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Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

- Optimised insulin-sucrose-polymer formulations designed for glass stability
- Insulin is shown to have a negative effect on formulation Tg and RHg values
- Interactive insulin-polymer and insulin-sucrose effects enhance glass stability

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Design of Co-lyophilised Ternary Insulin-Sucrose-Polymer Systems with Enhanced Amorphous Glass stability

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KEYWORD: Lyophilisation, Glass transition temperature, Mixture design of
experiments, polymer, humidity, dynamic vapour sorption

ABSTRACT

The glass stability of lyophilized amorphous peptide formulations, intended for incorporation into solid oral dosage forms, require stabilisation against the challenges of manufacturing, storage and handling temperature and humidity. High glass transition temperature (T_g) polymers, polyvinylpyrrolidone (PVP) and polyvinylpyrrolidone-vinyl acetate (PVPVA), were added to insulin-sucrose formulations to enhance glass stability when exposed to temperature and humidity. T_g and onset glass transition humidity (RH_g) parameters were experimentally determined as indicators of formulation glass stability with respect to temperature and humidity, respectively. A mixture design of experiment approach was employed to determine the influence of insulin, sucrose and polymer composition on formulation T_g and RH_g. Statistical regression models were established to evaluate the relationship between formulation composition and the corresponding glass transition parameters, T_g and RH_g. Phase separation noted for PVPVA-containing formulations, undermined regression model goodness of fit. Insulin content was shown to have a negative effect on both formulation T_g and RH_g. Formulation T_g appeared to be influenced by insulin's dynamical temperature rather than a previously reported insulin T_g value. Insulin-sucrose and insulin-polymer interactive effects resulted in increased T_g and RH_g values, indicating enhanced formulation glass stability. Formulation optimization for maximized T_g and RH_g identified a formulation composed of 26% w/w insulin, 40% w/w sucrose, and 34% w/w PVP, with a predicted T_g of 82°C and RH_g of 60% RH. The enhanced glass stability of the ternary insulin-sucrose-polymer formulations offers potential advantages for the manufacture, storage and handling of peptide containing oral dosage forms.

1. INTRODUCTION

Therapeutic peptides are an important class of drugs for the treatment of various diseases (1,2). Peptides are predominantly administered parenterally, as subcutaneous or intravenous injections. This approach is favoured as it facilitates peptide systemic delivery. Despite its efficacy, the parenteral route of administration presents drawbacks, including the costs associated with aseptic sterile production. These costs, coupled with patient discomfort and the risks of infection posed by parenteral administration (3,4), have motivated the development of non-parenteral dosage forms for systemic peptide delivery. Among these non-parenteral dosage forms, oral dosage forms have received considerable interest due to patients' preferences for oral administration (5).

To date, research into oral peptide delivery has predominantly focused on overcoming barriers to bioavailability, such as peptide metabolism within the gastrointestinal tract (6,7) and poor permeation across the intestinal epithelial barrier (8). Advances in formulation strategies, such as the use of protective excipients, enzyme inhibitors, permeation enhancers, nanoparticles, microcapsules, and enteric-coated dosage forms, have addressed these challenges (9,10). However, peptide stability can also be undermined during drug product manufacture, storage, and during patient handling prior to administration. These potential challenges during drug product development warrant investigation as they also pose a barrier to the development, commercial manufacture, and clinical translation of solid oral peptide dosage forms.

Peptides are incorporated into solid oral dosage forms in a solid state. In the solid state, reduced molecular mobility can enhance peptide stability (11). Lyophilization, also referred to as freeze drying, is a commonly employed process to convert protein and peptide solutions into solid formats. Non-reducing disaccharides, such as sucrose and trehalose, are commonly added to lyophilised peptide formulations to mitigate the destabilisation stresses experienced during lyophilisation (12). These disaccharides preserve peptide conformational stability during freezing (cryoprotection) and drying (lyoprotection) (13,14). Two key mechanisms are proposed which contribute to disaccharide stabilisation of peptides during lyophilisation and in solid-state (15). One mechanism is the classic vitrification theory, based on the formation of an amorphous glass disaccharide matrix which immobilizes peptide molecules, retarding physical and chemical degradation (16). Peptide stabilisation via the vitrification theory is highly dependent on the relationship between an amorphous solid's glass transition temperature (T_g) and the temperature exposure during processing or storage. T_g is the temperature at which the amorphous solid changes from a high viscosity, rigid, glassy state to

a lower viscosity, softer, rubbery state with increased molecular mobility. Increased matrix molecular mobility at temperatures above the system's T_g can undermine peptide stability in the solid matrix due to increased peptide molecular mobility, and potential for crystallisation of the thermodynamically unstable amorphous disaccharide state. Therefore, maintaining a disaccharide glass matrix formed via lyophilisation is considered critical for stabilisation of peptide and proteins (15). In this study the maintenance of an amorphous glassy state is referred to as 'glass stability'.

The resulting solid-state formed by lyophilisation (amorphous or crystalline) is determined by the interplay of formulation chemistry and process parameters. Amorphous solid phase arises when molecular mobility is restricted during the solution freezing and drying stages, producing a disordered amorphous matrix. Crystalline solid phases arise when molecules have sufficient mobility during processing to organize into ordered lattices, often influenced by excipients with strong crystallization tendencies such as mannitol or certain salts. Therefore, the formulation constituents, the thermal and pressure profile applied during lyophilization, and the level of residual moisture all contribute to the final solid-state phases obtained (14).

During storage environmental stressors such as elevated temperature and humidity can accelerate peptide chemical degradation pathways, compromising peptide structural integrity in the solid state (18). To mitigate the destabilising potential of environmental humidity, commercial peptide oral tablets, such as Rybelsus® (oral semaglutide), are supplied in aluminium-aluminium blister packaging with the patients advised to retain tablets in their original packaging until use (19). Environment humidity can also undermine the amorphous glass stability of lyophilised disaccharide systems. These amorphous disaccharide systems are hygroscopic due to their polar chemistry combined with their high surface area architecture, a feature of lyophilised products (20). Absorbed water acts as a plasticizer increasing the free volume within the amorphous matrix, thereby lowering the systems glass transition temperature (T_g) (21). This plasticizing effect "loosens" the system, enhancing molecular mobility and accelerating degradation reactions (22). Kilburn et al. (23) demonstrated that even small amounts of sorbed water disrupt hydrogen-bonded carbohydrate networks, producing a marked expansion of nanoscopic free volume elements and increasing matrix mobility well below the macroscopic T_g . More recent work on polysaccharide-disaccharide mixtures has reinforced these mechanistic insights. Li et al. (24) reported that water sorption behaviour, monolayer hydration capacity, and T_g depression in dextran-sugar blends depend strongly on molecular compatibility and the extent to which water perturbs packing density.

The plasticising effects of absorbed moisture are minimised for lyophilised parenteral peptide products due to their packaging operation in sealed primary packaging conducted in the extremely low humidity environment of the lyophiliser chamber (25). Lyophilised formulations incorporated into solid dosage formats, are exposed to environmental humidity during manufacture and patient handling which can undermine their amorphous glass stability (26). Primary packaging materials, such as aluminium–aluminium blister packaging, can protect against humidity stress during long term storage. However, environmental humidity exposure during manufacturing and packaging operations, in-process storage, and patient handling prior to administration, is more challenging to eliminate or control. Therefore, the motivation for this study was to design optimised lyophilised peptide-disaccharide formulations that would stabilise their amorphous glass solid-state during tablet manufacture (typically performed at ambient temperature and 30–65% RH) (27), and patient handling between removal from packaging and administration.

To mitigate these environmental challenges to amorphous glass stability, ternary lyophilised systems were evaluated consisting of insulin, as a model peptide, sucrose, as a cryo- and lyo-protectant, and a high T_g polymer, polyvinylpyrrolidone (PVP) or polyvinylpyrrolidone-vinyl acetate (PVPVA). The inclusion of these polymers has been shown to increase the T_g of lyophilised sugar matrices, making them more resistant to collapse or phase separation when exposed to heat or humidity (28). A previous study by our group demonstrated that incorporation of these high T_g polymers in lyophilized formulations effectively stabilized the glassy state of binary lyophilised polymer-disaccharide matrices by reducing the systems' hygroscopicity, enhancing T_g , and through disaccharide-polymer hydrogen-bonding interactions (29). The hypothesis evaluated in this study was that this stabilisation effect would also be observed in ternary systems containing a peptide component. To design ternary formulations with optimised glass stability, a mixture design of experiments (DOE) was employed. The effects of varying proportions of insulin, sucrose, and polymer (PVP or PVPVA) on key glass stability parameters was determined. Specifically, the DOE was designed to maximize the T_g and the onset glass transition humidity (RH_g). The RH_g represents the transition point humidity where absorbed water acts as a plasticizer, causing a shift in sorption characteristics due to increased bulk absorption as material mobility increases above its glass transition (20).

2. MATERIALS AND METHODS

2.1 Materials

Sucrose and polymers (PVP K30 and PVPVA 64) were donated by Pfanstiehl Inc. (USA), and BASF Inc. (Germany), respectively. Recombinant human insulin with a molecular weight (Mw) of 5.8 KDa was supplied by SAFC (Switzerland). Lyophilization 10 mL ISO Clear Type I Tubular glass vials, and 20 mm grey silicone stoppers were supplied by Adelphi Healthcare Packaging (UK). All other chemicals were reagent grade and solvents HPLC grade.

2.2. Methods

2.2.1 Ternary formulation selection

To determine the optimum ratio of insulin, sucrose, and polymer that maximizes glass stability, an extreme vertices mixture DOE was designed using Minitab 20.2.0.0 Software. An individual mixture DOE was conducted for both PVP and PVPVA-containing formulations. All solution formulations for lyophilisation had a fixed total solute concentration of 4% w/v. A total solute concentration of 4% w/v selected to achieve insulin solubility at all the insulin:sucrose:polymer ratios studied in the selected solvent 10 mM citric acid buffer at pH 2. A pH 2 buffer was selected due to the higher insulin solubility at acidic pH compared to neutral pH (30). The mixture DOE was constrained. Insulin solution concentration was limited to a range of 0.4% to 1.6% w/v, with the upper limit determined by its solubility in the selected buffer pH 2. A minimum sucrose concentration of 1.6% w/v was employed to ensure a minimum 1:1 sucrose-to-insulin ratio, since this threshold has been previously recommended to prevent lyophilization-induced protein unfolding and provide good stability (31). As a result of the insulin and sucrose constraints, the polymer content was also constrained 0% - 2% w/v (Table S1 Supplementary Materials), The composition of individual formulations post-lyophilisation is detailed in Table 1.

The glass transition temperature of maximally freeze-concentrated amorphous phase (T_g') of each formulation was determined by differential scanning calorimetry (DSC) employing the Q1000 instrument, TA Instruments Inc. (USA). Formulation volumes of 7.5 μ l were transferred to aluminium hermetically sealed pans. DSC analysis was performed by cooling samples to -60°C and reheating to 20°C at a ramp rate of $5^\circ\text{C}/\text{min}$. Thermograms were analysed using Universal Analysis 2000 software, TA Instruments Inc. (USA). All samples were analysed in triplicate.

2.2.2 Lyophilisation cycle

Formulations were freeze-dried with a laboratory-scale freeze dryer (VirTis AdVantage Pro). Sample aliquots (2.5 mL) were transferred into 10 mL lyophilization vials and placed on the lyophilizer shelf at room temperature. Samples were frozen by ramping down the shelf temperature to -40°C at $0.5^{\circ}\text{C}/\text{min}$ and holding for 3.5 h. Subsequently, the chamber pressure was reduced to 50 mTorr and shelf temperature increased to -30°C , initiating primary drying. After the samples were held at these parameters for 62h, secondary drying (50 mTorr chamber pressure) was initiated by increasing shelf temperature to 35°C at a rate of $0.5^{\circ}\text{C}/\text{min}$ for 6h.

2.2.3 Characterization of lyophilised formulations

Glass transition temperature (T_g): Modulated DSC was used to determine the T_g of lyophilized formulations. To minimise moisture uptake during sample preparation for DSC analysis, lyophilised samples were transferred from lyophilisation vials to DSC aluminium pans in a humidity-controlled glove box (relative humidity < 10%) and hermetically sealed prior to removal. During DSC analysis, samples were heated at $3^{\circ}\text{C}/\text{min}$ with an amplitude of $\pm 1^{\circ}\text{C}$ and 40 s period of modulation under dry nitrogen purge at 50 mL/min. The glass transition temperature was determined using Universal Analysis software. All samples were analysed in triplicate.

Onset glass transition humidity (RH_g): Moisture sorption was determined by dynamic vapor sorption (DVS) using a DVS Intrinsic Instrument and DVS Intrinsic Control software (Surface Measurement Systems, UK). The instrument was placed in a humidity-controlled glove box (relative humidity < 10%) to minimise any absorption of ambient moisture by the lyophilised powder during sample removal and handling. Initially, samples were dried at 0% RH for 6 h to establish a dry mass at a constant temperature ($25 \pm 0.1^{\circ}\text{C}$) and then exposed to a linear increase in relative humidity from 10 to 90% RH at a ramping rate of 10% RH/h. For each formulation, the onset glass transition humidity (RH_g) was identified from the sorption isotherm (mass change vs RH) by fitting linear tangents to the low and high RH regions. The intersection of these tangents marked the RH_g , corresponding to the inflection point where water uptakes shifted from predominately surface adsorption to bulk absorption (20).

Residual moisture determination: The residual moisture content in the lyophilised samples was determined by Karl Fischer titration using a Moisture Meter (CA-31, Mitsubishi Chemical Analytech Co., Ltd, Japan) by external extraction using anhydrous methanol. The samples were analysed in triplicate.

2.2.4 Mixture DOE analysis and formulation optimisation

Statistical regression modelling of the mixture DOE data was conducted using Minitab version 20.2.0.0 (Minitab LLC, State College, PA, USA). The objective of this analysis was to evaluate the influence of individual formulation components and their interactions on glass stability as determined by T_g and RH_g measurements. Subsequently, a mixture response optimization was conducted using the Response Optimizer tool in Minitab software to identify the optimal proportions of insulin, sucrose, and polymer within these ternary systems that maximize T_g and RH_g .

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Table 1. Lyophilised formulation compositions investigated, their respective average T_g and water content values \pm standard deviation ($n=3$), and RH_g values

#	Formulation composition (%w/w)			T_g ($^{\circ}C$)		Water content (%)		RH_g (%)	
	Insulin	Sucrose	Polymer	PVP-formulations	PVPVA-formulations	PVP-formulations	PVPVA-formulations	PVP-formulations	PVPVA-formulations
1	32.50	48.75	18.75	75.04 ± 1.98	102.96 ± 4.03	1.44 ± 0.49	0.43 ± 0.11	59.00	61.50
2	10.00	40.00	50.00	87.34 ± 0.97	63.90 ± 1.91	1.59 ± 0.25	0.52 ± 0.10	47.00	47.00
3	40.00	60.00	0.00	68.03 ± 1.68	68.03 ± 1.68	0.98 ± 0.63	0.59 ± 0.21	61.00	60.00
4	10.00	65.00	25.00	71.44 ± 1.13	53.45 ± 3.75	0.62 ± 0.34	0.44 ± 0.37	45.00	46.00
5	32.50	58.75	8.75	69.28 ± 1.35	63.84 ± 1.81	0.84 ± 0.26	0.71 ± 0.12	61.00	60.00
6	40.00	50.00	10.00	70.97 ± 0.58	69.30 ± 1.67	1.48 ± 0.35	0.99 ± 0.10	62.00	63.00
7	10.00	90.00	0.00	60.79 ± 3.10	60.79 ± 3.10	1.07 ± 0.06	0.83 ± 0.11	23.00	24.50
8	40.00	40.00	20.00	68.23 ± 1.33	106.48 ± 2.31	1.07 ± 0.10	1.00 ± 0.53	64.50	62.50
9	17.50	73.75	8.75	67.97 ± 2.09	64.84 ± 0.51	0.91 ± 0.12	0.83 ± 0.11	56.00	55.50
10	17.50	48.75	33.75	81.88 ± 2.21	63.67 ± 2.34	1.03 ± 0.26	1.10 ± 0.31	54.00	55.00
11	25.00	75.00	0.00	69.34 ± 2.45	69.34 ± 2.45	1.10 ± 0.26	1.10 ± 0.40	61.50	62.00
12	25.00	40.00	35.00	84.35 ± 0.27	68.09 ± 5.46	0.53 ± 0.16	0.84 ± 0.44	59.00	58.50
13	25.00	57.50	17.50	71.78 ± 1.43	66.08 ± 2.71	0.91 ± 0.34	0.76 ± 0.18	61.00	60.00

3. RESULTS

3.1. T_g' of pre-lyophilised formulation

The T_g' was determined prior to lyophilization, marking the temperature below which the frozen formulation becomes glassy and stable. Maintaining product temperatures below T_g' during the freezing and primary drying lyophilisation stages prevents cake collapse, melt back, and preserves the structural integrity of the lyophilised formulation (32,33). An exothermic event corresponding to crystallisation were observed during cooling related to ice crystallisation and a corresponding endothermic ice melting event on heating. Figure 1 illustrates the relationship between formulation composition and T_g' .

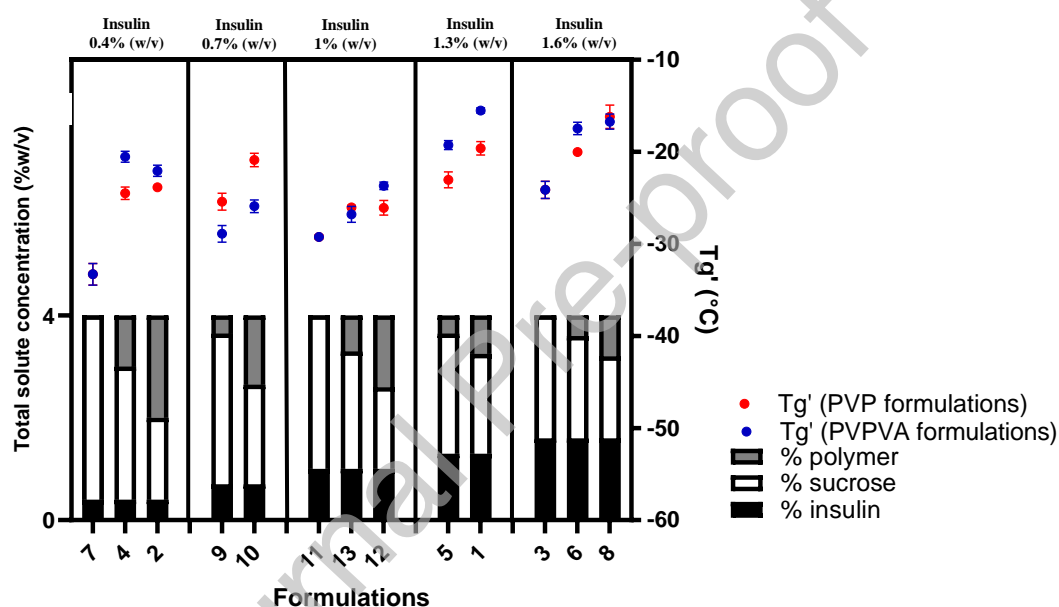


Fig. 1 Effect of formulation composition and polymer type (PVP vs PVPVA) on T_g' . Formulations are grouped by insulin concentration and ordered by increasing polymer content within each group. Formulation numbers refer to number assigned in DOE (Table 1), Bars show the percentage composition of each formulation: red and blue dots represent the measured T_g' values for PVP and PVPVA formulations, respectively ($n = 3$).

The lowest T_g' value (-33.3 ± 1.2) was noted for formulation #7, composed of the lowest concentration of insulin 0.4%w/v, with the highest concentration of sucrose 3.6%w/v and no polymer. Formulations with equivalent insulin concentration exhibited a clear increase in T_g' with polymer increase. This indicates that at fixed insulin content, the polymer component plays a significant role in enhancing the thermal stability of the frozen matrix. This effect was attributed to the polymer's anti-plasticizing properties, restricting molecular mobility and increasing T_g' . PVP and PVPVA both demonstrated this positive effect in T_g' . However, the

magnitude of this effect was composition dependent. Similar behaviour was previously observed for disaccharide-polymer systems (29). The T_g ' values obtained informed lyophilisation cycle design (section 2.2.2).

3.2. Impact of formulation composition on T_g

Analysis of DSC thermograms revealed T_g events for all formulations indicating the presence of an amorphous phase. The T_g values of individual lyophilised formulations are detailed in Table 1. T_g values ranged from 54°C to 106°C, indicating significant variations depending on the formulation composition. Phase separation was also noted from the thermograms of both PVP- and PVPVA-containing formulations #4, #7 and #11. A sucrose endothermic melting peak was noted around 180°C as shown in Figure 2a. These formulations contained a high sucrose content, with low insulin, and no or a low polymer content, indicating the stabilising influence of polymer.

The highest T_g values were detected for PVPVA-containing formulations #1 and #8, which were in the region of that of PVPVA alone, 107–110°C (34,35) (Figure 2b). These high T_g values determined by DSC be caused by phase separation with the thermogram displaying the T_g the PVPVA component. It is acknowledged that DSC has inherent limitations in assessing miscibility and to determine phase separation higher-resolution techniques such as solid-state nuclear magnetic resonance are required (22). This behaviour was not noted for the corresponding PVP formulations. This can be explained by PVPVA's lower hydrogen bonding capacity due to the presence vinyl acetate compared to PVP. Previously, immiscibility of PVPVA and sucrose has been noted for binary solid dispersions (29).

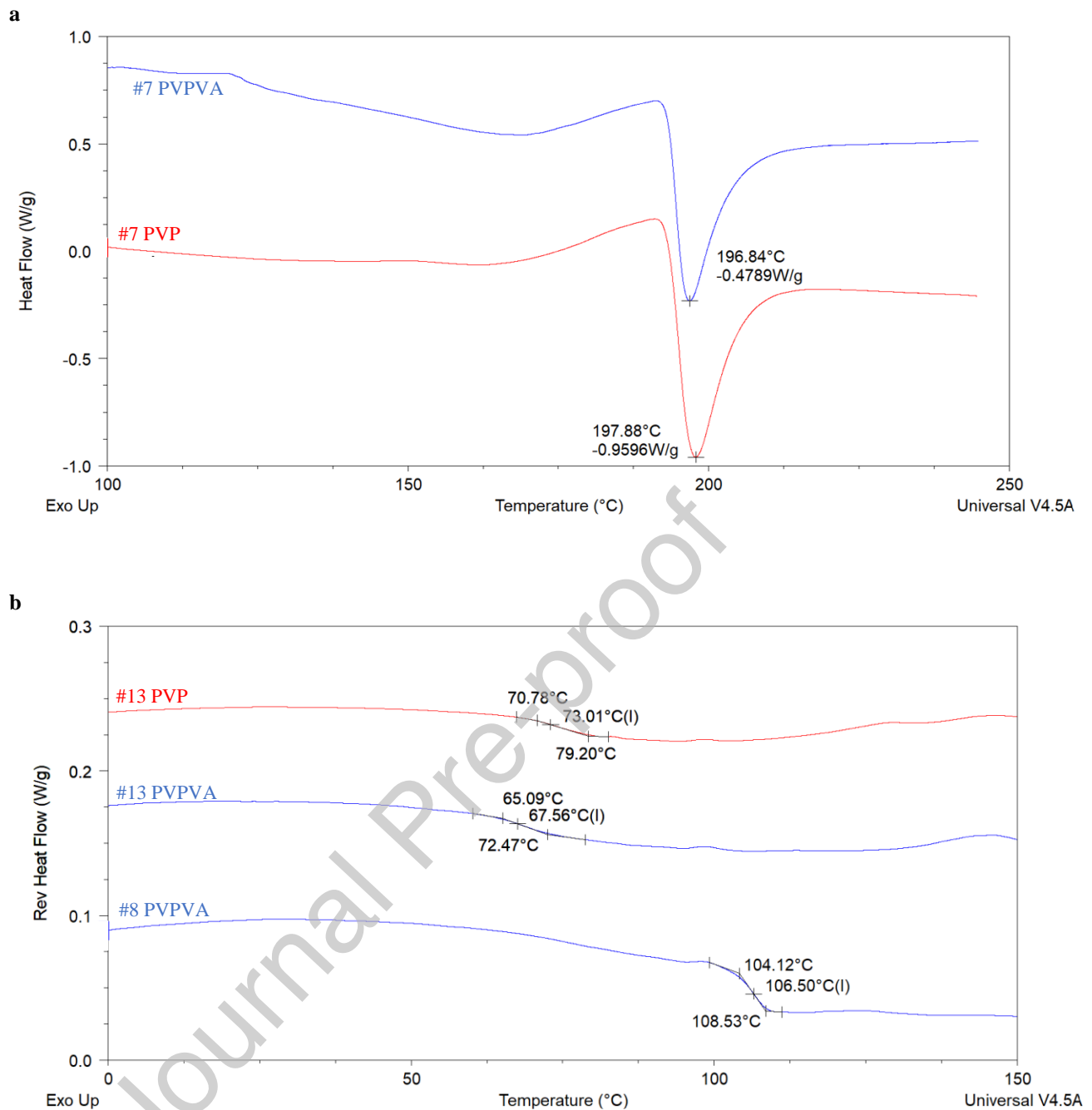


Fig. 2 Representative DSC thermograms showing (a) the sucrose melting endotherm observed for formulations #7 containing 10% w/w insulin, 90% w/w sucrose and no polymer and (b) the T_g event for formulation #13 which represents an example of homogenous system, while formulation #8 illustrates a PVPVA system with a high T_g .

As water content has been reported to reduce T_g by plasticizing amorphous matrices (36), the relationship between formulation residual water content and T_g was investigated, Figure 3. No consistent trend was observed between T_g and residual water content.

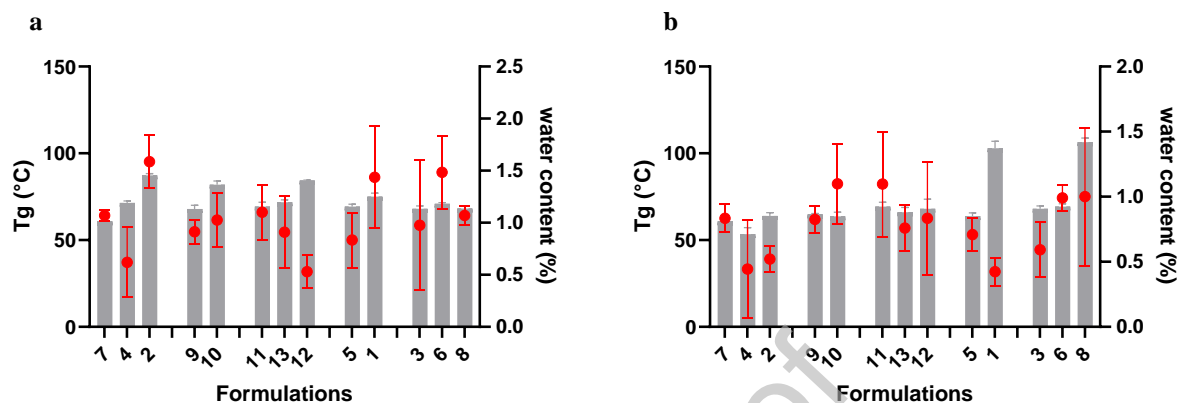


Fig. 3 Relationship between glass transition temperature (T_g) and residual water content (%) of lyophilised samples. Formulations are grouped by insulin concentration and ordered by increasing polymer content within each group. T_g values (grey bars, left y-axis) are compared with corresponding water content values (red dots, right y-axis). (a) Insulin-sucrose-PVP formulations and (b) Insulin-sucrose-PVPVA formulations.

To gain a deeper understanding of the relationships between individual formulation composition and T_g , statistical regression analysis was performed. Several regression models were evaluated. While higher-order models such as the full cubic and quartic showed slightly improved fit statistics (e.g., $R^2 > 87\%$), they introduced substantial multicollinearity ($VIFs > 10^5$), unstable coefficients, and overfitting, making them unsuitable for meaningful interpretation of individual component effects (Table S2 Supplementary Materials). Of the models evaluated, a “quadratic model” proved the most suitable model for analysing the contribution of formulation components to system T_g , for the PVP-containing formulations. The quadratic model provided a strong fit ($R^2 = 91.56\%$), with an adjusted R^2 of 90.28% and a predicted R^2 of 88.09%. The standard error of the regression (S) was 2.32°C, indicating good predictive performance. Table 2 lists the quadratic model regression coefficients.

Table 2. Regression model coefficients for the quadratic model describing the relationship between the composition of PVP-containing formulations and T_g .

PVP-formulations	
Term	Coef.
Insulin	-12.03
Sucrose	12.14
PVP	35.11
Insulin*Sucrose	14.95
Insulin*PVP	7.04
Sucrose*PVP	-3.56

Considering the main effects, insulin exhibited a negative impact, with a negative coefficient indicating that increasing insulin content significantly lowered T_g . In contrast, sucrose and PVP had positive effects. These findings align with the well-established roles of sucrose and polymers as stabilizers enhancing T_g by promoting glass formation and reducing molecular mobility (37). The model also revealed statistically significant interactions. Positive interactions between insulin-sucrose, and insulin-PVP, suggest that both components offset the negative effect of insulin on T_g . The sucrose-polymer interaction coefficient was smaller and negative.

For PVPVA-containing formulations, none of the regression models evaluated proved suitable for meaningful interpretation of individual component effects. A special cubic model provided a best scientific interpretability but low goodness-of-fit ($R^2 = 58.79\%$, adjusted $R^2 = 51.07\%$, predictive $R^2 = 42.46\%$, $S = 10.77$). The lower goodness-of-fit of regression models to PVPVA-containing formulation data may reflect the phase separation noted from the amorphous phase separation noted for formulations #1 and #8 highlighted above.

To visualize the relationship between PVP-containing formulations composition and T_g , a contour plot representing the regression model was generated, Figure 4. Notably, higher T_g values (darkest green regions) are concentrated in areas with elevated PVP content. Conversely, formulations with higher proportions of sucrose or insulin trend toward lower T_g values, depicted by the lighter green colour near those compositional vertices. Intermediate blend ratios exhibit moderate T_g values $> 70^\circ\text{C}$, revealing synergistic effects between components. The results indicate for insulin-sucrose formulations, the addition of PVP enhances T_g .

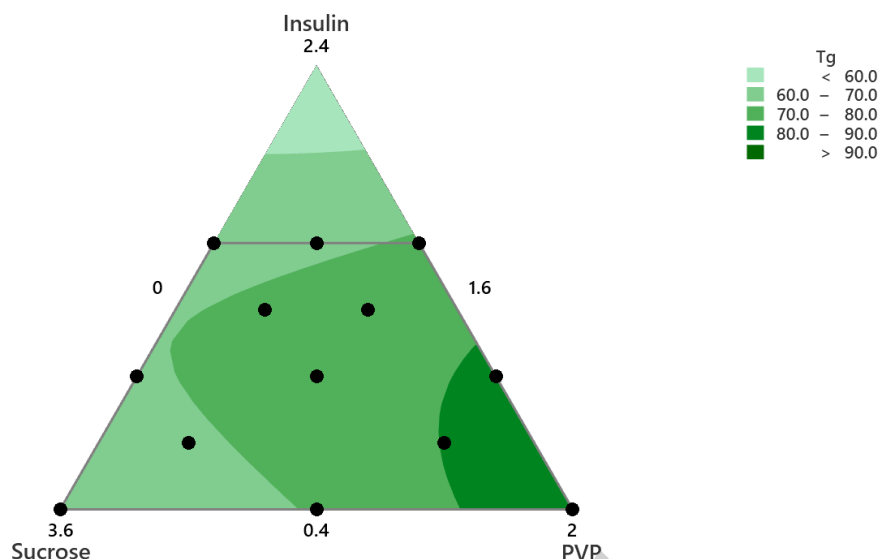


Fig.4 Contour plot representing the quadratic model of PVP-containing formulations. The plot shows predicted response values across varying proportions of the ternary mixture components. Black dots indicate formulation compositions used to establish the model. The colour gradient from dark green to light green indicate a decrease in T_g values across the composition range.

3.3. Impact of formulation composition on RH_g

To evaluate the lyophilised formulation glass stability with respect to humidity exposure during storage or handling environments, the lyophilised formulations were exposed to a ramping humidity profile; 0% to 90% RH at 10% RH per hour, at 25°C. RH_g , the humidity transition point at which absorbed water acts as a plasticizer causing an increase in absorption behaviour, was determined. The RH_g values for all formulations are summarized in Table 1, and representative individual moisture sorption plots are shown in Figure S2 Supplementary Materials. RH_g values ranged from a low humidity level of approx. 24% RH for formulation #7 composed of the lowest concentration of insulin 10%w/w, highest concentration of sucrose 90%w/w and no polymer, to 65% RH for formulation #8 with 40% insulin and sucrose and 20% PVP.

To interrogate the influence of individual formulation components and their interactions on RH_g , a number of regression models were explored to identify a model which balanced goodness-of-fit with interpretability (data shown in Table S3, in Supplementary Materials). A quadratic model was selected as it provided a balance between predictive accuracy and interpretability for both the PVP and PVPVA-containing formulations. The models' goodness-of-fit for the PVP-containing formulations was a R^2 of 90.31% and an adjusted R^2 of 83.40%,

and for PVPVA-containing formulations R^2 of 81.27% and an adjusted R^2 of 67.90%. The regression coefficients for each quadratic model are listed in Table 3. In terms of main effects, insulin exhibited a strong negative coefficient for both PVP and PVPVA-containing formulations. Sucrose and polymer, on the other hand, had relatively minor effects on RH_g . Interaction terms showed positive effects on RH_g . The insulin-sucrose interaction was strongly positive. Similarly, the interaction between insulin and polymer was positive. The sucrose-polymer interactions were also positive but less influential compared to the insulin-related interactions.

Table 3. Regression model coefficients for the quadratic models describing the relationship between the formulation composition and RH_g

PVP-formulation		PVPVA-formulation	
Term	Coef.	Term	Coef.
Insulin	-35.93	Insulin	-30.45
Sucrose	-1.41	Sucrose	-0.14
PVP	1.01	PVPVA	-3.02
Insulin*Sucrose	32.29	Insulin*Sucrose	28.96
Insulin*PVP	19.92	Insulin*PVPVA	17.46
Sucrose*PVP	7.54	Sucrose*PVPVA	9.49

Contour plots representing the quadratic models for both polymer systems facilitate visualisation of the relationship between the formulation composition and RH_g values, Figure 5. For both PVP and PVPVA-containing formulations, the RH_g values vary smoothly across the design space, with the highest RH_g values ($> 60\%RH$) generally located in the central insulin-rich regions, and the lowest values ($<30\% RH$) occurring near the sucrose-rich vertex. Despite these shared features, the contour plots reveal minor differences in the size and location of the high- RH_g regions. However, these differences may reflect prediction error due to model fit, as it should be noted that the measured RH_g values were similar for corresponding formulations for both polymers (Table 1).

a

b

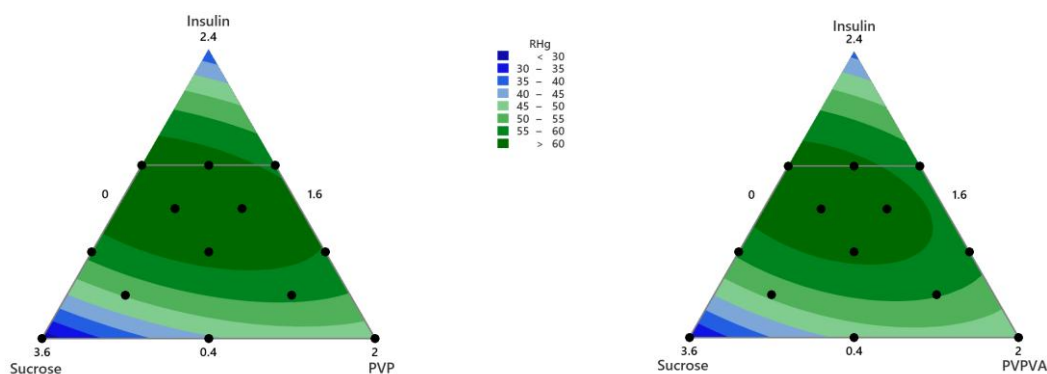


Fig.5 Contour plots representing the quadratic models of a) PVP-containing formulations and b) PVPVA-containing formulations. The plots show predicted response values across varying proportions of the ternary mixture components. Black dots indicate formulation compositions used to establish the model. The colour gradient from dark green to light green indicate a decrease in RH_g values across the composition range.

3.4. Selection of the optimum formulation based on T_g and RH_g responses

Mixture response optimization was conducted using Minitab software to identify the optimal composition of insulin, sucrose, and PVP that simultaneously maximized both T_g and RH_g values. Due to the inability to obtain a suitable regression model for T_g with both goodness-of-fit and interpretability, optimisation in terms of T_g and RH_g was not possible for PVPVA-containing formulations. The optimised PVP-containing formulation identified consisted of 26% w/w insulin, 40% w/w sucrose, and 34% w/w PVP, yielding a predicted T_g of 82°C and RH_g of 60% RH values. The response optimization plot, Figure 6, illustrates component levels where the formulation responses (T_g and RH_g) are optimised, maximised, and fall outside the optimal range.

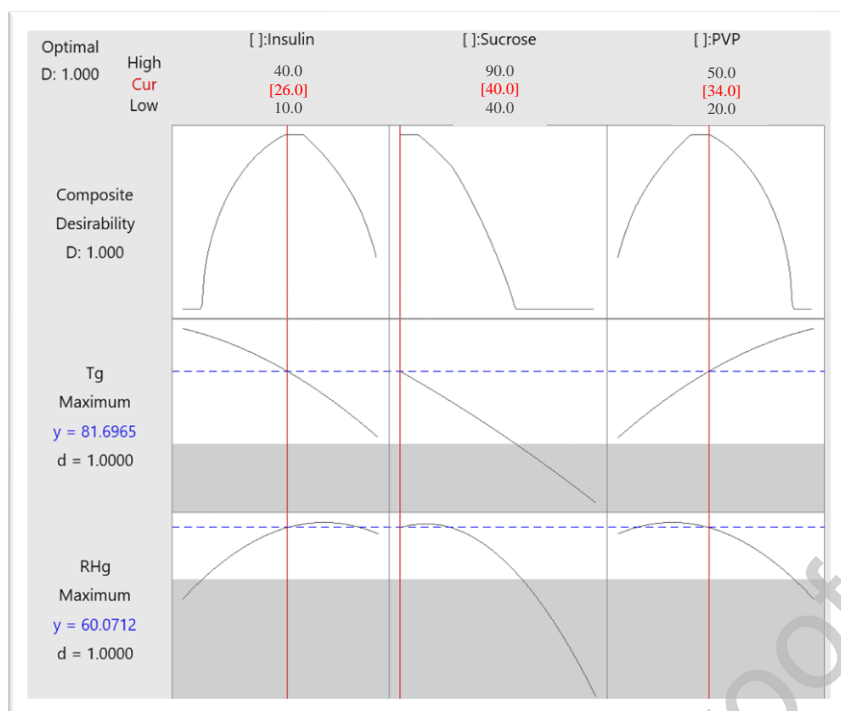


Fig. 6 Response optimization plot of insulin-sucrose-PVP formulations. The grey area represents regions where the formulation's responses (T_g and RH_g) fall outside the optimal range. The red vertical lines mark the optimal concentrations of insulin, sucrose, and polymer which are also indicated in red font. The blue dashed horizontal lines represent the theoretical maximum values for T_g and RH_g , which are also indicated in blue font. Black curves show how the increase of individual excipients affects the response.

The response optimization plot shows increasing insulin concentration beyond the optimum level would further reduce T_g , with minimal increase for RH_g . Increasing sucrose content would decrease both T_g and RH_g . This can be attributed to its plasticization effects, hygroscopic nature and moisture-binding capacity (38). PVP had a positive impact on T_g , increasing thermal stability up to the identified optimal level. However, for RH_g an opposite trend was noted. Above the optimum PVP level RH_g reduced possibly due to its hygroscopicity (29) and water uptake disrupting hydrogen bonding networks.

4. DISCUSSION

The focus of this study was to develop a lyophilised peptide formulation with optimised amorphous glass stability using a mixture DOE approach. Polymers (PVP and PVPVA) were added to formulations of a model peptide (insulin) and cryo- and lyo-protectant (sucrose) to increase glass thermal stability (indicated by T_g) and mitigate against the plasticisation effects

of environmental humidity (indicated by RH_g). The study results demonstrate that inclusion of both polymers increased glass stability with respect to temperature and humidity exposure.

All formulations investigated exhibited T_g values greater than ambient storage temperature (Table 1). Storage at temperatures below the T_g offsets the thermodynamic instability of the amorphous state by significantly reducing diffusive molecular mobility, providing kinetic stability against nucleation and crystal growth (39,40). It is generally considered that the greater the difference between T_g and storage temperature, the greater the reduction molecular mobility and the greater glass stability. At T_g-50K, diffusive α relaxation times for most organic molecules are in the order of years (41). A number of the PVP and PVPVA-containing formulations had T_g values in excess of 75°C, indicating good glass stability with respect to temperature. The optimised PVP formulation, containing > 25% insulin, had predicted T_g values > 90°C indicating good glass stability under dry conditions as the T_g values are > 50°C above the ICH Q1 recommended long-term storage temperature for ambient products, 25°C (42).

A basic hypothesis of the formulation approach employed, was that including a high-T_g polymer would increase the formulation T_g. Assuming a homogeneous amorphous system, T_g values can be predicted based on the additive effects of component T_g values and weight fraction, as described by models such as the Gordon Taylor equation (43). The reported T_g of PVP K30 is 149–166°C (34,44), PVPVA is 107–110°C (34,35), and sucrose is 60–70°C (45). The T_g of protein is difficult to determine due to degradation events in the region of its T_g. However, a T_g value of approximately 140°C has been reported based on the extrapolation of thermal analysis data from protein:disaccharide mixtures (38,39). Based on the T_g values of the individual components, the T_g of formulations investigated should be > 60°C due to the anti-plasticising effects of insulin and polymer. However, the T_g regression model for PVP-containing formulations showed insulin to have a negative effect on T_g, despite its high theoretical T_g. Also, the T_g of insulin-sucrose systems without polymer (formulations #3, #7, #11) T_g did not exceed 70°C. These observations suggest that factors other than the theoretical T_g of insulin may dominate its contribution to measured T_g in these amorphous matrices. One possibility is the pre-T_g endothermic event reported for many proteins between 40–80°C, referred to as the dynamical temperature (Td). The Td has been shown to mark a threshold above which increased internal protein mobility occurs via local residue fluctuations (48,49). While our data cannot confirm this mechanism, the proximity of Td to the measured T_g range raises the possibility that protein-specific mobility transitions could contribute to the apparent

negative coefficient. Alternative explanations, such as insulin-induced disruption of excipient hydrogen-bond networks or localised plasticisation effects, also remain plausible. We therefore interpret the regression result empirically and highlight that further molecular-level characterisation would be required to resolve the underlying cause.

Glass stability of multicomponent amorphous systems is not solely reliant on T_g , as molecular interactions can also inhibit nucleation and hence augment glass stability. The strong positive insulin-PVP interactive effect can be attributed to hydrogen bonding between PVP's pyrrolidone groups and insulin's peptide backbone, restricting molecular mobility and enhancing glass stability. The positive insulin-sucrose interaction implies that sucrose mitigates insulin's destabilizing influence, potentially through the formation of a protective glass network that reduces internal protein motions and enhances stability (50). The reduced capacity of PVPVA to hydrogen bond with sucrose and insulin compared to PVP would explain the possible phase separation observed for some insulin-sucrose-PVPVA formulations. Attempts to probe component miscibility, molecular interactions and phase separation utilizing FTIR spectroscopy were attempted. However, spectral complexity and overlapping component bands hindered the resolution of specific molecular interactions. Further, investigations employing solid-state NMR are warranted to provide greater insights into competing intermolecular interactions.

Residual water content and absorption are key factors that can undermine the stability of amorphous glass formulations exposed to humidity. The plasticisation effect of water in multicomponent amorphous formulations is complex and can be related to both individual component hygroscopicity and competing water-component and component-component interactions (22) and structural features such as surface area (20). This complexity may explain the lack of relationship between the residual water content and formulation T_g , Figure 1. Exposing formulations to a humidity ramp under isothermal conditions was employed to evaluate the sensitivity of formulation glass stability to humidity. The parameter used to screen formulation stability, RH_g , represents the humidity level at which the absorbed water acts as a plasticizer, causing an increase in water sorption due to increased material mobility (20). RH_g values ranged from 23% to 65% RH (Table 1), reflecting the significant influence of formulation composition. The contribution of differences in surface area between samples could also be a contributing factor and it is a limitation of this study that the surface area of samples was not determined due to experimental constraints. Despite the initial hypothesis that the inherent reduced hygroscopicity of PVPVA compared to PVP (51) would enhance stability

with respect to humidity, similar RH_g values were determined for both formulation sets, Table 1. Insulin content was shown to have the strongest negative effect on RH_g , suggesting that water disrupts intermolecular interactions between insulin molecules, causing an increase in free volume, molecular mobility and decrease packing density, observed by a resultant increase water uptake at the RH_g . Conversely, component-component interactions were shown to have a positive effect on RH_g , suggesting that these interactions can mitigate against water-induced free volume increases, by competing with water-component interactions, and offset the increase in free volume and molecular mobility to higher humidity levels, indicated by the increase in RH_g .

Increased formulation RH_g would indicate increased formulation amorphous glass stability when exposed to humid conditions (52,53). The optimised formulation containing > 25% w/w insulin had a predicted RH_g of 60% RH. However, stability during long-term exposure may be undermined given its proximity to the upper humidity levels during recommended manufacturing of 55% RH (27) and recommended long-term storage humidity level of 60% RH (42). Also, it should be noted that the RH_g values were determined under non-equilibrium conditions as a screening approach in this study. To determine the glass stability with respect to specific humidity levels, isothermal equilibrium moisture sorption studies would be required;

The study findings demonstrate good formulation glass stability and potential for enhanced glass stability during the manufacture of solid oral dosage forms and patient handling prior to administration. Further studies are warranted to evaluate insulin chemical stability in these lyophilized formulations and to establish their suitability for solid oral dosage forms manufacture.

5. CONCLUSION

Ternary insulin–sucrose–polymer formulations were identified as a strategy to enhance the glass stability when exposed to processing and handling temperatures and humidity. T_g and RH_g parameters were determined as indicators of formulation glass stability with respect to temperature and humidity. A mixture DOE approach enabled the identification of insulin, sucrose and polymer main and interactive effects on formulation T_g and RH_g values. Insulin content was shown to have negative effects on both T_g and RH_g . Formulation T_g appeared to be influenced by insulin's dynamical temperature rather than a previously reported insulin T_g . Insulin-sucrose and insulin-polymer interactive effects were shown to enhance formulation

glass stability. An optimized lyophilized formulation composed of 26% w/w insulin, 40% w/w sucrose and 34% w/w PVP predicted T_g values of 82°C and RH_g of 60% RH. The ternary formulations' enhanced glass stability offers potential advantages for the manufacture, storage and handling of peptide containing oral dosage forms and warrants further evaluation.

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Graphical Abstract

