Preparation of respirable nanoparticle agglomerates of the low melting and ductile drug ibuprofen: impact of formulation parameters

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ABSTRACT

Ductile and low melting point drugs exhibit challenging behaviour during both particle size reduction and spray drying as considerable amount of heat is involved in both processes. In this study, a systematic approach was employed to understand the preparation and *in-vitro* performance of respirable nanoparticle agglomerates by coupling wet milling and spray drying for ibuprofen, which is a drug with a low melting point and challenging mechanical properties. Wet milling in the presence of two stabilizers differing in their thermal properties and subsequent spray drying of the suspensions were employed after the addition of mannitol and/or leucine. The effects of the stabilizer type and the amounts of mannitol (matrix former) and leucine (dispersibility enhancer), on the yield of the process, the particle size, the redispersibility (i.e. reformation of nanoparticles upon rehydration) and the aerosolization (fine particle fraction, FPF%) of the nanoparticle agglomerates were evaluated using standard least squares model and a 2^3 full factorial design (3 factors at 2 levels plus four centre points). All factors investigated were found to have a significant effect on the yield of nanoparticle agglomerates ($p<0.05$). The size of the nanoparticle agglomerates was mainly dependent on the leucine to drug ratio and the type of stabilizer ($p<0.05$), while mannitol to drug ratio was the only significant factor affecting the redispersibility of the formulations ($p<0.05$). The FPF%, determined using a fast screening impactor, was found to be dependent on both the leucine and mannitol to drug ratio ($p<0.05$). This study demonstrates the successful preparation of respirable nanoparticle agglomerates of low melting point and ductile ibuprofen and the usefulness of the design of experiments as a tool to understand the impact of the formulation parameters on their fabrication and *in-vitro* performance.

1.   Introduction

Nanocrystals are nanosized drug particles. Nanocrystals are typically produced in the form of nanosuspensions, which are submicron, colloidal dispersions of nanosized drug particles, stabilised by surfactants, polymers, or a mixture of both [1]. Nanocrystals have been suggested as a beneficial formulation approach for Class IIa drugs of the Biopharmaceutics Classification System (BCS), for which the dissolution rate is the rate-limiting step of absorption [2]. Considering the various nanosizing techniques (i.e. top-down and bottom-up), wet milling is a reproducible, cost-effective and scalable way of preparing nanosuspensions with a typical size ranging from 200-500 nm [3]. Wet milling is the technique for the preparation of the majority of the nanosuspension-based pharmaceutical formulations that are either on the market or currently under development [4,5].

Solidification of the nanosuspensions has been explored to combine the advantages of liquid nanosuspensions (i.e. enhanced dissolution and solubility) with the benefits of solid formulations (i.e. stability, easier handling, enhanced patient compliance) producing nanoparticle agglomerates suitable for oral and pulmonary delivery. Spray drying is a single-step process for the conversion of a liquid feed into a dried particulate form. It is a popular process from an industrial perspective as it is more cost- and time-effective compared to freeze drying [6]. Therefore, preparation of nanosuspensions by wet milling followed by solidification, using spray drying, has been suggested as a formulation approach for nanoparticle agglomerates with enhanced dissolution and aerosolization efficiency [7–10].

Spray drying is also a fundamental particle engineering technique for pulmonary drug delivery due to its simplicity, adaptability and scalability. Spray drying is a rapid solidification procedure and the obtained particles (at least from solution feed) are usually amorphous. Amorphicity is regarded as a disadvantage for respirable particles as it is associated with the danger of recrystallisation upon storage, which may influence adversely the stability, dissolution, absorption and aerosolisation efficiency of the product [11]. Moreover, during spray drying nanocrystals are exposed to thermal stresses, which may cause irreversible

aggregation to the resultant formulations [12]. Irreversible aggregation is linked with poor redispersibility of the nanoparticle agglomerates, which cannot reform nanoparticles upon rehydration leading to the loss of the advantages of the nanoformulations [12,13].

Addition of generally recognised as safe (GRAS) excipients in the liquid feed before spray drying is a common strategy to manipulate the properties and thus the performance of the dry powders. Mannitol is a non-reducing sugar, which has been approved by regulatory authorities for use in inhalable pharmaceutical products [14]. In particular, mannitol exhibits excellent spray-drying properties as the size and morphology of the particles can be modified by varying the process parameters [15]. The crystallinity of the spray-dried mannitol and the non-hygroscopic nature of this excipient are advantageous for the long-term stability of the dry powders [16]. Moreover, mannitol has been used as a matrix former during the solidification of nanosuspensions [7,10,17–19]. As a water-soluble compound, mannitol forms a matrix around the nanoparticles, which upon rehydration dissolves allowing the reformation of the primary nanoparticles.

Leucine is an endogenous amino acid, which exhibits aerosolization-enhancing properties [14,16]. Leucine was found to influence particle formation and to reduce the cohesiveness of spray-dried mannitol particles, which subsequently resulted in higher emitted fractions [20,21]. According to Sou et al. [20], "*it is a combination of a high surface activity during the drying process, mass transport within the droplet, followed by subsequent self-assembly packing on the particle surface which may explain the dispersibility enhancing effect of leucine*". Modification of particle morphology in response to higher concentrations of leucine has been previously reported [20,22]. More specifically, when leucine was co-spray dried with mannitol and trehalose it was found that a leucine concentration <5% w/w was insufficient for discrete particle formation, a concentration of 5-20% w/w resulted in reduced particle aggregation while a concentration >20% w/w led to increased surface corrugation [21]. According to Mangal et al. [23], the surface concentration of leucine governs particle formation and optimum surface concentration of this amino acid results in optimum surface and bulk properties of the spray-dried powders.

69   Ibuprofen was selected as a poorly water-soluble model drug with a low melting

70   point (75-78 °C) and challenging mechanical properties as it exhibits a high brittle-

71   ductile transition point of 854 μm [24]. Below this point, it is hard to reduce the

72   size of particles by dry milling, as the particles tend to deform rather than fragment.

73   Despite the high ductility of ibuprofen, it was reported that wet milling using a

74   Micros Ring Mill resulted in particles with a diameter about 8-11 μm (much

75   smaller than the critical diameter of 854 μm) [25]. The superior size reduction

76   performance of wet milling was attributed to the contribution of shear forces,

77   which cause ductile fracture of crystals. Besides the ductility of ibuprofen, its low

78   melting point poses additional challenges to conventional nano-comminution

79   techniques. The heat generated in the mill may result in partial melting of the drug

80   particles and thereby formation of large aggregates or drug amorphisation [26–

81   28]. In a recent study by Lestari et al. [29], ibuprofen was classified as a drug with

82   poor millability as high concentrations of stabilizers and extended milling times

83   were required to produce stable nanosuspensions by wet milling.

84

85   Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) for treating fever,

86   pain and inflammation; it was recently reported that ibuprofen acts synergistically

87   with antibiotics and thus might play a multifunctional role in the treatment of

88   cystic fibrosis infections [30]. Based on this new evidence, Yazdi et al. [31]

89   formulated carrier-free dry powder inhalations of micronized (jet-milled)

90   ibuprofen as an attractive alternative to oral administration of the drug in cystic

91   fibrosis. Therefore, preparation of respirable nanoparticle agglomerates of

92   ibuprofen with increased dissolution may be used as an alternative formulation

93   approach for the targeted delivery of ibuprofen to the lungs.

94

95   In this study, wet milling of ibuprofen using two different stabilizers, namely

96   hypromellose (HPMC) and D-α-tocopherol polyethylene glycol 1000 succinate

97   (TPGS), followed by spray drying, after the addition of excipients, was assessed.

98   Different grades of HPMCs have been found to be the most effective stabilizers

99   in terms of particle size reduction and short-term physical stability of ibuprofen

100   nanosuspensions [32] while TPGS due to its low viscosity and high surface

101   activity has been identified as the surface modifier with the highest success rate

102   on stabilizing nanosuspensions of various drugs [33]. These two stabilizers differ

103 with respect to their thermal properties; HPMC has a glass transition temperature

104 of 125.5 ^{o}C [34] while TPGS is a thermosensitive surfactant with low melting

105 point (m.p. 38 ^{o}C). The effect of these two stabilizers on the size reduction of

106 nanosuspensions was investigated. The solid state, particle morphology and the

107 dissolution profiles of the spray-dried nanoparticle agglomerates were also

108 assessed. A full factorial design and standard least squares model were employed

109 to understand the critical formulation parameters as well as any interactions

110 between them involved in the wet milling and spray drying process and finally in

111 the formation of respirable nanoparticle agglomerates. The critical formulation

112 parameters investigated were: type of stabilizer, mannitol to drug ratio and leucine

113 to drug ratio. The yield of the process, the particle size distribution, the

114 redispersibility and the fine particle fraction were investigated as responses. To

115 the best of our knowledge, it is the first time that such a study is carried out

116 focusing on respirable nanoparticle agglomerates of a low melting drug.

117

2. Materials and methods

2.1. Materials

Ibuprofen (IBU, Shasun Pharmaceuticals, India) with volume mean diameter $D_{4,3}$: 64.5 ± 8.3 μm was used. Pharmacoat 603 (low viscosity hypromellose, HMPC 2910, Shin-Etsu Chemical Co., Japan) and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS, Sigma Aldrich, USA) were used as stabilizers. Mannitol (Pearlitol® 160C, Roquette Frères, Lestrem, France) and L-leucine (Sigma Aldrich, USA) were used as a matrix former and a dispersibility enhancer of the nanoparticle agglomerates, respectively. Hyclone™ Water for Injections (Thermo Scientific, UK) was used for the preparation of nanosuspensions. Methanol and acetonitrile were HPLC grade and all other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of nanosuspensions

Nanosuspensions were prepared by wet bead milling using a laboratory planetary mill (Pulverisette 5, Fritsch Co., Germany). 0.5 g IBU, the stabilizer (10% w/w of IBU) and 10 g of milling beads (0.5 mm diameter aluminium borosilicate glass grinding beads, Gerhardt, UK) were weighed into each glass vial of 14 mL capacity and suspended in 10 mL Water for Injections. The vials were placed into a stainless steel milling pot with a maximum loading capacity of 8 vials. Rotation speed (200 rpm), milling duration (6 cycles) and stabilizer concentration (10% w/w of IBU) were selected based on preliminary studies. Each milling cycle comprised 30 min rotation followed by 20 min pause. At each pause, the nanocrystal size was determined and at the end of the milling procedure, the nanosuspensions were allowed to cool to room temperature and collected by withdrawal using a pipette for separation from the milling beads.

2.2.2. Particle size distribution of nanosuspensions

Malvern Nano ZS (Malvern Instrument, UK) was used for size measurements by dynamic light scattering (DLS) yielding the intensity-weighted mean hydrodynamic diameter of the bulk population (z-average) and the polydispersity index (PI) as a measure of the width of size distribution. 20 μL of nanosuspension was diluted with 10 mL of saturated IBU solution, prepared by filtration of a

151 suspension through a 0.1 μm disposable syringe filter to avoid extensive

152 dissolution and was then shaken vigorously for 30 s by hand before being

153 transferred to disposable sizing cuvettes. The measuring parameters were:

154 dispersant refractive index of 1.338 and viscosity of dispersion medium 0.89 cP.

155 All measurements were performed in triplicate.

156 **2.2.3. Preparation of nanoparticle agglomerates**

157 The obtained nanosuspensions were solidified by spray drying immediately after

158 preparation. 10 mL of nanosuspension were diluted to 100 mL with an aqueous

159 solution of mannitol and/or leucine to obtain the proportions reported in Table 1.

160 Spray drying was performed using a laboratory scale spray dryer (Mini B-290,

161 Buchi Labortechnik, Switzerland) fitted with a high performance cyclone. On the

162 basis of preliminary experiments, the following parameters were employed: inlet

163 temperature of 70 $^\circ$C, outlet temperature of 50 $\pm$ 2 $^\circ$C, feed rate of 5 mL min^{-1} and

164 atomizing gas flow rate of 0.5 L s^{-1}. The collected nanoparticle agglomerates were

165 weighed and stored in a desiccator over silica gel for subsequent testing.

166 **2.2.4. Determination of yield**

167 Yield was calculated as the ratio of the mass of the particles collected after spray

168 drying to the mass of solids (drug and excipients) introduced in the feed suspension.

169 The drug quantity used in the calculations was the amount weighed in the milling

170 pots before the wet-milling step.

171

172 **2.2.5. Characterization of nanoparticle agglomerates**

173 **2.2.5.1. Particle size analysis**

174 Particle size distributions of the nanoparticle agglomerates were determined by

175 laser diffraction using a HELOS/ BR laser diffractometer (Sympatec, Germany)

176 which was fitted with the micro-dosing unit ASPIROS and the dry disperser

177 RODOS. Samples were placed in the feeder and pressurized air at 4 bar was used

178 to disperse them in the measurement chamber, while the feeding velocity was kept

179 constant at 50 mm s^{-1}. An R2 lens detector (0.25- 87.5 μm) and the particle size

180 distribution analysis software Windox 5 (Sympatec, Germany) were used. The

181 D_{10}, D_{50} and D_{90} particle sizes (i.e. the size in microns at which 10%, 50% and

182 90% of the particles are smaller) were recorded. Measurements were carried out
183 in triplicate.
184

185 **2.2.5.2. Scanning electron microscopy**
186 The morphology of the starting materials and the nanoparticle agglomerates was
187 investigated using scanning electron microscopy (SEM). Samples were placed on
188 to double-sided electro-conductive adhesive tape, which was fixed onto an
189 aluminium stub and then sputter-coated with gold (10 nm thickness). SEM
190 micrographs were taken using a FEI Quanta 200 FEG ESEM (FEI, Netherlands),
191 at 5.00 kV.
192

193 **2.2.5.3. X-ray powder diffraction**
194 X-ray powder diffraction (XRPD) was employed to assess the crystallinity of the
195 starting materials and the nanoparticle agglomerates. XRPD patterns were
196 obtained with a bench-top diffractometer (Rigaku Miniflex 600, Japan). Cu Kα
197 radiation at 15 mA and 40 kV with a step of 0.02 deg and a speed of 5 deg min^{-1}
198 was used, covering a 2 θ of 5-40 $^\circ$. Miniflex Guidance (Rigaku, Japan) was the
199 analysis software.

200 **2.2.5.4. Differential scanning calorimetry**
201 Differential scanning calorimetry (DSC) was performed using a TA DSC Q200
202 calorimeter (TA Instruments, USA) previously calibrated with indium. Accurately
203 weighed powder samples (1-3 mg) were sealed into crimped standard aluminium
204 pans (TA) and heated under nitrogen flow (50 mL min^{-1}) from 25 $^\circ$C to 30 $^\circ$C
205 above the expected melting point at a heating rate of 10 $^\circ$C min^{-1}.

206 **2.2.5.5. Thermogravimetric analysis**
207 Thermogravimetric analysis (TGA) was used for determining the residual
208 moisture content of the spray-dried formulations. TGA was performed with a
209 Discovery TGA (TA Instruments, USA) controlled by TRIOS (TA) software.
210 Weighted powder samples (1-5 mg) were placed into aluminium cups (TA) and
211 heated under nitrogen flow (50 mL min^{-1}) from 25 to 120 $^\circ$C at a heating rate of
212 10 $^\circ$C min^{-1}. The residual moisture content was calculated as the weight loss
213 between 25 and 120 $^\circ$C.

2.2.5.6. Drug loading

5 mg of nanoparticle agglomerates were dissolved in 50 mL methanol, and ibuprofen concentration was assayed using an HPLC system (Agilent 1100 Series, Agilent technologies, Germany). The stationary phase was a Luna® (150 x 4.60 mm, 5 micron) column (Phenomenex Co., California, USA) kept at 30 °C. The mobile phase comprised acetonitrile and aqueous trifluoroacetic acid solution (0.1% v/v) at 50/50 volumetric ratio. The mobile phase flow rate was 1 mL min^{-1}, the injection volume was 10 μL and the detection wavelength 214 nm. The retention time for ibuprofen was 7.4 min. The correlation coefficient of the calibration curve was R^2=0.9999 for a concentration range of 5-600 μg mL^{-1}, indicating acceptable linearity.

2.2.5.6. Redispersibility

Redispersibility index (RDI%) was determined according to Yue et al. [13]:

$$RDI\% = \frac{z-average}{z-average_0} *100 .$$

where, *z-average$_0$* is the intensity-weighted mean particle diameter of the nanosuspensions prior to spray drying measured by DLS and *z-average* is the corresponding value of nanosuspension reconstituted from nanoparticle agglomerates upon rehydration. For the measurement of redispersibility, around 100 mg of each spray-dried powder was added to a glass vial containing 10 mL of an aqueous saturated IBU solution and it was shaken vigorously for 30s by hand before being transferred to disposable sizing cuvettes. The saturated solution of ibuprofen was prepared by filtration of a drug suspension through a 0.1 μm disposable syringe filter in order to avoid extensive dissolution. A RDI value close to 100% indicates that the spray-dried nanoparticle agglomerates exhibit complete reconstitution after rehydration to particles of similar size as the primary nanocrystals after nanomilling and before the solidification step.

2.2.5.7. *In-vitro* dissolution testing

The paddle method was applied by using USP apparatus type II (Pharma Test, Germany), at 37 °C and 50 rpm stirring speed. The dissolution medium was 500 mL of deionised water (freshly boiled and cooled, pH: 6-7). At specific time

intervals up to 120 min, 5 mL of dissolution medium was withdrawn, filtered
through a 0.1 μm disposable syringe filter and placed in HPLC vials for assay,
whilst being immediately replaced with 5 mL of fresh medium. The HPLC
conditions for the assay were as for drug content determination. Dissolution tests
were conducted in triplicate for each formulation.

2.2.5.8. *In-vitro* aerosol performance

The aerodynamic assessment of the nanoparticle agglomerates was carried out
using the fast screening impactor, FSI (MSP 185 FSI, Copley Scientific, UK). The
FSI was developed based on the abbreviated impactor measurement concept. It
divides the particles discharged from the inhaler into two parts: the coarse fraction
and the fine fraction (aerodynamic diameter less than 5 μm). The coarse fraction
collector was equipped with an insert that enables a cut-off of 5 μm at 30 L min^{-1}.
The particles not captured in the coarse fine collector followed the airstream and
deposited in the fine fraction collector where a filter captured all of them. The FSI
was connected to a high-capacity vacuum pump (Model HCP5, Copley
Instruments, UK). Based on results from preliminary studies the bottom plate of
the pre-separator was coated with 1% w/v silicone oil in hexane in order to reduce
particle bounce that is created from the additional 5 μm cut-off plate. The actual
flow rate was measured using a calibrated flow meter (Flow Meter Model DFM
2000, Copley Instrument Ltd, UK) prior to each run, to ensure that a flow at 30 L
min^{-1} was achieved. Gelatin hard capsules (size 3) were filled with accurately
weighed amounts of product (ranging from 12.5 to 28 mg depending on the drug
loading of each formulation) corresponding to about 10 mg of IBU. The capsules
were placed in the inhaler device (Cyclohaler®) fitted to the impactor via an
airtight rubber adaptor and tested at 30 L min^{-1} for 8 s (total volume: 4 L). The
capsules were discharged into the FSI and after dispersion the particles were
collected on a glass fiber filter (76 mm, Pall Corporation, USA) and extracted in
methanol. Analysis of the extracts from the capsules, mouthpiece and each part of
the FSI was performed with HPLC. The HPLC conditions for the assay were as
for drug content determination. Each formulation was tested in triplicate. The fine
particle fraction (FPF%) of the formulations was the ratio of the drug mass
depositing on the fine fraction collector divided by the recovered dose. The fine

277   particle dose (FPD) was calculated as the total mass deposited on the fine fraction

278   collector divided by the number of doses (n=3).

279

280   **2.2.5.9. Design of Experiments**

281   A full factorial design 2^3 (3 factors at 2 levels) was used allowing the estimation

282   of the main effects and the two-way interactions. The three independent variables

283   used at two levels in the design were: type of stabilizer (X_1), mannitol to drug ratio

284   (X_2) and leucine to drug ratio (X_3). Dependent variables: yield, volume median

285   diameter (D_{50}), redispersibility index (RDI%) and fine particle fraction (FPF%),

286   were selected as responses (Table 1). The design matrix included 8 runs plus four

287   centre points (Fig. 1). Centre points were added to the design space to identify any

288   non-linearity in the responses. The design space was constructed and analyzed

289   using the JMP 12.1.0 software (SAS Institute, USA). To reduce systematic errors,

290   all the experiments were completely randomized. The standard least squares model

291   (including multiple linear regression analysis and ANOVA) was fitted in to model

292   the data. The significance and validity of the model was estimated by ANOVA.

293   The parameter estimates and the probability values (p-values) of the effects and

294   two-way interactions of each response are given. p-values less than 0.05 were

295   deemed to be statistically significant.

296

3. Results and discussion

3.1. Preparation and characterization of nanosuspensions

Both stabilizers were able to produce nanosuspensions of ibuprofen after 180 min of wet milling. The results of z-average size and polydispersity index (PI) of nanosuspensions obtained with both stabilizers as a function of milling time are presented in (Fig. 2). More specifically, after 180 min nanosuspensions stabilized with HPMC and TPGS exhibited a z-average size of 533 ± 28 nm and 663 ± 12 nm, respectively.

The starting material, with a volume mean diameter $D_{4,3}$: 64.5 ± 8.3 μm (Fig. 3) initially showed rapid size reduction during milling, especially with HPMC as stabilizer. In the case of HPMC, submicron particles of ibuprofen were produced in 60 min while for TPGS this occurred in 90 min. The breakage rate of crystals was high initially and with further milling time the size continued to decrease, but at a slower rate for both stabilizers. This is a common profile as breakage rate kinetics have been found to follow a first-order exponential decay [35].

In the study of Nihei et al. [27], thymoquinone, a low melting compound (m.p. 46 °C) with antioxidant properties had to be nanosized using a cold wet-milling system in order to avoid the formation of large aggregates that were caused by the partial melting of the compound when a wet mill without a heat exchanger was used. In this study, no aggregation of ibuprofen was observed during milling indicating that cautious selection of the process parameters (i.e. a lower milling speed and an increased milling time with intervals so as to cool down the vessels) could allow the use of wet mills which are not equipped with temperature control accessories for the nano-comminution of low melting drugs. This could be especially applicable to preformulation research at preclinical and clinical studies were limited resources are available (i.e. time, equipment, investment, compound).

The results reported in this study can be favorably compared with those obtained by rapid expansion of supercritical solutions [36] and high pressure homogenization [37], as both methods could not produce ibuprofen crystals with size in the submicrometre scale.

330

Comminution of ibuprofen in water using a Lena DM 100 nanoparticle production machine, equipped with a heat exchanger and operating in the recirculation mode, resulted in nanosuspensions with z-average size around 450 nm [38]. The smaller size of nanocrystals reported may be attributed to the use of a combination of polymers and surfactants as stabilizers, and this also indicates the scalability of wet milling in industrial settings [39].

3.2. Yield

The yield was selected as a response characterizing quantitatively the overall productivity of the process. For the experimental conditions applied the yield ranged from 27.3% to 72.5% (Table 1). The generated model was significant and the response was modeled with high accuracy (adjusted R^2: 0.96, Table 2).

All the three independent variables were identified as significant with a positive effect on the yield of the process ($p < 0.05$, Table 2). The positive effect of increasing the leucine and mannitol to drug ratios may be attributed to the increased concentration of the solids dissolved in the feed suspension, prior to the spray-drying step. Spray drying of nanosuspensions stabilized with TPGS led to the lowest yield of 27.3%. This low yield may be attributed to the low melting point of TPGS resulting in melting and adhesion of the nanoparticle agglomerates to the drying chamber and cyclone. Replacing TPGS with HPMC, which is a non-thermolabile stabilizer increased the yield. The yield of the process was found to maximize by increasing the leucine to drug ratio. This may be explained by the fact that leucine accumulates on the surface of the particles forming a coating around them and thus protecting them from high temperatures during spray drying. Similar results were reported regarding the spray drying of hydro-alcoholic solutions of β-estradiol where the powder yield increased with increasing leucine content in the formulation [40]. The explanation proposed by the authors was that when increasing leucine content was used, the drug was encapsulated in micelle-like structures of leucine and thus was protected from the relative harsh spray-drying conditions.

As shown in the surface plots (Fig. 6a,b) higher yields are obtained when HPMC was used as the stabilizer of ibuprofen nanosuspensions, and when high leucine

364 and mannitol to drug ratios were used in the formulations prior to the spray-drying

365 step.

366

367 **3.3. Characterization of nanoparticle agglomerates**

368 **3.3.1. Particle size and morphology**

369 The SEM images of the formulations prepared based on the full factorial design

370 are shown in Fig. 4,5. Spray drying of ibuprofen nanosuspensions stabilized either

371 by HPMC or TPGS in the absence of excipients resulted in aggregated particles of

372 irregular morphology with size outside the acceptable range for pulmonary drug

373 delivery (Table 1 and Fig.4,5).

374

375 Addition of mannitol and/or leucine resulted in the promotion of spherical particles

376 with mean size approximately 2-3 μm that is suitable for pulmonary drug delivery.

377 The surface of the spray-dried particles appears not to be smooth and a closer

378 inspection reveals the presence of nanoparticles indicating the composite structure

379 of the particles where ibuprofen nanocrystals are embedded in a matrix of mannitol

380 and/or leucine (i.e. nanoparticle agglomerates).

381

382 The nanoparticle agglomerates containing leucine consist of individual particles

383 (e.g. patterns 1-+, 2-+, Fig.4,5). This may be attributed to the accumulation of

384 leucine at the surface of the particles preventing any particle fusion. A high leucine

385 to drug ratio resulted in wrinkled particles (patterns 1++, 2++, Fig.4,5). A wrinkled

386 morphology was interpreted as an indication of hollow particles as the particle

387 density was found to decrease as the "wrinkleness" of the particles was increased

388 [41].

389

390 The nanoparticle agglomerates appear to be porous with holes and dimples in the

391 particle surface due to the evaporation of liquid that escapes from the inner of the

392 droplet through the solid crust built up in the course of the drying process on the

393 surface of the droplet [15,42]. Engineering of porous particles is considered

394 beneficial for pulmonary drug delivery as particles with high porosity have smaller

395 aerodynamic diameter compared to non-porous particles of the same physical size,

396 increasing the probability for deposition in the lower respiratory tract [43].

397

398   The particle size of the nanoparticle agglomerates obtained was measured by laser

399   diffraction as a volume diameter (Table 1). The dried powders obtained exhibited

400   a median diameter D_{50} between 2.15 and 16.04 µm and the data are in good

401   agreement with the particle size observed by SEM. The results were analyzed in

402   the experimental design performing ANOVA for particle size, focusing on the D_{50}

403   value and the model was found significant ($p < 0.05$, Table 2).

404

405   Leucine to drug ratio and the type of stabilizer used were identified as formulation

406   variables with the most significant effects on the D_{50} (Table 2). Their "negative"

407   effect is interpreted as size reduction, which is desirable for pulmonary drug

408   delivery. The observation regarding the influence of leucine on particle size is in

409   agreement with the study of Sou et al. [20] in which it was reported that leucine in

410   contrast to glycine and alanine was the only amino acid that reduced the D_{50} when

411   added into the mannitol formulations. Also, it is likely that increased leucine to

412   drug ratio enhanced surface coating around the particles, which prevented the

413   particles from melting and sintering, as observed for the process yield.

414

415   A significant interaction was identified between leucine to drug ratio and the type

416   of stabilizer, with a positive parameter estimate, despite the fact that the factors

417   had individually negative effects on the D_{50} (Table 2), indicating a synergistic

418   rather than an additive interaction between HPMC and leucine to drug ratio. Use

419   of the non-melting HPMC as a nanosuspension stabilizer and the addition of a high

420   leucine to drug ratio leads to further particle size reduction than the individual

421   factors alone.

422   As shown in the surface plot, spray drying of ibuprofen nanosuspensions stabilized

423   with HPMC and containing a high leucine to drug ratio is able to produce

424   nanoparticle agglomerates with particle size around 2-3 µm that is suitable for

425   pulmonary drug delivery (Fig. 6c). On the other hand, spray drying of ibuprofen

426   nanosuspensions stabilized with TPGS results in larger particles, while addition of

427   both high leucine and mannitol to drug ratios was required in order to produce

428   particles smaller than 4 µm (Fig. 6d). This indicates that the selection of stabilizer

429   is vital not only for the step of nanosuspension production, but it may also

influence the downstream process of spray drying by affecting the properties of the nanoparticle agglomerates produced.

3.3.2. Redispersibility

Redispersibility is an important quality attribute of nanoparticle agglomerates as it is a prerequisite for the reformation of nanoparticles upon rehydration of the larger particles with potential enhancement of therapeutic efficacy. Particularly, for nanoparticles of low melting drugs such as ibuprofen, thermal stresses during spray drying may lead to phase and composition changes of formulations causing irreversible aggregation and loss of the advantages of nanoformulations [12]. An RDI% value close to 100% means that the nanoparticle agglomerates reformed nanoparticles with z-average size close to the z-average size of the primary nanoparticles prior to the solidification step. For the experimental conditions applied, the redispersibility index (RDI%) ranged from 148 % to 938% (Table 1). The redispersibility results were analyzed in the experimental design performing ANOVA for particle size focusing on the RDI% value and the model was found significant ($p < 0.05$, Table 2).

The mannitol to drug ratio was identified as the only significant factor affecting redispersibility ($p < 0.05$, Table 2) with higher mannitol to drug ratio leading to RDI% values closer to 100%. The role of mannitol as a redispersibility enhancer can be explained by the formation of a continuous matrix around the nanocrystals during the spray-drying step, preventing their irreversible aggregation. Upon rehydration, mannitol as a hydrophilic excipient dissolves and the nanosuspensions are reconstituted.

As shown in the surface plots (Fig. 7a,b), nanoparticle agglomerates of ibuprofen with enhanced redispersibility (RDI% value close to 100%) were obtained only when high mannitol to drug ratios are present in the formulations prior to the spray-drying step.

3.3.3. Drug loading

The results of assayed ibuprofen content in the nanoparticle agglomerates are given in Table 3. Spray drying of nanosuspensions without mannitol and/or leucine appeared to have lower drug loading than the nominal. This may be attributed to

463   the melting of TPGS during spray drying that led to drug loss due to deposition on

464   the walls of the drying chamber and cyclone. For the spray-dried nanosuspensions

465   containing mannitol and/or L-leucine the assayed ibuprofen content is close to the

466   nominal content indicating that the addition of these excipients prevented

467   ibuprofen loss or powder segregation during the production process.

468

469   **3.3.4. Solid state characterization**

470   The XRPD patterns of the starting materials are shown in Fig.8a. Raw ibuprofen

471   exhibited sharp peaks in the range of 2 theta: 15-25 ^{o} that are characteristic of the

472   drug [38,44]. Mannitol starting material exhibited characteristic peaks of the β-

473   form (2 theta: 10.6^o, 14.7^o, 16.9^o, 21.2^o, 23.9^o, 29.5^o) [45] while the diffractogram

474   of L-leucine indicated a highly crystalline structure (2 theta: 6°, 12°, 24°, 31°, 37°)

475   [46].

476

477   The diffractograms of all runs prepared according to the DoE are shown in Fig.

478   8b. The diffractograms of patterns 1-- and 2-- (without matrix former and

479   dispersibility enhancer) showed peaks at similar 2 theta positions to those of the

480   raw ibuprofen. For the nanoparticle agglomerates of ibuprofen containing

481   mannitol and/or leucine, the diffractograms were a summation of the patterns of

482   their components. No new peaks or halo could be detected in the XRPD patterns

483   indicating the absence of generated amorphous content during the process.

484

485   The DSC was used to assess the thermal behaviour of the starting materials and

486   nanoparticle agglomerates of ibuprofen (Fig. 9). The DSC thermogram of

487   ibuprofen showed an endothermic peak at 76 ºC corresponding to the melting of

488   the drug. The nanoparticle agglomerates of ibuprofen without mannitol and

489   leucine exhibited the same endothermic peak shifted to a slightly lower

490   temperature (Fig. 9a), while those containing mannitol exhibited thermal

491   behaviour depending on the stabilizer.

492

493   More specifically, the nanoparticle agglomerates of ibuprofen containing mannitol

494   and stabilized with TPGS exhibited two endothermic peaks (patterns 2++, 2+-,

495   Fig. 9b) as expected: the melting peak at around 70 º C, which relates to the melting

496   of the drug and a sharp endothermic peak at 168 ºC which relates to the melting of

497   mannitol (Pearlitol: 160 °C). For the nanoparticle agglomerates of ibuprofen
498   containing mannitol and stabilized with HPMC, apart from the melting of
499   ibuprofen, an endothermic peak at 150 °C was followed by an exothermic event
500   and then an endothermic melting at 168 °C (patterns 1++, 1+-, Fig. 9b). The
501   thermal events observed in the DSC of patterns 1++ and 1+- could be attributed to
502   the formation of the metastable δ-form of mannitol (m.p. 150-158 °C) that is
503   followed by crystallization to the α- or/and β-form, and the melting of the
504   respective crystal form [47]. Both α- and δ-form of mannitol were found to be
505   chemically and physically stable for at least 5 years when stored at 25 °C and 43%
506   relative humidity [47]. Recently, co-spray drying of an aqueous solution of
507   mannitol with PVP in a ratio 4:1 was reported to produce the δ-form of mannitol
508   [48].
509
510   Overall, the XRPD and DSC data suggest that the engineered nanoparticle
511   agglomerates retain their crystallinity during wet bead milling followed by spray
512   drying. The preservation of the crystalline state is advantageous, ensuring the long-
513   term physical stability of the formulations during storage.
514

515   **3.3.5. Residual moisture content**
516   Thermogravimetric analysis of the spray-dried powders indicated that the moisture
517   content of the powders ranged from 1.1 to 4.7% w/w (Table 3). These values
518   compare favourably with other studies which report moisture content of spray-
519   dried powders in the region of 5-10% w/w [49,50]. Specifically, residual moisture
520   content ranged from 2.3 to 4.7% for the nanoparticle agglomerates stabilized with
521   HPMC and from 1.1 to 2.3% for those stabilized with TPGS and it was reduced
522   for the agglomerates with high mannitol to drug ratio (Table 3). This is in
523   agreement with the results reported by Yamasaki et al. [7], that increasing mannitol
524   content in nanomatrix powders of ciclosporin A reduced the residual moisture
525   content of the formulations, which was attributed to its non-hygroscopic nature.
526   Thus, despite the low inlet (70 °C) and outlet temperature (50 °C), selected to
527   prevent melting of the drug during spray drying, addition of mannitol can
528   minimize the moisture content of the nanoparticle agglomerates that is required
529   for quality reasons (e.g. physical and chemical stability, reduced cohesiveness
530   during storage, non-aggregation) [51].

3.3.6. *In-vitro* dissolution tests

The dissolution profiles of ibuprofen and the nanoparticle agglomerates prepared according to the matrix of the full factorial design are shown in Fig. 10. Nanoparticle agglomerates stabilized with either HPMC or TPGS exhibited enhanced dissolution profiles compared to ibuprofen. In the case of the raw ibuprofen, less than 40% was released in the first 20 min, while the nanoparticle agglomerates achieved complete dissolution in less than 5 min. The exceptions to this were the spray-dried nanosuspensions of ibuprofen without matrix former (patterns 1-- and 2--) which exhibited a higher dissolution rate compared to ibuprofen but slower than the nanoparticle agglomerates containing mannitol and/or leucine. In the case of TPGS, this may be associated with the formation of large aggregates with size around 50 μm and poor redispersibility (Fig. 5). Thus, the selection of suitable process and formulation parameters is of paramount importance in order to ensure that the dissolution benefit of nanoparticles is retained after spray drying.

3.3.7. *In-vitro* aerosol performance

Fine particle fraction (FPF) was selected as a quality attribute describing the aerodynamic performance of a dry powder for inhalation. The European Pharmacopoeia (2.9.18 preparations for inhalation: aerodynamic assessment of fine particles, Ph. Eur. 8.0) suggest that a pressure drop over the inhaler of 4 kPa is broadly representative of the pressure drop generated by the patients using dry powder inhalers during inhalation [52]. In this study, operating a 'medium' resistance device as Cyclohaler® at 30 L min^{-1} leads to a low pressure drop across the inhaler. While this low pressure drop across the inhaler reduces the probability of establishing comparable *in-vitro* performance and *in-vivo* drug deposition, it is considered acceptable for comparing the *in-vitro* aerosolisation performance of formulations prepared in this study. The FPF values of the nanoparticle agglomerates produced ranged from 5.84 to 68.55% (Table 1) and the model generated was found to be significant ($p < 0.05$, Table 2).

Leucine to drug ratio and mannitol to drug ratio were identified as the most significant factors on the FPF (Table 2.). The positive effect of leucine can be linked with its properties as a dispersibility and aerosolization enhancer. In

565 contrast to other amino acids such as alanine and glycine, leucine has been found

566 to reduce capsule retention and increase both the emitted and the fine particle

567 fraction of formulations [20,22]. For spray-dried particles of salbutamol sulfate

568 and lactose containing increasing amount of leucine (5-20% w/w), Seville et al

569 [22] observed an increase of the FPF% (from 50% to 80%, respectively). In

570 another study, Sou et al. [20] reported the highly significant and positive effect of

571 leucine on improving the FPF% of spray-dried mannitol particles.

572

573

574 Regarding the positive effect of mannitol to drug ratio, it may be attributed to the

575 good spray-drying properties of mannitol which facilitates the formation of

576 spherical particles with narrow and unimodal particle size distribution [15,53].

577

578 As illustrated in the surface plots (Fig. 7c, d), both leucine and mannitol to drug

579 ratio have a significant effect on the aerodynamic performance of the nanoparticle

580 agglomerates, resulting in a large FPF increase from 10% to over 65%. Therefore,

581 a combination of high leucine and mannitol to drug ratios is required in order to

582 maximise the FPF of the nanoparticle agglomerates of the low melting and ductile

583 ibuprofen.

584

585 **4. Conclusions**

586 Nanosuspensions of the poorly water-soluble, low melting point and ductile drug

587 ibuprofen stabilized with HPMC and TPGS were successfully produced and were

588 further spray dried with or without the addition of excipients (mannitol and/or L-

589 leucine) employing a full factorial design. Design of experiments is a useful

590 approach in order to gain insight into the formation of inhalable nanoparticle

591 agglomerates using wet milling followed by spray drying. Leucine to drug ratio,

592 mannitol to drug ratio and the type of stabilizer were found to be significant

593 ($p < 0.05$) factors affecting the yield of the particles obtained by combining wet

594 milling and spray drying. The particle size response was mainly dependent on the

595 leucine to drug ratio and the type of stabilizer employed ($p < 0.05$). Mannitol to

596 drug ratio was found to be the only critical parameter affecting redispersibility of

597 nanoparticle agglomerates ($p < 0.05$), and both leucine to drug ratio and mannitol

598 to drug ratio were found to be significant factors affecting FPF ($p < 0.05$). While

599 the importance of the type of stabilizer on the formation of nanosuspensions has
600 been previously reported [33,54,55], in this study it was observed that the selection
601 of stabilizer could also influence the downstream process of spray drying by
602 affecting the yield and the particle size distribution of the resultant nanoparticle
603 agglomerates. Moreover, the nanoparticle agglomerates were found to be
604 crystalline which is advantageous for their physical stability upon storage, and they
605 exhibit enhanced dissolution compared to the ibuprofen starting material. Overall,
606 it appears that by selecting the stabilizer and adjusting the mannitol and leucine to
607 drug ratio during the spray drying of nanosuspensions can result in nanoparticle
608 agglomerates with enhanced dissolution and aerosolization behaviour despite the
609 challenging properties (thermal and mechanical) of a drug as ibuprofen.

614

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Tables

Table 1 Matrix of full factorial design, yield of process and characteristics of spray-dried nanoparticle agglomerates: volume diameter, redispersibility (RDI %), fine particle fraction (FPF%) and fine particle dose (FPD).

Table 2 Summary of the regression analysis and ANOVA results.

Table 3 Nominal content and assayed ibuprofen content (% w/w) of nanoparticle agglomerates together with calculated drug loading efficiency (%) and residual moisture content (mean ± SD, n=3).

Table 1 Matrix of full factorial design, yield of process and characteristics of spray-dried nanoparticle agglomerates: volume diameter, redispersibility (RDI %), fine particle fraction (FPF%) and fine particle dose (FPD).

Formulation number	Pattern	Stabiliser	Mannitol to drug ratio	Leucine to drug ratio	Yield (%)	Volume diameter (µm)			RDI (%)	FPF (%)	FPD (mg)
						D10	D50	D90			
1	100	HPMC	0.5	0.25	58.2	0.92 ± 0.02	3.02 ± 0.04	7.02 ± 0.23	420	58.20 ± 4.2	5.41 ± 0.6
2	200	TPGS	0.5	0.25	46.6	0.85 ± 0.01	6.20 ± 0.05	9.02 ± 0.13	250	46.62 ± 5.6	4.42 ± 0.7
3	1++	HPMC	1	0.5	72.5	0.85 ± 0.00	3.57 ± 0.03	6.82 ± 0.07	283	68.55 ± 3.8	6.23 ± 0.6
4	100	HPMC	0.5	0.25	56.4	0.83 ± 0.01	2.27 ± 0.01	6.23 ± 0.16	400	45.11 ± 6.1	4.03 ± 0.6
5	2+−	TPGS	1	0	41.1	1.13 ± 0.05	9.88 ± 0.39	24.39 ± 2.70	181	9.32 ± 2.5	0.79 ± 0.3
6	1−+	HPMC	0	0.5	60.1	0.99 ± 0.02	2.30 ± 0.08	3.83 ± 0.11	751	22.93 ± 1.2	2.11 ± 0.2
7	2−−	TPGS	0	0	27.3	1.56 ± 0.04	16.04 ± 0.33	53.22 ± 1.51	765	6.68 ± 2.7	0.58 ± 0.2
8	2−+	TPGS	0	0.5	53.8	0.88 ± 0.02	2.15 ± 0.02	3.81 ± 0.01	600	40.00 ± 2.3	3.66 ± 0.2
9	1+−	HPMC	1	0	47.9	1.02 ± 0.06	3.22 ± 0.04	5.92 ± 0.34	307	29.56 ± 3.4	2.61 ± 0.5
10	2++	TPGS	1	0.5	60.0	0.77 ± 0.01	2.23 ± 0.04	6.21 ± 0.01	148	43.63 ± 4.1	4.23 ± 0.6
11	200	TPGS	0.5	0.25	43.2	0.87 ± 0.01	5.20 ± 0.04	7.31 ± 0.30	280	40.23 ± 2.7	3.64 ± 0.4
12	1−−	HPMC	0	0	37.7	0.96 ± 0.03	4.29 ± 0.09	6.87 ± 1.01	938	5.84 ± 2.8	0.46 ± 0.2

Table 2 Summary of the regression analysis and ANOVA results.

Term	Yield			D_{50} (µm)			RDI (%)			FPF (%)		
	Estimate	Std Error	p-value	Estimate	Std Error	p-value	Estimate	Std Error	p-value	Estimate	Std Error	p-value
Intercept	50.4	0.71	<0.0001	5.03	0.37	<0.0001	443.59	33.74	<0.0001	31.27	2.57	<0.0001
Main effects												
X_1: Stabiliser	5.07	0.71	**0.0008*****	-1.92	0.37	**0.0033****	72.93	33.74	0.0830	2.73	2.57	0.3366
X_2: Mannitol to drug ratio	5.33	0.87	**0.0017*****	-0.74	0.45	0.1617	-266.89	41.32	**0.0013****	9.45	3.14	**0.0298***
X_3: Leucine to drug ratio	11.55	0.87	**<0.0001*****	-2.90	0.45	**0.0013****	-51.14	41.31	0.2708	15.48	3.14	**0.0044****
Two-way interactions												
X_1*X_2	0.33	0.87	0.7231	0.79	0.45	0.1400	-7.89	41.31	0.8561	7.87	3.14	0.0543
X_1*X_3	0.2	0.87	0.8267	2.49	0.45	**0.0026****	-1.64	41.31	0.9699	-1.44	3.14	0.6649
X_2*X_3	-0.68	0.87	0.4714	1.07	0.45	0.0620	32.89	41.31	0.4129	2.84	3.14	0.4071
ANOVA												
Model			**0.0004*****			**0.0028****			**0.0181*****			**0.0251***
R^2	0.98			0.96			0.91			0.89		
Adj R^2	0.96			0.91			0.80			0.76		

* p< 0.05, ** p< 0.01, *** p< 0.001

Table 3 Nominal content and assayed ibuprofen content (% w/w) of nanoparticle agglomerates together with calculated drug loading efficiency (%) and residual moisture content (mean ± SD, n=3).

Pattern	Content (% w/w)					Drug Loading Efficiency (%)	Residual Moisture Content (%)
	Nominal				Assayed IBU		
	IBU	STAB	MAN	LEU			
100	54.05	5.41	27.03	13.51	50.8 ± 1.3	94.0 ± 2.4	3.5 ± 0.5
200	54.05	5.41	27.03	13.51	51.8 ± 1.7	95.9 ± 3.2	1.1 ± 0.2
1++	38.46	3.85	38.46	19.23	36.7 ± 0.2	95.4 ± 0.5	2.3 ± 0.4
100	54.05	5.41	27.03	13.51	49.2 ± 1.3	91.0 ± 2.6	3.0 ± 0.4
2+-	47.6	4.76	47.6	-	44.4 ± 0.8	93.2 ± 1.7	1.6 ± 0.1
1-+	62.5	6.25	-	31.25	59.4 ± 1.2	95.0 ± 1.8	3.4 ± 0.5
2--	91	9	-	-	77.4 ± 1.4	85.1 ± 1.6	2.3 ± 0.5
2-+	62.5	6.25	-	31.25	58.3 ± 1.1	93.3 ± 2.1	1.9 ± 0.1
1+-	47.6	4.76	47.6	-	42.1 ± 2.1	88.5 ± 3.6	2.7 ± 0.2
2++	38.46	3.85	38.46	19.23	35.4 ± 0.7	92.0 ± 1.9	1.6 ± 0.3
200	54.05	5.41	27.03	13.51	50.4 ± 2.1	93.2 ± 3.5	1.3 ± 0.3
1--	91	9	-	-	80.3 ± 1.9	88.2 ± 2.6	4.7 ± 0.4

Figures

Figure 1 The three-dimensional design space of the 2^3 full factorial design.

Figure 2 z-average size and polydispersity index (PI) of ibuprofen nanosuspensions with increasing wet-milling time (mean + SD, n=3).

Figure 3 SEM image of ibuprofen starting material.

Figure 4 SEM images of ibuprofen nanoparticle agglomerates included in the full factorial design. The stabiliser is HPMC.

Figure 5 SEM images of ibuprofen nanoparticle agglomerates included in the full factorial design. The stabiliser is TPGS.

Figure 6 Surface plots indicating the effect of mannitol to drug ratio and leucine to drug ratio on the yield and D_{50} particle size of the nanoparticle agglomerates stabilised with HPMC (a,c) and TPGS (b,d). The arrows indicate the direction of increasing values of the variables.

Figure 7 Surface plots indicating the effect of mannitol to drug ratio and leucine to drug ratio on the redispersibility and the FPF% of the nanoparticle agglomerates stabilised with HPMC (a,c) and TPGS (b,d). The arrows indicate the direction of increasing values of the variables.

Figure 8 XRPD diffractograms of (a) starting materials and (b) nanoparticle agglomerates included in the full factorial design.

Figure 9 DSC thermograms of (a) ibuprofen and mannitol starting materials and (b) nanoparticle agglomerates included in the full factorial design. A magnified thermogram between $130\,^{\circ}C$ and $170\,^{\circ}C$, for the pattern 1++, is given in the insert.

Figure 10 Dissolution profiles of ibuprofen starting material and nanoparticle agglomerates included in the full factorial design (mean + SD, n=3). The stabiliser is (a) HPMC and (b) TPGS.
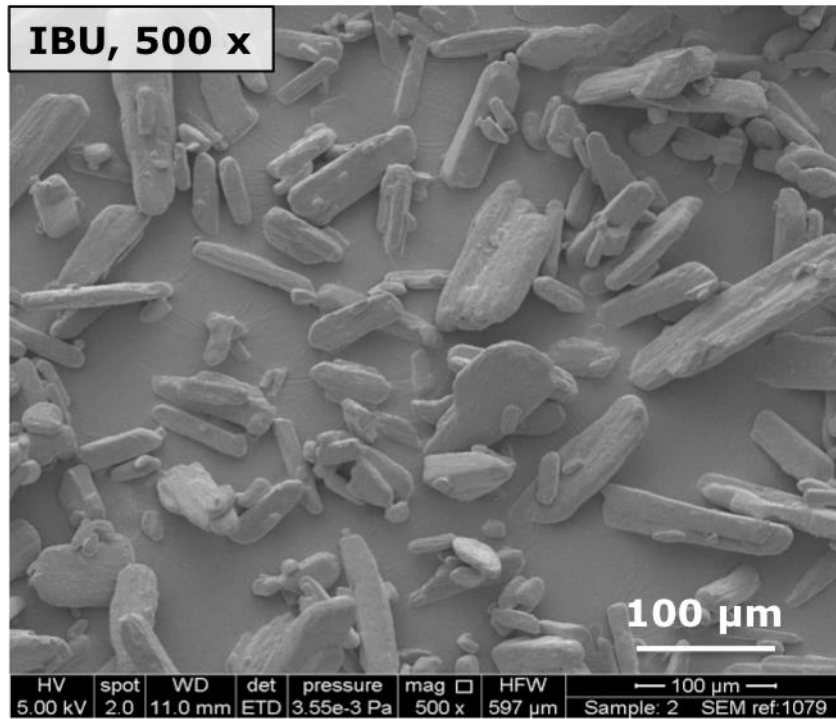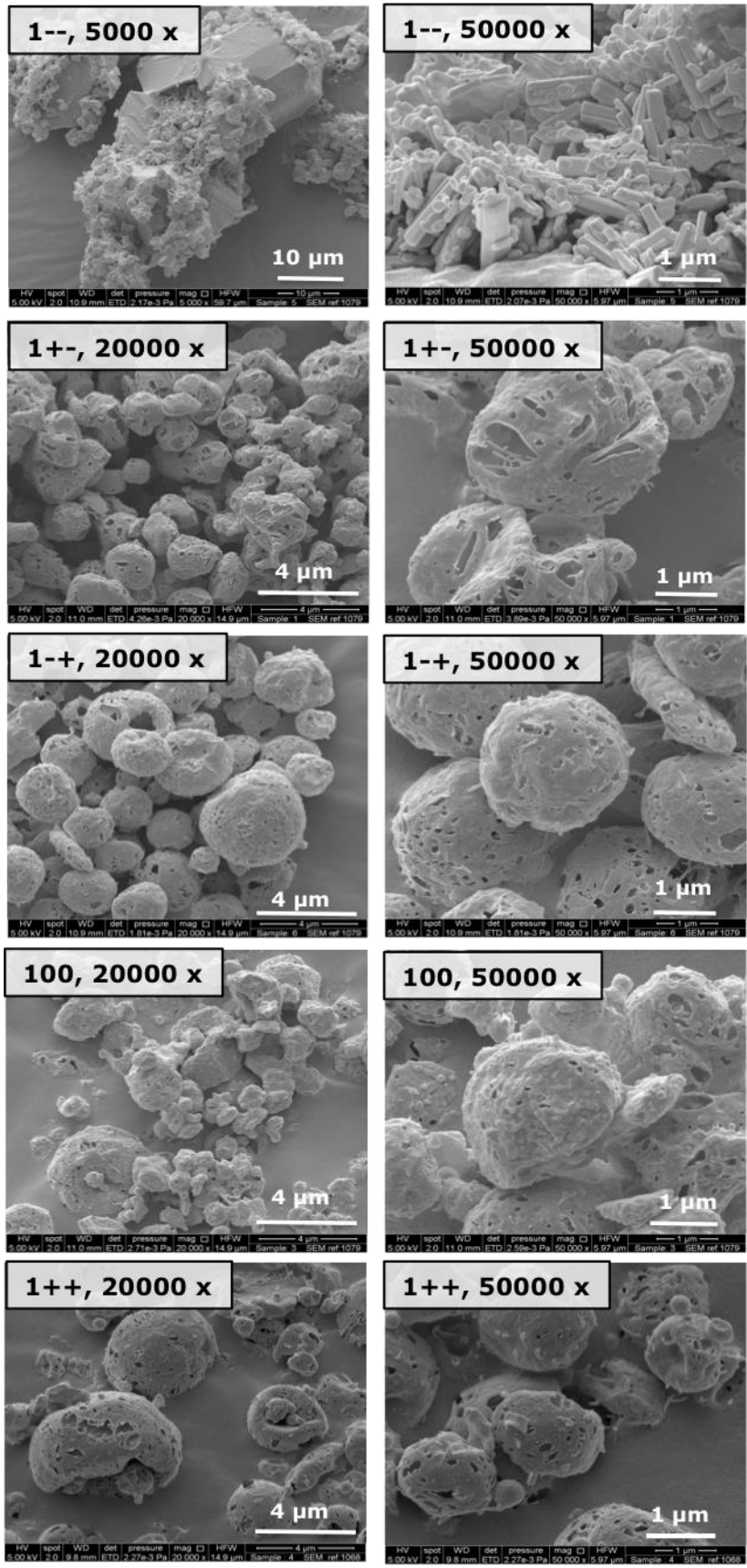
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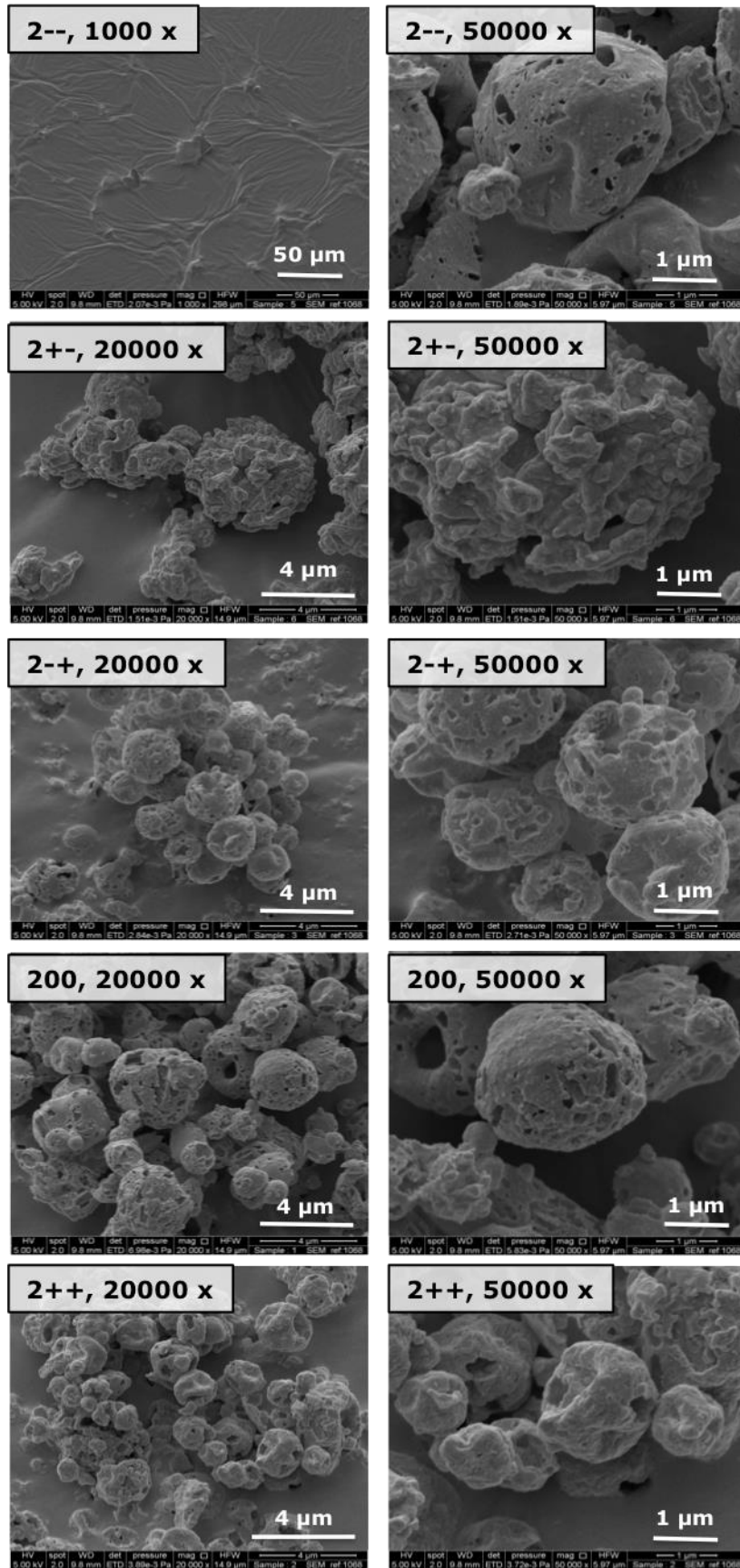
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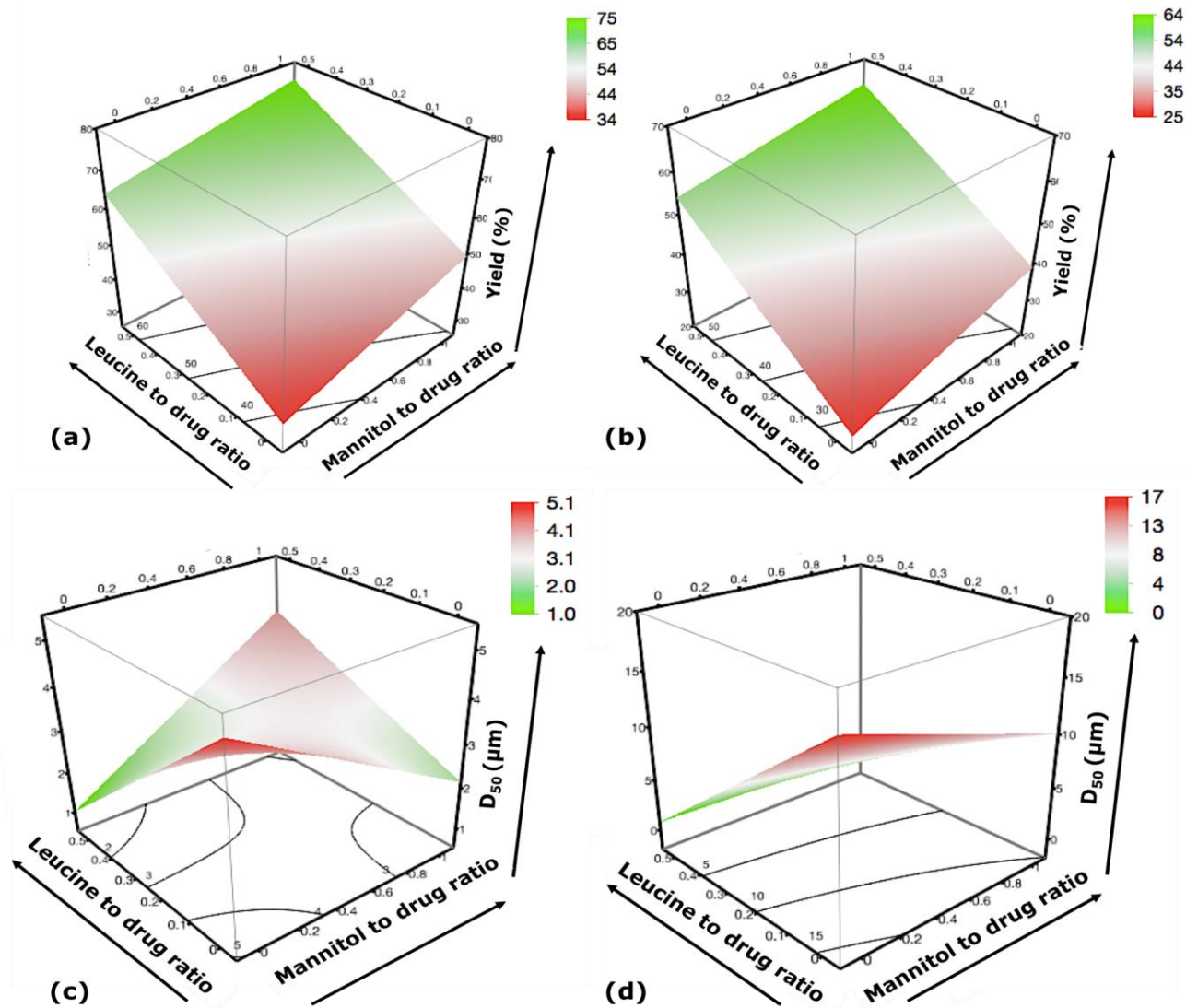
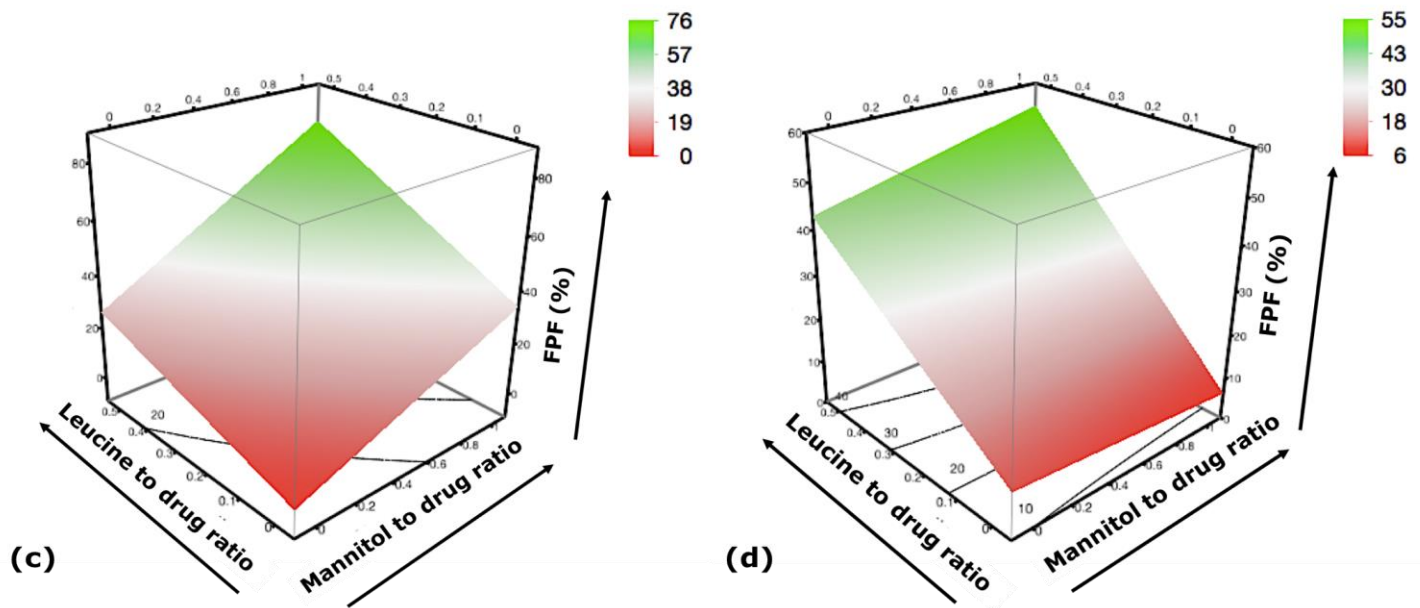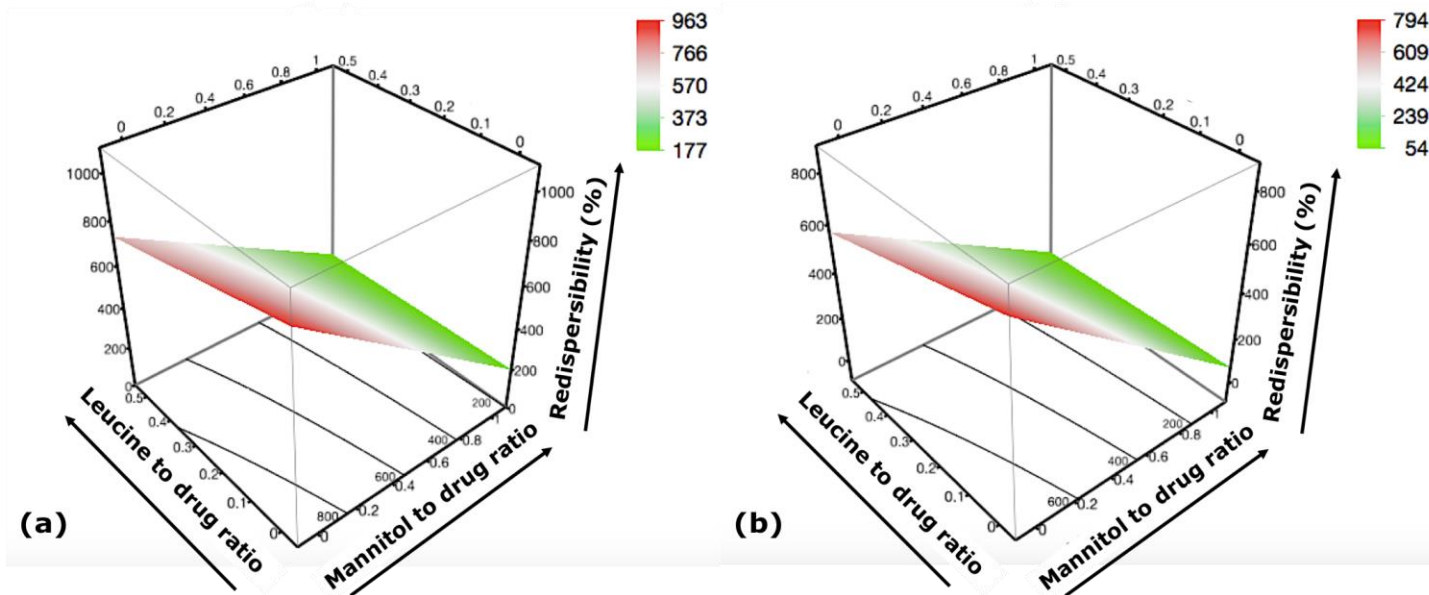**Figure 3** SEM image of ibuprofen starting material.

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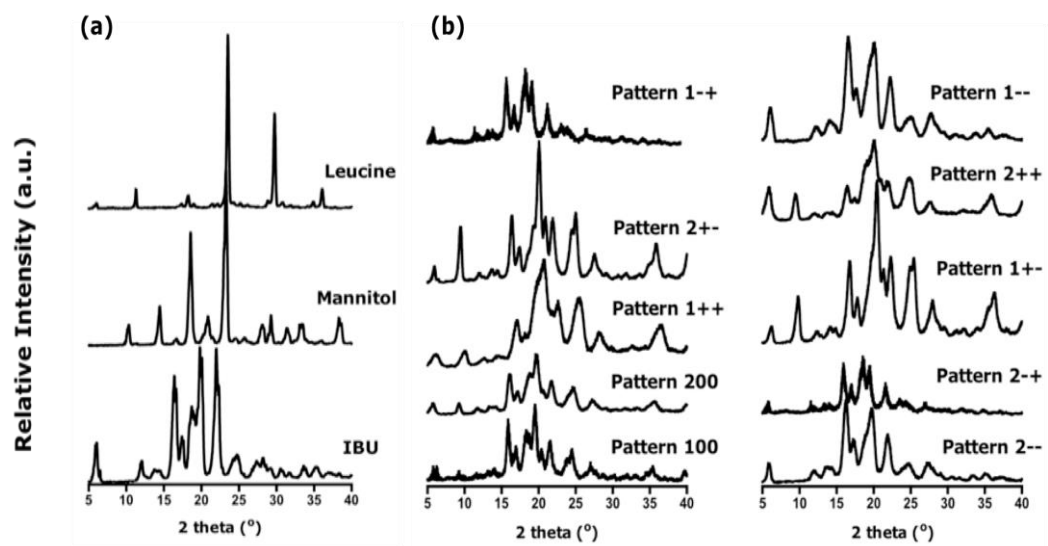
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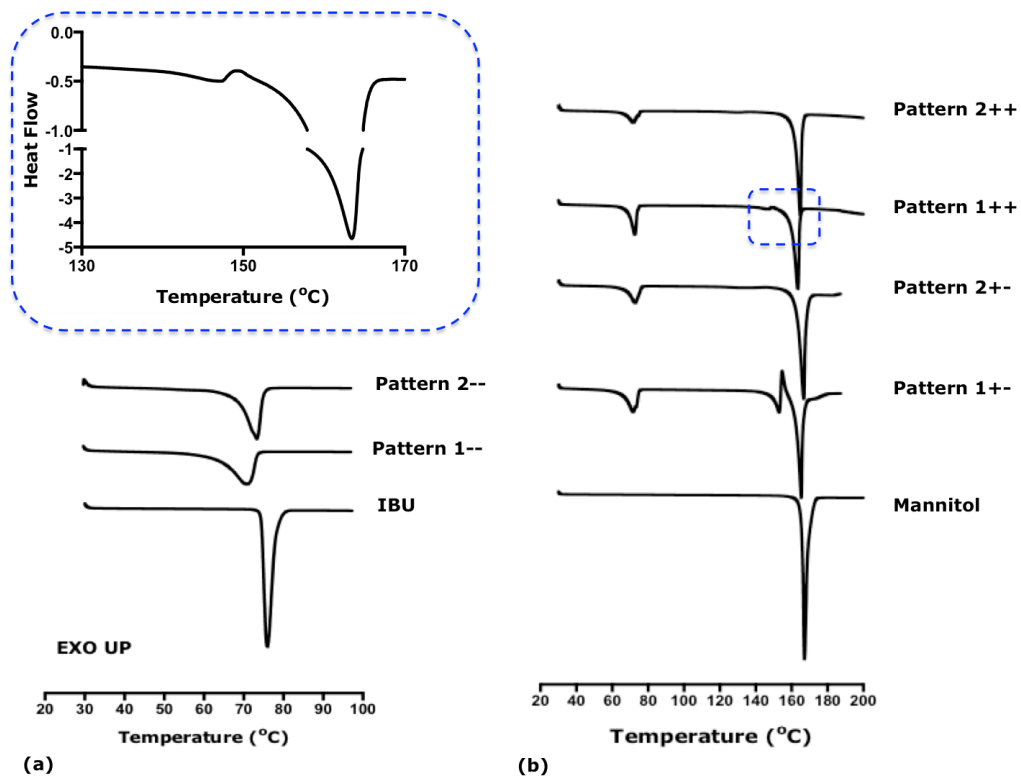
(a)

(b)

(c)

(d)

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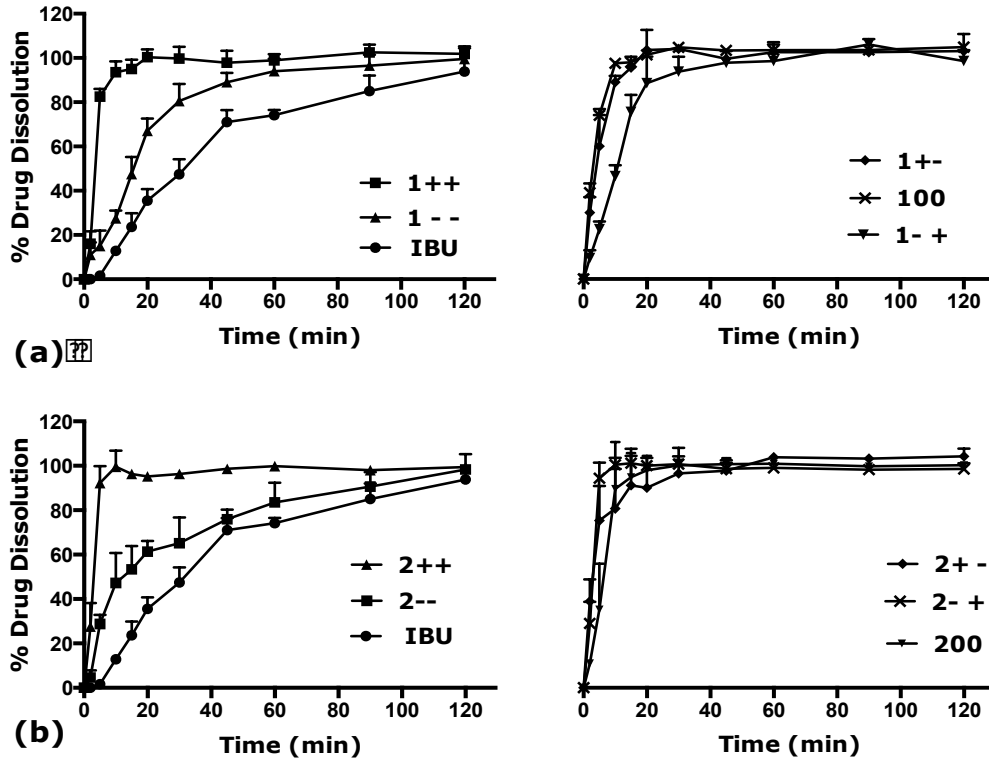
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