Faculdade de Farmácia, Universidade de Lisboa

Evaluation of the parameters of a bead milling process to produce a non-aqueous nanosuspension

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

O crescente número de moléculas pouco solúveis em solventes aquosos exige abordagens inovadoras na formulação de modo a atingir a biodisponibilidade necessária para que se obtenha o efeito terapêutico desejado. As nanosuspensões apresentam muitas vantagens, sendo de destacar o aumento na solubilidade e biodisponibilidade das substâncias ativas, pelo que constituem uma abordagem alternativa para solucionar este problema. O processo de moagem utilizando esferas é frequentemente utilizado devido à sua simplicidade e aplicação abrangente a diversas moléculas, independentemente das suas propriedades. Este processo tem sido largamente estudado em meio aquoso. No entanto, a sua utilização em meios lipofílicos tem sido limitada, sendo necessário desenvolver mais estudos de forma a explorar o grande potencial destas formulações para o desenvolvimento de uma forma farmacêutica final tendo em vista o perfil de libertação de fármacos.

Este trabalho tinha como objetivo a produção de nanopartículas em meio lipofílico, utilizando moinhos de esferas para moagem. Com esta finalidade, foram utilizados triglicéridos de cadeia média (miglyol® 812) como meio de dispersão, diclofenac sódico como fármaco modelo com propriedades hidrofílicas, tensioativo (maioritariamente span® 85) e esferas com cerca de 0,3 milímetros de diâmetro.

Neste estudo, procedeu-se à variação da quantidade de substância ativa, meio de dispersão, esferas e tensioativo, assim como o tipo de tensioativo, o tipo de substância ativa e a velocidade de agitação. Após análise das propriedades das formulações preparadas, selecionou-se a melhor combinação, a qual foi posteriormente utilizada nos estudos subsequentes executados tendo em vista a identificação dos parâmetros mais relevantes e a avaliação da sua influência na redução do diâmetro médio das partículas e na suspensão final. A melhor combinação incluía o span® 85 como tensioativo e o diclofenac sódico como substância ativa, pelo que estes foram maioritariamente utilizados nas experiências subsequentes. No entanto, para testar a influência da variação do tipo de tensioativo e do tipo de substância ativa no processo, o tensioativo KF-6105 e a dexametasona foram também utilizados em algumas experiências. As nanosuspensões obtidas no final do processo foram analisadas por espectroscopia de correlação fotónica e por microscópia ótica.

Foi possível obter nanosuspensões de diclofenac sódico em meio lipofílico, após um processo de moagem de 6 horas. O tamanho médio das partículas variou entre 326,63 e 836,50 nanómetros. Os resultados obtidos propõem que diferentes substâncias ativas influenciem de forma distinta a suspensão final, devido à natureza das diferentes interações da molécula com o tensioativo e com o meio de dispersão, como consequência das diversas estruturas moleculares e grupos químicos presentes. O tipo e a quantidade de tensioativo isoladamente parecem não influenciar diretamente a redução do diâmetro médio das partículas, embora ambos tenham um papel crucial na prevenção da aglomeração de partículas. Dentro dos limites estudados, a quantidade de substância ativa não mostrou afetar diretamente o processo de redução do diâmetro médio das partículas. No entanto, a diminuição da razão tensioativo/substância ativa parece aumentar o diâmetro médio das partículas e o número de aglomerados. Neste trabalho é proposto que a diminuição da razão sólido/líquido, representada maioritariamente pela razão esferas/meio de dispersão, permite o aumento da velocidade de agitação do processo, sem que resulte na instabilidade do sistema, como acontece quando esta razão é muito elevada. O aumento da velocidade de agitação e uma maior quantidade de esferas normalmente favorecem a diminuição de tamanho das partículas. No entanto, é igualmente expectável que o excesso de energia, que pode ser provocado pela elevada velocidade e/ou pela menor quantidade de esferas devido à diminuição da energia necessária para as mover, possa também levar à formação de aglomerados. A duração do processo é um fator fundamental na avaliação dos parâmetros do processo, tendo o seu aumento um impacto positivo na redução do tamanho das partículas e da homogeneidade da suspensão final. Pela avaliação destes resultados entende-se que é crucial a otimização dos parâmetros em conjunto, tendo em consideração as razões entre si e não apenas a otimização individual de cada um, para que se consigam obter os melhores resultados possíveis.

Palavras-chave: Nanosuspensões não aquosas; Moinhos de esferas para moagem; Triglicéridos de cadeia média; Diclofenac sódico

Abstract

The increasing number of poorly soluble drugs requires innovative formulation approaches to reach high drug bioavailability. Nanosuspensions have many advantages, including improved solubility and bioavailability of drugs. Bead milling process is a method frequently used to produce nanosuspensions due to the simple use and wide application, independently of drug properties. Wet bead milling in aqueous medium has been extensively studied, although, bead milling process in lipophilic medium is not so common and requires further research to assess the impact on release profiles and simplification of the formulation process.

This work aimed to produce nanoparticles in lipophilic medium using bead milling process, to identify the relevant parameters and understand how they influence the final nanosuspension. Medium-chain triglycerides (Miglyol® 812) were used as milling medium and diclofenac sodium as a hydrophilic drug model. In each experiment the amount of API, milling medium, beads and surfactant were varied, as well as the type of surfactant, type of API and the stirring speed. The final nanosuspensions were characterized by photon correlation spectroscopy and optical microscopy.

Non-aqueous nanosuspensions of diclofenac sodium were successfully prepared during a 6-hour process. The mean particle size of the obtained nanoparticles ranged from 326.63 to 836.50 nm. The results of this research work indicate that the use of distinct drugs influences the final nanosuspension properties. The type and amount of surfactant seem not to influence particle size reduction when considered alone, although, they have played a major role in avoiding particle agglomeration. In the studied range, the API amount did not directly influence particle size reduction. However, the decrease in surfactant/drug ratio appears to increase both particle size and agglomeration of particles. Increasing speed and beads amount usually favours particle size reduction, although, an excess of energy input may also favour the development of agglomerates. Milling time is a very important factor, which decreases MPS and particle size distribution. It is crucial to evaluate all parameters together and not individually to optimise the process and obtain the best results.

Keywords: Non-aqueous nanosuspension; Bead milling; Medium-chain triglycerides; Diclofenac sodium

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Acronyms

API – Active pharmaceutical ingredient

DEXA – Dexamethasone

DICL – Diclofenac sodium

EXP – Experiment

GAS - Gas anti-solvent recrystallisation

HPH - High-pressure homogenisation

LD - Laser diffraction

MPS – Mean particle size

NP – Nanoparticles

NS - Nanosuspensions

PCS – Photon correlation spectroscopy

PDI - Polydispersity index

PSR – Particle size reduction

RELGS - Rapid expansion of liquefied gas solution

SONS – Solid-in-oil nanosuspensions

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1 Introduction

1.1 Definition of nanosuspensions

The increasing number of poorly soluble drugs requires innovative formulation approaches to reach drugs high bioavailability. There are many conventional methods for increasing the solubility of poorly soluble drugs, which include micronisation, solubilisation using co-solvents, salt form, surfactant dispersion and lipophilic solution (1). Other techniques like liposomes (2), emulsions (3), microemulsions (1), solid dispersions (4) and cyclodextrins (5) show sensible achiever, but they lack universal applicability to all drugs (6). All these approaches are mostly limited by the physicochemical properties of the drug, such as solubility in oil, the need for a certain molecular size to fit into the cyclodextrin ring or the toxicity associated with some organic solvents. Micronisation of drug powders to sizes between 1 and 10 µm to increase the drug surface area, and thus improve overall dissolution profile, is used for Biopharmaceutical Classification System (BCS) class II drugs, i.e. drugs having a good permeability and poor solubility. Nowadays, many of the new drugs exhibit such a low solubility that micronisation does not lead to a sufficiently high bioavailability (7). Consequently, the next step was to move from micronisation to nanonisation, producing drug nanocrystals, which are nanoparticles (NP) being composed of 100% drug without any matrix material (7).

In the past decades, the number of therapeutics based on nanoparticles, which are particles between 1 and 1000 nm, has increased in several areas, such as cancer, vaccination, inflammatory diseases and others due to its large advantages (8–10). Nanosuspensions (NS), defined as colloidal dispersions of nanosized drug particles (11), provide the opportunity to increase solubility and bioavailability of BCS class II and class IV drugs (12,13) due to the higher surface area of the particles, which enables a faster dissolution rate in accordance with the Noyes-Whitney law (14). Considering the selective accumulation of NP in certain organs depending on their surface properties, improvement of the active pharmaceutical ingredient (API) selectivity and consequent reduction of the adverse effects and the dose needed to produce the desired bioactive effect can also be achieved with NS (15). Comparing with other pharmaceutical dosage forms, NS have the major advantage of being applicable to drugs which are poorly soluble in both aqueous and organic media (16). Regarding the intravenous (IV) administration of poorly soluble drugs, NS are an interesting approach since they can reduce the injection volume, compared to a

solution of the same drug (17). NS are able to extend drug half-life by enhancing its stability (18) and increase drug loading (19). In order to improve drug stability, solvents can be removed by evaporation or lyophilisation to obtain solid dosage forms (20).

NS are used in several administration routes, such as oral (21,22), parenteral (23,24), ocular (25,26), pulmonary (27), transdermal (28) and topical (29) delivery systems.

1.2 Nanotechnology-based production methods

NP can be obtained either by top-down or bottom-up techniques. Top-down techniques are based on the reduction of larger particles into smaller particles, whereas bottom-up techniques consist of the growth of small particles from individual molecules, achieved by supersaturation (30).

1.2.1 Top-down technology

Top-down approaches for drug nanocrystal production comprise media milling and high-pressure homogenisation (HPH). Typically, these production processes are conducted in liquid, hence forming a NS (31).

Media milling

The first-generation disintegration technique is pearl milling, developed by Liversidge, leading to the product Nanocrystals® (32). In this method, the components include a suspension of the drug in medium (usually water) with a stabiliser and the milling pearls or beads (typically made from glass, zircon oxide, or polystyrene resin), which rotate at high speed (6). High energy and shear forces generated as a result of friction and collisions among pearls and the drug particles provide the necessary energy input to disintegrate the drug microparticles into NP (33).

During this process it will occur particle size reduction (PSR) due to fragmentation, but at the same time, particle growth could also happen due to collisions between particles (34). Therefore, the probability of collisions between drug NP should be reduced to a minimum. Having this into consideration, increased drug amount, higher speed and longer milling time are parameters that could enhance particle growth and agglomeration because of this effect (35). Large number of milling pearls/beads could be beneficial considering the extended contact points with the API particles,

although an excessive amount will increase weight and promote collisions between the pearls/beads, consuming more energy (34).

The characteristics of the API, specially its molecular arrangement (porosity, amorphous/crystal state, polymorphic forms), have also influence on particle size reduction, for example an amorphous state and higher porosity will favour this process (36).

As the total particle surface area of the resulting NS is typically orders of magnitude larger compared to a coarse suspension, additives may be necessary to ensure adequate stabilisation. Therefore, a careful evaluation of the type and concentration of the stabiliser used is needed towards the successful production of NS. Both polymeric and surfactant stabilisers can be used for this purpose (37). Low temperature is able to modify the drug crystal structure and increase its friability, in this way, this factor could have a positive impact on particle size reduction (38). Since optimal values for the different parameters change with drug characteristics, surfactant type, medium, requested particle size and other factors, every process should be optimised to obtain the best results (35).

This technique is applicable to drugs insoluble in both aqueous and non-aqueous media, is suitable for large-scale production and therefore commercialisation, enables formulation of very diluted, as well as, highly concentrated drug NS and a narrow particle size distribution with small batch-to-batch variation can be obtained once the optimal formulation and progress is achieved (6,11,39). The main disadvantage of this method is the erosion of pearls, which may lead to contamination of the final product. This may constitute a problem when NS are intended to be administered as a chronic therapy. However, the severity of this problem is reduced with the use of polystyrene resin-based milling medium (11). Other drawbacks are the cost and duration of the process, which may reduce product efficiency and increase the risk of contamination (6).

<u>High-pressure homogenisation</u>

Homogenisation methods include microfluidisation and piston gap homogenisation. For the preparation of a nanosuspension using these methods, it is essential to prepare a presuspension of the micronized drug in a surfactant solution using a high-speed stirrer. Microfluidiser technology uses a chamber where a stream of suspension is divided into two or more parts. The frontal collision of the two fluid

streams under pressure leads to particle collision, shear and cavitation forces, which results in particle size reduction. The major disadvantage of this technique is the high number of cycles through the microfluidiser and the relatively high fraction of microparticles that the obtained product contains (7,40).

Regarding piston gap homogenisation methods, the pre-suspension is pumped from a pipe into a thin gap. The pressure provided by the pump converts to kinetic energy as the suspension passes through the narrow gap. In accordance with Bernoulli's equation, the static pressure of the fluid simultaneously decreases below the vapour pressure of water causing the boiling of the fluid. As the suspension leaves the gap, the pressure suddenly rises to the atmospheric pressure and the vapour bubbles implode vigorously. The combination of this phenomena named cavitation, fluid shear and particle collision, lead to the transformation of microparticles into NP (17,40,41). If the homogenisation occurs in aqueous media, it is called Dissocubes®. On the other hand, if it is a water-free media or a combination media with water it is called Nanopure®. The first operates at high pressure causing the water to boil at room temperature and suffer cavitation after passing through the gap, while the latter is conducted at low temperature to compensate the high boiling point and low vapour pressure of lipophilic fatty acids and oils, insufficient for cavitation (39).

Higher homogenisation pressure will result in increased velocity of the fluid in the gap. Therefore, it will extend the reduction of the static pressure and boost the cavitation process. Consequently, it is expected that the higher the homogenisation pressure, the smaller the particle size. The number of cycles depends on the hardness of the drug and the desired particle size. The flow rate of the fluid varies accordingly to the zone inside the pipe (in the centre or near the wall). Therefore, different extension in the cavitation will occur and the production of a nonhomogeneous particle size distribution will be expected. Taking this into account, an increased number of cycles will reduce the particle size and produce a narrow size distribution (41–43). There is an increase of temperature in the homogenisation progress, which should be strictly controlled in case of temperature-sensitive drugs, although, this problem can be avoided by placing a heat exchanger ahead of the homogenizer valve (44). Similarly to the previous method, the total surface area of the resulting NS particles is largely increased, therefore, surfactants and stabilisers may be needed to ensure adequate stabilisation (31).

Production of NS by high-pressure homogenisation possesses high reproducibility (16) and high productivity can be obtained with a very low microparticle content in the

product, which is favourable for industrial implementation (6). Furthermore, metal contamination due to the erosion is less pronounced than in media milling (45). The main drawback of this method is the need for pre-treatment to obtain microparticles before starting the homogenisation process (46).

1.2.2 Bottom-up technology

Bottom-up approaches hold good potential with respect to improve bioavailability by obtaining smaller particle sizes and amorphous drug particles. The driving force for the growth of a crystal from individual molecules is supersaturation. Supersaturation of a drug in a solution can be obtained by decreasing the temperature or adding an anti-solvent. The size of the formed crystals depends on the balance between nucleation rate and crystal growth (30). The basic advantage of precipitation techniques is the use of relatively simple and low cost equipment (6). However, particle growth to microcrystals is an important problem associated with precipitation techniques, which needs to be avoided (40).

Hydrosol

This technique takes advantage of the variation in the solubility of the same drugs in different but miscible liquids. In this method, the drug is dissolved in a solvent (usually organic solvent) and then this solution is mixed with a large amount of anti-solvent that is miscible with the first solvent (generally water) in the presence of a surfactant (47). Mixing the organic solution with the anti-solvent should be performed rapidly to assure fast nucleation and thereby small particles (30). As drawbacks, this method requires the drug to be soluble at least in one solvent and this solvent needs to be miscible with a non-solvent, high nucleation rate and low crystal growth rate of the drug to reach a stable suspension with minimum particle size and the use of organic solvents, which could potentially have human safety concerns (6,7).

To obtain uniform NP, stirring rate, drug content, temperature and volume ratio antisolvent/solvent should be optimised. The increase in stirring rate favours the decrease of the particle size by enhancing the rate of diffusion of drugs between the two phases, promoting a rapid nucleation. A larger volume ratio of antisolvent to solvent and lower temperature also contribute to a faster nucleation, due to a higher supersaturation. The increased drug concentration will hinder the diffusion between the two phases consequently, thereby leading to a non-uniform supersaturation and increase the probability of particle aggregation (6,48).

Emulsion - solvent diffusion method

Apart from the use of emulsions as drug delivering vehicles, emulsions can also be used to produce NS. This is applicable for those drugs that are soluble in either volatile organic solvent or partially water-miscible solvent. The first step is the dispersion of the drug in an organic solvent or mixture of solvents. The organic phase loaded with the drug is then dispersed in the aqueous phase, containing surfactants, to form an emulsion (3). The obtained emulsion is further homogenised by high-pressure homogenisation or other techniques. After homogenisation, the emulsion is diluted with water, and again homogenised to diffuse the organic solvent and convert the droplets into solid particles. Since one particle is formed in each emulsion droplet, it is possible to control the particle size of the nanosuspension by controlling the size of the emulsion (49). This technique cannot be used for drugs that are poorly soluble in both aqueous and organic media. The selection of solvent and stabiliser is critical to obtain drug particles within the nanometre range. Optimising both parameters will increase the intake of organic phase and ultimately the drug loading in the emulsion. The use of organic solvents and the high amount of surfactant/stabiliser required are major drawbacks of this technology due to potential environmental hazards and human safety issues (11,50).

Melt emulsification method

In this method, the drug is dispersed in the aqueous solution with a stabiliser. In a second step, the NS is heated above the melting point of the drug and homogenised to produce an emulsion. During this procedure, the temperature must be controlled and maintained above the melting point of the drug. The final step is cooling off the emulsion to a suitable temperature, either at room temperature or in an ice bath (49,50). Factors affecting particle size include drug and stabiliser concentrations, type of stabiliser, and cooling conditions. Solvent-free prepared NS are particularly important from the safety point of view. Therefore, the advantage of this method over solvent diffusion method is the avoidance of organic solvents (46).

Supercritical fluid methods

Although there are currently many different processes to prepare drug nanocrystals based on supercritical fluid technologies, they are mainly based on gas anti-solvent recrystallisation (GAS) and rapid expansion of liquefied gas solution (RELGS). In GAS, the supercritical fluid acts as an anti-solvent (30). A solution of the lipophilic

drug in an organic solvent is saturated with supercritical fluid (such as supercritical carbon dioxide), thereby decreasing the solubility of the drug in the solvent and consequently causing the drug to precipitate (51). The other supercritical fluid technique, RELGS, the supercritical fluid acts as a solvent. The lipophilic drug is dissolved in the supercritical fluid and stabilised by the presence of surface modifiers. After this step, the pressure is rapidly decreased, reducing the solvent power and causing rapid drug precipitation from the supercritical fluid (52,53).

1.2.3 Combination of methods

Top-down processes have mainly two limitations: the need for micronized drug as starting material and the long processing time (54). Combinative methods have been developed to overcome these limitations and improve particle size reduction effectiveness (55). These methods usually involve a pre-treatment step followed by a high-energy process for particle size reduction. Pre-treatment of drug, before top-down processes, can produce very small particles with a narrow size distribution, with less time or fewer cycles, compared with the unmodified drug (36,56,57).

NANOEDGE® was created by Baxter to avoid the growth of drug nanocrystals in the precipitation technique, adding an homogenisation step after the precipitation process (31). In this method, crystals are precipitated, and the obtained suspension is then subjected to high-pressure homogenisation (58). The main drawback comparing to top-down technologies is the use of organic solvents, due to the precipitation pre-step.

There are also different combination processes involving freeze-drying, spray-drying, counter flow precipitation or parallel flow precipitation combined with HPH (59).

1.3 Nanosuspension characterisation techniques

Since particle size, morphology, crystalline state and surface characteristics of a NP have a key role in its biodistribution, target ligand, cellular uptake, overall drug efficiency and toxicity *in vivo*, it is very important to use the appropriate techniques to characterise NP (60–64).

The most important characteristics of NS are particle size and polydispersity index (PDI). According to Noyes-Whitney equation, decreasing particle size increases the respective surface area, which in turn will increase drug solubility and consequently enhance dissolution rate (14). As it is observed in Figure 1, NP present a marked

increase in surface area compared to microparticles. This is an important point to consider, which justifies the large improvement in the dissolution rate and consequently bioavailability of some drugs (46).

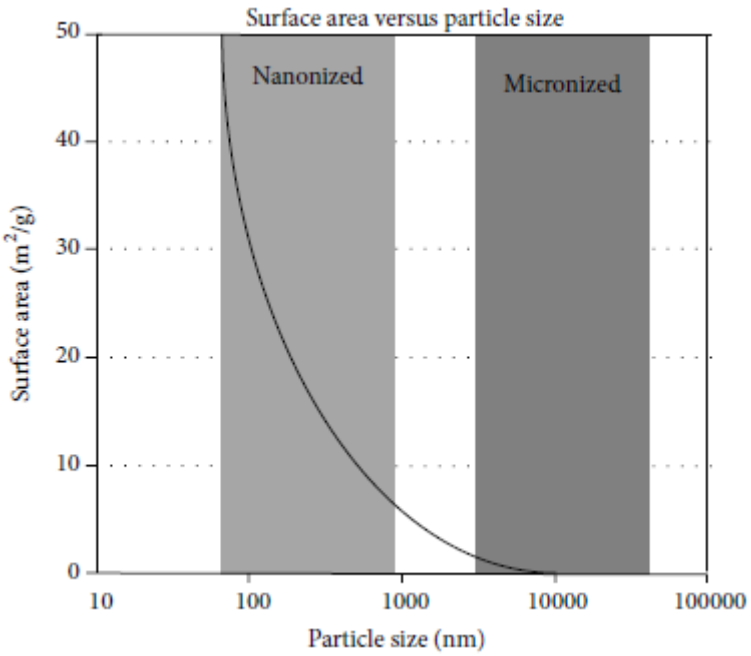

Figure 1 Ratio between particle size and particle surface area in microparticles and nanoparticles. Adopted from (46)

Size

The optimum particle size of NS depends on the target and the desired biopharmaceutical properties. When a very fast dissolution is required, a NP size between approximately 100 to 200 nm is preferred. On the other hand, if prolonged dissolution is desired, the mean particle size (MPS) should be around 900 nm (41).

The MPS and width of distribution named PDI can be determined by a variety of techniques, such as laser diffraction (LD), photon correlation spectroscopy (PCS), microscope and coulter counter although it is typically determined by PCS (35,36,38,65). PDI is an important parameter that governs the physical stability of NS and should be as low as possible for the long-term stability of NS. A PDI value between 0.1 to 0.25 shows a narrow size distribution, while above 0.5 indicates a broad distribution (49).

PCS, also known as dynamic light scattering, is a rapid and accurate method. In this technique, the Brownian motion, which is the movement in random direction, of

particles is measured as a function of time. Larger particles move with lower velocity than smaller particles and may settle out of the measurement zone. Hence, the measuring range is limited between 3 nm and 3 µm approximately. Therefore, laser diffraction is used as a complement to PCS to detect any content of particles in the micrometre range or agglomerates of drug NP. Depending on the type of equipment employed, the measuring range of LD is approximately from 0.05 to 80 µm up to maximum of 2000 µm (41,65).

LD data are volume data, typical characterisation parameters are the diameters 50, 90, 95, and 99%, meaning the percentage in volume of the particles below the given size in micrometres, being the diameters 90, 95 and 99% sensitive markers for the presence or the disappearance of large particles during the process (40).

For NS to be administered intravenously, an additional analysis by Coulter counter technique is essential, since it gives the absolute number of particles per volume unit for the different size classes, in contrast to the LD providing only a relative size distribution (49). The size of the smallest blood capillary is about 5 µm, so even a small content of particles greater than 5 µm may cause capillary blockade and embolism. Therefore, the content of microparticles in NS needs to be controlled by Coulter counter analysis (6).

Optical and electron microscopy are routinely used for measuring particle size, although optical microscopy cannot be used when the particles are smaller than the wavelength of visible light (46). But in this range, other imaging, spectroscopic and separation techniques may also be used for measuring particle size (31,66).

Morphology

Regarding the shape of NP, spherical particles have a higher dissolution rate than irregular particles (64). Morphology of nanocrystals can be determined using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). A wet sample of suitable concentration is needed for the TEM analysis. On the other hand, SEM uses the dried powder as sample (6). For this reason, it is commonly used when the original NS undergo through a drying process, such as spray drying or lyophilisation. The water removal process may lead to agglomeration and increased particle size, which should be monitored through SEM analysis (67).

SEM produces images of the surface of a sample by scanning it with a focused beam of electrons and detecting the various signals produced when the electron beam

interacts with electrons in the sample. In TEM, the electron beam passes through the sample and only NP with a sufficient combination of electron density and size can be detected, being imprecise for cores smaller than 2 nm (66).

Crystalline state

The evaluation of crystalline state is necessary to understand the polymorphic changes that a drug might undergo during nanosizing process. Some drugs are converted to the amorphous state during HPH process. Although it is a state with higher solubility compared with crystalline state, it is also more instable and should be controlled (68). In addition, some drugs exist in different polymorphic forms, and these isoforms also need to be controlled (41). X-ray powder diffraction (XRPD) is the most convenient way to determine crystal forms, since different crystal forms produce different diffraction patterns. Although a relatively large amount of drug is usually required for detailed investigation, the sample is reusable (69). Differential scanning calorimetry (DSC) can supplement X-ray diffraction by detecting crystalline and amorphous fractions (11). It measures the temperatures and heat flows associated with the transition in drugs from crystalline to amorphous state as a function of time and temperature in a controlled atmosphere. Applying this technique, few milligrams of the bulk sample are needed for the measurement (70). Terahertz (71), Infrared (72) and raman (73) spectroscopies are other methods that can be used in the evaluation of the crystalline structure of drug nanocrystals.

Surface charge

Particle's surface charge is typically measured by electrophoresis, upon application of an electric field, and expressed as electrophoretic mobility, which can be converted to zeta potential by using the Helmholtz-Smoluchowski equation (74). Zeta potential characterises the surface charge of particles and thus it gives information about repulsive forces between particles and droplets (49).

The measurement of zeta potential allows predictions about the storage stability of colloidal dispersions. In general, particle aggregation is less likely to occur for charged particles, with high zeta potential, due to electric repulsion (75). To obtain a physically stable nanocrystal suspension, preventing flocculation and coalescence, a zeta potential of at least ±30 mV is required, in case the NS is solely stabilised by electrostatic repulsion and ±20 mV for a combined electrostatic and steric stabilisation (41,76).

Saturation solubility and dissolution velocity

The determination of the saturation solubility and dissolution rate is very important as these two parameters are improved in NS and influence the in-vivo performance (blood profiles, plasma peaks and bioavailability) of the drug (11). Theoretically, saturation solubility is a compound-specific constant only depending on the temperature and the properties of the dissolution medium. Nevertheless, below a size of approximately 2 µm, the saturation solubility increases with a decrease in the particle size (6,64,77). Dissolution rate of drug NS should be determined in a suitable buffer according to dissolution test procedures reported in Pharmacopoeia monographs (78). To determine the saturation solubility of the drug, shake methods at different temperatures and distinct physiological buffers (e.g. artificial gastric or intestinal juice) should be performed until equilibrium has been reached (41).

In-vivo biological performance

The establishment of an *in-vitro/in-vivo* correlation and the monitoring of the *in-vivo* performance of the drug is an essential part of the study, irrespective of the route and the delivery system employed. Although, different *in-vitro* and *in-vivo* tests should be performed according to the different routes of administration to understand the behaviour and biodistribution of the drug in that specific tissue. Examples of these tests are muscular irritation study and skin permeation using modified Franz-type diffusion cells (79,80). Intravenous injectable NS require the determination of additional parameters since drug effectiveness depends on its biodistribution and target ligand, which consequently depends on NP surface properties, such as hydrophobicity and plasma protein interactions (81,82). Methodologies such as hydrophobic interaction chromatography can be used to determine surface hydrophobicity (83), whereas 2-D PAGE (84) can be employed for the quantitative and qualitative measurements of protein adsorption after IV injection of drug NS in animals. Basic correlations could be established between the protein adsorption patterns and the organ distribution, which can facilitate the production of target-specific NS in a controlled way (41).

1.4 Applications of lipophilic nanosuspensions

Ophthalmic drug delivery

Local ocular formulations are the most common and safe route of administration for the treatment of various eye disorders, considering the lower side effects compared

to the systemic route (85). However, drug bioavailability following an ophthalmic application is very poor due to the rapid removal of the formulation from the surface of the eye, through blinking, baseline and reflex lachrymation and drainage. To counteract this effect, frequent instillations of eye drops are used, although this may induce toxic side effects and cellular damage at the ocular surface (86). Furthermore, the relatively impermeable corneal barrier restricts the entry of foreign substances, resulting in a drug penetration of less than 5% of the administered drug. Several approaches have been proposed, although none of them was optimal (87).

Nanoparticulate suspensions constitute an alternative to improve the drug delivery upon an ophthalmic application by enhancing the sustained release and preventing tear washout, due to their adhesion properties and increase ocular bioavailability over aqueous solutions (88,89). Considering their liquid state, they do not interfere with vision, thus increasing their potential as drug delivery systems for ocular tissues (87). In contrast to the polymeric NP, NS for ocular use have also a regulatory advantage, as many polymers are not approved by official authorities and NS are purely composed of drug, medium and comparatively small amount of stabiliser (55).

Lipophilic NS might even increase drug delivery through the cornea over aqueous NS, by sustaining the drug release, since oil act as drug reservoirs. Lipophilic NS might also provide higher penetration of the drug into the deeper layers of the eye (90).

Oral drug delivery

Granath and Sigfridsson compared the bioavailability of an API following the oral administration of an aqueous nanosuspension, lipophilic solutions (mainly constituted by miglyol® 812) and a fat diet. The two latter strategies resulted in significantly higher *in vivo* exposure after the oral administration compared to the aqueous NS (91).

Persson *et al.* observed that the intestinal absorption and bioavailability of certain poor water-soluble drugs are increased when those drugs are administered as NS in lipid-containing medium. Furthermore, the absorption is decreased when administered as a solution in the same medium (92). An explanation for the last observation may be the increased adhesiveness and penetration properties of nanocrystals compared to the dissolved particles (55). These results demonstrate not

only the importance of the concomitant lipid administration in drug bioavailability, but also the importance of lipid NS over lipid solutions.

Another advantage of the concomitant drug and lipids administration, is the possibility for drug delivery by lymphatic transport through intestine avoiding hepatic first-pass metabolism (93). Lipid excipients may influence gastrointestinal drug absorption in many ways, including improving drug solubility, membrane permeability in gastrointestinal tract, inhibition of P-glycoprotein and CYP enzymes, influence the production and secretion of intestinal lipoproteins. These will result in increased bioavailability (94–96).

Intramuscular drug delivery

Intramuscular long-acting formulations are currently available on the market in several areas, such as contraception and psychiatric disorders. Most long-acting formulations are based on the incorporation of the API in either an oil-based solution or suspension, an aqueous suspension or a suitable matrix from which the API is slowly released (97–99).

Wei et al. prepared a lipophilic nanosuspension of curcumin decanoate by wet ball milling. Although the lipophilic nanosuspension appeared to exhibit slower clearance from the injection site compared to the lipophilic microsuspension, the NS achieved higher plasma and brain concentrations during longer periods of time (100). Hu et al. obtained similar results (101). These studies demonstrate that lipophilic NS are a viable option as a delivery system for long-acting intramuscular administration (100,101).

Subcutaneous drug delivery

Kraft et al. developed a lipid stabilised nanosuspension, for subcutaneous delivery, which enabled persistent drug levels in lymph nodes, blood cells and plasma. Significant enhancements were observed in overall intracellular drug levels of a hydrophilic drug, compared to the non-lipid-based formulation. The long-acting behaviour is of great interest to improve patient adherence (102). In most of the other delivery routes previously described, the improvement in drug bioavailability resourcing to lipid-based formulations was proved for lipophilic drugs. However, this study demonstrates the advantages of using lipid-based formulations as dispersion medium for a hydrophilic drug.

Topical and transdermal drug delivery

Topical treatment of skin diseases or local treatment of other pathologies have the advantage related to the possibility for high drug levels at the site of disease and thus lower systemic side effects compared to oral or parenteral drug administration (103). Nevertheless, skin constitutes an excellent barrier and presents difficulties for the transdermal delivery of drugs, since few drugs are able to permeate across the stratum corneum in levels sufficient to reach a therapeutic concentration in the blood or the site of action (104). Due to this barrier, permeation of hydrophilic drugs through the skin is generally low. However, it can be enhanced by appropriate dispersion medium and surfactant (79).

Nanocrystals have received considerable attention in dermal application due to their ability to enhance delivery through the skin and overcome bioavailability issues caused by poor drug solubility in water and oil (86). Vidlárŏvá *et al.* showed that a drug suspended in a dermal formulation yields superior penetration compared to a drug in solution, as when a molecule is dissolved in a favourable environment, the stimulus to leave this environment and penetrate in the skin is smaller, due to the lower solubility (86).

Piao *et al.* successfully enhanced dermal/transdermal permeability of diclofenac sodium, a hydrophilic drug, resourcing to solid-in-oil NS (SONS) (105). SONS improve the dispersity of hydrophilic drugs into a lipophilic phase and proved to be a good approach for enhancing the percutaneous absorption of diclofenac sodium. The present formulation is applicable to enhance permeation of other hydrophilic drugs and proteins into the skin (105,106).

Low viscosity of dermal formulations seems to promote penetration into the skin, therefore the use of low viscous oils, such as miglyol® 812, might be a good approach (86,91).

General advantages of lipid-based nanosuspensions

Some drugs suffer a very fast degradation in water, which constitutes a major drawback when formulating an aqueous nanosuspension. Lipophilic NS can protect the API against hydrolysis and limit its oxidation due to its lower water content and use of surfactants, which will increase stability of the product (107,108).

Frequently, nanocrystals are produced with the purpose to be incorporated into dermal formulations. In order to achieve more stable formulations, with less water content, aqueous NS are lyophilized for subsequent incorporation into creams and gels (100,109). Lipophilic NS could improve this process, leading to a more cost-effective, less time-consuming and less toxic process, by producing the nanocrystals directly in lipophilic medium.

Prolonged release is a major advantage of lipophilic NS, observed in several different routes of administration. The long-acting behaviour is of great interest to improve patient adherence (100,102).

Lipid-based formulations have been shown to enhance bioavailability of some drugs administered by oral, dermal, ophthalmic, intramuscular and subcutaneous delivery routes (90,94,100,102,105). Lipid-based formulations induce drug transport through the gastrointestinal lymphatic system, which may bypass the liver and avoid hepatic first-pass metabolism (93,110). Along with this advantage, lipids may also inhibit efflux transporters and achieve higher membrane permeability, increasing drug absorption, and consequently better bioavailability (111,112).

Lipid coating of ionically charged NP increased blood-brain barrier crossing 3- or 4-fold compared with uncoated particles. These lipid-coated NP were non- toxic toward the endothelial cell integrity, and crossed it without degradation (113).

Due to its structural properties, some lipids promote adsorption of TLR ligands to aluminium salts, enhancing immune response to vaccine antigens (114).

2 Aims of the project

Particle size reduction of slightly soluble drugs in aqueous media is frequently used, although particle size reduction in non-aqueous media is not so common and may have positive impact in the release profiles, drug bioavailability and offer the possibility for a final dosage form or an intermediate product that could easily be formulated into the ultimate product. Since preparation of a drug NS by bead milling is a simple and easy method, it can be used to develop a formulation to improve the bioavailability of drugs, by selecting the appropriate dispersion medium and surfactant.

The major aims of this work were to produce NP in lipophilic medium using bead milling process, identify relevant process and formulation parameters and understand their influence in the obtained nanosuspension.

For this purpose, medium-chain triglycerides-based compound (Miglyol® 812) was used as milling medium and diclofenac sodium (DICL) as a model hydrophilic API.

3 Materials and Methods

3.1 Materials

Dexamethasone (DEXA) was provided by Bausch & Lomb (Berlin, Germany); Diclofenac sodium (BASF SE, Ludwigshafen, Germany); Medium-chain triglycerides (Miglyol® 812, Caesar & Loretz GmbH, Hilden, Germany); Sorbitan trioleate (Span® 85) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland); Lauryl polyglyceryl-3 polydimethylsiloxyethyl dimethicone (KF-6105) (Shin-Etsu Chemical Co. Ltd., Tokyo, Japan); 0.25-0.35mm Zirconium oxide beads yttrium stabilised (Sigmund Lindner GmbH, Warmensteinach, Deutschland). All other chemicals and reagents used in the study were of analytical grade.

3.2 Methods

3.2.1 Nanosuspensions preparation

NS were prepared by bead milling. Surfactant was completely dissolved in miglyol® 812 using a magnetic stirrer (neoLab, Heidelberg, Germany), after weighting both components directly on the beaker. The grinded API was added to the previous mixture and stirred to obtain a homogenous suspension, or until saturation. Following the subsequent addition of the beads to the beaker, the suspensions were stirred for 6 hours. Samples were acquired every 1.5 hours, starting immediately after the beginning of the process. At the end of the process, the suspension was filtered through a sieve to retain the beads.

In each experiment (EXP) the amount of API, medium, beads and surfactant were varied, as well as the type of surfactant and the stirring speed. Among several combinations, the best one (number 0) was chosen and some variations were applied to understand how the process and formulation parameters influence the produced NP. The best combination used contained span® 85 as surfactant, therefore, this surfactant was selected for further experiments. However, to observe the impact of changing the type of surfactant on the produced NS, KF-6105 was tested in one experiment. Diclofenac sodium was the most used API and dexamethasone was tested in one experiment to validate the influence of the API in this process. The instability caused in some experiments when higher speed was used, forced the use of lower speed in these experiments. Beads were reused between experiments after a drying process in the oven with absorbing paper,

containing a small amount of remaining oil and surfactant molecules. Beads used for different surfactants or API were not reused. Washing of the beads was not applied to avoid remaining detergent molecules, as this might interfere with experiment results.

The table below demonstrates the differences applied to each factor in each experiment, to study the influence of the parameters on the obtained NS.

Table 1. Parameters used to understand the impact of each variable on NS development

EXPERIMENT	API	SURFACTANT	SURFACTANT (mg)	MIGLYOL (g)	API (g)	BEADS (g)	SPEED (rpm)
0	DICL	Span 85	0.160	4.75	0.50	38.07	750
1	DICL	Span 85	0.320	9.50	0.50	38.07	1250
2	DICL	Span 85	0.160	4.75	0.25	38.07	750
3	DICL	Span 85	0.160	9.50	0.25	38.07	1250
4	DICL	Span 85	0.160	4.75	0.50	23.8	1250
5	DICL	Span 85	0.080	9.50	0.50	38.07	1250
6	DICL	Span 85	0.190	9.50	0.19	23.8	1250
7	DEXA	Span 85	0.160	9.50	0.25	38.07	1250
8	DICL	KF-6105	0.160	9.50	0.25	38.07	1250
9	DICL	Span 85	0.056	9.50	0.19	38.07	1000
10	DICL	Span 85	0.140	9.50	0.19	38.07	1250
11	DICL	Span 85	0.120	9.50	0.19	38.07	1250
12	DICL	Span 85	0.120	9.50	0.19	38.07	1000
13	DICL	Span 85	0.181	9.50	0.19	38.07	1000
14	DICL	Span 85	0.056	9.50	0.19	38.07	1000
15	DICL	Span 85	0.056	9.50	0.19	38.07	1000
16	DICL	Span 85	0.016	9.50	0.19	38.07	1000
17	DICL	Span 85	0.000	9.50	0.19	38.07	1000
18	DICL	Span 85	0.320	9.50	0.05	38.07	1000
19	DICL	Span 85	0.320	9.50	0.05	38.07	750
20	DICL	Span 85	0.320	9.50	0.01	38.07	1000

To support the discussion, a table with ratios and the molecular structures of diclofenac sodium, span® 85 and KF-6105 are presented in Table 2 and Figure 2.

Table 2 Ratios between certain parameters

EXPERIMENT	BEADS/OIL	SURF/API	SURF/API/OIL	BEADS/API	BEADS/SPEED
0	8.014736842	0.32	0.067368421	76.14	0.05076
1	4.007368421	0.64	0.067368421	76.14	0.030456
2	8.014736842	0.64	0.134736842	152.28	0.05076
3	4.007368421	0.64	0.067368421	152.28	0.030456
4	5.010526316	0.32	0.067368421	47.6	0.01904
5	4.007368421	0.16	0.016842105	76.14	0.030456
6	2.505263158	1	0.105263158	125.263158	0.01904
7	4.007368421	0.64	0.067368421	152.28	0.030456
8	4.007368421	0.64	0.067368421	152.28	0.030456
9	4.007368421	0.294736842	0.031024931	200.368421	0.03807
10	4.007368421	0.736842105	0.077562327	200.368421	0.030456
11	4.007368421	0.631578947	0.066481994	200.368421	0.030456
12	4.007368421	0.631578947	0.066481994	200.368421	0.03807
13	4.007368421	0.95	0.1	200.368421	0.03807
14	4.007368421	0.294210526	0.030969529	200.368421	0.03807
15	4.007368421	0.294210526	0.030969529	200.368421	0.03807
16	4.007368421	0.084210526	0.008864266	200.368421	0.03807
17	4.007368421	0	0	200.368421	0.03807
18	4.007368421	6.4	0.673684211	761.4	0.03807
19	4.007368421	6.4	0.673684211	761.4	0.05076
20	4.007368421	32	3.368421053	3807	0.03807

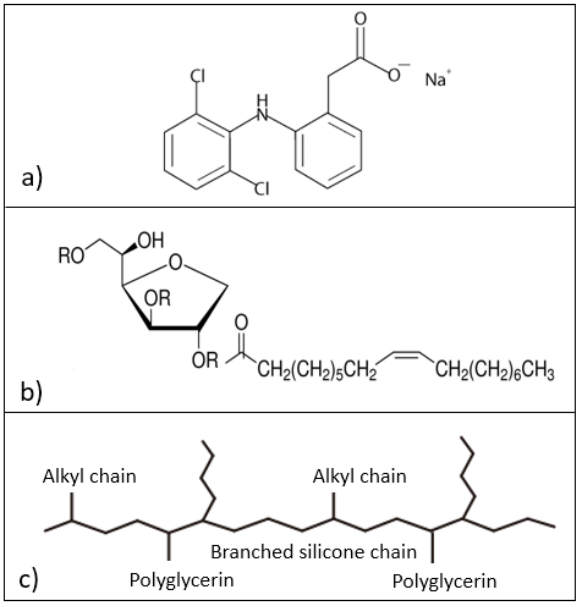

Figure 2 Molecular structures of the a) Diclofenac sodium b) Span® 85 c) KF-6105. Adopted from (115–117)

3.2.2 Particles characterisation

Microscopic images were obtained for all samples, immediately after sampling, using an optical microscope (Motic BA210, Motic Deutschland GmbH, Wetzlar, Germany) fitted with Moticam 3.0 M and Motic Images Plus 2.0 software (Motic Deutschland GmbH, Wetzlar, Germany). To simplify, only the microscopic pictures of the last sample, acquired at the end of the process (6 hours) from each experiment, are shown. As a demonstrative example of one experiment, it is shown the microscopic pictures of all samples acquired every 1.5 hours.

At the end of the process, MPS and PDI of NP were determined by PCS using a Zetasizer® Nano ZS (Malvern Instruments Ltd., Malvern, UK). The particle size and PDI of NP dispersed in the suspension were determined after dilution of 10 µl of suspension in approximately 2 mL of miglyol® 812. Ten measurements of each sample were made, and the mean value was used for both MPS and PDI.

4 Results

The characterisation of the final NS is represented in this section. The MPS and PDI index were determined by PCS and the microscopic pictures were acquired using an optical microscope.

Table 3. Mean particle size (MPS) and polydispersity index (PDI) values obtained with PCS

EXPERIMENT	API	SURFACTANT	SURFACTANT (mg)	MIGLYOL (g)	API (g)	BEADS (g)	SPEED (rpm)	MPS 6h (nm)	PDI
0	DICL	Span 85	0.160	4.75	0.50	38.07	750	506.71	0.214
1	DICL	Span 85	0.320	9.50	0.50	38.07	1250	525.92	0.326
2	DICL	Span 85	0.160	4.75	0.25	38.07	750	609.26	0.301
3	DICL	Span 85	0.160	9.50	0.25	38.07	1250	543.18	0.368
4	DICL	Span 85	0.160	4.75	0.50	23.8	1250	690.83	0.918
5	DICL	Span 85	0.080	9.50	0.50	38.07	1250	836.50	0.343
6	DICL	Span 85	0.190	9.50	0.19	23.8	1250	391.50	0.546
7	DEXA	Span 85	0.160	9.50	0.25	38.07	1250	276.40	0.860
8	DICL	KF-6105	0.160	9.50	0.25	38.07	1250	480.81	0.696
9	DICL	Span 85	0.056	9.50	0.19	38.07	1000	383.46	0.286
10	DICL	Span 85	0.140	9.50	0.19	38.07	1250	490.02	0.237
11	DICL	Span 85	0.120	9.50	0.19	38.07	1250	411.27	0.242
12	DICL	Span 85	0.120	9.50	0.19	38.07	1000	438.33	0.315
13	DICL	Span 85	0.181	9.50	0.19	38.07	1000	420.32	0.282
14	DICL	Span 85	0.056	9.50	0.19	38.07	1000	326.63	0.346
15	DICL	Span 85	0.056	9.50	0.19	38.07	1000	428.70	0.240
16	DICL	Span 85	0.016	9.50	0.19	38.07	1000	477.85	0.260
17	DICL	Span 85	0.000	9.50	0.19	38.07	1000	427.56	0.280
18	DICL	Span 85	0.320	9.50	0.05	38.07	1000	414.40	0.400
19	DICL	Span 85	0.320	9.50	0.05	38.07	750	460.52	0.410
20	DICL	Span 85	0.320	9.50	0.01	38.07	1000	453.76	0.390

MPS and PDI results are displayed in Table 3. The obtained results for the MPS of experiments 4, 6, 7 and 8 should not be considered, as the respective PDI values are superior to 0.5, which represents a non-homogenous final NS and may suggest a false MPS value.

As determined by PCS, the MPS of the obtained particles in the prepared suspensions ranged from 326.63 to 836.50 nm, in the valid experiments, i.e. excluding experiments with PDI above 0.5 (Table 3).

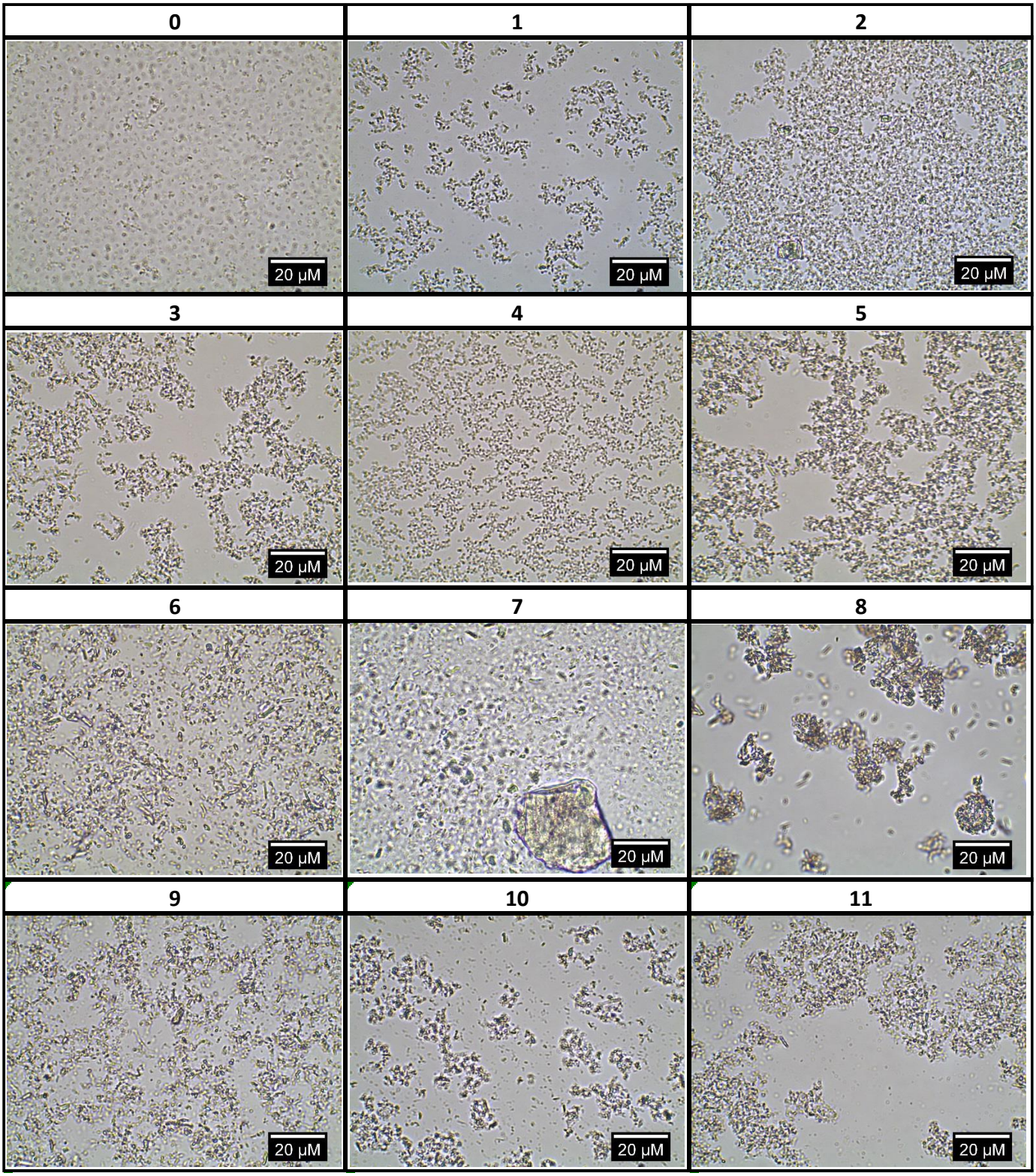

Figure 3 Microscopic pictures of the samples acquired at the end of the process (6 hours) from experiments 0 to 11. The magnification used was 100x10.

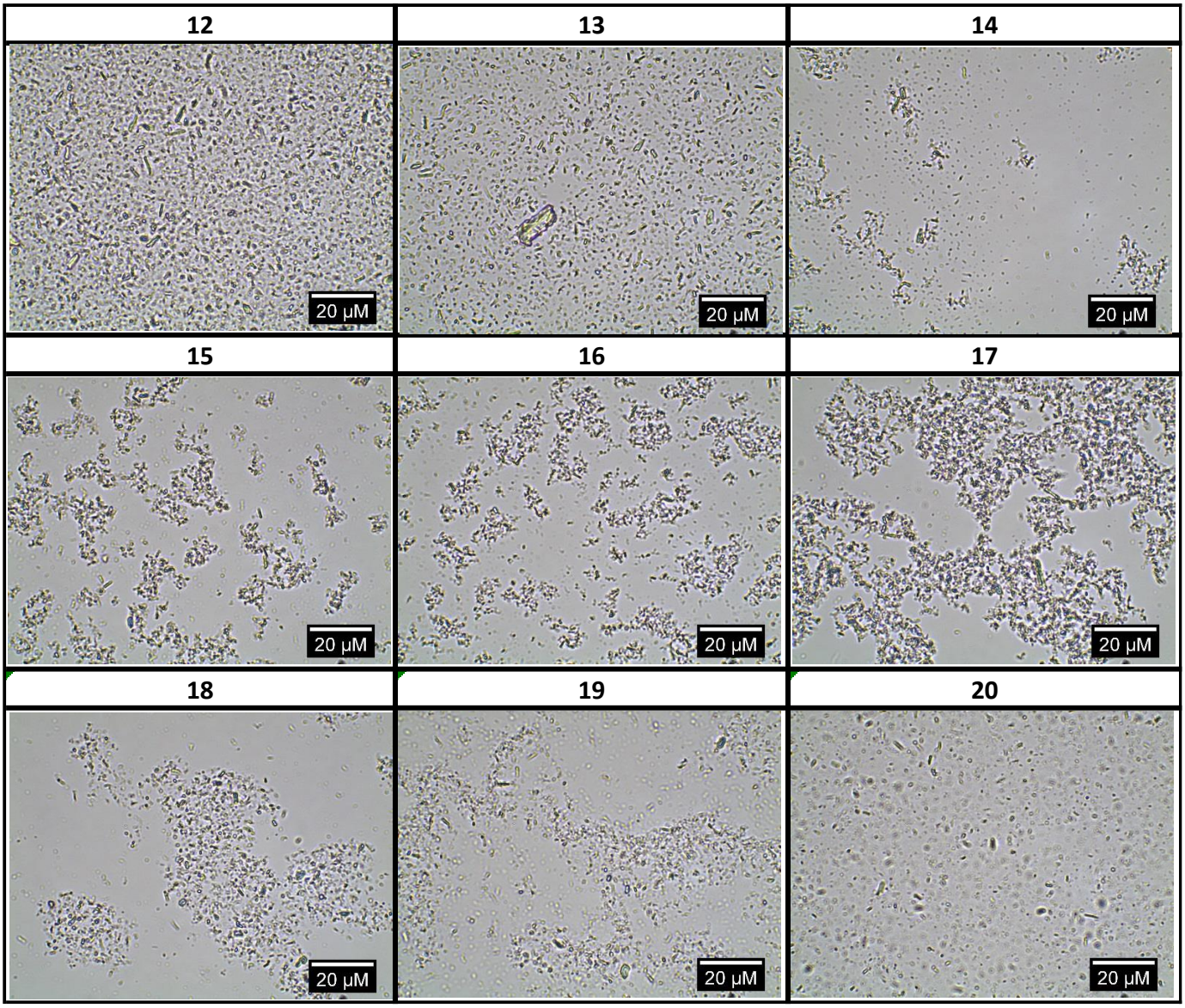

Figure 4 Microscopic pictures of the samples acquired at the end of the process (6 hours) from experiments 12 to 20. The magnification used was 100x10.

Figure 3 and Figure 4 represent the microscopic pictures of the NS obtained at the end of the process (6 hours) of each experiment. Experiments 0, 1, 3, 4, 5, 8, 10, 11, 12 and 14 to 20 show a narrow particle size distribution, in contrast to experiments 2, 6, 7, 9 and 13, which evidence remaining bigger particles, suggesting a higher distribution on NP size within each batch of NS.

It is clear a progressively increased amount of agglomerates in experiments 15 to 17 (Figure 4). A very strong agglomeration effect with the presence of many clusters of particles is observed in experiment 8, which contained KF-6105 as surfactant (Figure 3).

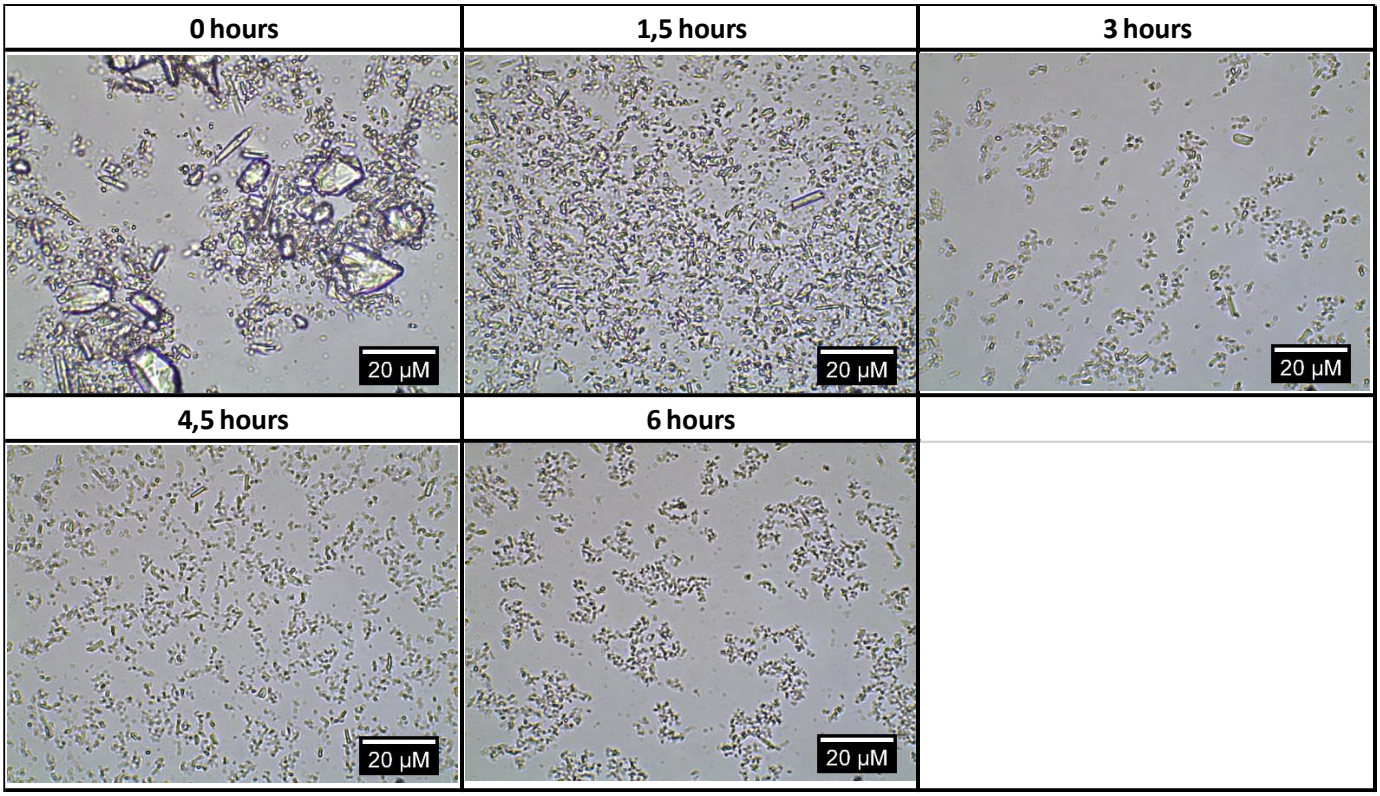

Figure 5 Microscopic pictures of the samples acquired along the process of experiment 1. The magnification used was 100x10.

As an example, microscopic pictures of the samples acquired during the process of experiment 1 are represented in Figure 5. Most of the other experiments have an identical evolution along the process (data not presented).

In these five microscopic pictures (Figure 5), representing five sequential time points of the process, two distinct events are clearly shown: the decrease in particle size of most of the particles and the increased homogeneity in the particle size along time.

In the first microscopic picture of Figure 5 (0 hours), representing the raw API in the prepared suspension is observed the presence of large particles in the micron range. After 1.5 hours, it is evidenced a clear reduction in the MPS, with the disappearance of the bigger particles. Three hours after the beginning of the process, it is still visible the reduction of particle size in most of the particles, in comparison to the previous sampling. In the microscopic pictures obtained after 4.5 hours and 6 hours after the process began, it is mainly observed a decline in the number of particles above the MPS, resulting in a higher homogeneity in each NS batch.

5 Discussion

Experiments 3 and 7 are comparable as the conditions and ratios used were the same, only differing in the API used, which were respectively diclofenac sodium and dexamethasone (Table 1). The microscopic pictures of both experiments (Figure 3) evidence a very broad size distribution with big particles in EXP 7, a scenario that is not present in experiment 3. Besides this clear evidence, it is also possible to identify a smaller particle size in experiment 3 compared to EXP 7, when analysing most of the particles present in the microscopic pictures. In this case, the PCS results for the MPS of EXP 7 should not be considered since the PDI value is above 0.5 (Table 3). However, this PDI and the microscopic pictures evidence the broad particle size distribution and the presence of big particles, absent in EXP 3 (Table 3, Figure 3). The direct comparison between experiments enables to infer that not every drug acts equally when exposed to the same process and formulation parameters, wherefore the optimal conditions for each drug should be studied. Possible reasons for this may be the different drug interactions with surfactant and medium, explained by the different molecular arrangement and chemical groups present, differences in drug's brittleness and other drug properties, which affect the milling time and the resultant particle size (118).

Comparing the microscopic aspect of experiments 3 and 8, a strong agglomeration effect is noticed in EXP 8, not present in EXP 3 (Figure 3). Considering that the only difference between these two experiments is the surfactant used, this agglomeration effect in EXP 8 should be a consequence of using KF-6105 as surfactant, instead of using span® 85 (Table 1). KF-6105 is a silicone-based surfactant with a chemical structure distinct from span® 85 (Figure 2). The different molecular chains present in each surfactant will allow distinctive interactions with the drug and with the triglycerides medium, which will influence the dispersion and stabilisation of the drug in the medium and may affect particle size reduction (31). In this case, it is not clear that particle size reduction has been affected by this parameter, since microscopic pictures do not evidence a marked difference in the particle size between the two experiments (Figure 3). However, it is obvious the agglomeration of the particles in the presence of a suboptimal surfactant, compared to a good surfactant.

To study the impact of decreasing the amount of surfactant, EXP 13, 15, 16 and 17 will be considered as this is the only differentiating factor between them (Table 1). The MPS values obtained for these four experiments are very identical, as well as

the low PDI values, representing narrow particle size distributions with valid MPS values (Table 3). These similar values of MPS between experiments may infer that the surfactant concentration did not affect the obtained particle size, as well as suggested the more appropriate type of surfactant. The observation of the microscopic pictures (Figure 4) evidences an increased agglomeration effect from EXP 13 to 17, which is explained by the decreasing amount of surfactant from EXP 13 to EXP 17 (Table 1). In the EXP 15, it is already visible an agglomeration effect (Figure 4), therefore, the ratio surfactant/API/oil should not be lower than 0.10, which is the ratio in EXP 13. These results suggest that a certain amount of surfactant is very important to avoid particle agglomeration. In addition, it indicates that the surfactant concentration by itself does not have a significant influence on particle size reduction.

High drug loading is of great interest to achieve an increased dose of the active substance and a marked therapeutic effect. Therefore, a considerable API amount should be used. On the other hand, an excessive API amount could lead to important problems, such as agglomeration and increased particle size due to the bigger probability of collisions between drug particles (34). EXP 18 and 20 permit the evaluation of the influence of the API amount on NS (Table 1). There is no marked difference in the MPS of both experiments (Table 3). However, a better dispersity and homogenisation of the particles in the suspension is evidenced in EXP 20 (Figure 4). Increased collisions between drug particles may explain this phenomenon (34). Although, the increased surfactant/API ratio may also play an important role on this effect. This can be clarified comparing EXP 1 and 3, which have the same surfactant/API ratio but different API amounts (Table 1, Table 2). Both experiments show similar particle size and microscopic aspect (Table 3, Figure 3), suggesting that, between certain limits, API amount does not directly influence particle size reduction or agglomerate formation, in case of similar surfactant/API ratio.

Surfactant/API ratio is an important factor to have in consideration to avoid agglomeration effects, as noted before in the study of drug amount influence on PSR. The comparison between EXP 3 and EXP 5 indicates a markedly increase in particle size when a significant decrease in surfactant/API ratio is applied (Table 2, Table 3). These results propose that the ratio between drug and surfactant not only has an impact on particle agglomeration, but it also influences particle size reduction.

Speed is a very important parameter as it is the source of energy of the bead milling process (6). Comparing EXP 18 and 19, the latter shows a slight increase in the MPS

because of the less energy input, justified by the lower speed (Table 3). On the other hand, an excess of energy input can be also problematic as it may create agglomerates (35), which can be observed in EXP 11 compared with EXP 12 (Figure 3, Figure 4), with decreased speed (Table 1). Less beads amount, represented in EXP 4 and 6, as well as an increased Miglyol® 812 amount, represented in EXP 1, 3 and 5, seem to enable the use of higher speed in the process, compared to experiments with more beads or less Miglyol® 812 amount, such as EXP 0 and 2 (Table 1). The instability caused in some experiments when higher speed was used can be possibly explained by an increased solid/liquid ratio, in agreement with the higher beads and lower Miglyol® 812l amount leading to this instability (Table 1, Table 2).

On the one hand, less beads amount enables the use of higher speed, which in turn may result in smaller particle sizes. On the other hand, higher number of beads will favour the collisions between the beads and the drug particles, also leading to reduced particle size (119). It is possible to observe higher dispersity and smaller particle size in EXP 0 than in EXP 4 (Figure 3). According to these results, it is proposed that the smaller number of beads was partially responsible for the increased MPS in EXP 4. In addition, the higher speed together with a small quantity of beads produces an excess of energy, due to the need for a lower energy to move the total amount of beads, which may be responsible for the agglomerates formed (Table 2).

Milling time is also a very important factor to consider. The microscopic pictures acquired during the process show a clear particle size reduction along time (Figure 5). Additionally, the results suggest the presence of two stages during the process. The first is a clear size reduction in most of the particles transforming a microparticle suspension into a suspension with mainly NP, while the second is the size reduction of the few remaining bigger particles resulting in a narrow particle size distribution, and therefore, an increased homogeneity. In this experiment, the duration of the process (6 hours) was sufficient to produce a homogeneous NS (Figure 5 – 6 hours), although other experiments that contained remaining bigger particles, such as EXP 2, 6, 7, 9 and 13 (Figure 3, Figure 4), might have benefited with an increased milling time. The results propose that after a certain process time, there will be no additional size reduction in most of the particles, which have already achieved a small size. On the other hand, this size reduction will focus on the larger particles leading to narrow size distributions and lower PDI values. This may be justified by the increased

probability for collision of larger particles with beads, compared to smaller particles, due to the larger volume.

Reproducibility of the production and sampling methods are important aspects to improve in future experiments, since results obtained from similar experiments are not always identical, especially in microscopic pictures. This can be analysed comparing EXP 9, 14 and 15 (Figure 3, Figure 4).

The different formulation and process parameters influence each other, therefore, to obtain the best results in the final NS, all parameters must be carefully evaluated considering their interactions and not only their role as individual factors.

6 Conclusions

Non-aqueous NS of diclofenac sodium were prepared by grinding the API with zirconia beads using Miglyol® 812 as the dispersion medium. Among several suspensions, the best one was selected, and some variations were applied to the different parameters to evaluate their influence on particle size reduction. The mean particle size of the obtained particles in the prepared suspensions ranged from 326.63 to 836.50 nm, in the valid experiments, i.e. with a PDI less than 0.5, as determined by PCS.

Different drugs appeared to influence the final NS, due to the different drug properties. The type and amount of surfactant seem not to influence particle size reduction when considered alone, although, these two factors have the major responsibility in the particle agglomeration effect. In the studied range, the API amount did not directly influence particle size reduction. However, the surfactant/drug ratio appears to influence both MPS and agglomeration of particles. The decreasing of this ratio increases MPS and agglomeration, leading to poor results. Increasing speed and higher beads quantity usually favour PSR and smaller particle size. Nevertheless, an excess of energy input, represented by higher speed or less beads amount, may also lead to the development of agglomerates and consequently reduce the quality of the NS. In this work, we propose that lower solid/liquid ratio, mainly represented by beads/ Miglyol® 812 ratio, will enable to use higher speed during the process, without favouring the instability of the system. Milling time is also a very important factor, which strongly influences particle size reduction. It is suggested that increased milling time favours particle size reduction, leading to smaller particle sizes, until a certain limit is achieved. After this limit, the small particles do not continue to reduce their size, being the size reduction mainly observed in the large particles, narrowing the particle size distribution. It is crucial to evaluate all parameters as global and not as individual factors to optimise the process and obtain the best results.

This work was the beginning of a NS development procedure, therefore there is plenty of room for improvements. One of the first future approaches to be performed would include the optimisation of the process by increasing the knowledge on how the process and formulation parameters influence the particle size reduction, which could be done resourcing to a design of experiments software. This will allow a better understanding of the design space, where it is possible to work to achieve the

smallest NP possible. Reproducibility of the production and sampling methods should also be improved, to reduce batch-to-batch variation. Another step forward would be to formulate the obtained NS and later evaluate its stability along time. To provide deeper knowledge of this production process, it would be interesting to evaluate other API's and oils, as well as the influence of the temperature in the process.

After having a good knowledge of the process, the scale-up is of upmost interest to have impact in the production of medicines.

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