TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

1 INTRODUCTION 1

2 FREEZE-DRYING OF PROTEINS 3

2.1 THE FREEZE-DRYING PROCESS IN BRIEF 3

2.2 FREEZE-DRYING OF PROTEIN PHARMACEUTICALS 6

3 THE COLLAPSE PHENOMENON 9

3.1 DEFINITION 9

3.2 MECHANISM 10

3.3 THE COLLAPSE TEMPERATURE 12

3.4 DETERMINANTS OF COLLAPSE 14

3.4.1 Viscosity 14

3.4.2 Time scale 15

3.4.3 Pore radius 17

3.5 THE OCCURRENCE OF COLLAPSE 17

3.5.1 Collapse during freeze-drying 17

3.5.2 Collapse during storage 18

3.6 EFFECTS OF COLLAPSE: CURRENT OPINION 18

3.7 PRO & CONTRA OF DRYING CLOSE TO THE COLLAPSE TEMPERATURE: WHY IT IS WORTH INVESTIGATING THE COLLAPSE PHENOMENON IN DETAIL 21

4 STABILITY OF PROTEIN PHARMACEUTICALS IN THE SOLID STATE 24

4.1 FACTORS AFFECTING STABILITY OF PROTEINS IN THE SOLID STATE 24

4.1.1 Water replacement hypothesis 24

4.1.2 Vitrification hypothesis 25

4.1.3 Temperature 25

4.1.4 Moisture 26

4.1.5 Hydrogen ion activity 26

4.2 IMPLICATIONS OF COLLAPSE FOR THE CONCEPTS OF SOLID STATE PRESERVATION 26

4.2.1 Effect of collapse on thermodynamic stabilization concepts 26

4.2.2 effect of collapse on kinetic stabilization concepts 26

4.3 CHARACTERISTICS OF GLASSY SYSTEMS 27

4.3.1 Temperature dependence of molecular mobility 28

4.4 STABILITY OF GLASSY SYSTEMS 31

4.5 CORRELATION OF MOLECULAR MOBILITY AND PROTEIN STABILITY 33

4.5.1 Global mobility 34
4.5.2 Local mobility 34
5 REFERENCES 36

CHAPTER 2: OBJECTIVES OF THE THESIS

OBJECTIVES OF THE THESIS 47

CHAPTER 3: MATERIALS AND METHODS

1 MATERIALS 51
1.1 IgG_{01} 51
1.2 L-LACTIC DEHYDROGENASE (LDH) 52
1.3 PA_{01} 52
1.4 FREEZE-DRYING EXCIPIENTS 53
1.5 FURTHER CHEMICALS AND REAGENTS 54
2 METHODS 55
2.1 FREEZE-DRYING 55
2.1.1 Process monitoring 55
2.1.2 Preparation of solutions for freeze-drying 55
2.1.3 The collapse-cycle 56
2.1.4 Conservative freeze-drying cycles 57
2.2 SAMPLE PROCESSING AFTER FREEZE-DRYING 61
2.2.1 Annealing in the solid dried state 61
2.2.2 Stability studies 61
2.2.3 Liquid storage stability 62
2.3 CHARACTERIZATION OF PROTEIN STABILITY 62
2.3.1 High pressure size-exclusion chromatography (HP-SEC) 62
2.3.2 One-chain/two-chain material PA_{01} 63
2.3.3 Asymmetric flow field flow fractionation (AF4) 63
2.3.4 Dynamic light scattering (DLS) 66
2.3.5 Light obscuration 66
2.3.6 Turbidity 67
2.3.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 67
2.3.8 Transmission Fourier Transform infrared (FTIR) spectroscopy 68
2.3.9 Intrinsic protein fluorescence spectroscopy 68
2.3.10 High resolution UV 2nd derivative absorbance spectroscopy (2DUV) 69
2.3.11 Color of reconstituted solution 69
2.3.12 Activity assays 70

2.4 CHARACTERIZATION OF LYOPHILIZATE CHARACTERISTICS 71
2.4.1 Differential scanning calorimetry (DSC) 71
2.4.2 Freeze-dry microscopy (FDM) 72
2.4.3 X-ray powder diffraction (XRD) 72
2.4.4 Karl Fischer residual moisture determination 72
2.4.5 Near infrared spectroscopy (NIRS) 73
2.4.6 Scanning electron microscopy (SEM) 75
2.4.7 Digital microscopy 75
2.4.8 Macroscopic classification of the extent of collapse 75
2.4.9 Specific surface area (SSA) measurement 76
2.4.10 Mercury (HG) porosimetry 76
2.4.11 Helium pycnometry 76
2.4.12 Glucose quantification (Trinder assay) 77
3 REFERENCES 78

CHAPTER 4: CONTROLLED GENERATION OF COLLAPSED LYOPHILIZATES

1 INTRODUCTION 81
2 COLLAPSIBILITY 82
2.1 DETERMINATION OF CRITICAL MATERIAL PROPERTIES 82
2.2 TENTATIVE EXPERIMENTS 85
2.2.1 Effect of process parameters and formulation variables in formulations comprising a crystalline bulking agent 85
2.2.2 Effect of formulation variables in formulations lacking a crystallinebulking agent 87
2.3 THE COLLAPSE-CYCLE 89
2.3.1 Collapse-drying protocol 89
2.3.2 Appearance of collapsed cakes 89
3 CHARACTERIZATION OF THE COLLAPSE CYCLE AND THE DRYING BEHAVIOR OF COLLAPSED LYOPHILIZATES 91
3.1 FACTORS AFFECTING THE DRYING BEHAVIOR OF COLLAPSED LYOPHILIZATES 91
3.1.1 Formulation variables 91
3.1.2 Matrix structure: Partially crystalline and amorphous cakes 93
3.2 DRYING KINETICS 94
3.2.1 Effect of N_2-injections 94
3.2.2 Mass-spectrometric monitoring of drying in the collapsed state 95
3.2.3 Monitoring the drying process by gravimetric determination 98
3.2.4 Monitoring the drying process by visual inspection 99

3.3 CONCLUSION: OPTIMIZATION OF THE COLLAPSE-CYCLE 100

4 SUMMARY AND CONCLUSION 100

5 REFERENCES 102

CHAPTER 5: METHODS FOR THE QUANTIFICATION OF THE EXTENT OF COLLAPSE IN FREEZE-DRIED CAKES

1 INTRODUCTION 105

2 MACROSCOPIC AND MICROSCOPIC EVALUATION OF THE CAKE APPEARANCE 106

2.1 PARTIALLY CRYSTALLINE LYOPHILIZATES: FROM NON-COLLAPSED TO COMPLETELY COLLAPSED CAKES 106

2.2 AMORPHOUS LYOPHILIZATES 109

3 SPECIFIC SURFACE AREA (SSA) DETERMINATION 110

3.1 PARTIALLY CRYSTALLINE LYOPHILIZATES 110

3.2 AMORPHOUS LYOPHILIZATES 111

4 MERCURY POROSIMETRY 112

4.1 PARTIALLY CRYSTALLINE LYOPHILIZATES 112

5 HELIUM PYCNOMETRY 113

5.1 EFFECT OF COLLAPSE ON THE INTACT CAKE: PARTIALLY CRYSTALLINE LYOPHILIZATES 114

5.1.1 Porosity 114

5.1.2 Density 116

5.2 EFFECT OF COLLAPSE ON THE PARTICLE DENSITY: AMORPHOUS LYOPHILIZATES 117

6 SUMMARY AND CONCLUSION 118

7 REFERENCES 119

CHAPTER 6: EFFECT OF RESIDUAL MOISTURE ON IgG_{01}-STABILITY IN THE LYOPHILIZED STATE

1 INTRODUCTION 121

2 PHYSICOCHEMICAL MATERIAL-PROPERTIES 122

2.1 CAKE APPEARANCE 122

2.2 RESIDUAL MOISTURE 122

2.3 GLASS TRANSITION & CRYSTALLIZATION 126

3 PHYSICAL PROTEIN STABILITY OF IgG_{01} IN RECONSTITUTED LYOPHILIZATES 128

4 CONFORMATIONAL STABILITY OF IgG_{01} IN RECONSTITUTED LYOPHILIZATES 132

5 EXCURSUS: DISTRIBUTION OF RESIDUAL MOISTURE IN COLLAPSED AND ELEGANT CAKES 133

6 SUMMARY AND CONCLUSION 135

7 REFERENCES 137

CHAPTER 7: EFFECT OF COLLAPSE ON PROTEIN STABILITY I: STABILITY AFTER FREEZE-DRYING

1 INTRODUCTION 139

2 IgG_{01} 140

2.1 AMORPHOUS SYSTEMS 140

2.1.1 Physicochemical characteristics of lyophilizates 141

2.1.2 Physical protein stability of IgG_{01} in reconstituted lyophilizates 143

2.1.3 Binding activity of IgG_{01} in reconstituted lyophilizates 148

2.1.4 Conformational stability of IgG_{01} in reconstituted lyophilizates 149

2.1.5 Conformational stability of IgG_{01} in freeze-dried cakes 154

2.2 PARTIALLY CRYSTALLINE: FROM ELEGANT TO COMPLETELY COLLAPSED 155

2.2.1 Physicochemical characteristics of lyophilizates 158

2.2.2 Physical protein stability of IgG_{01} in reconstituted lyophilizates 162

2.2.3 Binding activity of IgG_{01} in reconstituted lyophilizates 167

2.2.4 Conformational stability of IgG_{01} in reconstituted lyophilizates 168

2.2.5 Conformational stability of IgG_{01} in freeze-dried cakes 170

2.2.6 Is there a delayed effect? short term stress stability of reconstituted IgG_{01} lyophilizates 171

3 L-LACTIC DEHYDROGENASE (LDH) 173

3.1 PHYSICOCHEMICAL PROPERTIES OF COLLAPSED AND NON-COLLAPSED LYOPHILIZATES 173

3.2 ENZYME ACTIVITY OF LDH IN RECONSTITUTED LYOPHILIZATES 176

3.3 PHYSICAL PROTEIN STABILITY OF LDH IN RECONSTITUTED LYOPHILIZATES 178

3.4 CONFORMATIONAL STABILITY OF LDH IN RECONSTITUTED LYOPHILIZATES 181

3.5 EXCURSUS I: COMPARISON OF DIFFERENT DISACCHARIDES 185

3.6 EXCURSUS II: IS THERE A MINIMUM MOLECULAR WEIGHT REQUIRED? EFFECT OF MOLECULAR WEIGHT OF PEG ON THE PRESERVATION OF LDH STABILITY IN COLLAPSED AND NON-COLLAPSED LYOPHILIZATES 188

3.6.1 Residual moisture 189

3.6.2 Physical protein stability 190

3.6.3 Conformational stability 191

3.6.4 Enzyme activity 192

3.7 SUMMARY AND CONCLUSION 193

4 TISSUE-TYPE PLASMINOGEN ACTIVATOR (PA_{01}) 194

4.1 PHYSICO-CHEMICAL PROPERTIES OF COLLAPSED AND NON-COLLAPSED LYOPHILIZATES 194
4.2 CATALYTIC ACTIVITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 195
4.3 PHYSICAL STABILITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 196
4.4 CONFORMATIONAL STABILITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 199

5 FINAL SUMMARY AND CONCLUSION 201

6 REFERENCES 203

CHAPTER 8: EFFECT OF COLLAPSE ON PROTEIN STABILITY II: STABILITY OF LYOPHILIZATES DURING STORAGE AT ELEVATED TEMPERATURES

1 INTRODUCTION 213

2 IgG_{01} 215

2.1 AMORPHOUS SYSTEMS I - TREHALOSE-BASED LYOPHILIZATES: THE EFFECT OF COLLAPSE DURING LYOPHILIZATION 215
2.1.1 Physicochemical characteristics of lyophilizates 215
2.1.2 Physical protein stability of IgG_{01} in reconstituted lyophilizates 217
2.1.3 Binding activity of IgG_{01} in reconstituted lyophilizates 221
2.1.4 Conformational stability of IgG_{01} in reconstituted lyophilizates 222
2.1.5 Summary and conclusion 223
2.2 AMORPHOUS SYSTEMS II – SUCROSE-BASED LYOPHILIZATES: THE EFFECT OF COLLAPSE DURING STORAGE 224
2.2.1 Physicochemical characteristics of lyophilizates 224
2.2.2 Physical protein stability of IgG_{01} in reconstituted lyophilizates 238
2.2.3 Binding activity of IgG_{01} in reconstituted lyophilizates 247
2.2.4 Conformational stability of IgG_{01} in reconstituted lyophilizates 248
2.2.5 Excursus: In-depth investigation of crystallization and non-enzymatic browning in initially collapsed and initially non-collapsed sucrose-based lyophilizates 250
2.2.6 Summary and conclusion 255
2.3 PARTIALLY CRYSTALLINE SYSTEMS: THE EFFECT OF PARTIAL COLLAPSE 257
2.3.1 Physicochemical characteristics of lyophilizates 258
2.3.2 Physical protein stability of IgG_{01} in reconstituted lyophilizates 259
2.3.3 Binding activity of IgG_{01} in reconstituted lyophilizates 262
2.3.4 Conformational stability of IgG_{01} in reconstituted lyophilizates 263
2.3.5 Summary and conclusion 265

3 L-LACTIC DEHYDROGENASE (LDH) 266

3.1 PHYSICOCHEMICAL PROPERTIES OF COLLAPSED AND NON-COLLAPSED LYOPHILIZATES 267
3.2 ENZYME ACTIVITY OF LDH IN RECONSTITUTED LYOPHILIZATES 272
3.2.1 Collapsed versus non-collapsed lyophilizates 272
3.2.2 Collapsed versus partially collapsed lyophilizates 273
3.3 PHYSICAL PROTEIN STABILITY OF LDH IN RECONSTITUTED LYOPHILIZATES 274
3.3.1 Collapsed versus non-collapsed lyophilizates: Trehalose-based formulations 275
3.3.2 Collapsed versus partially collapsed lyophilizates: Sucrose-based formulations 278
3.4 CONFORMATIONAL STABILITY OF LDH IN RECONSTITUTED LYOPHILIZATES 281
3.4.1 Collapsed versus non-collapsed lyophilizates: Trehalose-based lyophilizates 281
3.4.2 Collapsed versus partially collapsed lyophilizates: Sucrose-based lyophilizates 285
3.5 SUMMARY AND CONCLUSION 288
4 TISSUE-TYPE PLASMINOGEN ACTIVATOR (PA_{01}) 290
4.1 PHYSICO-CHEMICAL PROPERTIES OF COLLAPSED AND NON-COLLAPSED LYOPHILIZATES 290
4.2 CATALYTIC ACTIVITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 292
4.3 PHYSICAL PROTEIN STABILITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 293
4.4 CONFORMATIONAL STABILITY OF PA_{01} IN RECONSTITUTED LYOPHILIZATES 299
4.5 SUMMARY AND CONCLUSION 301
5 FINAL SUMMARY AND CONCLUSION 303
6 REFERENCES 306

CHAPTER 9: GLASSY DYNAMICS OF COLLAPSED AND NON-COLLAPSED LYOPHILIZATES

1 INTRODUCTION 315
2 SPECIAL MATERIALS AND METHODS 318
2.1 FREEZE-DRYING 318
2.1.1 Solutions for freeze-drying 318
2.1.2 Collapsed lyophilizates 318
2.1.3 Non-collapsed lyophilizates 318
2.2 ANNEALING AFTER FREEZE-DRYING 319
2.3 ISOTHERMAL MICROCALORIMETRY (THERMAL ACTIVITY MONITOR (TAM)) 319
2.4 DIELECTRIC RELAXATION SPECTROSCOPY (DRS) 321

2.5 DIFFERENTIAL SCANNING CALORIMETRY (DSC) 325
2.5.1 Determination of T_g and Δc_p (T_g) 325
2.5.2 Determination of fragility 326
2.6 POWDER DENSITY MEASUREMENTS 326
2.7 KARL FISCHER RESIDUAL MOISTURE DETERMINATION 326
2.8 SOLID STATE FOURIER TRANSFORM INFRARED (SS-FTIR) SPECTROSCOPY 327
2.9 SPECIFIC SURFACE AREA (SSA) MEASUREMENT 327
2.10 HIGH PRESSURE SIZE-EXCLUSION CHROMATOGRAPHY (HP-SEC) 327
2.11 POLARIZED LIGHT MICROSCOPY 328
3 GLOBAL DYNAMICS OF COLLAPSED AND NOT COLLAPSED LYOPHILIZATES 329
3.1 GLOBAL DYNAMICS FROM CALORIMETRIC DATA 329
3.1.1 Sucrose-based freeze-dried systems 329
3.1.2 Trehalose based freeze-dried systems 332
3.2 GLOBAL DYNAMICS FROM SPECTROSCOPIC DATA 333
3.2.1 Experimental Set-Up: Benefit of the insertion of a Teflon-Sheet 333
3.2.2 The α-relaxation process 336
4 LOCAL DYNAMICS OF COLLAPSED AND NOT COLLAPSED LYOPHILIZATES 342
5 FRAGILITY OF COLLAPSED AND NOT COLLAPSED LYOPHILIZATES 354
5.1 FRAGILITY FROM CALORIMETRIC METHODS 355
5.2 FRAGILITY FROM SPECTROSCOPIC METHODS 355
6 IS IT JUST ANNEALING OR IS THERE A "COLLAPSE EFFECT"? 357
6.1 EFFECT OF ANNEALING ON GLASSY DYNAMICS OF NON-COLLAPSED CAKES 357
6.2 CORRELATION OF GLASSY DYNAMICS TO STORAGE STABILITY 358
7 SUMMARY 364
8 REFERENCES 366

CHAPTER 10: FINAL SUMMARY AND CONCLUSION

FINAL SUMMARY AND CONCLUSION 371