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LYOPHILISATION OF HIGH CONCENTRATION PROTEIN FORMULATIONS

Thesis presented by

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for the degree of

Doctor of Philosophy

Unversity College Cork

School of Pharmacy

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a Mario Guarasci, un secondo padre per me,
ti prometto che saremo felici...
ti dedico questa tesi e l'infinità dei paesaggi Irlandesi
che tanto avresti voluto visitare.

a mia Nonna Ida, punto di riferimento e
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Table of Contents

Declaration ..... **i**
Acknowledgements ..... **ii**
List of Publications ..... **vi**
List of Figures ..... **viii**
List of Tables ..... **xiv**
List of Equations ..... **xvi**
List of Abbreviations ..... **xvii**
Abstract ..... **xx**
Chapter 1: General Introduction **1**
1.1. Background 2
1.1.1 Therapeutic proteins 2
1.1.1.1 Proteins: structure and stability 2
1.1.1.2 Antibodies: structure and stability 4
1.1.1.3 High concentration protein formulations 7
1.1.1.4 Parenteral protein formulations 8
1.1.2 Lyophilisation process 11
1.1.2.1 Freezing 14
1.1.2.2 Primary drying 17
1.1.2.3 Secondary drying 20
1.1.3 Primary packaging components 21
1.1.4 Scale-up and Technical transfer 21
1.1.5 Quality by Design in lyophilisation 22
1.1.6 Process Analytical Technology Tools 24
1.1.7 Product Critical Quality Attributes 27
1.2 Thesis hypothesis 28
1.2.1 Aim 28
1.2.2 Primary objectives 28
1.3 Graphical Thesis outline (next page) 29
Chapter 2: Parenteral protein formulations: An overview of approved products within the European Union **31**
2.1 Introduction 32
2.2 Methodology 34
2.3 Results and Discussion 37
2.3.1 Approved protein formulations 37
2.3.2 Excipients included in approved protein formulations 40

2.3.2.1 Excipient categories 40
2.3.2.2 Non-amino acids buffers 45
2.3.2.3 Amino acids buffers/stabilisers/bulking agents 48
2.3.2.4 Non-amino acids stabiliser/bulking agents 52
2.3.2.5 Surfactants 57
2.3.2.6 Preservatives 59
2.3.2.7 Tonicifiers 61
2.3.2.8 Other excipients 62
2.3.2.9 Additional considerations in excipient selection 65
2.3.3 Approved antibody products 66
2.3.3.1 Antibody concentration and relationship with the route of administration 66
2.3.3.2 Qualitative and quantitative excipient composition 70
2.4 Conclusions 80
Chapter 3: Application of a mixture DOE for the prediction of formulation critical temperatures during lyophilisation process optimisation 81
3.1 Introduction 82
3.2 Materials 85
3.3 Methods 86
3.3.1 Pre-formulation studies 86
3.3.1.1 Tg′ and crystallisation events determination of placebo solutions 86
3.3.1.2 Viscosity of BSA formulations 87
3.3.2 Formulation studies 87
3.3.2.1 Empirical models development 87
3.3.2.2 Empirical models analysis and verification 88
3.3.2.3 Fox-Flory equation 90
3.3.2.4 Preparation of BSA formulations 91
3.3.2.5 Preparation of IgG1 formulations 91
3.3.2.6 Tg′ determination 92
3.3.2.7 Tc determination 92
3.4 Results 93
3.4.1 Tg′ and crystallisation of placebo solutions 93
3.4.2 Viscosity of BSA formulations 94
3.4.3 Empirical models development 96
3.4.4 Empirical models verification 107
3.5 Discussion 110
3.6 Conclusions 117
Chapter 4: Comparison of Thermal Sensors for Product Temperature Monitoring during Lyophilisation Process Optimisation 118

4.1 Introduction 119
4.2 Materials 123
4.3 Methods 123
4.3.1 Protein formulations preparation 123
4.3.2 Lyophilisation cycles 124
4.3.3 Temperature monitoring systems positioning and design 127
4.3.4 Heat transfer coefficient (Kv) calculation 131
4.3.5 Product dry layer resistance (Rp) calculation 132
4.3.6 Lyomodelling for primary drying prediction 132
4.4 Results 134
4.4.1 Vial heat transfer coefficient (Kv) 134
4.4.2 Product dry layer resistance (Rp) 137
4.4.3 Lyomodelling tool 138
4.4.4 Comparison of IgG1 and BSA formulation optimised lyo-cycles 143
4.4.5 Aggressive lyo-cycle 144
4.5 Discussion 145
4.6 Conclusions 150
Chapter 5: Solid state and moisture characterisation of lyophilised high concentration protein formulations 152
5.1 Introduction 153
5.2 Materials 157
5.3 Methods 157
5.3.1 Lyophilisation of protein formulations 157
5.3.2 Macroscopic visual appearance 159
5.3.3 Scanning electron microscopy (SEM) 159
5.3.4 Brunauer-Emmett-Teller (BET) 160
5.3.5 Secondary drying optimisation and moisture correlation model development. 160
5.3.6 FMS-moisture/pressure headspace analyser 161
5.3.7 Karl Fischer titration (KF) 163
5.3.8 Modulated Differential Scanning Calorimetry (mDSC) 163
5.3.9 Dynamic vapour sorption (DVS) 164
5.3.10 Powder X-ray diffraction (pXRD) 164
5.4 Results 165
5.4.1 Macroscopic/Microscopic visual appearance and correlation with the BET specific surface area 165
5.4.2 Secondary drying optimisation 167
5.4.3 Solid state stability 168
5.4.3.1 Tg and moisture 168

5.4.3.2 Correlation models: Tg, product moisture and vial headspace water vapour 171
5.4.3.3 Physical solid state 172
5.5 Discussion 176
5.6 Conclusions 179
Chapter 6: Biophysical stability of high concentration antibody formulations pre and post lyophilisation 181
6.1 Introduction 182
6.2 Materials 185
6.3 Methods 185
6.3.1 IgG1 formulations preparation 185
6.3.1.1 Formulation pre-lyophilisation 185
6.3.1.2 Lyophilisation process 186
6.3.2 Reconstitution procedure 186
6.3.3 Biophysical stability 187
6.3.3.1 Circular dichroism 187
6.3.3.2 UV-visible spectroscopy 188
6.3.3.3 Size Exclusion High Performance Liquid Chromatography (SE-HPLC) 189
6.3.3.4 Dynamic Light Scattering (DLS) 189
6.3.3.5 Modulated Differential Scanning Calorimetry (mDSC) 190
6.3.4 Accelerated biophysical stability 190
6.3.4.1 Thermal stability 190
6.3.4.2 Photostability 190
6.4 Results 191
6.4.1 Biophysical stability 191
6.4.1.1 Secondary structure 192
6.4.1.2 Tertiary structure 194
6.4.1.3 Aggregation and degradation 194
6.4.1.4 Z-Average and PdI 197
6.4.1.5 Thermal unfolding 199
6.4.2 Accelerated biophysical stability 200
6.4.2.1 Thermal stability 200
6.4.2.2 Photostability 200
6.5 Discussion 201
6.6 Conclusions 206
Chapter 7: General Discussion 207
7.1 Introduction 208
7.2 Interpretation and implications of the thesis findings 208
7.2.1 Formulation selection and design 210

7.2.2 Lyophilisation process design....213
7.2.3 Other QbD approaches....215
7.2.4 Primary packaging configuration....216
7.3 Advantages and disadvantages of key aspects investigated....217
7.4 Summary of key findings....217
7.5 Limitations....219
7.6 Recommendations for future work....220
7.7 Conclusions....222
References....223
Appendix....238
Supplementary Information Chapter 2 and 3....238
Supplementary Information Chapter 4....238
Supplementary Information Chapter 5....242
Supplementary Information Chapter 6....243

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signed: Valeria Gervasi

Valeria Gervasi

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List of Publications

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Oral Presentations

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List of Figures

Figure 1.1 Scheme describing mechanism of physical protein aggregation (Revised (6)). 3

Figure 1.2 Protein structural levels (Revised (11)). 4

Figure 1.3 Schematic representation of a typical IgG structure. 5

Figure 1.4 Schematic representation of the theoretical mechanisms of interaction occurring between protein and stabilising excipients in solution (liquid) and after dehydration (lyophilised). In the liquid state, preferential hydration occurs when the protein preferentially interacts with water. In contrast, preferential interaction occurs when the excipient preferentially interacts with the protein. In the lyophilised state, once the water is removed through dehydration, the excipient interacts with the protein replacing water (water replacement) or the excipient creates a glassy matrix around the protein (vitrification). 10

Figure 1.5 Schematic representation of a Lyophiliser. 12

Figure 1.6 Diagram of the triple point of ice and water phase transitions. 13

Figure 1.7 Schematic illustration of the impact of the cooling rate on the freezing and primary drying steps. 16

Figure 1.8 Schematic illustration of heat and mass transfer processes occurring during sublimation. 18

Figure 2.1 Trend of liquid and lyophilised parenteral protein products approved per year by the EMA between 1995 and 2018 (June) (*Last updated: 18/06/ 2018). 37

Figure 2.2 Classes of therapeutic proteins included in liquid and lyophilised parenteral products. (a) Pie chart of the total number of products; (b) Pie charts showing the split between liquid and lyophilised products. *'Other' class includes types of therapeutic proteins present in a percentage of commercial products <3% (analgesic peptide, antiangiogenic agent, anticoagulant, antiplatelet, antithrombin/thrombolytic, growth factor, HIV infusion inhibitor, muramyl peptide derivative, toxin, calcimimetic peptide). 39

Figure 2.3 The percentage of liquid and lyophilised parenteral protein products that contain excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/bulking agents); Surfactants; Preservatives; Tonicifiers and Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products *'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations. 40

Figure 2.4 The percentage of approved liquid and lyophilised parenteral protein products containing individual non-amino acids buffers. 46

Figure 2.5 The percentage of approved liquid and lyophilised parenteral protein products containing individual amino acids buffers/stabilisers/bulking agents. Note: The function of these excipients as bulking agents is only relevant for lyophilised products. 49

Figure 2.6 The percentage of approved liquid and lyophilised parenteral protein products containing individual non-amino acids stabilisers/bulking agents. Note: The function of these excipients as bulking agents is only relevant for lyophilised products. 52

Figure 2.7 Trend of liquid and lyophilised parenteral antibody products approved per year by the EMA between 1995 and 2018 (June) (*Last updated: 18/06/ 2018). 67

Figure 2.8 Range of therapeutic concentrations (mg/mL) of liquid and lyophilised parenteral antibody products. *for lyophilised formulations intended after reconstitution. 68

Figure 2.9 Amount of antibody per vial (mg/vial) for lyophilised parenteral antibody products. 69

Figure 2.10 Relationship between ranges of antibody concentrations (mg/mL) and route of administration. *for lyophilised formulations intended after reconstitution.....70

Figure 2.11 The percentage of liquid and lyophilised parenteral antibody products that contain excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/bulking agents); Surfactants; Preservatives; Tonicifiers and Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products *'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations. 71

Figure 2.12 The percentage of liquid and lyophilised parenteral antibody products that contain individual excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/ bulking agents); (SURF) Surfactants; (T) Tonicifiers; (OE*) Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products * (OE) 'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations. 73

Figure 3.1 Plasticising effect of arginine and HCl counter ion on the Tg′ of sucrose solutions. Arginine and HCl counter ion both have a significant plasticising effect on the Tg′ of placebo formulations with varied sucrose:arginine ratios and a fixed total solute concentration (10% w/v) (n=3).....93

Figure 3.2 Viscosity of BSA formulations. (a) Effect of protein concentration on viscosity for formulations with fixed Protein: Sucrose: Arg/Arg-HCl ratio (P1:S0.8:A0.2) and increased protein concentration (5–40% w/v); (b) Effect of Arg/Arg-HCl on viscosity for formulations with fixed protein (10% w/v) and total solute concentration (20% w/v), increased Arg/Arg-HCl concentration (0–8% w/v) and reduced sucrose concentration (10–2% w/v); (c) Effect of solute concentration (10%, 15%, 20% w/v) for formulations with fixed protein concentration and increased total solute concentration with and without Arg/Arg-HCl in ratios (P1:S0.8:A0.2 or P1:S1) (n=2). 95

Figure 3.3 Examples of the limitations observed in determining Tg′ of high concentration protein formulations. The effect of protein concentration on the overlapping of Tg′ and melting of the protein depressed by the presence of excipients. Tg′ of formulations with different protein concentration 2–15% (w/v) are shown: Formulation A: BSA 2% (w/v), Sucrose 6.5% (w/v), Arg/Arg-HCl 1.5% (w/ v); Formulation B: BSA 5% (w/v), Sucrose 12% (w/ v); Arg/Arg-HCl 3% (w/v); Formulation C: BSA 10% (w/v), Sucrose 8% (w/v), Arg/Arg-HCl 2% (w/v); Formulation D: BSA 12.5% (w/v), Sucrose 5.3% (w/v), Arg/Arg-HCl 2.3% (w/v), Formulation E: BSA 15% (w/v), Sucrose 3% (w/v), Arg/Arg-HCl 2% (w/v). 99

Figure 3.4 Representative FDM image of the collapse events observed for high concentration protein formulations. (1) Drying front; (2) Onset collapse (Toc), (3) Microcollapse (Tmc); (4) Full collapse (Tfc). Drying rate of 20°C/min was selected in order to show all the events occurring in a single image, but no quantitative information of the real temperature was extrapolated from this picture. 100

Figure 3.5 Effect of arginine/arginine-HCl and protein concentration on the collapse behaviour of high concentration protein formulations. (a) Formulation A: BSA 5% (w/v), Sucrose 5% (w/v); (b) Formulation B: BSA 5% (w/v), Sucrose 12% (w/v), Arg/Arg-HCl 3% (w/v); (c) Formulation C: BSA 10% (w/v), Sucrose 10% (w/v); 5d) Formulation D: BSA 2% (w/v), Sucrose 6.4% (w/v), Arg/Arg-HCl 1.6% (w/v). T_{oc} (yellow), T_{fc} (orange/red). 101

Figure 3.6 Cox response plots of the empirical models generated for critical temperatures prediction. (a) 10%(w/v) total solute concentration, (b) 15%(w/v) total solute concentration; (c)

20%(w/v) total solute concentration. Cox response plots show the effect of formulation components on the response Tg′. Cox response plots for the response Toc are reported in the supplementary information since they follow the same trend as for the response Tg′. 104

Figure 3.7 Plots of the empirical models generated by mixture DOEs for critical temperatures prediction and optimisation. (a–c) mixture contour plots; (d-f) contour plots of the overlaid responses for formulations with different total solute concentrations: 10% (w/v), 15% (w/v) and 20% (w/v). Mixture contour plots show black dots which indicate formulations provided by the software to build the design space, black lines to identify constraints used in the design and coloured bands to specify the formulation regions at specific Tg′ intervals; Contour plots of overlaid responses (Tg′ and Tc) identify the region selected by performing a response optimisation. Mixture contour plots for the response Toc are reported in the supplementary information since they follow the same trend as for the response Tg′. 106

Figure 3.8 Critical temperatures of 11 formulations selected. Formulations are ordered by ascending values of protein concentration and total solute concentration. Tfc (°C), Toc (°C) and Tg′ (°C) are reported for the 11 formulations selected and used to verify the empirical models (n=2). 107

Figure 3.9 The effect of the type of protein (BSA and IgG1) on the critical temperatures of high concentration protein formulations. Critical temperatures of equivalent formulations with different type of protein are not significantly different (Two-way ANOVA, Tukey's multiple comparison, n=3). Formulations contain 10% w/v protein (IgG1 or BSA), S=Sucrose, A=Arg/Arg-HCl. Difference in the Mean of Tg′ and Toc for formulations showed in the figure ranges between 2.88°C and 4.81°C. 108

Figure 3.10 Verification of the three DOEs and the Fox-Flory models by using BSA and IgG1 formulations. (a) Tg' and (b) Toc for DOE 10%-Low, n=8 observations (BSA); (c) Tg′ and (d) Toc for DOE 15%-Low, n=4 observations (BSA); (e) Tg′ and (f) Toc for DOE 20%-High, n=22 observations (n=13 BSA and n=9 IgG1). Black dots represent values of critical temperatures for formulations used to build the empirical models. Black line is the fit of the empirical regression models generated. Coloured dots represent critical temperatures of formulations used to verify the empirical models. Grey triangles and line represent the Fox-Flory model. 109

Figure 4.1 Example of 5 mL vials layout on shelf used in the lyo-cycles. Each numbered rectangle represents the area occupied by each vial. Empty vials and Ellab shuttles are indicated in white and black, respectively. Probed vials were 4 per temperature monitoring system: Red – Thin wire thermocouples, Blue - Ellab RTDs, Green – Tempris. Probed vials were distributed and scattered at the centre of the vial pack. 125

Figure 4.2 Pictures of the three different temperature monitoring systems used to determine product temperature. 4.2a) A-red) Thin Wire Thermocouple (TWTC); B-blue) Resistance temperature detectors RTDs, Ellab®; C-green) Temperature Interrogation Systems, Tempris®. Stoppers, holder devices and positioning of the systems are also shown in the figure. 4.2b) Shuttle device used to position Ellab RTDs on the tray and in the vial pack; 4.2c) Set-up of the Temperature Interrogation System (Tempris®). 128

Figure 4.3 Product temperature profiles of the sublimation phase for lyo-cycle conducted to determine Kv (5mL vials). Profiles display the difference in the water temperature reading during sublimation monitored using different temperature monitoring systems: (A) Thin wire thermocouples (TWTC)- red (n=8), (B) Ellab, RTDs- blue (n=12), and (C) Tempris- green (n=8). Max □T= 3 °C (TWTC vs Tempris). 134

Figure 4.4 Calculated Kv values determined using the three different temperature monitoring systems: TWTC (n=8), Ellab, RTDs (n=12) and Tempris (n=8). Comparison of the different Kv calculated for vials located at the centre, at the edge and on the first row close to Ellab shuttle 1 (front) and Ellab shuttle2 (back). One-way ANOVA, Tukey's post-hoc test, **** P< 0.0001. Y-error bars indicate standard deviation. 135

Figure 4.5 Heat map of the Kv values calculated relative to vial location on the shelf. Comparison of the Kv values calculated using temperature data acquired with the three different temperature monitoring systems. The heat map is a schematic representation, the rectangular shape does not account for the actual alternate position of the vials on the shelf. 136

Figure 4.6 Product dry layer resistance (Rp) determined for the 20% (w/v) total solute concentration material using the different temperature monitoring systems: TWTC (red) n=8, Ellab RTDs (blue) n=8 and Tempris (green) n=8. Lyo-cycle for Rp determination was conducted below T_{oc} of the two selected formulations (at a T_{shelf} of -25 °C). P:S indicates formulation with 10% protein, 10% sucrose; P:S:A indicates formulation with protein 10%, sucrose 8% and arg/arg-HCl 2%. Y- error bars indicate standard deviation. 137

Figure 4.7 Measured and predicted primary drying time for lyo-cycles conducted at a shelf temperature of -25 °C, -20 °C and -15 °C, respectively. Primary drying time prediction was conducted with the aid of a lyomodelling tool and is expressed by the symbols. The lyomodelling tool was informed with Kv values calculated using the different temperature monitoring systems. End of primary drying was measured from the different lyotraces: (1) when Pirani gauge was halfway to approaching the capacitance manometer (CM) reading, (2) when Pirani gauge reading was equivalent to be CM and (3) when product temperature monitored using the different systems was equal to the shelf temperature: TWTC (red, n=8), Ellab RTDs (blue, n=8), Tempris (green, n=8), Mean±SD. The secondary Y-axis shows the primary time reduction (%) for lyo-cycles conducted at increased shelf temperatures (-25 °C, -20 °C and -15 °C). 139

Figure 4.8 Measured and predicted product temperature over the primary drying conducted at three shelf temperatures (-25 °C, -20 °C and -15°C). For each cycle, product temperature was measured during the primary drying step with the use of the different temperature monitoring systems: TWTC (red, n=8), Ellab, RTDs (blue, n=8) and Tempris (green, n=8). Product temperature was determined: (1) at the primary drying steady state, (2) when Pirani is halfway reaching the CM (Pirani halfway), (3) when Pirani equalise CM (Pirani=CM). Predicted product temperature over primary drying is expressed using symbols. Predicted product temperature was estimated by the lyomodelling tool employing Kv values determined using temperature data from the different temperature monitoring systems. Two-way ANOVA $p<0.0001$, Tukey's multiple comparison. Toc1 (black dashed line) is the collapse temperature of formulation P:S containing protein 10% w/v and sucrose 10 % w/v, Toc2 (grey dashed line) is the collapse temperature of formulation P:S:A containing protein 10 % w/v, sucrose 8 % w/v and arg/arg-HCl 2% w/v. ..140

Figure 4.9 Primary drying time and maximum product temperature for the lyo-cycles conducted at three shelf temperatures (Tshelf= -25 °C, -20 °C and -15 °C). The lyomodelling prediction tool was informed with Kv values calculated using the three temperature monitoring systems. Product temperature was measured using: TWTC- red (n=8), Ellab, RTDs- blue (n=8) and Tempris- green (n=8). Symbols indicate predicted end of primary drying expressed as % of water removed over the primary drying step (purple), and predicted product temperature profiles over the primary drying step (red- TWTC, blue- Ellab, RTDs, green- Tempris).....142

Figure 4.10 Lyotrace of the optimised lyo-cycle (lyo-cycle C) conducted at a shelf temperature of -15 °C for IgG1 and BSA formulations. TWTC (n=4) per type of protein were used to monitor product temperature over the process. Formulations composition is indicated as P:S (protein: sucrose, line) and P:S:A (protein: sucrose: arg/arg-HCl, dashed line) where protein can be IgG1 or BSA. Green- IgG1 formulations, red- BSA formulations. 143

Figure 4.11 Lyotrace of the aggressive lyo-cycle (lyo-cycle D) conducted at a shelf temperature of 35°C during primary drying. Product temperature was monitored over the process using TWTC (n=4). Formulations composition is indicated as P:S (protein: sucrose, line) and P:S:A (protein: sucrose: arg/arg-HCl, dashed line) where protein is BSA. 145

Figure 5.1 Pressure detected in the vials headspace after backfilling and used to determine container closure integrity of vials intended for stability studies. Stability studies were conducted at two storage conditions 2-8 °C and 25°C (RH 60%). The total number of vials analysed in the

study design was 104; 52 vials per storage condition. Vials containing both formulations P:S and P:S:A with either BSA or IgG1 protein were stored at each storage condition (21 BSA-P:S vials, 5 IgG1-P:S vials, 21 BSA-P:S:A vials, 5 IgG1-P:S:A vials).....162

Figure 5.2 Relationship between visual appearance and specific surface area of selected lyophilised high concentration protein (BSA) formulations with and without arg/arg-HCl. Macroscopic (A) and microscopic cake appearance (B & C) were assessed by visual inspection and SEM, respectively. SEM images were taken at two magnifications: B) 50 μm, C) 200 μm. Specific surface area was determined by BET analysis, n=3 samples per formulation and freezing rate protocol were analysed, (Two-way ANOVA, Sidak's multiple comparison test, *p<0.05). 166

Figure 5.3 Secondary drying endpoint determination conducted with the aid of a 'samples thief' tool. Vials (n=5) were stoppered at each time point over the secondary drying step. Three vials were removed, and two vials were stoppered and left in the lyo chamber at each time point while secondary drying proceeded. Tg was determined by mDSC and product residual moisture by Karl Fischer titration (KF). Black line indicates the shelf temperature (Tshelf °C), blue line indicates a general upper limit of residual moisture for a product at the end of secondary drying, green line indicates the conventional room temperature (20 °C).....168

Figure 5.4 Tg, vial headspace water vapour pressure and product residual moisture of selected high concentration protein formulations upon stability at two temperature conditions (2-8 °C and 25 °C, RH 60%). Hashed bars indicate P:S formulations, solid bars indicate P:S:A formulations. Two-way ANOVA, Sidak's multiple comparisons test **** p< 0.0001, ** p< 0.001 and * p< 0.05.....170

Figure 5.5 Correlation models built between vial headspace water vapour pressure vs product residual moisture, and between glass transition temperature (Tg) vs product residual moisture. The model was used to monitor decrease in Tg and increase in residual moisture over storage for 6 months. WVP indicates water vapour pressure. RM indicates residual moisture.....172

Figure 5.6 pXRD diffractograms showing the physical solid state of the lyophilised formulations a) during vials storage (6 months, 2-8 °C and 25 °C, RH 60%), b) after exposure of the lyophilised material to elevated relative humidity (RH) conditions (50, 60 and 70%) in DVS and c) reference crystalline excipients as supplied compared to representative lyophilised samples. P:S indicates formulation containing protein 10%, sucrose 10%; P:S:A indicates formulation containing protein 10%, sucrose 8%, arg/arg-HCl 2%. 173

Figure 5.7 Sorption behaviour of selected formulations containing sucrose and arg/arg-HCL (P:S:A) after removal form the vial and exposure to a range of relative humidity conditions (50-90%, at 25 °C) in DVS, A) Visual appearance of the lyophilised material after exposure to the range of relative humidity in DVS, B) Water vapour sorption isotherm of the lyophilised material after exposure to the range of relative humidity in DVS, C) pXRD graphs of the lyophilised material after exposure to the range of relative humidity in DVS. NA indicates that pXRD analysis was not possible to the liquid nature of the material exposed to high residual humidity (RH) at 80% & 90%). 175

Figure 6.1 Reconstitution time of selected lyophilised high concentration IgG1 formulations. Unpaired t-test (n=4), no significant difference.....192

Figure 6.2 Far-UV CD spectra of the IgG1 formulations (with and without arg/arg-HCl) pre and post lyophilisation and stored at two temperature conditions (2-8 °C) and (25 °C, RH 60%). .193

Figure 6.3 Near-UV CD spectra of the IgG1 formulations (with and without arg/arg-HCl) pre and post lyophilisation and stored at two temperature conditions (2-8 °C) and (25 °C, RH 60%). .194

Figure 6.4 Monomer area (%) of selected IgG formulations (IgG10:S10 and IgG10:S8:A2) determined by SE-HPLC pre formulation, pre and post lyophilisation and after storage at 2-8 °C

and 25 °C, 60% RH. Two way ANOVA, Sidak's multiple comparison (95% CI, not significant different). 196

Figure 6.5 Z-average and PdI of selected IgG formulations measured pre and post lyophilisation and after storage at two temperature conditions (2-8 °C and 25 °C, RH 60%). Two-way ANOVA test, Tukey's multiple comparison p<0.0001 (****). 198

Figure 6.6 Thermal unfolding of the two formulations (IgG10:S10 and IgG10:S8:A2) pre and post lyophilisation at T0 (Heat Capacity (J/g×°C)- line, Heat Flow (W/g)- dotted line). 199

Figure 6.7 Effect of temperature on the tertiary structure of the IgG1 formulated at high concentrations. Protein concentration in formulation is 100 mg/mL, protein concentration during the analysis is 50 mg/mL. Formulation analysed is formulation IgG10:S8:A2. 200

Figure 6.8 Aggregation profiles of selected formulations in presence and absence of arg/arg-HCl measured by SE-HPLC after exposure to high intensity light (photostability cabinet). HMWS indicates high molecular weight species in %. 201

Figure 7.1 Flow diagram showing the workflow of the thesis incorporating the Quality by Design approaches (QbD) applied to each step. 209

List of Tables

Table 1.1 Summary of the excipient categories included in liquid and lyophilised protein formulations. 9

Table 1.2 Summary of the PAT tools used to monitor critical process parameters during the primary drying step of the lyophilisation process. 25

Table 1.3 General product CQAs tested for lyophilised drug products (110). 27

Table 2.1 More common individual excipients classified into functional categories and listed by descending frequency of use in liquid and lyophilised parenteral protein products (percentage and number of formulations containing each excipient and range of excipient concentrations included in approved products). 43

Table 2.2 Examples of other excipients with specific functions included in liquid and lyophilised parenteral protein products. 63

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration (124-126, 130). (Continue to next page) 74

Table 3.1 DOE name, formulation constraints applied and number of runs for each DOE set. 88

Table 3.2 Composition of the protein formulations used to verify the empirical models for each DOE set. 90

Table 3.3 Predicted and measured critical temperatures of formulations used to develop the empirical model for each DOE set. 96

Table 3.4 Summary of the statistical parameters required to assess the suitability of the empirical models generated from the different DOE sets. 102

Table 3.5 Regression coefficients of the three formulation components in amounts. 103

Table 4.1 Formulation composition, glass transition of the maximally freeze concentrated solution (Tg') and onset collapse temperature (Toc) (Mean ± SD, n=3) of selected formulations. 124

Table 4.2 Lyophilisation process parameters for lyo-cycles performed A-D. 126

Table 4.3 Summary of the three temperature monitoring systems key features (63, 64, 256, 257). 129

Table 4.4 Overview of the product temperature and primary drying time for the optimised lyo-cycle (lyo-cycle C) performed at a shelf temperature of -15 °C for equivalent protein formulations containing BSA or IgG1 (Mean±SD). 144

Table 5.1 Lyophilisation cycle parameters used to lyophilise selected high concentration protein formulations. 158

Table 5.2 Number of lyophilised product vials showing cracking defects and containing either BSA or IgG1 in presence and absence of arg/arg-HCl. 165

Table 5.3 Features of the model used to correlate vials headspace moisture, Tg and product residual moisture. 171

Table 6.1 IgG1 formulations composition.....186

Table 6.2 Lyophilisation cycle parameters used to lyophilise the high concentration IgG1 formulations.....186

Table 6.3 Methods selected to determine biophysical stability of the IgG formulations (pre and post lyophilisation) and listed by ascending order of protein concentration used for the analysis. 187

Table 6.4 CDDN-secondary structure of the IgG1 193

Table 6.5 Absorbance at 350 nm and aggregation index (n=3). 195

Table 7.1 Summary of advantages and disadvantages of key aspects studied in the thesis. 217

List of Equations

Equation 1.1: Kv contributors.....18

Equation 3.1: Polynomial equation for building the regression model.....88
Equation 3.2: Fox-Flory.....90

Equation 4.1: Kv determination.....131
Equation 4.2: Rp determination.....132
Equation 4.3: Primary drying time determination.....133

Equation 5.1: BET surface area equation.....160

Equation 6.1: Aggregation Index.....188

List of Abbreviations

Abs	Absorbance
ADC	Antibody-drug conjugate
Anti-IL8	Anti-interleukin 8
API	Active Pharmaceutical Ingredient
Arg/arg-HCl	Arginine/Arginine-Hydrochloride
BET	Brunauer-Emmett-Teller
BSA	Bovin Serum Albumin
C	Constant region (antibody)
CDR	Complementarity-determining region (Mab region)
CHO	Chinese hamster ovary (cells)
CM	Capacitance Manometer
CMC	Critical Micelle Concentration
CPP	Critical Process Parameter
CQAs	Critical Quality Attributes
DLS	Dynamic Light Scattering
DMA	Dynamic Mechanic Analysis
DOE	Design of Experiment
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
DVS	Dynamic Vapour Sorption
EDTA	Edetic acid or edetate salt
EI	Elctrical Impedence
EMA	European Medicine Agency
EPAR	European Public Assessment Report
Fab	Antigen binding fragment (antibody)
Fc	Crystallizable fragment (antibody)
FDA	Food and Drug Administration
FDM	Freeze Drying Microscopy
FMS	Frequency Modulation Spectroscopy
GSSG	Glutathione disulphide
H	Heavy chains (antibody)

HAMA	Human anti-mouse antibodies
H-bonds	Hydrogen bonds
HCl	Hydrochloric Acid
ICH	International Conference of Harmonization
IgG	Immunogobulin
IRU	Interrogation Unit
IV	Intravenous
k	Kappa region (antibody)
Kc	Conductive heat transfer
KCl	Potassium chloride
kDa	Kilodalton
KF	Karl Fischer
Kg	Convective heat transfer
Kg	Kilogram
Kr	Radiative heat transfer
Kv	Heat transfer coefficient
L	Liquid (Dosage form)
λ	Lambda region (antibody)
L	Light chains (antibody)
LYO	Lyophilised (Dosage form)
Lyo-cycle	Lyophilisation cycle
Mab	Monoclonal antibody
mg	milligram
mL	Milliliters
mM	Millimolar
mOsm	Milliosmolal
MTM	Manometric Temperature Measurement
Mw	Molecular weight
NaCl	Sodium Chloride
NIBRT	National Institute for Bioprocessing Research and Training
OCT-FDM	Optical Coherence Tomography-FDM
PAT	Process Analytical Technology
PdI	Polydispersity Index
pI	Isoelectric point

pXRD	X-ray Powder Diffraction
q	Kwei factor (for intermolecular interactions)
QbD	Quality by Design
RH	Relative Humidity
rHuPH20	Recombinant human hyaluronidase
Rp	Product dry layer resistance to the water vapour
RTD	Resistance Temperature Detector
SC	Subcutaneous
SDS-PAGE	SodiumDodecylSulphate-PolyAcrilamide Gel Electrophoresis
SE-HPLC	Size Exclusion High Peformance Liquid Chromatography
SEM	Scanning Electron Microscopy
TASC	Thermal Analysis by Surface Characterization (TASC)
Tc	Collapse temperature
TDLAS	Tunable Diode Laser Absorption Spectroscopy
Teu	Eutectic temperature
T_{fc}	Full collapse temperature
Tg	Glass transition temperature of the solid material
Tg'	Glass transition of the maximally freeze concentrated solution
Tm	Thermal unfolding
T_{mc}	Microcollapse temperature
Tm_{onset}	Onset thermal unfolding
T_{oc}	Onset collapse temperature
Tp	Product temperature
T_{shelf}	Shelf temperature
TVIS	Through Vial Impedance Spectroscopy
TWTC	Thin Wire Thermocouples
V	Variable region (antibody)

Abstract

Antibodies form the main class of commercial therapeutic proteins and are intended for the treatment of several chronic diseases. The current trend is to formulate antibodies at high concentrations in order to deliver a large therapeutic dose in small volumes (1-2 mL) subcutaneously (SC). Thus, enabling patients to self-administer these medications at home rather than in a hospital setting through intravenous (IV) infusions. However, several challenges can be encountered when formulating proteins at high concentrations. In the liquid state, high concentration protein formulations are prone to aggregation and exhibit high viscosities. In the lyophilised state, they show high total solute concentrations and product dry layer resistance, that can prolong primary drying, increase overall process time, costs of manufacturing and extend reconstitution time.

The overall aim of this thesis was to obtain a stable, lyophilised, high concentration antibody (immunoglobulin 1, IgG1) formulation via rational selection of the excipients (type and ratio) and optimisation of the lyophilisation process with the aid of Quality by Design (QbD) approaches.

Formulations selected include BSA as model protein and an IgG1 as the target protein. Arginine/arginine-HCl (arg/arg-HCl) and sucrose were selected as excipients as a result of an overview of the composition of liquid and lyophilised protein products approved in the European Union since 1995. Polysorbate 80 was also employed as a surfactant at a fixed concentration. The ratio of protein:excipients relative to the total solute concentration was determined with the aid of a mixture Design of Experiment (DOE) tool. The DOE was used to generate empirical models for critical temperatures optimisation and prediction. The lyophilisation cycle optimisation was conducted using a lyomodelling tool for primary drying prediction and the process was monitored using a

range of temperature monitoring systems. CQAs of the optimised lyophilised formulations (glass transition temperature (Tg), residual moisture, product appearance, reconstitution time and biophysical stability) were assessed and stability was monitored over a six month period in the presence and absence of cold chain storage.

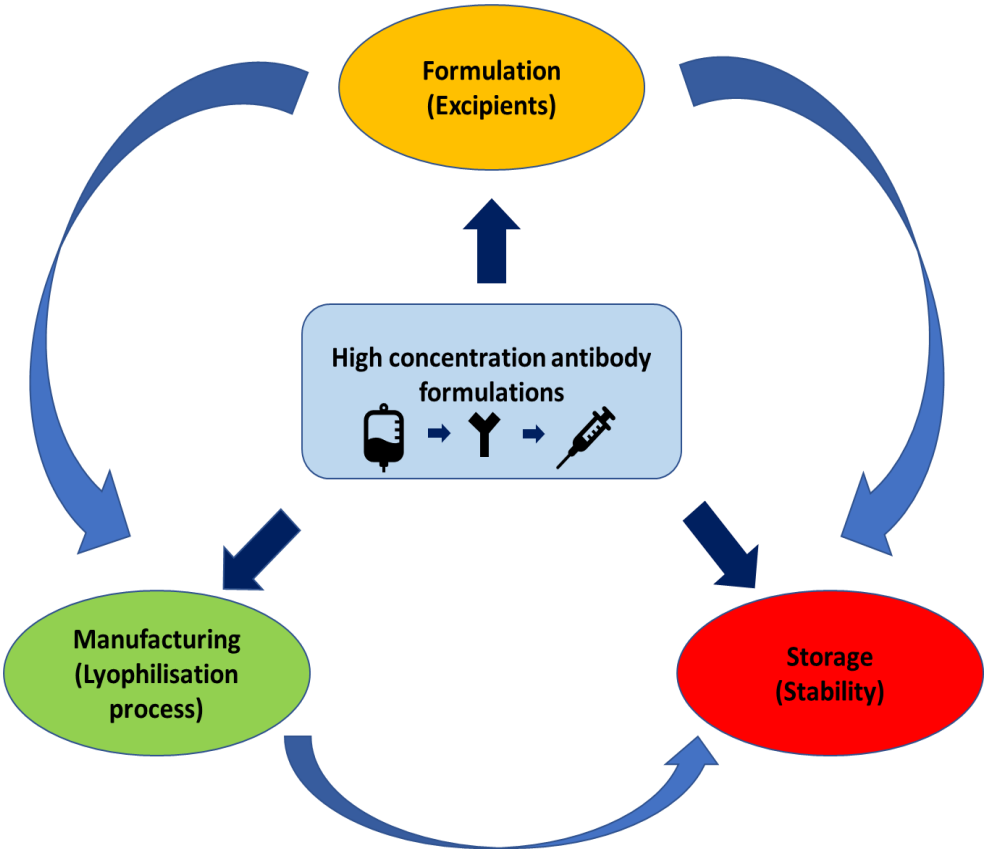
The empirical model generated from the DOE provided formulations containing 100 mg/mL of protein with maximised critical temperatures. The empirical model was also capable of accurately predicting both critical temperatures (glass transition of the maximally freeze concentrated solution, Tg' and collapse temperature, Tc) within the design space. The lyophilisation process was successfully optimised. A significant reduction in primary drying time (45%, -11h) was obtained when shelf temperature (T_{shelf}) was increased from -25°C to -15 °C. Additionally, an aggressive lyo-cycle conducted at a T_{shelf} of 35 °C provided an extremely short primary drying time (4.75h). The lyophilised products obtained by this lyo-cycle did not show any collapse and had similar Tg and residual moisture to the products lyophilised at conservative conditions (95-100 °C and 0.1-0.3% respectively).

The high Tg renders these products good candidates for the elimination of cold chain during storage. Formulations selected were amorphous and maintained their physical solid state over six months storage and exposure to high relative humidity (up to 70%) conditions. The high concentration of the protein was capable of inhibiting excipient recrystallisation, providing a stable amorphous product. A non-destructive technique was used to determine the vial headspace residual moisture which correlated with product moisture and Tg. A correlation model was developed to estimate product moisture and Tg by performing a non-destructive technique and retaining the samples at each time point of the stability programme.

The lyophilised formulations were biophysically stable over six months in the presence and absence of cold chain. Aggregation was not detected. The challenge of prolonged reconstitution times for high concentration protein formulations was overcome by lyophilising the product at a low fill volume (1.1 mL). Finally, the addition of arg/arg-HCl to the lyophilised formulations showed some advantages; reducing the specific surface area and improving the product visual appearance of lyophilised formulations. Furthermore, arg/arg-HCl provided a stabilising effect, reducing protein aggregation in formulations exposed to high intensity light.

This study provides a rational approach, insights and strategies that can be applied to overcome some of the challenges encountered during formulation and manufacturing of lyophilised high concentration protein formulations. The use of QbD approaches aids the development of stable, lyophilised, high concentration antibody formulations. The formulation strategy identified allows reduction of the manufacturing time and enables storage of these formulations at ambient conditions rending the development of lyophilised high concentration protein formulations more cost effective.

Chapter 1: General Introduction



1.1. Background

1.1.1 Therapeutic proteins

Therapeutic proteins are an emerging class of drugs utilised for the treatment of several chronic diseases such as diabetes, end stage renal disease, viral hepatitis, cancer, neutropenia, clotting disorders and inborn errors of metabolism (1-3). Therapeutic proteins included in commercial parenteral products are mainly antibodies, hormones, enzymes, cytokines, coagulation factors and fusion proteins, which are formulated in the liquid or solid formats (3-5).

1.1.1.1 Proteins: structure and stability

Proteins are large molecules, consisting of one or more chains of amino acids (polypeptides) that once folded in a specific three-dimensional structure provide a specific activity or function. The shape in which a protein naturally folds is defined as its native conformation, on the contrary the loss of the protein native conformation is defined as unfolding (6). The conversion of a protein from a folded to an unfolded state occurs through an intermediate or transition state (**Figure 1.1**). At this stage, the protein has a higher tendency to aggregate forming reversible or irreversible aggregates. The reversibility of protein aggregation is generally associated to the stage of the aggregation process (6, 7). The initial formation of soluble aggregates can be reversible, however, once the aggregates exceed certain sizes and solubility limits at a later stage they become irreversible, unless aggregation is artificially induced (6, 7). The conversion of the native protein to the initial aggregate is defined as nucleation. Higher hydrophobicity of the protein is generally associated with higher tendency to aggregation (6, 8).

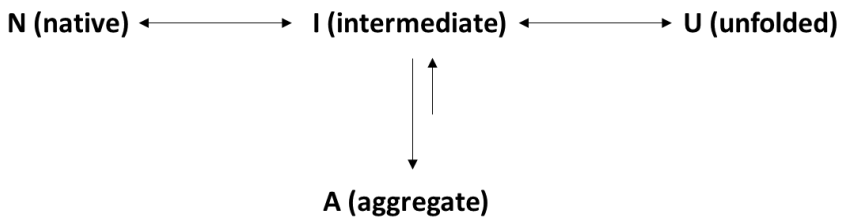


Figure 1.1 Scheme describing mechanism of physical protein aggregation (Revised (6)).

Proteins can be organised in different structural levels: a) Primary structure – linear amino acid sequence which specify the final protein native structure (peptide bonds); b) Secondary structure – first step of the folding process that a protein takes to assume its native structure (through intramolecular hydrogen bonds). The most common secondary structure elements include: α-helix, β-sheet, β-turns and random coils; c) Tertiary structure – protein is folded in its three-dimensional structure (through hydrophobic interactions and covalent bonds e.g. disulphide bridges); d) Quaternary structure – folded subunits of a protein or multiple polypeptides that once assembled form a fully functional protein (6, 9, 10) (**Figure 1.2**).

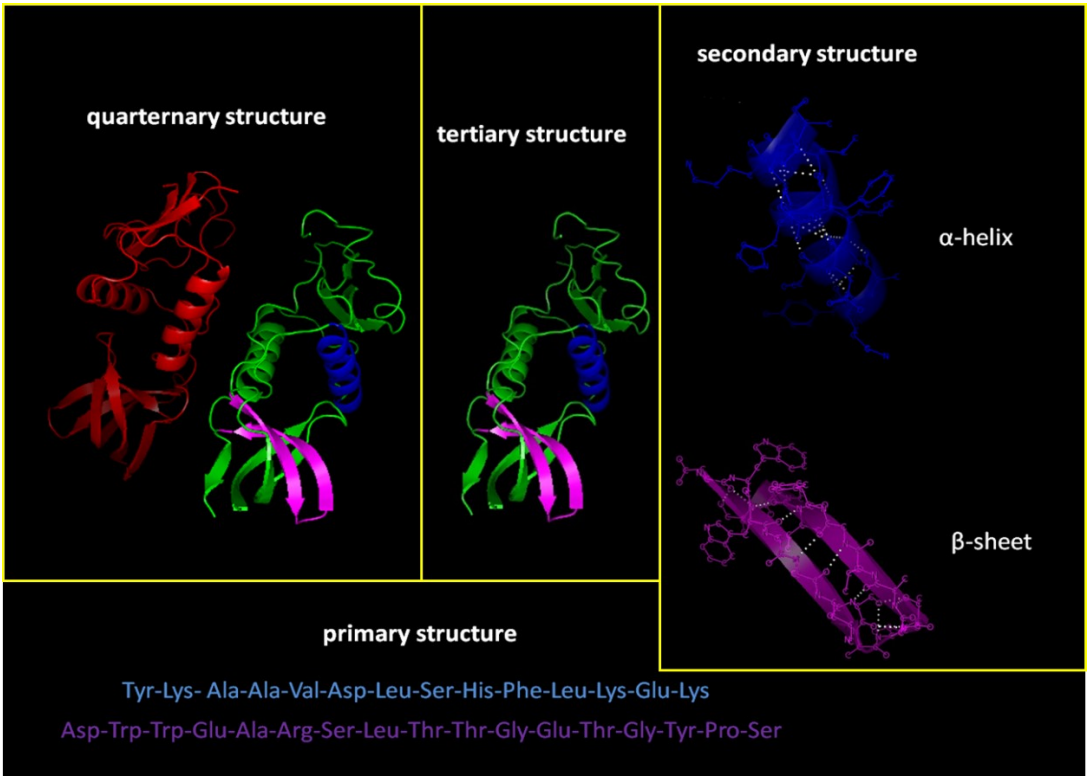


Figure 1.2 Protein structural levels (Revised (11)).

1.1.1.2 Antibodies: structure and stability

Monoclonal antibodies are the largest class of therapeutic proteins (1, 3, 5, 12-14). Antibody, also known as immunoglobulin (Ig) is a globular Y-shaped protein. Antibodies' structure is characterised by two identical heavy (H) chains and two identical light (L) chains with a typical molecular weight of 50 and 25 kDa, respectively. Therefore, the overall protein molecular weight is approximately 150 kDa. Two types of light regions can form the antibody: lambda (λ) and kappa (κ). Their ratio varies in mice (20:1) and humans (2:1) (12). The heavy and light chains are linked together by disulphide bonds. Additionally, the antibody structure possesses two main regions: the variable region (V) which includes the antigen binding fragment (Fab) and the constant region forming the crystallizable fragment (Fc) for recognition and binding of the effectors (e.g. receptors). The hinge region provides a certain flexibility to guarantee the binding to the antigen (Fab) and the effector function (Fc) (**Figure 1.3**).

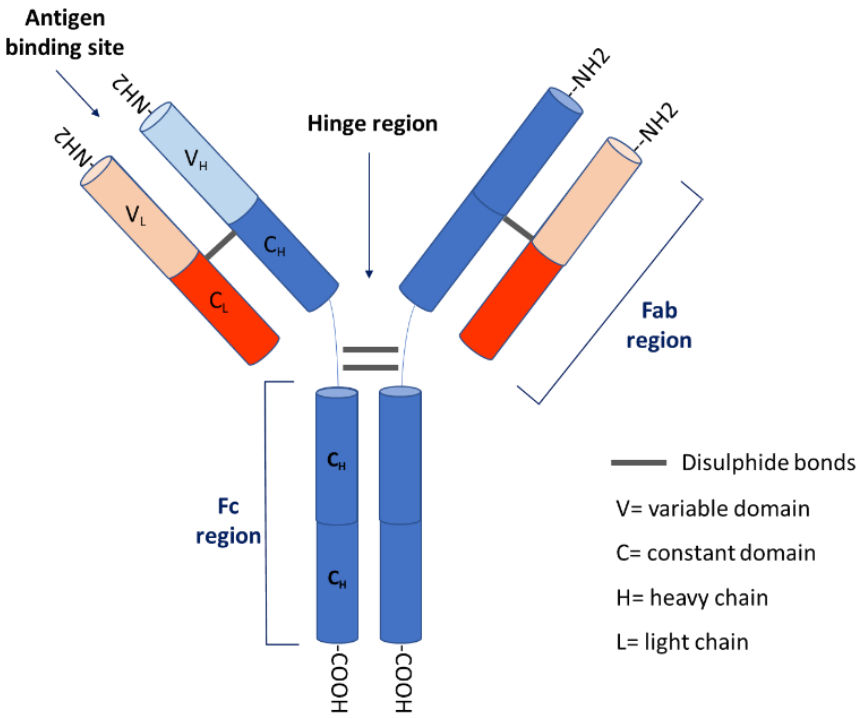


Figure 1.3 Schematic representation of a typical IgG structure.

Antibodies can be divided into five classes depending on their constant region (C): IgA, IgD, IgE, IgM and IgG. IgGs are monomers, whereas IgMs and IgAs are dimers and pentamers, respectively. IgGs can be further divided in subclasses: IgG1, IgG2, IgG3 and IgG4, due to differences in their heavy chains (γ1, γ2, γ3 and γ4) (12, 15). The main structural differences are related to the number and location of disulphide bonds and the length of their hinge region (12). Most IgGs have four interchain disulphide bonds, two of which link the H chains and two the L chains, with some exceptions (12, 15). In addition, one oligosaccharide chain is included in the IgG structure, usually on the constant region. The oligosaccharide chain, fucosylated in some cases, is critical in relation to the conformation, function and antigenicity of the antibody (12, 16, 17). The secondary structure of antibodies is mainly characterized by the formation of anti-parallel β-sheets (two domains- L chains, four domains- H chains) (12, 15). All these domains are folded in β-barrel structures stabilised by disulphide bonds and hydrophobic interactions

(12). Disulphide bonds and strong non-covalent interactions between the two H chains and H-L chains contribute to maintaining the characteristic Y-shape (12).

The increased use of antibodies as therapeutic proteins is related to several advantages: 1) their high specificity which enables a reduction of side effects, 2) their possible conjugation with other entities to guarantee an efficient delivery, 3) their possible use in diagnostic when conjugated with radioisotopes and 4) a reduction of the risk of immunogenicity due to significant improvements in the technology employed for their production (12). In early stage development, antibodies were mainly extracted from murine sources. Hence, murine antibodies were highly prone to induce an immune response in humans through the formation of human anti-mouse antibodies (HAMA). Subsequently, humanised chimeric antibodies (60-70% human) and highly humanised antibodies (90-95% human) were developed reducing the risk of immune response when administered (12, 16). Antibody fragments and nanobodies are novel therapeutic approaches in development (18).

Although antibodies are generally more stable than other proteins, they can undergo physical and chemical degradation through a variety of pathways (6, 7, 12, 19, 20). Antibodies are an heterogenous class of proteins, hence IgGs belonging to the same subclass can have different stability behaviours and sensitivity to degradation pathways when exposed to the same conditions (12). Differences in their polypeptide sequence, glycosylation, extraction or purification, and terminal processing can all impact the production of a unique and sometimes unpredictable antibody entity (1, 12). As well as other proteins, antibodies can undergo physical degradation through two main pathways (aggregation and denaturation) or chemical degradation through several pathways (cross-linking, deamidation, isomerization, oxidation and fragmentation) (6, 7, 12, 19, 20). Antibody degradation can have a detrimental impact on therapeutic activity and efficacy,

but also provokes an immune response that may lead to complete neutralization of the therapeutic antibody as well as of the human endogenous proteins (12, 21-23). pH, ionic strength, buffering agents, formulation composition, shaking/shearing, choice of the container, processing strategies (e.g. freeze/thaw or lyophilisation) (6, 24-26) are all factors that can negatively impact the rate of degradation of the specific antibody/protein in formulation (6, 12, 19, 27).

Despite the common use of platform approaches, individual formulation and processing strategies can contribute synergistically to overcome these challenges and provide a stable antibody formulation through manufacturing and storage (12, 28).

1.1.1.3 High concentration protein formulations

Proteins such as monoclonal antibodies are often administered at high doses and with frequent regimens for a variety of therapeutic indications (12, 13, 28). In general, the current preferential route of administration for protein commercial products is intravenous (IV) infusion (29). However, pharmaceutical companies are increasingly showing interest in formulating high protein doses required at high concentrations (usually ≥ 100 mg/mL) and in small volumes (1-2 mL) to promote their delivery through subcutaneous (SC) administration. The main advantages in developing protein formulations intended for subcutaneous use are: (a) self-administration, especially in the context of home medication, (b) ease of use, (c) reduction of hospitalization and thus treatment costs, and therefore increased patient compliance (28, 29). However, the development of high concentration protein formulations can pose several challenges in a liquid format due to high viscosity (30, 31), high aggregation propensity and low solubility (12, 13). In a lyophilised format, high concentration protein formulations also present some challenges due to high solid content, cryo concentration during freezing, and high resistance to the water vapour flow which can prolong primary drying and

reconstitution times (12, 13, 32). A number of studies have been published in relation to high concentration protein formulations (28-31, 33-35), but very little is published in relation to these formulations in the lyophilised state (32, 36-38).

1.1.1.4 Parenteral protein formulations

Parenteral protein products are generally formulated in two dosage forms: liquid or lyophilised. Depending on the format, the excipients included in the formulation can vary to promote the optimal stability of the therapeutic protein over its shelf-life. Liquid dosage forms are usually preferred as they are easier to administer and less expensive to manufacture (39, 40). On the contrary, lyophilisation is generally performed for APIs that are not adequately stable in the liquid dosage form, since lyophilisation is a time consuming and expensive process (39-42). Lyophilisation is also used to accelerate introduction of new products on the market, which would otherwise require a number of formulation optimisation studies to be commercialised in the liquid state. Recently, an increasing number of lyophilised products have been developed to ensure product stability, to prolong shelf-life and in some cases to eliminate the requirement for cold chain during storage (43, 44). The main excipient categories included in liquid and lyophilised parenteral protein formulations are summarised in **Table 1.1**.

Table 1.1 Summary of the excipient categories included in liquid and lyophilised protein formulations.

Excipient Class	Liquid	Lyophilised
Buffering agent	Yes	Yes
Stabilisers	Yes	Yes
Bulking agent	No	Yes
Surfactants	Yes	Yes
Preservatives	Yes	No
Tonicifiers	Yes	Yes

Chapter 2 provides a comprehensive and detailed overview of the excipients included in commercial parenteral protein products and approved in the European Union between 1995-2018. The overview particularly focuses on antibody products, since they form the main class of commercialised therapeutic proteins.

The minimum number of excipients capable to have a beneficial role and to improve protein stability should be included in protein formulations, providing a simple, but effective formulation strategy (liquid or lyophilised) (40). The mechanism by which some excipients are capable of stabilising proteins is not completely understood, however different hypothesis have been proposed (**Figure 1.4**)

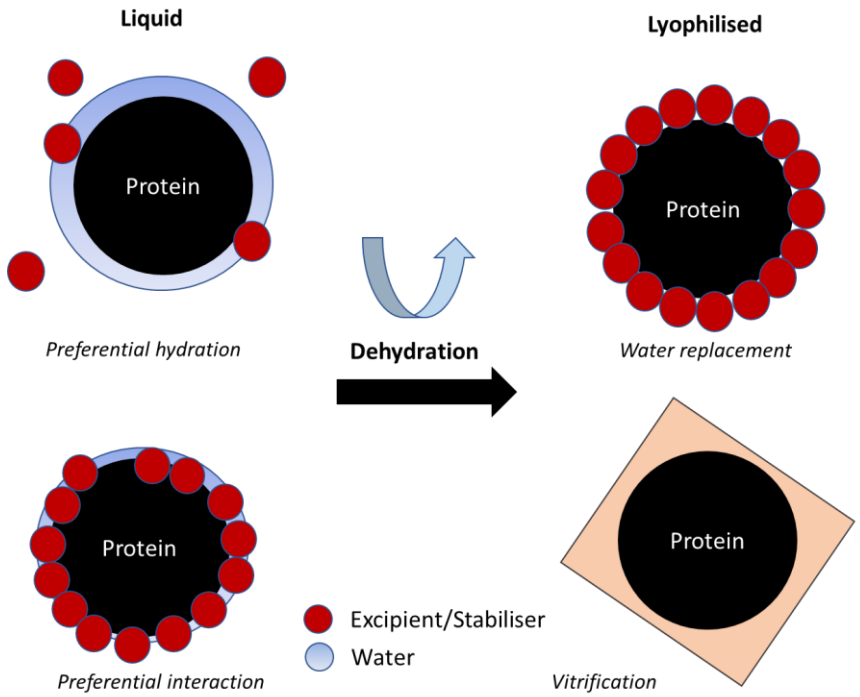


Figure 1.4 Schematic representation of the theoretical mechanisms of interaction occurring between protein and stabilising excipients in solution (liquid) and after dehydration (lyophilised). In the liquid state, preferential hydration occurs when the protein preferentially interacts with water. In contrast, preferential interaction occurs when the excipient preferentially interacts with the protein. In the lyophilised state, once the water is removed through dehydration, the excipient interacts with the protein replacing water (water replacement) or the excipient creates a glassy matrix around the protein (vitrification).

Two are the most common hypothesis identified for protein stabilisation in the liquid state, preferential hydration or preferential interaction. Firstly, preferential hydration, which implies exclusion of the excipient from the protein surface considering that any molecule is larger than water (45). In this case, the excipient is excluded due to its larger hydrodynamic radius and water directly interacts with the protein which maintains its native structure. Secondly, preferential interaction, according to which water is partially excluded due to preferential interactions of the excipient with the protein (e.g. hydrogen bonds, H-bonds) (45). In this case, a certain excipient concentration is required to ensure formation of the bonds on the protein surface (45). During lyophilisation, the dehydration process removes free water, first during freezing due to water crystallisation and then during drying due to sublimation. The two most common stabilising hypotheses for lyophilised formulations include: 1) water replacement - the water is replaced by the

excipient which directly interacts with the protein and 2) vitrification - the amorphous excipient (stabiliser) creates an amorphous glassy matrix around the protein, reducing protein mobility and the probability of protein-protein interactions leading to aggregation (45).

1.1.2 Lyophilisation process

Lyophilisation, also referred to as freeze-drying, is a process employed to manufacture pharmaceutical, biopharmaceutical and food products not sufficiently stable in the liquid or frozen forms (40, 46). Lyophilisation is generally employed to extend the shelf-life and stability of a product; however, it is a time consuming and expensive process (39-42). Hence, pharmaceutical companies execute lyophilisation process optimisation, aiming to reduce the process time while maintaining product critical quality attributes (CQAs) within specifications (39).

The lyophiliser (or freeze-dryer) is the equipment used to conduct the lyophilisation process. Lyophilisers can have different configurations, designs, dimensions and capacity (47, 48). Laboratory and pilot freeze-dryers are employed at small manufacturing scale, whereas commercial freeze-dryers are used at the large manufacturing scale. In both cases, the main lyophiliser components (**Figure 1.5**) include:

- *Lyo chamber* - where the shelves and the product vials are located.
- *Shelves* - situated in the lyo chamber, shelves contain a thermal fluid (silicone oil) that circulates in a serpentine for temperature control.
- *Vacuum pump* - compresses non-condensable gasses to the condenser.
- *Condenser* - refrigerated coils or plates, internal or external to the lyo chamber. Differences between product temperature (Tp) at the sublimation interface and condenser temperature is the driving force of the primary drying step. The condenser traps the water during processing. To maximise the

process efficiency, the condenser temperature should be at least 20 °C lower than the product temperature at the sublimation interface.

- *Isolation valve* - separates the lyo chamber from the condenser chamber.
- *Refrigeration system* - to cool shelf and condenser by using compressors or a refrigerant fluid.
- *PC/ Control system* - to set up controlled values of critical process parameters (chamber pressure, temperature, ramp rate and soak time).

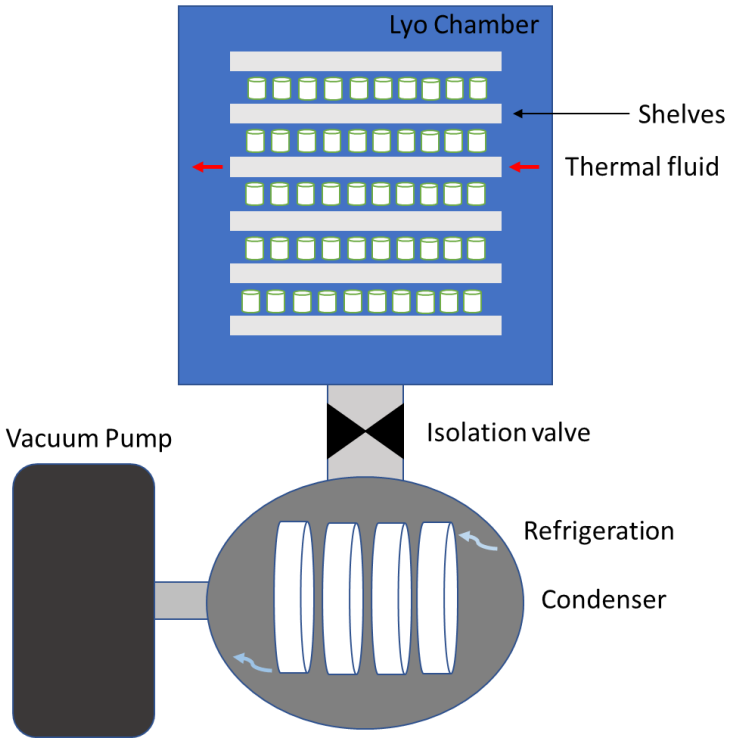


Figure 1.5 Schematic representation of a Lyophiliser.

The lyophilisation process (or lyo-cycle) consists of three steps:

- *Freezing* is the step where temperature is reduced to completely freeze the bulk product solution below its critical temperatures. If the formulation includes amorphous and crystalline components, a crystalline ice phase and a freeze concentrated amorphous phase will form.

- *Primary drying* is the step where the majority of the ice is converted into water vapour, by sublimation. Sublimation is a phase transition where the material changes from a solid to a vapour state, by-passing the intermediate liquid state. To guarantee sublimation completion, a reduction in pressure (below the triple point of ice at the specific product temperature, **Figure 1.6**) is required.

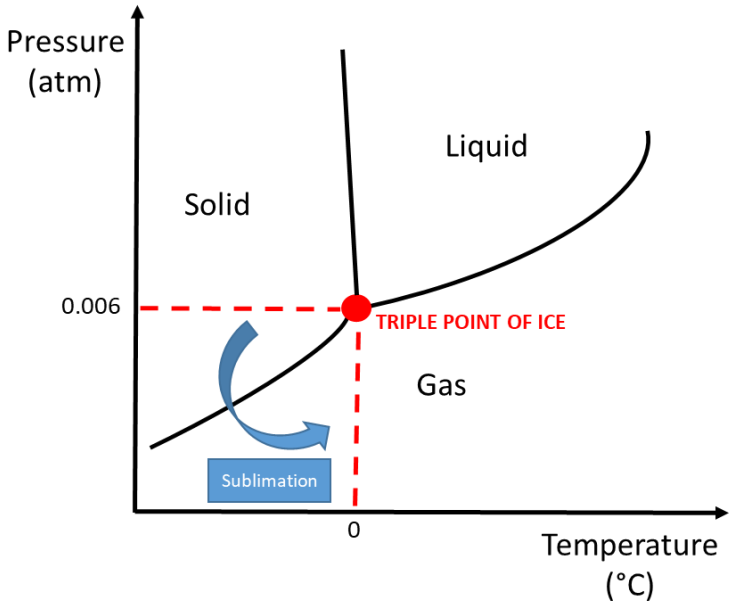


Figure 1.6 Diagram of the triple point of ice and water phase transitions.

- *Secondary drying* is the step where the residual unfrozen (bound) water is removed by desorption. During this step, temperature is increased to reduce moisture content in the lyophilised product to values typically below 1-3% (49-51).

Additional steps in the overall process include loading (pre lyophilisation), vial backfilling in the lyo chamber with inert gas, vials stoppering and unloading (post lyophilisation). Vials backfilling is generally executed under partial vacuum (800-900 mbar) to enable and preserve the sealing of the vials.

1.1.2.1 Freezing

Freezing is a stochastic process, difficult to control. Freezing is achieved by reducing the temperature from the loading temperature to a temperature value below the formulation critical temperatures. Critical temperatures include glass transition temperature of the maximal freeze concentrated solution (Tg') of the amorphous components or below the eutectic temperature (Teu) of the crystalline components. Each formulation has a critical temperature below which it needs to be frozen and dried for complete solidification and prevention of physical/structural collapses that can compromise the quality of the product (52, 53).

Tg' is defined as the temperature at which the frozen amorphous material changes from a brittle to a flexible structure. Tc is the temperature at which the amorphous material softens to the point of not being able to support its own structure, whereas Teu is the temperature at which the eutectic mixture melts (54). Critical temperature values are dependent on the formulation components, their physical nature (amorphous or crystalline) and their ratio in the mixture. Typically, a difference of 1°C to 3°C has been reported between the Tg' and Tc of a protein formulation (Tc>Tg') (52, 55), however, at higher protein concentrations this difference can progressively increase up to 10 °C (32).

If excipients used in the formulation have a tendency to crystallise, an annealing step is required to prevent occurrence of crystallisation during processing or upon storage (38, 56, 57). If an amorphous excipient re-crystallises, provoking molecular re-arrangement and release of bound water in the lyophilised product, the stability of the product can be compromised (57, 58). Annealing is performed by conducting a stochastic freezing and re-heating the product to a set temperature above Tg' of the amorphous fraction (56). The induced annealing increases mobility and restructures ice crystals to larger sizes (Ostwald ripening reaction), reducing primary drying time (56, 59). The re-crystallisation event

also removes the excipient from the amorphous phase modifying formulation Tg (56). However, a certain period of time at the desired temperature is required to ensure complete conversion of the amorphous material to its crystalline form, which often results in prolonged freezing times.

The freezing step is essential and can impact on the subsequent drying steps. The nucleation temperature is defined as the temperature at which ice crystals first form. The degree of supercooling is defined as the difference between the equilibrium freezing point and the nucleation temperature. The degree of supercooling is affected by the solution properties and process conditions (60). The freezing ramp rate has a significant impact on the formation and size of the ice crystals, that once removed through sublimation can leave pores of different sizes impacting primary and secondary drying time (39, 60, 61). The modality of freezing can affect the degree of supercooling and ice crystals size, hence the resistance of the material to the flow of water vapour during sublimation (Rp) (**Figure 1.7**). The degree of supercooling governs the rate of nucleation and thus determines the number and size of ice crystals formed, which, in turn, affects the porosity/surface area of the lyophilised cake (60).

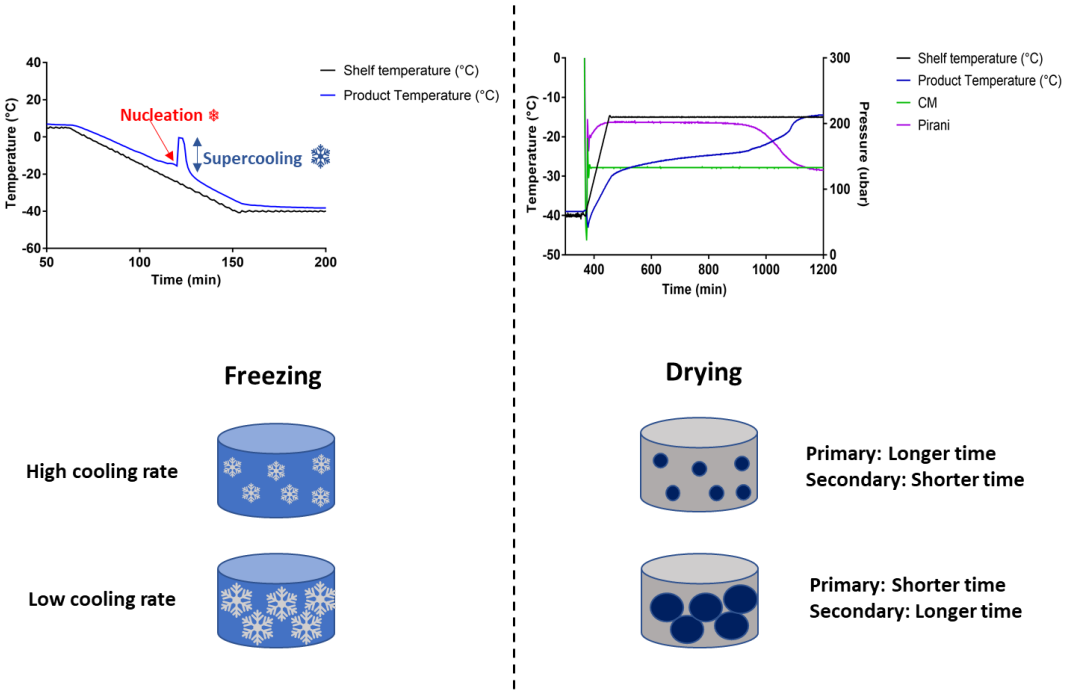


Figure 1.7 Schematic illustration of the impact of the cooling rate on the freezing and primary drying steps.

As shown in **Figure 1.7**, high freezing rates induce a high degree of supercooling (low nucleation temperature), small ice crystal formation, higher Rp and longer primary drying time (shorter secondary drying). Lower freezing rates induce a low degree of supercooling (high nucleation temperature) larger ice crystal formation, lower Rp and shorter primary drying time (longer secondary drying). The decrease of nucleation temperature by 1 °C has been observed to increase primary drying by 3% (60). Therefore, the stochastic freezing is highly responsible for vial to vial variability during freeze-drying.

To reduce the typical intra and inter batch variability and increase homogeneity of the freezing step, controlled nucleation can be employed. Controlled nucleation is a process that enables nucleation of the vials at the same time and temperature across the batch, resulting in a uniform starting point for ice crystals growth. Several technologies and methodologies are currently available to perform controlled nucleation during freeze-drying (61).

Finally, the optimal soak time at the minimum temperature can be selected considering the cake height (generally 1 h per cm) and evaluating the impact of total solute concentration. Higher total solute concentrations generally require longer times and lower temperatures for complete solidification (42).

1.1.2.2 Primary drying

Primary drying is the longest phase of the lyophilisation process, it is the step where the ice crystals formed during freezing are removed by sublimation. The critical process parameters in this step are shelf temperature and chamber pressure that will then define the product temperature (44, 62). Product temperature (Tp) is a critical parameter that has to be monitored to ensure the success of the manufacturing process (62-64). Conventionally, Tp should be maintained below critical temperatures of the product to prevent cake defects (e.g. collapse or melting). However, in some cases it has been observed that the use of aggressive conditions, resulting in a short-term increase in Tp above critical temperatures, does not negatively impact the cosmetic appearance and stability of the final product (65-68). Several Process Analytical Technology (PAT) tools can be used to monitor product temperature, however, the ones currently used in commercial manufacturing show some disadvantages (39, 63, 69).

The sublimation occurring during primary drying is governed by heat and mass transfer events (**Figure 1.8**).

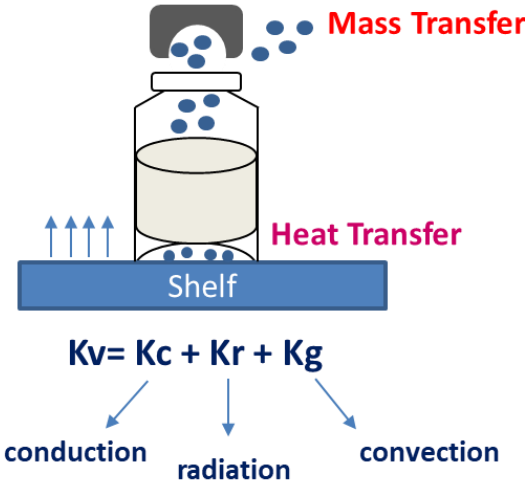


Figure 1.8 Schematic illustration of heat and mass transfer processes occurring during sublimation.

The heat transfer coefficient (Kv) is defined as the ratio of the area normalised heat flow to the temperature difference between heat source (shelf) and heat sink (product vial) (70). The overall Kv is given by the sum of three contributing factors: Kc which is the conductive heat transfer from the shelf to the vial, Kr which is the radiative heat transfer coefficient and Kg which is the gas convective heat transfer coefficient, given by the gas molecule located in the gap between shelf and the bottom of the vial and in the lyo chamber (**Equation 1.1**) (71).

Equation 1.1: Kv contributors

$$Kv = Kc + Kr + Kg$$

Kg provides the largest contribution and it is highly influenced by chamber pressure, unlike the other two contributors (71). Kr is widely reported to cause an increase of the overall heat transfer for vials located at the edges, known as 'edge effect' (71, 72). Kv trends non-linearly as a function of chamber pressure and can vary from freeze-dryer to freeze-dryer due to differences in equipment type, configuration and size (39, 47, 73).

The mass transfer is the mass of water vapour that will be transferred from the product to the lyo chamber during sublimation. Mass transfer is controlled by the equipment capacity, stoppers, processing conditions and product characteristics (74). During primary

drying, the product receives heat from the shelf, part of which will be consumed by ice sublimation. During mass transfer, an important role is carried out by the Rp. Rp is the resistance of the product dry cake to the water vapour flow during sublimation (74). Hence, Rp generally increases non-linearly as primary drying proceeds until the ice is sublimed and the maximum dry layer thickness is achieved. Rp is strictly linked to the ice nucleation and crystal growth (60, 61) (Section 1.1.4.1) as well as the total solute concentration. Products having high total solute concentrations tend to have higher Rp values (74). Rp plays a key role in determining sublimation rate and product temperature, hence primary drying time (70).

Considering the significant impact of primary drying time on the lyophilisation process duration, often the optimisation of the lyophilisation process is focused on reducing and optimising this step (75). End point of primary drying can be determined by using different methods. The two most common and simple methods include the identification of the points where: (1) the Pirani gauge reading matches the capacitance manometer (CM), and (2) the product temperature reaches the shelf temperature. The first method is generally more accurate and provides information on the end of primary drying for the overall batch rather than on the single vial. The Pirani gauge measures the thermal conductivity of the gas in the chamber. Considering that during primary drying the chamber contains 99% of water vapour, and the thermal conductivity of water vapour is 1.6 times larger than that one of the nitrogen gas, the Pirani gauge reads higher pressure levels while sublimation is occurring. Towards the end of primary drying, when the last fractions of ice undergo sublimation, the gas composition in the chamber changes from water vapour to nitrogen, and the Pirani pressure decays as a function of time until it reaches the same pressure level as the CM (76). At the end of primary drying, the moisture

content can vary from product to product, but for an amorphous product usually ranges between 5 to 20% (42).

1.1.2.3 Secondary drying

Secondary drying is the final step of the lyophilisation process where the unfrozen water is removed through desorption. This step involves increasing the shelf set point under vacuum to achieve optimal moisture levels (usually below 1-3%) in the product (49-51). High moisture levels can have a negative impact on product stability and storage. In amorphous materials, high moisture levels can lower the glass transition temperature (Tg), inducing excipient re-crystallisation and phase separation during the product storage over the shelf-life (77, 78). Tg is an important CQA parameter, it represents the temperature limit below which lyophilised amorphous products should be stored (36, 77, 78). Many biopharmaceutical products having low Tg require low storage temperature conditions (2-8 °C). The necessity for cold chain during storage is a significant cost investment for pharmaceutical companies, which contribute to further increasing the costs of high value products (e.g. IgGs). High moisture levels can also increase the rate of chemical degradation of the protein. However, it has been observed that for some products an optimal moisture level is required, and a certain amount of bound water is preferred to preserve product stability (36, 79, 80).

For amorphous products, it is important to select a T_{shelf} during secondary drying that assures a Tp below the Tg. Typically, amorphous products are more difficult to dry than crystalline products (42). Furthermore, crystalline products can withstand more drastic ramp rates and aggressive shelf temperatures during secondary drying. Usually, secondary drying times longer than 6 hours are not required. However, amorphous products having higher total solute concentrations may need extra secondary drying time (42).

1.1.3 Primary packaging components

Primary packaging components should be considered as intrinsic and critical elements of the lyophilised product (81). Changes in the primary packaging components can impact the manufacturing process and the stability of the lyophilised products. In particular, vials and rubber stoppers type, materials and treatments can impact the product CQAs. Type I glass vials are generally selected for parenteral protein products, and tubing vials are preferred over moulded vials (73). Tubing vials show less variability in the glass thickness which translates in more constant Kv values than moulded vials (73, 82). Therefore, the use of tubing vials can guarantee a less variable drying of the product during lyophilisation. Stopper rubber type, presence of silicone, sterilization and dryness can all affect the product stability (83-85). Extractable and leachable components (86), as well as moisture (83-85) release from the stoppers can compromise product stability and quality (81). The effect of stopper moisture and the dynamic of water exchange between stoppers and lyophilised products have been reported to significantly impact product stability (83-85).

Currently, the majority of high concentration protein formulations are in a liquid format in vials or pre-filled syringes to facilitate the subcutaneous administration (87, 88).

1.1.4 Scale-up and Technical transfer

The lyophilisation process transfer or scale-up between different freeze-dryers represents a significant challenge for pharmaceutical companies. In some cases, differences due to geometry (e.g. horizontal vs vertical condenser) or size (e.g. pilot vs commercial scale) of the equipment in use can drastically impact the lyophilisation process dynamics, increasing the risk of batch failure during transfer or scale-up (47, 48, 60, 89, 90). For this reason, it is good practice to collect information on the heat and mass transfer

performance relative to the specific equipment (60, 90). In particular, Kv, maximum sublimation rate and minimum controllable pressure studies are useful tests that can be performed to characterise the equipment, de-risking the technical transfer process. This information in combination with QbD approaches can facilitate the process transfer, reducing the number of lyophilisation cycles required to build confidence and to address data driven decisions for a successful lyophilisation process optimisation. If differences in the equipment performance are identified during transfer, operation conditions can be modified accordingly to provide a process that provides products with equivalent CQAs. Finally, the use of QbD approaches enables the establishment of proven acceptable ranges and ensures robustness of the lyophilisation process when variations in the Critical Process Parameters (CPPs) occur (deviations) within certain limits.

1.1.5 Quality by Design in lyophilisation

The term QbD is defined by ICH Q8 as *"a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management"*. QbD aims to achieve the quality of the product by applying a scientific approach where the design space is the key element. As per ICH Q8 guidelines, *"a design space is the multidimensional combination of input variables and process parameters that have been demonstrated to provide assurance of quality"*. This scientific approach allows evaluation of the impact of CPPs on the CQAs of the product, minimising the use of trial and error approaches. When a robust design space is built the change of CPPs within a proven acceptable range will not negatively impact the product CQAs. During lyophilisation process design, the use of QbD is generally applied to the primary drying step prediction and optimisation (48, 72, 89, 91-93). Pikal et al. developed a mathematical model based on the heat and mass transfer, which allows estimation of Tp and end point

of the primary drying, accounting for the sublimation flow of the specific product (72). This modelling tool aids to select optimal lyo-cycle conditions, while operating safely below the critical temperature of the formulation. This approach can also provide the user with information regarding the robustness of the cycle performance, which enables investigation into the potential effect of process deviations on product quality. However, the key element for the success of the prediction is the capability to insert correct input parameters (75, 94)

The input parameters required for development of the design space are reported in **Appendix, Table A3** and **Table A4**. Input parameters include mainly formulation and container information, Kv and Rp data. Kv and Rp data can be estimated or experimentally calculated with the aid of several PAT tools (Section 1.1.6). The most common method used to calculate Kv is the gravimetric method (95). Rp can be determined by the use of Manometric Temperature Measurement (MTM), or by using experimental Tp information obtained from a conservative lyo-cycle where Tp is maintained below the formulation critical temperature (74). Performing primary drying above the collapse temperature is known to increase Rp due to an increase in viscosity of the product and the occurrence of micro-collapse (74, 96). Rp data for some standard formulations is available in a general database and can be used in a preliminary stage to estimate Rp when product temperature data is not available (94).

Statistical uncertainty and variability in the Kv and Rp parameters estimation should be considered in the building of the design space. For instance, the Kv distribution due to differences in the heat transfer of vials located at different positions on the shelf can be a significant source of bias, compromising the accuracy of the prediction (93). The model and building of a design space have a relevant impact on the development of lyophilisation processes for products that cannot withstand aggressive process conditions,

but less impact on products that can be dried above their critical temperatures. Hence, it is advisable to understand the effect of micro or macro collapse on the other product CQAs to optimise the process time, avoiding product defects or excessively long primary drying steps. Chapter 4 reports the equations required for estimation of Kv, Rp and primary drying time.

Regarding the freezing step, a novel mechanistic model has been proposed to predict the ice crystals size distribution during freezing of lyophilised formulations (97). Finally, in 2013, Sahni and Pikal proposed a modelling approach for the prediction of desorption during secondary drying (98). The possibility to model the overall lyophilisation process (not only primary drying) would have a relevant impact for lyophilisation scientists engaged in the development of lyophilisation cycles.

1.1.6 Process Analytical Technology Tools

Process analytical technology (PAT) has been defined by the United States Food and Drug Administration (FDA) as *'a mechanism to design, analyse and control pharmaceutical manufacturing processes through the measurement of Critical Process Parameters (CPP) which affect Critical Quality Attributes (CQA).'* Several PAT tools can be used to monitor the lyophilisation process.

CPPs are *'process parameters whose variability has an impact on a CQA and therefore should be monitored or controlled to insure the process produces the desired quality'* (39). During the lyophilisation process, chamber pressure and shelf temperature are the main operating process parameters which define the resulting Tp (62). Several PAT tools have been used to monitor the lyophilisation process, particularly over the primary drying step. Some examples of PAT tools reported in literature are listed in **Table 1.2**.

Table 1.2 Summary of the PAT tools used to monitor critical process parameters during the primary drying step of the lyophilisation process.

Critical process parameter monitored	PAT tool	Target	References
Chamber pressure (end of primary drying)	Capacitance Manometer	Batch	(39, 63, 69)
	Pirani gauge (thermal conductivity type gauge)	Batch	(39, 63, 69)
	Mass spectrometry	Batch	(63)
Product temperature (Kv, Rp, end of primary drying)	Thermocouples	Single vial	(39, 63, 69)
	Resistance temperature detectors (RTDs)	Single vial	(39, 63, 69)
	Temperature remote interrogation system (Tempris)	Single vial	(39, 63, 64, 69)
	Pressure rise test (PRT)	Batch	(63)
	Manometric temperature measurement (MTM)	Batch	(39, 63, 69)
	Soft sensors	Single vial	(99, 100)
	Infrared Thermography	Batch	(101)
	Through Vial Impedance Spectroscopy (TVIS)	Single vial	(102, 103)
	Valvless monitoring system (VMS)	Batch	(39)
	Temperature measurement by sublimation rate (TMbySR)	Batch	(39)
	Near Infrared Spectroscopy (NIR)	Single vial	(69, 104)
Mass vapour flow rate (Kv, Rp)	Tunable Diode Laser Absorption Spectroscopy (TDLAS)	Batch	(39, 63, 69)
	Heat flux sensors	Batch	(63)

Primary drying is the longest and most critical phase of the lyophilisation process. Therefore, the majority of the existing PAT tools are designed to monitor parameters related to this step. In contrast, a small number of PAT tools are available to monitor freezing or desorption over the secondary drying step. Some of these PAT tools can be used to monitor more than one step. For instance, Through Vial Impedance Spectroscopy (TVIS) (105) or infrared camera (106) are two novel technologies which can both be employed in the monitoring of the freezing step as well as primary drying in pilot scale freeze-dryers. Additionally, the use of Raman spectroscopy has been proposed as a novel alternative and non-destructive technique to monitor in-line the freezing step (104) and off-line the protein product stability after lyophilisation (107). During the secondary drying step, a vial headspace moisture/pressure analyser can be used in-line and off-line

as a non-destructive technique to monitor container closure integrity and moisture in the vial headspace allowing moisture shelf mapping (Chapter 5) (108, 109). Fissore et al. proposed the use of an in-line system for monitoring of the secondary drying step. This tool couples mathematical models and solvent desorption rate, that is determined by measuring the vapour flux in the drying chamber (49).

The advantage of the use of PAT tools is the possibility to monitor and control the process, reducing the risk of batch failure and ensuring final product quality (69). However, some disadvantages should be considered: a) difficulties during the PAT tool implementation related to scale-up or technical transfer, b) interference of the PAT tool with the monitored system, c) use of single vial vs batch monitoring systems and d) use of in-line vs off-line tools. An example is provided by the temperature monitoring systems used to measure Tp over the lyophilisation process. The most common temperature monitoring tool is the thermocouple that provides single vial measurement and is an invasive system. Once placed in the vials, thermocouples induce a change in the dynamics of the freezing and drying steps, generating bias in the measurement (63, 64). Invasive temperature monitoring systems can act as sites of nucleation, inducing lower degree of supercooling, larger ice crystals formation and faster primary drying in probed vials relative to the un-probed vials (63, 64). Additionally, the design of thermocouples used in manufacturing and pilot scale equipment can vary impacting the Tp output information (63, 64).

1.1.7 Product Critical Quality Attributes

Product critical quality attributes (CQA) are defined as a '*physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality'* (39).

The product CQAs are tested to ensure the quality of lyophilised biopharmaceutical products which can vary depending on the product and its manufacturing process. As reported in the ICH Q6B - Test Procedures and Acceptance Criteria for Biotechnological/Biological Product "*Selection of tests to be included in the specifications is product specific. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency, and data from stability studies, and relevant development data*''(110). Product CQAs commonly tested for lyophilised drug products are reported in **Table 1.3** (110).

Table 1.3 General product CQAs tested for lyophilised drug products (110).

Product CQA
Sterility
Endotoxin
Microbiological limit
Particulate matter
Volume in the container
Uniformity of dosage units
Moisture content/ Loss on drying
Reconstitution time
Appearance of the lyophilised product
Appearance of the reconstituted solution
Identity (physicochemical, biological, immunochemical)
Purity and Impurities
Potency (physicochemical and biological)
Quantity
General testing (e,g. pH, osmolarity) Required in some cases
Additional testing for unique dosage forms

1.2 Thesis hypothesis

Pre-formulation and formulation development studies, combined with statistical/mathematical and QbD tools, can enable rational formulation design to achieve optimum protein stability and reduced lyophilisation times for high concentration antibody (IgG1) formulations.

1.2.1 Aim

The aim of this study is to develop a stable lyophilised high concentration IgG1 formulation intended for subcutaneous use, rending formulation and process development more cost effective for pharmaceutical companies and the administration more patient compliant. In particular, this work focuses on building new knowledge in the formulation and manufacturing process design for high concentration protein formulations.

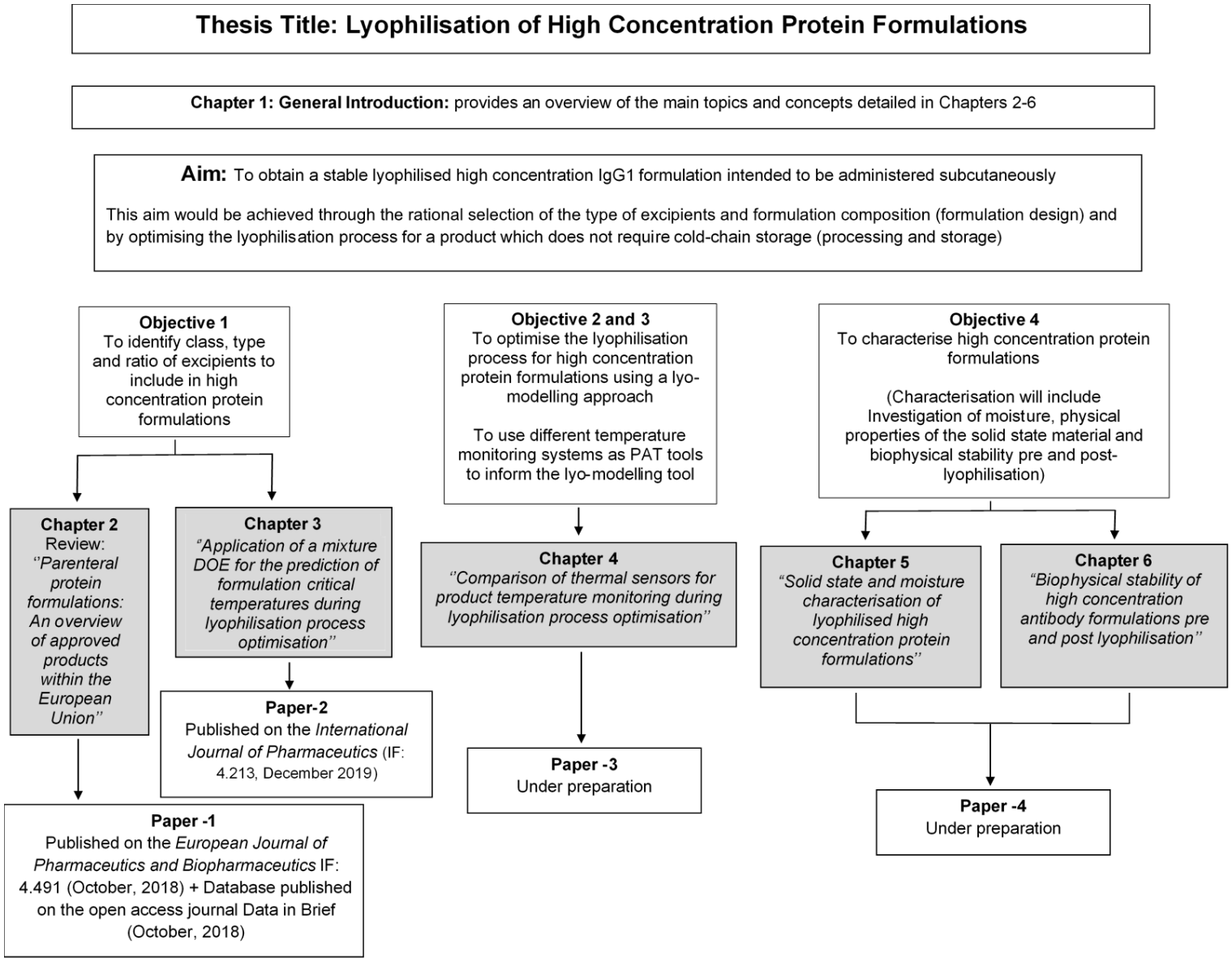
1.2.2 Primary objectives

A number of objectives were identified:

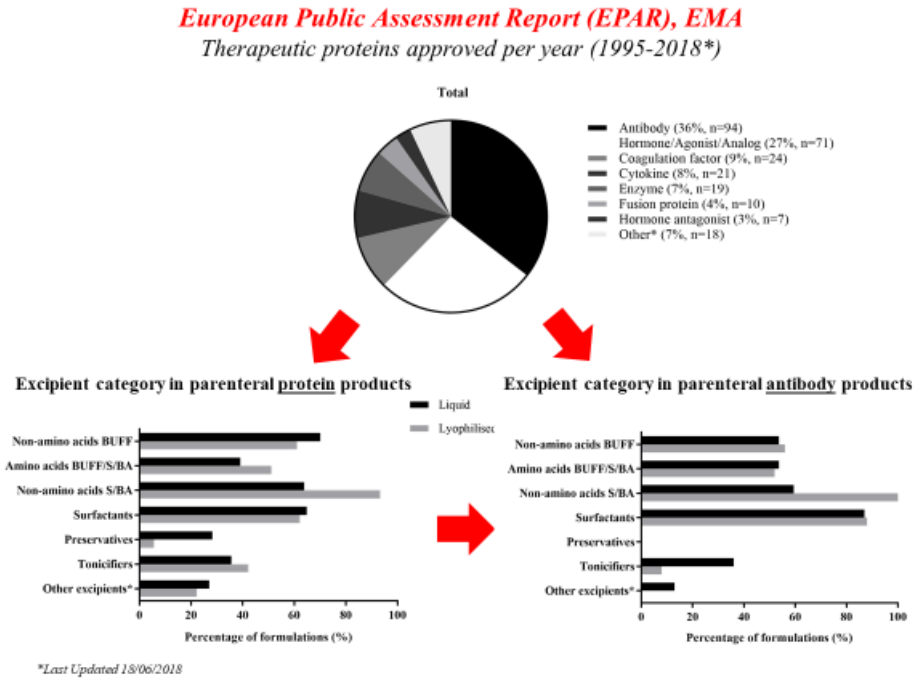
- To compile a systematic quantitative review, analysing formulation strategies of commercial parenteral protein formulations in the liquid and lyophilised formats, approved in Europe (European Medicine Agency, EMA) between 1995-2018. (**Chapter 2**)
- To develop an empirical model (based on a DOE) for selection of formulations with maximised critical temperatures to facilitate lyophilisation process optimisation. (**Chapter 3**).
- To optimise the lyophilisation process (reducing time and costs) with the aid of different PAT tools for product temperature determination and QbD tools for primary drying prediction. (**Chapter 4**)

- To analyse the physical/solid state characteristics of the high concentration protein formulations designed (Chapter 3) and lyophilised (Chapter 4) over stability at different storage temperature conditions, with the aim of eliminating cold chain during storage. (**Chapter 5**).
- To analyse biophysical stability pre and post lyophilisation of the high concentration antibody formulations designed (Chapter 3) and lyophilised (Chapter 4) at different storage temperature conditions, with the aim of eliminating cold chain during storage. (**Chapter 6**)
- To summarise overall findings and to evaluate how this body of work contributes to improve current knowledge on the topic. Recommendations for future work are also included in this chapter. (**Chapter 7**)

1.3 Graphical Thesis outline (next page)


Thesis Title: Lyophilisation of High Concentration Protein Formulations
Chapter 1: General Introduction: provides an overview of the main topics and concepts detailed in Chapters 2-6
Aim: To obtain a stable lyophilised high concentration IgG1 formulation intended to be administered subcutaneously
This aim would be achieved through the rational selection of the type of excipients and formulation composition (formulation design) and by optimising the lyophilisation process for a product which does not require cold-chain storage (processing and storage)
Objective 1
To identify class, type and ratio of excipients to include in high concentration protein formulations
Objective 2 and 3
To optimise the lyophilisation process for high concentration protein formulations using a lyo-modelling approach
To use different temperature monitoring systems as PAT tools to inform the lyo-modelling tool
Objective 4
To characterise high concentration protein formulations
(Characterisation will include Investigation of moisture, physical properties of the solid state material and biophysical stability pre and post-lyophilisation)
Chapter 2
Review: "Parenteral protein formulations: An overview of approved products within the European Union"
Chapter 3
"Application of a mixture DOE for the prediction of formulation critical temperatures during lyophilisation process optimisation"
Chapter 4
"Comparison of thermal sensors for product temperature monitoring during lyophilisation process optimisation"
Chapter 5
"Solid state and moisture characterisation of lyophilised high concentration protein formulations"
Chapter 6
"Biophysical stability of high concentration antibody formulations pre and post lyophilisation"
Paper-2
Published on the International Journal of Pharmaceutics (IF: 4.213, December 2019)
Paper -3
Under preparation
Paper -4
Under preparation
Paper -1
Published on the European Journal of Pharmaceutics and Biopharmaceutics IF: 4.491 (October, 2018) + Database published on the open access journal Data in Brief (October, 2018)
Chapter 7: General Discussion: highlights novel findings, contributions to the field of study from results Chapters 2-6

Chapter 2: Parenteral protein formulations: An overview of approved products within the European Union



Revised from publication doi: https://doi.org/10.1016/j.ejpb.2018.07.011

European Journal of Pharmaceutics and Biopharmaceutics

(October, 2018)

2.1 Introduction

The focus of parenteral protein formulation design is to identify an excipient composition that will stabilise proteins against stresses experienced during processing, storage, and administration. Excipients also aid reconstitution of lyophilised formulations, maintain sterility of multi-dose products, provide isotonicity, and in a small number of cases alter pharmacokinetics (111, 112). The majority of parenteral protein formulations consist of proteins and excipients in an aqueous-based solution or suspension. Processing conditions and external factors such as shifts in pH, changes in temperature, surface interactions and extraneous impurities can destabilise proteins, provoking their chemical and physical structural degradation (113, 114). In some cases, aqueous formulations of therapeutic proteins do not provide adequate stability and therefore, a dried state formulation is a favoured, alternative approach which can aid the stability and prolong the shelf-life of protein products (115). Lyophilisation, the process of subliming water from frozen solutions under low pressure (vacuum), is a widely employed technique for the manufacture of dried biological materials (116). However, lyophilisation has the potential to cause protein damage due to stresses during both the freezing and drying phases (24, 113). Hence, an appropriate excipient composition is required to protect proteins from stresses experienced during the lyophilisation process.

The function and behaviour of excipients in protein formulations is widely reported (28, 117-122). Despite the wide range of formulation information available, it can be challenging for a formulation scientist to get an overview of how frequently excipients are included in commercial protein products. For example, prior publications focus on the function of excipients and provide a range of products as examples of their application. What differentiates this overview from previous work is that it builds on earlier literature and provides up to date, comprehensive information of the frequency of excipients usage

in approved protein products. It also presents an analysis of the quantitative excipient composition in the majority of the commercial protein products. The compilation of such information creates a valuable source for formulation scientists regarding the regulatory acceptance of excipients and their prior history in commercial formulations.

Approval of therapeutic protein products for use within the European Union is via a centralised procedure (123). The European Medicines Agency (EMA) publishes a European Public Assessment Report (EPAR) for every medicine assessed (approved or refused), providing the public with information regarding the product. The EPAR is not a single document but a resource containing a set of regulatory documents related to authorisation details, product information and assessment history. The EPAR is one of the most informative and up to date public sources of information on a large number of commercial therapeutic proteins. The overview presented is a summary of the wealth of the formulation data available in the EPARs in relation to approved parenteral protein formulations. Specifically, data was gathered and analysed to provide a breakdown of products according to protein type, formulation approach (aqueous-based liquid or lyophilised formulations), the most frequently included excipients classified in functional categories, with a more detailed look at antibody formulations.

In reviewing EPARs' data, the split of protein formulations between the liquid and dried (lyophilised) state was investigated and the types of excipients incorporated in both formulation approaches (liquid or lyophilised) are discussed. Qualitative and quantitative composition of protein formulations can be influenced by the process selected for manufacturing products in a certain dosage form (liquid or lyophilised). The overview provides details of the excipient concentrations employed in approved protein products in the European Union (EMA). Due to the limited data related to the excipient quantitative composition in the EPARs, this information has been supplemented by using FDA

sources (124) and product information documents published by the Marketing Authorisation Holder (125). The excipient quantitative composition is provided for 215 out of 264 protein products and 88 out of 94 antibody products approved in the European Union.

The final part of the overview focuses specifically on monoclonal antibody (Mab) products, since Mabs are currently the largest class of therapeutic proteins (1, 3, 5, 14). Mab doses required for the treatment of chronic diseases are relatively high (usually 50–200 mg) compared to the majority of the other therapeutic proteins (126). Intravenous infusion administration is mostly used for the delivery of a number of these products. However, an alternate treatment approach is to formulate Mabs at high concentrations to enable administration of the required dose in smaller volumes (1–2 mL) subcutaneously. The selection of the appropriate excipient composition is required to address challenges when designing high concentration protein formulations (14, 127).

2.2 Methodology

A review of the EPARs available on the EMA website was conducted for all parenteral protein products authorised centrally in the European Union between 1995 and 2018 (up to June 2018) (126). For each of these products, the following information was collected: commercial name, active pharmaceutical ingredient (API) and related quantitative composition, class of therapeutic protein, therapeutic area, dosage form, route of administration, excipient composition, date of issue of marketing authorisation and marketing authorisation holder (pharmaceutical company). Information was compiled in a Microsoft Excel database (included in supplementary information). The accuracy of data transferred to the data base was assured by two researchers checking 100% of the data entries against the EPARs.

Formulations were divided into two groups: liquid (L) and lyophilised (LYO), based on their manufacturing process detailed in the EPARs. Commercial products having the same name but different excipient composition, different formulation approaches (e.g. liquid versus lyophilised) or a different liquid formulations' format (e.g. concentrate, solution or suspension) were considered as distinct products in this overview.

All excipients were categorised considering their potential roles in protein formulations and were assigned to one of seven functional categories. In the case of multifunctional excipients, they were listed in one category but referred to in the discussion under all relevant categories. Excipients can possess diverse roles when added at different concentrations or to different formulation approaches. This multifunctional nature of excipients influenced their classification in this publication. For example, amino-acids were designated as a single category with a range of functions (buffers, stabilisers, bulking agents). Non-amino acids stabilisers/bulking agents category comprise excipients (mainly carbohydrates) that in the liquid dosage form serve as stabilisers. However, some of these excipients in the lyophilised dosage form, have the potential to act also as bulking agents. Furthermore, as formulation data does not specify the excipients' solid form (amorphous or crystalline) it was not possible to separate the stabilising and bulking functions of some excipients (amino acids and carbohydrates) in lyophilised formulations. Therefore, the following seven categories were designated (1) non-amino acids buffers (2) amino acids buffers/stabilisers/bulking agents; (3) non-amino acids stabilisers/bulking agents; (4) surfactants; (5) preservatives; (6) tonicifiers and (7) other excipients.

The 'other excipients' category consisted of excipients present in a relatively low number of products, this included complexing agents, antioxidants, solubilising agents and excipients exclusively present in specific types of formulations, for example zinc salts in insulin products. The hypothesis and assumptions regarding the role of these excipients

in a specific product were stated only in presence of available information (124, 126, 128, 129) or individual specific references which are detailed in Section 2.3.2.8.

The analysis of the percentage and frequency at which individual excipients were included in the products was conducted using IBM SPSS Statistics v.23 software. Percentage of products containing a specific excipient or excipient category is calculated as percentage within the type of dosage form (liquid or lyophilised).

Quantitative composition of protein products is also reported in this overview as a guideline for the range of protein and excipients concentrations employed in marketed formulations. This information was gathered from accredited sources: FDA label (FDA) (124) and product information document (Marketing Authorisation Holder) (125), when data was not provided in the EPAR's scientific discussion (Assessment history, EMA) (130). These primary information sources are dynamic databases; hence this overview is a snapshot of the marketed products information available in the specific timeframe. Furthermore, to guarantee accuracy of the dataset, only information that could be verified against the primary sources was included in this overview. Again, the accuracy of data transferred was assured by two researchers checking 100% of the products' information against these sources. It is important to note that information listed in this overview relates only to products approved by the EMA. Other formulations with the same product name may be approved in other jurisdictions, for example, products with different strengths or excipient compositions.

Analysis of monoclonal antibody products was also conducted to show the relationship between concentration of protein administered and route of delivery.

2.3 Results and Discussion

2.3.1 Approved protein formulations

EPAR data showed that approximately 440 parenteral products were approved by the EMA via its centralised procedure in the period 1995–2018 (June). More than half of these products (n=264) contained therapeutic proteins and a greater number of them were formulated as liquid dosage forms (66%; n=174), compared to lyophilised forms (34%; n=90). **Figure 2.1** shows that the number of liquid parenteral protein formulations is consistently higher than the number of lyophilised formulations approved in the timeframe studied. Since 2013, approximately 20 parenteral protein products have been approved per year.

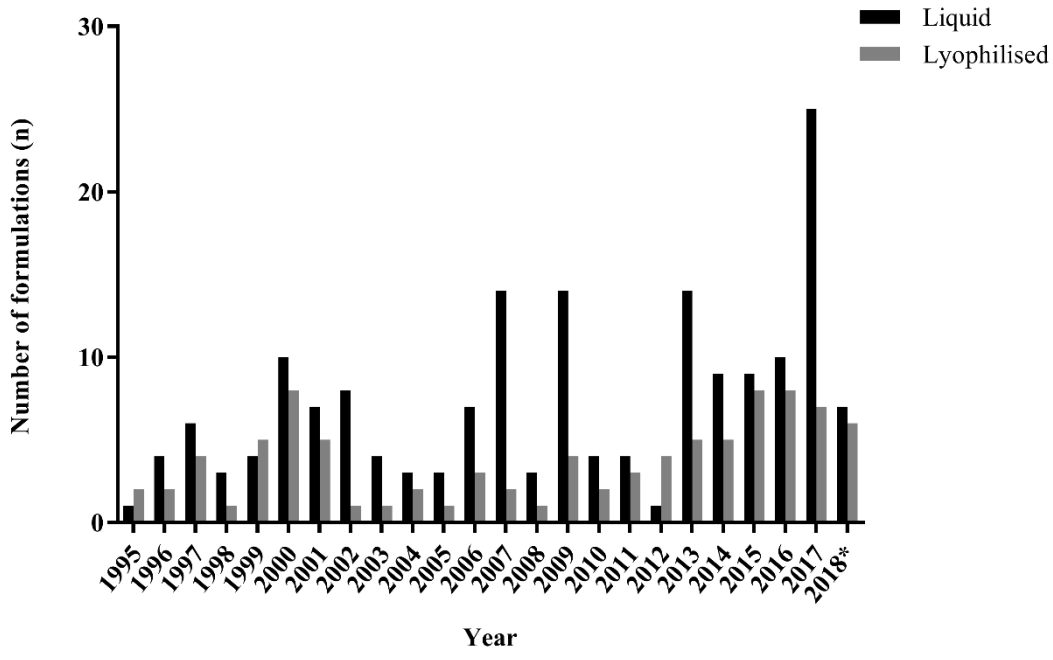


Figure 2.1 Trend of liquid and lyophilised parenteral protein products approved per year by the EMA between 1995 and 2018 (June) (*Last updated: 18/06/ 2018).

Therapeutic proteins included in liquid and lyophilised products, were divided into their functional classes, **Figure 2.2a and Figure 2.2b**. Antibodies (36%; n=94) and hormones

(27%; n=71), represent the two main classes, and are more commonly formulated as liquid formulations (antibodies: n=69 and hormones: n=61) compared to lyophilised formulations (antibodies: n=25 and hormones: n=10). Insulin and its analogues are the most frequent therapeutics in the class of hormone products (n=26 out of 61), and they are formulated exclusively as liquid dosage forms (solutions for infusion or injection, and suspensions for injection). In contrast, coagulation factors are manufactured only as lyophilised formulations (n=24) and represent the second largest class of therapeutic proteins in this dosage form following antibodies.

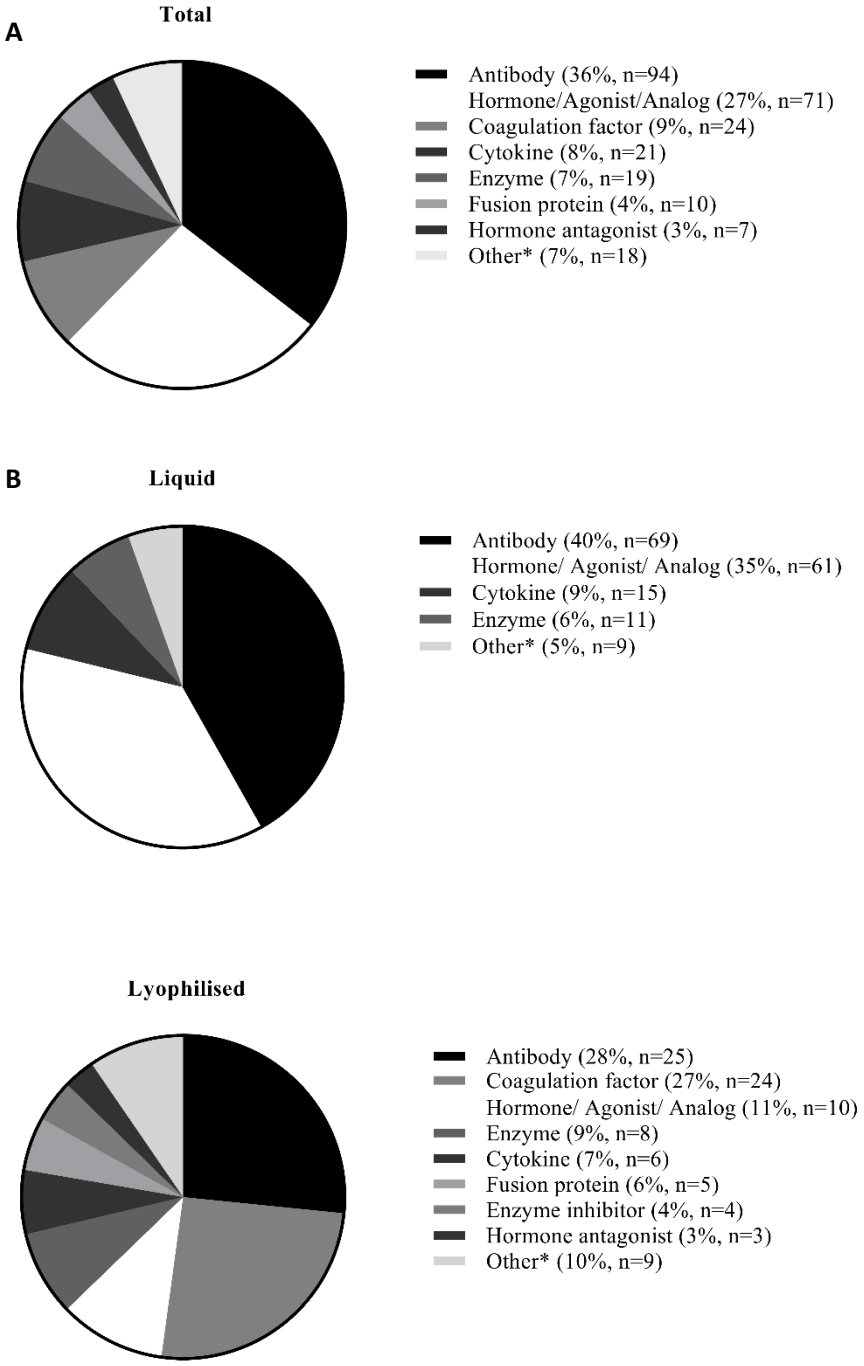


Figure 2.2 Classes of therapeutic proteins included in liquid and lyophilised parenteral products. (a) Pie chart of the total number of products; (b) Pie charts showing the split between liquid and lyophilised products. *'Other' class includes types of therapeutic proteins present in a percentage of commercial products <3% (analgesic peptide, antiangiogenic agent, anticoagulant, antiplatelet, antithrombin/thrombolytic, growth factor, HIV infusion inhibitor, muramyl peptide derivative, toxin, calcimimetic peptide).

2.3.2 Excipients included in approved protein formulations

2.3.2.1 Excipient categories

Excipients were subdivided into seven categories: non-amino acids buffers; amino-acids buffers/stabilisers/bulking agents; non-amino acids stabilisers/bulking agents; surfactants; preservatives; tonicifiers and other excipients as detailed in Section 2.2. The percentages of liquid and lyophilised products containing excipients which fall into each of these categories are shown in **Figure 2.3**.

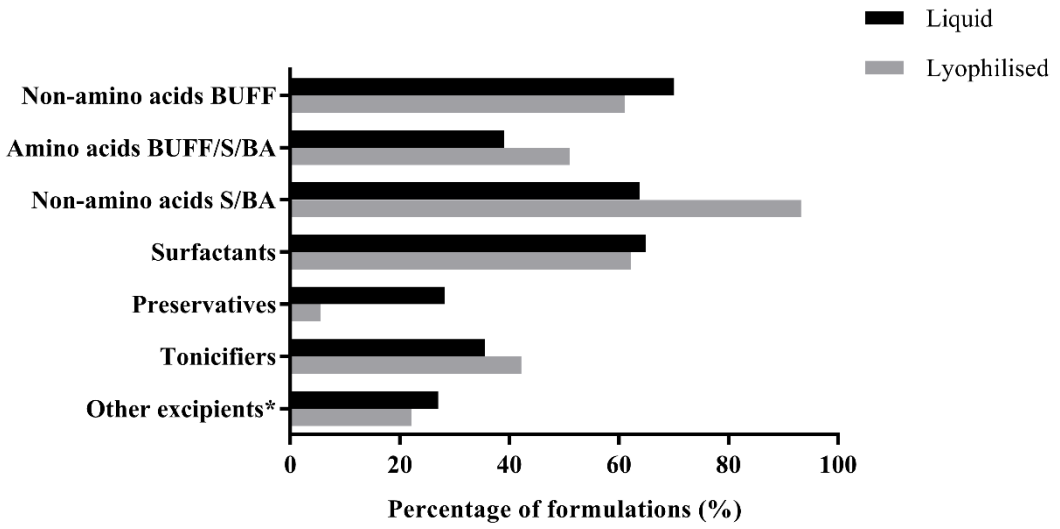


Figure 2.3 The percentage of liquid and lyophilised parenteral protein products that contain excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/bulking agents); Surfactants; Preservatives; Tonicifiers and Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products *'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations.

Most categories of excipients are frequently employed in both liquid and lyophilised products, whereas others, such as preservatives, are more specific to one formulation approach (e.g. multi-dose liquid formulations).

The majority of the formulations: liquid (70%; n=122) and lyophilised (61%; n=55) contain non-amino acids buffers. However, amino acids are present in a large number of

products (L: 39%; n=68; LYO: 51%; n=46) and can have different functions as buffering agents or stabilisers/bulking agents depending on the concentration incorporated. A small number of products are formulated in the absence of buffering agents. This may be due to the ability of the protein to maintain the critical pH, especially if formulated at high concentrations (e.g. FLEBOGAMMA DIF® (human normal immunoglobulin)) (131).

Non-amino acids stabilisers are present in a large number of liquid products (64%, n=111). Excipients included in the non-amino acids' stabilisers/bulking agents' category are also incorporated in most of the lyophilised products (93%, n=84). Surfactants are present in a similar percentage of liquid (65%; n=113) and lyophilised (62%; n=56) formulations. Preservatives are included in liquid (29%, n=50) and lyophilised (6%, n=5) products. As expected, all multi-dose products (n=53) contain preservatives. Multi-dose products comprise 20% of all products (liquid and lyophilised) and are more commonly formulated in the liquid dosage form (n=48). All lyophilised formulations intended for multiple use contain preservatives in the diluent for reconstitution and none in the lyophilised product. The reason for including preservatives directly in the diluent for reconstitution is to minimise any possible preservative-protein interactions (114, 132). Antibodies are exclusively formulated in a single dosage form, whereas insulin in a multiple dosage form. Tonicifiers are incorporated in a high percentage of both liquid (36%; n=62) and lyophilised (42%; n=38) formulations to achieve the iso-osmolarity recommendations for parenteral administration (133-135). Excipient classified in the 'other excipients' category was present in a similar percentage of liquid (27%; n=47) and lyophilised (22%; n=20) products.

Table 2.1 summarises the most common excipients included in liquid and lyophilised products for each functional category. This table also shows the concentration ranges

employed for each of these excipients in the liquid and lyophilised protein products analysed.

Table 2.1 More common individual excipients classified into functional categories and listed by descending frequency of use in liquid and lyophilised parenteral protein products (percentage and number of formulations containing each excipient and range of excipient concentrations included in approved products).

Excipient class	Liquid	Lyophilised
Non-amino acids Buffering agents	Sodium phosphate (33%; n=58) *(0.2-14.8 mg/mL)*	Sodium phosphate (32%; n=29) *(0.2-14.4 mg/mL)*
	Sodium acetate (19%; n=33) *(0.4-6.8 mg/mL)*	Sodium citrate (20%; n=18) *(1.1-34.8 mg/mL)*
	Sodium citrate (17%; n=29) *(0.5-14.7 mg/mL)*	Tris (8%; n=7) *(0.8-3 mg/mL)*
Amino acids Buffering agents/stabilisers/bulking agents	Histidine (16%, n=27) *(0.9-4.3 mg/mL)*	Histidine (34%; n=31) *(0.7-8.9 mg/mL)*
	Methionine (12%; n=21) *(0.1-3 mg/mL)*	Glycine (13%; n=12) *(0.2-25 mg/mL)*
	Glycine (9%; n=15) *(0.1-18.8 mg/mL)*	Methionine (6%; n=5) *(0.06-0.5 mg/mL)*
	Arginine (8%; n=14) *(4.4-42.1 mg/mL)*	Arginine (4%; n=4) *(5.4-52 mg/mL)*
Non-amino acids Stabilisers/bulking agents	Glycerol (17%; n=29) *(16-20 mg/mL)*	Sucrose (59%; n=53) *(1.9-160 mg/mL)*
	Sucrose (16%; n=28) *(10-200 mg/mL)*	Mannitol (33%; n=30) *(10.6-80 mg/mL)*
	Mannitol (14%; n=24) *(1.9-54.6 mg/mL)*	Trehalose (10%; n=9) *(8-70 mg/mL)*
	Sorbitol (10%; n=17) *(20-50 mg/mL)*	Human albumin (7%; n=6) *(8-15 mg/mL)*
Surfactants	Polysorbate 80 (32%; n=55) *(0.01-2 mg/mL)*	Polysorbate 80 (41%; n=37) *(0.05-0.7 mg/mL)*
	Polysorbate 20 (26%; n=45) *(0.01-2 mg/mL)*	Polysorbate 20 (18%; n=16) *(0.04-0.4 mg/mL)*
	Poloxamer 188 (7%; n=12) *(0.1-8 mg/mL)*	Poloxamer 188 (3%; n=3) *(1-1.2 mg/mL)*
*Preservatives**	Metacresol (19%; n=33) *(1.5-3.2 mg/mL)*	Benzyl alcohol (4%; n=4) *(9-14.9 mg/mL)*
	Phenol (14%; n=24) *(0.8-5.7 mg/mL)*	Benzalkonium chloride (1%; n=1)
	Benzyl alcohol (3%; n=6) *(9-10 mg/mL)*	Metacresol (1%; n=1) *(3.2 mg/mL)*
Tonicifiers	Sodium chloride (36%; n=62) *(0.6-11.7 mg/mL)*	Sodium chloride (42%; n=38) *(0.3-27 mg/mL)*
	Potassium chloride (1%; n=2) *(0.2 mg/mL)*	-

**Preservatives in lyophilised products are added to the diluent for reconstitution*

Note: the function of these excipients as bulking agents is only relevant for lyophilised products

Non-amino acids buffers are usually included in a range of concentrations between 0.2 and 14.8 mg/mL (usual ionic strength: 5–25 mM). However, ATRYN® (antithrombin alfa) contains sodium citrate at a higher concentration of 135 mM (34.8 mg/mL). Amino acids can have different functions; hence, they can be used in a wide range of concentrations (0.1 and 52 mg/mL) within the formulations. The usual range of concentrations at which amino acids are employed is between 0.1 and 25 mg/mL, with an ionic strength of 5–260 mM. However, arginine is added in higher concentrations compared to other amino acids, acting mainly as a stabiliser. For example, METALYSE® (tenecteplase) contains arginine at a concentration of 52 mg/mL to increase the solubility of the protein, as reported in the product EPAR's scientific discussion (136). Histidine at low concentrations between 0.7 and 8.9 mg/mL is included in the majority of the products, serving mainly as a buffer. Histidine and arginine are frequently used in their hydrochloride salt form in protein products. Glycine is often employed as a stabiliser/bulking agent besides its role as a buffering agent at concentrations between 0.1 and 25 mg/mL, whereas methionine at low concentrations (0.06–3 mg/mL) is usually selected for its antioxidant properties. Non-amino acids stabilisers and bulking agents are incorporated at concentrations up to 200 mg/mL to maintain the required molar ratio with the protein. Surfactants are generally present in a different range of concentrations in liquid (0.01–2 mg/mL) and lyophilised (0.04–1.2 mg/mL) products. However, the liquid formulation ORENCIA® (abatacept), with a high concentration of the fusion protein (125 mg/mL), contains 8 mg/mL of Poloxamer 188. Preservatives are employed in concentrations between 0.7 and 14.9 mg/mL. Sodium chloride and potassium chloride are included as tonicifiers and/or stabilisers in a variable range of concentrations between 0.2 and 27 mg/mL.

The following sections provide greater details and discussions regarding individual excipients divided into each functional category.

2.3.2.2 Non-amino acids buffers

Buffers are required to adjust and maintain the pH to a value at which the specific protein has maximal stability. The optimum pH value is selected considering the protein's isoelectric point (pI) as a critical physical parameter that can affect protein solubility, aggregation and degradation (137). Furthermore, the selected pH needs to be in a physiological range (pH6-7.4) in order to avoid irritation, pain or extravasation during injection into the patients. For intramuscular and subcutaneous administration the acceptable pH range is between pH 4 and 9 (138). Of the protein products where pH is reported, the values range from pH 3.1 for JETREA® (ocriplasmin) to pH 8.15 for XULTOPHY® (insulin degludec), which are respectively administered through intravitreal and subcutaneous routes.

The percentage of approved liquid and lyophilised parenteral products containing non-amino acids buffers are shown in **Figure 2.4.**

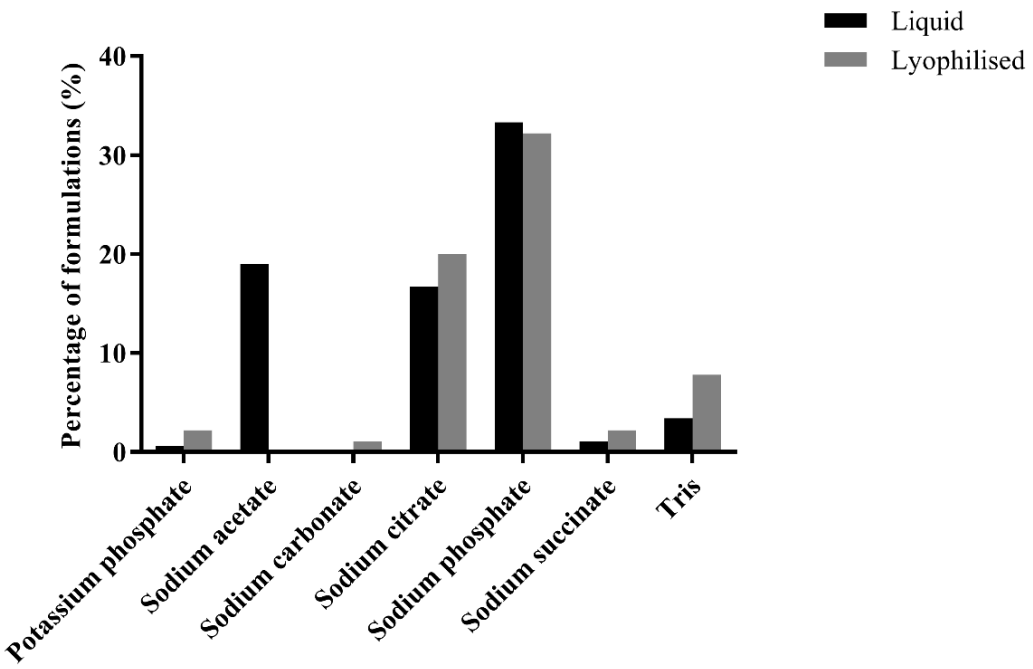


Figure 2.4 The percentage of approved liquid and lyophilised parenteral protein products containing individual non-amino acids buffers.

The most common buffers (excluding amino acids) employed in liquid protein formulations are sodium phosphate (33%; n=58), sodium acetate (19%; n=33) and sodium citrate (17%; n=29). Sodium phosphate (32%, n=29), and sodium citrate (20%; n=18) are also frequently added to lyophilised protein products (120). However, acetate buffer was found not to be incorporated in any lyophilised products identified as part of this overview. Acetic acid is volatile and can be converted to a gaseous state and hence be lost from the formulation during lyophilisation (139, 140). Sodium phosphate buffers employed in commercial products are usually composed of two salt forms: sodium dihydrogen monohydrate and disodium dihydrate. The different sodium phosphate components were grouped together under the name of sodium phosphate in this overview.

Despite their wide use as buffers, sodium phosphate salts present some drawbacks, especially when included in formulations intended to be lyophilised. Highly concentrated buffer systems can crystallize and provoke changes in pH during freezing (139). Sodium

phosphate is prone to crystallize during freezing, leading to a pH shift of up to four units. Furthermore, sodium phosphate crystallisation and precipitation are severely influenced by salt components solubility and concentration, temperature, cooling rate, volume of solution and initial pH value (25, 141). The presence of other compounds can influence buffer crystallisation, precipitation and consequently cause pH shifts which can accelerate drug degradation in frozen solutions. For example, crystallizable solutes, such as glycine, mannitol or sodium chloride can modify ion activity, facilitating crystallisation of buffers; while non-crystallizable excipients such as sucrose or trehalose can inhibit buffer crystallisation (139). The citrate salts remain in an amorphous state upon freezing and drying, minimizing pH shifts, compared to succinates and tartrates (131, 142).

A concept that should also be taken into account during pre-formulation studies is that the pH of the solution before drying has an influence on the rate of chemical reactivity in the resulting dried material (142). In lyophilised formulations, buffers tend to depress critical temperatures for the lyophilisation process; glass transition of the maximally freeze-concentrated solution (Tg') and collapse temperature (Tc). However, buffering agents are usually used in low concentrations (5–25 mM) (143). TRIS buffer which is included in a small number of liquid (3%; n=6) and lyophilised (8%; n=7) formulations has been shown to release formaldehyde in peptide formulations stored at 70°C. However, this does not occur at the lower temperatures normally employed for formulation processing or storage (142).

Individual acids and bases can also be incorporated as pH modifiers to adjust the pH to a target value. Sodium hydroxide (L: 46%; n=80; LYO: 20%; n=18), hydrochloric acid (L: 36%; n=62; LYO: 11%; n=10) and phosphoric acid (L: 3%; n=5; LYO: 6%; n=5) are frequently added to liquid and lyophilised formulations, to modify their pH to a desired value and/or to create a salt form in combination with other buffer components.

Key considerations: The choice of the optimal buffer system for a specific formulation needs to be conducted by performing compatibility studies between the excipient and the specific protein. However, some general rules need to be considered, especially for lyophilised protein products. Salts that can provoke significant pH shifts during freezing should be used with caution (e.g. sodium phosphate, sodium acetate). Despite reported disadvantages, sodium phosphate is present in a high number of liquid and lyophilised products. Most of the commercial lyophilised products contain amino acids (histidine) or salts with low pH shift tendency (sodium citrate) as buffer systems. The absence of buffers in protein formulations can be considered when this does not negatively impact the quality and stability of the product.

2.3.2.3 Amino acids buffers/stabilisers/bulking agents

Amino acids play several roles in parenteral protein formulations as buffers, stabilisers or bulking agents. They can influence tonicity of the formulation and some have antioxidant properties (144-147). The solid state (amorphous or crystalline) and concentration of an amino acid can determine its role in a specific product. Amino acids use as excipients in biopharmaceutical products has increased in recent years, due to their multi-functionality (108 products (2007–2018, June) vs 54 products (1995–2007) contain amino acids). Amino acids tend to stabilise proteins by hydration or direct interactions (145). However, the mechanism of interaction between the different amino acids with proteins is complicated and not always completely understood. Furthermore, amino acids have a complex chemistry (acidic or basic, multiple functional groups) and can be included in formulation as different salt forms; all these factors can further impact the type of protein-amino acid interactions. Most of the amino acids are included in formulations in their salt form, in order to increase their solubility. The effect of the counter ion, can also impact the stability of the protein (148).

As shown in **Figure 2.5** the most commonly employed amino acids in liquid and lyophilised commercial products are histidine (L: 16%; n=27; LYO: 34%; n=31), methionine (L: 12%; n=21; LYO: 6%; n=5) and glycine (L: 9%; n=15; LYO: 13%; n=12), followed by arginine (L: 8%; n=14; LYO: 4%; n=4).

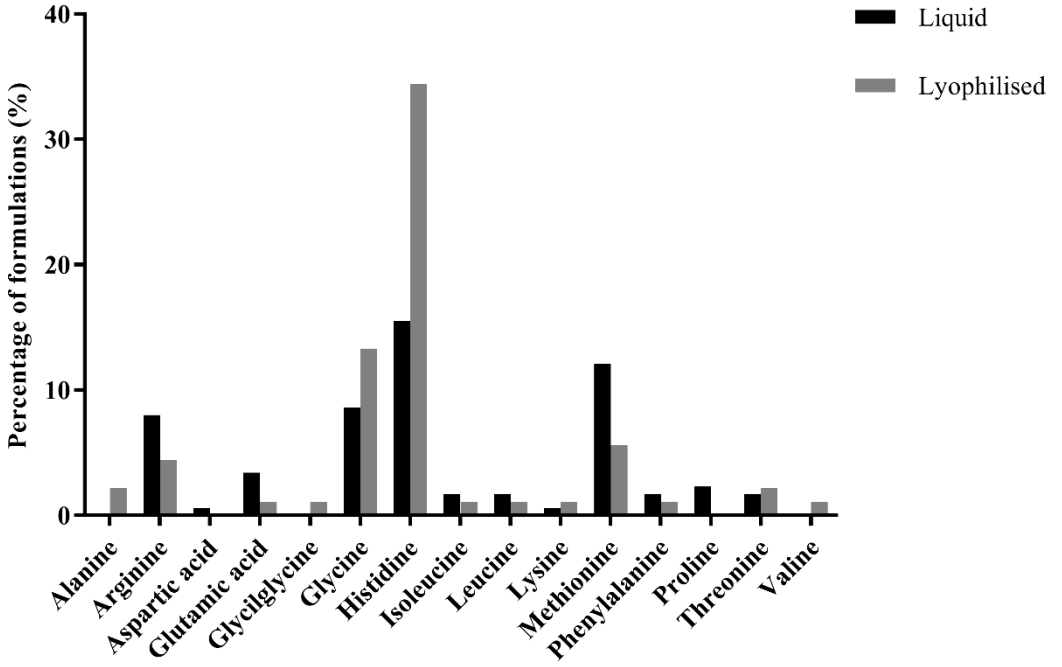


Figure 2.5 The percentage of approved liquid and lyophilised parenteral protein products containing individual amino acids buffers/stabilisers/bulking agents. Note: The function of these excipients as bulking agents is only relevant for lyophilised products.

Histidine as a buffer is reported to provide maximal stability (149), provoking minimal pH shifts during freezing (40). Histidine, is a multi-functional excipient, capable in some cases of reducing protein aggregation and functioning as cryo/lyo-protectant during the lyophilisation process in addition to acting as a buffer (150, 151). Al-Hussein et al. revealed how the role of histidine as a stabiliser was particularly important when formulated in combination with sugars and when maintained in the amorphous state (146). The concentration at which the excipient is used in different formulations determine its main role, i.e. as a stabiliser/bulking agent when present in high concentrations or as a buffer in low concentrations. Furthermore, histidine has antioxidant

properties, it can act as scavenger of hydroxy radicals in solution (144, 145). Methionine is present in a high number of liquid and lyophilised products; this excipient can be selected for its antioxidant properties (20, 145). Methionine is added to commercial products often in combination with other amino acids (e.g. histidine or arginine). Glycine is present in lyophilised products where it can act as a bulking agent, in addition to its potential buffering properties in liquid and lyophilised formulations (145).

The capability of positively charged amino acids to particularly enhance the stability of protein formulations and suppress aggregation is reported (152). Arginine is present in a relatively low number of products. However, its trend of use has increased between 2014 and 2018. Amongst the 18 products containing arginine, 10 were approved since 2014. Arginine and its salt forms have been shown to be capable of reducing protein aggregation, increasing protein solubility and reducing viscosity of protein solutions in some cases (145, 153, 154). This effect is particularly important when formulating proteins at high concentrations. However, the mechanism of interaction between arginine and proteins is not completely understood. Trout et al. (155-157) proposed a hypothesis according to which arginine molecules self-associate in clusters. These amino acidic clusters create weak hydrophobic interactions (hydrogen, electrostatic, cation- or Van der Waals) with guanidinium and aromatic groups of the protein, crowding out protein–protein interactions and avoiding aggregates formation. Furthermore, arginine and glutamic acid mixtures are shown to have a synergistic effect in increasing the solubility of proteins; this is due to the formation of additional hydrogen bonds with the protein in the presence of both excipients (158).

Other amino acids such as alanine, isoleucine, leucine, lysine, phenylalanine, proline, threonine and valine are added to a small number of commercial products; they can contribute to the stability of the protein in formulation through specific interactions (20,

145). Arginine, histidine and lysine are reported to be amorphous in the solid state, while all the other amino acids are observed to be in a crystalline form after lyophilisation (147, 159). Concentrations of amorphous amino acids in formulations intended to be lyophilised should be carefully selected, since they tend to suppress critical temperatures (Tg′ and Tc) for lyophilisation process, increasing time and processing costs (148).

Key considerations: Amino acids are a varied class of excipients with multi-functional roles and mechanisms, which are not yet completely understood especially when formulated in the dried state. The role of these excipients in protein formulation can be altered by adjusting the concentrations employed. From the analysis of this database, the trend of use of amino acids in protein formulations has increased in recent years probably due to their multi-functionality. In particular, the effect of combining different amino acids can provide a synergistic effect. Furthermore, some amino acids (basic amino acids) have properties that could be noteworthy when formulating proteins at high concentrations (e.g. to increase solubility, to reduce aggregation and viscosity) (145, 152-154, 160).

2.3.2.4 Non-amino acids stabiliser/bulking agents

The percentage of approved liquid and lyophilised parenteral products containing individual stabilisers and bulking agents are shown in **Figure 2.6**.

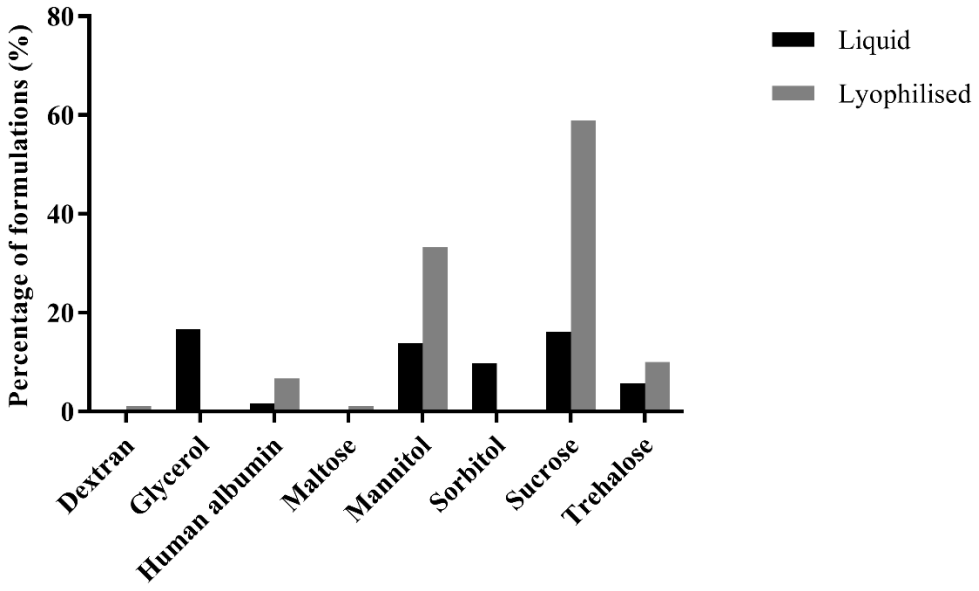


Figure 2.6 The percentage of approved liquid and lyophilised parenteral protein products containing individual non-amino acids stabilisers/bulking agents. Note: The function of these excipients as bulking agents is only relevant for lyophilised products.

The excipients included in this category are predominantly carbohydrates that can function as stabilisers in liquid and lyophilised protein products. Additionally, some of these excipients can act as bulking agents, maintaining the structure of lyophilised cakes (e.g. mannitol) (142, 161). In this case, the solid state of the excipient within the formulation determines its role. For example, mannitol which tends to crystallise is used as a bulking agent, while sucrose, which maintains its amorphous state, acts mainly as a stabiliser in lyophilised products. Due to the lack of this solid-state information in the EPAR data, it was difficult to determine the specific role of these excipients in commercial products. For this reason, stabilisers have been combined with bulking agents in a single category. The list of excipients that have been referenced as bulking agents (excluding

amino acids) is broad, consisting of human albumin, maltose, mannitol, sorbitol, sucrose and trehalose (138, 142, 144, 162-164). However, sucrose and trehalose are predominately incorporated in lyophilised products as stabilisers in an amorphous state, rather than bulking agents in their crystalline state.

Three different hypotheses have been proposed to explain the mechanism by which excipients are able to physically stabilise proteins (vitrification, exclusion and water replacement theories) (45, 58, 165). For many formulations the stabilisation may be due to the cumulative effect of these three different mechanisms. Excipients contribute to protein stabilisation by a range of mechanisms including direct interactions, minimising protein-protein interaction and aggregation, and stabilising the folded state of the protein (166-168).

Bulking agents are employed in lyophilised formulations of low dose (high potency) drugs that do not have the necessary bulk to support their own structure (e.g. total solid content < 2% (w/v)) (142, 161). While protein integrity and stability are not necessarily related to the cake structural defects, requirements for an intact cake appearance tend to be observed for commercial lyophilised products (65, 169). A review by Patel et al. (169) establishes some guidelines of what is acceptable from a product quality and regulatory perspective in terms of visual cake appearance. The presence of a crystalline compound in formulation can also reduce the reconstitution time of lyophilised products containing high concentrations of protein (37).

The most common stabilisers included in liquid formulations are glycerol (17%; n=29), sucrose (16%; n=28), and mannitol (14%; n=24). Due to the increased requirement for physical stabilisation of the protein during the lyophilisation process, non-amino acids stabilisers/bulking agents are added to the 93% (n=84) of the lyophilised formulations. Non-amino acids stabilisers/bulking agents most frequently present in lyophilised

products are sucrose (59%; n=53), mannitol (33%, n=30) and in a lower number of products trehalose (10%; n=9) and human albumin (7%; n=6). Glycerol is exclusively included in liquid products where it can have multiple roles; this excipient is a co-solvent/solubilising agent, which can serve as a tonicifier as well. Absence of glycerol in lyophilised commercial products may be attributed to its plasticising effect on the product's Tg and to the stability issues observed in some formulations, where the increase in protein mobility provokes deamidation (58). Sucrose can act as a cryo/lyo-protectant in lyophilised formulations; it is maintained in an amorphous state after lyophilisation with moisture contents lower than 2.5% (58). On the contrary, mannitol is widely present in lyophilised products as a bulking agent due to its capability to crystallize and support the lyophilised cake.

Sugars and polyols are frequently used as stabilisers (cryo/lyo protectants) and bulking agents in lyophilised products. However, carbohydrates with low Tg′ values, such as sorbitol (Tg′=−45°C) (170) can increase lyophilisation process time. This explains the absence of sorbitol as an excipient in lyophilised commercial formulations. Furthermore, sorbitol can crystallize over time and this needs to be considered in formulating a product intended to be lyophilised (170).

Despite several reported advantages, trehalose use is not widespread amongst commercial products compared to other sugars (L:6%; n=10; LYO: 10%; n=9). However, eight new products containing trehalose were approved between 2017 and 2018. Trehalose has good aqueous solubility, low hygroscopicity, high hydration number (due to its hydrophilicity), good hydrolytic stability in extreme pH and upon betaglycosidase action. This sugar has a higher Tg compared to other carbohydrates; Tg value in presence of 0.3% residual water content was reported to be approximately 111°C for trehalose, while it is approximately 65°C for sucrose (45, 142, 165). However, the following reasons may limit trehalose use

as an excipient. Firstly, business reasons can determine a company's choice of excipient; trehalose is more expensive than other stabilising sugars, such as the more commonly employed sucrose (171). Where comparative stability can be achieved with sucrose, logistic and business rationale would influence excipient choice. Secondly, the use of trehalose as stabiliser in some cases was observed to be less effective in comparison to sucrose. Jovanović et al. (172) reported significant changes in the tertiary structure of lysozyme and myoglobin in formulations containing trehalose. Finally, trehalose can be present in formulation as an heterogenous system formed by different crystalline phases. This polymorphism renders its behaviour in formulation difficult to predict, with a potential impact on stability (172, 173). The transformation paths that trehalose can undergo depend on several factors, including the solid state (amorphous or crystalline) and the dosage form (liquid or solid) as well as the dehydration process and residual moisture levels in the product (173-175). The use of trehalose could be preferred over other carbohydrates if there is a significant improvement in terms of product stability or process efficiency.

Reducing sugars (e.g. lactose and maltose) should be avoided due to potential interactions with amino acid side chains, which can cause chemical alteration of the proteins (Maillard reaction or glycation) (58). Indeed, maltose is present in only one lyophilised commercial product (ORENCIA® (abatacept)) at a concentration of 50 mg/mL and at a ratio of 2:1 maltose to abatacept in the lyophilised form prior to reconstitution (124). Maltose is reported on the EPAR's scientific discussion to be used as a stabiliser/bulking agent at the concentration in formulation (176). This sugar is included in the ORENCIA® (abatacept) lyophilised product intended to be administered intravenously but replaced by sucrose in the corresponding subcutaneous liquid product. It is important to note that the Maillard reaction is favoured in alkaline or acidic conditions, and that the pH of this

product after reconstitution is maintained neutral (pH 7.2–7.8). Trace level reducing sugars can also be found in non-reducing excipients such as mannitol, maltitol and sucrose. Hence, care should be taken regarding the quality of the excipients selected (58, 177).

Recrystallisation of sugars and polyols during manufacture and storage should be avoided, because conversion between amorphous and crystalline states can compromise the protein stability. Mannitol at high concentrations can provoke vial breakage due to recrystallisation during the lyophilisation process (primary drying phase) and storage (58). For these reasons, the introduction of an annealing step in the lyophilisation cycle is required when crystalline components are present in protein formulations. In some formulations, different sugars/ polyols are used in combination, so one excipient behaves as a stabiliser and the other as a bulking agent, for example ENBREL® (etanercept) contains sucrose and mannitol. The former is capable of stabilising the protein, whereas the latter prevents the collapse of the cake. Pikal et al. (178) reported similar examples of formulations containing glycine as a stabiliser and mannitol as a bulking agent. The main advantage in the employment of a mixture of amorphous and crystalline compounds is the possibility to reduce the lyophilisation cycle time, conducting the primary drying above the Tg' value of the amorphous phase (179). The recommended molar ratio of protein to stabiliser is 360:1 (weight ratio 1:1), whereas it is usually higher for bulking agents (40, 143, 161, 180).

Human albumin can have a number of functions in parenteral formulations, as a stabiliser (138, 144), bulking agent and tonicifier (138), but it is not employed in recent products possibly due to its potential risk of introducing contaminants (e.g. viruses) (138). Dextran 40 is included in one lyophilised antibody-drug conjugate product MYLOTARG®

(gentuzumab ozogamicin), approved in 2018. Dextran at concentrations of 9.1 mg/mL is reported to act as a bulking agent (EPAR's scientific discussion) (181).

Key considerations: The physical state of the excipient and the dosage form (liquid or lyophilised) need to be evaluated in the selection of a stabiliser/bulking agent. Sorbitol and glycerol can be used in liquid formulations, but they are not recommended in lyophilised products. Reducing sugars should be avoided in both liquid and lyophilised products because of the possibility to undergo Maillard reaction, altering the chemistry and the activity of the protein. Mannitol and sucrose are widely used especially in lyophilised commercial products. Mannitol as a bulking agent is in a crystalline form, hence the introduction of an annealing step in the lyophilisation process is required. Trehalose is a promising excipient, but its employment is limited partially due to its relative high costs.

2.3.2.5 Surfactants

Surfactants in both liquid and lyophilised formulations stabilise the protein, increasing its solubility and minimising interface interactions (182). Surfactants stabilise proteins by the following mechanisms; a) direct interactions of the surfactant molecules with hydrophobic domains exposed on the protein surface and b) interfacial competition, i.e. surfactant occupancy of the surface is more thermodynamically favoured compared to the protein occupancy (182). The use of surfactants in lyophilised products reduces the surface tension at the ice-water interface, promotes protein refolding and prevents aggregation (33, 45). High concentration protein formulations require surfactants in order to improve solubility of the protein and overcome problems related to their high tendency to form aggregates. Surfactants can also protect highly concentrated proteins from

mechanical agitation and manipulation (e.g. during syringeability) and reduce the reconstitution time of lyophilised products (28, 31).

A relatively small number of surfactants are included in the liquid and lyophilised products. The main excipients are polysorbate 80 (L: 32%, n=55; LYO: 41%, n=37), polysorbate 20 (L: 26%, n=45; LYO: 18%, n=16) and poloxamer 188 (L: 7%, n=12; LYO: 3%, n=3). Several of both formulation types (L: 35%; n=61; LYO: 38%; n=34) do not contain any surfactants, most of which are insulin-based products. The reason for the absence of surfactants in these commercial products was investigated. However, no clear trend was observed when evaluating products by year of approval, class of therapeutic protein or dosage form. The majority of surfactants included in protein formulations are non-ionic. Non-ionic surfactants are preferred over ionic surfactants which can denature proteins (182); they are also selected for their low toxicity and reduced sensitivity to the presence of electrolytes (183). Non-ionic surfactants are normally employed in a concentration range between 0.0003 and 0.3% (w/v) (45). Polysorbates are composed of fatty acid esters of polyoxyethylene sorbitan monolaurate (polysorbate 20) and polyoxyethylene sorbitan monooleate (polysorbate 80). The main disadvantage of polysorbates is their ability to undergo hydrolysis and autoxidation of the side-chains, resulting in hydrogen peroxide formation and development of short chain acids (e.g. formic acid). These sub-products can compromise the stability of a biopharmaceutical formulation (e.g. increasing the oxidation rate of proteins) and the safety of the product if accumulated in high amounts (182-184). The concentration of polysorbates in a pre-formulation stage is selected considering: a) critical micelle concentration (CMC) and b) the possibility of degradation through the manufacturing process or during storage of the product.

The concentration of surfactant needs to be carefully determined at the pre-formulation stage because of these disadvantages. An alternative surfactant is poloxamer 188, a triblock copolymer, included in a small number of liquid and lyophilised protein products (182). In comparison to polysorbates, poloxamer 188 inhibits protein adsorption through a different mechanism; which is independent from the interface affinity and allows formation of protein surfactant complexes (185). The mechanism by which surfactants reduce protein adsorption can also impact the concentration used in formulation, which does not always depend exclusively on the CMC (185). Poloxamer 171 was added to INSUMAN® solution for infusion (400 IU/mL) or injection (100 IU/mL) in the new formulation (EPAR updated 04/06/2018).

Key considerations: The most commonly employed surfactants are polysorbates. However, the impact of the formation of degradation sub/products on protein stability should be evaluated at the pre-formulation stage. If their employment is required, the minimum functional concentration should be included and the impact of their variability on product and process stability be assessed. Poloxamers represent an alternative to the polysorbates.

2.3.2.6 Preservatives

Parenteral liquid products in multi-dose vials require the presence of a preservative to minimise microbial contamination. One of the main drawbacks of antimicrobial preservatives is their significant volatility and reactivity. Furthermore, many examples of interactions between various preservatives and drugs, excipients, packaging and filter materials are reported in literature (58, 132, 186). Preservatives are usually used in low amounts (0.002–1% (w/v)) (132), however, concentrations above 1% (w/v) have been noted in commercial products. Metacresol, phenol, benzyl alcohol and benzalkonium chloride were identified as the main preservatives included in protein products.

Preservatives are mainly incorporated in multi-dose products (L: n=48; LYO: n=5) and in multi-dose lyophilised products they are always added to the solvent for reconstitution. Metacresol (L: 19%, n=33; LYO: 1%, n=1) and phenol (L: 14%, n=24; LYO: 0%) are the most frequently used preservatives, they are included respectively in 69% and 50% of the liquid multi-dose products. Metacresol is more active against gram +ve than gram −ve bacteria (144).

Phenol is the most common preservative in liquid insulin-based formulations and is active against a broader spectrum of microorganisms including viruses and mycobacteria. Phenol activity increases in acidic and concentrated solutions as well as at higher temperature. It has been reported that monoclonal antibody formulations containing phenol can lead to soluble and insoluble aggregates formation (114, 138, 144). Benzyl alcohol is present in four liquid multiple use products (8% of the multi-dose liquid products). However, it is also added to two liquid single use products (PEGASYS® (peginterferonalfa-2a) and REBIF® (interferon beta-1a)), where it acts as stabiliser to prevent oxidation (EPAR's scientific discussion) (187). Benzyl alcohol is also added to four lyophilised formulations (80% of the multi-dose lyophilised products). Benzalkonium chloride (L:1%, n=1; LYO:1%, n=1) is a quaternary ammonium compound active against gram +ve and gram −ve bacteria. Both products containing benzalkonium chloride are intended for multiple use.

Key considerations: Metacresol and phenol are the most common preservatives employed in liquid commercial products. The use of preservatives is particularly required in multi-dose preparations. Metacresol, benzyl alcohol and benzalkonium chloride were observed to be added to the diluent for reconstitution of a low number of lyophilised products (all for multiple use).

2.3.2.7 Tonicifiers

Tonicifiers are added to protein formulations to create isotonic solutions for parenteral administration. The delivery of a non-isotonic solution through a parenteral route of administration can cause damage to the tissue and pain at the site of administration. Osmolality values between 280 and 300 mOsm/Kg and <600 mOsm/Kg for intravenous and subcutaneous administration respectively represent the osmolality limitations in developing parenteral protein formulations (135). The usual range of osmolality observed in commercial protein products is between 210 and 440 mOsm/Kg (126). The achievement of iso-osmolar biopharmaceutical products is particularly challenging for high concentration protein formulations due to the high amount of protein and the overall high solute concentration. Sodium chloride (NaCl) and potassium chloride (KCl) are the two main tonicifiers, however all formulation components can contribute to the product tonicity. NaCl is used in a relatively high percentage of liquid (36%; n=62) and lyophilised (42%; n=38) commercial products and is added to formulations, especially in the liquid form, also as a stabiliser. Sodium chloride was reported to reduce the viscosity of a reconstituted high concentration protein formulation (31) and to have a stabilising effect on insulin based formulations (188). This excipient was also observed to inhibit mannitol crystallisation in frozen solutions (189). However, the use of NaCl is not optimal for lyophilised formulations, due to the ability of water and NaCl to form an eutectic mixture at −21°C that can enhance protein mobility (138, 174). This could be the justification for six lyophilised products, including BETAFERON® (interferon beta-1b) and ALPROLIX® (eftrenonacog alfa), where in sodium chloride is added to the diluent provided for reconstitution rather than to the lyophilised product (190). KCl is exclusively present in a low number of liquid formulations (L: 1%, n=2). The inclusion of sugars, polyols, amino acids and salts all increase the tonicity of a protein formulation. Hence,

excipients reported in Sections 2.3.2.3 and 2.3.2.4 contribute to the tonicity of the product. Furthermore, the impact of these excipients on the ionic strength of the formulation should be evaluated, since a high ionic strength can compromise the protein stability, promoting protein aggregation (7).

Key considerations: Achieving iso-osmolarity is recommended for products intended to be administered through parenteral routes. Tonicity of a product can be adjusted using NaCl or KCl, but also by using specific concentrations of sugars, polyols and amino acids in formulation. NaCl is present in a high number of lyophilised products, however care should be taken during lyophilisation process design due to its suppression effect on the eutectic temperature of the formulation.

2.3.2.8 Other excipients

The 'other excipients' category contains mainly complexing agents, antioxidants and solubilising agents. All these excipients have stabilising properties, but are not included in the main category stabilisers/ bulking agents for two reasons: (1) they are not bulking agents (usually used in low concentrations); (2) they are present in a low number of products and/or they are stabilising agents via specific mechanisms. The percentages of approved liquid and lyophilised parenteral products containing 'other excipients' are listed along with their main functions in Table 2.2 (138, 144, 188, 191-197).

Table 2.2 Examples of other excipients with specific functions included in liquid and lyophilised parenteral protein products.

Excipient	Function	Liquid	Lyophilised	Reference
Calcium chloride	Complexing agent; preservative	3%; n=5	19%; n=17	(138, 144)
EDTA	Complexing agent (chelating agent)	3%; n=6	0%; n=0	(144)
Glutathione	Antioxidant	0%; n=0	2%; n=2	(191)
Nicotinamide	Antioxidant; solubilizing agent	1%; n=1	0%; n=0	(144)
Pentetic acid	Complexing agent (chelating agent)	2%; n=3	0%; n=0	(144)
*Protamine sulphate**	Complexing agent to prolong insulin activity	5%; n=9	0%; n=0	(192, 193)
Recombinant human hyaluronidase	Bioavailability enhancer following SC administration	2%; n=3	0%; n=0	(195, 196)
Urea	Stabiliser (dissolving aggregates)	1%; n=1	1%; n=1	(194)
*Zinc acetate** *Zinc chloride** *Zinc oxide **	Complexing agent to prolong insulin activity	3%; n=5 10%; n=17 3%; n=5	0%; n=0 0%; n=0 0%; n=0	(138, 188)
1-palmitoyl, 2-oleoylphosphatidylcholine (POPC), 1,2-dioeloylphosphatidylserine (OOPS)	Liposomal targeting	0%; n=0	1%; n=1	(197)

**Specific for products containing insulin*

Edetic acid or edetate salt (EDTA) is used as a complexing agent in liquid formulations (L: 3%, n=6;). EDTA can form complexes (chelates) with metal ions which are removed from the solution in a process defined as sequestering. Heavy metals have the capability to catalyse autoxidation, so their removal can be required for stabilisation purpose. Usually employed EDTA concentrations are between 0.005 and 0.1% (w/v) (144). EDTA also possess antioxidant and antimicrobial properties and it can be used in combination with other antioxidants and preservatives for a synergistic effect (144). Calcium chloride (L: 3%; n=5; LYO: 19%; n=17) is usually employed as a complexing agent in lyophilised products containing coagulation factors. Coagulation factors is a class of therapeutic proteins whose activity and stability is promoted in presence of calcium ions (138).

Antioxidants minimise oxidative reactions of the API or excipients over the shelf-life of the product. Glutathione is an antioxidant found exclusively in two lyophilised products,

ADVATE® (octocog alfa) and ADYNOVI® (rurioctocog alfa pegol), both based on coagulation factors. Glutathione behaves as a reducing agent creating disulphide bonds with cysteine residues of proteins, preventing their oxidation. This process is aided by the thiol groups of glutathione which are oxidised forming glutathione disulphide (GSSG) (198-200).

Nicotinamide is included in one recent liquid product, FIASP® (insulin aspart). This excipient is reported to reduce the self-association of insulin, promoting the rapid absorption of the monomeric form, which results in a faster action. Protamine sulphate (L: 5%, n=9) is a specific excipient employed exclusively in liquid insulin products to prolong the action of the therapeutic protein. Zinc acetate (L: 3% n=5), zinc chloride (L: 10% n=17) and zinc oxide (L: 3% n=5) are also additives used specifically in liquid formulations based on insulin. The presence of zinc ions in the formulation at specific concentrations promote the association of insulin molecules in hexamers, increasing the protein stability and prolonging its activity (188).

Finally, recombinant human hyaluronidase (rHuPH20) is a novel excipient that enhances protein bioavailability following subcutaneous administration. It modifies and creates conduits in the interstitial matrix to promote dispersion of molecules including proteins (196). This excipient, exclusively employed in three liquid antibody products intended to be administered subcutaneously, is reported to reduce administration times in comparison to the corresponding intravenous products (201).

Key considerations: The employment of ‘other excipients’ can be due to the necessity to further improve the performance of the product, overcoming specific issues. Their inclusion in formulations should be evaluated case by case.

2.3.2.9 Additional considerations in excipient selection

Data analysis showed how qualitative and/or quantitative composition can vary for protein products according to different factors: (1) formulation approach (liquid or lyophilised); (2) route of administration; (3) API concentration; (4) primary packaging container; (5) different formats of liquid formulations (concentrate vs solution). Commercial examples are provided for each of the listed factors. BENLYSTA® (belimumab), COSENTYX® (secukinumab), ENBREL® (etanercept) and XOLAIR® (omalizumab) are all examples in which the excipient composition differs between the liquid and lyophilised product. In particular, a replacement of stabilisers (amino acids or trehalose) with sucrose in the lyophilised form was observed in BENLYSTA® (belimumab), COSENTYX® (secukinumab) and XOLAIR® (omalizumab). In ENBREL® (etanercept), the sodium phosphate salt included in the liquid form is replaced by the TRIS salt in the lyophilised form. Mannitol is also added, in combination with sucrose, to the lyophilised product. HERCEPTIN® (trastuzumab) contains methionine and rHuPH20, which are not present in the lyophilised product. The route of administration of a specific product can also impact the choice of the excipients; MABTHERA® (rituximab) is an antibody product whose subcutaneous formulation contains rHuPH20. This excipient is excluded from the product for intravenous use for reasons related to its function and discussed in Section 2.3.2.8. The different API concentrations can have varied qualitative and quantitative excipient compositions, this is the case of HUMIRA® (adalimumab) and OMNITROPE® (somatropin). Furthermore, LANTUS® (insulin glargine) and TOUJEO® (insulin glargine) liquid products do not contain polysorbate 20 in the cartridges; however, the surfactant is present in the vials. Some excipients can be added to specific types of liquid formulations, for instance, STELARA® (ustekinumab) concentrate for solution for IV infusion (20 mg/mL) contains EDTA, which is not

included in the highly concentrated (80 mg/mL) subcutaneous liquid solution. Therefore, excipients should be carefully selected considering several factors with the aim of improving the stability of a target/final product with specific characteristics.

Regarding lyophilised products, the addition of specific excipients to the diluent for reconstitution may improve stability of the product during its life span between reconstitution and administration. As discussed before, sodium chloride or preservatives can be included directly in the diluent for reconstitution. Furthermore, REFIXIA® (nonacog beta pegol) contains 4.2 mL of 10 mM histidine solution in a prefilled syringe as diluent for reconstitution which was observed to improve the stability of the final product (202). BLINCYTO® (blinatumomab) includes a stabiliser solution (containing lysine, citric acid, polysorbate 80 and sodium hydroxide) to prevent adsorption of blinatumomab to the surfaces of administration; hence, it is added to the IV infusion bag (203).

2.3.3 Approved antibody products

2.3.3.1 Antibody concentration and relationship with the route of administration

The number of antibody products approved up to June 2018 was 94 (L: 73%, n=69; LYO: 27%, n=25). **Figure 2.7** shows the trend in the number of liquid and lyophilised parenteral antibody products approved per year from 1998, when the first antibody product was approved, to June 2018.

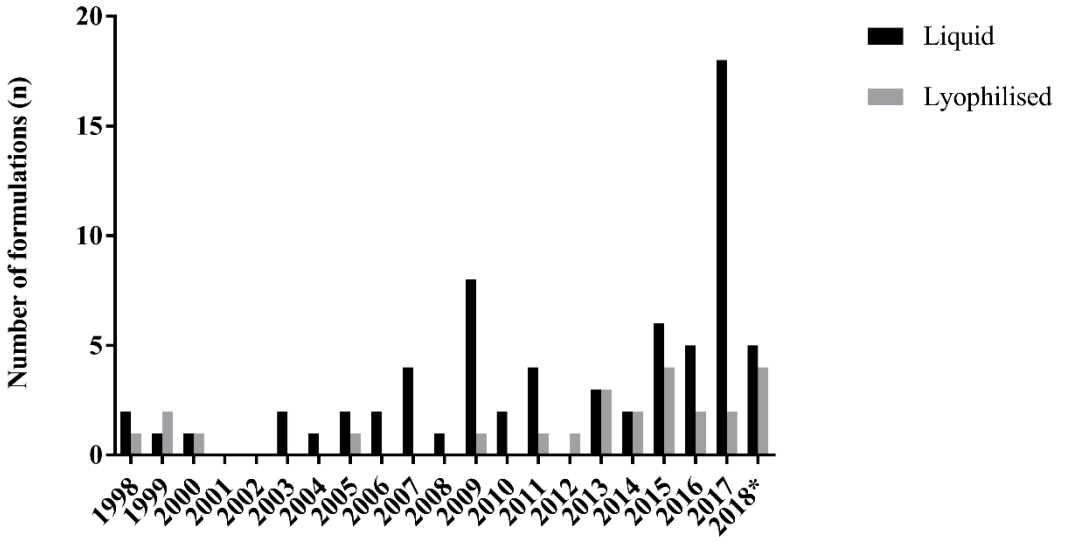


Figure 2.7 Trend of liquid and lyophilised parenteral antibody products approved per year by the EMA between 1995 and 2018 (June) (*Last updated: 18/06/ 2018).

Liquid formulations are more common than lyophilised formulations (119) and the number of marketed products per year has significantly increased in 2017. The most common class of antibodies in approved products is IgG1 (L: 70% n=48; LYO: 84% n=21). A lower number of liquid products contain IgG4 (L: 10% n=7), human normal IgG (L: 9% n=6), and IgG2 (L: 7% n=5) as API. Two liquid products contain Fab fragments (PRAXBIND® (idarucizumab) and CIMZIA® (certolizumab pegol)) and one liquid product contains an IgG2/4 (SOLIRIS® (eculizumab)). Lyophilised products are predominately based on IgG1 with three products containing IgG4. Only two bispecific antibody products are currently approved by the EMA. One HEMLIBRA® (emicizumab) is formulated as a liquid and the second BLINCYTO® (blinatumomab) as a lyophilised product. The four approved antibody-drug conjugates (ADC) are all in a lyophilised dosage form. No particular trend was observed in the use of specific excipients for the different classes of antibody. However, human normal immunoglobulin products tend to have a low number of excipients in their formulations (1 or 2).

Figure 2.8 shows the antibody concentration (mg/mL) in liquid products before dilution and in lyophilised products following reconstitution. Approximately half of these products (L: 48%; LYO: 68%) contain antibodies at a concentration ≤ 50mg/mL.

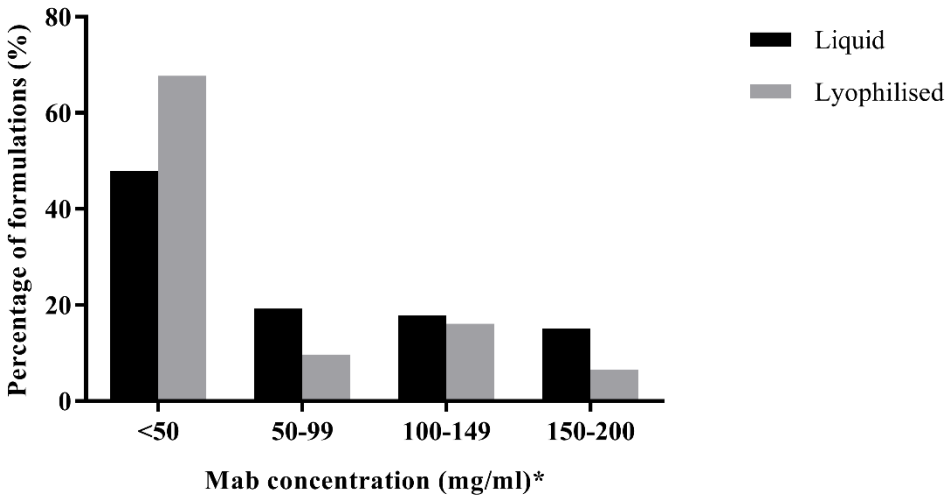


Figure 2.8 Range of therapeutic concentrations (mg/mL) of liquid and lyophilised parenteral antibody products. *for lyophilised formulations intended after reconstitution.

Figure 2.9 reports the amount of therapeutic protein per vial (mg/vial) for lyophilised products. Information regarding the fill volume of the vials before lyophilisation was not provided in the EPAR data, therefore it is not possible to determine the initial concentration of protein or the total solute concentration prior to lyophilisation. However, a higher percentage of products contain protein in amounts ≥ 100mg/vial.

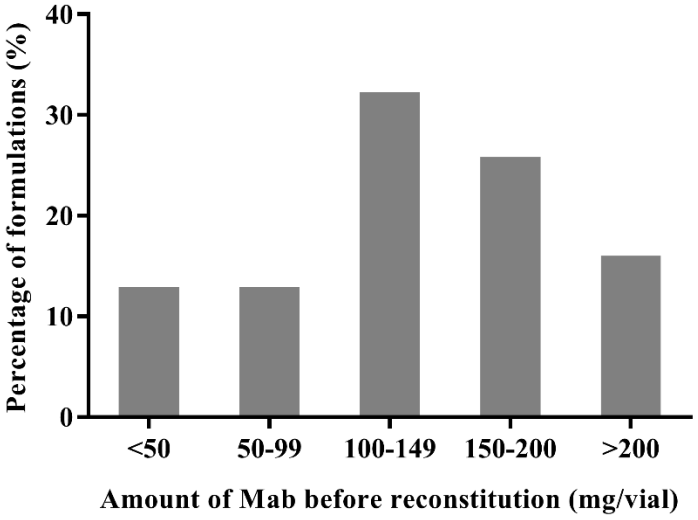


Figure 2.9 Amount of antibody per vial (mg/vial) for lyophilised parenteral antibody products.

Analysis of products' protein concentration (lyophilised products after reconstitution) reveals a clear relationship with the route of administration, **Figure 2.10.** More than half of the commercial antibody products are intended to be administered intravenously (60%). Antibody formulations with high concentrations of protein (>50 mg/mL) are more commonly administered subcutaneously. The antibody concentrations in formulation depends on the therapeutic effect and the route of administration selected to deliver the dose. For example, LUCENTIS® (ranibizumab), a product for the treatment of macular degeneration and edema, myopia and diabetes complications, is formulated at a relatively low concentration (10 mg/mL) and is intended to be administered by intravitreal route (204).

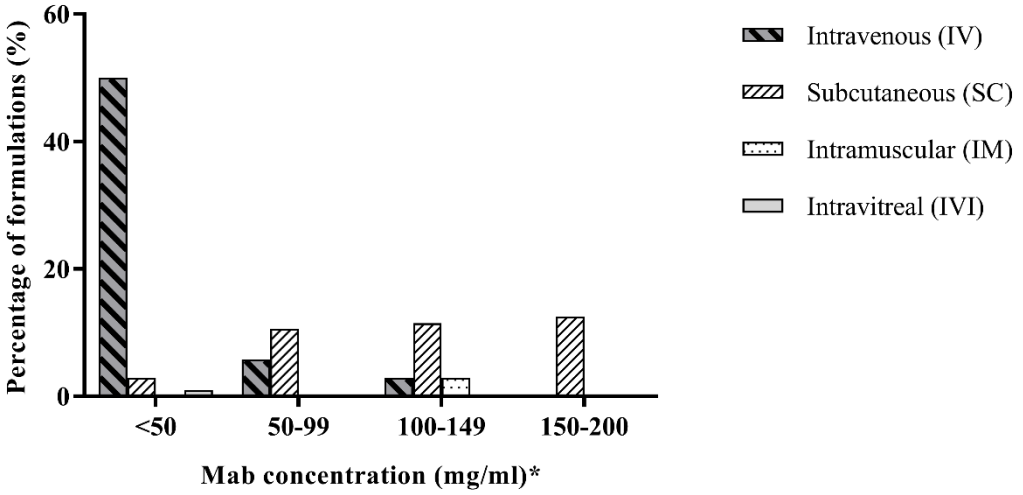


Figure 2.10 Relationship between ranges of antibody concentrations (mg/mL) and route of administration. *for lyophilised formulations intended after reconstitution.

2.3.3.2 Qualitative and quantitative excipient composition

The categories of excipients included in liquid and lyophilised antibody products are shown in **Figure 2.11**. Antibody formulations contain non-amino acids buffers in approximately half of both liquid (54%; n=37) and lyophilised (56%; n=14) commercial products. Amino acids are also added to approximately 50% of the products in both dosage forms (L: 54%; n=37; LYO: 52%; n=13). A high number of liquid formulations contain non-amino acids stabilisers (L: 59%, n=41), whereas all lyophilised products contain non-amino acids stabilisers/bulking agents (LYO: 100%, n=25). Most of the liquid and lyophilised products include surfactants (L: 87%, n=60; LYO: 88%, n=22). Preservatives are not present in any antibody formulations, which are always provided in a single use dosage form. Sodium chloride and potassium chloride are more frequently added to liquid products (L:36%, n=25) where they can act as tonicifiers and/or stabilisers. Excipients classified as 'other excipients' are only included in a small number of liquid products (13%; n=9).

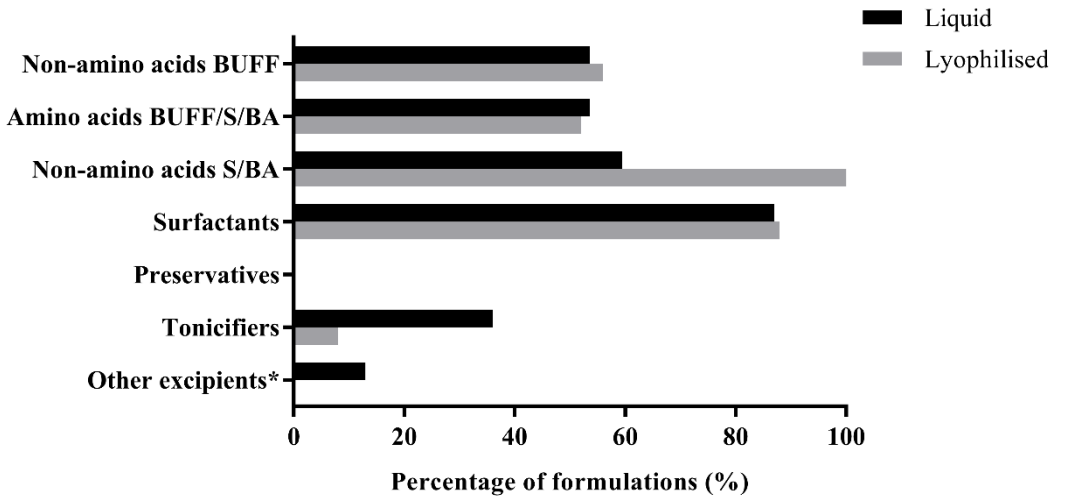


Figure 2.11 The percentage of liquid and lyophilised parenteral antibody products that contain excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/bulking agents); Surfactants; Preservatives; Tonicifiers and Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products *'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations.

Figure 2.12 shows the percentage of liquid and lyophilised antibody formulations that contain individual excipients. Amongst non-amino acids buffers the most commonly employed excipients are: sodium citrate (L: 20%, n=14; LYO: 20%, n=5) and sodium phosphate (L: 12%, n=8; LYO: 28%, n=7). As for protein products, sodium acetate (L: 22%, n=15) is observed to be included exclusively in liquid products. Histidine (L: 39%; n=27; LYO: 40%; n=10) is the most common amino acid added to liquid and lyophilised antibody products, followed by the amino acid glycine (L: 12%; n=8; LYO: 8%; n=2). Arginine is more frequently used in the liquid forms (10%; n=7) than the lyophilised forms (4%; n=1). Methionine is included exclusively in liquid products (9%; n=6).

Sucrose is the most common non-amino acid stabiliser and is present in most of the lyophilised products (L: 20%, n=14; LYO: 72%, n=18). Sorbitol is included exclusively in liquid products (10%; n=7). Surfactants employed comprise polysorbate 80 (L: 55%, n=38; LYO: 64%, n=16), polysorbate 20 (L: 29%, n=20; LYO: 24%, n=6) and to a lesser extent poloxamer 188 (L: 3%, n=2; LYO: 0%). Sodium chloride is present in a high

number of liquid products (L:36%, n=25) and only three lyophilised products with low concentrations of antibodies, two of which are ADC. Finally, excipients classified as 'other excipients' and employed in antibody formulations include chelating agents (EDTA and pentetic acid), calcium chloride as complexing agent and rHuPH20. rHuPH20 is exclusively present in MABTHERA® (rituximab), HERCEPTIN® (trastuzumab) and HYQVIA® (human normal immunoglobulin), all liquid products based on antibodies.

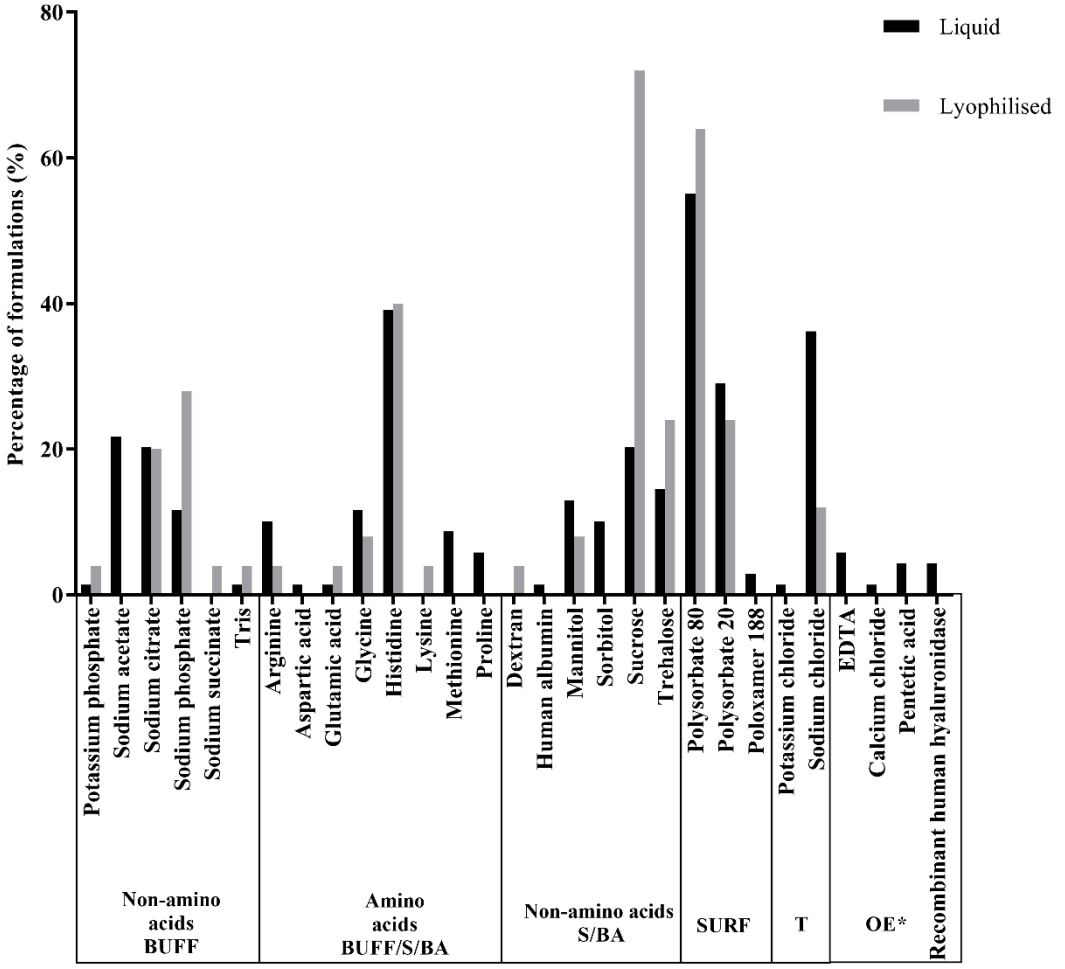


Figure 2.12 The percentage of liquid and lyophilised parenteral antibody products that contain individual excipients from each of the excipient categories: Non-amino acids BUFF (buffers); Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents); Non-amino acids S/BA (stabilisers/ bulking agents); (SURF) Surfactants; (T) Tonicifiers; (OE*) Other excipients. Note: The function of these excipients as bulking agents is only relevant for lyophilised products * (OE) 'Other excipients' category consists of complexing agents, antioxidants, solubilising agents and excipients specific to individual formulations.

The quantitative composition of 88 out of 94 liquid and lyophilised antibody commercial products was investigated consulting accredited sources and summarised in **Table 2.3** (Next page) (28, 117, 118, 205).

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration (124-126, 130). (Continue to next page)

Excipient name	Excipient quantitative composition of approved LIQUID antibody products	Excipient quantitative composition of approved LYOPHILISED antibody products
Potassium phosphate (Non-amino acids BUFF)[1]	**LEMTRADA® (alemtuzumab)** 0.2 mg/mL	**SIMULECT® (basiliximab)** 1.44 mg/mL
Sodium acetate (Non-amino acids BUFF) [1]	**AMGEVITA® (adalimumab)** 0.6 mg/mL acetic acid; **BAVENCIO® (avelumab)** 0.6 mg/mL; **SOLYMBIC® (adalimumab)** 0.6 mg/mL acetic acid; **TECENTRIQ® (atezolizumab)** 0.83 mg/mL acetic acid; **DUPIXENT® (dupilumab)** 1 mg/mL; **REPATHA® (evolocumab)** 1.2 mg/mL; **CIMZIA® (certolizumab pegol)** 1.36 mg/mL **PROLIA® (denosumab)** 17 mM (1.4 mg/mL); **XGEVA® (denosumab)** 18 mM (1.5 mg/mL); **OCREVUS® (ocrelizumab)** 20 mM (EMA); 2.14 mg/mL, 0.25 acetic acid (FDA); **CINQAERO® (reslizumab)** 2.45 mg/mL; 0.12 mg/mL acetic acid; **PRAXBIND® (idarucizumab)** 2.95 mg/mL, 0.20 mg/mL acetic acid; **DARZALEX® (daratumumab)** 2.96 mg/mL, 0.18 mg/mL acetic acid; **CYLTEZO® (adalimumab**) 3 mg/mL; 0.16 mg/mL acetic acid, **ARZERRA® (ofatumumab)** 6.8 mg/mL; **VECTIBIX® (panitumumab)** 6.8 mg/mL	None
Sodium citrate (Non-amino acids BUFF) [1]	**HUMIRA® (adalimumab, 50 mg/mL)** 1.3 mg/mL citric acid, 0.3 mg/mL; **ERBITUX® (cetuximab)** 10 mM (1,92 mg/mL) citric acid; **PORTRAZZA® (necitumumab)** 10 mM (EMA), 2.55 mg/mL, 0.26 mg/mL citric acid (FDA); **ZINPLAVA® (bezlotoxumab)** 4.75 mg/mL; 0.8 mg/mL citric acid, **TALTZ® (ixekizumab)** 5.11 mg/mL, 0.51 mg/mL citric acid; **OPDIVO® (nivolumab)** 5.88 mg/mL; **BLITZIMA® (rituximab)** 25 mM (7.35 mg/mL) tri-sodium dihydrate; **RITEMVIA® (rituximab)** 25 mM (7.35 mg/mL) tri-sodium dihydrate; **RITUZENA® (rituximab)** 25 mM (7.35 mg/mL) tri-sodium dihydrate; **TRUXIMA® (rituximab)** 25 mM (7.35 mg/mL) tri-sodium dihydrate; **MABTHERA® (rituximab, 10 mg/mL)** 7.35 mg/mL	**BLINCYTO® (blinatumomab)** 1.20 mg/mL citric acid; **EMPLICITI® (etoluzumab)** 1.38 mg/mL; 0.20 mg/mL citric acid; **BENLYSTA® (belimumab)** 2.7 mg/mL, 0.16 mg/mL citric acid; **ADCETRIS® (brentuximab vedotin)** 20 mM*** (EMA); 5.6 mg/mL, 0.21 mg/mL citric acid (FDA))
Sodium phosphate (Non-amino acids BUFF) [1]	**TYSABRI® (natalizumab)** 1.13 mg/mL monobasic monohydrate; 0.48 mg/mL dibasic heptahydrate; **LEMTRADA® (alemtuzumab)** 1.15 mg/mL dibasic dihydrate; **HUMIRA® (adalimumab, 50 mg/mL)** 1.53 mg/mL dibasic dihydrate, 0.85 mg/mL monobasic dihydrate; **HYQVIA® (human normal Ig)** 1.78 mg/mL dibasic; **SOLIRIS® (eculizumab)** 1.78 mg/mL dibasic, 0.46 mg/mL monobasic; **AVASTIN® (bevacizumab)** 51 mM (EMA), 5.8 mg/mL monobasic monohydrate, 1.2 mg/mL dibasic anhydrous (FDA); **MVASI® (bevacizumab)** 5.8 mg/mL monobasic monohydrate, 1.2 mg/mL dibasic anhydrous; **ROACTEMRA® (tocilizumab, 20 mg/mL)** 15 mM*	**SIMULECT® (basiliximab)** 0.20 mg/mL; **FLIXABI® (inflixabi)** 0.56 mg/mL monobasic monohydrate, 0.26 mg/mL dibasic heptahydrate; **MYLOTARG® (gemtuzumab ozogamicin)** 0.6 mg/mL dibasic anhydrous, 0.1 mg/mL monobasic monohydrate; **INFLECTRA® (infliximab)** 0.61 mg/mL dibasic dihydrate; 0.22 mg/mL monobasic monohydrate; **REMSIMA® (infliximab)** 0.61 mg/mL dibasic dihydrate, 0.22 mg/mL monobasic monohydrate; **REMICADE® (infliximab)** 0.61 mg/mL dibasic dihydrate, 0.22 mg/mL monobasic monohydrate; **NUCALA® (mepolizumab)** 7.14 mg/mL dibasic heptahydrate
Sodium succinate (Non-amino acids BUFF) [1]	None	**KADCYLA® (trastuzumab emtansine)** 10 mM (1.18 mg/mL)

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration. (Continued)

Excipient name	Excipient quantitative composition of approved LIQUID antibody products	Excipient quantitative composition of approved LYOPHILISED antibody products
Tris (Non-amino acids BUFF) [1]	**YERVOY® (ipilimumab)** 3.15 mg/mL HCl	**BESPONSA® (inotuzumab ozogamicin)** 2.4 mg/mL
Arginine (Amino acids BUFF/S/BA) [2]	**DUPIXENT® (dupilumab)** 5.25 mg/mL HCl monohydrate; **BENLYSTA® (belimumab)** 5.3 mg/mL HCl monohydrate; **KEVZARA® (sarilumab)** 7.84 mg/mL; **ARZERRA® (ofatumumab)** 10 mg/mL; **HEMLIBRA® (emicizumab)** 150 mM (26.13 mg/mL); **XOLAIR® (omalizumab)** 42.1 mg/mL HCl monohydrate	**ENTYVIO® (vedolizumab)** 125 mM (26.34 mg/mL) HCl monohydrate
Glycine (Amino acids BUFF/S/BA) [2]	**SYNAGIS® (palivizumab)** 0.1 mg/mL; **ERBITUX® (cetuximab)** 100 mM (7.5 mg/mL); **LARTRUVO® (olaratumab)** 7.5 mg/mL; **CYRAMZA® (ramucirumab)** 133 mM **(**9.98 mg/mL); **PORTRAZZA® (necitumumab)** 133 mM (9.98 mg/mL); **KIOVIG® (human normal Ig)** 250 mM (18.77 mg/mL); **HyQVIA® (human normal Ig)** 250 mM (18.77 mg/mL)	**SYNAGIS® (palivizumab)** 0.2 mg/mL; **SIMULECT® (basiliximab)** 8 mg/mL
Glutamic acid (Amino acids BUFF/S/BA) [2]	**KYNTHEUM® (brodalumab)** 4.33 mg/mL	None
Histidine (Amino acids BUFF/S/BA) [2]	**SIMPONI® (golimumab)** 0.87 mg/mL; **PRALUENT® (alirocumab, 75 mg/mL)** 8 mM (1.24 mg/mL); **PRALUENT® (alirocumab, 100 mg/mL)** 6 mM (0.93 mg/mL); **STELARA® (ustekinumab, 90 mg/mL)** 1 mg/mL; **STELARA® (ustekinumab, 5 mg/mL)** 1.04 mg/mL HCl monohydrate, 0.77 mg/mL,; **BENLYSTA® (belimumab)** 1.2 mg/mL HCl monohydrate, 0.65 mg/mL; **CYRAMZA® (ramucirumab)** 10 mM (EMA), 1.22 mg/mL HCl monohydrate, 0.65 mg/mL (FDA); **FASENRA® (benralizumab)** 1.4 mg/mL, 2.3 mg/mL HCl monohydrate; **TREMFYA® (guselkumab)** 1.5 mg/mL HCl monohydrate, 0.6 mg/mL; **KEYTRUDA® (pembrolizumab)** 1.55 mg/mL; **CRYSVITA® (burosumab)** 1.55 mg/mL; **LARTRUVO® (olaratumab)** 1.7 mg/mL HCl monohydrate, 0.3 mg/mL; **ILARIS® (canakinumab)** 2.1 mg/mL, 1.3 mg/mL HCl monohydrate; **XOLAIR® (omalizumab)** 2.34 mg/mL HCl monohydrate, 1.37 mg/mL; **COSENTYX® (secukinumab)** 20 mM (3.1 mg/mL); **DUPIXENT® (dupilumab)** 3.1 mg/mL; **HEMLIBRA® (emicizumab)** 20 mM (3.1 mg/mL); **QARZIBA® (dinutuximab beta)** 20 mM (3.1 mg/mL); **TECENTRIQ® (atezolizumab)** 20 mM (3.1 mg/mL); **KEVZARA® (sarilumab)** 3.25 mg/mL; **MABTHERA® (rituximab, 120 mg/mL)** 3.47 mg/mL HCl monohydrate, 0.53 mg/mL; **SYNAGIS® (palivizumab)** 3,9 mg/mL; **PERJETA® (pertuzumab)** 20 mM (4.28 mg/mL) acetate; **LUCENTIS® (ranibizumab)** 10 mM**; **GAZYVARO® (obinutuzumab)** 20 mM**; **HERCEPTIN® (trastuzumab)** 20 mM**	**HERCEPTIN® (trastuzumab)** 0.48 mg/mL HCl monohydrate, 0.31 mg/mL; **HERZUMA® (trastuzumab)** 0.48 mg/mL HCl monohydrate, 0.31 mg/mL; **KANJINTI® (trastuzumab)** 0.48 mg/mL HCl monohydrate, 0.31 mg/mL; **ONTRUZANT® (trastuzumab)** 0.48 mg/mL HCl monohydrate, 0.31 mg/mL; **SYLVANT® (siltuximab)** 0.74 mg/mL; **KEYTRUDA® (pembrolizumab)** 1.55 mg/mL; **XOLAIR® (omalizumab)** 2 mg/mL HCl monohydrate, 1.3 mg/mL; **ILARIS® (canakinumab)** 2.8 mg/mL, 1.7 mg/mL HCl monohydrate; **COSENTYX® (secukinumab)** 30 mM (4.66 mg/mL); **SYNAGIS® (palivizumab)** 7.3 mg/mL; **ENTYVIO® (vedolizumab)** 50 mM (EMA), 4.6 mg/mL, 4.28 mg/mL HCl monohydrate (FDA)
Lysine (Amino acids BUFF/S/BA) [2]	None	**BLINCYTO® (blinatumomab)** 8.30 mg/mL HCl monohydrate

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration. (Continued)

Excipient name	Excipient quantitative composition of approved LIQUID antibody products	Excipient quantitative composition of approved LYOPHILISED antibody products
Methionine (Amino acids BUFF/S/BA) [2]	**STELARA® (ustekinumab, 5 mg/mL)** 0.4 mg/mL; **COSENTYX® (secukinumab)** 5 mM (0.75 mg/mL); **HERCEPTIN® (trastuzumab)** 10 mM (1.49 mg/mL); **CRYSVITA® (burosumab)** 1.49 mg/mL; **MABTHERA® (rituximab, 120 mg/mL)** 1.49 mg/mL	None
Proline (Amino acids BUFF/S/BA) [2]	**KYNTHEUM® (brodalumab)** 24 mg/mL; **REPATHA® (evolocumab)** 25 mg/mL; **PRIVIGEN® (human normal Ig)** 250 mM (28.78 mg/mL); **HIZENTRA® (human normal Ig)** 250 mM (28.78 mg/mL)	None
Dextran (Non-amino acids S/BA) [3]	None	**MYLOTARG® (gentuzumab ozogamicin)** 9.1 mg/mL
Human albumin (Non-amino acids S/BA) [3]	**HYQVIA® (human normal Ig)** 1 mg/mL	None
Mannitol (Non-amino acids S/BA) [3]	**PORTRAZZA® (necitumumab)** 50 mM (9.1 mg/mL); **YERVOY® (ipilimumab)** 10 mg/mL; **HUMIRA® (adalimumab, 50 mg/mL)** 12 mg/mL; **LARTRUVO® (olaratumab)** 13.7 mg/mL; **DARZALEX® (daratumumab)** 25.5 mg/mL; **OPDIVO® (nivolumab)** 30 mg/mL; **HUMIRA® (adalimumab, 100 mg/mL)** 42 mg/mL; **ILARIS® (canakinumab)** 49.2 mg/mL; **BAVENCIO® (avelumab)** 51 mg/mL	**SIMULECT® (basiliximab)** 16 mg/mL; **SYNAGIS® (palivizumab)** 56.3 mg/mL
Sorbitol (Non-amino acids S/BA) [3]	**PRAXBIND® (idarucizumab)** 40.08 mg/mL; **SIMPONI® (golimumab)** 41 mg/mL; **CRYSVITA® (burosumab)** 45.91 mg/mL; **XGEVA® (denosumab)** 46 mg/mL; **PROLIA® (denosumab)** 47 mg/mL; **FLEBOGAMMA DIF® (human normal Ig, 50 mg/mL)** 50 mg/mL	None
Sucrose (Non-amino acids S/BA) [3]	**PERJETA® (pertuzumab)** 120 mM (41.08 mg/mL); **TECENTRIQ® (atezolizumab)** 120 mM (41.08 mg/mL); **DUPIXENT® (dupilumab)** 50 mg/mL; **KEVZARA® (sarilumab)** 50 mg/mL; **QARZIBA® (dinutuximab beta)** 50 mg/mL; **ROACTEMRA® (tocilizumab, 20 mg/mL)** 50 mg/mL; **CINQAERO® (reslizumab)** 70 mg/mL; **KEYTRUDA® (pembrolizumab)** 70 mg/mL; **STELARA® (ustekinumab, 90 mg/mL)** 76 mg/mL; **TREMFYA® (guselkumab)** 79 mg/mL **STELARA® (ustekinumab, 5 mg/mL)** 85 mg/mL; **AMGEVITA® (adalimumab)** 90 mg/mL; **SOLYMBIC® (adalimumab**) 90 mg/mL; **PRALUENT® (alirocumab)** 100 mg/mL	**SIMULECT® (basiliximab)** 4 mg/mL; **MYLOTARG® (gemtuzumab ozogamicin)** 15.5 mg/mL; **SYLVANT® (siltuximab)** 33.8 mg/mL; **EMPLICITI® (etoluzumab)** 42.5 mg/mL; **BESPONSA® (inotuzumab ozogamicin)** 50 mg/mL; **FLIXABI® (infliximab)** 50 mg/mL; **INFLECTRA® (infliximab)** 50 mg/mL; **REMICADE® (infliximab)** 50 mg/mL; **REMSIMA® (infliximab)** 50 mg/mL; **KADCYLA® (trastuzumab emtansine)** 60 mg/mL; **KEYTRUDA (pembrolizumab)** 70 mg/mL; **BENLYSTA® (belimumab)** 80 mg/mL; **ILARIS® (canakinumab)** 92.4 mg/mL; **COSENTYX® (secukinumab)** 270 mM (EMA), 92.4 mg/mL (FDA); **ENTYVIO® (vedolizumab)** 100 mg/mL; **XOLAIR® (omalizumab)** 104 mg/mL; **NUCALA® (mepolizumab)** 160 mg/mL

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration. (Continued)

Excipient name	Excipient quantitative composition of approved LIQUID antibody products	Excipient quantitative composition of approved LYOPHILISED antibody products
Trehalose (Non-amino acids S/BA) [3]	**OCREVUS® (ocrelizumab)** 106 mM (EMA), 40 mg/mL (FDA); **AVASTIN® (bevacizumab)** 60 mg/mL**; MVASI® (bevacizumab)** 60 mg/mL; **COSENTYX® (secukinumab)** 200 mM (EMA), 75.67 mg/mL (FDA)**; HERCEPTIN® (trastuzumab)** 210 mM (71.88 mg/mL); **MABTHERA® (rituximab, 120 mg/mL)** 79,45 mg/mL; **CYLTEZO® (adalimumab)** 81.25 mg/mL; **GAZYVARO® (obinutuzumab)** 240 mM (82.15 mg/mL); **FASENRA® (benralizumab)** 95 mg/mL; **LUCENTIS® (ranibizumab)** 100 mg/mL	**HERCEPTIN® (trastuzumab)** 19.05 mg/mL; **KANJINTI® (trastuzumab)** 19.05 mg/mL; **ONTRUZANT® (trastuzumab)** 19.05 mg/mL; **BLINCYTO® (blinatumomab)** 34.11 mg/mL; **ADCETRIS® (brentuximab vedotin)** 63 mg/mL (EMA), 70 mg/mL (FDA)
Polysorbate 80 (Surfactants)	**HIZENTRA® (human normal Ig)** 0.02 mg/mL (EMA), 0.008-0.03 mg/mL (FDA); **STELARA® (ustekinumab, 90 mg/mL)** 0.04 mg/mL; **BENLYSTA® (belimumab)** 0.1 mg/mL; **ERBITUX® (cetuximab)** 0.1 mg/mL; **YERVOY® (ipilimumab)** 0.1 mg/mL; **LEMTRADA® (alemtuzumab)** 0.1 mg/mL; **CYRAMZA® (ramucirumab)** 0.1 mg/mL; **REPATHA® (evolocumab)** 0.1 mg/mL; **PORTRAZZA® (necitumumab)** 0.1 mg/mL; **SIMPONI® (golimumab)** 0.16 mg/mL; **ARZERRA® (ofatumumab)** 0.2 mg/mL; **COSENTYX® (secukinumab)** 0.2 mg/mL; **KEYTRUDA® (pembrolizumab)** 0.2 mg/mL; **OPDIVO® (nivolumab)** 0.2 mg/mL; **TYSABRI® (natalizumab)** 0.2 mg/mL; **SOLIRIS® (eculizumab)** 0.22 mg/mL; **ZINPLAVA® (bezlotoxumab)** 0.25 mg/mL; **TALTZ® (ixekizumab)** 0.3 mg/mL; **ILARIS® (canakinumab)** 0.4 mg/mL; **STELARA® (ustekinumab, 5 mg/mL)** 0.4 mg/mL; **CRYSVITA® (burosumab)** 0.5 mg/mL; **ROACTEMRA® (tocilizumab, 20 mg/mL)** 0.5 mg/mL; **TREMFYA® (guselkumab)** 0.5 mg/mL; **MABTHERA® (rituximab, 120 mg/mL)** 0.6 mg/mL**; MABTHERA® (rituximab, 10 mg/mL)** 0.7 mg/mL; **BLITZIMA® (rituximab)** 0.7 mg/mL; **RITEMVIA® (rituximab)** 0.7 mg/mL; **RITUZENA® (rituximab)** 0.7 mg/mL; **TRUXIMA® (rituximab)** 0.7 mg/mL; **AMGEVITA® (adalimumab)** 1 mg/mL; **CYLTEZO® (adalimumab)** 1 mg/mL; **HUMIRA® (adalimumab, 50 mg/mL)** 1 mg/mL; **HUMIRA® (adalimumab, 100 mg/mL)** 1 mg/mL; **SOLYMBIC® (adalimumab)** 1 mg/mL; **DUPIXENT® (dupilumab)** 2 mg/mL	**FLIXABI® (infliximab)** 0.05 mg/mL; **INFLECTRA® (infliximab)** 0.05 mg/m; **REMICADE® (infliximab)** 0.05 mg/mL; **REMSIMA® (infliximab)** 0.05 mg/mL; **BESPONSA® (inotuzumab ozogamicin)** 0.1 mg/mL; **SYLVANT® (siltuximab)** 0.16 mg/mL; **ADCETRIS® (brentuximab vedotin)** 0.2 mg/mL; **KEYTRUDA® (pembrolizumab)** 0.2 mg/mL; **BLINCYTO® (blinatumomab)** 0.23 mg/mL; **EMPLICITI® (etoluzumab)** 0.28 mg/mL; **BENLYSTA® (belimumab)** 0.4 mg/mL; **ILARIS® (canakinumab)** 0.6 mg/mL; **ENTYVIO® (vedolizumab)** 0.6 mg/mL; **COSENTYX® (secukinumab)** 0.6 mg/mL; **NUCALA® (mepolizumab)** 0.67 mg/mL
Polysorbate 20 (Surfactants)	**FASENRA® (benralizumab)** 0.06 mg/mL; **LUCENTIS® (ranibizumab)** 0.1 mg/mL; **KYNTHEUM® (brodalumab)** 0.1 mg/mL; **PROLIA® (denosumab)** 0.1 mg/mL; **PRALUENT® (alirocumab)** 0.1 mg/mL; **QARZIBA® (dinutuximab beta)** 0.1 mg/mL; **XGEVA® (denosumab)** 0.1 mg/mL; **OCREVUS® (ocrelizumab)** 0.2 mg/mL; **PERJETA® (pertuzumab)** 0.2 mg/mL; **PRAXBIND® (idarucizumab)** 0.2 mg/mL; **LARTRUVO® (olaratumab)** 0.2 mg/mL; **AVASTIN® (bevacizumab)** 0.4 mg/mL; **DARZALEX® (daratumumab)** 0.4 mg/mL; **HERCEPTIN® (trastuzumab)** 0.4 mg/mL; **TECENTRIQ® (atezolizumab)** 0.4 mg/mL; **MVASI® (bevacizumab)** 0.4 mg/mL; **XOLAIR® (omalizumab)** 0.4 mg/mL; **BAVENCIO® (avelumab)** 0.5 mg/mL **KEVZARA® (sarilumab)** 2 mg/mL	**HERCEPTIN® (trastuzumab)** 0.08 mg/mL; **HERZUMA® (trastuzumab)** 0.08 mg/mL; **KANJINTI® (trastuzumab)** 0.08 mg/mL; **ONTRUZANT® (trastuzumab)** 0.08 mg/mL; **KADCYLA® (trastuzumab emtansine)** 0.2 mg/mL; **XOLAIR® (omalizumab)** 0.36 mg/mL

Table 2.3 Quantitative composition of individual excipients included in 88 approved liquid and lyophilised parenteral antibody products, listed by ascending values of concentration. (Continued)

Excipient name	Excipient quantitative composition of approved LIQUID antibody products	Excipient quantitative composition of approved LYOPHILISED antibody products
Poloxamer 188 (Surfactants)	**GAZYVARO® (obinutuzumab)** 0.2 mg/mL; **HEMLIBRA® (emicizumab)** 0.2-0.5 mg/mL	None
Potassium chloride (Tonicifiers)	**LEMTRADA® (alemtuzumab)** 0.2 mg/mL	None
Sodium chloride (Tonicifiers)	**PORTRAZZA® (necitumumab)** 40 mM (2.34 mg/mL); **LARTRUVO® (olaratumab)** 2.9 mg/mL; **OPDIVO® (nivolumab)** 2.92 mg/mL; **ARZERRA® (ofatumumab)** 2.98 mg/mL; **DARZALEX® (daratumumab)** 3.5 mg/mL; **CYRAMZA® (ramucirumab)** (75 mM) 4.38 mg/mL; **VECTIBIX® (panitumumab)** 5.8 mg/mL**; ERBITUX® (cetuximab**) 100 mM (5.84 mg/mL); **YERVOY® (ipilimumab)** 5.85 mg/mL; **HUMIRA® (adalimumab, 50 mg/mL)** 6.18 mg/mL**; BENLYSTA® (belimumab)** 6.7 mg/mL; **CIMZIA® (certolizumab pegol)** 7.31 mg/mL; **LEMTRADA® (alemtuzumab)** 8 mg/mL; **TYSABRI® (natalizumab)** 8.2 mg/mL; **HYQVIA® (human normal Ig)** 8.5 mg/mL; **SOLIRIS® (eculizumab)** 8.77 mg/mL; **ZINPLAVA® (bezlotoxumab)** 8.77 mg/mL; **MABTHERA® (rituximab, 10 mg/mL)** 9 mg/mL; **TRUXIMA® (rituximab)** 154 mM (9 mg/mL); **BLITZIMA® (rituximab)** 154 mM (9 mg/mL); **RITEMVIA® (rituximab)** 154 mM (9 mg/mL); **RITUZENA® (rituximab)** 154 mM (9 mg/mL); **TALTZ® (ixekizumab)** 11.69 mg/mL	**SIMULECT® (basiliximab)** 0.32 mg/mL; **BESPONSA® (inotuzumab ozogamicin)** 0.6 mg/mL **MYLOTARG® (gemtuzumab ozogamicin)** 5.8 mg/mL
Calcium chloride (Other excipients)	**HYQVIA® (human normal Ig)** 0.4 mg/mL	None
EDTA (Other excipients)	**STELARA® (ustekinumab, 5 mg/mL)** 0.02 mg/mL; **ARZERRA® (ofatumumab)** 0.02 mg/mL; **LEMTRADA® (alemtuzumab)** 0.02 mg/mL; **HYQVIA® (human normal Ig)** 1 mg/mL	None
Pentetic acid (Other excipients)	**OPDIVO® (nivolumab)** 0.01 mg/mL; **ZINPLAVA® (bezlotoxumab)** 0.01 mg/mL; **YERVOY® (ipilimumab)** 0.04 mg/mL	None
Recombinant human hyaluronidase (rHuPH20) (Other excipients)	**MABTHERA (rituximab, 120 mg/mL)** 2000 units/mL; **HERCEPTIN® (trastuzumab)** 2000 units/mL; **HYQVIA® (human normal Ig)** 160 units/mL	None

Note: The function of these excipients as bulking agents is only relevant for lyophilised products.
a- Disodium phosphate dodecahydrate/Sodium dihydrogen phosphate dihydrate.
b- Histidine/Histidine-HCl.
c- Sodium citrate/Citric acid.
d- Non-amino acids BUFF (buffers).
e- Amino acids BUFF/S/BA (buffers/stabilisers/bulking agents).
f- Non-amino acids S/BA (stabilisers/bulking agents).

Some of the products for which the quantitative composition was not available are biosimilars to other reference products. Biosimilars in some cases can have the same qualitative and/or quantitative excipient composition. For example, MVASI® (bevacizumab) is a biosimilar, having the same formulation of the reference product AVASTIN® (bevacizumab) (206). On MVASI®'s scientific discussion (EMA) it is reported: '*The finished product was developed to have the same formulation, route of administration, dosage form and strength as the reference product Avastin*' (207). Furthermore, some biosimilars possess identical excipient composition (e.g. rituximab biosimilars: RITUZENA®, RITEMVIA® and BLITZIMA®) and have been approved by the EMA following a multiple marketing authorisation application (208).

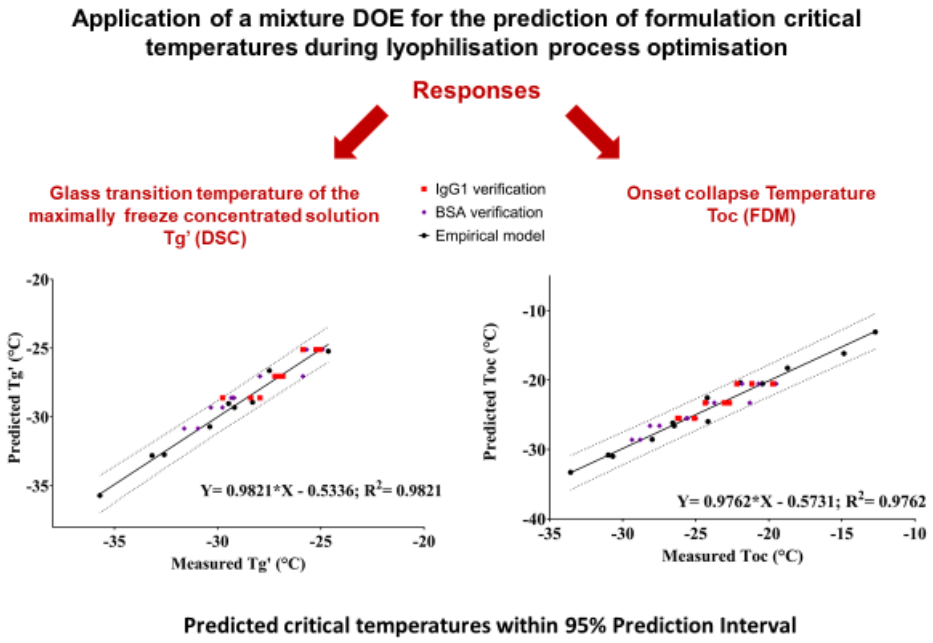
The pH values at which listed antibody formulations were buffered are in a range between pH 4.6 and 8.2. The trend observed in the use of excipients for antibody formulations matches with the results obtained for protein formulations, probably because antibodies form the main class of therapeutic proteins. Hence, additional information is reported below only for other excipients added to antibody products and not discussed in previous sections. For example, proline (24–28.78 mg/mL) is reported in the EPAR's scientific discussion of some liquid antibody products to be a viscosity and tonicity modifier or a stabiliser (209, 210). EDTA and pentetic acid are added as chelating agents only to liquid products at concentrations usually in a range between 0.02 and 1 mg/mL and 0.01–0.04 mg/mL, respectively. Calcium chloride is reported to be at concentrations of 0.4 mg/mL in the antibody product HYQVIA® (human normal Ig). Finally, rHUPH20 is added at concentrations of 2000 units/mL in MABTHERA® (rituximab) and HERCEPTIN® (trastuzumab). However, in HYQVIA® (human normal Ig) rHUPH20 (160 units/mL) is provided in a separate vial containing other excipients and added to the antibody product prior to administration.

2.4 Conclusions

The information summarised in this overview aims to update formulation scientists on the trends of excipients' use in approved protein products, aiding them in the selection of excipients for the development of new formulations. The data presented details the most common excipients included in liquid and lyophilised formulations classified into functional categories, focusing in particular on antibody products. The discussion also provides information and key considerations in the use of specific excipients, analysing their role and rationale of use in protein formulations with different dosage form (liquid or lyophilised). A shortcoming of the EPARs data is the limited quantitative compositional information on approved products. However, this information was collected for most of the protein formulations using other publicly available sources (FDA and Marketing Authorisation Holder). Of note from this overview is the low number of 'novel' excipients introduced within the products approved over the period analysed. This may be due to many factors; such as the employment of an usual 'platform approach' formulation strategy (28), the regulatory requirements of approving new excipients for parenteral formulations (166), as well as companies' marketing/commercial reasons that can drive the choice of one excipient over another (28, 211).

Supplementary data associated with this thesis chapter can be found online at: htTps://doi.org/10.1016/j.ejpb.2018.07.011

Chapter 3: Application of a mixture DOE for the prediction of formulation critical temperatures during lyophilisation process optimisation



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3.1 Introduction

As discussed in Chapter 1, each formulation has a critical temperature below which it needs to be frozen and dried for complete solidification and prevention of physical/structural collapses that can compromise the quality of the product (52, 53). Critical temperatures include glass transition of the maximally freeze concentrated solution (Tg') and collapse temperature (Tc) in the case of the amorphous fraction, and eutectic temperature (Teu) in the case of the crystalline fraction. Critical temperature values are related to the formulation components, their physical nature (amorphous or crystalline) and their ratio in the mixture. Typically a difference of 1°C to 3°C has been reported between Tg' and Tc of a protein formulation (Tc > Tg′) (52, 55) however, at higher protein concentrations this difference can progressively increase up to 10°C (32). Furthermore, the temperature range between the onset collapse temperature (T_{oc}) and the full collapse temperature (T_{fc}) could also widen as protein concentration increases (32, 212). The first step in lyophilisation process design of a protein formulation is the determination of the thermal properties of its frozen solution, specifically its critical temperatures.

Critical temperatures determination is commonly conducted by performing differential scanning calorimetry (DSC) to determine Tg′ and freeze-drying microscopy (FDM) to determine Tc and Teu. Additionally, a broad number of alternative techniques are currently available to determine these parameters, including dynamic mechanical analysis (DMA), electrical impedance (EI)/differential thermal analysis (DTA) and optical coherence tomography freeze-drying microscopy (OCT-FDM) (54, 213-217). Previously, Meister and Gieseler reported unconventional collapse events in formulations analysed by FDM and showed how a careful evaluation of the critical temperatures needs to be conducted to avoid bias in their measurement. In addition, Meister and Gieseler

recommended a new collapse classification and the need for further examples in literature to provide a full understanding of unconventional collapse events (212).

An optimised formulation intended to be lyophilised can be considered as a formulation with maximum critical temperatures and preserved protein stability following processing, storage and reconstitution. The possibility to lyophilise materials at high shelf temperatures is particularly valuable considering that each degree increased on the product temperature during primary drying, can produce a 13% reduction in drying time (72, 212). It is reported that formulations containing high concentration of proteins tend to have high critical temperatures (32, 65). This effect of the protein concentration on critical temperatures promotes the development of efficient lyophilisation cycles at higher shelf temperatures, with a product temperature maintained below Tg′ and/or Tc, and a significant reduction in time and costs of processing. However, for formulations with high protein concentrations, the high total solute concentration can negatively impact the freezing process (prolonging the time for complete material solidification), drying process (resulting in high product dry layer resistance) and drastically increase the viscosity and osmolality of the liquid or reconstituted protein formulations. High concentration protein formulations tend to exhibit relatively high viscosities, that can negatively impact protein aggregation, syringeability and injection into the patient (30).

In pharmaceutical freeze-drying, Quality by Design (QbD) is widely proposed to inform the rational design of formulations and process parameters, minimising the use of iterative trial and error approaches (42, 92, 218-220). The two most common theoretical models for prediction of Tg′ of amorphous mixtures are the Fox-Flory and Gordon-Taylor equations (221). However, these models are limited in their prediction of Tg′, since they do not consider the intermolecular interactions, such as hydrogen bonds occurring (222, 223), the variations in the conformational entropy (224), the size and shape or free

volumes of the molecules in the mixture (225). Weng et al. showed the inability of the conventional Gordon and Taylor equation to predict Tg for any of the protein/sugar mixtures analysed in their study (221).

The aim of this study was to aid formulation selection by predicting and maximising critical temperatures, and to investigate the effect of each formulation component on the critical temperatures. As an alternative to theoretical models, empirical models generated from mixture design of experiments (DOEs) were employed to predict both critical temperatures (Tg′ and Tc). While theoretical and mathematical models have been used to predict Tg and Tg' (148, 221) to our knowledge this is the first time that models for Tc prediction are reported in literature. Critical temperatures were analysed for formulations with a wide range of protein concentrations (1 to 10% w/v) and total solute concentrations (10, 15 and 20% w/v). The models were generated for formulations containing BSA, and subsequently verified with equivalent IgG1 formulations. The excipients included in formulations studied were sucrose and arginine/arginine-HCl (arg/arg-HCl). Sucrose was selected because it is the most commonly employed cryo/lyo-protectant, and arg/arg-HCl as its use has increased in recent years in commercialised, lyophilised protein products (Chapter 2). Sucrose is an amorphous disaccharide capable of stabilising the protein by forming H-bonds and replacing water (replacement theory) or by forming a rigid amorphous matrix (vitrification theory) which reduces mobility and degradation of the protein (226). Arginine was selected as excipient for its multifunctional role, since it can act as protein aggregation suppressor, viscosity and solubility enhancer as well as pH modifier (152, 227-232). In particular, protein stability was observed to increase when arginine is included in formulation in the presence of the chloride counter ion (148). The mechanism by which arginine contributes to reducing protein aggregation is not completely understood. The main hypothesis involves the occurrence of preferential

interactions between the guanidinium groups of arginine with the aromatic groups (π-electrons) of the amino acids in the protein (155, 233). Extensive studies have been conducted on the role of arginine as an aggregation suppressor in solution, but very little has been published on its role in lyophilised products (234-236).

3.2 Materials

Heat shock fractionated BSA (*Sigma Aldrich, Ireland*) with a purity ≥ 98% and Mw of 66 KDa was used. The humanized anti-IL8 IgG1 was produced by Chinese hamster ovary cell lines (CHO cells) in NIBRT (*Ireland*) and purchased as a frozen solution (−80°C) in tris buffer 50mM, pH7. The Mw of humanized IgG1 was ~140–150 KDa (characterisation conducted by SE-HPLC and SDS-Page). Sucrose and arginine were both purchased from *Sigma Aldrich, Ireland*. Hydrochloric acid (HCl) 37% (*Acros Organics, Fisher Scientific, Ireland*), diluted with ultrapure water (*Type I, MilliQ*) to 1M and 5M solutions, was used to adjust pH to desired values and to form the arg/arg-HCl salt. Polysorbate 80 (*Sigma Aldrich, Ireland*) was included in formulations as a surfactant. Ultrapure water (*Type I, MilliQ*) was used for preparation of the formulations. Syringe filters 0.2µm, PTFE membrane and 13mm diameter (*VWR International, North American, USA*) were used to filter excipient solutions prior to protein formulation under aseptic conditions. Formulation concentrations expressed in percentages are always considered as % (w/v) in this thesis.

3.3 Methods

3.3.1 Pre-formulation studies

In advance of developing the empirical models based on a DOE approach, pre-formulation studies were conducted to determine any formulation constraints.

3.3.1.1 Tg′ and crystallisation events determination of placebo solutions

Placebo solutions with 10% (w/v) total solute concentration were prepared using a range of arginine:sucrose ratios (5:0, 4:1, 3:2, 2:3, 1:4, 0:5) dissolved in ultrapure water. Sucrose (292 mM) and arginine stock solutions (574 mM) were prepared and HCl was used to titrate the arginine stock solution to pH5, pH6, and pH7. Equivalent formulations were prepared in absence of HCl to study the effect of the counter ion on Tg′ and crystallisation of arginine. Formulations were analysed by DSC (*Q2000, TA Instrument, Inc., Delaware, USA*), once volumes of 20µL were transferred into Tzero aluminium pans and hermetically sealed. Tg′ analysis was performed by freezing the samples to −80°C and reheating them to +5°C at a rate of 2°C/min with a temperature modulation of ± 1°C every 60s. For crystallisation detection, samples were cooled to −80°C, heated to −10°C and held at his temperature for 60 min, then re-cooled to −60°C and re-heated to +5°Cat a rate of 2°C/min and a modulation of ± 1°C every 60s. Data was processed by using the Universal Analysis Software (version 4.5A).

DSC experiments were performed in triplicate. Two-way ANOVA and Tukey's multiple comparison tests were conducted to investigate the effect of arginine and HCl on the Tg′ of placebo solutions.

3.3.1.2 Viscosity of BSA formulations

The effect of BSA and arginine concentrations on formulation viscosity was investigated at 25°C and in a wide range of shear rates 100–10,000 s^{-1} using the Fluidicam TM RHEO (*Formulaction, Inc., Toulouse, France*). A glass chip with a channel gap of 50 μm and a sample size of 1.5 mL were employed during the analysis. An aqueous dye solution with a viscosity of 0.95 cP was used as a reference. Two groups of formulations were analysed; (1) formulations with a fixed ratio of protein:sucrose:arg/arg-HCl (P1:S0.8:A0.2) and increased protein concentration (5 to 40% w/v) to observe the effect of protein concentration, and (2) formulations having a fixed protein concentration (10% w/v) and total solute concentration (20% w/v), but varied sucrose: arg/arg-HCl ratios (S1:A0, S0.8:A0.2, S0.6:A0.4, S0.4:A0.6, S0.2:A0.8) to observe the effect of arg/arg-HCl. Two replicates for each formulation were analysed.

3.3.2 Formulation studies

3.3.2.1 Empirical models development

A mixture DOE was developed by using Minitab®18 Software with the aim to select the ratio of formulation components and to predict critical temperatures for formulations included in the design space. An extreme vertices mixture design of experiments with three components and augmented degree two design (in presence of centre and axial points) was selected.

An individual DOE was constructed for four formulation sets with fixed total solute concentrations and protein concentration ranges (**Table 3.1**). Formulation constraints were applied and a total number of 49 BSA formulations with different ratios of protein:sucrose:arg/argHCl were characterised to generate the empirical models.

Table 3.1 DOE name, formulation constraints applied and number of runs for each DOE set.

DOE name	Total solute % (w/v)	Protein (% w/v)	Sucrose (% w/v)	Arg/Arg-HCl (% w/v)	Runs (N)
10%-Low	10	1-5	1-9	0-8	13
15%- Low	15	1-5	1-14	0-13	13
15%-High	15	5-14	1-10	0-9	10
20%-High	20	5-15	1-15	0-14	13

The constraints were selected considering the high concentration of protein desired and the necessity to include sucrose as cryo/lyo-protectant in all formulations. Detailed composition of the 49 BSA formulations is reported in the Results (Section 3.4.3).

3.3.2.2 Empirical models analysis and verification

Statistical analysis of the empirical models based on DOEs was performed by using Minitab®18 Software with the aim of predicting critical temperatures for formulations included in the design space, and thereby selecting the ratio of formulation components to achieve maximised critical temperatures. The advantage to select formulations with maximised critical temperatures is to facilitate the development of shorter and more cost effective lyophilisation cycles, conducted at more aggressive conditions. The model employed was based on a regression equation with more than one term (**Equation 3.1**)

Equation 3.1: Polynomial equation for building the regression model

$$y = b_0 + b_1X_1 + b_2X_2 + \ldots + b_kX_k$$

where: y is the response variable, b_0 is the constant (intercept), b_1,b_2, …,b_k are the regression coefficients (slope) for the different terms (X_1, X_2, …,X_k). This was used for prediction of the responses by introducing the regression coefficient of each component into the equation (Minitab®18 Software). Subsequently, a response optimiser control, provided by the software, was used to select formulations by maximizing the responses (critical temperatures). The DOE model was then verified by predicting Tg′ and Tc of

additional optimal formulations included in the design space and correlating the predicted critical temperatures with the measured values (Tg′ and Tc). Verification of the model 15%-High was not performed since the model could not be developed and used for prediction (Section 3.4.3). The additional optimal formulations used for verification include 25 observations for BSA formulations (3 formulations analysed in triplicate and 8 formulations analysed in duplicate), and 9 observations for IgG1 formulations (3 formulations analysed in triplicate) (**Table 3.2**).

Table 3.2 Composition of the protein formulations used to verify the empirical models for each DOE set.

10%- (w/v)-Low				
F	**Protein % (w/v)**	**Sucrose % (w/v)**	**Arg/Arg-HCl % (w/v)**	**Total solute % (w/v)**
1	2.0	8.0	0.0	10.0
2	2.0	6.4	1.6	10.0
3	5.0	5.0	0.0	10.0
4	5.0	4.0	1.0	10.0
15% (w/v)-Low				
F	**Protein % (w/v)**	**Sucrose % (w/v)**	**Arg/Arg-HCl % (w/v)**	**Total solute % (w/v)**
5	5.0	10.0	0.0	15.0
6	5.0	8.0	2.0	15.0
20% (w/v)-High				
F	**Protein % (w/v)**	**Sucrose % (w/v)**	**Arg/Arg-HCl % (w/v)**	**Total solute % (w/v)**
7	5.0	15.0	0.0	20.0
8	5.0	12.0	3.0	20.0
*9**	10.0	10.0	0.0	20.0
*10**	10.0	8.0	2.0	20.0
*11**	10.0	6.0	4.0	20.0

**All formulations (F) contained BSA as protein and were analysed in duplicates except for formulations 9, 10 and 11 20% (w/v)-High that were prepared including either BSA or IgG1 as proteins and were analysed in triplicates; Polysorbate 80 is added to the protein formulations at concentrations of 0.05% (w/v) and is not considered in the total solute concentration.*

3.3.2.3 Fox-Flory equation

Theoretical Tg' for all formulations was also estimated by using the Fox-Flory equation (**Equation 3.2**).

Equation 3.2: Fox-Flory

$$\frac{1}{Tg} = \frac{w^1}{Tg^1} + \frac{w^2}{Tg^2} + \frac{w^3}{Tg^3}$$

where Tg^1, Tg^2, Tg^3 and w^1,w^2,w^3 are Tg' and weight fractions of the components 1, 2 and 3, respectively. The Tg′ value of the excipient components (sucrose Tg′–32°C and arg/arg-HCl Tg′=−44°C) in aqueous solution was determined by DSC, whereas a previously reported Tg' value was used for the protein BSA (237, 238).

3.3.2.4 Preparation of BSA formulations

BSA formulations were prepared by dissolution of the protein in the excipient solutions. Excipient solutions were obtained by dilution of the sucrose and arginine stocks (20% w/v). For the stock solutions, the desired mass of excipients was dissolved in ultrapure water. The arginine stock solution (1148 mM) was titrated to pH7 ± 0.3 using 1 M HCl.

3.3.2.5 Preparation of IgG1 formulations

The IgG1 was formulated at the desired concentration by performing ultracentrifugation using AMICON ultra-15 centrifugal filter units (*Merck, Darmstadt, Germany*). The IgG1 at an initial concentration of 0.5% (w/v) was concentrated to a final concentration of 20% (w/v). The concentration and buffer exchange were conducted by performing three runs of ultracentrifugation (*Rotanta 460R, Hettich, Germany*) at 3800 rpm (30 min per run). The protein was then formulated by dilution 1:1 with the excipient solution (pH6) to obtain a final protein concentration of 10% (w/v). pI of the IgG1 was determined to be > 7 (as per NIBRT information).

Polysorbate 80 at fixed concentration of 0.05% (w/v) was added to the final formulations by dilution from the stock solution 1% (w/v). Polysorbate 80 is not considered in the total solute concentration of the formulations.

3.3.2.6 Tg′ determination

Tg′ of the formulations was measured using DSC (*Q2000, TA Instrument Inc., Delaware, USA*). Formulation volumes of 20µL were transferred into Tzero aluminium pans, and hermetically sealed.

DSC analysis were performed by cooling the samples to −60°C and re-heating them to +5°C at a ramp rate of 5°C/min. Data was processed by using the Universal Analysis Software (version 4.5A). Tg′ values were measured as midpoint temperature of the thermal event showed in the reversing heat flow thermogram **(Supplementary Information (SI), Figure 1).**

3.3.2.7 Tc determination

Tc was determined by carrying out FDM analysis (*Lyostat 3, equipped with a Zeiss Axio imager, A1 microscope and a Linksis32 Software, Linkam Scientific Instrument, Biopharma Process Systems Ltd, Winchester, UK*). All samples were cooled at a freezing rate of 10°C/min to −40°C. After 10 min of isothermal equilibration, a vacuum (0.1 mbar) was applied to initiate the sublimation. Once the drying front was approximately 1–2 inches on the monitor, the temperature was increased at a ramp rate of 0.5°C/min under vacuum until the full collapse of the material was reached.

Pictures were captured at 5s intervals during drying. The collapse temperature was reported as T_{oc} which is the first observable loss of material structure, and T_{fc} which indicates the complete loss of material structure, both occurring during the drying step.

All formulations included in the DOEs used to verify the empirical models were analysed using the selected DSC and FDM protocols.

3.4 Results

3.4.1 Tg′ and crystallisation of placebo solutions

The Tg′ of placebo solutions containing sucrose and arginine at a range of ratios and fixed total solute concentration (10% w/v) was analysed to investigate the effect of excipient components on the critical temperatures in absence of the protein. As shown in **Figure 3.1** arginine has a significant plasticising effect on the Tg′ of sucrose solutions ($p < 0.0001$ ANOVA, Tukey's multiple comparison). This effect was enhanced in the presence of HCl (pH 5-7), which significantly decreased the Tg' of the corresponding formulations containing arginine in absence of the counter ion (max $\Delta Tg' = 6.26°C$). The plasticising effect of arg/arg-HCl follows a linear trend, however, the same trend was not observed in absence of HCl. The Tg′ of analysed formulations was not affected by pH at the range (pH5-7) investigated.

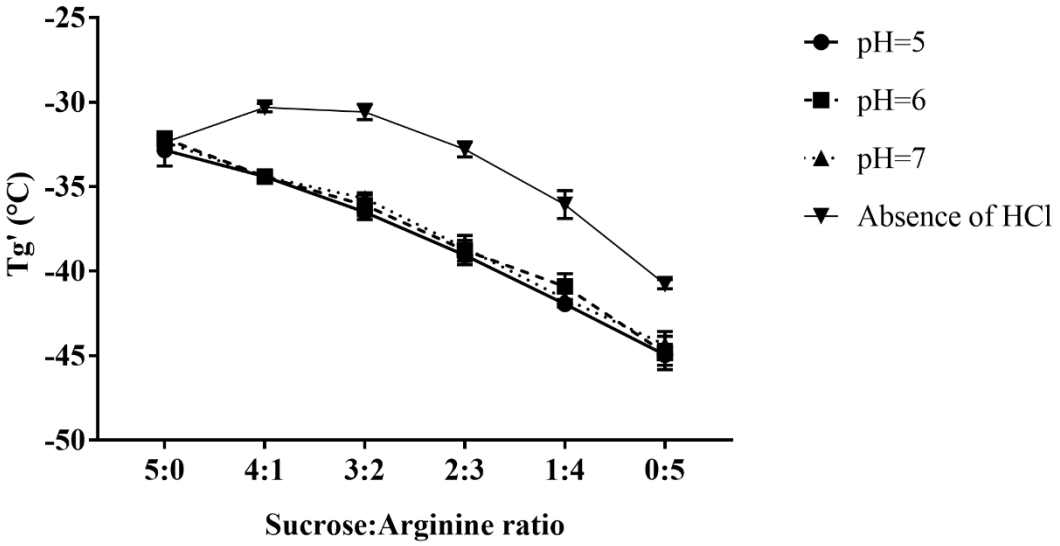


Figure 3.1 Plasticising effect of arginine and HCl counter ion on the Tg′ of sucrose solutions. Arginine and HCl counter ion both have a significant plasticising effect on the Tg′ of placebo formulations with varied sucrose:arginine ratios and a fixed total solute concentration (10% w/v) (n=3).

No significant difference in the Tg′ values and absence of crystallisation were observed by DSC analysis following annealing **(SI, Figure 2)**.

3.4.2 Viscosity of BSA formulations

Viscosity is one of the main limitations encountered when formulating proteins at high concentrations. Hence, it is necessary to investigate the effect of both protein and arg/arg-HCl concentration on the viscosity of high concentration BSA formulations studied herein. **Figure 3.2a** shows an increase in viscosity with increase in protein concentration, in particular for protein concentrations higher than 20% (w/v). For this reason, our study was focused on formulations with protein concentrations constrained between 1 and 15% (w/v). **Figure 3.2b** shows the effect of the increased amount of arg/arg-HCl on viscosity of formulations with fixed protein concentration (10% w/v) and total solute concentration (20% w/v). As previously reported arg/arg-HCl reduced viscosity (154), however, for fomulations studied this effect was significant up to arginine concentrations of 4% (w/v). Additionally, an increase in viscosity was observed for formulations with higher total solute concentration and equivalent protein concentrations (**Figure 3.2c**).

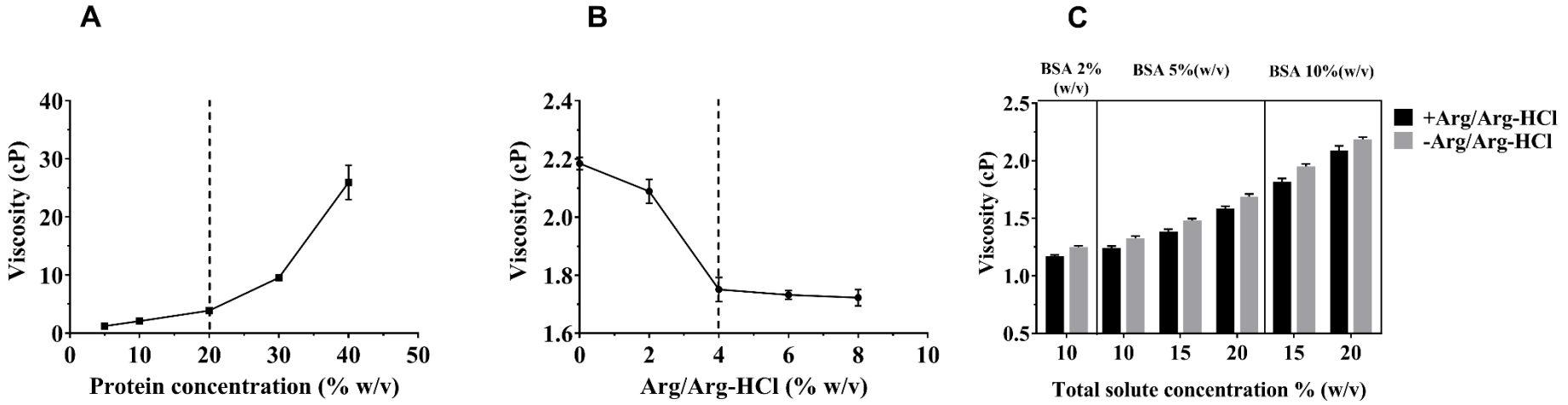


Figure 3.2 Viscosity of BSA formulations. (a) Effect of protein concentration on viscosity for formulations with fixed Protein: Sucrose: Arg/Arg-HCl ratio (P1:S0.8:A0.2) and increased protein concentration (5–40% w/v); (b) Effect of Arg/Arg-HCl on viscosity for formulations with fixed protein (10% w/v) and total solute concentration (20% w/v), increased Arg/Arg-HCl concentration (0–8% w/v) and reduced sucrose concentration (10–2% w/v); (c) Effect of solute concentration (10%, 15%, 20% w/v) for formulations with fixed protein concentration and increased total solute concentration with and without Arg/Arg-HCl in ratios (P1:S0.8:A0.2 or P1:S1) (n=2).

3.4.3 Empirical models development

Tg′ and Toc were determined for the formulations included in each DOE set (**Table 3.1**) with the aim of building an empirical model for each total solute concentration. Detailed formulation composition and corresponding critical temperature values are provided for each DOE set in **Table 3.3**.

Table 3.3 Predicted and measured critical temperatures of formulations used to develop the empirical model for each DOE set.

10% (w/v)-Low								
Run	**P**	**S**	**A**	**Tg' (DSC)**	**Tg' (Fox-Flory)**	**Tg' (DOE Pred)**	**Toc (FDM)**	**Toc (DOE Pred)**
1	2.0	6.5	1.5	-30.4	-30.0	-30.6	-29.2	-29.0
2	4.0	2.5	3.5	-28.7	-28.1	-27.9	-26.9	-26.0
3	5.0	3.0	2.0	-25.0	-24.1	-24.5	-23.1	-22.1
4	2.0	2.5	5.5	-35.5	-34.7	-33.8	-34.2	-32.5
5	5.0	1.0	4.0	-24.0	-26.5	-24.6	-22.2	-22.8
6	3.0	1.0	6.0	-31.0	-33.2	-31.1	-29.4	-29.6
7	4.0	4.5	1.5	-26.1	-25.7	-26.6	-23.2	-24.1
8	1.0	9.0	0.0	-30.8	-30.3	-30.6	-29.9	-29.9
9	5.0	5.0	0.0	-22.4	-21.7	-22.3	-18.4	-18.6
10	3.0	7.0	0.0	-26.9	-26.0	-26.7	-24.6	-24.0
11	1.0	5.0	4.0	-34.2	-35.1	-34.4	-33.3	-33.6
12	3.0	4.0	3.0	-28.7	-29.6	-30.1	-26.7	-28.4
13	1.0	1.0	8.0	-37.1	-39.9	-37.6	-36.0	-36.5
15% (w/v)-Low								
Run	**P**	**S**	**A**	**Tg' (DSC)**	**Tg' (Fox-Flory)**	**Tg' (DOE Pred)**	**Toc (FDM)**	**Toc (DOE Pred)**
1	4.0	3.8	7.3	-34.4	-32.5	-33.6	-32.3	-31.1
2	3.0	12.0	0.0	-30.1	-28.2	-29.7	-27.5	-27.1
3	2.0	10.3	2.8	-32.1	-31.8	-32.1	-30.0	-30.0
4	5.0	5.5	4.5	-31.6	-28.9	-32.0	-28.7	-29.1
5	2.0	3.8	9.3	-36.3	-37.0	-35.7	-34.6	-34.0
6	4.0	8.3	2.8	-31.6	-28.9	-31.5	-28.6	-28.8
7	3.0	6.5	5.5	-32.9	-32.5	-33.6	-30.6	-31.3
8	3.0	1.0	11.0	-34.6	-36.9	-35.3	-32.3	-33.2
9	5.0	10.0	0.0	-28.1	-25.3	-28.3	-25.9	-25.9
10	5.0	1.0	9.0	-32.4	-32.5	-32.2	-30.0	-29.9
11	1.0	1.0	13.0	-38.8	-41.4	-38.7	-36.9	-36.8
12	1.0	7.5	6.5	-34.2	-36.2	-34.3	-33.1	-33.2

13	1.0	14.0	0.0	-30.9	-31.0	-31.0	-28.3	-28.3
15% (w/v)-High								
Run	**P**	**S**	**A**	**Tg' (DSC)**	**Tg' (Fox-Flory)**	**Tg' (DOE Pred)**	**Toc (FDM)**	**Toc (DOE Pred)**
1	14.0	1.0	0.0	ND	-12.4	ND	NM	NM
2	5.0	1.0	9.0	-32.5	-32.5	ND	NM	NM
3	8.0	4.0	3.0	-24.5	-23.4	ND	NM	NM
4	5.0	10.0	0.0	-28.6	-25.3	ND	NM	NM
5	11.0	2.5	1.5	ND	-17.9	ND	NM	NM
6	6.5	2.5	6.0	-27.6	-27.9	ND	NM	NM
7	6.5	7.0	1.5	-24.6	-24.4	ND	NM	NM
8	5.0	5.5	4.5	-31.6	-28.9	ND	NM	NM
9	9.5	5.5	0.0	ND	-18.9	ND	NM	NM
10	9.5	1.0	4.5	-22.5	-22.5	ND	NM	NM
20% (w/v)-High								
Run	**P**	**S**	**A**	**Tg' (DSC)**	**Tg' (Fox-Flory)**	**Tg' (DOE Pred)**	**Toc (FDM)**	**Toc (DOE Pred)**
1	10.0	1.0	9.0	-30.4	-27.1	-30.7	-28.0	-28.6
2	15.0	1.0	4.0	ND	-18.8	-23.9	-18.7	-18.3
3	5.0	15.0	0.0	-29.2	-27.1	-29.3	-26.5	-26.6
4	12.5	3.3	4.3	-27.5	-21.6	-26.6	-24.2	-22.6
5	10.0	10.0	0.0	-25.2	-21.7	-25.1	-20.4	-20.5
6	10.0	5.5	4.5	-28.3	-24.4	-28.9	-24.2	-26.0
7	7.5	3.3	9.3	-33.2	-29.9	-32.8	-30.7	-31.0
8	5.0	8.0	7.0	-32.6	-31.3	-32.7	-31.0	-30.8
9	7.5	10.3	2.3	-29.5	-25.8	-29.0	-26.6	-26.2
10	12.5	5.3	2.3	-24.6	-20.4	-25.2	-21.9	-20.4
11	15.0	5.0	0.0	ND	-16.4	-20.7	-12.7	-13.1
12	15.0	3.0	2.0	ND	-17.6	-22.7	-14.9	-16.2
13	5.0	1.0	14.0	-35.7	-35.5	-35.7	-33.6	-33.3

P= Protein (BSA), S= Sucrose, A= Arg/Arg-HCl. ND not detected and NM not measured. Not detected indicates the incapability to detect the event, NM indicates that the analysis was not performed

For six formulations it was not possible to experimentally determine the Tg′ (3 formulations in DOE 15%-High and 3 in DOE 20%High). As a result, for the DOE 15%-High, an empirical model could not be generated and used to predict Tg′ of formulations within the design space. For the DOE 15%-High, some of the formulations selected to

build the model contain high protein concentration and low excipient concentrations with consequently high Tg′ values, which can overlap with the melting region in the thermogram (**Figure 3.3**). However, the undetected values for the DOE 20%-High did not impact the capability to build and verify the model. The possibility to develop the DOE 20%-High model, regardless of missing values, was due to the higher number of runs (13 instead of 10). In addition, the composition of the formulations with missing values in the DOE 20%-High design space is limited to a restricted region.

Since the empirical model for the DOE set 15%-High could not be built due to the inability to detect Tg′ values for certain formulations, the T_{oc} model for corresponding formulations was not further developed.

Examples of the limitations observed in determining Tg′ of high concentration protein formulations (14–15% (w/v), Tg′ > –22°C) is presented in **Figure 3.3**. This behaviour was also observed for a number of formulations with lower protein concentrations (9.5 and 11% (w/v) combined with excipients at specific ratios). In all these cases the predicted Tg′ determined using the Fox-Flory equation was higher than −19°C.

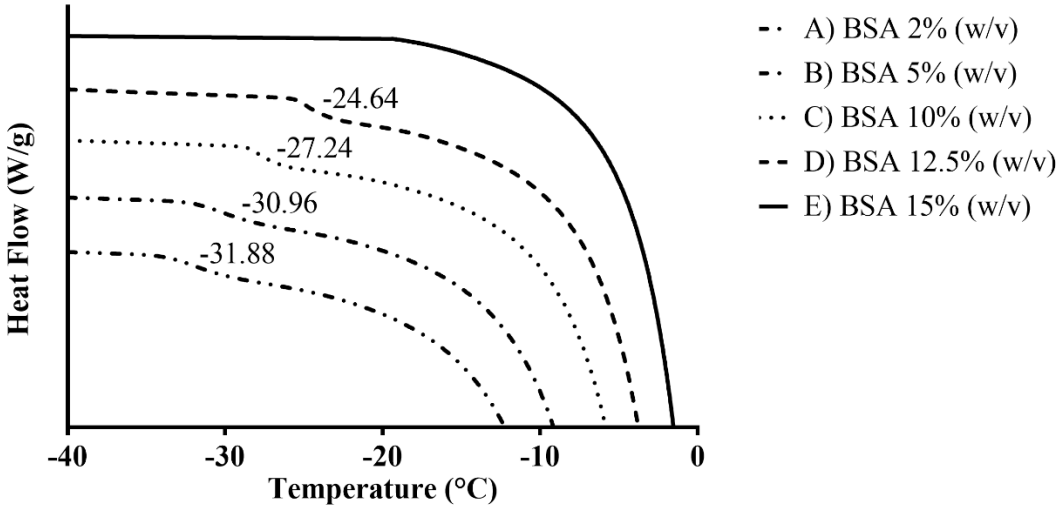


Figure 3.3 Examples of the limitations observed in determining Tg′ of high concentration protein formulations. The effect of protein concentration on the overlapping of Tg′ and melting of the protein depressed by the presence of excipients. Tg′ of formulations with different protein concentration 2–15% (w/v) are shown: Formulation A: BSA 2% (w/v), Sucrose 6.5% (w/v), Arg/Arg-HCl 1.5% (w/v); Formulation B: BSA 5% (w/v), Sucrose 12% (w/ v); Arg/Arg-HCl 3% (w/v); Formulation C: BSA 10% (w/v), Sucrose 8% (w/v), Arg/Arg-HCl 2% (w/v); Formulation D: BSA 12.5% (w/v), Sucrose 5.3% (w/v), Arg/Arg-HCl 2.3% (w/v), Formulation E: BSA 15% (w/v), Sucrose 3% (w/v), Arg/Arg-HCl 2% (w/v).

For formulations investigated across the DOE sets an unconventional collapse behaviour (e.g. microcollapses or fissures) was observed. Hence, the classification of the collapse events was conducted observing three different phases of structural loss, i.e. onset collapse (T_{oc}), microcollapse (T_{mc}) and full collapse (T_{fc}). **Figure 3.4** shows all the possible events occurring during the collapse of high concentration protein formulations. During FDM analysis the heating rate was increased (20°C/min) with the aim of showing all the collapse events in a single picture. **Figure 3.4** is exclusively included to summarize the type of events occurring, but not to collect temperature data for any of the formulations, since the high heating rate was considered suboptimal for accurate measurement.

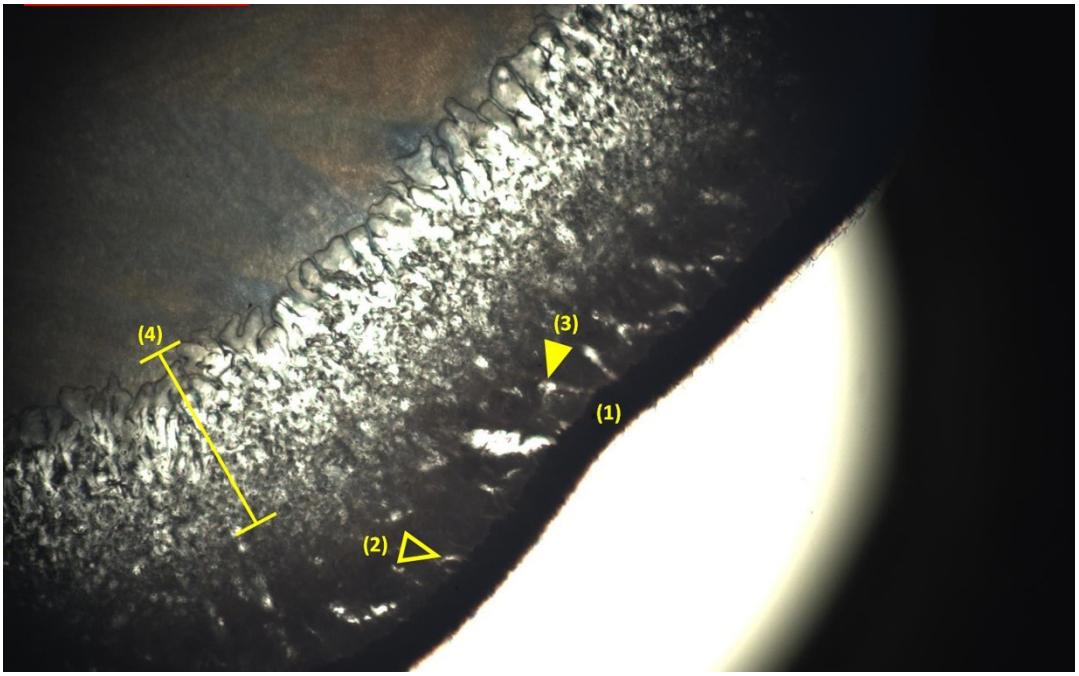


Figure 3.4 Representative FDM image of the collapse events observed for high concentration protein formulations. (1) Drying front; (2) Onset collapse (Toc), (3) Microcollapse (Tmc); (4) Full collapse (Tfc). Drying rate of 20°C/min was selected in order to show all the events occurring in a single image, but no quantitative information of the real temperature was extrapolated from this picture.

For some of the formulations analysed the interval between T_{oc} and T_{fc} was found to be large (0.20–5.86°C). The effect of protein and arg/arg-HCl concentration on the collapse behaviour is shown in **Figure 3.5**. Microcollapse events were usually observed for high concentration protein formulations (≥5% (w/v)), whereas they were less frequent in formulations with increased arg/arg-HCl concentration, which showed a more conventional collapse behaviour.

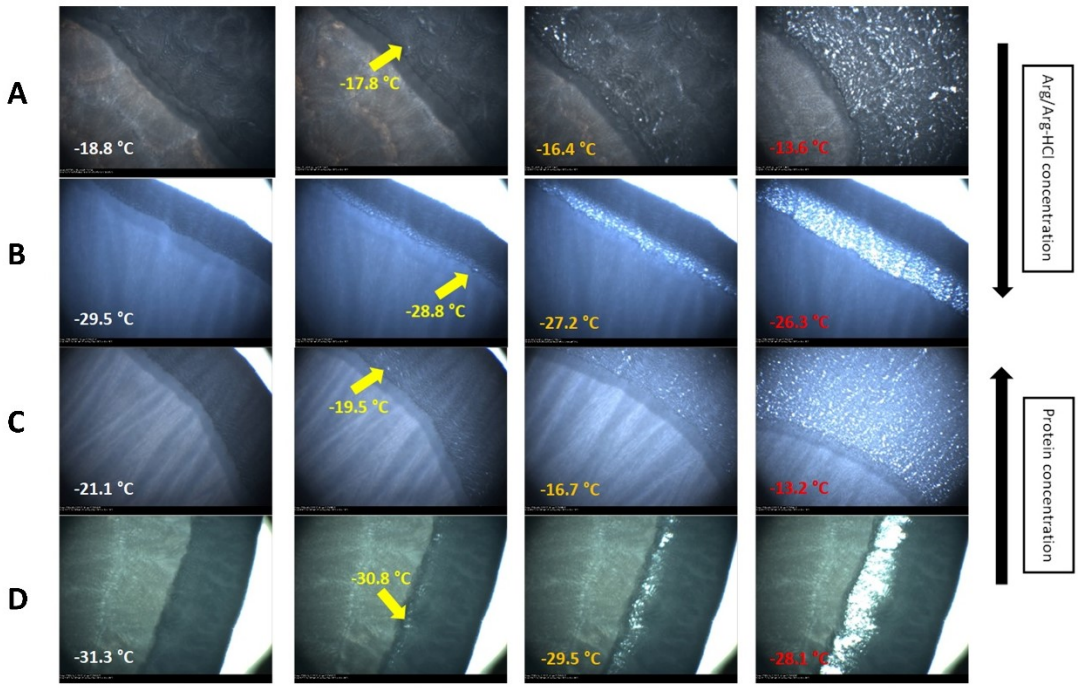


Figure 3.5 Effect of arginine/arginine-HCl and protein concentration on the collapse behaviour of high concentration protein formulations. (a) Formulation A: BSA 5% (w/v), Sucrose 5% (w/v); (b) Formulation B: BSA 5% (w/v), Sucrose 12% (w/v), Arg/Arg-HCl 3% (w/v); (c) Formulation C: BSA 10% (w/v), Sucrose 10% (w/v); 5d) Formulation D: BSA 2% (w/v), Sucrose 6.4% (w/v), Arg/Arg-HCl 1.6% (w/v). T_{oc} (yellow), T_{fc} (orange/red).

Data used to inform the empirical models were observed to be normally distributed (Anderson-Darling test, $p > 0.05$), and in absence of outliers (Grubbs' Test, $p > 0.05$). The type of model established was a linear regression model (analysis of variance, $p < 0.05$). It is considered that the absence of large residuals (within 2 and −2) and the normal distribution of the residual plots make the model adequate for prediction (**SI, Figure 3**). A summary of the statistical parameters that explain the suitability of the model for the three DOE sets analysed is shown in **Table 3.4**.

Table 3.4 Summary of the statistical parameters required to assess the suitability of the empirical models generated from the different DOE sets.

Response	DOE	R^2 (%)	R^2 (adj.) (%)	SD*
Tg'	Low-10%	97.17	94.33	1.07
	Low-15%	97.40	94.81	0.63
	High-20%	98.21	94.63	0.81
Toc	Low-10%	97.10	94.20	1.25
	Low-15%	96.99	93.99	0.75
	High-20%	97.62	95.24	1.36

The predicted sum of squares (PRESS) ranges between 9.36 and 59.59. Analysis of variance, linear regression model $p < 0.05$. SD represents the standard deviation of the distance between the data values and the fitted values; SD was measured in the units of the response.*

Firstly, the models described the effect of each formulation component on the critical temperatures expressed by the regression coefficients once incorporated in the general equation (**Equation 3.1**). The three formulation components have an additive effect with no level of interactions ($p > 0.05$). Several regression coefficients are available for the three different DOEs considering the responses (Tg′ and Toc), the formulation components and the ratio between protein and excipient, which varies with the total solute concentration.

Regression coefficients of each DOE model are reported in **Table 3.5**. In all cases, regression coefficients for the T_{oc} model were higher than those for the Tg′ model. Hence, the T_{oc} was always higher than the Tg′ for corresponding formulations, as expected.

Table 3.5 Regression coefficients of the three formulation components in amounts.

Response	DOE	P	S	A	P-S*	P-A*	S-A*	P-S-A*
Tg'	Low-10%	-0.90	-3.24	-4.23	-0.06	-0.07	-0.04	-0.06
	Low-15%	-1.34	-2.11	-2.78	-0.01	0.05	0.04	-0.03
	High-20%	-0.81	-1.67	-2.00	-0.01	-0.03	0.04	-0.01
Toc	Low-10%	-0.77	-3.31	-4.13	-0.07	-0.09	-0.06	-0.08
	Low-15%	-1.29	-1.93	-2.63	0.00	0.06	0.00	-0.01
	High-20%	-0.21	-1.56	-1.66	-0.03	-0.10	0.04	-0.01

*P=Protein (BSA), S=Sucrose, A=Arg/Arg-HCl. * P values of the coefficients for all the formulation components interaction were > 0.05.*

The contribution of protein and excipients to the responses was clearly observed in the Cox-response plots (**Figure 3.6a-c**) where each line describes the component impact on critical temperatures, once compared to a reference composition blend. In all DOE data sets the formulation components follow the same trend. Increased protein concentration has a positive effect on the Tg′, whereas sucrose and arg/ arg-HCl both decrease the responses in a concentration dependent manner. However, arg/arg-HCl had a more significant negative effect than sucrose.

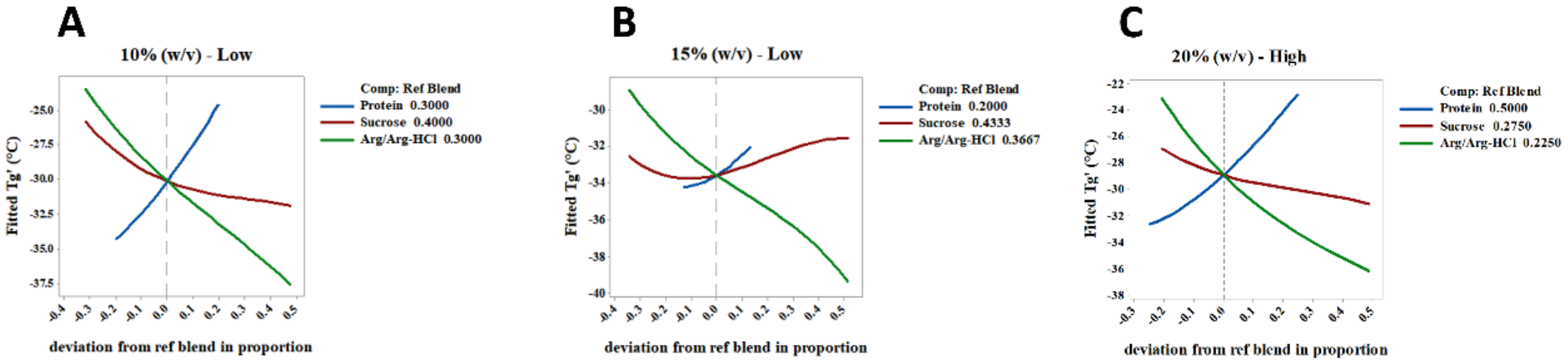


Figure 3.6 Cox response plots of the empirical models generated for critical temperatures prediction. (a) 10%(w/v) total solute concentration, (b) 15%(w/v) total solute concentration; (c) 20%(w/v) total solute concentration. Cox response plots show the effect of formulation components on the response Tg′. Cox response plots for the response Toc are reported in the supplementary information since they follow the same trend as for the response Tg′.

The mixture contour plots (

Figure 3.7a-c) indicate the composition of the formulations (black dots) used to build the model and the critical temperature intervals (coloured bands) of formulations included in the design space. A response optimisation command was applied to each model with the aim of selecting formulation compositions with maximised critical temperatures. Constraints to the temperature range were applied to identify the region of the design space in which these formulations are more likely to be located (

Figure 3.7d-f). The rational choice of the constraints applied was conducted based on preliminary results (Tg′ and T_{oc}) obtained for the analysed formulations and considering formulations with Tg′ values between −20 to −30°C and T_{oc} values between −15 to −28°C.

Cox response and mixture contour plots for the T_{oc} response followed the same trend as for the response Tg′ and are reported in the supplementary information (**SI, Figure 4**).

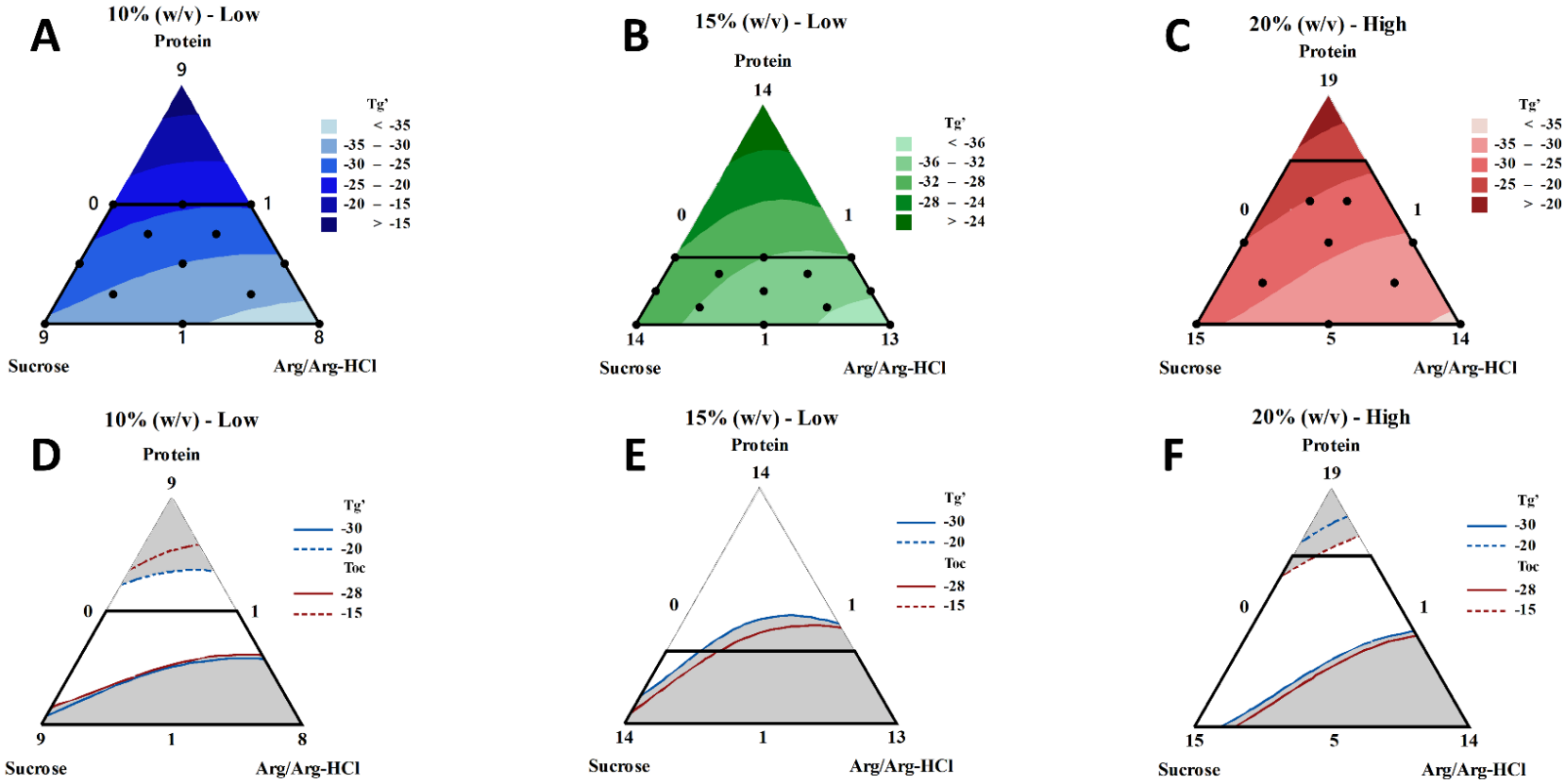


Figure 3.7 Plots of the empirical models generated by mixture DOEs for critical temperatures prediction and optimisation. (a–c) mixture contour plots; (d-f) contour plots of the overlaid responses for formulations with different total solute concentrations: 10% (w/v), 15% (w/v) and 20% (w/v). Mixture contour plots show black dots which indicate formulations provided by the software to build the design space, black lines to identify constraints used in the design and coloured bands to specify the formulation regions at specific Tg′ intervals; Contour plots of overlaid responses (Tg′ and Tc) identify the region selected by performing a response optimisation. Mixture contour plots for the response Toc are reported in the supplementary information since they follow the same trend as for the response Tg′.

3.4.4 Empirical models verification

Additional two constraints were applied to select specific formulations in the narrowed region of the design space for model verification: (1) low concentrations of arg/arg-HCl (<4%) were chosen due to the plasticising effect of this excipient and the minimal reduction in viscosity above this concentration (**Figure 3.2**), protein concentrations (2 – 15% w/v) were included in formulations since this was the concentration range of interest.

Considering applied constraints, 11 formulations were selected to validate the models. **Figure 3.8** shows the composition of the 11 formulations, and their corresponding Tg′, T_{oc} and T_{fc}. Formulations with equivalent protein concentrations showed reduced critical temperatures as the total solute concentration increased. Furthermore, as protein concentration increases, an obvious difference between $Tg' < T_{oc} < T_{fc}$ was observed. The maximum difference in temperature observed between Tg′ and T_{oc}, T_{oc} and T_{fc}, and Tg′ and T_{fc} was 5.9°C, 5.0°C, and 8.9°C, respectively. Critical temperature values of the additional formulations are detailed in **SI, Table 1**.

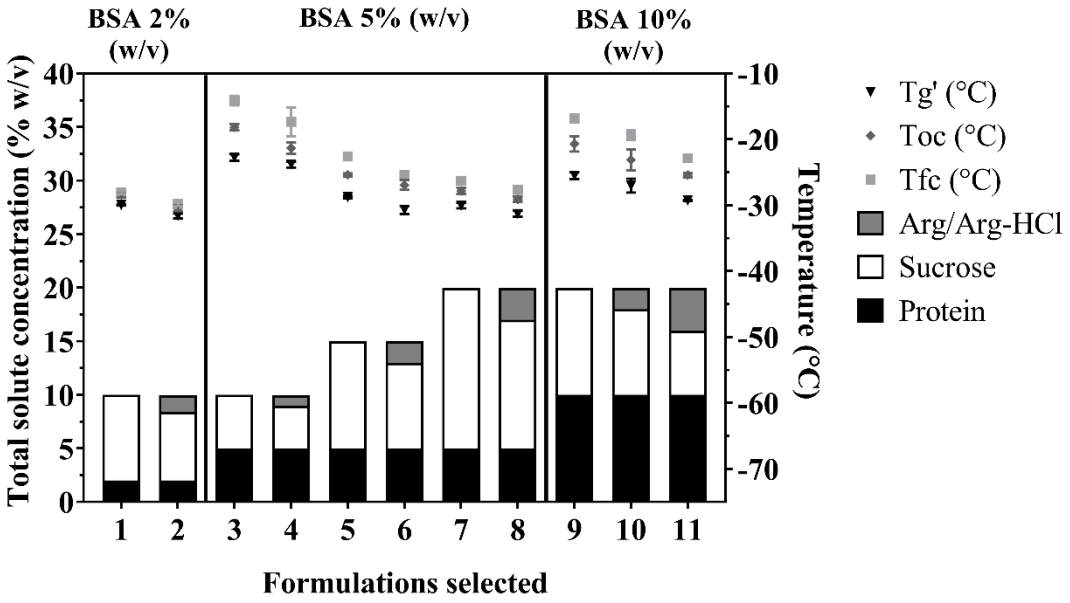


Figure 3.8 Critical temperatures of 11 formulations selected. Formulations are ordered by ascending values of protein concentration and total solute concentration. Tfc (°C), Toc (°C) and Tg′ (°C) are reported for the 11 formulations selected and used to verify the empirical models (n=2).

In order to obtain the highest protein concentration in formulation and maximised critical temperatures, formulations with BSA concentrations of 10% w/v were selected. Consequently, formulations having 10% w/v of the protein IgG1 were also analysed.

It was observed that the difference in critical temperatures of equivalent BSA and IgG1 formulations was not statistically significant (**Figure 3.9**). Hence, the empirical model 20%-High based on the BSA formulations was also verified for the IgG1 formulations.

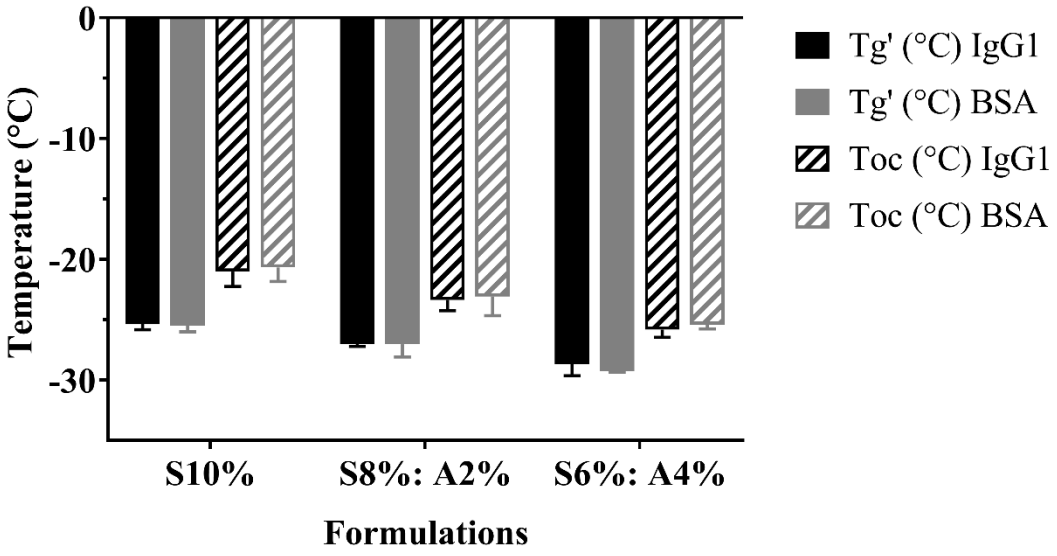


Figure 3.9 The effect of the type of protein (BSA and IgG1) on the critical temperatures of high concentration protein formulations. Critical temperatures of equivalent formulations with different type of protein are not significantly different (Two-way ANOVA, Tukey's multiple comparison, n=3). Formulations contain 10% w/v protein (IgG1 or BSA), S=Sucrose, A=Arg/Arg-HCl. Difference in the Mean of Tg′ and Toc for formulations showed in the figure ranges between 2.88°C and 4.81°C.

The model verification was conducted by detecting Tg′ and Toc for the additional BSA and IgG1 formulations. **Figure 3.10** shows how the additional data points fall within the 95% prediction interval (PI) for each model. In particular, **Figure 3.10e** and **f** display the capability of the DOE 20%-High model to accurately predict critical temperatures for the IgG1 formulations. Therefore, the model built on the BSA formulations can be used to predict critical temperatures of equivalent IgG1 formulations.

Furthermore, the empirical models provided were found to be more accurate in predicting Tg′ relative to the theoretical Fox-Flory model (i.e. R^2 values were always lower and 95% PI larger for the Fox-Flory model), as shown in **Figure 3.10a, c** and **e**.

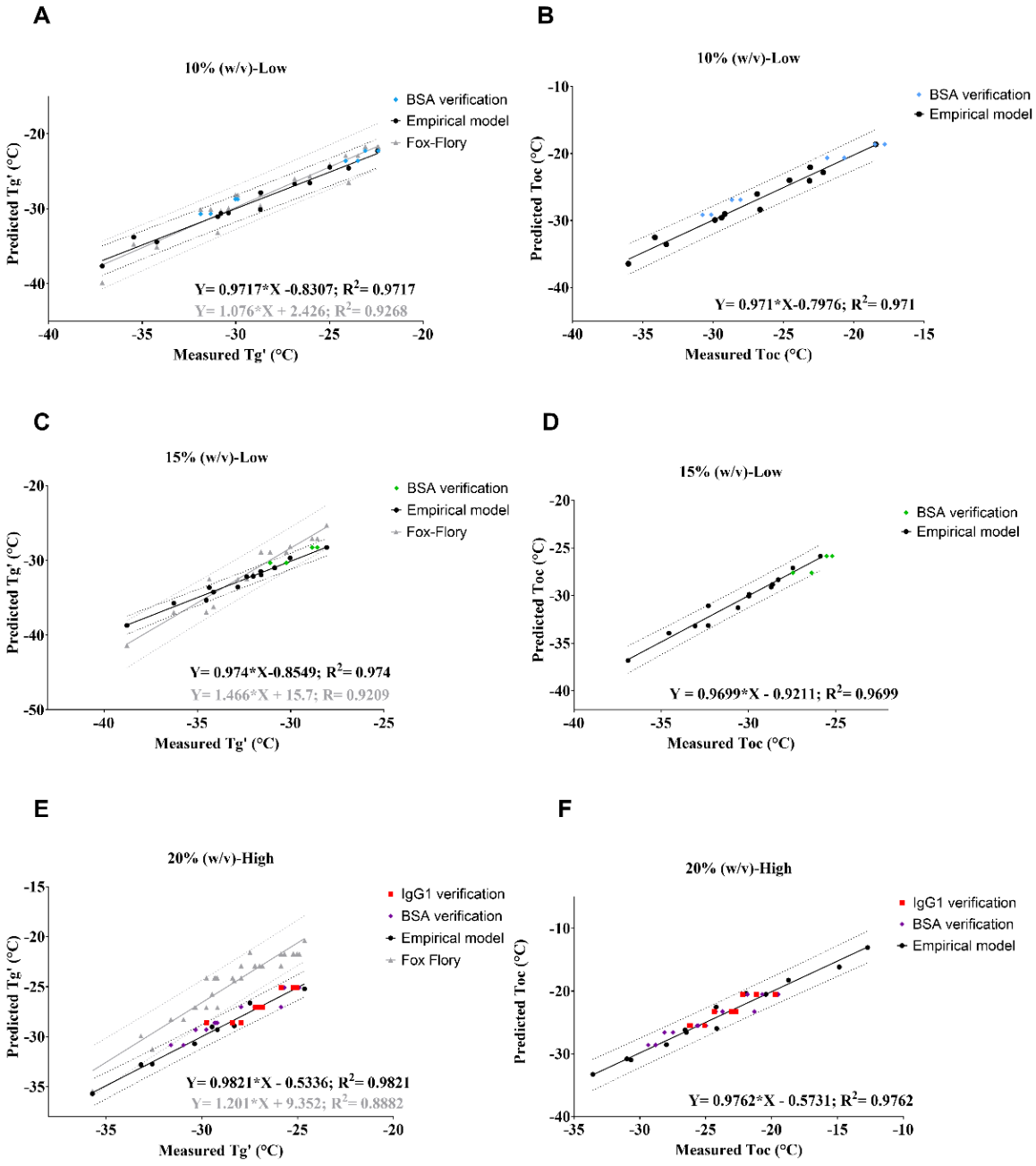


Figure 3.10 Verification of the three DOEs and the Fox-Flory models by using BSA and IgG1 formulations. (a) Tg' and (b) Toc for DOE 10%-Low, n=8 observations (BSA); (c) Tg′ and (d) Toc for DOE 15%-Low, n=4 observations (BSA); (e) Tg′ and (f) Toc for DOE 20%-High, n=22 observations (n=13 BSA and n=9 IgG1). Black dots represent values of critical temperatures for formulations used to build the empirical models. Black line is the fit of the empirical regression models generated. Coloured dots represent critical temperatures of formulations used to verify the empirical models. Grey triangles and line represent the Fox-Flory model.

3.5 Discussion

Initial studies were conducted on placebo solutions containing the selected excipients of interest, arginine and sucrose to analyse their impact on Tg′. Previously, Startzel et al. investigated the impact of arginine, pH and arginine counter ion on the Tg′ and Tc of sucrose rich formulations (148). The impact of arginine counter ions on protein stability was evaluated, showing the greatest stability for formulations containing arg/arg-HCl (148). Additionally, Tischer et al. demonstrated that L-ArgHCl increases the solubility of both, the native and the unfolded protein, which is linked to a reduction of the free energy of the protein species in solution (239). The capability of arg-HCl to increase protein solubility was previously explained by a shift of a series pre-equilibria towards dissociation, which results in a reduction of the concentration of nucleation seeds without impacting the overall mechanism of aggregation (239). Consequently, in this study, the effect of the chloride salt form of arginine was investigated along with the impact of sucrose and protein on the critical temperatures. In agreement with Startzel et al., our findings show that the use of arginine at high concentrations in formulations represents a limitation due to its plasticising effect on the critical temperatures (240). As previously reported, both excipients (sucrose and arg/arg-HCl) were observed to be in the amorphous state and to remain predominantly amorphous following annealing (148). The amorphous nature of both excipients induces a significant reduction of the formulation critical temperatures. However, this effect is counterbalanced by the high concentration of protein in formulations, which tend to increase critical temperatures.

In addition, arg/arg-HCl is widely reported to reduce viscosity of protein formulations, by acting as a salt and reducing the occurrence of protein-protein electrostatic interactions (154, 160). However, because of its plasticising effect, its concentration should be limited in protein formulations intended to be lyophilised. Therefore, arg/arg-HCl concentration

should be optimised to minimise its impact on Tg′ and maximise its positive effect on formulation viscosity. The rheological behaviour of high concentration protein formulations analysed in this study was in agreement with previous publications (154, 160). Protein had a major effect on formulation viscosity when included at high concentrations. However, this effect would be influenced by the total solute concentration, and the protein: excipient ratio. In this study, viscosity was determined for BSA formulations, which can show a similar trend, but different rheological behaviour and viscosity values than the corresponding IgG1 formulations (241). Hence, in an attempt to balance the positive effect on viscosity and the negative effect as plasticizer, results from this study show that the use of arg/arg-HCl is recommended at low concentrations 1–4% (w/v) for high concentration protein formulations.

Critical temperatures were determined for formulations provided by each DOE set, however six formulations provided by the DOEs have undetected values for Tg′ (**Table 3.3**). DSC is the most common method used to determine Tg′ of protein formulations, however, in some cases detection of the thermal event can be challenging. This drawback shows the necessity to explore the use of alternative techniques based on different physical principles to detect high Tg′ values, characteristic for high concentration protein formulations. For instance, DMA and DTA/Impedance can be two valid options, since they are based on mechanical and electrical inputs respectively, and therefore can provide information which are not exclusively based on thermal changes in the material (54, 215).

Regarding the FDM analysis, most published studies refer to the onset collapse (T_{oc}) as collapse temperature (Tc). Toc is the event which precedes the full collapse, and it is the safest point to consider as critical during lyophilisation process optimisation (52, 212). For some of the formulations studied the material undergoes collapse of a small region, and then proceeds until the region of full collapse is reached a few degrees later. This

event is denoted as microcollapse in this study (**Figure 3.4**). The trend observed in **Figure 3.5,** highlights how this unconventional collapse behaviour was less frequent in formulations with high concentration of arg/arg-HCl. This effect might be due to the capability of arg/arg-HCl to reduce the viscosity (and increase solubility) of protein formulations facilitating the freezing and drying step, and provoking more conventional collapses at lower temperature values.

Additionally, high concentration protein formulations present a high total solute concentration and dry layer resistance that can provoke structural losses of the material during drying before a real collapse event occurs (212). This might explain the reason why high concentration protein formulations can often be lyophilised above their critical temperatures with no impact on the physical collapse of the cake. Hence, the conventional classification of the collapse events should be revised for these formulations, and the collapse temperature might be more accurately reported as a range of temperature rather than a single value. Furthermore, the high dry layer resistance of high concentration protein formulations can affect significantly both the freezing and drying process. FDM analysis of Tc can be difficult to perform for formulations with high total solute concentrations (15 or 20% w/v). Some of the formulations with high total solute concentration became 'very dark' after freezing, making it difficult to adjust the light and the focus of the FDM camera. The same formulations during drying can lose their structure (microcollapses or fissures) at lower temperatures than their actual collapse.

The importance of the freezing protocol used in the FDM analysis and its impact on the collapse temperature behaviour have been reported by Meister and Gieseler (212). High freezing rates may generate a less homogenous matrix determining more variability in the velocity of sublimation and different observation times for the viscous flow. The high freezing rates can also increase the dry layer resistance provoking breakages of the

material before the occurrence of the actual collapse (212). Hence, for samples showing this behaviour the use of slower cooling rates might be more appropriate. In order to address these limitations and exclude any variability in determining Tc, the collapse information included in the DOEs are exclusively related to the Toc of the formulations and other structure losses are not considered at this stage in the models. The differences between Tg′, T_{oc} and T_{fc} for high concentration protein formulations (**Figure 3.8**) justify the necessity to establish which Tc should be considered in a process development stage, since the large difference observed can have a significant impact on the primary drying time of an optimised lyo-cycle.

The capability of an empirical model to predict T_{oc} was slightly lower compared to the model for Tg′ prediction (larger standard deviation from the fit, **Table 3.4**). This could be due to the fact that the FDM used to detect T_{oc} is a microscopy technique based on the visual observation of the operator, which generates a inherent variability. An alternative and novel method to overcome these difficulties in FDM data interpretation could be the use of a Thermal Analysis by Surface Characterization (TASC) software (Biopharma Technology Ltd., Winchester, UK) (242). This software addition to the Lyostat equipment (FDM, Biopharma Technology Ltd., Winchester, UK) can increase data accuracy and reproducibility, reducing operator error and providing a quantitative interpretation of the collapse events (242). The TASC software analyses changes in the successive images by monitoring brightness and colour of each pixel in the region of interest. Hence, it produces a normalised TASC trace overlay to the data graph and shows a change in the line based on the degree of collapse.

Finally, the difference in sample morphology, thickness and dry layer resistance between the FDM stage and the vial in the freeze-dryer might also have a significant impact on the Tc. The OCT-FDM is an alternative technique that can be used to detect Tc directly in

the vials, reducing bias in the measurement related to the sample size, preparation and treatment (216, 217).

Despite a similar trend across the different DOEs for each solute content and response, the contribution of each component varies considering its concentration in formulation, the total solute concentration and the ratio between the protein and excipients. For instance, for the DOE 15%-Low in which protein concentration is maintained between 1 and 5% (w/v) and the total solute concentration is 15% (w/v), the effect of protein is minimal if compared to the depressing effect of excipients (in high amounts, maximum 10% w/v). Additionally, as shown in the Cox-response plots (**Figure 3.6a-c**), the effect of protein at concentrations 1–5% is more significant for the DOE 10%-Low (lower excipient amounts) than the DOE 15%-Low (higher excipient amounts), hence, the protein:excipient ratio as well as the ratio between excipients has a significant impact on critical temperatures. The possibility to use the model to investigate the effect of different formulation components on the critical temperatures may be of particular interest for excipients with unknown behaviour.

Results shown in **Figure 3.9** support the concept that the concentration of protein rather than the type of protein affects the formulations critical temperatures. This finding is supported by previous unpublished data generated in our group on the thermal characterisation of high concentration protein formulations. The previous work showed that formulation critical temperatures for three types of proteins (BSA, Lysozyme and γ-globulin) at high concentrations had comparable thermal characteristics regardless of the protein type and molecular weight. The use of less expensive model proteins, such as BSA, to build an empirical model for predicting IgG1 formulation critical temperatures makes the process development of costly protein formulations more effective and less time consuming. The empirical models give the possibility to pharmaceutical companies

to construct a database of their commercial products and predict their critical temperatures in a preliminary process development stage, aiding also re-formulation of specific products when required. The creation of empirical models capable of predicting critical temperatures for specific formulations platforms could facilitate the lyophilisation process design and optimisation.

The theoretical Fox-Flory, Gordon and Taylor and Kwei (221) equations are very useful methods to calculate Tg′ values of specific formulations prior to their empirical determination in a preformulation stage. However, these theoretical models are limited in terms of applicability and accuracy. The Fox-Flory equation is based on a weighted averaged relationship, whereas the Gordon and Taylor equation considers an unequal distribution of the components including their density in the equation. If the densities of the components are equal the Gordon-Taylor can be simplified to the Fox-Flory equation. However, neither equation considers the intermolecular interactions occurring in the mixture blend or the plasticising effect of water on Tg′. On the contrary, the Kwei model implements the Gordon and Taylor equation by including an additional factor (q) in the equation which counts for the intermolecular interactions in the mixture (221). Hence, the Kwei model is more realistic and can complement the routinely used Fox-Flory and Gordon and Taylor models, improving their accuracy (221). However, the factor q is not easily obtainable for some components, especially proteins, which can have different sites of interaction. For this reason, the Kwei model was not applied to this study. The limitations observed in these theoretical/ mathematical models can be overcome by empirical/statistical models. The empirical models generated from the DOEs intrinsicly consider factors contributing to the responses (e.g intermolecular interactions) in the specific mixture, providing outcomes with a reduced level of uncertainty. Some

excipients can create preferential interactions (e.g sucrose: H-bonds, arginine: aromatic groups-guanidinium group) with the proteins which can impact the Tg′ regression.

Previously, a similar and successful approach was employed to generate a model for prediction of flow behaviour of pharmaceutical blends (243). These models establish the type of regression (linear, cubic or quadratic) for the specific system and return regression coefficients that once applied to the equation provide the best fit. The empirical models presented in this study follow a linear regression, but they can also be applied to mixtures containing components with a higher level of interaction, that can be accurately described by a different type of regression. For the first time, empirical models are reported to predict not only the Tg′, but also the T_{oc} of protein formulations. This is an important step considering the large interval that can elapse between these two critical temperatures when high concentration protein formulations are analysed. The reliability of the model depends on the capability to accurately determine the responses for specific mixtures. Hence, techniques and constraints need to be rationally selected to support a successful prediction model.

The main contribution of this manuscript to the current knowledge in the field are related to the possibilities: (1) to predict both critical temperatures by using the empirical model, (2) to aid formulation selection or reformulation when required, understanding the impact of excipients with unknown behaviour, (3) to build the model by using inexpensive model proteins and estimate critical temperatures of expensive high concentration protein formulations such as IgG1. Furthermore, the manuscript highlights the difficulty in determining critical temperatures for high concentration protein formulations in some cases and opens a discussion about the necessity to identify new techniques for the determination of these parameters.

The manuscript provides learnings in relation to arg/arg-HCl which has a depressing effect on the critical temperatures, but it has a positive effect on viscosity and solubility. Hence, the use of the empirical models in presence of rational constraints can aid the selection of optimal concentrations of this excipient in formulations. Additionally, the presence of arg/arg-HCl affects the way in which the material freezes and dries, reducing the occurrence of unconventional collapse events (fissures/ microcollapses) observed in some cases for high concentration protein formulations. This effect of arg/arg-HCl may be attributed to the known capability of this excipient to increase solubility and reduce viscosity of high concentration protein formulations.

3.6 Conclusions

The empirical models generated in this study were capable of establishing the effect of each formulation component on Tg′ and T_{oc}. Critical temperatures of formulations included in the design space were succesfully predicted and the models represent useful tools to select high concentration protein formulations with detectable maximised critical temperatures. The empirical models showed a more accurate prediction of Tg′ if compared to the conventional Fox-Flory equation. In addition, this is the first time that an empirical model has been reported to predict T_{oc}. The types of protein studied in formulations (BSA vs. IgG1) did not impact the critical temperatures of equivalent formulations. Hence, models built using BSA formulations can be successfully applied to predict critical temperatures of equivalent IgG1 formulations, making the formulation selection and lyophilisation process design more cost effective and less time consuming.

Supplementary Data to this chapter can be found online at:

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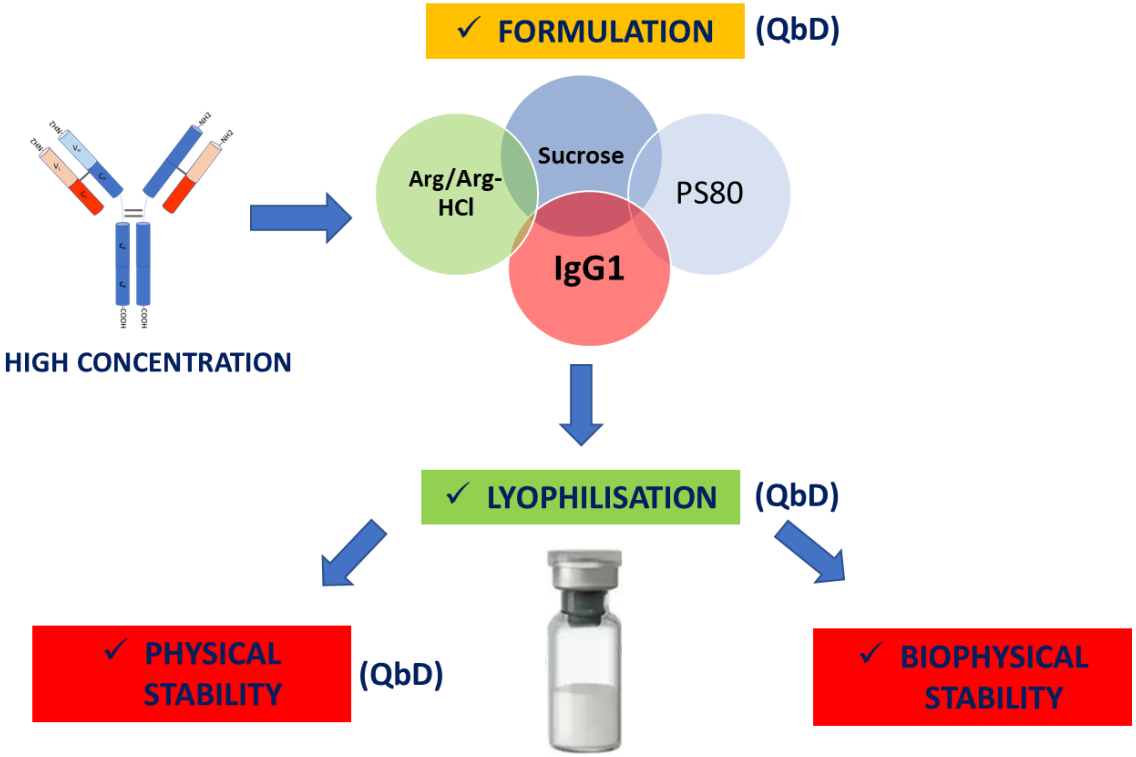


Gervasi, V. 2020. Lyophilisation of high concentration protein formulations. PhD Thesis, University College Cork.

Please note that Chapters 4, 5 & 6 (pp. 118-206) are unavailable due to a restriction requested by the author.

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Chapter 7: General Discussion



7.1 Introduction

The aim of this thesis was to design a stable, lyophilised, high concentration antibody (IgG1) formulation using a Quality by Design (QbD) approach. In this chapter, the impact of the thesis key findings on the current knowledge in the field is discussed and interpreted. In addition, the strengths and limitations of the thesis are summarised. To conclude, recommendations for future work are also provided.

7.2 Interpretation and implications of the thesis findings

The development of high concentration antibody formulations is a key topic of interest for pharmaceutical companies and healthcare providers. The protein market is currently moving from the IV administration of low concentration/high volume products to the more patient compliant SC administration of high concentration/low volume products. As discussed in Chapter 2, the majority of these formulations are currently commercialised in a liquid format, however, lyophilisation is the process of choice to enhance stability and prolong shelf-life of pharmaceutical products. Therefore, the design of stable high concentration antibody formulations intended for lyophilisation represents a novel and noteworthy research topic. This thesis provides a comprehensive overview of the overall process to follow for the design of high concentration protein formulations, including formulation selection (Chapter 2 and 3), lyophilisation cycle development (Chapter 4), physical stability (Chapter 5) and biophysical stability (Chapter 6).

The value of this research is that it contributes substantially to the field providing recommendations and insights on the design of lyophilised high concentration antibody formulations. In particular, this thesis is a comprehensive and detailed study of the process flow required to develop and optimise high concentration protein formulations in the lyophilised state. The work explores formulation, lyophilisation and stability which are

the three key areas in the development of lyophilised products, employing rationale and systematic approaches with the use of QbD strategies (**Figure 7.1**).

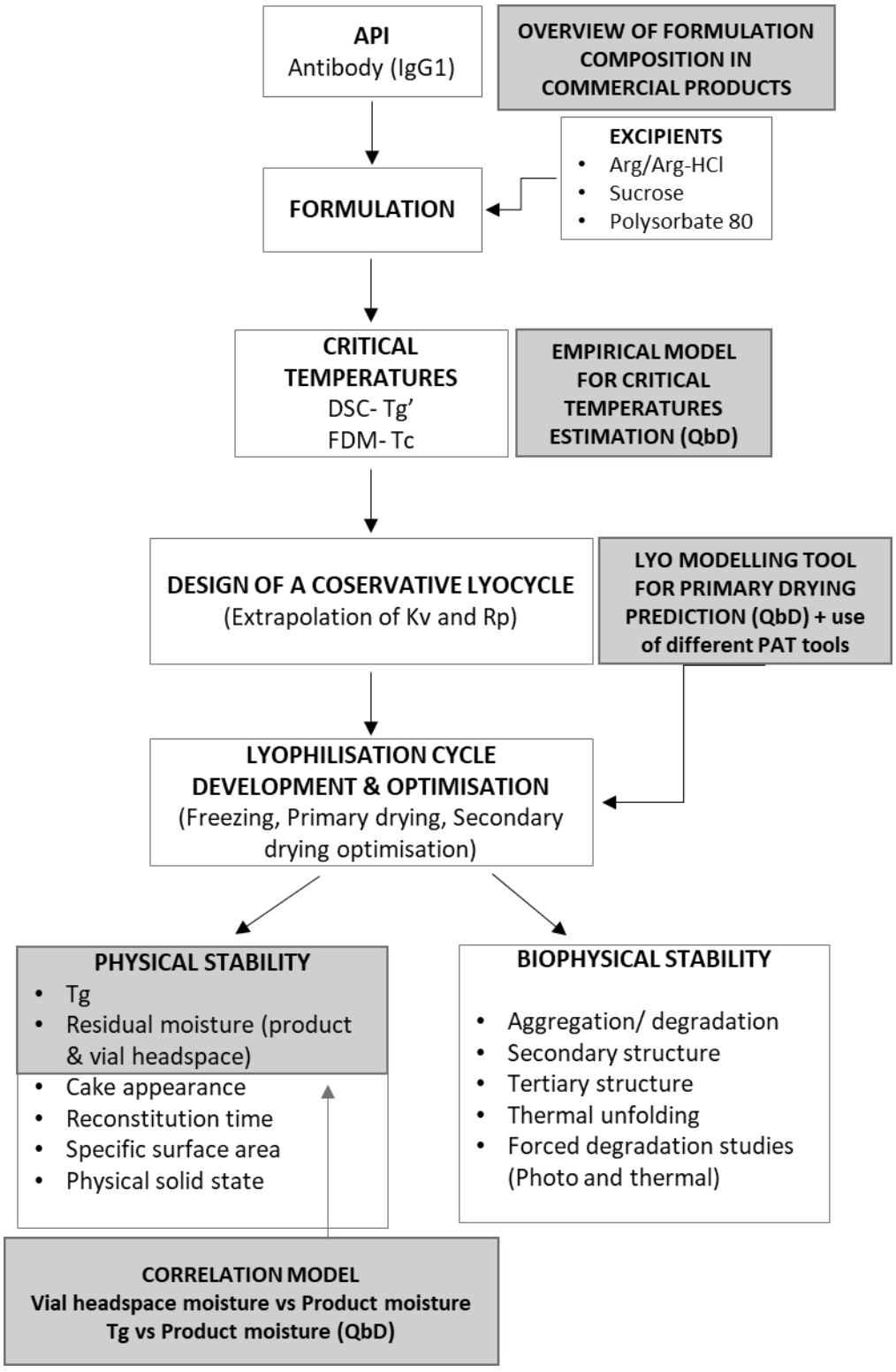


Figure 7.1 Flow diagram showing the workflow of the thesis incorporating the Quality by Design approaches (QbD) applied to each step.

Lyophilisation of high value products can be expensive and time consuming for pharmaceutical companies. Therefore, the employment of systematic strategies to reduce the number of trials and errors is a high impact contribution to the development of biopharmaceutical products. In this regard, this thesis is a crucial starting point and opens new avenues for future work in the field of lyophilisation of high concentration IgG formulations.

In this thesis QbD approaches contributed to aid three essential steps of the work: a) formulation selection (Chapter 3), b) lyophilisation cycle development and optimisation (Chapter 4), and c) physical solid state (Chapter 5).

7.2.1 Formulation selection and design

As a first step, the selection of excipients was conducted by studying the formulation composition of 264 parenteral protein formulations approved in the European Union (Chapter 2). Sucrose was included in formulations investigated since it is the most common stabiliser in lyophilised products (Chapter 2). On the contrary, arg/arg-HCl, included in a low number of commercial products, was selected for its multi-functional role and series of properties that can enhance stability of high concentration protein formulations (154, 233, 235, 236). Arg/arg-HCl is reported in literature to reduce protein aggregation (155, 230, 232, 292), reduce solution viscosity (154, 277) and enhance protein solubility (229, 239). These properties are critical for high concentration protein formulations, which tend to show high viscosity and aggregation profiles (13, 30). Despite its tendency to reduce formulation critical temperatures (Chapter 3), arg/arg-HCl was observed to be a valuable excipient in the design of high concentration protein formulations. Results show the capability of arg/arg-HCl to reduce viscosity of protein formulations (Chapter 3), light induced aggregation of high concentration protein formulations (Chapter 6) and in the solid state to reduce specific surface area, providing

a more elegant cake appearance in comparison to equivalent sucrose rich formulations (Chapter 5). Therefore, the first key finding concerns the use of arg/arg-HCl as excipient. Arg/arg-HCl at specific concentrations (≤ 4% w/v for selected formulations) can positively contribute to the development of high concentration protein formulations in the liquid and lyophilised formats.

The second main contribution is related to the use of high concentration of protein in formulations. The challenges encountered during the development of high concentration protein formulations have been widely reported in literature (13, 28, 37). However, several advantages have also been identified when formulating proteins at high concentrations. The high protein concentration tends to increase formulation critical temperatures (Chapter 3) allowing the execution of shorter lyophilisation cycles at more aggressive conditions. In Chapter 5, the high concentration of protein was shown to inhibit excipient crystallisation in the solid state material when exposed to elevated relative humidity conditions (up to 70% RH at 25ºC) and during storage with and without cold chain. Additionally, the protein at high concentrations can act as bulking agent (32) and buffering agent (291, 293), contributing to its own stability within the formulation and allowing design of less complex formulations with a lower number of excipients.

As a novel aspect in our study, the high protein concentration (100 mg/mL) was formulated in a ratio 1:1 with the excipients. The ratio 1:1 between protein and excipients involves a high total solute concentration (200 mg/mL) of the formulations pre lyophilisation. Generally, total solute concentrations ≤10% are selected in lyophilisation and a ratio 1:1 between protein and cryo/lyo protectant is recommended to guarantee complete protein stabilisation during lyophilisation (40, 138). For high concentration protein formulations, the high total solute concentration is an additional challenge. A conventional approach is to formulate the protein at lower concentration pre-

lyophilisation and to reconstitute the lyophilised powder in a smaller volume of diluent to achieve the target higher concentration. In this study, an extremely high protein concentration and consequent high total solute concentration pre lyophilisation have been successfully lyophilised (Chapter 4) without negatively impacting the biophysical stability of the protein (Chapter 6).

The proposed hypothesis is that the ratio 1:1 between protein and excipients contribute to ensure protein stability. A key aspect of these formulations was that they displayed a completely amorphous nature when analysed by pXRD. The high total solute concentration aided the creation of a glassy matrix where the amorphous formulation was capable to reduce protein mobility ensuring prolonged stability and preventing excipient crystallisation (Chapter 5 and 6). Generally, amorphous formulations tend to be less stable than crystalline formulations. Amorphous materials can undergo phase transitions over storage impacting protein stability (57). The principal advantage of the selected protein formulations is the capability of the protein at high concentrations to prevent excipient crystallisation, providing a stable lyophilised formulation. The lyophilised formulations were observed to remain stable in presence and absence of cold chain storage and after exposure to high humidity conditions. The possibility to eliminate the requirement for cold chain storage further reduces the costs for shipping and storage of these formulations (43, 294).

The high protein and total solute concentration, however, can negatively impact the lyophilisation process by increasing the cryo concentration effect (295, 296), favouring phase separation (295, 296) and increasing product dry layer resistance (Rp), hence primary drying time (297). However, the effect of the increased Rp was mitigated by the low fill volume which contributed to the achievement of short lyophilisation cycles (Chapter 4). Cryo concentration and phase separation were not a focus of this study,

however, IgG1 formulations were observed to be stable pre and post lyophilisation, showing that freezing and drying steps did not negatively impact the biophysical stability of the formulated IgG1 (Chapter 6).

The biophysical stability of the IgG formulations was studied at stressed conditions post lyophilisation, thermal stress and photo induced aggregation were investigated (Chapter 6). The thermal stress experiments show a thermal unfolding (~ 75 °C) of the protein measured by DSC in line with changes of the tertiary structure monitored by CD. In this thesis, DSC was the only technique used to monitor biophysical stability that did not require sample dilution and was performed at the initial formulation concentration (100 mg/mL). The limited number of techniques available to monitor biophysical stability at high protein concentration is a key challenge in the development of high concentration protein formulations (13). This highlights the requirement for the identification and development of novel techniques to study high concentration protein formulations.

7.2.2 Lyophilisation process design

Regarding the lyophilisation process, the freezing step was initially optimised. It was observed that lower freezing temperature, slower ramp and prolonged freezing improved the cake appearance of the product. Therefore, process parameters (Chapter 4) were selected to provide the optimal cake appearance (Chapter 5).

During primary drying, the effect of the high Rp was counterbalanced by the low fill volume, allowing the development of lyophilisation cycles with short primary drying times (11-12h conservative cycle, 5h aggressive cycle, Chapter 4). The possibility to successfully lyophilise high concentration protein formulations with relatively short lyophilisation cycles is the third significant contribution to this study. Considering that lyophilisation is generally a time consuming and expensive process (40, 42), the

possibility to reduce total cycle times is a key objective for pharmaceutical companies. In this study, the lyo-cycle development was conducted with the use of a lyomodelling tool for estimation of the primary drying step (product temperature, time and sublimation flow) (72) (Chapter 4).

The lyomodelling QbD tool was informed with input parameters extrapolated from three process analytical technology (PAT) tools used to monitor product temperature as critical process parameter (CPP). The combination of QbD and PAT tools, was selected as a favoured strategy to better understand the lyophilisation process and estimate primary drying. This approach enables the development of a lyophilisation cycle and construction of a design space around the cycle to evaluate and ensure process robustness, when CPPs are modified in a certain range (72, 248). The use of lyomodelling is therefore particularly useful during the development stage, where the prediction tool can minimise the number of pilot, engineering and validation cycles required to target the optimal cycle parameters. However, to ensure accurate output information, precise input parameters should be used to inform the model. Input parameters include formulation and vials configuration, heat transfer coefficient (Kv) and Rp parameters. Kv and Rp can be mathematically calculated using measured product temperature and the accuracy of their determination critically affects the accuracy of the model (71, 72, 248). In Chapter 4, a thorough comparison of three commonly used product temperature monitoring systems (63, 64) was conducted. For the first time, Kv and Rp for each system were experimentally calculated, compared and used as inputs in the model. Differences in the design, operating principle and ease of use can all impact the selection of one PAT tool over another. Understanding existing differences between the operation and applications of these tools represents a key learning.

7.2.3 Other QbD approaches

In addition to the lyomodelling tool, two additionally novel QbD approaches have been employed in this thesis: the empirical model for formulation critical temperatures prediction (Chapter 3) and the correlation models between both headspace residual moisture and product residual moisture, and product residual moisture and Tg (Chapter 5). The use of QbD approaches enabled formulation and process development saving costs and time and reducing the number of iterative experiments.

The main advantage of developing an empirical model for formulation critical temperatures prediction is the capability of this model to accurately estimate not only Tg' but also Tc of formulations included in a design space. The correlation model established between vial headspace water vapour and product moisture provided a non-destructive method to accurately estimate product residual moisture. This model enables analysis of 100% of the batch retaining samples, which can be critical during the development stage when API supply can be limited.

It is important to note that the significant correlation established between product residual moisture and Tg was linked to the stability of selected amorphous formulations (phase transitions such as recrystallisation is inhibited for these formulations). Phase transitions can potentially disrupt the robustness of the model due to a change in equilibrium moisture uptake by the formulation. However, for the formulation developed in this thesis, when vial headspace moisture was determined by performing a single, non-destructive technique, then product moisture and Tg could be accurately predicted.

For these QbD approaches, a model protein BSA was used to develop the models, which were then successfully verified using equivalent IgG1 formulations. The possibility to use model proteins to conduct preliminary studies and estimate the formulation behaviour is

a cost effective strategy. This strategy becomes particularly important for high value products (e.g. IgG formulations). Limited availability and high costs of these materials can represent a challenge in a formulation development stage, especially during lyophilisation of high concentration protein formulations, where a large amount of material is required. In particular, in this thesis, the model protein BSA was effectively employed in the estimation of critical temperatures and physical stability which were shown to be similar for equivalent IgG1 formulations. On the contrary, biophysical stability which is strictly protein dependent was evaluated on the protein formulation containing the target protein, IgG1.

7.2.4 Primary packaging configuration

Primary packaging configuration and components should be considered as integral parts of the formulation that can contribute to the processing and stability of a lyophilised formulation. Therefore, the type, size of vials and the stoppers (83-85) should be appropriately selected and processed ensuring sterilisation and dryness. In this study, 5 mL tubing vials were used for several reasons: a) to enhance equipment capacity- a larger number of smaller vials can be lyophilised in the same cycle determining higher productivity; b) the low fill volume selected (1.1 mL) could be accommodated in 5 mL vials (<50% of the vial fill volume); c) the use of tubing vials which are generally less variable than moulded vials and provide more accurate Kv parameters. The use of a low fill volume was also observed to be a successful formulation strategy. The low fill volume allowed a 1:1 dilution to achieve short reconstitution times (37) and to obtain short lyophilisation cycles mitigating the effect of high product dry layer resistance.

7.3 Advantages and disadvantages of key aspects investigated

Table 7.1 summarises the advantages and disadvantages of the key aspects studied in the thesis and discussed in Section 7.2.

Table 7.1 Summary of advantages and disadvantages of key aspects studied in the thesis.

Key aspect	Advantages	Disadvantages
Use of arg/arg-HCl as excipient	• Reduction of light induced aggregation • Reduction of viscosity • Reduction of specific surface area • Improvement of product cake appearance	• Depression of formulation critical temperatures
High protein concentration	• Inhibition of excipients crystallisation • Bulking agent • Buffering agent	• High tendency to aggregate is protein dependent • High viscosity
High total solute concentration	• Solid and rigid cake structure (reduced mobility) • Lower hygroscopicity	• High product dry layer resistance • Longer freezing (at lower soak temperature, slow ramp, prolonged time) • Longer primary drying • Longer reconstitution time
Use of model protein	• More cost effective formulation and process development	• Not representative of the biophysical stability which is highly protein dependent
Completely amorphous formulation	• At specific ratios, absence of excipients recrystallisation even when formulations are exposed to drastic humidity conditions	• More extensive stability investigation required
No requirement for cold chain storage	• Reduced costs • Products can be stored at temperatures ≥ 25 °C (High Tg)	• More studies required to establish the limitations of storage conditions (temperature, time)
Use of 5 mL vials	• Higher production capacity	• More difficult to handle
Use of low fill volume	• Shorter reconstitution time • Shorter lyophilisation cycles (counterbalancing the high product dry layer resistance) • Higher protein concentration pre lyophilisation (dilution 1:1)	• Larger headspace containing water vapour that could potentially be transferred to the product increasing residual moisture over time (dryness of the stoppers is important)
QbD approaches & PAT tools	• Reduced number of trials and errors • Improved process understanding	• Accurate input information to avoid bias in the estimation

7.4 Summary of key findings

The overall aim of this thesis was to provide insights into the development of high concentration antibody formulations in the lyophilised state and to obtain a formulation capable to protect the IgG from stresses experienced during concentration, formulation,

lyophilisation process and storage. The selection of the appropriate formulation components at critical concentrations was essential for the achievement of a stable lyophilised formulation.

The main novel aspect was the possibility to lyophilise formulations with high concentrations of IgG1 (100 mg/mL) and consequent high total solute concentrations (200 mg/mL), finding formulation and process strategies able to avoid impairment of the product quality and stability. As outcome of this study, the key findings to consider in formulating lyophilised high concentration of IgGs are summarised as follows:

I. Arg/arg-HCl was shown to be an excipient extremely useful in the formulation of high concentration protein formulations since it reduces light induced aggregation and viscosity in the liquid state, and reduces specific surface area, improving the cake appearance of lyophilised products. In contrast, arg/arg-HCl at high concentrations can significantly depress critical temperatures for lyophilisation process, however, this effect of arg/arg-HCl can be mitigated by the high concentration of protein which increases the critical temperatures. Therefore, it is particularly important to identify the optimal ratio protein: excipient to use in formulation.

II. The empirical model (based on DOE) was an essential tool to aid selection of the excipient ratio and to predict and optimise critical temperatures.

III. The primary drying optimisation was conducted using a lyomodelling tool for primary drying estimation. The tool predicted lyophilisation cycles with accurate and significantly shorter primary drying times.

IV. The correlation model developed enables estimation of product moisture and Tg using a non-destructive technique.

V. The optimised lyophilisation process did not compromise the biophysical stability of the IgG1 formulations after six months with and without cold chain storage and can be potentially employed to prolong the shelf-life of selected IgG1 formulations. The high Tg and low residual moisture achieved make these lyophilised products potential good candidates for the elimination of cold chain, rending their storage and supply more cost effective.

7.5 Limitations

The limitations of this study are mainly related to the restricted availability of the IgG1 material which limited the number and type of protein formulation approaches that could be investigated. As a consequence, a limited number of well-established techniques which often require dilution were used to evaluate physical and biophysical stability, reducing the possibility to investigate additional and novel methods for high concentration protein formulations in the liquid or solid state. Finally, in Chapter 4, the impact of different primary drying shelf temperatures on the lyophilisation cycle were evaluated. On the contrary, the effect of pressure variation during primary drying was not studied. This represents a limitation considering the significant impact of pressure on Kv and therefore on Tp and primary drying time. Evaluating the impact of pressure allows the construction of a complete design space around the target cycle, establishing proven acceptable ranges. Finally, the limited amount of IgG did not allow the execution of some experiments that could have added value to this thesis. For instance, the activity of the protein pre and post lyophilisation was not analysed, therefore this study is limited to the investigation of aggregation and structure of the IgG pre and post lyophilisation.

7.6 Recommendations for future work

This thesis provides new insights into the development of lyophilised high concentration antibody formulations. This work can be considered a starting point for future research in the field of lyophilisation of high concentration antibody formulations.

Future work is recommended in the following areas:

I. Investigation on the effect of different amino acids as 'novel' excipients in high concentration protein formulations. Amino acids can act as multi-functional excipients; they are included in a low number of recent commercial protein products and some amino acids seem to have synergistic effects if combined in formulation.

II. Identification of new techniques and methods for the characterisation of high concentration protein formulations in the liquid and lyophilised state. Aggregation can be underestimated when dilution of high concentration protein formulations is executed during the analysis at operative conditions; a limited number of techniques is available to monitor biophysical stability of lyophilised protein formulations.

III. Further optimisation of the proposed lyophilisation cycle can be conducted, considering the impact of pressure during primary drying and creating a complete design space. The impact of shelf temperature during primary drying was evaluated in the study, however, pressure excursion can also impact the process and should be assessed to ensure robustness of the lyophilisation process. The impact of more aggressive lyophilisation cycles on the biophysical stability of the protein could also be explored as future work.

IV. Exploration of the effect of different fill volumes to develop the best strategy. In this study, the use of low fill volumes at high protein and solid concentrations was identified as a successful strategy for the development of high concentration protein formulations. The effect of larger fill volumes at lower protein concentration pre lyophilisation could be investigated as an alternative formulation approach.

V. Further investigation into the root causes that can induce cake defects during lyophilisation of high concentration formulations (e.g. cracking) could be investigated. Generally, root causes responsible for cosmetic defects of the lyophilised cakes are not clearly identified. In this study, arg/arg-HCl was observed to reduce cracking defects in lyophilised cakes of sucrose rich formulations. Additional studies would be required to understand if other excipients can play a similar role, identifying their mechanistic behaviour.

VI. Investigation on the reconstitution time of high concentration protein formulations. More objective and reproducible methods as well as strategies and excipients to reduce reconstitution time are required. In this study, the reconstitution time of selected IgG1 formulations was approximately 5 min. In literature, however, high concentration protein formulations can show prolonged reconstitution times, up to 40 min in some cases (37). Finally, methods currently used to determine reconstitution time are extremely operator dependent and poorly reproducible.

7.7 Conclusions

The overall aim of the thesis was to develop a stable lyophilised high concentration IgG formulation. The primary objectives of this thesis were achieved by selecting a successful formulation strategy. In particular, the identification of suitable excipients (types and ratios) was conducted by a thorough study of the formulation approaches used in commercial protein products. Arg/arg-HCl was selected as excipient due to its positive roles in the formulation of high concentration protein formulations, also demonstrated in this study (e.g. Arg/arg-HCl preserves protein from photo induced aggregation). The use of QbD approaches was extremely useful in the selection of formulations with high critical temperatures, but also in the development of an optimal lyo-cycle and in the estimation of physical parameters (Tg and product moisture). The selected formulation showed preserved biophysical stability pre and post lyophilisation and after storage for 6 months with and without cold chain.

The use of a model protein (BSA) and QbD tools enabled a more cost effective development of stable IgG formulations. The short lyophilisation cycles and the absence of cold chain requirement render manufacturing and storage of lyophilised high concentration IgG formulations more convenient for pharmaceutical companies, healthcare providers and patients. Therefore, this thesis provides a significant contribution to the field and improves current knowledge on lyophilisation of high concentration protein formulations. Finally, it provides recommendations and new points of discussion that could be further investigated, such as the identification of novel techniques to monitor biophysical stability of proteins at high concentrations in the liquid and solid state.

References

1. Dingermann T. Recombinant therapeutic proteins: production platforms and challenges. Biotechnology journal. 2008;3(1):90-7.
2. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nature Reviews Drug Discovery. 2005;4(4):298-306.
3. Carter PJ. Introduction to current and future protein therapeutics: a protein engineering perspective. Experimental cell research. 2011;317(9):1261-9.
4. Descotes J, Gouraud A. Clinical immunotoxicity of therapeutic proteins. Expert opinion on drug metabolism & toxicology. 2008;4(12):1537-49.
5. Cui Y, Cui P, Chen B, Li S, Guan H. Monoclonal antibodies: formulations of marketed products and recent advances in novel delivery system. Drug development and industrial pharmacy. 2017;43(4):519-30.
6. Wang W. Protein aggregation and its inhibition in biopharmaceutics. International Journal of Pharmaceutics. 2005;289(1):1-30.
7. Wang W, Nema S, Teagarden D. Protein aggregation—Pathways and influencing factors. International journal of pharmaceutics. 2010;390(2):89-99.
8. Calamai M, Taddei N, Stefani M, Ramponi G, Chiti F. Relative influence of hydrophobicity and net charge in the aggregation of two homologous proteins. Biochemistry. 2003;42(51):15078-83.
9. Breen C. Protein Structure and Function-Gregory A. Petsko, Dagmar Ringe (Eds.), Blackwell Scientific Publications, Oxford,@ $29.95, Paperback, ISBN 1405119225. International Journal of Biochemistry and Cell Biology. 2004;7(36):1365.
10. Schulz GE, Schirmer RH. Principles of protein structure: Springer Science & Business Media; 2013.
11. 87 H. Protein structure 2019 [updated 26/01/2020; cited 26/01/2020. Available from: https://www.bing.com/images/search?view=detailV2&ccid=lQvP0Hkw&id=C273CB5476F28878EEC3F05E97BE251834084C3A&thid=OIP.lQvP0HkwJPqSXq7ypEutfAAAAA&mediaurl=https%3a%2f%2fupload.wikimedia.org%2fwikipedia%2fcommons%2fthumb%2f0%2f05%2fProtein_structure.png%2f350px-Protein_structure.png&exph=263&expw=350&q=Protein+structure&simid=608017680344484332&selectedIndex=16&ajaxhist=0.
12. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability, and formulation. Journal of pharmaceutical sciences. 2007;96(1):1-26.
13. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. Journal of pharmaceutical sciences. 2004;93(6):1390-402.
14. Shire S. Monoclonal Antibodies: Meeting the Challenges in Manufacturing, Formulation, Delivery and Stability of Final Drug Product: Woodhead Publishing; 2015.
15. Janeway C, Travers P, Walport M, Schlomchik M. Immunobiology: The Immune System in Health and Disease Garland Publishing. New York. 2001.
16. Roque ACA, Lowe CR, Taipa MÂ. Antibodies and genetically engineered related molecules: production and purification. Biotechnology progress. 2004;20(3):639-54.
17. Harris RJ, Shire SJ, Winter C. Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies. Drug development research. 2004;61(3):137-54.
18. Van Audenhove I, Gettemans J. Nanobodies as versatile tools to understand, diagnose, visualize and treat cancer. EBioMedicine. 2016;8:40-8.
19. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. Journal of pharmaceutical sciences. 2009;98(9):2909-34.
20. Jeong SH. Analytical methods and formulation factors to enhance protein stability in solution. Archives of Pharmacal Research. 2012;35(11):1871-86.
21. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. Nature reviews Drug discovery. 2014;13(9):655.

22. Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. Pharmaceutical research. 2004;21(6):897-903.
23. Patten P, Schellekens H. The immunogenicity of biopharmaceuticals. Lessons learned and consequences for protein drug development. Developments in biologicals. 2003;112:81-97.
24. Bhatnagar BS, Bogner RH, Pikal MJ. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharmaceutical development and technology. 2007;12(5):505-23.
25. Kolhe P, Amend E, K Singh S. Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation. Biotechnology progress. 2010;26(3):727-33.
26. Hawe A, Kasper JC, Friess W, Jiskoot W. Structural properties of monoclonal antibody aggregates induced by freeze–thawing and thermal stress. European Journal of Pharmaceutical Sciences. 2009;38(2):79-87.
27. Schreiber G. Kinetic studies of protein–protein interactions. Current opinion in structural biology. 2002;12(1):41-7.
28. Warne NW. Development of high concentration protein biopharmaceuticals: The use of platform approaches in formulation development. European Journal of Pharmaceutics and Biopharmaceutics. 2011;78(2):208-12.
29. Garidel P, Kuhn AB, Schäfer LV, Karow-Zwick AR, Blech M. High-concentration protein formulations: How high is high? European Journal of Pharmaceutics and Biopharmaceutics. 2017;119:353-60.
30. Neergaard MS, Kalonia DS, Parshad H, Nielsen AD, Møller EH, van de Weert M. Viscosity of high concentration protein formulations of monoclonal antibodies of the IgG1 and IgG4 subclass–Prediction of viscosity through protein–protein interaction measurements. European Journal of Pharmaceutical Sciences. 2013;49(3):400-10.
31. Liu JS, Steven J, inventor; Novartis AG, Genentech Inc, assignee. Method of reducing viscosity of high concentration protein formulations2010.
32. Colandene JD, Maldonado LM, Creagh AT, Vrettos JS, Goad KG, Spitznagel TM. Lyophilization cycle development for a high-concentration monoclonal antibody formulation lacking a crystalline bulking agent. Journal of pharmaceutical sciences. 2007;96(6):1598-608.
33. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Advanced Drug Delivery Reviews. 2006;58(5):686-706.
34. Garidel P, Blume A, Wagner M. Prediction of colloidal stability of high concentration protein formulations. Pharmaceutical development and technology. 2015;20(3):367-74.
35. He F, Woods CE, Litowski JR, Roschen LA, Gadgil HS, Razinkov VI, et al. Effect of sugar molecules on the viscosity of high concentration monoclonal antibody solutions. Pharmaceutical research. 2011;28(7):1552-60.
36. Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ. Effect of Moisture on the Stability of a Lyophilized Humanized Monoclonal Antibody Formulation. Pharmaceutical Research. 2001;18(9):1345-53.
37. Cao W, Krishnan S, Ricci MS, Shih L-Y, Liu D, Gu JH, et al. Rational design of lyophilized high concentration protein formulations-mitigating the challenge of slow reconstitution with multidisciplinary strategies. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(2):287-93.
38. Kulkarni SS, Suryanarayanan R, Rinella JV, Bogner RH. Mechanisms by which crystalline mannitol improves the reconstitution time of high concentration lyophilized protein formulations. European Journal of Pharmaceutics and Biopharmaceutics. 2018;131:70-81.
39. Kawasaki H, Shimanouchi T, Kimura Y. Recent Development of Optimization of Lyophilization Process. Journal of Chemistry. 2019;2019.
40. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharmaceutical research. 1997;14(8):969-75.
41. Franks F, Auffret T. Freeze-drying of Pharmaceuticals and Biopharmaceuticals: royal Society of Chemistry; 2008.
42. Tang X, Pikal MJ. Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice. Pharmaceutical Research. 2004;21(2):191-200.

43. Bishara RH. Cold chain management–an essential component of the global pharmaceutical supply chain. American Pharmaceutical Review. 2006;9(1):105-9.
44. Kasper JC, Winter G, Friess W. Recent advances and further challenges in lyophilization. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(2):162-9.
45. Ohtake S, Kita Y, Arakawa T. Interactions of formulation excipients with proteins in solution and in the dried state. Advanced drug delivery reviews. 2011;63(13):1053-73.
46. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. Rational design of stable protein formulations: Springer; 2002. p. 109-33.
47. Patel SM, Pikal MJ. Emerging freeze-drying process development and scale-up issues. Aaps Pharmscitech. 2011;12(1):372-8.
48. Pisano R, Fissore D, Barresi AA, Rastelli M. Quality by design: scale-up of freeze-drying cycles in pharmaceutical industry. AAPS PharmSciTech. 2013;14(3):1137-49.
49. Fissore D, Pisano R, Barresi AA. Monitoring of the Secondary Drying in Freeze-Drying of Pharmaceuticals. Journal of Pharmaceutical Sciences. 2011;100(2):732-42.
50. Schneid SC, Gieseler H, Kessler WJ, Luthra SA, Pikal MJ. Optimization of the secondary drying step in freeze drying using TDLAS technology. Aaps Pharmscitech. 2011;12(1):379-87.
51. Pikal MJ, Shah S. Intravial distribution of moisture during the secondary drying stage of freeze drying. PDA journal of pharmaceutical science and technology. 1997;51(1):17-24.
52. Pikal MJ, Shah S. The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy phase. International Journal of Pharmaceutics. 1990;62(2):165-86.
53. Franks F. Freeze drying: from empiricism to predictability. Cryo-Lett. 1990;11:93-110.
54. Kett V, McMahon D, Ward K. Freeze-drying of protein pharmaceuticals–the application of thermal analysis. Cryo letters. 2004;25(6):389-404.
55. Chatterjee K, Shalaev EY, Suryanarayanan R. Partially Crystalline Systems in Lyophilization: II. Withstanding Collapse at High Primary Drying Temperatures and Impact on Protein Activity Recovery. Journal of Pharmaceutical Sciences. 2005;94(4):809-20.
56. Searles JA, Carpenter JF, Randolph TW. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg′ in pharmaceutical lyophilization. Journal of pharmaceutical sciences. 2001;90(7):872-87.
57. Anko M, Bjelošević M, Planinšek O, Trstenjak U, Logar M, Ahlin Grabnar P, et al. The formation and effect of mannitol hemihydrate on the stability of monoclonal antibody in the lyophilized state. International Journal of Pharmaceutics. 2019;564:106-16.
58. Akers MJ. Excipient–drug interactions in parenteral formulations. Journal of pharmaceutical sciences. 2002;91(11):2283-300.
59. Searles JA. Freezing and annealing phenomena in lyophilization. Freeze-drying/lyophilization of pharmaceutical and biological products: CRC Press; 2016. p. 66-95.
60. Rambhatla S, Ramot R, Bhugra C, Pikal MJ. Heat and mass transfer scale-up issues during freeze drying: II. Control and characterization of the degree of supercooling. AAPS PharmSciTech. 2004;5(4):54-62.
61. Konstantinidis AK, Kuu W, Otten L, Nail SL, Sever RR. Controlled nucleation in freeze-drying: Effects on pore size in the dried product layer, mass transfer resistance, and primary drying rate. Journal of pharmaceutical sciences. 2011;100(8):3453-70.
62. Hardwick LM, Paunicka C, Akers MJ. Critical factors in the design and optimization of lyophilisation processes. Innov Pharm Technol. 2008;26:70-4.
63. Nail S, Tchessalov S, Shalaev E, Ganguly A, Renzi E, Dimarco F, et al. Recommended best practices for process monitoring instrumentation in pharmaceutical freeze drying—2017. AAPS PharmSciTech. 2017;18(7):2379-93.
64. Schneid S, Gieseler H. Evaluation of a new wireless temperature remote interrogation system (TEMPRIS) to measure product temperature during freeze drying. Aaps Pharmscitech. 2008;9(3):729-39.
65. Depaz RA, Pansare S, Patel SM. Freeze-Drying Above the Glass Transition Temperature in Amorphous Protein Formulations While Maintaining Product Quality and Improving Process Efficiency. Journal of pharmaceutical sciences. 2015.

66. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins III: collapse during storage at elevated temperatures. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(2):240-52.
67. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: Stability after freeze-drying. Journal of Pharmaceutical Sciences. 2010;99(5):2256-78.
68. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: stability during storage at elevated temperatures. Journal of pharmaceutical sciences. 2012;101(7):2288-306.
69. Patel SM, Pikal M. Process analytical technologies (PAT) in freeze-drying of parenteral products. Pharmaceutical development and technology. 2009;14(6):567-87.
70. Patel SM, Jameel F, Pikal MJ. The effect of dryer load on freeze drying process design. Journal of pharmaceutical sciences. 2010;99(10):4363-79.
71. Pikal M, Roy M, Shah S. Mass and heat transfer in vial freeze-drying of pharmaceuticals: Role of the vial. Journal of pharmaceutical sciences. 1984;73(9):1224-37.
72. Pikal M. Use of laboratory data in freeze drying process design: heat and mass transfer coefficients and the computer simulation of freeze drying. PDA Journal of Pharmaceutical Science and Technology. 1985;39(3):115-39.
73. Hibler S, Wagner C, Gieseler H. Vial freeze-drying, part 1: New insights into heat transfer characteristics of tubing and molded vials. Journal of pharmaceutical sciences. 2012;101(3):1189-201.
74. Kuu WY, Hardwick LM, Akers MJ. Rapid determination of dry layer mass transfer resistance for various pharmaceutical formulations during primary drying using product temperature profiles. International journal of pharmaceutics. 2006;313(1-2):99-113.
75. Patel SM, Pikal MJ. Lyophilization process design space. Journal of pharmaceutical sciences. 2013;102(11):3883-7.
76. Patel SM, Doen T, Pikal MJ. Determination of end point of primary drying in freeze-drying process control. Aaps Pharmscitech. 2010;11(1):73-84.
77. Duralliu A, Matejtschuk P, Williams DR. Humidity induced collapse in freeze dried cakes: A direct visualization study using DVS. European Journal of Pharmaceutics and Biopharmaceutics. 2018;127:29-36.
78. Burnett D, Thielmann F, Booth J. Determining the critical relative humidity for moisture-induced phase transitions. International journal of pharmaceutics. 2004;287(1):123-33.
79. Pikal M, Dellerman K, Roy M. Formulation and stability of freeze-dried proteins: effects of moisture and oxygen on the stability of freeze-dried formulations of human growth hormone. Developments in biological standardization. 1992;74:21-37; discussion -8.
80. Hsu C, Ward CA, Pearlman R, Nguyen H, Yeung D, Curley JG. Determining the optimum residual moisture in lyophilized protein pharmaceuticals. Developments in Biological Standardization. 1992;74:255-70; discussion 71.
81. DeGrazio FL. 16 Closure and Container Considerations in Lyophilization. Freeze Drying/Lyophilization of Pharmaceutical and Biological Products. 2016:396.
82. Scutellà B, Passot S, Bourlés E, Fonseca F, Tréléa IC. How Vial Geometry Variability Influences Heat Transfer and Product Temperature During Freeze-Drying. Journal of Pharmaceutical Sciences. 2017;106(3):770-8.
83. Sasaki H, Kikuchi J, Maeda T, Kuboniwa H. Impact of different elastomer formulations on moisture permeation through stoppers used for lyophilized products stored under humid conditions. PDA journal of pharmaceutical science and technology. 2010;64(1):63-70.
84. Donovan P, Corvari V, Burton M, Rajagopalan N. Effect of stopper processing conditions on moisture content and ramifications for lyophilized products: comparison of “low” and “high” moisture uptake stoppers. PDA journal of pharmaceutical science and technology. 2007;61(1):51-8.
85. Templeton AC, Placek J, Xu H, Mahajan R, Hunke WA, Reed RA. Determination of the moisture content of bromobutyl rubber stoppers as a function of processing: implications for

the stability of lyophilized products. PDA journal of pharmaceutical science and technology. 2003;57(2):75-87.
86. Danielson JW, Oxborrow GS, Placencia AM. Chemical leaching of rubber stoppers into parenteral solutions. PDA Journal of Pharmaceutical Science and Technology. 1983;37(3):89-92.
87. Sacha G, Rogers JA, Miller RL. Pre-filled syringes: a review of the history, manufacturing and challenges. Pharmaceutical development and technology. 2015;20(1):1-11.
88. Ingle RG, Agarwal AS. Pre-filled syringe – a ready-to-use drug delivery system: a review. Expert Opinion on Drug Delivery. 2014;11(9):1391-9.
89. Nail SL, Searles JA. Elements of quality by design in development and scale-up of freeze-dried parenterals. Biopharm international. 2008;21(1).
90. Rambhatla S, Pikal MJ. Heat and mass transfer scale-up issues during freeze-drying, I: atypical radiation and the edge vial effect. Aaps Pharmscitech. 2003;4(2):22-31.
91. Mockus LN, Paul TW, Pease NA, Harper NJ, Basu PK, Oslos EA, et al. Quality by design in formulation and process development for a freeze-dried, small molecule parenteral product: a case study. Pharmaceutical development and technology. 2011;16(6):549-76.
92. Pisano R, Fissore D, Barresi AA, Brayard P, Chouvenc P, Woinet B. Quality by design: optimization of a freeze-drying cycle via design space in case of heterogeneous drying behavior and influence of the freezing protocol. Pharmaceutical development and technology. 2013;18(1):280-95.
93. Koganti VR, Shalaev EY, Berry MR, Osterberg T, Youssef M, Hiebert DN, et al. Investigation of design space for freeze-drying: use of modeling for primary drying segment of a freeze-drying cycle. AAPS PharmSciTech. 2011;12(3):854-61.
94. Scientific S. LyoModelling Calculator, SP Scientific 2019 [updated 17/05/2019. Available from: https://www.spscientific.com/LyoCalc/Lyocalculator.html.
95. Hottot A, Vessot S, Andrieu J. Determination of mass and heat transfer parameters during freeze-drying cycles of pharmaceutical products. PDA Journal of Pharmaceutical Science and Technology. 2005;59(2):138-53.
96. Overcashier DE, Patapoff TW, Hsu CC. Lyophilization of protein formulations in vials: Investigation of the relationship between resistance to vapor flow during primary drying and small-scale product collapse. Journal of pharmaceutical sciences. 1999;88(7):688-95.
97. Arsiccio A, Barresi AA, Pisano R. Prediction of ice crystal size distribution after freezing of pharmaceutical solutions. Crystal Growth & Design. 2017;17(9):4573-81.
98. Sahni EK, Pikal MJ. Modeling the Secondary Drying Stage of Freeze Drying: Development and Validation of an Excel-Based Model. Journal of Pharmaceutical Sciences. 2017;106(3):779-91.
99. Bosca S, Fissore D. Design and validation of an innovative soft-sensor for pharmaceuticals freeze-drying monitoring. Chemical Engineering Science. 2011;66(21):5127-36.
100. Bosca S, Barresi AA, Fissore D. Use of soft sensors to monitor a pharmaceuticals freeze-drying process in vials. Pharmaceutical development and technology. 2014;19(2):148-59.
101. Lietta E, Colucci D, Distefano G, Fissore D. On the use of infrared thermography for monitoring a vial freeze-drying process. Journal of pharmaceutical sciences. 2019;108(1):391-8.
102. Smith G, Polygalov E, Arshad M, Page T, Taylor J, Ermolina I. An impedance-based process analytical technology for monitoring the lyophilisation process. International journal of pharmaceutics. 2013;449(1-2):72-83.
103. Smith G, Arshad MS, Polygalov E, Ermolina I. An application for impedance spectroscopy in the characterisation of the glass transition during the lyophilization cycle: the example of a 10% w/v maltodextrin solution. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(3):1130-40.
104. De Beer T, Vercruysse P, Burggraeve A, Quinten T, Ouyang J, Zhang X, et al. In-line and real-time process monitoring of a freeze drying process using Raman and NIR spectroscopy as complementary process analytical technology (PAT) tools. Journal of pharmaceutical sciences. 2009;98(9):3430-46.
105. Arshad MS, Smith G, Polygalov E, Ermolina I. Through-vial impedance spectroscopy of critical events during the freezing stage of the lyophilization cycle: the example of the impact

of sucrose on the crystallization of mannitol. European Journal of Pharmaceutics and Biopharmaceutics. 2014;87(3):598-605.
106. Colucci D, Maniaci R, Fissore D. Monitoring of the freezing stage in a freeze-drying process using IR thermography. International journal of pharmaceutics. 2019.
107. Fontana LK. Development of a Raman Spectroscopic Method to Detect Protein Conformation in Amorphous Solids: Application to Protein Formulation and Stability. 2019.
108. Lighthouse. FMS-Moisture/Pressure Headspace Analyzer 2019 [updated 26/05/2019. Available from: http://www2.lighthouseinstruments.com/l/302881/2018-01-09/zcm3.
109. Cook IA, Ward KR. Applications of headspace moisture analysis for investigating the water dynamics within a sealed vial containing freeze-dried material. PDA journal of pharmaceutical science and technology. 2011;65(1):2-11.
110. EMA. ICH Q6B Specifications: test procedures and acceptance criteria for biotechnological/biological products 01/09/1999 [updated 08/12/2019. Available from: https://www.ema.europa.eu/en/ich-q6b-specifications-test-procedures-acceptance-criteria-biotechnologicalbiological-products.
111. Buggins TR, Dickinson PA, Taylor G. The effects of pharmaceutical excipients on drug disposition. Advanced drug delivery reviews. 2007;59(15):1482-503.
112. Loftsson T. Excipient pharmacokinetics and profiling. International Journal of Pharmaceutics. 2015;480(1):48-54.
113. Hovgaard SFFaL. Pharmaceutical Formulation Development of Peptides and Proteins. United States of America: CRC Press; 2000.
114. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharmaceutical research. 2003;20(9):1325-36.
115. Moeller EH, Jorgensen L. Alternative routes of administration for systemic delivery of protein pharmaceuticals. Drug Discovery Today: Technologies. 2008;5(2):e89-e94.
116. Franks F. Freeze-drying of bioproducts: putting principles into practice. European Journal of Pharmaceutics and Biopharmaceutics. 1998;45(3):221-9.
117. Jameel F, Hershenson S. Formulation and process development strategies for manufacturing biopharmaceuticals: John Wiley & Sons; 2010.
118. Angkawinitwong U, Sharma G, Khaw PT, Brocchini S, Williams GR. Solid-state protein formulations. Therapeutic delivery. 2015;6(1):59-82.
119. Uchiyama S. Liquid formulation for antibody drugs. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. 2014;1844(11):2041-52.
120. Meyer BK. Therapeutic Protein Drug Products: Practical Approaches to Formulation in the Laboratory, Manufacturing, and the Clinic: Elsevier; 2012.
121. Schwegman JJ, Hardwick LM, Akers MJ. Practical formulation and process development of freeze-dried products. Pharmaceutical development and technology. 2005;10(2):151-73.
122. Drugs.com. Physician's Desk Reference (PDR), Drug information [updated 31/03/2018. Available from: https://www.drugs.com/pdr/.
123. Regulation (EC) No. 726/2004 Laying down community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency, 726/2004 (2004).
124. FDA. FDA Drugs [updated 18/06/2018. Available from: https://www.fda.gov/Drugs/default.htm.
125. Product Information Document. Marketing Authorisation Holder 2018.
126. EMA. European public assessment reports (EPAR) for human medicines published by the European Medicines Agency (EMA). 1995 [updated 18/06/2018. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/epar_search.jsp&mid=WC0b01ac058001d124.
127. Kuhn AB, Kube S, Karow-Zwick AR, Seeliger D, Garidel P, Blech M, et al. Improved Solution-State Properties of Monoclonal Antibodies by Targeted Mutations. The Journal of Physical Chemistry B. 2017;121(48):10818-27.

128. FDA. Inactive Ingredients Database 2016 [updated 23/03/2018. Available from: http://wayback.archive-it.org/7993/20170112022245/http:/www.fda.gov/Drugs/InformationOnDrugs/ucm113978.htm.
129. FDA. GRAS Substances (SCOGS) Database 2018 [updated 23/03/2018. Available from: https://www.accessdata.fda.gov/scripts/fdcc/?set=SCOGS.
130. EMA. Scientific Discussion, Assesment history, EPAR 2018 [updated 18/06/2018. Available from: www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/003766.
131. Garidel P, Pevestorf B, Bahrenburg S. Stability of buffer-free freeze-dried formulations: A feasibility study of a monoclonal antibody at high protein concentrations. European Journal of Pharmaceutics and Biopharmaceutics. 2015;97:125-39.
132. Meyer BK, Ni A, Hu B, Shi L. Antimicrobial preservative use in parenteral products: past and present. Journal of pharmaceutical sciences. 2007;96(12):3155-67.
133. EMA. A guideline on summary of product characteristics (SmPc) 2009 [updated 21/03/2018. Available from: https://ec.europa.eu/health//sites/health/files/files/eudralex/vol-2/c/smpc_guideline_rev2_en.pdf.
134. EMA SAG. Section 3. Pharmaceutical Form (SmPC training presentation) 20 [updated 01/2013. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Presentation/2013/01/WC500137013.pdf.
135. Wang W. Tolerability of hypertonic injectables. International Journal of Pharmaceutics. 2015;490(1):308-15.
136. EMA. METALYSE, EPAR Scientific Discussion, Assessment history 23/02/2001 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000306/WC500026888.pdf.
137. Pelegrine DHG, Gasparetto CA. Whey proteins solubility as function of temperature and pH. LWT - Food Science and Technology. 2005;38(1):77-80.
138. Henry R. Costantino MJP. Lyophilization of Biopharmaceuticals. R.T. Borchardt CRM, editor. United States of America: American Association of Pharmaceutical Scientists Arlington; 2004.
139. Sundaramurthi P, Shalaev E, Suryanarayanan R. "pH Swing" in Frozen Solutions-Consequence of Sequential Crystallization of Buffer Components. Journal of Physical Chemistry Letters. 2010;1(1):265-8.
140. Gero L, Smyrl T. Behavior of Low Molecular Weight Organic Acids During Freeze Drying. Journal of Food Science. 1982;47(3):954-7.
141. Gomez G, Pikal MJ, Rodríguez-Hornedo N. Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions. Pharmaceutical research. 2001;18(1):90-7.
142. Mehmood Y, Farooq U. Excipients Use in Parenteral and Lyophilized Formulation Development. Open Sci J Pharm Pharmacol. 2015;3:19-27.
143. Padilla A. Formulation Development in Freeze Dried Pharmaceuticals 2009 [updated 10/12/2017. SP Scientific Webinar]. Available from: https://spscientific.adobeconnect.com/_a944471687/p3il0v6w449/.
144. Complete M. Handbook of Pharmaceutical Excipients 2016 [updated 10/12/2017. Available from: https://www.medicinescomplete.com/mc/excipients/current/.
145. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein–excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development. Advanced Drug Delivery Reviews. 2011;63(13):1118-59.
146. Al-Hussein A, Gieseler H. Investigation of histidine stabilizing effects on LDH during freeze-drying. Journal of pharmaceutical sciences. 2013;102(3):813-26.
147. Arakawa T, Tsumoto K, Kita Y, Chang B, Ejima D. Biotechnology applications of amino acids in protein purification and formulations. Amino Acids. 2007;33(4):587-605.
148. Stärtzel P, Gieseler H, Gieseler M, Abdul-Fattah AM, Adler M, Mahler HC, et al. Freeze Drying of l-Arginine/Sucrose-Based Protein Formulations, Part I: Influence of

Formulation and Arginine Counter Ion on the Critical Formulation Temperature, Product Performance and Protein Stability. Journal of pharmaceutical sciences. 2015;104(7):2345-58.
149. Santana H, González Y, Campana PT, Noda J, Amarantes O, Itri R, et al. Screening for stability and compatibility conditions of recombinant human epidermal growth factor for parenteral formulation: Effect of pH, buffers, and excipients. International Journal of Pharmaceutics. 2013;452(1):52-62.
150. Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM. Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. Pharmaceutical research. 2003;20(12):1952-60.
151. Wei Wang CJR. Aggregation of therapeutic proteins. New Jersey, United States of America: John Wiley adn Sons; 2010.
152. Forney-Stevens KM, Bogner RH, Pikal MJ. Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: I. Screening of 15 Amino Acids in Two Model Proteins. Journal of pharmaceutical sciences. 2015.
153. Shah D, Li J, Shaikh AR, Rajagopalan R. Arginine–aromatic interactions and their effects on arginine-induced solubilization of aromatic solutes and suppression of protein aggregation. Biotechnology progress. 2012;28(1):223-31.
154. Inoue N, Takai E, Arakawa T, Shiraki K. Specific decrease in solution viscosity of antibodies by arginine for therapeutic formulations. Molecular pharmaceutics. 2014;11(6):1889-96.
155. Baynes BM, Wang DI, Trout BL. Role of arginine in the stabilization of proteins against aggregation. Biochemistry. 2005;44(12):4919-25.
156. Shukla D, Schneider CP, Trout BL. Complex Interactions between Molecular Ions in Solution and Their Effect on Protein Stability. Journal of the American Chemical Society. 2011;133(46):18713-8.
157. Vagenende V, Han AX, Mueller M, Trout BL. Protein-associated cation clusters in aqueous arginine solutions and their effects on protein stability and size. ACS chemical biology. 2012;8(2):416-22.
158. Shukla D, Trout BL. Understanding the Synergistic Effect of Arginine and Glutamic Acid Mixtures on Protein Solubility. Journal of Physical Chemistry B. 2011;115(41):11831-9.
159. Mattern M, Winter G, Kohnert U, Lee G. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. Pharmaceutical development and technology. 1999;4(2):199-208.
160. Inoue N, Takai E, Arakawa T, Shiraki K. Arginine and lysine reduce the high viscosity of serum albumin solutions for pharmaceutical injection. Journal of Bioscience and Bioengineering. 2014;117(5):539-43.
161. Staertzel P. Principles of Formulation Design for Pharmaceutical Freeze Drying - " The Art of Cooking" Part I 2014 [updated 10/12/2017. Available from: https://spscientific.adobeconnect.com/_a944471687/p6tp17ump4h/?launcher=false&fcsContent=true&pbMode=normal.
162. Wang W. Lyophilization and development of solid protein pharmaceuticals. International Journal of Pharmaceutics. 2000;203(1):1-60.
163. Baheti A, Kumar L, Bansal AK. Excipients used in lyophilization of small molecules. Journal of Excipients and Food Chemicals. 2016;1(1).
164. Tarelli E, Mire-Sluis A, Tivnann HA, Bolgiano B, Crane DT, Gee C, et al. Recombinant human albumin as a stabilizer for biological materials and for the preparation of international reference reagents. Biologicals. 1998;26(4):331-46.
165. Jain NK, Roy I. Effect of trehalose on protein structure. Protein Science. 2009;18(1):24-36.
166. Jorgensen L, Hostrup S, Moeller EH, Grohganz H. Recent trends in stabilising peptides and proteins in pharmaceutical formulation–considerations in the choice of excipients. Expert opinion on drug delivery. 2009;6(11):1219-30.
167. Shental-Bechor D, Levy Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. Proceedings of the National Academy of Sciences. 2008;105(24):8256-61.

168. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharmaceutical research. 2010;27(4):544-75.
169. Patel SM, Nail SL, Pikal MJ, Geidobler R, Winter G, Hawe A, et al. Lyophilized Drug Product Cake Appearance: What Is Acceptable? Journal of Pharmaceutical Sciences. 2017;106(7):1706-21.
170. Piedmonte DM, Summers C, McAuley A, Karamujic L, Ratnaswamy G. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharmaceutical research. 2007;24(1):136-46.
171. Ohtake S, Wang YJ. Trehalose: current use and future applications. Journal of pharmaceutical sciences. 2011;100(6):2020-53.
172. Jovanović N, Bouchard A, Hofland GW, Witkamp G-J, Crommelin DJA, Jiskoot W. Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying. European Journal of Pharmaceutical Sciences. 2006;27(4):336-45.
173. Cesaro A, De Giacomo O, Sussich F. Water interplay in trehalose polymorphism. Food chemistry. 2008;106(4):1318-28.
174. Shire SJ. Formulation and manufacturability of biologics. Current Opinion in Biotechnology. 2009;20(6):708-14.
175. Willart J, De Gusseme A, Hemon S, Descamps M, Leveiller F, Rameau A. Vitrification and polymorphism of trehalose induced by dehydration of trehalose dihydrate. The Journal of Physical Chemistry B. 2002;106(13):3365-70.
176. EMA. ORENCIA, EPAR Scientific Discussion, Assessment history 2007 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000701/WC500048938.pdf.
177. Ohrem HL, Schornick E, Kalivoda A, Ognibene R. Why is mannitol becoming more and more popular as a pharmaceutical excipient in solid dosage forms? Pharmaceutical development and technology. 2014;19(3):257-62.
178. Pikal MJ, Dellerman KM, Roy ML, Riggin RM. The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharmaceutical research. 1991;8(4):427-36.
179. Johnson RE, Kirchhoff CF, Gaud HT. Mannitol–sucrose mixtures—versatile formulations for protein lyophilization. Journal of pharmaceutical sciences. 2002;91(4):914-22.
180. Cleland JL, Lam X, Kendrick B, Yang J, Yang Th, Overcashier D, et al. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. Journal of pharmaceutical sciences. 2001;90(3):310-21.
181. EMA. MYLOTARG, EPAR, Assessment history [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/004204/WC500248705.pdf.
182. Khan TA, Mahler H-C, Kishore RS. Key interactions of surfactants in therapeutic protein formulations: A review. European Journal of Pharmaceutics and Biopharmaceutics. 2015;97:60-7.
183. Lee HJ, McAuley A, Schilke KF, McGuire J. Molecular origins of surfactant-mediated stabilization of protein drugs. Advanced Drug Delivery Reviews. 2011;63(13):1160-71.
184. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. Journal of pharmaceutical sciences. 2008;97(8):2924-35.
185. Kim HL, McAuley A, Livesay B, Gray WD, McGuire J. Modulation of Protein Adsorption by Poloxamer 188 in Relation to Polysorbates 80 and 20 at Solid Surfaces. Journal of Pharmaceutical Sciences.103(4):1043-9.
186. Bin T, Kulshreshtha AK, Al-Shakhshir R, Hem SL. Adsorption of benzalkonium chloride by filter membranes: mechanisms and effect of formulation and processing parameters. Pharmaceutical development and technology. 1999;4(2):151-65.
187. EMA. PEGASYS, EPAR Scientific Discussion, Assessment history 20/06/2002 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000395/WC500039192.pdf.
188. Brange J, Langkjaer L. Chemical stability of insulin. 3. Influence of excipients, formulation, and pH. Acta pharmaceutica nordica. 1992;4(3):149-58.

189. Telang C, Yu L, Suryanarayanan R. Effective inhibition of mannitol crystallization in frozen solutions by sodium chloride. Pharmaceutical research. 2003;20(4):660-7.
190. Costantino HR. Excipients for Use in Lyophilized Pharmaceutical Peptide, Protein. Lyophilization of biopharmaceuticals. 2004;2:139.
191. Pramanick S, Singodia D, Chandel V. Excipient selection in parenteral formulation development. Pharma Times. 2013;45(3):65-77.
192. DeFelippis MR, Dobbins MA, Frank BH, Li S, Rebhun DM, inventors; Eli Lilly and Co Ltd (GB), assignee. Stable insulin formulations2003.
193. Brader ML, Sukumar M, Pekar AH, McClellan DS, Chance RE, Flora DB, et al. Hybrid insulin cocrystals for controlled release delivery. Nature biotechnology. 2002;20(8):800-5.
194. Vlugt-Wensink K, Meijer Y, Van Steenbergen M, Verrijk R, Jiskoot W, Crommelin D, et al. Effect of excipients on the encapsulation efficiency and release of human growth hormone from dextran microspheres. European Journal of Pharmaceutics and Biopharmaceutics. 2007;67(3):589-96.
195. Frost GI. Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration. Expert opinion on drug delivery. 2007;4(4):427-40.
196. Bookbinder L, Hofer A, Haller M, Zepeda M, Keller G-A, Lim J, et al. A recombinant human enzyme for enhanced interstitial transport of therapeutics. Journal of Controlled Release. 2006;114(2):230-41.
197. EMA. MEPACT, EPAR European Public Assessment Report, Product Information 2009 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000802/WC500026565.pdf.
198. Exner R, Wessner B, Manhart N, Roth E. Therapeutic potential of glutathione. Wiener Klinische Wochenschrift. 2000;112(14):610-6.
199. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomedicine & Pharmacotherapy. 2003;57(3):145-55.
200. Baek M, Choy J-H, Choi S-J. Montmorillonite intercalated with glutathione for antioxidant delivery: synthesis, characterization, and bioavailability evaluation. International journal of pharmaceutics. 2012;425(1):29-34.
201. Ltd FH-LR. MabThera® SC/ Rituxan Hycela™ (rituximab/ hyaluronidase human) 2018 [updated 13/04/2018. Available from: https://www.roche.com/products/product-details.htm?productId=8abc3f49-f39f-4c6e-93ae-235c95c7efc6.
202. EMA. REFIXIA, EPAR Scientific Discussion, Assessment history 23/03/2016 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/004178/WC500232819.pdf.
203. EMA. BLINCYTO, EPAR Assessment report, Assessment history 24/09/2015 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/003731/WC500198227.pdf.
204. EMA. LUCENTIS EPAR European Public Assessment Report, Product Information [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000715/WC500043546.pdf.
205. Site W. Drugs Rx List (FDA source) 2018 [updated 07/03/2018. Available from: https://www.rxlist.com/drugs/alpha_a.htm.
206. initiative Gab. Biosimilars of bevacizumab 2014 [updated 13/10/2017. Available from: http://gabionline.net/Biosimilars/General/Biosimilars-of-bevacizumab.
207. EMA. MVASI, EPAR Assessment report, Assessment history 2017 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/004728/WC500242877.pdf.
208. initiative Gab. Biosimilars of rituximab 2015 [updated 10/11/2017. Available from: http://gabionline.net/Biosimilars/General/Biosimilars-of-rituximab.
209. EMA. REPATHA, EPAR Assessment report, Assessment history 2015 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/003766/WC500191400.pdf.

210. EMA. PRIVIGEN, EPAR Assessment report, Assessment history 2008 [updated 25/06/2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/000831/WC500043081.pdf.
211. Chang BS, Hershenson S. Practical approaches to protein formulation development. Rational Design of Stable Protein Formulations: Springer; 2002. p. 1-25.
212. Meister E, Gieseler H. Freeze-dry microscopy of protein/sugar mixtures: Drying behavior, interpretation of collapse temperatures and a comparison to corresponding glass transition data. Journal of pharmaceutical sciences. 2009;98(9):3072-87.
213. Liu J. Physical characterization of pharmaceutical formulations in frozen and freeze-dried solid states: techniques and applications in freeze-drying development. Pharmaceutical development and technology. 2006;11(1):3-28.
214. Hajare AA, More HN, Walekar PS, Hajare DA. Optimization of freeze drying cycle protocol using real time microscopy and integrated differential thermal analysis-electrical impedance. Research Journal of Pharmacy and Technology. 2012;5(7):13.
215. Gearing J, Malik KP, Matejtschuk P. Use of dynamic mechanical analysis (DMA) to determine critical transition temperatures in frozen biomaterials intended for lyophilization. Cryobiology. 2010;61(1):27-32.
216. Mujat M, Greco K, Galbally-Kinney KL, Hammer DX, Ferguson RD, Iftimia N, et al. Optical coherence tomography-based freeze-drying microscopy. Biomed Opt Express. 2012;3(1):55-63.
217. Greco K, Mujat M, Galbally-Kinney KL, Hammer DX, Ferguson RD, Iftimia N, et al. Accurate prediction of collapse temperature using optical coherence tomography-based freeze-drying microscopy. Journal of pharmaceutical sciences. 2013;102(6):1773-85.
218. Boss EA, Filho RM, de Toledo ECV. Freeze drying process: real time model and optimization. Chemical Engineering and Processing: Process Intensification. 2004;43(12):1475-85.
219. Sadikoglu H, Ozdemir M, Seker M. Freeze-drying of pharmaceutical products: Research and development needs. Drying Technology. 2006;24(7):849-61.
220. Dixon D, Tchessalov S, Bhatnagar B. Lyophilization: Process Design, Robustness, and Risk Management. Challenges in Protein Product Development: Springer; 2018. p. 407-39.
221. Weng L, Vijayaraghavan R, MacFarlane DR, Elliott GD. Application of the Kwei equation to model the Tg behavior of binary blends of sugars and salts. Cryobiology. 2014;68(1):155-8.
222. Nagoe A, Oguni M. Hydrogen-bond network formation of water molecules and its effects on the glass transitions in the ethylene glycol aqueous solutions: failure of the Gordon–Taylor law in the water-rich range and absence of the Tg= 115 K rearrangement process in bulk pure water. Journal of Physics: Condensed Matter. 2010;22(32):325103.
223. Shamblin SL, Taylor LS, Zografi G. Mixing behavior of colyophilized binary systems. Journal of pharmaceutical sciences. 1998;87(6):694-701.
224. Pinal R. Entropy of mixing and the glass transition of amorphous mixtures. Entropy. 2008;10(3):207-23.
225. Seo J-A, Kim S, Kwon H-J, Yang Y, Kim HK, Hwang Y-H. The glass transition temperatures of sugar mixtures. Carbohydrate research. 2006;341(15):2516-20.
226. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WLJ. How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. European Journal of Pharmaceutics and Biopharmaceutics. 2017;114:288-95.
227. Das U, Hariprasad G, Ethayathulla AS, Manral P, Das TK, Pasha S, et al. Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. PloS one. 2007;2(11):e1176.
228. Miyatake T, Yoshizawa S, Arakawa T, Shiraki K. Charge state of arginine as an additive on heat-induced protein aggregation. International journal of biological macromolecules. 2016;87:563-9.
229. Reddy K RC, Lilie H, Rudolph R, Lange C. L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. Protein Science. 2005;14(4):929-35.

230. Arakawa T, Tsumoto K. The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. Biochemical and biophysical research communications. 2003;304(1):148-52.
231. Arakawa T, Ejima D, Tsumoto K, Obeyama N, Tanaka Y, Kita Y, et al. Suppression of protein interactions by arginine: a proposed mechanism of the arginine effects. Biophysical chemistry. 2007;127(1-2):1-8.
232. Shukla D, Trout BL. Interaction of arginine with proteins and the mechanism by which it inhibits aggregation. The Journal of Physical Chemistry B. 2010;114(42):13426-38.
233. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T. Role of arginine in protein refolding, solubilization, and purification. Biotechnology progress. 2004;20(5):1301-8.
234. Hackl E, Darkwah J, Smith G, Ermolina I. Effect of Arginine on the Aggregation of Protein in Freeze-Dried Formulations Containing Sugars and Polyol: 1—Formulation Development. AAPS PharmSciTech. 2018;19(2):896-911.
235. Hackl E, Darkwah J, Smith G, Ermolina I. Effect of Arginine on the Aggregation of Protein in Freeze-Dried Formulations Containing Sugars and Polyol: II. BSA Reconstitution and Aggregation. AAPS PharmSciTech. 2018;19(7):2934-47.
236. Stärtzel P. Arginine as an Excipient for Protein Freeze-Drying: A Mini Review. Journal of pharmaceutical sciences. 2018;107(4):960-7.
237. Chang BS, Randall CS. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology. 1992;29(5):632-56.
238. Izutsu K-i, Yomota C, Okuda H, Kawanishi T, Randolph TW, Carpenter JF. Impact of heat treatment on miscibility of proteins and disaccharides in frozen solutions. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(2):177-83.
239. Tischer A, Lilie H, Rudolph R, Lange C. L-Arginine hydrochloride increases the solubility of folded and unfolded recombinant plasminogen activator rPA. Protein Science. 2010;19(9):1783-95.
240. Stärtzel P, Gieseler H, Gieseler M, Abdul-Fattah AM, Adler M, Mahler H-C, et al. Freeze-drying of L-arginine/sucrose-based protein formulations, Part 2: optimization of formulation design and freeze-drying process conditions for an L-arginine chloride-based protein formulation system. Journal of pharmaceutical sciences. 2015;104(12):4241-56.
241. Binabaji E, Ma J, Zydney AL. Intermolecular interactions and the viscosity of highly concentrated monoclonal antibody solutions. Pharmaceutical research. 2015;32(9):3102-9.
242. Ward KR, Matejtschuk P. Characterization of Formulations for Freeze-Drying. Lyophilization of Pharmaceuticals and Biologicals: Springer; 2019. p. 1-32.
243. Wang Y, Snee RD, Meng W, Muzzio FJ. Predicting flow behavior of pharmaceutical blends using shear cell methodology: A quality by design approach. Powder technology. 2016;294:22-9.
244. Kasper JC, Wiggenhorn M, Resch M, Friess W. Implementation and evaluation of an optical fiber system as novel process monitoring tool during lyophilization. European Journal of Pharmaceutics and Biopharmaceutics. 2013;83(3):449-59.
245. Schneid SC, Gieseler H, Kessler WJ, Pikal MJ. Non-invasive product temperature determination during primary drying using tunable diode laser absorption spectroscopy. Journal of pharmaceutical sciences. 2009;98(9):3406-18.
246. Wang DQ, Hey JM, Nail SL. Effect of Collapse on the Stability of Freeze-Dried Recombinant Factor VIII and α-amylase. Journal of Pharmaceutical Sciences. 2004;93(5):1253-63.
247. Passot S, Fonseca F, Barbouche N, Marin M, Alarcon-Lorca M, Rolland D, et al. Effect of product temperature during primary drying on the long-term stability of lyophilized proteins. Pharmaceutical development and technology. 2007;12(6):543-53.
248. Giordano A, Barresi AA, Fissore D. On the use of mathematical models to build the design space for the primary drying phase of a pharmaceutical lyophilization process. Journal of Pharmaceutical Sciences. 2011;100(1):311-24.
249. Schneid S. Investigation of novel process analytical technology (PAT) tools for use in freeze-drying processes. 2009.

250. Read E, Shah R, Riley B, Park J, Brorson K, Rathore A. Process analytical technology (PAT) for biopharmaceutical products: Part II. Concepts and applications. Biotechnology and bioengineering. 2010;105(2):285-95.
251. Tang XC, Nail SL, Pikal MJ. Freeze-drying process design by manometric temperature measurement: design of a smart freeze-dryer. Pharmaceutical research. 2005;22(4):685-700.
252. Tang X, Nail SL, Pikal MJ. Evaluation of manometric temperature measurement, a process analytical technology tool for freeze-drying: Part I, product temperature measurement. AAPS PharmSciTech. 2006;7(1):E95-E103.
253. Tang XC, Nail SL, Pikal MJ. Evaluation of manometric temperature measurement, a process analytical technology tool for freeze-drying: part II measurement of dry-layer resistance. AAPS PharmSciTech. 2006;7(4):E77-E84.
254. Kuu WY, Nail SL, Sacha G. Rapid determination of vial heat transfer parameters using tunable diode laser absorption spectroscopy (TDLAS) in response to step-changes in pressure set-point during freeze-drying. Journal of Pharmaceutical Sciences. 2009;98(3):1136-54.
255. Pisano R, Fissore D, Barresi AA. Heat transfer in freeze-drying apparatus. Developments in Heat Transfer: InTechOpen; 2011.
256. Ellab. Ellab Thermal Validation Solutions 2019 [updated 01/05/2019. Available from: http://www.ellab.com/.
257. GmbH i-ms. Tempris sensor technology 2019 [updated 01/05/2019. Available from: https://www.tempris.com/.
258. Horn J, Schanda J, Friess W. Impact of fast and conservative freeze-drying on product quality of protein-mannitol-sucrose-glycerol lyophilizates. European Journal of Pharmaceutics and Biopharmaceutics. 2018;127:342-54.
259. T. McCoy BA, D. Khamar. Considering Residual Moisture Measurement in Lyophilized Drug Product 2019 [updated December 2019. Available from: https://www.americanpharmaceuticalreview.com/Featured-Articles/559534-Considering-Residual-Moisture-Measurement-in-Lyophilized-Drug-Product/.
260. Duncan D. Headspace Moisture Analysis for Determination of Residual Moisture Content in Lyophilized Pharmaceutical Products. BIOPHARM INTERNATIONAL. 2016;29(6):36-40.
261. Jameel F, Khan M. Quality-by-design as applied to the development and manufacturing of a lyophilized protein product. Am Pharm Rev. 2009;12(7).
262. Awotwe-Otoo D, Agarabi C, Wu GK, Casey E, Read E, Lute S, et al. Quality by design: Impact of formulation variables and their interactions on quality attributes of a lyophilized monoclonal antibody. International Journal of Pharmaceutics. 2012;438(1):167-75.
263. Kasper JC, Friess W. The freezing step in lyophilization: Physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. European Journal of Pharmaceutics and Biopharmaceutics. 2011;78(2):248-63.
264. Beech KE, Biddlecombe JG, van der Walle CF, Stevens LA, Rigby SP, Burley JC, et al. Insights into the influence of the cooling profile on the reconstitution times of amorphous lyophilized protein formulations. European Journal of Pharmaceutics and Biopharmaceutics. 2015;96:247-54.
265. Rasmussen DH, MacKenzie AP. Glass transition in amorphous water. Application of the measurements to problems arising in cryobiology. The Journal of physical chemistry. 1971;75(7):967-73.
266. Qi P, Volkin DB, Zhao H, Nedved ML, Hughes R, Bass R, et al. Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. Journal of pharmaceutical sciences. 2009;98(9):3117-30.
267. Demeule B, Lawrence MJ, Drake AF, Gurny R, Arvinte T. Characterization of protein aggregation: the case of a therapeutic immunoglobulin. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2007;1774(1):146-53.
268. Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. Journal of molecular medicine. 2003;81(11):678-99.

269. Baert F, Noman M, Vermeire S, Van Assche G, D'Haens G, Carbonez A, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. New England Journal of Medicine. 2003;348(7):601-8.
270. West R, Zelinkova Z, Wolbink G, Kuipers E, Stokkers P, Van Der Woude C. Immunogenicity negatively influences the outcome of adalimumab treatment in Crohn's disease. Alimentary pharmacology & therapeutics. 2008;28(9):1122-6.
271. Li Y, Williams TD, Topp EM. Effects of excipients on protein conformation in lyophilized solids by hydrogen/deuterium exchange mass spectrometry. Pharmaceutical research. 2008;25(2):259-67.
272. Sinha S, Li Y, Williams TD, Topp EM. Protein conformation in amorphous solids by FTIR and by hydrogen/deuterium exchange with mass spectrometry. Biophysical journal. 2008;95(12):5951-61.
273. Moorthy BS, Zarraga IE, Kumar L, Walters BT, Goldbach P, Topp EM, et al. Solid-state hydrogen–deuterium exchange mass spectrometry: correlation of deuterium uptake and long-term stability of lyophilized monoclonal antibody formulations. Molecular pharmaceutics. 2017;15(1):1-11.
274. Moorthy BS, Schultz SG, Kim SG, Topp EM. Predicting protein aggregation during storage in lyophilized solids using solid state amide hydrogen/deuterium exchange with mass spectrometric analysis (ssHDX-MS). Molecular pharmaceutics. 2014;11(6):1869-79.
275. Maity H, O'Dell C, Srivastava A, Goldstein J. Effects of arginine on photostability and thermal stability of IgG1 monoclonal antibodies. Current pharmaceutical biotechnology. 2009;10(8):761-6.
276. Schneider CP, Shukla D, Trout BL. Arginine and the Hofmeister Series: The Role of Ion-Ion Interactions in Protein Aggregation Suppression. Journal of Physical Chemistry B. 2011;115(22):7447-58.
277. Borwankar AU, Dear BJ, Twu A, Hung JJ, Dinin AK, Wilson BK, et al. Viscosity reduction of a concentrated monoclonal antibody with arginine· HCl and arginine· glutamate. Industrial & Engineering Chemistry Research. 2016;55(43):11225-34.
278. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2005;1751(2):119-39.
279. Kim NA, Lim DG, Lim JY, Kim KH, Shim WS, Kang N-G, et al. Evaluation of protein formulation and its viscosity with DSC, DLS, and microviscometer. Journal of Pharmaceutical Investigation. 2014;44(4):309-16.
280. Pelton JT, McLean LR. Spectroscopic methods for analysis of protein secondary structure. Analytical biochemistry. 2000;277(2):167-76.
281. Dutta U, Cohenford MA, Dain JA. Monitoring the effect of glucosamine and glyceraldehyde glycation on the secondary structure of human serum albumin and immunoglobulin G: An analysis based on circular dichroism, thermal melting profiles and UV–fluorescence spectroscopy. Analytica chimica acta. 2006;558(1-2):187-94.
282. Aghaie A, Pourfathollah A, Bathaie S, Moazzeni S, Khorsand Mohammad Pour H, Banazadeh S. Structural study on immunoglobulin G solution after pasteurization with and without stabilizer. Transfusion Medicine. 2008;18(1):62-70.
283. Katayama DS, Nayar R, Chou DK, Campos J, Cooper J, Vander Velde DG, et al. Solution behavior of a novel type 1 interferon, interferon-τ. Journal of pharmaceutical sciences. 2005;94(12):2703-15.
284. Capelle MA, Gurny R, Arvinte T. A high throughput protein formulation platform: case study of salmon calcitonin. Pharmaceutical research. 2009;26(1):118.
285. Kim NA, Lim DG, Lim JY, Kim KH, Jeong SH. Comprehensive evaluation of etanercept stability in various concentrations with biophysical assessment. International journal of pharmaceutics. 2014;460(1-2):108-18.
286. Werk T, Huwyler J, Hafner M, Luemkemann J, Mahler H-C. An impedance-based method to determine reconstitution time for freeze-dried pharmaceuticals. Journal of pharmaceutical sciences. 2015;104(9):2948-55.
287. Vermeer AW, Norde W. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. Biophysical journal. 2000;78(1):394-404.

288. Hawe A, Hulse WL, Jiskoot W, Forbes RT. Taylor dispersion analysis compared to dynamic light scattering for the size analysis of therapeutic peptides and proteins and their aggregates. Pharmaceutical research. 2011;28(9):2302-10.
289. Luik A, Naboka YN, Mogilevich S, Hushcha T, Mischenko N. Study of human serum albumin structure by dynamic light scattering: two types of reactions under different pH and interaction with physiologically active compounds. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 1998;54(10):1503-7.
290. Jachimska B, Wasilewska M, Adamczyk Z. Characterization of globular protein solutions by dynamic light scattering, electrophoretic mobility, and viscosity measurements. Langmuir. 2008;24(13):6866-72.
291. Bahrenburg S, Karow AR, Garidel P. Buffer-free therapeutic antibody preparations provide a viable alternative to conventionally buffered solutions: From protein buffer capacity prediction to bioprocess applications. Biotechnology journal. 2015;10(4):610-22.
292. Lange C, Rudolph R. Suppression of protein aggregation by L-arginine. Current pharmaceutical biotechnology. 2009;10(4):408-14.
293. Gokarn YR, Kras E, Nodgaard C, Dharmavaram V, Fesinmeyer RM, Hultgen H, et al. Self-buffering antibody formulations. Journal of pharmaceutical sciences. 2008;97(8):3051-66.
294. Krämer I, Tredree R, Vulto AG. Points to consider in the evaluation of biopharmaceuticals. EJHP Practice. 2008;14(1):73-6.
295. Heller MC, Carpenter JF, Randolph TW. Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation. Biotechnology and bioengineering. 1999;63(2):166-74.
296. Izutsu K-i, Kojima S. Freeze-concentration separates proteins and polymer excipients into different amorphous phases. Pharmaceutical research. 2000;17(10):1316-22.
297. Liu J, Viverette T, Virgin M, Anderson M, Dalal P. A study of the impact of freezing on the lyophilization of a concentrated formulation with a high fill depth. Pharmaceutical development and technology. 2005;10(2):261-72.

Appendix

Supplementary Information Chapter 2 and 3

Supplementary information for Chapter 2 and 3 are available online at the doi links below:

Chapter 2: htTps://doi.org/10.1016/j.ejpb.2018.07.011;

Chapter 3: htTps://doi.org/10.1016/j.ijpharm.2019.118807.

Supplementary Information Chapter 4

Table A1 Input parameters for Kv determination using the gravimetric method.

Kv input information	Centre vials	Edge vials	Shuttle vials (1 & 2)
Vial capacity, mL	5	5	5
External diameter (Dout), cm	2.2	2.2	2.2
Weight loss, g	0.88 ± 0.04	1.087 ± 0.098 (Factor: 1.24)	Shuttle 1: 1.078 ± 0.094 (Factor: 1.23)
			Shuttle 2: 1.055 ± 0.118 (Factor: 1.21)
Pressure, mTorr	99.73 ± 0.32 (CM, 0.133 mbar)	99.73 ± 0.32 (CM, 0.133 mbar)	99.73 ± 0.32 (CM, 0133 mbar)
Shelf inlet temperature	Lyo shelf inlet	Lyo shelf inlet	Lyo shelf inlet
Product temperature, °C	Measured using TWTC, Ellab RTDs, Tempris	Measured using TWTC, Ellab RTDs, Tempris	Measured using TWTC, Ellab RTDs, Tempris
Time, hrs	every minute during the ice sublimation step	every minute during the ice sublimation step	every minute during the ice sublimation step

Table A2 Input parameters for Rp determination.

Rp input information	Value
Vial capacity, mL	5
Internal diameter (Din), cm	2.2
External diameter (Dout), cm	2
Ice density, g/cm^3	0.918
Solution density, g/cm^3	1.2
Fill volume, mL	1.1
Water content, g	1.06 (80% of total weight)
Dry cake thickness, cm	0.35
Time, hrs	every minute over primary drying (when CM pressure is stabilised until the end of primary drying)
Shelf inlet temperature, °C	Lyo shelf inlet temperature over primary drying
Product temperature, °C	Measured using TWTC, Ellab RTDs, Tempris systems over primary drying
Pressure, mTorr	CM pressure measured over primary drying (0.99 mTorr → 0.133 mbar)

Table A3 Lyomodelling input parameters (1st part) – Kv and Rp information.

Kv and Rp input information		TWTC (Mean±SD)		Ellab, RTDs (Mean±SD)		Tempris (Mean±SD)	
Kv (W/m^2 °C)		Centre vials: **16.12±0.14**		Centre vials: **15.19±0.19**		Centre vials: **14.70±0.18**	
		Edge vials: **20.08±0.17**		Edge vials: **18.85±0.23**		Edge vials: **18.25±0.22**	
		Ellab shuttles vials: **19.50±0.17**		Ellab shuttles vials: **18.29±0.22**		Ellab shuttle vials: **17.72±0.21**	
Rp (Conservative cycle, Tshelf= -25 °C)		*P:S*	*P:S:A*	*P:S*	*P:S:A*	*P:S*	*P:S:A*
	Rp_0	**1.05**	**0.92**	**0**	**0**	**0.13**	**0.19**
	A_1	**67.59**	**69.79**	**56.99**	**61.92**	**71.98**	**66.10**
	A_2	**0.38**	**0.30**	**0**	**0**	**0.58**	**0.88**

Rp values for a general material with high dry layer resistance which increases non-linearly with dry layer thickness Rp_0= 2, A_1= 90, A_2=5 (94);

Rp values for a general material with high dry layer resistance which increases linearly with dry layer thickness Rp_0= 2, A_1= 40, A_2=0 (94).

Table A4 Lyomodelling input parameters (2nd part) – vial, formulation and lyo-cycle information.

Vial input information	**Value**	
Vial capacity, mL	5	
Internal diameter (din), cm	2	
External diameter (dout), cm	2.2	
Formulation input information	**Value**	
Ice density, g/cm^3	0.918	
Solution density, g/cm^3	1.2	
Fill volume, mL	1.1	
Water content, g	1.06	
Dry extract, (% w/w and % w/v)	15.41 and 20	
Dry layer thickness, cm	0.35	
Number of vials	60 (filled), 312 (total)	
Collapse temperature, °C	P:S	P:S:A
	-20.68 ± 1.17	-23.09 ± 1.57
Lyo-cycle input information	**Value**	
Shelf inlet temperature, °C	Lyo shelf inlet	
Pressure, Torr	CM pressure measured over primary drying (0.099 Torr → 0.133 mbar)	
Time, hrs	every minute over primary drying (when CM pressure is stabilised until the end of primary drying)	

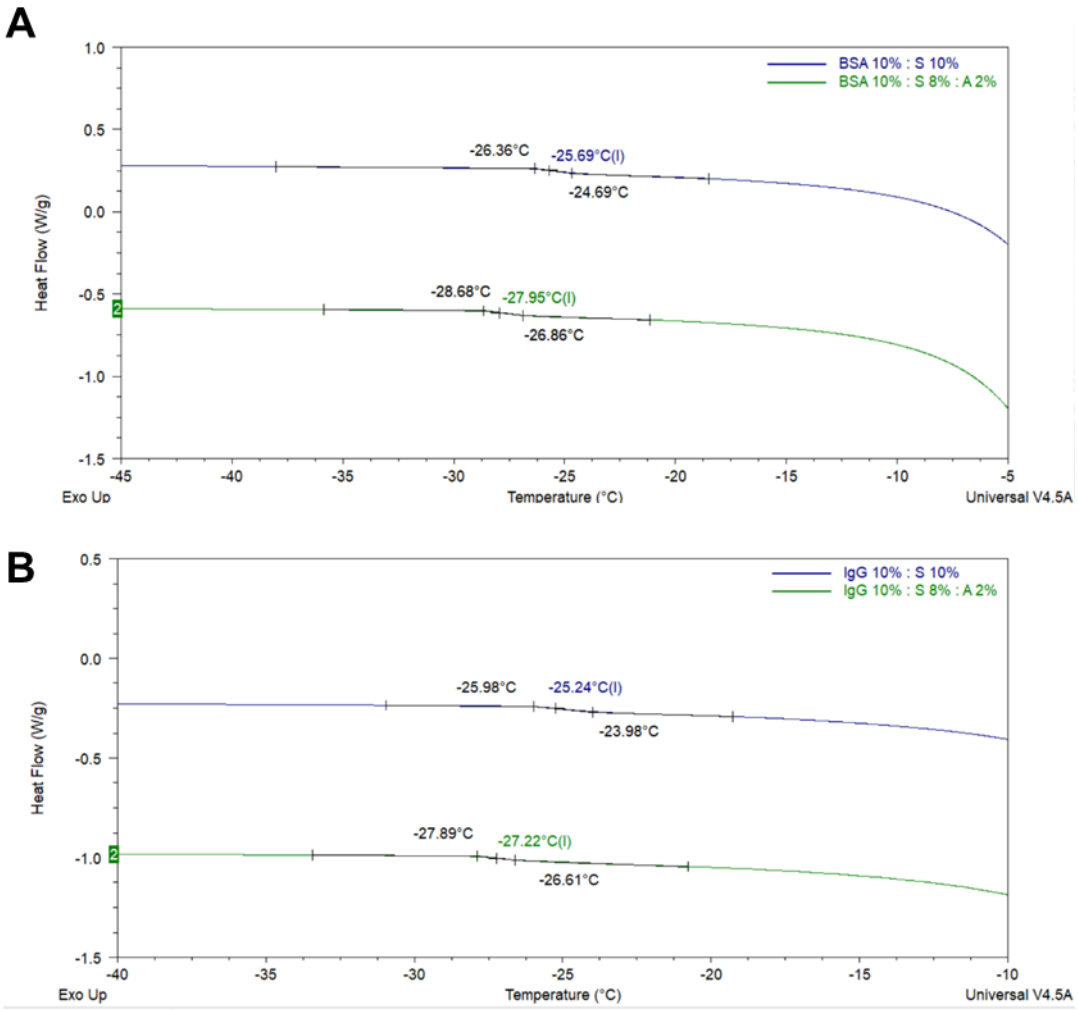
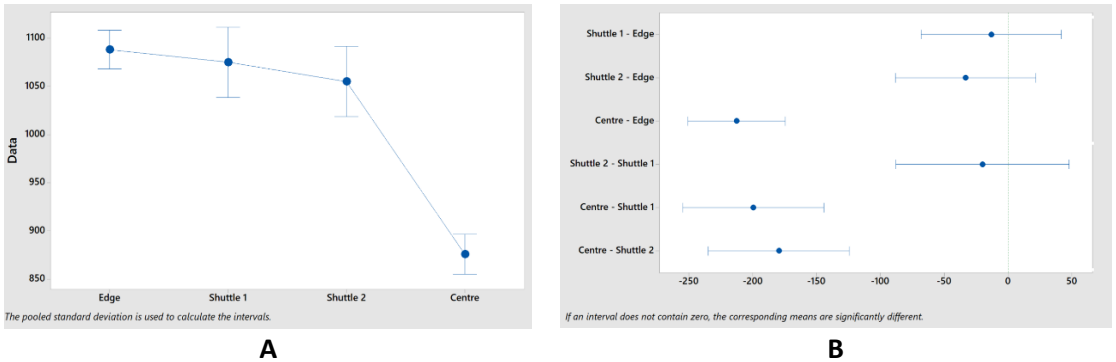


Figure A1 Examples of the Tg' determination for 1a) BSA formulations and 1b) IgG formulations in presence (green) and absence (blue) of arg/arg-HCl.



A **B**

Figure A2 Differences observed in the weight loss of Edge, Shuttle 1, Shuttle 2, Center vials. 2a) Mean and SD of the weight loss for each group of vials, 2b) Tukey's multiple comparison of the weight loss determined for each group of vials. Weight loss of the centre vials is significantly different if compared to the vials located on the first row close to the Ellab shuttles and on the first row at the edges, due to radiation effects.

Supplementary Information Chapter 5

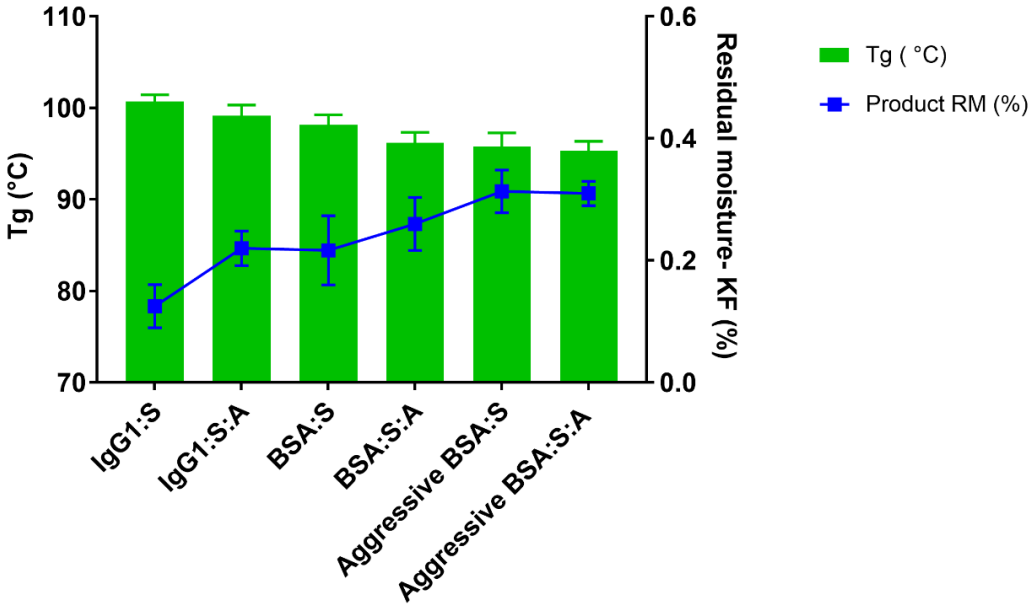


Figure A3 Comparison of the Tg and product residual moisture levels of selected formulations containing BSA and IgG1 as proteins and lyophilised at optimised (Tshelf=-15 °C) and aggressive conditions (Tshelf=35 °C). Formulation BSA:S (or IgG1:S) contain sucrose at concentrations of 10% w/v; formulation BSA:S:A (or IgG:S:A) contains sucrose at concentrations of 8% w/v and arg/arg-HCl at concentrations of 2% w/v. Both formulations contain protein at concentrations of 10% w/v. Samples analysed were n=3 for BSA formulations and n=3 for IgG1 formulations.

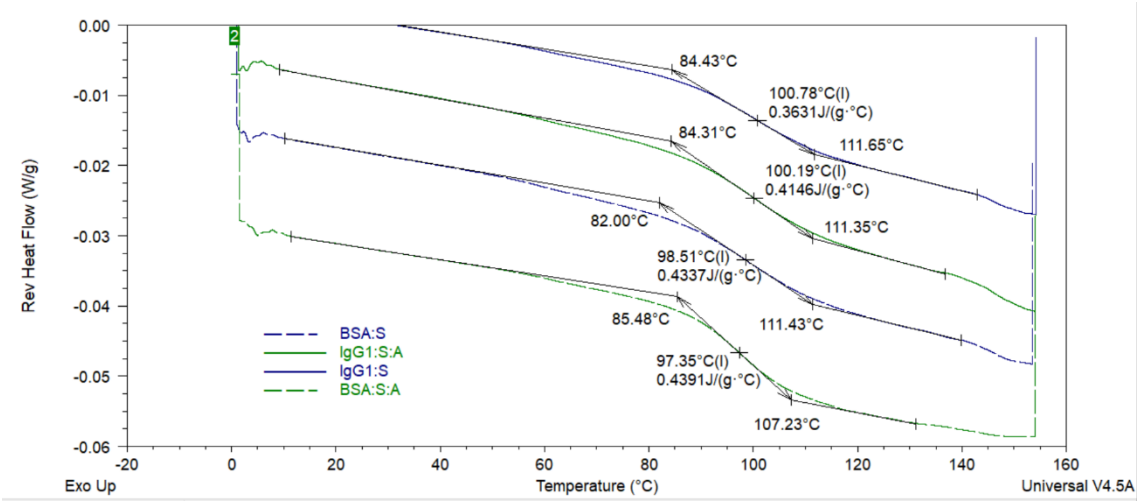


Figure A4 Reversing heat flow thermogram showing glass transition temperature (Tg) of the BSA and IgG1 formulations selected. BSA:S (blue, dotted line), BSA:S:A (green, dotted line), IgG1:S (blue, solid line), IgG1:S:A (green, solid line).

Table A5 Tg and residual moisture of selected IgG1 and BSA formulations. Mean and SD n=3 samples for the BSA formulations, n=2 samples for the IgG1 formulations.

Formulation	Tg (°C) (Mean ± SD)	Residual moisture (%) (Mean ± SD)
IgG1:S	100.7 ± 0.7	0.13 ± 0.03
IgG1:S:A	99.2 ± 1.2	0.22 ± 0.03
BSA:S	98.13 ± 1.1	0.22 ± 0.06
BSA:S:A	96.17 ± 1.2	0.26 ± 0.04
BSA:S Aggressive cycle (T_{shelf} = 35 °C)	95.8 ± 1.5	0.31 ± 0.04
BSA:S:A Aggressive cycle (T_{shelf} = 35 °C)	95.3 ± 1.0	0.31 ± 0.02

Supplementary Information Chapter 6

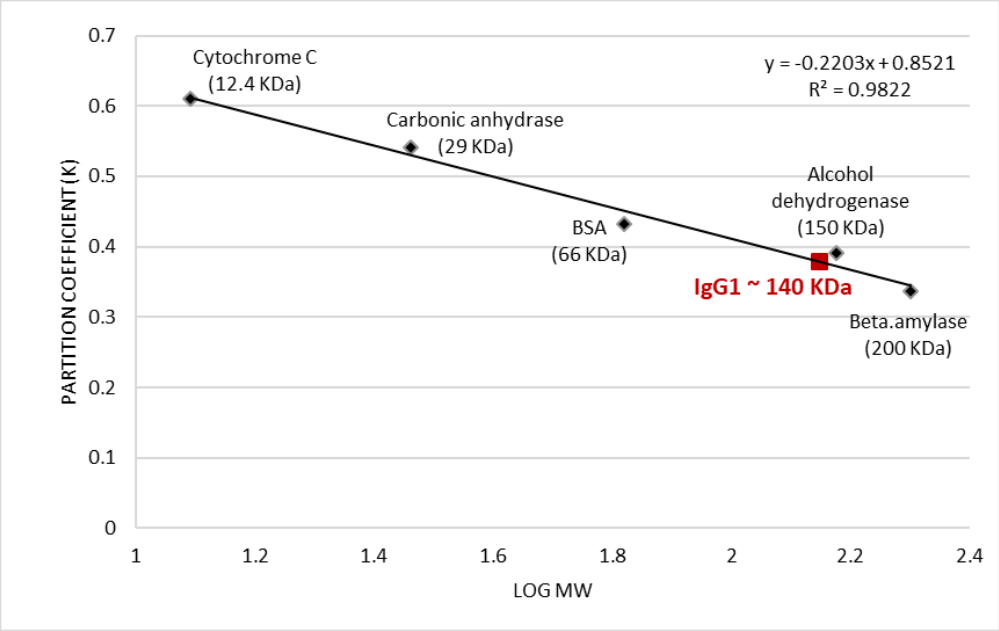


Figure A5 SE-HPLC calibration curve, partition coefficient versus Log Mw for a range of proteins including IgG1.

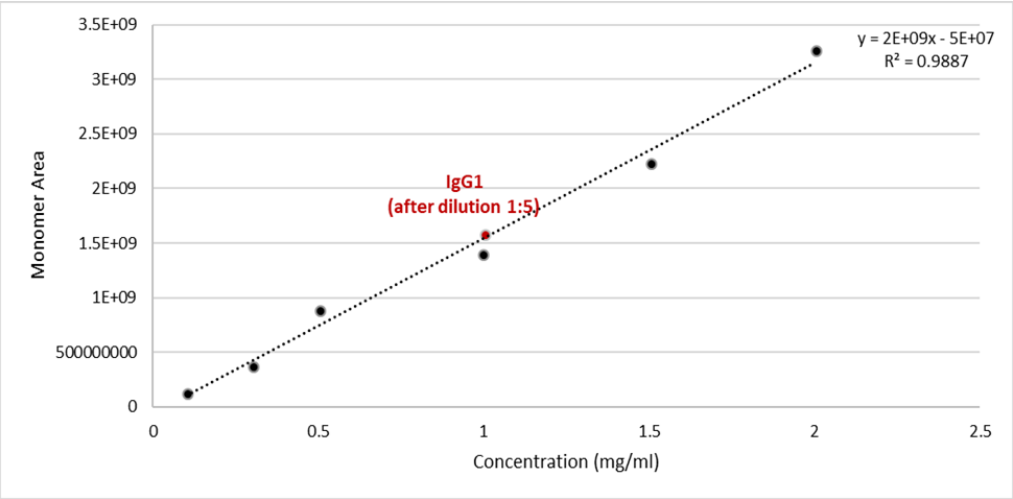


Figure A6 SE-HPLC calibration curve monomer area versus IgG1 concentration, showing IgG1 after dilution.

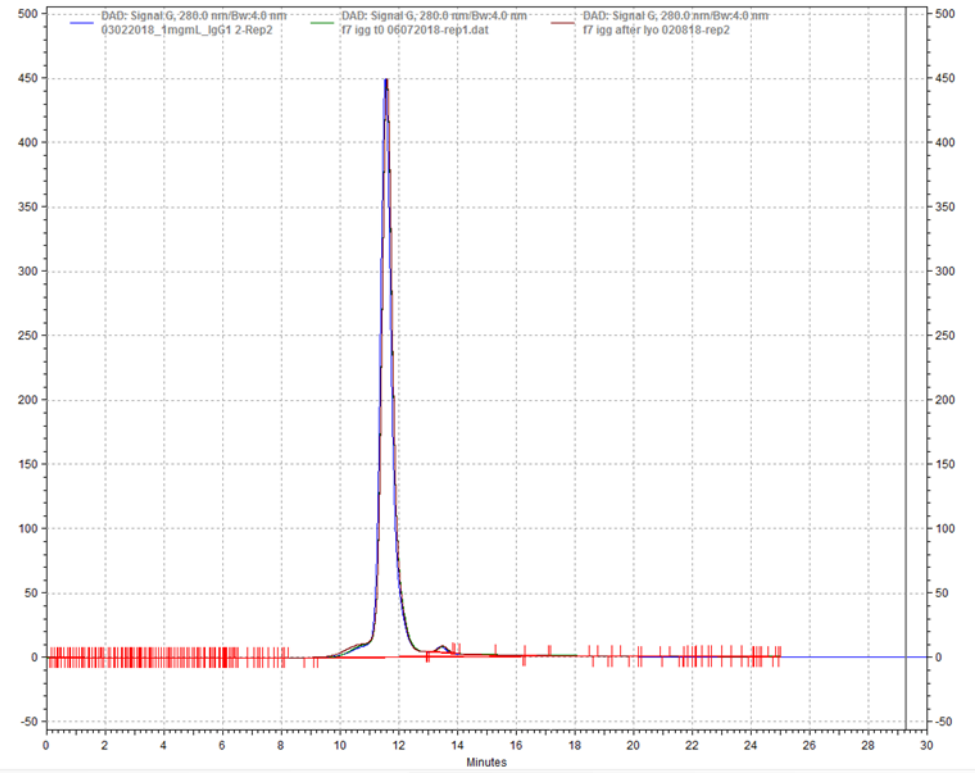


Figure A7 Overlap of the SE-HPLC chromatogram of the IgG1 pre formulation, pre and post lyophilisation (Example given is for IgG10:S10 formulation).

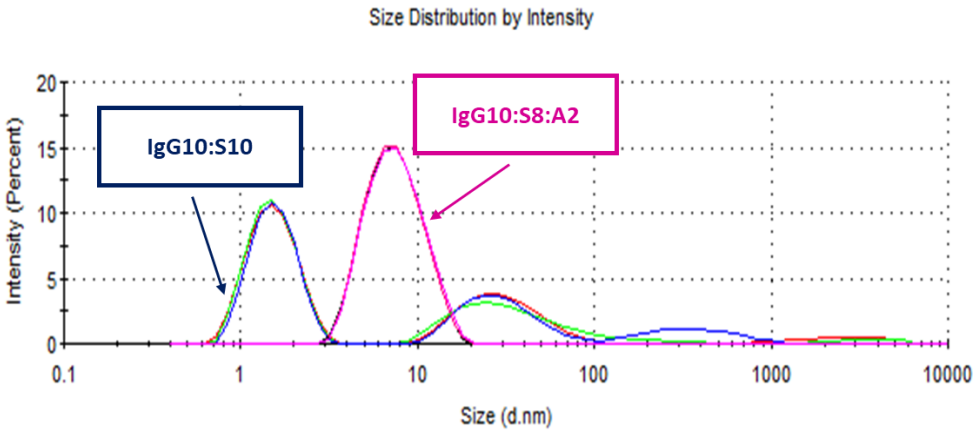


Figure A8 DLS graph of the size distribution by intensity for the two IgG1 formulation with and without arg/arg-HCl.

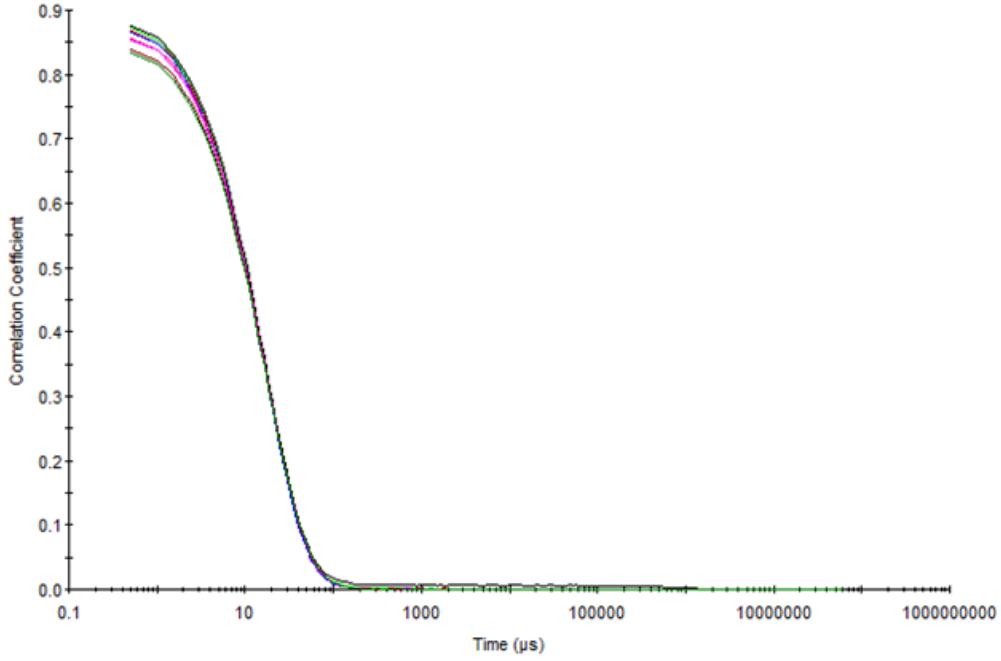
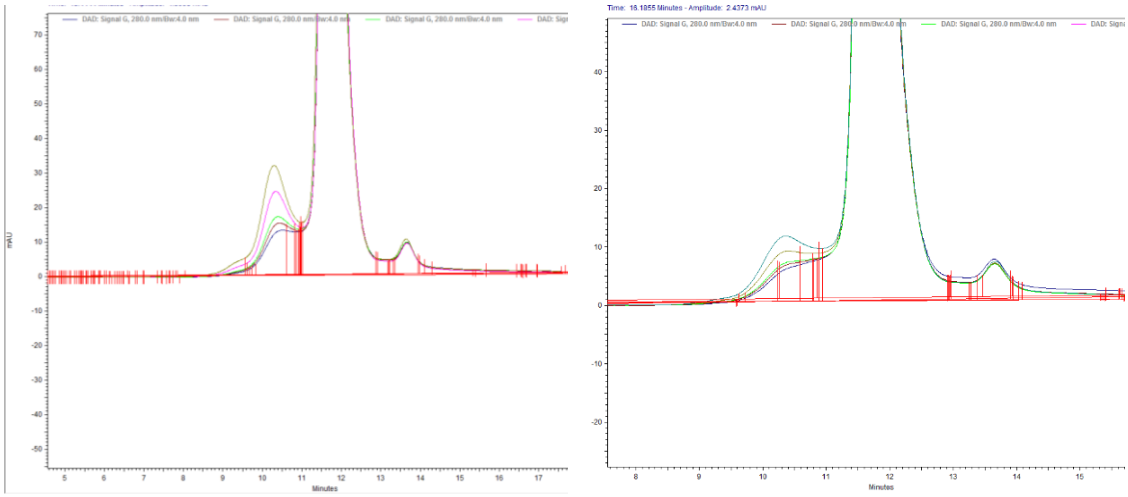
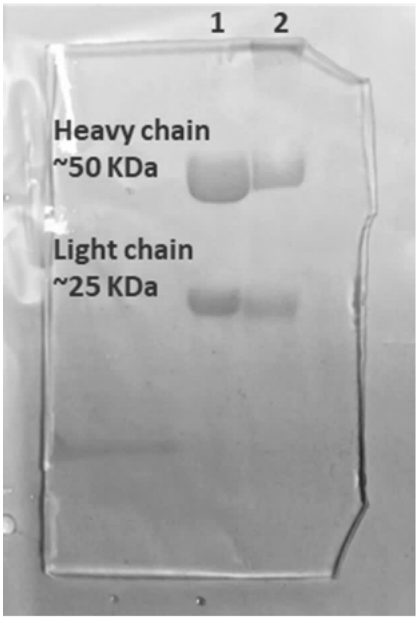


Figure A9 Correlogram for the DLS analysis conducted on the two IgG1 formulations in presence and absence of arg/arg-HCl.



A B

Figure A10 SE-HPLC chromatograms of the IgG1 formulations exposed to light at different time intervals. A) without Arg/Arg-HCL B) with Arg/Arg-HCl.



Legend:

IgG1 (NIBRT) Post concentration, buffer exchange and lyophilization

IgG1 (CHO cells)

Figure A11 Reduced SDS Page of the formulation IgG10:S8:A2 (1) post formulation and lyophilisation in comparison to (2) an aggregated IgG1 (CHO cells produced, as negative control).