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Developpement and characterization of new dosage forms based on drug containing aqueous colloidal polymer dispersion

Cristina Loira-Pastoriza

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**DEVELOPMENT AND
CHARACTERIZATION OF NEW DOSAGE
FORMS BASED ON DRUG CONTAINING
AQUEOUS COLLOIDAL POLYMER
DISPERSION**

Dissertation zur Erlangung des akademischen Grades des Doktors der
Naturwissenschaften (Dr. Rer. Nat)

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der Freien Universität Berlin

vorgelegt von

Cristina LOIRA-PASTORIZA
aus Marin (SPAIN)

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1. Gutachter: Prof. DR. Roland BODMEIER
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par Cristina LOIRA PASTORIZA

**Development and characterization of new dosage forms based on drug
containing aqueous colloidal polymer dispersion**

Le 28 septembre 2012

Membres du jury :

Rapporteurs :

M Hatem FESSI PRU, Université de Lyon 1, Lyon, France

M Alf LAMPRECHT PRU, Universität Bonn, Bonn, Allemagne

Examineurs :

M Roland BODMEIER PRU Freie Universität Berlin, Berlin, Allemagne, Directeur de thèse

M Philippe MAINCENT PRU, EA 3452, Université de Lorraine, Nancy, France ; co-Directeur de thèse

M Rainer H. MÜLLER PRU Freie Universität Berlin, Berlin, Allemagne

Mme Anne SAPIN-MINET MCF, EA 3452, Université de Lorraine, Nancy, France

**EA 3452 CITHEFOR ; Cibles Thérapeutiques, Formulation et Expertise Préclinique du
Médicament – 5, rue Albert Lebrun, BP 80403, 54001 Nancy Cedex, France**

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AIM OF THE THESIS

Aqueous polymeric nanosuspensions are currently used in pharmaceutical technology as excipient during coating process. These products have been developed as an alternative to organic polymer solutions, which were classically used for coating purposes.

Aqueous polymeric nanosuspensions present many advantages in terms of ecological, toxicological and manufacturing safety compared to organic-based polymer solutions.

The aim of this PhD project is to obtain new applications for these polymeric dispersions. The presence of preformed nanoparticles may allow the incorporation of different types of drug into their structure.

Depending on drug nature, different hypothesis have been developed:

- The lipophilic character of polymers was used to incorporate lipophilic drugs. Indeed, the selected drugs, which are poorly water soluble, present a certain affinity for polymer structure and thus, depending on drug-polymer interactions, drug will be incorporated into polymer matrix.
- The polycationic character of Eudragit[®] RS 30D, a copolymer of ethyl acrylate and methyl methacrylate containing quaternary ammonium groups, will be used to form a physical gel by electrostatic interaction. The selected model drugs were different types of Low Molecular Weight Heparins (LMWH), which are known to be anionic polysaccharides.

Based on the two hypotheses, two types of dosage form will be optimized. A liquid polymeric dispersion containing lipophilic drugs may increase oral bioavailability of drugs. Moreover, the liquid form will facilitate the observance of medicines especially in children and aged patients.

A gel administered topically, which contains an anticoagulant (LMWH) may be interesting in the treatment of superficial thromboses and haematomas. The local application may increase the drug activity in the site of action avoiding systemic side effects.

PART 1:

DRUG-POLYMER NANOPARTICLES FOR ORAL BIOAVAILABILITY ENHANCEMENT

1. Introduction

1.1 *Drug administration evolution*

Since the origin of human life, drugs were administered to cure or treat different illness or symptoms. The origins of the first drugs were medicinal plants (roots, stem and leaf). Drugs were incorporated in infusion, decoction...

Sumerians were the first civilization to establish the first pharmaceutical operations such as milling, drying, filtration... They had already prepared some pharmaceutical forms (i.e. creams, lotions, ointments, cataplasms...). Egyptians have conserved a codex describing symptoms and remedies; drug formulation and dosage have been also reported. In Rome civilization, tablet uses were developed by the discovery of terra sigillata. Galen proposed the basis to prepare pharmaceutical forms.

During centuries drugs were incorporated in vehicles to facilitate their administration. The distribution of drug in the organism was not a field of study since XX century.

The importance of dissolution processes in physiological bioavailability of drugs was not realized until the last 50 years (Dokoumetzidis and Macheras 2006). During the XIX century, Noyes and Whitney conducted the first dissolution experiments by the study of two sparingly soluble compounds, benzoic acid and lead chloride. They have observed that the rate of dissolution is proportional to the difference between the instantaneous concentration, C at time t , and the saturation solubility (Equation 1)

$$\frac{dC}{dt} = k(C_s - C) \quad \text{Equation 1}$$

Nevertheless, the first laws established for dissolution were not specifically for drug development until 1957 when Nelson relates the blood levels of theophylline salts to their *in vitro* dissolution rates (Dokoumetzidis and Macheras 2006). During the sixties, it was observed that differences in product formulation were related with drug response differences.

The term of bioavailability was then employed to describe the fraction of drug dose able to reach the general circulation. Further studies demonstrated the relationship between dissolution studies and drug bioavailability. It was observed that dissolution rate in gastrointestinal fluids and permeability through biological membranes are the most important factors in determining the bioavailability of orally administered drugs (Amidon, Lennernas et al. 1995; Löbenberg and Amidon 2000).

1.2 Drug classification

In 1995, Amidon et al. (Amidon, Lennernas et al. 1995) proposed a system to classify the drugs depending on their solubility range and intestinal permeability. This system was called the Biopharmaceutics Classification System. This classification provides a basis for *in vitro-in vivo* correlations. Drugs are divided in class depending on their aqueous solubility and their permeability determined by Fick's First Law (Equation 2):

$$J_w = P_w \cdot C_w = \frac{dM}{dt} \cdot \frac{1}{A} \quad \text{Equation 2}$$

where, J_w is the drug flux (mass, area, time) through the intestinal wall at any position and time, P_w is the permeability of the membrane, and C_w the drug concentration at the membrane surface (Amidon, Lennernas et al. 1995).

According to this classification, drugs are classified in four classes:

| | |
|---|--|
| Class I High solubility High permeability | Class II Low solubility High permeability |
| Class III High solubility Low permeability | Class IV Low solubility Low permeability |

Figure 1: Biopharmaceutics Classification System

In this table are reported some examples of drugs belonging to the 4 classes (Lindenberg, Kopp et al. 2004; 2005; Zaki, Artursson et al. 2010).

| Class I | Class II | Class III | Class IV |
|----------------------|-----------------|--------------------|-----------------|
| Acetylsalicylic acid | Celecoxib | Aciclovir | Cyclosporine A |
| Amiloride | Carbamezepine | Atenolol | Furosemide |
| Diazepam | Diclofenac | Chloramfenicol | Ivermectine |
| Digoxin | Griseofulvine | Codeine | Nelfinavir |
| Fluconazole | Ibuprofen | Colchicine | Ritonavir |
| Metronidazole | Nifedipine | Enalapril | |
| Paracetamol | Rifampicin | Ethambutol | |
| Prednisolone | Warfarin | Hydrochlorotiazide | |
| Propranolol | | Neostigmine | |
| Riboflavin | | Penicillamine | |
| Salbutamol sulphate | | Ranitidine | |
| Valproic acid | | | |

Table 1: Examples of the Biopharmaceutics Classification System

The Biopharmaceutics Classification System (BCS) proposes a basis to correlate in vitro dissolution and in vivo bioavailability. The objective of the BCS is to predict the in vivo bioavailability of drugs based on solubility and permeability data. Though drug bioavailability is limited by i) permeability if the drug has a low permeability and ii) by the dissolution rate in the case of a poorly soluble drug, FDA used this classification to establish a “Waiver of in vivo bioavailability and bioequivalence studies for immediate release solid oral dosage forms” (Lindenberg, Kopp et al. 2004). A biowaiver means that in vivo bioavailability or

bioequivalence studies may not be necessary for product approval. Instead of conducting in vivo studies, a dissolution test could be adopted to decide if two pharmaceuticals are bioequivalent. This procedure can be only used in case of drugs with high solubility and high permeability formulated in a solid form for immediate release. The major advantage of the Biowaiver procedure is the simplification and reduction of time required for product approval. To represent the mechanisms of the fundamental processes of permeation, drug dissolution and dose, Amidon determined three dimensionless numbers (Amidon, Lennernas et al. 1995; Löbenberg and Amidon 2000). These numbers are combinations of physicochemical and physiological parameters and represent the most fundamental view of GI drug absorption. Absorption number (An), is the ratio of permeability and the gut radius times and residence time in the small intestine (Equation 3).

$$An = \frac{P_{eff}}{R} \cdot t_{res} \quad \text{Equation 3}$$

Dissolution number (Dn) is the ratio of the residence time to the dissolution time, which includes solubility, diffusivity, density and the initial particle radius (Equation 4)

$$Dn = \frac{3DC_s t_{res}}{r^2 \rho} \quad \text{Equation 4}$$

Dose number (Do) is a dimensionless parameter used as a measurement of solubility/dissolution potential. A Do -value of 1 implies that the expected highest GI concentrations is similar to the solubility, and a high Do implies a low dissolution potential (Fagerholm 2007). It is calculated by (Equation 5)

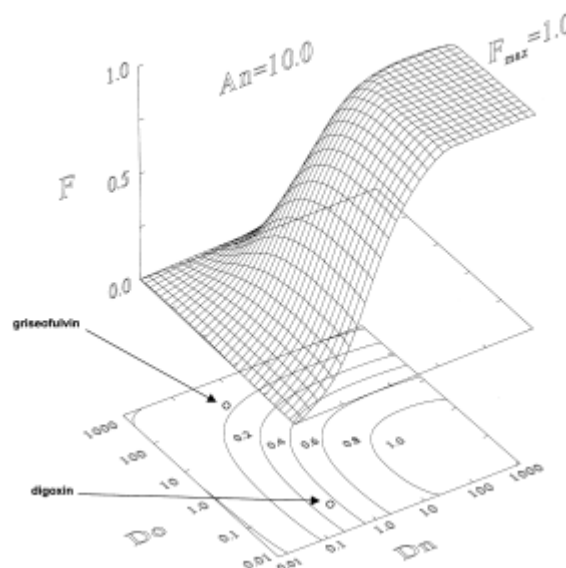
$$Do = \frac{M_0 / V_0}{C_s} \quad \text{Equation 5}$$

P_{eff} : permeability (cm/s), t_{res} : mean residence time (h); R : tube radius (cm); D : diffusion coefficient (cm²/s), C_s : solubility (mg/mL); r : particle radius (µm); ρ : density (mg/mL); M_0 : dose (mg); V_0 : Dissolution volume.

The dissolution of a poorly soluble drug is normally low ($D_n < 1$), while for many poorly soluble compounds A_n and D_o are usually high (Corresponding to a Class II drugs). Under the assumption that dissolution is not limited, the fraction dose absorbed of a suspension can be calculated as (Equation 6).

$$F = \frac{2A_n}{D_o} \quad \text{Equation 6}$$

When A_n , D_o and D_n are known values, the absorbed fraction (F_a) can be estimated by a three-dimensional graph:



| Drug | Dose (mg) | Cs (mg/mL) | Vsol mL | Do | Dn |
|--------------|-----------|------------|---------|------|------|
| Digoxin | 0.5 | 0.024 | 20.8 | 0.08 | 0.52 |
| Griseofulvin | 500 | 0.015 | 33333 | 133 | 0.32 |

Figure 2: Example of an estimation of fraction dose absorbed vs. Dissolution number (Al Omari, Daraghme et al.) and Dose number (Do) for a high permeability compound. $A_n=10$ corresponds to a drug with a permeability similar to glucose. Adapted from (Löbemberg and Amidon, 2000)

1.3 Drug permeability study

According to the BCS, a drug substance is considered highly permeable when the extent of the absorption in humans is determined to be $\geq 90\%$ of an administered dose (Wu and Benet 2005).

1.3.1 Mechanism of drug absorption

Human intestine is divided in two parts, small intestine and large intestine. Most of oral drug absorption occurs in the intestine, primarily in the small intestine. It is divided in duodenum, jejunum and ileum. The inner wall, or mucosa, of the small intestine is lined with simple columnar epithelial tissue. Drug absorption surface is about 200 m². To obtain this large surface, intestinal mucosa is covered by different specific structures: The plicae circulares increase 3 times the absorption surface. They are covered with small fingerlike projections called villi which increase intestine surface in 30 times. Each villus, in turn, is covered with microvilli that increase the surface 600 times. Drug absorption is located in enterocytes. There are two main mechanisms for drug absorption (Blanchette, Kavimandan et al. 2004):

A paracellular mechanism where molecules cross the epithelium between two cells. This mechanism is considered for small (<300 Da) and hydrophilic molecules.

A transcellular mechanism where drug substance cross the endothelial cells. It exists different approaches for this mechanism:

- Transcellular passive mechanism: Molecules diffuse from the apical membrane, through the cell to the other side of membrane. This is a passive mechanism which concerns small molecules with a low charge. This mechanism is modulated by the Fick's Law, so it depends on a gradient of concentration.
- Carrier mediated transport: Drug is recognized by carriers present in the membrane. This transport could need energy or could be passive.
- Endocytosis: By this mechanism, drug substance interacts with cell membrane. The membrane forms a pocket of lipid bilayer containing the drug called vesicle. The vesicle can migrate to the basal membrane and release the drug in the other side or can be degraded into the cell.

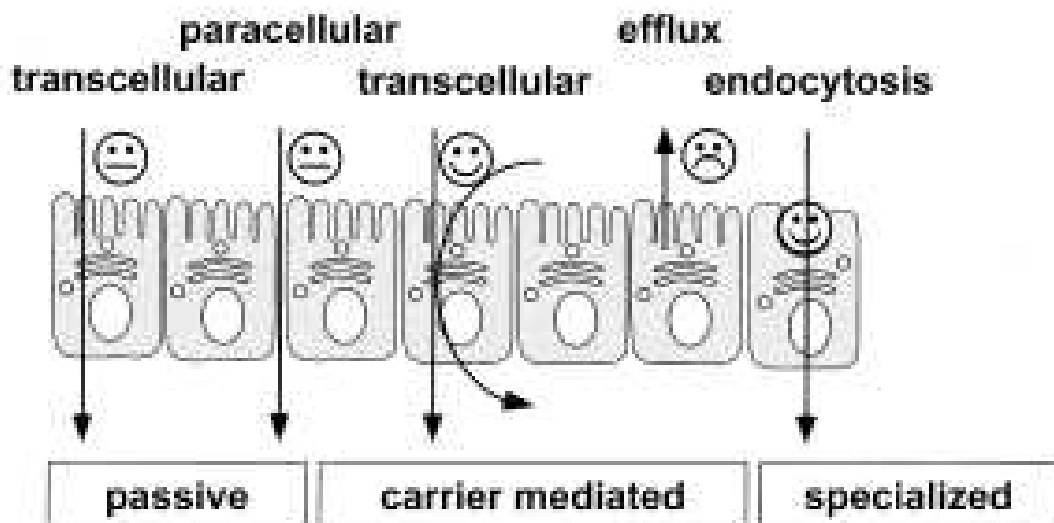


Figure 3: Different mechanisms of drug absorption (Löbenberg and Amidon 2000).

Mechanisms of drug absorption depend on the physico-chemical properties of drugs. The rule of 5 (Lipinski, Lombardo et al. 2001) develops the critical parameters to predict drug absorption. Therefore a drug presents a poor absorption if there are more than 5 H-bond donors (expressed as the sum of OHs and NHs), MW is over 500, Log P is over 5 (or MlogP over 4.15), there are more than 10 H-bond acceptors (expressed as the sum of Os and Ns) and compound classes that are substrates for biological transporters are exceptions to the rule. Lipinski rule is also used to detect the “drugability” of different compounds in early research drug development.

1.3.2 Permeability determination

Permeability studies allow to determinate the extent of absorption (fraction of dose absorbed, not bioavailability). There are several methods allowing the absorption rate of drugs to be determined. Following are reported different techniques to study drug permeability across intestinal barrier.

1.3.2.1 Cell culture

Cell lines are the in vitro models used for the drug permeability studies in the preclinical and clinical phases of the drug discovery. Cell line models are simple and quick to use and avoid the use of animal models. They are cost effective, produce reliable and reproducible results for evaluating drug permeability.

Caco-2 cells

Caco-2 cells, a human colon adenocarcinoma, undergo spontaneous enterocytic differentiation in culture. When they reach confluency on a semipermeable porous filter, the cell polarity, and tight junctions are well established. Caco-2 cell culture is classically used (Lindenberg, Kopp et al. 2004; Ku 2008). This cell line is the most used predictor of drug absorption (Rinaki, Valsami et al. 2003); in addition, such cell cultures are easy to perform. This cell line presents multiple similarities with intestinal epithelium such as the microvili, tight junctions, presence of enzymes and carriers (Shah, Jogani et al. 2006). Caco-2 cells are suitable for the study of molecules which presents a passive transcellular transport (Tavelin, Milovic et al. 1999).

The preparation of a fully functional cell monolayer generally requires a 3-week cell culture period with eight to nine laborious cell feeding with a high risk of contamination during culture.

TC-7 cells

TC-7 is one of the subclones isolated from Caco-2. They have a similar behaviour concerning morphological characteristics, apical brush border, microvilli, tight junctions and polarisation of the cell line. However it was shown to be more homogeneous in term of size cell (Gres, Julian et al. 1998). Permeability values of passively absorbed drugs obtained in TC-7 clone correlated equally well as in parental Caco-2 cells to the extent of absorption in humans.

Madin Darby Canine Kidney (MDCK) cells

Madin–Darby canine kidney (MDCK) cells differentiate into columnar epithelial cells and form tight junctions (like Caco-2 cells) when cultured on semipermeable membranes. Caco-2 and MDCK cell lines present a similar P_{app} values for passively absorbed drugs (Taub, Kristensen et al. 2002). MDCK cells present a shorter time to grow in culture (confluent cell monolayers are obtained after 5-7 days of culture) that allows minimizing potential contamination.

2/4/A1 Cells

The 2/4/A1 cell line (obtained from foetal rat intestine) presents a paracellular permeability similar to the human intestinal epithelium in vivo (Zaki, Artursson et al. 2010). This immortalized cell line forms viable differentiated monolayers with tight junctions, brush border membrane enzymes as well as the transporter proteins. The optimization of this cell line to study the paracellular transport of drugs has been studied by Tavelin. They have concluded that 2/4/A1 cell lines form intact monolayers on matrix-coated permeable supports. 2/4/A1 cells mimic the human jejunum permeability better than Caco-2 and are well suited for rapid screening of intestinal drug absorption (Tavelin, Milovic et al. 1999).

1.3.2.2 Artificial membranes

In order to mimic the lipid composition of the enterocyte membrane, artificial membranes were designed (Teksin, Seo et al. 2010). As an example, the PAMPA method (Parallel Artificial Membrane Permeation Assay) is based on the separation of two compartments (one containing the drug and the other one containing a fresh buffer) by artificial lipid membrane. This artificial membrane is prepared by coating a hydrophobic filter material with a mixture of lecithin and an inert organic solvent. Due to the absence of intestinal enzymes (Fortuna, Alves et al. 2012), this system allows predicting drug absorption by passive transcellular

diffusion. The limitation of this model is that PAMPA underestimates the absorption of compounds that are actively transported or hydrophilic compounds with low molecular weight.

1.3.2.3 Animal models

Excised animal tissue models have been used since the 1950s to explore the mechanism of absorption of nutrients from the intestine. However, the viability of the excised tissues is difficult to maintain since the tissues are devoid of direct blood supply and need constant oxygenation. Some of the more widely used methods for absorption and permeability studies are described below.

Everted gut technique

This technique was used as early as the 1950s in the transport study of sugars and amino acids from the mucosal to serosal side. In this model, gut animal is removed. It is widely washed and placed in an oxygenated medium. Pieces of intestine are prepared as a sac and placed in a dissolution medium containing the studied drug. After the incubation time, drug is assayed inside the pocket (Lassoued, Khemiss et al. 2011).

Ussing chamber

Transport studies across intestinal segments from animals are also a widely used in vitro method to study drug absorption. This method involves the isolation of the intestinal tissues, cutting it into strips of appropriate size, clamping it on a suitable device and then the rate of drug transport of across this tissue is measured (Lennernas 1998). The Ussing chamber technique is an ideal method to study regional differences on the absorption of drugs by mounting intestinal tissues from various intestinal regions.

The drawbacks of this technique include: lack of blood circulation, rapid loss of viability of the tissues during the experiment, and changes in morphology and functionality of transporter proteins during the process of surgery and mounting of tissues (Balimane, Chong et al. 2000).

1.3.2.4 In silico models

Computational or virtual screening has received much attention in the last few years. In silico models that can accurately predict the membrane permeability of test drugs based on lipophilicity, H bonding capacity, molecular size, polar surface area (PSA), and quantum properties has the potential to specifically direct the chemical synthesis and therefore, revolutionize the drug discovery process. Such an in silico predictive model would minimize extremely time consuming steps of synthesis as well as experimental studies of thousands of test compounds.

Lipinski et al. have proposed an in silico computational method for qualitatively predicting the developability of compounds. They can predict the permeability of compounds based on the rules of 5 they have proposed. Using this completely empirical model, useful permeability predictions were achieved for closely related series of compounds (Lipinski, Lombardo et al. 2001).

Takano et al. have developed a computer system based on miniscale dissolution tests using a simulated intestinal fluid (FaSSIF) to predict oral bioavailability of class II compounds (Takano, Sugano et al. 2006).

Quantitative structure–property relationship (QSPR) has been recommended to predict human intestinal absorption. By the use of different molecular descriptors, drug bioavailability can be estimated. Turner et al. have developed a QSPR based on a high number of molecular descriptors. They have observed that molecular size, polar surface area, octanol-water partition, hydrogen bonding, solubility and electronic effects significantly influence drug bioavailability after oral administration. The model developed was able to predict several

drugs' bioavailability compared with experimental results (Turner, Glass et al. 2003; Turner, Maddalena et al. 2004)

1.4 Solubility study

A drug is considered highly soluble when the highest dose strength is soluble in 250 mL or less of aqueous media over a pH range of 1-7.5 at 37°C. The objective of the BCS approach is to establish the equilibrium solubility of a drug at physiological pH conditions when determined at 37°C. To obtain an accurate pH-solubility profile, an adequate number of pH conditions should be evaluated. At least the $\text{pH}=\text{pK}_a$ value, $\text{pH}=\text{pK}_a+1$ and $\text{pH}=\text{pK}_a-1$. To determine drug solubility, several methods have been developed.

1.4.1 Saturation flask method

The saturation shake-flask method is a classic approach to measure solubility. An excess amount of drug is placed in vials containing buffer solutions. The vials are placed at 37°C and shaken for 24-48 h, or further to obtain equilibrium. After filtration or centrifugation, drug concentration is assayed in the clear solution (Avdeef 2007). Drug assay could be performed by different analytical methods such as UV spectroscopy, HPLC...

1.4.2 DMSO-stock solution

A stock solution of DMSO-drug could also be used to determine solubility. In early development stage, drug is typically stored in DMSO solutions. This stock solution is added carefully into a buffer solution until compound precipitation. The light scattering effect produced by the precipitation is evaluated by spectroscopic measurements. The solution can also be filtered and drug concentration directly assayed (Ku 2008).

1.4.3 Facilitated dissolution method

A facilitated dissolution method (FDM) was described by Higuchi et al. (Avdeef 2007). In this case, an excess of solid is needed to saturate the solution. A small amount of immiscible organic solvent is added to the aqueous solution. Even if the saturated system contains three phases (solid, organic solvent and water) the presence of an organic solvent does not modify the drug solubility. This method is not satisfactory for ionized compounds (Avdeef 2007).

1.4.4 Dissolution template titration method

The dissolution template titration method (DTT) uses a pH electrode to determine the intrinsic solubility. This technique takes pK_a and $\log P_{oct}$ as input parameters. These values are used to estimate the intrinsic solubility using the Hansch-Yalkowsky type lipophilicity equation (Equation 7).

$$\log S_a = 1.17 - 1.38 \log P \quad \text{Equation 7}$$

1.4.5 Chasing equilibrium method

To ensure that the phase equilibrium is attained, Stuart and Box developed a method called “chasing equilibrium” to measure the intrinsic solubility of ionisable compounds. This method is divided in 5 stages (dissolution, seeking precipitation, additional precipitation, chasing equilibrium and redissolution) as described following: the solute is dissolved by adding either an acid or a base. The pH is adjusted to obtain a totally dissolved solute in its ionized form. This solution is back-titrated to obtain a cloudy “solution”, which means the precipitation of the poorly water-soluble neutral species. Precipitation is detected by a spectroscopic probe. After precipitation, the “solution” is repeatedly changed from supersaturated to subsaturated by pH modifications. These modifications allow obtaining the point of equilibrium by changing the concentration of the neutral form (Stuart and Box 2005).

1.4.6 Miniaturized methods

Miniaturized and automated methods of the techniques described above have been also reported in an effort to decrease sample and time consumption (Glomme, März et al. 2005).

1.4.7 Rat dilution model

This model is able to predict the solubility of drugs in simulated gastro-intestinal conditions. The rat dilution model mimics the two compartments of GI tract of a fasted rat: the stomach and the intestine. It is a dual method with different pH in each compartment. In the stomach compartment, the primary dilution came from basal acid output (BAO). To simulate stomach pH, HCl solution was used at a pH of approximately 4. In case of intestine simulation, a fasted simulated small intestine fluid (FaSSIF) with a pH of 6.5 was used (Gao, Carr et al. 2010).

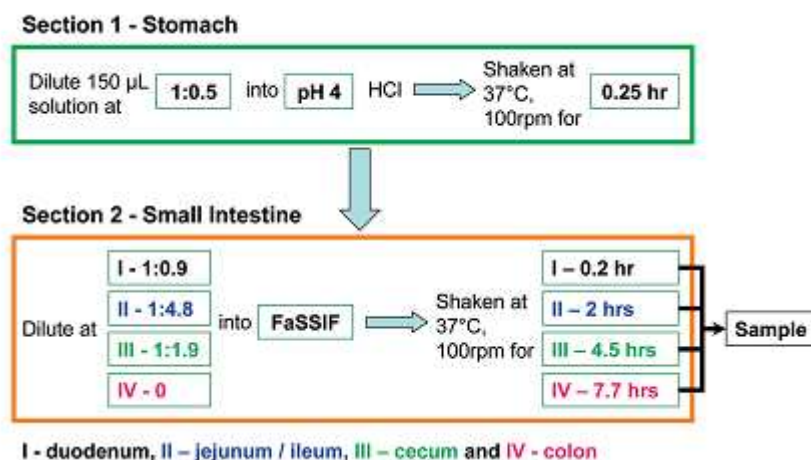


Figure 4: Rat dilution model (Gao, Carr et al. 2010).

1.4.8 In Silico models

Aqueous solubility can be predicted by QSPR studies. In silico methods for predicting the aqueous solubility of drug candidates provides a valuable tool to speed up the process of drug discovery and development. Duchowicz et al. have used different molecular descriptors as well as the parameters involved in the Lipinski's rules to predict aqueous solubility of different drugs. Predictions were compared to experimental results. They have succeeded in

establishing a QSPR model to study the aqueous solubility of 148 organic compounds (Duchowicz, Talevi et al. 2007; Duchowicz, Talevi et al. 2008).

1.5 BCS modifications

The BCS rules to classify drug solubility (solubility of the higher dose strength in 250 mL of aqueous media over a pH range of 1-7.5 at 37°C) may not be applied for all drugs.

Indeed, lipophilic compounds solubility could be more increased in intestinal fluid than the value obtained with a pH range of aqueous solution. The presence of lipids and bile salts may lead to a higher solubilisation. New approaches for pH-dissolution profiles are needed to both determine the solubility in GI tract and to perform a better prediction about in vivo drug solubility and absorption.

Likewise, weakly acidic drugs present a low solubility in stomach but a much higher solubility in intestinal fluid due to pH close to pKa and the presence of lipids and bile salts.

The solubility of several compounds was studied in two simulated small intestine fluids: FaSSIF (pH= 6.5 and ionic strength = 0.16) simulating a fasted state and FeSSIF (pH=5.0 and ionic strength= 0.32) simulating a fed state. These solubility tests were performed at 37°C. In all cases, it was found that solubility was more important in the simulated gastric fluid than in an aqueous solution (Phosphate buffer pH=6.5) (Yazdanian, Briggs et al. 2004; Zaki, Artursson et al. 2010).

1.5.1 Quantitative Biopharmaceutics Classification System (QBCS)

The study of the solubility depending on dissolution media allows the proposition of modified BCS. In 2003 (Rinaki, Valsami et al. 2003) have proposed a Quantitative Biopharmaceutics Drug Classification System (QBCS). This proposition is based on the parameters controlling the rate and extent of drug absorption and also permeability. It was observed that drug absorption rate can also be affected by the dose/solubility ratio. The authors have described

the quantity value (q) as a dimensionless number relating the administered dose and its solubility (Equation 8).

$$q = \frac{M_0}{(C_s \cdot V)} \quad \text{Equation 8}$$

Where q is quantity; M_0 the dose; C_s the solubility and V the intestinal volume.

When $q < 1$, drug is not completely solubilised; otherwise if $q > 1$, drug is totally solubilised.

The Caco-2 monolayer permeability (P_{app}) was selected to perform permeability studies. The QBCS classifies drugs in 4 classes according to their permeability (P_{app}) and their dose/solubility ratio. There are not so many differences between the BCS and the QBCS. Next figure shows the four categories of drugs according with the QBCS.

| | |
|---|--|
| <p>Category I</p> <p>$q \leq 0.5$ $P_{app} > 10^{-5}$ cm/s</p> | <p>Category II</p> <p>$q > 1$ $P_{app} > 10^{-5}$ cm/s</p> |
| <p>Category III</p> <p>$q \leq 0.5$ $P_{app} < 2 \times 10^{-6}$ cm/s</p> | <p>Category IV</p> <p>$q > 1$ $P_{app} < 2 \times 10^{-6}$ cm/s</p> |

Figure 5: Quantitative Biopharmaceutics Classification System

1.5.2 Biopharmaceutical absorption classification

Another BCS modification was suggested by Bergström et al. (Bergstrom, Strafford et al. 2003); the authors have studied whether the molecular surface properties could allow predicting drug solubility and permeability accurately. This prediction should allow classifying different drugs as a function of their absorption as represented in figure 6. Six classes were defined, Class I: High solubility, high permeability; Class II: Low solubility,

high permeability; Class III: High solubility, low permeability; Class IV: Low solubility, low permeability; Class V: High solubility, intermediate permeability and Class VI: Low solubility, intermediate permeability. Some examples of this classification are reported in table 2.

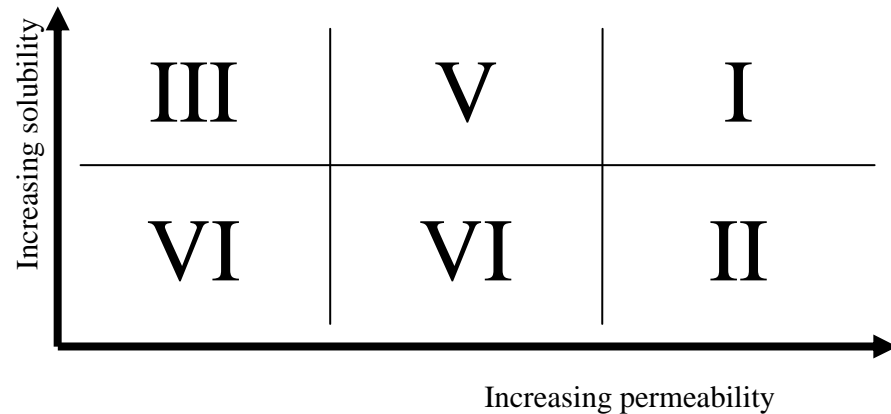


Figure 6: Biopharmaceutical Absorption Classification

| Drug | Biopharmaceutics Classification system | Biopharmaceutical absorption classification |
|-------------------|--|---|
| Aciclovir | III | I |
| Amiloride | IV | V |
| Amitriptiline | I | I |
| Chlorpromazine | III | II |
| Erythromycin | III | V |
| Ethynil estradiol | III/I | I |
| Folic Acid | III/I | I |
| Primakine | I | I |
| Verapamil | II | III |
| Warfarin | II | II |
| Zivoduvine | I | I |

Table 2: Comparaison de la BCS et d'Absorption Classification System.

1.5.3 Biopharmaceutics Drug Disposition Classification System (BDDCS).

Other studies were interested not only in solubility and drug intestinal permeability but also in drug metabolism and elimination. Wu et al. (Wu and Benet 2005) have predicted drug absorption extent as a function of metabolism and elimination route. The influence of

transporters, food and drug interaction on the bioavailability have been studied. They have suggested a new Biopharmaceutics Drug Disposition Classification System. The permeability criteria to classify drugs is based on the major route of elimination (metabolized vs. unchanged). Figure 7 represents the four different drug classes according with the Biopharmaceutics Drug Disposition Classification System. Some drug examples are reported in table 3.

| | |
|---|---|
| Class I High solubility Extensive metabolism | Class II Low solubility Extensive metabolism |
| Class III High solubility Poor metabolism | Class IV Low solubility Poor metabolism |

Figure 7: The Biopharmaceutics Drug Disposition Classification System (BDDCS) (Wu and Benet 2005).

| Drug | BCS | BDDCS |
|-------------------|-------|-------|
| Aciclovir | III | IV |
| Amiloride | IV | III |
| Amitriptiline | I | I |
| Chlorpromazine | III | II |
| Erythromycin | III | V |
| Ethynil estradiol | III/I | I |
| Folic Acid | III/I | I |
| Primakine | I | I |
| Verapamil | II | III |
| Warfarin | II | II |
| Zivoduvine | I | I |

Table 3: Comparison between BCS and BDDCS.

The Developability Classification system (DCS) aims to incorporate new concepts to the compound classification system: 1) An estimate of human fasted intestinal solubility as the primary measure of in vivo solubility to predict drug absorption. 2) A solubility limited

absorbable dose (SLAD) concept. This concept is based on the idea that for class II drugs, permeability and solubility are compensatory. 3) Dissolution rate expressed as particle size rather than dose/solubility ratio. Incorporation of concepts 1 and 2 are represented in figure 8. This revised classification system will be most reliable for non ionised drugs in gastrointestinal tract (Butler and Dressman 2010).

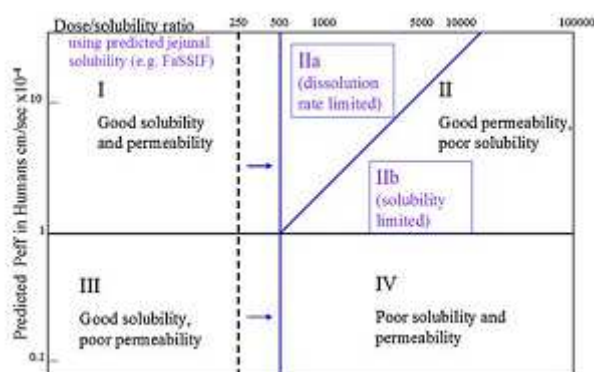


Figure 8: BCS modifications according to more realistic volumes in GI tract and the compensatory nature of permeability on low solubility (DCS modifications are represented in blue) (Butler and Dressman 2010).

1.6 Solubility enhancement methods

Many strategies were proposed during the last decades to increase drug solubility. The most significant ones are reported in the next table:

| Crystal engineering | Chemical modification | Particle Size reduction | Amorphization | Solvent composition | Carrier Systems |
|------------------------|-----------------------|-------------------------|-------------------|---------------------|----------------------------------|
| -Metastable polymorphs | -Pro-drug formation | -Micronization | -Solid dispersion | -pH-adjustment | -Nanoparticles |
| -Co-crystal formation | -Salt formation | -Nanosized drugs | | -Co-solvent | -Cyclodextrins |
| | | <i>NanoCrystal</i> | | | -Lipid formulations |
| | | <i>DissoCubes</i> | | | (<i>SLN, liposomes, SEDDS</i>) |

Table 4: Different strategies to enhance drug solubility.

1.6.1 Crystal formation

Crystals can be used to increase drug solubility. Metastable polymorphs and co-crystal formation will be developed below.

1.6.1.1 Metastable polymorphs

Polymorphism is the occurrence of crystalline forms of the same pure compound in which the molecules have different arrangement and/or conformation. Due to the different free energy between polymorphs, physicochemical properties may depend on the polymorph form (Park, Kim et al. 2010). Metastable polymorphs usually present a higher aqueous solubility (Blagden, de Matas et al. 2007). This characteristic could be exploited in drug development, to enhance drug absorption by solubility increase.

To select and stabilise a metastable polymorph, additives (impurities) or solvents can be used (Blagden, de Matas et al. 2007).

As an example, to form a polymorph of glutamic acid, trimesic acid was used as impurity. The reason is that trimesic acid mimics the stable conformation of glutamic acid.

The major inconvenient of metastable forms is the evolution of the structure to a stable form during processing or storage.

1.6.1.2 Co-crystals

A co-crystal is a crystalline form which contains at least two compounds in the structure (Shan and Zaworotko 2008). The components are solid under ambient conditions. They co-exist as a stoichiometric ratio of a target molecule or ion and a neutral molecular co-crystal former (Shan and Zaworotko 2008) They are typically formed by the strong hydrogen-bonds between carboxylic acid and N-heterocyclic hydrogen-bond acceptor (Kawakami 2012).

Carbamazepine, a model Class II drug was selected to study solubility enhancement by the co-crystal formation. Nicotinamide (the amide of niacin), was employed as the co-crystal

former. Nicotinamide has an active amide group and also the high electronegativity of lone paired nitrogen in order to give rise to a strong intermolecular hydrogen bonding required for co-crystals. Supercritical processing has been used to form co-crystals. Carbamazepine and nicotinamide were solubilised in ethanol. Supercritical carbon dioxide was used as anti-solvent to induce the co-crystal formation by precipitation. Dissolution rate was enhanced 2.5 fold by this method (Shikhar, Bommana et al. 2010).

1.6.2 Chemical-modification

The chemical modification of drugs is a classical approach in drug development to increase drug solubility as well as bioavailability. Two examples of this chemical modification are the salt formation and the pro-drug development.

1.6.2.1 Pro drug

A pro drug is a biologically inactive derivative of a parent drug which needs enzymatic transformation in the body to release the active drug. The use of a pro drug allows the increase of drug water-solubility as well as the reduction of the first pass effect. These two effects allow increasing drug bioavailability. The most common pro drug approach is the inclusion of a polar or ionisable structure in the molecule (Stegemann, Leveiller et al. 2007).

As an example, warfarin was conjugated with poly(ethylene glycol). The attachment of macromolecular carrier was performed by a covalent link. The interest of using PEG is its physico-chemical and biological properties. It is an amphiphilic molecule which dissolves either in aqueous and organic solvent. It is also well accepted by the regulatory authorities worldwide for human applications. It was observed that the prodrug PEG-warfarin is freely soluble in water and physiological buffers; in vivo it also presents a sustained release of the parent drug (Zacchigna, Luca et al. 2004).

Ezetimibe co-crystals were engineered with different crystal forming agents; benzoic acid and salicylic acid by solution crystallization technique. A dissolution rate enhancement of 222 and 480 times respectively was reported. The functional groups (carboxylic and phenolic) presented in the coformers participate in intermolecular hydrogen bonding between ezetimibe and coformer (Mulye, Jamadar et al. 2011).

1.6.2.2 Salt formation

Salt formation is a two-step process that includes the release of protons in solution and their transfer during a re-crystallization process. It is important to identify the adequate solvent which allows sufficient attractive forces of the salt. The major inconvenient of the salt formation is that their use is limited to weakly acidic or basic drugs. Neutral molecules are not able to form salts.

1.6.3 Particle size reduction

Particle reduction was used traditionally to increase solubility. Drug solubility is enhanced due to the increased drug surface, thus allowing a better solubilisation. Classification is based on particle size, micronization refer to particles in a micrometer range whereas nanosized drugs have particles sizes below one micrometer.

1.6.3.1 Micronization

For many years the micronization of poorly water-soluble drugs was a classic approach to increase drug solubility. The standard microparticles produced by air-jet mill were about 2-5 μm (Pouton 2006). Further size reduction represents a challenge due to the risk of agglomeration. In 1990 decade, two size reduction methods under the micrometer range were developed.

1.6.3.2 Nanosuspension

Nanosizing refers to a reduction of API (active pharmaceutical ingredient) under a sub-micron size. To prepare nanosized drugs, two techniques have been developed: top down and bottom up technologies. The top down approach is the most popular. It consists in transforming a crystalline drug in nanoparticles by mechanical processes. NanoCrystals and DissoCubes are the major examples of this type of technique. The bottom up approach relies on controlled precipitation/ crystallization (Kesisoglou, Panmai et al. 2007).

In 1990, Liversidge et al. developed a technology called NanoCrystals®. Large crystals of drug are wet milled in the presence of grinding media and a surface modifier. The average size obtained by this method is 400 nm (Hu, Johnston et al. 2004). Drug is first dispersed in an aqueous-based surfactant solution, then, the resulting suspension is wet milled with the grinding media. Poorly soluble drugs are reported to be in crystalline state (Merisko-Liversidge, Liversidge et al. 2003).

DissoCubes were developed by Müller in 1994. They are drug nanoparticles prepared by high pressure homogenisation in presence of an adequate surfactant. The cavitation forces disintegrate the micronized drug to obtain nanoparticles. The poorly soluble drug is first dispersed in an aqueous surfactant solution by stirring. The suspension is forced through a high pressure homogenizer applying a typical pressure of 1500 bar for several cycles (Müller, Jacobs et al. 2001). Resulting nanoparticle suspensions have an average size comprised between 40 to 500 nm. Nanosuspension size depends on the hardness of the drug, the processing pressure and the cycles number (Hu, Johnston et al. 2004).

Kocbek et al. have prepared ibuprofen nanosuspensions by two different methods: solvent diffusion method and melt emulsification method. In the solvent diffusion method, a solvent in water emulsion (with partially water-miscible solvents) is used with drug dissolved in the dispersed phase i.e. solvent phase. The selection of solvent and stabilizer is critical to obtain

particles in the nanometer range. After solvent diffusion, nanoparticles are formed by a nucleation growth (Dolenc, Kristl et al. 2009). In both cases, drug nanoparticles are stabilized by different compounds. Nanosuspensions present a size comprised between 160-190 nm. Dissolution rate of ibuprofen from nanosuspensions is increased for 65%. The rate proved to be higher for smaller drug particles (Kocbek, Baumgartner et al. 2006).

To stabilize nanoparticles in a colloidal system, excipients are needed. Stabilization can be performed by steric techniques or by electrostatic interactions. Steric stabilization is obtained by adsorbing polymers onto the drug surface. The use of charged molecules can facilitate the particle stabilization by electrostatic interactions.

Common pharmaceutical excipients are suitable for use as stabilizers such as cellulosic compounds like hydroxypropylcellulose (HPC) or hydroxypropylmethylcellulose (HPMC). These polymers present a molecular weight between 50 and 100 kDa. The stabilization method is by steric stabilization. Surfactants can also be used as stabilizers (non-ionic such as polysorbate, anionic such as sodium lauryl sulphate (SLS)). Cationic surfactants are avoided due to their antiseptic properties (Kesisoglou, Panmai et al. 2007).

Doroumis et al. (Doroumis and Fahr 2006), have developed a precipitation technique to form drug nanosuspensions. Drug is dissolved in a water miscible solvent, this solution is mixed with an aqueous phase with or without surfactants. By this way, the tested drug precipitates as micro or nanoparticles. Depending on drug the authors have observed a drug solubility increase up to ten times.

A secondary step, such as spray-drying, is needed to include the drug in a solid dosage form (i.e. tablets).

Moreover, dense gas technologies, such as CO₂ are also used to produce nanoparticles (Chattopadhyay and Gupta 2002).

1.6.4 Amorphization

Amorphization consists to transform a crystalline compound into its amorphous form. The amorphous solid state presents several advantages comparing to crystalline form. The major advantages are the increase of the compound wettability (which is necessary to its dissolution) and the solubility itself. An example of amorphization is the preparation of solid dispersions.

1.6.4.1 Solid dispersions

A solid dispersion is defined as a dispersion of one or more API in an inert matrix or carrier, prepared by the melting, solvent or melting-solvent method. This term refers usually to a hydrophilic matrix and a lipophilic drug. The physical state of the drug in solid dispersions is often transformed from crystalline to amorphous state. Amorphous formulations are rarely eutectic mixtures and therefore are usually metastable in the solid state (Pouton 2006). The main advantage of solid dispersions is the particle reduced size which increases the dissolution surface. Moreover, it was demonstrated that wettability is also improved in drugs contained in solid dispersions. Polymers increase the physical stability of amorphous drugs by increasing the T_g of the mixture (Karavas, Ktistis et al. 2006).

The melting method was developed by Sekiguchi et al. in the sixties. Accordingly, drug is melted within the carrier followed by cooling and pulverization (Karavas, Ktistis et al. 2006). One condition to prepare solid dispersions by this method is the miscibility of drug and carrier in the molten form. Melting method is not appropriate for thermo-labile compounds. In the solvent method, drug and carrier are dissolved in a common solvent. The solvent is then evaporated under vacuum to obtain a solid dispersion (Leuner and Dressman 2000).

Itraconazole was formulated with Kollicoat IR (a polyvinyl alcohol-polyethylene glycol graft copolymer). The technique used to prepare this solid dispersion is the hot extrusion method. Different drug-polymer ratios were evaluated (between 15 to 80 % of itraconazole in the solid

dispersion). A physical mixture was also prepared to compare dissolution rates. It was demonstrated an important drug solubility enhancement related to the polymer proportion (Janssens, de Armas et al. 2007).

Sustained-release solid dispersions were prepared by Wan et al. (Wan, Sun et al. 2011). A class II drug, i.e. curcumin, was used to prepare a solid dispersion in cellulose acetate. Drug and polymer were solubilised in acetone. Acetone was then evaporated and the obtained film was dried under vacuum. The film was mixed with mannitol and triturated to obtain a uniform dispersion of the film. The obtained mixture sizes around 150 μm . Crystalline curcumin was transformed in its amorphous state which allows increased water solubility.

The influence of surfactants in the dissolution rate and stability of solid dispersions was studied by Ghebremeskel (Ghebremeskel, Vemavarapu et al. 2006). Solid dispersion was prepared by hot melt extrusion. Drug was included in hydrophilic polymer matrices and different surfactants such as Tween-80 and sodium Docusate were incorporated into the solid dispersion. Powder X-ray diffraction shows the amorphous state of drug. The surfactants contribute to increase the dissolution rate by creating a favourable microenvironment for the drug at the dissolving surface. The authors have also demonstrated that the drug solubilisation rate is maintained after six months of storage in stress conditions.

1.6.5 Solvent composition

An approach to increase bioavailability of poor water-soluble drugs is the selection of appropriate solvents which allows the drugs to solubilise.

1.6.5.1 pH Adjustment

pH adjustment is the simplest and most commonly method to solubilise ionizable compounds and increase dissolution rate. The pH change significantly influences the saturation solubility of an ionisable drug by dissociation. The incorporation of pH modifiers in the dosage form

can alter the microenvironmental pH. There are several studies demonstrating the controlled release of drugs by pH modification technology (Tatavarti and Hoag 2006).

1.6.5.2 Co-solvent

Cosolvents are a mixture of miscible solvents able to increase poor-soluble drug dissolution rate. Co-solvents, such as organics, surfactants, hydrophilic macromolecules, and clathrate compounds, are commonly used in the pharmaceutical industry. Several commercial dosage forms include water insoluble drugs incorporated in co-solvents to increase their drug solubility and hence their bioavailability (Matsuda, Kaburagi et al. 2009). Some examples of commercial dosage forms are reported in table 5:

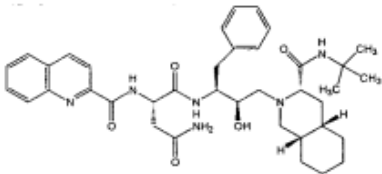
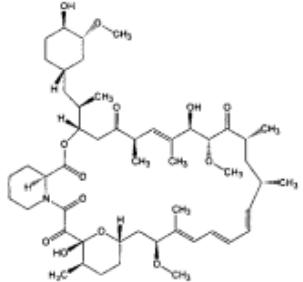
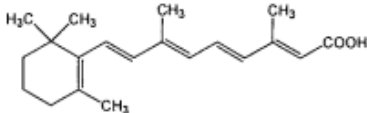
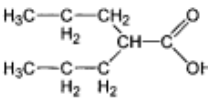
| Molecule/ Trade Name/ Company/ Indication | Chemical structure | Water solubility | Commercial oral formulation | Excipients |
|---|---|---|--|--|
| Saquinavir/ Fortovase/ Roche/ HIV (protease inhibitor) |  | Insoluble in aqueous medium (2) | Soft gelatin capsule 200 mg | Medium-chain mono- and diglycerides Povidone <i>dl</i> - α -tocopherol |
| Sirolimus/ Rapamune/ Wyeth-Ayerst/ Immunosuppressant |  | Insoluble in water (2) | Oral solution 1 mg/ml Bioavailability = 14% (2) | Phosal 50 PG (Phosphatidylcholine) Propylene glycol Mono- and diglycerides Ethanol 1.5–2.5% Soy fatty acids Ascorbyl palmitate) Polysorbate 80 |
| Tretinoin/ Vesanoid/ Roche/ Antineoplastic |  | | Soft gelatin capsule 10 mg | Beeswax BHA EDTA Hydrogenated soybean oil flakes Hydrogenated vegetable oils Soybean oil |
| Valproic acid/ Depakene/ Abbott/ Antiepileptic |  | Slightly soluble in water (1.3 mg/ml) (2) | Capsule 250 mg | Corn oil |

Table 5: Commercial oral dosage forms of poorly soluble water drugs, solubilised with adapted cosolvents (Strickley 2004).

1.6.6 Drug carriers

Poorly water-soluble drugs are included in different carriers (polymeric, lipophilic...) to increase their bioavailability. This enhancement may be related with an increase of drug solubility or a drug protection from the GI medium. The large spectra of drug carriers possessing various physico-chemical properties allows the control of drug formulation to modulate dissolution rate and drug delivery. Drug carriers range from micro to nanometer size; they included microparticles, nanoparticles, liposomes... Most common drug carriers are reported below. Only most common polymeric and lipophilic carriers will be described in this review.

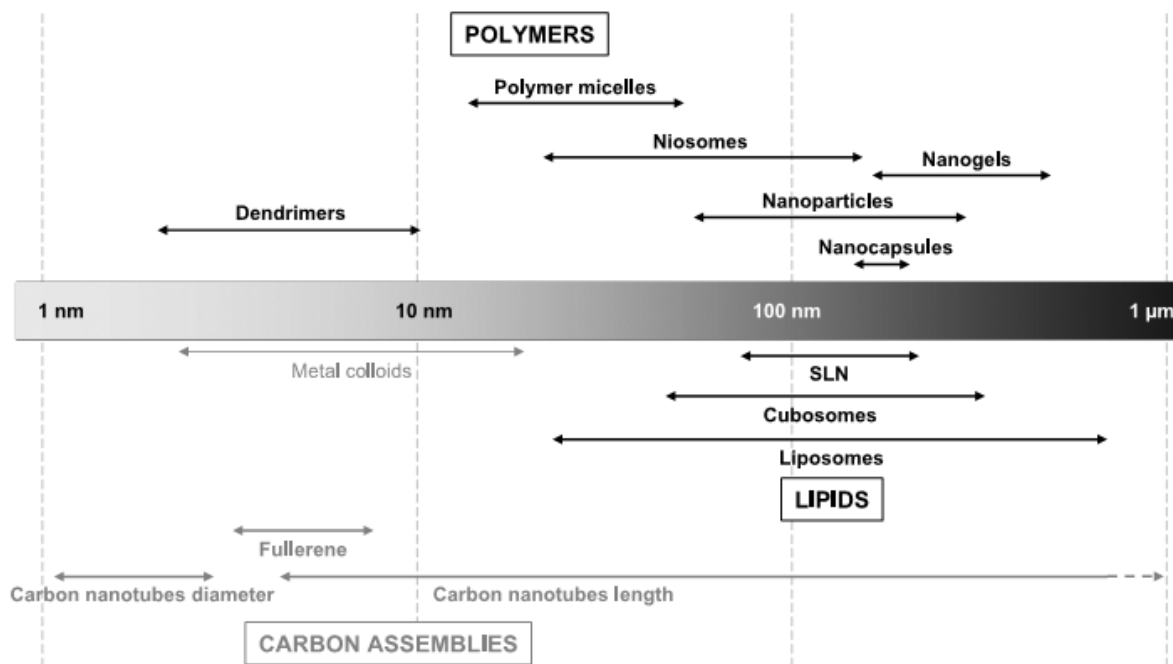


Figure 9: Types of nanotechnology used for drug delivery (Couvreur and Vauthier 2006).

1.6.6.1 Polymeric carriers

Polymeric carriers are largely used to manufacture controlled drug delivery systems.

1.6.6.1.1 Polymeric nanoparticles

Polymeric nanoparticles have been extensively studied and well established as useful drug carriers. Their major advantage is the bioavailability enhancement by different mechanisms such as drug solubility or dissolution rate, protection against acidic pH and enzymes in GIT, permeability enhancement... Polymer carriers should be biocompatible. The origin of these polymers could be natural as alginate or synthetic such as polylactid-co-glycolide or methacrylic polymers.

Nanoparticle preparation depends on drug and polymer properties. Different techniques to prepare nanoparticles were developed during the last decades.

| Technique | Drug | Advantages | Inconvenients | Ref |
|-------------------------------------|--------------------|-----------------------------|--|-----------------------------|
| Salting-out | Thermolabile drugs | High incorporation rate | A purification step is needed | (Mishra, Patel et al. 2009) |
| Emulsification-evaporation | Lipophilic drugs | | An important energy source is necessary Not adapted for hydrophilic drugs | (Tewa-Tagne 2007) |
| Nanoprecipitation | Lipophilic drugs | Energy source is not needed | | (Mishra, Patel et al. 2009) |
| Double emulsion-solvent evaporation | Hydrophilic drugs | | | (Tewa-Tagne 2007) |

Table 6: Different techniques for drug nanoparticles preparation.

Polymeric nanoparticles are used to increase drug bioavailability of highly lipophilic drugs. Celecoxib loaded into ethylcellulose nanoparticles were prepared. A rapid release of the drug in intestinal environment allows the drug to be highly available (Morgen, Bloom et al. 2012). Drug polymer nanoparticles can target different administration routes.

Ophthalmic controlled delivery can be reached by the use of polymeric nanoparticles suspensions. Eudragit® RS is a copolymer of poly(ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammonioethyl metahcrylate). A nanosuspension loaded with ibuprofen was prepared by nanoprecipitation technique. Obtained nanoparticles present a mean size comprise between 35 and 120 nm which is highly suitable for ocular administration. It was

observed that the dispersion of the drug in the polymer increase its distribution in the ocular tissues and consequently its pharmacological activity (Bucolo, Maltese et al. 2002; Pignatello, Bucolo et al. 2002). Biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) and poly[Lac(Glc-Leu) (PLDL) were used to prepare diclofenac nanoparticles for ocular administration. A particle size of about 130 nm was observed. The suspensions allowed a good corneal adhesion and a good solubility profile (Agnihotri and Vavia 2009).

Oral administration is the classical route for polymeric nanoparticles development. The incorporation of drugs presenting a low solubility and permeability into polymeric nanoparticles is able to increase drug absorption rate. Econazole, a low oral bioavailable drug, was loaded into alginate nanoparticles by the principle involving cation-induced controlled gelification of alginate. Econazole nanoparticles were administered to Laca mice orally. An increase of drug bioavailability was observed when compared with intravenous econazole solution (Pandey, Ahmad et al. 2005).

1.6.6.1.2 Polymeric micelles

Polymeric micelles are nanosized (between 20-100 nm) supramolecular structures formed from the self-assembly of amphiphilic block copolymers in aqueous environment (Chen, Khemtong et al. 2011). They are suitable nanocarriers for drugs, proteins, genes and imaging agents. Polymer micelles present a hydrophobic inner core surrounded by an hydrophilic outer shell architecture. Polymer micelles present a high ability to solubilise lipophilic drugs due to the presence of an inner hydrophobic core. Micelles are formed spontaneously in aqueous media. Figure 10 shows the structure of polymeric micelles. This structure type shows a long-term circulation in the blood stream, low toxicity and high stability (Ohya, Takeda et al. 2011). Furthermore, drug targeting is possible by attaching specific ligand molecules such antibodies, transferrin or folate onto the micelle surface (Torchilin 2007). By

varying micelle composition, it is possible to modify the size, loading capacity and micelle longevity in blood stream.

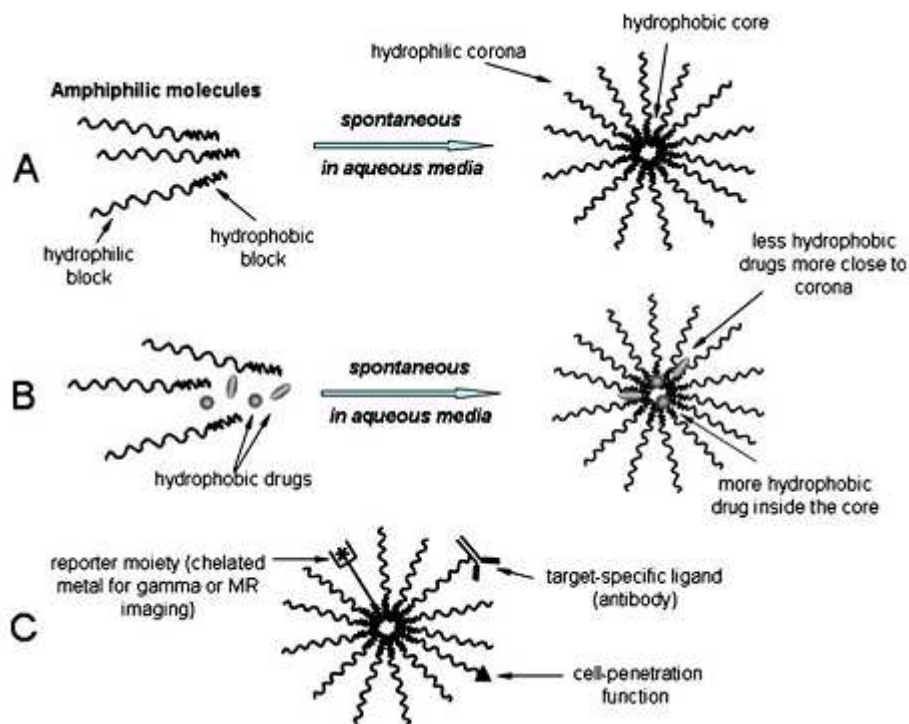


Figure 10: Formation and structure of polymeric micelles. (A) Micelle formation from amphiphilic molecules. (B) Micelle loading with hydrophobic drug. (C) Multifunctional micelle (Torchilin 2007).

Usually, amphiphilic micelle-forming unimers include poly(ethylene glycol) (Betz, Nowbakht et al.) blocks as hydrophilic corona former block (Torchilin 2007). Micelles can be also prepared by the use of polysaccharide main chain grafted with lipophilic side chains (Francis, Cristea et al. 2005). Polymeric micelles of PEG-OCL (poly (ethylene glycol)-b-oligo (ϵ -caprolactone)) were prepared to increase quercetin solubility. Micelles were prepared by the film hydration method. An increase of drug solubility from $9\mu\text{g/mL}$ to 1 mg/ml in PBS was demonstrated when drug was included in micelles (Khonkarn, Mankhetkorn et al. 2011).

1.6.6.2 Lipid carriers

Lipid based formulations are interesting to enhance the absorption of poorly water-soluble drugs by increasing drug solubility in GI tract and decreasing the activity of cytochrome P450 and drug efflux processes mediated via P-gp. Drug absorption could also be increased by stimulating intestinal lymphatic transport (Dollo, Le Corre et al. 2003).

1.6.6.2.1 Self-emulsification drug delivery systems (SEDDS)

Self-emulsification delivery systems (SEDDS) have been used in the recent years to enhance the oral bioavailability of poorly-water soluble drugs. SEDDS are colloidal dispersions of oil in water (or water in oil) stabilized by an interfacial film of surfactants and co-surfactants formed spontaneously by mixing the constituent solutions. There are thermodynamically stable carriers which presents a low viscosity and transparency (Date and Patravale 2004; Mehta, Kaur et al. 2008). They can be classified into self-microemulsification drug delivery systems (SMEDDS) and self-nanoemulsification drug delivery systems (SNEDDS) according to the size range of the droplets. Therefore, SMEDDS form microemulsions with a droplet size ranging from 100-250 nm and SNEDDS form nanoemulsions with a size droplet below 100 nm (Kawabata, Wada et al. 2011).

Oil phase of microemulsion can be formed by different oils such as Castor oil or polymeric solutions. Cloxinic acid was incorporated in a microemulsion containing Castor oil/Tween 20/Tween85 in a ratio of 5:12:18. It was observed an increase of drug solubility up to 3.2mg/mL (Lee, Park et al. 2002). Microemulsions prepared with PLGA-paclitaxel by Self-emulsifying drug delivery system (SMEDDS) present spherical shape and a good drug release profile. PLGA-paclitaxel microemulsions show an increased activity in tumor growth reduction in mice (Kang, Chon et al. 2004).

Recently, researchers have obtained oil-free microemulsions. Diallyl-trisulfide (a lipophilic compound present in garlic oil) was incorporated in a (o/w) microemulsion prepared with

Cremophor EL/ethanol:propylene glycol/saline. Microemulsion was designed for intravenous application. The oil-free microemulsion present better pharmacokinetic profile and less toxicity than the commercial available form Chentian[®] (Li, Yue et al. 2011).

All-trans-retinol acetate was incorporated in a SNEDDS using soybean oil as oil phase and Cremophor EL (surfactant) and Capmul MCM-C8 (co-surfactant). Nanosuspensions with a size droplet comprised between 40-100 nm were obtained depending on surfactant/co-surfactant ratio. An increase of dissolution rate in nanoemulsions prepared with a ratio 1:1 and 1:2 was observed (Taha, Al-Saidan et al. 2004).

The droplet size of emulsion may influence the extent of drug absorption after oral administration. As an example, Neoral[®], a cyclosporine SNEDDS formulation presents a better bioavailability compared with Sandimmune[®], a coarse SMEDDS formulation in humans (Humberstone and Charman 1997; Kawabata, Wada et al. 2011).

1.6.6.2 Liposomes

Liposomes are artificial vesicles formed by a lipidic bilayer. Lipids contained in the liposomes are phospholipids and lipid chains containing surfactant properties. Liposomes were largely used to encapsulate hydrophilic drugs. The biphasic character of liposomes is also interesting in lipophilic drugs encapsulation (Mohammed, Weston et al. 2004). There are different types of liposomes depending on their size (varying from few nanometers to micrometer range) and the bilayers number. Therefore, unilamellar vesicles contain one lipidic bilayer. They are named small (SUV) or large (LUV) depending on size vesicle. Large multilamellar vesicles (LMV) contains two or more lipidic bilayers as shown in figure 11.

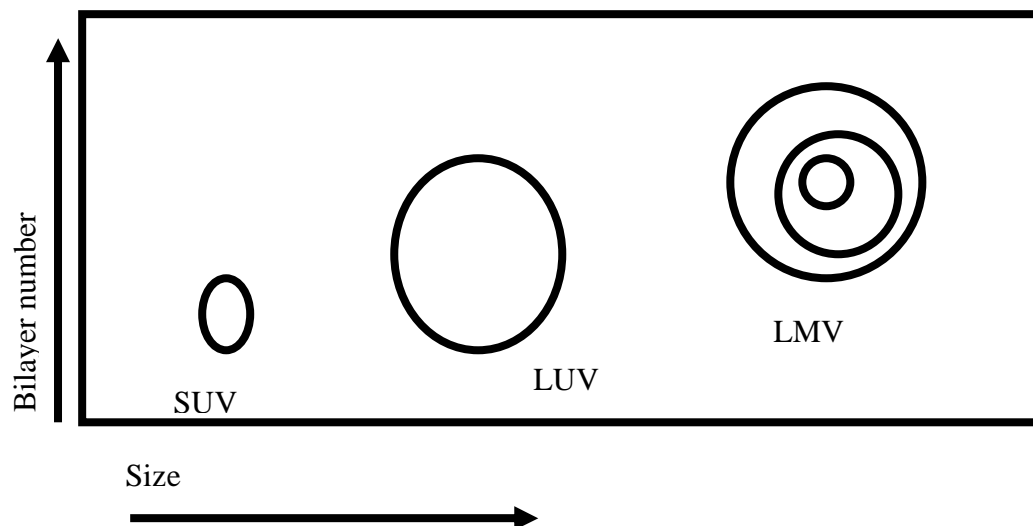


Figure 11: Schematic structure of liposomes.

Liposome formulations are administered by different routes. Betz et al have developed liposomes by the ethanol injection method loaded with heparin for topical application (Betz, Nowbakht et al. 2001). Ibuprofen liposomes incorporated in a poloxamer gels were injected for epidural analgesia (Paavola, Kilpeläinen et al. 2000).

1.6.6.2.3 Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles (SLNs) are a stable colloidal carrier system in which melted lipid is dispersed in an aqueous surfactant by high-pressure homogenization (similar to the DissoCubes) or microemulsification. They are generally made up of a solid hydrophobic core (at room temperature as well as body temperature) containing the drug dissolved or dispersed. They were first introduced by Müller et al. in 1993 and produced both by high pressure homogenization or in parallel by Gasco by diluting warm microemulsion. SLN have been exploited for delivery of actives via the dermal, peroral, parenteral, ocular, pulmonary and rectal route. Upon administration of SLN via the parenteral route of administration, improved bioavailability, targeting, enhanced cytotoxicity against multidrug resistant cancer cells have been observed (Joshi and Müller 2009).

A broad range of drugs, mainly with lipophilic properties, has already been incorporated into dispersions of solid lipid nanoparticles but the drug-loading capacity is indeed quite low (<5–10%) in most cases (Bunjés 2010).

1.6.6.3 Cyclodextrin complexation

Cyclodextrins are a series of cyclic oligomers consisting of six, seven and eight D-glucopyranose units named α -, β - and γ -cyclodextrin respectively. Hydroxypropyl derivatives of β -cyclodextrin have a much higher water solubility than the native β -cyclodextrin. Cyclodextrins form a ring with a relatively hydrophobic central cavity. Hydrophobic drugs can easily be included in this cavity to enhance their solubility.

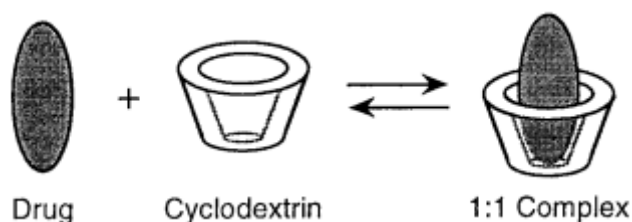


Figure 12: Example of drug inclusion into a cyclodextrin (Rajewski and Stella 1996).

Cyclodextrins complexes are suitable for all administration routes as well as for irritation reduction in mucosae and drug taste masking (Rajewski and Stella 1996).

Warfarin was included in β -Cyclodextrin in a ratio 1:1. An increase in drug solubility as well as wettability and an amorphization was demonstrated (Zingone and Rubessa 2005).

Ibuprofen presents an irritant effect in the oral cavity, throat and pharynx. This characteristic makes ibuprofen dosage forms unpleasant and therefore contributes to the non compliance of treatment. To solve this problem, ibuprofen, has been included in β -cyclodextrins to eliminate the bad taste and to produce a oral dosage form. By forming a tromethamine salt included in a cyclodextrin ring, researchers have been able to increase drug solubility (Al Omari, Daraghmech et al. 2009).

1.7 Polymeric nanosuspensions

A colloidal dispersion is a mechanical mixture where one substance (dispersed phase) is divided into particles and dispersed throughout a continuous phase. Colloidal particles present a size in nanometer range.

Colloidal particles can be prepared by different polymerization methods, such as polymerization in solution. By this method, monomers are dissolved in an organic solvent, the polymerization process occurs in presence of catalyst and heat. This process may take several hours or days and further polymer purification is necessary. The most common method to obtain colloidal particles is the emulsion polymerization method. Monomers are first emulsified in water by stirring in presence of an emulsifier, which stabilizes monomer droplets. Polymerization starts by the addition of a catalyst. Polymer formed precipitates as a new phase (latex particles) and stabilized by the emulsifier molecules. An aqueous dispersion of the colloidal particles is then obtained (Dittgen, Durrani et al. 1997).

Colloidal dispersions can be used in pharmaceutical technologies as excipients during coating process. An excipient is defined as a compound without pharmacological activity included in a dosage form. Its safety should be demonstrated. Aqueous dispersions of water-insoluble polymers are extensively used for coating of pharmaceutical tablets and pellets (Al-Zoubi, Al-Khatib et al. 2008). The role of this kind of compound is to promote stability and processability of dosage form. In some cases, coating can modify drug delivery or enhance drug bioavailability (Villanova, Ayres et al. 2012). Coating a dosage form means the formation of a polymeric film onto the dosage form surface by different techniques as spraying coating. In case of film formation based on organic solvents, when the organic solution is sprayed onto a surface, after solvent evaporation, polymer chains approach each other and finally forms a continuous film. In contrary, after spraying aqueous polymer dispersion onto a dosage form, water evaporates, particle approach each other and under

optimal conditions (in terms of temperature, plasticizer content...) particles coalesce to form a film. In some cases, an additional thermal step (i.e. curing), is needed to enhance the degree of polymer coalescence obtaining thereby a homogeneous polymeric film. Therefore, aqueous polymeric dispersions were developed in coating as an alternative to organic solutions (Frohoff-Hülsmann, Schmitz et al. 1999). Aqueous-based coating systems have numerous advantages over organic solvent-based systems with respect to ecological, toxicological and manufacturing safety (Guo, Heinämäki et al. 2008). The major advantages of these aqueous dispersions are their safety and their capabilities in drug delivery control, taste masking... They also present a low viscosity, low tackiness and reduced coating times as reported with polymeric organic solutions.

Aqueous-based polymer dispersions are currently available on the market including acrylic resins, cellulosic derivatives and vinyl polymer. Table 7 summarizes the major physico-chemical characteristics of commercial aqueous polymeric dispersions.

Since 1958, ethylcellulose has been used as coating material for sustained release forms. It is widely used in solid dosage forms coating due to its capacity to form films. It is non toxic, non allergenic and non irritant. It is widely used for moisture protection and drug delivery. The major inconvenient is its poor wettability that is translated in a slow release rate (Siepmann, Muschert et al. 2008). A polymer dispersion of ethyl cellulose (Aquacoat[®] ECD) is commercially available.

Cellulose acetate phthalate is a cellulose ester derivative used for enteric coating. This polymer dissolves in buffered solutions above pH 5.5. Polymer dispersion is commercially available under the name of Aquacoat[®] CPD.

Acrylic polymers prepared by copolymerization of methacrylic acid and methacrylic esters are largely used as enteric coating in dosage forms. Poly(methacrylates) have a very stable carbon skeleton which includes ester groups in side chains in contrast with cellulose ethers

whose glucose units are linked via oxygen bound hydrolysable by acids and basis. Nevertheless, films prepared with acrylic polymers are more flexible than cellulosic derivatives (Dashevsky, Kolter et al. 2004). There are different aqueous polymeric dispersions prepared with different co-polymers, which are commercialized under different names. Eudragit[®] is the commercial name of a wide range of acrylic polymers destined to sustained release, taste masking... They are composed of different co-polymers such as Ethylacrylate/methylmethacrylate (Eudragit[®] NE 30D, a neutral polymer); Ethylacrylate/Methylacrylate (Eudragit[®] L 30D-55, an anionic polymer) or Ethylacrylate/methylmethacrylate/trimethylammoniomethylmethacrylate (Eudragit[®] RL 30D or RS 30D, cationic polymers). Kollicoat[®] MAE 30DP is an acrylic co-polymer of Ethylacrylate/Methylacrylate with similar properties as Eudragit[®] L 30D-55. Quaternary ammonium groups present in Eudragit[®] RL and RS increase film permeability due to their hydrophilic nature, ionized in physiological pH range. Eudragit[®] NE and anionic acrylic polymers are more stable than cationic polymers.

Kollicoat[®] SR 30D is an aqueous colloidal polyvinyl acetate dispersion used for extended release coatings. Kollicoat[®] SR is very stable against sedimentation. It forms a flexible film. Drug release is independent of pH and ionic strength. The advantage of this polymer is that curing and plasticizer use are not necessary. To avoid the diffusion of a lipophilic drug into polymer film, an intermediate hydrophilic coating may be needed between drug and Kollicoat film (Dashevsky, Wagner et al. 2005).

| Polymer | MW | Additives | pH | Z-potential | MFT | Tg | Uses | Manufacturer | Reference | |
|-----------------------------------|--|-----------|--|-------------|-------|------|-------|-------------------|-----------------|---|
| Aquacoat [®] ECD | Ethylcellulose | 80000 | Na lauryl sulphate 0.9-1.7% Cetylalcohol 1.7-3.3% | 6.1 | -55mV | 81°C | 129°C | Sustained release | FMC biopolymers | (Schmidt and Bodmeier 1999; Schmid, Müller-Goymann et al. 2000) |
| Aquacoat [®] CPD | Cellulose Acetate Phtalate | | Poloxamer 7% | 2-3 | | 29°C | | Enteric coating | FMC biopolymers | (Williams Iii and Liu 2000) |
| Eudragit [®] FS 30D | Poly(methylacrylate, methylmethacrylate, methacrylic acid) | 220000 | Na laurylsulphate 0.3% Polysorbate 80 1.2% | 2-3.5 | | 14 | 48°C | Colon delivery | Evonik | (2011) |
| Eudragit [®] L 30D-55 | Poly(methacrylic acid,ethyl acrylate) 1:1 | 250000 | Na dodecylsulphate 0.2% Polysorbate 80 0.7% | 2.3 | -53mV | 27°C | 76°C | Enteric coating | Evonik | (Schmidt and Bodmeier 1999; 2011) |
| Eudragit [®] NE 30D | Poly(ethylacrylate,methylmethacrylate)1 :2 | 800000 | Nonoxynol100 1.5% | 6.7 | -39mV | 5°C | -8°C | Sustained release | Evonik | (Schmid, Müller-Goymann et al. 2000) |

| | | | | | | | | | | |
|---------------------------|--|--------|--|-----|-------|------|------|-------------------|--------|--|
| Eudragit® RL 30D | Poly(ethylacrylate, methylmethacrylate) trimethylammoniomethylmethacrylate 1:2:0.2 | 150000 | Sorbic acid 0.25% NaOH | 4.6 | +43mV | 39°C | 55°C | Sustained release | Evonik | (Schmid, Müller-Goymann et al. 2000) |
| Eudragit® RS 30D | Poly(ethylacrylate, methylmethacrylate) trimethylammoniomethylmethacrylate 1:2:0.2 | 150000 | Sorbic acid 0.25% NaOH | 4-6 | +50mV | 45°C | | | | (Dashevsky, Wagner et al. 2005) |
| Kollocoat® MAE 30DP | Poly(methacrylic acid, ethylacrylate) 1:1 | 250000 | Na dodecylsulphate 0.7% Polysorbate 80 2.3% | | | | | Enteric coating | BASF | (Bühler 2007) |
| Kollocoat® SR 30D | Poly(vinyl acetate) | 450000 | Povidone 2.7% Na laurylsulphate 0.3% | 3-5 | -23mV | 18°C | | Sustained release | BASF | (Dashevsky, Wagner et al. 2005; Bühler 2007) |

Table 7: Overview of physico-chemical properties of polymer aqueous dispersions.

1.8 Active principles

The next study is based on the use of different lipophilic drugs (used as a model drugs) incorporated into commercial polymeric nanosuspensions to increase their solubility. Selected drugs are defined as Class II (low solubility and high permeability) and Class IV (Low solubility and low permeability) of the BCS. The next table summarizes some characteristics of the different drugs that will be tested.

| Drug | Pharmacological activity | Biopharmaceutics Classification system |
|-------------------|--------------------------------|--|
| Celecoxib | Non-steroidal antiinflammatory | II |
| Sodium diclofenac | Non-steroidal antiinflammatory | II |
| Econazole nitrate | Antifungal | IV |
| Ibuprofen | Non-steroidal antiinflammatory | II |
| Ivermectin | Antiparasitic | IV |
| Warfarin | Anticoagulant | II |

Table 8: Overview of selected drugs used in the present study.

1.8.1 Anticoagulant: Warfarin

Warfarin is the most used coumarin anticoagulant in the world and it is considered the molecule of choice for the treatment and prevention of thrombosis and thromboembolism.

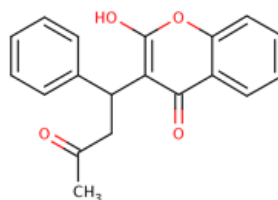


Figure 13: 2-hydroxy-3-(3-oxo-1-phenylbutyl)-4H-chromen-4-one or 3-(α -acetylbenzyl)-4-hydroxycoumarin

Warfarin is a racemic mixture of R and S enantiomer, which are metabolised by cytochrome P450. Both isomers are metabolised via different pathways, being more important the

metabolism of S-isomer (this isomer is 5 fold more active than the R one) (Moreau, Siguret et al. 2010; Nutescu, Chuatrisorn et al. 2011).

Due to its structural analogy with Vitamin K, warfarin inhibits the Vitamin K epoxy reductase avoiding the regeneration of reduced vitamin K (Vitamin K hydroquinone). Vitamine K hydroquinone acts as a coenzyme for carboxylase. The inhibition of this coenzyme does not allow the γ -carboxylation of the proteins. γ -carboxyglutamic acid enhances the binding of calcium ions. Coagulation factors undergo a conformational change which is necessary to interact with platelets surface to initiate the coagulation process in the presence of calcium ions. Coagulation factors II, VII, IX and X as well as protein C, S and Z are inhibited by this mechanism (Pineo and Hull 2005; Moreau, Siguret et al. 2010).

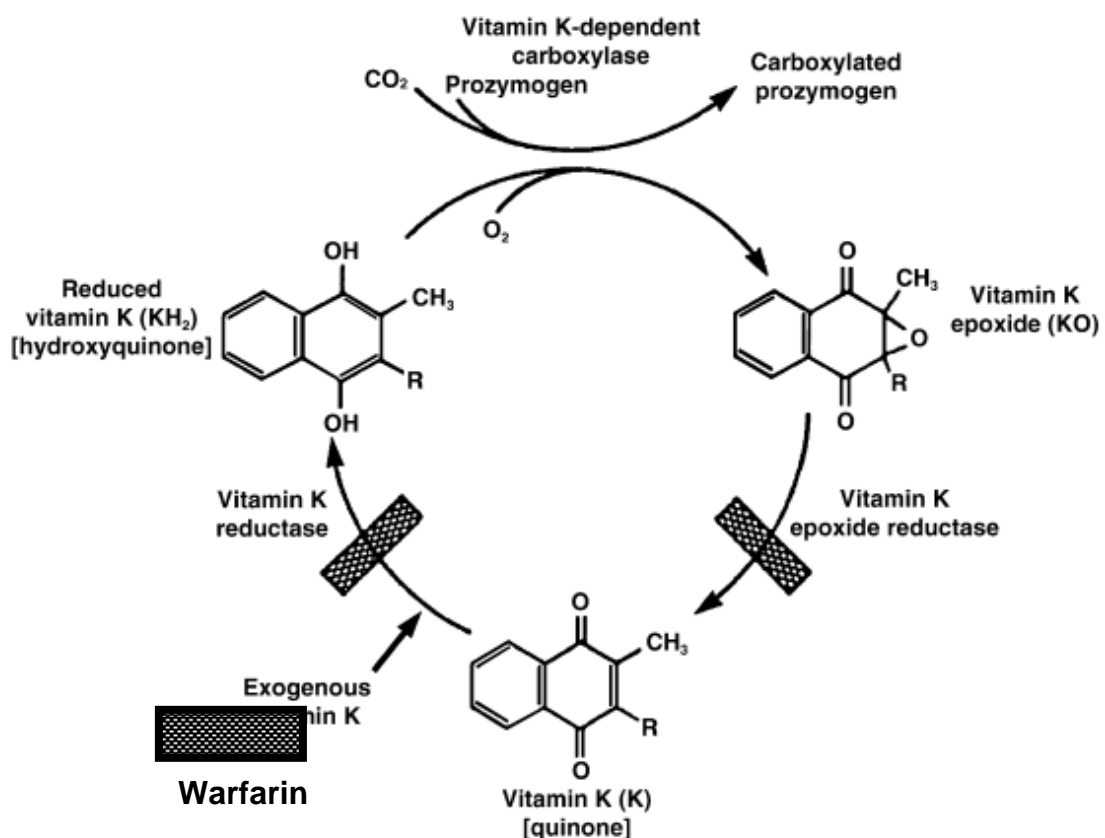


Figure 14: Vitamin K cycle and warfarin site of action. Adapted from Pineo et al (Pineo and Hull 2005).

Warfarin is rapidly absorbed after oral administration. Protein binding is very important (97%) primarily albumin. Maximal concentration in plasma is obtained 2-6 hours after administration. Antithrombotic activity appears 36-72 hours after treatment beginning and is prolonged until 4 days of the treatment stop. Warfarin is metabolized in liver by cytochrome P450. Phase I metabolism corresponds to hydroxylation and reduction. Some of the metabolites present an anticoagulant activity. Warfarin is eliminated primarily in urine (80%) and faeces (20%). Elimination of unchanged warfarin is found less than 5%. Half life of warfarin is in order of 35-45 hours (Dictionnaire 2007; Scala-Bertola 2009).

Warfarine is commercialized by the name of Coumadine.

1.8.2 Antifungal: Econazole

Econazole nitrate is an imidazole drug with antifungal activity for the treatment of skin infections and vaginal candidiasis (Albertini, Passerini et al. 2009).

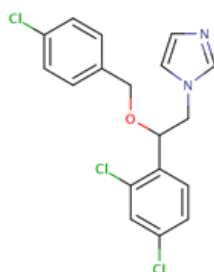


Figure 15: 1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole.

Imidazoles have been traditionally used against eukaryotic organism since they interfere in the synthesis of ergosterol by inhibiting the cytochrome oxidase group of enzymes (Vera-Cabrera, Campos-Rivera et al. 2010) and causing defects in fungal membrane. Gram positive antibacterial activity is also reported with imidazoles (Kokjohn, Bradley et al. 2003). After topical application, absorption is very low. In plasma, protein binding is about 98%. Econazole is metabolized in the liver by oxidation and O-desalkylation (Dictionnaire 2007).

Econazole is available commercially in topical forms as Dermazol[®], Gyno-Pevaryl[®], Myleugyn[®], Pevaryl[®] and Pevisone[®]. It is presented as a cream, lotion or vaginal ovules.

1.8.3 Antiparasitic: Ivermectin

Ivermectin is widely used for the control of lymphatic filariasis due to *Wuchereria bancrofti* and onchocerciasis (Edwards 2003).

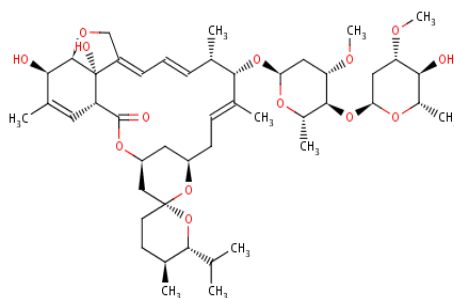


Figure 16: (22,23-dihydroavermectin B1a + 22,23-dihydroavermectin B1b)

Ivermectin is a semi-synthetic derivative of avermectin, a macrocyclic lactone derived from the soil bacterium *Streptomyces avermitilis*. Ivermectin binds selectively and with high affinity to glutamate-gated chloride ion channels present in invertebrate muscle and nerve cells. The binding to these channels favours the membrane permeability to chloride ions, causing an hyperpolarization of the cell, leading to paralysis and death of the parasite. Ivermectin also is believed to act as an agonist of the neurotransmitter gamma-aminobutyric acid (GABA), thereby disrupting GABA-mediated central nervous system (CNS) neurosynaptic transmission.

1.8.4 Non-steroidal anti-inflammatory drugs (NSAIDs): Celecoxib, Diclofenac and Ibuprofen.

1.8.4.1 Mechanism of action

NSAIDs are are drugs with analgesic and antipyretic (fever-reducing) effects and which have, in higher doses, anti-inflammatory effects.

NSAIDs act as inhibitors of enzyme cyclooxygenase (COX). The isoenzyme COX-2 participates in inflammatory processes. Most of NSAIDs are non selective inhibiting both isoenzymes COX-1 and COX-2.

COX catalyzes the synthesis of prostaglandins from arachidonic acid. Prostaglandin (PG) production is initiated by the Phospholipase A2 enzyme which, liberates the COX substrate arachidonic acid from membrane phospholipids. There are 2 isoenzymes of COX. COX-1 is a constitutive enzyme present mainly in gastric mucosa, kidney and platelets which is involved in homeostatic functions. The isoenzyme COX-2 is inducible in case of inflammatory processes. Both isoenzymes participate in PG synthesis. In case of COX-1, PG are involved in physiological process whereas PG synthesized by COX-2 are involved in inflammatory process.

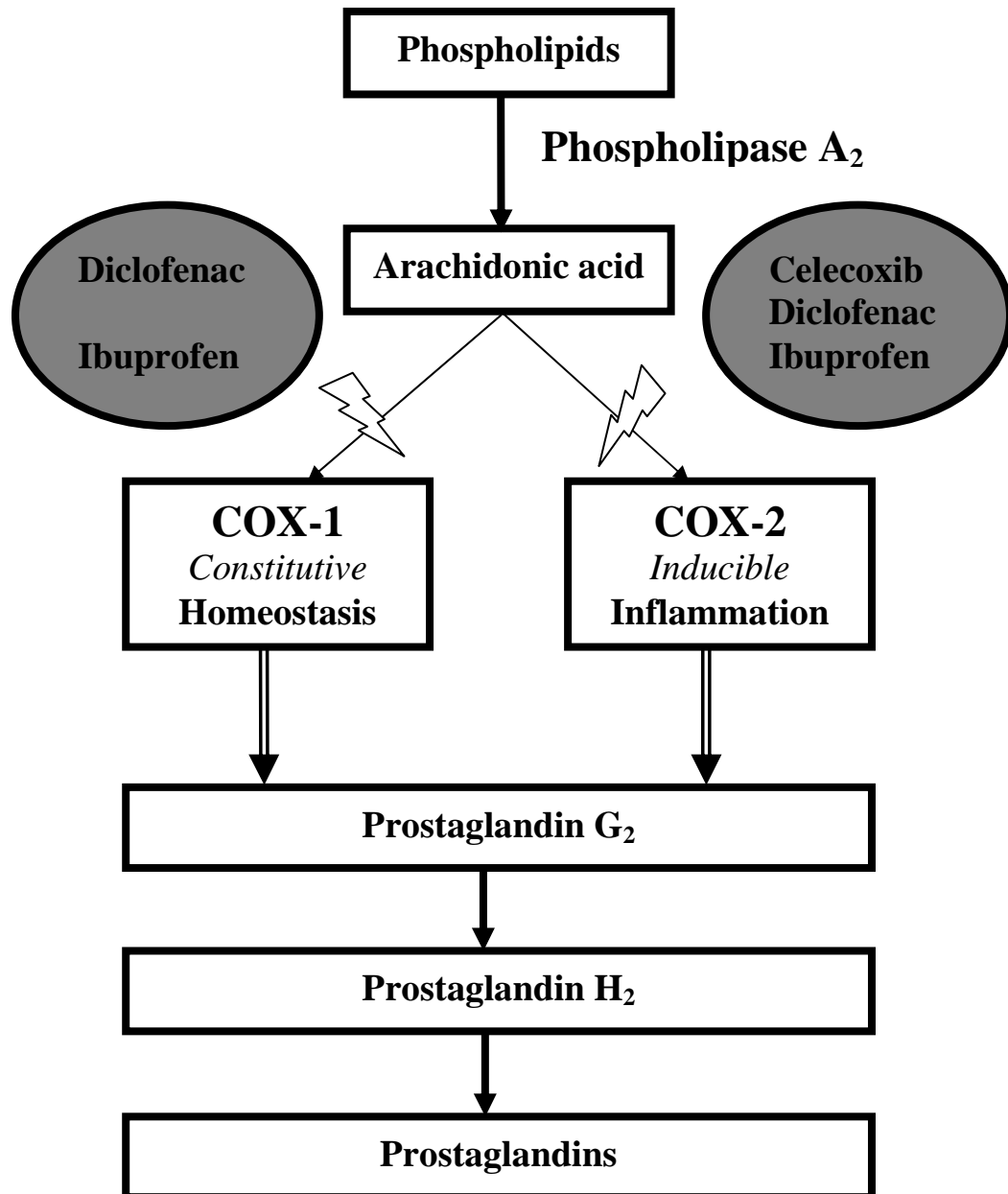


Figure 17: Mechanism of action of NSAIDs.

1.8.4.2 Celecoxib

Celecoxib belongs to a new generation of non-steroidal anti-inflammatory drugs (NSAID) used for treatment of osteoarthritis and rheumatoid arthritis.

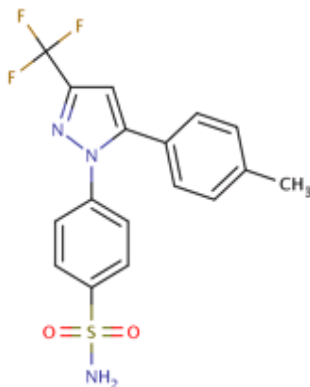


Figure 18: (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene-1-sulfonamide).

Celecoxib is a selective inhibitor of cyclooxygenase 2 (COX-2) expressed during inflammatory processes (Chandran, Ravi et al. 2006). Drug has similar efficacy than others NSAID in improvement of arthritic symptoms, but with a reduced incidence in gastrointestinal ulceration due to the non inhibition of COX-1. It was reported that celecoxib also presents analgesic and anticancer properties (Manzoori, Abdolmohammad-Zadeh et al. 2005).

Celecoxib is an acidic weakly with a pKa of 11.1 (Dolenc, Kristl et al. 2009). It is considered a BCS Class II drug due to its low solubility. It is reported to be 20-40% available with conventional capsule dosage form (Ali, Arora et al. 2007). The limiting factor of drug absorption is its poor solubility. Celecoxib is well absorbed and maximal plasmatic concentrations are attained after 2-3 hours of administration. Celecoxib is almost totally eliminated via hepatic metabolism. Drug is metabolised in the liver by oxidation, hydroxylation and partially glucuronization. Metabolism is basically catalyzed by Cytochrome CYP2C9. Resulting metabolites are inactive. Celecoxib plasmatic binding is about 97% principally albumin. Half life is comprised between 8-12 hours (Dictionary 2007).

Celecoxib is marketed in capsules as crystalline form by the name of Celebrex[®] with a dosage of 100 and 200 mg.

1.8.4.3 Diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID). Molecule has shown an anti-inflammatory, analgesic and antipyretic activity.

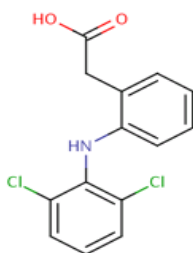


Figure 19: 2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid

The mechanism of action is still not well known but it seems to be related with the inhibition of prostaglandins synthesis by the inhibition of cyclooxygenase. It is a non selective inhibitor of COX which acts in cyclooxygenase 1 and 2 inhibitions. Diclofenac is used in the treatment of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. Due to its low solubility, it is commercially available as a salt (i.e. sodium salt, potassium salt). It is reported that potassium salt it solubilised faster than sodium salt. Diclofenac is 100% absorbed after oral administration. Only 60% of dose reaches to the systemic circulation due to the first pass effect. Plasmatic protein binding is very high (> 99%), primarily albumin. Diclofenac is rapidly metabolised in liver by hydroxylation and glucuroconjugation in inactive metabolites that are eliminated in urine and faeces. Half life is about 1-2 hours (Dictionnary 2007; Chuasuwana, Binjesoh et al. 2009).

Diclofenac is commercially found in different dosage forms such as eye drops, gels, granules and tablets. Commercial dosage forms are: Flector, Voltaren, Xenid...

pKa value of diclofenac is 4.9 due to the carboxylic acid (Arancibia, Boldrini et al. 2000).

1.8.4.4 Ibuprofen

Ibuprofen is a well known and widely used NSAID. It is used in treatment of moderated pain related to dysmenorrhoea, headache, migraine, post operative and dental pain and in the management of spondylitis, osteo -arthritis, rheumatoid arthritis, and soft tissue disorders. Ibuprofen has also antipyretic properties. Ibuprofen is considered the safest NSAID available. Ibuprofen is administered as a racemic mixture. The R-enantiomer undergoes extensive interconversion to the S-enantiomer *in vivo*. The S-enantiomer is believed to be the most pharmacologically active enantiomer.

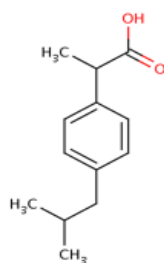


Figure 20: 2-[4-(2-methylpropyl)phenyl]propanoic acid

Ibuprofen is a non-selective inhibitor of cyclooxygenase. Antipyretic effects may be due to action on the hypothalamus, resulting in an increased peripheral blood flow, vasodilatation, and subsequent heat dissipation. Inhibition of COX-1 is thought to cause some of the side effects of ibuprofen including GI ulceration.

Ibuprofen is totally available after oral administration and maximum plasma values are obtained after 1-2 hours. Protein plasma binding is very important with ibuprofen (>99%). Hepatic metabolism results in inactive metabolites, mainly a glucuronic derivative. Urine is the major route of elimination. Ibuprofen presents a half life of 2 hours (Potthast, Dressman et al. 2005; Dictionnary 2007).

Ibuprofen is considered a BCS class II according to its high permeability and pH depending solubility.

Several commercial dosage forms are available for ibuprofen. It is presented as gel, solution, tablet... Advil, Brufen, Nurofen... are some of the commercial names of ibuprofen.

2. Objective of the work

This part of the PhD project is based on the incorporation of lipophilic drugs into polymeric nanoparticles. These nanoparticles are available as commercial aqueous suspensions which are classically used for dosage forms coating and film formation.

Our hypothesis is that the inclusion of lipophilic model drugs which are poorly water soluble into the polymeric nanoparticles may enhance their bioavailability due to a concentration gradient if such nanoparticles were able to coat the GIT (increase in contact surface)..

This project is developed in different steps:

Drug incorporation:

The first step of the work was to evaluate the incorporation of the drugs into the various nanoparticles dispersed in the suspension. Selected drugs (celecoxib, diclofenac, econazole, ibuprofen, ivermectin and warfarin) were incorporated into different polymer nanosuspension.

The incorporation extent and rate depends on drug and polymer structure and compatibility. A physico-chemical study was performed to characterize the drug-polymer nanosuspensions in terms drug incorporation into polymeric nanoparticles, drug-polymer interactions, size and zeta potential, drug release from nanoparticles and stability during storage.

Study of drug-polymer interactions:

The study of drug –polymer interactions may explain the formulation behaviour in terms of drug loading, *in vitro* drug release and oral bioavailability.

Applicability:

The last step of the work will be based on the possibility to develop a commercial dosage form. *In vivo* experiments will be able to evaluate the capability of our system to deliver drug in a biological medium. The efficiency of our system will be compared with already existing commercial forms. Moreover, in order to develop a more comfortable oral dosage form (by reducing the administered suspension volume), drug loading will be optimised.

3. Materials and methods

3.1 Materials

3.1.1 Drugs

Celecoxib (Matrix, India), Sodium diclofenac (Heumann Pharma GmbH, Nürnberg, Germany), Ibuprofen (BASF, Ludwigshafen, Germany), Econazole Nitrate, Ivermectin and Warfarin were purchased from Sigma (Saint Quentin Fallaver, France).

3.1.2 Polymers

Aquacoat[®] ECD (FMC BioPolymers Newark, USA), Eudragit[®] FS 30 D, Eudragit[®] NE 30D, Eudragit[®] L 30D-55, Eudragit[®] RL 30D, Eudragit[®] RS 30D (EVONIK Industries, Essen, Germany), Kollicoat[®] MAE 30DP, Kollicoat[®] SR 30D (BASF, Ludwigshafen, Germany) were generously provided.

3.1.3 Solvents and reactives

Acetonitrile and Methanol, both of HPLC grade (Carlo ERBA Val de Reuil, France), Isopropanol, Acetone (Fluka Saint Quentin Fallaver, France), Triethylamine, N-Methylimidazole, Trifluoroacetic anhydride, glycerol formal (Sigma, Saint Quentin Fallaver, France).

3.1.4 Animals

Male Wistar rats (mean body weight 700 ± 100 g) (Janvier, Le Genest-Saint-Isle, France). New Zealand rabbits (mean body weight 2500 ± 250 g) (Charles Rivers Laboratories, L'Arbeste, France).

3.2 Methods

3.2.1 Drug loaded nanoparticles preparation.

Each investigated drug was added into 15 mL of aqueous polymer suspension. Mixture was magnetically stirred at 350 rpm during appropriate times. The amount of drug added into the nanosuspensions was selected according to the usual therapeutic dose as reported in table 10.

| Drug | Drug amount in commercially oral dosage forms (mg) | Amount of added drug (mg) | Aqueous dispersion volume (mL) |
|-------------------|---|---------------------------|--------------------------------|
| Celecoxib | 50, 100, 200, 400 mg | 100 | 15 |
| Diclofenac | 50, 75, 100 mg | 50 | 15 |
| Econazole nitrate | No oral form available Topical administration (1%) | 45 | 15 |
| Ibuprofen | 200, 400, 600, 800 mg | 200 | 15 |
| Ivermectin | 200 µg/kg | 14 | 15 |
| Warfarin | 1, 2, 2.5, 3, 4, 5, 6, 10 mg | 10 | 30 |

Table 9: Drug added into polymeric nanoparticles suspension.

3.2.2 Polarising light microscopy

Drug incorporation rate (measured macroscopically as visible crystals) was followed by polarising light microscopy with a MOTIC B2 series system microscope (MOTIC, Wetzlar, Germany) mounted with a polarising light filter. At selected times (5, 15, 30 minutes 1, 2, 3, 4, 5, 6, 8, 10, 24... hours) a drop of nanosuspension was observed at different magnifications.

3.2.3 Size and Zeta potential measurements

Nanoparticles size was measured with a Nano ZS (Malvern Instruments, Worcestershire, UK). The principle of measurement is the Dynamic Light Scattering. This technique measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship.

Zeta potential of empty and loaded polymeric nanoparticles was measured by Laser Doppler Micro-electrophoresis. Molecules move with a velocity related to their zeta potential through an electric field applied to a dispersion of particles.

3.2.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out using a Hitachi FEG S4800 (Hitachi, Velizy, France) operating between 1 and 3 kV under nitrogen atmosphere.

3.2.5 Drug assay

The amount of drug incorporated into nanoparticles was assayed both directly and indirectly.

3.2.5.1 Indirect assay

1 mL of drug-polymer nanoparticles was ultracentrifuged (Ultracentrifuge Sorvall, Thermo Scientific, Saint Herblain, France) at 104,000 g during 30 minutes. Supernatant was appropriately diluted in water 15 MΩ.cm (Veolia Water, Antony, France) with a final dilution in HPLC mobile phase to conserve analytical conditions. Furthermore, samples were analyzed by HPLC. The HPLC system was a Shimadzu Prominence LC-20AD with the following components: DGU-20A degasser, SIL-20AC auto sampler, SPD-20A UV/VIS detector, CTO-20A column oven. The column is a UP5CN 250x4.6 mm (Interchim, Montluçon, France). Analytical method was adapted to each drug as reported in table 10.

| Drug | Celecoxib | Sodium diclofenac | Econazole nitrate | Ibuprofen | Ivermectin | Warfarin |
|----------------|--|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Column | UP5CN, 5µm particle size, 250x4.6 mm Interchim (Montluçon, France) | | | | | |
| | Triethylamine phosphate | Triethylamine phosphate | Triethylamine phosphate | Triethylamine phosphate | Triethylamine phosphate | Triethylamine phosphate |
| Mobile phase | buffer 10 ⁻² M | buffer 10 ⁻² M | buffer 10 ⁻² M | buffer 10 ⁻² M | buffer 10 ⁻² M | buffer 10 ⁻² M |
| | pH 6.5/ | pH 6.5/ | pH 6.5/ | pH 6.5/ | pH 6.5/ / | pH 6.5/ |
| | Methanol | Methanol | Methanol | Methanol | Methanol | Methanol |
| | 30/70 | 45/55 | 30/70 | 45/55 | 30/70 | 45/55 |
| Flow (mL/min) | 0.6 | 0,8 | 0.6 | 0.8 | 0.6 | 0.8 |
| T°C | | | | 45 | | |
| λ (nm) | | | | 220 | | |
| tr (min) | 6.5 | 4.6 | 8 | 4 | 7.2 | 4.5 |
| R ² | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 |
| LOQ (µg/ml) | 1.0 | 0.05 | 0.1 | 0.2 | 0.7 | 0.4 |
| LOD (µg/ml) | 0.4 | 0.02 | 0.03 | 0.05 | 0.2 | 0.1 |

Table 10: Indirect assay of lipophilic drugs by HPLC

3.2.5.2 Direct assay

Due to technical difficulties, direct assay was not performed for all drugs and it was adapted for each drug.

• Celecoxib

10 mg of freeze-dried nanosuspension were dissolved in 100 ml of an appropriate solvent (Methanol to dissolve Aquacoat[®] ECD and Kollicoat[®] SR 30D nanoparticles and Acetonitrile to dissolve Eudragit[®] NE 30D nanoparticles). The resulting solution was analyzed fluorometrically (Fluorescence spectrophotometer F-2000, HITACHI, Krefeld, Germany) at $\lambda_{exc}= 262\text{nm}$ and $\lambda_{em}=360\text{ nm}$. Calibration curve was performed using empty polymeric solution as blank. Calibration curve was linear between 0.5-7 $\mu\text{g/ml}$ with a $R^2=0.999$ and LOQ and LOD of 0.3 and 0.09 $\mu\text{g/ml}$, respectively.

• Ibuprofen

10 mg of freeze-dried nanosuspension were dissolved in 100 ml of an appropriate solvent (Methanol to dissolve Aquacoat[®] ECD, Eudragit[®] RL 30D and Kollicoat[®] SR 30D nanoparticles and Acetonitrile to dissolve Eudragit[®] NE 30D nanoparticles). The resulting solution was filtered (cellulose acetate, pore size 0.20 μm , Macherey Nagel, Hoerd, France) and then analyzed fluorometrically (Hitachi Fluorescence spectrophotometer) at $\lambda_{exc}= 224\text{nm}$ and $\lambda_{em}=286\text{ nm}$. Calibration curve was performed using empty polymeric solution as blank. Calibration curve was linear between 0.5-6 $\mu\text{g/ml}$ with a $R^2=0.999$ and LOQ and LOD of 0.25 and 0.07 $\mu\text{g/ml}$ respectively.

- **Warfarin**

Aquacoat ECD, Eudragit NE 30D and Kollicoat SR 30D

80 mg of freeze dried nanoparticles were dissolved in 10 mL of dichloromethane (DCM). 20 mL of NaOH 1M was then added to the mixture. Afterwards DCM was evaporated under reduced pressure. After evaporation of DCM the remaining solution was completed with demineralized water to 20.0 mL, filtered (cellulose acetate, pore size 0.20 μm , Macherey Nagel, Hoerd, France) and assayed via UV-spectrometry (UV-1700, Shimadzu, Japan) at 308 nm. Calibration curve was linear between 2-20 $\mu\text{g/ml}$ with a $R^2=0.999$ and LOQ and LOD of 0.8 and 0.26 $\mu\text{g/ml}$ respectively.

Eudragit RS and RL 30D

20 mg of freeze-dried nanoparticles were dissolved in 25 mL of a solvent blend (Isopropanol-Methanol 80:20). The resulting solution was analyzed fluorometrically (Fluorescence spectrophotometer F-2000, HITACHI, Krefeld, Germany) at $\lambda_{\text{exc}}=310$ nm and $\lambda_{\text{em}}=390$ nm. Calibration curve was performed using empty polymeric solution as blank. Calibration curve was linear between 0.2-1.5 $\mu\text{g/ml}$ with a $R^2=0.999$ and LOQ and LOD of 0.104 and 0.034 $\mu\text{g/ml}$ respectively.

3.2.6 Differential scanning calorimetry (DSC)

The thermal properties of nanoparticles were determined by differential scanning calorimetry (DSC) measurements (Q10 DSC, TA Instruments, Guyancourt, France). The instrument was calibrated using indium as the standard. Samples of 5 mg were heated and cooled in sealed aluminum pans from at a scanning rate of 20°C/min under nitrogen purge, with an empty aluminum pan as reference.

3.2.7 Films preparation

The compatibility between polymers and drugs was evaluated by preparing and evaluating casted films.

300 μL of unloaded nanosuspensions were dissolved in one milliliter of acetone to obtain a final polymer concentration of 1 mg/ml.

In parallel, drug was dissolved in acetone at a concentration of 100 $\mu\text{g}/\text{ml}$.

One milliliter of polymer solution was mixed with a volume of drug solution. The obtained solution was poured in a glass plate (24 cm^2) and allowed to evaporate at room temperature.

Next table summarizes the conditions of film preparation.

| Drug | Polymer solution (mL) | Drug solution (μL) | Final concentration (μg drug/mg of polymer) |
|------------|-----------------------|---------------------------------|---|
| Celecoxib | 1 | 220 | 22 |
| Ibuprofen | 1 | 440 | 44 |
| Ivermectin | 1 | 30 | 3 |

Table 11: Conditions for drug-polymer film formation.

3.2.8 In vitro release study

Drug release from nanoparticles was performed in sink conditions. These conditions were selected after determination the drug solubility in various dissolution media. An excess amount of drug was poured in a flask containing the selected dissolution medium. Drug was stirred for 24-48 h and then centrifuged (Biofuge Stratos, Heraeus, Buckinghamshire, UK) at 42,000 g for 20 minutes; Supernatant was assayed by HPLC as described in 2.4.1.

Two different methods were tested:

3.2.8.1 Pharmacopeia method

Two USP Pharmacopeia apparatus Type 2 (Dissolution Test System, Distek, North Brunswick, USA and Dissolution Tester, PharmaTest, Hainburg, Germany) were used to perform kinetic drug release. Briefly, 2 or 5 ml of drug-polymer nanosuspension were dispersed in 500 mL of dissolution medium, stirred at 50 rpm and maintained at $37\pm 1^\circ\text{C}$. At

selected times, samples of 1.5 mL were withdrawn and replaced with 1.5 mL of fresh buffer. Samples were centrifuged at 42,000 g for 20 minutes at room temperature. Supernatants were assayed spectrophotometrically or fluorimetrically at appropriated wavelengths. Table 13 summarizes drug release test conditions.

| Drug | Sample volume (mL) | Dissolution medium | Assay method | Wavelength (nm) |
|-------------------|--------------------|-----------------------------|--------------|---|
| Celecoxib | 5 | Phosphate buffer-Tween80 2% | UV | 255 |
| Econazole Nitrate | 5 | PBS pH 7.4 | UV | 220 |
| Ibuprofen | 5 | PBS pH 7.4 | UV | 222 |
| Ivermectin | 5 | Water SDS 2% | UV | 243 |
| Warfarin | 9 | PBS pH 7.4 | Fluorescence | $\lambda_{exc}= 310$ $\lambda_{em}= 390$ |

Table 12: In vitro drug release conditions.

3.2.8.2 Dialysis method

A dialysis membrane (Cellulose MW 10395, purchased from Sigma-Aldrich, Saint Quentin Fallavier, France) was also used to perform the in vitro release test. Dialysis tubes with an average width of 10 mm and average length of 10 cm were filled with 0.7 ml of nanoparticle suspension. Dialysis tube was placed in a plastic tube containing 12 mL of dissolution medium and stirred at 200 rpm at 37°C. At selected times, 0.5 ml were withdrawn and replaced by fresh buffer. After appropriate dilution, samples were analyzed spectrophotometrically (Shimadzu) or fluorimetrically at appropriated wavelengths (cf. Table 12).

3.2.9 Stability tests

Samples of loaded nanosuspensions were stored both at 4 and 25°C. At selected times, 1, 2, 3 weeks and 1, 2, 3, 4, 5, 6, 9, 12, 18 months, one drop was withdrawn and observed under polarised light microscopy to verify whether drug crystals were present or not.

3.2.10 In vivo tests

3.2.10.1 Animal housing

Male Wistar rats were housed in cages containing groups of 4 animals. New Zealand rabbits animals were housed in separated cages. Animals were maintained under standard conditions: stable room temperature, light/dark cycle (12/12 h). They have free access to food and water.

The day before experiments, animals were fasted overnight with water ad libitum. Guidelines and legislative regulations on the use of animals for scientific purposes were followed.

Two types of animal models were used for in vivo experiments. The choice of animal was performed as a function of the administered dose and analytical assay.

The next table summarizes the different in vivo experiments:

| Drug | Animal model | Administered dose | Drug assay method |
|------------|--------------------|-------------------|-------------------|
| Celecoxib | New Zealand rabbit | 2 mg/kg | UV-HPLC |
| Ivermectin | Male Wistar rats | 0.5 mg/kg | Fluorescence-HPLC |
| Warfarin | New Zealand rabbit | 1 mg/kg | UV-HPLC |

Table 13: In vivo experiments summary.

3.2.10.2 Wistar rats experiments: Ivermectin.

Ivermectin was administered orally as nanosuspension in rats at a dose of 200 µg/kg of body weight. Rats were divided in groups of 2-3 animals. Experience was repeated 2-3 times to obtain at least n=6. In case of rats it is not possible to take all blood samples from the same animal. Thus, animals were separated in two main groups as reported in table 15.

| Formulation (dose 200µg/kg) | | Number of animals | Sampling times | | Number of animals | Sampling times |
|--|----------------|----------------------|--------------------|----------------|----------------------|--------------------|
| IVOMECE [®] | | 2 | | | 2 | |
| IVM solution | Group A | 2 | 30 minutes | Group B | 2 | 1 hour |
| IVM-Eudragit [®] NE 30D nanosuspension | | 3 | 2 hours 6 hours | | 3 | 4 hours 8 hours |
| IVM-Eudragit [®] RL 30D nanosuspension | | 3 | 24 hours | | 3 | 48 hours |

Table 14: Example of sampling times during rat experiments.

Drug solutions and drug loaded nanosuspensions were administered orally to rats by gavage with the use of a steel cannula.



Figure 21: Left image shows the cannula used to oral administration. Right image shows the syringe after drug administration.

At selected times, 500 µl of blood were withdrawn from tail vein into vials containing citrate buffer as anticoagulant. Vials were gently homogenised to avoid blood clotting and then centrifuged 15 minutes at 5000 rpm (MiniSpin Plus, Eppendorff, Ville, Pays). Clear plasma was recovered and stored at -20°C until HPLC analysis.

- **HPLC analysis of ivermectin in plasma**

Ivermectin was assayed by HPLC with a fluorescence detector. IVM was first extracted from plasma and then derivatized to obtain a fluorescent compound. The assay method was first described by Camargo (Camargo 2010).

- **IVM extraction from plasma**

100 μ l of plasma were mixed with 500 μ l of methanol (HPLC grade) which was stored at -30°C. Mixture was vortexed during 1 minute and then maintained at -30°C during 15 minutes to precipitate proteins. The organic phase was separated by centrifugation (16,000 g during 15 minutes at 4°C).

The supernatant was recovered and evaporated at 45 \pm 5°C under nitrogen flux. Evaporation residues were redissolved by vortexing 2 minutes with 150 μ l of a blend (1:1, V/V) of N-methylimidazole and acetonitrile. The obtained solution was analysed by HPLC.

- **HPLC analysis**

HPLC analysis was performed in a HPLC (Shimadzu HPLC 10A VP, Japan) in reversed phase with fluorimetric detection.

Ivermectin is not a fluorescent compound. In presence of trifluoroacetic anhydride and N-methylimidazole (NMI) ivermectin is transformed in a fluorescent compound (according to the following reaction) which is analysed by HPLC.

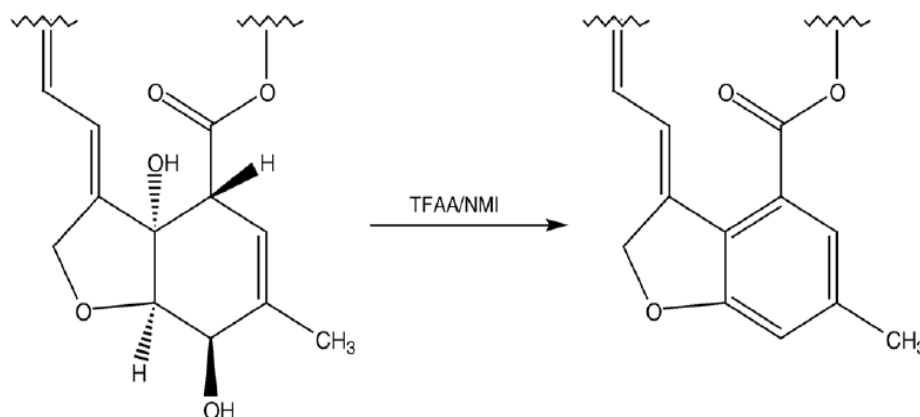


Figure 22: Derivatization reaction of ivermectin.

50 μ l of the previous solution was mixed automatically with 100 μ l of a blend (1:2, v/v) of trifluoroacetic anhydride and acetonitrile. After two minutes of reaction, 80 μ l were injected in the system. A column Uptisphere[®] (120 Å, ODB-C18, 5 μ m, 3x150 mm, Interchim, Montluçon, France) was used. Mobile phase was composed of Acetonitrile, methanol and an aqueous solution of acetic acid (0.2% V/V) in proportions 52:45:3. Flow was fixed to 1.5 ml/min and temperature was set to 37°C. Detection was performed fluorometrically at $\lambda_{exc}=365$ and $\lambda_{em}=475$ nm. Calibration curve was linear between 2-100 ng/ml and recovery is 85.5 ± 10 %.

3.2.11 New Zealand rabbits experiences

Nanosuspensions loaded with two different drugs (Celecoxib and Warfarin) were administered to rabbits.

- **Celecoxib**

Celecoxib (solution or loaded nanosuspension) was administered orally at a dose of 2 mg/kg of body weight. Administration was performed with the help of a plastic cannula. Groups of 3-4 animals were used for each condition.

At selected times (30 min, 1, 2, 4, 6, 8, 10, 24, 48 hours), a sample of 1.5 ml of blood was withdrawn from the marginal ear vein. Samples were recovered in vials containing citrate buffer as anticoagulant. Vials were gently homogenized and centrifuged during 15 minutes at 5,000 rpm. Clear plasma was recovered and stored at -20°C prior to analysis. Celecoxib was analyzed by HPLC after drug extraction from plasma. Assay method was adapted from (Homar, Ubrich et al. 2007).

Celecoxib extraction from plasma

200 µl of rabbit plasma were added to 200 µl of acetonitrile. Mixture was vortexed during 2 minutes and then centrifuged during 15 minutes at 16,000 g at 4°C. Supernatant was recovered and 30 µl was injected into HPLC system.

HPLC plasma assay for celecoxib

The HPLC system was a Shimadzu Prominence LC-20AD (Shimadzu, Japan). Celecoxib was assayed in reversed phase using a UV-detector.

A column Nucleodur C18 HTec, 5µm of particle size, 150 x 4.6 (Macherey Nagel, France) was used. Mobile phase was composed of an ammonium acetate 10⁻² M buffer pH 5 and acetonitrile (50:50, V/V). Flow was fixed to 1 ml/min and temperature was set to 40 °C. Detection was performed at $\lambda = 255$ nm. Calibration curve was linear between 40-400 ng/ml and recovery is 87.5 ± 10 %.

- **Warfarin**

Warfarin (solution or loaded nanosuspension) was administered orally at a dose of 100 µg/kg of body weight. Administration was performed by the help of a plastic cannula. Groups of 3-4 animals were used for each condition.

At selected times (30 min, 1, 2, 3, 4, 6, 8, 10, 24, 48 hours), a sample of 1.5 ml of blood was withdrawn from the marginal ear vein. Samples were recovered in vials containing citrate buffer as anticoagulant. Vials were gently homogenized and centrifuged during 15 minutes at 5,000 rpm. Clear plasma was recovered and stored at -20°C prior to analysis. Warfarin was analyzed by HPLC after drug extraction from plasma. Assay method was adapted from Sun et al (Sun, Wang et al. 2006).

Warfarin extraction from plasma

300 µL of blood plasma were added to 150 µL of hydrochloric acid 1M to convert warfarin in its neutral form. Afterwards, 3 mL of dichloromethane: n-hexane (9:1) were added to extract warfarin. The samples were vortexed for 60 seconds and centrifuged for 10 min at 3000 g. A volume of 2.5 mL of the organic layer was transferred into a glass tube and evaporated in a water bath at 55°C under nitrogen flux. The remainder was reconstituted with 100 µL of mobile phase and 20 µL of it were analyzed via HPLC.

HPLC plasma assay for warfarin

The analysis was carried out on a HPLC system LC-20 AD (Schimadzu, Japan). Separation was performed on a RP-18 column (Zorbax SB-C18, 5 µm particle size, 250 x 4.6 mm, Interchim, Montluçon, France) at 40 °C. The mobile phase consisting of ammonium acetate buffer 50 10⁻²M, pH 3.74 and methanol (25:75, V/V) was applied at a flow rate of 0.6 mL/min. Detection was recorded uv-spectrometrically (SPD-20 A, Schimadzu, Japan) at 308 nm. The warfarin calibration curve was linear from 40-300 ng/ml.

4. Results and discussion

Lipophilic drugs corresponding to BCS class II and IV were incorporated into aqueous polymer nanosuspensions. These drugs are poorly water soluble and their incorporation into nanoparticles may increase their solubility. A deep characterization of the different formulations was performed with the aim to developing a new oral dosage form. This study was designed with the aim to use pharmacopeia study methods to develop an industrialisable dosage form.

4.1 Study of the compatibility between drug and polymer

4.1.1 Drug incorporation into nanoparticles

The drugs selected for this study were poorly water soluble drugs with the exception of Diclofenac. Diclofenac is available as a sodium salt which increases its aqueous solubility. In the present study, the amount of drug incorporated into nanoparticles was chosen based on oral solid dosage forms used in clinics and which are reminded in the next table:

| Drug | Drug amount in commercially oral dosage forms (mg) | Drug incorporated (mg) | Aqueous dispersion volume (mL) |
|-------------------|---|------------------------|--------------------------------|
| Celecoxib | 50, 100, 200, 400 mg | 100 | 15 |
| Sodium diclofenac | 50, 75, 100 mg | 50 | 15 |
| Econazole nitrate | No oral form available Topical administration (1%) | 45 | 15 |
| Ibuprofen | 200, 400, 600, 800 mg | 200 | 15 |
| Ivermectin | 200 µg/kg | 14 | 15 |
| Warfarin | 1, 2, 2.5, 3, 4, 5, 6, 10 mg | 5 | 30 |

Table 15: Drug amount incorporated into polymeric nanoparticles.

Selected amount of drug was added to polymeric nanosuspension and stirred during different times until drug dissolution (in the limit of 7 days). Drug dissolution into polymeric nanoparticles was followed by observation of nanosuspensions under polarized light microscopy.

4.1.2 Observations with polarized light microscopy

Polarized light microscopy is a well known technique used to observe crystallization process mainly for polymer crystallization (Laihonen, Gedde et al. 1997; Krumme 2004). Moreover this technique has been used to study drug crystals evolution (Morgen, Bloom et al. 2012).

Crystals behaviour as well as drug miscibility with additives has been studied by other techniques such as DSC, wide angle X-ray scattering, TEM... (Laihonen, Gedde et al. 1997; Chawla and Bansal 2008).

We have selected the polarised light microscopy to observe the drug crystals evolution in the nanosuspension system because it is a simple and rapid method. The high number of combinations between drug and polymer necessitates a method which could provide a great number of results in a short time.

Depending on drug and polymer characteristics, it is possible to evaluate the drug dissolution or not, and also the possible drug-polymer incompatibility.

Immediately after drug addition into the nanoparticles suspension, a lot of crystals were observed. Then, drug crystals disappear as a function of time as shown in the next figure:

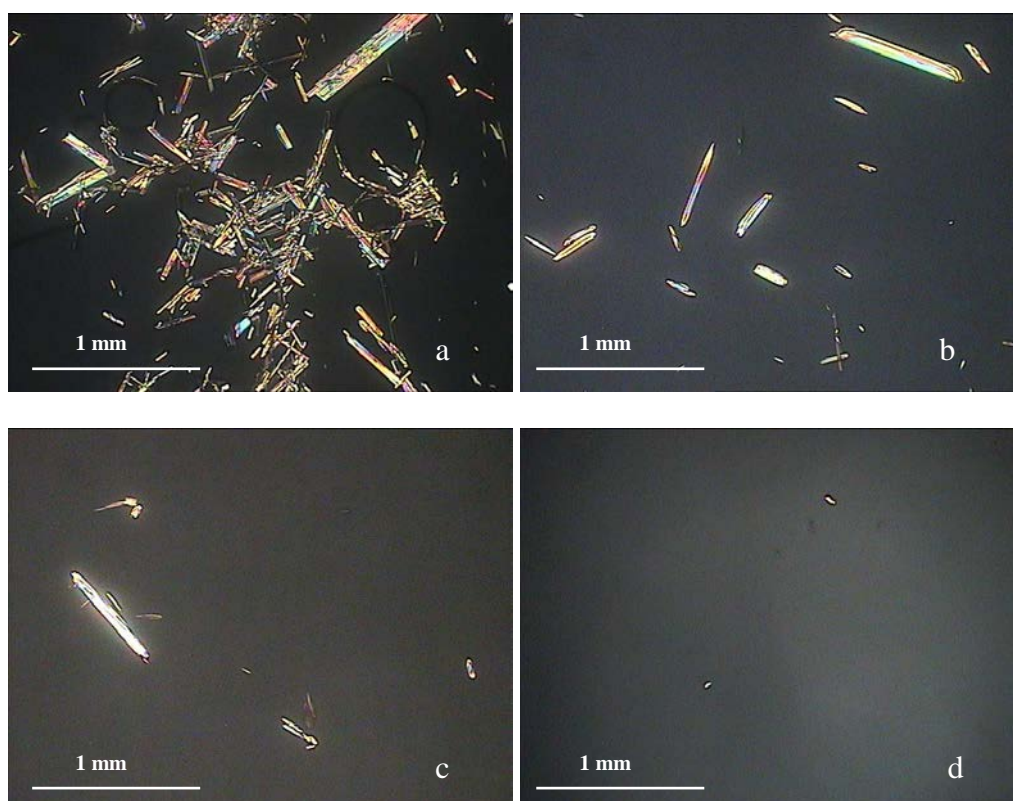


Figure 23: Incorporation of Celecoxib into Eudragit® NE 30D as a function of time: a) 5 minutes, b) 1h, c) 2h, d) 5h. Observations performed with amplification x10.

Five minutes after the drug addition to the nanosuspensions, a high amount of celecoxib crystals can still be observed (a). One hour of stirring lead to an important reduction of crystals amount (b). Celecoxib is rapidly solubilised in the polymeric phase (c and d).

The same observations were performed for all selected drugs in presence of different polymers. These observations are reported in Appendix A.

Drug-polymer incompatibility can also be observed with polarised light microscopy. In this case, polymer aggregates can be observed as dark shapes. Drug crystals can be entrapped into polymer aggregates as observed in figure 24, picture a, which shows diclofenac crystals entrapped into Eudragit® FS aggregates. Regardless polymer aggregation upon drug addition, it can be solubilised as observed in figure 24, picture b for diclofenac included in Eudragit® NE.

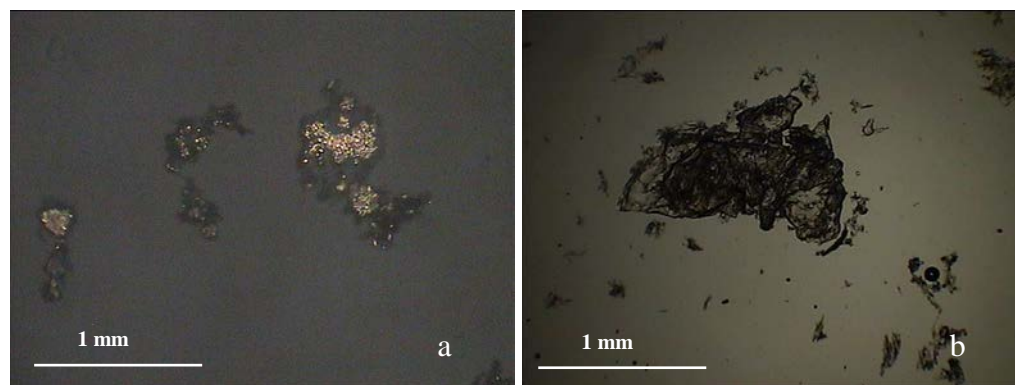


Figure 24: Polymer aggregates due to a drug-polymer incompatibility. a) Sodium diclofenac loaded into Eudragit® FS 30D and b) Sodium diclofenac loaded into Eudragit® NE 30D nanoparticles.

Results of observations obtained for the different drugs are described in the following section. Experiments were performed at two different temperatures (20 and 40°C). The first temperature was selected as room temperature. It was decided to increase the manufacturing temperature (40°C) in order to study the potential solubility enhancement due to temperature. Indeed, it was demonstrate that solubility may be dependent on temperature (Nordström and Rasmuson 2009). This temperature was selected to ensure the stability of both drugs and polymers

4.1.2.1 Celecoxib

The next table shows the results obtained after celecoxib (100mg in 15ml of nanosuspension) incorporation into the different polymeric nanosuspensions.

| Drug | Water solubility ($\mu\text{g/mL}$) | Polymer | Incorporation time | | Observations |
|-----------|---------------------------------------|------------------------------------|--------------------|--------|--|
| | | | 20°C | 40°C | |
| Celecoxib | 1 $\mu\text{g/ml}$ | Aquacoat [®] ECD | 48h | 48h | Dissolved |
| | | Eudragit [®] FS 30D | - | - | Polymer agglomerated |
| | | Eudragit [®] L 30D-55 | - | - | Not dissolved after 1 week of stirring |
| | | Eudragit [®] NE 30D | 6h | 6h | Dissolved |
| | | Eudragit [®] RL 30D | 6 days | 6 days | Dissolved |
| | | Eudragit [®] RS 30D | - | - | Not dissolved after 1 week of stirring |
| | | Kollicoat [®] MAE 30DP | - | - | Not dissolved after 1 week of stirring |
| | | Kollicoat [®] SR 30D | 6 days | 6 days | Dissolved |

Table 16: Incorporation times into polymeric nanoparticles for celecoxib.

In case of celecoxib, dissolution times are very long. It was noticed that the increasing temperature does not enhance the dissolution rate. Celecoxib is mainly solubilised into neutral polymer (Aquacoat[®] ECD, Eudragit[®] NE 30D and Kollicoat[®] SR 30D). In case of anionic polymers, the interaction between Celecoxib with Eudragit[®] FS 30D induces a polymer agglomeration immediately after drug addition. Celecoxib is not solubilised in Eudragit[®] L and Kollicoat[®] MAE after one week of stirring. Moreover, the two cationic polymers show a different behaviour after drug addition. Indeed, celecoxib is solubilised into Eudragit[®] RL but not into Eudragit[®] RS nanosuspension during the time of study. It should be noticed that celecoxib is solubilised into Eudragit[®] RS nanoparticles after 9 days of stirring. These two

polymers present the same chemical structure (copolymers of ethyl acrylate, methyl methacrylate containing quaternary ammonium groups). Quaternary ammonium groups which are present in a higher proportion in Eudragit[®] RL comparing to Eudragit[®] RS (10-12% and 4-6% respectively) is the only difference between these two polymers as reported in table 7.

4.1.2.2 Sodium diclofenac

The next table shows the behaviour of sodium diclofenac (50 mg in 15 ml of nanosuspension) after its addition to polymer nanosuspension.

| Drug | Water solubility (µg/mL) | Polymer | Incorporation time | | Observations |
|----------------------|-----------------------------|------------------------------------|--------------------|------------|--------------------------|
| | | | 20°C | 40°C | |
| Sodium diclofenac | | Aquacoat [®] ECD | 30 minutes | 30 minutes | Dissolved |
| | | Eudragit [®] FS 30D | - | - | Polymer agglomeration |
| | | Eudragit [®] L 30D-55 | - | - | Polymer agglomeration |
| | | Eudragit [®] NE 30D | 60 minutes | 60 minutes | Dissolved |
| | | Eudragit [®] RL 30D | - | - | Polymer agglomeration |
| | | Eudragit [®] RS 30D | - | - | Polymer agglomeration |
| | | Kollicoat [®] MAE 30DP | - | - | Polymer agglomeration |
| | | Kollicoat [®] SR 30D | 30 minutes | 30 minutes | Dissolved |

Table 17: Sodium diclofenac incorporated into different commercial nanosuspensions.

Sodium diclofenac is only solubilised into neutral polymers (Aquacoat[®] ECD, Eudragit[®] NE and Kollicoat[®] SR). In all cases, dissolution is very rapid (30 to 60 minutes). In case of charged polymers, both cationic and anionic, drug addition induces immediately polymer aggregation.

Sodium diclofenac presents quite solubility in water due to the sodium salt. In these conditions, it is not surprisingly that it is incompatible with most of polymers. It is only

solubilised into neutral polymers. Moreover, experiences performed at higher temperature (40°C) did not show differences in the dissolution rate or extent.

Sodium diclofenac entrapped or adsorbed in iron/ethylcellulose nanoparticles were prepared by an emulsion solvent evaporation method. It was noticed that the presence of surfactants such as sodium dodecyl sulphate contributes to create spaces within polymer network in which drug could be included (Arias, Lopez-Viota et al. 2009). Aquacoat[®] ECD, is composed of ethylcellulose nanoparticles and stabilised by sodium lauryl sulphate, as well as Kollicoat[®] SR. This composition can explain the rapid incorporation of the drug into particles according to Arias et al. (2009).

It was observed that nanosuspensions could be prepared with sodium diclofenac and biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) by the emulsion method. The ionization of carboxylic groups existing in polymer structure conduces to a polymer negative charge. Despite this negative charge, drug-polymer incompatibility was no observed, on the contrary to our observations in which the drug addition to anionic nanosuspensions induce polymer aggregation (Agnihotri and Vavia 2009).

4.1.2.3 Econazole nitrate

Econazole nitrate (45 mg of econazole nitrate into 15 ml of nanosuspension) was added to polymeric nanosuspensions. Drug dissolution into polymeric nanoparticles is reported in table 18.

| Drug | Water solubility ($\mu\text{g/mL}$) | Polymer | Incorporation time | | Observations |
|-------------------|---------------------------------------|---------------------------------|--------------------|------|-----------------------|
| | | | 20°C | 40°C | |
| Econazole Nitrate | 400 $\mu\text{g/ml}$ | Aquacoat [®] ECD | - | - | Polymer agglomeration |
| | | Eudragit [®] FS 30D | - | - | Polymer agglomeration |
| | | Eudragit [®] L 30D-55 | - | - | Polymer agglomeration |
| | | Eudragit [®] NE 30D | 2h | 2h | Dissolved |
| | | Eudragit [®] RL 30D | 2h | 2h | Dissolved |
| | | Eudragit [®] RS 30D | 2h | 2h | Dissolved |
| | | Kollicoat [®] MAE 30DP | - | - | Polymer agglomeration |
| | | Kollicoat [®] SR 30D | - | - | Polymer agglomeration |

Table 18: Econazole nitrate added to polymeric nanosuspensions.

Econazole nitrate is solubilised in 2h in acrylic polymers but only in cationic (Eudragit[®] RL and RS) and neutral (Eudragit[®] NE) ones. The addition of the drug into anionic acrylic polymers (Eudragit[®] FS, Eudragit[®] L, Kollicoat[®] MAE), neutral ethylcellulose (Aquacoat[®] ECD) and neutral vinylic polymer (Kollicoat[®] SR) induces its aggregation.

Econazole drug delivery is mainly developed for cutaneous application and vaginal administration. To increase drug bioavailability, econazole was formulated into microspheres (Albertini, Passerini et al. 2009), ethosomes (Verma and Pathak 2011), cyclodextrin complexes (Pedersen, Edelsten et al. 1993; Pedersen, Bjerregaard et al. 1998; Jacobsen,

Bjerregaard et al. 1999), emulsions (Youenang Piemi, Korner et al. 1999), and different types of nanoparticles (Ahmad, Pandey et al. 2008; Yordanov 2012).

Positively and negatively charged submicron emulsions of econazole were prepared by Youenang Pieni et al. (1999). The aim of these preparations was to increase econazole permeability through the skin. They have demonstrated that positively charged emulsions prepared with stearylamine were more effective in terms of topical penetration. They have suggested that the steric repulsion of charges present in stearylamine contributes to stabilize the colloidal system. The steric repulsion, which stabilizes the quaternary ammonium groups existing in Eudragit RS and RL may explain the easy incorporation of econazole into the nanoparticles as well as its good stability (Youenang Piemi, Korner et al. 1999).

4.1.2.4 Ibuprofen

The time needed to solubilise ibuprofen (200 mg of ibuprofen into 15 ml of nanosuspension) into the various polymer nanosuspensions is reported in table 19.

| Drug | Water solubility ($\mu\text{g/mL}$) | Polymer | Incorporation time | | Observations |
|-----------|---------------------------------------|---------------------------------|--------------------|------|---|
| | | | 20°C | 40°C | |
| Ibuprofen | 45 $\mu\text{g/ml}$ | Aquacoat [®] ECD | 24h | 24h | Dissolved |
| | | Eudragit [®] FS 30D | 4h | 4h | Polymer agglomeration |
| | | Eudragit [®] L 30D-55 | 24h | 24h | Dissolved but drug precipitation at 48h |
| | | Eudragit [®] NE 30D | 2h | 2h | Dissolved |
| | | Eudragit [®] RL 30D | 5h | 5h | Dissolved |
| | | Eudragit [®] RS 30D | 5h | 5h | Dissolved |
| | | Kollicoat [®] MAE 30DP | 24h | 24h | Dissolved but drug precipitation at 48h |
| | | Kollicoat [®] SR 30D | 1h | 1h | Dissolved |

Table 19: Incorporation time of ibuprofen into polymer nanosuspensions.

Ibuprofen is rapidly solubilised into all polymers. In case of Eudragit[®] FS 30 D, polymer aggregation occurs after drug addition but drug is completely solubilized. In other anionic polymers, two phenomena were observed: In a first time, drug is completely solubilized into Eudragit[®] L and Kollicoat[®] MAE but drug reprecipitation is observed two days after manufacturing.

Ibuprofen is a classical lipophilic model drug which has been encapsulated in a large number of drug delivery systems to increase its solubility and bioavailability. Ibuprofen can be incorporated in cyclodextrins (Hergert and Escandar 2003; Al Omari, Daraghmeh et al. 2009) The amphiphilic behaviour described by Khan et al., 2011 of ibuprofen (weak acid) could explain its favourable interaction with neutral and cationic polymers. Ibuprofen interaction with anionic polymers is weak, due to an anionic-anionic repulsion between drug and polymer in the same way than surfactants (Khan, Anjum et al. 2011). Authors have suggested that hydrophobic and electrostatic forces play a role in the interaction between polymers and amphiphilic drugs such as ibuprofen or others non steroidal antiinflammatory drugs. Moreover increasing manufacturing temperature does not modify the solubilsation profile.

4.1.2.5 Ivermectin

Ivermectin (14 mg of ivermectin into 15 ml of nanosuspension) was added to different polymer nanosuspensions. Next table reports the incorporation times needed to solubilise the drug into the polymer nanoparticles.

| Drug | Water solubility ($\mu\text{g/mL}$) | Polymer | Incorporation time | | Observations |
|------------|--|------------------------------------|-----------------------|--------|---|
| | | | 20°C | 40°C | |
| Ivermectin | 7 | Aquacoat [®] ECD | 24h | 24h | Dissolved |
| | | Eudragit [®] FS 30D | - | - | Polymer agglomeration |
| | | Eudragit [®] L 30D- 55 | 6h | 6h | Dissolved |
| | | Eudragit [®] NE 30D | 6h | 6h | Dissolved |
| | | Eudragit [®] RL 30D | 6 days | 6 days | Dissolved |
| | | Eudragit [®] RS 30D | - | - | Not dissolved after 1 week of stirring |
| | | Kollicoat [®] MAE 30DP | 6h | 6h | Dissolved |
| | | Kollicoat [®] SR 30D | 4h | 4h | Dissolved |

Table 20: Incorporation times of ivermectin into polymer nanosuspensions.

Ivermectin is dissolved into all polymer nanosuspensions except Eudragit[®] FS where drug addition induces polymer aggregation. In the other cases, drug is solubilised very rapidly, between 4 and 24 h. After addition to Eudragit[®] RL and Eudragit[®] RS suspensions, ibuprofen is solubilised after one week or 9 days of stirring respectively.

4.1.2.6 Warfarin

Warfarin (5mg of warfarine in 15 ml of nanosuspension) was added to polymeric nanosuspensions. The time needed to solubilise the drug is reported in the next table:

| Drug | Water solubility ($\mu\text{g/mL}$) | Polymer | Incorporation time | | Observations |
|----------|---------------------------------------|---------------------------------|--------------------|------|-----------------------|
| | | | 20°C | 40°C | |
| Warfarin | 17 | Aquacoat [®] ECD | 24h | 24h | Dissolved |
| | | Eudragit [®] FS 30D | - | - | Polymer agglomeration |
| | | Eudragit [®] L 30D-55 | 24h | 24h | Dissolved |
| | | Eudragit [®] NE 30D | 24h | 24h | Dissolved |
| | | Eudragit [®] RL 30D | 3h | 3h | Dissolved |
| | | Eudragit [®] RS 30D | 3h | 3h | Dissolved |
| | | Kollicoat [®] MAE 30DP | 48h | 48h | Dissolved |
| | | Kollicoat [®] SR 30D | 24h | 24h | Dissolved |

Table 21: Incorporation times of warfarin into polymer nanosuspensions.

Warfarin is dissolved in all polymer nanosuspensions with the exception of Eudragit[®] FS and Kollicoat[®] MAE (two anionic polymers). Surprisingly, drug is easily solubilised in Eudragit[®] L, which presents the same chemical structure than Kollicoat[®] MAE. Drug solubilisation is more rapid in presence of cationic polymers. Drug dissolution after addition into neutral polymeric nanosuspensions needs between 24-48h.

The solubility of warfarin was evaluated in acetone in presence of different methacrylic polymers: Eudragit[®] E (and Eudragit[®] RL both cationic polymers) and Eudragit[®] S, an anionic polymer currently used in colon delivery were tested (Lin, Cheng et al. 1994). It was observed that warfarin solubility increased in presence of polymer linearly with the polymer concentration. A higher solubility enhancement was reported in presence of cationic polymers

as we observed in our case. This behaviour suggests that cationic methacrylate copolymers easily interact with warfarin. The main interaction is probably due to hydrogen bonding but other weak intermolecular forces such as electrostatic, induction or London dispersion forces may contribute to complex formation.

Kotiyan et al. have observed that the increase in Eudragit[®] RL and RS amount in an estradiol patch allows the reduction of drug crystallization. The incorporation of Eudragit[®] RL and Eudragit[®] RS prevent drug crystallization probably by the weak association of the proton of quaternary amine with the hydroxyl group of the drug (Kotiyan and Vavia 2001). The presence of hydroxyl groups in the studied drugs may explain the solubilisation of most of them in Eudragit[®] RS-Eudragit[®] RL polymer nanosuspension.

Kollicoat[®] MAE 30DP is reported to be a more hydrophilic polymer than ethylcellulose. It is an enteric polymer, which entraps water during films formation (Kranz and Gutsche 2009). Its hydrophilic character can explain the incompatibility with lipophilic drugs, as observed in case of studied drugs. Indeed, when blended with Kollicoat[®] MAE (and Eudragit[®] L, which presents the same structure), most of drugs induce a polymer agglomeration.

As observed in the tables above, general characteristics can be reported:

Celecoxib and sodium diclofenac are mainly solubilised into neutral polymers.

Econazole nitrate, ibuprofen and warfarin solubilises easily in acrylic neutral and cationic polymers.

Ivermectin is principally dissolved into anionic and neutral polymers.

The next table shows the solubility enhancement of each tested drug after its addition into nanosuspensions.

| Drug | Aqueous solubility | Polymer | Solubility in suspension (mg/ml) | Increase of solubility |
|-------------------|--------------------|---|----------------------------------|------------------------|
| Celecoxib | 1 µg/ml | Aquacoat [®] ECD Eudragit [®] NE 30D Kollicoat [®] SR 30D | 6.6 | x 6600 |
| Sodium diclofenac | 9.5 mg/ml | Aquacoat [®] ECD Eudragit [®] NE 30D Kollicoat [®] SR 30D | 3.3 | No increased |
| Econazole nitrate | 400 µg/ml | Eudragit [®] NE 30D Eudragit [®] RL 30D Eudragit [®] RS 30D | 3 | x 7.5 |
| Ibuprofen | 45µg/ml | Aquacoat [®] ECD Eudragit [®] NE 30D Eudragit [®] RL 30D Eudragit [®] RS 30D Kollicoat [®] SR 30D | 13.3 | x 300 |
| Ivermectin | 7µg/ml | Aquacoat [®] ECD Eudragit [®] L 30D-55 Eudragit [®] NE 30D Kollicoat [®] MAE 30DP Kollicoat [®] SR 30D | 0.93 | x 130 |
| Warfarin | 17 µg/ml | Aquacoat [®] ECD Eudragit [®] NE 30D Eudragit [®] RL 30D Eudragit [®] RS 30D Kollicoat [®] SR 30D | 0.33 | x 20 |

Table 22: Drug solubility enhancement after its incorporation into polymeric nanosuspensions.

Except Sodium diclofenac, which is already soluble in aqueous media, due to the sodium salt, other drugs increase its solubility between 7.5 to 6600 times. These results demonstrate that our system is able to enhance the aqueous solubility of lipophilic drugs.

To increase drug bioavailability, one of the methods is to increase its solubility. Preparation of Gelucire[®] microparticles by spray congealing process allow increasing 15 times the econazole solubility comparing to the free drug. (Albertini, Passerini et al. 2009). In our case, the solubility increase is about 7.5 times. As observed by Albertini, the presence of polymers such as chitosan or sodium carboxymethylcellulose decreases the solubility enhancement rate.

They have suggested that drug wettability is decreased and in consequence its solubility is decreased too.

4.1.3 Hydrodynamic diameter measurements

In this part, the hydrodynamic diameter measurements were evaluated with the aim to verifying if drug inclusion modify particles' diameter. The next table reports the size measurements obtained for the commercial nanoparticles.

| | Empty NP | Drugs | | | | | |
|---------------------------------------|-----------|-----------|-------------------|-------------------|-----------|------------|-----------|
| | | Celecoxib | Sodium Diclofenac | Econazole Nitrate | Ibuprofen | Ivermectin | Warfarin |
| Aquacoat[®] ECD | 207.3±6.9 | 194.5±4.0 | 212.9±2.2 | NP | 202.8±1.6 | 199.1±5.7 | 208.6±2.2 |
| Eudragit[®] FS 30D | ND | NP | NP | NP | NP | NP | NP |
| Eudragit[®] L30D-55 | 112.5±1.2 | NP | NP | NP | ND | 115.2±1.9 | 115.5±3.1 |
| Eudragit[®] NE 30D | 141.8±1.0 | 152.4±4.5 | 148.9±1.0 | 145.2±4.5 | 145.5±1.0 | 146.0±1.9 | 146.9±1.9 |
| Eudragit[®] RL 30D | 160.0±6.2 | ND | NP | 154.1±9.5 | 152.3±5.4 | 169.5±3.7 | 127.6±0.5 |
| Eudragit[®] RS 30D | 130.1±3.2 | NP | NP | 143.4±1.8 | 128.7±6.2 | 136.5±2.9 | 127.6±0.5 |
| Kollicoat[®] MAE 30DP | 123.2±2.5 | NP | NP | NP | NP | NP | NP |
| Kollicat[®] SR 30D | 185.7±3.6 | 190.6±9.3 | NP | ND | 181.1±2.1 | 189.0±3.6 | 197.8±2.2 |

ND: not determined; NP: not possible

Table 23: Hydrodynamic diameter measurements of empty and drug loaded nanoparticles

Empty and drug-loaded nanosuspensions present a size comprised between 112 and 207 nm (Eudragit[®] L 30D-55 and Aquacoat[®] ECD respectively). It was observed for the whole particles that the drug addition into nanosuspensions did not increase particle diameter. The non modification of this parameter can be explained by the low proportion of drug added into the nanoparticles suspension. Indeed, drug proportion into nanosuspensions varies from 0.1 to 4% in case of warfarin and ibuprofen nanoparticles, respectively.

In literature, polymer manufactured nanoparticles characterization does not present the results of unloaded nanoparticles (Pandey, Ahmad et al. 2005). Indeed, there are only few studies which compare (loaded and unloaded) nanoparticles. Nevertheless, it was reported that drug incorporation does not increase significantly regardless the drug loading and manufacturing method (Leroueil-Le Verger, Fluckiger et al. 1998; Lamprecht, Ubrich et al. 1999; Damgé, Maincent et al. 2007).

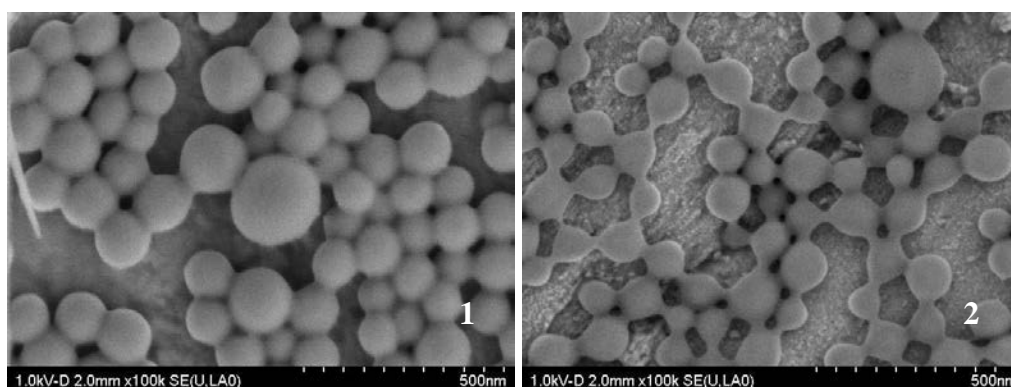
4.1.4 Scanning Electron Microscopy

Nanosuspensions were diluted in purified water. A drop of diluted suspension was allowed to dry and the obtained film was observed with SEM.

Three types of polymer nanosuspensions were observed:

- Eudragit[®] L 30D-55 and the ibuprofen loaded nanosuspension.
- Eudragit[®] NE 30D and the ibuprofen and warfarin loaded nanosuspensions
- Kollicoat[®] SR 30D and the ibuprofen loaded nanosuspension.

Different observations are reported in the next pictures:



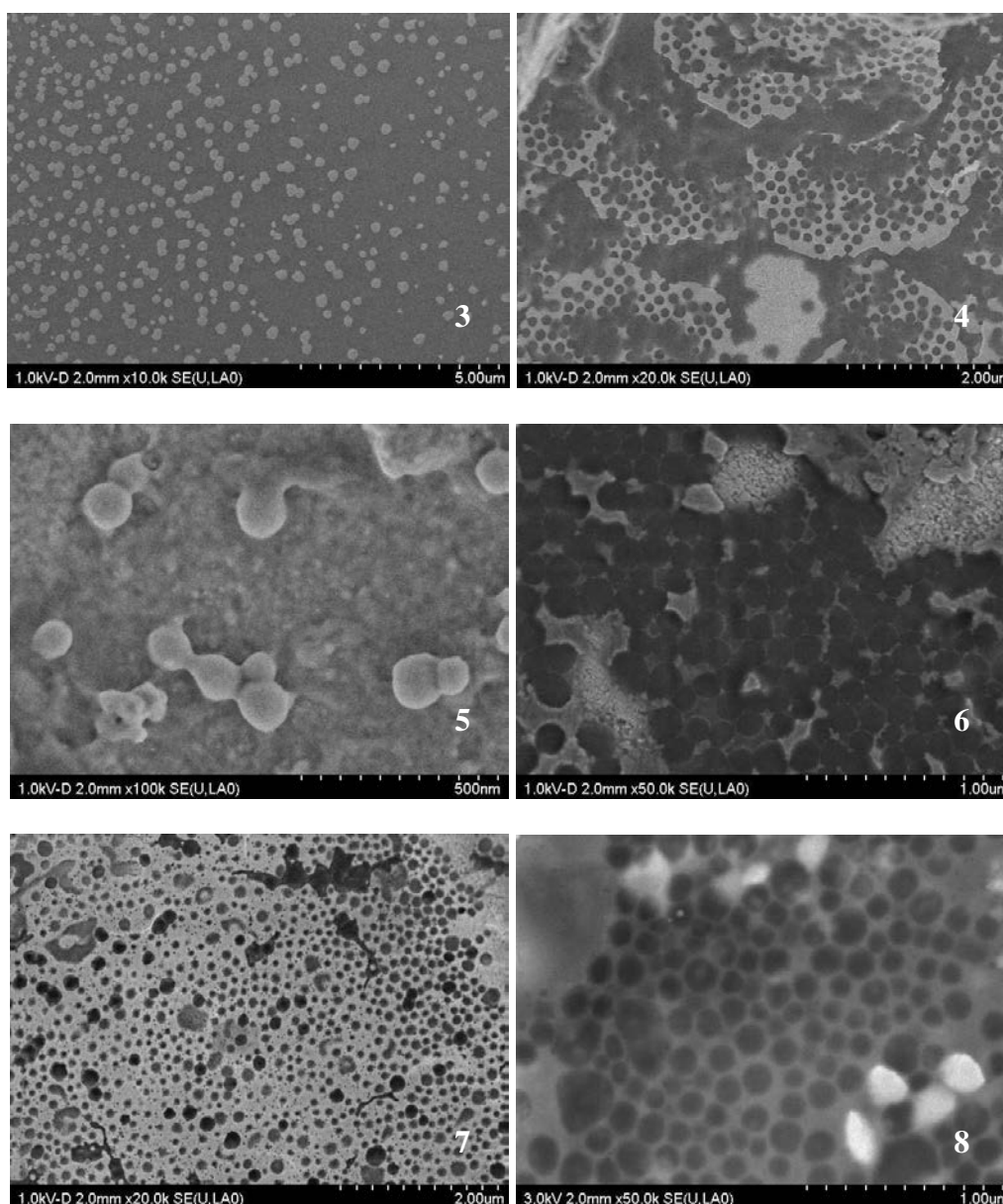


Figure 25: SEM observations of different polymer nanosuspensions: Empty Eudragit[®] L 30D-55 (1), Ibuprofen-loaded into Eudragit[®] L 30D-55 (2), empty Eudragit[®] NE 30D (3), Warfarin loaded into Eudragit[®] NE 30D (4), Ibuprofen-loaded into Eudragit[®] NE 30 D (5-6), empty Kollicoat[®] SR 30D (7) and ibuprofen-loaded into Kollicoat[®] SR 30D (8).

Nanoparticles (loaded with ibuprofen or not) are only observed in the case of Eudragit[®] L 30D-55. The nanoparticles present a size of 110 nm, as also determined by dynamic light scattering. Nanoparticles are stucked together and it was noticed during the observations that these nanoparticles melt when submitted to microscope beam to form a homogeneous film.

In case of empty Eudragit[®] NE suspension, a non homogeneous film was observed as well as the presence of small spherical particules. As for Eudragit[®] NE loaded with warfarin was no

nanoparticles were observed. A non homogeneous film containing holes of nanoparticle size was observed. Film seems to be broken in different layers. In case of Eudragit[®] NE loaded with ibuprofen two types of observations are reported: spherical nanoparticles which a mean size of 100 nm and on the other hand, a film containing holes. The distribution of nanoparticles and holes are not homogeneous.

In case of Kollicoat[®] SR nanoparticles, the same type of non-homogeneous film containing holes was observed in empty and ibuprofen loaded nanosuspensions.

4.1.5 Zeta potential measurements

As well as hydrodynamic diameter measurements, the zeta potential of nanosuspensions was measured. This characterization is necessary to determine the risk of nanosuspension instability if loaded nanoparticles get neutrally charged. The obtained results are reported in the next table:

| | Polymers | Drugs | | | | | | |
|--|---------------------------------|-----------|-----------|-------------------|-------------------|-----------|------------|-----------|
| | | Empty NP | Celecoxib | Sodium Diclofenac | Econazole Nitrate | Ibuprofen | Ivermectin | Warfarin |
| | Aquacoat [®] ECD | -48.3±2.6 | -49.7±1.3 | -46.1±0.6 | NP | -50.0±0.6 | -48.7±0.5 | -46.8±0.2 |
| | Eudragit [®] FS 30D | ND | NP | NP | NP | NP | NP | NP |
| | Eudragit [®] L30D-55 | -55.2±1.3 | NP | NP | NP | -59.2±0.8 | -58.0±0.3 | NP |
| | Eudragit [®] NE 30D | -55.9±0.7 | -44.2±2.8 | -50.5±3.1 | -50.3±0.5 | -49.1±0.2 | -44.3±4.1 | -41.1±1.5 |
| | Eudragit [®] RL 30D | 55.6±2.0 | NP | NP | 52.9±0.9 | ND | 52.6±0.7 | 55.6±1.3 |
| | Eudragit [®] RS 30D | 51.3±1.4 | NP | NP | 53.5±2.3 | 54.4±1.2 | 42.5±1.5 | 52.9±0.7 |
| | Kollicoat [®] MAE 30DP | NP | NP | NP | NP | ND | ND | ND |
| | Kollicat [®] SR 30D | -27.3±0.8 | -17.2±0.5 | NP | NP | -20.0±0.2 | -20.9±1.1 | -29.5±0.5 |

ND: not determined, NP: not possible.

Table 24: Zeta potential measurements of empty and drug loaded nanoparticles.

Aquacoat[®] ECD zeta potential is about -48 mV. However ethylcellulose is reported to be a neutral polymer. The negative zeta potential is explained by the polymer stabilizers adsorbed onto nanoparticles such as sodium lauryl sulphate which is an anionic surfactant. Drug-addition does not modify the zeta potential of the nanosuspension.

Eudragit[®] L 30D-55 presents a zeta potential of -55 mV. It is reported to be anionic and it is also stabilized by sodium lauryl sulphate, an anionic surfactant. Drug contained into nanoparticles does not modify the zeta potential of nanosuspension.

Eudragit[®] NE 30D is a neutral polymer stabilized with nonoxynol, a neutral surfactant. Measurements show a zeta potential of -56 mV. This result has also been reported by several authors (Schmid, Müller-Goymann et al. 2000).

Eudragit[®] RL and RS 30 D are cationic polymers due to the presence of quaternary ammonium groups. The zeta potential measured for the empty nanoparticles of these polymers are 55 and 51 mV respectively. Drug-loaded nanoparticles zeta potential is similar to unloaded nanoparticles, between 52 and 55 mV with the exception of Eudragit[®] RS 30D nanoparticles loaded with ivermectin which present a zeta potential of 42 mV.

The zeta potential of empty Kollicoat[®] SR 30D nanoparticles is -27mV. This polymer is reported to be neutral but it is stabilized with sodium lauryl sulphate, an anionic surfactant. Drug-loaded nanoparticles present a zeta potential ranging from -17 and -29 mV.

To obtain stable colloidal nanosuspensions, zeta potential should not be neutral i.e. not close to zero. It is considered that at least +/- 25 mV in Zeta potential measurements is necessary to obtain stable nanocolloids (Leroueil-Le Verger, Fluckiger et al. 1998) and the optimal conditions are reported to be +/- 60mV (Freitas and Müller 1998). In their experiments Freitas et al. prepared SLN dispersions containing Compritol stabilized with Poloxamer 188. The influence of light in formulation stability was studied by zeta potential measurements. After manufacturing, a zeta potential of -25 mV was reported. Depending on storage conditions

(artificial light, day light or darkness) the zeta potential decreases until -15mV, -19 mV and -18mV respectively after one week of storage leading to particles aggregation (Freitas and Müller 1998). Under -15 mV system coalescence was observed as also reported by (Müller and Heinemann 1994) for parenteral nutrition emulsions.

The next figure shows the zeta potential requirements to obtain stable colloids.

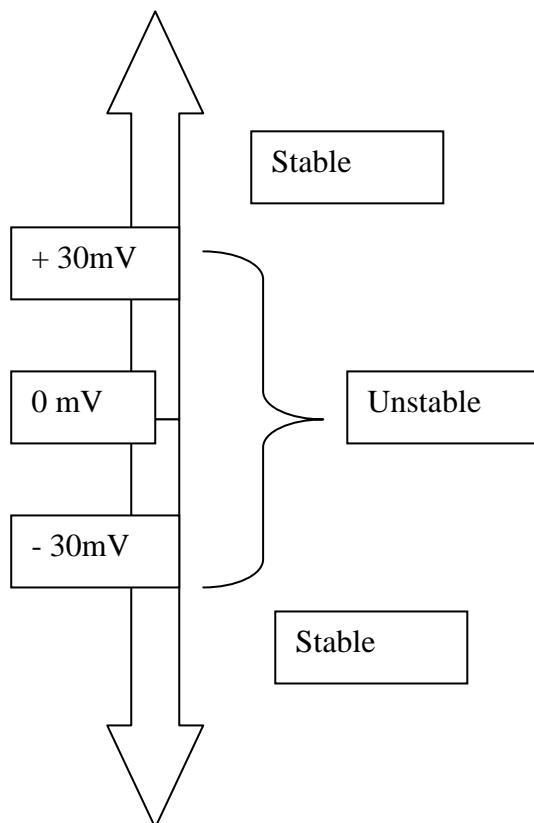


Figure 26: Potential range to obtain stable nanosuspensions.

The zeta potential of the majority of nanosuspensions is greater than -30 or + 30mV. For this reason nanoparticle stability is presumed. The obtained zeta potentials were according with the polymer structure. Indeed, cationic polymers such as Eudragit[®] RL and RS present a positive zeta potential. Moreover, the anionic polymers present a negative zeta potential. However, in case of neutral polymers, negative zeta potentials were also observed. This may be explained by the presence of anionic surfactants (Sodium lauryl sulphate) in case of

Aquacoat[®] ECD and Kollicoat[®] SR. Eudragit[®] NE, a neutral polymer which contains nonoxynol 100, a neutral surfactant also present a negative zeta potential. This behaviour has also been observed by (Schmidt and Bodmeier 1999). Thus, the nanosuspensions formed with Kollicoat[®] SR 30D shows a zeta potential below -30 mV. This zeta potential is reported to be the limit value of colloids suspension stability according to (Freitas and Müller 1998) and (Leroueil-Le Verger, Fluckiger et al. 1998). Nevertheless, Kollicoat[®] SR nanosuspensions remains stable during at least one year of storage.

4.1.6 Stability of drug loaded nanosuspensions.

Stability tests were performed at two different temperatures (+4 and +25°C). Drug recrystallization in the medium (observed under polarised microscopy) as well as the formation of polymer agglomerates was selected as the parameters indicating poor stability. It was considered that the drug recrystallizes after the observation of at least one crystal between 2 observations. In case of polymer aggregates, it was consider instability after the observation macroscopically of aggregates as well as the observation microscopically of at least one aggregate of few micrometers in two consecutive observations. In any case, a phase separation or polymer flocculation was observed.

The next figure shows the observations corresponding to celecoxib stability experiments:

| Drug | Polymer | Stability | | Observations |
|-----------|------------------------------------|-------------|-------------|--------------------------|
| | | 4°C | 25°C | |
| Celecoxib | Aquacoat [®] ECD | > 12 months | < 1 month | Polymer agglomeration |
| | Eudragit [®] FS 30D | - | - | |
| | Eudragit [®] L 30D-55 | - | - | |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | - | - | |
| | Eudragit [®] RS 30D | - | - | |
| | Kollocoat [®] MAE 30DP | - | - | |
| | Kollocoat [®] SR 30D | > 12 months | > 12 months | |

(-) *not performed.*

Table 25: Celecoxib loaded into nanosuspensions stability study.

The three studied nanosuspensions studied were stable during one year, except for celecoxib loaded into Aquacoat[®] ECD nanoparticles and stored at 25°C. In this case, polymer aggregates were observed.

The stability of polymer nanosuspensions loaded with sodium diclofenac was also studied.

The polarised light microscopy observations are reported in the next table:

| Drug | Polymer | Stability | | Observations |
|-------------------|------------------------------------|-------------|-------------|--------------------------|
| | | 4°C | 25°C | |
| Sodium diclofenac | Aquacoat [®] ECD | > 12 months | > 12 months | |
| | Eudragit [®] FS 30D | - | - | |
| | Eudragit [®] L 30D-55 | - | - | |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | - | - | |
| | Eudragit [®] RS 30D | - | - | |
| | Kollicoat [®] MAE 30DP | - | - | |
| | Kollicoat [®] SR 30D | < 1 month | < 1 month | Polymer agglomeration |

Table 26: Stability study of sodium diclofenac loaded into polymeric nanosuspensions.

Concerning sodium diclofenac, the drug was only included into neutral polymers. Aquacoat[®] and Eudragit[®] NE nanosuspensions are stable during at least one year. In case of Kollicoat[®] SR 30D, a rapid instability was observed. Indeed, polymer aggregates were observed after two weeks of storage at both temperatures, 4 and 25 °C.

The stability of polymer nanosuspensions loaded with econazole nitrate was studied with polarised light microscopy. The observations are reported in the next table:

| Drug | Polymer | Stability | | Observations |
|----------------------|------------------------------------|-------------|-------------|--------------|
| | | 4°C | 25°C | |
| Econazole nitrate | Aquacoat [®] ECD | > 12 months | > 12 months | |
| | Eudragit [®] FS 30D | - | - | |
| | Eudragit [®] L 30D-55 | - | - | |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | - | - | |
| | Eudragit [®] RS 30D | - | - | |
| | Kollicoat [®] MAE 30DP | - | - | |
| | Kollicoat [®] SR 30D | > 12 months | > 12 months | |

Tableau 27: Econazole nitrate loaded into polymer nanosuspensions stability study.

Econazole nitrate stability was studied with neutral nanosuspension. Instability was not observed in any formulation at both temperatures.

The following table displays the stability experiment results concerning ibuprofen loaded into polymeric nanoparticles.

| Drug | Polymer | Stability | | Observations |
|-----------|------------------------------------|-------------|-------------|---------------------------|
| | | 4°C | 25°C | |
| Ibuprofen | Aquacoat [®] ECD | > 12 months | > 12 months | |
| | Eudragit [®] FS 30D | < 1 month | < 1 month | Drug recrystallization |
| | Eudragit [®] L 30D-55 | < 1 month | < 1 month | Drug recrystallization |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | > 12 months | > 12 months | |
| | Eudragit [®] RS 30D | > 12 months | > 12 months | |
| | Kollicoat [®] MAE 30DP | < 1 month | < 1 month | Drug recrystallization |
| | Kollicoat [®] SR 30D | > 12 months | > 12 months | |

Table 28: Stability tests of ibuprofen loaded nanosuspensions.

It was observed that neutral and cationic polymers are more stable when compared to anionic polymers. Indeed, ibuprofen loaded into anionic polymers such as Eudragit[®] FS 30D, Eudragit[®] L 30D-55 and Kollicoat[®] SR 30D recrystallizes rapidly, a few days after manufacturing. Depending on the formulation, the recrystallization occurs from 2 to 7 days.

The potential recrystallization of ivermectin loaded into polymeric nanosuspensions was also studied.

The observations are reported in the table below:

| Drug | Polymer | Stability | | Observations |
|------------|------------------------------------|-------------|-------------|------------------------|
| | | 4°C | 25°C | |
| Ivermectin | Aquacoat [®] ECD | > 12 months | > 12 months | |
| | Eudragit [®] FS 30D | - | - | |
| | Eudragit [®] L 30D-55 | < 2 months | < 2 months | Polymer aggregation |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | > 12 months | > 12 months | |
| | Eudragit [®] RS 30D | > 12 months | > 12 months | |
| | Kollicoat [®] MAE 30DP | < 2 months | < 2 months | Polymer aggregation |
| | Kollicoat [®] SR 30D | > 12 months | > 12 months | |

Table 29: Ivermectin loaded into polymer nanosuspensions stability.

Nanosuspensions loaded with ivermectin present the same behaviour as ibuprofen. Neutral and cationic nanosuspensions are more stable than anionic nanoparticles. In this case, the instability of Eudragit[®] L 30D-55 is noticed by the observation of polymer aggregates.

The stability of nanosuspensions loaded with warfarin was also studied.

The obtained results are reported in the next table:

| Drug | Polymer | Stability | | Observations |
|----------|------------------------------------|-------------|-------------|------------------------|
| | | 4°C | 25°C | |
| Warfarin | Aquacoat [®] ECD | > 12 months | > 12 months | |
| | Eudragit [®] FS 30D | - | - | |
| | Eudragit [®] L 30D-55 | < 2 months | < 2 months | Polymer aggregation |
| | Eudragit [®] NE 30D | > 12 months | > 12 months | |
| | Eudragit [®] RL 30D | > 12 months | > 12 months | |
| | Eudragit [®] RS 30D | > 12 months | > 12 months | |
| | Kollicoat [®] MAE 30DP | < 2 months | < 2 months | Polymer aggregation |
| | Kollicoat [®] SR 30D | > 12 months | > 12 months | |

Table 30: Stability study of polymer nanosuspensions loaded with warfarin.

The nanosuspensions loaded with warfarin which are the most stable are the neutral and cationic ones. This behaviour has been also observed for the others drugs.

The presence of stabilizers in the nanosuspensions allows the physical stability of the colloids. Most of loaded nanosuspensions were stable for at least one year of storage.

Fungal proliferation was observed in some of formulations stored at 4°C. This proliferation was observed (regardless the drug) in Eudragit NE 30 D nanosuspensions which were not hermetically sealed. When this phenomenon is observed, nanosuspensions were discarded for the next observations.

Sodium lauryl sulphate crystallizes at low temperature. This surfactant is present in the composition of Eudragit[®] FS 30D (0.3%), Eudragit[®] L 30D-55 (0.7%), Kollicoat[®] MAE 30DP (0/7%) and Kollicoat[®] SR 30D (0.3%). This crystallization can participate to nanosuspension instability by different mechanisms: the diminution of surfactant

concentration into the colloidal system may decrease the drug solubility. Moreover, the thermodynamical stability of the colloidal system can be affected by this precipitation.

Anionic polymers (Eudragit[®] L 30D-55 and Kollicoat[®] MAE) present less stability. Both, drug recrystallization and polymer aggregates formation are observed.

Films prepared with Eudragit[®] NE 30D presents surfactant (nonoxynol 100) crystals after films storage (Lin, Muhammad et al. 2001). It was observed that the crystal formation depends on temperature. Surprisingly, this effect was not observed in Eudragit[®] NE 30D nanosuspensions loaded with lipophilic drugs. Indeed, the suspension of nanoparticles seems to be more stable than after polymer coalescence to form a film. This behaviour could be explained by the steric arrangement of drug-polymer molecules into the solvent.

4.2 Study of drug-polymer interactions

The following part of this discussion is based on the study and the determination of molecular interactions between drugs and polymers. Film formation, thermal studies, drug loading as well as the in vitro kinetic release will be developed.

4.2.1 Films formation

The objective of the films' preparation was to determine the compatibility between drugs and polymers. A solution of polymer in which drug was added was allowed to dry in a glass plate. A thin film was obtained. The compatibility between drug and polymer was shown by the presence of a transparent and homogeneous film. On the other hand, a heterogeneous surface demonstrated incompatibility.

The next pictures correspond to Eudragit[®] L 30D-55 films:

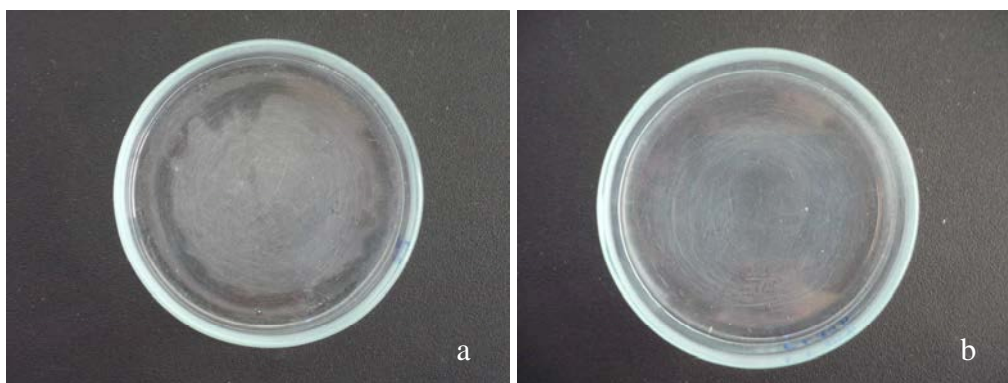


Figure 27: Films of polymer with solubilised drug. a) Eudragit[®] L 30D-55 including celecoxib and b) Eudragit[®] L 30D-55 including ivermectin.

In case of films incorporating celecoxib, a white layer is observed in the middle of the plate. This behaviour shows a drug-polymer incompatibility because the drug and the polymer are separated. On the other hand, a film prepared with a polymer solution including ivermectin shows a transparent and homogeneous film. So it can be concluded that Eudragit[®] L 30D-55 and ivermectin are compatible.

Films were prepared by solubilisation of polymer and drug in acetone. A concentration corresponding to drug concentration in loaded nanosuspensions: (44 µg/mg of polymer for ibuprofen; 22 µg/mg for Celecoxib and 3 µg/mg in case of Ivermectin).

In case of ibuprofen, a transparent film was observed for all polymers (Ibuprofen is soluble in almost all polymers). In case of Eudragit[®] L30D-55 some aggregates were observed on the film surface.

In case of celecoxib, homogeneous films were observed for all polymers. Transparent films, with some aggregates in case of Eudragit[®] RS 30D, were observed. Celecoxib is rapidly soluble in Aquacoat[®] ECD, Eudragit[®] NE and Kollicoat[®] SR. To be solubilised in Eudragit[®] RS and RL, it takes at least one week for Eudragit[®] RL and more time for Eudragit[®] RS. The long time of incorporation may explain the presence of aggregates.

In case of Ivermectin a transparent film was observed for all polymers (Ivermectin is soluble in all polymers).

The film preparation shows that drug and polymer are compatible and in most of cases, the inclusion of the different drugs into polymer nanosuspensions is possible.

4.2.2 DSC studies

For a better understanding of drug-polymer interactions in drug delivery systems, the thermal properties of the systems can be measured.

DSC studies were performed with freeze-dried nanosuspensions as well as with polymer films containing the same drug proportion than polymeric nanosuspensions. The interest of studying freeze-dried nanosuspensions and films was to compare the two structures. Indeed, freeze-drying may modify nanoparticles' structure.

Unfortunately, the results obtained by DSC are not exploitable. Indeed, drug-polymer interactions studies reported in literature are referred to formulations containing a high proportion of drugs. These ratios vary between 5 to 95% in drug content as reported in table 32. In our case, drug contents are 0.1, 0.3, 0.9, 1.1, 2.1 and 4.2 % for warfarin, ivermectin, econazole nitrate, sodium diclofenac, celecoxib and ibuprofen, respectively. The low drug content does not allow performing accurate DSC measurements.

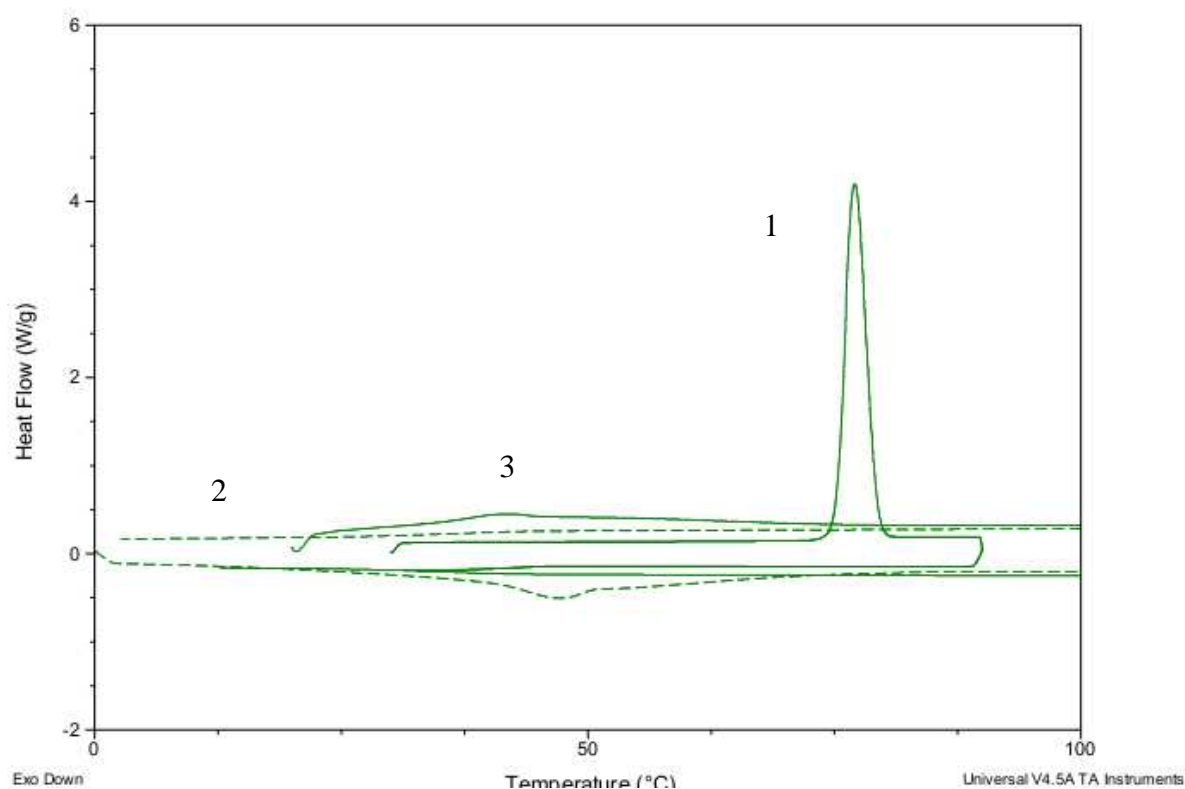


Figure 28: DSC thermograms corresponding to Kollicoat[®] SR nanosuspension loaded with ibuprofen. (1) Ibuprofen, (2) Kollicoat[®] SR and (3) Ibuprofen loaded nanosuspension.

In the previous figure, the peak corresponding to ibuprofen is not observed in loaded nanosuspension. The reason may be that ibuprofen is in its amorphous state. Nevertheless, due to the low proportion of drug contained into the polymeric system, drug crystallinity could not be detected. We have selected the most concentrated nanosuspension to show that DSC measurements may not give accurate information.

The next table summarizes different studies which used DSC to characterize drug-polymer interactions. As shown in this table, drug content into polymeric matrix is always higher than the concentrations used in this study.

| Author | Formulation type | Drug | Polymer | Drug content (%) | Ref |
|----------|------------------|--|--|--|--------------------------------------|
| Janssens | Solid dispersion | Itraconazole | Kollicoat [®] IR | 5-80% | (Janssens, de Armas et al. 2007) |
| Sarisuta | Films | Erythromycin | Eudragit [®] L100 Shellac [®] Polyvinylacetate ftalate Cellulose acetate ftalate Hydroxy propyl methylcellulose acetate ftalate Hydroxypropyl methylcellulose | 50% | (Sarisuta, Kumpugdee et al. 1999) |
| Schimd | Film | Ibuprofen | Aquacoat [®] ECD | 66%, 85% | (Schmid, Müller-Goymann et al. 2000) |
| Glaessl | Films | Tartaric acid, Metoprolol free base, Metoprolol tartrate | Eudragit [®] RL PO Eudragit [®] RS PO | 5-30% | (Glaessl, Siepmann et al. 2009) |
| Albers | Extrudate | Celecoxib | Eudragit [®] EPO | 50% | (Albers, Alles et al. 2009) |
| Lin | Solution Films | Warfarin | Eudragit [®] E Eudragit [®] S Eudragit [®] RL | Solution : 10, 20, 40, 50, 60, 80, 100 % Film : 66, 40, 25% | (Lin, Cheng et al. 1994) |
| Lin | Films | Nonoxynol 100 (surfactant) | Eudragit [®] NE 30D | 5, 10% | (Lin, Muhammad et al. 2001) |

Table 31: Overview of drug contents used in characterization of drug-polymer interactions by DSC.

The interaction between Ibuprofen and Aquacoat[®] ECD has already been described by Schmid et al. (2000). More precisely, DSC studies have shown the existence of an eutectic mixture between ibuprofen and cetyl alcohol, one of the component of Aquacoat[®] ECD which is responsible of a reduction of ibuprofen melting point (Schmid, Müller-Goymann et al. 2000).

Sarisuta et al have studied the interaction between erythromycin and various polymer films. The selected polymers were Eudragit[®] L100, Shellac[®], Polyvinylacetate phtalate, Cellulose acetate phtalate, Hydroxypropyl methylcellulose acetate phtalate and Hydroxypropyl methylcellulose. In each case they have suggested that the interaction between drug and polymer is based on the protonation of amine group by the carboxylic groups existing in polymers (Sarisuta, Kumpugdee et al. 1999). The same behaviour can be expected by the interaction of celecoxib and the carboxylic groups of the studied polymers such as Eudragit L 30D-55.

To study the interaction between drug and polymer, different techniques can be considered. X-ray diffraction, infrared studies, combined with DSC can be used to determine these interactions. Despite the utility of these tools, their sensitivity is not sufficient due the low proportion of drug included in our particles.

4.2.3 Drug loading into nanoparticles

To determine the drug content into nanoparticles, different analytical methods have been developed. The first approach to determine drug entrapment was to centrifuge drug-polymer nanosuspension to separate nanoparticles and unloaded drug. Centrifugation step should be optimised to obtain a clear supernatant. Regardless the considered nanosuspension, a classical method of drug assay such as spectrophotometry was not adapted to our systems. Indeed, the presence of polymer residues (which interferes in drug assay) as well as the low amount of unloaded drugs prevents the use of this technique due to its low sensitivity. A HPLC method

has been developed in order to assay all nanosuspension types with the same technique. The advantages of HPLC are a higher sensitivity as well as the ability to separate the drug molecule from polymer residues and other compounds existing in polymer nanosuspensions (surfactants, preservatives...). Experiments were carried out with the same HPLC column, wavelength and mobile phase. In order to optimize the drug assay, the mobile phase proportion as well as flow was adapted for each drug. Accordingly, two general methods were used: one allows the assay of Sodium diclofenac, Ibuprofen and Warfarin whereas the second one allows the assay of Celecoxib, Econazole nitrate and Ivermectin.

The important proportion of polymer existing in the nanosuspensions causes damages in HPLC columns. For this reason, a direct assay method was also developed. In this case, drug concentration in nanoparticles was much higher than in supernatant. Most of the tested model drugs are fluorescent in appropriate conditions whereas polymer structures are not fluorescent. A fluorescent assay was developed with the aim to correlate direct and indirect assays.

Unfortunately, the direct and indirect assays were not always available to assay the entire group of formulations due to problems related with sensitivity, matrix effect, interferences...

The next table summarizes the obtained results concerning direct and indirect assay.

| | Drug | | | | | | | | | | | | |
|----------|------------------------------------|--------------|-------------------|----------|-------------------|----------|-------------|--------------|--------------|----------|--------------|--------------|--------------|
| | Celecoxib | | Sodium diclofenac | | Econazole nitrate | | Ibuprofen | | Ivermectin | | Warfarin | | |
| | Direct | Indirect | Direct | Indirect | Direct | Indirect | Direct | Indirect | Direct | Indirect | Direct | Indirect | |
| Polymers | Aquacoat [®] ECD | 100% | NP | NP | NP | - | - | 49.5% | NP | NP | NP | 102% | NP |
| | Eudragit [®] FS 30D | - | - | - | - | - | - | - | - | - | - | - | - |
| | Eudragit [®] L 30D-55 | - | - | - | - | - | - | - | - | NP | 99.4% | NP | 99.8% |
| | Eudragit [®] NE 30D | 78.2% | NP | NP | 64.1% | NP | 100% | NP | 94.5% | NP | 100% | 69.6% | 79.3% |
| | Eudragit [®] RL 30D | NP | NP | - | - | NP | 100% | 93.6% | NP | NP | NP | 92.2% | NP |
| | Eudragit [®] RS 30D | - | - | - | - | - | 100% | NP | NP | NP | NP | 50.5% | NP |
| | Kollicoat [®] MAE 30DP | - | - | - | - | - | - | - | - | NP | 99.4% | NP | 99.8% |
| | Kollicoat [®] SR 30D | 100% | 99.9% | NP | NP | NP | NP | 54.3% | 99.7% | NP | 99.7% | 95.9% | 93.1% |

NP indicates problems during drug assay. (-) corresponds to non-solubilized nanosuspensions.

Table 32: Overview of drug entrapment into polymeric nanosuspensions.

As observed in table 32, direct-indirect correlation is only possible in three cases: Celecoxib loaded into Kollicoat[®] SR 30D and warfarin loaded into Kollicoat[®] SR 30D. In case of warfarin loaded into Eudragit[®] NE 30D, a difference of 10% between direct and indirect assay was observed which can be considered as acceptable. In case of ibuprofen loaded into Kollicoat[®] SR nanosuspensions, correlation is not possible (54 and 99% for direct and indirect assay respectively). In the other cases, only one of two analytical methods could be applied. In general, entrapment efficiency higher than 70% was obtained, except for Sodium diclofenac loaded into Eudragit[®] NE (64%), Ibuprofen loaded into Aquacoat[®] ECD (49.5%) and warfarin loaded into Eudragit[®] RS 30D (50.5%).

Polymer nanoparticles are dispersed into an aqueous medium containing surfactants. It can be considered that drug is partially solubilised in the aqueous media. Then, it was decided to measure the saturation concentration of each drug in the aqueous media of each polymer nanosuspension. The aim of this experiment is to explain the non total incorporation of the drug into nanoparticle matrix. Indeed, by polarised light microscopy, drug is reported to be solubilised. It could be considered that solubilised drug is incorporated into nanoparticles. Nevertheless, the obtained encapsulation rate shows that in some cases, drugs are not completely incorporated into nanoparticles and thus their solubilisation on the aqueous phase could be assumed.

The next table shows the different saturated concentrations as well as the maximum percentage of drug which could be dissolved in the aqueous phase.

| Drug | Polymer | Drug entrppment (%) | Drug amount into nanosuspension (mg/ml) | Supernatant solubility (mg/ml) | Drug in aqueous phase (%) |
|-------------------|---------------------------------|---------------------|---|--------------------------------|---------------------------|
| Celecoxib | Aquacoat [®] ECD | 100 | 6.6 | 0.479±0.06 | 7.3 |
| | Eudragit [®] NE 30D | 78.2 | | 0.158±0.06 | 2.4 |
| | Eudragit [®] RL 30D | - | | - | - |
| | Kollicoat [®] SR 30D | 100 | | - | - |
| Sodium diclofenac | Aquacoat [®] ECD | - | 3.3 | 65±6 | >100% |
| | Eudragit [®] NE 30D | 64.1 | | 8±0.2 | |
| | Kollicoat [®] SR 30D | - | | - | |
| Econazole nitrate | Eudragit [®] NE 30D | 100 | 3 | - | - |
| | Eudragit [®] RL 30D | 100 | | 0.120±0.001 | 4% |
| | Eudragit [®] RS 30D | 100 | | 0.120±0.001 | 4% |
| Ibuprofen | Aquacoat [®] ECD | 49.5 | 13.3 | 1.5±0.06 | 11.3% |
| | Eudragit [®] NE 30D | 94.5 | | 0.161±0.019 | 1.2% |
| | Eudragit [®] RL 30D | - | | - | - |
| | Eudragit [®] RS 30D | - | | - | - |
| | Kollicoat [®] SR 30D | 54.3 | | 0.170 | 1.3% |
| Ivermectin | Aquacoat [®] ECD | - | 0.93 | 13±2 | >100 |
| | Eudragit [®] L 30D-55 | 99.4 | | 0.18 | 19.3% |
| | Eudragit [®] NE 30D | 100 | | 5.9±0.01 | > 100 |
| | Kollicoat [®] MAE 30DP | 99.4 | | 0.18 | 19.3% |
| | Kollicoat [®] SR 30D | 99.7 | | 1.5± 0.05 | > 100% |
| Warfarin | Aquacoat [®] ECD | 102 | 0.3 | 0.218±0.028 | 72.6% |
| | Eudragit [®] NE 30D | 79.3 | | 0.202±0.008 | 67.3% |
| | Eudragit [®] RL 30D | 92.2 | | - | - |
| | Eudragit [®] RS 30D | 50.5 | | - | - |
| | Kollicoat [®] SR 30D | 95.9 | | 0.018±0.006 | 6% |

Table 33: Saturation concentrations of different aqueous medium of nanosuspension.

In case of celecoxib, a small proportion of drug can be solubilised in the aqueous media. This may be the reason of the very high entrapment rate, near 100 %. The same behaviour was observed with econazole nitrate, which can explain the total incorporation of the drug into nanoparticles.

Ibuprofen is quite soluble in the aqueous medium of Aquacoat[®] ECD, which contains sodium lauryl sulphate and cetyl alcohol. Schmid et al. (2000) have already demonstrated an important interaction between ibuprofen and cetyl alcohol (Schmid, Müller-Goymann et al. 2000) which can explain the relatively low entrapment rate of drug in Aquacoat[®] ECD nanosuspensions (49.5%).

In case of sodium diclofenac, which is soluble in water, the total amount of drug could be solubilised in the aqueous medium and not internalised in the particles, nevertheless an entrapment efficiency of 64 % was found. It can be considered that sodium diclofenac presents some affinity for the polymer, which facilitates its incorporation into the nanoparticles. Other drugs such as ivermectin and warfarin are very soluble in the aqueous medium too and as observed with sodium diclofenac, drug are incorporated into nanoparticles with a higher proportion (close to 100%) indicating a high affinity for polymeric matrix.

4.2.4 Drug release from nanoparticles

To study the drug release profile from nanoparticles, the experiment was carried out using a USP dissolution apparatus II (paddle).

Dissolution media were selected as a function of drug solubility and stability. It was necessary to adapt the dissolution media in order to fulfill sink conditions for each tested drug.

Celecoxib and Ivermectin solubility was increased by the addition of Tween[®] 80. Phosphate buffer pH 7.4 was selected for the others drugs.

A first step of burst was observed for all preparations. The extent of drug released depends on drug type. In case of ibuprofen, drug is not released when using phosphate buffer as

dissolution medium. Increasing drug solubility in dissolution medium (by using PBS-Tween[®] 80 (2%)) did not show any increase in release. In case of econazole, ivermectin and warfarin, a total release of drug was observed. The different observed behaviours in release may be explained by the different interactions between polymers and tested drugs.

4.2.4.1 Celecoxib

Celecoxib was incorporated into neutral polymers as well as Eudragit[®] RL 30 D an acrylic cationic polymer. The release profiles are reported in the next figure.

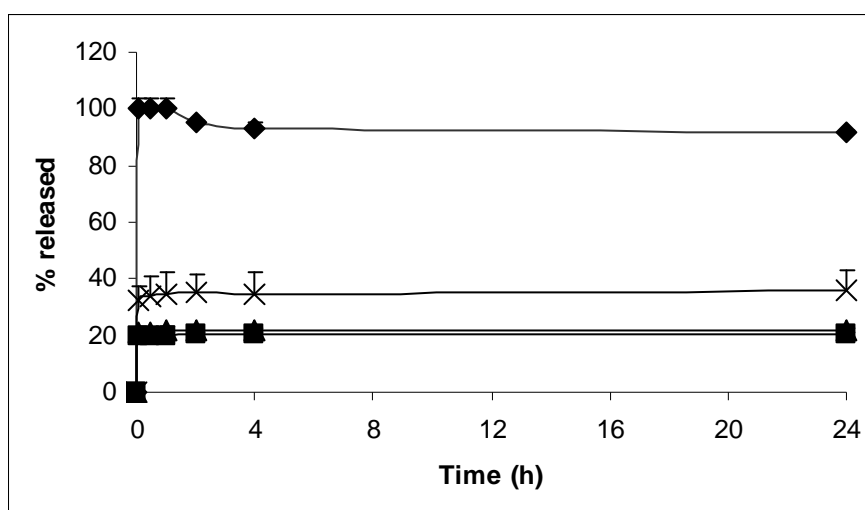


Figure 29: Celecoxib released from nanoparticles. (♦) Drug solution, (■) Aquacoat[®] ECD, (▲) Eudragit[®] NE 30D, (X) Kollicoat[®] SR 30D. Results are expressed in $n=3 \pm SD$.

The release experiments performed with Eudragit[®] RL 30D could not be assayed due to polymer interference during assay.

In the other cases, a partial release was observed. In all samples a double phase profile was obtained; a burst release followed by a plateau. Celecoxib control shows that the molecule is stable in the dissolution medium.

In case of Celecoxib released from Kollicoat[®] SR 30 D nanoparticles, a partial release, up to 37%, was obtained. On the other hand, drug release from Aquacoat[®] ECD and Eudragit[®] NE 30D was only 20%.

4.2.4.2 Econazole nitrate

Econazole nitrate was incorporated in Eudragit[®] NE 30D, a neutral polymer and Eudragit[®] RL and RS 30D, two cationic polymers. The dissolution medium used to study the drug release was pH 7.4 phosphate buffer. The drug release study performed with Eudragit[®] RL and RL was not assayed. Indeed, spectrophotometric drug assay was not possible due to polymer interferences. The next figure reports the release profile of Econazole nitrate control solution and drug release from Eudragit[®] NE 30D suspension.

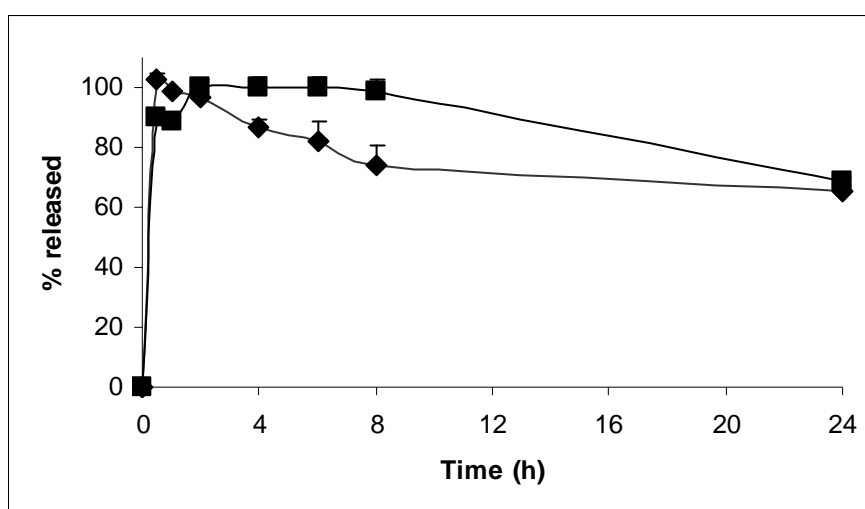


Figure 30: Econazole released from nanoparticles. (♦) Drug solution, (■) Eudragit[®] NE. Results are expressed in $n=3 \pm SD$.

Control solution shows a sustained degradation of the molecule in the medium. This degradation is almost immediate after drug addition into the dissolution medium. Econazole released from Eudragit[®] NE nanosuspensions shows a burst release of 90 % during the first minutes of experiments, then followed by a plateau. The presence of polymer in dissolution medium seems to have a protective effect against drug degradation, at least during the first hours of experiment. After 8 h of stirring, drug degradation occurs in the same way as for the solution i.e. 30% degraded at 24 h.

4.2.4.3 Ibuprofen

Ibuprofen was loaded into all types of nanosuspensions. Only the formulations prepared with Aquacoat[®] ECD, Eudragit[®] NE and Kollicoat[®] SR were analysed. In the other cases, ibuprofen released from nanoparticles cannot be assayed. As already stated before, polymer interferences were observed during drug assay in case of Eudragit[®] RL and RS, Kollicoat[®] MAE and Eudragit[®] L 30D-55.

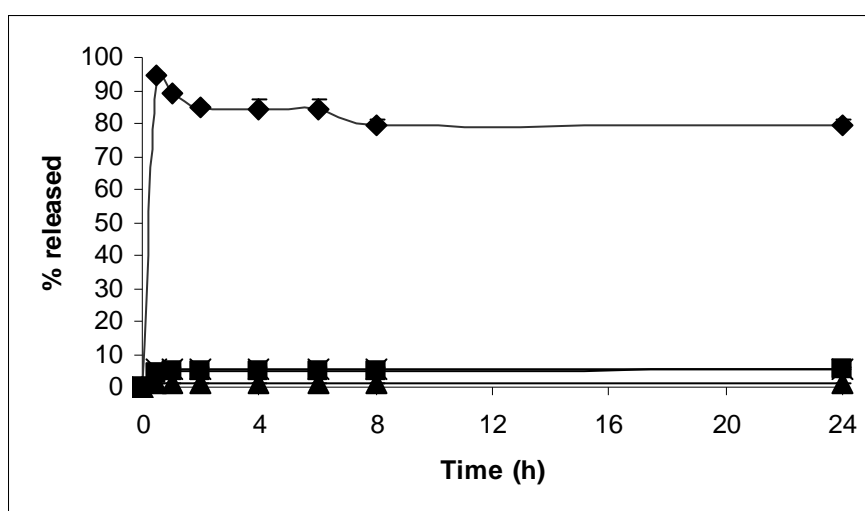


Figure 31: Ibuprofen released from nanoparticles. (♦) Drug solution, (■) Aquacoat[®] ECD, (▲) Eudragit[®] NE 30D and (X) Kollicoat[®] SR 30D. Results are expressed in $n=3 \pm SD$.

In case of nanosuspensions prepared with Eudragit[®] L 30D-55 and Kollicoat[®] MAE 30 DP, polymer was solubilised after its addition into the dissolution medium (Phosphate buffer, pH 7.4). Thus, it could be considered that all drug was released after polymer dissolution. Nevertheless, this result could not be confirmed. Indeed, the important proportion of polymer contained in the system interferes with drug assay.

Drug release from Eudragit[®] RL and RS was also not analysed. In this case, polymer interference (if spectrophotometric assay was tested) or sorbic acid interference (if HPLC assay was performed) did not allow obtaining satisfactory results.

Nanosuspensions prepared with the neutral polymers (Aquacoat[®] ECD, Eudragit[®] NE 30D and Kollicoat[®] SR 30D) were not able to release ibuprofen into the dissolution medium. A release between 2 and 7 % was obtained. As observed in figure 30, drug release occurred during the first minutes, and then a plateau was obtained.

In order to enhance drug solubility, drug release was also carried out by using PBS-Tween 80 (2%) as a dissolution medium. In this case, drug was also not released from nanoparticles.

The low release of drug may be explained by strong interactions between drug and polymers. The rapid solubilisation of the drug into nanosuspension can indicate a high affinity between these molecules. As reported above, Ibuprofen interacts easily with neutral polymers (Khan, Anjum et al. 2011). This interaction can explain the lower drug release.

A kinetic release of ibuprofen loaded into Eudragit[®] NE 30 D was also performed in different conditions. In this experiment, release from drug polymer nanosuspension in colloidal form was compared to the same formulation after freeze-drying. In this case, dissolution medium was 20 mL of an aqueous solution of sodium lauryl sulphate 0.25% and the experiment was carried out in sink conditions at 37°C under magnetic stirring at 200 rpm. The obtained results are represented in the next figure.

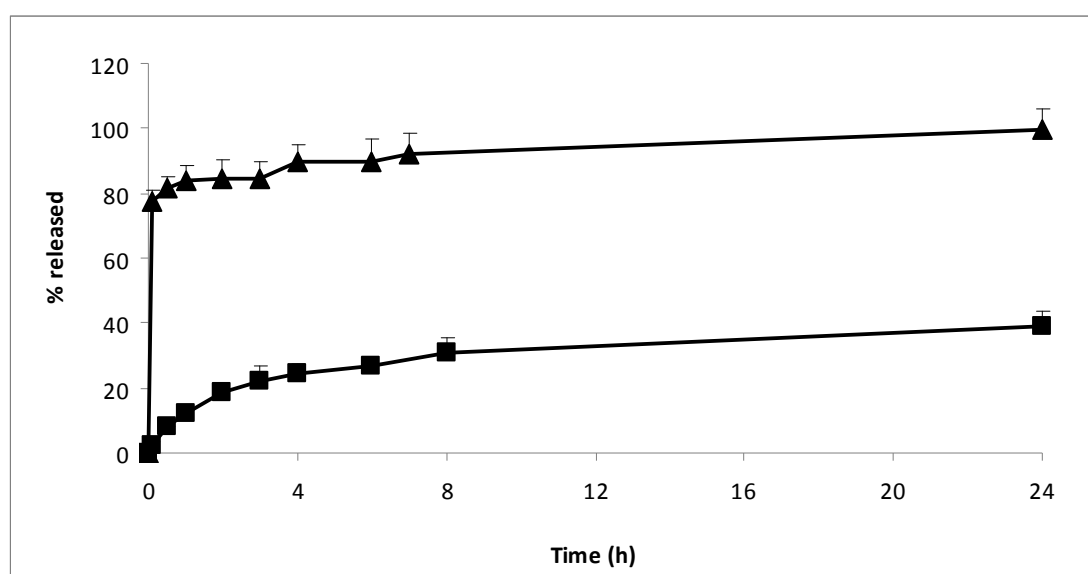


Figure 32: Kinetic release of ibuprofen loaded into Eudragit[®] NE 30D. (▲) Colloidal nanosuspension, (■) freeze dried nanosuspension.

This figure shows the kinetic release of ibuprofen in an aqueous solution of SDS 0.25 %. In case of colloidal nanosuspensions, drug is completely released after 5 minutes of experience. The freeze-dried nanosuspension showed a sustained release reaching 40% after 24 hours. After freeze-drying, Ibuprofen loaded into Eudragit[®] NE 30D is not available as a powder but as a kind of sticky gum. It is not surprising that this “form” provides a more sustained release. It could be considered that this texture did not allow the wettability of the formulation and consequently the drug release.

4.2.4.4 Ivermectin

Ivermectin release from polymer nanosuspensions was evaluated with Aquacoat[®] ECD and Eudragit[®] NE 30D. As for ibuprofen, drug release was not evaluated for all nanosuspensions due to polymer interactions.

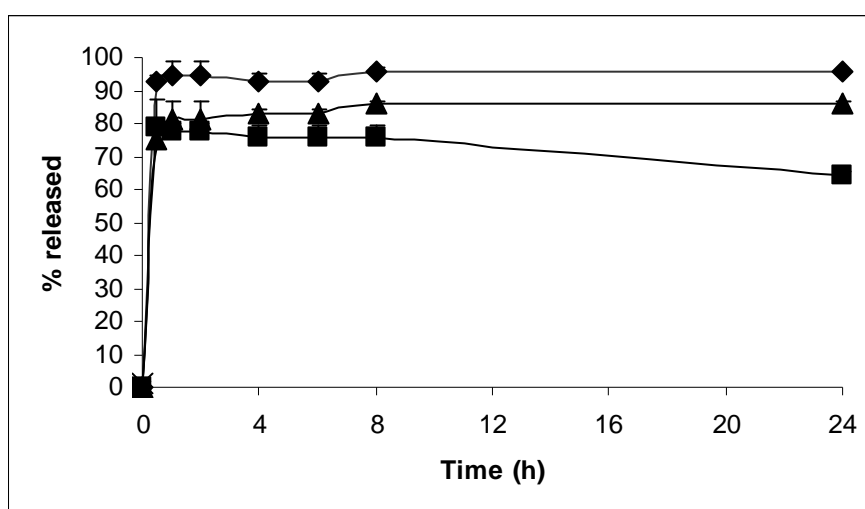


Figure 33: Ivermectin released from nanoparticles. (◆) Drug solution, (■) Aquacoat[®] ECD, (▲) Eudragit[®] NE 30D. Results are expressed in $n=3 \pm SD$.

The release profile of ivermectin from nanoparticles showed a burst and a very important release (78 % and 75% for Aquacoat[®] and Eudragit[®] NE respectively). In case of Aquacoat[®], drug release did not change during time and a partial degradation of the drug in the

dissolution medium was detected. On the other hand, ivermectin released from Eudragit[®] NE nanoparticles was slightly increased during time to attain a maximum of 86% of drug release. In the presence of this polymer, drug degradation was not observed.

4.2.4.5 Warfarin

Neutral nanosuspensions loaded with warfarin were used to evaluate drug release. Drug assay was not possible with Eudragit[®] RL, RS; Eudragit[®] L and Kollicoat[®] MAE due to polymer interactions.

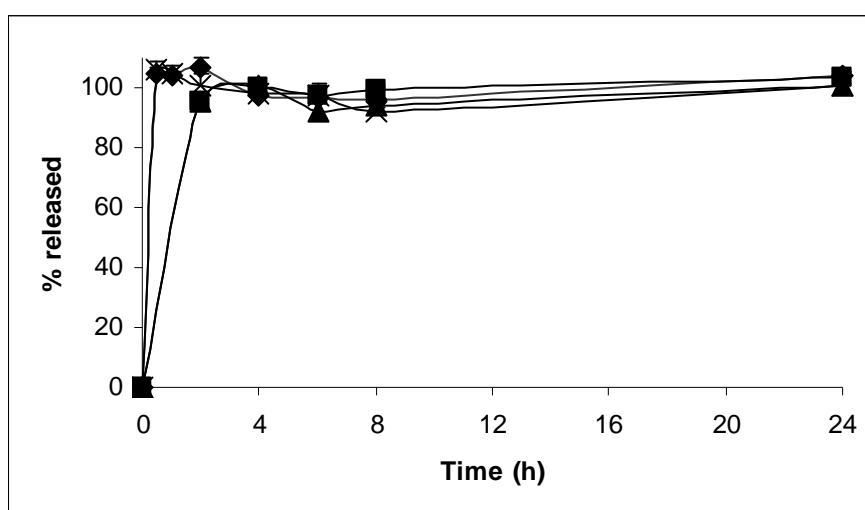


Figure 34: Warfarin released from nanoparticles. (♦) Drug solution, (■) Aquacoat[®] ECD, (▲) Eudragit[®] NE 30D and (X) kollicoat[®] SR 30D. Results are expressed in $n=3 \pm SD$.

Warfarin release was complete after the first minutes of the experience. Dissolution rate in case of warfarin was very high, comprising between 24-48h. The low affinity between drug and polymers may explain the total drug release in vitro. Drug degradation was not detected.

4.2.4.6 Discussion

It should be noticed that the dissolution medium plays an important role in drug release. We have decided to carry out the entire dissolution experiments pH 7.4 phosphate buffer with or without surfactant. The objective was to compare the dissolution profile of the different drugs.

Depending on the tested drug, different profiles were observed. In case of ibuprofen there was no release in these conditions. A higher release was observed with celecoxib but again non total. On the other hand, regardless the type of polymer, drug is totally released in case of econazole, ivermectin and warfarin.

The reasons for such differences in release profiles are difficult to explain. It can be considered that ibuprofen and celecoxib present stronger interactions between drug and polymer or the drug is more internalized into polymeric matrix. This internalization could avoid the drug wettability and in consequence drug is not solubilized. In the other cases, drug could be dispersed on the particle surface.

The methods used above to evaluate drug release from nanoparticles present some limitation. Indeed, a small volume of nanosuspension was directly dispersed into a large volume of dissolution medium. At selected times, samples were withdrawn and centrifuged to separate polymer residues from drug solution. A clear supernatant was not obtained in every case; Eudragit[®] L and Kollicoat[®] MAE nanosuspension could not be assayed. As reported above, these polymers are solubles in pH above 5. So drug-polymer separation was not possible. In case of nanosuspensions prepared with Eudragit[®] RS and RL, polymer residues seem to be precipitated during centrifugation; nevertheless, during drug assay, important interferences were observed. These interferences may be due to polymer molecules in solution and/or the presence of sorbic acid, a preservative present in the commercial nanosuspension.

Two other methods to evaluate drug release from nanoparticles were tested. In both cases, a dialysis membrane was used:

4.2.4.7 Glass baskets method

Glass cells containing nanosuspension were used to evaluate the drug release from nanoparticles. This method has been published by Abdel-Mottaleb et al. (2011). Briefly, a glass tube corresponding to the dimensions of the European Pharmacopeia basket (apparatus

I) was prepared. At the bottom of the tube, a dialysis membrane was fixed and the entire system was fixed in the European Pharmacopeia apparatus support as displayed in figure 35.



Figure 35: Nanosuspension located in the glass cell. Polymeric nanosuspension is separated from dissolution medium by a dialysis membrane (CE, 14000 kda, SpectraPor, Spectrum Labs, Breda, Netherlands).

The position of the cell in the dissolution medium was also evaluated. Indeed, as observed in figure 36, glass cell was completely immersed in the dissolution medium (left picture) or placed on the medium surface (right picture). The objective of the second position was to avoid leakage. Dissolution medium was maintained at 37°C and glass cells were rotated at 50 rpm.

Different parameters were modified to optimize the system. The different tests are reported in table 34.

| Experiment number | Volume of dissolution medium (mL) | Volume of nanosuspension (mL) | Glass cell position* |
|--------------------------|--|--------------------------------------|-----------------------------|
| 1 | 100 | 2 | Surface |
| 2 | 100 | 5 | Surface |
| 3 | 500 | 2 | Surface |
| 4 | 500 | 5 | Surface |
| 5 | 500 | 2 | Immersed |
| 6 | 500 | 5 | Immersed |

Table 34: Glass basket experiments summary.

Nanoparticles leakage was observed when drug release was evaluated with the glass baskets immersed into the dissolution medium. Glass baskets placed on medium surface did not allow drug release. Obtained results are not reproducible due to the impossibility to really standardize the method. Abdel-Mottaleb et al. (2011) have studied ibuprofen release from different types of nanoparticulate system (liposomes, polymeric nanoparticles and lipid nanocapsules). The authors have studied two different systems: a classic dialysis bag and glass basket dialysis method. A more sustained drug release was obtained with glass baskets when compared with dialysis bag (Abdel-Mottaleb and Lamprecht 2011). In our case, results could not be reproduced. It could be considered that all parameters are not controlled and deeper studies should be considered.

The next figure summarizes the two types of *in vitro* release studies performed in a Pharmacopeia apparatus.



Figure 36: Two different techniques to perform the kinetic drug release. (1 and 3) represent direct dispersion of nanosuspension in the dissolution mediums, (2 and 4) represent the glass cells closed with dialysis membrane containing polymeric nanosuspension.

4.2.4.8 Dialysis bag method

The second tested method to study the drug release was the dialysis bag method. According to this method, 2 ml of formulation were introduced into a dialysis bag and then introduced into a plastic tube with a magnetic stirrer. System was maintained at 37°C and stirred at 200 rpm. Warfarine was the model drug used to evaluate this dialysis bag method. The other drugs was also tested by this method, nevertheless drug remains adsorbed onto the dialysis membrane.

Next figure displays the dialysis bag method.



Figure 37: Dialysis bag method. Left picture shows the dialysis bag containing drug-loaded nanosuspension. Right picture shows the plastic tube containing the dialysis bag into a water bath.

At selected times, samples of 1ml were withdrawn and assayed with fluorescence spectrophotometer. Dialysis method was used to study warfarin release from nanosuspensions. Different profiles were observed.

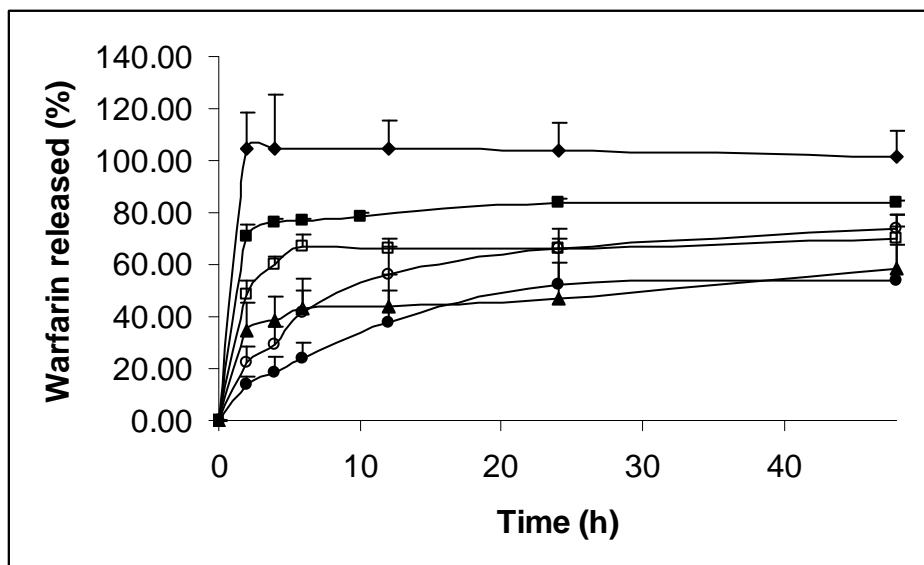


Figure 38: warfarin release from polymer nanosuspension using a dialysis bag. (◆) warfarin solution, (■) Aquacoat® ECD, (▲) Eudragit® NE 30D, (●) Eudragit® RL 30D, (○) Eudragit® RS 30D, (□) Kollicoat® SR 30D.

Different profiles were obtained depending on polymer nanosuspension. Warfarin solution was used as a control: all drug was released from dialysis bag. The release profile from Aquacoat® ECD shows a double phase profile, a burst followed by a plateau (80% of drug was released). Kollicoat® SR 30D also shows a burst release followed by a plateau. The burst is less pronounced than in case of Aquacoat® ECD only 60% of drug was released. Eudragit® RS 30D presents a sustained release, which attains 75% of drug release after 24h of experiment.

4.3 Applications

This part of the discussion describes the application of the drug-polymer nanosuspensions. Selected nanosuspensions were tested in vivo to evaluate the bioavailability. Furthermore, the maximum amount of drug which can be incorporated was evaluated in order to reduce the volume of nanosuspension in case of oral administration.

4.3.1 In vivo tests

The in vivo experiments were carried out for three different drugs. The investigated drug-loaded nanosuspensions investigated were selected according to parameters of stability (>12 months) and loading charge. At least one neutral polymer was selected as well as a cationic polymer in case of ivermectin and warfarin.

The next table summarizes the overall in vivo experiments carried out to evaluate the oral bioavailability of drug-loaded nanosuspensions.

| Drug | Animal model | Formulation tested | Administration route | Dose (mg/kg) |
|------------|-----------------|---|----------------------|---------------------|
| Celecoxib | New | Celecoxib solution Celebrex [®] | SC and oral route | SC: 0.5 mg/kg |
| | Zealand Rabbit | Aquacoat [®] ECD | Oral route | Oral route: 2 mg/kg |
| | | Kollicoat [®] SR 30D | Oral route | |
| Ivermectin | Wistar rat male | Ivermectin solution IVOMEC [®] | SC and oral route | 0.5 mg/kg |
| | | Aquacoat [®] ECD | Oral route | |
| | | Eudragit [®] NE 30D | Oral route | |
| | | Eudragit [®] RL 30D | Oral route | |
| Warfarin | New | Warfarin solution | Oral route | 0.1 mg/kg |
| | Zealand rabbits | Eudragit [®] RL 30D | Oral route | |
| | | Kollicoat [®] SR 30D | Oral route | |

Table 35: Overview of *in vivo* experiments.

4.3.1.1 Celecoxib

The next figure represents the plasma concentration profile of celecoxib after oral and subcutaneous administration.

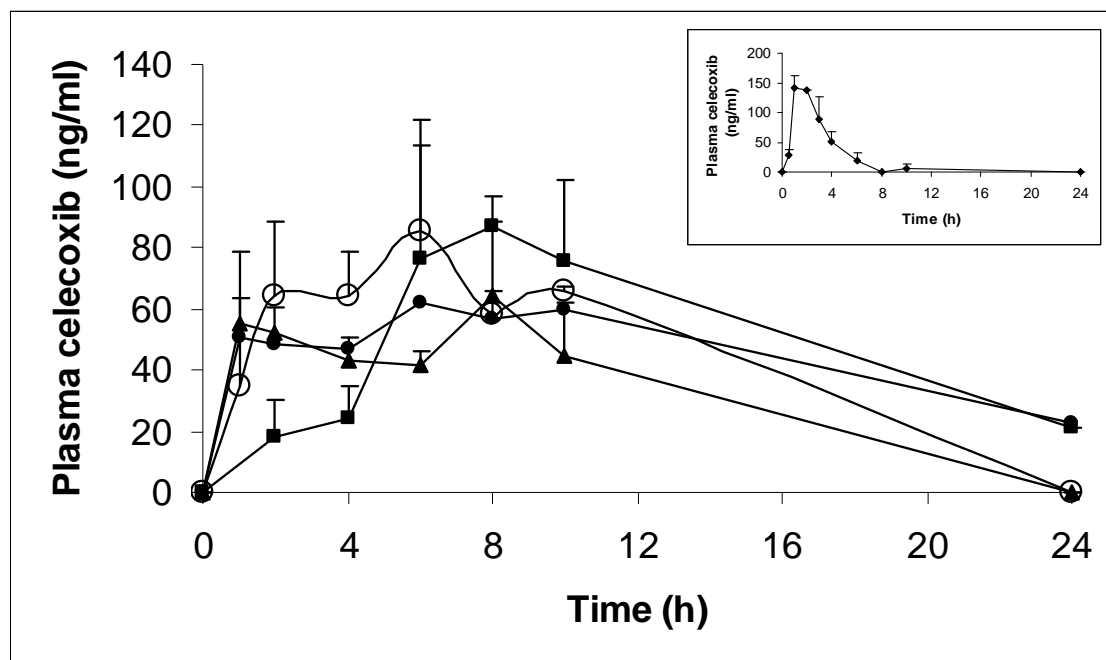


Figure 39: In vivo release of celecoxib at a dose of 2 mg/kg. (■) Celebrix[®] oral route, (▲) Celecoxib solution, oral route, (○) Aquacoat[®] suspension loaded with celecoxib, oral route, (◆) Kollicoat[®] SR suspension loaded with Celecoxib, oral route. Insert: (◆) Celecoxib subcutaneous injection at a dose of 0.5 mg/kg.

The plasma concentrations of celecoxib administered as Celebrix[®], the commercial dosage form, showed a classic profile. Drug concentration increased to attain a maximum at 10 hours and then decreased. At 24 h, celecoxib was still present in plasma. In case of Celecoxib solution, a more sustained release was observed. T_{max} was determined at 8 hours. Aquacoat[®] ECD nanosuspension showed higher plasma concentrations during the first hours of experiments. A shorter T_{max} was also observed (6 hours). In case of Kollicoat[®] SR 30D, plasma concentration profile was similar to Celecoxib solution during the 10 first hours of experiment. A sustained release until 24 hours was also observed.

Celecoxib is a class II drug according to BCS classification. In this case, the limiting factor for drug absorption is its solubility. The aim of this projet was to increase drug solubility to

facilitate absorption. Plasma profile of drug loaded into nanosuspensions, show a shorter t_{\max} compared to the commercial dosage form. Celecoxib contained into nanosuspensions was more easily available to absorption.

To carry out the experiments, a relatively low number of animals (n=3-4) was used. This may explain the important deviation expressed in the figure above.

Plasmatic concentrations were used to calculate the main pharmacokinetic parameters. These parameters are displayed in the table below:

| Formulation | Dose (mg/kg) | C. _{max} (ng/ml) | t _{max} (h) | AUC (ng.mL ⁻¹ .h) | F rel vs SC | F. rel vs Celebrex oral | F. rel vs. Celecoxib oral |
|--|--------------|---------------------------|----------------------|------------------------------|-------------|-------------------------|---------------------------|
| Celecoxib (SC) | 0.5 | 141.9±20.6 | 1 | 502.9 | - | - | - |
| Celebrex [®] (Gallardo, Morales et al.) | 2 | 86.7±10.4 | 10 | 1161.4 | 57.7 | - | - |
| Celecoxib (oral) | 2 | 77.1±24.1 | 8 | 812.8 | 40.4 | 69.9 | - |
| Aquacoat [®] ECD | 2 | 85.6±28.0 | 6 | 1072.5 | 53.3 | 92.2 | 131.9 |
| Kollicoat [®] ECD | 2 | 62.8±33.9 | 6 | 1095.5 | 54.5 | 94.3 | 134.7 |

Table 36: Kinetic parameters obtained after administration of drug-loaded nanosuspensions. Relative bioavailability was calculated by comparing a subcutaneous injection as well as the commercial oral dosage form and a drug solution.

The comparison between Celebrex[®] (the commercial form) and a celecoxib solution shows that the commercial form is more reproducible. Furthermore, the pharmacokinetic parameters (AUC, t_{\max} , c_{\max} and relative bioavailability) show the advantages of commercial form. In case of drug-loaded nanosuspensions, pharmacokinetics parameters are similar to the commercial form. The T_{\max} is increased in case of Celebrex[®] (10 hours), when compared with Aquacoat[®] and Kollicoat[®] nanosuspensions. An increase in drug dissolution rate in GI tract may explain the shorter T_{\max} when compared with the commercial form.

Homar et al. (2007), have noticed that the presence of sodium lauryl sulphate may enhance the bioavailability of lipophilic drugs (Homar, Ubrich et al. 2007). In our case, Sodium Lauryl Sulphate is present in the composition of Aquacoat[®] ECD (0.9-1.7 %) and in Kollicoat[®] SR 30D (0.3 %). Compared to commercial form, the presence of surfactant did not influence the oral bioavailability.

4.3.1.2 Ivermectin

Ivermectin plasma concentrations after subcutaneous and oral administration are reported in the next figure:

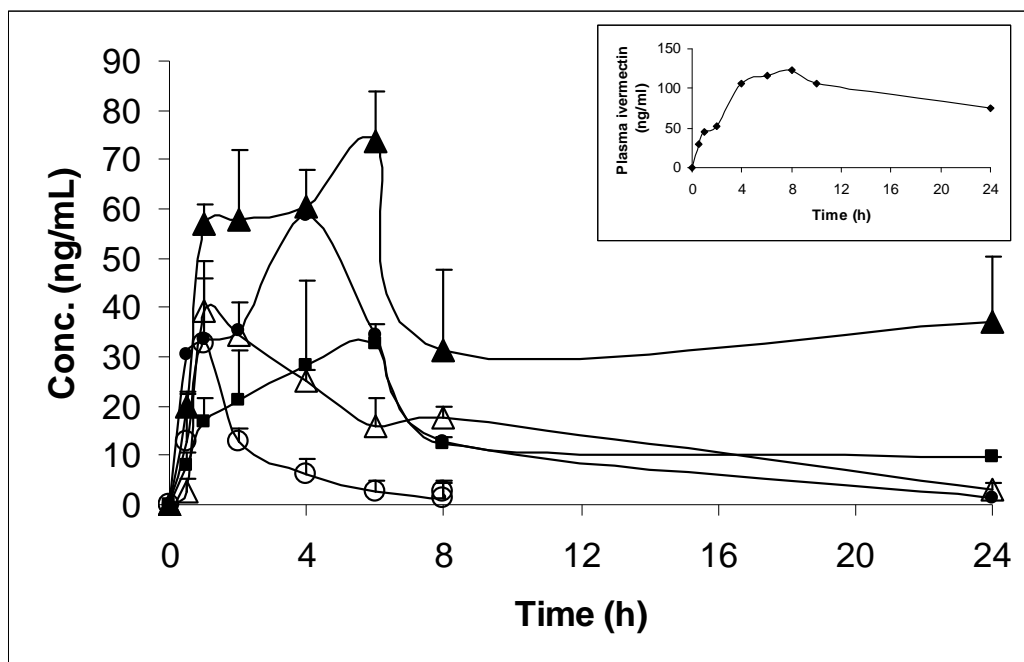


Figure 40: In vivo release of Ivermectin at a dose of 500 µg/kg., (■) IVOMECE[®], (Δ) Ivermectin solution, (○) Aquacoat[®] suspension loaded with ivermectin, (●) Eudragit[®] NE 30D suspension loaded with ivermectin and (▲) Eudragit[®] RL 30D loaded with ivermectin, oral route. In the insert, (◆) Ivermectin subcutaneous injection.

Ivermectin plasma concentrations show different profiles, which depend on formulation. When comparing Ivomec[®] (the commercial form) with Ivermectin solution, two different profiles are observed: In case of ivermectin solution, the T_{max} is reached 1 hour after administration. On the contrary, the plasma concentrations after Ivomec[®] administration increase gradually up to a maximum obtained after 6 hours. Ivomec[®] solution is prepared in a

mixture of glycerol formal and propylenglycol (40:60 V/V). Ivermectine solution was prepared by dissolving the ivermectine powder into propylene-glycol. The difference in solvents can explain the bioavailability differences between the two drug solutions.

The administration of Aquacoat[®] loaded nanosuspensions produced a rapid increase of ivermectin concentration ($T_{\max}=1\text{h}$) followed by a quick decrease.

Ivermectin loaded in Eudragit[®] NE 30D shows a classic profile with a T_{\max} of 4 hours.

A sustained release of ivermectin was observed after the administration of Ivermectin-Eudragit[®] RL nanosuspension. This sustained release was observed during 24 hours.

| Formulation | Dose (mg/kg) | C. max (ng/ml) | t. max (h) | AUC (ng.mL ⁻¹ .h) | F.relative vs SC | F.relative vs IVOMECE [®] oral | F. relative vs. oral Ivermectin |
|-------------------------------|--------------|----------------|------------|------------------------------|------------------|---|---------------------------------|
| Ivermectin (SC) | 0.5 | 123.2±2.3 | 8 | 3529.9 | - | - | - |
| IVOMECE [®] (orally) | 0.5 | 32.9±3.7 | 6 | 573.9 | 16.3 | - | 144.7 |
| Ivermectin (orally) | 0.5 | 39.1±6.9 | 1 | 396.6 | 11.2 | 69.1 | - |
| Aquacoat [®] ECD | 0.5 | 32.5±16.9 | 1 | 193.3 | 5.5 | 33.7 | 48.7 |
| Eudragit [®] NE 30D | 0.5 | 58.6±10.5 | 4 | 425.6 | 12.1 | 74.2 | 107.3 |
| Eudragit [®] RL 30D | 0.5 | 73.8±9.9 | 6 | 1469.6 | 41.6 | 256.09 | 370.5 |

Table 37: Pharmacokinetic parameters obtained after the oral administration of drug loaded nanosuspensions. Relative bioavailability was calculated by comparing a subcutaneous injection as well as the commercial dosage form and a drug solution administered orally.

As observed in the table above, Aquacoat[®] ECD and Ivermectin in solution present the lower t_{\max} (1h), followed by Eudragit[®] NE (4h) and Eudragit[®] RL and Ivomec[®] (6h). The low t_{\max} obtained with Aquacoat[®] ECD can indicate that ivermectin is not incorporated into the nanoparticles. Drug is immediately available to be absorbed after oral administration in the same manner as the ivermectin solution. The much higher t_{\max} obtained with Eudragit[®] RL shows a sustained release. Such a behaviour was expected. Indeed, Eudragit[®] RL is a non soluble but swellable polymer which is reported to allow drug sustained release. A higher

bioavailability compared with the others nanosuspensions as well as the commercial dosage form (Ivomec[®]). Nevertheless, Ivomec[®] is a commercial dosage form for subcutaneous administration in the treatment of animal parasitosis. It was observed that in case of ivermectin solutions, the obtained drug bioavailability is different depending on the solvent in which the drug is solubilised. Indeed, ivermectin was solubilised in propylene glycol and the commercial form Ivomec is solubilised in a mixture of propylene glycol and glycerol formal. Indeed, glycerol formal is present into the solution to enhance the residence time of ivermectin in the absorption site (Clement 2005). The difference in bioavailability obtained between the two solutions can be explained by the solvent difference. It can be suggested that the presence of glycerol formal allows a more sustained release of the drug

4.3.1.3 Warfarin

Plasma concentrations of warfarin after the oral administration of different nanosuspensions are reported in the next figure:

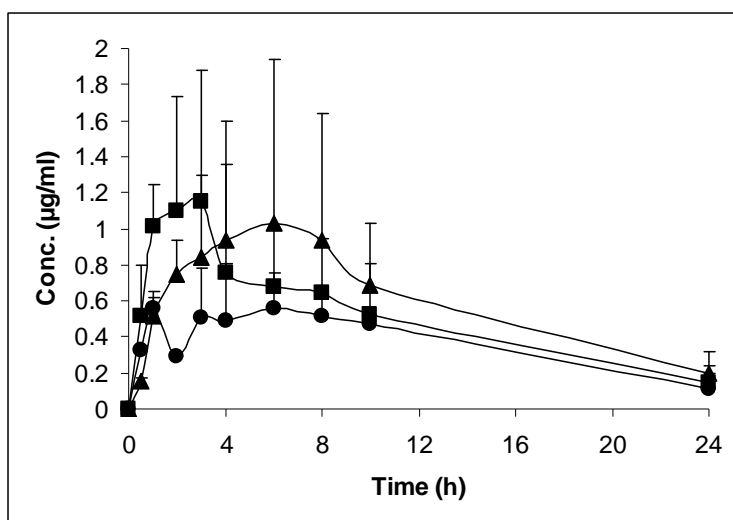


Figure 41: In vivo release of Warfarin at a dose of 100 µg/kg. (■) Warfarin solution oral route, (▲)Eudragit[®] RL 30D suspension loaded with warfarin, oral route, (●) Kollicoat[®] SR 30D suspension loaded with warfarin,oral route.

After the oral administration of a warfarin solution, a plasmatic peak was obtained at 3 hours.

For the polymer nanosuspensions, T_{max} was obtained at 6 hours. Eudragit[®] RL 30D

nanosuspension offered a sustained release of warfarin during at least 10 hours. In case of Kollicoat® SR 30D a sustained release profile was also observed but plasmatic concentrations were lower than for Eudragit® RL and warfarin solution.

| Formulation | Dose (µg/kg) | C _{max} (µg/ml) | t _{max} (h) | AUC (µg.mL ⁻¹ .h) | F rel vs. Warfarin oral |
|-------------------|--------------|--------------------------|----------------------|------------------------------|-------------------------|
| Warfarin (orally) | 100 | 1.15±0.73 | 3 | 14.8 | - |
| Eudragit® RL 30D | 100 | 1.03±0.91 | 6 | 17.6 | 118.63 |
| Kollicoat® SR 30D | 100 | 0.562±0.19 | 6 | 10.8 | 73.07 |

Table 38: Pharmacokinetic parameters obtained after the oral administration of drug loaded nanosuspensions. Relative bioavailability was calculated by comparing a subcutaneous injection as well as a drug solution administered orally.

Table 38 shows the main pharmacokinetics parameters obtained after the oral administration of warfarin. The t_{max} of loaded nanosuspensions is increased until 6 hours compared with warfarin solution. This behaviour can be explained by the sustained release character of the polymers. Oral bioavailability is highly increased in case of Eudragit RL 30D. The same behaviour was previously observed with ivermectin.

4.3.2 Perspectives in dosage form industrialisation

Selected amounts of drug to be included in nanoparticles were decided with regards to the dosage forms available in the market. The next step was to determine the maximum amount of drug which can be included into the nanoparticle with the aim to decrease the administered volume. The reduction of the dosage form volume allows reducing the administered polymer amount. By this way, the possibility of side effects related to polymers will be avoided or reduced.

Experiments were performed at room temperature and the maximum time of stirring was set to 72 hours to minimize the risk of drug degradation and with the aim to facilitate a potential

industrialised method. The next table represents the maximum amount of drug which can be included in the nanoparticles

| | | Drugs | | | | | |
|----------|---------------------------------------|-----------|-------------------|-------------------|-----------|------------|----------|
| | | Celecoxib | Sodium Diclofenac | Econazole Nitrate | Ibuprofen | Ivermectin | Warfarin |
| Polymers | Aquacoat[®] ECD | <110 | <200 | <20 | <700 | <150 | <15 |
| | Eudragit[®] FS 30D | <10 | <10 | <20 | <150 | <20 | <5 |
| | Eudragit[®] L30D-55 | <20 | <10 | <20 | <150 | <20 | <5 |
| | Eudragit[®] NE 30D | <100 | <50 | <100 | <600 | <20 | <15 |
| | Eudragit[®] RL 30D | <30 | <20 | <100 | <350 | <20 | <10 |
| | Eudragit[®] RS 30D | <30 | <20 | <100 | <350 | <20 | <10 |
| | Kollicoat[®] MAE 30DP | <20 | <10 | <20 | <150 | <20 | <5 |
| | Kollicat[®] SR 30D | <30 | <20 | <50 | <350 | <20 | <15 |

Table 39: Maximum amount of drug which can be included into nanosuspensions.

Loading capacity of polymer nanosuspensions is reported to be at its maximum regardless the drug. In other words, most of polymer nanosuspension cannot incorporate a higher amount of drug. The exception is ibuprofen. In this case, a higher amount (which can be twice or three times higher) can be incorporated into selected polymers such as Aquacoat[®] ECD or Eudragit[®] NE 30D.

PART 2:

**LOW MOLECULAR WEIGHT
HEPARIN GELS BASED ON
POLYMERIC
NANOSUSPENSIONS FOR
CUTANEOUS APPLICATION**

1. Introduction

1.1 Skin

The human skin is the body's largest organ. It covers an area of approximately 1.5 to 2 m² and contributes at about 16 % of body weight. It provides an effective barrier between body and the external environment. Skin barrier controls the water loss and the permeability of foreign substances (Wickett and Visscher 2006).

Furthermore, it regulates the body temperature, participates in the adjustment of the blood pressure and prevents the penetration of ultraviolet rays. Lastly, the skin is an important sense organ that perceives stimuli like temperature, pressure and pain.

1.1.1. Skin physiology

Histologically, skin is composed by different layers: Stratum Corneum (SC), epidermis, dermis and lower layers of adipose tissues (from outside to inside). Figure 1 represents the global skin structure.

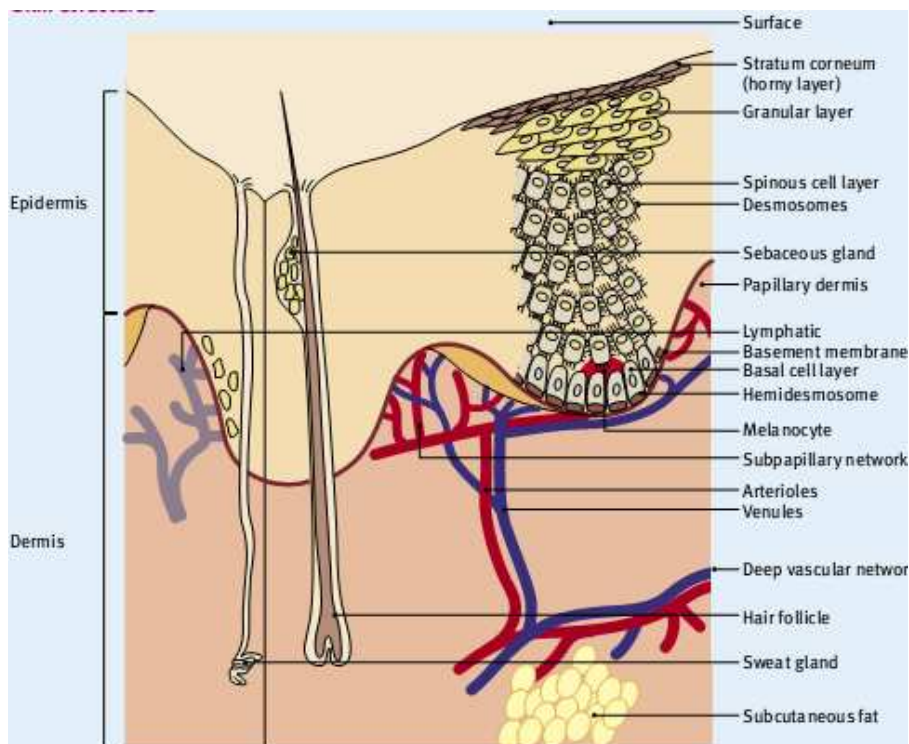


Figure 42: Skin structure (Powell 2006).

1.1.1.1 Stratum corneum (SC)

The SC or horny layer is the uppermost layer of the skin. SC thickness is comprised between 5-20 μm . It is composed of a multiple layers (15-20) of dead cells named corneocytes. They are flat, roughly and hexagonally shape cells. Corneocytes are filled with insoluble bundled-keratins and surrounded by a cell envelope stabilized through covalently bound lipid and cross-linked proteins. Each corneocyte is attached to its neighbours by desmosomes which contributes to the cohesion of SC layers. SC is a lipophilic barrier which contains 13% of water.

Stratum corneum is described by the mortar and brick model. In this model, corneocytes correspond to the bricks and the intercellular lipids (composed by ceramides, fatty acids, cholesterol and cholesterol esters) represent the mortar.

The principal function of SC is to protect the inner body, mainly composed by water, from the external environment (Asbill and Michniak 2000; Powell 2006).

1.1.1.2 Viable epidermis

Viable epidermis is the second layer of the skin. It is composed of three parts: Stratum granulosum, stratum spinosum and stratum basale. Viable epidermis contains keratinocytes at various stages of differentiation; melanocytes, Langerhans cells which participate in antigen presentation and immune response and Merkel cells which are involved in sensory perception.

The cells of the SC are originated in viable epidermis and undergo many morphological changes before desquamation. The origin of these cells lies in the stratum basale. To form SC, the keratinocytes undergoes several steps of differentiation. The cells progress from the stratum basale through the stratum spinosum and the stratum granulosum to the stratum corneum. During migration process, cells flatten, loose their nuclei and become more keratinized. This process is estimated to be in order of 21 days (Forslind 1995; Walters 2002).

Melanocytes are a functional cell type of stratum basal, also present in eyes and hair. Melanocytes contain organelles called 'melanosomes' that synthesize the pigment melanin from an essential amino acid phenylalanine. Melanosomes are transferred to keratinocytes, probably by a process involving fagocytosis. The size and number of melanosomes in the keratinocytes (and not the melanocytes) govern pigmentation. The major role of skin pigmentation is protecting the organism against harmful environmental effects such as ultraviolet radiation.

Langerhans cells are dendritic cells derived from bone marrow and are present in stratum basal. Their main function is to detect and collect exogenous antigens in the skin and present them to T lymphocytes in lymph nodes. The activate Langerhans cells migrate from the epidermis to the dermis and from there to the regional lymph nodes where they sensitize T cells. The mobilization of Langerhans cells is mediated through the interaction of their cell-surface receptors with cytokines and chemokines. Langerhans cells are part of the immune surveillance system against viral and tumour antigens.

The final type of cells present in the stratum basal is the Merkel cells. These cells can be distinguished from the keratinocytes by their clear cytoplasm. Merkel cells appear to be attached to the basement membrane separating the epidermis from the dermis. These cells are associated with nerve endings present on the dermis which suggests they function as sensory receptors. It was suggested that Merkel cells are a mechanoreceptor which detects tissue deformations with its microvilli. Merkel cells may have a possible chemosensitive function, in particular nociceptive function (Lucarz and Brand 2007).

1.1.1.3 Dermis

The region below epidermis is called dermis. It consists of the outer papillary and the inner reticular dermis, which taken together are usually 5-20 times thicker than epidermis and normally measure up to 2 mm in thickness. Dermis provides nutrition and immune support to

epidermis and plays a role in temperature, pressure and pain regulation. The main cells are fibroblasts which produce the collagen and mast cells which are involved in the immune and inflammatory responses. Collagen is a triple helix comprising three polypeptide chains. Fibres are packed in bundles and give enormous tensile strength. The dermis also contains elastin fibres that provide stretch. The fibres sit within a matrix of amorphous mucopolysaccharide ground substance. This binds water to facilitate passage of nutrients and other chemicals, acts as a lubricant to allow skin movement and provides bulk to aid shock absorption. Blood vessels form a deep plexus in the subcutaneous fat to supply the sweat glands and the hair papillae, and a superficial plexus in the papillary dermis.

The lymphatic system is an important component of the skin in regulating interstitial pressure, mobilization of defense mechanisms, and in waste removal.

Sensory nerves are abundant in the skin and are used for touch, heat, pain and itch. Mechanical, chemical and cytokine stimuli affect fine, free nerve endings at the dermo–epidermal junction (Powell 2006).

There are four skin appendages situated in the dermis: the hair follicles with their associated sebaceous glands, eccrine and apocrine sweat glands and the nails.

Hair follicles are distributed across all skin surface excepting the sole of feet, the palms of the hands and lips. The base of the hair follicle consists of the dermal papilla, which is richly supplied by blood vessels and sensory nerves. Within the hair papilla resides the hair bulb, which is the site where the growing hair originates. The hair bulb is the site of insertion of the arrector pili muscle. Contraction of the arrector pili muscle leads to ‘goosebumps’. Each follicle is associated to a sebaceous gland. The sebum secreted by this gland protects and lubricates the skin as well as maintains skin pH of about 5 (Lai-Cheong and McGrath 2009).

Eccrine sweat glands are distributed all over the body surface but not on the lips, external ear canal, clitoris, or labia minora. They secrete hypotonic (pH 5) sweat consisting mostly of

water and electrolytes. Their main function is the control of body temperature. Sweat lowers body temperature by dissipation of heat by evaporation. Eccrine sweat gland is a single tubular structure consisting of a secretory portion and a ductal portion. Apocrine sweat glands are larger than eccrine sweat glands. They are distributed on the axillae, areola of nipple, and genital areas. Apocrine sweat glands become active at the puberty and secrete proteinaceous viscous sweat. Apocrine sweat glands are composed of three segments; the secretory portion, the intradermal duct, and the intraepithelial duct. Ducts of apocrine sweat glands open to hair follicles whereas ducts of eccrine sweat glands open to the surface of the skin through the epidermis (Saga 2002).

The human nail, equivalent to claws and hooves in other mammals, evolved as our manual skills developed and protects the delicate tips of fingers and toes against trauma, enhances the sensation of fine touch and allows one to pick up and manipulate objects. The nail plate is the most visible part of the nail apparatus, consists of tightly packed dead cells and is highly keratinised (Murdan 2002).

1.1.1.4 Hypodermis

The deepest layer of the skin is the subcutaneous tissue or hypodermis. Hypodermis is mainly used for fat storage in adipose cells. Apart from storing energy, this tissue serves as a mechanical cushion and protects the body from the cold. This layer is a network of fat cells arranged in lobules and linked to the dermis by interconnecting collagen and elastine fibers.

1.2 Skin drug delivery

Drug delivery in the skin can be decided for a topical (local effects) or transdermal (systemic effects). Local effects may be interesting for the treatment of a local affection or to avoid some undesirable effects. In case of systemic effects, skin delivery may be an alternative to other routes.

1.2.1 Skin penetration

Drug penetration across the skin has three possible pathways: Hair follicles and their associated sebum glands, sweat glands and permeation across the stratum corneum. Intact stratum corneum presents an important barrier against hydrophilic and ionized drugs. Its 'brick and mortar' structure is analogous to a wall.

Viable layers may metabolise a drug, or activate a prodrug. The dermal papillary layer is so rich in capillaries that most drugs clear within minutes. Usually, deeper dermal regions do not significantly influence absorption (Barry 2001; Hadgraft 2001).

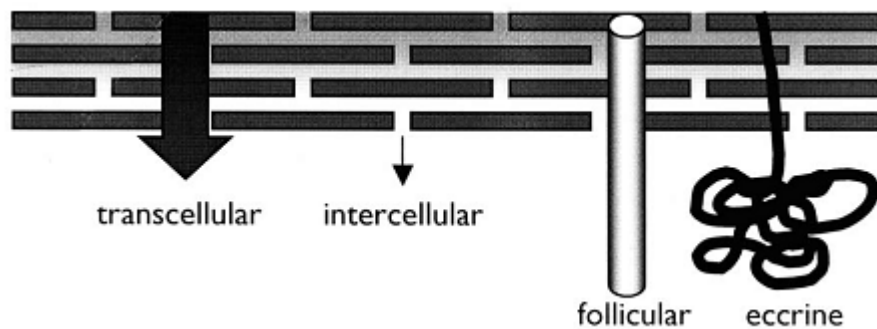


Figure 43: Schematic representation of possible penetration routes through the stratum corneum (Hadgraft 2001).

1.2.2 Transdermal drug delivery possibilities

Transdermal drug uptake is described by the Fick's first law of diffusion (equation 9).

$$J = \frac{DK\Delta C}{h} \quad \text{Equation 9}$$

J: flux per unit area

D: diffusion coefficient in the skin

K: stratum corneum-vehicle partition coefficient

ΔC : Concentration difference across the skin

H: skin thickness

According to this equation, good drug candidates to transdermal delivery should present a molecular weight below 500 Da, moderated lipophilicity (logP 1-3), a melting point below 200°C, an aqueous solubility higher than 1 mg/ml and an important pharmacological activity. Based on that, only few drugs are suitable for skin permeation (Barry 2001; Hadgraft 2001; Benson and Namjoshi 2008). Due to the poor permeability of the skin, several strategies have been developed to enhance drug passage across the skin.

Different methods have been studied such as physical methods, chemical methods and formulation methods using drug carriers.

1.2.2.1 Physical methods

The mechanism of action of this kind of methods is the barrier disruption by the formation of pores on the SC or by the lipidic bilayer perturbation.

- **Electroporation**

Electroporation causes a transitory structural perturbation of lipid bilayer membranes by the application of high voltage pulses. Electrical exposures typically involve electric field pulses of 100-150 V that generate transmembrane potentials of 0.5–1.0 V during a short time (10 μ s-10ms). It has been hypothesized that these rearrangements consist of temporary aqueous pathways, with the electric field inducing pore formation and providing a local driving force for molecular transport (Denet, Vanbever et al. 2004).

- **Iontophoresis**

Transdermal iontophoresis is a technology that enhances drug transport across the skin barrier with the assistance of an electric field (Yan, Li et al. 2005). The application of a low level electric current either directly to the skin or indirectly via a dosage form enhances drug permeability. For iontophoretic drug delivery, charged drugs are contained in an electrolyte

solution. The generated electric field forces charged molecules to move down their electromotive gradient (Riviere and Heit 1997).

- **Laser radiation**

Laser radiation is a method to remove SC. Laser ablation or removal of SC enhances transdermal delivery of hydrophilic drugs. Laser radiation can be controlled in terms of intensity and exposure which allows a precise ablation of SC. Moreover, the duration of laser operation is in the range of nano to microseconds, indicating that transdermal delivery is performed in a very short time scale

- **Microneedles**

A more sophisticated approach to physically overcome the stratum corneum's barrier is the use of microneedle systems. These devices, included into a patch, are equipped with a multitude of microneedles that insert the drug underneath the stratum corneum. Microneedles can be defined as solid or hollow cannula with an approximate length of 50–900 μm and an external diameter of not more than 300 μm (Bariya, Gohel et al. 2012). Either drug coated silicone needles or hollow metal needles (filled with drug solution) can be used for that purpose (Gill and Prausnitz 2007). The needles penetrate the horny layer without destroying it and release the drug. Clinical studies report only minimal patient discomfort, low skin irritation and low erythema incidence (Prausnitz 2004).

- **Sonophoresis**

This method uses ultrasounds to increase drug permeability. The mechanism of action is the creation of gaseous cavities within intercellular lipids on the exposure to ultrasounds resulting in a SC disruption. Transdermal transport enhancement induced by low-frequency ultrasound

($f < 100$ kHz) has been found to be more significant than that induced by high frequency ultrasound (Mitragotri and Kost 2004).

1.2.2.2 Chemical methods

Penetration enhancers are widely used to increase transdermal delivery. Penetration enhancers are chemicals that interact with skin constituents to promote drug flux. A good penetration enhancer should have some properties such as: non toxic, non irritant, non allergenic; ideally should work rapidly and the duration of action and activity should be predictable and reproducible; should not have pharmacological activity, should be compatible with other excipients and drugs. When a penetration enhancer is removed, skin barrier should keep intact (Williams and Barry 2004). There are different chemical structures which can act as penetration enhancers.

- **Alcohols**

Ethanol is commonly used in many transdermal formulations. Ethanol acts as a solvent (it can increase drug solubility in the vehicle). It can also alter solubility properties in the tissue, modifying the partition of drug in the membrane (Williams and Barry 2004).

- **Sulphoxides**

Dimethyl sulphoxide (DMSO) is a powerful aprotic solvent with a high dielectricity constant because of the S-O bond. The dissolving power of DMSO is able to generate solvent filled spaces in the Stratum corneum where drug solubility is increased. Otherwise, the lipidic structure of stratum corneum is disturbed by the action of DMSO when administered in concentrations above 60% (Trommer and Neubert 2006).

- **Azone**

Azone and its derivatives are the first molecules, which were specifically designed as penetration enhancers. It is a highly lipophilic compound ($\log P=6.2$). Azone has low toxicity and irritating power and no pharmacological activity. The mechanism of action is probably an intercalation into structured lipids in stratum corneum, thus perturbing its structure (Niazy 1996).

- **Terpenes**

Terpenes are non-aromatic compounds only made of carbon, hydrogen and oxygen. They are found in essential oils. Numerous terpenes have long been used as medicines, flavourings and fragrance agents. Terpenes and its derivatives are highly lipophilic compounds with a high partition coefficient. They increase the percutaneous penetration of drugs by increasing the diffusivity of the drug across the stratum corneum. They are a popular choice for formulators because the FDA classifies them as GRAS (generally regarded as safe), which facilitates their approval (Walker and Smith 1996).

- **Urea**

Urea is used most commonly as a hydrating agent and is employed in the treatment of ichthyosis, psoriasis, and other hyperkeratotic skin conditions. It has been suggested that urea increases the water content of the skin and also acts as a mild keratolytic agent, which could affect the stratum corneum corneocytes. These actions led researchers to believe that urea would increase the penetration of drugs through the skin. However, the application of urea and its derivatives as penetration enhancers is limited by their inadequate chemical stability, and irritating effects (Godwin, Player et al. 1998).

- **Water**

Water is one of the most effective and safest penetration enhancer. By simple hydration of the stratum corneum, the penetration of most drugs can be increased. Normally in the stratum corneum the water content is 5–10%. The water content can be increased up to 50% under occlusive conditions. Furthermore, moisturizers can be used to increase the hydration of the stratum corneum and in consequence to improve the diffusion of hydrophilic drugs (Trommer and Neubert 2006).

1.2.2.3 Drug carriers

Controlled and reproducible drug delivery across the skin barrier can be achieved by the use of nanosized carriers. Drugs are entrapped within a phospholipid or polymeric particle delivery system. These carriers must have a semi-solid consistence adapted to dermal application (Lippacher, Müller et al. 2004).

- **Lipid carriers**

Liposomes are mostly made of cholesterol and phospholipids but other amphiphilic compounds can be included in their formulation. They are highly biocompatible and biodegradable due to their composition. These carriers have the ability to deliver molecules with different physicochemical properties (Neubert 2011).

Niosomes are non-ionic surfactant vesicles, formed from the self-assembly of non-ionic amphiphiles in aqueous media. Niosomes presents a bilayer structure similar to liposomes. They are able to entrap both lipophilic and hydrophilic drugs. Topical application of niosomes can increase residence time of drugs in stratum corneum. Niosomes have been used in cosmetics, topical delivery and diagnostics (Uchegbu and Vyas 1998; Azeem, Ahmad et al. 2008; Cosco, Celia et al. 2008).

Transfersomes are ultradeformable vesicles consisting of phospholipids and an edge activator. An edge activator is often a single chain surfactant able to destabilize the lipid bilayer of the vesicles and increase its deformability by reducing the interfacial tension. Transfersomes allow efficient permeability due to their high deformability. They penetrate spontaneously the skin barrier allowing drug transport (Cevc 2004; Li, Zhai et al. 2008).

Ethosomes are soft vesicles made of phospholipids (phosphatidylcholine), ethanol at relatively high concentration and water. Ethosomes present high encapsulation efficiency for both hydrophilic and lipophilic compounds. Ethanol allows the fluidity, malleability and stability of ethosomes. These characteristics produce a disorganization of lipidic bilayer in the stratum corneum. The penetration of soft vesicles through the disorganized SC lipid bilayers can more easily occur, and as such, a pathway can be forced through the skin to allow fusion of ethosomes with the cells in the deeper skin layers (Touitou, Godin et al. 2000).

• **Polymeric carriers**

Polymer nanoparticles have potential applications in improving the transport of drugs and biomacromolecules through the skin. There are not a lot of studies concerning the transdermal delivery of drugs which can explain that skin permeation mechanism by polymeric nanoparticles is not yet clear (Li, Zhai et al. 2008). The use of a large series of polymers is limited by their bioacceptability. In fact, the most used polymers in particle formulation are chitosan, poly(lactic acid) (PLA), poly(glycolic acid) and their copolymers, poly(lactide-co-glycolide) (PLGA) which are recognised as biocompatible (Cosco, Celia et al. 2008).

1.3 Gels

A semisolid preparation such as a gel may be suitable for topical use due to the application facility which can increase patient compliance.

A gel is defined as a viscoelastic solid-like material comprised of an elastic three-dimensional cross-linked network and a solvent which is the major component (Sangeetha and Maitra 2005). Gels can be classified in different ways depending on their origin, constitution, crosslinking agent and solvent nature. In this review gels will be classified as a function of solvent nature.

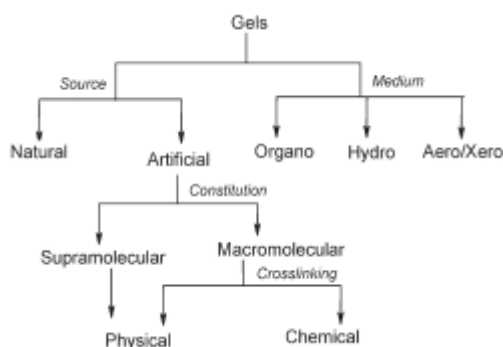


Figure 44: General classification of gels (Sangeetha and Maitra 2005).

1.3.1 Hydrogels

Hydrogels are polymeric networks, which absorb and retain large amounts of water. Hydrophilic groups are hydrated in an aqueous environment thereby creating the hydrogel structure. Crosslinker agents have to be present to avoid the dissolution of hydrophilic chains in the aqueous phase. The formation of a gel can be made either for chemical reactions or physical interactions. When gels are chemically cross-linked, they are formed by strong chemical bonds, they cannot be dissolved and are thermally irreversible. Whereas, physical gels, which are formed by non covalent interactions such as H-bonding, hydrophobic forces or van der Waals interactions, can be readily transformed in fluids by heating and are generally thermally reversible. There are large panels of crosslinkers, which allow the formation of gels.

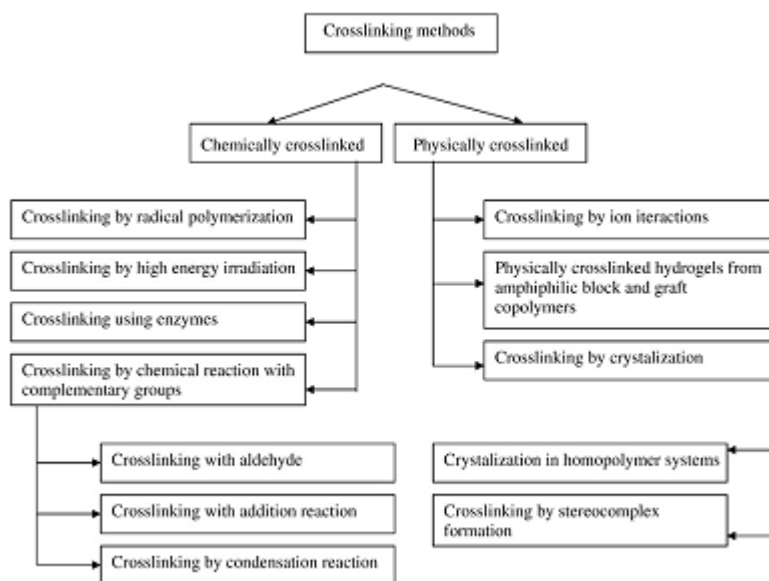


Figure 45: Different crosslinking methods available for gels formation (Hamidi, Azadi et al. 2008).

1.3.1.1 Chemically crosslinked gels

Chemically crosslinked gels can be obtained by radical polymerization of low molecular weight monomers in the presence of crosslinking agents. Crosslinked HEMA (2-hydroxyethyl methacrylate) hydrogel was first described by Whichterle and Lim in 1960.

Crosslinking can also be achieved by the chemical reaction of complementary groups. By this method, functional groups such as carboxylic groups or amines present in polymers can react with functional groups with complementary reactivity.

High energy radiation, especially gamma and electron beam, can be used to polymerize unsaturated compounds. Radiation techniques, due to the additive-free initiation and easy processability, are very suitable tools for the synthesis of the hydrogels, used in biomedical applications (Abd El-Mohdy and Safrany 2008; Singh and Kumar 2008).

Crosslinking can also be obtained by the use of enzymes. By the action of oxidative enzymes as lactase or peroxydase, peptidic hydrogels can be obtained. The use of this kind of gels have

been investigated for drug delivery, cell culture as well as in food engineering (Kuuva, Lantto et al. 2003; Bakota, Aulisa et al. 2011).

1.3.1.2 Physically crosslinked gels

Chemical crosslinkers are often toxic compounds, which should be usually removed from gels after preparation. They can also degrade drug molecules during gel manufacturing. Physical crosslinking gels have received an increased interest during last years because the use of chemical crosslinkers is avoided. To create physically cross linked gels, different approaches have been investigated.

Ionic interactions between a cationic molecule and an anionic compound allow the formation of physical gels. There are several polymers, which can be crosslinked by this method. Alginate (anionic copolymer obtained from sea weed) is a well-known example where cross-linking can be carried out at room temperature and physiological pH. Chitosan (biopolymer consisting in glucosamine units) can be cross-linked by the addition of metallic ions (Van Tomme, van Steenberg et al. 2005).

Amphiphilic block and graft copolymers are able to self-assemble in water to form organized structures like polymeric micelles and hydrogels. Block polymers forms mainly in situ hydrogels, able to be injected for delivery purposes (He, Kim et al. 2008). They are usually composed by a PEG block due to its biocompatibility. PEG is grafted to poly(ϵ) caprolactone (PCL), poly(lactide-co- glycolyde acid) (PLGA) (Shim, Kim et al. 2006).

Crosslinking can be achieved by cristallization. There are two different methods to obtain gels: Crystalization in homopolymer systems or crosslinking by stereocomplex formation.

Homopolymers like poly(vinylalcohol) or dextran can form hydrogels. Spontaneous crystallization in water has been observed for both polymers. It is suggested that hydrogen bonds form physical cross-links that contribute to the gel formation (Stenekes, Talsma et al. 2001).

Stereocomplexes are formed by a stereoselective interaction between two complementing stereoregular polymers that interlock and form a new composite, demonstrating altered physical properties compared to the parent polymers. The main interactions, resulting in the complexation, are suggested to rely on stereoselective Van der Waals forces. The complementing polymer molecules can be a pair of isotactic and syndiotactic, not necessarily optically active polymers, or two opposite enantiomeric, optically active polymer chains with identical chemical compositions. A third group consists of hetero-stereocomplexes formed by polymers from different polymer families (like polyesters and polyamides) having opposite stereoregular configuration (Slager and Domb 2003; Van Tomme, Storm et al. 2008).

1.3.2 Organogels

Organogels are semi-solid systems, in which an organic solvent is entrapped by a three-dimensional network composed by self-assembled gelator fibers.

An organogel is usually prepared by heating a gelator into an organic liquid until solid dissolution. Solution is then cooled below gelation transition temperature (Terech and Weiss 1997).

In the pharmaceutical field, organogels can be used for drug and vaccine delivery but only a few numbers of these gels are still studied. Most of the existing organogels are composed of pharmaceutically unacceptable organic liquids and/or gelators.

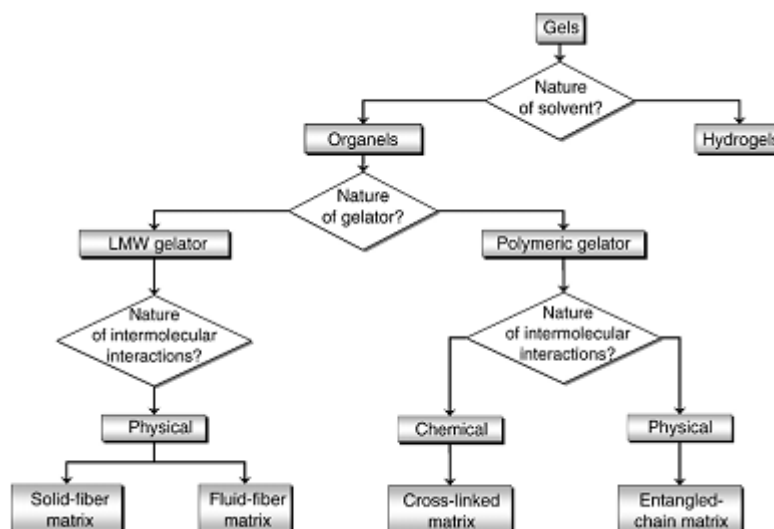


Figure 46: Classification of organogels (Vintiloiu and Leroux 2008).

Depending on intermolecular interactions, organogels can be described as physical or chemical gels. To prepare organogels there are two types of organogelators: low molecular weight organogelators and polymeric gelators.

1.3.2.1 Low molecular weight gelators

Low molecular weight gelators are characterized by good solubility upon heating and inducement of smooth gelation of organic fluids at a low concentration. Since gelation occurs as a result of noncovalent interactions, the physical gels from low molecular weight gelators always exhibit thermally reversible behaviour (Hanabusa, Matsumoto et al. 2000).

Organogels prepared by LMW gelators should be divided in solid and fluid fiber matrix. The solid fiber forms strong organogel due to their permanent solid-like networks in which junction points are relatively large (pseudo)crystalline microdomains. Fluid-matrix gels are formed upon the incorporation of polar solvents to organic solutions of surfactants. In these networks, junctions' points are often simple chain entanglements (Vintiloiu and Leroux 2008).

Low molecular weight organogelators include fatty and amino acids (Hanabusa, Matsumoto et al. 2000; Pal, Ghosh et al. 2007), steroids, organometallic compounds (Terech and Weiss

1997), amide or urea compounds (Akazawa, Uchida et al. 2008), nucleotides and dendrimers (Xu, Chen et al. 2011).

1.3.2.2 Polymeric gelators

One of the important factors for organogelation by polymers is to make physical crosslinking points by relatively strong supramolecular interactions. Such crosslinking points are formed by a conformational change in the polymer backbone or by the addition of crosslinking agents. Polymer organogelators were classified into two categories: supramolecular crosslinking and self-assembling systems. Polystyrene and poly(methyl methacrylate), as well as poly-(ester)s, have organogelation abilities for organic solvents (Suzuki and Hanabusa 2010).

1.3.3 Aerogels

Aerogels are transparent, highly porous and low density foams. Aerogels are structures in which the solvent (organic or inorganic) is replaced by a gas. The microstructure, comprised of nano-sized pores and linked primary particles, as well as the elemental composition can be tailored by solution chemistry via a process known as the sol-gel method. As a result of this unique microstructure, these light-weight materials exhibit many interesting and unusual properties (Fricke and Tillotson 1997). Silica aerogels and carbon aerogels are the most studied aerogels (Loira-Pastoriza, Sapin-Minet et al. 2012).

1.4 *Low Molecular Weight Heparin*

1.4.1 Historical background

Heparin was discovered by McLean in 1916. After early clinical trials in 1930s and 1940s, heparin has been used clinically as an antithrombotic agent for several decades. Heparin is an inexpensive and highly effective drug for the prophylaxis and treatment of various thrombotic disorders. The therapeutic benefits of heparin are limited by an increased risk of bleeding.

Low molecular weight heparins were developed for clinical use in 1980s with the intention of overcoming the therapeutic limitations of heparin. Pre-clinical data suggested that LMWH was superior to heparin from biologic and pharmacokinetic standpoints. Biologically, animal experiments suggested that the therapeutic window would be larger for LMWH, resulting in a lower risk of bleeding than an equally effective dose of heparin. Nevertheless, clinical trials of venous thromboembolism treatment have not demonstrated statistically significant differences in bleeding rates with the use of LMWH or heparin. Furthermore, it was suggested that LMWH has a more predictable and favourable pharmacokinetic profile than heparin (Morris 2003; Gray, Mulloy et al. 2008).

1.4.2 Obtention of LMWH

Low molecular weight heparin is obtained by depolymerisation of unfractionated heparin obtained from porcine intestinal mucosa or bovine lung heparin. The depolymerisation of heparin to prepare LMWH can be accomplished by chemical, enzymatic, physical and radiochemical methods. The resulting LMWH exhibit marked differences in their chemical and biological activity (Bick, Frenkel et al. 2005). As an example, chemical depolymerisation results in partial desulfation, reduction in charge density and lowering of antithrombin III binding sites. Only tinzaparin is obtained by enzymatic depolymerisation by heparinase.

| INN | Trade Name | Manufacturer/Supplier | Method of Preparation |
|------------|-------------------|--------------------------------------|---|
| Nadroparin | Fraxiparin | Sanofi (Paris, France) | Optimized nitrous acid depolymerization |
| Enoxaparin | Clexane, Lovenox | Rhône Poulenc (Paris, France) | Benzoylation and alkaline hydrolysis |
| Dalteparin | Fragmin | Pharmacia/Upjohn (Stockholm, Sweden) | Nitrous acid depolymerization |
| Ardeparin | Normflo | Wyeth Ayerst (Philadelphia, PA, USA) | Peroxidative cleavage |
| Tinzaparin | Logiparin/Innohep | Novo and Leo (Copenhagen, Denmark) | Heparinase digestion |
| Certoparin | Sandoparin | Novartis (Nürnberg, Germany) | Isoamylnitrate digestion |
| Reviparin | Clivarin | Knoll AG (Ludwigshafen, Germany) | Optimized nitrous acid digestion |

INN = international nonproprietary name.

Table 40: Example of depolymerisation methods (Fareed, Jeske et al. 1998).

Unfractionated heparins present a molecular weight comprised between 17-20kDa. A large variety of LMWH are obtained by depolymerisation. The obtained molecular weight

corresponds to one third of original heparin. Typically, these molecules have molecular masses of 4 – 8 kDa and each one has distinct structural and functional properties depending on polymerization method (Fareed, Jeske et al. 1998; Gray, Mulloy et al. 2008).

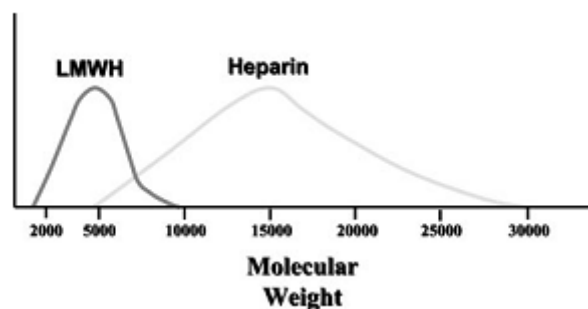


Table 41: Molecular weight distribution of LMWH and Heparin (Hirsh and Raschke 2004).

1.4.3 Chemical structure

Heparins are polydispersed anionic mucopolysaccharides owing to glycosaminoglycans family. They are formed by saccharidic linear chains with variable size and strongly acidic. Heparin is formed by repeating disaccharide groups formed by glucosamine and glucuronic and iduronic acid. The anticoagulant activity of heparin is reported to be dependent on a specific sequence with high affinity for Antithrombin III. This sequence is characterized by a pentasaccharide containing three glucosamins and two uronic acids. This sequence is not present in all heparin molecules. Furthermore, LMWH contains the specific pentasaccharide sequence but in lower proportion than the parent heparin (Hirsh 1995; Gray, Mulloy et al. 2008).

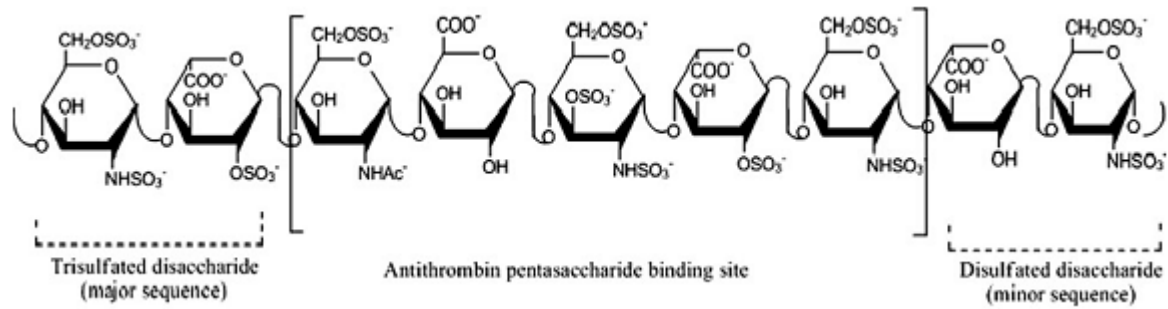


Figure 47: General structure of heparin containing the Antithrombin pentasaccharide binding site (Bhaskar, Sterner et al. 2012).

1.4.4 Mechanism of action

The mechanism of action of LMWH is analogue to the UH. As well as UH, the LMWH anticoagulant effect is not directed but mediated by a cofactor, the physiological coagulation inhibitor antithrombin III (ATIII).

Administered in pharmacological doses, 30% of UH binds to ATIII with high affinity, thus leading to a conformational change, which converts ATIII from a slow to a very rapidly acting inhibitor of thrombin. After thrombin fixation, the affinity between heparin and its cofactor decreases and thus heparin is released to interact with a new ATIII molecule.

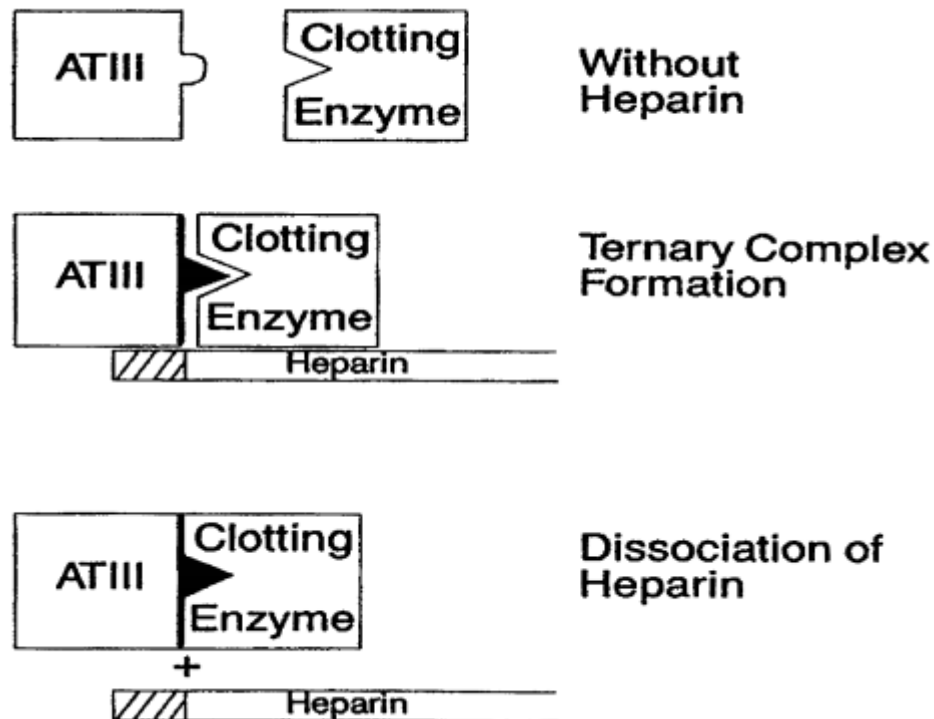


Figure 48: Inactivation of clotting enzymes mediated by heparin: Top panel: ATIII is a slow inhibitor without heparin. Middle panel, heparin binds ATIII through pentasaccharide sequence inducing a conformational change. ATIII becomes then a rapid inhibitor. Bottom panel, ATIII binds covalently to the clotting enzyme and the heparin dissociates from the complex and can be reutilized (Hirsh, Warkentin et al. 2001).

Apart from thrombin, ATIII interacts with coagulation factor Xa, and other components of plasmatic haemostasis such as factors IXa, XIa and XIIa, plasmin, kallikrein and trypsin. The pentasaccharide sequence is necessary for binding heparin to ATIII. The inactivation of thrombin by the heparin–ATIII complex needs a heparin molecule composed of at least 18 monosaccharides. In contrast, smaller molecules containing the pentasaccharide sequence are sufficient to inhibit factor Xa. This may be the reason for the increased activity of LMWH in Xa inhibition (Hetzl and Sucker 2005).

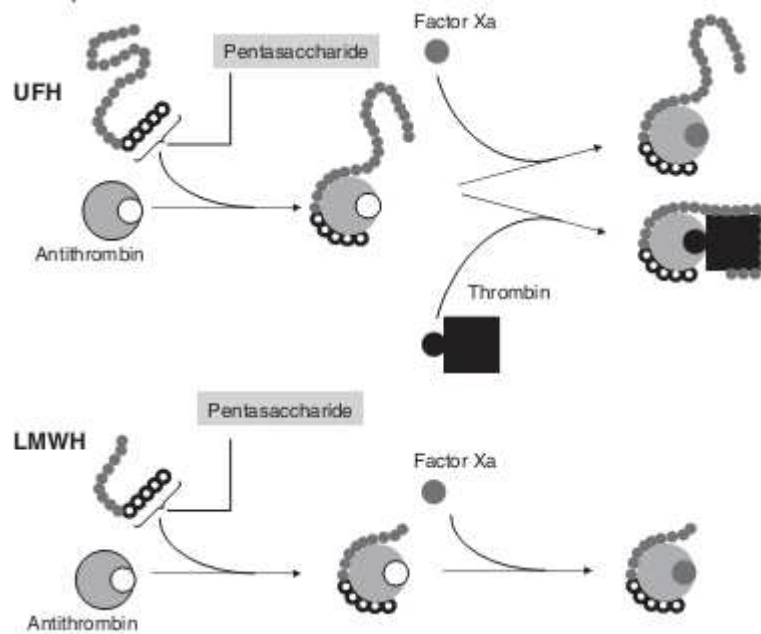


Figure 49: Mechanism of action of LMWH vs. UFH (Hetzl and Sucker 2005).

1.4.5 Heparin activity

Heparin has a large number of pharmacological properties. Moreover, the most important activities of heparin are the anticoagulant and antithrombotic effects. Other reported biological activity is related with antitumoral, antiviral and antibacterial activity.

Heparin is used in the therapy of several cardiovascular disorders including prevention and treatment of arterial and venous thromboembolism, treatment of instable angina, acute myocardial infarction, cardiac and vascular surgery, coronary angioplasty and stent implantation. Heparin is also the anticoagulant of choice during pregnancy because it does not cross the placenta (Bick, Frenkel et al. 2005).

Heparin binds a multitude of physiological substances due to its large and negatively charged structure. Heparin has been proposed to have a regulatory role in limiting inflammation. Moreover, the binding to several proteins such as chemokines, growth factors, adhesion and cytotoxic molecules, has been reported to play anti-inflammatory effects in asthma, ulcerative colitis and interstitial cystitis (Ragazzi and Chinellato 1995; Day, Landis et al. 2004).

Heparin presents an antitumoral activity. In patients with cancer, coagulation proteases play a significant role in tumor biology. The anti-cancer activity of heparins depends more on inhibition of metastasis formation than on the effects on primary tumour growth (Niers, Klerk et al. 2007). It was suggested that heparins alter the distribution pattern of cancer cells by their strong negative charge. As a result of the binding of anionic heparins to cancer cells, adherence to negatively charged endothelium would be inhibited. Heparins can inhibit the proliferation of various cell types including vascular smooth muscle cells, fibroblasts and epithelial cells. The antiproliferative effects of heparins are related to the inhibition of expression of proto-oncogenes (Castelli, Porro et al. 2004).

Heparin activates lipoprotein lipase (LPL), an enzyme responsible for clearing postprandial lipemia, by enhancing conversion of chylomicrons and low-density lipoproteins to free fatty acids (FFAs) and high-density lipoproteins. Heparin and LMWH have been shown to increase LPL release with a corresponding increase in FFA, but the plasma lipolytic effect of LMWH is significantly weaker than that of heparin (Day, Landis et al. 2004).

Heparin also exhibits antibacterial and antiviral effects.

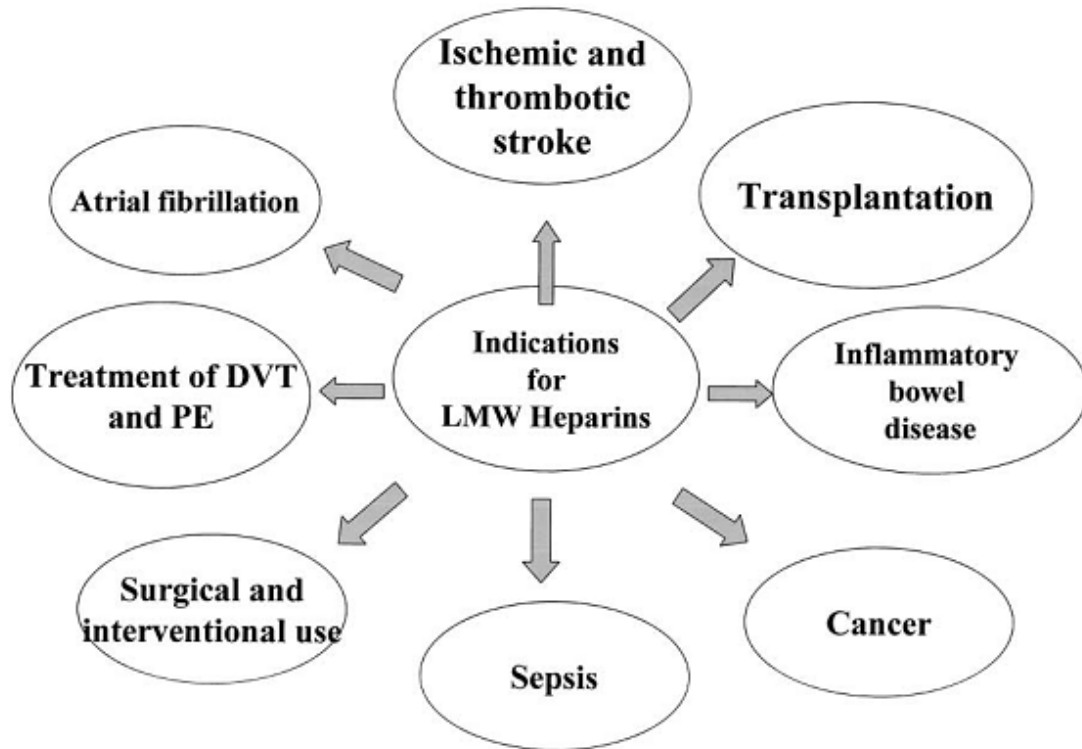


Figure 50: Indications for LMWHs (Hoppensteadt, Walenga et al. 2003).

1.4.6 Topical administration

Heparin and LMWH are typically administered subcutaneously. They are not bioavailable by oral route. The lack of oral bioavailability is mainly due to their large molecular size, hydrophilicity and surface charge. Sublingual, nasal and pulmonary routes for heparin delivery have also been investigated. However, these approaches have not been successful in delivering heparin. Transdermal route is an attractive alternative for drug delivery. Several approaches have been studied to enhance drug delivery into the skin (Lanke, Strom et al. 2009; Lanke, Kolli et al. 2009). Furthermore, transdermal heparin could be interested to the treatment of superficial thrombosis and haematomas. In addition, local application may avoid the possible side effects.

Physical methods such as iontophoresis (Pacini, Punzi et al. 2006), electroporation (Prausnitz, Edelman et al. 1995; Weaver, Vanbever et al. 1997), low frequency ultrasounds (Mitragotri

and Kost 2001) or a combination of two of the previous physical methods (Le, Kost et al. 2000) were largely studied to increase drug permeability.

Chemical methods by the use of penetration enhancers were also studied. Three penetration enhancers (laurocapram, 1,8-cineole and neurolidal) were incorporated into hydrogels containing LMWH. It was reported that these three compounds provide significant enhancing actions, azone (laurocapram) being the most effective (Xiong, Quan et al. 1996). Furthermore, non-toxic penetration enhancers such as Transcutol, soybean lecithin or d-limonene were investigated on the in vitro percutaneous absorption of heparin. Except Transcutol, the other compounds were all able to increase heparin permeability (Bonina and Montenegro 1994).

A different approach to increase drug delivery across the skin barrier is the use of lipidic carriers. Liposomes were used to increase UH and LMWH transport through the skin. However, Betz et al reported that liposomes containing Phospholipon® 80 and sphingomyelin did not have any positive effect on UH and LMWH permeation across the skin (Betz, Nowbakht et al. 2001). Nevertheless, the treatment of superficial venous thromboembolism with liposomal heparin-sprayed gel combined with compression therapy is comparable with SC injection of LMWH combined with compression therapy. Moreover, liposome therapy is less invasive than SC injections and thus more patient-friendly (Katzenschlager, Ugurluoglu et al. 2003). Overall, it is worth noting that heparin liposome based therapeutic products are commercially available (Cevc 2004).

Heparin and LMWH can be transported through the skin to obtain a systemic effect. Nevertheless, local effect may be interesting in the treatment of superficial venous thromboembolism (SVT). Effective treatment of peripheral vascular disorders is important not only for resolutions of local symptoms but also for preventing the development of systemic diseases such as deep vein thrombosis (Vecchio and Frisinghelli 2008). Topically applied heparin plays a role in the anticoagulant action and the modulation of skin

microcirculation. Moreover, heparin participates on skin permeability, allowing other drugs to diffuse better and faster into the skin (Cesarone, Belcaro et al. 2007; Cesarone, Belcaro et al. 2007).

Topical application of LMWH was not largely studied for the moment. Only few carriers such as liposomes (Song and Kim 2006) or gels (Loira-Pastoriza, Sapin-Minet et al. 2012) have been studied for LMWH topical application.

1.5 Eudragit[®] RS 30D

Eudragit[®] RS 30D (ERS) is a copolymer of Ethylacrylate /methylmethacrylate /trimethylammoniomethylmethacrylate marketed by EVONIK Industries (Essem, Germany).

The general formula is represented in figure 49.

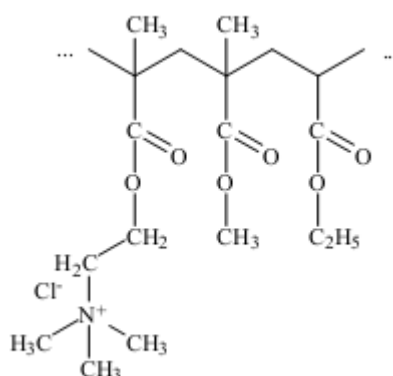


Figure 51: Chemical structure of Eudragit[®] RS 30D.

Eudragit[®] RS 30D is prepared by the copolymerization of ethyl acrylate, methyl methacrylate and trimethyl-ammonioethyl-methacrylate chloride with a mole ratio 1:2:0.1 (Lin and Yu 2000). Ester groups in polymethacrylic polymers are stable against hydrolytic attack by dilute acids or bases. Moreover, the quaternary ammonium groups in side-chains of acrylic polymers are hydrophilic in nature. The hydrophilicity of cationic quaternary groups is nearly independent of pH, within the physiological range from 1 to 8. According to its chemical structure, Eudragit[®] RS 30D is insoluble in water but swells in physiological fluids with

independency of pH. It becomes water permeable allowing drug release (Dittgen, Durrani et al. 1997; Wittaya-areekul, Prahsarn et al. 2006).

Eudragit[®] RS 30D is available as an aqueous dispersion of polymeric nanoparticles. Dispersions are composed of a 30% of solid content. Eudragit[®] RS 30D does not contain any stabilizer, however, the presence of quaternary cationic groups stabilize the dispersion (Lin and Yu 2000). Sorbic acid (0.25 %) is used as preservative and NaOH is used for pH adjustment. The polymer molecular weight is 150,000 Da. Nanoparticle dispersion present a pH comprised between 4-6 and a mean size of 130nm. Eudragit[®] RS 30D is a cationic polymer therefore its zeta potential is + 50mV. It presents a low glass transition temperature which allows the formation of very flexible films (Wittaya-areekul, Prahsarn et al. 2006).

These kind of polymeric aqueous dispersions are widely used in oral dosage forms. Eudragit[®] RS 30D is used to prepare controlled-release drug formulations, matrix tablets and as coating agents in oral solid forms. Small particles such as pellets can also be coated with the polymeric dispersion and included later in capsules or granulated to form tablets (Chen, Tsay et al. 2000; Wu and McGinity 2003). The functional groups are cationic in nature and therefore are expected to interact with anionic species (Omari, Sallam et al. 2004). The interaction of cationic polymer with ionic drugs has been studied, as well as the release behaviour through the polymer layer in coated forms (Sun, Hsu et al. 2001).

Eudragit[®] RS used as a powder or in aqueous dispersion is able to form gels for different purposes such as transdermal delivery (McGinity 1989). Gels can be obtained by the mixture either of polymers or polymeric particles with a gelling agent such as hydroxypropylmethylcellulose (HPMC) (Lucero, García et al. 1995; Labouta and El-Khordagui 2010). Ionic interactions between quaternary ammonium groups and anionic polyelectrolytes lead to hydrogels formation (Babak, Baros et al. 2008; Babak, Baros et al. 2008).

2. Objective of the work

The aim of this part of the work is to manufacture gels to be applied onto the skin. The gels prepared with LMWH, an anticoagulant may be interesting in the treatment of superficial thromboembolism and haematomas

The gel formation is based on the electrostatic interaction between Eudragit[®] RS 30, a cationic polymer) and LMWH, an anionic polysaccharide. This interaction allows the formation of physical gels.

Different concentrations of four LMWH (bemiparin, enoxaparin, nadroparin and tinzaparin) were mixed with a fixed amount of polymer nanosuspension. The interaction between polycations existing in Eudragit[®] RS and polyanions of LMWH lead to a gel of different textures depending on charges concentration.

The obtained gels will be characterized in terms of incorporation rate, in vitro release, and rheological behaviour.

Moreover, the capability to cross the skin was also studied by ex-vivo experiments such as Franz Cells technique and tape stripping.

The last step is to verify that drug remains in the skin layer. A systemic effect is not desirable in order to avoid anticoagulant effects. This effect is evaluated by in vivo experiments.

3. Materials and methods

3.1 Materials

3.1.1 Low molecular weight heparins (LMWH)

Sodium bemiparin powder (MW 3600 Da) (ROVI Pharmaceutical Laboratories (Madrid, Spain)), calcium nadroparin [Fraxiparin[®] (MW 4300 Da)] GlaxoSmithKline (Marly-le-Roi, France), sodium enoxaparin [Lovenox[®] (MW 4500 Da)] Sanofi-Aventis (Paris, France) and sodium tinzaparin [Inohep[®] (MW 6500 Da)] Leo Pharma (Vernouillet, France), (Gray, Mulloy et al. 2008) were used as model drugs.

3.1.2 Polymer

Eudragit[®] RS 30D (copolymer of ethyl acrylate, methyl methacrylate and a low content of a methacrylic acid ester with quaternary ammonium groups (trimethylammonioethyl methacrylate chloride)) (Evonik Industries, Essem, Germany) was used for its poly-cationic properties.

3.1.3 Anti-Xa activity measurement

Stachrom[®] Heparin kit (Diagnostica Stago (Asnières-Sur-Seine, France)) contains the reagents (Antithrombin III, coagulation factor Xa and substrate) necessary for the measurement of the anti-Xa activity of LMWH.

3.1.4 Animals

Male Wistar rats (mean body weight 700±100 g, Janvier, Le Genest-Saint-Isle, France) and New Zealand rabbits (mean body weight of 2500±250 g, Charles Rivers Laboratories, L'Arbresle, France) were the animal models. Guidelines and legislative regulations on the use of animals for scientific purposes were followed.

3.2 Methods

3.2.1 Gel preparation

Gels were manufactured with various concentrations of LMWH (400, 600, 800, 1000, 2000, 3000, 4000 and 5000 IU/mL): they were prepared by mixing, under magnetic stirring (200 rpm for 3 hours) at room temperature, an equal volume of an aqueous solution of LMWH with the Eudragit[®] RS 30D suspension.

3.2.2 Rheological study

The rheological measurements were performed with a Rheo Stress AR 600 rheometer (Thermo Electron Corporation, Saint Herblain, France) equipped with a cone-and-plate geometry (plate diameter 35 mm and cone angle 2°). A solvent trap was used to minimize the water evaporation. All samples were equilibrated for 30 minutes before rheological measurements. All experiments were carried out with enoxaparin from Sanofi-Aventis unless otherwise specified.

Viscoelasticity properties were determined by applying an oscillating shear stress for a given frequency of 1 Hz. The storage modulus G' and loss modulus G'' , as well as the loss angle δ ($\tan \delta = G''/G'$) were measured for each stress and frequency. When $G' > G''$ (which means $\tan \delta < 1$ or $\delta < 45^\circ$), the elastic properties are more pronounced than viscous ones and conversely. So, the values of $\tan \delta$ (or δ) are good and precise indicators of the viscoelastic nature of the gel. The lower the $\tan \delta$ or δ values, the more elastic and cross-linked the gel.

Two kinds of oscillatory tests were successively performed:

- 1) A stress sweep was carried out at a frequency of 1 Hz in a stress range of 0-100 Pa to determine the gel formation kinetic. Various gels (corresponding to 1000 to 5000 IU/mL of LMWH) were studied in order to determine the influence of LMWH concentration on viscoelastic behaviour. Measurements were performed at 20°C and 32°C in order to study

possible changes in stability or consistency as a function of temperature. These stress sweeps also allowed the linear viscoelastic region (i.e. where moduli are independent of the stress) to be identified.

2) A frequency sweep was performed by varying the angular frequency from 0.01 to 100 Hz, at a constant shear stress of 1 Pa at 20°C (this value fell within in the linear viscoelastic region).

3.2.3 LMWH incorporation into gel network

The amount of LMWH involved in the cross-linking with Eudragit[®] RS 30D was determined indirectly by measuring the amount of free LMWH contained in the aqueous phase, which was recovered after centrifugation of the gel. Thus, immediately after preparation, gels were centrifuged at 42,000g for 20 minutes at room temperature. The supernatant was recovered and the concentration of LMWH was determined by a turbidimetric assay. This method is based on the quantitative precipitation at 1:1 stoichiometry occurring between sulphate and carboxyl groups of heparin at pH 6.8 and the amine groups of cetylpyridinium chloride (Javot, Lecompte et al. 2009). All experiments were performed in triplicate. Briefly, 500 µL of supernatant were reacted for 1h at 37°C with 500 µL of acetate buffer (1 M, pH 5), followed by the addition of 2 mL of cetylpyridinium chloride (2.9 mM) in NaCl aqueous solution (168.8 mM). Precipitation was assayed by spectrophotometry at 500 nm (Uvikon 922, Kontron, Eching, Germany). The LOQ and LOD for this analytical method are 0.85 and 0.25 IU/mL respectively. The cross-linked drug was expressed as the percentage of LMWH incorporated in gels with respect to the initial LMWH concentration.

3.2.4 Release study

In-vitro release kinetics of LMWH were studied with gels that had been dried at room temperature for 48 h. Gel drying was performed to ensure that a constant mass was used for

the release study. Briefly, 50 mg of dried gel were poured into 20 mL of phosphate-buffered saline (KH_2PO_4 4.4 mM, Na_2HPO_4 045.1 mM, NaCl 100 mM, pH 7.4 adjusted by H_3PO_4) at 37°C under magnetic stirring at 200 rpm. Dissolution tests were performed in sink conditions. At selected times (5, 15, 30 minutes and 1, 2, 4, 8 and 24 h) aliquots of 1.5 mL were withdrawn and replaced by fresh buffer.

Moreover, the *in-vitro* release of LMWH from the gel structure was also evaluated by the use of different ionic strengths of phosphate buffered saline (NaCl 0.1, 0.5 and 1.0 M) as acceptor medium.

The drug released *in vitro* was evaluated by the turbidimetric assay described above and the results are presented as the percentage of drug released with respect to the initial LMWH concentration in the gel.

3.2.5 LMWH gel topical application

LMWH gel was applied topically to rats and rabbits. The localization of the drug into the skin layers as well as its plasma concentration were evaluated.

3.2.6 LMWH localization into the skin: Tape stripping

Hairless male Wistar rats (n=3) were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg). One hundred microliters of LMWH gel was applied to the square test area (2x2 cm) on the ventral skin with an approximate LMWH amount of 65 IU/cm². Application time was fixed at 1 hour. Before performing the tape stripping, the animals were sacrificed with a lethal dose of pentobarbital. Excess formulation was removed by a cotton swab. To determine the LMWH concentration in the stratum corneum (SC), 20 tapes (Scotch[®] Crystal Clear Tape, 3M, Cergy-Pontoise, France) were stuck onto the test area and carefully peeled away. The tapes were pooled in 10 mL of phosphate buffer at pH 7.4. Then samples were vortexed for 5 minutes to extract the LMWH from the SC. The resulting solution was

centrifuged at 42000 g for 20 minutes. LMWH extracted from the SC was assayed by the turbidimetric method described above.

3.2.7 Plasma determination

The gel was applied on the hairless shaved skin of male New Zealand rabbits (n=6). Eight hundred microliters of LMWH gel were applied onto a square test area (6x4 cm) of the dorsal skin with an approximate amount of 90 IU/cm² LMWH under occlusive conditions.

Blood samples were collected from the marginal ear vein at selected times (2, 4, 6, 8, 12 and 24 hours) into vials containing sodium citrate as anticoagulant. Samples were then centrifuged for 10 minutes at 3000 g to obtain clear plasma which was frozen at -20°C until further analysis.

The concentration of LMWH (bemiparin in this experiment) was determined automatically (STA Compact automate, Diagnostica Stago, France) with a chromogenic assay for factor Xa. Plasma samples (25 µL) were mixed with 50 µL of antithrombin III solution. This solution was mixed with 100 µL of factor Xa and incubated for 30 s at 37°C. Factor Xa substrate (100µL) was added to the solution and incubated at 37°C. The absorbance was determined at 405 nm every 2 s for 10 to 30 s of incubation. A linear relationship between absorbance/min and the concentration of bemiparin in the range of 0.1 to 0.8 anti-Xa UI/mL was obtained. The LOQ and LOD are 0.06 and 0.02 IU/mL respectively.

4. Results and discussion

4.1 *Gel properties*

The first step to characterise our formulations it is to verify its macroscopic appearance. Indeed, white and brilliant gels were obtained. It was noticed a sandy feeling upon skin spreading with a smooth texture.

Gels are reported to be semisolid structures although consistency depends on drug concentration added to the Eudragit[®] RS 30D suspension. Gels are much more fluids for lower concentrations of LMWH. Moreover, in case of a formulation prepared with 400 IU, a milky suspension, rather than a gel, was obtained. When the amount of LMWH was increased, the gel consistency increased up to a maximum of about 1000 IU. Above 3000 IU the gel consistency declined. These observations suggest that gel formation could be explained by electrostatic interactions between the positive charges carried by the polymer (Eudragit[®] RS 30D) and the negative charges of the active ingredient, as already reported by Hoffart et al. in the case of LMWH nanoparticles (Hoffart, Lamprecht et al. 2006). In other words, it appeared that a larger amount of negatively charge carried by LMWH would form stronger gels with the constant amount of charge in the Eudragit[®] RS 30D. Furthermore, electrostatical interactions have been largely used to form gels (Van Tomme, van Steenbergen et al. 2005; Ferstl, Strasser et al. 2011). The obtained hydrogels produce a rapid release of drug allowing the drug availability onto the skin.

4.2 *Rheological behaviour*

Rheological studies are necessary to confirm the gel structure. It is known that polymer gels build a three-dimensional network. Solvent molecules are bounded to the polymeric network

and thus, due to reduced mobility of the molecules into the structured system with increased viscosity, exhibit viscoelastic properties (Valenta and Auner 2004).

To perform oscillatory tests, linear viscoelastic region was first determined. It was shown that linear viscoelastic region was located between 0.1 to 5 Pa at a constant frequency of 1 Hz at 20°C.

The elastic or storage, G' , (reflecting the solid-like component of viscoelastic behaviour) and loss or viscous, G'' , (reflecting the liquid-like component) modulus were monitored during the gel formation (Tamburic and Craig 1995). For all concentrations (except 400 IU/mL) G' was always higher than G'' , as recorded in table 42. As indicated previously, the very low value of $\tan \delta$ (~0.18), which corresponds to a very low value of δ (~10°) for all concentrations except 400 IU/mL, show a very good elastic behaviour of the gel, which means the formation of a cross-linked gel.

| LMWH concentration (IU/mL) | G' (Pa) | G'' (Pa) | δ (degrees) | Tan δ (1Hz) |
|----------------------------|-----------|------------|--------------------|--------------------|
| 400 | 0.09 | 0.181 | 62.28 | 1.922 |
| 600 | 836 | 148 | 10.03 | 0.173 |
| 800 | 2534 | 556.8 | 12.39 | 0.190 |
| 1000 | 4609 | 860.8 | 10.58 | 0.143 |
| 3000 | 1617 | 262 | 14.02 | 0.289 |
| 5000 | 668.7 | 225.5 | 18.63 | 0.443 |

Table 42: Viscoelastic parameters for gels with different LMWH concentrations.

According to the previously described macroscopic observations, the gel was formed at concentrations higher than 400 IU/mL: the elastic and viscous moduli increased with LMWH concentration to finally reach a maximum at 1000 IU/mL. Lucero Muñoz et al. have prepared hydrogels containing a constant amount of Eudragit® RS 30D and various proportions of HPMC (Lucero Muñoz, Garcia Andréu et al. 1998). They have shown an increase in apparent viscosity when the proportion of HPMC was increased. This observation was expected but may be compared to our study since in both cases the amount of Eudragit® RS 30D was fixed.

In our case, the increase in the ionic interactions between the two oppositely charged polymers plays the role of the increasing proportion of HPMC. Gels prepared with higher concentrations of LMWH (3000 IU/mL) showed a decrease in these parameters (G' , G'' , δ). This behaviour could be explained by a progressive saturation of the positive charges present in the polymer by the LMWH sulphate groups. When saturation was attained (1000 IU/mL), the strongest gel was obtained as shown in Table 1 ($G' > G''$). The most concentrated gels (600 to 5000 IU/mL) can be considered as typical three-dimensional networks established by interparticular bounds.

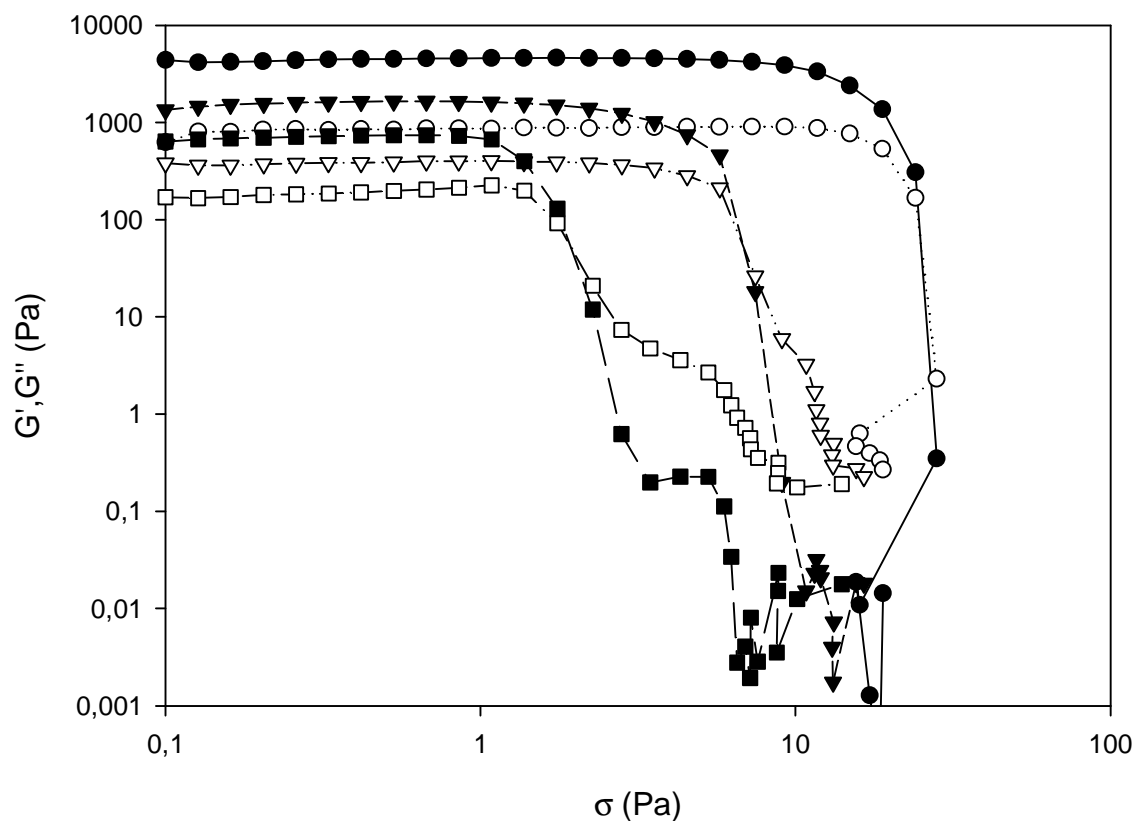


Figure 52: Evolution with time of a stress sweep representing G' at 2h (\bullet), 3h (\blacktriangledown), 5h (\blacksquare) and G'' at 2h (\circ), 3h (\triangledown), 5h (\square) as a function of stress (Pa) of enoxaparin gels prepared with 1000 IU/ml of LMWH.

Figure 50 shows the gel formation at a concentration of 1000 IU/mL as a function of time and stress. It can be observed that gel is already structured after 2 hours of stirring since $G' > G''$. Nevertheless, an additional hour of stirring (3 hours total) leads to the complete formation of

the gel since G' and G'' values are at their maximum. Total stirring of 5 hours before performing the G' and G'' measurements does not change the gel overall structure. However, the resistance to stress was better for the 3-h mixing time since the breakdown point was around 40 Pa whereas it was only about 20 Pa for 5 hours. This observation confirms that a mixing time of 3 hours for the two oppositely charged polymers corresponds to the optimal preparation conditions, which were adopted for all the gel preparations used in this study. The 40 Pa threshold is very high, enough to insure very good stability at rest. Results of literature show that a yield about 1 to 2 Pa is often sufficient to stabilize colloidal systems as emulsions against sedimentation or creaming (Benna-Zayani, Kbir-Ariguib et al. 2008).

When different LMWH concentrations (1000, 3000 and 5000 IU/mL) were compared at 20°C (Figure 51a), it appears that the gel prepared with the lowest LMWH concentration presented a more solid-like behaviour (G' of 1000 IU/mL is the highest value of the graph). This gel is also the most resistant to strain. The magnitude order of solid-liquid behaviour is 1000 > 3000 > 5000 IU/mL. As also shown in Figure 2a the resistance to rupture is lower for the most concentrated gels (i.e. 20 Pa and 10 Pa for the 3000 and 5000 IU/mL respectively). At 32°C (Figure 51b), gels present the same solid-liquid behaviour order but a more solid-like appearance for the three concentrations, which could be explained by the increase in viscosity of Eudragit[®] RS 30 D with temperature. Indeed, Bonacucina et al. have observed an increase of G' with gels prepared with Carbopol. This module was varied from 79 to 300 Pa at 20 and 60°C respectively (Bonacucina, Cespi et al. 2006). Regardless of concentration, G' and G'' were higher at 32°C than 20°C. The gels also showed a more resistant network for all concentrations compared with 20°C.

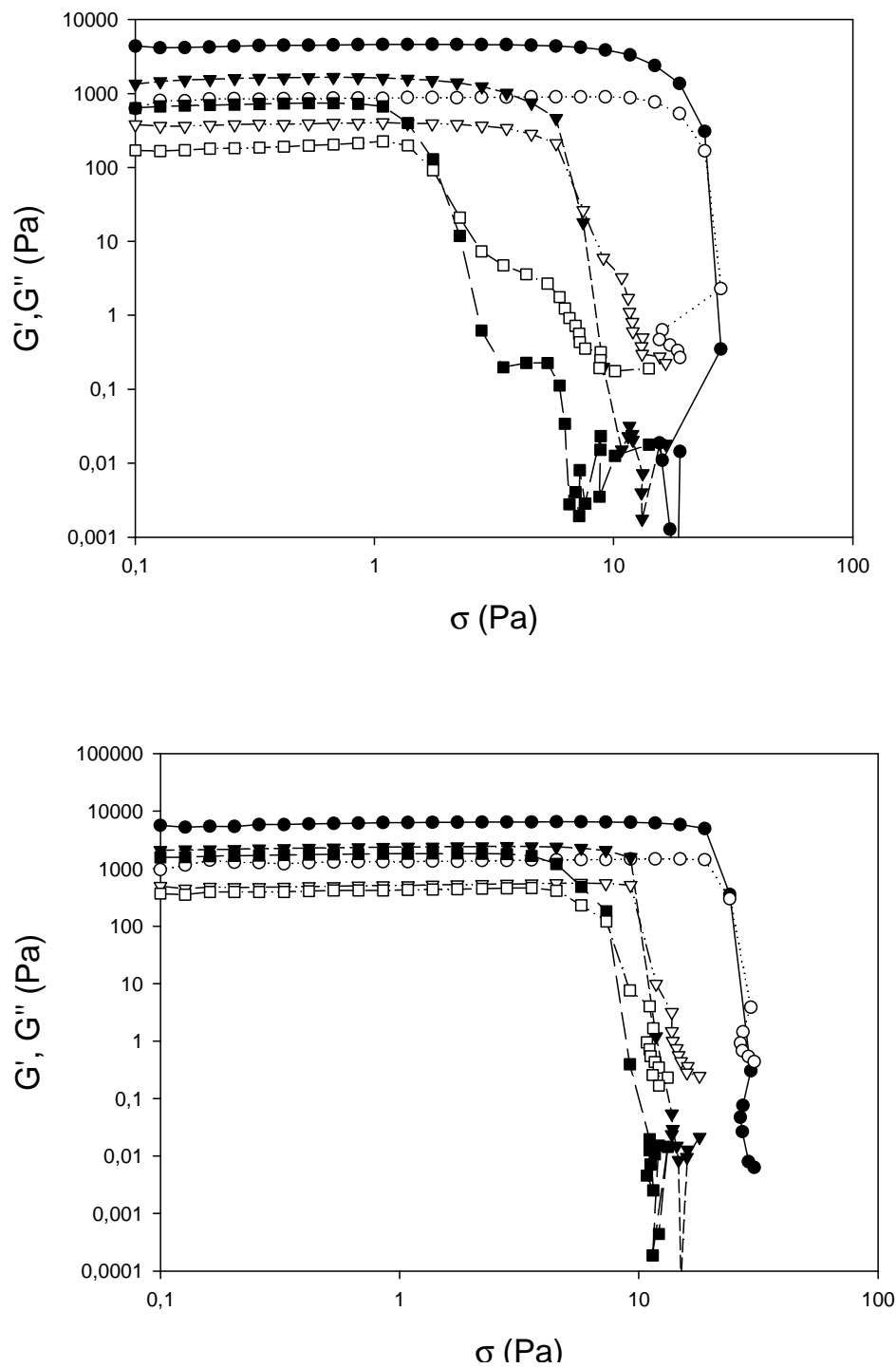
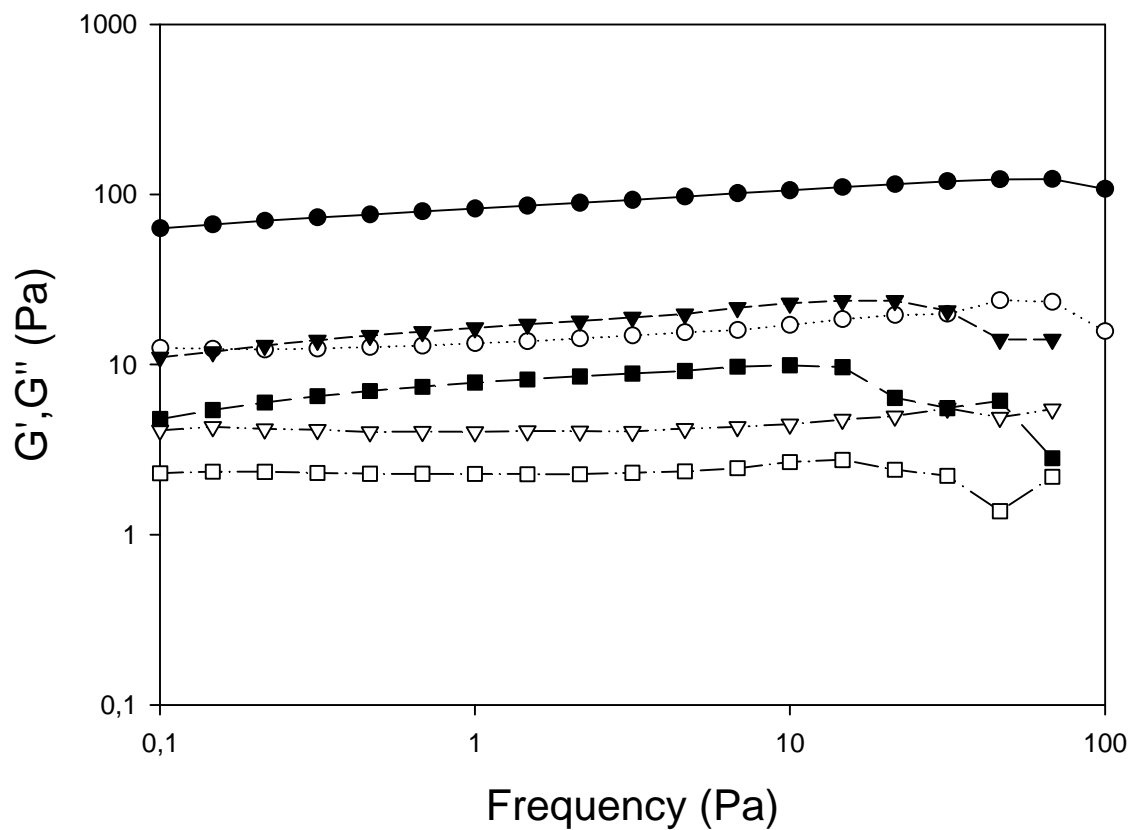


Figure 53: Comparison of stress sweep performed at 20 °C (a) and 32 °C (b). G' (1000 IU/mL (●), 3000 IU/mL (▼), 5000 IU/mL (■)) and G'' (1000 IU/mL (○), 3000 IU/mL (▽), 5000 IU/mL (□)). Results are expressed as mean \pm SD (n=3).

A frequency sweep test was performed at constant stress of 1 Pa. G' and G'' were measured. As observed in figure 52a both the dynamic storage modulus (G') and the viscous modulus (G'') did not depend on the frequency. Similar behaviour was observed at 32°C (figure 52b). This confirms the viscoelastic behaviour of the gels prepared, irrespective of their concentration (Ma, Xu et al. 2008).



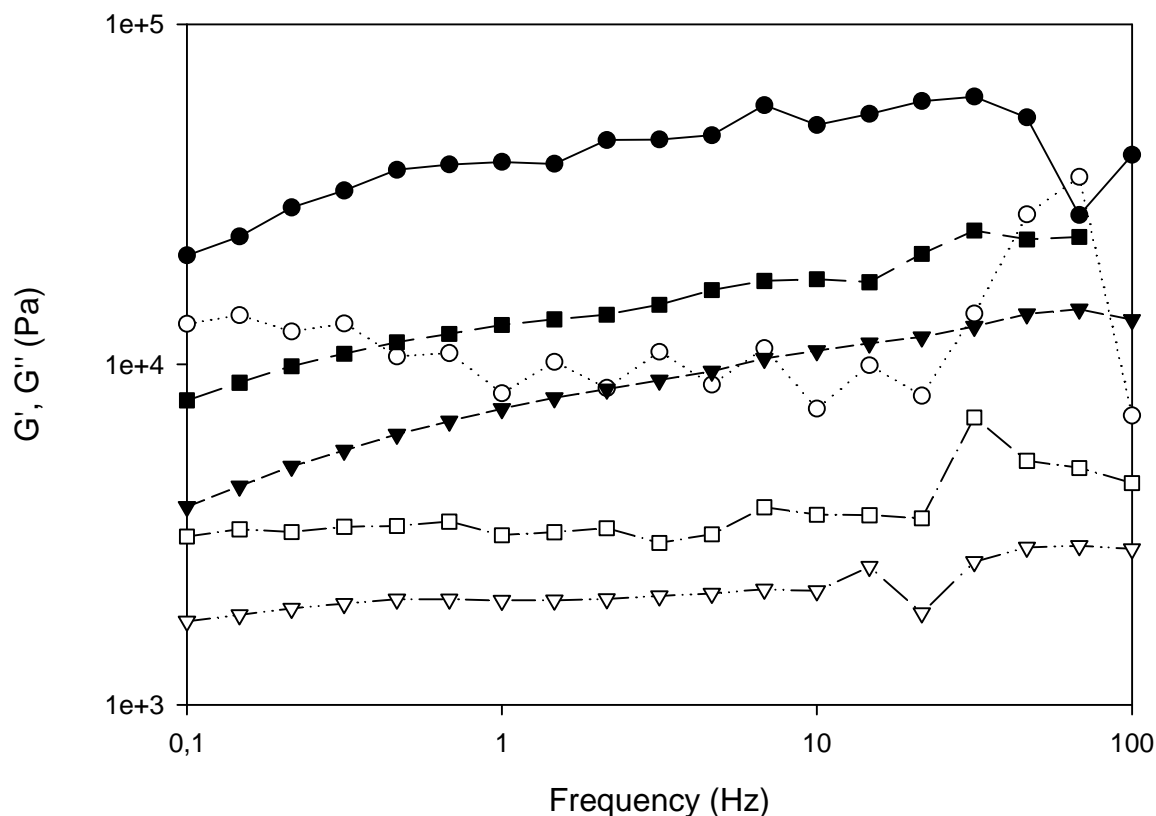


Figure 54: (a) Frequency sweep performed at 20°C. G' (1000 IU/mL (●), 3000 IU/mL (▼), 5000 IU/mL (■)) and G'' (1000 IU/mL (○), 3000 IU/mL (▽), 5000 IU/mL (□)). Results are expressed as mean \pm SD ($n=3$). (b) Frequency sweep performed at 32°C. G' (1000 IU/mL (●), 3000 IU/mL (▼), 5000 IU/mL (■)) and G'' (1000 IU/mL (○), 3000 IU/mL (▽), 5000 IU/mL (□)). Results are expressed as mean ($n=2$).

4.3 Drug incorporation into the network

The LMWH amount incorporated in the gel network was studied by indirect assay, which have been already validated in the laboratory. As shown in figure 53, an increase of LMWH incorporated in the gel network is observed when the amount of LMWH added to the gel during preparation was increased. AThe whole added drug (100%) was incorporated into the gel prepared with LMWH concentrations of 1000 to 3000 IU/mL.

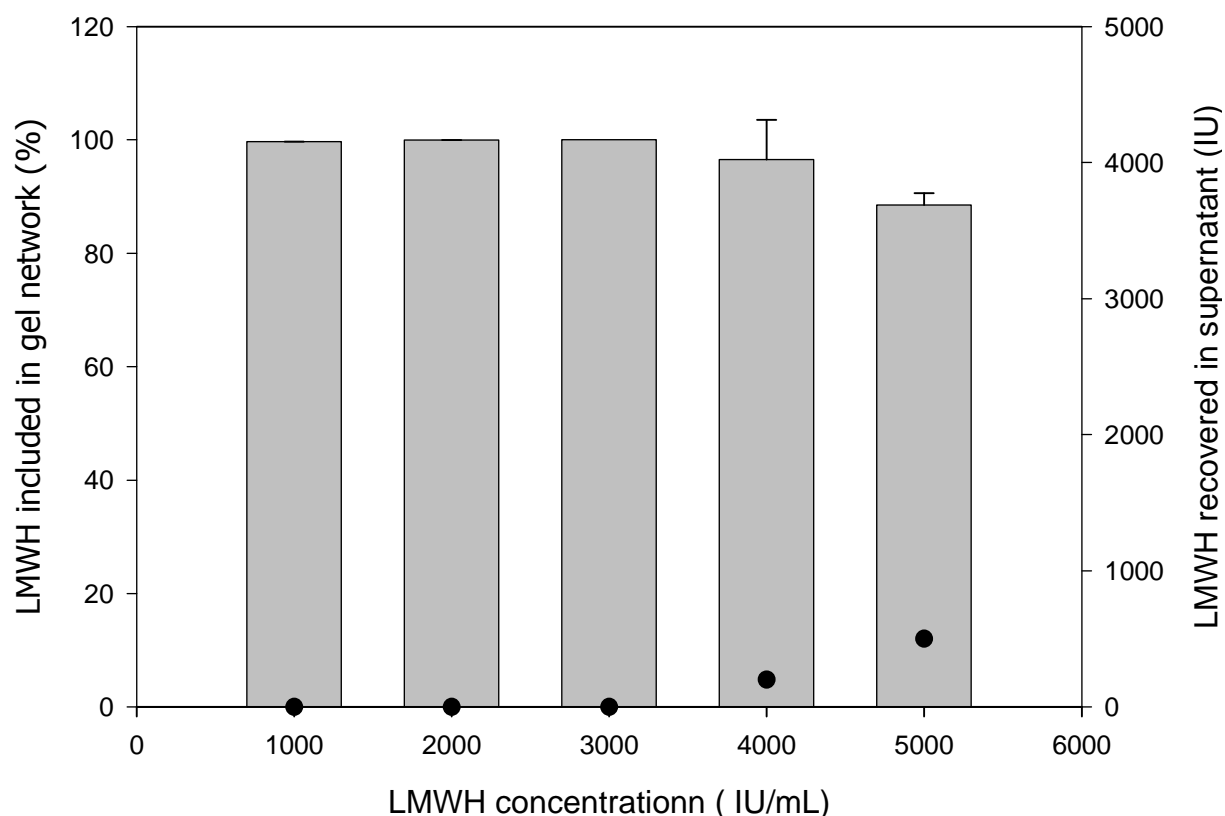


Figure 55: Amount of cross-linked LMWH with Eudragit® RS 30D gels as a percentage (bars) as well as the amount of LMWH recovered in supernatant in IU (black spots).

The percentage of cross-linked drug slightly decreased with higher concentrations, and finally reached 96 % and 90% for 4000 and 5000 IU/mL respectively. In other words, all the added LMWH molecules were not involved in the gel network formation since a small amount (200 and 500 IU for 4000 and 5000 IU/mL respectively) was found in the clear aqueous supernatant after centrifugation. The ionic interactions between the cationic groups in Eudragit® RS 30D and the negatively charged LMWH explain the high amount of LMWH cross-linked to form the gel. It can be considered that the binding sites of the Eudragit® RS are totally occupied by the LMWH molecules up to 3000 IU/mL for a constant amount of Eudragit® RS. A saturation of the binding sites appears at a concentration just below 4000 IU/mL of LMWH since 200 IU of the LMWH were found in the supernatant. However, based

on this assumption, adding 5000 IU/mL of LMWH onto the same amount of Eudragit[®] should lead to approximately 1200 IU of free LMWH, which was not the case. Some steric rearrangement could probably explain this difference. As observed by Song et al (Song and Kim 2006), a similar behaviour was obtained when preparing cationic flexosomes with LMWH. These authors have prepared liposomes with anionic, neutral and cationic lipids and obtained entrapment efficiency close to 92 % in their liposomes prepared with cationic lipids. On the other hand, the entrapment efficiency was limited to 30% for neutral and anionic liposomes. They also concluded that an ionic interaction between the negatively charged LMWH and the positively charged liposomes occurred, which may improve the adsorption of the LMWH to the liposomal membrane.

4.4 LMWH kinetic release

If the drug is able to cross the skin barrier, it must first be released from the gel network. So, the release kinetics of the four commercial LMWH from the gel containing 5000 IU/mL were evaluated (figure 54a). This gel was selected because it contains the largest amount of drug included within the gel. Thereafter, the release of the drug was performed for sodium tinzaparin gels with LMWH concentrations between 1000 to 5000 IU/mL (figure 54b). Tinzaparin was selected because the four LMWH showed the same release profile at 5000 IU/mL. Tinzaparin was not released at the lowest concentration (1000 IU/mL). When the amount of LMWH in the gels was increased from 1000 to 5000 IU, the percentage of drug released increased from zero to 32%. More precisely, a drug release of 7.8% (corresponding to 150 LMWH IU) in gels prepared with 2000 IU/mL was observed whereas the drug release was 17.6 % (corresponding to 470 IU in the medium) for an initial concentration of 3000 IU/mL. Higher initial concentrations of drug leads to a better drug release: 1100 IU (27.5%) were released from a gel prepared with 4000 IU/mL. The maximum amount of drug released

in the dissolution medium was obtained with a gel prepared with 5000 IU/mL: in this case, a maximum release of 32 % (corresponding to 1800 IU) was obtained.

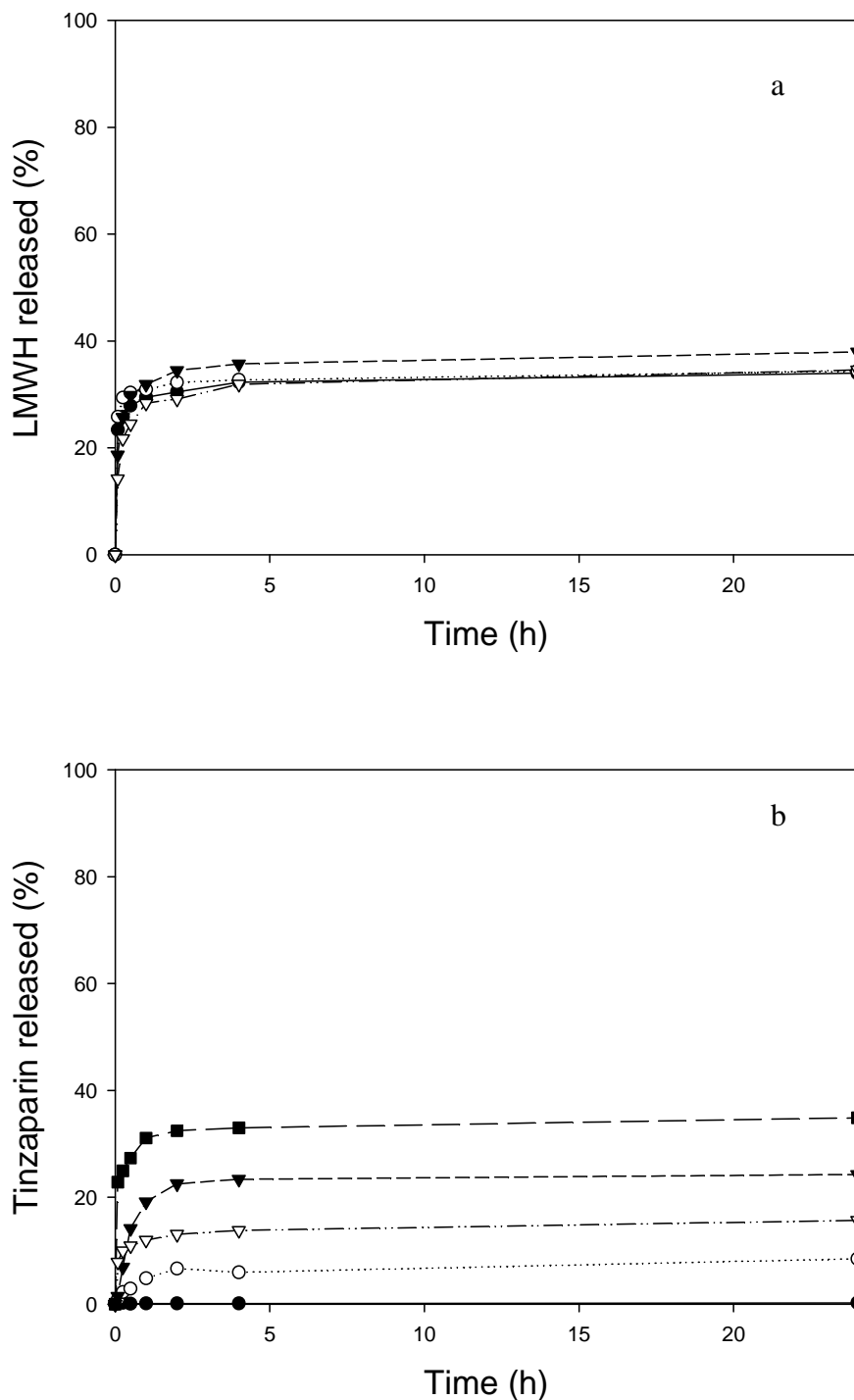


Figure 56: 57a: Release kinetics of four LMWH in gels at 5000 IU/mL. Bemiparin (●), enoxaparin (○), nadroparin (▼) and tinzaparin (▼). 57b: Release kinetics of sodium tinzaparin (Innohep®) at different concentrations: 1000 IU/mL (●), 2000 IU/mL (○), 3000 IU/mL (▼), 4000 IU/mL (▼) and 5000 IU/mL (■).

From the rheological studies, it was shown that the 1000 IU/mL gels were the strongest ones in terms of viscoelastic behaviour. That probably means that the electrostatic interactions were at a maximum, thus preventing LMWH release from the gel network. Nevertheless, gels made with 3000 IU/mL or 5000 IU/mL were also very strong, which finally is a better option in terms of drug release due to a potential concentration gradient. As shown in figure 54a, the same double phase profile was obtained for the different commercial LMWH preparations. An initial burst (between 5 to 15%) was observed during the first 30 minutes of the experiment followed by a plateau, which was reached after about 4 hours. Regardless of LMWH type, release of drug was incomplete since the plateau was stable between 4 to 24 hours. The burst drug release may allow the drug to be rapidly incorporated into the skin. Furthermore, a gradient could be formed between the gel and the skin to promote the complete and progressive release of the drug. This burst could be explained by the release of LMWH weakly or not involved in the electrostatic interactions with the cationic groups of the polymer. On the other hand, the non-total release of drug could be explained by the strong electrostatic interactions between drug and polymer. Similar behaviour was observed by Hoffart et al. when preparing Eudragit[®] nanoparticles loaded with LMWH. They found an important burst and an incomplete drug release explained by the strong electrostatic interaction between the negatively charged LMWH and the positively charged polymer (Hoffart, Lamprecht et al. 2006). To confirm this hypothesis, the LMWH release was measured (with a gel prepared with 5000 IU/mL of drug) in dissolution media of different ionic strength (NaCl 0.1, 0.5 and 1.0 M). As observed in figure 55, drug release increases with the increase of ionic strength to reach a maximum of 65% in NaCl 1M. This result confirms the strong influence of ionic interactions between drug and polymer in the formation of the polymer network. Ionic strength has an important influence on drug release. For example, Bodmeier et al. (Bodmeier, Guo et al. 1996) observed that increasing the NaCl concentration

in the buffer (acetate buffer pH 3.5 and phosphate buffer pH 7.4) decreased the polymer hydration and consequently reduced the drug release. On the other hand, Holgado et al (Holgado, Iruin et al. 2008) observed an increase of morphine release on increasing the ionic strength of the medium: they found that ionic strength values higher than physiological range also produced a faster release of the drug. A high affinity of hydrophilic drug for the Eudragit[®] hydrogel could also contribute to a non-total drug release.

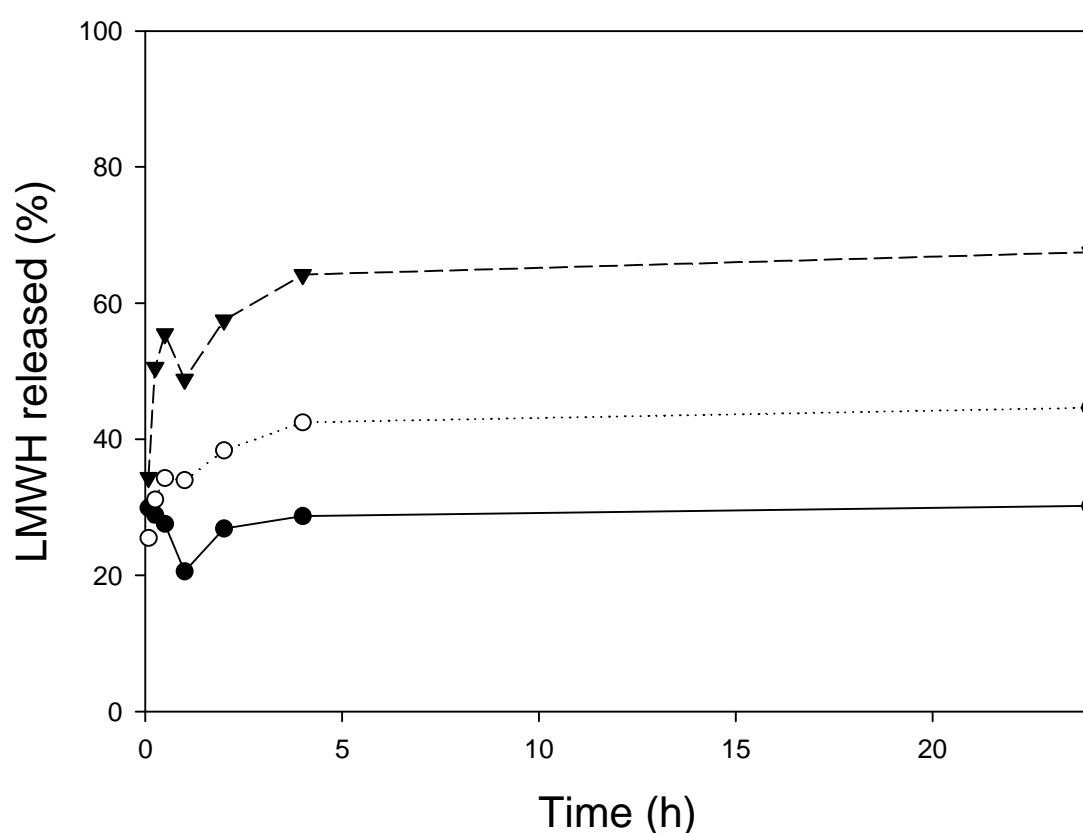


Figure 57: Release kinetics of LMWH performed in dissolution media of different ionic strength: NaCl 0.1M (●), NaCl 0.5M (○) and NaCl 1.0M (▼). Results are expressed as mean \pm SD (n=3).

4.5 LMWH localization in the skin after topical application

The method of tape stripping was used to determine the amount of LMWH deposited in the SC. The SC is the first barrier of skin. This method is an easy and non invasive technique to study the drug permeation into the skin. Stratum corneum is removed by the application of successive tapes. As observed in figure 57, a high amount of corneocytes is stucked to the

scotch tape. After 15 strippings, no more cells appear in the scotch tape. It can be considered that stratum corneum is completely removed. To observe the corneocytes cells, tapes were coloured with trypan blue before microscopic observation. Indeed, trypan blue is widely used in cell culture to observe the presence of death cells and dead corneocytes.

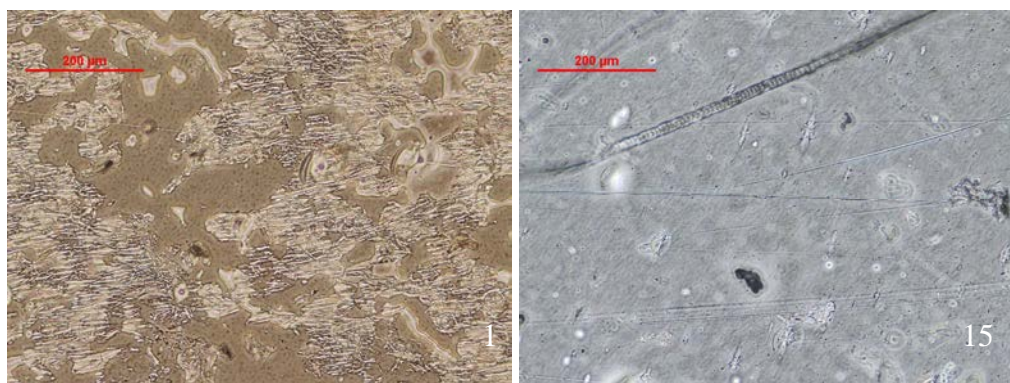


Figure 58: Rat stratum corneum pictures. Left image (corresponding to the first strip) shows the presence of a high amount of corneocytes. Right image (corresponding to the 15th strip) shows a few number of corneocytes. These observations are necessary to know how many tapes should be used to remove the entire stratum corneum.

The analytical method allows 80% of the applied drug to be recovered. LMWH solution was used as control, and it was found that the absorption of LMWH through the SC was 2-fold higher with the LMWH solution than with the gel as shown in table 43.

| Formulation used | Administered dose (IU/cm ²) | LMWH recovered in the SC (%) |
|-------------------------------------|---|------------------------------|
| LMWH solution (5000 IU/mL) (n=1) | 65 | 68 |
| LMWH gel (5000 IU/mL) (n=3) | 65 | 32 ±5 |

Table 43: LMWH (Tinzaparin) recovered in the SC after topical application.

Actually, this reflects the low release of LMWH from the gel as it was observed *in vitro* (figure 5b). A similar observation was reported by Song et al. They obtained a higher amount of LMWH (25%) in the skin, when LMWH was applied as a solution rather than a cationic flexosomes (10%). A slight increase of drug transported by the cationic flexosomes was also observed in viable skin (Song and Kim 2006). In our case, 32% of LMWH was found in the SC. This result is in agreement with the amount of drug released during the *in-vitro* test (also

32%). It can be hypothesized that a gradient of drug is established that allows the LMWH to cross the skin barrier. The cationic charge of the gel could improve the intradermal retention of the drug due to the negative charge in the skin surface at physiological pH (Song and Kim 2006).

4.6 LMWH quantification in plasma after topical application

For topical application of LMWH, drug should not pass into the systemic blood circulation to avoid a systemic effect. After 24 hours of occlusion application, LMWH was not detected in plasma so no systemic effect would be expected. Similar results have been obtained by Song et al. (Song and Kim 2006). These authors prepared flexible liposomes to deliver LMWH into the skin and observed a small accumulation of drug within the skin without any systemic effect, which they explain by the large size of liposomes, thus preventing them to entering into the blood capillaries. A similar effect could be expected for the LMWH gel. Polymer nanoparticles are entrapped in the network by electrostatic bonds. The size of the nanoparticles (around 130 nm) as well as their entrapment in the gel network does not allow the nanoparticles to penetrate in local blood circulation but allows the drug to be accumulated in the skin probably as a result of a concentration gradient. It cannot be excluded that a very low amount of heparin is absorbed since the LOD of our method is 0.02 IU/mL. Nevertheless, if this was the case, such a low absorption would have no therapeutic consequence. Although this result should be confirmed (and also in other animal species), it is a strong argument to continue developing heparin gels for local treatment. Indeed, local heparin would really be very benefit for treating haematomas with no, or very low, systemic absorption.

4.7 Conclusions

Physical gels containing different low molecular weight heparin were prepared and evaluated. The negative charges existing in the drug allows the interaction with a polycationic polymer (Eudragit ® RS 30D) to form a semisolid structure with viscoelastic properties. Moreover, the drug release ability from the gel was evaluated as well as the potential to drug delivery into the skin. It was observed that our gel presents a capability to release the drug in a dissolution medium, but a non-total release was noticed, indicating the strong interactions between drug and polymer. Although the ability to cross the stratum corneum was not total, this system presents the basis to optimize the formulation.

Moreover, this type of structure (gels prepared with electrostatic interactions) may be studied with other molecules to obtain gels with different capacities and used as drug carriers.

Summary of the thesis

English

The aim of this work is the use of commercial nanosuspensions largely used in dosage forms coating to obtain new applications. From these nanosuspensions new dosage forms have been developed to

- i) An oral administration of lipophilic drugs and poorly water solubles (celecoxib, econazole, diclofenac, ibuprofen, ivermectin and warfarin) as liquid suspension dosage form and
- ii) A cutaneous application of Low Molecular Weight Heparins (LMWH) as a gel.

Indeed, these polymeric nanosuspensions have different chemical structure and charge (as reported in the following table), which are able to solubilise the selected drugs.

| Commercial name | Chemical structure | Charge |
|---------------------------------|--|----------|
| Aquacoat [®] ECD | Ethylcellulose | Neutral |
| Eudragit [®] FS 30D | Poly(methylacrylate, methylmethacrylate, methacrylic acid) | Anionic |
| Eudragit [®] L 30D-55 | Poly(methacrylic acid, ethyl acrylate) | Anionic |
| Eudragit [®] NE 30D | Poly(ethylacrylate, methylmethacrylate) | Neutral |
| Eudragit [®] RS 30D | Poly(ethylacrylate, methylmethacrylate) trimethylammoniomethylmethacrylate | Cationic |
| Eudragit [®] RL 30D | | |
| Kollicoat [®] MAE 30DP | Poly(methacrylic acid, ethyl acrylate) | Anionic |
| Kollicoat [®] SR 30D | Poly(vinyl acetate) | Neutral |

In one hand, model lipophylic drugs were mixed with polymeric nanosuspensions. Because of lipophylic characteristics of model drugs, our initial hypothesis lies on a certain affinity between drugs and polymers, which are also lipophylic. Based on this behaviour, an increase of drug solubility directly in the polymeric structures can be expected.

On the other hand, the polycationic nature of Eudragit ® RS 30D nanosuspension was used to manufacture gels by electrostatic interactions with different low molecular weight heparins (bemiparin, enoxaparin, nadroparin and tinzaparin).

The two types of formulations have been characterized and *in vivo* experiences have been performed to evaluate its efficiency. The obtained results have been presented in two distinct parts.

Polymeric nanosuspensions

In the case of lipophylic drugs loaded into polymeric nanosuspensions, a large panel of formulations has been studied. Depending on drug and polymer characteristics, three different behaviours have been observed as the total incorporation of drug into polymeric matrix, polymer agglomeration after drug addition or the non solubilisation of drug into the polymer. A physical-chemical characterization of the obtained loaded nanosuspensions has been performed. Thus, neutral polymers are the most suitable structures to model drug incorporation. Indeed, these polymers are able to solubilise all model drugs with an incorporation rate greater than 70%. Deeper studies have shown that stable formulations (> 1 year), which are able to release the drug *in vitro* and *in vivo* could be easily obtained.

The major advantages of this type of formulation are:

- An easier administration of dosage form. It is a liquid form to be taken orally, which can enhance the treatment observance in children and aged persons.
- An adaptation to other lipophylic and/or poorly water soluble drugs.

- A transposition to the industry. Indeed, a simple manufacturing method consisting in the mixture between drug and polymer by gentle stirring is able to be transferred to pharmaceutical industry.

Polymeric gels

Second part of this work is based on gels manufacturing by electrostatic interactions. The gel is formed between a cationic polymer and a LMWH, which is negatively charged. Due to the anticoagulant activity of LMWH, gels are destined to a cutaneous application in the treatment of superficial thrombosis and haematomas.

The mixture of a Eudragit[®] RS 30D suspension with LMWH solutions at different concentrations gave rise to semi-solid preparations. Depending on LMWH concentration, different textures have been obtained (from a milky solution to a semi-solid preparation).

Rheological measurements have demonstrated the gel structure for most of tested formulations. The ability to drug release have been studied by in vitro, ex vivo and in vivo methods. These studies have shown that LMWH is released from the gel network and is partially incorporated in the stratum corneum. In vivo studies have confirmed the absence of systemic effect avoiding the potential side effects.

New applications for commercial nanosuspensions have been obtained during this work. Moreover, the obtained formulations may be rapidly used in therapeutics because the products used during manufacturing (drugs and polymers) are recognized by Health authorities as safe and they are already approved for other medicines.

Deutsch

Das Ziel dieser Arbeit war es, Nanosuspensionen, die bereits heute kommerziell zum Überziehen fester oraler Arzneiformen verwendet werden, für neue Applikationswege zu verwenden. Im Rahmen der experimentellen Arbeit wurden dementsprechend neue pharmazeutische Arzneiformen entwickelt

- i) für die orale Applikation lipophiler und wenig wasserlöslicher Wirkstoffe (Celecoxib, Diclofenac, Econazol, Ibuprofen, Ivermectin und Warfarin) in Form flüssiger Suspensionen und
- ii) für die kutane Anwendung niedrig molekularer Heparine in Form von Gelen.

Die verwendeten polymeren Nanosuspensionen unterscheiden sich sowohl in ihrer chemischen Struktur als auch in ihrer Ladung (siehe unten stehende Tabelle), wodurch es möglich ist, verschiedene Arzneistoffe löslich zu machen.

| Handelsname | Chemische Struktur | Ladung |
|--|--|------------|
| Aquacoat [®] ECD | Ethylcellulose | neutral |
| Eudragit [®] FS 30D | Poly(methylacrylat, methylmethacrylat, methacrylsäure) | anionisch |
| Eudragit [®] L 30D-55 | Poly(methacrylsäure,ethylacrylat) | anionisch |
| Eudragit [®] NE 30D | Poly(ethylacrylat,methylmethacrylat) | neutral |
| Eudragit [®] RS 30D Eudragit [®] RL 30D | Poly(ethylacrylat,methylmethacrylat) trimethylammoniomethylmethacrylat | kationisch |
| Kollicoat [®] MAE 30DP | Poly(methacrylsäure,ethylacrylat) | anionisch |
| Kollicoat [®] SR 30D | Polyvinylacetat | neutral |

In einem Teil der Arbeit wurden lipophile Arzneistoffe mit polymeren Nanosuspensionen vermischt. Auf Grund der lipophilen Eigenschaften der pharmazeutischen Wirkstoffe wurde angenommen, dass zwischen ihnen und den ebenso lipophilen Polymeren eine gewisse Affinität existiert. Davon ausgehend wurde angenommen, dass sich die Löslichkeit der lipophilen Wirkstoffe in den polymeren Strukturen erhöht.

In einem weiteren Teil der Arbeit wurde der polykationische Charakter der Suspension Eudragit[®] RS 30D genutzt, um Gele auf der Basis elektrostatischer Wechselwirkungen herzustellen, die verschiedene niedrigmolekulare Heparine enthalten (Bemiparin, Enoxaparin, Nadroparin und Tinzaparin).

Diese beiden Applikationsformen – polymere Nanosuspensionen und polymere Gele – wurden hinsichtlich verschiedener Eigenschaften charakterisiert. Für die Verifizierung ihrer Effizienz dienten in vivo-Studien. Die entsprechenden Resultate wurden in zwei eigenständigen Kapiteln diskutiert.

Polymere Nanosuspensionen

Mit lipophilen Arzneistoffen beladene polymere Nanosuspensionen wurden in vielzähligen Formulationen studiert. In Abhängigkeit der Eigenschaften sowohl des Polymers als auch des Wirkstoffs konnten unterschiedliche Verhaltensweisen der Nanosuspensionen beobachtet werden – wie beispielsweise die komplette Inkorporierung des Wirkstoffs in der Nanosuspension, die Agglomeration des Polymers beim Kontakt mit der aktiven Substanz oder die Unlöslichkeit des Arzneistoffs in der polymeren Matrix. Die gewonnenen Nanosuspensionen wurden auf ihr physiko-chemisches Verhalten hin systematisch geprüft. So haben sich die neutralen Polymere als günstigste Nanosuspension für die Inkorporation der verwendeten Arzneistoffe herausgestellt. Diese Polymere machen es möglich, mehr als 70 Prozent des jeweiligen Arzneistoffs in die Nanosuspension einzuschließen. Vertiefende

Studien haben gezeigt, dass die Arzneiformulierungen auch über einen längeren Zeitraum (mehr als ein Jahr) stabil sind und dass sie den Arzneistoff sowohl in vitro als auch in vivo freisetzen.

Die hauptsächlichen Vorteile dieser Formulierungen sind:

Eine einfache Administration durch die orale Einnahme, die es insbesondere für ältere Menschen und Kinder vereinfacht, therapeutische Empfehlungen einzuhalten.

Eine mögliche Anpassung der Formulierung an andere Wirkstoffe mit lipophilen und/oder wenig wasserlöslichen Eigenschaften.

Die unkomplizierte Übertragung auf einen industriellen Maßstab. Denn die Formulierungen basieren auf einer sehr einfachen Herstellungsmethode, die daraus besteht, aktiven Wirkstoff und Polymer unter leichtem Rühren miteinander in Kontakt zu bringen. Daher kann eine Herstellung auf industriellem Niveau einfach geplant werden.

Polymere Gele

Der zweite Teil dieser Arbeit beruhte auf der Herstellung von Gelen durch elektrostatische Wechselwirkungen. Das Gel wird zwischen einem polykationischen Polymer und niedrig molekularem Heparin gebildet, welches polyanionsich geladen ist. Auf Grund der antikoagulativen Eigenschaften des Heparins können diese Gele kutan angewendet werden, um oberflächliche Thrombosen und Hämatome zu behandeln.

Durch die Mischung einer Suspension von Eudragit® RS 30D mit Heparin-Lösungen verschiedener Konzentrationen wurden halb feste Arzneiformen hergestellt. Deren Textur zeigte sich abhängig von der Konzentration der Heparin-Lösung und reichte von milchähnlichen Lösungen bis zu dickflüssigen Formulierungen. An Hand von rheologischen Untersuchungen wurde bei den meisten Zubereitungen ein Gelzustand nachgewiesen. Die Höhe der Wirkstofffreisetzung wurde mittels in vitro-, ex vivo- und in vivo-Methoden

getestet. Diese Studien haben gezeigt, dass Heparin aus dem Gel freigesetzt wird und dass ein bestimmter Anteil des Wirkstoffs vom Stratum corneum aufgenommen wird. Die in vivo-Studien haben bestätigt, dass es sich bei dieser Art von Applikation um eine lokale Wirkstofffreisetzung handelt; es wurde keine systemische Aktivität des Heparins nachgewiesen. Auf diese Art können unerwünschte Arzneimittelwirkungen vermieden beziehungsweise reduziert werden.

Mit dieser Arbeit wurden neue Formulierungsmöglichkeiten für polymere Nanosuspensionen, die sich bereits im Handel befinden, vorgeschlagen. Darüber hinaus können die Formulierungen einfach auf einen industriellen Maßstab übertragen werden. Die Arzneiformen sind dazu geeignet, als alternative Therapiemöglichkeiten eingesetzt zu werden, da die verwendeten Materialien (Polymere und Wirkstoffe) den entsprechenden Gesundheitsbehörden als biokompatibel bekannt sind und bereits in Form anderer Fertigarzneimitteln zugelassen sind.

Français

L'objectif de ce travail est l'utilisation de nanosuspensions commerciales utilisées traditionnellement dans l'enrobage des formes orales solides pour des applications nouvelles.

A partir de ces nanosuspensions, des nouvelles formes pharmaceutiques ont été développées

i) pour une administration orale de principes actifs lipophiles et peu hydrosolubles (celecoxib, diclofenac, econazole, ibuprofène, ivermectine et warfarine) sous forme de suspension liquide et

ii) également une administration cutanée d'héparines de bas poids moléculaire (HBPM) sous forme de gel.

En effet, ces nanosuspensions polymériques présentent des structures chimiques et charges différentes (cf tableau ci-dessous) permettant de solubiliser les principes actifs testés.

| Nom commerciale | Structure chimique | Charge |
|---------------------------------|--|---------------|
| Aquacoat [®] ECD | Ethylcellulose | Neutre |
| Eudragit [®] FS 30D | Poly(methylacrylate, methylmethacrylate, methacrylic acid) | Anionique |
| Eudragit [®] L 30D-55 | Poly(methacrylic acid, ethyl acrylate) | Anionique |
| Eudragit [®] NE 30D | Poly(ethylacrylate, methylmethacrylate) | Neutre |
| Eudragit [®] RS 30D | Poly(ethylacrylate, methylmethacrylate) | Cationique |
| Eudragit [®] RL 30D | trimethylammoniummethylmethacrylate | |
| Kollicoat [®] MAE 30DP | Poly(methacrylic acid, ethyl acrylate) | Anionique |
| Kollicoat [®] SR 30D | Poly(vinyl acetate) | Neutre |

D'une part, les principes actifs lipophiles modèles ont été mélangés avec les nanosuspensions polymériques. En raison du caractère lipophile de ces principes actifs, l'hypothèse de départ réside sur une certaine affinité des principes actifs par les polymères qui sont eux aussi lipophiles. De ce fait, une augmentation de la solubilité des principes actifs, directement dans les structures polymères, peut être espérée.

D'autre part, le caractère polycationique des suspensions d'Eudragit[®] RS 30D a été mis à profit pour fabriquer des gels par interaction électrostatique avec différentes héparines de bas poids moléculaire (bemiparine, enoxaparine, nadroparine et tinzaparine).

Ces deux types de formulation ont été caractérisées et des essais *in vivo* ont été réalisés pour vérifier leur efficacité. Les résultats correspondant ont été présentés en deux parties distinctes.

Nanosuspensions polymériques

Dans le cas des nanosuspensions polymériques chargées de principes actifs lipophiles, un panel assez large de formulations a été étudié. En fonction des caractéristiques des principes actifs et des polymères, différents comportements ont pu être observés comme l'incorporation totale du principe actif au sein de la nanosuspension, l'agglomération du polymère au contact avec le principe actif ou une non solubilisation du principe actif dans la matrice polymérique. Une caractérisation physico-chimique systématique des nanosuspensions chargés a été menée. Ainsi, les polymères à caractère neutre se sont révélés les plus convenables pour l'incorporation des principes actifs modèles. En effet, ces polymères permettent de dissoudre l'ensemble des principes actifs avec un taux d'incorporation supérieur à 70%. Des études plus approfondies ont montré que des formulations stables au cours du temps (> 1 an) et capable de libérer du principe actif *in vitro* comme *in vivo*, pouvaient être facilement obtenues.

Les avantages majeurs de ce type de formulations sont :

Une facilité d'administration, il s'agit d'une forme orale susceptible d'améliorer l'observance du traitement notamment chez les patients âgés ou des enfants.

Une possible adaptation à d'autres principes actifs lipophiles et/ou peu hydro-solubles.

Une transposition facile en milieu industriel. En effet, ces formulations possèdent une méthode de fabrication très simple qui consiste uniquement à la mise en contact entre le principe actif et le polymère sous agitation légère. De ce fait, une fabrication au niveau industriel peut être facilement envisagée.

Gels polymériques

La deuxième partie de cette étude a porté sur la fabrication des gels par interaction électrostatique. Le gel est formé entre un polymère polycationique et une HBPM, qui est polyanionique. Grâce à l'activité anticoagulante des HBPM, ces gels sont destinés à une application cutanée pour le traitement des thromboses superficielles et hématomes.

Le mélange d'une suspension d'Eudragit® RS 30 D avec des solutions d'HBPM de concentration différentes, a donné naissance à des préparations semi-solides. En fonction de la concentration en HBPM, des textures différentes (d'une solution laiteuse à une préparation semi-solide épaisse) ont été ainsi obtenues. Des études rhéologiques ont montré la présence d'un gel pour la plupart des concentrations testées. La capacité de libérer le principe actif a été évaluée par des méthodes *in vitro*, *ex-vivo* et *in vivo*. Ces études ont montré que l'héparine se libère à partir du gel et qu'une certaine proportion est capable de s'incorporer au stratum corneum. Des études *in vivo* ont confirmé l'absence d'activité systémique ce qui réduirait les effets indésirables potentiels.

L'ensemble de ce travail a permis de proposer des nouvelles applications à des nanosuspensions polymériques déjà existantes sur le marché. De plus, les formulations

obtenues sont faciles à mettre en œuvre au niveau industriel et susceptible d'être utilisées en thérapeutique rapidement puisque les produits utilisés lors de la fabrication (principes actifs et polymères) sont reconnus par les autorités de santé comme biocompatibles et déjà approuvés dans d'autres médicaments.

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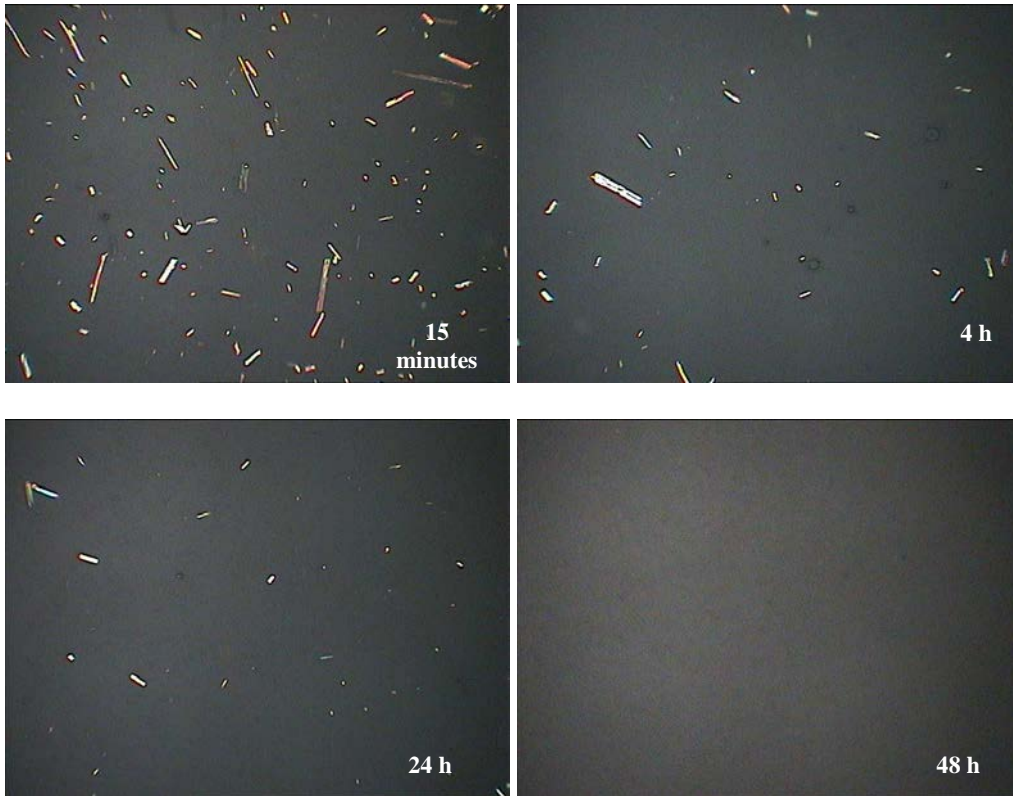
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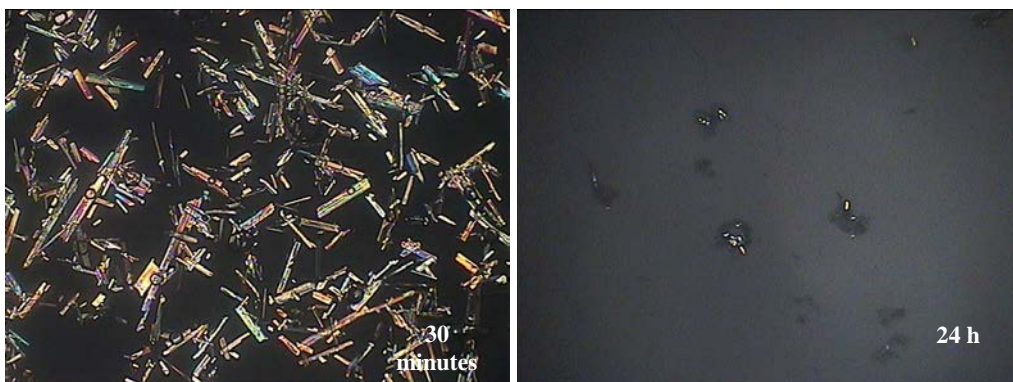
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Celecoxib

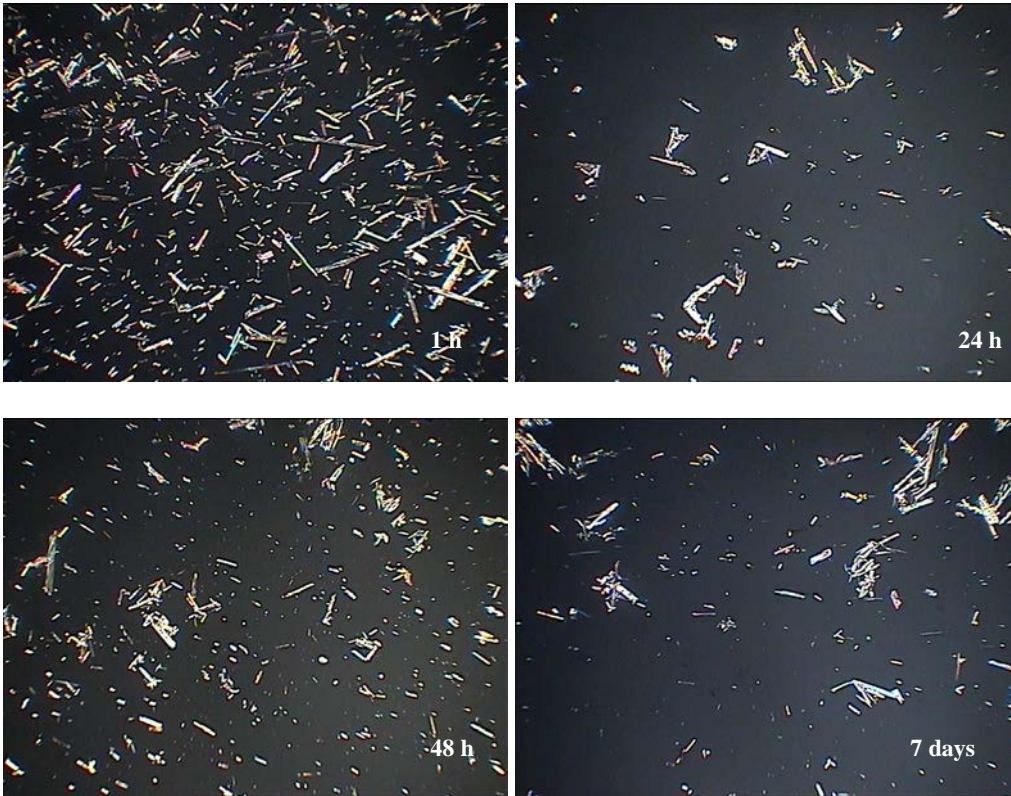
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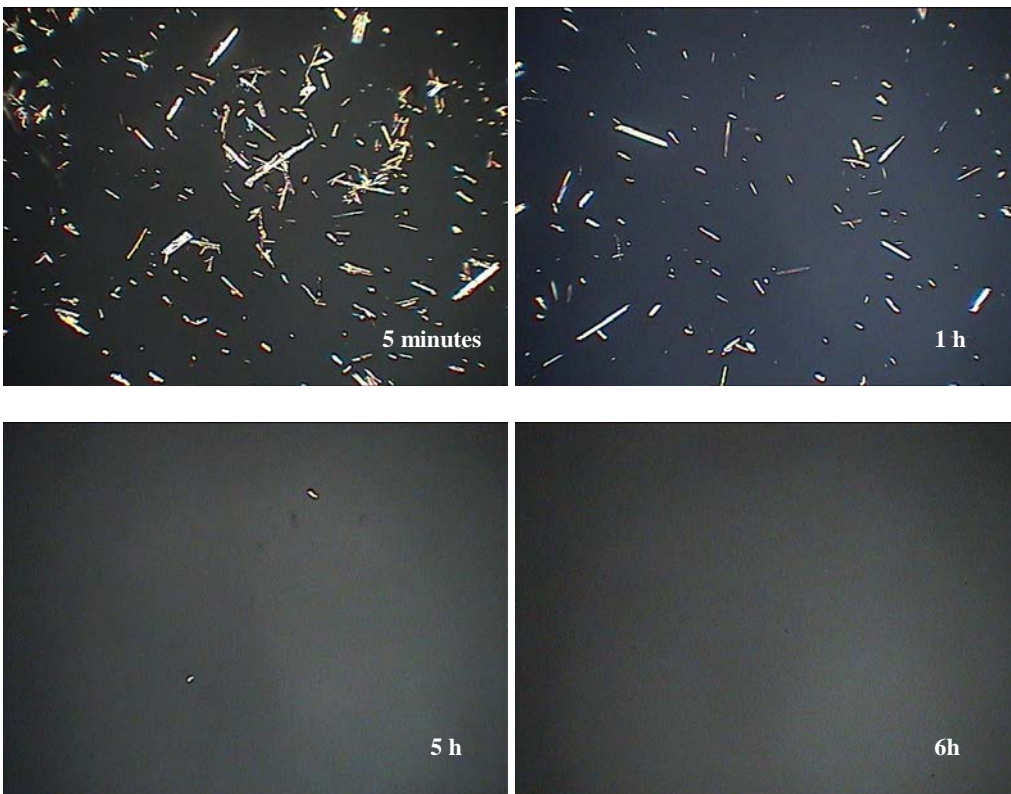
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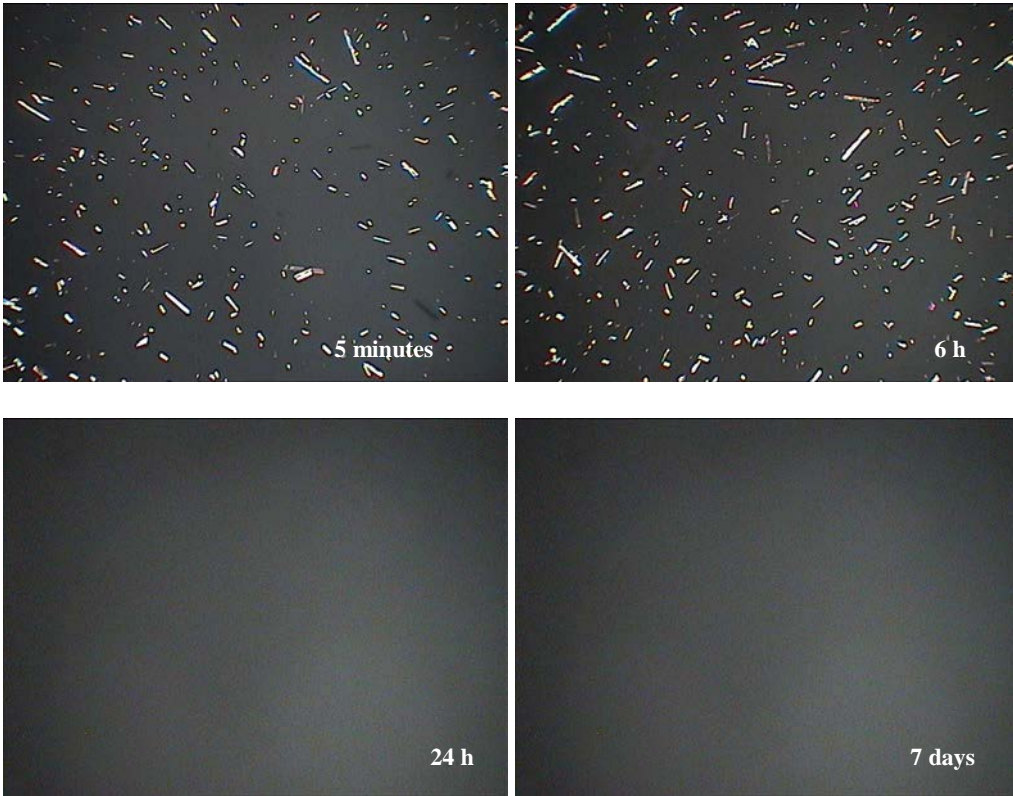
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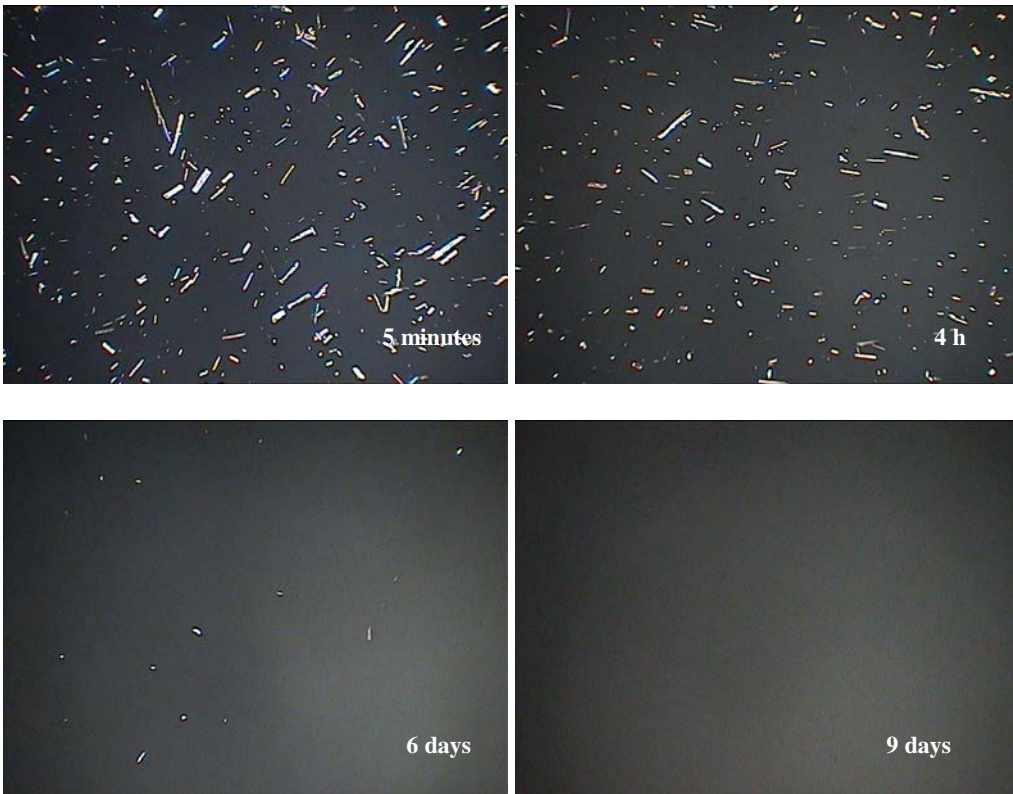
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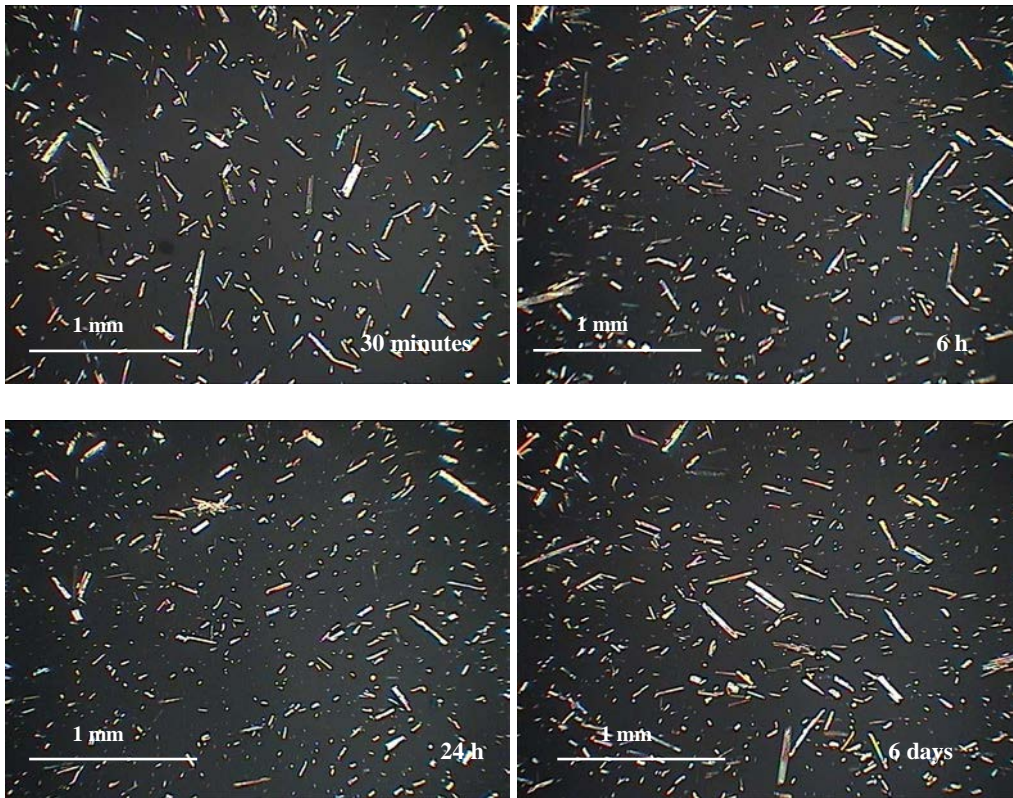
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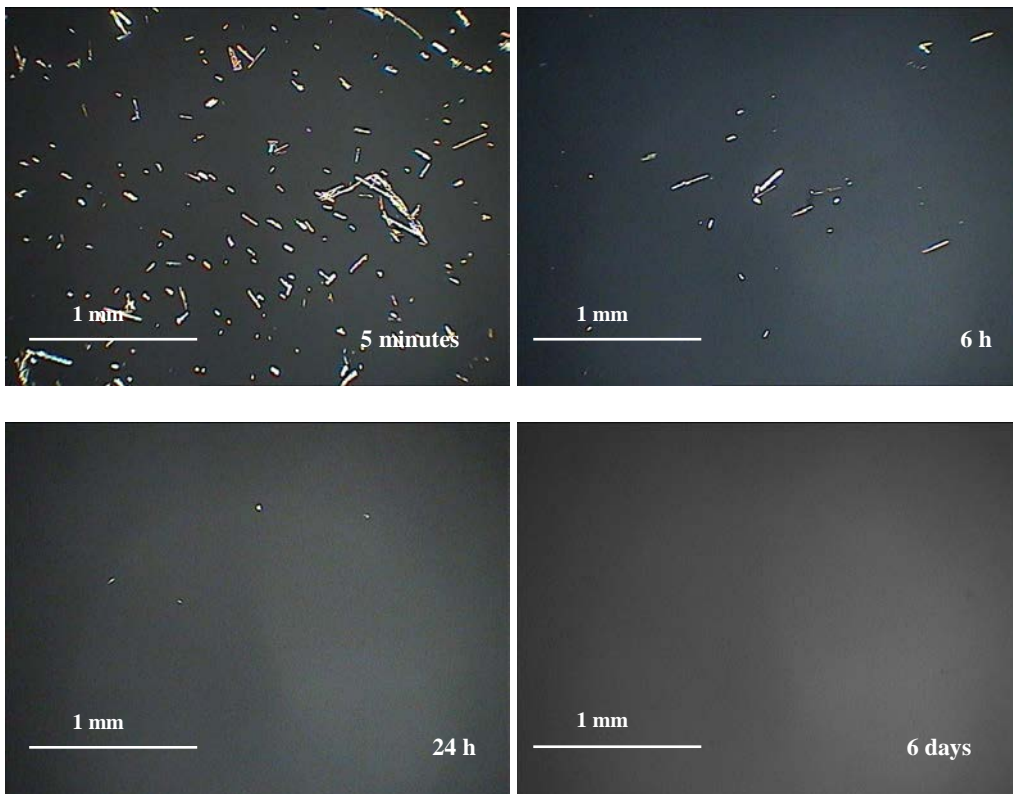
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Kollicoat[®] MAE 30DP

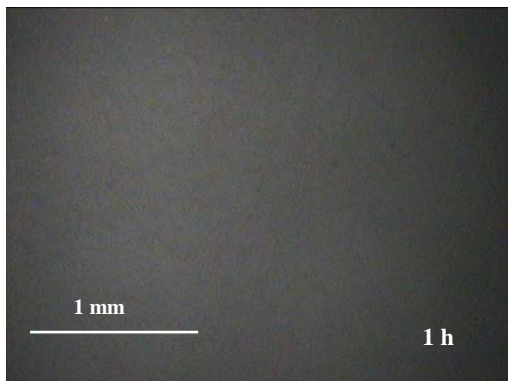
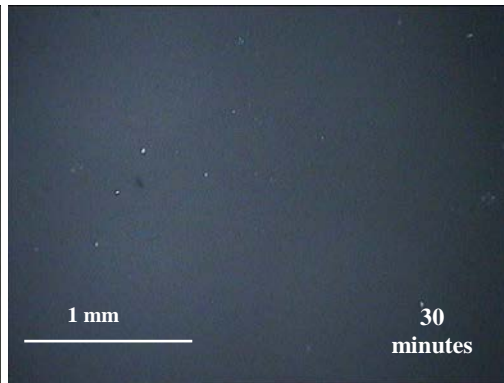
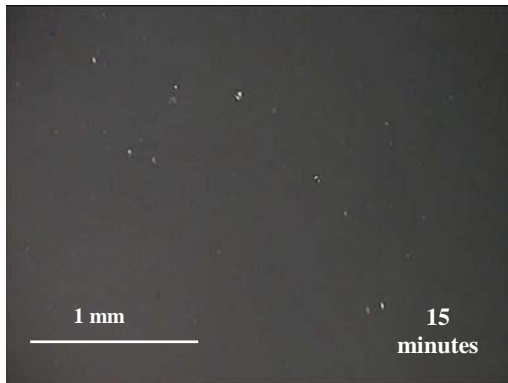


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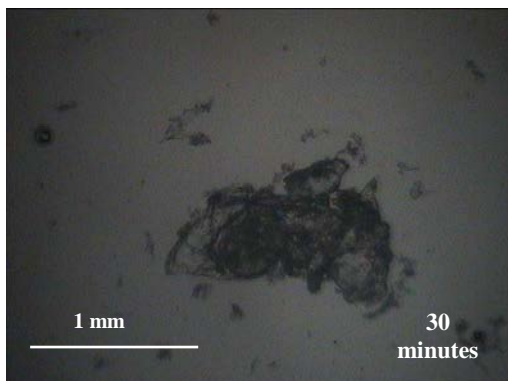


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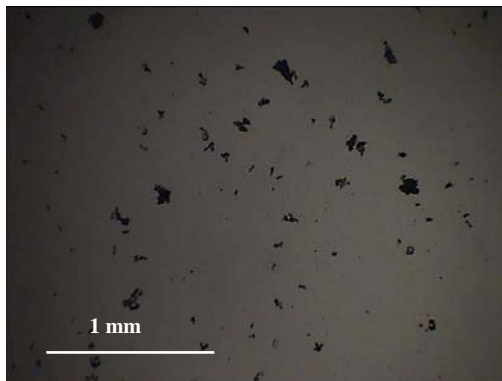
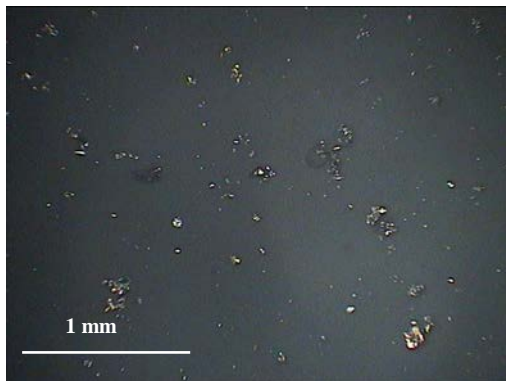
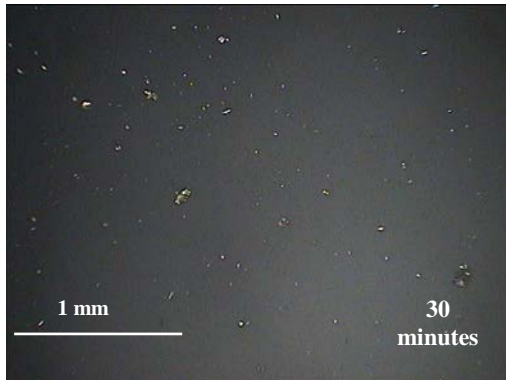
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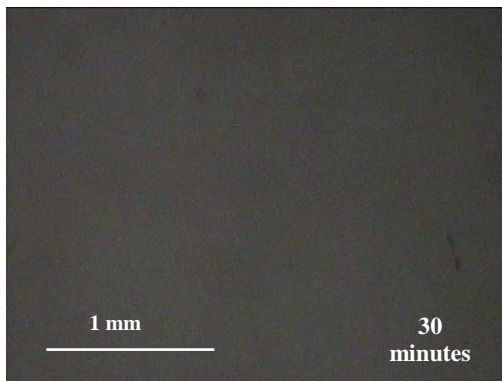
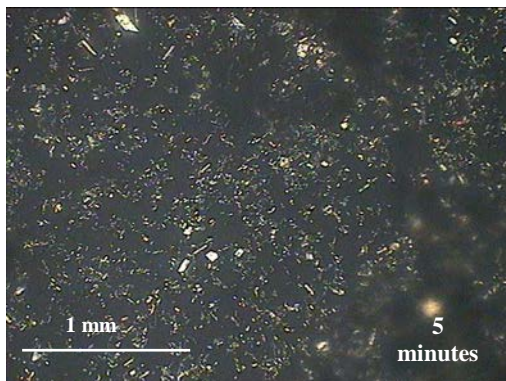


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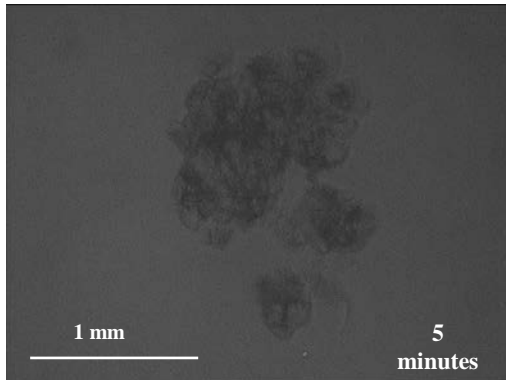


2h of stirring. Left picture was taken under polarized light, right picture was taken without polarized light.

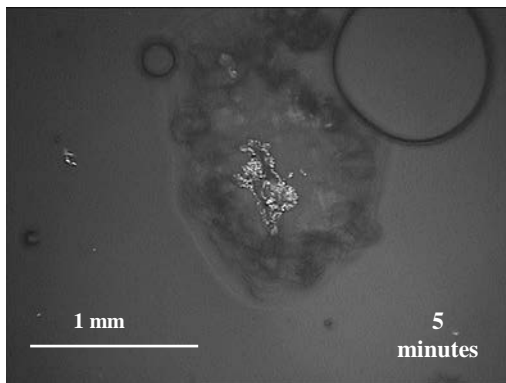
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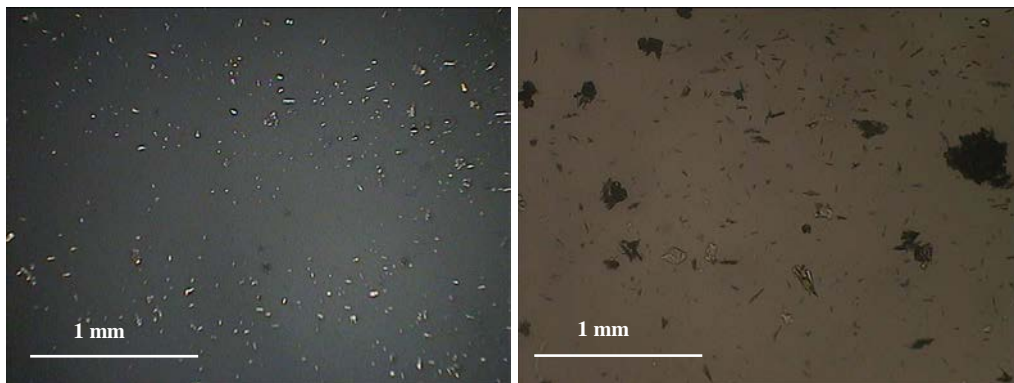
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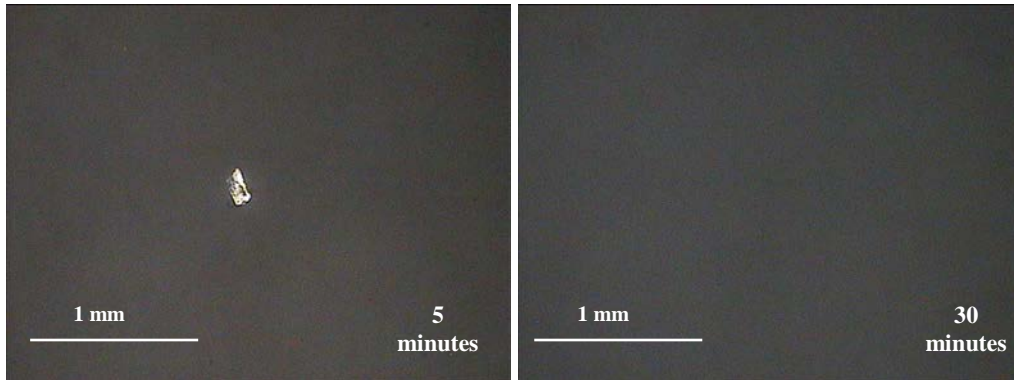


Kollicoat® MAE 30DP



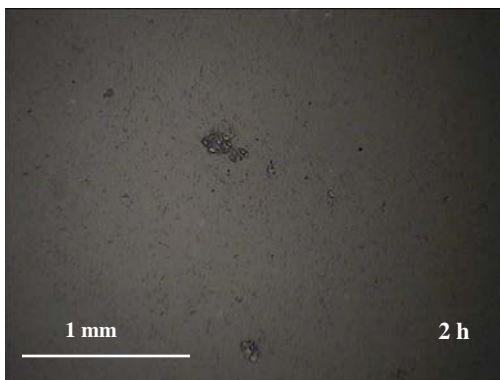
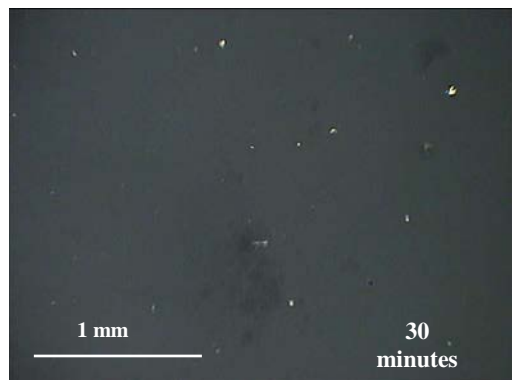
30 minutes of stirring. Left picture was taken under polarized light, right picture was taken without polarized light.

Kollicoat[®] SR 30D

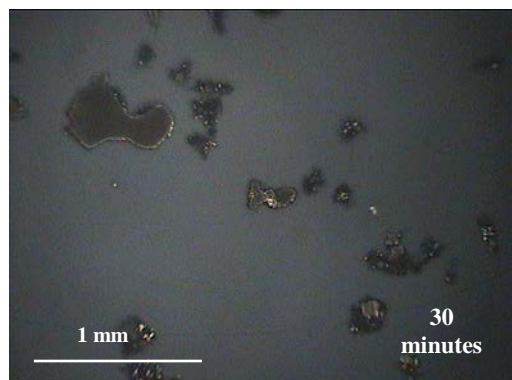


Econazole

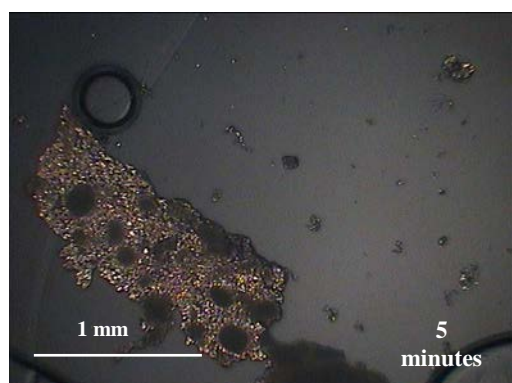
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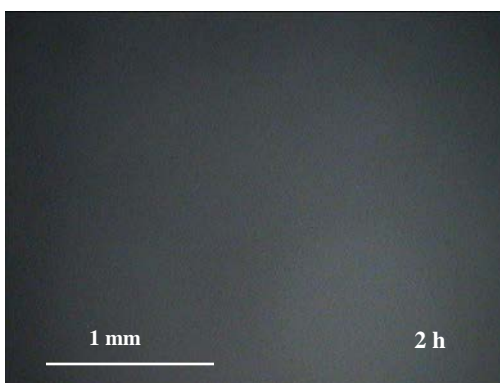
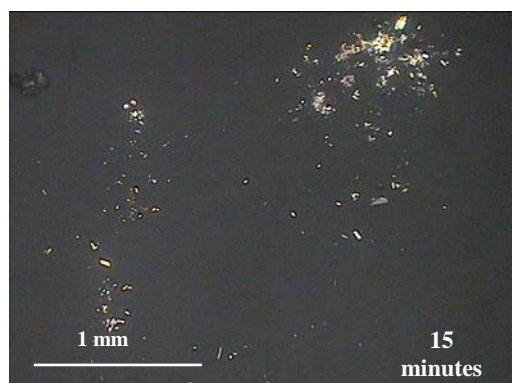
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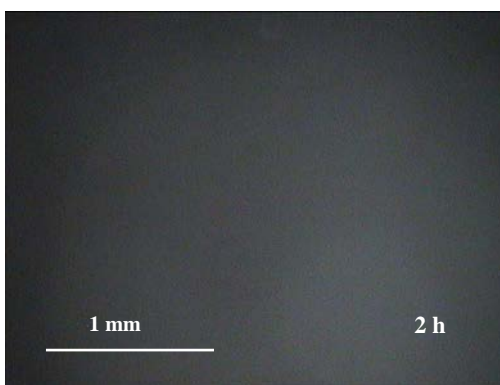
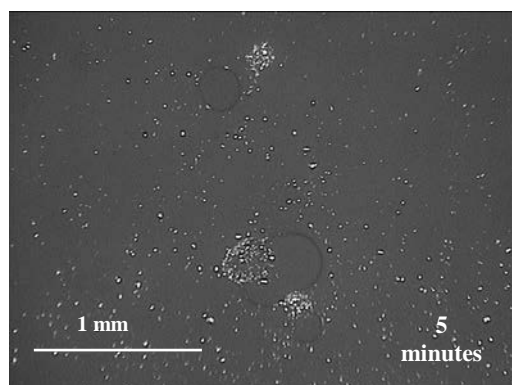
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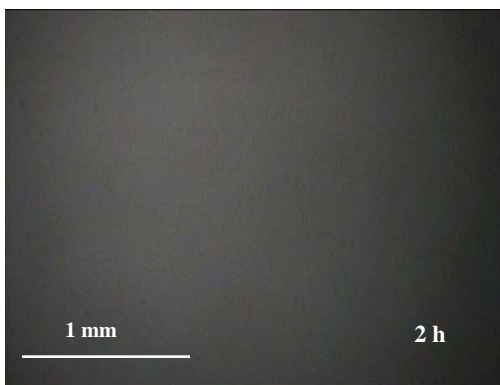
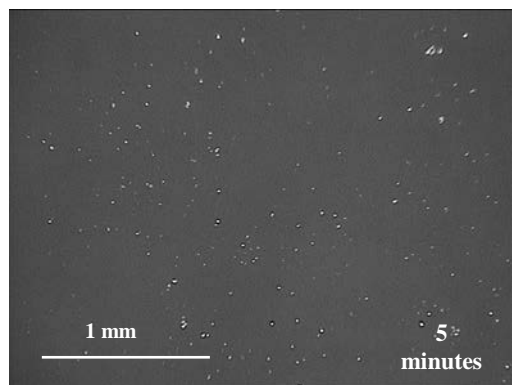
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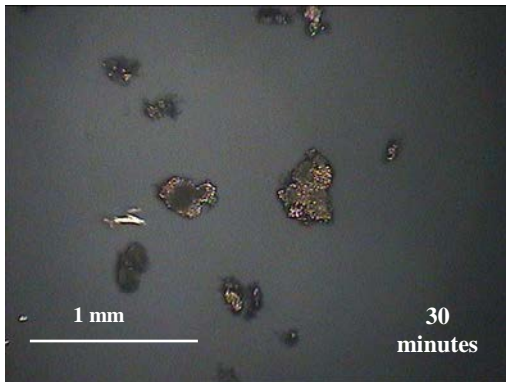
Eudragit® RL 30D



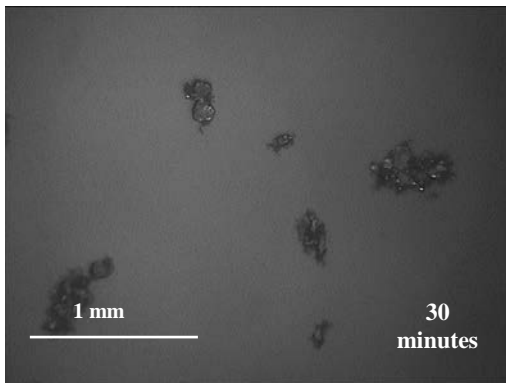
Eudragit® RS 30D



Kollicoat[®] MAE 30DP

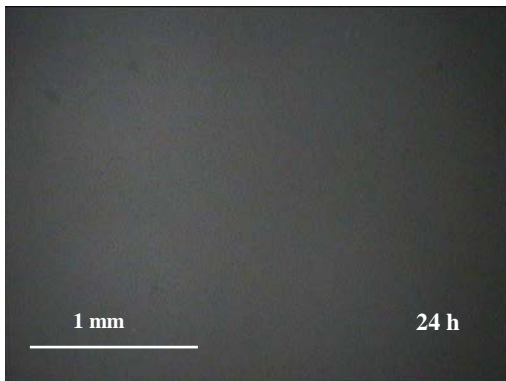
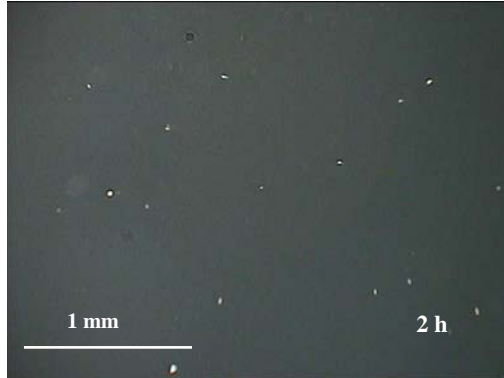
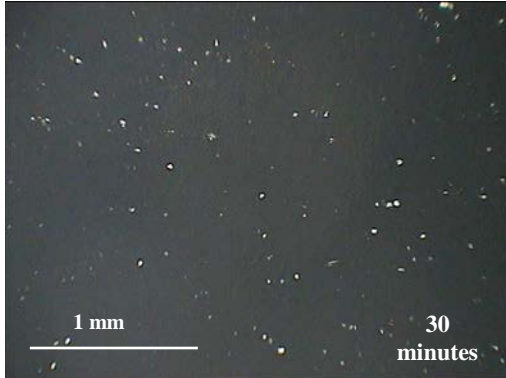


Kollicoat[®] SR 30D

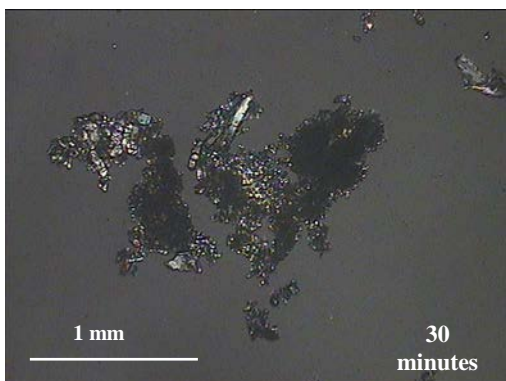


Ibuprofen

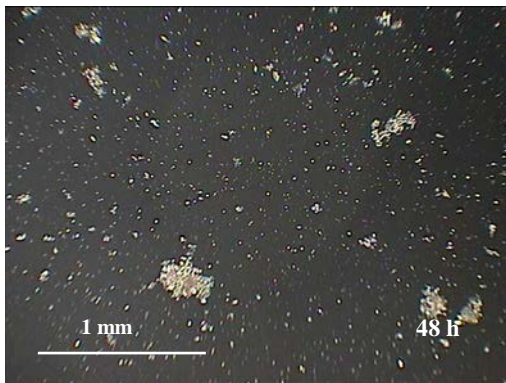
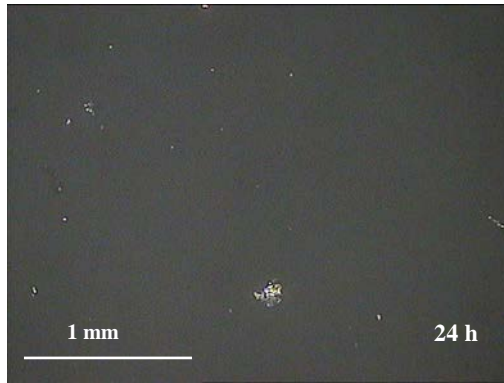
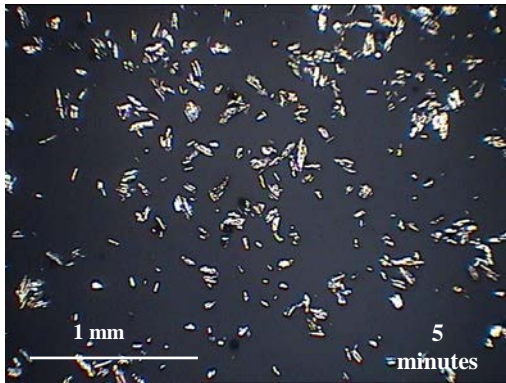
Aquacoat[®] ECD



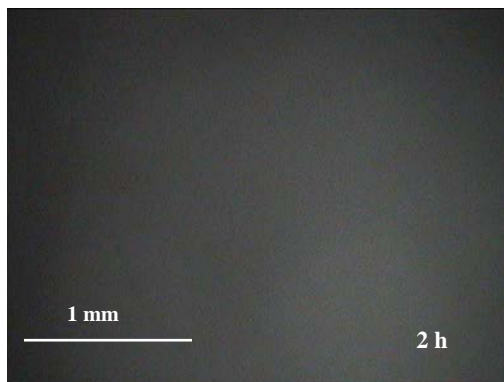
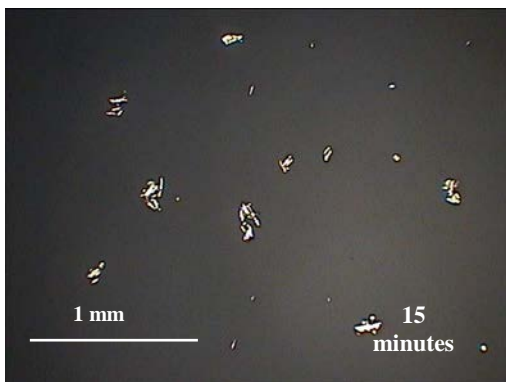
Eudragit[®] FS 30D



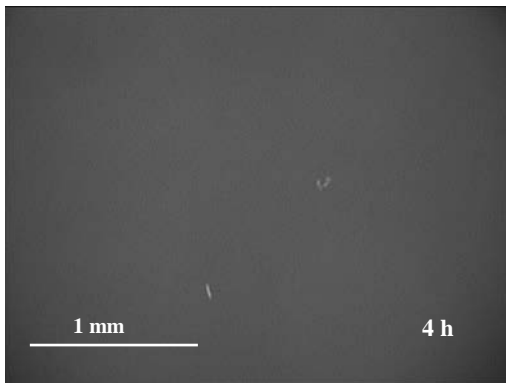
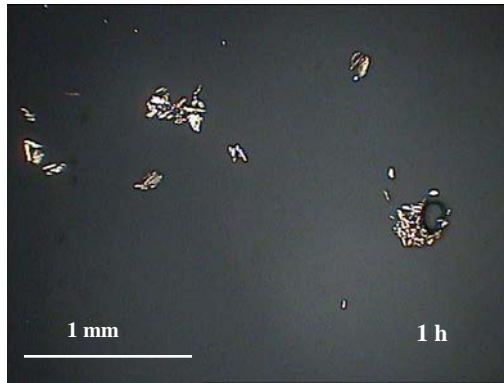
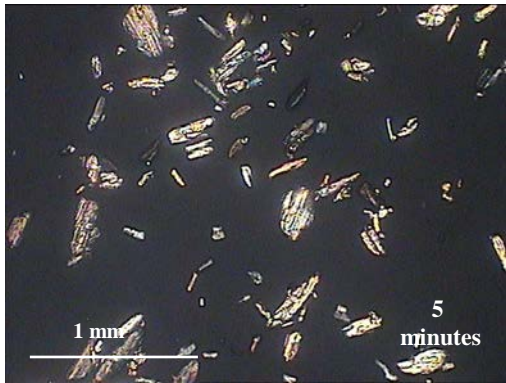
Eudragit® L 30D-55



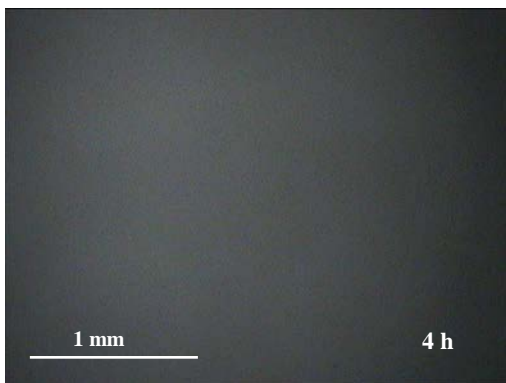
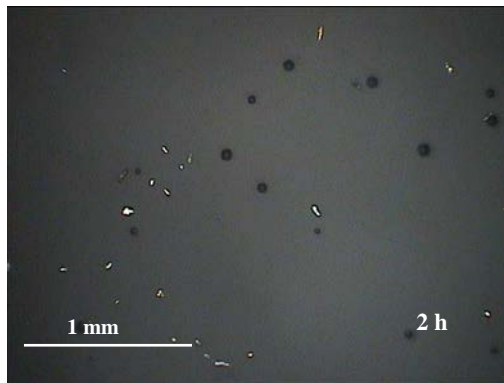
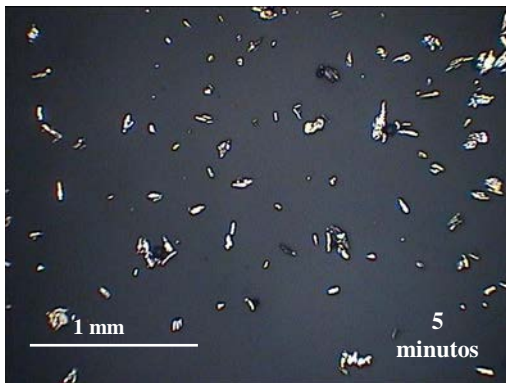
Eudragit® NE 30D



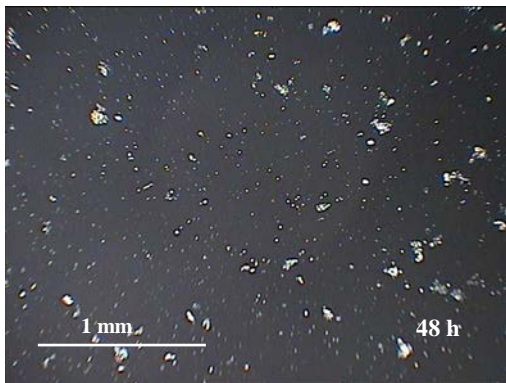
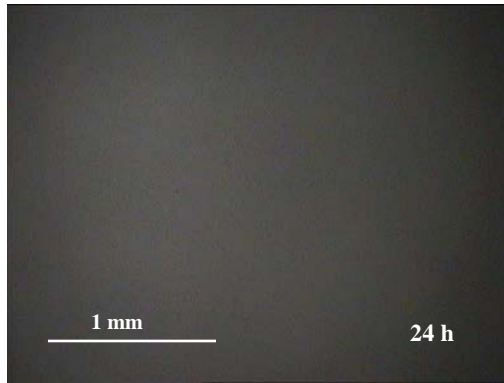
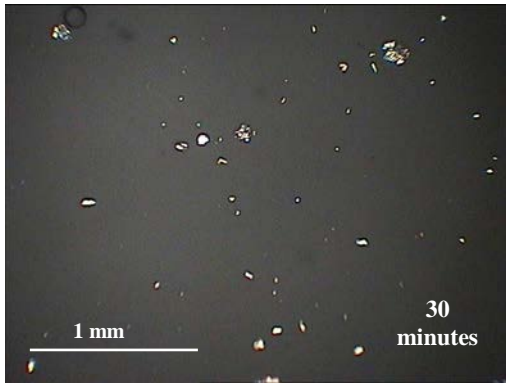
Eudragit® RL 30D



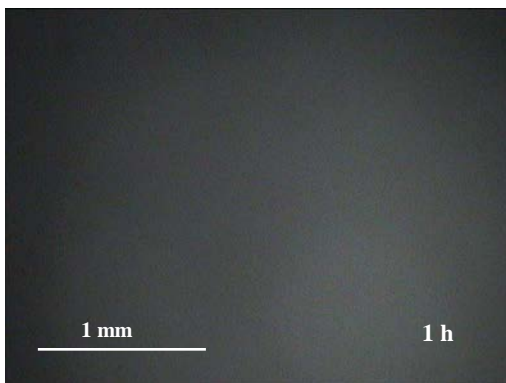
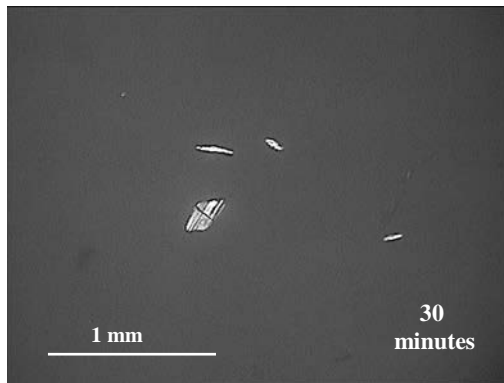
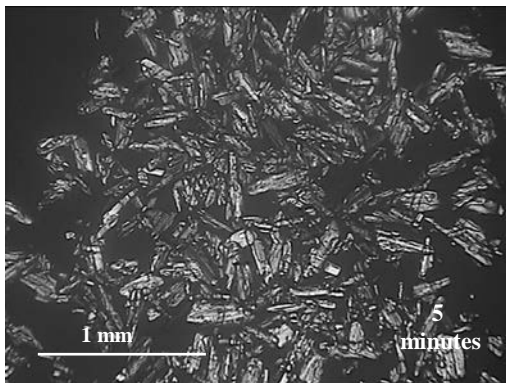
Eudragit® RS 30D



Kollicoat[®] MAE 30DP

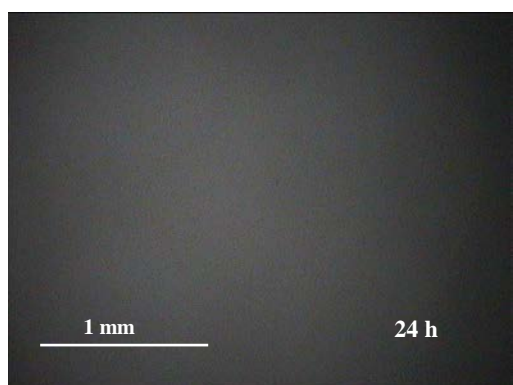
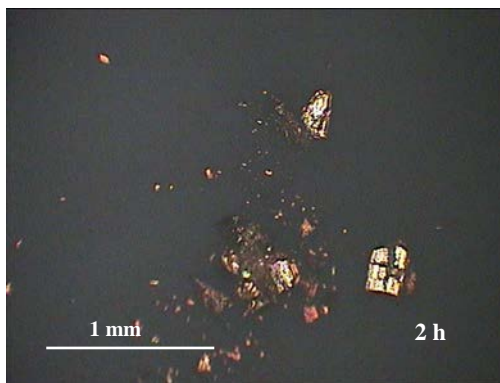
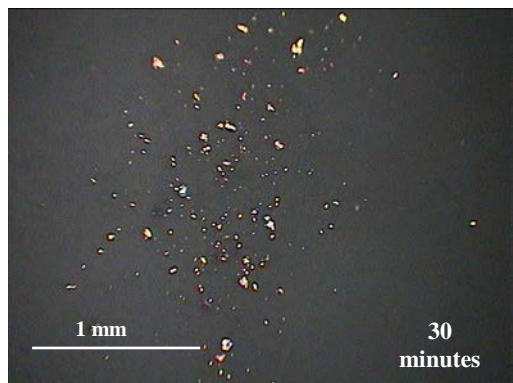


Kollicoat[®] SR 30D

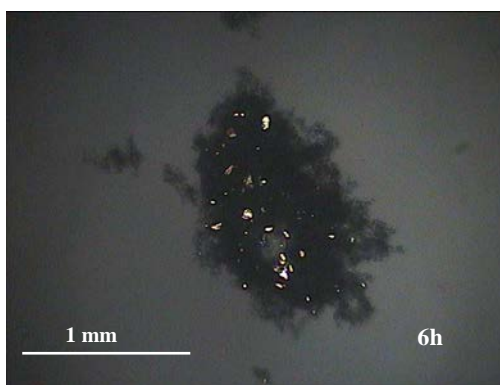
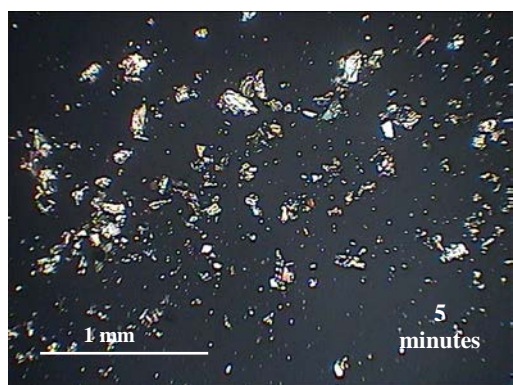


Ivermectin

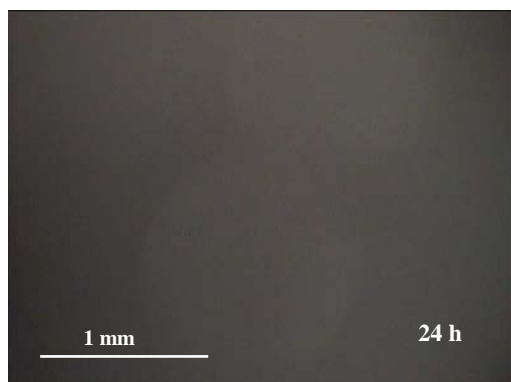
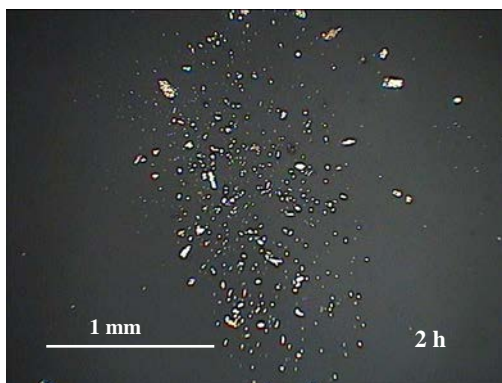
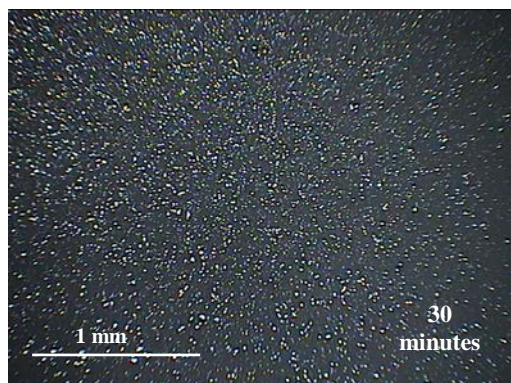
Aquacoat[®] ECD



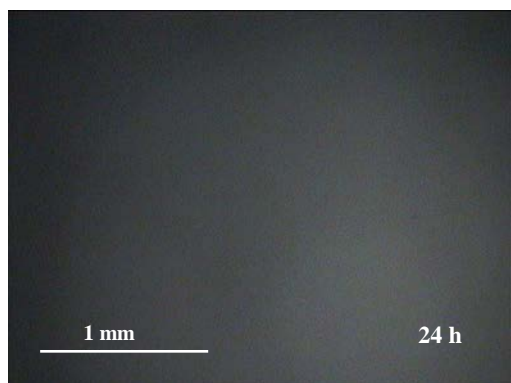
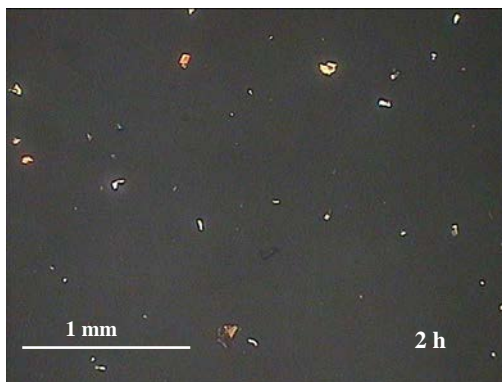
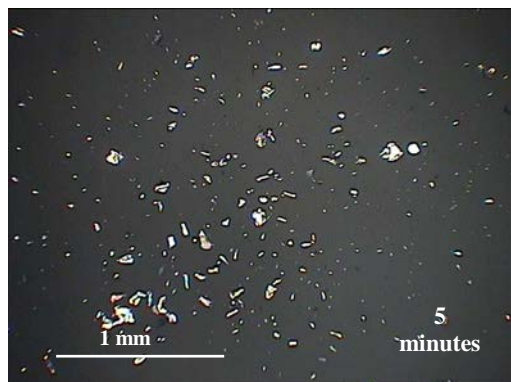
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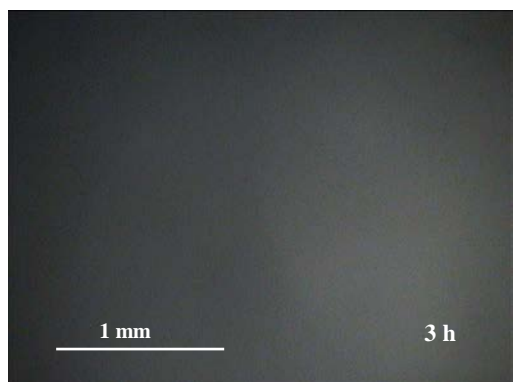
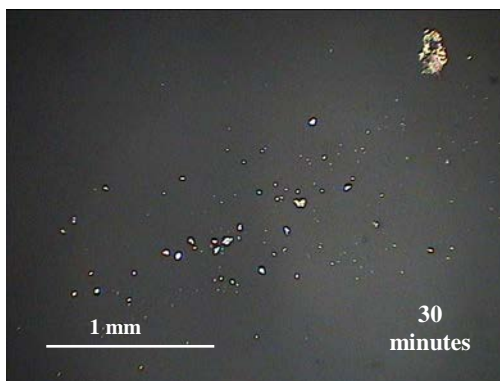
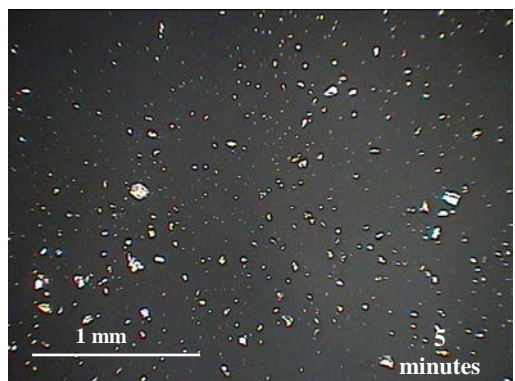
Eudragit® L 30D-55



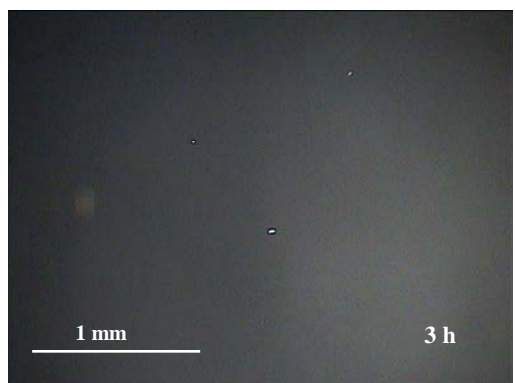
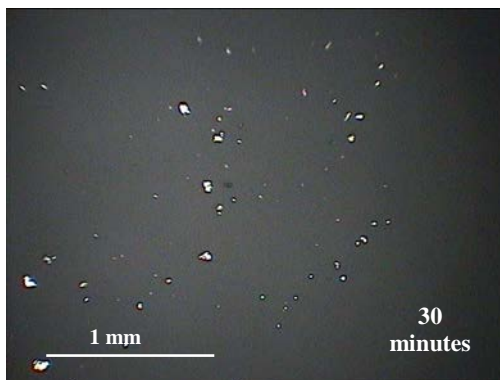
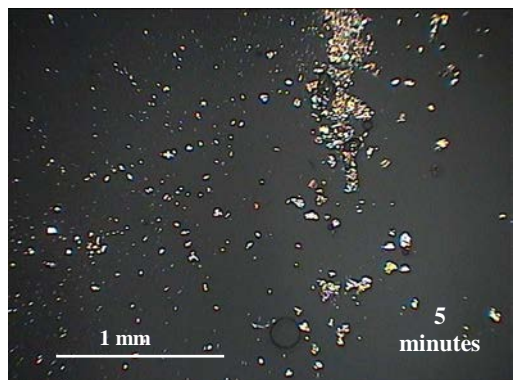
Eudragit® NE 30D



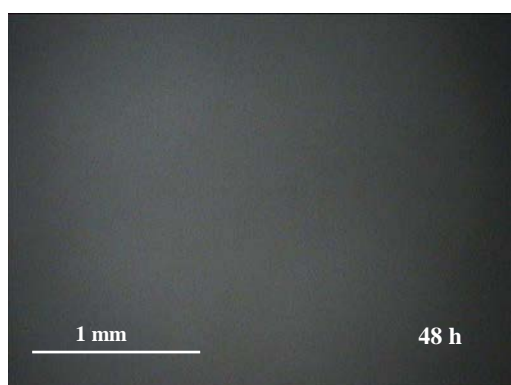
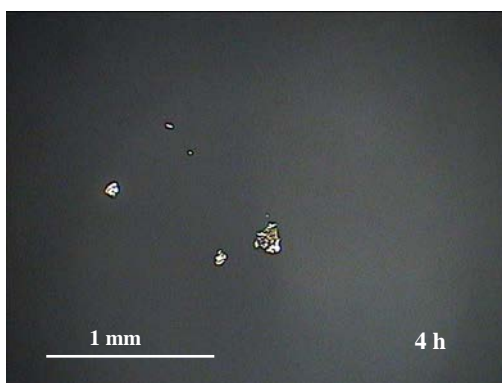
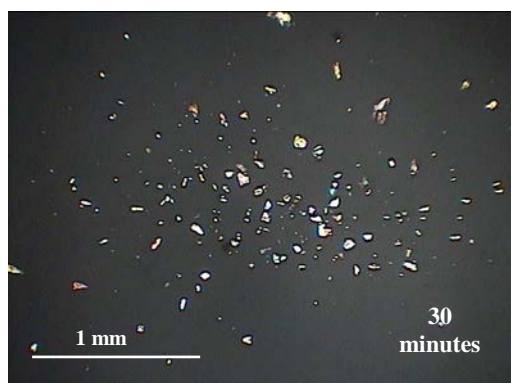
Eudragit[®] RL 30D



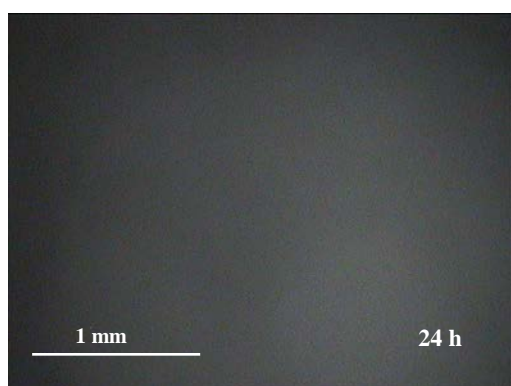
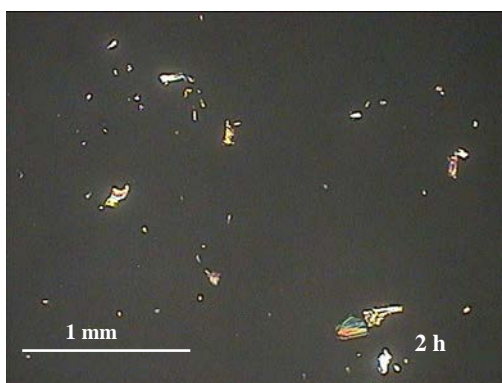
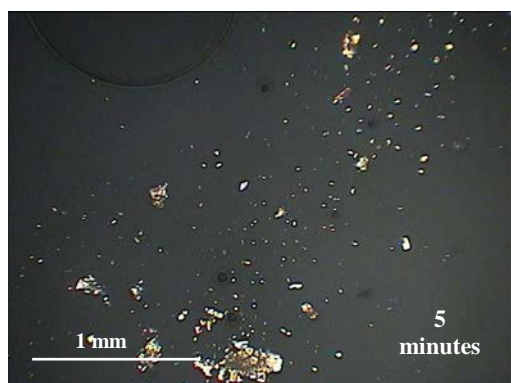
Eudragit[®] RS 30D



Kollicoat[®] MAE 30DP

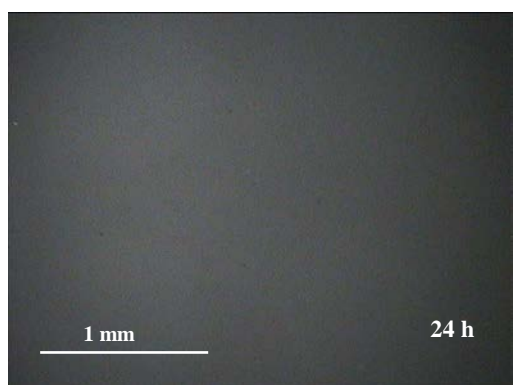
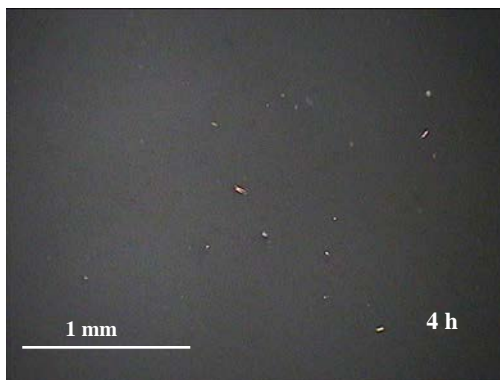
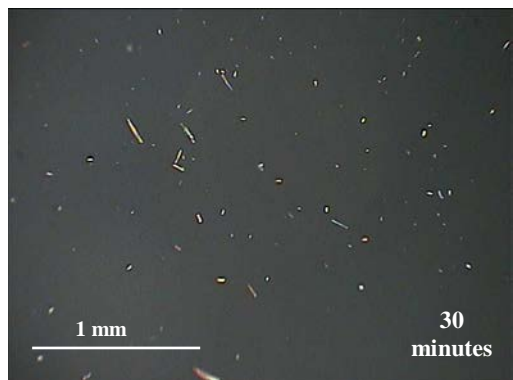


Kollicoat[®] SR 30D

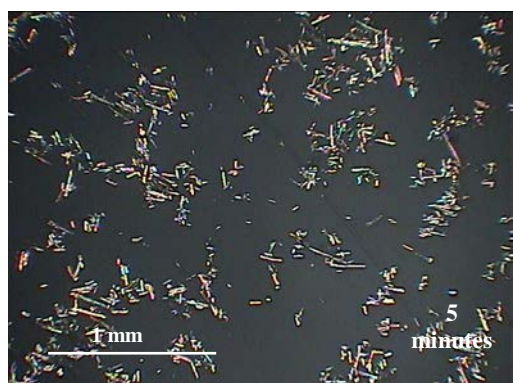


Warfarin

Aquacoat ECD:

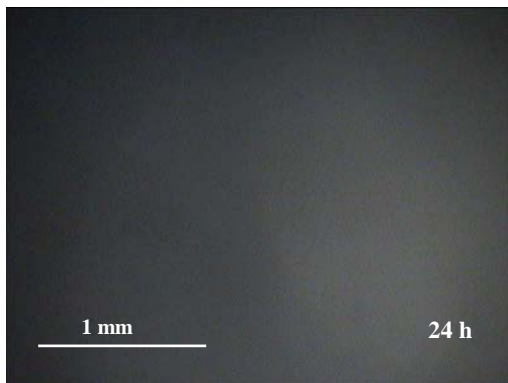
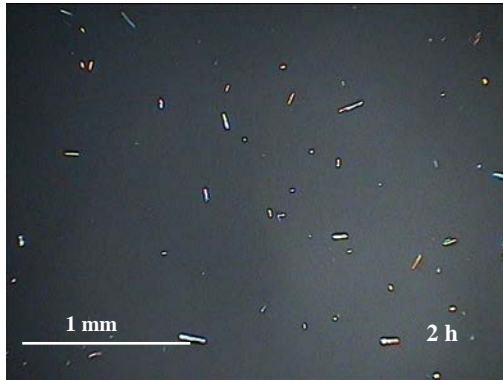
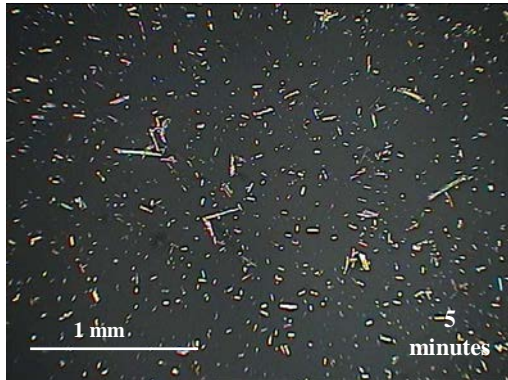


Eudragit® FS 30D

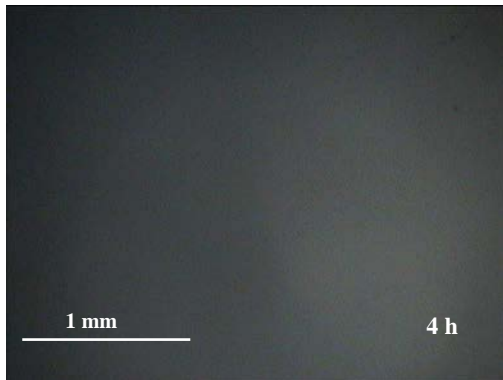
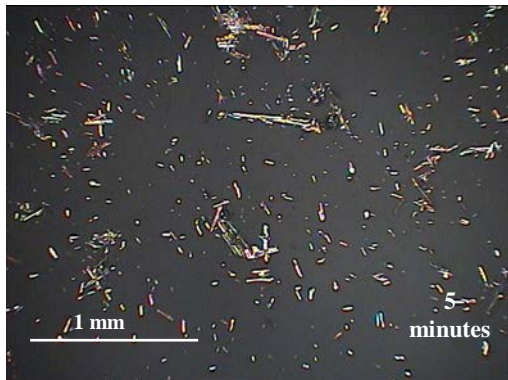


The picture of the flask shows polymer agglomerates, which are visible macroscopically.

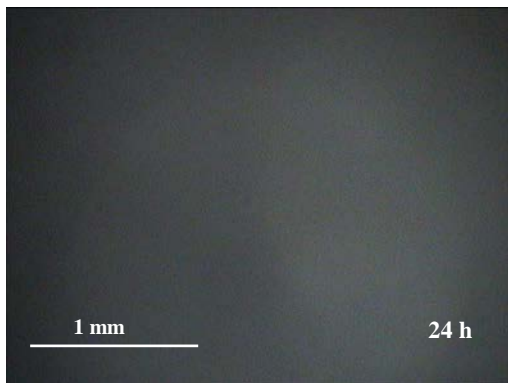
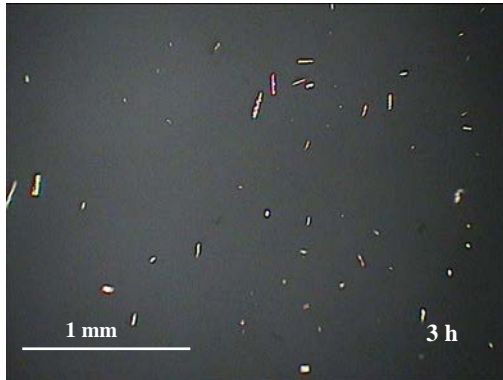
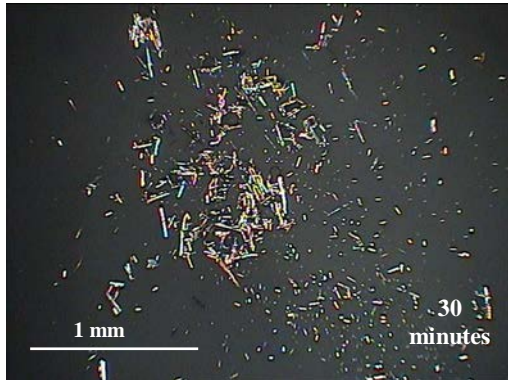
Eudragit® L 30D-55



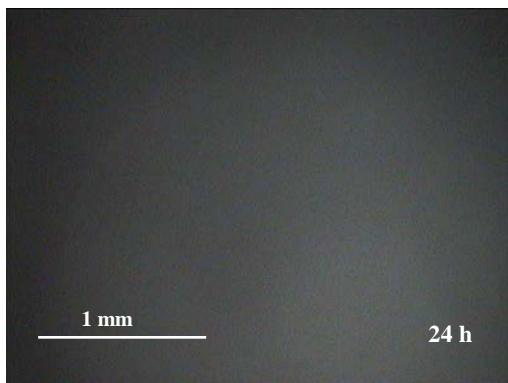
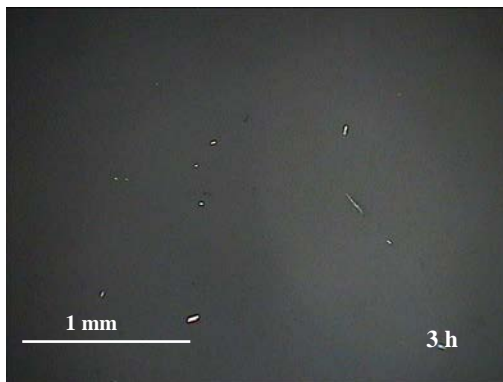
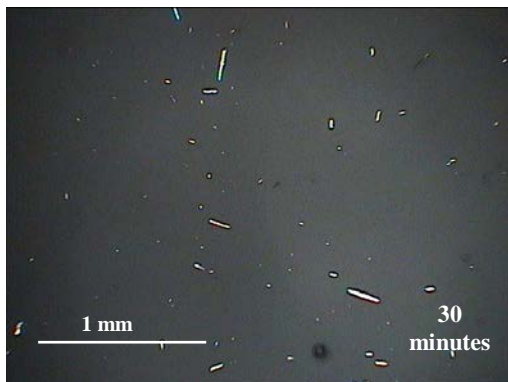
Eudragit® NE 30D



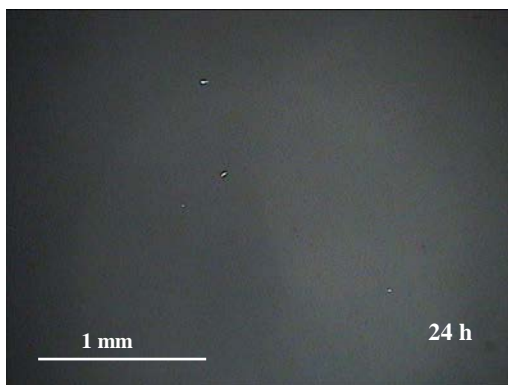
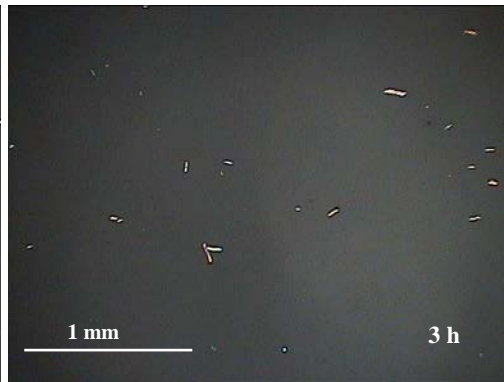
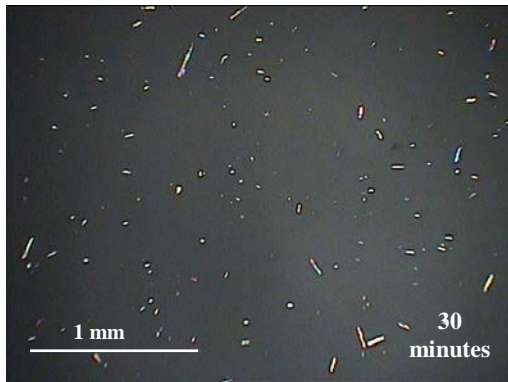
Eudragit® RL 30D



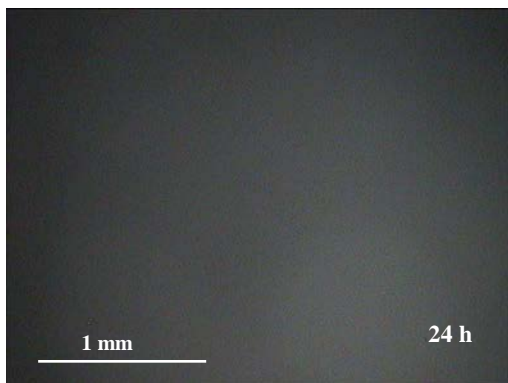
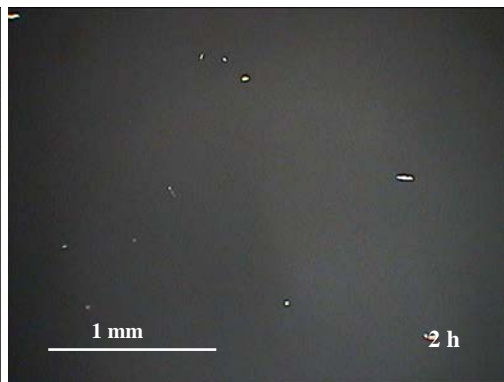
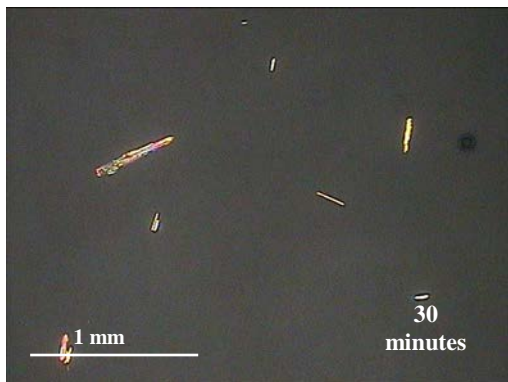
Eudragit® RS 30D



Kollicoat[®] MAE 30DP



Kollicoat[®] SR 30D



Curriculum vitae

LOIRA-PASTORIZA, Cristina

Date of birth July 20th, 1982
Place of birth Marin (Spain)

EDUCATION

02/2009-09/2012 PhD studies in “co-tutelle” between Freie Universität Berlin, College of Pharmacy (Germany) and Université de Lorraine, College of Pharmacy (France).
Supervisors: Prof. Dr Roland BODMEIER and Prof. Dr Philippe MAINCENT.
09/2000-04/2006 Pharmacy degree. Universidad de Santiago de Compostela, Spain.
06/2000 High School graduation, I.E.S. Sanchez Canton, Pontevedra, Spain.

PROFFESIONAL EXPERIENCE AND INTERNSHIPS

05/2007-06/2008 Laboratorios ROVI, Spain, Research and Development department.
11/2005-04/2006 Leonardo da Vinci Internship. Laboratoire de Pharmacie Galénique et Biopharmacie, Université Henri Poincaré-Nancy I, France.
03/2005-09/2005 Erasmus Internship. Laboratoire de Pharmacie Galénique et Biopharmacie, Université Henri Poincaré-Nancy I, France.

Publications

Journal publication

C LOIRA-PASTORIZA, A SAPIN-MINET, R DIAB, JL GROSSIORD, P MAINCENT
(2012). “Low molecular weight heparin gels, based on nanoparticles, for topical delivery”
International Journal of Pharmaceutics, 2012, 426 (1-2):p. 256-262

Poster

C LOIRA-PASTORIZA, A SAPIN-MINET, R DIAB, JL GROSSIORD, P MAINCENT
(2010). “Heparin gels based on nanoparticle suspension for topical application”. 25th Annual
Meeting of the G.T.R.V., Toulouse, France.

Development and characterization of new dosage forms based on drug containing aqueous colloidal polymer dispersion

L'utilisation de certains principes actifs dans le traitement et prévention de diverses maladies peut être limitée à cause de leur faible biodisponibilité. Cette faible biodisponibilité peut être liée à une solubilité limitée du principe actif et/ou une faible perméabilité à travers les barrières biologiques. La BCS (Biopharmaceutical Classification System), permet de classer les différents principes actifs en fonction de leur solubilité aqueuse et leur perméabilité intestinale. Le but de ce travail est d'augmenter la biodisponibilité de principes actifs peu biodisponibles et appartenant aux classes II et IV.

D'une part, des nanosuspensions polymériques chargés en principes actifs peu hydrosolubles ont été préparées et caractérisées. En effet, une faible solubilité aqueuse, empêche le principe actif de se trouver sous forme moléculaire au site d'absorption, limitant ainsi son passage à travers les barrières biologiques. L'augmentation de la solubilité des principes actifs au sein d'un vecteur polymérique permettrait une mise à disposition accrue du principe actif au niveau du site d'absorption et en conséquence une augmentation de la biodisponibilité. L'incorporation de ces principes actifs (celecoxib, diclofenac, econazole, ibuprofène, ivermectine et warfarine) dans la matrice polymérique a permis d'augmenter fortement leur solubilité. Une caractérisation physico-chimique de ces nanosuspensions a été effectuée et des études pharmacocinétiques *in vivo* ont été réalisées afin de démontrer notre hypothèse. Les résultats obtenus ne permettent pas de confirmer cette hypothèse sauf dans le cas des formulations préparées avec l'Eudragit® RL 30D.

D'autre part, un gel d'héparine destiné à l'administration topique a été préparé. Ce gel est obtenu par l'interaction électrostatique entre l'héparine (polyanion) et une suspension polymérique polycationique. Ce gel a démontré une grande capacité à incorporer de l'héparine. Des études de passage cutané ainsi que des études *in vivo* ont montré que ce gel peut permettre une action locale de l'héparine en évitant ses effets systémiques.

Mots clés : Dispersion polymérique, nanosuspension, solubilité, biodisponibilité, gel.

Development and characterization of new dosage forms based on drug containing aqueous colloidal polymer dispersion

Currently, the poor bioavailability of some drugs may limit their use in clinics. The poor bioavailability can be related to a low solubility of the drug and/or a low permeability through biological barriers. BCS (Biopharmaceutical Classification System) allows drugs classification as a function of their aqueous solubility and intestinal permeability. The aim of this work is to enhance the bioavailability of poorly available drugs.

On one hand, nanosuspensions containing poorly soluble drugs were prepared and characterised. To be absorbed, the drug should be available in its molecular form at the site of absorption; so a sufficient solubility is needed. The hypothesis of our work is to consider that the incorporation of poorly soluble drugs into a polymeric carrier may increase drug solubility and consequently enhance drug bioavailability. The incorporation of different lipophilic drugs (celecoxib, diclofenac, econazole, ibuprofen, ivermectin and warfarin) shows a great enhancement of drug solubility. Physico-chemical characterization as well as *in vivo* pharmacokinetics studies have been performed. The obtained results, does not allow to confirm our hypothesis except formulations prepared with Eudragit® RL 30D.

On the other hand, a heparin gel destined to a topic application has been prepared. The gel is obtained by electrostatical interaction between heparin (polyanion) and a polymeric polycationic nanosuspension. Heparin has been successfully incorporated into the gel and drug may be delivered to obtain a local action of heparin and thus, avoiding its systemic effects.

Keywords: Polymer dispersion, nanosuspension, solubility, bioavailability, gel.