8 and 4 (Figure 4a). A shift to a higher λ_{max} is an indication of protein unfolding in these formulations. There was no change in the observed λ_{max} for samples in formulation 10 and the fluorescence data for such samples was almost identical to the standard sample (Figure 4a). In addition, we undertook guanidine hydrochloride denaturation of the high concentration model mAb that had been stored under different formulation conditions (formulations 4, 9 and 10) to further investigate the conformational stability under the different conditions. The denaturation of the mAb with increasing guanidine hydrochloride concentrations resulted in a small shift in the fluorescence emission maximum when intrinsic fluorescence was excited at 280 nm (Figure 4b). The curves all showed one phase of unfolding with the denaturation midpoint occurring at approximately 1.75 M guanidine hydrochloride (Figure 4b). All samples will therefore have more-or-less the same $\Delta G°$ (standard free energy change between native and denatured states) and little or no difference in their stability as determined by standard denaturation analysis.

ESI-MS analysis of reduced mAb under different formulation conditions for the presence of protein modifications. For mass spectrometry analysis, mAb was initially subjected to reduction and alkylation to allow intact analysis of the heavy and light chains. The major heavy chain (HC) and light chain (LC) masses and relative abundance of each are reported in Figure 5. **T**he major HC mass observed in each sample was at approximately 51171 Da, which corresponds to the expected mass of the mAb heavy chain. A number of other heavy chain species were also present in the standard and formulated samples (Figure 5A). Analysis of samples from the different formulations also revealed HC mass peaks not observed in the standard sample, indicative of either loss or gain of mass and therefore protein modification. For example, the HC spectra of samples in formulation 10 contained a peak of mass 47372 Da, a mass loss of 3797 Da. For the light chain analysis (Figure

5B), the major peak observed had a mass of 22899 Da in the standard sample and a number of higher mass species were also present. As in the case of the heavy chain analyses, samples formulated in formulations 4, 9 or 10 had species of light chain with a different mass to that observed in the control, indicative of protein modification(s) (Figure 5B).

Do mAb samples stored under different formulation conditions have different activity? To determine whether there was any effect on the activity of the model mAb in the high concentration formulations, potency assays were undertaken. Due to the limited amount of antibody in solution after storage in formulations 1, 8 and 12, potency assay measurements were only undertaken after storage in formulations 4, 9 and 10. The resulting dose response curves are shown in Figure 6. The data shown reveals that there was no significant change in the activity of the antibody in formulation conditions 4, 9 and 10 compared to the control sample. Thus, under the conditions and formulations of 4, 9 and 10, mAb samples maintained their physical and structural stability and antibody potency/activity.

Statistical analysis of all mAb data. Design-Expert 7.1.6 was used to statistically analyse and correlate the data from all mAb samples and formulations studied here to identify any variable(s) impacting on the stability and integrity of the mAb. Temperature, as expected, was found to be the most important factor of all the responses recorded (solubility, visual appearance, SDS-PAGE analysis, fluorescence, reduced heavy and light chain mass spectrometry analysis and potency of the mAb) after accelerated storage in the appropriate conditions (Table 1). For some of the responses, the effect of temperature was extremely prominent such as solubility (97.2% increase in protein precipitation as the temperature increases), heavy and light chain mass changes (99.9% change in the mass as the temperature increases), aggregation (41.1% change in band intensity as the temperature increases) and potency (43.1% decrease in the activity as the temperature increases) (Table 1). Buffer concentration was the next most influential factor affecting the presence of non-covalent aggregates as indicated by structural stability fluorescence analysis (Table 1). From the data generated, Design Expert was used to predict the formulation condition to be the most appropriate for maintaining the mAb protein integrity at 4 and 25°C and this is reported in Table 2.

The influence of buffer concentration on a model antibody single chain fragment (ScFv). The effect of buffer concentration on the stability of an antibody single chain fragment from a variable region (ScFv) was undertaken. The initial formulation used was that based upon the results

generated with the model intact mAb and consisted of 250 mM sodium acetate, 0.2 M NaCl and 0.13 M glycine at pH 6.3 and filtered and stored at 4°C and is hence forth referred to as formulation A. Buffer (sodium acetate) concentrations of 10, 100, 250 and 500 mM were investigated (with NaCl, glycine and pH kept constant), with ScFV samples formulated in the varying buffer concentrations and the absorbance at 280 nm and fluorescence profiles of the resulting ScFv solutions generated after 1 week of storage at 37°C investigated. The ScFv fragment was soluble in the high buffer concentration formulation (500 mM), but became more insoluble as the buffer concentration was lowered (10 and 100 mM) and in PBS (Table 3). After a week of storage at 37°C, the 500 mM buffer concentration formulation maintained the ScFv in solution as determined by visual analysis while the remaining buffer concentrations did not appear to deteriorate upon storage any further than observed upon formulation (Table 3). Despite the small precipitate observed in the PBS, 10 and 250 mM buffer concentration formulations, there were no large apparent structural changes as determined from the fluorescence curves and the λ_{max} values (data not shown). Mass spectrometry analysis of the material from the 100, 250 and 500 mM samples revealed no major mass changes compared to the control sample (Figure 7). In the resolubilised pellets of the PBS and 10 mM buffer concentration formulations, the most abundant mass peaks upon mass spectrometry analysis were twice the expected mass of the protein. It is likely that dimerization had occurred during storage under these conditions (Figure 7). Other mass peaks were also observed that were not in the control sample (Figure 7).

Discussion

A potential issue in developing high protein concentration formulations is protein aggregation that compromises quality control and biological activity, whilst the administration of such aggregates can lead to immune-related adverse effects (Harn et al 2007; Shire et al 2004; Kameoka et al 2007). The effect of different formulations variables including protein concentration, pH, buffer composition, time of storage, storage, temperature, glycine and salt (NaCl) concentration was therefore investigated on a model mAb. Visual analysis showed that the antibody precipitated out of solution after storage in formulations 1, 8 and 12 with the most significant factor in terms of accelerating aggregation, unsurprisingly, being temperature. The impact of temperature in promoting aggregation events for monoclonal antibodies (and proteins in general) has been well documented (e.g. Cleland et al 2001; Chen et al 2003; Breen et al 2001). At elevated temperatures, proteins can partial/completely unfold and for this reason storage temperature is set well below

the thermal melting for long term stability at 2-8°C (Perico et al 2008). However, temperature is used during formulation studies to accelerate stability studies and identity formulations that are less stable, or in this case formulation components.

The protein concentrations in solution were determined in the different formulations spectrophotometrically at 280 nm before and after storage and statistical analysis was then applied to the results to identify the variables in terms of aggregation that appeared most significant. This analysis showed that protein concentration and temperature of storage were statistically significant with respect to changes in protein concentration. Much work has been undertaken into this area, for example a study by Perico et al. using an IgG2 monoclonal antibody demonstrated that the aggregation of this antibody was temperature dependent. This finding agrees with the analysis here where temperature positively impacted upon the precipitation of mAb. In particular, under storage conditions of 2-8°C at pH 4.0 there was minimal aggregation while at elevated temperatures (37°C) the acidic formulations lead to the presence and detection of high order aggregates. Ejima *et al.* did not detect a significant increase in aggregation of a recombinant humanized IgG4 after storage at pH 2.7 and 3.5 at 4°C (Ejima et al 2007) in agreement with the data presented here where after storage in formulation 4 at pH 4.0 and 4°C there was no evidence of aggregation based on the A_{280} measurements, SDS-PAGE or SEC-HPLC analysis. Thus, low pH alone was not enough to destabilize the antibody. On the other hand, after storage in formulation 8 at pH 4.0 at elevated temperature (55°C) there was a significant increase in aggregation events. This agrees with others who have reported the formation of aggregates of a chimeric monoclonal antibody at pH values below 5 at 60°C (Paborji et al 1994). However, Kuetzo *et al.* reported an increase in aggregation at decreased pH of an IgG2 monoclonal antibody during freeze-thawing after storage at 4°C for up to 6 weeks, and moreover, that the aggregation was most prevalent at pH 3 and 4 (Kueltzo et al 2008). This contrasts with the data presented here where formulation 4 is very similar to those in the report by Kuetzo et al but minimal aggregation of the MAb in this formulation was observed. In a further study two mouse monoclonal antibodies were stored at 37°C, pH 3 and pH 4, and at 4°C, pH 3.0 and insoluble aggregates were observed (Jiskoot et al 1990). All of these studies demonstrate the importance of temperature in the formation of aggregates, especially at low pH, but importantly the different behaviour of different antibodies and the fact this can be prevented by manipulating formulation conditions.

The presence of non-covalent and covalent aggregates in solution was investigated by reduced and non-reduced SDS-PAGE. Unfortunately, analysis of the aggregates in high

concentration formulations where changes were observed in solution was difficult due to the amount of aggregation. As a result, the precipitate was resolubilized with 8 M Urea/Tris/EDTA, but this can disrupt the aggregates present and give misleading results. This method was successful for analysis of formulation 8 where less precipitate was observed and in the subsequent SEC analysis high molecular weight aggregates were observed. Surprisingly, in the low mAb concentration formulation 2, the same high molecular weight aggregates were observed and a smaller molecular weight peak that corresponds to disassembled antibody. The temperature of storage in this formulation was high (55°C) and this might explain the formation of aggregates. These data show that even in a clear solution aggregates can be present. Long term storage of such samples could result in the early aggregate material leading to further aggregates or fibril formation and gelation with time as has been previously described (Demeule et al 2007a; Demeule et al 2007b).

Protein aggregation usually involves structural and conformational changes to the protein (Demeule et al 2007a; Demeule et al 2007b). The structural integrity of the model mAb here after storage in high concentration formulations was therefore investigated by fluorescence analysis. The λ max curves revealed that the mAb in majority of high concentration formulations remained folded although in formulations 1, 8 and 12 that had high storage temperature (55°C) there was a shift in λ_{max} to higher values indicating structural change and unfolding. This red shift indicates that there are local changes in the environment of tryptophan, tyrosine and phenylalanine residues and suggests that there may be a decrease in the hydrophobicity around these residues i.e. local environment around tryptophan molecules is more hydrophilic.

The conformational stability of the model mAb after storage in different formulations was also investigated by guanidine hydrochloride denaturation studies. The change in the λ_{max} was used to follow the denaturation of the protein with increasing guanidine concentrations (Williamson et al, 1994). Due to limitations in amount of sample, the analysis was only carried out for the high concentration formulations 4, 9 and 10. Previous analysis of these formulations showed no or minimal evidence of aggregation and a single-state unfolding was observed for the denaturation curves. Little difference in the concentration of guanidine required to initiate and complete the unfolding between samples in the different formulations was observed, suggesting that tertiary structure stability was more-or-less the same in all samples investigated. The potency of these samples was also investigated and statistical analysis suggested there was a significant difference in the potency of the material from these formulations.

There are now a number of different antibody formats on the market and in development, including single chain fragments from variable regions (ScFv, ~20-30 Da). These smaller molecules can also have stability issues, particularly due to the absence of sections of the constant region of the light or heavy chain resulting in the exposure of hydrophobic side chains (Worn et al 1999; Barthelemy et al 2008). Therefore, we investigated the impact of buffer concentration and composition, as in the case of the model mAb, on a model ScFv. In contrast to the full length mAb, the fragment did not easily go into solution at 10 mM buffer concentration or in PBS and after 1 week of storage at 37°C there was much insoluble material present. The best buffer concentration formulation for solubility of the ScFv of those investigated was that at a concentration of 500 mM, where the fragment went easily into solution and remained so after the appropriate storage. A possible explanation might be that at this concentration the fragment had reached its isotonicity (where the solute, in this case the ScFv, concentration is the same as the concentration of the solution surrounding it). Changes in the overall charge of the fragment that could result in an increase/decrease of the hydrophobicity might be another factor. Regardless, the work here demonstrates that potency may not necessarily be impacted by modifications (as determined by mass spectrometry) of a model mAb and that despite the large amount of work undertaken to further our understanding of how formulation conditions can impact upon mAb and general protein stability, the use of 'platform' formulation been adopted across the industry, it is still necessary to screen excipients and formulations to identify appropriate formulations for biotherapeutics and prediction of formulation performance remains a challenging area.

Acknowledgements

We thank Mr Kevin Howland for his help with mass spectrometry analysis work. This research was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) Industrial CASE studentship for EG.

REFERENCES

Almeida AG, Pinto RCV, Smales CM, Castilho LR (2017) Investigations into, and development of, a lyophilized and formulated recombinant human factor IX produced from CHO cells. Biotechnol Lett 39:1109-1120.

Barthelemy PA, Raab H, Appleton BA, Bond CJ, Wu P, Wiesmann C, Sidhu SS (2008) Comprehensive analysis of the factors contributing to the stability and solubility of autonomous human VH domains. J Biol Chem 283:3639-3654.

Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ (2001) Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. Pharm Res 18:1345-1353.

Carpenter JF, Kendrick BS, Chang BS, Manning MC, Randolph TW (1999) Inhibition of stress-induced aggregation of protein therapeutics. Methods Enzymol 309:236-255.

Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM (2003) Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. Pharm Res 20:1952-1960.

Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks D, Hsu C, Carpenter JF (2001) A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci 90:310-321.

Cudd A, Arvinte T, Das RE, Chinni C, MacIntyre I (1995) Enhanced potency of human calcitonin when fibrillation is avoided. J Pharm Sci 84:717-719.

Dani B, Platz R, Tzannis ST (2007) High concentration formulation feasibility of human immunoglubulin G for subcutaneous administration. J Pharm Sci 96:1504-1517.

Demeule B, Gurny R, Arvinte T (2007a) Detection and characterization of protein aggregates by fluorescence microscopy. Int J Pharm 329:37-45.

Demeule B, Lawrence MJ, Drake AF, Gurny R, Arvinte T (2007b) Characterization of protein aggregation: the case of a therapeutic immunoglobulin. Biochim Biophys Acta 1774:146-153.

Ejima D, Tsumoto K, Fukada H, Yumioka R, Nagase K, Arakawa T, Philo JS (2007) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. Proteins 66:954-962.

Ford JA, Jones R, Elders A, Mulatero C, Royle P, Sharma P, Stewart F, Todd R, Mowatt G (2013) Denosumab for treatment of bone metastases secondary to solid tumours: systematic review and network meta-analysis. Eur J Cancer 49: 416-430.

Goi T, Nakazawa T, Hirono Y, Yamaguchi A (2014) Anti-Prokineticin1 (PROK1) Monoclonal Antibody Suppresses Angiogenesis and Tumor Growth in Colorectal Cancer. Ann Surg Oncol 21:S665-671.

Gourbatsi E, Povey J, Uddin S, Smales CM (2016) Biotherapeutic protein formulation variables influence protein integrity and can promote post-translational modifications as shown using chicken egg white lysozyme as a model system. Biotechnol Lett 38:589-596.

Harn N, Allan C, Oliver C, Middaugh CR (2007) Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. J Pharm Sci 96:532-546.

Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W (2004) Structure-immunogenicity relationships of therapeutic proteins. Pharm Res 21:897-903.

Ivanov A, Beers SA, Walshe CA et al (2009) Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. J Clin Invest 119:2143-2159.

Jiskoot W, Beuvery EC, de Koning AA, Herron JN, Crommelin DJ (1990) Analytical approaches to the study of monoclonal antibody stability. Pharm Res 7:1234-1241.

Kameoka D, Masuzaki E, Ueda T, Imoto T (2007) Effect of buffer species on the unfolding and the aggregation of humanized IgG. J Biochem 142:383-391.

Kueltzo LA, Wang W, Randolph TW, Carpenter JF (2008) Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing. J Pharm Sci 97:1801-1812.

Kurata T, Nakagawa K (2012) Efficacy and safety of denosumab for the treatment of bone metastases in patients with advanced cancer. Jpn J Clin Oncol 42:663-669.

Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J, (2008) Heterogeneity of monoclonal antibodies. J Pharm Sci 97:2426-2447.

Paborji M, Pochopin NL, Coppola WP, Bogardus JB (1994) Chemical and physical stability of chimeric L6, a mouse-human monoclonal antibody. Pharm Res 11:764-771.

Perico N, Purtell J, Dillon TM, Ricci MS (2008) Conformational implications of an inversed pH-dependent antibody aggregation. J Pharm Sci 98:3031-3042.

Povey JF, Perez-Moral N, Noel TR, Parker R, Howard MJ, Smales CM (2009) Investigating variables and mechanisms that influence protein integrity in low water content amorphous carbohydrate matrices. Biotechnol Prog 25:1217-1227.

Shire SJ, Shahrokh Z, Liu J (2004) Challenges in the development of high protein concentration formulations. J Pharm Sci 93:1390-1402.

Smales CM, Pepper DS, James DC (2002) Protein modification during anti-viral heat-treatment bioprocessing of factor VIII concentrates, factor IX concentrates, and model proteins in the presence of sucrose. Biotechnol Bioeng 77:37-48.

Wang W, Singh S, Zeng DL, King K, Nema S (2007) Antibody structure, instability, and formulation. J Pharm Sci 96:1-26.

Worn A, Pluckthun A (1999) Different equilibrium stability behavior of ScFv fragments: identification, classification, and improvement by protein engineering. Biochemistry 38:8739-8750.

Williamson RA, Bartels H, Murphy G, Freedman RB (1994) Folding and stability of the active N-terminal domain of tissue inhibitor of metalloproteinases-1 and -2. Protein Eng 7:1035-1040.

Zhu D, Ravindranath MH, Terasaki PL, Miyazaki T, Pham T, Jucaud V (2014) Suppression of allo-human leucocyte antigen (HLA) antibodies secreted by B memory cells in vitro: intravenous immunoglobulin (IVIg) versus a monoclonal anti-HLA-E IgG that mimics HLA-I reactivities of IVIg. Clin Exp Immunol 177:464-477.

Table 1: Analysis to determine the significant variables for the measured responses on mAb after storage in the different Plackett-Burman high (1, 4, 8, 9, 10 and 12) protein concentration formulations.

MAb CAT3 after incubation				
Response	**High protein conc. formulations**			
	Variable	**Effect**	**% Response change**	**Significant**
[MAb] in solution	Temperature	positive	**97.2**	yes
Visual appearance	Temperature	positive	39.5	yes
Non-reduced SDS-PAGE	Temperature	negative	**41.1**	yes
Native-PAGE	Temperature	negative	37.2	yes
	Glycine	negative	35.1	no
	Buffer conc.	negative	33.5	no
Fluorescence (λmax)	Temperature	positive	1.8	yes
	Buffer conc.	positive	1.2	yes
Heavy chain MS	Temperature	negative	**99.9**	yes
Light chain MS	Temperature	negative	**99.9**	yes
Potency	Temperature	negative	**43.1**	yes

Table 2: Predicted most appropriate formulation for the maintenance of the model mAb integrity based on the data generated and reported previously for high protein concentration formulations at 4 and 25°C.

	After incubation						
High [mAb] formulations	**Protein conc. (mM)**	**pH**	**Buffer conc. (mM)**	**Temp (°C)**	**Glycine (mg/ml)**	**NaCl (M)**	**Desirability**
	1.04	6.3	250	**4**	20.0	0.19	0.96
	1.04	6.3	250	**25**	10.0	0.24	0.96

Table 3: ScFv visual appearance, precipitation and fluorescence analysis before and after a week of storage at 37°C in 10, 100, 250 and 500 mM buffer concentration formulations (n=1).

ScFv Buffer conc. (mM) pH 6.25	**Upon formulation**			**After 1 week incubation at 37°C**		
	Appearance	**[ScFv] mg/ml from A_{280}**	**λ_{max} (nm)**	**Appearance**	**[ScFv] mg/ml from A_{280}**	**λ_{max} (nm)**
STD PBS pH 7.2	white precipitate	1.8	341	white precipitate	2.0	338
10	white precipitate	2.0	339	white precipitate	2.2	339
100	small white precipitate	2.2	337	small white precipitate	2.3	340
250	clear solution	2.3	337	clear solution	2.4	338
500	clear solution	2.4	337	clear solution	2.4	341

Figure Legends

Figure 1: **(A) 7.5% non-reduced SDS-PAGE analysis of mAb samples in formulations 1-12, (B) 12% SDS-PAGE analysis of reduced mAb samples in formulations 1-12. Key**: R stands for resolubilized sample in 8 M urea solution, Std is the standard mAb before formulation.

Figure 2: Size exclusion chromatography analysis of mAb samples after incubation in various formulations. (A) standard antibody, (B) formulation 1R, (C) formulation 2, (D) formulation 4, (E) formulation 8R, (F) formulation 9, (G) formulation 10, (H) formulation 12R. The chromatograph shown of formulation 1R (B) and 12R (H) shows that there was only a very small amount of protein in solution. **Key:** R stands for resolubilized sample in 8 M urea solution.

Figure 3: The % of each of the four major peaks observed by SEC-HPLC analysis of mAb samples in different formulations 1-12. RES = resolubilized sample in 8 M urea solution. All values represent the average of three independent experiments (n=3).

Figure 4. (A) Fluorescence curves of high concentration mAb samples in formulations 1, 4, 8, 9, 10 and 12 after incubation along with a standard MAb sample or comparison (n=3). The wavelength at which maximum intensity was observed was approximately 336 nm for all samples except formulation 1, 8 and 12 whereby the λ_{max} was shifted to 344, 339 and 346 nm respectively. **(B)** Denaturation curves for mAb in standard solution PBS, mAb in Formulation 4, mAb in formulation 9, mAb in formulation 10.

Figure 5: (A) Heavy (HC) and **(B)** Light chain (LC) mass analysis of mAb samples after incubation in high concentration formulations 4, 9 and 10 by ESI-MS. The figure shows the abundance of each peak as a percentage of the most abundant peak which was assigned a value of 100%.

Figure 6: Dose response curves for the mAb in formulations 4, 9 and 10. (A) mAb in formulation 4 (B), mAb in formulation 9 and (C) mAb in formulation 10. The table shows the reference potency to the mAb STD. There was no significant change in the activity of the mAb after incubation in these formulations although in formulation 4 the potency was slightly elevated and in formulation 10 slightly reduced.

Figure 7: Mass analysis of ScFv samples after incubation in PBS and 100 mM, 250 mM and 500 mM buffer concentration formulations (Res = resolubilized pellet). The figure shows the abundance of each peak as a percentage of the most abundant peak which was assigned a value of 100%.

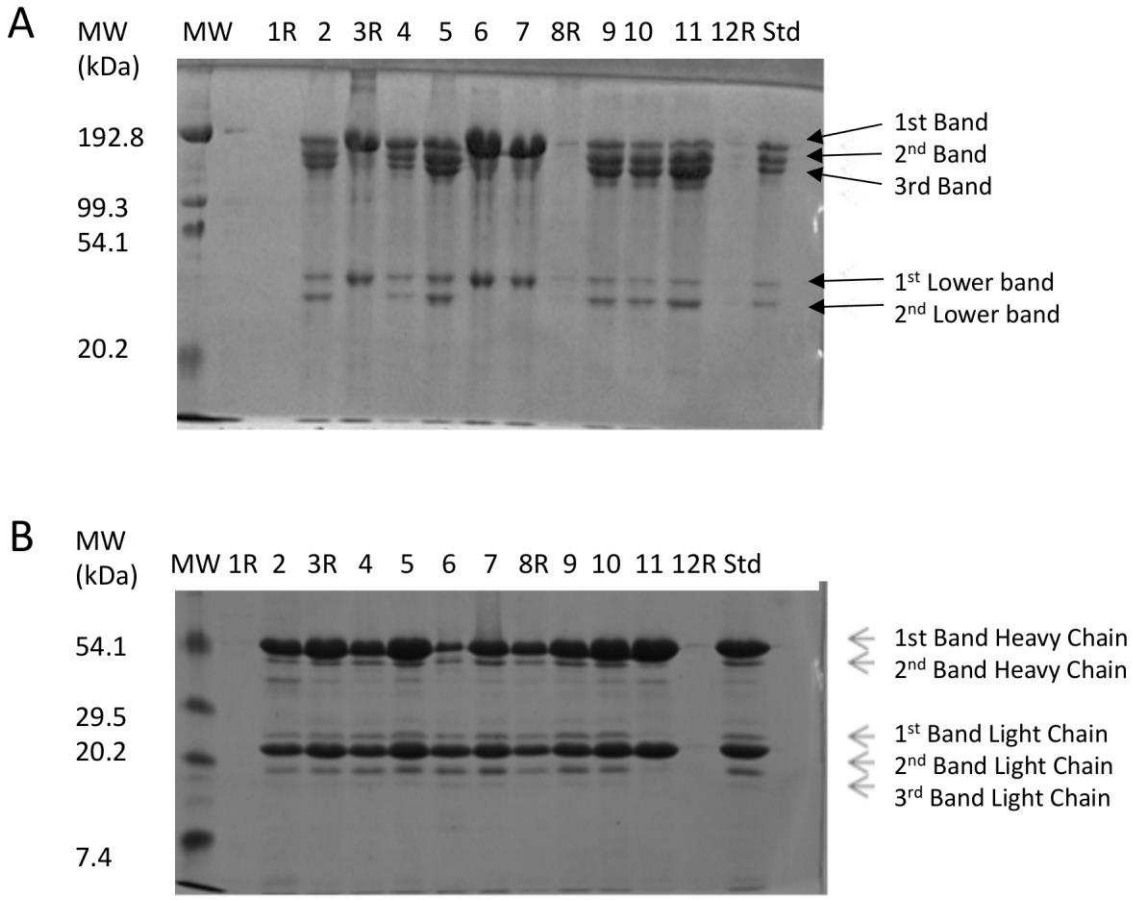


Figure 1: **(A) 7.5% non-reduced SDS-PAGE analysis of mAb samples in formulations 1-12, (B) 12% SDS-PAGE analysis of reduced mAb samples in formulations 1-12. Key**: R stands for resolubilized sample in 8 M urea solution, Std is the standard mAb before formulation.

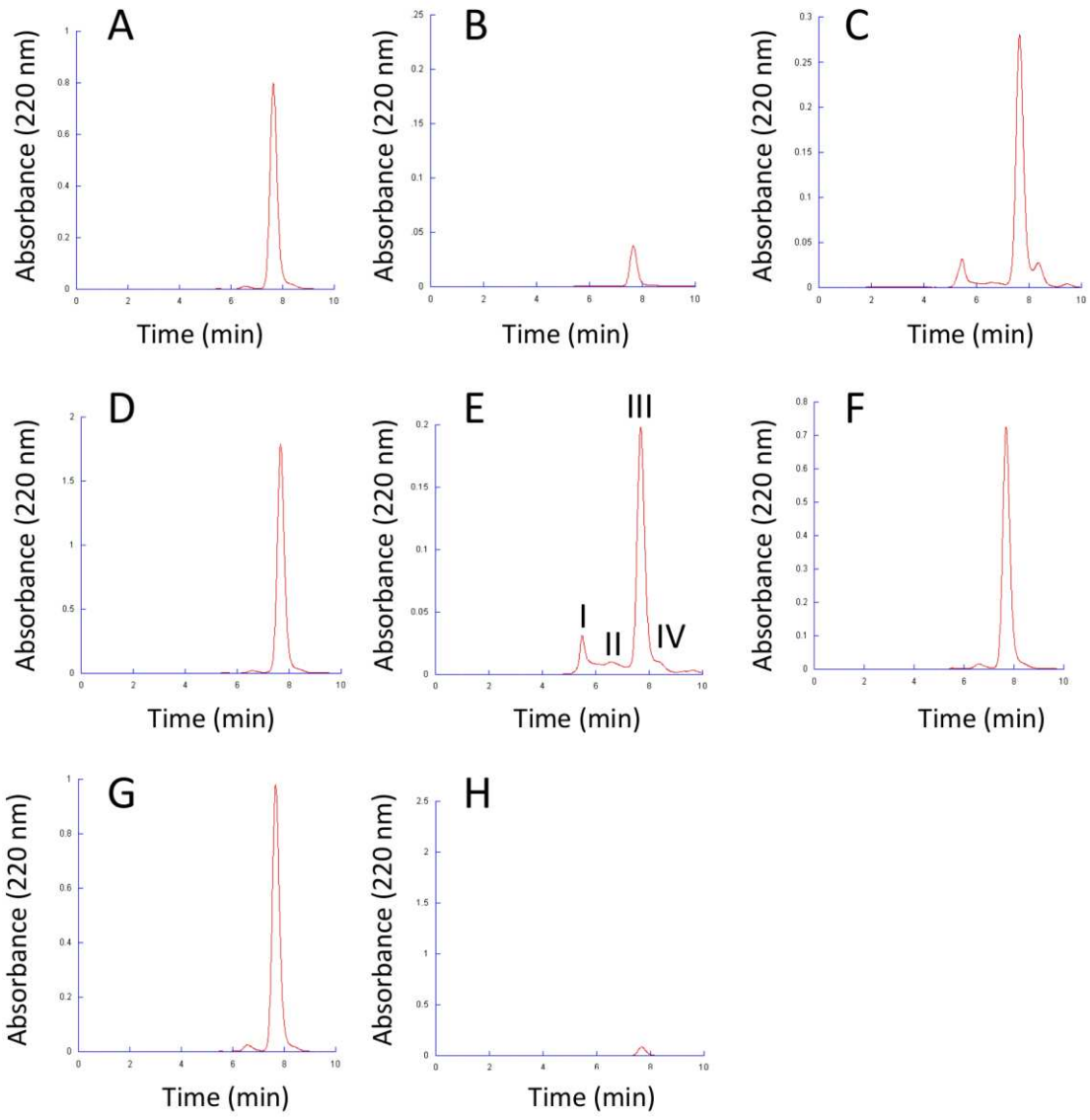


Figure 2: Size exclusion chromatography analysis of mAb samples after incubation in various formulations. (A) standard antibody, (B) formulation 1R, (C) formulation 2, (D) formulation 4, (E) formulation 8R, (F) formulation 9, (G) formulation 10, (H) formulation 12R. The chromatograph shown of formulation 1R (B) and 12R (H) shows that there was only a very small amount of protein in solution. **Key:** R stands for resolubilized sample in 8 M urea solution.

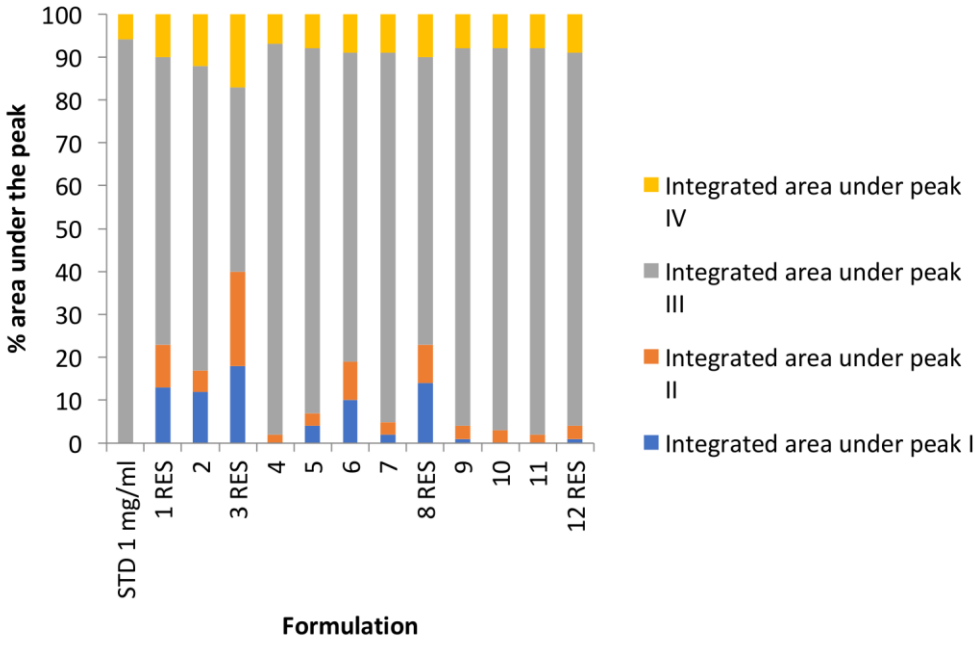
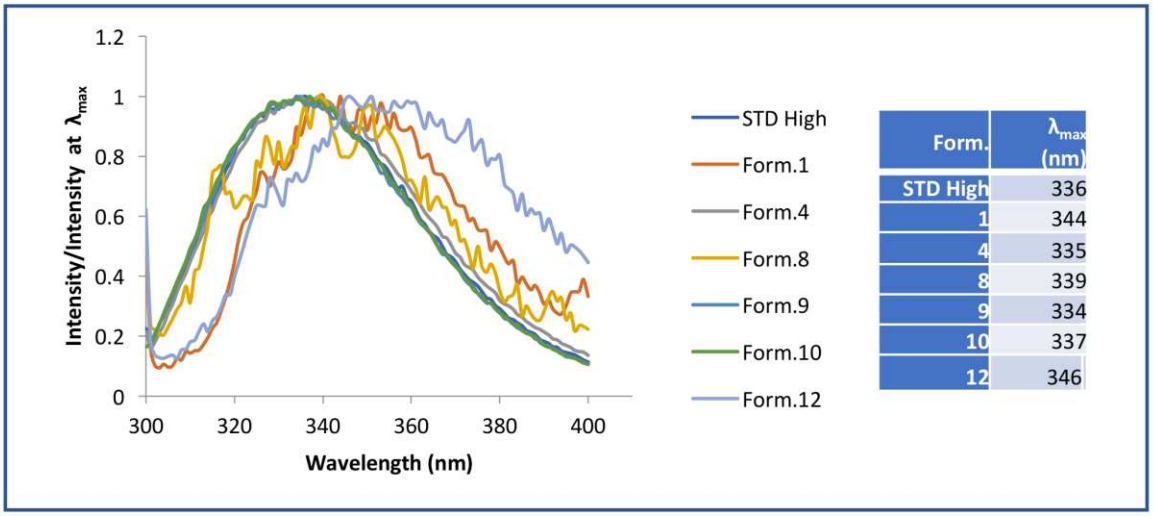


Figure 3: The % of each of the four major peaks observed by SEC-HPLC analysis of mAb samples in different formulations 1-12. RES = resolubilized sample in 8 M urea solution. All values represent the average of three independent experiments (n=3).



Form.	λ_{max} (nm)
STD High	336
1	344
4	335
8	339
9	334
10	337
12	346

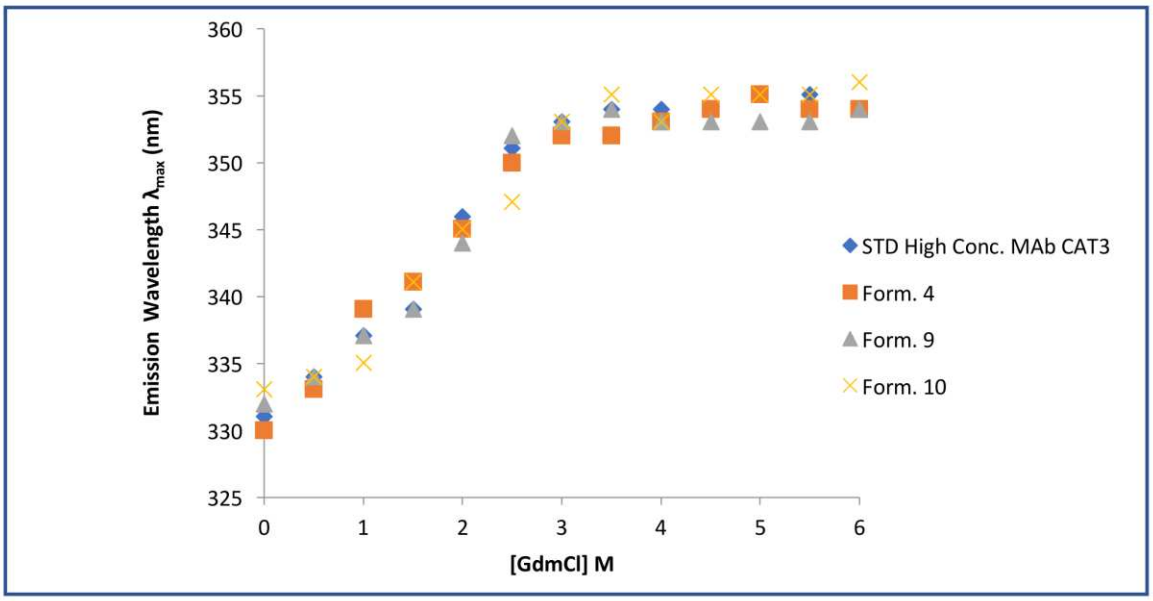
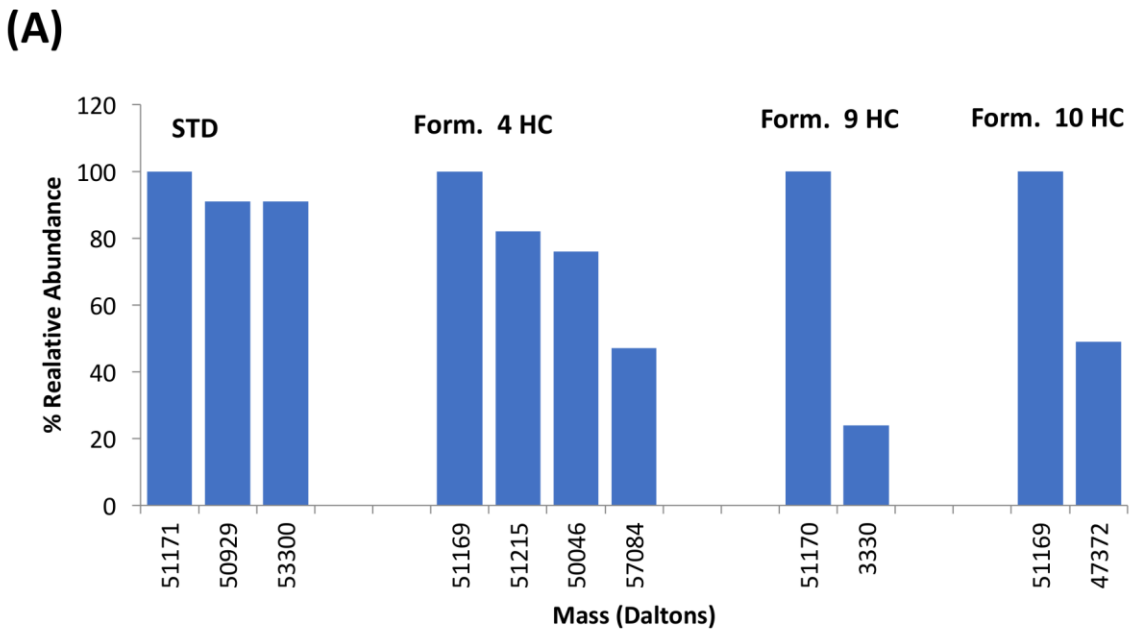


Figure 4. (A) Fluorescence curves of high concentration mAb samples in formulations 1, 4, 8, 9, 10 and 12 after incubation along with a standard MAb sample or comparison (n=3). The wavelength at which maximum intensity was observed was approximately 336 nm for all samples except formulation 1, 8 and 12 whereby the λ_{max} was shifted to 344, 339 and 346 nm respectively. **(B)** Denaturation curves for mAb in standard solution PBS, mAb in Formulation 4, mAb in formulation 9, mAb in formulation 10.



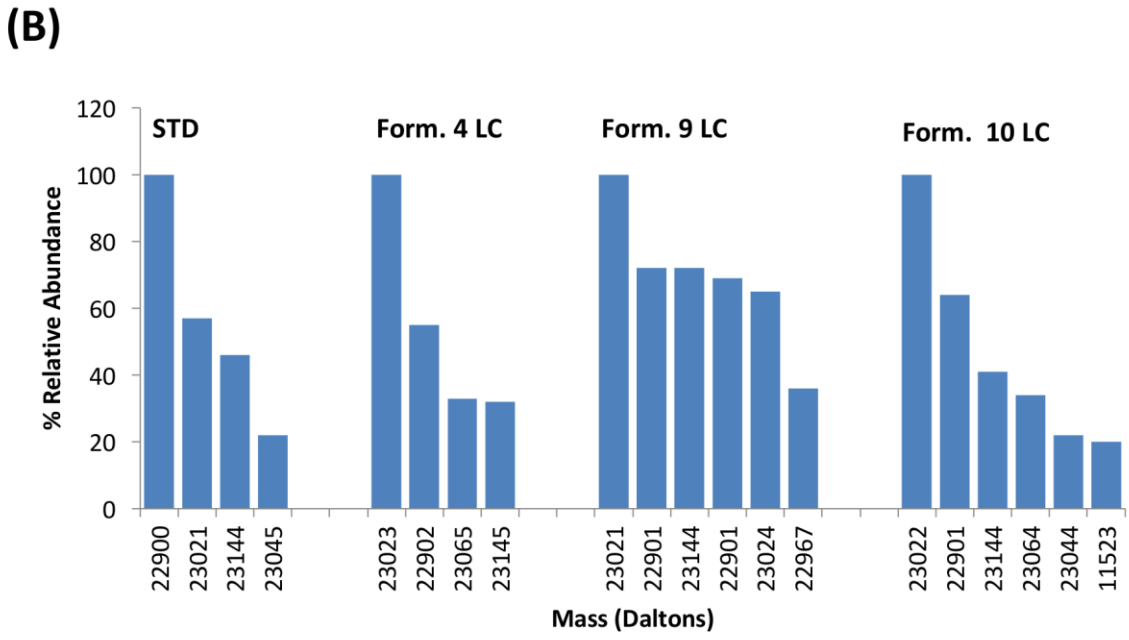
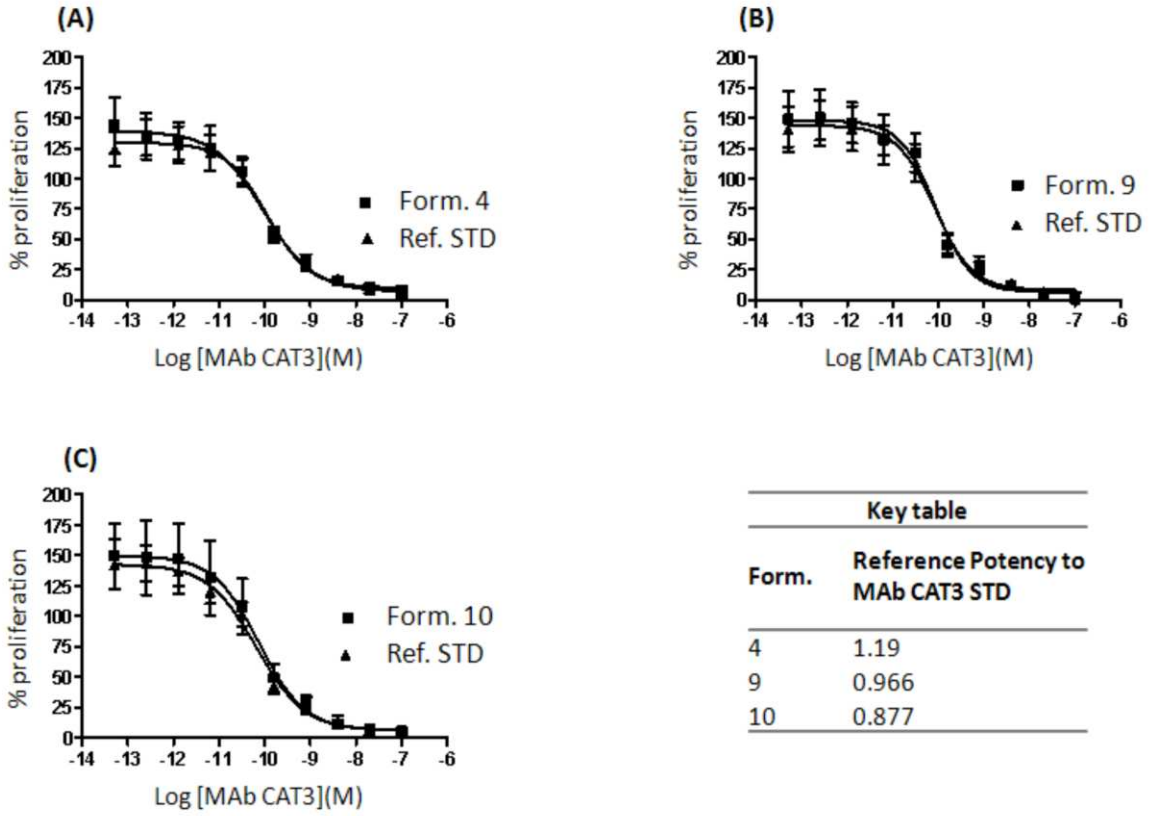


Figure 5: (A) Heavy (HC) and **(B)** Light chain (LC) mass analysis of mAb samples after incubation in high concentration formulations 4, 9 and 10 by ESI-MS. The figure shows the abundance of each peak as a percentage of the most abundant peak which was assigned a value of 100%.



Key table	
Form.	**Reference Potency to MAb CAT3 STD**
4	1.19
9	0.966
10	0.877

Figure 6: Dose response curves for the mAb in formulations 4, 9 and 10. (A) mAb in formulation 4 (B), mAb in formulation 9 and (C) mAb in formulation 10. The table shows the reference potency to the mAb STD. There was no significant change in the activity of the mAb after incubation in these formulations although in formulation 4 the potency was slightly elevated and in formulation 10 slightly reduced.

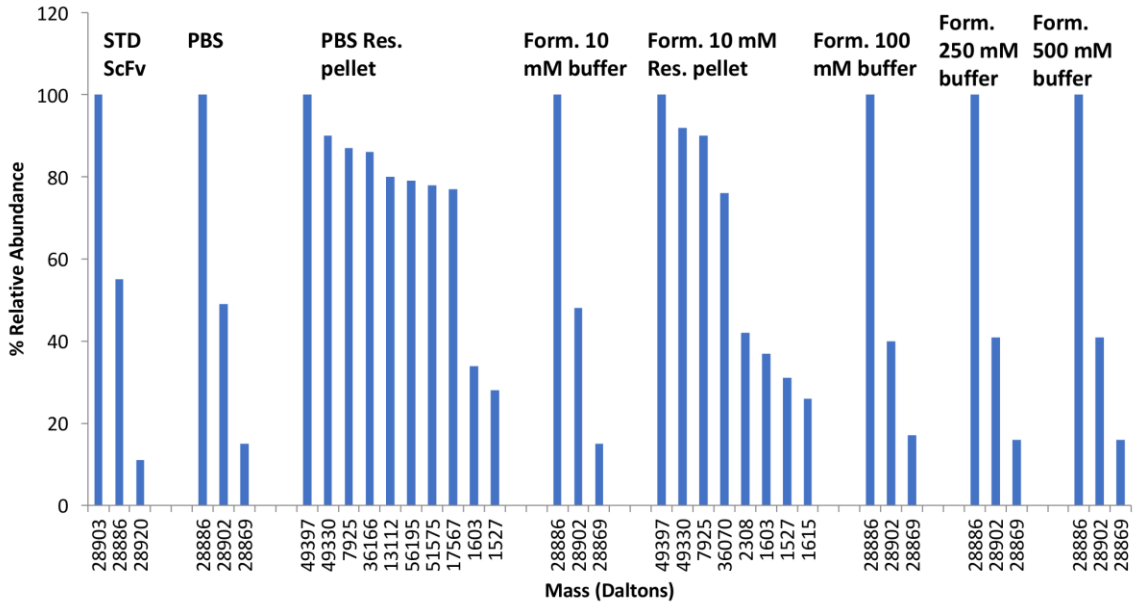


Figure 7: Mass analysis of ScFv samples after incubation in PBS and 100 mM, 250 mM and 500 mM buffer concentration formulations (Res = resolubilized pellet). The figure shows the abundance of each peak as a percentage of the most abundant peak which was assigned a value of 100%.

Supplementary Table S1. Variables investigated and the low/high amounts of each during Plackett-Burman Design analysis

Variables For lysozyme	High level of variable	Low level of variable
Protein conc. (mM)	0.61 and 0.81 (mAb/ScFv)	0.070
pH	8.5*	4*
Buffer conc. (mM)	500	10
Time (h)	168	2
Temperature (°C)	55	4
Glycine (M)	0.4	0.07
NaCl (M)	0.25	0.05

*For pH 8.5 sodium phosphate buffer, for pH 4.0 trisodium citrate buffer

Supplementary Table S2. The Plackett-Burman seven variable factor design used to investigate the effects of formulation variables on protein integrity

Form. No.	Protein Conc. (mM)	pH	Buffer Conc. (mM)	Time (hrs)	Temp (°C)	Glycine (mg/ml)	NaCl (M)
1	High	High	High	High	High	High	High
2	Low	High	Low	High	High	High	Low
3	Low	Low	High	Low	High	High	High
4	High	Low	Low	High	Low	High	High
5	Low	High	Low	Low	High	Low	High
6	Low	Low	High	Low	Low	High	Low
7	Low	Low	Low	High	Low	Low	High
8	High	Low	Low	Low	High	Low	Low
9	High	High	Low	Low	Low	High	Low
10	High	High	High	Low	Low	Low	High
11	Low	High	High	High	Low	Low	Low
12	High	Low	High	High	High	Low	Low

Supplementary Table S3: Determination of the model mAb concentration in solution upon formulation and after incubation in formulations 1-12 by A280 nm measurement. The percentage in solution after incubation was calculated by comparison to the pre-incubation value.

Formulation	[mAb] before incubation (mg/ml)	[mAb] after incubation (mg/ml)	Standard deviation (n=3)	% [mAb] in solution after incubation	% [mAb] precipitated after incubation
STD high*	116.00	116.00	0.00	100	0
1 (high)	111.11	2.05	0.01	2	98
2 (low)	9.80	7.92	0.52	81	19
3 (low)	9.72	0.29	0.02	3	97
4 (high)	110.83	105.89	1.57	96	4
5 (low)	10.97	11.13	0.17	101	-1
6 (low)	10.95	10.85	0.11	99	1
7 (low)	10.00	9.87	0.20	99	1
8 (high)	110.83	0.46	0.15	0	100
9 (high)	107.69	104.27	0.75	97	3
10 (high)	110.26	109.40	1.73	99	1
11 (low)	10.17	10.14	0.06	100	0
12 (high)	60.11	0.53	0.46	1	99

***Standard (STD) refers to standard formulation in supplied antibody.**

Supplementary Table S4: Statistical analysis of the effect of formulation variables on mAb loss (precipitation) as determined by A_{280} analysis. The statistical significance of changes in protein concentration (mg/ml) and % change amounts relative to a PBS control for each variable are shown upon formulation and after incubation.

Significance of any change in soluble protein levels					
Formulation variable		**Upon Formulation**		**After Incubation**	
		mg/ml	**%**	**mg/ml**	**%**
Protein conc. (mg/ml)	**P**	0.496	0.431	0.086	0.271
	d.o.f	5	5	5	9
	% sign.	50.4	56.9	**91.4**	**72.9**
Buffer comp. (mM)	**p**	0.375	0.404	0.724	0.321
	d.o.f	5	5	9	9
	% sign.	62.5	59.6	27.6	67.9
pH	**p**	0.377	0.375	0.673	0.286
	D.F.	5	5	9	9
	% sign.	62.3	62.5	32.7	**71.4**
Temp. (°C)	**p**	-	-	0.082	0.017
	d.o.f	-	-	5	5
	% sign.	-	-	**91.8**	**98.3**
Time (hours)	**p**	-	-	0.757	0.899
	d.o.f	-	-	9	9
	% sign.	-	-	24.3	10.1
Glycine (mg/ml)	**p**	0.375	0.461	0.804	0.894
	d.o.f	5	5	9	9
	% sign.	62.5	53.9	19.6	10.6
NaCl (M)	**p**	0.324	0.367	0.759	0.901
	d.o.f	5	5	9	9
	% sign.	67.6	63.3	24.1	9.9

Key: d.o.f. = degrees of freedom, % sign. = level of statistical significance, P= p value from two-tailed student t-test.