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Nanocrystal formulation for poorly soluble drugs

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ACADEMIC DISSERTATION

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Abstract

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Poorly soluble drugs are often a challenging problem in drug formulation. Reducing the particle size of the drug to a nano-scale leads to an increased surface area-to-volume ratio, increased dissolution velocity and adhesiveness, and improved *in vivo* performance of poorly soluble drugs. Wet media milling is one of the most popular techniques to prepare the nanocrystals. The aim of this thesis was to optimize the preparation conditions and characterization methods of nanosuspensions for poorly water-soluble drug compounds, then formulate the nanosuspensions into suitable pharmaceutical dosage forms, and finally test the efficacy in *in vivo*.

Firstly, basic research on the preparation and characterization of nanosuspensions was done using the wet milling technique and poorly water-soluble drugs as model compounds. The various milling parameters, including the milling time, diameter of milling balls, stabilizer type and concentrations, were investigated. The particle size, size distribution and stability of nanosuspensions were considered as the important parameters to evaluate the milling conditions. The drug nanocrystals manufactured at the optimal milling environment exhibited significantly increased dissolution rates, good stability and kept a crystalline state.

Secondly, the importance of particle size on dissolution was determined. The conventional dissolution method can effectively discriminate the milled and unmilled samples, but it fails to discriminate the dissolution profiles of nanosuspensions with different particle sizes. A novel condition, i.e. the sample concentration in the dissolution medium is close to the apparent saturation solubility of the drug, were found to discriminate the dissolution profiles of nanosuspensions.

Thirdly, “nanos-in-micros” structure for suitable pharmaceutical formulations, e.g. tablet or pulmonary products was set up. The structure is that drug nanocrystals are buried inside microparticle carriers, in which the mannitol and crystalline L-leucine were as matrix and outer layer formers, respectively. “Nanos-in-micros” structure avoids problems in further formulation of nanopowders, such as poor flowability and aggregation/sinter, meanwhile functionality of individual nanoparticles in microparticles are remained, such as fast dissolution.

Finally, the efficacy of nanocrystal formulations *in vivo* was tested. Brinzolamide nanosuspensions were prepared in buffers and formulated for ocular administration in order to reduce the intraocular pressure in rats. The nanocrystal formulations exhibited a low cytotoxicity and significant reduction in intraocular pressure compared to the physiologic salt solution and untreated group.

In conclusion, the nanosuspensions of poorly water-soluble drug can be easily produced by the wet milling technique when the choice of stabilizers and milling parameters are appropriate. The nanosuspensions were successfully formulated and exhibited a good *in vivo* performance. More efficient formulations based on drug nanosuspensions should be further researched and developed.

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Helsinki, October 2013

Peng Liu

Contents

List of original publications

This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (**I-IV**).

I Liu P, Rong X, Laru J, Van Veen B, Kiesvaara J, Hirvonen J, Laaksonen T, Peltonen L. Nanosuspensions of poorly soluble drugs: Preparation and development by wet milling. Int J Pharm 2011, 411: 215-222.

II Laaksonen T, Liu P, Rahikkala A, Peltonen L, Kauppinen EI, Hirvonen J, Järvinen K, Raula J. Intact nanoparticulate indomethacin in fast-dissolving carrier particles by combined wet milling and aerosol flow reactor methods. Pharm Res 2011, 28: 2403-2411.

III Liu P, De Wulf O, Laru J, Heikkilä T, Van Veen B, Kiesvaara J, Hirvonen J, Peltonen L, Laaksonen T. Dissolution studies of poorly soluble drug nanosuspensions in non-sink conditions. AAPS PharmSciTech 2013, 14(2): 748-756.

IV Sarnes A, Liu P, Puranen J, Rönkkö S, Laaksonen T, Kalesnykas G, Oksala O, Ilkka J, Laru J, Järvinen K, Hirvonen J, Peltonen L. Brinzolamide nanocrystal formulations for ophthalmic delivery: Reduction of elevated intraocular pressure *in vivo*. Submitted manuscript, 2013.

Abbreviations and symbols

AFR	Aerosol flow reactor
API	Active pharmaceutical ingredient
AUC	Area under the plasma concentration time curve
BAC	Benzalkonium chloride
BBB	Blood-brain barrier
BDD	Biphenyl dimethyl dicarboxylate
BRA	Brinzolamide
C_{max}	Maximum peak concentration of the drug in plasma
CMC	Critical micelle concentration
c_s	Apparent saturation solubility
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
EPR	Enhanced permeability and retention
F68	Poloxamer 188 (Pluronic® F68)
F127	Poloxamer 407 (Pluronic® F127)
GIT	Gastrointestinal tract
g.s.d.	Geometric standard deviation
HPC	Hydroxypropylcellulose
HPH	High-pressure homogenization
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methyl cellulose
IND	Indomethacin
IOP	Intraocular pressure
ITR	Itraconazole
i.v.	Intravenous
MC	Methylcellulose
MM	Media milling
MPS	Mononuclear phagocyte system
NPs	Nanosuspensions
PBS	Phosphate buffered saline
pcs.	Pieces
PI	Polydispersity index
PEG	Polyethylene glycol
PVA	Polyvinyl alcohol
PVP	Polyvidone (polyvinylpyrrolidone)
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
Vitamin E TPGS	D-alpha tocopheryl polyethylene glycol 1000 succinate
XRPD	X-ray powder diffraction
ΔG	Change in Gibbs free energy (potential energy)

1 Introduction

In drug discovery the combinatorial chemistry and high throughput screening often leads to new chemical entities with high molecular weight and increasing lipophilicity and therefore decreasing aqueous solubility [1]. It is estimated that nearly 40% of the drugs in the pipeline have solubility problems and 60% of new drugs are poorly water-soluble [2]. To achieve its pharmacological activity, drug must be present in the dissolved state at the site of absorption in oral administration. The poor aqueous solubility of drugs resulting in poor oral bioavailability has always been a challenging problem in pharmaceutical research.

Many approaches have been developed to improve the drug solubility in aqueous phase, such as salt formation, co-solvents, complexes with cyclodextrins, changing its solid state. Decreasing the particle size into nanometre is a promising approach to improve the apparent saturation solubility, dissolution rate and oral bioavailability of hydrophobic drugs (BCS (biopharmaceutical classification system) Class II, in some cases also with BCS Class IV drugs). Drug nanocrystals, consisting of pure drugs and a minimum of surface active agents required for stabilization, are carrier-free submicron colloidal drug delivery systems with a mean particle size in the nanometre range, typically between 10 and 1000 nm [3]. Compared to other nanotechnological approaches, nanocrystals have a very high drug loading, as the particle core is composed of pure drug material.

The wet pearl milling technique is an important top-down method for preparing nanosuspensions. At the moment, approximately twenty nanocrystal products are on the market and most of them are made by the wet milling technique. However, there are still problems with the wet milling. There is no single versatile stabilizer suitable for all drug compounds and different drugs require their optimal stabilizers. Inadequate systematic understanding on the interactions between stabilizers and drugs is available. Although the wet milling technique has been thought as a simple milling process for size reduction, actually multidisciplinary knowledge, including knowledge of grinding mechanism, breakage kinetics of nanocrystals, the physical background of crystal stability, formulation processing and factors affecting the drug fate *in vivo* are all necessary to fully understand the technique [4]. In addition, with the number of nanoformulations increasing, conventional dissolution methods are not good enough for discriminating the dissolution profiles between nano-products since the very rapid dissolution in sink conditions masks the differences.

Nanocrystals are considered as versatile platform for administration through various routes, such as oral, parenteral, ophthalmic, transdermal and pulmonary delivery. It was reported that pharmaceutical nanocrystals have good performances *in vivo*, such as improved bioavailability, potential site-special drug delivery, fed/fasted state independent bioavailability, and suitability for drugs with a narrow absorption window.

This thesis focuses on manufacturing and formulating the nanosuspensions for poorly water-soluble drug compounds. Our aims were to find out the optimal manufacturing conditions and basic properties of nanosuspensions. The effect of preparation parameters on particle size, size distribution and stability, including milling time, stabilizer type and concentration, and the diameter of the milling balls, were evaluated. The basic properties of nanosuspensions such as morphology, dissolution rate and solid state were characterized. Slowing down the dissolution rate was used in this thesis to discriminate between the dissolution profiles of nanosuspensions with different particle sizes. Furthermore, the formulations of nanosuspensions were developed. The nanosuspensions were dried in an aerosol flow reactor to generate the microparticles, which can be used for example for tablet or pulmonary drug delivery. Finally, brinzolamide nanocrystal formulations were made for ocular drug delivery in order to reduce the intraocular pressure *in vivo*.

2 Review of the literature

2.1 Poorly water-soluble drug compounds

When drug discovery and medicinal chemistry moved from wet chemistry to combinatorial chemistry and high throughput screening in the mid-1990s, the properties of newly developed chemical entities shifted towards higher molecular weight and increasing lipophilicity [5]. Drug dissolution is a prerequisite to drug absorption and clinical response for orally administered drugs [6]. However, it was estimated that 40% of the drugs in the pipelines have solubility problems and 60% of new drug molecules are poorly water soluble [7, 8], which generates many problems in drug research and development (Table 1).

Table 1 *Major issues associated with poorly water-soluble compounds [9].*

− Poor bioavailability
− Inability to optimize lead compound selection based on efficacy and safety
− Fed/fasted variation in bioavailability
− Lack of dose-response proportionality
− Suboptimal dosing
− Use of harsh excipients, i.e., excessive use of cosolvents and other excipients
− Use of extreme basic or acidic conditions to enhance solubilization
− Uncontrollable precipitation after dosing
− Noncompliance by the patient, i.e., inconvenience of the dosage platform

To overcome the problems arising from the limited solubility and dissolution rate, intrinsic modification of chemical or physical properties of drug molecules and extrinsic modification of drug formulations can be used. The former includes salt complexes, prodrug formation, changes in the solid state, and particle size reduction. The formulation strategy includes cosolvents (e.g. water-ethanol), solubilization in surfactant systems, solid dispersions and formation of water-soluble complexes (e.g. β-cyclodextrins). Good overviews of these strategies for poorly water soluble drugs have been presented in recent reviews (refs. [10] and [11]). However, excipient-related toxicity or unwanted side-effects should be cautioned when the excipients, such as surfactants, polymers and organic solvents, are used to enhance the solubility of the drug. For example, in the formulation of Taxol®, anti-cancer drug paclitaxel is dissolved in the blend of ethanol and Cremophor® EL (polyoxyethylated castor oil) (1:1), which causes serious hypersensitivity reactions [12].

2.2 Nanocrystalline particles

In pharmaceutical sciences term drug nanocrystals means particles having a solid, usually crystalline drug core in the nanometer size range and an outer layer consisting of a stabilizer. Nanocrystals can also be named as drug nanoparticles and sometimes even amorphous drug nanoparticles are called nanocrystals. Since pharmaceutical nanocrystals are normally prepared in either an aqueous solution or non-aqueous solvent medium, the term nanosuspension is also often

used as synonymous to nanocrystals, meaning a sub-micron colloidal dispersion of pure drug particles, which are stabilized by surfactants, polymers, or both [13].

2.2.1 Properties of nanocrystals

2.2.1.1 Increased solubility and dissolution velocity

Solubility is determined by balance of intermolecular forces between the solvent and solute, and the entropy change that accompanies the solution. According to an IUPAC (International Union of Pure and Applied Chemistry) definition, solubility is the analytical composition of a saturated solution expressed as a proportion of a designated solute in a designated solvent [14]. The extent of the solubility of a substance in a specific solvent is measured as the saturation concentration. Under certain conditions, the saturation concentration can be exceeded to give a so-called supersaturated solution, which is metastable. Apparent solubility is the solubility for the thermodynamically less stable form in a special situation, which differs from the thermodynamic solubility; e.g. amorphous or metastable polymorphs, nanocrystals, cyclodextrin complexes. And, correspondingly, apparent saturation solubility is the saturation solubility for these systems. In dissolution, a solute forms a solution in a solvent. The dissolution rate quantifies the speed of the dissolution.

How the apparent saturation solubility and dissolution velocity are increased in the case of nanocrystals is illustrated in Fig.1. Assuming that the drug nanoparticles have spherical shape, the surface area-to-volume ratio of a particle is 3/r, where, *r* is the radius of the particle [15]. Thus, the reduction in particle size increases the surface area-to-volume ratio (Fig.1.1). Meanwhile, the total surface area of particles increases when one large particle is divided into many nanoparticles. The increased surface area accelerates the dissolution rate according to the Noyes-Whitney equation (see below).

The apparent saturation solubility (c_s) of drugs not only depends on the drug compound, the dissolution medium and temperature, but also on the particle size when it is below 1μm [16]. According to the Ostwald-Freundlich equation, the vapor pressure of liquid droplets changes due to the increasing curvature of the droplet/vapor interface with decreasing droplet radius. As the droplet radius decreases, the high droplet surface curvature leads to a higher vapor pressure. The process of molecule transfer from liquid droplet to a gas phase is similar with the process of molecule transfer from solid phase (nanocrystals) to liquid phase (dissolution medium). The vapor pressure is equivalent to the dissolution pressure [16]. Thereby the apparent saturation solubility of nanocrystals is increased by the high surface curvature and dissolution pressure (Fig.1.2). Increased apparent saturation solubility further accelerates the dissolution rate.

In Fig.1.3, the diffusional distance (h) around the particles decreases for very small particles, leading to a high concentration gradient around particles by $(c_s-c_x)/h$. Combining all influencing factors and according to the Noyes-Whitney equation, the dissolution velocity of nanocrystals is remarkably improved. Anderberg *et al.* [17] reported that at the particle size of 1 μm the intrinsic dissolution rate is very fast and that further decrease in size did not markedly increase oral adsorption. Jinno *et al.* [18] estimated the 50% dissolution times ($T_{50\%}$) of nanosuspensions based on the simulated curves. The results indicated that the dissolution rates of nanosuspensions with particle size of 0.22 μm would be 5100-fold greater than that of 13 μm in the same dissolution conditions. Particle size reduction is an effective and safe approach to improve the apparent

saturation solubility and dissolution velocity of poorly water-soluble drug compounds, while avoiding a large amount of solubilizing agents.

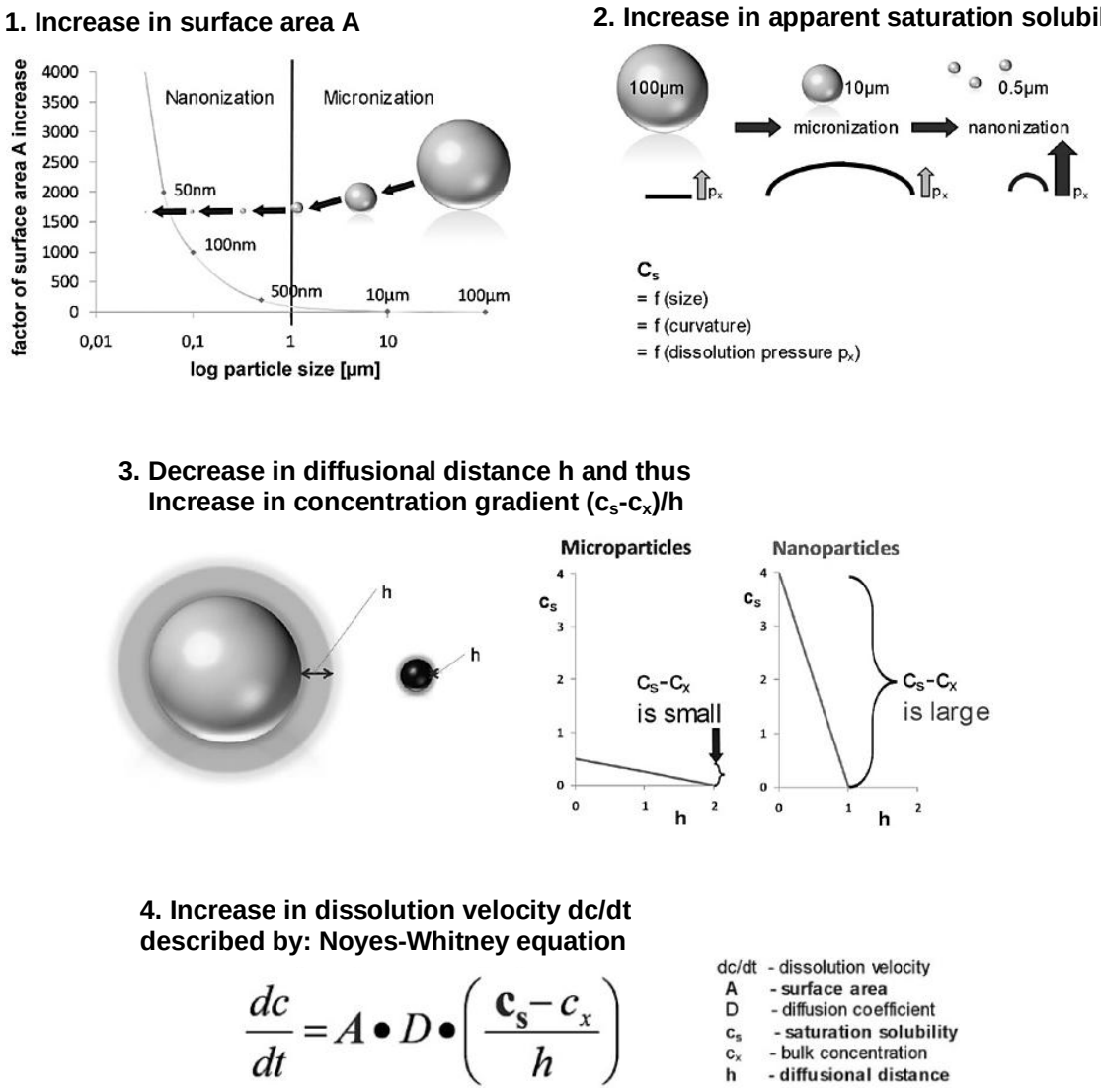

Figure 1 *Transfer of microcrystals to nanocrystals leads to an increase in surface area (upper). Increase in* apparent *saturation solubility c_s, decrease in diffusional distance h and increase in the concentration gradient $c_s - c_x$ /h all increase the dissolution velocity dc/dt [19].*

2.2.1.2 Increased adhesiveness

Once nano- and microparticles are taken orally, particles are faced with at least three different pathways: (i) capture by gut-associated lymphoid tissue; (ii) mucoadhesion; and (iii) direct faecal elimination after gastrointestinal transit [20]. For nanoparticles, the mucous gel layer is a porous structure (Fig.2 A). Nanoparticles can quickly penetrate deeply into the gel layer and closely contact with the mucous network. The adsorption isotherm shows linear increase with particle concentration. However, for microparticles, the mucosal gel layer resembles more like a smooth surface, rather than porous, since the particle size is too large to enter the mucosal gel layer (Fig.2

B). The adsorption follows a Langmuir isotherm [20]. The size dependency of the particle deposition has been found [21]. Durrer et al. [22] quantified adsorbed poly (styrene) latexes on rat intestinal mucosa. The adsorption results showed that 90% of equilibrium was reached after 10 min for a particle size of 230 nm, 20 min for a size of 320 nm and 30 min for a size of 670 nm. Therefore, nanoparticles increased the adhesiveness capability and shortened the adsorption time. The increased adhesiveness of nanoparticles prolongs the contact time and retention time to gastrointestinal tract (GIT) membrane [23, 24]. Meanwhile, the diffusion distance of nanoparticles to the GIT membrane is decreased, and then the concentration gradient becomes larger, which enhances the passive transport of the drug molecules [25]. In addition, particles captured by the mucosal layer are protected from denaturation in the gastro-intestinal lumen [20].

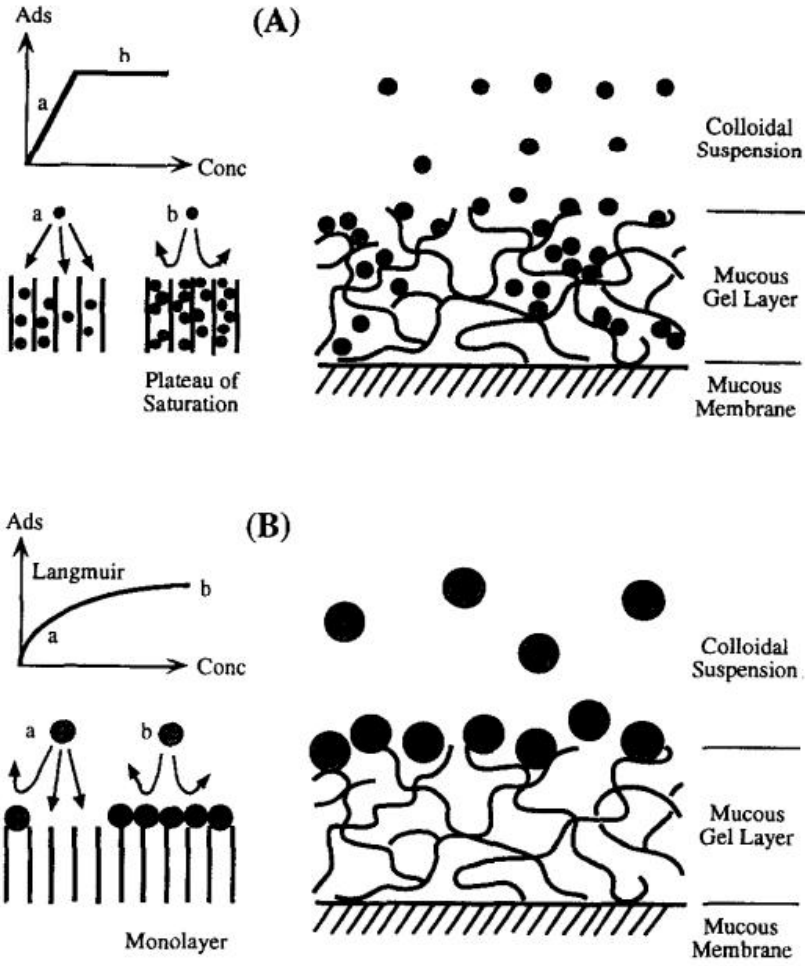

Figure 2 *Adsorption isotherm shapes and corresponding adsorption models. A: case of particles < 1 μm. B: case of particles > 1μm [20].*

2.2.2 Nano-sized techniques

There are two approaches to produce nanocrystals: top-down approaches and bottom-up approaches. In top-down methods large particles are broken down to small ones (e.g. wet milling and high pressure homogenization (HPH)), while bottom-up methods rely on a process of dissolved

drug molecules building up to nano-sized particles (e.g. precipitation). Table 2 shows an overview of various technologies for nanocrystal preparations.

Table 2 *Overview of the technologies utilized in nanocrystal formulations (modified after the reference [26])*

Technique		Nanocrystal	Company
Precipitation	Bottom-up	Hydrosol®	Sandoz/Novartis
Precipitation	Bottom-up	Nanomorph®	Soligs/Abbott
Wet milling	Top-down	Nanocrystal®	NanoSystems/élan
HPH	Top-down	IDD-P®	SkyePharma Canada Inc.
HPH	Top-down	Dissocubes®	SkyePharma
HPH	Top-down	Nanopure®	PharmaSol
Combination	-	NanoEdge®	Baxter
Combination	-	smartCrystal®	PharmaSol Berlin/Abbott

2.2.2.1 Bottom-up techniques

The basic idea of bottom-up techniques for preparation of nanosuspensions is that dissolved drug molecules precipitate from the solvent and grow up to nanoparticles. Nucleation and crystal growth are the two main steps for nanocrystal formation. List and Sucker in 1988 first reported the preparation of "hydrosol" by controlled precipitation method, with the intellectual property owned by Novartis (previously Sandoz) [27]. Later, Nanomorph® technology as another precipitation method was reported to be able to prepare stable amorphous drug nanoparticles. This technique is owned by the company Soliqs/Abbott (previously Knoll/BASF) [16] (Table 2).

Precipitation technologies can be broadly classified into four categories: precipitation by liquid solvent-antisolvent addition, precipitation in presence of supercritical fluid, precipitation by removal of solvent and precipitation in presence of high energy processes [28]. Among them, precipitation by liquid solvent-antisolvent addition is the simplest and most common method. The drug compound dissolved in a solvent is mixed with a miscible antisolvent under stirring or sonication. The supersaturated drug in the antisolvent quickly creates a large amount of nuclei, and then the nuclei grow to the nanoparticles. Agitation and ultrasound is a feasible mixing method to accelerate molecular diffusion and mass transfer, which controls the nucleation and crystallization processes [29]. Stabilizers should be added in solvent or antisolvent to prevent the molecular association and crystal growth. Many experimental parameters can affect the results of nanocrystals, such as the drug concentration in solvent, volume ratio of antisolvent to drug solution, power input (the stirring speed or ultrasonication time), precipitation temperature and stabilizers [30, 31]. The choice of the antisolvent is important since it determines the supersaturation degree of the drug. The high level of supersaturation would lead to a fast nucleation rate and small nucleus size. That large amount of nuclei are created at the same time and that the nuclei grow simultaneously, are preconditions to produce the small crystals with the same size [32]. Water is mostly used as the antisolvent for poorly water soluble drugs, but in some cases both the solvent and the antisolvent can be organic in nature [33]. Precipitation in the presence of supercritical fluids can be performed by RESS (rapid expansion of supercritical solution), RESOLV (rapid expansion of a supercritical solution into a liquid solvent) or SAS (supercritical antisolvent) techniques [28]. Mostly utilized supercritical fluid for pharmaceutical applications is CO_2.

Other bottom-up techniques for preparing the nanosuspensions have been reported. Mou *et al.* [34] utilized an acid-base neutralization reaction to prepare itraconazole nanosuspensions, which is based on the pH-dependent solubility of the drug. Briefly, the drug dissolved in hydrochloric acid solution and ethanol (1.10, v/v) were added to the sodium hydroxide solution under stirring until the solution was neutralized. Ali *et al.* [35, 36] prepared hydrocortisone nanosuspensions using a microfluidic nanoprecipitation process. The drug dissolved in organic solvent and antisolvent phases (e.g. water) containing stabilizers were flowed in parallel from the microchannels and nucleoli occurs in the diffusion layer when the molecules diffuse across the interface between fluids. Liquid atomization based techniques, such as spray drying [37], electrospraying [38] and aerosol flow reactor [39], can be used for producing drug nanoparticles. In aerosol flow reactor method [39], the drug solution was atomized using a collision-type air jet atomizer and the droplets were dried at high temperature companying the solvent evaporation. However, the bottom-up techniques are not widely applied for drug nanocrystal production nowadays.

2.2.2.2 Top-down techniques

Wet pearl milling is a typical top-down technique. The pearl milling technique (Nanocrystal®) was developed by Liversidge *et al.* in 1990 [40] and was formerly owned by company NanoSystems, currently owned by Élan Drug Delivery Systems [41]. Milling chamber filled with milling pearls, drug, stabilizer and dispersion media (e.g. water, buffer or organic solvent) are rotated in a milling machine at a very high speed. Under the movement of milling pearls, the collisions of the drug particles with milling pearls, milling chamber and other drug particles, together with high shear forces, fracture the drug crystals into nanosized particles. The common materials for the milling pearls include zirconium oxide, stainless steel, glass or highly cross-linked polystyrene resin. The process can be performed in batch or continuous modes. The range of milling time is from 30-60 min to several days for a batch of nanosuspensions, depending on the milling devices, milling parameters, batch size and components. The long duration of milling process increases the risk of microbial growth, instability (e.g. degradation) and costs.

Many factors affect the milling results, including the properties of the drug (e.g. hardness and density), concentration and type of stabilizer, viscosity of suspensions, amount of drug in the milling vessel, amount, diameter and density of milling pearls, and milling parameters (milling speed and time) [42-44]. The hardness and density of the milling pearls and milling chamber must be greater than the materials being ground. Luckily, most of active pharmaceutical ingredient (API) crystals are relatively brittle. The size of the milling pearls vary from less than 0.1 mm to 20 mm. Generally, the smaller milling pearls can induce finer nanoparticles because of the large number of contact points; but too small pearls, e.g. 0.03 or 0.015 mm, are not good for milling, because their light weight cannot supply with the sufficient impact energy for the drugs [45]. Larger number of milling pearls with high density is beneficial for getting fine particles. Longer milling time and higher speed increase the percentage of fine particles, but in some cases no change or even increase in the particle size were found with the longer milling times. Higher speed reduces the milling time. A minimum drug filling quantity is required to avoid the wear to the grinding balls and chamber, while a high drug concentration is good for milling attributing to the increased collision probability between the drug particles. A normal drug concentration in the milling chamber ranges from 1 to 400 mg/ml.

The merits and demerits of the wet milling technique are listed in Table 3. This technique is suitable for commercialization due to its advantages, e.g. avoid organic solvents, ease of scale-up and high efficiency of drug loading. Polymorphic transition of drug during the wet milling process is in most cases not an issue and the drug crystallinity remains intact, providing a good stability and shelf life [46]. Contamination from the erosion of grinding pearls is the main problem in the milling process. Grinding media fragments were found in the milled samples [31]. Using milling pearls coated with highly cross-linked polystyrene resin can minimize the erosion [47], which is currently applied in NanoCrystal Technology. Another problem is that the drug product adheres to the surface of milling vessel and milling pearls, which cause undesirable drug losses. The drug loss is a big issue for very expensive drugs and limited quantities of new chemical entities, like the drug development in preformulation phase [48].

Table 3 *The merits and demerits of wet milling, HPH and precipitation techniques.*

	Merits	Demerits
Milling	- Useful for drugs that are poorly soluble in aqueous and non-aqueous solvent - Avoids organic solvents - Ease of scale-up - Little batch to batch variation - Narrow size distribution of nanoparticles - High efficiency of drug loading	- Expensive manufacturing equipment - Intensive-energy use with long milling times - Potential instability of drugs induced by high shear and temperature - Contamination from the grinding media - Undesirable drug loss
HPH	- Same as with wet milling	- Micronized drug particles needed - Suspension formation need - Potential erosion and contamination from the machine - Energy intensive technique
Precipi-tation	- Low cost - Low energy-input - Simple operation - Common equipment - Possible for continuous production and scale-up	- Incomplete removal of toxic solvents - Difficult to choose the solvent and antisolvent - Potential particle growth - Potential production of amorphous drug state

The high-pressure homogenization (HPH) is another basic disintegration technology for nanocrystal production. Two homogenizer types normally applied are the piston-gap homogenizer (e.g. APV Gaulin, Avestin, etc.) and microfluidizer homogenizer (Microfluidics, Inc.)

In the piston-gap homogenizer, the homogenization in water (DissoCubes® technology) was invented by Müller *et al.* in 1994 [49]. Before homogenization, the particle size of raw drug powders (or dispersed in an aqueous surfactant solution) is firstly reduced into microparticles using jet-mill [41], basic homogenizer [50, 51], sonication [52] or mortar and pestle [53], which effectively avoids blocking the very small homogenization gap (approximately 25 µm). When the micronized suspensions contained in a cylinder with a diameter of 3 cm (for APV LAB 40) pass through a very thin gap (about 25 µm), the liquid boils because the static pressure of liquid is

decreased to a lower value than the vapor pressure of the liquid at room temperature. As a result, formation and implosion of gas bubbles, cavitation, in the homogenization gap produces particle breakage. The strong cavitation forces, high shear forces and the collision of the particles against each other act as the main forces to produce nanocrystals. The particle size and size distribution of nanosuspensions are related to homogenization pressure, cycle number and hardness of the drugs. Pardeike *et al*. [54] prepared PX-18 (2-N,N-Bis(oleoyloxythyl)amino-1-ethanesulfonic acid) nanosuspensions using the HPH method. An exponential decrease rather than a linear decrease in particle size was found with the increasing homogenization cycle number. It indicated that the raw material containing more weak points (imperfections) is easy to break. When smaller particles are formed, the particles are more perfect and more energy is needed to break them.

The second generation of piston-gap homogenizers was named Nanopure® technology, which homogenizes in non-aqueous media. For some purposes and administration routes, the drug nanocrystals are needed to be dispersed in water-free media (oils) to be used as soft gelatin capsules or isotonic suspensions (water-glycerol mixtures) for intravenous (*i.v.*) injection, or water-ethanol mixtures for easy drying. The cavitation effect is limited in this case since the static pressure is not sufficiently lower than the vapour pressure of dispersion media. The details of HPH method were described in a recent review [26].

Microfluidizer homogenizers are based on a jet stream principle, in which the drug suspension passes through the designed interaction chambers at a high pressure. Two types of homogenization chambers divided by the flow direction of suspensions that have been used are Z-type and Y-type. The liquid streams with supersonic velocities turbulent in channels with cross-sections smaller than a human hair and impinge against each other and against the chamber wall [55]. The particle size is reduced by the high-shear forces, turbulence, and impaction, as well as cavitation. The disadvantage of this technology is wide size distribution, i.e. nanosuspensions contain a relatively large fraction of microparticles (especially for hard drugs) [56]. However, Hao et al. [57] studied the preparation of Amoitone B nanocrystals using the microfluidization technology. Fine nanosuspensions with a diameter of 256 nm and polydispersity index (PI) of 0.206 were obtained after the homogenization. Verma et al. [58] investigated microfluidization for nanosuspension preparation using a quality by design approach. The milling time, microfluidization pressure, stabilizer type, processing temperature and stabilizer concentration were the critical parameters affecting the formation of indomethacin nanocrystals. Xiong *et al*. [59] investigated the effects of production parameters, such as pressure, cycle numbers and crushing principles, on the mean particle size and polydispersity of nanosuspensions. The results showed that the cavitation forces produced from piston-gap homogenizers are more powerful and suitable crushing force to prepare the nanocrystals than the shear forces in the Microfluidizer processor. The increased pressure leads to smaller and homogenous particles. Until now, microfluidization has been popular in the preparation of emulsions, liposomes and microcapsules, rather than in the preparation of crystalline drug nanosuspensions.

2.2.2.3 Combination techniques

Combination of multiple techniques can overcome the shortages of each single technique. Baxter International Inc. (Deerfield, IL) invented a combination technology called NANOEDGE, which is precipitation followed by a second high energy step [60]. The second energy-addition step avoids further crystal growth and aggregation after precipitation and converts the amorphous and semi-

unstable crystalline form arising from precipitation process to the crystalline form. Moreover, the subsequent energy-addition step becomes quicker and more efficient since the crystals are friable after the first step. The precipitation-high pressure homogenization was studied in the preparation of 10-hydroxycamptothecin nanosuspensions [61] and nitrendipine nanocrystals [62]. They found out that the first size-controlling step using microprecipitation decreases the energy required and made the homogenization process easier. And the homogenization process promoted an annealing effect and helped the surfactant to adsorb onto the nanocrystal surface, which increased the stability of nanosuspensions. Other combination method, such as melt emulsification - high pressure homogenisation, were used to prepare orlistat nanosuspensions [63].

SmartCrystal® technology, which is considered as a second generation technology of nanocrystals, is a series of combination technologies rather than only one technology. SmartCrystal® technology combines the pre-treatment and subsequent main treatment (high pressure homogenization, HPH), and includes spray drying-HPH, precipitation-HPH, lyophilization-HPH and pearl milling-HPH. These combination technologies generate faster production processes, smaller nanocrystals (lower than 100 nm) and improved physical stabilities [64, 65].

2.2.3 Stability of nanosuspensions

Stability is a very important property for nanosuspensions. Nanoparticles can for example agglomerate, aggregate or sinter during the production or storage. Agglomerates are clusters of primary particles held together by weak physical interactions. Aggregation forms stronger particle clusters and it is often irreversible process. In sintering, individual particles are merged irreversibly to larger particles. Increased particle size can change the apparent saturation solubility and dissolution rate, and consequently change the blood plasma concentration and bioavailability for oral drug delivery; change the tissue distribution or cause the vascular blockage for intravenous injection. The decomposition/ degradation of the drug can cause the loss of potency of the drug and even generate undesirable and toxic chemical agents.

In top-down techniques, a large number of newly formed surfaces with high surface energy tend to agglomerate/aggregate to reduce the Gibbs free energy in the system. In bottom-up techniques, nucleation and growth in supersaturated solution occur spontaneously with a decrease in free energy. These aggregation and growth behaviors related to the change of free energy are causing thermodynamic instability of colloidal systems.

At the same time, nanoparticles are subject to kinetic instability in colloidal particle systems. There are various interaction forces between the nanoparticles. Russians Deryagin and Landau and the Dutch scientists Verwey and Overbeek considered the electrostatic repulsion and van der Waals attraction as two main forces in colloidal dispersions. Their theory looks at the balance between these two opposing forces, known as the DLVO theory of colloid stability. The energy curves of van der Waals attraction (V_A), electrostatic repulsion (V_R) and total ($V_{total} = V_A+V_R$) plotted against the distance of two spherical particles (H) are shown in Fig.3. The maximum and minimum energy states are showed in the curve of total energy. Van der Waals force promotes aggregation of particles, while potential existing on the charged particle surface expels the particles by repulsive inter-particle forces. The primary maximum state creates an energy barrier for aggregation, providing the stability of charged colloidal particles. The larger the barrier, the longer the system will remain stable. If the primary maximum is too small, the particles tend to agglomerate,

aggregate and sinter depending on the depth of primary minimum. The depth of the secondary minimum is important in determining the stability of a hydrophobic dispersion. As we know, particles tend to aggregate spontaneously by thermodynamic drive to decrease free energy. If the secondary minimum is smaller than the thermal energy, the particles will repel each other and keep stable [66, 67].

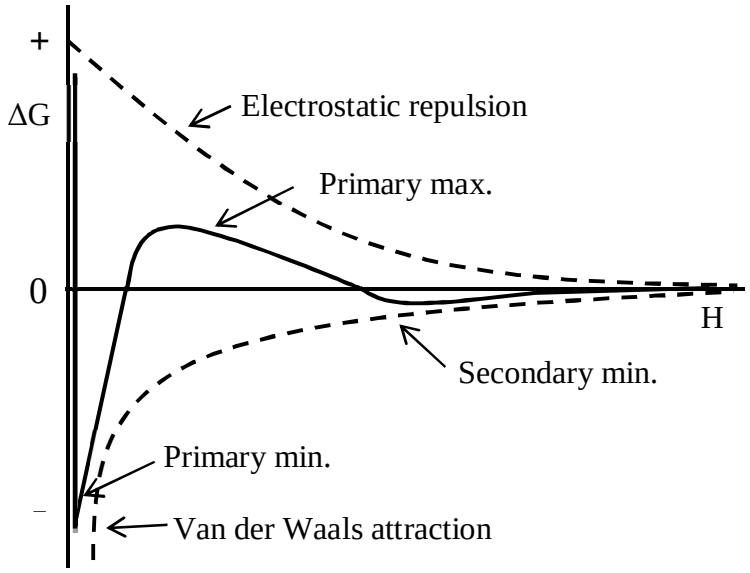

Figure 3 *The curves of van der Waals attraction, electrostatic repulsion and total potential energy against distance of surface separation (H) for interaction between two particles.*

Besides van der Waals attraction and electrostatic repulsion, there are many other non-DLVO surface forces that control the colloidal kinetic stability, such as hydrophobic forces, hydration forces, oscillatory structural forces, neutral polymer-mediated surface forces [68]. For hydration forces, the particle surface groups bind with water molecules and become solvated. Two hydrated surfaces generate the repulsive hydration forces. In an aqueous medium, hydrophobic interactions between hydrophobes are spontaneous. Hydrophobic forces are much stronger than the van der Waals attraction forces and hydrogen bonds [68]. Depending on the particle size, size deviation and surface properties, the importance of different forces can vary. The situation is further complicated, when nanosuspensions are dried for formulation purposes. In nanocrystalline systems the balance between attractive and repulsive forces are adjusted by adding stabilizers on the particle surfaces.

2.2.3.1 Stabilizers

Stabilizer plays a very important role in particle size reduction and stability of nanosuspensions. Stabilizers can spontaneously adsorb on and cover the newly formed particle surface to (a) decrease the free energy of system and interfacial (surface) tension of particles; (b) form a dense hydrophilic layer around hydrophobic particles, provide steric hindrance and steep repulsions between the particles (steric stabilization); (c) charge the particle surface if the stabilizer has ionizable groups, which increase the repulsive force (electrostatic stabilization); (d) combine the steric and

electrostatic stabilization (Fig. 4). The stabilizers, including polymers, surfactants and their combinations, are widely used in the nanosuspension preparation.

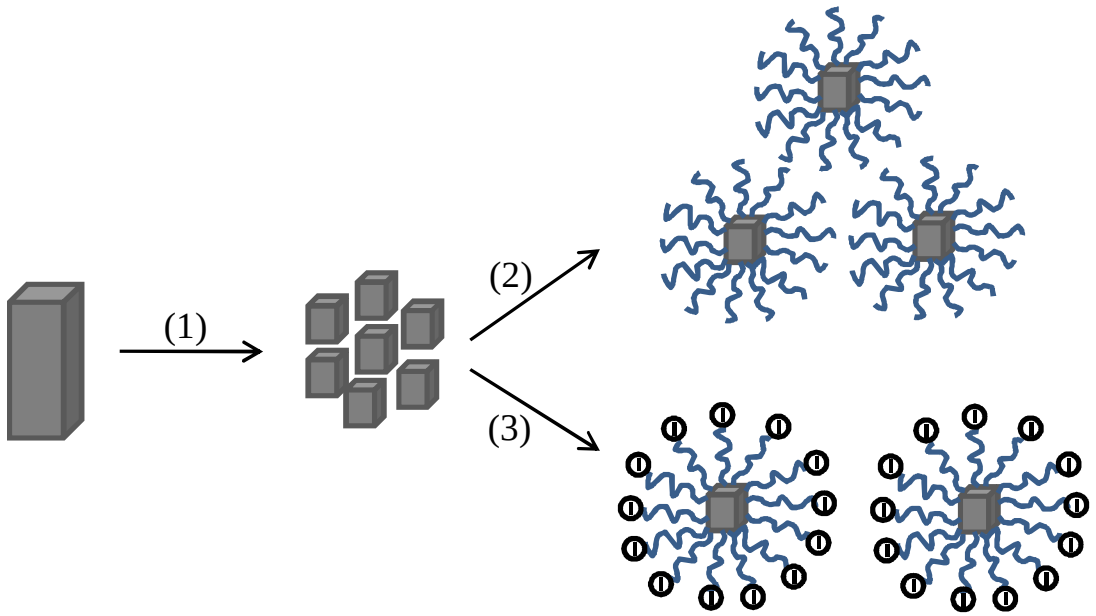

Figure 4 *The mechanism of nanocrystal stabilization during particle reduction (1): steric stabilization (2), and electrostatic stabilization (3).*

Homopolymers used as stabilizers always contain hydrophilic backbone chains, such as polyvidone (PVP), polyvinyl alcohol (PVA), hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC) and methylcellulose (MC). These polymers can adsorb on the particle surface by hydrogen bonds to form a hydrodynamic boundary layer. Douroumis *et al.* [69] screened various stabilizing agents for carbamazepine using the cosolvent technique. Cellulose ethers (HPMC and MC) contain a high degree of substitution as methoxy or hydroxypropoxy groups, which can form hydrogen bonds with the drug and inhibit the crystal growth. The stability of the nanosuspensions is dependent on the degree of substitution. PVP has only one hydrogen bonding carbonyl group per molecule unit, showing a weak crystal inhibition because of less favorable drug-polymer association. Opposite to the above results, Choi *et al.* [70] found out that hydrophobic drug surfaces without polar functional groups are ideal for HPC and PVP to physically adsorb and produce steric stabilization, since the hydrogen bonding between polymer and drug tends to interfere the stabilization activity of polymers.

Amphiphilic block/graft polymers comprising of hydrophobic and hydrophilic chains are more promising stabilizers compared to homopolymers. The hydrophobic segments adsorb on the surfaces of the drug crystals by hydrophobic interactions, while the hydrophilic segments cover the drug particles and stick out into the solution, giving the steric hindrance and preventing particle aggregation and growth. The most common amphiphilic polymers are poloxamer 407 (F127) and poloxamer 188 (F68). Several factors can affect the nanosuspension particle size and stability, such as hydrophilic/hydrophobic ratio, molecular weight, functional groups and morphology of polymers. Lee *et al.* [71] synthesized a series of amphiphilic copolymers with the same hydrophobic component but different hydrophilic components using various amino acids. The particle size of

naproxen after milling indicated that an effective stabilization performance of copolymers required the hydrophobic moiety content to be higher than 15%. The hydrophobic moiety of copolymer is a driving force for polymer diffusion and absorption onto the drug particles. The higher the hydrophobicity of polymer, the faster diffusion, stronger absorption and longer time for desorption of polymer are obtained. Hydrophilic polymers (such as PVP) are worse stabilizers for poorly soluble drugs compared to the amphiphilic polymers and surfactants, because they lack the thermodynamic driving force to adsorb on the hydrophobic drugs [72]. In another publication of Lee *et al.* [73], a series of amino acid copolymers as stabilizers were studied on seven model drugs during the wet milling. When 100% hydrophilic stabilizer polylysine was used, nano-sized particles could still be successfully obtained since the polylysine has a strong hydrogen bonding capability. The authors concluded that the existence of special interactions between the drug and polymer are more important than hydrophobic interactions in nanomilling. Similar to amphiphilic polymers, a surfactant contains a hydrophilic head and hydrophobic tail(s). The surfactants are smaller molecules compared to polymers, and have faster diffusion and adsorption kinetics on the particle surface [72]. Furthermore, a surfactant, like amphiphilic polymers, can reduce the interfacial tension and increase the wettability of nanoparticles.

Ionic polymer or surfactant adsorbed on particle surfaces leads to higher energy barrier by electrostatic repulsive forces and results in good stability. For example, sodium dodecyl sulfate (SDS) attaches to the particles by hydrocarbon tails, and the anionic sulfate groups stay outside (Fig.4). A polymer such as poly (lactic-co-glycolic acid) (PLGA) can shift the particle surface charge to negative values because of the carboxylic acid groups in the polymer structure [74]. Chitosan has a number of free amine groups and can therefore form a positively charged layer on the surface of particles [75]. Dai *et al.* [76] used the ionic interaction between sulfate groups of carrageenan (stabilizer) and oppositely charged drugs to prepare stable nanosuspensions by wet milling. Dextran sulfate having negative charges, acts as a stabilizer during wet milling and is also effective for producing nanosized suspensions.

Combinations of polymer-surfactant or polymer-polymer are considered for synergistic effects for nanosuspension preparation and stability. Bilgili *et al.* [77] checked the role of polymers/ surfactants on breakage rate of the griseofulvin suspensions using the wet media milling. The narrowest particle size distribution and smallest particle size of nanosuspensions were found at the combination of HPC-SDS as stabilizers compared to using HPC alone and SDS alone as stabilizer. Ryde *et al.* [78] invented the combination usage of at least one polymeric stabilizer and anionic surfactant dioctyl sodium sulfosuccinate in nanosuspension production and subsequently drying process. The solid dose nanoparticulate compositions exhibited superior redispersibility after administration to mammals. Lee *et al.* [79] studied the particle sizes of 11 different drugs after nanocomminution in the presence of polymeric stabilizer and ionic surfactant (anionic surfactant SDS and cationic surfactant benzethonium chloride). The results indicated that the addition of surfactant is not always beneficial for particle size reduction. Surfactant addition might hinder the interactions between the polymer and drugs and increase the particle size for some drugs.

Choice of stabilizer type and concentration is critically important for particle size reduction and stabilization of the formulation. Every drug compound has typically its own optimal stabilizer. The stabilizer amount is usually in the range of 1:20 to 20:1 (stabilizer weight: drug weight) [80]. The effect of stabilizer concentration is described in detail in the physical stability part. Having a surface tension value of the polymer close to the one of drug is beneficial for the size reduction. High viscosity of polymers have inherent disadvantages, such as decreasing the diffusion velocity of polymer molecules, slowing down the movement of milling pearls and hindering the energy

delivery, and delaying the preparation procedure [81]. A stabilizer with a low melting point makes problems in manufacturing process with the high temperature, such as spray drying [82]. The toxicity of stabilizers should be considers in different drug delivery routes. For instance, cationic surfactants lead to hemolysis in *i.v.* injection and anionic surfactants are toxic for oral application. Commercialized stabilizers shorten the approval time and decrease the risk of new formulation application rejection, e.g. surfactants of lecithin and Span 85® have been approved for pulmonary inhalation products [83]. Meanwhile some new synthesized polymers or natural stabilizers have been found [84]. Furthermore, stabilizers have other functions in formulations except the stabilization. SDS was reported to open tight junctions and enhance the transport of molecules across epithelial barriers, consequently increasing the drug passage through gastrointestinal membrane and blood-brain barrier (BBB) [85]. Polysorbate 80 can also facilitate the drug to overcome the BBB [86]. The common stabilizers used in nanocrystals are listed in Table 4.

Table 4 *Examples of stabilizers for nanosuspension preparation used in media milling (MM) or high pressure homogenization (HPH) process.*

	Stabilizer	API	Method	Ref.
Homopolymer	PVA	nitrendipine	HPH	[62]
		rutin	HPH	[87]
		ucb-35440-3	HPH	[88]
	PEG	BDD, paclitaxel	MM	[79]
	PVP	naproxen, anthracene	MM	[70]
		indomethacin	HPH	[50]
		glimepiride, BDD, digitoxin, naproxen, paclitaxel	MM	[79]
		halofantrine	MM	[89]
	HPC	ibuprofen, glimepiride, BDD, digitoxin, naproxen, paclitaxel, nifedipin, itraconazole,	MM	[79]
		naproxen, ibuprofen, nifedipin, anthracene, itraconazole	MM	[70]
	HPMC	nabumetone, halofantrine	MM	[89]
		ibuprofen	HPH	[90]
		nitrendipine	HPH	[51]
		indomethacin	HPH	[58]
	Inutec SP1	hesperetin	HPH	[91]
	Tyloxapol	budesonide	HPH	[92]
		brinzolamide	MM	[93]
Copolymer	Poloxamer 188	glimepiride, BDD, digitoxin, naproxen, paclitaxel, nifedipin, itraconazole, hydrocortisone acetate, prednisolone acetate	MM	[79]
		piroxicam	HPH	[52]
		oridonin, diclofenac, omeprazole, asulacrine, revaprazan hydrochloride	HPH	[94-98]
		hydrocortisone, prednisolone, dexamethasone	HPH	[99]
	Poloxamer 407	glimepiride, BDD, digitoxin, naproxen, paclitaxel, itraconazole	HPH	[79]
		danazol, itraconazole	HPH	[100]

		etoposide, paclitaxel	MM	[101]
		simvastatin	HPH	[50]
	Poloxamer 338	probucol	MM	[45]
Non-ionic surfactant	Tween 80	celecoxib	HPH	[82]
		hesperetin	HPH	[91]
		ascorbyl palmitate	HPH	[102]
		rutin	HPH	[87]
		PX-18	HPH	[54]
	Tween 20	folic acid	HPH	[53]
	Vitamin E TPGS	cinnarizine, griseofulvin, indomethacin, itraconazole, loviride, mebendazole, naproxen, phenylbutazone, phenytoin	MM	[103]
		fenofibrate	HPH	[104]
		probucol	MM	[45]
	Plantacare 2000	hesperetin	HPH	[91]
		lutein	HPH	[105]
	Gelucire	probucol	MM	[45]
		itraconazole, febantel	MM	[106]
Anionic	SDS	rutin	HPH	[19, 87]
	Dioctylsulfo-succinate	spironolactone	HPH	[107]
Cationic	Benzethoni-um chloride	glimepiride, nifedipin, hydrocortisone acetate	MM	[79]
Zwitterionic	Lecithin	buparvaquone	HPH	[83]

2.2.3.2 Physical stability

Because of thermodynamic and kinetic instability, drug nanoparticles in the colloidal system tend to agglomerate, aggregate and sediment, which appears in the form of particle size growth and phase separation. The steric or ionic barriers provided by stabilizer are the main forces to prevent the drug nanoparticles from getting into contact with each other. The higher the barrier energy, the more stable nanosuspensions are obtained. For an electrostatically stabilized nanosuspension, a minimum zeta potential of ±30 mV is needed. For a combined electrostatically and sterically stabilized nanosuspensions, a minimum zeta potential of ±20 mV is required [108].

Ostwald ripening is another important factor influencing the physical stability of nanosuspensions. Ostwald ripening is caused by the differences in dissolution pressure/apparent saturation solubility between small and large particles. The smaller particles have a higher solubility compared to the larger particles. Smaller particles are dissolved faster and free molecules diffuse to the larger particles possessing lower drug solubility. A supersaturated environment around the larger particles is formed, which consequently results in the growth of larger particles. To simplify, Ostwald ripening is the process of shrinking of smaller particles and growth of larger particles. There are two preconditions for Ostwald ripening: (i) the particle size in nanosuspensions should not be uniform, i.e. the nanosuspension should be polydisperse and (ii) the dispersed phase (drug nanoparticles) should have finite solubility in the dispersion medium [109]. Pharmaceutical drug

nanoparticles mostly fit these two requirements of Ostwald ripening. Therefore, Ostwald ripening finally leads to the larger average size of nanosuspensions during the storage.

Given the preconditions of Ostwald ripening, homogenous nanosuspensions can effectively decrease the chance of Ostwald ripening; the drug nanocrystals having the lower solubility (e.g. drug crystals) should be more stable than the ones having the higher solubility (e.g. amorphous drug nanosuspensions). Introducing a second component as an inhibitor, which is miscible with the drug compound but essentially insoluble in the continuous phase, to the nanosuspensions can inhibit the Ostwald ripening. For example, the addition of Miglyol has been shown to effectively inhibit the Ostwald ripening of felodipine, nifedipine and bicalutamide amorphous nanosuspensions [110].

The concentration of the stabilizer has a pronounced effect on Ostwald ripening. A low concentration of stabilizer could result in an insufficiently/partially covered particle surface, which cannot impart the repulsive forces to prevent particle aggregation. Meanwhile, more than one drug particle can adsorb on the same polymer chain, which leads to the polymer bridging attraction and thereby to aggregation [111]. A sufficiently high concentration of stabilizer provides a thicker coating layer on the nanocrystals and better long-term stability [112]. However, the high concentration of surfactant/polymer also can enhance the particle growth. Deng *et al.* [113] investigated the effect of stabilizer F127 concentration in the stability of paclitaxel nanocrystals. When the stabilizer concentration was below its critical micelle concentration (CMC), smaller and stable paclitaxel nanocrystals were formed because of the high affinity of F127 on drug particles. When the amount of stabilizer was increased above the CMC, the competition between polymer-polymer and polymer-particles occurred and the polymer-polymer interaction to form micelles was stronger than polymer-particles, which worsened the stability of nanocrystals. The particles tended to aggregate in the higher F127 concentration. In addition, the micelle formation improves the drug solubility and thereby enhances the particle growth by the Ostwald ripening process [28].

Temperature is another important influencing factor in the physical stability of nanosuspensions. Higher temperature can increase the saturation solubility of drug, hydrophobic interactions between particles and the dehydration of polymer. Therefore, the nanoparticles tend to aggregate or grow in storage conditions with higher temperature [109, 113]. Polymorphic transitions during the storage should be considered as another instability factor.

Energy-addition, such as ultrasonication after the precipitation, and HPH after the precipitation, can enhance the stability. Xia *et al*. [30] combined the precipitation-ultrasonication to prepare the stable nitrendipine nanosuspensions. The results showed that the particle size of non-sonicated nanosuspensions increased in 24 h, while the sonicated sample was stable for 6 months. Sonication is a kind of annealing step, which converts the thermodynamically unstable regions into a more stable form. The sonication reorders the surfactant molecules and prompts the surfactant absorption rate on the particle surface as well as disrupts the crystal agglomeration. Meanwhile, the sonication energy input transfers the instable polymorph form to stable crystal form and changes the crystal habit [29, 30]. However, if the energy input goes over a critical value, the particles will collide and agglomerate because of the extra kinetic energy [28, 29]. In addition, transferring the suspensions to dried solid state is another approach to improve the physical stability of nanosuspensions [103].

Under the critically optimal storage conditions, nanosuspensions exhibit a long-term stability. The commercialized Megace® ES nanoparticle dispersion formulation of megesterol acetate shows a 2 year shelf life in an ambient temperature. It was reported by Merisko-Liversidge *et al.* [80] that the drug nanosuspensions prepared in their laboratory exhibited excellent physical stability for 5 years at 5 ^{o}C, which provided the sufficient time to prepare a formulation, perform preclinical testing and a good shelf life.

2.2.3.3 Chemical stability

Many drugs are susceptible to decomposition or degradation during manufacture, package and storage, depending on environmental factors, such as temperature, pH, ionic strength, humidity, oxygen and light [66]. Such decomposition causes a loss of potency of the drug, difficulties in predicting the administration dosage, changes in physical appearance of dosage form (such as discoloration), and can even generate the undesirable and toxic chemical agents.

Formulating a chemical compounds into nanoparticulate compositions is a promising way to decrease the degradation and improve the chemical stability [114]. For a drug solution, drug molecules dissolve in the medium, which leads easily to degradation by oxidation or hydrolysis. For nanocrystals, chemical reactions take place just on the crystal surface, and the degraded outer monolayer can form a protecting shell to shield the inner core of crystals [115]. Besides the crystal structure, the stabilizer layer around the particle surface can also form a relatively closed micro-environment to reduce the contact of the drug with the outer water environment and decrease the chance of degradation. In the presence of a surfactant polysorbate 80, a 7-fold increase in the stability of indomethacin has been reported [116]. Pu *et al.* [61] reported that 90% of 10-hydroxycamptothecin (10-HCPT) in the lactone form was preserved in the nanosuspension formulation at physiological pH values after 24 h incubation, while less than 30% of 10-HCPT in the solution was left in the same conditions. An increased chemical stability of nanosuspension compared to solutions was also seen in other publications [96, 117].

Converting a drug solution or nanosuspensions to dried solid particles is another promising way to increase the chemical stability and decrease hydrolysis, which is the main problem for liquid formulations. The drying methods and advantages of nanopowders are presented in the next chapter (2.3.1).

2.3 Pharmaceutical application of nanosuspensions

Conversion of liquid nanosuspensions into solid dosage forms, such as tablets, capsules, pellets and granules, can improve the commercialization, allow better handling and avoid the potential physical instability (agglomeration and Ostwald ripening) and chemical instability (e.g. hydrolysis, oxidation).

2.3.1 Solidification of nanosuspensions

There are two different types of drying technologies to solidify nanosuspensions. First method is drying the nanosuspension to a powder using lyophilisation, spray drying, or oven drying, and then further formulating the powder into e.g. tablets and capsules with other excipients, such as dispersants, disintegrates and so on. The second solidification method, including granulation and pelletization, is the combination of drying and shaping processes together. The nanosuspensions are used as a granulating liquid in the granulation process or as layering dispersion in a fluidized bed process [118, 119].

During the drying process the water is removed from the nanocrystals suspensions by evaporation or sublimation and the motions of stabilizer chains around the nanocrystals are decreased because of the dehydration or possible partial crystallization. The steric repulsion

supplied by the stabilizer ceases and then the polymer chains adsorbed on the particles can entangle each other, which hinder particle separation and prompts particle aggregation. In addition, the contact points among nanocrystals are increased with water removal. Under mechanical stresses (such as crystallization of ice, the stress of freezing and dehydration) and additional thermal stresses occurring in the drying process, nanocrystals are subject to reversible agglomeration or irreversible aggregation/fusion. The agglomeration/aggregation can compromise the advantages of nanocrystals based on the large surface area, e.g. decreased dissolution rate, the lag time in the beginning of the dissolution, and unpredictable variations in bioavailability [120]. Therefore, re-dispersibility of dried nanopowders in the dissolution medium with a short time (a few minutes) is an important standard to evaluate the drying step.

The parameters of the drying procedure, the properties of drug compounds, the choice of dispersed matrix and stabilizer can affect the nanocrystal re-dispersibility. In freeze drying process, fast freezing and low API concentration are beneficial for re-dispersibility [121]. In spray drying process, high gas flow rate and feed temperature lead to formation of donut shaped particles [122]. Spray drying at high inlet temperature causes powders with higher lag time as well as lower dissolution rates [120]. Eerdenbrugh *et al.* [103] showed that regardless of the drying methods or other properties of drug compounds, such as molecular weight, melting point, solubility and density, the hydrophobicity of drug surface, which in general corresponds to log P values (partition coefficient) of the drug, is a key parameter for nanocrystal re-dispersibility. More hydrophobic compounds resulted in harder–to–disintegrate agglomeration. Chaubal *et al.* [120] showed that using a charged surfactant as a stabilizer and sugar as excipient can effectively prevent irreversible aggregation compared to non-charged polymeric surfactants and sugars. Excipients are usually added during the freeze drying process to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) [123]. The commonly used excipients include water-soluble sugars such as mannitol [124, 125], sucrose [126], lactose [125], trehalose [102, 127] and water-insoluble microcrystalline cellulose (MCC) as a matrix-former [128] to protect the nanocrystals from aggregation. The results showed that the reconstituted nanocrystals dried with excipients had smaller mean particle size than the ones dried without added lyoprotectant. The preferable amount of cryoprotectant used is about 2%-25%, based on the total weight of the nanoparticulate suspension [129]. Sometimes, dried powders without any excipients exhibit good re-dispersibility since stabilizers used in nanocrystal production act lyo/cryprotectant in a certain extent.

2.3.2 Drug delivery routes

2.3.2.1 Oral delivery

Oral dosage forms are usually the first choice for drug delivery due to their convenience, good patient compliance and low production costs. The efficacy of the drug by oral administration is dependent on the solubility and absorption in the gastrointestinal tract. Poor solubility and low dissolution rate of the drug in human GI lumen can lead to a large amount of undissolved drug escaping from the adsorption window and therefore poor bioavailability. To reach the therapeutically relevant drug concentrations, a high drug dose is required, which can cause undesirable side effects to the normal tissue, poor patient compliance and costly therapy. The nanocrystals can effectively improve bioavailability. Using nanocrystals can lead to e.g. higher

maximum peak concentration of the drug in plasma (C_{max}) and faster onset of action, faster dissolution in the narrow absorption window, reduced fast/fed variation, improved dose proportionality and reduced inter-subject variability, which are described in detail below. For oral administration of nanocrystals, they can be formulated into tablets, grain-filled capsules, suspension-filled capsules, pellets and oral suspensions.

The C_{max} and oral bioavailability of the drug can be improved by the particle size reduction. There are two main reasons. Firstly, decreasing the particle size increases the surface area-to-volume ratio. According to the Noyes–Whitney and Ostwald–Freundlich's equations, the dissolution velocity and apparent saturation solubility are increased. The high drug concentration gradient between the gastrointestinal contents and blood enhances the drug absorption. Secondly, the bioadhesion property of nanoparticles to gastrointestinal mucosa prolongs the retention time, decreases diffusion distance, and increases local drug concentrations. Xia *et al.* [25] prepared five different particle sizes of nitrendipine nanosuspensions using precipitation-ultrasonication method and administrated the nanosuspensions to rats orally. The dissolution testing and simulated results having good agreements showed that the simulated dissolution rate at $T_{50\%}$ (50% dissolution time) of nitrendipine nanocrystals with particle sizes of 200 nm, 620 nm, 2.7 μm, 4.1 μm and 20.2 μm were calculated to be 5.1×10^4-, 1×10^4-, 237-, 64- and 11-fold greater than that of the raw crystals, respectively. The *in vivo* testing showed that the absolute bioavailability of five samples were 61.4, 51.5, 29.4, 26.7 and 24.7 %, respectively. The absolute bioavailability of raw crystals was only 9.9%. In addition, a good linear relationship was observed between the Log ($T_{50\%}$) and the absolute bioavailability. Liversidge *et al*. [130] compared the pharmacokinetic parameters between conventional suspensions (particle size of 10 μm) and nanoparticle dispersion (169 nm) prepared by a ball milling process. The results showed that the C_{max} and oral absolute bioavailability of conventional suspensions of danazol in dogs were 0.2 μg/ml and 5.1%, while for nanosuspensions they were 3.94 μg/ml and 82.3%. Quan *et al.* [62] prepared nitrendipine nanosuspensions using precipitation-homogenization process and converted the nanosuspensions to a solid form using spray drying. The results of *in vivo* testing indicated that the C_{max} of the nanocrystals was about 15-fold and 10-fold greater than that of physical mixture and commercial tablet, respectively. Meanwhile, The AUC (Area under the plasma concentration time curve) of the nanocrystals was about 41-fold and 10-fold greater than that of physical mixture and commercial tablet, respectively. Improved bioavailability with nanocrystal formulations can be found in a number of other references as well [131]. The improved bioavailability leads to a decrease in the administrated dose and times, decreased side effect and is good for patient convenience.

A faster onset of action (shorter T_{max} (The time that C_{max} happens)) can be obtained by nanoformulations compared to the microparticles. Li *et al.* [98] prepared revaprazan hydrochloride nanosuspensions using high pressure homogenization and researched the *in vitro/in vivo* evaluations of drug nanosuspension (particle size of 562 nm), microsuspension (1.5 μm) and coarse suspension (2 μm). The T_{max} of nano-, micro- and coarse suspensions were 45, 360 and 230 min, respectively. Ravichanran [132] compared 3 kinds of lyophilized albendazole nanosuspensions formulations with a slightly varied particle sizes but very much lower size than the control (an unmilled albendazole sample). The T_{max} of sample-control was 5.95 h, but the T_{max} of the three nanoformulations were 4.63, 3.80 and 3.67 h, respectively.

Food affects the drug absorption and consequently alters the pharmacokinetic and pharmacodynamic profiles of drugs [133]. Nanoformulations can effectively reduce the fed/fasted state variability since the similar rapid dissolution was obtained in fasted/fed conditions and the dissolution/absorption become less dependent on the diet and physiological state [134]. Jinno *et al.*

[135] investigated the absolute bioavailability of wet-milled cilostazol nanocrystals tablet in beagle dogs in fasted and fed conditions. The commercial tablet provided 5% bioavailability of cilostazol in the fasted state and 20% in the fed state, while wet milled nanocrystals in a tablet formulation exhibited 64% bioavailability in the fasted state and 69% in the fed state. The cilostazol nanocrystals not only remarkably enhanced the bioavailability, but also eliminated the food effect on absorption. Shono *et al.* [136] used an *in silico* simulation technology to forecast *in vivo* oral absorption of micronized and nanosized aprepitant formulations in the pre- and post- prandial states. The simulated plasma profiles generated for a dose of 125 mg aprepitant having various particle sizes demonstrated that the ratio of C_{max} and AUC in fed/fasted states decreased with decreasing particle size, meaning that the food effect on absorption of aprepitant was diminished with the decreasing particle size. The simulated results were in good agreement with the available *in vivo* data in humans.

The gastric irritancy can be eliminated with the decreasing particle size of the drugs. Liversidge *et al.* [46] reduced the particle size of naproxen material from 20 µm to 270 nm using a roller mill. The results of stomach irritations indicated that the milled naproxen formulation had significantly lower irritation scores than the unmilled naproxen. No significant difference was found in stomach irritation following administration of nanoparticle naproxen comparing the oral and intravenous routes.

There are other effects of nanoformulations on drug pharmacokinetics and therapeutic efficacy. The fast dissolution can overcome the challenges of a narrow absorption window [134], improve dose proportionality and reduce inter-subject variability [65]. The bioadhesion property of nanoparticles can not only improve the bioavailability, but also reduce the variability of absorption and can be used for targeted drug delivery in the GIT [24, 137]. The efficiency of mucoadhesion process depends on the surface properties of the particles and the polymer [20]. A controlled release pellet formulation was developed using the spray dried nanocrystal nanosuspensions dispersed in the hydrophobic matrix pellets [138].

2.3.2.2 Ophthalmic delivery

There are three main routes for drug delivery to the eye: topical, systemic and intraocular injection. Nearly 90% of ophthalmic products on the market are in the form of eye drops for topical administration, including solutions, ointments and suspensions, which mainly target the anterior segment eye diseases [139].

Ocular drug delivery has remained as one of the most challenging research topics, because of the unique structure and effective removal mechanisms of the eye (keep the ocular surface free from foreign substances). The fate of instilled dose can be seen in Fig.5. Firstly, The cul-de-sac of the eye normally holds about 7-9 µl of tear fluid but can retain up to 20-30 µl of liquid if care is taken not to blink [140]. However, the volume of a drop delivered from normal commercial eye dropper is approximately 25-56 µl (average 39 µl) [141]. The excess volume of instillation into the lower cul-de-sac will be spilled out of the eye. Also, the drops can be drained from the eye by various mechanisms, such as blink reflex and rapid tear secretion after irritation. The human tear flow rate is 1 µl/min under resting conditions [142]. It was reported that an eye-drop is often eliminated quickly within 5-6 minutes after administration [142] and has a short residence time of about 2 minutes in the tear film [143]. Secondly, physiological factors can affect the ocular delivery of topical drugs, such as protein binding and drug metabolism by the enzymes in tear fluid. Thirdly,

the special structure of the eye acts as a physiological barrier. The cornea comprising of epithelium, stroma and endothelium, acts as a protective barrier to prevent the drug absorption into the eye [144]. Finally, the drops can go through the nasolacrimal duct into the nasal cavity and then down to the throat and gastrointestinal tract and ultimately enter the systemic absorption. The absorption of drops by conjunctiva and sclera will also enter the blood circulation. More than 75% of applied ophthalmic solution is lost or absorbed systemically by nasolachrymal drainage and conjunctiva [145], which cause undesirable systemic side effects, e.g. timolol may cause severe (up to patient death) systemic side effects in the form of reduced heart rate and blood pressure [146]. Because of the rapid precorneal drug elimination and corneal epithelial barrier, less than 5% of the drug dose can penetrate through the cornea and reach intraocular tissues [147].

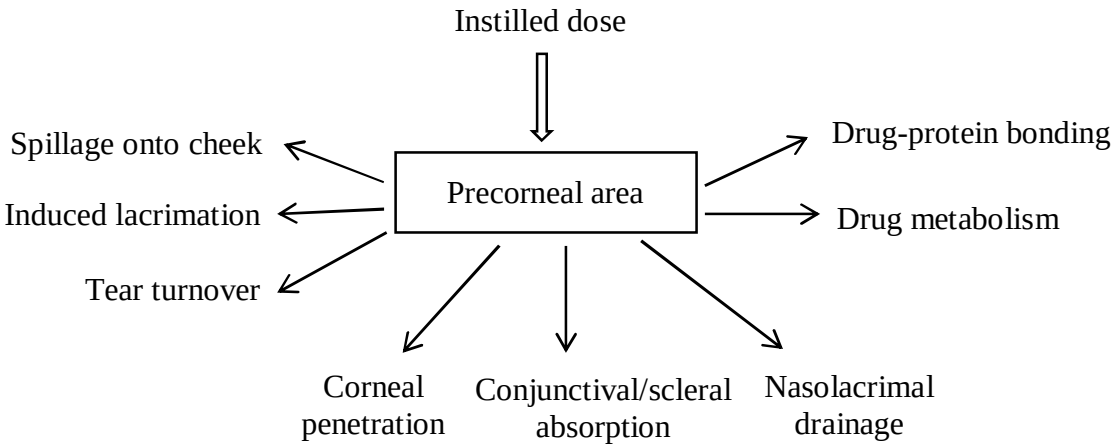

Figure 5 *Fate of ophthalmic drug delivery systems (modified after reference [144]).*

The poor ocular bioavailability of eye drops leads to problems in drug delivery. A large ophthalmic dose is needed to reach the effective therapeutic concentration of the drug, which results in poor compliance and, potentially, serious systemic toxicity. Another problem for ophthalmic delivery is a short duration of action for some drugs. It was reported that the duration of lowering of intraocular pressure caused by pilocarpine lasted only about 3 hours. As a consequence, a high administration frequency of 3-6 times/day is required which results in undesirable side effects such as myopia and miosis [148].

To overcome the disadvantage of conventional eye drops, novel ocular drug delivery systems have been developed in the past several decades, such as prodrugs, cyclodextrins, microemulsions [149, 150] colloidal drug delivery systems (nanoparticles [151], nanosuspensions, micelles [152] and liposomes), intraocular implants [153], soft contact lenses [154, 155], hydrogel systems, ocular iontophoresis. The basic ideas of these novel drug delivery systems are prolonging the precorneal residence time, enhancing corneal drug penetration and achieving a sustained drug release to finally improve the duration of action and bioavailability. To prolong the precorneal drug residence time, viscosity enhancers such as viscosity-increasing polymers [156], mucoadhesive agents [157], *in situ* gels and soft contact lenses can be used. Drug carriers with positively charged surfaces are preferentially captured at the cornea, since corneal surface has a negative charge [158]. To enhance the drug corneal penetration, penetration enhancers, such as cetylpyridinium chloride, Tween 20, Brij® 35, among others, have been used to increase the permeability of the corneal epithelial

membrane [159]. The formation of prodrug obtained by chemical modification of the active agents is another approach to enhance the partitioning in corneal. For example, changing the epinephrine (EPI) to a more lipophilic compound dipivalyl epinephrine (DPE), the partition coefficients of DPE is 100-600 times higher than that of EPI [160], since the epithelium of the cornea are rich in lipid content. Transporter-targeted prodrug is another way to improve the absorption and bioavailability [161]. For sustained drug release, gels, nanoparticle-loaded contact lenses and intraocular implants have been widely researched [153, 162, 163].

With the development of the nanocrystal technique, nanosuspensions have been researched and formulated in the form of eye drops. Drugs with poor solubility in aqueous or organic solvent can be transferred into nanosuspensions with a high dissolution rate and apparent saturation solubility, which supply a higher concentration gradient in precornea and a higher drug flux through the cornea. The nano-scale particle size and a narrow size range ensure low irritation of the eye. Particle size in an eye drop must be less than 10 µm to avoid the irritation of the eye surface [156]. Moreover, nanosuspensions are more comfortable for the eye since less drug is in the solution [164]. Nanoparticles show a higher bioadhesion [41, 165] which prolongs the residence time of nanoparticles in the eye and thus provide a longer time for the dissolution in tears. On the other hand, (nano-sized) particles tend to retain in the cul-de-sac [166] and act as a reservoir for sustained release of the drug, which can extend the duration of drug action and decrease the administration frequency [36]. The importance of particle size in ophthalmic bioavailability was investigated [167] and nanosuspensions were shown to improve the ophthalmic bioavailability [168]. In addition, the mild pH range of 3.5-9 (prefer as close to physiological pH as possible) and simple formulation of nanosuspensions avoid the pH-induced or excipient-induced irritation, lacrimation and discomfort.

Ali *et al.* [36] prepared hydrocortisone nanosuspensions using microfluidic nanoprecipitation and wet milling for ophthalmic delivery. The results of tests *in vivo* indicated that the nanosuspensions had an onset of drug action as fast as the drug solution, but a longer duration of drug action and higher AUC value. Kassem *et al.* [99] reported that nanosuspensions allowed lower doses and less frequent instillation in ophthalmic drug delivery, since the extended duration of drug action and higher bioavailability were found in nanosuspensions compared to the solution. Moreover, the maximum percentage of increase in intraocular pressure (IOP_{max}) is dependent on the particle size of nanosuspension, e.g. dexamethasone micro- and nanosuspensions with the particle sizes of 930 nm, 2.46 µm and 4.89 µm exhibited the significantly different IOP_{max} values 24.97, 19.95 and 17.35%, respectively.

The drug of particular interest in this thesis was brinzolamide. It was first marketed in 1998 with the trade name Azopt® by Alcon Inc. using the nanocrystal technique for the treatment of open-angle glaucoma and ocular hypertension [169]. Many comparisons of IOP-lowering efficacy, safety and tolerability were done between brinzolamide 1.0% suspension (Azopt®) and dorzolamide 2.0% solution (Trusopt®) [169, 170]. The data [171] collected from patients showed that brinzolamide 1.0% (dosed two times a day) and dorzolamide 2.0% (dosed three times a day) equaled each other in IOP-lowering efficacy and brinzolamide was significantly more comfortable than dorzolamide, which generated ocular burning and irritation.

2.3.2.3 Parenteral/Intravenous administration

Water insolubility of the drug compound has always been a challenge in parenteral administration. To solubilize the drug, surfactants (e.g. polysorbates), cosolvents (e.g. ethanol, glycerin and

propylene glycol), emulsions, nonaqueous vehicles (e.g. sesame oil) and cyclodextrin inclusion complexes can be used [172]. However, large quantities of solubilizing excipients are associated with undesirable adverse effects. For example, Cremophor® EL (polyethoxylated castor oil) in Taxol® is added to enhance the solubility of anti-cancer drug paclitaxel. The surfactant of Cremophor EL causes hypersensitivity reactions in certain patients [173]. Adjusting the pH of dissolved medium to solubilize the ionic API can lead to a local tissue irritation, necrosis and possible chemical instability of the drug. Compared to the drug solutions, nanocrystals are a smart approach exhibiting many advantages for *i.v.* administration. The small amount of excipients causes only limited intravenous irritation [59] and thus they are well tolerated at higher doses [101, 174]. The high drug loading makes the drug administration possible with a high dose but relatively small injection volume.

Drug nanoparticles also show potential for targeted drug delivery, sustained release and improved drug efficacy. Once nanosuspensions are intravenously injected, the fate of nanocrystals is controlled by the particle size, surface properties (such as zeta potential, hydrophilic/hydrophobic ratio) and particle shape. Smaller particles will be rapidly dissolved in blood stream and the pharmacokinetics are similar to the drug solution, while larger particles with lower dissolution velocity will circulate in bloodstream, much like with any other particulate drug carriers (e.g. polymeric nanoparticles) [175, 176]. Particles will be taken up by mononuclear phagocyte system (MPS) as foreign matter, typically within 5 min [177] to a few hours [178] depending on the particle size and composition. This clearance process can be considered as a passive targeting to macrophages or tissues abundant in macrophages, such as liver, spleen and lungs [179]. Meanwhile, after the uptake of phagocytic cells of MPS, drug molecules can be dissolved in phagolysosomes, released from phagocytic cell and then enter the blood circulation again. This phenomenon maintains the drug blood concentration for a longer duration [97].

On the other hand, nanocrystals coated with hydrophilic layers and neutral zeta potential surfaces can reduce the opsonizing effect, avoid clearance by MPS and obtain a long circulation time in blood (stealth property), which provide efficient delivery time of nanocrystals to the target tissue [180]. The nanoparticles can be passively targeted and accumulated into solid tumor because of the enhanced permeability and retention (EPR) effect in tumor site [181, 182]. Active targeting can be achieved by modifying nanocrystal surfaces with targeting molecules, such as antibody, peptide, small molecule ligand and oligonucleotide [183]. Shegokar *et al.* [184] modified a nevirapine nanocrystal surface with serum albumin, dextran and polyethylene glycol to target macrophages. The cellular uptake studies showed decreased cellular uptake for PEGylated nanosuspension because of its hydrophilic protective layer. Dextran and serum albumin modified nanocrystals showed higher intracellular content of nevirapine compared to the un-modified nanocrystals due to the specific receptor-mediated macrophage endocytosis. The results of gamma imaging demonstrated that surface modified nanosuspensions showed comparable accumulation in MPS organs, especially in liver and spleen as compared to drug solution and un-modified particles since the modified nanocrystals with the larger particle size can be easily phagocytosed by macrophages. Through surface modifications of nanocrystals, drug target accumulation and bioavailability were enhanced, and the residence of the drug at the target site was prolonged.

2.3.2.4 Other delivery routes

Pulmonary administration is a non-invasive and promising drug delivery route for the treatment of local lung diseases or systemic circulations for non-respiratory diseases (especially for peptides and proteins) since lungs have an enormous surface area, a relatively low enzymatic activity and good blood supply. Aerosol particle size is one of the important influencing factors for dose deposition, distribution and therapy efficacy within the lungs. Aerosol particles > 10 μm are most likely deposited in the mouth and throat; 5-10 μm are mainly deposited in the upper airways or oropharyngeal region; Particles with a diameter of 1-5 μm are deposited in the small airways and alveolar region [185]. Currently, micron-sized inhalation powders are prepared by jet mill, which produces particles with a wide particle size distribution. Large particle size with a wide particle size distribution in inhalation formulation has certain disadvantages, such as low solubility and dissolution rate, unwanted deposition in pharynx and mouth rather than lungs, and quick clearance from the lungs by ciliary movements [186]. Nanosuspension is a versatile formulation and can be nebulized using commercially available nebulizers. The adhesiveness property of nanocrystals to mucosal surface prolongs the drug retention and release time. Nanoparticles exhibit more reliable delivery to the lungs since homogenous nanosuspensions contain more particles in each aerosol droplet than micro-suspensions and the drug content in each droplet will be more consistent [92]. The nanoparticles require smaller aqueous droplets generated by nebulizers, and the smaller droplets are important as they can carry the drug to the smaller airways, especially for young children [187]. With the same nebulizer (a Pari LC Jet nebulizer), ultra compressor and formulation volume, a significantly shorter nebulization time was found for nanobudesonide (with the size range of 75-300 nm) than Pulmicort Respules® (4400 nm), which improves the patient compliance [188]. Compared to a conventional formulation, a quick onset of action and higher therapeutic drug concentration of nanosuspensions were found, contributing to rapid diffusion, dissolution and adsorption of the nanosized nanocrystals [187-189]. Zhang *et al.* [189] prepared a baicalein nanocrystal dispersion using a combination method of precipitation and high pressure homogenization. The *in vivo* bioavailability study indicated that the pulmonary baicalein nanocrystals had rapid absorption and almost identical pharmacokinetic parameters to intravenous baicalein injection.

Skin as the largest organ provides huge opportunities for drug delivery. The application of nanocrystals for dermal delivery was started in 2006 [190]. There are two cosmetic products on the market which contain nanocrystals. The first one contains rutin nanocrystal for age-decoder face cream and fluid and has been on the market since 2007 (Juvedical by Juvena). Another one contains hesperidin nanocrystals developed by Platinum Rare in 2009 [65]. The fast dissolution, high solubility, adhesiveness and sustained release properties of nanocrystals are still working in transdermal drug delivery system [191]. Simple formulation of nanocrystals avoiding the solubilization agent and organic solvent decreases the skin irritation. Venkataraman *et al.* [192] prepared silver sulfadiazine (SSD) nanosuspensions with a particle size of 367 nm using microprecipitation-high-pressure-homogenization technique and further formulated the nanosuspensions in gel matrix (nanogel) for infection in burn therapy. Bacterial inhibition studies showed that the bacterial inhibitory efficiency of SSD nanosuspension was as good as that of SSD solution against bacteria. The results of treating hot water-induced burn wounds in rats revealed that a nanogel containing 0.5% SSD was more effective in wound healing compared to 0.5% and 1% marketed cream, since the small size and large surface area of released nano silver promote closer interaction with bacteria.

2.3.3 Nanocrystals on the market

It was reported that approximate 28 nano-drugs are on the market by 2009. These products include liposome based formulations, polymeric based (PEG) nanodrugs, nanocrystals and albumin-bound platform [193]. With the drug nanocrystals invented at the beginning of the 1990s, drug nanocrystals have been developed in pharmaceutical industry very fast during the last twenty years based on their outstanding advantages. Until now, approximately 20 nanocrystal drug products have been launched on the market (Table 5) and more than twenty products are currently under clinical trials.

Among these nanocrystal products, almost all are prepared using the wet pearl milling technology. Rapamune® was marketed as a nanocrystal product in 2000 by Wyeth Pharmaceuticals. The drug nanocrystals are released from the tablets and from nanosuspension. No crystal aggregation takes place during the tablet production because of the very low drug nanocrystal percentage in the whole tablet (1 mg nanocrystals in 365 mg of the total tablet weight and 2 mg of nanocrystals in a 370 mg tablet). In comparison to the oral solution, nanocrystals have enhanced bioavailability (21% higher) and are user friendly. Emend® was introduced to the market in 2003. Aprepitant having a narrow absorption window is only absorbed in the upper gastrointestinal tract. The nanosized aprepitant reached improved bioavailability because of the high solubility and dissolution rate. Tricor® and Triglide® have the same API (fenofibrate) but are made by different preparation methods. The bioavailabilities of Tricor® and Triglide® are independent on the fed/fasted state since the dissolution rate of nanocrystals in water (fasted state) is as high as in fats/oils (fed state). In addition, the adhesiveness of nanocrystals to the gut wall avoids the effects of fed/fasted condition. For Megace ES®, the volume of a single dose is decreased by 4 times compared to the oral solution. Furthermore, the bioavailability variation of Megace ES® is decreased and the viscosity of oral suspensions is decreased to increase patient compliance [16].

Table 5 *Examples of nanocrystal products on the market (modified after the reference [118]). MM and HPH stand for media milling and high-pressure homogenization, respectively.*

Product/Company	Drug	Indication	Technology	Route	Approval date
Gris-Peg®/Novartis	Griseofulvin	Anti-fungal	Precipitation	Oral	1982
Cesamet®/Lilly	Nabilone	Anti-emetic	Precipitation	Oral	2005
Verelan PM®/Schwarz Pharma	Verapamil	Anti-arrhythmia	MM	Oral	1998
Azopt®/Alcon	Brinzolamide	Glaucoma	MM	Eye drops	1998
Rapamune®/Wyeth	Sirolimus	Immunosuppressant	MM	Oral	2000
Focalin XR®/Novartis	Dexmethyl-phenidate HCl	Anti-psychotic	MM	Oral	2001
Avinza®/King Pharm	Morphine sulfate	Anti-chronic pain	MM	Oral	2002
Skelaxin/King Pharm	Metaxolone	Skeletal-muscular pain	MM	Oral	2002
Ritalin LA®/Novartis	Methylphenidate hydrochloride	Anti-psychotic	MM	Oral	2002
Herbesser®/Mitsubishi Tanabe Pharma	Diltiazem	Anti-angina	MM	Oral	2002
Zanaflex™/Acorda	Tizanidine hydrochloride	Muscle relaxant	MM	Oral	2002
Emend®/Merck	Aprepitant	Anti-emetic	MM	Oral	2003
Tricor®/Abbott	Fenofibrate	Hypercholesterolemia	MM	Oral	2004
Megace® ES/Par Pharma	Megestrol acetate	Appetite stimulant	MM	Oral	2005
Naprelan®/Wyeth	Naproxen sodium	Anti-inflammation	MM	Oral	2006
Theodur®/Mitsubishi Tanabe Pharma	Theophylline	Bronchial dilation	MM	Oral	2008
Tridlide®/Skye Pharma	Fenofibrate	Hypercholesterolemia	HPH	Oral	2005
Invega Sustenna/J & J	Paliperidone palmitate	Anti-depressant	HPH	Injection	2009
Zyprexa® Relprevv™/Lilly	Olanzapine	Schizophrenia	MM	Injection	2009
Xeplion®/J & J	Paliperidone palmitate	Schizophrenia	MM	Injection	2011

3 Aims of the study

The aim of the thesis project was to optimize the preparation (wet media milling technique) and characterization methods of nanosuspensions for poorly water-soluble drug compounds, then formulate the nanosuspensions into suitable pharmaceutical dosage forms, and finally test the efficacy in *in vivo*.

The more specific aims of the present study were:

1. In order to have the smallest particle size, most homogeneous and stable nanosuspensions, the most critical process parameters such as milling time, milling pearl diameter, rotational speed, stabilizer type and concentration were studied with model drugs **(I, III, IV)**.

2. In order to determine the importance of particle size on dissolution, the discriminating dissolution conditions for poorly water-soluble drug nanosuspensions using simulation and experimental methods were developed **(III)**.

3. To set up a "nanos-in-micros" structure for suitable pharmaceutical formulation, e.g. tablet or pulmonary products. This structure avoids problems in further formulation of nanopowders, meanwhile functionality of individual nanoparticles are remained though particles are micron sized **(II)**.

4. To test the efficacy of nanocrystal formulations *in vivo*. Brinzolamide nanosuspensions for ocular administration were formulated in order to reduce the intraocular pressure in rats **(IV)**.

4 Experimental part

4.1 Materials

All chemicals used in the studies were obtained from standard sources and were used without further purification. In all experiments water was ultrapurified Millipore® water (Millipore, Molsheim, France). The main materials including model drug compounds and stabilizers can be seen below.

4.1.1 Model drug compounds

Three poorly water-soluble drug compounds were chosen as model drugs in the studies. The most important physicochemical properties are given in Table 6.

Table 6 *List of the model drug compounds, their basic properties and corresponding publications.*

API	Mw (g/mol)	pKa	Solubility	Reference	Used in
Indomethacin (IND)	357.8	4.5	14 µg/ml (pH 5)	[194]	**I, II, III**
Itraconazole (ITR)	705.6	3.7	< 1 µg/ml	[195]	**I**
Brinzolamide (BRA)	383.5	5.9 & 8.4	0.9% (w/v) (pH 5) 0.05% (w/v) (pH 7.4)	[164]	**IV**

4.1.2 Stabilizers

Five different stabilizers were chosen for the milling process. Poloxamer 188 (Pluronic® F68) **(I-IV)** and poloxamers 407 (Pluronic® F127) **(I, IV)** were obtained from BASF Co. (Ludwigshafen, Germany). Polysorbate 80 (Tween® 80) **(I, IV)** was from Fluka Chemika (Buch, Switzerland). Polyethylene glycol (PEG) of M_w 6000 g/mol was from Sigma (St. Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC) (Methocel®, E5 Premium LV EP) **(IV)** was from Dow Chemical Company (Michigan, USA).

4.2 Preparation of nanosuspensions (I-IV)

Nanosuspensions were prepared using the wet pearl milling technique. The stabilizer was dissolved in milling medium to form a stabilizer solution. A certain amount of drug compound was dispersed in the stabilizer solution. Then the drug dispersion was poured into a milling vessel containing milling pearls (zirconium oxide). Two milling vessels having an identical weight or one milling vessel with its weight counter were fixed in a milling machine (Pulverisette 7 Premium, Fritsch Co., Idar-Oberstein, Germany). The maximum pearl amount and maximum milling speed were used

depending on the size of the milling pearls. For milling pearls with the diameters of 1, 5 and 10 mm, the maximum speeds were 1100, 1000 and 850 rpm, respectively. One milling cycle was set to 3 minutes and there was a 15 min pause between each milling cycle for cooling down the milling vessels. Nanosuspensions were obtained after grinding and separated from the milling pearls by pipetting. Information about the amount of drug and stabilizer, and milling parameters can be seen in Table 7.

Table 7 *Detailed information about the amount of API and stabilizer, and milling parameters used in the process of nanosuspension preparation.*

API and amount (g)	Stabilizer type and amount (g)	Milling parameters					Used in
		Medium and volume (ml)	Vessel volume (ml)	Ball size (mm)	Ball amount	Number of milling cycle	
IND (2)	PEG, Tween 80, F68, F127 (0.2, 0.5, 1.2, 1.6)	Water (10)	45	1	70 g	2, 6 and 10	**I**
ITR (2)	PEG, Tween 80, F68, F127 (0.2, 0.5, 1.2, 1.6)	Water (10)	45	1	70 g	2, 6 and 10	**I**
IND (2)	F68 (1.2)	Water (10)	45	1	70 g	10	**II**
IND (1)	F68 (1.2)	Water (5)	20	1 5 10	30 g 80 pcs. 10 pcs.	10	**III**
BRA (1)	Tween 80 (0.5), HPMC (0.5), F68 and F127 (1.2)	PBS (pH 4.5 & 7.4) (5)	20	1	30 g	6 or 10	**IV**

4.3 Formulation of nanosuspensions

Indomethacin nanosuspensions were converted to dried powders for inhalation or oral drug delivery **(II)**. Mannitol (Alfa Aesar) 20 g/l and/or L-leucine (Alfa Aesar) 10 g/l were dissolved in the diluted nanosuspensions with the final drug concentration of 5 g/l. The mixed precursor was dried using an aerosol flow reactor (AFR) method [196, 197]. Briefly, the precursor suspensions were atomized using a modified air-jet atomizer (Collision atomizer, TSI model 3076) (TSI Inc. Particle Instruments, St. Paul, USA). To produce micron-sized droplets, the opposing impaction wall in atomizer was removed. The precursor suspensions were passed through a heated tubular aerosol reactor by nitrogen gas with a flow rate of 3 l/min. The total residence time of the aerosol in the reactor was 9 s. The temperature of the reactor was controlled with four separated heating blocks and two sets of temperature profiles from aerosol entry to exit were tested to dry the suspensions: 100-100-100-100 ^{o}C (AFR 100) and 100-100-100-160 ^{o}C (AFR 160). The residence time of the aerosol in the zone at 160 ^{o}C was 1.5 s. The dried particles were diluted in a porous tube diluter with nitrogen (20 ^{o}C) in a ratio of 1:10 before collecting the size-exclusively particles with a Berner-type low pressure impactor onto aluminum foils.

Brinzolamide nanosuspensions milled in two different pHs of phosphate buffered saline (PBS) were formulated into 3 kinds of eye drops for ocular drug delivery **(IV)**. Excipients, including benzalkonium chloride (BAC) as preservative and possible polysorbate 80 as absorption enhancer

were added in the diluted nanosuspensions with PBS solutions. In three formulations, the final concentrations of brinzolamide, HPMC, BAC and Polysorbate 80 were 1, 0.25, 0.01 and 0.25 w/v%, respectively. The physical mixtures of bulk drug and stabilizer in PBS (pH = 7.4) was prepared for *in vitro* negative control.

4.4 Characterization techniques and dissolution modeling

4.4.1 Size and size distribution (I-IV)

The particle size and size distribution of nanosuspensions were measured by dynamic light scattering (DLS) using a Malvern Zetasizer 3000HS (Malvern Instrument, Malvern, UK) **(I-IV)**. Polydispersity index (PI) was used to indicate the width of particle size distribution. The lower the PI value, the more uniform and monodisperse the particles are. Before the DLS measurement, a part of nanosuspensions were sonicated for 3-4 min and diluted by saturated drug solution to a suitable concentration for DLS measurement. The drug saturated solution was prepared by filtration through a 0.22 μm filter membrane (Pall Co. Mexico) of the raw drug suspensions in the same milling medium containing 0.1% (w/v) same stabilizer with the nanosuspensions after 24 h of shaking equilibration. The analyses were performed with a dispersant refractive index of 1.33, and the viscosity of the dispersion medium was 0.89 cP. The measurements were repeated 3 times for each sample.

The particle size distributions of the aerosols were measured from gas phase at the aerosol reactor downstream using a TSI scanning mobility particle sizer equipped with a long differential mobility analyzer (TSI model 3081) (TSI Inc. Particle Instruments, St. Paul, USA) and a condensation particle counter (TSI model 3022) (TSI Inc. Particle Instruments, St. Paul, USA) **(II)**. Geometric standard deviation (g.s.d.) was used to describe how spreads out the values are in the distribution. The low values of geometric standard deviation means the monodisperse aerosols having narrow size distributions.

4.4.2 Morphology (I, II and IV)

Morphological evaluation of drug nanoparticles was conducted through transmission electron microscopy (TEM) (FEI Tecnai F12, Philips Electron optics, Holland) and scanning electron microscopy (SEM) (JSM-7500F, JEOL Ltd., Japan). For TEM sampling, diluted samples of IND and ITR nanosuspensions **(I)** were pipetted on formvar film-coated 300 mesh copper grids (Agar Scientific, Essex, UK) and dried at the room temperature before analyses. For SEM sampling, the dried IND nanopowders **(II)** and BRA nanosuspensions **(IV)** were placed or pipetted on a carbon-coated double-sized tape. The nanosuspension samples were dried at an ambient condition before next step. The samples were coated with platinum in a sputter coater (Q150T Quomm, Turbo-Pumped Sputter Coater, China) and then imaged by SEM.

4.4.3 Drug content (II, III and IV)

After milling, collecting and drying of nanosuspensions, the true content of drug compound was different from the theoretical one. To quantify the exact drug content in the nanosuspensions or dried powders, a certain amount of IND nanosuspensions **(I)** were dried in an oven and then dissolved in ethanol **(I)**, directly dissolved in ethanol without drying **(III)**, or a known amount of dried IND powder **(II)** was dissolved in ethanol: water mixture (1:1, v/v). After complete dissolution, the IND solution was diluted by water **(I)** or ethanol/water (1:1, v/v) **(II, III)** for high performance liquid chromatography (HPLC) analysis. A certain amount of ITR nanosuspensions **(I)** were dried in an oven and dissolved in methanol, and finally diluted by methanol: water (1:1, v/v) prior to HPLC analyses. For BRA content determination **(IV)**, a constant volume of suspensions was pipetted, dissolved in PBS (pH 7.4) and diluted by the same solution for HPLC analysis.

HPLC instrument (Agilent 1100 series, Agilent Technologies, Germany) was used for quantification the drug concentrations. The parameters can be seen in Table 8. The injection volume of sample was 20 µl for all sample measurements.

Table 8 *Parameters of HPLC analyses for drug concentrations.*

API	Column	Mobile phase A	Mobile phase B	A : B (v/v)	Flow rate (ml/min)	λ_{max} (nm)
IND	Luna 3µ C18 100A	0.2% phosphoric acid in water (pH 2.0)	acetonitrile	60:40	1.5	320
IND	Gemini NX 3µ C18 110A	0.2% phosphoric acid in water (pH 2.0)	acetonitrile	65:35	1	320
ITR	Gemini-NX 3µ C18 110A	0.1% trifluoroacetic acid in water	acetonitrile	55:45	1	261
BRA	Zorbax Eclipse XPD-C18	acetonitrile/acetic acid/MQ water/ 1-octanesulfonic acid sodium salt (24/2/74/0.025)	acetonitrile /water (90/10)	96:4	1	254

4.4.4 Dissolution studies (I-IV)

Different dissolution conditions were set up for dissolution tests with different purposes. Sink conditions were used for comparisons of nanoformulations and raw materials. The dissolution profiles of IND and ITR suspensions after milling, before milling and pure bulk drugs were studied in phthalate buffer (pH = 5.0) and 0.1 M HCl aqueous solution, respectively **(I)**. The dissolution behaviors of IND nanosuspensions, dried nanosuspensions, physical mixtures and raw IND compound were compared in phthalate buffer (pH = 5.0) **(II)**. Dissolution tests were performed using a rotating paddle apparatus according to the European Pharmacopoeia with an Erweka DT-06 dissolution system (Heusentamm, Germany) at 37 ^{o}C and rotating speed of 100 rpm in 600 ml of their corresponding dissolution medium. A known amount of samples were introduced to the dissolution medium to maintain sink conditions. At predetermined time intervals, 5 ml of dissolution medium was withdrawn and the same volume of pre-thermostated fresh medium was added. The 1 ml of samples were immediately centrifuged at 13 000 rpm for 8 min to remove the undissolved particles. The drug concentration in the supernatant was analyzed by HPLC. All

experiments were performed at least three times, and the average values and standard deviations were calculated to form the dissolution profiles.

Dissolution tests for BRA nanocrystal formulations were performed using the same paddle equipment **(IV)**. PBS buffer (0.9% (w/v) sodium chloride in 10 mM phosphate buffer, pH 7.4) of 500 ml at 37 ^{o}C was used as the dissolution medium. The exact amounts of the nanoformulations (I-III) were dispersed in the dissolution medium with an agitation speed at 100 rpm. The sink conditions were maintained during the dissolution process. At specific time points, a constant volume of the dissolution medium was withdrawn and replaced by a fresh medium. The dissolution profiles of commercial product Azopt® and formulation with bulk BRA were investigated as controls. The BRA concentration was determined using HPLC method.

To discriminate the dissolution profiles of nanosuspensions with different particle sizes, various dissolution conditions were investigated, such as the dissolution medium (hydrochloric acid medium, pH = 1.2 and phthalate buffer, pH = 5.0), agitation speed (50 and 120 rpm) and sample amount ratio (1/4, 1 and 3) **(III)**. Here, the sample amount ratio was defined as the total sample amount introduced in the dissolution medium to its apparent saturation solubility. The dissolution equipment was the same one with the experiments described above. A certain amount of sample was transferred into the dissolution medium of 600 ml at 37 ^{o}C. At certain times (0.5, 1, 2, 4, 6, 15 and 30 min), 5 ml of dissolution medium was pipetted out and replaced with the same volume of pre-thermostated fresh medium. The samples were filtered through Acrodisc® syringe filters with 0.2 μm GHP membranes (PALL Life Science, Ann Arbor, MI, USA) to remove the undissolved particles. To avoid the possible absorption of IND molecules on the filter membrane, the first 4.5 ml of filtrate was discarded. The last 0.5 ml filtrate was analyzed by HPLC.

To chart the dissolution profiles in non-sink conditions, the dissolved drug amount (%) is calculated according to the equation (1):

$$Dissolved\ amount\ (\%) = 100 \times c_b/c_{max} \qquad (1)$$

In conventional dissolution tests (sink conditions), the maximum concentration value (c_{max}) is equal to the total drug amount introduced to the dissolution medium (c_{tot}). In our developed method (non-sink conditions), if $\varphi \leq 1$ the c_{max} is equal to c_{tot}; and if $\varphi > 1$ *the* c_{max} is equal to the apparent saturation solubility (c_s). c_b is the bulk concentration of the drug. The φ introduced here is calculated according to the equation (2), which means the sample amount ratio, i.e. a ratio of the sample concentration in the dissolution medium to the apparent saturation solubility of the sample.

$$\varphi = \frac{W_0/V}{c_s} \qquad (2)$$

where W_0 is the total drug mass in the dissolution medium, and V is the volume of the dissolution medium. c_s was determined for each particle size fraction by shake flask method.

4.4.5 Dissolution modeling (III)

Based on a shrinking-core model and after a series of equation conversions, a relationship between particle radius and sample amount ratio was established. The equation (3) is introduced as follows:

$$\frac{dR_i}{dt} = \frac{2Dc_s}{\rho}\frac{1}{R_i}\left(1-\left(1-\frac{W}{W_0}\right)\varphi\right) \quad (3)$$

where, R_i is the radius of the particle, D is the diffusion coefficient of the drug in water, ρ is the density of the drug particles and W is the mass of the particles in the system at a given time point. The mass was calculated as a sum of the masses of particles.

The equation were evaluated for a simulated normal size distribution for 10000 particles for 1800 times steps (1 step = 1 s, total simulation time was 30 min). In all calculations, D was assumed to be 2×10^{-6} cm^2s^{-1}, c_s were 1 µg/ml, and ρ was 1.37 g/cm^3. The particle population with radius of 200 nm (R_0) and standard deviation ($\Delta R = 0.15R_0$) was simulated. To model the bimodal size distribution particle populations were simulated as above, but at the same time 5% of particles with twice as large a radius were added to the overall population. All calculations were done using standard mathematical software (Matlab) by numerically evaluating the equation using the Euler method for all the simulated particles in each time step.

4.4.6 Stability of nanosuspensions (I)

Stability of nanosuspensions was studied after storage for 2 months at room temperature (25 ^oC) and 4 ^oC. For physical stability, the particle size, size distribution and morphology of nanosuspensions were checked during the storage period by DLS and TEM methods. For chemical stability, the drug concentrations of nanosuspensions were monitored during the storage by HPLC. Any decrease in the area of the drug peak or occurrence of extra drug peak in chromatograms was considered as chemical instability.

4.4.7 Solid state analyses (I, II and IV)

For solid state analysis nanosuspension were freeze-dried (FTS Lyostar II freeze drying system, SP Industries Inc., Warminster, USA). Primary drying was performed in -30 ^oC for 17 hours and secondary drying was done stepwise from -25 ^oC to 45 ^oC. Thermal analyses of the dried nanosuspensions were carried out with a differential scanning calorimeter (DSC) (DSC 823e, Mettler Toledo Inc., Greifensee, Switzerland). Before measurements, the DSC was calibrated using gallium, indium and tin. Sample powders from 2 to 6 mg were placed in perforated aluminum sealed pans. An identical pan was used in the machine as reference pan. The samples were heated in a temperature range from 25 ^oC to 180 ^oC (IND) or 200 ^oC (ITR) or 310 ^oC (BRA), depending on the melting points of drug compounds and excipients with a heating rate of 10 ^oC /min. The measurements were performed under the nitrogen flow of 50 ml/min. Pure drug, each excipient and physical mixtures were also tested as controls under the same conditions corresponding to its dried nanosuspensions. The data were analyzed with STAR^e software (Mettler Toledo, Greifensee, Switzerland).

X-ray powder diffraction (XRPD) diffractograms of each of the excipients, pure drug, dried powder before milling and after milling were recorded using an X-ray diffractometer (Bruker AXS D8, Karlsruhe, Germany) **(I and II)**. The measurements were performed in symmetrical reflection mode with Cu-Kα radiation λ = 1.54Å (40 kV voltage and 40 mA current). The samples was placed

on a flat aluminum sample holder and scanned from 5° to 40°, 2θ with a step size of 0.02°, and the measuring time was 0.5 s/step.

4.4.8 *In vivo* experiments (IV)

Seven-month old male Wistar rats (n = 44, weight 406-601 g; Harlan Laboratories B.V., Venray, The Netherlands) were used in animal study. The experimental procedures were approved by the Finnish National Animal Ethics Committee in State Provincial Office of Southern Finland. The animal study was performed according to the European Communities Council Directive (86/609/EEC) and the guidelines published by the Institute for Laboratory Animal Research.

Prior to *in vivo* tests, ocular hypertension in one eye of each rat was induced as laser method [198]. Finally 36 rats successfully infected a reasonable range of intraocular pressure (IOP) were used for *in vivo* tests. Fifteen hours after the laser treatment, the rats were anesthetized with the mixtures of ketamine and medetomidine, and a dose of 10 µl from each formulation was instilled in the lower conjunctival sac of laser-treated eyes. The treatment groups were as follows: formulation I (n = 5 rats), formulation II (n = 5), formulation III (n = 6), Azopt® (n = 6), 0.9% NaCl (n = 8) and non-treated group (NT, n = 6). The IOPs were measured prior to drug administration and after the administration at different time intervals (7.5, 15, 30, 45 and 60 min) using an Icare Tonolab tonometer (Icare Finland Oy, Helsinki, Finland). The samples were coded and administered in blind fashion.

5 Results and discussion

5.1 Preparation and characterization of nanosuspensions (I, III)

Though nanocrystal technologies, like wet milling, are considerably simple processes for particle size reduction, nanocrystal development requires multidisciplinary knowledge, including the breakage kinetics of nanocrystals, dynamic equilibrium between the particle breakage and aggregation, stabilization mechanisms of stabilizers, formulation processing and affecting fates *in vivo [4, 77]*. The fundamental and essential complementary research should be developed and performed in order to obtain the nanocrystals successfully. In this thesis, the effects of milling parameters, such as the milling time, diameter of milling pearls, stabilizer type and concentration, on the particle size, size distribution and stability were investigated on three different model drugs.

5.1.1 Influencing factors on particle size

*5.1.1.1 Effect of milling time (**I, IV**)*

The mean particle sizes and PIs of milled indomethacin and itraconazole suspensions with different milling cycles are shown in Fig. 6. For all stabilizers, the concentration was kept 25% relative to the drug amount. Generally, decreasing trends in particle sizes and PIs were found with increasing milling time, except for the case of PEG as stabilizer. After only 2 milling cycles, the average particle sizes of both drugs were dramatically decreased below 1 µm since pharmaceutical compounds have low hardness, which is easy for grinding. Fine nanosuspensions with smaller and more uniform particle sizes were obtained with longer milling times. Long milling time provides more energy input to break the particles into smaller ones and provides sufficient diffusion and absorption time for stabilizer molecules to attach onto the particle surfaces. Further increase in milling time did not remarkably decrease particle sizes or they even increased in the case of indomethacin nanosuspensions with F127 as the stabilizer. The same phenomenon was also found in milling process of 10-hydroxycamptothecin suspensions using the high pressure homogenization [61]. For brinzolamide compound, the milling process was stopped after 6 cycles since the good nanosuspensions were obtained at that milling time and prolonging the milling time was not beneficial.

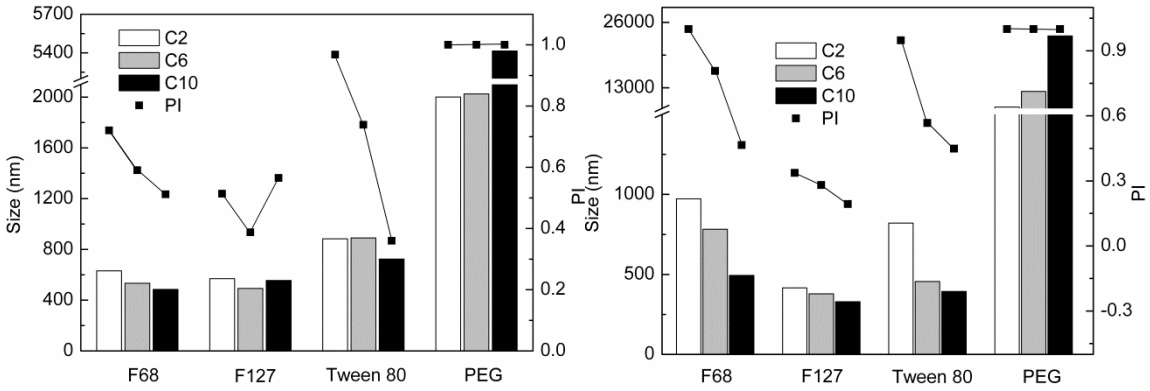

Figure 6 *Mean particle sizes and PIs of the suspensions with the same stabilizer concentration (25% drug content), as a function of milling cycle. Indomethacin suspensions (left) and itraconazole suspensions (right). C_2, C_6 and C_{10} in the figure mean after 2, 6 and 10 milling cycles of 3 min each (from paper* ***I****).*

*5.1.1.2 Effect of stabilizer type and concentration (**I, IV**)*

The choices of stabilizer type and concentration are important in nanosuspension preparation. The mean particle sizes and PIs of indomethacin and itraconazole as functions of stabilizer type and concentration after 10 milling cycles are shown in Fig. 7. For both drugs, PEG failed to stabilize the nanosuspensions. PEG is a linear hydrophilic homopolymer. Interactions between the drug and stabilizer, such as hydrophobic interactions, hydrogen bonds and ionic interactions, are the main driving forces for stabilizer adsorbing on the drug particle surfaces. Shortage of hydrophobic moieties and other functional groups in PEG chains are major problems for absorption. It was reported that for a hydrophobic drug, an effective stabilization performance of copolymers requires the hydrophobic moiety content to be higher than 15 mol%, if there are no functional groups in the polymer suitable for interactions with the drug [71, 73]. In addition, PEG is a hydrogel polymer, which can imbibe water and swell to gel. Thereby, the slurry of the drug, PEG and water is in paste form and exhibits a very high viscosity. The high viscosity of suspensions hinders the movement of milling pearls and energy transfer [81], thereby reducing the milling efficiency.

For indomethacin nanosuspensions, the finest nanosuspensions were obtained with the mean particle sizes of 345 nm, 375 nm and 723 nm stabilized by F68 (80%), F127 (60%) and Tween 80 (25%), respectively (Fig. 7 a). F68 was the most effective stabilizer, followed by F127 and Tween 80. Pluronic® F68 and F127 are linear triblock ABA non-ionic copolymers (A stands for hydrophilic polyethylene oxide (PEO) segment and B stands for hydrophobic polypropylene oxide (PPO) segment). It was clearly confirmed that the hydrophobic PPO chains act as driving forces for absorption on particle surfaces, while the PEO chains form hydrophilic layers surrounding drug particles to sterically protect against aggregation [79]. In contrast to F127, F68 was more effective for particle size reduction since F68 has a lower molecular weight which may exert less kinetic restriction in the adsorption process and faster diffusion [79]. Moreover, nanosuspensions exhibited a good stability for two months with both polymers as stabilizers. For the surfactant Tween 80, the nanosuspensions with a larger particle size and PI were achieved compared to the polymers. Furthermore, the nanosuspensions were not stable during the 2 months storage, i.e. the mean particle size was decreased from 986 nm to 736 nm and the PI was increased from 0.37 to 0.62. Tween 80 is a smaller surfactant molecule, which effectively decreases the interfacial tension of the

particles but forms a thin protective outer layer. The thin layer is insufficient for the particle stability. In addition, Tween 80 can remarkably increase the solubility of indomethacin and thus prompt the Ostwald ripening [28].

For itraconazole nanosuspensions, the smallest particle sizes were generated when 60% of F127 was used as stabilizer, and then followed by 25% Tween and 25% F68. Here, F68 was worse than F127 for particle size reduction and stability, which might be contributed to stronger hydrophobic interactions between F127 and drug particles. Moreover, a slurry with a high viscosity was formed when F68 and itraconazole were mixed, which is not beneficial for milling. Comparing the two drugs, the different milling results were potentially caused by the intrinsic properties of the drugs, such as hardness, hydrophobicity and crystal shape [42].

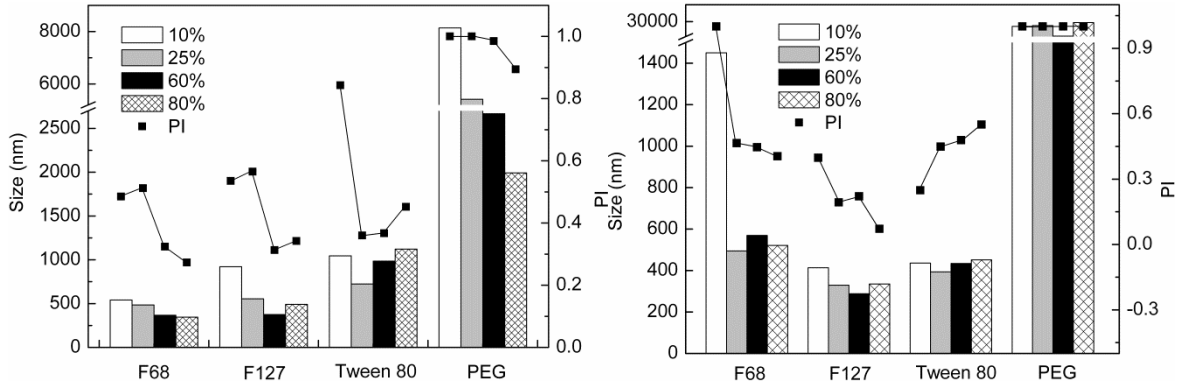

Figure 7 *Mean particle sizes and polydispersity indexes (PIs) of the suspensions with different stabilizers and stabilizer concentrations after milling for 10 cycles. Indomethacin suspensions (left) and itraconazole suspensions (right) (from paper **I**).*

For preparation of brinzolamide nanosuspensions, four types of stabilizers, including F127, F68, polysorbate 80 and HPMC, were screened. The most optimal stabilizer conditions were HPMC with a concentration of 25% relative to the drug amount. HPMC containing a high degree of substitution of the methoxy and hydroxypropoxy groups can effectively adsorb on the drug particles by hydrogen bonding, which prompts the particle size reduction during the milling and protects the particles against aggregation [69].

*5.1.1.3 Effect of milling ball diameter (**III**)*

As shown in Table 9, the indomethacin nanosuspensions with different particle sizes and PIs were prepared depending on the diameters of milling pearls. The particle size and PIs of suspensions were decreased with the decreasing diameter of milling pearls. Small size of milling pearls supplies more contact points and larger contact surface area between milling pearls and drug crystals, which enhance the milling efficiency and then produce fine nanosuspensions [199].

Table 9 *Particle size, polydispersity index, and apparent saturation solubility of indomethacin nanosuspensions milled with different sized milling balls (n = 3, milling for 10 cycles) (from paper **III**).*

			Solubility (μg/ml)	
Milling ball diameter (mm)	Size (nm)	PI	pH 1.2	pH 5.0
1	340 ± 4	0.24 ± 0.06	1.29 ± 0.03	21.23 ± 0.19
5	560 ± 11	> 0.7	1.23 ± 0.06	19.25 ± 0.39
10	1300 ± 111	> 0.7	1.15 ± 0.01	18.06 ± 0.43

In summary, the particle sizes decreased fast within the first 2 cycles as the large micron-sized drug particles were easier to break. As milling continued, the apparent breakage rate decreased, and the particles became smaller and more homogeneous. The smaller diameter of milling pearls leaded to fine nanosuspensions. Stabilizers play a major role in particle reduction and stabilization of the milled drug particles.

5.1.2 Morphology evaluation (I, IV)

The TEM images of itraconazole micro/nanosuspensions milled with four different stabilizers can be seen in Fig. 8 (A-D). Microparticles were observed when PEG was used as the stabilizer in the milling process, and this result is well in agreement with the DLS results. For stabilizer F127, itraconazole nanocrystals kept their original rod-like shape as bulk materials. However, for stabilizer Tween 80, the crystal shape changed from rod-like to cubic-like, which might be attributed to the surfactant molecules adsorbing on crystal surfaces and then changed the crystal shape. The similar findings reported by Mou *et al.* [34] showed that itraconazole nanocrystals prepared using an acid-base neutralization method exhibited a rod shape with F127 as a stabilizer but a spherical shape with HPMC. For stabilizer F68, the nanocrystals were found to aggregate.

The morphologies of indomethacin nanocrystals with stabilizers F68 and Tween 80 are shown in Fig. 8 (E-F). The angular surfaces of crystals were transformed to smooth round surfaces after milling. The gray layers around the particles were considered to be due to the polymer or surfactant. No different shapes of indomethacin nanocrystals with the four stabilizers was seen.

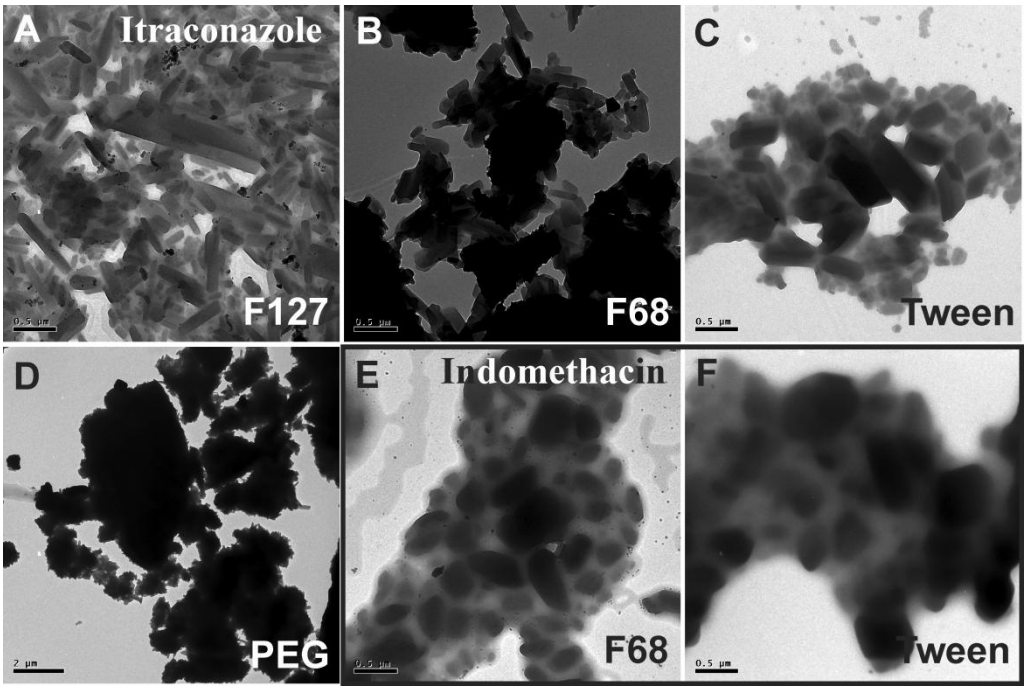

Figure 8 *TEM images of suspensions: (A-D) itraconazole stabilized by the F127, F68, Tween 80 and PEG; (E-F) indomethacin stabilized by the F68 and Tween 80. The stabilizer concentration is 25% relative to the drug amount (from paper **I**).*

The morphology of brinzolamide nanosuspensions stabilized by HPMC in pH 7.4 was imaged using SEM (Fig. 9). The mean particle size of brinzolamide nanosuspensions detected by SEM (Fig. 17 A-B) is well in agreement with the findings of the particle size measurements based on DLS. A rod-like big crystal in Fig.9A was considered to the crystallization of free BRA from the liquid phase during the sample preparation.

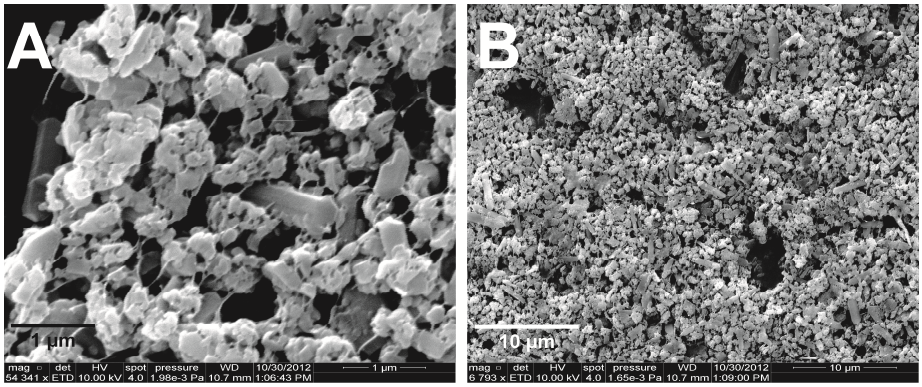

Figure 9 *SEM (images with different magnifications) of brinzolamide nanocrystal suspension (pH 7.4) stabilized by HPMC, scale bars being 1 µm and 10 µm, respectively (from paper **IV**).*

5.1.3 Dissolution studies

*5.1.3.1 Comparisons of milled and unmilled suspensions (**I, IV**)*

In order to compare the dissolution profiles of milled and unmilled suspensions, sink conditions were used to enhance the dissolution rate of poorly water-soluble drug compounds. The dissolution profiles of the milled suspensions, physical mixtures and pure drug materials are shown in Fig. 10. Compared to the suspensions of physical mixtures and pure drugs, milled suspensions of both drugs displayed a significant increase in dissolution rate and the dissolutions were completed in a few minutes. A large surface area-to-volume and higher apparent saturation solubility of nanocrystals pronouncedly enhance the dissolution rate according to the Noyes-Whitney equation. It is worth noting that the milled suspensions with quite different average particle sizes (300 nm to several micrometres) depending on the stabilizers used generated very similar dissolution profiles. Sink conditions cannot discriminate between the dissolution profiles of the milled samples. The same dissolution behaviours were found for brinzolamide samples.

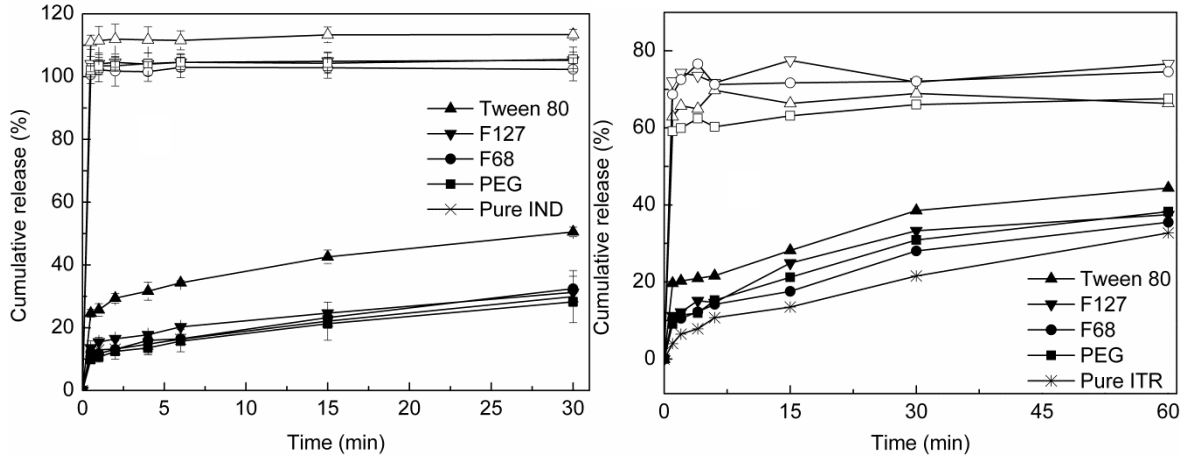

Figure 10 *The dissolution profiles of indomethacin (left) and itraconazole (right) from nanosuspensions (open symbols), physical mixtures and pure drug (solid symbols) with 25% stabilizer (from paper **I**).*

*5.1.3.2 Comparisons of milled suspensions (**III**)*

As mentioned above, sink conditions can effectively discriminate between the dissolution profiles between nanosuspensions and raw materials, but fail to see differences between nanosuspensions. Therefore, a discriminating dissolution method using non-sink conditions was investigated. Firstly, the dissolution behaviours of nanosuspensions were simulated *in silico*. The changes in particle mass fraction profiles at various dissolution times and with different sample amount ratio (φ) are shown in Fig. 11. For the monomodal particle population (average radius was 200 nm) (Fig. 11, left), all particles vanished after around 1.5 min in sink conditions (φ = 0.1). With increasing φ values, the completion of dissolution process needed a longer time. The longest dissolution time was occurred when φ = 1. Further increased the φ value to 3, the peak of profiles shifted to left side, which indicated that just a part of particles was dissolved and the average particle size became

smaller after dissolution. The profiles at dissolution time points of 1.5, 3, 7.5 and 15 min overlapped, implying that the dissolution process was stopped in a short time (1.5 min). For the bimodal particle populations (200 nm/ 400 nm, 95%/5% of the particle population) (Fig. 11, right), the slowest dissolution rate was observed at φ = 1. At sink conditions (φ = 0.1), the smaller particles dissolved fast and the larger particles became smaller. When φ = 3, only a part of the smaller particles dissolved and the larger particles never changed in size. The dissolved smaller particles saturated the dissolution medium and large particles could not dissolve anymore in a saturated solution.

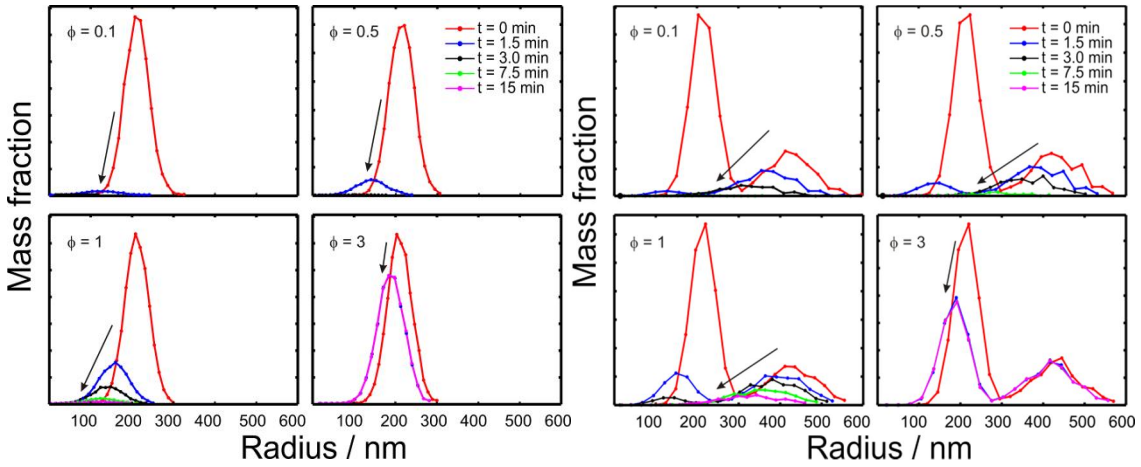

Figure 11 *Mass weighted graphs of particle populations during the dissolution simulations. Parameters for the simulation of monomodal were $\Delta R = 0.15R_0$ and R_0 = 400 nm (left). Parameters for the simulation of bimodal were $\Delta R = 0.15R_0$ and R_0 = 200 nm (95% of the population)/400 nm (5% of the population) (right). The arrows indicate the curves at later time points (from paper **III**).*

The time to achieve 90% of the maximum extent of dissolution/saturation for particle populations with average sizes of either 200 nm or 400 nm and bimodal distribution as a function of φ are shown in Fig. 12. In the case of φ < 1, i.e. the concentration of sample in the dissolution medium is below its apparent saturation solubility, all particles in three samples would be completely dissolved, but the dissolution time was different. The smaller the particle size, the shorter dissolution time was needed. The time curve of the bimodal sample was between the ones of samples with 200 nm and 400 nm. In the case of φ > 1, i.e. the amount of sample in the dissolution medium is above its apparent saturation solubility, the dissolution medium finally becomes saturated during a dissolution experiment. The trend of bimodal curve was close to the sample with the radius of 200 nm since the dissolution was mostly due to the small particles and the presence of the larger particles did not make a difference for the dissolution rate, which was confirmed by Fig.11 (right). The longest dissolution time occurred when φ = 1.

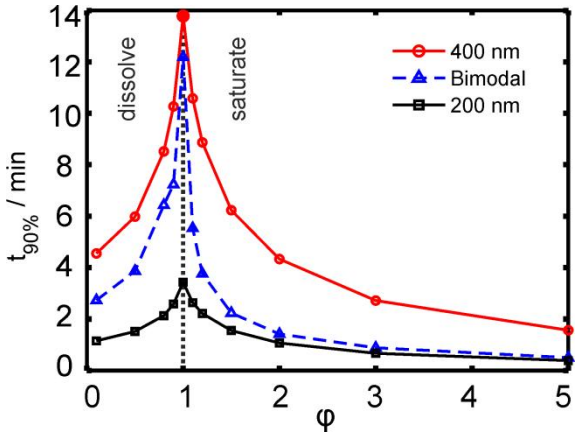

Figure 12 *Plots of simulated times to achieve 90% of dissolution/saturated solubility for 200 nm, 400 nm (particle radius), and bimodally distributed particles (5% larger particles) as a function of φ (from paper **III**).*

Secondly, the dissolution tests of nanosuspensions with three different particle sizes were carried out as a function of sample amount ratio using paddle apparatus (Fig. 13). The sample amounts introduced into the dissolution media were calculated based on their solubilities (Table 9). As expected based on the simulations, the most discriminating dissolution profiles between the three nanosuspensions were found at φ = 1, since the slowest dissolution rate occurred at this condition. Significant differences were found especially at the beginning of the dissolution processes.

The weak discrimination in dissolution profiles were obtained at sink conditions because the rapid dissolution masked the differences that resulted from particle sizes. The same weak discrimination was found when the value of φ was larger than 1, since the fast dissolution was mainly caused by the large number of smaller particles and the solution became saturated quickly, which was already proofed by the simulations (Figure 11). Besides the sample amount ratio, the effects of the dissolution medium pH and agitation speed on dissolution rate were investigated. Lower solubility by choosing a proper pH of the dissolution medium was helpful in getting discriminating dissolution profiles, whereas the agitation speed had little influence on the dissolution profiles of nanoparticles.

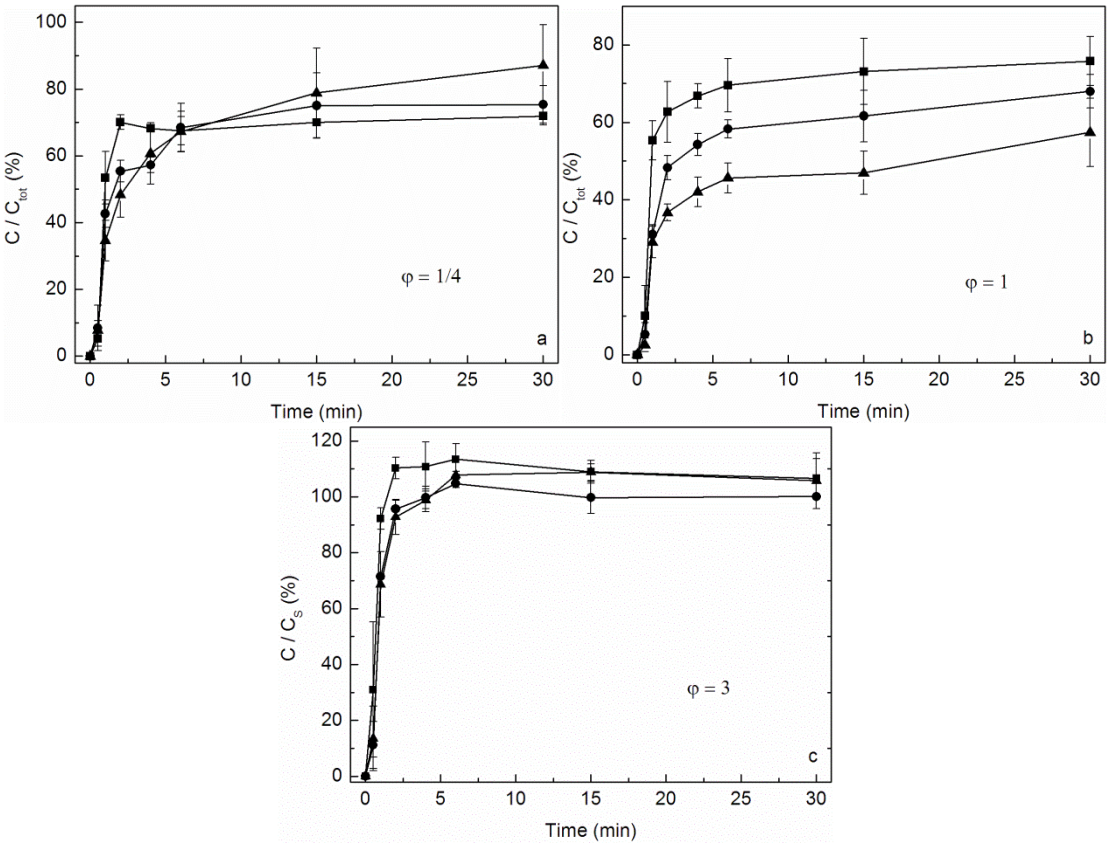

Figure 13 *Dissolution profiles of different suspension amounts (φ) when the dissolution medium and agitation speed were fixed at pH 1.2 and 120 rpm at 37°C. (a) φ = 1/4, (b) φ = 1, (c) φ = 3. Square, round, and triangle symbols stand for the suspensions with particle size 340, 560, and 1300 nm, respectively (n = 3 – 6) (from paper **III**).*

5.1.4 Evaluation of the crystalline state (I, II and IV)

To characterize the solid state of freeze-dried nanosuspensions, DSC and XRPD studies of the drugs, stabilizers, physical mixtures and freeze-dried nanosuspensions were performed. The DSC thermographs of indomethacin samples are shown in Fig. 14 (left). The curve of pure indomethacin exhibited a single sharp endothermic peak at onset temperature of 160 ^{o}C, due to its melting point. Stabilizer F127 showed also a single melting peak with onset temperature at around 54 ^{o}C. Compared to the physical mixtures, indomethacin melting peak of milled samples showed a little bit shift to a lower temperature, which caused by the reduction of particle size or the presence of the stabilizer [30, 200]. No baseline shift corresponding to glass transition or peaks for recrystallization appeared, indicating that amorphous form of indomethacin was not produced during the milling and freeze drying processes.

The X-ray diffractograms are shown in Fig.14 (right). Raw indomethacin exhibited crystalline state. There are no apparent differences in the X-ray patterns between the physical mixtures and milled samples. Low diffraction peak intensities in the milled samples were attributed to the dilution of the particles with the stabilizer [51]. For the samples of itraconazole and brinzolamide, the crystalline state was kept after milling. As a conclusion, combining the results of DSC and XRPD, the crystalline state of the drug compounds was not interfered with by the wet milling processes.

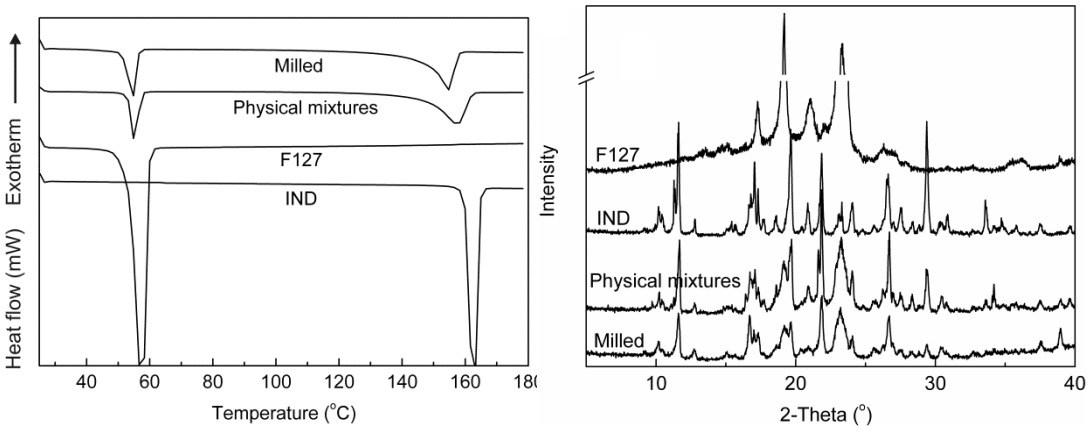

Figure 14 *(Left) the DSC patterns of IND, from up to down: milled sample, physical mixtures, pure F127 and bulk drug. (Right) X-ray analyses of IND from up to down: F127, bulk drug, physical mixtures and milled sample (from paper **I**).*

5.2 Formulation of nanosuspensions (II, IV)

The merits of nanocrystals, including increased surface-to-volume ratio, improved solubility, fast dissolution, enhanced adhesiveness and potential targeted drug delivery, provide huge opportunities for drug delivery. In the past twenty years, there has been a considerable research interest in various nanocrystal technologies related to the production of drug nanocrystals and their *in vitro* physical and chemical properties. Nowadays, *in vivo* behaviours of the nanocrystals have been generally studied [201]. The drug nanocrystals can be used as a versatile formulation platform, such as oral, parenteral, ophthalmic, transdermal and pulmonary delivery, to alter and improve the pharmacokinetic, pharmacodynamics and targeting properties of poorly soluble drugs [201]. In this thesis, the microparticulate powders consisting of drug nanocrystals for oral or pulmonary drug delivery were formulated. In addition, the formulations of the brinzolamide nanosuspensions for ophthalmic administration were studied.

5.2.1 Nanocrystals in micron-sized formulation (II)

Nanocrystallization is a promising way to improve the dissolution rate of poorly water-soluble drugs, but handling the nanopowders in the production of the final formulation is problematic, due to their physical properties such as poor flowability, low density and aggregation tendency. Assembling the nanocrystals into microparticulate carriers is a potential way to solve the problems related to the small particles and at the same time to keep the high dissolution rate. The concept of “nanos-in-micros” is illustrated in Fig. 15. The carrier matrix of mannitol provides stable powder formulations, which are suitable for inhalation products or automated tablet manufacturing. The surface-active L-leucine forms crystalline coating layers around the microparticles during the aerosol drying process, which provides a protection for inner core, excellent aerosolization properties [202], improved flowability and dispersibility of powders [203].

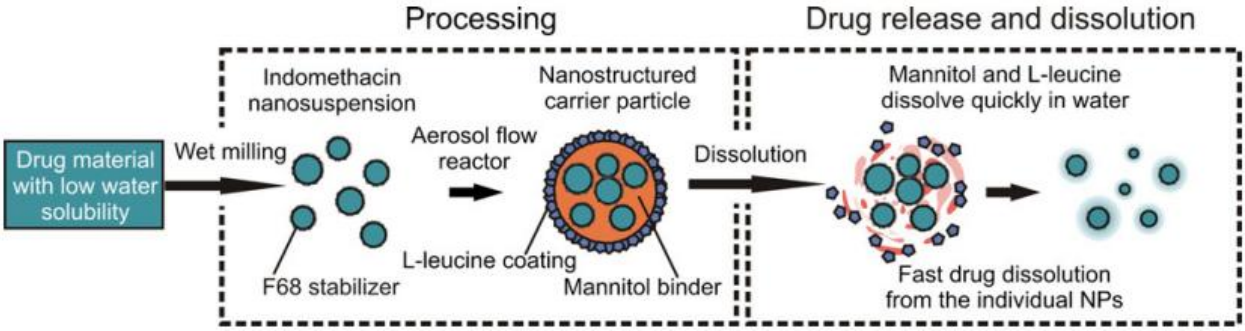

Figure 15 *Illustration of the concept for producing and using nanostructured microparticles, nanos-in-micros. Wet-milled nanosuspensions are dried and embedded into a mannitol matrix and coated with a crystalline L-leucine layer in an aerosol flow reactor. Microparticle structure dissolves rapidly in water, and primary nanoparticles are released. Fast drug dissolution is due to the small size of the initial particles (from paper* ***II****).*

The compositions of precursor suspensions used for drying into microparticles are listed in Table 10. The amounts in compositions were based on previous studies and should result in micron-sized particles with a crystalline L-leucine coating [203, 204]. Samples were dried using two different temperatures. The mass mean diameters of the six formulations ranged from 576 nm to 956 nm.

Table 10 *Precursor Solution Compositions for AFR and Dry Powder Sizes of the Prepared Powders. Sizes are Mass Mean Diameters (from paper* ***II****).*

	Drying	Precursor solution content (g/l)				Powder size	
	T (^{o}C)	IND	F68	Mannitol	Leucine	Size (nm)	g.s.d
AFR 100-1	100	5	1.25	-	-	956	1.7
AFR 100-2	100	5	1.25	20	-	673	1.6
AFR 100-3	100	5	1.25	20	10	824	1.5
AFR 160-1	160	5	1.25	-	-	714	1.6
AFR 160-2	160	5	1.25	20	-	665	1.6
AFR 160-3	160	5	1.25	20	10	576	1.4

The images of size-excluded samples with D_{50} (mass-median-diameter) of 3.9 µm were evaluated by SEM (Fig. 16). The aerosol particles dried from indomethacin nanosuspensions without mannitol and L-leucine (AFR 100-1 and AFR 160-1) were physically unstable, i.e. aggregation/sintering of particles in AFR 100-1 (Fig. 16A) and re-crystallization of indomethacin in AFR 160-1 (Fig. 16D) was seen. The indomethacin nanocrystals with the onset melting temperature of 146 ^{o}C melted in the high aerosol temperature (160 ^{o}C) and then re-crystallized at the lower temperature. Mannitol alone (AFR 100-2 and AFR 160-2) protected the particles from aggregation to some extent, but still could not sufficiently stabilize the particle integrity, especially for AFR 160-2 (Fig. 16E). With the L-leucine coating (AFR 100-3 and AFR 160-3), individual and integrated particles were obtained. L-leucine is a surface-active material which diffuses to the droplet surfaces and thus forms a crystalline surface layer around the particles after drying. In AFR 160-3 samples (Fig. 16F), long needle-like crystals distributed around microparticles was owned to

the partial re-crystallization of indomethacin during the drying process. For the samples of AFR 100-3 and AFR 160-3, the indomethacin kept the crystalline state in the microparticles which was confirmed by DSC and XRPD.

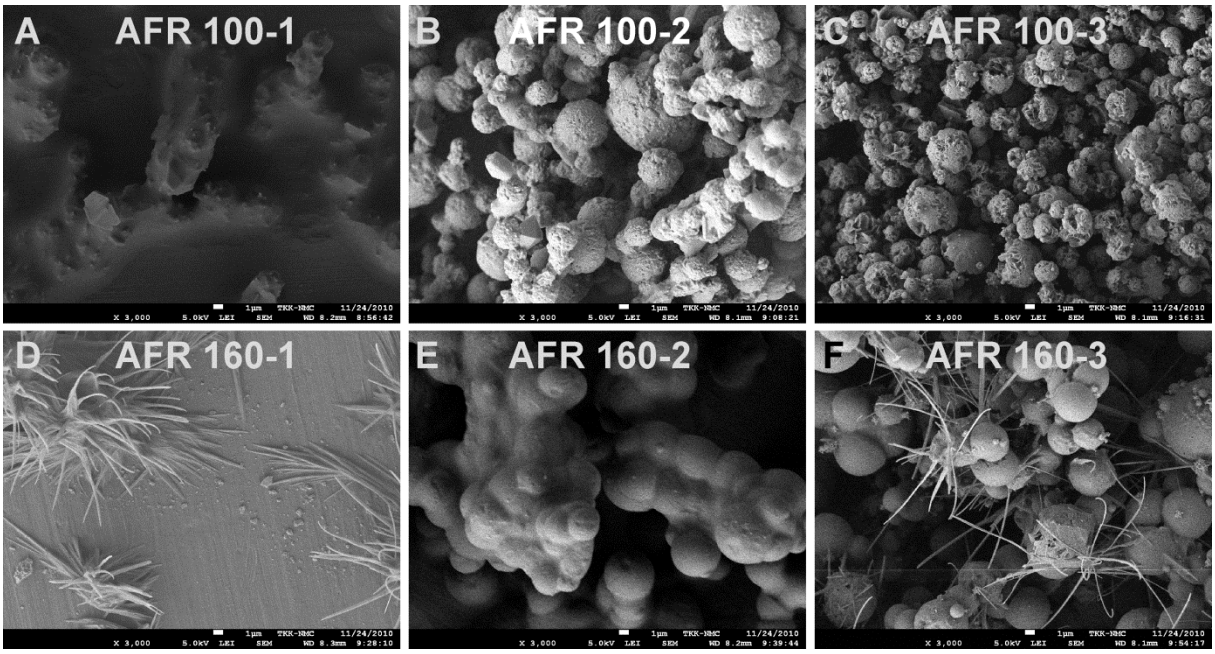

Figure 16 *SEM images of aerosol flow reactor produced microparticles. Size-excluded samples with D_{50} of 3.9 μm: AFR 100 – 1 (A), AFR 100 – 2 (B), and AFR 100 – 3 (C) and AFR 160 – 1 (D), AFR 160 – 2 (E), and AFR 160 – 3 (F). Needle-like particles are indomethacin crystals after being melted and recrystallized in the aerosol process (F) (from paper* ***II****).*

Drug loading in fractionated particle sizes of AFR 100-3 and AFR 160-3 samples are shown in Fig. 17. The AFR 100-3 had much lower drug amount in the smaller size fractions (<422 nm), while AFR 160-3 had higher drug amount in the smaller size fractions (<241 nm). It is reasonable and easier to understand for AFR 100-3 samples that the smaller particles contained low drug amounts as the particle sizes there were smaller than the particle size of the nanocrystals used. For the sample AFR 160-3, long and thin strips protruding from the particles were formed owing to the recrystallization of melted nanocrystals in 160 ^{o}C (seen in Fig. 16F). These crystalline strips were fragmented into smaller particles by the gas turbulence and particle collision. Small segments of strips were located in the impactor layer of the particles below 241 nm and lead to a high apparent drug contents. Total drug loadings in the two samples were not significantly different. For the AFR 100-3 sample, the drug loading was 8.7% (w/w) and in the AFR 160-3 sample the loading was 8.0% (w/w), which corresponded to their encapsulation efficiencies of 63% and 58%, respectively.

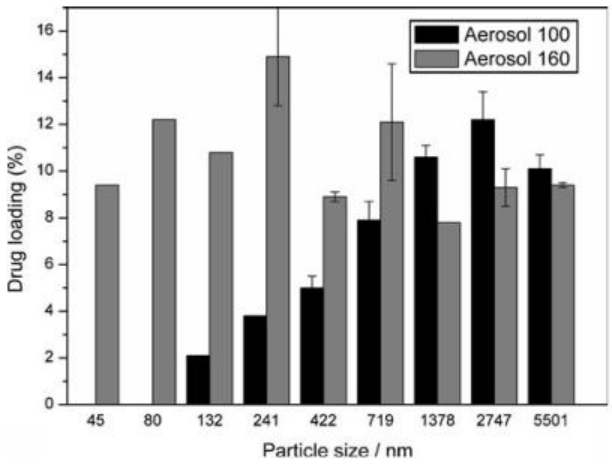

Figure 17 *Indomethacin loadings (w-%) in the microparticles of different sizes (n =1-3) (from paper **II**).*

Dissolution curves of the nanosuspensions, aerosol dried nanosuspensions, physical mixtures of drug and stabilizer, and the pure indomethacin are given in Fig. 18. Both aerosol flow reactor dried samples appeared to have as fast dissolution as the fresh nanosuspensions and significantly improved dissolution rate compared to bulk indomethacin. The slightly slower initial dissolution (at first 2 min) of the drug from the aerosol samples was attributed to the disintegration of microstructure. The re-dispersion testing supported the dissolution results. The particle size of fresh nanosuspensions was 485 nm with PI = 0.51. The re-dispersions of AFR 100-3 and AFR 160-3 in drug saturated solution showed the sizes of 490 nm (PI = 0.45) and 1330 nm (PI = 0.94), respectively. The AFR 100-3 preserved the nanoparticles and dissolved as fast as nanocrystals in the aqueous medium.

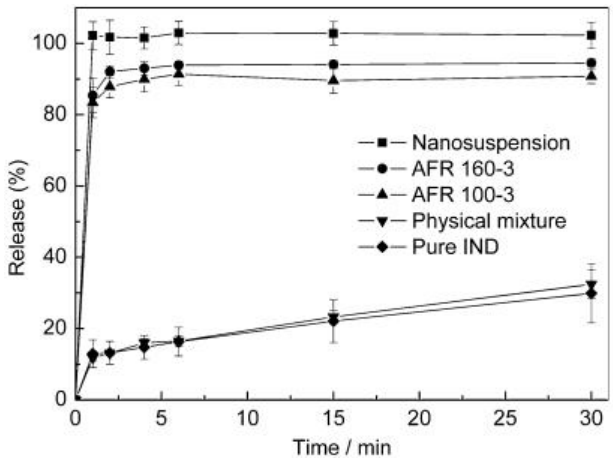

Figure 18 *Dissolution profiles of aerosol dried samples, indomethacin nanosuspension, physical mixtures and pure indomethacin (n=3) (from paper **II**).*

In summary, intact microparticles containing drug nanocrystals were achieved when mannitol and L-leucine as excipients were added and the precursor suspensions were dried in a low temperature. The dried powders (in low temperature) can disintegrate into nanoparticles and

dissolve as fast as nanocrystals in the aqueous medium. The “nanos-in-micros” powder is a novel tool for pharmaceutical formulations (e.g. tablet and pulmonary drug delivery) with fast-dissolving property.

5.2.2 Nanosuspensions for ophthalmic delivery (IV)

HPMC was chosen as the stabilizer for the preparation of brinzolamide nanosuspensions (BRA-NPs) since it could effectively reduce the particle size and make the nanosuspensions stable. Moreover, HPMC as an inactive ingredient has been approved for ocular drug delivery, ophthalmic lubricant and tear fluid substitute [205]. As the solubility of brinzolamide is affected by pH, the nanosuspensions were prepared in two different pHs of PBS media (pH = 4.5 and 7.4). The particle sizes and PIs of nanosuspensions are shown in Table 11. The nanosuspensions were formulated in three different formulations. The compositions of the formulations are listed in Table 11. In formulation II, polysorbate 80 was added to enhance the drug permeation. The bulk drug was used as a negative control.

Table 11 *The compositions of the BRA nanocrystal formulations (I-III) and the negative control (the final composition concentrations in formulations are BRA, 1 w/v%; HPMC 0.25 w/v%; BAC, 0.01 w/v%) (from paper **IV**).*

	Composition	Particle size (nm)	PI
Formulation I	BRA-NPs[a], pH 7.4 BAC PBS, pH 7.4	460 ± 10	0.21 ± 0.17
Formulation II	BRA-NPs[a], pH 7.4 Polysorbate 80 (0.25 w/v%) BAC PBS, pH 7.4	460 ± 10	0.21 ± 0.17
Formulation III	BRA-NPs[a], pH 4.5 BAC PBS, pH 4.5	530 ± 2	0.12 ± 0.02
Negative control[b],	bulk BRA, HPMC BAC PBS, pH 7.4	micronsized[c]	-

([a]BRA; 16 w/w%, [b]physical mixture, [c]particle size > 10 µm, outside the DLS detection limits)

The IOP reductions as a function of time in the laser-treated eyes after topical administration of different formulations are shown in Fig. 19. Compared to the 0.9% NaCl and NT groups, the administration of formulation III significantly lowered the IOP when measured after 60 min in the laser-treated eyes (Mann-Whitney U test, $P \leq 0.004$). The IOP lowering effects of formulations I and II and Azopt® in the laser-treated eyes after 60 min were equally significant when compared to the 0.9% NaCl group ($P \leq 0.019$), but not statistically significant when compared to the NT group. Comparing to the formulations I and II, formulation II showed a little bit higher IOP than formulation I at each measurement time point, which is in contrast to our expectations. The

surfactant polysorbate 80 added in formulation II did not enhance the drug permeation and reduce the IOP further. Formulation III reduced IOP after 60 min more effectively than formulation II (P = 0.017) and Azopt (P = 0.004), but the difference to formulation I was insignificant (P = 0.126). In a summary, the brinzolamide nanocrystal formulations were as good as, or even better than the commercial product Azopt® in IOP reduction.

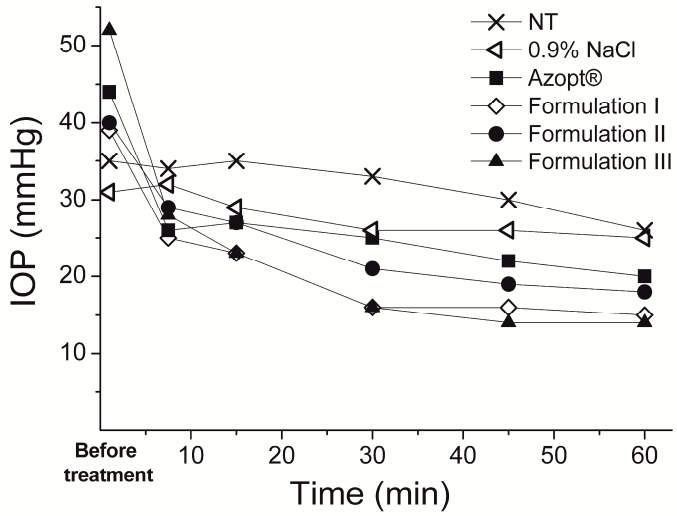

Figure 19 *The IOP values as a function of time in the laser-treated eyes after topical application (BRA; 100 µg) of BRA nanocrystal formulations I - III and Azopt®. For comparison, the results after the topical application of 0.9% NaCl as well as the values for the non-treated group (NT) are presented (from paper **IV**).*

6 Conclusions

The wet pearl milling technique is an effective and powerful tool to reduce the particle size of pharmaceutical drug compounds and manufacture nanosuspensions. For different drug compounds, the manufacturing parameters are different and should be optimized in order to obtain the smallest, most homogeneous and stable particles. The milling process, stabilizer and drug properties play important roles in milling results. Generally, the longer milling time leads to a smaller particle size with narrower particle size distribution. The stabilizer molecules fully covering drug particle surfaces through the hydrophobic or other interactions provide steric or electrostatic stabilization. Once the optimal preparation conditions are found out, the nanosuspensions were easily obtained with the small variation. The nanosuspensions exhibited a significantly increased dissolution rate compared to the micron-sized particles and good stability during storage period. The crystalline state was kept after milling.

A discriminatory method for nanosuspensions was developed based on the compendial rotating paddle apparatus. The results of simulation and experiments indicated that when the sample amount in dissolution medium was close to the apparent saturation solubility of the drug, the slowest dissolution rate and the most discriminating dissolution curves could be obtained. This condition was possible to apply to nanoparticles with various particle sizes. Sink conditions lead to a relatively rapid dissolution, which masks the differences between dissolution profiles caused by particle sizes. An excessive amount of sample also produces rapid dissolution rate since the dissolution medium is quickly saturated by the quickly dissolving smaller particles. This discriminatory method is simple to perform and can be potentially used in any nano-product development and quality control studies.

The “nanos-in-micros” particles were prepared using the combination of wet milling technique and aerosol flow reactor. Mannitol was used as the matrix and L-leucine as the coating layer, which protected the particles from aggregating/sintering. The drying process was temperature dependent. The primary nanoparticles did not survive at the higher processing temperature. The sample dried at the lower temperature would be suitable for further processing (e.g. tablet) and also suitable for pulmonary drug delivery after size fractionation. This “nanos-in-micros” concept can be as a tool for pharmaceutical formulation.

Another drug compound (brinzolamide) in the form of nanocrystals was formulated for ophthalmic drug delivery to reduce the IOP. Nanosuspensions were successfully prepared in two different pH values with HPMC as the stabilizer using the wet milling method. The brinzolamide nanocrystal formulation exhibit a rapid dissolution rate *in vitro*, and a good ocular bioavailability *in vivo*. The efficacy in IOP reduction of nanoformulations is as good as, even better than the commercial product.

As a summary, this thesis represents different milling conditions for nanosuspensions of three poorly water-soluble drug compounds. The wet milling technique offers the advantages of a high drug loading and less excipients requiring process and is a promising method for preparing drug nanosuspensions. Secondly, the pharmaceutical dosage forms of nanosuspensions, including dried powder for tablet or plumary delivery, and ocular nanosuspension were successfully developed. These formulations having the improved dissolution rates provide a potential opportunity in improving bioavailability, decreasing frequency of administration and improving patient compliance.

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