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Nanosuspensions of a poorly soluble investigational molecule ODM-106: Impact of milling bead diameter and stabilizer concentration

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ABSTRACT

Aqueous solubility of a drug substance is an important attribute affecting oral bioavailability. Nanonization, particle size reduction to submicron level, is an elegant approach to improve drug solubility and dissolution by increasing the surface energy, which in turn necessitates the use of stabilizers. The purpose of this study was to develop a nanosuspension of a practically water-insoluble investigational molecule by nanomilling approach using wet media milling. A variety of polymeric and surface active excipients were tested for their wettability. A combination of hydroxypropyl methylcellulose and sodium lauryl sulfate (SLS) were selected as stabilizers on the bases of compatibility studies and efficient wettability behaviour in contact angle measurements ($\approx 80^{\circ}$). A factorial design set-up was used to study the effect of milling bead diameter and stabilizer concentration on the efficiency of particle size reduction. Nanonization outcome was different when milling beads of 0.5 mm and 1 mm diameter were used at different concentrations of the stabilizers, which demonstrated the complex nature of the whole system. Storage of the nanosuspensions under different temperature conditions resulted only in minor changes of the particle size fractions.

1. Introduction

Development of nanosized pure drug crystals (nanonization) is one of the approaches to improve dissolution behaviour of poorly water-soluble drugs and it has made the fastest breakthrough from development to an industrial scale (Medarevic et al., 2018). Nanosuspensions are considered as sub-micron (usually 200–500 nm) colloidal suspensions of drug particles, which are stabilized by surfactants, polymers or the mixture of the both (Liu et al., 2011; Malamatari et al., 2018; Medarevic et al., 2018). The basic idea behind this approach is that particle size reduction leads to increased drug dissolution rate as well as increased saturated solubility of the poorly soluble drugs (Medarevic et al., 2018; Oktay et al., 2018). According to Noyes-Whitney equation, dissolution rate is increased due to the higher interfacial area that is associated to decreased particle size. The increase in saturation solubility is explained by the Ostwald-Freundlich theory, which suggests that this happens due to particle size being reduced to approximately smaller than 1 μm. Apart from these advantages, nanosuspensions have many other benefits: a high drug loading, low incidence of side effects, low cost, better adhesion to biological membranes, potential of targeted drug delivery, reduced fed/fasted state variability, possibility of many administration routes etc. (Liu et al., 2011; Liu et al., 2015; Medarevic et al., 2018).

It is well established that nanosuspensions are associated with physical instability (Azad et al., 2016; Knieke et al., 2013; Patel et al., 2018). This is due to the high interfacial free energy, which is associated with their large interfacial area (Malamatari et al., 2018; Wang et al., 2013). Particle agglomeration and crystal growth can result in losing many important properties of nanosuspensions; therefore, stability must be ensured during the milling process as well as during the subsequent storage (Azad et al., 2016; Knieke et al., 2013; Wang et al., 2013). This is achieved by either steric or electrostatic stabilization, provided by various ionic and non-ionic stabilizers, which are therefore a necessary component of nanosuspensions (Knieke et al., 2013; Malamatari et al., 2018). In fact, the right choice of stabilizers is one of the most important factors for both physical stability as well as in achieving the target particle size (Peltonen and Hirvonen, 2010). The most commonly used stabilizers that have been reported are different

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kinds of polymers, such as poloxamers, polyvinyl pyrrolidone (PVP), cellulose derivates (e.g. hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), hydroxyethyl cellulose (HEC), methylcellulose (MC)), and vitamin E TPGS, acting mainly via steric stabilization. Also surfactants, either ionic like sodium lauryl sulphate (SLS) providing electrostatic stabilization or non-ionic like polysorbates stabilizing sterically, are widely utilized (Peltonen and Hirvonen, 2018; Tuomela et al., 2016; Wang et al., 2013). A common way to achieve stability is also the combination of both steric and electrostatic stabilizers, which is suggested to give a synergistic stabilizing effect, usually referred to as electrosteric stabilization (Malamatari et al., 2018).

Nanosuspensions can be produced via reduction of particle size (top-down techniques) or via precipitation of dissolved molecules into solid particles (bottom-up techniques) (Ghosh et al., 2011; Liu et al., 2011; Oktay et al., 2018). By far the most typical and widely used top-down approach is wet-ball milling, also referred to as wet milling (Möschwitzer, 2013). This technique employs a certain grinding medium to mill the microcrystalline aqueous suspension into nanosuspension (Chin et al., 2014). During the process, high energy and shear forces are generated due to the stirring of drug particles, aqueous solution of stabilizers and milling media (beads/pearls, often yttrium stabilized zirconium oxide) at high speed, which provide the energy necessary for particle breakage and, thus, formation of nanosized particles (Afolabi et al., 2014; Peltonen, 2018; Peltonen and Hirvonen, 2010). Wet milling has many benefits, which makes it such a widely used technique; it is rather simple, it avoids organic solvents, it is easy to change or scale-up, the process repeatability is very high, it is cost-efficient etc. (Liu et al., 2011; Möschwitzer, 2013; Peltonen and Hirvonen, 2010, 2018). However, there are several parameters affecting the efficiency of achieving optimal particle size reduction, such as drug amount, quantity and size of the milling beads, milling speed, milling time and temperature (Möschwitzer, 2013). Milling beads have been found to be a crucial and complex parameter in achieving optimal particle size in different studies (Ghosh et al., 2011; Ghosh et al., 2013; Ghosh et al., 2012). Commonly milling beads, which are used to produce nanocrystals, are 0.5 mm and 1 mm in diameter. Milling beads larger than 1 mm in diameter are not very efficient in producing nanocrystals. On the other hand, beads smaller than 0.5 mm are too small to generate sufficient kinetic energy for production of nanocrystals, which are smaller than what is achievable using 0.5 mm beads (Kakran et al., 2012; Medarevic et al., 2018; Toziopoulou et al., 2017)

As stated above, there is an interplay between many variables which affect particle size and stability of nanosuspensions, and the importance of each of them is challenging to evaluate. In previous studies, attempts have been made to estimate the influence of different parameters on the formation of nanosuspensions using different approaches (e.g. Design of Experiment, dynamic equilibrium curve etc.) (Ghosh et al., 2013; Nekkanti et al., 2015; Oktay et al., 2018). However, it is still not possible to predict an optimal stabilizer type and concentration for a given drug that ensures an effective particle size reduction and proper stability. Furthermore, it is still unclear, how different bead sizes influence particle size in relation to different stabilizer concentrations and amounts of drug in the milling vessel, where both the parameters are affecting the viscosity of the system.

The primary aim of our study was to gain an insight into how two different bead sizes in the wet-ball milling process affect particle sizes when different viscosity affecting stabilizer concentrations are used; particularly of interest was how smaller beads perform in highly viscous media compared to larger beads. Our secondary aim was to investigate the stability of prepared nanosuspensions in different storage conditions. Nanosuspensions were prepared by wet-ball milling using two different bead sizes; 0.5 mm and 1.0 mm. For both the bead sizes, several combinations of different concentrations of HPMC and SLS were used utilizing factorial design as the study plan. Amount of HPMC is affecting to the viscosity of the system, which reflects to the milling efficiency. Particle size and polydispersity index (PDI) were compared between the bead sizes as well as among the different stabilizer concentrations. Stability of nanosuspensions was assessed in three different storage conditions during the time period of three weeks.

2. Materials and methods

2.1. Materials

ODM-106, IUPAC name 3-(4-bromobenzyl)-7-fluoro-1-methylquinazoline-2,4(1H,3H)-dione (Orion, Espoo, Finland), an investigational poorly water soluble active pharmaceutical ingredient (API), was used as a model drug for wet-ball milling. HPMC (Pharmacoat 603) from Shin-Etsu Chemical (Tokyo, Japan) and SLS (Merck KGaA, Darmstadt, Germany) were used as the stabilizers. SLS was used as an electrostatic stabilizer and HPMC was used as a steric polymeric stabilizer. PVP and vitamin E TPGS were procured from Sigma, St. Louis, MO, USA. Methanol (HPLC grade, VWR International, Pennsylvania, USA), monobasic potassium phosphate (Sigma, St. Louis, MO, USA), sodium hydroxide (Sigma, St. Louis, MO, USA), hydrochloric acid (Merck KGaA, Darmstadt, Germany), and Polysorbate 80 (Tween® 80, Fluka Chemika, Buch, Switzerland) were used for the characterization of nanosuspensions. In all the experiments ultrapurified Millipore® water (Millipore, Molsheim, France) was used.

2.2. Wettability of ODM-106

The dynamic contact angle of the ODM-106 with water and with aqueous solutions of the surface active excipients (0.1% w/v) and suspending agents (1% w/v) was determined using DCAT 11 (DataPhysics Instruments GmbH, Filderstadt, Germany). The ODM-106 micronized drug was attached to the rectangular microscopic slide partially covered with standard double-sided adhesive tape. The velocity of plate's feed was 200 μm/s, the immersion depth was 10 mm. The measurements were run in triplicate.

2.3. Wet-ball milling

Nanosuspensions were prepared by the wet-ball milling technique. A custom central composite design was utilized to statistically investigate the interaction between three independent variables: (1) HPMC concentration (5 level numeric factor), (2) SLS concentration (3 level numeric factor), and (3) milling beads diameter (2 level category factor), on two dependent variables: drug particle size (nm) and PDI. Design matrices were comprised of 26 runs.

Stabilizer solutions were made by dissolving HPMC (0.625 – 5.0% w/w) and SLS (0.0625 – 0.1875% w/w) in water. The API (1.25 g), aqueous stabilizer solution (5 g) with different proportions of HPMC and SLS, and zirconium oxide milling beads (30 g, diameter 0.5 mm or 1.0 mm) were placed into the milling vessel (vessel volume 20 ml) and dispersed. With the addition of API, final concentrations of HPMC were 0.5, 1, 2, 3 and 4% w/w (level: −1.5, −1, 0, +1, +2, respectively) and final SLS concentrations were 0.05, 0.1 and 0.2% w/w (level: −1, 0, +1, respectively) (Table 1). Amounts of the API, stabilizer solution and milling beads were determined based on preliminary studies and initial screening experiments. The milling speed and quantities of the beads were fixed and used to the recommended levels from the equipment supplier. The milling vessels were then inserted into the planetary ball mill (Pulverisette 7 Premium, Fritsch Co., Idar-Oberstein, Germany) and the grinding was performed at 1100 rpm. There were 10 milling cycles of 3 min and there was a 15 min break after each cycle to allow the system to cool down. These parameters were also determined in preliminary studies. After the milling process, nanosuspensions were collected and separated from the milling beads by pipetting from the vessel. Unless otherwise specified, all the concentrations refer to the mass of the substance with respect to the total content of suspension, w/w (API, water and stabilizers).

Table 1
Experimental runs according to the central composite design.

Run	HPMC % w/w	SLS % w/w	Milling beads diameter (mm)
1	2	0.2	1.0 mm
2	0.05	0.1	1.0 mm
3	0.05	0.1	0.5 mm
4	4	0.1	1.0 mm
5	3	0.1	0.5 mm
6	4	0.1	0.5 mm
7	3	0.1	1.0 mm
8	1	0.1	0.5 mm
9	1	0.1	1.0 mm
10	2	0.05	0.5 mm
11	2	0.2	0.5 mm
12	1	0.2	0.5 mm
13	1	0.2	1.0 mm
14	2	0.05	1.0 mm
15	1	0.05	0.5 mm
16	1	0.05	1.0 mm
17	2	0.1	1.0 mm
18	2	0.1	0.5 mm
19	2	0.2	0.5 mm
20	3	0.2	0.5 mm
21	2	0.2	1.0 mm
22	3	0.2	1.0 mm
23	2	0.05	0.5 mm
24	3	0.05	0.5 mm
25	2	0.05	1.0 mm
26	3	0.05	1.0 mm

2.4. Particle size distribution

The mean particle sizes and polydispersity indexes (PDIs) of the nanosuspensions were analyzed by photon correlation spectroscopy (PCS) with Malvern Zetasizer 3000HS (Malvern Instrument, Malvern, UK). PDI means the width of the particle size distribution. The lower the PDI value, the more monodisperse in size the particles are. If the PDI is higher than 0.7, the particles in suspensions are polydisperse. A part of the fresh nanosuspension was diluted with saturated solution containing ca. 0.1% w/w of HPMC, in order to achieve a suitable concentration for the measurements by PCS. The nanosuspensions were sonicated for 4 min before the size measurements. The analyses were performed with a dispersant refractive index of 1.33. The measurements were performed 3 times for each sample.

2.5. Transmission electron microscopy (TEM)

The selected suspensions were pipetted on formvar film-coated copper grids with mesh size of 300 (Agar Scientific, Essex, UK) and dried at ambient temperature. The morphological evaluation of the particles was done using TEM (Jeol JEM-1400, Jeol Ltd., Tokyo, Japan).

2.6. Solid state characterization

To determine the polymorphic transformation(s) during wet milling, if any, differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD) analyses were performed. Nanosuspension containing 0.1% of SLS and 2% of HPMC, centre point of all the studied stabilizer concentrations, was chosen for this evaluation and compared with the unmilled form of ODM-106.

2.6.1. DSC

Thermal properties of room temperature dried samples were analyzed with a DSC 823e (Mettler Toledo Inc., Columbus, USA). The samples were placed in sealed aluminium pans perforated in the lid. The temperature range for the measurements was from 0 to 200 °C with the heating rate of 10 °C/min. The measurements were performed under nitrogen flow of 50 ml/min. Pure bulk drug, HPMC, and physical mixtures of the drugs and the stabilizers (in the same weight ratio as nanocrystals) were tested as controls. The data were analyzed with STARe software (Mettler Toledo, Columbus, USA).

2.6.2. XRPD

XRPD patterns were determined from dried nanomilled samples, pure drugs and physical mixtures of drugs and stabilizers by an X-ray diffractometer (Bruker AXS D8, Karlsruhe, Germany). The XRPD was performed in symmetrical reflection mode using Cu-Kα radiation with $\lambda = 1.54$ Å (40 kV and 40 mA). The sample was placed on a flat aluminium sample holder. Data were collected by scanning from 5° to 50° with 0.01° steps.

2.7. Stability testing

Nanosuspensions were envisioned to be dried for final formulation development within a two weeks period. However, the physical stability of nanosuspensions was tested for a longer period of time, up to three weeks, by monitoring particle size distribution to better understand the stabilization efficiency at different SLS concentrations (HPMC concentration was fixed at 2%) under several temperature settings: refrigerated condition (4 ± 2 °C), ambient room temperature (25 °C), and 40 ± 2 °C. Samples were taken at the interval of 1 week and were diluted as per instructions for particle size distribution analysis after 30 s of vortex shaking of nanosuspension vials, followed by 1 min of sonication, and once more 30 s of additional vortexing.

2.8. Statistical analysis

Interactions between stabilizers' concentration and milling beads diameters and their effects on particle sizes, were statistically studied using design of experiment approach (Design-Expert ver. 11, Stat-Ease, Inc.). ANOVA test was performed to get information on significant parameters. The level of significance was fixed at $p = 0.05$.

3. Results and discussion

3.1. Contact angle of ODM-106

One stabilizer can be enough for stabilizing drug nanocrystals (Liu et al., 2011), but often two stabilizers, typically a mixture of a polymer and a surfactant, are used in order to further improve the stabilization effect, which combines the advantages of both steric and electrostatic stabilizations. (Ghosh et al., 2013; Leung et al., 2014; Van Eerdenbrugh et al., 2008). Excipients increasing drug wettability offer better stabilization of nanosuspensions (Cerdeira et al., 2010; Li et al., 2018). Wettability of a drug substance by a stabilizer solution is typically determined by measuring the contact angle. HPMC and PVP were selected as suspending agents, and SLS, Tween 80, and Vitamin E TPGS were chosen as surfactants to make different mixtures of stabilizer solutions. The average contact angle (CA) values are reported in the Table 2. The smaller the contact angle between drug and stabilizer the better the wettability. ODM-106 showed poor wettability properties in water

Table 2
Summary of contact angle (CA) data of drug in water and aqueous suspending/surfactant solutions.

Suspending agent (1% w/v)	Surfactant(0.1% w/v)	CA (°)
–	–	140 ± 3
HPMC	SLS	82 ± 2
HPMC	Tween 80	89 ± 1
HPMC	Vitamin E TPGS	97 ± 4
PVP	SLS	76 ± 1
PVP	Tween 80	86 ± 4
PVP	Vitamin E TPGS	122 ± 3

considering a CA scale 0-180°, where 0° denotes completely wettable and 180° completely unwettable. At the selected concentrations, SLS and Tween 80 seemed to improve wettability more than TPGS. PVP-SLS combination greatly reduced the CA and, therefore, was the first choice as stabilizing agent for nanomilling. However, compatibility studies between PVP and ODM-106 revealed high degradation risks. PVP usually has hydroxyl and aldehyde groups in the terminal ends of the macromolecule, which underlie its redox reactivity (Amri and Roger, 2020). Also, PVP typically contains peroxides as impurities and therefore it is often discarded from the early stages of formulation development based on the results of incompatibility studies (Wu et al., 2011). These incompatibility results indicated that other stabilizers like polyethylene glycol (Hemenway et al., 2015) and polyoxyethylene-polyoxypropylene block copolymer will also not be suitable since trace levels of peroxides, aldehydes and organic acids are also present in these, which are linked to instability of active ingredient (Wasylaschuk et al., 2007). It is well known that the degradation risk is higher in the liquid formulation such as nanosuspension, moreover, high energy involved in the wet milling process will increase the rate of the API degradation. Therefore, HPMC was chosen as the suspending agent and SLS as the surface active excipient to form the most appropriate stabilizer solutions.

3.2. Nanomilling

3.2.1. Effect of milling time

Milling time at constant milling speed is a direct measurement of energy input to the system (Peltonen, 2018; Singh et al., 2011). Given that required nanomilling energy is specific to drug crystals properties, there is no specific value applicable to all drugs. In general, longer milling time is likely to support comminution because of higher energy input with increased numbers of collisions between drug particles and milling beads (Toziopoulou et al., 2017). However, longer milling time may also increase the temperature of the milling vessel, which in turn can cause crystal growth due to Ostwald ripening in the system, and may also lead to contamination from milling beads or vessel.

The required milling time for ODM-106 was determined by dispersing bulk ODM-106 (1.25 g) in 5 ml of aqueous stabilizer solution containing HPMC (125 mg; 10% relative to the drug amount) and SLS (6.25 mg; 0.5% relative to the drug amount) (Fig. 1). Routinely, the mean diameters of bulk crystalline drug materials are tens of micrometers and the size distributions are broad (particle size distribution of ODM-106 micronized drug was d_{10} = 5.8, d_{50} = 17 and d_{90} = 80 μm). After milling for only 6 min (2 milling cycles, each of 3 min), the mean size of the drug was decreased dramatically below 1 μm. With increasing milling times, the sizes and the PDI were decreased further. The size-decreasing trend for the model drug was observed due to the long milling time that splits bigger particles into smaller ones and provides adequate time for the stabilizers to cover the drug surfaces. No

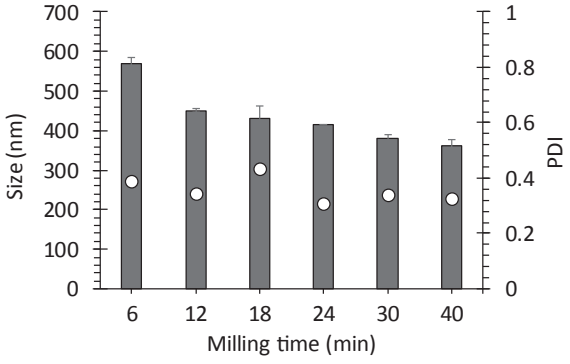

Fig. 1. The effect of milling time on mean particle size (bars) and polydispersity index (circles).

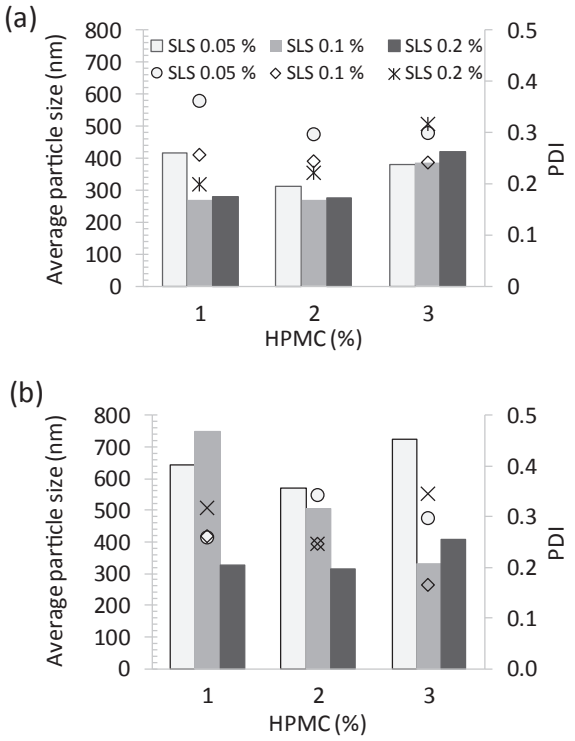

Fig. 2. The effect of different HPMC and SLS concentrations on media milling using (a) 0.5 mm and (b) 1 mm milling beads. Average particle sizes in bars and PDI values in symbols.

significant reduction in particle sizes and size distributions were observed after further prolonging the milling times beyond 30 min of media milling (10 cycles).

Particle size reduction with an increase in milling time can be attributed to the increase in the energy and shear forces produced due to impact between the grinding balls and the drug that provides high energy to break the microparticles to nanosized particles (Liu et al., 2011). Additional mechanical energy by prolonging milling could have a negative impact on the stability of the system by breaking the repulsive forces and causing aggregation of the small particles (Liu et al., 2011). Given that further continuation of milling was not beneficial, the milling time was fixed at 30 min for all further experiments.

3.2.2. Effect of stabilizer concentration and bead size

Particle size and PDI of nanosuspensions at different stabilizer concentrations and milling beads are presented in Fig. 2. The measured PDI was in most cases close to or below 0.3, which is an indicator of good particle size uniformity (Patel et al., 2014).

It is well established that increasing amount of steric stabilizer, such as HPMC, results in more efficient particle size reduction (Azad et al., 2016; Knieke et al., 2013; Peltonen and Hirvonen, 2010). Usually one stabilizer can be enough for stabilizing drug nanocrystals (Liu et al., 2011), but often two stabilizers, typically a mixture of a polymer like HPMC and an ionic surfactant like SLS are used in order to further improve the stabilization effect, which combines the advantages of both steric and electrostatic stabilizations (Ghosh et al., 2013; Leung et al., 2014; Van Eerdenbrugh et al., 2008).

Stabilization due to electrostatic stabilizers alone is sensitive to pH, ionic strength, *etc.*, as in drying. Therefore, combined stabilization effect is much needed where the objective is to develop a dry nanoformulation for oral dosing (Beirowski et al., 2011). After stabilizer selection based on the contact angle and compatibility data, optimal concentration needs to be decided for stabilizing the smaller sized particles and for maintaining the shelf-life stability of the final product

(Ghosh et al., 2011; Ghosh et al., 2012; Van Eerdenbrugh et al., 2009).

In this study, we noticed that optimum proportions of HPMC and SLS were needed for maximum particle size reduction. As can be seen from Fig. 2, the effect of HPMC on particle size reduction depended on the amounts of SLS. Nanomilling using 1 mm beads demonstrated decrease in particle size with the increase in HPMC concentration from 1% to 2% at 0.05% and 0.1% of SLS concentrations (Fig. 2(b)). Similar decrease in particle size was also observed using 0.5 mm beads at the lowest studied SLS concentration (0.05%).

As the particles are becoming smaller during milling, their surface area as well as number concentration is increasing, leading to an increase in collision frequency and decrease in inter-particle distances (Azad et al., 2016). Therefore, it is important to sufficiently protect particles from possible agglomeration. Higher HPMC concentrations help in particle size reduction by covering more surfaces and providing sufficient steric stabilization (Knieke et al., 2013; Peltonen and Hirvonen, 2010). With an increase in HPMC concentration i.e., 1% to 3% (2 mPa s to 5 mPa s, respectively at 20 °C in aqueous solution (Shin-Etsu, 2004)), the efficiency of particle size reduction of the milling process is reduced, which is probably because of an increase in viscosity of the suspension. In general, grinding efficiency increases with increased solids concentration and viscosity (Kwade, 1999), which is also consistent with the results for 1.0 mm beads at 1 and 2% HPMC concentrations. However, if suspension viscosity is too high, the grinding efficiency drops due to a decrease of movement between the grinding media and the product suspension (Kwade, 1999).

In comparison to 1 mm beads, 0.5 mm beads have a higher surface area and thus higher probability of contact with the API and thereby reducing particle size by impaction forces. However, smaller beads are more sensitive to viscosity changes since smaller and lighter beads do not have enough kinetic energy to break the particles in more viscous media (Peltonen, 2018) and, therefore, particle size reduction is not as effective anymore (Sepassi et al., 2007). Thus, increasing milling speed might be a way to achieve efficient comminution of drug particles in a highly viscous dispersion medium (Medarevic et al., 2018). At 0.2% concentration of SLS, the small difference between 0.5 and 1.0 mm beads might be because the minimal size has already been achieved at lower concentrations with 0.5 mm beads and further increase of SLS concentration does not reduce the particle size further. However, for 1.0 mm beads, adding SLS still contributed to reaching smaller particle size, hence it came closer to the particle size achieved by using 0.5 mm beads.

When HPMC concentration was increased to 4%, it was observed that the obtained suspension was very viscous (6 mPa s, 20 °C in aqueous solution), and after some days of storage at room temperature, it solidified in both 1.0 mm and 0.5 mm batches. Interestingly, lowering the HPMC concentration also resulted in the formation of very unstable suspensions, which had a strange morphology even right after the milling process, as depicted in Fig. 3. Also, it was observed that only after a few days, there was a very distinct phase separation when the concentration of HPMC was 1% or lower with 1.0 mm beads, and 0.5% with 0.5 mm beads. The reason behind this behaviour might be that at some concentration point, the amount of HPMC was not sufficient to prevent the beads from generating more energy by collisions and thereby heating the suspension over a certain threshold, above which the morphological changes were observed. With 0.5 mm beads, which most likely have less kinetic energy than 1.0 mm beads at the same milling conditions, this threshold occurs at lower HPMC concentration and suspension viscosity, where the kinetic energy is still sufficient.

Furthermore, HPMC is a polymer with lower critical solution temperature (LCST) between 75 and 90 °C and, therefore, with increasing temperature it forms reversible gels via hydrophobic interactions occurring between the hydrophobic sections of the polymer chains (Marefat Seyedlar et al., 2014). It was expected to observe such sol to gel transitions in this study as well, due to the heat generated with high kinetic energy in less viscous solutions where HPMC concentration was below 1% for 1 mm beads and 0.5% for 0.5 mm beads. Also, the presence of water has a significant effect on the glass transition temperature (T_g) of a polymer (Perfetti et al., 2012). Perfetti et al. (2012) observed a decrease in T_g from 120 °C to approximately 70 °C, when the water content in HPMC (Pharmacoat 603) was increased from 0 to 12%. Considering that the milling vessel felt unusually warm after the experiment with the lower HPMC concentration, it is possible that T_g was reached, which might be related to the observed effect on the consistency of the nanosuspensions. Nevertheless, to prove that with more certainty, more studies are required to further examine the effect of temperature associated behaviour of nanosuspensions stabilized by HPMC.

The addition of SLS as an electrostatic stabilizer in combination with HPMC is well known to be beneficial for the stabilization effect (Knieke et al., 2013; Knieke et al., 2015; Parmentier et al., 2017; Peltonen, 2018). The stabilizing effect is due to the adsorption on particle surfaces and, thereby, formation of surfaces with a charge sufficient for stabilization (Peltonen and Hirvonen, 2010). Fig. 2 shows a very similar phenomenon as was noticed with HPMC; with 1.0 mm beads, the particle size seemed to decrease with an increase in SLS concentration, whereas with 0.5 mm beads, there were no significant changes. It has been reported in literature that an increase in SLS content above a certain point does not further influence the particle size, because a minimum for the applied grinding operation has already been reached (Knieke et al., 2013). Although more experiments would be needed to claim that also in this case, this could also be the reason for the observation for 0.5 mm beads.

3.2.3. Morphology of nanocrystals

The SEM image of micronized API is presented in Fig. 4**(a)**. Before nanomilling, raw drug materials consisted of micron sized crystals. After wet milling, the ODM-106 crystals in the presence of the stabilizers were transformed into nanocrystals. The morphology of drug nanosuspension was observed using TEM (Fig. 4**(b)**). The angular surfaces of the drug nanocrystals were now much smoother.

3.3. Crystalline state evaluation

The DSC thermograms are shown in Fig. 5. The extrapolated onset temperature was used to define the melting point (T_m). Pure drug showed a sharp endothermic melting peak at 161 °C and HPMC showed a broad endothermic peak in the range 40–100 °C. The endothermic peak of the physical mixture showed slight broadening, which was expected due to the solubility of drug with the polymeric stabilizer. Comparing the curves of physical mixtures and nanocrystal samples implies that no significant differences were found and ODM-106 exists in crystalline form after the milling. In the DSC thermogram, a larger shift in the nanocrystals peaks to lower melting temperatures (157 °C) can be seen, which could be due to the smaller drug particle size, resulting in more solid state solubility of API in polymeric stabilizer. Melting transition, being a colligative property, decreases with the increase in solubility of drug in the polymer, HPMC in this case, and will depend on the amount of polymer present (Chokshi et al., 2005). Such decrease in melting transition after nanonization has also been reported previously (Gahoi et al., 2012; Liu et al., 2011; Teeranachaideekul et al., 2008).

The X-ray diffractograms are shown in Fig. 6. The diffraction pattern of micronized drug exhibited sharp peaks at 12°, 14°, 15°, 17°, 20°, 22°, 24° and 27° 2θ. The position of all characteristic ODM-106 peaks remained in the diffractograms of its physical mixtures with HPMC and SLS, and also in the nanocrystals. However, low peak intensities in nanoformulations were observed, owing to the smaller size of the drug and covering of the particles surface with the stabilizer that resulted in the broadening of the peaks and appearance of weak amorphous halo in the background instead of intense narrow peaks (Hecq et al., 2005). Combining the results of DSC and XRPD, it was established that the

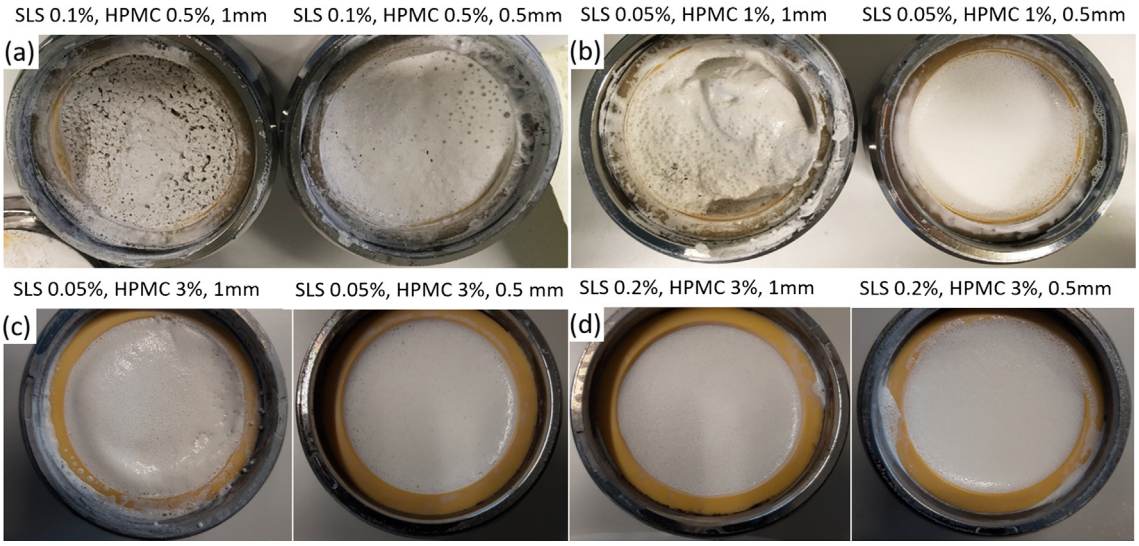

Fig. 3. Appearance of the suspensions in milling bowl immediately after milling. Compositions with (a) 0.1% SLS, 0.5% HPMC resembles a gel at both milling beads diameter and (b) compositions with 0.05% SLS, 1% HPMC resembles gel at only 1 mm milling bead. Other compositions (c) and (d) with higher HPMC concentrations resulted in good nanosuspension. Inner diameter of the milling bowl is 48 mm.

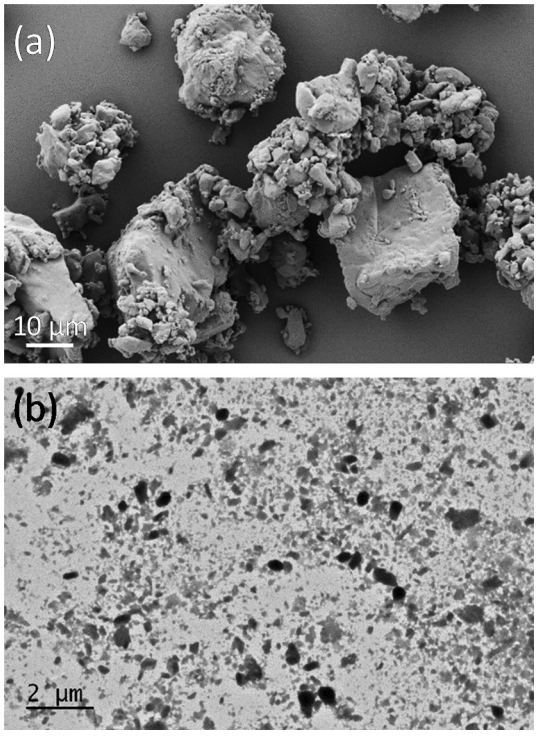

Fig. 4. Morphology of drug crystals. (a) SEM image of micronized bulk ODM-106 and (b) TEM image of ODM-106 nanocrystals made with 0.05% SLS and 2% HPMC using 0.5 mm beads.

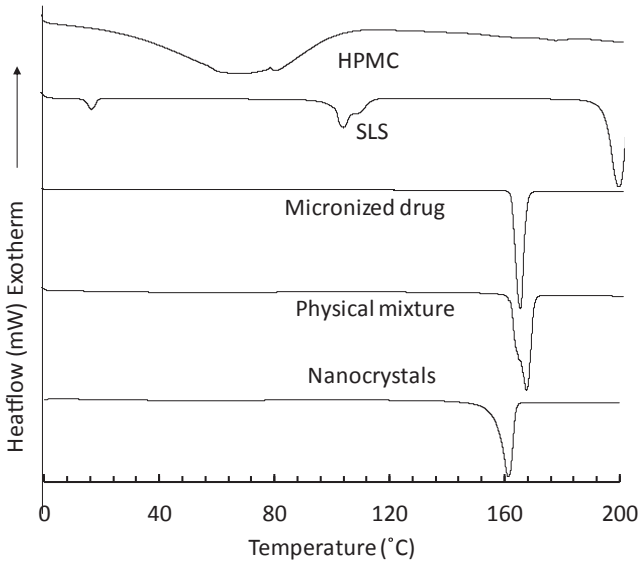

Fig. 5. The DSC patterns of nanocrystals (SLS 0.1% and HPMC 2%), physical mixture, micronized bulk drug, SLS and HPMC.

milling does not interfere with the crystallinity or polymorphic form of the drug compound.

3.4. Physical stability of suspensions

For testing the stability of the prepared nanosuspensions, three different formulations in triplicates were made with both beads sizes. Given that at 2% HPMC concentration both the milling beads sizes demonstrated efficient milling, the concentration of HPMC was fixed at 2% and three different SLS concentrations were selected (0.05, 0.1 and 0.2%). All chosen formulations were stored in three different conditions: ambient temperature (25 °C), 4 °C and 40 °C. Stability was assessed via particle size and PDI value measurements, where particle size increase and/or increase in PDI was interpreted as an indicator of physical instability (Ghosh et al., 2013; Knieke et al., 2015). At first, the effect of temperature was studied on nanosuspensions prepared using the centre point conditions (Fig. 7).

The stability study results of formulation prepared with 2% of HPMC and 0.1% of SLS using 0.5 mm beads revealed that there was a significant increase in particle size after 7 days of storage at all temperature conditions, and after that no further significant changes in particle sizes were noticed (Fig. 7**(a)**). The initial increase in particle size could be associated with relaxation of nanoparticles after high shear milling; since the energy inputs are very high during milling, the suspension is likely to be far away from equilibrium state after milling, and needs some time to achieve it (Deng et al., 2008). However, stability results from nanosuspensions prepared using 1 mm beads demonstrated no significant changes in particle sizes at all storage

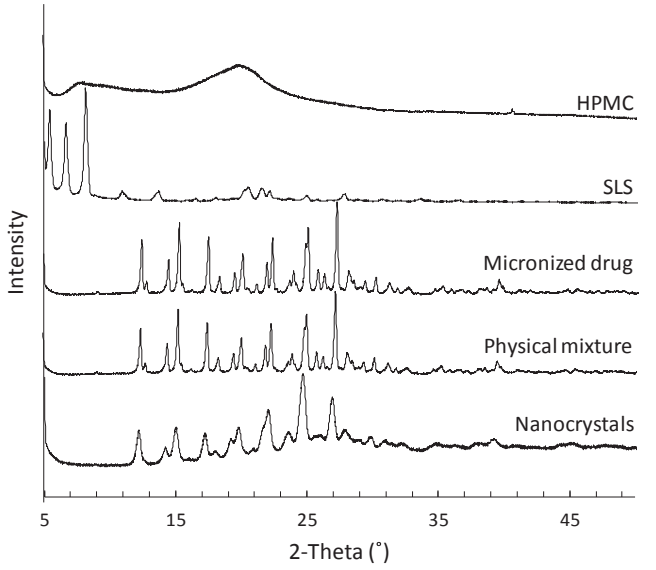

Fig. 6. X-ray diffraction analyses of nanocrystals (SLS 0.1% and HPMC 2%), physical mixture, micronized bulk drug, SLS and HPMC.

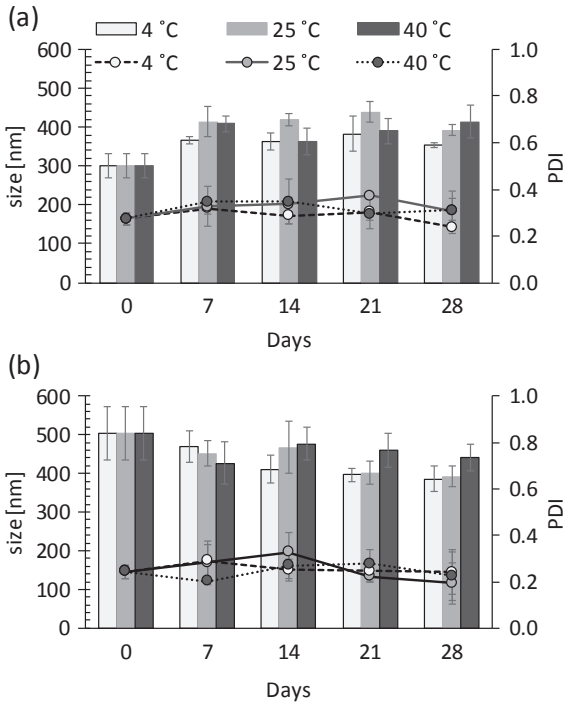

Fig. 7. Effect of various stability temperature conditions on the growth of ODM-106 nanocrystals prepared with 2% HPMC and 0.1% SLS as a stabilizer. (a) Mean particle size using 0.5 mm beads, and (b) mean particle size using 1 mm beads. Particle sizes in bars and PDI values in symbols.

conditions (Fig. 7**(b)**). PDI values for all batches remained below 0.5, which is considered as an index for good particle size uniformity for nanosuspensions (Mohammadi et al., 2011), meaning the stability was satisfactory from this point of view. However, there is quite a lot of fluctuation in particle sizes as well as in PDIs throughout the whole time period of observation seen in all the figures. That might be due to the nature of sample preparation and measurements; since only a small part of the suspension is taken for one analysis, it can hardly account for the state of the whole sample, although the results are given as an average of three measurements.

Given the fact that least fluctuations in the size and PDI were

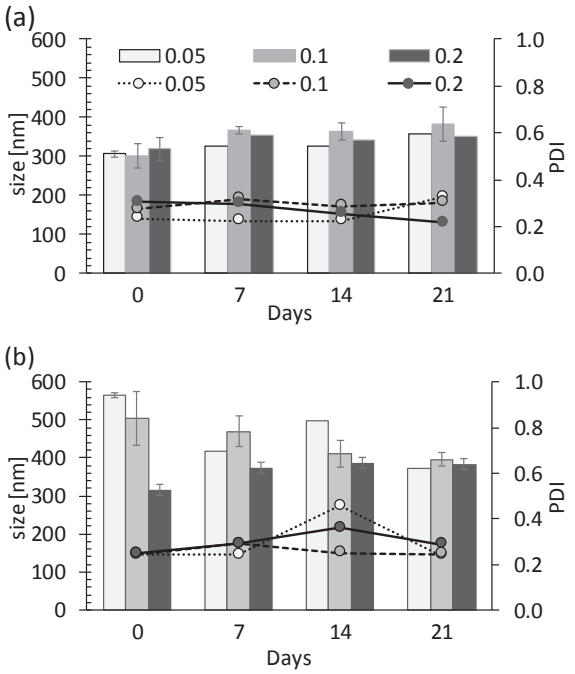

Fig. 8. Effect of various concentrations of SLS (%) to inhibit the growth of ODM-106 nanocrystals during stability study at 4 ˚C. (a) Mean particle size using 0.5 mm beads, and (b) mean particle size using 1 mm beads. HPMC concentration fixed at 2%. Particle sizes in bars and PDI values in symbols.

noticed from samples stored in refrigerated conditions, the effect of SLS concentration on 3 weeks stability was studied only from those samples (4 ± 2 ˚C). In Fig. 8**(a)** it can be seen that for 0.5 mm beads and SLS concentration of 0.1% there is some initial increase in particle size from day 0 to day 7 ($p = 0.017$), after which no distinct trend was visible anymore in either of the storage conditions. For 1.0 mm beads and 0.1% or 0.05% SLS, on the other hand, the average particle size seemed to be decreasing throughout the observation period. A similar behaviour, however, has been reported by Deng et al. (2008), who noticed particle size reduction after initial increase, as well as a change in surface morphology throughout the testing period (Deng et al., 2008). They recognized the observation to the process of reaching the equilibrium state by reorganization of the stabilizer molecules. After the high shear milling process, the system is far from the thermodynamic equilibrium and thus evolves to a new organized state. This phenomenon can happen within a few days and apparent particle size reaches a maximum. Thereafter, the polymeric structure in the nanosuspension is relaxed spontaneously with an apparent size reduction and disassociation of clusters into individual particles. Ultimately, the nanosuspensions are aimed to transform into dried nanocrystals to make an oral solid dosage form.

When the applied concentration of SLS was 0.2%, slight increase in the particle size was noticed with both the bead sizes. That could be due to the fact that the critical micelle concentration (CMC) might have been exceeded in this case. This could have a significant deleterious effect on physical stability of the suspension, since the presence of charged micelles could facilitate particle aggregation in the suspension (Richetti and Kékicheff, 1992). Furthermore, when SLS concentration is higher than CMC, the drug starts entering the micelles, but at the same time, it also starts precipitating from them. That results in the formation of solid drug particles without any stabilizers, where the Ostwald ripening can occur (Azad et al., 2016; Ghosh et al., 2011). The CMC value for SLS is 0.23% (Azad et al., 2016; Knieke et al., 2013), which is lower than the concentration in this study, but it also has to be acknowledged that the presence of polymers in the solution can affect the CMC.

Kumar et al. (2013) found out that an increase in the percentage of

various PEG polymers led to an increase in CMC in cationic polymers (Kumar et al., 2013). Although the applied polymer as well as the surfactant were not the same in this experiment, CMC would have to be determined by an appropriate method to claim with certainty that CMC has really been exceeded. Furthermore, SLS in the nanosuspensions is not free in the solution, but is rather attached to the solid drug surfaces, which also indicates that the amount of SLS to reach CMC should be higher. Since the observed increase in particle size over time was not very drastic, exceeding CMC seems unlikely.

4. Conclusion

The present study aimed at investigating the impact of using different milling beads sizes in the wet-ball milling process on drug particle size when different stabilizer concentrations were used. It has been concluded that the effect of bead size on particle size is complex and highly dependent on the properties of the whole system, particularly the concentration of both the used stabilizers, i.e. HPMC as well as SLS. Although it has been observed that smaller bead size generally leads to smaller particle sizes, as expected from the literature, the complexity of the whole system needs to be considered. Especially the concentration of HPMC seemed to be of particular importance here, since it greatly affected the suspension viscosity. Firstly, it has been seen that higher HPMC concentration and, thereby, more viscous suspension resulted in a smaller difference between the 0.5 mm and 1 mm milling beads under a given SLS concentration. Secondly, lowering HPMC concentration has been shown to have a significant effect not only on particle size, but also on the consistency of the suspensions, most probably due to the associated temperature changes during the process. Also in this case, the behaviour of suspensions differed between the two bead sizes. The suspensions tested on three-week stability at different storage conditions generally showed satisfactory physical stability, regardless of the storage temperature.

CRediT authorship contribution statement

Mayank Singhal: Conceptualization, Validation, Investigation, Writing - original draft, Visualization. **Ana Baumgartner:** Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Elina Turunen:** Conceptualization, Writing - review & editing. **Bert van Veen:** Conceptualization, Writing - review & editing. **Jouni Hirvonen:** Writing - review & editing, Supervision. **Leena Peltonen:** Conceptualization, Project administration, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2020.119636.

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