Trileucine Improves Aerosol Performance and Stability of Spray-Dried Powders for Inhalation

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ABSTRACT: For particles to be useful medicinal aerosols, not only their aerodynamic diameter has to be on the order of a few micrometers but also they have to be chemically and physically stable. Manufacture of respirable particles is a technical challenge because as particles are reduced in size by conventional milling techniques, their cohesiveness greatly increases and physical and chemical stability is often compromised by the formation of amorphous material. In the present study, we describe the use of trileucine for the preparation of dry powders suitable for inhalation via spray drying of a wide range of drugs (i.e., asthma therapeutics such as albuterol and cromolyn, and anti-infectives such as netilmicin and gentamicin, as well as therapeutic proteins and peptides such as human growth hormone and salmon calcitonin). The glass transition of spray-dried trileucine is dependent on the pH and can be correlated with the proportion of the anion, cation, and zwitterion concentration in solution. Trileucine glass transition is relatively high (≈104°C) enabling long-term room temperature stability. The solubility of trileucine is dependent on the pH and is lowest at neutral pH (≈6.8 mg/mL). Trileucine's low aqueous solubility enables the formation of low-density corrugated particles and promotes the formation of trileucine coated spray-dried particles, resulting in superior aerosol performance. Trileucine is surface active and promotes the formation of spray-dried powders with a reduced cohesiveness as demonstrated by a decrease in the measured surface energy which correlates with an observed improvement in aerosol performance. Additionally, trileucine competes with the protein on the air/water interface resulting in an additional depression of surface tension in solution which correlates with a decreased denaturation and aggregation in the solid state.

Keywords: dry powder inhalers; spray drying; formulation; stability; protein; antibiotic; asthma; aggregation; surface activity; solubility; aerosol; emitted dose; dispersibility; size distribution; surface concentration; surface energy; trileucine; glass transition

INTRODUCTION

The use of spray drying to prepare dry powders for inhalation of small molecules, peptides and proteins has been established in recent years. The main aim has been to produce powders that can be easily dispersed to form an aerosol, without the use of a carrier, to enable the delivery of pharmaceuticals into the lung for local or systemic absorption into the human body. This is typically achieved by producing a low-density fine powder with a mass median aerodynamic diameter (MMAD), usually less than 3 μm. The fine powders can be filled into capsules or blister packages, and then redispersed using an aerosolization

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device.[1,2] It is known that hollow and corrugated particles packed in a loose powder bed are advantageous for aerosol drug delivery because of their relatively small MMAD when compared to the volume mean diameter (VMD) and their reduced interparticle interactions.

A key aspect to the successful development of spray-dried powders for inhalation has been the ability to quantitatively measure the properties that directly impact aerosol performance and stability (particle size, particle density, bulk density, specific surface area or rugosity, surface activity, surface concentration, cohesivity, van der Waals forces, charge density, zeta potential, crystallinity, hygroscopicity, glass transition and melting temperatures, and reactivity to water vapor). Finally, because physical properties greatly depend on the intrinsic properties of each drug molecule and because small and large molecules exhibit a rich variety of solid-state behavior, a successful technological platform must rely upon novel excipients to enable the preparation of stable, low-density dispersible powders that covers a wide range of drugs.

Spray drying is an attractive process because it is a scalable, single-step unit operation. Knowledge of the particle formation mechanism, which ultimately leads to the control and scalability of the manufacturing process, is essential. In addition, formulation challenges have emerged due to the inherent fast drying of the particles which promotes the formation of amorphous material.

Amino acids and short peptide sequences have been explored as useful excipients in a variety of formulations.[3–13] Furthermore, formulation of proteins for injection have utilized amino acids as buffering systems, lyoprotectants, stabilizers against aggregation and oxidation, and modifiers of viscosity and hygroscopicity.[3–25]

Amino acids have been used as buffers for both proteins[25,26] and antibodies.[17,19,20,24] For example, rhuMAb HER2 was purified as drug substance in 5 mM histidine, pH 6.0.[19,20] The antibody was then further formulated into the final drug product, using histidine as a buffering system. Histidine was chosen for its buffer capacity at pH 7.0 with recombinant Factor VIII.[25] Glycine has also been used as a buffer in the formulation of human Growth Hormone (hGH; Nutropin, Genotropin, Humatrope, Norditrope) and antibodies (Gammagard, BayRho-D, Gamimune), and is a compendial excipient.[15]

Amino acids have been utilized as lyoprotectants of therapeutic proteins increasing the stability to lyophilization and decreasing aggregation upon reconstitution.[21–29] Incorporation of histidine into recombinant Factor VIII formulations was found to stabilize the protein and maintain activity during formulation, lyophilization and storage (2 years at 7°C and 1 year at 25°C).[25] Lysine was also investigated, however the recovery and stability of Factor VIII was not as effective. Similarly, incorporation of glycine into formulations of Chimeric BR96-doxorubicine immunoconjugate resulted in a decrease in aggregation upon lyophilization.[23] Glycine has been used to decrease aggregation of hGH and growth hormone releasing factor.[10,30,31] Histidine, glycine, glutamic acid, and arginine were screened for their lyoprotectant ability in formulations of a human anti-IL8 monoclonal antibody (ABX-IL8).[24] A lead formulation containing glycine, mannitol, glutamic acid, and histidine, pH 5.7–6.3, was chosen, where the stability of ABX-IL8 was strongly dependent on histidine concentration. Specifically, increasing the histidine concentration in the bulk solution inhibited formation of high molecular weight species. In addition, histidine enhanced solution stability upon freezing and thermal stress conditions, where its cryoprotectant ability was comparable to that conferred by sucrose. Lysine, glutamic acid and glycine were determined to reduce aggregation of Interleukin-2 and Ribonuclease A upon lyophilization and reconstitution.[21] Poly-Lys and poly-Glu were found to be most effective stabilizers due to their high charge density.[21] Similarly, histidine, glycine, aspartic acid, glutamic acid, and lysine were determined to be effective at decreasing aggregation of keratinocyte growth factor upon lyophilization.[22] Poly-Glu and poly-Lys also produced a significant reduction in aggregation, suggesting that poly-ions provided a benefit.

Amino acids, such as lysine, asparagine, histidine, valine, leucine, and isoleucine have also been used to improve stability of hGH to deamidation, oxidation and cleavage products.[8,9,11,32–35] Specifically, amino acids have more commonly provided increased stability as antioxidants.[14,16,17] Both methionine and cysteine have been evaluated as antioxidants in the stabilization of Ciliary Neurotrophic Factor (hCNTF) and Nerve Growth Factor (hNGF) at pH 7.6 against degradation caused by alkyl hydroperoxides and hydrogen peroxide.[14] Antioxidants were ranked for their effectiveness, where thiols (cysteine) were more effective than thioethers (methionine), specifically for prevention of dimer formation of hCNTF.

Furthermore, the authors point out the effectiveness of the amino acid antioxidant is dependent on the pH of the solution. Literature indicates that at basic pH (pH >7.0), there is significant net negative charge density of the reactant species, and cysteine reactivity increases with increasing pH. Similarly, at acidic pH (pH <5.0), methionine has been reported to be more reactive than cysteine. Formulations of rhuMAb HER2 have also used 14.5 mM methionine, pH 5.0, to minimize temperature-induced oxidation caused by free radicals generated by the presence of metal ions and peroxide impurities.[16] Adenovirus vaccine Ad5 used 7.5–10 mM histidine, pH 7.4–8.0, not only as a buffer, but also an antioxidant.[17] Not surprisingly, L-carnosine (Ala-His) has been documented to have antixoidative properties in biological systems.[18]

Utility is emerging in the ability of amino acids to improved viscosity, hygroscopicity, surface activity and charge density.[3,12,24] The addition of histidine to bulk solution (prior to lyophilization) of ABX-IL8 resulted in a decrease in viscosity, most probably by disruption of charge–charge interactions between antibody molecules.[24]

More recently, isoleucine was spray-dried with a model protein for pulmonary delivery with decreased MMAD, increased dispersibility, improved particle characteristics and good storage stability.[3] Leucine has been shown to improve the dispersibility of sodium cromolyn spray-dried powders.[12]

Fewer examples are available utilizing short amino acid sequences as excipients; however, Leu-His-Leu and Lys-Gly-Asp-Ser have been used in a lyophilized formulation of growth hormone as a buffering agent and as a stabilization agent against deamidation, oxidation and peptide cleavage.[6,7] Leu-Leu and Leu-Leu-Leu have been utilized to formulate spray-dried pulmonary formulations with improved aerosol performance (dispersibility) and stability.[4,5,10]

Delivery of pharmaceutical aerosols to the deep lung presents additional significant challenges to a formulator, who must consider stability in the solution and solid states, as well as control of particle morphology, size, surface energy, and composition that will result in highly dispersible spray-dried particles. Formulation studies with a variety of pharmaceutically active molecules, many of which have been challenging to formulate using other excipients, show that an amino acid based excipient, trileucine, provides many of these benefits.

There are multiple competing factors that must be considered in the development of a successful pulmonary formulation, including surface chemistry (composition), hydrophobicity, surface energy, and morphology (and thus bulk and particle density) all of which makes it challenging to design experiments in which only a single parameter changed.

The present study describes the use of Leu-Leu-Leu (trileucine), to design low-density, noncohesive spray-dried particles, that are physically and chemically stable at room temperature, and have a sufficiently small aerodynamic diameter to enable efficient delivery to the lung as a dry powder aerosol.[4,36] The use of characterization techniques to measure key properties of the powders as well as the description of the main physicochemical factors that determine the particle formation process during spray drying are also described.

MATERIALS AND METHODS

Materials

Lyophilized Met-hGH (hGH) containing glycine and mannitol (hGH:glycine:mannitol) (1:1:5) was obtained from BresaGen Limited (Adelaide, Australia). Salmon Calcitonin (sCT) was obtained from Peninsula Labs (San Carlos, CA). Netilmicin sulfate, gentamicin sulfate, and albuterol sulfate were obtained from Sigma Co. (St. Louis, MO). Sodium cromolyn was obtained from Orion Pharmaceuticals (Kuopio, Finland). Sodium phosphate, potassium phosphate, Tris buffers and Na_2SO_4 were obtained from J.T. Baker (Phillipsburg, NJ). Trileucine was obtained from Bachem (Torrance, CA).

Solution and Powder Preparation

A Büchi 190 spray dryer (Brinkman), with a custom-made atomizer nozzle was used for spray drying. Solutions of hGH (pH 7.8 and pH 3.6) or sCT (pH 7.0) were spray-dried under the following conditions: liquid feed rate of 5 mL/min, atomizing pressure of 60 psi and the (controlled) inlet and (measured) outlet air temperatures were 125 and 70°C, respectively. Netilmicin sulfate, gentamicin sulfate, albuterol sulfate, and sodium cromolyn were spray dried from 1 wt% aqueous solutions under conditions as outlined above.

Solution Characterization

The ionization constants of trileucine at 25°C were determined from a titration curve generated by the addition of either HCl or NaOH to 25 mL of 14 mM trileucine (initial pH 5.7).

The solubility of trileucine in water and its pH dependence was determined at 25°C. The pH was maintained by using sodium phosphate buffers. The effect of buffer concentration (50, 100, and 200 mM) was found to be negligible.

Equilibrium surface tension of trileucine aqueous solutions prior to spray drying was measured using the Wilhelmy plate method using a Krüss model K-12 tensiometer at 25 and 40°C. The surface tension of water was 72 and 66 mN/m at 25 and 40°C, respectively.

Dynamic surface tension of trileucine and hGH solutions were performed utilizing a pendant droplet apparatus (FTA 100, First Ten Angstroms, Portsmouth, VA). The surface tension at the air/solution interface was calculated from the size and shape of a droplet hanging from the tip of a syringe with a blunt end metal or Teflon coated needle (Hamilton Micolitre Syringes, Hamiltom Nonaduz AG, Switzerland). The surface tension of water measured with the pendant drop method was 72 mN/m (25°C). A detailed method can be found in application notes found at www.firsttenangstroms.com.

Powder Characterization

Scanning electron microscopy (SEM) was used to observe the morphology of the spray-dried particles. Samples were mounted on silicon wafers that were then mounted on top of double-sided carbon tape on an aluminum SEM stub. The mounted powders were then sputter-coated with gold:-palladium in a Denton sputter-coater for 60–90 s at 75 mTorr and 42 mA. This produces a coating thickness of approximately 150 Å. Images were taken with a Philips XL30 ESEM using a LaB_6 source operated in high vacuum mode using an Everhart–Thornley detector to capture secondary electrons for the image composition. The accelerating voltage was set at 20 kV and the beam current was set at 33 mA. The working distance was between 5 and 6 mm.

Primary particle size distributions were determined using static laser light scattering ("laser diffraction"). Powder samples were measured using a Sympatec HELOS unit (with an R2 lens) equipped with a VIBRI vibratory feeder and a RODOS/M dry powder-dispersing unit (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The following settings were applied for analysis of samples: a sample mass of approximately 10 mg, an optical concentration of approximately 1%, and suction and driving pressures of 70–75 mbar and 2 bar, respectively. Data were collected over a measurement-duration of 10 s. Particle size distributions were calculated by the instrument software using the Fraunhofer model. Prior to measurement of samples, the system suitability was assessed by measurement of the primary particle size distribution of a silicon carbide reference standard supplied by Sympatec GmbH.

Aerodynamic particle size distributions and emitted dose (ED) determinations were gravimetrically determined using an active dry powder inhaler, the Pulmonary Delivery System (PDS, Nektar Therapeutics, San Carlos, CA) on an Andersen cascade impactor (ACI, Series 20–800 Mark II, Thermo Scientific, Waltham, MA) at flow rate of 28.3 LPM at 1.4 L airflow volume (3 s). Gravimetric ED was evaluated at a flow rate of 30 LPM at 1.25 L airflow volume (2.5 s). Both analyses are performed at ambient laboratory conditions (21°C, 40 ± 5%RH). The PDS was operated with blister packages that contained 3 mg of powder. The ED is the mass of powder emitted from the device relative to the total mass in the blister package, and the fine particle mass <3.3 μm ($FPM_{<3.3\ \mu m}$) is the mass of powder with a MMAD less than 3.3 μm. The ED is a measure of device emptying efficiency. The FPM is a measure of efficiency for deep-lung delivery.

Powder surface composition was determined by X-ray photoelectron spectroscopy (XPS) also known as electron spectroscopy for chemical analysis (ESCA). This a nondestructive spectroscopic technique used to study surfaces of solids (penetration depth is about 50 Å). A sample is bombarded by soft X-rays, which causes the surface molecules to emit electrons of binding energies characteristic of each element in the molecule (except for hydrogen, which is not detected using this technique). The intensity of the photoelectron signal is proportional to the number of atoms of a particular element present in the sample as well as the photoelectric cross section of that particular element (i.e., the atomic sensitivity factors). The area of the peak at a unique binding energy for that element is used to determine its atomic concentration. To convert the atomic concentrations to molecular concentrations, a linear least squares model is used to relate the

elemental atomic concentrations data to the theoretical atomic concentrations of each element in the sample. In doing so, the molecular composition of the particle surface is determined.

To prepare a sample for an XPS experiment, a glass plate (precleaned with a 10% hydrofluoric acid solution) was used to press each powder sample onto a glass substrate that had been sandblasted to improve the adhesion of the powder to the glass. A monochromatic Al X-ray source with a take-off angle of 45° was directed to a spot size of 2 mm × 3 mm on the sample. Survey (pass energy = 89.45 eV) and high-resolution (pass energy = 17.90 eV) spectra were collected with a Physical Electronics PHI 5000 Series Spectrometer. Peak areas from the high-resolution spectra were converted to atomic concentrations on the surface of each sample. JMP software (SAS Institute) was used to fit a linear least squares model to the atomic concentrations to obtain the concentration of each molecular species on the surface. These concentrations were normalized to a total value of 100%.

Surface energy of the spray-dried powders was calculated from the acetone and octane adsorption isotherms at 25°C, which were gravimetrically determined using a dynamic vapor sorption (DVS) instrument (Surface Measurement Systems, London, UK). This instrument gravimetrically measures uptake and loss of water or organic solvent vapor by a material. The DVS system is equipped with a recording microbalance with a resolution of ±0.1 μg and a daily drift of approximately ±1 μg. In the first step of the experimental run, the sample was dried at 25°C and 0%RH for at least 600 min to bring the sample to a constant mass. Then, the relative vapor pressure was increased in steps of 5% (P/P_o) and decreased in steps of 5% P/P_o from 90% to 0% P/P_o (where P_o is the vapor pressure of the solvent at the measurement temperature). An equilibration criterion of $dm/dt = 0.005\%$/min was chosen for the system to achieve at each step before automatically proceeding to the next step. Sample masses between 10 and 15 mg were used in this study. Calculation of the surface energy is described in SMS DVS application note 17 found at www.smsuk.co.uk.

Glass transition temperatures of trileucine spray-dried powders was measured using modulated differential scanning calorimetry (DSC 2920, TA instruments, New Castle, Delaware). A mass of 5–15 mg of the powdered sample was filled and pressed into a compact pellet in aluminum DSC pans and hermetically sealed using a sample encapsulation press. Sample preparation was performed in a glove box, with RH maintained below 2%. Heating rate was 2°C/min and modulation was ±0.318°C every 60 s. Helium was used as a purge gas at 30 cm^3/min; the Refrigerated Control System (RCS) used helium at a flow rate of 110 cm^3/min.

Chemical stability of hGH was monitored by reversed phase high performance liquid chromatography (RP-HPLC) and Size Exclusion Chromatography (SEC). RP-HPLC was used for hGH chemical analytes, where the hGH main peak and the early eluting degradation products were monitored. Early eluting peaks by SEC were used to monitor hGH soluble aggregates. RP-HPLC was performed on a Waters 2690 HPLC system with a Waters 996 photodiode array detector (Milford, MA) and a Vydac C-4 column (5 μ, 300 Å, 250 mm × 4.6 mm) (Hesperia, CA). Samples were eluted isocratically with 27.5% n-propanol/72.5% 50 mM Tris, pH 7.5 at 1 mL/min, and analytes were detected at 220 nm. SEC was performed on a Waters 2690 HPLC system with a Waters 996 detector and a TosoHaas TSK G2000SWXL column (5 μ, 300 mm × 7.8 mm) (Montgomeryville, PA). Samples were eluted isocratically with 3% isopropanol in 63 mM potassium phosphate, pH 7.0 at 0.6 mL/min at 214 nm. The percentage of total monomer (TM) was calculated from the amount of soluble aggregate (SA) and insoluble aggregate (IA).[37]

$$\%\mathrm{TM} = \frac{(100 - \%\mathrm{SA})(100 - \%\mathrm{IA})}{100}$$

Stability of sCT was determined by RP-HPLC and SEC. The chemical stability was monitored by RP-HPLC using a Vydac C-18 column, at 40°C, with an acetonitrile gradient (0.1% TFA) at a flow rate of 1 mL/min and detection at 210 nm. Samples were analyzed for monomer content onto a TosoHaas TSK-GEL G2000SWXL column. Samples were eluted isocratically with 0.25 M Na_2SO_4 at 0.7 mL/min at 210 nm.

RESULTS AND DISCUSSION

Correlation between Solution and Powder Properties

The morphology of spray-dried particles is largely determined by the solubility of the formulation components in solution, their ability to form a supersaturated solution and their diffusivity

(which is inversely proportional to molecular weight).[38] The spray drying process starts with the atomization, or formation of droplets, of an under saturated solution. As the droplets dry in stream of gas, the square of the droplet size, d^2, decreases linearly with time (with d being the droplet diameter), which follows from combining the diffusional flux caused by the evaporation, proportional to the droplet diameter, and the droplet volume (proportional to d^3), which changes linearly with the mass flux.[38,39] It also follows that solute concentration changes are inversely proportional to volume changes.

Formulation components with lower solubility precipitate earlier in the drying process, leading to the formation of a solid shell that eventually collapses as drying continues, resulting in a corrugated particle.[40,41] In contrast, highly water-soluble components continuously recede as the liquid droplet dries, resulting in a smooth, spherical particle. Besides reaching the solubility limit, the ability to form a supersaturated solution will determine the state of the solid particle. Formulation components such as mannitol will tend to crystallize, in spite of its high aqueous solubility, whereas sugars, such as sucrose, raffinose, trehalose, lactose, will tend to precipitate as an amorphous solid. In the case of an amorphous solid, a high glass transition temperature (T_g) is desired to ensure acceptable physical stability. For two or more component systems, the least soluble component will determine the particle morphology.

Trileucine is surface active (Fig. 1) and its surface activity was not affected by the presence of non-surface active formulation components, such as raffinose. The surface activity of trileucine is even higher at 45°C, which is the temperature close to the wet bulb temperature of the drying droplet.

The solubility of trileucine, a zwitterionic compound, is a function of pH (Fig. 2a). When hydrochloric acid and sodium hydroxide are used to adjust pH, trileucine exists as the hydrochloride salt, the zwitterion, and the sodium salt at low, neutral and high pH, respectively (Fig. 2b).

The pH dependence of the solubility can be predicted if the solubility (line in Fig. 2a) at the isoelectric point (pH 5.7) and the pK_a values are known. The predictive equations are based on acid base equilibria, and are given by:

$$\mathrm{pH} - \mathrm{p}K_{a1} = \log\left(\frac{S}{S - S_o}\right) \qquad (\mathrm{pH} < 5.7) \qquad (1)$$

$$\mathrm{pH} - \mathrm{p}K_{a2} = \log\left(\frac{S - S_o}{S_o}\right) \qquad (\mathrm{pH} > 5.7) \qquad (2)$$

where S_o is the minimum solubility (6.8 mg/mL), taken here as the average of the solubility values at pH values within 1 pH unit of the isoelectic point (pH 5.7). The calculated pH solubility profile was calculated using the pK_a's of trileucine, 3.6 and 7.8, determined by titration at 25°C. A third pK_a was estimated to be greater than >12 from the titration curve but was not used for solubility calculation.

Spray drying solutions of trileucine at various pH values rendered noncrystalline powders whose glass transition temperature (T_g) is dependent on the various trileucine species in solution (Fig. 2b).

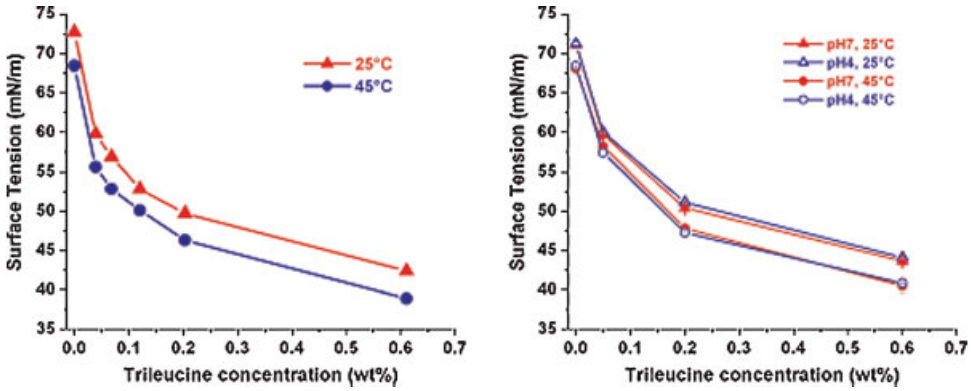

Figure 1. Equilibrium surface tension of aqueous trileucine at 25°C and 45°C (left) and in the presence of raffinose at 25 and 45°C (right). Raffinose was added to the solutions to give a total concentration of 1 wt% solids. At the concentrations studied, raffinose had no significant effect on surface tension. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

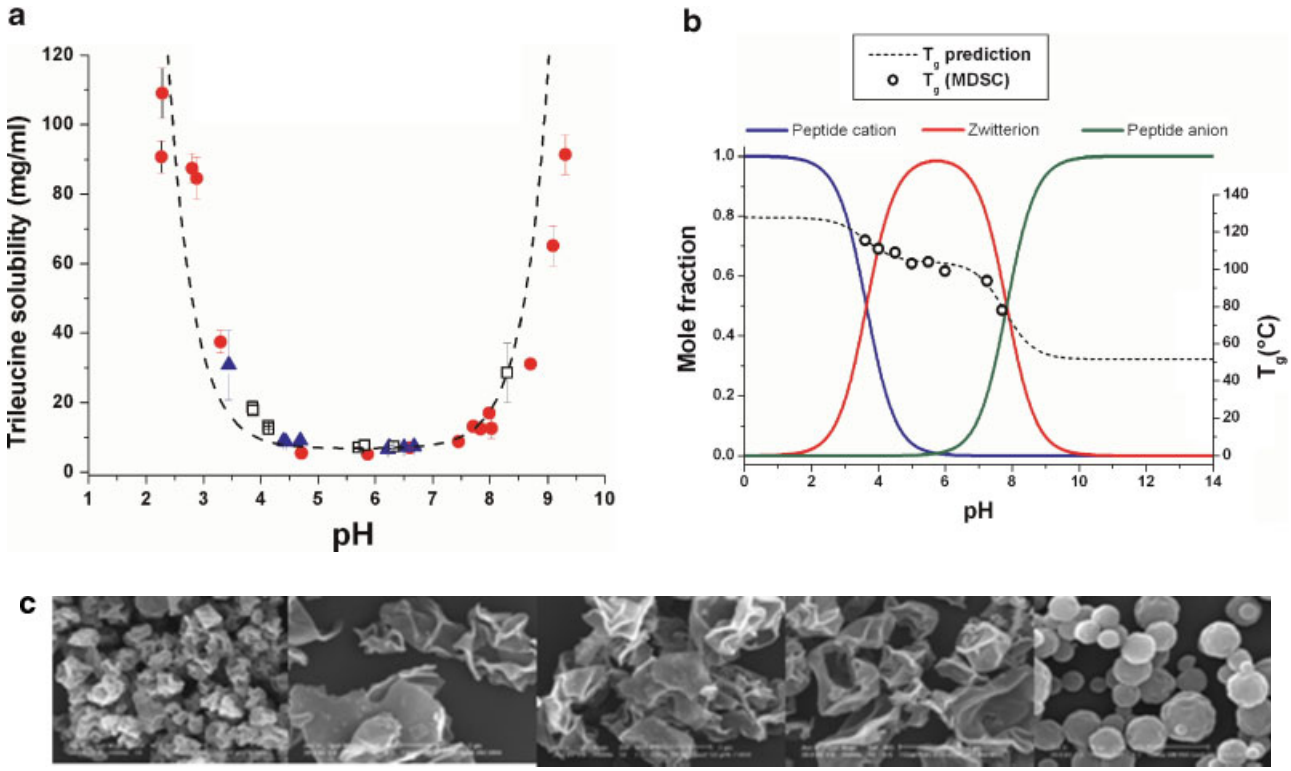

Figure 2. (a) Trileucine pH-solubility curve for trileucine, solid line is calculated using $pK_{a1} = 3.6$ and $pK_{a2} = 7.8$, and average solubility (6.8 mg/mL) at isoelectric point of 5.7 ± 1 pH units. pH adjustments were made by addition of HCl or NaOH. (b) Trileucine glass transition temperature as a function of pH (measured between pH 4–8), superimposed on the relative abundance diagram as a function of pH, peptide cation (left curve), zwitterion (center curve), and anion (right curve). Open circles represent experimentally measured glass transition on spray-dried powders with about 1 wt% moisture content, broken line is calculated glass transition using Fox equation and using glass transition values of 128°C (pH <1.6), 104°C (pH ≈5.7), and 55°C (pH >9.8) for cation, zwitterion, and anion, respectively. (c) Trileucine particle morphology changes when spray dried from solutions at various pH values: 1.8, 4.0, 5.7, 7.0, and 9.7 from left to right. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

Spray-dried trileucine containing mainly the hydrochloride salt has the highest T_g followed by the zwitterion form (i.e., spray dried at neutral pH) and the lowest T_g was observed for the sodium salt of trileucine. This result may be regarded as unusual since often the glass comprising sodium salt of an organic compound has a higher T_g than the corresponding weak acid.[42]

From a physical stability perspective, the peptide cation is the most desirable species due to its high T_g. However, other considerations, such as pH requirements for the active pharmaceutical ingredient of the formulation, may take precedence over T_g alone.

The Fox equation is the simplest model to predict the T_g of amorphous mixtures. It is an inverse linear relationship whose general form for n components is given by

$$\frac{1}{T_g} = \sum_{i=1}^{n} \frac{w_i}{T_{g(i)}} \tag{3}$$

where w_i and $T_{g(i)}$ are the mole fraction and the T_g of component i (+, cation; ±, zwitterion; −, anion), respectively. The Fox equation written for trileucine is given by

$$\frac{1}{T_g} = \frac{w_+}{T_{g(+)}} + \frac{w_\pm}{T_{g(\pm)}} + \frac{w_-}{T_{g(-)}} \tag{4}$$

where, at a given pH, the w_i are readily calculated from the acid–base equilibria. Thus, experimental T_g (pH) data can be converted into $T_g(w_i)$ data.

These results can then be fit to Eq. (4). The adjustable parameters are the T_g values of the pure species, $T_{g(i)}$. From Eq. (4) the T_g of the cation (pH <1.6), zwitterion (pH ≈5.7), and anion (pH >9.8) are predicted to be 128, 104, and 55°C, respectively.

Excellent agreement between the measured T_g values and the prediction of Eq. (4) is shown in Figure 2b. The root-mean-square error of the predicted and measured values is 2.1°C, which is comparable to the reproducibility of T_g measurements on a given sample. Note that no effort has been made to correct for the water content of the powders (approximately 1 wt%). That is, both the T_g data and the model prediction are for powders with about 1 wt% residual H_2O.

It was also noted that the morphology of the resulting particles was influenced by the solubility of trileucine at the various pH values. Particles formed from a neutral pH solution were corrugated due to low trileucine solubility and earlier precipitation during drying. Particles formed from a high or low pH solution were much less corrugated due to the higher trileucine solubility (Fig. 2c).

When spray dried in binary mixtures at low concentration (2 wt%) with compounds of greater water solubility (such as gentamicin, netilmicin, cromolyn, and albuterol), trileucine was found not to have a significant effect on the primary particle size distribution (Fig. 3). However a dramatic increase of the ED and the fine particle fraction of these powders when tested in the Nektar PDS (Tab. 1 and Fig. 4a and b) is observed. Results from surface analysis by XPS show an enhanced surface concentration of trileucine. It was found that trileucine surface concentration increases threefold to tenfold with respect to the nominal bulk concentration (Tab. 1 and Fig. 4b). Cromolyn and 98% raffinose/2% albuterol sulfate, as well as netilmicin and gentamicin, tend to form spherical particles when spray dried from their aqueous solution (Fig. 5a–c). Upon addition of trileucine, the particles eventually adopt the morphology of the least soluble compound, trileucine, as can be seen by the formation of the corrugated particles as the concentration of trileucine increases. The resulting corrugated particles had a increased fine particle fraction (Tab. 1 and Fig. 4b), presumably due to a lower enveloping density and corresponding lower aerodynamic diameter.

As stated above, particle morphology is largely determined by the solubility of the formulation components in solution, where solutes with lower

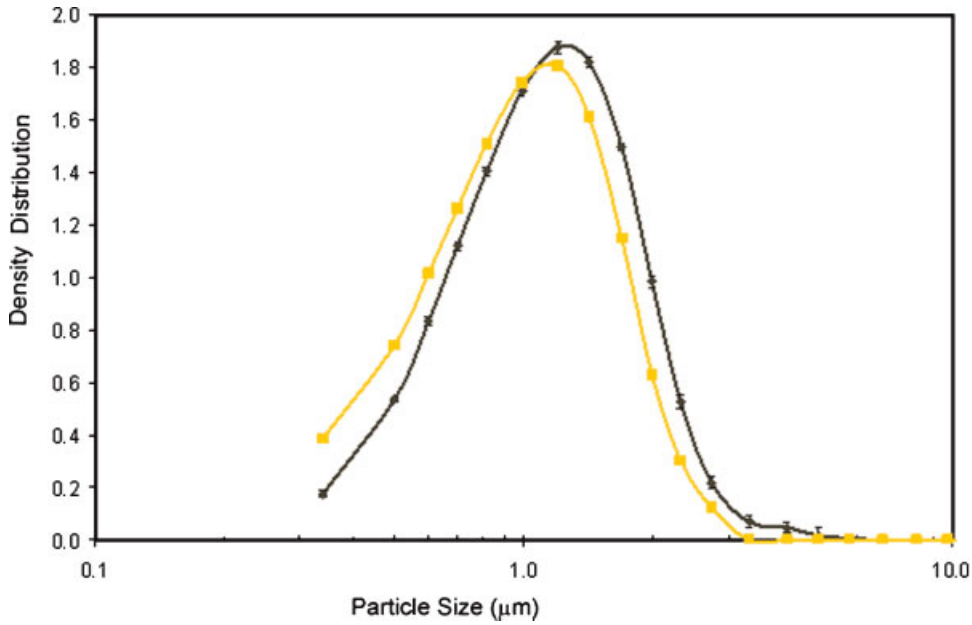

Figure 3. Addition of 2% trileucine did not significantly affect the primary particle size distribution measured by laser diffraction. A median size of 1.1 and 1.0 μm was obtained for gentamycin (diamond symbols) powders and 2% trileucine (square symbols), with a calculated volume mean diameter of 1.2 and 1.1 μm and a geometric standard deviation of 1.6 and 1.7, respectively. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

Table 1. Aerosol Characterization and Trileucine Surface Concentration of Spray-Dried Powders Containing Antibiotic and Trileucine.

	Emitted Dose (%)		Fine Particle Fraction (%)[a]		Trileucine Surface Concentration (%)[a]	
Trileucine (wt%)	Gentamicin	Netilmicin	Gentamicin	Netilmicin	Gentamicin	Netilmicin
0	34 (9.1)[b]	54 (4.3)	26.5	28.5	0	0
2	65 (5.5)	76 (3.3)	46.8	48.8	ND	20
5	75.7 (5.4)	82.4 (2.9)	ND	ND	ND	ND
15	79 (4.3)	89 (5.5)	49.3	62.4	56	45
25	93.9 (4.7)	91.3 (4.4)	ND	ND	ND	ND
45	87.3 (5.5)	90.4 (3.0)	ND	ND	ND	ND

[a] $n = 1$; technique error is within 10%.
[b] $n = 3$ standard deviation.

solubility precipitate earlier in the drying process, tending to produce a corrugated particle, while solutes with high solubility form spheres. Mixtures of low and high solubility solutes result in a variety of particle morphologies. As the relative amount of the less soluble component (trileucine) increases, the morphology changes from spherical to corrugated particles.

If particle size and morphology are kept constant, the ED can be a comparator for the cohesiveness of the particles. The addition of low concentrations of trileucine (2 wt%) to water-soluble antibiotics, such as gentamicin and netilmicin, resulted in an increase in ED, even though the morphology of the spray-dried gentamicin particles and the particle size distribution were not dramatically altered. However, the addition of trileucine at levels greater than 5% induced a morphology change and resulted in a considerably ED increase (Fig. 5b and c). Addition of 15% trileucine also correlates with an increase in the fine particle fraction (FPF) and with an increase in trileucine surface concentration as shown for netilmicin in Table 1. Gentamicin powders containing 25% trileucine observed further improvement in aerosol performance; no additional improvement was observed at 45% trileucine. Maximum improvement in netilmicin powders aerosol performance was observed at 15% trileucine.

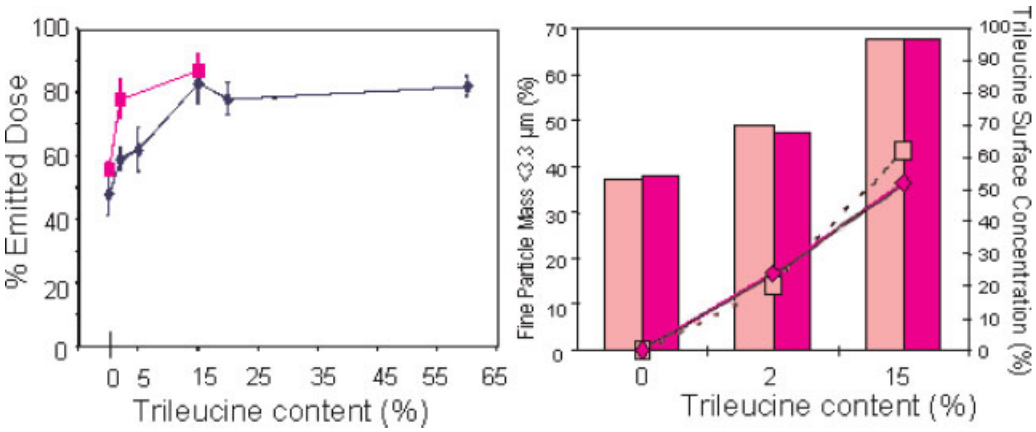

Figure 4. Influence of trileucine content on the emitted dose of anti-asthmatics, 2% albuterol/raffinose (diamond symbol) and cromolyn (square symbol). (a) Left panel: Emitted dose for a 3 mg blister package tested using the Nektar PDS. (b) Right panel: influence of trileucine content on the fine particle mass less than 3.3 µm by Andersen Cascade Impactor (albuterol, left column and cromolyn, right column) and surface concentration by XPS (albuterol, square symbols with doted line and cromolyn, solid line with solid squares). [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

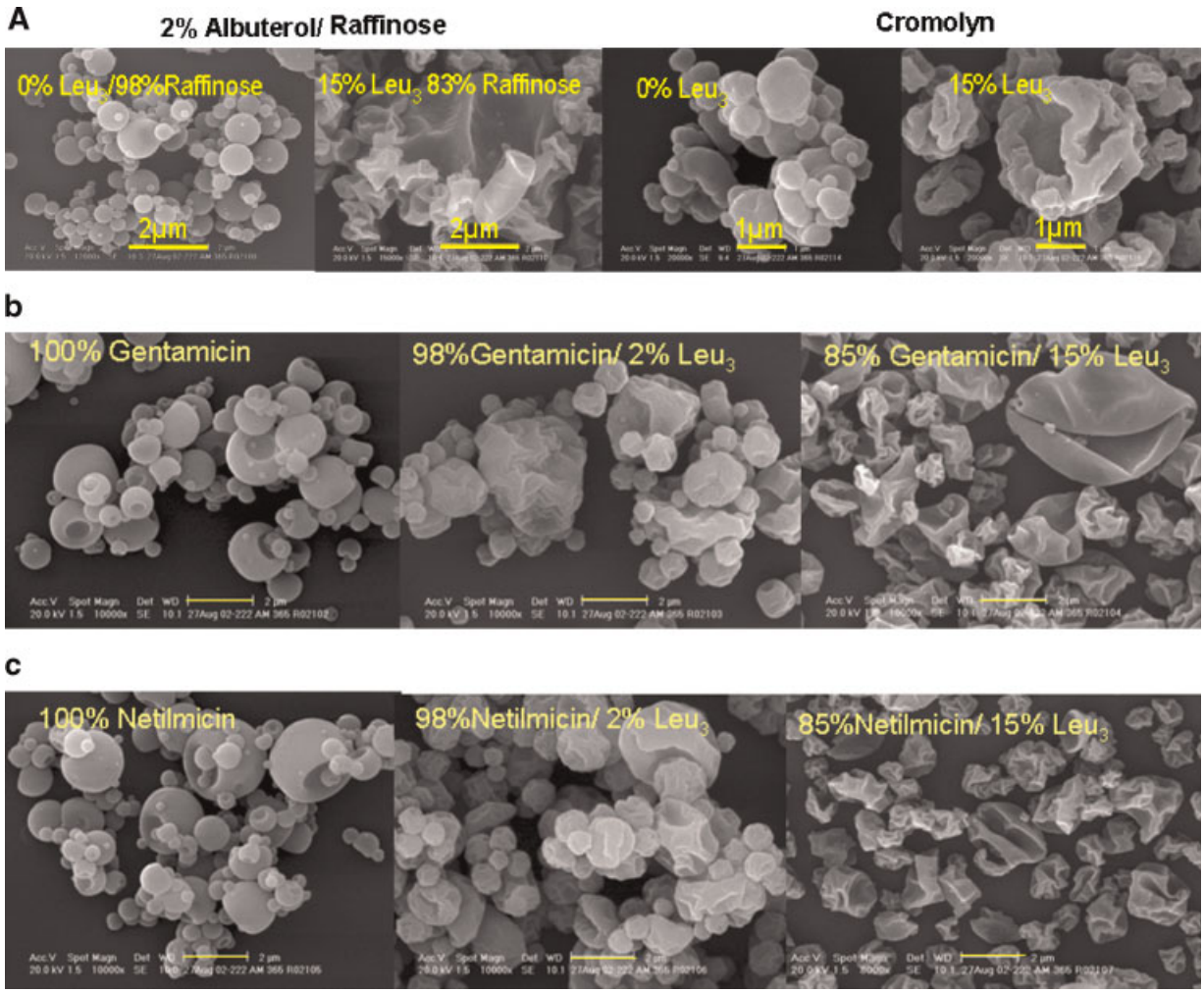

Figure 5. (a) Morphology of spray-dried particles of albuterol/raffinose (two left images) and cromolyn (two right images) at indicated levels of trileucine. (b) Morphology of netilmicin spray-dried particles at indicated levels of trileucine. (c) Morphology of gentamicin spray-dried particles at indicated levels of trileucine. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

The surface energy of spray-dried powders (γ_s) containing 2% albuterol with 5, 20, and 60% trileucine and 93, 78, and 38% raffinose was calculated from the gravimetrically determined acetone and octane adsorption isotherms at 25°C. Results in Table 2 describe the decrease in γ_s, where $\gamma_s = (\gamma_s^d \gamma_s^p)^{1/2}$, and γ_s^d is the dispersive (nonpolar) and γ_s^p is the polar component of the surface energy. Surface energy significantly decreased with increasing trileucine concentration,

Table 2. Surface Energy of Albuterol/Raffinose/Trileucine Powders Measured by Gravimetric Sorption Isotherms of Acetone and Octane (25°C)

Trileucine (wt%)	Emitted Dose (%)	Dispersive Component, γ_s^d (mN m^{-1})	Polar Component, γ_s^p (mN m^{-1})	Surface Energy, γ_s (mN m^{-1})
5	62	30.0	2140.3	254
20	78	30.9	1194.2	192
60	82	30.7	974.4	173

The surface energy decreases as trileucine content, and coverage, increases. In all cases, the albuterol concentration is 2% (w/w).

providing further support that trileucine decreases particle cohesivity

The observed enrichment in the trileucine surface concentration and corresponding decrease in the powder surface energy correlated with a depression of solution surface tension before spray drying. For example, addition of only 0.6% trileucine depressed the surface tension from 72 to 42 $mN \cdot m^{-1}$ at 25°C (Fig. 1). Similar results from pendant droplet data were observed trileucine a synergistic effect in the presence of hGH (Fig. 6).

As a result of its surface activity, trileucine molecules can orient the hydrophobic groups towards the air at the air/liquid interface during the drying process, providing a hydrophobic surface to the dry particle,[43] therefore contributing to the observed improved aerosol efficiency.

The modification of the surface properties of corrugated particles can be also inferred considering the effect of trileucine in the spray-dried powders containing salmon calcitonin (sCT). sCT has a low aqueous solubility and formed corrugated particles when spray dried alone (Fig. 7). Addition of 5 wt% of sCT altered the spherical morphology of spray-dried raffinose, producing corrugated particles (Fig. 7) and improved its dispersibility from about 30% to 48%. The ED of raffinose powders containing 5% sCT is furthered increased from 48% to 85% upon the addition of 60% trileucine (Fig. 8) even though the morphology is not dramatically altered (Fig. 7). Therefore, assuming that no further changes in the particle density and bulk density have been induced in the already corrugated particles the increase in ED can only be explained by a trileucine-induced decrease in the particle cohesiveness.

The effect of trileucine on peptide formulations was also tested. Formulations containing 30% trileucine and 70% hGH were prepared at pH 3.6 and 7.8. Trileucine is less soluble at pH 3.6, compared to 7.8, and should provide preferentially coated particles. This was confirmed by ED results which demonstrated EDs at 90 and 79% for pH 3.6 and 7.8, respectively. Therefore, increasing trileucine content resulted decreased surface tension, decreased MMAD, and increased ED.

As shown above, the powders discussed here may be considered to have similar particle size distributions. Thus the two factors determining their dispersion are morphology and cohesiveness. A qualitative analysis of the morphology of the spray-dried particles suggests that corrugated particles disperse better than spherical ones, consistent with the literature.[44] This is counter-intuitive because the number of inter-particle contacts of a random powder bed is minimized for spherical particles.[45] A plausible explanation

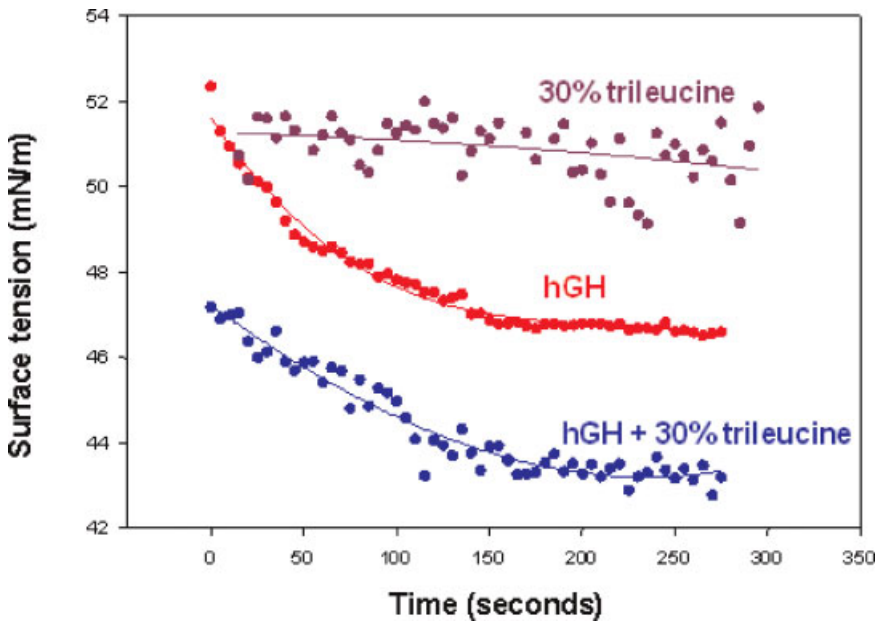

Figure 6. Dynamic surface tension of trileucine, human growth hormone, and trileucine/human growth hormone aqueous solutions measured with the pendant drop method at 25°C. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

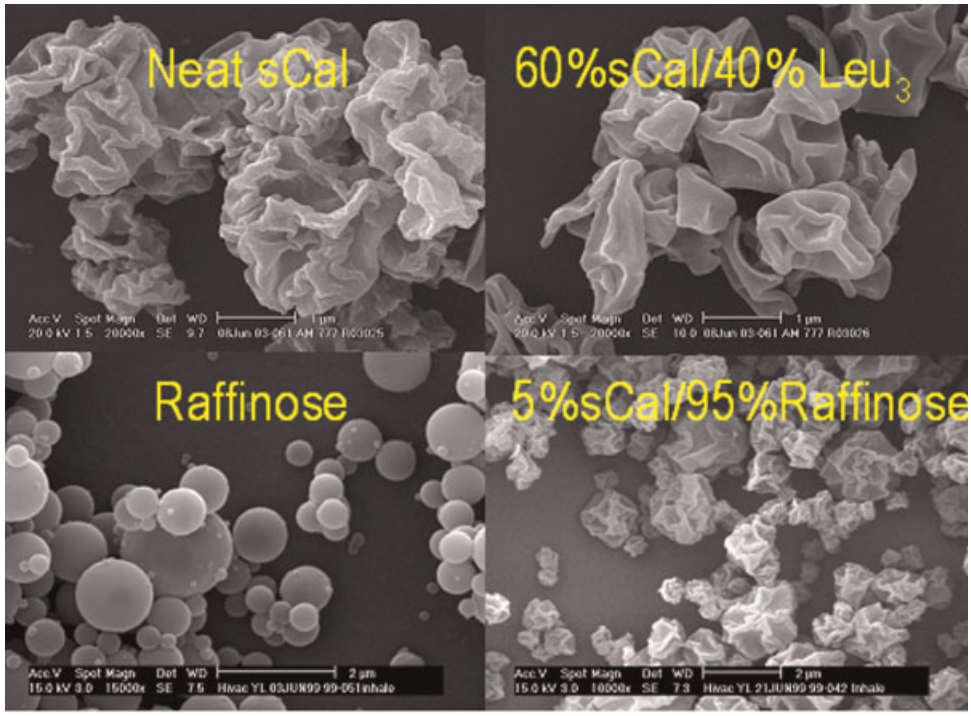

Figure 7. Morphology of spray-dried salmon calcitonin, neat and in raffinose, and trileucine containing formulations. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

why corrugated particles disperse better than spherical ones may be that corrugation increases the fraction of void volume, thereby reducing particle density and bulk density of the powder. This facilitates their aerosolization as more air is available to lift the particles. However, particle cohesiveness plays also an important role since the addition of trileucine at levels which did not significantly alter the morphology significantly increased their dispersibility. In addition trileucine also increased the dispersibility of already corrugated particles, highlighting the changes in particle cohesiveness.

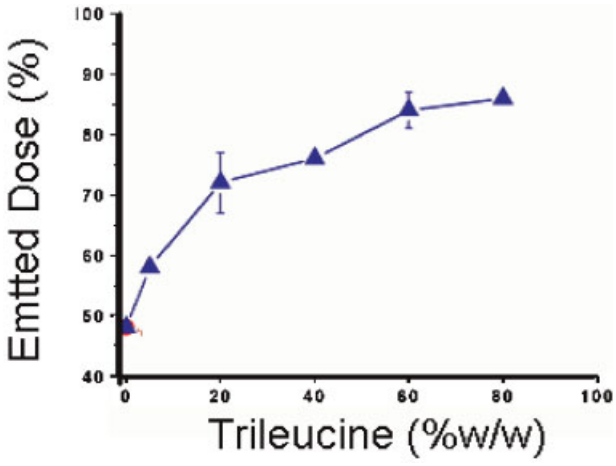

Figure 8. Effect of trielucine on the emitted dose of spray-dried salmon calcitonin/raffinose powders. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

Chemical and Physical Stabilization

Besides governing the surface properties, there are additional benefits of using trileucine to protect peptides both during atomization (creation of air/liquid interfaces), during drying, and in the solid state. Improved chemical and aggregation stability of sCT in high humidity environments is observed with the addition of trileucine (Fig. 9). Significant degradation (nearly 80% with respect to initial concentration) and decrease in monomer content (nearly 70% monomer loss) was observed when a spray-dried powder of 100% sCT was stored at 25°C and 75% RH for 7 days. In contrast, the initial sCT concentration and monomer content were nearly unchanged for a spray-dried powder of sCT formulated with 60% (wt/wt) trileucine. This is in striking contrast with spray-dried powders of sCT formulated with mannitol,

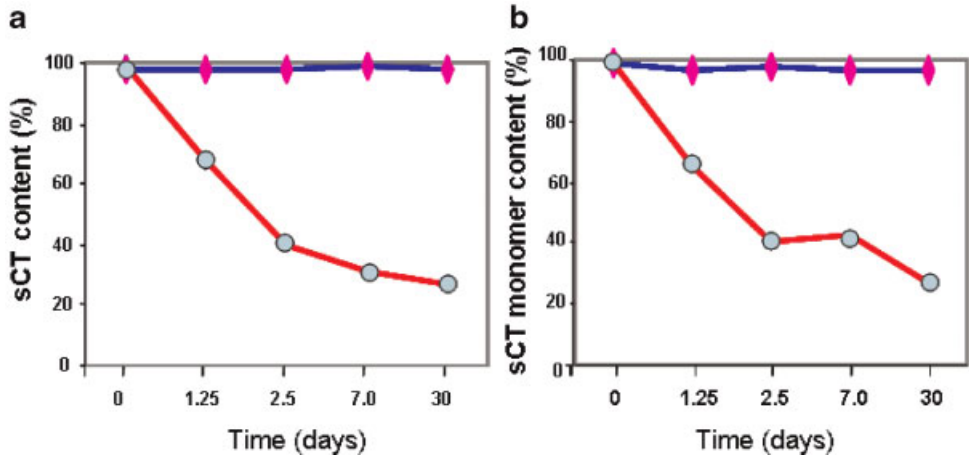

Figure 9. Effect of trileucine on spray-dried salmon calcitoinin (a) chemical stability and (b) aggregation. Salmon calcitonin neat (circle symbols) and formulated with 40%w/w trileucine (diamond symbols). [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

which are unstable when exposed to elevated humidity regardless of the concentration of mannitol.[46]

Similar results were obtained with hGH, where the primary degradation pathways include oxidation, deamidation and both covalent and noncovalent aggregation.[47–49] Aerosolized hGH has revealed sensitivity to mechanical stress in a nebulizer, where nebulization-induced stress resulted in extensive aggregation, and loss of monomer content from 98% to 10%.[50] When formulated with trileucine, hGH showed increased stability during the drying step, where 97% of the hGH was intact.[36] Formulations containing 30% trileucine and 70% hGH were prepared at pH 3.6 and pH 7.8. When hGH aggregation was compared to 100% hGH spray-dried formulations, a dramatic decrease in aggregation was observed due to trileucine preferential protection of hGH from air/liquid interfacial denaturation (Fig. 10a). This correlates well with the surface energy depression observed in solutions containing mixtures of trileucine and hGH. Furthermore, when the formulations were placed on stability, the trileucine containing formulations demonstrated better stability at 40°C/75RH for 6 months (Fig. 10b). For hGH, trileucine formulations at pH 7.8 provided increased stability, while formulations at pH 3.6 provided improved aerosol characteristics (as previously discussed), indicating that a balance must often be achieved to optimize a formulation.

CONCLUSIONS

The addition of small amounts of trileucine to a formulation produces stable dry powders for inhalation enrichment. This result in superior aerosol performance of otherwise difficult-to-formulate antibiotics, asthma therapeutics and peptide hormones. Overall, addition of trileucine to spray-dried particles for inhalation produced corrugated particles of low cohesivity. A threefold to tenfold enrichment of trileucine on the surface of the spray dried powders was observed. Physical stability of trileucine containing powders result from its relatively high glass transition temperature.

Water-soluble molecules tend to produce spherical particles and less soluble molecules produce corrugated particles. Trileucine-containing particles are corrugated, owing to trileucine's low aqueous solubility. This result in significantly improved aerosol performance. However, corrugation is not the only factor for improving aerosol performance, which seems to be also influenced by the low surface energy, noncohesive nature of trileucine particles, which is a reflection of its surface activity in solution.

Finally, peptide and protein aggregation and chemical degradation (deamidation and hydrolysis) can be reduced by the addition of trileucine in the formulation, presumably related to trileucine surface activity in solution.

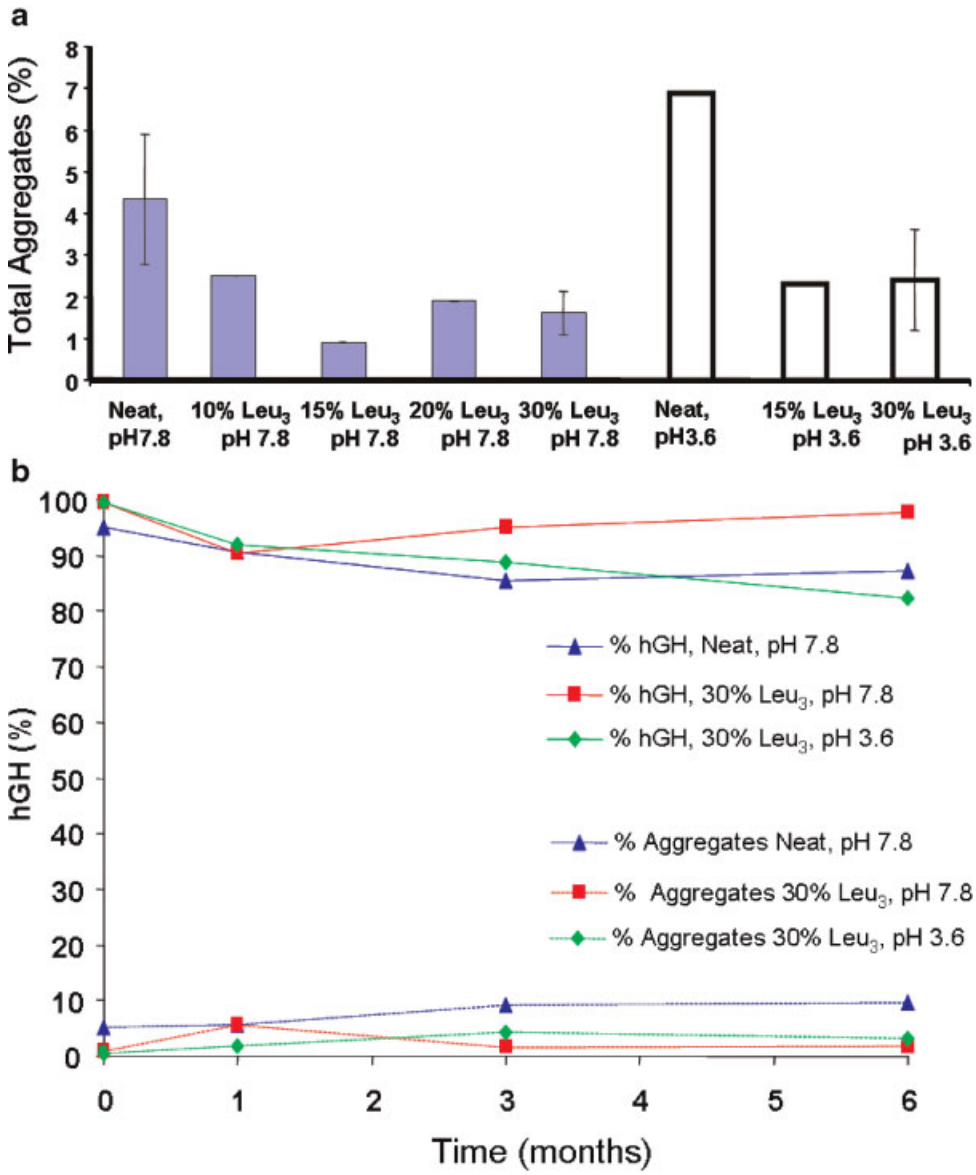

Figure 10. Effect of trileucine on human growth hormone (a) aggregation stability against spray drying interfacial denaturation and (b) stability at 40°C/75RH for 6 months. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

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