Development and evaluation of nanocrystal formulations and their incorporation into topical semisolid vehicles

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To my family

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Declaration of authorship

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This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

Berlin, 1st August 2023

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1 General introduction

1.1 Nanocrystals

Recently, the development of new active pharmaceutical ingredients (API) has made significantly progress in research and development through the application of high-throughput screening and combinatorial chemistry technology. However, up to 40 % of the developed active drug molecules have poor water solubility, resulting in low bioavailability. Usually, the biopharmaceutical classification system (BCS) is used to categorize these drugs based on their physicochemical properties, such as solubility, permeability, and pharmacokinetic behaviour in the human body [1]. The BCS classifies drugs into four classes according to their solubility and permeability: drugs, which are categorized in class I, are highly soluble in water and have high permeability; class II drugs have low water solubility and high permeability; class III is used to classify the drugs which have high water solubility and low permeability; drugs in class IV have both low solubility and permeability. Most of the APIs are class II drugs, which are poorly water-soluble resulting in a delay or limited drug absorption. Therefore, it is challenging to increase the dissolution rate of drug upon administration. There are many strategies to improve the dissolution rate. Nowadays, nanosization is given more and more attention.

In the pharmaceutical industry, particles with sizes less than 1 μm are classified as nanoparticles. The FDA has approved 100 nanomedicine applications and products, showing the importance of nanotechnology in current pharmaceutical and biomedical science. Nanotechnology plays a significant role in the field of drug delivery, mainly due to the major advantages that affect conventional active pharmaceutical ingredients (APIs) and finished dosage formulations [2]. Nanocrystals are pure drug particles with stabilizers. They are designed to improve the dissolution rate and bioavailability of poorly soluble drugs. Upon nanosizing, undesirable surface amorphization might happen. The increased dissolution rate results from the enlarged surface area and the surface amorphization (Figure 1.1) [3, 4]. Usually, nanocrystals are prepared as

nanosuspensions, which have the dispersed nanocrystal stabilized by the stabilizers in different media. Once prepared, the nanocrystals can be loaded into various formulations according to the administration routes. The use of nanocrystals in drug delivery enhances the pharmacokinetics and pharmacodynamics of drugs in the pharmaceutical industry [5-8].

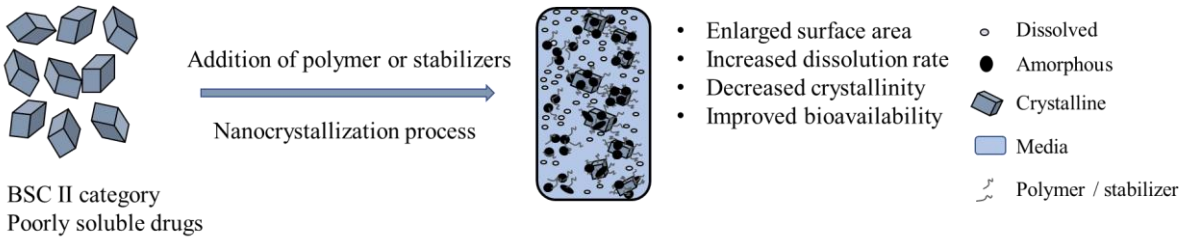

Figure 1.1 A schematic diagram of nanocrystallization of poorly – soluble drugs and their physicochemical properties

1.2 Pharmaceutical-based nanosizing production methods

Usually, pharmaceutical nanosizing techniques produce nm-size particles, which are broadly categorized into “top-down” (ball milling, wet beads milling, high-pressure homogenization (HPH)) and “bottom-up” (nanoprecipitation) technologies (Figure 1.2) [9-11]. These methods have different processes to produce nanocrystals with pros and cons. The selection of nanosizing techniques is crucial for the lateral performance of nanocrystals among stability and *in vitro* release etc. Additionally, some novel methods are also invented to achieve efficient nanosizing for commercial usage.

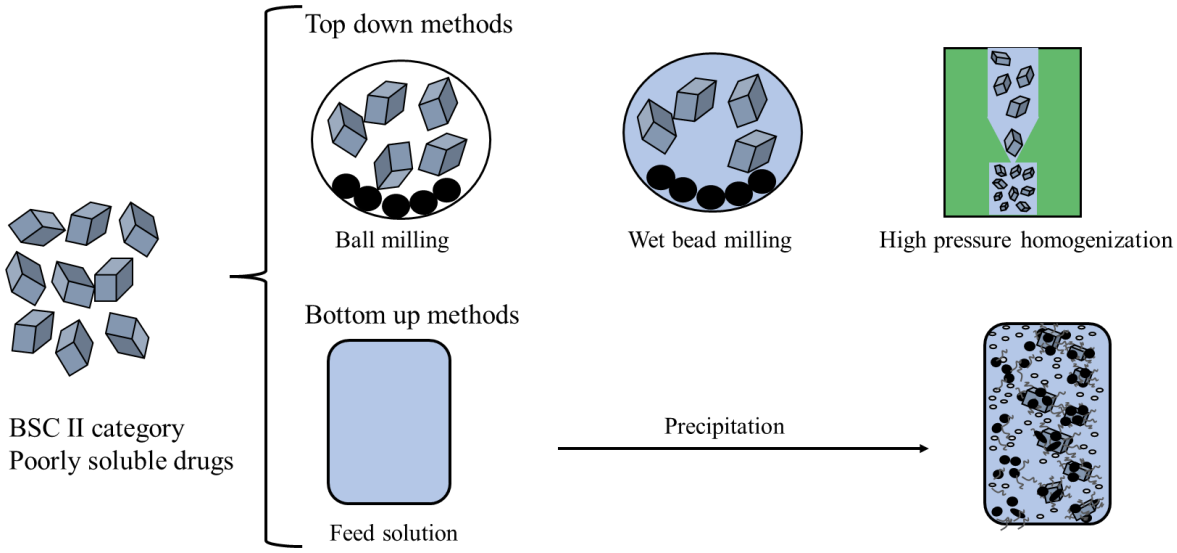

Figure 1.2 Scheme of top-down and bottom-up technology

"Top-down" techniques

"Top-down" techniques reduce the μm or mm size particles into the nm size range by using mechanical forces (attrition, cavitation, and others) [5]. Milling techniques are commonly used in the "Top-down" method, which includes the ball milling and wet bead milling. Two basic requirements should be met in the milling process: (i) the flowability of drug crystals into the milling zoom; (ii) the shear force to break the crystals [12]. For the ball milling, drug powders are ground in a dry state without any liquid media. Even though this process is highly energetic, the flowability of drug powders during the milling process is not preferable. Due to the insufficient flowability, firstly the narrow particle size distribution is not achievable and then amorphous formation is not negligible resulting in the lateral potential of instability. Additionally, due to the strong collision force, the damage of milling ball is unavoidable. Therefore, the contamination of the milling ball should be carefully monitored [13]. For wet bead milling, liquid media are involved in the milling process which could improve the flowability upon milling. Currently, this is a widely used technique to produce nanosuspensions. Numerous researchers investigate the preparation parameters to

achieve high milling efficiency. Quality by design (QbD) is used to evaluate this approach [14]. The milling speed, milling time, drug loading, amount and size of used beads must be controlled to obtain particles in the desired size range [15]. Another alternative top-down method is high-pressure homogenization which is introduced and patented in 1899 by Auguste Gaulin. Later Müller together with his coworkers improved the techniques into a piston gap homogenizer, where the drug suspension is passed through a small orifice resulting in the particle size reduction by the collision of crystals though the small orifice [16]. The homogenization cycles, temperature and the given pressure should be carefully investigated to achieve sufficient milling efficiency.

"Bottom-up" techniques

"Bottom-up" techniques have been utilized since the late 1980s for the preparation of micronized particles. The bottom-up technique initiates from a molecular state of the drug (drug dissolved in organic solvent) and subsequently, nanoprecipitation is governed by solvent evaporation, ultrasonic waves, supercritical fluid and others [17]. Usually, solvents, which are used in the nanoprecipitation technique have miscibility with antisolvent. Precipitation occurs during the solvent evaporation process. Due to the tedious precipitation process, the rate of precipitation and prevention of μm crystal growth should be taken into account. Additionally, the residual organic solvent is potentially toxic for the patients. It can also impact the formulation and stability of nanosuspensions. Therefore, bottom-up methods are rarely used in the industry field due to the risk of residual organic solvents and the complexity to control the process to inhibit the crystal growth [18].

Combination techniques

Usually, "Top-down" and "Bottom-up" methods can be used for most drugs. However, the high nanosizing efficiency of drugs, which are extremely hard or thermal unstable, cannot be achieved by the sole selection of "Top-down" and "Bottom-up" methods. For

the industrialization of nanocrystal formulations, high scalability and narrow particle size distribution are required (Figure 1.3) [19]. Nanocrystals can be loaded into various formulations after the solidification of the nanosuspensions. Usually, spray-drying and freeze-drying are the common techniques to solidify the nanocrystals. Therefore, some novel combinative nanosizing and solidification methods are invented to increase the size reduction efficiency and have higher feasibility in large-scale production [20, 21]. The combined technologies mainly include freeze-drying-high-pressure-homogenization, spray-drying-high-pressure homogenization, and rotary evaporation-high-pressure homogenization [22-24].

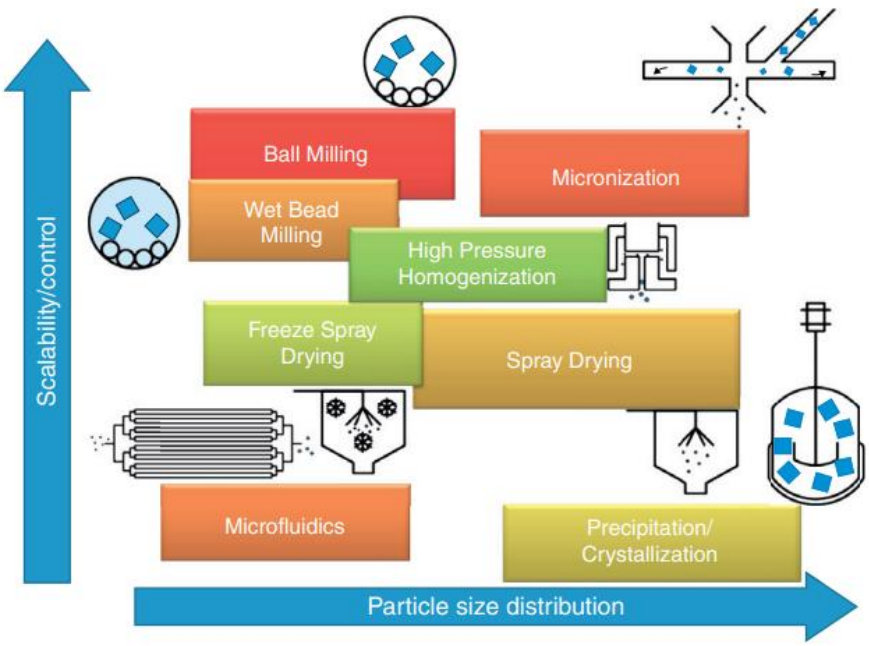

Figure 1.3 Preparation of nanocrystals depending on scalability/control and particle size distribution requirements [19]

Freeze-drying-high-pressure homogenization and spray-drying-high-pressure homogenization are preferrable for drugs with high hardness, which require long milling time by single wet-bead milling methods and have easy clogging of the instrument upon high-pressure homogenization. Freeze-drying technology is used to

freeze the prepared nanosuspensions under an ultra-low temperature and subsequently the liquid phase of frozen nanosuspensions sublimates under a vacuum. Spray drying technology uses a nebulizer to spray prepared nanosuspensions into small mist droplets and has a circulated hot air stream to remove the liquid media quickly. Briefly, freeze-drying and spray-drying can be used to dry nanosuspensions into powders, which ensure the long-term stability of nanocrystals under storage, transportation, and subsequent loading into other formulations.

1.3 Stabilization of nanocrystals

During the nanosizing process, drug nanocrystals have the new surface area increasing interfacial tension. Due to the increment of free energy, nanocrystals prefer to reduce this increased surface tension by fusing into larger crystals. Eventually, the instability of nanosuspensions, which include agglomeration, aggregation, and sedimentation, will occur. This instability of nanosuspensions will not only lead the low bioavailability but also the potential toxicity upon administration [25]. Usually, researchers inhibit the higher free energy and interfacial tension by adding surface-active agents. Those surface-active agents are categorized into charged surfactants and polymers. According to different classifications of stabilizers, the two main mechanisms of nanosuspensions stability are electrostatic repulsion and steric hindrance, respectively [26].

1.3.1 Stabilization by electrostatic repulsion

Usually, the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory explains the electrostatic repulsion process to stabilize the nanosuspensions [27]. This theory explains the equilibrium between attractive and repulsive forces of particles. According to DLVO theory, the stability of nanosuspensions is determined by the sum of the

attractive and repulsive forces acting on the particles. When the attractive forces are stronger than the repulsive forces, the particles tend to aggregate and form larger clusters. In contrast, If the repulsive forces are stronger than the attractive forces, the particles remain dispersed. Usually, the attractive forces in nanosuspensions are Van der Waals forces, which are weak, short-range forces between atoms and molecules. The repulsive forces are the electrostatic repulsion between particles that have the same charge (Figure 1.4).

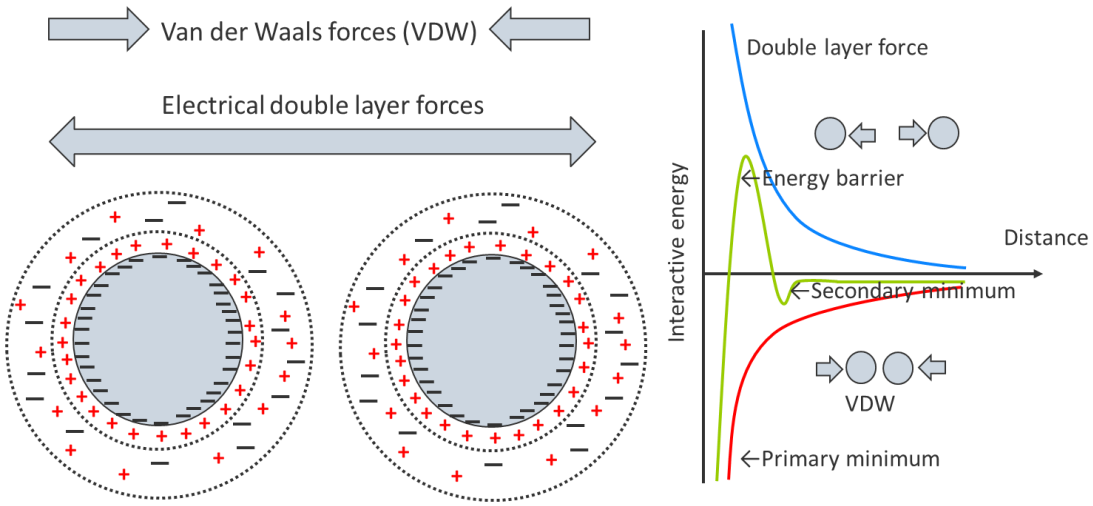

Figure 1.4 Schematic interaction energy versus distance profiles of DLVO interaction

1.3.2 Stabilization by steric hindrance

Polymeric stabilizers are used to stabilize nanosuspensions by steric hindrance mechanism. According to the Brownian motion, particles collide randomly with surrounding particles. Usually, polymeric stabilizers have a hydrophobic head and hydrophilic tail. Most poorly water-soluble drugs have a hydrophobic surface. The hydrophobic heads of polymeric stabilizers attach to the surface of the newly created surface of nanocrystals and the hydrophilic tails stretch to the dispersions (Figure 1.5). When two stabilizer-attached nanoparticles collide, the hydrophilic tails of the attached

polymers are overlapping. More water medium prefers to penetrate within the hydrophilic tails to reduce the increased osmotic pressure between the overlapped chain of the polymers, resulting in the steric hindrance force [26, 28]. TPGS (D-alpha-Tocopheryl polyethylene glycol 1000 succinate) is a nonionic surfactant that can stabilize nanocrystals through the amphiphilic structure of lipophilic alkyl tail and hydrophilic polar head. Poloxamers are even a special case. These amphiphilic copolymers are composed of hydrophilic polyethylene oxide (PEO) and hydrophobic polypropylene oxide (PPO) blocks. The general structure of a poloxamer can be represented as PEO-PPO-PEO. The PEO block is usually positioned at the ends of the molecule, while the PPO block is located in the centre. The PEO blocks are water-soluble and can form hydrogen bonds with water molecules, while the PPO blocks are insoluble in water and can interact with the hydrophobic surfaces of nanocrystals. The PEO-PPO-PEO chains act as the loops adsorbing on the surface of nanocrystals. The collision of the loops reduces entropy and the overlapping of loops increased osmotic pressure resulting in the stabilization function of nanocrystals.

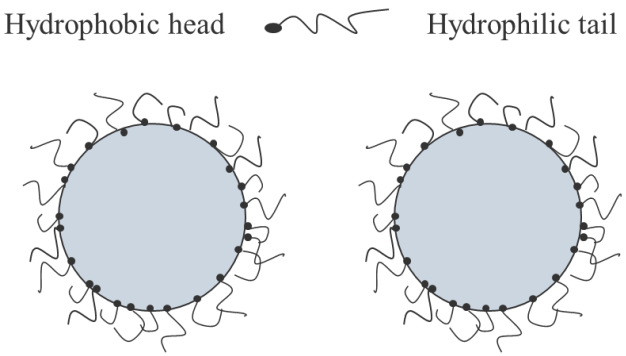

Figure 1.5 Schematic steric hindrance stabilization mechanism of nanocrystals

1.4 Characterization of nanocrystals

After the successful preparation of nanocrystal formulations, the characterization of the formulations is important to guarantee the performance of nanocrystals under the specified limits. Usually, three parts are required to be guaranteed in nanocrystal-loaded formulations: particle size, solid state and the *in vitro* release. The following sections will discuss in detail the various characterization methods for the evaluation of nanocrystals.

1.4.1 Particle size measurement

Various techniques are used to analyze the size of nanocrystals. Currently, the most frequently used techniques for particle size measurements are dynamic light scattering techniques, laser diffraction and microscopy. Each method has advantages but also disadvantages [29] and the sample preparation methods also influence the relevant results. Usually, the combination of these techniques can clarify the size and morphology of nanocrystals.

1.4.1.1 Photon correlation spectroscopy

Dynamic light scattering has become a standard tool for the measurement of diffusion coefficients. It is also known as photon correlation spectroscopy (PCS), which yields accurate results and the measurements are fast and easy to perform. The key theory of PCS is to evaluate the intensity changes of the scattered lights which are measured as a function of time [30]. Nanoparticles have undirected motion in the suspension. The fluctuations in the intensity of the scattered light are related to the movement of the nanocrystals in the suspension. By analyzing the fluctuations in the intensity, it is possible to determine the size of the particles and their size distribution. However, the

detective limitation of this technique is to measure the particle size between 0.3 nm to 10 µm. Therefore, other techniques are required to ensure the detection of possible larger particles. However, the interpretation of the data can be complex and may require expertise in the field of particle sizing and characterization. Usually, the samples for PCS measurement require to be diluted to a certain extent. The solution used to dilute the samples and the dilution time should be carefully investigated. Different dilution media and times lead to different results. For the critical measurement of particle size, the standard validation of particle size measurement methods of PCS should be done. Additionally, the morphology of nanocrystals could not be clarified by PCS. Nevertheless, PCS is a widely used technique in the field of nanosuspensions, and it can be performed using commercially available instruments.

1.4.1.2 Laser diffraction

Laser diffraction (LD) is another technique commonly used to measure the particle size of nanosuspensions. A laser beam passes through the suspension, and the intensity of the scattered light is collected and measured at different angles, and the data is analyzed to determine the particle size distribution [31]. This is done using a mathematical model called the Mie theory, which relates the size and refractive index of the particles to the scattering pattern [32]. Laser diffractometry is a robust technique and has the advantage over all the other techniques to be able to analyze large particle size range within only one single measurement. It is a fast and convenient method for measuring the particle size distribution of nanosuspensions. However, laser diffraction is not suitable for measuring nanoparticles below 70 nm as the sensitivity and accuracy of the technique are limited. Compared to PCS, the sample preparation for laser diffraction measurement should be considered carefully, especially for nanocrystals to dissolve immediately upon dilution. It assumes the shape of the particle is round, therefore, the morphology of the nanoparticle could not be illustrated by laser diffraction. However, laser

diffraction is still widely used due to the feasibility of measurement for liquid and powder samples.

1.4.1.3 Electron microscopy

Electron microscopy is a powerful tool for characterizing the morphology and structure of nanocrystals. It is used together with PCS and LD to present more accurate information about the nanoparticles. Usually, there are two types of electron microscopy: scanning electron microscopy (SEM) and transmission electron microscopy (TEM) [33].

SEM is a powerful technique that is widely used to study the properties and structure of nanocrystals. A focused electron beam is used to scan the surface of samples and a variety of signals are generated, including secondary electrons, backscattered electrons, and X-rays. These signals can be detected and used to create an image of the sample with high spatial resolution [34]. In the case of nanocrystals, SEM provides valuable information about their size, shape, and structure. However, it is important to note that the SEM technique requires the nanocrystals to be stable under high vacuum conditions and the measured samples need to be diluted to a certain extent. Therefore, the solidification of the nanosuspensions is required and the dilution method needs to be investigated. These extra processes might vary the morphology and structure of nanocrystals. Additionally, the electron beam will also damage the sample, which can affect the observed properties of the nanocrystals. To minimize this effect, low electron beam energies and short exposure times are typical during the measurement.

TEM is another powerful technique that is widely used to study the properties and structure of nanocrystals. For nanosuspensions, samples are frozen under high-pressure suppresses. Then, a beam of electrons is transmitted through a thin frozen sample, and the resulting interactions between the electrons and the sample provide information about the sample's properties [35]. The disadvantages are the formation and

morphology of nanocrystals would be varied under freezing. In addition to imaging, TEM can also be used to identify the elements and the composition of nanocrystals, e.g., energy-dispersive X-ray spectroscopy (EDS) and electron energy-loss spectroscopy (EELS). However, this analysis is usually for inorganic compounds. Most of the APIs are organic compounds, and the high energy of the beam damages the drugs during measurement.

In summary, particle size measurement is crucial to the evaluation quality of nanosuspension upon the preparation, storage and administration. Microscopy techniques are powerful tools to investigate the size, morphology and structure of nanocrystals. They can provide high-resolution imaging and detailed structural and compositional information. However, careful sample preparation and specialized operation processes are required to obtain accurate and reliable results.

“Liquid-cell TEM” is a powerful imaging technique that can be used to visualize the structure and properties of nanosuspensions without the solidification process at high resolution [36]. Conventional SEM and TEM require high vacuum conditions, which are generally incompatible with liquid samples. However, as mentioned previously, the crystal growth of nanocrystals during water evaporation needs to be clarified. Liquid-cell TEM is more suitable for this investigation. It involves encapsulating a small amount of the nanosuspension in a thin liquid layer between two layers of thin-film support, typically made of materials such as graphene, silicon nitride, or carbon. The liquid layer can be introduced using a specialized microfluidic device, which allows for precise control over the amount and composition of the liquid (Figure 1.6) [37]. It is a specialized technique that allows the imaging of nanosuspensions in a liquid environment, providing insight into their behaviour under conditions that are closer to their intended use.

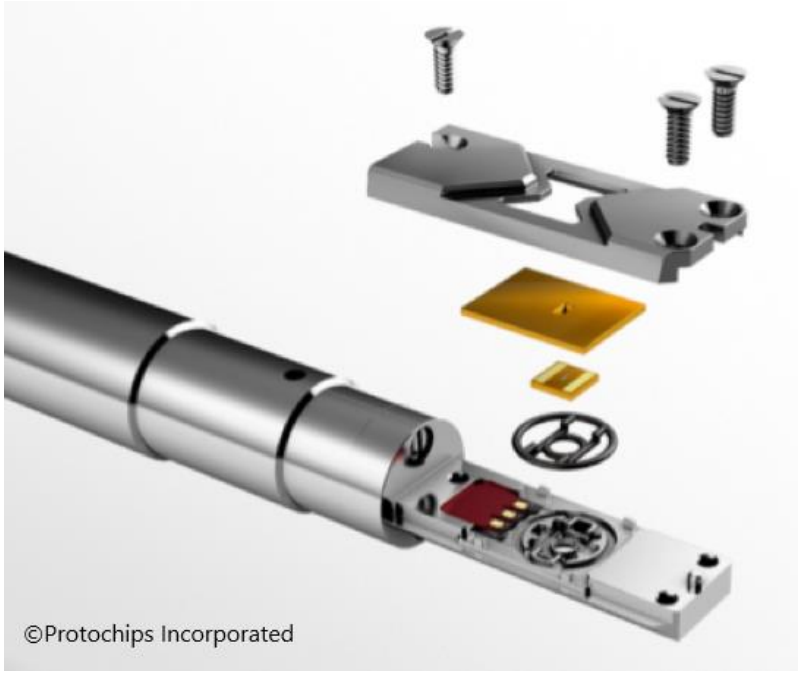

Figure 1.6 The diagram of liquid-cell chip: Protochips Poseidon 300

1.4.2 Solid state measurement

Usually, nanocrystals benefit from the faster dissolution rate due to the enlarged surface area. Currently, there is another reason for that, the amorphization of nanocrystals after milling or drying. Parts of the advantages of nanosuspensions might come from decreased crystallinity [38]. Only slightly decreased crystallinity was found due to water present in the nanosuspensions acting as a trigger for recrystallization. However, even a small change in the crystallinity might influence both chemical and physical stability or affect the bioavailability if uncontrolled crystallization occurs during storage. Therefore, the solid-state measurement is crucial for evaluating nanosuspensions. Commonly the used techniques for solid-state measurement are differential scanning calorimetry (DSC) and X-ray diffraction (XRD).

1.4.2.1 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is frequently used in the pharmaceutical thermal analysis field due to provided information about both the physical and energetic properties of drugs [39]. It provides quantitative information about exothermic, endothermic and heat capacity changes as a function of temperature and time (such as melting, purity and glass transition temperature) [40]. Nanocrystals, which are solidified by freeze-drying or spray-drying, are heated at a constant heat flow and temperature changes are measured. The resulting data can be used to determine the thermal properties of the nanocrystals. As mentioned before, understanding the crystalline and amorphous state of drugs is critical to evaluate the physicochemical property of nanocrystals. In DSC scans, crystallization is observed as an exothermic transition, and samples that undergo crystallization during heating necessarily have some amorphous content. However, there are amorphous compounds that do not recrystallize. The amount of energy released on crystallization is related to the lattice energy of the crystalline compound, and thus DSC can be used to quantify the crystallinity of lyophilized or milled pharmaceutical compounds.

Even though DSC is an easy and useful tool for solid-state measurement of nanocrystals. Some factors might induce the artefacts. The commonly used stabilizers melt before the drug, the nanosized drugs potentially dissolve in the melt stabilizers. The solidification of nanosuspensions might change the solid state of drugs. Additionally, the dried samples consist of drug and stabilizers, the melting endotherm temperature (Tm) and enthalpy are the results of the interaction among drug and stabilizers. Therefore, DSC could be used the qualify the solid-state of nanocrystals. To quantify the crystallinity more accurately, X-ray diffraction should be used.

1.4.2.2 X-ray diffraction analysis

X-ray diffraction (XRD) is a widely used analytical technique for studying the crystal structure of materials, including nanocrystals. In contrast to DSC, X-ray diffraction is a non-destructive technique and requires little solid or semisolid sample preparation, enabling it as a valuable tool for characterizing nanocrystals in both research and industrial fields. It can determine the atomic and molecular structure of a crystal, in which the crystalline structure causes a beam of incident X-rays to diffract into many specific directions, measuring the angles and intensities of these diffracted beams [41]. The technique is based on the principle of Bragg's law, which relates the diffraction angle of X-rays to the interatomic spacing of the crystal lattice This information can be used to determine the crystal structure of the nanocrystals [42]. In conclusion, XRD is a powerful technique for investigating the crystal structure and properties of nanocrystals, and it is a key tool for understanding their behaviour and optimizing their performance in various applications.

1.4.3 *In vitro* release

In vitro release measurement is an important method used to assess the quality of formulations and to estimate the relevant *in vivo* performance. The release kinetics of nanosuspensions depend on various factors, such as the size and surface area of the nanoparticles, the composition and concentration of the nanosuspension, and the properties of the dissolution medium. Therefore, it is important to carefully design and control the experimental conditions to ensure that the results are reliable and reproducible from *in vitro* release facilitating a scientific and predictive approach to the design and development of sustained delivery systems with desirable properties [43]. However, no compendial or regulatory standards exist for *in vitro* release model of drug release kinetics for nanocrystal formulations. According to the topical research topic,

dialysis bags, Franz-diffusion cells and some novel release tests are used. The following parts will discuss these methods in detail.

1.4.3.1 Dialysis bag

Dialysis bag release is a widely used method for studying the release of drugs and other molecules from nanoparticles, including nanosuspensions. This method involves placing the nanosuspension inside a semi-permeable dialysis bag and suspending it in a dissolution medium, such as a buffer solution. The membrane of the dialysis bag is a molecular weight cut-off membrane (MWCO), which can be used for particular and dissolved drugs during release [44]. Float-A-Lyzer®, which has a cylindrical tubing geometry that avoids sample dilution and provides open access for full-volume sampling, is widely used in academic research (Figure 1.7) [45]. Dialysis bag release is a simple and low-cost method for studying the release of nanosuspensions, and it can provide valuable information about the release kinetics and mechanism. However, most of the nanocrystal-loaded topical formulations are semisolids, which have relevant higher viscosity compared to nanosuspensions. Add there is a potential to have an artefact that the membrane conflicts with the release. Therefore, a more mimic topical administration method - Franz-diffusion cell is used for nanocrystal-loaded semi-solid formulations.

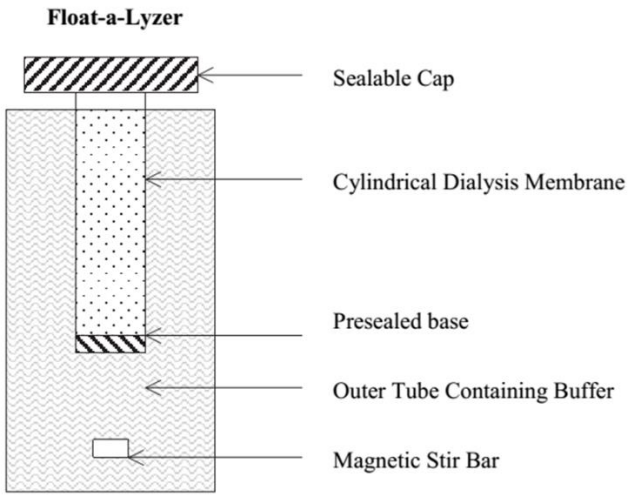

Figure 1.7 Illustration of set-up for *in vitro* release by using the Float-a-Lyzer [45]

1.4.3.2 Franz-diffusion cell method

The first proposed Franz diffusion cell was in 1975, which is used for evaluating the specification agreement qualitatively and quantitatively [46]. After that, the Franz-diffusion cell is widely used for studying the *in vitro* release and permeation of topical formulations. This method is especially designed to simulate the penetration and permeation of the drug into skin, allowing for the evaluation of the efficacy and safety of topical formulations [47]. Usually, Franz diffusion cell consists of two compartments separated by a semi-permeable membrane (Figure 1.8) [48]. The donor compartment contains semi-solid formulations, while the receptor compartment contains a suitable release buffer. The semipermeable membrane acts as a barrier and is mounted between the receptor and donor compartment. There are different kinds of membranes, which can be used to investigate the penetration and permeation rate of formulations. Samples are withdrawn from the sampling port at predetermined time intervals. The physiological temperature of skin will be mimicked by the water jacket. The stir bar is used to rotate the dissolution medium to mix the diffused drug uniformly.

Several criteria should be investigated in advance to validate the Franz-diffusion cell methods: (i) sink conditions; (ii) incubation time; (iii) incubation temperature; (iv)

stirring speed; (v) membrane type and hydration time; (vi) sampling amount. All these factors must be addressed in the design of the test to acquire valid dermal penetrability data.

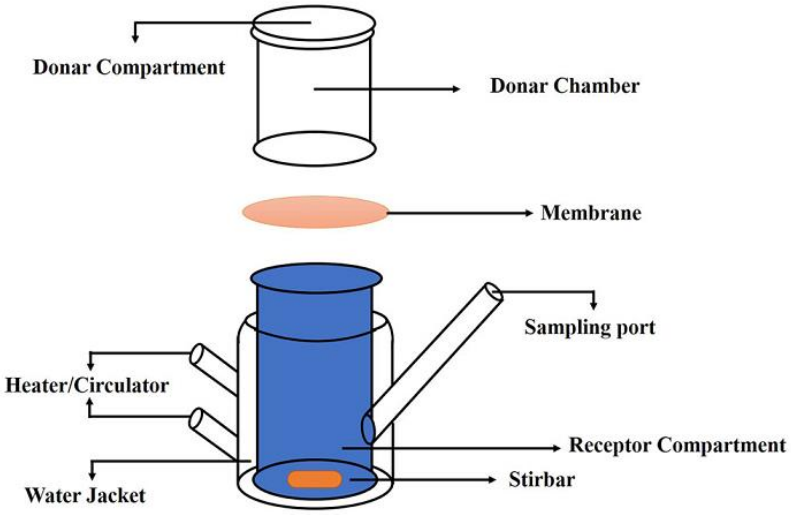

Figure 1.8 Representative diagram of Franz diffusion cell [48]

1.5 Nanocrystal-loaded formulations

Poorly water-soluble drugs are attractive candidates for the development of nanocrystal formulations. Nanocrystal technology enabled the delivery of hydrophobic drugs through multiple administration routes, e.g., oral, parenteral and topical delivery. Nanocrystal-loaded formulations have emerged as a promising approach for increasing the biopharmaceutical performance of drugs [8].

1.5.1 Nanocrystal-loaded oral formulations

Nanocrystal-loaded oral formulations incorporate nanocrystals into oral dosage forms such as suspensions, capsules and tablets. Currently, there are around 20 commercialized nanocrystal products for oral administration (Table 1.1) [49]. For

nanocrystal-loaded capsules and tablets, the solidified nanocrystal powder from nanosuspensions should be solidified by freezing-drying or spray drying and then filled or compressed into capsules and tablets respectively.

Freeze drying is a commonly used technique to solidify the nanosuspension. It consists of the freezing and primary drying and secondary drying steps to ensure the final moisture content of the sample is below 1 %. This dry powder can be formulated immediately into oral dosage forms. Cryoprotectants must be added to nanosuspensions to inhibit the damage to nanocrystals by freezing.

Spray drying is routinely used to transfer the liquid formulation into dry solid powder. Usually, nanosuspension is atomized to fine droplets in a preheated chamber. Heat and mass transfer occur between air and the droplets. The water is removed by the heated gas. This method might lead to slight stickiness depending upon the formulation composition. This stickiness or aggregation can be circumvented by adding adsorbents such as lactose, mannitol etc.

Table 1.1 Overview of drug nanocrystals for oral application in the current market [49]

Drug compound	Product	Nanosizing approach	Company
Fenofibrate (BCS II)	Tridlide®	High-pressure homogenization (HPH)	Skye Pharma
Theophylline (BCS I)	Theodur®	Media-milling	Mitsubishi Tanabe Pharma
Naproxen sodium (BCS II)	Naprelan®	Media-milling	Wyeth
Megestrol acetate (BCS II)	Megace® ES	Media-milling	Par Pharma
Fenofibrate (BCS II)	Tricor®	Media-milling	Abbott
Aprepitant (BCS IV)	Emend®	Media-milling	Merck
Tizanidine hydrochloride (BCS II)	Zanaflex™	Media-milling	Acorda
Diltiazem (BCS I)	Herbesser®	Media-milling	Mitsubishi Tanabe Pharma
Methylphenidate hydrochloride (BCS II)	Ritalin LA®	Media-milling	Novartis
Morphine sulfate (BCS III)	Avinza®	Media-milling	King Pharm
Dexmethylphenidate hydrochloride (BCS II)	Focalin XR®	Media-milling	Novartis
Verapamil (BCS I)	Verelan PM®	Media-milling	Schwarz Pharma
Metaxalone (BCS II)	Skelaxin	Media-milling	King Pharm
Olanzapine (BCS II)	Zyprexa® Relprevv™	Media-milling	Lilly
Sirolimus (BCS II)	Rapamune®	Media-milling	Wyeth
Griseofulvin (BCS II)	Gris-PEG®	Coprecipitation	Novartis
Nabilone	Cesamet®	Coprecipitation	Lilly
2-Methoxyestradiol	Penzem®	Media-milling	EntreMed
Celecoxib (BCS II)	Celebrex®	Media-milling	Pfizer
Paliperidone Palmitate (BCS II)	Invega Sustenna®	Media-milling	Johnson & Johnson

As mentioned above, both methods will lead to a temperature challenge for nanosuspensions. Therefore, the cytoprotectant for freeze-drying and the condition for spray-drying need to be investigated to ensure the stable nm size of drugs. The redispersibility of nanosuspensions from spray drying and freeze-drying is investigated. Spray-dried nanosuspension has a better redispersibility compared to freeze-drying samples. The dried nanocrystals were matrix-like structures after freeze-drying. However, the spherical particles formed by spray drying resulted in a minimal aggregation of nanocrystals compared to freeze-drying [50].

Researchers found the advantages of nanoformulation compared to conventional formulations. The *in vitro* dissolution profile of the optimized nanoformulation compared to the pure drug and marketed formulations (Canditral Capsule) showed

higher drug release. A significant enhancement of bioavailability of nanocrystal-loaded capsules or tablets was superior compared to the marketed formulation and microcrystal-loaded formulations which were demonstrated from *in vitro* dissolution and *in vivo* studies [51-53]. Two hypotheses are used to explain this phenomenon: the increased dissolution rate due to the enlarged surface area and the effective intestinal permeability. The rate of dissolution of a solid drug compound is directly proportional to the surface area available for dissolution. The reduction of API particle size from 50 μm to 159 nm led to a surface area enhancement of 333-fold resulting in a significant increase in the dissolution rate of nanosuspension [54]. Previously, effective intestinal membrane permeability (Peff) was tacitly assumed to be independent of particle size. However, many reports suggested that nm drug particles have an unstirred water layer (UWL). In this case, the effective thickness of the UWL could be smaller than the nominal thickness, increasing effective intestinal permeability. It was suggested that when the dose ratio mg/particle size (μm) ratio exceeds 20, the particle drifting effect would become more significant [55]. In summary, these nanoformulations are designed to improve the dissolution rate and absorption of the drug in the gastrointestinal tract, thereby enhancing its therapeutic effectiveness [49].

1.5.2 Nanocrystal-loaded parenteral formulations

The widespread attention of nanocrystal-loaded parenteral formulations is raised not only due to increased solubility and dissolution rate but also due to the potential of improved drug targeting to the site of tumours as well as improved solubility/partition in the bloodstream without using several excipients. Several nanoparticle products for parenteral delivery have been approved by FDA [56]. In 2005, FDA approved the first “nano” particle-based delivery product: a 130 nm albumin-bound paclitaxel nanoparticle for breast cancer. Encapsulating drug nanoparticles into carriers such as liposomal and polymer-mediated delivery systems, can potentially improve drug targeting, increase residence time and reduce their side effects.

The use of nanosuspensions in parenteral drug delivery is a new concept. Currently, there are no commercial nanocrystal products for parenteral delivery. For many decades, coarse microsuspensions of poorly water-soluble drugs (10–100 μm) have been commercialized for intramuscular or subcutaneous delivery e.g., the penicillin G benzathine (BICILLIN L-A, registered trademark by Wyeth-Ayerst), drug product prepared by the reaction of dibenzylethylene diamine with two molecules of penicillin G, dexamethasone acetate (DECADRON-LA,5 a registered trademark by Merck), and methylprednisolone acetate (DEPO MEDROL, a registered trademark by Pfizer), which are administered intramuscularly. Usually, the inner diameter of the smallest blood vessels ranges from approximately 5 to 7 μm. Hypotension has been reported that it related to the size of particles. Thus, conventional microsuspensions might have a side effect on the patients. However, nanoparticles are surprisingly well tolerated. Researchers found that the hypotensive effect decreased as particle diameter was reduced from 200 to 100 nm, and was absent at 50 nm [57]. Thus, nanocrystal-loaded formulations for parenteral delivery are urgently required. They enhance the bioavailability and increase therapeutic efficacy of drugs. Nanocrystals have a large surface area to volume ratio, which enhances the dissolution rate and bioavailability of poorly soluble drugs compared to conventional drug delivery systems [58]. Additionally, nanocrystals increase penetration into tissues and cells, leading to improved drug targeting and therapeutic efficacy [59].

1.5.3 Nanocrystal-loaded topical formulations

Usually, the transport of drugs across the skin barriers takes place by passive diffusion, which is governed by Fick's first law, which states that the rate of diffusion or transport across a membrane (dC/dt) is proportional to the difference in active concentration on both sides of the membrane. Nanocrystals improve the dissolution velocity (Noyes-Whitney equation) of poorly soluble drugs and the high drug loading results in an

increased concentration gradient, which will promote passive diffusion through biological membranes. Additionally, nanocrystals increase the adhesiveness to membranes due to the surface-to-volume ratio increases with decreasing size (Gecko effect) [60]. All these benefits give nanocrystals more attention to be applied for topical applications. However, until now, no pharmaceutical products for dermal application are commercialized. There are still some difficulties with nanocrystal-loaded formulations. These conflicts are in the preparation, incorporating nanocrystals into semisolids and during the administration. The detailed information will be discussed in the following sections [61].

1.6 Topical drug delivery

1.6.1 Structure and function of skin

The human skin is the largest organ of the human body and is against external assault. It consists of epidermis, dermis and hypodermis, which are separated by a basement membrane. Hairs, sebaceous and sweat glands are regarded as derivatives of skin (Figure 1.9). Epidermis is the outermost layer of the skin and consists of four to five layers of cells. The topmost layer of the epidermis is the stratum corneum, which consists of dead skin cells that are constantly shedding and being replaced by new cells. The dermis is the middle layer of the skin and is made up of connective tissue containing blood vessels, nerves, hair follicles, and sweat glands. The dermis is responsible for providing strength and elasticity to the skin. The subcutaneous tissue is the innermost layer of the skin and is made up of adipose tissue (fat). It serves as a cushion and insulator and helps regulate body temperature [62, 63].

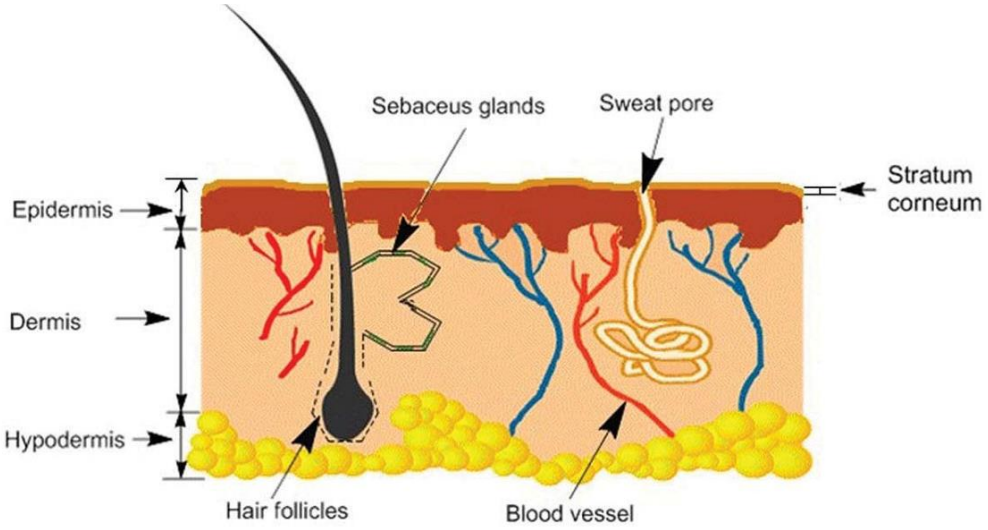

Figure 1.9 Schematic diagram of human skin structure [63]

Due to the specific structure of human skin, it acts as a remarkably efficient barrier, controlling the unregulated loss of water, electrolytes and solutes [64-66]. Furthermore, the skin absorbs IR and UV irradiation, regulates the temperature, prevents dehydration and defends the body from entering chemicals and biological agents. The immune and enzymatic systems of the skin are additional cellular and molecular barriers to neutralize, attack or degrade everything that is not physically kept outside [67, 68]. However, such a strong protective function of the skin also presents a challenge to deliver drugs across the skin into the body.

1.6.2 Penetration pathway into skin

The skin is a selectively permeable barrier. Different drugs permeate through the skin at different rates. Three general pathways exist to permeate the skin: (i) hair follicles, sweat ducts and sebaceous glands can provide transappendageal route (Figure 1.10a); (ii) skin cells such as keratinocytes can provide the intracellular routes for drug molecules (Figure 1.10b); (iii) the lipid matrix between the intercellular spaces can

provide the intercellular route (Figure 1.10c), which is the principal pathway to cross skin. Most penetration through the skin of the actives depends on passive diffusion. Among the three described appendageal follicular penetration is the most important one. However, the effective use of these pathways is limited due to the barrier stratum corneum [63, 66, 67].

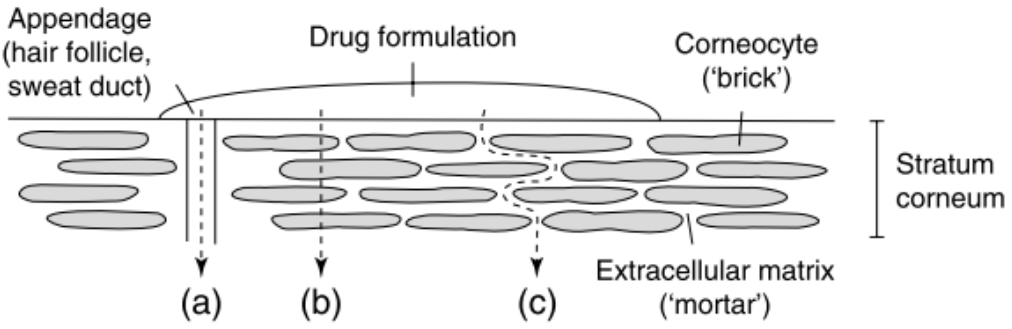

Figure 1.10 Drug permeation pathways in the skin (stratum corneum shown): (a) appendageal route; (b) intracellular route; (c) intercellular route [66]

1.7 Challenges of nanocrystal-loaded topical formulations

After understanding the physicochemical properties of nanocrystals and knowledge of topical administration, some difficulties arise in nanocrystal-loaded topical formulations. Detailed information will be discussed in the following parts

1.7.1 Stabilizer-free nanosuspensions

Usually, nanocrystals are produced in aqueous phase. A suitable stabilizer is often identified by trial and error since it can avoid aggregation, decrease the surface energy, create steric hindrance or facilitate electrostatic repulsion. Optimizing the stabilizers and their toxicities is time-consuming. Furthermore, sterilization is required for the

scale-up of nanosuspensions. Usually, the terminal sterilization methods are autoclave and gamma radiation. Additionally, thermocycle stability tests including freeze-thaw and long-term storage stability are unavoidable for the commercialization of nanocrystal-loaded formulations. Hitherto, there are, however, only limited possibilities for the preparation of monodisperse samples after these tests. Additionally, there are no accepted guidelines for stabilizer selection. The self-stabilized nanosuspensions should be desired to reduce the stabilizer-based toxicity and simplifying the formulation development process are also necessary. In order to design a suitable stabilizer-free nanoformulation, some fundamental knowledge regarding the stability measurement in nanoformulation is discussed below:

Sterilization

Autoclave is a commonly used method for sterilization in the pharmaceutical industry. For most applications, the standard temperature and pressure for sterilization in an autoclave are 121 °C at 2 bar of pressure for 15-20 minutes. However, when it comes to nanosuspensions, such high temperatures and pressure may cause aggregation and destabilization of nanosuspensions. Different stabilizers result in different changes in particle size and distribution. For example, an increase in the mean particle size with a simultaneous decrease in polydispersity index was observed after autoclaving of poloxamer 188-stabilized supercooled smectic cholesteryl myristate nanoparticles [69]. However, for HPMC-stabilized nanocrystals, the HPMC precipitates out of the nanosuspension due to a phenomenon called thermal gelation. Even though HPMC is a thermo-reversible polymer, nanocrystals have aggregated together resulting in sedimentation afterwards. The selection of used stabilizers should be given more attention.

Gamma radiation involves the use of high-energy gamma rays to kill microorganisms and other pathogens that may be present in a product. It is an effective method of

sterilization. However, it is important to consider the potential effects of gamma radiation on the stability and properties of the nanosuspension. The size, shape and stability of the nanosuspension might change due to the high-energy input. Therefore, it is important to carefully evaluate the impact of gamma radiation on the nanosuspension and to validate the chosen method of sterilization to ensure that it does not impact the stability or efficacy of the nanosuspension. Additionally, the potential residual radiation in the nanosuspensions after sterilization should be evaluated. Any residual radiation must be within acceptable limits to ensure product safety and efficacy.

Thermocycling stability tests

Numerous researches investigated the stability mechanism of aqueous nanosuspensions [70]. Long-term particle size stability of aqueous nanosuspensions was achieved in this literature [25, 70]. However, there was no successful case that particle sizes in the nm range after the freeze-thaw test, which is crucial for commercialization. For aqueous nanosuspensions, it is assumed that freezing of colloidal formulations changes the interactions between stabilizing layer and particle, leading to agglomeration [71]. Furthermore, dissolved drug portions during the freezing process were in a supersaturated state and re-crystallization easily occurs. Upon the thawing process, the precipitated stabilizers may not dissolve immediately upon the melting of the ice. The nanocrystals lost the stabilization function from the stabilizers, inducing aggregation. Solubility would be greatly altered during these sterilization and stability tests, leading to crystal growth. Additionally, electric or polymeric stabilizers have a cloud point with changed temperatures. Once the stabilizer precipitates out during the stability tests, nanocrystals lose stability. Thus, it suppresses the commercialization of nanoformulations. A novel nanocrystal vehicle, which has satisfying stability for commercialization is needed.

Recently, some novel nanocrystal carriers were investigated. Kamiya et al. successfully formulated a solid-in-oil nanosuspension (SONS) as a novel delivery nanocarrier for sparingly soluble drugs and cancer vaccines [72, 73]. In 2014, Zhai et al. investigated the production of caffeine nanocrystals in an aqueous mixture. The stability of caffeine nanosuspension was more than half a year under accelerated test [74]. Therefore, milling in different media might improve the stability of nanocrystals. Media with a lower dielectric constant (DI) reduce the attractive force of hydrophobic particles. Notably, most oils have a low dielectric constant below 5 (water=80) and a high viscosity, which improves the inherent stability of nanosuspensions. Only a few publications and patents investigated the preparation of oily nanosuspensions [75, 76]. The obstacle to the preparation of oily nanosuspensions is the high viscosity limiting the nanosizing efficiency. Nowadays, the use of dual centrifugation (DC) for effective and rapid drug-nano milling was described. DC differs from normal centrifugation by an additional rotation of the samples during centrifugation, resulting in a very fast and powerful movement of the samples inside the vials, which in combination with milling beads results in effective milling (Figure 1.11) [77].

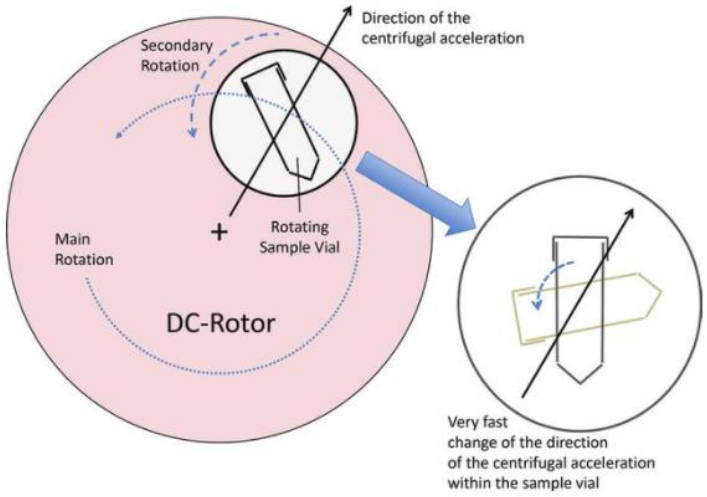

Figure 1.11 Principles of dual centrifugation (DC). Left: Dual rotor with a vial turning around its axis (only one vial is shown). Right: Visualization of the changing direction of the centrifugal forces in a sample vial [78]

Nanotechnology is widely used for hydrophobic drugs, and stabilizers must be used to prepare aqueous nanosuspensions. The surface energy of particles is increased during the milling process. The stabilizers reduce surface energy by improving wettability. Oils have a low dielectric constant improving the wettability of hydrophobic drugs compared to water. In summary, there is the potential to prepare oily nanosuspensions without stabilizers which can have the potential to achieve monodisperse nanosuspension after the sterilization and thermocycling tests for commercialization. Until now, there is a lack of systematic investigations on the preparation, stability, and *in vitro* release of oily nanosuspensions. For *in vitro* release, the surface area of samples has a crucial influence on the release rate. Thus, self-made fixed surface area release equipment has to be used to evaluate the influence of particle size, oil type and drug loading on the release rate without sample emulsification.

1.7.2 Incorporation of nanocrystals into semisolid vehicles

For topical drug delivery, nanocrystals need to be formulated into a final semisolid dosage form in order to apply to the patient's skin site. Usually, pharmaceutical semisolids include gels, ointments and emulsions. Different semisolids consist of different excipients. Ideally, the nanocrystals should maintain their properties (including drug content, drug release and particle size) after incorporation. However, interactions of the nanocrystals with ingredients in the final dosage form or certain process conditions could result in changes in nanocarrier performance.

"Gels" are semisolids, containing the gelling agent and other ingredients in water or other media (oils or solvents). The simplest nanocrystal-loaded hydrogels consist of nanocrystals, stabilizers, gelling agents and water. For oleogels, oily nanosuspensions are prepared for the lateral gelling process. However, some gelling agents need to interact with the media at certain conditions, e.g., temperature and pH. Usually, the stability of nanocrystals will be influenced by the temperature and pH changes.

Furthermore, the gel-formers might also have interacted towards stabilizers, resulting in the aggregation of the nanocrystals. Therefore, systematic investigation on the influence of types of stabilizers and different gel vehicles should be investigated.

“Creams” are a mixture of oil and water, along with an emulsifying agent. Different phases will have different hydrophobicity. After loading nanocrystals into cream, the hydrophobicity of nanocrystals governs the distribution of the drugs. Principally, nanocrystals, which are prepared from water, have a hydrophilic property due to the hydrophobic head of the stabilizer attaching to the surface of the nanocrystal and the hydrophilic branches stretching into the water. The nanocrystals, which are prepared from oils, have a hydrophobic property due to the surrounding oil layer on the surface of nanocrystals. However, high temperatures used during the preparation of emulsion and the homogenization force might vary the surface property of nanocrystals influencing the distribution of nanocrystals in the oil or water phase of cream. These changes will result in a different performance of nanocrystal-loaded cream.

Therefore, the type of stabilizers and gelling agents should be systematically investigated. The distribution of nanocrystals, which are prepared from the water or oil phase, is caused in different, whereas this phase of the cream needs carefully evaluating.

1.7.3 Physical stability of nanosuspension upon topical administration

Drug nanocrystals (NC) have a size below 1000 nm with pure drugs, were introduced in the 1990s [10]. The high bioavailability and faster release are due to the decreased crystallinity and enlarged surface area [7, 38, 79]. Reduced dose not only benefits the patients in terms of reduced side effects and toxicity but also leads to cost savings. Stability is the main issue of NC due to the thermodynamical instability with a tendency toward agglomeration or crystal growth in an aqueous medium. Normally, Ostwald ripening is used to explain this phenomenon. Indeed, due to Ostwald ripening, the

average particle size may increase over time. The driving force is the higher solubility of smaller particles compared to the larger ones. The following approach can minimize Ostwald ripening. In order to limit the monomer diffusion, the difference in drug solubility among large and small particles should be maintained as low as possible [6, 80]. However numerous previous reports have investigated the long-term physical stability of nanocrystals [25, 26, 64, 67, 70, 79, 80], and fewer publications investigate the stability of the nanocrystal upon administration. For topical administration, a well-defined particle size is especially important since the particle size can affect not only the dissolution rate but also alter the penetration route of the drug penetrating the skin. Therefore, the size of the particles remaining in the nano dimension is requirable. Despite recent progress, crystal growth mechanisms during topical administration are still not well understood due to the lack of a suitable simulation model and *in situ* measurement techniques.

The skin absorption of nanocrystals is mainly from the transappendageal pathway and the maximum size was below 600 nm [63, 66, 67, 72, 81]. However, water evaporation occurs immediately after the formulation has been applied to the skin. This might lead to the destabilization of the nanocrystals. Size changes will happen during the administration, and this will also lead to reduced drug efficacy [62, 82]. Current knowledge of crystallization is that nucleation and crystal growth occur simultaneously. Nucleation requires activation energy, attributed to interfacial tension between the initial nucleus and the stabilizer solution. Crystal growth happens when the activation energy is above a certain extent. According to the solid-state of drug in the system, it could be categorized into different pathways to crystallization. For the molecules, monomer by monomer classical nucleation theory (CNT) of crystal growth will be observed. Nanosuspension consists of amorphous state of drugs surrounding the crystalline drugs and dissolved drugs, different states of the drugs have different crystal growth mechanisms. It has been recognized that “nonclassical” crystallization (NCC) from solution involving transient amorphous precursors is ubiquitous [83-85]. Thus,

crystallization starts from the initial densification of the precursors. Crystallographic fusion is gradual with the optimization of molecular ordering and morphology. Upon the water evaporation, the concentration of water, stabilizer, dissolved drug and nanocrystal experience dynamic changes. It is not only varying the solubility, the amphiphilic and polymeric stabilizers used DLVO theory and the steric hindrance mechanism also will affect the stability and lead to different precipitation phenomena by different crystallization mechanisms [25, 26, 28]. Thus, the solid-state of drugs, type and concentration of stabilizers are crucial to the precipitation.

Therefore, the particle size and solid-state stability of nanocrystals need to be investigated during topical administration. Hydrocortisone and dexamethasone two topical corticosteroids were selected as the model drug. Normally, during topical administration, the diffusion of nanocrystals in water and the penetration into the skin happen simultaneously. Due to analytical limitations and difficulties to simplify the complex administration process, such studies need research. The stability of nanocrystals upon administration needs to be investigated by with/without penetration process dividedly. Without penetration, a simulation experiment was performed by incubating samples on the petri dish to present the diffusion influence, monitoring by microscopy, laser diffraction and XRD. With penetration simulation experiment can be the *ex vivo* investigation of selected formulations on porcine skin. Liquid TEM can be used for imaging the intermediate mesocrystal surrounded surface of the nanocrystal. These findings can contribute to the explanation of the crystal growth mechanism of nanocrystals upon topical administration.

1.8 Research objectives

The main objective was to evaluate the performance of nanocrystals upon preparation, incorporation into semisolids and topical administrations: (i) for the preparation part, stabilizer-free oily nanosuspensions will be investigated including the studying of milling efficiency, *in vitro* release and physical stability; (ii) nanocrystals will be incorporated into semisolids and the influence of different types of stabilizers and gelling agents and the distribution of nanocrystal in creams will be studied together with particle size stability, viscosity and *in vitro* release; (iii) different simulated topical methods will be applied to nanosuspensions and the solid-state and particle size changes will be discussed.

Specific objectives are as follows:

a) To investigate the preparation and *in vitro* release of oily nanosuspensions

b) To investigate the loading nanosuspensions into semisolid dosage forms

c) To clarify particle growth mechanisms of nanosuspension after simulated topical administration

2 Materials and methods

2.1 Materials

Dexamethasone (Dv50 = 6.2 µm) (Fagron GmbH & Co.KG, Barsbüttel, Germany) and hydrocortisone (Dv50 = 4.2 µm) (Tokyo Chemical Industry Co., LTD, Tokyo Japan), Triglycerides, Medium-Chain (Miglyol® 812 N, MCT ~14 mPa·s), Low viscosity paraffin (~22 mPa·s), Span 80 (Caelo Loretz GmbH, Hilden, Germany), Tocofersolan (TPGS) (BASF SE, Ludwigshafen, Germany), Poloxamer 407 (P407) (BASF SE, Ludwigshafen, Germany), Hydroxypropyl methylcellulose E5 (HPMC-E5) (Colorcon, Dartford Kent, UK), Sodium dodecyl sulfate (SDS) (Carl Roth GmbH & Co.KG, Karlsruhe, Germany), Polyvinyl pyrrolidone K30 (PVP) (BASF SE, Ludwigshafen, Germany). Hydroxypropyl methylcellulose E4M (HPMC-E4M) (Colorcon, Dartford Kent, UK), Carbopol® 980 NF (Lubrizol, Humberg, Germany), Triethanolamine (TEA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Ethyl cellulose (EC) (Ethocel® Standard 4 Premium, Colorcon Ltd., Dartford, UK), Polysorbat 60 (Caelo Loretz GmbH, Hilden, Germany), Cetostearyl alcohol (Caelo Loretz GmbH, Hilden, Germany), Glycerol (Caelo Loretz GmbH, Hilden, Germany), 0.1-0.2 mm, 0.25-0.35 mm zirconium beads (SiLibeads®, Sigmund Lindner GmbH, Warmensteinach, Germany), Ultra-purified water (Milli-Q-apparatus, Millipore GmbH, Darmstadt, Germany), NaH_2PO4, Na_2HPO4, HCl, NaOH and NaCl (Carl Roth GmbH Co. KG, Karlsruhe, Germany).

2.2 Methods

2.2.1 Preparation of nanosuspensions

2.2.1.1 Preparation of oily nanosuspensions

Micronized drugs were weighed into oils and mixed with an Ultra Turrax T-25 (8,000 rpm, 10 min, IKA®-Werke GmbH & Co. KG, Staufen, Germany) to disperse large particles. Then the 10 ml microsuspensions (oily suspensions without/with stabilizer) were added into a 15-ml DC-Twist-Top-Vial (Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany). The oily nanosuspensions with stabilizer have a ratio of 1:5 (span 80: drug). Dual centrifugation was performed using a ZentriMix 380 R (Andreas Hettich GmbH and Co. KG, Tuttlingen, Germany) at 1,500 rpm. For every milling trial, the cooling device was set to 8 °C. The milling times for hydrocortisone and dexamethasone were 2 h and 1.5 h, respectively.

2.2.1.2 Preparation of aqueous nanosuspensions

Drug powders were firstly added into the 10 ml 0.4 % stabilizer solutions. They were mixed with an Ultra Turrax T-25 (8,000 rpm, 10 min) to disperse large particles, then preprepared 10 ml suspensions and 20 g beads were added into 15 ml DC-Twist-Top-Vial. Sealed vials were milled at 1,000 rpm for 2 h (hydrocortisone) and 1.5 h (dexamethasone) with a surrounding temperature of 0 °C.

All milled oily and aqueous nanosuspensions were separated from beads by filtration through paper filters (pore size of ~20 μm).

2.2.2 Preparation of nanocrystal-loaded semisolid vehicles

The prepared nanosuspensions from 2.2.1 (Table 2.1) were loaded into gels and O/W creams.

Table 2.1 Particle sizes of aqueous and oily nanosuspensions after wet bead milling

Sample		Z-average, nm ± SD	PDI ± SD
Hydrocortisone	1.2 % HPMC 3 % drug	210 ± 6	0.11 ± 0.01
	0.8 % SDS 3 % drug	189 ± 5	0.12 ± 0.00
	5 % drug in MCT	316 ± 2	0.25 ± 0.04
	5 % drug in paraffin	212 ± 9	0.21 ± 0.07
Dexamethasone	1.2 % HPMC 3 % drug	209 ± 4	0.13 ± 0.02
	0.8 % SDS 3 % drug	217 ± 2	0.11 ± 0.07
	5 % drug in MCT	385 ± 6	0.20 ± 0.03
	5 % drug in paraffin	326 ± 3	0.11 ± 0.06

2.2.2.1 Preparation of nanocrystal-loaded gels

1 % nanocrystal 3 % HPMC-E4M gels:

300 mg HPMC-E4M was added into 3.3 ml distilled water at 80 ℃ After the polymer dispersed homogeneously, 6.7 ml 3 % aqueous nanosuspensions were added into the transparent pre-prepared gels at room temperature.

1 % nanocrystal 0.25 % Carbopol gels:

25 mg Carbopol was added into 6.7 ml distilled water. After the polymer was dispersed homogeneously, 10 % TEA solution was used to adjust the pH to 7.4. 3.3 ml 3 % aqueous nanosuspension was added to the transparent gels. The final homogeneous nanocrystal-loaded gels were produced by mortar mixing.

1 % nanocrystal 8 % SiO_2 gels:

800 mg SiO_2 was added into 8 ml MCT/paraffin. After the SiO_2 was dispersed homogeneously, 2 ml 5 % MCT/paraffin nanosuspensions were added into pre-prepared transparent gels. The final homogeneous nanocrystal-loaded gels were produced by

mortar mixing.

1 % nanocrystal-loaded 10 % ethyl cellulose gels:

1 g ethyl cellulose was added into 8 ml MCT with the mixing under 160 ℃. After the ethyl cellulose was dispersed and dissolved homogeneously, 2 ml 5 % MCT nanosuspensions were mixed into pre-prepared transparent gels at 90 ℃ for 5 min. The 1 % nanocrystal-loaded 10 % ethyl cellulose gels were obtained after cooling to room temperature.

2.2.2.2 Preparation of nanocrystal-loaded O/W creams

The O/W creams were produced according to the composition in Table 2.2

Creams with 1 % nanocrystal in oil phase:

5 % paraffin nanosuspensions were diluted into 4 %, melting together with the cetystearylalcohol at 80 ℃. Then the oil phase of the creams was poured into the water phase, which was also preheated at 80 ℃. The oil and water phase were subsequently homogenized under 8000 rpm for 5 min. These samples were cooled to room temperature.

Creams with 1 % nanocrystal in aqueous phase:

3 % aqueous nanosuspensions were diluted into 2 %, preheating with glycerol and polysorbate 60 at 80 ℃. The oil phase of the creams, which was also preheated at 80 ℃, was poured into the water phase. homogenization under 8000 rpm for 5 min. These samples were cooled to room temperature.

Table 2.2 Composition of O/W creams

Formulation	Excipients	Parts
Nonionic	Low viscosity paraffin	25

cream	Cetystearylalcohol	10
	Polysorbate 60	5
	Glycerol 85 %	10
	Water	50

2.2.3 Simulated water evaporation study

2.2.3.1 Petri dish method

To prepare drug-saturated solutions and saturated stabilizer solutions, an excess amount of drug was added to 10 ml water and different stabilizer solutions, stirring for 48 h under room temperature. The mixtures were centrifugated at 12,000 rpm (Biofuge, Thermo Electron Corporation, Langenselbold, USA) for 2 h, and the clear supernatant was filtered through a 0.45 μm nylon filter (Tianjin Jinteng Experiment Equipment Co. Ltd, Tianjin, China) to separate undissolved drug.

To achieve the possibility of particle size measurement by laser diffraction (LD) at different time intervals, 2.5 ml drug-saturated solutions, saturated stabilizer solutions and nanosuspensions were dropped onto 19.5 cm^2 petri dishes. Samples were incubated in an oven at 45 ± 5 % RH and 30 ± 2 °C. The water-evaporated samples were observed by polarized microscopy.

The solubility changes of drugs in the saturated solution, saturated stabilizer solution and nanosuspensions upon water evaporation were evaluated. 0.5 ml samples were taken at 0, 1, 3, 5 and 7 h and filled into molecular cut-off membrane tube (Vivaspin 500, 3kDa MWCO, Sartorius Lab Instruments GmbH & Co. KG, Goettingen Germany). The 3 kDa MWCO membrane can exclude all stabilizers except TPGS (MW ≈ 1000 Da). The samples were centrifugated at 12,000 rpm for 30 min. The filtrates were appropriately diluted with water, and diluted filtrates were assessed by UV (Agilent HP 8453, Agilent Technologies Inc., Santa Clara, USA).

2.2.3.2 *Ex vivo* porcine skin method

The administered amount to patients is usually 0.2 cm length of 1 % drug ointment (drug amount ≈ 1 μg/cm^2) [86]. Therefore, 1 μg/cm^2 drug was applied on the porcine skin which is the same amount for the petri dish method. The samples were carefully covered at the back part of the porcine skin with aluminium paper to maintain hydration. Samples were incubated in the oven at 45 ± 5 % RH and 30 ± 2 °C. After water evaporation, 3M transparent scotch tape was used to take off the surface crystals. The crystals on the scotch tape were observed by polarized microscopy.

2.2.4 Particle size and morphology determination

2.2.4.1 Optical light microscopy and polarized light microscopy

Microscopic and cross-polarized light microscopic images of samples were taken using polarized light microscopy (Zeiss Axioskop, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an Axiocam 105 colour camera.

2.2.4.2 Photon correlation spectroscopy (PCS)

The average particle size was determined by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments, Malvern, United Kingdom). As the viscosity of nanocrystal-loaded gels was too high for the measurement, the samples were diluted with saturated drug solutions (ethyl acetate solution for oily nanosuspensions; water solution for aqueous nanosuspensions). Each sample was stirred overnight to have a homogenous and suitable viscosity for the measurement. The average size and polydispersity index (PDI) were obtained by averaging 3 measurements at 25 °C, each measurement consisting of 10 runs.

2.2.4.3 Laser diffraction (LD)

The particle sizes of nanocrystals were measured by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). Due to the solubility of hydrocortisone and dexamethasone, each measurement was done in their saturated solution to limit the dissolution of nanocrystals.

2.2.4.4 Liquid-cell scanning transmission electron microscopy (LC-STEM)

0.4 % TPGS stabilized 1 % dexamethasone nanosuspension was diluted 10 times. 0.5 µl diluted sample was dropped on the liquid cell and dried for 30 s at room temperature. LC-STEM experiments were performed to assess the nanocrystal shape, size, and stability under the beam, while particles were dispersed in the aqueous medium. Measurements were carried out at an acceleration voltage of 200 kV using liquid cells consisting of two silicon wafers (E-chips) assembled onto a sample holder (Protochips Inc., Poseidon 300). Imaging of the sample in the native aqueous phase was performed in STEM mode by using a high-angle annular dark-field detector (STEM-HAADF) on a FEI Tecnai™ G2 F20 X-Twin TEM equipped with a field-emission gun electron source.

2.2.4.5 Conventional transmission electron microscopy (TEM)

TEM samples were prepared by depositing a few µl of the suspension on a petri dish, then a holey carbon Cu TEM grid was immersed in the drop for different time intervals during the drying process and subsequently immediately dried on an absorbing paper to quench the drying reaction. Due to the fast removal of water, some of the residual precipitates/particles on the Cu-grid were imaged successfully by conventional TEM

(FEI Tecnai™ G2 F20 X-Twin TEM) equipped with a field-emission gun electron source. The grids were then kept at room temperature and mounted on Gatan double-tilt holder. Images were acquired at an acceleration voltage of 200 kV as energy-filtered images on a Gatan GIF Tridiem detector.

2.2.5 Solid state measurements

2.2.5.1 Differential scanning calorimetry (DSC)

The solid-state of drugs as received and their formulations were analyzed by differential scanning calorimetry (DSC) using a DSC 6000 (Perkin-Elmer, Inc. Waltham, USA). Oily nanosuspensions were centrifugated at 12,000 rpm for 2 h. The centrifuge tube was inverted overnight to remove as much oil as possible. Accurately weighed 5 mg oil-removed pellets from centrifugation were sealed in aluminium pans with pinholes. They were heated over a range of 20-300 °C with a scanning rate of 10 °C/min under a nitrogen flow rate of 20 ml/min.

2.2.5.2 X-ray powder diffraction (XRD)

The X-ray powder diffraction data were recorded on a PANalytical Empyrean diffractometer (Malvern Panalytical, Almelo, Netherlands) equipped with a PIXcel1D-Medipix3 detector, using CuKα radiation (λ = 1.54060 Å) at 40 kV and a tube current of 40 mA. Nanosuspensions were centrifuged at 17,000 rpm for 2 h. The resulting pellets (nanocrystal 0 h) were measured after removing the supernatant. The dried nanosuspensions were scrapped from the petri dishes after complete water evaporation. Samples were put on the holders to have an amount of drug equal to 20 mg. The analyses started only when the samples were dried on the holders (room temperature drying). Samples were scanned on a rotating stage at 2°2θ/min (step size 0.013°2θ) in

the range 10–40°2θ. Mean crystallite sizes (D) were calculated by the Scherrer equation:

$$D = (K * \lambda)/(\Delta 2\theta * cos\theta)$$

Where K is a shape factor with a value of 0.9, λ is the X-ray wavelength of the Cu anode, Δ2θ is the full-width at half-maximum (FWHM, in radians) of the X-ray diffraction peak corrected for instrumental peak broadening using a well crystalline Si reference material, and θ is the Bragg angle. Diffractograms were analyzed with PeakFit 4 (Systat Software). Voigt profiles were fitted to the most intense reflections at 2θ = 13.7° for dexamethasone and 2θ = 14.6° for hydrocortisone samples. The shift of the most intense reflections was due to the surface roughness of the sample on the holder.

$$Degree\ of\ Crystallinity\ (DC) = \frac{D(sample)}{D(NC\ 0h)} * 100\%$$

2.2.6 *In vitro* release

2.2.6.1 Fix surface area release

Oily micro- and nanosuspensions were investigated under a fixed surface area method with self-made glass tubes. The surface area was approximately 1.33 cm^2. The two-side opened glass tube was fixed into a 100 ml glass bottle filled with 80 ml PBS buffer. By this, samples (corresponding to 2 mg drug) could be dropped inside the double-side opened tube, maintaining the surface area and avoiding emulsification. The experiments were evaluated in a water bath at 37 °C and stirred at 300 rpm. 2 ml media was removed and replaced with the same volume of fresh PBS buffer. Sink conditions were maintained. The concentration of drugs was determined using UV (hydrocortisone at 245 nm, dexamethasone at 242 nm).

2.2.6.2 Franz diffusion cell

Nanocrystal-loaded semisolid vehicles were investigated by Franz diffusion cells. The surface area was approximately 2.54 cm^2. 200 mg samples (≈ 2 mg drug) were added to the cellulose acetate membrane. The experiments were evaluated in a water bath with 30 ± 2 °C and stirred at 600 rpm. 2 ml media was removed and replaced with the same volume of fresh PBS buffer. 0.1 % SDS was used in the PBS buffer to maintain sink conditions. The concentration of drugs in each sample was determined using UV.

2.2.7 Viscosity measurements

The viscosity of nanocrystal-loaded gels was measured using the controlled stress mode of double gap connected with a computer interface (Rheostress RS 100, Haake Mess-Technik GmbH, Karlsruhe, Germany) at 20 ± 0.2 °C, with a DG40 rotor with fixed shear stress (10 Pa) for a constant time (30 s). All measurements were performed in triplicate and data values are presented as means ± SD.

2.2.8 Stability tests

2.2.8.1 Freeze-thaw test

Freeze-thaw cycling of oily nanosuspensions was investigated by filling the samples in 2-ml glass vials and sealed with rubber stoppers and aluminium caps. Samples were stored at -20 °C for 24 h and the particle size was characterized by PCS, and polarized microscopy (after thawing for 24 h at room temperature. All oily samples were in the frozen (solid) state at -20 °C.

2.2.8.2 Dry heat sterilization

Dry heat sterilization of oily nanosuspensions was evaluated by filling the samples in

2-ml glass vials, sealed with rubber stoppers and aluminium caps. Those samples were stored at 160 ± 5 °C for 1 h. The particle size was observed by polarized microscopy. 3-month stability of hydrocortisone oily nanosuspensions was performed by storing the samples in glass bottles sealed with aluminium caps at room temperature. Particle size changes were investigated by PCS. Sampling time points were 0 and 3 months.

2.2.8.3 Storage stability

3-month stability of formulations was performed by storing the samples in glass bottles sealed with plastic caps at room temperature. Particle size changes were evaluated by polarized microscopy.

2.2.9 Drug solubility measurement

An excess amount of drugs was added to 5 ml water and oils and stirred for 72 h at room temperature. The mixtures were centrifugated under 12,000 rpm (Biofuge, Thermo Electron Corporation, Langenselbold, USA) for 2 h, then the clear supernatant was filtered through a 0.45 μm nylon filter (Tianjin Jinteng Experiment Equipment Co., Ltd, Tianjin, China) to separate undissolved drug. For the solubility in water, the filtrates were appropriately diluted with water. As the UV absorbance of triglycerides interferes with the drug absorbance, directly measuring the solubility by UV was not possible. Thus, 100 μl saturated drug oil solutions were added into 5 ml water, vortexing for 1 min. Concentration of the drugs in the aqueous phase was determined after separation of oil and water phase by UV (Agilent HP 8453, Agilent Technologies Inc., Santa Clara, USA). The solubilities of drugs in the oils were indirectly calculated by the extraction ratio of oil to water.

2.2.10 Oil/water partition coefficient of drugs

The partition coefficients were determined indirectly by measuring the amount of drugs dissolved in the aqueous phase. 4 ml of 100 µg/ml DEX/HC MCT were added to 4 ml water, and incubated in a shaker for 72 h with 80 rpm at room temperature. Then, the concentration of drugs partitioned into the aqueous phase was measured by UV.

2.2.11 Contact angle measurement

Drug compacts (diameter: 13 mm, weight: 300 mg) were prepared by direct compression using a hydraulic press (P/N 25.011, Specac, England) at a compression force of 5 tons for 5 min. Measurements were performed with a contact angle goniometer (G1, Kruess GmbH, Hamburg, Germany) by the sessile drop method at room temperature (n = 5). 10 µl MCT and paraffin were applied to the compressed drug with a microsyringe. The contact angle was measured 10 s after drop formation.

3 Results and discussion

3.1 Preparation and characterization of directly milled stabilizer-free oily nanosuspensions with prolonged release

3.1.1 Background

Oily formulations are used parenterally, orally or topically and often result in a prolonged release [87, 88]. They are grouped into solutions and suspensions. Suspensions are preferable due to their higher drug loading and usually have particle size in the µm range. However, sedimentation occurs during storage which is problematic for administration. Pharmaceutical manufacturers use various techniques to improve the stability. Wetting agents are added to prevent agglomeration and aggregation. Furthermore, lipophilic thickeners are used to increase the viscosity [89, 90]. Another approach would be the use of oily nanosuspensions.

The common way to obtain oily nanosuspensions is to prepare an aqueous nanosuspension in the first step since the high viscosity of oils hinders effective milling. Subsequently, the aqueous nanosuspension is lyophilized and the resulting powder is dispersed in the oil phase. Therefore, direct nanomilling in oils is preferable since it excludes additional preparation steps and only a few publications and patents investigated this kind of preparation [75, 76]. However, in these studies, stabilizers are used.

Regarding the *in vitro* release there is limited knowledge about the mechanism of oily suspensions. Under sink conditions, particles experience mass loss, according to the drug solubility in oils. Two possible mechanisms describe drug liberation from oily suspensions (Figure 3.1). First, the suspended drug dissolves in oil before being released into the aqueous phase by partitioning [91-93]. The other mechanism is a three-step release: (i) particles are transported to the interface by sedimentation; (ii) pass through the interface; (iii) dissolve in the aqueous layer [94, 95]. Therefore, the

dissolution rate and the sedimentation rate of the drug in oil influence the release. Thus, e.g., the particle size and type of oil need careful evaluation. Regarding the sustained release of oily microsuspensions, oily nanosuspensions may benefit for example from their decreased sedimentation rate in the oil phase prolonging the release.

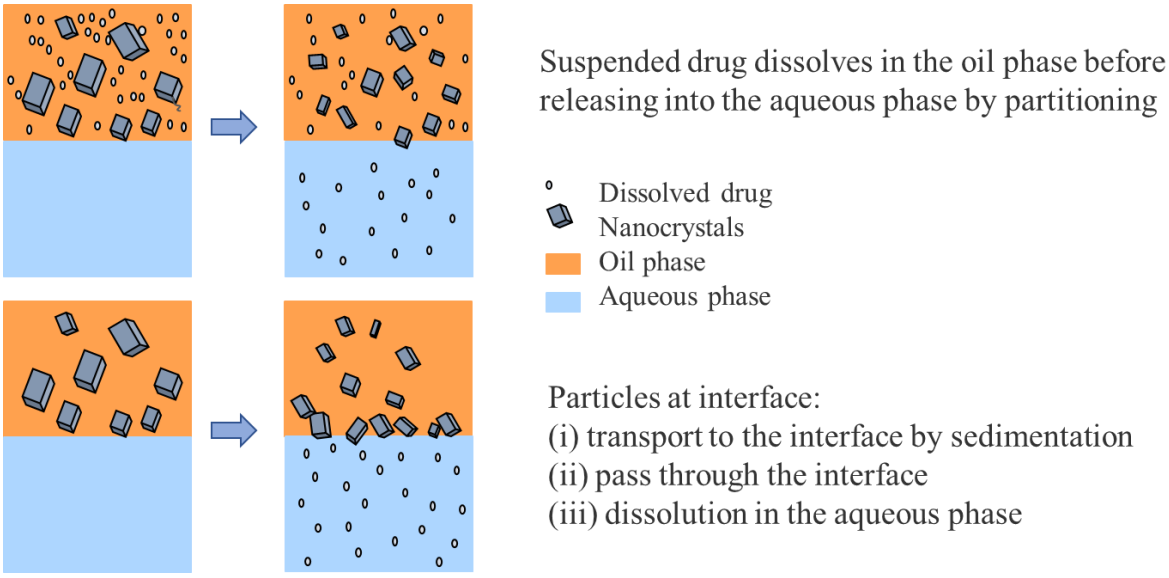

Figure 3.1 The illustration of the two release mechanisms of oily suspensions

Usually, the physical stability of nanosuspensions is problematic due to the increased total surface energy resulting in particle growth. However, the enlarged total surface area increases the viscosity of the suspension with decreasing particle size and increasing drug loading [7, 79, 96], whereas this trend is pronounced in oils [97]. This will decrease the particle growth and the sedimentation rate and an additional thickener in oily nanosuspensions is not needed. Furthermore, particle growth is usually prevented by stabilizers, whereas the stabilizer screening is costly. Notably, most oils have a low dielectric constant (DI), better wettability and a high viscosity, which improves the inherent stability of nanosuspensions. Therefore, there is the potential to prepare oily nanosuspensions without stabilizers. Furthermore, these stabilizer-free oily

systems might possess a favourable freeze-thaw stability since the precipitation of the stabilizer during freezing is circumvented. Additionally, these oily suspensions may benefit from a promising dry heat sterilization stability. Together with a sufficient particle size stability upon storage time, these two processes are crucial for potential commercialization [98-100].

In the present study, stabilizer-free oily nanosuspensions were prepared by dual centrifugation wet bead milling [77]. Dexamethasone (DEX) and hydrocortisone (HC) were selected as model drugs. Miglyol 812 (DI = 2.5) and paraffin (DI = 1.6) were chosen as dispersion media. They not only have a different dielectric constant and chemical structure but also different solubilization potentials to dexamethasone and hydrocortisone [101, 102].

In summary, this study aimed to prepare and characterize directly milled oily nanosuspensions without stabilizer which potentially have a prolonged release and promising stability. The influence of drug loading and type of oils on the milling efficiency, *in vitro* release and stabilities among freeze-thaw cycling, dry heat sterilization and storage were systematically investigated.

Usually, microsuspensions have particle sedimentation resulting in poor bioavailability and potential toxicity. Thickeners and stabilizers are added to stabilize the microsuspensions. Nanocrystals (NC) in oils have enlarged surface area increasing the interfacial force resulting in increased viscosity, potentially improving the stability. Additionally, oils have better wettability to lipophilic drugs. Therefore, a system without stabilizer may be achievable. Stabilizer-free oily nanosuspensions were evaluated regarding the preparation, solid state, *in vitro* release and particle size stability.

3.1.2 Preparation of stabilizer-free nanosuspensions

Numerous researches investigated the crucial parameters influencing drug milling efficiency, which include milling time, speed, and milling beads. Usually, smaller sized

and higher amount of beads together with longer milling time led to a higher milling efficiency [81, 103]. Therefore, the particle size distribution and the degree of crystallinity depended on milling conditions [104, 105]. In this study, milling time, speed, and beads were optimized. The low dielectric constant of oils decreases the attractive forces and increases milling efficiency [25, 106]. Additionally, a higher mechanical breaking force will have higher milling efficiency. During the milling process, the number of drug particles (different drug loadings) influences the mechanical force, since the more particle collisions the higher the mechanical force. Thus, a systematic investigation on preparation of stabilizer-free oily nanosuspensions according to bead size, type of oils and drug loading is presented in the following sections.

During the milling process, the first stage of fast breakage of crystals is attributed to the existence of cracks and crystal defects from larger crystals. As the particle size decreases further with increasing milling time, the shear stress of the suspension increases. Thus, the second size reduction is dominated by the mechanism of comminution, which relates to the coordination of the entire system [107, 108]. To induce breakage of the drug particles, two conditions have to be met: (i) The drug particles need to be trapped in the active grinding zone between approaching milling beads; (ii) The milling beads need to transfer sufficient energy to introduce breakage [12]. The kinetic energy of the milling beads is dissipated by displacement of the media. Oils have an inherent high viscosity and low dielectric constant inhibiting these two conditions. In addition, drug particles in oils potentially convert the fluid to a colloidal gel. Smaller sized particles in the oily system lead to higher viscosity and varying the bead size results in different milling efficiency. Thus, the size reduction influence upon milling process by using 0.1 mm and 0.25 mm beads was compared. Below 0.5 h milling time, drug particles had larger size reduction with 0.1 mm beads compared to 0.25 mm beads. After 2 h milling, hydrocortisone paraffin nanosuspensions had particle size around 200 nm with 0.25 mm, which was a larger size reduction compared to 0.1

mm beads (Figure 3.2). The viscosity of samples in the milling chamber increased with milling time. 0.1 mm beads had larger size reduction inducing higher viscosity increase before 0.5 h compared to 0.25 mm beads. Thus, the higher viscosity of 0.1 mm beads samples had more inhibition force of the drug particles to move into the active milling zone after 0.5 h when compared to 0.25 mm beads. Additionally, the higher weight of a single 0.25 mm bead promoted a faster movement through the viscous liquid due to a higher centrifugal force of the bead during milling. Thus, 0.1 mm beads had less size reduction compared to 0.25 mm beads.

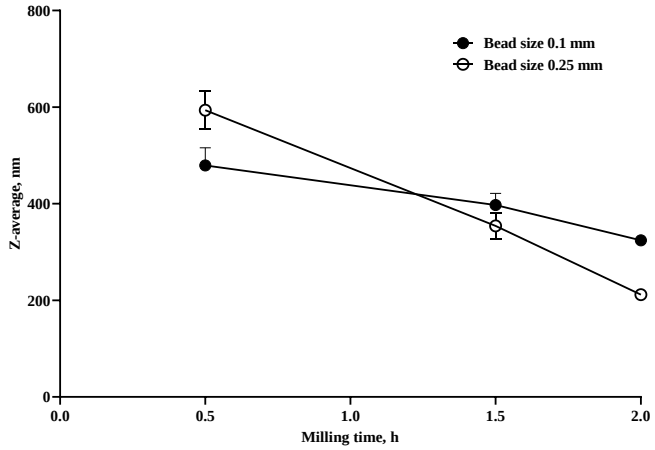

Figure 3.2 Effect of beads size and milling time on the particle size of 1 % hydrocortisone paraffin nanosuspensions

After optimizing the milling process with different bead sizes, hydrocortisone and dexamethasone stabilizer-free oily nanosuspensions were prepared with 0.25 mm beads (Table 3.1). All formulations displayed a Z-average below 400 nm and a narrow particle size distribution (< 0.4). Paraffin nanosuspensions had higher milling efficiency than MCT. The lower dielectric constant of paraffin (1.6) shielded the particles more than MCT (2.5). Furthermore, hydrocortisone and dexamethasone have a solubility of 0.54 mg/ml and 0.33 mg/ml in MCT and are insoluble in paraffin. Thus, a greater number of

particles in paraffin nanosuspensions leads to a higher collision force. Oswald ripening is also reduced for drugs with lower solubility. The higher the drug loading, the better the milling efficiency. The force to break the large particles into small particles not only depends on the external force (beads and vessel wall) but also is influenced by the internal force (particles) [109]. Thus, the higher drug loading induced higher mechanical breaking forces by particle-particle interactions.

Table 3.1 Effect of drug loading and oil types on the particle size after milling drugs into oily nanosuspensions

Drug loading, %	Hydrocortisone		Dexamethasone	
	MCT	Paraffin	MCT	Paraffin
	Z-average, nm ± SD	Z-average, nm ± SD	Z-average, nm ± SD	Z-average, nm ± SD
1 %	370.2 (24.0)	278.6 (3.1)	456.3 (5.1)	503.4 (9.7)
3 %	345.5 (3.9)	247.4 (7.6)	396.4 (7.5)	332.6 (8.0)
5 %	316.2 (2.3)	211.7 (9.3)	385.4 (5.7)	326.4 (3.3)

During and after milling, colloidal drug particles with strong, short-ranged attractions potentially form a colloidal gel [97, 110]. The investigated oily suspensions transferred into a colloidal gel after milling. According to the Brownian motion, high viscosity inhibits movement of particles and slows down the surface nucleation process of Ostwald ripening. Thus, viscosity changes upon milling were systematically investigated. With size reduction, the viscosity increased to a different extent according to drug loading and oil types. The higher the drug loading, the more increased viscosity was found in both oils. However, paraffin samples had a more increased viscosity compared to MCT (Figure 3.3). Hydrocortisone was poorly soluble in MCT and insoluble in paraffin. There were more undissolved particles in paraffin resulting in more short-ranged attractions between these particles, which induced a higher increase in viscosity. Overall, the higher viscosities are one reason enabling the preparation of

stabilizer-free oily nanosuspensions. However, the gel formation after milling has also drawbacks. The processability is reduced, e.g., pumping ability through pipes between different vessels. Furthermore, the application as liquid dosage form is limited, however, the direct use as a semi-solid dosage form is advantageous.

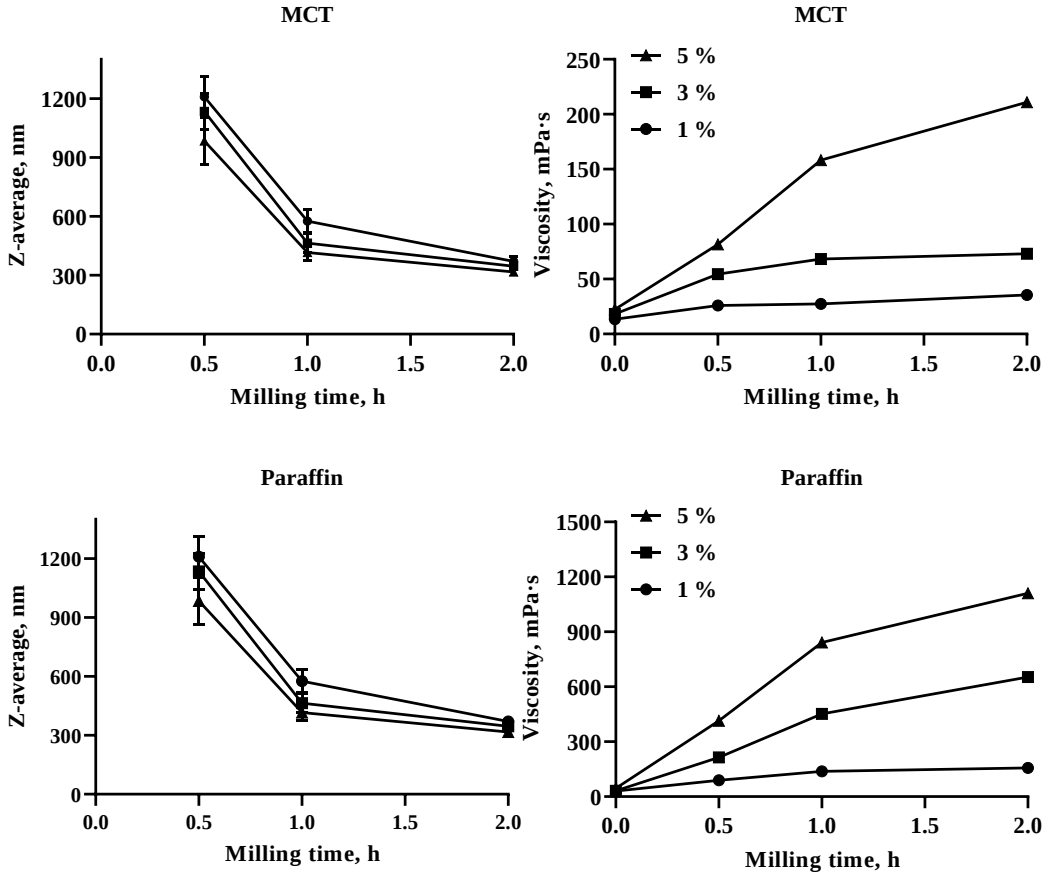

Figure 3.3 Effect of particle size reduction and its relevant viscosity changes of hydrocortisone upon milling in MCT and paraffin with different drug loading

Furthermore, the wetting ability of MCT and paraffin to hydrocortisone and dexamethasone was evaluated by contact angle measurements. A more decreased contact angle of the medium to the drug represents a higher potential to reduce the

surface energy upon milling. Generally, the adequate contact angle for milling nanocrystals is below 45° [111]. The contact angle of paraffin and MCT to these two drugs were all ≤ 20° (Table 3.2). Oils had a promising wettability to the drugs. Therefore, another reason for the success of milling hydrocortisone and dexamethasone into oily nanosuspensions without stabilizer besides the increased viscosity is the high wettability of oils.

Table 3.2 Drug contact angle of different media (mean values ± SD, n = 3)

Drugs	Contact angle, ° ± SD	
	MCT	Paraffin
Hydrocortisone	11 ± 2	15 ± 1
Dexamethasone	11 ± 1	20 ± 1

3.1.3 Solid-state of drugs in oily nanosuspensions

The solid-state of nanocrystals is decisive for their physical stability. Wet bead milling will produce primarily nanocrystals in crystalline state, as shear forces breakaway parts of the larger particles at crystal lattice imperfections [4]. However, there is no information about the solid-state of drugs in oily nanosuspensions. Thus, the following investigation will focus on solid-state changes before and after milling hydrocortisone and dexamethasone into oily nanosuspensions by differential scanning calorimetry. To have a systematic evaluation of the solid-state of oily nanosuspensions, the Tm changes among unprocessed drug powder from oil dispersions and nanosized drugs were investigated.

The drug powders as received had a Tm at 226 °C (HC) and 273 °C (DEX), respectively, in accordance with literature values [112, 113]. Milling hydrocortisone with 1 %, 3 % and 5 % drug loading into paraffin nanosuspensions displayed Tm of 205 °C, 210 °C and 213 °C, respectively. With MCT nanosuspensions, the Tm of 1 %, 3 % and 5 % hydrocortisone were depressed from 225 °C (microcrystals from oil dispersions) to

198 °C, 206 °C and 210 °C (Figure 3.4a). For dexamethasone, similar phenomena were found before and after milling into oily nanosuspensions (Figure 3.4b). The Tm of dexamethasone microcrystal in paraffin and MCT were 262 °C and 252 °C, respectively. Tm was depressed after milling dexamethasone with 1 %, 3 % and 5 % drug loading when compared to microcrystals. The Gibbs–Thomson equation was used to explain the decreased Tm [114], where decreased Tm might be due to size reduction and decreased crystallinity. Nevertheless, all drugs were still mainly in a crystalline state after milling.

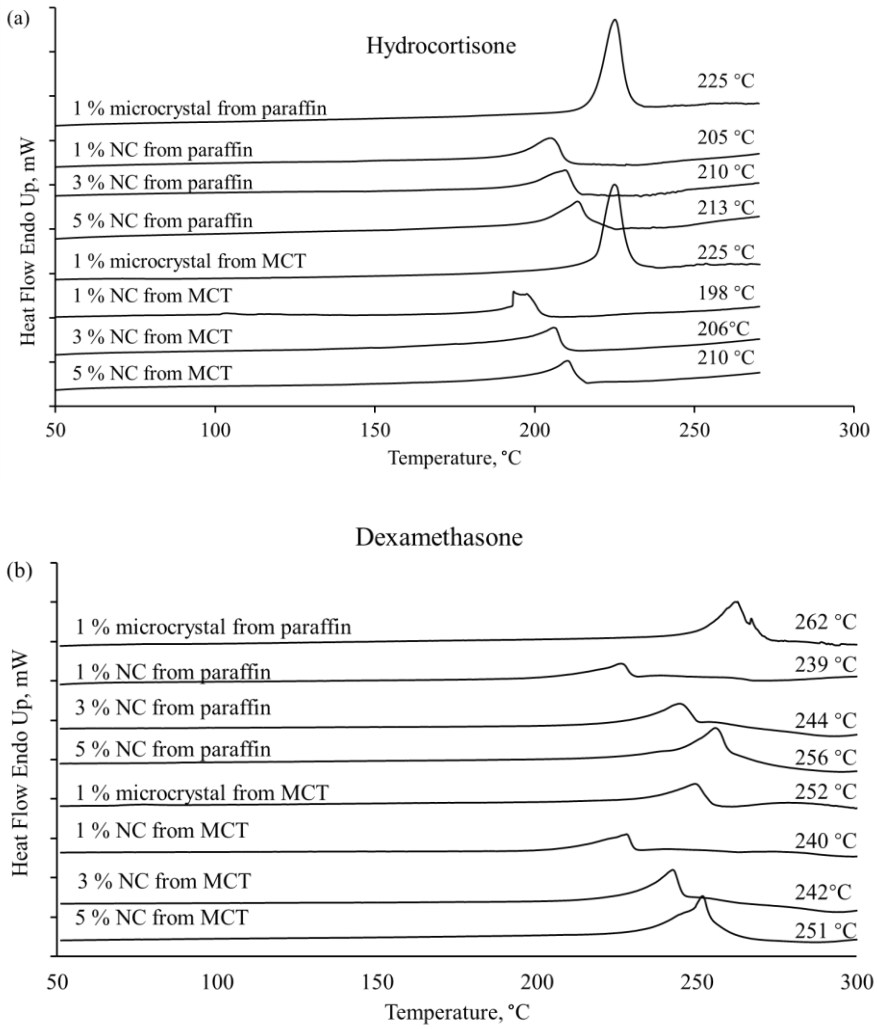

Figure 3.4 Thermogram of hydrocortisone (a) dexamethasone (b) before and after milling with different drug loading

3.1.4 *In vitro* release

Nanosizing of oily nanosuspensions significantly increased the viscosity compared to microsuspensions. Usually, nanosized particles have a faster dissolution rate than microparticles according to the Noyes-Whitney theory [115]. However, an increased viscosity slows down the dissolution of nanocrystals in oils, additionally, leading to a slow sedimentation rate of particles to the oil/water interface according to the Stokes law [116]. The release rate of oily nanosuspensions is influenced by the competition between the faster dissolution of particles with a higher surface area and the slower release due to the increased viscosity. Thus, the influence of size potentially results in an opposite phenomenon for *in vitro* release as expected, i.e. smaller particles result in a slower release since smaller particles resulted in a higher viscosity of the oily nanosuspensions. Therefore, the influence of particle size, oil type and drug loading on release was investigated.

To compare the particle size influence on the *in vitro* release, 1 % hydrocortisone/dexamethasone MCT nano- and microsuspensions were evaluated (Figure 3.5a, b). Drug nanosuspensions had a strongly slower release than their relevant microsuspensions due to the greatly increased viscosity after nanosizing. Highly viscous oily nanosuspensions had less sedimentation of drug particles and slower drug dissolution, compared to oily microsuspensions. Furthermore, hydrocortisone had a faster release than dexamethasone in all samples. Hydrocortisone had a lower MCT/water partition coefficient (0.78) than dexamethasone (2.34). The higher the partition coefficient, the more hydrophobic the drugs. Thus, dexamethasone had a slower release than hydrocortisone.

For the influence of oil types, 1 % hydrocortisone/dexamethasone oily nanosuspensions were evaluated (Figure 3.5c, d). Drug MCT nanosuspensions had complete release in 2 days. However, the paraffin nanosuspensions had strongly longer release compared to MCT. Solubility and viscosity differences are considered to explain this phenomenon.

Drugs were insoluble in paraffin. However, dexamethasone has a solubility of 0.33 mg/ml and hydrocortisone has a solubility of 0.54 mg/ml in MCT. Due to the difference in solubility, the drug release mechanism in these two oils is different. Particles experience a three-step release mechanism in paraffin: (i) particles are transported to the interface of paraffin/water; (ii) pass through the interface; (iii) dissolve in the aqueous phase. In contrast to paraffin, particle dissolution in MCT and MCT/water interface are combined. Thus, the different release mechanisms and higher viscosity of paraffin nanosuspensions resulted in a slower release.

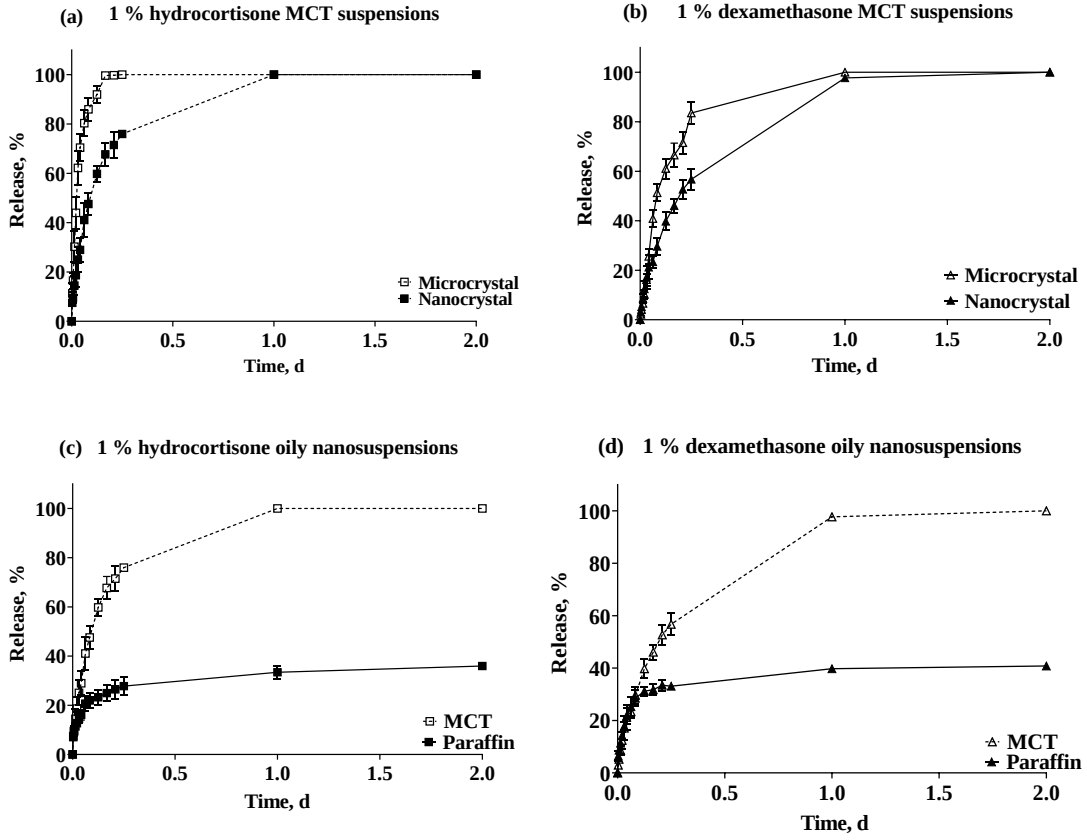

Figure 3.5 Influence of particle size and oil types on the *in vitro* release of oily suspensions under sink condition (mean values ± SD, n = 3)

To systematically differentiate the drug loading influence on the release, oily micro- and nanosuspensions were compared. For microsuspension, 3 % drug loading samples

had faster release than 1 % in both oily systems (Figure 3.6a, b). 3 % drug loading samples had more drug particles accumulated at the oil/water interface. However for nanosuspensions, the drug loading influence was varied according to the oil types (Figure 3.6c, d). 1 % MCT nanosuspensions had a faster release than 3 %, which was the opposite phenomenon of microsuspensions. The higher the drug loading the longer the complete release time, which is due to the increased viscosity after milling. 3 % nanosuspensions had higher increased viscosity than 1 %, leading to slower particle sedimentation. Surprisingly, 3 % paraffin nanosuspension, which had higher increased viscosity compared to 1 %, had faster release in 2 days. One reason was that more particles accumulated at the interface of paraffin/water, leading to a faster release at the beginning. Notable, drugs were insoluble in paraffin, which resulted in a higher release in the aqueous phase compared to MCT. Surface accumulated particles together with the drugs partition coefficient led to the faster release of 3 % paraffin nanosuspensions.

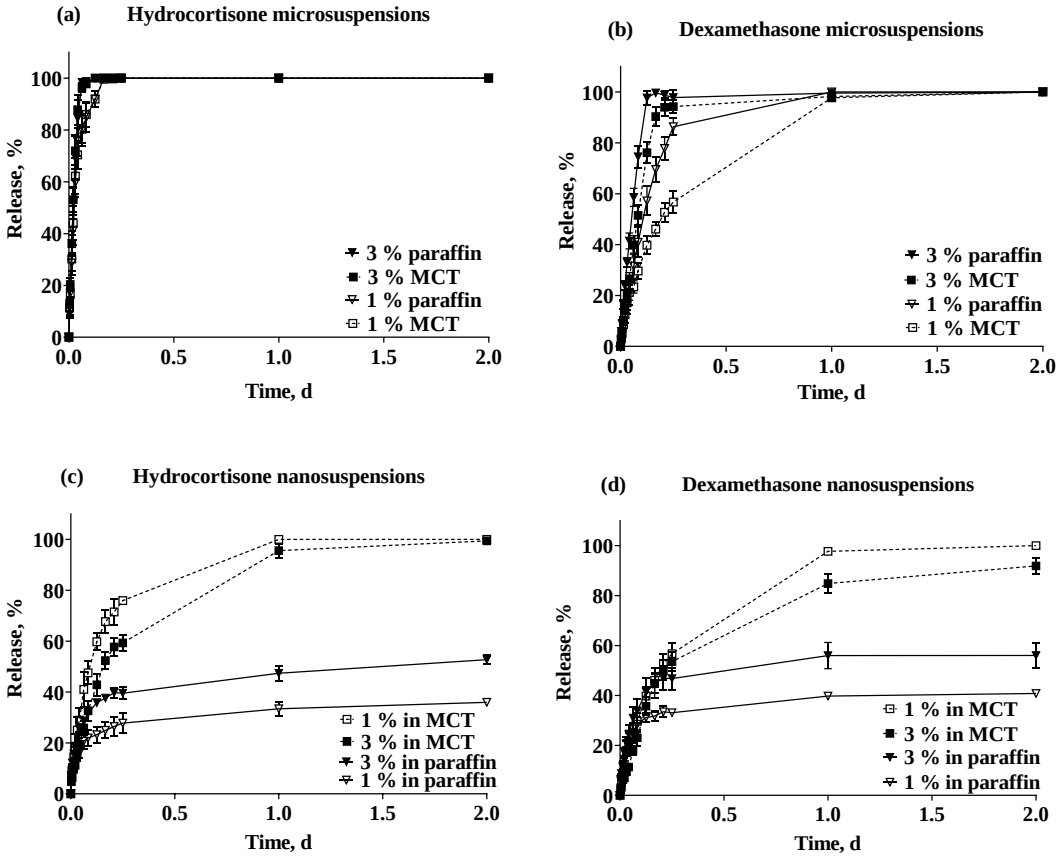

Figure 3.6 Influence of drug loading on the *in vitro* release of oily suspensions under sink condition (mean values ± SD, n = 3)

3.1.5 Stability tests

The stabilizer-free oily nanosuspensions were investigated by freeze-thaw test and they were still in the nm range with particle sizes below 800 nm and PDI below 0.5 after 10 cycles (Figure 3.7). Oily nanosuspensions were highly viscous colloidal gel systems with strong, short-range attractions. According to the Brownian motion, high viscosity inhibits movement of particles and slows down the surface nucleation process of Ostwald ripening. The crystal growth kinetics in nanosuspension is governed by two basic processes: (i) diffusion of the solute molecules; (ii) attachment or detachment (crystal growth and dissolution) to and from the particle surface [117]. The two crucial processes for the crystal growth of nanosuspension were limited by increased viscosity.

Thus, the paraffin nanosuspensions had higher stability than MCT nanosuspensions. The significantly higher viscosity of paraffin samples limited the movement and dissolution of particles and slowed down the diffusion rate of solute to the surface of nanocrystals. Additionally, stabilizer precipitation was avoided, since no stabilizer was required for these oily nanosuspensions.

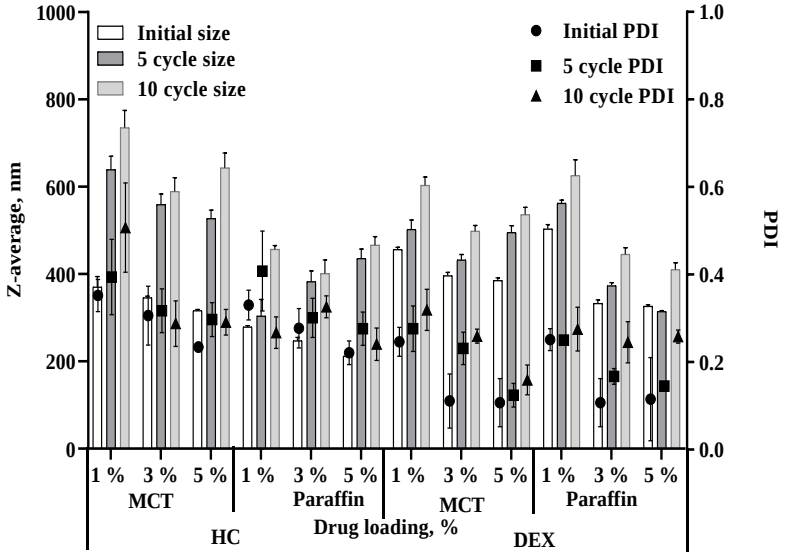

Figure 3.7 Freeze-thaw test of oily nanosuspensions, particle size and distribution changes of hydrocortisone/dexamethasone oily nanosuspensions after 10 cycles freeze-thaw test (mean values ± SD, n = 3)

For nanosuspension development, product sterilization represents a major challenge. Usually, sterile filtration and terminal steam sterilization are used for nanoformulations [118]. Sterile filtration is difficult due to the high solid content and high viscosity resulting in poor filtration rate and clogging. Terminal steam sterilization is the most desirable method regarding sterility assurance but potentially leads to significant increases in particle size. Usually, the sterilization of oily suspensions is performed by dry heat sterilization (160 °C for 1 h). Crystal growth on heating and cooling referred

to Ostwald ripening, is known to be crucially dependent on the solubility-temperature profile of the drug. Thus, the stability of oily nanosuspensions under dry heat sterilization was investigated.

The size of 1 %, 3 % and 5 % hydrocortisone oily nanosuspensions after the dry heat sterilization was imaged by polarized microscopy. No significant crystal growth to the μm size range was observed in hydrocortisone paraffin nanosuspensions. However, the particles of hydrocortisone MCT nanosuspension changed with drug loading (Figure 3.8). The higher the drug loading, the smaller the crystal growth. Hydrocortisone was insoluble in paraffin and its nanocrystal had a melting point around 200 ℃, no dissolved/melted hydrocortisone at 160 ℃ could recrystallize on the surface of nanocrystals. Thus, there was no crystal growth. Hydrocortisone was soluble in MCT and its solubility increased with increasing temperature. 1 % hydrocortisone nanocrystals completely dissolved in MCT at 160 ℃, 3 % and 5 % hydrocortisone MCT nanosuspensions were still partially undissolved and particular system. Usually, nucleation is an important process in crystal growth [119], and the amount of particles in the system also affects the crystal growth phenomena. Particles in 1 % hydrocortisone MCT nanosuspensions precipitated from solutions. The dissolved hydrocortisone in 3 % and 5 % nanosuspensions did not only grow from the nucleus but also attached to the surface of the undissolved nanocrystals resulting in smaller particles compared to particles which grew from the nucleus. Thus, with increased drug loading, the smaller crystal growth of hydrocortisone MCT nanosuspension after dry heat sterilization was observed.

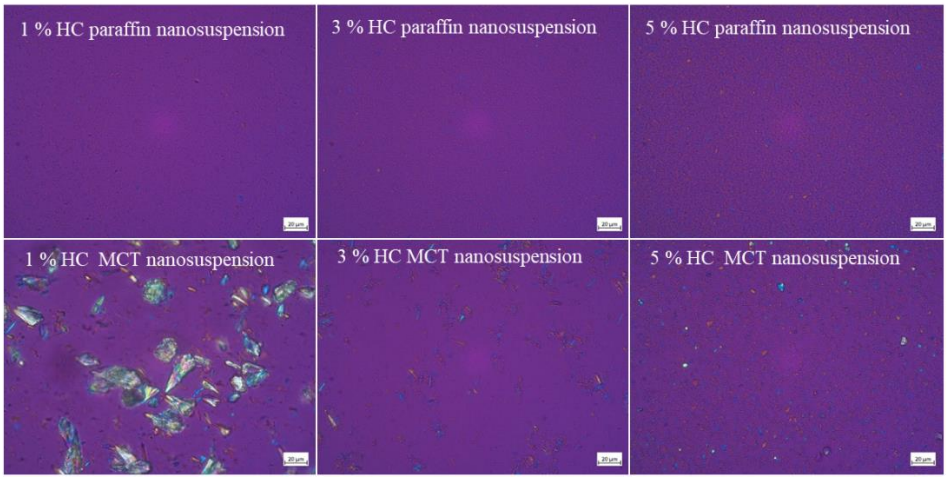

Figure 3.8 Microscopy of hydrocortisone nanocrystals in oily nanosuspensions after dry heat sterilization test (scale bar 20 µm)

The stability of oily nanosuspensions with and without stabilizer was evaluated at room temperature. The Z-average of all hydrocortisone oily nanosuspensions was still in the nm range after 3-month storage. Span 80 used as stabilizer had no significant influence on the stability results. However, with higher drug loading, a higher stability was observed with all oily nanosuspensions (Figure 3.9). The pronounced higher viscosity with increased drug loading after milling improved the stability of stabilizer-free oily nanosuspensions.

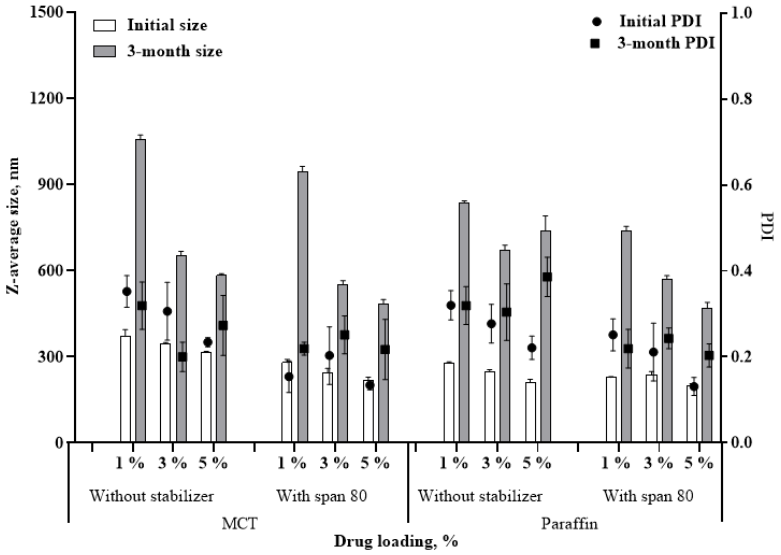

Figure 3.9 Particle size and distribution of hydrocortisone oily nanosuspensions at room temperature (mean values ± SD, n = 3)

3.1.6 Conclusion

The time and cost-consuming screening for thickeners and stabilizing agents for oily drug suspensions was eliminated by the successful preparation of oily nanosuspensions without stabilizers. In addition, the drugs were directly milled in the oils avoiding additional preparation steps for the nanocrystals such as freeze drying. After the direct milling, the drugs were still in a crystalline state. Oily nanosuspensions had a prolonged release when compared to oily microsuspensions. The drug release could be modified by varying the drug loading and oil type. Oily nanosuspensions displayed a great freeze-thaw cycling physical stability because of a greatly increased oil suspension viscosity due to the increased drug surface area after drug milling an inherently low dielectric constant. Additionally, a good stability upon dry heat sterilization and a favourable 3 months storage stability were achieved. The addition of the stabilizer Span 80 resulted in no improved storage stability for oily nanosuspensions. Thus, an alternative one step preparation process without the use of stabilizers for oily nanosuspension formulations with prolonged release were successfully introduced.

3.2 Incorporation of drug nanocrystals into various semisolid vehicles and their release

3.2.1 Background

Topical drug delivery provides advantages over the systemic route to reduce toxicity due to a minimum or exposure to non-target organs [120]. There are various drawbacks associated with conventional topical delivery systems including high doses, poor permeation, frequent application, local irritation or allergy and stickiness in the case of ointments. Commonly, drugs, which have a low molecular weight (< 500 Da) and balanced lipophilicity (log P = 1 ~ 3) are easier to penetrate into skin [121]. However, BCS II drugs, which have poor solubility, need other strategies to improve the skin penetration. Nanonization of drugs is a technique to overcome the bioavailability problems of poorly soluble drugs due to the enlarged surface area, leading to a faster dissolution rate and higher adhesion [4, 122]. Therefore, more attention is given to nanocrystal-loaded semisolid formulations.

Commonly used semisolid vehicles are gels and creams. A gel is a polymer network swollen in a liquid medium, which could be water, oils and organic solvents. Creams are composed of a mixture of oil and water combined with emulsifiers. Drugs from gels tend to have better penetration into the skin due to their higher water content, while creams may be better suited for conditions that require occlusion or hydration of the skin. Nanocrystal-based cosmetic products were introduced in 2007 [123, 124]. The type of gel vehicles can have a tremendous influence on dermal penetration efficacy [8, 125-127]. Dexamethasone and acyclovir nanocrystal-loaded creams showed a significantly increased drug amount that penetrated into skin compared to the base cream formulations [128, 129]. The viscosity of the vehicle, as well as the lipophilicity, are crucial parameters to affect the penetration efficacy of nanocrystals [130-132].

Hydrogels and oleogels are commonly used for topical delivery. Aqueous

nanosuspensions with stabilizers are used for nanocrystal-loaded hydrogels. Stabilizers, which stabilize the aqueous nanosuspensions by electrostatic and polymeric stabilization mechanisms, also have the potential to interact with the gelators resulting in changes in particle size stability and performance of hydrogels. Currently, there is no information about nanocrystal-loaded oleogels. In our previous findings, stabilizer-free oily nanosuspensions were successfully prepared [133]. Therefore, nanocrystal-loaded oleogels can be achieved. Different types of gel have different preparation conditions, which will affect the performance of nanocrystals. Hydroxypropyl methylcellulose (HPMC) gels are prepared at 80 ℃ [134]; Carbopol requires a neutralization process; SiO_2 oleogel is prepared under room temperature [135] and the preparation of ethyl cellulose miglyol oleogel should be done under 160 ℃ to melt the ethyl cellulose [136]. This leads to differences in the number of nanocrystals and physical state of drug in the formulation, inducing the variety of particle size stability, viscosity and release performance. For nanocrystal-loaded creams, there is no information about the distribution of nanocrystals after loading into creams. Nanocrystals, which are prepared from oils or water have different surface hydrophobicity. For the aqueous nanosuspensions, the hydrophobic head of stabilizers attaches to the hydrophobic surface of nanocrystals and the hydrophilic branches stretch into the aqueous phase, resulting in hydrophilic nanoparticles. In contrast, stabilizer-free oily nanosuspensions have hydrophobic nanoparticles. Theoretically, nanocrystals prepared in aqueous phase stay in the water phase of cream and nanocrystals prepared in oils distribute in the oil droplets. However, the high shear force of homogenization process and high temperature to prepare the cream might vary the surface hydrophobicity of nanocrystals, resulting in different localizations. The distribution of nanocrystals in creams might have a significant influence on stability, viscosity and relevant *in vitro* release. However, there is little information available for formulators on the key factors in how to select the suitable vehicle to load the nanocrystals.

In the present study, dexamethasone (DEX) and hydrocortisone (HC) were selected as

model drugs due to their topical relevance. Aqueous and oily nanosuspensions were prepared by dual centrifugation wet bead milling [77]. Nanocrystal-loaded hydrogels, oleogels and O/W creams were prepared to investigate of particle size stability, viscosity and *in vitro* release. The influence of different nanoparticle stabilizers and the distribution of nanocrystals after loading into cream were simultaneously evaluated.

For topical delivery, nanosuspensions need to be incorporated into semisolids after preparation. Nanocrystals can be produced either from the aqueous or oily wet bead milling process. Nanocrystal-loaded hydrogels and oleogels can be achieved by adding these nanocrystals. Additionally, the nanocrystals can be incorporated into the different phases of O/W creams. The study was divided into two parts according to the type of semisolids. For the nanocrystal-loaded gels, the influence of stabilizers on the particle size of nanocrystals and the performance of gels were investigated. For the nanocrystal-loaded O/W creams, the distribution of nanocrystals and relevant *in vitro* release were evaluated.

3.2.2 Nanocrystal-loaded gels

3.2.2.1 The particle size of nanocrystals after incorporation

HPMC E5-stabilized and SDS-stabilized nanosuspensions were used to prepare 1 % nanocrystal-loaded 3 % HPMC E4M and 0.25 % Carbopol gels. The particle size of both drugs had a slight increase without exceeding the nm range (Figure 3.10). HPMC and Carbopol are high molecular weight gelling agents. HPMC is first dispersed and stirred in water with heating to 80 °C to disperse the HPMC and promote the gelation process upon cooling. Carbopol powders are poured into water, stirring until the powders are fully dispersed. The solution is then neutralized to pH 7.4. At this pH, the carboxyl groups on the Carbopol polymer chains become ionized and repel each other, leading the polymer chains to extend and form a loose network. The low molar mass

stabilizers e.g., SDS, can significantly change the interaction of these polymers, leading to the breaking or rebuilding of the interpolymer network, which affects the viscosity of lateral gels [137]. Even though nanocrystals were incorporated into the pre-prepared HPMC and Carbopol gels, gelators were still needed to complete the hydration processes to achieve homogenous nanocrystal-loaded gels. Therefore, the incorporation influenced the particle size of nanosuspensions.

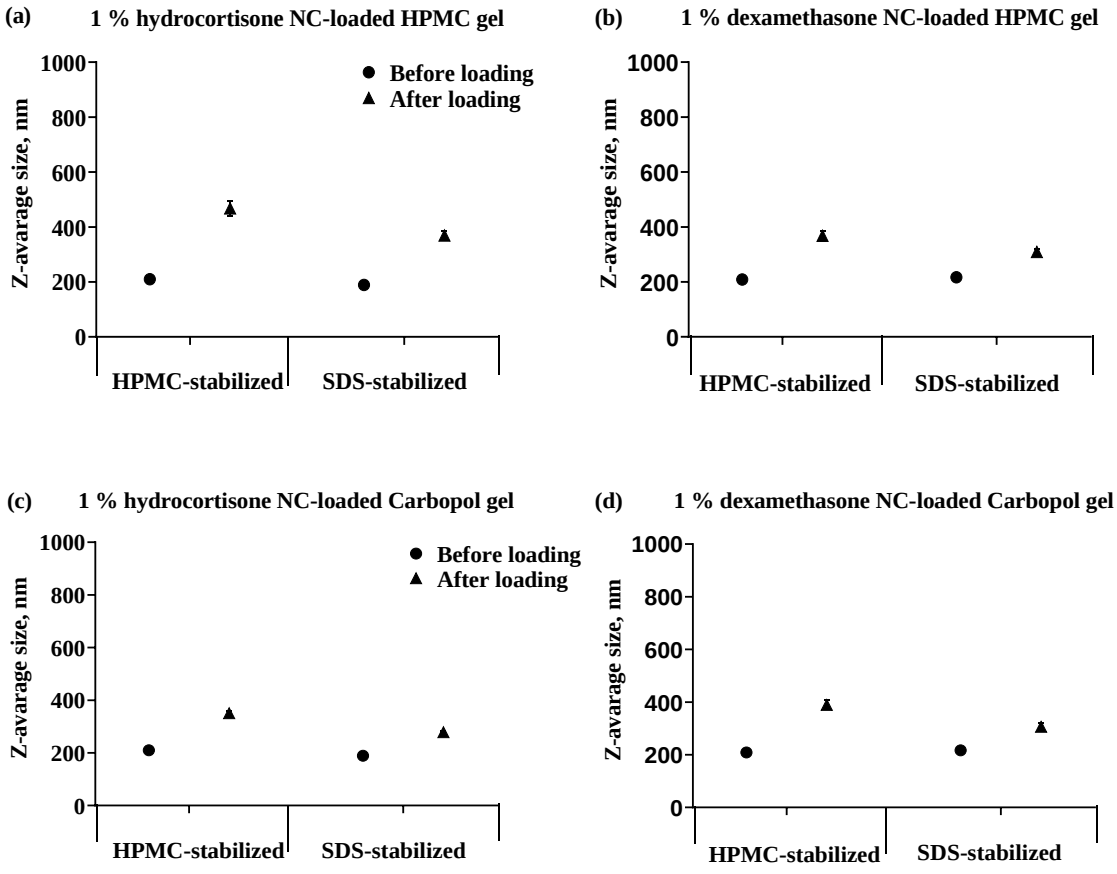

Figure 3.10 Effect of stabilizers on the particle size of nanocrystals after loading into 3 % HPMC and 0.25 % Carbopol hydrogels (± SD, n = 3)

1 % hydrocortisone and dexamethasone stabilizer-free oily nanosuspensions were loaded into oleogels. No significant particle growth to the μm-size range was observed (Figure 3.11). For SiO_2 oleogels, fumed silica nanoparticles are typically dispersed into

the oils with stirring. The silica nanoparticles become wetted by the oils, forming a three-dimensional network. The loaded nanocrystals could potentially promote the gelling process. Hydrocortisone and dexamethasone nanocrystals were either part of or caught by the three-dimensional network. Therefore, nanocrystals had no significant crystal growth after incorporating into SiO_2 oleogels. Ethyl cellulose oleogels also form a three-dimensional network of ethyl cellulose polymer chains within the Miglyol. However, ethyl cellulose had to be dissolved in the Miglyol, which was heated at 160 ℃. Oily nanosuspensions were added into the pre-prepared oleogel at 90 ℃. The stable particle size of hydrocortisone and dexamethasone nanocrystals at 90 ℃ might have two reasons: (i) nanocrystals were either the composition of or caught by the three-dimensional network; (ii) the dissolved nanocrystals did not only grow from the nucleus but also attach to the surface of the undissolved nanocrystals resulting in smaller particles compared to particles which grew from the nucleus. Thus, there was no significant particle growth of nanocrystals after incorporation into oleogels.

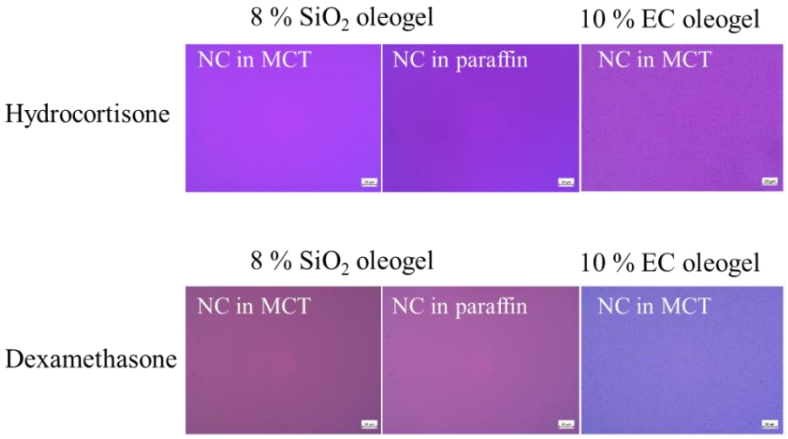

Figure 3.11 Particle size observations of nanocrystal after loading into oleogels, characterized by polarized microscopy (scale bar 20 μm)

3.2.2.2 Viscosity changes of gels after the incorporation of nanocrystals

Nanosuspension is a colloidal system which has a gel-like consistency. The high surface area to volume ratio of nanocrystals and their ability to form strong interparticle bonds

result in increased viscosity. Due to this inherent property of nanosuspensions, the three-dimensional gelling network might be influenced by the nanocrystals. Additionally, as mentioned in the previous paragraph, the stabilizer could disturb the gelling process. Therefore, the viscosity changes of nanocrystal-loaded gels should be systematically investigated. The viscosity of 3 % HPMC gel and 0.25 % Carbopol gel had a viscosity of around 20 Pa·s and 9 Pa·s, respectively. The viscosity of nanocrystal-loaded hydrogels varied to different extent after loading the nanocrystals according to the stabilizers (Figure 3.12a, b). For nanocrystal-loaded HPMC gels, the increased viscosities were evaluated compared to the blank HPMC gel. The nanosized particles can strengthen the gel network by the interparticle bonds or be trapped in the spaces of the network. However, SDS-stabilized nanocrystals had a less increased viscosity compared to HPMC. Usually, the dissolved SDS in aqueous phase is forming micelles. The hydrophilic sulfate head groups face aqueous phase and the hydrophobic alkyl tail groups are in the core of the micelle. The hydrophilic sulfate head might have interacted with the hydroxypropyl (HPMC), which might influence the process of gelling to form a gel network. For nanocrystal-loaded Carbopol gel, HPMC-stabilized nanocrystals also increased the viscosity of Carbopol gels. Surprisingly, SDS-stabilized nanocrystals decreased the viscosity compared to the blank Carbopol gel. Carbopol gelling process involves the neutralization of carboxylic acid groups of this polymer. SDS is an anionic surfactant which can disturb the neutralization process resulting in a decreased viscosity. The viscosity of all nanocrystal-loaded oleogels had an increased viscosity compared to their blank oleogels (Figure 3.12c, d). For SiO_2 oleogels, different increases in viscosities were observed depending on the type of oils. Paraffin nanosuspension-loaded SiO_2 oleogels had a higher increased viscosity compared to MCT. Hydrocortisone and dexamethasone were insoluble in paraffin, and a greater number of nanocrystals in the oleogels contributed to the relevant higher viscosity. For ethyl cellulose oleogels, a higher increased viscosity was observed compared to SiO_2 oleogels. When ethyl cellulose was added to the MCT, it formed a non-covalent network of

entangled polymer chains, trapping the oil and nanocrystals in a three-dimensional structure, resulting in a more rigid formation. Due to the stronger interaction of polymer and nanocrystals, ethyl cellulose oleogels had a higher viscosity compared to SiO_2 oleogels.

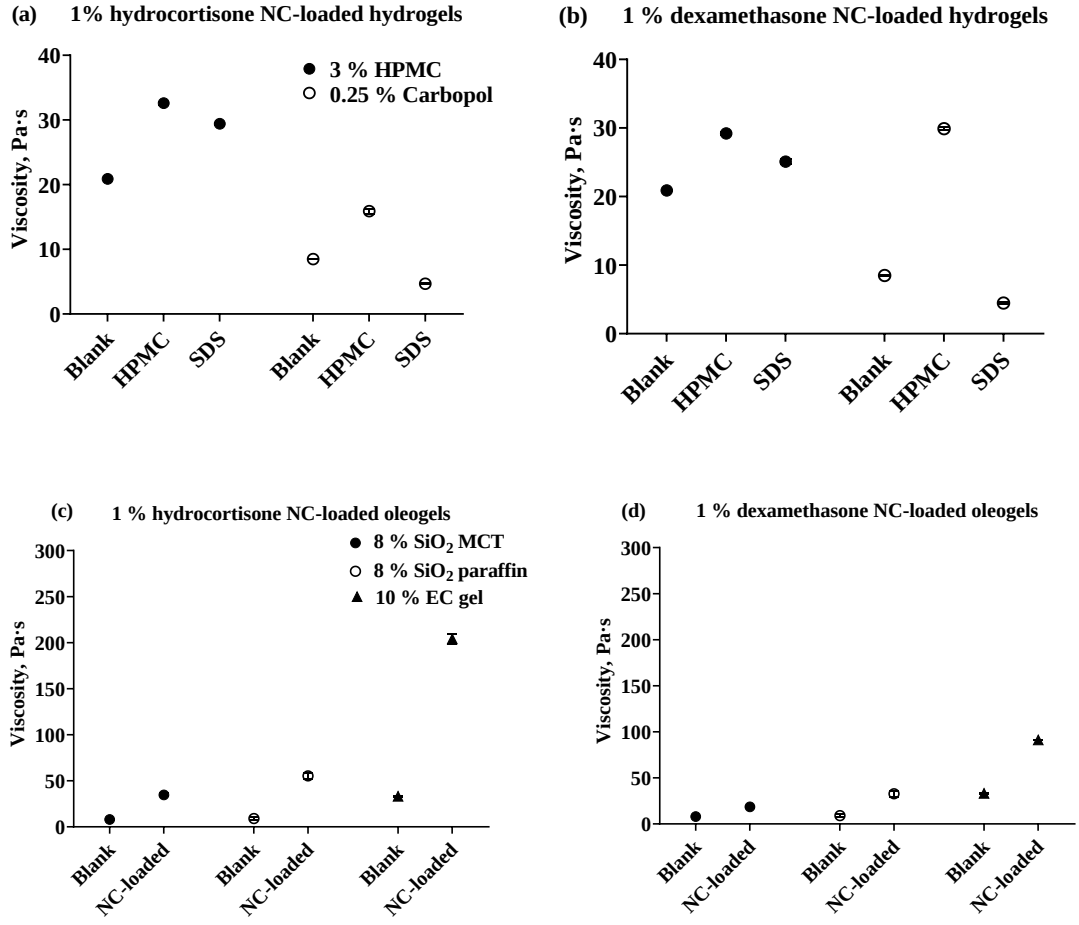

Figure 3.12 Influence of nanocrystals on the viscosity of gels (± SD, n = 3)

3.2.2.3 *in vitro* release of nanocrystal-loaded hydrogels

1 % hydrocortisone and dexamethasone nanocrystal-loaded gels were investigated to clarify the influence of stabilizer and gelling agents on the *in vitro* release. For nanocrystal-loaded hydrogels, hydrocortisone and dexamethasone had around 400 µg/cm^2 and 150 µg/cm^2 cumulative amount of release in 7 h, respectively. HPMC and

SDS as the stabilizer had no significant influence on the release rate. However, nanocrystal-loaded Carbopol gels had a faster release compared to HPMC gels (Figure 3.13a, b). This might be due to the lower viscosity of Carbopol gels compared to HPMC gels. For nanocrystal-loaded oleogels, hydrocortisone and dexamethasone had around 200 μg/cm^2 and 100 μg/cm^2 cumulative amount of release respectively. The type of oils in SiO_2 oleogel had no significant influence on the release. However, the gelling agents to form the oleogels had a significant influence on the release (Figure 3.13c, d). Nanocrystal-loaded ethyl cellulose oleogels had a faster release in the first 3 h compared to nanocrystal-loaded SiO_2 oleogels, especially for dexamethasone nanocrystal-loaded ethyl cellulose oleogels. Oily nanosuspensions were incorporated into ethyl cellulose oleogels at 80 °C, such high temperature inducing the increased solubility of drug in the oleogels. In contrast to ethyl cellulose oleogels, nanocrystals were loaded into SiO_2 oleogel at room temperature. The varied solubility in the preparation process induced the different concentrations of dissolved drugs in the gels, therefore, nanocrystal-loaded oleogel had a faster release.

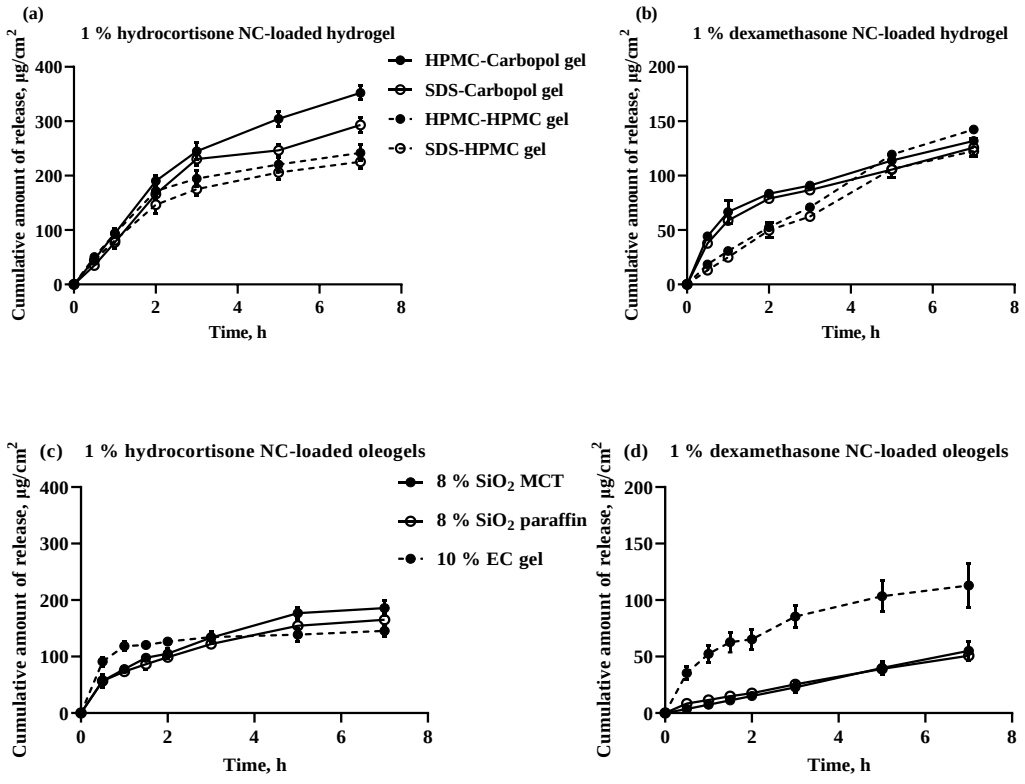

Figure 3.13 The cumulative amount release of 1 % hydrocortisone and dexamethasone nanocrystal-loaded gels (± SD, n = 3)

3.2.2.4 Storage stability of nanocrystal-loaded gels

3-month storage stability was investigated among the nanocrystal-loaded gels. All the 1 % nanocrystal-loaded gels had a stable particle size for 3 months at room temperature, except the 1 % hydrocortisone and dexamethasone nanocrystal-loaded ethyl cellulose oleogels (Figure 3.14). The nanocrystals in oleogels grew into μm crystals after 1 month, since the solubility of drugs changed by incorporating process at 90 ℃ inducing Ostwald ripening.

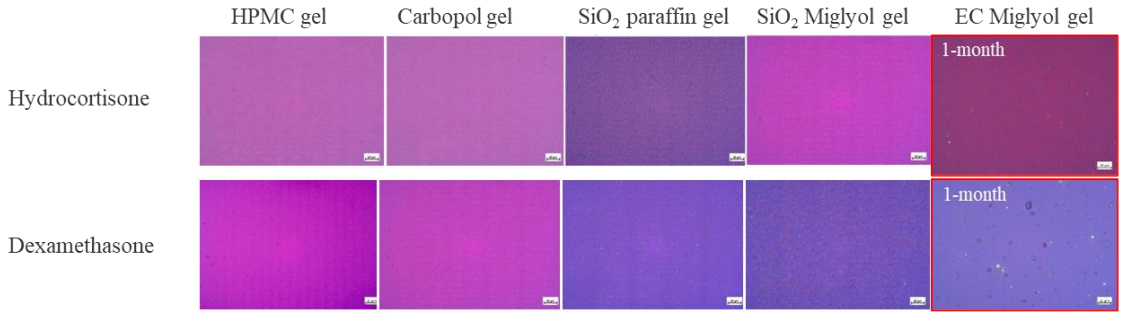

Figure 3.14 Observation of 3-month particle size stability of 1 % nanocrystal-loaded gels at room temperature by polarized microscopy (scale bar 20 μm)

3.2.3 Nanocrystal-loaded O/W creams

3.2.3.1 Preparation of nanocrystal-loaded O/W creams

Stable hydrocortisone and dexamethasone aqueous and oily nanosuspensions were achieved. The O/W creams were loaded with aqueous and oily nanosuspensions. Due to the complexity of creams, the observation of nanocrystals was difficult, therefore, microcrystal-loaded O/W creams were used as comparison. The size of the oil droplets in the nanocrystal-loaded cream was smaller than that in the blank cream. However,

microcrystal-loaded creams did not have this phenomenon. Hydrocortisone and dexamethasone nanocrystals had no significant particle size increase after loading into O/W cream (Figure 3.15). Nanoparticles can act as stabilizers to stabilize the oil droplets upon emulsification process due to their ability to adsorb at the oil-water interface and form a monolayer to inhibit the growth of oil droplets. The high surface area to volume ratio of nanoparticles allows them to interact more strongly with the interface than microparticles. Usually, polymeric and inorganic nanoparticles were used as stabilizers to stabilize the emulsion, which have a size below 50 nm [138]. Pharmaceutical nanocrystals have a Z-average size of around 200 nm. Currently, drug nanocrystals could be used as stabilizer to stabilize the Pickering emulsion [139]. Therefore, hydrocortisone and dexamethasone nanocrystals might act as an extra stabilizer to stabilize the oil droplet during the emulsification process.

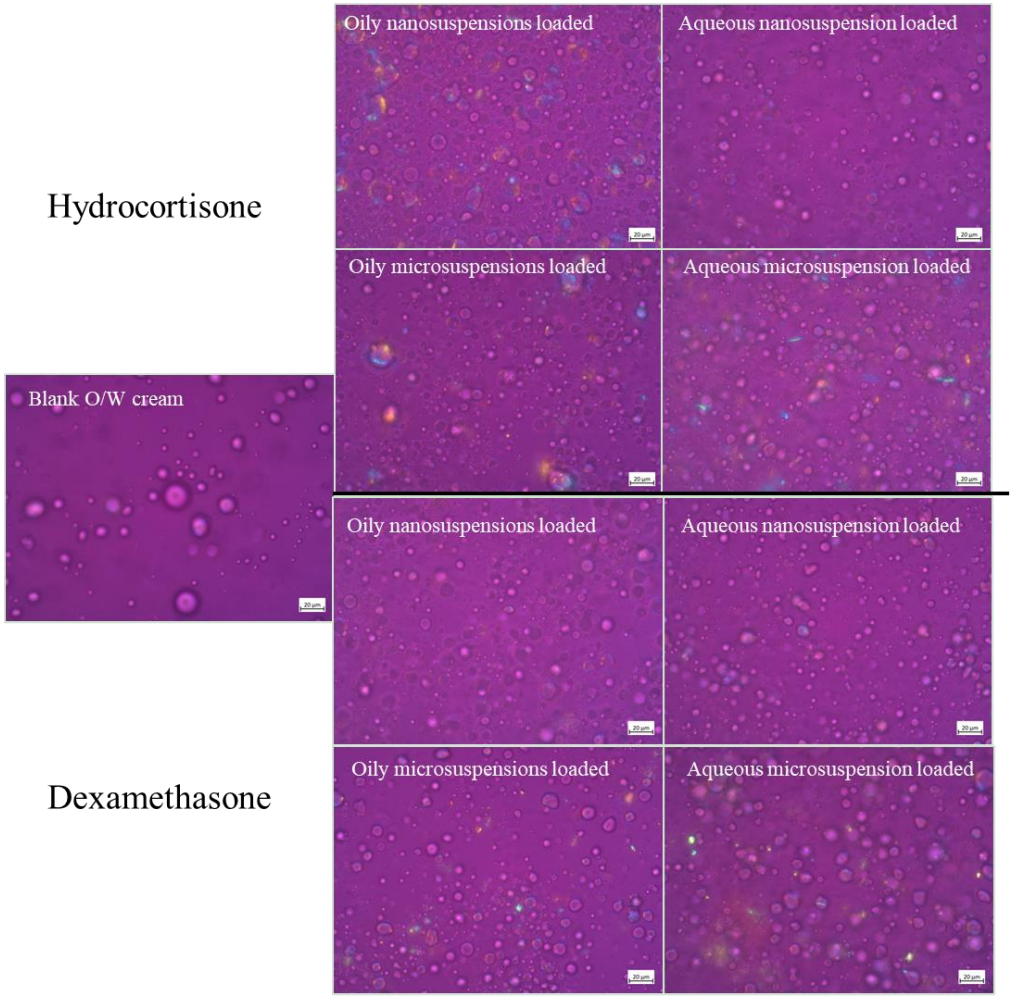

Figure 3.15 Observation of oil droplet size of 1 % nano-/microcrystal O/W cream by polarized microscopy sample diluted with water (scale bar 20 µm)

3.2.3.2 Viscosity changes of O/W cream after incorporating nanocrystals

Nanocrystal-loaded cream had a higher viscosity compared to the blank cream. Additionally, nanocrystals in the water phase of O/W cream had a higher increased viscosity compared to the nanocrystal in the oil phase (Figure 3.16). Usually, the viscosity of creams is influenced by the oil phase composition, the emulsifier and the size of the oil droplets after emulsification. For nanocrystal-loaded O/W creams, the nanocrystals can not only act as emulsifier to stabilize the oil droplet upon the

homogenization process which can reduce the size of droplet but also the enlarged surface area to volume ratio of nanocrystals enabling the interaction among particle and dispersion system. Notably, the oily nanosuspensions had a significantly increased viscosity [133]. All these factors increased the viscosity of nanocrystal-loaded O/W cream.

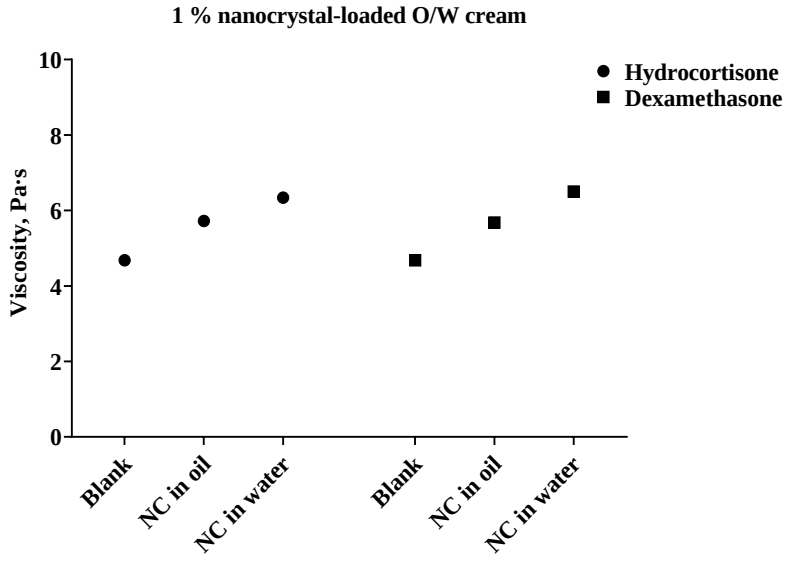

Figure 3.16 Influence of nanocrystals on the viscosity of O/W creams (± SD, n = 3)

3.2.3.3 The *in vitro* release of nanocrystal-loaded O/W creams

The *in vitro* release mechanisms of nanocrystal-loaded O/W cream can be complex according to the distribution of nanocrystals. Usually, diffusion is the primary mechanism of drug release from creams and involves the diffusion of the drug molecules through the cream matrix and into the release medium. There are two possible release mechanisms for the nanocrystals located in the oil droplet: (i) if the drug has a solubility in oil droplets, the drug particle experiences dissolution in the oil and sedimentation to the water/oil interface, then diffuses from the water phase of the

cream into the release media; (ii) if the drug is insoluble in the oil droplets, it only releases by the sedimentation to the water/oil interface, then diffusing in the water phase of the cream into the release media. For the nanocrystals distributed in the water phase of the cream, release occurs when the nanocrystals dissolve in the water phase and the drug molecules diffuse out into the release medium. Therefore, the distribution of nanocrystals in the O/W cream should be evaluated to clarify the release mechanism.

Pure drug nanocrystals have an inherent hydrophobic surface. Notably, the surface property of nanocrystals changed by producing in different media. In the oily nanosuspensions, no extra stabilizer was required to stabilize the nanocrystal. Therefore, nanocrystals kept the hydrophobic surface property. However, nanocrystals, which were produced in water, required the stabilizer to inhibit the aggregation. The hydrophobic head of stabilizers attached to the surface of nanocrystals, and their hydrophilic branch stretched into the aqueous phase. Therefore, these nanocrystals had a hydrophilic surface. The surface hydrophobicity might result in a different distribution of nanocrystals. Principally, the nanocrystals, which are produced from oil, distribute in the oil phase. However, the surface hydrophobicity might be changed due to the high shear force in the homogenization process. Cetyl stearyl alcohol, which is an emulsifier, blends oil and water-based ingredients in the cream. It helps to prevent the separation of cream. This property limits the possibility to observe the distribution of nanocrystals in O/W cream. Therefore, O/W emulsions without the cetyl stearyl alcohol were investigated, in which the distribution of nanocrystals can be observed after the phase separation. The blank emulsion had a transparent water phase and a milky oil phase (Figure 3.17a). Nanocrystals, which were produced from HPMC solutions, had sedimentation in the water phase of the O/W emulsion after 1 month. Interestingly, the nanocrystals, which were originally dispersed in the oil phase of O/W emulsion, also sedimented to the bottom of the tube (Figure 3.17b, c). This might be due to the glycerol and polysorbate 60 in the water phase of the emulsion. Upon emulsification, the nanocrystals from the paraffin had the potential to contact the water phase caused by

high shear force. The hydrophobicity of nanocrystals from paraffin was varied by the wettability of these two excipients in the water phase. Therefore, the distribution of nanocrystals in the O/W cream is related to the high shear force upon the homogenization process.

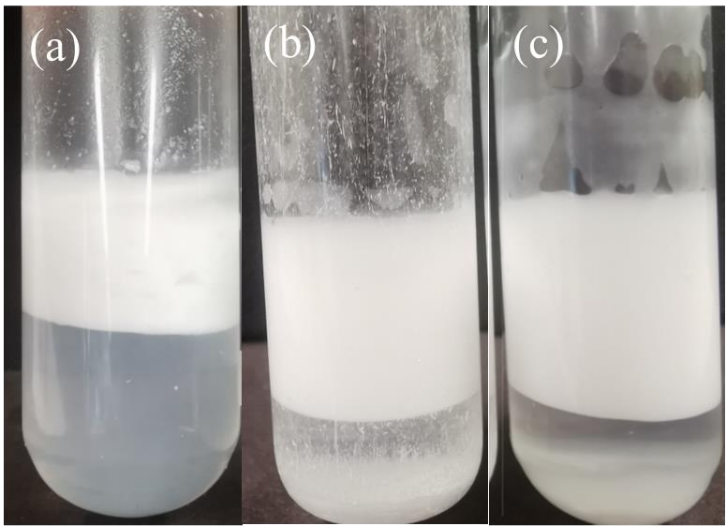

Figure 3.17 Phase separation and distribution of nanocrystals in O/W emulsion without cetyl stearyl alcohol (a) blank O/W emulsion; (b) nanocrystal in water O/W emulsion; (c) nanocrystal in oil O/W emulsion (picture taken 1 month after preparation)

1 % hydrocortisone/dexamethasone nanocrystal-loaded O/W creams were investigated on the *in vitro* release. The nanocrystal in oil O/W creams had a faster release compared to nanocrystals in water (Figure 3.18). The number of nanocrystals located in the water or oil phase should be different after preparation. Additionally, the viscosity of O/W creams with nanocrystals in the oil phase had lower viscosity compared to the O/W creams with nanocrystals in the aqueous phase. Therefore, these factors induced faster release.

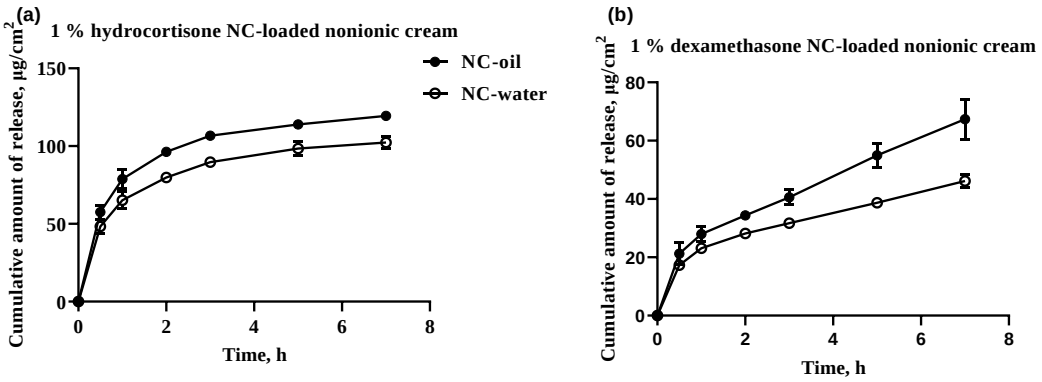

Figure 3.18 Cumulative amount release of 1 % hydrocortisone and dexamethasone nanocrystal-loaded gels (± SD, n = 3)

3.2.3.4 The storage stability of nanocrystal-loaded O/W creams

3-month storage stability was investigated for the nanocrystal-loaded O/W cream. For the O/W creams, all samples had no phase separation after the stability test. Nanocrystals had a different crystal growth phenomenon. For the nanocrystals, which were produced from aqueous nanosuspensions, dexamethasone had better stability compared to hydrocortisone nanocrystals in the O/W cream. Hydrocortisone had more µm particles after 3-month storage stability. For nanocrystals, which were produced from paraffin, dexamethasone also had better stability compared to hydrocortisone nanocrystals in the O/W cream. The size of the oil droplets was influenced by the particles. The hydrocortisone nanocrystals from paraffin deformed the round-shaped oil droplets of the cream compared to dexamethasone (Figure 3.19). Usually, for Ostwald ripening, the growth of particles resulted from the dissolved smaller drug particles. Hydrocortisone had a higher solubility compared to dexamethasone. Therefore, there were more dynamic changes in the dissolution of smaller particles and recrystallization on the surface of larger particles.

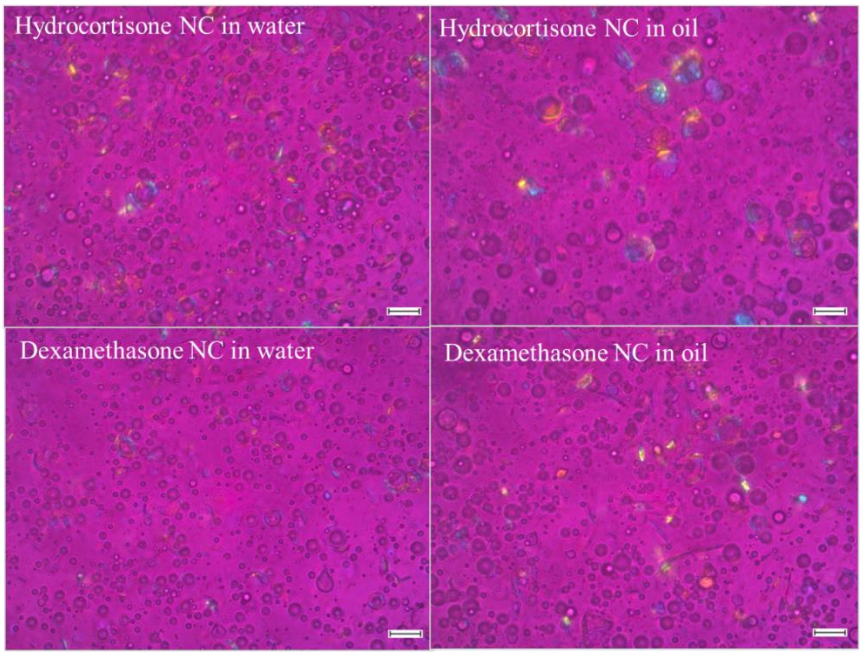

Figure 3.19 Observation of 3-month particle size of 1 % nanocrystal-loaded O/W cream at room temperature by polarized microscopy (scale bar 20 μm)

3.2.3.5 *in vitro* release comparison of nanocrystal-loaded gels and O/W creams

For formulators, it is important to select suitable semisolid vehicles to load nanocrystals. Nanocrystal-loaded semisolids were prepared and investigated in the previous part. The *in vitro* release comparison of the selected nanocrystal-loaded semisolids was investigated in the following part. For nanocrystal-loaded hydrogels, HPMC-stabilized nanocrystals were selected due to a smaller influence on the gelling process compared to SDS. Nanocrystal-loaded MCT/paraffin SiO_2 gels and nanocrystals in water O/W creams were selected due to their storage stability.

Hydrogels (HPMC and Carbopol) had a significantly faster release compared to the oil-containing semisolid vehicles (Figure 3.20). The release of drugs from gels occurs through various mechanisms, including diffusion, swelling and erosion. Diffusion is the most common mechanism, whereby the drug molecules diffuse out of the gel matrix

and into release media. The drug can also be released as the result of the increased pore size or the decreased polymer density. Erosion-controlled release occurs when the gel degrades or dissolves in the surrounding medium, leading to the release of the drug. For nanocrystal-loaded HPMC/Carbopol gels, the fastest release profiles were a synergistic phenomenon among diffusion, swelling and erosion mechanism. However, for oil-containing semisolids (oleogels and O/W creams), mainly diffusion of the drug and SiO_2 erosion influenced the release rate. The diffusion of drug in the oil phase was slower than in the water phase. Therefore, nanocrystal-loaded oily semisolids had a slower release compared to hydrogels.

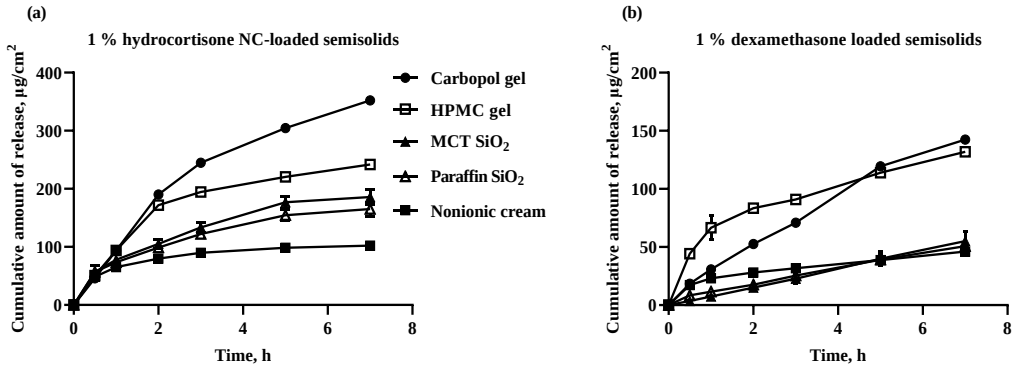

Figure 3.20 Cumulative amount release of 1 % hydrocortisone and dexamethasone nanocrystal-loaded gels and O/W creams (± SD, n = 3)

3.2.4 Conclusions

Nanocrystal-loaded gels and O/W creams were investigated. For nanocrystal-loaded gels, the stabilizer to stabilize nanosuspensions had to be carefully selected in order to avoid undesirable interactions with the gelling agent. Furthermore, the added nanocrystals increased the viscosity of the gels by the enlarged surface area to volume ratio, giving the possibility to reduce thickeners in the gel formulations. Nanocrystal-loaded hydrogels had a faster *in vitro* release when compared to oleogels. Particle size

stability over a 3 months period was achieved. For nanocrystal-loaded O/W cream, nanocrystals added either to the water or oil phases acted as extra stabilizers to stabilize the oil droplet upon the homogenization process. This decreased the oil droplet size and increased the viscosity of the cream. Comparing different nanocrystal-loaded semisolids, hydrogels had a faster release compared to other types of vehicles. A systematic investigation of the incorporation of drug nanocrystals into various semisolid vehicles and their drug release was achieved.

3.3 Particle growth mechanism of nanosuspensions after water evaporation upon simulated topical administration

3.3.1 Background

Drug nanocrystals, pure solid particles with stabilizers, were introduced in the 1990s [10]. Their decreased crystallinity and enlarged surface area result in faster dissolution rate enabling a higher topical bioavailability [7, 38, 79]. The reduced dose not only benefits the patients in terms of lower side effects and toxicity but also leads to cost savings for manufacturers. However, stability is the main challenge of nanocrystals due to the thermodynamical instability with a tendency toward agglomeration or crystal growth. Usually, Ostwald ripening is used to explain this phenomenon. The smaller particles dissolve and recrystallize on the surface of larger ones [6, 80]. Previous reports have investigated the long-term physical stability of nanocrystals [25, 26, 64, 67, 70, 79, 80]. Fewer publications investigated the stability of nanocrystals upon topical administration. There are three topical penetration routes of drug: transcellular, intercellular and transappendageal route [140]. Numerous research studies reported that nanocrystal skin absorption is mainly governed by the transappendageal pathway. For the current understanding, the advantageous penetration rate is attributed to nm size (< 600 nm) and the high surface-area-to-volume ratio [63, 66, 67, 72, 81]. The nanosuspensions enhance the permeation flux with no lag phase both *in vitro* and *in vivo*, compared to coarse suspension and physical mixture. However, water evaporation occurs immediately after applying the formulation on the skin. This might lead to destabilization of nanocrystals, reducing drug efficacy [62, 82]. Thus, the question arises whether the size of nanocrystals is still in nm range after water evaporation. Therefore, the particle growth mechanism of nanosuspensions during water evaporation needs investigation.

Upon water evaporation, the concentration of water, stabilizer, dissolved drug, and nanocrystals experience dynamic changes (Figure 2.21). The fate of nanosuspensions experiences two processes: i) dissolution of nanocrystals and ii) precipitation of drug. The concentration of stabilizer is increasing upon water evaporation and the stabilizer usually solubilizes the drugs. Therefore, drug nanocrystals could partially dissolve during the water evaporation process. However, when the concentration of dissolved drug exceeds saturation of the concentrated stabilizer system, nucleation and precipitation of drug can occur. Different types of stabilizers have different solubilization and stabilization functions to drug nanocrystals [25, 26, 28].

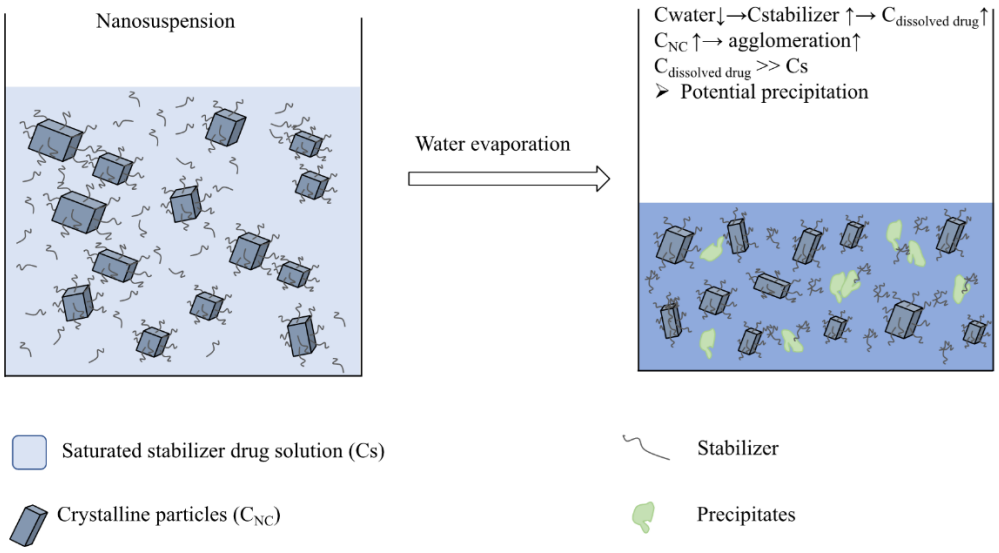

Figure 3.21 Fate of nanosuspensions during topical administration

Current knowledge of drug crystallization is that nucleation and crystal growth occur simultaneously. Nucleation requires activation energy, attributed to interfacial tension between the initial nucleus and the media. Crystal growth happens when the activation energy is above a certain threshold. The crystal growth mechanism depends on several factors: morphology of crystals, state of the material (dissolved or particular) and physicochemical properties of media. Usually, it is categorized into classical nucleation

theory (CNT) and non-classic crystal growth (NCC) (Figure 3.22). CNT starts with the ordered drug nucleus, subsequently crystallizing into bulk crystals by monomer-attaching-monomer method [141]. Quasi-planar shaped particles are usually found in the CNT process, which ensures the faster kinetics of nucleation due to the high number of intermolecular contacts and low energy barriers that have to be overcome [142]. In contrast, NCC is governed by nearly oriented particle-particle attachment, resulting in meso-crystals. Another NCC pathway is that the growth starts from initial densification of round-shaped amorphous precursors, which subsequently grow to polycrystals with complex shapes [83-85, 143]. NCC has been recognized in the pharmaceutical field, etoricoxib crystallization started from well-defined round-shaped amorphous intermediates [144].

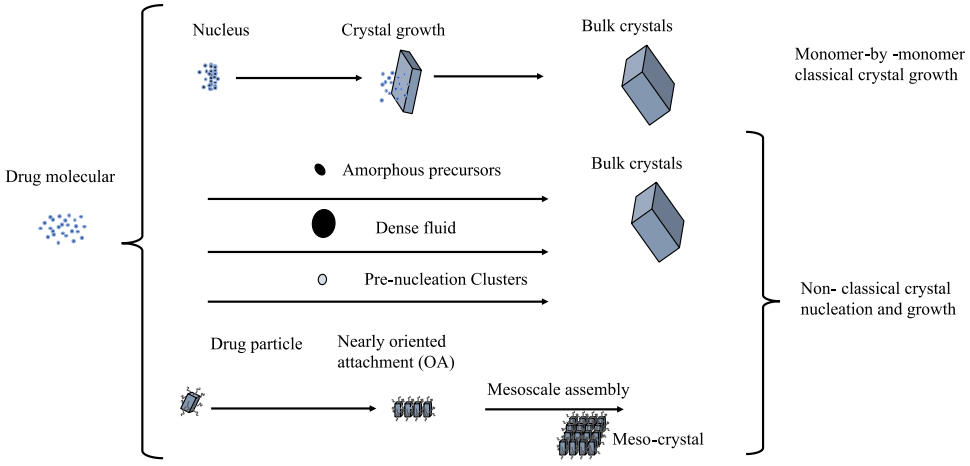

Figure 3.22 Schematic illustration of different crystal growth mechanisms

Despite recent progress, particle growth mechanism of nanosuspensions during topical administration is still not well understood due to the lack of a suitable simulation model and *in situ* measurement techniques. As the crystallization process of nanosuspension

upon topical administration is inside liquid phase, direct observation of the nucleation process is impossible. The limited temporal and spatial resolution of experimental techniques cannot observe dynamic processes in a solution. In recent studies, liquid-cell TEM illustrated the crystal growth of inorganic particles [145, 146]. However, there are few investigations for the *in situ* crystal growth process of organic nanocrystals, especially for drugs [147].

In this work, hydrocortisone (solubility 322.05 ± 4.29 μg/ml) and dexamethasone (solubility 72.05 ± 0.42 μg/ml) were selected as model drugs, due to their low water solubility and their relevance for topical administration. The stability of nanocrystals upon topical administration was investigated by incubating samples on petri dishes and porcine skin. Liquid-cell TEM together with conventional TEM were used to clarify the particle growth mechanism. It is hypothesized that the state of drugs and type of stabilizers will influence the particle growth mechanism.

For the administration of topical nanosuspensions, water evaporating varies the concentration of water, stabilizer and nanocrystals. Additionally, the increasing stabilizer concentration increases the solubility of drug. The water evaporation process disrupts the stability also by increasing the particle concentration. Due to the complexity of the nanosuspensions, saturated solutions and saturated stabilizer solutions were used as comparison in two different water evaporation studies. Subsequently, the solid-state and morphology of nanocrystals were investigated.

3.3.2 Particle growth in a simulated water evaporation study

3.3.2.1 Petri dish method

Precipitation is a 2-step process: nucleation and crystal growth. Water evaporation will disrupt the thermodynamic equilibrium. The resulting supersaturated system has an increased chemical potential compared to the corresponding saturated or unsaturated

systems. The increased chemical potential makes the system thermodynamically unstable and acts as a driving force for precipitation [148, 149]. Nanosuspension consists of crystalline drug with different degrees of crystallinity and dissolved drug. Various solid-states of drugs have different crystal growth mechanisms. To figure out the crystal growth mechanism in such complex systems, firstly drugs in saturated solutions without and with stabilizers were used to clarify the influence of stabilizers.

All saturated solutions displayed particle precipitation after complete water evaporation. The hydrocortisone/dexamethasone saturated solutions without stabilizers had strong particle growth (≈ 20 μm). For the hydrocortisone/dexamethasone saturated solutions with stabilizers, crystal growth phenomena (< 15 μm) were limited by the stabilizers to a different extent (Figure 3.23). Stabilizers are usually used for limiting particle aggregation and growth by steric and electrostatic stabilization. Due to surface-attached stabilizers, precipitation had a higher energy barrier, which was an obstacle for crystal growth compared to saturated solution without stabilizers. For the type of stabilizers, TPGS and P407 had more and larger precipitation after water evaporation, compared to HPMC and PVP solutions (Figure 3.23). In summary, dissolved drugs displayed crystal growth from molecular dispersed to μm size. However, saturated solution with stabilizers had less crystal growth compared to saturated solution without stabilizers. Stabilizers decreased the particle growth upon water evaporation.

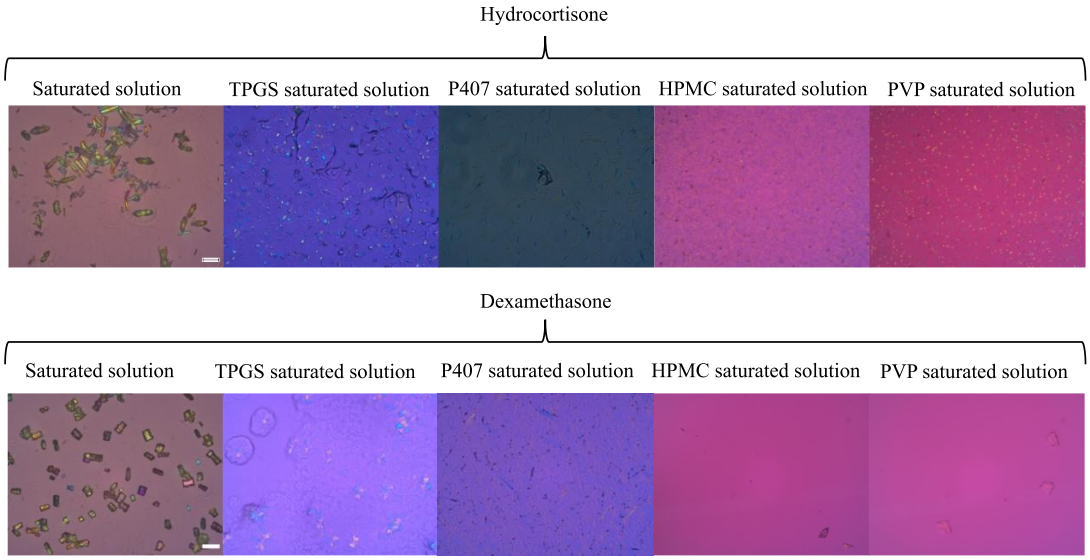

Figure 3.23 Water evaporation study of the completely dried saturated drugs solutions without and with stabilizers on petri dishes by polarized microscopy (scale bar 20 µm)

After clarifying the influence of stabilizers, 0.4 % stabilizer / 1 % drug nanosuspensions were simulated on petri dishes. Surprisingly, there were no microcrystals observed after complete water evaporation on the petri dishes (Figure 3.24). Therefore, the existence of stabilizer together with nanoparticles is the reason that no formation of large crystals was observed.

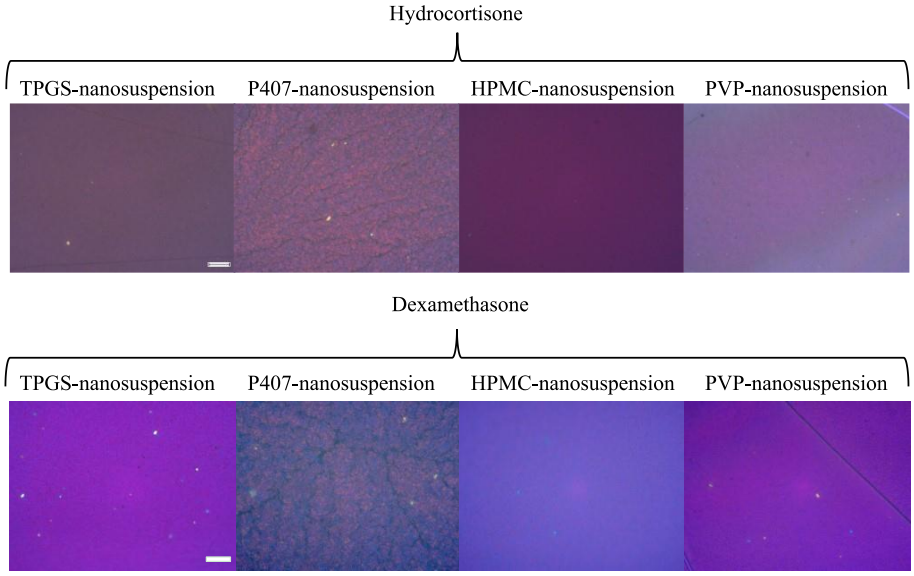

Figure 3.24 Water evaporation study of the completely dried drugs nanosuspensions on petri dishes by polarized microscopy (scale bar 20 µm)

Even though the nanocrystals did not grow to µm size after complete water evaporation, however, there was still the possibility that nanocrystals grew in the lower size range. Thus, in the next step, further particle size investigation of nanocrystals upon water evaporation was performed. The particle size changes of nanosuspensions upon water evaporation were evaluated at 0, 1, 3 and 5 h. Nanocrystals were still dispersed in aqueous phase at these time intervals. Nanosuspensions had D90 particle sizes in the nm range after 5 h water evaporation (Figure 3.25). Even though there was a slightly different particle size growth trend among the used stabilizers with lower molecular weight and polymeric stabilizers, nevertheless, the particle size of nanocrystals was always in the nm range.

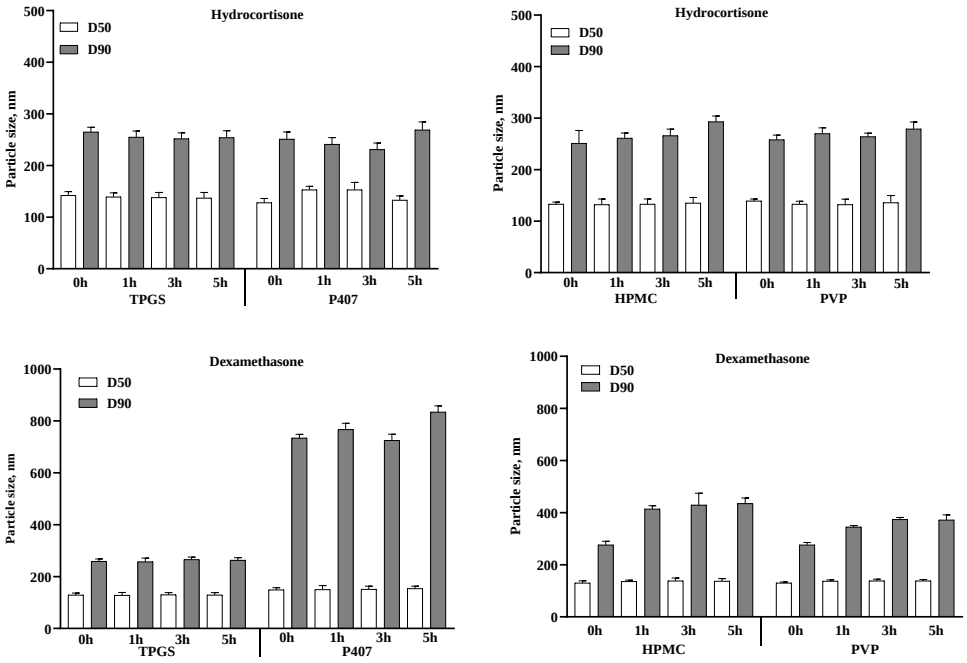

Figure 3.25 D50, D90 particle size changes of 0.4 % stabilizer / 1 % drugs nanosuspensions upon water evaporation at different time intervals

Drugs in different solid states have different fates. In saturated solution without stabilizer, solutions are always in the saturated condition upon the water evaporation. Drug nuclei appear once the concentration of dissolved drug is above its supersaturated solubility. Subsequently, the drug experiences the nucleation and crystal growth process [150]. For the saturated solutions with stabilizer, the systems are unsaturated at the earlier stage of water evaporation. This is due to the solubilization function of the stabilizers. At a certain degree of the water evaporation, nucleation and crystal growth start. Nanosuspensions have dynamic processes between redissolution of drug particles and precipitation of redissolved drugs due to the solubilization function of stabilizers. In summary, drugs in saturated solution without and with stabilizers and nanosuspensions experience different processes. Solubility changes are the main reason to clarify the crystal growth mechanism. MWCO membrane tubes were used to separate the nuclei and later precipitates upon the water evaporation process. As mentioned above, the dynamic changes of solubility among unsaturated, saturated and

supersaturated conditions appeared in these different systems. Usually, crystal growth is controlled by the nucleation process [119]. The more nucleation appears at the same time the less crystal growth happens. Consequently, an increased solubility in the initial stage of water evaporation and a decreased solubility due to precipitation should be observed.

Solubility changes of hydrocortisone in saturated solutions, 0.4 % saturated stabilizer solutions and nanosuspensions upon the water evaporation were evaluated. The solubilities of hydrocortisone saturated and saturated stabilizer solutions were increasing until 7 h of water evaporation. For the saturated solutions, the ratio of increased solubility of hydrocortisone was 1.86 (290 - 540 µg/ml). TPGS saturated hydrocortisone solution also had an increased solubility with the ratio of 1.93 (387 – 746 µg/ml) (Figure 3.26a). The solubilities of hydrocortisone samples at 0 h presented the thermodynamic solubility. The increased solubilities upon the water evaporation represented the kinetic solubilities, whereas any possible precipitates, which always start from the beginning from nm size and decreased crystallinity, will contribute to the kinetic solubility. However, the temporal stability of the kinetic solubility and the extent of the increase of the kinetic solubility is influenced by the surrounding conditions (solutions versus stabilized nanosuspensions). The increased solubility of hydrocortisone in TPGS saturated solution derived mainly from its solubilization function. P407, HPMC and PVP had increased solubility ratios with 1.83 (350 – 640 µg/ml), 1.98 (297 – 587 µg/ml) and 2.05 (301 – 618 µg/ml) respectively (Figure 3.26b-d). Polymers acted as precipitation inhibitors which resulted in the increased kinetic solubilities upon water evaporation [151, 152]. After milling hydrocortisone into nm size range, the solubility at 0 h varied with the stabilizer type (TPGS: 628 µg/ml; P407: 519 µg/ml; HPMC: 552 µg/ml; PVP: 564 µg/ml). The increased solubility was due to the decreased crystallinity and enlarged surface area of particles after nanosizing [38, 153]. For nanosuspensions, the kinetic solubilities of hydrocortisone were increasing until a certain time, then had a decreasing trend due to the precipitation (Figure 3.26).

Notably, the increased kinetic solubility ratios were lower than solution systems. TPGS, P407, HPMC and PVP stabilized hydrocortisone nanosuspensions had an increased kinetic solubility ratio of 1.38 (628 – 867 μg/ml), 1.30 (519 – 678 μg/ml), 1.42 (552 – 788 μg/ml) and 1.29 (564 – 730 μg/ml), respectively. Additionally, there was a sudden decrease in solubility (arrows). As mentioned above, at the earlier stage of water evaporation, the increased stabilizer concentration induced the redissolution of nanocrystals which resulted in the increased solubility. However, at a certain water loss, particle growth occurred. The particle growth in nanosuspensions was governed by two possible methods: (i) precipitates appear from the dissolved drug by nucleation and crystal growth; (ii) precipitates come from the diffusion of the solute molecules (dissolved and redissolved drugs) attaching to the surface of the undissolved nanoparticles [117]. At this stage of water evaporation, a high amount of undissolved nanoparticles provides a large solid surface for further precipitation, which exits in nanosuspensions, but not in drug solutions. These resulted in the fast decrease in solubility. In contrast, the saturated solution without and with stabilizers only had the surface of the few developing nuclei. However, at the end of the water evaporation process (around 11 h), these few nuclei grew to large μm crystals, whereas precipitation inside the nanosuspensions was distributed over the surface of many undissolved nanoparticles. This could also be the reason that there were no microcrystals in case of nanosuspensions after the complete water evaporation process observed.

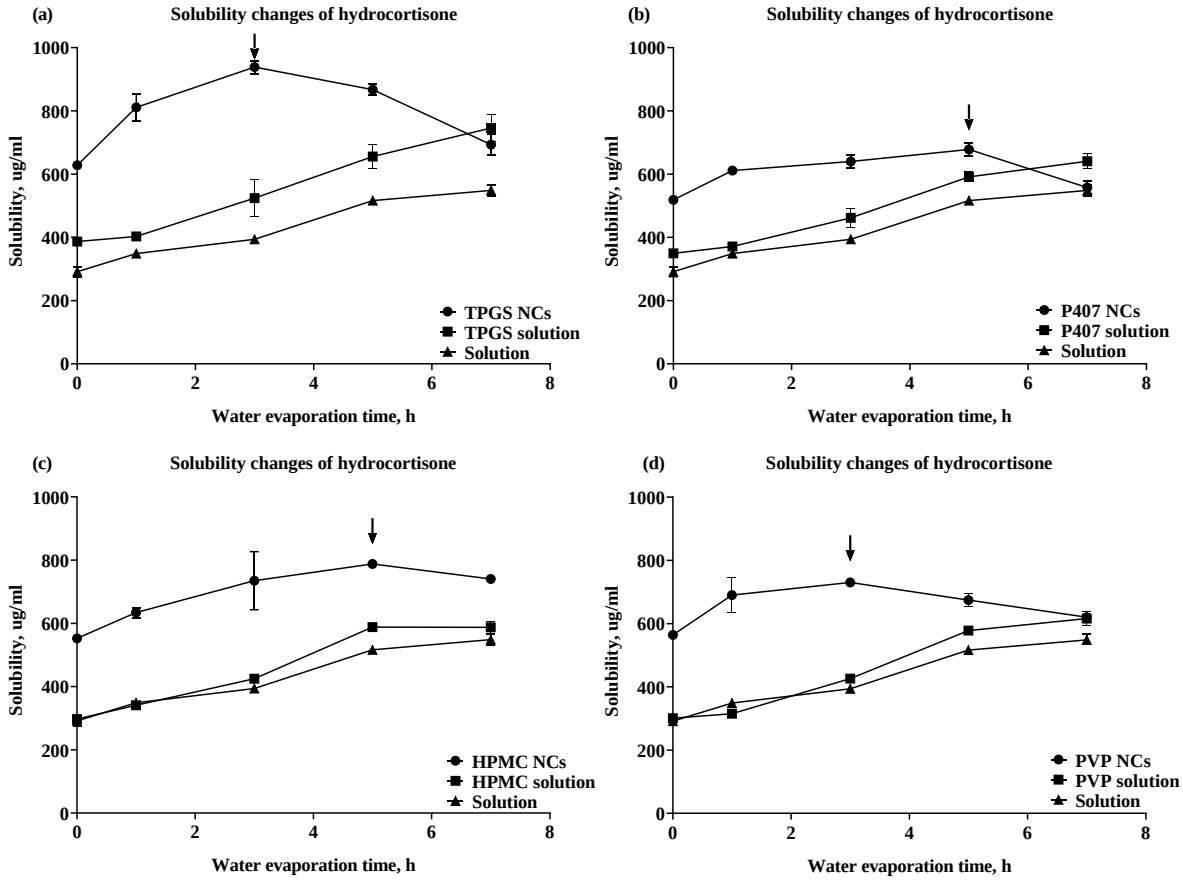

Figure 3.26 Solubility changes of hydrocortisone in saturated solutions, 0.4 % saturated stabilizer solutions and nanosuspensions upon water evaporation

3.3.2.2 E*x vivo* porcine skin method

After the investigation of nanocrystal size stability upon administration without penetration on the petri dishes, porcine skin, which involved penetration was investigated. 0.4 % TPGS/PVP stabilized 1 % HC/DEX nanosuspensions were selected due to their water evaporation stability in petri dish experiments. The drugs saturated solution and the 0.4 % TPGS/PVP drugs saturated solutions were also dropped on the porcine skin with the same drug amount as nanosuspensions. After the water evaporated from the nanosuspensions applied to the porcine skin, no particle growth to µm range was observed in accordance with results from the petri dish method. In contrast, as for

the petri dish method drugs saturated solutions and drugs saturated stabilizer solutions displayed microcrystals on the porcine skin (Figure 3.27).

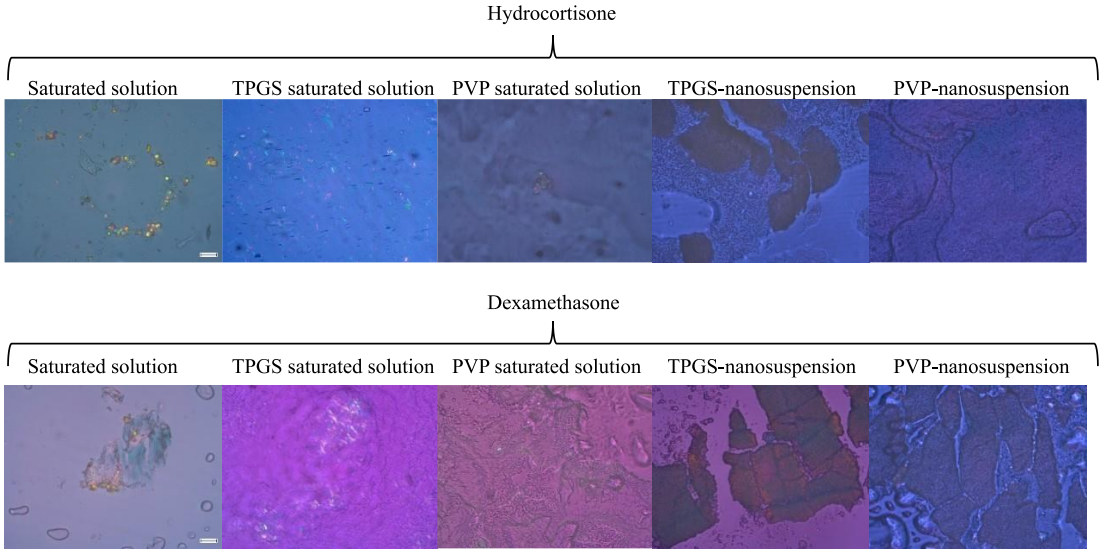

Figure 3.27 Water evaporation study on porcine skin characterized by polarized microscopy, scale bar 20 µm

The crystal growth of samples by simulated petri dish and porcine skin studies corresponded to each other. The nanosuspensions had a stable particle size during the whole drying process, in contrast to drug solutions. It is assumed that there are different crystallization mechanisms between solution and nanosuspension during water evaporation. Usually, the solid-state and morphology differ after experiencing different crystallization processes. The NCC has crystallization from an amorphous intermediate with well-defined round-shaped particles. If the particle growth mechanism is followed by the NCC, the crystallinity decreases and the morphology of the particles differs from the initial nanocrystals. Thus, the solid-state and morphology of particles during the water evaporation process were investigated in the next steps.

3.3.3 Solid-state changes of nanosuspensions upon water evaporation

The solid-state of nanocrystals is commonly studied by X-ray diffraction (XRD) and thermal analysis such as differential scanning calorimetry (DSC) [154, 155]. Nanosuspensions consist of nanocrystals and relatively high concentration of stabilizers. Upon the heating process during the DSC measurement, the drug thermal behaviour is influenced by melting of stabilizers. XRD, which is a non-invasive measurement, achieves the solid-state of nanocrystals upon water evaporation more appropriately [156]. To investigate whether the crystallinity changes during water evaporation process, nanosuspensions were evaporated with the petri dish method and the dry samples were scrapped off from the petri dish for the measurement.

After water evaporation, 0.4 % TPGS and PVP stabilized 1 % hydrocortisone nanocrystals showed broader peaks than their initial nanosuspensions (diffractogram not shown) and decreased crystallinity of 66.36 % and 71.72 %respectively (Table 3.3a). The water evaporated dexamethasone nanocrystals had a similar phenomenon. The crystallinity of TPGS and PVP stabilized dexamethasone nanocrystal was decreased to 64.07 % and 89.31 % respectively (Table 3.3b). As mentioned before, NCC involves transient amorphous precursors, this might lead to a decreased crystallinity. However, the stabilizer condition between nanocrystal (0 h) and nanocrystal dry was different, since there was no removal of the stabilizer by centrifugation for the dried samples. Due to the physicochemical properties of stabilizers, the particle formation of an amorphous solid dispersion might be also a reason for the decreased crystallinity after water evaporation. In order to clarify this mechanism further, the direct imaging of the crystal morphology is investigated in the next step.

Table 3.3 Crystallinity parameters of 0.4 % TPGS/PVP stabilized 1 % nanosuspension upon water evaporation: (a) hydrocortisone, (b) dexamethasone

(a)

Sample	FWHM	Crystallite size, nm	Crystallinity, %
0.4 % TPGS 1 % HC NC 0h	0.19	41.55	100.00
0.4 % TPGS 1 % HC NC dry	0.29	27.58	66.36
0.4 % PVP 1 % HC NC 0h	0.17	48.49	100.00
0.4 % PVP 1 % HC NC dry	0.23	34.78	71.72

(b)

Sample	FWHM	Crystallite size, nm	Crystallinity, %
0.4 % TPGS 1 % DEX NC 0 h	0.15	53.24	100.00
0.4 % TPGS 1 % DEX NC dry	0.23	34.11	64.07
0.4 % PVP 1 % DEX NC 0 h	0.19	41.96	100.00
0.4 % PVP 1 % DEX NC dry	0.21	37.48	89.31

3.3.4 Imaging crystal growth mechanism of nanosuspension during water evaporation by liquid-cell and conventional TEM

As the simulated topical administration involved the ratio variation among water, stabilizer, dissolved and crystalline drugs, the crystal growth mechanism was highly dependent on these dynamic changes. However, samples used for liquid-cell and conventional TEM, may be diluted. Additionally, the particles have to be fixed on the liquid-cell to inhibit the fast movement [157]. Thus, dynamic equilibrium among water, stabilizer, and drugs of the original sample during the water evaporation was influenced by the dilution and additional material to fix the particle movement. Therefore, the sample preparation methods were evaluated carefully to achieve the imaging of the crystal growth for the nanosuspension. For this purpose, 0.4 % TPGS stabilized 1 % dexamethasone nanosuspension was selected for this investigation due to its high particle size stability and its pronounced decreased crystallinity. The sample preparation methods were investigated to check the possibility of imaging particles without and with dilution by liquid-cell and conventional TEM.

In preliminary studies, it was impossible to image the morphology of dexamethasone nanocrystals in high water content samples due to fast movement of particles. In addition, the complete dried original sample also could not be imaged. The electron beam was not able to image the particles through the thick layers of dried sample (data not shown). Thus, 10 times diluted samples dried in the liquid cell for 30 s were used to distinguish original drug nanocrystals and possible precipitates.

Due to the sample preparation method, the dried phase and water phase of sample were on the liquid cell simultaneously. The changes of crystal morphology and size in both phases were used to explain the crystal growth mechanism of nanosuspension during water evaporation. The water phase was firstly recognized by the shrinkage area of greyish phase upon measurement (Figure 3.28a-b). The shrinkage was due to the water removal in the liquid cell. To distinguish dexamethasone, energy-dispersive spectroscopy (EDS) was used. The element fluorine (-F) of dexamethasone was found (Figure 3.28c). The original dexamethasone nanocrystal was around 200 nm and consistent with previous LD results. Smaller particles appeared upon water evaporation. The size of these particles was less than 50 nm, which had an oval shape. (Figure 3.28d). By zooming on these particles (possible precipitates), agglomerates were found (Figure 3.28e). Some particles still moved during the measurement (Figure 3.28f-j). However, they did not fuse to large crystals and separation between particles was still clear in Figure 3.28k. The attachment and detachment of possible precipitates might be explained by the “energy landscape”, controlled by the depths and shapes of the energy minima [158]. Precipitated dexamethasone had shapes far from the compact, smooth, and polyhedral dexamethasone initial particle. The curved surface of precipitates had a free energy significantly higher than conceivable compact faceted shapes (initial dexamethasone particles). The crystal growth of oval shaped precipitates was slowed down by higher surface energy. Thus, reduced intermolecular contacts from the surface of oval-shaped precipitates resulted in the inhibition of fusing into larger crystals due to the obstacle from the higher energy barriers [159].

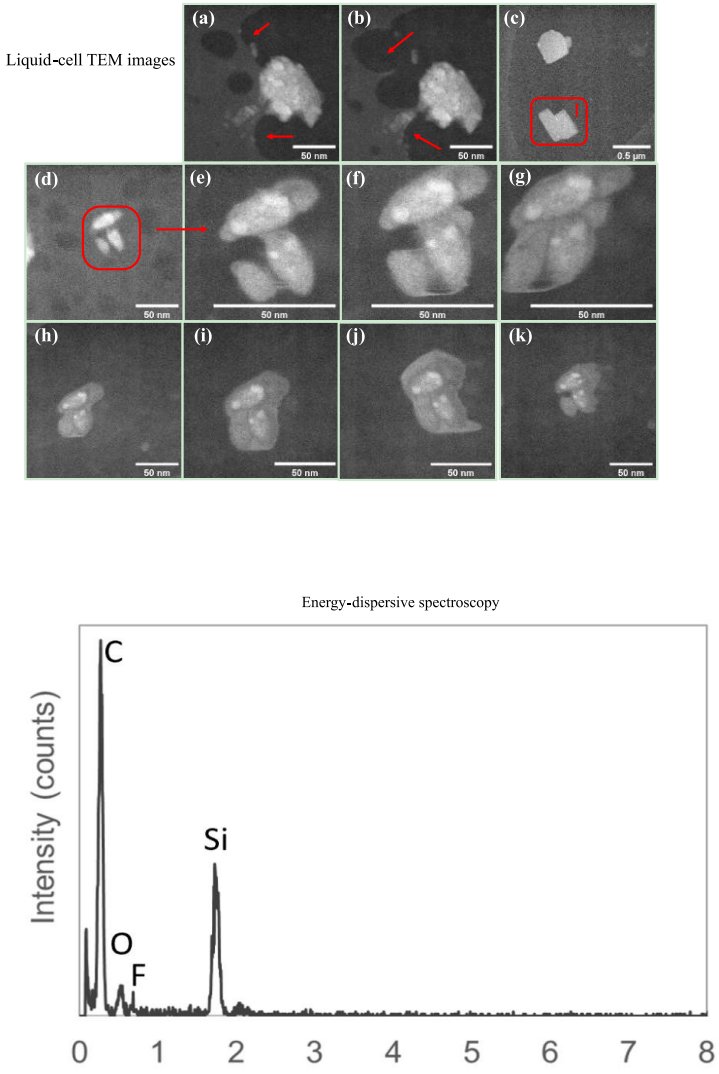

Figure 3.28 Liquid-cell TEM images: (a-b) water flow tracing upon time; (c) the confirmation of initial dexamethasone nanocrystal by EDS; (d) precipitates of dexamethasone during the water evaporation; (e-k) zoomed precipitates from images within the acquisition time; Energy-dispersive spectroscopy of I

Due to the limitation to image nanocrystals without dilution by liquid-cell TEM, conventional TEM was used as a supplement to clarify the crystal growth mechanism. 0.4 % TPGS stabilized 1 % dexamethasone nanosuspension was dropped on the glass petri dish. The Cu-grid was dipped into the samples at different times during the drying

process and the water phase was carefully and quickly removed from the grid. Due to the fast removal of water, some of the residue particles on the Cu-grid were imaged successfully by conventional TEM.

The completely dried 0.4 % TPGS solution in absence of dexamethasone nanocrystals was used as the blank. The element of oxygen element (-O) was found by EDS, which certified that the TPGS polymer covered the carbon of the TEM grid (Figure 3.29a, EDS of TPGS solution). Notable, beam damage from the incident electron beam degraded the TPGS polymer (Figure 3.29a4). For the initial DEX nanocrystals, nanosized cubes with a sharp edge corresponded to particles imaged by liquid-cell TEM and the average particle size was 272 ± 133 nm (n = 33) (Figure 3.29b). After 2 min of drying, there were electron denser particles inside the lower contrasted “flaky traces”. The denser particles had a pronounced oval shape with sizes below 50 nm (Figure 3.29c). TPGS was used as the stabilizer in the nanosuspensions, which would also precipitate out at certain decreased water concentrations. However, combining the morphology of blank TPGS and appeared “flaky traces” (the mixture of dissolved and precipitated dexamethasone together with TPGS) confirms the non-classical crystal growth mechanism. After the complete water evaporation process of dexamethasone nanosuspension, the dried drug nanocrystal powder, which was scrapped from the petri dish was imaged (Figure 3.29d). Interestingly, the average size of particles changed from initial 272 ± 133 nm to 216 ±103 nm (n = 100) after complete drying. Additionally, fluorine (-F) of dexamethasone was found by EDS (EDS of dried dexamethasone precipitates). Particles were still in nm range after water evaporation. Many rod-shaped smooth-surfaced particles were in the aggregate, which could be the growth of amorphous precursors (denser fluid particles). Clearly identifiable precipitated agglomerates which did not fuse to larger crystals confirmed the non-classical crystal growth mechanism further.

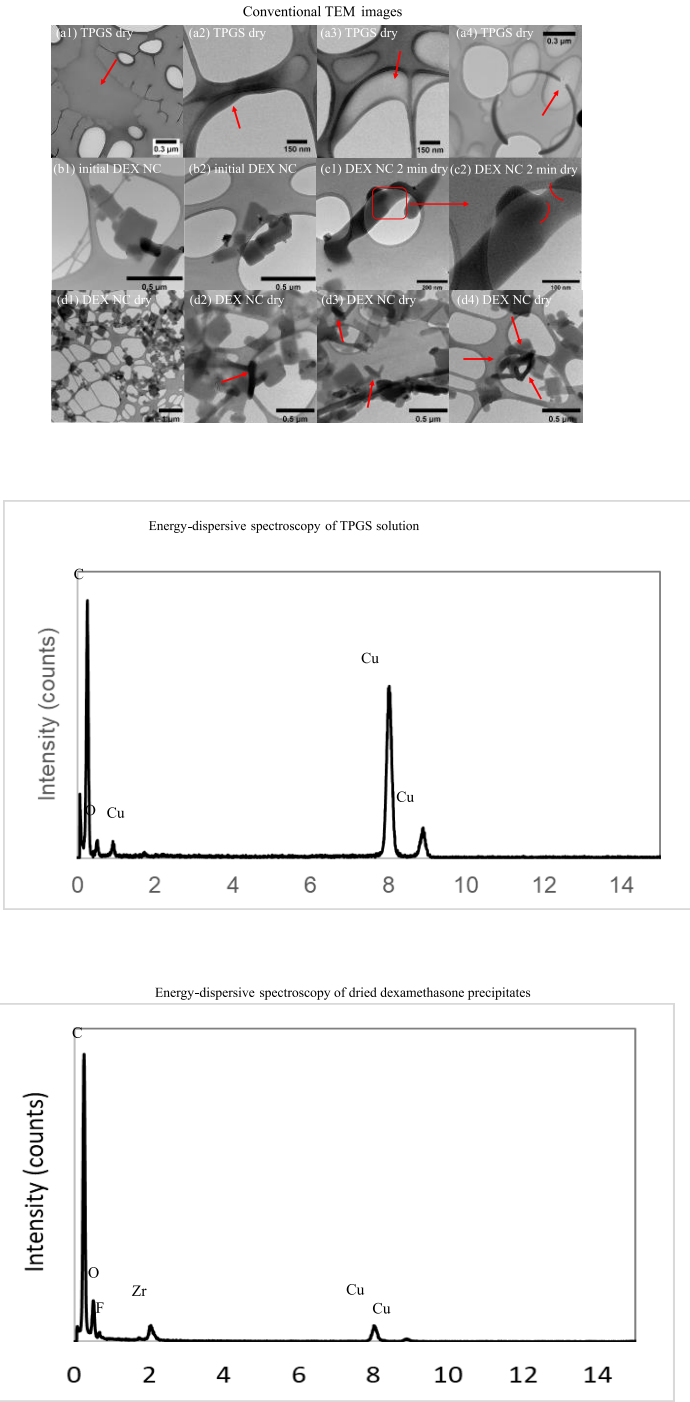

Figure 3.29 Conventional TEM images: (a) complete dried 0.4 % TPGS solution; (b) initial dexamethasone nanocrystals; (c) dexamethasone precipitates from nanosuspension after drying 2 min; (d) complete dried 0.4 % TPGS 1 % dexamethasone nanosuspension; Energy-dispersive spectroscopy of TPGS solutions

Since particles were not in regular cuboid shape, the previous diameters to present the average size might not fully reflect the size changes of the particles. Thus, frequency distribution data of particle area changes before and after water evaporation were evaluated. The % frequency of dried particle area distribution shifted to the smaller area, compared to the initial particles (Figure 3.30). This also corresponded to the results of average size changes.

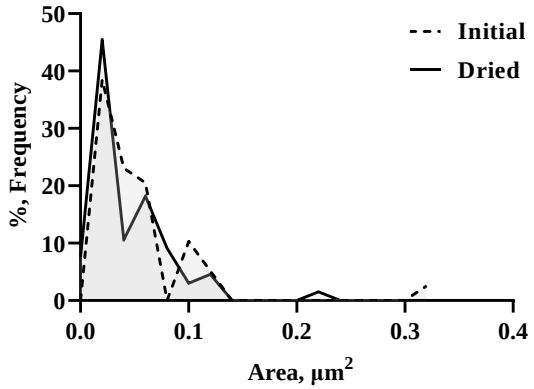

Figure 3.30 Frequency distribution data of particle area changes before and after water evaporation, characterized by conventional TEM

The fate of dexamethasone nanocrystals experienced dissolution at the beginning of water evaporation due to the increased TPGS concentration. When the concentration of dissolved drug exceeded the solubility of dexamethasone, precipitation occurs at the later drying stage. During this stage, particles were formed from the dense fluid, which consisted of dissolved dexamethasone, TPGS and water. Usually, significant crystal fusion happens when the crystals have quasi-planar shape. Therefore, the oval spheres of drug precipitates slowed down the kinetics of surface nucleation due to the lower number of intermolecular contacts and the high energy barriers that have to be overcome. Thus, the precipitated dense oval spheres could not fuse to each other. Thus, the average size and area % frequency of particles both decreased after water evaporation due to unfused smaller precipitates. This confirms the nanosize of particles

from the previous simulated topical administration experiments. With these direct findings from liquid-cell and conventional TEM, the non-classical crystal growth mechanism of nanosuspension during water evaporation was verified.

3.3.5 Conclusion

The crystal growth mechanism of nanosuspension upon simulated topical administration was investigated by petri dish and porcine skin methods. For the petri dish method, large μm crystal precipitation was observed in saturated hydrocortisone / dexamethasone solutions and stabilizer solutions, in contrast to stable nanosuspension after water evaporation. Results obtained with porcine skin correlated to results of the petri dish method. These two different crystal growth phenomena were in accordance with the physical state of the drug (dissolved versus nanoparticular system). As shown with XRD studies, the decreased crystallinity after water evaporation of nanosuspension was due to stabilizer-matrix effects and crystallinity-decreased dense fluid particles. Directly visible nanocrystal changes in size and morphology as studied with liquid-cell and conventional TEM also proved the non-classical crystal growth of nanosuspension during the water evaporation process. The cubic- and faceted-shaped dexamethasone nanocrystals dissolved during the initial water evaporation stage due to the more concentrated stabilizer TPGS. Afterwards, around 50 nm oval drug spheres precipitated from the dense fluids (water, stabilizer, and drug) within the precipitated stabilizer after complete water evaporation, resulting in a matrix similar to a drug solid dispersion. After complete water evaporation, the particles were still in the nanometer size range. Based on these findings, the promising high bioavailability of nanosuspension for topical administration could not only benefit from the nm particle size, but also from faster redissolution and higher solubility of the dense fluid/decreased crystallinity particles formed upon water evaporation. The clarified crystal growth mechanism for nanosuspensions upon simulated topical administration is not the

classical crystal growth, which was found for solutions, but the non-classical crystal growth mechanism. This might improve the design of nanocrystal loaded formulations, e.g., by selecting optimized stabilizers to improve the dense fluid matrix towards a higher solubility and fast dissolution of drug particles in the nanometer range with reduced crystallinity.

4 Summary

Nanosuspension technology is a strategy to increase the bioavailability of poorly soluble drugs and has been used for various routes of drug administration. Nanocrystals consist of pure drug, thus allowing the administration of a high drug dose in a small volume. This is advantageous for topical administration to increase the concentration gradient for the drug uptake into the skin. Moreover, the nm size of the drug (< 1µm) allows improved skin penetration and enhanced drug delivery to the target site. Nanosuspensions can be used as prepared or be loaded into semisolid vehicles. There are some challenges, which need to be solved. Stabilizers are unavoidable for aqueous nanosuspensions. However, stabilizers can also be a limiting factor for stability, e.g., during freeze-thaw cycling or sterilization. In addition, stabilizers might interact with the excipient in semisolid dosage forms, resulting in a different performance of the nanocrystals. The nanocrystal stability upon topical administration is also still an open question. Therefore, a systematic investigation of nanocrystal-loaded topical formulations was performed upon preparation, incorporation into semisolid vehicles and administration.

For the preparation of stabilizer-free nanosuspensions, this study aimed to prepare stabilizer-free oily nanosuspensions and to characterize their *in vitro* release and physical stability. The nanosuspensions were prepared by wet bead milling with different oils and drug loadings. They were characterized by photon correlation spectroscopy, differential scanning calorimetry and *in vitro* release. The particle size stability was evaluated under freeze-thaw cycling, dry heat sterilization and as a function of storage time. Hydrocortisone and dexamethasone could be successfully formulated into oily nanosuspensions without stabilizers. Drugs after milling remained in the crystalline state. For in vitro release, nanosuspensions had a longer release than microsuspensions. Increasing the drug loading from 1 % to 3 % resulted in a longer release due to a significantly higher viscosity. Different release periods were observed for paraffin and medium-chain triglycerides (MCT) nanosuspensions due to different

drug solubilities in the oils. Drug particles were released by settling to the paraffin-water interface. In contrast, particles partially dissolved in MCT before drug release to the aqueous medium, which promoted the faster release. Stabilizer-free oily nanosuspensions had an improved physical stability among freeze-thaw cycling and dry heat sterilization, and a similar stability compared with Span 80-stabilized oily nanosuspensions after 3 months. In summary, stabilizer-free oily nanosuspensions possessed promising stability and a prolonged in vitro release properties. By varying drug loadings and oil types, “tailored” release profiles can be obtained for stabilizer-free oily nanosuspensions.

For incorporating the nanosuspension into different semisolid vehicles, the nanosuspensions were incorporated into various gels and creams to investigate the interaction between the nanoparticle stabilizers and hydrogel formers, the distribution of nanocrystals in O/W creams and their difference in *in vitro* release. The aqueous and oily nanosuspensions were prepared by wet bead milling and subsequently they were loaded into gels and O/W creams. They were characterized using photon correlation spectroscopy, polarized microscopy, viscosity measurements and *in vitro* release. The particle size stability was evaluated after loading into semisolids and after storage. For the stabilizer influence on hydrogels, sodium dodecyl sulfate had a greater influence on the viscosity of hydrogels compared to hydroxypropyl methylcellulose. Nanocrystal-loaded gels had a 3 months particle size stability except for nanocrystal-loaded ethyl cellulose oleogels. For nanocrystal-loaded creams, nanocrystals acted as additional stabilizers decreasing the oil droplets size and increased the viscosity through the enlarged surface area to volume ratio. Hydrogels had the fastest release when compared to other types of vehicles because their release was not only governed by diffusion but also by swelling and erosion. A systematic investigation of the incorporation of drug nanocrystals into various semisolid vehicles and their drug release was achieved.

The investigation of the performance/ stability of nanosuspensions upon topical administration aimed to understand the particle growth mechanism of nanocrystals during topical application. Nanosuspensions were produced by wet bead milling. Simulated water evaporation experiments were performed by incubating samples on petri dishes and porcine skin. Saturated drug solutions without and with stabilizers were used as comparison. Particle size was characterized by microscopy and laser diffraction. X-ray diffraction quantified the degree of crystallinity. The particle growth process was imaged by liquid-cell and conventional transmission electron microscopy. For both simulation experiments, hydrocortisone and dexamethasone saturated solutions had a significantly higher particle growth than their nanosuspension formulations. TPGS-stabilized nanocrystals had a larger decrease in crystallinity, compared to nanosuspensions stabilized with Poloxamer 407, HPMC and PVP. Nanocrystals might experience a non-classical particle growth mechanism since the rhombohedral shape of initial nanocrystals changed to oval, spherical dense fluid particles during water evaporation. In conclusion, the nanocrystals after simulated topical administration did not grow into larger crystals. The promising high bioavailability of nanosuspensions for topical administration could not only benefit from the nm size, but also from the higher solubility of the less crystalline particles, whereby drug and stabilizer partially formed a solid drug dispersion upon water evaporation. This could improve the design of nanocrystal-loaded formulations with optimized stabilizer selection.

5 Zusammenfassung

Nanosuspensionen bieten die Möglichkeit zur Erhöhung der Bioverfügbarkeit von schwerlöslichen Arzneistoffen, welche für verschiedene Verabreichungswege angewandt werden. Nanokristalle bestehen aus reinem Arzneistoff, was die Verabreichung einer hohen Wirkstoffdosis in einem kleinen Volumen ermöglicht. Dies ist für die topische Verabreichung vorteilhaft, um den Konzentrationsgradienten des Arzneistoffes zu erhöhen, welcher in die Haut aufgenommen wird. Darüber hinaus ermöglicht die Nanometer-Größe des Arzneistoffes (< 1 µm) eine verbesserte Hautpenetration und eine erhöhte Arzneistoffkonzentration im Zielgewebe. Nanosuspensionen können direkt verwendet oder in halbfeste Grundlagen eingearbeitet werden. Es gibt einige Herausforderungen, die gelöst werden müssen. Die Zugabe von Stabilisatoren zu wässrigen Nanosuspensionen ist unvermeidlich. Stabilisatoren können jedoch ein limitierender Faktor für die Stabilität bedeuten, z. B. beim Gefrier-Tau-Wechsel oder bei der Sterilisation. Darüber hinaus kann der Stabilisator mit den Hilfsstoffen in halbfesten Darreichungsformen interagieren, was zu veränderten Nanokristalleigenschaften führt. Die Stabilität der Nanokristalle bei topischer Verabreichung ist immer noch eine strittige Frage. Daher wurde eine systematische Untersuchung der mit Nanokristallen beladenen topischen Formulierungen durchgeführt, bezogen auf die Herstellung, die Einarbeitung in die halbfesten Arzneiformen und Verabreichung dieser.

Für die Herstellung von Stabilisator-freien Nanosuspensionen zielte die Studie darauf ab, stabilisatorfreie ölige Nanosuspensionen herzustellen und ihre in-vitro-Freisetzung und physikalische Stabilität zu charakterisieren. Die Nanosuspensionen wurden durch Naßperlmahlung mit verschiedenen Ölen und Wirkstoffkonzentrationen hergestellt. Sie wurden mittels Photonenkorrelationsspektroskopie, Differentialthermoanalyse und in-vitro-Freisetzung charakterisiert. Die Partikelgrößenstabilität wurde bei Gefrier-Tau-Zyklen, Sterilisation mit trockener Hitze und Langzeitlagerung bewertet. Hydrocortison und Dexamethason können erfolgreich ohne Stabilisator zu öligen

Nanosuspensionen formuliert werden. Die Arzneistoffe blieben nach dem Mahlen im kristallinen Zustand. Für die in-vitro-Freisetzung hatten Nanosuspensionen eine längere Freisetzung als Suspensionen mit mikronisiertem Arzneistoff. Bei einer Erhöhung der Wirkstoffbeladung von 1 % auf 3 % führten Nanosuspensionen aufgrund einer signifikant höheren Viskosität zu einer längeren Freisetzung. Aufgrund der verschiedenen Löslichkeiten in diesen Ölen wurden in Nanosuspensionen aus Paraffin und mittelkettigen Triglyceriden (MCT) unterschiedliche Freisetzungsperioden beobachtet. Wirkstoffpartikel wurden durch das Absetzen an der Paraffin-Wasser-Grenzfläche freigesetzt. Im Gegensatz dazu lösten sich Partikel teilweise in MCT auf, bevor sie in das wässrige Medium freigesetzt wurden, was die schnellere Freisetzung förderte. Stabilisatorfreie ölige Nanosuspensionen hatten eine verbesserte physikalische Stabilität bei Gefrier-Tau-Zyklen und Sterilisation mit trockener Hitze und eine ähnliche Stabilität im Vergleich zu Span 80 stabilisierten öligen Nanosuspensionen nach 3 Monaten. Zusammenfassend zeigten stabilisatorfreie ölige Nanosuspensionen eine vielversprechende Stabilität und eine verlängerte in-vitro-Freisetzung. Durch unterschiedliche Wirkstoffbeladungen und Öltypen können gezielte Freisetzungsprofile für Stabilisator-freie ölige Nanosuspensionen erhalten werden.

Für die Einarbeitung der Nanosuspensionen in verschiedene halbfeste Grundlagen wurden die Nanosuspensionen in verschiedene Gele und Cremes eingearbeitet, um die Wechselwirkung zwischen den Nanopartikelstabilisatoren und Hydrogelbildnern, die Verteilung von Nanokristallen in Cremes und ihrer unterschiedlichen in-vitro-Freisetzungen zu untersuchen. Die wässrigen und öligen Nanosuspensionen wurden durch Naßperlmahlung hergestellt und anschließend in Gele und O/W-Cremes eingearbeitet. Sie wurden mittels Photonenkorrelationsspektroskopie, polarisierter Mikroskopie, Viskositätsmessung und in-vitro-Freisetzung charakterisiert. Die Partikelgrößenstabilität wurde nach dem Einbringen in halbfeste Grundlagen und nach der Lagerung bewertet. Bezüglich des Stabilisatoreinflusses auf Hydrogele hatte

Natriumdodecylsulfat im Vergleich zu Hydroxypropylmethylcellulose einen größeren Einfluss auf die Viskosität von Hydrogelen. Mit Nanokristallen beladene Gele hatten eine Partikelgrößenstabilität von 3 Monaten, mit Ausnahme von mit Nanokristallen beladenen Ethylcellulose-Oleogelen. Bei mit Nanokristallen beladener Creme verringerten Nanokristalle, welche als zusätzlicher Stabilisator fungierten, die Größe der Öltröpfchen und erhöhten die Viskosität durch das vergrößerte Verhältnis von Oberfläche zu Volumen. Hydrogele zeigten im Vergleich zu anderen Vehikeltypen die schnellste Freisetzung, da ihre Freisetzung nicht nur durch Diffusion, sondern auch durch Quellen und Erosion bestimmt wurde. Es wurde eine systematische Untersuchung der Einarbeitung von Arzneistoff-Nanokristallen in verschiedene halbfeste Grundlagen und der entsprechenden Arzneistoff-Freisetzungen durchgeführt.

Die Stabilitätsuntersuchungen der Nanosuspensionen bei topischer Verabreichung in zielten dieser Studie darauf ab, den Partikelwachstumsmechanismus von Nanokristallen während topischer Anwendung zu verstehen. Die Nanosuspensionen wurden durch Naßperlmahlung hergestellt. Wasserverdunstung wurde simuliert, indem Proben auf Petrischalen und Schweinehaut inkubiert wurden. Als Vergleich dienten die gesättigten Wirkstofflösungen mit und ohne Stabilisator. Partikelgrößen wurden durch Mikroskopie und Laserbeugung charakterisiert. Röntgenbeugung quantifizierte den Grad an veränderter Kristallinität. Der Partikelwachstumsprozess wurde durch Flüssigzellen- und herkömmliche Transmissionselektronenmikroskopie abgebildet. Bei beiden Simulationsexperimenten hatten gesättigte Lösungen von Hydrocortison und Dexamethason ein signifikant höheres Partikelwachstum als ihre Nanosuspensionen. TPGS-stabilisierte Nanokristalle zeigten eine stärkere Abnahme der Kristallinität im Vergleich zu Poloxamer 407, HPMC und PVP stabilisierten Nanosuspensionen. Nanokristalle könnten einen nicht-klassischen Partikelwachstumsmechanismus besitzen, da sich die rhomboedrische Form der anfänglichen Nanokristalle während der Wasserverdunstung in ovale, kugelförmige, dichte Flüssigkeitspartikel veränderte.

Zusammenfassend wuchsen die Nanokristalle nach simulierter topischer Verabreichung nicht zu großen Kristallen. Durch diese Ergebnisse könnte die vielversprechende hohe Bioverfügbarkeit von Nanosuspensionen bei topischer Verabreichung nicht nur von der Größe im Nanometerbereich profitieren, sondern auch von der höheren Löslichkeit von weniger kristallinen Flüssigkeitspartikeln, wobei Wirkstoff und Stabilisator bei der Wasserverdunstung teilweise eine feste Arzneimitteldispersion bilden. Dies wird das zukünftige Design nanokristallbeladener Formulierungen durch optimierte Stabilisatorauswahl verbessern.

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7 Publications and conference contributions

Publications:

- Preparation and characterization of stabilizer-free oily nanosuspensions (in progress)

- Incorporation of drug nanocrystals into various semisolid vehicles and their releases (in progress)

- Non-classical crystal growth mechanism of nanosuspensions upon simulated topical administration (in progress)

Published:

- Design, synthesis and pharmacological evaluation of novel N-(2-(1, 1-dimethyl-5, 7-dioxo-4, 6-diazaspiro [2.4] heptan-6-yl)ethyl) sulfonamide derivatives as potential anticonvulsant agents, Eur. J. Med. Chem, 2015. 92(6): p. 370-376

Seminars, Conferences & Workshops

2022 12th Scientific Symposium on the 'Day of Pharmacy' Deutsche Pharmazeutische Gesellschaft (Berlin)

- Preparation and characterization of a stabilizer-free dexamethasone oily nanosuspension **(Poster)**

13th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (Rotterdam)

- Degree of crystallinity as indicator for the long-term stability of nanosuspensions **(Poster)**

8 Curriculum vitae

12/2018 –06/2023 PhD - Freie Universität Berlin (DE)

10/2017 – 01/2019 MSc Pharmaceutical Research - Freie Universität Berlin (DE)

07/2013 – 05/2017 Wuhan Union Hospital (CN)

10/2011 – 06/2013 MSc Pharmaceutical Chemistry - Wuhan University (CN)

10/2007 – 06/2011 BSc Pharmacy - Hebei University of Science and Technology (CN)