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Formulation, lyophilization and solid-state properties of a pegylated protein

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Abstract

In this paper the importance of formulation and process parameters on the solid-state properties of a lyophilized, pegylated growth hormone antagonist (pegvisomant) was studied. The degree of solid-state disorder (amorphicity), protein/polyethylene glycol (PEG)/sucrose interactions, and dissolution characteristics of the resultant cakes were examined. Using isothermal microcalorimetry (IMC) and differential scanning calorimetry (DSC), it was shown that in co-lyophilized pegylated protein/sucrose systems there was an interaction between sucrose and pegylated protein molecules. This interaction was evidenced by a decrease in the melting temperature (T_m) and melting enthalpy of PEG as a function of sucrose concentration. It was also shown that the sum of the heat of interaction with water for the individual constituents, lyophilized pegylated protein and lyophilized sucrose, was higher than the heat of interaction for the co-lyophilized system. As the concentration of sucrose was increased, the degree of solid-state disorder increased and the solid dissolved faster. A correlation was found among heat of interaction with water, degree of solid-state disorder, and dissolution time. Pegylation caused a shorter dissolution time, lower moisture content, increased amorphicity, and a more rapid moisture-induced crystallization of sucrose.

Keywords: Lyophilization; Pegylated protein; Growth hormone; Polyethylene glycol; Amorphous; Isothermal microcalorimetry

1. Introduction

Despite progress in the field of protein crystallization (e.g. Durbin and Feher, 1996), supercritical fluid technology (e.g. Moshashaee et al., 2000), spray drying (e.g. Elversson, 2005), evaporative drying, and spray freeze-drying, lyophilization remains the most common method of preparing solid dosage forms of proteins. The properties of lyophilized cakes should ideally be controlled to guarantee that the bioactivity of the protein in the formulation remains intact and is stable over the shelf life of the product. In order to increase stability, the protein is usually lyophilized with a lyoprotectant such as a carbohydrate to produce an amorphous or partially amorphous stabilizing matrix. Since the mobility and reactivity of the protein in such a glassy solid state is reduced (Franks et al., 1991), protein–protein interactions and aggregation may also be reduced (Liu et al., 1991), resulting in increased protein stability. Protein stability in lyophilized cakes may also be enhanced by molecular interaction via hydrogen bonding between the carbohydrate and the protein in the solid state (Carpenter and Crow, 1989). Sugars such as sucrose and trehalose are potentially useful excipients for lyophilization, because they tend to produce amorphous cakes and enhance protein stability during freeze-drying and storage (Izutsu et al., 1991). All of these considerations mean that characterizing the solid-state structure of the lyophilized cakes of a protein is an important aspect of formulation development.

The solid-state structure of the cake is the result of the net effect of formulation and process parameters. Formulation variables such as the choice of excipients, the ratio of protein to excipients (e.g. Costantino et al., 1998b), and pH (e.g. te Booy et al., 1992; Ohtake et al., 2004) all affect the solid-state properties. The solid-state structure of the cake is also affected by different lyophilization process parameters, such as cooling rate (Chongpresart et al., 1998), degree of supercooling (Rambhatla

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Table 1
Thermal transitions of sucrose

Material	Frozen solution	Amorphous state				Crystalline state		
	T'_g (°C)	T_g (°C)	T_c (°C)	ΔH_c (J/g)	$\Delta H_{solution}$ (cal/g)	T_m (°C)	ΔH_m (J/g)	$\Delta H_{solution}$ (cal/g)
Sucrose	−32[a]	62.8–65.7[b]	103–110[b]	60[c], 56[d]	−6.74 (0.08)[e]	190[b]	120[c]	5.46 (0.15)[e]

[a] Kasraian et al. (1998).
[b] Elamin et al. (1995).
[c] Costantino et al. (1997).
[d] te Booy et al. (1992).
[e] Souillac et al. (2002).

et al., 2004), and product temperature during primary drying (Chang and Fischer, 1995). The effects of cooling rate on ice formation and porosity of the cakes have been thoroughly discussed in the literature (e.g. Heller et al., 1999a).

Another important consideration is storage stability of the cakes. It is known that moisture uptake by amorphous solids increases molecular mobility and consequently facilitates the recrystallization process (Ahlneck and Zeografi, 1990), which can affect product stability. Reductions in glass transition temperature (T_g) (Ahlneck and Zeografi, 1990) and crystallization temperature (T_c) (Elamin, 1994) as a result of moisture uptake have been discussed thoroughly in the literature.

It is also well known that in several cases polymers or proteins inhibit the heat- or moisture-induced recrystallization of the amorphous solid, resulting in a delayed recrystallization event or none at all (e.g. Costantino et al., 1998a; Berggren and Alderborn, 2004). Since the solid-state structure of sucrose and its recrystallization from the amorphous state has been the focus of many studies, including the present one, the thermal characteristics of this compound are summarized in Table 1 for reference.

While many have related the retarding effect of high molecular weight compounds such as dextran-40 on the crystallization of sucrose to an increase in glass transition temperature (te Booy et al., 1992), others have shown that in many cases delayed crystallization (i.e., an increase in T_c) was not accompanied by a change in T_g (e.g. Costantino et al., 1998a; Berggren, 2003). Specific interactions via H-bonding between a polymer and a sugar (e.g. poly(vinylpyrrolidone) (PVP) and sucrose) (Shamblin et al., 1998) or a polymer and the model drug indomethacin (Matsumoto and Zografi, 1999) in co-lyophilized systems, have been shown to be responsible for preventing the sucrose or the indomethacin from nucleation and crystal growth. The inhibition presumably occurs by reducing the molecular mobility of sucrose (Van Scoik and Carstensen, 1990; Shamblin and Zografi, 1999). More effective H-bonding between sugars and polymers is known to result in better phase mixing and less separation (Hill et al., 2005) than when there are no specific interactions involved.

While most of the research in this field has been conducted using polymer-sugar systems or protein-sugar systems (Souillac et al., 2002), the interactions in systems containing protein/polymer/sugar, and particularly pegylated protein/sugar systems, are less explored. In pegylated proteins, polyethylene glycol (PEG) chains are covalently attached to specific residues on the protein molecule. There is an increased interest in therapeutic pegylated proteins because of their perceived decreased immunogenicity (Baumann, 1991), longer circulation half-life (Harding et al., 1996), and lower dose-frequency (Greenwald et al., 2003) than their non-pegylated counterparts. One example is pegylated recombinant human growth hormone (rhGH) analog, a highly selective growth hormone receptor (GH-R) antagonist that is used for long-term treatment of acromegaly (Trainer et al., 2000; van der Lely et al., 2001).

It is also known that pegylation alters the physico-chemical properties of the protein, such as molecular conformation (Bailon and Berthold, 1998), hydrophobicity, isoelectric point, binding properties (Thorner et al., 1999), aqueous solubility (Chang et al., 2005), steric interference (Pradhananga et al., 2002), and electrostatic binding (Bailon and Berthold, 1998). Pegylation may also alter the interaction between excipients and protein (Heller et al., 1999a). Such changes may have important implications on the solid-state structure of the lyophilized cakes of a pegylated protein and the way the protein is preserved in the solid-state.

The objective of this work was to study the lyophilization of a pegylated protein with a focus on the effect of formulation and process parameters on the solid-state properties such as degree of solid-state disorder (amorphicity), protein/PEG/excipient interactions, and dissolution characteristics of the resultant cakes. Special attention was paid to the degree of solid-state disorder, which was determined by isothermal microcalorimetry (IMC). It was also shown how this method could be used to study protein/excipient interactions and moisture-induced recrystallization of the amorphous samples containing pegylated protein and sucrose, or sucrose co-lyophilized with PEG and/or protein. The importance of cooling rate for the solid-state properties of the cakes was also studied.

2. Materials

rhGH and a pegylated rhGH analog, PEG-hGH G120k (PEG-B2036, with pegvisomant as the generic name (Thorner et al., 1999; Muller et al., 2004)), were used as the non-pegylated and pegylated model proteins. PEG-B2036, a GH receptor anatagonist, is an analog of GH that is PEG-modified to prolong its action (Thorner et al., 1999).

Molecular structures of hGH (Conova-Davis, 1998) and pegvisomant (Pradhananga et al., 2002) have been discussed in detail in the literature (e.g. Muller et al., 2004). Pegviso-

Table 2
Formulation compositions

Interaction model	Formulation	ID	Weight ratio (w/w/w), protein:PEG[a]:sucrose	Total solute content (%, w/w)
Sugar	Sucrose	1	0:0:25	2.5
Sugar	Sucrose	2	0:0:50	5.0
Polymer	PEG 6000	3	0:30:0	3.0
Protein/sugar	rhGH/sucrose	4	30:0:25	5.5
Polymer/sugar	PEG 5000[a]/sucrose	5	0:30:25	5.5
Protein/polymer	rhGH/PEG 6000[b]	6	30[c]:30[d,e]:0[d]	6.0
Protein/polymer/sugar	rhGH/PEG 6000/sucrose	7	30:30:50	11.0
Pegylated protein	Pegylated rhGH analog	8	30:30:0[d]	6.0
Pegylated protein/sugar	Pegylated rhGH analog/sucrose	9	30:30:5	6.5
Pegylated protein/sugar	Pegylated rhGH analog/sucrose	10	30:30:25	8.5
Pegylated protein/sugar	Pegylated rhGH analog/sucrose	11	30:30:50	11.0

[a] PEG 5000, chemically activated to attach to proteins.
[b] In this case PEG 6000 was used as non-activated PEG.
[c] In all cases the concentration of protein (without PEG) was 30 mg/ml. The protein concentration in the original pegvisomant bulk was 59 mg/ml and for each ml solution made 0.51 ml bulk solution was added.
[d] This amount is equal to a molar ratio of protein/PEG/sucrose of 1:5:0.
[e] In pegylated rhGh analog protein; pegvisomant, one molecule of protein (M_w = 22,000 Da) is covalently bound to five molecules of activated PEG5000. The protein is thus pegylated with 5 PEG/protein. Since the no. of PEG 5000 chains/protein molecules is 5:1, the theoretical concentration of PEG in this solution was calculated to be 30 mg/ml.

mant is the pegylated form of mutant growth hormone (B2036) (Pradhananga et al., 2002). rhGH and its analog both have a relatively small size (i.e. 22 kDa) (Muller et al., 2004), and are composed of 191 amino acid residues, but in rhGH analog, nine of these residues have been substituted (Chen et al., 1995; Zhou et al., 1997; Thorner et al., 1999; Ross et al., 2001). Additionally, in pegvisomant, an average of 5 PEG 5000 are covalently bonded to the protein molecule at the lysine residues (Fuh et al., 1992; Pradhananga et al., 2002). In this paper this pegylated protein will be referred to as pegylated rhGH analog.

The average molecular weight of growth hormone and pegylated rhGH analog are 22 and 47 kDa (i.e. 22 kDa for rhGH analog protein + 25 kDa for five conjugated PEG 5000), respectively.

Both rhGH and pegylated rhGH analog were produced by Pfizer Health by fermentation in *Escherichia coli* cells, cell lysis and subsequent down-stream processing, and were supplied as concentrated, frozen, bulk solutions. In all the studies presented in this paper, a single well characterized lot of pegylated rhGH analog was used.

Sucrose (Merck, France) was used as bulking agent and lyoprotectant. The buffer salts, monobasic and dibasic sodium phosphate, were obtained from VWR International (Sweden). Sodium bromide (Sigma, Germany) was used to obtain 57.5% relative humidity (RH) in microcalorimetry experiments. All chemicals were of p.a. grade or higher.

PEG 6000 (VWR International, Sweden) was used as the free PEG reference (non-conjugated PEG) to prepare lyophilized PEG formulations with rhGH.

3. Methods

3.1. Formulation preparation for lyophilization

Formulations used in this study are listed in Table 2. Stock solutions containing the materials (Table 2) were prepared in 5 mM phosphate buffer (pH 7.0). In all cases the protein concentration in the formulation was 30 mg/ml.

The solutions were filtered using 0.22 μm Millipore sterile PVDF hydrophobic filters into dual-chamber glass cartridges to a volume of 1.0 ml/cartridge and freeze-dried using an Edward freeze drier (Edward 28, Edward, Kiniese & Co, Germany). Thermocouples were positioned in representative cartridges for monitoring the product temperature during the process.

3.2. Lyophilization process

All formulations were lyophilized using two different lyophilization cycles with slow and fast cooling rates during the freezing step. Formulations were cooled to −45 °C either at a "fast" cooling rate of 1.1 °C/min (process A) or a "slow" cooling rate of 0.11 °C/min (process B). The primary drying was performed at a shelf temperature of −30 °C for 40 h at 0.1 mbar and the secondary drying was performed at a shelf temperature of 10 °C for 10 h at 0.05 mbar in both A and B processes. These lyophilization cycles are shown in Fig. 1. After lyophilization, the dual-chamber cartridges were sealed and stored at 2–8 °C, until analysis. The stability of the product was maintained at this storage temperature (results not shown).

When manufacturing amorphous cakes, it is critical to conduct primary drying below the glass transition temperature of the maximally freeze-concentrated amorphous phase (Carpenter et al., 1997) (T_g') in order to avoid collapse of the system. The T_g' of pure sucrose is −32 °C (Kasraian et al., 1998). The T_g' of a sucrose-protein formulation was higher than −32 °C and was determined to be around −20 °C by freeze-drying microscopy (results not shown).

3.3. Quenched and crystalline sucrose samples

The starting material of sucrose was used as the totally crystalline (ordered) reference.

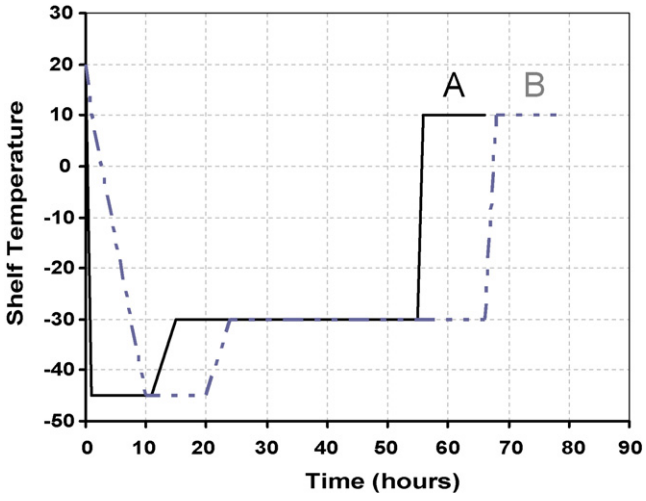

Fig. 1. Freeze-drying cycles for freeze-drying process A (continuous line) and process B (dashed line) with cooling rates of 0.11 and 1.1 °C/min, respectively during the freezing steps.

In order to prepare a highly amorphous sucrose reference by a method other than freeze-drying, quenched amorphous sucrose was prepared by melt-quenching crystalline sucrose in liquid nitrogen. It is known that melt-quenching or rapid cooling of a molten material results in a totally disordered amorphous sample (Anantharaman and Suryanarayana, 1971; Yoshioka et al., 1994) and has been used in several studies for preparing amorphous references (e.g. Elamin et al., 1994; Shamblin et al., 1999; Mosharraf and Nystrom, 2003). After preparation, quenched sucrose was stored at room temperature in a desiccator at 0% RH (over P_2O_5) and was analyzed immediately. The solid-state structure of crystalline and quenched sucrose were determined using X-ray powder diffraction (XRPD) and IMC.

3.4. Determination of solid-state properties

3.4.1. Internal cake structure and porosity

In order to determine the internal structure and porosity of the lyophilized cakes, each cake was sliced into three pieces. The middle slice was then scanned using a Stereoscan 420 scanning electronic microscope under an acceleration voltage of 20 kV with magnifications of 75× and 300×.

3.4.2. Determination of residual moisture

The percentage of residual moisture in each formulation was determined using a 684 Karl Fischer coulometer (Metrohm, Germany). A solution of methanol and formamide (70:30) was used as solvent, to extract the moisture from the solid cake. For each formulation, three cartridges were analyzed, and the average percent residual moisture and standard deviation (S.D.) were calculated.

3.4.3. X-ray powder diffraction

The solid-state structures of the lyophilized pegylated protein/sucrose formulation and samples of quenched and crystalline sucrose were investigated using XRPD. Diffraction patterns of the materials were obtained using a Siemens D5000 diffractometer (Siemens, Germany), with Cu Kα radiation at 45 kV and 40 mA. The samples were scanned in steps from 5° to 45 ° (2θ).

3.4.4. Differential scanning calorimetry

The differential scanning calorimetry (DSC) experiments were performed on a DSC 220 (Seiko Instruments, Japan). Special attention was paid to minimizing the exposure of the samples to moisture prior to DSC analysis. Rubber-stoppered cartridges containing samples were opened in a nitrogen flushed chamber. Immediately after opening, the freeze-dried cake was gently disintegrated and a sample of approximately 2.5–3.5 mg was accurately weighed in an aluminium pan and sealed hermetically. An empty pan was used as reference. The sample and the reference were exposed to a linear heating ramp in the temperature range 20–225 °C at a heating rate of 5 °C/min in an atmosphere of (100%) nitrogen. For each formulation, duplicate samples were analyzed ($n = 2$). Exothermic signals were given positive values.

In cases for which no recrystallization exotherm for sucrose was detectable by DSC (i.e. in (rhGH/sucrose) and (pegylated rhGH analog/sucrose) 30:30:25) formulations), the degree of order (crystallinity) of sucrose was estimated from normalized enthalpy of melting (ΔH_m) values (Grant et al., 1986; Grosvenor and Staniforth, 1996) by dividing the ΔH_m value of sucrose (in J/g) by that of the 100% crystalline standard. Subtraction of this value from 100 gave the degree of amorphicity (disorder) as a percentage. The value of enthalpy of melting of pure crystalline sucrose used in this study was 120 J/g (see Table 1) according to the literature (Costantino et al., 1997). The degree of disorder of PEG was calculated in the same way. The value for enthalpy of melting for crystalline PEG 5000 was determined to be 186 J/g.

3.4.5. Isothermal microcalorimetry

The IMC experiments were performed in a 2277 thermal activity monitor (TAM) (Thermometric AB, Sweden) using the ampoule method, according to Angberg et al., 1992a; Sebhatu et al., 1994; Mosharraf, 2004. Three to six freeze-dried cakes were removed from the cartridges, disintegrated, and pooled. A sample of about 100 mg of the disintegrated cake (powder) was placed into a vial, weighed carefully, and analyzed in the microcalorimeter. Samples of crystalline sucrose and quenched sucrose were also tested.

Since disordered material absorbs water vapor and recrystallizes under storage at high relative humidity (Ahlneck and Zeografi, 1990; Sebhatu et al., 1994), the experiments were performed at an RH of 57.5 %. In order to obtain this RH, a glass tube containing saturated NaBr solution (Nyqvist, 1983) was placed into the vials, which were then sealed. An empty, freshly sealed vial was used as reference. The sample and reference vials were equilibrated in the TAM for 15 min before starting the experiment. All experiments were performed at 25 °C. The heat flow signal (dQ/dt in μW) was monitored as a function of time. The heat flow curve obtained for a pure amorphous reference usually consists of a moisture absorption phase and a recrystallization phase (Sebhatu et al., 1994). Because of over-

lapping moisture absorption and recrystallization peaks, the heat of interaction with water (Q, measured in J) was calculated by integrating the total area under the heat flow curve (Buckton and Darcy, 1999). The values were normalized for eventual weight differences. These values were used to calculate the degree of solid-state disorder according to Sebhatu et al. (1994).

Three samples were run for each formulation except for lyophilized sucrose (formulation 1, obtained at a cooling rate of 0.11 °C/min) and lyophilized pegylated rhGH analog (formulation 8, obtained at a cooling rate of 0.11 °C/min), which were single experiments. This method was not used for the samples of formulations 1, 8, and 3 obtained at a cooling rate of 1.1 °C/min, because of sample limitations.

The degree of disorder in the remaining formulations was calculated using the Q value of quenched sucrose as the 100% amorphous reference. The 100% amorphicity of this sample was verified by XRPD. The degree of disorder was determined by dividing the Q value obtained for the sample by that of quenched sucrose and multiplying by 100.

3.5. Dissolution studies

The rear compartment of the cartridges was filled with 1 ml water for injection (WFI) and sealed. The cakes were then reconstituted using a threaded mixing device. The cartridges were turned over every 30 s manually until a clear solution was obtained. The time taken for dissolution of the cake was measured. For each formulation, dissolution time was recorded for three cakes and the mean dissolution time and S.D. were calculated.

4. Results

4.1. Freeze-drying process

The freeze-drying product thermocouple data showed that the formulations were supercooled to between −4 and −12 °C when the cooling rate was 1.1 °C/min and between −8 and −15 °C with a cooling rate of 0.11 °C/min. The temperature difference between the supercooled solution and the frozen solution was greater with the slow (0.11 °C/min), more homogeneous cooling than with the fast (1.1 °C/min) cooling rate.

In the freeze-drying runs, the formulations completed primary drying within 32–36 and 40–44 h when the cooling rates were 1.1 and 0.11 °C/min, respectively. This was consistent with the differences in pore size, since smaller pores would be expected to introduce greater resistance to water vapor during sublimation, resulting in slower sublimation (Pikal, 1985; Searles et al., 2001). The product temperatures were maintained below the T_g's of the formulations, and solid cakes were obtained for all the formulations.

4.2. Solid-state characterization

XRPD, IMC, and DSC results are shown in Figs. 2–4, respectively. The data are summarized in Tables 3–6.

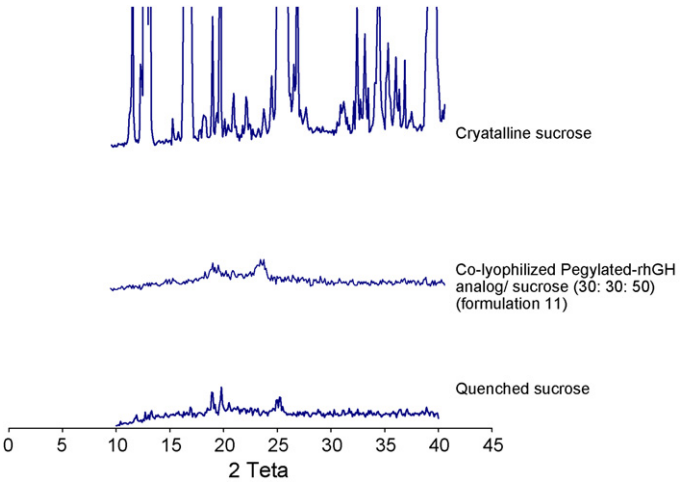

Fig. 2. XRPD patterns of crystalline sucrose (top), co-lyophilized pegylated-rhGH analog/sucrose (formulation 11) with a weight ratio of protein:PEG:sucrose of 30:30:50 (middle) and quenched sucrose (bottom).

4.2.1. XRPD

The XRPD pattern for crystalline sucrose (Fig. 2) shows sharp peaks indicative of long-range order, confirming the crystalline ordered structure. The patterns for the lyophilized formulation of pegylated rhGH analog in 50 mg/ml sucrose and quenched sucrose both have broad, diffuse spectra typical of a highly disordered (amorphous) structure.

4.2.2. Moisture-induced crystallization as determined by IMC

In all cases except that of crystalline sucrose, the samples interacted with water vapor, resulting in exothermic peaks. Representative heat flow curves for lyophilized pegylated rhGH analog alone and co-lyophilized with 5, 25, and 50 mg/ml sucrose at a cooling rate of 0.11 °C/min are shown in Fig. 3. Similar exothermic peaks were also obtained for other samples.

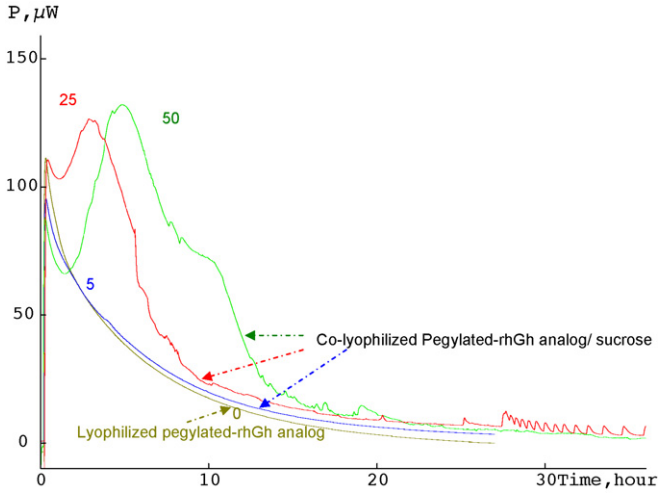

Fig. 3. Representative heat flow curves for lyophilized pegylated rhGH analog alone (formulation 8) and co-lyophilized with 5 (formulation 9), 25 (formulation 10), and 50 (formulation 11) mg/ml sucrose at a cooling rate of 0.11 °C/min obtained at 57.5% RH and 25 °C. The amount of sucrose (mg/ml) in each formulation is shown next to the corresponding curve.

Table 3
Solid-state properties of formulations lyophilized by a cooling rate of 0.11 °C/min

Formulations	Weight ratios of protein:PEG:sucrose (w/w/w)	Total solids (%, w/w)	Heat of interaction with water (Q)[a] (mJ/mg) mean ± S.D.	Degree of disorder (D)[a,b] (%)	Moisture content (%), mean ± S.D.	Dissolution time(t) (min), mean ± S.D.
Sucrose	0:0:25	2.5	21.6[c]	52	3.53 ± 0.12	<0.1 ± 0
Sucrose	0:0:50	5.0	33.3 ± 5.3	80	1.66 ± 0.12	<0.1 ± 0
PEG	0:30:0	3.0	4.8 ± 0.6	nd	0.54 ± 0.02	0.2 ± 0.0
rhGH/sucrose	30:0:25	5.5	23.7 ± 2.7	57	0.86 ± 0.14	5.2 ± 1.8
PEG/sucrose	0:30:25	5.5	24.0 ± 4.5	58	0.91 ± 0.10	0.6 ± 0.3
rhGH/PEG	30:30:0	6.0	12.1 ± 0.2	nd	0.67 ± 0.05	12.5 ± 2.7
rhGH/PEG/sucrose	30:30:50	11.0	19.6 ± 2.6	47	0.48 ± 0.05	3.7 ± 0.9
Pegylated rhGH analog	30:30:0	6.0	18.5[b]	45	0.45 ± 0.02	4.0 ± 0.1
Pegylated rhGH analog/sucrose	30:30:5	6.5	20.7 ± 1.4	50	0.22 ± 0.01	3.8 ± 0.3
Pegylated rhGH analog/sucrose	30:30:25	8.5	36.3 ± 0.7	88	0.18 ± 0.01	2.4 ± 0.4
Pegylated rhGH analog/sucrose	30:30:50	11.0	44.5 ± 2.3	107	0.17 ± 0.02	2.2 ± 0.5

[a] Determinded by isothermal microcalorimetry.
[b] Using the heat flow of quenched sucrose (41.5 ± 9.2 mJ/mg) as 100% amorphous sucrose standard, the degree of disorder in other formulations that contained sucrose was calculated from: $(Q_{(sample)} \times 100)/Q_{(quenched\ sucrose)}$.
[c] This value is based on a single measurement. All other values are mean value of three measurements.

Table 4
Solid-state properties of formulations lyophilized by a cooling rate of 1.1 °C/min

Formulations	Weight ratios of protein:PEG:sucrose (w/w/w)	Total solids (%, w/w)	Heat of interaction with water (Q)[a] (mJ/mg), mean ± S.D.	Degree of disorder (D)[a,b] (%)	Moisture content (%), mean ± S.D.	Dissolution time(t) (min), mean ± S.D.
Sucrose	0:0:25	2.5	nd	nd	3.37 ± 0.12	<0.1 ± 0
Sucrose	0:0:50	5.0	nd	nd	3.43 ± 0.34	<0.1 ± 0
PEG	0:30:0	3.0	nd	nd	0.56 ± 0.05	0.2 ± 0.1
rhGH/sucrose	30:0:25	5.5	31.4 ± 10.5	71	0.77 ± 0.25	5.4 ± 0.2
PEG/sucrose	0:30:25	5.5	15.3 ± 3.8	34	0.16 ± 0.02	0.5 ± 0.1
rhGH/PEG	30:30:0	6.0	nd	nd	0.76 ± 0.06	13.0 ± 2.4
rhGH/PEG/sucrose	30:30:50	11.0	nd	nd	1.02 ± 0.50	4.6 ± 1.0
Pegylated rhGH analog	30:30:0	6.0	nd	nd	0.32 ± 0.02	3.5 ± 0.6
Pegylated rhGH analog/sucrose	30:30:5	6.5	34.6 ± 3.9	83	0.15 ± 0.02	3.3 ± 0.9
Pegylated rhGH analog/sucrose	30:30:25	8.5	39.7 ± 1.4	96	0.12 ± 0.01	3.2 ± 0.7
Pegylated rhGH analog/sucrose	30:30:50	11.0	41.0 ± 9.7	99	0.10 ± 0.01	2.8 ± 0.3

This value is based on a single measurement. All other values are mean value of three measurements.
[a] Determinded by isothermal microcalorimetry.
[b] Using the heat flow of quenched sucrose (41.5 ± 9.2 mJ/mg) as 100% amorphous sucrose standard, the degree of disorder in other formulations that contained sucrose was calculated from: $(Q_{(sample)} \times 100)/Q_{(quenched\ sucrose)}$.

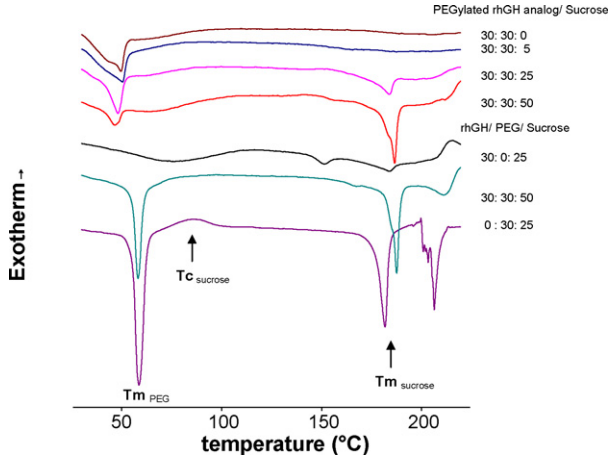

Fig. 4. Representative DSC thermograms of lyophilized pegylated rhGH analog, co-lyophilized pegylated rhGH analog/sucrose, rhGH/sucrose and PEG 6000/sucrose. These formulations were lyophilized at a cooling rate of 0.11 °C/min. The ratio of protein/PEG/sucrose was varied as indicated.

These exothermic peaks were caused by the interaction of powder with water vapor, demonstrating the amorphous character of the lyophilized and quenched samples.

The Q values (Angberg et al., 1992a) were considered as heat of recrystallization and were calculated by integration of heat flow curves (Buckton and Darcy, 1999) and normalized for the weight of the samples. The normalized values are listed in Tables 3 and 4. The degree of disorder in the formulations containing sucrose was calculated using the Q value for quenched sucrose (41.5 ± 9.2 mJ/mg) as the 100% amorphous reference. The crystalline sucrose sample did not react with moisture, confirming a totally crystalline structure for this sample.

4.2.3. Degree of solid-state disorder in lyophilized sucrose without additive

The crystallization of lyophilized sucrose formulations that consisted solely of 25 and 50 mg/ml sucrose, was studied only in the formulations obtained at the cooling rate of 0.11 °C/min. The

Table 5
DSC data for formulations lyophilized by a cooling rate of 0.11 °C/min

Formulations	Weight ratios of protein:PEG:sucrose (w/w/w)	PEG melting endotherm		PEG Degree of disorder[a]	Sucrose Crystallization Exotherm		Sucrose Melting Endotherm		Sucrose Degree of disorder[b]
		$T_{m_{(PEG)}}$ (°C)	ΔH_m (J/g_{PEG})	(%)	$T_{c_{(sucrose)}}$ (°C)	ΔH_c (J/$g_{sucrose}$)	$T_{m_{(sucrose)}}$ (°C)	ΔH_m (J/$g_{sucrose}$)	(%)
rhGH/sucrose	30:0:25	–[c]	–[c]	–[c]	–[c]	–[c]	184	31	74
PEG/sucrose	0:30:25	59	155	0	85	15	182	109	–[c]
rhGH/PEG/sucrose	30:30:50	58	160	0	–[c]	–[c]	187	88	nd[d]
Pegylated rhGH analog	30:30:0	50	68	63	–[c]	–[c]	–[c]	–[c]	nd[d]
Pegylated rhGH analog/sucrose	30:30:5	49	64	68	–[c]	–[c]	–[c]	–[c]	nd[d]
Pegylated rhGH analog/sucrose	30:30:25	48	83	55	150	1	185	64	47
Pegylated rhGH analog/sucrose	30:30:50	46	44	76	143	4	186	79	nd[d]

[a] The degree of disorder of PEG was calculated from $100 - [\Delta H_{m(\text{PEG in lyophilized pegylated formulations})}/\Delta H_{m(\text{crystalline PEG 5000})} \times 100]$. The values for enthalpy of melting and melting temperature for crystalline PEG 5000 were determined to be 186 J/g and 63 °C, respectively.
[b] The degree of disorder of sucrose was calculated only in those cases that heat-induced recrystallization of sucrose was not observed (rhGH/sucrose) or was not significant (Pegylated rhGH analog/sucrose 30:30:25). The following equation was used $100 - [(\Delta H_{m(\text{sucrose in formulation})}/\Delta H_{m(\text{1 pure crystalline sucrose})}) \times 100]$ assuming that the value of enthalpy of melting of 100% crystalline standard is 120 J/g as reported by Costantino et al. (1997).
[c] Not detected.
[d] Not determined.

Table 6
DSC data for formulation frozen by a fast cooling rate of 1.1 °C/min

Formulations	Weight ratios of protein:PEG:sucrose (w/w/w)	PEG melting endotherm		PEG degree of disorder[a]	Sucrose crystallization exotherm		Sucrose melting endotherm		Sucrose degree of disorder[b]
		$T_{m_{(PEG)}}$ (°C)	ΔH_m (J/g_{PEG})	(%)	$T_{c_{(sucrose)}}$ (°C)	ΔH_c (J/$g_{sucrose}$)	$T_{m_{(sucrose)}}$ (°C)	ΔH_m (J/$g_{sucrose}$)	(%)
rhGH/sucrose	30:0:25	nd[c]	nd[c]	nd[c]	–[d]	–[d]	184	26	79
PEG/sucrose	0:30:25	58	130	30	123	29	182	118	–[d]
rhGH/PEG/sucrose	30:30:50	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd	nd	–[d]
Pegylated rhGH analog	30:30:0	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd	nd	–[d]
Pegylated rhGH analog/sucrose	30:30:5	49	68	63	–[d]	–[d]	–[d]	–[d]	–[d]
Pegylated rhGH analog/sucrose	30:30:25	47	64	66	150	1	185	67	44
Pegylated rhGH analog/sucrose	30:30:50	46	48	74	147	10	186	83	–[d]

[a] The degree of disorder of PEG was calculated from $100 - [\Delta H_{m(\text{PEG in lyophilized pegylated formulations})}/\Delta H_{m(\text{crystalline PEG 5000})} \times 100]$. The values for enthalpy of melting and melting temperature for crystalline PEG 5000 were determined to be 186 J/g and 63 °C, respectively.
[b] The degree of disorder of sucrose was calculated only in those cases that heat-induced recrystallization of sucrose was not observed (rhGH/sucrose) or was not significant (pegylated rhGH analog/sucrose 30:30:25). The following equation was used $100 - [(\Delta H_{m(\text{sucrose in formulation})}/\Delta H_{m(\text{1pure crystalline sucrose})}) \times 100]$ assuming that the value of enthalpy of melting of 100% crystalline standard is 120 J/g as reported by Costantino et al. (1997).
[c] Not determined.
[d] Not detected.

heats of moisture-induced crystallization for these samples (21.6 and 33.3 mJ/mg, respectively) were less than that obtained for quenched sucrose (41.5 mJ/mg). This suggested that quenched sucrose was more reactive than the lyophilized sucrose samples. Quenching uses a faster cooling rate than lyophilization, and the sample is brought to much lower temperatures using liquid Nitrogen. Thus the quenched sucrose was more amorphous and in a higher energy state than the lyophilized sucrose. Recently Surana et al. (2004) studied the effect of preparation method on the physical properties of amorphous trehalose. According to this study, the resistance to crystallization for trehalose could be rank-ordered as (trehalose prepared by dehydration) < (freeze-dried approximately spray-dried) < (melt-quenched).

To date, most published literature on protein lyophilization is based on the assumption that lyophilized sucrose is always 100% amorphous. Although in most of these reports (e.g. te Booy et al., 1992; Shamblin et al., 1999; Kasraian et al., 1998; Hatley, 1997) the amount of sucrose in the pre-lyo solution is 50–70 mg/ml (i.e. 5–7%, w/v), amounts of 5 mg/ml (0.5%, w/v) (Costantino et al., 1998a) or 100 mg/ml (10% w/v) (Souillac et al., 2002) have also been used. In most of these cases, the solution was cooled to −50 °C but the rate of cooling varied between 0.2 °C/min (Costantino et al., 1998a) and 0.3 °C/min (Kasraian et al., 1998) to a cooling rate of 0.4 °C/min (te Booy et al., 1992). Shamblin et al. (1999) used a faster rate of 1 °C/min.

The present study shows that when a cooling rate of 0.11 °C/min is used, the lyophilized sucrose formulation containing 2.5% (w/v) sucrose (25 mg/ml) was only partially amorphous (52%). As the amount of sucrose in the pre-lyo solution was increased to 5.0% (w/v) sucrose (50 mg/ml) the degree of amorphicity of the obtained cakes was increased 1.5 times to 80%. At higher sucrose concentrations (≥50 mg/ml) or cooling rate (1.1 °C/min), a highly amorphous sucrose would be expected (see Fig. 7). It is thus concluded that, under the conditions applied in the present study, at a cooling rate of 0.11 °C/min the degree of solid-state disorder of sucrose is dependent on sucrose concentration in the pre-lyo solution at concentrations ≤5% (w/v).

To our knowledge, this dependence of degree of disorder of lyophilized sucrose on the sucrose concentration in the pre-lyo solution has not been reported in the literature before. The assumption that lyophilized sucrose is always amorphous is not necessarily valid. However, the results of the present study are from lyophilization in dual chamber syringes and solid-state properties of the cake may differ when similar formulations are lyophilized in a vial.

4.2.4. Degree of disorder in pegylated rhGH analog alone

When pegylated protein was lyophilized without excipients at a cooling rate of 0.11 °C/min (formulation 8), the resulting cakes were partially crystalline and the degree of solid-state disorder was estimated by IMC to be 45% (Table 3). The higher degree of crystallinity in these cakes seems to be due to the presence of PEG molecules. PEG increases the crystalline character of the cake (Carpenter et al., 1993). This result is in good agreement with DSC results. Representative DSC curves for the formulations are shown in Fig. 4 and the data are listed in Tables 5 and 6.

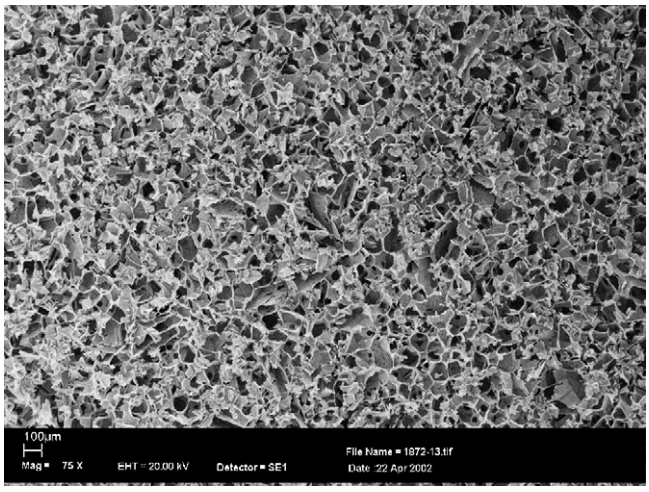

Fig. 5. A representative scanning micrograph of the middle slice of a lyophilized cake of pegylated rhGH analog (formulation 8, see Table 2 for composition), obtained at a cooling rate of 0.11 °C/min.

DSC scans of the lyophilized formulations containing PEG suggested the presence of crystalline PEG in these samples, as evidenced by the characteristic crystal melting endotherm (Carpenter et al., 1993; Heller et al., 1999a). It appears that the process of pegylation causes a decrease in the melting temperature of PEG 5000 from 63 °C (for pure crystalline PEG 5000) to 50 °C (for pegylated rhGH analog, Table 5). Covalent binding of PEG chains to the protein molecule also resulted in a lower enthalpy of melting than that of 100% crystalline standard. The degree of disorder of PEG in lyophilized pegylated protein prepared at a cooling rate of 0.11 °C/min was 63% by DSC (see pegylated rhGH analog in Table 5). Thus both IMC and DSC indicate that the lyophilized pegylated protein is in a partially crystalline state.

When a 30 mg/ml PEG 6000 was lyophilized under similar conditions (cooling rate of 0.11 °C/min), the obtained cakes were almost completely crystalline, as indicated by the very low heat of interaction with water obtained by IMC (4.8 mJ/mg, Table 3). This is in good agreement with other reported data. For example, Hu et al. (2003) reported that melt-quenched PEG 8000 was 94% crystalline when quenched by a cooling rate of 1 °C/min. It thus seems that conjugation of the protein retards complete crystallization of PEG. It is concluded that the coupling of PEG to protein causes both protein and PEG to alter their physico-chemical characteristics, and the resulting molecule exhibits characteristics that are intermediary between the two. In Fig. 5, a representative scanning micrograph of the middle slice of a lyophilized cake of pegylated rhGH analog obtained at a cooling rate of 0.11 °C/min is shown.

4.2.5. Solid-state structure in co-lyophilized pegylated protein/sucrose systems

When pegylated protein was co-lyophilized with sucrose, the melting temperature (T_m) of PEG was reduced from 50 °C for PEG in the lyophilized formulation without sucrose to 49, 47

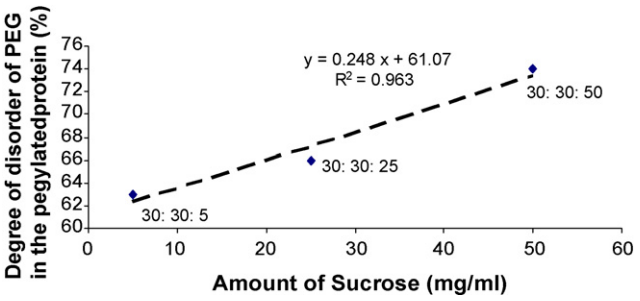

Fig. 6. Degree of solid-state disorder of PEG 5000 in co-lyophilized pegylated protein/sucrose at different proportions of PEG/protein/sucrose, when a cooling rate of 1.1 °C/min was used in the freezing step.

and 46 °C as the concentration of sucrose was increased to 5, 25, and 50 mg/ml, respectively (Tables 5 and 6). This reduction in T_m as a function of sucrose concentration is independent of the cooling rates during freezing. Similarly, the melting enthalpy of PEG was reduced as the amount of sucrose co-lyophilized with the pegylated protein was increased. This occurred in all samples except the formulation containing 25 mg/ml sucrose prepared at a cooling rate of 0.11 °C/min, suggesting that in this formulation PEG was probably more crystalline. The lowest melting enthalpy of PEG was observed when pegylated rhGH analog was co-lyophilized with 50 mg/ml sucrose.

It is thus shown (Table 5) that PEG is partially amorphous in the cakes of pegylated protein/sucrose and its amorphicity is increased at the highest amount of sucrose. The reduction of melting temperature and enthalpies of melting may suggest a possible interaction between PEG and sucrose molecules, especially at higher sucrose concentrations. There is a linear relationship between degree of disorder of PEG in co-lyophilized pegylated rhGH analog/sucrose cakes and amount of sucrose in the pre-lyo solution (Fig. 6).

It is also interesting to consider the sucrose melting endotherm in all formulations that contained more than 5 mg/ml sucrose in the temperature range of 182–187 °C (Tables 4 and 5). This melting endotherm is in good agreement with the reported data in the literature (e.g. Elamin et al., 1995, Table 1). The enthalpy of melting of 100% crystalline sucrose is reported to be 120 J/g (Costantino et al., 1997). The enthalpy of melting of sucrose in co-lyophilized pegylated protein/sucrose samples in the present study was much lower than this value 64 and 79 J/g for samples with sucrose concentrations of 25 and 50 mg/ml, respectively, lyophilized at 0.11 °C/min, and 67 and 83 J/g for those with 25 and 50 mg/ml sucrose, respectively, lyophilized at 1.1 °C/min. The observation confirms that a large portion of the sample remains amorphous.

Sucrose recrystallization in pegylated rhGH analog formulations containing 25 and 50 mg/ml sucrose occurred at higher temperatures (150 and 147 °C, respectively for cakes obtained by 1.1 °C/min lyophilization and 150 and 143 °C, respectively, for cakes obtained by 0.11 °C/min lyophilization). These also resulted in only a small heat of crystallization (see Tables 5 and 6) compared to pure amorphous sucrose (60 J/g, as reported by Costantino et al., 1997, see Table 1). This suggested only a partial recrystallization of sucrose, because of the retarding effect of protein (e.g. Sarciaux and Hageman (1997)) and polymer (e.g. Taylor and Zografi, 1998) on sucrose recrystallization. (It is difficult to see this broad recrystallization exotherm in Fig. 4).

It is thus suggested here that solid-state interactions such as H-bonding might exist between sucrose, PEG, and protein, preventing heat-induced crystallization of the samples. The extent of this interaction is dependent on both the sucrose concentration and the freezing conditions. This postulate is in agreement with other literature in this field. Polymers such as PVP and PEG have been reported to retard the recrystallization of spray-dried lactose (e.g. Stubberud and Forbes, 1998; Berggren and Alderborn, 2004).

The heat of recrystallization obtained by DSC (Tables 5 and 6) increased with higher amounts of sucrose, indicating an increase in the amorphous character of the cakes. This is also in agreement with the IMC results (Tables 3 and 4). In Fig. 7, average heat values for the interaction of powder with water vapor (i.e. heat of moisture-induced crystallization) are plotted against the amount of sucrose in the lyophilized sucrose formulations alone, or co-lyophilized with 30 mg/ml pegylated rhGH analog. According to this figure and IMC data listed in Table 4, when the cooling rate was 1.1 °C/min, the heat of crystallization (and thus the corresponding degree of disorder) was less dependent on sucrose concentration, reaching the heat of crystallization for quenched sucrose (41.5 mJ/mg) at sucrose concentrations of 25 and 50 mg/ml (with corresponding average heat values of 39.7 and 41.0 mJ/mg, respectively) (see also Table 4).

When the cooling rate during the freezing step was 10 times slower (i.e. 0.11 °C/min) the heat of crystallization and thus the degree of reactivity and solid-state disorder increased as the amount of co-lyophilized sucrose was increased until, at 50 mg/ml sucrose, the crystallization energy level reached that of quenched sucrose alone. This behavior was very similar to that observed for lyophilized formulations of sucrose alone, obtained under the same lyophilization conditions (see Fig. 7). The difference between the two plots seems to be equal to the initial value for the plot for co-lyophilized pegylated protein without sucrose ($Q_{\text{pegylated protein}}$). At first the contribution of sucrose (Q_{sucrose}) to the total heat of crystallization (or degree of disorder) (Q_{total}) seems to be independent of the presence of pegylated protein, and the contribution of pegylated protein to the total degree of solid-state disorder seems to be more or less constant at each sucrose concentration, since the amount of protein in the formulation is constant. This suggests that there may be two phases formed in these cakes, (1) the pegylated protein partially crystalline phase and (2) the amorphous or partially amorphous sucrose phase.

If there is no interaction taking place between these two constituent phases, at each mixture, the sum of Q values for individual phases, i.e. that of pegylated protein alone and that of lyophilized sucrose alone (Table 3), should be equal to the total heat value for the co-lyophilized system (Angberg et al., 1992b). In an ideal mixture, the following relationship exists:

$$Q_{\text{total}} = Q_{\text{sucrose}} + Q_{\text{pegylated protein}} \quad (1)$$

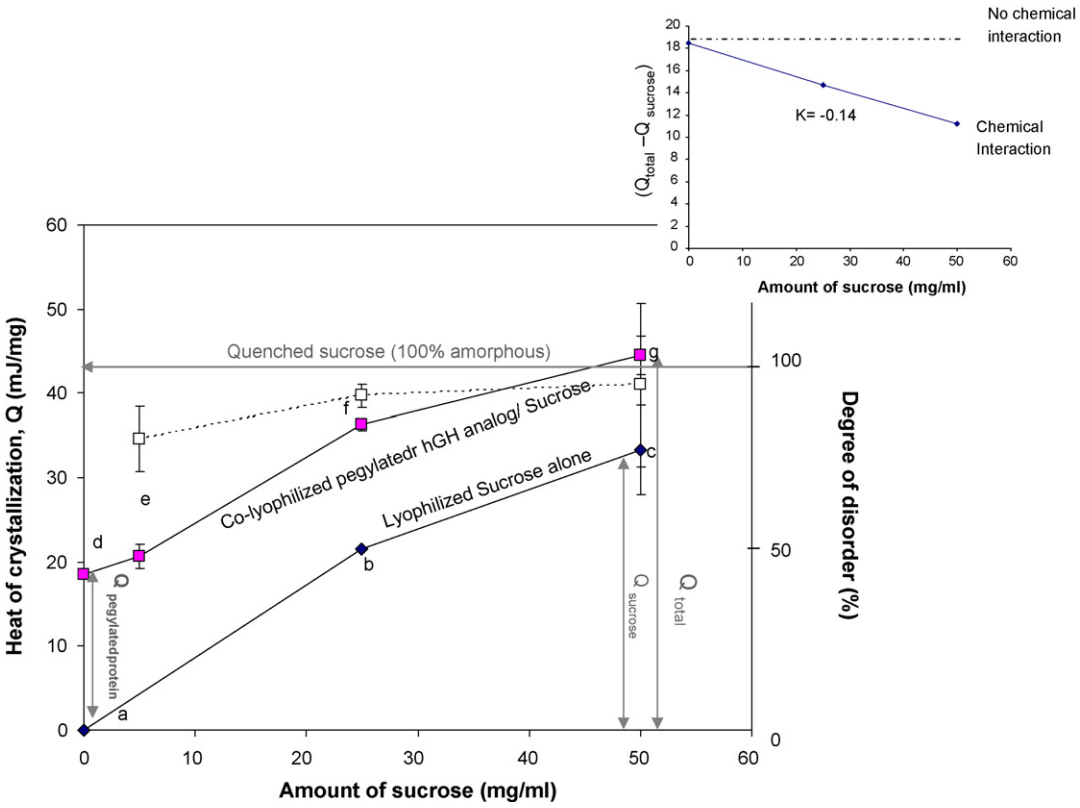

Fig. 7. The effect of sucrose concentration on degree of disorder in the lyophilized cakes of pegylated protein, using a cooling rate of 1.1 °C/min (□) or 0.11 °C/min (■) compared to lyophilized sucrose formulations without any additive lyophilized at a cooling rate of 0.11 °C/min (♦). See Table 2 for formulation descriptions. Q_{total} denotes the total heat of interaction of co-lyophilized pegylated protein/sucrose formulations with water, $Q_{sucrose}$ denotes the heat of interaction of sucrose with water in the reference lyophilized formulations containing only sucrose, $Q_{pegylated\ protein}$ denotes the heat of interaction of lyophilized pegylated rhGH analog with water when lyophilized at a cooling rate of 0.11 °C/min. The solid line shows the heat of interaction of quenched sucrose (100% amorphous sucrose reference) with water. The upper figure shows the graph of the difference between Q_{total} and $Q_{sucrose}$ as a function of sucrose concentration. The dashed line shows the theoretical values that would obtain if there were no interaction taking place between the sucrose and pegylated protein phases. The solid line shows the actual values. *K* denotes the slope of the line.

Any deviation from this relationship will suggest a specific interaction between the molecules in the system. This is applied to the results of present study as follows.

The sum of $Q_{(25\ mg/ml\ lyophilized\ sucrose)}$ (21.6 mJ/mg) and $Q_{pegylated\ protein}$ (19.6 mJ/mg) was 41.2 mJ/mg. The $Q_{(co\text{-}lyophilized\ sucrose/pegylated\ protein)}$ at the corresponding sucrose concentration (25 mg/ml) was 36.3 which, even when accounting for three times S.D., is lower than the theoretical value (41.2 mJ/mg), suggesting a slight interaction between sucrose and pegylated protein. When the co-lyophilized system is studied at 50 mg/ml sucrose concentration, the sum of heat of crystallizations for sucrose alone (33.3 mJ/mg) and pegylated protein alone (19.6 mJ/mg) is 52.9 mJ/mg. This is almost 8.4 mJ/mg higher than the actual value obtained experimentally for the co-lyophilized system (44.5 mJ/mg). It is interesting to note that the ratio between $Q_{total} - (Q_{sucrose} + Q_{pegylated\ protein})$ for the two sucrose concentrations, 8.4/3.8, is 2.2, which is the same as the sucrose concentration ratios (50/25). This shows that since the concentration of protein in the formulation is constant, the more sucrose in the formulation, the greater the deviation from an ideal behavior for the co-lyophilized mixture, i.e., the greater the interaction between the phases.

It seems also that in these cakes partially amorphous pegylated protein co-exists with a totally or partially amorphous sucrose. Additionally, the presence of PEG-conjugated protein molecules with a total molecular weight of 47 kDa challenges the degree of disorder in the system further than that of 100% disordered sucrose alone. (The degree of disorder in pegylated rhGH analog/sucrose system was calculated to be 107% using amorphous quenched sucrose as 100% amorphous standard (Table 3).)

The interaction between sucrose and pegylated protein was explored further using linear regression analysis of the plots in Fig. 7 obtained for the samples lyophilized at a cooling rate of 0.11 °C/min.

4.2.6. *Solid-state interactions in co-lyophilized pegylated protein/sucrose systems*

Linear regression analysis of the plots of heat flow versus amount of sucrose revealed that there was a linear relationship between heat of interaction with water (recrystallization) and amount of sucrose in the pre-lyo solution. Considering the linear regression analysis results listed in Table 7, it can be concluded that the following relationships exist:

$$Q_{total} = (K \times [sucrose]) + Q_{pegylated\ protein} \quad (2)$$

where Q_{total} is the total heat flow of the co-lyophilized system obtained experimentally or calculated using such equation, *K* is

Table 7
Linear regression analysis of the plots in Fig. 7

Interaction model	Linear regression equation
Sucrose (0.11 °C/min)	$Y = 0.7056x$, $R^2 = 0.9657$
Pegylated rhGH analog/sucrose (0.11 °C/min)	$Y = 0.5394x + 19.213$, $R^2 = 0.9621$
Pegylated rhGH analog/sucrose (1.1 °C/min)	$Y = 0.1385x + 34.739$, $R^2 = 0.8524$

a constant which depends on cooling rate and can be determined from the slope of the plot of Q_{total} versus sucrose concentration, and $Q_{pegylated\ protein}$ is the intercept of such plot or the heat flow obtained for the pegylated protein in the absence of sucrose.

When sucrose is lyophilized alone, since the intercept is zero (i.e. no pegylated protein in the formulation), heat flow depends only on sucrose concentration. This linearity will reach a plateau when 100% amorphicity is reached. The total heat flow for pegylated protein co-lyophilized with sucrose also reflects the contribution of each phase to the total heat flow. Subtraction of the heat flow obtained for co-lyophilized pegylated protein/sucrose (Q_{total}) from that obtained for lyophilized sucrose alone (at the same sucrose concentration and condition) ($Q_{sucrose}$) would give the heat flow of pegylated protein in that formulation. The curve corresponding to the crystallization of sucrose can be subtracted from the heat flow curve for co-lyophilized sucrose/pegylated protein (Fig. 7), to get an approximate curve for the interaction of water with pegylated protein.

4.2.7. Appearance of the heat flow curve

The appearance of the heat flow curve (Fig. 3), reveals that a biphasic curve is obtained for co-lyophilized pegylated protein/sucrose, whereas only the first peak is observed for the lyophilized pegylated protein alone. This peak overlaps with the first peak obtained for all other pegylated protein formulations co-lyophilized with sucrose. The appearance of the heat flow curve for a powder mixture may give indications of the order in which the water vapor interacts with the powder constituents (Angberg et al., 1992b). Considering the heat flow curve in Fig. 3, it may be concluded that pegylated protein interacts with water first and co-lyophilized sucrose second. This is in good agreement with other reported data observed for another polymer (micro-crystalline cellulose) and lactose (Angberg et al., 1992b). It thus can be presumed that amorphous or partially amorphous sucrose is embedded in a partially amorphous matrix of pegylated protein.

4.2.8. Solid-state disorder in co-lyophilized PEG/sucrose, rhGH/sucrose and PEG/protein/sucrose systems

In co-lyophilized PEG/sucrose samples, the recrystallization of sucrose is clearly apparent at 123 °C in this formulation. PEG has a retarding effect on heat-induced recrystallization of sucrose and delays it from 103 to 110 °C (Elamin et al., 1995, see Table 1) to 123 °C, after lyophilization at 1.1 °C/min (Table 6). This is in good agreement with data reported for other polymers such as PVP (e.g. Taylor and Zografi, 1998) and is possibly related to the antiplastisizing effects of polymers. At a slower cooling rate (0.11 °C/min) during the freezing step in lyophilization, T_c was reduced to 85 °C and a lower enthalpy of crystallization (15 J/g) was observed, suggesting that these cakes were more crystalline than those obtained at 1.1 °C/min with the corresponding values of 123 °C and 29 J/g (Table 5).

When rhGH was co-lyophilized with sucrose, no recrystallization exotherm was observed on DSC scans. When heat of interaction with water (Q) (see Table 3) for co-lyophilized formulation rhGH/PEG/sucrose 30:30:50 (19.6 mJ/mg) and lyophilized sucrose alone (33.3 mJ/mg) (0:0:50) were compared, it was concluded that moisture-induced crystallization was not completed for co-lyophilized rhGH/PEG/sucrose. However, at a lower concentration of sucrose (25 mg/ml), moisture-induced recrystallization of sucrose was completed (compare PEG/sucrose and sucrose 0:0:25), i.e. heat of interaction with water for co-lyophilized PEG/sucrose (24.0 mJ/mg) was similar to that of lyophilized sucrose alone (21.6 mJ/mg).

When non-pegylated protein (rhGH) was co-lyophilized with PEG/sucrose (30:30:50), the heat of interaction with water was much less (19.6 mJ/mg) than that obtained for pegylated rhGH analog/sucrose (30:30:50) (44.5 mJ/mg, see Table 3), suggesting that in the latter, the cakes were much more disordered.

4.2.9. The effect of non-conjugated and protein-conjugated PEG on moisture-induced recrystallization peak time of sucrose

Quenched sucrose crystallized after approximately 9 h (8.7 ± 0.3 h) exposure to an RH of 57.5%. The crystallization occurred more slowly for the lyophilized sucrose formulations of 25 and 50 mg/ml; i.e. after 14.2 and 19.8 (± 5.1) h exposure to 57.5% RH, respectively. In co-lyophilized PEG/sucrose systems, crystallization peak occurred after 22.6 (±0.6) and 31.4 (±1.7) h for the cakes prepared at 1.1 °C/min and 0.11 °C/min cooling rates, respectively. These results suggest that in both cases PEG delayed the moisture-induced crystallization of sucrose. Similar results have been reported in the literature for moisture-induced crystallization of amorphous spray-dried lactose-PVP particles using IMC (Berggren and Alderborn, 2004). It is known that crystallization of an amorphous material is an overall process consisting of nucleation and growth of nuclei (Tamman, 1926). If nucleation is inhibited by the addition of small amounts of additives, crystallization can be delayed (Van Scoik and Carstensen, 1990).

In contrast, in pegylated rhGH analog formulations co-lyophilized with 25 and 50 mg/ml sucrose, not only was the retarding effect of PEG on sucrose crystallization lost, but crystallization occurred much faster than with the quenched and lyophilized sucrose samples alone (8.7 and 14.2–19.8 h, respectively). For co-lyophilized pegylated rhGH analog/sucrose formulations with sucrose amounts of 25 and 50 mg/ml, the peak times (at 57.5% RH) were 2.5 (±0.2) and 5.3 (±0.6) h, respectively (when lyophilized at a cooling rate of 0.11 °C/min) and 4.8 (±0.2) and 7.1 (±1.0) h, respectively (when lyophilized at a cooling rate of 1.1 °C/min). From the results of the present study it is not clear why these co-lyophilized samples were more prone to crystallization than quenched and lyophilized sucrose.

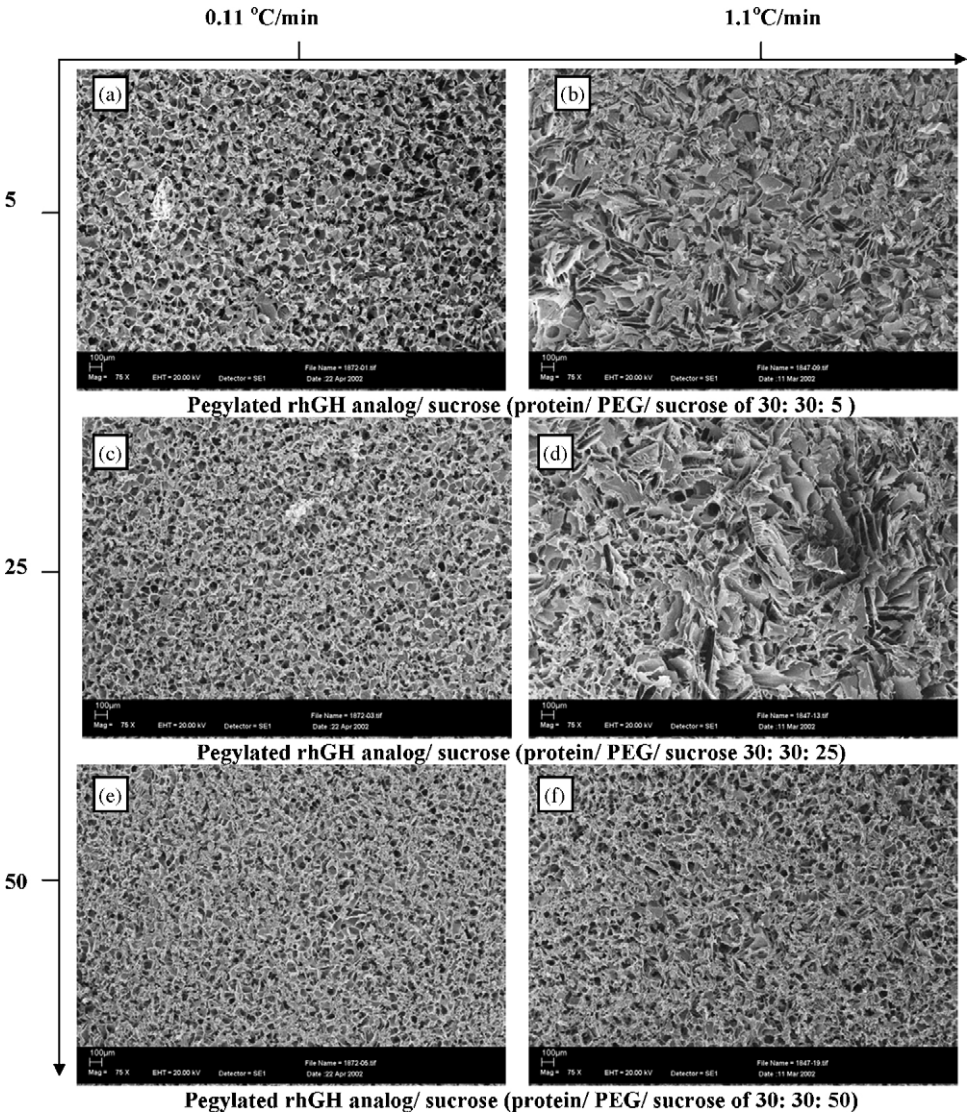

Fig. 8. Representative SEM photomicrographs of the middle slice of the lyophilized cakes of pegylated rhGH analog formulations (9, 10 and 11) at 5 (a and b), 25 (c and d) and 50 (e and f) mg/ml sucrose, lyophilized at cooling rates of 0.11 °C/min (a, c and e) or 1.1 °C/min (b, d and f).

It can be speculated that the presence of pegylated protein promotes mobility of the sucrose molecules, and the presence of sucrose has a similar effect on pegylated protein, increasing the nucleation rate of these phases.

4.3. The relationship between cooling rate, solid-state structure and cake porosity

4.3.1. The effect of cooling rate and degree of supercooling on porosity

Representative scanning electron microscopy (SEM) photomicrographs (Figs. 8 and 9) of the lyophilized cakes showed that the cakes of pegylated rhGH analog/sucrose that were lyophilized at a cooling rate of 0.11 °C/min (Fig. 8, SEM micrograph a, c and e), had a smaller pore size and a more homogeneous pore distribution within the cake structure than in the corresponding formulations cooled 10 times faster at 1.1 °C/min (Fig. 8, SEM micrographs b, d and f). A similar result was obtained for co-lyophilized rhGH/sucrose (Fig. 9, SEM micrograph g and i compared to h and j, respectively). It is known that the size and number of ice nuclei are related to the degree of supercooling, with higher degree of supercooling leading to smaller crystals (e.g. Craig et al., 1999; Pikal, 2001; Tang and Pikal, 2004), Other investigators in this field (e.g. Strambini and Gabellieri, 1996; Heller et al., 1999b) have suggested that faster cooling rates lead to higher rate of nucleation and hence smaller ice crystals. However, in the present study, the degree of supercooling was greater in solutions lyophilized at a cooling rate of 0.11 °C/min than those lyophilized at 1.1 °C/min, resulting in more nucleation of ice in the former. This in turn would result in a large number of small pores in the cakes obtained at 0.11 °C/min after water removal. This study is the first to address the effect of cooling rate on the pore size of a lyophilized pegylated protein.

Most studies published in the literature discuss the results obtained from lyophization in vials. In the present study, the samples were lyophilized in a cartridge. It is not known whether

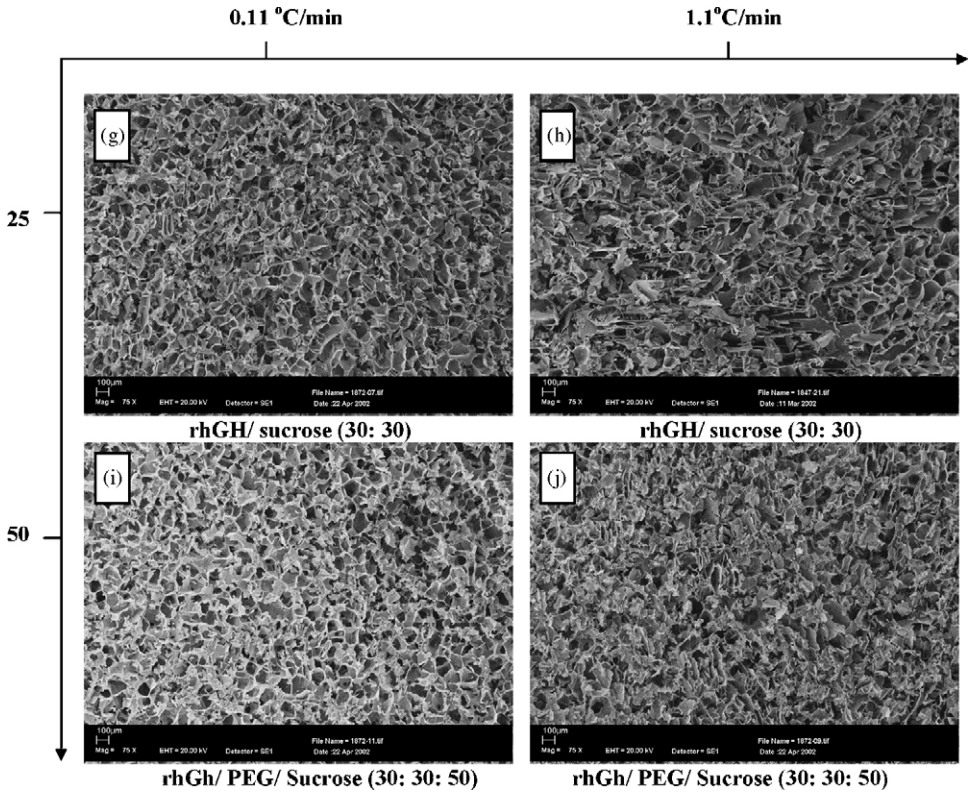

Fig. 9. Representative SEM photomicrographs of the middle slice of the lyophilized cakes of rhGH formulations 4 and 7 at 25 (g and h) and 50 (i and j) mg/ml sucrose, lyophilized at cooling rates of 0.11 °C/min (g and i) or 1.1 °C/min (h and j).

lyophilization in a cartridge might affect the solid-state structure of the lyophile differently from lyophilization in vials.

4.3.2. The effect of sucrose content on water structure and cake porosity

When co-lyophilized rhGH analog/sucrose formulations were compared at different amounts of sucrose (5, 25, and 50 mg/ml) obtained at the same cooling rate (0.11 or 1.1 °C/min), a decrease in pore size was observed with increasing amounts of sucrose. Decrease in pore size as a result of increase in sucrose concentration is an observation that has not been reported before.

As the sucrose concentration was increased to 50 mg/ml, the cakes exhibited a more defined pore structure with a large number of small pores and the effect of cooling rate on porosity was less pronounced. This behaviour may be due to the effect of sucrose on structuring of water. Sucrose is known to be a kosmotropic cosolvent that enhances the structuring of water and increases the formation of hydrogen bonds (Galinski et al., 1997; Moelbert et al., 2004). As a result, at this concentration (50 mg/ml), the effect of cooling rate is less pronounced, because the amount of sucrose is sufficiently high to cause a homogeneous dispersion of water in the mixture, which after removal, would result in a cake with a large number of small pores.

When the solutions used contained less solute (for example for pegylated rhGH analog co-lyophilized with 5 and 25 mg sucrose), water was less structured. When these solutions were cooled at 1.1 °C/min, the solution froze before the water molecules had rearranged themselves to more ordered structures, i.e., the resulting ice was less ordered (nucleation rate of ice < freezing rate of water). It has been claimed that solution heterogeneity (phase separation) is directly proportional to ice crystal growth inhibition (Regand and Goff, 2002).

When the solution was cooled slowly (0.11 °C/min), the water molecules had time to rearrange themselves into more ordered structures, and thus smaller pores were obtained. These results are supported by considering the degree of disorder of the solids obtained by microcalorimetry (Table 3) and SEM pictures (Fig. 8) of each representative cake.

4.4. Residual moisture content

The residual moisture content results are summarized in Tables 3 and 4. Residual moisture contents in lyophilized sucrose formulations alone (formulations 1 and 2), were approximately 1.7–3.5%. In all other lyophilized formulations, the water content was <1%, indicating efficient drying.

Comparison of moisture content results for different cooling rates (Fig. 10), show that cooling rate did not affect the moisture content. On the other hand, if the results obtained for formulations 8, 9, 3, 4, 5, and 1 are compared, it seems that as soon as PEG was incorporated in a formulation, the moisture content in the resulting cake was reduced remarkably (Tables 3 and 4). Similar behavior was observed when rhGH was co-lyophilized with sucrose. The amount of residual moisture was very small also when pegylated rhGH analog was lyophilized alone. These results suggest that PEG and protein influence the amount of

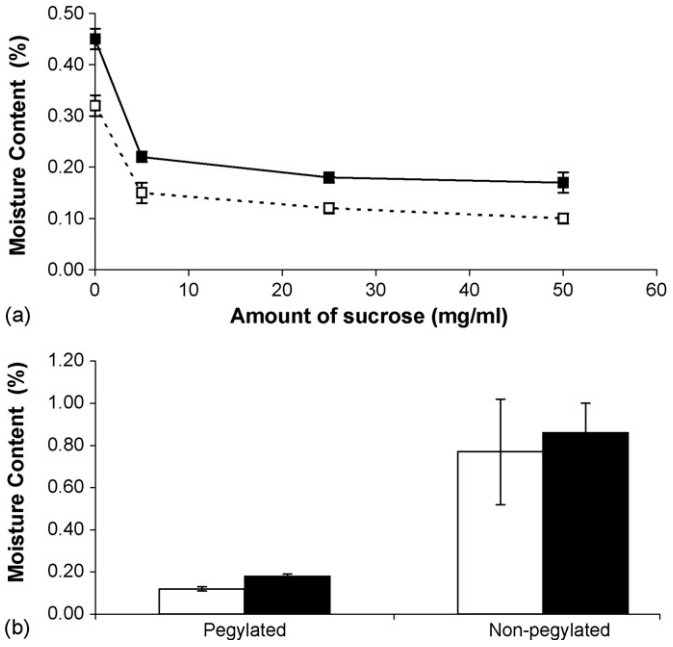

Fig. 10. (a) The effect of sucrose on residual moisture content for the cakes lyophilized at cooling rates of 1.1 °C/min, (□) or 0.11 °C/min (■). (b) The effect of pegylation on residual moisture content of the lyophilized cakes, lyophilized at cooling rates of 1.1 °C/min, (□) or 0.11 °C/min (■).

residual moisture in formulations containing sucrose. However, when PEG is covalently bound to protein the effect is more pronounced.

When the water content in the pegylated protein formulations was compared with that in the non-pegylated protein formulations (Fig. 10b), it was found that pegylation caused a decrease in water content. In order to see whether differences between the residual moisture content for each formulation were significant, the results for pegylated formulations in varying concentrations of sucrose and rhGH were evaluated using an *F*-test. The results (not shown) confirmed that the difference between data sets for co-lyophilized pegylated rhGH analog/sucrose formulations (30:30:5, 30:30:25 and 30:30:50) and the formulation containing co-lyophilized non-pegylated protein (rhGH)/sucrose (0:30:25), was statistically significant ($p = 0.02$). It is concluded that pegylation causes a decrease in residual moisture content. Pegylation may alter the ability of protein to bind to water, thus increasing the tendency of water to leave the lyophile during secondary drying.

When the moisture content in cakes of co-lyophilized pegylated rhGH analog/sucrose formulations were compared with each other or with that obtained for co-lyophilized PEG/sucrose formulation (0:30:25), the *F*-test showed that there was no significant difference in moisture content among pegylated rhGH analog formulations or between these and co-lyophilized PEG/sucrose formulation (i.e. $p > 0.05$ in all cases).

4.5. Dissolution time

The influence of formulation on dissolution time was investigated as described in the methods section. Dissolution times of samples are listed in Tables 3 and 4. Lyophilized sucrose without additives dissolved very quickly, i.e., in less than 0.1 min. Lyophilized free PEG also dissolved rapidly, i.e., in 0.2 min.

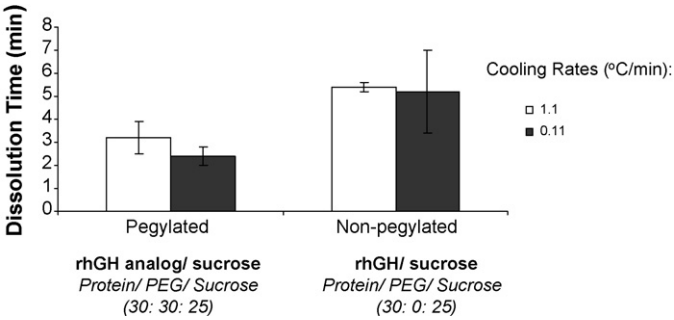

Fig. 11. The effect of pegylation on dissolution time of the lyophilized cakes, lyophilized at cooling rates of 1.1 °C/min, (□) or 0.11 °C/min, (■).

4.5.1. The effect of pegylation on dissolution time

The dissolution time for cakes containing pegylated rhGH analog was reduced to a third of that for cakes containing non-pegylated protein and free PEG (rhGH/PEG) (Tables 3 and 4). In both cases the total amount of solid was 6.0% and the weight ratios of PEG: protein were 30:30, and considering molecular similarities between the two proteins, a decrease in dissolution time in these cases is likely to be caused by pegylation. It is known that pegylation changes the surface properties of protein and increases protein solubility (e.g. Chang et al., 2005).

Similarly, when dissolution times of these proteins after co-lyophilization with 25 mg/ml sucrose were compared (Fig. 11 and Tables 3 and 4), it was concluded that the dissolution time of co-lyophilized pegylated protein (pegylated rhGH analog/sucrose (30:30:25) was 1.7–2 times shorter than co-lyophilized non-pegylated protein (rhGH) in the same amount of sucrose (rhGH/sucrose (30:0:25)), with the former being 1.5 times more disordered than the latter. Hence, the results show that pegylation reduces the dissolution time of the cake by increasing the degree of solid-state disorder.

Another interesting comparison is between rhGH/PEG/sucrose (30:30:50) and pegylated rhGH/sucrose (30:30:50), in which the total amounts of solid were also the same (i.e. 11.0%). During dissolution of co-lyophilized rhGH/PEG/sucrose (30:30:50) cakes, a gel was formed on the cartridge walls. This formulation took longer on average (i.e. 3.7 and 4.6 min at 0.11 and 1.1 °C/min, respectively) than the formulation containing pegylated rhGH analog/sucrose (30:30:50) (i.e. 2.2 and 2.8 min, respectively) to dissolve in WFI. These constitute reductions of 1.7 and 1.6 times, respectively. Recently, it was reported that in suspensions of globular proteins there is a competition between gelation and crystallization (Dixit and Zukoski, 2003). Gelation was described as a consequence of incomplete rearrangement of molecules in solution to non-equilibrium configurations, yielding amorphous clusters of protein aggregates in solution (Dixit and Zukoski, 2003). In other words gelation, like crystallization, is a result of particle aggregation and dissociation processes. However, its occurrence depends on whether a particle diffuses to its local free energy minimum (crystalliza-

tion) or remains trapped in amorphous locations (gelation) (Dixit and Zukoski, 2003). In the present study, since the dissolution of pegylated protein was faster than the non-pegylated reference and no gelation was observed when the protein was pegylated, it was suggested that pegylation prevented the gelation and aggregation behavior of the protein—presumably because of a steric hindrance caused by covalently bound PEG chains.

4.5.2. *The effect of sucrose on dissolution time*

The co-lyophilized rhGH/PEG cakes dissolved in 12.5–13.0 min. When these formulations were co-lyophilized with 50 mg/ml sucrose, the average dissolution times of the cakes were decreased markedly (from 13.0 to 4.6 min for those lyophilized at 1.1 °C/min and from 12.5 to 3.7 min for those lyophilized at 0.11 °C/min) (see Tables 3 and 4). Dissolution was much more rapid (within 4 min) in co-lyophilized pegylated protein/sucrose formulations. In these formulations, the dissolution time was decreased slightly from 3.5 to 2.8 min (when cooling rate was 1.1 °C/min) and from 4.0 to 2.2 min (when cooling rate was 0.11 °C/min) as the sucrose concentration was increased from 0 to 50 mg/ml. It seemed that an increase in cooling rate from 0.11 to 1.1 °C/min was not sufficient to have a significant effect on dissolution time, whereas the effect of sucrose concentration on dissolution time was much more significant, perhaps because of effect of sucrose on solid-state disorder. It was shown that the more sucrose in the formulation, the more amorphous the lyophilized solid, and the shorter the dissolution time. It is suggested here that sucrose reduced the dissolution time of the lyophilized cakes because it increased the level of disorder and thus, their apparent solubility.

4.6. *The relationship between solid-state structure and dissolution time of the lyophilized cakes*

Fig. 12 shows the correlation between dissolution time and degree of solid-state disorder and heat of interaction with water vapor, for the pegylated protein formulations lyophilized by a cooling rate of 0.11 °C/min. The higher the heat of interaction

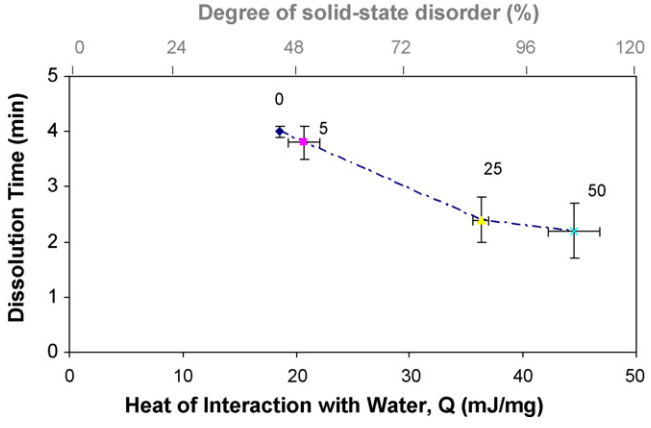

Fig. 12. The relationship between heat of interaction with water, amount of sucrose, degree of solid-state disorder and dissolution time of lyophilized pegylated rhGH analog alone and co-lyophilized with protein lyophilized at a cooling rate of 0.11 °C/min.

with water (i.e. degree of amorphicity), the shorter the dissolution time, until the dissolution time seemed to reach a plateau, when the solid was completely disordered. When a cooling rate of 1.1 °C/min was used, since the cakes were already at least 80% amorphous, no difference was observed in amorphicity, and dissolution time as the amount of sucrose was increased.

Noyes and Whitney (1897), Nernst (1904), and Brunner (1904) described the dissolution rate of a suspended solid drug particle in a diffusion-controlled process, by the well-known equation

$$\left(\frac{dm}{dt}\right) = \left(\frac{D}{h}\right) A_I(C_s - C_t) \quad (3)$$

where h is the thickness of a stagnant boundary layer (diffusion layer), and A_I is the interfacial surface area, i.e. the surface area of the undissolved solid in contact with solvent. This equation was developed under two basic assumptions: (1) the mass flux is given by Fick's first law of diffusion, and (2) the concentration gradient within the diffusion layer is constant (Grant and Higuchi, 1990).

From Eq. (3), it can be concluded that parameters such as surface area and solubility are two of the most important parameters for dissolution rate (and thus dissolution time) of drugs. Increases in the solubility of drugs after freeze drying (Frömming et al., 1986) and preparation of solid dispersions (Kai et al., 1996), spray-drying (Kawashima et al., 1974), dry mixing (Mosharraf and Nystrom, 1999) and milling (Florence and Salole, 1976; Elamin et al., 1994; Phillips and Byron, 1994; Mosharraf et al., 1999) have been reported to be related to a change in the solid-state structure of the material, usually from a more ordered structure to a less ordered one. It has also been suggested that the extent of solubility enhancement is closely related to the amount of amorphous material in the product (Kawashima et al., 1974; Mosharraf and Nystrom, 2003). An increase in apparent solubility would lead to an increase in initial dissolution rate (and thus a decrease in initial dissolution time) (Mosharraf et al., 1999). On the other hand, heat of interaction with water obtained by IMC (Q), is a reflection of degree of solid-state disorder, wetting ability and specific surface area (Mosharraf, 2004). A correlation between Q and dissolution time provides the possibility of predicting dissolution time of a formulation from heat of interaction with water.

5. Conclusions

In this study the use of IMC to study interactions in multicomponent systems was demonstrated. Using IMC and DSC, it was shown that in co-lyophilized pegylated protein/sucrose systems, there was an interaction between sucrose molecules and pegylated protein molecules. This intermolecular interaction was shown, by both cooling rates, as evidenced by a decrease in T_m, and enthalpy of the melting of PEG, to be a function of sucrose concentration in the lyophilized cakes. It was also shown that the sum of heat of interaction with water for individual constituents (lyophilized pegylated protein and lyophilized sucrose alone) was higher than the heat of interaction obtained for the co-lyophilized system when the samples were lyophilized at a

cooling rate of 0.11 °C/min. The extent of such interaction was found to be directly proportional to the amount of sucrose in the formulation.

As the concentration of sucrose was increased in the formulation, the degree of solid-state disorder was increased and the solid dissolved faster. A correlation was found among heat of interaction with water, degree of solid-state disorder, and dissolution time. It was also shown that when the concentration of sucrose was high (50 mg/ml), the effect of cooling rate on the solid-state structure was less pronounced and the degree of amorphicity of quenched sucrose was reached.

Pegylation affected the solid-state properties of the cake remarkably, causing a shorter dissolution time and lower moisture content. It also increased the amorphicity of the cake compared to a cake consisting of sucrose alone (at a cooling rate of 0.11 °C/min). Another interesting observation was that moisture-induced crystallization of sucrose occurred much faster when sucrose was co-lyophilized with a pegylated protein than when it was co-lyophilized with free unbound PEG alone.

Although pegylated rhGH analog partially prevented thermally induced recrystallization of the lyophilized cake samples, exothermic recrystallization peaks were obtained by IMC, indicating that pegylated rhGH analog did not prevent moisture-induced recrystallization of the lyophilized cakes. Thus, in contrast to DSC, IMC was capable of demonstrating a recrystallization event, indicating a disordered and/or partially disordered solid-state structure for all pegylated rhGH analog/sucrose samples. It is suggested that amorphous or partially amorphous sucrose was embedded in the partially amorphous pegylated-protein phase.

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