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DSC reveals the excipient impact on aggregation propensity of pharmaceutical peptides during freezing

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ABSTRACT

Pharmaceutical peptides are susceptible to aggregation in solution, making stabilization by addition of suitable excipients essential. To investigate this stabilization, lengthy and cost-intensive experiments are often necessary. In this work, a differential scanning calorimetry (DSC) based method was developed that allows a rapid assessment of the stabilization properties of excipients regarding the aggregation of pharmaceutical peptides. The stabilization properties of investigated excipients are derived from the thermal behavior around T_g', the glass-transition temperature of the excipient-rich phase after freezing, as a function of repeated freeze-thaw cycles.

The pharmaceutical peptide glucagon was investigated in combination with the excipients trehalose and lactose. In addition to the type of excipient, the concentration ratio of peptide/excipient was also varied. Lactose proved to better stabilize glucagon solutions compared to trehalose. On the one hand, the onset of aggregation could be delayed and after aggregation started the aggregation kinetics were slowed down. In addition, it was shown that a high excipient to peptide ratio, regardless of the type of excipient tested, reduces the aggregation tendency of glucagon.

1. Introduction

Aside from classical biopharmaceuticals such as monoclonal antibodies, pharmaceutical peptides have become increasingly important. Although being smaller representatives of biopharmaceuticals, peptides face the same or even increased challenges regarding their short and long-term stability in aqueous solution. With degradation often observed after hours, peptides are classically either stored in the frozen state, or freeze-dried (Joshi et al., 2000; Matilainen et al., 2008). Storage in the frozen state is employed during early stages of formulation and drug product development, if freeze-drying is not applicable, or if the stability of the biopharmaceutics in the refrigerated liquid is not sufficient (Avis and Wagner, 2010). Although degradation of peptides is slowed down in the frozen state, aggregation of the peptides can be amplified due to the crystallization of water during freezing. The peptide is subjected to interfacial stress during crystallization through the formation of ice/solution interfaces (Authelin et al., 2020). To inhibit aggregation during storage in the frozen state, excipients such as disaccharides, e.g., sucrose are added to the peptide solution (Connolly et al., 2015). During freezing, these excipients retain a liquid phase, also called freeze concentrate, to stabilize the peptide, subsequently termed amorphous phase. As long excipients do not crystallize and are kinetically stabilized, this amorphous phase is in meta-stable equilibrium with the solid water, i.e., ice phase (Steven L. Nail et al., 2002). The peptides, the excipient, and the residual unfrozen water are confined to this amorphous phase, with the residual water concentration in the amorphous phase given by the solubility of water (Steven L. Nail et al., 2002). As soon as the temperature is lowered below the glass transition temperature of the amorphous phase (classically termed T_g'), further crystallization of water is kinetically inhibited, and no change of the composition in the amorphous phase occurs. The corresponding concentration of solutes (excipient and peptide combined) at and below T_g' is called w_g'. T_g' is dependent on the composition of excipients and peptides used. A phase with peptide in the monomeric form has a

Abbreviations: DSC, Differential scanning calorimetry; PC-SAFT, Perturbed-Chain Statistical Association Fluid Theory.

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different T_g' than a phase with peptides in aggregated form, due to its different mean molar mass (Levine and Slade, 1988).

Three possible stabilization theories have been proposed to describe the behavior of excipients used as stabilizers during freezing and thawing: (1) the vitrification theory, (2) the water replacement theory, or (3) a combination of the two aforementioned mechanisms. For the vitrification theory, kinetic stabilization of the peptide in the amorphous phase is assumed. Within this theory, aggregation tendency of the biopharmaceutical is connected to the molecular mobility governed by global and local relaxation within the lyophilizate. (Chang et al., 2005; Cicerone and Douglas, 2012; Groël et al., 2021; B. Wang, Tchessalov, Cicerone, et al., 2009; Yoshioka and Aso, 2007). For the water replacement theory, interactions such as hydrogen bonding between biopharmaceutical and excipients are assumed to be responsible for stabilization in solution. The interactions with the water molecules need to be replaced by interactions with the excipient molecules when the amorphous phase is formed upon water removal/crystallization (Andya et al., 1999; Arsiccio and Pisano, 2018; Cleland et al., 2001; Pisano et al., 2024; B. Wang, Tchessalov, Cicerone, et al., 2009, 2009).

Few works describing the stabilizing capabilities of excipients on peptides are reported in literature. Fang et al. investigated the influence of excipients on the stability of the pharmaceutical peptide glucagon during freeze-drying and subsequent storage using mass spectrometry and chromatography. They showed a superior stability for formulations containing trehalose in comparison to those including β-cyclodextrins and hydroxyethyl starch. However, the impact of freezing-induced stresses on peptide stability was not investigated alone.(Fang et al., 2012) Wewer Albrechtsen et al. studied the aggregation behavior of glucagon and the glucagon-like peptide 1 in human plasma after short and long-time storage and after freeze-thawing using activity assays. During their studies no significant effect of freeze-thawing on the aggregation of glucagon was observed.(Wewer Albrechtsen et al., 2015) Matilainen et al. studied the influence of various cyclodextrins on the chemical stability of glucagon in liquid and freeze-dried state using mass spectrometry and chromatography. They found stabilizing capabilities of cyclodextrins to be superior to lactose.(Matilainen et al., 2008, 2009)

The aim of the work is to present a simple and easy approach to assess the stability of peptides during freezing, by monitoring the change in the glass-transition temperature of the amorphous phase (T_g') over repeated freeze-thawing cycles using differential scanning calorimetry (DSC). This method allows for simultaneous application of freeze-thaw stressing, detection of aggregation, evaluation of cryo-protective properties of excipients, and determination of the solubility of water, all in one measurement. Furthermore, this method only requires a minimal amount of material (few µ-liters). Experiments were performed using a model peptide in combination with two excipients. Glucagon was used as model peptide, as it is known to aggregate under stresses and mishandling (Onoue et al., 2004; Pedersen, 2010). Sucrose was not considered as excipient, as at low pH, this excipient induces hydrolysis (US Pharmacopeia 24, 1999). Instead, trehalose was chosen as excipient, as it does not undergo acid hydrolysis. Lactose was considered as excipient as recent studies found its capabilities in stabilizing lyophilized glucagon through molecular dynamics and microfluidic modulation spectroscopy (Pisano et al., 2024). The solubility of water of the peptide/excipient formulations was also modeled using the equation of state Perturbed-Chain Statistical Association Fluid Theory (PC-SAFT). This method allows for a low effort and fast differentiation of stabilizing capabilities of excipients and may aid in future preliminary formulation screenings.

2. Materials and methods

2.1. Chemicals

Lactose-anhydrate with a purity of ≥99 %, D-(+)-Trehalose-dihydrate with a purity of ≥99 %, Tri-sodium citrate dihydrate with a purity of ≥99 %, lyophilized Glucagon with a purity of ≥95 %, and Polysorbate 20 were all purchased from Sigma-Aldrich Co. LLC (Milano, Italy). For the preparation of formulations, water for injection was used.

2.2. Solid-liquid equilibrium

The solubility of water was calculated using Eq. (1), assuming an equilibrium of the chemical potential in the ice phase and the amorphous phase and the immiscibility in the solid phase (Stephan and Mayinger, 1999).

$$x_w \cdot \gamma_w = \exp\left(-\frac{h_w^{SL}}{R T_w^{SL}} \cdot \left(1 - \frac{T}{T_w^{SL}}\right)\right) \tag{1}$$

The solubility of water, expressed by the mole fraction x_w, can be calculated as a function of the melting temperature T_w^{SL} and enthalpy h_w^{SL}, the temperature T and the activity coefficient γ_w of water. Specific interactions such as van der Waals forces and hydrogen bonds are considered by the activity coefficient.

2.3. PC-SAFT

The activity coefficients necessary for the calculation of the solid-liquid equilibrium are derived from the residual Helmholtz energy A^{res}. A^{res} is calculated with the Perturbed-Chain Statistical Association Fluid Theory (PC-SAFT) (Gross and Sadowski, 2001, 2002). In this process, A^{res} is composed of different fractions. $A^{hard-chain}$ accounts for hard-chain repulsions, $A^{dispersion}$ accounts for attractive interactions, and $A^{association}$ accounts for hydrogen bonding.

$$A^{res} = A^{hard-chain} + A^{dispersion} + A^{association} \tag{2}$$

For the calculation of A^{res}, each molecule is described as a chain of m_i^{seg} segments, with each segment having a diameter σ_i. In addition, the dispersion-energy parameter u_i/k_B, the association-energy parameter ϵ^{AiBi}/k_B, and the association volume k^{AiBi} are considered. These quantities are calculated using Berthelot–Lorentz (Lorentz, 1881) mixing rules, introducing an adjustable binary interaction parameter k_{ij}.

$$\sigma_{ij} = 1/2\left(\sigma_i + \sigma_j\right) \tag{3}$$

$$u_{ij} = \sqrt{u_i u_j}\left(1 - k_{ij}\right) \tag{4}$$

The binary interaction parameter k_{ij} can be temperature dependent with a constant value $k_{ij,0K}$ at 0 K and a temperature slope $k_{ij,T}$.

$$k_{ij} = k_{ij,T} \cdot T + k_{ij,0K} \tag{5}$$

For the calculation of the association energy and volume, mixing rules of Wolbach and Sandler (Wolbach and Sandler, 1998) were used.

$$\varepsilon^{AiBj} = 1/2\left(\varepsilon^{AiBi} + \varepsilon^{AjBj}\right) \tag{6}$$

$$\kappa^{AiBj} = \sqrt{\kappa^{AiBi}\kappa^{AjBj}}\left(\frac{\sqrt{\sigma_i \sigma_j}}{1/2\left(\sigma_i + \sigma_j\right)}\right)^3 \tag{7}$$

The PC-SAFT pure component parameters are listed in Table 2. The three parameters m_i^{seg}, σ_i, and association scheme of glucagon are derived from the peptides primary structure and are composed of the sum of the respective amino acids. The amino acid sequence can be found in literature (Joshi et al., 2000). The remaining dispersion-energy parameter u_i/k_B, the association-energy parameter ϵ^{AiBi}/k_B, the association volume k^{AiBi} and the binary interaction parameter k_{ij} were fitted to the solid-liquid equilibria.

For the calculation of the solubility of water binary interaction parameters were used and are listed in Table 3. The binary interaction parameters of trehalose/water, lactose/water and glucagon/water were fitted to the solubility of water for the sub-zero temperature range. The binary interaction parameters of glucagon/trehalose and glucagon/

lactose were fitted to the solubility of water for the systems glucagon/trehalose/water and glucagon/lactose/water, respectively.

2.4. Peptide/excipient formulation sample preparation

The solute concentration (both peptide and excipient) was 30 wt% in total. Therefore, a total solute mass of 10 mg was measured and introduced into 1.5 ml Eppendorf tubes. Water for injection was used to prepare the buffer. 50 mM sodium citrate buffer (pH 3) was prepared and the corresponding amount of buffer to achieve the total solute concentration was added to the Eppendorf tubes. Tubes were not shaken to prevent mechanical stressing. Analysis started as soon as all solutes were dissolved.

2.5. Measuring the thermal behavior of peptide systems using DSC

For the measurement of the peptide aggregation and the solubility of water, (modulated) differential scanning calorimetry (DSC) was used. A Q200 DSC from TA Instruments (New Castle, DE, USA) was used. The instrument was calibrated using indium. For the measurement, 20–40 mg of the peptide/excipient solutions were hermetically sealed in an aluminum pan. An empty aluminum pan was used as reference. The pan was cooled to −60 °C with a rate of 5 K/min. Temperature was held for 2 min. It was then heated with 1 K/min to 5 °C to prevent thermal stressing. For freeze-thaw stress testing, this cycle was repeated 12 times. Solubilities of water were evaluated according to literature. (Mohan et al., 2002) The modulated DSC measurement were conducted with a modulation rate of 0.159 K/min. T_g' was interpreted as the infliction point of the non-reversing heat flow and was analyzed using the software TA universal analysis from TA Instruments. The expected deviation in the measurement of T_g' is around 0.5 K for two separate samples where the mean deviation of T_g' between the high and the low concentrated sample is 0.42 K. The sensitivity within one sample/-measurement is much greater, where also smaller deviations of T_g' can be detected and quantified, as can be seen in Table 1.

2.6. Visual observations on peptide aggregation post freeze-thaw cycling in a freeze-dryer

For the visual inspection of aggregation of glucagon formulations containing trehalose or lactose the pilot-scale freeze-dryer LyoBeta 25 (Telstar, Terrassa, Spain) was used. 1 ml of sample with a total solute concentration of 5 wt%, consisting of a) 1:1 ratio of glucagon:excipient and b) 1:3 ratio of glucagon:excipient was pipetted into 5 ml glass vials and put in the freeze-dryer together with blank samples containing water. Shelf temperature was lowered with 1 K/min to −60 °C and held for 30 min. It was then raised with 1 K/min to 25 °C and held for 30 min. This cycle was repeated 12 times. The samples were observed using a camera attached to the outside of the freeze-dryer.

Table 1
T_g' of the freeze-thawing cycles of 1:3 glucagon:trehalose mixture, 1:1 glucagon:trehalose mixture, 1:3 glucagon:lactose mixture, and 1:1 glucagon:lactose mixture. Indicated as 1:1 glucagon:trehalose lower concentration is the lower concentrated mixture to compare to the effect of storage on aggregation of the same mixture at 5 °C.

Cycle	T_g' / °C				
	1:3 glucagon: trehalose	1:1 glucagon: trehalose	1:1 glucagon: trehalose lower concentration	1:3 glucagon: lactose	1:1 glucagon: lactose
1	−30.61	−28.84	−28.77	−29.27	−27.45
2	−30.58	−28.92	−28.69	−29.56	−27.55
3	−30.53	−28.85	−28.69	−29.45	−27.45
4	−30.63	−28.77	−28.92	−29.48	−27.12
5	−30.68	−29.08	−28.99	−29.45	−27.58
6	−30.68	−28.70	−29.12	−29.43	−27.57
7	−30.81	−29.54	−29.02	−29.48	−27.37
8	−30.61	−31.54	−29.58	−29.43	−27.37
9	−30.83	−30.87	−31.09	−29.45	−28.11
10	−30.86	−31.90	−31.76	−29.60	−29.24
11	−31.22	−31.26	−32.20	−29.84	−29.12
12	−32.30	−31.62	−31.76	−30.02	−28.73

2.7. Aggregation detection using size-exclusion chromatography

The size-exclusion chromatography set-up used for the qualitative detection of glucagon aggregation consisted of an Infinity II Quarternary system from Agilent Technologies (Santa Clara, California, USA) with an Superdex 200 Increase 10/300 GL SEC column from Cytiva (Marlborough, USA), a G7115A diode array detector, a G7162A refractive index (RI) detector, and a miniDawn light scattering detector (Wyatt Technology Corporation, Santa Barbara, USA). A flowrate of 0.75 ml min^{-1} of the above characterized citrate buffer was set for analysis. An injection volume of 10 µl of sample was used. The glucagon concentration for the unstressed/native reference sample was 1 wt%. To check for freeze-induced aggregation, a 1:1 glucagon/trehalose sample was stressed using DSC as described above. The hermetic pan was then opened and diluted with citrate buffer to match the 1 wt% glucagon of an unstressed reference sample. Before analysis the sample was centrifuged at 5000 RPM for 2 min to separate the solution from the formed gel-phase. For the calculation of the molecular weight of the glucagon samples the specific refractive index (dn/dc) of 0.182 was used (Hoppe et al., 2008).

3. Results

3.1. Multiple freeze-thaw cycling using DSC to determine the aggregation propensity

In order to assess the peptides stability against aggregation, the peptide sample was stressed by repeated freeze-thaw cycles within the DSC measurements. The samples were prepared as described in the method section. A typical result of these repeated T_g' measurements is shown in Fig. 1. Starting after a certain number of freeze-thaw cycles, a distinct shift to lower temperatures in the position of T_g' can be observed. This shift is caused by peptide aggregation in the course of the freeze-thaw cycles. The cycle number, at which the shift occurs, is a measure for the peptide's aggregation propensity. The lower the number, the higher the aggregation propensity in the given system. The evaluation of the aggregation kinetics is based on the number of cycles from the start of aggregation until no further changes in heat flow are

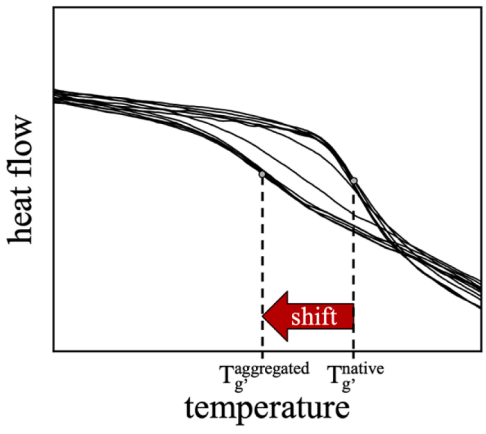

Fig. 1. Schematic diagram of the effect of repeated stressing of a peptide formulation in the DSC. Temperature range around T_g' is shown. Indicated by the dotted lines and the red arrow is the shift in T_g' from the T_g' of the native peptide to the T_g' of the aggregated peptide.

measured, indicating that aggregation is complete.

3.2. DSC based assessment of peptide aggregation

The newly developed method was used to assess the aggregation behavior of glucagon in combination with excipients trehalose and lactose. The ratio of glucagon:excipients was varied to investigate the effect of glucagon concentration on the aggregation behavior.

3.2.1. Trehalose as excipient

Experimental results on T_g' for a 1:3 glucagon:trehalose mixture are shown in Fig. 2 (left). Thawing cycle one is shown in green. Thawing cycle twelve is shown in red. The cycles in between are gradually colored. This color code is used for all following figures. Thermograms for the starting eight cycles are almost identical. Consequently, the measured T_g' as well as its position in the thermogram are also nearly identical. After nine cycles, a shift of T_g' to lower temperatures can be observed. In addition, there is a widening of the glass transition temperature region. T_g' is reduced by 0.22 K after cycle nine compared to cycle eight. The shift after cycle ten, eleven, and twelve is even more pronounced with a difference in T_g' to cycle eight of 0.25 K, 0.61 K, and 1.69 K respectively. T_g' of the individual cycles of all experiments are given in Table 1. Even after twelve cycles, the heat flow curve in the thermogram was still shifting, and no superimposition of the last two cycles was observable.

Experimental results on T_g' for a 1:1 glucagon:trehalose mixture are shown in Fig. 2 (right). For this ratio, thermograms for the starting five cycles were almost identical. The first shift of T_g' was measured for cycle six. In addition to the shift of T_g' occurring earlier (lower number of cycles), the shift of the glass transition to lower temperatures was larger. For the two subsequent cycles seven and eight, measurements revealed a shift in T_g' of 0.46 K and 2.46 K compared to cycle five. After eight freeze-thaw cycles, no further change in the thermogram and T_g' position was measured. All subsequent thermograms are identical and indicate that the equilibrium state was reached in this case. A second visualization of the DSC data, in which the individual cycles are stacked on top of each other, is provided in the SI.

3.2.2. Lactose as excipient

Experimental results on T_g' for a 1:3 glucagon:lactose mixture are shown in Fig. 3 (left). For this ratio, thermograms for the starting ten cycles are almost identical. The subsequent freeze-thaw cycles showed a small shift in the glass transition compared to the previous cycles. A maximum deviation of 0.40 K in T_g' was measured. The last two thermograms did not overlap indicating that the equilibrium state was not reached in this case.

Experimental results on T_g' for a 1:1 glucagon:lactose mixture are shown in Fig. 3 (right). For this ratio, thermograms for the starting eight cycles are almost identical. Starting with cycle nine, a progressive shift to lower temperatures for T_g' was measured with a maximum shift of 1.44 K in T_g' after cycle twelve compared to cycle eight. Again, the last two thermograms did not overlap indicating that the equilibrium state was not reached in this case.

Since the stress tests by freeze-thawing with twelve cycles required about 12 h, an experiment was designed to confirm, that instabilities/aggregates were not induced by storage for 12 h alone. Therefore, two samples of a 5 wt% solutes concentration (1:1 glucagon:trehalose mixture) were prepared. The first sample was stressed via DSC like the samples before. The measured T_g' are listed in Table 1. The second sample was stored at 5 °C for the same duration (12 h). 5 °C represents the highest temperature undergone by the stressed sample during freeze-thawing cycles. The relevant thermograms are shown in Fig. 4a. The thermogram of the unstressed sample is shown in green and thus represents the initial state of the sample. The blue curve shows the thermogram after storage in the refrigerator at 5 °C for 12 h No deviation from the initial (green) state is observed. The red curve illustrates the thermogram of the freeze-thawed sample. As expected, a shift in the position of the glass transition of the stressed sample can be observed, confirming that the shift was induced by freeze-thawing.

3.2.3. Multiple thermal events within DSC measurements

In addition to the shift in the glass transition, an exothermic event prior to the glass transition was observed in all samples once the shift in T_g' occurred. Modulated DSC was performed to better investigate the nature of this thermal event, as illustrated in Fig. 4b. In addition to the heat flow signal (bottom), the reverse heat flow (top) is also shown. The affected temperature range is highlighted in gray. A broad exothermic peak can be seen starting at the cycle where aggregation occurs and thus the glass transition is shifted. To exclude a second glass transition, the reverse heat flow of the sample was analyzed. No signal can be seen in the gray shaded area of the reverse heat flow.

3.3. Visual determination of peptide aggregation

For optical validation of aggregation, a freeze-thaw stress test in a pilot-scale freeze-dryer was performed. The drying chamber of the freeze dryer can be viewed through the plexiglass door, thus enabling visual examination of the samples during freeze-thawing. As described in the methods section, a 1:3 and a 1:1 glucagon:excipient ratio was prepared for trehalose and lactose. In addition, pure water samples were also analyzed to provide a reference. Fig. 5 (top) shows the initial states of the liquid formulations. All liquids were colorless, clear and showed no sign of turbidity. Formulations after one freeze-thaw cycle are shown in Fig. 5 (middle). It was noticeable that 1:1 glucagon:lactose and glucagon:trehalose samples showed turbidity, suggesting peptide aggregation, while the 1:3 glucagon:excipient samples were still clear after one cycle. Fig. 5 (bottom) shows the formulations after twelve freeze-thaw cycles. All samples containing glucagon were opaque.

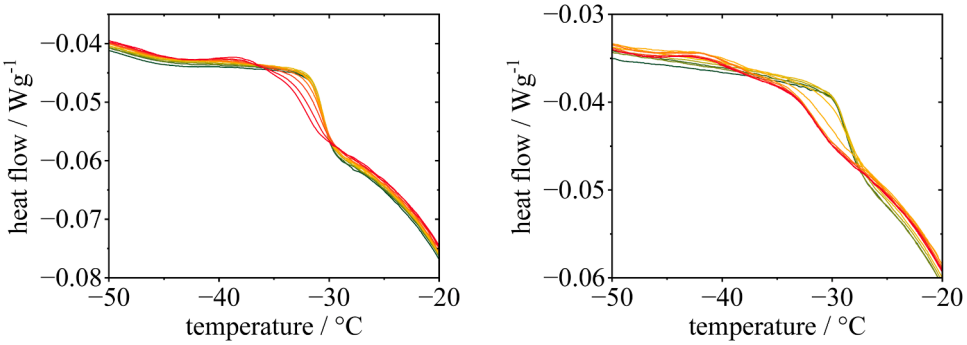

Fig. 2. Freeze-thawing thermograms around the region of T_g' shown. Thawing cycle one is shown in green. Thawing cycle twelve is shown in red. The cycles in between are gradually colored. Solute concentration is 30 w%. left) 1:3 glucagon/trehalose mixture. right) 1:1 glucagon/trehalose mixture.

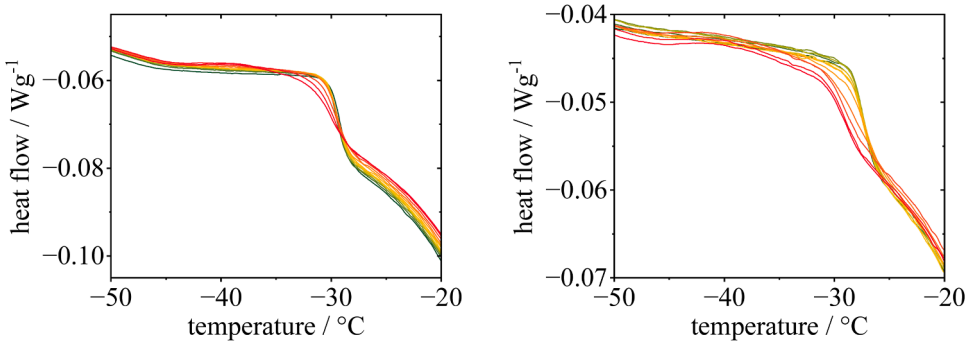

Fig. 3. Freeze-thawing thermograms around the region of T_g' shown. Thawing cycle one is shown in green. Thawing cycle twelve is shown in red. The cycles in between are gradually colored. Solute concentration is 30 w%. left) 1:3 glucagon/lactose mixture. right) 1:1 glucagon/lactose mixture.

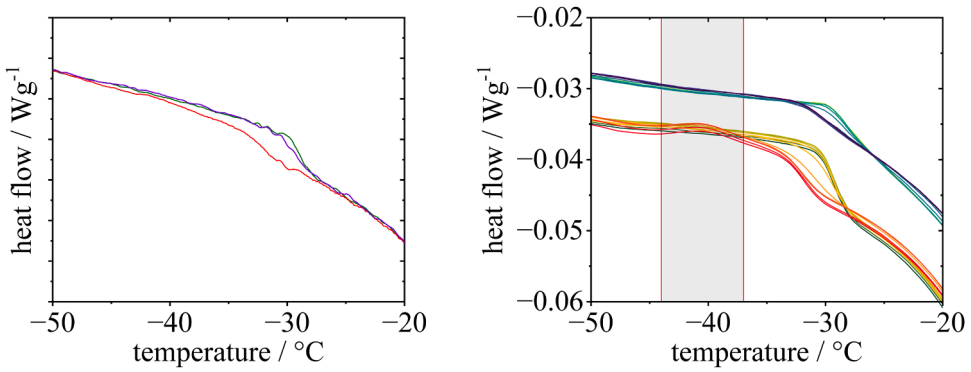

Fig. 4. Freeze-thawing thermograms around the region of T_g' shown. left) Green line shows thawing cycle one of 1:1 glucagon/trehalose mixture. Solute concentration is 5 w%. Red line shows thawing cycle twelve. The blue line shows a thawing cycle after 12 h of storing at 5 °C. right) Thermograms of 1:1 glucagon/trehalose mixture. Solute concentration is 30 w%. Bottom: Thawing cycle one is shown in green. Thawing cycle twelve is shown in red. The cycles in between are gradually colored. Top: The reverse heat flow of thawing cycle one is shown in light green. The reverse heat flow of thawing cycle twelve is shown in dark blue. The cycles in between are gradually colored.

3.4. Size-exclusion chromatography of peptide solutions

After stressing glucagon using DSC, analyses was performed using size-exclusion chromatography. A 1:1 glucagon:trehalose sample was stressed using DSC and then prepared for size-exclusion chromatography analysis as described in the methods section. As a reference, a non-stressed 1:1 glucagon:trehalose sample was also measured. The results of the size-exclusion chromatography analysis are shown in Fig. 6. The light scattering signal of the non-stressed sample is shown in black and the light scattering signal of the stressed sample is shown in red. The average molecular weight of the native sample for the second peak is 3590 Da The average molecular weight of the stressed sample for the second peak is 3656 Da These values match the native glucagon molecular weight. The integrated peak area of the non-stressed sample is factor 2.2 higher than the integrated peak area of the stressed sample. This implies that the concentration of glucagon in the stressed sample is lower than in the non-stressed sample. Prior to the native peptide a higher molecular weight species elutes, presumably being soluble peptide aggregates.

3.5. Solubility of water in peptide/excipient mixtures

3.5.1. Experimental

Besides measuring the aggregation behavior, the solubility of water of the respective systems was measured simultaneously using DSC (within the same experiment), as described in the materials and methods section. Knowledge of the solubility of water allows for temperature dependent determination of residual water content (1 –solutes weight fraction) in the amorphous phase during and after freezing. The solubility of water was measured for binary systems containing either trehalose, lactose, or glucagon in combination with water. Additionally, ternary systems containing the mixtures glucagon/trehalose and glucagon/lactose in combination with water were also investigated. In Fig. 7 the solubility of water in solutions with for increasing glucagon: excipient ratios is illustrated. The results show the solubility of water in the respective mixtures spans over a broad concentration range from a solute weight fraction of 0.4 up to 0.9.

By gradually increasing the glucagon:trehalose ratio from pure trehalose to pure glucagon (system water/glucagon/trehalose) the solubility of water decreases continuously. According to the evaluation method described in the material and methods section, the last experimental data point for each glucagon:trehalose ratio at the highest measurable concentration represents w_g' at T_g'. Experimental results thus also reveal that T_g' increases with increasing glucagon:trehalose ratio from −32 °C in pure trehalose, over −31 °C in the 1:3 glucagon: trehalose mixture, −28 °C in the 1:1 glucagon:trehalose mixture, −26 °C in the 3:1 glucagon:trehalose mixture, to −15 °C in pure glucagon. The characteristic value of w_g' of trehalose was determined to be 0.775.

A comparable behavior was observed when using lactose as excipient. By gradually increasing the glucagon:lactose ratio from pure lactose to pure glucagon (system water/glucagon/lactose) the solubility of water decreases continuously. Again, T_g' increases with increasing glucagon:lactose ratio from -32 °C in pure lactose, over −30 °C in the 1:3 glucagon:lactose mixture, to −28 °C in the 1:1 glucagon:lactose mixture. W_g' for lactose was determined to be 0.873.

3.5.2. Solubility modeling using PC-SAFT

To reduce experimental efforts in the development of new

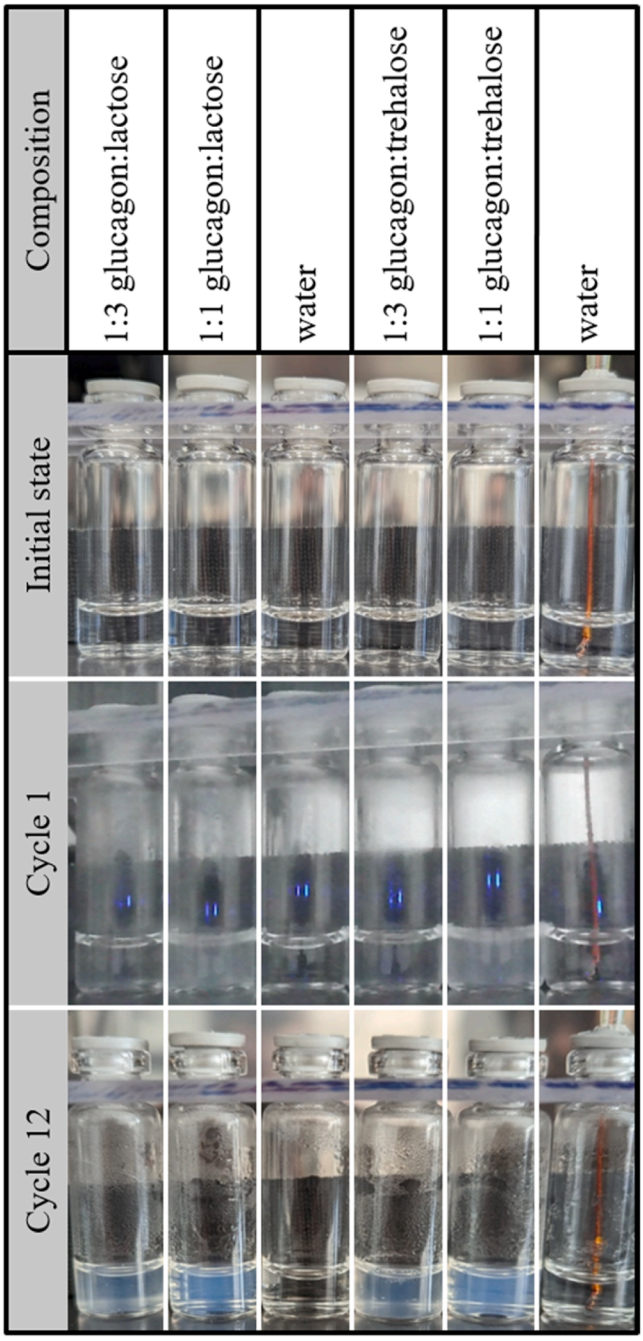

Fig. 5. Pictures of liquid glucagon/excipient formulations at 25 °C at different time points: top/initial state) directly after preparation; middle/Cycle 1) after one freeze-thawing cycle; bottom/Cycle 12) after twelve freeze-thawing cycles.

formulations, water solubility was modeled using the equation of state PC-SAFT. Pure-component parameters for glucagon and the interaction parameter between glucagon and water were fitted to experimental solubility data measured for the respective binary systems within this work (Fig. 7), as described in the materials method section. The binary interaction parameters between glucagon and trehalose as well as glucagon and lactose were fitted to the solubility of water in glucagon/trehalose and glucagon/lactose mixtures. All parameters used within this work are listed in Table 2 and Table 3.

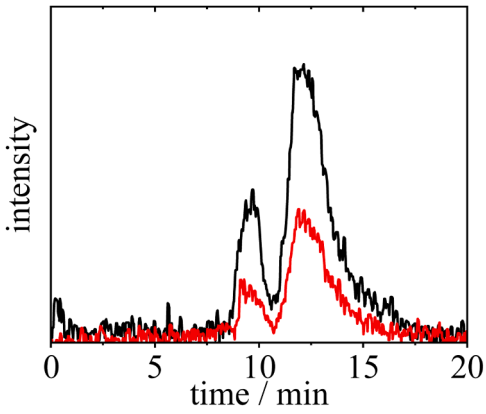

Fig. 6. Light scattering signal after size-exclusion chromatography of two glucagon/trehalose samples. Shown in black is the light scattering signal of a non-stressed 1:1 glucagon/trehalose sample. Shown in red is the light scattering signal of a stressed 1:1 glucagon/trehalose sample.

4. Discussion

4.1. Rapid assessment of aggregation propensity of frozen peptide formulations

The DSC enabled assessment of peptide aggregation propensity for samples containing glucagon is reliable and in agreement with results obtained using orthogonal measuring techniques. Additional analysis was performed with optical investigation after freeze-thaw cycles performed in a pilot-scale freeze-dryer and size-exclusion chromatography-multi angle light scattering measurements after stressing by DSC. For the optical investigations, a turbidity/gelation of the sample was detected after one freeze-thaw cycles for the 1:1 excipient:glucagon formulations, but not the 3:1 excipient:glucagon formulations. After 12 cycles both formulations showed turbidity. This matches the DSC enabled stabilization investigation, since there the 1:1 excipient:glucagon samples also first showed aggregation during stressing and both formulations aggregated eventually. This does qualitatively overlap with the DSC results, but not quantitatively, since different attributes are measured in the DSC and in the freeze-drier leading to different cycle numbers for the onset of aggregation in the DSC and the occurrence of turbidity in the freeze-drier. Decrease in the light scattering signals detected in the size-exclusion chromatography-multi angle light scattering measurements (see Section 3.4) indicate a decreasing peptide/aggregate concentration in the samples, presumably through liquid-liquid phase separation/precipitation of the aggregates throughout the multiple freeze-thaw cycles. This is plausible as the T_g' shifts to lower temperatures. The peptide concentration thus decreases in the amorphous phase. In term, the concentration of excipient increases and approaches the T_g' of pure excipient in water. If soluble (high-molecular weight) aggregates would occur, a T_g' shift to higher temperatures would be expected. The overlay of the thermograms after the first DSC cycle with a fresh sample and after 12 h of storage at 5 °C (Section 3.2.2) confirms that aggregation is not caused by simple storage but by freeze-thaw stressing.

The pre-T_g' thermal event (Section 3.2.3) was shown not to be a second glass transition. Results indicate it to be a reversible process that occurs just before the glass transition in samples with aggregated peptide, as the event continues to be seen even in fully aggregated samples. Thus, aggregation itself can be ruled out as the causative mechanism. The phenomenon will be further elaborated in future works.

4.1.2. Stabilization of glucagon in frozen formulations

By comparing trehalose and lactose used for the stabilization of glucagon (results Section 3.2.1 and 3.2.2), two conclusions can be drawn. Firstly, it is advantageous if a high excipient to peptide ratio is

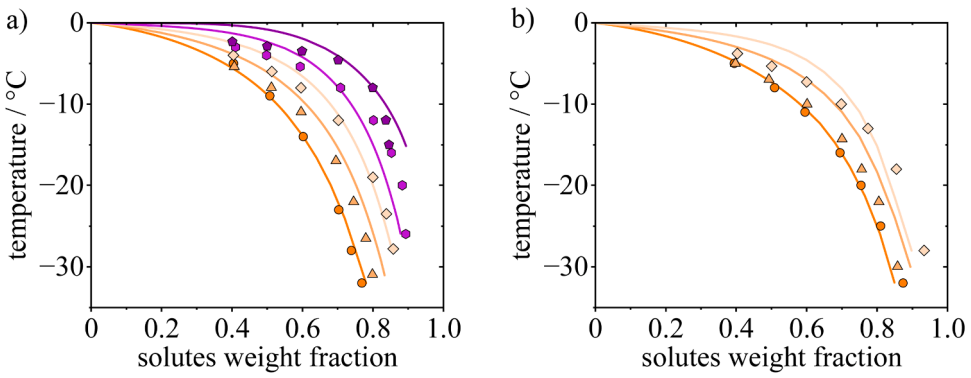

Fig. 7. Phase behavior of glucagon/excipient mixtures. a) Solubility of water in trehalose (orange circles), 1:3 (light orange triangles), 1:1 (cream diamonds), 3:1 (light violet hexagons) glucagon/trehalose mixtures, and in glucagon (violet pentagons). The according colored lines show the modeling of the solubility of water using PC-SAFT. b) Solubility of water in lactose (orange circles), 1:3 (light orange triangles), and 1:1 (cream diamonds) glucagon/lactose mixtures. The according colored lines show the modeling of the solubility of water using PC-SAFT.

Table 2
PC-SAFT pure-component parameters of the substances used in this work.

Substance	M_i / g mol^{-1}	m_i^{seg} / mol g^{-1}	σ_i / Å	$u_i\ k_b^{-1}$ / K	$\varepsilon^{AiBi}\ k_b^{-1}$ / K	k^{AiBi}	N_i^{assoc}	Ref.
trehalose	342.30	0.04000	2.856	319.85	5000.0	0.100	8/8	(Held et al., 2013)
lactose	342.30	0.04188	2.811	319.21	5000.0	0.100	8/8	(Held et al., 2013)
glucagon	3482.70	0.05466	2.673	330.00	1850.0	0.100	8/3	this work
water	18.02	1.20147	[a]	353.94	2425.7	0.045 [b]	1/1	(Cameretti and Sadowski, 2008)

[a] $\sigma = 2.7927 + 10.11 \cdot \exp(-0.01775 \cdot T) - 1.417 \cdot \exp(-0.01146 \cdot T)$. [b]Typo in reference (Cameretti and Sadowski, 2008).

Table 3
PC-SAFT binary interaction parameters used and fitted in this work.

Substances	$k_{ij,T}$ / K^{-1}	$k_{ij,0K}$	Temperature range / K
trehalose/water	0.000350	−0.140	238–273
lactose/water	−0.000239	0.020	238–273
glucagon/water	−0.000320	0.020	238–273
glucagon/trehalose	0.000003	−0.029	238–273
glucagon/lactose	−0.000500	0.100	238–273

present. The better stabilization at a low glucagon concentration can either be attributed to a decreased stress of glucagon at the ice/amorphous phase interface and/or to beneficial interactions between glucagon and the excipients at low glucagon concentrations. Secondly, our method indicates that lactose is superior over trehalose if used as excipient for stabilizing glucagon in applications with freeze-thawing involved. If used at the same peptide:excipient ratio, lactose can stabilize glucagon for one additional cycle at low glucagon concentration (1:3 glucagon:excipient) and two cycles at a high glucagon concentration (1:1 glucagon:excipient). These results were also confirmed by visual observation of aggregation in freeze-thawed samples.

4.1.3. Predicting water solubility and w_g' in frozen formulations

Results show that the solubility of water for the systems containing trehalose, lactose, and glucagon is closely matched and can reliably be predicted for sub-zero temperatures. The measured w_g' of trehalose corresponds to the values from literature of 0.833 (Hatley and Franks, 1991) and more recently 0.780 (Zäh et al., 2023). The w_g' for lactose is also much higher than the first ever published value of 0.592 (Levine and Slade, 1988) but corresponds to the more recent value of 0.853 (Zäh et al., 2023).

The knowledge about the influence of peptide on w_g' can be used for the design of freeze-drying processes, w_g' can be used to estimate the mass of ice and thus the primary drying time. The PC-SAFT parameters for glucagon can be used to determine the influence of the peptide on the interactions between peptide, excipients, and water in the amorphous phase, which can be crucial for the stability in frozen formulations.

5. Conclusion

In this work we presented a novel approach for the fast assessment of excipient impact on peptide aggregation during freezing. We investigated the aggregation behavior of the model peptide glucagon in combination with trehalose and lactose.

The advantage of this new DSC method is that statements about the stabilizing effects of excipients on peptides can be made in a short time without the need of freeze-drying in a conventional freeze-dryer and subsequent storage. The number of freeze-thawing cycles that the peptide and excipient solution can withstand without aggregating can be used to assess how the excipients in the amorphous phase affect glucagon stability under freeze-thawing stress. It also enables the efficient and rapid screening of different excipients, by combining stressing and the simultaneous detection of aggregation. The small amount of sample allows a material-friendly and cost-effective analysis. In contrast to the classic method, in which large-volume samples are repeatedly freeze-thawed in the freeze-dryer to make qualitative statements about aggregation, the method presented in this work allows for making quantitative statements, e.g., determining the number of freeze-thawing cycles that do not compromise peptide stability. It is also possible to detect incomplete/gradual aggregation.

It was shown that low peptide/high excipient concentration is beneficial for glucagon and that lactose is able to stabilize glucagon better and longer during freeze-thaw cycles than trehalose. The validation tests using a pilot-scale freeze-dryer and size-exclusion chromatography further supported the previously obtained findings. Additionally, we investigated the phase behavior of glucagon in combination with trehalose and lactose. We fitted PC-SAFT parameters for glucagon, allowing to model the impact on phase behavior for future formulation development.

Using the newly developed approach, it is now possible to judge the stabilizing potential of excipients efficiently at a very early stage in formulation development (where low amounts of drug are available), reducing material and labor cost.

Nomenclature

Latin Symbol	Description	Unit
A_{assoc}	Fraction association	J/mol
A_{disp}	Fraction dispersion	J/mol
A_{hc}	Fraction hard chain	J/mol
A_{res}	Residual Helmholtz Energy	J/mol
k_{ij}	Interaction parameters	–
m_{seg}	Number of segments	mol g^{-1}
p	Pressure	Pa
R	General gas constant	J/mol·K
T	Temperature	K
T_g'	Maximum glass transition temperature of amorphous phase achievable through freezing	K
T_{0i}^{SL}	Melting point	K
u_i/k_B	Dispersion energy	K
x	Mole fraction liquid phase	mol mol^{-1}
Δh_{0i}^{SL}	Melting enthalpy	J/mol
w	Weight fraction	–
w_g'	Weight fraction of amorphous phase at and below T_g'	–
Greek Symbol	**Description**	**Unit**
γ	Activity coefficient	–
ε^{AiBj}/k_B	Energy of association	K
κ^{AiBj}	Association volume	–
ρ	Density	kg m^{-13}
σ	Segment diameter	Å

CRediT authorship contribution statement

Maximilian Zäh: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christoph Brandenbusch:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Fiora Artusio:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Gabriele Sadowski:** Resources, Project administration, Funding acquisition. **Roberto Pisano:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106954.

Data availability

Data will be made available on request.

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