Nanocrystals for Drug Delivery Applications

Annika Tuomela

ACADEMIC DISSERTATION

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Abstract


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Low oral bioavailability and general delivery problems related to poorly water soluble drugs are major challenges in pharmaceutical formulation development. Nanocrystal technologies have been introduced as advantageous, universal formulation approaches for these molecules. Nanocrystals, with greater surface to volume ratio, can effectively increase both the dissolution rate and saturation solubility of active ingredients.

The aim of this thesis was to obtain detailed knowledge about the dissolution characteristics of drug nanocrystals, and to develop feasible nanocrystal applications for ocular and oral drug delivery purposes. Hence, developability issues from the analytical difficulties of the drug dissolution behavior to the scale-ability of the nanocrystals were covered. A new approach in dissolution testing of nanocrystals, UV imaging, was introduced and applied for the first time in order to study drug dissolution from solid nanocrystal compacts. The focus was detailed understanding of the real-time dissolution behavior of different nanosized particle fractions, and the possibly generated supersaturated states. Moreover, both liquid and solid nanocrystal dosage forms were formulated and studied in vitro and in vivo.

High-quality nanocrystal suspensions were successfully prepared using the rapid and industrially relevant top-down wet milling technique. Nanocrystal dissolution analyses were performed in real-time during the dissolution process, which enabled a close level insight into the occurring phenomena. The relevance of the nanosized particle fraction was established in these experiments. With regards to the formulation development, an in vivo effective nanocrystal suspension formulation was developed for ocular delivery, to treat elevated intraocular pressure. Furthermore, nanocrystal suspensions were converted into dry powders by both freeze-drying and granulating techniques. The dry powders were further processed
into tablet and capsule formulations and tested *in vitro* and *in vivo*. As a result, the great difficulties in predicting the *in vivo* behavior based on the *in vitro* results, was addressed. Finally, the feasibility parameters for nanocrystal compositions of solid formulations at higher scale were optimized by screening powder and tablet properties. Hence, optimal compositions for nanocrystalline tablet compositions were indicated with regards to the higher scale process-ability. It was concluded that the methods applied and studied provided valuable knowledge and important tools for the pharmaceutical formulation development in order to solve the current problems related to the delivery of poorly soluble drugs.
Acknowledgements

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This thesis is based on the following publications of Annika Tuomela (né Sarnes). The publications are referred to in the text by their respective roman numerals (I-IV).


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**Abbreviations and symbols**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>API</td>
<td>Active pharmaceutical ingredient</td>
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<tr>
<td>AUC</td>
<td>Area under the plasma concentration curve</td>
</tr>
<tr>
<td>BAC</td>
<td>Benzalkonium chloride</td>
</tr>
<tr>
<td>BRA</td>
<td>Brinzolamide</td>
</tr>
<tr>
<td>C$_{\text{max}}$</td>
<td>Maximum plasma peak concentration</td>
</tr>
<tr>
<td>CSD</td>
<td>Colloidal silicon dioxide</td>
</tr>
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<td>DC</td>
<td>Direct compression</td>
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<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>F68</td>
<td>Poloxamer 188</td>
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<tr>
<td>F127</td>
<td>Poloxamer 407</td>
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<tr>
<td>G</td>
<td>Granulation</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HPMC</td>
<td>Hydroxypropyl methylcellulose</td>
</tr>
<tr>
<td>HPH</td>
<td>High pressure homogenization</td>
</tr>
<tr>
<td>IND</td>
<td>Indomethacin</td>
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<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
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<tr>
<td>ITC</td>
<td>Itraconazole</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>MCC</td>
<td>Microcrystalline cellulose</td>
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<tr>
<td>NC</td>
<td>Nanocrystal</td>
</tr>
<tr>
<td>NPS</td>
<td>Nanocrystal suspension</td>
</tr>
<tr>
<td>NT</td>
<td>Non-treated group</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>PI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone, Povidine</td>
</tr>
<tr>
<td>PVPP</td>
<td>Cross-linked-PVP</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMCC</td>
<td>Silicified microcrystalline cellulose</td>
</tr>
<tr>
<td>WBM</td>
<td>Wet ball milling</td>
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<tr>
<td>XRPD</td>
<td>X-ray powder diffraction</td>
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1 Introduction

Pharmaceutical drug formulation development encounters tremendous problems related to poorly soluble compounds. Most of the drugs in the development pipelines and drugs coming from synthesis or high throughput screening, *i.e.* new chemical entities, are poorly soluble in aqueous media, many as well simultaneously in organic solvents (Heimbach et al., 2007). Poorly soluble drugs, possessing very limited solubility and dissolution velocity, consequently display many biopharmaceutical issues in oral drug delivery: low bioavailability, high fasted/fed state variation, retarded onset of action, lack of dose proportionality, high interpatient variation and local irritation (Gao et al., 2013).

Novel, advantageous possibilities to improve the bioperformance of poorly soluble drug compounds, are offered by the rapidly emerging field of nanoscience, and more precisely nanocrystal technology (Jinno et al., 2006; Ige et al., 2013). The diminution of the particle size to nanometer range contributes to an increased particle surface area and curvature, and thus to enhanced saturation solubility, dissolution velocity, and increased adhesiveness to surface/cell membranes and, further, acceptable bioavailability after oral administration (Müller et al., 2011a). Hence, nanocrystals possess outstanding features enabling to overcome the solubility problems of poorly soluble drugs. Nanocrystal formulations provide an enhanced bioavailability, high drug load, low incidence of side effects due to the excipients, fast onset of action, versatile administration routes (*i.e.* oral, parenteral, ocular, pulmonary and dermal), no fasted/fed state variation, and an overall improvement of efficiency and safety.

Even though the nanocrystals were launched in the 1990s, there exist still a limited number of commercially available nanocrystal products, despite the promising potential of the technology (Junyaprasert and Morakul, 2015). At present the drug nanocrystals are paid a lot of attention as a promising approach due to an increasing number of poorly soluble drugs in the drug development process, pharmacoeconomic value, straightforward production and safe composition. The pharma companies can benefit from the nanocrystal approach also due to the possibility of product line extension offered by the regulatory authorities (FDA) for the already existing drug formulations (Singare et al., 2010; Raghava Srivalli and Mishra, 2014).
Various technologies are developed for the production of drug nanocrystals, which are roughly divided in top-down, bottom-up and combinatorial techniques. The focus of this thesis is in one of the most beneficial and profitable top-down technique, rapid wet media milling, producing nanocrystal suspensions. The liquid nanocrystal suspensions can be employed as a liquid dosage form or transformed into solid dry powders for further production of tablets or capsules.

The flexibility of both up-scaling and down-scaling offered by nanocrystals, is a great advantage during manufacture process (Van Eerdenbrugh et al., 2009b). In this emerging reign of nanocrystal technology, increasing resources are applied in the development of effective solid nanocrystal formulations. There exists an urge to enable a universal shift of the production of the solid nanocrystal formulations from laboratory scale to industrially feasible scale. The scale-ability, or up-scaling, is essential for the future of the pharmaceutical development by nanocrystals. The success of any formulation development depends on its transfer-ability to large scale manufacture (Raghava Srivalli and Mishra, 2014). In order for the up-scaling to be successful, a detailed characterization of process parameters, design and choice of equipment, development of a robust formulation including effective excipients and satisfactory stability surveillance results, are all required (Raghava Srivalli and Mishra, 2014).

Nanotechnology is expected to facilitate revolutionary innovations in the fields of biomedical sciences, such as drug therapy, diagnostics and imaging. In drug delivery and clinical applications nanotechnology is one of the key factors for modern drug therapy, now and in the years to come. In this thesis, approaches to detailed investigations of dissolution properties and formulation development of drug nanocrystals, are taken. It is essential to establish the significance of nanocrystal particle size when dealing with particles below one micron. Since the smaller the particle size, the more there will be stability issues to be dealt with. Which is why in this thesis, a novel dissolution method, UV imaging, was applied for the first time to investigate these rapidly dissolving samples. Later on, the advantageous characteristics of drug nanocrystals were utilized as both suspension and solid dosage forms, and thus provided important information about the industrially and therapeutically feasible nanocrystal formulations.
2 Review of the literature

2.1 Pharmaceutical Nanoparticles

Poorly-water soluble drug compounds comprise in general a significant problem in pharmaceutical formulation development. Currently about 40% of the drugs in the industries development pipelines, and even up to 60% of the new chemical entities are poorly soluble (Gao et al., 2013). Dissolution of the drug from the formulation is a requirement for drug absorption (Dressman and Reppas, 2000; Wang and Thanou, 2010; Gao et al., 2013). Especially in oral drug delivery, which is the major drug administration route due to its numerous benefits, dissolution plays an important role. This explains the significance of the issues related to poorly soluble drugs, which possess limited solubility and dissolution rate, and consequently exhibit several biopharmaceutical problems, e.g. low bioavailability, high fed/ fasted variation, retarded onset of action, lack of dose proportionality, high interpatient variation and local irritation (Gao et al., 2013). Thus, a significant amount of attention has been focused on formulation development strategies for the poorly soluble drugs, concerning molecules in biopharmaceutics classification system (BCS) class II (poorly soluble, permeable) and class IV (poorly soluble, impermeable) (Benet et al., 2008; Merisko-Liversidge and Liversidge, 2011). These poorly soluble drugs have been sub-classified into two types of molecules: grease ball and brick dust compounds (Bergstrom et al., 2007). Grease ball molecules represent highly lipophilic compounds (log P > 4, melting points < 200 °C), which cannot form bonds with water molecules. Thus, their solubility is limited by the solvation process. Whereas, brick dust molecules show lower log P ( < 2) values and higher melting points (> 200 °C). Their water solubility is restricted due to the strong intermolecular bonds within the crystal structure.

Several techniques have been applied to overcome the solubility problems of poorly soluble drugs, such as co-solvents, salt/pro-drug formation, amorphous formulations, solubilization, complexation and approaches that employ inclusion of solid dispersions (Serajuddin, 1999; Leuner, 2000), cyclodextrins (Davis and Brewster, 2004), melt extrusion (Breitenbach, 2002), emulsions (Davis et al., 1987), microemulsions (Kawakami et al., 2002), liposomes (Fenske et al., 2008), micellar systems (Torchilin, 2012) and soft gelatin capsules including liquid formulations.
(Gullapalli, 2010). However, as numerous times evidenced, these approaches offer no universal solution. Novel possibilities are offered also by the rapidly emerging field of nanoscience. Nanotechnology is a broad interdisciplinary area of research, development and industrial activity that has been growing rapidly worldwide during the past decades (Aitken et al., 2006). The use of reduced particle size approach to form stable nanometer size drug formulations has been shown to be an advantageous formulation strategy. Nanotechnology can be considered to comprise the areas of nanomedicine, nanofabrication, nanometrology and nanomaterials, including nanoparticles (Aitken et al., 2006). The concept of nanoparticles covers also nanodevice systems, e.g. nanopores, fullerenes, dendrimers, nanotubes, nanowires, generally exhibiting particle sizes below 100 nanometers (Aitken et al., 2006; Longmire et al., 2008; Parveen et al., 2012).

Formulating poorly-water soluble actives as nanometer-sized drug particles, generally referred to as pharmaceutical nanoparticles, have shown great promise in terms of biomedical applications, ranging from novel drug delivery platforms and drug targeting to diagnostics and tissue engineering. The nanoparticulate systems are roughly categorized into matrix nanoparticles, consisting e.g. of a polymeric matrix (polymeric nanoparticles (Couvreura et al., 1977) or a lipidic matrix (nanoemulsions (Collins-Gold et al., 1990), liposomes (Crommelin and Storm, 2003) and lipid nanoparticles (Müller et al., 2011b)), and drug nanocrystals (NCs) (Figure 1). The matrix particles have the drug distributed throughout, possibly inside, the matrix and/or adsorbed onto their surface (drug loading << 100%), whereas the drug nanocrystals consist of 100% drug, typically stabilized by surfactants or polymeric steric stabilizers [1,2]. High drug loading of NCs makes them very efficient in transporting the drug to or in the cells, reaching a sufficiently high therapeutic concentration for the pharmacological effect. Often drug NCs are referred to as drug nanoparticles, which might lead to confusion with polymeric nanoparticles. The NCs are typically produced in a liquid dispersion medium, which contains stabilizers for the colloidal state; therefore these systems can also be referred to as nanocrystal suspensions (NPS). In the field of pharmacy the definition of NCs covers particles below one micron (Merisko-Liversidge et al., 2003; Gao et al., 2013), however the NCs in the size range between 100 nm and <1000 nm may sometimes be more suitably classified as submicron particles (Müller et al., 2011a). In order to give some idea about the size scale, bulk drug materials possess typically particle sizes of tens to hundreds of microns, i.e. indomethacin ~ 80 µm, whereas micronized drug
particles are around tens of microns. In this thesis, the particles below one micron are referred to as NCs.

![Figure 1](image.png)

**Figure 1** Pharmaceutical nanoparticles and their structural arrangement with emphasis on drug distribution: matrix particles, including polymeric nanoparticles (A), nanoemulsions (B), solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) (both derived from C), versus drug nanocrystals (NC) (D) (modified after Müller et al., 2011a; Pawar et al., 2014).

### 2.2 Nanocrystals (NCs) of poorly water soluble drugs

Novel advantageous possibilities for pharmaceutical research are offered by the rapidly emerging field of drug nanocrystal technology, the major contributions of which are the benefits for formulating poorly soluble drugs (Rabinow, 2004). The
special characteristics of drug NCs are discussed here in detail, followed by the nanocrystallization methods and the analytical specificities of NCs.

2.2.1 Special features of NCs

The dissolution rate of a drug is of special importance when it is the rate-limiting step for the uptake of drugs after oral administration. This is the case for many hydrophobic, especially BCS class II, drugs with extremely low aqueous solubility. Basically, the solubility is considered low if the highest dose solubility volume is more than 250 ml. Drug with poor solubility and dissolution rate will provide a slow and erratic dissolution that limits the in vivo absorption and achievement of an effective therapeutic concentration. Therefore, investigations of different ways to enhance the dissolution of these drugs are of special interest. Compared to the micron-sized drug powders the special features of NCs display a series of benefits on the bioperformance of the poorly soluble drugs, including improved absorption, enhanced bioavailability, rapid onset of action, reduced fed/fasted state variability, reduced intersubject variability and ability for dose escalation and hence improved efficacy and safety (Shegokar and Müller, 2010; Merisko-Liversidge and Liversidge, 2011). The mechanisms contributing for the enhanced oral bioavailability of drug NCs are based on enhanced bioadhesion to the surfaces/cell membranes, and most importantly on increased saturation solubility and dissolution velocity, which lead to high drug concentration gradient between gastrointestinal tract and blood vessel, and thus contribute to improved absorption and high bioavailability (Müller et al., 2011a). Furthermore, besides for the improvement of the dissolution features of poorly soluble molecules, due to their simple structure and versatile features the NCs can also be utilized in controlled and targeted drug delivery for example by surface modification.

2.2.1.1 Enhanced oral bioavailability

The essence of nanosizing poorly water soluble compounds rests primarily in the Noyes-Whitney and Prandtl equations, which describe how the reduction of the particle size facilitates an increase of the surface area and a decrease of the diffusion layer thickness, and thus provide an enhanced dissolution rate (Noyes and Whitney,
Whereas the enhancement in saturation solubility with nanosized particles is described by the Ostwald–Freundlich and Kelvin equations, which correlate particle size and particle curvature with solubility (von Helmholtz, 1886; Noyes and Whitney, 1897; Wu and Nancollas, 1998; Godec et al., 2009; Kesisoglou and Mitra, 2012). Thus, with particles below one micron, the saturation solubility depends not only on the drug compound, the dissolution medium and temperature but also on the particle size (Junghanns and Müller, 2008). The special features of the NCs are summarized in Figure 2.

According to the Noyes–Whitney equation (Figure 2) the dissolution velocity \((dc/dt)\) is determined by the rate of change of mass dissolved with time \((t)\), related to the diffusion coefficient \((D)\) through a static layer of liquid of thickness \(h\), and \(Cs\), the equilibrium solubility (the concentration of the solute required to saturate the solvent) and the amount dissolved in the bulk medium at time \(t\) \((C_t)\) (Buckton and Beezer, 1992). The concentration around the particles at time \(t\) \((C_t)\), can be influenced by a changing particle size. According to the equation an increase in the surface area \((A)\) of a drug will result in a more rapid dissolution process, particularly under sink conditions \((C<<<Cs)\). According to Prandtl boundary layer equation \((k\) denotes a constant):

\[
h = k\left(L^{1/2}/V^{1/2}\right)
\]  

(1)

in dispersed solid-liquid systems, particle size reduction results in an increase of the dissolution rate of a sparingly soluble material by decreasing the thickness of the diffusion layer \((h)\) around each particle (Prandtl, 1904; Mosharraf and Nyström, 1995). When the \(h\) decreases, both the \(Cs\) and the concentration gradient \(((Cs-C_t)/h)\) will increase and hence the dissolution rate will increase according to Noyes-Whitney. Basically, according to Prandtl, a decrease in particle size leads to a decrease in both the length of the surface in the direction of flow \((L)\) and in the relative velocity of the flowing liquid surrounding the particles parameters \((V)\), where the net effect is a thinner diffusion layer \((h)\) around the particles and an increase in the surface specific dissolution rate (Bisrat and Nyström, 1988).
Figure 2 Special features of nanocrystals: (1) increased saturation solubility ($C_s$) due to the increased dissolution pressure of strongly curved small NCs, (2) increased dissolution velocity ($dc/dt$) due to increased surface area ($A$) and decreased diffusional distance ($h$) and (3) increased adhesiveness of NCs due to the increased contact area (modified after Mauludin et al., 2009; Müller et al., 2011a).

With submicron particles, saturation solubility is a function of particle size: the saturation solubility increases with the decreasing particle size. This is based on an increase in the curvature of the particle surface, and thus enhanced dissolution pressure (Figure 2). The phenomenon can be explained by the Kelvin (Eq. 2) and the
Ostwald–Freundlich (Eq. 3) equations (von Helmholtz, 1886; Wu and Nancollas, 1998; Mauludin et al., 2009; Müller et al., 2011a). Initially the Kelvin equation defines the vapor pressure over a curved surface of a liquid droplet in gas phase (Thomson, 1871; Jungmanns and Müller, 2008; Junyaprasert and Morakul, 2015); A decline in the particle size of liquid droplet provides an increase in curvature of the surface and the increasing vapor pressure. The transfer of molecules from a liquid phase (droplet) to a gas phase is in principle identical to the transfer of molecules from a solid phase (nanocrystal) to a liquid phase (dispersion medium). Thus, the vapor pressure is considered equivalent to the dissolution pressure. Therefore, the Kelvin equation is also applicable to explain the relationship between the dissolution pressure and the curvature of the solid particles in liquid (von Helmholtz, 1886; Wu and Nancollas, 1998; Junyaprasert and Morakul, 2015):

\[
\ln \frac{P}{P_0} = \frac{2\gamma V_m}{rRT} \quad (2)
\]

where \(P_r\) is the dissolution (vapor) pressure of a particle with the radius \(r\), \(P_0\) is the saturated dissolution pressure of the surrounding medium or flat surface, \(\gamma\) is the surface tension, \(V_m\) is the molecular volume of the solute particle, \(R\) is the gas constant, \(T\) is the absolute temperature and \(r\) is the radius of the particle. In a saturated solution, there exists an equilibrium between dissolving and recrystallizing molecules. The equilibrium is transferred when the dissolution pressure increases (decreasing particle size), and thus the saturation solubility increases. Additionally, the Ostwald–Freundlich equation, which is an equivalent to the Kelvin equation, precisely expresses the relationship between the saturation solubility and the particle size (von Helmholtz, 1886; Wu and Nancollas, 1998; Junyaprasert and Morakul, 2015):

\[
\ln \frac{S}{S_0} = \frac{2\gamma V_m}{rRT} \quad (3)
\]

where \(S\) is the saturation solubility, \(S_0\) is the solubility of the solid consisting of large particles, \(\gamma\) is the interfacial tension of substance, \(V_m\) is the molar volume of the particle material, \(R\) is the gas constant, \(T\) is the absolute temperature and \(r\) is the radius. From the Ostwald–Freundlich equation, it is clearly shown that the saturation
solubility (S) of a drug increases with a decrease in the particle size (r). However, this effect is pronounced solely for submicron particles.

Drug NCs possess also an outstanding feature of distinctly increasing adhesiveness to biological mucosa including gastrointestinal (GI) mucosa (Figure 2) (Ponchel et al., 1997; Junyaprasert and Morakul, 2015). The increased adhesiveness is usually due to an increased contact area of NCs, which lead to an improvement of oral absorption of poorly soluble drugs apart from the increased saturation solubility and dissolution rate (Möschwitzer and Müller, 2007; Gao et al., 2012). For NCs, the mucous gel layer is a porous structure, into which they can quickly and deeply penetrate and reach a close contact with the mucous network. Also, the adsorption isotherm shows linear increase with particle concentration (Ponchel et al., 1997). Different theories of mucoadhesion mechanisms of nanoparticles have been proposed (Gao et al., 2013): the electronic theory (electrostatic attraction forces between the surfaces of particles and mucus), the adsorption theory (secondary forces such as hydrogen and van der Waals bonds between the surfaces of particles and mucus), the diffusion theory (interpenetration and physical entanglement of the protein of the mucus and polymer chains) and the trapping theory (retention of nanoparticles by the uneven mucosa surface). The GI mucoadhesion of drugs enables the drug release exactly at the absorption sites, which leads to a high concentration gradient and prolonged retention time (Li et al., 2009). In order to strengthen the mucoadhesion, further processing, e.g., incorporation of mucoadhesive polymer or surface modification with cationic polymers, which facilitate stronger adhesiveness to the negative mucin of the mucosal surface, can be performed (Kayser, 2001).

Another factor affecting the dissolution behavior of drugs is the crystalline state. Depending on the preparation process of drug NCs, it may induce the transformation of crystalline structure, increase an amorphous fraction or even create completely amorphous particles. Compared to equally sized drug NCs in the crystalline form, drugs in the amorphous state possess higher solubility and faster dissolution rate due to the higher inner energy (Sarkari et al., 2002; Gao et al., 2011). However, the utilization of the amorphous state in pharmaceutical products has to be carefully considered that it can maintain the amorphous state for the shelf life of the product (Junghanns and Müller, 2008; Van Eerdenbrugh et al., 2009a). Thus, although conversion to the amorphous state can markedly improve solubility and dissolution characteristics, the amorphous state may revert to a lower energy state, typically crystalline form during storage. Unfortunately the behavior of such conversions is
not easy to predict, and thus NC at crystalline state are mainly preferred (Vogt et al., 2008).

Despite these advantageous features of NCs, the greatest challenge arises however along the small particle size. The nanosize induces physical stability problems presented more in detail in the following chapter.

2.2.1.2 Stability

Since the preparation of NC suspensions generates an increase in the particle surface area and hence, the surface interactions, they have an extremely high tendency to aggregate. Due to the increased Gibbs free energy in the system, associated with the formation of additional interfaces, the NC suspensions as colloidal systems exhibit thermodynamically unstable states and have a tendency to minimize the total free energy by agglomeration/aggregation (Van Eerdenbrugh et al., 2008). Thus, the long-term stability of NC formulations is strongly affected by the particle aggregation, which mostly depends on the stabilizer, its type and concentration used (Sinha et al., 2013). Still, the long-term stability is considered a special feature of drug nanocrystals (Junyaprasert and Morakul, 2015). The NC suspension can show good physical stability by an absence of aggregation and Ostwald ripening phenomenon.

Kinetically, the aggregation process depends on its activation energy, which can be increased by including stabilizers to the system (Van Eerdenbrugh et al., 2008). The appropriate stabilizer provides thus a barrier to aggregation. Theoretically, the principle of an energetic barrier can be explained by the DLVO theory, according to Russians Deryagin and Landau and the Dutch scientists Verwey and Overbeek. The theory basically describes the interaction of solid particles in a liquid medium in terms of attractive and repulsive interactions between the electric double layers surrounding the particles in solution (Derjaguin and Landau, 1993; Van Eerdenbrugh et al., 2008). The balance between the attractive and repulsive forces is adjusted by the stabilizers in the nanocrystalline systems. Additionally, the kinetic stability of a suspension may also be controlled by some surface forces, such as hydrophobic forces.

The Ostwald ripening phenomena (Jacobs et al., 2000; Müller et al., 2001) has been described for highly dispersed systems, and means a reduction in size of the finest particles fraction and the final disappearance combined with simultaneously
growth of larger particles. Reasons for the phenomena are the different saturation solubilities in the vicinity of differently sized particles and the concentration gradient existing between them. The molecules surrounding the small particles will diffuse to surround the large particles driven by the concentration gradient. Due to the supersaturation, recrystallization occurs on the surface of the larger particles leading to the formation of microparticles. Simultaneously the concentration in the vicinity of the smaller particles will decrease below the saturation concentration, thus leading to further drug dissolution, and the fine particles getting even smaller. This continuous process leads finally to the disappearance of the fine particles. Due to the uniform particle size of NCs, created by the homogenization process, the difference in saturation solubility due to different particle sizes and thus, an absence of Ostwald ripening phenomena, can be avoided. Additionally, the stabilizer layer surrounding the particles also prevents Ostwald ripening.

The stabilization is based on the stabilizer absorption on the particle surfaces in order to decrease the free energy of system and the interfacial tension of particles. The principle mechanisms consist of electrostatic and steric stabilization. The selection of the suitable type of stabilizer and its optimal concentration are very important for stabilizing the smaller sized particles and to maintain the shelf life stability of the final product (Van Eerdenbrugh et al., 2009a). The stabilizer used may be: (1) semisynthetic non-ionic polymers (HPMC, MC, HEC, HPC) (Choi et al., 2005;Zuo et al., 2013;Tuomela et al., 2014), (2) semisynthetic ionic polymers (NaCMC, NaAlg) (Sinha et al., 2013), (3) synthetic linear polymer (PVPs, PVAs) (Ghosh et al., 2013), (4) synthetic copolymers (poloxamers, polyvinyl alcohol–polyethylene glycol graft copolymer) (Liu et al., 2011;Kumar et al., 2014), (5) surfactant of ionic type (SDS, sodium docusate, sodium deoxy cholate), or (6) non-ionic type surfactant (polysorbate esters, sorbitan esters) (Sinha et al., 2013). Additionally, for instance an application of a combination of ionic surfactants with polymeric stabilizers may provide an enhanced stabilization which combines the advantages of both the electrostatic and steric stabilization. So far, the selection of suitable stabilizer for nanocrystallization has been mostly based on trial and error. However, recently surface plasmon resonance (SPR) and contact angle techniques were successfully applied as tools for monitoring stabilizer-drug interactions for nanocrystal research (Liu et al., 2015). Three fundamental requirements for efficient stabilizer were concluded: firm attachment to the solid surface, coverage of the nanocrystal and the hydrophilic/lipophilic balance of the stabilizer structure. Besides the physical stability, drug NC can be used for a chemical stabilization of
chemically labile drug (Junyaprasert and Morakul, 2015). The increased stability of drug NCs can be explained by a shield effect of surfactants and a monolayer of degraded drug molecules, which act as the surface of drug NCs for protecting the drug underneath its surface from degradation.

Despite the generally straightforward and yet safe compositions of drug NCs, similarly with any drug formulations, the possible toxic effects have to be still carefully considered. This aspect is covered in the following section.

2.2.1.3 Toxicity of NCs

Nanomaterials, including NCs, often behave differently from their bulk counterparts. The possible magnetic, optical and complicated structural properties make a direct transition of the understanding about bulk toxicity impossible to the corresponding nanomaterial. Thus, the determination of the safety of nanomaterials is a difficult and ever ongoing process. Besides the size factor, there exist a number of parameters contributing to the possible toxic effect that should be considered; surface area, surface charge, chemical composition and reactivity, solubility, shape and agglomeration potential (Oberdorster et al., 2005a). Nanomaterials enter the body via various traditional routes (orally, through the respiratory system, through blood circulation, through the skin), through which they can interact with the body greatly differently than their corresponding micronsized versions (Oberdorster et al., 2005b; Leslie-Pelecky, 2007). The nanoscale materials can penetrate deeper into the tissues than larger particles. They have the potential to produce oxidative stress, inhibit cell proliferation, reduce phagocytosis and decrease cell viability (Leslie-Pelecky, 2007). Nanoparticles can also remain in the body longer, due to the ability to escape the reticuloendothelial system. These effects are the stronger the smaller the particles are in question.

Despite that, NCs with the size in the range of about 100-1000 nm can only be taken up by a limited number of cells with phagocytic activity, e.g. the macrophages of the mononuclear phagocytic system (MPS), which are not easy to access (Müller et al., 2011b). This is different for particles with a size below 100 nm. These particles can be taken up by all cells by endocytosis. Therefore, they can be considered as high risk nanoparticles. A nanotoxicological classification system (NCS) is applied to assess the toxicity risk of nanoparticles (Müller et al., 2011a). The size and biopersistence related risks are combined to classify the nanoparticles
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according to the toxicity risk as follows: class I (nanoparticles >100 nm and biodegradable), class II (nanoparticles >100 nm and non-biodegradable), class III (nanoparticles <100 nm and biodegradable), and class IV (nanoparticles <100 nm and non-biodegradable). The NCs are a priori low risk or non-risk nanoparticles, due to their particle size (generally > 100 nm) and biodegradable nature (dissolution occurs in sufficient water amount). Consequently, the toxic risk of NCs is limited. However, NCs may cause undesired systemic effects in the body or intracellularly until the complete dissolution, which is generally not an issue because of the rapid dissolution behavior of NCs. In case NCs significantly improve the bioavailability, the therapeutic dose has to be reconsidered in order to avoid toxicity caused by overdosing. Thus, the development of NCs requires the detailed investigation in order to facilitate the potential effects with low toxicity, even with biodegradable nanoparticles.

Generally drug nanocrystals are reported safe and well tolerated in many administration routes compared with the conventional products (Junyaprasert and Morakul, 2015). The toxicity of the material is mostly the issue, not the toxicity of the NCs.

2.2.2 Methods for nanocrystallization

Drug nanocrystals are commonly prepared in a liquid dispersion medium producing nanocrystal suspensions (NPS) (Müller et al., 2011b). The nanocrystallization techniques are mainly divided into bottom-up and top-down approaches (Figure 3). Additionally, there exist combinations of the previous.

The top-down nanocrystallization approaches are high energy processes, comprising wet media milling and high-pressure homogenization (HPH), where micronsized drug crystals are diminished to nanodimension under mechanical attrition or high pressure, respectively. In the wet media milling method the drug particles are dispersed in a surfactant/stabilizer solution and the obtained microsuspension is then subjected to milling energy (Müller et al., 2011a). The particle size is reduced by the shear forces generated by the movement of the milling media. The crystals are ground between the moving pearls, moved by an agitator, resulting in a NPS. Generally, after wet media milling process crystalline structures have been reported (Müller et al., 2001; Liu et al., 2011). During wet milling of crystalline drugs the water is acting as an inhibitor of the formation of amorphous
material due to the reduced glass-transition temperature (Sharma et al., 2009). Different sized milling pearls of zirconium dioxide, stainless steel, glass or highly crosslinked polystyrene resin-coated beads may be used. The erosion from the milling material during the process may be a problem of this technology. The milling time varies from minutes to hours or days, according to the hardness of the drug, viscosity, temperature, energy input, size of the milling medium and surfactant concentration used (Shegokar and Müller, 2010; Müller et al., 2011a). Wet milling is considered as a standard method to produce NPS and as a platform technology for formulating poorly soluble compounds. Generally, the brick dust molecules, which are not only poorly soluble in water, but also in oils, have shown to benefit from the development of NPS and amorphous systems (Bergstrom et al., 2007; Pu et al., 2009). Thus, they are considered suitable substances for milling. Whereas, the oral bioavailability of grease ball molecules can be increased, for instance, by the inclusion into cyclodextrines, application of micelles and lipid-based formulations. Thus, milling is most likely not the effective method to be applied for the grease ball molecules.

The versatility of the technique and the achievable particle sizes are the most important aspects for the success of this technology. The reported particle sizes of the various APIs illustrate the universal applicability of this particle size reduction method.

High pressure homogenization (HPH) can be regarded as the second most important technique to produce drug NPS (Möschwitzer, 2013). For HPH there exist three basic processes: 1) the jet stream principle (Microfluidizer, IDD-P™ (insoluble drug delivery microparticle technology) (Keck and Müller, 2006), where high energy fluid streams of the suspension collide, 2) the piston-gap homogenization either in water (Dissocubes® technology,) (Müller et al., 2003), or 3) alternatively in water-reduced/non-aqueous media (Nanopure® technology), in which a drug/surfactant microsuspension is forced with a high velocity by a piston under pressure (Radtke and Müller, 2001; Shegokar and Müller, 2010). HPH techniques usually promote the formation of amorphous state, which may be prone for recrystallization (Müller et al., 2001). However, this is dependent on the process parameters and conditions used. The obtained solid state has been reported to be strongly affected by the presence of water (Sharma et al., 2009). The role of water in inhibiting the amorphization during HPH of crystalline drugs was significant.

The bottom-up approaches are generally based on the drug precipitation from a supersaturated solution of the drug. These precipitation approaches can be
categorized in four groups: precipitation by liquid solvent-antisolvent addition, precipitation in the presence of supercritical fluid, precipitation by the removal of solvent and precipitation in the presence of high energy processes (Sinha et al., 2013). The precipitation based methods have commonly the potential to produce amorphous material (Sinha et al., 2013). However, the amorphous form may be converted to crystalline state in the presence of water. Recrystallization is a common phenomenon with amorphous material.

![Nanocrystals](image)

**Figure 3** The basic principle of nanocrystallization techniques (modified after Rabinow, 2004).

Combination of the bottom-up and/or top-down approaches provides an effective method to produce smaller particle sizes and also to overcome the deficiencies of the techniques, *i.e.* clogging of the equipment and relatively long process times. Basically the combination methods consist of a pre-treatment step followed by a high energy top-down process. The first combinative method, Nanoedge™ technology, consists of a classical micro-precipitation pre-phase followed by HPH (Möschwitzer, 2013). Usually, the second phase inhibits further crystal growth and aggregation after precipitation, and converts the amorphous and semi-unstable crystalline form arising from precipitation process to the crystalline form. However, this is not necessarily always the case.
Later on, the SmartCrystal® technology presents a series of combination approaches. SmartCrystal® technology combines the pre-treatment and subsequent main treatment (HPH) (Shegokar and Müller, 2010). The methods comprise of the following pre-treatment techniques combined with the HPH: Nanopure (no pre-treatment), H42 (spray-drying), H69 (precipitation), H96 (lyophilization) and CT (media milling) (Shegokar and Müller, 2010). Even though the combination technologies improve the particle size reduction and the effectiveness of the process, the fact is that any pre-treatment step increases the complexity of the overall process and can significantly increase the costs. Therefore it is obvious that combinative particle size reduction methods will be only used in cases that the more established methods, like wet ball milling or standard high pressure homogenization, cannot be used to reach the desired results (Möschwitzer, 2013).

When considering the final formulations of drug NCs, solid dosage form is usually the preferred. Therefore, the NPS are converted into dry powders, which are further processed into tablets, capsules, or pellets.

### 2.2.3 Conversion into solid form

Different drying and granulation methods are mainly utilized to convert the aqueous NPS into dry powders. Firstly, the aqueous NPS can be powdered through some drying processes, such as the lyophilisation (i.e. freeze-drying), spray drying or oven drying (Badawi et al., 2011; Lai et al., 2011). The resultant powders, blended with other excipients, are further processed into solid dosage forms through shaping and handling, such as direct compression and capsule filling (Mauludin et al., 2009; Juhnke et al., 2012). Spray-drying process is the cost effective approach to transform the NPS into dry powders under appropriate conditions. Freeze-drying is recommended for e.g. intravenous products in order to avoid aggregation or caking of the settled drug NCs. Particle aggregation should be inhibited during the drying process since the benefits of NCs will be lost if aggregation occurs. Therefore, an addition of lyoprotectants (usually sugars) may reduce the growth of particle size during the solidification process. However, often the stabilizer alone provides adequate protection against the aggregation during freeze-drying.

Secondly, a granulation method may be used for solidification. The aqueous NPS is used as granulation fluid in the granulation process or as layering dispersion in a fluidized bed process (Kocbek et al., 2006; Basa et al., 2008). Independent of the
chosen solidification technology, aggregation of the drug nanoparticles is a phenomenon that has been reported to be able to profoundly impact the properties of products intended for a diversity of applications (Figure 4) (Wang et al., 2005b). If irreversible aggregation takes place, the gained profits of large surface of the original nanosized particles would be greatly jeopardized since the surface area advantage of NCs would be lost. Additionally, the redispersion of solid drug NCs in the GI fluids should be concerned (Junyaprasert and Morakul, 2015). The stabilizers attached to the NC surfaces that provide efficient ionic or steric repulsion and have no effect from the GIT environment should be used. However, generally the steric stabilization is the most common mechanism with the NCs stabilized with non-ionic stabilizers such as poloxamers.

![Diagram](image)

**Figure 4** The solidification of NC suspensions is aimed to facilitate reversible aggregation of dry powders, which thus rapidly reconstitute into the individual NCs when dispersed in an aqueous medium (modified after Gao et al., 2013).
2.2.4 Dissolution testing

The determination of a dissolution profile of any drug formulation/compound is a prerequisite for the investigation of the drug release and absorption behavior. Moreover, it is the most important primary evidence about the profitable nature of NCs of poorly soluble drugs. Dissolution studies are widely utilized in pharmaceutical formulation development processes, ranging from the characterization of API until the prediction of the in vivo performance of the compound/formulation (Tong et al., 2009). Especially the dissolution study is essential in showing the bioequivalency between formulations, (Amidon et al., 1995). An optimized, possibly differentiating, experimental set-up is desired in general for dissolution studies (Nikolic et al., 1992). The experimental conditions may have a drug release regulating effect. Thus, careful consideration should be made in the selection of e.g. dissolution medium, pH of the medium, volume of the medium and prevailing temperature.

When considering the existing traditional, oftentimes material and time consuming, pharmacopoeial dissolution methods (USP, European and Japanese Pharmacopoeia) that are mainly developed for quality control purposes, they are often regarded inadequate and not evolved enough to correspond to the needs of modern dissolution studies, i.e. to study in detail rapidly dissolving samples like NCs (Dokoumetzidis and Macheras, 2006; McAllister, 2010). The traditional methods, e.g. paddle (USP II) method combined with UV spectroscopy or HPLC, do not provide real-time, spatially and temporally resolved information of the dissolution process, since the monitoring of the process immediately next to the surface of the dosage form is impossible with those techniques (Windbergs et al., 2009; Greco and Bogner, 2012). Despite that, they are still valid methods to explore, compare and obtain information about dissolution behavior of different compounds/formulations, including nanocrystalline samples, both suspensions and solid formulations. In addition to the traditional methods, today there exist several methods to study the dissolution of solid dosage forms, which are based on imaging, e.g., UV, FT-IR, NIR, and MRI imaging, CARS and Raman spectroscopy, in order to cover those missing aspects of traditional methods (van der Weerd and Kazarian, 2005; Aaltonen et al., 2006; Kazarian and van der Weerd, 2008; Metz and Mader, 2008; Nott, 2010; Østergaard et al., 2010; Kowalczuk and Tritt-Goc, 2011).
2.3 Applications of NCs

There exist several subjects within the field of pharmaceutical drug delivery, which can greatly benefit from the NC approach. Hence, the wide suitability of NCs to different administration routes and biomedical applications are here presented, followed by the examples of the currently marketed NC products.

2.3.1 Delivery routes of NCs

2.3.1.1 Oral delivery

NCs are studied mainly through oral, ocular, pulmonary, parenteral, and dermal routes, all showing their high therapeutic applicability (Pawar et al., 2014). Due to the several advantages, the oral route is the most preferred and is considered as the safest and most suitable route for drug delivery. NCs offer solutions to the solubility related problems, such as low/variable bioavailability, a retarded onset of action, a variation in bioavailability resulting from fed/fast state and a large oral dose usage. NCs facilitate an increased dissolution rate, higher saturation solubility (Noyes-Whitney and Ostwald-Freundlich principles) and bioadhesion to the intestinal wall, thus impressively improving the bioavailability of orally administered poorly soluble drugs. This is shown as changes in pharmacokinetic parameters of blood profiles, including an increase in area under the blood concentration-time curve (AUC), an increase in maximum plasma concentration (Cmax) and a decrease in time to reach maximum plasma concentration (Tmax), describing quick onset of action (Liversidge and Conzertino, 1995; Xia et al., 2010; Li et al., 2011). For instance, a 16-fold increase in bioavailability of danazol, together with reduced Tmax and 15-fold increase in Cmax, were obtained by the conversion of the micronsized particles into NCs (Liversidge and Cundy, 1995).

Poorly soluble drugs often exhibit increased or accelerated absorption when they are administered with food, due to the enhancement of the dissolution rate in the GIT caused by factors such as delayed gastric emptying, increased bile secretion, larger volume of the gastric fluid, increased gastric pH (for acidic drugs) and increased splanchnic blood flow (Jinno et al., 2006). Also, the fat within the food
itself may act as a dissolving agent. Applying NPS, the variation in bioavailability resulting from fasted/fed state can be minimized (Gao et al., 2013; Junyaprasert and Morakul, 2015). The reason is that the dissolution rate of NCs is fast enough even under the fasted condition. Therefore, the absorption in both fasted and fed state can be a permeability-limited process, and the absorption difference between the fasted and fed conditions due to the dissolution difference is eliminated (Figure 5).

**Figure 5** The fasted/fed state variation of drug NCs is reduced due to their rapid dissolution (modified after Merisko-Liversidge et al., 2003; Gao et al., 2013). With NCs permeation might be the limiting factor for drug absorption.

Due to the fine particle sizes of the NCs, the distribution uniformity in the GI fluid is enhanced and high, and prolonged local concentrations are avoided (Liversidge and Conzentino, 1995). Thus, NCs are also better tolerated in the mucosal delivery by reduced gastric irritancy. Finally, the NCs offer an opportunity to escalate dose and reduce solvent-related adverse effects due to the safe compositions, since no organic solvents or extreme pH ranges for solubilization of poorly soluble drug are required (Merisko-Liversidge and Liversidge, 2011).

Moreover, fine particle size and safe composition are beneficial safety aspects for orally delivered NCs. Additionally, the tolerance of NCs to various sterilizations provide important benefits regarding other delivery routes where sterility is a prerequisite. Various sterilization approaches (Konan et al., 2002; Rabinow, 2004)
can be applied to NC suspensions, including gamma radiation (Junyaprasert and Morakul, 2015), filtration sterilization (Zheng and Bosch, 1997) and thermal sterilization (Na et al., 1999). Besides oral drug delivery, also other administration routes are often required. The special aspects of parenteral, ocular, pulmonary and dermal delivery are reviewed subsequently.

2.3.1.2 Parenteral delivery

Subsequently, intravenous (i.v.) administration of poorly soluble compounds, using *i.e.* cosolvents, surfactants, liposomes, or cyclodextrines, is often associated with large injection volumes or toxic side effects. Carrier-free NPS empower high loading capacity compared to other parenteral application systems. Applying NPS the administration volume can be clearly reduced compared to micro-solutions (Möschwitzer et al., 2004). Due to the small particle size and safe composition of NCs, NPS can be *i.v.* injected to give 100% bioavailability, immediate action and reduced dosing (Müller and Keck, 2004; Ganta et al., 2009; Pawar et al., 2014). NPS may also show passive targeting similar to colloidal drug carriers after *i.v.* administration (Peters et al., 2000). Furthermore, also special targeting can be achieved by a surface modification using the concept of differential protein adsorption. When considering the parenteral administration route, it must be kept in mind that the carrier system shall not be phagocytosed by reticuloendothelial system. Thus, the size of parenteral NCs should be ≤100 nm (Jinno et al., 2006). Additionally, in order to avoid the rapid removal of the NCs and/or nanocarriers from the circulation and to endow nanosystems with long circulation properties, the long circulating nanocarriers, “stealth” systems, have been introduced (Salmaso and Caliceti, 2013). Stealth particles can be obtained by surface coating with hydrophilic polymers, thus preventing the opsonisation process (Moghimi et al., 1993; Moghimi et al., 2001). Typically stealth approach is applied to liposome systems. However, NC surface can also be modified analogue to the stealth liposomes generating stealth NCs (Gao et al., 2008). The consequence of avoiding opsonisation is the prolongation and permanence in the bloodstream from few seconds to several hours.
2.3.1.3 Ocular, pulmonary and dermal delivery

Ophthalmic drug delivery is a challenging task due to the critical pharmacokinetic environment and physiological barriers of the eye hindering the delivery of drugs (Geroski and Edelhauser, 2000; Duvvuri et al., 2003; Koevary, 2003; Dey and Mitra, 2005; Thakur and Kashiv, 2011). Topical instillation is by far the most widely preferred, noninvasive route of drug administration to treat diseases affecting the anterior segment of the eye (Tangri and Khurana, 2011). Numerous anatomical and physiological constraints such as rapid precorneal drug elimination, tear turnover, nasolachrymal drainage, reflex blinking, systemic absorption from the conjunctival sac and ocular static and dynamic barriers pose a challenge and impede deeper ocular drug permeation (Figure 6a) [3]. Hence, less than 5% of topically applied dose reaches to intraocular tissues [4] (Urtti et al., 1990; Keister et al., 1991; Kaur and Kanwar, 2002).

To overcome the ocular drug delivery barriers and improve ocular bioavailability, various conventional (e.g. emulsions, ointments, suspensions, gels) and novel, especially nanotechnology based drug delivery systems, e.g. nanomicelles, nanoparticles, liposomes, dendrimers, implants, contact lenses, in-situ thermosensitive gelling systems and microneedles, have been developed for the earlier mention ocular diseases (Ali et al., 2011; Patel et al., 2013). Additionally, for instance, by increasing the viscosity and mucoadhesivity of the system, the residence time on the mucosa can be prolonged and sustained drug release obtained (Kaur and Kanwar, 2002). Furthermore, the pH of eye drops can be adjusted, approx. pH 3–10, to maximize the unionized fraction of the drug in order to optimize its ocular absorption (Alcon Laboratories, 2004; Gibson, 2009; Wu et al., 2013). Subsequently, nanocrystal technology plays an advanced role in ophthalmic drug delivery by solving dispersibility issues of poorly soluble drugs, such as budesonide, dexamethasone, hydrocortisone, prednisolone (Kassem et al., 2007) [17] and fluorometholone (Gupta et al., 2010). The development of such colloidal delivery systems for ophthalmic use aims at droppable dosage forms with a high drug loading, improved bioavailability and an extended, long-lasting drug action, when compared to micronsized bulk material (Ali et al., 2011). The significance of both the nanosized particles and the properties of topical nanocrystal suspensions, such as the absence of irritation due to the small size, proper viscosity and mucoadhesivity, have been well documented in terms of improved ocular bioavailability (Hui and Robinson, 1986; Kassem et al., 2007). In addition, the use polymeric nanoparticle
suspensions, loaded with the salt form of a drug, provided a gradual and prolonged release profile compared to an aqueous drug solution of the salt form (Pignatello et al., 2002). The nanocrystal suspensions both improved the ophthalmic drug absorption and increase the intensity and duration of the drug action (Figure 6c).

**Figure 6** Ocular drug delivery. (A) Precorneal factors that influence bioavailability of topically applied ophthalmic drugs, (B) simplified ocular pharmacokinetic model describing the movement of a topically applied drug to the eye, (C) intraocular pressure of rabbits eyes following administration of hydrocortisone (Hc) solution and Hc nanosuspensions (NS) produced by wet milling and precipitation methods (modified after Kaur and Kanwar, 2002; Ali et al., 2011; Patel et al., 2013).

Kinetically, corneal absorption is a much slower process than elimination (Kaur and Kanwar, 2002). Figure 6b presents a simplified ocular pharmacokinetic model describing the transfer of a topically applied drug. For most drugs $K_{loss}$ is approximately 0.5–0.7/min and $K_{abs}$ is about 0.001/min. These rate constants control the fraction of the applied dose absorbed into the eye, and thus the ocular
bioavailability (Lee and Robinson, 1986). The $K_{loss}$ can be decreased by modifying the ocular dosage forms and the $K_{abs}$ increased by formulating ocular dosage forms containing lipophilic prodrugs or by adding penetration enhancers.

Furthermore, the delivery of poorly soluble drugs, such as corticosteroids like budesonide or beclomethasone dipropionate, to the respiratory tract is very important for both the local and systemic treatment of lung related diseases. These drugs could however be inhaled as drug NPS. Lungs are highly perfused organs with an expanded surface area. Due to the lack of hepatic portal drainage, molecular dispersion of drug is rapidly transported into the systemic circulation with high efficiency (Pawar et al., 2014). The tendency of the NCs to attach to mucosal surface offer a beneficial prolonged residence time at the site of absorption, and thus increase the drug absorption (Jacobs and Müller, 2002). An undesired particle deposition in mouth and pharynx, and thus the local and systemic side effects can be avoided with NCs. The lungs deposition can be controlled via the size distribution of the generated NCs. Compared with microparticles, the drug is more evenly distributed within NPS. Recently, it has been demonstrated that pulmonary NCs have the ability to rival pharmacokinetics offered by intravenous administration of baicalin (Zhang et al., 2011). Pulmonary route thus comes across as a viable option for delivery of therapeutics.

Finally, the skin is a therapeutic barrier, limiting delivery of many drugs (Foldvari, 2000). Success in dermal delivery depends upon the permeation of drugs across stratum corneum (Saunders et al., 1999; Mathur et al., 2010). Due to their small size, NCs are expected to pack closely to form an occlusive layer which hydrates the skin increasing penetration and permeation of drugs. Also, the mode of dermal action of nanocrystals is explained via the increased saturation solubility, leading to an increased concentration gradient, which subsequently promotes penetration into the skin (Müller et al., 2011a). This effect may be further enhanced by the use of positively charged polymers as stabilizers for the drug NCs. The opposite charge may lead to an increased affinity of the NCs to the negatively charged stratum corneum. NCs have been largely utilized and applied by the cosmetic industry. Several cosmetic products exist in the markets, especially as facial creams.
2.3.2 Marketed NC products

Increasing resources are applied in the development of effective solid nanocrystal formulations. The scale-ability, or up-scaling is essential for the future of the pharmaceutical development of nanocrystals. The success of any formulation development depends on its transferability to large scale manufacture (Raghava Srivalli and Mishra, 2014). In order for the up-scaling to be successful, a detailed characterization of process parameters, design and choice of equipment, development of a robust formula including effective excipients and satisfactory stability surveillance results, are all required (Raghava Srivalli and Mishra, 2014). The NC approach may benefit the companies also due to the possibility of a product line extension offered by the FDA for the already existing drug formulations (Singare et al., 2010; Raghava Srivalli and Mishra, 2014). Finally, the essential prerequisite for entry to the pharmaceutical market is the availability of large scale production methods at sufficiently low cost and simultaneously meeting the regulatory requirements. The existing commercially available NC products and their characteristics are summarized Table 1.

Principally, the advantages of the NC technology can be applied to wide range of poorly soluble drugs. As presented, NC approach is not applied solely for improving dissolution properties, but also for facilitating sustained drug release, *i.e.* Invega® Sustenna® product. Figure 7 presents some of the particular benefits of the specific NC products, summarizing simultaneously the general advantages of drug NCs (Junghanns and Müller, 2008). Besides the already marketed products, there exist several products close to being marketed or in clinical trials. Information about these products is sparingly available due to the risks for knowledge leaks and fear of competitors in the pharmaceutical industry, but the following examples give an idea of potential future products. Products in the pipeline, at clinical phases II-III, are such as Semapimod®/Cytokine Pharmasciences (guanylhydrazone, TNF-α inhibitor), Paxeed®/Angiotech (paclitaxel, anti-inflammatory), Theralux®/Celmed (thymectacin, anti-cancer) and Nucrust®/Nucryst Pharmaceuticals (silver, anti-bacterial) (Junghanns and Müller, 2008).
Table 1 Examples of the available drug NC products and their key characteristics (WBM = wet ball milling, HPH = high-pressure homogenization) (modified after Junghanns and Müller, 2008; Gao et al., 2013; Möschwitzer, 2013).

<table>
<thead>
<tr>
<th>Product/Company</th>
<th>API</th>
<th>Indication</th>
<th>Method</th>
<th>Route</th>
<th>Formulation</th>
<th>Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gris-Peg®/Novartis</td>
<td>Griseofulvin</td>
<td>Anti-fungal</td>
<td>Precipitation</td>
<td>Oral</td>
<td>Tablet</td>
<td>1982</td>
</tr>
<tr>
<td>Cesamet®/Lilly</td>
<td>Nabilone</td>
<td>Anti-emetic</td>
<td>Precipitation</td>
<td>Oral</td>
<td>Capsule</td>
<td>2005</td>
</tr>
<tr>
<td>Verelan PM®/Schwarz Pharma</td>
<td>Verapamil HCl</td>
<td>Anti-arrhythmia</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>1998</td>
</tr>
<tr>
<td>Azopt®/Alcon</td>
<td>Brinzolamide</td>
<td>Glaucoma</td>
<td>WBM</td>
<td>Ocular</td>
<td>Suspension</td>
<td>1998</td>
</tr>
<tr>
<td>Rapamune®/Wyeth</td>
<td>Sirolimus</td>
<td>Immunosuppressant</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2000</td>
</tr>
<tr>
<td>FocalinXR®/Novartis</td>
<td>Dexamethyl-phenidate HCl</td>
<td>Anti-psychotic</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2001</td>
</tr>
<tr>
<td>Avinza®/King Pharm</td>
<td>Morphine sulfate</td>
<td>Anti-chronic pain</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2002</td>
</tr>
<tr>
<td>Ritalin LA®/Novartis</td>
<td>Methyl-phenidate HCl</td>
<td>Anti-psychotic</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2002</td>
</tr>
<tr>
<td>Herbesser®/Mitsubishi</td>
<td>Diltiazem</td>
<td>Anti-angina</td>
<td>WBM</td>
<td>Oral</td>
<td>Tablet</td>
<td>2002</td>
</tr>
<tr>
<td>Tanabe Pharma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zanaflex™/Acorda</td>
<td>Tizanidine HCl</td>
<td>Muscle relaxant</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2002</td>
</tr>
<tr>
<td>Emend™/Merck</td>
<td>Aprepitant</td>
<td>Anti-emetic</td>
<td>WBM</td>
<td>Oral</td>
<td>Capsule</td>
<td>2003</td>
</tr>
<tr>
<td>Tricor®/Abbott</td>
<td>Fenofibrate</td>
<td>Hyper-cholesterolemia</td>
<td>WBM</td>
<td>Oral</td>
<td>Tablet</td>
<td>2004</td>
</tr>
<tr>
<td>Megace® ES/Par Pharma</td>
<td>Megestrol acetate</td>
<td>Appetite stimulant</td>
<td>WBM</td>
<td>Oral</td>
<td>Suspension</td>
<td>2005</td>
</tr>
<tr>
<td>Naprelan®/Wyeth</td>
<td>Naproxen sodium</td>
<td>Anti-inflammation</td>
<td>WBM</td>
<td>Oral</td>
<td>Tablet</td>
<td>2006</td>
</tr>
<tr>
<td>Theodur®/Mitsubishi Tanabe Pharma</td>
<td>Theophylline</td>
<td>Bronchial dilation</td>
<td>WBM</td>
<td>Oral</td>
<td>Tablet, Capsule</td>
<td>2008</td>
</tr>
<tr>
<td>Triglide®/Skye Pharma</td>
<td>Fenofibrate</td>
<td>Hyper-cholesterolemia</td>
<td>WBM, HPH</td>
<td>Oral</td>
<td>Tablet</td>
<td>2005</td>
</tr>
<tr>
<td>Invega Sustenna®/Johnson &amp; Johnson</td>
<td>Paliperidone palmitate</td>
<td>Anti-depressant</td>
<td>WBM, HPH</td>
<td>Parenteral</td>
<td>i.m. injection</td>
<td>2009</td>
</tr>
</tbody>
</table>
Figure 7 Summary of the benefits of drug NCs specified to certain marketed NC products (modified from Junghanns and Müller, 2008).

Besides the products already mentioned, there are drugs such as naproxen, which are also being investigated for formulation as NC suspension e.g. for fast action onset and reduced gastric irritancy (Junghanns and Müller, 2008). However, when
incorporating drug NCs into a tablet in relatively high concentration (single dose 250 mg), it requires developed formulation technology to ensure the drug release. Related to this, the size of a tablet should be carefully evaluated with regards to patient compliance. In addition to these marketed NC products, the NC technology facilitates evolved solutions for novel biomedical applications, covered in the following section.

Finally, as earlier presented, the NCs show their versatile nature by being able to be utilized not solely in dissolution enhancement purpose, but also in controlled drug delivery. This element is discussed subsequently.

2.3.3 Controlled delivery

In order to provide a broader perspective about the NC approach within the field of pharmaceutics, the subject is here widened also to other biomedical applications benefiting from the technology. Nanotechnology, including nanocrystals, offers numerous opportunities for pharmaceutical applications. Besides drug therapy, nanotechnology facilitates solutions for diagnostic and imaging purposes. Imaging is an important factor in early detection of diseases.

The simple structural aspects of NCs allow them to be applied in controlled drug delivery systems facilitating \textit{i.e.} sustained drug release, which is a great advantage. For instance highly porous nanocellulose aerogels were introduced as NC reservoirs for oral drug delivery systems (Valo et al., 2013). Since the release of the drug was controlled by the structure and interactions between the NCs and the cellulose matrix, modulation of the matrix formers enabled a control of the drug release rate. As carriers for controlled drug delivery these nanocomposite systems can facilitate novel possibilities for NC applications. A well-known, marketed NC product, Invega® Sustenna®, showing established sustained drug release profile, is a good example about other than dissolution related, profitable nature of drug NCs (Section 2.3.2, Marketed NC Products).

Furthermore, applications like magnetic nanoparticles and nanowires can be utilized for instance in biosensing purposes (Reiss et al., 2005; Wang et al., 2005a) and intracellular drug and gene delivery (Salem et al., 2004; Salem et al., 2005). Magnetic targeting approach provides solutions for both nucleic acid and localized drug delivery (Scherer et al., 2002; Schillinger et al., 2005). Together with molecular targeting, the magnetic nanoparticle technology has especially empowered appealing
approaches to early cancer detection, imaging and treatment (Romanus et al., 2002; Brannon-Peppas and Blanchette, 2004). Moreover, NCs can also solely be employed for targeting purposes. Targeting ligands or other functionalizing groups can be incorporated on the NC surfaces. This way the NCs can be attached onto different matrix structures or targeted into a specific, desired site of action. For example a hydrophobin fusion protein, where the hydrophobin was coupled with two cellulose binding domains, was utilized in order to facilitate drug nanoparticle binding to nanofibrillar cellulose (Valo et al., 2011). The functionalized protein coated itraconazole nanoparticles were enclosed to this external nanofibrillar cellulose matrix, which provided protection for nanoparticles during the formulation process and storage. The versatility of NCs and ease of commercial production enables the development of commercially viable NPS for targeted drug delivery.

Another interesting application to provide significant solutions for drug delivery and other biomedical applications are the nanogel systems, gel macromolecules in the size range of tens to hundreds of nanometers, loaded with different types of therapeutics (Yallapu et al., 2007). Nanogels are in special interest due to their slow degradation nature, biocompatibility, stimuli-reactiveness (e.g. pH- or temperature-sensitive) and the ability to develop targeted drug delivery systems by surface functionalization.

Numerous medical applications of gold nanoparticles are enabled due to their biocompatibility, dimensions and ease of characterization (Daniel and Astruc, 2004). Application of targeted gold nanoparticles has gained success within both detection and therapy of different diseases, especially within cancer treatment. As imaging agents, due to their photophysical properties, they offer technologies like computer tomography, optical coherence and photoacoustic tomography. Whereas therapy wise photothermal therapy, i.e. hyperthermia, and X-ray- and radiotherapy are facilitated, mostly utilized in cancer treatment. In conclusion, it is clear that nanotechnology will have a major impact on the future of novel imaging and therapeutic systems fighting against diseases including cancer.
3 Aims of the study

The aim of this thesis was to obtain detailed knowledge about the dissolution characteristics of drug nanocrystals and to study the applicability of the nanocrystal approach for different drug delivery formulations.

The specific objectives of this study were:

1. To assess the effect of different nanocrystal particle size fractions to the dissolution behavior. Thus, the real-time, spatially and temporally resolved, dissolution behavior of nanocrystals was investigated using UV-Vis spectroscopy and a novel UV imaging technique (I).

2. By utilizing the wet milling technique, to develop nanocrystal formulation to be administered as a suspension. This was carried out by preparing ocular, intraocular pressure reducing, nanocrystal formulations, whose effect was investigated with an in vivo ocular hypertension model (II).

3. To examine formulation approaches for solid oral nanocrystal formulations, prepared by the rapid wet milling technique, and to evaluate their bioavailability in vivo (III).

4. To increase the batch size of a wet milling process producing nanocrystal suspensions, and to screen and to optimize parameters for feasible nanocrystal compositions in tablet formulation (IV).
4 Experimental

Complete experimental details are reported in the original publications (I-IV).

4.1 Materials

Model compounds and excipients used in this work are summarized together with their essential properties or functions in the formulations in Table 2. The corresponding publications are referred to accordingly.

Table 2a Model compounds used in this work (I-IV).

<table>
<thead>
<tr>
<th>API</th>
<th>Solubility in aqueous media</th>
<th>Molecular weight</th>
<th>pKa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (IND)</td>
<td>5 μg/ml pH=4, 14 μg/ml pH=5, 3540 μg/ml pH=7.6</td>
<td>357.8</td>
<td>4.4</td>
<td>I, IV (Jain, 2008)</td>
</tr>
<tr>
<td>Itraconazole (ITC)</td>
<td>1 ng/ml pH=7, 4 μg/ml pH=1</td>
<td>705.6</td>
<td>3.7</td>
<td>III, IV (Peeters et al., 2002; Ghazal et al., 2009)</td>
</tr>
<tr>
<td>Brinzolamide (BRA)</td>
<td>9000 μg/ml pH=5, 500 μg/ml pH=7.4</td>
<td>383.5</td>
<td>5.9/8.4</td>
<td>II (DeSantis, 2000)</td>
</tr>
</tbody>
</table>


**Experimental**

Table 2b *Stabilizers and other excipients used in this work (I-IV).*

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Function in formulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poloxamer 188 (F68)</td>
<td>Stabilizer</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Poloxamer 407 (F127)</td>
<td>Stabilizer</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>Stabilizer, Absorption enhancer</td>
<td>I, II</td>
</tr>
<tr>
<td>Hydroxypropyl methylcellulose (HPMC)</td>
<td>Stabilizer</td>
<td>II</td>
</tr>
<tr>
<td>Benzalkonium chloride (BAC)</td>
<td>Preservative</td>
<td>II</td>
</tr>
<tr>
<td>Microcrystalline cellulose (MCC; PH-101, PH-102)</td>
<td>Filler</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Silicified microcrystalline cellulose (SMCC)</td>
<td>Filler</td>
<td>IV</td>
</tr>
<tr>
<td>Lactose monohydrate (200M, 80M)</td>
<td>Filler</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>Binder</td>
<td>III, IV</td>
</tr>
<tr>
<td>Cross-linked-PVP</td>
<td>Disintegrant</td>
<td>III, IV</td>
</tr>
<tr>
<td>Colloidal silicon dioxide (CSD)</td>
<td>Mass lubricant</td>
<td>III, IV</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>Mould lubricant</td>
<td>III, IV</td>
</tr>
</tbody>
</table>

4.2 **Production techniques**

4.2.1 **Nanocrystal suspensions (I-IV)**

The starting point for this thesis was to produce NPS with wet media milling method. Thus, indomethacin (IND), brinzolamide (BRA) and itraconazole (ITC) nanocrystal suspensions (NPSs) were prepared using a rapid top-down wet milling technique. Stabilizer (25-80 w/w% in relation to drug amount) was dissolved in milling medium (5-22 ml) and, thereafter, drug powder (1-8 g) was dispersed in the stabilizer solution. The drug dispersion was inserted into a milling vessel (zirconium oxide or stainless steel) containing the maximum, varying amount of milling pearls (zirconium oxide or stainless steel), depending of the size of the pearls (Ø 1, 5 or 10
Experimental

mm) and the vessel (V=20, 40 or 80 ml) used. Milling bowl was placed in a planetary ball mill (Pulverisette 6 (80 ml vessel, stainless steel) (IV) or Pulverisette 7 Premium (20-40 ml vessels, zirconium oxide) (I-III), Fritsch Co., Idar-Oberstein, Germany) using an appropriate counter balance. Milling was performed in 3-6 min cycles repeated in total 6-10 times. After each cycle there was a 15 min pause for the system to cool down. The grinding speed was adjusted according to the milling pearl size (Ø 1 mm, 5 mm or 10 mm corresponded to 1100 rpm, 600 (max. for Pulverisette 6)/1000 rpm or 850 rpm, respectively). After milling, the NPS was separated from the pearls by pipetting or sieving. Detailed milling protocols for each of the model compounds are presented in the original publications (I-IV).

4.2.2 Formulation of NPS (I-IV)

After obtaining the NPS, the further formulation of the NPS was to follow. Three brinzolamide (BRA) NPS formulations (I-III) for ocular delivery (II) were prepared by diluting exact amounts of the BRA NPSs (pH=4.5 and pH=7.4), stabilized with HPMC (25 w/w% in relation to drug amount), with appropriate phosphate buffered saline (PBS, pH=4.5 or pH=7.4) solutions, including benzalkonium chloride (BAC) and a possible permeation enhancer (polysorbate 80) (Inoue and Shah, 2011), in a way that the desired formulation concentrations (BRA 1 % (w/v); HPMC 0.25 % (w/v); BAC 0.01 % (w/v)) were obtained.

For both analytical (I) and formulation purposes (III, IV) the NPSs were transformed into solid nanocrystal powders by freeze-drying (I, III, IV) or granulating (III). Freeze-drying was performed either using an automatic, single phase 72 h freeze-drying cycle (I, III) (LyoPro 3000, Heto-Holten A/S, Allerød, Denmark) or a 48 h multiphase method (IV) (Lyostar II, SP Industries Inc., Warminster, USA).

NC powders were formulated into both capsules (III) and tablets (III, IV) for per oral administration (Table 3). The NC powders could be utilized as direct compression (DC) tableting mass when combined with suitable tableting excipients (Table 2) (III, IV). Alternatively, the NCs could be processed into tablets via granulation. The granulation was performed either manually (III) or using a miniaturized, motorized mixer (Orion Pharma Oy) (IV). In order to manually convert the NPSs into dry powders, the NPS was used as a granulation medium in producing fast dissolving micro-granules. The granules were produced by manual
Experimental grinding using mortar, pestle and sieve. Whereas the motorized granulation process comprised of the miniaturized high-shear kind of wet granulation set-up, where the freeze-dried NC powders where sprayed with granulation fluid. In this thesis the tableting was performed both by manual compaction (I, III, IV) and using motorized, automatized (IV) mode of an instrumented (Single Station DAAS Measure, software version 1.2) single punch tablet press (Korsch, EK-0, Korsch, Germany) with rounded surface punches (Ø 9 mm and 5 mm).

**Table 3** The developed solid NC formulations divided according to production method (III, IV).

<table>
<thead>
<tr>
<th>Tableting* and Granulation*</th>
<th>Drug/ NPS</th>
<th>Excipients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tablets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIRECT COMPRESSION</td>
<td>manual*</td>
<td>Freeze-dried ITC</td>
<td>lactose monohydrate, MCC, PVP, CSD, cross-linked PVP, magnesium stearate</td>
</tr>
<tr>
<td></td>
<td>automated</td>
<td>Freeze-dried IND</td>
<td>lactose monohydrate, MCC, PVP, CSD, cross-linked PVP, magnesium stearate</td>
</tr>
<tr>
<td><strong>Tablets</strong></td>
<td>manual*</td>
<td>Granulated ITC</td>
<td>lactose monohydrate, MCC, PVP, CSD, cross-linked PVP, magnesium stearate</td>
</tr>
<tr>
<td>GRANULES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>automated</td>
<td>Granulated ITC Granulated IND</td>
<td>PVP, cross-linked-PVP, SMCC, magnesium stearate</td>
</tr>
<tr>
<td><strong>Capsules</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>manual*</td>
<td>Freeze-dried ITC</td>
<td></td>
</tr>
</tbody>
</table>

\footnote{\*}{Tableting and Granulation are indicated as manual or automated.}
Experimental

4.3 Characterization techniques

The methods for characterization of the NCs and the developed NC formulations are summarized in Table 4. The suppliers of the equipment can be found in the original publications (I-IV).

Table 4 The key characterization methods applied in this thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photon Correlation Spectroscopy (PCS)</td>
<td>Particle size and polydispersity (PI) analysis of the fresh and freeze-dried NPS</td>
<td>I- IV</td>
</tr>
<tr>
<td>Differential Scanning Calorimetry (DSC)</td>
<td>Solid state analysis, confirming the crystalline form of the milled material</td>
<td>I-IV</td>
</tr>
<tr>
<td>X-ray Powder Diffraction (XRPD)</td>
<td>Solid state analysis</td>
<td>I-IV</td>
</tr>
<tr>
<td>Scanning Electron Microscopy (SEM)</td>
<td>Morphology, particle size and shape</td>
<td>I-IV</td>
</tr>
<tr>
<td>Channel Flow Method</td>
<td>Dissolution</td>
<td>I (Peltonen et al., 2003)</td>
</tr>
<tr>
<td>UV Imaging</td>
<td>Dissolution</td>
<td>I</td>
</tr>
<tr>
<td>Paddle Method</td>
<td>Dissolution, according to European Pharmacopoeia standard</td>
<td>II-IV</td>
</tr>
<tr>
<td>HPLC assays</td>
<td>Quantification of BRA, ITC and IND concentrations in vitro and in vivo</td>
<td>II-IV</td>
</tr>
<tr>
<td>Cell viability assay</td>
<td>Cellular toxicity of BRA NC formulations</td>
<td>II</td>
</tr>
<tr>
<td>Ocular in vivo hypertension model</td>
<td>Elevated intraocular pressure reducing effect of the BRA NC formulations</td>
<td>II (Kalesnykas et al., 2007)</td>
</tr>
<tr>
<td>Per oral in vivo model</td>
<td>Oral administration of ITC NC formulations</td>
<td>III</td>
</tr>
</tbody>
</table>


4.3.1 Dissolution assay

After the determination of the quality of the prepared NCs according to particle size, PI, morphology and solid state form, the characterization of the dissolution behavior of the nanocrystalline samples had a great importance in this thesis. In the sense of analytical development, a channel flow method (Peltonen et al., 2003) was applied together with UV imaging (Østergaard et al., 2010; Boetker et al., 2011; Østergaard et al., 2011; Ye et al., 2011) (I). In order to eliminate the effect of increased surface area on the dissolution testing, all the tests were performed from a flat surface of compressed nanocrystalline powders. Whereas the paddle method, according to European Pharmacopoeia standard (Erweka DT-06, Heusenstamm, Germany and Sotax AT7, Sotax Corporation, Horsham, England), was used to study the dissolution of the developed BRA, ITC and IND NC formulations (II-IV).

In the channel flow dissolution method (I) one face of the compacted sample was exposed to the dissolution medium (acetate buffer, pH 5.0), which circulated by a peristaltic pump (medium flow rate of 8.1 ml/min, Watson-Marlow, Cornwall, UK) through the channel flow cell, medium reservoir and UV–Vis spectrophotometer (analytical wavelength 318 nm) with a flow-through cuvette (UV-1600PC, VWR International, Leuven, Belgium). The parts of the system were interconnected in a closed-loop fashion using silicone tubings. UV–Vis data was collected and analyzed with M. Wave Professional software (v 1.0, VWR International, Leuven, Belgium). Dissolution rate results were calculated as a released drug amount in time unit per constant area.

Additionally, an Actipix SDI300 dissolution imaging system (I) (Figure 8) (Paraytec Ltd., York, UK) with an Actipix flow-through type dissolution cartridge (CADISS-3) was also applied with following parameters: imaging area 3.64 mm ×
8.12 mm, pixels (7 µm × 7 µm) binned 4 × 4, pulsed Xe lamp as light source, quartz flow cell light path 4.0 mm and detection wavelength of 265 nm. Images were recorded (2.6 images per s) and analyzed with Actipix D100 software version 1.3 (Paraytec Ltd., York, UK). The dissolution of the compacted nanocrystals was studied both with a solution phase (acetate buffer, pH 5.0; absence and presence of flow: 0.5 ml/min to 0.1 ml/min) and a gel matrix (agarose/acetate buffer, pH 5.0) as the dissolution medium. After measurements, the conversion of pixel intensities into absorbance values (A) was done using the Actipix software based on Equation 4 (Østergaard et al., 2010):

$$A = \log \left( \frac{I_{ref} - I_0}{I_{sig} - I_0} \right)$$  \hspace{1cm} (4)

where $I_0$, $I_{ref}$, and $I_{sig}$ are the pixel intensity due to the dark current (electronic noise measured with the lamp turned off), pixel intensity measured with the dissolution medium in the cell (reference signal), and pixel intensity measured during the experiment, respectively. Thus, allowing the determination of the apparent IND concentration within the imaging area as a function of position and time. For the correct conversion of pixel intensities into analyte concentrations it is a premise that Beer’s law is obeyed (UV absorbance for each pixel read is within the linear range) and that other UV absorbing species do not interfere with the quantification (Østergaard et al., 2010; Østergaard et al., 2011). These preconditions were fulfilled on the study.
4.3.2 *In vivo* models (II-III)

Following the *in vitro* characterization, two *in vivo* models, ocular and oral, were applied to prove the bioavailability of the developed NC formulations. All the animal experiments were approved by the Finnish National Animal Ethics Committee in State Provincial Office of Southern Finland. The experiments were conducted in accordance with the guidelines set by the Finnish Act on Animal Experimentation, Statute of Animal Experimentation, other animal protection legislation (62/2006, 36/2006 and HE32/2005), the European Union Directive 2010/63/EU and the European Union Commission recommendations 2007/526/EC (European Communities Council Directive 86/609/EEC).

For the ocular *in vivo* hypertension model (II), ocular hypertension was induced unilaterally to seven-month old male Wistar rats (Harlan Laboratories B.V., Venray, The Netherlands) using an Iris Medical argon laser (Oculight GL, Device Optical,
Miami, USA) (Kalesnykas et al., 2007). The contralateral eye served as an untreated control. 15 hours after the laser treatment, single doses (10 µl) of each suspension sample were applied into the laser-treated eyes using the following treatment groups: (1) formulation I (n = 5 rats), (2) formulation II (n = 5), (3) formulation III (n = 6), (4) Azopt® (n = 6), (5) 0.9% NaCl (n = 8) and (6) non-treated group (NT, n = 6). The IOP-values were measured both before the administration of each sample and at predetermined times (7.5, 15, 30, 45, 60 min) after the administration. The samples were coded and administered in a blinded fashion.

For per oral in vivo experiment (III) the investigated suspension and solid samples, containing 2 mg of ITC, were divided into five treatment groups: 1) ITC-NPs (n = 6, suspension); 2) freeze dried ITC-NPs (n = 6, capsule); 3) granulated ITC-NPs (n = 5, capsule); 4) Sporanox® (n = 6, capsule) and 5) physical mixture (n = 5, suspension), which were intragastrically administered to male Sprague–Dawley rats (Laboratory Animal Centre of the University of Oulu, Finland). Blood samples were drawn from saphenous vein at predetermined time points: prior (0), 30 min, 1, 2, 3, 5, 8, 12 and 24 h after the administrations. The maximum plasma concentration (C$_{\text{max}}$) and the time to reach the C$_{\text{max}}$ (t$_{\text{max}}$) for ITC and hydroxyl-ITC (OH-ITC), an active metabolite of ITC, were assayed using LC–MS/MS (Agilent Technologies, Palo Alto, CA, USA) method (Decosterd et al., 2010; Valo et al., 2011) and further analyzed with appropriate data acquisition and quantification softwares (Agilent MassHunter Workstation, Agilent Technologies, Palo Alto, CA, USA and GraphPad Prism 5.04 for Windows, GraphPad Software Inc.).
5 Results and Discussion

5.1 Benefits of NCs (I-IV)

5.1.1 Characterization of the NCs (I-IV)

Firstly, in order to give an overview about the quality of the developed NPS, the results about the key characterization results are presented in this section. The developed NPS are summarized in Table 5. The obtained particle sizes depend on e.g. the stabilizer, the amount of it, size of the used milling pearls (Ø 1, 5, 10 mm), milling time and used milling apparatus (Pulverisette 6 or 7)(Liu et al., 2011). With constant milling parameters, the smaller the pearl size, the smaller and homogenous particles (PI < 0.5) are produced (Müller and Jacobs, 2002; Yadav et al., 2012). Polydisperse particles are considered to have PI-values larger than 0.7.

Table 5 The compositions (% (w/w) in relation to drug amount), average particle sizes and polydispersity indices (PI) of the developed IND, BRA and ITC NPSs.

<table>
<thead>
<tr>
<th>API</th>
<th>Stabilizer</th>
<th>% (w/w)</th>
<th>Particle size (nm)/PI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IND</td>
<td>F68</td>
<td>60</td>
<td>580 ± 30/0.4 ± 0.1</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>950 ± 190/0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>micronsized</td>
<td></td>
</tr>
<tr>
<td>IND</td>
<td>F127</td>
<td>60</td>
<td>970 ± 30/0.30 ± 0.02</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>580 ± 20/0.6 ± 0.06</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>740 ± 30/0.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>micronsized</td>
<td></td>
</tr>
<tr>
<td>IND</td>
<td>Polysorbate 80</td>
<td>60</td>
<td>460 ± 10/0.21 ± 0.17</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>530 ± 2/0.12 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>BRA</td>
<td>HPMC</td>
<td>25</td>
<td>315 ± 5/0.07 ± 0.02</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>550 ± 20/0.40 ± 0.04</td>
<td>IV</td>
</tr>
<tr>
<td>ITC</td>
<td>F127</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results and Discussion

Media milling was proven to produce crystalline drug material (IND, ITC, BRA) (I-IV) according to DSC and XRPD analyses, as extensively reported also earlier (Liu et al., 2011; Ali et al., 2011; Sarnes et al., 2013; Sarnes et al., 2014) (Figure 9). Milling and freeze-drying did not induce formation of amorphous IND, ITC or BRA nor changes in the crystalline form, supported by the absence of glass transition or recrystallization events (Chamarthy and Pinal, 2008). During wet milling of crystalline drugs the water is acting as an inhibitor of the formation of amorphous material due to the reduced glass-transition temperature (Sharma et al., 2009). In general, the NC samples showed endothermic melting events approximately at the same temperatures as their bulk counterparts. However, the endothermic melting peaks (Tm) of the NCs showed slightly shifted and broader compared to the bulk materials. This size of change in Tm has been reported to be related to particle size reduction (Xia et al., 2010; Xu et al., 2012). Also the mixtures of materials in the formulations have an impact to the slightly altered melting endotherms. The lower peak intensities of the NC samples are reported to be derived from the presence of the stabilizers surrounding the particles after milling (Hecq et al., 2005).

![Figure 9 Solid state analysis (DSC, XRPD) of ITC (A, B) and IND (C, D) NPS confirming the crystalline form of the drug after wet milling and freeze-drying.](image-url)
In addition to the PCS particle size measurements, the SEM images provided understanding about both the morphology and confirmed the nanosized structures of the different NC formulations in Figure 10, which summarizes the SEM analysis of ITC and IND NPS (A, D), freeze-dried NPS (B, E) and granulated NPS (C, F). Additionally, the morphology of BRA NPS is shown (G-H), which revealed that the NCs were stabilized by HPMC, seen as a mass around the crystals. Some crystallization of the free drug occurred during the sample preparation, which was evident in the images as some needle-like micronsized crystals in the images (G, H). Commercial BRA product, Azopt®, (I) was imaged as reference. After ensuring these quality characteristics of the prepared NPS, the desired dissolution properties were closely investigated.

**Figure 10** SEM images showing the typical morphological characteristics of (A-C) ITC and (D-F) IND NPS, freeze-dried NPS and granulated NPS, respectively. Additionally, the morphology of (G-H) BRA NC formulations and (I) Azopt® are presented.
5.1.2 Enhanced dissolution rate and solubility of NCs (I)

Dissolution properties of IND were improved by preparing different sized NCs using rapid wet milling technique with three different stabilizers, poloxamers F68 and F127 and polysorbate 80 (Table 5). The dissolution properties of the freeze dried and compacted NCs were studied using the channel flow dissolution method and novel UV imaging technique, used in this study for the first time in the dissolution testing of fast-dissolving nanoscale samples. The effect of the variation in the area available for dissolution was eliminated by studying even, constant surfaces instead of particulate samples. Thus, according to the Noyes–Whitney equation, the dissolution rate was affected only by the concentration gradient, which in turn is influenced by the small particle size and the decreased diffusion layer thickness of the NCs. The channel flow method distinguished clearly both the particle size fractions and the used stabilizers (Figure 11).

![Figure 11 Channel flow method: dissolution of IND from the NC compacts. Micronized bulk IND samples were used as corresponding references. Dissolution rate decreases along increasing particle size according to the arrows.](image)

In addition to supporting the results from the channel flow method about the increased dissolution rate of the NCs, UV imaging provided valuable new information about the concentration of the dissolved drug next to the sample surface (Figure 12): with the smallest NCs the concentration next to the particle surface exceeded fivefold the thermodynamic solubility. The apparent IND solubilities are
Results and Discussion

presented in Table 6 for supporting the data interpretation. This indicates that the solubility improvement itself, and not only the increased dissolution area, has a crucial role in higher dissolution rates of NC formulations. Even the micronsized particles showed substantially faster dissolution than the bulk material. Hence, it is clear that different nanocrystal size fractions provide divergent dissolution profiles. Based on this study it was evident that UV imaging is a promising technique to analyze the dissolution properties of nanoscale formulations in detail.

Table 6 The effect of stabilizer (stabilizer:drug ratio 0.6:1, the total concentration of stabilizer being 0.5 mg/ml) on the apparent solubility of bulk IND in acetate buffer (pH 5.0, after 12 h of shaking, n=3, T = 19-22°C).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Solubility</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>IND/F68</td>
<td>6.43 ± 0.06</td>
<td>0.018 ± 0.00</td>
</tr>
<tr>
<td>IND/F127</td>
<td>4.80 ± 0.00</td>
<td>0.013 ± 0.00</td>
</tr>
<tr>
<td>IND/polysorbate 80</td>
<td>10.9 ± 1.54</td>
<td>0.030 ± 0.00</td>
</tr>
<tr>
<td>Bulk IND</td>
<td>4.89 ± 0.18</td>
<td>0.014 ± 0.001</td>
</tr>
</tbody>
</table>
Figure 12 Apparent concentration-distance profiles obtained from UV imaging of IND NCs at time points 5, 15 and 30 min: (A) F68/580 ± 30 nm, (B) F68/micronized particles, (C) F127/580 ± 20 nm, (D) F127/micronized particles and (E) bulk IND/tens of µm.

5.2 NC Suspension formulation (II)

The beneficial character of NCs was next aimed to be utilized in formulation development. Three ophthalmic BRA NC formulations (BRA, 16 w/w%) in PBS (pH 4.5 and 7.4), stabilized with HPMC, were successfully developed using the straightforward rapid wet media-milling technique (Table 7). In addition to the beneficial characteristics of HPMC, such as low toxicity, prolonged contact with the mucosa and, most of all, its highly swellable nature (Toda et al., 1996; Ludwig, 2005; El-Sousi et al., 2013), HPMC is also approved for ocular drug delivery as an inactive ingredient, and has been used as an ophthalmic lubricant and tear substitute (FDA; El-Sousi et al., 2013). Polysorbate 80 was added after the milling to
formulation II because of the well-known permeation enhancing effect in ocular drug delivery (Kaur and Kanwar, 2002; Inoue et al., 2006). The pH values were selected in order to study the importance of the free drug amount for the efficacy of the formulations. The aqueous phase of the NC suspensions was saturated with the drug, and with BRA, the solubility at pH 4.5 was considerably higher when compared the situation at pH 7.4. An acidic pH increases the solubility of the ampholytic BRA (pKa 5.9/amine, 8.4/primary sulfonamide) compared to its solubility at physiological pH, where the drug is significantly less protonated; the solubility of BRA increases from 0.4 mg/ml (pH 7.4) to >30 mg/ml (pH 3.8) (Hall et al., 1999).

Table 7 The properties (n=3) of the prepared BRA nanocrystal formulations (I-III) and the negative control (BRA, 1 w/v%; HPMC 0.25 w/v%; BAC, 0.01 w/v%).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Particle size (nm)</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation I</td>
<td>BRA-NPs&lt;sup&gt;a&lt;/sup&gt;, pH 7.4</td>
<td>460 ± 10</td>
</tr>
<tr>
<td></td>
<td>BAC PBS, pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Formulation II</td>
<td>BRA-NPs&lt;sup&gt;a&lt;/sup&gt;, pH 7.4</td>
<td>460 ± 10</td>
</tr>
<tr>
<td></td>
<td>Polysorbate 80 (0.25 w/v%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAC PBS, pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Formulation III</td>
<td>BRA-NPs&lt;sup&gt;a&lt;/sup&gt;, pH 4.5</td>
<td>530 ± 2</td>
</tr>
<tr>
<td></td>
<td>BAC PBS, pH 4.5</td>
<td></td>
</tr>
<tr>
<td>Negative control&lt;sup&gt;b&lt;/sup&gt;,</td>
<td>bulk BRA HPMC BAC</td>
<td>micronized&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>in vitro</td>
<td>PBS, pH 7.4</td>
<td></td>
</tr>
</tbody>
</table>

(<sup>a</sup>BRA; 16 % (w/w), <sup>b</sup>physical mixture, <sup>c</sup>particle size > 10 µm, outside the PCS detection limits)

The ophthalmic NPS formulations (I–III) dissolved immediately in vitro in PBS pH 7.4 (Figure 13a.). Neither the pH 4.5 (formulation III) nor the added solubilizing agent, polysorbate 80 (formulation II), had any major influence on the dissolution profiles.
The laser treatment was successfully performed based on the mean IOP values of the treated (39.6 ± 2.7 mmHg) and contralateral control eyes (10.8 ± 0.2 mmHg) (independent samples t-test, $P \leq 0.001$). The in vivo rat ocular hypertension model showed the significantly decreased intraocular pressure values by all the formulations (Figure 13b). The IOP reduction was enhanced at pH 4.5, when the free drug in the NPS (formulation III) was at its highest. The acidic pH improves BRA solubility excessively when compared to the neutral pH (Hall et al., 1999). The low pH of formulation III was able to facilitate an advantageous IOP response even though the unionized form of a drug, present at pH 7.4 for BRA does favor the ocular absorption (Järvinen et al., 1994). This can be explained by the fact that acids tend to become buffered in the eye. The greatest relative IOP reduction (72.2%) was observed with formulation III at 45 min after administration. The actions of the formulations were proven against the commercial BRA product, Azopt®, used as a control. In conclusion, formulation III reduced IOP after 60 min more effectively than formulation II ($P = 0.017$) and Azopt® ($P = 0.004$), whereas the difference to formulation I was insignificant ($P = 0.126$). The results revealed that NPS are valid tools for ophthalmic drug delivery and valid therapeutic approaches.

![Figure 13](https://example.com/image13.png)

**Figure 13** (A) The in vitro dissolution profiles of BRA NPS formulations (I-III), Azopt® and negative control in PBS pH 7.4 using the paddle method. (B) The elevated IOP reducing effect of the same samples (NT, non-treated group).
5.3 Solid NC Formulation (III-IV)

5.3.1 Superior *in vitro* dissolution – Absence of *in vivo* correlation (III, Supplement I)

As presented, solid dosage form is generally preferred in drug delivery. Hence, itraconazole (ITC) nanocrystal suspensions, prepared by media milling (section 5.1.1, Table 5) were transformed into solid form by both freeze drying and granulation, and further developed both into tablets (25 mg; 1.2 mg ITC, 40 N) and capsules (25 mg; 1 mg ITC). The freeze dried ITC formulation proved to be equally advantageous as the NC suspension formulation according to both *in vitro* dissolution and *in vivo* bioavailability analyses (Figure 14a). Even after a compaction process of both freeze-dried and granulated NC suspensions, the rapid *in vitro* dissolution characteristics were maintained. The solid ITC NC formulations were *in vitro* superior to Sporanox® capsules, a marketed itraconazole product, which was used as a reference.

Tablet formulations were excluded from the *in vivo* experiments due to their large size and thus unreliable dosing to the rats. Additionally, the dosage form was different. Thus, the ITC-NPS and capsulated freeze-dried and granulated NPSs were administered to the rats (Section 4.3.2). When the *in vivo* bioavailability was compared, however, Sporanox® capsules significantly outperformed all the NC formulations (Figure 14b-c). This unexpected result indicates the difficulty to predict the *in vivo* behavior on the basis of the *in vitro* analyses: superior *in vitro* dissolution enhancement was not realized in *in vivo* drug absorption. In this particular case, the sugar beads in the Sporanox® formulation (Kapsi and Ayres, 2001) may have prolonged its retention time in the stomach, where ITC is dissolved slowly and emptied into the intestine for enhanced absorption. On the other hand, the ITC nanocrystal formulations may have passed through the stomach rapidly like a liquid formulation due to increased dissolution. The solubility of ITC is considerably much lower in the intestine than in the stomach, and thus, the high concentration of ITC from NCs may have precipitated in the small intestine (Van Speybroeck et al., 2010). The peak drug concentration of the freeze dried formulation occurred in the first minutes (Figure 14b), indicating rapid dissolution, followed by precipitation in the intestine. An efficient way to deliver ITC may be to facilitate rapid highly concentrated solution in the stomach and stabilize the solution when entering the
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small intestine. The stomach serves thus as a reservoir, from which the highly concentrated solution ITC can be delivered to the small intestine, and to be absorbed (Matteucci et al., 2009). Matrix type of structures, utilized for instance in Sporanox®, which remain longer times in the stomach, could be applied here in wider sense. Because of this unique behavior of ITC in the gastrointestinal tract, its in vivo behavior could not be predicted on the basis of the in vitro analysis.

Figure 14 (A) The in vitro dissolution profiles of the ITC NC formulations, and in vivo pharmacokinetic profiles of (B) ITC and (C) its active metabolite, OH-ITC, after per oral administration of the NC formulations.

Despite the fact that the NC formulations did not exhibit advantageous in vivo behavior in comparison to the marketed product, the results provided valuable information and starting points in order to further improve the in vivo
pharmacokinetics of ITC with the NC approach. For the further formulation research, there exists a need to obtain detailed information about the reasons explaining the \textit{in vivo} advantageousness of Sporanox®. Especially the solubilization and precipitation inhibition as well as the stabilized supersaturation are mechanisms that are to be closely investigated. There may be many other drugs that show similar behavior as ITC. This may be one of the reasons why only a very small number of clinically used nanocrystal formulations have been developed. Each drug may have to be analyzed differently based on its pH-dependent solubility. If a drug is less soluble in the intestine, rapid dissolution in the stomach may not increase the bioavailability. For the drugs like ITC having pH-dependent solubility and the drugs having a window for absorption, prolonged retention in the stomach may be as important as improving the drug solubility. It is vital to consider various formulation parameters carefully to develop clinically useful formulations, rather than relying on a single parameter such as water solubility alone. Finally, these tools could be combined with the nanocrystal approach and hopefully utilized to offer a valuable approach for delivering poorly soluble drugs like ITC.

\subsection*{5.3.2 Feasibility of NC approach for tableting applications (IV)}

Finally, the concept of solid NC formulation was further processed in order to expand the knowledge for higher amounts of NC formulations. Thus, ITC and IND NPSs were successfully manufactured by wet milling, increasing the batch scale 2-4 times, freeze-dried, and further developed into both direct compression (DC1-DC3) and granulated (G) tableting masses (Table 8). In order to find the optimal formulations for tableting, the powder and tablet properties of the ITC and IND, both DC and G compositions, were screened and compared in detail according to true density, powder flowability, dose uniformity, maximum upper punch force, tablet crushing strength, dissolution and disintegration behavior, and stability testings.
Results and Discussion

Table 8 The compositions of test formulae (% w/w) of the ITC and IND NC formulations for direct compression (ITC; DC1, DC2, DC3 // IND; DC1, DC2, DC3), the final ITC and IND DC compositions (fDC), the ITC (ITCG) and IND (INDG) NC granulation formulas for 250 mg tablets, and the physical mixtures (PM).

<table>
<thead>
<tr>
<th>Composition (w/w%)</th>
<th>DC1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DC2</th>
<th>DC3</th>
<th>ITCG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>INDG&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-dried NPS</td>
<td>18</td>
<td>40</td>
<td>40</td>
<td>71</td>
<td>73</td>
</tr>
<tr>
<td>MCC</td>
<td>41</td>
<td>29</td>
<td>19</td>
<td>73</td>
<td>57</td>
</tr>
<tr>
<td>Lactose</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>PVP</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>cross-linked PVP</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>CSD</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>SMCC</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>18.5</td>
<td>34.5</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>PMs, NPS replaced with bulk ITC/IND, <sup>b</sup>Therapeutic dose included, <sup>c</sup>fDC compositions.

The critical tableting parameters were optimized by manually compacting both ITC and IND DC1-DC3 compositions and PMs (n = approx. 30 tablets), whereas the G and fDC compositions were compressed using the automated process (n = approx. 300 tablets), which explains the differences in the forces and in the comparisons made in the data analysis. The tableting experiments with the ITC and IND DC1-DC3 compositions demonstrated informatively the effect of the varying amounts of NC powders. The existence of NCs improved the compressibility; with lower force, sufficiently hard tablets were provided. Figure 15 shows intriguing information about the compression force and tablet crushing strength with different compositions. An important factor explaining the results is the particle size. The smaller the particle size, the more there exists contact surfaces, and thus the greatest potential for bond formation, which in turn increases the hardness, i.e. crushing strength, of the tablets (MCKennan and MCCafferty, 1982; Velasco et al., 1999). The increased number of contact surfaces and thus potential for bond formation explains the fact that the greater the load of nanocrystals in the composition, the lower force is required to produce hard tablets. Both the granulated compositions,
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ITC G and IND G, facilitated significantly harder tablets, compared to the final ITC DC ($P < 0.001$) and IND DC compositions ($P < 0.0001$), with lower forces ($P < 0.0001$). This supports the well-known profitable properties of granulated tableting masses (Iveson et al., 2001), which can be compressed more easily and consume less energy (Faure et al., 2001; Hansuld and Briens, 2014).

**Figure 15** The maximum upper punch forces (N, column) with each (A) ITC and (B) IND compositions (DC1-DC3, final (f) DC, G, PM) in comparison to the average crushing strengths (N, after 48 h from tableting, line) of the compressed 250 mg tablets.

Furthermore, the dissolution behavior of IND was dependent on the load of nanocrystal powder in the IND DC1-DC3 tablet formulations (Figure 16a). At 15 min detection time-point the released drug load from the IND DC1 tablets was clearly higher than from the DC2 and DC3 tablets ($P < 0.0001$). The drug release from DC2 was in turn significantly above DC3 tablets ($P < 0.05$). Taking into account the tablet properties, processability, inclusion of adequate drug concentrations and dissolution profiles, the IND DC1 showed to be the best candidate for the final DC formula. ITC DC1 mini-tablets (30 mg) enabled the ITC dissolution study in *sink*-conditions, and thus related the ITC dissolution to its disintegration behavior. This relation between disintegration and dissolution was utilized to describe the dissolution behavior of the highly concentrated ITC DC2 and DC3 tablets, which were otherwise excluded from the dissolution analyses due to the high drug content.
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Figure 16 The dissolution profiles of (A) IND DC1-DC3 tablets (250 mg) and powders, (B) IND NPS compared to final IND DC (fDC), G and PM tablets (250 mg), and (C) ITC NPS compared to DC1 tablets (30 mg) and powder and PM tablets (30mg).

The disintegration results demonstrated also the effect of the amount of nanocrystal powders in the tablets (Figure 17): the less the formulation included nanocrystal powder (ITC DC1 // IND DC1), thus the more porous and less dense the structure, the faster was the medium penetration (diffusion), and the tablet disintegration. ITC and IND DC2s, and moreover the DC3s, provided more prolonged medium diffusion times. The disintegration times correlated in general well with the crushing strength values. The compositions of both the model substances showed comparable disintegration behavior in descending order according to the disintegration times (DC3 > DC2 > DC1 > PM and G > fDC, \( P \leq 0.001 \)). The disintegration results proposed the choice of ITC DC2 and IND DC1 for the final DC compositions. ITC DC2 offered the most suitable choice, since it provided an opportunity of inclusion of adequate drug concentrations in reasonable sized tablets, which would be disintegrated within acceptable time frames.
Figure 17 The disintegration times (min, column) versus crushing strengths (N, line) of the (A) ITC and (B) IND tablets.

In conclusion, the amount of the nanocrystal powder is critical for the formulation. When including excessive amounts of nanocrystals in the formulation, the benefits of the nanosized powders will be finally lost. Thus, there exists a maximum concentration, which provides good processability resulting in tablets with suitable strengths and drug release properties, *i.e.* dissolution behaviors and disintegration times. The DC designs of both the model drugs with compositions including 40% of freeze-dried nanocrystalline drug powder (ITC DC2 and IND DC1) outperformed the corresponding granulated tablets in all parameters after the stability surveillance. In the light of these evidences, both the model substances would benefit from nanocrystal tablet formulation approach.
Conclusions

6 Conclusions

Dissolution properties of the poorly soluble drugs (indomethacin, brinzolamide, itraconazole) were improved by preparing drug nanocrystals with universally applicable and industrially relevant, rapid wet media milling technique. High-quality nanocrystal suspensions, regarding particle size, size uniformity, morphology, stability and solid state were obtained. Dissolution study of freeze-dried nanocrystal compacts with the channel flow dissolution method and the novel UV imaging technique revealed the significance of the particle size for dissolution. UV imaging provided valuable new information about the concentration of the dissolved drug next to the sample surface: with the smallest nanocrystals the concentration next to the particle surface exceeded five-fold the thermodynamic solubility, creating supersaturated states. Even though the effect of the variation in the area available for dissolution was eliminated by studying smooth, constant surfaces instead of particulate samples, the differences between particle sizes were evident. This difference will be increased even further in actual drug formulation. This indicates that the solubility improvement itself, and not only the increased dissolution area, have a crucial role in higher dissolution rates of nanocrystal formulations.

Three ophthalmic BRA nanocrystal suspension formulations in PBS (pH 4.5 and 7.4) were successfully developed using HPMC as an effective stabilizer. The ophthalmic nanocrystal suspensions dissolved immediately in vitro and were homogenous and stable. The in vivo rat ocular hypertension model showed the significantly decreased intraocular pressure values by all the formulations. The IOP reduction was enhanced the most at pH 4.5, when the amount of free drug in the nanosuspension (formulation III) was at its highest. The actions of the formulations were proven against the commercial BRA product used as a control. The results revealed that nanocrystal suspensions are valid tools for ophthalmic drug delivery and therapeutic approaches.

Lastly, the results of the set of performed experiments answered to a wide range of questions regarding the feasibility and the preparation of solid oral nanocrystal formulations. Freeze-drying was proven over granulation to be an effective method to convert the nanocrystal suspension into dry powders. The compaction process of nanocrystal powders was performed successfully, maintaining the original characteristics of the nanocrystals, i.e. rapid dissolution. The up-scaling of wet milling was a straightforward process, and regarding the further formulation
Conclusions

development, it was clear that the amount of the nanocrystal powder in the solid formulation was critical in order to be able to fully utilize the benefits of the nanocrystals. Therefore, nanocrystal tablet formulation is an advantageous design with a fairly simple production process. However, the difficulty to predict the \textit{in vivo} behavior on the basis of the \textit{in vitro} analyses was demonstrated.

To conclude, NC suspensions offers a versatile platform to deliver poorly water soluble drugs via liquid and solid formulation. However, the formulation should be carefully considered in order to obtain an \textit{in vitro-in vivo} correlation and, thus the transfer of the \textit{in vitro} benefits to an \textit{in vivo} environment.
References


References


References


References


