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Short Communication

The relation between moisture-induced aggregation and structural changes in lyophilized insulin

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Abstract

Objectives Long-term stability is a critical factor in the successful development of protein pharmaceuticals. Due to the relative instability of proteins in aqueous solutions, they are formulated frequently and stored as lyophilized powders. Exposure of such powders to moisture constitutes a substantial storage problem leading to aggregation and inactivation. We have investigated the structural consequences of moisture sorption by lyophilized insulin under controlled humidity conditions by employing Fourier transform-infrared (FT-IR) microscopy.
Methods Lyophilized insulin samples were stored in humidity chambers under controlled conditions at 50°C. Protein aggregation studies were carried out by redissolving the insulin samples and measuring the amount of both soluble protein and insoluble aggregates. Near-UV circular dichroism spectra were collected to assess the tertiary structure. FT-IR microscopy studies were carried out to investigate secondary structural changes in solid-state insulin after incubation at different relative humidities.
Key findings It was found that sorption of moisture was accompanied by small structural changes in lyophilized insulin at low levels of relative humidity (i.e. 11%). At higher relative humidity levels, structural changes were becoming more pronounced and were characterized by a loss in the α-helix and increase in β-sheet content. The magnitude of the structural changes in tendency paralleled the solid-state instability data (i.e. formation of buffer-insoluble aggregates and loss in tertiary structure upon reconstitution).
Conclusions The results support the hypothesis that water sorption by lyophilized proteins enables structural transitions which can lead to protein aggregation and other deleterious phenomena.
Keywords Fourier transform-infrared microscopy; lyophilization; moisture-induced aggregation; protein stability; protein therapeutics

Introduction

During the past two decades, the employment of proteins as biotherapeutic and biocatalytic agents has gained increasing interest due to advances in recombinant DNA technology and biotechnology.[1] This has become a major challenge because of their relatively fragile nature, which makes them susceptible to detrimental events during processing, storage and delivery.[2] Due to this, proteins are often formulated lyophilized. This formulation method combines superior stability properties promoting an acceptable shelf-life with reduced operational costs.[3] Unfortunately, solid protein pharmaceuticals can also suffer from long-term instability problems due to moisture sorption, thus hampering their successful therapeutic application.

The residual moisture content after lyophilization often controls long-term chemical and physical protein stability. During the transformation from a dry solid protein to the fully hydrated one, profound changes are observed in protein structure, conformational dynamics, and folding–unfolding equilibrium that have direct repercussions for stability in the solid state.[2,4] To obtain a successful protein formulation, proteins must not only retain their chemical stability, but must also retain their three dimensional structure (conformation) to be effective therapeutic agents.[5,6] Loss of the three dimensional (native) conformation leads not only to loss of biological activity but to increased susceptibility to subsequent deleterious processes, such as covalent or non-covalent aggregation.[5,7] Aggregates may have lower activity (decreasing the efficiency of the pharmaceutical protein) and increased immunogenicity.[8] Recent studies have demonstrated that a US Food and Drug Administration-approved dry formulation of insulin has led to abnormal lung function,

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possibly due to insulin aggregation.[9] This lung abnormality disappears after the treatment is discontinued. This highlights that the presence of insoluble aggregates (>1%) in a protein pharmaceutical makes it unsuitable for product release and must be avoided.

Various studies of moisture-induced aggregation of lyophilized proteins have been reported.[5,10–17] In those investigations, the extent of protein aggregation had been related to the amount of water sorbed by the protein and to residual water bound to charged and polar protein surface groups and the peptide backbone.[18,19] It is commonly assumed that the hydration of solid proteins leads to increased rates of deleterious processes due to greater conformational dynamics of the protein molecule and higher mobility of reactive species.[5,19,20] Recent investigations on the effects of moisture-induced aggregation on whey protein conformation have been performed by amide I FT-IR spectroscopy.[10] Those authors found structural changes as the result of aggregation after three months of storage.

In contrast, in this work we have focused on structural changes of insulin upon exposure to moisture before and after aggregation occurred at various levels of relative humidity (RH). Insulin was chosen as a model for investigating denaturation induced by exposure of the solid protein powder to humidity. The main question we tried to answer was how far did protein structural perturbations induced by moisture adsorption contribute to solid-phase protein instability.

Materials and Methods

Preparation of insulin samples

Preparation of samples followed the procedure described by Costantino *et al*.[5] In brief, porcine Zn-insulin (Sigma-Aldrich, St Louis, MO, USA) was dissolved in deionized water by stirring for 20 min at pH 3. The solution was clarified by filtration and the pH was adjusted to 7.3 with NaOH before lyophilization. These samples were frozen in liquid N_2 and lyophilized for 48 h using a Labcono FreeZone 6-l freeze drier (Labconco, Kansas City, MO, USA) at a condenser temperature of −45°C and a pressure of < 60 μmHg. The final moisture content of the lyophilized powders was 4.3 ± 1.2% (w/w) as determined by Karl Fisher titration. The samples were prepared and analysed in triplicate for all experiments.

Moisture-induced aggregation

To study the effect of moisture on insulin solid-phase instability, the lyophilized powder (0.5 mg protein) was exposed to accelerated storage conditions i.e. controlled humidity and 50°C. Incubation of the lyophilized powders at constant levels of RH was performed by vapour equilibration in dessicators over salt slush: LiCl (11% RH), NaBr (51% RH), NaCl (75% RH), and K_2SO_4 (96% RH).[21,22] Following the desired length of time, the incubated protein sample was removed and 1 ml phosphate-buffered saline (PBS, 10 mM, pH 7.3) was added followed by 2 h gentle stirring to ensure dissolution of the soluble fraction of the sample. The soluble and insoluble (aggregated) protein fractions were separated by centrifugation in a microcentrifuge at 5000 rev/min for 10 min. To determine the amount of aggregated protein 1 ml 6 M urea was added to the pellet and left stirring overnight, a buffer solution with urea and a reducing agent (10 mM dithiothreitol) was added to further dissolve any covalent aggregates left. The protein concentration in the resulting clear solution was determined by absorption measurements at 280 nm. The experiments were performed in triplicate and the results averaged. Error bars in the figure are the calculated standard deviations (SD).

Circular dichroism measurements

Near-UV circular dichroism (CD) measurements were acquired with an Olis DSM-10 UV-vis CD spectrophotometer. The protein concentration was adjusted to 0.6 mg/ml in 10 mM potassium phosphate buffer at pH 7.1 and 25°C. Spectra were recorded from 260 to 320 nm using a 1.0-cm path length quartz cell. Each spectrum was obtained by averaging six scans at 2 nm resolution. Solvent reference spectra were digitally subtracted from protein CD spectra.

Fourier transform infrared microscopy

Fourier transform-infrared (FT-IR)-microscopy studies were performed using a Nicolet Magna-IR 870 bench (Thermo-Nicolet, Madison, WI, USA) and a Thermo Spectra-Tech Continuum microscope (Thermo-Spetratech, Shelton, CT, USA) using a diamond cell (Thermo Spectra-Tech, Diamond windows) to avoid exposing the moist protein powders to the high pressure encountered during the formation of KBr pellets usually employed in such studies.[23] Spectra were recorded in the transmission mode at 4 cm^{-1} resolution and 256 scans were averaged to obtain one spectra. To avoid artifacts by water vapour and sorbed water, the amide III region (1220–1330 cm^{-1}) was used for the analysis of protein secondary structure since the absorbance of water is negligible in this spectral region.[24]

Determination of water sorption isotherms

Water sorption isotherms were determined following the procedure by Costantino *et al*.[5] In brief, measurements of protein-bound water were conducted by Karl Fisher titration using hydranal solvent with a Metrohm 831 KF Coulometer (Metrohm, Herisau, Switzerland). Insulin protein powders (0.5 mg) in triplicate were stored at various levels of RH until equilibrium uptake of water by the lyophilized powder was achieved and no additional water was absorbed (after 10 h at 50°C). Such rapid absorption of water has been reported for insulin and other proteins.[5,25] Following storage, 1.0 ml anhydrous dimethylsulfoxide was added to each sample. This solution was then sonicated for 30 s and injected into the titrator. All water contents are reported as percentages (w/w).

The Guggenheim–Anderson–de Boer model was used to calculate the water vapour sorption isotherm. The Guggenheim–Anderson–de Boer equation is:

$$m = \frac{(m_0 c k_b a)}{\{(1-k_b a)(1-k_b a + c k_b a)\}} \quad (1)$$

where m is the moisture content on wet basis, m_0 is the Guggenheim–Anderson–de Boer monolayer moisture content,

k_b and c are constants, and a is the activity of the sorbed water (which is equivalent to the % RH). The Guggenheim–Anderson–de Boer model has been used previously to describe the water vapour sorption isotherm and monolayer water sorption for proteins.[26] The Guggenheim–Anderson–de Boer sorption model was calculated using the water analyser program developed by Labuza and Nelson.[27]

Statistical methods

Statistical analysis of the effect of the water content (11, 51, 75, and 96% RH) on the quantity of insoluble aggregates formed after 24, 48, and 96 h, and after one and two weeks was performed using the Kruskal–Wallis test, and the individual differences between the various samples were then examined using a post-hoc test (Dunn's test). All experiments were performed in triplicate.

Results

Insulin samples were stored after lyophilization in humidity chambers under controlled conditions at 50°C. Water-sorption isotherms demonstrated that all samples were completely equilibrated after 24 h. As described by Costantino *et al.*,[5] all insulin samples exhibited a significant solubility loss during incubation at different RH levels due to aggregation. This was indeed due to moisture-sorption because insulin stored in sealed vials maintained full solubility after re-dissolution. Aggregation levels increased during storage of the protein under controlled humidity conditions, being more pronounced at high RH values (Figure 1). In agreement with previous results, exposure to 96% RH caused the most pronounced aggregation.[5]

The Kruskal–Wallis test was employed to statistically investigate whether aggregate formation was significantly different at the various RH levels employed. The analysis revealed that there were marked differences in the amount of aggregates formed at the various RH levels. To compare the effect of the RH on aggregate formation quantitatively, we performed the Dunn's test. This test revealed that aggregation was markedly different at RH of 11, 51, and 75% when compared with the 96% RH samples, in which we found by far most aggregation. In addition, aggregation at 11% RH (the samples with the lowest amount of aggregates formed) was markedly different when compared with that of the 75% RH samples. Statistically significant differences in aggregation were also found when comparing the samples exposed to 11 and 51% RH and 51 and 75% RH, respectively, but differences were smaller than the Z-value cut-off. In summary, statistical analysis supported the finding that aggregation increased at increasing levels of RH.

Water vapour sorption isotherm for insulin was determined using the Guggenheim–Anderson–de Boer sorption isotherm model, which was calculated with the water sorption data. This model has been used previously for other proteins and the experimental data exhibited a fair fit to the model (the correlation coefficient was 0.97 for the Guggenheim–Anderson–de Boer plot).[26] The Guggenheim–Anderson–de Boer isotherm is depicted by the solid curve in Figure 2, which shows that the 11 and 51% RH samples had a minimal water uptake by the lyophilized powder. The 75 and 96% RH samples exhibited enhanced water sorption. As previously reported by others, this water does not exist as a contiguous monolayer surrounding the protein surface, but it is clustered at the polar sites on the protein surface and any additional water sorbed by the protein will form multilayers.[5,28]

We investigated the possible changes in the tertiary structure of the soluble insulin fraction after exposure to moisture by performing near-UV CD spectroscopy. Insulin showed a negative CD signal at approximately 275 nm (Figure 3). Exposure of insulin to moisture caused a decrease in the amplitude of this signal demonstrating loss of tertiary structure. This effect was most significant for the samples stored at 96% RH, which exhibited rapid loss of tertiary structure following just 24-h incubation.

The fact that we noted irreversible structural changes occurring to the soluble fraction of insulin after exposure to moisture prompted us to investigate the structure of solid-phase insulin after exposure to moisture. To do so, we conducted noninvasive FT-IR microscopy using diamond windows rather than pressing the powders into KBr pellets,

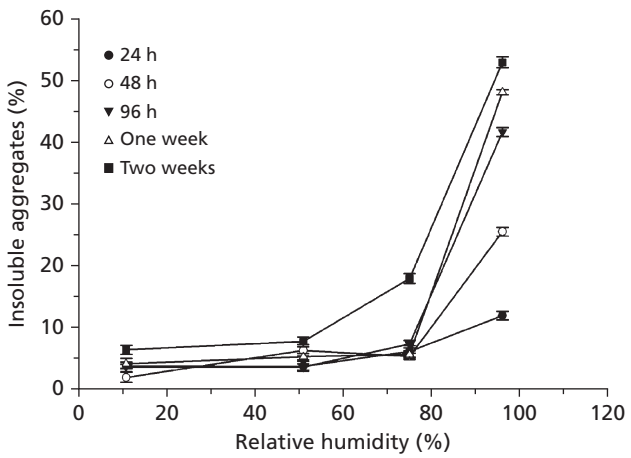

Figure 1 Aggregation of lyophilized insulin. Incubation was at 50°C at different levels of relative humidity.

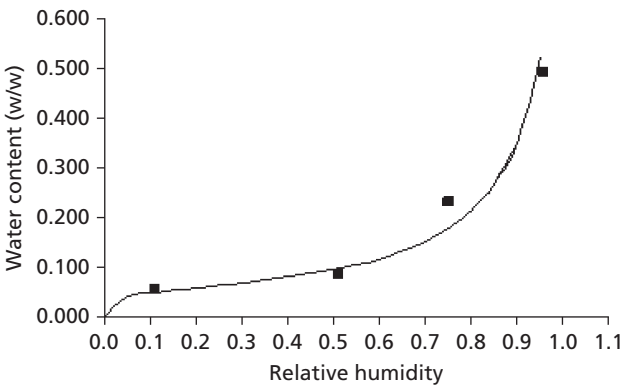

Figure 2 Water sorption isotherm for insulin at 50°C. The solid curve represents the fit to the Guggenheim–Anderson–de Boer model with m_0 = 0.054 water/g total protein.

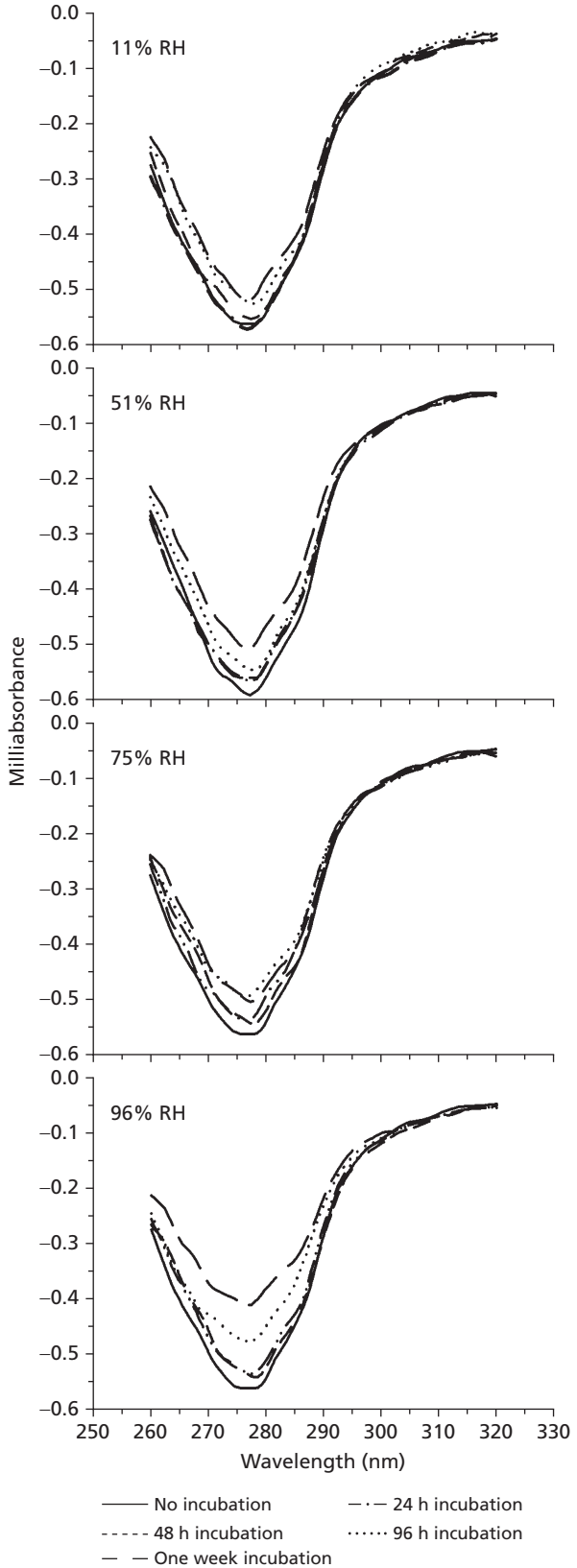

Figure 3 Near-UV circular dichroism spectra of insulin. Insulin was incubated at different relative humidity levels at 50°C and reconstituted in phosphate buffer, pH 7.1.

which is usually done to investigate the structure of lyophilized proteins.[29–31] The latter was unsuitable for the study of moist powders for the following reasons. Firstly, the moisture makes proteins more dynamic and less stable and thus pressing of KBr pellets could potentially cause structural changes. Secondly, the dry KBr powder will dehydrate the moist protein powders and thus the amount of water bound to the protein will change. Finally, moisture will be detrimental to the quality of the KBr pellets. In contrast, the diamond cell is sealed and thus the moisture content of the protein powder will not be influenced and the sample is not mixed with any other compound, making this an ideal noninvasive technique to study this question.

FT-IR analysis was performed in the amide III spectral region because water and water vapour have an insignificant contribution in this spectral area.[31] The amide III spectral region has the advantage that structural transitions can be monitored easily without spectral deconvolution because the absorption of α-helix (ca. 1330–1290 cm^{-1}), other and β-sheet (ca. 1250–1215 cm^{-1}) secondary structures are well separated.[31]

First, we obtained FT-IR spectra of the lyophilized powder using the usual KBr-pellet method and by FT-IR-microscopy.[29,31] The spectra obtained were very similar (Figure 4) and demonstrated that the method of FT-IR spectral acquisition and sample preparation did not influence the result substantially in agreement with literature data.[3]

Qualitative analysis of the spectra (Figure 4) revealed that some spectral changes occurred in the amide III region after incubation of all powders under the RH conditions described. It was obvious that the shape of the spectra obtained at 11% RH was somewhat different from those obtained without incubation. An increase in FT-IR absorption was visible in the region corresponding to α-helix structure (ca. 1330–1290 cm^{-1}), while β-sheet structure contributions to the spectra (ca. 1250–1215 cm^{-1}) decreased. This demonstrated that some structural changes occurred to insulin powder upon exposure to even low levels of moisture. The structural transition was characterized by an increase in the α-helix content and decrease in the β-sheet content, thus to some extent reversing the structural changes occurring upon lyophilization.[31,32] A somewhat similar result was obtained when exposing the lyophilized powder to 51% RH. It was evident that structural transitions over the time course of the experiment were not very pronounced at RH levels of 11 and 51%. In contrast, incubation of the samples at the two higher RH levels resulted in marked spectral changes characterized by an increase in the components at approximately 1230 cm^{-1}, indicating an increase in the β-sheet content while the α-helix content suffered a drastic decrease. Such an increase in the β-sheet content has been related to aggregation in proteins.[31]

Discussion

Proteins and peptides have become a major focal point in the development of pharmaceuticals. However, the complex nature of proteins that enhances their potential as pharmaceuticals also adds to their fragility. This is one major reason why solid formulations are usually preferred over liquid ones.

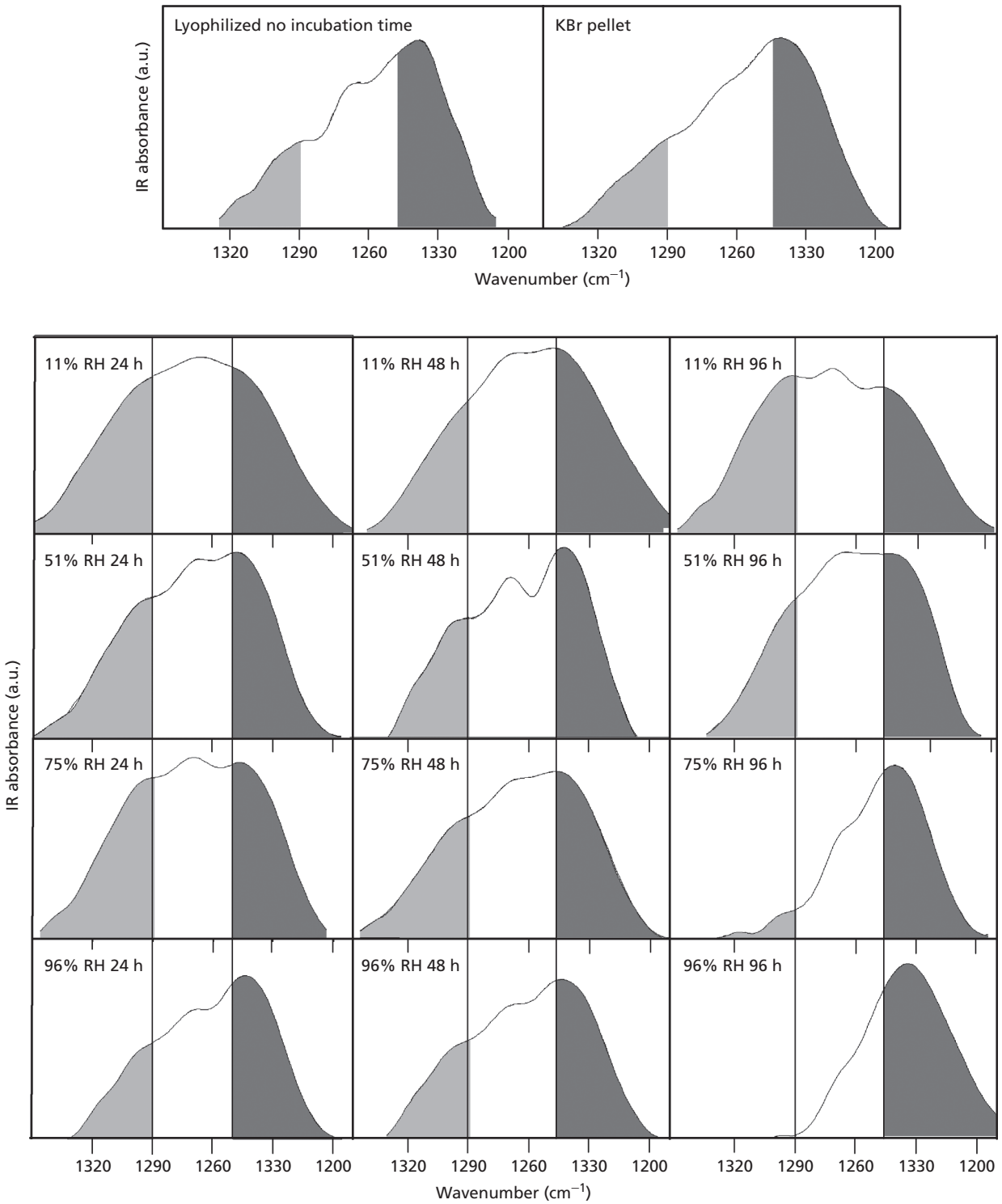

Figure 4 Amide III IR spectra of lyophilized insulin after incubation at different relative humidity levels. The area shaded in light grey was due to α-helix secondary structure, the dark grey area was due to β-sheet secondary structure.

Typically, proteins are relatively stable in the solid-state but exposure of lyophilized proteins to excess moisture has been reported to cause undesirable physical and chemical changes.[11] These effects will have a direct impact on solid therapeutic protein formulations, which become hydrated upon storage at elevated humidities or during delivery from a sustained-release device.[5] The role of water in the stability of proteins is quite critical because it has been related to deleterious processes, in particular aggregation and structural changes. Upon reconstitution, these aggregates, which have an

altered structure, display increased immunogenicity and are thus unsafe for use.[13] Therefore, it is important to examine the stability of solid proteins under pharmaceutically relevant conditions.[2]

The goal of our study was to examine the structural changes that a protein undergoes after moisture sorption and correlate these changes with the aggregation of the protein. Aggregation of solid pharmaceutical protein powders has been observed for various proteins, but while those reports provided information on decomposition of proteins, less is known about irreversible conformational changes of proteins which result in loss of biological activity and formation of insoluble aggregates.[5,10,11,13,14,33] For our study we chose insulin as an important and well studied therapeutic protein model which exhibits instability in various formulations and it is also a candidate for use in a sustained-release device.[2] The formation of insoluble aggregates in lyophilized insulin at elevated temperature and high humidity has been investigated and mechanistically rationalized, showing that insulin aggregates via non-covalent cross-linking and by formation of intermolecular disulfide bonds, involving the formation of free thiols from existing, intact disulfide bonds, followed by intermolecular disulfide exchange catalysed by these free thiols.[5] Our results showed that approximately 50% of insulin aggregated under high RH conditions (i.e. 96%). The trend of the graph (Figure 1) follows the generally reported observation that the amount of aggregate increased at increasing RH and storage time. A buffered solution containing urea and dithiothreitol was able to dissolve all aggregates as seen in previous studies, confirming the mechanism of aggregation for this protein. Significant differences for the insoluble aggregate formation for the different RH levels were found by Kruskal–Wallis and Dunn's test statistical analysis. These findings were important since insulin is a therapeutic protein which is used in the control of diabetes mellitus and aggregate formation can impair the therapeutic effect of this protein. Furthermore it can lead to ill effects in patients as reported for Food and Drug Administration-approved formulations.[9]

To account for the observed aggregation of the protein near-UV CD and FT-IR studies were performed to observe changes in the tertiary and secondary structure of insulin. CD studies revealed changes in the tertiary structure of the protein after incubation at different RH levels for various storage times, especially at higher RH and storage time (Figure 3). These structural changes, while minimal, could be related to the increase in conformational dynamics of the protein due to the adsorption of water. It seemed likely that such structural changes of proteins were linked to exposure of specific amino acid side chains leading to degradation conditions.[11] For insulin this would increase the accessibility of sulfhydryl groups and disulfide bonds and contribute to the formation of more intermolecular disulfide bonds for samples that are stored at higher RH.[17]

IR spectra of proteins as a function of hydration have been collected for lysozyme, α-lactalbumin, bovine serum albumin, immunoglobulin G and whey protein.[10,34] Those studies were performed using different techniques, such as mulletization or production of KBr pellets in conjunction with standard FT-IR measurements or attenuated total reflection (ATR) FT-IR, amongst others.[34] These techniques display disadvantages or disparity in the preparation or the analysis of the sample. For example, it has been demonstrated that ATR spectra differ from transmission spectra in the peak positions and intensities, which investigators attributed to an effect introduced by the wavelength dependence or penetration depth and the refractive index dispersion of the sample, and which needs to be corrected using algorithms. To avoid these and before-mentioned disadvantages, FT-IR microscopy was used in our study of partially hydrated insulin. As shown in Figure 4, the secondary structure of insulin changed after incubation. For the samples incubated at low RH (i.e. 11 and 51% RH), there was an increase in the region corresponding to α-helix structure while β-sheet structure contributions to the spectra decreased. We could conclude from this that some structural changes occurred to insulin powder upon exposure to even low levels of moisture. The structural transition was characterized by an increase in the α-helix content and decrease in the β-sheet content, thus to some extent reversing structural changes occurring upon lyophilization.[31,32] In contrast, incubation of the samples at the two higher RH levels resulted in notable spectral changes, showing an increase in the β-sheet content while the α-helix content decreased. Such an increase in the β-sheet content has been related to aggregation in proteins.[26] The data for the higher RH showed no evidence for the commonly held belief that refolding of reversibly unfolded protein should be complete at relatively high hydration. It has been suggested that while complete dissolution results in refolding, high water content in the 'solid' does not provide either the thermodynamic or the dynamic conditions that mimic a dilute aqueous solution.[34]

Conclusions

Exposure of lyophilized insulin to moisture caused structural changes in the solid-state. However, depending on the RH value, these structural changes had a different direction: while incubation at low moisture levels of 11 and 51% RH caused an increase in the α-helix contributions (thus reversing to some extent lyophilization-induced structural changes), incubation at higher humidity levels caused an increase in β-sheet secondary structure. The magnitude of these structural changes in tendency paralleled the solid-state instability data (i.e. formation of buffer-insoluble aggregates and loss in tertiary structure upon reconstitution). Thus, our work has provided proof that water sorption by lyophilized proteins enabled structural transitions which could lead to protein aggregation and other deleterious phenomena.

Declarations

Conflict of interest

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

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References

1. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003; 2: 214–221.
2. Costantino HR *et al*. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. *J Pharm Sci* 1994; 83: 1662–1669.
3. Costantino HR *et al*. Deterioration of lyophilized pharmaceutical proteins. *Biochemistry (Mosc)* 1998; 63: 357–363.
4. Hageman MJ. The role of moisture in protein stability. *Drug Dev Ind Pharm* 1988; 14: 2047–2070.
5. Costantino HR *et al*. Moisture-induced aggregation of lyophilized insulin. *Pharm Res* 1994; 11: 21–29.
6. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. *Nat Rev Drug Discov* 2005; 4: 298–306.
7. Carrasquillo KG *et al*. On the structural preservation of recombinant human growth hormone in a dried film of a synthetic biodegradable polymer. *J Pharm Sci* 1999; 88: 166–173.
8. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS J* 2006; 8: E501–507.
9. Stote RF, Feldman F. Intranasal insulin: a potential new treatment for diabetes mellitus. *Drug Delivery Technol* 2007; 7: 86–92.
10. Zhou P *et al*. Effects of moisture-induced whey protein aggregation on protein conformation, the state of water molecules, and the microstructure and texture of high-protein-containing matrix. *J Agric Food Chem* 2008; 56: 4534–4540.
11. Chang BS *et al*. Stabilization of lyophilized porcine pancreatic elastase. *Pharm Res* 1993; 10: 1478–1483.
12. Hsu CC *et al.* Determining the optimum residual moisture in lyophilized protein pharmaceuticals. *Dev Biol Stand* 1992; 74: 255–270; discussion 271.
13. Jain NK, Roy I. Role of trehalose in moisture-induced aggregation of bovine serum albumin. *Eur J Pharm Biopharm* 2008; 69: 824–834.
14. Liu WR *et al*. Moisture-induced aggregation of lyophilized proteins in the solid state. *Biotechnol Bioeng* 1991; 37: 177–184.
15. Schwendeman SP *et al*. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. *Proc Natl Acad Sci U S A* 1995; 92: 11234–11238.
16. Sharma VK, Klibanov AM. Moisture-induced aggregation of lyophilized DNA and its prevention. *Pharm Res* 2007; 24: 168–175.
17. Zhou P *et al*. Moisture-induced aggregation of whey proteins in a protein/buffer model system. *J Agric Food Chem* 2008; 56: 2048–2054.
18. Gregory RB *et al*. The influence of hydration on the conformation of bovine serum-albumin studied by solid-state C-13-NMR spectroscopy. *Biopolymers* 1993; 33: 1871–1876.
19. Separovic F *et al*. A solid-state NMR study of protein hydration and stability. *Pharm Res* 1998; 15: 1816–1821.
20. Hageman MJ. The role of moisture in protein stability. *Drug Dev Ind Pharm* 1988; 14: 2047–2070.
21. Greenspan L. Humidity fixed points of binary saturated aqueous solutions. *J Res NBS* 1977; 81A: 89–96.
22. Young JF. Humidity control in the laboratory using salt solutions. *J Appl Chem* 1967; 17: 241–245.
23. Carrasquillo KG *et al*. Relationship between conformational stability and lyophilization-induced structural changes in chymotrypsin. *Biotechnol Appl Biochem* 2000; 31: 41–53.
24. Griebenow K *et al*. Secondary structure of proteins in the amorphous dehydrated state probed by FTIR spectroscopy. Dehydration-induced structural changes and their prevention. *Intern J Vibr Spectrosc [www.ijvs.com]* 1999; 3: 3.
25. Hageman MJ *et al*. Prediction and characterization of the water isotherm for bovine somatotropin. *J Agric Food Chem* 1992; 40: 342–347.
26. Zhou P, Labuza TP. Effect of water content on glass transition and protein aggregation of whey protein powders during short-term storage. *Food Biophys* 2007; 2: 108–116.
27. Labuza TP, Nelson KA. Water Analyzer Program. Saint Paul, MN: University of Minnesota, 1993.
28. Rupley A *et al*. Water and globular proteins. *Trends Biochem* 1983; 8: 18–22.
29. Lam XM *et al*. Replacing succinate with glycolate buffer improves the stability of lyophilized interferon-gamma. *Int J Pharmaceutics* 1996; 142: 85–95.
30. Carpenter JF, Crowe JH. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* 1989; 28: 3916–3922.
31. Griebenow K, Klibanov AM. Lyophilization-induced reversible changes in the secondary structure of proteins. *Proc Natl Acad Sci U S A* 1995; 92: 10969–10976.
32. Prestrelski SJ *et al*. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys J* 1993; 65: 661–671.
33. Townsend MW, DeLuca PP. Stability of ribonuclease A in solution and the freeze-dried state. *J Pharm Sci* 1990; 79: 1083–1086.
34. Luthra *et al*. Effect of hydration on the secondary structure of lyophilized proteins as measured by fourier transform infrared (FTIR) spectroscopy. *J Pharm Sci* 2007; 96: 2910–2921.