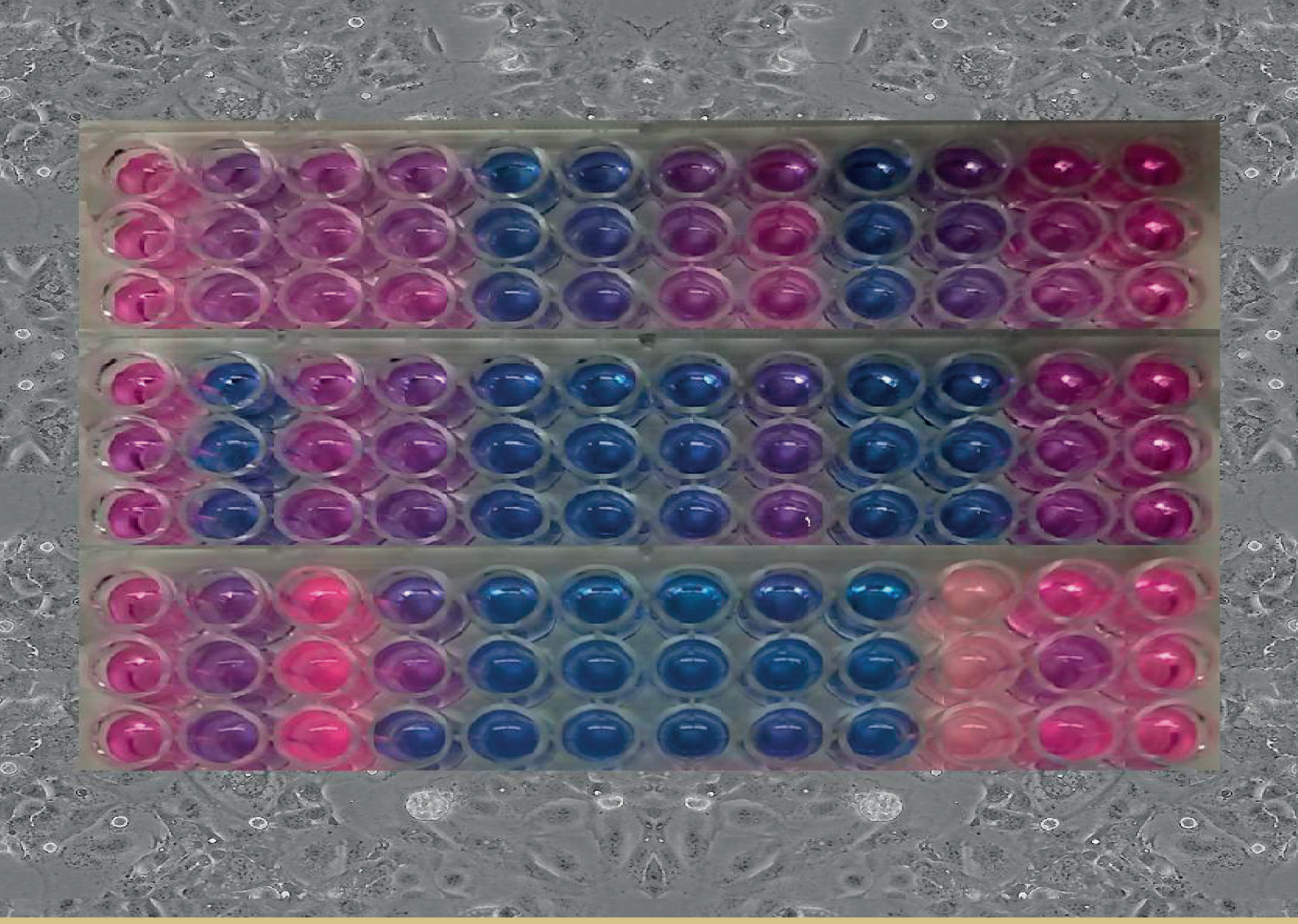

Ana Rita Venceslau Fernandes

ENHANCING THE SOLUBILITY OF IBUPROFEN USING BIOPOLYMERS-BASED NANOENCAPSULATION

Dissertação de Mestrado em Tecnologias do Medicamento, orientada pela Professora Doutora Eliana Maria Barbosa Souto, apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2016

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Universidade de Coimbra

"Focus on your dream and never quit.

It is always too soon to quit.

If you quit, you can't succeed.

By achieving your dream you will be an inspiration to others.

You will set the example and make an enormous impact on the world."

Rudy Ruettiger

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ABBREVIATIONS

AFM: Atomic Force Microscopy

BCS: Biopharmaceutics Classification System

DoE: Design of Experiment

DSC: Differential Scanning Calorimetry

DLS: Dynamic Light Scattering

ELS: Electrophoretic Light Scattering

ESEM: Environmental Scanning Electron Microscopy

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

HI: Heat Inactivated

HPH: High Pressure Homogenization

NSAIDs: Nonsteroidal Anti-Inflammatory Drugs

PBS: Phosphate-Buffered-Saline

PI: Polydispersity Index

PVA: Polyvinyl Alcohol

PVP K30: Polyvinylpyrolidone K30

SEM: Scanning Electron Microscopy

SDS: Sodium Dodecyl Sulphate

S80: Span 80

TEM: Transmission Electron Microscopy

T80: Tween 80

UV: Ultraviolet

ZP: Zeta Potential

ABSTRACT

The clinical use of poorly water-soluble drugs has become a big challenge in pharmaceutical development due to the compromised bioavailability of the drugs *in vivo*. Nanocrystals have been proposed as a formulation strategy to improve the dissolution properties of these drugs. The reduction of the particle size down to the nano-sized range has dramatically changed the physicochemical properties of drugs. Drug nanocrystals show particle sizes varying from 100 nm to 400 nm, and can be produced by top-down, bottom-up or via a combination of these techniques. The core of the nanocrystals consists of pure drug, which is covered by a layer of a suitable surfactant. The benefits of using nanocrystals in drug delivery, when compared to other nanoparticles, are related to their production facility, simple structure, and suitability for a variety of administration routes. The greatest disadvantage of nanocrystals is their inherent instability, due to the risk of crystal growth (a process so-called Ostwald ripening). Thus, the selection of an appropriate stabilizer is crucial to obtain long-term physicochemically stable nanocrystals. High pressure homogenization is the most promising production process, which can employed at low or high temperatures. This technique have advantages, including the possibilities of scaling up, lack of organic solvents and the production of small particles diameter with low polydispersity index, usually below 0.2. The sequential use of high shear homogenization followed by high pressure homogenization, can modulate nanoparticles size for different routes of administration. The present study focuses on the optimization of the production process of two formulations composed with different surfactants, and produced by High Shear Homogenization and High Pressure Homogenization. To build up the surface response charts, a 2^2 full factorial design experiment, based on 2 independent variables, was used to obtain an optimized formulations. The effects of the production process on the mean particle size, polydispersity index were investigated. The *in vitro* ibuprofen release from the optimized formulations were determined using Franz diffusion cells. Cell viability was assessed for the formulations and different controls on human epithelial colorectal cells (Caco-2). Evaluation of cell viability was performed by a colorimetric assay, i.e., AlamarBlue® assay. The cell viability assay was performed based on the cell capacity to metabolize resazurin, at pre-determined time-intervals, 3, 6 and 24 hours. In both formulations, Caco-2 cells viability was shown to be dependent, both on the drug concentration and time of exposure.

RESUMO

O uso clínico de fármacos pouco solúveis em água é um grande desafio no desenvolvimento farmacêutico, uma vez que a biodisponibilidade *in vivo* destes fármacos se encontra comprometida. Os nanocristais têm sido usados como estratégia de formulação para melhorar as propriedades de dissolução desta classe de fármacos. A redução do tamanho de partícula para a gama de tamanhos nano modifica drasticamente as propriedades físico-químicas dos fármacos. Os nanocristais apresentam tamanhos de partícula que variam de 100 nm a 400 nm, e podem ser produzidos de cima para baixo ("top-down"), de baixo para cima ("bottom-up") ou através da combinação destas técnicas. O núcleo interno dos nanocristais é formado por fármaco puro, e de seguida é coberto por uma camada de agente tensioativo adequado. Os benefícios da utilização de nanocristais na cedência de fármacos, estão relacionados com a sua facilidade de produção, estrutura simples e possibilidade de serem utilizados em várias vias de administração quando comparados com outras nanopartículas. A maior desvantagem dos nanocristais é a sua instabilidade inerente, devido ao risco de crescimento de cristais (um processo designado por maturação de Ostwald). Assim, a escolha de um tensioativo adequado é fundamental para obter nanocristais físico-quimicamente estáveis a longo prazo. A homogeneização a alta pressão é o processo de produção mais promissor, uma vez que pode ser utilizado a baixas ou elevadas temperaturas. Esta técnica tem vantagens, incluindo a possibilidade de transposição de escala a nível industrial, o facto de não se utilizarem solventes orgânicos e a possibilidade de produção de partículas de pequeno diâmetro, com baixo índice de polidispersão, geralmente abaixo de 0.2. O uso sequencial da homogeneização de alta velocidade de corte, seguida da homogeneização a alta pressão, pode modular o tamanho dos nanocristais permitindo a sua utilização em diferentes vias de administração. O presente estudo centra-se na optimização do processo de produção de duas formulações compostas por diferentes agentes tensioativos, produzidas por homogeneização de alta velocidade de corte seguida da homogeneização a alta pressão. Para construir os gráficos de superfície de resposta, utilizou-se o desenho factorial 2^2 completo, baseado em 2 variáveis independentes, conseguindo assim obter formulações optimizadas. Foi investigado o efeito da variação dos constituintes do processo de produção no tamanho de partícula médio e índice de polidispersão. A libertação *in vitro* do ibuprofeno a partir das formulações optimizadas foi determinada usando células de difusão de Franz. A viabilidade celular foi avaliada para as diferentes formulações e diferentes controlos em células epiteliais colorrectais humanas (Caco-2). A avaliação da viabilidade celular

foi realizada através de um ensaio colorimétrico, isto é ensaio de AlamarBlue®. O ensaio de viabilidade celular foi realizado com base na capacidade das células para metabolizar a resazurina, em intervalos de tempo pré-determinados, 3, 6 e 24 horas. Em ambas as formulações, a viabilidade das células Caco-2 mostrou-se dependente tanto da concentração do fármaco, bem como do tempo de contacto com as células.

1. Introduction

Nanotechnology is one of the most popular strategic approaches currently in use in modern drug delivery and therapy. Pharmaceutical nanocrystals are materials produced by down-sizing from the microscale to nanoscale with the aim to dramatically change the physicochemical properties of compounds. Nanocrystals are solid particles surface-stabilized with a surfactant layer featuring a size in the nanometer range (i.e. mean diameter below 1 μm) (1).

There are many methods to solubilize poorly water-soluble drugs, however these are limited due to certain properties with respect to the drugs' chemistry, e.g. their solubility in organic media, their conformation or the molecular size. While it is possible the use of surfactants or co-solvents, these can lead to increased adverse side effects (2).

When the particle size of a crystal decreases down to 100 nm, the properties of the materials are dramatically changed. The most important changes result from the enhancement of the surface area which contributes to the improved drug solubility. Thus, there is a proportionate increase in the bioavailability of poorly water soluble drugs. Another benefit of nanocrystals is their simplicity, given the inexistence of many excipients in their structure. While the micronization of powders of poorly water soluble drugs, down to sizes of 1-10 μm is used to increase dissolution velocity, this is not sufficient to increase the drugs' bioavailability. The next step was therefore to move from micronization to nanonization.

In the beginning of the 90s, Elan Nanosystems (San Francisco, CA, USA) preferentially propagated the use of nanocrystals for oral bioavailability enhancement instead of the use of microcrystals, by the use of nanocrystals suspended in water, i.e., nanosuspensions (3). Nanosuspensions consist of the dispersion of drug nanocrystals in liquid media. Surfactants or polymeric stabilizers are used usually to stabilize dispersed particles. Water, aqueous or non-aqueous solutions can be used as dispersion media (4).

Nanonization increases simultaneously the surface area and the saturation solubility, when compared to micronization. The solubility is a compound specific constant in the case of particles with sizes above 1 μm, only depending on the temperature and on the solvent the particles are exposed to. When the saturation solubility is also a function of particle size it means that the particle size of the crystal is lower than 1-2 μm. As a consequence of the increase in the saturation solubility, there is an increase in the dissolution rate and, thus, an increase of the concentration gradient between the gut lumen and the blood, due to the formation of a supersaturated solution. This phenomenon promotes the drug absorption because the supersaturated solution accelerates the diffusion (5). Thus, nanocrystals are

responsible for an earlier onset due to faster dissolution and absorption. Comparing to conventional dosage forms, the bioavailability of nanocrystals increases significantly, because in this case the particles are made of 100% of drug. This property contributes to achieve a high therapeutic concentration in the site of action to exhibit the pharmacological effect. In addition, due to their high loading, nanocrystals are very efficient in transporting drugs (3).

Liversidge et al. patented the first formulation (6). Rapamune by Wyeth was the first product accepted in 2000 by the US Food and Drug Administration (FDA). The reason for a fast acceptance and entry to the market was attributed to their simple structure, without to many excipients. Rapamune® was formulated as oral tablet when it entered in the market. Later, nanocrystals have been further exploited for other administrations routes, such as parenteral, ocular, dermal, buccal and pulmonary drug delivery (7), (8), (9), (10), (11).

Due to their capacity to increase the solubility and the bioavailability of poorly water soluble drugs, nanocrystals can be supplied in oral solid dosage forms, such as tablets and capsules, which are more patient friendly. Nanocrystals of poorly water soluble drugs feature high penetration power through dermal application, thus it is also possible their incorporation in cosmetic products (12). Since the particle size (around 200-400 nm), is smaller than the size of blood capillaries, they may also be used for intravenous injection, as long as the polydispersity index remains below 0.24. On the other hand, if nanocrystals are intended for intravenous injection, the content of particles larger than 5 μm needs to be closely monitored since these may block the 5-6 μm blood capillaries, leading to embolism. This advantage provides a bioavailability of 100% and simultaneously prevents the use of co-solvents to dissolve the drug or the use of toxic surfactants that would be very harmful to health and expensive (5).

Nanocrystals are usually stabilized by electrostatic and/or steric stabilization by surfactants such as lecithin, alone or in combination with sodium cholate or non-ionic surfactants, e.g., Tween 80, poloxamer 188 and polyvinylpyrrolidone. These surfactants are accepted for intravenous injection, while using binary or ternary mixtures of electrostatic and steric surfactants was found to be effective for long-term stability. For other administration routes, e.g., oral administration, several other surfactants can be used.

Several methods have been described to solubilize poorly water soluble drugs, however the selection depends on the chemical properties of the drug, such as its solubility in organic media, conformation and/or molecular size. Although it is possible the use of surfactants or co-solvents, these options can lead to adverse side effects and toxicity (13), (14).

Nanocrystals can solve the biopharmaceutical delivery problems of poorly water soluble drugs, as: (i) too low bioavailability after oral administration; (ii) low dermal bioavailability (reduced penetration into the skin); (iii) need of too large injection volume for intravenous administration, which are responsible for undesired side effects; (iv) need of several administrations to achieve the required effect, such as traditional drug solutions formulations. This problem makes the treatment not friendlier to the patient. Nanocrystals have the ability to solve these problems due to their special features, such as (i) enhanced dissolution rate; (ii) enhanced solubility and, consequently, increased saturation velocity; (iii) increased adhesion to surfaces/cell membranes. Gao *et al.*, have shown that these three combined effects have indeed considerably amplified the *in vivo* performance of various poorly water soluble drugs when compared to micron-sized drug powders (15).

The increased saturation solubility c_s, the increased dissolution velocity dc/dt, and the adhesive properties of ultra-fine particles are the most important features of drug nanocrystals. While theoretically the saturation solubility is generally described as a compound-specific constant depending on the temperature, for particles of sizes below 1-2 μm the saturation solubility is also dependent on the particle size. As shown from the Kelvin equation (Eq. 1), the dissolution pressure of a compound increases with the decrease of the particle size attributed to the increase of the curvature of the particle surface:

$$ln\frac{p}{p_0} = \frac{2\gamma V_m}{rRT} \qquad \text{(Eq. 1)}$$

where p stands for the vapor pressure, p_0 for the saturated vapor pressure, γ for the surface tension, V_m is the molar volume of the liquid, r is the particle radius, R is the universal gas constant, and T is the temperature. Surrounding the nanosized particles, a higher saturation solubility occurs in comparison to particles sized above 1-2 μm. This effect is described by the Ostwald-Freundlich equation.

Particle size reduction is a non-specific formulation approach because independently of its solid state or other physicochemical properties, it can be applied for every poorly water soluble drugs (16). According to the Noyes–Whitney equation, the surface-to-volume ratio increases significantly when particles are broken down to the micrometer range, leading to an increase of dissolution velocity. Subsequently, there is an enhancement of absorption in the gastrointestinal tract. However, if the drug has a very poor solubility, the definite increase of

dissolution velocity is not enough to obtain the expected bioavailability, being therefore crucial to downs size the particles to the nanometer range (17). To achieve a significant increase in the surface area, the particle size of the compounds can be reduced. An increase in the surface area results in faster dissolution rates, as shown in Noyes–Whitney equation (Eq. 2) (18).

$$\frac{dX}{dt} = \frac{DA}{h_D}(Cs - Ct) \quad \text{(Eq. 2)}$$

where $\frac{dX}{dt}$ is the dissolution velocity, D is the diffusion coefficient, A is the surface area, h_D is the diffusional distance, C_S is the saturation solubility and C_t is the concentration around the particles (19).

The most direct effect of particle size decrease is the enhanced dissolution rate of nanocrystals. The surface area accessible for drug release per mass of drug increases inversely proportional to the radius of particle. This occurs when the particle size decreases. This relation is easily obtained from the consideration of spherical surface area versus volume through the equation $\frac{A}{V} = \frac{3}{r}$, where A is the surface area, V is the volume and r is the radius of the particle. Thus, a reduction in the particle size from 40 μm to 400 nm would increase the dissolution rate by 100 fold. This is the mechanism of action to increase the oral bioavailability. It is possible to dissolve a higher amount of drug during the gastro-intestinal tract time-limited transit, enhancing the concentration gradient that, consequently, improves the permeability and, thus, the bioavailability of drugs (20).

The Ostwald-Freundlich theory sustains the hypothesis of solubility increase. This theory was firstly derived for liquid droplets in a gas phase but it also has been considered valid to describe the solubility increase for very small solid particles in liquids. According to equation 3 (Eq. 3), especially in the lower nanometer range, the saturation concentration at the surface of lower sized-particles is higher when compared to the saturation concentration in the surface of major particles (21).

$$C_{NP} = C_b \, exp\left(\frac{2V_m\gamma}{RT_r}\right) \quad \text{Eq. 3)}$$

where C_{NP} is the solubility of nanoparticles with a radius r, C_b is the solubility of bulk material, V_m is the molar volume of the drug, γ is the interfacial tension between the solvent and the drug material, R is the gas constant, and T is the temperature (22).

Since dc/dt (dissolution velocity) depends on the difference $c_s - c_x$ (where c_x is the concentration in the suspending medium), the increased saturation solubility c_s leads to the increased dissolution velocity. In the same way, since the diffusional distance h is decreased, (h is the distance over which the concentration gradient $c_s - c_x$ is formed, described by the Prandtl equation), the intrinsic dissolution rate is increased. The increased dissolution pressure and velocity, due to the increase of c_s and decrease of h is described in Figure 1.

Based on these properties, the main benefit of drug nanocrystals in terms of drug release and delivery is the increased surface area per mass. In general, submicron-meter particles exhibit increased adhesiveness in comparison to larger particles. As a result, an increase of the contact area occurs with improved bioavailability. The best drug candidates for the production of nanocrystals are those depicting high permeability but low solubility.

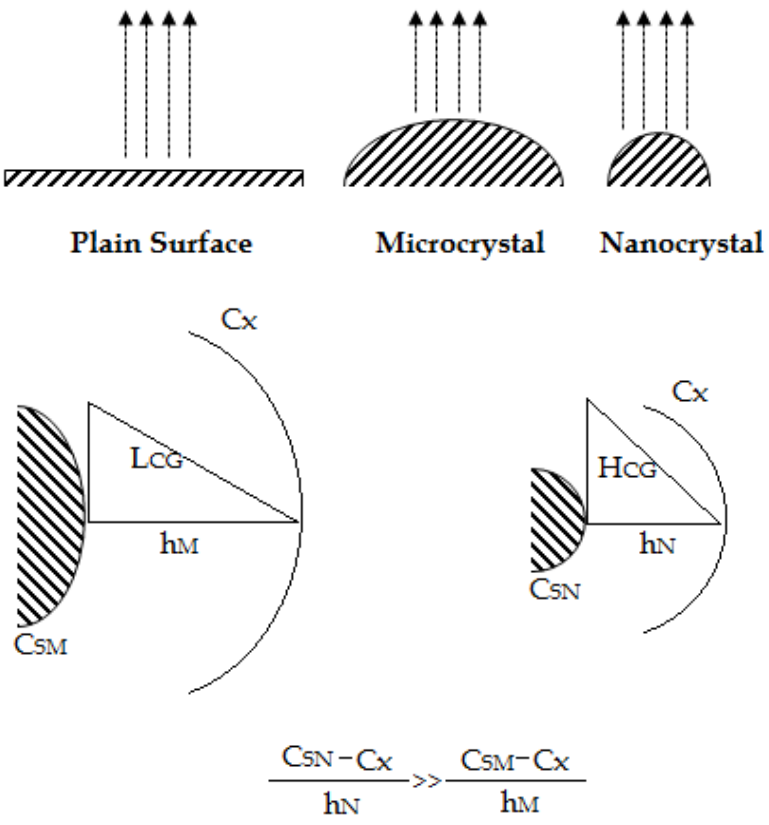


Figure 1: Schematic representation of the increase in the saturation solubility C_S and dissolution velocity for drug nanocrystals, suspended in liquid medium (Cx, Concentration in the surrounding medium; L_{CG}, Lower concentration gradient; H_{CG}, higher concentration gradient; C_{SM}, surface saturated solution layer onto microcrystal; C_{SN}, surface saturated solution layer onto nanocrystal; h_M, high of saturation layer surrounding microcrystal; h_N, high of saturation layer surrounding nanocrystal; $C_{SN} >$

C_{SM}; $h_N < h_M$) (Fernandes, A.R., Santos, A.C., Veiga, F.J., Kovačević, A.B., Souto, E.B. Formulating pure nanocrystals for enhanced dissolution of poorly water-soluble drugs, in: Six-Volume Book Series on, "NanoBioMedicine", published by M/s Studium Press LLC, USA Executive Editor - Dr. Bhupinder Singh Bhoop, Studium Press LLC, USA (2016) (accepted on 03/08/2016).

1.1. Biopharmaceutics Classification System (BCS)

The BCS was developed by Amidon *et al.* and is based on fundamental parameters that control the rate and the extent of oral drug absorption (23). Considering these parameters, this system categorizes the drugs in four classes, according to the solubility and permeability. The main objective is to predict the *in vivo* pharmacokinetic performance of numerous compounds based on the measurements of permeability and solubility. If the drug in study is administered orally, its efficacy is dependent on the extent of absorption and, consequently, on the oral bioavailability. In turn, oral absorption is dependent on the intestinal drug solubilisation and on the drug permeability. Concentration, temperature, surface area, affinity to a membrane transporter, time and viscosity, are the factors that affect the amount of drug that crosses the membrane (24).

Compounds are considered highly soluble when the highest marketed dose strength is soluble in 250 mL in a media at 37°C over pH range of 1-6.8. On the other hand, to be considered highly permeable, the drug needs to have extent of absorption greater or equal to 85% of the administered dose (25, 26).

The compounds belonging to Class I show both high solubility and high permeability (e.g., diazepam, propranolol). In Class II, drugs are highly permeable but poorly water soluble (e.g., ibuprofen, carbamazepine). Class III shows opposite characteristics of the Class II, namely poor permeability and high solubility (e.g., atenolol, metformin). Class IV compounds possess low permeability and low solubility (e.g., sulfasalazine, bifonazole) (27).

When the oral bioavailability of poorly water soluble drugs is limited by their dissolution velocity, their low solubility can be increased by different strategies. The increase in the saturation solubility, promotes the enhancement of the penetration into the skin, due to the saturation solubility that creates a larger concentration gradient between creams with nanocrystals and the skin (23).

Some drugs belonging to the BCS Class II can be injected in a sufficiently small volume when they are complexed with cyclodextrins or when solubilized with surfactants (3).

1.2. Nanocrystals technology

The standard methodologies to decrease the particle size of poorly water soluble drugs are bottom-up and top-down techniques and, more recently, a combination of both approaches. The latter is based on the use of a bottom-up process followed by a top-down stage (28). The starting point is the bulk material using top-down methods, followed by a decrease in the particle size, by milling or high-pressure homogenization (HPH), e.g., particles are formed from pre-dissolved molecules through various techniques, like precipitation and bottom-up techniques. Pre-milling followed by HPH represents a combination of different technologies which can also be used. For industrial purposes, all the products are prepared by a top-down method (29), (30).

1.2.1. Bottom-up technique (Precipitation method)

In the precipitation method, also known as "*via humida paratum*", the drug and the stabilizer are dissolved in a solvent and then this solution is added to a non-solvent, which induces a fast decrease in the drug solubility, triggering the drug precipitation on finely dispersed drug nanocrystals. The precipitation method is simple and requires low cost equipment (5), (4).

However, this technique presents some disadvantages for newly developed drugs which are generally insoluble in both aqueous and organic media. This way, the drug needs to be soluble in at least one solvent. Another problem of this method is that the solvent needs to be miscible with at least one non-solvent. It is difficult to dissolve this type of compounds in any solvent, especially due to the miscibility requirement with the anti-solvent. Also, solvent residues need to be removed, and this issue can poses problems, namely increasing production costs, particularly when large solvent volumes are required. This occurs when the drug exhibits low solubility in water and also in organic solvents (3). The control over the particle size is often difficult to achieve, but it is necessary to ensure that crystals do not grow and remain within the nano-range. To preserve the submicron-size range, recommended techniques include spray drying and lyophilisation (31), (32). Alternatives to preserve the size of nanocrystals consist of processing precipitation under a high-energy zone, e.g., in a microfluidizer, or the use of polymeric growth inhibitors, which can contribute to delay particle growth. To prepare

nanocrystals, several stabilizers can be used, like sodium dodecyl sulphate (SDS), polyvinyl alcohol (PVA), Tween® 80 and Poloxamer® 188 (33). Another problem is the fact that many poorly water soluble compounds are very difficult to solubilize, not only in aqueous but also in organic media.

The precipitation method can also produce amorphous particles, which may be a benefit for the case of solubility, however, it is problematic for the case of stability. Facing this, Soliqs/Abbott developed another precipitation method to enhance the dissolution rate and solubility, namely Nanomorphs (34).

Based on these facts, bottom-up technologies are currently not used in the production of commercial formulations. These techniques take the advantage of the variation in the solubility of drugs in diverse but miscible liquids. There are several parameters which should be optimized to obtain uniform nanocrystals, namely: (i) stirring rate; (ii) drug content; (iii) the volume ratio of anti-solvent to solvent, and (iv) the temperature.

The increase in the stirring rate favors the particle size enhancement of nanocrystals, due to the intensification of the micromixing between the two parts. A high micromixing efficiency increases the rate diffusion of compounds between the two phases, and, as a consequence, induces a fast and a high homogenous supersaturation, with the creation of a higher number of smaller drug particles (35).

A higher drug content will increases the probability of particles aggregation, thus a moderate drug content is required for the precipitation process. The reduction of particle size is favored when using lower temperatures. Together, a lower saturation solubility and a lower temperature makes the supersaturation easier to reach (17).

To produce nanocrystals using the precipitation method, some prerequisites should be satisfied (17), namely: (i) the solvent needs to be miscible with a non-solvent, (ii) the drug must be soluble at least in one solvent, (iii) the solvents should be eliminated to an acceptable level. The main advantages of the bottom-up technique (precipitation technique) include the production of small sized particles, good control of the desired size (monodispersity) and the production of finely dispersed drug nanosuspensions. The main limitations are related to the risk of solvent residues, time-consuming process during optimization, difficulty to scale-up, and the need of stabilizers.

Ostwald ripening describes the crystal growth by differences in saturation solubility of differently sized particles. In order to avoid it, the content of microcrystals needs to be as

lowest as possible, since the homogeneous size of the suspended particles avoids differences in saturation solubility, therefore delaying Ostwald ripening.

In the bottom-up techniques, such as the precipitation technique, nanocrystals can be built from bulk compounds. Using top-down methods, such as the homogenization method and the pearl milling, nanocrystals can be fragmented step by step from the coarse powder (17).

1.2.2. Top-down methods

The most used top-down techniques are pearl milling and HPH. In both methods, it is easy to achieve particle sizes around 100 nm, however smaller particles are more difficult to be obtained. In comparison to the bottom-up techniques, nanocrystals are often more polydispersed, but with longer processing times, e.g., longer milling or increased number of homogenization in order to increase the homogeneity of the product (36).

Pearl/ball milling was developed by Merisko-Liversidge *et al.*, and it occurs into the milling chamber where the drug is placed along with the milling media, dispersion media (usually water) and the stabilizer. The drug is dispersed in an aqueous stabilizer solution, and the milling is completed with the aid of milling pearls (1). The particle size reduction is a consequence of the movement of milling media that generate high shear forces of impact. The pearls or balls can be composed of ceramic, glass, stainless steel or to be highly cross-linked polystyrene resin coated beads. For the milling process there are two basic principles that can be used. In one of the cases, the milling material is moved by an agitator, or alternatively the complete container is be moved in a complex movement. In the latter, it is difficult to process large batches, therefore mills using agitators for large batches are usually preferred (37).

Depending on various factors, such as drugs hardness, viscosity, surfactant contents, temperature, particle size of milling media and energy input, it is possible to determine the milling time. This period can last from 30 minutes to several hours. Some advantages of pearl milling include (i) low cost; (ii) simple technology and ability for large scale production. Pearl milling methods have, however, some disadvantages, namely, (i) erosion from the milling material can lead to product contamination; (ii) adherence of the product to the inside surface of the mill and to the surface of the milling pearls; (iii) long milling times, e.g., in case of hard drugs; (iv) potential growth of germs in the water phase, e.g., for long-time milling processes; (v) time and costs associated with the separation procedure of the milling material from the

drug nanoparticle suspension, especially when producing parenteral sterile products. The problem of erosion from the glass beads could be reduced when these are covered with a highly cross linked polystyrene resin (1). In general, several process parameters should be explored to obtain an optimal formulation (17), namely, drug amount, number of milling pearls, milling speed, milling time, and temperature.

The first four marketed products containing nanocrystals were prepared by Pearl Mill methods by Elan nanosystems, namely Rapamune®, Emend®, Tricor®, Megace ES®. The HPH technique has been applied for the production of emulsions and suspensions. A different advantage of this technology is the easier scale up. To produce nanocrystals using the HPH technique, there are three important technologies:

(a) The microfluidizer technology (IDD-P™ technology) is based on the jet-stream principle. Under high pressures (≥ 1700 bar), two streams of liquid with high velocity (≥1000 m/sec) collide frontally. Due to the high shear force, particle collision and cavitation, the particle size is reduced. Same results can be achieved using jet stream homogenizers, such as Microfluidizer®. The collision chamber can be Y-type or Z-type in shape. Phospholipids or surfactants are essential to stabilize particles into the desired particle size (38).

(b) The production with piston gap homogenization in water (Dissocubes® technology) under high pressure, the powdered drug is dispersed in an aqueous surfactant solution which is forced by a piston through a tiny homogenization gap. The gap breadth is adjusted according to the viscosity of the suspension and the applied pressure. The gap width shows generally the size range of 5 to 20 µm. The high streaming velocity of the suspension of nanocrystals induces an increase in the dynamic pressure which is compensated by a reduction in the static pressure. This static pressure in the gap decreases, at room temperature, the vapor pressure of water. To lead the formation of gas bubbles, water starts boiling in the gap, at room temperature. These gas bubbles lead to pressure waves promoting the crystals disintegration. The static pressure increases to normal air pressure when the liquid leaves de homogenization gap, and gas bubbles collapse (39). This process of formation and implosion of gas bubbles is called cavitation. The particle size diminution occurs due to high shear forces, turbulent flow and the power of shock waves (40). In this method, the use of water as dispersion medium has disadvantages, e.g., the hydrolysis of water sensitive drugs and problems during the drying step. Sometimes, in the case of thermolabile compounds or drugs with a low melting point, removal of water requires the use of other techniques, such as lyophilization. The lyophilization is another disadvantage of this method, since it is quite expensive (41).

(c) The piston gap homogenization in water mixtures or in non-aqueous medium (Nanopure® technology) uses non-aqueous phase or phases with reduced water content as dispersion media. An advantage of this technology is the use of non-aqueous media with drugs, undergo hydrolysis in water (42). Oils, water-glycerol, mixtures, polyethylene glycols, hydro-alcoholic mixtures are some different media used for homogenization, since these dispersion media show a low vapor pressure. In this method, the cavitation does not occur because the static pressure in the homogenization gap does not fall under the vapor pressure of the liquid, so the liquid does not boil. However, even without cavitation, there is a particle size reduction to the nano-range, since, in this case, the forces responsible for the decrease of particle size are the particle collision and shear forces that occur in the highly turbulent fluid into the gap (43).

1.2.3. Combined techniques

These approaches combine a pre-treatment step followed by a high energy homogenization, e.g., a pre-precipitated sample can be further homogenized by HPH (cavi-precipitation technique) (44), or a pre-milled material can be still homogenized (43).

Combination techniques show several advantages, e.g., the capacity of production time shortening (by decreasing the number of passes through the homogenizer), and the production of very small nanocrystals (≤ 100 nm) that can be difficult to obtain by using only one method. Comparing milling, HPH and cavi-precipitation as techniques for the production of nanocrystals, it is possible to conclude that is possible to achieve successful nanocrystallizations is using any of these techniques. However, the smallest particles were achieved using milling process, and the lowest polydispersity index was achieved with HPH (45). These two referred methods showed to promote the more stable nanocrystals. In the case of cavi-precipitation, stability problems were caused by remaining solvent residues of the precipitation step, resulting from their competition with the surfactant for hydration. These problems caused partial surfactant dehydration and decreased the stability of the nanocrystals (44).

(a) The NANOEDGE® Technology combines the method of microprecipitation and homogenization (precipitation followed by an annealing process). The annealing process makes use of high energy, such as high shear forces and thermal energy (46). When produced by only

a precipitation method, nanoparticles have the tendency to grow, and may be amorphous or partially amorphous. In addition, amorphous particles may recrystallize and this may leads to a decreased bioavailability of the drug. These problems can be solved using combination technologies, due to their capacity to prevent the crystal growth and also to reduce the uncertainty formation of either crystalline or amorphous state nanocrystals, once the annealing phenomenon allows the conversion of all precipitated elements into the crystalline state. Combination techniques are particularly appropriate for compounds that are soluble in non-aqueous media with low toxicity.

(b) The SmartCrystal technology enables the use of different combination processes depending upon the physical characteristics of the compounds. There are multiple combination variants of this technology (5), such as: (i) the spray-drying and HPH (much faster in one to a few homogenization cycles), (ii) the precipitation and HPH, (iii) the lyophilization and HPH, and the (iv) the pre-milling followed by HPH. Nanocrystals production by SmartCrystal® technology is faster and economically more feasible than when using only HPH. Another advantage is the creation of smaller particle size, which is an indicator of a better physical stability. Small nanocrystals are problematic to access via pearl milling or via high pressure homogenization alone, particularly in large scale industrial production (3). Advantages and disadvantages of different methods for the production of nanocrystals are summarized in Table 1.

Table 1: Advantages and disadvantages of different methods for the production of nanocrystals.

Technologies	Advantages	Disadvantages
Precipitation	Finely dispersed drug; Well control of the desired particle size; Low-energy requirement.	Not universally applicable, restricted for drugs with specific properties; Organic solvent residues presence; Necessity of stabilization; Risk of particle growth.
Milling	First four marketed products; Low-energy requirement.	Presence of impurities, due to the erosion of milling material; Can be a slow process (several days) Wastage of the drug due to adherence to the milling pearls; Difficult to transpose to the large scale production, due to the size of the milling chamber; Need of stabilizers.
High Pressure Homogenization (HPH)	Universally applicable; Large scale production possibility; Fast process (several minutes, possibly); Continuous process; Possibility of water-free production.	High-energy requirement; Great experience required.

A dry dosage form is usually preferred, for oral administration, due to convenience, need to achieve a controlled drug delivery, prevent drug degradation, allow for better drug targeting, enhancement of the physical stability for long-term storage, and achieve a fine and non-aggregated suspension in the gastro-intestinal tract after oral administration.

There are various techniques used to transform nanosuspensions into solid forms such as spray drying, freeze drying, pelletization or granulation. Some particularities of each of these approaches are discussed next.

Spray drying is used for drug nanosuspension production using HPH, allowing for an aqueous solution of water-soluble matrix materials obtainment. In the following phase, the aqueous nanosuspension can be spray dried under suitable conditions. Drug nanocrystals

remain fixed within the matrix - which is one of the advantages of this method -, and their physical contact is avoided, allowing for the minimization of long-term physical instability (5).

Freeze drying uses a mixture of the compound, solvent and mannitol that is cooled quickly, resulting in the separation of the drug in a nanocrystal form covered by a matrix of mannitol. The stability is increased due to the presence of this matrix. However, this method show some disadvantages, e.g., it is a complex and expensive process and the resultant product is highly sensitive to process parameters, being not appropriate for industrial production (47).

Pelletization results in a multi-particulate dosage form such as coated pellet system. These forms have some advantages over single unit dosage forms, because they are faster and show a more predictable gastric emptying and also a more uniform drug distribution in the gastrointestinal tract (48).

There are many alternative technologies that are industrially less relevant, e.g., solution enhanced dispersion by the supercritical fluids (49); spray freezing into a liquid (50); rapid expansion of supercritical solution (51); rapid expansion of supercritical solution into an aqueous solution (5); evaporative precipitation into an aqueous solution process (52).

1.3. Administration routes for nanocrystals

Drug nanocrystals can be formulated in several traditional dosage forms, such as tablets, capsules, creams, gels, solutions, among others, meaning that a new drug delivery system can be combined with dosage forms well-recognized by the patient.

For mouth application, nanocrystals can be suspended in aqueous media, or be jellified if necessary by adding a viscosity enhancer (e.g. bioadhesive polymer) to increase the stickiness to the mouth mucosa.

Nanosuspensions for oral administration are one of the most important application area of nanocrystals enabling effective therapeutic blood concentrations by overcoming the absorption problems in the gastrointestinal tract by particle size reduction (53). The oral route, due to its numerous advantages (e.g. safety, convenience, compliance), is the most attractive and, thus, the first choice for drug delivery. Most nanosuspension-based products on the market are actually for oral delivery. Drug nanocrystals can be incorporated in tablets, pellets, and in hard or soft gelatine capsules. Nanosuspensions can be used as granulation media for the production of tablets, or as wetting liquid for the extrusion mass for the production of pellets.

Nanocrystals can be freeze-dried or spray-dried for the production of powders for further filling of hard capsules. If a non-aqueous medium is required (e.g. highly water-soluble drugs) the described Nanopure® production technology can be used for filling soft capsules, using low molecular weight polyethylene glycols or medium chain triglycerides as suspending media.

After administration, the drug is absorbed from the gut and enters into the blood circulation in order to be further distributed to various tissues. The solubility in the digestive juice and the passage through the gastrointestinal tract depend on the performance of the compound. The poor solubility and the dissolution rate of poorly water soluble drugs, belonging to the BCS class II, limit *in vivo* absorption and do not reach optimal therapeutic concentrations. Moreover, drugs belonging to the BCS class II often exhibit increased or accelerated absorption when are administered with food (22). There are many problems associated with these poorly water soluble drugs, such as the low bioavailability, the necessity of high oral doses, the variation in the bioavailability resulting from fasted states and the retarded onset of action. A high drug concentration gradient between the gastrointestinal tract and blood vessels is achieved when the drug is administered by a nanosuspension. This occurs due to the enhanced saturation solubility and the dissolution velocity in the digestive juice, increasing absorption and thus a high bioavailability, as previously referred. Variations in bioavailability of poorly water soluble drugs resulting from the fed/fasted state can be minimized when these are formulated as a uniform nanosuspension (54). This occurs because the dissolution rate of nanocrystals is increased significantly due to the enhanced solubility and enormous particle surface. Absorptions, both in fasted and fed state, can show permeability limited progress. Other advantages of nanosuspensions is quickly onset of action achieving for drugs with a slow dissolution rate. If particle aggregation occurs in the gastrointestinal tract, the characteristics of nanosuspensions and their associated actions may be compromised, reducing, thus, the oral bioavailability (55). Thus, the use of a stabilizer is important to prevent aggregation of the compounds under *in vitro* and *in vivo* conditions.

The parenteral administration route provides a quick onset of action, especially the intravenous, and enables the use of a reduced dosage of the drug. Intravenous injection is requested to meet some treatment purposes, such as an immediate effect, a targeting effect, an undergoing first-pass metabolism and for those drugs that are not absorbed or degraded in the gastrointestinal tract. Drugs can reach a 100% of bioavailability in the body by using intravenous administrations. However, if the drug is soluble in the blood, the pharmacokinetic profile and the tissues distribution of nanocrystals-based suspensions are similar to that of drug solutions

(4). This intravenous route is an exigent route, for which many requirements should be achieved, as like as: the products should be sterile, and the constituents should not cause any biological problem such as toxic and allergic reactions. The particle size is also crucial factor to determine whether or not a formulation can be used by the parenteral route. Particles larger than 5 μm should be controlled severely for intravenous injection, due to the fact that the smallest size of blood capillaries is about 5 μm. A high content of particles larger than 5 μm can lead to an embolism and capillary obstruction (56).

The formulation of drug nanocrystals in topical/dermal dosage forms (such as creams, hydrogels) can improve the skin bioavailability of the drug due to the increased saturation solubility of the drug. Transdermal patches may also be produced containing drug nanocrystals. The retention time of applied drug nanocrystals in the eye may be enhanced by the increased contact area with improved drug bioavailability. A depot effect may be created by the change of a water-soluble compound in a poorly water-soluble counter-part, being formulated in a final dosage form. Preparations such as ointments and suspensions have been investigated for the ocular delivery of poorly water soluble drugs. These formulations have some advantages, such as a prolonged time in the site of action and a relative higher drug dosage. The limited intrinsic solubility of the drug in lachrymal fluids is responsible for their actual performance. The low drug intrinsic solubility in those fluids leads to a low level of drug concentration in the local of action, being difficult to obtain an effective performance (17). Intrinsic adhesive features of nanocrystals reduce the precorneal loss of drug produced by the outflow of lachrymal fluids and allow for a sustained release of the drug. Actually it is possible to incorporate nanocrystals into a suitable mucoadhesive base or ocular inserts to achieve a sustained release of the drug for a specific time period. All these options guarantee a high bioavailability and an effective performance. These topical formulations are capable of promoting drug penetration and maintaining therapeutic levels with a rational frequency of application and have the capacity, at the same time, to reduce the unwanted side effects. In order to prove their security to be used for ocular delivery, nanosuspensions have been characterized for particle size, zeta potential, drug loading, *in vitro* drug release and ocular tolerability (57).

Nanosuspensions can also be nebulized using mechanical or ultrasonic nebulizers for lung/pulmonary delivery. These nanosuspensions show a great potential for the pulmonary delivery of poorly water soluble drugs. Some of these compounds have been effectively made into aerosols in the form of microparticles to be nebulized for pulmonary administration, however microparticles have some disadvantages, such as the unwanted sedimentary of the

microparticles in the pharynx and/or mouth. Moreover, together with the clearance of the drug by cilia movement those phenomena are the main causes leading to the loss of the drug in the lungs, and also constitute another disadvantages of microparticles. All these disadvantages cause a lower bioavailability, affecting hardly the performance. The solution for these problems can be the substitution of microparticles by nanosuspensions for pulmonary delivery. The enhanced dissolution velocity and saturation solubility promoted by nanosuspensions can rapidly create a larger concentration of the drug in the lungs and, consequently, lead to higher local drug levels at the absorption site. Another advantage of the nanosuspensions is the natural tendency of the nanoparticles to stick to mucosal surfaces at the absorption local over a prolonged period of time. Compared with microparticles, the longer residence time on the mucosal surface reduces the loss of the drug due to the clearance by cilia movement, and also contribute to a larger extent absorption, due to an increased dissolution time (58). The increased adhesiveness and prolonged pulmonary residence time are the fundamental advantages of nanocrystals (20).

Targeted delivery is another possibility for nanosuspensions, due to their surface properties and *in vivo* behavior that can be easily altered by the choice of adequate stabilizers. The concept of "differential protein absorption" has raised by Muller *et al.* (59). Drugs have the tendency to absorb proteins from the blood when administered by intravenous injection, so after administration the absorbed proteins can determine the *in vivo* destiny of compounds (56).

Depending on the physicochemical surface properties of the drugs, the qualitative and quantitative composition of the proteins adsorption pattern are altered (60). With the change of the stabilizer, it is easily to alter the *in vivo* behavior and drug targeting can be possible, by means of surface properties control of nanoformulations.

1.4. Physicochemical characterization of nanocrystals

The characterization of nanocrystals is essential to have repeatability of the formulation process and stability of the formulation. Particle size and shape, surface charge and crystal morphology are essentials in the formulation behavior and, thus, in the drug efficacy and *in vivo* safety profiles (61).

The size distribution of the nanosuspensions is an important step of the characterization, because it is responsible for other characteristics, like the saturation solubility, the dissolution velocity, the biological performance and the physical stability. There are many

techniques to evaluate the particle size, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM) (62), environmental scanning electron microscopy (ESEM) (63), sedimentation, atomic force microscopy (AFM) (64) and capillary hydrodynamic fractionation. It is possible to measure simultaneously the morphology and shape of the particles using electron microscopy techniques.

Generally, the shape and the morphology of the nanocrystals can be determined using a TEM and/ or a SEM, and also AFM, depending on the particle size, although the latter is clearly a more demanding technique. A wet sample of appropriate concentration is needed for the TEM analysis. Nanosuspensions are required to be processed into a dried power. SEM analysis is crucial to monitor modifications of the particle size before and after the progress of the water removal. The form of drug crystals depends on their crystalline structure (17), (20).

Zeta potential (ZP) determinations are based on the electrophoretic mobility measurements of particles (65), and this analysis allows the prediction about the storage stability of colloidal dispersions (66). Particle aggregation is less probable to occur if particles possess a ZP capable of providing sufficient electric repulsion, or enough steric barrier for a sufficient steric repulsion. Jacobs *et al.*, specify a ZP of at least -30 mV for electrostatic and -20 mV for sterically stabilized systems necessary to obtain physically stable nanocrystal suspensions (14). The ZP of a nanosuspension is fundamental, because it gives an idea about the physical stability of the solution. The ZP of a nanosuspension is dependent on the stabilizer and the drug itself (67). To evaluate the crystalline structure of the drug nanocrystals differential scanning calorimetry (DSC) and X-ray diffraction can be used (54).

The determination of the saturation solubility and the dissolution velocity is essential, because not only these parameters can help to assess the benefits compared to the microparticle formulation, but also may help to anticipate the *in vivo* performance. Previously of the investigation of the dissolution behavior, nanosuspensions should be moved into a dried power (68). Diverse temperatures can be used, in the experiments to determine the saturation solubility of a dehydrated powder in a different artificial medium (17). In the case of the determination of dissolution velocity, the methods described in the Pharmacopoeia might be used. Light-scattering has proved to be very useful for accurate determination of the nanocrystals solubility (69).

The chemical composition of the particle surfaces and the surface analysis of nanocrystals can be evaluated by X-ray photoelectron spectroscopy. The knowledge of the surface parameters of nanosuspensions is essential, especially if the objective is to the

intravenous administration (70). DSC is used to analyze thermal behavior of crystalline and amorphous materials. This technique has the capacity to detect the transition temperature, the melting point, polymorphic changes and gives precise quantitative data of the energy that is adsorbed or released during the experiment (71). The infrared spectra is normally used to recognize specific functionalities of chemicals and to determine the interactions between different compounds (72). The *in vivo* behavior of nanocrystals after injection depends on their surface properties. These properties include the surface hydrophobicity and the interaction of nanocrystals with plasma proteins (73).

1.5. Ibuprofen

Ibuprofen is a non-steroidal analgesic and an anti-inflammatory drug, has a suitable molecule size of about 1.0–0.6 nm (74). Is an acidic poorly soluble compound (pKa=4.49) that has frequently used as a model. Ibuprofen ((RS)-2-(4-(2-methylpropyl) phenyl)propanoic acid) present more solubility in a neutral-basic environment (75).

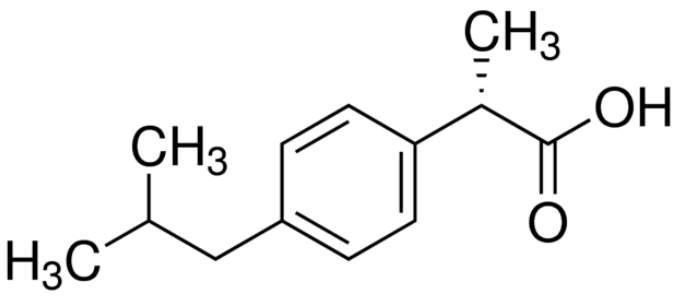


Figure 2: Ibuprofen structural formula. (www.sigmaaldrich.com accessed on June 2016).

Although ibuprofen is practically insoluble in water, which limits the oral bioavailability, is quite soluble in organic solvents, as can be seen from the data presented in Table 2.

Table 2: Ibuprofen solubility values in different solvents at 25°C solvents.

Solvents	Solubility / mg mL^{-1}		References
	R-(-)- enantiomer	S-(+)- enantiomer	
Water	0.12	0.08	(76)
Ethanol	0.20		(77)
***n*-octanol**	394.00		(78)
Chloroform	618.00		(78)
Cyclohexane	193.00		(78)

The solubility of a substance may be described in a variety of ways. The USP/NF generally defined the solubility in terms of the volume of solvent required to dissolve 1 gram of the drug at a specified temperature. Other references may use more subjective terms to describe solubility, such as those given in Table 3.

Table 3: Descriptive terms of approximate solubility of substances (79, 80).

Descriptive terms	**Parts of solvent needed for 1 part solute**
Very soluble	<1
Freely soluble	1-10
Soluble	10-30
Sparingly soluble	30-100
Slightly soluble	100-1000
Very slightly soluble	1000-10000
Practically insoluble or insoluble	>10000

Usually, nonsteroidal anti-inflammatory drugs (NSAIDs), in this case ibuprofen, are usually prescribed in managing rheumatoid arthritis, post-operative pain, chronic pain associated with cancer and to treat fever. Ibuprofen have good therapeutic effect and in high doses can cause gastric irritation, consequently, should be delivered via parenteral route only on the site of the infection (81).

NSAIDs inhibit the production of prostaglandins from arachidonic acid by the cyclooxygenase (COX), then there is a suppression of inflammation in most patients (82). NSAIDs can be prescribed as analgesics, anti-inflammatory and antipyretic for a number of indications.

Ibuprofen is a racemic mixture of the (S)-(+)- and (R)- (-)-enantiomers, the (S)-(+)-ibuprofen is the active enantiomer, both *in vitro* and *in vivo*. Is possible to administered ibuprofen through several pharmaceutical formulations (83). In the liver, approximately 65% of the R-(-)-enantiomer is transformed in the (S)-(+) – ibuprofen. Some of it is pre-systemically inverted in the gut in the presence of Acyl CoA thioester, where alpha-methylacylcoenzyme A racemase acting as the catalyst. Both enantiomers are quickly metabolised by phase I detoxification enzymes in human liver (84). However, the metabolic pathways of its enantiomers differ significantly. Although the (S)-(+)- enantiomer is metabolised frequently by CYP2C9, the (R)-(-)-ibuprofen is metabolised mostly via CYP2C8 (85). Almost total of ibuprofen is metabolised and the principal route of ibuprofen excretion is through the kidney and just a small percentage of the drug consumed is excreted without alteration in urine.

The risk of toxicity is associated to the binding of ibuprofen-glucuronide to plasma proteins, the highest risk is related in patients with renal impairment (86). Ibuprofen was too confirmed as beneficial in reducing the colorectal cancer risk, no variant modified their protective effects (87). In therapeutic, ibuprofen seems to be the first choice, due do its higher safety profile, as it is associated with rarer gastrointestinal and renal side effects when compared with others drugs, for example, indomethacin (88).

Ibuprofen has been frequently used as a model drug on the purpose of sustained/ controlled release, once is a compound with low solubility (intrinsic solubility of approximately 0.06 mg $mL^{(-1)}$) (89), high permeability and is considered a class II compound according to The Biopharmaceutical Classification System (BCS) (90). How it is practically insoluble in water, consequently its oral absorption is the dissolution rate limited. So, ibuprofen because of ductile nature and associated challenge in particle size reduction, was used as a model drug in this work.

Table 4: Physicochemical properties of ibuprofen (79, 91).

Properties of ibuprofen	
CAS no.	15687-27-1
Molecular weight	206.29
Formula	$(CH_3)_2CHCH_2C_6H_4CH(CH_3)COOH$
Indication	Non steroid anti-inflammatory drug
Physical-state	White crystalline powder
Melting point	75-78°C
Pka	pKa_1: 4.40; pKa_2: 5.20
Solubility	Practically insoluble in water, readily soluble in most organic solvents

The aim of this work has been the development by Design of Experiment (DoE) and the physicochemical characterization of optimized formulations of poorly-soluble drug nanocrystals, using ibuprofen as model drug. In the present work, nanosuspensions stand for aqueous dispersion of drug nanocrystals stabilized by hydrophilic surfactants in aqueous medium.

2. Materials and Methods

2.1. Materials

Polysorbate 80 (Tween 80®) was purchased from Uniqema, Everberg, Belgium. Ibuprofen was kindly donated from Medinfar-Amadora, Portugal. Polyvinylpyrrolidone (PVP) K30 was acquired from Fluka (Switzerland). Phosphate buffered saline, pH 7.40 and sorbitan monooleate (Span 80®) were from Sigma-Aldrich (Steinheim, Germany). Cellulose membrane Millipore® HA with an average pore size of 0.22µm. RPMI 1640, BioWhittaker®, was acquired from Lonza (Belgium), Fetal Bovine Serum was purchased from Biowest (South America origin), Trypsin EDTA 0.25% was from Sigma-Aldrich (Steinheim, Germany), 96-well microplates (VWR, USA). Ultra-purified water was obtained from Milli® Q Plus system, home supplied.

2.2. Production of drug nanocrystals by melt emulsification

The melt-emulsification process was used for the production of drug nanocrystals (i.e. nanosuspensions) composed of 0.25% (m/v) ibuprofen and aqueous solution of surfactants. Ibuprofen was added to the aqueous solution of surfactants, Tween 80® (T80) and Polyvinylpyrrolidone (PVP) K30 or T80 and Span 80® (S80). The surfactants (or their mixture) should exhibit sufficient affinity for the droplet surface to enable preparation of emulsion and should present affinity for the particle surface in order to stabilize the nanosuspension. In preliminary experiments were used different concentrations of stabilizers (Table 5) to stabilize the nanosuspension during the production process.

Table 5: Emulsions containing different amounts of stabilizers and fixed concentration of melted drug (0.25% (m/v)).

Formulation	% T80 (m/v)	%S80 (m/v)	Formulation	% T80 (m/v)	% PVP K30 (m/v)
S1	0.125	0.125	**P1**	0.125	0.125
S2	0.50	0.125	**P2**	0.50	0.125
S3	0.125	0.50	**P3**	0.125	0.50
S4	0.50	0.50	**P4**	0.50	0.50
S5	0.25	0.25	**P5**	0.25	0.25

The obtained drug suspension was heated up to 80°C to melt ibuprofen (melting point is approximately 76°C). The suspension obtained followed by high shear homogenization (Ultra-Turrax ®, T25, IKA) for 10 minutes to obtain a coarse emulsion. It was transferred to a high pressure homogenizer (EmulsiFlex®-C3, Avestin), and homogenized at 1000 bar for 20 minutes in the continuous mode, operated at 80°C.

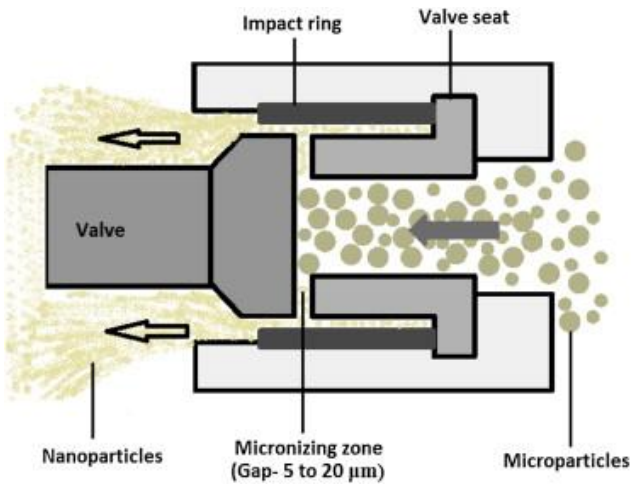


Figure 3: Diagram of the high pressure homogenization process. The microparticles are forced through a minute gap in the micronizing zone, which creates conditions of high turbulence and shear, combined with compression, acceleration, pressure drop and impact. All of these conditions lead to the formation of a nanosuspension (92).

The hot emulsion was then cooled down, by placing it in an ice-bath for, approximately, more 20 minutes.

2.3. Characterization of drug nanocrystals

2.3.1. Particle size analysis

The particle size and polydispersity index (PI) were investigated by dynamic light scattering (DLS) using a particle size analyzer (DelsaNano C Submicron, Beckman Coulter Delsa, Krefeld, Germany). Mean diameter, size distribution, and PI of nanosuspensions were determined in triplicate. Values are presented as means of triplicate runs per sample. For each measurement, the nanosuspension was diluted in Milli-Q water to an appropriate concentration to avoid multiple scattering.

2.3.2. Zeta potential

Zeta potential (ZP) measurements were taken by electrophoretic light scattering (ELS) using a Nano Zeta Potential Analyzer (DelsaNano C Submicron, Beckman Coulter Delsa, Krefeld, Germany). Measurements were taken in a Flow Cell (Beckman Coulter Delsa) at 25 °C, and Milli-Q water was used to dilute the nanosuspensions to a proper concentration. The ZP was calculated using the Helmholtz −Smoluchowsky equation included in the software of the system. Values are presented as means of triplicate runs per sample.

2.3.3. Factorial design

The influence of the concentration of both surfactants in both mixtures was evaluated using a 2^2 factorial design with triplicate of central point for estimating the experimental error, composed of 2 variables for each formulation which were set at 2-levels each. The dependent variables were the mean particle size, PI and ZP. The design required a total of 7 experiments for each formulation. As summarized in Table 6, each factor, the lower and higher values of the lower and upper levels, was represented by (-1) and a (+1), respectively, and the central point was represented by (0).

Table 6: Initial full factorial design to both formulations, providing the lower (-1), upper (+1) and (0) central point level for each variable.

Variables	Levels		
	-1	**0**	**+1**
Combination 1:			
T80	0.125	0.25	0.50
S80	0.125	0.25	0.50
Combination 2:			
T80	0.125	0.25	0.50
PVP K 30	0.125	0.25	0.50

These values were chosen on the basis of the tested lower and upper values for each variable according to literature research. A factorial design approach was applied to maximize the yield of production on the basis of the production possibility curves. The data were analyzed using STATISTICA 7.0.

2.3.4. *In vitro* ibuprofen release

In vitro release studies of ibuprofen from nanocrystals were performed using Franz glass diffusion cells (Figure 4). These cells consisted of donor and receptor chambers between which a cellulose membrane Millipore HA with an average pore size of 0.22µm is positioned. The utilized membrane had diameter sufficient to cover the effective diffusion area of the receptor, and was soaked in receptor fluid for at least 2 hours.

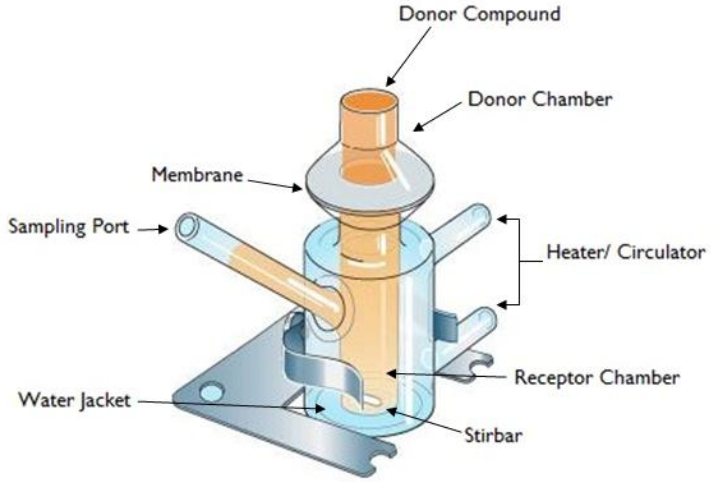


Figure 4: Schematic diagram of Franz glass diffusion cells.

The prehydrated membrane was mounted between the matched donor and receptor compartment with ibuprofen nanocrystals was placed on the membrane surface in the donor compartment. All openings including donor top and receptor arm were occluded with parafilm to prevent evaporation. At determined times, using a syringe, 200µL of the samples were collected and the same volume was replaced with buffer. The ibuprofen collected was analyzed for ultraviolet (UV) (at 264 nm) assay by Synergy™ HT Multi-Mode Microplate Reader. Air bubbles formed below the membrane were removed by carefully tilting the Franz cells for the air bubbles to escape via the sampling arm. Phosphate-buffered saline (PBS) at pH 7.40 is used as receptor fluid and maintained at 37°C. A volume of 1mL of nanocrystals (0.25% Ibuprofen) was applied to the donor compartment and the receptor chamber contained 5 mL of buffer. During all the experiment, a magnetic bar was stirring in each cell.

2.3.5. *In vitro* cell viability evaluation

2.3.5.1. Cell culture

The human epithelial colorectal cell line (Caco-2) was purchased from ATCC® HTB-37™ and kindly provided by Prof. Dr Marco Lemos (MARE/IPL). The Caco-2 cells were cultured in RPMI 1640 and supplemented with 10% fetal bovine serum (FBS) heat inactivated (HI). The cells culture was kept at 37°C in 5% CO_2 in a wet incubator.

In preliminary tests, Cos-7 cell line derived from the kidney of the African green monkey, *Cercopithecus aethiops*, was employed but due to its higher sensitivity and both formulations in study are for oral administration, their use in the following *in vitro* testing was discarded.

The Caco-2 cell line was previously thawed with suitable media at 37°C and centrifuged. After resuspend the cell culture with media was incubated for 24 hours. Trypsin EDTA 0.25% was added to the cells to remove them the bottom of the flask then were again incubated for some minutes. Then cells were resuspended with the appropriate media and were transferred to a new flask to have the required space and facilitate the healthy growth. Incubated for more 24 hours.

2.3.5.2. Cell viability assays

For cell viability assays cells were seeded in 96-well microplates (VWR, USA) at a density of $0.05x10^6$ cells/ µl in 200 µl of medium. After 24 hours of cell attachment, microplates were aspirated and the Caco-2 cells were treated with decreasing concentrations of each formulation of nanocrystals. Three replicate wells were used for each control and test concentration per microplate. Cell viability was assessed using resazurine assay.

2.3.5.3. Resazurin (AlamarBlue)®

Based on the literature, between the multiple cytotoxicity assays used, the resazurin assay was found to be the most sensitive (93).

Cell viability was assessed for the formulations and controls (cellular control, T80, S80, T80+S80, PVP K30, T80+PVP K30) on human epithelial colorectal cells, Caco-2. Evaluation of cell viability was performed by a colorimetric assay, AlamarBlue® assay, where resazurin, the active ingredient of AlamarBlue® reagent, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert

resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells.

Briefly, when confluence reached 80-90%, a cell suspension of 0.05x106 cells/mL was prepared and was left to stabilize in 96-well microplates with a final volume of 200µL. After 24 hours, the medium was removed and the different formulations were added in a final volume of 200µL.

After 3h, 6h and 24h, the medium was completely removed and new medium containing 10% resazurin was added per well. Cells were further incubated during 3 hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After this incubation time, the quantification of resorufin was performed using an ELISA microplate reader at 570 nm with a reference wavelength of 620 nm.

Three independent experiments were performed with the formulations. The results were expressed as percentage of resazurin reduction relatively to control cells.

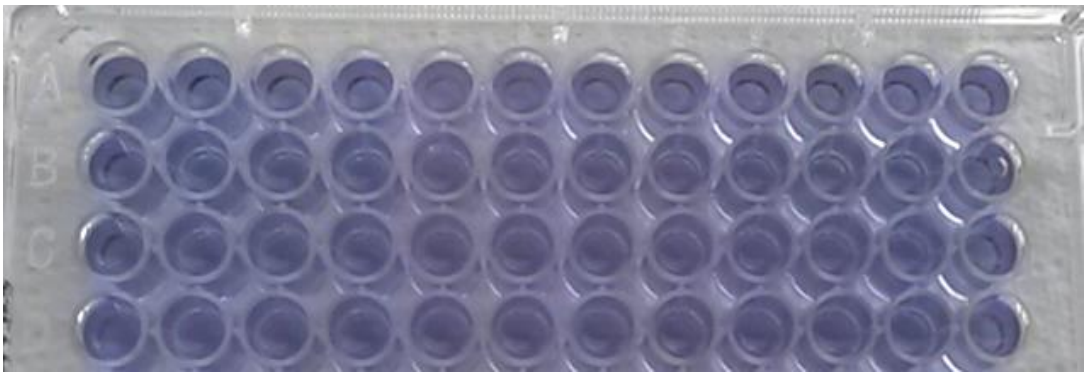


Figure 5: Cell cultures with resazurin.

The second and third assays was preformed after 6 and 24 hours respectively, repeating the method describe above.

The absorbance was analyzed by Synergy™ HT Multi-Mode Microplate Reader.

2.4. Data analysis

All the cells experiments were performed in triplicate, being the results expressed as mean ± SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA, with a Dunnett's multiple comparisons test. The statistical tests were applied using GraphPad Prism, version 6.00 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. Production of drug nanocrystals by melt emulsification

In the production of nanosuspensions by melt emulsification method, the first step is the preparation of a hot emulsion with melted drug as dispersed phase by Ultra-Turrax ®, T25, IKA. The primary emulsion was homogenized in HPH and cooled to solidify the droplets of melted drug. In this technique, particle formation is the consequence of the transformation of the melted drug into the solid state.

Figure 6: High pressure homogenizer (EmulsiFlex®-C3, Avestin) and Ultra-Turrax ®, T25, IKA with the respective heating baths.

The size of the drug particles is dependent mostly on the size of dispersed droplets and the surfactants figuring in the formulation. To prepare smaller drug particles from hot emulsion, the collision of droplets can be prevented by fast cooling once it causes very fast solidification of melted drug droplets, resulting in smaller drug particles. The mean particle size (Z-AVE) and PI of ibuprofen suspensions obtained from emulsions containing different amounts of surfactants and different mixtures of these surfactants were determined by DLS (see section 3.2).

3.2. Particle size analysis

In preliminary experiments different concentrations and mixtures of stabilizers were used to stabilize the nanosuspension during the production process and their influence on mean Z-AVE and PI was studied by DLS. The preliminary factorial design study, required a total of 7 experiments for each formulation, as summarized previously. After production, the results of the mean Z-AVE and PI were described below (Table 7 and 8).

Table 7: Mean particle size and polydispersity index of different concentrations to the mixture of T80 and S80.

Codes (combinations)	T80 (%, m/V)	S80 (%, m/V)	Z-AVE (nm)	PI
S1	**0.125**	**0.125**	169.60	0.19
S2	**0.50**	**0.125**	150.70	0.34
S3	**0.125**	**0.50**	160.30	0.19
S4	**0.50**	**0.50**	160.90	0.26
S5	**0.25**	**0.25**	149.50	0.17
S6	**0.25**	**0.25**	150.90	0.20
S7	**0.25**	**0.25**	146.40	0.19

In all the measurements, Milli-Q water was used to dilute the nanosuspensions to a proper concentration.

Table 8: Mean particle size and polydispersity index of different concentrations of the mixture of T80 and PVP K30.

Codes (combinations)	T80 (%, m/V)	PVP K30 (%, m/V)	Z-AVE (nm)	PI
P1	**0.125**	**0.125**	1204.00	0.97
P2	**0.50**	**0.125**	120.60	0.27
P3	**0.125**	**0.50**	157.60	0.19
P4	**0.50**	**0.50**	83.70	0.19
P5	**0.25**	**0.25**	157.30	0.33
P6	**0.25**	**0.25**	145.50	0.20
P7	**0.25**	**0.25**	68.80	0.15

All the results were used to make possible the study of factorial design in way to optimized both formulations and increasing their stability.

3.3. Factorial design

To design a new formulation, it is of paramount importance to identify the influencing parameters, since these might affect the properties of the final dosage form. The experimental design method analyses the influence of different variables on the properties of the drug delivery system. To evaluate the optimum experimental conditions for both formulations of nanocrystals produced with different concentrations of surfactants (independent variables), a 2-levels 2-factors factorial design has been employed to assess the effects of the independent variables in the dependent factors that are involved in the characteristics of nanocrystals, such as, Z-AVE and PI.

The ZP (i.e., the electrical charge at the nanocrystals surface) translates the long-term physical stability and the particle adhesive properties. Higher ZP values, either positively or negatively charged, mean that nanocrystals will have greater long-term stability (94).

The influence of concentrations of each surfactant are shown in Tables 7 and 8 after 20 minutes in HPH with the constant pressure, 1000 bar. Table 7 shown the results for the

formulation 1, where the surfactants used were T80 and S80 with the fixed concentration of ibuprofen (0.25%), while the results for the formulation 2, with T80 and PVP K30 as surfactants were studied and with the fixed concentration of ibuprofen (0.25%) in all the combinations, are shown in Table 8. Figure 7 shows Pareto charts of the standardized effects for formulation with T80 and S80, Figure 8 shows the surface response charts of experimental design.

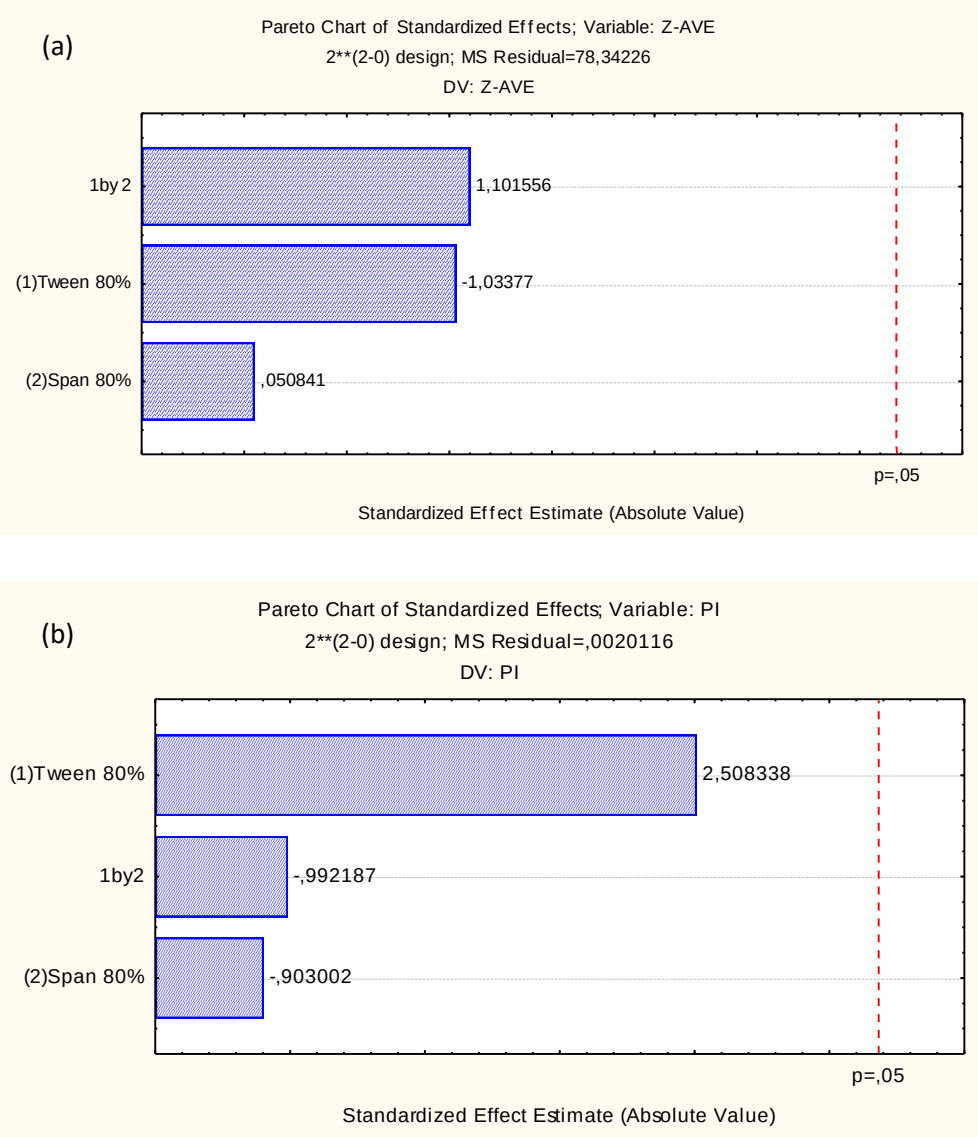
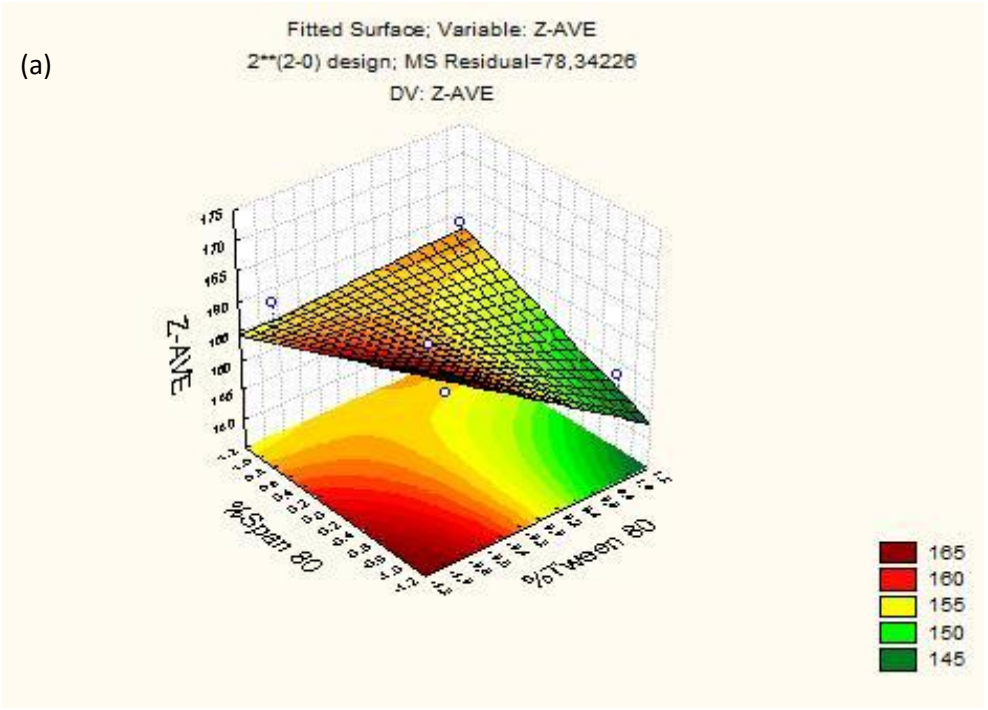


Figure 7: Pareto charts of the standardized effects for nanocrystals obtained for formulation 1 (a) particle size (Z-AVE); (b) polydispersity index (PI).

The results obtained for the formulation 1 (T80 and S80) demonstrate that the concentrations of both surfactants were found not to be statistically significant (Figure 7). However, in the surface response charts of experimental design, Figure 8(b), the PI is strongly affected by the increase concentration of T80.

(a)



(b)

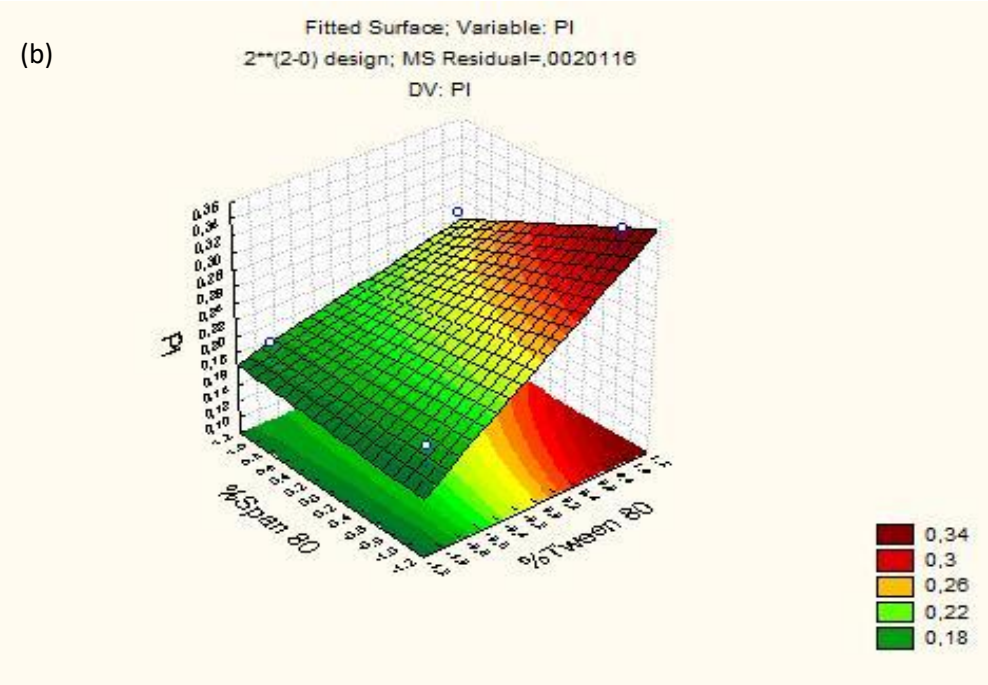


Figure 8: Surface response charts of experimental design of nanocrystals obtained for formulation 1: (a) particle size (Z-AVE); (b) polydispersity index (PI).

There is still a committed relationship that needs to be established between the values of Z-AVE and PI. The response surface graphs (Figure 8(a)), shows that increasing the %T80 decreases the average size, however, it increases the PI.

Thus, in this formulation, although none variable is statistically significant, when the formulation have a smaller concentration of T80 and a larger amount of S80, the nanocrystals tend to be larger, i.e., approximately 160nm, and the PI is <0.20.

Based on this findings, the formulation that we selected as optimal to follow *in vitro* studies have the characteristics presents in the Table 9.

Table 9: Physicochemical characterization of optimized Formulation 1.

Formulation 1 (day of production)	**% T80**	**% S80**	**Z-Ave (nm)**	**PI**	**ZP**
	0.20	1.20	174.10	0.18	-28,10

Usually, particle aggregation is less likely to occur for charged particles with ZP >|20|, once there is electrostatic repulsion between particles with the same electrical charge (95).

In the followed days, the Z-AVE and PI of the formulation was analysed, once, visually the nanosuspension was perfectly homogeneous which did not happens before the factorial design.

Table 10: Physicochemical characterization of Formulation 1 after 1 and 7 days of the production.

Formulation 1	**% T80**	**% S80**	**Z-Ave (nm)**	**PI**
Day 1	0.20	1.20	181.40	0.22
Day 7	0.20	1.20	178.50	0.19

The influence of each independent variable and their interactions on formulation 2, with T80 and PVP K30, were also assessed using Pareto charts (Figure 9).

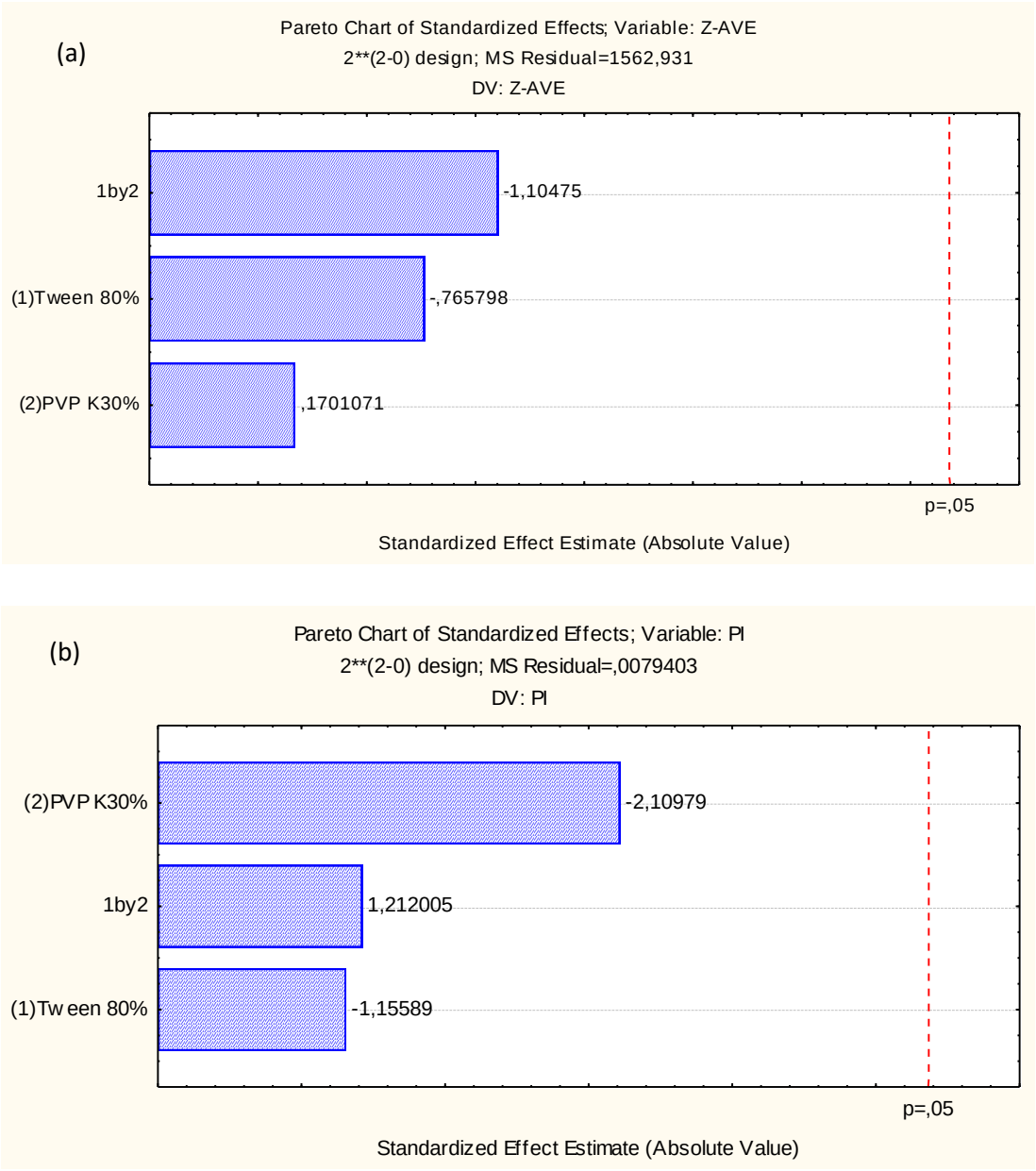


Figure 9: Pareto charts of the standardized effects for nanocrystals obtained for formulation 2 (a) particle size (Z-AVE); (b) polydispersity index (PI).

As shown in Figure 9, the Z-AVE of crystals and their PI were not shown to be significantly influenced by the tested parameters, neither were the interaction between variables.

Similar results obtained with formulation 1 were also observed in formulation 2 (Figure 10). Over again, a committed relationship need to be establish between the values of Z-AVE and PI.

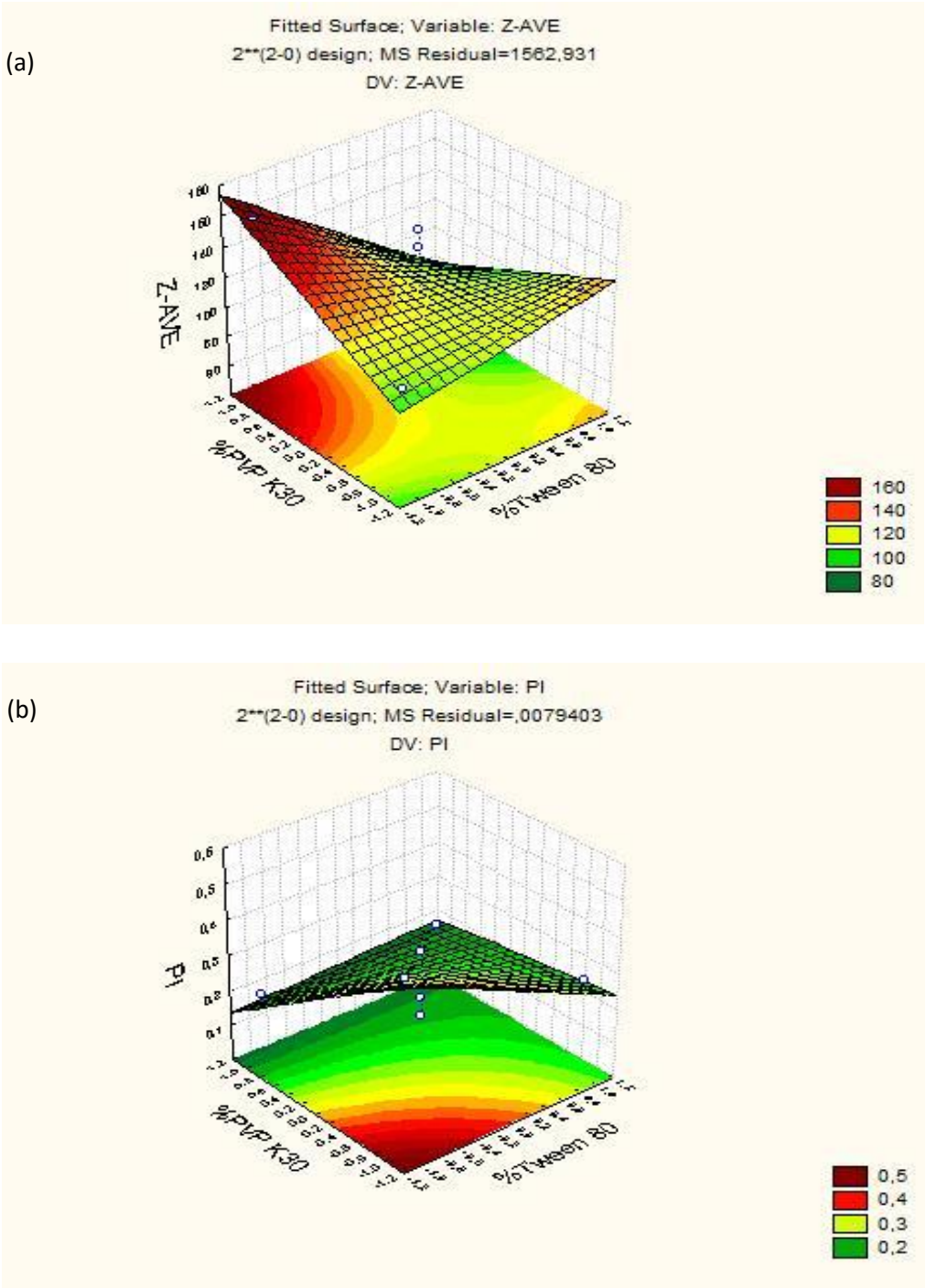


Figure 10: Surface response charts of experimental design of nanocrystals obtained for formulation 2: (a) particle size (Z-AVE); (b) polydispersity index (PI).

Thus, in this formulation, again, although none of the variables were statistically significant, when the formulation has a smaller concentration of T80 (~ 0.20%) and a larger amount of PVP K30, the nanocrystals tend to be smaller. Additionally, in these range of concentrations the PI is <0.20 indicating homogeneity between the particles.

Based on these findings, the formulation selected as optimal to follow *in vitro* studies has the characteristics presents in Table 11.

Table 11: Physicochemical characterization of optimized Formulation 2.

Formulation 2 (day of production)	**% T80**	**% PVP K30**	**Z-Ave (nm)**	**PI**	**ZP**
	0.20	1.20	79.00	0.12	-12.70

The mean Z-AVE and PI was recorded after 1 and 7 days. Results are shown in Table 12.

Table 12: Physicochemical characterization of Formulation 2 after 1 and 7 days of the production.

Formulation 1	**% T80**	**% PVP K30**	**Z-Ave (nm)**	**PI**
Day 1	0.20	1.20	82.72	0.14
Day 7	0.20	1.20	94.58	0.07

The results obtained in this study clearly demonstrate the added value of using factorial design for the development of stabilized drug nanocrystal formulations.

3.4. *In vitro* ibuprofen release

In vitro release studies of ibuprofen from nanocrystals were performed using Franz glass diffusion cells (Figure 11). At pre-determined time-intervals, 15 minutes, 30 minutes, 1, 2, 4, 6, 12, 24 hours samples were collected and the same volume was replaced with PBS.

Figure 11: Franz cells (2 formulations; n=3 each formulation).

The collected samples were analysed by UV (at 264 nm) assay by Synergy™ HT Multi-Mode Microplate Reader. Previously was made in the same equipment at the same wavelength the calibration curve (Figure 12), where ibuprofen was dissolved in PBS (pH=7.40).

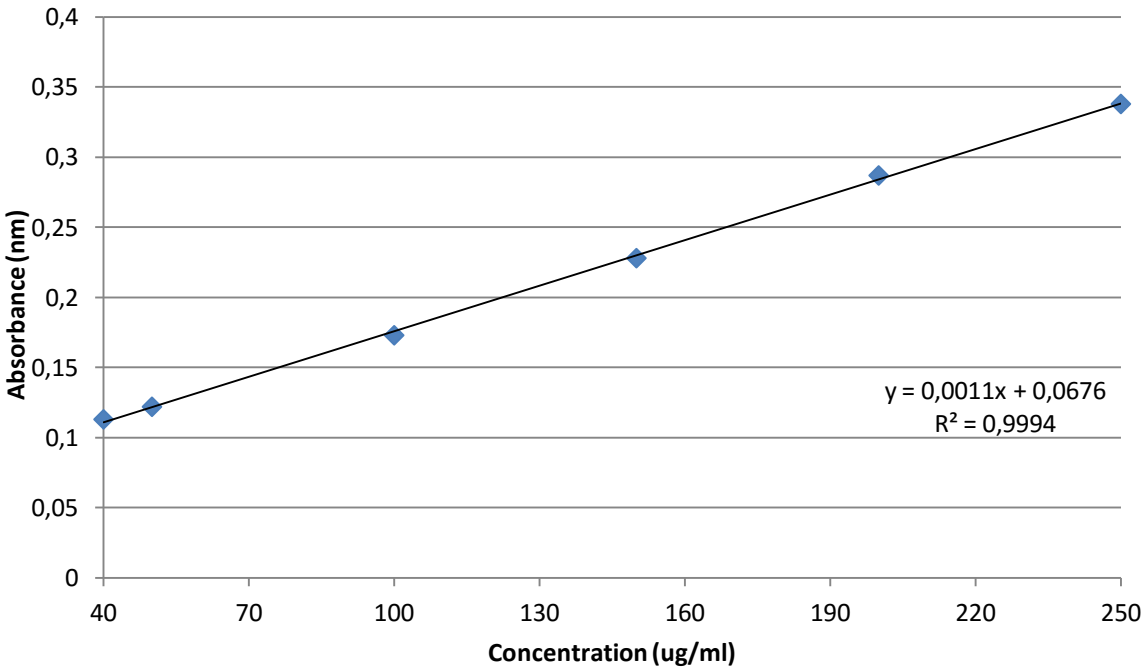


Figure 12: Calibration curve [40-250 µg/ml] of Ibuprofen at 264 nm.

The results obtained for the release of ibuprofen from the formulation 1 are shown in Figure 13.

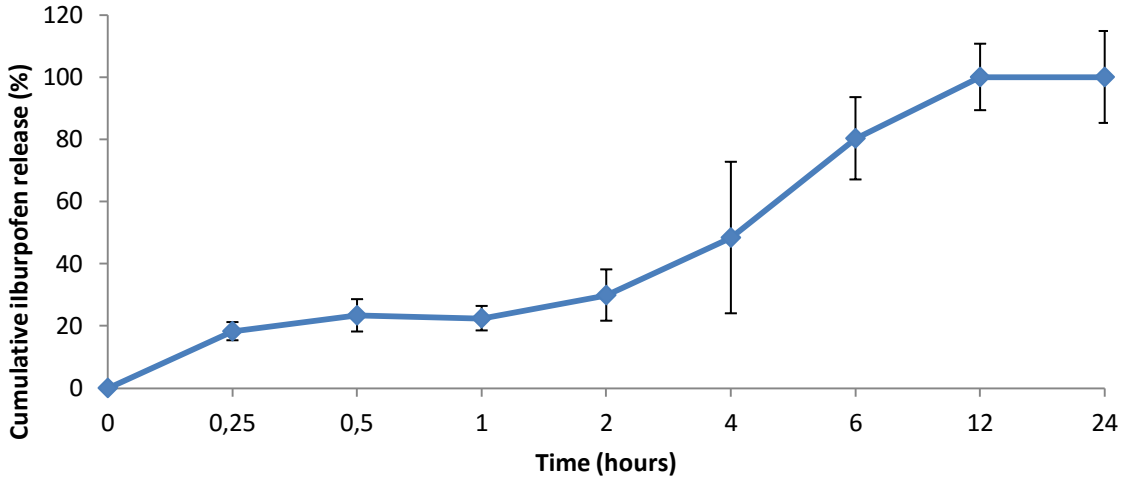


Figure 13: In vitro release of Ibuprofen during 24 hours, recorded for formulation 1.

Within the first 12 hours, formulation 1 released about 100% of drug. The profile shown in Figure 13 also allowed the identification of a burst release within the first 15 minutes, during which 20% drug was immediately released. Burst release has been attributed to a variety of physical, chemical and processing parameters (96).

The obtained release profile has been adjusted to kinetic models, i.e., zero order, first order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas, Baker-Lonsdale, Weibull (97). These methods describe the release profile based on different mathematical functions. Depending on the derived model parameters and if a suitable function has been selected, the release profiles are evaluated. Results are summarized in Table 13.

Table 13: Mathematical modelling for ibuprofen release from formulation 1 at pH 7.4.

	Higuchi	**Zero-order**	**First-order**	**Baker-Lonsdale**	**Weibull**	**Hixson-Crowell**
K	0.1009204	0.0204	0.5039	4.50E-11	1.6870	0.0329
b	-0.1388138	-0.0261	-6.4458	-1.35E-10	-6.0784	0.2262
R^2	0.9786022	0.9697	0.7460	0.948	0.9080	0.9505

For the formulation 1 and based on the data presented in Table 13, the best fitting model is the Higuchi. This model was initially conceived for planar systems and was extended to different geometrics and porous systems (98).

Higuchi can be used to describe the drug dissolution from several types of modified release pharmaceutical dosage forms. Is possible to simplify the Higuchi model as (known as the simplified Higuchi model): $f_t = Q = K_H \times t^{1/2}$, where, K_H is the Higuchi dissolution constant (97).

The results obtained at determined time, for formulation 2 was analysed. The result obtained for the release of ibuprofen from the formulation 2 is represented in Figure 14.

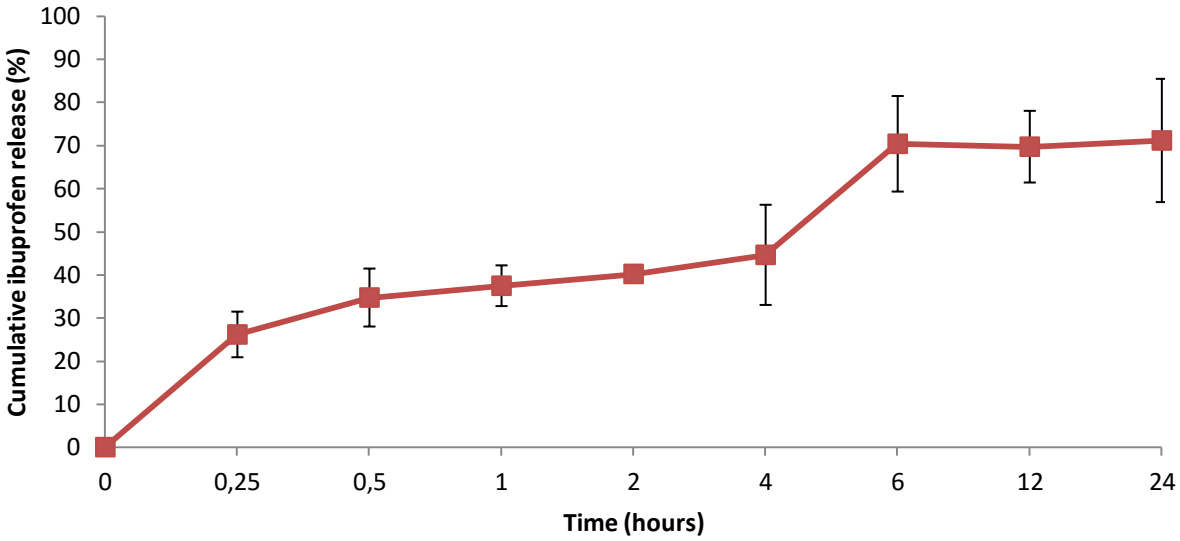


Figure 14: In vitro release of Ibuprofen during 24 hours, recorded for formulation 2.

Within the first 6 hours, formulation 2 released about 70% of drug. The profile shown in Figure 14 also allowed the identification of a burst release within the first 15 minutes, during which 20% drug was immediately released. Formulation 2 showed a similar profile as that recorded for formulation 1. However, lower amount of drug was released within the same time-period. The strongest interaction between the surfactants and ibuprofen in formulation 2 may be the cause of the slower drug release in comparison to formulation 1.

To describe the dissolution profile of the nanocrystals, mathematical models, i.e., zero order, first order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas, Baker-Lonsdale, Weibull (97) have also been used (Table 14). Depending on the derived model parameters and if a suitable function has been selected, the release profiles are evaluated.

Table 14: Mathematical modelling for ibuprofen release from formulation 2 at pH 7.4.

	Higuchi	Zero-order	First-order	Baker-Lonsdale	Weibull	Hixson-Crowell
K	0.0850267	0.0160	0.2506	4.93E-11	1.5891	0.0268
b	-0.0808804	-0.0056	-5.7038	-9.39E-11	-6.0798	0.1694
R^2	0.9545803	0.9465	0.6193	0.963	0.9227	0.8174

For the formulation 2 and based on the data presented in Table 14, the best model was found to be Baker-Lonsdale. Baker-Lonsdale model was developed from the Higuchi model, describing the drug release from spherical matrices according to the equation

$$f_1 = \frac{3}{2}\left[1 - \left(1 - \frac{M_t}{M_\infty}\right)^{2/3}\right] \frac{M_t}{M_\infty} = k_t \qquad \text{(Eq. 4)}$$

where $\frac{M_t}{M_\infty}$ is a fraction of drug released at time t and the release rate constant, k, corresponds to the slope (99). Baker-Lonsdale model has been used for the linearization of release data from several formulations of microcapsules or microspheres and formulations within the nano range.

Comparing the profiles obtained for both formulations (Figure 15) it is possible to draw some conclusions. Within the first 15 minutes, formulation 2 depicted a faster release profile, which may infer a faster therapeutic effect. However, the same formulation released only 70% of the drug within the first 6 hours, which means that 30% of the administered ibuprofen does not have any therapeutic effect. On the other hand, formulation 1 released 100% of the drug within the first 12 hours, offering the same burst effect as formulation 2. In addition, a modified release profile could also be attributed to formulation 1 given the slower release in the first 4 hours.

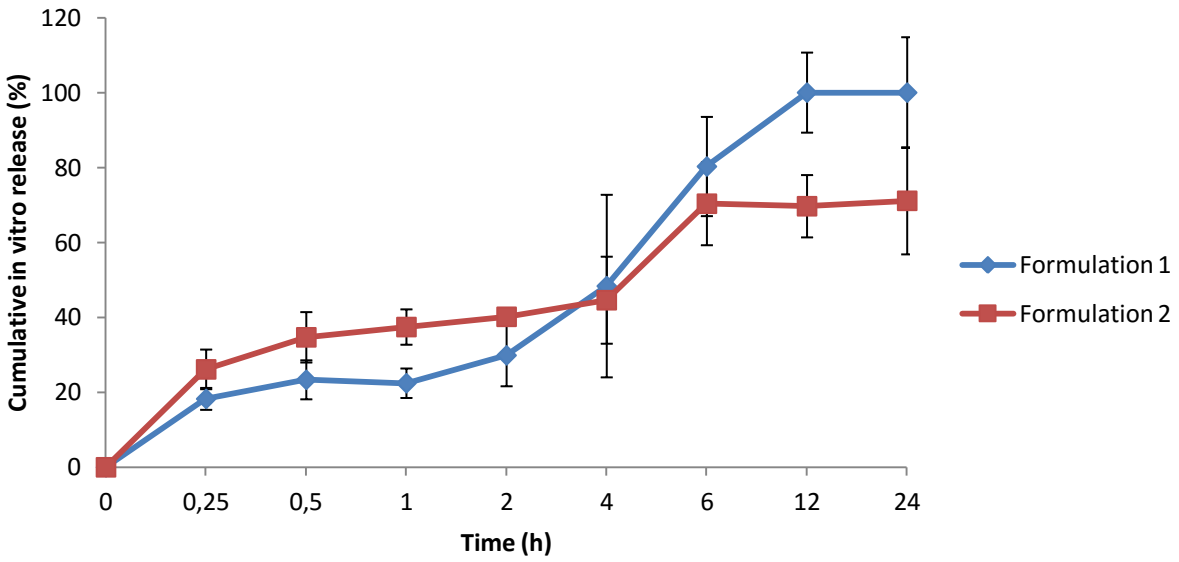


Figure 15: *In vitro* release of Ibuprofen during 24 hours, recorded for formulations 1 and 2.

The *in vitro* ibuprofen release assay allowed to conclude that both formulations have advantages and disadvantages. Depending on the therapeutic purpose, immediate action or modified release over time is possible to achieve by choosing the most appropriate formulation.

3.5. Cell viability assays

The cell viability assay was performed based on the cell capacity to metabolize the resazurin, at pre-determined time-intervals, i.e. 3, 6 and 24 hours. Changes in the cell capacity to metabolize the formulations in each well, during the culture of Caco-2 cells, are shown in Figures 16, 18 and 20.

Only with the observation of the wells in the study there is clearly a difference in cell viability. If the cells variability are 80-100%, all resazurin is metabolized and thus the medium will pass from blue to red, means that the cells are viable, as can see in control 1. All the wells with blue medium are strongly affected by the formulations.

All the experiments were performed in triplicate, being the results expressed as mean ± SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA, with a Dunnett's multiple comparisons test (Figure 17).

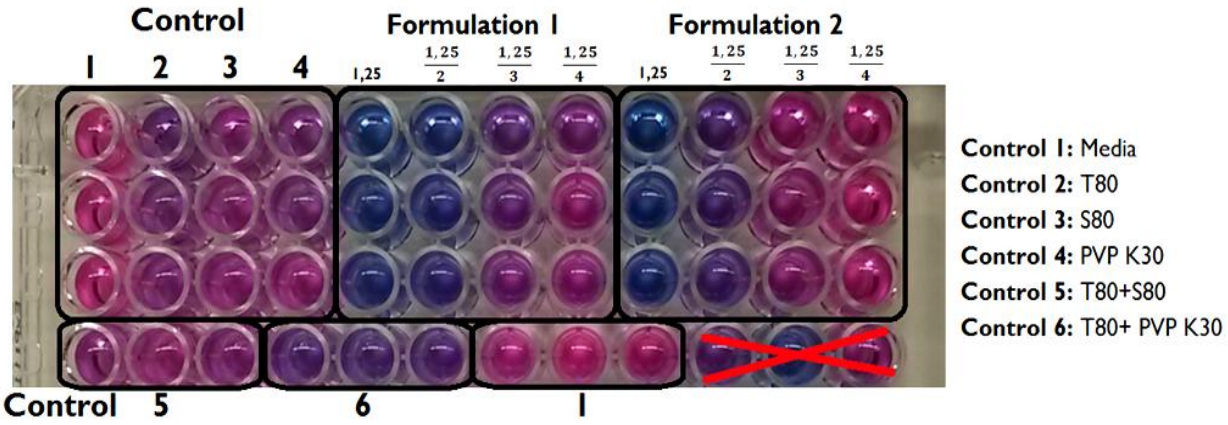


Figure 16: Cell cultures after 3 hours incubated with both formulations in four different concentrations (n=3).

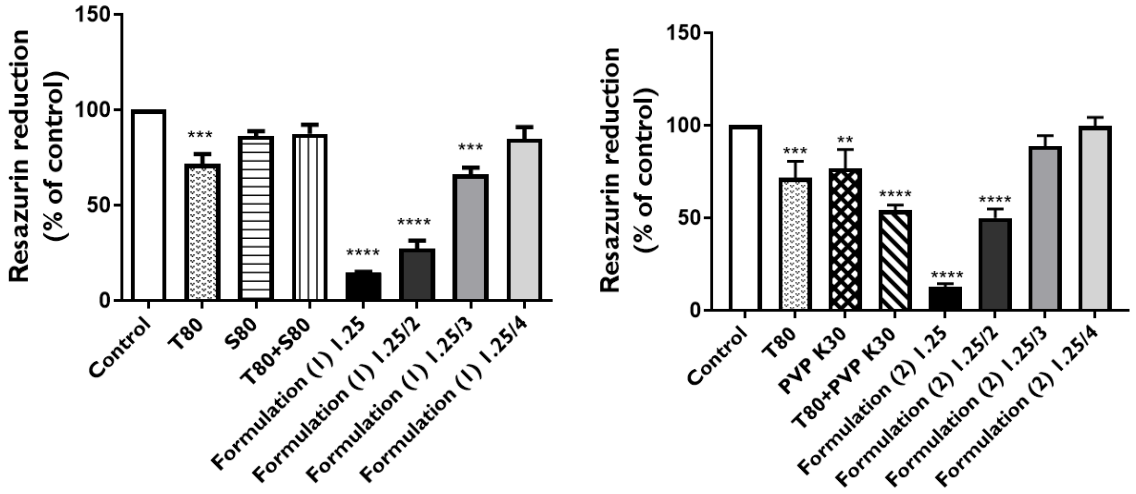


Figure 17: Effect of 3h incubation with the formulation 1 and formulation 2 and controls, on human epithelial colorectal cells (Caco-2) viability by Alamar blue assay. Results are expressed as a percentage of resazurin reduction by control cells. Each value represents the mean ± SEM of three independent experiments performed in triplicate (**** p<0.0001, *** p <0.001, ** p <0.01, compared to control).

Although all the surfactants affected formulation 2, in lower concentration, the cell viability was not affected significantly during the assay of 3 hours. However, the formulation 1 affects significantly in majority of the concentrations which does not coincide with the effect of control of surfactants (S80 and T80+S80). The interaction between the surfactants was shown

to be cytotoxic, i.e., the drug released significantly affected cell viability. To describe the effect of the formulations further tests over the time were made at 6 and 24 hours.

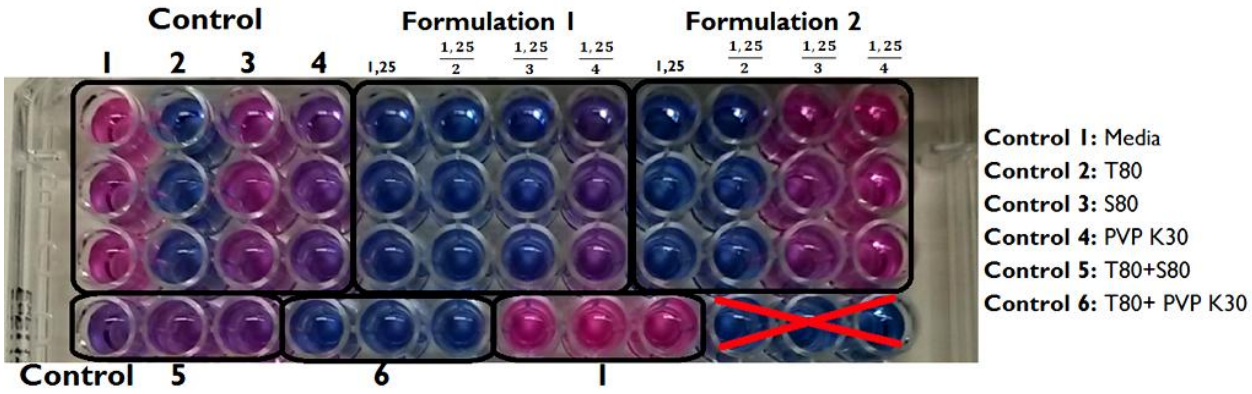


Figure 18: Cell cultures after 6 hours incubated with both formulations in four different concentrations (n=3).

Within the first 6 hours of the assay, the viability of the cells treated with formulation 1 was strongly affected, i.e,. all the wells turned blue. In the wells treated with formulation 2, some appeared to keep cell viability. The data analyses evidence if there are significant differences (Figure 19).

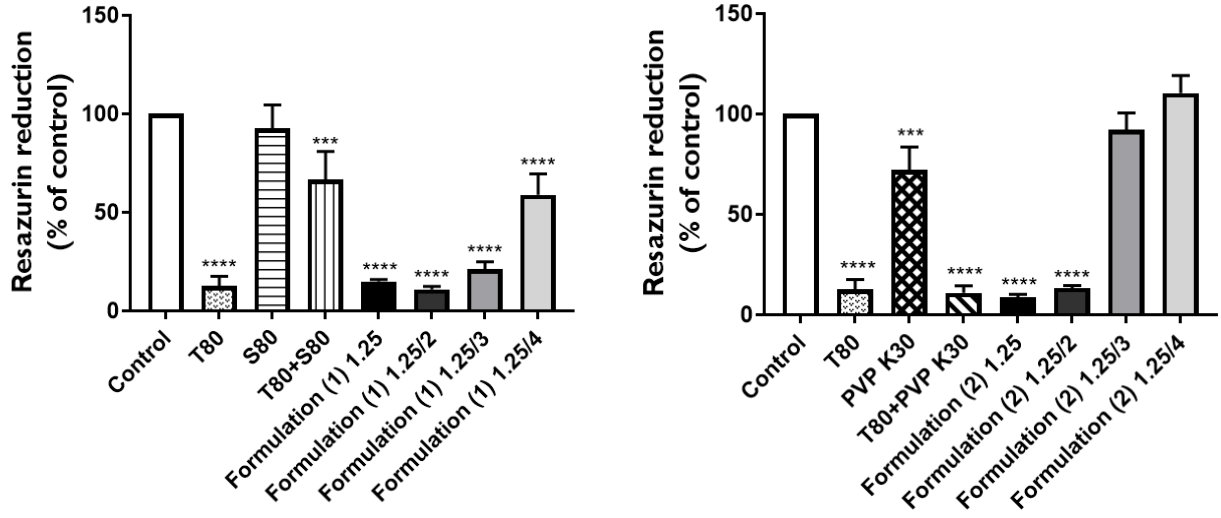


Figure 19: Effect of 6h incubation with the formulation 1 and formulation 2 and controls, on human epithelial colorectal cells (Caco-2) viability by Alamar blue assay. Results are expressed as a percentage of resazurin reduction by control cells. Each value represents the mean ± SEM of three independent experiments performed in triplicate (**** $p<0.0001$, *** $p<0.001$, compared to control).

As shown in Figure 19, cells lost viability when treated with formulation 1. Indeed, within 3 hours of exposure, cells remained viable upon contact with combined surfactants. However, the longer the incubation time larger the risk of cell toxicity.

In case of cells treated with formulation 2, there were no large differences between the results obtained after 3 and 6 hours.

As shown in the section 3.1.4, formulation 1 depicted the highest drug release compared to formulation 2. Within 6 hours after the beginning of the in vitro ibuprofen release, formulation 1 released ~80% of the drug whereas formulation 2 released the maximum drug available, i.e., ~70%. Thus, it is possible to estimate that the higher drug content released from formulation 1 is more harmful to the cells, when compared to the drug concentration present in the cells when these are treated with formulation 2 at same concentrations. After 24 hours of assay, the obtained results are shown in Figure 20.

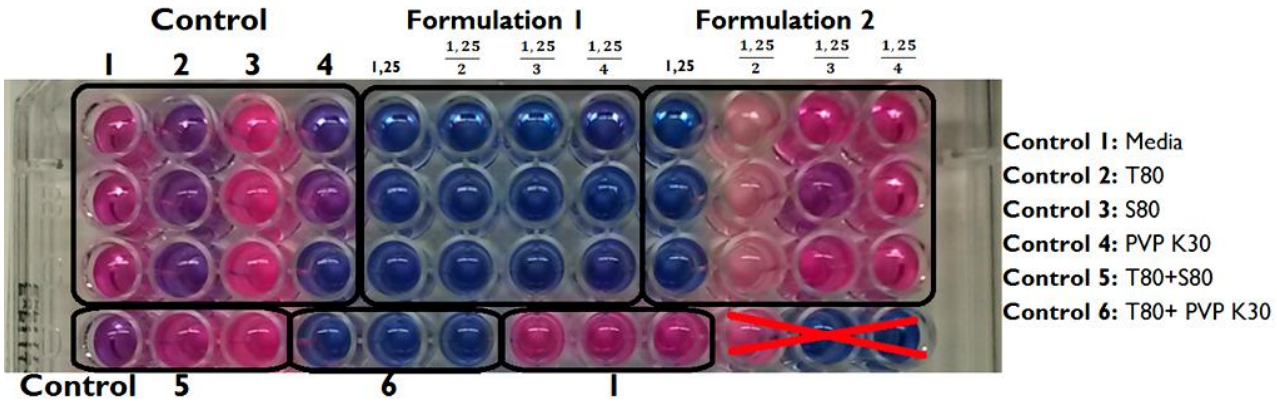


Figure 20: Cell cultures after 24 hours incubated with both formulations in four different concentrations (n=3).

Within 24 hours after the beginning of the assay, the viability of the cells treated with formulation 1 remained strongly affected, i.e. all the wells turned blue. The results obtained for formulation 2 were not conclusive only with analyse of Figure 20.

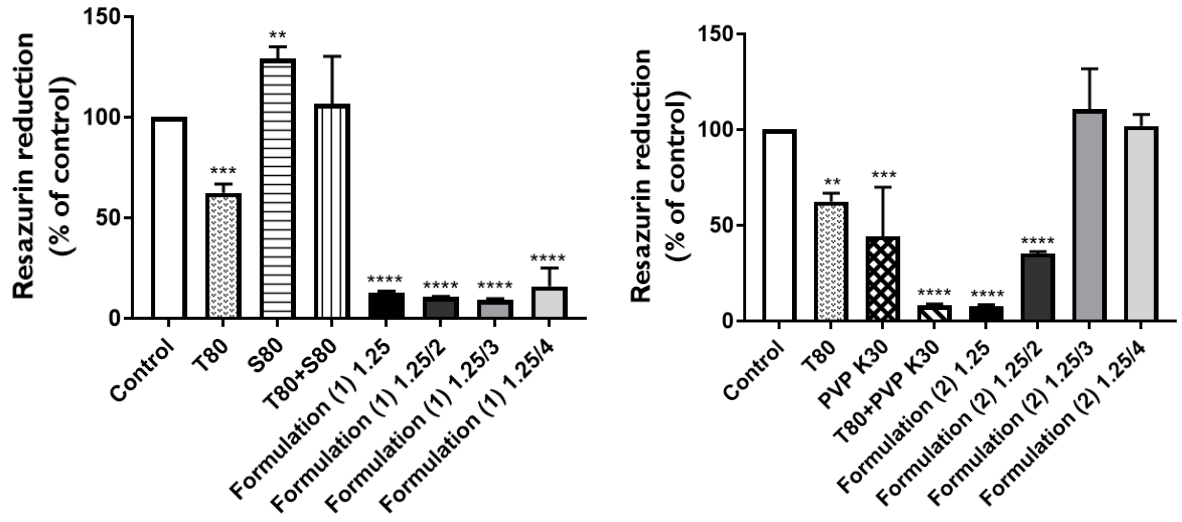


Figure 21: Effect of 24h incubation with the formulation 1 and formulation 2 and controls, on human epithelial colorectal cells (Caco-2) viability by Alamar blue assay. Results are expressed as a percentage of resazurin reduction by control cells. Each value represents the mean ± SEM of three independent experiments performed in triplicate (**** p<0.0001, *** p <0.001, ** p <0.01, compared to control).

In case of the formulation 1, as seen previously, longer incubation time increased significantly the risk of cell toxicity. The drug release continued between 6 and 12 hours, approximately, until it stabilized. After 24 hours of exposed to the formulation, the cell viability decreased further. The results of formulation 2 is more or less constant over time, once the maximum concentration released had already been achieved after 6 hours.

Formulation composed of S80, T80+S80, formulation 2 with the concentration of $\frac{1,25}{3}$ and concentration $\frac{1,25}{4}$ presented cell viability greater than 100%. This reflects the beginning of the cells saturation. Cell saturation can be caused by the stimulation of cell metabolism and/or exceeding time of the test compounds in contact with the cell culture.

In fact, no drug administrated orally is in contact with the cells of human body during 24 hours. This cell metabolic stress is ordinary, even because after 24 hours, cells that are viable will continue to be divided into the well, with increase of the density of cells. This also affects the cell viability and increases the level of stress.

4. Conclusions

Since the beginning of the 90s, drug nanocrystals have been exploited for the enhancement of the dissolution properties of poorly water-soluble drug materials and to solve bioavailability problems. Nanocrystals are composed of 100% of drug which is covered by a stabilizer layer. Drug nanocrystals have shown to promote biological activities, such as sustained drug release and targeting to specific tissues and/or organs. The great advantage of these formulations is that they can be applied to various administration routes, namely oral, parenteral, pulmonary and ocular routes.

Production techniques, especially the high-pressure homogenization, have been employed in large-scale production of nanocrystals.

This study reports an approach to use a 2-level 2-factor factorial design in the optimization of nanocrystal formulations produced with different surfactants. The dependent variable values for the preparation of optimum formulations with desired mean particle size, PI and ZP was obtained, optimal parameters were obtained using 0.20% T80 and 1.20% S80 for formulation 1 and 0.20% T80 and 1.20% PVP K30 for formulation 2. The decrease of the concentration of T80 contributed for the decrease of the mean particle size in both formulations. The factorial design experiment demonstrated the correlation between various production parameters.

In the *in vitro* ibuprofen release assay, formulation 2 depicted a faster release profile in the first 15 minutes, which may infer a faster therapeutic effect. However, the same formulation released only 70% of the drug within the first 6 hours, which means that 30% of the administered ibuprofen does not have any therapeutic effect. On the other hand, formulation 1 released 100% of the drug within the first 12 hours, offering the same burst effect as formulation 2 during the same time-period. In addition, a modified release profile could also be attributed to formulation 1 given the slower release in the first 4 hours. For the formulation 1 and based on mathematical models, the best fitting model is the Higuchi. For the formulation 2, the best model was found to be Baker-Lonsdale.

In cell viability assays, in case of the formulation 1, longer incubation time increased significantly the risk of cell toxicity. The drug release continued between 6 and 12 hours, approximately, until it stabilized. After 24 hours of exposed to the formulation, the cell viability decreased further.

The results recorded for formulation 2 were more or less constant over time, since the maximum concentration released had already been achieved after 6 hours. Formulation 2

presented cell viability greater than 100% in some wells, after 24 hours of assay. This reflects the beginning of the cells saturation. Cell saturation can be caused by the stimulation of cell metabolism and/or exceeding time of the test compounds in contact with the cell culture.

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