A study of some lipid-based drug delivery systems

Alyaa Adel Ramadan

UNIVERSITE D'ANGERS **Année 2010**
U.F.R. des Sciences Pharmaceutiques N° 1083
UNIVERSITE D'ALEXANDRIE
Faculté de Pharmacie

ETUDE DE SYSTÈMES LIPIDIQUES DE DÉLIVRANCE DE PRINCIPES ACTIFS

THESE DE DOCTORAT
EN PHARMACOLOGIE EXPERIMENTALE
Présentée à

ECOLE DOCTORALE D'ANGERS
ET
ECOLE DOCTORALE D' ALEXANDRIE

à soutenir publiquement
Le 23 Septembre 2010
à Alexandrie

par

Alyaa Adel Ramadan
M. Pharm. Sci., Alexandria University

Devant le Jury ci-dessous :

Prof .Jean-Pierre BENOIT, Université d'Angers, Directeur de thèse
Prof .Labiba El KHordagui, Université d'Alexandrie, Codirecteur de thèse
Prof .Igor Choupra, Université de Tours, Examinateur
Prof .Nahed Mortada, Université du Caire, Examinateur
Prof .Fatma Ismail, Université d'Alexandrie , Examinateur
Prof .Magda El-Massik, Université d'Alexandrie, Rapporteur

INSERM U646, Ingénierie de la Vectorisation Particulaire
IBT, 10 rue André Boquel, 49100 Angers, France

Faculté de Pharmacie, Université d Alexandrie
Place de Khortoum, Azarita, Alexandrie, Egypt.

A STUDY OF
SOME LIPID-BASED DRUG DELIVERY SYSTEMS

A Thesis Presented

To

THE GRADUATE SCHOOL

FACULTY OF PHARMACY, ALEXANDRIA UNIVERSITY
AND
ANGERS UNIVERSITY

In Partial Fulfillment of the
Requirements for the Degree

Of

Doctor of Philosophy

In

Pharmaceutics

By

Alyaa Adel Abdel Aziz Ramadan

M. Pharm. Sci., Alexandria University

2010

A STUDY OF
SOME LIPID-BASED DRUG DELIVERY SYSTEMS

Presented by

Alyaa Adel Abdel Aziz Ramadan

M. Pharm. Sci., Alexandria University

For the Degree

Of

Doctor of Philosophy

In

Pharmaceutics

Examiners' Committee Approved

Prof. Dr. Nahed Mortada ……

Prof. Dr. Fatma Ismail ……

The French Prof. Dr. Igor Choupra ……

Advisors' Committee

The Late Prof. Dr. Nabila Abdel Hamid Daabis ----------

Professor of Pharmaceutics
Department of Pharmaceutics
University of Alexandria

Prof. Dr. Labiba Khalil El-Khordagui ----------

Professor of Pharmaceutics
Department of Pharmaceutics
University of Alexandria

Prof. Dr. Jean Pierre Benoit ----------

Professor of Pharmaceutics
Department of Pharmaceutics
University of Angers

Prof. Dr. Magda Abdel Sameea El-Massik ----------

Professor of Pharmaceutics
Department of Pharmaceutics
University of Alexandria

Acknowledgement

At the beginning, my deepest gratitude goes to Allah for giving me the strength to accomplish this amazing journey with all the difficulties and triumphs I experienced. The work accomplished in this thesis was completed partly in the Department of Pharmaceutics, Faculty of Pharmacy, Alexandria University in Egypt and the Institute Inserm U 646 , University of Angers in France. I owe my gratitude to both.

To my professors and colleagues at the University of Alexandria:

My deep sincere indebtedness are first addressed to the late ***Professor Nabila Daabis,*** *may God bless her soul who passed away at the beginning of this work but I will never forget her infinite support and continuous encouragement during my master's thesis.*

My deepest thanks, gratitude and everlasting appreciation to ***Professor Labiba El Khordagui****, who was always of great support throughout my career. I learned a lot from Prof. Labiba, but most of all, how to be a professional woman in science. Her valuable instructions and guidance make her a guide for all of us in her love for students' life as well as research life. Her generosity and kindness with every one of us can never be forgotten.*

My great appreciation to ***Prof. Magda EL Massik*** *, for her continuous support, great encouragement, precious advice and sincere suggestions throughout the development of this work. I was lucky to have her as supervisor throughout my postgraduate career.*

My deepest indebtedness, respect and great appreciation to Professor ***Nawal M. Khalafallah****; I was lucky under her official supervision at the beginning of this thesis and I will never be able to describe how much I learned from her valuable instructions, whole-hearted guidance, patience and continuous encouragement. Although she didn't officially continue to be my supervisor but she never stopped helping me to achieve this work.*

Appreciation is also expressed to my late Prof Youssef Ezz El-Din, May God bless his soul. Appreciation to all my dear professors in the Department and special thanks to Prof Zeinab El-Gholmy for her support, Prof Amal El-Kamel for her help in bibliography, Prof Osama Abdullah and Prof Mohamed Etman for the supply of materials. My special thanks to Dr Abir Kassem for her help with the Arabic summary, Dr Ragwa Farid for her help in statistics and endnote,Dr Gihan Salah and Dr Safaa Soliman for their precious friendship. Furthermore, Many thanks to all my colleagues especially my dear Sally for her help and (Dr Azza, Magda, Noha, Ayman, Heba, Allam, Riham, Hoda, Mohamed, Mariam, Yosra, Nesma, Asmaa, Ahmed and May). My great wishes to my colleagues abroad, May god help them all (Rania, Mohamed Essa, Iman, Mohamed Nounou, Kamilia, Noha, Mostafa, Iman, Yassmine and Hagar). Special thanks to Mostafa and Hagar for their collaboration and help at the beginning of this thesis. I would like to thank also my great professors in other Departments of the

Faculty who never hesitated to support me: Prof M Hafez and Prof M Abou sheaaer for the HPLC and statistics. The Deans of Faculties of Pharmacy and , Beirut Arab University (2004-2005) for allowing me to initiate my work in the industrial Pharmacy laboratory. Special thanks to Mme Sohayla, Beirut industrial lab for zeta potential measurements.

I wish to extend my warmest thanks to the secretarial staff, technicians and workers at the Department of Pharmaceutics as well as the technical staff of the Central Lab (Inas, Mohamed and Fatma) for their help with HPLC, DSC, and LD. Special thanks to Dr Omaiyma El-Gazaerly for image analysis at the Faculty of Pharmacy, Cairo university.

*For the time spent in the **French University of Angers**, I would first like to thank the University and the Egyptian government for the financial support during my stay in France.*

*I would like to express my deepest thanks and gratitude to **Professor Jean Pierre Benoit**, Professor of Pharmaceutics, Faculty of Pharmacy, Angers University, France, Director of Inserm U646, who had accepted to be my supervisor for the thesis in France and had allowed me to work in his lab with all the financial support. I am very grateful for his kindness, acceptance and support. Prof. Benoit made me feel at home and allowed me to achieve such work, Thank you for giving me the opportunity to learn a lot in a short period of time. Prof benoit "Veuillez trouver ici le témoignage de ma profonde reconnaissance"*

I would like to thank Prof. Frédéric Lagarce, Professor of pharmaceutics, Faculty of Pharmacy, Angers University for his precious advice, help, and valuable supervision of the in vivo part of the thesis where the oral bioavailability study was done. Thank you Frederic for your time and guidance to obtain such results in a short period of time and for the poster presentation.

I am very grateful to Olivier Thomas, who helped me a lot through my work in the lab. Without his precision, quick conclusions, great ideas, and active planning, the patent would have never been attained. I would like to thank Anne Tessier Marteau, University hospital, Angers, for her help with conducting the biological assay of heparin at the hospital. Special thanks to Prof Laurent Macchi for his discussion and help. Special thanks to Pierre le Gras, in the animal house for his help with blood sampling for the in vivo study. I am really grateful to Prof Patrick Saulnier, Faculty of Pharmacy, Angers University, France who helped me understand a lot of physical phenomena, Nathalie Faisant for her friendship and help, Frank Boury for his kindness and help, Edith Greleau for her support and help, Emilie Roger for conducting the Caco-2 transport study, Katrin, l'allemagne who worked a lot on the formulations of the microemulsions as main part of her undergraduate project, Ahmed El Sheik and Kaies Saudi for their help specially in the last days and Thomas Perrier for helping me understand chemical interactions. I would like to thank all the professors and colleagues in the IBT and all the others (Laurence, Emilien,, Sandy, Erika, Marie, Nolwenn, Catherine Chapon, Emile Allard, Than, Maud, Archibald, Gaeton, Pascal, for their kindness, help and support to make me feel at home!!

At last and most important, my family whom without them nothing would have really been achieved, My Great Parents (Mother and father): "thank you both for your help, patience and continuous support and for taking care of me and my children all the time". Above all, I will always be grateful and never forget how my husband stood beside me, encouraged me in every step of the thesis, without his support and patience, I would have never have continued that far. He has been a great support, and a great father for our kids. Finally, my two little boys:"Thank you for your patience, I love you"

My Great Mother & Father

The Soul of My grand mother

My Sincere Husband And
My Lovely Sons

TABLE OF CONTENT

PART I

CORTICOSTEROID-LOADED SOLID LIPID NANOPARTICLES GEL FOR

DERMAL DELIVERY

LIPID NANOCAPSULES

CHAPTER 1: LIPID NANOCAPSULES INCORPORATING FONDAPARINUX MICROEMULSIONS

CHAPTER 2
FONDAPARINUX LOADED LIPID NANOCAPSULES FOR ORAL ANTI-FACTOR XA EFFECT

LIST OF TABLES

GENERAL INTRODUCTION

I. OVERVIEW OF LIPID–BASED DRUG DELIVERY SYSTEMS

There is always a growing need for novel drug delivery systems to deal with the vast majority of the new chemical drug entities that have poor solubility or permeability and to improve the delivery of existing drugs. Polymeric drug delivery systems have been widely developed and provide an attractive alternative for progressive and long-term delivery of therapeutic agents as well as their wealth of possible chemical modification. Nevertheless, the number of products based on polymeric micro and nanoparticles in the market is still limited because of the toxicity of polymers and of solvent residues used in their production, high cost of biodegradable polymers, potentially toxic/allergic end products of biodegradable polymers and the lack of suitable large-scale production methods [1].

In order to overcome these problems, a great deal of interest has focused on lipid-based carriers such as lipid emulsions, liposomes or lipid nanoparticles [2]. Lipid-based delivery systems (LBDDS) are an accepted approach and an emerging field for drug delivery and have attracted different research groups because of their inherited properties, their biocompatibility and biodegradability of physiologically tolerated lipids, physiochemical diversity, lower toxicity, high incorporation efficiency for lipophilic drugs, protection of drugs from degradation, improved bioavailability, and controlled-release characteristics, challenges in stability and manufacturing at the commercial scale and suitability for drug delivery at different sites of administration [3].

The physicochemical stability of the lipid carriers showed variations due to their numerous compositions and structures. The mechanisms of the destabilization (chemical, physical, and interfacial mechanisms), the particle interfacial behavior, techniques used to detect any destabilization, the inductors of destabilization (composition, storage, and process parameters) and the methods to optimize the stability of lipid nanoparticulate systems are reviewed by Heurtault et al.[4].

LBDDS formulations can be tailored to meet a wide range of product requirements dictated by disease indication, route of administration and considerations of cost, product stability, toxicity, and efficacy[5].

LBDDS are attractive carriers for oral formulations [6, 7] since it was proven, from many years ago, that the co-administration of poorly water-soluble drugs (PWSD) with a meal rich in fat enhanced their oral bioavailability through enhancing gastrointestinal solubilization and absorption via selective lymphatic uptake [8]. Further mechanistic understanding of their impact on drug disposition is emerging[9]. The maximum advantage from a lipid formulation could only be drawn if the drug remains in lipid solution throughout its residence in the GI tract. The choice of lipid formulations according to the Lipid Formulation Classification System (LFCS) in relation to the physicochemical properties of the drug as well as the properties of excipients, criteria for their selection for lipid-based formulations and the fate of these materials during dispersion and digestion, and the likely consequences of their use in formulations have been also reviewed elsewhere[10].

Lipid-based formulations can hence be applied to influence the absorption of active ingredients via various mechanisms, such as modifying the release of active ingredients, improving their bioavailability, changing the composition and hence the character of the intestinal environment, stimulating the lymphatic transport of active ingredients, interacting with enterocyte-based transport processes and reducing unwanted drug side effects. Understanding the role of lipid in the process of bioavailability of lipophilic drugs, the *in vivo* fate of lipids (digestion, absorption, circulation), mechanism of digestive process, transcellular transport, if any and challenges of formulation with emphasis on solid dosage forms as well as example of some lipid-based oral drug delivery systems available on the market are well reviewed elsewhere [(11)]. Moreover, special emphasis on phospholipids–based formulations, the role of phospholipids in protecting active ingredients from degradation in the gastrointestinal tract, increasing lymphatic uptake of some drugs or decreasing their side effects or masking their taste have been also reviewed lately [(12)].

Encapsulating lipophilic drugs into oil droplets was first introduced in 1960 using the submicron emulsion o/w (Intralipid®) for parentral nutrition via injection. Microemulsions were reviewed for their parenteral use[(13)]. The low viscosity of the droplets causing fast release and susceptibility of the incorporated actives towards degradation by the aqueous continuous phase were the main drawbacks from such systems. More interesting, injectable liposomal products were commercialized such as AmBisome® (Amphotericin B), Doxil®/Caelyx® (Doxorubicin) and DaunoXome® (Daunorubicin) and a large array of investigational products which clearly indicates the potential advantages of liposomes as novel lipid carriers [(14)]. Other LBDDS such as lipid nanoparticles have also proven efficacy in parenteral delivery of a wide variety of drugs such as anticancer agents, imaging agents, anti-parkinsonism, antiHIV, antipsychotics, anti-rheumatoid arthritic agents, antiparasitics, antihypertensives and antibiotics[(14)]. With their controlled drug release , site specifying targeting, accepted parenteral lipid excipients generally recognized as safe (GRAS), stable formulations ease of manufacturing in a sterile form and cost effectiveness may expand the array of targetable parenteral lipid-based systems [(14-16)].

The use of LBDDS for the topical treatment of skin diseases is very attractive, since epidermal lipids are found in high amounts within the penetration barrier thus decreasing their systemic absorption by localization in skin layers. Besides liposomes[(17)],solid lipid nanoparticles and nanostructured lipid carriers showed potentials in dermal targeting as well as cosmetic products by overcoming the stability problems of liposomes[(18, 19)].

Moreover, transdermal drug application has gained increasing importance for systemic treatment, e.g. with drugs subject to extensive first-pass elimination such as glyceryl trinitrate or estrogens as well as for the sustained suppression of chronic pain. Extensive research has been conducted on the use of nanoemulsions, microemulsions, liposomes and most importantly lipid nanoparticles for the transdermal delivery of various drug groups [(20)].

The use of lipids in parenteral, dermal/ transdermal, and ocular drug delivery systems has been reviewed[(21)]. Specific needs, considerations, approaches, and

limitations in lipid-based drug delivery were also discussed on the basis of the anatomical and physiological diversity of the target organs and systems.

Currently, strategies for controlling the release of inhalation therapy include molecular dispersions (liposomal-based systems), solid lipid microparticles as well as coating or encapsulating drug particles in a lipid outer shield[22]. Basic advantages of drug release from lipid nanoparticles and lipid microparticles in the lung are control of the release profile, and faster biodegradation compared to particles made from some polymeric materials.

Different strategies and considerations for successful LBDDS have been reviewed[5]. Successful drug design with lipids depends largely on understanding the physical–chemical and physiological factors that promote or inhibit bioavailability. A prerequisite to formulating with lipids is the ability to differentiate among the classes of lipids in relation to their physical–chemical properties and the mechanism of absorption enhancement. Appropriate selection of lipid excipients and formulation strategies at the earliest stages of drug development can lead to considerable savings in cost and time to reach market.

Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds. Lipid-based carriers are well tolerated in living systems because they are made of physiological compounds which are expected to pass by metabolic pathways that exist in the human body decreasing the risk of acute and chronic toxicity[23]. In fact, the most important issue remains in the toxicity of the emulsifiers [11, 16, 24].

However, further research is still needed to better understand the absorption enhancing mechanisms of lipids; to find better predictive tools for assessing the *in vivo* behavior of various lipids with different types of drug molecules; development of regulatory guidelines for characterization of lipid based formulations; and techniques for enhancing the stability of lipid based systems [5].

II. CLASSIFICATION

A. LIQUID LIPID-BASED DRUG DELIVERY SYSTEMS

The idea of encapsulating lipophilic drugs into oil droplets has been introduced since the use of submicron sized parenteral fat o/w emulsion for parenteral nutrition Intralipid® in 1960s. Lipid emulsions (LE), suspensions, solutions, have all been used to enhance the oral bioavailability of poorly water soluble drugs (PWSD). Examples of studies investigating the oral bioavailability of PWSD from oil-in-water emulsion formulations were reviewed [9].

One of the reasons preventing a broad introduction of LEs is their physical instability which can be exacerbated by the incorporated drug [2]. The low viscosity of the droplets causes fast release and susceptibility of the incorporated actives towards degradation[25].

1. NANOEMULSION

Lipid nanoemulsions (LNE) are fine o/w dispersions, having droplets covering the size range between 50 and 200 nm. They were introduced during the 50ies for the purpose of parenteral nutrition and are also referred to mini-emulsions, fine-dispersed emulsions or submicron emulsions[26]. The lipid phase (10%–20% of the emulsion) could be fatty vegetable oils (eg, soy oil) or middle chain triacylglycerols. Other ingredients such as phospholipids (stabilizers, 0.6%–1.5%) and glycerol (osmolarity regulation, 2.25%) could be added.

Nanoemulsions are transparent or translucent kinetically stable systems, (no thermodynamic equilibrium). They are characterized by great stability due to their very small size and hence significant steric stabilization between droplets [27]. Due to their small particle size, large surface area and free energy, they do not undergo the inherent creaming, flocculation, coalescence and sedimentation associated with macroemulsions. They can be formulated in a variety of formulations such as liquids, sprays, foams, creams. More advantages of nanoemulsions include toxicological safety and a high content of the lipid phase, as well as the possibility of large scale production by high pressure homogenization, as ointments and gels [15]. Their main problems are membrane stability and drug-leaching. Ostwald ripening is the only adapted droplet destabilization process[28]. The partitioning of the drug in emulsions could result in a burst release. For most drugs, a rapid release will be observed with limited controlled release properties (except for very lipophilic drugs), due to the small size and the liquid state of the carrier.

The generation of nanoemulsions is mainly based on three different groups of methods: high-energy methods like high-pressure homogenization and microfluidization or ultrasonication, low-energy spontaneous emulsification or solvent-diffusion methods, and the low-energy phase inversion temperature (PIT) method.

During recent years, LNE have been used as drug carriers for mainly lipophilic drugs and several formulations are nowadays commercialized like etomidate (Etomidat-Lipuro®) and diazepam (Diazepam- Lipuro®). A reduction of the local and systemic side effects (eg, pain during injection) has been found in comparison to previous solubilization-based formulations of these drugs[11]

2. MICROEMULSIONS

Microemulsions are thermodynamically stable isotropic dispersions, transparent, of low viscosity, containing water and/or oil nano-domains coexisting in thermodynamic equilibrium due to the presence of a surfactant film adsorbed at the oil/water interface, typically in conjunction with a co-surfactant[26]. Usually, the inner phase either oil (o/w emulsions) or water (w/o emulsions) has globule sizes ranging

from 5 to 100 nm. Oil-in-water (o/w) microemulsions are called Winsor Type I microemulsions, water-in-oil (w/o) are Winsor Type II, and microemulsions bicontinuous in oil and water are called Winsor Type III or Type IV if there are no excess phases. Microemulsions offer several advantages for pharmaceutical use including ease of preparation, thermodynamic stability, high solubilization capacity for lipophilic and hydrophilic drugs, and their ability to facilitate the transport of drugs through biological membranes[29]. Amongst the various drug delivery systems, the microemulsion system is considered an ideal alternative for the oral delivery of poorly water-soluble compounds as well as proteins and peptides [11, 30].

Microemulsions have been also in use for transdermal drug delivery for a long time; their popularity for dermal applications is second only to liposomes, as they can ensure an unusually intimate contact between the epicutaneous drug reservoir and skin lipids[31]

On the other hand microemulsions offer only limited, if any, protection of the peptide or protein against chemical and enzymatic degradation in the gastrointestinal tract. In contrast, particulate delivery systems can protect these problem drugs, against degradation [32]. Other problems reported for microemulsions include: their cytotoxicity due to high amounts of surfactants and cosurfactant (alcohol) and their low stability.

3. SELF –EMULSIFYING SYSTEMS (SMEDDS, SNEDDS)

Self-emulsifiying drug delivery systems (SEDDS) are particular examples of nanoemulsions[33]. They are isotropic mixtures of an oil, surfactant, cosurfactant and drug . These components form fine o/w nanoemulsions when introduced into aqueous media under mild agitation or after *in vivo* administration. SEDDS typically produce emulsions with a droplet size between 100 and 300 nm, while self-microemulsifying drug delivery system (SMEDDS) form transparent microemulsions with a droplet size of less than 50 nm. When compared to emulsions, which are sensitive and metastable dispersed forms, SEDDS are physically stable formulations that are easy to manufacture[11]

Their ability to form small droplets of oil and the polarity of the oil droplets to promote faster drug release into the aqueous phase as well as their surfactant content control their in vivo performance[34]. These systems may offer an improvement in the rate and extent of absorption and result in more reproducible blood/time profiles for lipophilic drug compounds that exhibit dissolution rate limited absorption[35]

A marketed formulation of cyclosporine (Sandimmune®, Neoral®), a microemulsion pre-concentrate with self-emulsifying properties, is reported to improve oral bioavailability and reduce inter- and intra-subject variability in cyclosporine

pharmacokinetics[9]. The nonaqueous microemulsions, also known as microemulsion pre-concentrate, contains polar solvent ethanol and not water. Upon contact with aqueous media, such as gastrointestinal fluids, the nonaqueous microemulsion spontaneously forms a fine dispersion or aqueous microemulsion.

Furthermore, SNEDDS have a great potential for oral protein delivery[36]. In order to produce peptides- or proteins-loaded SEDDS, these bioactives are first formulated in a w/o microemulsion pre-concentrate, and then dispersed into an aqueous phase to form a w/o/w microemulsion prior to administration[37]. The clinical usefulness of the SEDDS is evident from the commercially available formulations containing cyclosporin A, ritonavir and Saquinavir[9].

However, since a relatively high concentration of surfactants is generally employed in the SEDDS formulation, toxicity of the surfactant being used should be taken into account. In fact, a compromise must be reached between the toxicity and self-emulsification ability of the surfactant that is considered for use.

B. SOLID LIPID-BASED DRUG DELIVERY SYSTEMS

4. LIPOSOMES

Liposomes are enclosed vesicles composed of one or more phospholipid bilayers enclosing an aqueous phase [38]. They can be classified as 100nm–20 mm large multilamellar vesicles (MLV), 10–50 nm small unilamellar vesicles (SUV) or 50–1000 nm large unilamellar vesicles (LUV), depending on their size and the number of lipid bilayers. They are capable of carrying either hydrophilic drugs in their inner aqueous phase or hydrophobic drugs in their hydrophobic lipid bilayers. The techniques of preparation have often been described and reviewed in literature[17]. As their composition is similar to the biomembranes, they are biodegradable and non-toxic. The use of liposomes as oral drug delivery system is difficult due to the poor stability of the vesicles under the physiological conditions typically found in the GI tract. Nevertheless, there are many studies and recent publications that indicate the potential of phospholipid-based liposomes to enhance the oral bioavailability of poorly soluble and low-bioavailability drugs, including peptides and proteins[39]. Oral cyclosporine A and calcitonin as well as vaccination with liposomes were successful.

Liposomes offered several advantages increasing most of which not offered by submicronic emulsions[17]. Drug release, in vivo stability, and biodistribution, are determined by the size of the vesicles, their surface charge, surface hydrophobicity, and membrane fluidity[11]. It is possible to construct a wide range of liposomes varying in size, phospholipids composition and surface characteristics to suit the specific application for which they are intended. Membrane permeability can be adapted by the selection of the phospholipids composition, and by the presence of additives, such as cholesterol molecules. It is possible to avoid a rapid reticuloendothelial uptake of the

liposomes by the incorporation of natural compounds or by the use of chemical modified polyethylene glycols (PEG′s)[11]. The development of such sterically stabilized systems (‘stealth liposomes’) allows the development of drug targeting strategies (eg, by incorporation of specific antibodies)[11]. So, their biocompatibility, and ease of surface modification rendered them suitable candidate delivery systems for peptides and proteins molecules[11]. Liposome-based systems encapsulating drugs are already used in some cancer therapies (e.g. Myocet®, Daunoxome®, Doxil®).

Liposomes also allow the intravenous injection of lipophilic drugs with very low water solubility, eg, amphotericin B (Ambisome®)[40], reducing therefore the toxicity of such drugs. Liposomes have been investigated for the delivery of vaccine, toxoids, gene, anticancer, and anti-HIV drugs. Their blood circulation time can be increased through surface modification[41], (eg, by attaching PEG or dextran to the lipid bilayer). Furthermore, conjugation with targeting ligands, like monoclonal antibodies or aptamers, can enhance their tissue specificity[42].

Advantages of liposomes in dermal as well as transdermal drug delivery were well proved [17, 20, 43]. They were first introduced to the cosmetic market by Dior in 1986 as an anti-aging product [44]. Liposomes may serve as penetration enhancers facilitating the transport of compounds through the epidermis; help in reducing skin irritation by sustaining the release of drugs and by hydration of the epidermis. They also have the potential to target drugs into the pilosebaceous structures and hence they have an additional advantage for treatment of hair follicle-associated disorders [17].

However, chemical and physical stability problems have been described leading to liposome aggregation, drug leakage and drug degradation during storage [23]. Liposome technology has existed for the past four decades, but they do not have enough market share due to some of their potential drawbacks, like batch-to-batch variation in manufacturing, low drug loading efficiency, and poor stability[42]. Further liposomes drawbacks include: low capacity to encapsulate lipophilic drugs complex manufacturing processes involving organic solvents, difficulties in scale-up, instability in biological fluids and more generally in aqueous solutions and enormous cost of the liposomal formulation [14, 44, 45].

5. LIPID MICROPARTICLES

Colloidal lipid suspensions represent aqueous dispersions of solid lipids at room temperature. The use of solid lipids instead of liquid oils is a very attractive idea to achieve controlled drug release, as drug mobility in a solid lipid should be considerably lower compared with liquid oil. They are prepared by emulsification of a molten lipid in an aqueous medium creating an oil-in-water emulsion which is supposed to transform into an aqueous colloidal suspension by recrystallization of the dispersed lipids upon cooling. These systems have been regarded as o/w emulsions with solid globules [46].

The production of solid lipid microparticles (SLM) by spray congealing was described earlier as “Lipospheres” by Speiser at the beginning of the eighties followed by lipid nanopellets for peroral administration [44]. The nanopellets and lipospheres (in

μm range) were produced by dispersing a melted lipid in a surfactant solution with high speed mixers and/or ultrasound. The obtained particle size is determined by the power density of the stirrer. Other solvent evaporation techniques or a melt dispersion techniques could be used for their preparation[47], and they are suitable for SC or IM injection.

Lipid microparticles could be an alternative to polymeric microparticles as a parenteral controlled release device for peptides[47]. Mucosolvan® retard capsules are well-known example for lipid pellets for oral drug delivery. Additional advantages in dermal application of lipid microspheres is their high affinity to the stratum corneum where sunscreen UV filters intended to act , proper size for reduced skin absorption, high entrapment efficiency and their solid matrix protects loaded labile substances against decomposition.

6. LIPID NANOPARTICLES AS DRUG DELIVERY SYSTEMS

Lipid nanoparticles gain more interest than microparticles, they combine both technology of lipid sciences and nanosciences. Generally, the size is considered as a major issue for pharmaceutical applications since it greatly influences the *in vitro* and *in vivo* studies . The role of particle size and recent progress for the rational design of nanoparticles have been discussed in a recent review [48]. One fundamental advantage of nanoparticles as compared to other colloidal drug delivery systems (liposomes, niosomes, microemulsions etc.) as well as nanoemulsions is their great kinetic stability and rigid morphology.

The term nanoparticles refers to nanospheres or nanocapsules that are, respectively, matricial having a homogeneous structure in the whole particle, or vesicular exhibiting a typical core-shell structure varying in size from 10 to 1000nm [27, 30]. They have liquid core or solid core.

These lipophilic colloidal delivery systems have attracted increasing attention over the last years as efficient and non-toxic drug carriers. Their colloidal dimensions and their controlled release behavior enable drug protection and administration by parenteral and non-parenteral routes thus emphasizing the versatility of this nanoparticulate carrier. A main challenge of the formulation of nanoparticles is adapting the choice of their own structure to the final aims of drug delivery[27]. Their composition, structure and formulation process could be optimized to increase their physico-chemical stability[4]. The special features of lipid matrix nanoparticles, in particular for controlled release purposes as well as pharmacokinetic and biopharmaceutic results achieved by different research groups have been reviewed[49].

An overview of these lipid nanoparticles, their characteristics, applications, advantages and disadvantages comparing the lipid nanoparticles with solid core (SLN), partial solid and liquid lipid core (NLC) and those with liquid lipid core known as lipid nanocapsules (LNC) is provided in the next section with more emphasis on LNC.

1. TYPES OF LIPID NANOPARTICLES

4) SOLID LIPID NANOPARTICLES

Solid lipid nanoparticles (SLN) were developed at the beginning of the 1990s. They are colloidal lipid suspensions or submicron sized aqueous dispersions of solid lipids called also lipospheres or nanospheres and produced by replacing the liquid lipid (oil) of an o/w emulsion by a solid lipid or a blend of solid lipids, i.e. the lipid particle matrix being solid at both room and body temperature [46, 50]. They are stabilized by surfactants. They are attractive because they combine advantages of various traditional carriers. Identical to polymeric nanoparticles they possess a solid matrix, protective for chemically labile actives and giving the ability to modulate drug release. Identical to nanoemulsions and liposomes they are composed of well-tolerated, regulatorily accepted lipids and can be produced easily on a large industrial scale [14]

5) NANOSTRUCTURED LIPID NANOCARRIERS

Nanostructured lipid carriers (NLC) are the second generation of SLN, composed of a solid lipid matrix with a certain content of a liquid lipid (oils). To obtain the blends for the particles matrix, solid lipids are mixed with liquid lipids, preferably in a ratio of 70:30 up to a ratio of 99.9:0.1[18, 51]. They possess improved properties for drug loading especially lipophilic one, modulation of the delivery profile, and stable drug incorporation during storage [2, 23]. There are three types of NLC depending on their lipid composition and oil content : (i) the imperfect type, (ii) the multiple type, and (iii) the amorphous type which could affect drug loading [52].

6) LIPID NANOCAPSULES

Lipid nanocapsules (LNC) are novel lipidic systems in a nanometer-size range also but made of an oily liquid core surrounded by a 2 -10nm thickness solid or a semisolid surfactant shell (i.e., a nanocapsule system)[53, 54] **(Figure 1)**. These nanoparticulate carriers were primarily developed since 2000 to combine the colloidal stability of solid particles suspensions in biological fluids and the solubilizing properties of liquids.They are considered particular nano-emulsions characterized by a great stability in suspension which is fundamental for many industrial applications [55]

Analysis of the interfacial behaviour of lipid nanocapsules compared with the interfacial behaviour of pure constituents allowed studying their structure. The advantages of such a structure are: Firstly, the high drug encapsulation efficiency due to the optimized drug solubility in the nanoparticle core and low polymer content compared to polymeric nanospheres. Secondly, since the drug is ‘protected’ within the LNC core, tissue irritation at the administration site as well as the burst effect are lowered, and the drug itself remains protected against degradation.

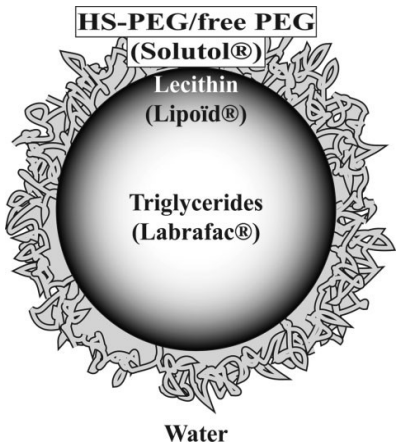


Figure 1 : Schematic representation of lipid nanocapsules formed by a tensioactive shell composed by the association of hydroxystearate of poly(ethyleneglycol) and phosphatidylcholine protecting an oily core (medium chain triglycerides)[53, 56]

2. COMPOSITION OF LIPID NANOPARTICLES

SLN are dispersions having typically water contents of 70–99.9% and are composed of 0.1 % to 30% (w/w) solid lipid dispersed in an aqueous medium and if necessary stabilized with preferably 0.5% to 5% (w/w) surfactant [19, 23]. Further advantage of the second generation of lipid nanoparticles, **NLC,** is that the overall solid content could be increased up to 95% with a better practical outcome.

Only defined mixtures lead to **LNC** formulations; oil (10%-25%w/w), hydrophilic surfactant (10%-40% w/w) and water (35%-80% w/w) [53]. Lecithin as lipophilic surfactant 1.5 % (w/w) and NaCL 1.75% (w/w) were added to the mixtures from the beginning. Similar to nanoemulsions[27], a ternary diagram is established to optimize the constituent proportions (w/w) before the cooling dilution step in their preparation, which could favor the formation of LNC. The region was termed the (Ob domain) **(Figure 2)** and determine the feasibility region (ob domain) of nanocapsules. Using a high quantity of hydrophilic surfactant in water, micellar systems were formed which were not stable by dilution and were characterized by a particle size of <10 nm. On the other hand, the formed emulsion was not stable (phase separation) for a low hydrophilic surfactant concentration, again preventing the formation of nano-objects. Furthermore, inside the feasibility domain, each formulation was characterized by a wide range of diameters that were reproducible with the same composition[57].

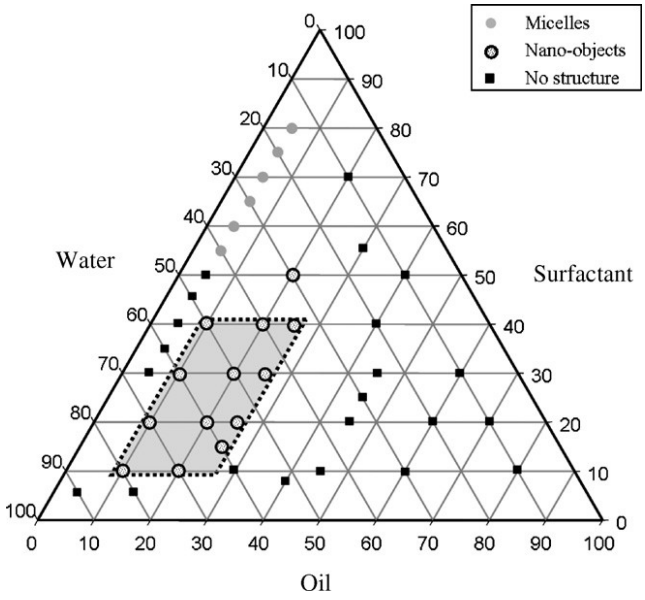


Figure 2 Ternary diagram allowing the determination of the feasibility zone (Ob domain) of nanocapsules[53].

The Lipid phase

The lipid matrix is made of physiological biocompatible lipids which are well tolerated by the body and possess excellent biodegradability and low toxicity.

The lipids used for **SLN** and **NLC** include highly purified triglycerides, complex glyceride mixtures, partial glycerides (e.g. Imwitor), fatty acids (e.g. stearic acid), steroids (cholesterol) or even waxes (e.g. cetyl palmitate)[23]. Furthermore, SLN based on para-acyl- Calixarenes have been prepared and studied [58]. The presence of oil mixed with the solid lipid n **NLC**, resulted in melting point depression compared to the pure solid lipid but the blends obtained are also solid at body temperature

It was found from many studies that the average particle size, particle size distribution, drug loading , drug release, quality and stability of the SLN dispersions are affected by the lipid content [59], type of lipid matrix and their properties such as melting point, viscosity of the triglyceride melts[60], velocity of lipid crystallization, lipid hydrophilicity and shape of the lipid crystals (and therefore the surface area)[23]. Lipid composition (e.g. impurities), amounts of mono-and diglycerides which possess surface active prrpoerties, crystal packing, degradation velocities, crystallinity of lipid phase were also found to affect the SLN characteristics[44]. All methods of SLN preparation involve melting the lipid component and subsequent rapid cooling to solidify the SLN. The lipid components solidify with various degrees of crystallinity which will affect drug loading and stability of the dispersion[61]. The properties of colloidally dispersed glycerides differ from those of their bulk materials due to their colloidal size. This especially applies to the melting and crystallization behavior and to the kinetics of the polymorphic transitions. Crystallization temperature and polymorphic transitions are important parameters for the preparation of triglyceride nanoparticles [62].

In **LNC**, medium-chain triglyceride such as caprylic and capric acid triglycerides (Labrafac®) are mainly used as the oil phase. But other lipids like Miglyol®812[63], isopropyl myristate, or ethyl oleate, etc can also be used[54]. It was found that the HLB value of the fatty substances does not appear to have an influence on the size of the nanocapsules but affect the temperature of the phase inversion during their preparation. In other words, an oil in-water emulsion is more readily obtained when the oil is more lipophilic. Moreover, the carbon chain length of the fatty acid does not influence the particle size, or the phase inversion temperature (between C14 and C18). It appears, however, that the double bond present in ethyl oleate substantially increases the phase inversion temperature[54]. The oil content affects the drug payload, depending on physicochemical properties of both. The droplet diameters reached with the stabilization are governed by the water/oil ratio, which also governs the microemulsion structure (more or less swollen by oil)[55]

Surfactants

The choice of the suitable surfactant or surfactant mixtures and their concentrations have great impact on the quality of **SLN**, since surfactant affects surface properties of SLN[23]. The best choice contributes to higher stability by preventing particle aggregation more efficiently.

In SLN, all classes of surfactants (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion, including phospholipids, bile salts, poloxamers and other ionic and nonionic surfactants[11]. Different emulsifier compositions might require different homogenization parameters [23]. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently and reduce particle size of the SLN [23]. The choice of emulsifier depends on administration routes. Alternatively, they can be produced surfactant-free using steric stabilizers (e.g.poloxamer188) or an outer phase of increased viscosity (ethyl cellulose solution)[23]. The particle size of the SLN dispersion produced with the ionic surfactants was considerably smaller compared to the nonionic formulation[64]. The type of emulsifier used in the formulation may not only affect the physical stability of the dispersion but also influence the crystallization and polymorphic behavior of the nanoparticles[62], degradation stabilization[65] as well the quality of the dispersion. The concentration of the emulsifier influences the particle size of the SLN dispersions. High concentrations reduce the surface tension and facilitate the particle partition during homogenization. The excessive emulsifier molecules might be present in different forms e.g.molecular form (emulsifier monomers), in form of micelles (sodium dodecyl sulphate, SDS) or liposomes (lecithin) that differ in the time scale for redistribution processes and it is often combined with considerable water solubility and toxicity. The addition of co-surfactant e.g. sodium glycocholate to the aqueous phase decreases the particle size too[23].The hydrophilic co-stabilizer is able to modify the basic crystal shape of the triglycerides in dependence of its chemical structure and concentration[62, 66]. It was also found that the amount of butanol as non-ionic co-emulsifier (molecule with oxygenated function) affects the polymorphism of stearic acid (lipid phase). A part of

the butanol may stay at interface of microemulsion, partially replacing the anionic surfactant (Tween 80), resulting in formation of polymorph B of stearic acid

In LNC: Nonionic polyethoxylated (PEO) surfactants are used as they allow performing the emulsion inversion, with regards to the formulation variables, and particularly the temperature[27], but the affinities of the surfactant for the aqueous and oily bulk phases have to be relatively balanced, right from the start. PEG chain length affects the phase inversion temperature (PIT) which is not possible when increasing the molecular weight of the non ionic SAA with very long PEG chain up to 1500 or 2000 g/mol)[54, 67] The Solutol®HS is the most commonly used nonionic surfactant which is a polyethoxylated PEO model, a macrogol 15 hydroxystearate (its major component is polyoxyethylene-660-12-hydroxy stearate). Other nonionic surfactants such as Cremophor®[63] were successful as well. An additional, somewhat neutral component, the lipophilic surfactant Lipoid®, composed of 69% phosphatidylcholine soya bean lecithin, is used in small proportions and has been shown to significantly increase LNC stability creating a 'framework' in the shell [68-70] which is especially necessary in the case of 50–100nm LNC formulations.

In the region of feasibility, increasing the percentage of hydrophilic surfactant (Solutol®), leads to a considerable decrease of average particle diameter. The Solutol® exhibits high surfactive properties due to its amphiphilic structure consisting of a big hydrophilic polyethylene glycol moiety associated to a big hydrophobic hydroxystearate moiety. This affects its properties at the triglyceride/water interface [71]. The concentration of Solutol® used in the formulation of LNC is above the critical micellar concentration of the surfactant[71]. Therefore, interface structures would be micellar-like organizations in which amphiphilic drugs could be easily incorporated[72]. Further studying of the electrokinetic properties of blank LNC [70], it was shown that about 30% of Solutol® could be lost from the particle surface by dialyzing LNC, resulting in a better surface organization of the LNC[72]. Moreover, the LNC diameter depends also on both $Fraction_{oil}$/$Fraction_{Solutol®}$, and the Solutol® / Lipoid® ratios[57].

Another cosurfacatnt or lipophilic surfactant used in LNC is Lipoid® which exhibits lower surfactive properties than Solutol®. Moreover, since it is an ionic surfactant, the presence of sodium chloride in the water phase screens the electric charges of the molecule, leading to a decreased surfactive power. Nevertheless, Lipoid® has an influence on LNC diameter. Interestingly the mean diameter of LNC is smaller for Lipoid®-enriched formulations. A possible explanation is that, when introduced in higher amount in the formulation, the Lipoid® may be better combined to Solutol® to stabilize the emulsion as a co-surfactant. On the other hand, the use of charged molecules did not allow nanocapsules to be obtained[54] . In a recent study[63], cetyl alcohol, stearyl alcohol were used instead of Lipoid® with much lower melting temperature and did not affect the solid state properties of the LNC shell which is reflected in the release profiles[63]. On the contrary, increasing the concentration of Lipoid® up to 5% (w/w) was found to have no influence on particle size distribution [63, 73]. Indeed, the increase of the percentage of lecithin in the outer layer leads to a thicker and more rigid shell, with anticipated improvement of the aptness to freeze-drying and the prevention of the leakage of the oily phase[57]. Furthermore, since the

melting temperatures of Solutol® components are 18.4°C and 31.0°C while the melting temperature of Lipoid®is 83.5°C, it can be assumed that the rigidity of the shell increases when the S/L ratio decreases[57]. Therefore, the S/L ratio should be compromised to preserve both the stability of LNC in a dried form and the biopharmaceutical performances of LNC.

Other ingredients

SLN dispersions, which are stabilized by electrical charge, are sensitive to the addition of electrolytes. Whereas, high salt concentrations induce an increase in viscosity and lead to formation of gels, low salt concentrations, in contrast reduce the dispersion viscosity. The observed viscosity effect in dispersions with ionic stabilizers is probably due to a shrinking of the particle electrical double layer by the influence of added electrolytes and corresponds to the reduction of the particles'effective volume fraction[66]

In LNC, increasing the NaCL concentration before dilution (from 1 to 5% w/w),shifts the phase inversion zone (PIZ) from high to lower temperatures but the PIZ remains virtually unchanged [53]. This may be due to modification in the solubility of the PEO surfactant (salting out effect) [45, 74].

3. METHODS OF PREPARATION AND PRINCIPLES FOR LIPID NANOPARTICLES

SLN are produced by several methods extensively described in the literature. The principle of all methods is based on generation of nanoemulsions by substituting oil for melt lipid using both high- and low-energy methods. Then additional cooling gives rise to lipid crystallization and the generation of SLNs.

The high energy approach for SLN production [23, 44], in general consists in (i) keeping the lipid phase (plus potentially solubilized drug) 5–10 °C above its melting point, (ii) premixing it with the aqueous surfactant solution at the same temperature, (iii) nano-emulsifying the preemulsion using a high-energy method (high pressure homogenizer or high shear sonication [23] and finally (iv) cooling it down to room temperature to crystallize the lipids.

The low energy approaches for SLN are either based on generation of nanoemulsions by spontaneous emulsification [75, 76](lipid melt instead of oil) generated by solvent evaporation or diffusion so rapid displacement of the surfactants from the oily to aqueous phase[77], or other based on formation of a microemulsion above

melting point of the lipid (lipid melt/SAA/coSAA/water system) followed by cooling to precipitate the nanoparticles[78].

In brief, methods used for SLN production include:

- **High pressure homogenization** (HPH) , either hot or cold homogenization methods, [23] the latter being used for highly thermosensitive or hydrophilic molecules [23, 62]
- Breaking of o/w **microemulsion** [23, 44, 79].
- **Solvent emulsification-evaporation** using water immiscible solvents [80-82], or **solvent emulsification–diffusion** using water miscible solvents [75, 76, 83]
- **Solvent injection or solvent displacement** [84, 85].
- Preparation via water-in-oil-in-water double emulsion (w/o/w) or multiple emulsion technique, this is more specific for hydrophilic molecules. [82].
- **High shear homogenization** or high speed stirring and/or **ultrasound dispersion** [16, 86-88].
- Preparation by using **membrane contactor** as a new reported technique for SLN production[89].

Some of these methods involve organic solvents to dissolve the lipid or large amounts of surfactants to increase quality of dispersions and decrease the particle size, which might be correlated with toxicological problems after parenteral administration. Studies have been performed by various research groups in order to improve the stability of the obtained SLN dispersions[87, 88]. Other methods use low concentration of lipid (<1%) resulting in diluted dispersions. One of the best techniques used, is the high pressure homogenization technique which has many advantages compared to the other methods, e.g. easy scale up, avoidance of organic solvents and short production time [19].

The same methods could be used for the generation of **NLC**, but the HPH is the most commonly used [90]. The addition of oil with the lipids in the formulation process of NLC at temperatures above the lipid melting point will create a porous lipid matrix as with the nanoparticle structure, in which the porosity will be controlled by the amount of oil. The methods used to produce NLC for peptide and protein are the hot HPH technique and w/o/w double emulsion[11].

Article I. **The formulation process of LNC is in fact another low energy method based on the phase inversion temperature (PIT) method plus a temperature cycling treatment [53]. Similar to the microemulsion method of Gasco et al [78]for production of SLN, the method for LNC production is also based on the generation of a microemulsion which upon dilution gives highly monodispersed nanoemulsions under certain conditions. The most important is the inclusion of a lipophilic PEG-surfactant that is able to generate a microemulsion within the phase-inversion zone.**

The PIT method was introduced in the sixties by Shinoda and Saito [91, 92], using the particular ability of emulsions stabilized by polyethoxylated (PEO) nonionic

surfactants to undergo a phase inversion following a variation of temperature. The so-called *transitional* phase inversion occurs, when, at a fixed composition, the relative affinity of the surfactant for the different phases is changed and controlled by the temperature, "temperature or temperature range at which the hydrophilic and lipophilic properties of a nonionic surfactant just balance". Within the transitional region between both macro-emulsions, for the temperatures at which the nonionic surfactants show very close affinities for the two immiscible phases, the ternary system shows an ultra low interfacial tension and curvature, typically creating microemulsions, bicontinuous and nanoscaled systems [45, 93-95] **(Figure 2)**. This is followed by rapid cooling at PIT or by a sudden dilution in water or oil which will suddenly break-up the chosen bicontinuous microemulsion (a point of zero spontaneous curvature maintained at the PIT), nano-emulsions or submicron-scale particles are immediately generated [27, 55, 96]

Figure 3: Variation of the conductivity as a function of tempertature before cooling or dilution at T_{cd}; *(The amounts of Labrafac® , solutol and NaCl were 20.6%, 16.9% and 3% respectively):*The PIZ (in purple strips on the graph) is characterized by the change of conductivity from measurable values to 0µS/cm via a narrow peak of conductivity [53]

This method is essentially based on the changes in solubility of polyoxyethylene-type, nonionic surfactants according to the temperature. These types of surfactant become lipophilic with increasing temperature as a consequence of the dehydration of polyoxyethylene chains due to the breakdown of hydrogen bonds with water molecules. At low temperatures, the surfactant monolayer has a large, positive, spontaneous curvature forming O/W emulsions, characterized by high conductivity values (35mS/cm). By increasing the temperature, the spontaneous curvature becomes negative, leading to W/O emulsion formation as a rapid decrease in conductivity of nearly 0 mS/cm at 84 ◦C is observed. This suggests a phase-inversion process from an O/W to a W/O emulsion which takes place in the phase inversion zone (PIZ) **(Figure3).**

An additional stage to this PIT method, consists in performing a **cycling on the temperatures** crossing the PIZ [53, 55, 74, 97]. Three temperature cycles of heating and cooling at the rate of 4 °C/min are usually applied between 85 and 60 °C [53]. This temperature cycling process has been linked with improving quality of the nanometric

dispersions, that is to say decreasing the size and polydispersity index (PDI). In certain cases, it even has been shown as a condition for generating such a nanometric suspension [55]. Accordingly, this temperature cycling process appears as a solution to reduce the quantity of nonionic surfactant needed to generate nano-dispersions[55]. Furthermore, two irreversible phenomena induced by temperature cycling were reported: the salting-out effect, strongly linked to the concentration of the electrolyte and water/oil ratio (WOR), create a shift of the emulsion inversion toward lower temperatures, and an effect, linked to the surfactant amount, which enlarges the transitional microemulsion zone. These two effects are proved to be independent since they are generated by distinct composition and/or formulation parameters[74] [55];Thus, the temperature cycling process forces the interfacial concentration of surfactant to increase. The thermal cycles act on the surfactants, forcing them to migrate to the interface, where they reach a thermodynamically more stable state. The latter are trapped, overconcentrated, and therefore generate an increase of the global water/oil interfacial area. As a result, the microemulsion stability is enhanced, the ability to form the macroemulsions gradually decreases, and the transitional region becomes larger [74]. furthermore, The bicontinuous network is more and more structured along the cycling process, and its fineness is gradually improved, progressively giving rise to the nanometer template generation [53]. Concretely, the less the amount of surfactant added, the higher the number of temperature cycles required to stabilize nanometric dispersion. For the larger amounts of surfactant, several cycles do not really appear to be necessary [55]. An increase of the number of temperature cycles to over 3 does not really seem to be beneficial to LNC size and polydispersity index reduction[45]

In brief, the generation of nano-emulsions by the PIT method plus temperature cycling leads, in fact, to droplets exhibiting an important quantity of surfactants in the interfacial region. In addition to the fact that the final formulation is reached by a sudden dilution with water at temperature below the nonionic surfactant melting point (~30 °C), indicate that **shell crystallization** could occur and this leads to the formation of **lipid nanocapsules**. The structure of these LNC is being specific and typical of the formulating method and of the properties of the nonionic surfactants used.

This last dilution stage is considered as an irreversible process since it leads to the generation of the kinetically stable oil droplets, *i.e.* the o/w **nanoemulsions** or **nanocapsules**. This great stability is due to the fact that the steric stabilization prevents the droplets flocculation and therefore coalescence. Although both nanoemulsions and nanocapsules could be obtained by the PIT method, they differ in stability. The lipid nanocapsules are more stable owing to their rigid shell structure reducing the inter-droplets oil diffusion (Ostwald ripening) rate that is involved with the nanoemulsions under storage [45]. Complementary insights into the phenomena governing the emulsion phase inversion could be found in the literature [74]. To further understand the difference, it is useful to review the ternary diagram for the different constituents and phases either aqueous, oily, microemulsion, or macroemulsion. The effect of temperature on the behavior of the non ionic surfactant could be a key element. Under equilibrium conditions, different Winsor systems may exist according to the temperature. This could be more illustrated by the fish-cut diagram introduced by Winsor in 1954 [98] **(Figure 4).**

In brief, with small amounts of nonionic surfactant, the three-phase region is observed. Near the PIT, this system has the minimum degree of interfacial tension. A

sudden dilution in cold water of this system produces nanoemulsions. Conversely, with large quantities of surfactant (>10wt.%), when combined with a temperature cycling process, the rapid cooling of the Winsor IV system leads to the formation of lipid nanocapsules[27] .

Water solubility of nonionic surfactant, investigations on the cloud point, and investigations on the phase inversion temperature (PIT) of water/nonionic surfactant/oil system are closely related phenomena [74].

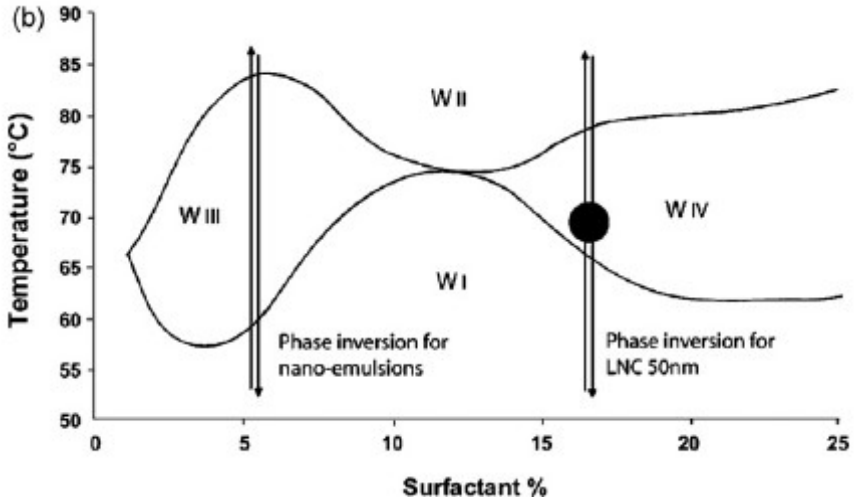


Figure 4 : Fish-cut diagram at a constant water/oil ratio under equilibrium conditions as a function of temperature (°C) and of surfactant amount (%). (W_I=o/w emulsion, W_{II}= w/o emulsion, W_{III}= water, microemulsion, and oil phases in equilibrium, W_{IV}=single phase microemulsion)[45, 95]

4. Physicochemical characteristics of lipid nanoparticles:

4.1. *Particle size*

The exact knowledge of size and size distribution is essential for such nanoparticulate drug delivery systems, since these parameters can significantly modify physicochemical as well as biopharmaceutical behavior for example changing drug release kinetics or transport phenomena across biological barriers [4, 97]. Well-formulated systems should display a narrow particle size distribution in the submicron range. Particle size is relevant if lipid nanoparticles dispersions are intended for intravenous

administration. Injections of highly polydispersed particle dispersions can cause embolism due to the risk of particle aggregation during injection [11]

The mean particle size of **SLN** and **NLC** is in the submicron range, ranging from about **40 to 1000nm** [19] but mostly >150nm. A finer dispersion could be obtained by imputing higher energy such as elevated production temperature, higher stirring rate, longer emulsification time, stronger ultrasound power and so on. Besides the production parameters, lipid matrix, surfactant blend and also viscosity of lipid and aqueous phase influenced the outcome of the procedure[99].

The average hydrodynamic diameter of **LNC** is controllable and ranges from **20nm to 100nm** according to their composition[73], with a low polydispersity index (<0,01) and showing a monomodal particle size distribution.

INSTRUMENTS AND PRINCIPLE OF MEASUREMENT:

Photon correlation spectroscopy (PCS) and laser diffraction (LD) are the most powerful techniques for routine measurements of particle size of dispersed particles when the size approaches a nanometre.

PCS (also known as dynamic light scattering, DLS) is a non invasive technique which measures the fluctuation of the intensity of the scattered light which is caused by particle movement. PCS is a good tool for measuring the size and size distribution of molecules and particles dispersed or dissolved in a liquid, typically, over a size range from a few nanometers to about 3 µm (submicron region). The measurement of the scattering intensity, also called backscatter detection, provides the following advantages: an improved sensitivity, a wider range of sample concentrations and a simplified sample preparation[100]. The diameter that is measured in Dynamic Light Scattering is called the hydrodynamic diameter and refers to how a particle diffuses within a fluid. The diameter obtained by this technique is that of a sphere that has the same translational diffusion coefficient as the particle being measured. Since DLS is sensitive to the intensity of light scattered by particles, and larger particles scatter more light than small particles, then the DLS is very sensitive to the presence of aggregates, and hence this technique is an excellent basis for studying the stability of submicron particle dispersions. In order to assure a convenient scattered intensity on the detectors, dilutions of the nanoparticle dispersions might be necessary because the Brownian agitation of the measured LNC is affected and cannot be detected across the fluctuation of the scattered intensity[69].

LD measurement is based on the dependency of the diffraction angle on the particle radius (Fraunhofer spectra)[101]. Smaller particles cause more intense scattering at high angles compared to the larger ones. A clear advantage of LD is the coverage of a broad size range from the nanometer to the lower millimeter range (40nm to 2000µm). However, despite this progress, it is highly recommended to use PCS and LD simultaneously. It should be kept in mind, that both methods are not `measuring' particle

size. Rather they detect light scattering effects which are used to calculate particle sizes. For example, uncertainties may result from non spherical particle shapes. Platelet structures commonly occur during lipid crystallization. Additional techniques might be useful. For example, light microscopy is recommended, although it is not sensitive to the nanometer size range.

FACTORS AFFECTING PARTICLE SIZE

The effect of production method, temperature used, the type of lipid, its properties and its content as well as the surfactant type and concentration on the final particle size of SLN have been extensively studied [23]

In **LNC**, The composition of the samples influenced not only size distribution, but decided if there were particles formed at all: For instance, no objects could be located when hydrophilic surfactant concentrations were low; on the other hand, high concentrations led to micellar systems [53]. when the proportion of the surfactant increases, the average particle diameters strongly decreased. This could be explained by the stabilizing properties of Solutol® which confer great stability to the oil-in-water system, thus allowing lower-size particle formation. This result may be attributed to both the strong hydrophilic properties of Solutol® due to the PEG groups and the strong hydrophobic characteristic conferred by the stearate chains. Furthermore, the strong interactions in-between the Solutol® molecules themselves probably contributed to the interfacial stability as well [71]. On the contrary, increasing the proportions of oil (Labrafac®) in the mixture had a lesser influence on the size distribution, and the size increased when oil proportions were increased. This result confirmed the nanocapsule structure, described by microscopy studies and differential scanning calorimetry[53], with the oil component concentrated in the centre of the particle. Lastly, changing the proportions of water had no effect on particle diameter[73] **(Figure 1)**

4.2. *Zeta and surface properties*

The zeta potential represents the electric potential at the nanoparticles shear plane. The shear plane is an imaginary surface separating the thin layer of liquid (liquid layer constituted of counter-ions) bound to the solid surface in motion. Almost all particles in contact with a liquid acquire an electric charge on their surface. This is an important and useful indicator to predict and control the colloidal dispersion stability [4, 44, 73]. In general, particle aggregation is less likely to occur for charged particles (high zeta potential-30mv) due to electric repulsion. However, this rule cannot be strictly applied to systems which contain steric stabilizers, because the adsorption of steric stabilizer will decrease the zeta potential due to the shift in the shear plane of the particle [44] Moreover, one has to take care about strong dependence of the zeta potential or ions present in the medium[4].

The zeta potential and the electrophoretic mobility measurements of the different nanoparticles were performed using the Zetasizer (same instrument used for particle size measurement) equipped with an AZ-4 cell and were based on the laser Doppler effect.

In general, **SLN** and **NLC** have a highly negative surface charge (-20mV to -40mV). Furthermore, the zeta potential is a good predictor of gelation phenomena seen with **SLN** dispersions. Stable samples had a zeta of -25mV, while a zeta of -15mV indicated the beginning of gelation phenomena[102]. Moreover, The zeta potential was remarkably high (-45 mV) for poloxamer stabilized dispersions[23].

LNCs have a slightly negative surface charge (-1mV to -2mV) due to the negative contribution of phospholipids molecules [103] and the presence of PEG dipoles in their shell [70]. One single peak is observed only in the case of nanocapsules without Lipoid, which corresponds to only one population LNC with similar electro-kinetic properties. Those with more negative values of the Zeta potential are due to the population LNC II (containing Lipid molecules) [69, 97]

It could be observed that surface of LNC is generally less negatively charged if compared to SLN. This could be attributed further to the PEG (from Solutol®) on their surface. The presence of PEG on the surface of SLN partially masked the negative charge of the uncoated particles. This effect is common for PEG-coated nanoparticles due to an extension of the plane of shear of the nanoparticles [50, 104].

4.3. ***Microscopic aspects***

The particle size distribution is essentially disclosed by dynamic light scattering (DLS), but also by scanning electron microscope (SEM), transmission electronic microscopy (TEM) coupled with negative staining, or cryo-TEM [27] and other imaging techniques, such as atomic force microscopy (AFM). These produce a scan that looks like an image of the material and can be used for submicron particle size measurement. An additional benefit of using these imaging techniques is that other information can be obtained, such as shape and surface structure. But there are special precautions in the preparation of samples for TEM, not to affect their real size and shape[53].

The shape and size of **SLN** were extensively described by many authors using the three different techniques; being mostly nanospheres with smooth surface and solid matrix.

LNCs possessed a stability that was sufficient to avoid any fusion when samples were prepared for AFM. The rigidity of the nano-object surface seemed directly inherent to the surfactant shell structure[53, 62]. TEM of LNC revealed that the lipid core of LNC was homogeneous, limited by an electron dense interface, and an external clear layer. Capsules were surrounded by a large continuum which seemed to correspond to the large quantities of added water and free polyethylene glycol[105].

4.4. ***Structural investigation***

In addition to particle size , the degree of crystallinity and the modification of the lipids are of relevance of drug incorporation and release [44]. Lipid crystallization and a change of the modification can be delayed with very small particles and in the presence of emulsifiers[62, 106]. Differential scanning calorimetry (DSC) and X-ray diffraction are well established as means to characterize the crystallinity of lipids. **DSC** uses the fact

that different lipid modifications possess different melting points and melting enthalpies. By means of **X-ray** scattering it is possible to assess the length of the long and short spacings of the lipid lattice as well as the influence of storage on this parameter. The colloidal size of the lipid particles (**SLN)** and the addition of emulsifiers may change the features of nanodispersions as compared to the bulk lipid.

The DSC study on freeze-dried particles provided some answers about how compounds interacted in LNC. A study comparing individual phase transition temperature and their enthalpy from a mixture with other component and their behavior in LNC was conducted[53]. DSC results concurred to define a nanocapsule structure as described in **Figure 1.** The decrease of Lipoid enthalpy in the nano-objects and in the presence of pure Labrafac indicated an interaction between these two components. Inside the objects, Lipoïd® was obviously in contact with Labrafac®. Therefore, it could be concluded that the system was constituted by an oily liquid core corresponding to free Labrafac® that was surrounded by a tensioactive rigid shell that was made from a mixture of Lipoïd® and Solutol®. The first component (Lipoid®) was anchored in the oily phase, whereas the second component (Solutol®) was oriented toward the water phase[53].

4.5. ***Drug candidates***

A very important point to judge the suitability of a drug carrier system is its loading capacity [44]. Many different drugs have been incorporated in SLN, mostly lipophilic as well as hydrophilic and insoluble drugs . **SLN** and **NLC** are usually applicable for entrapping lipophilic drugs. Many kinds of drugs (mainly lipophilic) were reported to be incorporated into the nanospheres prepared by the microemulsion method, such as, timolol[107] and paclitaxel[108].

Lower drug entrapment efficiency was obtained for hydrophilic drugs due to their limited solubility in the lipid matrix. Many approaches were considered to be adoptable to solve this problem [8]. For example: Increasing drug lipophilicity by forming an ion-pair complex with pilocarpine[109],tobramycin[110] or by preparing a dispersion of magnetite[111] or by ionic complexation of doxorubicin and verapamil, with an anionic polymer like dextran sulphate [112] or by dissolving the hydrophilic molecule in the internal phase of an w/o/w microemulsion for e.g. Thymopentin[113], contrast agents[114] and hydrophyllic macromolecules[115]. Many studies investigated the incorporation of peptide and protein into SLN[11]. For example: SLN based on soybean phosphatidylcholine and lectin-modified SLNs were selected as carrier systems for insulin[116]. *In vitro* studies showed these carrier systems to deliver insulin with greater protection from enzymatic decomposition. An *in vivo* study demonstrated reduced blood glucose levels with both SLN formulations in rats [12]. Encapsulation rates of the hydrophilic molecules were also increased using the **NLC** or or lipid-drug conjugates [117].

Examples of bioactives associated to **NLC** for oral delivery are calcitonin and cyclosporine A. NLC for the delivery of calcitonin have been produced by w/o/w double emulsion, resulting in an association efficiency higher than 90% [118]. NLC with

cyclosporine A have been produced by hot HPH [11, 90]. Potential applications of NLC as drug delivery system have been described[42, 90]

Lipid drug conjugates (**LDC**) were developed especially for hydrophilic drug molecules, wherein an insoluble drug–lipid conjugate bulk is synthetically prepared either by salt formation (e.g., with a fatty acid) or by covalent linking (e.g., to esters or ethers) [16]. LDC bulk is then homogenized in the presence of a stabilizer in water using high pressure homogenization similar to SLN preparation[119]. Transforming water-soluble hydrophilic drugs into LDC and formation of nanoparticles allows prolonged drug release and targeting to specific sites by i.v. injection. These results provide a first basis of using LDC-polysorbate 80 nanoparticles for brain delivery of diminazene to treat second stage human African trypanosomiasis (HAT)[117]. The efficiency of apolipoprotein E coated LDC to selectively target brain was also shown as determined by fluorescence microscopy[120].

LNCs were found to be good candidates as novel carriers for lipophilic drugs as well as amphiphilic drugs [97, 121]. They provide considerable drug encapsulation capacity (over 90%) which is higher than liposomes (usually 50%)[45] and also exhibit sustained-release functions at the site of action. It was shown that they exhibit a relatively long half disappearance time after i.v. injection in rats (21 minutes)[122]. Since the process for LNC production involves multiple thermal cycling across 80°C; it may not be suitable for thermolabile drugs. At least, the integrity of the active molecule encapsulated has to be checked after the encapsulation. Indeed, many studies precisely utilizing this system and process (three temperature cycles across 80 ◦C.), have proved the stability, integrity and activity of encapsulated drugs[55].

Lipophilic drugs are loaded easily in the lipid core of the LNC whereas the amphilic drugs could settle at the oil–water interface of formulated LNCs. Examples of amphiphilic drugs encapsulated efficiently in the LNC include: amodiarone, an antiarrythmic drug[97], ibuprofen, incorporated into LNCs for pain treatment by intravenous administration[121] and indinavir, an inhibitor of HIV-1 protease [123] and various hydrophobic anticancer agents such as: etoposide[124], paclitaxel [125], tripentone[72], docetaxel and derivatives of 4-hydroxy tamoxifen combined with ferrocen [126].

In preclinical studies, etoposide and paclitaxel-loaded LNC showed a higher cytotoxicity effect than free drugs after systemic administration[124]. This was explained by sustained drug release and P-glycoprotein (P-gp) inhibition. Interestingly, radioactive components such as 99mTc and 188Re can be used to label LNC allowing the imaging of their distribution and for diagnostic and therapeutic uses[127].

Furthermore, LNCs are also adequate systems to encapsulate lipophilic complexes of hydrophilic molecules[128]. An alternative could be the encapsulation of free hydrophilic molecules inside an aqueous core[129].

4.6. ***Model of drug encapsulation or loading:***

The solubility of the drug in the lipid melt or its miscibility determines whether the crystallization of the melt results in a solid solution or a solid dispersion containing the drug in a homogenous distribution or in clusters[44]. Decreasing the water solubility of drugs by lowering production temperature and surfactant concentration used will avoid this repartitioning and forms homogenous solid solution of the drug (the drug is molecularly dispersed in the lipid matrix. Depending on the melting point of the lipid and the drug as well as its solubility and concentration, the drug could enrich the core or the outer layers of the particles. The drug-enriched core will be formed if the drug is precipitated before the lipid recrystallizes. The drug-enriched shell-type contains an outershell enriched with drug, which covers a lipid core. It is formed when phase separation occurs during the cooling process from the liquid oil droplet to the formation of **SLN** at hot high-pressure homogenization [18]. Of course a combination of the models may be relevant too In summary the particle structure obtained (**Figure 5**) is a function of the ingredients and the production conditions [23, 44].

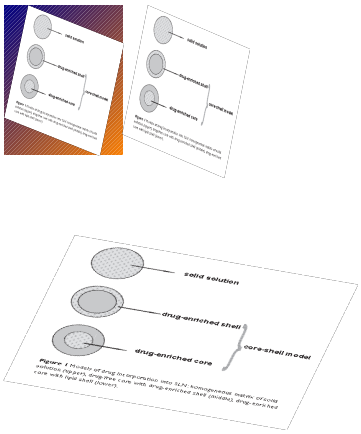


Figure 5 : Models of drug incorporation into SLN, modified [50]

Formation of an almost perfect crystalline structure in SLN by identically shaped molecules limits the loading capacity for actives. Neverthless, formation of a solid particle matrix of **NLC** with many imperfections comparable to building a wall from very differently shaped stones, the increased number of imperfections leads to an increased loading capacity for active compounds [18].

For LNC, the model of drug incorporation should follow drug enriched core in case of highly lipophilic compounds or drug enriched shell in case of amphiphilic or hydrophilic drugs depending on the degree of interaction with the PEG groups of the LNC surface [130].

4.7. ***Model of drug release:***

The drug incorporation model of **SLN** affects the drug release pattern. In other words, the release studies may be used to indirectly evaluate the incorporation model of a drug substance in SLN. It is related to the composition and production method of SLN as explained in the last section. For instance, in case of solid solution incorporation model, drug release is prolonged over several weeks since mobility of the drug molecularly dispersed in colloidal particles is very limited. Fast initial drug release (burst effect) exists in the drug-enriched shell model as a result of drug deposition near the particle surface [131]. The burst release is reduced with increasing particle size while prolonged release could be obtained by increasing particle size, i.e lipid microparticles[132]. The type of surfactant and its concentration, which will interact with the outer shell and affect its structure, should be noted as an important factor, because a low surfactant concentration leads to minimal burst and prolonged drug release[50]. It was also found that production parameters (e.g. temperature) affect the drug release profile. Higher temperature and higher surfactant concentrations increase the burst, production at room temperature avoids partitioning of drug into the water phase and subsequent re-partitioning to the oil phase, thus showing no burst[44]. Furthermore, crystallinization behaviour of the lipid carrier and high mobility of the drug lead to fast drug release[50].

In the case of **NLC,** the oil content of the particles dissolves the drug and combines controlled release characteristics with high drug loading capacity[18]. **NLC** offer a fine arrangement of the release profiles[27]. The imperfect type and amorphous type of NLC, in particular, provide much more flexibility to achieve the desired prolonged release.

In brief, the particle size that affects drug release rate directly depends on various parameters such as composition of SLN formulation (such as surfactant/surfactant mixture, amount of drug incorporated, structural properties of lipid and drug), production methods and conditions (such as time, production temperature, equipment, sterilization and lyophilization and also surface modification by PEG coating [50].

For the **LNC**, the oily core is liquid and thus ensures a homogenous dispersion of the drug throughout the whole particle. Therefore, the rate of drug release will only be affected by the extent of drug-oil interaction and sustained or fast drug release rates could be achieved by changing the oil used as a core material[63]. A common release profile for all encapsulated drugs from LNCs showed a relatively small initial burst effect followed by slow sustained release of the encapsulated drugs (e.g.: amodiarone, ibuprofen)[121] and tripentone[72]. It was found in a recent study on in vitro drug release mechanism from LNC that the physicochemical properties of the drug (e.g. Ibuprofen)as well as the oil phase (composition and viscosity) have a high impact on the

drug release rate while the surfactant (lipophilic surfactant) type, composition or density exerted only a minor effect[63].

5. Routes of administration of lipid nanoparticles:

SLN, derived from physiologically compatible lipids, with controlled drug delivery, enhancement of bioavailability of entrapped drugs via modification of dissolution rate and/or improvement of tissue distribution and targeting of drugs have been extensively investigated in various application routes [19, 50, 133, 134]

Upon administration of SLN via the **parenteral route** of administration (intravenously, intramuscularly, subcutaneously), improved bioavailability, targeting, enhanced cytotoxicity against multidrug resistant cancer cells have been observed with concomitant reduction in side-effects [14, 135] .More advantages of SLN for parenteral application are their excellent physical stability, protection of incorporated drugs from degradation, controlled drug release depending on the incorporation model, prolonged circulation time in blood, good tolerability and high toxicological acceptance [14, 16]. The injectable SLNs loaded with anticancer agents, imaging agents, anti-parkinsonism, antiHIV, antipsychotics, anti-rheumatoid arthritic agents, antiparasitics, antihypertensives and antibiotics have shown promising results as well as being used successfully as adjuvants for protein antigens and DNA for immunization [11, 14, 108, 136-138]

In vivo studies performed with **orally** administered solid lipid nanoparticles containing drugs such as cyclosporine A [139], camptothecin, idarubicin[140], tobramycin [110], rifampicin, isoniazid and pyrazinamide, praziquantel [141] and proteins such as chitosan-coated nanoparticles containing calcitonin[142] and insulin [143] have shown promising results in bioavailability enhancement, attributable to their lipid nature, their small particle size or protection of incorporated drugs from lipolytic enzymes[144-146]. Surface modification with PEG coating [115] improved the stability and resistance to lipolytic enzymes which is beneficial especially in delivery of peptide drugs and calcitonin [147]. Some oral SLN products have found their way to the market for the delivery of cyclosporine and rifampicin (Rifamsolin™ by AlphaRx)[50].

SLN are successful delivery systems for **topical** application of cosmetic and pharmaceutical products [19, 148]. Their short time to market products potential have made them second generation after the liposomes for delivery of dermal especially cosmetic products. This is due to their non-irritant and non-toxic lipids, small particle size that increases their adhesiveness to the stratum corneum similar leading to film formation and their occlusive properties [149], increasing skin hydration[150]. Furthermore, by modifying drug release, they increase skin penetration of encapsulated drugs reducing systemic uptake [151]. SLN and NLC have been studied with active compounds such as vitamin E [152], tocopherol acetate [153], retinol[154], clotrimazole [155], triptolide [156], phodphyllotoxin [157] and a nonsteroidal antiandrogen RU 58841[158]for topical application. About thirty cosmetic products containing lipid nanoparticles are on the market in 2009 [19]. Solid lipid nanoparticles are commercialised by Skyepharma (London, UK). SLN offer potential as well for **transdermal delivery** of drugs by

enhancing skin permeation [19], mainly via a (semi)occlusive superficial film formation [44, 159].

SLN have shown also potential for **pulmonary** delivery of drugs due to their high tolerability and drug targeting to the lung macrophages[44, 50]. Nebulization of solid lipid particles carrying antitubercular drugs was observed to be successful in improving drug bioavailability and reducing the dosing frequency for better management of pulmonary tuberculosis [160].

A few reports are available on **rectal drug** administration via SLN in the literature [161]. Hydrophilic coating of SLN permits the interaction and transport of SLN through the **nasal** mucosa and therefore bring great benefits and compliance as nasal drug carriers especially for vaccines[162]. Biocompatibility and mucoadhesive properties of SLN improve their interaction with **ocular** mucosa and prolong corneal residence time of the drug, with the aim of ocular drug targeting [163]. SLN significantly enhanced tobramycin bioavalability in the aqueous humor [163]. Ocusolin™ from AlphaRx is a gentamicin loaded-SLN product in the form of ophthalmic solution. It is still under preclinical development [50]

The major advantage of **NLC** over SLN is increased drug load[90]. NLC can be administered, e.g., via the oral [164],ocular[163],and intravenous routes[14]. By now NLCs are mainly investigated for dermal application[19, 165]as they can increase skin hydration like SLN and reinforce damaged skin film [151] . The increase in hydration further leads to an increased penetration of active which can be exploited in dermal drug targeting[166] and in cosmetics, e.g., for coenzyme Q10[167] Nonetheless, the increase in skin hydration also leads to a reduction in wrinkle depth. Therefore, NLC can be considered an allover interesting carrier system for pharmaceuticals, cosmetics and personal care. A NCL formulation containing black currant seed oil was introduced in the German market for regenerative care of scaly and aged skin (NanoLipid RestoreTM). At present, more than 40 NLC products are available on the cosmetic market,and NLC for pharmaceutical and nutritional use are in development[168].

LNCs offer a good alternative to polymeric and other lipid systems for pharmaceutical applications. Their easy, solvent-free and low-energy preparation method preventing the drug to be encapsulated from degradation during processing made them most suitable for application in the fields of nano-medicine, pharmaceutical sciences and cosmetics[45]. Furthermore, thanks to their monodisperse controllable nanosize, their biocompatible FDA approved ingredients, GRAS surfactants, their pegylated surface with low complement activation, low macrophage uptake and by inhibiting the P-glycoprotein efflux pump (P-gp), LNCs offer strong potential as **injectable drug** carriers, reaching target tissues without being rapidly eliminated. Further trials to increase their residence time in blood [127] was even made possible by simple post insertion of longer and denser PEG chains . Up to 50% of the injected dose was still present in the blood 8 h after administration for LNC containing 6 mol% PEG 5000 [169] or 10 mol% PEG 2000. Such long-circulating LNC of controlled sizes with their stealth properties could prove useful for the passive delivery of many lipophilic

anticancer drugs to solid tumors [124, 170, 171]. Other adaptations of the LNCs standard formulation for their i.v. administration in animals were lately proposed by possible use of GMO-free components and buffering the dispersions to avoid metabolic acidosis. Freezing LNC in liquid nitrogen avoids significant modifications to their main characteristics for a 6-month storage period was well adapted for paclitaxel-loaded LNCs[24, 172] .LNC provided the possibility for an intravenously and orally administered ibuprofen formulation which could be useful in the treatment of postoperative pain [173].

LNC provided another promising new formulation to enhance the **oral** bioavailability of paclitaxel (Ptx) while avoiding the use of pharmacologically active P-gp inhibitors, such as verapamil. After oral administration of Ptx -loaded LNC or Ptx associated with verapamil, the area under the plasma concentration time curve was significantly increased (about 3-fold) in comparison to the control group ($p < 0.05$)[173]. The effect may be attributed to stability in gastric medium and fasted state intestinal medium[174] and by its Pgp inhibiting properties owing to Solutol®[45] . Further *in vitro* transport studies showed that LNCs improved Ptx transport across the intestinal barrier [175]. In addition, LNCs were uptaken by Caco-2 cells mainly via active endocytic processes and more particularly via clathrin-dependent and caveolae-dependent transport mechanisms that likely allow transcytosis and thus improved gastrointestinal crossing of the drug[176]. Further studies demonstrated an effect of P-gp on the transport of Ptx when loaded in LNCs and support a direct effect of P-gp on their endocytosis in Caco-2 cells[175].

The **pulmonary** drug delivery of Ptx by nebulisation of the Ptx-loaded LNC as dose-intensification strategies in severe, malignant lung diseases showed promising results [177]. LNC dispersions were made into aerosols by using mesh nebulisers without altering the LNC structure nor modifying their drug payload and no cytotoxicity effects of Ptx -loaded LNC were observed. Further scaled-up procedure and a mid-term conservation procedure have been developed for preclinical and clinical studies[177].

LNC showed good biocompatability and potentially suitable carrrier for **inner ear** drug delivery[178]. LNCs proved as potential vectors for drug delivery into the spiral ganglion cells, nerve fibers, hair cells, and spiral ligament.[179]

LNC may also offer good potentials for **dermal as well as transdermal** applications due to their small particle size, high encapsulation of lipophilic compounds, biocompatible and safe ingredients, physical stability and protection of incorporated drugs but haven t been explored as yet. A recent study showed that Nile red loaded LNC showed a major decline in Nile red concentration from stratum corneum to epidermis and dermis following their application similar to nile red distribution following Nile red loaded SLN[180], Unexpectedly, the influence of particle size (60nm to 200nm) on the dye penetration into the skin was marginal and not significant ($p > 0.05$)

6. Special applications of lipid nanoparticles

Strategies for targeting

The interesting properties of SLN and LNC, allow their use in many therapeutic applications, not only for drug delivery, cancer diagnosis and therapy, but also for gene and cell therapy.

Altering the surface characteristics of **SLN** by coating with hydrophilic molecules (PEG) improves their plasma stability and biodistribution, and subsequent bioavailability of drugs entrapped as well as storage stability [(50)] (**Figure 6a),** thus mimiking liposomes in drug targeting to cell populations, while exhibiting the advantages of greater stability[(50)]. In vitro studies on Pgp-over expressing human cancer cells using doxorubicin and paclitaxel-loaded SLN have shown to overcome multidrug resistance via Pgp inhibition and ATP depletion which indicate their potential in improving the therapeutic efficacy of drugs[(11)]**.**

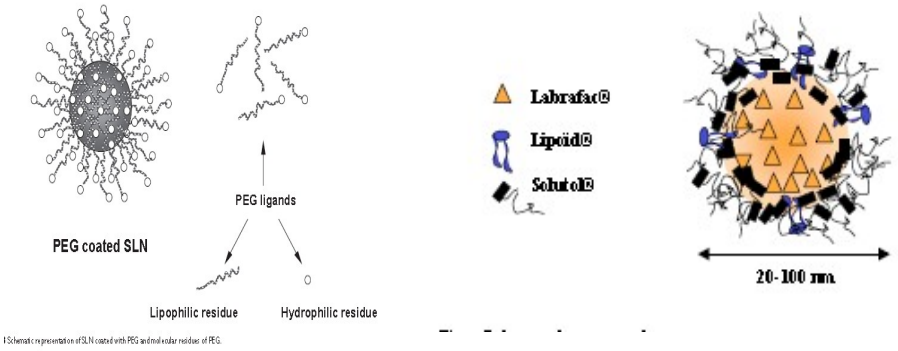


Figure 6: Schematic representation of a) SLN coated with PEG[(50)] and b) LNC (Shell consists of PEG 660 hydroxystearate (Solutol®)) [(171)]

LNC, with their nanoscale size range and their shell consisting of a PEG 660-surfactant at high density **(Figure 6b)**, can be used to overcome the P-gp dependent multidrug resistance (MDR) being the major limiting factor for achieving therapeutically successful drug concentrations at the site of action[(175)].Moreover, they display a P-gp inhibitory effect harmonized with a stealth effect versus the complement system as well as mononuclear phagocytic system (MPS) uptake, as an interesting new concept in cancer treatment and an efficient approach in cell culture [(124)]. LNCs exhibited a blood half-life of ~21 minutes for 99mTc and 188Re in rats[(127)]. Lamprecht et al. [(124)] demonstrated the interaction between LNC and P-gp, resulting in the negative regulation of ATPase activity. Anti-cancer agents as well as radionuclides [(127)]were packaged into the oily core, with a high loading efficiency. Etoposide and paclitaxel-loaded LNC contributed to a significant reduction in the growth of glioma cell lines due to intracellular drug delivery combined with P-gp inhibition [(124, 171)]. Solutol® is able to block P-gp related drug efflux with a very low level of *in vitro* toxicity[(181)].

Moreover, grafting longer PEG chains, such as PEG 1500 stearate instead of PEG 660 stearate, to the LNC surface provided an opportunity to prolong their circulation residence time up to 5.5 h, with 20% of total dose still present in the blood 24 h after an

intravenous injection into healthy rat [169].This could be attributed to PEG chain length[182]. Indeed, LNCs retained longer in systemic circulation by post-inserting distearoylphosphatidylethanolamine (DSPE)-PEG 2000 or DSPE-PEG 5000 at their surface with half-life times of over 6 h after intravenous administration [170]. In a recent study, a method was proposed for the setting-up of post-insertion feasibility domains[183].Thus, these pegylated nanocapsules are considered to be potential carriers for docetaxel delivery to the sites of action, particularly into solid tumors due to the enhanced permeability and the retention effect (EPR) [184]. It was possible to produce long-circulating LNC of controlled sizes. Such LNC could prove useful for the passive delivery of lipophilic anticancer drugs to solid tumors[170].

Nanoparticles can accumulate in target tissues based on their natural host cell tropisms and on their biophysical properties (passive targeting). In practice, active targeting to cells other than the mononuclear phagocytic system (MPS) cells is often insufficient for rapid and specific accumulation in target tissues. Further improvement of tissue selectivity can be achieved by engineering the surface of lipid carriers with hydrophilic polymers [185] or coupling targeting ligands [186].

Moreover, colloidal carriers can be coated with hydrophilic polymers or albumin to actively target the incorporated drugs to specific cells, tumors and/or organs. This strategy can be successfully applied to concentrate drugs in such target tissues minimizing systemic side effects and therefore toxicity[11].

Particulate carriers (including **SLN**) usually accumulate in the liver by passive targeting on parenteral administration. However, passive targeting leads to entrapment of the drug in the Kupffer cells and not in the hepatocytes which is the major target for the treatment of hepatic diseases such as cancers. Hence, for liver targeting, SLN containing galactosylated or mannosylated lipids are employed. To date, there are very few studies which have systematically explored the liver targeting of SLN[14].

LNC also can be grafted with ligands for the purpose of actively targeting drug delivery. LNC used for active targeting were modified using site specific ligands to allow drug delivery to the CNS. OX26 murine monoclonal antibodies (OX26 MAb) that recognize transferrin receptor (TfR) were conjugated to LNC (OX26-immunonanocapsules). This was facilitated by the incorporation of PEG 2000, functionalized with reactive maleimide groups (DSPE–PEG 2000–maleimide), into LNC shells by a post-insertion procedure allowing the covalent attachment of the ligands to LNCs. This antibody is able to cross the BBB via a receptor-mediated transcytosis mechanism [187]. Furthermore, a combination of nanotechnology with the convection-enhanced delivery(CED) technique emerged[188]. Promising as an approach for direct drug delivery for the treatment of brain tumors (The increase in the median survival time was about 80%compared to the control group, and 33% of the animals were long-term survivors (over 100 days).

LNCs ensured a prolonged therapeutic effect and can be considered as a promising radio-pharmaceutical carrier for internal radiotherapy of brain tumors. The same method was recently used for the local delivery of LNCs loaded with ferrocenyl diphenol molecules called ‘ferrociphenol’ (Fc-diOH) followed by external beam

irradiation on a rat brain tumor model[189]. LNC were better internalised by glioma cancer cells, they were less toxic to healthy cells in culture and they could be loaded by hydrophobic molecules with high drug loading levels. Promising in vivo results were obtained after intratumoural subcutaneous administration of this new drug-carrier as it dramatically reduced the tumour mass and glioma volume [126]

Various drugs ranging from antipsychotics, anti-Parkinson, antieschemic to antibiotics have been encapsulated in **SLN** with the aim to either modify the biodistribution or for brain targeting [190, 191]. Recently, a comprehensive review [192]covering various aspects of brain targeting using SLN has been published. A series of experiments evaluated biodistribution of radiolabelled non-stealth and stealth SLN after intravenous injection[193]. Transferring-conjugated SLN were studied for their ability to target quinine hydrochloride to brain by studying biodistribution as first example to active targeting for the delivery of SLN in the treatment of brain diseases such as cerebral malaria[194]. SLNs exhibit certain potential advantages over Polymeric NPs. They are safely taken up by brain and exhibit the least toxicity due to the biodegradable nature of the carrier lipid [195]. Smaller size (around 10 to 200 nm) and narrow size range (100 to 200 nm) allows them to cross tight endothelial cells of the blood-brain barrier (BBB), escape from the reticuloendothelial system (RES), and bypass the liver.

Mode of cell uptake

LNCs were rapidly accumulated within cells (from 2 min exposure) through active and saturating mechanisms involving endogenous cholesterol with a major contribution of clathrin/caveolae-independent pathways [175] Although initially present in endosomes, LNCs can bypass the endo-lysosomal compartment with only 10% of the cell-internalized fraction found in isolated lysosomes after 2 h exposure[196].As demonstrated by use of lysosomal probes, LNCs reverted lysosome integrity similarly to V-ATPase inhibitors and in a size-dependent fashion with best efficiency for small nanoparticles. When loaded with paclitaxel, smallest LNCs also triggered the best cell death activity. LNC properties are ascribed to the proportion of HS-PEG they provided to the cell. They are important to consider toward the development of nanomedicines that use drugs sensitive to lysosomal degradation or that need to reach extra endo-lysosomal targets[196]

Gene delivery (examples of peptides or proteins associated to those systems)

The search for “perfect vector” for systemic gene delivery especially against cancer are needed for therapeutic application to organs that are inaccessible by percutaneous injection[197].

Cationic **SLN** have been demonstrated to bind genes directly via electrostatic interactions, and to have potential benefits in targeted gene therapy in treatment of cancer[136]. The charge of particles can also be modulated via the composition, thus allowing binding of oppositely charged molecules [198]. Moreover, coating of SLN with

PEG increases stability and plasma half life of SLN in order to decrease phagocytic uptake, and therefore improves the biovailability of drugs. Cationic SLN formed stable complexes with DNA and were found to protect DNA against DNAase I ligation. They were found to have very low toxicity and were found to promote transfection of liver cancer cell. Cationic solid lipid nanoparticles (SLN) for gene transfer, **TransoPlex®** was produced by PharmaSol DDS(Germany)[14].

In fact, hydrophilic DNA molecules were encapsulated into the oily core of **LNCs** via formation of lipoplexes, leading to the formation of neutral, 110-nm DNA nanocapsules[199] demonstrate that the fabrication of nanocapsules encapsulating hydrophilic DNA in an oily core that meet criteria for blood injection is possible. But *in vivo* stability, blood half life was ill-adapted to efficient in vivo transfection. Increasing systemic delivery have been possible consequently, by making DNA LNCs stealthy[200]. DNA LNCs were coated with amphiphilic and flexible PEG polymers [PEG lipid derivative (DSPE-mPEG 2000) or F108 poloxamer. In fact, tumor accumulation assessment was evidenced for the coated LNC as passive targeting without causing any hepatic damage. Furthermore, to overcome the internalization difficulties encountered with PEG shields, active targeting using galactose has also been designed for the purpose of efficient hepatocyte targeting [201] and provide a promising systemic gene-delivery system[202].

7. Application in imaging and radiotherapy of lipid nanoparticles

Magnetite[203], iron oxide [204], and Luminescent lipophilic CdSe/ZnS core shell quantum dots (QDs) encapsulated into **SLN** have great potential in biological imaging [205].

Similarly, **LNCs** could be loaded with different radionuclides in their oily core or at their surface such as 99mTc, 188Re, 125I or 111In [127, 206, 207]. These LNCs provide opportunities for their application in imaging and radiotherapy. Original lipidic nanoparticulate systems radiolabeled with Iode-125 and Technetium-99m allows biodistribution studies by scintigraphy and counting. Radiolabeling was carried out with a good yield and a satisfactory in vitro stability. In vivo, the relatively long remanence in blood and the digestive excretion of the tracers seem promising for the use of these solvent free lipidic nanocapsules as carrier of lipophilic drugs.

8. Stability

SLN Long-term stability as aqueous dispersions was shown over three years [102, 208, 209]. Common disadvantages of SLN include particle growth, unpredictable gelation tendency, unexpected dynamics of polymorphic transitions, inherently low incorporation capacities due to crystalline structure of solid lipid [23, 46, 210], drug expulsion after polymorphic transition during storage and relatively high water content of the dispersions (70-99.9%) have been observed[11, 16]. The preparation method, choice

of lipids and surfactants could be optimized to increase their stability. It was found that the stability of the lipid dispersions decreases as stability of the lipid modification increases [23, 211]. Furthermore, coating of SLN with PEG provides good physical stability and dispersability of SLN dispersions.

NLC increase the loading capacity of the active compounds in the particles and also avoids or minimizes the expulsion of the active compound during storage[19].

LNC showed improved stability with time, in the same range as that of SLN [212]. The sample stored at 4°C was perfectly stable for at least 18 months but at 37°C, the same sample was only stable for 1.5 months[53]. LNC are more stable than nano-emulsions prepared by the same PIT method [213]. Such a difference of stability is attributed to the difference in the droplet structures having the thick interfacial layer potentially created with the temperature cycling process and reinforced by the neutral phospholipidic framework [69, 70]. This could represent a real barrier around the nano-emulsion droplets against the passage of the oil across the interface, which significantly reduces the inter-droplet oil diffusion, *i.e.* the Ostwald ripening[27]. It is actually for this reason, that these particular nano-emulsions formulated by temperature cycling, are often denominated as *nanocapsules* or *lipid nano-capsules* (LNC)[45]. Indeed, a loss of mechanical stability at air–water interface is observed for the fraction without lipid molecules (lipoid), which undergo a rapid destabilization, disaggregation and formation of a surface film [69]. It contains mainly Labrafac and Solutol®[68]. Moreover, the high stability at the air/water interface of these nanocapsules was found to be different from the liposome or high density lipoprotein (HDL) behavior [56].The physical stability of lipid nanocapsules is primarily dependent on i) the thickness and ii) the rigidity (i.e., the crystallinity) of the shell [45, 57]. LNC do not expect to show signs of drug expulsion, supercooled melts or gelation commonly seen with SLN [23]. Further stability studies may be of benefit for the commercialization of these lipidic nanocarriers.

9. Toxicological concerns

To formulate parental lipid-based carriers, surfactant of GRAS status must be employed, eg, lecithin, Tween 80, Poloxamer 188, Span 85, and sodium glycocholate. It can be assumed that the cytotoxicity of the **SLN** can be mainly attributed to components of the aqueous phase, especially to non-ionic emulsifiers and preservatives that have eventually been used [214]. The interactions of SLN and their respective cytotoxicities were studied with human granulocytes, showing low uptake by phagocytosis resulting in prolonged duration time in blood. Similar results obtained with HL60 cell lines showing lower cytotxicity compared to polymeric nanoparticles. Other studies with RAW 264.7 macrophage and murine peritoneal macrophages showed similar results [14, 215]. Furthermore, when performing bolus injections into mice good tolerability was found for most of the surfactants coating SLN. After autopsy and histopathology no significant evidence was documented that SLN were acutely toxic to tested animals [16].

LNCs, prepared by a simple, solvent free process using GRAS surfactants and biocompatible lipids are characterized by low toxicity[53, 177]. In comparison with paclitaxel-solvent formulation Taxol®, the paclitaxel-loaded LNCs dispersion tolerance

in mice was increased such that the LD50 and MTD were eightfold and eleven fold the commercial formulation, respectively [24].

10. Secondary production steps:

Lyophilisation and spray drying

SLN have shown liability for lyophilization[208] and spray drying [209]. In general, SLN may be either administered as an aqueous dispersion or after incorporation into a traditional dosage form, i.e. tablets, pellets, capsules or powders in sachets. The aqueous SLN dispersion may be used as the granulation fluid in the granulation process or can be spray-dried into a powder and mixed with the necessary excipients before compression into tablet[23]. SLN dispersion can also be used as wetting agent in the extrusion process for the production of pellets. The pellets disintegrate and release the SLN completely non aggregated[216]. Alternatively, SLN powders can be filled in hard gelatin capsules or incorporated in liquid PEG and filled into soft gelatin capsules. The spray-dried or lyophilized powders can even be filled into sachets. The physical characteristics of the resultant SLN powder like flow property, compressibility, bulk density, waxy nature and strength to withstand the compression force and temperature need to be carefully addressed before production of the final dosage form[217].

Thanks to **LNC** rigid shell, such suspensions allow freeze-drying to take place by means of adding a cryoprotectant, such as trehalose 5% w/w [53, 57]. These results were in complete agreement with other studies on liposomes and solid lipid nanoparticles. The aptness to freeze-drying and the physical stability under storage of the dried form depend not only on the thickness but also on the physical quality of the LNC shell [57]. Lecithin, being the structure-enforcing component having a high melting point and crystallinity, allows the shell to get hard and freeze-dried. Furthermore, DSC studies allowed pointing out complexation between lecithin and trehalose. This complex was described as a stabilizer of phospholipid bilayers in freeze-dried liposomes formulations [57].

Scale up feasibility

For the introduction of a product to the market, scaling up feasibility is of utmost importance. A striking advantage of **SLN** is the feasibility of large industrial scale production by a high-pressure homogenization technique similar to emulsions[218]. For the production via microemulsions, a system has been developed allowing the production of 100 ml microemulsion which is then poured in excess cold water forming SLN. The dispersing water ratio ranged from 1:1 to 1:10, leading to batch sizes up to 1.1 L[219].

LNC likewise, since its production process by PIT and temperature cycling technique is relatively simple and low-energy consuming, it allows easy industrial scale-

up[27]. Scaled-up procedure and a mid-term conservation procedure have been developed for preclinical and clinical studies for paclitaxel loaded LNC for pulmonary delivery[177].

Sterilization

Sterilization of SLN is an important issue in the case of pulmonary or parenteral administration. Aseptic production, filtration, γ-Irradiation and heating (autoclave) are normally used to achieve sterility. The sterilization should not change the properties of the formulation with respect to physical and chemical stability and the drug release kinetics[23, 44]. Aqueous **SLN** dispersions can be sterilized by autoclaving or alternatively γ- irradiation [23]. However, autoclaving is not possible when a certain structure has been given to the SLN to modulate release profile. It would be lost when the particles melt again during the autoclaving and recrystallize in a non-controlled way. Moreover, autoclaving is not possible if a poloxamer is used in SLN dispersion[23, 44, 220]. The physical stability during autoclaving depends very much on the composition of the SLN formulation[44, 88]. Steam sterilization did not cause changes in particle size and zeta potential of drug loaded SLN [220]

LNC are produced aseptically if they are sterilized by filtration similar to emulsions for parenteral nutrition

From the above, LNC, with their special features summarized, offer another generation of lipid nanoparticles, having similarities with other LBDDS and offering new biological implications with great potential therapeutic outcomes. More studies are expected to be conducted for direct comparison between all LBDDS and more specifically between SLN and LNC in different fields of application. Any single change in composition, formulation technique will have tremendous effects on the performance of these colloidal lipid based carriers. Special attention should be drawn for the delivery of challenging biopharmaceuticals (recombinant proteins and monoclonal antibodies or nucleic acid based products) and biotechnological drugs (drugs produced by biotechnological products such as fermentation, enzyme, tissue and cell culture technology and genetic engineering) as well as well known therapeutic drugs used.

AIM OF THE WORK

This thesis aimed at improving the physicochemical and/or biopharmaceutical properties of some drugs such as solubility, stability, poor bioavailability and/or short duration of action, etc., using lipid-based delivery systems for dermal, parenteral and oral applications.

Part I: Corticosteroid-Loaded Solid Lipid Nanoparticles Gel for Dermal Delivery

Part one of the thesis dealt with the investigation of solid lipid nanoparticles (SLN) as lipid nanocarrier for the dermal delivery of the high-potency corticosteroid, clobetasol propionate (CP). The study included preparation and characterization of CP-loaded SLN. A topical composite gel containing 0.05% CP as CP-loaded SLN was assessed using *in vitro* release studies and skin permeability through *ex-vivo* whole thickness human skin. The pharmaceutical performance of the formulated gel was compared to that of a conventional CP gel marketed in the USA.

Part two of the thesis dealt with the investigation of lipid nanocapsules (LNC), formulated by a phase inversion temperature (PIT) method plus a temperature cycling treatment, as carriers for a hydrophilic drug, the pentasaccharide "fondaparinux". This part is further divided into two chapters:

Chapter 1: Lipid Nanocapsules Incorporating Fondaparinux Microemulsions.

This chapter dealt with the development of a new strategy for the formulation of fondaparinux-loaded lipid nanocapsules (LNC) using two microemulsions. Phase diagrams for different ternary and pseudo ternary mixtures were constructed. The impact of the first microemulsion (w/o) on the physicochemical properties of the final fondaparinux-loaded LNC dispersion such as particle size, zeta potential, encapsulation efficiency and drug bioactivity using the anti Xa chromogenic assay was investigated.

Chapter 2: Fondaparinux-Loaded Lipid Nanocapsules for Oral Anti-Factor Xa Effect.

This chapter part dealt with the development of fondaparinux-loaded LNC by cationic interaction strategy based on the physicochemical structure of both the drug and LNC to achieve high association efficiency. The impact of this formulation on the physicochemical properties of the fondaparinux-loaded LNC and on the pharmacokinetics of the drug after oral administration to rats was studied.

Part I*

CORTICOSTEROID-LOADED SOLID LIPID NANOPARTICLES FOR ENHANCED DERMAL DELIVERY

***** *This part was presented in a GTRV poster, at Angers, France in November, 2008*

*"***DEVELOPMENT OF CLOBETASOL PROPIONATE SOLID LIPID NANOPARTICLES GEL FOR DERMAL DELIVERY"**

Ramadan A., El-massik M., El-Khordagui L, Khalafallah N., and Daabis N.

Article II. INTRODUCTION

For effective topical treatment of skin diseases, it is imperative that the topically applied drug or drug loaded particles permeate the non viable stratum corneum or via follicular ducts to reach therapeutically relevant concentrations in the skin layers or appendages without leading to high serum levels and systemic exposure[221].

This depends on the physicochemical properties of the drug candidates and vehicles and on the function of the skin as a transport barrier. In general, beside acting as a permeation barrier, the horny layer is also a reservoir for topically applied substances. Lipophilic compounds can permeate the skin more easily than the hydrophilic ones. Moreover, highly lipophilic compounds can penetrate the skin via the hair follicles. Follicular uptake may be most relevant with rigid drug crystalline particles and particulate carriers with a size up to 10µm. These factors, along with the ability of the drug carrier system to provide protection against skin first pass metabolism and hemodynamic parameters of the cutaneous tissue determine the permeation of topically applied drugs [222]. Furthermore, the ability of these carriers to control and sustain cutaneous drug release would increase clinical efficacy [223].

Drug levels following topical application using conventional vehicles systems, such as creams and ointments, within the diseased skin may be sub-therapeutic in some patients while inducing unwanted local or systemic side effects in others [224]. Topical delivery carriers such as liposomes[17], niosomes [225], microemulsions, polymeric and lipid micro and nanoparticles [226, 227] have proved to increase drug transport into the skin and consequently reduce variability in drug permeation. The influence of carrier system on drug uptake by the skin has already been studied for each of these systems and reviewed by many authors [19, 224] with a focus on nanocarriers, as particle size may affect drug targeting to specific skin layer [19, 180, 228]. Among these, lipid-based formulations seem to be the most appropriate and promising for topical application of actives. They can attach themselves to the skin surface and allow lipid exchange between the outermost layers of the stratum corneum (Horney layer) and the carrier [224, 229].

Beside liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been studied intensively. The production, characterisation as well as topical use of lipid nanoparticles for skin diseases have been reviewed in detail [18, 19, 44]. Because of their physiological and biodegradable lipid ingredients, these formulations resemble the skin' structure and therefore no or little disturbances would occur when applied topical [230].

Both NLC and SLN have many features that are advantageous for dermal application[18]. They are colloidal carriers providing controlled release profiles for many substances. They are composed of physiological and biodegradable lipids exhibiting low toxicity and excellent tolerability. Their small size ensures a close contact to the stratum corneum and can increase the amount of drug penetrated into the skin [231]. Further, the occlusive properties of lipid nanoparticles make them very successful in cosmetic and pharmaceutical applications. This can increase the water content of the skin and favor drug permeation. SLN formulations have enough viscosity to be spread on the skin with an elastic film and give an emollient effect reducing irritation [151, 231-233]. The choice of the lipids and surfactants will affect the degree of emolliency. SLN can be also produced

with an optimum PH 4 to 7 or even buffered, they also show suitable isotonicity with body fluids although the horney layer is tolerant to osmotic changes[230]. SLN have a whitening effect , acting as a UV screening system [18, 234] and having a solid matrix that enhances the chemical stability of compounds sensitive to light, oxidation and hydrolysis [149]. In addition, SLN provide sustained release to supply the skin over a prolonged period of time with drug and to reduce systemic absorption [131, 154, 157].

In general, lipid nanoparticles do not penetrate the horny layer [224]. Improved dermal uptake of active compounds loaded to lipid nanopartiles can be attributed mainly to follicular uptake[235]. Increased contact surface of the active compound with the corneocytes, a rapid or a steady release , surfactants [236] and the distribution of the active compound within the lipid particles can vary considerably and hence exert different effects on the percutaneous uptake [233, 237]. The rate and extent of release of the active ingredient and its permeation through skin layers depend on the SLN production procedure, their matrix structure, lipid composition, lipophilicity as well as the solubililty of the drug in the lipid surfactant mixture [230]. These features have been of benefit for skin targeting potential [155, 157, 233] not only in pharmaceutical applications but in the cosmetic field which lead to the market introduction of a number of cosmetic products[19, 230, 238].

SLN dispersions are of superior stability as compared to liposomes [18, 148]. Uptake enhancement, efficacy and local tolerability of SLN were shown for various hydrophilic and lipophilic topical agents[180, 239] such as antiacne drug [237] , anti mitotic (podophyllotoxin[157]), anti inflammatory such as triptolide[156], prednicarbate [232]and psoralens [240], antifungal such as clotrimazole [155] and miconazole nitrate [226] and finally antialopecia such as Minoxidil [241].Similarly, NLC [90], composed of a mixture of oil and solid in their lipid core, showed potential topical carrier for clotrimazole [155] clobetasol propionate [242], and anti-inflammatory drugs such as indomethacin [243] and psoralens [240]. NLC can be considered an interesting carrier system for pharmaceuticals, cosmetics, personal care and also nutrition [168].

Among the inflammatory chronic skin disorders, psoriasis, that can have a profound impact on the quality of life of patients. The treatment of psoriasis is complicated by the availability of numerous topical agents, systemic agents, and phototherapy. Topical glucocorticoids (TG) are the most frequently prescribed drugs for this disease as well as for the majority of other skin disorders [244]. Their clinical effectiveness is related to their vasoconstrictive, anti-inflammatory, immunosuppressive and anti-proliferative effects. Despite their benefit in the therapy of inflammatory diseases, TG are associated with a number of side effects that limit their use[245]. Most TG are absorbed in quantities that can produce both systemic and topical side effects.

Topical corticosteroids are available in different strengths measured by their vasoconstrictive effect. Class I steroids are 1000-1500 times stronger than class VII steroids. Topical corticosteroids of classes I, II, and III are referred to as fluorinated steroids because they usually have fluorine groups added to their structure that increase their strength and skin permeation. Several attempts have been made to increase the safety of TG treatment, including new application schedules, special vehicles and new synthesized agents [246].

The efficacy of topical corticosteroid depends on two properties. Their intrinsic anti-inflammatory activity and skin permeability[247]. In addition , the degree of epidermal hydration can affect the penetration of steroids into skin[248]. It was also reported that the concurrent use of salicylic acid with corticosteroids may improve

treatment of psoriasis by enhancing skin penetration. Studies have shown that CP formulated in various cream bases released the drug and penetrated skin at different rates, causing various degrees of skin blanching response [249].

Long term topical glucocorticoid treatment can induce several side effects, including skin atrophy, telangiectasia, purpura, and striae formation. Skin atrophy occurs by the inhibition of fibroblasts. The overuse of topical steroids resulted in the formation of clinically unapparent but dermoscopically apparent red lines in the treated plaques and or skin adjacent to treated plaques [250].

Lipid-based delivery systems have been extensively investigated for the dermal delivery of TC. For instance, phospholipids liposomes were demonstrated to increase the skin content of topically applied corticosteroids [251, 252] and improve the use of potent glucocorticoids by enhancing their therapeutic activity and minimizing their systemic and local side effects [253-255]. Of greater scientific interest is the improved disposition of corticosteroids within the skin layers of rabbit which was claimed following the topical application of preparations containing triamcinolone acetonide incorporated into liposomes[256]. Similarly, solid lipid microspheres containing CP were also recently prepared representing an alternative system to conventional dosage forms in the treatment of oral lichen planus [257].

In addition to liposomes and solid lipid microspheres, the potential of SLN as drug carriers for topical glucocorticoids was investigated. SLN improved prednicarbate (PC) absorption by the skin by 30 % as compared to PC cream[232]. SLN were shown to contribute to a reduction of skin atrophy caused by glucocorticoid therapy increasing benefit/risk ratio of topical steroids. This was attributed to glucocorticoid targeting to the viable epidermis where the inflammatory reaction takes place and by lowering drug concentrations in the dermis which is most susceptible for irreversible damage [166, 258].

Clobetasol-17-propionate (CP) is a synthetic, fluorinated glucocorticoid, considered to be the most potent of the currently available corticosteroids. CP is more than 1800 times more potent than hydrocortisone[248]. A number of controlled clinical studies of CP have demonstrated its favorable safety profile and efficacy in treatment of patients with psoriasis. In comparison with other topical corticosteoids including betamethasone dipropionate 0.5 % ointment, hacinonide 0.1% cream, and fluocinonide 0.05% cream, CP produced significantly greater improvement, as measured by the degree, rate, and duration of healing[248]. Clobetasol propionate as superpotent corticosteroid, is more likely to be associated with adverse effects than less potent steroids and clinical and histochemcial signs of relapse are often evident within 6 weeks of discontinuing CP treatment [259]. Selective persistent of dermal CD8+Tcells in lesional plaque psoriasis after CP treatment was observed , which must be associated with disease relapse [249, 260].

(a)

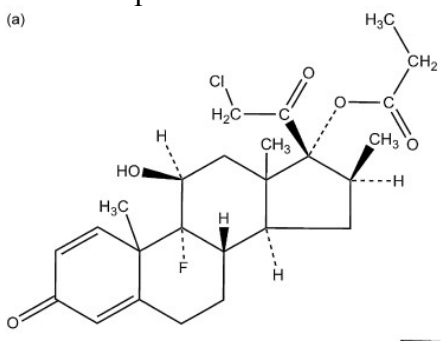


Figure 7: Clobetasol Propionate (CP) from [246]

CP has been available traditionally as ointment and cream for the treatment of inflammatory skin conditions, particularly psoriasis. The drug has also been formulated in other conventional dermatological vehicles such as solution, lotion, and gel, all at 0.05% strength [248, 261]. In general, the vehicle used substantially affects clinical action, potency, and acceptability to the patient. For example, ointments are optimal for the trunk and extremities, and gels and mousses (foams) best for the scalp [262, 263]. A fundamental shortcoming of conventional formulations is the provision of topically active agents in relatively high concentrations to the skin with a limited duration of action, resulting in cycles of short-term overmedication and long-term undermedication. Few attempts have been made to incorporate CP in newer delivery systems to enhance its dermatological performance. These include lipid-based[75, 264].and polymer-based [252] particulate systems. However, further studies are needed for more dermatological formulations with different characteristics.

The **objective** of the present study was to prepare a controlled release clobetasol propionate (CP) gel for dermal delivery using solid lipid nanoparticles (SLN) as lipid nanocarrier. Interest in the gel formulation has been initiated by poor compliance of Egyptian patients with the usually long term clobetasol therapy using ointments and creams as the only available clobetasol dermatological preparations on the Egyptian market as well as lack of information on the *in vitro* performance of CP-SLN formulated in a gel base. Skin permeation of CP from the formulated composite gel in comparison to a conventional CP gel available commercially in the USA was assessed using ex vivo full thickness human skin.

Article III. EXPERIMENTAL

MATERIALS:

- Clobetasol Propionate was a kind gift from EL-AMRYIA, Pharmaceutical Ind.Co., Alexandria, Egypt.
- Tripalmitin (Dynasan®116) generously supplied by Sasol Germany GmbH.
- Soy phosphatidylcholine 95% (Epikuron® 200) donated by Lipoid GmbH, Germany
- Poloxamer 188 (Pluronic® F68) was supplied by Sigma, St. Louis, MO (USA).
- Chloroform, methanol, Tween 80 , ADWIC, EL-NASR Pharmaceutical Chemicals Co., Cairo, Egypt.

- PEG 400 supplied from El-Amryia, Pharmaceutical Ind. Co., Alexandria, Egypt.
- Methanol HPLC, Hypersolv, Prolabo
- Carbopol®934 (Goodrich Chemicals Co., Ceveland, Ohio, USA), supplied by El-Amryia, Pharmaceutical Ind.Co., Alexandria, Egypt.
- Spectra/Por 2 dialysis membrane (12,000-14,000 Da molecular weight cut-off) was purchased from Spectrum Laboratories Inc, USA.
- Embeline™ gel (0.05% CP), Healthpoint, Ltd, USA was purchased from the USA market.
- The other chemicals were of analytical grade.

EQUIPMENT:

- Rotavapor, Buchi, Germany
- Ultra turrax homogenizer, T25, Germany
- Ultrasonic Bath JULABO USR3, JULABO LABORTECHNIK GMBH, Seelbatch / West Germany
- Laser Diffraction particle size analyzer, model 1064 liquid (Cilas , France).
- TEM (JEOL, 100TX, Japan)
- Zeta meter, 3.0 + zeta-Meter Microscope module, USA.
- Ultracentrifuge: Sigma laborzentrifugen GmbH. Germany
- Differential Scanning Calorimetry, Perkin Elmer model, DSC 6 (Perkin Elmer Instruments, USA)
- Leica Image Analyzer Model Q5501W equipped with Leica DMLB microscope, Cambridge (England) connected to a Camera Model TK-C1380JVC, Victor Company (Japan); Shaking water bath
- HPLC: Perkin Elmer series 200 equipped with series 200 LC pump, Series autosampler, series 20UV/V detector, series 600interface and total Chrom Navigator 6.2.0.0 computerized chromatography analysis software.
- Column: 5, RP-18 column (Perkin Elmer with a particle size of 5 um, 220mm x 4.6mm ID

METHODS:

1. Preparation of solid lipid nanoparticles:

Clobetasol Propionate (CP) loaded SLNs were prepared by the hot emulsification homogenization method [88, 265, 266]. Typically, CP (0.2% w/v of final dispersion, 80 mg) was dissolved with the lipids such as tripalmitin (Dynasan®116) (2% w/v from final dispersion, 800mg), Epikuron 200 (1% w/v of final dispersion, 400mg) in 20ml of a mixture of chloroform and methanol (1:1). Organic solvents were then removed using

rotary evaporator (at 60°C). The drug embedded lipid residue was then melted by heating at 5°C above melting point of the lipid. An aqueous phase, containing poloxamer (1% w/v of final dispersion, 400mg)) was heated to the same temperature and added to the molten lipid phase; The coarse 0/W emulsion was homogenized by ultra turrax homogenizer at 6000 rpm for 3 min at a temperature maintained 5 °C above the melting point of the lipid. The globule size was further reduced by ultrasonification at the same temperature for 25 min. The CP-SLNs were obtained by allowing the hot nanoemulsion to cool at room temperature. The dispersion was kept in refrigerator at 4-7°C in a well closed and covered bottle for protection against light. Final dispersion volume was 40 ml. Blank-SLN (unloaded) were prepared as control using the same procedure.

The formation of non-aggregated lipid spheres with no drug crystals was confirmed by light microscopy.

2. *Characterization of solid lipid nanoparticles:*

1. *Particle size and shape analysis*

1.1. *Laser diffraction (LD):*

Particle size analysis was performed using Laser Diffraction particle size analyzer [155, 267]. LD data were evaluated using D90%, D50%, D10% (the given percentage values are the percentage of particles smaller than the given sizes). LD describes the maximum size of 95% of the particle population in a given volume and is thus influenced by larger particles, so number distribution was used. Prior to size analysis by LD, the SLN dispersion was diluted with distilled water (100 times), and sonicated for 1 minute outside and 120s during measurement. Particle size results are the mean of three different batches each in triplicate. The size distribution was measured in terms of span value [268] as follows:

A stability study was conducted for one batch for a three month-study period of SLN dispersion stored in refrigerator away from light.

1.2. *Transmition Electron Microscopy (TEM):*

SLN samples were prepared by placing a drop of CP-SLN dispersions on cupper grid, air dried and negatively stained with a drop of phosphomolybdate solution (1%) for contrast enhancement [269, 270]. The air-dried samples were then examined directly under the transmition electron microscope.

1.3. *Image Analysis*

Particle shape and size analysis was performed also by photomicroscopic analysis, where sample (a drop) was placed on a microscopic slide after suitable dilution, examined and then photographed. The particles were characterized for size using Leica image analyzer equipped with special computer software.

2. *Zeta Potential:*

The determination of zeta potential was performed in aqueous SLN dispersions stored at room temperature by using a zeta meter. Necessary dilutions were done with distilled water (200 times). Results were the average of 3 determinations.

3. *The Drug Percent Entrappment Efficiency (%EE):*

The percentage of entrapped drug was calculated after separation of the free drug by the following two methods and its determination by **HPLC**.

3.1. *Dialysis:*

The unentrapped drug was removed by dialysis: An aliquot of CP-SLN dispersion (0.5 ml) in a dialysis bag at 4 ° C for 2 hrs in the release medium (15ml) with no stirring. Some runs were extended to 4 hours (no further release was observed from 2 to 4 hours). The amount of free drug released (unentrapped) was determined.

3.2. *Ultracentrifugation:*

An aliquot of CP-SLN dispersion (0.5 ml) was subjected to ultracentrifugation [155, 267] at 20.000 rpm at 5°C for 1 hour. The amount of free drug in the supernatant was estimated after necessary dilutions with the mobile phase. The entrappment efficiency was determined using the following equation:

Where the free drug amount is the mass of the free drug detected in the supernatant or that released from dialysis bag and the total drug amount or drug content was measured dissolving 0.5 ml of the SLN dispersion in 1:1 mixture of methanol and chloroform, slightly heating the mixture till solution becomes clear then completing to a constant volume (10ml). An aliquot of this solution (1ml) was further diluted with 2 ml mobile phase (75% methanol) followed by centrifugation and filtration for HPLC assay. Data are the average of three determinations. Entrappment efficiency of the drug in SLN dispersions was checked during storage for a two months period.

4. *Differential Scanning Calirometry (DSC):*

DSC analysis was performed using 5 mg of different samples which were placed in hermetically sealed pans. A heating rate of 5°C /min was employed after 1 min stabilization at 25°C and the temperature range 25-220°C was scanned then temperature held at 220°C for 15 min. inert atmosphere was maintained by purging nitrogen at a flow rate of 20ml/min. An empty pan was used as reference.

The recrystallization indices (RI) were calculated as follows[211]:

$$RI\% = \frac{Enthalpy_{SLN\ dispersion}\ Jg^{-1} - Enthalpy_{bulk\ material}\ Jg^{-1}}{Concentration_{lipid\ phase}\%} \times$$

5. *In Vitro Drug Release Study:*

In vitro release of CP from nanoparticles was evaluated using a dialysis bag diffusion technique[270, 271]. Dialysis bags, with a molecular weight cut-off 12,000-14,000Da (Spectrum laboratories Inc, USA), were soaked overnight and filled with 500 µl of CP-SLN dispersion, then immersed in 30 ml dissolution medium (composed of 40% PEG 400 aqueous solution containing 0.5% Tween 80 in pH 7.2 phosphate buffer) [75]. In vitro release was performed at 37°C ± 0.5°C using thermostatically controlled shaking water bath at 50 strokes/min. Samples (1ml) were taken from the outer solution at different time intervals for 5 days, and compensated with the same volume of fresh solution at same temperature. The samples were diluted with mobile phase then injected into HPLC column for analysis. Sink conditions were verified from solubility study data of CP in the release medium. Solubility data were generated by equilibrating excess drug with release medium for 24 hrs. Data are the average of 5 determinations.

An aliquot (500ul) of drug solution in buffer/methanol (1:1), filled in dialysis bag, subjected to the same release study, was used to confirm the dilyzability of the drug.

6. *HPLC Assay for Clobetasol Propionate:*

A previously reported HPLC method for the drug [75, 272] has been used for the *in vitro* determination of the drug as well as the in *ex vivo* permeation study using excised human skin. The chromatographic system consisted of Perkin Elmer series 200 equipped with series 200LC pump, series autosampler, series 20UV/V detector, series 600 interface and total Chrom Navigator 6.2.0.0 computerized chromatography analysis software. Separation was carried out on sphere 5, RP-18 column (Perkin Elmer with a particle size of 5 um, 220mm x 4.6mm ID). The mobile phase was a mixture of methanol: water (75:25v/v) at a flow rate of 1 ml/min. UV detection was performed at a wavelength of 242nm. The injection volume was 10 µl and retention time was 4-5min, Calibration curve was constructed over a concentration range (0.04-10ug/ml) in methanol. The method was revalidated for selectivity, linearity, accuracy and precision in presence and in absence of skin.

3. *Formulation of SLN in gel preparations:*

1. *Gel Formulation:*

CP-SLNs were formulated into a gel preparation using 1% w/w Carbopol®934P as gelling agent. The required quantity of Carbopol®934P was spread in a small quantity of water to prepare an aqueous dispersion and allowed to hydrate

overnight. Propylene glycol (10%w/w) and glycerol (30%w/w) were added subsequently to the aqueous dispersion and 0.5 ml triethanolamine was added under gentle stirring to avoid inclusion of air. The pH of the gel was 6. The equivalent amount of CP-SLN dispersion was added to the gel by geometric dilution using gentle mechanical stirring. The final concentration of CP in gel was 0.05%. The gel was allowed to stand overnight to remove entrapped air. Microscopical examination showed no aggregation of nanoparticles. Samples of CP-SLN gel were examined for particle size and zeta potential.

A gel containing 0.05% free CP, prepared by dissolving the drug in hot propylene glycol then prepared as previously (upon cooling, CP crystals were observed dispersed in the gel) and a commercial gel product containing 0.05% CP were used for comparison . The CP content in gel formulations was estimated by HPLC. Exactly 400 mg of the gel accurately weighed were dissolved in methanol and further diluted with mobile phase followed by filtration before HPLC injection.

2. *In Vitro Drug Release from Gel Formulations:*

In vitro release of CP from the prepared gel formulations was performed using a ring and cup with a dialysis membrane method[273] **(Figure 8)**. An accurately weighed quantity of freshly prepared gel (400mg) containing a total of 0.2 mg of CP was placed in a circular stainless steel assembly designed to hold the gel. The cup containing the gel was then carefully covered with a dialysis membrane fixed with a stainless steel ring. Cups were then immersed in prewarmed 15 ml phosphate buffer containing 40% PEG and 0.5% Tween 80, pH 7.2 in a 20 ml bottles which were shaken in a thermostatically controlled shaking water bath at 35 ± 0.5°C at 50 strokes/min. Samples (500µl) were withdrawn at 1, 6, 24 and 72 hrs, diluted with mobile phase, filtered and injected into HPLC column. Data are the mean of 5 determinations. The effect of the gel on the release of the drug from SLN was determined using the same method and conditions for CP from SLN dispersion (0.1ml).

Figure 8: The stainless steel assembly designed to hold the gel (cup & ring)

4. *Ex vivo skin permeation study:*

Full-thickness human abdominal skin of female patients (40-50 years old) was obtained from the Department of Plastic surgery, Faculty of Medicine, Alexandria University according to institutional ethical guidelines. Subcutaneous fat was excised using surgical scissors and scalpel, shortly after surgical removal of the human skin. The skin was then washed with normal saline to eliminate blood, and other impurities present on skin surface, blotted dry between two filter papers, then was cut into 4x4-cm pieces, wrapped in aluminum foil and stored in polyethylene bags at -20°C in a deep freezer until further use (within 2-3 weeks).

Before storage, the thickness of the different skin pieces was measured between two slides using a micrometer. The average thickness of skin samples was (2.92 ± 0.34 mm). Before the experiment, skin samples were allowed to thaw and were soaked in phosphate buffer saline (PBS) for 1 hour. Permeation of CP through human skin was assessed using the test SLN gel formulation in comparison with the free drug gel and commercial gel.

Experiments were run in **modified vertical Franz diffusion cells (Figure 9)** having a receptor compartment volume of 8 ml and a donor compartment with an effective diffusion area of 3.14 cm^2. The prepared human skin was fastened carefully between the donor and receptor compartments in the diffusion cells, with the stratum corneum side up and held in place with a clamp. The dermal side of the chamber contained a receptor solution consisting of phosphate buffer saline, pH 7.4 containing 40% PEG and 0.5% Tween 80. Care was taken to minimize air bubbles under skin. Accurately weighed gel formulations (400mg) were gently placed in the donor chamber, spread over skin surface. Diffusion cells were maintained at 37°C ±2 in order to maintain skin surface at 32°C throughout the experiment using a shaking water bath adjusted at 50 strokes /min. Following exposure time of 6 hr shaking was stopped, the skin removed and the receptor solution collected. Then donor compartment and skin surface washed many times with methanol. CP content in the donor receptor solution was determined by HPLC. The percentage drug retained in the skin was calculated based on the mass balance assumption that the initial amount of CP in the donor phase was the sum of the drug amounts remaining in the donor phase, drug retained in skin and drug in receptor phase. Each gel was investigated in 4 cells. The whole experiment was repeated three times (n=12 for each gel preparation). Data were expressed as mean value ± SD. Statistical significance was checked by student 's t test for the ex vivo permeation study and considered to be granted at $P<0.01$ unless otherwise indicated.

Figure 9: Modified Franz diffusion cells and human skin used for the ex vivo permeation study

Article IV. RESULTS

1. *PREPARATION AND CHARACTERIZATION OF CP-LOADED SOLID LIPID NANOPARTICLES*

The first part of the work deals with the preparation and characterization of CP-loaded SLN prepared by hot emulsification homogenization method using tripalmitin and lethicin as lipid matrix and Poloxamer 188 as surfactant [87, 88]. A preliminary study was undertaken using other preparation methods including hot homogenization emulsification[274], microemulsion [220, 275], solvent emulsification-diffusion [75, 76]using other lipids (stearic acid, glyceryl monostearate, Softisan®, Compritol, cetyl palmitate) and surfactants (Tween 80) and polyvinyl alcohol as stabilizer (data not shown). However, the hot emulsification homogenization method generated SLNs with a higher yield, better particle size distribution, with no lump formation and ease of filtration and therefore, this method was used throughout the study.

1. Particle Size, Shape and Zeta Potential:

The **particle size** of SLN prepared was measured using laser diffraction. Data are shown in **Table 1**. The mean particle size of blank SLN was 150 nm while that of CP-loaded SLN ranged from 90 to 250 nm with a mean 173 nm ±59. Low span values (Table1) indicate narrow size distribution and low polydispersity. A sample histogram and cumulative frequency curve is shown in **Figure 10**.

The mean particle size of the loaded and unloaded SLN showed a relatively small increase in size distribution after storage at 8°C (210nm) and 30, 80, 510 for the D10%, D50% and D90% respectively and with a span value 5.6 [102].

TEM and **image analysis (Figure 11 and 12)** showed that CP-SLN were nanospheres, generally with a diameter of <200nm and a narrow size distribution. These observations are consistent with laser diffraction data.

The **zeta potential** measurements were done in triplicate **(Table 2**). The SLN were highly negatively charged (average ~ -22mv). The values lie between -14.2mv and -35.5mv. The incorporation of the drug into SLN had no influence on the zeta potential of nanoparticles. The particles had high negative charge although the presence of Poloxamer 188 as steric stabilizer may decrease zeta potential negative values as seen in some samples, due to the shift in the shear plane of the particles [44, 75].

Table 1: Mean diameters and particle size distribution in terms of span value of SLN dispersion loaded with CP

Batch*	Mean diameter, nm (n=3)	D10%	D50%	D90%	Span value
Blank-SLN	150 ± 12	30	60	210	3
CP-SLN1	240 ± 10	30	50	310	5.6
Cp-SLN2	110 ± 34	30	60	166.6	2.2

Cp-SLN 3	170 ±0	30	50	263.3	4.6

*Particle size of each batch was determined in triplicate, mean diameter of 3 batches (**173 nm ±. 59.2**, n=9)

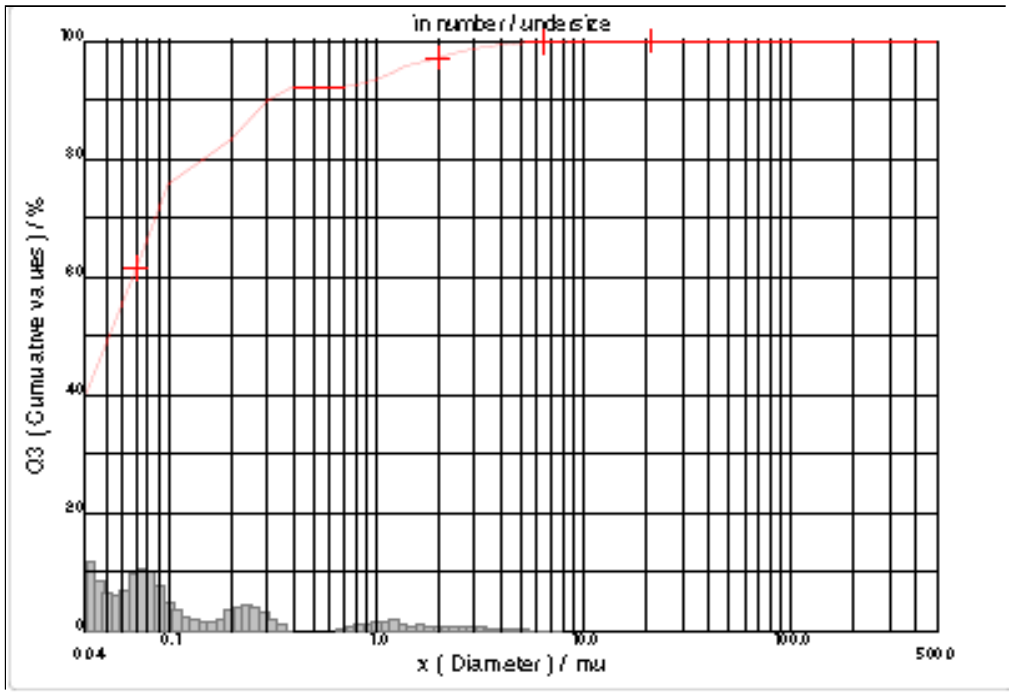


Figure 10: Selected nanoparticle size ditribution histogram and cumulative frequency curve of CP-SLN 1(<1.0µm).

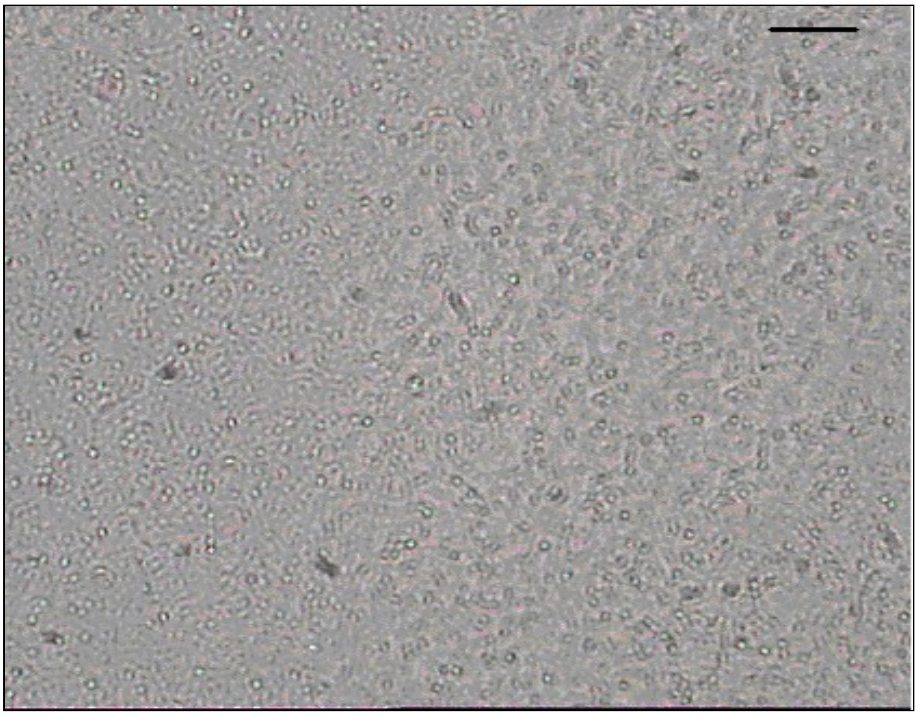


Figure 11: Image analysis showing CP-SLN.

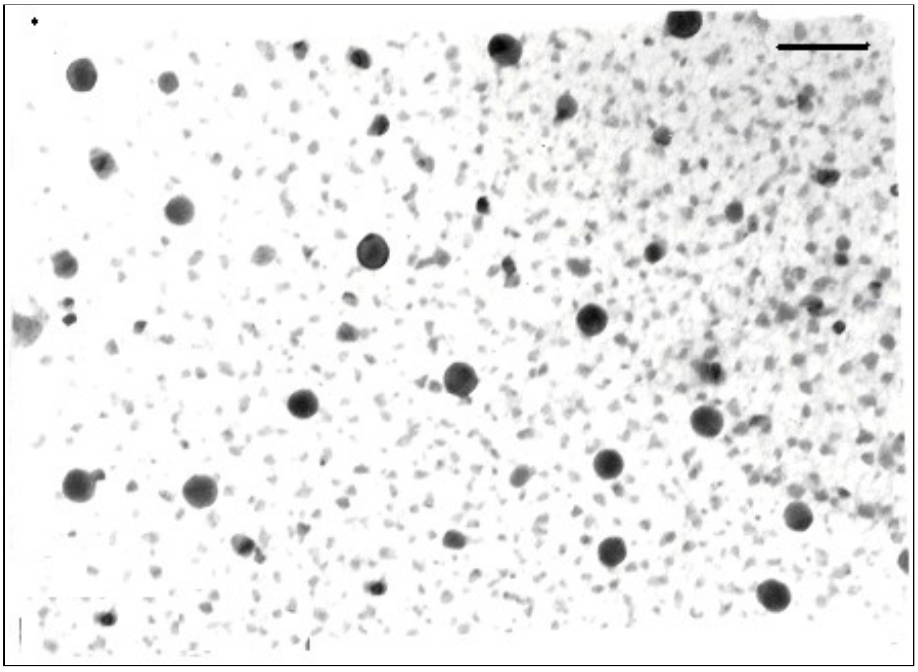


Figure 12: TEM of CP-SLN. (The bar on the photograph means 200nm)

Table 2: Zeta potential results for Blank and SLN dispersion loaded with CP

Sample	Z1	Z2	Z3	Mean ± S.D.
Blank-SLN	-25 9	-30.0	-18.9	-24.9±7.85
CP-SLN1	-35.5	-14.9	-15.7	-22.0±11.6
CP-SLN2	- 14.2	-34.2	-29.6	-26.0±10.47

Table 3: DSC results

Sample	Type	Melting Point (Peak)	ΔH (J/G)	M.P	onset	% Crystallinity (RI% for the lipid)
Drug	bulk	198.034	82.885		196.97	
	PM	196.137	1.027		194.8	
	SE	No peak				
	SLN*	No peak				
Lipid	Bulk	**67.010**	**211.453**		**63.865**	**100**
	PM**	66.815	178.215		62.86	93.6
	SE*	66.049	112.033		63.679	58.8
	SLN	64.772	62.817		61.932	50.79

*SLN sample was lyophilized prior to DSC

**The ratio of drug to lipid in the physical mixture and solvent evaporated is 1:10 as in SLN.

2. HPLC Assay Validation:

The assay was selective as seen from HPLC chromatogram **(Figure 13)**, the peak of CP free from interference from degradation products or endogenous components of the skin. The assay was linear in the concentration range 0.05 to 10 µg/ml with a good correlation coefficient (r =0.999) and an accuracy of 6.53 (coefficient of variation). The smallest concentration having good RSD was 0.05µg/ml, and the coefficient of variation for intra and inter-assay precision of the calibration samples (0.05-1-10ug/ml) were 1.38-5.8-1.5% and 19.35-12.86- 4.48% respectively.

3. The Drug Percent Encapsulation Efficiency (%E.E.):

Drug content data showed that the total concentration of clobetasol propionate in the final SLN dispersion (encapsulated and free CP) ranged from 1.01 to 1.28 mg/ml (n=7) with a mean of 1.16 mg/ml ± 0.155.Theoretical concentration of the drug was 1 mg/ml.

The % E.E. value obtained using the dialysis method (89 % ± 3.01, n=8) was similar to that obtained by ultracentrifugation (88.8 ± 5.36, n=6). It is worth noting that the concentration of the free drug and hence the % E.E. did not change after storage for three months at 40°C in a well closed container away from light.

4. Differential Scanning Calorimetry (DSC):

DSC was used to study the melting and recrystallization behavior of the prepared SLN systems. DSC thermograms of prepared SLN formulations lyophilized together with the bulk lipids (Dynasan®116) and the drug (CP) each alone as well as the physical mixture (PM) and the solvent evaporated co precipitate (SE) of the drug : lipid ratio 1:1 are presented in **Figure 14**. Clobetasol propionate is characterized by an endothermic peak at 198.03°C corresponding to the melting of the crystalline form of the drug This peak was slightly shifted in the physical mixture thermogram (at 196.14 °C) but disappeared in the test SLN formulations and the 1:1 drug:lipid solvent evaporated coprecipitate indicating molecular dispersion of the drug in the lipid matrix. These data are in agreement with those reported previously [266, 270] .

Regarding the lipid components, pure Dynasan116® showed a melting point of 67.0°C which was decreased to 64.7°C upon formulation as nanoparticles. A similar decrease in enthalpy was also shown indicating a reduced degree of crystallinity of the lipid in SLN as well as in the solvent evaporated coprecipitate (lower recrystallization index, **Table 3)**. Appearance of a new peak at 53.67°C was also observed in the thermograms of SLN. On the other hand, physical mixing of the drug and the lipid did not influence their respective thermal behavior.

Figure 13: Calibration curve of CP with the corresponding HPLC chromatograms for the different concentrations of CP in methanol, concentration (a,b,c,d,e corresponds to 0.5, 1, 2, 5 and10µg/ml).

5. *In Vitro* Release Study:

The CP in vitro release study was carried out at 37°C±2 in 30 ml of 40% PEG aqueous solution pH 7.2 containing 0.5% Tween 80 using a dialysis method. The solubility of CP determined in the release medium was found to be 156 µg/ml± 21.5 (n= 4). This is much higher than the concentration of CP in the release medium after 100% release (33.3µg/ml) which ensures sink conditions. Furthermore, to confirm dilyzability of the drug, it was found that 98% of the drug was released after 24 hours, when the drug solution in buffer/methanol (1:1) was filled in the dialysis bag and subjected to the same release study.

The release of CP from the prepared SLN under sink conditions is shown in **Figure 15**. A biphasic release pattern with ≈17% burst release in the first hour was observed. The burst effect corresponds to the dissolution of free (unentrapped) drug calculated by dialysis and ultracentrifugation methods. The extent of drug released in 24 hours was about 25%. This was followed by the release of about 70% of CP over 6 days. (Higuchi diffusion model, R^2=0.995,Y= 4.99X+9.44)

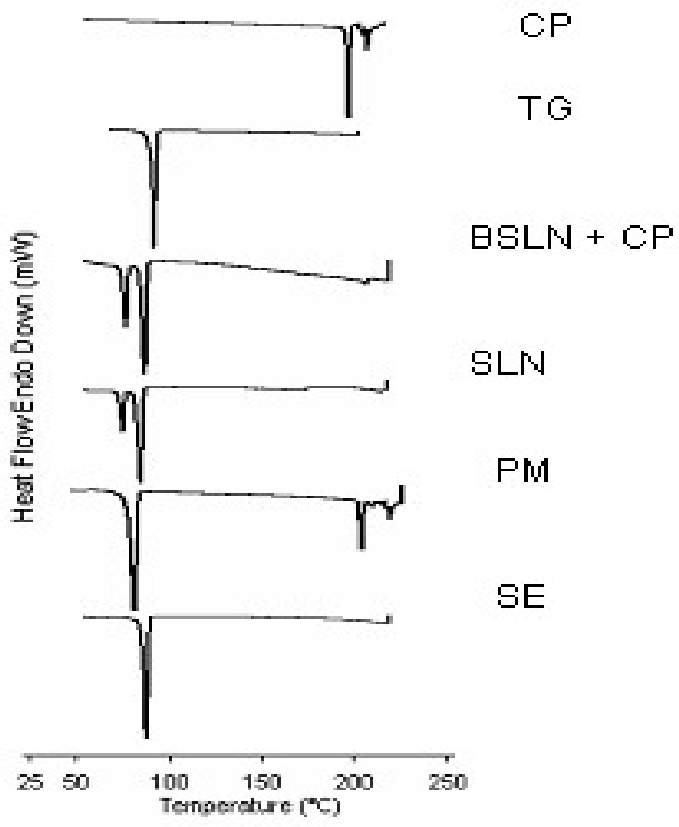

CP
TG
BSLN + CP
SLN
PM
SE
Heat Flow Endo Down (mW)
25
50
100
150
200
250
Temperature (°C)

Figure 14: DSC thermograms of : The drug (CP), the lipid triglycerides (TG), blank SLN with the drug physically mixed (BSLN + CP), SLN, physical mixture of all ingredients (PM), and a solvent evaporated coprecipitate (SE) with drug : lipid ratio (1:1)

Figure 15 : Release profile of CP from SLN dispersion in 40% PEGaqueous solution containing 0.5% Tween 80, pH 7.2 at 37°c±2 using a dialysis method .

2. CLOBETASOL PROPRIONATE SOLID LIPID NANOPARTICLE GEL FOR DERMAL DELIVERY:

1. Release study of the drug from SLN in gel

A SLN-based CP gel was prepared by incorporating CP-loaded SLN in 1% w/w carbopol 934 P. Incorporation of SLN into the gel base did not result in aggregation or change in particle size (120 nm) and zeta potential (-14.72mv ± 2.88) of the nanoparticles. The drug content of CP-SLN gel was determined to be 0.425 ± 0.025 mg/g gel (0.042%). The in vitro release profile of the CP-SLN test gel formulation in comparison with that of a formulated gel containing CP dispersed in the gel base (0.05%) and of a commercial conventional gel (0.05%) at 35°C is shown in **Figure 16.**

The release profile of CP crystals dispersed in the gel base was relatively slow (about 48% release in 24 hrs) and showing a biphasic pattern. Release of CP from the freshly prepared SLN-based gel was slighly slower (42.6% at 24 hr) and according to a similar pattern. On the other hand, CP release from the commercial gel product was the fastest, the % CP released in 24 hrs was about 76%. The in vitro release profile of CP from SLN aqueous dispersion for 24 hrs in comparison with CP released from SLN in gel is shown in **Figure 17.** The % CP released from SLN dispersion was (28% at 24 hr) slower than that from SLN in gel. The mechanism of drug release from SLN-containing gel could be considered to involve two steps, namely controlled release of SLN containing CP into the gel followed by drug diffusion through the gel barrier to the release medium.

The release data were analyzed according to zero order, first order and Higuchi's square root of time mathematical models. Release of CP from the three types of gel best fitted the Higuchi equation, indicating a diffusion controlled release mechanism. The correlation coefficient values (r) were (0.999, 0.962, 0.999) for the free CP, CP-SLN and commercial gels release profiles respectively.

Figure 16 : Release profile of CP from different gel formulations (freshly prepared) in phosphate buffer pH 7.2 containing 40% PEG and 0.5% Tween 80 at 35ºc,(n= 5 ± SD) using the cup method.

Figure 17 :Release profile of CP from SLN dispersion in comparison to its release from SLN in gel in phosphate buffer pH 7.2 containing 40% PEG and 0.5% Tween 80, at 35°c±2 using the cup method.

2. *Ex Vivo* Skin Permeation Study:

The ability of CP in a test SLN gel formulation to permeate human skin was assessed *ex vivo* using modified Franz diffusion cells. A control gel containing free CP (0.5 mg/g) and a commercial CP gel "Embeline™" with the same drug content were used for comparison. An HPLC method was used to determine drug concentration in the donor and receiving compartments.

The calibration curve obtained by plotting relative peak area against concentration of standard solutions of the drug was linear in the concentration range 0.05 to 10µg/ml. Accuracy, intra and interday assay were determined and were not affected in the presence of skin. No skin residues interference with the peak of the drug. The retention time did not change (**Figure 18**).

The amount of CP retained in the skin samples was determined by difference as the complete extraction of the drug from the skin did not give satisfactory recovery. Data presented as percentage of the total applied dose are shown in **Figure 19**.

Treatment of the skin samples with the commercial gel for 6 hrs resulted in skin retention of 3.69 % of the amount of CP used while application of the test SLN-gel resulted in 21.46% drug retention indicating enhanced skin permeation by SLN. About 10% of the amount of drug used was found in the receiving compartment in both cases**(Table 4).**

SLN formulation showed a significant increase in skin deposited CP ($P<0.01$), relative to CP dispersed in commercial gel after 6 hours of non-occlusive application.

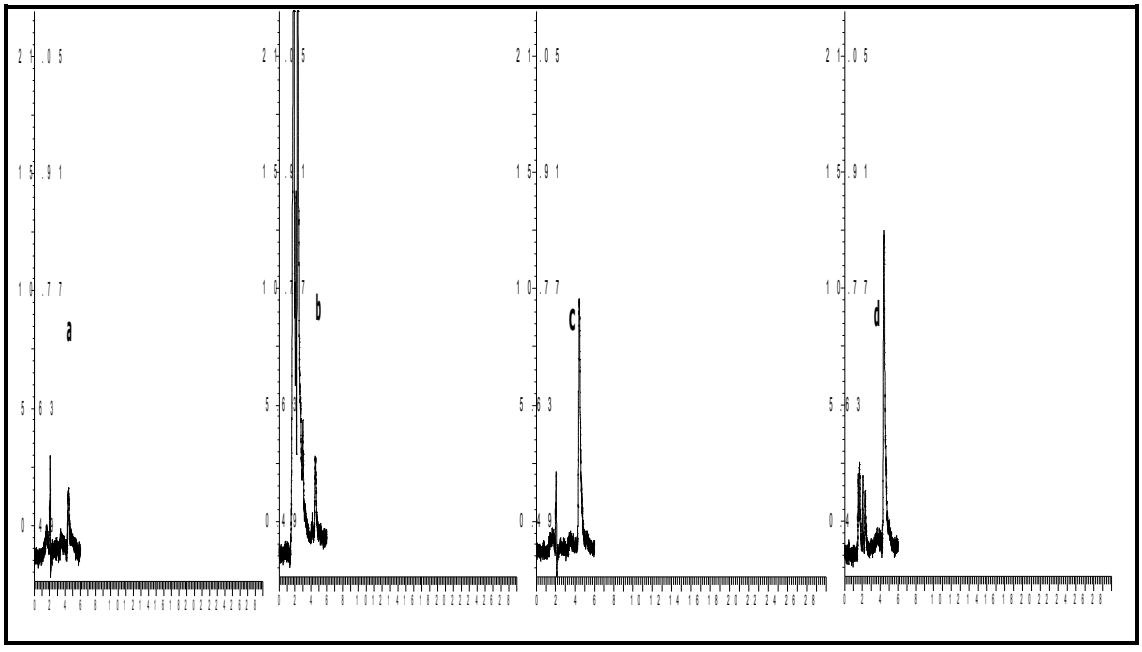


Figure 18: Selected chromatograms of different standard CP solutions in presence or absence of skin (**a**, 2µg/ml in methanol, **b**, same concentration in receptor solution **, c**, st 10µg/ml in methanol and **d,** same concentration in donor solution

Table 4: Amounts of CP (%) permeated, remaining and retained in the skin after in vitro transport study using full thickness human skin (Franz diffusion) after 6 hours study.

Time (hours)	Gel	Cumulative CP amount permeated (receptor) in µg (%)	Cumulative **CP** amount remaining (donor) in µg (%)	Amount retained by the skin in µg ** (%)
6	**CP-SLN**	21.83± 0.21 (10.75± 0.10)(n=12)	135.25± 0.42* (67.63±0.21)(n=11)	42.92 (21.46)
	Embeline ®	22.7± 0.06 (11.36±0.04)(n=11)	169.91± 0.74 (84.91± 0.35)(n=11)	7.39 (3.69)

*highly significant compared to Embeline gel (P<0.01)

** The CP amount retained by the skin is calculated by difference.

Figure 19: *Exvivo* skin permeation study of CP from different gel formulations at 6 hrs (* statistically different, p <0.01), ± SD, n=11.

Article V. DISCUSSION

The objective of the present work was to prepare clobetasol propionate (CP) - loaded solid lipid nanoparticles (SLN) with adequate pharmaceutical attributes for the formulation of a composite SLN-based gel for the dermal delivery of clobetasol propionate. A gel was selected as a dermatological vehicle based on better patient acceptability of clobetasol propionate gel and the suitability of a gel base for lipid delivery systems in general drug release into the vehicle upon storage[252, 263]. For instance, liposomes for topical drug are usually formulated in a gel form[276, 277]. The release profile of a solid lipid microparticle-based cream of a lipophilic sunscreen agent indicated loss of sustained release properties due to drug release into the cream base [278].

The main target characteristics of SLN used for the gel formulation include drug entrapment in SLN to minimize direct skin contact with a large amount of the drug, high drug loading to reduce the amount of SLN needed, adequate nanoparticle size to enhance skin permeation and provide acceptable aesthetic properties of the final gel, and controlled release of the drug according to a biphasic pattern extending over an in-use relevant period. For dermal application both burst release and sustained release features are of interest. Burst release might promote a rapid clinical effect by enhancing skin penetration of active compounds. Sustained release becomes important for active ingredients that are irritating at high concentrations or those used to supply the skin over a prolonged period of time such as antimycotics[19, 279].

Formulation and *in vitro* characterization of CP-SLN:

Based on data obtained in preliminary trials using different materials and methods of preparation, tripalmitin as lipid base, lecithin and poloxamer 188 as surfactant combination were selected for SLN using the hot emulsification/ homogenization method.

Tripalmitin was selected based on reported data indicating that tripalmitin generates SLN with smaller size and intermediate crystallinity compared to other lipids such as tristearin and trimyristin[88, 271]. Further, tripalmitin SLNs loaded with the lipophilic drug clotrimazole showed optimum physicochemical characteritics and occlusive properties[155]. SLN prepared with fast crystallizing lipids glycerides with long chain fatty acids as tripalmitin (Dynasan®116) and surfactants not disturbing the crystallization process of lipid (like poloxamer) do not show changes in degradation velocity during storage time [60, 280].

Moreover, it was found that SLN stabilized with a **surfactant** mixture such as lecithin and poloxamer 188 as the surfactant combination used in the present study have lower particle size ranges and high storage ability compared to formulations with only one surfactant[23] as this prevents particle agglomeration more efficiently. The type of surfactant and its concentration affect the product as well[88]. In general in SLN, high concentrations of surfactants reduce the surface tension and facilitate the particle partitioning during homogenization. The decrease in particle size is connected with a tremendous increase in surface area. The excess emulsifier molecules might be present in different forms e.g. monomers, micelles (SDS) or liposomes (lecithin). The time scale for redistribution processes of emulsifier molecules between particle surfaces, water-solubilized monomers and micelles or liposomes is different. Redistribution processes will take a longer time for high molecular weight surfactants (poloxamers and lecithin) [23].

Homogenization followed by **ultrasonication** was reported to be an economic, simple and reproducible method for the preparation of SLN [88]. In the present study, solvents used in small amounts to disperse the drug in the lipid homogeneously were completely removed as seen from DSC. Similar to HPH method, it applies high shear stress disrupting lipid particles down to the submicron range.

Processing of the materials selected in this study by the hot emulsification / homogenization method generated CP-loaded SLN in the form of uniform nanospheres with average particle diameter of 173 nm, negative surface charge with an average zeta potential of ~ -22 mv and ~ 89% incorporation efficiency. CP proved to be distributed in the lipid matrix in the amorphous form. Drug release from SLNs showed a biphasic pattern with ≈ 17% burst effect at one hr. Drug release was sustained over a 6 day-study period. The % release at the end of the study was 70%.

A finer dispersion is usually obtained by imputing high energy such as high production temperature, high stirring rate, longer emulsification time, stronger ultrasound power and so on. Besides, processing parameters such as lipid matrix, surfactant blend and viscosity of the lipid and aqueous phase influenced outcome of the procedure.

Particle size (PS) analysis was done by three methods because results obtained by Laser diffraction (LD) were not enough to give the exact particle diameter of the particles. It s just a tool to demonstrate a stable nanoemulsion and covers a broad size range. Laser diffraction is based on dependency of the diffraction angle on the particle radius (solid radii) contrary to the photon correlation spectroscopy which measure the

hydrodynamic radii. Smaller particle cause more intense scattering at high angles compared to the larger ones in LD measurement [87, 281]. The nanodispersion had to be suitably diluted prior to measurements with LD, sometimes a drop of Tween 80 was used in the medium to increase particle suspendability. The particle size was homogenously distributed as seen from the span values calculated

The lipid concentration used to prepare SLN in this study was only 2% w/v of final dispersion. Increasing lipid content (over 5-10%) in most cases result in larger particles and broader particle size distributions. This may be due to a decrease in homogenization efficiency and increase in particle agglomeration[23].

Furthermore, loading the SLN with the drug resulted in a slight increase in the PS which might reflect the dissolution of the drug in the lipid phase.

It has been found that the average particle size of SLN dispersions is increasing with higher melting lipids and with triglyceride chain length[282]. This can be explained by the higher viscosity of the triglyceride melts[60]. Different lipids will also differ in the velocity of lipid crystallization, the lipid hydrophilicity (influence on self-emulsifying properties) and the shape of the lipid crystals (and therefore the surface area)[23]. Furthermore, most of the lipids used represent a mixture of several chemical compounds. Small differences in the lipid composition (e.g. impurities) may have considerable impact on the quality of the dispersion (by changing the zeta potential, crystallization processes etc..)[23]. Lipids with shorter fatty acid chains and considerable amounts of mono-and diglycerides which possess surface active properties [283]give smaller size dispersions.

Relatively low span values were obtained. A narrow particle size distribution is a great indication of nanoparticles stability and homogeneous dispersion. After three months of **storage**, no further increase in PS was observed (slight increase in span value might indicate some aggregations and particle growth). In another study, the size of Dynasan® 116 SLN increased from 260nm to 330 after 28 days storage at different temperature which may be also affected by the shape of the particles when the fat crystallizes[280]. The type of the surfactant used and storage time affect the crystallinity of SLN and consequently the degradation velocity.

Particle size also affects the dermal delivery of LBDDS[180]. Nanosized particles are of growing interest for topical treatment of skin diseases to permit site-specific delivery to the skin and to reduce side effects. Effects of the particle structure and size were studied loading Nile red to dendritic core-multishell (CMS) nanotransporters (20-30 nm) and solid lipid nanoparticles (SLNs, 150-170 nm). CMS nanotransporters can favour the penetration of a model dye into the skin even more than SLN which may reflect size effects[228]. In another study, the superiority of the CMS nanotransporters seems to be attributed to the character of the nanoparticles and not to its smaller size[180]. Loading rhodamin B onto SLN (250-340 nm) and CMS nanotransporters (20-30 nm), the dye amount increased significantly in viable epidermis and dermis as compared to a conventional cream. CMS nanotransporters were most efficient. Creating nanoparticles of 50-200 nm demonstrated only marginal size effect for the skin penetration[180] .

The average **zeta potential** of the prepared CP-loaded SLN was -22 mV. The negative charge is conferred by the lipid used tripalmitin. Lipid nanoparticles generally have a negatively charged surface[217, 284]. Zeta potential is an important and useful tool to indicate particle surface charge, which could be used to predict and control the stability of colloidal suspensions. Larger zeta potential enhances dispersion stability. A

high zeta potential (> 30 mV) can provide electric repulsion to avoid the aggregation of particles[285]. However, the physical stability of SLN is dependent on both the charge conferred by the lipid in addition to steric stabilization provided by the surfactant used [23]. This explains lack of aggregation of SLN prepared in the study despite relatively low zeta potential values in some samples. However, it is worth noting that non-ionic surfactants may result in lower zeta potential as a result of coverage of the diffuse layer by a layer of the uncharged surfactant and/or a shift in the shear plane of the particles [44]. The steric stabilizer (poloxamer 188) coat may have sufficient thickness and density to provide physical stability of the SLN formulations despite relatively low zeta potential values in some samples.

The CP-loaded SLN obtained had relatively **high drug entrapment efficiency** (89%). This can be attributed to the physicochemical properties of the drug, most importantly, its lipophilic nature (logP;3.98) [224] and those of the lipid base. Drug lipophilicity enhances dissolution in the melted lipid matrix and entrapment in the lipid core following emulsification and cooling. % E.E. data obtained in this study were consistent with those obtained for the incorporation of CP into SLN using a solvent diffusion method [75, 264]. Other lipophilic drugs such as the antifungal miconazole nitrate [226]were incorporated in SLN at high % E.E. However, in addition to the lipophilicity of CP, its structural parameters such as the ester side chain were reported to enhance its entrapment in SLN compared to other corticosteroids [75] . Another physical parameter of importance in the entrapment of drugs in lipid matrices is the melting point of the drug relative to that of the lipid used. In the present study, CP having a higher melting point (199°C) than the lipid base (67°C) was expected to solidify first upon cooling during the hot homogenization production process with the drug forming a core in the lipid phase, an observation reported previously [149] .

Beside the physico-chemical features of the drug, the structure of the lipid influences the drug incorporation capacity of SLN as drug delivery system[224]. For instance, lipids allowing for a limited space in the formed crystal lattice such as triglycerides, may result in expulsion of the drug from the lipid matrix during crystallization [286]. Drug expulsion is reduced when mixed lipids (mono and diglycerides) are used as they allow for more space in the crystal lattice formed upon crystallization. Furthermore, the lipid polarity[282], the interaction of the drug with the lipid structure as well as the use of high temperature in production and high surfactant concentration might influence the drug loading and the shape of the loading profile [44].Indeed, the crystallization of the melt will result in a solid solution or a solid dispersion of the drug in a homogeneous distribution or in clusters. Either a drug-enriched core or drug-enriched outer layers of the particles may be obtained. A drug enriched core is generally formed when the drug precipitates before crystallization of the lipid. In brief, the internal structure of the drug-loaded lipid particles is a function of the formulation ingredients and the production conditions[44].

DSC measurements generally offer a valuable tool for studying the interaction between the lipid and the drug incorporated in SLN, where the lipid within the nanoparticles is in a less ordered arrangement compared to the bulk lipid form, resulting in lower melting enthalpies.

DSC data for CP-loaded SLN, their components and related systems indicated disappearance of the endothermic peak of the drug in the thermograms for SLN and solvent evaporated coprecipitate. This can be explained by molecular distribution of CP in the lipid matrix. Moreover, a shift was observed in the melting peak of the lipid

(67.0ºC) to a lower value (64.7ºC) in SLN samples, which can be attributed to the less orderly arrangement of the lipid. Appearance of a new peak at 54ºC was also observed in the thermograms of SLN. This can be attributed to a change in properties of the lipid upon melting and crystallization during SLN preparation. Distribution of drug in the amorphous form in SLN has been reported for other drug-loaded SLNs such as those containing clozapine [88] and camptothecin [270]. Moreover, a depression in the melting point of lipids upon processing into SLN and inclusion of drug molecules has been attributed to defects in the crystalline lattice of the lipid cores [87, 157, 266], probably as a result of the small particle size of the prepared SLNs, their high specific surface area and the presence of surfactants [62, 106, 240, 271]. It has been shown that the properties of colloidal dispersions of glycerides differ from those of their bulk form. This especially applies to the melting and crystallization behavior and to the kinetics of the polymorphic transitions [60, 211, 280, 287]. Changes in the degree of crystallinity of the lipid are of relevance to the drug incorporation and release from SLN.

In vitro release data obtained under sink conditions indicated biphasic release pattern of CP from SLN with a 17% burst release in one hour and sustained release of 70% of the drug over a 6 day-study period. These are data are generally consistent with drug release from different SLN [23, 44, 155] . The burst effect could be attributed to the presence of a small fraction of unentrapped drug or drug embedded near the SLN surface. The slower release phase can be attributed to drug diffusion through the lipid matrix into the release medium. Generally, factors affecting drug release from SLN include mainly the large surface area of SLN, high diffusion coefficient of the entrapped drug, viscosity of the lipid matrix as well as the presence of surfactants adsorbed and incorporated in the surface during the production process [288]. So the drug release is affected by lipid composition and the drug solubility in the lipid.

The magnitude of burst release from SLN depends on various formulation and processing parameters. An increase in burst effect could be promoted by a higher surfactant concentration [286], lower lipid content[155]and higher homogenization temperature[286]. As shown with prednisolone SLN[131], surfactant and higher temperature enhancing drug solubility in the aqueous phase favour the enrichment of the steroid in the superficial layers during cooling of the preparation and crystallisation of the lipid. Superficially entrapped prednisolone is available for the initial burst release. Clotrimazole release from solid lipid nanoparticles [155] was faster at 10% lipid content and slower at 20% lipid content [155]. Results have been explained by a drug-enriched shell or drug-enriched core model at lower lipid content respectively. For lower lipid content systems, during the cooling process after homogenization, the lipid solidifies and the drug repartitions into the shell of the particles. At the higher lipid level (20%), a drug-enriched core is formed where lower release is observed. Etoposide SLN exhibited a high sustained effect caused probably by the high lipophilicity of tripalmitin[271].

Preparation of CP-loaded SLN gel for topical delivery

The CP-loaded SLN prepared showed good pharmaceutical attributes in terms of size, surface charge, entrapment efficiency and drug release characteristics. These nanoparticles were used to prepare a clobetasol propionate gel for topical delivery. A gel vehicle has been selected in view of the good acceptability of CP gel by patients[252, 262, 263, 289], good physical stability of lipid nanocarriers in different gel formulations including xanthan gum, hydroxyethylcellulose 400, Carbopol 943 and chitosan [155, 290] and the questionable physical stability of lipid nanocarriers in dermatological vehicles containing lipids or surfactants such as creams [154]. The lipids of the SLN or NLC may

dissolve into the cream and destroy their structures. The presence of surfactants may also alter their stability or enhance drug release[154].

Different methods have been proposed for the preparation of gels based on SLN or the related carriers NLC [154, 290, 291]. These include simple admixing of SLN/NLC with preformed gel products, addition of viscosity enhancers to the aqueous phase of SLN/NLC to obtain a gel or direct production of a final product containing only nanoparticles in a one-step process using high lipid concentrations [19, 292]. In the present study, CP-loaded SLN were incorporated in carbopol gel 1% containing 10% w/w propylene glycol and 30% w/w glycerol by simple mixing, a composition reported previously for a SLN gel formulation [293]. CP concentration in the test gel was 0.05%. Incorporation of CP-SLN into the prepared gel did not considerably affect their particle size (120 nm), or zeta potential (-14.72mv) upon storage at 8°C for 2 months. The in vitro performance of the test CP-SLN gel was compared to that of a control gel containing free CP 0.05% prepared using the same gel base and to a commercial conventional CP gel "Embeline Gel®" 0.05% formulated with carbopol/propylene glycol gel base.

The *in vitro* release study in phosphate buffer pH 7.2 containing 40% PEG and 0.5% Tween 80 as release medium at 35°C indicated slower release of CP from the test SLN-based gel. The release profile was biphasic with 28% burst release in 1hr followed by sustained release of ≈ 40% of CP over the 24 hr-study period. This indicated efficiency of the reservoir action of SLN and lack of rapid discharge of the drug into the gel vehicle, an effect previously seen in other studies[157, 221, 222, 249, 267]. Although faster than CP release from the SLN gel, drug release from the conventional control gel was relatively slow with an apparent biphasic pattern. This can be explained at least in part by the different rates of dialysis of the cosolvent-solubilized drug and dissolution rates of CP crystals of different size dispersed in the gel base. Rapid release of the solubilized drug and dissolution of smaller crystals may account for the faster release phase which was followed by slower dissolution of larger crystals. Such a crystal size distribution rendered the gel a reservoir for drug release. CP release from the commercial gel was much faster (~75% release in 24 hr), probably as a result of a different particle size distribution of the drug crystals and/or a greater hydrophilizing effect imparted by gel components [154, 157, 166, 249]. The biphasic release pattern can similarly be explained by the different release rates of small and larger CP crystals dispersed in the gel. The hydrophilizing properties of the gel base and its release enhancing effect were demonstrated by comparing the release profiles of CP from SLN dispersion and SLN-gel respectively. Faster CP release from the gel was observed (**Figure 17).**

Although the control gels containing free CP sustained the release of CP because of the relatively slow dissolution of drug crystals dispersed in the gel, yet they do not meet other essential requirements for enhanced topical corticosteroid performance. These include mainly reduction of direct contact of CP with the skin to minimize cutaneous reactions and absorption, particularly by inflamed skin[293] , possible skin irritation by the drug crystals, and a release profile from the gel affected by the crystal size distribution of the drug. Accordingly, the SLN-based gel showed essentially better drug release performance compared to the two control conventional gels. Nevertheless, CP release from SLN-based gel could be further modulated using different formulation approaches concerning the SLN delivery system (lipid type and content, etc.), the gel base (e.g. using different gel matrices, modifying the gel viscosity, etc.) or the drug (e.g. incorporation of a small fraction of free drug.

The *ex vivo* skin permeation study:

As the penetration of corticosteroids through the stratum corneum and their retention in skin layers or appendages is imperative for effective topical corticosteroid treatment of skin diseases[166, 232, 258], the skin permeability of the CP-SLN composite gel and the conventional commercial gel was assessed. Ex-vivo abdominal human skin from female subjects aged around 40 was used and the thickness of skin samples measured to ensure reproducibility of data. The average skin thickness was 2.92 ± 0.34 mm. CP permeation was assessed using Franz diffusion cell after 6hr of skin exposure to the gels.

Treatment of skin samples with the control conventional gel for 6 hr resulted in skin retention of ≈ 4 % CP and permeation of ≈ 11 % CP through full thickness skin. Despite slower in vitro drug release from the composite CP-SLN gel, treatment of the skin samples with this gel for the same period resulted in a six fold enhancement of CP skin uptake (≈ 22 %) while the % of drug permeating through the full thickness skin was more or less similar (≈ 10 %). The difference in CP skin retention was statistically significant ($p<0.01$). This provides further evidence to the role of SLN as skin penetration enhancers. Enhanced skin uptake of CP from the SLN gel probably result from the solid matrix of the nanoparticles facilitating their penetration into follicular openings of the skin and sticking to the stratum corneum where they act as reservoir for drug release[154]. Data obtained in this study using human skin support those reported for enhanced uptake of CP from a cream base by porcine skin and may explain the improvement in therapeutic response recorded in patients with chronic eczema [264]. Data are also consistent with those reported for a wide variety of drugs like Prednicarbate[166], Vit A[231], Miconazole nitrate[226] Isotretinoin[269] and cosmetic actives[294] .

Glucocorticoid targeting to the viable epidermis where the inflammatory reaction takes place is the key element in a successful topical corticosteroid therapy with low drug concentrations in the dermis which is most susceptible for irreversible damage [166, 224, 232, 258]. It is of importance in the treatment of skin diseases to localize the drug in the upper skin layers like stratum corneum and epidermis with minimal access to the dermis and the circulation[224]. Adequate association of drug and carrier is essential for epidermal targeting [157]. SLN with small diameters are advantageous to improve the penetration of nanoparticles into skins[75, 166]. Both the burst and controlled release of SLN may induce the increase of drug accumulation in the skin[269].The association of the drug with particle surface and the dose of the drug to the site of action in relevance to the % encapsulated of the drug are essential for drug targeting [233, 237, 295]. The type and concentration of the lipids and surfactants used in SLN may have an influence on the uptake of drug in the skin by affecting its release and affinity or partitioning, as well as their occlusive properties affecting skin hydration[224, 233, 258]. Even though, the skin targeting mechanism is unclear and the relative mechanism needs further investigation in future [269, 290] as the literature exhibits high variation in results from drug permeation experiments across human skin and other skin models[296].

Article VI. CONCLUSION

Solid lipid nanoparticles incorporating the highly potent corticosteroid clobetasol propionate were prepared using a hot emulsification /homogenization method. CP-SLN

were characterized for particle size, surface charge, entrapment efficiency, physical form of the incorporated drug and in vitro drug release. SLN prepared using the selected lipid and surfactants showed good pharmaceutical attributes. CP-loaded SLN were used to prepare a gel formulation for controlled topical delivery. The *in vitro* release characteristics and *ex vivo* human skin permeability of the CP-SLN gels were assessed in comparison with those of a conventional CP gel available commercially in the USA. Data obtained indicated the efficiency of the reservoir action of SLN as a nanocarrier for CP release. Skin permeability data provided evidence of enhanced skin uptake of CP during a 6 hr-incubation period with full thickness skin. Results obtained in this study contribute to the research efforts aiming at improving the performance of corticosteroids in the topical treatment of skin conditions. However, further investigation is needed to substantiate the data obtained.

Part II

LIPID NANOCAPSULES

Part II in brief:

The lipid nanocapsules (LNC), previously discussed in the introduction of this thesis with their numerous advantages are investigated in this part (chapter 1 & 2) as a potential delivery system for a hydrophilic macromolecule model drug fondaparinux (F) which is an anti factor Xa. With their controllable nanosize, ease of production, biocompatibility, stability for over 18 months, a better anticoagulant therapy by different administration routes could be an expected outcome. But, the encapsulation of such a hydrophilic molecule in the lipid based delivery system is a challenging task. This is the aim of the **chapter 1** of this part. Further assessment of this F-loaded LNC formulation administered via oral route will be carried out in an *in vivo* pharmacokinetic study in animals to prove its ability to increase the drug GIT stability as well as its intestinal transport and hence could lead to a new oral anticoagulant strategy. The latter is the aim of **chapter 2** of this part.

CHAPTER 1*

LIPID NANOCAPSULES INCORPORATING FONDAPARINUX MICROEMULSIONS

*This chapiter has been granted a patent :

« **Procédé de préparation de nanoparticules lipidiques à partir d'au moins deux micro-émulsions »**

Inventors(s): Benoit Jean-Pierre; Thomas Olivier; Alyaa Ramadan; Patrick Saulnier.

Applicant(s): UNIV ANGERS
France : N° de dépôt : FR 08 0058547; date de dépôt : 12/12/2008 N° de brevet: FR2939699 (A1), Date de pub: 2010-06-10. PCT : N° Dépôt : PCT/FR2009/052500 – date dépôt : 11/12/2009

INTRODUCTION

Anticoagulant use is recommended for the prevention and treatment of several thromboembolic disorders. They are widely used for the prevention and treatment of venous thromboembolism (VTE), for the acute management of patients with acute coronary syndromes (ACS) and for stroke prevention in patients with atrial fibrillation (AF) or mechanical heart valves[297-299].

Heparin, a naturally occurring glycosaminoglycan, remains one of the most important anticoagulant drugs in current clinical use and is the drug of choice when rapid effect is desired[300]. Commercial unfractionated heparin (UFH) , with a molecular weight 8,000 to 40,000Da) is a heterogeneous mixture of polysaccharides comprising alternating 1-to-4 linked sulfated monosaccharide residues of D-glucosamine and a uronic acid (90% L-iduronic acid, 10% D-glucuronic acid) **(Figure20a).** The pentasaccharide binding site for antithrombin occurs in approximately one-third of heparin chains (**Figure 20b)** [301, 302].

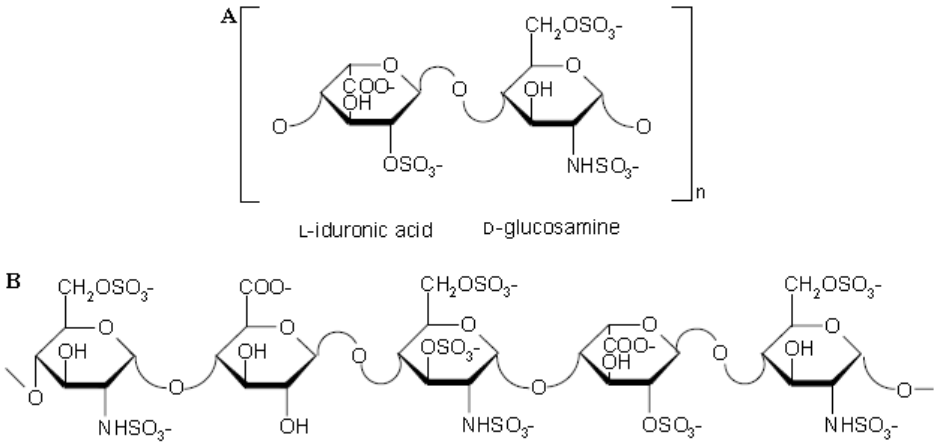


Figure 20A&B: Structure of unfractionated heparin (UFH)

In addition to its anticoagulant effect, heparin is also implicated in an ever growing number of physiological and pathological processes such as inflammation, immune cell migration, tumor cell metastasis, and smooth muscle cell (SMC) proliferation among others [300, 303]

Heparin is a potent inhibitor of coagulation, primarily through formation of a complex with antithrombin (AT), resulting in indirect inhibition of factor Xa, factor IIa, and other AT-dependent coagulation factors in addition to other AT-independent pathways **(Figure 21)**[304]. It must be given parenterally, by continuous i.v. infusion or by s.c. injection[305]. LMWHs (derived from UFH) by enzymatic or chemical depolymerization resulting in shorter heparin chains (4,500-6,000Da), have an enhanced affinity for antithrombin-mediated inhibition of Factor Xa relative to thrombin inhibition. They have improved pharmacokinetic properties and a decreased toxicity profile after subcutaneous injection [300, 306, 307]. They are only able to bind the coagulation

factors **(figure 21)** because of their shorter chain length and therefore, their effects can better be predicted, thus do not require laboratory monitoring. They have replaced UFH as drugs of choice for the surgical prophylaxis of DVT and the management of ACS and are being developed for therapeutic applications in cancer, transplantation, and immunology **(Table 5)** [308-312]

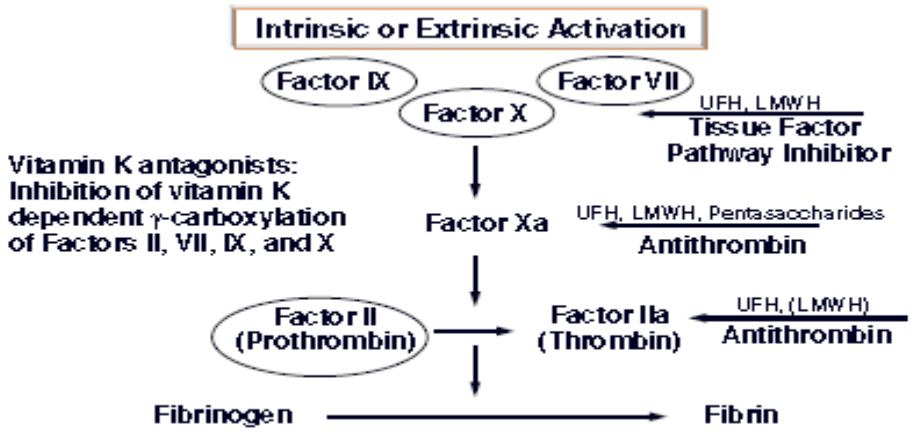


Fig. 1 Anticoagulant effects of conventional anticoagulants

Figure 21 : Anticoagulant effects of conventional anticoagulants ;from [313]

To improve the predictability of the effects of anticoagulants, focus on the direct inhibition of specific factors in the coagulation cascade such as the inhibition of thrombin (FIIa) and FXa has received particular attention.

Fondaparinux (F) **(Figure 22)** was the first in a new class of selective factor Xa inhibitors to be developed followed by idraparinux a synthetic, long-acting, highly sulfated analogue with an exceptionally long half-life designed for weekly, rather than daily, administration [299] and idrabiotaparinux with an attached biotin moiety at the non-reducing end unit, which allows its neutralisation with avidin**.** These heparin derivatives are synthetic analogues of the pentasaccharide sequence present in UFH and LMWHs that mediates their interaction with AT [306, 314]. Comparison of F with the conventional anticoagulants have been revised extensively in literatures and are shown in **Table 5**.

Fondaprinux has the potential for a highly specific (anti-Xa activity of F is about seven-fold higher than that of LMWHs) and a selective mode of action (no effect on AT-mediated thrombin inhibition) that could obviate the unpredictability and adverse effects currently associated with the heparins and oral anticoagulants replacing them in most indications (**Figure 21, Table 5**) [308, 315].

Fondaparinux sodium is found on the market as **Arixtra®** injectable solution (5 mg/0.4 ml, 7.5mg/0.6ml and 10 mg/0.8ml) to be taken by subcutaneous route once daily depending on body weight (<50Kg, 50-100Kg and >100Kg, respectively). F was reported to be rapidly and completely absorbed (100%) after SC injection. Its long half-life allows a once daily regimen. It does not require dose adjustment for age or gender, has predictable effects over the defined therapeutic dose range, and does not require laboratory monitoring[315-317].

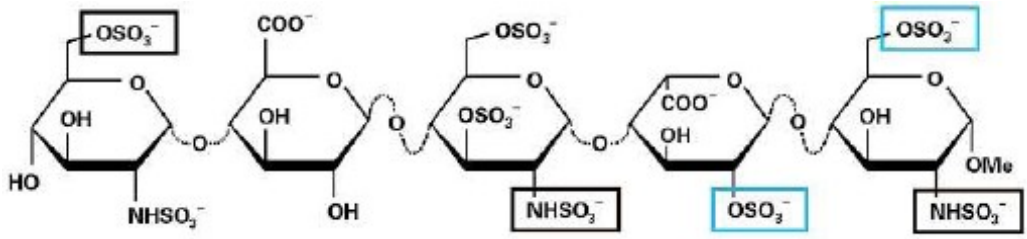


Figure 22: Sulfated and carboxylated carbohydrate-sequence of *Fondaparinux,* from [318].

It was approved in the US and in Europe in 2001 for thromboprophylaxis in patients undergoing major orthopedic surgery (MOS) and in 2004 for treatment of VTE, and recent trials have confirmed its efficacy and safety in ACS [298, 313, 315, 319-322]. F may be a better choice for patients with a history of HIT [323, 324]. F is associated with significantly less bleeding [41]. A single dose of F was well tolerated in mouse, rat and monkey at doses up to 40 mg/kg. Few non-haemorrhage related effects were observed. The thrombin generation test (TGT) could be a good option for monitoring fondaparinux and recombinant activated factor VII (rFVIIa) to reverse the inhibitory effect of fondaparinux on TG in patients presenting severe bleeding complications [320].

New anticoagulant agents that target specific factors in the coagulation cascade, primarily Factor (FXa) and thrombin, are in phase II clinical testing and several are moving onto phase III. The results thus far are promising, but more data are needed to assess the long-term efficacy and safety of these drugs [325, 326]. They are parenterally administered direct thrombin inhibitors (DTIs) such as bivalirudin and argatroban, or orally administered DTIs evaluated in phase III such as dabigatran etexilate and the orally active direct factor Xa inhibitors such as razaxaban, apixaban .

All data indicate that F is a safe and effective alternative to anticoagulants currently utilized in patients with ACS or mostly whenever clinical anticoagulation needed. Incorporation of such a molecule in a lipid nanoparticulate carrier system could have beneficial clinical outcome. These nanoparticulate carrier systems, in general, can efficiently protect fragile macromolecules against enzymatic degradation in the harsh environment of the GI tract if administered orally, can provide high transfer of drugs across the epithelial mucosa, control the release rate, and can target drug delivery to specific intestinal sites [327, 328]. Certain particles can be taken up by epithelial cells or the lymphoid tissues in Peyer's patches without employing additional penetration enhancers [329, 330]

Table 5 : Comparative properties of unfractionated heparin, LMWH and fondaparinux (showing advantages and disadvantages of each) adapted from [299, 308, 311, 313, 331-334]

	UFH	LMWH	FONDAPARINUX
Source	Animal	Animal	Synthetic (no contamination)
Av MWt (Da)	15,000	5,000	1,728
Structure	Heterogeneous	Heterogeneous	Homogeneous (well defined)
Targets	Multiple	Multiple	Single (Factor Xa) (high affinity to AT)
Pharmacological effect	Activity expressed as IU (anti Xa=anti IIa)	Activity expressed as IU (anti Xa>anti IIa)	Activity expressed gravimetrically as µg or µmol (no anti IIa activity)
Administration	IV, SC (2-3 times daily)	SC (1-2 times daily)	SC Once daily
Bioavailability	Variable	High	High (up to 100%sc)
Half-life	Dose- dependent (~1-5 hours (IV))	~4 hours	~17 hours
Mode of excretion	Reticuloendothelial mainly, urinary	urinary	urinary
Antidote	Protamine sulfate	Protamine sulfate (partial neutralization)	rFVIIa*
HIT response	----------	~80% crossreactivity with heparin-induced thrombocytopenia (HIT) antibodies	No crossreactivity with HIT antibodies
Monitoring	Required using aPTT	Sometimes required using aPTT	Not required (no prolongation of the aPTT)**

Bleeding	Not predictable	predictable	Minimal bleeding risk (predictable dose- effect)
Osteoporosis	High risk	High risk	No risk
Clinically	In renal insufficiency, cardiao-pulmonary by-pass	surgical prophylaxis of DVT & management of ACS	Thromboprophylaxis in MOS &treatment of VTE & ACS

* rFVIIa is recombinant activated factor VII (rFVIIa) to reverse the inhibitory effect of fondaparinux on thrombin generation in patients presenting severe bleeding complications

**Monitoring using TGT(thrombin generation test)

Lipid-based carriers do not entrap hydrophilic macromolecules with high efficiency due to the latter limited solubility in the lipid matrix. Conventional liposomes and microemulsions have not met with much success in the mucosal delivery of hydrophilic macromolecular drugs[330]. SLN, NLC, and LNC being novel carriers for controlled drug delivery entrap lipophilic drugs with high encapsulation efficiency. Four approaches were considered basically to be adoptable for encapsulating hydrophilic molecules into such LBDDS [8]. First, **increasing the lipophilicity** by hydrophobic ion pairing with molecules of opposite charges, chemical conjugation or the noncovalent interaction with hydrophobic component [330]. For example, hydrophilic DNA molecules were encapsulated into the oily core of LNCs via the formation of lipoplexes, leading to the formation of neutral DNA nanocapsules having a mean size of 110nm [199]. Second; **non-aqueous techniques** during encapsulation so as to reduce drug leakage, for example, compressed gas anti-solvent precipitation techniques[335], oil-in-oil (O/O) emulsion-evaporation method[336]. Third; increasing **the hydrophilicity of nanoparticles** by using hydrophilic materials or introducing functional groups that can be easily substituted or conjugated to the particle matrix. Lastly and most important; the **double-emulsion** solvent evaporation methods basically applied for encapsulation of most water soluble peptides, e.g., **water-in-oil-in-water (w/o/w)** technique [336]. **A**n example of this method is the successful loading of gonadorelin into SLN [142, 143, 337]. Nanostructured lipid carriers (NLC) for the delivery of calcitonin have been produced by this double emulsion method, resulting an association efficiency higher than 90% [118, 142]. NLC with cyclosporine A have been produced by hot high pressure homogenization [51]. The incorporation of salmon calcitonin into trimyristin SLN by w/o/w emulsion technique was found to be a key factor for the improvement of the efficiency of such carriers for oral delivery of proteins [338]. However, the main reported problems were that, by this method, the instability of water-in-oil pre-emulsion induced the lower drug encapsulation efficiency of the peptide or protein drug [8, 11, 339].

Recently, much attention has been paid to the administration of microemulsions as drug delivery systems, since they are thermodynamically stable and are formed spontaneously by simple mixing of the various components, they have high solubilization capacity for lipophilic and hydrophilic drugs, and they are able to facilitate the transport of drugs through biological membranes [13, 30, 340-342]. Water-in-oil

(w/o) microemulsions have been described in the literature as drug carriers of water-soluble molecules and peptides [343, 344]. They afford some protection against enzymatic or acidic degradation when administered orally[343] increasing the oral bioavailability of hydrophilic model drugs up to 64% [343]. Sandimmun Neoral®, an example of a marketed formulation, is a ME pre-concentrate containing a surfactant, lipophilic and hydrophilic solvents and ethanol [345]. The development of lipid based ME [344] and of "biocompatible microemulsions" [346] are interesting areas of research for *in vitro* and *in vivo* studies. As these ME systems are diverse in composition, extensive studies are necessary for their standardization and generalization so that these systems could be used in the form of "drug formulation" for clinical use. According to the formulations, the water-oil-surfactant mixture present, at equilibrium, one or more phases [98, 347]: Simply, type I and type II MEs are two-phase systems (o/w) or (w/o) MEs respectively, Type III MEs exhibit three phase system in which the middle phase MEs are in equilibrium with both excess oil and excess aqueous phases. Type IV MEs result from the expansion of the middle phase Type III MEs with increasing surfactant concentration such that all the excess oil and excess water are incorporated into a single phase system (bicontinuous ME). From a microstructure perspective, the type of emulsion formed as well as the transition from o/w to w/o ME is dependent on the phase ratio of the aqueous and lipid phases, together with the type, concentration, and preferred solubility of the emulsifying agents used. Transition can be accomplished via bicontinuous ME or via the LC phase[348].

One main issue in microemulsion formulations is to identify surfactant combinations that would produce a thermodynamically stable formulation. Thus, it is essential to establish the phase behavior of the particular combination of chosen components. Many important theories and concepts are to be understood and considered when dealing with MEs in order to predict their stability and phase behavior [349]. Different concepts have been established to approach formulation: Bancroft's rule of thumb describes that the external phase is the one in which the surfactant preferentially partitions, viz. the phase for which it exhibits a greater affinity. The value of the **HLB** (Hydrophilic Lipophilic Balance) by Griffin considers this amphiphilic behaviour and serves as an instrument to compare non-ionic surfactants of the same family within the same formulation conditions. The **HLD** (Hydrophilic Lipophilic Deviation) is another concept which includes the contributions of the physicochemical environement of the formulation. It reflects the deviation from an optimal formulation. It is a numeric expression used to calculate or compensate for the effects of different formulation variables. More details are well elaborated in [350]. The **phase inversion temperature (PIT)** by Shinoda [92] at which a polyethoxylated nonionic surfactant switches its dominant affinity from the aqueous phase to the oily phase to produce a change in emulsion type depends also on the physicochemical environment of the surfactant. The PIT describes the temperature at which the **Winsor R ratio** shows the value 1 , at this point, the amphiphile neither prefers the oily nor the aqueous phase [98], Winsor proposed the R ratio to explain the interactions of surfactants located at the interface with oil or water molecules, respectively. Depending on whether the R ratio is inferior, superior or equal to 1, one obtains **ternary phase diagrams** of the types Winsor I, II or III, represented in **figure 23.** Another interesting concept in formulation of ME, similar to the HLD, is the so-called "surfactant affinity difference" (SAD) which, as well as the terminology of Winsor, describes the affinity of the surfactant for the oil or the water

phase, respectively. A value of zero in the SAD concept is equal to R = 1 in the Winsor terminology and can thus be taken as a kind of reference state to compare different systems, which are in the same physicochemical state, although no single formulation variable possesses a common value [93].

In some ME systems, the surfactant concentration can be as high as 70% [351] . This may limit the ME application as well as the use of cosurfactants, mainly medium-chain alcohols, which may cause skin irritationin topical or transdermal drug delivery[352]. Decreasing surfactant concentrations and the use of Lecithin based ME or ME without cosurfactants have been the aim of recent studies[353]. Despite the geometric packing theory and the accumulation of experimental data on microemulsions, their formulation is still highly empirical and time consuming. Full characterization of such systems is a tedious task and requires a large number of experiments. In a recent study, an artificial neural network (ANN) modeling was proposed and allowed better understanding of the process of microemulsion formation and stability within ternary and pseudoternary colloidal systems and thus minimize experimental effort [354].

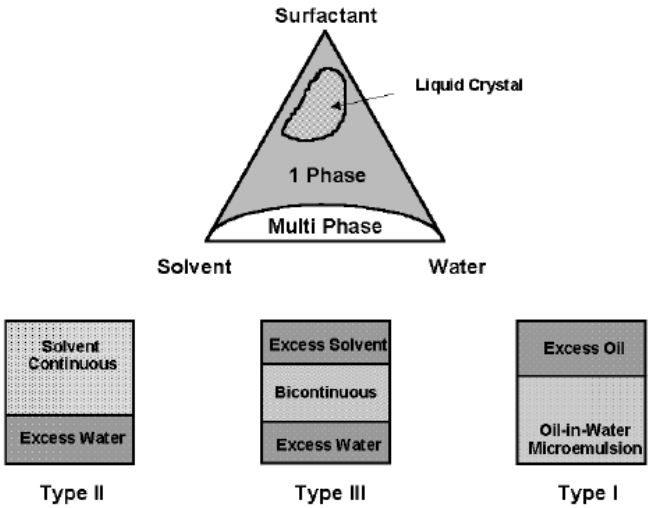


Figure 23: Hypothetical **pseudo-ternary phase diagram** (fixed cosurfactant concentration) showing multiphase system at low concentrations (white), single-phase ME (gray), and liquid-crystal region at high surfactant concentrations (mottled) from [355].

The **objective of this chapter*** was to prepare lipid nanocapsules loaded with the pentasaccharide fondaparinux. The described microemulsions (w/o) were chosen as a "precarrier" for the hydrophilic drug for allowing incorporation into the core of the lipid nanocapsules. The possible incorporation of a hydrophilic molecule in a lipid based delivery system was investigated using two MEs similar to the well known w/o/w technique. Initially, different compositions of oils, surfactants (mainly lipophilic, lecithin –based), and polar phases (non aqueous or partially aqueous) were tested. Their states were defined by the creation of ternary diagrams. From these mixtures, only clear w/o MEs were chosen for further drug incorporation. This was followed by the addition

of this "precarrier" ME_1 into the second ME_2 during a phase-inversion based preparation process followed by sudden dilution with cold water to obtain lipid nanocapsules loaded with fondaparinux (F-LNC). These were further characterized for their particle size, zeta potential and encapsulation efficiency.

EXPERIMENTAL

MATERIALS:

Materials for the production of lipid nanocapsules:

- **Lipoid®S75–3**:(soybean lecithin at 69% phosphatidylcholine and 10% phosphatidylethanolamine, with an average molecular mass of 780 g/mol or average molecular weight of 800) was a gift from Lipoïd (Ludwigshafen, Germany).
- The lipophilic **Labrafac®** CC (caprylic-capric acid triglycerides, European Pharmacopoeia, IVth, 2002, with an average molecular mass of 470 g/mol or average molecular weight of 512) was provided by Gattefossé S.A. (Saint- Priest, France)
- **Solutol®HS-15** (70% PEG 660 hydroxystearate and 30% free PEG 660, European Pharmacopoeia, IVth, 2002, with an average molecular mass of 870 g/mol or average molecular weight of 911) kindly provided by BASF (Ludwigshafen, Germany);
- NaCl was obtained from Prolabo (Fontenay-sous-Bois, France).
- Deionized water was obtained from a Milli-Q plus system (Millipore, Paris, France) and saturated with N2. The specific conductivity(18.2 $m\Omega cm^{1}$) and the surface tension (72 mN/ m) of this quality of water indicated that it was free of surface-active impurities.

Materials for the production of microemulson:

- Propylene glycol (from Sigma-Aldrich, Steinheim, Germany) and deionized water as the aqueous phase,
- The lipophilic Labrafac® and the surface active agent Lipoïd® S 75-3(mentioned above)
- Epikuron®200 (from Laserson, Etampes, France), (95% phosphatidylcholine)
- Span® 20 and 60 and Tween® 80 (from Sigma-Aldrich, Steinheim, Germany)
- Imwitor® 308 (Glycerylmonocaprylate allocated by Sasol Germany GmbH, Witten, Germany), (Monoester >80 %)

Drug and other materials:

- Fondaparinux sodium was purchased in the form of ARIXTRA® subcutaneous injection (10mg/0.8ml) by GlaxoSmithKline (Marly-le-Roi, France).
- The liquid Fondaparinux injection solution was lyophilized prior to encapsulation in the microemulsion.
- The dye (Azure A) and the nonionic detergent (Triton X 100) were from (Sigma-Aldrich, Germany)
- The anti-Xa chromogenic assay was performed using the Biophen Heparin 6 kit STA-Fondaparinux Calibrator and Sta-Fondaparinux Control kits (Hyphen Biomed, Neuville-Sur-Oise, France) (Diagnostica Stago, France)

The brand names of materials will be used throughout the following chapter

EQUIPMENT:

- Hot plate with magnetic stirrer (IKA MAG Ret control visc, IKA Gmbh & Co KG, 79219 Staufen, Germany)
- Centrifuge (JOUAN, Germany)
- Malvern Zetasizer® Nano ZS Serie DTS 1060 at a fixed angle (173°) at 25 °C furnished by a 4 mW He-Ne_laser at 633 nm (Malvern Instruments S.A., Worcestershire, UK)
- Conductimeter (WTW 330i, Weilheim, Germany)
- UV-visible spectrophotometer (Uvikon 940, Kontron KG Instruments, Montigny Le Bretonneux, France)
- Automatic analyzer (STA-R®, STAGO, Asnières sur Seine, France).
- A freeze-dryer STERIS Lyovac GT2, coupled to a pump Alcatel and a cryothermostat Huber CC-505

METHODS:

1. PREPARATION OF LIPID NANOCAPSULES FROM TWO MICROEMULSIONS

The present method of preparation of LNC from two microemulsions has been granted a patent during 2009[356].

1. The "precarrier" w/o microemulsion (ME_1):

(component screening, phase diagram construction and preparation of ME_1)

Principle:

The "precarrier" microemulsion was intended to be prepared as w/o system to achieve the solubilization of the hydrophilic drug fondaparinux into the polar core of the emerging structures.

Initially, the w/o ME was prepared by combining lipophilic Labrafac® **(35%-70% w/w) with the aqueous phase (10-30%w/w) deionized water alone or water: propylene glyol (W:PG) in 1:3 ratio or PG alone (non aqueous ME) and the**

lipophilic surfactant mixture (6% -50%w/w) either lecithin-based (Lipoid® S75 or Epikuron® 200), or not lecithin-based like (Imwitor ®308) and sometimes using small amounts of a non ionic surfactants as co-surfactant (Span® 20, 60, Tween®80 and Solutol® HS, which has a major role in the formulation of lipid nanocapsules[53]). Existence of a clear, one-phase or two-phase ME region in a three- or four-component mixture was identified in the constructed ternary and pseudo-ternary phase diagrams, respectively.

Procedure:

The PG or aqueous phase, the surfactant and the oil, and when applicable a second surfactant, accurately weighed, were mixed together in a closed vial to a final weight ranging from (100 - 550 mg) or sometimes double that weight (with or without F). The whole mixture was then left in a water bath at 65°C to 85°C, to allow homogenization of the mixture with regular mixing by vortex. This temperature is recommended if one of the components of the ME is solid at room temperature for example like in the case of Lipoid® (melting at 83°C). The mixture was then left for 2 to 4 hours for equilibrium, or left overnight. Occasionally, the tubes containing the mixtures were vortexed to guarantee homogeneity. **Visual inspection of the resultant mixtures allowed the detection of any sign of turbidity.**

Only one-phase, clear, non-birefringent (lamellar crystalline, LC) phases were classified as MEs. In most cases the limit of the region of ME existence was signaled by the appearance of a cloudy solution. Phase diagrams were constructed from the experimental data obtained using weight percent (wt %) of the components (figure 25, 26, 27). This process was repeated on three separate occasions, and the values thus obtained were estimated to be accurate to within a few percent. The temperature of the "precarrier microemulsion" was maintained till incorporated into the second microemulsion. Two MEs (o/w) were prepared as control using the same procedure, as no drug encapsulation was expected when the polar phase is outside, allowing the drug escape to the aqueous phase during LNC preparation.

2. Preparation of "precarrier" microemulsion loaded with fondaparinux:

After construction of the phase diagrams, MEs (one phase translucent or sometimes two phase systems) were chosen to prepare F-loaded systems suitable to be incorporated into the LNC system. A known amount of the lyophilized drug (0.5mg, 1,2,3 or 4 mg) was then dissolved in the PG or aqueous phase using vortex occasionally, then the PG or aqueous phase, the surfactant and the oil, and when applicable a second surfactant, accurately weighed, were added on the drug solution. Then, the process was pursued as aforementioned.

3. Preparation of the second microemulsion and formation of LNC:

Principle:

The second ME is obtained at the phase inversion zone (PIZ) of a macroemulsion containing the non ionic surfactant Solutol® (10%- 40%w/w). As a polyethoxylated surfactant, it has the property to change its hydrophilic lipophilic balance with the increase in temperature, thus invert an o/w macroemulsion into an w/o one at higher temperature. The phase between the two macroemulsions is characterized by the bicontinuous Winsor IV ME intended. Temperature cycling of the system through its PIZ three times was found to increase the interfacial stability of the droplets, which upon sudden cooling, at a temperature at the beginning of the PIZ, would form lipid nanocapsules characterized by a rigid shell of the non ionic surfactant [53]. The components and their amounts in the second ME were determined according to a previous ternary diagram aiming to obtain the LNC with a 50 nm particle size [54, 73].

This phase inversion technique (PIT) with the temperature cycling and other formulation parameters affecting the formation of these LNCs were discussed in detail in the general introduction of this thesis.

Procedure:

To prepare LNC loaded with F, the first microemulsion (ME_1) is introduced into the second one (ME_2) at a temperature just before the dilution step. The optimal amounts of ingredients for preparation of 50nm LNCs were: 504 mg Solutol®, 504 mg Labrafac®, 44 mg NaCl and 1.538 mg demineralized water which were mixed in a closed container and heated under magnetic stirring up to 92°C at a constant rate (4°C/min), above the phase inversion temperature. In the next step, the temperature was decreased down to 60°C, below the inversion zone. This temperature cycle was applied two other times. The last cooling ramp was performed down to 72°C± 8°C, close to the lower limit of the phase inversion zone (ME_2 zone), measured by conductimetry. At this point, 100-200µl corresponding to different weights in mg of the ME_1 loaded with F and maintained at the same temperature of ME_2, was quickly added. Fast cooling was then operated by addition of 8ml of water at 0–2°C to obtain the nanoparticles. Stirring was maintained for 5 minutes at a slower rate after LNC formation followed by filtration through a 0.20µm Sartorius filter.

When the two MEs were mixed, the whole system remained translucent (Figure 24). If any cloudiness was observed at this point, this reflects the disturbance or destruction of the interfacial film of surfactants or the lipophilic hydrophilic balance, the LNC were not obtained and the dispersion was then discarded and the whole process was repeated. The different ME_1systems used ,their phases, their different weights added to ME_2, their different drug content as well as changes in the temperature of addition of ME_1 or in the quench temperature are shown in (Tables 7,9,11).

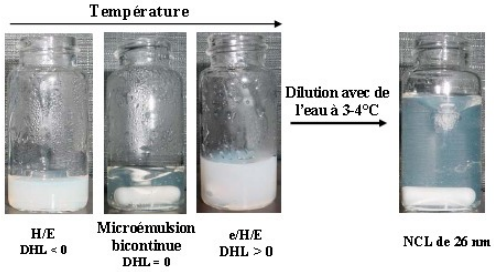
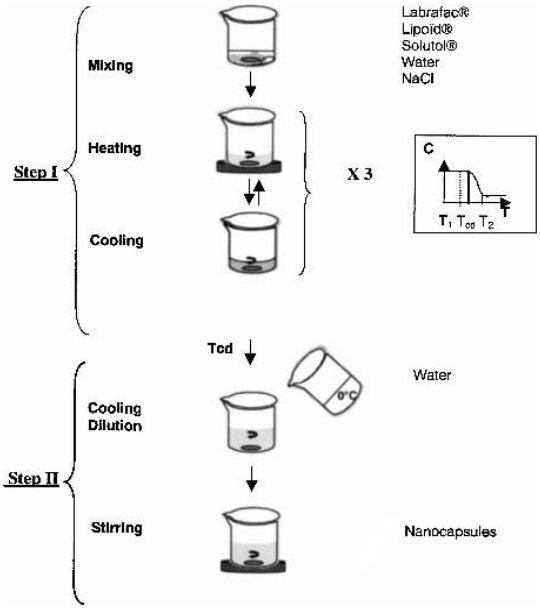


Figure 1. Observation de la préparation des NCL au cours du chauffage et après dilution

LNC (50-100nm)



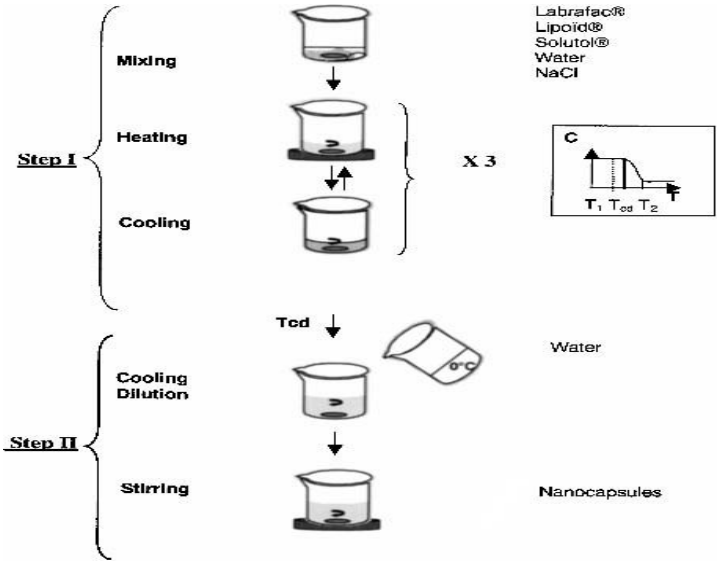


Figure 24: ***Preparation steps for the LNC from two microemulsions, adapted from[53]***

2. CHARACTERIZATION OF THE F-LOADED LNC:

1. Particle Size:

The average hydrodynamic diameter and the polydispersity index (PDI) of LNCs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer® at a fixed angle (173°) at 25 °C[72]. A polydispersity index above 0.3 indicates a broad distribution. Prior to size analysis by DLS, a (1:60 v/v) dilution of the nanocapsule suspension in deionized water enabled measurements and ensured a convenient scattered intensity on the detector. Data are the average of three determinations.

2. Zeta Potential :

The zeta potential (ζ) and the electrophoretic mobility measurements of the different nanoparticles dispersions were performed using the same instrument for particle size measurement equipped with an AZ-4 cell and were based on the laser Doppler effect[4, 70]. Zeta potential measurements were made in water at 25 °C, with dielectric constant of 79, refractive index of 1.33, viscosity of 0.89 cP, cell voltage 150 V and current of 5 mA. Nanocapsule suspension was diluted 1:60 (v/v) in deionized water prior to measurements in order to have the same conductivity of the solutions, ~0.2mScm−1. Data are the average of three determinations.

3. Conductivity:

The conductivity of the LNC preparation medium was measured to determine the time for the first ME addition, ascertained by a conductimeter with two platinum plate attachments between 60°C and 90°C after two cycles of heating and cooling under magnetic stirring. All conductivity values were expressed in microsiemens per cm (μS/cm).

4. Drug Percent Encapsulation Efficiency (% EE) and Drug Content :

4.1. ***Assay of fondaparinux by azure method:***

PRINCIPLE:

Many methods are used for the determination of heparin or its derivatives in solutions or in biological fluids. The most important techniques are photocolorimetric polarographic, chromatographic, electrophoretic, turbidimetric [307] and most commonly used, biological [357]. Unfortunately, these approaches require sophisticated instrumentation and expensive reagents, which frequently make them hardly realizable in practice and not practical for routine work.

Like any heparin derivative, F is an acidic mucopolysaccharide containing carboxyl and sulfonic groups in D-glucuronic and L-iduronic acid residues possessing optical activity. This could be a base for the spectrophotometric method of F determination. The UV spectra of heparin solutions exhibit an absorption maximum at 257 nm irrespective of the solution pH. But this method is inapplicable for the determination of the heparin concentration in solutions of nanocarriers and in stained biological fluids (e.g., unpurified blood plasma, operative filtrate, etc.), which poses a problem for heparin determination in these media. We have used the ability of heparin to form stained complexes with Azure A (in the ratio 1 : 3) that exhibit absorption in the visible range. Azure A assay was performed to quantify unentrapped F as well as F adsorbed on the surface of the nanocapsules [358, 359] Azure A has a characteristic absorption at 633 nm. When azure A forms a complex with heparin, the intensity of this absorption diminishes proportionally with the amount of heparin, which obeys Beer-Lambert law. It was established that the heparin-Azure A complex absorbs in the visible range with a maximum at 516 nm (at pH 5.9) [357]. The absorption was measured by spectrophotometer.

PROCEDURE:

By analogy, quantitative determination of the complexes (Azure-F) was based on a calibration plot at 516 nm constructed using different concentrations of F solution prepared from an initial stock solution (100 µg/ml) (The F concentration in ARIXTRA® vials is 12,500 µg/ml). A freshly prepared Azure solution (0.04mg/ml) was added to all samples in 3:1 v/v ratio (azure: sample respectively). The assay was validated according to the ICH guidelines. This was used to quantify the loading of F in the LNC after dissolving the LNC in a solution of Triton X 100.

Another standard curve was generated with known F concentration at 633nm. This was used to quantify indirectly the concentration of unentrapped F in the external aqueous solution recovered after ultrafiltration and separation of the LNC and the complex of free F with Azure

4.2. *Drug percent encapsulation efficiency (%EE)*

Unentrapped or free drug was separated from the F-loaded LNC dispersions by complexation with Azure A dye followed by ultrafiltration at 4,000 RPM for 30 minutes at 20°C using (Millipore® Centricon YM-100 filters, USA) to remove the complex and most of the LNC. The concentration of the uncomplexed dye in the filtrate was then measured colorimetrically at 633 nm, which is indirectly proportional to the concentration of the free drug. Data are the average of three determinations .

Typically, aliquots (500 µl) of LNC samples (100 µl of the F-loaded LNC or unloaded LNC (as a blank) were diluted 5-fold with 400 µl deionized water) and 1500 µl Azure solution (0.04 mg/ml) and mixed thoroughly (1: 3 v/v) at room temperature followed by ultrafiltration and the dye solution in the filtrate was assayed in triplicate at 633 nm and referred to a standard calibration performed with different concentrations of F standard solution and ME-loaded F-free LNC dispersions. The drug entrapment efficiency was expressed as the percentage of F entrapped with respect to the total drug content.

The encapsulation efficiency was calculated as follow:

$$\textbf{Encapsulation efficiency\% EE} = \frac{\textbf{C total-C unentrapped}}{\textbf{C total}} \times 100$$

where C unentrapped is the concentration of free F in a volume of LNC suspension (µg/ml)(by indirect azure method) and C total is the total concentration of the F in the same volume of LNC suspension(µg/ml)

Prior to using this indirect technique for routine assay purposes, it was verified that the results were correlated with the biological assay (anti X A) of the free F measured in presence of the L N C (no ultrafiltration).

4.3. ***Drug content:***

The **total amount of F** ***(****C total)* in final volume of LNC dispersion was either calculated theoretically based on concentration of F in the final dispersion (total F fraction added in the fraction of ME (µg)/final volume of dispersion measured (ml)) or determined by the **Triton method**[360] after disruption of the nanocapsules completely and release of the encapsulated drug.

Typically, 50 µl of the F-loaded LNCs (in triplicate) were diluted 5-fold with 200 µl of a solution of Triton X 100 (10% w/w), a non-ionic detergent that is often used in biochemical applications. The closed tubes were heated at 80°C for 1 minute in a water bath. This is done to disrupt the nanocapsules completely and release the encapsulated drug (seen as clear solution and particle size measurement confirmed destruction of the LNC) [360]. After cooling down, tubes were vortexed, 250 µl of deionized water were added and vortexed for a second time (further 2-fold dilution). 250 µl of the destroyed LNCs were diluted with 750 µl of Azure solution (0.04 mg/ml) (1:3 v/v) and mixed again by vortex. The dye Azure formed a complex with the present heparin whose maximum absorbance could be detected at 510 nm, whereas ME-loaded F-free LNCs (blank) treated the same way served as reference during colorimetric measurement.

The concentration of *F* was assessed by comparison with a calibration curve which was established by means of blank LNCs mixed with F concentrations prepared from standard solution (100 µg/ml) and the same procedure were carried out.

5. BIOLOGICAL ASSAY :

PRINCIPLE:

The anti-Xa activity is a measure of the ability of fondaparinux , like heparin, to inhibit or neutralize, through plasma antithrombin III, the activated Factor Xa (Factor Xa) active coagulating enzyme.

The assay was performed according to the protocol supplied by the manufacturer[317]. The principle of this assay is based on the fact that the anticoagulant effect of F is achieved via its ability to bind antithrombin and potentialize its natural inhibitory activity for coagulant serine esterases as factor Xa. Biophen Heparin 6 is a kinetic method based on the inhibition of a constant amount of factor Xa by the tested anticoagulant in presence of endogenous antithrombin, and hydrolysis of a factor Xa specific chromogenic substrate (SXa-11) by the factor Xa in excess, thus liberating the chromophoric group, paranitroaniline (pNA). The amount of pNA released relates to the residual factor Xa activity. There is an inverse relationship between the concentration of F and color development measured at 405 nm.

[AT-fonda] + Xa excess → [AT-fonda-Xa] + Xa residual

Xa residual + substrate→ pNA

PROCEDURE:

The anti-Xa activity was measured from the calibration curve of absorbance (A/min) vs F standard concentrations (µg/ml) with Sta-Fondaparinux Calibrator and Sta-Fondaparinux Control kits according to the protocol supplied by the manufacturer

CALIBRATION AND CONTROL SOLUTIONS

Calibration curves were prepared at the Hematology lab at the University hospital of Angers : dilution in series of a solution of fondaparinux (from a syringe ARIXTRA® (GSK, France) 10 mg/0.8 ml) in normal pooled human plasma (Sta®-Pool Norm, Diagnostica Stago, France); 6 points de calibration : 0-0.125-0.25-0.5-1-2 µg/ml in duplicates. A new calibration with each new heparin lot. Calibration curve obtained was comparable to that obtained with the kit STA®-Fondaparinux Calibrator (Diagnostica Stago, France). Two control solutions (0.25 ± 0.10µg/ml and 1 ±0.15µg/ml) were prepared and measured with each measurement. STA®-Fondaparinux Control kit (Diagnostica Stago, France) have been tested, validated with the commercial calibration curve and the ones prepared during the course of this study.

6. Stability (Shelf:)

STABILITY OF MICROEMULSION

Was monitored by visual inspection of the MEs prepared with different systems and left at room temperature for 3 to 4 weeks. Heating in a water bath at 70°c just before inspection was carried out because lecithin is solid at room temperature. The vials were checked for clarity.

STABILITY OF LNC

Physicochemical stability of LNC formulations was followed by the determination of the change in the particle size of the LNC after storage at 4 °C and at room temperature (25°C) for 3 months. Particle size and zeta potential were measured for every preparation once every month.

RESULTS

The results are presented according to the first "precarrier" ME composition, using the same oil (Labrafac) as that used in the preparation of the LNC:

1. LECITHIN-BASED "PRECARRIER" MICROEMULSIONS:

1. LIPOID® S75-3 as surfactant:

The first MEs were lecithin-based, in which the ratio of the oil was higher than the polar phase to prepare w/o MEs enabling entrapment of the hydrophilic F. Heating the mixtures was absolutely necessary because simple vortex at room temperature did not yield clear MEs. In the case of Lipoïd®, one could also notice that the MEs became quickly solid as soon as they were removed from the water bath to room temperature. It was also evident that a higher amount of surfactant led to faster adjustment of the equilibrium of the ME. However, contrary to the definition of a spontaneous formation of a ME, time was necessary to obtain a translucent system (>4 hours).

1.1. ***Propylene glycol (PG) as polar phase :***

The focus of interest in this chapter was the system consisting of Lipoid®, propylene glycol and Labrafac®,

The phase diagram of the ternary system Labrafac®, Lipoid® and propylene glycol as the polar phase is represented in **figure 25**. Two regions were clearly identified in the phase diagram; the first represents an isotropic region (seen as yellow dots) where this formulation was stabilized and formed a clear and transparent w/o ME seen visually after equilibrium at 70°C in a water bath for 1 to 4 hours at least. The other represents biphasic region (two immiscible fluid phases, seen as black dots), one phase of w/o ME and the other a simple oil or polar phase as the amount of lecithin, as a surfactant, is too low to solubilize one of the phases in the other. Thus, it was possible to form three-component lecithin - based MEs by replacing water with propylene glycol. In contrast, it was not possible to form lecithin-based MEs using water and the same oil without the addition of a cosurfactant(354).

Three systems were chosen for further encapsulation of the drug and preparation of F-loaded LNC; **Point A** as seen in **figure 25**, representing a system with Labrafac® (66.66%), Lipoïd (6.66%) and propylene glycol (26.66%) in *w/w* ratio, and which presents two liquid phases. Another **point B** increasing the Lipoid® (to 15% w/w) and reducing the PG (to 15% w/w) and almost the same for Labrafac® (70% w/w) to obtain one phase, clear ME system.

More trials involved **Point X** as another clear region using smaller oil concentration (50% Labrafac®) and more Lipoid® (30%) and PG (20%).

Two points were chosen as a **control** (no expected drug encapsulation), using much smaller concentration of the Lipoid® (15%) to form o/w system where the drug should be found in the external continuous hydrophilic phase and the incorporation into the lipophilic core of the LNCs should be excluded. The first (control 1) with 15% Lipoid® and PG in excess (70%) resulted in two phase system (seen as black dot, **figure 25**) and the other (control 2) seen as clear one ME (o/w) (yellow dot), composed from 30% Lipoid® and 55% PG. More details of formulations shown in **table 7.**

The impact of the differences in the ME_1 formulation parameters presented in each of these points (A, B, X) on the final physicochemical properties of LNC was further assessed **(Table 8**). The LNC ingredient amounts were chosen to prepare 50nm nanocarriers with a neutral to slightly negative surface. Any increase in particle size could reflect destabilization of the single systems.

As seen in **figure 25**, PG allowed the formation of ME using Lipoid® as a single surfactant, showing a region with two clear immiscible phases (**point A**) which is composed mainly from w/o ME and another phase of excess oil. Using this system to further encapsulate F was one of the first successful results obtained as seen with formulations A1 (n=5) having a mean particle size 103.24nm with a good particle size distribution (PDI>0.3), ζ (-3.46mV) and EE% (20.5) up to 40% seen with only one of the 5 formulations. Further trials, aiming to decrease the weight of ME_1 added to ME_2 with a slight increase in the temperature of ME_1 addition and the quench temperature, resulted in formation of F-LNC (A2) with smaller PS having a mean of 64.6 nm, PDI (<0.3), mean ζ (-1.83mV) and mean EE% (32.5% ± 25.8) but these formulations were of very small drug content (< 37.1µg/ml). Other trials with higher ME_1 amount added but still low F content, gave formulations (A3) having more or less same PS and ζ (63.2nm, -2.2mV respectively) with no further improvement in EE% (19%) still showing high variability (± 26.8) **(table 8)**.

Other trials to prepare F-LNC were further investigated using ME_1 formulations at **point B** where mixing PG (15%), Lipoid®(15%) and Labrafac®(70%) resulted in one clear phase **(figure 25**). B1 formulations which were similar to A3 were more promising in terms of % EE (40.6% ± 9.07) but still had limitation because of their PS (ø 97.5 nm). Increasing the temperature of ME_1 addition and quench temperature up to (82°C/80°C respectively), involved both smaller size B2 formulations (64.7nm) and high EE% (43.6%). At this temperature (82°C/80°C), the LNCs system might exist as w/o emulsion just before the phase inversion. Further increase in the temperatures (92°C/90°C) and the weight of ME_1 added resulted in unstable formulations (B3) as shown from PS results and lower EE% (11.5%). Further trials (B4,B5,B6) changing the temperatures, the ME1 weight added as well as F content did not further improve the EE% or the PS (**Table 8**).

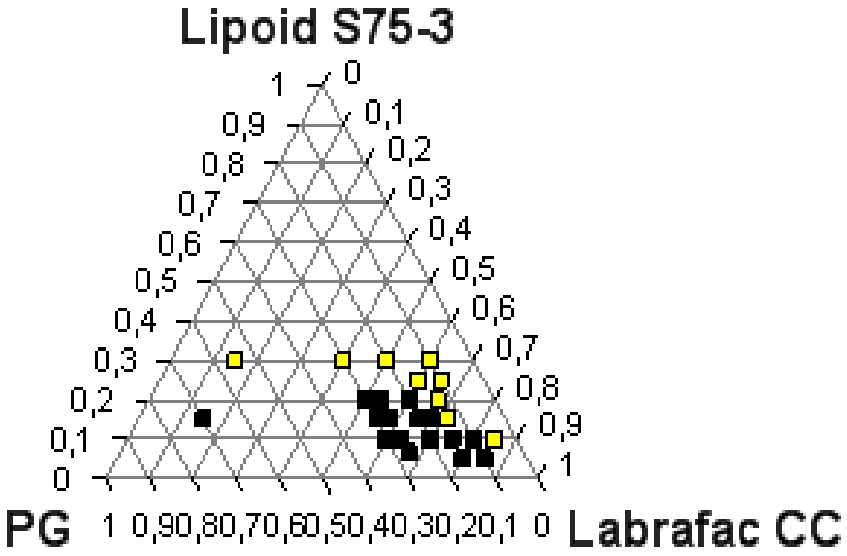


Figure 25 : Ternary diagram of propylene glycol, Labrafac® CC, and Lipoid® S75-3 at 75°C (points A, B, X and control are points of interest for encapsulation of F **Yellow:** one phase (clear), **black**: two phases (no turbidity).

Table 6 : Composition % (w/w) Lipoid®-based ME_1 using Propylene glycol as polar phase

Formulation code (total n)	Labrafac® (%)	Polar phase (%) (PG)	Lipoïd® (%)
A (11)	66.66	26.66	6.66
B (13)	70	15	15
X (3)	50	20	30

Table 7: Formulation parameters of F- LNC using Lipoid® –based ME_1 , (*propyleneglycol, Lipoïd® and Labrafac®)*

code (n*)	Number of phases in ME_1	Concentration F in ME_1 (%w/w)	Weight (mg) of ME_1 added to 1 g ME_2	µg of drug added to 1 g ME_2	Temp. of addition of ME_1 (°C)	Quench Temp. (°C)
A1 (5)	2	0.67	133.5 ± 26.16	891.1± 85.2	72	70
A2(4)	2	0.67	60.5± 14.17	371.8±31.1	77	75
A3 (2)	2	0.22	169±14.14	405.3±94.9	75	74
B1 (3)	1	0.19	187.66±9.86	488±18.7	72	70
B2 (3)	1	0.19	197±11.86	374.3±27.5	82	80
B3 (2)	1	0.09	286±102	257.4±129.8	91	89
B4 (1)	1	0.09	181	165.6	72	70
B5(2)	**1**	0.19	156.5±0.07	297.3±1.3	92	90
B6(2)	1	0.19	227±8.49	431.3±1.61	74.5±6.36	71±7.07
X1 (2)	1	0.25	178	445 ±2.82	72	70
X2 (1)	1	0.42	97	404	77	75
Control 1	1	0.66	47	310.2	82	80
Control 2	2	0.19	395	750.5	82	80

*n (number of batches prepared)

Table 8 : Physicochemical properties of F-LNC formulations prepared from Lipoid®-based ME_1 systems(*propyleneglycol, Lipoïd® and Labrafac®)*

code (n*)	P.Size (nm)	PDI	Zeta(mV)	Conductivity **(mS/cm)	Calculated F content (theoretical) (µg/ml)	F Content by Triton method (µg/ml)	% EE***
A1 (5)	103.24 ± 27.3	0.18 ± 0.06	-3.46± 2.59	0.22 ± 0.07	61.75±32.31	63.65±20.17	20.5± 13.02[XA]
A2(4)	64.13± 1.42	0.06± 0.02	- 1.83± 1.17	0.16± 0.01	37.15± 9.25	14.25± 5.79	32.5± 25.8[XA]
A3 (2)	63.23± 2.58	0.30± 0.21	-2.22	0.36	34.5± 2.4	31.15±12.37	19± 26.87
B1 (3)	97.53± 2.93	0.16± 0.02	-3.77± 2.57	0.17± 0.01	32.83± 1.87	ND	40.66± 9.07
B2 (3)	64.7± 3.10	0.26± 0.02	- 2.79± 0.36	0.17± 0.01	34.83± 2.16	ND	43.66± 14.33
B3 (2)	66.25± 2.05[2]	0.39± 0.16	- 2.86± 0.75	0.15± 0.01	25.4± 9.1	ND	11.57± 11.57
B4 (1)	107.4	0.10	-2.79	0.166	16.1	ND	42. 32
B5 (2)	72.25± 3.74	0.22± 0.05	- 3.5± 0.42	0.14± 0.01	27.4± 0.14	ND	16.2± 3.81
B6 (2)	47.6± 6.64	0.21± 0.12	- 4.37± 0.23	0.15± 0.01	42.45± 0.77	ND	18± 0
X1 (2)	102.15± 1.31	0.12± 0.01	-3.34± 0.33	0.18	36.75± 1.16	ND	21± 0.70
X2(1)	77.19(91.7%)	0.23	-4.12	0.16	39.1	35.8	8
Control 1	95.2	0.21	-2.87	0.16	29.6	ND	0
Control 2	110.8 (3peaks)	0.86	-2.89	0.17	70.4	ND	27

ND (not determined), [XA] (anti Xa activity measured for this sample to determine the concentration of the free drug and confirm its biological activity)
* n (nimber of batches prepared)
**Conductivity measurements ensure consistency in LNC dispersions
*** EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6),
[2] Two peaks

Other ME_1 composition (20% PG, 30% Lipoïd®, 50% Labrafac®) at point X (**Figure 25**), resulted in LNC formulations either with low EE% (8%) seen with X1 or larger PS (102 nm) and better EE% (21%) seen with X2 (**Table 8**).

Control formulation (control 1) prepared with (o/w) ME1 using small weight of ME1 added and showing small drug content (<30µg/ml) was of large PS (95.2nm) showing no drug encapsulation. Unexpectedly, the other control formulation (control 2) with higher drug content (70µg/ml) and higher weight of ME_1 added (395mg) had EE% (21%) but a destabilisation of the system was seen resulting in three populations of different particle sizes**(Table 8)**.

1.2. ***PG/water (1:3) as polar phase:***

Once water was added to the polar phase, a large clear ME (w/o) phase could be obtained with (9-21%) / (9-30)% / (48-80)% ratio for the polar phase (25% water, 75% propyleneglycol), Lipoïd® and Labrafac® , respectively (**point C**, **Figure 26, table 9**). The clear two immiscible phases area (point A, **Figure 25**) obtained with PG alone as polar phase has been substituted in this phase diagram (**Figure 26**) with a large area of cloudy points (violet) or one cloudy lower phase and another supernatant fluid phase (brown) which may represent liquid crystals (LC) phase (birefringence appearance) or multiphase regions as phosphatidylcholine one of major Lipoid® components tend to rearrange themselves into the form of bilayers in the presence of water [(344)].

LNC formulations C1, representing a series of formulations similar to A1 in terms of the weight of ME_1 added, F content and temperatures used for addition of ME_1 and quenching, did not show any improvement in the EE% (18.5%). Increasing temperature of addition of the ME1 up to 92°C and quench temperature up to 89°C, resulted in formulations C2 with optimum PS (59nm), small PDI (0.06), ζ (-1.3) and relatively higher EE% (30%). Repeating these formulations but at 82°C/80°C was not successful (EE%, 8% only).

Other F- LNC formulations (C4) were prepared using high weight (mg) of F added in ME_1 to ME_2 (the drug content ~4 folds higher) and were added to the LNCs at a relatively high temperature (92°C) when the LNCs were expected to be in w/o emulsion phase from conductivity measurement**(Figure 30).** They showed good PS (63nm) with good PDI, ζ (-3.6). Elevated concentrations of the F implicated raised sums of NaCl, however, which were comprised in the original solution of F. The salt could lead to the dehydration of the surfactant's polar heads which caused in turn, that the ME became more solid and difficult with its addition to the LNCs. An encapsulation efficiency of almost 10% at an average for the three samples was obtained **(Table 10&11).**

Further formulations (C5, C6) using lower temperatures, smaller F content and smaller weight of ME_1 added were not successful. The increase in Labrafac® concentration (80%) in C5 or decreasing its concentration (C6) to 50% resulted in a

Table 9: Composition % (w/w) Lipoid®-based ME_1 using Propylene glycol/ water (3:1) as polar phase

Code (n*)	Labrafac® (%)	Polar phase (%) PG (75%) / water (25%)	Lipoïd® (%)
C1 (2)	66.08±2.23	10.065±1.04	23.85±1.202
C2 (4)	65.725±1.08	9.775±0.48	24.5±0.63
C3 (1)	66.5	9.5	24
C4(3)	65.77±1.56	14.16±1.03	23.2±0.69
C5(2)	49.15±0.77	20.9±1.27	29.95±0.49
C6(2)	79.9±0.14	9.95±0.64	10.15±0.49

**(number of batches prepared)*

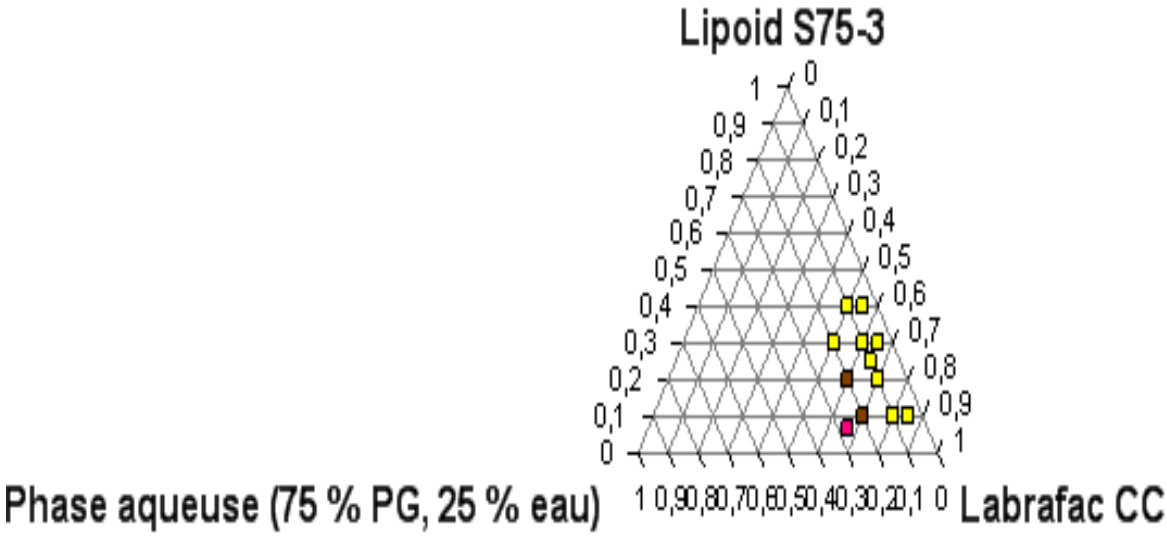


Figure 26 : Pseudo-ternary diagram of Lipoid® S75-3and Labrafac® CC with propylene glycol/water (3:1 w/w) containing F, at 75°C, (point C) is a point of interest for encapsulation of F

Yellow: one phase (clear) , **black**: two phases (no turbidity), **violet:** cloudy, **brown**: one cloudy lower phase and another supernatant fluid phase

Table 10: Formulation parameters of LNC using Lipoid® –based ME_1 systems (*polar phase* (*75% propylene glycol/25%water) , Lipoïd® and Labrafac®)*

code (n*)	Number of phases in ME_1	Concentration F in ME_1 (%w/w)	Weight (mg) of ME_1 added to 1 g ME_2	µg of drug added to 1 g ME_2	Temp. of addition of ME_1 (°C)	Quench Temp. (°C)
C1 (2)	1	0.67	90±14.85	603±94.7	72	70
C2 (4)	1	0.67	104.16±104	697±70.3	92	89
C3 (1)	1	0.67	90	603	82	80
C4(3)	1	1.24±0.03	258.4±39.5	3215.9±449	89	86
C5(2)	1	1	67±5.65	670 ±56.5	72	70
C6(2)	1	0.67	90±8.48	603 ±56.8	72	70

Table 11: Physicochemical properties of F-LNC formulations prepared from Lipoid® – based ME_1 systems (*polar phase* (*75% propylene glycol/25%water), Lipoïd® and Labrafac®)*

code (n*)	P.Size (nm)	PDI	Zeta(mV)	Conductivity **(mS/cm)	Calculated F content (theoretical) (µg/ml)	F Content by Triton method (µg/ml)	% EE***
C1 (2)	ND	ND	ND	ND	55.75± 7.84	56.5± 9.33	18.5± 7.77
C2 (4)	59.32± 8.19	0.067± 0.02	-1.39± 1.21	0.194± 0.01	65.2± 5.68	68.9± 7.53	30± 4
C3 (1)	ND	ND	ND	ND	54.1	49.4	6
C4(3)	63.76± 0.71	0.122± 0.08	-3.68± 1.01	0.259± 0.01	488.1± 54.0	488.4± 57.3	9.66± 2.49
C5(2)	ND	ND	ND	ND	64± 7.64	63.05± 6.72	0.5±0.70
C6(2)	ND	ND	ND	ND	69.25± 6.85	64.95± 16.05	0

ND (not determined),
* n (nimber of batches prepared)
**Conductivity measurements ensure consistency in LNC dispersions
*** EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6),

complete failure to encapsulate the drug unlike X1 with PG alone in the polar phase which have shown some encapsulation (20%) (**table 8**).

2. EPIKURON® 200 AS SURFACTANT:

Figure 27a and b show the ternary and pseudo-ternary phase diagrams of another lecithin-based, Labrafac® and polar phase systems but this time using Epikuron® 200 as surfactant which contains mainly 95% phosphatidylcholine. In comparison with Lipoid® phase diagrams (**Figure 25, 26**), the region of one phase clear ME is larger. Heating the mixtures in a water bath at 75°C was still absolutely indispensable like Lipoid® because simple vortex at room temperature does not yield clear MEs, but shorter time was needed for equilibrium and the ME formed remained a stable fluid not solidifiying for longer times (> 24 hrs).

Using the same formulations as in point X but with Epikuron® ME resulted in LNC (E1) with better PS (~85nm) but no further improvement in the % EE (~28%).

Replacing some of the PG with water in the polar phase like in C2 but with much lower temperature of addition of both ME_1 or cold water, gave same encapsulation results (31%) but 2 populations of nanocapsules appeared from PS measurement. This may be because the ME used was somewhat little turbid. While, further increase in % Labrafac®, similar to C6 , resulted in almost no encapsulation of F (5%).

To summarize, results obtained with the lecithin- based MEs using Lipoid® or Epikuron® as surfactants, PG or PG/water as polar phase, and Labrafac® as oil phase were of limited success. Although the particle size of LNC was more or less controllable (<100nm) with good particle size distribution (<0.3), the encapsulation of F could not reach higher than a mean of 26.72 % ± 14.26. Changing formulation parameters like the amount of fondaparinux added, the weight of the ME_1 added to the LNCs, the temperature of the addition of the ME_1 and of the dilution process as well as the amount of the cold water were not sufficient to achieve desirable goals of therapeutic concentrations.

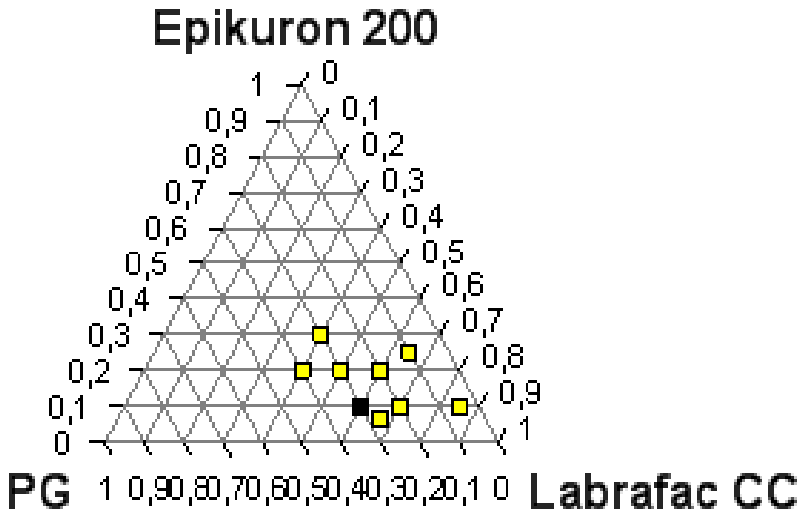


Figure 27a : Ternary diagram of propylene glycol, Labrafac® CC, and Epikuron ® 200 at 75°C

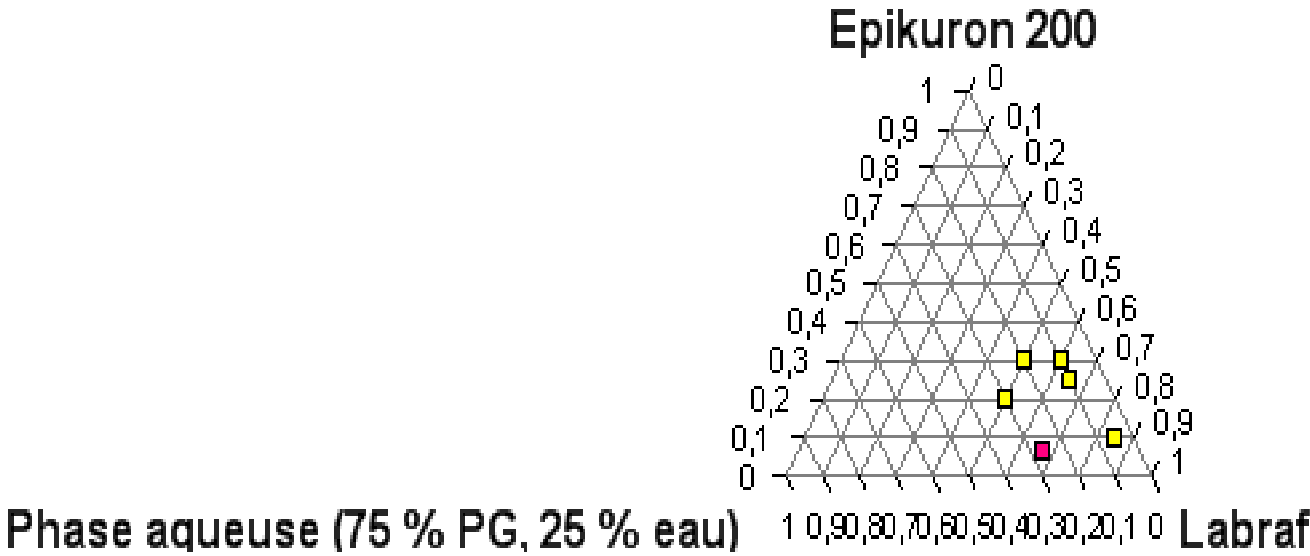


Figure 27b : Pseudo-ternary diagram of Epikuron® 200 and Labrafac® CC with propylene glycol/water (3:1 w/w) , at 75°C,

Yellow: one phase (clear), **black**: two phases (no turbidity), **violet:** cloudy

Table 12 : Composition % (w/w) Epikuron®-based ME_1 using Propylene glycol or Propylene glycol/ water (3:1) as polar phase

Code(n*)	Labrafac® (%)	Polar phase (%) (PG)	Polar phase (%) PG (75%) / water (25%)	Lipoïd® (%)
E1 (3)	46.8±0.19	25.16±2.77		28.01±2.57
E2 (1)	66.30		10.25	23.44
E3 (1)	73.6		7.5	18.9

Table 13: Formulation parameters of LNC using Epikuron® –based ME_1 systems

code (n*)	Number of phases in ME_1	Concentration F in ME_1 (%w/w)	Weight (mg) of ME_1 added to 1 g ME_2	µg of drug added to 1 g ME_2	Temp. of addition of ME_1 (°C)	Quench Temp. (°C)
E1 (3)	1	0.53±0.05	57.23±21.11	303.319	72	70
E2 (1)	1	0.69	109.4	754.86	92	89
E3 (1)	1	0.5	172.98	864.9	82	80

code (n*)	P.Size (nm)	PDI	Zeta(mV)	Conductivity **(mS/cm)	Calculated F content (theoretical) (µg/ml)	F Content by Triton method (µg/ml)	% EE***
E1 (3)	85.15±28.7	0.16± 0.12	-1.84± 0.82	0.163± 0.06	20.16± 8.57	24.75± 8.36	28± 3.03
E2[t] (1)	72.23[2] (87.6 %)	0.275	0.494	0.188	N D	63.02	31.71
E3 (1)	69.25 (86.7 %)	0.275	-2.46	0.151	N D	86.67	5.14

Table 14: Physicochemical properties of F-LNC formulations prepared from Epikuron® –based ME_1 systems

ND (not determined), [t] The ME was little turbid, [2] Two peaks
*n (number of batches prepared)
** Conductivity measurements ensure consistency in LNC dispersions
*** EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6),

Partial Replacement of Epikuron® 200 or Lipoid® with non ionic cosurfactants:

The addition of a second surfactant has been reported in some cases to promote ME formation[361]. Other lecithin–based ME_1 systems using a cosurfactant (Tween®80, Span®20, Solutol®) were tested for their clarity and stability (**Table 15)**.

A microemulsion with 30% Epikuron® 200, 15% Span® 20, 15% water and 40% Labrafac® CC was used further to prepare F-LNC. Typically, 100 mg of this ME_1 containing 500 µg F was added to ME_2 at 75°C followed by dilution with cold water at 72°C to obtain the nanocapsules. Unfortunately, no encapsulation of the drug was detected.

More formulations and maybe other MEs systems presenting one phase could be of better incorporation results. The composition of the ME may have been the cause for the low encapsulation of F and therefore, surfactants other than lecithin were investigated to prepare the precarrier ME_1.

2. NON LECITHIN–BASED PRECARRIER MICROEMULSION

IMWITOR ® 308/SPAN® 60 (SAA/COSAA):

Identifying the surfactant combinations that would produce a thermodynamically stable microemulsion formulation has always been a challenging task. In a study [354], ME could be obtained using an Imwitor / Span® 60 - mixture in a 7:3 ratio with extraordinary stability that possessed above all a large area of existence in an isotropic region. Thereby, the liquid crystalline region (LC) remains small and only expands, if the amount of the cosurfactant Span® 60 is raised.

Based on the phase diagram reported in **figure 28**, MEs were formulated from mixtures containing 15% water, 35% Imwitor® 308 (surfactant), 15% Span®60 (cosurfactant) and 35% Labrafac®. The MEs were spontaneously formed no need for heating unlike the lecithin-based.

The ME with the Imwitor® / Span ®60 - surfactant combination, in contrast to lecithin-based, were very stable, stayed almost transparent, did not disturb the second ME when added in the PIZ and the resulting LNCs showed a significantly lower mean particle size (59.87 ± 7.25 nm) and a very low PDI (0.04-0.2) (**Table 17)** which is also a sign that the system had not been destabilized.

In these formulations, the temperatures used for the addition of the ME_1 and the cold water to the nanocapsules were not high (77°C and 75°C respectively)(**Table 16**). It was obvious from physicochemical characterization of F-LNC prepared from these MEs that their particle size declines when the addition of $ME_1$was accomplished at temperatures during the phase inversion zone in consequence of the conductivity

measurements. The optimal moment for the addition of the precarrier ME_1 was at the end of the PIZ that means the second ME is in w/o state.

Table 15: Other lecithin-based microemulsions using different composition and different cosurfactants.

Lecithin (%w/w)	Cosurfactant (%w/w)	Water (%w/w)	Labrafac® (%w/w)	Visual observation
Epikuron® 20%	Tween® 80 :10%	15%	55%	cloudy
Epikuron® 20%	Tween ® 80 :10%	20%	50%	cloudy
Epikuron® 20%	Tween® 80 :10%	10%	60%	liquid + little clot
Epikuron® 30%	Tween ®80 :15%	15%	40%	liquid + little clot
Epikuron® 26.66%	Tween® 80 : 13.33%	10%	50%	liquid + little clot
Epikuron® 12.5%	Tween ®80 :12.5%	15%	60%	liquid + little clot
Epikuron® 30%	Tween ® 80 :10%	15%	45%	liquid + little clot
Lipoïd® : 30%	Span® 20: 15%	15%	40%	2 ph, cloudy and clear fraction
Epikuron® : 30%	Span ® 20: 15%	15%	40%	clear
Lipoïd® : 30%	Solutol® : 15%	15%	40%	cloudy
*Epikuron® : 30%	Solutol® : 15%	15%	40%	almost clear, water drop on the ground
Epikuron® : 30%	Solutol ® : 15%	10%	45%	limpid
Epikuron® : 25%	Solutol® : 10%	15%	50%	2 ph, cloudy and clear fraction
Lipoïd® : 12%	Tween® 80: 32%	21%	35%	2 ph, cloudy and clear fraction
Epikuron® : 12%	Tween® 80: 32%	21%	35%	clear

*This ME system was further used to prepare F-LNC

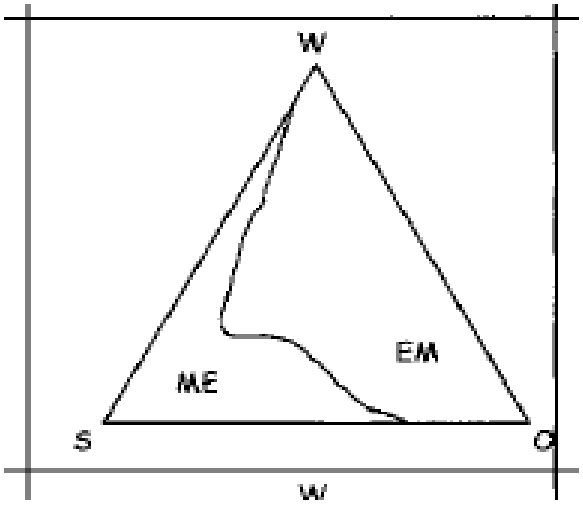


Figure 28: pseudo-ternary diagram of Imwitor® 308:Span® 60 (7:3w/w) and Labrafac® CC (O)with water (W),from [354]

Table 16: Formulation parameters of LNC using ME_1 (35% Imwitor® 308, 15% Span® 60 , 15% water and 35% Labrafac®)

code (n*)	Number of phases in ME_1	Concentration F in ME_1 (%w/w)	Weight (mg) of ME_1 added to 1 g ME_2	µg of drug added to 1 g ME_2	Temp. of addition of ME_1 (°C)	Quench Temp. (°C)
W1 (1)	1	0.25	159.7	399.25	77	75
W2 (8)	1	0.5	75.9±6.23	379.5±31.1	77.4±1.14	76±1.58
W3 (1)	1	1	66.7	667	76.5	76
W4 (1)	1	2	78.8	1576	77	75.5
W5 (3)	1	3.33±0.57	71.86±1.35	2392.9±41.16	77.16±1.04	76.5±1.8
W6 (1)	1	4.19	71.6	3000	77	75
W7 (1)	1	5.46	54.9	3000	77.4±1.14	76±1.58
W8 (1)	1	6.86	58.3	4000	76.5	76
W9 (6)**	1	0.64±0.04	78.2±4.57	500	77	75.5

*n (number of batches prepared)
**The ME_1 used in these formulations varied in the temperature of ME equilibrium (37°C, 50°C, 78°C) and time incubation in WB before addition to ME_2

Table 17: Physicochemical properties of F-LNC formulations prepared from Imwitor® 308:Span® 60 (7:3w/w) –based ME_1 systems

code (n*)	P.Size (nm)	PDI	Zeta(mV)	Conductivity **(mS/cm)	Calculated F content (theoretical) (µg/ml)****	% EE*[4]
W1 (1)	52.82	0.09	-6.28	0.166	37.6	44.6
W2 (8)	69.94±20.31	0.117±0.08	- 2.26±0.43	0.15±0.01	34.92±3.05	45.65[XA]±36.5
W3 (1)	57.3	0.051	-1.12	0.147	62	49.3
W4 (1)	79.8	0.207	-1.59	0.163	141.7	71.3
W5 (3)	56.03±0.94	0.0526±0.02	-1.44 ±0.23	0.160±0	223.26±40.04	25.6±36.53
W6 (1)	58.6	0.059	-2.40	0.163	200.4	89.3
W7 (1)	58.6	0.059	-2.4	0.163	158	74.3
W8 (1)	59.04	0.042	-1.65	0.197	268.7	70.4
W9 (6)	ND[v]	ND	ND	ND	36.3±20.31	58.85±20.31

***n (number of batches)**
**Conductivity measurements ensure consistency in LNC dispersions
***F content has not been determined by Triton method, only based on theoretical calculations.
*[4]EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6),
[v]ND not determined but visually was similar to formulations (W6-W8)
[XA] some of the samples were analysed for the free F by biological anti-XA assay

W1 represented the first formulation with the new surfactant combination which assimilated 159 mg ME_1 to the translucent ME_2 state during LNC preparation

(77°C/75°C). Unlike lecithin-based ME_l formulations and with relatively small quantities of ME_l added as well as higher F content, Imwitor® 308:Span® 60 (7:3w/w) – based ME_l systems achieved highest encapsulation of fondaparinux in the final LNC (ø48.62 ± 31.95%) up to 90% seen one batch of W2 formulations and confirmed by the coagulation factor anti-Xa method (**Table 17**).

In the other formulations (W3-W8), the amount of F was increased from 500 µg up to 4 mg per 100 mg ME keeping the weight of ME added the same . Their particle size was not affected by increased drug content (~53nm) and the encapsulation of the drug was also satisfactory resulting in formulations with higher drug content (up to 268µg/ml for W8).

In addition, the equilibrium state of the MEs was investigated for W6-W9 formulations. MEs were heated at different temperatures for a different period of time. From 50°C to higher temperatures, quite encouraging encapsulation rates were observed. Moreover, formulations were more successful when the ME stayed longer time for equilibrium in the water bath.

Summing up all formulations of LNC from two microemulsions, the Imwitor® /span® -based ME resulted in LNC with better size and better encapsulation than lecithin-based ME as shown in **(Figure 29)**

STABILITY RESULTS :

Three formulations of LNC; lecithin-based ME_l (B1) and non lecithin-based ME_l (W2 and W6) were further studied for stability of their physicochemical properties at 4°C and at 25°C (**Table 18**). It was found that at both temperatures, the LNC particle size increased by time showing two PS populations in some samples. The particle size distribution and the zeta potential did not show major changes.

Figure 29 : Physicochemical properties of LNC formulations (particle size in nm and EE %) prepared from two MEs using different ME_1 precarrier systems (error bar ±SD, n variable 4 to 15)

TABLE 18: Stability study of some formulations of ME-F-loaded LNC

LNC Formulations	Temperature	Time of storage	Particle size (nm)	PDI	Zeta potential (mV)	Conductivity (mS/cm)
B1	25°C	T_0	94.2	0.18	-2.34	0.157

		$T_{1\ month}$	116.3 (99.3%)	0.186	-2.52	0.151
		$T_{3months}$	133.5	0.179	ND	ND
	4°C	T_0	94.2	0.18	-2.34	0.157
		$T_{2\ months}$	112.7 (98.2%)	0.202	-3.05	0.160
		$T_{4\ months}$	123.3	0.175	ND	ND
W2	25°C	T_0	57.70	0.051	-2.01	0.142
		$T_{1\ month}$	64.22	0.058	-2.88	0.151
		$T_{3months}$	66.20	0.068	ND	ND
	4°C	T_0	57.70	0.051	-2.01	0.142
		$T_{2\ months}$	63.88	0.065	ND	ND
		$T_{4\ months}$	65.81	0.059	ND	ND
W6	25°C	T_0	58.87	0.052	-1.26	0.160
		$T_{1\ month}$	65.50	0.062	-1.59	0.155
		$T_{3months}$	69.54	0.064	ND	ND
	4°C	T_0	58.87	0.052	-1.26	0.160
		$T_{2\ months}$	66.25	0.073	ND	ND
		$T_{4\ months}$	68.16	0.042	ND	ND

Conductivity measurement:

The conductivity decreases with higher temperature which indicates a phase inversion from o/w to w/o. Furthermore, a shift between the phase inversion of the system was determined depending on whether it was heated or cooled down (**figure 30**).

Fondaparinux assay:

Azure assay validation results

The calibration curve at 516nm was linear over the concentration range of 3–20 µg/ml (R^2 =0.9958 ± 0.0013, n =4) **Figure 31a**. The error of determination of these complexes in solution does not exceed 0.06, and the detection limit is 3 µg/ml. Interday precisions of the method were less than 20%, and accuracies were over the range of 96.23–103.76%.

The calibration curve at 633nm was linear with a negative slope over the concentration range of 3-15 µg/ml (R^2 =0.9982 ±0.0057, n = 4) **Figure 31b**. The error of determination does not exceed 0.06, the limit of drug detection was 3µg/ml. Interday precisions of the method were less than 20%, and accuracies were over the range of 86.05–113.94%.

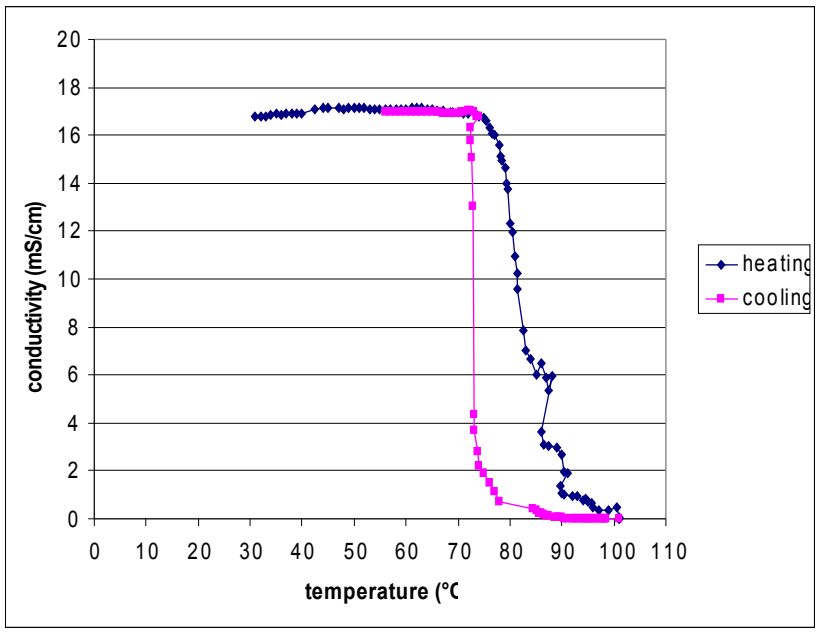


Figure 30: Conductivity measurement of the emulsion medium prior to the LNC formation with varying temperature.

Figure 31a : Calibration curve of fondaparinux at 516nm (Azure-F complex) for the determination of total drug content in LNC by Triton method, average values (n=15) (blank is unloaded broken LNC, Triton mixture)

Figure 31b: Calibration curve for fondaparinux at 633 after the removal of complex (Azure-F) by ultrafiltration , used for the determination of drug encapsulation efficiency in LNC, average values (n=20) (blank water

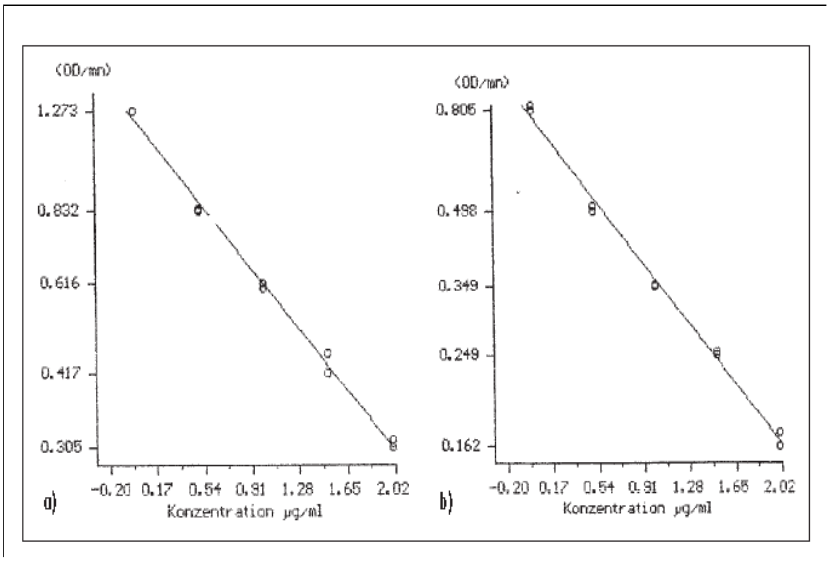


Figure 31c: Calibration curve for fondaparinux for its anti-Fxa activity (biological assay), c= (-2.5839*Log (d) +0.3855), R^2 = 0.996. This assay had a coefficient of variation of <7 % at a limit of detection of 0.01 anti-Xa U/ml.

DISCUSSION

Formulation strategy:

Lipid nanocapsules have shown potential as a nanocarrier for many lipophilic drugs as such as amodiarone, tripentone, paclitaxel and other anticancer drugs**.** In the present chapiter, the encapsulation of a hydrophilic molecule as the anticoagulant pentasaccharide fondaparinux in the core of such a lipid-based system was not simple due to rapid escape of the drug to the aqueous continuous phase of the dispersion. Simple addition of the drug to the Labrafac® , Solutol® and salted water from the beginning of the formulation technique or just before their dilution with cold water to form the LNC according to the previous discussed phase inversion method did not

reveal any encapsulation, even with longer time of stirring or changing the ionic strength of the dispersion, or decreasing the volume of dilution. Such an active hydrophilic principle could not be attached to the surface of the nanocapsules by simply introducing it into the aqueous phase during the first step of initial oil/water preparation or in the stable nanocapsules dispersion obtained after the process. The presence of a nonionic hydrophilic surfactant did not promote the interaction bonds between the water-soluble active principle and the free surface of the nanocapsules .

Other strategies seemed little promising by using a reversed micelle system prepared from Labrafac® and span® 80 that did succeed with a fluorescent dye, but the incorporation ratios were not important. Similar to reversed micelles are the w/o MEs, which seemed worth trying for the encapsulation of the drug and be used as a "precarrier" for F for further incorporation into the LNC.

This strategy of using w/o ME to encapsulate the hydrophilic drug is similar to the w/o/w technique which is well known in the literature for encapsulation of hydrophilic molecules in a LBDDS [343]. For example, few commercial products related to some formulations using nonaqueous MEs, also known as ME pre-concentrate, where the polar solvent is ethanol have been reported, in order to produce peptides- or proteins-loaded SEDDS [362].

Initially, it was essential to establish the phase behavior of the particular combination of chosen components to identify surfactant combinations that would produce a thermodynamically stable formulation. Phase diagrams were utilized in the development of MEs to determine their macroscopic phase behavior and to compare the efficiency of different surfactants used in terms of water incorporation. The goal in the formulation of MEs is to have the lowest possible surfactant content with optimal solubilization of hydrophilic components (in this case F).

The components of this ME were chosen so as to be similar or comparable to the components of the LNC and to favor the formation of w/o ME. The same triglyceride (Labrafac® CC) was chosen as the lipid of choice in the phase mapping because of the solubility profiles and for its accepted use in pharmaceutical preparations. ME containing less than approximately 20 wt.% water is expected to be an oil phase continuous with isolated water droplets, a w/o ME. It is not possible, however, to establish the existence of a ME solely from visual studies. Usually, MEs containing surfactants with HLB values in the range of 3–6 were designated as water in oil (w/o), while those in the 8–18 range were oil in water (o/w) MEs. One should consider HLD, SAD and R ratio in the design of a ME as previously mentioned in the introduction of this chapter.

The type of microemulsion formed as well as the transition from o/w to w/o ME is dependent on the phase ratio of the aqueous and lipid phases, together with the type, concentration, and preferred solubility of the emulsifying agents used. Transition can be accomplished via bicontinuous ME or via the LC phase [354]. The structure and composition of ME are highly interrelated. MEs are single phase systems which are distinguished by thermodynamic stability, translucence, low viscosity and which contain labile microdomains of water and oil separated by a pliable film of surfactant

molecules. Moreover, ME domains constantly fluctuate in size and shape, so that the detected structures are always just pictures of a current situation [349]

In this unique phase, it is the surfactant that enables the coexistence of both the aqueous and oily phase at quasi-molecular sizes by the formation of micelles, if the so-called critical micelle concentration (CMC) is exceeded; ME cannot be considered as dispersions consisting of extremely small droplets as formerly assumed, but rather as percolated or bicontinuous structures in which there is no internal or external phase and no possibility of dilution as in normal emulsions.

Substances that are incompatible with the solvent can be solubilized in the core of micelles. As a result of this process, micelles become swollen and may attain a size of a small droplet, i.e., 1000 Å or 0.1 μm. If another phase is present, e.g., oil if the solvent is water, and provided that the physicochemical formulation is appropriate, the micelles would solubilize large amounts of this phase until they start interacting in a phenomenon called percolation.

Lecithin-based "Precarrier" microemulsions

Since the selection of the surfactants to be used to identify ME regions was the critical criterion , it was given the most attention. Lipoid®, as an important lipophilic surfactant, used in LNC formulation, was chosen as the major surfactant for ME formation. Recently, considerable dosage form development activity has focused on the formulation of lecithin-based MEs [214, 363, 364].They have proven to be desirable in biocompatible formulations due to their tendency to mimic the phospholipids' nature of cell membranes. Furthermore, lecithin is a naturally occurring, non-toxic and safe material to use as biological surfactant[365]. Recent studies have evaluated the applications of lecithin such as rhamnolipid and sophorolipid as biosurfactants in cosmetics and pharmaceuticals [353]. Furthermore, Lipoid®was found to increase the rigidity of the shell of the LNC, hence increase their stability up to 18 month and increase their aptness to freeze drying[57]

Propylene glycol alone or with water as polar phase:

Unfortunately, it was not possible to form MEs from a ternary mixture of Lipoid®, oil and water [366, 367] without the addition of a cosurfacatnt, short chain alcohol. Interestingly, it was found that propylene glycol could possibly be used as a nonaqueous polar phase without water or with small concentration of water to form lecithin-base ME [344]. Glycol as a polar solvent, in most cases, negates the requirement for a cosurfactant in the production of lecithin-based MEs. However, as propylene glycol is unfavorable in large quantities, another study was performed to determine how much water could be added to the propylene glycol and still produce a large clear, one-phase ME region[344]. It was found that 25%water and 75% PG increases the ME region and might increase the solubility of the fondaparinux in the inner core of the w/o ME. The increase in the extent of the LC phase was, however, expected due to the natural

tendency of phosphatidylcholines, the main component of Epikuron® 200, to rearrange themselves into the form of bilayers in the presence of water.

Interestingly, the addition of water to PG did not greatly alter the extent of the cloudy one-phase region. It is well-known that triglyceride oils (such as soybean) are incorporated at only very low levels into phospholipid bilayers [368] so Epikuron® with same concentration of Lipoid® could solubilize more Labrafac® resulting in larger areas of ME.

Using ME, with the two immiscible clear phase system (point A) for the preparation of the first "precarrier "ME, may have been beneficial, if one of the phases is excess oil which facilitates its mixing with the second ME.

Addition of a cosurfactant to the lecithin-based system

The addition of a second surfactant, Tween® 80 [polyoxyethylene (20) sorbitan monooleate], as second surfactant (HLB 15) with Epikuron ®200 primarily in an attempt in an attempt to increase encapsulation of the drug was not successful (**table 15**). It has been reported in some cases that the addition of the micelle-forming nonionic ethoxylated sorbitan ester as second surfactant promotes ME formation in aqueous systems using a phospholipid as the primary surfactant and can reduce the extent of the LC phases formed in the three-component systems in which water is used as the polar phase[354].

Temperature

Heating the mixtures up to 72°C for at least 4 hours was absolutely indispensable to reach the equilibrium in the ternary or pseudo-ternary mixture to give one clear area of ME. The melting point of Lipoid® is 82°C, so it solidifies at room temperature unlike Epikuron containing 90% phosphatidylcholine was found to be easily liquified giving more quick equilibrium resulting in a large clear ME region. The latter could not show any further increase in the incorporation of the drug. Though, contrary to the definition of a spontaneous formation of a ME, time was necessary to obtain a really translucent composition. It also seemed that a higher amount of surfactant led to faster adjustment of the equilibrium of the ME. Furthermore, this precarrier ME should have the same temperature of the second ME before its addition which corresponds to the end of the PIZ just before the dilution steps. Drug degradation was expected to be limited and this was confirmed for some formulations by the biological test. Trials to do the addition step of the precarrier ME in the w/o macroemulsion phase of the second mixture for LNC preparation showed also limited success in terms of both particle size and encapsulation efficiency. Typical LNC are formed when dilution occurs at the bicontinuous phase of the microemulsion [53].

The complete understanding of what happens when both ME are unified is still unclear and needs further investigation to know exactly the phases that might have risen due to their mixture at certain temperature and this would of course explain the

tendency of F to escape at this moment as well as the low repeatability of the formulations with high variation in results.

Impact of the formulation parameter on physicochemical characteristics of LNC

Particle size of the blank LNC is ~50nm (PDI<0.3) according to composition components amounts previously chosen according to the reported data from [73]. Preparation of LNC from two MEs led to slight increase in PS (~60nm, sometimes 100nm) especially with the Lipoid®-based ME system. This might be due to the increase in the oil phase, change in WOR, and disturbance in the bicontinuous phase of the second ME, seen as whitish changes upon the addition step which disappears instantaneously. The PIZ also may have been shifted but the moment of dilution was shifted similarly. In case of Imwitor®- based ME, the two MEs remain transparent upon mixing and there were no further change in the particle size **(Figure 32).**

Sometimes, appearance of a second peak in particle size measurement output, indicating other population which might be a reflection for the disturbance of the system upon addition of the first ME to the second.

The moment of addition is rather important and affects final particle size (PS), ideally at the beginning of the last cycle just before dilution to give nice distribution of PS. Furthermore, it was found that the quicker the quenching, the smaller the time for F to escape and the higher might be the encapsulation rate. The more the concentration of surfactant and cosurfactant, the smaller the PS of LNC, and since oil concentration is only 45% with Imwitor® system thus the PS was found to be smaller.

Furthermore, the F is extremely soluble in water (> 12.5 mg/ml, concentration of F in its commercial form Arixtra®).Therefore, its hydrophilic properties increase its liability to escape and thus explain the low incorporation rate sometimes observed.

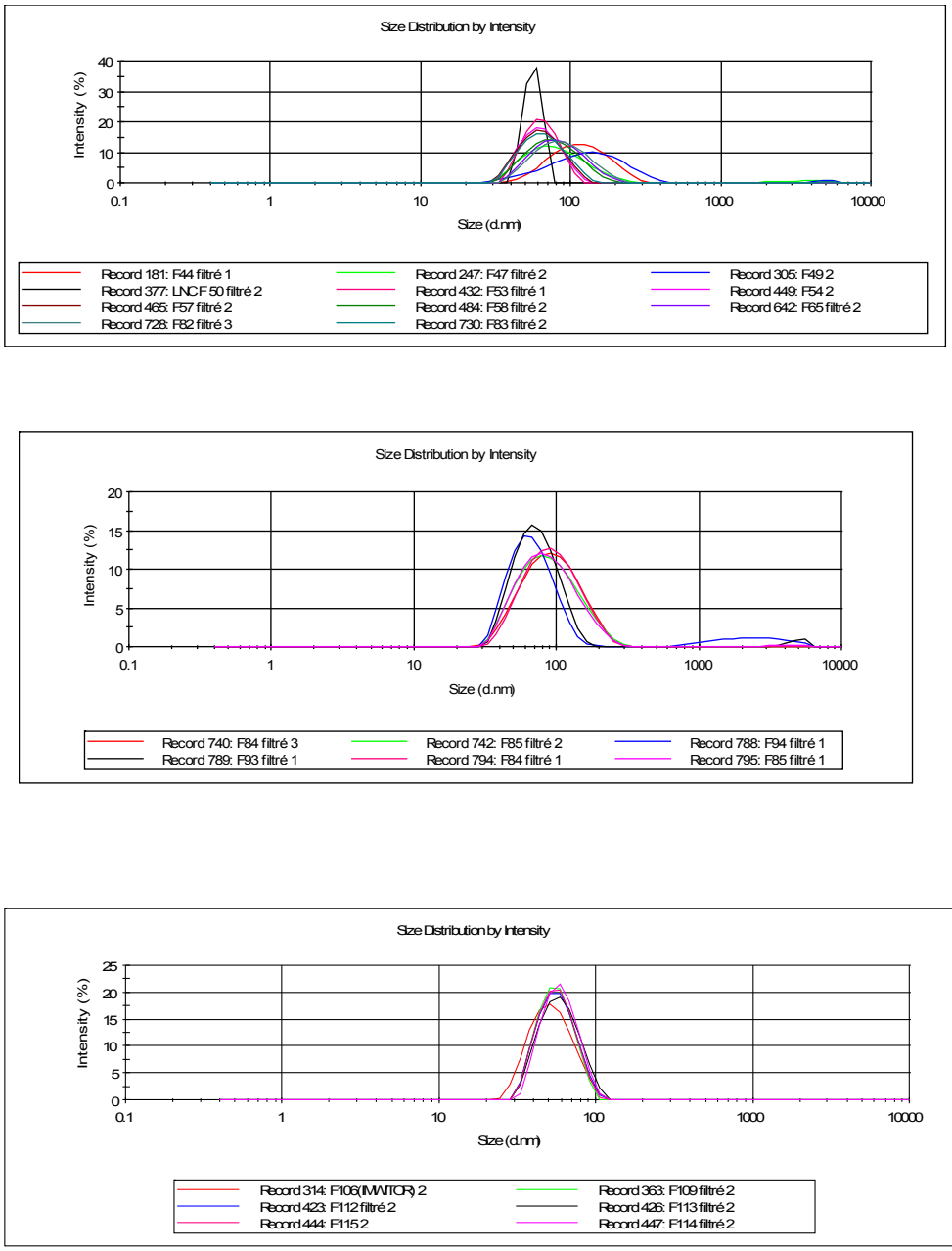


Figure 32: Particle size histograms for different F-LNC from two microemulsions using different ME_1 formulations; A (lecithin-based ME_1 at point A), B(lecithin-based ME_1 at pointB) and W (non lecithin-based ME_1 using Imwitor/Span 60)

Non Lecithin-based "Precarrier" microemulsions

Systems with Imwitor® 308 and Span® 60 as surfactant combination proved to be very stable, incorporating 25–30% of the oil, an intermediate quantity of surfactant and maintaining homogeneity on dilution. The formation of a lamellar liquid crystalline phase in the case of surfactant/cosurfactant blend was prevented by the inclusion of the cosurfactant. Incorporation of Span 60 as a cosurfactant in a system containing Imwitor®380 inhibits the tendency of the cosurfactant- free system to form ordered LC systems and promotes the formation of balanced ME. The necessity of a cosurfactant to stabilize the interfacial film and to introduce the degree of flexibility required for microemulsification [354]. Systems with imwitor® 308 and Span® 60 as surfactant combination proved to be very stable up to 2 weeks period in different temperature, in comparison with lecithin being a surfactant of natural origin, is unstable itself, with oxidation of the acyl chains and hydrolysis of the polar head a problem from a formulation stability point of view[369]. Furthermore, the main benefit in using a second surfactant as cosurfactant derives from the fact that the resultant ME can be diluted without altering or destroying the ME.
In conclusion, lecithin-based MEs were found to be less efficient than the ME with the Imwitor ®/ Span® 60 - surfactant combination in terms of encapsulation efficiency.

Summarizing all these trials, it was found that to obtain LNC with good physicochemical properties, formulation parameters such as the optimum timing and temperature of addition of the precarrier ME or the temperature of quenching as well as the minimum volume of the first ME required to be added to the second ME without its disturbance (white system) should be considered. Last but not least, the speed of the quenching step could further decrease the amount of F to escape to the external continuous phase.

Conductivity:

Electrical conductivity measurements [74]is another aspect of the characterization. It is done following the emulsion inversion and structure by electrical conductivity through the temperature scan. It allows determining the location of the emulsion inversion zone following the conductivity variations according to the temperature. In fact, a conductivity value lower than 10µS cm−1 and essentially zero on the illustrated scales, means that the continuous phase is oil, whereas high steady state reached reflects that a water continuous phase is established [55, 74]**(figure30)**

Through the phase inversion region, the conductivity gradually changes, continuously, linking the two macro-emulsions states. Such a continuous variation of the electrical conductivity suggests the existence of different microemulsion structures. All along the emulsion phase inversion. The hydrophilic/ lipophilic balance of Solutol was probably changed with temperature, as was the case for the ethoxylated surfactant , becoming less hydrophilic on heating. This change took place in the phase inversion zone (PIZ). In this region, the system appeared translucent with blue glints, which is representative of microemulsions. At this moment, in the third cycle descending temperature, the addition of the second w/o microemulsion followed by rapid cooling to obtain the LNC. Sometimes, a conductivity peak was reported in the PIZ,

corresponding to a narrow domain where a liquid crystalline lamellar phase could be present [53].

The encapsulation efficiency :

Determination of the concentration of a drug entrapped in a colloidal delivery system for example liposomes is often complicated. Initially, the unentrapped drug has to be separated from the delivery system entrapping the drug then this is followed by the quantification of the free drug or the entrapped drug after disruption of the nanocarrier. Several methods have been commonly used for the removal of the unencapsulated drugs from these nanocarriers such as:

a) Dialysis
   The suspension of drug loaded nanocarriers is placed into a dialyzing tube and dialyzed against an isotonic solution. Unencapsulated Drug is discharged into the isotonic solution outside the tube.
b) Gel Filtration
   The suspension of drug-loaded nanocarriers is passed through a column filled with a gel filtration carrier under isotonic saline. The nanocarriers will pass through first and unencapsulated drug will be eluted later. The gel has to be selected (e.g. Sephadex G-50 for low molecular weight, Sepharose 4B for high molecular weight) media according to the molecular weight of the drug to be removed.
c) Centrifugation
   Centrifugation is carried out by high rotation (up to 100,000 rpm) with isotonic saline solution. Drug-loaded nanocarriers will settle to the bottom. Unencapsulated drug remains in the supernatant. This is followed by washing.

Many methods have been investigated to separate the free unencapsulated drug from the F-LNC dispersion. Gel filtration using Sephadex® G50, phosphate buffer or water as eluent and azure to measure colorimetrically the free F in all the collected tubes. The LNC were first to appear as shown by measurement of turbidity at 600nm then followed by the free drug which was much diluted. This technique could not be used as a routine assay for the number of formulations to be tested.

Furthermore, dialysis of the LNC was found to be able to pass the free drug and not the LNC which remain inside the bag. The concentration of the drug was again very diluted and measured in the external medium surrounding the bag. This method was found also not to be practical.

The most commonly used method to separate nanocarriers from their mixture, is ultracentrifugation which is often used like with liposomes in very high speed followed by washing several times. As a consequence, unstable nanocarriers can suffer from some destruction or release of the active ingredient or aggregation and larger particle size or even inability to be redispersed.

In this work, the combination of ultracentrifugation with filtration at the same time allowed using lower speeds of centrifugation and hence decreasing possibility of the destruction or the loss of the nanosystems. Although, some LNC could be detected in the filtrate (< 2%, data not shown), possible control with blank nanocapsules allowed

good determination of the free drug in the filtrate. Taking advantage of the complexation of the F with azure and possible precipitation of this complex rendered the assay more accurate by measuring the excess unreacted dye in the filtrate, hence determination of the unencapsulated drug by an indirect way. As control, a physical mixture of the standard solution with the blank LNC was always used.

Stability:

The increase of the particle size within 2-4 months at different storage temperature could be attributed to the absence of the Lipoid® from the shell of the LNC. Although it is used in the precarrier ME but it may not distribute itself on the surface of the nanocapsules. Further interfacial studies, DSC or TEM should be carried out to reveal the distribution of the Lipoid® in the LNC prepared from two MEs.

CONCLUSION

In summary, the lipid nanocapsules formulated according to Heurtault by a phase inversion and temperature cycling process could be loaded in their phase inversion zone with an w/o ME carrying a hydrophilic molecule in its core. This formulation strategy, resembling the well known w/o/w technique for the encapsulation of hydrophilic molecules into the lipid delivery systems, offered great potential for the encapsulation of the antifactor Xa, fondaparinux in the lipid nanocarriers. The produced formulations could serve as parenteral dosage forms for intravenous injection to avoid the first-pass metabolism of the active agent or as oral dosage form for an anticoagulant therapy for long term administration. Furthermore, more application of this strategy for the encapsulation of other water soluble active principle including more important proteins, peptides oligonucleotides and DNA plasmids should be with beneficial therapeutic outcomes.

CHAPTER 2*

FONDAPARINUX LOADED LIPID NANOCAPSULES FOR ORAL ANTI-FACTOR XA EFFECT

*This chapter was presented as a poster at the AAPS meeting, USA in November, 2009

" Oral Fondaparinux : Encapsulation in lipid nanocapsules and In Vivo Absorption Study of the pentasaccharide for oral anticoagulant effect"

Ramadan, F. Lagarce, A.Tessier-Marteau, O.Thomas, P.Legras, L.Macchi , P. Saulnier and JP.Benoit

INTRODUCTION

Therapeutic oral anticoagulation is still commonly achieved by administration of warfarin or other vitamin K antagonists. These have been associated with unwanted side effects due to their narrow therapeutic range and drug or food interaction potential [297, 370].

New synthetic and selective agents have been designed and developed for an improved risk/benefit ratio in oral antithrombotic therapy [299] as well as new strategies for commonly used anticoagulants like unfractionated heparin (UFH) and low molecular weight heparin (LMWH) [371, 372]. Several of these new targeted oral anticoagulants currently in development (oral direct factor Xa inhibitors such as Rivaroxaban and Apixaban and oral direct thrombin inhibitors such as Dabigatran etexilate) have demonstrated at least equivalent efficacy and a similar safety profile to enoxaparin sodium (a LMWH) for the prevention of VTE after orthopedic surgery. However, various challenges remain, such as reducing the incidence of bleeding side effects, thereby increasing the available therapeutic window [297, 309].

Undoughtly, the use of the widely accepted and proven anticoagulants like UFH, LMWH that are still essentially administered only via intravenous IV or SC injection, would be more desirable clinically for effective oral pharmacotherapy of thrombosis [300, 373]. However, they are not efficiently absorbed through the gastrointestinal tract when taken orally and have relatively short half-lives. This low bioavailability, explained by Heparin's high molecular weight and highly negative charge, results from its repulsion from the negatively charged mucosal layer and epithelial cells and its inability to cross membranes due to its hydrophilicity. Furthermore, its gastrointestinal instability has been reported due to its chemical instability in the stomach acidity or possible enzymatic degradation.

The pulmonary, nasal, buccal and other routes have been investigated as delivery options for Heparin therapy, but none result in improved patient compliances and patient quality of life as the oral route [371, 374]. Pegylated cationic liposomes were highly effective in enhancing encapsulation efficiency and pulmonary absorption of LMWH[375]. Furthermore, LMWH could be delivered deeply into the skin by topical application of cationic flexosome for the treatment of superficial thrombosis, subcutaneous wounds, bruises, and burns[358].

Different oral strategies proved to be effective when used and could provide higher reproducible bioavailability **(Table 19)**. Among these, the use of permeation enhancers or protease inhibitors as additives such as surfactants as bile salts [376], chelating agents [377], fatty acids like sodium caprate [371] or proteolytic enzymes such as papain[378, 379] is well reported. The use of polymers of high molecular weight with mucoadhesion properties such as chitosan derivatives or thiolated polymers alone or with glutathone demonstrated a powerful capacity to increase Heparin oral absorption. However, potentially, safety concerns have always been considered [327, 330]. Another successful strategy used is to increase membrane permeability by increasing the lipophilicity of Heparin by Covalent and noncovalent drug modifications [306, 378, 380]. The most promising was a non covalent complex with sodium N-[8-(2-

hydroxybenzoyl)amino] caprylate (SNAC) and decanoate (SNAD) [300, 371] that was extended to clinical studies[381]. Another study based on the synthesis of new heparin derivatives by coupling LMWH with deoxycholic acid (DOCA) mediated also the intestinal absorption of LMWH [382, 383].

Among all the strategies, the use of a particulate delivery carrier system seems to provide high transfer of the drug across the epithelium mucosa and protect against enzymatic degradation. So far, polymeric drug delivery systems based on hydrogels, nanoparticles, microspheres [380, 384, 385], as well as lipid-based drug delivery systems (e.g. microemulsions, liposomes, and solid lipid nanoparticles) have been developed and employed for oral macromolecular drug delivery in general and specifically for UFH or LMWH delivery.

The combination of nanoparticles and biological molecules is of intense interest because of the synergistic properties offered by such newly synthesized composites. Heparin, conjugated to nanomaterials, has recently been investigated for its chemical and biological properties [42, 386]. However, the low incorporation efficiency of hydrophilic drugs, the precise control of drug release and the possible accumulation of non-degradable particles in tissues or the use of unreasonably high quantities of the carrier (even with degradable particles) with toxicity outcomes all represent difficulties that must be overcome to achieve success in carrier delivery systems[327].

Furthermore, it is possible to increase bioavailability to some extent using pharmaceutical technologies, or using micro and nanoparticles being good candidates for incorporation in hard capsules [387]. Many authors used also enteric coating to protect the Heparin from degradation at stomach pH [304, 388] and others confirmed the oral absorption of LMWH from pellets [389] or granules [390] loaded with polycationic polymer.

The role of lipid dosage forms in intestinal absorption was extensively discussed [9, 11, 146, 314] and the lipid-based oral drug delivery system has attracted considerable academic attention [9, 10, 12, 146]. There are several examples of biomolecules used nowadays in therapeutics showing improved oral bioavailability when delivered by means of microemulsions, nanoemulsions, liposomes and lipid microparticles or nanoparticles [11]. A medium-chain glyceride/phosphatidylcholine (3:1) mixture (MCG/PC) increased approximately 10-fold the transport of a low molecular weight heparin through Caco-2 cell layer [378]. Oil in water emulsions (o/w) of Heparin administered orally had the ability to slightly increase the gastro intestinal absorption [391] but their stability in the intestine limited their use. Heparin entrapped in liposomes showed an anticoagulant activity that was surprisingly only 2 to 3 times the activity of a similar dose of Heparin in aqueous solution. However, the lipid-based particles, generally, do not entrap hydrophilic macromolecular drugs with high efficiency. In addition, they have low stability in the GI tract. Conventional liposomes and microemulsions have not met with much success in the mucosal delivery of hydrophilic macromolecular drugs except for fusogenic liposomes [392] or that are coated with a mucoadhesive polymer [393]. Solid particles such as solid lipid nanoparticles seemed to be better than liquid lipid-based particles for oral delivery[146].

TABLE 19: Different strategies for administering heparin and its derivatives via oral route

Strategy	Delivery system	Heparin type	Study	Outcome and mechanism	Reference
Permeation enhancer & enzyme inhibitor	-Surfactant as Labrasol	LMWH	Oral BA (rats &dogs) in situ	↑A by active transport,BA 4 -18,8%	(388, 394, 395)
	-Tetradecylmaltoside (TDM)	LMWH Enoxaparin	Oral BA (rats)	RB 2 - 8%	(396)
	-Dendrones	LMWH	Oral BA (rats)	Cmax↑2 -3 fold, AUC2fold lipophilic ion pair	(306)
	-Bile salts& Chelating agents &Fatty acids like sod caprate	UFH		↑PE	(371, 376, 377)
	-l-arginine	LMWH (ardeparin)	Oral BA (rats)	4 % BA & 13% RB	(397)
	-Proteolytic enzymes such as papain	LMWH	Oral BA (rats)	RB (2,4- 9%),open TJ	(378, 379)
	-Polymers of high Mwt, mucoadhesve such as chitosan der.	LMWH	Oral B(rats)+ transport study	**Bioadhesion**, open TJ, AUC ↑7 times,> control& BA ↑up to 9%	(377, 398, 399)
	-Carbopol	LMWH (opocrin)	Oral BA (rats& pigs)	**Mucoadhesion;** AUC↑ up to 10 times	(400)
	-Thiolated polymers alone or with glutathone	LMWH	Oral BA (rats)	BA 19,9% ,prolonged PE, **mucoadhesion**	(310, 401)
Modifying physicochemical structure (prodrug and lipidization)	-Sodium N-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC) and decanoate (SNAD)	UFH & LMWH	Oral BA (rats & monkeys) -clinical studies	non covalent complex↑lipophilicity ↑ permeability, ↑ in RB	(300, 371, 381, 402).
	-Deoxycholic acid (DOCA)	LMWH	Oral BA (rats & monkeys &mice)	new heparin derivatives (conjugation), ↑lipophilicity ↑ permeability, ↑BA up to 17.6% with	(382, 383, 387, 403-406).

				solubilizers	
Using particulate delivery carrier systems	**-Polymeric** microparticles and nanoparticles with biodegradable and non-biodegradable polymers (PLC, PLGA,ERS)	UFH & LMWH	Oral BA (rabbits)	↑ electrostatic int with mucosa, ↑ contact, ↑BA up to 51%	(373, 380, 383-385, 389, 390, 407)
	-Lipid-based such as liposomes **(IV not oral)**	LMWH	IV BA(rats)	prolonged anticoagulant activity	(408)

(TDM) tetradecylmaltoside is a nonionic surfactant that has been previously shown to be a potent absorption enhancer in studies with peptide drugs, (PLA) polylactic acid, (BA) bioavailability, (RB) relative bioavailability, (A)absorption,(AE) absorption enhancer, TJ tight junction, PE (permeation enhancer),IV intravenous.

Lipid nanocapsules (LNC), recently developed, combine the advantages of colloidal particles and lipid delivery systems. They are prepared by a simple phase inversion technique without using any solvents and are composed of rigid surfactant shell surrounding an oily liquid core [53]. They have shown potential for the delivery of many anticancer drugs, more specifically paclitaxel by different routes mainly parenteral [125]as well as pulmonary and more interesting the oral route [173]. Their gastrointestinal stability [174] and their P-gp inhibiting properties were attributed to Solutol HS15 which is a key excipient in their composition [181]; Both may have facilitated the intracellular uptake of the anticancer drug resulting in three fold increase in its oral bioavailability [173]. Further transport studies on Caco 2 cell culture demonstrated that paclitaxel transport across the intestinal barrier was mainly via active endocytic process [175, 409].

Fondaparinux (Arixtra®) shares all the pharmacological and biological advantages of LMWHs over UFH. However, compared with LMWHs, fondaparinux, an entirely synthetic molecule, has a high affinity to the natural factor Xa inhibitor antithrombin III (ATIII)(7 fold higher than LMWH), thus catalyzing ATIII-mediated inactivation of factor Xa [410, 411]. It is highly standardized and has no antigenic properties. In contrast to enoxaparin, fondaparinux sodium combines a rapid onset of action with a long terminal half-life that permits once-daily administration and results in complete 24-hour coverage [317]. Several clinical trials demonstrated the efficacy of this anticoagulant in various clinical situations [315, 321, 412]. One of its major advantages is the lack of antifactor IIa activity, which has been associated with bleeding in UFH and LMWH in clinical trials. Furthermore, because F does not bind to PF4 and does not interact with platelets, it is used in patients with Hp-induced thrombocytopenia [316] and it was shown to be a more cost-effective antithrombotic agent than enoxaparin in non–ST-elevation acute coronary syndrome [413]. Therefore, F should be an ideal agent for home therapy due to its safety/efficacy profile [414]. Even dabigatran and rivaroxaban , which are new effective oral drugs in patients undergoing major orthopaedic surgery (MOS), it is not clear if they are more effective than F because there are no comparative studies conducted till now [325, 415]. Recently, a study showed that the thrombin generation test (TGT) could be a good option for monitoring F and also for the assessment of the efficacy of reversal agents in patients receiving Arixtra®. Some preliminary data have been reported on the ability of recombinant activated factor VII

(rFVIIa) to reverse the inhibitory effect of F on TG in patients presenting severe bleeding complications [416] (**Table 5, chapiter 1 in part II**).

Since a medical need still remains for a safe and effective oral anticoagulant that is easier than warfarin for physicians and patients to use on a long-term basis, an orally active F would undoubtedly effectively reduce chronic thrombotic events. To achieve such a goal, its delivery using a lipid nanocarrier might be of high benefit outcome. High association efficiency between this bioactive molecule and the lipid nanocapsules will depend on the physicochemical properties of both, as well as the production procedure applied.

On the basis of the above mentioned considerations, **the aim of this chapter** is to combine the advantages of LNCs as oral delivery systems with the benefit of introducing a positive charge on their surfaces to both encapsulate the anionic hydrophilic macromolecule F and to increase electrostatic interaction with the intestinal mucosa in an attempt for the oral delivery of F as an alternative to currently used subcutaneous (sc) delivery. The preparation of F-loaded cLNC and its *in vitro* characterization followed by the assessment of the impact of this delivery system on the pharmacokinetics of the drug after oral administration in rats were investigated.

EXPERIMENTAL

MATERIALS:

- Fondaparinux sodium injectable solution (ARIXTRA® 10mg/0.8ml) was purchased from GlaxoSmithKline (gsk pharmaceutical company), France.
- The lipophilic Labrafac WL 1349 (caprylic-capric acid triglycerides; European Pharmacopeia, IVth, 2002) was kindly provided by Gattefossé S.A. (Saint-Priest, France),
- Lipoïd S75-3 (soybean lecithin at 69% of phosphatidylcholine,) provided by Lipoïd Gmbh (Ludwigshafen, Germany)
- Solutol HS 15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate, European Pharmacopeia, IVth, 2002) provided by BASF (Ludwigshafen, Germany).
- NaCl was obtained from Prolabo (Fontenay-sous-Bois, France).
- Water was obtained from a Milli RO System (Millipore, Paris, France).
- Azure A powder, Triton X100, octadecyl amine (stearylamine) and hexadecyltrimethyl ammonium bromide (CTAB) were purchased from Sigma Aldrich (St Quentin-Fallavier, Yvelines, France).
- Biophen Heparin 6 kit (HYPHEN BioMed, Neuville-sur-Oise, France) and Sta-Fondaparinux Calibrator and Sta-Fondaparinux Control Kits (Diagnostica Stago, Asnières sur Seine, France)
- Pooled normal plasma for dilution (STA®-POOL NORM, DIAGNOSTICA STAGO, France)

The brand names of materials will be used throughout the following chapter

EQUIPMENT:

- Malvern Z sizer® Nano ZS Serie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK) at a fixed angle (173°) at 25 °C furnished by a 4 mW He-Ne_laser at 633 nm.
- Hot plate with magnetic stirrer (IKAMAG Ret control visc, IKA Gmbh & Co KG, 79219 Staufen, Germany)
- Centrifuge (JOUAN, Germany)
- UV-visible spectrophotometer, Uvikon 940, Kontron KG, instruments Montigny Le Bretonneux, France)
- Automatic analyzer for biological assay (STA-R®, STAGO, Asnièressur Seine, France).
- 96-well microplate photometer (Multiskan Ascent-Thermo scientific, USA)

METHODS:

1. PREPARATION OF LIPID NANOCAPSULES

The lipid nanocapsules (LNCs) were prepared by the phase inversion method previously described in chapter 1 of this part [(53)]: Briefly, the emulsion is first prepared by mixing all the components under magnetic stirring at room temperature, with a progressive heating to 85°C followed by a progressive cooling from 85 to 60°C at a rate of 4 °C/min. Three temperature cycles are applied to obtain the inversion process defined by a temperature range. This temperature range depends on the composition of the mixture[(417)]. The temperature of the mixture before dilution was set 1 to 3°C lower than the one corresponding to the beginning of the phase inversion. Then, an irreversible shock is induced by dilution with cold deionised water (2°C) added to the mixture (3.5-fold by dilution). This fast cooling dilution process leads to the formation of stable nanocapsules [(53)]. Afterwards, slow magnetic stirring was applied to the suspension for 5 min followed by filtration (0.2µm syringe filter, Sartorius, Gottingen, Germany) to remove any lipid lumps.

To prepare LNC loaded with F, Cationic surfactants (hexadecyltrimethyl ammonium bromide (CTAB) or stearylamine (SA) were added from the beginning to the other components in different concentrations ranging from 0.125 to 1% w/v of the final volume of the dispersion. The concentration of the cationic surfactant was chosen based on an adsorption experiment and on the targeted final drug concentration; higher cationic surfactant concentrations were used for higher drug load **(Table 20).**

The effect of different concentrations of cationic surfactants (0.125 to 1 % w/v) on the LNC s characteristics was determined by an adsorption experiment in which the following test samples were prepared: (1) 1.5 ml of the different F solutions containing increasing concentrations (0, 10, 20, 25, 50, 100µg/ml) of the drug; (2) various cLNC (CTAB or SA) dialyzed to remove excess surfactants mixtures containing same increasing concentrations of the drug as in (1) and the same volume of cLNC (25µl). In each sample of F-cLNC the particle size and ζ potential were determined. The interactions between F and cLNC were further tested by the colorimetric assay using azure A by mixing a fixed amount of the dye with all the test samples in (1 & 2) and proceed as in the assay section to calculate the encapsulation efficiency (%.). In each case, 300 µl (0.04mg/ml) of azure A dye was mixed with 100 µl of test sample, followed by ultrafiltration and measuring the dye in the filtrate in a 96-well microplate photometer at 580 nm.

Lipoid concentration was slightly variable in all formulations, and its ratio with the cationic surfactants differed being the least with SA (1:1.5). Different volumes of fondaprinux solution (ARIXTRA®) were rapidly injected at the zip region when bluish tinge of the microemulsion appeared (a temperature close to 70°C, at the third cycle) before the final dilution with cold water. The final F concentrations in cationic LNC dispersions (F-cLNCs) corresponded to (100µg/ml to 1000µg/ml). Blank cLNCs with CTAB and SA were also prepared as control.

Table 20: Variation in composition of cationic lipid nanocapsule formulations

Formulation	LNC blank	LF-LNC-CTAB (0.5%w/v)*	Variable composition of other formulations**
Ingredients	**Weight (mg)[V1]**	**Weight in mg[V2] (% w/w)**	**(% w/w)**
Solutol®	846	515 (4.72)	--------
Labrafac®	1028	557 (5.11)	--------
Lipoid®	75	76 (0.69)	0.39, 0.7 for CTAB f.*** 0.45, 0.63, 0.68 for SA f.
Deionized water	2962	1532 (14.04)	-------
NaCL	89	46(0.42)	---------
CTAB or SA	_	50 (0.46)	0.23, 0.45 for CTAB f. 0.23, 0.35, 0.92 for SA f.
Sod bicarbonate*[4]	_	120 (1.10)	----------
Deionized water **for dilution (ml)**	12.5	8.5 (73.35)	----------
F ondaparinux	_	10 (0.09)	0.01, 0.04, 0.18 for CTAB LNC

			Same for SA

*** An example for LNC-CTAB formulation used orally (dose 5mg/kg) for *in vivo* study; LF (low fondaparinux content), 0.5% w/v is the concentration of CTAB in the final dispersion**

****Compared to LF-LNC-CTAB (0.5%w/v), the other formulations of CTAB and SA-LNC only differed in the concentration (%w/w) of CTAB or SA, F and Lipoid®.**

***f. (formulation), [v1] volume of the final dispersion 17.5ml, [v2] volume of the final dispersion 11ml

*[4] The bicarbonate is used only in the formulations used for the in vivo study in rats (it did not affect the physicochemical characteristics of the F-cLNC)

2. IN VITRO CHARACTERIZATION:

1. Particle Size and Zeta Potential Measurements:

The cLNCs were analyzed for their average particle size and the polydispersity index (PI) by photon correlation spectroscopy (PCS) using a Malvern Z sizer® at a fixed angle (173°) at 25 °C furnished by a 4 mW He-Ne_laser at 633 nm. The polydispersity index was used as a measurement of the size distribution. A small value of PI (< 0.1) indicates a unimodale size distribution, while a PI > 0.3 indicates a higher heterogeneity. A 1:60 v/v dilution of the nanoparticle suspensions in deionised water was achieved in order to enable measurements and to ensure a convenient scattered intensity on the detector.

PCS data is presented as the mean (volume) intensity–particle size distribution, and standard deviation of the mean of particle size measurements performed in triplicate was calculated. The samples were kept in polystyrene cuvettes.
The zeta (ζ) potential and the electrophoretic mobility measurements of the different nanoparticles were performed using the same Malvern instrument, equipped with an AZ-4 cell and were based on the laser Doppler effect. ζ potential measurements were made in water at 25 °C, with dielectric constant of 79, refractive index of 1.33, viscosity of 0.89 cP, cell voltage 150 V and current of 5 mA. Nanocapsule suspension was diluted in deionized water as for size measurement considering that NaCl was present in the formulation of the nanocapsules. Data analysis of ζ potential is presented as mean and standard deviation of triplicate runs.

2. Drug Encapsulation Efficiency and Drug Payload:

The amount of F unentrapped within the LNC was determined with a modified Azure A colorimetric method [357]. The amount of free dye in the blue external aqueous solution was indirectly proportional to the amount of unentrapped drug. Typically, aliquots (500 µl) of LNC samples were reacted with 1.5 ml of the Azure A solution (0.04 mg/ml) at room temperature followed by ultrafiltration at 4000 rpm for 30 minutes using Millipore® Centricon YM-100 filters (Millipore ,USA) to separate the purple complex of azure with free F as well as the F-cLNC . The blue excess dye solution in the filtrate was then measured in triplicate at 633 nm by UV spectroscopy in reference to a standard calibration performed with different concentrations of F solution and empty cLNC dispersions. The drug entrapment efficiency was expressed as the percentage of F entrapped with respect to the total drug content.
The encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency } \% = C_{total} - C_{unentrapped} C_{total} \times 100$$

where C unentrapped is the concentration of F free in a volume of cLNC suspension (µg/ml) and C total is the total concentration of the F in the same volume of cLNC suspension (µg/ml) either calculated based on concentration of F in the final dispersion (total F added (µg)/final volume of dispersion(ml)) or determined by the Triton method after disruption of the nanocapsules completely and release of the encapsulated drug[418].
Mean drug payload (milligrams of drug per gram of cLNC dispersion) was also calculated as follows:

$$\textbf{Drug payload (mg/g)} = \text{mg total} - \text{mg unentrapped} \text{Dry weight of the cLNC dispersion (g)} \times 100$$

Prior to using the indirect technique for routine assay purposes, it was verified the results were correlated with the biological assay of the drug to prove that encapsulation in cLNCs did not affect its anticoagulant effect.

3. *In Vitro* Release Study and The Stability Of cLNC in Different Release Media:

Fondaprinux release from two formulations prepared in deionized water (LF-LNC-CTAB (0.5%w/v) and (LF-LNC-SA (0.375%w/v)) was performed using the dialysis bag method in deionized water (pH 6.5). The dialysis bag retains LNC and allows the free drug into the dissolution media with a cut-off of 100.000K. The bags containing 3ml of F-cLNC dispersion or F solution (250µg/ml) and cLNC blank dispersion as well as physical mixture of F solution with blank cLNC were placed in 75ml receiving phase (deionized water). The conical flasks were shaked at 37 °C at a rate of 125 times per min. The concentration of released F was analyzed by the azure UV-Vis method previously mentioned at predetermined time intervals (1, 2, 4, 6, 24, 48 h) using the medium of blank cLNC dispersion as reference in the calibration curve. All the operations were carried out in triplicate. The percent of drug release (Q) was calculated from the cumulative drug concentration calculated at predetermined time intervals in the dissolution medium versus the total drug concentration (if all released from the bags).

In order to investigate the stability of cLNC in deionised water and in GI fluid, the cLNC formed with CTAB (LF-LNC-CTAB (0.5%w/v) or SA (LF-LNC-SA (0.99%w/v) were diluted with deionized water (pH 6), or simulated gastric fluid (*SGF*, 0.32% (w/v) of pepsin; pH 1.2 (USP XXIV, 2006) or simulated intestinal fluid *(FaSSIF-V2* (fasted state, pH 6.5) and *FeSSIF-V2* (fed state, pH 5.8) fluids*)*[174, 419] in the volume ratio of 1:4 and incubated at 37°C and 125rpm for different time intervals 3 to 24 hrs. Separate tubes were used for each data point. Background readings were obtained using the supernatants from the blank LNC. Procedures for the assessment of the amount of F in the supernatant were the same as those described above using azure colorimetric method. The integrity of the cLNC at predetermined time intervals (2, 4, 6, 24, 48 h) for water samples and for different pH media was monitored by dynamic light scattering. Procedures for the assessment of the amount of F in the supernatant were the same as those described above using azure colorimetric method at (2, 4, 6, 24h) for water samples and (45, 90 and 150 min) for SIFs.

4. *In Vitro* Transport Study in CACO-2-Cells

The human colon adenocarcinoma cell line (Caco-2) were used as the transport model since they express bile acid transporters[383]. These were obtained from the American Type Culture Collection (Manassas, USA) and Passage (27). Cells were cultured in Dulbecco's modified Eagle medium (D-MEM, high glucose) supplemented with 15% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and 1% antibiotic solution (1×104UI/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphothericin B) in a humidified incubator 5% CO2/95% air atmosphere at 37 °C [176]. Cells were plated on 75 cm^2 flasks at a density of 1.2×10^6 cells/flask and were then harvested at 80% confluence with trypsin–EDTA and seeded onto polycarbonate membrane filters (0.4 µm pore size, 1.12 cm^2 growth area) inside

Transwell® cell culture chambers (Corning Costar, Cambridge, MA) at a density of 0.1×10^6 cells/insert. The culture medium (0.5 ml per insert and 1.5 ml per well) was replaced every two days for the first two weeks and everyday thereafter. After 22 days in culture, cell monolayers were used for the following assays.

Before experiments, Cell monolayer integrity was evaluated by monitoring transepithelial electrical resistance (TEER). Cell monolayers were washed twice with Hank's buffered salt solution (HBSS) for 15 min at 37 °C. The TEER of monolayers was checked before and after each experiment by using a Millicell®-ER system (Millipore Corporation, Bedford, MA). Only cell monolayers with TEER values over 250 Ω cm2 were used. Then, atenolol and propanolol transports were evaluated for 2h at 37 °C to control paracellular and transcellular transport: $20\times0.5.10^6$cm S^{-1} and $1.0\times0.2.10^6$cm S^{-1} for apparent permeability coefficient of propanolol and atenolol were accepted [176].

The transports of different formulations of fondaprinux (F): fondaparinux (F) solution, and F-loaded LNCs (HF-CTAB-LNC, LF-SA-LNC and HF-SA-LNC) were studied in the apical to basolateral direction on Caco-2 cells. The test solutions were diluted in HBSS at 5.714 x10 ^{3} µM F (final concentration from all solutions=10µg/ml after suitable dilutions as 1µM=1750x10 ^{6} µg/ml) concentrations. The experiment was started by adding 0.5 ml of test solution at the apical side and 1.5 ml of HBSS at the basolateral side. Then inserts were incubated at 37 °C. After 2 h of incubation, samples were taken from apical and basolateral sides and F content was determined indirectly using anti-FXa activity chromogenic assay. Control experiment was performed with HBSS for first incubation of cells.

3. IN VIVO EXPERIMENTS

Experiments were carried out according to the French legislation on animal experiments. Male Wistar rats (male, 310–340g) were fasted for 12 h. The rats were anesthetized with isoflurane (0.5%). The rats then typically received one of the following treatments in (4) groups: Of these groups, two groups received F in isotonic solution by IV bolus injection via the tail vein or SC (dorsal) (200µg/kg), remaining groups orally received F unloaded and F-loaded cLNCs suspensions as a single oral dose with different F concentrations. Oral administration was through an oral gavage that was carefully passed down the esophagus into the stomach using a stainless steel catheter with a blunt end to avoid causing lesions on the tissue surface. The F control solution and F-loaded cLNCs suspensions were prepared in a sodium bicarbonate buffer (1.5g/100ml, pH 8.2) so as to neutralize the gastric acidity[406]. The total orally administered volume of the F-loaded cLNCs suspensions was 1.5 to 2.5 ml. The dose amount was varied at 0.5, 2, 5, and 10 mg/kg. There were 5 rats in each group at the beginning of the study, some died during the study due to sampling procedure (n final=3-5). Blood (950 µl) was collected serially by capillary intracardiac sampling each time into pediatric citrated tubes containing a constant volume of sodium citrate (0.109M) (**Figure 33)**. Blood samples were started at 2 minutes in case of IV or SC injection and after 15 minutes in case of oral administration followed by sampling at 30

min, 1hr, 2, 4, 8, and 24hrs. Plasma was collected after centrifugation (4000 rpm, 15 min, 4°C) and stored at -20°C until analysis utilizing the chromogenic assay.

1. ***Measurement of Anti-factor Xa Activity (biological assay)***

F concentration in citrated plasma was determined by an anti-Xa chromogenic assay using the Biophen Heparin 6 kit using an automatic analyzer. The assay was performed according to the protocol supplied by the manufacturer. The principle of this assay is based on the fact that the anticoagulant effect of F is achieved via its ability to bind antithrombin and potentialize its natural inhibitory activity for coagulant serine esterases as factor Xa. In fact, this assay uses a pharmacodynamic response to estimate the absorption and bioavailability of F and thus monitor its therapeutic efficacy. Biophen Heparin 6 is a kinetic method based on the inhibition of a constant amount of factor Xa by the tested anticoagulant in presence of endogenous antithrombin, and hydrolysis of a factor Xa specific chromogenic substrate (SXa-11) by the factor Xa in excess, thus liberating the chromophoric group, paranitroaniline (pNA). The amount of pNA released is then a relation of the residual factor Xa activity. There is an inverse relationship between the concentration of F and color development measured at 405 nm[317, 399]. In case of *in vivo* plasma samples, they were diluted with human normal plasma at F concentrations above 0.7 µg/ml.

The anti-Xa activity was measured from the calibration curve of absorbance (A/min) vsF standard concentrations (µg/ml) with Sta-Fondaparinux Calibrator and Sta-Fondaparinux Control Kits according to the protocol supplied by the manufacturer

$$[\text{AT-fonda}] + \text{Xa xss} \rightarrow [\text{AT-fonda-Xa}] + \text{Xa residual}$$

$$\text{Xa residual} + \text{substrate} \rightarrow \text{pNA}$$

2. ***Determination of pharmacokinetic parameters***

Noncompartmental analysis (EquivTest/PK) Statistical Solutions Ltd, was performed for F absorption profiles. The following pharmacokinetic parameters were derived: peak concentration (Cmax); time to reach the peak concentration (tmax); area under the concentration-time curve. The area under the plasma concentration versus time curve ($AUC_{0\rightarrow\infty}$) was calculated by the trapezoidal method. The absolute bioavailability (F) was estimated by comparing the $AUC_{0\rightarrow\infty}$ for F after oral delivery with that of intravenously administered F. Calculations were made as follows:

$$AUC_{0\rightarrow\infty} = AUC_{0\rightarrow 24h} + C_{24h}/\lambda_z$$

where λ_z is the estimated elimination rate constant

For the estimation of the elimination rate λ_z, a point from which the slope is to be calculated is specified – this can be different for each subject.

AUMC0→∞ (Area Under the Moment Curve) calculates the area under the moment curve, i.e. under the curve for (concentration*time) versus time using the linear trapezoidal rule.

$$AUMC_{0\to\infty} = AUMC_{0\to 24h} + (T_{24h}.C_{24h}/\lambda_z) + (C_{24h}/\lambda_z^2)$$

The mean residence time in 24h ($MRT_{0\text{-}24h}$) and at infinity ($MRT_{0\text{-}\infty}$) was determined as follows :

$$MRT_{0\text{-}24h} = AUMC_{0\to 24h} / AUC_{0\to 24h}$$

$$MRT_{0\text{-}\infty} = AUMC_{0\to\infty} / AUC_{0\to\infty}$$

Half Life – during the elimination phase, the elimination half-life ($t_{1/2}$) is often studied. The plasma elimination half-life is the time taken for the plasma concentration to fall by one half.

$$t_{1/2} = \frac{\ln(2)}{\lambda_k}$$

The plasma clearance (Cl) and the apparent volume of distribution at steady state (V_{ss}) were calculating using the IV data set :

$$Cl = Dose / AUC_{0\text{-}\infty}$$

$$V_{ss} = (Dose \,.\, MRT_{0\text{-}\infty}) / AUC_{0\text{-}\infty}$$

The terminal elimination half-life ($t_{1/2}$) observed for F following IV administration, was calculated using the slope from linear regression analysis of log-transformed concentration values from the last three time points.

The absolute bioavailability BA (%) of oral F was calculated as the ratio of the AUC after oral and after IV administration with a correction for the difference in dose :

$$F = \frac{AUC_{0\text{-}\infty,oral}}{AUC_{0\text{-}\infty,IV}} \times \frac{Dose_{IV}}{Dose_{oral}} \times 100$$

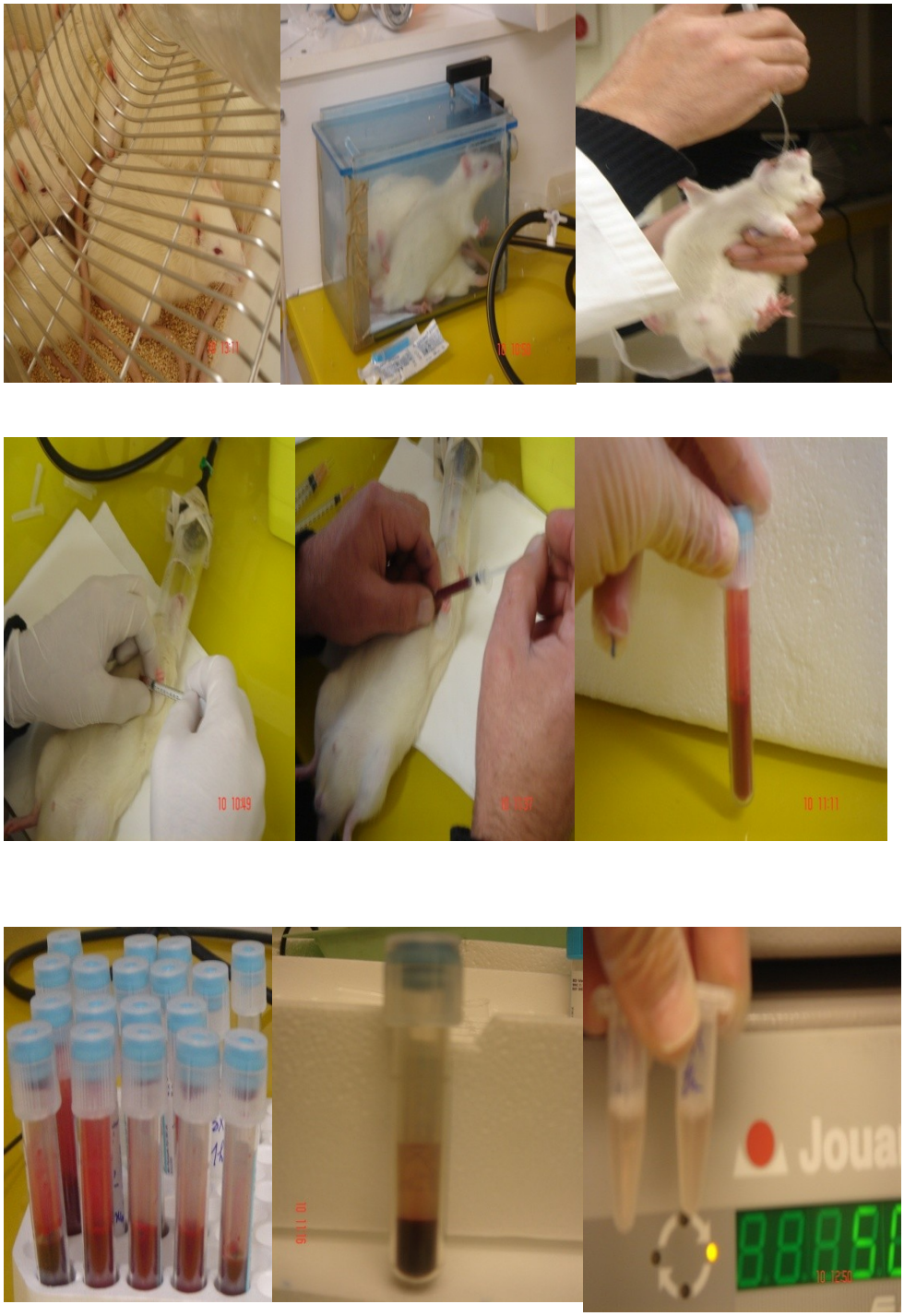


Figure 33 **:** Steps for the *in vivo* study: animal anesthesia (1-2), oral gavage(3), intracardiac sampling (4-7), plasma separation (8-9)prior to analysis of samples using the anti Xa biological assay.

4. STATISTICAL ANALYSIS

All studies were repeated a minimum of three times and parameters measured at least in triplicate. Results were reported as means ± SD (standard deviation). Statistical significance was analyzed using the Student's t-test or Anova (Excel). Differences between experimental groups were considered significant when the P-value was less than 0.05 ($P < 0.05$).

RESULTS

1. *IN VITRO* CHARACTERIZATION

1. Particle Size, Zeta Potential And Percent Encapsulation Efficiency:

The mean diameter of F-loaded cLNCs prepared with different cationic surfactant ranged from 45.2 ± 2.4 nm to 60.1± 2.3 nm with a mean 53.2±8.9 nm and 47.0±0.8 nm for F-LNC-CTAB and F- LNC-SA whereas that for the empty cLNC was 45.6 ± 2.4 nm. A narrow index of polydispersity (PDI) was shown for all the formulations (< 0.3) **(Table 21a&b)**. The particle size increased with the increase in drug load showing the highest (60.1±2.3 nm) in case of HF-LNC-CTAB (0.5%w/v) but remained unchanged with F-SA-LNCs. CTAB concentration range was (0.12-0.36 %w/w) of total LNC weight, higher CTAB or SA concentrations were needed to encapsulate higher drug concentration as shown in the table and was more obvious with SA.

The overall charge of the cationic LNCs was positive as shown from ζ potential data ranging from (3.9±0.4 to 42.9±0.5mv) showing the highest positive value with LF-LNC-CTAB (0.5%w/v) and the lowest with SA formulations.

For the determination of the concentration of F by azure assay, the calibration curves were linear with a negative slope over the concentration range of 3-15 µg/ml (R^2 =0.9989 ± 0.0057, n = 4). The error of determination did not exceed 0.06, the limit of detection was 3µg/ml for F. Interday precisions of the method were less than 20%, and accuracies were over the range of 86.05–113.94%.

For the biological assay using standard kit, the calibration curves with a negative slope over the concentration range of (0-0.125-0.25-0.5-1-2 µg/ml) (R2 =0.975). This assay had a coefficient of variation of <7% at a limit of detection of 0.01 anti-Xa U/ml.

Entrapment efficiency (% EE) for the anionic drug was high for all cLNCs ranging from 80% to 100% with a mean of 93.5%±6.0 and the highest values (100%) with LF-LNC-CTAB (0.25%w/v and 0.5%w/v). The mean drug payload (W) was (7.9±5.5 mg/g LNC) and (5.5±2.2 mg/g LNC) for CTAB-LNC and SA-LNC formulations respectively.

The concentrations of F used in the adsorption experiment were all adsorbed on the surface of the cationic LNC as shown in **Figure 34a** (no free drug to react with Azure), indicating the strong electrostatic interaction between the anionic drug and the cationic surface of the LNC.

Azure A is a positively charged dye which forms a purple colored complex with the negatively charged functional groups (sulphate) of F. The azure A assay quantitatively measures unreacted (free) functional groups of F and can be used as a measurement of free F. No change in absorbance was observed when an increasing concentration of F was added to a solution containing a fixed amount of azure A and a fixed amount of CTAB-LNC or SA-LNC dispersions **(Figure 34a)**.

Table 21a: The *in vitro* characteristics of CTAB- LNC formulations

Formulation	Conc** F (µg/ml)	Psize± SD (nm)	PDI± SD	Zeta ± SD (mV)	F Content by Triton method (µg/ml)	%EE ± SD*** (%)	Drug payload [4*] (mg/g)
LNC (blank)	0	49.78± 2 .63	0.12±0.01	-1.24±1.08			
LNC-CTAB (0.25%w/v)	0	45.21 ± 2.40	0.09± 0.01	33.02 ± 2.12			
LF-LNC-CTAB (0.25%w/v)	95	54.99± 2.48	0.16 ± 0.01	30.26±1.28	90.94±4.72	100 ±0	0.68
HF-LNC-CTAB* (0.25%w/v)	400	58.04 ± 0.64	0.08 ± 0.01	33.2 ±2.26	397.62 ± 20.90	98.18 ± 1.89	2.80
LF-LNC-CTAB (0.5%w/v)	900	58.17± 1.44	0.14± 0.00	42.86.±493	916.29±56.75	100 ±0	7.20
HF-LNC-CTAB (0.5%w/v)	1666	60.06± 2.33	0.22± 0.01	18.2 ±1.05	1517.74±74.2	96.48±0.44	13.84

*HF (high fondaparinux load in the formulation)
**The concentration of F (encapsulated) in the final dispersion based on theoretical calculations from the initial F concentration in the final volume of the dispersion
***EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6)
[4]*the drug payload was calculated for each formula from weight of the drug added to the total dry weight of the ingredients.

Table 21b: The *in vitro* characteristics of SA- LNC formulations

Formulation	Conc.** F (µg/ml)	Psize± SD (nm)	PDI± SD	Zeta ± SD (mV)	Content by Triton method (µg/ml)	%EE *** ± SD (%)	Drug payload[4*] (mg/g)
LNC-SA (0.25%w/v)	0	46.21±2.4	0.07±0.02	11.91±1.63			
F-LNC-SA (0.25%w/v)	95	46.54±2.06	0.12± 0.00	12.47±2.12	95	90 ±0. 01	0.67
F-LNC-SA (0.375%w/v)	400	46.75±0.62	0.06±0.03	11.42±1.21	395 ±6.85	95.59 ± 0.158	3.06
LF-LNC-SA (0.99%w/v)	900	48.11±2.4	0.15±0.01	3.95±0.36	912.56 ±34.65	80 .2± 0. 487	5.15
HF-LNC-SA*4 (0.83%w/v)	1500 (1100)	73.93±3.73 (47.54±2.09)	0.36±0.13 (0.06±0.13)	3.57±0.65 (6.33±1.39)	ND	96.48±0.45 (91%)	8.48 (5.43)

*This formulation, initially unstable, of high SA to Lipoid® ratio (2.6:1) was used *in vivo* (for an oral dose of 7mg/kg dose) , then further improved (values between brackets) using lower SA: Lipoid® ratio (1:1.5).
**The concentration of F (encapsulated) in the final dispersion based on theoretical calculations from the initial F concentration in the final volume of the dispersion
***EE% calculation was based on free drug determined by azure method and on theoretical total drug content (appearing in column 6)
4*the drug payload was calculated for each formula from weight of the drug added to the total dry weight of the ingredients.

This suggested that SA or CTAB interacts with all the negatively charged functional groups of F leaving no free drug available to interact with the dye in the concentration used in this experiment. On the other hand, with blank LNC, increasing concentration of F resulted in a decrease in the amount of free dye shown in the decrease in A at 633nm. These data also substantiate the data on particle size and ζ potential.

It is shown in **Figure 34b** that with the same formulation ingredients in CTAB-LNC or SA-LNC dispersions, a slight increase in the particle size of LNC upon the increase in F concentration was noted, the effect being more in case of CTAB-LNC. Furthermore, the ζ potential values were more positive in case of CTAB-LNC (+32 mV to +48mV) than in SA-LNC (+26mV to +29mV) although using the same concentration of cationic surfactants. ζ potential values decreased from +48 mv to +37mv upon increasing F concentration to 5µg/ml with the CTAB-LNC formulations. This effect could not be observed in case of SA formulations.

The stability of some cationic LNCs dispersion over a period of 120 days in storage at 4 °C or at 25°C is shown in **Table 22**. The CTAB formulations were more stable than the SA formulations in particle size, PDI, ζ potential and no release of drug was detected after 2 month storage at 4°C. The need for higher SA concentration with higher drug loading resulted in higher PDI value and unstable formulations.

Figure 34a: Determination of the free drug indirectly after adsorption experiment by azure method at 633nm in blank LNC (A), CTAB-LNC (A CTAB) and SA-LNC(A SA)

Figure 34a: Physicochemical characterization of different cLNCs formulations after adsorption experiment using fixed amount of CTAB or SA-LNC with increasing concentration of F.

Table 22 : Stability data for some CTAB and SA-LNC formulations up to 4 months at different storage temperature (4°C and 25°C)

Formulation	Storage time (months) and Temp (°C)	Psize ± SD (nm)	PDI ± SD	Zeta ± SD (mV)	Content by Triton method (µg/ml)	% EE (%)
LF-LNC-CTAB (0. 5%w/v)	Fresh	51.90 ± 2.4	0.12	44.44 ± 1.72	800	100
	3 m - 25°C	72.70 (99.6%)	0.15	**ND**	**ND**	**ND**
	2 m - 4°C	68.82	0.12	46.12	800	100
	4 m - 4°C	72.71(99.6%)	0.15	**ND**	**ND**	**ND**
LF-LNC-SA (0.25%w/v)	Fresh	48.61±1.5	0.04	13.73±1.45	250	88
	3 m - 25°C	65.51 (94%)	0.55	**ND**	**ND**	**ND**
	2 m - 4°C	56.21 (94.4%)	0.27	**ND**	**ND**	**ND**
	4 m - 4°C	60.42 (93%)	0.31	**ND**	**ND**	**ND**
LF-LNC-SA (0.75%w/v)	Fresh	45.24± 1.4	0.22	9.39	730	83
	2 m - 4°C	42.62 (42%)	0.93 ↑	3 .21	730	76.5

m (months), ND (not determined)

2. *In Vitro Release and Stability:*

In vitro release study was carried out at 37°C in 75 ml deionized water using a dialysis method. The high solubility of the drug in water (>12,500µg/ml, ARIXTRA® injection) is much higher than the concentration of the drug in the release medium after 100% release (10µg/ml) which ensures sink conditions. Furthermore , it was found that 78% of the drug was released after 48 hours , when the drug aqueous solution was filled in the dialysis bags with a with a cut-off of 100.000K, compared to 19% and 10% only with other dialysis membranes with cut off (50.000 and 15.000 respectively). The release of F from the cLNC under sink conditions is shown in **Figure 35a**. A biphasic release pattern with ≈ 7% burst release in the first hour was observed for SA-LNC. The burst effect corresponds to the dissolution of free (unentrapped) drug calculated by ultracentrifugation azure methods. The extent of drug released in 48 hours was about 18% compared to 10% only from CTAB formulations with no burst effect reflecting high association efficiency of the drug to the surface of the CTAB-LNC.

These results corresponded with of results ultracentrifugation method for studying the stability of F in deionized water showing less than 5% drug released in 24 hours from SA-LNC and less than 1% from CTAB formulation. Using the same method for further studying the stability of F following incubation in an updated simulated intestinal fluid in the fasted state (FaSSIF-V2) and in updated simulated intestinal fluid in a fed state (FeSSIF-V2) in comparsion with water for CTAB-LNC formula and SA-LNC did not give reproducible and reliable results due to interaction of the media components such as electrolytes and interference with the azure assay[420]. Furthermore, a bathochromic shift was well detected in acidic medium.

cLNC were stable regarding particle size and ζ potential after 48 hours in acidic medium, but in the presence of enzymes, measurements were not applicable. cLNCs become less positive in alkaline buffer medium and slightly larger in size with CTAB-LNC; this effect was not time dependent as seen in **(Figure 35b&c)**.

3. *Caco 2 Cell Transport Results:*

No TEER reduction confirmed absence of paracellular transport and tight junction opening except in case of highly concentrated formulation (HF-SA-LNC) where TEER values dropped to <200 indicating cell death.

Apparent permeability coefficient (Papp) could not be determined as no F was detected after biological analysis of the drug in the basolateral side. However, the concentration of F in the apical side was slightly reduced. Small drug concentrations, drug degradation or the unsuitability of the medium for biological test could be contributing factors. For all formulations, the transported amounts of drug were below 1% of the total initial amount applied to the apical side of the cell culture.

Figure 35a:*In vitro* release study of fondaparinux from cationic LNC in deionized water at 37°C using dialysis bags technique.

Figure 35b : Stability of the particle size of CTAB and SA-LNC at different pH for 48hrs

Figure 35c: Stability of the zeta potential of CTAB and SA-LNC at different pH for 48hrs

2. *IN VIVO* STUDY

Pharmacokinetics of F-loaded lipid nanocapsules:

Fondaparinux plasma concentrations in rats after intravenous administration of 200µg/kg dose is shown in **Figure 36**. The mean pharmacokinetic (PK) parameters analysed by the noncompartmental analysis using the **EquivTest/PK software** corresponding to all the different oral F-loaded LNC in comparison to the IV dose are shown in **Table 23** and the plasma concentration –time profiles for the different CTAB-LNC and SA-LNC oral formulations are plotted in **Figures 37a&b.**

It should be noted that the anti Xa activity of F can be expressed on a gravimetric basis (µg/ml or µmol/l) and not in international units like Heparin and LMWH [322] because fondaparinux is a synthetic molecule and each mole corresponds to its antifactor Xa unit activity [373]

A SC injection of a dose equal to the IV dose (200µg/Kg) resulted in an anti-FXa effect (0.836µg/ml) within 60 min. Formulations with CTAB-LNC had faster onset of action (30 min) and on the contrary, SA-LNC showed a much slower rate (Tmax 90 -210 min, $P<0.05$) and the anticoagulant effect lasted up to 16 hours (**Table 23**).

The oral administration of F alone (0.5, 2, 5 mg/kg) did not affect the anti-factor Xa level in rat plasma; all failed to attain the therapeutic anti-FXa levels in rat (0.2 IU/ml). Oral administration of F-loaded cLNCs at a 2 mg/kg dose produced a slight increase in the peak plasma concentration (0.18 µg/ml)) with both formulations and reached higher values with higher dose (5mg/kg) up to 0.98± 0.33 µg/ml with LNC-CTAB and more significantly with LNC-SA (1.31±0.37 µg/ml, $P<0.05$). Further increase in the doses up to 7mg/kg with LNC-SA and 10mg/kg with LNC-CTAB resulted in higher Cmax (1.8 & 1.6 µg/ml respectively, $P<0.002$).

The area under anti-factor Xa activity in rat plasma versus time curve ($AUC_{0-\infty h}$) in the dose of 2 mg/kg with CTAB-LNCs and SA-LNCs formula was 78.7µg.min /ml and 42.5 µg.min /ml respectively. Lower dose (0.5mg/kg) did not affect $AUC_{0-\infty h}$ but was significantly higher with the higher doses especially with SA-LNC (5mg/kg) (730.89±214.8 µg.min /ml, $p<0.05$) compared to 138.21 µg. min /ml for the IV dose (200µg/kg). This increase in AUC corresponds to 4 and 5 fold increase **(Figure 38a)** and up to 19 fold increase with SA-LNC 7 mg/ml (n=3, SEM>50%). The increase in the AUC_{tot} as well as the increase in plasma anti-FXa level after oral administration of F-loaded SA and CTAB-LNC different formulations was linear with the increase in dose, being with higher slope with SA formulations (**Figure 38b).**

The absolute bioavailability of orally administered F loaded in LNC at different doses ranged from 3 % to 21% and up to 57.17 % (n=2), compared to the iv administration, but in a dose that is 10 times to 50 times more than the iv dose, with a prolongation of the anticoagulant effect up to ~3 hrs (t_{half}) and even up to 21 hours (SA-

LNC 5 and 7mg/kg respectively) compared to 70.49 min after IV administration (**Figure 37b)**.

Table23: Pharmacokinetic parameters (±SD) following oral administration of F loaded CTAB-LNC and SA-LNC in comparison with a bolus IV injection of 200µg/kg

Study	Cmax	Tmax (median)	AUC_{tot}*	t_{half}	MRT	Clearance	Absolute bioavailability** (n***)
Unit	µg/ml	min	µg.min /ml	min	min	µl/min	%
Per os							
LNC SA 2mg/kg	0.18±0.96	90	42.75±28.47	204.13±149.41	239.80±112.41	465.94±5.85	3.09±2.044(4)
LNC SA 5mg/kg	1.31±0.37	210	730.89±214.89	188.54±9.69	338.44±69.44	485.86±13.76	**21.18±6.210(4)**
LNC CTAB 2 mg/kg	0.18±0.10	60	78.72±74.41	248.25±165.67	349.23±210.21	467.63±10.17	5.70±5.40(3)
LNC CTAB 5mg/kg	0.98±0.33	30	336.48±187.32	177.10±82.62	255.53±124.22	450.72±7.09	9.75±5.44(4)
Control oral F solution 5mg/kg	0.06±0.02	120	48.70± 37.16	51.7±36.43	81.39±58.69	455.43±14.51	1.41±1.07(3)
IV F solution 200µg/Kg	**1.42±0.12**	**2**	**138.21±46.32**	**70.49±17.63**	**108.17±26.07**	**490.52± 147.46**	
SC F solution 200µg/kg	0.836±0.19	60	154.01±123.92	88.46±83.94	232.21±78.66		115.84(4)

*AUC_{tot}= $AUC_{0\rightarrow\infty}$
**the bioavailability was calculated from the dose corrected areas under the curves for oral versus IV administration (EquivTest/PK software)
***n =3-5 (the final number of surviving animals)

Figure 36: Antifactor XA activity in plasma-time profiles of fondaparinux in rats after IV bolus injection of F (200µg/Kg, n=3-5)

*MEC: Minimum effective therapeutic concentration

Figure 37 (a&b): Anti-factor Xa activity in plasma-time profiles of fondaparinux in rats after oral administration of LNCs formulations (a=CTAB-LNCs, b=SA-LNCs). Data are shown as the mean concentration, and error bars represent the SEM (n= 3-5).

The mean residence time (MRT) increased with increasing the doses as well. Other authors have reported pharmacokinetic data after IV or SC administration of F that are similar to these findings[421]. Clearance values calculated from oral data were similar to IV clearance values (table 21 a) and did not increase with the increase in the dose reflecting no increase of fraction of free drug and that the saturation of the plasma protein (ATIII) did not occur in the dose range used.

In brief, cationic LNC significantly increased oral bioavailability of F and improved its pharmacokinetic profile in a dose-dependent fashion **(Figure 38c &d**). It is evident from the above pharmacokinetics that the F-loaded cationic LNC increased the oral bioavailability of the pentasaccharide significantly with 5mg/kg oral dose CTAB formulation (9.75%±5.4, $P<0.05$) and more with SA-LNC (21.18%±6.2, $P<0.001$) but non significantly with lower oral doses (2mg/kg) of the same formulations.

The computation of PK parameters with a non-compartmental model for the different groups showed high variation in each group from the mean especially in the high doses of F-loaded CTAB –LNC and SA-LNC (with 5, 7 or 10mg/kg). Data from SA-LNC (5mg/kg) were shown to be the least variable with SEM <30% and n=5.

Figure 38a&b: Effect of fondaparinux dose in different loaded cLNC on the area under the plasma concentration-time curve, AUC_{tot} (a) and absolute bioavailabilty (b**). (**mean ±SD, normalized for the dose difference between oral and iv dose)

Figure 38c&d : Dose-proportional increases of the area under the plasma concentration time curve from time zero to infinity ($AUC_{0-\infty}$) of F(**a**)) and the maximum plasma concentration (Cmax) (**b)** after single -dose administration of F-loaded cationic LNC respectively.

DISCUSSION

1. FORMULATION AND *IN VITRO* CHARACTERIZATION

Formulation Strategy:

The selection of the optimal encapsulation technique into a delivery system for a drug depends strongly on its physicochemical properties. For the entrapment of highly water-soluble molecules such as LMWH in microparticles, double emulsion (water/oil/water) followed by a solvent extraction has been established as the best technique for many years to prepare particulate delivery system [307, 422]. Nevertheless, low encapsulation ratios were reported when applying these standard designs [407]

Another strategy, described thoroughly, is to apply counter ions (SAA, polymers or lipids) to increase the drug entrapment by attaching the drug to the particle by electrostatic interactions[197, 375, 407, 423]. Hp-loaded polymeric microparticles and nanoparticles were prepared with positively charged polymers [384, 424, 425].

In this chapter, F entrapment within the lipid based delivery system, the lipid nanocapsules was a real challenge. The synthetic pentasacharride, similar to Hp and LMWH, being very hydrophilic and negatively charged due to sulfate and carboxylate groups, failed to be attached to the surface of the lipid nanocapsules by introducing it into the solution in which are dispersed the stable nanocapsules. The presence of a nonionic hydrophilic surfactant was not enough to promote the interaction bonds between the water-soluble active principle and the free surface of the nanocapsules. Furthermore, since heparins are water-soluble compounds, they have a tendency to leak into the aqueous external phase during the drug entrapment step (before phase inversion), which usually decreases the entrapment efficiency.

LNC easily entrap many lipophilic drugs, as well as amphiphilic drugs, with high efficiency [45, 124, 125]. Lately, hydrophilic DNA molecules were successfully encapsulated into the oily core of LNCs only after lipophilic complexation of DNA using the cationic lipid DOTAP [199, 202]. Furthermore, novel lipid nanocapsules with an aqueous core were formulated to encapsulate free hydrophilic molecules [426].

LNC have a negative surface charge due to the contribution of phospholipids molecules [45, 103] and the presence of PEG dipoles in their shell [70]. Introduction of a positively charged molecule into the structure of LNC was first examined using CTAB or SA in the primary emulsion. The hypothesis behind using these cationic surfactants was however to form ion pairs with the polyanionic F at the water/oil interface thus

stabilizing the primary emulsion before phase inversion and hence facilitate F encapsulation [424, 427]

The single-tailed cationic lipid cetyl trimethyl ammonium bromide (CTAB) has been widely used in gene delivery systems and shows good performance in loading DNA and promoting gene transfection [428-431], it has a hydrophilic lipophilic balance (HLB) value of 10; which was found to be optimal for formulation of stable mineral oil /water emulsions. SA , a cationic lipid with a pKa of 10.6 was found to contribute an overall positive charge to the oil droplet interfaces over a wide pH range owing to its primary amine group [432]. Positively charged submicron emulsion, liposomes, nanoparticles, SLN using SA have been extensively investigated for their potential therapeutic applications [433-436].

The presence of CTAB and SA in the formulation of LNC did not prevent the phase inversion, and it takes part in the microemulsion structuring, probably of bicontinuous type and it was found not to affect the phase inversion temperature based on conductivity results (data not shown).

Particle size of cLNC

LNC particle size and dispersity are strongly dependent on the proportions of the constituents, more specifically, the proportion of hydrophilic and lipophilic surfactants [45, 53, 56, 73]. Specifically, the surfactant brings about a decrease in the interface tension and thus a stabilization of the system, which promotes the production of small particles. In fact, the size of these nanocapsules could be adjusted between 25 and 95 nm according to their composition, especially Solutol®HS15 [73]

Blank LNC had an average diameter (~50 nm) based on the proportion of their component[73]. It could be noticed that the average particle diameter of CTAB-LNC was increased slightly (~60nm) especially with the increase in drug load up to (5.75±0.1 % w/w) (**Table 21a**). This could be attributed to the high encapsulation efficiency (100%) and the association of the drug on the surface of the cLNC. The positively charged quaternary ammonium groups of CTAB could be mainly directed toward the external aqueous phase, thus leading to an increase in the mean diameter, with the increase in drug load, compared with unloaded cLNC (**Figure 39**).

In SA formulations, the particle size remained more or less constant (47nm) although the proportions of total surfactant were higher than that in CTAB formulations (5.88%-7.27%w/w) compared to (5.41% w/w) respectively. This could be due to lesser interaction of F with the primary amine groups of SA as reflected by lower encapsulation efficiency (84-90%) or due to SA surfactant properties at the triglyceride/water interface. Adsorption of the drug on the surface could have compensated the decrease in particle size by the increase in SA concentration and the net result was no change in particle size. SA / Lipoid® ratio as well as its effect on the stability of the shell and the disturbance of the surfactant stabilizing concentrations could be other contributing factors.

In another study on the encapsulation of LMWH in microparticles using polycationic polymers, the increase in diameter was related to the hydrophilic nature of LMWH that may favor the uptake of water, thus conferring potential swelling of the particles [385]or due to decreased surfactant properties of Eudragit® RS, leading to a formation of larger microparticles, regardless of the polymer ratio [373].

It is important to notice that the preparation of F-cLNC in presence of bicarbonate solution for further in vivo study did not affect the physicochemical characterisitics of the final dispersion and it was thought better to only present the particle size and zeta potential results for the formulations used for the invivo study.

Zeta Potential of cLNC:

The zeta potential was dramatically changed for the cLNC if compared to blank LNC (~-1mv), being more positive in case of CTAB-LNC (+32 ±1.7 mV) than in case of SA-LNC (+11.946±0.5 mV). The positive surface potential value of the cLNC was probably due to the presence of the cationic agent in its ionized form at the oil/water interface[437]. Indeed, the CTAB presented higher potential surface which may be due to the quaternary ammonium groups while SA has a strong amine group at the end of a lipophilic chain. Similarly, Eudragit RL which carries 8.8 to 12% of ammonium groups presented higher potential surface than Eudragit RS characterized by 4.5 to 6.8% of positively charged groups[423]. The magnitude of the ζ potential decreased in an F concentration-dependent manner. The decrease in ζ potential agrees with the fact that the addition of a positively charged surface to a negatively charged drug will neutralize it[423]. Whether F was located on the surface of the LNC or in the core has to be further investigated. The steric effect of SA or CTAB or F chains at the surface of the complex can significantly improve the stability and prevent aggregation of the LNC even when the zeta potential is less than 10mV (e.g. HF-SA-LNC). Similar phenomenon was also observed by other researchers [438].

Drug percent Encapsulation efficiency , in vitro drug release and stability:

The **association efficiencies** of F were considerably high, at approximately 90%. Indeed, the encapsulation efficiency with CTAB-LNC was higher than with SA LNC and reached 100%. Although both bear positive charge, the physicochemical nature of both differs, the degree of interaction with F, the net charge, the molecular weight as well as place and orientation in the shell of the LNC all differ (**Figure 39).** The degree of association seemed dependent on CTAB or SA content [439]. Both SA and CTAB could act as complexing and emulsifying agents, depending on their affinity to the drug, the hydrophobicity of the complex formed, its ability to be in the emulsion or migrate to the water-oil interface and form stable primary emulsion[440, 441].

The **release** of a drug from a drug delivery system is strongly dependent on the properties of the delivery system and its composition i.e., unit composition, charge, as well as its physicochemical properties (crystallinity, hydrophilicity, hydrophobicity) which determines the permeability to solvent and drug [385]. In a recent study on the release mechanism from LNC[63], it was found that the physicochemical properties of

the drug as well as the oil phase had a high impact on the drug release rate while the surfactant type, composition or density exerted only a minor effect.

The *in vitro* release study of F-loaded cLNC, carried out at 37°C in deionized water using dialysis bag technique, was conducted to optimize the formulation of cLNC by investigating the effect of nature and ratio of cationic molecules components on the formulation results. A proper understanding of the release mechanism and of the influence of the surfactant monolayer barrier composition as well as the core material and drug properties in the drug release pattern is essential.

In vitro release data obtained under sink conditions indicated biphasic release pattern of F from SA-LNC with a 7% burst release in one hour and sustained release of 18% of the drug over 48 hours-study period. These data are generally similar to drug release profiles from different lipid nanoparticles [23, 44, 155, 286] . The burst effect could be attributed to the presence of a small fraction of unentrapped drug or drug embedded near the LNC surface (encapsulation efficiency of F-SA-LNC 80% - 96%). The slower release phase can be attributed to the slow drug desorption from the surface of LNC into the release medium[423]. This lack of release was also observed for amiodarone [97] and tripentone [72] loaded LNC but was attributed in particular to the low drug solubility in the aqueous phase [380].

On the other hand, the F release from CTAB-LNC was very slow (10% in 48 hrs) (**Figure 35a).** This could mainly be related to the strong ionic interactions between the drug and the polycationic surface of the LNC that are difficult to disrupt in the experimental conditions. The drug F may be attracted to CTAB molecules oriented outwards on LNC surface or included in micelles of the surfactant although concentrations used are lower than CMC for CTAB but the presence of the sodium chloride in the preparation might have decreased it. Surface tension of CTAB was found to decrease significantly in the presence of NaBr and NaCl, as has been reported [442]. Thus the degree of electrostatic interaction plays the major role in affecting the drug release from LNC, not related to the drug solubility in aqueous phase.

Similarly, the release of UFH or LMWH from loaded microparticles and nanoparticles prepared with the cationic polymer (Eudragit) and biodegradable polymers [373, 384, 407] was not complete after 24 hr and ranged from 2 to 10% .This incomplete drug release may have resulted from a decrease in the mobility of the polymeric chains as well as interactions between LMWH and the polymer(s), thus inducing the formation of an insoluble and stable complex that would slow down LMWH release [385]. Morover, similar release profiles were obtained when positively charged chitosan was used with LMWH for oral delivery. This was attributed to the electrostatic interaction between the ionized CS and Hp being very strong at gastric pH [425, 443]. The stability of NPs in response to pH had a significant effect on the release of Hp. In the presence of an enzyme, the release of Hp from test NPs became much more prominent.

The assessment of the **stability** of colloidal carriers in GI fluids is essential in order to predict their suitability for oral administration. Their stability upon contact with GI fluids since they are composed of biodegredable materials, their particle size if in

nano range which maximizes the surface area for enzymatic degradation [444] and their particle aggregation due to environmental conditions of the GI tract [445] are critical parameters for the design of new and efficient colloidal drug carrier systems for oral use.

In this study, the cLNC were stable for 48 hrs regarding their particle size and zeta potential when diluted in different pH media only in absence of enzyme (e. g pepsin) (**Figure 35b&c**). Slight changes in zeta and particle size in the alkaline medium that could be related to ionization of chemical groups on their surface and the difference in their orientation. The concentration of the released drug could not be determined due to interference of medium composition with the azure dye causing metachromic shifts. In another study[174], LNCs were stable in gastric media for 3 h and their size was conserved, and approximately 12% of initial amount of the encapsulated drug was released. This was related to their surface coating with PEG..

The shelf stability of F-LNC at different storage temperature was maintained for their encapsulated drug content (**Table 22**) . CTAB-LNC were more stable than SA formulations in their particle size and size distribution up to 4months at 4°C and 25°C.

The relevance of the *in vitro* media in predicting *in vivo* stability of LNCs was further assessed in the present chapter with *in vivo* experiments. The high drug payload in CTAB-LNC and SA –LNC (7.948 ± 5.5 mg/g LNC) and (5.53 ±2.232 mg/g LNC) respectively made them suitable for oral administration in rats in convenient doses and volumes. Most important, it should be noted that the encapsulation of F in CTAB-LNC and SA-LNC did not affect its biological activity as a good correlation was found between the amount of unencapsulated F (free drug) determined by the colorimetric method with Azure II and that evaluated with the biological method based on the antifactor-Xa activity.

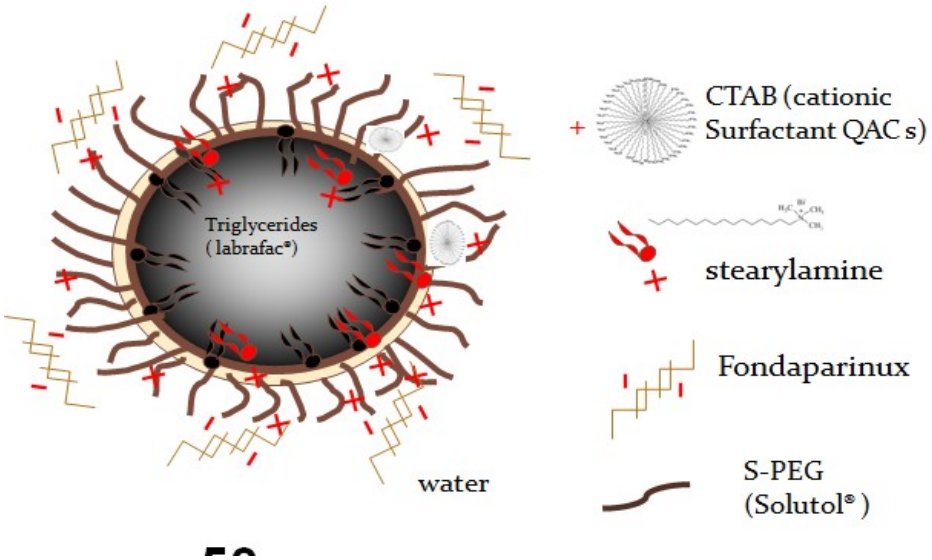


Figure 39 : Schematic representation of cLNC (CTAB or SA-LNC) loaded with the drug (fondaparinux), showing the nanocapsules core shell structure with possible loading of the drug on their surface by electrostatic interactions, (Lipoid® might be present at the inner face of the shell represented by the black head and tail)

2. *IN VIVO* RESULTS

Fondaparinux (F), a synthetic pentasaccharide, demonstrating efficacy after subcutaneous (SC) injection compared with LMWHs in randomized clinical trials was approved in 2001 for thromprophylaxis in patients undergoing major orthopedic surgery and in 2004 for treatment of thromboembolism and recently its efficacy was confirmed in acute coronary syndrome[317, 321, 325, 413]. It does not require dose adjustment for age or gender, has predictable effects over the defined therapeutic dose range, and does not require laboratory monitoring [315].

In a clinical study, F was reported to be rapidly and completely absorbed by the subcutaneous route: The maximum plasma concentration (Cmax) was reached between 1 and 3 hours and bioavailability was 100%. There was limited distribution volume, no evidence of metabolism of the drug and the pharmacokinetics were linear in the dose range 2 to 8 mg. Steady state was reached after 3 to 4 days 'treatment, and the terminal mean half-life was between 17 and 21 hours, dose-independent and optimal for once-daily administration[317]. A large phase II dose ranging study demonstrated a clear potential benefit over LMWHs and indicated that the 2.5mg once-daily dose should be selected for further clinical trials in the prevention of venous thromboembolism in patients undergoing major knee transplant therapy [322] The peak plasma level of F after SC injection and the area under the curve were reported to be linearly related to the dose administered[322]. Routine monitoring and dose adjustments are therefore not necessary during F therapy.

The study design:

Fondaparinux, like Hp, is presumed unstable under acidic enzymatic conditions[394]. Few studies support the observations that Hps are absorbed by the gastrointestinal tract but they do not reach a therapeutic concentration [446] and that active transport is involved and is pH dependant preferably around pH 7 [447, 448]. Thereby some *in vivo* studies of UFH and LMWHs were carried out using *in situ* intestinal administration[390, 395, 399, 449]. While, most were carried out by **oral gavage** where promising results were obtained[304, 402, 450] and some even extended to clinical trials[451]. The stability of F could thus be increased in the GIT , like in the case of UFH or LMWH studies, if administered with bicarbonate buffer [448, 452] by oral gavage to neutralize gastric acidity [306]. Another reason for using the buffer may be to maintain the pH above 5 in the micrenvironment of the cLNC thus increasing the dissociation of sulfate and carboxylic groups of the pentasaccharide and thus its maximum binding to the cationic surface of the cLNC and hence its gastric stability. In the present study, cLNC have been prepared using bicarbonate buffer resulting in dispersions having a final mean pH of 8.5± 0.5 for CTAB and SA formulations respectively. Furthermore,

their particle size, zeta potential, encapsulation efficiency as well as the biological activity of encapsulated F did not change in presence of the buffer (data not shown).

It has been stated in the literature that in oral studies, the detrimental effects of the oral route (e.g., degradation and dilution) are typically compensated for; by using a Hp **dose** at least one order of magnitude greater than that used for in situ intestinal studies. It can be stressed that when oral absorption of Hp is claimed, the dose is usually much larger than the iv dose by 10 times up to 50 at least [307]. In this study the F-cLNC were administered in doses that are somewhat high and much higher than the iv or SC doses for F.

Male Wistar **rats** with a mean weight of (325.875 ±14.1 g) were chosen as animal model which are commonly used for the in*vivo* studies of Hp , LMWH and F [453] . Other used rabbits [307, 384, 390, 407], or monkeys [382, 383, 454] as a non human primate model and few studies were extended to humans (clinical studies[451]).

The **anti-FXa activity** of F in the collected plasma samples were measured by anti-Xa assay using the appropriate calibrator (F) [320]. This indirect method of using pharmacodynamic response to assess absorption and bioavailability of F has been widely used to monitor therapeutic efficacy of LMWHs[455]. In case of F, which has only anti Xa activity[373], this method could be considered as pharmacokinetic study because it s a synthetic molecule and each mole corresponds to its antifactor Xa[322]. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) are relatively insensitive measures of F activity, and, unsuitable for monitoring because the pentasaccharide-AT complex may be unable to inhibit factor Xa at a rate equal to the rate at which factor Xa incorporates into the prothrombinase complex.

Furthermore, *in vivo* experiments have been carried out with the liquid colloidal suspension of F-loaded cLNC, but the technology may be also available for the production of solid preparation after freeze-drying of the nanocapsule suspension [57], allowing its use in capsules containing the powder or in tablets. Many authors used this strategy to protect the Hp from degradation at stomach pH[304, 387, 456, 457]

Unfortunately, all of the above variations in the different experimental setups such as animal species, analytical methods and administration pathway made impossible the direct comparison of the results of this chapter of the thesis with other results attempting oral administration of Hp. In addition, no in *vivo* study for F except for Arixtra ® has been reported which is mainly administered via SC route.

Preliminary trials were conducted to adjust a suitable IV **dose** of F to male wistar rats (av weight 300g) based on reported Vd of F after SC injection (7-11L)[317]. It was found that 200µg/kg (60µg) IV or Sc doses which are lesser 40 times than human doses were enough to give plasma concentration far above that reported for therapeutic anticoagulant effect (0.1- 0.2 IU/ml) with no bleeding side effect. It was reported that pentasaccharide, when administered at 200 to 20.000 anti-Xa U/kg IV doses to rats, produced only a mild increase in blood loss, whereas Hp administration in the same tail transaction model resulted in a five-fold increase in blood loss over placebo at a dose of 300 anti-Xa U/kg IV [458]

Fondaparinux, similar to UFH and LMWH was not absorbed orally from control solution in bicarbonate buffer in doses (0.5, 2 and 5mg/kg) and did not result in any measurable plasma antiXa levels. Only with very high doses of F solution (10 mg/kg), a low antifactor Xa plasma activity was shown (0.1µg/ml) with high inter individual variation [459]. Variability is well known in different studies conducted in vivo [173]. The **intracardiac method** for blood collection used in this study was sometimes difficult as some samples may be drawn from veins. This could contribute to the variability of results, than the most widely used methods for blood collection via the animal tail or through a catheter. .

Pharmacokinetic study results:

Oral administration of F -CTAB-LNC formulations solution in a bicarbonate buffer solution showed low absolute bioavailability (3% to 9%) although plasma levels reached therapeutic concentration for an anticoagulant effect (C max=C therapeutic). The absolute bioavailability of F from F-SA-LNC increased significantly to about 21%. Most of the pharmacokinetic parameters, such as $t_{1/2}$, CL, MRT, were not significantly different between F control solution and F-cLNC **(Table 23**). These results indicated that the F loaded in cLNC did not affect the pharmacokinetic properties of F except for its bioavailability.

F- cLNC oral formulations evaluated can be divided into two groups based on the resulting *invivo* profiles:

The first group: LNC formulated with CTAB as cationic surfactant, being highly positively charged LNC (+30 and +42mV), loaded with high amounts of F (EE 96-100%) most probably on the surface of LNC resulting in a relatively fast AntiXa plasma acitivity (30-60 min) but of relatively shorter duration of action compared to SA formulations **(Figure 37a)** and slight increase in absolute bioavailability (9%).

Higher doses with CTAB formulations (10 mg/kg) further increased Cmax and AUC slightly, (but not linearly) and did not further increase its bioavailability (% Fabs) (**Figure 37a).**

The second group: SA-LNC with less positively charged surface (+3mV), and less EE (80-95%) resulted in a significant slower onset of action (up to 3 hours) and longer anticoagulant effect in the blood up to 21 hours **(Figure 37b).** This was shown with the higher doses (5mg/kg) and resulted in significant increase in AUC (17 fold than smaller doses) ($P< 0.01$)) and absolute bioavailability significantly increased (up to 21%) (**Figure 38a&b**). The differences in HF-SA-LNC formulation used for the high dose (5mg/kg) besides its higher drug load, were the increased concentration of SA (0.94% w/w), with SA: lipoid concentration (1.5:1) and a lower encapsulation efficiency 80%.

The elimination of F from the circulation was reported to be significantly faster in rat, rabbit and monkey compared with man. The terminal half-life did not change with the dose in the animals. Half-life in rats, rabbits, and baboons ranged from 1 to 4 h [414, 458]. In the present thesis, F showed dose proportionality values of AUC as a function of

dose after oral administration of F-loaded cLNC (<10mg/kg) **(Figure 38c&d**) similar to that shown in healthy volunteers after SC or IV administration [317]. AT saturation by pentasaccharide at high doses has been confirmed in human volunteer studies [460]. Saturable plasma protein binding in rats occurs within the concentration range achieved with 5-10 mg F once daily (up to 3 mg/l plasma level) while, AT III binding is not saturated. Increasing the dose further may result in saturation of AT III binding. The dose-proportionality in the higher dose range for SA-cLNC formulations (<3 mg/l plasma level) was maintained as seen in **Figure 38C&d**. This was not the case with CTAB –LNC formulations of higher dose range (10mg/kg) although no change in clearance values was observed (no excess free fraction of drug).

The increase in AUC as well as the increase in plasma anti-FXa level after oral administration of F in different SA and CTAB- LNC formulas was linear with the increase in dose being of higher slope with SA formulations (**Figure 38b).** Linear pharmacokinetics were reported in the literature for Hp and LMWH and F after SC injection[317]

Concerning **the mechanism of absorption**, another study reported the higher bioavailability of Hp by miceller solubilization suggesting that the drug is transported by passive diffusion. This set of data is consistent with the fact that hydrophilic macromolecule, LMWH, is not a substrate for P-glycoprotein [396].

The two primary pathways for molecules to cross the epithelium and reach the bloodstream are the **paracellular** route and **transcellular** route. Hydrophilic and charged molecules primarily use the paracellular route; however, the size constraints presented by the tight junctions limit this route to molecules of a size less than 11Å. Three mechanisms that utilize the transcellular route exist (passive transport, transcytosis and carrier-mediated). Recent data suggest another pathway for penetration of substances from the intestinal lumen into **lymph** with possible subsequent entrance to blood circulation. This involves microfold M_cells, found in the follicle_associated epithelium of the Peyer's patches[327, 461, 462]. Some hydrophilic molecules of various sizes are absorbed transcellularly, but only through specific active or facilitated mechanisms [446, 463]. It is known that oil droplets can be easily absorbed orally via lipid absorption mechanisms such as endocytosis, passive diffusion, or pinocytosis[36].

The modifications in the nanocarrier delivery system such as altering nanoparticle zeta potential, as well as hydrophobicity, thus influencing formulation colloidal stability, nanoparticle mucoadhesion properties, and protein adsorption at their surface could affect their potentials for the oral delivery of drugs [464].

The role of the particle size in oral delivery of F-cLNC

In general, transport of nanoparticles by the transcellular pathway depends on several factors, among these: the physicochemical properties of particles and the physiology of the GI tract or the animal model used to study the uptake [464-466]. Nanoparticles, in general, have shown to be more stable than liposomes in biological fluids and more susceptible to endocytosis [464]. Due to their nanosize, they distribute

more uniformly in the GI tract, resulting in more uniform drug absorption and a reduced risk of local irritation [464]. They have a better uptake by the intestinal tissue than microparticles and can be translocated across the gut wall [176, 467, 468], which would be beneficial for hydrophilic macromolecular drugs [468]. A recent review examined the different possibilities of Hp-based nanoparticle composites and their medicinal or biological applications [236, 386]. Hp was successfully entrapped into biodegradable nanospheres [305] or CS nanoparticles [359, 443] with improved oral absorption[384, 388]. Hp loaded polymeric nanoparticles (NPs) prepared by double emulsion technique using biodegradable and nonbiodegradable positively charged polymers (Eudragit RS and RL), used alone or in combination [384, 423]achieved an absolute bioavailability of 22.7% in rabbits with doses that were similar to those administered by intravenous infusion or subcutaneous injection in humans[384]. Hoffart *et al.* [307, 388] reported that an oral polymeric nanoparticle delivery system of LMWH showed promising absolute bioavailability of more than 51% by a combination of nanoparticle system and mucoadhesive properties of the polymers used.

Lamprecht *et al.* [121] found that LNC diameter at around 50nm may exhibit relatively good prerequisites to be transported efficiently. LNC exhibited a prolonged efficiency after oral administration compared to ibuprofen solution. An assumption has been made that LNC might have been translocated across the gut wall retaining their controlled drug release properties and thus prolong the presence of the drug in the plasma [121].

The role of the positive charge in oral delivery of F-cLNC

Nanoparticle surface properties govern the extent of nanoparticle uptake[466]. The mucous layer is known to be negatively charged at physiological pH[384]. The charge exposed at the surface of NPs may significantly affect their internalization mechanism and intracellular route[468, 469]. Positively charged nanocarriers display better capacity for uptake by the enterocytes and interact more with the mucus[434, 466]. One of the most successful approaches toward increased electrostatic interaction with the intestinal mucosa has been the use of positively charged colloidal dispersions such as liposomes [375, 470], submicron emulsions [432, 471, 472], self-emulsifying oily formulations [473], and chitosan (CS)-coated lipid nanoparticles [142] cationic SLNs[3]. It was further hypothesized else, that core-modifying agents used to prepare microspheres may separate out from the microspheres and act as absorption promoters [474]. The presence of positive charge has been responsible for the enhancement of bioadhesion effect seen with different absorption enhancers or polycationic polymers strategies used for UFH and LMWH [307, 384, 390, 398]. This interaction may also lead to a higher local concentration of Hp helping its diffusion and absorption through the mucocellular wall.

In this study, the role of the positively charged F-cLNC formulations in the increase of permeability of the drug was not clear, as slightly positive charge (+3 to+11mV) shown with SA-LNC was sufficient to increase F bioavailability. Some residual and noncomplexed cationic charges, unmasked during drug release, may increase the residence time of cLNCs next to the absorption surface of the

gastrointestinal tract. This mechanism would reinforce the F gradient because of the natural gastrointestinal tract coating of LNCs.

On the other hand, the presence of an excess amount of unbound cationic charge in case of (CTAB-LNC) may inhibit endocytosis by competing for binding sites on cell surface proteogylcans (F) and may thus decrease its biological activity [475]. Indeed,

Both formulations, given with the same doses, gave more or less different responses; At low doses (10 times higher than IV dose) both gave more or less same Cmax (0.18µg/ml) and % F didn't differ much although CTAB formulations being more positive (30mV) compared to SA formulations (11mV) (P< 0;05). In higher doses (25 times higher than IV dose), much less positive charge in SA -LNC formulation (+3mV) gave better significant pharmacokinetics profile than that with CTAB-LNC formulations (+42mV) (**Table23**). These results are partly confirmed with a preliminary *in vitro* CACO-2- cells monolayer tranport study for these formulations. TEER values didn't differ after 2 hours incubation at 37°C which confirms that the tight junction opening did not occur and the mechanism mostly is not paracellular. These results are different from those reported for permeability changes induced by polycations which were more dependent on the amount of positive charges than on the type of cationic moiety.

CTAB, being of higher molecular weight, bearing a quaternary ammonium group as well as a hydrophobic group may have interacted in a different way with the drug , the negatively charged mucosa and the LNC shell compared with the less molecular weight SA bearing a primary amine group, resulting in lower F bioavailability with CTAB formulations. In another study, the reduction in biological activity is believed to occur due to the presence of an excess amount of unbound cationic charge that inhibits endocytosis by competing for binding sites on cell surface proteogylcans[475]. Absorption may require certain +ve charge concentration.

The role of the positive charge was reduced in another study, where nanoparticles of negative or neutral zeta potential were better transported by Peyer's patches, compared to positively charged nanoparticles[464]. Anionic chitosan derivatives have been found to increase permeation of anionic macromolecules though to a lesser extent than cationic derivatives. A combination of surface charges and matrix hydrophilicity seems favorable, the available polymeric carrier systems for oral macromolecular delivery still need further improvement[468].

The role of the lipophilicity of the LNC in oral delivery of F

Another factor affecting the permeability of the drug against mucosal membrane might be the increased lipophilicity imparted by the use of LNC. Strategies based on increasing Hp lipophilicity were successful as well, it is worth mentioning the good results obtained with the delivery agents SNAC or SNAD and DOCA for LMWH and UFH [383, 387, 398, 404, 446]. Although these two most advanced studies show the oral absorption of LMWH in a dose-dependent manner in amounts that would provide prophylaxis against DVT, the anticoagulant activity did not last more than 4 h and high doses of LMWH were orally administered compared to the subcutaneous route. Moreover, SNAD is not yet accepted by health authorities and gives a bitter taste to the

oral solution possibly involving compliance issues. The potential of phospholipids and lipid-based formulations in enhancing the oral bioavailability of drugs with low aqueous solubility or low membrane penetration potential has been discussed elsewhere[146]. Their effect on the drug release or uptake or instability in the GIT are some examples reported by others [12, 476].

The unique properties of LNC [45] as lipid delivery systems, their physiochemical diversity, biocompatibility, nanosize , their compatibility for large-scale industrial production, the stringency of registration requirements [477] and most important their proven ability to enhance oral bioavailability of paclitaxel [173]and ibuprofen [121] have made them very attractive candidates as carriers for oral formulations of the anticoagulant F. LNC constituted by medium-chain triglycerides (MCG) and hydrophilic/lipophilic surfactants. The MCG had the most potent absorption-enhancing effects for Hp or LMWH [314, 377, 478]. A medium length alkyl chain surfactant may penetrate the lipid bilayer easily [479]. A transcytosis (cholesterol-dependent caveolin pathway for transcytosis) transport of these nanovectors was suggested for paclitaxel-loaded LNCs after an *in vitro* transport study using CACO-2- cell culture [176].

Furthermore, *in vivo* experiments have been carried out with the liquid colloidal suspension of F-loaded cLNC, but the technology may be also available for the production of solid preparation after freeze-drying of the nanocapsule suspension,[57] allowing its use in capsules containing the powder or in tablets. Many authors used this strategy to protect the Hp from degradation at stomach pH [304, 387, 456, 457].

Furthermore, the increased stability of cLNCwould prolong therapeutic activity of drug in gastrointestinal region. Although LNC might be affected by gastric enzymes (after 1hr) increasing the release of the encapsulated drug , but it seems from the biavailability data obtained that the F-LNC passed into the intestin before (Tmax=1hr) being affected.

In conclusion, the successful oral delivery of F in cationic LNC may be because of high drug entrapment efficacy and favorable physicochemical characteristics of F-cLNC, which facilitate more drug transportation to the blood. A hypothesis, from a combination of the potential mechanisms mentioned above, could then be deduced. The nanosize of the F-cationic nanoparticles, their lipophilic properties and positive surface charge can facilitate binding and endocytosis by plasma membrane. Owing to the coating of the GIT with LNC [173]and the electrostatic interactions with the negatively charged mucus, F may be released close to the intestinal wall facilitating its absorption. Indeed, the effect being more pronounced with the SA-LNC formulations. In the latter; the F, probably, being highly attached to SA-LNC, its lipophilicity would be increased and could increase its permeability to cross the gut then dissociated later in blood (higher T max) resulting in the linear PK similar to SC administration with a much slower onset of action due to slower release of the drug from LNC.

One last point, LNCs are originally manufactured without the use of solvents and the ingredients used known to be very safe and no organic solvents have been used, but the effect of inclusion of CTAB or SA in the LNC needs to be evaluated. Nevertheless, CTAB has been used previously for a range of biomedical applications, including use as

an antibacterial agent in eye drops. It was given to male and female Sprague-Dawley rats in drinking water in dosages of approximately 10, 20, and 45 mg/kg/day for 1 year. The surfactant was well tolerated at the two lowest dose levels but at the highest dose level a reduction in body weight was detected. The bioavailability and the distribution of anti-psychotic clozapine in the brain were enhanced by the entrapment in CSLNs with cationic SA [435, 480]. Hence, although the cLNC needs to be evaluated, the toxicity of CTAB and SA is well defined (rat LD50 oral 410 and 1395 mg/kg respectively) and their level can be kept to a minimum (0.45 and 0.92 mg/mg LNC) respectively corresponding to (30 and 90 mg/kg) rat. Moreover, although CTAB was chosen as the initial surfactant, the work with SA clearly shows that CTAB can be replaced if necessary, by safer single-tailed cationic lipid moieties [481]without reducing the potency of the delivery system. The different types of cationic lipids and cationic polymers and their toxicity have been reviewed [482]. Non-viral vectors based on the use of cationic lipids or polymers appear to have promising potential, given the problems of safety encountered with viral vectors for DNA[481]

Finally, factor Xa can be a target for new oral anticoagulants, a potential indication for fondaparinux. Many other new anti factor Xa agents are in phase II clinical testing and several are moving onto phase III. The results thus far are promising, but more data are needed to assess the long-term efficacy and safety of these new drugs. Further identification of the mechanism of oral absorption of fondaprinux loaded on cLNC shall be the focus of future studies or histological evaluation of gastrointestinal tissues from rats to investigate site of absorption and the study of the role of these formulations (F-cLNC) in thrombus treatment in thrombotic rats (pharmacodynamic effect). Complementary in vitro studies shall be carried out to prove the cytotoxicity of the cationic LNC after their dialysis and removal of excess cationic lipids or surfactants; with further modification of the formulation if needed to optimize the bioavailability of F associated with these vehicles In addition, we have to further prove that cationic lipids remain stably bound to the LNC, providing a surface for adsorption of F or other anionic molecules such as DNA and that these drugs can remain associated with cLNC during their movement through a density gradient .

Is oral F better or the new generation of oral antifactor XA and IIA inhibitors? Interstingly, F-loaded cLNC gave similar pharmacokinetic characteristics to that of dabigatran etexilate (DTI) , a promising novel, oral small molecule that specifically and reversibly inhibits thrombin[483]. Could oral F compete having same advantages: including no requirement for anticoagulant monitoring, a low drug—drug interaction potential and the possibility to use in both the acute and chronic settings. These new drugs provided in a study that they can be an alternative to subcutaneous enoxaparin for the prevention of VTE after THR and TKR. Rivaroxaban demonstrated superior efficacy over enoxaparin even in symptomatic DVT, without significant differences in major bleeding. Dabigatran etexilate is the first anticoagulant registered and approved by the EMEA with two different doses and, for the first time, one reduced dose is defined for the elderly population or for patients with moderate renal insufficiency. Further studies and comparison should be carried out for better therapeutic outcome.

CONCLUSION

This study is considered an extension of the applicability of LNC as an oral delivery system. The results obtained in the *in vitro* characterization study demonstrated that this LNC system, in 50 nm particle size range, monodispersed, with a positively charged surface could markedly enhance the drug loading and GIT stability of the hydrophilic macromolecule F. Owing to the polyanionic nature of F, ionic interactions between the drug and the Quaternary ammonium compounds of the CTAB or primary amine groups of SA led to the increase of F immobilization in LNC compared to plain patent LNC. *In vivo* results showed that a significant anticoagulant activity in plasma was observed after oral administration of test F-cLNCs in rats, indicating that the intestinal absorption of the pure anti-factor Xa inhibitor was considerably enhanced.
This **first proof of concept** of enhanced oral bioavailability of fondaparinux, may be of great clinical outcome. This new simple formulation strategy for peroral form may replace the currently used injectable anticoagulant as well so as to improve patient acceptability and therapeutic effect, facilitating the development of chronic treatment schedules without the bleeding side effects seen with LMWH or the coagulation monitoring required with VKA treatment.

GENERAL SUMMARY

Lipid-based delivery systems (LBDDS) are a rapidly emerging approach to formulating pharmaceuticals for dermal, ocular, oral, pulmonary or parenteral delivery. Whether in the form of liposomes, emulsions, micelles, lipid-drug conjugates, microparticles or nanoparticles, LBDDS formulations can be tailored to meet a wide range of product requirements dictated by disease indication, route of administration and considerations of cost, product stability, toxicity, and efficacy.

LBDDS have attracted increasing attention because of the several advantages they offer: biocompatibility and biodegradability, small size, lower toxicity compared to polymeric nanoparticulate deliver systems, high incorporation efficiency, controlled drug delivery and suitability for drug delivery at different sites of administration.

The **general introduction** of the thesis provided an overview of the different types of LBDDS with their main classification as liquid or solid lipids and colloidal carriers. Their composition, advantages, drawbacks and applications were reviewed in comparison with polymeric delivery systems whenever possible. However, the introduction placed more emphasis on solid lipid nanoparticles (SLN) and lipid nanocapsules (LNC) as these two systems were the subject of the experimental part of the thesis. The overview included the potentials offered by these two systems in terms of size, controlled release and targeting ability of diverse drugs. The formulation strategies and characterization tests of these systems were also summarized.

Part 1*: Corticosteroid-Loaded Solid Lipid Nanoparticles gel for Dermal Delivery

Prolonged drug delivery associated with enhanced skin penetration and minimal systemic absorption is the main criteria for successful topical corticosteroid therapy. This could be achieved by using solid lipid nanoparticles (SLNs) carrier. In this part of the thesis, SLNs of the high-potency corticosteroid, clobetasol propionate (CP) were prepared, characterized and formulated as a hydrogel for dermal delivery of CP.

CP-SLN were prepared by the hot homogenization emulsification and characterized for particle size and shape (by laser diffraction, TEM and image analysis), zeta potential (using a zeta meter), drug physical state in SLNs (by DSC), encapsulation efficiency (by dialysis and ultracentrifugation methods), *in vitro* drug release (by dialysis) at 37^{o}C in pH 7.2 phosphate buffer containing 40% PEG 300 and 0.5% Tween 80 (a release medium proved suitable for maintaining sink conditions for the poorly soluble corticosteroid). A gel containing CP-loaded SLNs (0.05% CP, pH 6) was formulated using 1% w/w Carbopol 934. The gel base also contained 10% w/w propylene glycol and 30% w/w glycerol. The prepared gel was assessed for in vitro drug release in comparison with control conventional CP gel 0.05% and a commercial CP gel 0.05%. Ex-vivo skin permeability of CP from the formulated gel relative to the commercial gel was assessed using Franz diffusion cells fitted with intact human skin. HPLC was used to determine drug concentrations in both *in vitro* and *in vivo* studies.

Results indicated the formation of spherical SLNs with a mean diameter of 173 nm, narrow size distribution and a mean zeta potential value of -22 mV. CP entrapment efficiency was 89.0% and 88.8% as measured by dialysis and ultracentrifugation methods, respectively. DSC indicated molecular dispersion of CP in SLN. Release data for Cp loaded-SLNs at 37^{o}C showed a biphasic release pattern with ≈17% burst release in the first hour followed by 70% release of CP over 6 days.

Drug release from the three gels under study was sustained over 24 hrs, CP-loaded SLNs showing the slowest release rate. Franz diffusion cell studies indicated that the skin retention of CP form the SLN test formulation, measured at 6 hr, was six fold that of the commercial product, the difference being statistically significant ($p<0.001$)

In conclusion, formulation of CP as SLN-based gel offers better biopharmaceutical attributes, in terms of reduced contact with the skin, controlled delivery of CP greater skin retention and presumably less potential for skin absorption and side effects.

Part II of the thesis focused on a second example of LBDDS, the lipid nanocapsules (LNC) which combine the colloidal stability of suspensions of solid particles in biological fluids and the solubilizing properties of liquids. Their novel formulation process was in fact based on the phase inversion temperature (PIT) method plus a temperature cycling treatment.

Lipid-based drug delivery systems including liposomes and microemulsions generally show low entrapment efficiency of hydrophilic macromolecular drugs. In addition, they have low stability in the GI tract. This part of the thesis aimed at extending the applicability of LNC as a potential lipid delivery system for hydrophilic macromolecules. The pentasaccharide, fondaparinux (F), was selected as a model drug given that oral anticoagulant therapy is a major research challenge.

Part II of the thesis is divided into two chapters:

Chapter 1: Lipid Nanocapsules Incorporating Fondaparinux Microemulsions.

This chapter aimed at encapsulating the hydrophilic drug fondaparinux(F) into LNCs by a novel patented two microemulsion strategy.Ternary and pseudo-ternary diagrams were first constructed based on mixing the lipophilic triglyceride “Labrafac CC” with lecithin or nonionic surfactant and a polar solvent (aqueous or nonaqueous). Only clear w/o microemulsion (ME) regions, seen visually, were used for further loading of the drug. This “precarrier” ME was introduced into the second ME in the phase inversion zone of the last temperature cycle of the preparation process of the original LNC. Rapid cooling of the system at a predetermined temperature (from conductivity measurements) led to the formation of the F-loaded nanocapsules. Formulation parameters as the F concentration (% w/w) in the precarrier ME, the temperature of addition of the precarrier ME and cold water have been considered. LNCs were characterized for particle size, particle size distribution (PDI), zeta potential and encapsulation efficiency. The latter was measured indirectly following ultrafiltration by the spectrocolorimetric Azure method after assay validation. Moreover, bioactivity of the encapsulated drug was assessed using the anti Xa chromogenic assay.

Data for LNC prepared using Imwitor308 and Span 60 as non ionic surfactant mixture for the “precarrier” ME were better than those obtained using lecithin.

In conclusion, LNCs could be loaded with a w/o microemulsion carrying F in its core; this technique resembles the well known w/o/w technique for the encapsulation of

hydrophilic molecules into lipid delivery systems such as liposomes and SLN. However, the technique is simpler and solvent -free.

Chaptre 2: Fondaparinux-Loaded Lipid Nanocapsules for Oral Anti -Factor Xa Effect

This chapter aimed at encapsulating F by cationic interaction strategy based on the physicochemical structure of both the drug and LNC to achieve high association efficiency. The impact of this formulation on F pharmacokinetics after oral administration to rats was studied.

LNCs were prepared by the phase inversion method using the cationic surfactants hexadecyltrimethyl ammonium bromide (CTAB) or stearylamine (SA) as charge imparting agents. F-loaded cationic LNC-CTAB and LNC-SA showed a relatively small mean particle size (**46.9 and** -53.2nm respectively and low span value (0.3). **Particle size increased with increasing F loading. Zeta potential ranged from +3.9 to +42.9 mV. Entrapment efficiency ranged from 80 to 100%.** The release of F from both test LNC was very slow, <5% and <1% F release at 24 hrs from LNC-SA and LNC-CTAB respectively.

In vivo study in rats administered F-loaded LNCs orally in comparison with a solution market product, also administered SC and IV according to a parallel study design demonstrated that cationic LNCs significantly increased the bioavailability ($P< 0.05$) of fondaparunux and improved its bioactivity in a dose-dependent fashion.

In conclusion, F-loaded LNCs demonstrated potential for a formulation increasing the oral bioavailability of the indirect anti-factor Xa inhibitor as an alternative to the currently used subcutaneous injection. After this first proof of concept, the formulation has to be modified in order to enhance the oral bioavailability of the LMWH which is an urgent clinical need. This new simple formulation strategy offers great promise for a more convenient chronic LMWH therapy which may replace the currently used injections.

In summary, this thesis highlighted the importance of lipid-based colloidal carriers and their pharmaceutical implications in the delivery of drugs of different nature for dermal and oral administration. There are several examples of biomacromolecules used nowadays in therapeutics, which are promising candidates to be delivered by means of LBDDS, such as solid lipid nanoparticles (SLN) and lipid nanocapsules (LNC). Several production procedures or formulation strategies can be applied to achieve high association efficiency between the bioactives and the carrier, depending on the physicochemical properties of both, as well as on the production procedure applied. The thesis is a contribution to research efforts aiming at improving the performance of existing drugs which would definitely improve therapeutic outcomes and the patients' quality of life.

FRENCH SUMMARY

Les systèmes lipidiques de délivrance de principes actifs (Lipid Based Drug Delivery Systems (LBDDS)) sont une approche privilégiée pour la formulation de formes pharmaceutiques pour la voie cutanée, oculaire, orale, parentérale ou pulmonaire.

Que ce soit sous forme de liposomes, émulsions, micelles, le principe actif est conjugué à des lipides. Les formulations ainsi obtenues peuvent répondre à un grand nombre de besoins dictés par la maladie, la voie d'administration et des considérations de coût, de stabilité, d'absence de toxicité, et d'efficacité.

Les LBDDS sont l'objet d'une attention croissante en raison des avantages qu'ils offrent : la biocompatibilité et la biodégradabilité, leur petite taille, leur faible toxicité par rapport aux nanoparticules polymériques, leur rendement d'incorporation, la libération contrôlée de médicaments et leur administration possible à partir de différents sites.

L'introduction générale de cette thèse donne un aperçu des différents types de LBDDS avec leur classification. Leurs compositions, leurs avantages et inconvénients et leurs applications sont décrites. Ils sont comparés aux systèmes polymériques chaque fois que possible.

L'introduction a mis l'accent sur les nanoparticules lipidiques solides (Solid Lipid Nanoparticles, SLN) et les nanocapsules lipidiques (Lipid Nanocapsules, LNC), car ces deux systèmes sont l'objet principal de la thèse. Les potentialités offertes par ces deux systèmes en terme de taille, libération contrôlée, capacité de ciblage des divers principes actifs y compris les anticancéreux ainsi que la délivrance de gènes sont abordées. Les stratégies de formulation et les méthodes de caractérisation de ces systèmes sont également présentées.

Partie I :

« Nanoparticules lipidiques solides gel pour la délivrance de corticoïdes par voie cutanée »

Cette partie concerne les nanoparticules lipidiques solides (SLN) en tant que transporteur d'un médicament lipophile, le propionate de clobétasol, ainsi que leur administration par voie cutanée.

L'administration prolongée de principes actifs associée à la pénétration cutanée sans absorption systémique sont les éléments d'une corticothérapie topique réussie. Celle-ci pourrait être obtenue par l'utilisation de nanoparticules lipidiques solides (SLN) en tant que vecteur de médicament. Dans cette étude, les SLN chargées avec un corticostéroïde, le propionate de clobétasol (CP), ont été développées, caractérisées et formulées en un hydrogel pour la libération prolongée.

Les CP-SLN ont été préparées par homogénéisation-émulsification à température élevée et caractérisées en terme de taille des particules, de forme (par diffraction laser, TEM et analyse d'image), de potentiel zêta (à l'aide d'un zétamètre), d'état physique du principe actif dans les SLN (par DSC), d'efficacité d'encapsulation du principe actif (par dialyse et les méthodes d'ultracentrifugation), de libération *in vitro* (par dialyse) à 35° C dans un tampon phosphate pH 7,2 contenant 40% de PEG 300 et 0,5% de Tween 80 (maintien de conditions sink pour ce corticostéroïde de faible solubilité). Un gel contenant les SLN chargées de CP (0,05% CP, pH 6) a été formulé en utilisant 1% w / w de Carbopol 934. Le gel contient aussi 10 %w/w de propylène glycol et 30% w/w de glycérol. La pénétration de Cp à partir du gel CP-SLN dans la peau humaine intacte a

été évaluée en utilisant des cellules de diffusion de Franz par rapport à un contrôle CP gel sans SLN à 0,05% et un gel commercial de CP à 0,05%. La HPLC a été utilisée pour déterminer les concentrations du médicament dans *in vitro* et *ex vivo.*

Les résultats indiquent la formation de nanoparticules sphériques avec un diamètre moyen de 173 nm, une distribution de taille étroite et un potentiel zêta moyen de -22mV. Le rendement d'encapsulation est de 89,0% et 88,8% tel que mesuré respectivement par dialyse et par méthode d'ultracentrifugation. La DSC a montré la dispersion de CP dans les SLN à l'état amorphe. La libération du CP des SLN a montré un schéma de libération biphasique avec ≈ 17% libérés dans la première heure suivi de 60 % pendant 5 jours La libération du médicament à partir des trois gels a été soutenue pendant plus de 24 heures, les CP -SLN montrant une libération plus lente Les études du transport *in vitro* ont indiqué que le CP maintenu par la peau, mesuré à 6 h, était plus élevé (32%) pour les SLN gel par rapport au produit commercial (16%)($P<0.05$).

En conclusion, la formulation de CP-SLN dans un gel offre un meilleur profil biopharmaceutique, en terme de délivrance controlée de CP avec une plus grande rétention dans la peau en minimisant l'absorption systémique et probablement les effets secondaires.

La **Partie II** mettra l'accent sur un deuxième exemple de LBDDS, les nanocapsules lipidiques (LNC), qui combinent la stabilité colloïdale des suspensions de particules solides dans les liquides biologiques et les propriétés de solubilisation des liquides. Leur processus d'élaboration est en fait basé sur la méthode d'inversion de phase en jouant sur la température (TIP).

Les nanoparticules lipidiques ne peuvent généralement pas piéger les pricipes actifs macromoléculaires hydrophiles. En outre, elles ont une faible stabilité dans le tractus gastro-intestinal. Les liposomes classiques et les microémulsions n'ont pas, non plus, rencontré beaucoup de succès dans la délivrance des macromolécules hydrophiles.

Cette partie de la thèse vise à étendre l'applicabilité des LNCs pour accéder à des nanocapsules chargées en principes actifs, notamment de caractère hydrosoluble ou hydrodispersible et, qui plus est, avec un taux de charge intéressant. Le pentasaccharide, le fondaparinux (F), a été choisi comme modèle. Un traitement anticoagulant par voie orale pourrait constituer un second défi qui sera abordé dans ce travail.

La deuxième partie est divisée en **deux chapitres:**

Chapitre 1 :

« Nanocapsules lipidiques formulées à partir de microémulsions chargées en fondaparinux »

Ce chapitre vise à obtenir des LNC chargées dans leur cœur lipidique d'un principe actif hydrophile à partir de deux miroémulsions selon un brevet qui a été récemment déposé. Des diagrammes de phases ternaires et pseudo-ternaires ont d'abord été élaborés en prenant en compte des mélanges de triglycérides lipophiles (Labrafac CC), des surfactants à base de lécithine ou non ioniques et une phase polaire (aqueuse ou non). Seules les microémulsions (ME) E /H limpides, vues visuellement, ont été

utilisées pour l'encapsulation du principe actif. La formation de nanocapsules impose d'introduire la première microémulsion dans la seconde à une température qui corresponde à la température d'inversion de phase de la seconde microémulsion. Cela est suivi d'une trempe dans de l'eau froide selon les travaux d'Heurtault, *et al* pour la formulation des LNC. Les paramètres de formulation des F-LNC tels que le F % w/w dans la première ME, la température de la première ME et de l'eau froide, ont été pris en considération. Les LNC ont été caractérisées par leur taille, l'indice de polydispersité (IDP), leur charge et le rendement d'encapsulation (RE %). Ce dernier est calculé à partir de la concentration de F libre. Celui-ci a été mesuré après ultrafiltration indirectement par la méthode spectrocolorimétrique Azure après validation de l'essai. Par ailleurs, la bioactivité de la drogue encapsulée a été évaluée en utilisant le test chromogène anti-Xa.

Les résultats obtenus avec les LNC préparées à l'aide de tensio-actifs non ioniques (Imwitor308 et Span 60) en tant que mélange de tensioactifs pour la première ME ont été les meilleurs, montrant une taille moyenne de 59,87 ± 7,25 nm, un IDP <0,2, un potentiel zêta de -2,23 ± 1.16mv. Le rendement d'encapsulation atteint 48,62 ± av 31,95%**.** En plus, l'activité biologique du F encapsulé a été démontré par la mesure de l'activité anti-Xa par la méthode chromogénique en collaboration avec le CHU d'Angers.

En conclusion, les LNC ont pu être chargés par une autre microémulsion durant leur préparation. Cette dernière encapsule le F hydrophile. Cette technique ressemble à celle bien connue de l'émulsion multiple pour l'encapsulation des molécules hydrophiles dans les systèmes lipidiques tels que les liposomes et les SLN. Cependant, la préparation des LNC est plus simple et n'utilise pas de solvant organique.

Chapître 2 :

« Nanocapsules lipidiques de fondaparinux pour un effet anti-Xa par administration orale. »

Ce chapitre vise à encapsuler le F par la stratégie de l'interaction cationique basée sur la structure physico-chimique du F et des LNC pour réaliser une association efficace élevée. L'impact de cette formulation sur la pharmacocinétique du médicament après administration orale chez le rat a été étudié.

Les LNC ont été préparées par la méthode d'inversion de phase en ajoutant des molécules cationiques tensio-actives telles que le bromure d'hexadecyltrimethyl ammonium (CTAB) ou la stéarylamine (SA). Les LNCs ont été caractérisées de façon complète (taille, charge, taux d'encapsulation, libération *in vitro* et activité biologique du pentasaccharide encapsulé***).*** Pour l'étude in *vivo,* des rats (groupe de 5) ont reçus des doses orales uniques de F-LNC (0.5, 2, 5 et 10 mg /kg), la solution aqueuse de F (Arixtra®) servant de contrôle en utilisant les mêmes doses. D'autres groupes de rats ont reçu la solution de F par voie IV et SC à la dose 200µg/kg. La concentration de F dans le plasma a été mesurée par dosage biologique au CHU.

La taille moyenne des F-LNCs préparées avec les 2 agents de surface cationiques a varié de 45.2 à 60.0 nm, avec une valeur moyenne de 53.2 ± 8.8 nm et 46.9 ± 0.83nm pour respectivement les F-LNC-CTAB et F-LNC-SA. Un indice de polydispersité étroit (IPD) a été obtenu pour toutes les formulations (<0.3). La taille des particules augmente avec

l'augmentation des taux de charge du F, la valeur la plus élevée étant obtenue avec F-LNC-CTAB 0.5 (% w /v). La charge globale des LNC cationiques était de +3.955 à +42.860 mv montrant la valeur la plus élevée avec LF-LNC-CTAB (0.5% w /v). Les taux d'encapsulation du F (anionique) étaient pour toutes les LNC de 80% à 100% avec une valeur moyenne de 93.6%. Le taux de charge (W) est de 7.95 et 5.53 mg /g LNC pour respectivement les CTAB-LNC et SA-LNC. La libération de F à partir de la surface des LNC cationiques a été très lente, moins de 5% et <1% libérés à partir respectivement des SA-LNC et des CTAB-LNC après 24 heures.

Le fondaparinux en solution n'est pas absorbé par voie orale .L'aire sous la courbe (AUC) de 0 à ∞h qui décrit l'activité anti-facteur Xa dans le plasma du rat en fonction du temps à la dose de 2 mg /kg avec CTAB-LNC et SA-LNC a été respectivement de 78.7 et 42.5 min.mg /ml, et beaucoup plus élevé avec les doses les plus élevées à 730.8 ± 214.9 mg /ml * min ($p < 0.05$) comparativement à 138.21 mg. min /ml pour la dose IV. Le C max aux doses de 0.5, 2 et 5 mg / kg était respectivement de 0.02, 0.18, 0.98 UI /ml pour LNC-CTAB et de 0.02, 0.18 et 1.31 UI / ml pour LNC-SA, (le niveau thérapeutique d'activité anti-FXa est > 0.2 UI /ml), avec une demi-vie plus longue pour les préparations LNC-SA (jusqu'à 21 heures). Ces résultats *in vivo* ont démontré une biodisponibilité orale absolue allant de 3.09% à 21%. Les LNC cationiques ont démontré une biodisponibilité elevée ($P < 0.05$) de manière significative et une amélioration de la pharmacocinétique du médicament selon un mode dose-dépendant.

, les résultats de notre étude préliminaire montrent une amélioration de la biodisponibilité de principes actifs réputés non absorbables par l'utilisation des LNC. Les LNC cationiques chargées en F ont démontré une potentialité thérapeutique intéressante pour l'administration d'un pentasaccharide dérivé de l'héparine par voie orale, comme solution alternative à l'administration sous-cutanée utilisée actuellement. Après cette première preuve de concept, la formulation doit être modifiée afin d'améliorer la biodisponibilité orale du fondaparinux. Cette première preuve de concept peut générer des résultats cliniques intéressants. Cette formulation orale simple et nouvelle peut remplacer la voie injectable utilisée actuellement afin d'améliorer l'acceptabilité du traitement par les patients et l'effet thérapeutique, ce qui facilitera l'élaboration des calendriers de traitement chronique.

Enfin, Le développement de nouvelles formes pharmaceutiques telles que les LNC cationiques devrait permettre d'apporter des solutions à l administration parentérale ou orale, de principes actifs posant problème comme les peptides et les protéines .

En résumé, cette thèse met en évidence l'importance des transporteurs colloïdaux lipidiques et leurs implications pharmaceutiques dans la délivrance de principes actifs de nature différente, par voie orale et /ou par voie cutanée. Il existe plusieurs exemples de biomacromolécules utilisées de nos jours en thérapeutique, qui sont des candidats prometteurs pour une formulation sous forme de LBDDS, comme les nanoparticules lipidiques solides (SLN) et les nanocapsules lipidiques (LNC). Plusieurs méthodes de production ou de formulation peuvent être utilisées pour obtenir une efficacité élevée, due à une meilleure biodisponibilité orale et/ou cutanée.

Les mots clés : systèmes lipidiques de délivrance (LBDDS); nanoparticules lipidiques solides (SLN); nanocapsules lipidiques (LNC); transporteurs colloïdaux; propionate de clobétasol; pénétration cutanée ; hydrogel ; cutanée; température d'inversion de phase; fondaparinux; héparine; microémulsion; LNC cationiques; biodisponibilité; voie orale; anticoagulants; effet anti-Xa; pharmacocinétique.

REFERENCES

1. Muller, R.H. and Keck, C.M.," Challenges and solutions for the delivery of biotech drugs-a review of drug nanocrystal technology and lipid nanoparticles". ***J Biotechnol***, 2004,113.(1-3)151-70.
2. Chen, C., Tung-Hu, T., Zih-Rou, H., et al.," Effects of lipophilic emulsifiers on the oral administration of lovastatin from nanostructured lipid carriers: Physicochemical characterization and pharmacokinetics". ***Eur J Pharm Biopharm***, 2010,74 474–482.
3. Liu, D., Liu, C., Zou, W.*, et al.*," Enhanced gastrointestinal absorption of N3-O-toluyl-fluorouracil by cationic solid lipid nanoparticles". ***J Nano Res***, 2009,1.1-10.
4. Heurtault, B., Saulnier, P., Pech, B.*, et al.*," Physico-chemical stability of colloidal lipid particles". ***Biomaterials***, 2003,24.(23)4283-300.
5. Jannin, V., Musakhanian, J., and Marchaud, D.," Approaches for the development of solid and semi-solid lipid-based formulations". ***Adv Drug Deliv Rev***, 2008,60.734-746.
6. Porter, C., Trevaskis, N., and Charman, W.," Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. " ***Nat Rev Drug Discov***, 2007,6.231-248.
7. Pouton, C.," Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the Lipid Formulation Classification System." ***29***, 2006,Eur J Pharm Sci.278-287.
8. Yuan, H., Jiang, S.P., Du, Y.Z.*, et al.*," Strategic approaches for improving entrapment of hydrophilic peptide drugs by lipid nanoparticles". ***Coll Surf B:Biointerf***, 2009 70.(2)248-253.
9. Porter, C.J., Pouton, C.W., Cuine, J.F.*, et al.*," Enhancing intestinal drug solubilisation using lipid-based delivery systems". ***Adv Drug Deliv Rev***, 2008,60.(6)673-91.
10. Pouton, C.W. and Porter, C.J.," Formulation of lipid-based delivery systems for oral administration: materials, methods and strategies". ***Adv Drug Deliv Rev***, 2008,60.(6)625-37.
11. Martins, S., Sarmento, B., Ferreira, D.C.*, et al.*," Lipid-based colloidal carriers for peptide and protein delivery--liposomes versus lipid nanoparticles". ***Int J Nanomedicine***, 2007,2.(4)595-607.
12. Fricker, G., Kromp, T., Wendel, A.*, et al.*," Phospholipids and Lipid-Based Formulations in Oral Drug Delivery". ***Pharm Res***, 2010,6.97-99.
13. Date, A.A. and Nagarsenker, M.S.," Parenteral microemulsions: An overview. " ***Int J Pharm***, 2008,355 (1-2)19-30.
14. Joshi, M.D. and Müller, R.H.," Lipid nanoparticles for parenteral delivery of actives". ***Eur J Pharm Biopharm***, 2009,71 161-172.
15. Constantinides, P.P., chaubal, M.V., and Shorr, R.," Advances in lipid nanodispersions for parenteral drug delivery and targeting". ***Adv Drug Deliv Rev*** 2008,60.757-767.
16. Wissing, S.A., Kayser, O., and Muller, R.H.," Solid lipid nanoparticles for parenteral drug delivery". ***Adv Drug Deliv Rev***, 2004,56.(9)1257-72.
17. De Leeuw, J., DE Vijlder, H., Bjerring, P.*, et al.*," Liposomes in dermatology today". ***J Eur Acad Dermatol Venereol***, 2009,23.505-516.

18. Muller, R.H., Radtke, M., and Wissing, S.A.," Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations". ***Adv Drug Deliv Rev***, 2002,54 Suppl 1.S131-55.
19. Pardeike, J., Hommoss, A., and Muller, R.H.," Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products". ***Int J Pharm***, 2009,366.(1-2)170-84.
20. Cevc, G. and Vierl, U.," Nanotechnology and the transdermal route A state of the art review and critical appraisal". ***J Control Release***, 2009,1-23.
21. Gershkovich, P., Wasan, K., and Barta, C.," A review of the application of lipid-based systems in systemic, dermal/ transdermal, and ocular drug delivery". ***Crit Rev Ther Drug Carrier Syst***, 2008,25.(6)545-84.
22. Salama, R., Traini, D., Chan, H.-K.*, et al.*," Recent Advances in Controlled Release Pulmonary Therapy ". ***Curr Drug Deliv***, 2009, 6 (4)404-414.
23. Mehnert, W. and Mader, K.," Solid lipid nanoparticles: production, characterization and applications". ***Adv Drug Deliv Rev***, 2001,47.(2-3)165-96.
24. Hureaux, J., Lagarce, F., Gagnadoux, F.*, et al.*," Toxicological study and efficacy of blank and paclitaxel-loaded lipid nanocapsules after i.v. administration in mice". ***Pharm Res***, 2010,27.(3)421-30.
25. Collins-Gold, L., Feichtinger, N., and Wärnheim, T.," Are lipid emulsions the drug delivery solution?" ***Mod Drug Discov.***, 2000,3 44-48.
26. Bouchemal, K., Briançon, S., Perrier, E.*, et al.*," Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation". ***Int J Pharm*** , 2004,280.241-251.
27. Anton, N., Benoit, J.P., and Saulnier, P.," Design and production of nanoparticles formulated from nano-emulsion templates-a review". ***J Control Release***, 2008,128.(3)185-99.
28. Taylor, P.," Ostwald ripening in emulsions." ***Adv. Colloid Interface Sci.*** , 1998,75.107-163.
29. Lawrence, M.J. and Rees, G.D.," Microemulsion-based media as novel drug delivery systems". ***Adv Drug Deliv Rev***, 2000,45.(1)89-121.
30. Yin, Y.M., Cui, F.D., Mu, C.F.*, et al.*," Docetaxel microemulsion for enhanced oral bioavailability: preparation and in vitro and in vivo evaluation". ***J Control Release***, 2009,140.(2)86-94.
31. Kogan, A. and Garti, N.," Microemulsions as transdermal drug delivery vehicles". ***Adv.Colloid Interface Sci.***, 2006,123-126.369-385.
32. Graf, A., Rades, T., and Hook, S.," Oral insulin delivery using nanoparticles based on microemulsions with different structure-types: Optimisation and in vivo evaluation". ***Eur J Pharm Sci***, 2009,37.53-61.
33. Gursoy, R. and S., B.," Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. " ***Biomed Pharmacotherapy***, 2004,58.173-82.
34. Constantinides, P.P.," Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects". ***Pharm Res***, 1995,12.(11)1561-1572.
35. Balakrishnana, P., Leeb, B.-J., Oha, D.H.*, et al.*," Enhanced oral bioavailability of Coenzyme Q10 by self-emulsifying drug delivery systems". ***Int J Pharm*** 2009,374.66-72.

36. Rao, S.V., Yajurvedi, K., and Shao, J.," Self-nanoemulsifying drug delivery system (SNEDDS) for oral delivery of protein drugs: III. In vivo oral absorption study". ***Int J Pharm***, 2008,362.(1-2)16-9.
37. Zheng, J. and Fulu, M.-Y.," Decrease of genital organ weights and plasma testosterone levels in rats following oral administration of leuprolide microemulsion." ***Int J Pharm***, 2006,307.209-15.
38. Bangham, A., Standish, M., and Watkins, J.," The action of steroids and streptolysin S on the permeability of phospholipid structures to cations." ***J Mol Biol***, 1965,13.253-9.
39. Vemuri, S. and Rhodes, C.T.," Preparation and characterization of liposomes as therapeutic delivery systems: a review". ***Pharm. Acta Helvetiae*** 1995,70.95- 111.
40. Janknecht, R., Marie, S., Bakker-Woudenberg, I.*, et al.*," Liposomal and lipid formulations of amphotericin B". ***Clin Pharmacokinetic***, 1992,23.279-91.
41. Lasic, D.," Sterically stabilized liposomes in cancer therapy and gene delivery". ***Curr Opin Mol Ther***, 1999,1.177-85.
42. Mishra, B., Patel, B., and Tiwari, S.," Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery". ***Nanomed: Nanotech, Bio, and Med*** 2010,6 9-24.
43. Elsayed, M., Abdallah, O., Naggar, V.*, et al.*," Lipid vesicles for skin delivery of drugs: Reviewing three decades of research". ***Int J Pharm*** 2007,332 1-16.
44. Muller, R.H., Mader, K., and Gohla, S.," Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art". ***Eur J Pharm Biopharm***, 2000,50.(1)161-77.
45. Huynh, N.T., Passirani, C., Saulnier, P.*, et al.*," Lipid nanocapsules: A new platform for nanomedicine". ***Int J Pharm,*** 2009 379.(2)201-209.
46. Westesen, K. and Siekmann, B.," Investigation of the gel formation of phospholipid stabilized solid lipid nanoparticles". ***Int J Pharm,*** 1997,151. (May12)35-45.
47. 47. Reitmeier, H., Herrmann, J., and Gopferich, A.," Lipid microparticles as a parenteral controlled release device for peptides". ***Journal of Controlled Release*** 2001,73.339-350.
48. Petros, R.A. and Desimone, J.M.," Strategies in the design of nanoparticles for therapeutic applications". ***Nat Rev Drug Discov***, 2010.
49. Doktorovova, S., Gokce, E., Ozyazici, M.*, et al.*," Lipid matrix nanoparticles: Pharmacokinetics and biopharmaceutics". ***Current Nanoscience***, 2009 5.(3)358-371.
50. Uner, M. and Yener, G.," Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives". ***Int J Nanomedicine***, 2007,2.(3)289-300.
51. Muller, R., Radtke, M., and Wissing, S.," Nanostructured lipid matrices for improved microencapsulation of drugs." ***Int J Pharm***, 2002,242.121–8.
52. .Jenning, V., Mader, K., and Gohla, S.H.," Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a (1)H-NMR study". ***Int J Pharm***, 2000,205.(1-2)15-21.

53. Heurtault, B., Saulnier, P., Pech, B*., et al.*," A novel phase inversion-based process for the preparation of lipid nanocarriers". ***Pharm Res***, 2002 19. (6)875-880.
54. Heurtault B., Saulnier P., Pech B*., et al.* (2000) "*Lipidic nanocapsules: preparation process and use as Drug Delivery Systems*." WO02688000
55. Anton, N., Gayet, P., Benoit, J.P*., et al.*," Nano-emulsions and nanocapsules by the PIT method: an investigation on the role of the temperature cycling on the emulsion phase inversion". ***Int J Pharm***, 2007,344.(1-2)44-52.
56. Heurtault, B., Saulnier, P., Pech, B*., et al.*," Interfacial stability of lipid nanocapsules". ***Coll Surf B:Biointerf***, 2003,30.(3)225-235.
57. Dulieu, C. and Bazile, D.," Influence of lipid nanocapsules composition on their aptness to freeze-drying". ***Pharm Res***, 2005,22.(2)285-92.
58. Dubes, A., Parrot-Lopez, H., Abdelwahed, W*., et al.*," Scanning electron microscopy and atomic force microscopy imaging of solid lipid nanoparticles derived from amphiphilic cyclodextrins". ***Eur J Pharm Biopharm***, 2003,55. (3)279-82.
59. Patravale, V.B. and Ambarkhane, A.V.," Study of solid lipid nanoparticles with respect to particle size distribution and drug loading". ***Pharmazie***, 2003,58.(6)392-5.
60. Bunjes, H., Westesen, K., and Koch, M.H.," Crystallisation tendency and polymorphic transitions in triglyceride nanoparticles". ***Int J Pharm***, 1996,129.159-173.
61. Jenning, V., Thunemann, A.F., and Gohla, S.H.," Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids". ***Int J Pharm***, 2000,199.(2)167-77.
62. Bunjes, H., Koch, M.H., and Westesen, K.," Influence of emulsifiers on the crystallization of solid lipid nanoparticles". ***J Pharm Sci***, 2003,92.(7)1509-20.
63. Abdel-Mottaleb, M.M.A., Neumann, D., and Lamprecht, A.," In vitro drug release mechanism from lipid nanocapsules (LNC)". ***Int J Pharm,*** 2010,390.(2)208-213.
64. Cavalli, R., Caputo, O., Marengo, E*., et al.*," Effect of the components of microemulsions on both size and crystalline structure of solid lipid nanoparticles (SLN) containing a series of model molecules". ***Pharmazie,*** 1998,53.(6)392-396.
65. Olbrich, C. and Muller, R.H.," Enzymatic degradation of SLN-effect of surfactant and surfactant mixtures". ***Int J Pharm***, 1999,180.(1)31-9.
66. Illing, A. and Unruh, T.," Investigation on the flow behavior of dispersions of solid triglyceride nanoparticles". ***Int J Pharm***, 2004,284.(1-2)123-31.
67. Saulnier, P. and Benoit, J.P., *Lipidic Core Nanocapsules as New Drug Delivery Systems*, in *Nanoparticulates as drug carriers* 2006, Imperial college press: London. p. 213-224.
68. Minkov, I., Ivanova, T., Panaiotov, I*., et al.*," Reorganization of lipid nanocapsules at air-water interface. Part 2. Properties of the formed surface film". ***Coll Surf B:Biointerf***, 2005,44.(4)197-203.

69. Minkov, I., Ivanova, T., Panaiotov, I.*, et al.*," Reorganization of lipid nanocapsules at air–water interface I. Kinetics of surface film formation". ***Coll Surf B:Biointerf*** 2005,45.32–41.
70. Vonarbourg, A., Saulnier, P., Passirani, C.*, et al.*," Electrokinetic properties of noncharged lipid nanocapsules: influence of the dipolar distribution at the interface". ***Electrophoresis***, 2005,26.(11)2066-75.
71. Heurtault, B., Saulnier, P., Pech, B.*, et al.*," Properties of polyethylene glycol 660 12-hydroxy stearate at a triglyceride/water interface". ***Int J Pharm***, 2002,242.167-170.
72. Malzert-Freon, A., Vrignaud, S., Saulnier, P.*, et al.*," Formulation of sustained release nanoparticles loaded with a tripentone, a new anticancer agent". ***Int J Pharm***, 2006,320.(1-2)157-64.
73. Heurtault, B., Saulnier, P., Pech, B.*, et al.*," The influence of lipid nanocapsule composition on their size distribution". ***Eur J Pharm Sci***, 2003,18.(1)55-61.
74. Anton, N., Saulnier, P., Beduneau, A.*, et al.*," Salting-out effect induced by temperature cycling on a water/nonionic surfactant/oil system". ***J Phys Chem B***, 2007,111.(14)3651-7.
75. Hu, F.Q., Yuan, H., Zhang, H.H.*, et al.*," Preparation of solid lipid nanoparticles with clobetasol propionate by a novel solvent diffusion method in aqueous system and physicochemical characterization". ***Int J Pharm***, 2002,239.(1-2)121-8.
76. Trotta, M., Debernardi, F., and Caputo, O.," Preparation of solid lipid nanoparticles by a solvent emulsification-diffusion technique". ***Int J Pharm***, 2003,257.(1-2)153-60.
77. Anton, N. and Vandamme, T.F.," The universality of low-energy nano-emulsification". ***Int J Pharm***, 2009,377.(1-2)142-7.
78. Gasco, M.R.," Solid lipid nanospheres from warm microemulsions,". ***Pharm. Technol.Eur.*** , 1997,9.52-58.
79. Mao, S.R., Wang, Y.Z., Ji, H.Y.*, et al.*," [Preparation of solid lipid nanoparticles by microemulsion technique]". ***Yao Xue Xue Bao***, 2003,38.(8)624-6.
80. Sjostrom, B., Kaplun, A., Talmon, Y.*, et al.*," Structures of nanoparticles prepared from oil-in-water emulsions". ***Pharm Res***, 1995,12.(1)39-48.
81. Sjostrom, B., Westesen, K., and Bergenstahl, B.," Preparation of submicron drug particles in lecithin-stabilized o/w emulsions II. Characterization of cholesteryl acetate particles". ***Int J Pharm***, 1993,94.89-101.
82. Cortesi, R., Esposito, E., Luca, G.*, et al.*," Production of lipospheres as carriers for bioactive compounds". ***Biomaterials***, 2002,23.2283-2294.
83. Hu, F.Q., Jiang, S.P., Du, Y-Z, Yuang, H., Ye,Y-Q., Zeng, S.," Preparation and characterization of stearic acid nanostructuresd lipid carriers y solvent diffusion method in an aqueous system". ***Coll Surf B:Biointerf***, 2005,45.167-173.
84. Schubert, M.A. and Muller-Goymann, C.C.," Solvent injection as a new approach for manufacturing lipid nanoparticles--evaluation of the method and process parameters". ***Eur J Pharm Biopharm***, 2003,55.(1)125-31.

85. Pandita, D., Ahuja, A., Velpandian, T.*, et al.*," Characterization and in vitro assessment of paclitaxel loaded lipid nanoparticles formulated using modified solvent injection technique". ***Pharmazie***, 2009,64.(5)301-310.
86. Domb, A.," Long acting injectable oxytetracycline-liposphere formulation". ***Int J Pharm***, 1995,124.271-278.
87. Hou, D., Xie, C., Huang, K.*, et al.*," The production and characteristics of solid lipid nanoparticles (SLNs)". ***Biomaterials***, 2003,24.(10)1781-5.
88. Venkateswarlu, V. and Manjunath, K.," Preparation, characterization and in vitro release kinetics of clozapine solid lipid nanoparticles". ***J Control Release***, 2004,95.(3)627-38.
89. Charcosset, C., El-Harati, A., and Fessi, H.," Preparation of solid lipid nanoparticles using a membrane contactor". ***J Control Release***, 2005,108.112–20.
90. Muller, R.H., Radtke, M., and Wissing, S.A.," Nanostructured lipid matrices for improved microencapsulation of drugs". ***Int J Pharm***, 2002,242.(1-2)121-8.
91. Shinoda, K. and Saito, H.," The effect of temperature on the phase equilibria and the types of dispersions of the ternary system composed of water, cyclohexane, and nonionic surfactant". ***J Coll Interf Sci***, 1968,26.(1)70-74.
92. Shinoda, K. and Saito, H.," The Stability of O/W type emulsions as functions of temperature and the HLB of emulsifiers: The emulsification by PIT-method". ***J Coll Interf Sci***, 1969,30.(2)258-263.
93. Salager, J.L., Marquez, N., Graciaa, A.*, et al.*," Partitioning of ethoxylated octylphenol surfactants in microemulsion-oil-water systems: influence of temperature and relation between partitioning coefficient and physicochemical formulation". ***Langmuir***, 2000,16.(13)5534-5539.
94. Morales, D., Gutiérrez, J.M., García-Celma, M.J.*, et al.*," A study of the relation between bicontinuous microemulsions and oil/water nano-emulsion formation. " ***Langmuir***, 2003,19.7196–7200.
95. Forster, T., Von Rybinski, W., And Wadle, A.," Influence Of Microemulsion Phases On The Preparation Of Fine-Disperse Emulsions". ***Adv. Colloid Interface Sci.***, 1995,58.119-149.
96. Tadros, T., Izquierdo, P., Esquena, J.*, et al.*," Formation and stability of nano-emulsions". ***Adv. Colloid Interface Sci.*** , 2004,108.303-318.
97. Lamprecht, A., Bouligand, Y., and Benoit, J.P.," New lipid nanocapsules exhibit sustained release properties for amiodarone". ***J Control Release***, 2002 84.(1-2)59-68.
98. Winsor, P., Solvent Properties of Amphiphilic Compounds. 1954, Londres: Butterworth.
99. Liedtke, S., Wissing, S., Muller, R.*, et al.*," Influence of high pressure homogenisation equipment on nanodispersions characteristics". ***Int J Pharm***, 2000,196.183-185.
100. Yegin, B.A. and Lamprecht, A.," Lipid nanocapsule size analysis by hydrodynamic chromatography and photon correlation spectroscopy". ***Int J Pharm***, 2006 320.(1-2)165-170.
101. Jores, K., Mehnert, W., and Mader, K.," Physicochemical investigations on solid lipid nanoparticles and on oil-loaded solid lipid nanoparticles: a nuclear

magnetic resonance and electron spin resonance study". ***Pharm Res***, 2003,20.(8)1274-83.

102. Freitas, C. and Muller, R.H.," Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN) dispersions". ***Int J Pharm*** 1998,168.221-229.

103. Manconi, M., Aparicio, J., Vila, A.O.*, et al.*," Viscoelastic properties of concentrated dispersions in water of soy lecithin". ***Coll Surf A:Physico and Eng Asp*** 2003,222.(1-3)141-145.

104. Gref, R., Lück, M., Quellec, P.*, et al.*," 'Stealth' corona-core nanoparticle surface modifi ed by polyethylene glycol (PEG): infl uence of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption." ***Coll Surf B:Biointerf***, 2000,18.301-13.

105. Saulnier, P., Anton, N., Heurtault, B.*, et al.*," Liquid crystals and emulsions in the formulation of drug carriers". ***Comptes Rendus Chimie***, 2008,11.(3)221-228.

106. Westesen, K. and Bunjes, H.," Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix?" ***Int J Pharm*** 1995,115.129-131.

107. Gasco, M.R., Cavalli, R., and Carlotti, M.E.," Timolol in lipospheres". ***Pharmazie***, 1992,47.(2)119-121.

108. Cavalli, R., Caputo, O., and Gasco, M.R.," Preparation and characterization of dolid lipid nanospheres containing paclitaxel". ***European Journal of Pharmaceutical Sciences***, 2000,10.305-309.

109. Cavalli, R., Morel, S., Gasco, M.R.*, et al.*," Preparation and evaluation in vitro of colloidal lipospheres containing pilocarpine as ion pair". ***Int J Pharm***, 1995,117.243-246.

110. Cavalli, R., Paolozara, G., Caputo, O.*, et al.*," Transmucosal transport of tobramycin incorporated in SLN after duodenal administration to rats. Part 1-a pharmacokinetic study". ***Pharmacological research***, 2000,42.(6)541-545.

111. Igartua, M., Saulnier, P., Heurtault, B.*, et al.*," Development and characterization of solid lipid nanoparticles loaded with magnetite". ***Int J Pharm***, 2002,233.(1-2)149-57.

112. Wong, H.L., Bendayan, R., Rauth, A.M.*, et al.*," Development of solid lipid nanoparticles containing ionically complexed chemotherapeutic drugs and chemosensitizers". ***J Pharm Sci***, 2004,93.(8)1993-2008.

113. Morel, S., Ugazio, E., Cavalli, R.*, et al.*," Thymopentin in solid lipid nanoparticles". ***Int J Pharm***, 1996,132.259-261.

114. Morel, S., Terreno, E., Ugazio, E.*, et al.*," NMR relaxometric investigations of solid lipid nanoparticles (SLN) containing gadolinium(III) complexes". ***Eur J Pharm Biopharm***, 1998,45.(2)157-63.

115. Garcia-Fuentes, M., Torres, D., and Alonso, M.J.," Design Of Lipid Nanoparticles For The Oral Delivery Of Hydrophillic Macromolecules". ***Colloids and Surfaces B.:Biointerfaces***, 2002,27.159-168.

116. Zhang, N., Ping, Q., Huang, G.*, et al.*," Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin". ***Int J Pharm***, 2006,327.(1-2)153-9.

117. Olbrich, C., Gessner, A., Kayser, O., et al.," Lipid-drug-conjugate (LDC) nanoparticles as novel carrier system for the hydrophilic antitrypanosomal drug diminazenediaceturate". ***J Drug Target***, 2002,10.(5)387-96.

118. Garcia-Fuentes, M., Torres, D., and Alonso, M.J.," New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin". ***Int J Pharm***, 2005,296.(1-2)122-32.

119. Lim, S.J., Lee, M.K., and Kim, C.K.," Altered chemical and biological activities of all-trans retinoic acid incorporated in solid lipid nanoparticle powders". ***J Control Release***, 2004,100.(1)53-61.

120. Gessner, A., Olbrich, C., Schroder, W.*, et al.*," The role of plasma proteins in brain targeting: species dependent protein adsorption patterns on brain-specific lipid drug conjugate (LDC) nanoparticles ***Int J Pharm***, 2001,214.87-91.

121. Lamprecht, A., Saumet, J.L., Roux, J.*, et al.*," Lipid nanocarriers as drug delivery system for ibuprofen in pain treatment". ***Int J Pharm,*** 2004 278.(2)407-414.

122. Cahouet, A., Denizot, B., HindrÃ©, F.*, et al.*," Biodistribution of dual radiolabeled lipidic nanocapsules in the rat using scintigraphy and Î³ counting". ***Int J Pharm,*** 2002,242.(1-2)367-371.

123. Pereira de Oliveira, M., Garcion, E., Venisse, N.*, et al.*," Tissue distribution of indinavir administered as solid lipid nanocapsule formulation in mdr1a (+/+) and mdr1a (−/−) CF-1 mice. " ***Pharm. Res.***, 2005,22.1898-1905.

124. Lamprecht, A. and Benoit, J.P.," Etoposide nanocarriers suppress glioma cell growth by intracellular drug delivery and simultaneous P-glycoprotein inhibition". ***J Control Release***, 2006,112.(2)208-13.

125. Lacoeuille, F., Hindre, F., Moal, F.*, et al.*," In vivo evaluation of lipid nanocapsules as a promising colloidal carrier for paclitaxel". ***Int J Pharm,*** 2007 344.(1-2)143-149.

126. Allard, E., Passirani, C., Garcion, E.*, et al.*," Lipid nanocapsules loaded with an organometallic tamoxifen derivative as a novel drug-carrier system for experimental malignant gliomas". ***J Control Release***, 2008 130.(2)146-153.

127. Ballot S., Noiret N., Hindre F.*, et al.*," (99 m)Tc/(188)Re-labelled lipid nanocapsules as promising radiotracers for imaging and therapy: formulation and biodistribution". ***European Journal of Nuclear Medicine and Molecular Imaging***, 2006,1.6.

128. Morille, M., Montier, T., Legras, P.*, et al.*," Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting". ***Biomaterials***, 2010,31.(2)321-9.

129. Anton, N., Saulnier, P., Gaillard, C.*, et al.*," Aqueous-core lipid nanocapsules for encapsulating fragile hydrophilic and/or lipophilic molecules". ***Langmuir***, 2009,25.(19)11413-9.

130. Heurtault, B., Saulnier, P., Pech, B.*, et al.* (2000) *Lipidic nanocapsules: preparation process and use as Drug Delivery Systems.*

131. zur Muhlen, A., Schwarz, C., and Mehnert, W.," Solid lipid nanoparticles (SLN) for controlled drug delivery--drug release and release mechanism". ***Eur J Pharm Biopharm***, 1998,45.(2)149-55.

132. Muller, R.H., Mehnert, W., Lucks, J.S., *et al.*," Solid lipid nanoparticles (SLN)-An alternative colloidal carrier system for controlled drug delivery". ***Eur J Pharm Biopharm***, 1995,41.(1)62-69.
133. Uner, M. and Yener, G.," Importance of solid lipid nanoparticles (SLN) in various administration routes and future perspectives". ***Int. J. Nanomed.*** , 2007,2.289-300.
134. Sawant, K.K. and Dodiya, S.S.," Recent advances and patents on solid lipid nanoparticles". ***Recent Pat Drug Deliv Formul***, 2008,2.120-135.
135. Shenoy, V.S., Vijay, I.K., and Murthy, R.S.R.," Tumour targeting: biological factors and formulation advances in injectable lipid nanoparticles,". ***J. Pharm. Pharmacol.***, 2005,57 411-422.
136. Bondi, M.L., Azzolina, A., Craparo, E.F., *et al.*," Novel cationic solid-lipid nanoparticles as non-viral vectors for gene delivery". ***J Drug Target***, 2007,15.(4)295-301.
137. Yang, S.C., Lu, L.F., Cai, Y., *et al.*," Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain". ***J Control Release***, 1999,59.(3)299-307.
138. Heiati, H., Tawashi, R., and Phillips, N.C.," Solid lipid nanoparticles as drug carriers. Part 2. Plasma stability and biodistribution of solid lipid nanoparticles containing the lipophilic prodrug 3'-azido-3'-deoxythymidine palmitate in mice". ***Int J Pharm,*** 1998,174.(Nov 15)71-80.
139. Muller, R.H., Runge, S., Ravelli, V., *et al.*," Oral bioavailability of cyclosporine: solid lipid nanoparticles (SLN) versus drug nanocrystals". ***Int J Pharm***, 2006,317.(1)82-9.
140. Zara, G.P., Bargoni, A., Cavalli, R., *et al.*," Pharmacokinetics and tissue distribution of idarubicin-loaded solid lipid nanoparticles after duodenal administration to rats". ***J Pharm Sci***, 2002,91.(5)1324-33.
141. Yang, L., Geng, Y., Li, H., *et al.*," Enhancement the oral bioavailability of praziquantel by incorporation into solid lipid nanoparticles". ***Pharmazie***, 2009,64.86-9.
142. Garcia-Fuentes, M., Prego, C., Torres, D., *et al.*," A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery". ***Eur J Pharm Sci***, 2005,25.(1)133-43.
143. Zhang N, P.Q., Huang G, et al.," Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin". ***Int J Pharm***, 2006,327.153-9.
144. Porter, C.J.H., Kaukonen, A.M., Boyd, B.J., *et al.*," Susceptibility to lipase-mediated digestion reduces the oral bioavailability of danazol after administration as a medium-chain lipid-based microemulsion formulation". ***Pharm. Res.***, 2004,21.1405-1412.
145. Yang, S., Zhu, J., Lu, Y., *et al.*," Body distribution of camptothecin solid lipid nanoparticles after oral administration". ***Pharm Res***, 1999,16.(5)751-7.
146. Chakraborty, S., Shukla, D., Mishra, B., *et al.*," Lipid--an emerging platform for oral delivery of drugs with poor bioavailability". ***Eur J Pharm Biopharm***, 2009,73.(1)1-15.

147. Garcia-Fuentes, M., Torres, D., and Alonso, M.J.," New surface modified lipid nanoparticles as delivery vehicles for salmon calcitonin". ***Int J Pharm***, 2005,296.122-132.
148. Kuchler, S., Herrmann, W., Panek-Minkin, G.*, et al.*," SLN for topical application in skin diseases--characterization of drug-carrier and carrier-target interactions". ***Int J Pharm***, 2010,390.(2)225-33.
149. Jenning, V. and Gohla, S.H.," Encapsulation of retinoids in solid lipid nanoparticles (SLN)". ***J Microencapsul***, 2001,18.(2)149-58.
150. de Vringer, T. and de Ronde, H.A.," Preparation and structure of a water-in-oil cream containing lipid nanoparticles". ***J Pharm Sci***, 1995,84.(4)466-72.
151. Wissing, S.A. and Muller, R.H.," The influence of solid lipid nanoparticles on skin hydration and viscoelasticity--in vivo study". ***Eur J Pharm Biopharm***, 2003,56.(1)67-72.
152. Dingler, A., Blum, R.P., Niehus, H.*, et al.*," Solid lipid nanoparticles (SLN/Lipopearls)--a pharmaceutical and cosmetic carrier for the application of vitamin E in dermal products". ***J Microencapsul***, 1999,16.(6)751-67.
153. Wissing, S.A. and Muller, R.H.," Solid lipid nanoparticles (SLN)--a novel carrier for UV blockers". ***Pharmazie***, 2001,56.(10)783-6.
154. Jenning, V., Schafer-Korting, M., and Gohla, S.," Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties". ***J Control Release***, 2000,66.(2-3)115-26.
155. Souto, E.B., Wissing, S.A., Barbosa, C.M.*, et al.*," Development of a controlled release formulation based on SLN and NLC for topical clotrimazole delivery". ***Int J Pharm***, 2004,278.(1)71-7.
156. Mei, Z., Chen, H., Weng, T.*, et al.*," Solid lipid nanoparticle and microemulsion for topical delivery of triptolide". ***Eur J Pharm Biopharm***, 2003,56.(2)189-96.
157. Chen, H., Chang, X., Du, D.*, et al.*," Podophyllotoxin-loaded solid lipid nanoparticles for epidermal targeting". ***J Control Release***, 2006,110.(2)296-306.
158. Munster, U., Nakamura, C., Haberland, A.*, et al.*," RU 58841-myristate--prodrug development for topical treatment of acne and androgenetic alopecia". ***Pharmazie***, 2005,60.(1)8-12.
159. Lombardi Borgia, S., Regehly, M., Sivaramakrishnan, R.*, et al.*," Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy". ***J Control Release***, 2005,110.(1)151-63.
160. Pandey, R. and Khuller, G.," Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis". ***Tuberculosis***, 2005,85.227-34.
161. Sznitowska, M., Janicki, S., Gajewska, M.*, et al.*," Investigation of diazepam lipospheres based on Witepsol and lecithin for oral or rectal delivery." ***Acta Polon Pharm***, 2000,57.61-4.
162. Vila, A., Gill , H., McCallion, O.*, et al.*," Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density." ***J Control Release***, 2004,98.231-44.
163. Cavalli, R., Gasco, M.R., Chetoni, P.*, et al.*," Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin". ***Int J Pharm***, 2002,238.(1-2)241-5.

164. Muchow, M., Maincent, P., and Muller, R.H.," Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery". ***Drug Dev Ind Pharm***, 2008,34.(12)1394-405.

165. Müller, R.H., Petersen, R.D., Hommoss, A.*, et al.*," Nanostructured lipid carriers (NLC) in cosmetic dermal products". ***Adv. Drug Deliv. Rev.***, 2007,59.(6)522-530.

166. Maia, C.S., Mehnert, W., Schaller, M.*, et al.*," Drug targeting by solid lipid nanoparticles for dermal use". ***J Drug Target***, 2002,10.(6)489-495.

167. Pardeike, J. and Müller, R.H.," Coenzyme Q10-loaded NLCs: preparation, occlusive properties and penetration enhancement". ***Pharm. Technol. Eur.***, 2007,01.(online journal).

168. Obeidat, W.M., Schwabe, K., Müller, R.H.*, et al.*," Preservation of nanostructured lipid carriers (NLC)". ***Eur J Pharm Biopharm***, 2010.

169. Beduneau, A., Saulnier, P., Anton, N.*, et al.*," Pegylated nanocapsules produced by an organic solvent-free method: Evaluation of their stealth properties". ***Pharm Res***, 2006,23.(9)2190-9.

170. Hoarau, D., Delmas, P., David, S.*, et al.*," Novel long-circulating lipid nanocapsules". ***Pharmaceutical Research***, 2004 21.(10)1783-1789.

171. Garcion, E., Lamprecht, A., Heurtault, B.*, et al.*," A new generation of anticancer, drug-loaded, colloidal vectors reverses multidrug resistance in glioma and reduces tumor progression in rats". ***Molecular Cancer Therapeutics***, 2006 5.(7)1710-1722.

172. Hureaux J., Lagarce F., Gagnadoux F.*, et al.*," The adaptation of lipid nanocapsule formulations for blood administration in animals". ***Int J Pharm***, 2009,379.266–269

173. Peltier, S., Oger, J.M., Lagarce, F.*, et al.*," Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules". ***Pharmaceutical Research***, 2006 23.(6)1243-1250.

174. Roger, E., Lagarce, F., and Benoit, J.P.," The gastrointestinal stability of lipid nanocapsules". ***Int J Pharm***, 2009 379.(2)260-265.

175. Roger, E., Lagarce, F., Garcion, E.*, et al.*," Reciprocal competition between lipid nanocapsules and P-gp for paclitaxel transport across Caco-2 cells". ***European Journal of Pharmaceutical Sciences***, 2010.

176. Roger, E., Lagarce, F., Garcion, E.*, et al.*," Lipid nanocarriers improve paclitaxel transport throughout human intestinal epithelial cells by using vesicle-mediated transcytosis". ***J Control Release***, 2009,140.(2)174-81.

177. Hureaux, J., Lagarce, F., Gagnadoux, F.*, et al.*," Lipid nanocapsules: ready-to-use nanovectors for the aerosol delivery of paclitaxel". ***Eur J Pharm Biopharm***, 2009,73.(2)239-46.

178. Scheper, V., Wolf, M., Scholl, M.*, et al.*," Potential novel drug carriers for inner ear treatment: Hyperbranched polylysine and lipid nanocapsules". ***Nanomedicine***, 2009 4.(6)623-635.

179. Zou, J., Saulnier, P., Perrier, T.*, et al.*," Distribution of Lipid Nanocapsules in Different Cochlear Cell Populations After Round Window Membrane Permeation". ***J Biomed Mater Res Part B: Appl Biomater***, 2008,87B.10-18.

180. Kuchler, S., Abdel-Mottaleb, M., Lamprecht, A.*, et al.*," Influence of nanocarrier type and size on skin delivery of hydrophilic agents". ***Int J Pharm***, 2009,377.(1-2)169-72.

181. Coon, J.S., Knudson, W., Clodfelter, K.*, et al.*," Solutol HS 15, nontoxic polyoxyethylene esters of 12-hydroxystearic acid, reverses multidrug resistance". ***Cancer Res***, 1991,51.(3)897-902.

182. Vonarbourg, A., Passirani, C., Saulnier, P.*, et al.*," Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake". ***Journal of Biomedical Materials Research - Part A***, 2006,78.(3)620-628.

183. Perrier, T., Saulnier, P., Fouchet, F.*, et al.*," Post-insertion into Lipid NanoCapsules (LNCs): From experimental aspects to mechanisms". ***Int J Pharm***, 2010.

184. Khalid M., Simard P., Hoarau D.*, et al.*," Long Circulating Poly(Ethylene Glycol)-Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors". ***Pharmaceutical Research, Vol. 23, No. 4, April*** 2006,23.(4)752-758.

185. Goppert, T.M. and Muller, R.H.," Plasma protein adsorption of Tween 80- and poloxamer 188-stabilized solid lipid nanoparticles". ***J Drug Target***, 2003,11.(4)225-31.

186. Muller, R., Souto, E., Goppert, T.*, et al.*," Production of biofunctionalized solid lipid nanoparticles for site-specific drug delivery. " ***Nanotechnologies for the life sciences biological and pharmaceutical nanomaterials***, 2005,291-307.

187. Béduneau A., Saulnier P., F., H.*, et al.*," Design of targeted lipid nanocapsules by conjugation of whole antibodies and antibody Fab' fragments". ***Biomaterials*** 2007,28.4978-4990.

188. Allard, E., Passirani, C., and Benoit, J.P.," Convection-enhanced delivery of nanocarriers for the treatment of brain tumors". ***Biomaterials***, 2009,30.2302-2318.

189. Allard, E., Jarnet, D., VessiÃ¨res, A.*, et al.*," Local delivery of ferrociphenol lipid nanocapsules followed by external radiotherapy as a synergistic treatment against intracranial 9L glioma xenograft". ***Pharmaceutical Research***, 2010,27.(1)56-64.

190. Müller, R.H. and Keck, C.M.," Drug delivery to the brain-realization by novel drug carriers". ***J. Nanosci. Nanotechnol.***, 2004, 4.471-483.

191. Zara, G.P., Cavalli, R., Bargoni, A.*, et al.*," Intravenous administration to rabbits of non-stealth and stealth doxorubicin-loaded solid lipid nanoparticles at increasing concentrations of stealth agent: pharmacokinetics and distribution of doxorubicin in brain and other tissues". ***J Drug Target***, 2002,10.(4)327-35.

192. Kaur, I.P., Bhandari, R., Bhandari, S.*, et al.*," Potential of solid lipid nanoparticles in brain targeting". ***J. Control. Release***, 2008,127.97-109.

193. Podio, V., Zara, G.P., Carazzonet, M.*, et al.*," Biodistribution of stealth and non-stealth solid lipid nanospheres after intravenous administration to rats". ***J. Pharm. Pharmacol.*** , 2000,52.1057-1063.

194. Gupta, A., Jain, S., and Jain, K.," Transferrin-conjugated solid lipid nanoparticles for enhanced delivery of quinine dihydrochloride to the brain". ***J. Pharm. Pharmacol.*** , 2007,59 935-940.

195. Fontana, G., Maniscalco, L., Schillaci, D.*, et al.*," Solid lipid nanoparticles containing tamoxifen characterization and in vitro antitumoral activity". ***Drug Deliv.*** , 2005,12.385-392.
196. Paillard, A., Hindré, F., Vignes-Colombeix, C.*, et al.*," The importance of endo-lysosomal escape with lipid nanocapsules for drug subcellular bioavailability". ***Biomaterials***, 2010,1-13.
197. Morille, M., Passirani, C., Vonarbourg, A.*, et al.*," Progress in developing cationic vectors for non-viral systemic gene therapy against cancer". ***Biomaterials***, 2008,29.(24-25)3477-96.
198. Tabatt, K., Sameti, M., Olbrich, C.*, et al.*," Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer". ***Eur J Pharm Biopharm***, 2004,57.(2)155-62.
199. Vonarbourg, A., Passirani, C., Desigaux, L.*, et al.*," The encapsulation of DNA molecules within biomimetic lipid nanocapsules". ***Biomaterials***, 2009 30. (18)3197-3204.
200. Morille, M., Montier, T., Legras, P.*, et al.*," Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting". ***Biomaterials***, 2009,31. (2)321-329.
201. Morille, M., Passirani, C., Letrou-Bonneval, E.*, et al.*," Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting". ***Int J Pharm***, 2009,379.(2)293-300.
202. Morille, M., Montier, T., Legras, P.*, et al.*," Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting". ***Biomaterials***, 2010,31. (2)321-329.

203. Müller, R.H., Maaen, S., Weyhers, H.*, et al.*," Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles". ***Int J Pharm***, 1996,138 85-94.
204. Peira, E., Marzola, P., Podio, V.*, et al.*," In vitro and in vivo study of solid lipid nanoparticles loaded with superparamagnetic iron oxide". ***J Drug Target***, 2003,11.(1)19-24.
205. Liu, W., He, Z., Liang, J.*, et al.*," Preparation and characterization of novel fluorescent nanocomposite particles: CdSe/ZnS core-shell quantum dots loaded solid lipid nanoparticles". ***J. Biomed. Mater. Res.***, 2008,84.1018–1025.
206. Jestin, E., Mougin-Degraef, M., Faivre-Chauvet, A.*, et al.*," Radiolabeling and targeting of lipidic nanocapsules for applications in radioimmunotherapy". ***Nucl. Med. Mol. Imaging*** 2007, 51.51–60.
207. Allard, E., Hindre, F., Passirani, C.*, et al.*," 188Re-loaded lipid nanocapsules as a promising radiopharmaceutical carrier for internal radiotherapy of malignant gliomas." ***Eur. J. Nucl. Med. Mol. Imaging***, 2008a.,35.1838-1846.
208. Lim, S.J. and Kim, C.K.," Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid". ***Int J Pharm***, 2002,243.(1-2)135-46.
209. Freitas, C. and Mullera, R.H.," Spray-drying of solid lipid nanoparticles (SLN TM)". ***Eur J Pharm Biopharm***, 1998,46.(2)145-51.
210. Westesen, K., Bunjes, H., and Koch, M.H.," Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading

capacity and sustained release potential". ***Journal of controlled Release***, 1997,48.223-236.

211. Freitas, C. and Muller, R.H.," Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase". ***Eur J Pharm Biopharm***, 1999,47.(2)125-32.

212. Freitas, C., Mehnert, W., and Müller, R.H.," Effect of storage conditions on long-term stability of solid lipid nanoparticles (SLN) in aqueous dispersion." ***Eur. J. Pharm. Sci.***, 1994,177.(2).

213. Izquierdo, P., Esquena, J., Tadros, T.F*., et al.*," Phase behavior and nano-emulsion formation by the phase inversion temperature method". ***Langmuir***, 2004,20.(16)6594-8.

214. Schubert, M.A. and Muller-Goymann, C.C.," Characterisation of surface-modified solid lipid nanoparticles (SLN): influence of lecithin and nonionic emulsifier". ***Eur J Pharm Biopharm***, 2005,61.(1-2)77-86.

215. Olbrich, C., Scholer, N., Tabatt, K*., et al.*," Cytotoxicity studies of Dynasan 114 solid lipid nanoparticles (SLN) on RAW 264.7 macrophages-impact of phagocytosis on viability and cytokine production". ***J Pharm Pharmacol***, 2004,56.(7)883-91.

216. Pinto, J.F. and Muller, R.H.," Pellets as carriers of solid lipid nanoparticles (SLN) for oral administration of drugs". ***Pharmazie (Die-Pharmazie)***, 1999,54.(Jul)506-509.

217. Zimmermann, E., Muller, R.H., and Mader, K.," Influence of different parameters on reconstitution of lyophilized SLN". ***Int J Pharm***, 2000,196. (2)211-3.

218. Dingler, A. and Gohla, S.," Production of solid lipid nanoparticles (SLN): scaling up feasibilities". ***J Microencapsul***, 2002,19.(1)11-6.

219. Marengo, E., Cavalli, R., Caputo, O*., et al.*," Scale up of the preparation process of solid lipid nanospheres:part I." ***Int J Pharm***, 2000,205.3-13.

220. Cavalli, R., Caputo, O., Carlotti, M.E*., et al.*," Sterilization and freeze drying of drug-free and drug-loaded solid lipid nanoparticles". ***Int J Pharm***, 1997,148.(Mar14)47-54.

221. Billich, A., Aschauer, H., Aszodi, A*., et al.*," Percutaneous absorption of drugs used in atopic eczema: pimecrolimus permeates less through skin than corticosteroids and tacrolimus". ***Int J Pharm***, 2004,269.29-35.

222. Mueller, B., Anissimov, Y.G., and Roberts, M.S.," Unexpected clobetasol propionate profile in human stratum corneum after topical application in vitro". ***Pharma Res***, 2003,20.(11)1835-1837.

223. Tsai, J.-C.," content and transdermal delivery of clobetasol 17-propionate from commercial creams and ointments". ***J Food Drug Analys***, 2002,10.(1)7-12.

224. Schafer-Korting, M., Mehnert, W., and Korting, H.C.," Lipid nanoparticles for improved topical application of drugs for skin diseases". ***Adv Drug Deliv Rev***, 2007,59.(6)427-43.

225. Manconi, M., Sinico, C., Valenti, D*., et al.*," Niosomes as carriers for tretinoin. III. A study into the in vitro cutaneous delivery of vesicle-incorporated tretinoin." ***Int J Pharm***, 2006,311.(1-2)11-9.

226. Bhalekar, M.R., Pokharkar, V., Madgulkar, A*., et al.*," Preparation and evaluation of miconazole nitrate-loaded solid lipid nanoparticles for topical delivery". ***AAPS PharmSciTech***, 2009,10.(1)289-96.

227. Puglia, C., Blasi, P., Rizza, L.*, et al.*," Lipid nanoparticles for prolonged topical delivery: an in vitro and in vivo investigation". ***Int J Pharm***, 2008,357.(1-2)295-304.

228. Kuchler, S., Radowski, M.R., Blaschke, T.*, et al.*," Nanoparticles for skin penetration enhancement--a comparison of a dendritic core-multishell-nanotransporter and solid lipid nanoparticles". ***Eur J Pharm Biopharm***, 2009,71.(2)243-50.

229. Kuntsche, J., Bunjes, H., Fahr, A.*, et al.*," Interaction of lipid nanoparticles with human epidermis and an organotypic cell culture model". ***Int J Pharm***, 2008,354.180–195.

230. Souto, E.B. and Muller, R.H.," Cosmetic features and applications of lipid nanoparticles (SLN, NLC)". ***Int J Cosmet Sci***, 2008,30.(3)157-65.

231. Jenning, V., Gysler, A., Schafer-Korting, M.*, et al.*," Vitamin A loaded solid lipid nanoparticles for topical use: occlusive properties and drug targeting to the upper skin". ***Eur J Pharm Biopharm***, 2000,49.(3)211-8.

232. Maia, C.S., Mehnert, W., and Schafer-Korting, M.," Solid lipid nanoparticles as drug carriers for topical glucocorticoids". ***Int J Pharm***, 2000,196.(2)165-7.

233. Sivaramakrishnan, R., Nakamura, C., Mehnert, W.*, et al.*," Glucocorticoid entrapment into lipid carriers--characterisation by parelectric spectroscopy and influence on dermal uptake". ***J Control Release***, 2004,97.(3)493-502.

234. Wissing, S.A. and Muller, R.H.," Solid lipid nanoparticles as carrier for sunscreens: in vitro release and in vivo skin penetration". ***J Control Release***, 2002,81.(3)225-33.

235. Teichmann, A., Jacobi, U., Ossadnik, M.*, et al.*," Differential stripping: determination of the amount of topically applied substances penetrated into the hair follicles". ***J. Invest. Dermatol.*** , 2005,125.264-269.

236. Liu, T.Y., Huang, L.Y., Hu, S.H.*, et al.*," Core-shell magnetic nanoparticles of heparin conjugate as recycling anticoagulants". ***J of Biomed Nanotech***, 2007,3.(4)353-359.

237. Stecova, J., Mehnert, W., Blaschke, T.*, et al.*," Cyproterone acetate loading to lipid nanoparticles for topical acne treatment: particle characterisation and skin uptake". ***Pharm Res***, 2007,24.(5)991-1000.

238. Wissing, S.A. and Muller, R.H.," Cosmetic applications for solid lipid nanoparticles (SLN)". ***Int J Pharm***, 2003,254.(1)65-8.

239. Lv, Q., Yu, A., Xi, Y.*, et al.*," Development and evaluation of penciclovir-loaded solid lipid nanoparticles for topical delivery". ***Int J Pharm***, 2009,372.(1-2)191-8.

240. Fang, J.Y., Fang, C.L., Liu, C.H.*, et al.*," Lipid nanoparticles as vehicles for topical psoralen delivery: solid lipid nanoparticles (SLN) versus nanostructured lipid carriers (NLC)". ***Eur J Pharm Biopharm***, 2008,70.(2)633-40.

241. Atrux-Tallau, N., Denis, A., Padois, K.*, et al.*," Skin Absorption Modulation: Innovative Non-Hazardous Technologies for Topical Formulations". ***The Open Dermatology Journal***, 2010,4.3-9.

242. Hu, F.Q., Jiang, S.P., Du, Y.Z.*, et al.*," Preparation and characteristics of monostearin nanostructured lipid carriers". ***Int J Pharm***, 2006,314.(1)83-9.

243. Castelli, F., Puglia, C., Sarpietro, M.G*., et al.*," Characterization of indomethacin-loaded lipid nanoparticles by differential scanning calorimetry. " ***Int J Pharm***, 2005,304.(1-2)231-8.

244. Zhang, H.J., Wang, X.F., Wang, P*., et al.*," Spectroscopic study on the binding of a cationic porphyrin to DNA G-quadruplex under different K+ concentrations". ***Photochem Photobiol Sci***, 2008,7.(8)948-55.

245. Wiedersberg, S., Leopold, C.S., and Guy, R.H.," Bioavailability and bioequivalence of topical glucocorticoids." ***Eur. J. Pharm. Biopharm.*** , 2008,68.453-466.

246. Senyigit, T., Padula, C., Ozer, O*., et al.*," Different approaches for improving skin accumulation of topical corticosteroids". ***Int J Pharm***, 2009,380.155-160.

247. Lebwohl, M.," the role of salicylic acid in the treatment of psoriasis". ***Int J dermatol***, 1999,38.16-24.

248. Gordon, M.L.," The role of clobetasol propionate emoolient 0.05% in the treatment of patients with dry, scaly, corticosteroid-responsive dermatoses". ***clinical therapeutics***, 1998,20.(1)26-39.

249. Fang, J.Y., shen, K., Huang, Y.-B*., et al.*," Evaluation of topical application of clobetasol 17-propionate from various cream bases". ***Drug Dev Ind Pharm***, 1999,25.(1)7-14.

250. Vazquez-Lopez, F. and Marghoob, A.," Dermoscop[ic assessment of long term topical therapies with potent steoids in chronic patients". ***J Am Acad Dermatol***, 2004,51.(5)811-3.

251. Jacobs, M., Martin, G.P., and Marriot, C.," Effects of phosphatidylcholine on the topical bioavailability of corticosteoirds assessed by the human skin blanching assay". ***J Pharm Pharmacol***, 1988,40.829-833

252. Senyigit, T., Sonvico, F., Barbieri, S*., et al.*," Lecithin/chitosan nanoparticles of clobetasol-17-propionate capable of accumulation in pig skin". ***J Control Release***, 2010,142.(3)368-73.

253. Mezei, M., Touitou, E., junginger, H.E*., et al.*," Liposomes as carriers for topical and transdermal delivery". ***J.Pharm. sci***, 1994,9.1189-1203.

254. Krowczynski, L. and Stozek, T.," Liposomes as drug vehicles in percutaneous therapy". ***Pharmazie***, 1984,39.(9)627-9.

255. Cevc, G. and Blume, G.," Biological activity and characteristics of triamcinolone- acetonide formulated with the self-regulating drug carriers, Transfersomes". ***Biochim. Biophys. Acta*** 2003,1614.156-164.

256. Mezei, M. and Gulasekharam, V.," Liposomes, a selective drug delivery system for the topical route of administartion:lotion dosage from"". ***Life SCi***, 1980,26.(18)1473-1477.

257. Gampisi, G., Giandalia, G., De Caro, V*., et al.*," A new delivery system of clobetasol 17-propionate (lipid loaded microspheres 0.025%) compared with a conventional formulation (lipophilic ointment in a hydrophilic phase 0.02%) nin topical treatmet of atrophic/erosive oral lichen planus. a phase iv, randomized, observed-blinded, parallel group clinical trial". ***Br J Dermatol***, 2004,150.984-990.

258. Gysler, A., Kleuser, B., Sippl, W*., et al.*," Skin penetration and metabolism of topical orticosteroids in reconstructed epidermis and excisd human skin". ***Pharm Res***, 1999,16.(9)1386-1391.

259. Lebwohl, M., Lombardi, K., and Tan, M.-H.," Duration of improvement in psoriasis after treatment with tazarotene 0.1%gel plus clobetasol propionate 0.05% ointment: comparison of mainteneace treatments". ***Int J Dermatol***, 2001,40.(1)64.

260. Bovenschen, H.J. and *et al*., "Selective persistence of dermal CD8+Tcells in Lesional plaque Psoriasis after clobetasol -17-propionate treatment". ***Acta Derm Venereol.***, 2005,85.113-117.

261. Feldman, S.R. and Yentzer, B.A.," Topical clobetasol propionate in the treatment of psoriasis: a review of newer formulations". ***Am J Clin Dermatol***, 2009,10.(6)397-406.

262. Franz T.J.," Clobetasol propionate foam 0.05% a novel vehicle with enhanced delivery". ***Int J dermatol***, 2000,39.521-538.

263. Franz, T.J., Lehman, P.A., Feldman, S.R*., et al.*," Bioavailability of clobetasol propionate in different vehicles". ***Skin Pharmacol Appl Skin Physiol***, 2003,16.(4)212-6.

264. Kalariya, M., Padhi, B.K., Chougule, M*., et al.*," Clobetasol propionate solid lipid nanoparticles cream for effective treatment of eczema: formulation and clinical implications". ***Indian J Exp Biol***, 2005,43.(3)233-40.

265. Kumar, V.V., Chandrasekar, D., Ramakrishna, S*., et al.*," Development and evaluation of nitrendipine loaded solid lipid nanoparticles: influence of wax and glyceride lipids on plasma pharmacokinetics". ***Int J Pharm***, 2007,335.(1-2)167-75.

266. Castelli, F., Puglia, C., Sarpietro, M.G., Rizza, L., Bonina, F.," Characterization of indomethacin-loaded lipid annoparticles by differential scanning calorimetry". ***Int J Pharm***, 2005,304.231-238.

267. Jain, S.K., Chourasia, M. K., Masuriha, R., Soni, V., Lain, A., Jain, N.K., Gupta, Y.," Solid Lipid Nanoparticles bearing flurbiprofen for transdermal delivery". ***Drug Delivery***, 2005,12.207-215.

268. Garmise, R.J., Mar, K., Crowder, T.M*., et al.*," Formulation of a dry powder influenza vaccine for nasal delivery". ***AAPS PharmSciTech***, 2006,7. (1)E1-E7.

269. Liu, J., Hu, W., Chen, H*., et al.*," Isotretinoin-loaded solid lipid nanoparticles with skin targeting for topical delivery". ***Int J Pharm***, 2007,328.(2)191-5.

270. Yang, S.C. and Zhu, J.B.," Preparation and characterization of camptothecin solid lipid nanoparticles". ***Drug Dev Ind Pharm***, 2002,28.(3)265-74.

271. Reddy, L.H., Murthy, R.S.R.," Etoposide-loaded nanoparticles made from glyceride lipids:Formulation, characterization , in vitro drug release, and stability evaluation". ***AAPS pharmSciTech***, 2005,6.(2)E 158-166.

272. Santoyo, S., Jalon, E.G., Campanero, M.A., Ygartua, P.," Determination of cidovir in both skin layers and percutaneous penetration samples by HPLC". ***J. Pharm. Biomed. Analysis***, 2002,29.819-826.

273. Barakat,H.S., Darwish, I. A., El-Khordagui, L. K.,*et al*.,"Development of Naftifine Hydrochloride Alcohol-Free Niosome Gel". ***Drug Dev Ind Pharm,*** 2009,35.631-637.

274. Mao, S.R., Wang, P., and Bi, D.Z.," Investigations on 5-fluorouracil solid lipid nanoparticles (SLN) prepared by hot homogenization". ***Pharmazie***, 2005,60. (4)273-277.

275. Gasco, M.R. (1993) *Method for producing solid lipid microsphers having a narrow size distribution.*patent No.USS188
276. Masini, V., Bonte, F., Meybeck, A.*, et al.*," Cutaneous bioavailability in hairless rats of tretinoin in liposomes or gel". ***J. Pharm. Sci.***, 1993,82.17-21.
277. Patel, V.B., Misra, A., and Marfatia, Y.S.," Topical liposomal gel of tretionin for the treatment of acne: research and clinical implications". ***Pharm. Dev. Technol.*** , 2000,5 455–464.
278. Tursilli, R., Piel, G., and delattre, L.," Solid lipid microparticles containing the sunscreen agent , octyl-dimethylaminobenzoate:Effect of the vehicle". ***Eur J Pharm Biopharm***, 2007,66.483-487.
279. Sanna, V., Gavini, E., Cossu, M.*, et al.*," Solid lipid nanoparticles (SLN) as carriers for the topical delivery of econazole nitrate: in-vitro characterization, ex-vivo and in-vivo studies. " ***J. Pharm. Pharmacol.***, 2007, 59.1057-1064.
280. Olbrich, C., Kayser, O., and Muller, R.H.," Lipase degradation of Dynasan 114 and 116 solid lipid nanoparticles (SLN)--effect of surfactants, storage time and crystallinity". ***Int J Pharm***, 2002,237.(1-2)119-28.
281. Jores, K., Mehnert, W., Drechsler, M.*, et al.*," Investigations on the structure of solid lipid nanoparticles (SLN) and oil-loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy". ***J Control Release***, 2004,95.(2)217-27.
282. Jensen, L.B., Magnussson, E., Gunnarsson, L.*, et al.*," Corticosteroid solubility and lipid polarity control release from solid lipid nanoparticles". ***Int J Pharm***, 2010,390.(1)53-60.
283. Ahlin, P., Kristl, J., and Smid Korbar, J.," Optimization of procedure parameters and physical stability of solid lipid nanoparticles in dispersions". ***Acta Pharm.***, 1998,48.259-267.
284. Schwarz, C. and Mehnert, W.," Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization". ***J Microencapsul***, 1999,16.(2)205-13.
285. Levy, M.Y., Schutze, W., Fuhrer, C.*, et al.*," Characterization of diazepam submicron emulsion interface: role of oleic acid". ***J. Microencapsul.***, 1994,11.79- 92.
286. Zur Muhlen, A. and Mehnert, W.," Drug release and release mechanism of prednisolone loaded solid lipid nanoparticles". ***Pharmazie***, 1998,53.552-555.
287. Westesen, K. and Bunjes, H.," Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix?" ***Int J Pharm***, 1995,115.129-131.
288. Borgia, S.L., Regehly, M.,Sivaramakrishnan,R.,Mehnert, W., Korting, H.C., ..." Lipid nanoparticles for skin penetration enhancement-correleation to drug localization within particle matrix as determined y flurescence and paraelectric spectroscopy". ***J Control Release***, 2005,110.151-163.
289. Lebwohl, M., Sherer, D., Washenik, K.*, et al.*," a randomized, double-blind, placebo-controlled study of clobetasol propionate 0.05% foam in the treatment of nonscalp psoriasis". ***Int J dermatol***, 2002,41.269-274.
290. Souto, E.B., Wissing, S.A., Barbosa, C.M.*, et al.*," Evaluation of the physical stability of SLN and NLC before and after incorporation into hydrogel formulations". ***Eur J Pharm Biopharm***, 2004,58.(1)83-90.

291. Mei, Z., Wu, Q., Hu, S., Li, X., Yang, X.," Triptolide loaded solid lipid nanoparticle hydrogel for topical application". ***Drug Dev Ind Pharm***, 2005,31.161-168.
292. Lippacher, A., Muller, R.H., and Mader, K.," Semisolid SLN dispersions for topical application:influence of formulation and production parameters on microstructure". ***Eur J Pharm Biopharm***, 2002,53.(2)155-160.
293. Kalariya, M., Padhi, B., Chougul, M.*, et al.*," Methotrxate-loaded solid lipid nanoparticles for topical treatent of psoriasis: formulation and clinical implications". ***Drug Deliv tech***, 2005,.....7-19.
294. Gulbake, A., Jain, A., Khare, P.*, et al.*," Solid lipid nanoparticles bearing oxybenzone:in-vitro and in-vivo evaluation". ***J Microencapsul***, 2010,27.(3)226-33.
295. Santos Maia, C., Mehnert, W., Schaller, M.*, et al.*," Drug targeting by solid lipid nanoparticles for dermal use". ***J Drug Target***, 2002,10.(6)489-95.
296. Henning, A., Neumann, D., kostka, K.-H.*, et al.*," Influence of human skin specimens consisting of different skin layers on the result of in vitro permeation experiments". ***Skin Pharm Phys***, 2008,21.81-88.
297. Weitz, J.I.," New oral anticoagulants in development". ***J Thromb Haemost***, 2010,103.(1)62-70.
298. Weitz, D.S. and Weitz, J.I.," Update on heparin: what do we need to know?" ***J Thromb Thrombol***, 2009 1-9.
299. De Caterina, R., Husted, S., Wallentin, L.*, et al.*," Anticoagulants in heart disease: Current status and perspectives". ***Eur Heart J***, 2007 28.(7)880-913.
300. Arbit, E., Goldberg, M., Gomez-Orellana, I.*, et al.*," Oral heparin: Status review". ***J Thromb***, 2006,4.
301. Radoff, S. and Danishefsky, I.," Location on heparin of the oligosaccharides section essential for anticoagulant activity". ***J Biol Chem***, 1984,259.(1)166-172.
302. Rosenberg, R.D.," Biochemistry of heparin antithrombin interactions and the physiologic role of this natural anticoagulant mechanism". ***Am J Med***, 1989,1989.(Supp3B)2S-9S.
303. Wang, L., Brown, J., Varki, A.*, et al.*," Heparin's anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins." ***J Clin Invest***, 2002,110.127-136.
304. Mousa, S.A., Fuming, Z., Aljada, A.*, et al.*," Pharmacokinetics and pharmacodynamics of oral heparin solid dosage form in healthy human subjects". ***J Clin Pharmacol***, 2007 47.(12)1508-1520.
305. Dunn, J.M. and Hollister, A.S.," Oral bioavailability of heparin using a novel delivery system". ***Current Therapeutic Research - Clinical and Experimental***, 1995,56.(8)738-745.
306. Hayes, P.Y., Ross, B.P., Thomas, B.G.*, et al.*," Polycationic lipophilic-core dendrons as penetration enhancers for the oral administration of low molecular weight heparin". ***Bioorganic and Medicinal Chemistry***, 2006 14.(1)143-152.
307. Hoffart, V., Lamprecht, A., Maincent, P.*, et al.*," Oral bioavailability of a low molecular weight heparin using a polymeric delivery system". ***J Control Release***, 2006,113.(1)38-42.
308. Cheng, J.W.M.," Fondaparinux: A new antithrombotic agent". ***Clinical Ther***, 2002,24.(11)1757-1769.

309. Turpie, A.G.G.," Direct Factor Xa and direct thrombin inhibitors: A clinical trial update". ***Curr Opin Drug Discov Dev,*** 2009,12.(4)497-508.
310. Kast, C.E., Guggi, D., Langoth, N.*, et al.*," Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil". ***Pharm Res***, 2003 20.(6)931-936.
311. Hirsh, J., O'Donnell, M., and W. Eikelboom, J.," Beyond Unfractionated Heparin and Warfarin: Current and Future Advances". ***Circulation***, 2007,116.552-560.
312. Hirsh, J. and Raschke, R.," Heparin and low-molecular-weight heparin: The seventh accp conference on antithrombotic and thrombolytic therapy." ***Chest*** 2004,126.188S-203S.
313. Haas, S.," New oral Xa and IIa inhibitors: Updates on clinical trial results". ***J Thromb Thrombol***, 2008,25.(1)52-60.
314. Leonard, T.W., Lynch, J., McKenna, M.J.*, et al.*," Promoting absorption of drugs in humans using medium-chain fatty acid-based solid dosage forms: GIPETâ„¢". ***Expert Opinion on Drug Delivery***, 2006 3.(5)685-692.
315. Bauer, K.A., Hawkins, D.W., Peters, P.C.*, et al.*," Fondaparinux, a synthetic pentasaccharide: the first in a new class of antithrombotic agents - the selective factor Xa inhibitors". ***Cardiovasc Drug Rev***, 2002,20.(1)37-52.
316. Re, G. and Legnani, C.," Thrombocytopenia during fondaparinux prophylaxis: HIT or something different?" ***Intern Emerg Med***, 2010.
317. Donat, F., Duret, J.P., Santoni, A.*, et al.*," The pharmacokinetics of fondaparinux sodium in healthy volunteers". ***Clin Pharmacokinet***, 2002,41 Suppl 2.1-9.
318. Harenberg, J.," Development of idraparinux and idrabiotaparinux for anticoagulant therapy". ***J Thromb Haemost***, 2009,102.811-815.
319. Faaij, R.A., Burggraaf, J., Schoemaker, R.C.*, et al.*," Absence of an interaction between the synthetic pentasaccharide fondaparinux and oral warfarin". ***Br J Clin Pharmacol***, 2002,54.(3)304-8.
320. Desmurs-Clavel, H., Huchon, C., Chatard, B.*, et al.*," Reversal of the inhibitory effect of Fondaparinux on Thrombin generation by rFVIIa, aCCP and PCC". ***Thromb Res***, 2009,123.(5)796-798.
321. Schiele, F.," Fondaparinux and acute coronary syndromes: update on the OASIS 5-6 studies". ***Vasc Health Risk Manag***, 2010,6.179-87.
322. Samama, M.M. and Gerotziafas, G.T.," Evaluation of the pharmacological properties and clinical results of the synthetic pentasaccharide (fondaparinux)". ***Thromb Res***, 2003,109.(1)1-11.
323. Buller, H., Davidson, B., Decousus , H.*, et al.*," Fondaparinux or enoxaparin for the initial treatment of symptomatic deep venous thrombosis: a randomized trial". ***Ann Intern Med.***, 2003,140.867–873.
324. Buller, H., Davidson, B., Decousus , H.*, et al.*," Subcutaneous fondaparinux versus intravenous unfractionated heparin in the initial treatment of pulmonary emboli". ***N Engl J Med.***, 2003,349.1695-1702.
325. Santi, R., Contino, L., Gatto, S.*, et al.*," Fondaparinux in major orthopedic surgical patients". ***Inter Emerg Med***, 2010,5.(2)167-167.
326. Bates, S.M. and Weitz, J.I.," The status of new anticoagulants". ***Br J Haemat***, 2006 134.(1)3-19.

327. Khafagy el, S., Morishita, M., Onuki, Y.*, et al.*," Current challenges in non-invasive insulin delivery systems: a comparative review". ***Adv Drug Deliv Rev***, 2007,59.(15)1521-46.
328. Ponchel, G., Montisci, M.J., Dembri, A.*, et al.*," Mucoadhesion of colloidal particulate systems in the gastro-intestinal tract". ***Eur J Pharm Biopharm***, 1997,44.25-31.
329. Pauletti, G.M., Gangwar, S., Siahaan, T.J.*, et al.*," Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies". ***Adv Drug Del Rev***, 1997,27.235-256.
330. Morishita, M. and Peppas, N.A.," Is the oral route possible for peptide and protein drug delivery?" ***Drug Dis Tod***, 2006,11.(19-20)905-910.
331. WALENGA, J.M., FAREED, J., JESKE, W.P.*, et al.*," Development of a Synthetic Heparin Pentasaccharide: Fondaparinux". ***Turk J Haematol***, 2002,19.(2)137-150.
332. Heinzelmann, M. and Bosshart, H.," Fondaparinux sodium lacks immunomodulatory effects of heparin". ***Am J Surgery*** 2004,187.111-113.
333. Weitz, D. and Weitz, J.," Update on heparin: what do we need to know?" ***J Thromb Thrombol***, 2010,29.(2)199-207.
334. Bauer, K.A.," New anticoagulants". *Hematology / the Education Program of the American Society of Hematology.* ***American Society of Hematology. Education Program***, 2006,450-456.
335. Caliceti, P., Salmaso, S., Elvassore, N.*, et al.*," Effective protein release from PEG/PLA nano-particles produced by compressed gas anti-solvent precipitation techniques". ***J Control Release***, 2004,94.(1)195-205.
336. Carrasquillo, K.G., Stanley, A.M., Aponte-Carro, J.C.*, et al.*," Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly(lactide-co-glycolide) microspheres results in release of native protein". ***J Control Release***, 2001,76.(3)199-208.
337. Hu, F.Q., Hong, Y., and Yuan, H.," Preparation and characterization of solid lipid nanoparticles containing peptide". ***Int J Pharm***, 2004,273.(1-2)29-35.
338. Martins, S., Silva, A.C., Ferreira, D.C.*, et al.*," Improving Oral Absorption of Samon Calcitonin by Trimyristin Lipid Nanoparticles". ***J Biomed Nanotech***, 2009 5.76-83.
339. Poutona, c. and Charmanb, W.N.," The potential of oily formulations for drug delivery to the gastro-intestinal tract". ***Adv Drug Del Rev,*** 1997,25.1-2.
340. Hathout, R.M., Woodman, T.J., Mansour, S.*, et al.*," Microemulsion formulations for the transdermal delivery of testosterone". ***Eur J Pharm Sci***, 2010,40.(3)188-96.
341. Nornoo, A.O., Zheng, H., Lopes, L.B.*, et al.*," Oral microemulsions of paclitaxel: In situ and pharmacokinetic studies". ***Eur J Pharm and Biopharm***, 2009,71 7.310-31.
342. He, C.X., He, Z.G., and Gao, J.Q.," Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs". ***Expert Opin Drug Deliv***, 2010,7.(4)445-60.
343. Mrestani, Y., Behbood, L., Hartl, A.*, et al.*," Microemulsion and mixed micelle for oral administration as new drug formulations for highly hydrophilic drugs". ***Eur J Pharm Biopharm***, 2010,74.(2)219-22.

344. Patel, N., Schmid, U., And M.J., L.," Phospholipid-Based Microemulsions Suitable for Use in Foods". ***J. Agric. Food Chem***, 2006,54.7817-7824.
345. Tenjarla, S.," Microemulsions: an overview and pharmaceutical applications". ***Crit Rev Ther Drug Carrier Syst***, 1999,16.(5)461-521.
346. Gupta, S. And Moulik, S.P.," Biocompatible Microemulsions and Their Prospective Uses in Drug Delivery". ***J Pharm Sci***, 2008,97.22-45.
347. Attwood, D., Microemulsions. Colloidal Drug Delivery Systems, ed. H. Kreuter. 1994, New York: Marcel Decker.
348. Alany, R.G.T., Rades, S., Agatonovic-Kustrin, N.G*., et al.*," Effect of alcohols and diols on the phase behaviour of quaternary systems." ***Int J Pharm***, 2000,196.141-145.
349. Podlogar, F., Gašperlin, M., Tomši˘c, M*., et al.*," Structural characterisation of water–Tween 40®/Imwitor 308®–isopropyl myristate microemulsions using different experimental methods ". ***Int J Pharm***, 2004,276.
350. 358. Salager, J.L., Antòn, R., Andérez, J.M*., et al.*," Formulation des microémulsions par la méthode du HLD". ***Technique de l'ingénieur, Traité Génie des Procédés***, 2001,J2 157.1-19.
351. Kreilgaard, M.," Influence of microemulsions on cutaneous drug delivery". ***Advanced Drug Delivery Reviews***, 2002,54.(SUPPL.)S77-S98.
352. Yuan, J.S., Yip, A., Nguyen, N*., et al.*," Effect of surfactant concentration on transdermal lidocaine delivery with linker microemulsions". ***Int J Pharm***, 2010,392.(1-2)274-84.
353. Nguyen, T.T.L., Edelen, A., Neighbors, B*., et al.*," Biocompatible lecithin-based microemulsions with rhamnolipid and sophorolipid biosurfactants: Formulation and potential applications". ***J. Colloid Interface Sci***, 2010,1-7.
354. Agatonovic-Kustrin, S., Glass, B.D., Wisch, M.H*., et al.*,"Prediction of a stable microemulsion formulation for the oral delivery of a combination of antitubercular drugs using ANN methodology". ***Pharm Res***, 2003,20.(11)1760-5.
355. Klier, J., Tucker, C.J., Kalantar, T.H*., et al.*," Properties and Application of Microemulsions." ***Advanced Materials***, 2000,12.(23)1751-1757.
356. Benoit, J.-P., Thomas, O., Ramadan, A*., et al.* (2009) *Procédé de préparation de nanoparticules lipidiques à partir d'au moins deux microémulsions*. 08 0058547
357. Khokhlov, V.Y., Selemenev, V.F., Khokhlova, O.N*., et al.*," Heparin determination by spectrophotometric and photocolorimetric methods". ***J Pharm Chem***, 1999,33.(8)450-451.
358. Song, Y.-k. and Kim, C.k.," Topical delivery of low-molecular-weight heparinwith surface-charged flexible liposomes". ***Biomaterials*** 2006,27.271-280.
359. Sun, W., Mao, S., Mei, D*., et al.*," Self-assembled polyelectrolyte nanocomplexes between chitosan derivatives and enoxaparin". ***Eur J Pharm Biopharm***, 2008,69.(2)417-25.
360. Shabbitsa, J.A., Chiua, G.N.C., and Mayera, L.D.," Development of an in vitro drug release assay that accurately predicts in vivo drug retention for liposome-based delivery systems". ***J Control Release***, 2002,84.161-170
361. Aboofazeli, R., Lawrence, C.B., Wicks, S.R*., et al.*," Investigations into the formation and characterization of phospholipid microemulsions. III. Pseudo-

ternary phase diagrams of systems containing water-lecithin-isopropyl myristate and either an alkanoic acid, amine, alkanediol, polyethylene glycol alkyl ether or alcohol as cosurfactant." ***Int J Pharm***, 1994,111.63-72.

362. Zheng, J. and Fulu, M.-Y.," Decrease of genital organ weights and plasma testosterone levels in rats following oral administration of leuprolide microemulsion." ***Int J Pharm***, 2006,307.209-15.

363. Trotta, M., Cavalli, R., Gallarate, M.*, et al.*," Microencapsulation of insulin into lipid carriers." ***Proc 5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology.***, 2006.

364. Cilek A, Celebi N, and F., T.," Lecithin-based microemulsion of a peptide for oral administration: preparation, characterization, and physical stability of the formulation." ***Drug Deliv***, 2006,13.19-24.

365. Cilek, A., Celebi, N., Tirnaksiz, F.*, et al.*," A lecithin-based microemulsion of rh-insulin with aprotinin for oral administration: Investigation of hypoglycemic effects in non-diabetic and STZ-induced diabetic rats." ***Int J Pharm***, 2005,298.176-85.

366. Bergenstahl, B. and Fontell, K.," Phase-equilibria in the system soybean lecithin water." ***Prog. Colloid Polym. Sci.***, 1983,68.48-52.

367. Schurtenberger, P., Peng, Q., Leser, M.E.*, et al.*," Structure and phase behaviour of lecithin-based microemulsions: a study of the chain length dependence. " ***J. Colloid Interface Sci.***, 1993,156.43-51.

368. Von Corswant, C., Engstrom, S., and Soderman, O.," Microemulsions based oil soybean phosphatidylcholine and triglycerides. Phase behaviour and microstructure." ***Langmuir***, 1997,13.5061-5070.

369. Trotta, M., Gallarate, M., Pattarino, F.*, et al.*," Investigation of the phase behaviour of systems containing lecithin and 2-acyl lysolecithin derivatives." ***Int J Pharm,*** 1999,190.83-89.

370. Rosencher, N., Bellamy, L., and Arnaout, L.," Should new oral anticoagulants replace low-molecular-weight heparin for thromboprophylaxis in orthopaedic surgery?" ***Arch Cardiovas Dis***, 2009,102.(4)327-333.

371. Ross, B.P. and Toth, I.," Gastrointestinal absorption of heparin by lipidization or coadministration with penetration enhancers". ***Current Drug Delivery***, 2005 2.(3)277-287.

372. Ubrich, N., Hoffart, V., Vigneron, C.*, et al.*," Digestive absorption of heparin with alternative formulations". ***S.T.P. Pharma Sciences***, 2002,12.(3)147-155.

373. Javot, L., Lecompte, T., Rabiskova, M.*, et al.*," Encapsulation of low molecular weight heparins: Influence on the anti-Xa/anti-IIa ratio". ***J Control Release***, 2009 139.(1)8-14

374. Motlekar, N.A. and Youan, B.-B.C.," The quest for non-invasive delivery of bioactive macromolecules: A focus on heparins". ***J Control Release***, 2006,113.(2)91-101.

375. Bai, S., Gupta, V., and Ahsan, F.," Cationic liposomes as carriers for aerosolized formulations of an anionic drug: safety and efficacy study". ***Eur J Pharm Sci***, 2009,38.(2)165-71.

376. Aungst, B.J.," Intestinal permeation enhancers". ***J Pharm Sci***, 2000,89.(4)429-42.

377. Thanou, M., Verhoef, J.C., and Junginger, H.E.," Oral drug absorption enhancement by chitosan and its derivatives". ***Adv Drug Del Rev,*** 2001 52. (2)117-126.
378. Grabovac, V. and Bernkop-Schnurch, A.," Improvement of the intestinal membrane permeability of low molecular weight heparin by complexation with stem bromelain". ***Int J Pharm***, 2006,326.(1-2)153-9.
379. Grabovac, V., Schmitz, T., FÃ¶ger, F.*, et al.*," Papain: An effective permeation enhancer for orally administered low molecular weight heparin". ***Pharm Res***, 2007 24.(5)1001-1006.
380. Lamprecht, A., Ubrich, N., and Maincent, P.," Oral low molecular weight heparin delivery by microparticles from complex coacervation". ***Eur J of Pharm Biopharm***, 2007 67.(3)632-638.
381. Berkowitz, S.D., Marder, V.J., Kosutic, G.*, et al.*," Oral heparin administration with a novel drug delivery agent (SNAC) in healthy volunteers and patients undergoing elective total hip arthroplasty". ***J Thromb Haemost***, 2003 1.(9)1914-1919.
382. Lee, Y.K., Kim, S.K., Lee, D.Y.*, et al.*," Efficacy of orally active chemical conjugate of low molecular weight heparin and deoxycholic acid in rats, mice and monkeys". ***J Control Release***, 2006 111.(3)290-298.
383. Kim, S.K., Lee, D.Y., Lee, E.*, et al.*," Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anti-coagulant". ***J Control Release***, 2007 120.(1-2)4-10.
384. Jiao, Y., Ubrich, N., Marchand-Arvier, M.*, et al.*," In vitro and in vivo evaluation of oral heparin-loaded polymeric nanoparticles in rabbits". ***Circulation***, 2002,105.(2)230-235.
385. Hoffart, V., Ubrich, N., Lamprecht, A.*, et al.*," Microencapsulation of low molecular weight heparin into polymeric particles designed with biodegradable and nonbiodegradable polycationic polymers". ***Drug Del:*** *Journal of Delivery and Targeting of Therapeutic Agents*, 2003 10.(1)1-7.
386. Kemp, M.M. and Linhardt, R.J.," Heparin-based nanoparticles". ***Wiley Interdiscip Rev Nanomed Nanobiotechnol***, 2010, 2.(1)77-87.
387. Kim, S.K., Lee, D.Y., Kim, C.Y.*, et al.*," A newly developed oral heparin derivative for deep vein thrombosis: Non-human primate study". ***J Control Release***, 2007 123.(2)155-163.
388. Ito, Y., Kusawake, T., Prasad, Y.V.R.*, et al.*," Preparation and evaluation of oral solid heparin using emulsifier and adsorbent for in vitro and in vivo studies". ***Int J Pharm***, 2006 317.(2)114-119.
389. Scala-Bertola, J., Gajdziok, J., RabiÅ¡kovÃ¡, M.*, et al.*," Pellets for oral administration of low-molecular-weight heparin". ***Drug Dev Ind Pharm***, 2009 35.(12)1503-1510.
390. Scala-Bertola, J., Rabiskova, M., Lecompte, T.*, et al.*," Granules in the improvement of oral heparin bioavailability". ***Int J Pharm***, 2009 374.(1-2)12-16.
391. Guarini, S. and Ferrari, W.," Olive oil-provoked bile-dependent absorption of heparin from gastro-intestinal tract in rats". ***Pharmacol Res Commun***, 1985,17. (8)685-97.
392. Goto, T., Morishita, M., Nishimura, K.*, et al.*," Novel mucosal insulin delivery systems based on fusogenic liposomes". ***Pharm Res***, 2006,23.(2)384-91.

393. Takeuchi, H., Matsui, Y., Yamamoto, H., *et al.*," Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats". ***J Control Release***, 2003,86.(2-3)235-42.
394. Mori, S., Matsuura, A., Rama Prasad, Y.V., *et al.*," Studies on the intestinal absorption of low molecular weight heparin using saturated fatty acids and their derivatives as an absorption enhancer in rats". ***Bio Pharm Bull***, 2004 27.(3)418-421.
395. Prasad, Y.V.R., Minamimoto, T., Yoshikawa, Y., *et al.*," In situ intestinal absorption studies on low molecular weight heparin in rats using Labrasol as absorption enhancer". ***Int J Pharm***, 2004 271.(1-2)225-232.
396. Yang, T., Arnold, J.J., and Ahsan, F.," Tetradecylmaltoside (TDM) enhances in vitro and in vivo intestinal absorption of enoxaparin, a low molecular weight heparin". ***J Drug Target***, 2005 13.(1)29-38.
397. Motlekar, N.A., Srivenugopal, K.S., Wachtel, M.S., *et al.*," Modulation of gastrointestinal permeability of low-molecular-weight heparin by L-arginine: In-vivo and in-vitro evaluation". ***J Pharm Pharmacol***, 2006 58.(5)591-598.
398. Thanou, M., Nihot, M.T., Jansen, M., *et al.*," Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin across intestinal epithelia in vitro and in vivo". ***J Pharm Sci***, 2001 90.(1)38-46.
399. Thanou, M., Henderson, S., Kydonieus, A., *et al.*," N-sulfonato-N,O-carboxymethylchitosan: A novel polymeric absorption enhancer for the oral delivery of macromolecules". ***J Control Release***, 2007,117.(2)171-178.
400. Thanou, M., Verhoef, J.C., Nihot, M.T., *et al.*," Enhancement of the intestinal absorption of low molecular weight heparin (LMWH) in rats and pigs using carbopolÂ® 934P". ***Pharm Res***, 2001,18.(11)1638-1641.
401. Schmitz, T., Leitner, V.M., and Bernkop-SchnÃ¼rch, A.," Oral heparin delivery: Design and in vivo evaluation of a stomach-targeted mucoadhesive delivery system". ***J Pharm Sci***, 2005 94.(5)966-973.
402. Leone-Bay, A., Paton, D.R., and Weidner, J.J.," The development of delivery agents that facilitate the oral absorption of macromolecular drugs". ***Med Res Rev***, 2000 20.(2)169-186.
403. Lee, Y.K., Kim, S.H., and Byun, Y.," Oral delivery of new heparin derivatives in rats". ***Pharm Res***, 2000,17.(10)1259-1264.
404. Lee, Y.K., Nam, J.H., Shin, H.C., *et al.*," Conjugation of low-molecular-weight heparin and deoxycholic acid for the development of a new oral anticoagulant agent". ***Circulation***, 2001 104.(25)3116-3120.
405. Sang, K.K., Eun, H.L., Vaishali, B., *et al.*," Tricaprylin microemulsion for oral delivery of low molecular weight heparin conjugates". ***J Control Release***, 2005,105.(1-2)32-42.
406. Kim, S.K., Vaishali, B., Lee, E., *et al.*," Oral delivery of chemical conjugates of heparin and deoxycholic acid in aqueous formulation". ***Thromb Res***, 2006,117.(4)419-427.
407. Jiao, Y., Ubrich, N., Hoffart, V., *et al.*," Anticoagulant activity of heparin following oral administration of heparin-loaded microparticles in rabbits". ***J Pharm Sci***,, 2002 91.(3)760-768.
408. Kim, T.D., Sakon, M., and Kawasaki, T.," Studies on liposome-encapsulated heparin". ***Thromb Res***, 1986,43.(6)603-612.

409. Roger, E., Lagarce, F., Garcion, E.*, et al.*," Lipid nanocarriers improve paclitaxel transport throughout human intestinal epithelial cells by using vesicle-mediated transcytosis". ***J Control Release***, 2009,140.(2)174-181.
410. Turpie, A.G., Eriksson, B.I., Lassen, M.R.*, et al.*," Fondaparinux, the first selective factor Xa inhibitor". ***Curr Opin Hematol***, 2003,10.(5)327-32.
411. Herbert, J.M., Herault, J.P., Bernat, A.*, et al.*," Biochemical and pharmacological properties of SANORG 34006, a potent and long-acting synthetic pentasaccharide". ***Blood***, 1998,91.(11)4197-205.
412. Frank, R.D., Schabbauer, G., Holscher, T.*, et al.*," The synthetic pentasaccharide fondaparinux reduces coagulation, inflammation and neutrophil accumulation in kidney ischemia-reperfusion injury". ***J Thromb Haemost***, 2005,3.(3)531-40.
413. Sculpher, M.J., Lozano-Ortega, G., Sambrook, J.*, et al.*," Fondaparinux versus Enoxaparin in non-ST-elevation acute coronary syndromes: short-term cost and long-term cost-effectiveness using data from the Fifth Organization to Assess Strategies in Acute Ischemic Syndromes Investigators (OASIS-5) trial". ***Am Heart J***, 2009,157.(5)845-52.
414. Walenga, J.M., Jeske, W.P., Bara, L.*, et al.*," Biochemical and pharmacologic rationale for the development of a synthetic heparin pentasaccharide". ***Thromb Res***, 1997,86.(1)1-36.
415. Turpie, A., Bauer, K., Eriksson, B.*, et al.*," Efficacy and safety of fondaparinux in major orthopedic surgery according to the timing of its first administration". ***Thromb Haemost***, 2003,90.(2)364-6.
416. Desmurs-Clavel, H., Huchon, C., Chatard, B.*, et al.*," Reversal of the inhibitory effect of fondaparinux on thrombin generation by rFVIIa, aPCC and PCC". ***Thromb Res***, 2009,123.(5)796-8.
417. Heurtault, B., Saulnier, P., Pech, B.*, et al.*," The influence of lipid nanocapsule composition on their size distribution". ***Eur J Pharm Biopharm***, 2003 18.(1)55-61.
418. Shabbits, J.A., Chiu, G.N., and Mayer, L.D.," Development of an in vitro drug release assay that accurately predicts in vivo drug retention for liposome-based delivery systems". ***J Control Release***, 2002,84.(3)161-70.
419. Jantratid, E., Janssen, N., Reppas, C.*, et al.*," Dissolution media simulating conditions in the proximal human gastrointestinal tract: an update". ***Pharm Res***, 2008,25.(7)1663-76.
420. MacIntosh, F.," A colorimetric method for the standardization of heparin preparations". ***Biochem J***, 1941,35.776.
421. Hobbelen, P.M.J., Van Dinther, T.G., Vogel, G.M.T.*, et al.*," Pharmacological profile of the chemically synthesized antithrombin III binding fragment of heparin (pentasaccharide) in rats." ***Thromb Haemostasis***, 1990,63.265-270.
422. Lanke, S.S.S., Gayakwad, S.G., Strom, J.G.*, et al.*," Oral delivery of low molecular weight heparin microspheres prepared using biodegradable polymer matrix system". ***J Microencap***, 2009,26.(6)493-500.
423. Hoffart, V., Ubrich, N., Simonin, C.*, et al.*," Low molecular weight heparin-loaded polymeric nanoparticles: Formulation, characterization, and release characteristics". ***Drug Dev Ind Pharm***, 2002 28.(9)1091-1099.
424. Meissner, Y., Ubrich, N., Ghazouani, F.E.*, et al.*," Low molecular weight heparin loaded pH-sensitive microparticles". ***Int J Pharm***, 2007 335.(1-2)147-153.

425. Sun, W., Mao, S., Wang, Y.*, et al.*," Bioadhesion and oral absorption of enoxaparin nanocomplexes". ***Int J Pharm***, 2010,386.(1-2)275-81.
426. Anton, N., Saulnier, P., Gaillard, C.*, et al.*," Aqueous-core lipid nanocapsules for encapsulating fragile hydrophilic and/or lipophilic molecules". ***Langmuir***, 2009 25.(19)11413-11419.
427. Teixeira, H., Dubernet, C., Puisieux, F.*, et al.*," Submicron cationic emulsions as a new delivery system for oligonucleotides". ***Pharm Res***, 1999,16.(1)30-6.
428. Oster, C.G., Kim, N., Grode, L.*, et al.*," Cationic microparticles consisting of poly(lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination". ***J Control Release***, 2005,104.(2)359-77.
429. Basarkar, A., Devineni, D., Palaniappan, R.*, et al.*," Preparation, characterization, cytotoxicity and transfection efficiency of poly(DL-lactide-co-glycolide) and poly(DL-lactic acid) cationic nanoparticles for controlled delivery of plasmid DNA". ***Int J Pharm***, 2007,343.(1-2)247-54.
430. Sun, L., Sun, Y., Xu, F.*, et al.*," Atomic force microscopy and surface-enhanced raman scattering detection of DNA based on DNA-nanoparticle complexes". ***Nanotech***, 2009,20.(12).
431. Zheng, H.Z., Pang, D.W., Lu, Z.X.*, et al.*," Combing DNA on CTAB-coated surfaces". ***Biophys Chem***, 2004,112.(1)27-33.
432. Yang, S.C. and Benita, S.," Enhanced absorption and drug targeting by positively charged submicron emulsions". ***Drug Dev Res***, 2000,50.(3-4)476-486.
433. Meisner, D., Pringle, J., and Mezei, M.," Liposomal ophthalmic drug delivery. III. Pharmacodynamic and biodisposition studies of atropine". ***Int J Pharm***, 1989,55.(2-3)105-113.
434. Harush-Frenkel, O., Rozentur, E., Benita, S.*, et al.*," Surface charge of nanoparticles determines their endocytic and transcytotic pathway in polarized MDCK cells". ***Biomacromolecules***, 2008,9.(2)435-43.
435. Kuo, Y.C. and Chen, H.H.," Entrapment and release of saquinavir using novel cationic solid lipid nanoparticles". ***Int J Pharm***, 2009,365.(1-2)206-13.
436. Manjunath, K. and Venkateswarlu, V.," Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration". ***J Control Release***, 2005,107.(2)215-228.
437. Peira, E., Carlotti, M.E., Trotta, C.*, et al.*," Positively charged microemulsions for topical application". ***Int J Pharm***, 2008,346.(1-2)119-123.
438. Bae, K.H., Moon, C.W., Lee, Y.*, et al.*," Intracellular delivery of heparin complexed with chitosan-g-poly(ethylene glycol) for inducing apoptosis". ***Pharm Res***, 2009,26.(1)93-100.
439. Singh, M., Ugozzoli, M., Briones, M.*, et al.*," The effect of CTAB concentration in cationic PLG microparticles on DNA adsorption and in vivo performance". ***Pharm Res***, 2003,20.(2)247-51.
440. Rawat, A., Majumder, Q.H., and Ahsan, F.," Inhalable large porous microspheres of low molecular weight heparin: In vitro and in vivo evaluation". ***J Control Release***, 2008,128.(3)224-232.
441. Mbela, N., Verschueren, E., and Ludwig, A.," Influence of stearylamine and dicetylphosphate on the physical properties of submicron O/W emulsions". ***J Pharm Belg***, 1998,53.(2)81-86.

442. Bai, Y., Xu, G.Y., Xin, X.*, et al.*," Interaction between cetyltrimethylammonium bromide and β-cyclodextrin: Surface tension and interfacial dilational viscoelasticity studies". ***Coll Polymer Sci***, 2008,286.(13)1475-1484.
443. Chen, M.C., Wong, H.S., Lin, K.J.*, et al.*," The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin". ***Biomaterials***, 2009 30.(34)6629-6637.
444. Müller, R.H., Rühl, D., and Runge, S.A.," Biodegradation of solid lipid nanoparticles as a function of lipase incubation time". ***Int J Pharm***, 1996,144. (1)115-121.
445. Jani, P., Halbert, G.W., Langridge, J.*, et al.*," Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency". ***J Pharm Pharmacol***, 1990,42.(12)821-6.
446. Malkov, D., Wang, H.Z., Dinh, S.*, et al.*," Pathway of oral absorption of heparin with sodium N-[8-(2-hydroxybenzoyl)amino] caprylate". ***Pharm Res***, 2002 19. (8)1180-1184.
447. Moazed, B. and Hiebert, L.M.," Movement of heparins across rat gastric mucosa is dependent on molecular weight and pH". ***Pharm Res***, 2009,26.(1)189-95.
448. Moazed, B. and Hiebert, L.M.," Low molecular weight heparins cross rat gastric mucosa mounted in an Ussing Chamber". ***Int J Pharmacol***, 2008,4.(6)431-442.
449. Leone-Bay, A., Paton, D.R., Variano, B.*, et al.*," Acylated non-Î±-amino acids as novel agents for the oral delivery of heparin sodium, USP". ***J Control Release***, 1998 50.(1-3)41-49.
450. Baughman, R.A., Kapoor, S.C., Agarwal, R.K.*, et al.*," Oral delivery of anticoagulant doses of heparin. A randomized, double- blind, controlled study in humans". ***Circulation***, 1998 98.(16)1610-1615.
451. Berkowitz, S.D., Marder, V.J., Kosutic, G.*, et al.*," Oral heparin administration with a novel drug delivery agent (SNAC) in healthy volunteers and patients undergoing elective total hip arthroplasty". ***J Thromb Haemost***, 2003,1.(9)1914-9.
452. Moazed, B. and Hiebert, L.M.," Movement of heparins across rat gastric mucosa is dependent on molecular weight and pH". ***Pharm Res***,, 2009,26.(1)189-195.
453. Leone-Bay, A., O'Shaughnessy, C., Agarwal, R.*, et al.*," Oral low molecular weight heparin absorption: From solution and solid dosage forms in rat, dog, and monkey models". ***Pharma Tech***, 2002 26.(3)38-46.
454. Rivera, T.M., Leone-Bay, A., Paton, D.R.*, et al.*," Oral delivery of heparin in combination with sodium N-[8-(2- hydroxybenzoyl)amino]caprylate: Pharmacological considerations". ***Pharm Res***,, 1997 14.(12)1835-1837.
455. .Hirsh, J., Warkentin, T.E., Raschke, R.*, et al.*," Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing considerations, monitoring, efficacy, and safety". ***Chest***, 1998,114.(5 Suppl)489S-510S.
456. Kast, C.E., Guggi, D., Langoth, N.*, et al.*," Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil". ***Pharm Res***, 2003,20.(6)931-6.
457. Leonard, T.W., Lynch, J., McKenna, M.J.*, et al.*," Promoting absorption of drugs in humans using medium-chain fatty acid-based solid dosage forms: GIPET". ***Expert Opin Drug Deliv***, 2006,3.(5)685-92.

458. Peppas, N.A. and Carr, D.A.," Impact of Absorption and Transport on Intelligent Therapeutics and Nano-scale Delivery of Protein Therapeutic Agents". ***Chem Eng Sci***, 2009,64.(22)4553-4565.
459. Stangier, J.," Clinical pharmacokinetics and pharmacodynamics of the oral direct thrombin inhibitor dabigatran etexilate". ***Clin Pharmacokinet***, 2008,47.(5)285-95.
460. Boneu, B., Necciari, J., Cariou, R.*, et al.*, " Pharmacokinetics and tolerance of the natural pentasaccharide (SR90107/Org31540) with high affinity to antithrombin III in man". ***Thromb Haemost***, 1995,74.(6)1468-73.
461. Ipatova, O.M., Torkhovskaya, T.I., Medvedeva, N.V.*, et al.*," Bioavailability of oral drugs and the methods for its improvement". ***Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry***, 2010,4.(1)82-94.
462. Brayden, D.J., Jepson, M.A., and Baird, A.W.," Keynote review: intestinal Peyer'spatch M cells and oral vaccine targeting". ***Drug Discov. Today***, 2005,10.1145-1157.
463. Daugherty, A.L. and Mrsny, R.J.," Transcellular uptake mechanisms of the intestinal epithelial barrier. Part One." ***Pharm. Sci. Technol. Today***, 1999,2.144-151.
464. des Rieux, A., Fievez, V., Garinot, M.*, et al.*," Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach". ***J Control Release***, 2006,116.(1)1-27.
465. Florence, A.T.," Issues in oral nanoparticle drug carrier uptake and targeting". ***J Drug Target***, 2004,12.(2)65-70.
466. Roger, E., Lagarce, F., Garcion, E.*, et al.*," Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery". ***Nanomedicine (Lond)***, 2010,5.(2)287-306.
467. Desai, M.P., Labhasetwar, V., Amidon, G.L.*, et al.*," Gastrointestinal uptake of biodegradable microparticles: effect of particle size". ***Pharm Res***, 1996,13.(12)1838-45.
468. Jung, T., Kamm, W., Breitenbach, A.*, et al.*," Biodegradable nanoparticles for oral delivery of peptides: Is there a role for polymers to affect mucosal uptake?" ***Eur J Pharm Biopharm***, 2000,50.(1)147-160.
469. Rabinovich-Guilatt, L., Couvreur, P., Lambert, G.*, et al.*," Cationic vectors in ocular drug delivery". ***J Drug Target***, 2004,12.(9-10)623-33.
470. Rao, N.M.," Cationic lipid-mediated nucleic acid delivery: beyond being cationic". ***Chem Phys Lipids***, 2010,163.(3)245-52.
471. Jain, V., Prasad, V., Jadhav, P.*, et al.*," Preparation and performance evaluation of saquinavir laden cationic submicron emulsions". ***Drug Deliv***, 2009,16.(1)37-44.
472. Simovic, S., Hui, H., Song, Y.*, et al.*," An oral delivery system for indomethicin engineered from cationic lipid emulsions and silica nanoparticles". ***J Control Release***, 2010,143.(3)367-73.
473. Gershanik, T. and Benita, S.," Positively charged self-emulsifying oil formulation for improving oral bioavailability of progesterone". ***Pharm Dev Technol***, 1996,1.(2)147-57.
474. Yang, T., Hussain, A., Bai, S.*, et al.*," Positively charged polyethylenimines enhance nasal absorption of the negatively charged drug, low molecular weight heparin". ***J Control Release***, 2006,115.(3)289-97.

475. Bai, S., Thomas, C., and Ahsan, F.," Dendrimers as a carrier for pulmonary delivery of enoxaparin, a low-molecular weight heparin". ***J Pharm Sci***, 2007,96.(8)2090-106.
476. Hauss, D.J.," Oral lipid-based formulations". ***Adv Drug Deliv Rev***, 2007,59.(7)667-76.
477. Wawrezinieck, A., Pean, J.M., Wuthrich, P.*, et al.*," [Oral bioavailability and drug/carrier particulate systems]". ***Med Sci (Paris)***, 2008,24.(6-7)659-64.
478. Mori, S., Matsuura, A., Rama Prasad, Y.V.*, et al.*," Studies on the intestinal absorption of low molecular weight heparin using saturated fatty acids and their derivatives as an absorption enhancer in rats". ***Biol Pharm Bull***, 2004,27.(3)418-21.
479. Rama Prasad, Y.V., Minamimoto, T., Yoshikawa, Y.*, et al.*," In situ intestinal absorption studies on low molecular weight heparin in rats using labrasol as absorption enhancer". ***Int J Pharm***, 2004,271.(1-2)225-32.
480. Manjunath, K. and Venkateswarlu, V.," Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration". ***J Control Release***, 2005,107.(2)215-28.
481. Yu, W., Liu, C., Ye, J.*, et al.*," Novel cationic SLN containing a synthesized single-tailed lipid as a modifier for gene delivery". ***Nanotechnology***, 2009,20.(21)215102.
482. Lv, H., Zhang, S., Wang, B.*, et al.*," Toxicity of cationic lipids and cationic polymers in gene delivery". ***J Control Release***, 2006,114.(1)100-9.
483. Stangier, J., Rathgen, K., Stahle, H.*, et al.*," The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects". ***Br J Clin Pharmacol***, 2007,64.(3)292-303.

ARABIC SUMMARY