Formulation development of drug nanocrystals and nanoparticles for dermal delivery

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
MIRIAM COLOMBO
From Cantù, Italy

2018

Die vorliegende Arbeit wurde von März 2015 bis April 2018 unter der Leitung von Prof. Dr. Roland Bodmeier im Institut für Pharmazie angefertigt.

1. Gutachter:	Prof. Dr. Roland Bodmeier
2. Gutachter.	Prof. Dr. Philippe Maincent

Disputation am 08.06.2018

Alla mia famiglia

Acknowledgements

In first place, I would like to sincerely thank Prof. Dr. Roland Bodmeier for the opportunity he gave me to join his research group, for his being a concrete guide and supervisor, and for his support. I am very thankful for all what I have been learning from him and for what I have achieved during the last years under his supervision, on both scientific and personal aspects.

I kindly thank Prof. Dr. Philippe Maincent for reading and co-evaluating my thesis.

I am deeply grateful to Dr. Sven Staufenbiel for being a dedicated co-supervisor during the last two years, for the productive and motivating working atmosphere, for his scientific input in this work, and for being a friend, who supported me during many moments and celebrated with me every achievement.

I would also like to thank the committee members for their evaluation.

Thank you to the friends and colleagues of the research group, particularly to Marina, Prutha, Luisa and Lisa, for all the moments spent together thinking, solving problems, smiling, and supporting each other through good and bad times. A big “thank you” to them, for their friendship, and for all the wonderful memories which I will carry with me. Thanks also to Clara for her help in performing experiments for some time, and for the lovely friendship we made.
Thank you also to my lab-mates, for creating a nice atmosphere in room 133.

Many thanks also to Dr. Martin Körber, for his support and suggestions, to Dr. Andriy Dashevskiy, for the nice scientific discussions and for the enjoyable conversations in Italian, to Mr. Stefan Walter, for his kindness and for the flowers for my birthday every year, to Mrs. Eva Ewest, Mr. Andreas Krause and Mrs. Gabriela Karsubke for their assistance and help in experimental and bureaucratic matters.

My deepest gratitude goes to my parents Roberta and Luigi, who have dedicated their lives to my brother and to me, for letting us being the way we are, for giving us strength, and for helping us in every moment. To my brother Gabriele, for the warm love-filled hugs he always gave me, for accepting the physical distance despite of his age, and to my grandparents Franca and Attilio, for their never-ending support and for their lovely care.
Finally, a most sincere “thank you” goes to my beloved Maximilian, who has always been by my side during the last years, with his love, almost-infinite patience, and support, for the care and little thoughts which filled with serenity and happiness my daily-life.

Table of contents

1. Introduction

The present thesis deals with the preparation, characterization and investigation of properties of nanocrystals and nanoparticles of poorly soluble drugs for dermal application. In this introductory chapter, problems related to the increasing amount of new poorly soluble drugs are illustrated. Strategies to increase drug solubility are discussed next, with focus on drug amorphization and polymer-based nanoencapsulation. The strongest emphasis is given to nanosuspensions, which constitute the main part of the herewith presented work, their features and potential, manufacturing and characterization, but also their limitations and challenges. Finally, the applications of nanosuspension are discussed.

1.1. Poor aqueous solubility of novel drugs

The innovations in new target-specific drug development, high throughput screening and drug design pursued by pharmaceutical companies have resulted in new drugs characterized by poor water solubility, thereby low bioavailability [1, 2]. Any drug must indeed be absorbed in a sufficient amount in order to determine a pharmacological response and, hence, besides the case of cellular uptake, must be present as an aqueous solution at the site of absorption [3]. Insolubility in water is defined as more than 10,000 parts of solvent to dissolve 1 part of solute [4], *i.e.* a solubility of <0.1 mg/mL. The main reason for drug insolubility is the high hydrophobicity of the new compounds, thereby poor hydrogen bond forming ability with water, but potentially high affinity for the targeted receptors. A certain hydrophobicity is also a requirement to pass the lipidic domain of bilayer-phospholipid membranes [5]. Hydrophobicity is, however, often accompanied by high melting temperature, high molecular weight (>500 Da) and high LogP, which, according to Lipinski's rule [6], contribute to poor bioavailability due to poor water solubility. Interestingly, many of the new drugs have also poor solubility in organic solvents and oils [7].

Poorly soluble drugs belong to two classes of the biopharmaceutical classification system (BCS), which is a scientific framework that classifies drug substances based on their aqueous solubility and intestinal permeability, originally created by Amidon and co-workers [8], and whose use is strongly recommended by international agencies as the U.S. Food and Drug Administration (FDA) [9]. Drugs belong to class II if their permeability is high, while they are included in class IV in case of low solubility and also low permeability.

Poor solubility is reported to affect about 40% of marketed drugs and more than 70% of newly discovered drugs [10, 11], hence a very large portion of new substances, and solutions enabling the formulation of poorly water soluble compounds are necessary. Standard approaches in formulating these drugs are often, unfortunately, not successful, and result in

final products with suboptimal properties, characterized by poor bioavailability, lack of fed/fasted equivalence, toxicity related to large amounts of excipients, lack of optimal dosing, and, non-ultimately, poor patient compliance [12]. There is thus a growing need for novel and efficient formulation technologies able to overcome limitations associated with poorly soluble drugs and substantially improve discovery effectiveness and product performance [13].

1.2. Strategies to enhance drug solubility and/or dissolution rate

The selection of the proper technique to use in order to improve drug solubility and/or dissolution rate during product formulation is challenging and should be based on a careful consideration of the drug's physicochemical characteristics. A well-structured decision tree to utilize during the selection of the proper formulation approach was proposed by B. Rabinow (Fig. 1), who considered the drug's attributes contributing the most to poor solubility, and the correspondent strategy to use. This diagram suggests that a close interaction between chemical and pharmaceutical development is crucial during the early phase of product development [5].

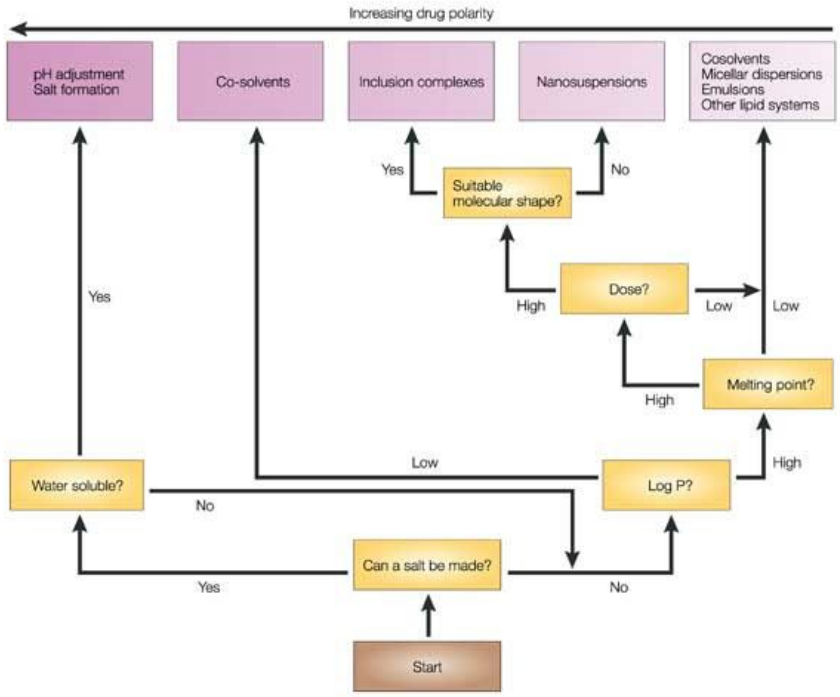

Figure 1 Decision tree for the selection of a formulation strategy based on drug's physicochemical characteristics (with permission from [14]).

The strategies to formulate poorly soluble drugs are based either on techniques to increase the drug dissolution rate or on techniques to achieve a sustained solubilization of the drug

[15]. The first group comprises micronization [16] and solid dispersions [17] technologies, while enhancement of drug solubility is achieved by salt formation [18], use of co-solvents and surfactants [19], inclusion complexes with cyclodextrines [20], lipidic systems [5] and polymeric nanoparticles [21]. Although the successful application of these techniques is reported in the literature, many are the cases of failure, where the increase in drug solubility/ dissolution rate was not high enough to generate the desired effects. Different drawbacks may be additionally encountered: drug precipitation due to dilution or to the physiological pH, limited dose escalation due to side effects caused by excipients' toxicity, *e.g.* with Cremophor® [22, 23], low drug loading due to solubility limit, need of large volumes, often incompatible with the intended administration route, ideal size to fit into cyclodextrines' cavity [2], extreme pH to dissolve the drug, accompanied with *e.g.* pain on injection, presence of solvent residues. Very promising formulation strategies which can overcome many of the aforementioned limitations, are the ones that combine increased solubility with enhanced dissolution rate, *i.e.* drug amorphization [24, 25] and nanosuspensions [13, 14].

1.2.1. Drug amorphization

The internal structure of a solid can exist in two different states, *i.e.* crystalline, which includes different polymorphic forms, cocrystals and solvates, or amorphous. The main difference among them is their internal organization: while crystalline compounds exhibit a long-range order, where the unit cell is repeated in the three space dimensions, the amorphous state is characterized by lack of the unit cell, hence a short-range molecular order which results in properties that strongly differ from those of crystalline materials. These properties have the potential to provide remarkable advantages, but also limitations.

The amorphous form represents the most energetic solid state of the material and thus results in enhanced properties. The main advantages of an amorphous drug in comparison to its crystalline form are the increased saturation solubility and thereby dissolution rate [26, 27], but favorable characteristics with regard to mechanical properties and tablettability are also reported [28]. The high internal disorder is, however, also responsible for a number of difficulties as their tendency to convert to the crystalline form upon storage (devitrification) [29, 30], which is also caused by their higher hygroscopicity [31], and a higher chemical and physical reactivity [30], potentially resulting in faster degradation. It should be mentioned, however, that phenomena like devitrification are dependent on parameters like storage conditions and glass transition temperature (Tg) and may be very slow, hence irrelevant for the efficacy and safety of a final product [30]. Nevertheless, the full understanding and knowledge of the product remains essential, and complete studies are necessary in order to move forward during product development.

1.2.1.1. Preparation and characterization of amorphous drugs

Three are the circumstances under which the amorphous state may arise [30]. Firstly, the amorphous form is deliberately produced to enhance the thermodynamic properties of the drug. The process for preparation of the amorphous form should be fast in order to give to the molecules only short time for interaction, hence no possibility to build optimal bonds which may lead to an organized crystal structure. The methods for preparation of amorphous drugs can be divided into two main groups: the ones where the starting material is the drug solution and the ones where the solid drug is used. Table 1 lists the currently available methods [28].

Table 1 Methods for preparation of the amorphous form of a drug based on initial dissolved or solid drug state.

Initial state	
Drug solution	**Solid drug**
• Spray-drying • Lyophilization (freeze-drying) • Rapid precipitation due to antisolvent addition • Precipitation of acids or bases due to sudden pH or T°C variation	• Quench cooling (also called melt-quenching) • Grinding, milling • Drying of solvated crystals • Extrusion

Secondly, co-processing a drug in combination with an amorphous material, *e.g.* with polymers like polyvinylpyrrolidone, may, under certain conditions, result in a final product where the drug is present in the amorphous state. The third case is the accidental generation of the amorphous form, for instance during processes involving high stress and harsh conditions as milling. This is relatively problematic because the drug may have undergone only partial amorphization, sufficiently large to cause changes in product performance, but too small to be easily detected during quality controls [30]. Although the confirmation of the drug amorphous solid state can be obtained by different techniques, a detection limit or the masking potential of co-processed drugs or excipients may impede the determination of the partial or total loss of crystallinity. The strategies used for characterizing amorphous drugs differ from those of crystalline compounds in the sense that the molecular-level structural elucidation is less applicable to amorphous solids, and greater emphasis is placed on structural mobility [28]. Standard techniques used for amorphous solid state characterization and information retrieved are listed in Table 2.

Table 2 Physical techniques for characterizing amorphous solids[a] (modified after [28]).

Technique	Information
X-ray Diffraction (XRD)	DOC, CK
Molecular Spectroscopy	SR (*e.g.* Raman and NMR)
Polarized light	Absence of birefringence
Differential Scanning Calorimetry (DSC)	DOC, CK, SR
Isothermal Calorimetry	SR, CK, DOC
Modulated DSC (MDSC)	Reversing vs. non-reversing heat flow, SR
Solution Calorimetry	Excess enthalpy, DOC
Solubility measurements	Saturation solubility, excess free energy
Density measurements	Density difference from crystalline solids
Viscometry	SR
Water Absorption (gravimetric)	Hygroscopicity, DOC, CK

[a]Key: DOC = degree of crystallinity, CK = crystallization kinetics, SR = structural relaxation

Although drug solubility and dissolution rate can be remarkably enhanced by preparation of the drug amorphous solid state, the physicochemical instability is the reason for the limited presence on the market of products based on this relatively old technology.

1.2.2. Drug polymer-based nanoencapsulation

The interest in the use of polymeric nanoparticles for drug delivery has kept on increasing during the last years. Nanoparticles are defined as solid particles with a diameter ranging between 1-1000 nm, generally 100-500 nm [32], which may, or may not be biodegradable, and where the drug may be dissolved, encapsulated, adsorbed or dispersed into them [33]. The term “nanoparticles” refers to particles with different morphologies, architectures and chemistry and it is, hence, quite general. Under this term, when speaking about polymer-based ones, nanospheres and nanocapsules are included [34]. The main difference between nanospheres and nanocapsules is their matricial or vesicular structure, respectively [35]. The two differently-structured particles are obtained by using different preparation methods [36]. The popularity of these systems is due to the several advantages they provide for drug delivery. These include versatility in controlling drug release [36], size-dependent re-circulation time, which is longer in case of particles <200 nm due to their reduced opsonization because of their high surface curvature, which prevents the efficient binding of opsonins [37], tumor-targeting potential [33], potentially improved drug stability [38], increased drug loading via solubilization within the hydrophobic particle core [34].

Nanocapsules are defined as nano-vesicular systems where the drug is confined within a cavity surrounded by a polymer layer [36]. Advantages of nanocapsules compared to

nanospheres, which are matrix systems where the drug is uniformly dispersed [36], are lower polymer content, higher encapsulation efficiency, better protection towards pH- and light-dependent degradation [34]. The drug confined within the cavity can be in a dissolved or solid state, or molecularly dispersed [34, 39]. Nanocapsules, as well as nanospheres, can be administered by different routes, *i.e.* oral, rectal, transdermal, ocular, nasal, subcutaneous, intraperitoneal, and intramuscular and they can also be injected directly into the systemic circulation without the risk of blocking blood vessels because of their small size [33, 34]. Nanocapsules are a promising drug delivery system and their use as drug carrier has been highlighted in order to achieve controlled drug release [40, 41], increased drug bioavailability [42], modification of drug biodistribution [43], potentially increased therapeutic effects [43], skin-barrier permeation and many other effects [34]. This formulation technology has, however, some limitations. The preparation method has to be selected depending not only on the type of polymer, but also on the physicochemical characteristics of the drug, and not all drugs can be processed to obtain nanocapsules [34, 44]. Concerns are also related to the use of organic solvents, and some preparation techniques are not able to provide a high drug encapsulation efficiency [44]. Additionally, in some cases, the non-reduction of toxic effects and the non-achievement of expectations related to drug-targeting performance have also been observed [45, 46].

1.2.2.1. Preparation methods and characterization of nanocapsules

The selection of the proper method to prepare stable nanocapsules with high drug encapsulation efficiency depends on the physicochemical characteristics of the drug and of the polymer selected [44]. Nanocapsules are generally prepared by using preformed polymers, while the polymerization technique, which is often used for preparing nanoparticles, is not used in this case. Generally, seven are the methods used for nanoencapsulation: nanoprecipitation, emulsion-evaporation, emulsion-diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer. Among the different methods, the nanoprecipitation method seems to be the most used [34]. It consists in the slow addition of the solvent (organic) phase consisting of a solution of the film-forming polymer, the active substance and, if necessary, other excipients as surfactants in a solvent or in a mixture of solvents to the non-solvent phase (generally water), which constitutes a non-solvent for the coating polymer. The addition is performed under moderate stirring. The main variables in this method are the injection rate of the organic phase into the aqueous phase, the agitation rate of the aqueous phase, the organic phase/ aqueous phase ratio and the method of addition [34]. The nanocapsule characteristics are also influenced by the nature and concentration of their components [47, 48]. The advantages of this method are that it is a simple, reproducible and low-cost procedure, and the obtained nanocapsules have

high drug encapsulation efficiency [34, 49]. The next two most commonly used methods are emulsion-diffusion and double emulsification [34].

Although the solvent emulsion-evaporation method is generally performed for microencapsulation [50, 51] rather than for nanoencapsulation, successful preparation of nanocapsules by this technique was reported [52]. This method consists in the rapid addition of the solvent (organic) phase, where the film-forming polymer and the drug are dissolved, to the non-solvent phase, generally water, followed by emulsification via energy input with an Ultra-Turrax or ultra-sonicator, and finally evaporation of the solvent under magnetic stirring or under vacuum [52]. The parameters affecting the process are the polymer concentration in the solvent phase, the quality of the solvent, the organic/ aqueous phase ratio, the rate of polymer precipitation, the energy input to generate the emulsion, the rate of solvent removal, the type and amount of stabilizer/ emulsifying agent and the physicochemical characteristics of drug and polymer [51, 53].

The properties and characteristics which are generally evaluated during and after nanocapsule preparation are: particle size, zeta potential, morphology, encapsulation efficiency, shell thickness, dispersion pH in case of pH-dependent polymers, *in vitro/ in vivo* drug release, physical and chemical stability. Table 3 summarizes the techniques used for characterization and the information retrieved.

Table 3 Techniques for nanocapsule characterization and information retrieved.

Technique	**Information**
Photon correlation spectroscopy (PCS)	Particle size, polydispersity index
Dynamic light scattering (DLS)	Zeta potential
Laser diffraction (LD)	Particle size distribution
Transmission/ surface electron microscopy (TEM/ SEM)	Structure and morphology, shell thickness
X-ray photoelectron spectroscopy (XPS)	Surface elemental analysis
X-ray Diffraction (XRD)	Solid state of surface
Differential Scanning Calorimetry (DSC)	Solid state of polymer and drug
High performance liquid chromatography (HPLC)	Drug content (relevant for encapsulation efficiency determination and during drug release experiments), chemical profile/ degradation
In vitro/ *in vivo* drug release	Drug release kinetics

Although the use of nanocapsules as drug delivery system has enabled the achievement of a variety of goals, including specific organ/ tissue targeting, controlled drug release, side-effect

reduction and increased drug bioavailability [34], the drawbacks related to opsonization, difficult scale-up, use of organic solvents, dependence of the preparation method on drug's and polymer's physicochemical characteristics, limited drug solubility and relatively low encapsulation efficiency if compared to other technologies have limited the launch to the market of polymer-based nanocapsules.

1.2.3. Drug nanocrystals (nanosuspensions)

Drug nanocrystals are pure drug particles in the nanometer size range (1-1000 nm) stabilized by proper type and amount of stabilizers (surfactants and/or polymers) [22]. When drug nanocrystals are dispersed in a liquid medium, generally water, nanosuspensions are obtained [14]. Formulating drugs as nanocrystals is the lead technology for compounds characterized by high LogP, high molecular weight, high melting temperature (Tm) and/or drugs insoluble in both water and oils, because the need of dissolving the drug is obviated by maintaining it in a crystalline solid state. Additionally, the utilization of the dense, solid state offers the advantage of a higher mass per volume loading, which is crucial in case of drugs which require a high dosing in small volumes, for instance for intramuscular (IM) or ophthalmic applications [14]. Nanocrystals have indeed a drug loading of 100% because of the absence of carrier material [22], oppositely to the case of polymeric nanoparticles, where the drug may be distributed in the particle core or on the surface. In addition to the high drug loading, nanocrystals are characterized by a set of favorable properties and advantages which justify the extensive research efforts pursued in this field and the presence on the market of products based on this recent technology. Drug nanocrystals are indeed one of the most important strategies to formulate and enhance the bioavailability of hydrophobic drugs [54].

1.2.3.1. Formulation theory, main properties and advantages

Nanocrystals can be prepared either by building crystals up from molecules in a solution, or by breaking particles until the nanometer size range is reached. In both cases, a new surface area is formed, which necessitates a free energy cost, as per the equation (1):

$$\Delta G = \gamma_{s/l} * \Delta A \quad (1)$$

where ΔG is the free energy, $\gamma_{s/l}$ is the interfacial tension between the solid particle and the liquid, and ΔA is the new surface area. The interfacial tension arises during nanocrystal preparation because the water molecules are energetically driven to leave the surface as they incur fewer attractive forces when in contact with a free surface [14]. This leads to a higher free energy and hence thermodynamic instability. The system counteracts the

increase in interfacial tension by reducing the surface area with two mechanisms: by dissolving crystalline nuclei or by agglomerating particles. The size stability of a final product is a critical aspect to ensure its safety and efficacy, especially if intravenously administered because the formation of large particles may result in capillary blockade and embolism. The formulators resist this tendency of instability by addition of agents which lower the $\gamma_{s/l}$ and hence the free energy of the system. The stabilization is more efficient if these agents are already present during the formation of new surface area, rather than added afterwards [14]. The two main mechanisms by which nanosuspensions can be stabilized are electrostatic repulsion and steric stabilization, which are achieved by adding respectively ionic or non-ionic stabilizers to the medium [55]. In addition to the type, two more aspects should be also carefully considered: the stabilizer amount and its effect on drug solubility [56]. The amount of stabilizer should be below the critical micelle concentration in order not to form micelles and incur thermal instability. Moreover, it should have no or only minor effect on drug solubility, as its increase would result in further instability due to Ostwald ripening. The stabilization via electrostatic repulsion has its fundament in the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory [57], which assumes that the forces acting on a particle include attractive Van der Waals (VDW) forces and repulsive electrostatic forces. The predominance of a certain type of force, hence the aggregation or repulsion between two particles, depends also on their distance: particles relatively far or very close will aggregate, while a proper distance will maintain them apart. The total potential energy (V_T) of a particle-particle interaction is the sum of a repulsion potential (V_R) generated by the electrostatic forces and an attractive potential (V_A) due to VDW forces. The repulsion potential is very sensitive to ion concentration in the medium, whose increase leads to loss of stabilization and aggregation. The measurement of the zeta potential, which is the electric potential at the shear plane, is a very successful tool used to predict the stability of nanosuspensions stabilized by electrostatic repulsion: a higher zeta potential correlates to a more stable suspension. A zeta potential of ± 30 mV is believed to provide good stability when using electrostatic stabilization [56]. The commonly used ionic stabilizers are sodium dodecyl/lauryl sulfate (SDS, SLS), chitosan and docusate sodium (DOSS).

Steric stabilization is achieved by introduction into the medium of amphiphilic non-ionic stabilizers, generally polymers, which are adsorbed onto the particle surface through a hydrophobic anchor segment, while the hydrophilic tails are well-solvated in the medium. The stabilization is obtained by a repulsion effect through a steric mechanism involving both enthalpic and entropic contribution [58]. The key to obtain a good stabilization and prevent agglomeration is indeed a strong solvation between the solvent which constitutes the medium and the stabilizing tails [55]. If the medium and the tails have similar characteristics,

meaning if the medium is a good solvent for the polymer, than a sufficient reduction in the depth of the potential well on contact may be achieved, and the Brownian motion may keep the system in a dispersed state. In case of a good solvent, the polymer chains will interpenetrate upon collision of two particles. This will increase the density of the polymer segments in the surrounding of the particles. The medium will consequently diffuse into the region between the surfaces to reduce the segment concentration and to bring the surfaces apart. Moreover, because the segments are linked together in a polymer chain, the increased density will constrain the chains, leading to a reduction of the configurations which they may adopt. This implies a reduction, *ΔS*, in the entropy of the system and an increase in the free energy. This contribution is called “entropic repulsion” [59]. The enthalpic repulsion is obtained by overlapping of the side chains of the polymer, hence local increase in osmotic pressure, thereby more water comes within the tails to reduce this increase. The tails should also be long enough to provide a steric barrier among the particles. Relevant is also the interaction of the hydrophobic portion of the stabilizer with the hydrophobic drug surface. This may constitute a drawback of steric stabilization, as the selection of the proper stabilizer type is highly dependent on the drug of interest [60]. The generally used steric stabilizers are non-ionic surfactants as Tweens or poloxamers and polymers like polyvinylpyrrolidone (PVP) hydroxypropylmethylcellulose (HPMC), Vitamin E TPGS 1000 and polyethylene glycole (PEG). Additionally, as the mechanism of interaction between steric stabilizers and dispersed particles is not yet well-understood [55], finding the right formulation may often be time-consuming and burdensome, and an empirical screening of different stabilizers may be necessary [61, 62].
The two approaches, electrostatic and steric stabilization, may also be used simultaneously, especially because steric stabilization is more affected by temperature changes rather than electrostatic repulsion [14, 55].

The main features of nanocrystals are their increased dissolution rate and saturation solubility, which are responsible for the remarkable advantages that this technology is able provide [22, 63]. The increased dissolution rate of nanocrystals is explained by the Noyes-Whitney equation (2) [64]:

$$\frac{dM}{dt} = \frac{DA(C_S - C)}{h} \qquad (2)$$

where *dM/dt* is the dissolution rate, *D* the diffusion coefficient, *A* the surface area, C_s the saturation solubility, *C* the solubility in the bulk medium, and *h* the thickness of the diffusional layer. The reduction to the nanometer size range results in an increased surface area (*A*), which is directly proportional to the dissolution rate (*dM/dt*), hence enhanced dissolution rate

is obtained. This feature is remarkably favorable for drugs whose slow dissolution rate is responsible for their low bioavailability, and has been particularly exploited for the oral administration of poorly soluble drugs. The increased saturation solubility finds its explanation in the Ostwald-Freundlich equation (3) [2], which hypothetically pertains to spherical particles and that describes the increase in solubility by particle size reduction:

$$\ln \frac{S}{S_0} = \frac{2M\gamma}{\rho r R T} \quad (3)$$

where S is the solubility at a given temperature T, S_0 is the solubility of a particle with infinite radius, M is the molecular weight of the solid, γ is the interfacial tension (solid/liquid), ρ is the density of the particle, r is the particle radius and R is the gas constant. The practical application of this equation for predicting the increase in solubility is, however, difficult because of the challenge of measuring the interfacial tension. Moreover, in order to have the parameter $S/S_0 > 2$, hence a substantial effect, the particle radius should be smaller than 200 nm [2], therefore a markedly high solubility increase should not be expected. The increased saturation solubility of nanocrystals is also supported by the Kelvin equation, which describes the vapor pressure over a curved surface of a liquid droplet in a gas [65], and whose theory can be applied for a solid drug particle in a liquid medium, where the vapor pressure is replaced by the dissolution pressure [22]. Based on the Noyes-Whitney equation, the enhanced saturation solubility additionally contributes to a further increase in dissolution rate because the two parameters are directly proportional.

Favorable properties of nanosuspensions are not limited to increased solubility and dissolution rate, but comprise a variety of attributes, many of which are related to their physicochemical characteristics. Table 3 summarizes the properties of nanosuspensions and the corresponding advantages obtained.

Table 3 Properties and corresponding benefits of nanosuspensions (modified after [14]).

Property/ attribute/ characteristic	Benefit
Solid state: high drug loading	Reduced administration volumes and/or units Increased patient compliance
Solid state: stability	Increased stability towards hydrolysis and oxidation
No need of harsh vehicles (extreme pH, co-solvents, high amounts of excipients)	Reduced toxicity and side effects
Reduced particle size	Increased dissolution rate and solubility Increased adhesiveness
Multiple-unit dosage form	Improved reproducibility after *e.g.* oral absorption Improved dose-bioavailability proportionality
Particulate dosage form	Potential for sustained release (IV, IM)

Nanosuspensions are thus a technology applicable for challenging drugs, as they offer a concrete formulation strategy, versatility in the administration route and a set of favorable properties related to their solid and physicochemical characteristics. However, their drawbacks should also be mentioned, as they may limit and/or prevent achieving a successful final formulation and product.

1.2.3.2. Drawbacks and limitations of nanosuspensions

Products based on drug nanocrystals should remain stable for their entire shelf life in order to comply with the safety and efficacy requirements. Following administration, nanocrystals should not aggregate, but remain distinct single units in order to exercise their properties and provide the expected and desired effects. The main drawback of nanosuspensions is, however, their physical instability due to the increase in free energy consequent to the formation of new surface area, which results in particle agglomeration, consequent loss of nanocrystal advantages and risk of side effects. This limitation can be overcome by utilizing the proper type and amount of stabilizer, as previously described (section 1.2.3.1.). Agglomeration is, however, not the only instability phenomenon, but other limitations and risks are also present. Unwanted and uncontrolled sedimentation is the extreme case of nanosuspension instability and takes place when the gravity of the drug particle is greater than the buoyancy provided by the dispersing system [55]. Irreversible sedimentation is hindered by the use of a proper formulation (stabilizer). Another event of instability is crystal growth, which is known as Ostwald ripening in colloidal suspensions and consists in the growth of larger particles to the expenses of the smaller ones [55]. This phenomenon is

caused by the different saturation solubilities of drug particles with different sizes, which results in a concentration gradient and diffusion of dissolved molecules from the smaller crystals, which have higher saturation solubility and dissolution rate, towards the larger particles. This creates a supersaturated solution in the vicinity of the larger particles with subsequent precipitation of the dissolved molecules on their surface, hence leading to crystal growth. Simultaneously, the diffusion of molecules produces an unsaturated solution around the still-intact smaller particles, causing their dissolution. This process results in a shift of particle size and size distribution. The tools for preventing Ostwald ripening are the following: a virtuous manufacturing process, resulting in narrow size distribution, hence only minor differences in the saturation solubility of differently-sized drug particles; the use of proper stabilizers, which should wet the particle surface but not increase the drug solubility; low storage temperatures, as drug solubility tends to increase with increasing temperatures [66].
With regard to solid state instability, the crystallinity of the drug particles should remain unchanged throughout the entire manufacturing process and storage, as different solid states and crystal packings remarkably affect drug solubility [26]. Particular attention should be paid to amorphous formation because it may reconvert to the most thermodynamically stable crystalline form upon storage. Increased medium viscosity and careful control of process temperature help avoiding solid state modifications [56]. Drying techniques as freeze-drying [67] or spray-drying [68] are often utilized to obtain a stable nanosuspension if no other way is available. The drying process may, however, also result in instability issues. Fundamental is avoiding aggregation and crystal growth during drying, otherwise the final powder cannot be redispersed. The key to prevent instability during drying is the quality of the formulation: proper excipients like matrix formers and cryoprotectants should be added.
Chemical stability should be also ensured. This is, however, mostly dependent on the drug. Esters and amides are indeed susceptible to hydrolysis, while amino groups may undergo oxidative degradation. Moreover, formulating drugs as nanosuspensions may also improve their chemical stability [69].
Relevant for intravenously-administered nanosuspensions is also their plasma stability, especially if an increased (prolonged) drug exposure is desired, as for the case of enhanced permeation and retention effect (EPR) [56]. Testing in plasma is hence generally performed before parenteral application [70].

Major concerns were brought up in the last years by international agencies with regard to toxicity of nanosuspensions. In addition to the burst release which may characterize a nanosuspension readily dissolving after administration, issues have raised related to the potential novel features that very small nanocrystals have. The European Commission and the European Medicines Agency (EMA) have given the following definitions [71, 72]:

- *Nanomaterial:* natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.
- *Nanotechnology*: the production and application of structures, devices and systems by controlling the shape and size of materials at nanometre scale. The nanometre scale ranges from the atomic level at around 0.2 nm (2 Å) up to around 100 nm.
- *Nanomedicine*: the application of nanotechnology in view of making a medical diagnosis or treating or preventing diseases. It exploits the improved and often novel physical, chemical and biological properties of materials at nanometre scale.

Agencies have hence taken 100 nm as upper size limit for defining a nanomaterial, and they acknowledge the fact that the latter has novel properties, not encountered with larger particles. The reason for the selection of 100 nm as threshold is that particles with a size ≤ 100 nm can enter any cell via endocytic process, while larger particles require a phagocytic process to be internalized, *e.g.* by liver macrophages. Due to the limited availability of these cells and to the difficulty of reaching some of them, for instance the macrophages of the liver, the risks related to particles larger than 100 nm is relatively low. 100 nm should, however, not be taken as strict limit during toxicity considerations because some mechanisms, even though rare, allow internalization of particles ≥100 nm by non-phagocytic processes [73]. Moreover, drug nanocrystals are generally larger than 100 nm, especially if prepared by wet bead milling and, since their dissolution happens very fast, their size reduction below 100 nm will not last for long. This aspect, however, became extremely relevant and a system was created for classifying nanoparticles according to the risks associated to their use: the nanotoxicological classification system (NCS) [74]. The latter divides nanoparticles in four classes (I-IV) according to their size and biodegradability. Each class is further divided based on the biocompatibility or not of the particle.
Drug nanocrystals are constituted of pure drug, thus they are biodegradable. However, excipients used for stabilization purposes may result in side effects, *e.g.* SDS-induced skin irritation [75].

1.2.3.3. Manufacturing techniques for drug nanocrystals

The methods for preparing drug nanocrystals can be divided into two main categories, *i.e.* bottom-up and top-down technologies [76], where, respectively, drug nanocrystals grow from a drug solution, or their dimensions are reduced until the nanometer size range is reached.

1.2.3.3.1. Bottom-up method

The bottom-up technique consists in a controlled precipitation process, whose conditions are chosen to minimize the particle size [77]. This method involves two main steps: i) the creation of crystal nuclei and ii) their successive growth. Rapid nucleation and slow crystal growth are necessary to obtain a stable nanosuspension of small-sized nanocrystals [14]. The process starts with dissolving the drug within an organic solvent, which has to be miscible with the non-solvent (generally water). High-supersaturation conditions are required for rapid nucleation, hence the organic solution should be highly-concentrated. The latter is afterwards added under rapid mixing to the non-solvent, where proper stabilizers are dissolved. Amorphous drug nanoparticles may also be obtained by bottom-up [69] because rapidly grown nuclei tend to be more imperfect. Although the precipitation approach holds a great potential with respect to improved drug bioavailability because of the possibility of manufacturing crystals characterized by a very small particle size (<100 nm), no products based on this technology are currently on the market [69]. The control of the process, which is a requirement for an industrially-feasible manufacturing technique, is indeed difficult [76].

1.2.3.3.2. Top-down methods

Top-down methods consists in the reduction of the crystal size to the nanometer range [69] and they include wet bead milling, high pressure homogenization and combination techniques. So far, all marketed products, with only one exception, are manufactured by wet bead milling [78]. This process was first developed at the beginning of the 90s by Gary Liversidge and his colleagues at Sterling Drug Inc./Eastman Kodak, who applied to drug particles a process commonly used in the paint and ink industry [76, 79]. The mills used have been later re-designed and sanitized to meet the needs of pharmaceutical companies. The process is now known in the pharmaceutical industry as NanoCrystal®.

The reduction of the particle size during wed bead milling is based on the high energy and shear forces generated as a result of the impact of the milling media (beads/ pearls) with the drug and with the milling chamber [80]. The milling media is rotated by a drive shaft attached to rotated disks in order to produce a very high shear rate and hence provide the energy input necessary to break the particles down [80]. Grinding mills are available in different sizes and set-ups to allow a relatively easier up-scalability. For all scales of operation, the wet bead milling process is similar: the grinding beads are first charged into the chamber, which can be vertical or horizontal depending on the scale, followed by addition of the drug dispersed in the stabilizer solution (Fig. 3). The process can be performed in either batch or re-circulation mode [76]. The milling process is affected by the following parameters: rotation speed, size and amount of grinding agents, milling time, suspension viscosity, drug amount and temperature [80, 81]. Generally, the residence time for most crystalline compounds to

reach a particle size <200 nm is approximately 30-120 min [70]. Indeed, after a certain time and by operating under a set of conditions, a milling limit will be reached and further energy and time will not effectively decrease the particle size of the suspension [70], which may even start increasing.

The main advantages of wet bead milling are its up-scalability and high reproducibility [13, 80], with very little batch-to-batch variation once the process has been optimized, the manufacturing of small nanocrystals with a monodisperse distribution, and that almost any API can be processed with this technology [79]. The particle sizes generally obtained range between 100 and 300 nm, irrespectively of the type of mill [76]. The major drawback of this technique is that it involves shearing of media against the drug and the milling chamber to achieve particle size reduction, hence contamination from beads and ball mill and wear of the set-up are issues [70, 80]. The increase of contaminants and wear of the set-up is directly proportional to the rotation speed [81]. The quality and durability of the milling media is also a concern. Chemical and physical (solid state, Ostwald ripening) integrity must be additionally controlled at the end of the process, as the high energy might have an effect on these aspects. Loss of drug due to adhesion onto the beads' surface may also occur [22]. However, the use of highly crosslinked polystyrene beads or yttrium-stabilized zirkonium beads and a ball mill of high quality, together with jackets which maintain low product temperatures throughout the process, prevent or reduce the extent of the abovementioned drawbacks. Overall, wet bead milling is a universally-applicable process which is considered superior over the other techniques for particle size reduction and it is currently extensively used in both the academic research [69, 76, 80], and industrial preparation of marketed products [22, 54, 70].

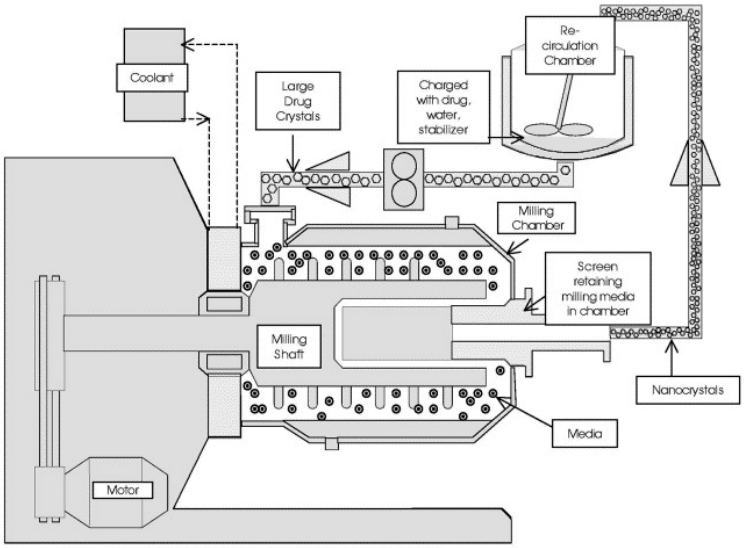

Figure 3 Schematic representation of a ball mill operated in the recirculation mode (with permission from [13]).

The second top-down method for particle size reduction is high pressure homogenization (HPH). Although only one marketed pharmaceutical product (Triglide®) is prepared by HPH, this technique is used in the cosmetic and food industry [82] and extensively in the pharmaceutical academic field [77, 83-85]. The term HPH comprises two different principles/ set-ups: microfluidics and piston-gap homogenization.

Microfluidization is based on a jet-stream principle, where the suspension is accelerated and passes through a homogenization chamber, whose shape can be “Z”, and in this case the particle size reduction happens because the suspension changes few times its direction, thus the particles collide against each other and against the wall, or “Y” shape, where a frontal collision between two fluid streams results in particle size reduction [77]. Limitations of this technique are the high number of passes necessary, hence long processing times, and the presence of a relatively large fraction of microparticles at the end of the process, especially in case of hard drugs [77].

The piston-gap homogenization technique for preparation of drug nanocrystals was first developed by Müller and his colleagues [86] and it involves the forcing of a suspension under pressure through a valve that has a narrow aperture [14]. This causes an increase in dynamic pressure compensated by a decrease in static pressure that leads to formation of bubbles of water vapor at room temperature, which collapse as they exit the valve (cavitation) [14, 22]. Cavitation-induced shockwaves are hence generated, resulting in particles crack. Crystals react differently to this process according to their strength. However, very hard crystals also inevitably possess defects in their structure, resulting in weak points where their breaking can start [14]. The main parameters affecting the process are powder density, homogenization pressure, number of cycles, temperature and drug’s characteristics *e.g.* hardness [77, 87]. The latter seem to affect the process much more than in case of wet bead milling [76]. High pressure homogenization is an up-scalable process and the contamination from the set-up is lower than in case of wet bead milling [76]. The process can additionally be performed with non-aqueous suspensions of the drug, for instance in media like PEG 400 or 600, other oils or even in molten PEG 1000 [77]. High pressure homogenization can also be combined with other techniques.

The third way of nanocrystal preparation consists in the combinative technologies. The latter were implemented considering the two main limiting factors of wet bead milling and high pressure homogenization once manufacturing at industrial scale: the necessity of the micronized-drug as starting material and the relatively long processing times [76]. The processing times can be considerably reduced by pre-treating the drug before the top-down process is performed. The pre-treatment can consist in different processes, for example micro-precipitation from a drug solution [2], spray-drying [76] or freeze-drying [14], and it is

followed by a top-down technique, generally high pressure homogenization [76]. The combinative technologies have, however, not found any application in the industry, yet.

1.2.3.4. Characterization of nanosuspensions

The quality and stability of nanosuspensions are essential parameters for the safe administration of the final product and are evaluated by proper characterization tests. While some methods are always applied to every nanosuspension, some tools are more specific for a certain intended administration route. The characterization tests can be divided into four main categories: physical, chemical, biological [14] and performance-related tests. A summary of available techniques for nanosuspension and nanocrystal characterization and information retrieved is presented in Table 4.

Physical tests are performed to determine the stability of nanosuspensions with regard to mean particle size, particle size distribution, zeta potential, particle shape and crystallinity. These tests should be performed directly after preparation and over time to assess the shelf-life of the product. Once the proper formulation with the desired shelf-life has been obtained, accelerated stability tests should be performed, which challenge the system both thermally and mechanically [14]. The mean particle size and the width of distribution (polydispersity index) of nanosuspensions are measured by photon correlation spectroscopy (PCS) [88]. The measuring range of PCS is, however, between 3 nm and 3 µm, hence the presence of larger particles and/ or aggregates would not be detected by this technique [89]. The certainty about the absence of large particles is fundamental in case of intravenously-administered nanosuspensions. The smallest blood capillaries have indeed a diameter of ~5 µm, hence particles with this (or larger) size may cause vessel blockade and embolism [89]. The measuring gap left from PCS can be fulfilled by additional techniques, namely laser diffraction (LD), light microscopy and Coulter counter. The measuring range of laser diffraction covers particles from 0.01 µm up to 3500 µm, depending on the equipment type [89, 90]. The information provided by this technique is in *e.g.* volume distribution, and typical characterization parameters are the diameters (D) 10, 50, 95 and 99. The latter values indicate that respectively 10, 50, 95 or 99% of the volume of the particles have a particle size lower than the given number. The drawback of laser diffraction is the need of diluting, sometimes strongly, the suspension in order to perform the measurement. This parameter depends on the machine model used and on the concentration of the nanosuspension, as higher volumes are needed for lower-concentrated ones. Light microscopy is a very useful tool to collect supportive information and exclude the presence of large aggregates and/or agglomerates. The last method for particle size analysis is the Coulter counter, whose use is recommended in case of intravenously-administered particles [89]. This analysis provides

absolute data, meaning the absolute number of particles per volume unit of a certain size class, while LD provides a relative size distribution. Another important parameter related to stability is the surface charge of the particles, which can arise from ionization of the particle surface or by adsorption of ions (*e.g.* ionic stabilizers) [91]. The surface charge can be measured by electrophoresis or by electrophoretic light scattering (zeta potential (mV)) [89]. Zeta potentials in the range of ±15-30 mV, depending on the type of stabilizers used, ensure proper particle size stability. The determination of the particle shape is generally performed by transmission or scanning electron microscopy (TEM or SEM, respectively) [87, 92], however the use of atomic force microscopy (AFM) is also reported [93].

The viscosity of nanosuspensions is measured as indicator of the extent of flocculation, hence indicator of physical stability. A well-stabilized nanosuspension is generally characterized by a Newtonian behavior, while shear-tinning is inherent to flocculating systems [91]. The difference among the two types of behavior is the constant or decreasing viscosity with increasing shear rate, respectively. The viscosity is determined as a function of shear rate by a controlled-stress or control-strain rheometer. The working shear rate range goes from 0.01 to 1000 s^{-1}, and the viscosity may vary from 1 cP for water or diluted nanosuspensions to 1000 cP (or larger values) for more concentrated nanosuspensions.

Another important aspect to consider during stability study is the solid state analysis of the drug formulated as nanosuspension. Process involving high shear stress, as wet bead milling, or bottom-up techniques followed by high pressure homogenization may result in conversion from one crystalline form to a different one (polymorphism), loss of crystallinity or drug amorphization [89, 91]. However, regulatory agencies stress the necessity to ensure the maintenance of one and the same drug solid state throughout the process [94] because different solid states have different physicochemical properties, hence different performances following administration [26, 30]. The solid state of nanocrystals can be evaluated via different techniques: differential scanning calorimetry (DSC), X-ray diffraction (XRD) and, in some cases, Fourier transform infrared spectroscopy (FTIR) [89, 91]. The solid state of protein nanoparticles has also been successfully determined by solid state NMR [95].

Depending on the intended administration route, nanosuspensions may need to fulfill additional tests. This is for instance applied when they are intended for IV administration. Physical properties required in this case include syringeability/ injectability. If lyophilized products are prepared, their resuspendability has to be evaluated as well [14].

The chemical stability of nanosuspensions is mostly related to the drug processed, its properties and degradation profile [69]. Chemical tests are performed to determine the purity of the active ingredient and other excipients (stabilizers, preservatives), absence (or controlled) presence of degradation products, moisture content in case of lyophilized and

solid dosage forms and pH stability, particularly for IV products [14]. Surface hydrophobicity/ hydrophilicity should be determined as well in case of IV-administered particles, as these parameters determine the extent of interaction with cells [89], adsorption of plasma proteins and, hence, organ/ tissue distribution [96, 97].
The parenteral administration route additionally requires pharmaceutical nanosuspensions to comply with certain biological requisites as sterility, pyrogenicity and isotonicity. The suspensions should be non-toxic, non-irritating and non-hemolytic [14].

The performance of a product is the key for its success in the therapy. Nanosuspensions are tested to evaluate their efficacy *in vitro* and afterwards *in vivo*. In addition to the advantages related to a specific administration route, for instance adhesiveness with regard to dermal products [89], the properties of nanosuspensions which are applicable for every administration route are their increased dissolution rate and saturation solubility [22, 78]. Testing these properties is relevant to assess the benefits of nanosuspensions in comparison to traditional drug formulations. The saturation solubility of a drug once formulated as nanosuspension can be determined by *in situ* [98, 99] or non *in situ* [100, 101] methods, although the latter are considered less accurate and precise than *in situ* methods [99]. The dissolution rate can be calculated by the slope of the curve obtained during standard dissolution experiments performed with USP apparatus [101] but also by calculating the released drug amount in time unit per constant area during, for instance, UV-imaging and channel-flow dissolution studies [102, 103]. However, although nanocrystals have achieved promising results during *in vitro* studies, their *in vivo* fate is not well understood, yet [104]. The data in the literature sometimes mismatch: while a great increase in drug bioavailability was obtained during *in vivo* studies in dogs [13, 70], in some other cases a lack of *in vitro-in vivo* correlation (IVIVC) was obtained, with nanocrystals performing worse than the marketed formulation, as in the case of itraconazole nanocrystals versus the commercially available Sporanox® [102]. The explanation given for this result was related to the shorter transit time of itraconazole nanocrystals from the stomach to the small intestine, where the solubility of the drug was lower due to the higher pH, resulting in instability of the highly supersaturated solution (generated because of the formulation as nanocrystals) followed by drug precipitation [102]. It should be also considered that while the pH used to mimic the stomach compartment during *in vitro* studies is ~1, the pH of the GI tract of the animal model used might be different, for instance it ranges from ~3.9 to ~6 in rats, and this might have strong implications on the results and their correlation [105]. Furthermore, the environment (amount of water, pH) that the nanocrystals meet following administration consistently varies depending on the administration route. Nanocrystals may survive immediate dissolution, potentially resulting in their phagocytosis, or, if their surface and size comply with the

required characteristics, EPR effect and tumor targeting may be obtained [104, 106]. Drug's physicochemical characteristics should also always be considered during the IVIVC studies as they affect the dissolution rate, which can be very low for extremely poorly soluble drugs. This feature has been exploited in the marketed product INVEGA® SUSTENNA® (Xeplion® in Europe), an intramuscular depot nanocrystalline formulation of paliperidone palmitate, whose sustained release is mainly due to the remarkably low drug solubility [104, 107]. More studies should thus be focused on establishing study conditions based on considerations related to drug's characteristics, nanocrystal size, shape and surface properties and administration route to obtain, potentially, generally applicable experiment set-ups and proper IVIVC.

Table 4 Techniques for nanosuspension/ nanocrystal characterization and information retrieved.

Technique	Information
Photon correlation spectroscopy (PCS)	Particle size, polydispersity index, resuspendability
Electrophoretic light scattering (ELS)	Zeta potential
Laser diffraction (LD)	Particle size distribution (relative), resuspensability
Coulter counter	Particle size distribution (absolute), resuspensability
Transmission/ surface electron microscopy (TEM/ SEM), atomic force microscopy (AFM)	Structure and morphology, shell thickness
Light microscopy	Presence of large aggregates/ agglomerates
X-ray Diffraction (XRD)	Drug solid state
Differential Scanning Calorimetry (DSC)	Drug solid state
Fourier transform infrared spectroscopy (FTIR)	Drug solid state
Solid state NMR	Drug solid state
Texture analyzer	Syringeability/ injectability
Rheometer	Viscosity
High performance liquid chromatography (HPLC)	Drug amount (*e.g.* during drug release experiments), chemical profile/ degradation
Hydrophobic interaction chromatography (HIC)	Surface hydrophobicity
Rabbit pyrogen test (RPT), bacterial endotoxin test (BET), monocyte activation test (MAT)	Pyrogenicity
Membrane filtration, direct inoculation	Sterility
In vitro drug release methods and *in vivo* studies	Drug release kinetics, therapeutic effect

1.2.3.5. Applications of nanosuspensions and marketed products

The formulation of drugs as nanosuspensions resulted in a remarkably positive impact on their performance [70, 108], which justifies the launch in the market of products based on nanosuspensions after only about 10 years from their discovery. The characteristics which

affect performance are many and can be differentially exploited based on the administration route: the small size of nanocrystals enhances solubility and dissolution rate, hence bioavailability following oral administration; simultaneously, the particulate nature and the surface properties dictate targeting and possible EPR effect after intravenous injection. In general, however, increased dissolution rate and saturation solubility are the properties which mainly justify the formulation of nanosuspensions for drugs of BCS classes II and IV. These properties result in high concentration gradient along the absorption path, which enhances the passage of dissolved molecules through the biological membrane. This mechanism is exploited by all administration routes, IV excluded. The general mechanisms, reasons and *in vivo* fate of nanosuspensions following different administration routes are showed in figure 4.

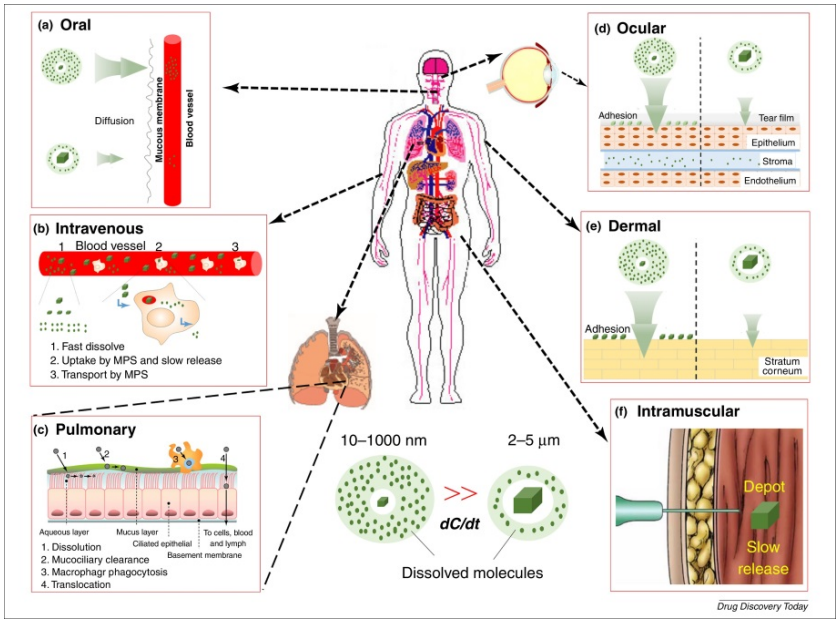

Figure 4 Mechanisms, properties and *in vivo* fate of nanosuspensions administered by different routes (with permission from [104])*.

*Reprinted from Formation, characterization, and fate of inhaled drug nanoparticles, Zhang J. et al, Adv. Drug Delivery Rev.(63), 441-455, 2011, with permission from Elsevier.

1.2.3.5.1. Oral route

The successful use of nanosuspensions for oral administration has to be addressed to the several beneficial properties that nanocrystals provide when administered via this route. The nanoparticulate form has a taste-masking effect and can be hence utilized to cover unpleasant tastes [14], as in case of the first marketed nanocrystal product Rapamune® (Table 5), containing the bitter poorly soluble drug sirolimus [70]. In addition to the taste-masking effect, nanocrystals enhance the oral bioavailability of drugs whose absorption is limited by their low solubility [14]. The practical manifestations of this effect are increased

maximum plasma level concentration (C_{max}), reduced time to reach maximum plasma level (T_{max}) and enhanced area under the curve plasma concentration–time (AUC) [108]. A good example of this effect in addition to the case of Rapamune® is the formulation of danazol as nanocrystals, where the dissolution-limited absorption of the regular danazol suspension was overcome by formulating the drug as nanosuspension and thereby improving the dissolution rate [109]. Other remarkable effects obtained by administering nanocrystals via oral route are the increased patient compliance because the nanosuspension can be dried and administered as solid dosage form and because the frequency of the administration can be reduced due to the high drug loading of nanocrystals. Furthermore, the differences in absorption between fed and fasted state are reduced with nanocrystals [14]. This advantage is the key for the large success of the marketed product Emend®. Emend® is a spray coated capsule formulation of a nanosuspension of aprepitant [70]. The conventional dosage form of the drug exhibited a significant food effect, which was more evident at higher doses. Formulating the drug as nanosuspension increased by 40 fold the surface area and resulted in a 4 fold increase in drug exposure in the fasted state [110], thereby eliminating the food/fasted effect on bioavailability and increasing its reproducibility [70]. Moreover, enhanced dose proportionality with the use of nanosuspensions was also obtained [14]. The increased bioavailability of nanocrystals after oral administration can be also explained by their increased muco-adhesion due to their increased surface area and particle size [111]. The consequence of higher muco-adhesion is an increased gastrointestinal transit time which can lead to increased bioavailability [14]. This property depends, however, on particle size, surface charge and on type of stabilizer used [14, 112].

Table 5 Currently marketed pharmaceutical products based on nanocrystals.

Trade name	Drug	Preparation method	Administration route
Avinza®	Morphine sulfate	Wet bead milling	Oral
Emend®	Aprepitant	Wet bead milling	Oral
Focalin® XR	Dexmethyl-phenidate HCl	Wet bead milling	Oral
Invega Sustenna®	Paliperidone palmitate	Wet bead milling	Intramuscolar
Megace ES®	Megestrol acetate	Wet bead milling	Oral
Rapamune®	Sirolimus	Wet bead milling	Oral
Ritalin® LA	Methylphenidate HCl	Wet bead milling	Oral
Tricor®	Fenofibrate	Wet bead milling	Oral
Triglide®	Fenofibrate	IDD-P® technology	Oral
Zanaflex Capsules ™	Tizanidine HCl	Wet bead milling	Oral

1.2.3.5.2. Parenteral delivery

The term “parenteral” includes different administration sites, *i.e.* intravenous (IV), intramuscular (IM), intradermal (ID) and subcutaneous (SC), and it indicates the injection of a formulation in the body. The main advantages that nanosuspensions provide with regard to parenteral administration are reduction of toxicity due to low excipient amount and absence of harsh conditions, reduction of injection volume due to the high drug loading, possibility of altering the pharmacokinetics of the drug leading to higher dosing and less frequent administration, thereby increased patient compliance, and possibly passive or active targeting [1, 2].

After IV administration, the fate of nanocrystals depends on factors such as their dissolution rate, particle size, surface properties and morphology [1, 104]. When nanocrystals are injected directly into the blood stream, they are subjected to an immediate sink condition and they may completely and rapidly dissolve. In this case, the pharmacokinetic profile is similar to that of a drug solution [1, 2]. If intravenously-administered particles do not readily dissolve due to proper surface and size characteristics, they may be recognized by the immune system and be phagocytized by the macrophages, or they could successfully avoid opsonization and phagocytosis. The internalization in the macrophages results in a depot effect and in a pharmacokinetic profile characterized by lower C_{max} but prolonged t_{max} [14].

This can be very advantageous for drugs whose toxicity is mediated by peak plasma levels, but whose efficacy is AUC driven. The modification of the particle surface [37], for instance by PEGylation or other means [113], could result in delay of protein adsorption and evasion of opsonization, thereby reduced macrophages uptake [1, 14]. This mechanism enables long circulating time and the particles have the opportunity to find discontinuities in the vasculature, particularly present in case of neoplasm, infections and inflammation, and thereby leak out. This phenomenon of passive targeting of tumors is termed enhanced permeation and retention effect (EPR) [1].

The most recent parenteral formulation based on nanosuspension technology is the marketed product INVEGA® SUSTENNA® (Xeplion® in Europe). This product is a once-monthly sustained release injectable nanosuspension of paliperidone palmitate, an atypical antipsychotic, indicated for the acute and maintenance treatment of adult patients with schizophrenia [70, 114]. The drug was formulated as nanosuspension using Elan's NanoCrystal® Technology and was engineered for sustained release, thereby facilitating the 1-monthly injection. The unique features of this product are: i) it is an injectable liquid suspension product in prefilled syringes; ii) syringeability is guaranteed also at high doses by a regular 23 G x 1″ or 22 G x 1 1/2″ gauge needles for deltoid or gluteal injection, respectively; iii) it has a shelf life of two years; iv) it is a sterile product; v) the particulate nature, together with drug's characteristics, generate sustained release [70]. The release of the drug from an IM suspension includes three steps: diffusion of water to the site of injection, dissolution of the particles and diffusion of the dissolved drug to the bloodstream [1]. Following IM injection, the particles of paliperidone palmitate dissolve very slowly due to the extreme poor solubility of the drug and to the limited amount of water present in the interstitial fluid [107]. The slow drug release combined with the high drug loading and the fact that paliperidone palmitate is a prodrug, whose active metabolite is paliperidone, obtained by hydrolysis by the muscle esterases, result in a one-month sustained release.

1.2.3.5.3. Pulmonary delivery

Nanocrystals aerosols constitute ideal carriers for poorly soluble drugs used for the treatment of pulmonary diseases [104] because the targeting of the deep lung requires an aerodynamic particle size in the range 1-5 µm [115]. Another important feature of nanocrystals with regard to pulmonary application is their increased adhesiveness in comparison to larger particles. Nanocrystals would hence better and longer adhere to the mucosal surfaces of the lungs, thereby enhancing drug absorption [111]. However, no studies have yet been done about the fate of aerosolized nanocrystals and many mechanisms seem to be involved [116].

1.2.3.5.4. Ocular delivery

Ocular drug delivery is one of the most interesting and challenging endeavors for formulation scientist [117] because of the difficulty of delivering drugs in a therapeutically significantly amount via this route. Conventional ophthalmic formulations result indeed in <5% bioavailability because of the presence of various ocular barriers and rapid elimination by lacrimal fluid draining, exacerbated by the poor solubility of many drugs [104]. Nanocrystal represent a promising technology for administration of drugs via ocular delivery because the increased saturation solubility and dissolution rate would lead to a high concentration gradient which would facilitate the permeation of the dissolved drug molecules across the corneal and conjunctival epithelium [104], whose effectiveness was proofed in some literature research [118, 119]. In addition, the increased adhesiveness of nanocrystals prolong the residence time in the cul-de-sac [104].

1.2.3.5.5. Dermal delivery

The dermal application of nanocrystals and, in general, nanoparticles, is currently a topic of high relevance because the skin is an organ which can be addressed by both cosmetic and pharmaceutical products. Currently, only cosmetic formulations based on nanosuspensions are on the market for dermal application [104]. Examples are Juvedical (Juvena) and Platinum Rare (la praire) [120], containing rutin and hesperidin nanocrystals, respectively. Indeed, while quite some research is available concerning the formulation of anti-oxidant, anti-aging and sun-protectant substances for dermal application [121-124], very few studies are available on the treatment of skin diseases by drug nanocrystals or in general on their dermal intended use [125, 126], and, in most cases, the research is focused on the use of inorganic materials with anti-inflammatory and antimicrobial effects [127, 128].

The nanocrystal properties that render this technology extremely promising with regard to dermal delivery are hereafter elucidated. As per other administration routes, the increased saturation solubility of nanocrystals results in a higher concentration gradient between the external and internal sides of the stratum corneum, hence the flux is increased [84] and the passage of the dissolved molecules across the membrane is accompanied by the immediate dissolution of other crystals. Moreover, the adhesive properties of nanocrystals result in better and longer stickiness [84]. Fundamental is the follicular pathway [129]: nanocrystal can penetrate the hair follicle and provide a depot, maintaining a constant concentration gradient and constant dermal penetration [84]. A proper size for optimal and deeper hair follicle penetration was determined to be ranging between ~650 and ~750 nm [130]. Furthermore, less viscous (semisolid) nanosuspensions resulted in better penetration through the skin and follicular targeting [84].

Inflammatory skin diseases like psoriasis and atopic dermatitis are examples of diseases whose topical treatment based on the use of conventional creams can be, in quite some cases, unsuccessful. In those cases, the oral administration of drugs is required, inevitably resulting in side effects, which can be severe when highly potent drugs are used. Nanocrystals and, more in general, nanocarriers characterized by an increased skin penetration efficacy would hence result in successful therapy and improved patients' life-quality. Combination of the release kinetics of different nanocarriers to have a first dose rapidly delivered followed by a sustained release may also be extremely advantageous.

1.3. Research objectives

The objective of this work was to prepare and characterize nanocrystals and nanoparticles of poorly soluble drugs for dermal application. The specific goals were:

- To determine *in situ* the increased saturation solubility of nanocrystals and to assess whether different nanocrystal excess conditions have an effect on the factor of solubility increase, as the latter could be relevant for dermal application because the concentration gradient between formulation and skin would thereby be higher (chapter 3.1);
- To evaluate whether the wet bead milling process, which is the most used preparation method for nanocrystals, affects the crystalline nature of the particles and what are the consequences of a different crystallinity degree (<100%) on the increased saturation solubility of nanocrystals (chapter 3.2);
- To prepare amorphous nanoparticles by aqueous wet bead milling and determine whether the combination of amorphous state and nanosize would have a synergistic effect on saturation solubility and dissolution rate (chapter 3.3);
- To prepare nanocapsules with a nanocrystal core and a polymer shell in order to combine the advantages of nanocrystals and polymeric nanoparticles and overcome their limitations, thereby obtaining a nanocarrier characterized by high drug loading and controlled drug release (chapter 3.4). This type of carrier would result in potentially high drug bioavailability with fewer dermal applications.

2. Discussion

2.1. Dependency of drug solubility on particle size and degree of crystallinity

Nanosuspensions are one of the latest, most effective and promising technologies for the delivery of poorly soluble drugs. The success of this formulation tool has to be mainly addressed to two of their features, which both come into play during administration of nanocrystals via any route: their increased dissolution rate and saturation solubility (paragraph 1.2.3.1). The enhanced dissolution rate of nanocrystals is due to their increased surface area in comparison to larger particles and it is explained by the Noyes-Whitney equation [64]. Their increased saturation solubility is described by the Ostwald-Freundlich and Kelvin equations [2, 65]. As increased dissolution rate was a concept already known from the micronization techniques [16], the dissolution rate enhancement obtained by reducing the particle size further down from micro- to nanometer range was a predictable achievement. Unexpected and highly-discussed was instead the increased saturation solubility obtained by nanonization, as it was generally believed that the drug solubility depends only on chemistry of the compound, type of solvent and temperature [22]. However, the physical state of the drug, including both the solid state and the particle size, was found having a remarkable effect on the solubility. Different polymorphs result in diverse solubilities [131], the highest one corresponding to the amorphous state [26], and particles whose size is <1 μm have increased saturation solubility [22]. The physical state was thus included among the parameters affecting drug solubility.

Although scientists currently agree on the fact that formulating drugs as nanocrystals increases their saturation solubility, the extent of the increase factors remains under discussion. There are two groups of techniques which can be used to determine the saturation solubility of nanocrystals: non *in situ* and *in situ* methods. The main difference among them is that the first ones measure the increased solubility indirectly, after a separation or filtration step, while the second ones allow the direct determination of the drug solubility, in the moment when the particles are dissolving and without the need for separation. Therefore, *in situ* methods are considered more accurate for solubility determination [99], and the values obtained are generally lower than non *in situ* methods. Non *in situ* methods include separation techniques as centrifugation and filtration [100], while *in situ* methods include dynamic light scattering [99] and solution calorimetry [98]. In this work, a novel method based on *in situ* UV-vis spectroscopy was utilized to determine the solubility of nanocrystals of three different poorly soluble drugs in comparison to their

micronized counterparts (chapter 3.1). Nanocrystals of dexamethasone, tacrolimus and ibuprofen were first prepared with proper type and amount of surfactant by wet bead milling. The milling process and the parameters influencing the particle size were studied in detail, with higher amount of beads, higher speed and longer milling times resulting in smaller particles. Particles with sizes ranging between ~300 nm and ~1 µm were obtained by using different process parameters and afterwards analyzed with regard to solubility in comparison to the solubility of micronized drug powder. The *in situ* solubility experiments were performed by adding controlled excess amounts (2-3 times excess over the previously determined saturation solubility of the micronized drug powder) of nanocrystals in the form of freeze-dried powder to the medium (water) and the UV absorbance was constantly measured over time (20 min) in order to detect possible phenomena like recrystallization. The presence of small surfactant amounts due to the addition of the freeze-dried powder was always analyzed prior the experiments and the only drug whose solubility was thereby affected was ibuprofen, hence the calculation for the amount to add in order to be in excess was made accordingly. A Tyndall-Rayleigh correction was applied to the obtained spectra in order to exclude the scattering of undissolved particles. Nanocrystals of dexamethasone, tacrolimus and ibuprofen with a size of ~300 nm resulted in an increased solubility compared to their micronized counterparts. Indeed, the kinetic solubility was achieved with particles of ~300 nm size, while the thermodynamic solubility was obtained with particles of ~1 µm. This result underlined the effect of size on saturation solubility, as its increase was obtained only when the particle size was markedly smaller than 1 µm. The factors of increase were, however, not remarkably high and they ranged between 1.3 and 2.8. These values were in accordance with previously published data using a different *in situ* method [99] and were lower than what obtained with non *in situ* methods [100]. The effect of excess nanocrystal amount available for dissolution and solubility enhancement was evaluated for tacrolimus because its extremely low solubility allowed the use of higher excess conditions (up to 10 times) without the problem of a too high background scattering, as in case of dexamethasone and ibuprofen. Increasing the excess conditions resulted in higher factors of solubility increase because of the presence of a larger amount of relatively small nanocrystals. The maximum increase obtained was 6.6. Moreover, drugs with lower solubility resulted in larger increases. No recrystallization was observed during the experimental time, indicating that nanocrystals form a relatively “stable” supersaturation state. However, considering the relatively low increase factors obtained, the enhanced saturation solubility cannot be considered as main reason for the great advantages obtained following administration of nanocrystals, which should be mostly addressed to their other characteristics, *e.g.* adhesiveness and dissolution rate. The latter parameter was hence investigated, and nanocrystals provided a remarkable increase in this regard, particularly when the difference in particle size among the original

powder and the nanocrystals was quite large, as in case of ibuprofen, where the dissolution rate of 0.14 µg/(mL*s) corresponding to 70 µm particles was increased to 4.02 µg/(mL*s) with ~300 nm crystals.

The second factor which belongs to the category of the physical state characteristics of a drug in addition to particle size, is the drug solid state, which remarkably contributes to its solubility. The solid state of a drug can exist in a crystalline (different polymorphs and solvates are also considered) or amorphous form (paragraph 1.2.1.). The latter corresponds to the most energetic state and results in favorable properties, particularly increased solubility and thereby dissolution rate. The techniques available to induce conversion from the crystalline to the amorphous state also include (dry) milling because the high energy involved in this process can damage or lead to changes of the crystal structure and the molecules may not be able to rearrange in the most thermodynamic stable form.
Wet bead milling is the most used preparation method for drug nanocrystals because of its several advantages as cost-efficiency and up-scalability [13, 79], and almost all currently marketed nanocrystal products are prepared via this technology (Table 5). This process, however, involves very high energy and shear rate [80], which may thus lead to change or reduction of the crystallinity of the processed drug, reason why the solid state of the final product should always be determined and compared to the initial one [70]. The effect of nanomilling time on the degree of crystallinity of dexamethasone and tacrolimus nanocrystals was analyzed during this work in order to evaluate whether this high-energy process resulted in reduction of crystallinity or changes in the solid state of the two drugs and if this phenomenon would have implications on drug solubility in addition to particle size reduction (chapter 3.2). Dexamethasone and tacrolimus were milled for 0.5 to 5 h, while the other process parameters (bead amount, milling speed) were kept constant. The degree of crystallinity was calculated based on the crystallite size, which was obtained by utilizing the previously measured full-width at half-maximum (FWHM) in the Scherrer equation [132]. The FWHM is the width of a reflection calculated at half of its maximum height, and its increase indicates a broader reflection, hence lower crystallinity. Amorphous compounds are indeed characterized by lack of reflection in their spectra and presence of a broad halo. The crystallite size of the unprocessed drug powder was taken as 100% crystallinity and used to calculate the degree of crystallinity of the milled samples. Nanomilling resulted in a reduction of the crystallite size for both drugs, indicating either a breakage of the crystallite into smaller ones, or a loss of crystallinity (partial amorphization). The degree of crystallinity decreased over time for both drugs until reaching a plateau, ~79% for dexamethasone and ~76% for tacrolimus, because the energy involved in the process was not high enough to induce further crystallinity loss. The time needed to achieve the plateau differed among the drugs: 2

h for dexamethasone, 3 h for tacrolimus. The difference in the time needed to reach the plateau could be explained by the brittleness of the drugs. The stress-strain curves of dexamethasone and tacrolimus were determined and compared to those of very brittle or elastic materials (dihydrate calcium phosphate and microcrystalline cellulose, respectively). Although the curves of both drugs were in between the two extremes, a trend was observed: dexamethasone was more brittle than tacrolimus, thus the drug particles started breaking as soon as the milling process was initiated, and after 2 h no more changes neither in size nor in crystallinity were observed. Tacrolimus was more ductile, it deformed plastically for a longer time and the particles started breaking only after 2 h of milling.

As freeze-drying is a commonly used method for nanosuspension drying [67], its effect on the degree of crystallinity of nanocrystals was also evaluated. Freeze-drying the nanosuspensions resulted in a reduction of their crystallinity and the effect of nanomilling time on the degree of crystallinity was thereby evened. The values obtained after drying scattered, indeed, close to average values of 35% and 45% degree of crystallinity for dexamethasone and tacrolimus, respectively. This result may be due to the equilibrium involved in the drying process of suspensions: the degree of crystallinity of the molecules recrystallizing from the dissolved fraction may change dramatically. Thus, the solid state of nanocrystals should also be considered and controlled during lyophilization, in addition to agglomeration and cake appearance.

The effect of nanomilling time and reduced degree of crystallinity on dexamethasone and tacrolimus solubility was finally investigated in combination with their reduced particle size. The highest solubility increase (factor 1.15 for dexamethasone and 1.7 for tacrolimus) was always obtained with the lowest degree of crystallinity (79 and 76% for dexamethasone and tacrolimus, respectively), but not with the smallest particle size, particularly in case of tacrolimus: its highest solubility was obtained with particles milled for 5 h, whose particle size was ~527 nm, hence larger than particles milled for 2 h with a size of ~240 nm, but their crystallinity was the lowest obtained.

2.2. Synergistic effect of nanosize and amorphous solid state: preparation of amorphous nanoparticles by aqueous wet bead milling

The combination of nanosize and reduced degree of crystallinity obtained by milling for long times (chapter 3.2) resulted in drug nanocrystals whose solubility was higher than what obtained with size reduction only (nanocrystals with high degree of crystallinity). This result suggested that combining amorphous state and nanosize could provide synergistic effect,

thus further increase in solubility and dissolution rate. Therefore, the aim of chapter 3.3 was to prepare amorphous nanoparticles of a poorly soluble drug, indomethacin, which is a stable glass former, via aqueous wet bead milling, in order to determine whether these two aspects have a synergism resulting in superior performance with regard to saturation solubility and dissolution rate.

Wet bead milling in aqueous medium was selected as preparation method because of its industrial relevance and feasibility. The presence of water was, however, the biggest challenge to tackle, as it promotes recrystallization. The preparation of amorphous indomethacin could be easily performed by quench cooling the molten compound because indomethacin is a stable glass former [133], however its stabilization during the milling process was difficult. Polyvinylpyrrolidone (PVP, Kollidon 30) was selected as stabilizer because it is generally used for amorphous solid dispersions due to its well-known anti-recrystallization effect [134], but it has also been utilized for nanosuspension stabilization [83]. Poloxamer 407, a commonly used polymeric particle size stabilizer, was used as comparison. While the latter was highly efficient in stabilizing the particle size in the nanometer range but the amorphous drug state was thereby not maintained, the opposite situation was obtained with PVP. Combining the two polymers together resulted in particle size stabilization, however the drug recrystallized to the thermodynamically stable crystalline state. It was hypothesized that this result was due to the competition among the two large polymers for surface coverage: PVP was not sufficiently protecting the surface of the particles from recrystallization because of the steric impediment of poloxamer 407. Thus, a smaller particle size stabilizer, sodium dodecyl sulfate (SDS), was selected and combined with PVP in order to obtain amorphous nanoparticles. This combination was effective in stabilizing both the nanosize and the amorphous state of the drug particles. Amorphous indomethacin nanoparticles were thus obtained by aqueous wet bead milling, and the importance of drug-polymer interactions for solid state stabilization was thereby demonstrated. The effect of amorphous state and nanosize on saturation solubility and dissolution rate was investigated *in situ*. The solubility of crystalline indomethacin powder of ~7 µg/mL was increased up to ~17 µg/mL by its amorphization. The dissolution rate of the amorphous powder was remarkably faster than the crystalline drug (0.085 µg/(mL s) vs. 0.003 µg/(mL s)), although its particle size was larger (x_{50}=78 µm vs. x_{50}=5.4 µm). Nanocrystals resulted in a factor of solubility increase comparable to drug amorphization, 2.6 times, and in a slight further increase in dissolution rate, which reached 1.138 µg/(mL s). The highest increase in saturation solubility and dissolution rate was, however, obtained with amorphous nanoparticles: the saturation solubility was ~35 µg/mL, with an increase factor of 5.2, while the dissolution rate obtained was 2.328 µg/(mL s). It was thus demonstrated that the combination of amorphous solid state and nanonization provides a remarkable

synergistic effect with regard to both saturation solubility and dissolution rate. This effect could be particularly favorable for drugs whose amorphization already consistently increases their dissolution rate and solubility because their nanonization would further enhance both aspects and be hence clinically relevant.

2.3. Overcoming limitations and drawbacks of nanocrystals by their nanoencapsulation within a polymer shell

Dissolution studies reported in the literature and performed during this work demonstrated that nanocrystals dissolve very fast upon dilution with water. This characteristic, which finds its fundament in the Noyes-Whitney equation, is particularly favorable in the therapy of many diseases because a drug formulated as nanosuspension is thereby directly available to be absorbed and to act. However, this poses also two main limitations/ drawbacks: i) risk of side effects due to a burst release, hence a readily-available (potentially) large amount of drug, particularly in case of drugs whose concentration range between efficacy and toxicity is small and ii) no controlled drug release, hence no possibility to adjust the drug release to meet specific therapeutic needs. The selection of a different type of nanocarrier to obtain controlled drug release, *i.e.* polymeric nanoparticles, would allow this aspect and reduce/ eliminate the burst release, but the drug loading of polymeric nanoparticles is very low, generally 5-10% [135], while drug nanocrystals have a "drug loading" of 100%, as they are constituted of only drug. A solution would consist in the combination of nanocrystals and polymeric nanoparticles to overcome their limitations and combine their advantages, hence in the encapsulation of drug nanocrystals within a shell of suitable polymers to obtain nanocapsules with high drug loading and controlled drug release. The preparation and characterization of nanocapsules with dexamethasone nanocrystal core and polymer shell was the aim of chapter 4.

The method selected for preparing the nanocapsules was solvent evaporation, which is commonly used to obtain microspheres and microcapsules [51, 136]. The first challenge to tackle was to prepare dexamethasone nanocrystals in an organic medium where the coating polymer was dissolved, but where the drug solubility was acceptable (~500 µg/mL). Wet bead milling was performed in dichloromethane and nanocrystals of ~350 nm size were obtained, underlining the potential use of organic solvents for nanosuspension preparation. Two different polymers were tested to form the outer shell: Eudragit® RS 100 and ethyl cellulose 4 cP. Nanocapsules were obtained after emulsification of the nanosuspension (organic phase) with the aqueous phase and evaporation of the solvent during overnight

stirring. No additional stabilizer in the aqueous phase was necessary in case of Eudragit® RS 100 because of its self-stabilizing properties, while polyvinyl alcohol was used in case of ethyl cellulose. The polymer amount markedly affected the formation of nanocapsules in case of Eudragit® RS 100: too low amounts did not provide sufficient stabilization during emulsification and solvent evaporation, while a too high quantity resulted in polymer precipitation. All tested concentrations were successful in case of ethyl cellulose. Nanocapsules with a size of ~250 nm were obtained for both polymers. The smaller size of nanocapsules compared to nanocrystals was due to the simultaneous formation of blank nanoparticles during the preparation process and to the presence of dissolved polymer covering the surface of nanocrystals, resulting in larger particle size if measured by PCS. The confirmation of the nanoencapsulation was obtained by morphology, FTIR, size stability upon dilution and drug release studies. Morphology investigations demonstrated the simultaneous presence of three different types of particles within the nanocapsules' sample. The particles had differences with regard to shape and contrast: drug nanocrystals were squared with dark contrast, blank polymer nanoparticles where round and light grey (low contrast), while nanocapsules were relatively round and their contrast was high, similar to the one of nanocrystal, confirming the presence of crystalline material (drug) in the core. FTIR studies showed that the drug reflections were masked because of the coating polymer in case of nanocapsules, similar to what obtained during previous studies with microcapsules [137]. Upon dilution with water, the particle size of the nanocapsules decreased instantaneously and remained afterwards constant. The initial reduction was due to the immediate dissolution of non-coated nanocrystals, while the nanocapsules remained stable over 2 h. The final confirmation of the successful nanoencapsulation of dexamethasone nanocrystals within a polymer shell was obtained by drug release studies performed with Franz diffusion cell. After an initial burst due to the dissolution of non-encapsulated nanocrystals, the drug release from nanocapsules happened in a controlled fashion, while nanocrystals showed a faster and complete drug release. Thus, the successful nanoencapsulation of drug nanocrystals within a shell of Eudragit® RS 100 or ethyl cellulose was confirmed.

The hereby obtained nanocapsules represent a very innovative nanocarrier which combines the high drug loading provided by nanocrystals with the controlled drug release obtained by the polymer coating. The potential of this carrier for the treatment of diseases which require prolonged exposure to the drug or for drugs whose toxicity is mediated by peak plasma values but efficacy is AUC driven could be remarkable because a high amount of drug could be loaded within small volumes, and the drug release could be controlled to meet specific therapeutic needs.

3. Results

3.1. "*In situ* determination of the saturation solubility of nanocrystals of poorly soluble drugs for dermal application"

Miriam Colombo[a], Sven Staufenbiel[a], Eckart Rühl[b], Roland Bodmeier[a]

[a] College of Pharmacy, Freie Universität Berlin, Kelchstr. 31, 12169 Berlin, Germany

[b] Physical Chemistry, Institute for Chemistry and Biochemistry, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany

M. Colombo, S. Staufenbiel, E. Rühl, R. Bodmeier, "*In situ* determination of the saturation solubility of nanocrystals of poorly soluble drugs for dermal application", International Journal of Pharmaceutics: Volume 521, Issues 1-2, 15 April 2017, Pages 156-166

https://doi.org/10.1016/j.ijpharm.2017.02.030

The author of this work developed and realized the experiments and the written manuscript independently. The characterization by scanning or transmission electron microscopy was performed by one of the co-authors.

3.2. "Influence of drug brittleness, nanomilling time, and freeze-drying on the crystallinity of poorly water-soluble drugs and its implications for solubility enhancement"

Miriam Colombo[1], Steven Orthmann[2], Marco Bellini[1], Sven Staufenbiel[1], Roland Bodmeier[1]

[1] College of Pharmacy, Freie Universität Berlin, Kelchstr. 31, 12169 Berlin, Germany

[2] Institut für Chemie, Technische Universität Berlin, Straβe des 17. Juni 135, 10623, Berlin, Germany.

M. Colombo, S. Orthmann, M. Bellini, S. Staufenbiel, R. Bodmeier, "Influence of Drug Brittleness, Nanomilling Time, and Freeze-Drying on the Crystallinity of Poorly Water-Soluble Drugs and Its Implications for Solubility Enhancement", AAPS PharmSciTech: Volume 18, Issue 7, October 2017, Pages 2437–2445.

https://doi.org/10.1208/s12249-017-0722-4

The author of this work developed and realized the experiments and the written manuscript independently. The determination of the brittleness of the drugs, the XRD analysis, and the related written parts were performed by the co-authors.

3.3. "Preparation of amorphous indomethacin nanoparticles by aqueous wet bead milling and *in situ* measurement of their increased saturation solubility"

Miriam Colombo[a], Clara Minussi[a], Steven Orthmann[b], Sven Staufenbiel[a], Roland Bodmeier[a]

[a] College of Pharmacy, Freie Universität Berlin, Kelchstr. 31, 12169 Berlin, Germany

[b] Institut für Chemie, Technische Universität Berlin, Straβe des 17. Juni 135, 10623, Berlin, Germany.

M. Colombo, C. Minussi, S. Orthmann, S. Staufenbiel, R. Bodmeier, "Preparation of amorphous indomethacin nanoparticles by aqueous wet bead milling and *in situ* measurement of their increased saturation solubility", European Journal of Pharmaceutics and Biopharmaceutics: Volume 125, April 2018, Pages 159–168.

https://doi.org/10.1016/j.ejpb.2018.01.013

The author of this work developed and realized the experiments and the written manuscript independently. The XRD analyses were performed by the co-authors.

3.4. Controlled release of dexamethasone from nanocapsules with a drug nanocrystal core and shell of water-insoluble polymers

1. Introduction

The recent advances in computational screening and drug design have led to the development of new drugs characterized by high lipophilicity, high molecular mass (> 500 Da) and poor aqueous solubility, with consequent problems related to absorption and bioavailability. It was calculated that 40% of the drugs on the market and 70% or more of the pipeline drugs are poorly water-soluble [1, 2]. Approaches available to overcome poor aqueous solubility consist in the use of solubilizers or co-solvents, inclusion into cyclodextrin complexes, drug amorphization and preparation of nanosuspensions [3]. The poor solubility of new drugs not only in water but also in organic solvents and oils, together with the problems associated with toxicity of excipients when used in large quantities for solubilizing a therapeutically relevant amount of drug, ideal size to form inclusion complexes and physicochemical stability of amorphous products, have underlined how promising nanosuspensions are [1, 4, 5]. The latter are sub-micron suspensions of pure drug particles stabilized by appropriate electrostatic or steric stabilizers and characterized by a remarkably increased dissolution rate in comparison to microcrystals due to their larger surface area, as described by the Noyes-Whitney equation, and by increased saturation solubility according to the Kelvin and Ostwald-Freundlich equations [5-8]. These properties may result in higher drug absorption and increased bioavailability. Additionally, nanosuspensions have other advantages such as high drug loading, better reproducibility after oral absorption, elimination of fed/fasted state effects, reduction of excipients, thereby lower toxicity and potentially increased patient compliance [9]. A big drawback of nanosuspensions is, however, their thermodynamic instability due to the high surface energy, which results in agglomeration and particle growth or, in the worst case, sedimentation [5, 10]. Moreover, as nanocrystals are characterized by rapid dissolution and immediate drug release, a burst effect may cause toxicity and/or severe side effects [11, 12].

Although a high Cmax is generally desired, drug classes whose toxicity is caused by high plasma values but whose efficacy is driven by AUC require controlled drug release. Nanosuspensions able to provide controlled drug release would thus represent the chance to treat several diseases which require longer exposure to the drug with less product applications, thereby additionally increasing patient compliance. Controlled drug release after intramuscular, subcutaneous or intradermal injection of nanosuspensions may be achieved

by depot formulations where drug nanocrystals do not dissolve instantaneously due to the extremely low drug solubility. If intravenously administered particles do not immediately dissolve, they may be taken up by the macrophages, thereby a depot effect may be obtained. Additionally, the optimization of particle size and surface characteristics, for example by PEGylation, of intravenously injected particles may result in longer circulation time due to absence of opsonization, thereby tumor tissue targeting may be also achieved. The latter, combined with a specific drug release mechanism, may provide significant improvement to the therapy. As regards oral formulations, nanosuspensions may be dried, tableted, and the tablets may be coated for *e.g.* colon targeting. Products based on these approaches are already on the market [5, 13, 14].

One method to modify the pharmacokinetics of drug nanocrystals is their encapsulation within a polymer (or lipid) shell and formation of nanocapsules. The latter are nano-vesicular systems that exhibit a core-shell structure in which the drug is confined within a cavity surrounded by a polymer membrane or coating [15]. The drug in the cavity can be in a liquid or solid form. If the drug in the solid form is present as nanocrystals (one or more), clear advantages of such a carrier would be the high drug loading, generally not achievable with other carriers such as polymeric nanoparticles, combined with a controlled drug release. Darunavir nanocrystals were successfully encapsulated within a shell of Eudragit® L100 using coaxial electrospraying and pH-dependent drug release was obtained [11]. The drug release from nanocapsules depends indeed primarily on the physicochemical characteristics of the coating polymer, which first gets in contact with the release media, and on the ones of the drug itself, but also on coating microstructure (porosity, tortuosity, thickness), release media and study conditions. The preparation method may also remarkably affect the release profile [15].
Another advantage of nanocapsules with nanocrystal core would be the increased physical and chemical stability that the polymer may confer to the nanocrystals, potentially protecting them from aggregation and degradation.

The aim of this study was to prepare nanocapsules of dexamethasone nanocrystals within a shell of water-insoluble polymers in order to achieve high drug loadings and controlled drug release. The process involved the preparation of nanocrystals by organic wet bead milling and their successive nanoencapsulation by the solvent evaporation method. This technique has often and successfully been used for microcapsule and microsphere preparation [16-18], and it has been also positively used for nanoencapsulation [19]. Dexamethasone was selected as model drug considering the potential of a depot formulation for the treatment of, for example, skin diseases like psoriasis via intradermal injection of nanocapsules with a nanocrystal core. Coarse dexamethasone had been additionally successfully reduced to the

nanometer size range during previous studies [20, 21]. Eudragit® RS 100 and ethyl cellulose were selected to form the outer shell because of their ability to provide controlled drug release [22-24]. Their use for preparation of nanoparticles was additionally already established in previous reports [25, 26]. Parameters affecting the nanocapsule formation were thoroughly investigated and different techniques were adopted for confirmation of the nanoencapsulation of dexamethasone nanocrystals.

2. Materials and methods

2.1. Materials

Dexamethasone (Fagron GmbH, Barsbüttel, Germany), poloxamer 407 (Kolliphor® P407, SE, Ludwigshafen, Germany), polyvinyl alcohol 4-88 (PVA) (Emprove®, Merck KGaA, Darmstadt, Germany), ammonio methacrylate copolymer type B (Eudragit® RS 100, Evonik Industries AG, Darmstadt, Germany), ethyl cellulose (Ethocel® Standard 4 Premium, Colorcon Ltd., Dartford, UK), 0.1-0.2 mm zirkonium beads (SiLibeads®, Sigmund Lindner GmbH, Warmensteinach, Germany), dichloromethane (Rotisolv® HPLC, Carl Roth GmbH und Co. KG, Darmstadt, Germany), ultra-purified water purified by a Milli-Q® apparatus (Millipore GmbH, Darmstadt, Germany).

2.2. Methods

2.2.1. Preparation of nanocrystals

Nanosuspensions of 1% (w/v) dexamethasone were prepared by wet bead milling in an organic solution of 0.5, 1, 2 or 4% (w/v) Eudragit® RS 100 or ethyl cellulose in dichloromethane. For comparison, regular nanocrystal suspensions of 1% (w/w) dexamethasone were prepared by aqueous wet bead milling with 1% (w/v) PVA as stabilizer. The drug was added to the polymer or stabilizer solution and milled for 3 h with ~800 rpm in a tightly closed Erlenmeyer immerged in an ice bath to keep low product temperatures. 0.1 – 0.2 mm zirkonium beads were used as grinding agents in a ratio 1:3 (w/w) suspension:beads. The nanosuspension was afterwards separated from the beads by filtration through a filter paper with a pore size of ~45 µm. The milling conditions were selected based on previous experiments [20]. The organic nanosuspensions were further processed in order to obtain nanocapsules of dexamethasone nanocrystals.

2.2.2. Preparation of nanocapsules of dexamethasone nanocrystals and of blank polymer nanoparticles

Dexamethasone nanocrystals were encapsulated within a shell of water-insoluble polymers by the solvent evaporation method. 5 mL of organic nanosuspension was added to 15 mL of water in case of Eudragit® RS 100 or, in case of ethyl cellulose, to 15 mL of 1% (w/v) PVA.

The emulsion was sonicated for 2 min with 25, 50 or 65% amplitude (Bandelin Sonopuls HD 3200, Bandelin electronic GmbH & Co. KG, Berlin, Germany) in an ice bath. The suspension was afterwards stirred with 600 rpm overnight at room temperature to let dichloromethane evaporate. The obtained suspension was filtered through 1.2 µm filter and stored at room temperature. Pictures of some batches where large aggregates/ agglomerates were present were taken with a photo camera (iPhone SE, Apple Inc., Cupertino, US). Some mL of the nanosuspensions selected for further studies were put in a freezer at 80°C and afterwards freeze-dried (Alfa® 2-4 LD Plus freeze-dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Primary drying was performed at 0.1 mbar and -42°C for 16 h followed by 3 h secondary drying at 0.05 mbar and -48°C.

Blank polymer nanoparticles were prepared with the same method described for the nanocapsules but without final filtration. The solutions of the polymers in dichloromethane were used as organic phase instead of the organic nanosuspensions.

2.2.3. Preparation of physical mixtures

Physical mixtures 1:2 (w/w) drug:polymer were prepared by mixing the powders with a pestle in a mortar. The mixtures were analyzed by Fourier transform infrared spectroscopy (Excalibur 3100 FTIR spectrophotometer, Varian Inc., Palo Alto, USA) and the obtained spectra were compared to the ones of the nanocapsules, unprocessed drug and excipients to confirm their structure.

2.2.4. Characterization of nanoparticles and freeze-dried powders

2.2.4.1. Particle size and zeta potential analysis

The particle size of nanocrystals, nanocapsules and blank polymer nanoparticles was measured in triplicate by photon correlation spectroscopy (PCS) using a Zetasizer® Nano ZS (Malvern Instruments Ltd., Malvern, UK). The samples were diluted with a saturated dexamethasone solution to a final concentration of ~1 mg/mL. During studies of particle size stability upon dilution, the samples were diluted with water to a final drug concentration of ~50 µg/mL and analyzed in triplicate by PCS.

The zeta potential of the nanocapsules used for the release experiments, whose drug concentration was ~0.08% (w/v), was measured in triplicate at 23°C using the Zetasizer® Nano ZS.

2.2.4.2. Drug content

The real drug content of the nanosuspensions was measured by UV spectrophotometry at λ=242 nm (Agilent HP 8453, Agilent Technologies Inc., Santa Clara, US). Aliquots (n=3) of nanocrystal suspension were diluted with water prior measurement, while a mixture 80:20 (v/v) isopropanol:water was used as solvent in case of the nanocapsules.

2.2.4.3. Light microscopy, transmission electron microscopy (TEM) and intensity analysis

Optical microscopy pictures of the suspensions were taken (Zeiss Axioskop, Carl Zeiss Microscopy GmbH, Jena, Germany) in order to determine if large agglomerates and/or aggregates were present. The morphology of nanocrystals, nanocapsules and blank polymer nanoparticles was analyzed by TEM (Hitachi SU8030 FESEM, Hitachi Ltd., Tokyo, Japan). 10 µL of sample was pipetted on a 400 mesh carbon support film coated copper grids (Quantifoil®, Quantifoil Micro Tools GmbH, Groβlöbichau, Germany) and afterwards analyzed. The intensity of the pixels related to the particles on the TEM images was analyzed with the software ImageJ. Squares of 2 mm length and 2 mm height were drawn in the center of the particles (n=3 per type of particle) and these areas were afterwards analyzed with the function “measure mean, minimum and maximum grey value”, which provided the integer values related to the intensity of the pixels. The values were averaged and compared.

2.2.4.4. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the freeze-dried powders, physical mixtures and raw materials were generated with an Excalibur 3100 FTIR spectrophotometer. The spectra were collected using a horizontal attenuated total reflectance (ATR) accessory with a single reflection diamond crystal (Pike Miracle, Pike Technologies, Madison, USA). Sixtyfour scans at 4 cm−1 resolution were averaged and analyzed.

2.2.4.5. Drug release studies with Franz diffusion cell

The drug release studies were performed in triplicate with Franz diffusion cell under non-sink conditions, at 30% of the drug solubility (Cs), with 100% drug release corresponding to a drug concentration of 21 µg/mL and at 25°C. The dexamethasone solubility (73 ± 0.4 µg/mL) was determined by centrifugation of aliquots (n=3) of a suspension of dexamethasone in water. The suspension was obtained by adding excess of drug to water and was stirred for 48 h at room temperature prior analysis. The centrifugation was conducted at room temperature (23°C) at 17000 rpm (HeraeusTM, Thermo Fisher Scientific Inc, Waltham, UK). The nanosuspensions used for the Franz diffusion cell experiment were diluted to a drug concentration of 0.06-0.08% (w/v) before the release study in order to add volumes > 300 µL to the donor compartment and cover the entire surface of the membrane. The donor compartment was separated from the acceptor compartment by a membrane of regenerated cellulose (Spectra/Por® 2 Dialysis Membrane, RC discs of MWCO 12–14 kDa, Spectrum Laboratories, Inc., CA, USA) which was conditioned in purified water prior to the experiment. The acceptor compartment was filled with purified water as release medium and was stirred at 600 rpm throughout the experiment. Calculated amounts of nanosuspensions were placed in the donor compartment, which was closed during the release study to avoid water

evaporation. 0.4 mL of sample was taken from the acceptor compartment at predefined time points (0.25, 0.75, 2, 3, 5, 7, 24 h) and replaced by fresh medium. The samples were analyzed spectrophotometrically for drug release at λ = 242 nm.

3. Results and discussion

3.1. Wet bead milling in organic media

Among the different methods available for the preparation of nanosuspensions, wet bead milling is an efficient way to prepare nanocrystals due to its up-scalability and cost-efficiency [27], proven by the presence on the market of nanocrystal products obtained by this technique [28]. Therefore, wet bead milling was selected as preparation method. The aqueous solution of the stabilizer is generally used as media. In order to obtain nanocapsules with a shell of insoluble polymers, nanomilling was performed in an organic solution of the polymers. In addition to the risk of residual solvent in the final formulation, the challenge of using organic solvents for wet bead milling was the drug solubility, as its increase due to the organic medium would enhance phenomena like Ostwald ripening, leading to instability and crystal growth [9, 10]. Eudragit® RS 100 and ethyl cellulose are water-insoluble polymers, thus dichloromethane was selected as dispersion medium. Dexamethasone solubility in dichloromethane was determined by addition of weighted amounts of drug to the solvent and was ~500 µg/mL. The addition of ethyl cellulose to the medium further increased the drug solubility up to ~600 µg/mL, while Eudragit® RS 100 did not have any effect. Nanocrystals with a particle size ranging between 250 and 500 nm were successfully obtained by nanomilling in dichloromethane with each polymer. This result underlines the potential use of solvents or low-viscosity oils for nanocrystal preparation.

3.2. Preparation of nanocapsules: influence of formulation and process parameters on particle size

Solvent evaporation is a very efficient method for the preparation of polymer microspheres [18] and nanoparticles [25, 26] and was thus selected for the preparation of nanocapsules. Different formulation and process parameters such as polymer concentration, stabilizer concentration, sonication time and power may affect the particle size and stability of thereby obtained particles [17, 18]. The influence of polymer concentration and sonication power on the particle size was first evaluated by preparation of blank polymer nanoparticles of Eudragit® RS 100 (Fig. 1). The organic phase was rapidly added to the aqueous phase and sonicated. The sonication time was kept constant at 2 min, and no additional stabilizer was added to the aqueous phase because of the self-stabilizing effect of Eudragit® RS 100 [29]. The quaternary ammonium groups of the polymer hence provide a positive zeta potential and thereby stabilize the system [26]. Blank polymer nanoparticles with an average particle size

of ~200 nm and PDI ≤ 0.2 were successfully obtained under all tested conditions (Fig. 1). Increasing the polymer concentration slightly increased the average particle size, in accordance with previous reports. This was attributed to a higher polymer concentration in the organic droplets or to larger droplets of organic phase in the emulsion [26]. A trend was observed also with regard to sonication power: 25% amplitude resulted in larger particles, irrespectively of polymer concentration, probably because a low sonication power provided larger droplets of organic phase, therefore more polymer forming the particles. 50 and 65% amplitude resulted in particles with a similar particle size, however smaller particles were always obtained with 50% sonication power, hypothetically a high sonication power promoted coalescence of the droplets formed. Thus, 50% was selected as sonication power during the preparation of nanocapsules. Interestingly, while further increase in particle size was obtained with 4% polymer in case of 50% sonication power, a plateau was obtained with concentrations ≥ 2% polymer in case of 25 and 65%, indicating that in these two cases the major impact on droplet size was provided by the sonication power, and not by the amount of polymer. Overall, the differences in size were, however, not remarkably pronounced.

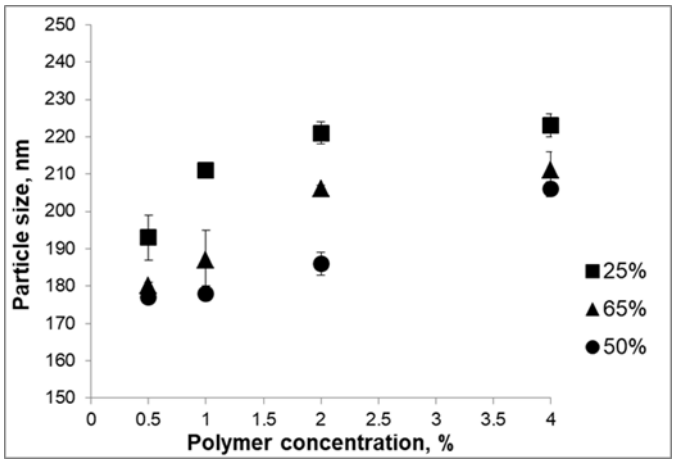

Figure 1 Effect of polymer concentration and sonication power on particle size of blank Eudragit® RS 100 nanoparticles.

In order to obtain nanocapsules with a nanocrystal core and polymer shell, dexamethasone nanocrystals were milled in dichloromethane and used as organic phase for the preparation of the nanoemulsion. Eudragit® RS 100 concentrations in the organic phase of 0.5, 1, 2 and 4% (w/v) were tested in order to determine the effect of the amount of polymer on the formation of nanocapsules and on their particle size. Based on the results obtained with blank polymer nanoparticles, the sonication power was set to 50%. Dexamethasone nanocrystals with an overall Z-average of ~300 nm were obtained with each polymer concentration. Low amounts of polymer were also enough to provide stability, perhaps because of the short time frame between the milling and the emulsification processes.

Longer processing time may have resulted in instability. The amount of Eudragit® RS 100 markedly influenced the particle size after emulsification. Nanocapsules could be obtained only with 2% (w/v) polymer concentrated organic phase, instead only large agglomerates were present with the other three tested concentrations (Table 1, Figs. 2 A, B, C and D). In case of 0.5 and 1%, the movement of the stirrer during overnight evaporation was completely blocked due to the presence of large aggregates. The formation of aggregates/ agglomerates could be due to the lack of polymer needed to stabilize the nanocrystals during emulsification with the aqueous phase, which resulted in a sudden drop in drug solubility due to addition of the organic suspension to water. In case of Eudragit® RS 100 4% (w/v) concentrated organic phase, the amount of polymer was too high and resulted in its exhaustive desolvation and extensive precipitation, with loss of the stabilization of nanocrystal, which aggregated together and with the polymer. 2% polymer resulted in a successful process and in preparation of nanocapsules with a particle size of ~230 nm (Table 1). Thus, if the polymer was present in an adequate amount, nanocrystals and nanocapsules were stabilized. The nanocrystals were hence stabilized by the dissolved polymer, while the nanocapsules were stabilized by the positive charge provided by the polymer. The zeta potential of the nanocapsules obtained with 2% polymer was indeed 47 ± 2 mV. The smaller size of nanocapsules compared to dexamethasone nanocrystals could be explained by the simultaneous formation of blank polymer nanoparticles during nanocapsule preparation, lowering the average particle size. Additionally, as PCS measures the hydrodynamic diameter, the presence of dissolved stabilizer covering the surface of nanocrystals (not present in case of nanocapsules) resulted in a larger particle size.

Table 1 Effect of different Eudragit® RS 100 concentrations on the particle size of nanocrystals (NC) after wet bead milling in dichloromethane and of nanocapsules (NCap) obtained by the solvent evaporation method.

Eudragit® RS100		
Polym. conc. (%)	**NC Z-average, PDI**	**NCap Z-average, PDI**
0.5	350 ± 21; 0.1	>1000
1	244 ± 2; 0.1	>1000
2	254 ± 1; 0.1	233 ± 2; 0.2
4	291 ± 4; 0.1	>1000

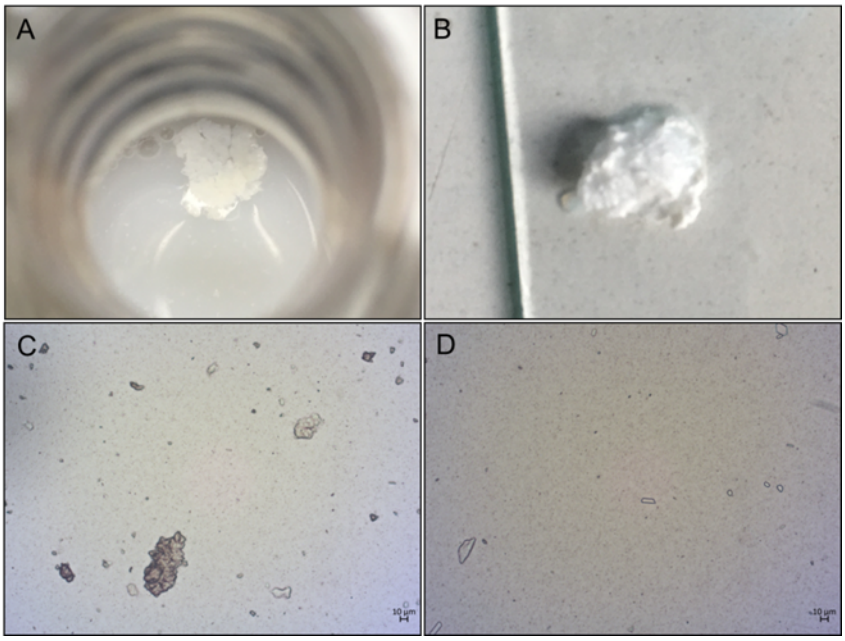

Figure 2 Photos and optical microscopy pictures of aggregates/ agglomerates and non-encapsulated nanocrystals obtained after nanocapsule preparation and overnight stirring for the samples prepared with A) and C) 1% and B) and D) 4% Eudragit® RS 100 in the organic phase.

The effect of formulation and process parameters on the particle size of blank nanoparticles was evaluated also for ethyl cellulose (Fig. 3). As ethyl cellulose lacks of self-stabilizing properties, the addition of a stabilizer to the aqueous phase was necessary. Poloxamer 407 was selected because of the good physical stability it provided to drug nanocrystals during previous studies [20, 30] and to the potential use of poloxamers as emulsifiers [31]. A solution of 1% (w/v) poloxamer 407 was used as aqueous phase. Blank nanoparticles with a size average of ~230 nm were obtained for all process conditions tested. In general, the particle size of ethyl cellulose nanoparticles was slightly larger than Eudragit® RS 100 nanoparticles. This could be due to the highly effective electrostatic repulsion between the nanoparticles provided by the positive charges of the quaternary ammonium groups of Eudragit® RS 100 exposed on the particle surface. The ethyl cellulose concentration had a more pronounced effect on particle size compared to sonication power: increasing the polymer concentration interestingly resulted in smaller particles, with a difference in size average of ~100 nm between 0.5 and 4% polymer. Perhaps, as dichloromethane has a water solubility higher than other water-immiscible solvents as chloroform or benzene, the mass transfer between the organic and aqueous phase happens relatively fast, resulting in small droplets [32]. Moreover, since dichloromethane is an appropriate solvent for ethyl cellulose, especially for low viscosity grade ones, as indicated by the interaction constant k′ determined during previous studies [33], it is hypothesized that as the solvent is removed, the polymer

chains aggregate fast together, thereby smaller particle size may be obtained with higher polymer amount (Fig. 3). A different trend compared to Eudragit® RS 100 was obtained also with regard to effect of sonication power on particle size: the smallest particles were obtained with 25% amplitude, followed by 65 and 50% power. The differences in size were overall, however, not pronounced, as already observed in case of Eudragit® RS 100.

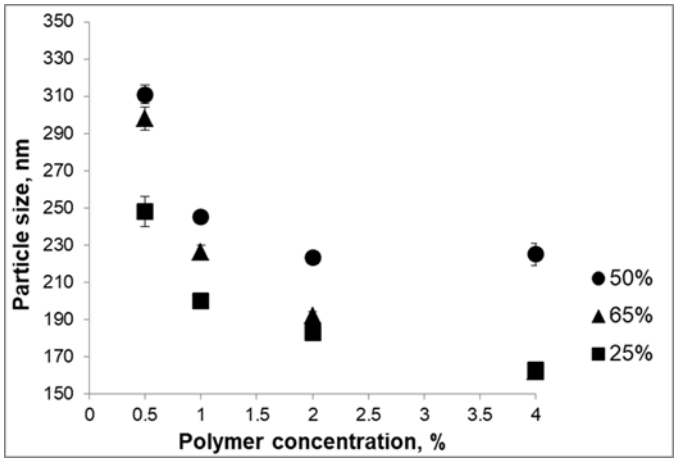

Figure 3 Effect of polymer concentration and sonication power on particle size of blank ethyl cellulose nanoparticles with poloxamer 407 as stabilizer.

The sonication power was set to 25% during the preparation of nanocapsules. Prior to the emulsification step, dexamethasone nanocrystals were prepared by wet bead milling in dichloromethane with 0.5, 1, 2, or 4% (w/v) ethyl cellulose. The obtained nanocrystal suspension was used as organic phase and added to the aqueous phase in a volume ratio 1:3. Nanocapsules could not be obtained with any of the tested polymer concentrations, and the process resulted in large aggregates/ agglomerates, blocking the magnetic stirrer. The system was highly unstable due to the sudden drop in drug solubility obtained during addition of the organic phase to the aqueous phase and to the incapacity of poloxamer 407 to adequately stabilize the emulsion. Thus, poloxamer 407 was replaced by PVA, a polymer commonly used during nanoparticle preparation and also used as stabilizer for nanosuspensions [10, 26]. First, blank polymer nanoparticles were prepared to evaluate the feasibility of the process. 1% (w/v) PVA solution was used as aqueous phase during emulsification. The influence of ethyl cellulose concentration in the organic phase and of sonication power was also determined (Fig. 4). Particles in the nanometer size range were obtained with all tested conditions. Neither sonication power nor ethyl cellulose concentration in the organic phase markedly affected the particle size, which scattered around a size average of ~220 nm. No clear trend was observed by changing the parameters. The nanoparticles were, in general, only slightly larger than Eudragit® RS 100 nanoparticles,

which could be explained by the strong particle-particle repulsion provided by the surface charge of Eudragit® RS 100.

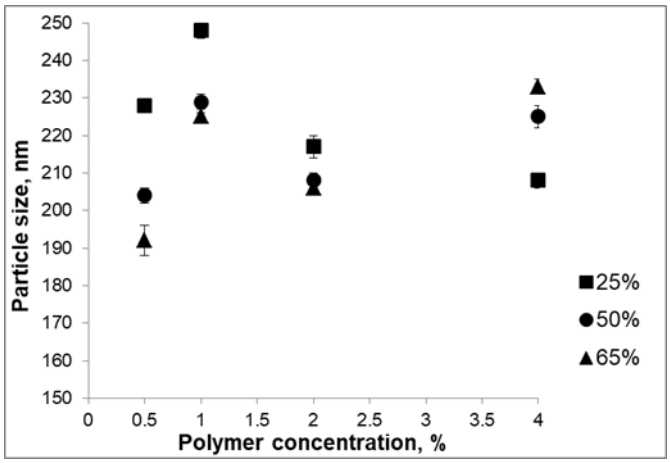

Figure 4 Effect of polymer concentration and sonication power on particle size of blank ethyl cellulose nanoparticles with PVA as stabilizer.

Next, nanocapsules with a nanocrystal core and ethyl cellulose shell were prepared. The sonication power was set to 25% in order to have the same process parameters used in case of poloxamer 407 as stabilizer, thereby comparing the results and evaluating PVA as emulsion stabilizer. Nanocrystals with a size average of ~360 nm were first prepared by wet bead milling in dichloromethane with different ethyl cellulose concentrations. Nanocrystals were obtained for each polymer concentration, indicating that the stabilization provided by ethyl cellulose was at least enough for the short time frame between the milling and emulsification processes. Increasing the polymer concentration resulted in larger nanocrystals. This could be explained by the larger hydrodynamic diameter of particles on whose surface more dissolved polymer is adsorbed. The nanosuspension was afterwards used as organic phase during emulsification. The water phase was an aqueous solution of 1% (w/v) PVA, as for the blanks. PVA was excellent both as emulsifier and particle size stabilizer. Nanocapsules with an average particle size of ~270 nm were obtained with each polymer concentration. While in case of Eudragit® RS 100 a polymer concentration in the organic phase lower than 2% resulted in large agglomerates, particles in the nanometer size range were obtained with ethyl cellulose also at low concentrations (Table 2). The smaller particle size of the nanocapsules compared to nanocrystals was attributed to the presence of blank nanoparticles, lowering the average particle size, and to the presence of dissolved polymer on the nanocrystal surface. The combination of ethyl cellulose and PVA thus provided a stable nanoemulsion and a stable nanosuspension after evaporation of the solvent.

Table 2 Effect of different ethyl cellulose concentrations on the particle size of nanocrystals (NC) after wet bead milling in dichloromethane and of nanocapsules (NCap) obtained by the solvent evaporation method.

Ethyl cellulose		
Polym. conc. (%)	**NC Z-average, PDI**	**NCap Z-average, PDI**
0.5	281 ± 3; 0.2	301 ± 6; 0.2
1	314 ± 3; 0.1	262 ± 2; 0.2
2	349 ± 1; 0.1	273 ± 3; 0.2
4	528 ± 148; 0.2	245 ± 1; 0.1

Solvent evaporation was therefore a suitable method for the encapsulation of dexamethasone nanocrystals within a shell of water-insoluble polymers. The formulations with 1% (w/v) drug and 2% (w/v) polymer resulted in stable nanocapsules for both Eudragit® RS 100 and ethyl cellulose. Thus, these formulations were selected for all further studies.

3.3 Characterization of nanocapsules and confirmation of their structure

3.3.1. Morphological evaluation of nanocrystals, blank polymer nanoparticles and nanocapsules

The morphological evaluation of nanocrystals, blank nanoparticles and nanocapsules was performed by TEM (Figs. 5 A-F). Differences in size, shape and intensity of the transmission (darkness of the contrast) were observed, indicating three different types of particles. Nanocrystals milled in Eudragit® RS 100 or ethyl cellulose organic solutions were characterized by a relatively squared shape (Figs. 5 A and D, respectively), comparable to results obtained during milling of dexamethasone in an aqueous stabilizer solution [20]. The particle size measured by PCS (434 ± 21 nm for nanocrystals with Eudragit® RS 100, 349 ± 1 nm in case of ethyl cellulose) was confirmed. Blank polymer nanoparticles of Eudragit® RS 100 and ethyl cellulose (189 ± 3 nm and 217 ± 3 nm, respectively) were prepared using the same polymer concentration as for nanocrystals and nanocapsules (2% (w/v) in the organic phase). The resulting particles were characterized by a spherical shape and smaller particle size than nanocrystals, confirming the PCS results (Figs. 5 B and E). The dark contrast of the particles on the image was lower than for nanocrystals: while nanocrystals were deeply black, blank nanoparticles were grey. This was in agreement with the fact that polymers/ amorphous materials have a lower density, therefore the scattering of incident electrons is primarily in the forward direction, with little or no contrast given by the objective aperture [34]. The particle size of nanocapsules measured by PCS was 216 ± 1 nm for the ones with Eudragit® RS 100 and 273 ± 3 nm with EC. In both cases, the analysis of shape, intensity and size of the visualized particles led to the conclusion that all three different types of particles were simultaneously present (Figs. 5 C and F). Blank nanoparticles could be

distinguished based on their lower contrast and smaller size, few squared nanocrystals were observed, while the other particles were quite spherical in shape, with relatively high contrast in the image, indicating presence of crystalline material in the particles. These characteristics were attributed to the nanocapsules. Majority of the nanocapsules on the image were relatively small, in accordance with the size measured by PCS. It is hence hypothesized that small and less squared nanocrystals are overall more efficiently coated than larger nanocrystals. The successful preparation of nanocapsules with a nanocrystal core and a polymer shell was thus supported by morphological evaluation via TEM analysis.

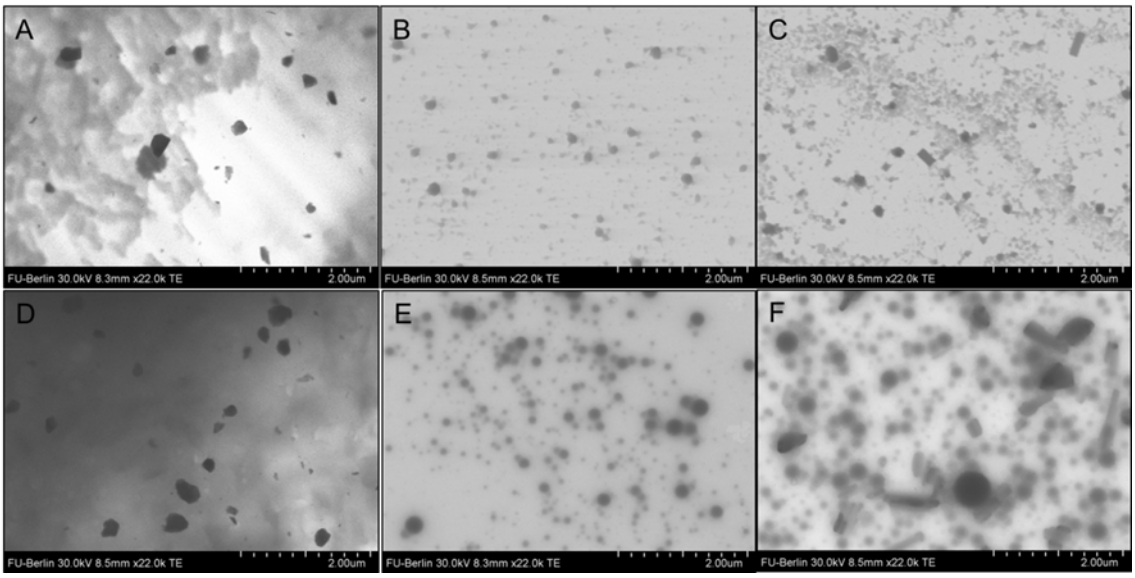

Figure 5 Transmission electron micrographs of A) and D) nanocrystals milled in dichloromethane with 2% (w/v) Eudragit® RS 100 or ethyl cellulose, respectively, B) and E) blank nanoparticles of Eudragit® RS 100 and ethyl cellulose, respectively, and of nanocapsules of dexamethasone nanocrystals within C) Eudragit® RS 100 or F) ethyl cellulose.

The contrast of the particles on the images was analyzed with the software ImageJ in order to obtain numerical values which could be compared among the particles. Each pixel in a digital image is associated with an integer value. These values can be interpreted as intensities, where 0 corresponds to a black pixel and no intensity. Higher values are associated to lighter pixels, lower values to darker pixel [35]. Thereby, the values related to the intensities of the different types of particles were compared (Table 3). Nanocrystals of both polymers resulted in dark pixels on the images, with intensity values of ~75. Blank polymer nanoparticles were, as observed, lighter and resulted in higher average values of ~125. The contrast of nanocapsules was also dark, however not as high as nanocrystals due to the presence of the coating polymers. The obtained values were, in this case, in between

the ones of nanocrystals and blank nanoparticles. The conclusions drawn by observation of the TEM images were hence further supported.

Table 3 Mean intensity values and related standard deviations determined for nanocrystals, blank polymer nanoparticles and nanocapsules (n=3) (EuRS100 = Eudragit® RS 100, EC = ethyl cellulose).

	Nanocrystals		**Blank nanoparticles**		**Nanocapsules**	
	EuRS100	**EC**	**EuRS100**	**EC**	**EuRS100**	**EC**
Average	83.4	69.4	135.1	119.4	113.9	93.9
Standard deviation	24.4	2.1	6.4	8.9	2.2	20.9

3.3.2. FTIR analysis

FTIR has become a widely used technique for the analysis of pharmaceutical solids and liquids and its applications include solid state analysis, discrimination of different polymorphic forms and identification of pharmaceutical substances [36]. In our study, FTIR was used to support the successful preparation of nanocapsules. During nanoencapsulation, dexamethasone nanocrystals were embedded within a layer of a water-insoluble polymer. The drug's functional groups were thereby not exposed on the outer surface of the particle and not remarkably susceptible to interaction with the IR light. A reduction of the absorption peaks when a material is encapsulated has been observed in previous studies where microparticles were analyzed [37, 38]. The drug's functional groups are indeed surrounded by the polymer, which is generally present in relatively higher amounts compared to the drug, thereby exercising a masking effect. However, if non-encapsulated nanocrystals are present in the sample in large amounts, the functional groups of the drug would be less covered by the polymer, thereby available for interaction, and drug representative absorption peaks should be present in the spectrum. The higher the amount of non-encapsulated nanocrystals, the higher the absorption of the drug's functional groups. IR spectra of nanocapsules prepared with Eudragit® RS 100 or ethyl cellulose were collected and compared to the spectra of physical mixtures drug:polymer and of pure drug and excipients (Figs. 6 A and B). The freeze-dried nanocapsules' powder was used for the experiments. The spectra of pure dexamethasone and of physical mixtures of drug and polymers were characterized by absorption peaks related to the vibration of the carbonyl C=O and to the double bond framework conjugated to the C=O bond of dexamethasone at 1703, 1660, 1616 and 1602 cm-1, in accordance with previous reports [39]. The intensity of the drug's absorption peaks in the spectra of the nanocapsules was reduced in comparison to physical mixtures or pure drug, suggesting that most of the drug was encapsulated within the polymer shell. These

results supported the conclusions drawn by observation of the TEM pictures, confirming that nanocapsules of dexamethasone nanocrystals within a polymer shell were obtained.

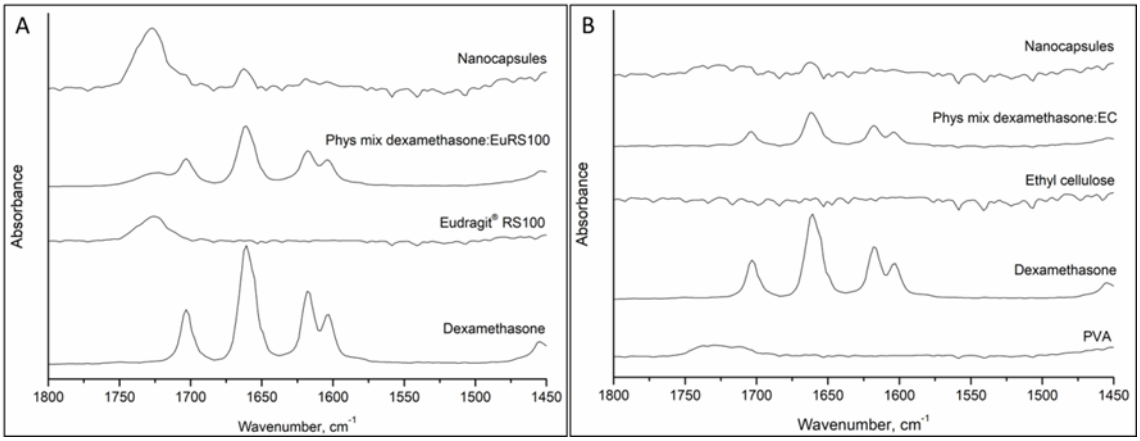

Figure 6 FTIR spectra of physical mixtures and nanocapsules prepared with A) Eudragit® RS 100 and B) ethyl cellulose and of pure drug and excipients.

3.3.3. Stability of particle size during dilution with water

Eudragit® RS 100 and ethyl cellulose are polymers extensively used for preparation of nanoparticles, as they can provide controlled drug release, high encapsulation efficiency due to higher drug solubility, stability and protection towards drug degradation. In our study, they were used to form the shell embedding dexamethasone nanocrystals and thereby forming nanocapsules with a solid core. The drug release from nanocapsules differs depending on parameters such as concentration and physicochemical characteristics of the active substance and of the polymer, solid microstructure of the polymer shell, particle size, release conditions and preparation method [15]. Overall, a controlled drug release is expected. In the present study, where nanocrystals constitute the nanocapsules' core as they are included within a shell of a water-insoluble polymer, drug release is expected to be controlled because the polymer prevents the drug nanocrystals from immediately dissolving. Hence, the particle size of such nanocapsules should remain stable over time during dissolution experiments. The particle size stability of nanocapsules was tested by dilution of the nanosuspensions with water until a final drug concentration of ~50 µg/mL was reached. The particle size was measured over time by PCS before dilution, directly after dilution, and at predefined time points (15, 30, 45, 60 and 120 min) (Figs. 7 A and B). The size of nanocapsules decreased instantly after dilution due to the rapid dissolution of non-encapsulated nanocrystals. After this initial decrease, the particle size remained stable within 2 h, indicating that the nanocapsules were experiencing neither swelling nor erosion, thereby their structure was maintained. The particle size was, however, fairly low, quite similar to the particle size of blank polymer nanoparticles. This confirmed what observed in the TEM images, where, in the samples of nanocapsules, blank nanoparticles and nanocrystals were also present. The

particle size of these samples was, therefore, the average of the differently-sized particles present.

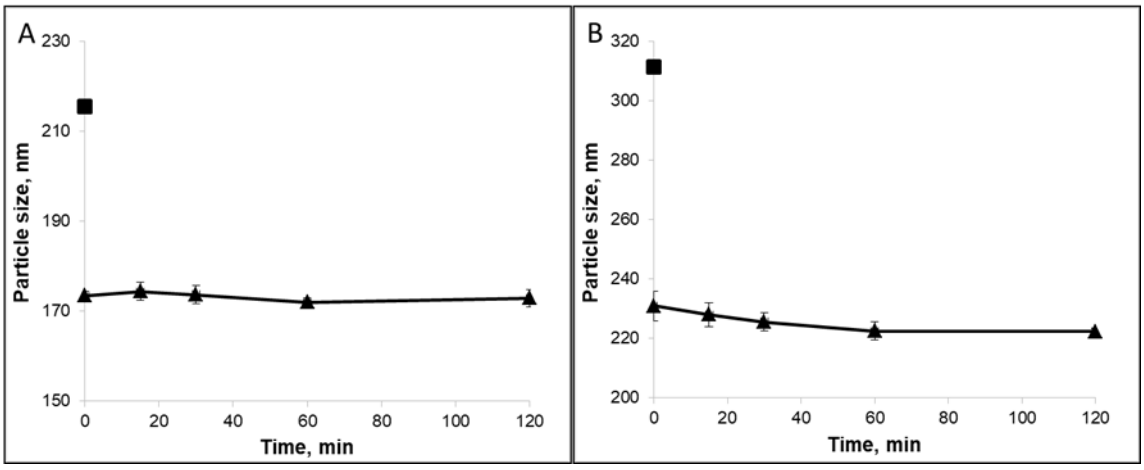

Figure 7 Trend over time of particle size of A) Eudragit® RS 100 and B) ethyl cellulose nanocapsules before (square) and after (triangle) dilution with water.

3.4. Drug release studies with Franz diffusion cell

The drug release from nanocapsules is expected to be controlled due to the presence of a polymer coating the inner drug core. The drug release profile can, therefore, be used to proof the successful encapsulation of drug nanocrystals, as non-encapsulated drug nanocrystals are characterized by a fast and immediate dissolution [28, 40]. The drug release profiles from regular nanocrystals prepared with 1% (w/v) PVA as stabilizer (354 ± 1 nm, PDI 0.2) and nanocapsules with a nanocrystal core and Eudragit® RS 100 (230 ± 1; PDI 0.3) or ethyl cellulose (364 ± 6 nm, PDI 0.2) shell were compared during dissolution studies with Franz diffusion cell (Fig. 8). The drug release from nanocapsules was slower than from nanocrystals, although the difference was observed only after ~3.5 h of release. The initial similarity to nanocrystals was due to the presence of non-encapsulated nanocrystals, which boosted the drug release and thereby masked the differences in release during the initial hours. Interestingly, no difference was obtained among the nanocapsules with Eudragit® RS 100 or with ethyl cellulose, although a faster release was expected from Eudragit® RS 100 nanocapsules due to the higher hydrophilicity of this polymer. The drug release from nanocapsules is, however, also dependent on the microstructure of the polymer shell: the PVA present in the formulation of ethyl cellulose nanocapsules may have acted as pore former, thereby increasing the release rate from ethyl cellulose nanocapsules.

The drug release profiles from nanocrystals and nanocapsules were hence discriminated by Franz diffusion cell, thereby proofing the successful coating of dexamethasone nanocrystals by water-insoluble polymers.

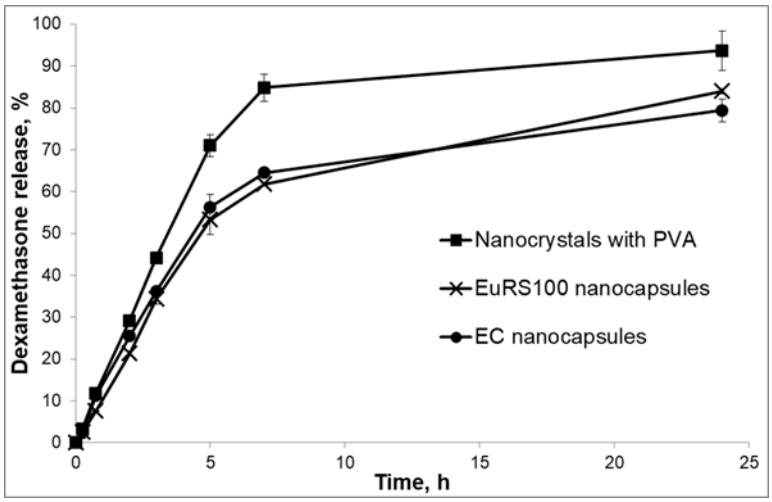

Figure 8 Dexamethasone release profiles from different nanocarriers investigated with Franz diffusion cells (n=3) (EuRS100 = Eudragit® RS 100, EC = ethyl cellulose).

4. Conclusions

Dexamethasone nanocrystals were prepared by wet bead milling in an organic solution of water-insoluble polymers in dichloromethane, underlining the potential of the use of organic solvents for nanocrystal preparation by nanomilling. The thereby obtained nanosuspension was afterwards used as organic phase during preparation of nanocapsules by the solvent evaporation method. The emulsification with the aqueous phase and consecutive evaporation of the solvent overnight resulted in precipitation of the polymer on the nanocrystals' surface. The successful encapsulation was supported by morphology, intensity analysis and FTIR studies, particle size stability upon dilution and drug release experiments. The drug release from the nanocapsules was controlled, instead the drug release from regular nanocrystals was faster and complete.

Nanocapsules with a nanocrystal core within a shell of insoluble polymers represent a very innovative nanocarrier which combines the high drug loading provided by nanocrystals with the controlled drug release obtained by the polymer coating. This type of nanocarrier could have a remarkable potential for the treatment of diseases which require prolonged exposure to the drug or for drugs whose toxicity is mediated by peak plasma values but efficacy is AUC driven because of the possibility of loading more drug inside a small volume and guaranteeing drug release for a longer period of time.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the Sonderforschungsbereich (SFB) 1112 in the context of Nanocarriers: Architecture, Transport, and Topical Application of Drugs for Therapeutic Use.

References

[1] J. Junghanns, R.H. Müller, Nanocrystal technology, drug delivery and clinical applications, Int J Nanomedicine, 3 (2008) 295-309.
[2] S. Kalepu, V. Nekkanti, Insoluble drug delivery strategies: review of recent advances and business prospects, Acta Pharmaceutica Sinica B, 5 (2015) 442-453.
[3] K.T. Savjani, A.K. Gajjar, J.K. Savjani, Drug solubility: importance and enhancement techniques, ISRN pharmaceutics, 2012 (2012).
[4] R.H. Müller, S. Gohla, C.M. Keck, State of the art of nanocrystals–special features, production, nanotoxicology aspects and intracellular delivery, Eur. J. Pharm. Biopharm., 78 (2011) 1-9.
[5] B.E. Rabinow, Nanosuspensions in drug delivery, Nature Reviews Drug Discovery, 3 (2004) 785.
[6] J.E. Kipp, The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs, Int. J. Pharm., 284 (2004) 109-122.
[7] A.A. Noyes, W.R. Whitney, The rate of solution of solid substances in their own solutions, J. Am. Chem. Soc., 19 (1897) 930-934.
[8] L. Skinner, J. Sambles, The Kelvin equation—a review, Journal of Aerosol Science, 3 (1972) 199-210.
[9] Y. Wang, Y. Zheng, L. Zhang, Q. Wang, D. Zhang, Stability of nanosuspensions in drug delivery, J. Control. Release, 172 (2013) 1126-1141.
[10] L. Wu, J. Zhang, W. Watanabe, Physical and chemical stability of drug nanoparticles, Advanced Drug Delivery Reviews, 63 (2011) 456-469.
[11] D.N. Nguyen, C. Clasen, G. Van den Mooter, Encapsulating darunavir nanocrystals within Eudragit L100 using coaxial electrospraying, Eur. J. Pharm. Biopharm., 113 (2017) 50-59.
[12] A.S. Hasan, M. Socha, A. Lamprecht, F.E. Ghazouani, A. Sapin, M. Hoffman, P. Maincent, N. Ubrich, Effect of the microencapsulation of nanoparticles on the reduction of burst release, Int. J. Pharm., 344 (2007) 53-61.
[13] E. Merisko-Liversidge, G.G. Liversidge, Nanosizing for oral and parenteral drug delivery: A perspective on formulating poorly-water soluble compounds using wet media milling technology, Advanced Drug Delivery Reviews, 63 (2011) 427-440.
[14] S. Gopal, C. Gassmann-Mayer, J. Palumbo, M.N. Samtani, R. Shiwach, L. Alphs, Practical guidance for dosing and switching paliperidone palmitate treatment in patients with schizophrenia, Curr. Med. Res. Opin., 26 (2010) 377-387.
[15] C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, Int. J. Pharm., 385 (2010) 113-142.

[16] R. Bodmeier, J.W. McGinity, Solvent selection in the preparation of poly(dl-lactide) microspheres prepared by the solvent evaporation method, Int. J. Pharm., 43 (1988) 179-186.
[17] R. Bodmeier, J.W. McGinity, Polylactic acid microspheres containing quinidine base and quinidine sulphate prepared by the solvent evaporation technique. II. Some process parameters influencing the preparation and properties of microspheres, J. Microencapsul., 4 (1987) 289-297.
[18] J. Herrmann, R. Bodmeier, Biodegradable, somatostatin acetate containing microspheres prepared by various aqueous and non-aqueous solvent evaporation methods, Eur. J. Pharm. Biopharm., 45 (1998) 75-82.
[19] C. Pinto Reis, R.J. Neufeld, A.J. Ribeiro, F. Veiga, Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles, Nanomedicine: Nanotechnology, Biology and Medicine, 2 (2006) 8-21.
[20] M. Colombo, S. Staufenbiel, E. Rühl, R. Bodmeier, In situ determination of the saturation solubility of nanocrystals of poorly soluble drugs for dermal application, Int. J. Pharm., 521 (2017) 156-166.
[21] M. Colombo, S. Orthmann, M. Bellini, S. Staufenbiel, R. Bodmeier, Influence of Drug Brittleness, Nanomilling Time, and Freeze-Drying on the Crystallinity of Poorly Water-Soluble Drugs and Its Implications for Solubility Enhancement, AAPS PharmSciTech, (2017) 2437-2445.
[22] R. Pignatello, C. Bucolo, P. Ferrara, A. Maltese, A. Puleo, G. Puglisi, Eudragit RS100® nanosuspensions for the ophthalmic controlled delivery of ibuprofen, Eur. J. Pharm. Sci., 16 (2002) 53-61.
[23] G.S. Rekhi, S.S. Jambhekar, Ethylcellulose - A Polymer Review, Drug Dev. Ind. Pharm., 21 (1995) 61-77.
[24] S.C. Porter, Controlled-Release Film Coatings Based on Ethylcellulose, Drug Dev. Ind. Pharm., 15 (1989) 1495-1521.
[25] B. Balzus, M. Colombo, F.F. Sahle, G. Zoubari, S. Staufenbiel, R. Bodmeier, Comparison of different in vitro release methods used to investigate nanocarriers intended for dermal application, Int. J. Pharm., 513 (2016) 247-254.
[26] B. Balzus, F.F. Sahle, S. Hönzke, C. Gerecke, F. Schumacher, S. Hedtrich, B. Kleuser, R. Bodmeier, Formulation and ex vivo evaluation of polymeric nanoparticles for controlled delivery of corticosteroids to the skin and the corneal epithelium, Eur. J. Pharm. Biopharm., 115 (2017) 122-130.
[27] L. Peltonen, J. Hirvonen, Pharmaceutical nanocrystals by nanomilling: critical process parameters, particle fracturing and stabilization methods, J. Pharm. Pharmacol., 62 (2010) 1569-1579.

[28] R. Shegokar, R.H. Müller, Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives, Int. J. Pharm., 399 (2010) 129-139.
[29] R. Bodmeier, H. Chen, P. Tyle, P. Jarosz, Spontaneous formation of drug-containing acrylic nanoparticles, J. Microencapsul., 8 (1991) 161-170.
[30] M. Colombo, C. Minussi, S. Orthmann, S. Staufenbiel, R. Bodmeier, Preparation of amorphous indomethacin nanoparticles by aqueous wet bead milling and in situ measurement of their increased saturation solubility, Eur. J. Pharm. Biopharm., (2018).
[31] I.R. Schmolka, A review of block polymer surfactants, Journal of the American Oil Chemists' Society, 54 (1977) 110-116.
[32] N.V.N. Jyothi, P.M. Prasanna, S.N. Sakarkar, K.S. Prabha, P.S. Ramaiah, G.Y. Srawan, Microencapsulation techniques, factors influencing encapsulation efficiency, J. Microencapsul., 27 (2010) 187-197.
[33] H. Arwidsson, M. Nicklasson, Application of intrinsic viscosity and interaction constant as a formulation tool for film coating II. Studies on different grades of ethyl cellulose in organic solvent systems, Int. J. Pharm., 58 (1990) 73-77.
[34] M.R. Libera, R.F. Egerton, Advances in the Transmission Electron Microscopy of Polymers, Polymer Reviews, 50 (2010) 321-339.
[35] V. Baecker, Workshop: Image processing and analysis with ImageJ and MRI Cell Image Analyzer, in, 2008.
[36] A. Salari, R.E. Young, Application of attenuated total reflectance FTIR spectroscopy to the analysis of mixtures of pharmaceutical polymorphs, Int. J. Pharm., 163 (1998) 157-166.
[37] K. Kesavan, S. Kant, P.N. Singh, J.K. Pandit, Effect of hydroxypropyl-β-cyclodextrin on the ocular bioavailability of dexamethasone from a pH-induced mucoadhesive hydrogel, Curr. Eye Res., 36 (2011) 918-929.
[38] W. Jincheng, C. Sihao, Preparation and characterization of microcapsules containing capsaicin., J. Appl. Polym. Sci., 116 (2010) 2234–2241.
[39] L.B. Rodrigues, H.F. Leite, M.I. Yoshida, J.B. Saliba, A.S.C. Junior, A.A.G. Faraco, In vitro release and characterization of chitosan films as dexamethasone carrier, Int. J. Pharm., 368 (2009) 1-6.
[40] B. Van Eerdenbrugh, G. Van den Mooter, P. Augustijns, Top-down production of drug nanocrystals: Nanosuspension stabilization, miniaturization and transformation into solid products, Int. J. Pharm., 364 (2008) 64-75.

4. Summary

Nanocrystals are pure drug particles in the nanometer size range stabilized by proper type and amount of stabilizers. They represent the leading technology for drugs, the formulation of which is problematic due to their low solubility in water and oils, high melting temperature and high molecular weight, because the need for solubilization is obviated by maintaining the drug in a solid crystalline state. Moreover, nanocrystals have a 100% drug loading because no carrier material is used, hence they allow the administration of a high dose of drug in a small volume, a factor which is crucial for *e.g.* IM or SC administration. With regard to dermal administration, other advantages related to the use of nanocrystals are their increased saturation solubility, resulting in a higher concentration gradient, faster dissolution rate, increased adhesiveness, and potential hair follicle targeting.

The increased saturation solubility of nanocrystals was an unexpected and highly-discussed outcome because this factor was considered dependent only on drug's chemistry, solvent, and temperature. As different increase factors are reported in the literature with regard to saturation solubility of nanocrystals, and since *in situ* methods are considered more accurate for its determination, a novel *in situ* method based on UV-vis spectroscopy was utilized to evaluate the extent of the enhanced solubility of nanocrystals of three poorly soluble drugs, dexamethasone, tacrolimus and ibuprofen (chapter 3.1). Wet bead milling was selected as preparation method, and factors affecting the particle size were analyzed. Increasing speed and bead amount and reducing the bead size resulted in smaller nanocrystals, and using different process parameters enabled the preparation of particles with different sizes. The increased saturation solubility of 300 nm-sized nanocrystals was analyzed *in situ* by adding 2-3 times excess amounts (with regard to the saturation solubility of the micronized drug) to water. The increase factors obtained ranged between 1.3 and 2.8. Particles with a size of ~1 µm were not characterized by increased solubility. The factors of increased solubility measured by our and other different *in situ* methods reported in the literature were comparable, and were smaller than what was obtained with non *in situ* techniques. Thus, the use of *in situ* methods for nanocrystal solubility determination is encouraged. The effect of nanocrystal excess conditions was analyzed with tacrolimus, the solubility of which was extremely low, thereby allowed the use of higher excess conditions without problems related to the background scattering. The maximum obtained factor of increase in solubility was 6.6. Thus, the enhancement with regard to saturation solubility when formulating drugs as nanocrystals is not remarkably high. Therefore, considering dermal application, the concentration gradient between formulation and skin would not markedly increase, and other features may be more relevant to explain the efficiency of nanocrystals in delivering drugs,

for instance their dissolution rate. The latter is indeed highly increased by particle size reduction to the nanometer range. This parameter was evaluated during the *in situ* solubility studies. The effect was extremely remarkable for ibuprofen: the dissolution rate of 0.14 µg/(mL*s) corresponding to 70 µm particles was increased to 4.02 µg/(mL*s) with ~300 nm crystals, thereby an increase factor of ~29 was obtained.

The solid state of a drug, *i.e.* crystalline or amorphous, markedly affects its solubility: the thermodynamically-unstable amorphous state is characterized by the highest saturation solubility and thereby dissolution rate. Milling (grinding) is one of the methods used to generate the amorphous form of a compound because the high energy and shear-rate involved in the process can damage the crystalline structure. In wet bead milling, very high energy forces are used to reduce the particle size. The effect of this process on the solid state of drug nanocrystals and its implications on solubility enhancement was evaluated (chapter 3.2). Wet bead milling for long times (≥2 h) resulted in a reduction of the degree of crystallinity of dexamethasone and tacrolimus nanocrystals. Taking the initial drug powder as 100% crystallinity, the values of degree of crystallinity of the milled particles reached plateaus of ~79 and ~76% for dexamethasone and tacrolimus after 2 and 3 h milling, respectively. The energy involved in the process was not enough for further reduction. The different time needed to achieve the plateaus depended on the brittleness of the drugs, which was determined by the stress-strain curves of the two drugs, which were compared to the ones of very brittle or ductile materials. In comparison to dexamethasone, tacrolimus was more ductile, hence it deformed plastically before it started to break. The effect of freeze-drying, a commonly used drying technique for nanocrystals, on the degree of crystallinity of nanocrystals was also evaluated. A further reduction in crystallinity was obtained, and the effect of the milling process on the solid state was thereby evened. Finally, the implications of reduced crystallinity on saturation solubility of nanocrystals were analyzed *in situ* in combination to the effect of nanosize. The highest increase in saturation solubility was always obtained with the lowest degree of crystallinity, and not with the smallest particle size. This was observed with tacrolimus, for which the highest solubility increase (factor 1.7) was obtained for particles milled for 5 h, the degree of crystallinity of which was the lowest obtained, 76%, but their average particle size was 527 nm, hence larger than particles milled for a shorter time, but the degree of crystallinity of which was higher and resulted in a lower solubility increase. Although a reduced crystallinity has positive implications on solubility enhancement, alterations to drug solid state during product shelf-life are not acceptable. Thus, the selection of proper parameters (*e.g.* reduced speed, increased medium viscosity) is suggested to preserve the solid state of the drug throughout the milling process and during product shelf-life.

The evidence that the reduced degree of crystallinity contributed to the increase in saturation solubility of nanocrystals suggested that the maximum solubility enhancement, the clinical relevance of which may be hence higher, could be obtained by combining amorphous solid state and nanosize by preparation of amorphous nanoparticles (chapter 3.3). Indomethacin was the model drug used because it is a stable glass former. Wet bead milling was selected as preparation method and the challenge represented by water as medium, promoting recrystallization, was tackled by finding a proper formulation stabilizing both the amorphous solid state and the particle size. Polyvinylpyrrolidone (PVP, Kollidon 30) was compared to poloxamer 407 as stabilizer. PVP was able to stabilize the amorphous solid state throughout the milling process, however, the particles did not remain in the nanometer range after redispersion of the freeze-dried powder. Poloxamer 407 acted oppositely: it stabilized the particle size, but not the amorphous solid state, and the particles recrystallized during milling. Combining the two polymers together was also not successful, as the amorphous state converted to the crystalline one upon nanomilling. Thus, it was hypothesized that the presence of two large polymers competing for surface coverage was the problem: PVP could not sufficiently cover the particle surface because of the steric impediment of poloxamer 407, thereby a lack of protection against recrystallization occurred. Poloxamer was thus replaced by a small surfactant, sodium dodecyl sulfate (SDS), which showed a synergism with PVP: this combination stabilized both the amorphous solid state and the particle size. Thus, the formulation necessary, and hence suggested, for stabilization of amorphous nanoparticles in a preparation process involving water, consists in the combination of a polymer acting as anti-recrystallization agent and a small molecule preserving the particle size in the nanometer range. The effect of amorphous state and nanosize on saturation solubility and dissolution rate was tested during *in situ* studies. The amorphous solid state and the nanonization of indomethacin alone had similar increase factors with regard to solubility (~2.4), while the dissolution rate was more enhanced in the case of nanocrystals. The greatest increase in both saturation solubility and dissolution rate was, however, obtained for amorphous nanoparticles, with a factor of increase of 5.2 for saturation solubility, while the dissolution rate reached 2.328 µg/(mL*s), markedly higher than what was obtained in the other cases. It was thus demonstrated that the combination of amorphization and nanonization provides a synergism with regard to both saturation solubility and dissolution rate. This effect has the potential for being therapeutically relevant in case of, but not limited to, dermal application, especially for drugs, the amorphization of which already consistently increases dissolution rate and solubility, which could be further enhanced by their nanonization.

Finally, a novel nanocarrier which would allow controlled drug release from nanocrystals was obtained by nanoencapsulating dexamethasone nanocrystals within a shell of water insoluble polymers by the solvent evaporation method (chapter 3.4). The polymers selected were Eudragit® RS 100 and ethyl cellulose. Wet bead milling of dexamethasone was performed in dichloromethane, and nanocrystals with a size of ~350 nm were obtained, demonstrating the feasibility of milling in organic solvents for nanocrystal preparation. After optimal process conditions were determined during preparation of blank polymer nanoparticles, the organic nanosuspension was emulsified with water or with an aqueous solution of polyvinyl alcohol (PVA) (for Eudragit® and ethyl cellulose, respectively) and the solvent was afterwards removed during overnight stirring. Polymer concentration of 2% (w/v) resulted in nanocapsules with a particle size of ~250 nm. The reason for the smaller particle size of nanocapsules compared to nanocrystals was the simultaneous formation of blank nanoparticles in the nanocapsule sample, and the presence of dissolved polymer covering the surface of nanocrystals, hence increasing the particle size measured by PCS. The successful nanoencapsulation of dexamethasone nanocrystals within a polymer shell was confirmed by morphology studies, FTIR, stability of particle size upon dilution below the drug solubility and, most relevant, dissolution studies. After an initial burst due to the presence of non-encapsulated nanocrystals, the drug release from nanocapsules was controlled over time in comparison to the one of a regular nanosuspension. A novel nanocarrier for the treatment of diseases which require prolonged exposure to the drug was thereby obtained and characterized. Indeed, a depot formulation for the treatment of, for instance, skin diseases like psoriasis via intradermal injection of this type of nanocarrier would be possible. Moreover, organic solvents may be replaced by oils or semisolids, and this method could thus be used for preparation of dermal nanocrystals directly in their final dosage form, saving time and costs.

5. Zusammenfassung

Nanokristalle sind reine Arzneistoffpartikel im Nanobereich, die durch geeignete Art und Menge eines Stabilisators stabilisiert werden. Sie repräsentieren die führende Technologie für Arzneistoffe, deren Formulierung aufgrund ihrer niedrigen Löslichkeit in Wasser und Ölen, hohen Schmelztemperaturen und hohen Molekulargewichten problematisch ist, zumal die Solubilisation vermieden wird, da der Arzneistoff in fester kristalliner Form erhalten bleiben muss. Zudem haben Nanokristalle aufgrund fehlendem Arzneistoffträger eine Arzneistoffbeladung von 100%, wodurch große Arzneistoffdosen in kleinen Volumina appliziert werden können, welches einen kritischen Faktor bei beispielsweise intramuskulärer oder subkutaner Administration darstellt. Insbesondere in Hinsicht auf eine dermale Applikation bieten sich weitere Vorteile wie höhere Konzentrationsgradienten, schnellere Freisetzungsraten, erhöhte Haftfähigkeit und ein potentielles Haarfollikel-Targeting.

Die erhöhte Sättigungslöslichkeit der Nanokristalle stellte ein unerwartetes und stark diskutiertes Ergebnis dar, da dieser Faktor ausschließlich durch die chemischen Eigenschaften des Arzneistoffs, des entsprechenden Lösungsmittels und der Temperatur beeinflusst werden sollte. Da von unterschiedlichen Erhöhungsfaktoren in der Literatur in Hinsicht auf Sättigungslöslichkeit von Nanokristallen berichtet wird, und da die Bestimmung mit *in situ* Methoden als genauer angesehen wird, wurde eine neue, auf UV-Vis Spektroskopie basierende *in situ* Methode verwendet, um das Ausmaß der erhöhten Löslichkeit von Nanokristallen der drei schwer löslichen Arzneistoffe Dexamethason, Tacrolimus und Ibuprofen zu bestimmen (Kapitel 3.1). Nassperlenvermahlung wurde als Herstellungsmethode gewählt, und Faktoren, die die Partikelgröße beeinflussen, wurden analysiert. Eine Erhöhung der Geschwindigkeit und der Menge an Mahlperlen und eine Verringerung der Mahlperlengröße führte zu kleineren Nanokristallen, und die Anwendung von verschiedenen Prozessparametern ermöglichte eine Herstellung von Partikeln mit verschiedenen Größen. Die erhöhte Sättigungslöslichkeit von Nanokristallen mit einer Größe von 300 nm wurde *in situ* analysiert, indem ein 2-3-facher Überschuss (basierend auf der Sättigungslöslichkeit von mikronisiertem Arzneistoff) in Wasser gegeben wurde. Die Erhöhungsfaktoren lagen im Bereich zwischen 1,3 und 2,8. Partikel mit einer Größe von ungefähr 1 µm wurden nicht durch eine erhöhte Löslichkeit charakterisiert. Die Faktoren der erhöhten Löslichkeit, die mit unserer und anderen verschiedenen in der Literatur veröffentlichten *in situ* Methoden gemessen wurden, waren vergleichbar, und waren gleichzeitig niedriger als die Werte, die mit nicht-*in situ* Methoden gemessen wurden. Daher wird die Verwendung von *in situ* Methoden für die Bestimmung von Nanokristall-Löslichkeiten empfohlen. Der Einfluss von Nanokristall-Überschuss-Bedingungen wurde mit

Tacrolimus analysiert, dessen Löslichkeit extrem niedrig ist und somit höhere Überschuss-Bedingungen möglich waren, ohne Probleme durch eine Hintergrundstreuung hervorzurufen. Der höchste erhaltene Faktor der Löslichkeitssteigerung war 6,6. Daher ist die Erhöhung in Hinsicht auf die Sättigungslöslichkeit bei Arzneistoffformulierungen basierend auf Nanokristallen nicht außergewöhnlich hoch. Bei dermaler Applikation würde der Konzentrationsgradient zwischen der Arzneistoffformulierung und der Haut nicht stark gesteigert werden, weshalb andere Faktoren relevanter sein müssen, um die Wirkstoffzufuhr-Effizienz von Nanokristallen erklären zu können, wie beispielsweise ihre Freisetzungsrate. Letztere wird tatsächlich deutlich durch Partikelgrößenverkleinerung im Nanobereich erhöht. Dieser Parameter wurde während den *in situ*-Löslichkeitsstudien untersucht. Der Einfluss war für Ibuprofen außergewöhnlich hoch: die Freisetzungsrate von 0.14 µg/(mL*s) von Partikeln einer Größe von 70 µm wurde auf 4.02 µg/(mL*s) mit Kristallen einer Größe von ~300 nm gesteigert, was einem Faktor von ~29 entspricht.

Der feste Zustand eines Arzneistoffes, genauer kristallin oder amorph, hat einen großen Einfluss auf seine Löslichkeit: der thermodynamisch-instabile amorphe Zustand wird durch die höchste Sättigungslöslichkeit und somit die höchste Freisetzungsrate charakterisiert. Mahlen (Schleifen) stellt eine der Methoden dar, um amorphe Formen eines Stoffes herzustellen, da der hohe Energieeintrag und Scherkräfte, die in den Prozess involviert sind, die kristalline Struktur beschädigen können. Bei der Nassperlenvermahlung werden sehr hohe Energiekräfte verwendet, um die Partikelgröße zu reduzieren. Die Auswirkung dieses Prozesses auf den Festzustand der Arzneistoff-Nanokristalle und seinen Einfluss auf Löslichkeitserhöhung wurde ausgewertet (Kapitel 3.2). Nassperlenvermahlung über einen längeren Zeitraum (≥2 Stunden) resultierte in einer Reduktion des Kristallinitätsgrades von Dexamethason- und Tacrolimus-Nanokristallen. Mit einer initialen 100%-Kristallinität des Arzneistoffpulvers erreichte der Kristallinitätsgrad der gemahlenen Partikel Plateaus von ~79 und ~76% für Dexamethason und Tacrolimus nach einer Mahldauer von 2 und 3 Stunden. Die eingebrachte Energie des Prozesses war nicht ausreichend, um die Kristallinität weiter zu reduzieren. Die abweichende Zeit, die benötigt wurde, um die jeweiligen Plateaus zu erreichen, ergab sich aus der Sprödigkeit der Arzneistoffe, die anhand von Spannungs-Dehnungs-Diagrammen der beiden Arzneistoffe bestimmt wurde, die mit Diagrammen von sehr spröden oder dehnbaren Materialien verglichen wurden. Im Vergleich mit Dexamethason war Tacrolimus duktiler, weshalb es plastisch verformt wurde, bevor es zum Bruch des Materials kam. Der Einfluss von Gefriertrocknung, die eine übliche Trocknungsmethode für Nanokristalle darstellt, auf den Kristallinitätsgrad wurde ebenfalls untersucht. Eine weitere Reduktion der Kristallinität wurde erhalten, und der Einfluss des Mahlvorgangs auf den Festzustand wurde somit ausgeglichen. Schließlich wurden die

Auswirkungen einer reduzierten Kristallinität auf die Sättigungslöslichkeit von Nanokristallen *in situ* analysiert, kombiniert mit dem Einfluss von Partikelgrößen im Nanobereich. Die höchste Steigerung der Sättigungslöslichkeit wurde immer mit dem niedrigsten Kristallinitätsgrad erhalten, und nicht mit der niedrigsten Partikelgröße. Dieser Effekt wurde mit Tacrolimus beobachtet, für das die höchste Löslichkeitssteigerung (Faktor 1,7) bei Partikeln erhalten wurde, die für 5 Stunden gemahlen wurden, den niedrigsten Kritallinitätsgrad aufwiesen (76 %), aber deren mittlere Partikelgröße bei 527 nm lag, was deutlich größer war als die von Partikeln, die für kürzere Zeit gemahlen wurden, aber deren Kristallinitätsgrad höher war und niedrigere Löslichkeitssteigerungen aufwiesen. Obwohl eine reduzierte Kristallinität positive Auswirkungen auf die Löslichkeitsverbesserung hat, sind Veränderungen des Festzustands des Arzneistoffes während der Haltbarkeitsdauer des Produktes nicht akzeptabel. Daher wird eine Auswahl von passenden Parametern (z. B. reduzierte Geschwindigkeit, erhöhte Viskosität des Mediums) empfohlen, um den Festzustand des Arzneistoffes während des Mahlprozesses und der Haltbarkeitsdauer zu erhalten.

Der Beweis, dass der reduzierte Kristallinitätsgrad zur Steigerung der Sättigungslöslichkeit beiträgt, legt nahe, dass die maximale Löslichkeitssteigerung, deren klinische Relevanz dann höher sein könnte, durch eine Kombination von amorphem Festzustand und Partikelgrößen im Nanobereich durch die Herstellung von amorphen Nanokristallen erreicht werden könnte (Kapitel 3.3). Indomethacin wurde als Modellsubstanz verwendet, da es ein stabiler Glasbildner ist. Nassperlenvermahlung wurde als Herstellungsmethode gewählt, und die Herausforderung, Wasser als Medium zu verwenden, das selbst die Rekristallisation fördert, wurde angegangen, indem eine passende Formulierung gesucht wurde, die neben dem amorphen Zustand auch die Partikelgröße stabilisiert. Polyvinylpyrrolidon (PVP, Kollidon 30) wurde mit Poloxamer 407 als Stabilisator verglichen. PVP konnte den amorphen Zustand über den gesamten Mahlprozess stabilisieren, allerdings gewährleistete es nicht die Aufrechterhaltung der Partikelgrößen im Nanobereich nach Redispergieren des gefriergetrockneten Pulvers. Poloxamer 407 hingegen zeigte die entgegengesetzte Wirkung: es stabilisierte die Partikelgröße, aber nicht den amorphen Zustand, wodurch die Partikel während des Mahlens rekristallisierten. Die Kombination der beiden Polymere war ebenfalls nicht erfolgreich, da durch den Mahlvorgang der amorphe Zustand in den kristallinen überging. Daher wurde die Hypothese aufgestellt, dass das Vorhandensein zweier großer Polymere, die um die Oberflächenanlagerung konkurrieren, das Problem darstellte: PVP konnte die Oberfläche durch die sterische Hinderung von Poloxamer 407 nicht ausreichend bedecken, wodurch es zu einem verringerten Schutz gegen Rekristallisation kam. Poloxamer wurde deshalb durch ein kleines Tensid ersetzt, Natriumdodecylsulfat (SDS), welches einen

Synergismus mit PVP aufwies: diese Kombination stabilisierte neben dem amorphen Festzustand auch die Partikelgröße. Daher ist es nötig und wird hiermit empfohlen, dass eine Formulierung zur Stabilisierung amorpher Nanopartikel in einem Herstellungsprozess, in dem Wasser beteiligt ist, eine Kombination aus einem Polymer als Anti-Rekristallisationsmittel und einem kleinen Molekül zur Erhaltung der Partikelgröße im Nanobereich enthält. Der Effekt des amorphen Zustands und die Partikelgröße im Nanobereich auf die Sättigungslöslichkeit und die Freisetzungsrate wurde mit *in situ* Studien getestet. Der amorphe Zustand und die Verkleinerung der Partikelgröße in den Nanobereich von Indomethacin hatten jeweils einen ähnlichen Einfluss auf die Löslichkeit (~2,4), während die Freisetzungsrate im Fall der Nanokristalle stärker erhöht wurde. Die größte Steigerung der Sättigungslöslichkeit und der Freisetzungsrate wurde für amorphe Nanokristalle erreicht, mit einem Steigerungsfaktor von 5,2 für die Sättigungslöslichkeit, während die Freisetzungsrate einen Wert von 2,328 µg/(mL*s) erreichte, was deutlich höher als in anderen Fällen war. Es wurde somit demonstriert, dass die Kombination von Amorphisierung und Nanonisation einen Synergismus bietet im Hinblick auf Sättigungslöslichkeit und Freisetzungsrate. Dieser Effekt hat das Potential, um im Fall von dermaler Applikation, aber nicht ausschließlich darauf beschränkt, therapeutisch relevant zu sein, insbesondere für Arzneistoffe, deren Amorphisierung bereits die Freisetzungsrate und Löslichkeit erhöht, die durch Nanonisierung weiter erhöht werden könnten.

Abschließend wurde ein neuer Nanoträger erhalten, der eine kontrollierte Arzneistofffreisetzung aus Nanokristallen erlauben würde, indem Dexamethason-Nanokristalle in wasserunlöslichen Polymeren anhand der Lösungsmittelverdampfungsmethode nanoverkapselt wurden (Kapitel 3.4). Die gewählten Polymere waren Eudragit® RS 100 und Ethylzellulose. Nassperlenvermahlung von Dexamethason wurde in Methylenchlorid durchgeführt, was die Machbarkeit des Mahlens in organischen Lösungsmitteln zur Herstellung von Nanokristallen zeigte. Nachdem optimale Prozessbedingungen für unbeladene Polymernanopartikel erhalten wurden, wurde die organische Nanosuspension in Wasser oder einer wässrigen Polyvinylalkohollösung (PVA) (für Eudragit® und Ethylzellulose) emulgiert und das Lösungsmittel anschließend über Nacht unter Rühren entfernt. Eine Polymerkonzentration von 2 % (w/v) resultierte in Nanokapseln mit einer Partikelgröße von ungefähr ~250 nm. Der Grund für die kleinere Partikelgröße von Nanokapseln im Vergleich zu Nanokristallen war die gleichzeitige Bildung von reinen Nanopartikeln in der Nanokapselprobe, und das Vorhandensein von gelöstem Polymer, das die Oberfläche der Nanokristalle bedeckt, wodurch die mit PCS gemessene Partikelgröße erhöht wurde. Die erfolgreiche Nanoverkapselung von Dexamethason-Nanokristallen in einer Polymerhülle wurde durch Morphologiestudien, FTIR, Stabilität der Partikelgröße nach

Verdünnung unterhalb der Arzneistofflöslichkeit und, insbesondere, durch Freisetzungsstudien bestätigt. Nach einem initialen Burst, hervorgerufen durch das Vorhandensein von nicht-verkapselten Nanokristallen, wurde die Arzneistofffreisetzung über die Zeit im Vergleich zu einer regulären Nanosuspension kontrolliert. Ein neuer Nanoträger für die Behandlung von Erkrankungen, die eine langfristige Arzneistofffreisetzung erfordern, wurde somit erhalten und charakterisiert. Eine Depot-Formulierung für die Behandlung von beispielsweise Hauterkrankungen wie Psoriasis via intradermaler Injektion dieses Nanoträgertyps wäre somit möglich. Außerdem könnten organische Lösungsmittel durch Öle oder halbfeste Zubereitungen ersetzt werden, wodurch eine direkte Herstellung von dermalen Nanokristallen in ihrer finalen Arzneiform und somit die Einsparung von Zeit und Kosten ermöglicht werden würde.

6. References

[1] J. Wong, A. Brugger, A. Khare, M. Chaubal, P. Papadopoulos, B. Rabinow, J. Kipp, J. Ning, Suspensions for intravenous (IV) injection: A review of development, preclinical and clinical aspects, Advanced Drug Delivery Reviews, 60 (2008) 939-954.

[2] J.E. Kipp, The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs, Int. J. Pharm., 284 (2004) 109-122.

[3] K.T. Savjani, A.K. Gajjar, J.K. Savjani, Drug solubility: importance and enhancement techniques, ISRN pharmaceutics, 2012 (2012).

[4] K. Gupta, Solutions and phase equilibria, in: A. Gennaro (Ed.) Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins, Baltimore, 2000, pp. 208-209.

[5] P. van Hoogevest, X. Liu, A. Fahr, Drug delivery strategies for poorly water-soluble drugs: the industrial perspective, Expert Opinion on Drug Delivery, 8 (2011) 1481-1500.

[6] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Advanced Drug Delivery Reviews, 23 (1997) 3-25.

[7] A. Singh, Z.A. Worku, G. Van den Mooter, Oral formulation strategies to improve solubility of poorly water-soluble drugs, Expert Opinion on Drug Delivery, 8 (2011) 1361-1378.

[8] G.L.L. Amidon, Hans; Shah, Vinod P.; Crison, John R., A Theoretical Basis for a Biopharmaceutic Drug Classification: The Correlation of in Vitro Drug Product Dissolution and in Vivo Bioavailability., Pharm. Res., 12 (1995) 413-420.

[9] FDA, Guidance for industry: dissolution testing of immediate release solid oral dosage forms, in: F.D.A. U.S. Department of Health, Center for Drug Evaluation and Research, Maryland, 1997.

[10] H.D. Williams, N.L. Trevaskis, S.A. Charman, R.M. Shanker, W.N. Charman, C.W. Pouton, C.J.H. Porter, Strategies to Address Low Drug Solubility in Discovery and Development, Pharmacol. Rev., 65 (2013) 315-499.

[11] S. Kalepu, V. Nekkanti, Insoluble drug delivery strategies: review of recent advances and business prospects, Acta Pharmaceutica Sinica B, 5 (2015) 442-453.

[12] M.M.-L. Elaine, G.L. Gary, Drug Nanoparticles: Formulating Poorly Water-Soluble Compounds, Toxicol. Pathol., 36 (2008) 43-48.

[13] E. Merisko-Liversidge, G.G. Liversidge, E.R. Cooper, Nanosizing: a formulation approach for poorly-water-soluble compounds, Eur. J. Pharm. Sci., 18 (2003) 113-120.

[14] B.E. Rabinow, Nanosuspensions in drug delivery, Nature Reviews Drug Discovery, 3 (2004) 785.

[15] A. Fahr, X. Liu, Drug delivery strategies for poorly water-soluble drugs, Expert Opinion on Drug Delivery, 4 (2007) 403-416.

[16] N. Rasenack, B.W. Müller, Micron-Size Drug Particles: Common and Novel Micronization Techniques, Pharm. Dev. Technol., 9 (2004) 1-13.
[17] C. Leuner, J. Dressman, Improving drug solubility for oral delivery using solid dispersions, Eur. J. Pharm. Biopharm., 50 (2000) 47-60.
[18] A.T.M. Serajuddin, Salt formation to improve drug solubility, Advanced Drug Delivery Reviews, 59 (2007) 603-616.
[19] K. Kawakami, N. Oda, K. Miyoshi, T. Funaki, Y. Ida, Solubilization behavior of a poorly soluble drug under combined use of surfactants and cosolvents, Eur. J. Pharm. Sci., 28 (2006) 7-14.
[20] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization, J. Pharm. Sci., 85 (1996) 1017-1025.
[21] S.M. Moghimi, Recent Developments in Polymeric Nanoparticle Engineering and Their Applications in Experimental and Clinical Oncology, Anticancer Agents Med. Chem., 6 (2006) 553-561.
[22] J. Junghanns, R.H. Müller, Nanocrystal technology, drug delivery and clinical applications, Int J Nanomedicine, 3 (2008) 295-309.
[23] L. Willems, R. Van Der Geest, K. De Beule, Itraconazole oral solution and intravenous formulations: a review of pharmacokinetics and pharmacodynamics, J. Clin. Pharm. Ther., 26 (2001) 159-169.
[24] D.E. Alonzo, G.G. Zhang, D. Zhou, Y. Gao, L.S. Taylor, Understanding the behavior of amorphous pharmaceutical systems during dissolution, Pharm. Res., 27 (2010) 608-618.
[25] B.C. Hancock, G. Zografi, Characteristics and significance of the amorphous state in pharmaceutical systems, J. Pharm. Sci., 86 (1997) 1-12.
[26] L.-F. Huang, W.-Q. Tong, Impact of solid state properties on developability assessment of drug candidates, Advanced Drug Delivery Reviews, 56 (2004) 321-334.
[27] B.C. Hancock, M. Parks, What is the true solubility advantage for amorphous pharmaceuticals?, Pharm. Res., 17 (2000) 397-404.
[28] L. Yu, Amorphous pharmaceutical solids: preparation, characterization and stabilization, Advanced Drug Delivery Reviews, 48 (2001) 27-42.
[29] B.C. Hancock, S.L. Shamblin, G. Zografi, Molecular Mobility of Amorphous Pharmaceutical Solids Below Their Glass Transition Temperatures, Pharm. Res., 12 (1995) 799-806.
[30] D.Q. Craig, P.G. Royall, V.L. Kett, M.L. Hopton, The relevance of the amorphous state to pharmaceutical dosage forms: glassy drugs and freeze dried systems, Int. J. Pharm., 179 (1999) 179-207.

[31] B.C. Hancock, G. Zografi, The Relationship Between the Glass Transition Temperature and the Water Content of Amorphous Pharmaceutical Solids, Pharm. Res., 11 (1994) 471-477.

[32] D. Quintanar-Guerrero, E. Allémann, H. Fessi, E. Doelker, Preparation Techniques and Mechanisms of Formation of Biodegradable Nanoparticles from Preformed Polymers, Drug Dev. Ind. Pharm., 24 (1998) 1113-1128.

[33] K. Letchford, H. Burt, A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes, Eur. J. Pharm. Biopharm., 65 (2007) 259-269.

[34] C.E. Mora-Huertas, H. Fessi, A. Elaissari, Polymer-based nanocapsules for drug delivery, Int. J. Pharm., 385 (2010) 113-142.

[35] L. Cruz, L.U. Soares, T.D. Costa, G. Mezzalira, N.P. da Silveira, S.S. Guterres, A.R. Pohlmann, Diffusion and mathematical modeling of release profiles from nanocarriers, Int. J. Pharm., 313 (2006) 198-205.

[36] K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, W.E. Rudzinski, Biodegradable polymeric nanoparticles as drug delivery devices, J. Control. Release, 70 (2001) 1-20.

[37] A. Vonarbourg, C. Passirani, P. Saulnier, J.-P. Benoit, Parameters influencing the stealthiness of colloidal drug delivery systems, Biomaterials, 27 (2006) 4356-4373.

[38] A.F. Ourique, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Tretinoin-loaded nanocapsules: Preparation, physicochemical characterization, and photostability study, Int. J. Pharm., 352 (2008) 1-4.

[39] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm., 55 (1989) R1-R4.

[40] T.-Y. Liu, K.-H. Liu, D.-M. Liu, S.-Y. Chen, I.W. Chen, Temperature-Sensitive Nanocapsules for Controlled Drug Release Caused by Magnetically Triggered Structural Disruption, Advanced Functional Materials, 19 (2009) 616-623.

[41] J. Whelan, Nanocapsules for controlled drug delivery, Drug Discovery Today, 6 (2001) 1183-1184.

[42] T. Nassar, A. Rom, A. Nyska, S. Benita, Novel double coated nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate drug, J. Control. Release, 133 (2009) 77-84.

[43] A. Vila, A. Sánchez, M. Tobío, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, J. Control. Release, 78 (2002) 15-24.

[44] C. Pinto Reis, R.J. Neufeld, A.J. Ribeiro, F. Veiga, Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles, Nanomedicine: Nanotechnology, Biology and Medicine, 2 (2006) 8-21.

[45] B. Stella, S. Arpicco, F. Rocco, V. Marsaud, J.-M. Renoir, L. Cattel, P. Couvreur, Encapsulation of gemcitabine lipophilic derivatives into polycyanoacrylate nanospheres and nanocapsules, Int. J. Pharm., 344 (2007) 71-77.

[46] V.B. Cattani, A.R. Pohlmann, T.D. Costa, Pharmacokinetic evaluation of indomethacin ethyl ester-loaded nanoencapsules, Int. J. Pharm., 363 (2008) 214-216.

[47] F. Lince, D.L. Marchisio, A.A. Barresi, Strategies to control the particle size distribution of poly-ε-caprolactone nanoparticles for pharmaceutical applications, J. Colloid Interface Sci., 322 (2008) 505-515.

[48] P. Legrand, S. Lesieur, A. Bochot, R. Gref, W. Raatjes, G. Barratt, C. Vauthier, Influence of polymer behaviour in organic solution on the production of polylactide nanoparticles by nanoprecipitation, Int. J. Pharm., 344 (2007) 33-43.

[49] M. Chorny, I. Fishbein, H.D. Danenberg, G. Golomb, Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics, J. Control. Release, 83 (2002) 389-400.

[50] A. Loxley, B. Vincent, Preparation of Poly(methylmethacrylate) Microcapsules with Liquid Cores, J. Colloid Interface Sci., 208 (1998) 49-62.

[51] R. Bodmeier, J.W. McGinity, Polylactic acid microspheres containing quinidine base and quinidine sulphate prepared by the solvent evaporation technique. II. Some process parameters influencing the preparation and properties of microspheres, J. Microencapsul., 4 (1987) 289-297.

[52] E. Pisani, E. Fattal, J. Paris, C. Ringard, V. Rosilio, N. Tsapis, Surfactant dependent morphology of polymeric capsules of perfluorooctyl bromide: Influence of polymer adsorption at the dichloromethane–water interface, J. Colloid Interface Sci., 326 (2008) 66-71.

[53] R. Arshady, Microspheres and microcapsules, a survey of manufacturing techniques: Part III: Solvent evaporation, Polymer Engineering & Science, 30 (1990) 915-924.

[54] H. Chen, C. Khemtong, X. Yang, X. Chang, J. Gao, Nanonization strategies for poorly water-soluble drugs, Drug Discovery Today, 16 (2011) 354-360.

[55] L. Wu, J. Zhang, W. Watanabe, Physical and chemical stability of drug nanoparticles, Advanced Drug Delivery Reviews, 63 (2011) 456-469.

[56] Y. Wang, Y. Zheng, L. Zhang, Q. Wang, D. Zhang, Stability of nanosuspensions in drug delivery, J. Control. Release, 172 (2013) 1126-1141.

[57] B. Derjaguin, L. Landau, Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes, Progress in Surface Science, 43 (1993) 30-59.

[58] S.M. Moghimi, I.S. Muir, L. Illum, S.S. Davis, V. Kolb-Bachofen, Coating particles with a block co-polymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1179 (1993) 157-165.

[59] D.H. Everett, Basic Principles of Colloid Science., London, Royal Society of Chemistry, 1988.

[60] J.-Y. Choi, J.Y. Yoo, H.-S. Kwak, B. Uk Nam, J. Lee, Role of polymeric stabilizers for drug nanocrystal dispersions, Current Applied Physics, 5 (2005) 472-474.

[61] B. Van Eerdenbrugh, J. Vermant, J.A. Martens, L. Froyen, J. Van Humbeeck, P. Augustijns, G. Van den Mooter, A screening study of surface stabilization during the production of drug nanocrystals, J. Pharm. Sci., 98 (2009) 2091-2103.

[62] J. Lee, J.Y. Choi, C.H. Park, Characteristics of polymers enabling nano-comminution of water-insoluble drugs, Int. J. Pharm., 355 (2008) 328-336.

[63] R.H. Müller, S. Gohla, C.M. Keck, State of the art of nanocrystals–special features, production, nanotoxicology aspects and intracellular delivery, Eur. J. Pharm. Biopharm., 78 (2011) 1-9.

[64] A.A. Noyes, W.R. Whitney, The rate of solution of solid substances in their own solutions, J. Am. Chem. Soc., 19 (1897) 930-934.

[65] L. Skinner, J. Sambles, The Kelvin equation—a review, Journal of Aerosol Science, 3 (1972) 199-210.

[66] K. Vijiai, T. Divya, Pharmaceutical Testing, Analysis and Control, in: D.B. Troy (Ed.) Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins pp. 672-690.

[67] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: formulation, process and storage considerations, Advanced drug delivery reviews, 58 (2006) 1688-1713.

[68] M.V. Chaubal, C. Popescu, Conversion of Nanosuspensions into Dry Powders by Spray Drying: A Case Study, Pharm. Res., 25 (2008) 2302-2308.

[69] B. Van Eerdenbrugh, G. Van den Mooter, P. Augustijns, Top-down production of drug nanocrystals: Nanosuspension stabilization, miniaturization and transformation into solid products, Int. J. Pharm., 364 (2008) 64-75.

[70] E. Merisko-Liversidge, G.G. Liversidge, Nanosizing for oral and parenteral drug delivery: A perspective on formulating poorly-water soluble compounds using wet media milling technology, Advanced Drug Delivery Reviews, 63 (2011) 427-440.

[71] E.M. Agency, Reflection paper on nanotechnology-based medicinal products for human use, in CHMP, 2006.

[72] E. Commission, COMMISSION RECOMMENDATION of 18 October 2011 on the definition of nanomaterial, 2011.

[73] J. Rejman, V. Oberle, I.S. Zuhorn, D. Hoekstra, Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis, Biochem. J., 377 (2004) 159-169.

[74] C.M. Keck, R.H. Müller, Nanotoxicological classification system (NCS) – A guide for the risk-benefit assessment of nanoparticulate drug delivery systems, Eur. J. Pharm. Biopharm., 84 (2013) 445-448.

[75] T.K.M. Le, P. de Mon, J. Schalkwuk, P.G.M. van der Valk, Effect of a topical corticosteroid, a retinoid and a vitamin D3 derivative on sodium dodecyl sulphate induced skin irritation, Contact Dermatitis, 37 (1997) 19-26.

[76] J.P. Möschwitzer, Drug nanocrystals in the commercial pharmaceutical development process, Int. J. Pharm., 453 (2013) 142-156.

[77] C.M. Keck, R.H. Müller, Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation, Eur. J. Pharm. Biopharm., 62 (2006) 3-16.

[78] R. Shegokar, R.H. Müller, Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives, Int. J. Pharm., 399 (2010) 129-139.

[79] E.R. Cooper, Nanoparticles: a personal experience for formulating poorly water soluble drugs, J. Control. Release, 141 (2010) 300-302.

[80] L. Peltonen, J. Hirvonen, Pharmaceutical nanocrystals by nanomilling: critical process parameters, particle fracturing and stabilization methods, J. Pharm. Pharmacol., 62 (2010) 1569-1579.

[81] S.L.A. Hennart, M.C. Domingues, W.J. Wildeboer, P. van Hee, G.M.H. Meesters, Study of the process of stirred ball milling of poorly water soluble organic products using factorial design, Powder Technology, 198 (2010) 56-60.

[82] E. Dumay, D. Chevalier-Lucia, L. Picart-Palmade, A. Benzaria, A. Gràcia-Julià, C. Blayo, Technological aspects and potential applications of (ultra) high-pressure homogenisation, Trends in Food Science & Technology, 31 (2013) 13-26.

[83] X. Li, L. Gu, Y. Xu, Y. Wang, Preparation of fenofibrate nanosuspension and study of its pharmacokinetic behavior in rats, Drug Dev. Ind. Pharm., 35 (2009) 827-833.

[84] L. Vidlářová, G.B. Romero, J. Hanuš, F. Štěpánek, R.H. Müller, Nanocrystals for dermal penetration enhancement – Effect of concentration and underlying mechanisms using curcumin as model, Eur. J. Pharm. Biopharm., 104 (2016) 216-225.

[85] J. Möschwitzer, G. Achleitner, H. Pomper, R.H. Müller, Development of an intravenously injectable chemically stable aqueous omeprazole formulation using nanosuspension technology, Eur. J. Pharm. Biopharm., 58 (2004) 615-619.

[86] R.H. Muller, R. Becker, B. Kruss, K. Peters, Pharmaceutical nanosuspensions for medicament administration as systems with increased saturation solubility and speed of dissolution, US Patent number US5858410A, 1999.

[87] R.H. Müller, K. Peters, Nanosuspensions for the formulation of poorly soluble drugs: I. Preparation by a size-reduction technique, Int. J. Pharm., 160 (1998) 229-237.

[88] M. Filella, J. Zhang, M.E. Newman, J. Buffle, Analytical applications of photon correlation spectroscopy for size distribution measurements of natural colloidal suspensions: capabilities and limitations, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 120 (1997) 27-46.

[89] R.H. Müller, C. Jacobs, O. Kayser, Nanosuspensions as particulate drug formulations in therapy: Rationale for development and what we can expect for the future, Advanced Drug Delivery Reviews, 47 (2001) 3-19.

[90] Malvern Analytical, Laser diffraction, accessed date 13.03.2018.

[91] F. Kesisoglou, S. Panmai, Y. Wu, Nanosizing — Oral formulation development and biopharmaceutical evaluation, Advanced Drug Delivery Reviews, 59 (2007) 631-644.

[92] H.S.M. Ali, P. York, N. Blagden, Preparation of hydrocortisone nanosuspension through a bottom-up nanoprecipitation technique using microfluidic reactors, Int. J. Pharm., 375 (2009) 107-113.

[93] H.G. Shi, L. Farber, J.N. Michaels, A. Dickey, K.C. Thompson, S.D. Shelukar, P.N. Hurter, S.D. Reynolds, M.J. Kaufman, Characterization of Crystalline Drug Nanoparticles Using Atomic Force Microscopy and Complementary Techniques, Pharm. Res., 20 (2003) 479-484.

[94] S. Byrn, R. Pfeiffer, M. Ganey, C. Hoiberg, G. Poochikian, Pharmaceutical Solids: A Strategic Approach to Regulatory Considerations, Pharm. Res., 12 (1995) 945-954.

[95] R.W. Martin, K.W. Zilm, Preparation of protein nanocrystals and their characterization by solid state NMR, J. Magn. Reson., 165 (2003) 162-174.

[96] M. Lück, B.R. Paulke, W. Schröder, T. Blunk, R.H. Müller, Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics, J. Biomed. Mater. Res., 39 (1998) 478-485.

[97] T. Blunk, E. Mak, R.H. Müller, Characterization of Colloidal Drug Carriers: Determination of surface hydrophobicity by hydrophobic interaction chromatography Pharm. Ind., 55 (1993) 612-615.

[98] P. Kayaert, B. Li, I. Jimidar, P. Rombaut, F. Ahssini, G. Van den Mooter, Solution calorimetry as an alternative approach for dissolution testing of nanosuspensions, Eur. J. Pharm. Biopharm., 76 (2010) 507-513.

[99] K. Anhalt, S. Geissler, M. Harms, M. Weigandt, G. Fricker, Development of a new method to assess nanocrystal dissolution based on light scattering, Pharm. Res., 29 (2012) 2887-2901.

[100] M. Mishra, R. Shegokar, S. Staufenbiel, R. Müller, Production, characterization and long-term stability study of glycyrrhetinic acid smartCrystals®, in: San Antonio: AAPS Annual Meeting, 2013.

[101] J. Hecq, M. Deleers, D. Fanara, H. Vranckx, K. Amighi, Preparation and characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine, Int. J. Pharm., 299 (2005) 167-177.

[102] A. Sarnes, J. Østergaard, S.S. Jensen, J. Aaltonen, J. Rantanen, J. Hirvonen, L. Peltonen, Dissolution study of nanocrystal powders of a poorly soluble drug by UV imaging and channel flow methods, Eur. J. Pharm. Sci., 50 (2013) 511-519.

[103] L. Peltonen, P. Liljeroth, T. Heikkilä, K. Kontturi, J. Hirvonen, Dissolution testing of acetylsalicylic acid by a channel flow method—correlation to USP basket and intrinsic dissolution methods, Eur. J. Pharm. Sci., 19 (2003) 395-401.

[104] Y. Lu, J. Qi, X. Dong, W. Zhao, W. Wu, The in vivo fate of nanocrystals, Drug Discovery Today, 22 (2017) 744-750.

[105] E.L. McConnell, A.W. Basit, S. Murdan, Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments, J. Pharm. Pharmacol., 60 (2008) 63-70.

[106] K. Fuhrmann, M.A. Gauthier, J.-C. Leroux, Targeting of Injectable Drug Nanocrystals, Mol. Pharm., 11 (2014) 1762-1771.

[107] S. Gopal, C. Gassmann-Mayer, J. Palumbo, M.N. Samtani, R. Shiwach, L. Alphs, Practical guidance for dosing and switching paliperidone palmitate treatment in patients with schizophrenia, Curr. Med. Res. Opin., 26 (2010) 377-387.

[108] L. Gao, G. Liu, J. Ma, X. Wang, L. Zhou, X. Li, Drug nanocrystals: In vivo performances, J. Control. Release, 160 (2012) 418-430.

[109] G.G. Liversidge, K.C. Cundy, Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs, Int. J. Pharm., 125 (1995) 91-97.

[110] Y. Wu, A. Loper, E. Landis, L. Hettrick, L. Novak, K. Lynn, C. Chen, K. Thompson, R. Higgins, U. Batra, S. Shelukar, G. Kwei, D. Storey, The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a Beagle dog model predicts improved bioavailability and diminished food effect on absorption in human, Int. J. Pharm., 285 (2004) 135-146.

[111] C. Jacobs, R.H. Müller, Production and Characterization of a Budesonide Nanosuspension for Pulmonary Administration, Pharm. Res., 19 (2002) 189-194.

[112] M.P. Desai, V. Labhasetwar, G.L. Amidon, R.J. Levy, Gastrointestinal Uptake of Biodegradable Microparticles: Effect of Particle Size., Pharm. Res., 13 (1996) 1838-18545.
[113] K.R. Reddy, Controlled-Release, Pegylation, Liposomal Formulations: New Mechanisms in the Delivery of Injectable Drugs, Ann. Pharmacother., 34 (2000) 915-923.
[114] S.M. Hoy, L.J. Scott, G.M. Keating, Intramuscular Paliperidone Palmitate, CNS Drugs, 24 (2010) 227-244.
[115] T.B. Martonen, I.M. Katz, Deposition Patterns of Aerosolized Drugs Within Human Lungs: Effects of Ventilatory Parameters, Pharm. Res., 10 (1993) 871-878.
[116] J. Zhang, L. Wu, H.-K. Chan, W. Watanabe, Formation, characterization, and fate of inhaled drug nanoparticles, Advanced Drug Delivery Reviews, 63 (2011) 441-455.
[117] J. Araújo, E. Gonzalez, M.A. Egea, M.L. Garcia, E.B. Souto, Nanomedicines for ocular NSAIDs: safety on drug delivery, Nanomedicine: Nanotechnology, Biology and Medicine, 5 394-401.
[118] M.A. Kassem, A.A. Abdel Rahman, M.M. Ghorab, M.B. Ahmed, R.M. Khalil, Nanosuspension as an ophthalmic delivery system for certain glucocorticoid drugs, Int. J. Pharm., 340 (2007) 126-133.
[119] H.S.M. Ali, P. York, A.M.A. Ali, N. Blagden, Hydrocortisone nanosuspensions for ophthalmic delivery: A comparative study between microfluidic nanoprecipitation and wet milling, J. Control. Release, 149 (2011) 175-181.
[120] R.H. Müller, C.M. Keck, Twenty years of drug nanocrystals: Where are we, and where do we go?, Eur. J. Pharm. Biopharm., 80 (2012) 1-3.
[121] P.R. Mishra, L.A. Shaal, R.H. Müller, C.M. Keck, Production and characterization of Hesperetin nanosuspensions for dermal delivery, Int. J. Pharm., 371 (2009) 182-189.
[122] L. Al Shaal, R. Shegokar, R.H. Müller, Production and characterization of antioxidant apigenin nanocrystals as a novel UV skin protective formulation, Int. J. Pharm., 420 (2011) 133-140.
[123] S. Kobierski, K. Ofori-Kwakye, R.H. Müller, C.M. Keck, Resveratrol nanosuspensions for dermal application - Production, characterization, and physical stability, Die Pharmazie - An International Journal of Pharmaceutical Sciences, 64 (2009) 741-747.
[124] K. Mitri, R. Shegokar, S. Gohla, C. Anselmi, R.H. Müller, Lutein nanocrystals as antioxidant formulation for oral and dermal delivery, Int. J. Pharm., 420 (2011) 141-146.
[125] N. Döge, S. Hönzke, F. Schumacher, B. Balzus, M. Colombo, S. Hadam, F. Rancan, U. Blume-Peytavi, M. Schäfer-Korting, A. Schindler, E. Rühl, P.S. Skov, M.K. Church, S. Hedtrich, B. Kleuser, R. Bodmeier, A. Vogt, Ethyl cellulose nanocarriers and nanocrystals differentially deliver dexamethasone into intact, tape-stripped or sodium lauryl sulfate-exposed ex vivo human skin - assessment by intradermal microdialysis and extraction from the different skin layers, J. Control. Release, 242 (2016) 25-34.

[126] X. Zhai, J. Lademann, C.M. Keck, R.H. Müller, Nanocrystals of medium soluble actives—Novel concept for improved dermal delivery and production strategy, Int. J. Pharm., 470 (2014) 141-150.
[127] K.C. Bhol, P.J. Schechter, Topical nanocrystalline silver cream suppresses inflammatory cytokines and induces apoptosis of inflammatory cells in a murine model of allergic contact dermatitis, Br. J. Dermatol., 152 (2005) 1235-1242.
[128] K.C. Bhol, J. Alroy, P.J. Schechter, Anti-inflammatory effect of topical nanocrystalline silver cream on allergic contact dermatitis in a guinea pig model, Clin. Exp. Dermatol., 29 (2004) 282-287.
[129] J. Lademann, N. Otberg, H. Richter, H.J. Weigmann, U. Lindemann, H. Schaefer, W. Sterry, Investigation of Follicular Penetration of Topically Applied Substances, Skin Pharmacol. Physiol., 14(suppl 1) (2001) 17-22.
[130] A. Patzelt, H. Richter, F. Knorr, U. Schäfer, C.-M. Lehr, L. Dähne, W. Sterry, J. Lademann, Selective follicular targeting by modification of the particle sizes, J. Control. Release, 150 (2011) 45-48.
[131] A. Llinàs, J.M. Goodman, Polymorph control: past, present and future, Drug Discovery Today, 13 (2008) 198-210.
[132] A.L. Patterson, The Scherrer Formula for X-Ray Particle Size Determination, Physical Review, 56 (1939) 978-982.
[133] D. Mahlin, C.A.S. Bergström, Early drug development predictions of glass-forming ability and physical stability of drugs, Eur. J. Pharm. Sci., 49 (2013) 323-332.
[134] Y. Aso, S. Yoshioka, S. Kojima, Molecular mobility-based estimation of the crystallization rates of amorphous nifedipine and phenobarbital in poly (vinylpyrrolidone) solid dispersions, J. Pharm. Sci., 93 (2004) 384-391.
[135] B. Balzus, F.F. Sahle, S. Hönzke, C. Gerecke, F. Schumacher, S. Hedtrich, B. Kleuser, R. Bodmeier, Formulation and ex vivo evaluation of polymeric nanoparticles for controlled delivery of corticosteroids to the skin and the corneal epithelium, Eur. J. Pharm. Biopharm., 115 (2017) 122-130.
[136] M. Li, O. Rouaud, D. Poncelet, Microencapsulation by solvent evaporation: State of the art for process engineering approaches, Int. J. Pharm., 363 (2008) 26-39.
[137] W. Jincheng, C. Sihao, Preparation and characterization of microcapsules containing capsaicin., J. Appl. Polym. Sci., 116 (2010) 2234–2241.

7. Publications and conference contributions

Publications

- **Colombo M.**, Staufenbiel S., Rühl E., Bodmeier R. "*In situ* determination of the saturation solubility of poorly soluble drugs for dermal application" *Int J Pharm 521 (2017) 156-166*
- **Colombo M.**, Orthmann S., Bellini M., Staufenbiel S., Bodmeier R. "Influence of drug brittleness, nanomilling time and freeze-drying on the degree of crystallinity of poorly water-soluble drugs and its implications for solubility enhancement" *AAPS PharmSciTech (2017) 2437-2445*
- **Colombo M.,** Minussi C., Orthmann S., Staufenbiel S., Bodmeier R. "Preparation of amorphous indomethacin nanoparticles by aqueous wet bead milling and *in situ* measurement of their increased saturation solubility" *Eur J Pharm Biopharm 125 (2018) 159-168*
- Balzus B., **Colombo M.**, Sahle F.F., Zoubari G., Staufenbiel S., Bodmeier R. "Comparison of different *in vitro* release methods used to investigate nanocarriers intended for dermal application" *Int J Pharm 513 (2016) 247-254*
- Döge N., Hönzke S., Schumacher F., Balzus B., **Colombo M.**, Hadam S., Rancan F., Blume-Peytavi U., Schäfer-Korting M., Schindler A., Rühl E., Skov P.S., Church M.K., Hedtrich S., Kleuser B., Bodmeier R., Vogt A. "Ethyl cellulose nanocarriers and nanocrystals differentially deliver dexamethasone into intact, tape-stripped or sodium lauryl sulfate-exposed *ex vivo* human skin - assessment by intradermal microdialysis and extraction from the different skin layers" *J Control Release 242 (2016) 25-34*

Conference contributions

- **Colombo M.**, Minussi C., Staufenbiel S., Bodmeier R. "Encapsulation of dexamethasone nanocrystals within a shell of Eudragit® RS100" (Poster number 12), 2018 PBP World Meeting, Granada, Spain
- **Colombo M.**, Staufenbiel S., Bodmeier R. "Amorphous indomethacin nanoparticles prepared by aqueous wet bead milling and *in situ* measurement of their increased solubility" (Poster No. M6052), 2017 AAPS Annual Meeting and Exposition, San Diego, California
- **Colombo M.**, Minussi C., Sahle F.F., Staufenbiel S., Bodmeier R. "Nanoencapsulation of dexamethasone nanocrystals within Eudragit® RS100 by solvent evaporation" 2017 SPhERe Symposium on Pharmaceutical Engineering Research, Braunschweig, Germany

- **Colombo M.**, Staufenbiel S., Bodmeier R. "*In situ* determination of the increase in saturation solubility of nanocrystals of poorly soluble drugs" (Poster No. 32W1030), 2016 AAPS Annual Meeting and Exposition, Denver, Colorado
- **Colombo M.**, Staufenbiel S., Bodmeier R. "*In situ* determination of the increased saturation solubility of dexamethasone nanocrystals for dermal application" 2016 International Conference on Dermal Drug Delivery by Nanocarriers, Berlin, Germany

8. Curriculum vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version.