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Keywords: nanoporous/nanoparticulate microparticles, spray drying, trypsin, stability, aerosol, inhalation

Abstract

Objectives: The aim of this study was to determine if spray drying could successfully produce microparticles containing the model protein trypsin, suitable for inhalation.

Methods: Trypsin was spray dried with raffinose from a methanol:n-butyl acetate solvent system (MeOH:BA). The solvent system was then adjusted to include water and trypsin was co-spray dried with raffinose, trehalose or hydroxpropyl-β-cyclodextrin. The spray dried products were characterised by SEM, XRD, DSC, TGA and FTIR. Protein biological activity and *in vitro* deposition of trypsin:excipient nanoporous/nanoparticulate microparticles (NPMPs) was also assessed.

Key findings: The inclusion of water in a MeOH:BA solvent system allowed for the successful production of NPMPs of trypsin:excipient by spray drying. Trypsin formulated as trypsin:excipient NPMPs retained biological activity on processing and showed no deterioration in activity or morphological characteristics when stored with desiccant at either 4 °C or 25 °C. HP-β-CD showed advantages over the sugars in terms of producing powders with appropriate density and with greater physical stability under high humidity conditions. Fine particle fractions of between 41 and 45% were determined for trypsin:excipient NPMPs.

Conclusions: NPMPs of trypsin:excipient systems could be produced by spray drying with adjustment of the solvent system to allow for adequate solubility of trypsin.

1. Introduction

The inhaled route is widely used to deliver low molecular weight active pharmaceutical ingredients (APIs) to treat lung disease such as asthma, chronic obstructive pulmonary disease and infections. More recently the potential of the pulmonary route for local and systemic delivery of biological macromolecules has been explored. Systemic delivery of proteins or peptides via inhalation can be a more patient-friendly and cost-effective alternative to the parenteral route. Peptides/proteins which have been formulated for delivery by inhalation include insulin [1], interleukin-1, interferons and calcitonin [2].

Factors determining successful inhalation therapy include physicochemical properties of the active and formulation, biological aspects of the active ingredient(s) and the performance of the delivery device. Dry powder inhalers offer advantages over other delivery systems in terms of stability, and delivery efficiency and are the most popular studied method of protein delivery to the lungs [3, 4]. Significant formulation challenges exist however, in terms of maintaining adequate protein stability during processing and storage, and ensuring efficient and reproducible delivery to the lungs.

Efficient delivery of dry powders to the peripheral lung for systemic delivery, requires aerodynamic diameters of ~ 1-3 μm [5]. Spray-drying is a widely applied method for producing fine powders of proteins. Non-reducing sugars or other stabilising excipients may be added to the formulation to improve the process and storage stability of proteins [5,6]. Excipients may also be employed as carriers in DPI systems, to improve lung delivery either in a passive or an active capacity [4]. Where the protein/peptide to be used for pulmonary delivery is a low dose, high potency material, it may be desirable, for dry powder inhalation, to formulate with a carrier material (inert excipient) to increase the volume of powder loaded and delivered from the dry powder inhaler (DPI) device.

Porous particles represent a particle engineering solution to improving lung deposition. They may have smaller aerodynamic diameters than represented by their geometric diameters, as a result of low particle densities due to void spaces [7]. Advantages of porous particles for

pulmonary delivery have been extensively described in the literature, principally improved dispersibility and reduced inter-particulate interactions compared to non-porous particles [7, 8, 9, 10, 11, 12].

A one-step spray-drying method for producing nano-porous/nano-particulate microparticles (NPMPs) of bendroflumethiazide by spray drying from a mixed solvent system was initially described by Healy et al. [7]. Nolan et al. [13] subsequently continued this work, developing the method for budesonide, a low molecular weight therapeutically relevant active which is delivered by oral inhalation and used in the preventive treatment of asthma. More recently we described the extension of the method for producing NPMPs to hydrophilic compounds [14]. We also successfully applied the method to the production of composite NPMPs comprising a model protein (lysozyme) with a carrier sugar (trehalose or raffinose) [6].

Processing methods for proteins and peptides are generally developed on an individual case basis, depending on their suitability to the process and the need for stabilising or other excipient(s). Thus, formulation development for proteins tends to be on an individual basis. Lactose, sucrose, trehalose and raffinose have been investigated as examples of stabilising sugars in spray-drying and other process drying experiments and against specific experimentally-induced stresses for example liquid-solid interfacial stress [5, 15, 16]. Sub-optimal excipient concentration(s) may not adequately stabilise the protein, while excessive excipient can lead to destabilisation [17].

Trypsin has a similar size (23.5 kDa) and isoelectric point (10.3-10.5) to lysozyme (size: 14.7 kDa; isolelectric point: 11.35). Literature data concerning spray-drying of trypsin gives an indication of its lability to spray-drying stresses (from aqueous or aqueous buffer solution), but without the additional stresses of the proposed methods, i.e. the use of organic solvents. Trypsin has been spray-dried from aqueous solution at an inlet temperature 127 ± 4 °C resulting in a measured activity of 89.7 ± 4.1% of original unprocessed trypsin [18]. This is similar to the reported activity of lysozyme after spray-drying [15, 19, 20]. Trypsin was also spray-dried with various carbohydrates, lactose, sucrose, mannitol, α-cyclodextrin and dextrin, at a low protein load (0.2:99.8, 1:99, 5:95) from aqueous solution (pH 7) at an inlet

temperature of 180 °C [21]. Residual activity was in all cases ≥ 82% of original unprocessed trypsin, which was significantly better than the residual activity for the similar ratio of freeze-dried trypsin:carbohydrate. In all cases, when activity was plotted versus trypsin % in solid, the residual activity reached a plateau when the trypsin content was 1% or higher and residual activity was 90% or higher, except for the mannitol system where the activity was 82%.

Previous studies do not describe spray drying trypsin from non-aqueous solvents. The aim of the current study was to evaluate the general applicability of the NPMP production process which involves spray drying from organic solvents, by determining if the process could be applied to a different protein (i.e. trypsin) to that previously employed. The micromeritic properties of NPMPs make them suitable for aerosolisation and thus it is important to establish if the process has broad applicability before conducting studies on more therapeutically relevant, and expensive, bioactive macromolecules.

The physicochemical characteristics of the spray dried particulate systems produced and post processing biological activity of the protein will be established.

2. MATERIALS AND METHODS

2.1 Materials

d-(+)-trehalose dihydrate, d-raffinose pentahydrate and hyroxypropyl-β-cyclodextrin (HP-β-CD) raw materials were purchased from Sigma, Ireland. Trypsin (from bovine pancreas lyophilised powder, salt free ~9000 units/mg) was purchased from Fluka, USA. Freeze dried trypsin was prepared, as previously described by Chin et al. [22] and Bromberg and Klibanov [23], from aqueous solution pH3, with a view to increasing its solubility in methanol. Potassium bromide (KBr) was purchased from Sigma, Ireland. Methanol (MeOH) was purchased from Lab Scan Analytical Sciences, Ireland, while n-butyl acetate (BA) was

purchased from Merck, Germany. Deionised water (H_2O) was purified using a Purite Prestige Analyst HP water purification system. All other reagents were analytical grade.

2.2 Spray drying

All solutions prepared were spray dried with a Büchi B-290 Mini spray dryer. A nozzle tip of 0.7 mm and nozzle screw cap of diameter 1.5 mm were used. The Büchi B-290 was operated in the closed loop configuration, whereby the drying gas (nitrogen) is recycled to the drying chamber after precooling in a preheat exchanger and solvent condensation in a refrigerator unit (B-295 inert loop). The high performance (HP) cyclone, designed to improve the separation rate and collection efficiency of particles [24], was used.

In all cases the gas flow rate was 670 Nl/h (based on air, 4 cm on the gas rotameter indicator), the pump setting was 30% and the aspirator setting was 100%.

Systems spray dried and spray drying conditions (other than those detailed above) are summarised in Table 1. Trypsin (initially freeze-dried) was spray-dried with raffinose at a mass ratio of 1:4, from an 80:20 MeOH:BA solvent system. The total solute content (t.s.c.) was 0.5% w/v.

Trypsin:excipient mixtures were also spray dried from MeOH:BA:H_2O solvent systems. In all cases, trypsin and the excipient were first dissolved in the H_2O component, followed by addition of MeOH and subsequently BA, resulting in a precipitation with a marked observable increase in turbidity of the feed for all systems,. Spray-drying was carried out with stirring of the feed material to avoid settling of the suspension.

A solvent ratio of 79:19:2 (MeOH:BA:H_2O) and a t.s.c. of 0.5% w/v was selected for spray-drying trypsin:raffinose composites, based on preliminary studies on spray-drying raffinose alone. Trypsin:raffinose were spray-dried in mass ratios of 1:9, 1:4, 1:1 and are hereafter referred to by this mass ratio.

A solvent ratio of 49:49:2 (MeOH:BA:H_2O) and a t.s.c. of 0.3% w/v was selected for spr-drying trypsin:trehalose composites, based on preliminary studies on spray drying trehalose

alone. Trypsin:trehalose were spray-dried at mass ratios of 1:9, 1:4, 1:1 and are hereafter referred to by this mass ratio.

A solvent ratio of 15:15:1 (MeOH:BA:H_2O) was selected for spray-drying trypsin:HP-β-CD composites, based on preliminary studies on spray-drying HP-β-CD alone. A t.s.c. of 1.61% w/v was selected for the 1:9 and 1:4 mass ratio samples. The t.s.c. was further decreased to 0.81% w/v for the 1:1 mass ratio to try to minimise precipitation and loss of protein material on the walls of the feed container, as was considerable with the higher concentrations.

2.3 Characterisation of physicochemical properties of materials

X-ray powder diffraction (XRD) measurements were made on samples in low background silicon mounts, which consisted of cavities 0.5 mm deep and 9 mm in diameter (Bruker AXS, UK). A Philips PW1720 XRD was used which consisted of a PW1050/80 goniometer with a Cu fine focus tube (1.5 kW) and 1.0º dispersion slit, a 1.0º anti-scatter slit and a 0.2º receiving slit, operated at 40 kV and 20 mA. Measurements were taken from 5 to 40° on the two θ scale at a step size of 0.05° per s and were made in duplicate.

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) measurements were carried out under nitrogen purge as previously described [7]. DSC was performed using a Mettler Toledo DSC 821e (Mettler Toledo Ltd., U.K.) calorimeter. Samples (approximately 4 – 10 mg) were accurately weighed using a Mettler MT50 microbalance into 40 µl aluminium pans, which were sealed with a lid into which three vent-holes were pierced. A heating rate of 10 °C/min and temperature range of 25 - 300 °C, was used for all measurements. Mettler Toledo STAR^e software was used for capture and analysis of data. At least two measurements were made for each sample.

TGA measurements were carried out using a Mettler TG50 module with attached Mettler MT5 balance (Mettler Toledo Ltd., U.K.). Samples (approximately 4 - 12 mg) were accurately weighed, using a Mettler MT5 microbalance, into 40 µl aluminium pans or lids. Aluminium pans or lids were left open for the duration of the analysis. A heating rate of 10 °C/min and

temperature range of 25-200/300 °C, was used for all TGA experiments. Mettler Toledo STAR^{e} software was used for capture and analysis of data. At least two measurements were made for each sample.

Bulk and tapped density measurements were performed as previously described [7]. Bulk density (bρ) was calculated by determining the weight of powder required to occupy a 1 ml volume in a graduated glass syringe (Lennox Laboratory supplies, Dublin, Ireland), by pouring under gravity. The tapped density (tρ) of the powders was determined by vertically tapping this sample onto a level bench-top surface from a height of 5 cm for 100 times. The tapped density was calculated as the ratio of the mass to the tapped volume of the sample. Analyses were limited to one run for trypsin:trehalose, and trypsin:raffinose composites due to powder quantity, but the standard deviation calculated from three replicate measurements of the trypsin:HP-β-CD composite, indicative of inter-batch sample variation, was low.

Powder samples were visualised using a Hitachi S-4300 field emission scanning electron microscope (Hitachi Scientific Instruments Ltd., Japan) as previously described [7]. Samples were fixed on aluminium stubs using double-sided adhesive tabs and then sputter-coated with gold. Samples were visualised at 5 kV.

Particle size was determined from scanning electron micrographs as previously described [14]. The mean particle diameter (Feret's diameter) was calculated using SEM photographs. The diameter of at least 150 particles was measured and the mean particle diameter was taken as the average of these measurements.

2.4 *In vitro* deposition studies using the Andersen Cascade Impactor (ACI)

Aerodynamic assessment of fine particles was carried out using an Andersen cascade impactor (ACI) and Handihaler device as previously described [13] and in the British and European Pharmacopeias [25, 26].

Briefly, a size 3 hard gelatin capsule (Farillon Ltd., U.K.) was filled with approximately 25 mg powder, and placed in the Handihaler device (Boehringer Ingelheim, Germany). A pressure

drop of 4 kPa was established over the Handihaler device using a Critical Flow Controller Model TPK (Copley Scientific Ltd. UK) and sample collection tube. Flow rate stability was ensured by measuring the absolute pressure on either side of the flow control valve (p2, p3), with a ratio of p3/p2 ≤ 0.5 indicating sonic flow. The flow rate, Q, required to produce a pressure drop of 4 kPa, was measured by attaching a flow meter model DFM2 (Copley Scientific Ltd. UK) in place of the inhaler. A 4 l inspiration volume was achieved by setting the timer so that t = [4*60/Q] s. Two actuations were used to empty the capsule from the Handihaler device, as per the inhaler manufacturer's instructions. The average measured flow produced was ~50 l/min.

After the experiment, retained/deposited powder was collected from the capsule shell, device, mouthpiece, induction port, and each individual stage plus the impaction plate/filter below it, by rinsing and making up to suitable volume with water.

Analysis of the protein was carried out using a Pierce Micro BCA protein assay kit® (Pierce, Rockford, IL, USA) (see section 2.6).

The "recovered dose", being the total amount of powder collected from the device, capsule and impactor was calculated, and the analysis was accepted if this fell within 75% - 125% of the nominal loaded dose [27]. If the measured recovery was outside the range 75%-125%, the experiment was excluded from the analysis. A table of cumulative mass% (as % of the total recovered emitted dose) versus effective cut-off diameter for each stage of the impactor was constructed. Fine particle fraction (fraction of the emitted dose having aerodynamic diameter < 5 µm) and particle fraction with aerodynamic diameter < 3 µm were calculated by interpolation from the linear portion of the log normal graph of this data. Experimental mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were similarly determined by interpolation from the linear portion of the log normal plot of cumulative mass% (as % of the total dose recovered from the impactor stages) versus effective cut-off diameter.

Analyses were run in triplicate and expressed as mean ± standard deviation.

2.5 Secondary protein structure as determined by FTIR

Solid state samples were prepared as KBr disks. Protein:excipient composite samples were mixed with KBr at a ratio of 1:100 or 1:200 using an agate mortar and pestle. Disks were compressed at 8 tonnes pressure for 2 minutes using a 13 mm KBr die set (Apollo scientific, U.K.). FTIR spectra were obtained using a Nicolet Magna 560 Spectometer and Omnic software Ver 4.1. 64 scans were run for each sample at resolution of 2 cm^{-1}.

The spectrum was baseline corrected, and smoothed using automatic functions in Omnic software 4.1. The area between 1720 – 1600 (amide 1) was truncated, as described by Van De Weert et al. [28], and exported into Origin software. Baselines of isolated peaks were corrected by subtraction of a straight line, across the base of the peak prior to area normalisation. Areas of comparative peaks were normalised to 100 by integration and simple math functions. The absolute difference spectrum of two area-normalised peaks was calculated. Integration of this absolute difference spectrum gave the area of non-overlap between two area-normalised spectra [29]. Area of overlap was then calculated from (100 – area of non-overlap), giving a semiquantitative measure of the similarity of the shape of the peaks.

2.6 Trypsin quantitation

Trypsin quantitation was carried out using a Pierce Micro BCA protein assay kit® (Pierce, Rockford, IL, USA), as previously described for lysozyme [6]. Standards were prepared in water and dilutions for at least 2 standard curves were prepared (range 0.04 – 0.005 mg/ml). The assay was performed following the plate assay protocol recommendations, as provided by the manufacturer. Briefly, 150 µl of appropriately diluted solutions of test/standard were transferred into a clear flat-bottom 96 well micro-plate (Sarstedt, Inc.) in duplicate. 150 µl of the working reagent was added to each well. Plates were covered, manually shaken and incubated in an oven (economy incubator with fan size 1, Gallenkamp) for 2 hours at 37 °C, and allowed to cool to room temperature. The samples were analysed for a colorimetric change measured as the optical density (O.D.) at 562 nm using a UV plate reader

(Microplate Autoreader EL311, Bio-Tek Instruments, U.S.A.). The O.D. of each sample was compared to the average standard curve, and the concentration of lysozyme was extrapolated from this standard curve.

2.7 Trypsin activity assay

Activity of trypsin samples was assessed by a continuous spectrophotometric rate determination assay as described by USP [27] for crystallised trypsin and by Forbes et al. [18]. Measurements were carried out at 25 °C, pH 7.6, using a heλios UV/Vis spectrometer with attached Thermo Spectronic Single Cell Peltier (Thermo Electron Corp., U.S.A.).

Protein concentration in the test solution was assessed by BCA protein quantitation using a Pierce Micro BCA protein assay kit (Section 2.6).

2.8 Stability studies

Solid state stability studies were conducted using the conditions of temperature and humidity recommended in the ICH protocol for long-term testing, i.e. 25 °C/60% relative humidity (RH) (ICH, 2003). Solid samples were placed in open jars in a glass chamber containing a saturated solution of sodium bromide to maintain a constant RH of 60% [30]. The glass chamber was stored at 25 ˚C in an incubator (Gallenkamp, UK).

Samples were also stored at refrigerator temperature of 4 ℃ in a sealed desiccator containing silica gel desiccant and at 25 ℃ in a sealed desiccator containing silica gel desiccant. At appropriate time intervals, samples were removed for subsequent analysis.

2.9 Statistical analysis

Comparison of means was conducted by one way ANOVA using Minitab v14 software. A difference or effect was considered to be significant at a significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1 Spray-dried trypsin:raffinose from methanol:n-butyl acetate

Trypsin (initially freeze-dried) was spray-dried with raffinose at a mass ratio of 1:4, from an 80:20 MeOH:BA solvent system, which was the solvent system that was used previously for lysozyme: raffinose in a similar 1:4 ratio [6]. Freeze-dried trypsin could not be dissolved with manual agitation or with stirring in the initial MeOH component (0.125% w/v). The feed mixture was spray-dried as a suspension. Resultant spray-dried material comprised of a mixed morphology of flakes and small fused spheres when viewed under SEM (Figure 1(a)). A small amount of water was therefore added to the MeOH:BA solvent system to enable protein dissolution, prior to addition of the organic solvents.

3.2 Spray-dried trypsin:excipient from methanol:n-butyl acetate:water

3.2.1 Particle morphology and micromeritic characteristics

Particle morphologies for the composites of 1:9 and 1:4 and 1:1 trypsin:raffinose mass ratios were similar, as seen from SEM analysis (Figure 1 (b), (c), (d)), being a mix of spherical to slightly irregular nano-particulate/nanoporous particles and nonspherical nano-particulate aggregates.

In the case of trypsin:trehalose composites, 1:9 and 1:4 trypsin:trehalose mass ratio systems consisted of spherical nano-particulate/nanoporous particles (Figure 2 (a), (b)). Particle morphology for the mass ratio 1:1 composite was a mixture of spherical nanoparticulate/ nanoporous particles and non-spherical nano-particulate aggregates (Figure 2 (c)).

Particle morphologies were similar for the three mass ratio composites of trypsin: HP–β–CD, 1:9, 1:4 and 1:1 (Figure 3). Spherical nano-particulate/nanoporous particles and non-spherical nano-particulate aggregates were observed. Some fusion/contact between smaller particles was observed.

Trypsin:excipient NPMP composites (spray-dried with a mass ratio of 1:4) were selected for preliminary particle characterisation, in terms of bulk density, tapped density and particle size analysis.

Composite powders had low measured bulk and tap densities as shown in Table 2. The highest bulk density value was calculated for the trypsin:HP-β-CD composite and the lowest

bulk density value calculated for the trypsin:raffinose composite. This suggests that of the composite systems prepared, that containing HP-β-CD might be more easily handled and would enable high powder loading in a DPI device. Estimation of particle size from SEM images indicated no statistically significant difference in average particle size between any of the trypsin:excipient (1:4) systems. In all cases the average particle size was less than 2 μm (Table 2). This indicates suitability of the process to produce particles of appropriate dimensions for pulmonary drug delivery. It is generally accepted that such particles should be less than 3 μm in aerodynamic diameter to achieve a systemic effect [5].

3.2.2 Process stability: Solid-state characterisation

3.2.2.1 Trypsin:raffinose composites

Glass transitions were detected in the DSC scans of mass ratio composites 1:9 and 1:4 at ~120 °C (Figure 4A). No recrystallisation peaks were identified, similar to raffinose spray-dried alone [6]. An endothermic peak was identified in all samples at ~190 °C, attributed to decomposition, which is slightly lower than for raffinose spray-dried alone (~ 220 °C, [6]). Initial mass losses over the temperature range 25 - 100 °C of ~ 4.2 – 4.6% were calculated. The shape of these mass loss steps (not shown) was similar to spray-dried raffinose alone (amorphous material) [6]. Further mass losses corresponding to decomposition starting at ~ > 180 °C were observed from TGA. As expected, spray-dried composites were also shown to be amorphous by XRD

3.2.2.2 Trypsin:trehalose composites

Broad endothermic peaks were observed from 25 - ~120 °C in DSC scans (Figure 4B). Glass transitions were identified for mass ratio composites 1:4, and 1:1 at ~125 °C. This area was obscured in the 1:9 mass ratio composite by the initial endothermic peak. An exotherm was identified in the DSC of the sample of mass ratio 1:4 and, although smaller, also in the 1:9 mass ratio composite, before a sharp endothermic peak (~ 213 °C) attributed

to melting of anhydrous trehalose. These exotherms most likely correspond to crystallisation of the sugar. Issues of amorphous/amorphous phase separation of sugars and globular proteins and experimental limitations to its detection have been discussed by Hill et al. [31]. Exothermic crystallisation peak(s) or a melting peak of anhydrous trehalose were not identified in the DSC scan of the composite of mass ratio 1:1. Decomposition was further noted from the DSC scans as endothermic areas starting at ~225 – 240 °C. Initial mass losses over the temperature range 25-100 °C of 4.5 – 6.7% were determined and further mass losses corresponding to decomposition starting at ~ 220 °C were observed from TGA. Spray-dried composites were XRD amorphous.

3.2.2.3 Trypsin:HP-β-CD composites

Broad endothermic peaks were observed from 25 - ~120 °C in DSC scans (Figure 4C), followed by small endotherms at ~ 220 °C, which may correspond to the melting point of the trypsin component. This peak enthalpy (J/g) increased with increasing trypsin content. Decomposition was further noted in DSC scans as endothermic areas starting at ~255 – 260 °C. Initial mass losses over the temperature range 25 - 100 °C of 5 – 6.6% were calculated, with further mass losses corresponding to decomposition starting at ~250 - 260 °C observed from TGA. The spray-dried composites were XRD amorphous.

3.2.3 Process stability: Secondary structure and biological activity

The percentage area of overlap (using the FTIR method) of the normalised amide 1 region of the trypsin:raffinose mass ratio composite 1:4 with that of unprocessed trypsin was 85%. The percentage overlap was 82% for the trypsin:trehalose 1:4 mass ratio composite and 80% for the trypsin:HP-β-CD 1:4 mass ratio composite. These values compare to an area of overlap of two batches of unprocessed trypsin of 97.5%.

Biological activity assays were carried out on the 1:9, 1:4 and 1:1 trypsin:excipient composites, and compared to unprocessed trypsin. Activity of spray-dried powders (units/mg

348 solid) and specific activity (units/mg protein) were calculated. Results are expressed in terms
349 of units and % of activity of control solution (Table 3). Test solutions were prepared so as to
350 contain a protein concentration of ~ 0.044 mg/ml. The amount of protein in the test solutions
351 were quantified by micro BCA protein analysis, and were similar to the expected
352 concentrations. There was no statistically significant decrease in specific activity compared
353 to control for any of the composites, except for a slight decrease for the trypsin:raffinose 1:9
354 mass ratio composite (92.0%). Thus, the process compares favourably to the previously
355 reported process of spray drying trypsin without excipient from aqueous solution, in which
356 case a significant reduction in trypsin activity was noted [18].
357
358 **3.2.4 Storage Stability**
359 Trypsin:excipient NPMP composites with a mass ratio of 1:4 spray-dried from
360 MeOH:BA:H_2O were selected for investigation of temperature-dependant storage stability.
361 The conditions 4 ^oC/desiccant and 25 ^oC/desiccant were selected for a 12-week stability
362 study. A small amount of the composite materials was also stored at 25 ^oC/60% RH, with
363 SEM and DSC analysis after storage for 24 hours. A similar study was carried out previously
364 for lysozyme:excipient composites spray-dried from a MeOH:BA system [6].
365
366 3.2.4.1 Storage under desiccant conditions at 4 ^oC and at 25 ^oC
367 Particle morphology of trypsin:raffinose, trypsin:trehalose and trypsin:HP-β-CD particles
368 were unaffected by storage for 12 weeks under desiccant conditions at 4 ^oC and at 25 ^oC, as
369 can be seen from SE micrographs taken at week 12 (Figure 5) and compared to SE
370 micrographs of freshly spray-dried composites (Figures 1(c), 2(b), 3(b)).
371 Trypsin:raffinose, trypsin:trehalose and trypsin:HP-β-CD composites were XRD amorphous,
372 with no change in the amorphous halos, after 12 weeks storage at 4 ^oC/desiccant and at 25
373 ^oC/desiccant. The distinctive peaks attributable to crystalline raffinose pentahydrate at
374 10.75^o, 13.65^o and 21.1^o [6] were not identified in any of composite samples.

No changes indicating crystallisation or increased moisture uptake were observed from DSC or TGA for any the composites stored under desiccant conditions at 4 °C, or at 25 °C for 12 weeks.

No decrease in % specific activity was observed for trypsin:excipient composites (1:4) stored at 4 °C/desiccant for 12 weeks compared to control (similar to the freshly spray-dried starting material batches) (Table 4). A decrease in % specific activity was observed for all composites stored at 25 °C/desiccant for 12 weeks compared to control however, and this was statistically significant ($p=0.002$), with no excipient statistically significantly better than another.

3.2.4.2 Storage at 25 °C / 60% R.H.

Storage of trypsin: raffinose and trypsin: trehalose (1:4) composites at 25 °C / 60% RH for 24 hours resulted in particle collapse – loss of particle structure and surface morphology when viewed under SEM (Figure 6(a) and (b)). Storage of typsin:HP-β-CD (1:4) composite at 25 °C / 60% RH for 24 hours did not exhibit this, with retention of particle structure and shape and porous surface morphology when viewed under SEM (Figure 6(c)).

Crystallisation of trypsin:trehalose and trypsin:raffinose (1:4) composites stored at 25 °C / 60% RH for 24 hours was indicated by sharp endothermic peaks observed in the temperature range 80 – 100 °C, suggesting loss of hydrate water, in DSC scans (Figure 7(a) and (b) – marked by boxes). No change was observed in the DSC scan of trypsin:HP-β-CD (1:4) composite stored at 25 °C / 60% RH for 24 hours (Figure 7(c)), compared to starting material. Thus the use of HP-β-CD as the stabilising excipient may provide greater protection under high humidity conditions than either of the sugar excipients. Previous studies have shown that peptide powders formulated with sugars are often sensitive to increased relative humidity and are too cohesive [32]. Trehalose is known to be hygroscopic and spray dried powders comprising trehalose were previously shown to adsorb about 5 and 11% water at 30 and 50% RH respectively, by DVS analysis [33], while spray dried HP-β-

CD adsorbed less at the same RH - about 4% and 7% water at 30% and 50% RH respectively [34]. Additionally, it has been shown that various β-cyclodextrin derivatives can function as aggregation suppressors for a wide range of proteins [35].

3.2.5 Aerosol characterisation

Trypsin:excipient NPMP composites (spray-dried with a mass ratio of 1:4) were selected for *in-vitro* deposition via Andersen cascade impactor analysis.

It is generally accepted that particles with an aerodynamic particle size of 1-5 μm are required to avoid deposition in the mouth or throat and reach the lungs. Small peptides and proteins appear to be best absorbed in the lowest stages (higher generation number) of the respiratory tract (36). Particles with aerodynamic diameters of 1-3 μm may thus be considered optimal, because they are sufficiently small to reach the deep lung and alveoli.

In vitro deposition experiments were conducted with two replicates on one batch each of trypsin:trehalose, trypsin:raffinose and trypsin:HP-β-CD. Results of *in vitro* deposition experiments were compared using ANOVA to determine statistical significance (significance level $\alpha = 0.05$). No statistically significant difference was determined between the trypsin:excipient composites for any of the calculated parameters (Table 5). Average fine particle fractions (< 5 μm) of 41.55 – 44.82% and average particle fractions < 3 μm of 26.21 – 29.60% were calculated from ACI analysis (Table 5).

Breakdown of deposition by stage of the apparatus showed high deposition in the mouthpiece adapter and induction port, and within the stages of the impactor, deposition in the mid and lower stages – particularly stages 3, 4, and 5 (Figure 8). Average MMAD values of 2.76 - 3.18 μm were calculated for the composites. High average GSD vales of 2.29 – 2.65 were calculated, suggesting variation in aerodynamic diameter.

The lower average particle size (Table 2) estimated from SEM analysis compared to MMAD suggests particle aggregation. Fusion was also observed in SEM analysis, between

particles, which may also have contributed to the high deposition in the mouthpiece adaptor/induction port.

Fine particle fractions (FPF) of NPMPs of lysozyme:excipient (1:4 ratio) [6] and trypsin:excipient (1:4 ratio) (Table 5) were compared by ANOVA. The analysis indicates that the lysozyme composite NPMPs spray-dried from MeOH:BA systems resulted in statistically significantly higher ($p<0.05$) FPF than trypsin composite NPMPs spray-dried from MeOH:BA:H_2O systems. The looser surface morphology and SEM evidence of deviation from sphericity [14] and particle-particle contact of trypsin composite systems may have influenced the lower FPF calculated.

4. Conclusions

A MeOH:BA solvent system, which had previously been used for producing composite NPMPs of lysozyme with sugar excipient by spray drying, could not be used to produce composite NPMPs of trypsin with excipients (trehalose, raffinose and HP-β-CD) and resulted in a mixed morphology of flake-like and spherical particles. The insolubility of trypsin was postulated to be problematic in this system. Water was therefore added to the solvent system and the MeOH:BA:H_2O solvent system was successfully used to produce porous particles of trypsin:excipient in mass ratios of 1:9, 1:4 and 1:1 with excellent retention of biological activity in all cases, comparable or better than the previously reported activity of trypsin spray dried from aqueous solution with or without carbohydrate stabilisers [15, 19]. This may be due to the low spray drying temperatures achievable with the organic solvent system used.

Particle characterisation of the 1:4 mass ratio trypsin:excipient systems showed favourable bulk and tapped density and particle size estimated from SEM analysis. *In-vitro* deposition experiments showed that the 1:4 mass ratio composites had good *in-vitro* deposition properties.

This method of spray-drying protein with excipient from a MeOH:BA:H_2O system may represent an alternative method of producing NPMPs for water soluble proteins/peptides. Of the excipients used, HP-β-CD showed advantages over the sugars, raffinose and trehalose in terms of maintaining physical stability under high humidity conditions and preparing powders with lower bulk volume.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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Table 1. Summary of systems spray dried and spray drying inlet and outlet temperatures.

System (mass ratio put into solution)	Solvent (volume ratio) MeOH:BA:H_2O	Solution concentration (% w/v)	Inlet/Outlet temperature ($^oC/^oC$)
Trypsin:Raffinose (1:4)	80:20:0	0.5	100/64
Trypsin:Raffinose (1:9)	79:19:2	0.5	100/57
Trypsin:Raffinose (1:4)	79:19:2	0.5	100/53
Trypsin:Raffinose (1:1)	79:19:2	0.5	100/50
Trypsin:Trehalose (1:9)	49:49:2	0.3	100/58
Trypsin:Trehalose (1:4)	49:49:2	0.3	100/52
Trypsin:Trehalose (1:1)	49:49:2	0.3	100/55
Trypsin:HP-β-CD (1:9)	15:15:1	1.61	100/59
Trypsin: HP-β-CD (1:4)	15:15:1	1.61	100/51
Trypsin: HP-β-CD (1:1)	15:15:1	0.81	100/55

Table 2. Average particle size, bulk and tapped densities for composites as shown (spray-dried mass ratio 1:4); *n=3; average ± standard deviation, **n=1

Composite (mass ratio 1:4)	Particle size (μm)	Bulk Density (g/cm^3)	Tapped Density (g/cm^3)
trypsin:trehalose	1.51 ± 0.74	**0.0878	**0.1596
trypsin:raffinose	1.79 ± 0.81	** 0.0649	**0.118
trypsin:HP-β-CD	1.84 ± 1.17	*0.0946 ± 0.0015	*0.1523 ± 0.0063

Table 3. Summary of biological activity assay for trypsin:excipient composites (mass ratios as shown, spray-dried from MeOH:BA:H_2O); n=3; results expressed as average ± standard deviation; *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

1:9 mass ratio	Units/mg protein	% Activity	% Specific activity
Trypsin:trehalose	9239 ± 333	8.5 ± 2.1*	95.4 ± 5.9*
Control	9682 ± 525	100 ± 2.1*	100 ± 5.9*
Trypsin:raffinose	8841 ± 247	11.2 ± 1.7*	92.0 ± 2.9*
Control	9606 ± 356	100 ± 1.7*	100 ± 2.9*
Trypsin:HP-β-CD	9499 ± 763	12.7 ± 2.1*	98.1 ± 5.9*
Control	9682 ± 525	100 ± 2.1*	100 ± 5.9*

1:4 mass ratio	Units/mg protein	% Activity	% Specific activity
Trypsin:trehalose	9120 ± 260	20.8 ± 1.7*	94.9 ± 2.9*
Control	9606 ± 356	100	100
Trypsin:raffinose	7181 ± 121	22.5 ± 1.0*	100.8 ± 1.5*
Control	7122 ± 41	100	100
Trypsin:HP-β-CD	9305 ± 218	20.0 ± 1.7*	96.9 ± 2.9*
Control	9606 ± 356	100	100

1:1 mass ratio	Units/mg protein	% Activity	% Specific activity
Trypsin:trehalose	7287 ± 170	52.6 ± 1.0*	102.3 ± 1.5*
Trypsin:raffinose	7238 ± 57	55.4 ± 1.0*	101.6 ± 1.5*
Control	7122 ± 41	100	100
Trypsin:HP-β-CD	6379 ± 88	36.2 ± 4.1*	94.2 ± 3.7*
Control	6770 ± 387	100	100

Table 4. Summary of biological activity assay for trypsin:excipient composites – freshly prepared, stored at 4 °C/desiccant or 25 °C/desiccant for 12 weeks (1:4 mass ratio, spray-dried from MeOH:BA:H_2O); n=2; results expressed as average (range); *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/mg protein	% Activity	% Specific activity
Starting			
Trypsin:trehalose	8012 (8353, 7671)	19.1 ± 2.1	102.8 ±3.3
Trypsin:raffinose	7949 (7922, 7976)	21.9 ± 2.1	102.0 ±3.3
Trypsin:HP-β-CD	7650 (7646, 7653)	19.0± 2.1	98.2 ±3.3
Control	7790 (7919, 7661)	100± 2.1	100 ±3.3
4 ℃/des. Wk 12			
Tryp:trehalose	7339 (7740, 6938)	18.1 ± 1.9	97.1 ± 4.4
Tryp:raffinose	7480 (7657, 7302)	21.6 ± 1.9	99.0 ± 4.4
Tryp:HP-β-CD	7398 (7503, 7292)	18.2 ± 1.9	97.9 ± 4.4
Control	7557 (7684, 7430)	100 ± 1.9	100 ± 4.4
25 ℃/des. Wk 12			
Tryp:trehalose	6499 (6488, 6510)	16.7 ± 1.8	86.0 ± 1.8
Tryp:raffinose	6221 (6318, 6123)	17.5 ± 1.8	82.3 ± 1.8
Tryp:HP-β-CD	6243 (6345, 6140)	16.0 ± 1.8	82.6 ± 1.8
Control	7557 (7684, 7430)	100 ± 1.8	100 ± 1.8

Table 5. Summary of results from Andersen cascade impactor analysis for trypsin:trehalose, trypsin:raffinose and trypsin:HP-β-CD NPMP composites spray-dried in a mass ratio of 1:4; n = 2, mean (range).

Composite (mass ratio 1:4)	% < 5 µm	% < 3 µm	MMAD (µm)	GSD	% emitted (of nominal dose)
trypsin:trehalose	42.71 (41.57, 43.86)	26.21 (24.80, 27.62)	2.83 (2.92, 2.74)	2.29 (2.23, 2.34)	77.6 (77.7, 77.4)
trypsin:raffinose	44.82 (39.96, 49.68)	29.60 (25.53, 33.68)	2.76 (2.99, 2.52)	2.51 (2.56, 2.46)	81.7 (85.0, 78.4)
trypsin:HP-β-CD	41.55 (43.08, 40.02)	27.82 (29.22, 26.42)	3.18 (3.33, 3.03)	2.65 (2.55, 2.76)	80.3 (79.8, 85.0)

Figure 1 SE micrographs showing trypsin:raffinose spray-dried (a) from MeOH:BA solvent system in a mass ratio of 1:4 and spray dried from MeOH:BA:H_2O solvent system in mass ratios (b) 1:9 (c) 1:4 (d) 1:1.

Figure 2 SE micrographs showing trypsin:trehalose spray-dried from MeOH:BA:H_2O solvent system in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.

Figure 3 SE micrographs showing trypsin:HP-β-CD spray-dried from MeOH:BA:H_2O solvent system in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.

Figure 4 DSC scans of (A) trypsin:raffinose, (B) trypsin:trehalose and (C) trypsin:HP-β-CD spray-dried in mass ratios of (i) 1:9 (ii) 1:4 (iii) 1:1.

Figure 5 SE micrographs of composite particles (spray-dried ratio 1:4) after 12 weeks storage at 4 ^{o}C/desiccant and at 25 ^{o}C/desiccant (a), (d) trypsin:raffinose; (b), (e) trypsin:trehalose; (c), (f) trypsin:HP-β-CD.

Figure 6 SE micrographs of composite particles after 24 hour storage at 25 ^{o}C/60% RH (spray-dried ratio 1:4) (a) trypsin:raffinose (b) trypsin:trehalose (c) trypsin:HP-β-CD.

Figure 7 DSC scans of composite particles (mass ratio 1:4) (a) trypsin:raffinose (b) trypsin:trehalose (c) trypsin:HP-β-CD taken after 24 hours storage at 25 ^{o}C/ 60% RH (solid line), freshly prepared (broken line).

Figure 8 Deposition patterns on the stages of the ACI apparatus for the composites as indicated (spray-dried mass ratio 1:4). Calculated as % of recovered emitted dose (mouth –

filter), used to calculate fine particle fraction. White bars: trypsin:trehalose, light grey bars: trypsin:raffinose and dark grey bars: trypsin:HP-β-CD.

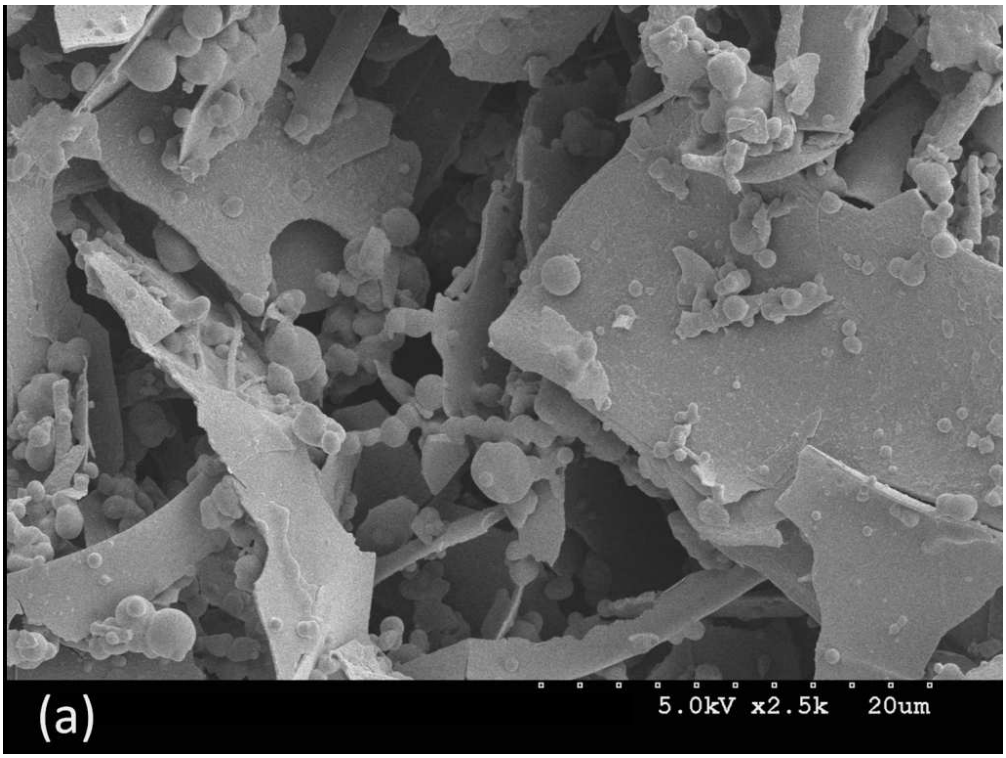
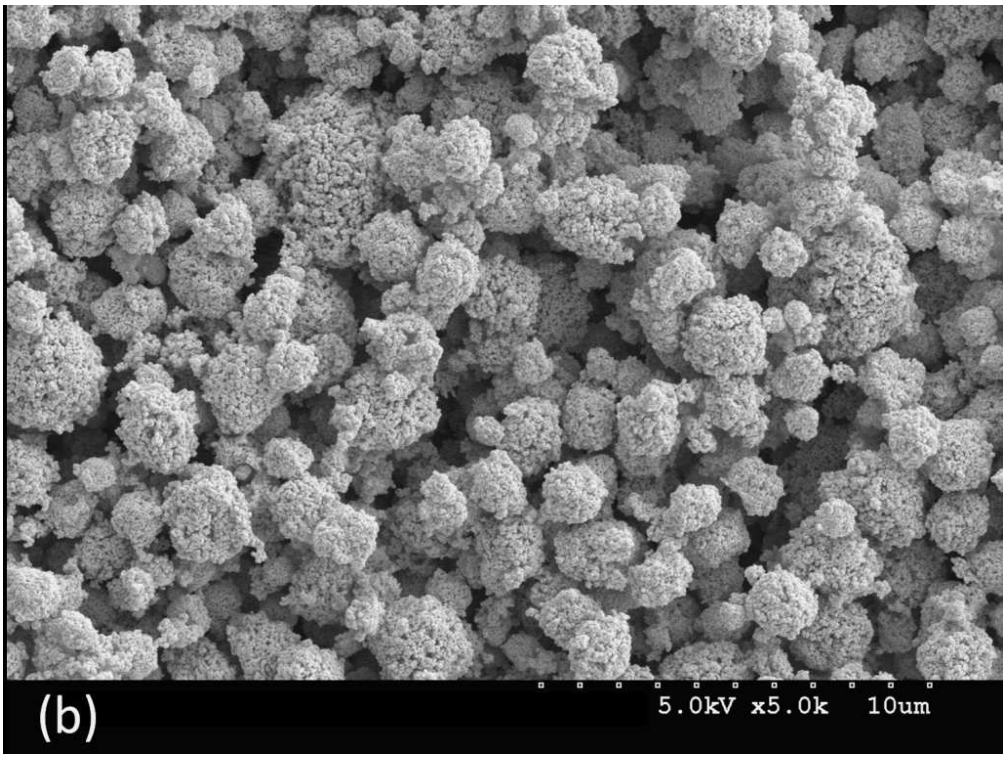


Figure 1 SE micrographs showing trypsin:raffinose spray-dried (a) from MeOH:BA solvent system in a mass ratio of 1:4 and spray dried from MeOH:BA:H_2O solvent system in mass ratios (b) 1:9 (c) 1:4 (d) 1:1.
173x129mm (150 x 150 DPI)



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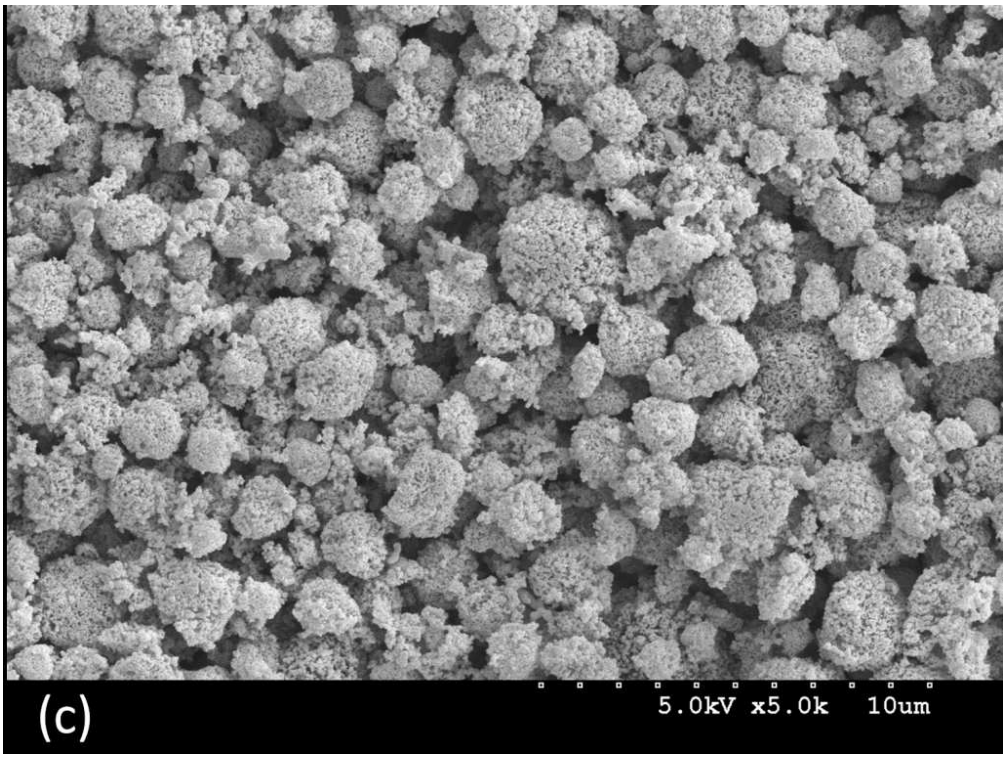

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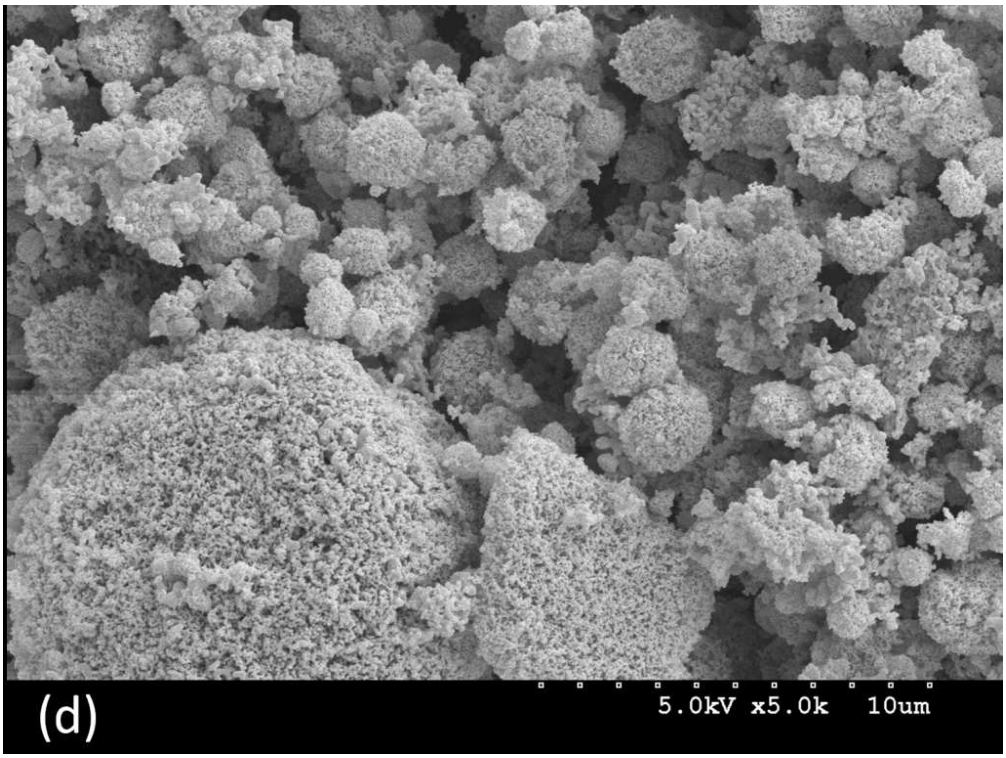


173x129mm (150 x 150 DPI)

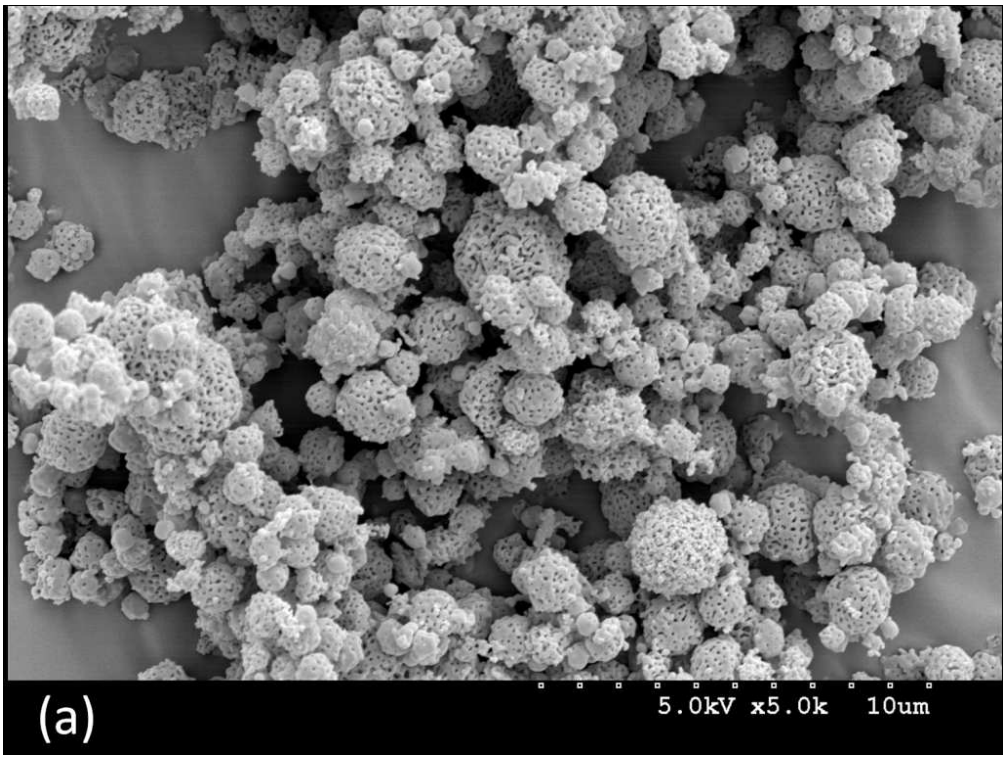
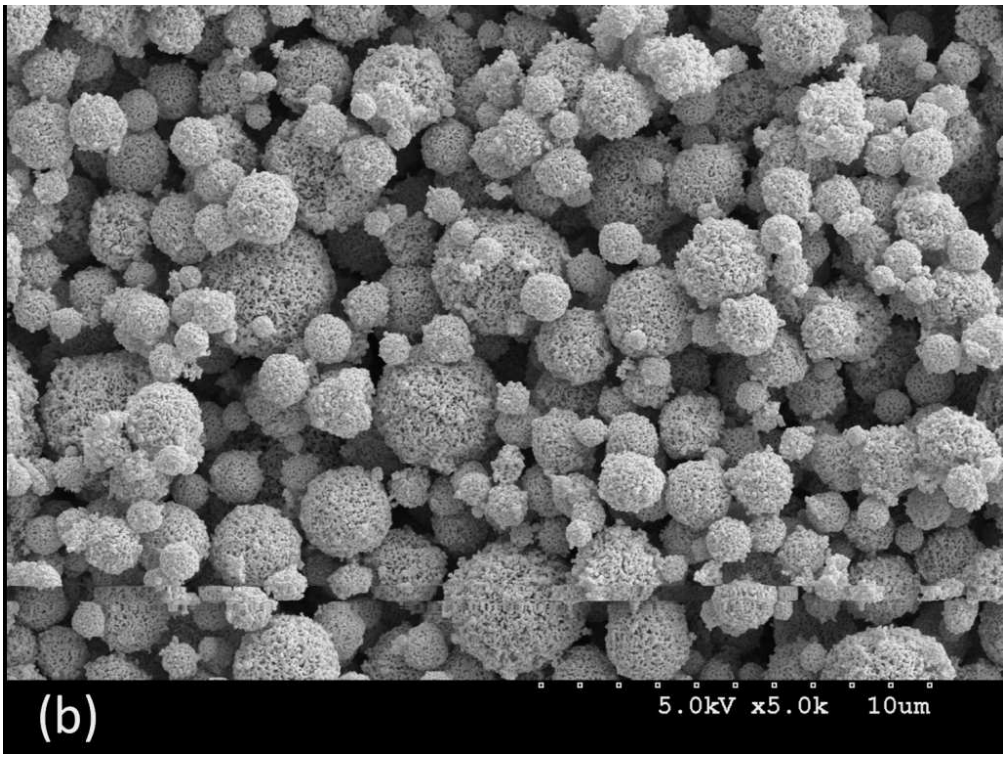


Figure 2 SE micrographs showing trypsin:trehalose spray-dried from MeOH:BA:H_2O solvent system in mass ratios (a) 1:9 (b) 1:4 (c) 1:1
173x129mm (150 x 150 DPI)



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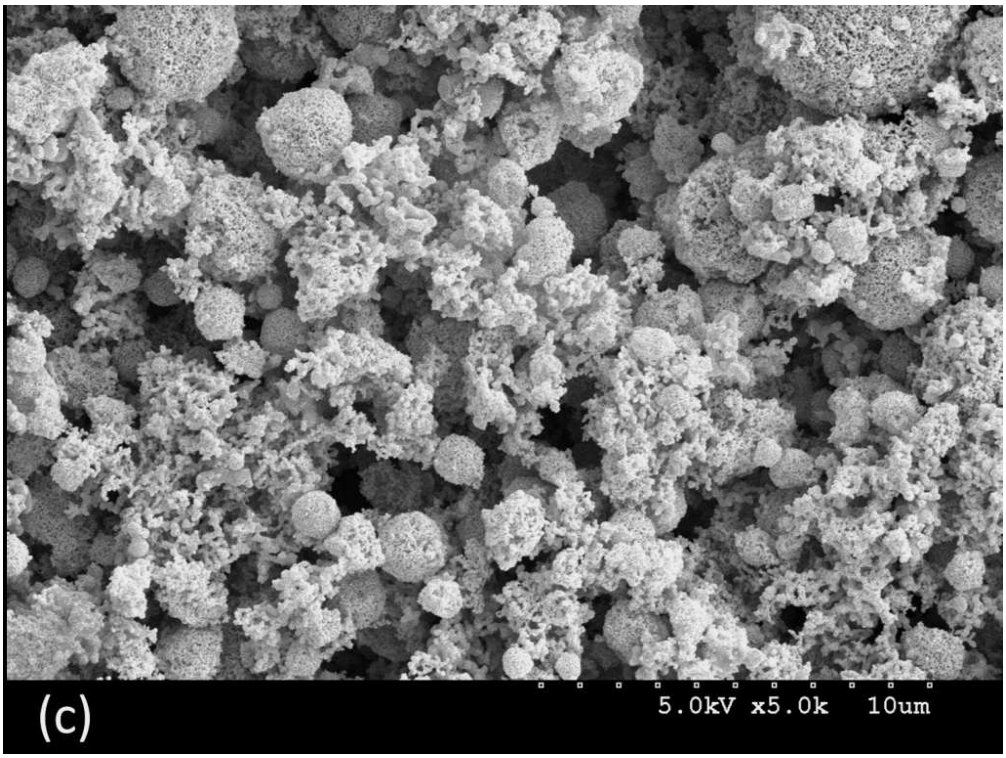

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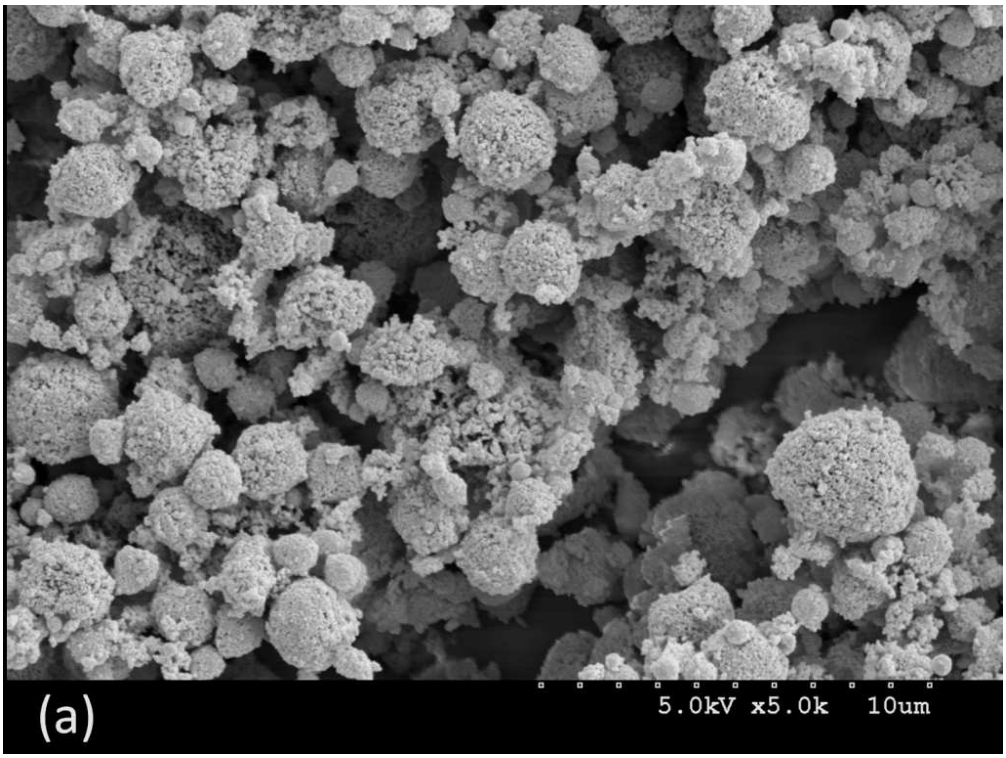

Figure 3 SE micrographs showing trypsin: HP-β-CD spray-dried from MeOH:BA:H_2O solvent system in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.
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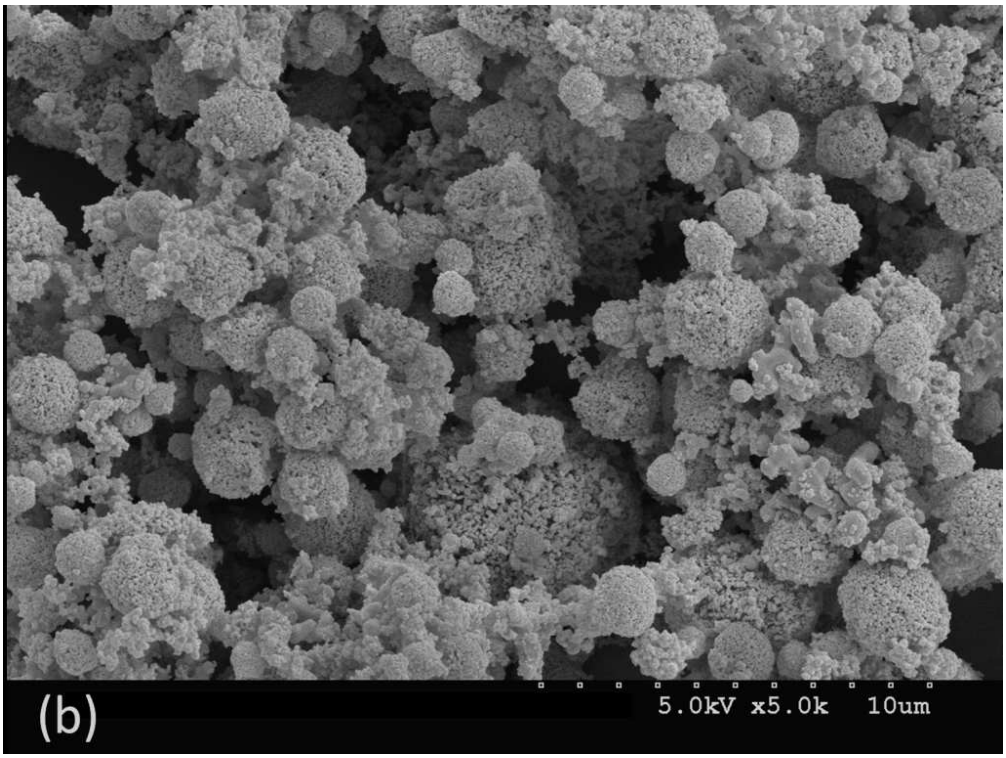

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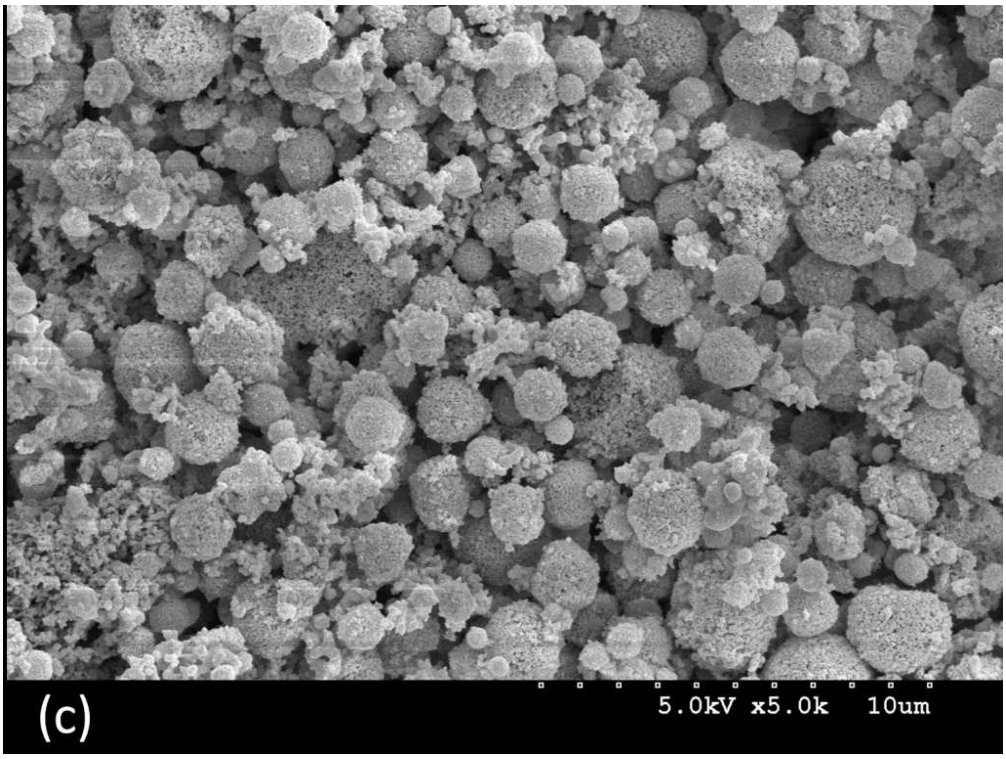

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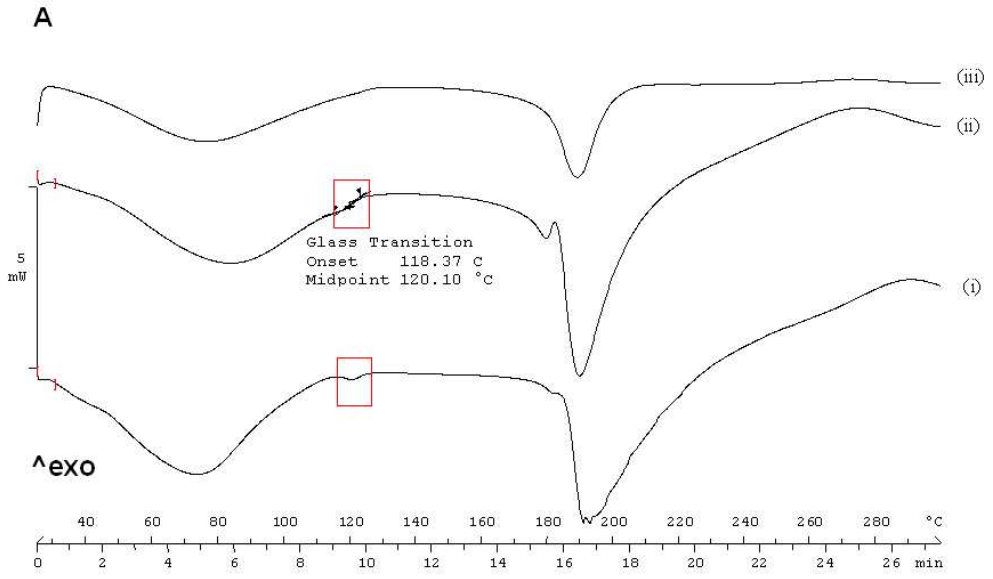
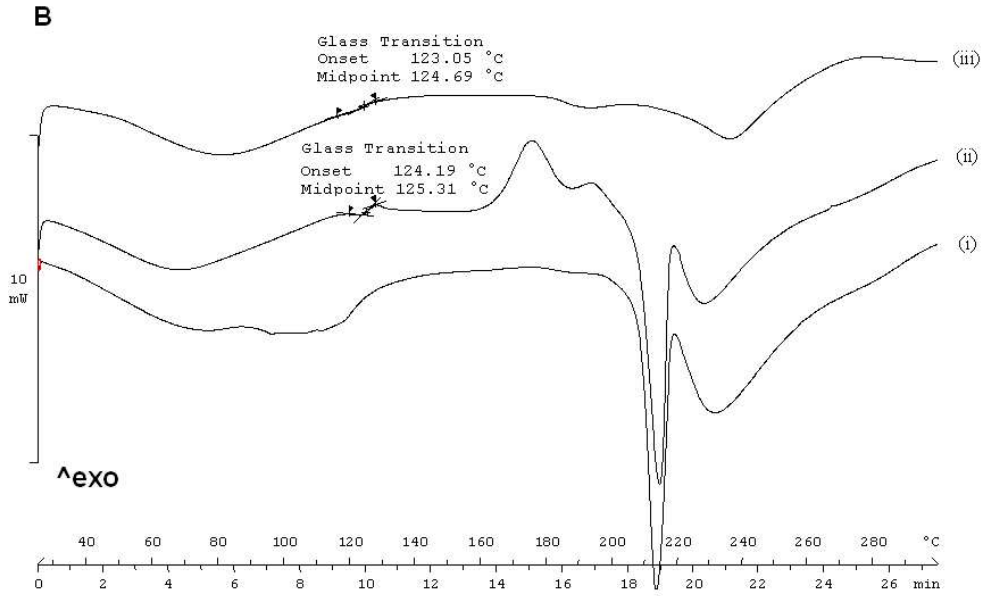


Figure 4 DSC scans of (A) trypsin:raffinose, (B) trypsin:trehalose and (C) trypsin:HP-β-CD spray-dried in mass ratios of (i) 1:9 (ii) 1:4 (iii) 1:1.
232x143mm (96 x 96 DPI)



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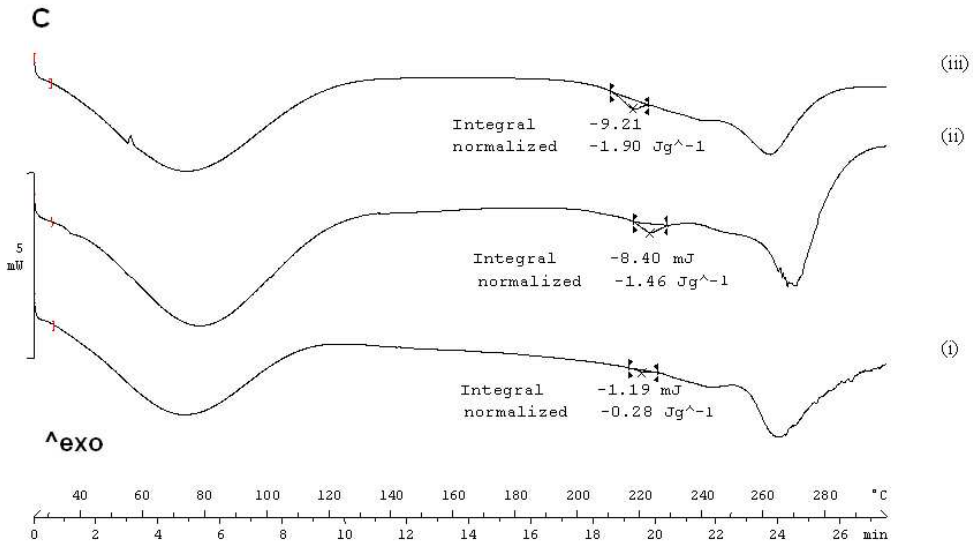


229x133mm (96 x 96 DPI)

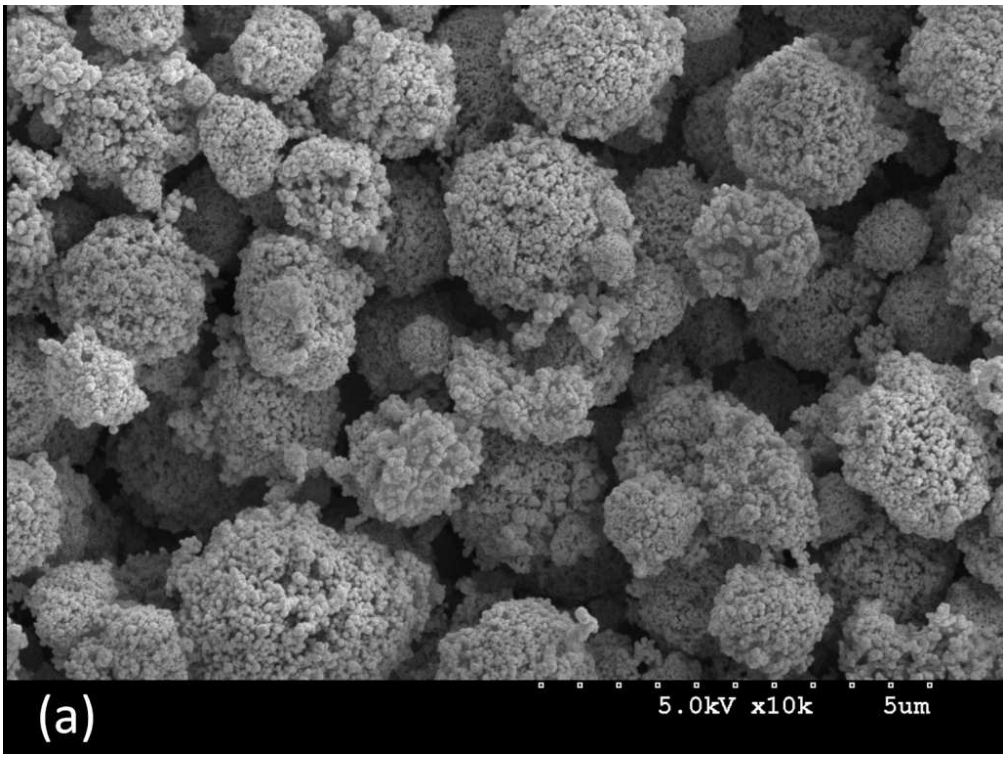
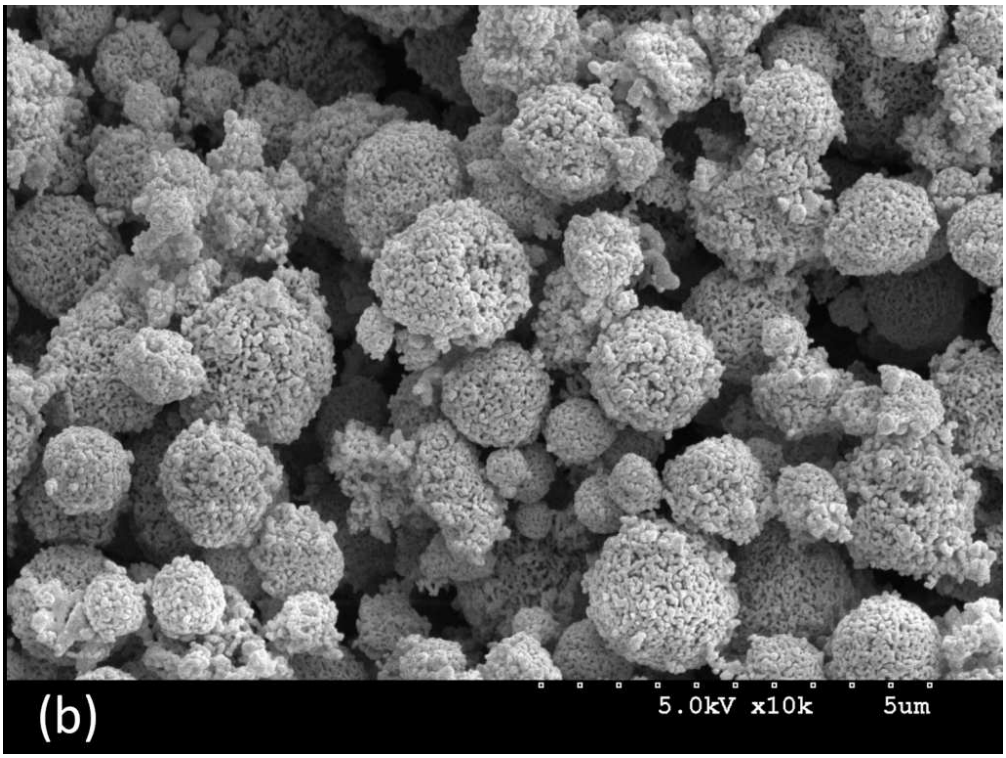
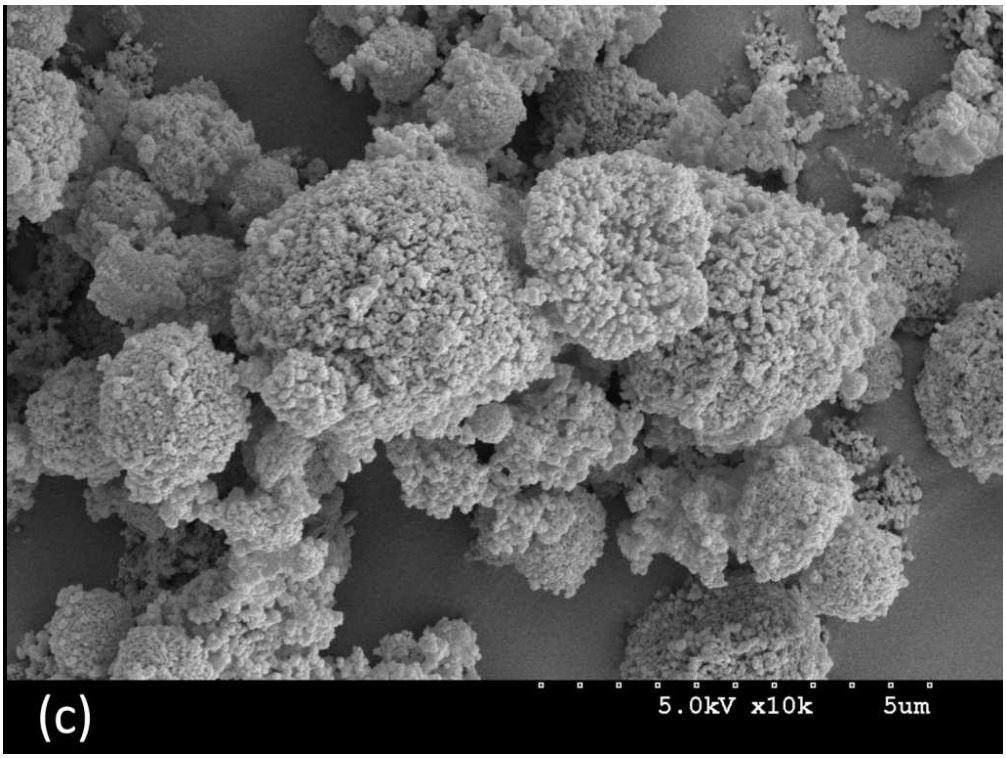


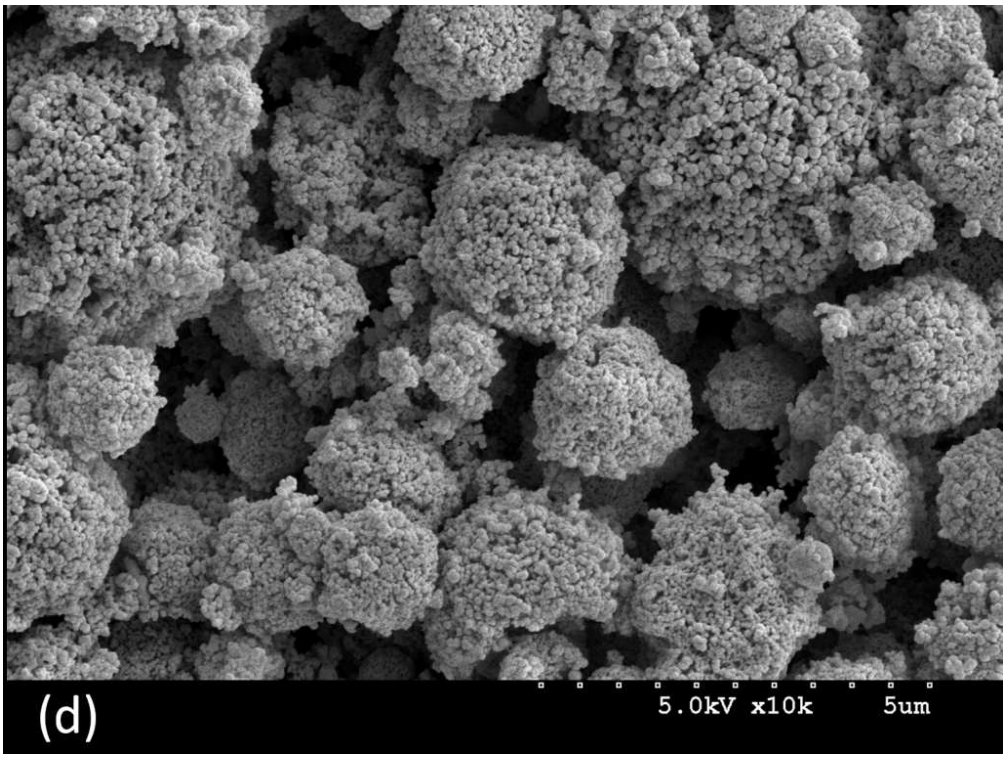
Figure 5 SE micrographs of composite particles (spray-dried ratio 1:4) after 12 weeks storage at 4 °C/desiccant and at 25 °C/desiccant (a), (d) trypsin:raffinose; (b), (e) trypsin:trehalose; (c), (f) trypsin:HP-β-CD.
173x129mm (150 x 150 DPI)



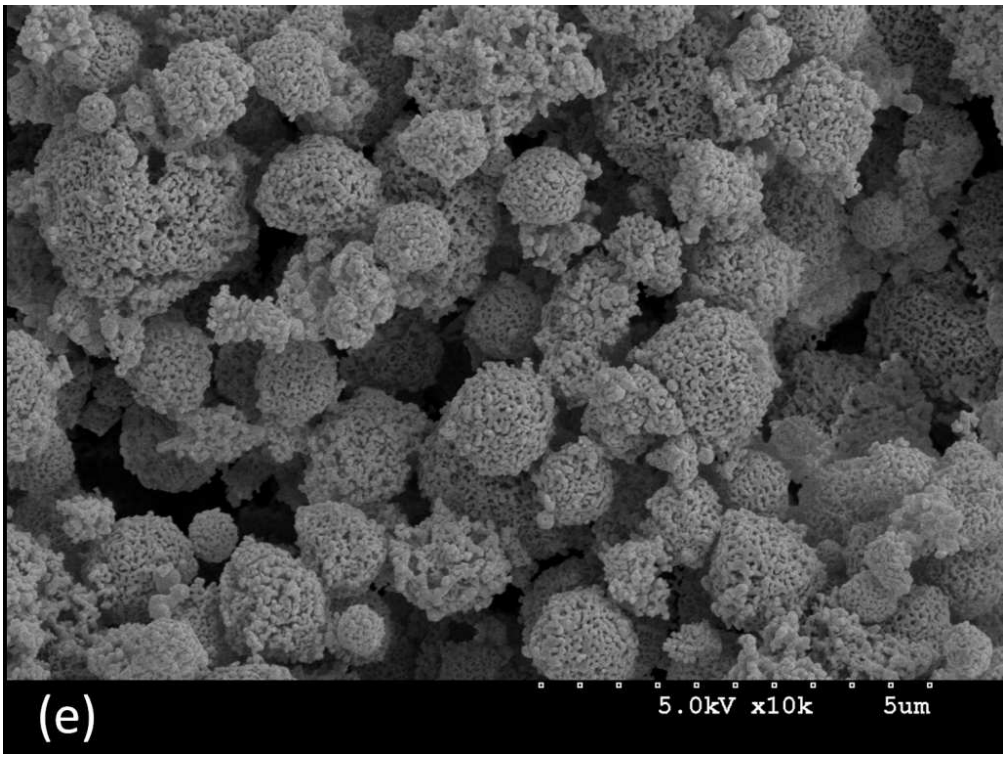
173x129mm (150 x 150 DPI)



173x129mm (150 x 150 DPI)



173x129mm (150 x 150 DPI)



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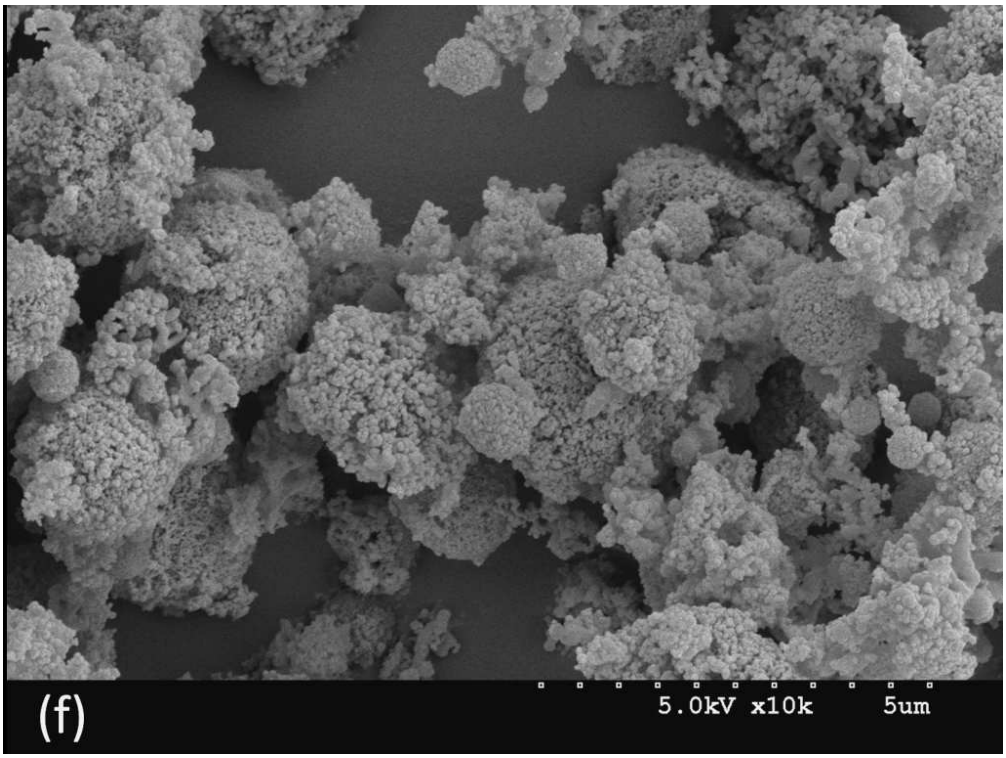


173x129mm (150 x 150 DPI)

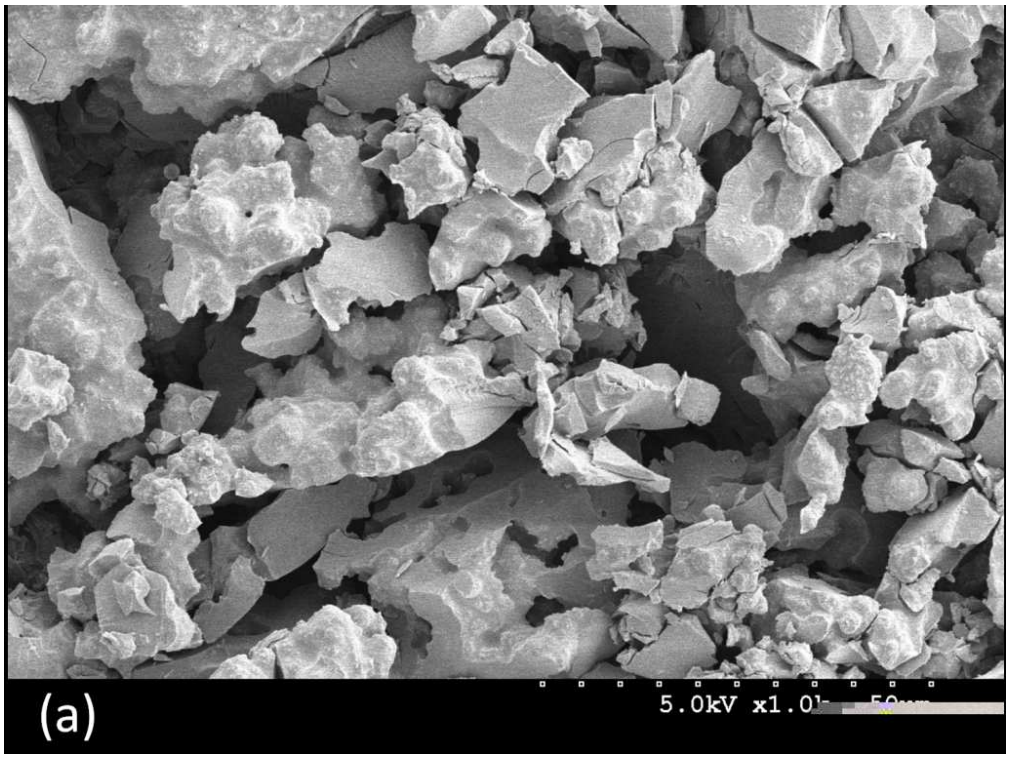
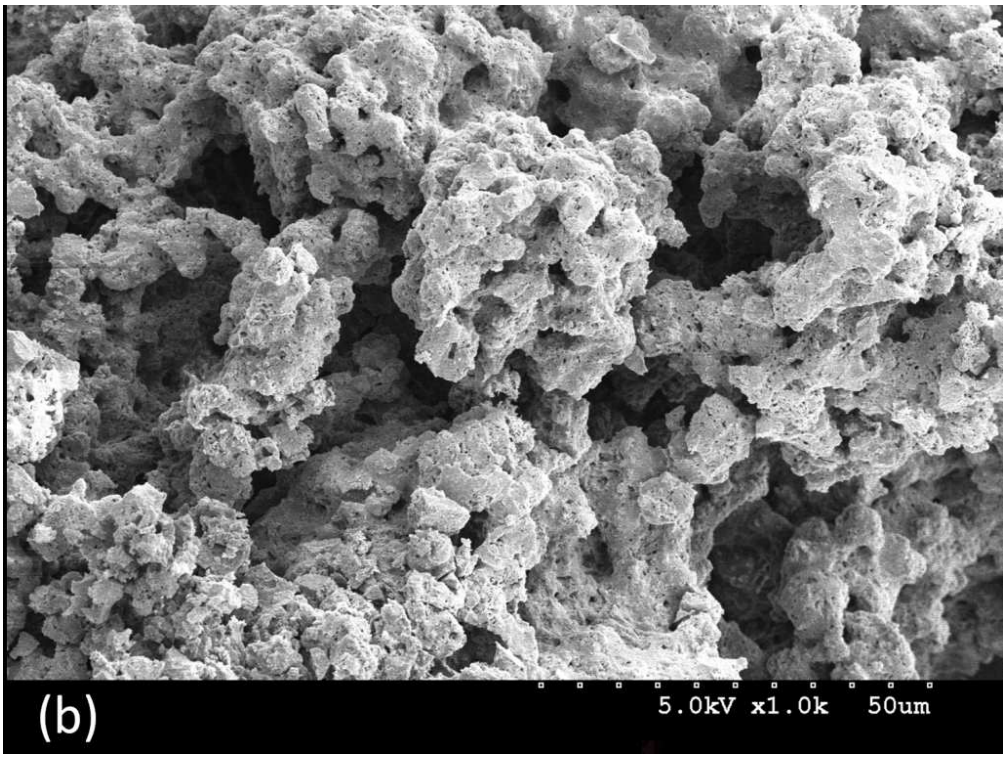
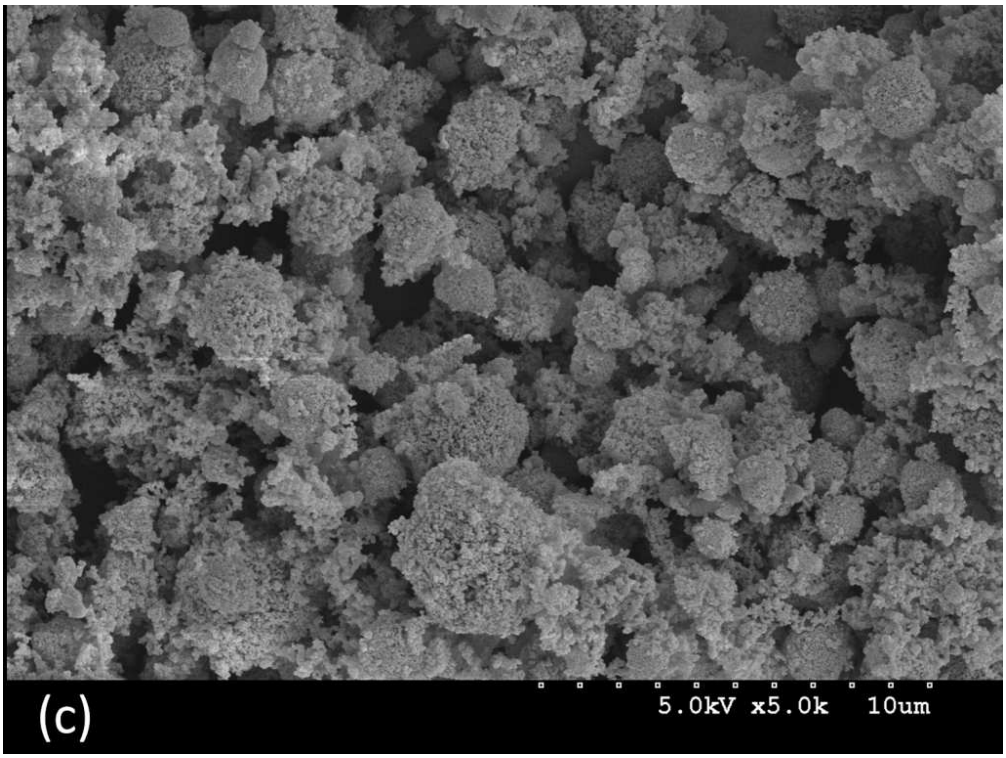


Figure 6 SE micrographs of composite particles after 24 hour storage at 25 °C/60% RH (spray-dried ratio 1:4) (a) trypsin:raffinose (b) trypsin:trehalose (c) trypsin:HP-β-CD.
173x130mm (150 x 150 DPI)



173x129mm (150 x 150 DPI)



173x129mm (150 x 150 DPI)

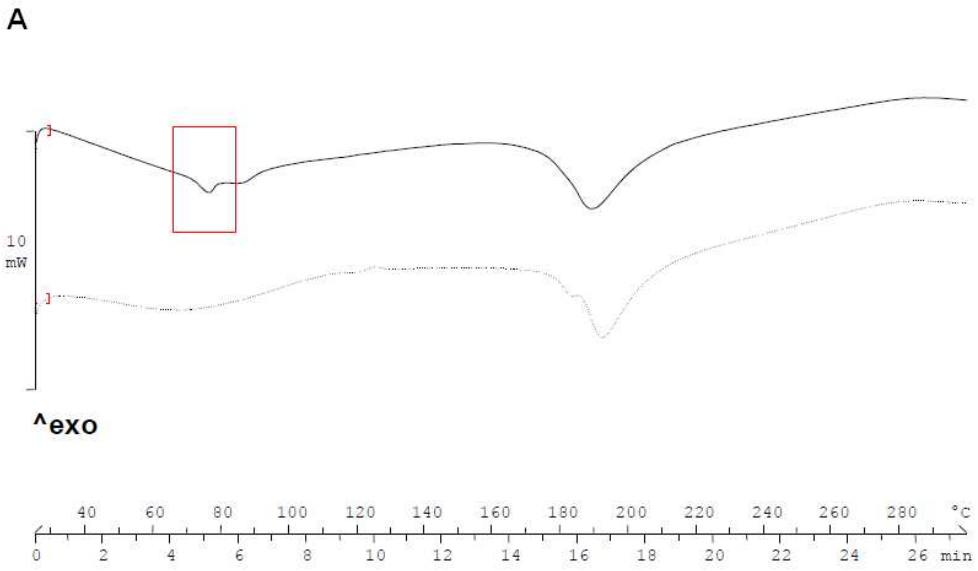
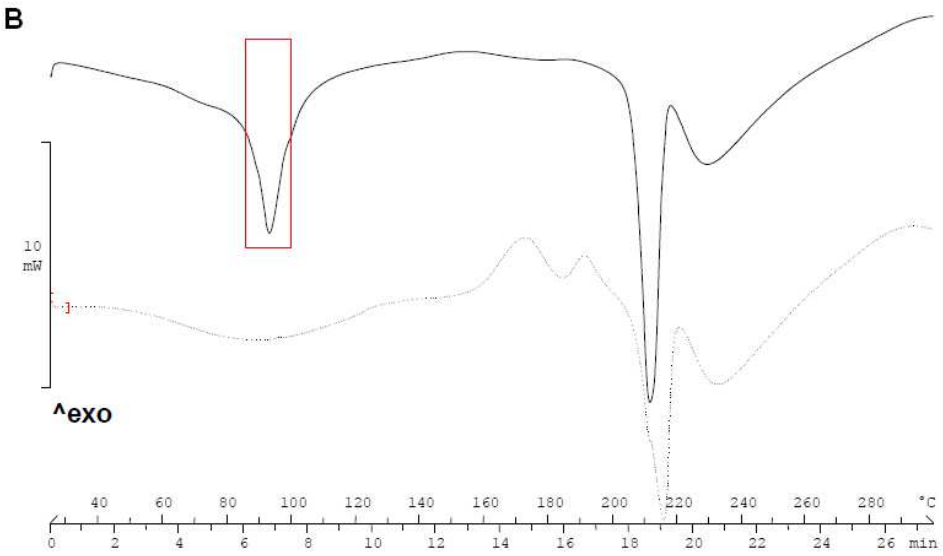
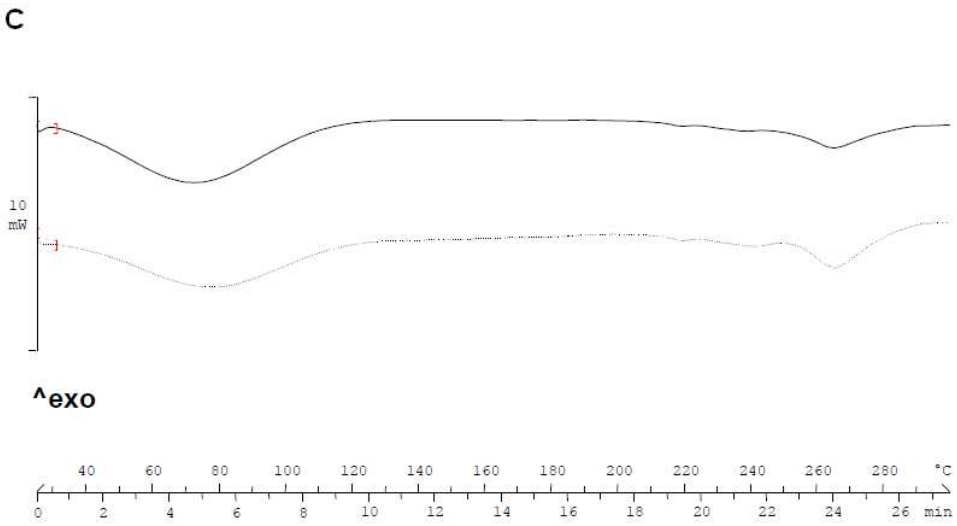


Figure 7 DSC scans of composite particles (mass ratio 1:4) (a) trypsin:raffinose (b) trypsin:trehalose (c) trypsin:HP-β-CD taken after 24 hours storage at 25 °C/ 60% RH (solid line), freshly prepared (broken line).
215x125mm (96 x 96 DPI)



215x125mm (96 x 96 DPI)



219x119mm (96 x 96 DPI)

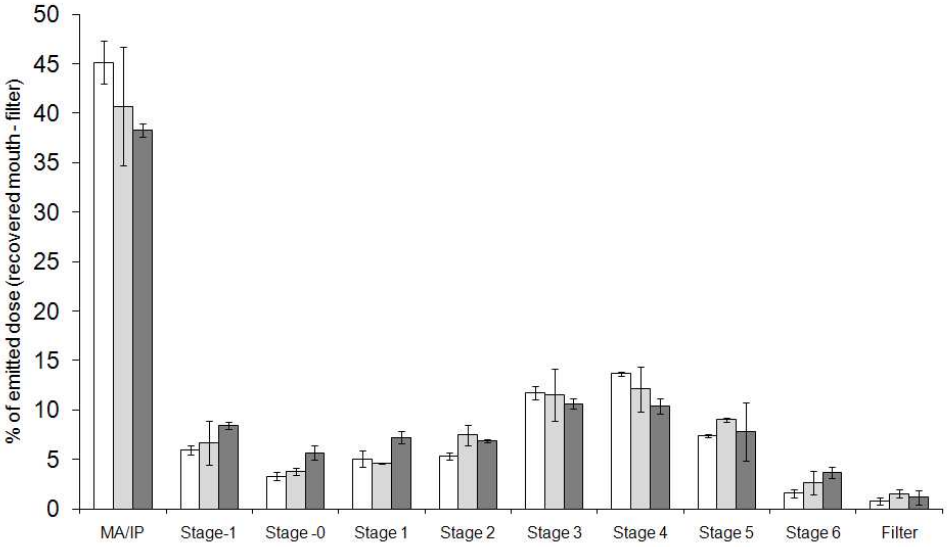


Figure 8 Deposition patterns on the stages of the ACI apparatus for the composites as indicated (spray-dried mass ratio 1:4). Calculated as % of recovered emitted dose (mouth – filter), used to calculate fine particle fraction. White bars: trypsin:trehalose, light grey bars: trypsin:raffinose and dark grey bars: trypsin:HP-β-CD.
258x169mm (96 x 96 DPI)