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Investigating the interactions of amino acid components on a mannitol-based spray-dried powder formulation for pulmonary delivery: A design of experiment approach

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ABSTRACT

Combining an amino acid and a sugar is a known strategy in the formulation of spray or freeze dried biomolecule powder formulations. The effect of the amino acid leucine in enhancing performance of spray-dried powders has been previously demonstrated, but interaction effects of several constituents which may provide multiple benefits, are less well-understood. A 3 factor 2 level (2^3) factorial design was used to study the effects of leucine, glycine and alanine in a mannitol-based dry powder formulation on particle size, aerosolisation, emitted dose and cohesion. Other qualitative tests including scanning electronic microscopy and X-ray powder diffraction were also conducted on the design of experiment (DoE) trials. The results show that the use of glycine and/or alanine, though structurally related to leucine, did not achieve similar aerosol performance enhancing effects, rather the particle formation was hindered. However, when used in appropriate concentrations with leucine, the combination of amino acids produced an enhanced performance regardless of the presence of glycine and/or alanine, yielding significantly modified particle properties. The results from the DoE analyses also revealed the lack of linearity of effects for certain responses with a significant curvature in the model which would otherwise not be discovered using a trial-and-error approach.

1. Introduction

Dry powder inhalation has been an attractive delivery method for pulmonary drug administration due to its many advantages: these include ease of administration, convenient portability, relatively simple formulation, low cost and inherent solid state stability, especially for proteins and peptides (Carpenter et al., 1997; Prime et al., 1997; Rave et al., 2004). Dry powder formulations must be readily dispersible and aerosolisable upon inhalation for efficient delivery. However, inter-particulate cohesive forces are particularly dominant in the finely micronised powders (i.e. 1–5 μm) required for pulmonary delivery (Forsyth et al., 2001). These forces influence flowability and de-agglomeration of micronised dry powders and therefore the aerosolisation properties and delivery efficiency of dry powder formulations (Ashurst et al., 2000; Prime et al., 1997).

Spray-drying has gained increased interest for engineering suitable small particles due to its simplicity, adaptability, cost-effectiveness and scalability (Fourie et al., 2008). Spray-drying is a process in which the compound(s) of interest are first prepared in a liquid form, which is then atomised into a drying chamber in which the droplets are dried with heated air. Particle formation is achieved by precipitation of the dissolved compounds as the solvent evaporates from the solution droplets in the drying chamber. The ability to incorporate various ingredients in a single step manufacturing process is a powerful strength of spray-drying. Particles can be engineered to contain various ingredients by adjusting the content of the feed solution. Excipients can therefore be incorporated which may, in principle, manipulate the properties of the dry powder formulation. For example, the amino acid glycine has been added to the sugar mannitol to modify the particle precipitation process, with the benefit of increasing its resulting solid state particle glass transition temperature (T_g) (Sadrzadeh et al., 2010; Schule et al., 2008).

Many excipients have also been considered to improve the aerosolisation properties and performance of inhalable dry powder formulations (Li et al., 2003, 2005a; Maa and Prestrelski, 2000; Rabbani and Seville, 2005; Staniforth et al., 2002). Leucine has been demonstrated to improve aerosolisation and performance of dry powder inhaler formulations and more specifically the inclusion of leucine as an additive in a precursor solution for spray-drying has been shown to improve the aerosolisation of the resulting powders (Kamlag et al., 2004; Li et al., 2003, 2005b; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007). However, this dispersibility

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enhancing property appears to be specific to leucine and is not necessarily the case with other amino acids (Chew et al., 2005; Minne et al., 2008; Seville et al., 2007). While effects produced from the use of a single amino acid on the performance of a dry powder formulation is more extensively studied, the specific interaction effects resulting from the combined use of different amino acids is not well understood. Such interactions may allow or prevent multiple powder functionalities being achieved.

Several studies investigating amino acids as spray-drying additives used concentrations based on mass ratios (Chew et al., 2005; Minne et al., 2008; Seville et al., 2007; Shur et al., 2008). However, it is the interactions between the amino acids and other formulation components at the molecular level that eventually determines the behaviour of the final formulation. The extent of molecular interactions may hence relate to the number of molecules, as opposed to mass, which does not provide the same number of molecules of each component for comparability. Compounds with a lower molecular mass obviously contain more molecules than larger compounds in the same mass. Molar concentrations were therefore used in the present study instead of mass ratio in order to investigate and compare the effects achieved with the same number of molecules of glycine, alanine and leucine.

Studying the effect of several formulations with multiple excipients in different compositions by trial-and-error or 'changing one separate factor at a time' (COST) approaches are often inefficient as the results from these experiments do not allow the identification of interaction effects between formulation ingredients (Naelapää et al., 2010). A previous study investigating the effect of leucine, glycine and alanine on the performance of dry powder formulations did not provide information on the combination use of these excipients (Minne et al., 2008). Recently, the design of experiment (DoE) approach has been successfully used to optimise spray-drying process conditions by identifying combination of parameters, including drying temperature, airflow rate, pump setting, aspiration setting, feed concentration and solution feed rate, that produced formulations with the best performance (Baldinger et al., 2011; Tajber et al., 2009). The DoE approach was therefore used in the present study to screen in a systematic manner a range of formulations, however, in this case with a focus on various compositions. The study utilised a 2^3 factorial design to investigate the effect of the three amino acids in various compositions.

In the current study, three amino acids, glycine, alanine and leucine were selected specifically with increasing hydrocarbon chain lengths respectively, in order to control specific properties of a mannitol-based dry powder formulation. It was proposed that the hydrocarbon chain length may influence the mass transport and self-assembly of each amino acid upon droplet drying. The subsequent influence of these amino acids on the physical properties of the spray-dried particles including particle size distribution, dispersibility, aerosolisation properties, inter-particle interaction, surface morphology and crystallinity were therefore investigated. The study aims to screen a range of formulations using a DoE approach to determine the potential utility of these amino acids as performance enhancing excipients for inhalable dry powder formulations. To the best of our knowledge, the effects produced from the combination use of these or similar additives on a spray-dried powder formulation for pulmonary delivery have not previously been investigated.

2. Materials and methods

2.1. Materials

D-Mannitol was obtained from VWR International Ltd. (Poole, BH15 1TD, England). L-Leucine (LEU), glycine (GLY) and L-alanine (ALA) were obtained from Sigma–Aldrich Chemicals (Castle Hill, NSW, Australia).

Table 1
2^3 Full factorial experimental design: constants, variables and responses.

Factors	Levels of factors used in the formulation		
	−1	0	+1
X_1 = leucine (molar%)	0	15	30
X_2 = glycine (molar%)	0	15	30
X_3 = alanine (molar%)	0	15	30

Responses	Process and formulation parameters kept constant
Y_1 = Mastersizer D_{50} (μm)	Mannitol content: 5 g
Y_2 = Spraytec D_{50} (μm)	Feed solution volume: 200 mL
Y_3 = Spraytec ED (mg)	Aspirator setting: 20
Y_4 = TSI fine particle fraction (%)	Pump setting: 5 (6.67 mL/min)
Y_5 = TSI ED (%)	Airflow: 800 L/h
Y_6 = cohesion value (kPa)	Outlet temperature: 75 °C

Abbreviations: ED, emitted dose; SD, spray-drying; TSI, twin-stage impinger.

2.2. Preparation of spray-dried powders

Aqueous solutions containing mannitol and selected amino acids (LEU, GLY, ALA) in various compositions as shown in Table 1 were dissolved in 200 mL of Milli-Q water. A small amount of methylene blue dye (10 mg) was incorporated in each formulation to allow a simple quantification of powder by UV–vis spectrophotometric analysis as described below. The prepared formulations were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow rate, 800 L/h; pump setting, 5 (6.67 mL/min); aspirator setting, 20; outlet temperature, 75 °C.

2.3. Powder characterisation

2.3.1. Particle size distribution analysis

The particle size distribution of the powders was determined by laser-light scattering using the Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) equipped with a Scirocco cell and a Scirocco 2000 dry powder dispersion unit. The powders were dispersed in air at a shear pressure of 3.0–4.0 bar, which was selected to achieve suitable de-agglomeration. The average particle size was measured in three replicates for each sample. The volume median diameter (D_{50}) was derived from the diffraction data using the in-built software for each sample.

2.3.2. Powder dispersibility by laser diffraction

A real-time laser-light diffraction particle sizer (Spraytec, Malvern Instruments Ltd, Worcestershire, UK) was used to determine the *in situ* aerosol particle size distribution. The powders were measured with the inhalation cell attachment at a flow rate of 60 L/min using the Monodose inhaler (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was controlled using a Critical Flow Controller Model TPK 2000 & Flow meter model DFM 2000 (Copley Scientific Limited, Nottingham, UK). Approximately 20 mg of each powder was filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed over 5 s at an air-conditioned laboratory (20 ± 2 °C, 50 ± 5% relative humidity). Emitted doses were measured by weighing the inhalers filled with capsules before and after the experiments. The measurements were performed in three replicates for each formulation (n = 3) in a random order to minimise the occurrence of any potential progressive error. The particle size distribution was derived from the laser diffraction data with the in-built software.

2.3.3. In vitro powder aerosolisation and particle deposition

The *in vitro* powder aerosolisation performance and particle deposition was assessed using a twin stage impinger (TSI, Apparatus, A; British Pharmacopoeia, 2000) with the Monodose inhaler (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was adjusted to 60 L/min using a Critical Flow Controller Model TPK 2000 & Flow meter model DFM 2000 (Copley Scientific Limited, Nottingham, UK). Approximately 20 mg of each powder was filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed at an air-conditioned laboratory (20 ± 2 °C, 50 ± 5% relative humidity). Each capsule was actuated from the inhaler over 4 s for each measurement (n = 5). The amount of powder deposited at different stages was determined using a UV–vis light spectrophotometer as described below. The cut-off diameter for the TSI at 60 L/min is approximately 6.3 μm (Hallworth and Westmoreland, 1987).

The total amount of powder deposited in the inhaler, stage 1 (S_1) and stage 2 (S_2) was the recovered dose (RD). The amount of powder deposited in stage 1 and 2 was the emitted dose (ED) and it was calculated as the percentage of the RD (Eq. (1)). The fine particle fraction (FPF) was defined as the percentage of RD deposited in stage 2 (Eq. (2)).

$$\text{ED\%} = \frac{(S_1 + S_2 \times 100)}{\text{RD}} \quad (1)$$

$$\text{FPF\%} = \frac{S_2 \times 100}{\text{RD}} \quad (2)$$

2.3.4. Inter-particulate cohesion by shear test

Inter-particulate cohesion in the powder samples was characterised by the Freeman FT4 powder rheometer (Freeman Technology, Worcestershire, UK) using its shear cell module configuration as previously described (Zhou et al., 2010). In brief, a shear head was attached to the module driver and shear stresses were measured against a series of normal stress exerted of 7, 6, 5, 4 and 3 kPa. A consolidation stress of 9 kPa was applied to the powder bed prior to each measurement. The shear stress under each normal stress was recorded from which the yield loci were obtained. The cohesion value of each formulation was extrapolated from the yield loci as the shear stress at zero normal stress. The measurements were performed at room temperature and humidity (20 ± 2 °C, 50 ± 5% relative humidity). A higher cohesion value represents higher inter-particulate forces and thus a more cohesive powder.

2.3.5. Scanning-electronic microscopy (SEM)

The morphology of the particles was visualised under a scanning electron microscope (Phanom™, FEI company, USA). Powder samples were gently poured onto a double-sided carbon tape mounted on a sample holder for examination under the SEM. Excessive powder was removed to leave a fine layer of particles on the surface of the tape. The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25 mA for 6 min with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the inbuilt image capturing software.

2.3.6. X-ray powder diffraction (XRPD)

Sample powders were sprinkled onto a quartz sample plate smeared with a small amount of Vaseline at room temperature. The sample was then analysed by the X-ray diffractometer (Philips 1140 vertical diffractometer, Philips, Holland) for scanning from 2° to 60°, with an angular increment of 2°/min. The crystalline status of the powders was qualitatively assessed from the diffraction patterns.

Table 2
Formulations as per the 2^3 full factorial experimental design.

Trial number	Formulation variable – X_1 (leucine, molar%)	Formulation variable – X_1 (glycine, molar%)	Formulation variable – X_1 (alanine, molar%)
T1	−1	−1	−1
T2	+1	−1	−1
T3	−1	+1	−1
T4	+1	+1	−1
T5	−1	−1	+1
T6	+1	−1	+1
T7	−1	+1	+1
T8	+1	+1	+1
CP	0	0	0

Abbreviations: CP, centre point; T, trials.

2.3.7. UV–vis spectrophotometric analysis

A UV–vis light spectrophotometer (Cary 3 Bio, Varian Instruments, Australia) was used to determine the amount of powder recovered from TSI studies using wavelength of 665 nm for detection of methylene blue. Calibration curve was generated for each formulation using linear regression over the range of 0.05–0.80 mg/mL using five concentrations. The regression coefficient (r^2) values were greater than 0.99 for all formulations demonstrating satisfactory linearity. The amount of powder deposited at each stage was determined from the calibration curve.

2.4. Experiment design—2^3 full factorial design

A 2^3 full factorial design (Design Expert, Version 7.1.3, Stat-Ease Inc., Minneapolis, MN) was used for conducting the experiments. The studied factors were: the amount of leucine (X_1, molar%), glycine (X_2, molar%) and alanine (X_3, molar%). The responses studied were Mastersizer D_{50} (μm, Y_1), Spraytec D_{50} (μm, Y_2), Spraytec ED (mg, Y_3), Twin-stage impinger fine particle fraction (%, Y_4), Twin-stage impinger ED (%, Y_5) and shear test cohesion value (kPa, Y_6). The levels of each variable were designated as −1, 0 and +1, respectively, and the corresponding actual values for each variable are listed in Tables 1 and 2. Other process and formulation parameters were kept constant in order to investigate exclusively the effect of the three amino acids on the response variables (Table 1). The influence of factors and their interactions, on each of the response are represented graphically (Fig. 1).

3. Results and discussion

3.1. Factorial design methodology and analysis

In the present study, the design of experiment methodology was employed to systematically evaluate the effect of varying the amount of glycine, alanine and leucine, as well as to identify any interaction among these excipients on the particle size distribution, dispersibility, aerosolisation and inter-particle interaction of the mannitol dry powder formulations. The summary of result data obtained of various responses is presented in Table 3. The DoE approach facilitated the identification of the most significant factors influencing the performance of the formulation.

A mathematical model was generated describing the relationship between the factors and responses for determining the levels of factors which yield optimum responses. For a 2^3 full factorial design, the following first order polynomial equation was fitted to the data:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3 \quad (3)$$

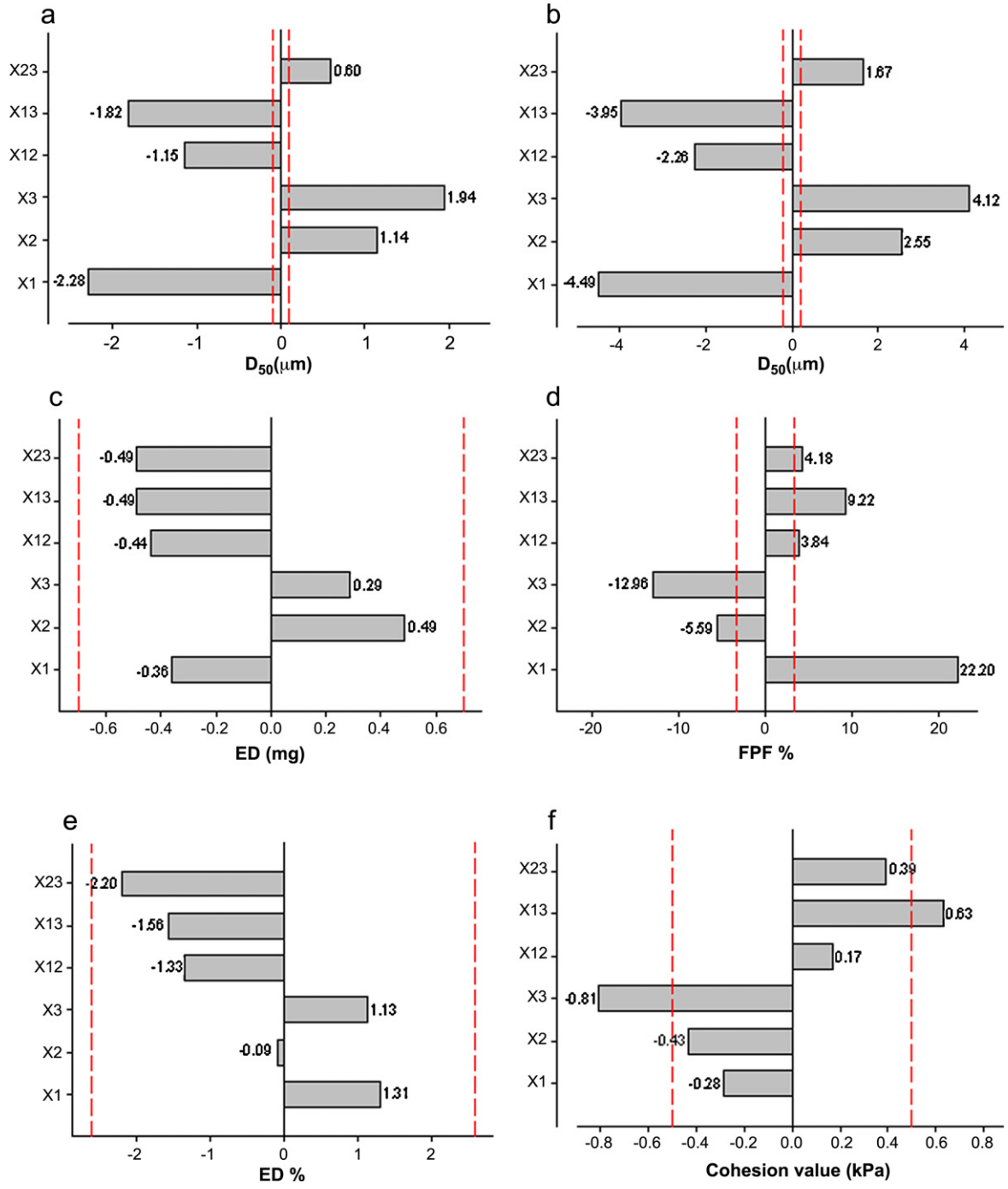

Fig. 1. Graphical representation of effect of factors on various responses (Y). (a) Mastersizer D_{50} (Y_1), (b) Spraytec D_{50} (Y_2), (c) Spraytec ED (Y_3), (d) fine particle fraction (Y_4), (e) Twin-stage impinger ED (Y_5) and (f) cohesion value (Y_6).

where b_0 is the intercept representing the arithmetic averages of all the quantitative responses of eight experimental runs; b_1–b_{123} are the regression coefficients computed from the observed experimental values (Y); and X_1, X_2 and X_3 are the coded levels of factors. The terms X_iX_j (i and j = 1, 2 and 3) represent the interaction terms. The equation represents the quantitative effect of factors (X_1, X_2 and X_3) upon each of the responses (Y_1–Y_6). Coefficients b_1–b_3 represent the effect of factor 1, 2 and 3 while the other coefficients represent the interaction between those factors.

Analysis of variance (ANOVA) was applied for estimating the significance. A p-value of less than 0.01 demonstrates the significance of the factor or the interaction (Table 4). In addition, graphical analysis of responses was performed as shown in Fig. 1. This analysis allowed the important factors for the considered responses to be identified and an estimated optimum factor level could be selected. The bar graphs were constructed in which the bars that exceed the two lines of limit of significance, calculated according to the experimental variance derived from the centre point results, correspond to the factors that are influential on the response. In particular, the influential factors are those where a level change triggers a response variation which is statistically different from the variation due to the experimental error (Pund et al., 2010).

Table 3
Result data of mean values of various responses: Mastersizer D_{50} (μm, Y_1), Spraytec D_{50} (μm, Y_2), Spraytec ED (mg, Y_3), fine particle fraction (%, Y_4), twin-stage impinger ED (%,Y_5) and cohesion value (kPa, Y_6).

Batch	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	0	0	0	1.87	2.83	15.80	66.20	78.04	4.53
2	30	0	0	1.75	2.70	17.50	80.10	91.11	2.21
3	0	30	0	3.75	5.52	19.20	34.62	89.59	2.39
4	30	30	0	2.05	3.50	18.00	72.62	88.00	1.04
5	0	0	30	6.69	12.02	18.90	9.11	92.47	0.71
6	30	0	30	2.27	3.24	17.50	68.64	89.99	1.21
7	0	30	30	13.97	28.55	19.20	2.96	85.90	0.43
8	30	30	30	1.97	3.58	17.20	69.13	87.40	1.32
9[a]	15	15	15	2.05	2.58	16.60	76.84	88.79	1.82
10[a]	15	15	15	1.95	2.49	17.40	76.40	86.97	1.79
11[a]	15	15	15	1.99	2.28	16.40	74.27	88.82	2.11
12[a]	15	15	15	n/a	2.40	17.0	n/a	n/a	1.32

Abbreviation: n/a, not available.
[a] Indicates the centre point of the design.

3.2. Particle size analysis

The volume median particle size (D_{50}) of all the formulations measured using Mastersizer 2000 are listed in Table 3. The regression equation for Mastersizer D_{50} is shown as Eq. (4). Spray drying mannitol alone produced small particles with D_{50} of 1.87 μm. This is not surprising as it contains the lowest solid loading. The addition of amino acids in all compositions, other than leucine only, increased the particle size of the mannitol formulation. However, relative changes in sizes did not follow a pattern of solid loading (Table 5). The presence of glycine and alanine, though incurring lower solid loadings owing to their lower molecular weights compared to leucine, increased particle size to the greatest extent as demonstrated by the largest D_{50} of the formulations containing glycine/alanine 30/30%, alanine 30% and glycine 30%. The significant effects of glycine and alanine in increasing D_{50} are evident as shown in graphical analysis (Fig. 1a). In contrast, leucine was the only amino acid that relatively reduced the D_{50} when added into the mannitol formulation, and all the formulations containing leucine have a D_{50} of <5 μm. The significant effect of leucine in reducing this particle size measure is evident from the highest negative value of coefficient of term X_1 (Eq. (1)) and longest bar length shown in graphical analysis (Fig. 1a).

Interestingly, the interaction terms X_1X_2, X_1X_3 and X_2X_3 all have a significant effect for the Mastersizer D_{50} (Fig. 1a). The term X_2X_3 indicates that the concurrent use of glycine and alanine increases D_{50} significantly in a synergistic manner. However, the negative influence of the terms X_1X_2 and X_1X_3 can be attributed to the initial increase of D_{50} associated with the addition of glycine and alanine, which in turn leads to a more pronounced apparent D_{50} reducing effect when leucine was present. This is consistent with the fact that although glycine and/or alanine increased particle size significantly when used alone, the addition of leucine appeared capable of overwriting the effect of glycine and alanine on D_{50} to produce smaller particles. The enhanced performance resulting from the combined use of these amino acids is demonstrated by the significant lack of fit from analysis of variance and shows the non-linearity effects of these amino acids within the study design space (Table 4). It should especially be noted that the results from the centre point formulations are highly consistent as demonstrated by the narrow experimental variance on graphical analysis (Fig. 1a).

$$Y_1 = 4.29 - 2.28X_1 + 1.14X_2 + 1.94X_3 - 1.14X_1X_2 - 1.82X_1X_3 + 0.60X_2X_3 - 0.75X_1X_2X_3 \quad (4)$$

where $p = 0.0001$ and $r^2 = 0.9168$.

The results are not only consistent with previous findings that leucine is an excipient that can be used to improve aerosolisation of spray-dried particles, but the results show that leucine also assists in the formation of suitable small-sized particles. However, glycine and alanine, although being structurally similar to leucine, do not achieve similar effects, but instead they significantly increase the particle size of the formulations. It is worth noting that while initial concentration in feed solution is a known determinant of particle size (Vehring, 2008), the range of solid loading used within the study design space did not appear to have a strong influence on geometric particle size as measured by laser diffraction. The total solid loading in the feed solution ranged from 2.50% to 3.72% in the present study (Table 5). It is proposed that the change in particle size within this relatively small range of solid loading was negligible compared to the effects of the formulation excipients on cohesion and shape. The 30 molar% ratio of leucine, glycine and alanine used in the present study amounts to mass ratios of 17.8%, 11.0% and 12.7% (w/w), respectively, in the final formulations. A previous study using 30% mass ratio of leucine or glycine or alanine as excipients in a spray-dried formulation produced particles with D_{50} within a particle size range of 4.0–4.7 μm (Minne et al., 2008). In contrast, the equivalent molar ratio of the three amino acids in the present study when used alone produced particles with very different D_{50} of 1.75, 3.75 and 6.69 μm from leucine, glycine and alanine, respectively. The scanning electronic microscope images discussed in Section 3.6 confirm that the latter two results show what appear to be fused particles, whereas the leucine formulation results in distinct spherical primary particles (Fig. 2). It is proposed that molar ratios, with respect to surface coverage, may be considered to be more appropriate than mass ratios as we propose that molecules of amino acids are assembling at the surface with their hydrocarbon chains aligning away from the interface in a manner reflecting molecules with surface active properties. This is discussed further in Section 3.3. Furthermore, considering the particle sizes produced from the mixed amino acids, it is indicated from this analysis that the combination use of these amino acids with leucine at

Table 4
A summary of *p*-values for coefficients of factor for response: Mastersizer D_{50} (μm, Y_1), Spraytec D_{50} (μm, Y_2), Spraytec ED (mg, Y_3), fine particle fraction (%, Y_4), twin-stage impinger ED (%,Y_5) and cohesion value (kPa, Y_6).

Coefficients	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
b_1	**<0.0001**	**<0.0001**	0.1038	**0.0005**	0.0277	0.0470
b_2	**0.0002**	**<0.0001**	0.0529	**0.0075**	0.8008	0.0124
b_3	**<0.0001**	**<0.0001**	0.1641	**0.0014**	0.0409	**0.0013**
b_1b_2	**0.0002**	**<0.0001**	0.0684	0.0156	0.0265	0.1659
b_1b_3	**<0.0001**	**<0.0001**	0.0529	**0.0028**	0.0175	**0.0033**
b_2b_3	**0.0009**	**<0.0001**	0.0529	0.0133	**0.0067**	0.0174
Lack of fit	**0.0002**	**<0.0001**	0.0297	**0.0013**	0.6485	0.8904

Significant effects of factors ($p < 0.01$) on individual responses are shown in bold type.

Table 5
Table of total solid loading (g) in each formulation versus Mastersizer D_{50} (μm).

Trial number	Amino acids (g)			Total solid loading (% w/v)	Mastersizer D_{50} (μm)
	Leucine	Glycine	Alanine		
T1	–	–	–	2.50%	1.87 ± 0.05
T2	1.08 g	–	–	3.04%	1.75 ± 0.01
T3	–	0.62 g	–	2.81%	3.75 ± 0.03
T4	1.08 g	0.62 g	–	3.35%	2.05 ± 0.04
T5	–	–	0.73 g	2.87%	6.69 ± 0.08
T6	1.08 g	–	0.73 g	3.41%	2.27 ± 0.04
T7	–	0.62 g	0.73 g	3.18%	13.97 ± 0.33
T8	1.08 g	0.62 g	0.73 g	3.72%	1.97 ± 0.02
CP1	0.54 g	0.31 g	0.37 g	3.11%	2.05 ± 0.02
CP2	0.54 g	0.31 g	0.37 g	3.11%	1.95 ± 0.04
CP3	0.54 g	0.31 g	0.37 g	3.11%	1.99 ± 0.01

Abbreviations: CP, centre point; T, trials. (Mean ± SD, $n = 3$).

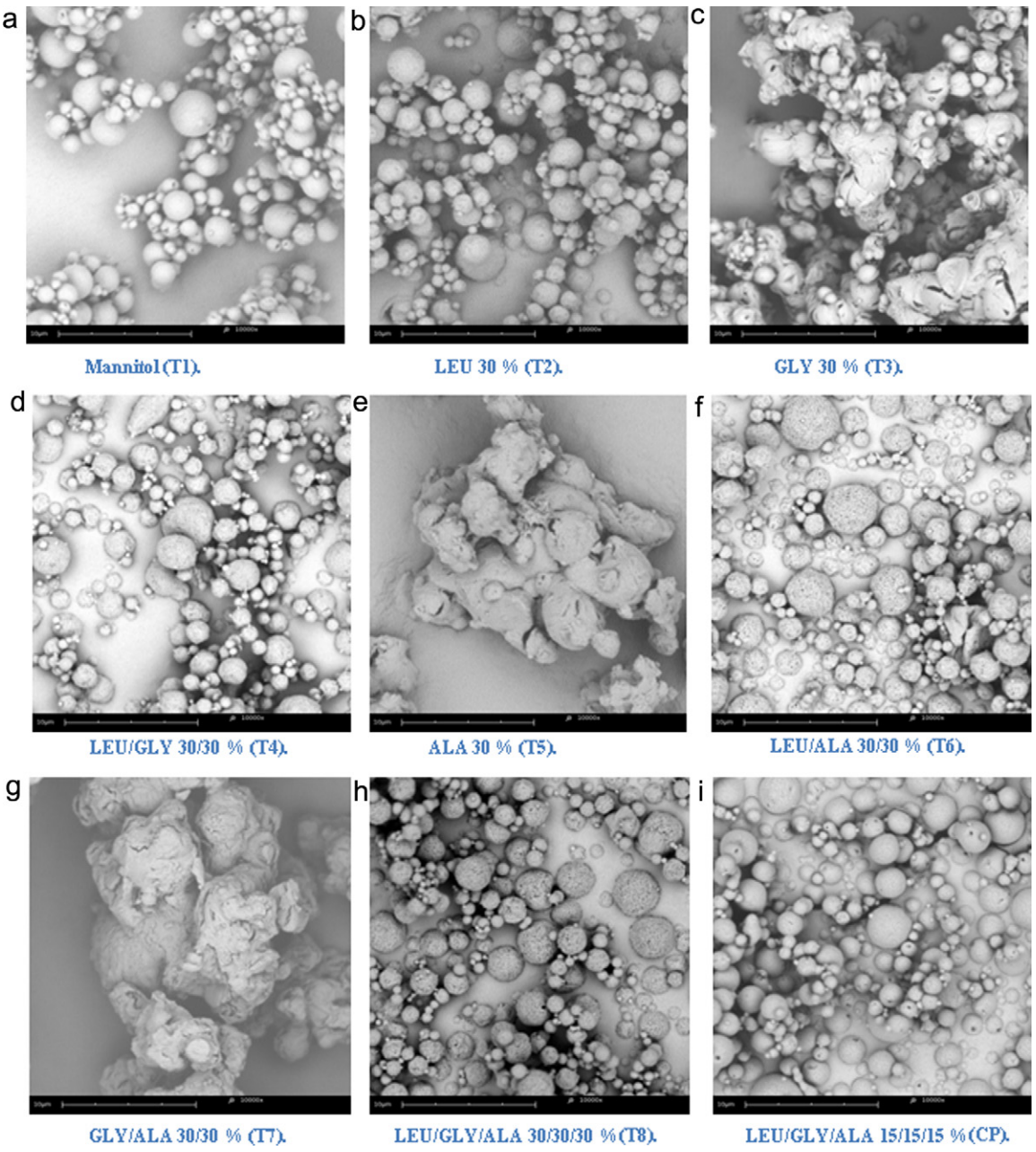

Fig. 2. Representative scanning electronic micrographs of the spray-dried mannitol formulations containing (a) mannitol (T1), (b) LEU 30% (T2), (c) GLY 30% (T3), (d) LEU/GLY 30/30% (T4), (e) ALA 30% (T5), (f) LEU/ALA 30/30% (T6), (g) GLY/ALA 30/30% (T7), (h) LEU/GLY/ALA 30/30/30% (T8) and (i) LEU/GLY/ALA 15/15/15% (CP) [magnification: 10,000×].

appropriate concentrations may also further influence particle size (and form) contrasting that achieved by leucine alone.

3.3. Powder dispersibility and de-agglomeration

The volume median diameters (D_{50}) measured with the Spraytec are listed in Table 4. The pattern of the results from the Spraytec study correlates well with the results from the Mastersizer study. Apart from mannitol as a foundation material alone, all the formulations without leucine had a D_{50} of greater than 5 μm. Mannitol alone had a D_{50} of 2.83 μm while the formulations containing glycine/alanine 30/30%, alanine 30% or glycine 30% as additives had D_{50} of 28.55 μm, 12.02 μm and 5.52 μm, respectively. Graphical analysis demonstrates the significant effect of leucine in reducing D_{50} and the significant effects of glycine and alanine in increasing D_{50} (Fig. 1b). It is worth noting that the experimental variance of the study, as shown in graphical analysis, is narrow indicating the consistency of the results from the centre point experiments. The regression equation for D_{50} and emitted doses measured by Spraytec are shown below as Eqs. (5) and (6), respectively.

$$Y_2 = 7.74 - 4.49X_1 + 2.55X_2 + 4.11X_3 - 2.26X_1X_2 - 3.95X_1X_3 + 1.67X_2X_3 - 1.79X_1X_2X_3 \quad (5)$$

where $p = <0.0001$ and $r^2 = 0.8820$.

$$Y_3 = 17.91 - 0.36X_1 + 0.49X_2 + 0.29X_3 - 0.44X_1X_2 - 0.49X_1X_3 - 0.49X_2X_3 + 0.29X_1X_2X_3 \quad (6)$$

where $p = 0.0692$ and $r^2 = 0.7274$.

The interaction effects of the amino acids on Spraytec D_{50} within the design space are significant as shown by the interaction terms X_1X_2, X_1X_3 and X_2X_3 on graphical analysis. This finding is not surprising considering the similar Mastersizer result outcome. These results are consistent with the particle size distribution data from the Mastersizer study and clearly demonstrate the significant effect of leucine on not only the formation of particle size, but also on the aerosolisation of the mannitol dry powder formulations. Interestingly, the four centre point formulations with leucine/glycine/alanine 15/15/15% as additives demonstrated the smallest aerosolised D_{50} of 2.28 μm, 2.49 μm, 2.58 μm and 2.40 μm. The curvature is demonstrated by the significant lack of fit from the analysis of variance (Table 4). This result further suggests the interesting finding that the combined use of these amino acids at appropriate concentrations may improve aerosol dispersibility better than with any of these amino acids alone, and hence deserves further investigation.

Spray-dried mannitol has been shown to be an effective non-reducing sugar stabilising agent to preserve the structure of spray-dried proteins in a number of studies (Andya et al., 1999; Maa et al., 2004; Schule et al., 2008). Spray-dried mannitol produced particles with D_{50} of 2.83 μm which appears to indicate a satisfactory dispersibility for inhalable dry powder formulations, but it should be noted that it also produces the lowest emitted dose during the Spraytec study (Table 3). The retention of powder in the device after the experiment was visually evident, and suggests a more cohesive powder than the other formulations here. The presence of amino acids in all combinations resulted in improved ED (Table 3) however, considering the experimental variability the effects of these amino acids do not appear to be significant as shown in graphical analysis (Fig. 1c). The ability of leucine to reduce surface cohesiveness resulting in enhanced dispersibility of spray-dried particles has been indicated previously (Muttil et al., 2010; Shur et al., 2008). In the present study, the beneficial effect of leucine was evident in its capacity to offset the effect of the other two amino acids on D_{50} and of improving both de-agglomeration and ED.

The mechanism of the performance enhancing effect of leucine remains unclear from the literature. The results here suggest that the effect of leucine is unlikely to be simply dependent on molecular weight. Leucine has been suggested to have some surface active properties (Glinski et al., 2000) and has a low water solubility of 0.22 mg/mL (Vehring, 2008). Amino acids with non-polar side chain such as leucine, phenylalanine, methionine and tryptophan have been shown to provide higher particle surface coverage after spray-drying than amino acids with polar side chain such as asparagine and arginine (Chew et al., 2005). Therefore, hydrophobicity may play a role in increasing surface affinity of leucine during droplet drying, leading to modified surface properties of resulting particles. However, the lack of direct correlation between the hydrocarbon chain lengths and effect on aerosolisation from these amino acids suggest that the low solubility and hydrophobicity are not the sole factors causing this. It has also been previously suggested that there is a preferential precipitation of leucine during the drying process and hence the subsequent surface enrichment of leucine on the particles (Kamlag et al., 2004). A lamellar-like self-assembly behaviour of leucine has been reported and this may be a contributing factor to its surface modifying properties (Harding and Howieson, 1976). Though self-packing of molecules has also been reported within the crystal structures of glycine and alanine, these amino acids do not appear to have the same degree of surface affinity compared to leucine (Albrecht and Corey, 1939; Iitaka, 1961; Simpson and Marsh, 1966). It is proposed here that a combination of a high surface affinity during the drying process, mass transport within the droplet, followed by the subsequent self-assembly packing on the particle surface may explain the dispersibility enhancing effect specific to leucine, and further work is ongoing to investigate this hypothesis.

3.4. In vitro aerosolisation and particle deposition

While the Spraytec is useful as a high throughput screening tool to study the real-time apparent particle sizing of dry powder formulations, it provides no direct information on the aerodynamic properties upon powder aerosolisation. While particles with a lower D_{50} are likely to deposit in the lower airway, the Spraytec results do not take into account the potential influence of particle density and morphology on aerosolisation and subsequent deposition. The TSI was therefore used as a preliminary screen of this range of formulations to provide aerodynamic aerosol information complementary to the Spraytec results.

Fine particle fraction results correlate well with the Spraytec D_{50} results, as all the formulations containing leucine, with D_{50} below 5 μm demonstrate the highest FPF of greater than 68% (Table 3). Powders containing amino acids without leucine, with D_{50} above 5 μm show significantly lower FPF as demonstrated by formulations containing glycine/alanine 30/30%, alanine 30% or glycine 30%, with FPF of 2.96%, 9.11% and 34.62%, respectively. While mannitol alone shows reasonable FPF of 66.20%, this formulation also demonstrates the lowest ED which is consistent with the Spraytec study findings (Table 3). The regression equations for FPF and ED% from the TSI study are shown as Eqs. (7) and (8), and graphical analyses shown in Fig. 1d and e, respectively. The highly significant effect of leucine ($p = 0.0005$, Table 4) on improving FPF is demonstrated by the longest bar length in graphical analysis (Fig. 1d) and the highest positive value of coefficient of term X_1 (Eq. 7). The significant negative effects of glycine ($p = 0.0075$, Table 4) and alanine ($p = 0.0014$, Table 4) on FPF are also evident on graphical analysis (Fig. 1d). It is interesting to note that the combined use of the three amino acids at 15% were more effective at improving FPF than the combination used at 30% (Table 3). The analysis of variance showed significant lack of fit to the model because of curvature which is consistent with the Mastersizer and Spraytec results (Table 4). Though further investigation is required to define the optimal concentration ranges, these results suggest that the inclusion of glycine and/or alanine with leucine produced the greatest improvement on aerosolisation performance within a critical concentration range and that higher concentration of these excipients may not be appropriate.

$$Y_4 = 50.42 + 22.20X_1 - 5.59X_2 - 12.96X_3 + 3.84X_1X_2 + 9.22X_1X_3 + 4.17X_2X_3 - 2.18X_1X_2X_3 \quad (7)$$

where $p = 0.0020$ and $r^2 = 0.8217$.

$$Y_5 = 87.92 + 1.31X_1 - 0.09X_2 + 1.13X_3 - 1.34X_1X_2 - 1.56X_1X_3 - 2.20X_2X_3 + 2.33X_1X_2X_3 \quad (8)$$

where $p = 0.0128$ and $r^2 = 0.9820$.

The results support previous studies in which leucine was consistently demonstrated to be the amino acid that had the most significant effect on the aerosolisation of dry powder formulations (Chew et al., 2005; Seville et al., 2007). Studies investigating the aerosolisation enhancing effect of various amino acids including leucine, phenylalanine, tryptophan, methionine, asparagine, arginine, aspartic acid and threonine on the dispersion of spray-dried powders found leucine to be the most effective amino acid that enhanced *in vitro* particle deposition, demonstrating the highest FPF and reproducibly high ED (Chew et al., 2005; Seville et al., 2007). The findings of the current study are also consistent with a previous study which demonstrated the effect of leucine, glycine and alanine on FPF with no correlations with hydrophobicity (Minne

et al., 2008). However, these previous studies did not investigate the effect achieved from the combination use of these amino acids. The results from the present study suggest that the combination use of leucine with glycine and/or alanine in the appropriate concentrations is able to produce particles within the suitable size range with good aerosolisation properties for pulmonary delivery. In addition, this current study also demonstrates the ability to incorporate multiple components (for multiple potential functionalities within the particle) into an inhalable spray-dried formulation while maintaining good aerosolisation, which might otherwise not be possible without the presence of leucine.

3.5. Inter-particle interaction

From the shear cell testing, mannitol alone was the most cohesive powder with a cohesion value of 4.53 kPa (Table 3). The regression equation for cohesion values is shown as Eq. (9). The high r^2 (>0.9) indicates a high correlation between the mathematical model and the experimental results. The presence of amino acids demonstrates a clear trend in reducing inter-particle cohesion of the dry powders as shown by the negative values of coefficients of term X_1, X_2 and X_3 (Eq. (9)) and the negative bars in graphical analysis (Fig. 1f). The presence of amino acids in all compositions reduced the cohesion of the mannitol alone formulations (Table 3). The incorporation of leucine, glycine and alanine all reduces inter-particle cohesion as concentration increases. These results indicate that these amino acids as excipients are effective at reducing inter-particle cohesion.

$$Y_6 = 1.74 - 0.29X_1 - 0.44X_2 - 0.81X_3 + 0.17X_1X_2 + 0.63X_1X_3 + 0.39X_2X_3 - 0.07X_1X_2X_3 \quad (9)$$

where $p = 0.0051$ and $r^2 = 0.9741$.

However, leucine was the only amino acid that reduced cohesion without substantially increasing particle size, maintaining the particle size range (1–5 μm), as suitable for pulmonary delivery. Inter-particle forces will increase for a given composition as particle size reduces (Forsyth et al., 2001). Although the inclusion of glycine and alanine reduced cohesion in the absence of leucine, this was evidently attributable to relative increase in particle size. The results suggest that even though glycine and alanine effectively reduced inter-particle cohesion of the mannitol formulations, the increase in particle size means the use of these amino acids is less appropriate for pulmonary delivery (and notably under the process conditions used here) without inclusion of dispersibility enhancing excipients such as leucine. This finding is further reflected by the poor aerosolisation performance of the formulations containing glycine/alanine 30/30%, alanine 30% and glycine 30% in both the Spraytec and TSI studies. Therefore, it is noted that the presence of leucine is required to maintain a suitable particle size range for inhalation.

3.6. Particle morphology and appearance

Spray-dried mannitol as a foundation material alone was observed to form small spherical particles that are heavily agglomerated (Fig. 2a). This result is consistent with the particle size distribution data from the Mastersizer and cohesion study. Upon addition of amino acids, spherical particles were preserved in all formulations containing leucine regardless of the presence of glycine and alanine (Fig. 2b, d, f, h, i). Other formulations containing glycine and/or alanine without the addition of leucine formed much larger particles of irregular shape with rough surfaces (Fig. 2c, e, g). It appears that these particles are created as a result of irreversible fusion of primary structures formed during the drying process of each isolated drying droplet. Amino acids with non-polar side chains including leucine have been shown to have higher affinity to the particle surface (Chew et al., 2005). It is therefore proposed that this fusion is prevented when leucine is present on the surface to form a protective shell. The increased particle size observed is consistent with the Mastersizer and SprayTec size data as discussed above.

So, the result suggests that the presence of leucine assists in the formation of spherical particles by forming a coating on the drying particle surface, hence providing a protective shell which preserves the individual particles as they are collected from the dryer and preventing any such fusion. In contrast, the presence of glycine and alanine enhanced fusion, and it is suggested that these amino acids therefore increase the relative hygroscopicity, compared to mannitol alone.

Leucine has also been shown to alter particle morphology and may produce either smooth or wrinkled particles depending on the concentration used in the feed solution (Kamlag et al., 2004). Such structures are proposed to result from the formation of hollow particles that are inflated during drying (Vehring, 2008). More recently, co-spray-drying of unfractionated heparin with L-leucine 1% (w/w) and disodium cromoglycate with L-leucine 5% (w/w) produced smooth spherical particles (Chew et al., 2005; Shur et al., 2008); while spray-drying of salbutamol sulphate and lactose with L-leucine 12% w/w produced particles of irregular surface (Seville et al., 2007). These results indicate that a relatively high concentration of leucine (i.e. >5%, w/w) tends to lead to corrugated particles. The morphology of leucine-containing particles in the present study appears to behave somewhat differently. The concentrations of leucine used within the study design space (15–30 molar%), which corresponds to roughly 10–18% (w/w), did not form corrugated particles. It is therefore speculated that the presence of glycine and/or alanine altered the core structure of the spherical drying particles, while leucine tended to reside on the particle surface, hence not only providing a coating to reduce surface cohesiveness and prevent fusion but also with the net result of reducing tendency to form corrugations in the drying process.

While surface asperities and rugosity on corrugated particles have been demonstrated to reduce powder cohesiveness and therefore improve dispersibility (Chew and Chan, 2001; Lechuga-Ballesteros et al., 2008), in the present study, leucine was able to enhance aerosolisation performance of the mannitol formulations without necessitating the formation of corrugated particles. Furthermore, the FPF results suggest that this combination may also be advantageous in terms of aerosolisation efficiency. This finding is consistent with previous studies in which spray-dried unfractionated heparin with L-leucine 1% (w/w) increased FPF by >4.5-fold without alteration of surface morphology (Shur et al., 2008).

3.7. Crystallinity

To investigate the feasibility of developing a dry powder carrier platform capable of stabilising proteins for pulmonary delivery, the glassy phase (amorphous or crystalline) status of the formulations was investigated by assessing their crystallinity using XRPD. All the formulations were sealed and stored in a refrigerator before the XRPD study. According to the glassy dynamics hypothesis, an amorphous glassy solid should provide a highly viscous "vitreous" environment which restricts the molecular mobility of biomacromolecules and thereby stabilises proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Any excipients combination used should ideally be a good glass former with high glass transition temperature (T_g) but otherwise inert. Mannitol alone has a relatively low T_g of 11 °C (Weers et al., 2007), therefore it is not surprising to find a high level of crystallinity from mannitol as a baseline material alone (Fig. 3). In a study of the salmon calcitonin-mannitol system, the spray-dried powders remained

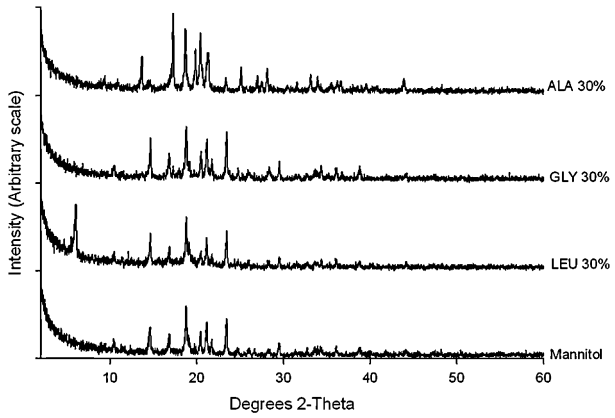

Fig. 3. XRPD profiles of mannitol and formulations containing 1 amino acid: mannitol with leucine 30% (molar%), mannitol with glycine 30% (molar%) and mannitol with alanine 30% (molar%).

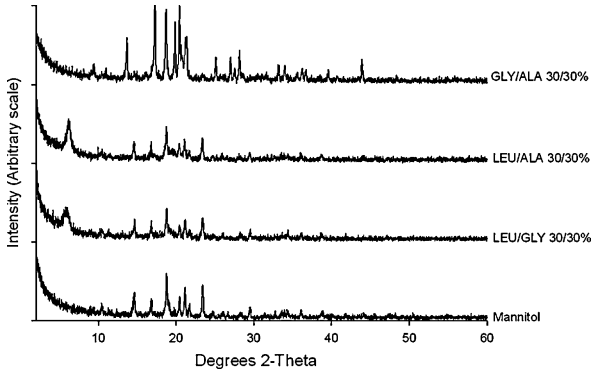

Fig. 4. XRPD profiles of mannitol and formulations containing 2 amino acids: mannitol with leucine/glycine 30/30% (molar%) mannitol with leucine/alanine 30/30% (molar%) and mannitol with glycine/alanine 30/30% (molar%).

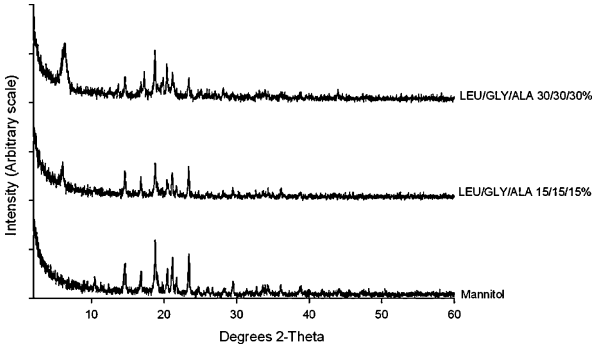

Fig. 5. XRPD profiles of mannitol and formulations containing 3 amino acids: mannitol with leucine/glycine/alanine 15/15/15% (molar%) and mannitol with leucine/glycine/alanine 30/30/30% (molar%).

amorphous in formulations containing less than 50% (w/w) of mannitol (Chan et al., 2004). Therefore, it was hypothesised that the addition of excipients in sufficient quantity could be used to prevent or delay crystallisation of mannitol after spray-drying. It is unclear from the literature how effective small molecule additives such as amino acids, may be at stabilising the amorphous status of mannitol. However, spray-dried powders containing mannitol and glycine have been successfully used to produce inhalable dry powder insulin formulation stable at room temperature without refrigeration (Sadrzadeh et al., 2010; Siekmeier and Scheuch, 2008; White et al., 2005). In particular, a study on spray-dried IgG has established that formulations containing mannitol/glycine combinations produced dry powders with T_g ranging from 14.1 to 53.8 °C with the 50/50% (w/w) ratio producing a dry powder with the highest T_g (Schule et al., 2008). In other words, in the absence of other excipients, a high level of glycine was required to modify the glassy state of a mannitol-based dry powder formulation. The present study examined the effect of leucine, glycine and alanine, both alone and in combination in the proportions defined in the study design space, on the amorphous status of the formulations.

The presence of either a single amino acid or combinations under the experimental conditions used did not appear to have much impact on reducing the crystallinity of the mannitol formulation. The presence of either leucine 30%, glycine 30% or alanine 30% did not appear to reduce the crystallinity profile of the mannitol formulations as demonstrated by the similar XRD profiles (Fig. 3). Similarly combinations of amino acids at other concentrations within the study design space did not appear to have much effect on the crystallinity of the formulations either (Figs. 4 and 5). The formulation containing leucine/glycine/alanine 30/30/30%, provided slightly reduced peaks compared to the background, but this was not quantified and crystalline peaks were still present (Fig. 5). XRPD patterns need to be interpreted with caution when evaluating the crystallinity of small particles. Considering the line broadening effect of particles at this small particle size range, there appears to be little alterations in crystallinity of the formulation examined. While the combination of mannitol/glycine in a 50/50% (w/w) ratio has been shown to retain high amount of amorphous content (Schule et al., 2008), the result from the present DoE study indicate that the amount of amino acids used within the study design space was not sufficient to prevent crystallisation. Hence, further study using higher concentrations of excipients should be investigated as potential to maintain the amorphous status of spray-dried mannitol based particles.

4. Conclusion

This study indicates that interactions between combinations of excipients used in spray dried formulations can be identified hence leading to a deeper study of such combination effects and greater understanding of the study design space. The results from the present study show that the use of glycine and/or alanine, though being structurally similar to leucine, provide detrimental rather than beneficial effects on particles both during as well as after spray-drying with mannitol. In addition, the combination of leucine with either (or both) glycine and alanine provides particles with the inherited benefits of the leucine. Although further study is required, this work indicates there is potential to investigate further ranges of concentrations of combined amino acids that may further lead to added benefits, such as particle morphology control or stable glass formation. The investigation also suggests there are some further aspects regarding the mechanisms leading to these effects that require more study. It is worth noting that the results from the DoE analysis also revealed the lack of linearity of the effects achieved from the combination use of these amino acids across the concentration range within the study design space. This information should be considered in future study design when investigating the optimal concentration and effect of these potential performance enhancing excipients.

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