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NANOCARRIERS FOR ORAL BIOAVAILABILITY ENHANCEMENT

NANOVECTEURS POUR L'AMELIORATION DE LA BIODISPONIBILITE ORALE

MEMBRES DU JURY

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NANOCARRIERS FOR ORAL BIOAVAILABILITY ENHANCEMENT

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This work was prepared under co-supervision of Prof. Dr. Rainer H. Müller and Prof. Dr. Philippe Maincent at Institut für Pharmazie of Freie Universität Berlin and Faculté de Pharmacie of Université Henri-Poincaré Nancy I (co-tutelle).

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Meinen Eltern und meiner Schwester

Content

1 Introduction

1.1 English

Testosterone replacement therapy still lacks a widely accepted drug delivery system. The systems currently used are either not very efficient (i.e. they have a very low bioavailability) or they are not convenient to use and they do not foster compliance of the patient.

Other systems like patches, gels and injections are not very much accepted by the patient. It seems obvious that carrying a patch for 24 hours is not a convenient approach. The same is valid for injections which need to be administered every 2 to 4 weeks. Generally, oral drug delivery systems are the most accepted ones.

Oral delivery is basically convenient to use but the testosterone system marketed is –from the patient's point of view – a sub-optimal solution: 2 capsules need to be taken several times a day. A meal has to be eaten every time with the medication.

In recent years there have been many promising developments in the nanoparticulate field. Some systems (i.e. solid lipid nanoparticles and drug nanosuspensions) increased oral bioavailability tremendously for some drugs (Liversidge & Cundy, 1995; Hanafy et al., 2007).

Developing a nanoparticulate oral testosterone drug delivery system seemed promising, especially taking into account the lymphatic absorption of both lipid nanoparticles and nanosuspensions which corresponds to the same pathway as the commercial oral delivery system.

First aim was to develop one or more oral testosterone drug delivery systems which are superior in bioavailability and hence are beneficial for the patient. The second aim was to establish an *in vivo* testing system to evaluate the developed systems with regard to their pharmacokinetic parameters.

Another goal was the design of fish oil-based lipid nanocarriers with a reduced smell and taste but improved stability.

1.2 Français

Il n'existe pas actuellement de système optimisé et largement accepté pour la thérapeutique de substitution à base de testostérone. Les formes pharmaceutiques de testostérone aujourd'hui commercialisées sont peu efficientes (leur biodisponibilité orale est faible) ou difficiles d'utilisation ce qui entraîne des problèmes d'observance chez les patients.

Les autres systèmes comme les dispositifs transdermiques, les gels voire les systèmes injectables sont peu appréciés par les patients. Dans le cas de la testostérone, le port d'un dispositif transdermique pendant 24 heures est généralement difficilement accepté par les malades. Le même réflexe est observé pour les injections même si le rythme d'administration est plus lent (toutes les 2 à 4 semaines). Il est reconnu que les systèmes de délivrance pour la voie orale sont, encore aujourd'hui, les mieux acceptés.

L'administration orale est très pratique mais les formes pharmaceutiques de testostérone représentent une solution non optimale du point de vue du patient. En effet, il est nécessaire d'ingérer 2 capsules molles jusqu'à plusieurs fois par jour. De plus un repas doit être pris en même temps que la médication.

Ces dernières années ont vu le développement prometteur des formes nanoparticulaires. Quelques uns de ces systèmes (et notamment les nanoparticules lipidiques solides et les nanosuspensions de principe actif) ont permis d'augmenter de façon très importante la biodisponibilité des principes actifs incorporés (Liversidge & Cundy, 1995; Hanafy et al., 2007).

Il nous est apparu que le développement d'une forme nanoparticulaire de testostérone, destinée à la voie orale, correspondait à un système prometteur particulièrement si on prend en compte l'absorption lymphatique préférentielle des lipides et des nanosuspensions qui correspondent au même type de passage que la forme pharmaceutique actuellement commercialisée.

Le but de ce travail a été de développer un ou plusieurs systèmes de délivrance orale de la testostérone dont la biodisponibilité serait supérieure et, en conséquence, profitable au patient d'un point de vue thérapeutique. L'autre objectif était le développement d'un

modèle animal permettant d'évaluer in vivo les systèmes développés et notamment de comparer les paramètres pharmacocinétiques entre les nouveaux systèmes et la forme actuellement commercialisée.

Parallèlement, un autre travail a été conduit dans le but de réaliser des nanoparticules lipidiques à base d'huile de poisson afin d'en réduire le goût et l'odeur tout en améliorant leur stabilité. Les huiles de poisson sont en effet une source importante d'acides oméga 3 dont l'utilisation thérapeutique pourrait être étendue.

1.3 Deutsch

Der Testosteronsubstitutionstherapie fehlt immer noch ein Arzneistoffträgersystem das allgemein akzeptiert wird. Die zurzeit verwendeten System sind entweder wenig effizient (das heißt die haben eine geringe Bioverfügbarkeit) oder sie sind unkomfortabel in der Anwendung und förder damit nicht die Compliance des Patienten.

Andere System wie Pflaster, Gele und Injektionen werden vom Patienten kaum akzeptiert. Das Tragen eines Pflasters für 24 Stunden ist offensichtlich kein bequemer Weg. Das gleiche gilt für Injektionen die alle 2 bis 4 Wochen appliziert werden. Die orale Arzneiform ist generell die am meisten akzeptierte.

Oral Trägersysteme sind grundsätzlich einfach anzuwenden, doch ist die auf dem Markt verfügbare Form nur suboptimal: Zwei Kapseln müssen mehrmals täglich eingenommen werden und das zusammenmit einer Mahlzeit.

In den letzten Jahren gab es einige vielversprechende Entwicklungen im Bereich der Nanopartikel. Einige Systeme (Solid Lipid Nanopartikel und Nanosuspensionen) erhöhen die Bioverfügbarkeit für einigen Arzneistoffe enorm (Liversidge & Cundy, 1995; Hanafy et al., 2007).

Die Entwicklung eines oralen Testosteronarzneiträgersystems schien vielversprechend, insbesondere wenn man bedenkt, dass Lipidnanopartikel und Nanosuspensionen ebenso lymphatisch aufgenommen werden, wie das System, das schon auf dem Markt ist.

Das erste Ziel war also die Entwicklung eines oder mehrerer Arzneistoffträgersysteme mit einer erhöhtem Bioverfügbarkeit, welches damit vorteilhaft für den Patienten ist. Das zweite Ziel war die Etablierung eines in vivo-Testsystem, um die entwickelten System in Hinblick auf ihre pharmakokinetischen Parameter bewerten zu können.

Ein weiteres Ziel war das Design von Fischöl-basierten Lipidnanopartikel mit geringem Eigengeruch und –Geschmack und hoher Stabilität.

2 Lipid carriers: state of the art

Parts of this section have been published in Drug Delivery and Industrial Pharmacy "Lipid Nanoparticles with a solid matrix" (Muchow, Maincent & Müller, 2008).

2.1 Overview: Lipid nanoparticles for oral drug delivery

Lipid based drug delivery systems gained increased attention during the last years[1]. Lipids are known to promote oral absorption of drugs (Charman et al., 1992; Charman, Porter, Mithani & Dressman, 1997; Charman, 2000; Porter & Charman, 2001b; Stuchlik & Zak, 2001; Holm, Porter, Mullertz, Kristensen & Charman, 2002). Vitamins, such as vitamin A and E are better absorbed in the presence of fats/lipids (Kuksis, 1987). There are quite a number of drugs for which an increased oral bioavailability is reported when they are administered in the presence of fat-rich food. Examples are testosterone and halofantrine (Charman & Porter, 1996; Porter, Charman, Humberstone & Charman, 1996; Khoo, Porter & Charman, 2000; Porter & Charman, 2001a; Holm, Porter, Mullertz, Kristensen & Charman, 2002; Khoo, Shackleford, Porter, Edwards & Charman, 2003; Shackleford et al., 2003). An excellent model drug to demonstrate this - which is at the same time of high commercial interest - is Cyclosporin A. Delivery of Cyclosporin A in form of a microemulsion (Sandimmun® Optoral/Neoral), the second generation product by Novartis, distinctly reduced the variation in bioavailability which was a major problem of the classic Sandimmun® emulsion. However, an undesired plasma peak occurred which is being held responsible for side effects such as nephrotoxicity (Martindale, 1989).

In a comparative *in vivo* study Cyclosporin A was administered as a drug nanocrystal suspension (so called "nanosuspension") and incorporated into solid lipid nanoparticles (SLN). Nanocrystals consist of 100% drug without any matrix material. The increases in oral bioavailability reported for drug nanocrystals are really impressive for certain drugs. An example is the increase in absolute bioavailability for the drug danazol from 5.1 ± 1.9% as a normal suspension to 82.3 ± 10.1% as a drug nanosuspension (Liversidge & Cundy, 1995). In

[1] AAPS Lipid-Based Drug Delivery Systems Focus Group: www.aaps.org/inside/focus_groups/Lipid/

contrast to this literature data, the results of the Cyclosporin A nanosuspension study were disappointing; the oral bioavailability was very low and far away from the required pharmacological levels (Müller, Runge & Ravelli, 1998; Runge, 1998; Penkler, Müller, Runge & Ravelli, 1999, 2003; Müller et al., 2006).

However, this first *in vivo* animal study of Cyclosporin A-loaded solid lipid nanoparticles (SLN) confirmed the theory of an absorption-enhancing effect of lipids. The oil used in Sandimmun® Optoral/Neoral was replaced by a solid lipid. The lipid particles were already administered in the nanosize range (in contrast to *in situ* generation of a nanosized microemulsion by breaking in the stomach as in the commercial formulation). The Cyclosporin A-loaded SLN showed a sufficiently high oral bioavailability, they remained within the therapeutic window equally long as the Sandimmun® Optoral/Neoral microemulsion formulation. However, they avoided the nephrotoxic peak above 1000 ng/ml due to controlled release from the solid lipid (Runge, 1998; Müller et al., 2006). This in vivo study demonstrates, that lipids can enhance oral bioavailability and that drug nanocrystals without lipids being present can potentially be of limited effect.

SLN were developed in the beginning of the 1990ies. Their characteristic property is that they are made from a solid lipid only (Gasco, 1993; zur Mühlen, Schwarz, Mehnert & Müller, 1993; Müller & Lucks, 1996). At the turn of the millennium the second generation, nanostructured lipid carriers (NLC), was developed (Müller, Mäder, Lippacher & Jenning, 2000). Identical to SLN, the particle matrix is still solid at body temperature, but is produced from a blend of a solid lipid with a liquid lipid (= oil) leading to certain advantages compared to SLN.

SLN and NLC can incorporate only lipophilic molecules efficiently. Loading with hydrophilic molecules can only be achieved to a very low extent by solubilisation in the lipid melt. This limits the use of SLN and NLC to very potent/low dose lipophilic drugs such as erythropoietin (EPO) or other potent peptides. Lysozyme was successfully incorporated as “model peptide” (Almeida, Runge & Müller, 1997). From this point, there was a definite need to create a lipid nano-delivery system for highly dosed hydrophilic actives having a substantial loading capacity (e.g. up to 30%) and exploiting the oral absorption enhancing effect of solid lipids.

An approach for improved oral drug delivery of hydrophilic molecules is the formation of lipid drug conjugates (LDC). Many hydrophilic drugs show a poor oral bioavailability due to their explicit hydrophilic character. The lipophilicity of the molecules is not high enough to sufficiently pass the gastrointestinal wall. Transferring them to a lipid drug conjugate and forming a nanoparticle has three major effects:

1. in case of chemically labile drugs: Transfer to an insoluble molecule/particle reduces distinctly the gastrointestinal enzymatic/non-enzymatic degradation,
2. due to the increase in lipophilicity the drug shows an improved permeation through the gastrointestinal wall, and
3. oral absorption is promoted by the matrix lipids present in the LDC nanoparticles

The presence of lipids in the absorption process (SLN, NLC, LDC) or transfer of hydrophilic molecules to more lipophilic molecules by conjugation (LDC) can generally increase the oral absorption. The lipid absorption enhancing effect can even be more pronounced when combining lipid technology with nanotechnology, which is creating lipidic solid nanoparticles. This section deals with the production, characterisation and performance of various types of lipid nanoparticles with a solid matrix, which are:

1. Solid lipid nanoparticles (SLN),
2. Nanostructured lipid carriers (NLC) and the
3. Lipid drug conjugate (LDC) nanoparticles

2.2 Definitions

There are basically three different types of lipid nanoparticles with a solid matrix: Solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and lipid-drug conjugate (LDC) nanoparticles.

2.2.1 Solid Lipid Nanoparticles (SLN)

SLN are particles made from a lipid being solid at room temperature and also at body temperature. The starting material is solely a solid lipid, e.g. Dynasan 112 (zur Mühlen, Schwarz & Mehnert, 1998) or other lipids such as Compritol 888 ATO (Souto, Mehnert & Müller, 2006) or Imwitor 900 (Müller et al., 2006). There are two main production methods for SLN: The high pressure homogenisation method (Müller, Schwarz, zur Mühlen & Mehnert, 1994; Müller et al., 1995; Müller & Lucks, 1996; Mehnert, zur Mühlen, Dingler, Weyhers & Müller, 1997; Müller, Dingler, Weyhers, zur Mühlen & Mehnert, 1997; Müller, Weyhers, zur Mühlen, Dingler & Mehnert, 1997; zur Mühlen, Schwarz & Mehnert, 1998; Müller, Mäder & Gohla, 2000; Mehnert & Mäder, 2001) and the microemulsion method (Cavalli, Caputo & Gasco, 1993; Gasco, 1993). The homogenisation method is described in detail in section 2.6 and 2.7 of this thesis. The microemulsion method after Gasco utilizes a basic mechanism of microemulsions: They transform into an ultra-fine emulsion by breaking (i.e. after addition of e.g. water). During production of the microemulsion the solid lipid is melted, the drug is dissolved in the melted solid lipid. Surfactant, co-surfactant and water are then added until a hot microemulsion is formed. This hot microemulsion is subsequently poured into cold water. The microemulsion breaks and ultrafine emulsion droplets develop which immediately crystallize to form solid lipid nanoparticles (SLN) (Gasco, 1993). Disadvantages of the microemulsion method are the partially required relatively high concentration of surfactants, the organic solvents used to form the microemulsion and the strong dilution of the particle suspension by pouring the microemulsion into water. This is not very production friendly concerning the subsequent processing to solid oral dosage forms (e.g. tablets, pellets etc.) as a lot of water needs to be removed to end up with a dry dosage form. However, both production processes – homogenisation and microemulsion method – yield an identical product: Solid lipid particles dispersed in an aqueous dispersion medium. As described in the patent (Müller & Lucks, 1996), SLN made by high pressure homogenisation can also be produced in non-aqueous dispersion media as long as the dispersion medium does not dissolve the lipid, e.g. liquid polyethylene glycol (PEG) or oils (e.g. mineral oil).

SLN are an interesting delivery system. However, there are some potential limitations: The relatively low loading capacity for a number of drugs and potential expulsion of the drug during storage. Especially SLN prepared from one highly purified lipid can crystallize in a more or less perfect crystalline lattice. Such a perfect crystalline structure leaves little room for the incorporation of drugs. Usually, drugs are incorporated between the fatty acid chains, alternatively in between lipid layers or in amorphous clusters in crystal imperfections. The more perfect densely packed the crystal is, the fewer drug can be incorporated.

After production, SLN crystallize in high energetic lipid modifications such as α and β'. During storage the lipid molecules undergo a time-dependent re-structuring process leading to formation of the low energetic modifications βi and β. Consequently, the more perfect lipid crystalline structure leads to expulsion of drug (Westesen, Bunjes & Koch, 1997). This phenomenon has been well known for a long time from suppositories (Müller B. W., 1986).

2.2.2 Nanostructured Lipid Carriers (NLC)

To overcome these limitations, nanostructured lipid carriers (NLC) have been developed (Fig. 2.1) (Müller, Mäder, Lippacher & Jenning, 2000). Characteristic feature of the NLC is a controlled nanostructuring of the lipid particle matrix, i.e. creation of a lipid particle matrix as imperfect as possible. To achieve this, spatially very different molecules are mixed. In general a solid lipid is mixed with a liquid lipid (oil). This blend is used to produce the lipid particles which are still solid at temperatures up to about 40° C.

It can be summarized that SLN are particles produced from a solid lipid only, NLC are particles produced from a blend of solid lipid with a liquid lipid (oil). This results in differences of the particle matrix structure. NLC posses many imperfections increasing drug loading capacity and minimizing or avoiding drug expulsion during storage. Fig. 2.2 depicts this mechanism.

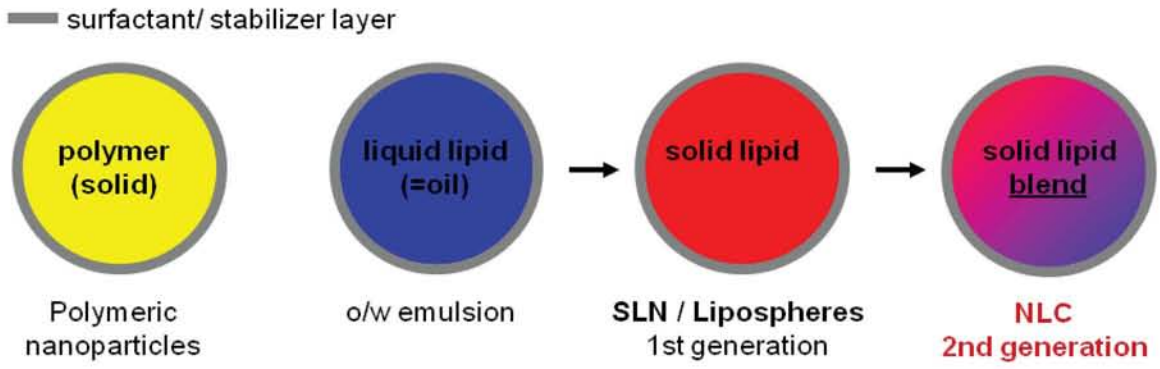


Fig 2.1: Graphical overview of the development of lipid nanoparticles

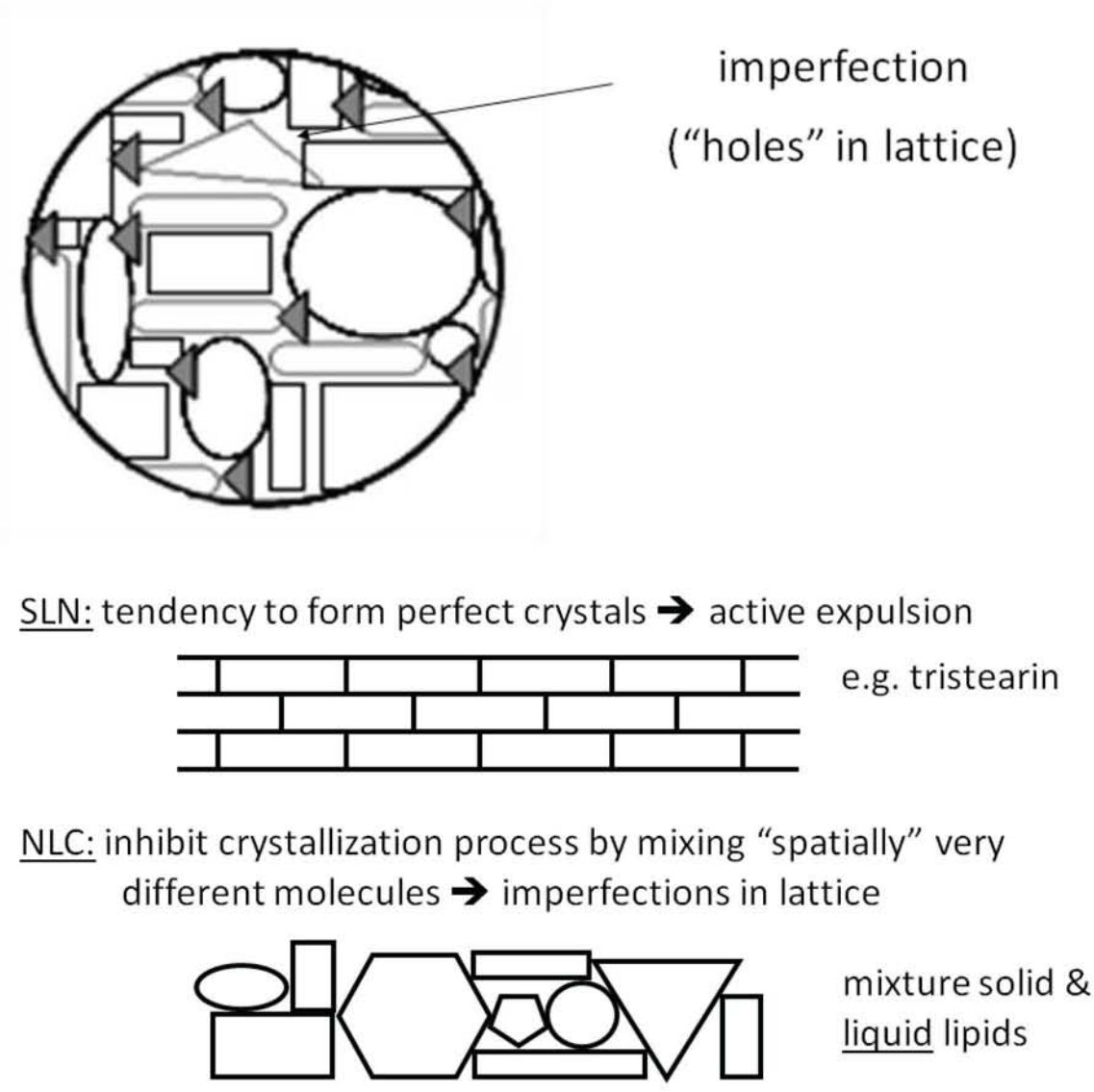


Fig. 2.2: Imperfections of the crystal lattice of NLC avoid the expulsion of drugs

2.2.3 Lipid-Drug Conjugates (LDC)

Based on their lipophilic character, SLN and NLC have only a limited loading capacity for hydrophilic drugs. This is not a problem in case of highly potent hydrophilic drugs such as erythropoietin (EPO): They can be solubilized in the melted lipid matrix using surfactant mixtures or just simply by the mono- and digylcerides present in the lipid anyway (e.g. Imwitor 900). However, a higher loading capacity was not achievable with this kind of lipid nanoparticles. To solve this problem, lipid-drug conjugate (LDC) particles were developed. The hydrophilic drug is transformed to a more lipophilic, insoluble molecule by conjugation with a lipidic compound. The conjugation can be performed by covalent linkage or simply by formation of a salt with a fatty acid (in case of drugs having e.g. protonisable functional groups).

The lipid drug conjugates are poorly water-soluble, they typically have a melting range of approximately 50-100° C and can be transformed to nanoparticles using a high pressure homogenisation method similar to the one described for SLN and NLC. Considering the molecular weight of the two fractions in the conjugate molecule, i.e. of the drug itself and the lipid part, a drug loading of approximately 30-50% is achievable (e.g. as reported for diminazene of about 33% formulated as diminazene diaceturate-acid conjugate with palmitic acid/stearic acid (Olbrich, Geßner, Kayser & Müller, 2002)). LDC nanoparticles can be made from the conjugated drug only or solid lipids can be additionally admixed to form a mixed matrix of LDC and lipid.

2.3 Loading capacity and drug incorporation mechanisms

It has to be differentiated between *loading capacity* and *entrapment efficiency*. *Entrapment efficiency* is defined as the percentage of drug incorporated into the lipid nanoparticles relative to the total drug added. It specifies how many percent of drug are included in the particles and how many percent of free drug are still present in the dispersion medium.

Loading capacity refers to the percentage of drug incorporated into the lipid nanoparticles relative to the total weight of the lipidic phase (i.e. lipid + drug). To give an example, we

assume a 10 % lipid nanoparticle dispersion (i.e. 10 % lipid, about 1 % surfactant and 89 % water). In this case the particle mass is equivalent to 10 %, the particle mass is defined as the sum of lipid and drug (= 100 %). If the drug had a *loading capacity* of 10 % in the lipid phase, this would correspond to 1 % in the total aqueous lipid nanoparticle dispersion.

Entrapment efficiencies in the literature are relatively high. They range from 80 % for the drug tetracaine to about 99 % for example for prednisolone (Schwarz, 1995). Of course, *entrapment efficiency* figures might look nice in case only very little drug is added. The more important point is the *loading capacity* of the lipid particles themselves. How many percent of drug can be incorporated into the total lipidic mass of the particles? The loading capacity depends on the solubility of the drug in the solid lipid (in case of SLN) or the lipid blend (in case of NLC). In case of LDC nanoparticles the *loading capacity* is determined from the ratio of the molecular fractions in the conjugate (e.g. drug and conjugated fatty acid). Examples of *loading capacities* reported for SLN are 1 % for prednisolone (zur Mühlen, Schwarz & Mehnert, 1998), 10 % for tetracaine (Schwarz, 1995), 20 % for Cyclosporine A by Runge (Runge, 1998), and even 25 % for Cyclosporine A by Radtke (Radtke, 2003). In case of very lipophilic compounds and a good solubility in the lipid, loading capacities up to 50 % can be achieved (e.g. vitamin E) (Dingler, Blum, Niehus, Müller & Gohla, 1999).

What are the mechanisms of drug incorporation? There are different models described in the literature. The major work has been done by the research group of Mehnert and co-workers (Müller et al., 1995; Schwarz, 1995; zur Mühlen, 1996; Mehnert, zur Mühlen, Dingler, Weyhers & Müller, 1997; Müller, Dingler, Weyhers, zur Mühlen & Mehnert, 1997; zur Mühlen & Mehnert, 1998; Mehnert & Mäder, 2001). Basically, there are three incorporation models (Fig. 2.3):

1. Homogenous matrix of solid solution

2. Core-shell model with drug enriched in the shell (= drug enriched shell)

3. Core-shell model with drug enriched in the core (= drug enriched core)

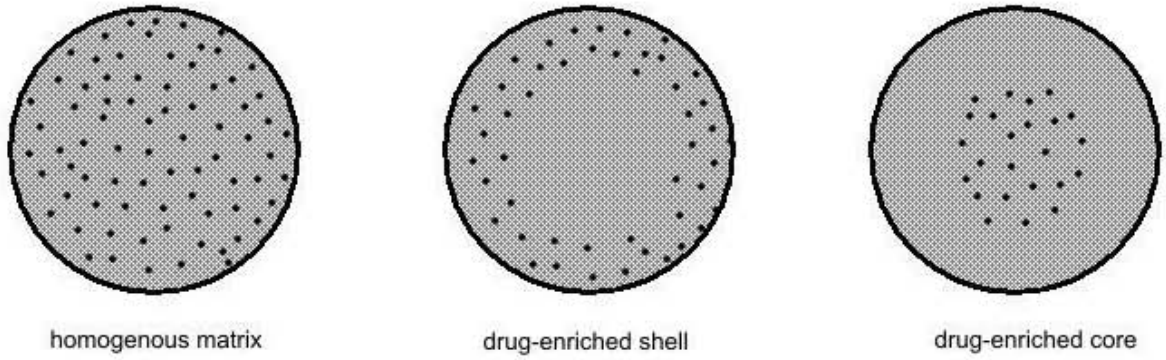


Fig. 2.3: Incorporation models for the three types of SLN (explanation in the text).

Modified after (Müller, Mäder & Gohla, 2000).

In case of a homogenous matrix (model 1, Fig. 2.3, left) the drug is molecularly dispersed evenly in the particle matrix. Drug release takes place by diffusion from the solid lipid matrix and additionally by lipid nanoparticle degradation in the gut.

In model 2 (Fig. 2.3, middle) the drug is enriched in the shell. This can be explained by a lipid precipitation mechanism occurring during particle production. After homogenisation there is a mixture of drug and lipid in each droplet. It is then being cooled. Depending on the TX solubility diagram[2] the lipid can precipitate earlier than the drug to form a drug-free core or at least a core with reduced drug content. Reaching the eutectic temperature and composition, lipid and drug precipitate simultaneously in the outer shell of the particles. Examples are coenzyme Q10 SLN. They possess a soft Q10-rich outer shell as proven by atomic force microscopy (AFM) (Fig 2.4) (Dingler, Lukowski, Gohla & Müller, 1997; Lukowski, Hoell, Kranold, Gehrke & Pflegel, 1997; Lukowski, Hoell, Dingler, Kranold & Pflegel, 1998). Drug enrichment in the shell is also a function of the solubility of the drug in the water-surfactant mixture at increased temperature during the production process. The drug partially leaves the lipid particle and dissolves in the aqueous phase during hot homogenisation. Reason for this is the increased solubility for many drugs in the outer phase

[2] TX diagram: A two-dimensional graphical representation, with temperature and concentration coordinates, of the isobaric phase relationship in a binary system

(surfactant solution) at elevated temperatures. Cooling of the o/w nanoemulsion reduces the drug solubility in the aqueous phase, drug tries to re-partition into the lipid particles leading to enrichment in the particle shell in case the particle core already started to solidify (Fig. 2.5). Such particles are known to lead to a burst release (Müller et al., 1995). In the case of the oral cyclosporine A formulation this was a desired effect. The dissolution rate needed to be sufficiently high to reach the therapeutic drug level but not too high to avoid nephrotoxic levels. In case of other applications it might be necessary to have a prolonged release formulation which can be achieved by varying the production conditions during the particle production (zur Mühlen & Mehnert, 1998). Such conditions are low production temperature (preferably "cold homogenisation") and low surfactant concentration.

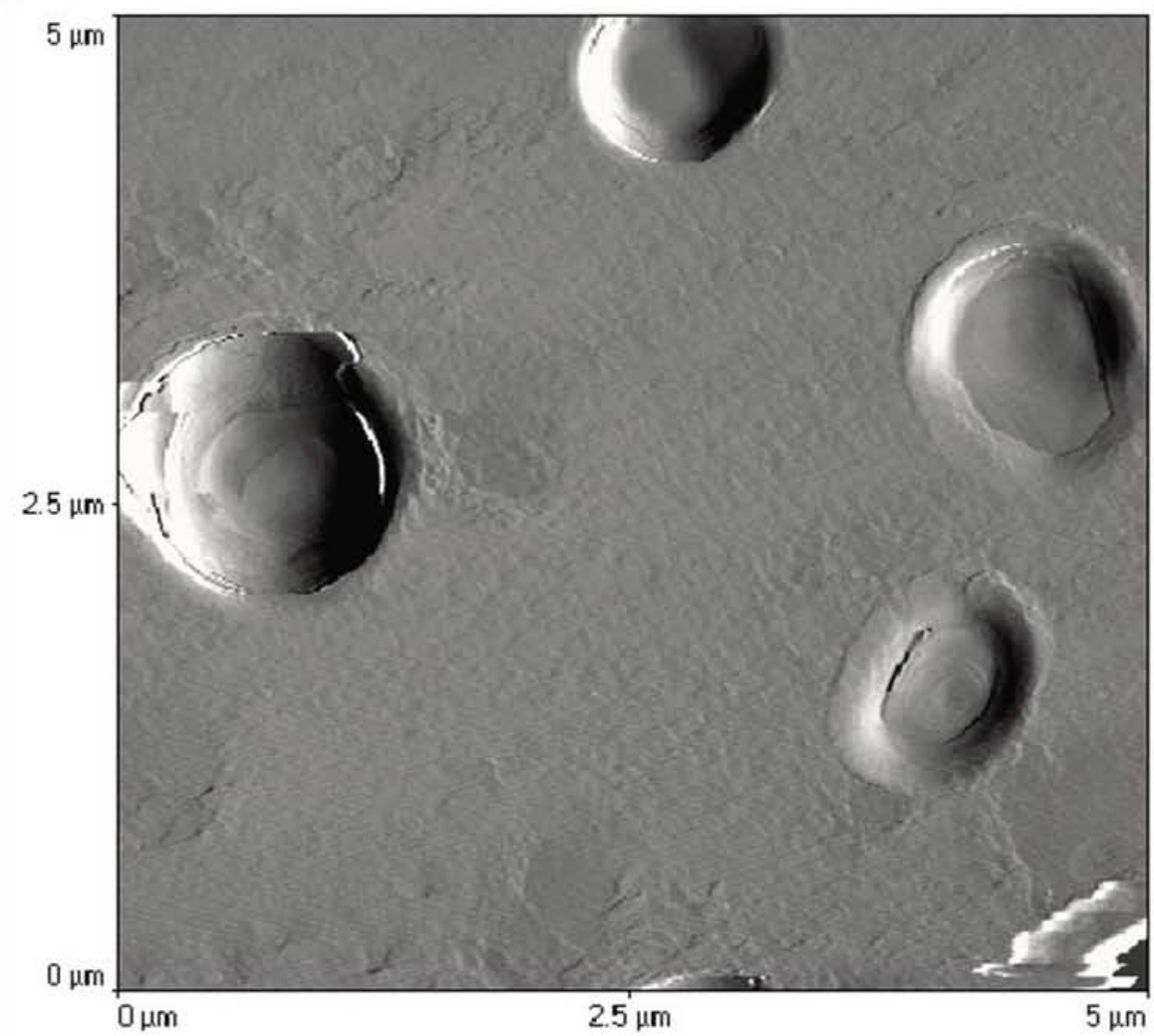


Fig. 2.4: Atomic force micrograph of Q-10-loaded SLN after (Dingler, Lukowski, Gohla & Müller, 1997)

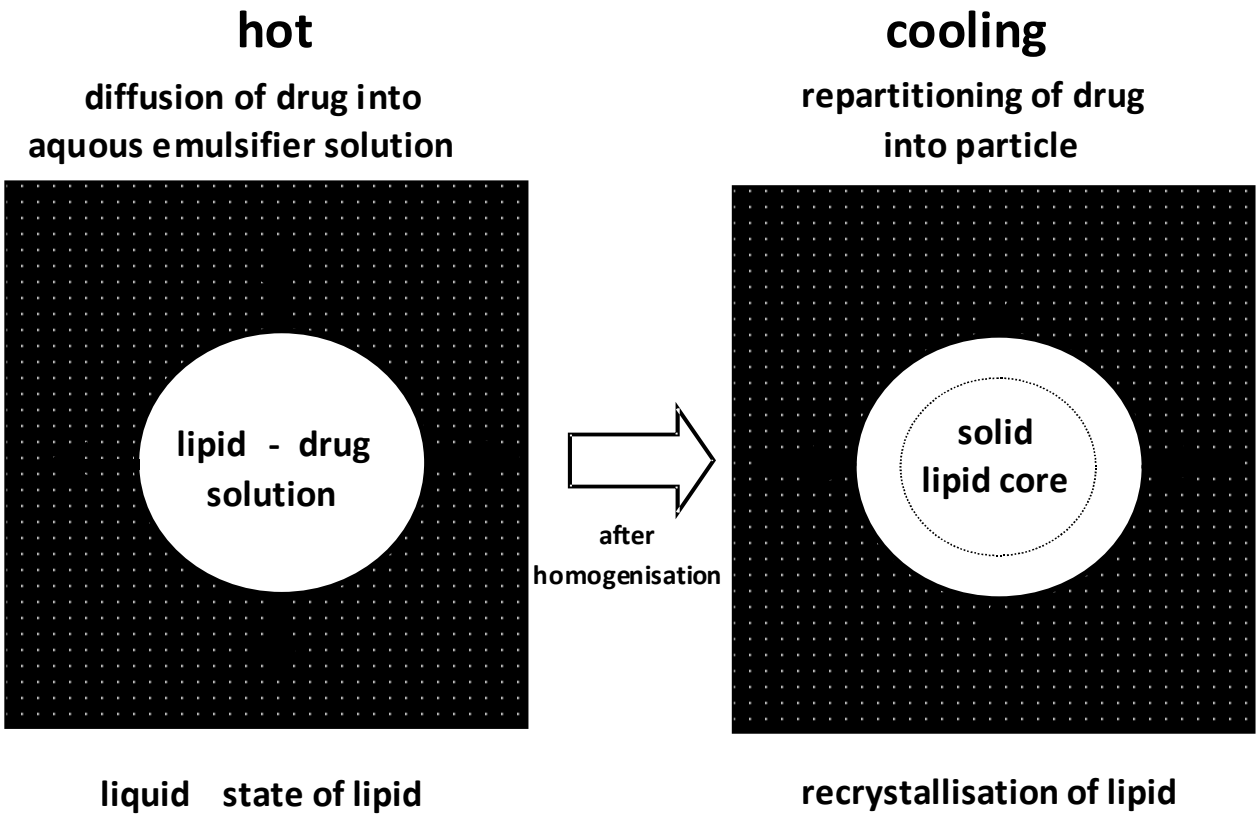


Fig. 2.5: Forming of SLN with drug enriched shell: During production drug leaves the liquid particle and diffuses into the outer phase, having an increased solubility for the drug because of the high temperature. During cooling, drug repartitions into the particle shell. The core of the particle is less/not accessible because lipid crystallisation has started. Modified after (Müller, Mäder & Gohla, 2000).

Enrichment in the shell (model 2) takes place when particles are produced by hot homogenisation method and the drug used shows a distinctly increased solubility in the aqueous surfactant phase at production temperature. In contrast, the drug-enriched core (model 3, Fig. 2.3, right) is formed in case cooling of the hot o/w emulsion leads to precipitation of the drug first. This takes preferentially place in lipid solutions with drug dissolved at its saturation solubility in the lipid at production temperature. During cooling a super saturation and subsequent drug precipitation is achieved. The prolonged release of prednisolone is explained by this model (zur Mühlen, 1996; zur Mühlen & Mehnert, 1998).

2.4 Oral delivery of drugs using lipid nanoparticles

The first drug intensively investigated to be formulated as SLN dispersion was cyclosporine A (Müller, Runge & Ravelli, 1998; Runge, 1998; Penkler, Müller, Runge & Ravelli, 1999, 2003; Müller et al., 2006; Müller et al., 2008). As mentioned above, it was known from the literature that the "old" Sandimmun® emulsion showed variations in the oral bioavailability ranging from 10-60 %. The second generation product, the microemulsion Sandimmun® Optoral/Neoral avoids this strong variation in bioavailability but possesses potential nephrotoxicity as an undesired side effect due to plasma peaks well above 1000 ng/ml (Meinzer, Müller & Vonderscher, 1998). The aim of formulating a cyclosporine A loaded SLN formulation was to avoid the undesired plasma peak and to achieve a similarly high reproducible oral bioavailability in the therapeutic window. Hence combination of the advantages of the "old" Sandimmun® (no nephrotoxic plasma peak) and the "new" Sandimmun® Optoral/Neoral (little variation in bioavailability) should be achieved by the SLN formulation. Three formulations were used in the study: Cyclosporine A-loaded SLN suspension, cyclosporine A drug nanocrystals and Sandimmun® Optoral/Neoral microemulsion as a reference. The SLN blood profile did not exhibit the undesired plasma peak and remained over a similar time period time in the therapeutic window as the microemulsion (Fig. 2.6).

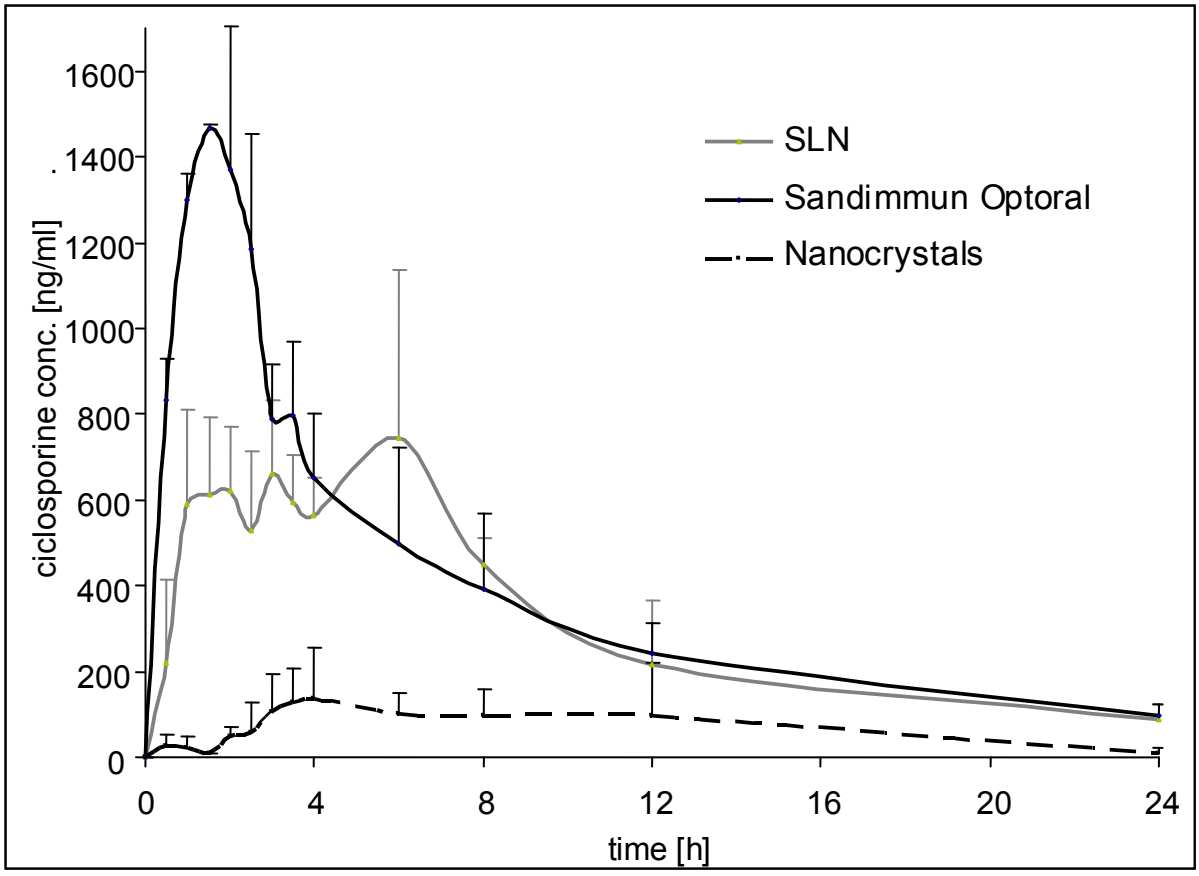


Fig. 2.6: Blood profiles of cyclosporine-loaded SLN suspension, drug nanocrystals and Sandimmun® microemulsion lipids. (from: Müller, R.H., Keck, C.M., Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles, 151-170, Copyright (2004), with permission from Elsevier).

At the first glance it was surprising that the cyclosporine A nanosuspension had such a low bioavailability. This is in contrast to increases in bioavailability reported for a number of drugs by the scientists of the company Nanosystems (nowadays Élan): As discussed above, the bioavailability of danazol could be increased from 5 % to 82 % by transferring the drug to a nanosuspension (Liversidge & Cundy, 1995). The reasons for the low bioavailability of cyclosporine A nanocrystals might be multi-factorial. Cyclosporine A is a substrate of p-glycoprotein, thus the absorption is limited a priori. In addition, it cannot be excluded that the drug nanocrystals aggregated in the gastrointestinal fluid. It is known that nanocrystal aggregation can reduce dissolution rate (Keck, Fichtinger, Viernstein & Müller, 2004; Keck, 2006) and subsequently oral bioavailability. Furthermore, it might be that the dissolution

rate of cyclosporine A from the crystals was not as high as from the lipid nanoparticles. The cyclosporine A was located – at least to a certain extent – in the outer shell of the SLN as indicated by the fast release observed in *in vitro* release studies (Runge, 1998).

To summarize: Drug nanocrystals, often seen as the ultimate, universal formulation approach for poorly soluble drugs, do not work in any case. For a number of drugs it seems to be beneficial to have lipids present during the absorption process. This is well in agreement with publications describing the positive effect of lipids on oral drug absorption (Porter, Charman, Humberstone & Charman, 1996; Charman, Porter, Mithani & Dressman, 1997; Charman, 2000; Porter & Charman, 2001b; Holm, Porter, Mullertz, Kristensen & Charman, 2002). Therefore, the first choice in screening might be drug nanocrystals but lipid nanoparticles are the essential back up formulation.

2.5 Mechanism of oral absorption enhancement

There are different mechanisms discussed which lead to an absorption enhancement and also to a reproducible bioavailability (little variation in bioavailability). These mechanisms are:

1. a general adhesiveness of nanoparticles
2. reproducibility of adhesion
3. an absorption enhancing effect of lipids

It is a general property of nanoparticles that they are adhesive (Tarr & Yalkowsky, 1989). The adhesiveness of particles to a surface increases with the surface area of the particles, the interaction forces can be calculated (Stieß, 1995). This is a general behaviour of all nanoparticles, not specific for lipid nanoparticles. After adhesion to the gut wall the drug is exactly released at its place of absorption (Liversidge & Cundy, 1995).

This adhesion process proved to be very reproducible. *In vivo* data obtained with drug nanocrystals show that there is little variability between the fasted and fed state of rats (Liversidge & Conzentino, 1995). The same is considered as valid for the lipid nanoparticles.

The low variation in bioavailability observed with cyclosporine A-loaded SLN suspension was similar to the Sandimmun® Optoral/Neoral microemulsion (Runge, 1998).

It is known that lipids can promote the absorption of active compounds, examples are lipid soluble vitamins such as vitamin A, D, E and K (Kuksis, 1987). The body absorbs the fat and simultaneously the drug is taken up, it can be considered as a kind of “Trojan Horse” effect.

The lipid absorption enhancing effect can be explained more specifically by the studies performed by W. Charman and co-workers (Charman et al., 1992; Charman, Porter, Mithani & Dressman, 1997; Charman, 2000; Porter & Charman, 2001b). To present it in a condensed form: The lipids are degraded by enzymes in the gut leading to the formation of surface active mono- and diglycerides on the surface of the lipid droplets or solid lipid particles. These molecules detach and form micelles. During the detachment and micelle forming process the drug dissolved in the lipid is taken up in the micelle (solubilized). Solubilisation is a well-known principle for solubility enhancement in pharmaceutical technology. The formed micelles interact with surface-active bile salts (e.g. sodium cholate) leading to the formation of so-called “mixed micelles”. In the subsequent absorption process of the lipid degradation product the drug is simultaneously absorbed (Fig. 2.7). Charman and co-workers could show that the absorption enhancing effect differs from one lipid to the other. For example it was observed that long-chain triglycerides (LCT) are more effective in promoting absorption of the drug halofantrine compared to medium-chain triglycerides (MCT) (Khoo, Shackleford, Porter, Edwards & Charman, 2003).

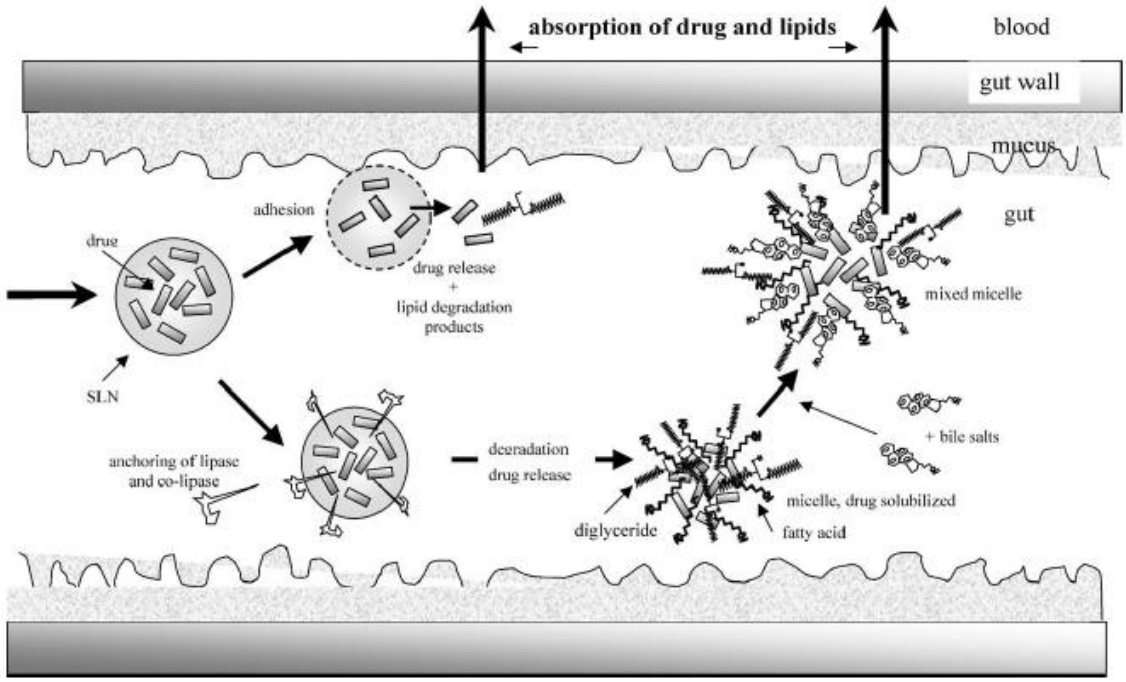


Fig. 2.7: Mechanisms of absorption promoting effect of lipids being formulated as a lipid nanoparticle (from: Journal of Biotechnology (113), Müller, R.H., Keck, C.M., Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles, 151-170, Copyright (2004), with permission from Elsevier).

Additionally, the length of the fatty acid chains affects the primary place of absorption. Fatty acids with C-14 chains to C-18 chains promote lymphatic absorption (Porter & Charman, 2001a). This is of interest for drugs such as testosterone, which undergo a strong first pass metabolism when they are absorbed through the gut wall and passing the liver.

Lymphatic absorption avoids this first pass metabolism to a certain degree and can be used to increase the oral absorption of testosterone or testosterone-undecanoate. A commercial formulation exploiting this concept is Andriol® capsules with testosterone-undecanoate dissolved in oleic acid (Shackleford et al., 2003). Oleic acid as a C-18 fatty acid is ideally suited as oil compound in capsules. The bioavailability of testosterone-undecanoate is reported to be 3.25 ± 0.48 % in dogs (Shackleford et al., 2003). As a kind of "second generation formulation" with improved chemical stability Andriol Testocaps® were launched by the company Organon. The capsules can be stored at room temperature, whereas the "first generation" needs to be stored below 8 °C. Andriol Testocaps® contain a blend of oleic

acid and castor oil (which major component is the C-18 fatty acid ricinoleic acid) as oil component and additionally a surfactant, lauroglycol/FCC (=propylene glycol monolaurate) (Shackleford et al., 2003). The bioavailability reported is about 2.88 ± 0.88 % (Shackleford et al., 2003). It can be imagined that the surfactant being present in this formulation will contribute or promote the formation of mixed micelles. Adding the surfactant moves this formulation to the direction of the self-emulsifying drug delivery systems (SEDDS) (Charman et al., 1992; Pouton, 2000).

Another important point is that for reaching the maximum absorption enhancing effect, the drug needs to be closely associated with the lipid. It is less efficient when drug and lipid are given separately (as it happens during normal food intake and simultaneous drug administration). If a separate administration of lipid and drug worked with similar efficiency, the easiest way to exploit the absorption enhancing effect would be simultaneous administration of a lipid-filled capsule with drug intake. This required very close association of lipid and drug has been realized with the three types of lipid nanoparticles. The drug can be incorporated in between the fatty acid chains or the lipid lamella (Runge, 1998) (Fig. 2.8) or alternatively be present as amorphous clusters in imperfections of the lipid crystal lattice. The surface active compounds in the gut disperse lipids from food to relatively fine droplets. Their diameter is typically in the range of 1-50 µm (Patton & Carey, 1979; Armand et al., 1996). Lipid nanoparticles represent an ultra-fine dispersion with typical diameters of about 250 nm (= 0.25 µm). Solubilisation can take place very fast and efficiently due to the large surface area of the particles. In case a slower degradation is required (e.g. for prolonged release) a fraction of the lipid nanoparticles can be stabilized with a high molecular weight steric stabilizer (e.g. Poloxamer). It could be shown that to some extent the nature of the lipid matrix but mainly the type of stabilizer affects the enzymatic degradation velocity (Olbrich, 1998, 2002; Olbrich, Kayser & Müller, 2002).

Stabilisation of the lipid particles with a mixture of sodium cholate and lecithin promotes the anchoring of the lipase/co-lipase complex and thus accelerates the degradation (Olbrich & Müller, 1999; Olbrich, Kayser & Müller, 2002). The use of high molecular weight stabilizers such as Poloxamer 407 leads to a steric hindrance of the anchoring of the complex, thus delaying the lipid degradation. It is also possible to adjust the degradation time between

"fast" and "slow" by using mixtures of degradation accelerating and degradation delaying stabilizers (Olbrich & Müller, 1999).

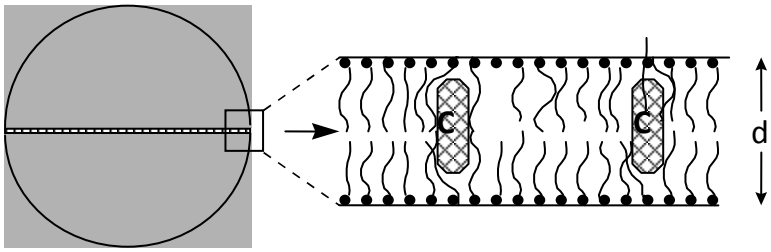


Fig. 2.8: Proposed incorporation of ciclosporin A (c) in the lipid matrix of Inwitor 900. (reprinted from Eur. J. Pharm. Biopharm, Müller, R.H., Runge, S.H., Ravelli, V., Thünemann, A.F., Mehnert, W., Souto, E.B., Cyclosporine-loaded solid lipid nanoparticles (SLN): drug-lipid physicochemical interactions and characterisation of drug incorporation, Copyright (2008), with permission from Elsevier).

2.6 Production on lab scale

The production method of first choice for the three types of lipid nanoparticles – SLN, NLC and LDC – is high pressure homogenisation. In case of SLN the drug is dissolved or dispersed in the melt of a solid lipid, typically approximately 5-10 °C above the lipid's melting point. In case of NLC the drug is dissolved in a melted blend of a solid lipid with a liquid lipid (oil), again slightly above the melting point of the lipid blend. LDC nanoparticles are commonly produced at room temperature. In the next production step the drug-containing lipid melt or lipid-drug conjugate is dispersed in a surfactant/stabilizer solution of identical temperature by high speed stirring. This yields an aqueous oil-in-water "pre-emulsion" (SLN and NLC) or suspension (LDC). To achieve a high dispersity in the subsequent homogenisation process it is recommended to use dispersion-efficient, fast diffusing, electrostatically stabilising, low molecular weight surfactants (e.g. sodium dodecylsulfate (SDS)). They diffuse very fast into newly formed interfacial layers and stabilize the formed small droplets efficiently minimizing subsequent coalescence phenomena. To achieve highest physical stability in gastrointestinal medium the combination with a steric stabilizer is recommended (e.g. Tween 80 or Poloxamer 188). Steric stabilisation is less impaired by the presence of electrolytes

compared to electrostatic stabilisation (high zeta potential). The pre-emulsion/suspension is passed through a temperature controlled high pressure homogenizer, either of the piston-gap type (APV Gaulin, APV Deutschland GmbH, Unna/Germany; Avestin Europe, Mannheim/Germany etc.) or of the jet-stream type (microfluidisation principle, Microfluidizer, Microfluidics Inc., Newton, MA, USA) Homogenisation temperature for SLN and NLC is again typically about 5 °C above the melting point of the lipid phase and room temperature for LDC. In principle for SLN and NLC production one homogenisation cycle at 500 bar is sufficient to yield a hot o/w emulsion with a particle size of approximately 250-300 nm. Cooling of the nanoemulsion during their production leads to crystallisation of the lipid and formation of solid nanoparticles.

Typical homogenisation parameters reported in the literature are 500 bar and up to 3 homogenisation cycles. The two additional cycles lead to a further slight reduction in the particle size to approximately 220 nm and a narrowing of the width of the distribution (reduction in the polydispersity index of photon correlation spectroscopy, PCS). However, for oral drug delivery such a small difference of 30 or 40 nm is not crucial. The same is valid for the homogeneity in the particle size distribution. Hence, one cycle at 500 bar is considered as being sufficient for particles intended for oral administration.

2.7 Large scale production

In most cases scaling up of a process encounters problems. This is different for the production of lipid nanoparticles based on high pressure homogenisation. On the contrary, using larger scale machines leads to an even better quality of the product with regard to a smaller mean particle size and its homogeneity (width of the size distribution). Basic advantage is that high pressure homogenisation is a production technique widely used, e.g. in pharmaceutical industry for the production of emulsions for parenteral nutrition (Klang, Parnas & Benita, 1998). High pressure homogenisation is also used in non-pharmaceutical areas, e.g. in food production, where homogenizers are running with a capacity of up to several tons of homogenisation volume per hour (e.g. for homogenized milk).

Typical machines for lab scale production are the Micron LAB 40 (batch size: 20 to 40 mL, APV Deutschland GmbH, Unna/Germany, Fig. 2.9) and the Avestin C5 (batch size: 7 mL to

1 L, capacity: 5 L/hour, Avestin Europe, Mannheim/Germany). In case of very expensive drugs or if there is a limited supply (e.g. new chemical entities) it is favourable to reduce the batch size (scaling down capability). Batch size reduction can be done by using the Avestin B3 (Avestin Europe, Mannheim/Germany). The batch volume is 0.5 mL to 3.2 ml.

Fig. 2.9: LAB 40 homogenizer with temperature control unit

A first scaling up step to 500 ml to 1 L can be realized by using the continuous version of the Micron LAB 40. It uses the same homogenisation tower as the discontinuous version with only slight modifications. Essential parts such as cylinder, piston and geometry of the homogenisation valve are identical. The continuous version is equipped with product

containers. The product is pumped through the valve by a number of piston movements (e.g. for 400 ml homogenisation volume ten piston movements with 40 ml each are required). In case of the continuous LAB 40 version the homogenisation tower and the two product containers are equipped with temperature control jackets. In theory, one could enlarge the product containers (e.g. to two kilograms), however the machine is originally not designed to run larger volumes in continuous mode.

The next scaling up step with a minimum batch size of 2 kg and a maximum of 10 kg was achieved using the LAB 60 (APV Deutschland GmbH, Unna/Germany). The LAB 60 has a homogenisation capacity of 60 litres per hour.

The two product containers of the LAB 60 are equipped with powerful high-speed stirring units. The feeding container has a dissolver disc built in to prepare the pre-emulsion in it. For the production of a 2 kg batch the pre-emulsion is passed through the homogenisation unit and then circulates back to the feeding container. It is a continuous homogenisation process in a loop. It can be calculated that it takes 15 minutes to ensure that - statistically - 99,9 % of the droplets have passed the homogenisation valve at least once (Leviton & Pallansch, 1959; Müller, Wissing & Radomska, 2001). For such a relatively small production volume of 2 kg it is not sensible to run the homogenisation with the LAB 60 in a discontinuous process. The void volume is relatively large (approx. 250 ml). Running discontinuous cycles would leave about 10-15 % of the batch each time in the void volume being non-homogenized. Running a 10 kg batch in continuous circulating required more than 90 minutes to ensure that 99.9 % of the droplets have passed the homogenisation gap at least once (Leviton & Pallansch, 1959). Therefore a discontinuous production is recommended. The 10 kg pre-emulsion is prepared in the feeding container, passed through the homogenizing unit and then collected in the second product container. When the pre-emulsion has passed the homogenizer it will be fed back from the second container by gravity via a temperature-controlled tube to the first container. Then the second homogenisation cycle starts. In general, two homogenisation cycles at 500 bar are fully sufficient to yield a relatively monodisperse product, the total homogenisation time for 10 kg is only 20 minutes (10 minutes per cycle). As already mentioned, for orally administered lipid nanoparticles even one single

homogenisation cycle is considered to be sufficient. In that case it does not make any difference if the particle size is 260 or 230 nm.

The LAB 60 unit was designed for a pharmaceutical company; that is why it can be used in a GMP area for clinical batch production. As pointed out above, the homogenisation results obtained with large-scale machines is typically better than with the lab scale LAB 40. There are several reasons for this: First of all, transfer of the process from a smaller capacity machine to a larger capacity machine is relatively easy because of the identical or similar geometry of the homogenisation valves. Secondly, the larger machines have two or three plungers leading to less fluctuation in the actual homogenisation pressure. The LAB 40 has only one plunger, therefore the pressure needs to build up; the first small fraction of the pre-emulsion is less efficiently homogenized than the rest. This effect is distinctly reduced when a homogenizer is running with three plungers smoothing the pressure fluctuation profile. Furthermore, the temperature control of the larger machines is much more effective. Machines can be ordered equipped with a temperature control unit for the homogenisation unit itself. Also, all required tubes and containers can be ordered double-walled for temperature control with a temperature controlling liquid. In addition, these homogenizers are equipped with two homogenisation valves in series, main (first) valve and a second valve. When the homogenized droplets leave the first homogenisation valve they possess a relatively high kinetic energy. Their surface might not yet be fully covered with stabilizer. Therefore limited coalescence can occur. A part of the coalesced or flocculated droplets is immediately re-dispersed when they pass the subsequent homogenisation valve. Typically, the second valve operates at one tenth of the pressure of the first valve, e.g. 500 bar for the first valve and 50 bar for the second valve.

Fig. 2.10: A typical arrangement for a Gaulin 5.5 with attached product container (right)

The next step in scale-up was the use of a Gaulin 5.5 (APV Deutschland GmbH, Unna/Germany, Fig. 2.10) with a homogenisation capacity of 150 litres per hour (approx. equivalent 150 kg). The pre-emulsion is prepared in larger product containers. Product containers and homogenizer are made from material of pharmaceutical grade. The product containers can be sterilized by autoclaving; preparation of the pre-emulsion under protective gas is possible. They also have the features of cleaning-in-place (CIP) and sterilisation-in-place (SIP). For the production, the lipid can be melted in the feeding container. Surfactant as well as sterile water from a sterile water supply system are then added. Advantageous is a hot storage supply system providing sterile water of 80°C which is ideal for the production process. This way of production leads also to a very low microbiological load. For oral administration one homogenisation cycle with the Gaulin 5.5 is

sufficient. The product is collected in the second product container and cooled in a controlled way under stirring. A batch size of about half a ton can be produced in approximately 3 hours homogenisation time with the Gaulin 5.5. For many products this is already a typical batch size. This way of production was used by the company “Chemisches Laboratorium Dr. Richter GmbH (CLR)” in Berlin to produce the first large scale batch of NLC for cosmetic industry to realize the first cosmetic NLC products (Müller, Rimpler, Petersen, Hommoss & Schwabe, 2007).

For even larger scales an Avestin EmulsiFlex C1000 (Avestin Europe, Mannheim/Germany) or a Rannie 118 (APV Deutschland GmbH, Unna/Germany) can be employed. Their capacity is 1000/2000 litres per hour at the low pressure required for lipid nanoparticle production.

In this case it is not sensible any more to prepare a 2 ton batch in a discontinuous way, i.e. preparing the pre-emulsion in a container. The heat exchange times are too long giving a too high temperature burden on the active (cosmetic active or drug). For production lines of this size static blenders are recommended. The drug containing melted lipid is admixed in a static blender to the hot surfactant/stabilizer solution (e.g. blenders from Sulzer Chemtech, Winterthur, Switzerland). The homogenized product needs to be cooled, the temperature controlled container will then be replaced by a heat exchanger to remove at least most of the heat. Fig. 2.11 shows a design for such an arrangement.

To sum up, the larger machines are more effective in dispersing and more sophisticated regarding their features to control the process. As a result, the product quality is in general better when moving to larger scale machines.

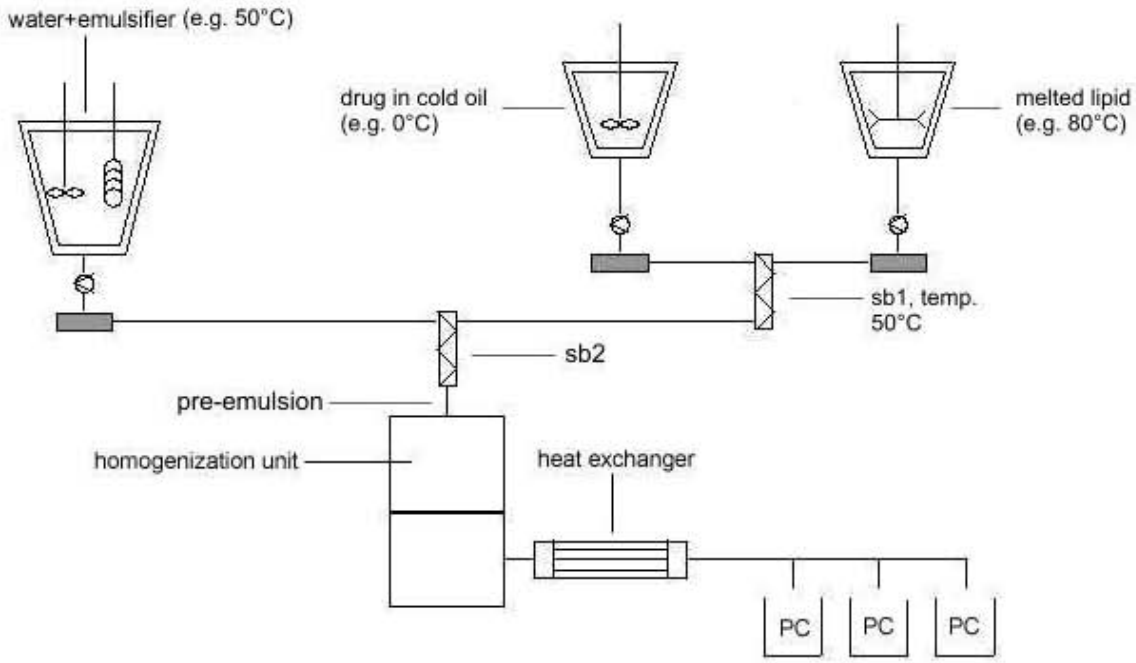


Fig. 2.11: Production flow chart for a production capacity of 1-2 tons per hour. First cold drug solution (drug dissolved in oil) and "overheated" melted lipid (e.g. 80 °C) are mixed in the first static blender (sb1). Mixing leads to a temperature of the melt of e.g. 50 °C, which is still above the melting point of the solid-liquid lipid blend. Then the hot surfactant solution and melted drug-containing lipid are mixed in static blender 2 (sb2) The pre-emulsion passes the homogenisation unit, the nanoemulsion is cooled by passing a heat exchanger, final cooling is performed in the product container (PC).

2.8 Production of final oral dosage forms

In principle the aqueous lipid nanoparticle suspensions can be used as an oral dosage form. Using optimal stabilizer formulations a physical stability of up to three years has been reported (Müller et al., 1995). Liquid dosage forms might be convenient for certain groups of patients (e.g. children and elderly patients). However, the dosage forms of first choice for delivery are dry forms: Tablets, capsules or fast dissolving drug delivery systems (FDDS) for the oral cavity. In some countries, sachets are also accepted. Pellets with incorporated SLN have been produced by Pinto and Müller (Pinto, 1996). The aqueous SLN dispersion was used as wetting agent in the production process of the pellets. It could be shown that the pellets released the SLN completely and without or very little aggregation. Such pellets can be filled into hard gelatine capsules or can be compressed to tablets (similar to pellet-containing tablets such as Beloc ZOK®).

Alternatively, the aqueous SLN dispersion can be used as granulation fluid in the production process of tablets or the SLN can be spray-dried (Freitas, 1998; Freitas & Müller, 1998) and the obtained powder added to the tabletting mixture (DirectCompress® technology) (Müller, 1997). As mentioned above, when producing oral dosage forms the loading capacity of each single dose needs to be considered. In case too much lipid mass is required to dissolve the required drug dose, the excipients necessary to produce pellets or tablets might lead to an unacceptable large volume. In such cases it might be considered to produce a spray-dried powder for oral administration.

Another interesting approach is the production of fast-dissolving delivery systems (FDDS). The adhesive properties of lipid nanoparticles could be exploited for delivery to the mouth cavity. Incorporation into such an FDDS would be relatively easy by simply lyophilising the lipid nanoparticle suspension under addition of cryoprotective agents such as mannitol or trehalose.

2.9 Regulatory aspects

One of the key pre-requisites for introducing a new technology and new products to the market is meeting regulatory requirements, not only with regard to excipients but also qualification and validation of production lines. In general, for the production of lipid nanoparticles only excipients accepted by the regulatory authorities are used, that means excipients with a GRAS status[3] (generally recognized as safe) or excipients which are already used in products on the pharmaceutical market. In the latter case these excipients need to be used in their commonly applied, regulatorily accepted concentrations. If distinctly higher concentrations are used a limited toxicity study might be necessary to prove the safety of the excipient in this concentration. All lipids, surfactants and stabilizers used in the production of capsules, pellets and tablets can be fully exploited for the production of oral lipid suspensions. There is definitely no lack of accepted excipients. In addition, all lipids, surfactants and polymeric stabilizers used in food industry can be employed. However, they need a registration for pharmaceutical purposes. One can refer to the toxicity data collected for registration as food or food additive. To sum up, a rich variety of excipients is available for oral lipid nanoparticles.

Another important point is the qualification of production lines within the general quality management (QM) (Müller, 2000). The production lines need to be made out of materials and compounds allowing a qualification of the production line. In addition, other QM steps such as validation need to be performed. Also each compound of the line needs to be capable of validation. Unfortunately many experimental production lines developed in academic research labs cannot meet these criteria. The production lines for lipid nanoparticles can be validated, for example a clinical batch production unit is available (Müller, 2000). A very important point is that the lipid nanoparticle technology uses production lines already established and existing in the pharmaceutical industry. The regulatory accepted production lines for emulsions can be used for lipid nanoparticle production because many are temperature controlled anyway. Therefore, it is possible to use the lines of parenteral emulsions for the production of lipid particle suspensions. The

[3] FDA Summary of GRAS Notices: http://vm.cfsan.fda.gov/~rdb/opa-gras.html (retrieved 08/09)

only “disadvantage” is that a certain minimum batch size will be required, which is approximately half a ton.

2.10 Perspectives on drug delivery using lipid nanoparticles

The three different types of lipid nanoparticles – SLN, NLC and LDC – represent a promising tool box for the oral delivery of lipophilic but also hydrophilic drugs, especially for hydrophilic drugs showing a reduced stability in the gut and a limited bioavailability. Lipid nanoparticles exploit the absorption enhancing properties of lipids, which is now commonly used for new improved delivery systems and oral dosage forms. Lipid nanoparticles fulfil essential prerequisites for entering the market with a new formulation. Such prerequisites are low cost production, clinical and large scale production facilities and accepted status of excipients. In contrast to e.g. liposomes, they are not only a low cost system but also physically more stable. Interesting for commercial exploitation is the exclusivity of the various lipid particle technologies; they are protected by issued patents or by patent applications in the major countries. By acquisition of the SLN technology by SkyePharma PLC in 1999 the technology of SLN has meanwhile entered pharmaceutical industry. The second generation technology of NLC is meanwhile present on the cosmetic market in more than 20 products worldwide (Müller, Rimpler, Petersen, Hommoss & Schwabe, 2007). The cosmetic products proved the feasibility of NLC products in general. Also large-scale production lines were established for these products in industry (e.g. Rimpler GmbH, www.rimpler.de), which can also be used for pharmaceutical products. In November 2007 the U.S. pharmaceutical company Brookwood acquired the worldwide exclusive rights for the NLC technology from Pharmasol to turn NLC into pharmaceutical products. The future will show if this is successful.

2.11 State of the art of testosterone drug delivery:

2.11.1 Overview

In general products for testosterone delivery can be divided into two groups: Products for injection and other products which include oral, buccal, and transdermal systems.

The crucial problem is the very high first pass effect of natural testosterone taken orally. 99 % of the drug are immediately metabolised by liver enzymes and do not reach circulation.

To bypass this problem several approaches have been made: The first attempt was the methylisation of testosterone, which hindered liver enzymes from inactivating it. Although application of methyltestosterone lead to a pronounced serum testosterone level, it was found to be very liver toxic and was therefore withdrawn (Wu, 1992; Wang et al., 2009).

However there is a variety of testosterone delivery systems on the market consisting of testosterone or its ester which all have more or less major disadvantages.

2.11.2 Products for injection

2.11.2.1 I.v. injection

Intravenous injection is performed with an aqueous suspension of testosterone. Due to rapidly degradation of the molecule by enzymes, several injections per day are necessary to maintain a constantly high serum level of the hormone. As a result the products have been discontinued (Androlan Aqueosus, Andesterone Suspension)

2.11.2.2 I.m. injection

Roughly half a dozen testosterone preparations for intramuscular injection are on the market in the U.S.A., France and Germany. Those products consist of an oily solution of a testosterone ester (mostly testosterone enantate or testosterone undecanoate). After intramuscular injection the hormone is liberated from the oil during a period of 6 to 12 weeks.

More than 10 % of the patients suffer from pain at the site of injection. Once administered, the dose cannot be adapted which carries the risk of overdosing - especially with patients that start with a testosterone replacement therapy (Mackey, Conway & Handelsman, 1995).

2.11.3 Other products

2.11.3.1 Oral route

Testosterone (T) has a poor oral bioavailability due to its extensive first pass metabolism by the liver. Therefore in commercial products lipidic derivatives of testosterone are used, e.g. testosterone undecanoate (TU) and testosterone enantate (TE). Still the oral bioavailability (BA) is very low, approx. about 7 % for TU in men and 3 % in beagle dogs (Köhn & Schill, 2003; Shackleford et al., 2003).

To overcome this problem, capsules filled with a solution of testosterone undecanoate in an oily solution have been developed (Andiol and Andriol Testocaps). The oil is partly taken up by the lymphatic system bypassing the liver and hence the first pass effect. Still, the bioavailability is quite low and therefore a total of up to four capsules have to be taken by the patient with one to four administrations per day.

The testosterone serum level resulting is irregular with peaks of short duration. Another problem of this formulation are the high inter- and intraindividual differences in pharmacokinetic parameters of the product (Behre & Nieschlag, 1998).

The discontinued product Andriol® was a solution of TU in oleic acid (Köhn & Schill, 2003). The successor commercial product Andriol Testocaps® is a solution of TU in castor oil and lauroglycol FCC (Shackleford et al., 2003). The rationale for replacing Andriol® by Andriol Testocaps® was the low stability and the very short shelf life (3 months) of the original product. It had to be stored at 8°C. Due to the low solubility of TU in the oil a single dose of 80 mg has to be administered in two capsules. Each Andriol Testocaps® capsule contains a total amount of 335 mg TU solution. Taking two capsules is not very convenient for the patient and reduces compliance. As mentioned above, the bioavailable TU is mainly absorbed via the lymphatic route (Horst et al., 1976; Horst & Erdmann, 1980; Houwing, Maris, Schnabel & Bagchus, 2003; Köhn & Schill, 2003; Shackleford et al., 2003). Minor parts

of TU are immediately de-esterified to testosterone at the gut wall. The major part is still absorbed across the gut wall but then immediately metabolised in the liver (first pass) (Horst et al., 1976; Horst & Erdmann, 1980; Köhn & Schill, 2003). This unavailable amount for therapy creates stress to the liver (Frey, Aakvaag, Saanum & Falch, 1979; Foye, Lemke & Williams, 2007).

2.11.3.2 Buccal

Buccal administration of testosterone is achieved by the use of a tablet (bioadhesive delivery system), which has to be placed on the gum. A buccal formulation (Striant) is currently marketed in the U.S., Great Britain and the Netherlands.

Sufficiently high serum levels can only be achieved if the product is constantly applied and changed every 12 hours (Zhang, Zhang & Streisand, 2002).

The tablet does not always stay in place. Tooth brushing, eating and drinking often loosens it. It is also unintentionally swallowed without even noticing it. Side effects that have been described are: Headache, gingivitis and irritations of the gum and dysgeusia (taste perversion). Regular gum examination is strongly advised.

(Striant patient information, www.striant.com/Consumer/patient_info/patient_info.asp, accessed 04/03/09)

2.11.3.3 Transdermal systems

Systems available for this route of application are gels and patches. They can be classified by their site of application: Systems which are applied on the torso and systems for the scrotum.

Gels are preparations of testosterone and sometimes contain also permeation enhancers like cyclopentadecanolide or high concentrations of alcohol. They are applied at least once a day on dry skin. The patient has to wait for 5 to 10 minutes after application for the formulation to dry. Hands have to be carefully washed and the site of administration has to

be covered to avoid other persons (especially children) to get into contact with the gel. If skin contact with untreated persons is expected, showering beforehand is strongly advised. Otherwise it bears the risk of transferring the drug to them and can result (in case of females) in androgenisation. Side effects are skin irritations.

Due to inter- and intraindividual differences in skin permeation the flux of drug can greatly vary and is almost impossible to predict.

The second transdermal delivery system are patches which are also widely used and available on the markets worldwide. Depending on the delivery system, one or even two patches have to by applied simultaneously and carried for two to four days. Severe skin irritations are a common phenomenon.

The systems for use on the torso are still available while the formulations for application on the scrotum have been discontinued: While absorption of testosterone via scrotum skin is better, it contains high concentration of esterases that lead to a high concentration of dihydrotestosterone after application. Furthermore compliance was low: Shaving of the scrotum was necessary, problems with adhesion of the patches and skin irritations occurred (Findlay, Place & Snyder, 1989).

While the problem of high dihydrotestosterone levels does not occur with non-scrotum patches, skin irritation is even more pronounced with this formulation.

2.11.3.4 Subcutaneous implants

There is only one subcutaneous product commercially available, which is only sold in the U.S.A.: Testopel pellets. They are implanted under the skin and release the drug continuously over a four to six months time period.

A microsurgery has to be performed to put the 3.4 x 9 mm pellets into their desired place of action. Furthermore once administered the release rate of testosterone is difficult to adjust. Risks and side effects are : Extrusion, bleeding, and inflammations/infections at the site of implantation (Jockenhovel et al., 1996).

2.11.3.5 Other formulation approaches

There are a variety of other formulation attempts like nasal spray, pulmonary delivery or ultrasound enhanced transdermal transport. Those attempts have not made it to the market yet.

3 Materials and Methods

3.1 Materials

3.1.1 Tetstosterone undecanoate NLC and testosterone (undecanoate) nanosuspensions

Tesotsterone undecanoate was bought from Jenapharm (Jena, Germany), testosterone from Caelo (Hilden, Germany). For the production of the lipid nanoparticles the following lipids and surfactants were used: Dynasan 118 was obtained from Condea (Witten, Germany). Stearic acid, sodium dodecylsulfate and Tween 80 were bought from Sigma-Aldrich (Deisenhofen, Germany), oleic acid from Caelo.

3.1.2 Fish oil NLC

Dynasan 118 (glyceryl tristearate) used as matrix lipid for the production of NLC was obtained from Condea (Witten, Germany). Sodium dodecyl sulfate (SDS) and polyvinyl pyrrolidon were purchased from Fluka Chemie GmbH (Buchs, Switzerland) and from Merck (Darmstadt, Germany), respectively. Tween 80 was bought from Uniqema (Eversberg, Belgium), whereas TPGS (D-alpha Tocopheryl Polyethylene Glycol 1000 Succinate) was a kind gift from Eastman (Anglesey, U.K.). The water used was produced by a MilliQ system (Millipore, Billerica, MA, United States). The fish oil was obtained as a gift from Pharmasol GmbH (Berlin, Germany). It contained 38 % omega-3 fatty acids including 19 % EPA (eicosapentaenoic acid) and 13 % DHA (docosahexaenoic acid) according to its analysis certificate.

For taste masking, aroma concentrates (lemon, orange, mango and strawberry flavour) from Symrise (Holzminden, Germany) were used which were a kind gift of the company. For colouring, Sicovit food colours (BASF AG, Ludwigshafen, Germany) were used.

All excipients were used as received.

3.2 Methods

Each chapter in the "results and discussion" part has a section describing the methods used in detail.

3.2.1 Nanostructured Lipid Carriers (NLC)

The production of the Nanostructured Lipid Carriers is explained in the respective results chapters (4.1.2 and 4.2.3).

3.2.2 Nanosuspensions

Nanosuspension production is described in section 4.1.4 on page 66.

3.2.3 Laser Diffractometry

Laser diffractometry is a technique for measuring particles sizes in the range of 40 nm to 2000 µm (depending on the system used). In can be applied to liquid particles dispersion in water and organic solvents but also to dry powders and is based on the diffraction of a laser beam on the particle surface.

The laser beam is expanded using an optical system. It then passes the particle dispersion or the powder and is then projected onto a detector (Fig. 3.1). Depending on the particle size the laser beam is diffracted by the particle surface, whereas small particle diffract the light in a greater angle than larger one, which can be explained by their more curved surface.

Every particle produces its own diffraction pattern.

A Fourier lens is used to project the pattern of particles identical in size to the same region of the detector no matter in which area of the sample unit it is located.

The detector is made of concentric rings (e.g. 32), which are located in the focus area of the Fourier lens. Since the detector is very expensive, in most LD machines it is not a whole circle but consists only of a segment of it.

As already mentioned, the diffraction pattern of a particle depends on its size. Hence the intensity of the diffracted laser light is more intense on the inner rings for small particles and on the outer rings for larger particles. In polydisperse systems these patterns are

superpositioned. That means, the pattern projected onto the detector is the sum of all projection patterns of the particles present in the dispersion.

To calculate the size distribution, two major theories can be applied. For particles larger than about 4 micrometers, the theory of Fraunhofer is used. For smaller particles the theory after Mie applies which takes into account also light scattering and absorption of the particles. The smaller the sizes the more important are those factors. With particles above 4 micrometers those effects can be ignored. To be able to apply Mie's theory, the refractive index of the particle and the dispersion medium has to be known. In this work, a real refractive index of 1.456 and an imaginary part of 0.001 was used which was found by Schumann in 1996 for intravenous fat emulsions (Müller & Schuhmann, 1996). The majority of research groups working in that field has used it since that time. This value is not necessarily correct for lipid nanoparticles. However, LD analysis was mainly applied to be sure to exclude particles in the micron range. For this purpose, the used values are sufficiently exact. There are slight variations in the indices depending on the nature of matrix lipid and stabilizer used (Keck, 2006) but for the envisaged development of an oral formulation (not an intravenous formulation) these effects can be neglected.

Laser diffractometry yields a volume distribution of particles sizes present in the entire population. As a matter of fact, larger particles (with a larger volume) are taken more into account than smaller ones.

Characteristic parameters of an LD measurement are the mean diameters 50 % (d50%), 90 % (d90%) and 95 % (d95%), whereas the number means that e.g. 50 % of the population have a size inferior to the size shown.

The laser diffractometers applied in this work were a Beckman Coulter LS-230 (Beckman-Coulter, Krefeld, Germany) and a Malvern Mastersizer X (Malvern Instruments, Malvern, UK).

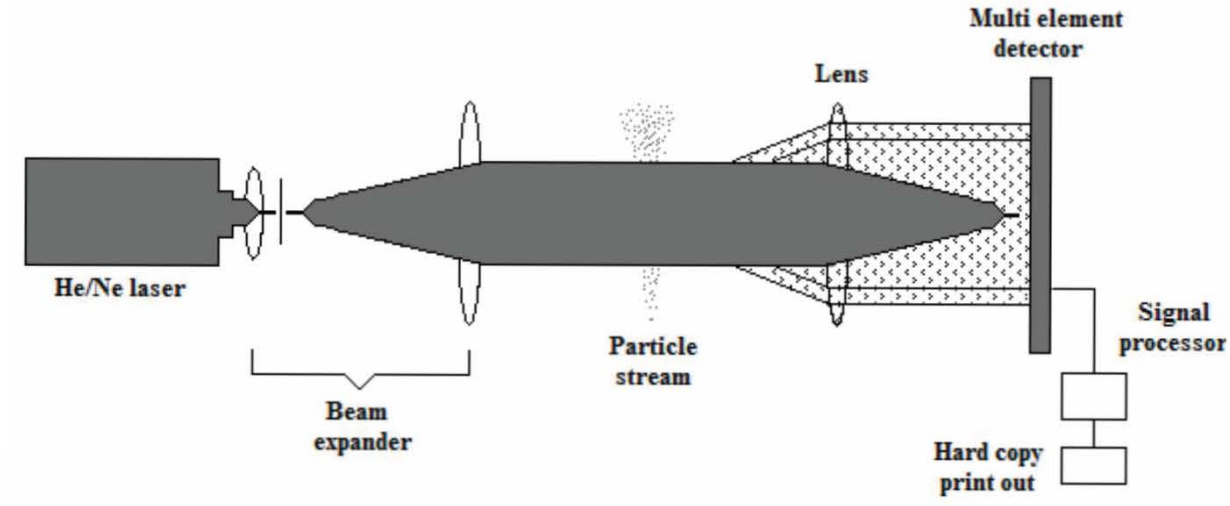


Fig. 3.1: Schematical set up of a laser diffractometer after (Müller & Schuhmann, 1996)

3.2.4 Photon correlation spectroscopy (PCS)

PCS is a widely used method to determine particles sizes in the range of 3 nm to 3 µm in colloidal systems. The measurement is based on the fluctuation of light intensity of laser light scattered by the sample dispersion. It is also called "dynamic light scattering". The fluctuation of the scattering is caused by the particles in the dispersion measured moved by the Brownian motion of the dispersion medium (most commonly water). PCS yields a mean diameter (z-average) and a polydispersity index (PI) as a measure for the width of the size distribution (Müller, 1991). A PI of 0 is theoretically indicating a monodisperse population, 0.10-0.20 indicates a relatively narrow distribution and values > 0.50 indicate a very broad distribution. A typical set up of a PCS machine is shown in Fig. 3.2

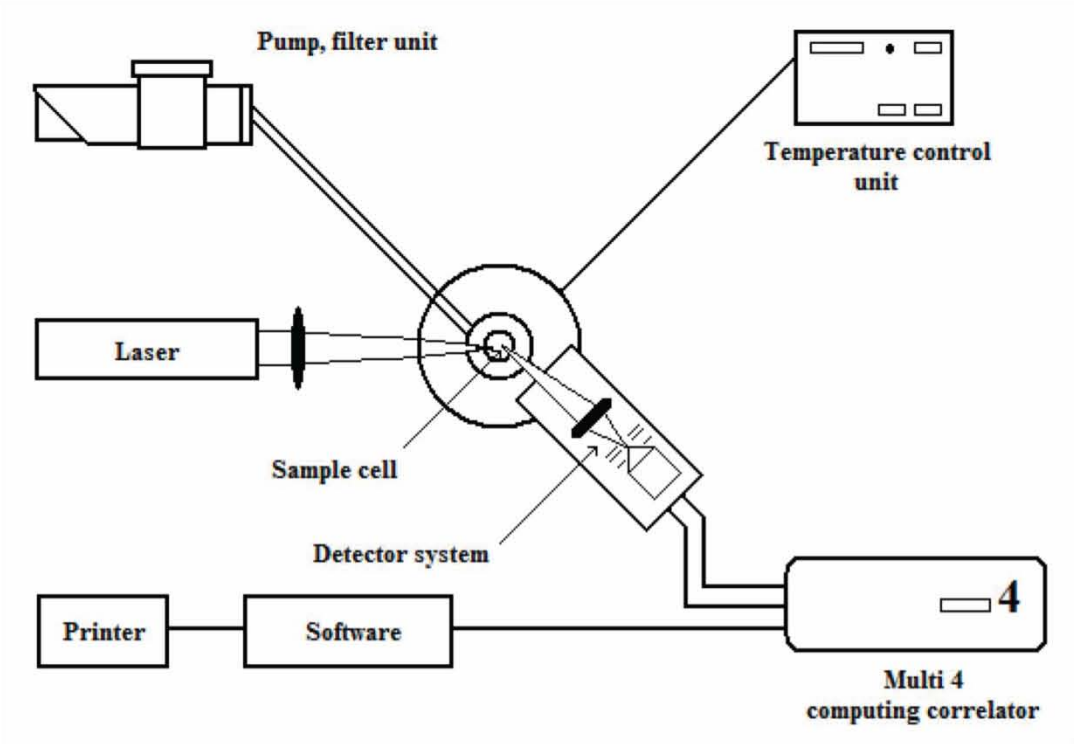


Fig. 3.2: A typical setup of a PCS apparatus. Modified after (Müller & Schuhmann, 1996).

The sample is illuminated by the laser. The scattered light is then passed through a lens which is located at an angle of typically 90° (other angles possible depending on the purpose of the measurement). A photodiode or a photomultiplier in older systems measures the scattered light's intensity (and thus the changing of the intensity).

Small particles move faster than larger ones and therefore cause a higher fluctuation in the intensity of the scattered light then larger ones.

A correlator (hardware or software) transfers the fluctuations into a diffusion coefficient D by means of a correlation function.

If the viscosity of the dispersion medium and its temperature are known, the mean particle radius can be calculated using the Stokes-Einstein equation (assuming perfectly round particles):

$$r = \frac{k\,T}{6\,\pi\,\eta\,D}$$

where r = mean particle radius, k = Bloltzmann's constant, T = absolute temperature, η = dynamic viscosity and D = diffusion constant

PCS measurements in this work were done using either a Zetasizer 2000HS, Zetasizer 4 or a Zetasizer Nano NS (both Malvern Instruments, Malvern, UK) applying a measuring angle of 90°. All samples were diluted with ultrapure water to an appropriate concentration.

3.2.5 High performance liquid chromatography (HPLC)

The drug content of the produced formulations was checked by HPLC. A previously published method for the quantification of testosterone and testosterone undecanoate was adopted (Claussen, Bose & Dittgen, 2000). NLC formulations and emulsions where dissolved in ethylene chloride and then analysed to determine the overall drug content of the formulation.

The HPLC system was a Shimadzu Prominence LC-20AD with the following components: DGU-20A degasser, SIL-20AC auto sampler, SPD-20A UV/VIS detector, CTO-20A column oven.

A gradient elution was applied using the following time scheme: 0-2 minutes: 10 % acetonitrile in water, 2-5 minutes: 10 % to 96 % in water linearly increasing, 5-17 minutes: 96 % in water. The flow rate was 0.8 ml/min at 40 °C.

The HPLC column was a reversed phase RP-18. UV detection was performed at a wavelength of 243 nm. The injection volume of the sample was 10 µl. The water and acetonitrile used was HPLC grade water purchased from Fisher Scientific.

The testosterone peak occurred after 10 minutes while the testosterone undecanoate peak appeared after 12 minutes.

3.2.6 Zeta potential

The zeta potentials (ZP) were determined using also the Malvern Zetasizer (Malvern Instruments). The applied field strength was about 20 V/cm, the electrophoretic mobility was converted to the zeta potential by applying the Helmholtz-Smoluchowski equation (Müller, 1996). Measurements were performed in distilled water with the conductivity adjusted to 50 µS/cm by using sodium chloride solution. Adjustment to 50 µS/cm avoids fluctuations of the zeta potentials due to changes in the quality of distilled water (i.e. the conductivity which can fluctuate between 1-10 µS/cm) (Lucks, Müller & Müller, 1990). In addition, measurements were performed in the original dispersion, i.e. in 1 % and 2 % Tween 80 solution.

3.2.7 X-ray diffraction

X-ray analysis was used to determine the crystalline status of the lipid nanoparticles, in addition to the DSC measurements. Diffraction patterns were measured using a Philips X-ray generator PW 1830 equipped with a copper cathode (λ = 1.5418 Å, 40 kV, 20 mA) coupled to a computer-interfaced Philips PW 1710 diffractometer control unit. The scattered radiation was measured with a vertical goniometer (Philips PW 1820) (Philips Industrial & Electro-Acoustic Systems Division, Almelo, the Netherlands). The system is a powder diffractometer – it can only analyse particles in suspensions if the viscosity of the dispersion medium is sufficiently enhanced. For analysis of highly fluid suspensions a viscosity enhancer is added (e.g. xanthan gum). In the case of the paste this was not necessary because it had already a sufficiently high viscosity.

3.2.8 Differential scanning calorimetry (DSC)

The particle matrix structure was investigated by differential scanning calorimetry (DSC) using a Mettler Toledo DSC821e (Mettler-Toledo, Giessen, Germany). For analysis the materials were weighted in 40 mg pans. Heating and cooling rates were 5 K/min., respectively. The thermograms were analysed using the STAR software.

3.2.9 Light microscopy

The NLC dispersions were also characterised by light microscopy using a microscope Leitz Orthoplan (Leitz, Wetzlar, Germany). Analysis was performed applying up to 1000 x magnification and oil immersion. The intention to use light microscopy was to screen for particles and aggregates being larger than 1 µm. To increase the probability of detecting such particles, the NLC suspensions were analysed undiluted.

3.2.10 Determination of serum testosterone levels

Serum analysis was done using an enzyme immunoassay (EIA) test No. 582701 from Cayman Chemicals (USA) (Ann Arbor, Michigan, USA)

Analysis was performed following the extraction procedure provided by the manufacturer. The test was used exactly as directed. Recovery rates were determined using a cold spike method and found to be approx. 90 %. The plate was read using a microplate reader (Tecan Sunrise, Tecan, Männedorf, Switzerland) at 412 nm wavelength.

Results were calculated following the instructions given by the manufacturer using the provided spreadsheet (MS Excel 2003).

The assay test is based on the competition of testosterone and a testosterone tracer for a limited amount of testosterone antiserum. The concentration of the tracer is held constant; the concentration of testosterone varies (depending on the serum level of the sample). Therefore the amount of tracer which can bind to the antiserum is inversely proportional to the testosterone concentration of the sample. Ellman‘s reagent is an substrate to the tracer and is used to determine the amount of bound tracer. The product of the enzymatic reaction has a yellow colour. Its concentration can be assessed using a photometer (microplate reader) (after (Cayman Chemicals, 2006))

A typical picture of a developed test can be seen in Fig. 3.3

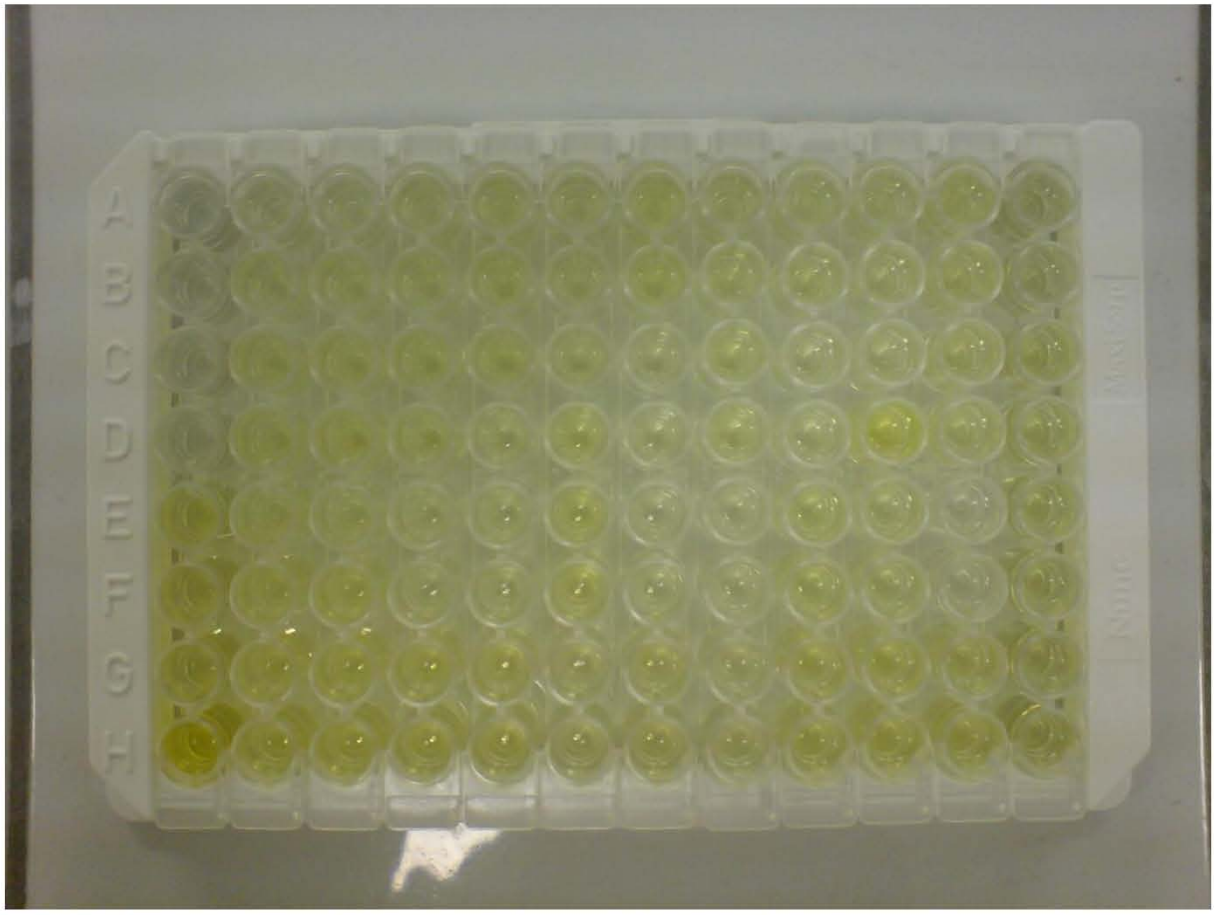


Fig. 3.3: Typical picture of a developed EIA test. The different colours indicate different concentration of the samples.

3.2.11 In vivo studies

Experiments were carried out in compliance with the French legislation on animal experiments under the personal experimentation authorisation No. 54–68.

3.2.11.1 Animals

Male Wistar rats (purchased from Charles River, Wilmington, MA, USA) were used for the *in vivo* studies.

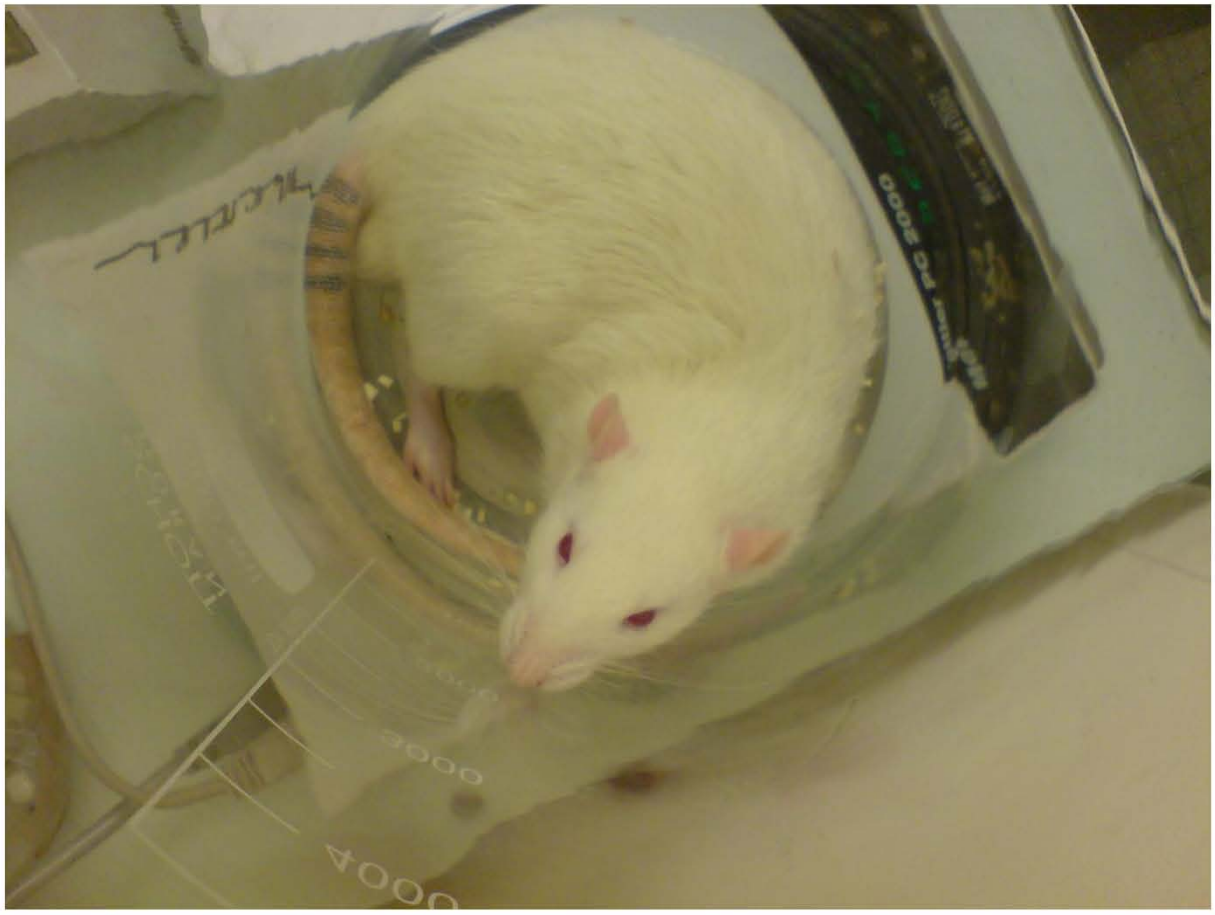


Fig. 3.4: A rat with marked tail used for the *in vivo* experiments during weighing prior to the sample administration.

3.2.11.2 Housing and treatment

Housing

Animals were delivered to the laboratory at least 5 days before the experiments during which time they are acclimatized to laboratory conditions.

Rats were housed in groups of 4 in cages under standard conditions: room temperature (21 ± 3 °C), light/dark cycle (12/12 h with lights on from 7 a.m. to 7 p.m.). They have free access to food and water. Each animal can be identified by a marking on the tail with a permanent pen (Fig. 3.4).

There is no reason to expect that any contaminant or nutritional component will be present in the feed, direct bedding or water at levels capable of interfering with the results of the tests.

Treatment

Animals were deprived of food 12 hours before the treatment but still had free access to water.

Prior to administration of the dose, a blood sample was taken to determine the testosterone level at t = 0.

For the *in vivo* tests with oral drug administration rats were divided into groups of four animals and one replacement animal. The replacement animal was treated exactly the same way as the other rats and was used in case of one of the "regular" group members could not be used.

The freshly prepared samples were administered to the rats by means of a feeding needle and a syringe. The dose applied was 10 mg TU per kg body weight for the oral in vivo tests and 1 mg TU per kg for the intravenous studies. In case of the administration of testosterone the dose was adapted to be equal to the TU value. Fig. 3.5 shows a dose just prior and after administration to one of the rats.

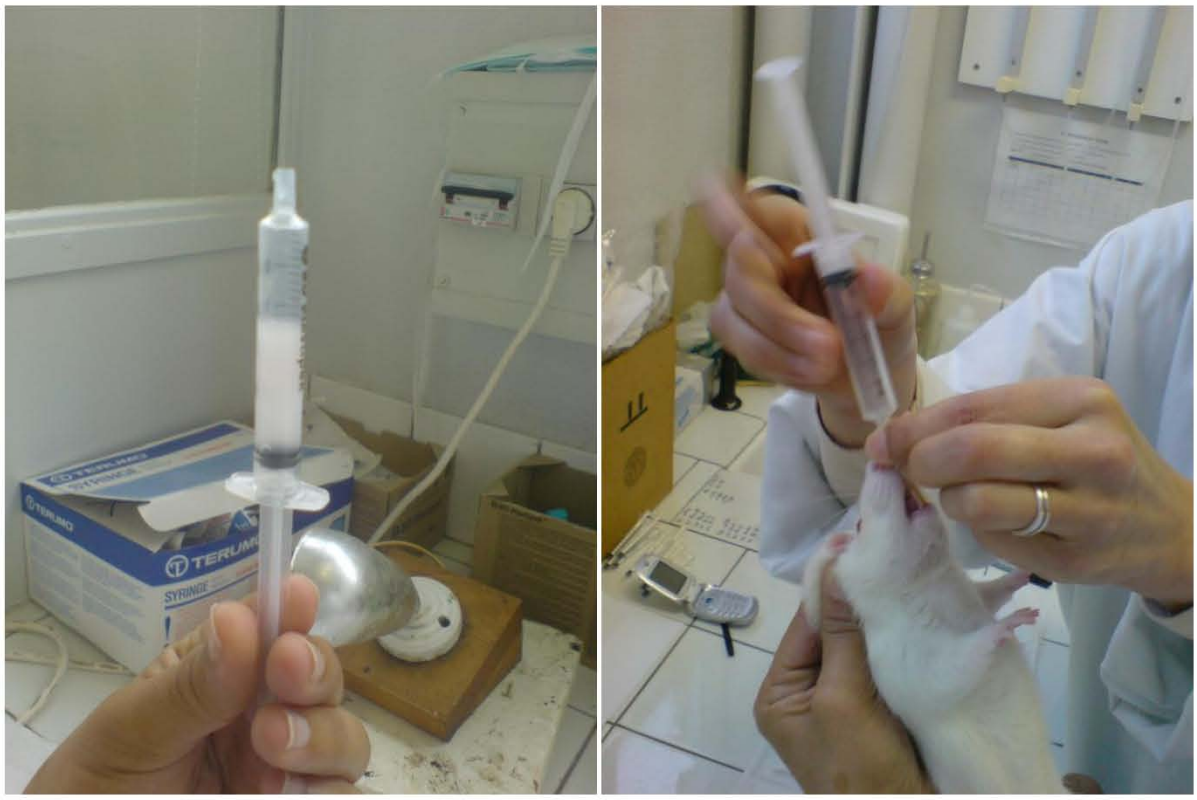


Fig. 3.5: Dose ready for administration (left). The right hand picture shows the animal and the empty syringe just after administration.

The volume of administration was typically between 300 and 500 µl depending on the weight of the animal. The dead volume of the feeding needle was determined for every sample to guarantee the exact dose to be delivered into the stomach of the rat.

After administration, rats were put back into the cages where they were allowed to move freely and had access to drinking water.

400 µl of blood were drawn from the animal by heart puncture. After 30 minutes of cold storage (4 °C) the samples were centrifuged at 6000 x g (also at 4 °C).

The obtained serum was then immediately transferred into polypropylene tubes which were stored at -80 °C until they were assayed for testosterone concentration.

3.2.11.3 Sample administration

Number of administration: One single administration per rat was performed.

Route of Administration: Oral administration with a feeding needle

Animal groups: 3 groups of 4 male rats each.

One additional rat per group was used to possibly replace an animal, which would have been judged ill during the habituation period.

Animals were deprived of food overnight prior to administration. Twelve hours post-administration, they were offered food *ad libitum*.

Body weight measurement was recorded for each animal prior to administration.

3.2.11.4 Blood sampling

Blood samples were collected before (T0) and then after the test item administration according to the schedule indicated in table 3.1.

Table 3.1: Blood sampling time schedule

Blood sampling	**Time of sampling**	**Rat n°**
1	T0 + 1 hours	1 to4
2	T0 + 2 hours	5 to 8
3	T0 + 3 hours	9 to 12
4	T0 + 4 hours	1 to4
5	T0 + 6 hours	5 to 8
6	T0 + 8 hours	9 to 12

A total of 3 blood samples per animal were thus collected for 8 hours for each formulation. Approximately 400 µL of blood were collected by heart puncture using sterile syringes with a needle.

The tubes were then gently mixed and held at 4 °C for 30 minutes and then centrifuged at 4 °C for 15 min at 6000 x g. The serum obtained were immediately transferred into 1.5 mL polypropylene vials and stored at -80 °C.

4 Results and Discussion

4.1 Testosterone and testosterone undecanoate nanocarriers for oral bioavailability enhancement

4.1.1 Rationale of development

As outlined before, the oral bioavailability of testosterone is relatively low (approximately 1 %) due to the pronounced first pass metabolism of the liver (Johnsen, 1978; Frey, Aakvaag, Saanum & Falch, 1979; Foye, Lemke & Williams, 2007). Lipophilic derivatives of testosterone (e.g. testosterone undecanoate (TU)) possess a higher bioavailability of about 3 % in beagle dogs and 7 % in man (Köhn & Schill, 2003; Shackleford et al., 2003). Therefore TU is used in commercial preparations, e.g. Andriol® and Andriol Testocaps® (Shackleford et al., 2003). The latter one is a solution of TU in castor oil and Lauroglycol FCC. The rationale for replacing Andriol® by Andriol Testocaps® was the low stability and the very short shelf life (3 months) of the original product. It had to be stored at 8 °C (Shackleford et al., 2003). The weight of the total solution in these two capsules is 770 mg. Obviously, two capsules are not patient-friendly; in addition multiple units reduce the compliance. In addition, the bioavailability of Andriol Testocaps® is strongly dependent on the non-fed/fed status of the patient (Bagchus, Hust, Maris, Schnabel & Houwing, 2003; Schnabel, Bagchus, Lass, Thomsen & Geurts, 2007).

Nanoparticulate delivery systems are known to increase the oral bioavailability (Liversidge & Conzentino, 1995; Liversidge & Cundy, 1995; Hanafy et al., 2007). In addition, lipids as an excipient are also promoting the oral absorption (Charman, 2000; Porter & Charman, 2001a; Shackleford et al., 2003). The nanostructured lipid carriers (NLC) unify in one delivery system both aspects being nanoparticulate and composed of lipids as particle matrix. The advantage of the nanoparticles is that they also show a lymphatic uptake when their size is below about 100 nm, the cutting edge size for lymphatic absorption (Liversidge & Cundy, 1995). The TU bioavailable in the blood is mainly a result of lymphatic absorption. Therefore a nanoparticulate delivery system could exploit the lymphatic route for leading to an improved bioavailability.

Solid lipids and oils were screened regarding their solubility properties for TU to be used in NLC production. NLC were produced with different matrix compositions and sizes to allow the study of these effects on the *in vivo* bioavailability. The particles were characterised regarding size, surface charge, structure of matrix and short-term stability with perspective of developing an improved oral formulation.

Another promising approach to increase the oral bioavailability of poorly soluble drugs are drug nanocrystals (Liversidge & Cundy, 1995; Keck & Müller, 2006; Möschwitzer & Müller, 2006a; Möschwitzer & Müller, 2007). Production of drug nanocrystals is simpler than producing NLC. Therefore and for reasons of convenience and costs, drug nanocrystals can be considered as carrier system of first choice. In case the drug nanocrystals do not work or do not work sufficiently, the NLC are the alternative delivery system. NLC are especially of value when the presence of lipid promotes drug absorption. This is the case for TU because the bioavailability is very much dependent on the fed state (Bagchus, Hust, Maris, Schnabel & Houwing, 2003; Schnabel, Bagchus, Lass, Thomsen & Geurts, 2007). Considering this, NLC were chosen as nanocarrier system. However, it appeared also worthwhile to run nanocrystals formulations as well.

Not only TU was investigated, also the "original" molecule testosterone. After absorption TU is fast metabolised into T, therefore it was a logic approach by companies to use TU instead of T because of its higher bioavailability. Nevertheless there is the general trend in pharmacy to use rather original molecules than synthetic derivatives. For this reason testosterone was also included. In addition, testosterone nanocrystals could contribute to better understanding of mechanisms and be used for exploring new potential opportunities. Furthermore, testosterone could not be formulated as NLC since its solubility in lipid mixtures is too low.

4.1.2 Production method of tetstosterone undecaonate nanostructured lipid carriers (NLC)

The NLC were produced by melting the lipid blend at 75 °C, dissolving the TU and homogenising the TU-containing blend in a hot Tween 80 solution of identical temperature. The Tween 80 concentrations used were 1 % and 2 %, respectively. The obtained coarse

macroemulsion was homogenised using a Micron Avestin emulsiflex B3 homogeniser (Avestin Europe, Mannheim, Germany). The applied production pressure and the number of cycles were varied to modify the particle size: 200 to 500 bars and 1 to 3 cycles. For homogenisation at 75 °C, the Avestin emulsiflex B3 was equipped with a temperature control jacket.

4.1.3 Testosterone undecanoate NLC: Production and characterisation

4.1.3.1 Lipid screening and testosterone undecanoate solubility

Two capsules of Andriol Testocaps® are required to deliver one single dose because the solubility of TU is limited in the capsule oil mixture (approx. 10 %, 80 mg TU in 770mg oil solution in two capsules). To load the NLC with a dose as high as possible, a lipid screening was performed determining the solubility of TU in oils and in melted solid lipids. Primary focus was on the lipids which appeared promising for delivery of TU according to theoretical considerations. According to Charman, glycerides with fatty acids with a carbon chain length of 16-18 atoms have the greatest potential to increase lymphatic absorption (Charman & Porter, 1996; Charman, 2000; Porter & Charman, 2001b). Therefore Dynasan 118 was chosen as triglyceride of stearic acid (which is a C-18 fatty acid). To look at the effect of the lipid structure, triglyceride versus fatty acid, stearic acid itself was chosen as another C-18 lipid. Admixing of oil to a solid lipid reduces the melting point. As a result, to achieve a high oil load, it is favourable to have lipids with a very high melting point. Carnauba wax with a melting point of 82-86 °C was chosen due to previous experiences. It allowed incorporation of up to 60 % oil and yielded NLC still solid at 40 °C. Oleic acid was included as oil component because it is contained in Andriol® and possesses very good solubility properties for TU. As an alternative, castor oil was also investigated. It is the oil component of Andriol Testocaps®. The screening was done in steps of 5 %.

Table 4.1.1 shows the different solubilities. Oleic acid was superior in dissolution of TU compared to castor oil having a 5 % higher solubility both at room temperature and at 75 °C. The solubility of TU in the melted stearic acid was 25 %, and 30 % for Dynasan 118 and carnauba wax. Dissolving TU in the mixtures of oil and lipid increased the solubility in all mixtures by 5 % compared to the best solubility in one of the single components. This can be explained by the difference in dissolution behaviour between ideal binary mixtures of two solvents and real mixtures. In ideal binary mixtures the solubility in a mixture is the sum of the solubility in both components, based on the mol fractions of each of the components. In real binary mixtures there can be a negative or positive deviation yielding lower or higher solubility. Fortunately in the investigated real mixtures the solubility of TU was higher which was favourable for the production of NLC.

In general the solubility of a component in a solid lipid is lower than in a liquid lipid. Therefore admixing an oil to the solid lipid to produce NLC leads to an increased drug loading compared to the first generation of lipid nanoparticles, the solid lipid nanoparticles (SLN) (Müller, Mäder, Lippacher & Jenning, 2000; Muchow, Maincent & Müller, 2008). In addition, the presence of the oil molecules distorts the formation of perfect lipid crystals of the solid lipid. This leads to imperfections, which is favourable for drug inclusion (Müller, Radtke & Wissing, 2002).

Table 4.1.1: Solubility (%) of testosterone undecanoate in oils at room temperature and in oils, molten lipids and blends of oils and molten lipids at 75°C after 30 minutes.

Lipid	***Solubility (25°C)***
Oleic acid	25%
Castor oil	20%
	Solubility (75°C)
Oleic acid	30%
Castor oil	25%
Dynasan 118	30%
Stearic acid	25%
Carnauba wax	30%
Oleic acid/Dynasan 118 (1:1)	35%
Oleic acid/stearic acid (1:1)	30%
Carnauba wax/oleic acid (1:1)	40%

4.1.3.2 Production of NLC: Size

For the production of NLC mixtures of Dynasan 118 with oleic acid (50:50) were chosen. The basic drug loading was 15 %. The rationale behind was to have still a high lipid to drug ratio (85:15) to exploit the absorption promoting properties of the lipid. Two different concentrations of Tween 80 were chosen (1 % and 2 %) to obtain particles with different sizes. This should allow investigating the size effect but maintaining the same composition of the particle matrix and drug loading. One formulation was produced with 30 % drug content to investigate *in vivo* the effect of a reduced lipid to drug ratio (70:30). Can a similarly high bioavailability still be achieved at the lower ratio? The forth formulation was chosen to compare the effect of glyceride versus fatty acid in the particle matrix, i.e. producing NLC with stearic acid/oleic acid (50:50) instead of using Dynasan 118. For these particles again 2 % Tween 80 were chosen, because from theoretical considerations small particles should

result in a higher bioavailability compared to larger NLC. Table 4.1.2 gives an overview of these four NLC formulations.

Table 4.1.2: Overview of the formulations used: All formulations contained a 10 % lipid phase (= solid lipid, oil and drug).

Formulation	Tween 80	Lipids (ratio solid lipid : liquid lipid)	TU content of lipid phase
NLC1	1 %	Dynasan 118/oleic acid (50/50)	15 %
NLC2	2 %	Dynasan 118/oleic acid (50/50)	15 %
NLC3	2 %	Dynasan 118/oleic acid (50/50)	30 %
NLC4	2 %	Stearic acid /oleic acid (50/50)	15 %

Table 4.1.3 shows the mean PCS diameters and the polydispersity indices (PI) of these four formulations. As expected, the formulation with only 1 % Tween 80 and homogenised with just one homogenisation cycle and 200 bars yielded a relatively large size of about 600 nm. Increasing the concentration to 2 % Tween 80 and applying 3 homogenisation cycles and 500 bars yielded a size of about 200 nm for both the Dynasan 118/oleic acid NLC loaded with 15 % and 30 %, respectively. This allowed comparing *in vivo* large size versus small size NLC (identical lipid: drug ratio). Furthermore, small size NLCs were compared with high and low lipid to drug ratio 85:15 and 70:30 (i.e. a lower and higher drug loading, 15 % and 30 % TU). In formulation 4 Dynasan 118 was replaced by stearic acid (15 % TU), also a size of about 200 nm was achieved. Therefore all target sizes could be produced for the envisaged *in vivo* study. The polydispersity index for all formulations was around 0.20 indicating a relatively narrow size distribution. This was confirmed by the laser diffractometry diameters 50 % and 90 %. For example, the diameter 90 % is 0.91 µm for the larger sized NLC1 and about 0.50 µm for the other three NLC formulations 2, 3 and 4 (Table 4.1.4). The LD diameters 50 % are in general higher than the PCS diameters, because laser diffractometry yields a volume size distribution. The diameters 50 % confirm also that NLC1 are larger than the other NLC formulations, NLC2 to 4 are similar in the diameter 50 % (0.25-029 µm).

Table 4.1.3: PCS sizes of the formulations directly after production (n=3)

Formulation	PCS size [nm]	PI
NLC1	592 (±7)	0.15 (±0.02)
NLC2	176 (±0)	0.23 (±0.01)
NLC3	215 (±3)	0.15 (±0.01)
NLC4	200 (±3)	0.23 (±0.02)

Table 4.1.4: Laser diffractometer measurements of the formulations directly after production

Formulation	LD50 % [µm]	LD90 % [µm]
NLC1	0.37	0.91
NLC2	0.25	0.40
NLC3	0.28	0.48
NLC4	0.29	0.54

4.1.3.3 Structural investigations

The bulk material of TU and the blends of the solid lipids with the oils were investigated. Fig. 4.1.1 shows exemplarily the DSC curve of TU and the blend of Dynasan 118 and oleic acid. The TU melts at 64.4 °C, the peak maximum of the lipid blend is at 66.6 °C. Fig. 4.1.2 shows exemplarily the DSC curves after incorporation of 15 % and 30 % TU in these matrices. The melting peaks are at 66.5 °C and 65.4 °C showing only a slide reduction in the melting point due to TU. From the distinct sharp shape of the melting peaks it can be concluded that TU is molecularly dispersed in the blend and not precipitated as a separate crystalline fraction. The cooling curves of the DSC show a recrystallisation of TU in two peaks, a minor peak at 47.0 °C and a major peak at 27.9 °C (Fig. 4.1.1, upper). In contrast to this, a bimodal peak is obtained with the lipid blend having peak maxima at 42.2 °C and 35.3 °C (Fig. 4.1.1, lower). The shape of this recrystallisation peak remains practically the same, apart from an increase of the peak height at about 42 °C when moving from 15 % TU to 30 % TU (Fig.4.1.2). A separate recrystallisation peak of TU is absent, supporting also the assumption that TU is molecularly dispersed. At least no crystalline TU is present.

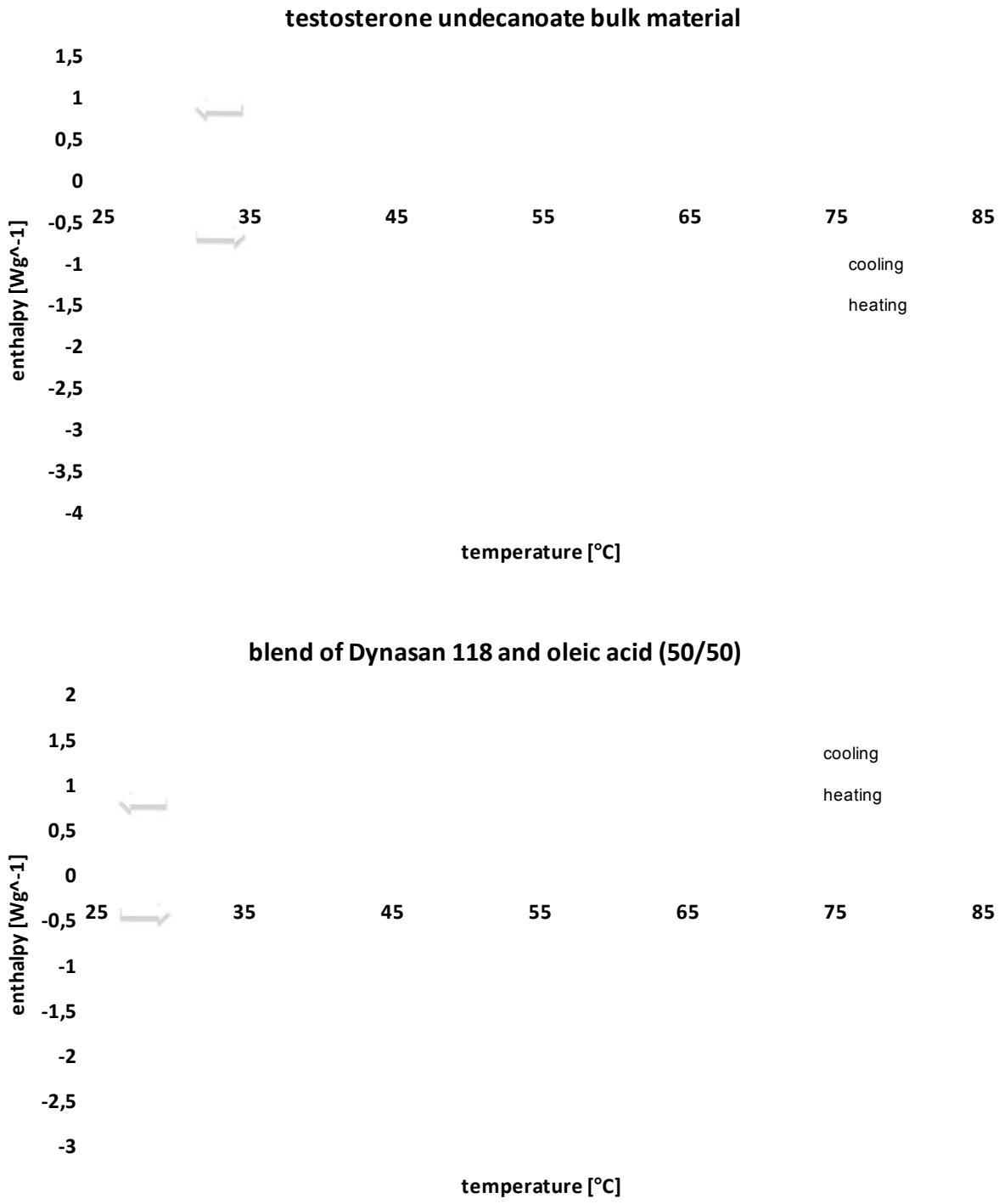


Fig. 4.1.1: DSC curve of the bulk material of testosterone undecanoate (upper) and a blend of Dynasan 118 and oleic acid (50/50) [m/m] (lower)

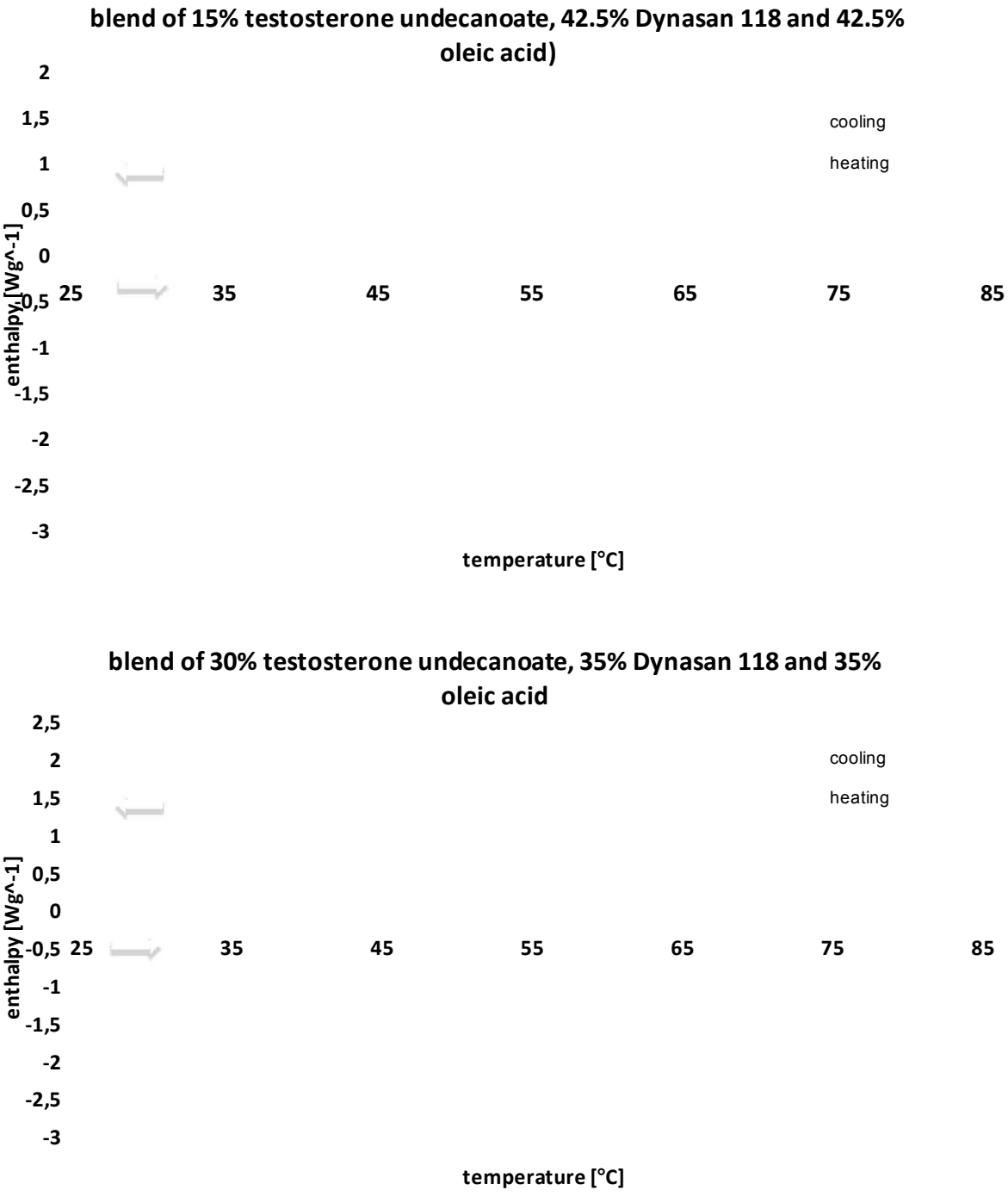


Fig. 4.1.2: DSC curve of the bulk material of a blend of 15 % testosterone undecanoate, 42.5 % Dynasan 118 and 42.5 % oleic acid (upper) and a blend of 30 % testosterone undecanoate, 35 % Dynasan 118 and 35 % oleic acid indicating the complete solution of TU in the lipids.

This is confirmed by the X-ray diffractograms. Fig. 4.1.3 shows the X-ray diffraction patterns of the bulk material of TU and the blend of Dynasan 118 and oleic acid. Analysis of the NLC dispersions revealed an amorphous halo due to presence of water, superimposed with the peaks of the lipid, the TU peaks were absent. Fig. 4.1.4 shows this exemplarily for 15 % and 30 % TU incorporated in a matrix of Dynasan 118/oleic acid. Crystalline TU is absent, or to be more precise: below 5 %, which is the detection limit of the applied X-ray diffraction.

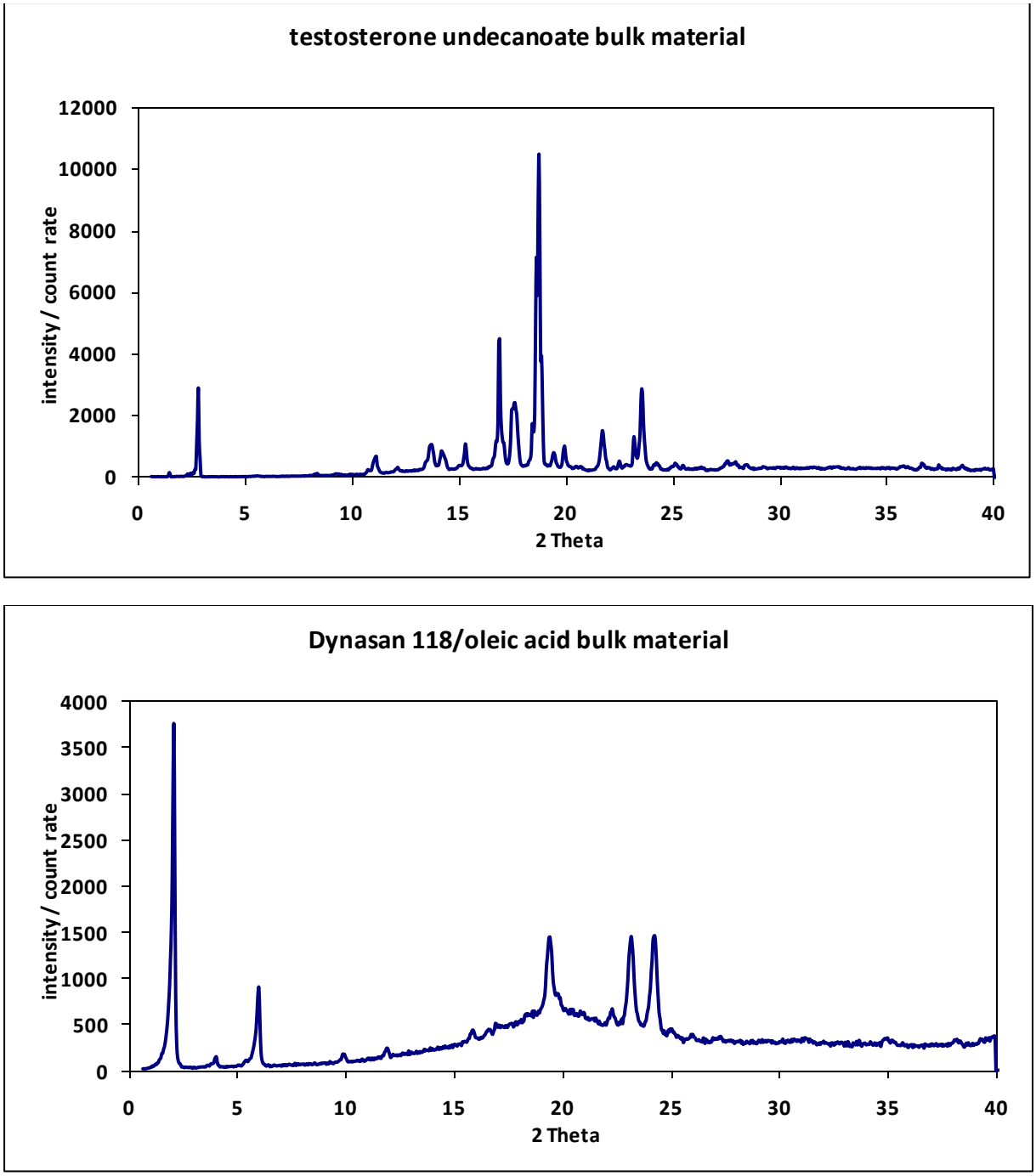


Fig. 4.1.3: X-ray diffraction pattern of the bulk material of testosterone undecanoate (TU) (upper) and a blend of oleic acid and Dynasan 118 (50/50) [m/m] (lower)

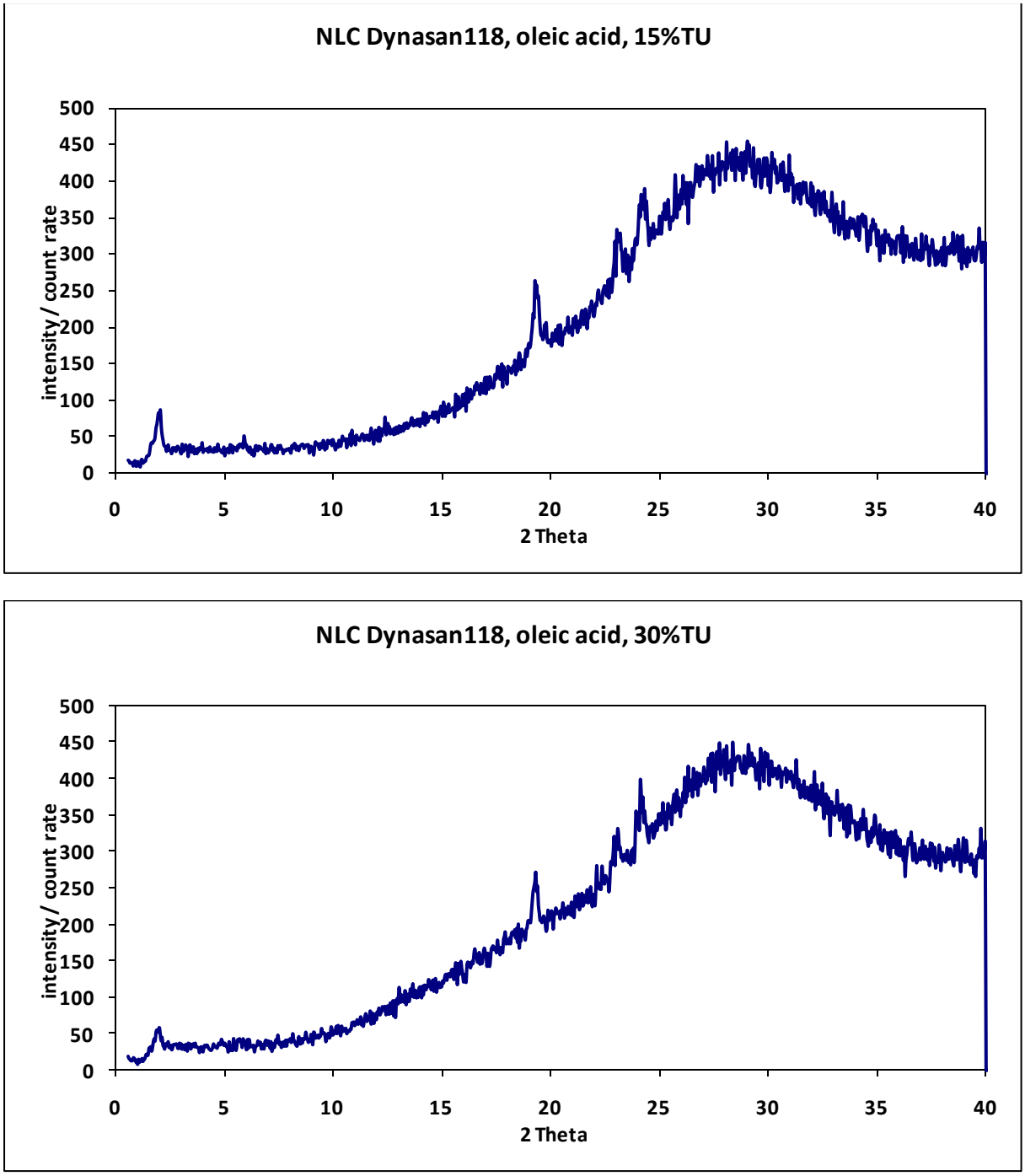


Fig. 4.1.4: X-ray diffraction pattern of a 10 % aqueous NLC dispersion made of 15 % TU (upper) and 30 % TU (lower) in Dynasan 118 and oleic acid (50/50) showing a solution of TU in both concentrations. None of the characteristic peaks of the TU bulk material can be found in the patterns

The NLC suspensions were also analysed by light microscopy using up to 1000 x magnification (Fig. 4.1.5). The pictures taken directly after production (400x magnification) show a more coarse suspension for the formulation NLC1 with a mean PCS diameter of 600 nm. The formulations NLC2 and NLC3 show a very fine appearance. With a mean PCS diameter of about 200 nm the main population is below the detection limit of the microscope. The larger particles in these two formulations detectable with the microscope reveal that they are relatively small. Formulation NLC4 shows a slighter coarse image, which is in agreement with the tendency to aggregate, found in the short-term stability study (see below).

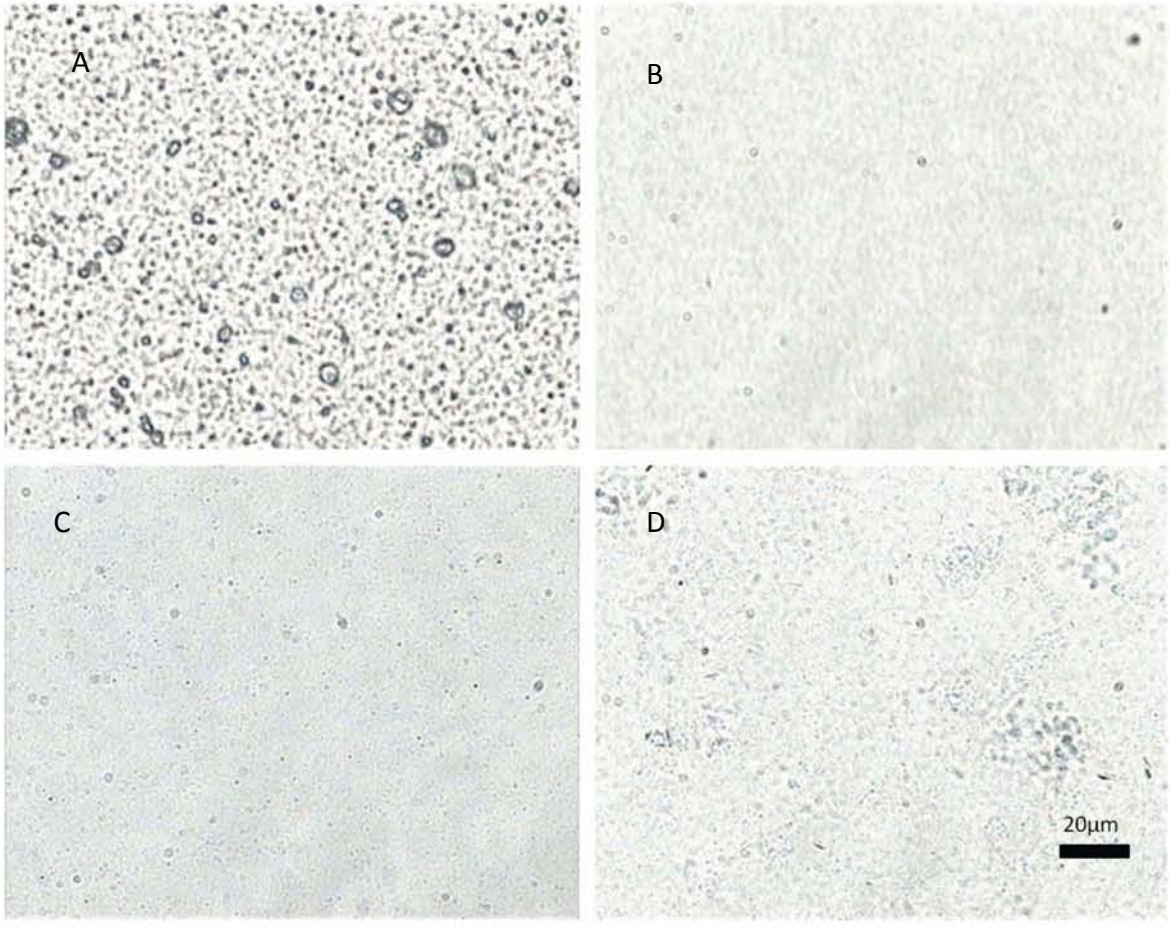


Fig. 4.1.5: Light microscopic pictures of the NLC formulations NLC1 (A), NLC2 (B), NLC3 (C), NLC4 (D). The formulation with the larger particles (NLC1) can be clearly distinguished from NLC2 and NLC3 (which have similar compositions). NLC4 has a different pattern due to a different solid lipid used (stearic acid).

4.1.3.4 Zeta potential

The zeta potential is a parameter to roughly estimate the physical stability of dispersions. The zeta potential measured in distilled water is set to be identical to the Stern potential. The Stern potential is a function of the Nernst potential of the surface. The higher the Nernst potential, the higher is the Stern potential. The zeta potential measured in the original dispersion medium is a measure of the actually stabilising force in the original dispersion, i.e. it is a measure of the thickness of the diffuse layer.

The zeta potentials measured in distilled water were lowest for NLC1 (-18.6 mV), about -30 mV for NLC2 and 3 and about -40 mV for NLC4 (table 4.1.5). The high zeta potential for NLC4 can be explained by the presence of stearic acid introducing additional charges to the particle surface, the Nernst potential. The zeta potentials in the original dispersion medium were slightly reduced. Based on these data, least stability was expected for formulation NLC1, but highest for formulation NLC4.

Table 4.1.5: Zeta potential results: Measurements were performed in ultrapure water adjusted to a conductivity of 50 µS/cm

Formulation	Zeta potential [mV]
NLC1	-18.6
NLC2	-29.3
NLC3	-29.8
NLC4	-38.5

4.1.3.5 Short term stability

The NLC suspensions were stored at room temperature and the size monitored as a function of storage time over 3 months. Table 4.1.6 shows the PCS and LD data. NLC1 was not stable, this is in agreement with the measured lower zeta potential. After one week the suspensions showed microscopically visible large aggregates and size measurements were not continued. The NLC formulations 2 and 3 were stable over a period of three months. There was little change in PCS mean sizes and LD diameters. Surprisingly formulation NLC4 was found to be also unstable at measuring point one week. Due to the highest zeta potential measured, a better stability was expected. However, these instabilities can be explained by bridging phenomena that some fatty acids and longer chain alcohols can cause (Kutz, 2001).

Nevertheless, it should be pointed out that for a pharmaceutical oral formulation a stability of just one day is fully sufficient because the aqueous suspensions will be transferred to a tablet or a spray-dried product anyway. That means immediately after NLC production the subsequent processing step to the final dosage form can be performed.

Table 4.1.6: Short term stability of the formulations.

Formulation	**Day 0**		**Day 3**		**1 week**		**4 weeks**		**3 months**	
PCS size in nm and polydispersity index (in brackets)										
NLC1	592±7 (0.15±0.01)		576±12 (0.55±0.05)		unstable		unstable		unstable	
NLC2	176±1 (0.23±0.01)		173±4 (0.23±0.03)		169±1 (0.30±0.17)		175±3 (0.22±0.01)		174±4 (0.23±0.02)	
NLC3	215±3 (0.15±0.01)		228±7 (0.13±0.11)		227±5 (0.13±0.08)		208±1 (0.15±0.02)		232±3 (0.11±0.01)	
NLC4	200±3 (0.21±0.01)		281±8 (0.35±0.23)		unstable		unstable		unstable	
Laser diffractometry size data (LD50 and LD90) in µm										
	LD50	LD90	LD50	LD90	LD50	LD90	LD50	LD90	LD50	LD90
NLC1	0.37	0.91	0.45	1.24	unstable					
NLC2	0.25	0.40	0.26	0.43	0.28	0.41	0.26	0.44	0.29	0.47
NLC3	0.28	0.48	0.29	0.53	0.30	0.55	0.29	0.51	0.33	0.55
NLC4	0.29	0.54	0.37	0.77	unstable					

4.1.4 Nanosuspensions

Nanosuspensions where prepared by ball milling the bulk product with a Bühler PML-2 mill (Bühler, Uzwil, Switzerland) for 30 minutes with pearls of 0.2 mm. The obtained crude suspension was then homogenized with an LAB 40 homogenizer (APV Deutschland GmbH, Unna, Germany) with 3 cycles at 1000 bars and 2 cycles at 1500 bars at room temperature. Particle sizes were measured as mentioned above.

4.1.5 Bioavailability studies

4.1.5.1 Bioavailability study of TU and T versus Andriol Testocaps®

Nanocrystals are a much simpler approach than NLC for the oral delivery of poorly soluble drugs. They can be considered as a formulation of first choice. Therefore in the first part of this in vivo study, nanocrystals of testosterone undecanoate (TU) and testosterone (T) were

prepared and compared regarding their bioavailability (BA) relative to Andriol Testocaps®. In general, the commercial pharmaceutical products for testosterone replacement therapy do not contain the original molecule testosterone, but lipophilic derivatives, such as testosterone undecanoate (TU) and testosterone propionate (TP). The reason for this is very simple: The bioavailability of testosterone is even much lower than of the lipophilic derivatives. TU has an oral bioavailability of about 7 %, TP much below that (Köhn & Schill, 2003). In addition, TU is very fast de- esterified to mainly T after absorption. Therefore in these *in vivo* studies, only T was analysed in the blood, not TU itself.

For reasons of market differentiation, there is still a demand of pharmaceutical industry to successfully deliver oral testosterone. That is why for this study nanocrystals of both TU and T were prepared as described in the methods section. The size of the T nanocrystals was about 800 nm. Further reduction by high pressure homogenisation was not possible since the material proved to be very hard. However, this size was sufficient for a first study for getting an impression how effective nanocrystal T formulations can be. TU nanocrystals were prepared with a size of 474 nm. The serum profiles show a very steep increase for both nanocrystal formulations at the first measuring point of one hour (Fig. 4.1.6, lower). The serum concentration is about double as high compared to Andriol Testocaps® (Fig. 4.1.6, upper). This is very interesting because:

1. It proves that absorption from the nanonised material is very fast.
2. Absorption of the 20 times larger sized T nanocrystals (865 nm) is as fast as the one for the small sized TU nanocrystals (474 nm)
3. It cannot be excluded that especially for T nanocrystals an even higher serum concentration is present at 0.5 hours (concluded from the fast decrease in serum concentration from one hour to two hours).
4. The fast decay of the T serum concentration is potentially attributed to the faster transit of the large nanocrystals through the gastrointestinal tract; the absorption window in the upper gut is passed faster.
5. The very small TU nanocrystals are more mucoadhesive, consequently they remain for a longer time in the absorption window.

6. It is very remarkable that such high serum concentrations were obtained despite the fact that no lipid at all was co-administered with the nanocrystals, considering that lipids are known to increase the TU absorption.

Based on these data conclusions can be made for optimising the nanocrystal formulations:

1. The T nanocrystals dissolve fast, but measures need to be taken to keep them longer in the absorption window.
2. Increased retention time can be achieved by reducing the size or alternatively stabilising them with a mucoadhesive polymer (e.g. chitosan HCl).
3. Lipid can be co-administered with the nanocrystals to exploit the absorption enhancing effect of lipids, thus further increasing the BA.

In the commercial product Andriol Testocaps®, TU is dissolved in oil. Based on the consideration that administering 10 mg TU per kg bodyweight will lead to serum concentrations of T which can be analytically quantified, this dose was chosen for all in vivo tests. Administering this oil solution would have required to administer an amount as small as 20 µl for an average rat of 250 g. This amount is too small for a proper, definite administration. Therefore the TU oil solution of Andriol Testocaps® was taken out of the capsule and dispersed in Tween 80 solution to obtain a macroemulsion. The main fraction of the oil droplets was in the range 5-10 µm. This resembles the dispersion of the oil after release from the capsule which takes place by movement of the gut, the reduced interfacial tension of the gut fluids (approx. 40 mN/cm (Kalantzi et al., 2006)) and the presence of surfactants such as bile salts. The administration volume of this macroemulsion was 500 µl. For a rat of 250 g, the emulsion contained 2.5 mg TU and 21 mg oil (lipid). (This lipid was not present when administering the nanosuspensions.)

The maximum serum concentrations (c_{max}) obtained with Andriol Testocaps® are half or a little bit more than half of the ones obtained with the nanocrystals. However, Andriol Testocaps® shows a distinctly prolonged serum level. The width of the major serum peak is up to double compared to the nanocrystals. It is assumed that absorption mainly takes place by two mechanisms:

1. The oil droplets are degraded by enzymes, surface-active monoglycerides are formed leading to the formation of TU-loaded micelles. Absorption can take place across the gut wall and via the lymphatic system.
2. The enzymatic degradation is a surface degradation, thus decreasing the size of the TU-loaded oil droplets. When the size is below 100 nm, the droplets are small enough for lymphatic uptake, as described in the literature (Olbrich, 1998; Müller & Olbrich, 1999; Olbrich & Müller, 1999; Olbrich, Kayser & Müller, 2002).

Potential explanations for the prolonged serum level of Andriol Testocaps® are:

1. For an unknown reason, the oil droplets remain longer in the absorption window.
2. The presence of fat allows absorption at sites where drug from nanocrystals cannot be absorbed anymore.

The shape of the observed Andriol Testocaps® serum profile is generally in accordance with the serum profiles published by the group of William Charman. However, it needs to be kept in mind that these studies were done in beagle dogs (Shackleford et al., 2003).

Fig. 4.1.6 (lower) shows the AUC values for the time period of the first 8 hours. Taking into account that the nanocrystals were tested without any formulation optimisation, the AUC values achieved are very promising. The TU nanosuspension is close to Andriol Testocaps®. This holds promise to match the Andriol Testocaps® bioavailability when optimising the TU nanosuspension formulation as outlined above. It seems possible to incorporate a single dose of TU nanocrystals into one tablet or capsule. Instead of optimising the formulation, one could also take the approach to administer a slightly higher dose to compensate for the lower oral bioavailability observed. Such an approach can only be followed in case the "access part of drug" is not absorbed and just passes the gut non-absorbed. In case of TU the problem is that this access drug would be absorbed, immediately be metabolised by the first pass effect of the liver, thus leading to higher TU and T concentrations in the liver which could cause undesired side effects. Therefore it is definitely a smarter approach to optimise the nanocrystals formulation.

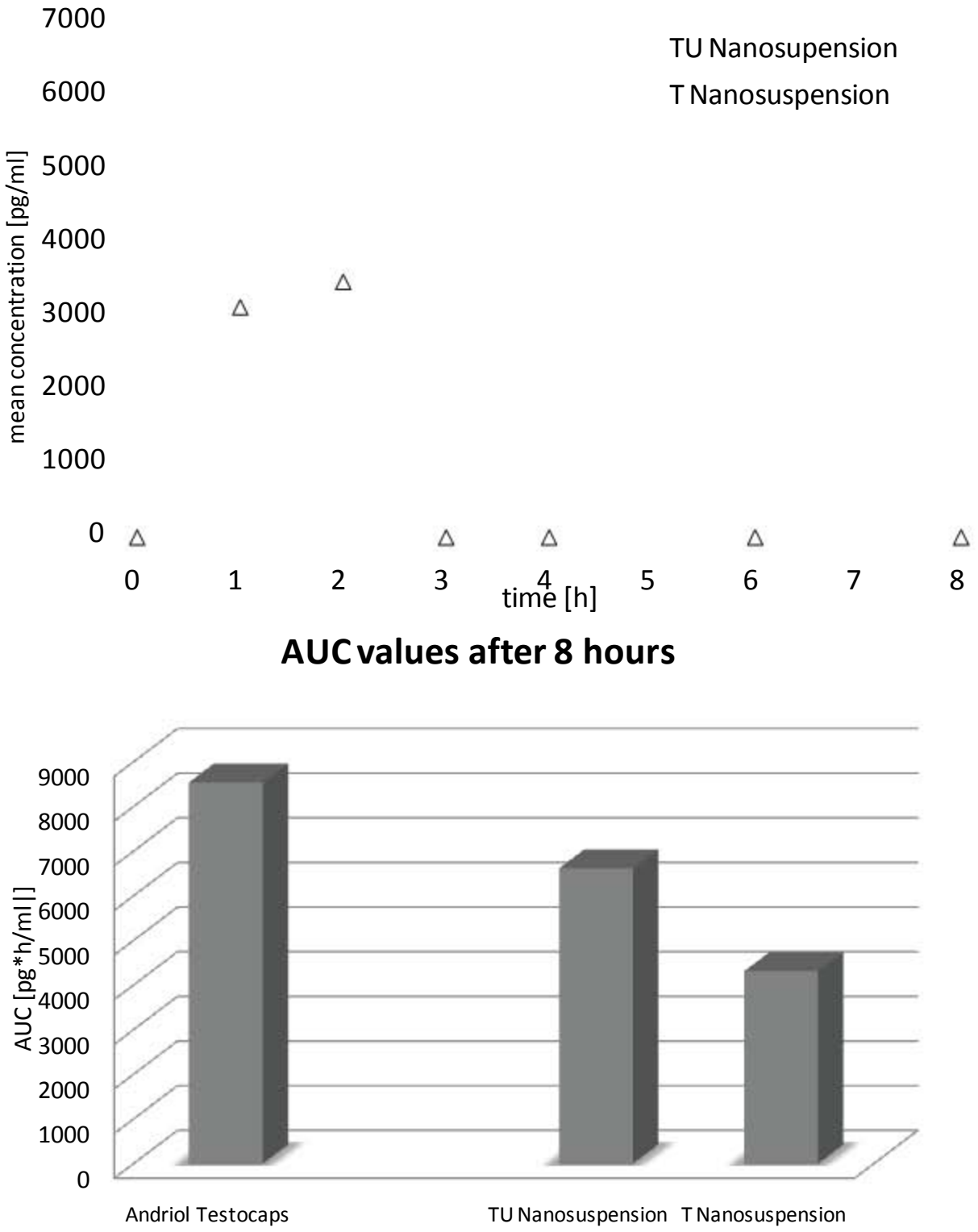


Fig. 4.1.6: Testosterone serum concentration plot after an oral administration of T nanosuspension, TU nanosuspension and Andriol Testocaps dispersed in Tween 80 (upper). Comparison of the area under the curve values of after 8 hours (lower).

Table 4.1.7 lists the bioavailability parameters calculated from the serum profiles. The nanocrystals possess higher c_{max} and shorter t_{max}, which is well described in the literature as a general feature of orally administered nanocrystals (Liversidge & Cundy, 1995). In case such higher c_{max} and short t_{max} are not required or even should be avoided, the nanocrystals need to be combined with a controlled release technology, e.g. incorporating the nanocrystals into pellets, a hydrocolloid matrix tablet or by coating the single nanocrystals as described by Möschwitzer et al. (Möschwitzer & Müller, 2006b). There is evidence in literature that the presence of lipid promotes absorption of TU (Horst et al., 1976; Bagchus, Hust, Maris, Schnabel & Houwing, 2003; Köhn & Schill, 2003; Shackleford et al., 2003; Schnabel, Bagchus, Lass, Thomsen & Geurts, 2007), which could be exploited to generally increase the AUC of nanocrystals by co-administration.

Table 4.1.7: Key pharmacokinetic parameters of the formulations. Explanations are given in the text.

Formulation	***C_{max} [pg/ml]***	***T_{max} [hours]***	***AUC after 8 h [pg*h/ml]***
Andriol in Tween 80 solution (“fasted”)	2,483	2	8,542
Andriol in castor Oil/Lauroglycol (“fed”)	6,408	1	13,105
TU Nanosuspension	3,481	2	6,667
T Nanosuspension	3,682	1	4,312
NLC 30 % Dynasan (approx. 600 nm)	5,533	1	10,208
NLC 30 % Dynasan (approx. 200 nm)	6,036	1	13,950
NLC 15 % Dynasan (approx. 200 nm)	8,589	1	12,933
NLC 15 % stearic acid (approx. 200 nm)	6,675	2	11,976

4.1.5.2 Bioavailability of Andriol Testocaps® versus Andriol Testocaps® with additional lipid (non-fed vs. fed state)

Serum concentrations after oral administration of TU are much higher in the fed state compared to the non-fed state. Bagchus et al. found an almost 10 times higher bioavailability in the fed state compared to the fasted state in men (Bagchus, Hust, Maris, Schnabel & Houwing, 2003). To study the effect of lipid on the absorption, in the second study the bioavailability of Andriol Testocaps® dispersed in Tween 80 solution was compared to Andriol Testocaps® with additionally added lipid, i.e. a mixture of Lauroglycol FCC (Propylene glycol monolaurate) and castor oil (40 %:60 %). This is exactly the composition of the liquid oil phase in the Andriol Testocaps® capsules. It should be investigated if the presence of more "lipophilic excipient" from the capsule formulation promotes absorption.

Fig. 4.1.7 (upper) compares the serum profiles of these two formulations. Surprisingly the shape of the serum profiles completely changed when Andriol Testocaps® was administered in an oil phase. The profile resembles very much the profiles obtained with T nanocrystals, a very high c_{max} and short t_{max}. The c_{max} is even about 50 % higher compared to the nanocrystals, the t_{max} is identically at one hour. It cannot be excluded that there would be differences at earlier time points of 15 minutes and 0.5 hours, but this early time points were not covered in these *in vivo* studies. Clear aim of these studies was to get a first overview, especially to assess if any relevant bioavailability can be obtained at all when administering the developed nanocarriers.

The initial very high serum peak can be explained by better dispersion of the administered oil due to the surfactant present in the oil phase and potential stimulation of bile salt secretion. Both can lead to the formation of finer, smaller oil droplets. Smaller droplets increase the bioavailability as this is well described for the microemulsion formulation of cyclosporine (Sandimmun Neoral, Optoral) (Meinzer, Müller & Vonderscher, 1998). After the initial peak a pronounced tailing of the profile occurs, which does not exist for the investigated nanocrystals. This kind of prolonged serum level is similar to the one of Andriol Testocaps® administered in aqueous Tween solution. It can be summarised that the serum profile obtained from Andriol Testocaps® administered in oil is a superposition of the serum

profiles of nanocrystals and Andriol Testocaps® administered in aqueous Tween solution: An initially high serum peak (identical to nanocrystals) followed by a prolonged serum level (identical to Andriol Testocaps® dispersed in aqueous surfactant solution).

The serum profiles of the fed state versus the non-fed state in men described in (Bagchus, Hust, Maris, Schnabel & Houwing, 2003) show an even more pronounced change in shape as observed in these studies.

Fig. 4.1.7 (lower) shows the AUC values of both formulations, the AUC of Andriol Testocaps® in oil is about 50 % higher. It demonstrates the effect of lipid present on the absorption of TU quite well. For a better understanding of the *in vivo* situation in humans, a model calculation is made. In Andriol Testocaps® capsules, there is 8 mg lipid phase per 1 mg testosterone (total weight of capsule content: 335 mg, drug content: 40 mg TU). When dispersing the required dose for the rat of 2.5 mg/animal TU in 250 mg oil mixture, this corresponds to 99 mg lipid per 1 mg drug TU. For a dose of 80 mg TU the amount of lipid equals almost 8,000 mg. When swallowing two capsules of Andriol Testocaps®, 7.3 g pure lipid (plus 0.7 g in the capsules) has to be co-administered to have similar conditions as tested in the animal model. This amount of lipid corresponds to a medium meal of about 100 calories for the lipid alone. Compliance of the patients would probably not be very high if such an amount of additional lipid has to be taken when administering Andriol Testocaps® capsules for improving the oral bioavailability.

Table 4.1.7 shows the pharmacokinetic parameters as discussed above, t_{max} of Andriol Testocaps® administered in oil are identical to the nanosuspensions of T but c_{max} and AUC are higher for the Andriol Testocaps® in oil. TU nanosuspension shows a c_{max} which is almost equal to the T nanosuspension but with a later t_{max} at 2h.

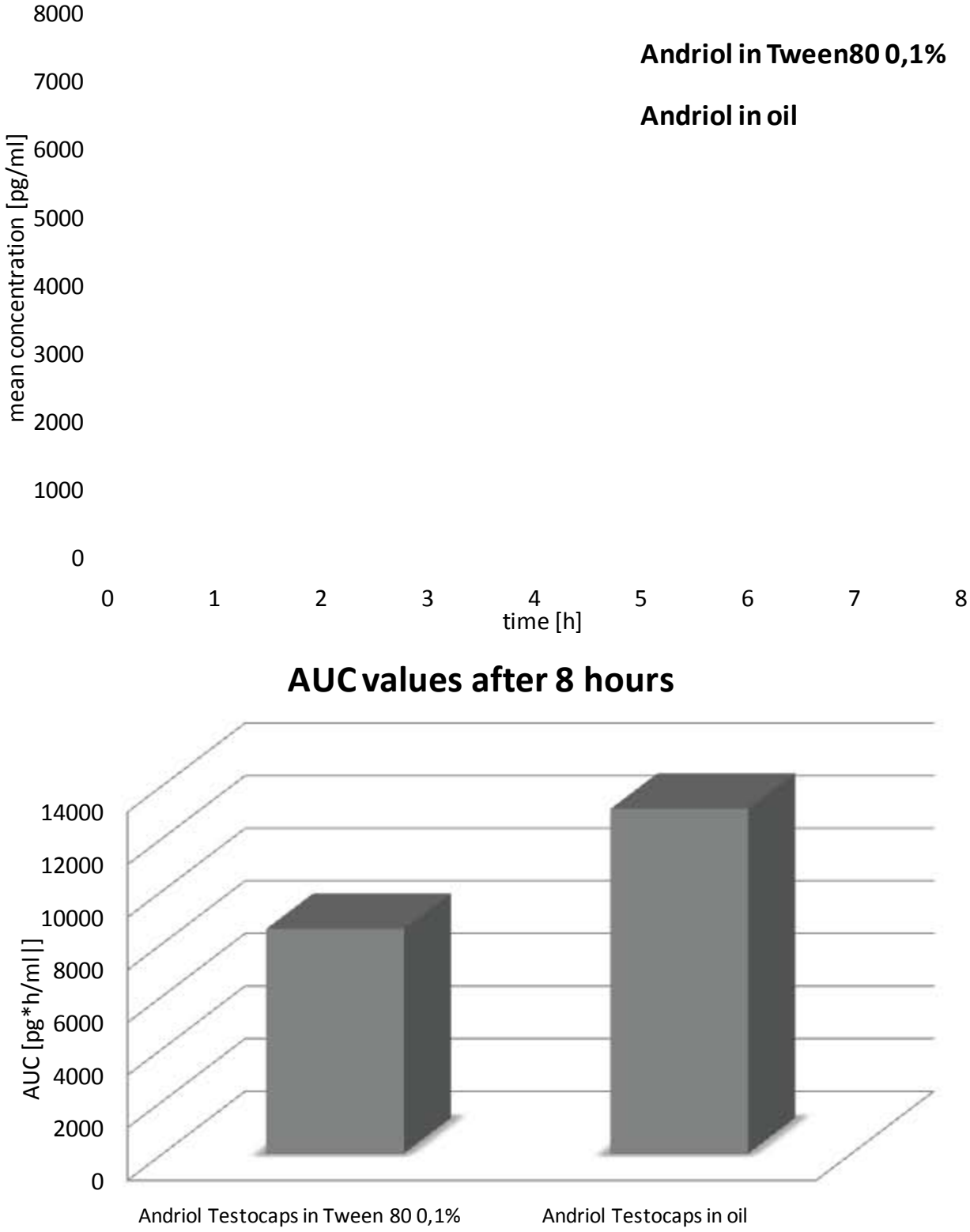
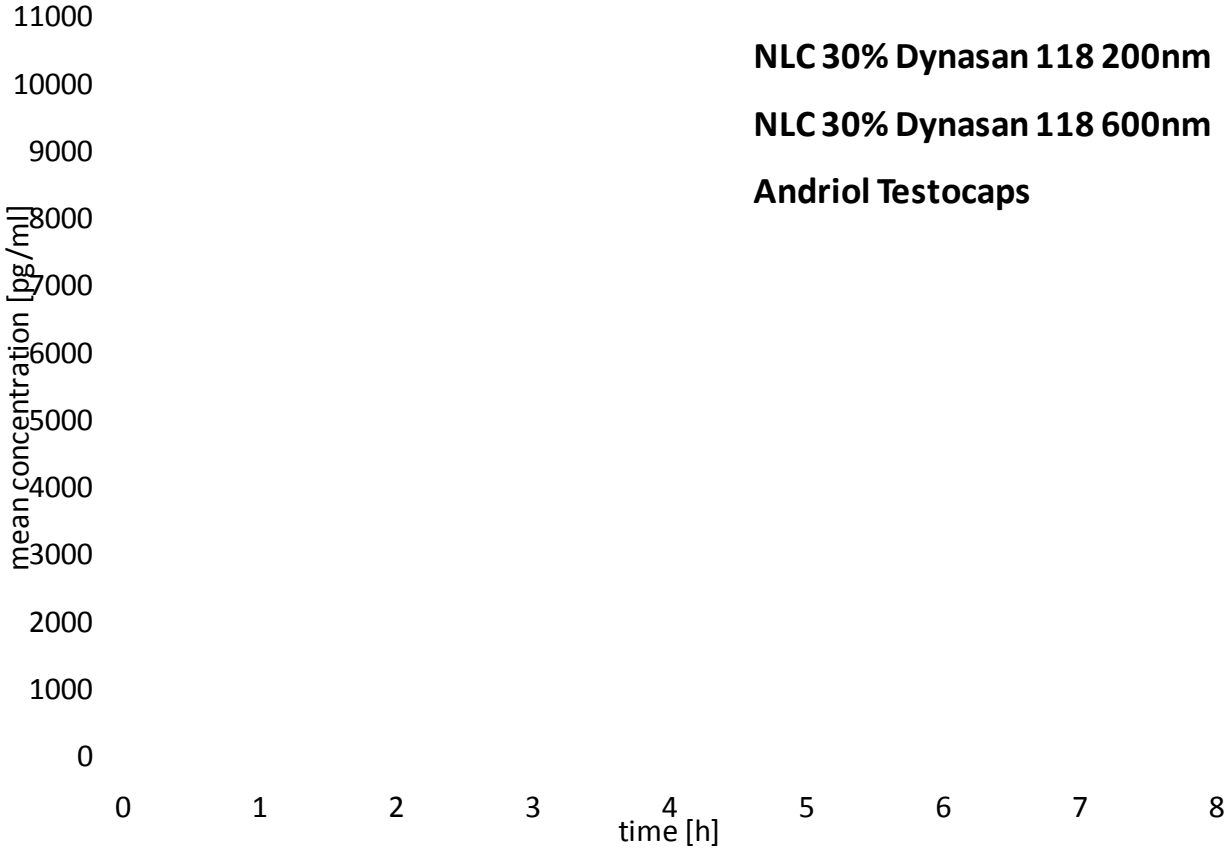


Fig. 4.1.7: Plot of serum testosterone levels after oral administration of Andriol Testocaps dispersed in Tween 80 0.1 % and Andriol Testocaps diluted with its own excipients (upper). The comparison of the AUC values can be found in the lower section.

4.1.5.3 Bioavailability of NLC as a function of size (600 nm vs. 200 nm)

In the next study, two NLC formulations were prepared using the same lipid (Dynasan 118), but with a different size: 600 nm versus 200 nm. The rationale behind was that the bioavailability of drugs dissolved in oils increases, the better the oil is dispersed in the gut (i.e. the smaller the oil droplet size is). Of course this study could also show whether nanosized lipid particles are superior in bioavailability compared to the oil solution of TU administered in capsules. An improvement was expected based on the data published for cyclosporine (Müller et al., 2006), when moving to smaller lipid nanoparticles.

Interestingly the two serum profiles of both NLC have an almost identical shape as Andriol Testocaps® administered in oil (= simulation of fed state) (Fig. 4.1.8, upper). For all the three formulations (NLC 600 nm, NLC 200 nm and Andriol Testocaps® in oil), c_{max} and t_{max} are alike. After the sharp initial peak a prolonged higher serum level occurs, which is most pronounced for the 200 nm NLC. The serum concentrations of the prolonged level of these 200 nm particles are up to 1.5-2.0 times higher compared to the 600 nm NLC. The data prove that NLC administered in a non-fed state exhibit a comparably high bioavailability as TU (Andriol Testocaps®) administered in oil (resembling the fed state condition). Calculated on a human dose of 80 mg TU, Andriol in fed state corresponded to simultaneous administration of about 8 g lipid. Administering the 80 mg dose in NLC (30 % drug load) corresponded to co-administration of only about 0.2 mg lipid.



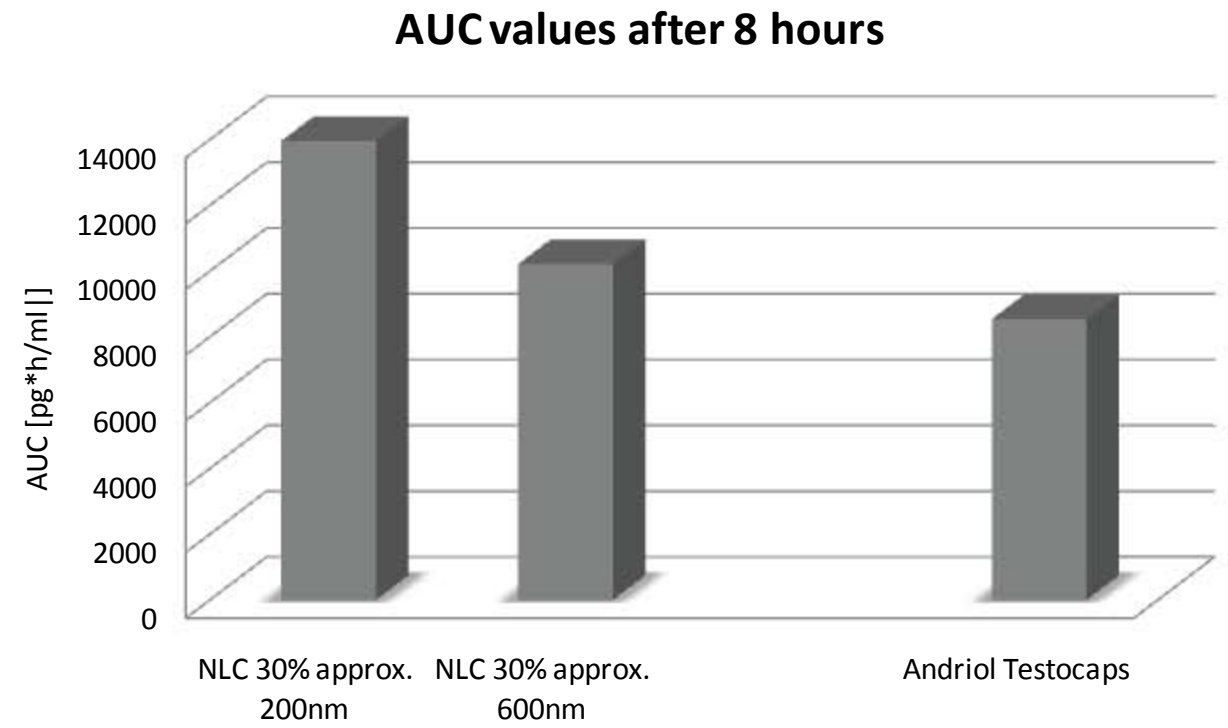


Fig. 4.1.8: Serum testosterone levels of NLC with 200 nm and 600 nm compared to Andriol Testocaps in Tween 80 (upper) and AUC values (lower).

The AUC values depicted in Fig. 4.1.8 (lower) show a higher bioavailability of the 600 nm NLC compared to Andriol Testocaps® (Andriol Testocaps® dispersed in Tween 80 solution). The highest AUC was found for the smaller sized 200 nm NLC which is in the same range as Andriol Testocaps® in oil (approx. 14,000 units for 200 nm NLC, 13,000 units for Andriol Testocaps® in oil). The pharmacokinetic parameters are given in table 4.1.7.

The conclusions for the study are:

1. The fast onset and initial high serum peak seems to be characteristic for all nanosized formulations (nanocrystals, NLC, ultra-fine dispersed oil).
2. The total AUC increases with decreasing size of the lipid nanoparticles.
3. Despite that the 200 nm NLC have a higher total AUC, the initial high serum concentration (c_{max}) and t_{max} of one hour is practically identical. Obviously the reduction in size has little influence on the absolute height of the first initial peak. There seems to be an uptake limitation for NLC or fatty materials.
4. The major difference is that for small sized 200 nm NLC, there is a higher prolonged serum level. A potential explanation is that the smaller sized NLC are more mucoadhesive. They remain longer in the absorption window in the upper gut, thus yielding higher serum levels. The passage velocity of the 600 nm NLC seems to be higher, causing less uptake in the absorption window.
5. Another explanation: For lymphatic uptake, the size should be smaller than approx. 100 nm. NLC are surface-degraded. They reduce in size during passage of the gut. Compared to the 600 nm NLC, a larger fraction of the 200 nm NLC was sufficiently degraded and reduced in size to be < 100 nm for lymphatic absorption.
6. The very high serum concentrations after one hour suggest again that there might be even higher concentrations at 15 minutes and 0.5 hours. This seems possible when the residence time in the stomach is sufficiently short. In contrast to food, stomach emptying happens fast after administration of a liquid (as it was done in this study).

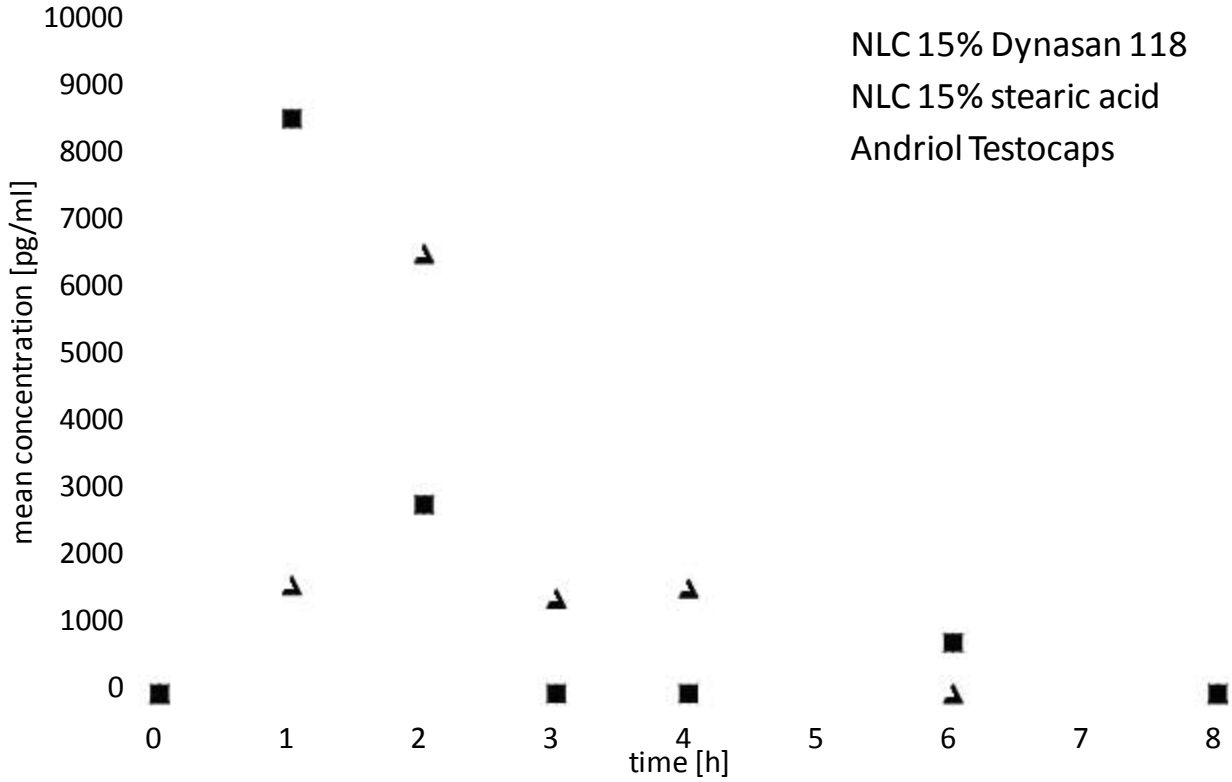
4.1.5.4 Bioavailability of NLC as a function of lipid nature

In this part of the study, the effect of the chemical nature of the lipid is discussed. The publications by Charman and co-workers report the length of the fatty acids in the oil have a pronounced effect on the extent of absorption of drugs dissolved in such oils. Long-chain fatty acids (C14 to C18) are more effective in promoting absorption than lipids with shorter fatty acid chains (Porter & Charman, 2001b). Based on these data two lipids were selected containing C18 fatty acids. To have additionally a difference in the molecular structure, a triglyceride (Dynasan 118 = stearic acid triglyceride) was compared with the fatty acid itself (stearic acid).

The serum profiles revealed a very interesting difference (Fig. 4.1.9, upper). The Dynasan NLC exhibit a c_{max} and t_{max} identical to all the other nanosized formulations at one hour. However, when using stearic acid as lipid matrix, t_{max} moved to two hours. The molecular structure of the lipid has a very distinct effect.

This difference is thought to be associated with the different degradation velocities of the particles and differences in solubilisation of the drug into micelles. *In vitro* degradation studies with lipase/co-lipase revealed a pronounced dependency of the degradation velocity on the lipid nature (e.g. within various triglycerides/glycerides mixtures, glycerides versus wax). Stearic acid is practically insoluble in water (saturation solubility is 0.3 mg/100 mL). It is not primarily a substrate for lipases. Triglycerides are degraded by lipases easily. Based on this the size decrease for a Dynasan NLC due to degradation and lipid solubilisation is faster than for the stearic acid NLC. They reach faster a sufficiently small size (< 100 nm) - small enough for lymphatic absorption.

Size decrease by degradation or dissolution of the fatty acid is slower for the stearic acid NLC. However, obviously they stay sufficiently long in the absorption window that a similar bioavailability (AUC) to the Dynasan NLC is reached.



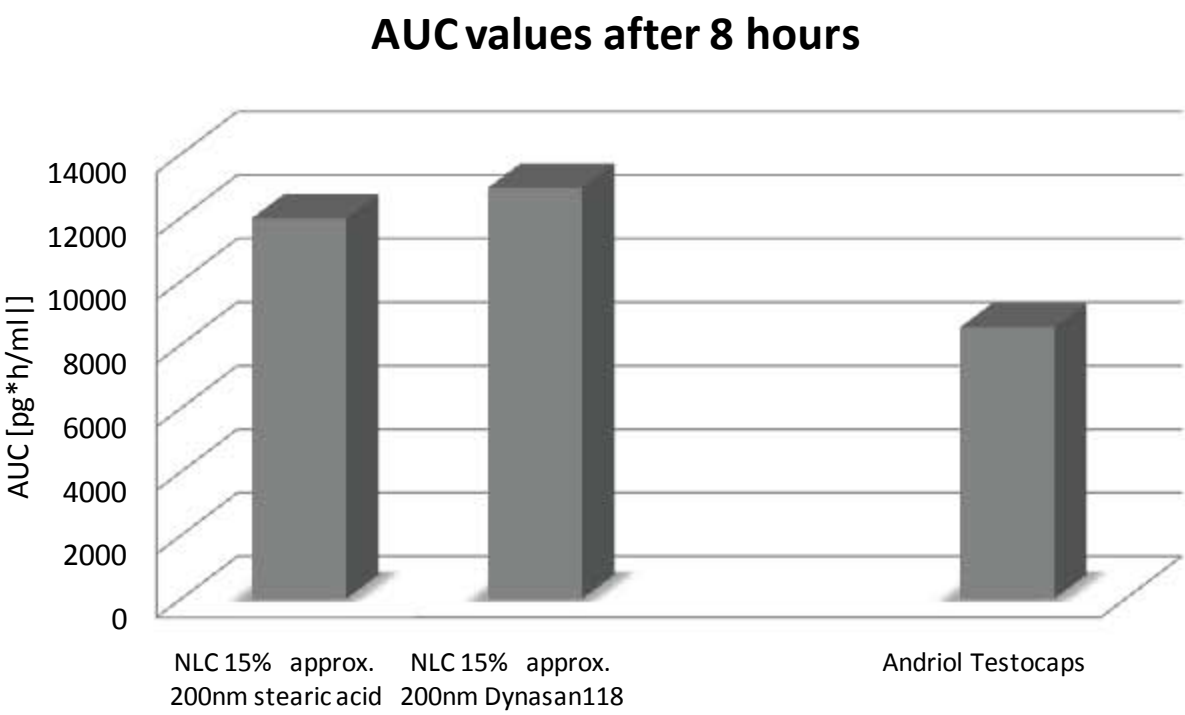


Fig. 4.1.9: Testosterone serum concentration levels after oral administration of identically sized NLC (200 nm) only differing in the lipid used compared to Andriol Testocaps in Tween 80 (upper) and AUC values (lower)

Fig. 4.1.9 (lower) shows that the AUC for both NLC formulations is practically identical (range of about 12,000-13,000 units). To be precise, the AUC is practically identical, but the shape of the serum profiles is different. For the stearic acid NLC, firstly c_{max} shifts from one hour to two hours; secondly there is a more pronounced tailing in the decrease of the serum concentration. Serum levels are more prolonged. In contrast to this, a fast decay occurs with Dynasan NLC. After three hours the "absorbable fraction" of the formulation has practically been taken up. The serum concentration was close to 0. The pharmacokinetic parameters are listed in table 4.1.7.

From the data it can be concluded:

1. It could be confirmed that small NLC (200 nm) provide highest AUC values.
2. The nature of the lipid matrix seems to have an effect on the absorption kinetics.
3. NLC from lipids which can be fast degraded and/or the lipid with drug which can be solubilised fast, lead to c_{max} at early times.
4. NLC made from lipids which are slower degraded or solubilised lead to a shift of c_{max} to a later time point, i.e. increased t_{max}.
5. The similar AUC values obtained suggest that small particles seem to remain sufficiently long in the absorption window (attributed to adhesive properties of the nanoparticles), providing time to be degraded to an adequate small size for lymphatic uptake.

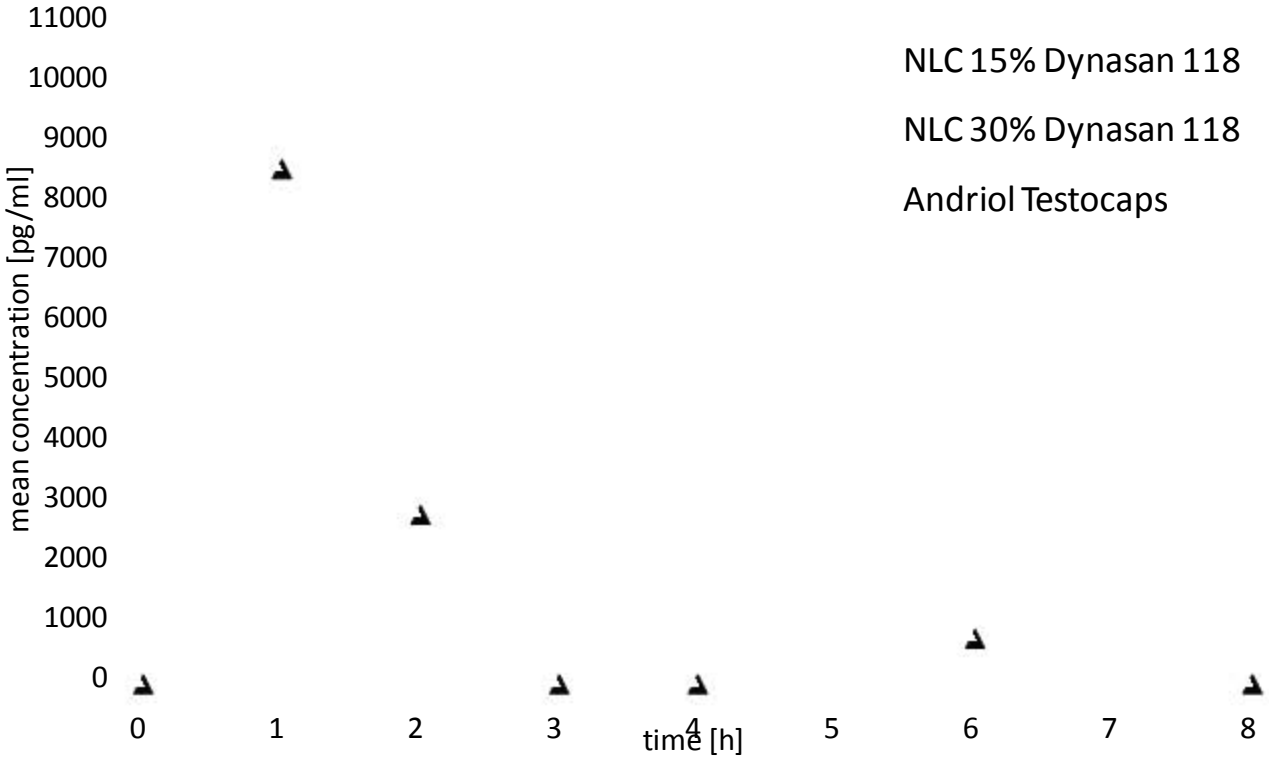
4.1.5.5 Bioavailability of NLC as a function of lipid:drug ratio

In a last comparison it was assessed if a larger amount of lipid in the NLC suspensions has an effect on the bioavailability. NLC were prepared with a reduced drug loading (15 % versus 30 %) that means more NLC needed to be administered for giving the same amount of drug to the animals. The NLC investigated up to this point contained a drug loading of 30 %. The particle mass (= 100 %) consisted of 70 % lipid and 30 % drug. The drug loading was reduced to 15 % (NLC particle mass consisted of 85 % lipid and 15 % drug). To administer the same amount of drug, in this case double the number of particles need to be administered. At the same time the amount of lipid per particle was increased (from 70 % to 85 %). When administering the NLC with 15 % TU, an about 2.5 times higher amount of lipid was administered (precisely 2.44 times higher). The total amount of lipid administered to a medium weight rat of 250 g in a single TU dose of 4 mg was:

9.3 mg lipid when administering the NLC with 30 % drug loading.

22.7 mg lipid when administering the NLC with 15 % drug loading.

Looking at the AUC values after eight hours, there is no significant difference between the NLC with 30 % drug load and NLC with 15 % drug load (Fig. 4.1.10, lower). This appears logic. Even when increasing the amount of lipid by a factor of about 2.5, the total amount of lipid is still very low. Therefore it could not be expected to see such an increase in bioavailability as it was observed when administering Andriol Testocaps® diluted with oil.



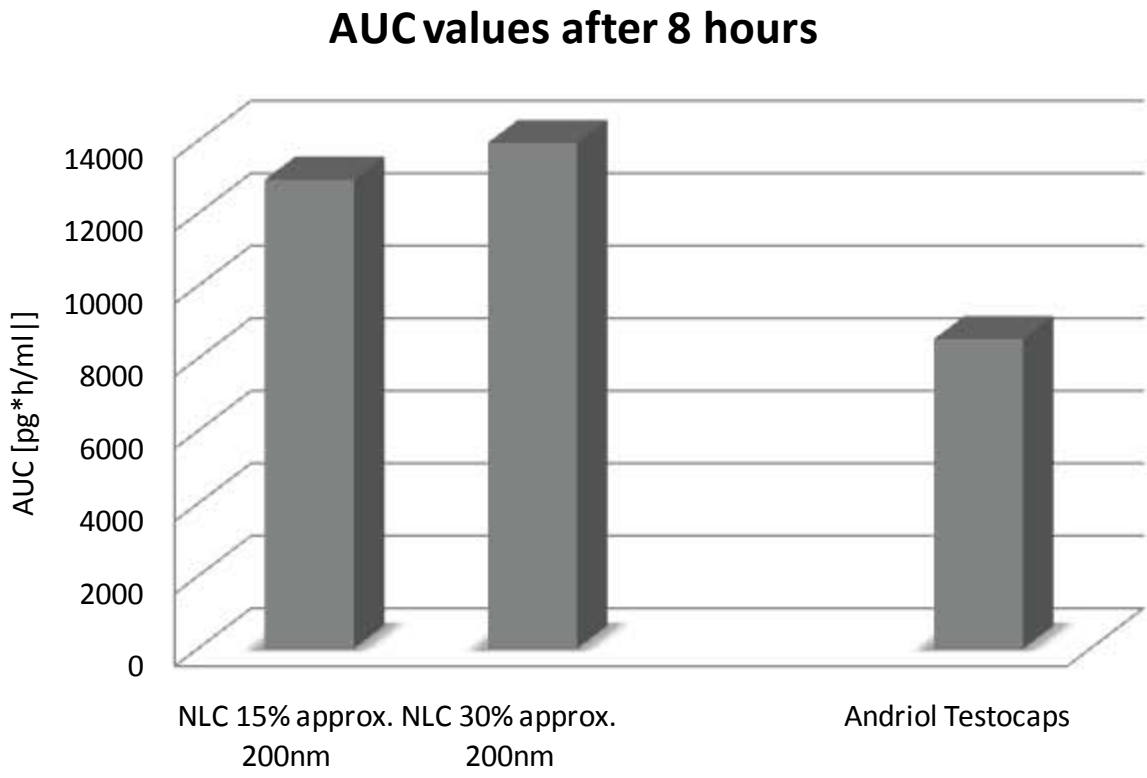


Fig. 4.1.10: Serum testosterone levels of NLC with 15 % TU and 30 % compared to Andriol Testocaps in Tween 80 (upper) and AUC values (lower).

However, again very interesting is the difference in the shape of the serum profiles between the two particles (Fig. 4.1.10, upper). The curves were already plotted in the previous figures, but they are again plotted in one figure together to better illustrate the differences. The NLC with the higher drug load of 30 % show a c_{max} of about 6,000 at one hour, then a prolonged decay in the serum concentration up to about 4-6 hours. In contrast to this the NLC with 15 % drug load (more lipid per particle) show a c_{max} of almost 9,000 at one hour, then a sharp decrease to 0 at three hours. From this it can be concluded that the shape of the serum profile can obviously also be modified by changing the drug load of the NLC. It can be imagined that the incorporated drug at a higher concentration slows down the degradation of the particle, thus leading to a slower, prolonged absorption. In case the drug loading is low (15 %), the degradation velocity is faster, less affected. The particles are fast reduced in size, get below 100 nm and can be lymphatically taken up. In addition, micelles formed during the particle degradation might contribute to lymphatic uptake.

4.2 Omega-3 Fatty Acids-loaded Lipid Nanoparticles for Patient-convenient Oral Bioavailability Enhancement

*Parts of this section have been published in Die Pharmazie, "*Omega-3 fatty acids-loaded lipid nanoparticles for patient-convenient oral bioavailability enhancement*"(Muchow, Schmitz, Despatova, Maincent & Müller, 2009)*

4.2.1 Introduction

The nutritional status of cancer patients is important; maintaining them in a good nutritional condition will support their battle against the disease (Chapkin, McMurray & Lupton, 2007; Dupertuis, Meguid & Pichard, 2007). Therefore various nutrition supplements for cancer patients are on the market, e.g. NT24 [Orcapharm, Pensberg / Germany]. The additional oral nutrition supply improves the health condition and prolongs the time until the necessity of parenteral nutrition.

Omega-3 fatty acids are important for the body because of various aspects:

1. Synthesis by the body of omega-3 fatty acids eicosapentaenic acid (EPA) and docosahexanic acid (DHA) from the essential alpha-linoleic acid is very limited. Nevertheless, EPA and DHA are considered the most valuable omega-3 fatty acids: providing them with nutrition is recommended (Harris, 2004; Hansen & Harris, 2007).

2. Omega-3 fatty acids are known to be cardioprotective (Hansen & Harris, 2007) and have a positive effect both on the blood lipid profile (Lemaitre et al., 2003; Anil, 2007) and on inflammatory processes (Kelley et al., 1999; Belluzzi, 2004).

There are a number of products on the market for additional supply with omega-3 fatty acids by the oral administration route. In general these products are soft gelatine capsules filled with fish oil. Most of the commercial products contain 500 mg of fish oils per capsule with an omega-3 fatty acid content of approximately 20-25 % (Lipiscor®, Sanum-Kehlbeck, Hoya, Germany), (Ameu®, Lichtwer Pharma, Germany), (Eicosan®, Stada, Germany). Disadvantage of this dosage form is that the patients actually need to swallow the capsule. In addition, administration of 3-6 capsules is recommended. Such a high number of capsules is necessary to meet the required dose considering the oral bioavailability of the omega-3

fatty acids. Cansell et al. have found a 72 ± 6 % oral bioavailability in a rat model, which was improved to 98 % using a very diluted liposome suspension (Cansell, Nacka & Combe, 2003). Increasing the oral bioavailability without enlarging the volume of the dosage form decreased the total dose to be administered, which is patient-friendly.

In special conditions, e.g. for the treatment of high triglyceride blood levels, the dose is even higher; in this case 10-20 capsules per day (which is equivalent to 1200-2400 mg omega-3 fatty acids) may have to be administered. At a certain stage, especially cancer patients develop the problem of swallowing capsules and tablets. Therefore a liquid or semi-solid dosage form will be definitely more appropriate for such patients. In addition, such a dosage form would be very convenient for elderly patients.

The soft gelatine capsules disintegrate in the stomach releasing the fish oil. Reported undesired side effects are a fishy odour in the breath and undesired regurgitation leaving an unpleasant fishy taste in the mouth. Enteric-coated fish oil capsules have also been developed for studies but are not commercially available (Belluzzi et al., 1996).

Based on these disadvantages of the present formulations, a novel semi-solid, paste-like formulation was developed. It consists of a highly concentrated lipid nanoparticle dispersion (70 % w/w). This paste can be administered via a teaspoon and swallowed directly; alternatively the paste can be dispersed in soft drinks or water, avoiding swallowing problems with solid dosage forms. The omega-3 fatty acids were incorporated in NLC (Müller, Mäder, Lippacher & Jenning, 2000). The solid matrix has a taste masking effect minimizing odour and taste problems. This section of the thesis describes the development and characterisation of this semi-solid oral formulation.

4.2.2 Rationale of development

The price of omega-3 fatty acids as a pure substance is fairly high, for example 340 Euro for 1 gram of purified docosahexaenoic acid. Therefore fish oils with a high percentage of unsaturated omega-3 fatty acids and plant oils (like flax seed oil) are used as nutrition supplements. The fish oil used for preparation of the nanostructured lipid carriers (NLC) had a relatively high content of 38 % omega-3 fatty acids (Schmitt). For chemical stabilisation,

inclusion of chemically labile compounds in a solid matrix can be protective. Therefore creation of a solid particle matrix in form of NLC was chosen to stabilise the fatty acids. In addition, a solid matrix can mask unpleasant taste and smell to a certain extent. Further reduction of the unpleasant smell was achieved by the solid-in-water dispersion system since the aqueous phase may additionally act as a barrier with the external environment. The fish oil-loaded NLC are surrounded by a water phase, which has a very low solubility for the lipophilic compounds, which further reduces evaporation. This is an old principal used in many pharmacopoeiae for taste masking, e.g. by producing oleum jecoris emulsions.

NLC are prepared by mixing a solid lipid with a liquid lipid (oil). In this case the oil compound is the fish oil. Admixing of the oil reduces the melting point of the solid lipid. To minimize unpleasant side effects from the stomach, the NLC should be solid at body temperature to slow down the degradation, that means degradation should mainly take place in the gut. Therefore a number of lipids was screened. The mixture should still melt above 40 °C but nevertheless contain the highest possible percentage of fish oil. This should minimise the total amount of NLC to be administered in a single dose. It was found that a lipid blend containing 20 % Dynasan 118 and 80 % fish oil was still solid at 40 °C.

To promote absorption, the NLC paste should be dispersible easily to yield a fine NLC suspension taking into account that aggregation reduces the bioavailability. The gut contains many electrolytes able to destabilise dispersions due to zeta potential reduction. Therefore a combination of electrostatic and steric stabilisation was chosen to minimize aggregation. Sodium dodecyl sulfate (SDS) is well known as an efficient dispersing agent; in addition it creates highly negative zeta potentials after adsorption to the surface (Lucks, Müller & Müller, 1990). As steric stabilisers TPGS and PVP were also selected. Both SDS and TPGS are known absorption enhancers. TPGS was used primarily as a steric stabilizer. It is also known as an absorption enhancer mainly due to inhibition of P-glycoprotein (Rege, Kao & Polli, 2002). The SDS was used in the first formulation attempts because it is an efficient surfactant for dispersing oils. In addition it was a challenge to produce a 70 % concentrated NLC suspension, which is fine in size and not too viscous. Generally, the SDS concentration in pharmaceutical preparations is up to 2 %, whereas 3 % were used in this formulation

approach. However, we have also shown that is was possible to replace SDS by Poloxamer 188, which can be used also at higher concentrations (data not shown).

The solid matrix of the NLC is chemically protective but additionally vitamin E can be added as anti-oxidant. It is also frequently used in commercial fish oil soft gelatine capsules (e.g. Ameu®).

4.2.3 Production and characterisation of the fish oil NLC

4.2.3.1 Fish oil NLC production

The NLC were prepared by high pressure homogenisation. The solid lipid Dynasan 118 was melted at 70 °C and the fish oil was then added. Both lipids where mixed at 70 °C at a ratio of 20 % Dynasan 118 and 80 % fish oil. Then a stabilizer solution containing 3.0 % (w/w) SDS, 0.1 % Polyvinylpyrrolidon (PVP), 0.1 % Tween 80 and 1 % Vitamin E TPGS at equivalent temperature was added under stirring (30 % stabiliser solution, 70 % oil phase). Alternatively a SDS-free stabiliser mixture containing Poloxamer 188 was used. Stirring (9000 rpm) was performed for 30 seconds using an Ultra-Turrax with a T-25 head (IKA Janke und Kunkel, Stauffen, Germany). The obtained pre-emulsion was then homogenized using a Micron LAB 40 (APV Deutschland GmbH, Unna, Germany) at 500 bar and one homogenisation cycle at 70 °C. The LAB 40 was equipped with a temperature control jacket; the temperature controlling fluid (water) was heated to 85 °C.

4.2.3.2 Particle size and charge

PCS covers a size range of approximately 3 nm-3 µm, thus it was employed to determine the size of the bulk population. The PCS diameter was 243 nm and the polydisperity index 0.064. Polydispersity indices around 0.1 indicate a relatively narrow particle size distribution. The NLC with 243 nm represent a highly dispersed ultra fine system compared to orally administered oils. Once administered, an oil is emulsified by the surfactants present in the gut and dispersed by gut movement resulting in oil droplets typically in the size of 1-50 µm (Patton & Carey, 1979; Armand et al., 1996). The degree of dispersity affects bioavailability as known from the first Cyclosporin A product Sandimmun (Meinzer, Müller & Vonderscher, 1998). With decreasing droplet/particle size, the absorption increases.

Laser diffractometry (LD) was performed to assess the potential presence of particles in the low micrometer range (measuring range of LD: 0.04-2000 µm). The LD yields a volume distribution, thus is very sensitive to detect even low amounts of particles larger than the bulk population. The 50 % diameter was 0.313 µm, 90 % diameter 0.950 µm and 99 % diameter 1.240µm. This indicates a product with particles almost completely in the nanometer range with a low amount of micrometer particles. Fig. 4.2.1 shows the LD size distribution curve.

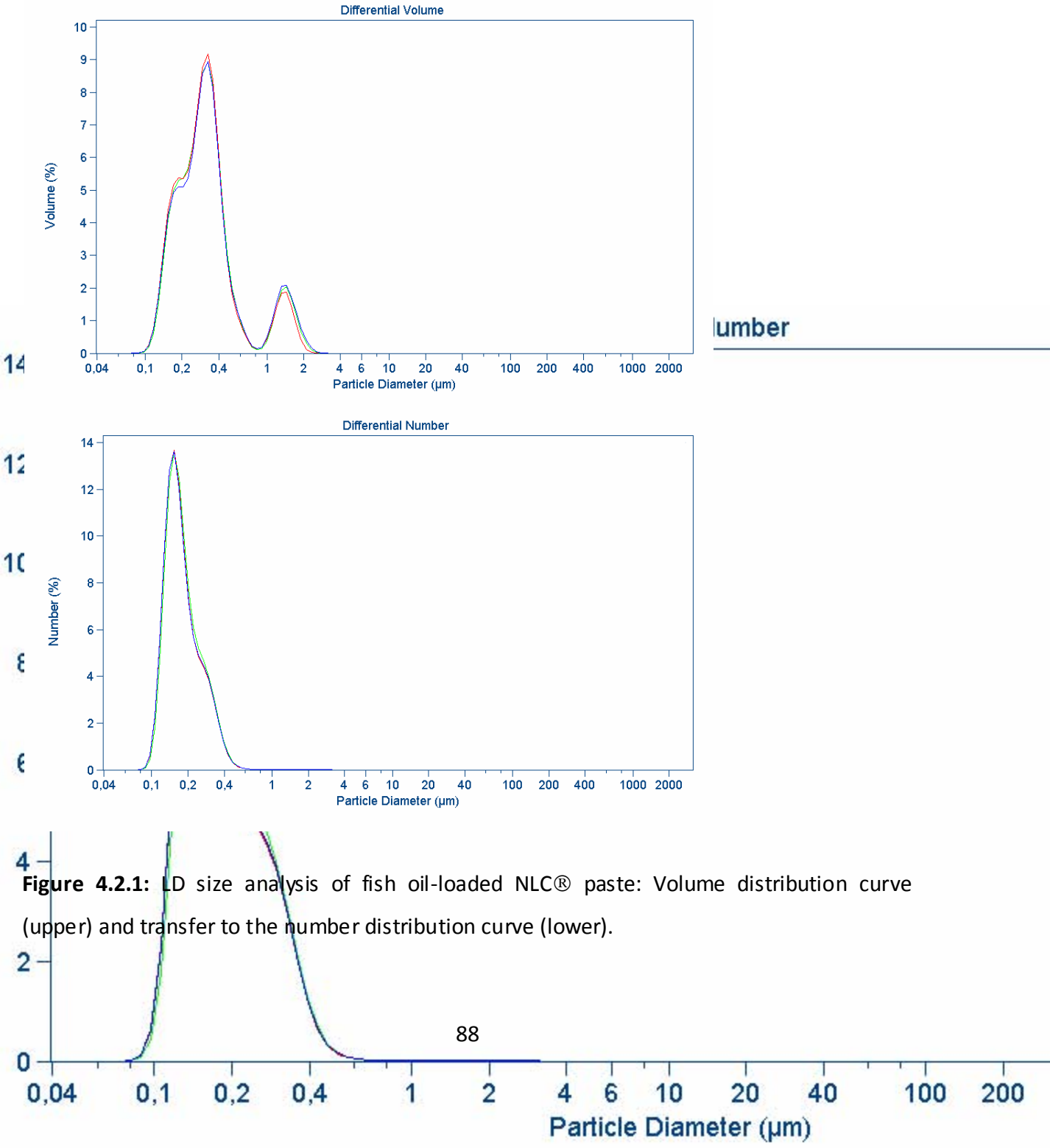


Figure 4.2.1: LD size analysis of fish oil-loaded NLC® paste: Volume distribution curve (upper) and transfer to the number distribution curve (lower).

The zeta potential measurement is a tool to foresee the physical stability of colloidal suspensions. Stability is higher in case of high electrostatic repulsion. The measurements in ultrapure water (conductivity adjusted to 50 µs/cm) yielded a value of -20 mV. This is a characteristic value for combined electrostatic (SDS) and steric stabilisation (TPGS, PVP). In addition, zeta potential measurements were performed in the original dispersion medium (water with 3.0 % SDS, 1.0 % TPGS and 0.1 % PVP). Measurement in the original dispersion medium provides information about the stability in the original dispersion. The high electrolyte concentration leads to compression of the diffuse double layer and subsequently to a reduction in the measured zeta potential. The zeta potential of -15 mV is relatively low but the rather high viscosity of the system additionally stabilises the dispersion. Furthermore, the high fraction of inner phase (70 %) promotes the formation of a pearl-like particle network also stabilising the system. This is well described for highly concentrated NLC dispersions. Considering the SDS adsorption onto the NLC surface (electrostatic stabilisation), the additional steric barrier by TPGS and PVP in combination with the pearl network structure, the NLC pastes are predicted to be physically stable.

4.2.3.3 Crystalline status

The NLC paste was analysed by differential scanning calorimetry (DSC) and Fig. 4.2.2 shows the DSC graph. Compared to lipid Dynasan 118 bulk material, the peak onset decreased from 72.7 °C to 55.1 °C (Fig. 4.2.3).

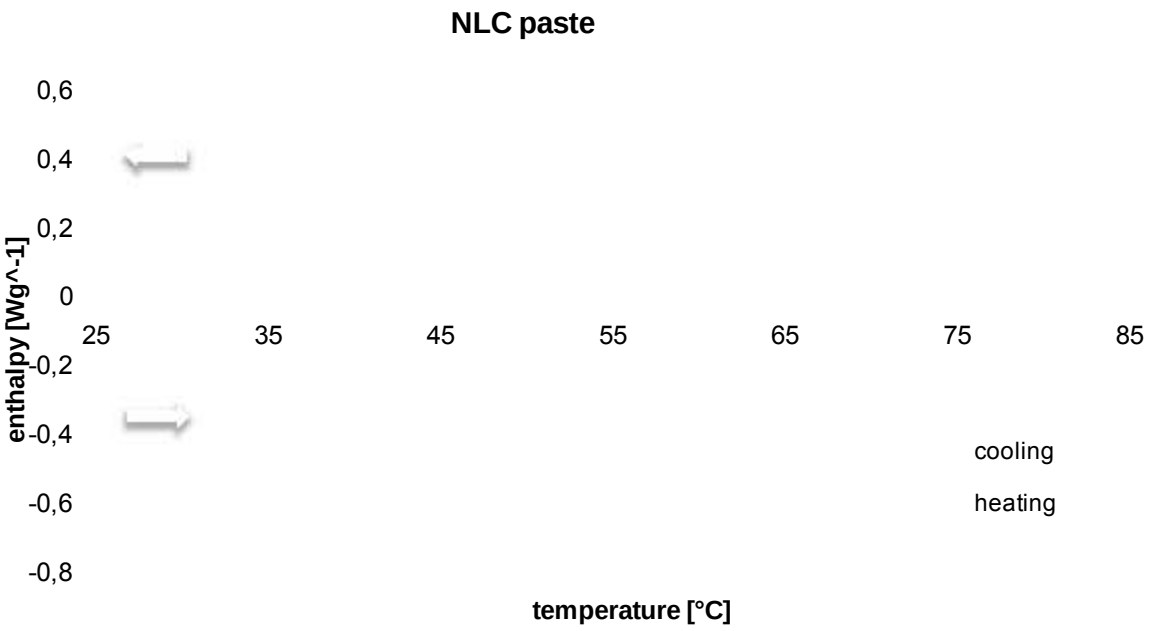


Fig. 4.2.2: DSC graph of the highly viscous 70 % lipid nanoparticle dispersion (= NLC paste), heating from 25 °C to 75 °C and subsequent cooling to 25 °C.

Dynasan 118 as a triacylglycerol can crystallise in three different modifications: α, β' and β (most thermodynamically stable). Running a DSC with two cycles reveals the three modifications. The heating curve of the first cycle shows the β modification with a peak maximum at 72.7 °C whereas cooling leads to cristallisation into the α modification (51.7 °C). Furthermore, the second cycle shows the β' modification with its melting point of 62.9 °C.

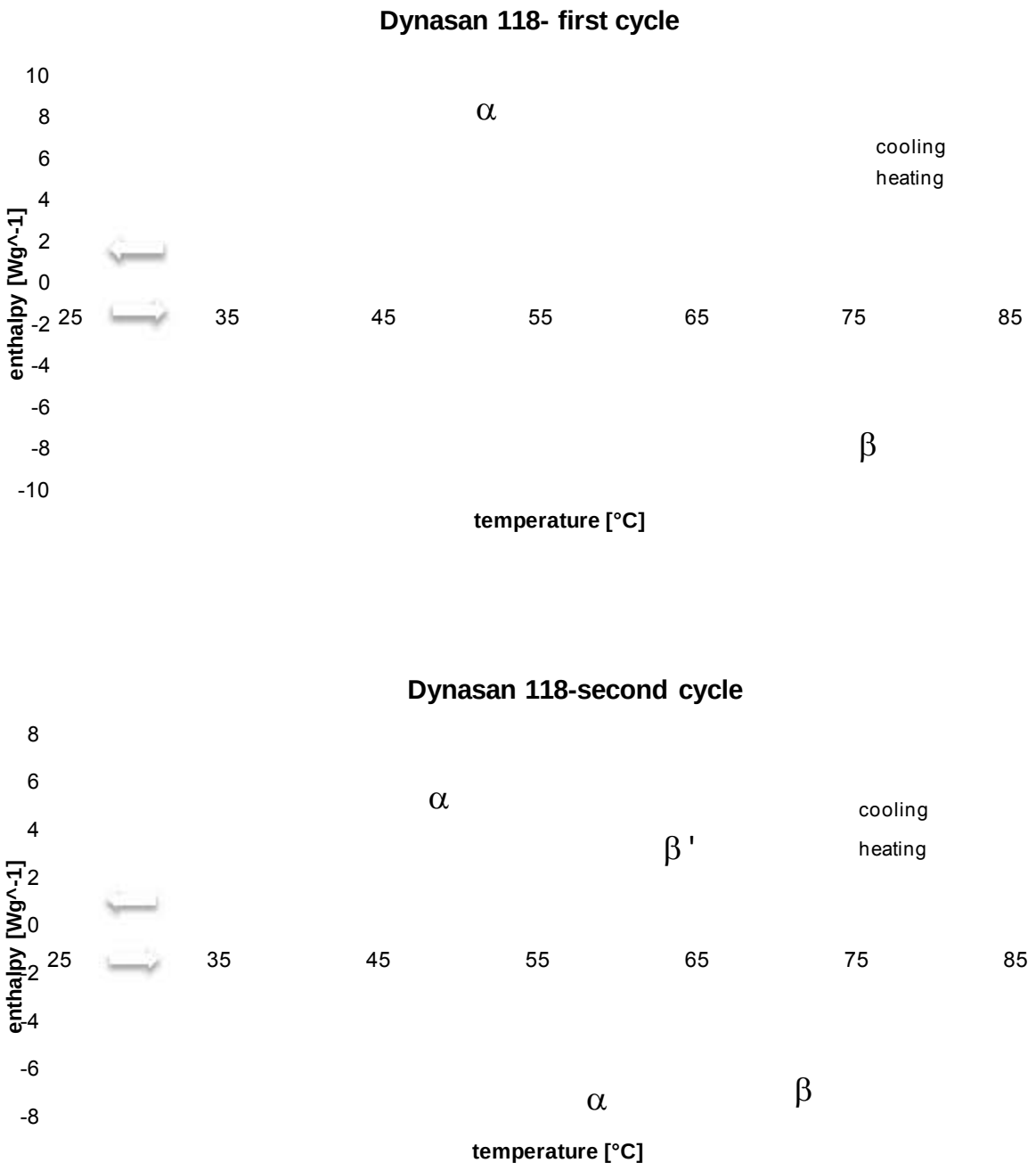


Figure 4.2.3: Two-cycle DSC graph of Dynasan 118 bulk material revealing the three different crystalline modifications of the lipid.

The melting enthalpy of the lipid blend in NLC (fishoil + Dynasan 118) was 41.47 J/g, compared to 201.04 J/g of the bulk lipid. Taking into account that only 20 % of the NLC

consists of Dynasan, the melting enthalpy increased to 207.35 J/g, which means that all the Dynasan in the particles is of crystalline status.

Cooling of the melted lipid dispersion lead to a delay in recrystallisation with a temperature shift from 61.1 °C to 41.4 °C (peak maximum). In addition, the melting enthalpy was distinctly reduced (6.26 J/g) indicating a delayed solidification process and increased α modification of the Dynasan.

The recorded X-ray diffraction spectrum (figure 4.2.4) of the bulk material Dynasan 118 shows the three expected peaks for triacylglycerols representing the three modifications of the lipid: 16.4 (2θ) for the α modification, 19.3 (2θ) for β and 23.1 (2θ) and 24.1 (2θ) for the β' modification (Hagemann, 1988).

The fish oil spectrum (figure 4.2.5) shows no significant peaks, which is also expected for a liquid.

The X-ray diffraction pattern of the paste exhibit mainly the peaks of the β' and β modifications of Dynasan 118. The peaks indicate the solid, crystalline character of the particles.

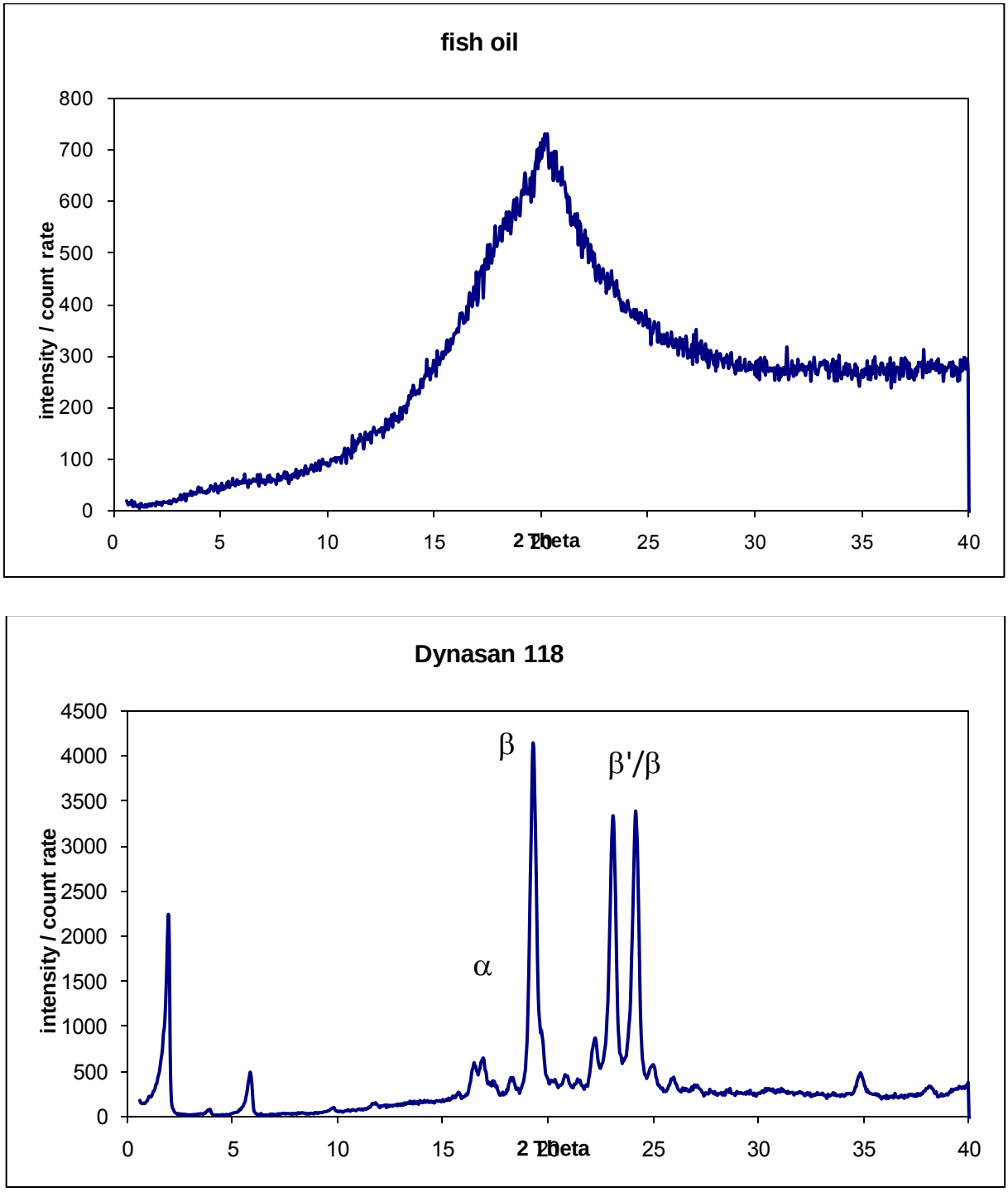


Figure 4.2.4: X-ray diffraction pattern of the bulk materials fish oil and Dynasan 118 (explanations are given in the text).

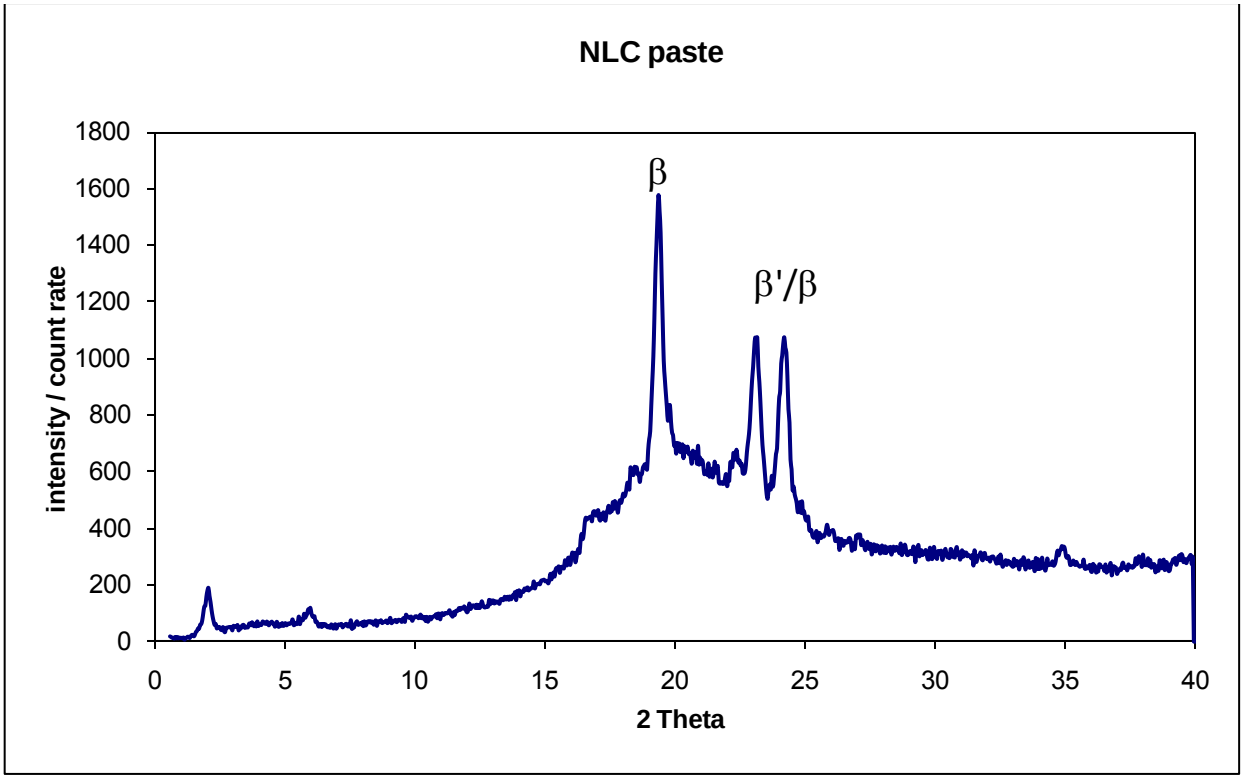


Figure 4.2.5: X-ray diffraction pattern of the NLC-paste with the β' and β modification peaks of Dynasan 118.

4.2.3.4 Re-dispersion properties

From the concept the fish oil NLC paste can be dosed via a spoon and swallowed directly. For this, the paste requires appropriate flavouring. Alternatively the non-flavoured paste can be dispersed for example in soft drinks. In both cases the paste should re-disperse easily, either in the gut fluids or in water. A 500 mg capsule of fish oil (Ameu®) contains approximately 125 mg of omega-3 fatty acids. Considering the higher omega-3 fatty acid content in the fish oil used for preparation of the NLC paste, despite addition of Dynasan 118 and water, 500 mg paste will contain approximately 110 mg omega-3 fatty acids. That means for a dose of approximately 500 mg omega-3 fatty acids a total of about 2.5 g NLC paste should be taken (equals 3-4 capsules of the commercial product).

The two grams of paste represent approximately one level teaspoon or alternatively an approximately 5 cm long strand squeezed out of a standard tube. To test the redispersibility, 5 cm paste were given into 200 ml of water (equivalent to a typical volume of a soft drink).

The paste was either dispersed by manual stirring with a spoon (Figure 4.2.6 left) or alternatively with a slow moving magnetic stirrer to imitate gut forces (Figure 4.2.6 right).

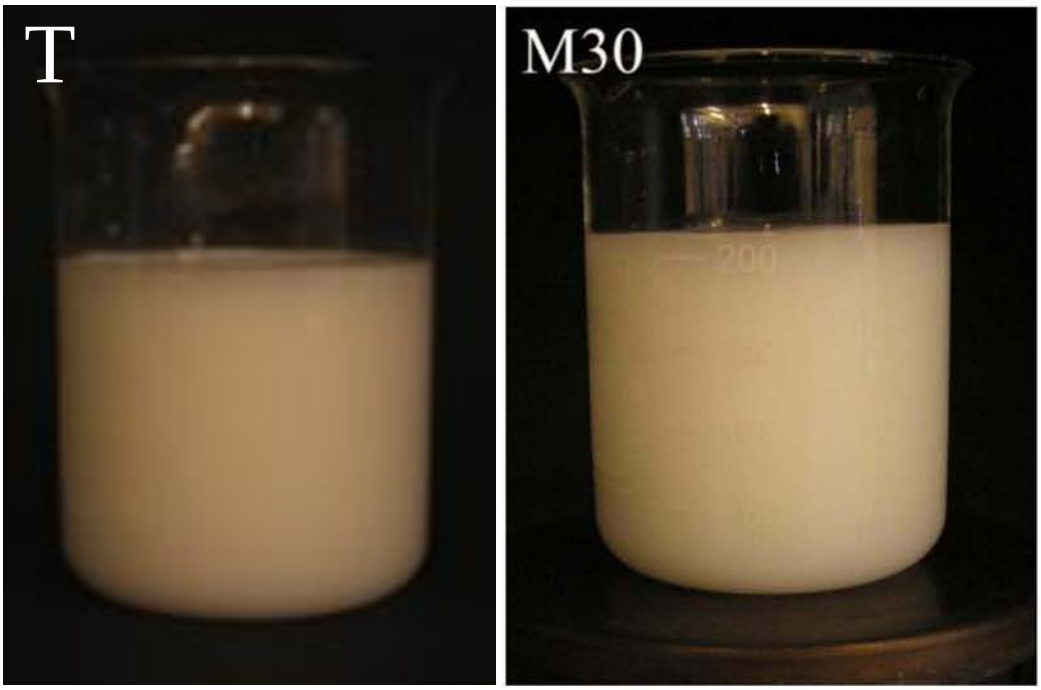


Figure 4.2.6: Appearance after 10 seconds of stirring with a teaspoon (T) and after magnetic stirring at 50 rpm for 30 seconds (M30) following administration of 5 cm NLC® paste (squeezed out of a tube) in 200 ml water.

The formulation is well dispersible by spoon stirring and shows also good redispersibility when applying weak forces. To admix the NLC paste to soft drinks instead of using a spoon, a tiny eggbeater can be used which eases even further dispersion.

4.2.3.5 Absorption mechanism

As already mentioned in the introductory part, it is well documented in the literature that the presence of lipids can promote the absorption of drugs (Charman, Porter, Mithani & Dressman, 1997; Porter & Charman, 2001a). To obtain maximum effect, the drug or active needs to be closely associated with the lipid, that means preferentially the active to be absorbed should be incorporated or dissolved in the lipid.

It was also found that the length of the fatty acid chains has an effect on the absorption. Fatty acids with a chain length of C14 to C18 promote absorption by lymphatic uptake

(Charman, Porter, Mithani & Dressman, 1997; Porter & Charman, 2001a). In addition, it is beneficial if the lipid is presented as finely dispersed as possible. Based on these considerations, the omega-3 fatty acids were incorporated into the solid matrix (close association). The triglyceride chosen (Dynasan 118 = glyceryl tristearate) contains C18 fatty acids and the size of the particles was as small as possible, that means in the nanometre range. Fulfilling all these three requirements should provide an optimal formulation, whereas of course the bioavailability enhancement has to be investigated in *in vivo* studies.

It could be shown that there is no major difference in the mechanism of degradation between oil droplets and solid lipid particles. The solid lipid particles are degraded by lipases, the degradation velocity is a function of the chemical composition of the particle matrix and the stabilisers used (Müller & Olbrich, 1999; Olbrich, Geßner, Kayser & Müller, 2002).

As mentioned in the introductory part, Fig. 2.7 shows a model of the degradation and the subsequent absorption promoting mechanism. The lipid nanoparticles enter the gut, lipase adsorbs onto the particle surface whereas the adsorption is promoted by the presence of co-lipase. Enzymatic degradation of the lipid particles leads to the formation of surface-active di- and monoglycerides forming micelles. In case of drug-loaded lipid nanoparticles, these micelles contain solubilised drug. In case of the fish oil-loaded NLC they consist of glycerides with various fatty acids including the omega-3 fatty acids. In the next step mixed micelles will be formed with bile salts leading finally to the lipid absorption.

4.2.3.6 Flavouring and colouring

Dilution of the fish oil in the solid particle matrix and surrounding the particles by a poorly diffusible medium (water) for the lipophilic fish oil components could not completely eliminate the unpleasant smell: A slight fishy smell remained which could irritate sensitive noses. Therefore various flavours were tested for masking: Orange, lemon, strawberry and mango. The flavours were applied in different concentrations. Most efficient proved to be orange flavour in a concentration of 1.25 %. The flavour was incorporated by adding it to the melted lipid blend just prior to homogenisation. Evaporation of the volatile flavour does not represent a problem because the processing time is relatively short. In addition, at large-

scale production, the homogenisation will take place in closed containers avoiding any relevant flavour loss.

A colour appropriate to the flavour was chosen for colouring the product: Food colour yellow ZLT3 in a concentration of 0.24 % and 0.013 % food colour red ZLT2.

4.3 Conclusions

4.3.1 Oral testosterone formulations

NLC could be produced with a drug loading of up to 30 %, which is superior to the solubility of TU in Andriol Testocaps® (about 10 %). TU is obviously present in the particle matrix as molecular dispersion. Sizes can be tailor-made depending on surfactant concentration and production conditions

The NLC showed a superior ability to increase the bioavailability of TU compared to the commercial product Andriol Testocaps®. Reducing the particle size led to higher AUC values, as expected from data, e.g. published with microemulsions. The total AUC with the best formulations was roughly double as high compared to Andriol Testocaps® in non-fed state. Taking into account the reduced volume of the NLC particles compared to Andriol Testocaps® oil solution, administration of a single dose in one oral unit seems to be feasible.

The highest c_{max} concentrations found with the best formulations were very similar or practically identical. This suggests that there might be a maximum absorption capacity. Absorption might not be further increased, even when further optimising the particles. However, this needs to be verified in additional studies.

Very interesting is that despite the total AUC was similar for the best formulations; the shape of the serum profiles was very different depending on particle characteristics. Main parameters determining the shape of the serum profile are the chemical nature of the lipid matrix (differences in degradation of the particles are assumed) and the drug loading. Lower drug loading and more lipid present (= faster degradation assumed) lead to high c_{max} at short t_{max} and an uptake very fast completed. Higher drug loadings lead also to an early c_{max} at t_{max} of one hour, but the c_{max} is distinctly lower and the decrease in the serum concentration much more prolonged (tailing of profile).

This opens the opportunity of a controlled design of the serum profile by varying the particle characteristics, the nature of the lipid and drug loading (ratio of drug to lipid in the particle). The *in vivo* data were collected in rats, however the best model (and of course the most expensive) for T and TU are beagle dogs. Therefore the best formulations screened in this

study should be tested in the dog model. To justify such studies, the *in vivo* animal studies described in this publication provide the first evidence to distinctly increase the bioavailability when using nanocarriers.

4.3.2 Fish oil formulations

The produced fish oil-loaded NLC paste possesses an ultra fine particle size, which is favourable for the absorption in the gut. It is easily dispersible in fluid media. The formulation represents an alternative to persons, especially cancer patients, who have problems swallowing solid dosage forms. Encapsulation in the particle matrix and formulation as O/W system minimized undesired odour and taste. Apart from pharmaceutical preparations, the formulation seems ideal for enriching food and beverages with omega-3 fatty acids and makes it a promising tool for “functional food” which is quite popular.

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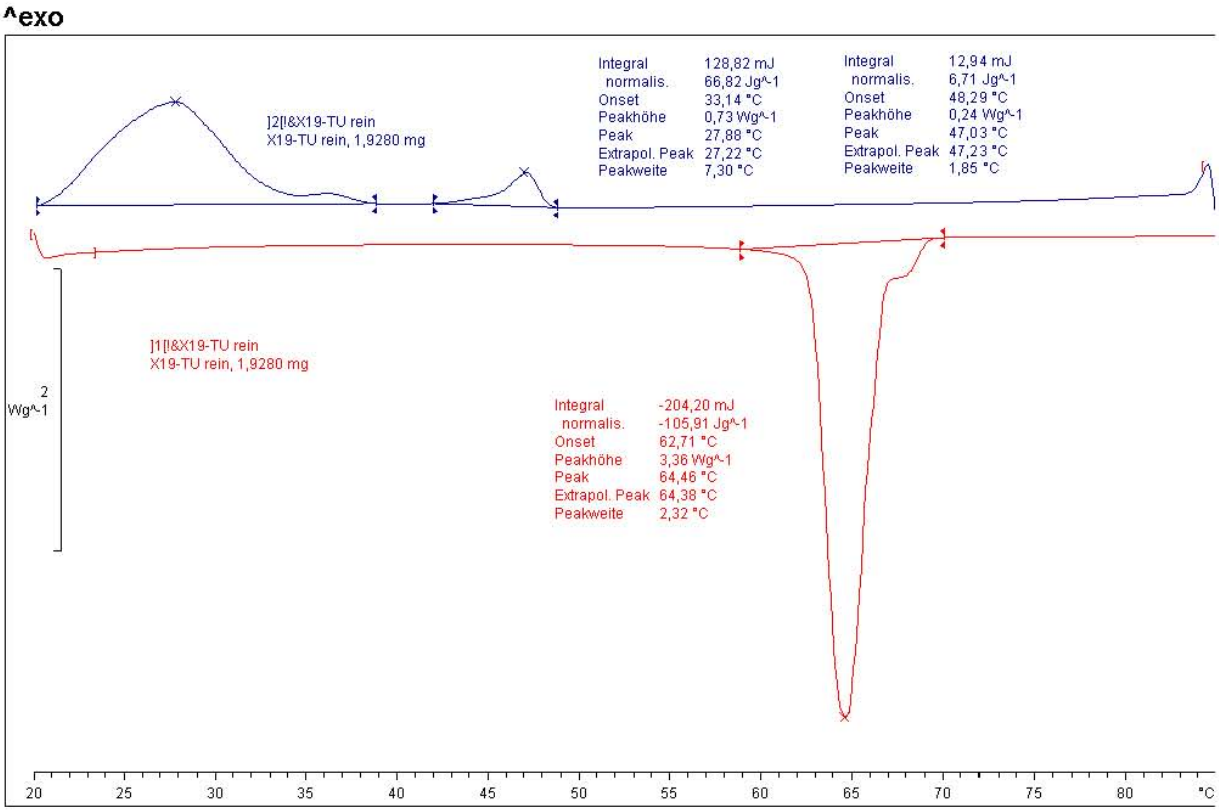
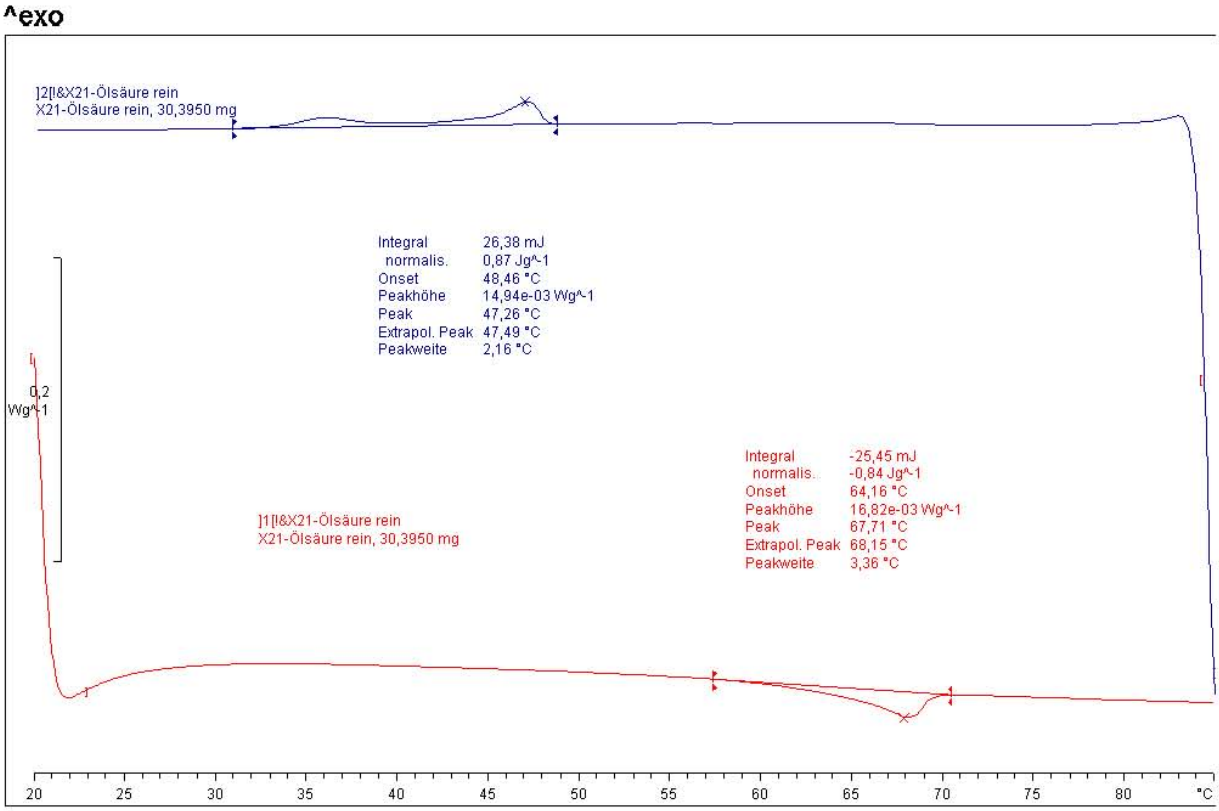
6 Appendix

6.1 Differential scanning calorimetry figures

6.1.1 Bulk materials

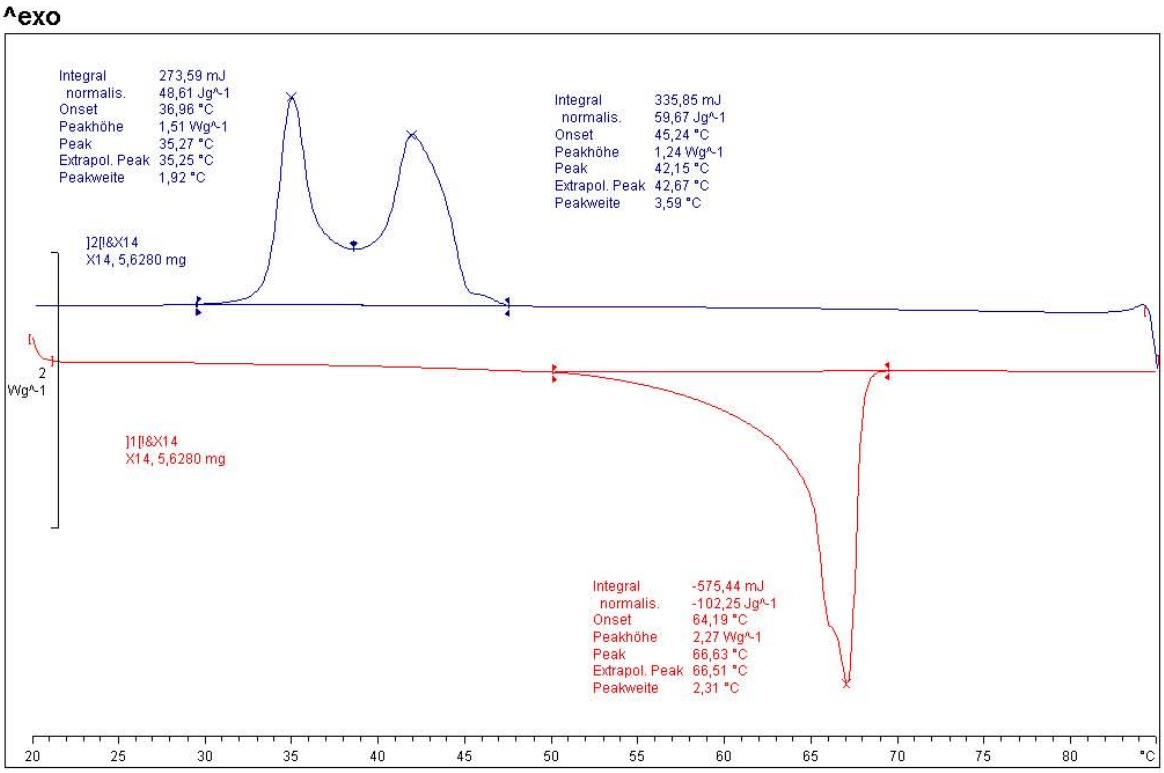


bulk material: Testosterone undecanoate

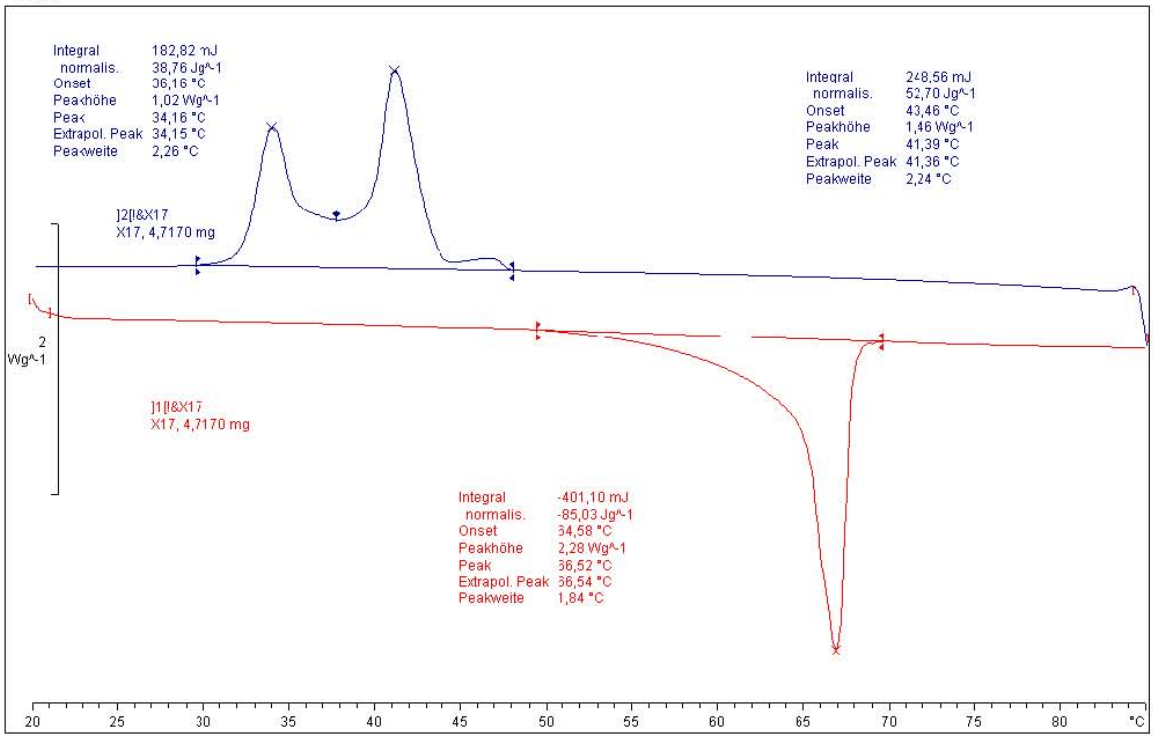


bulk material: Oleic acid

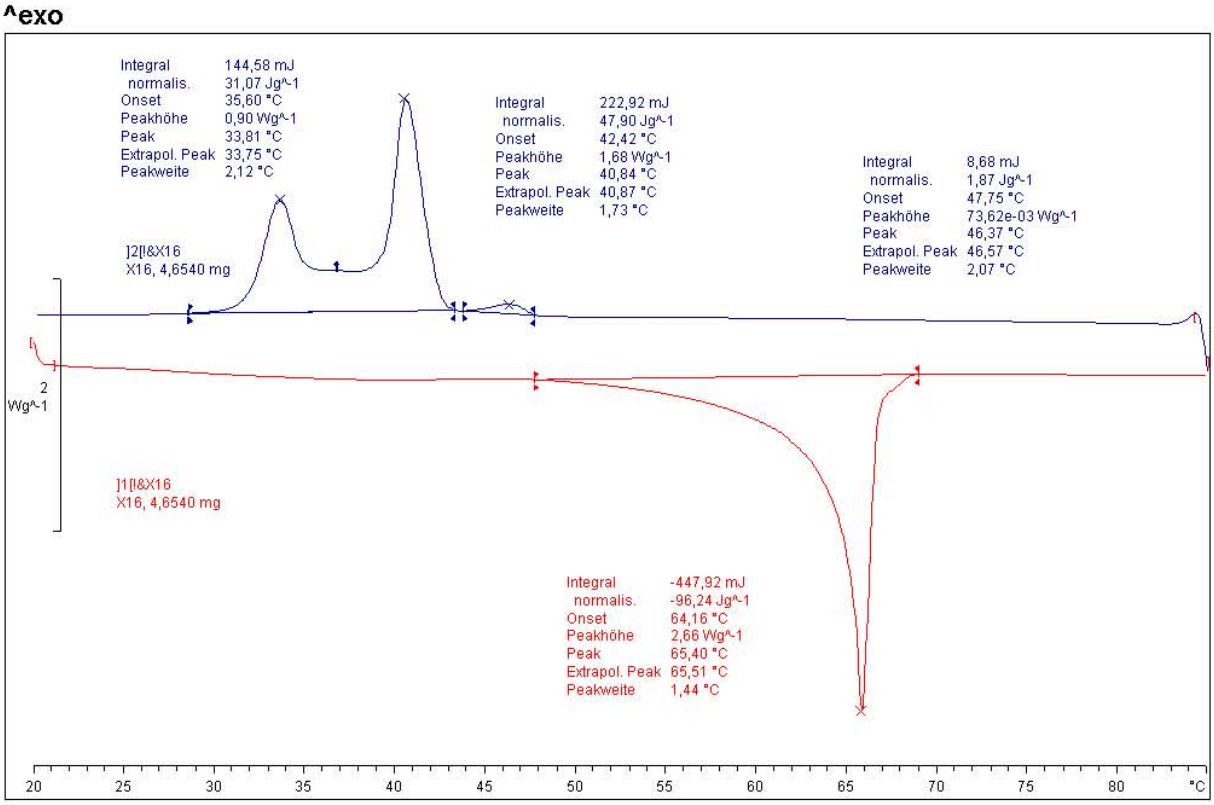
6.1.2 Bulk materials: Lipid blends



bulk material: Dynasan 118, oleic acid (50/50) (m/m)

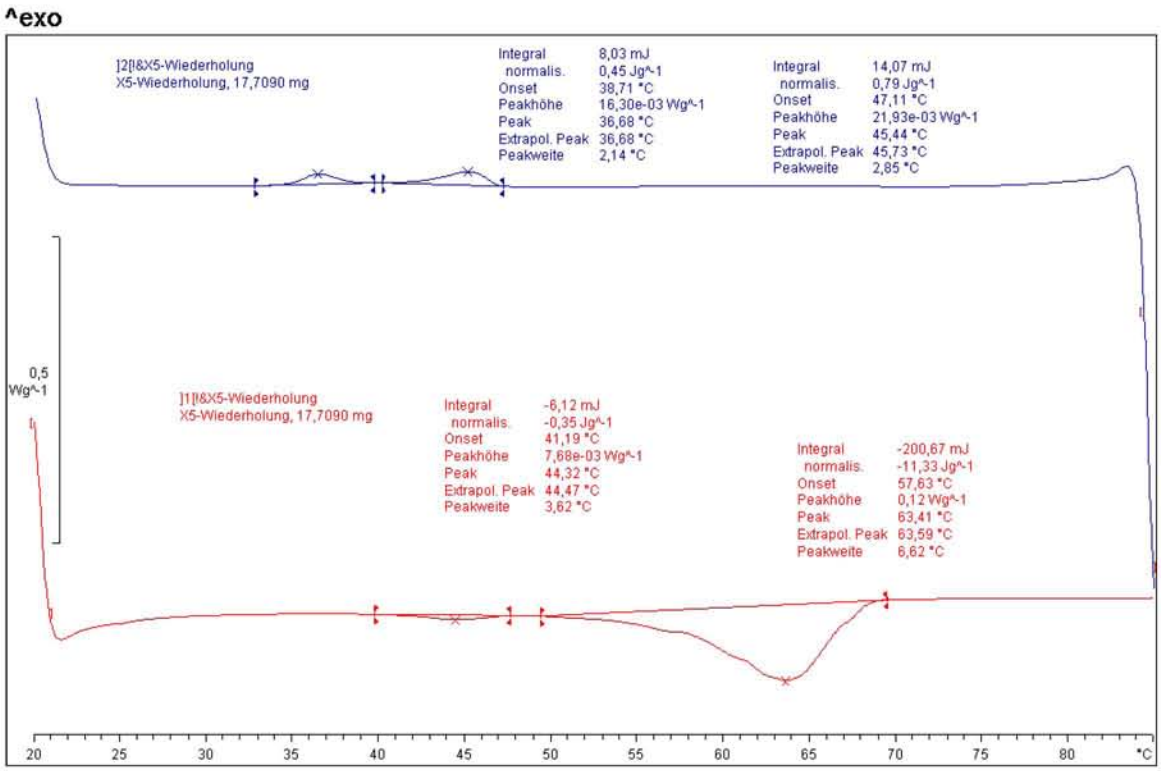


bulk material: Dynasan 118, oleic acid, TU (42,5/42,5/15) (m/m)

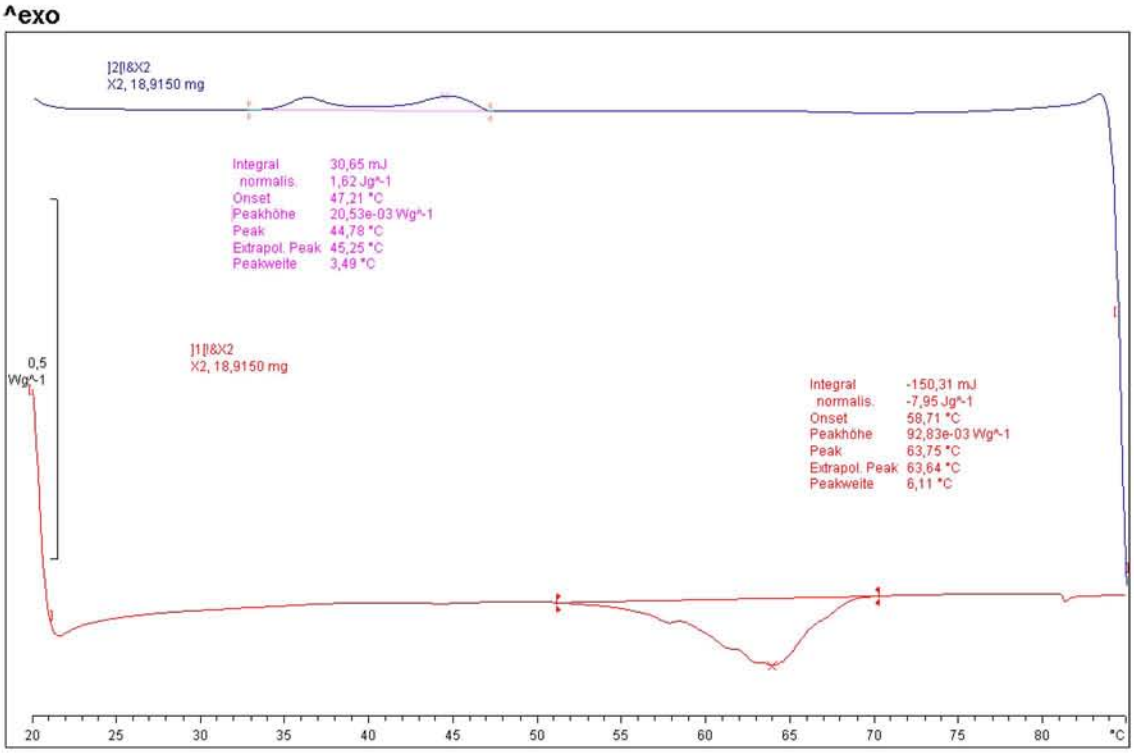


bulk material: Dynasan 118, oleic acid, TU (35/35/30) (m/m)

6.1.3 Dynasan 118-based Nanostructured Lipid Nanoparticles (NLC)



NLC 10 % lipid phase (0 % TU, 50 % Dynasan 118, 50 % oleic acid), 2 % Tween 80



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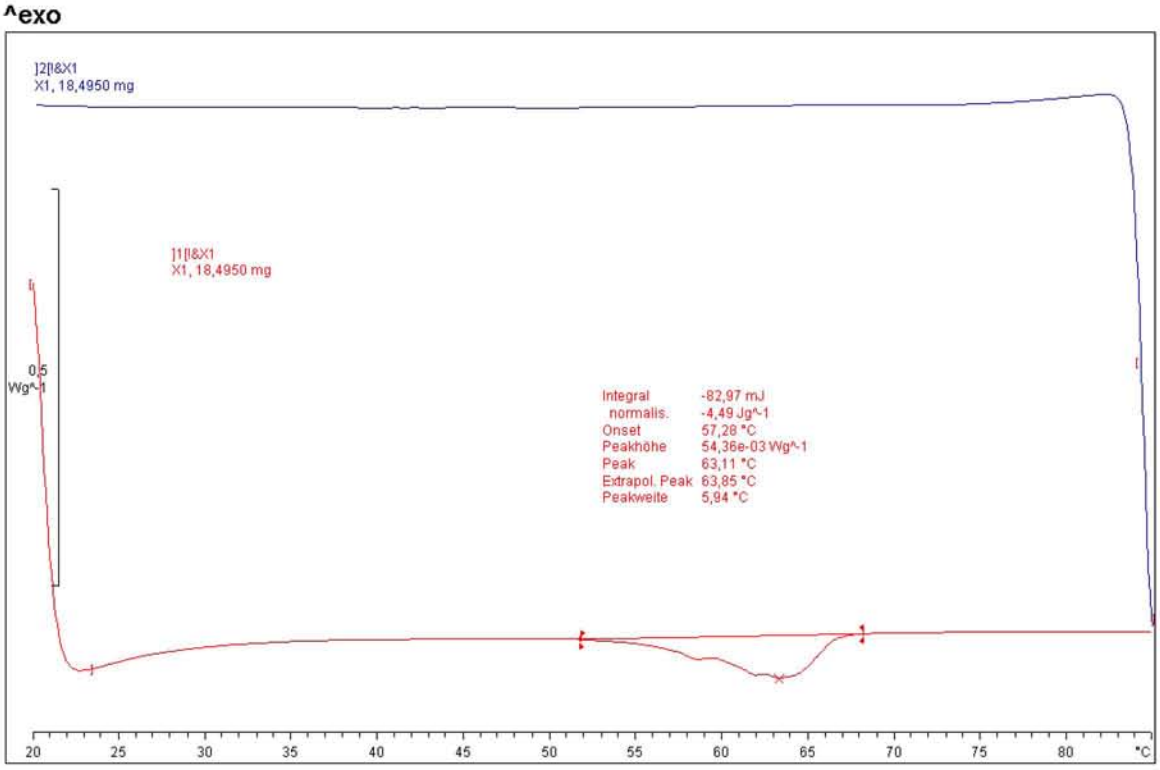
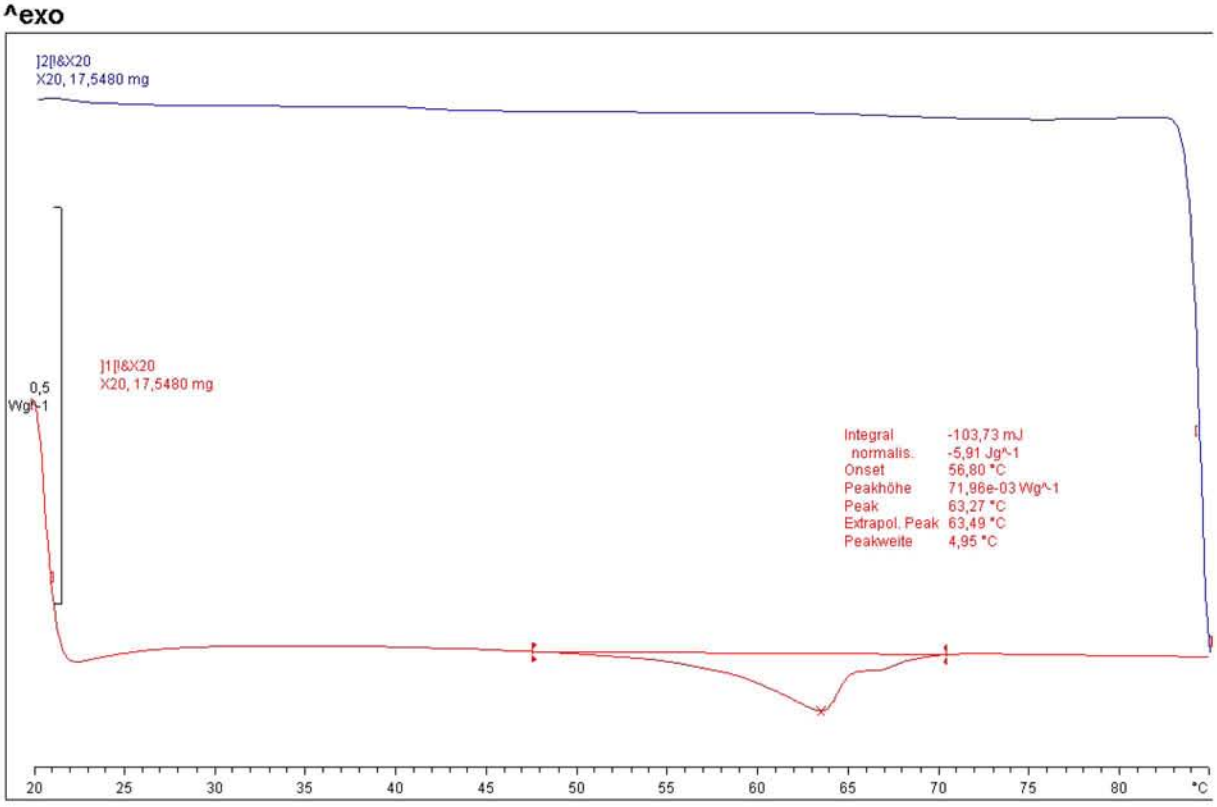
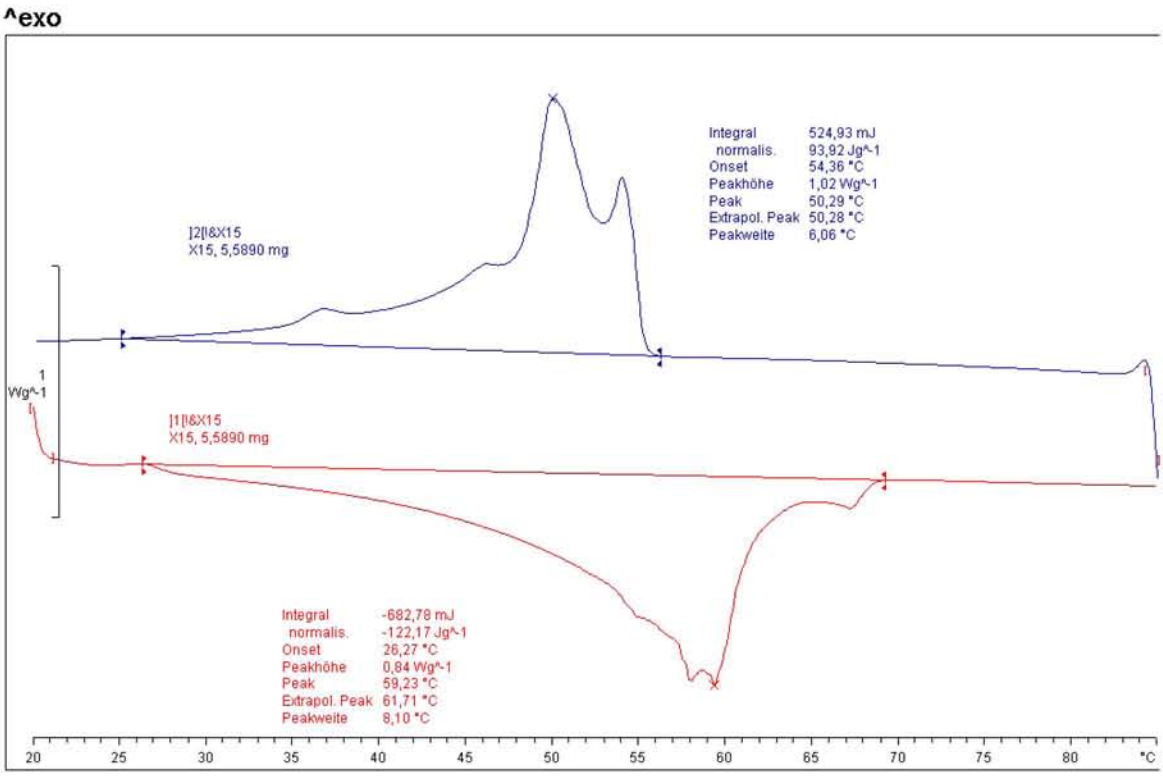


NLC 10 % lipid phase (30 % TU, 35 % Dynasan 118, 35 % oleic acid), 2 % Tween 80

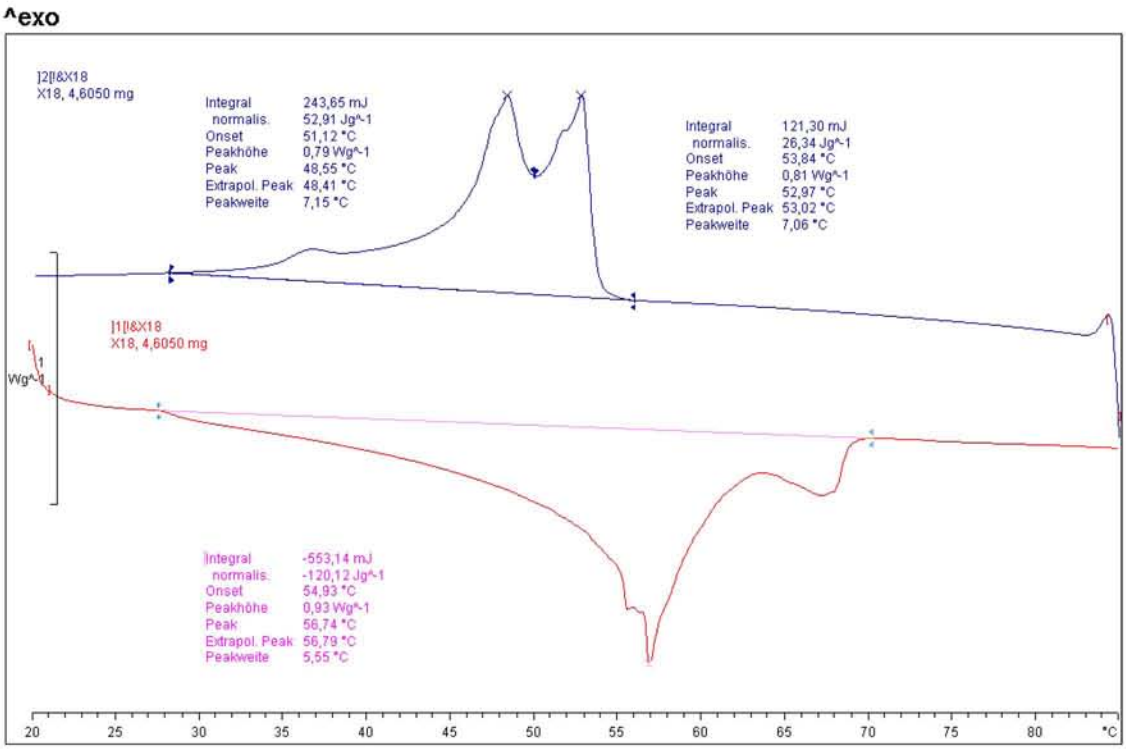


NLC 10 % lipid phase (30 % TU, 35 % Dynasan 118, 35 % oleic acid), 1 % Tween 80

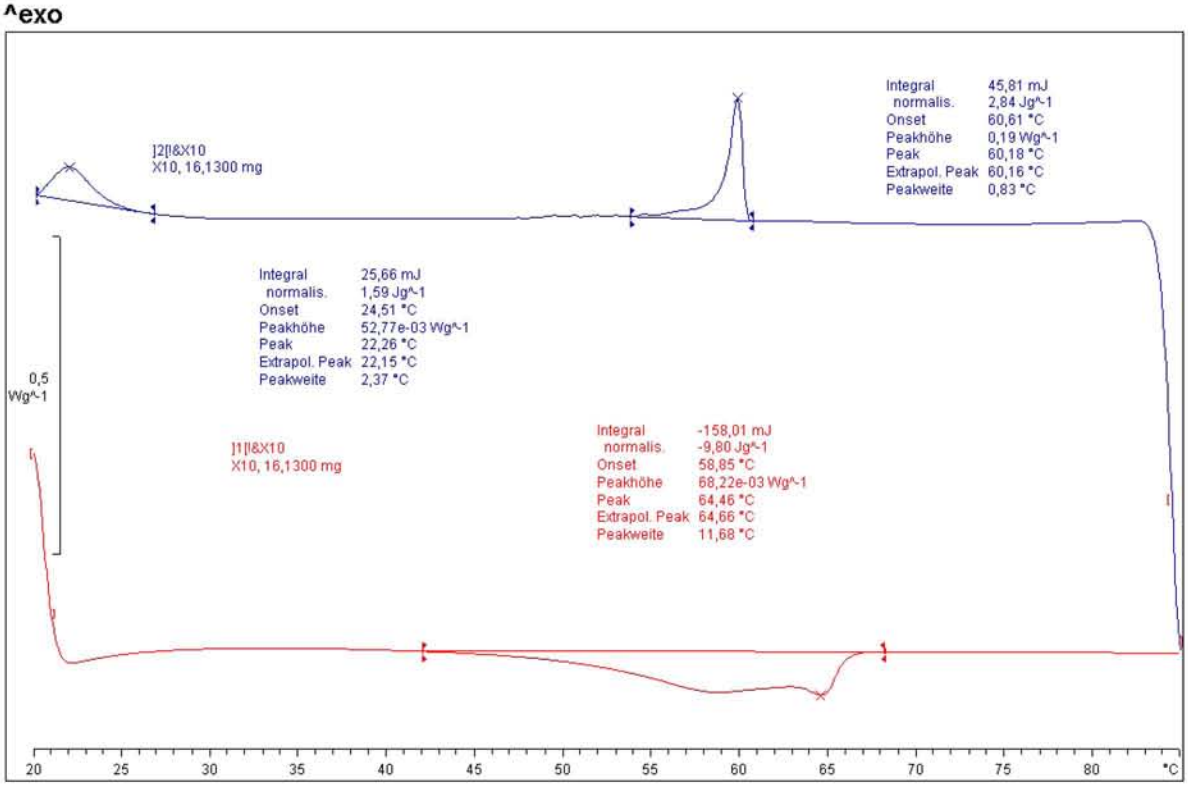
6.1.4 Stearic acid-based NLC



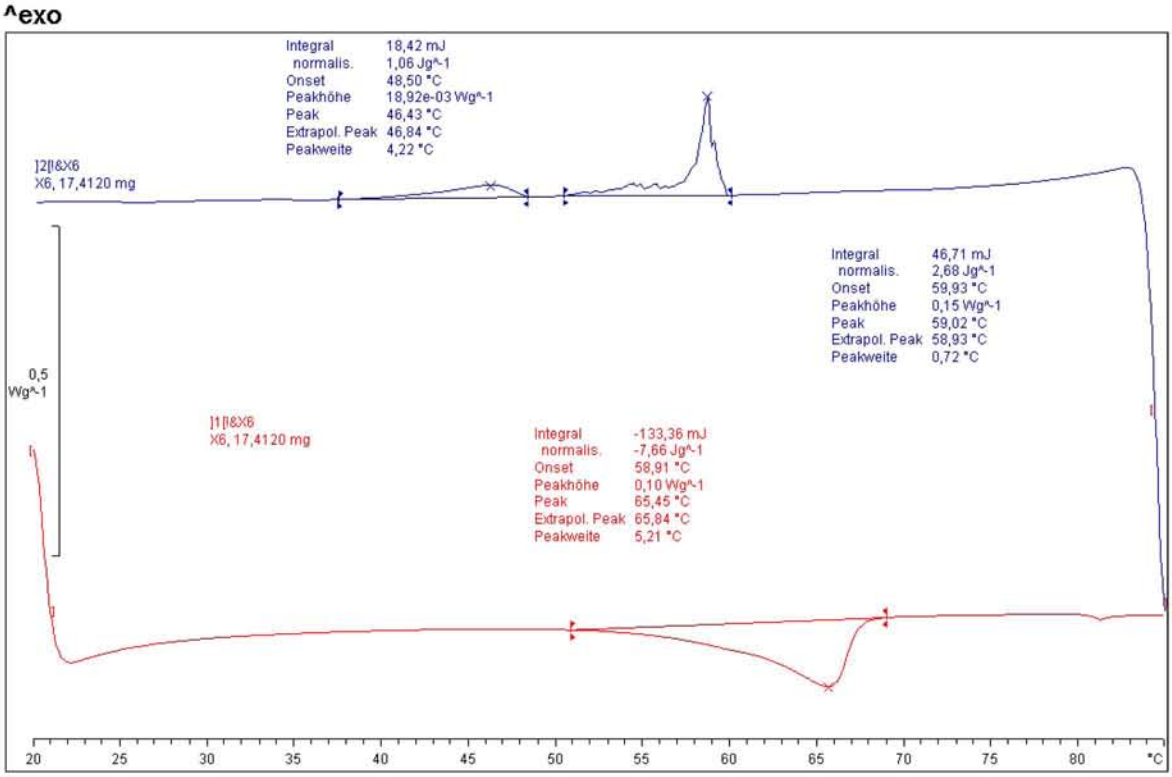
bulk material: Stearic acid, oleic acid (50/50) (m/m)



bulk material: Stearic acid, oleic acid, TU (42,5/42,5/15) (m/m)



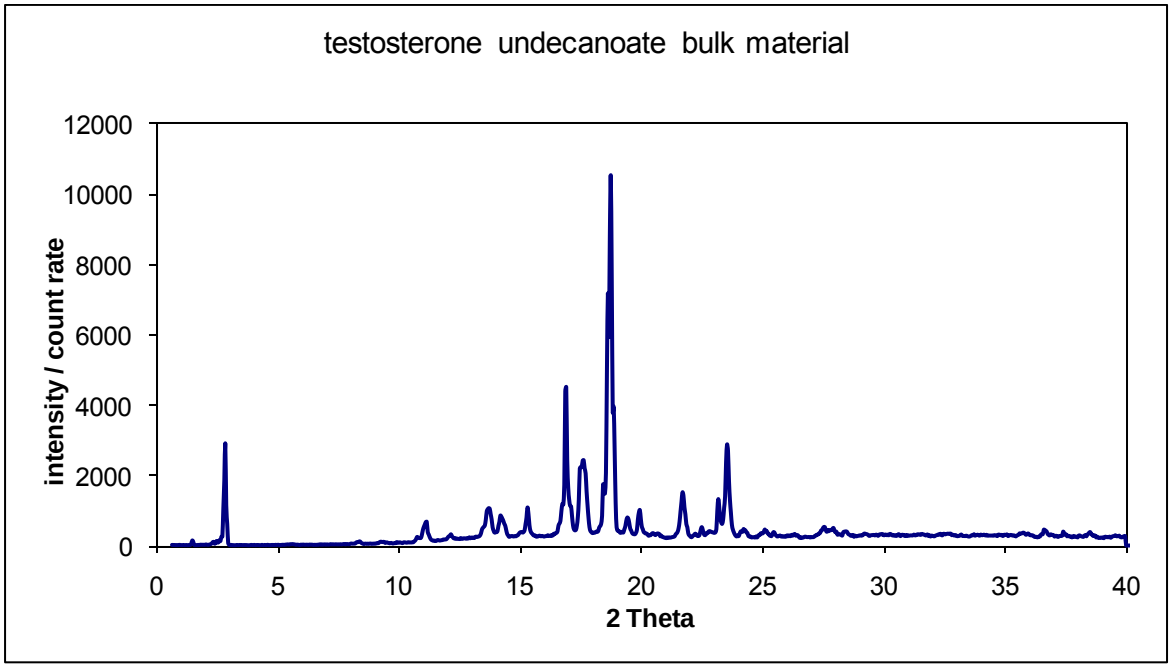
NLC 10 % lipid phase (0 % TU, 50 % stearic acid, 50 % oleic acid), 2 % Tween 80



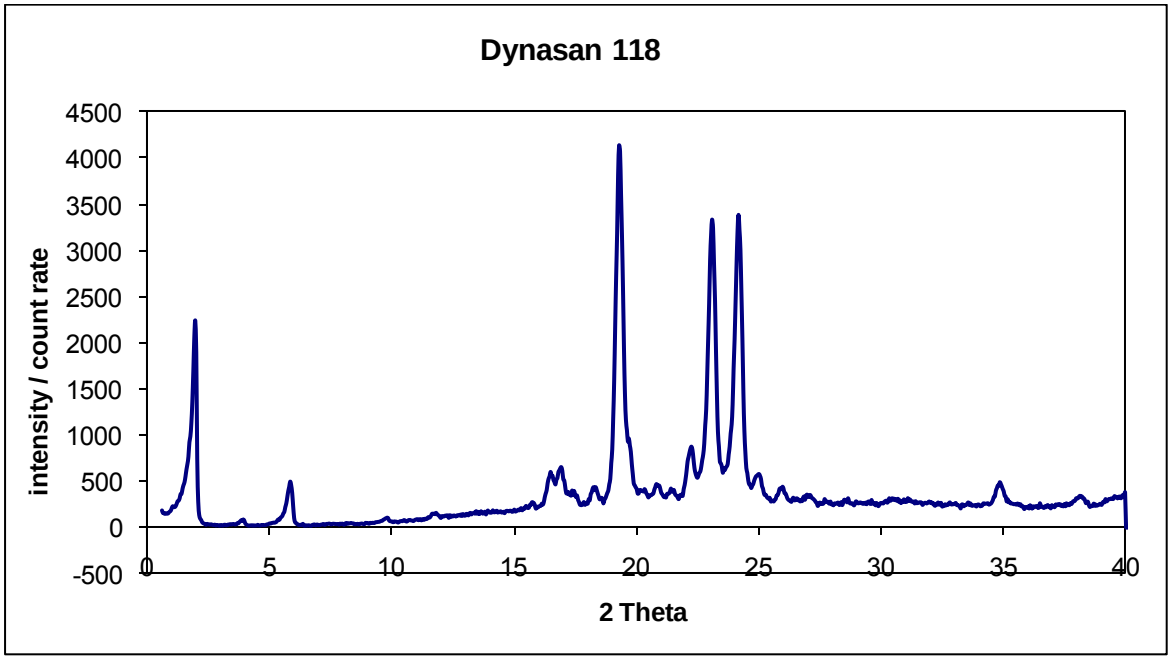
NLC 10 % lipid phase (15 % TU, 42,5 % stearic acid, 42,5 % oleic acid), 2 % Tween 80

6.2 Wide angle X-ray diffraction figures

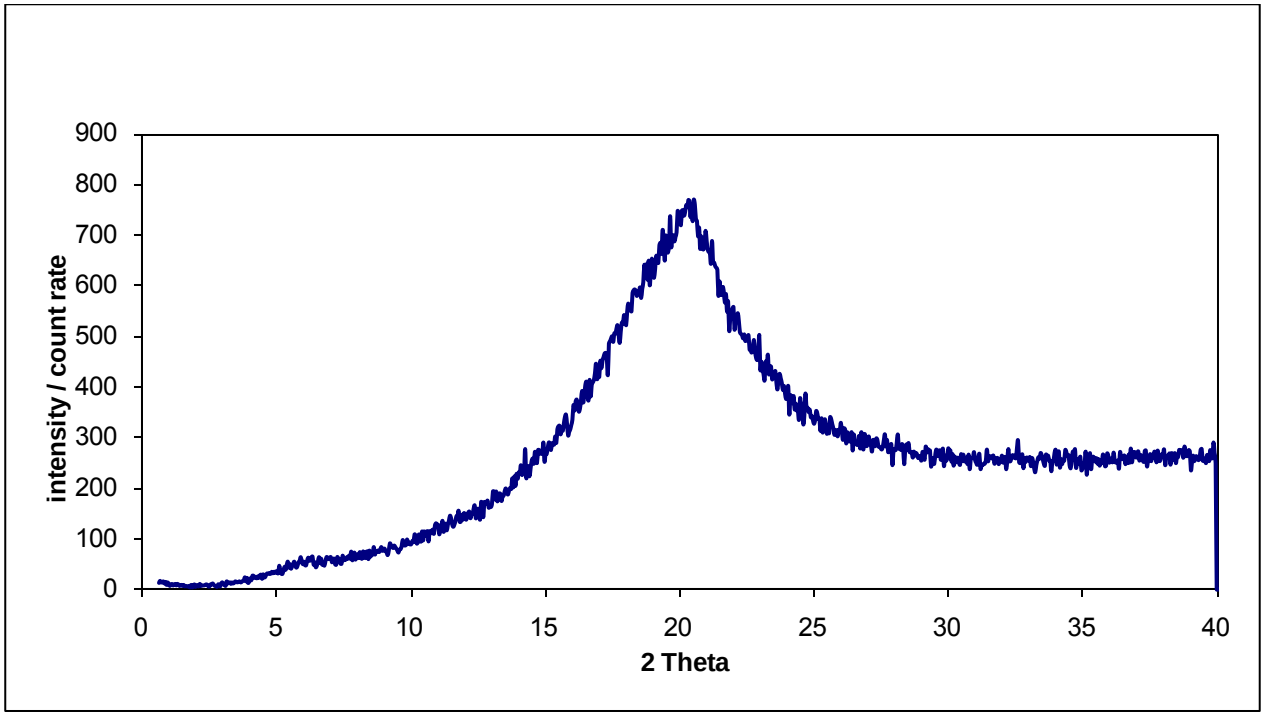
6.2.1 Bulk materials



bulk material: Testosterone undecanoate

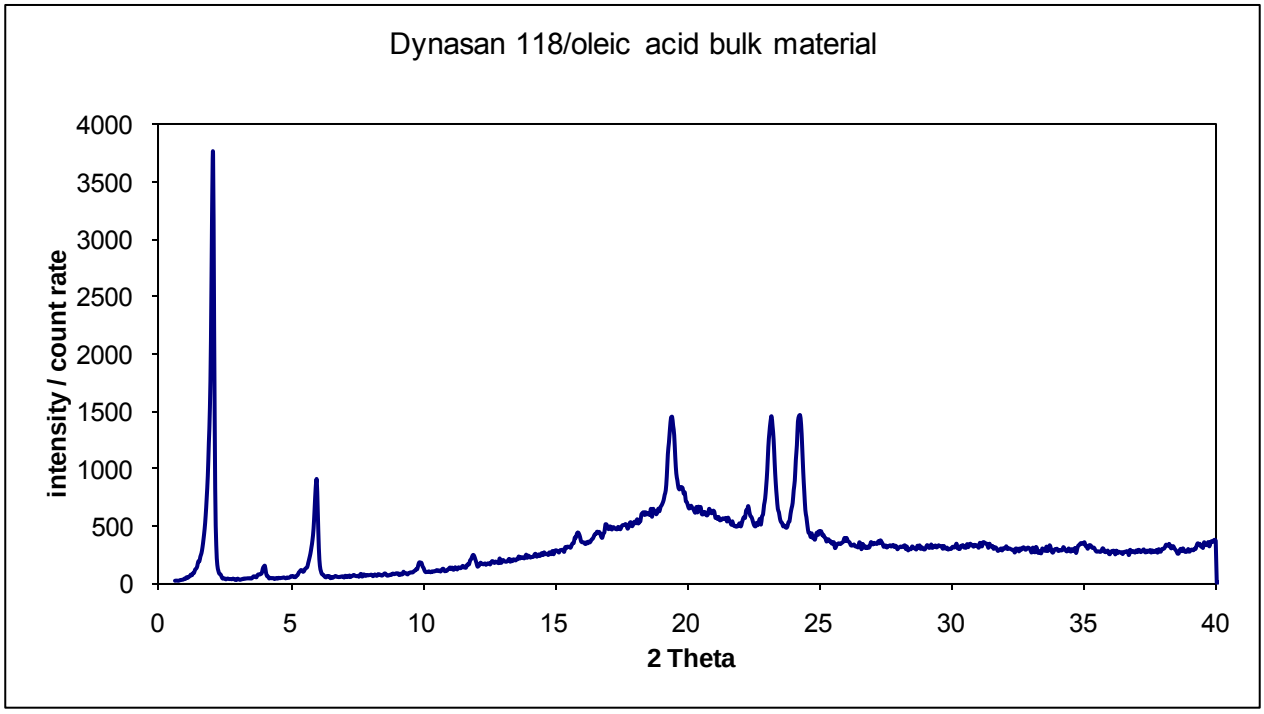


bulk material: Dynasan 118

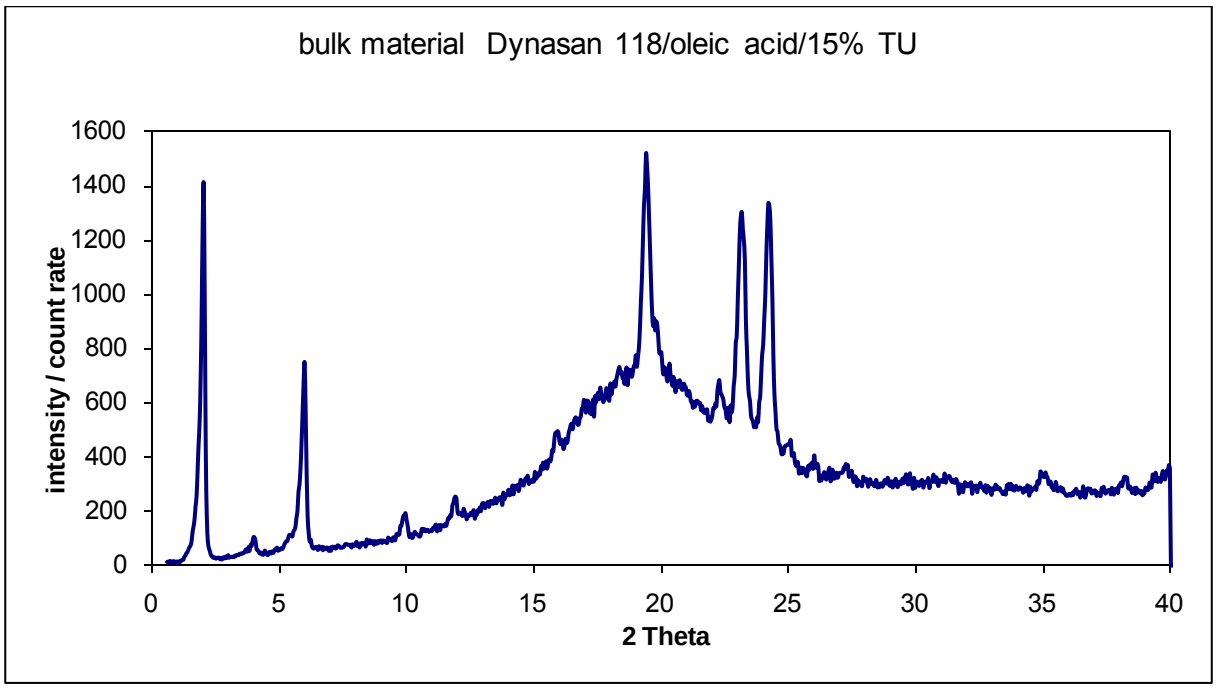


Bulk material: Oleic acid

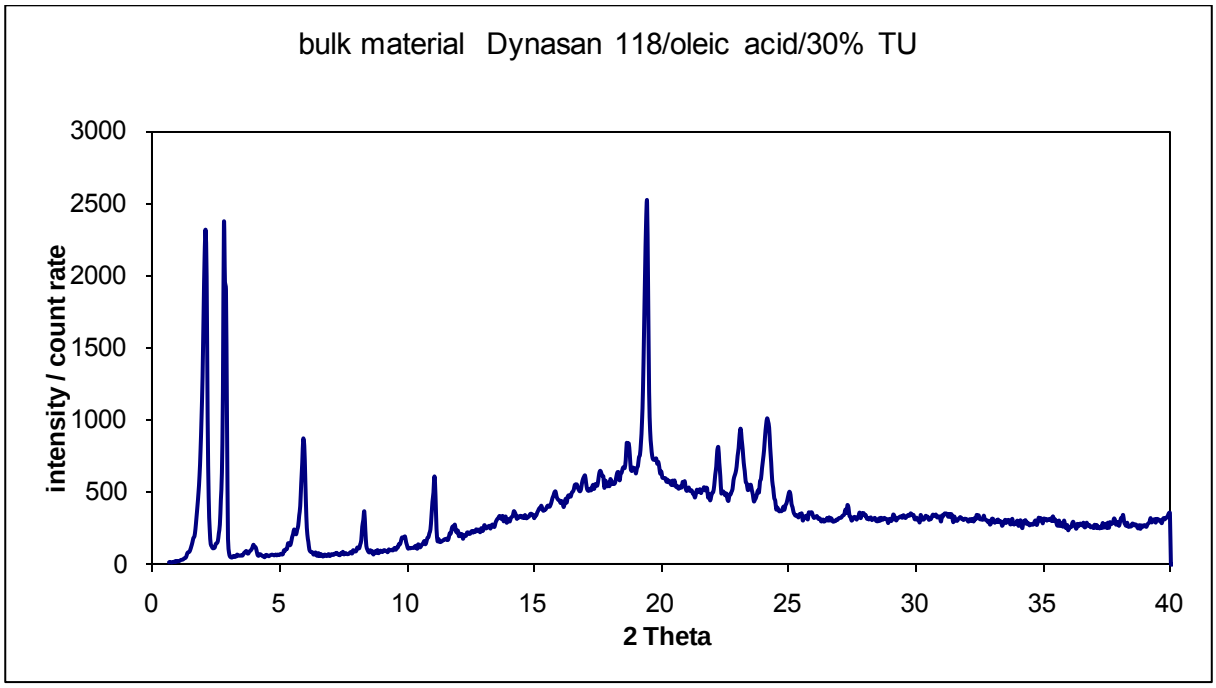
6.2.2 Bulk materials: Lipid blends



Bulk material: Dynasan 118/oleic acid melt (50/50) (m/m)

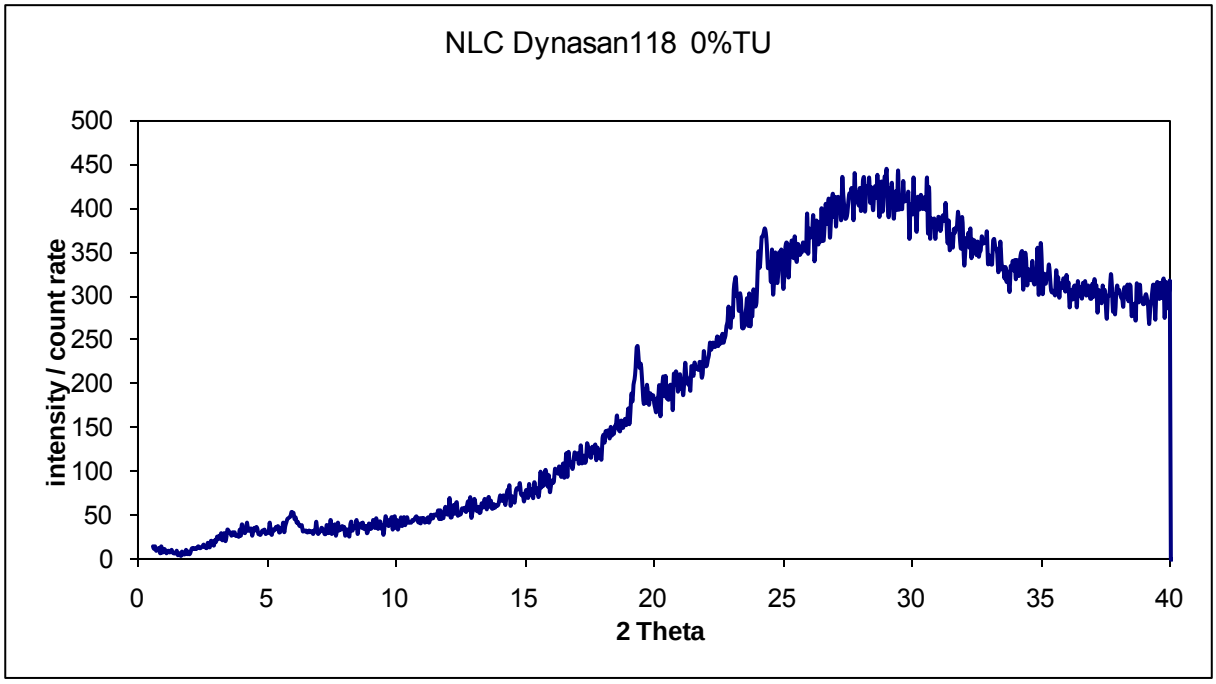


Bulk material: Dynasan 118/oleic acid/TU (42,5/42,5/15) (m/m)

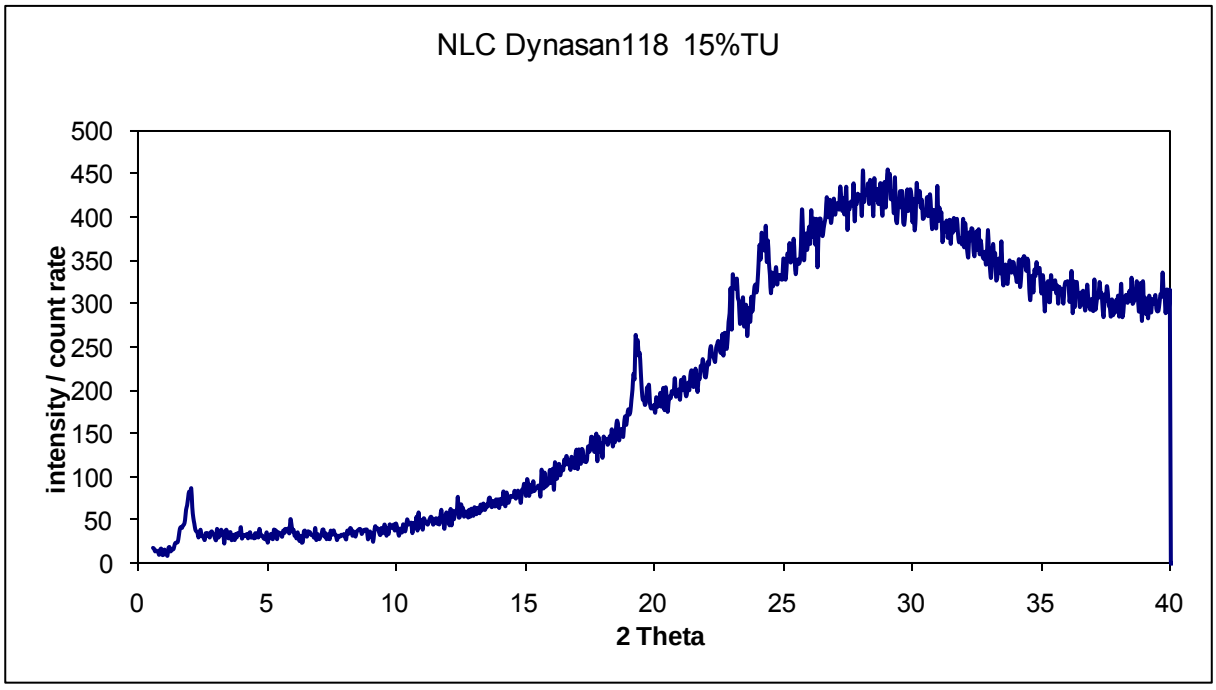


Bulk material: Dynasan 118/oleic acid/TU (35/35/30) (m/m)

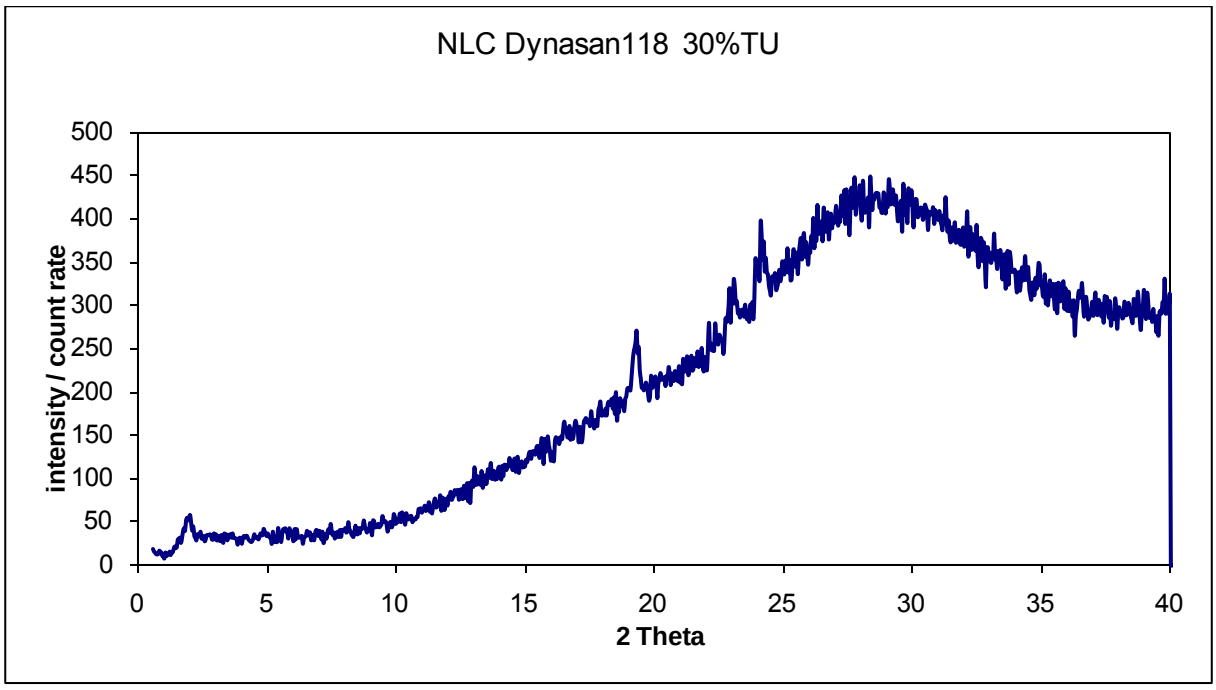
6.2.3 Dynasan 118-based Nanostrucutred Lipid Carriers



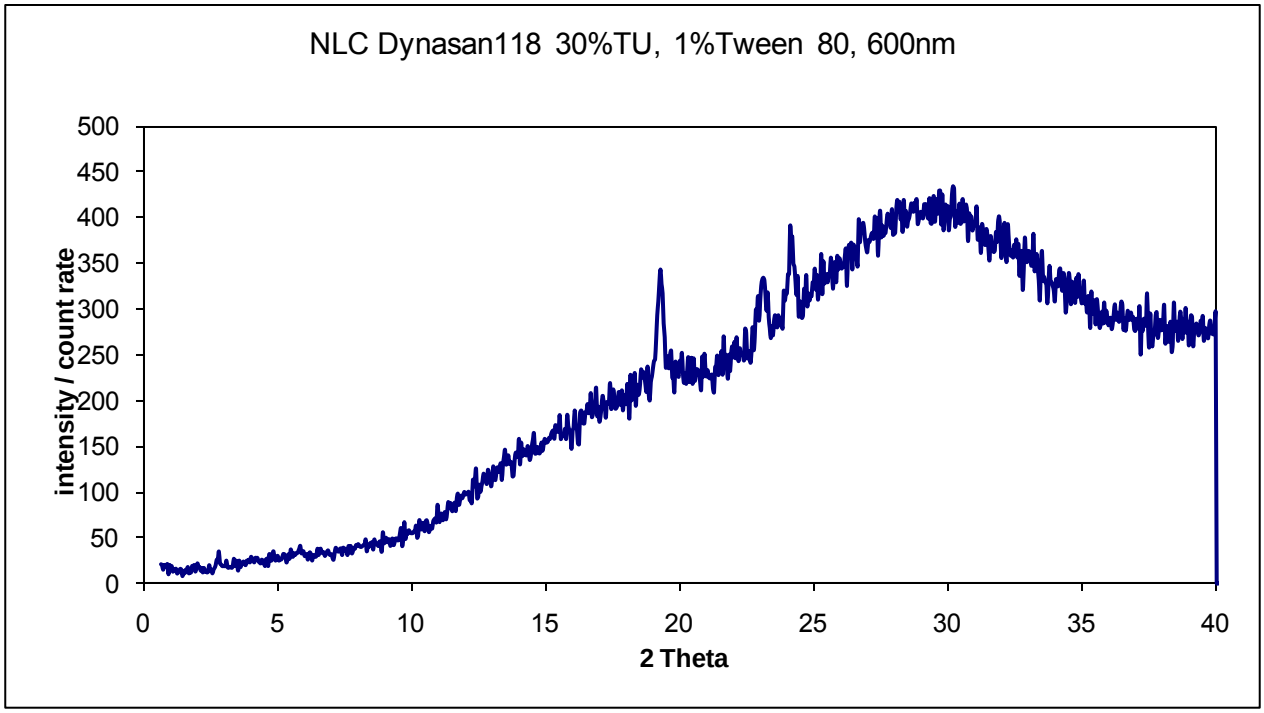
NLC 10 % lipid phase (0 % TU, 50 % Dynasan 118, 50 % oleic acid), 2 % Tween 80



NLC 10 % lipid phase (15 % TU, 42,5 % Dynasan 118, 42,5 % oleic acid), 2 % Tween 80

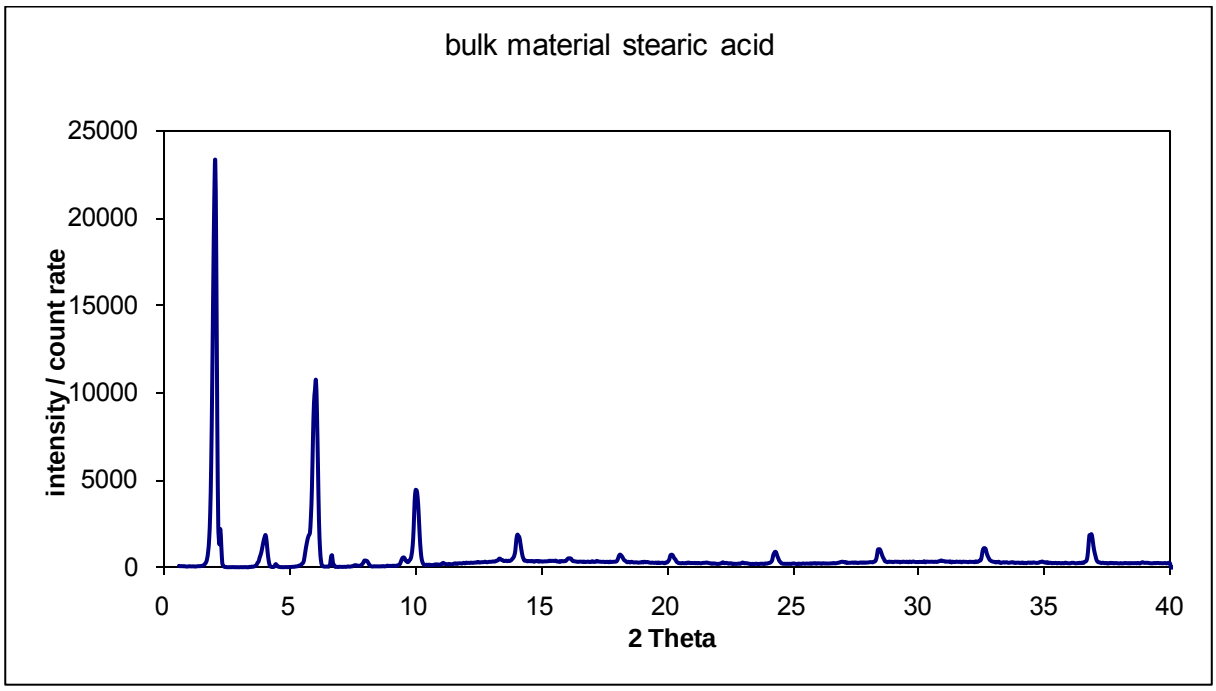


NLC 10 % lipid phase (30 % TU, 35 % Dynasan 118, 35 % oleic acid), 2 % Tween 80

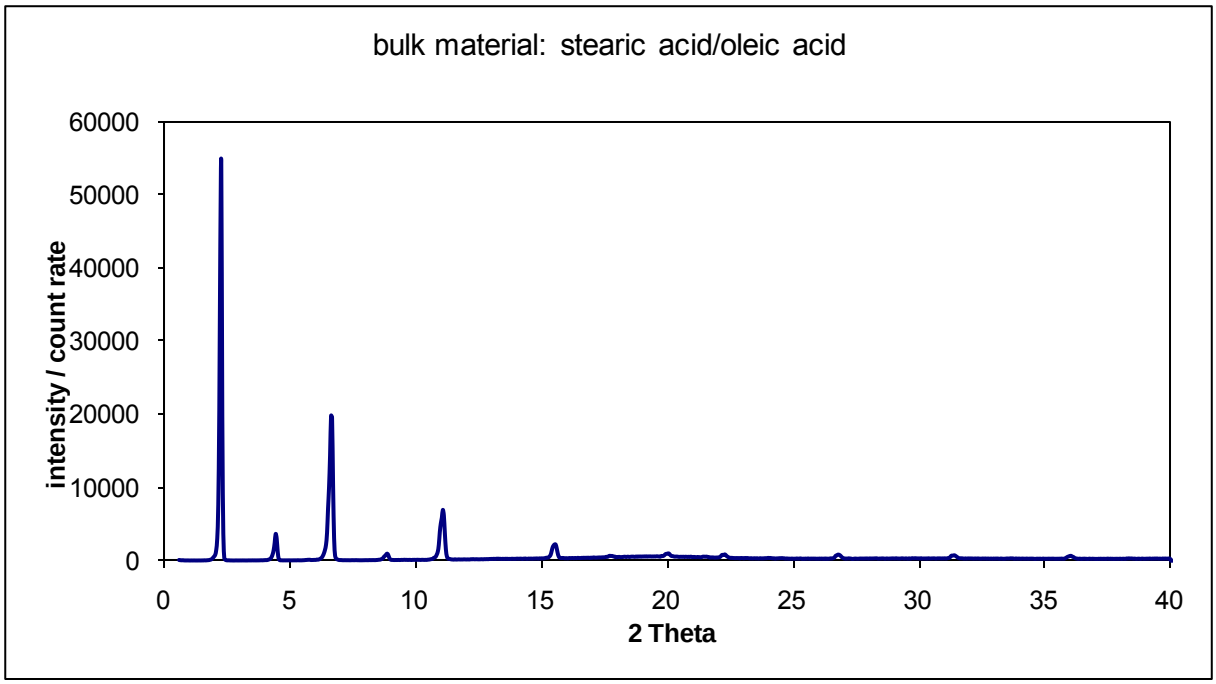


NLC 10 % lipid phase (30 % TU, 35 % Dynasan 118, 35 % oleic acid), 1 % Tween 80

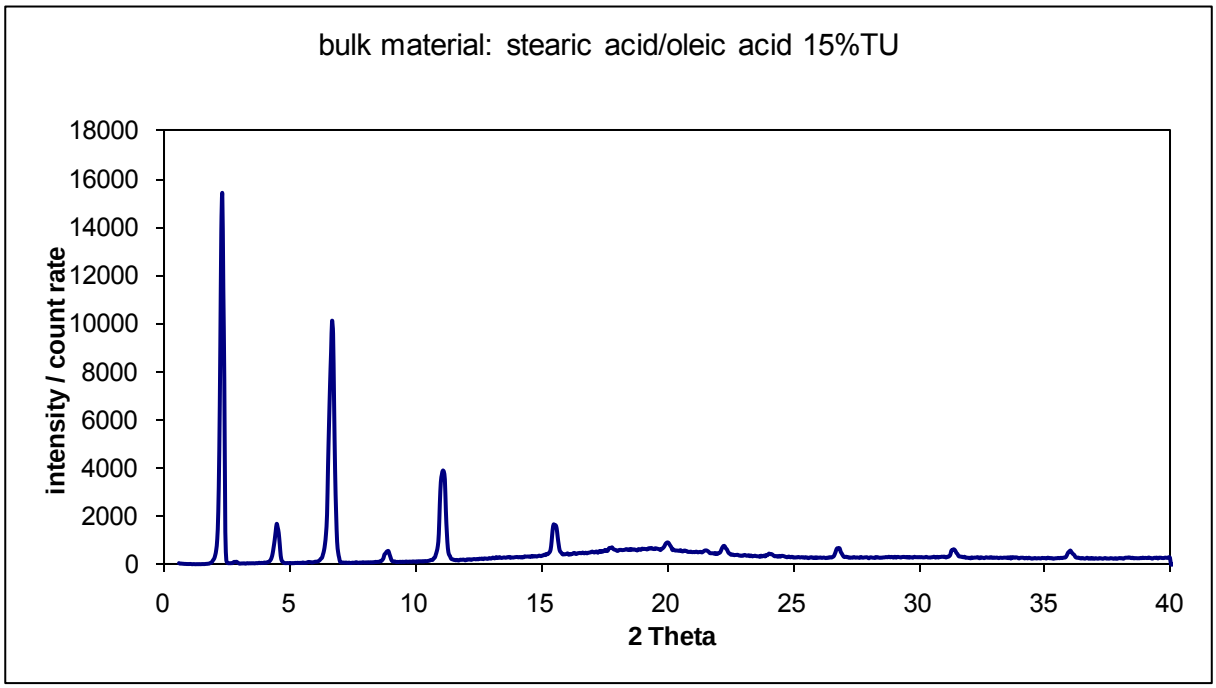
6.2.4 Stearic acid-based NLC



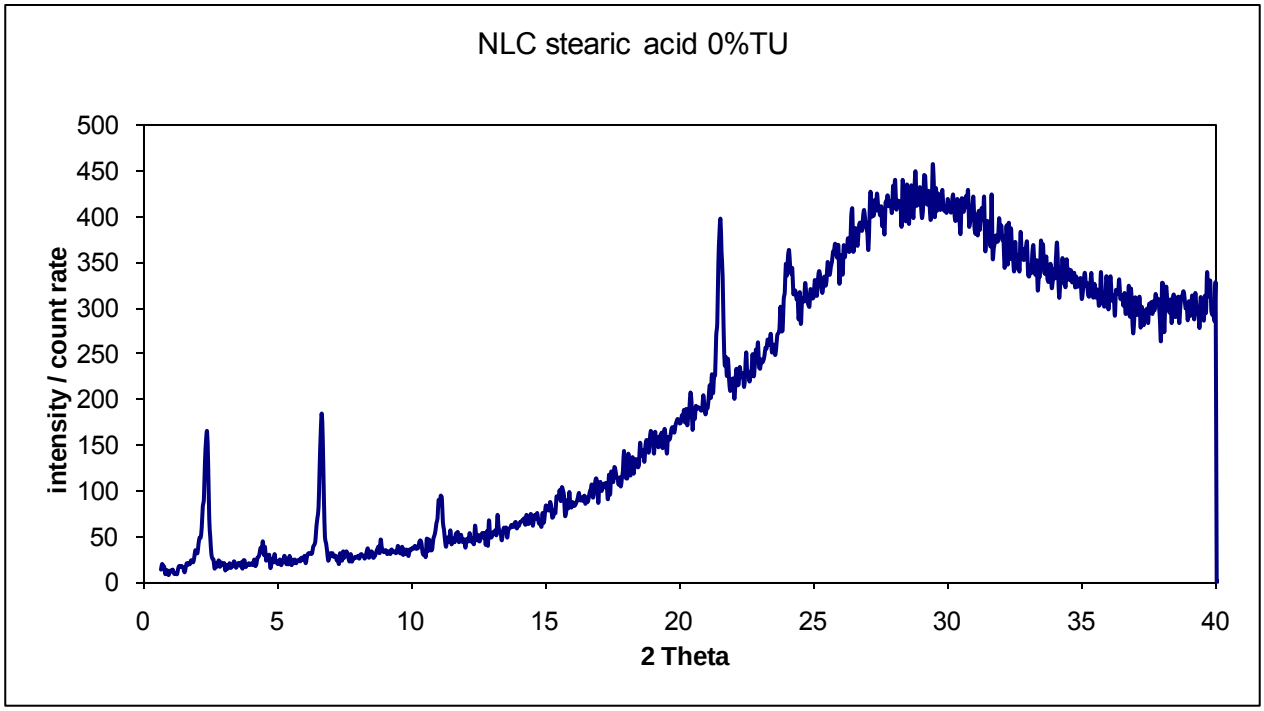
Bulk material: Stearic acid



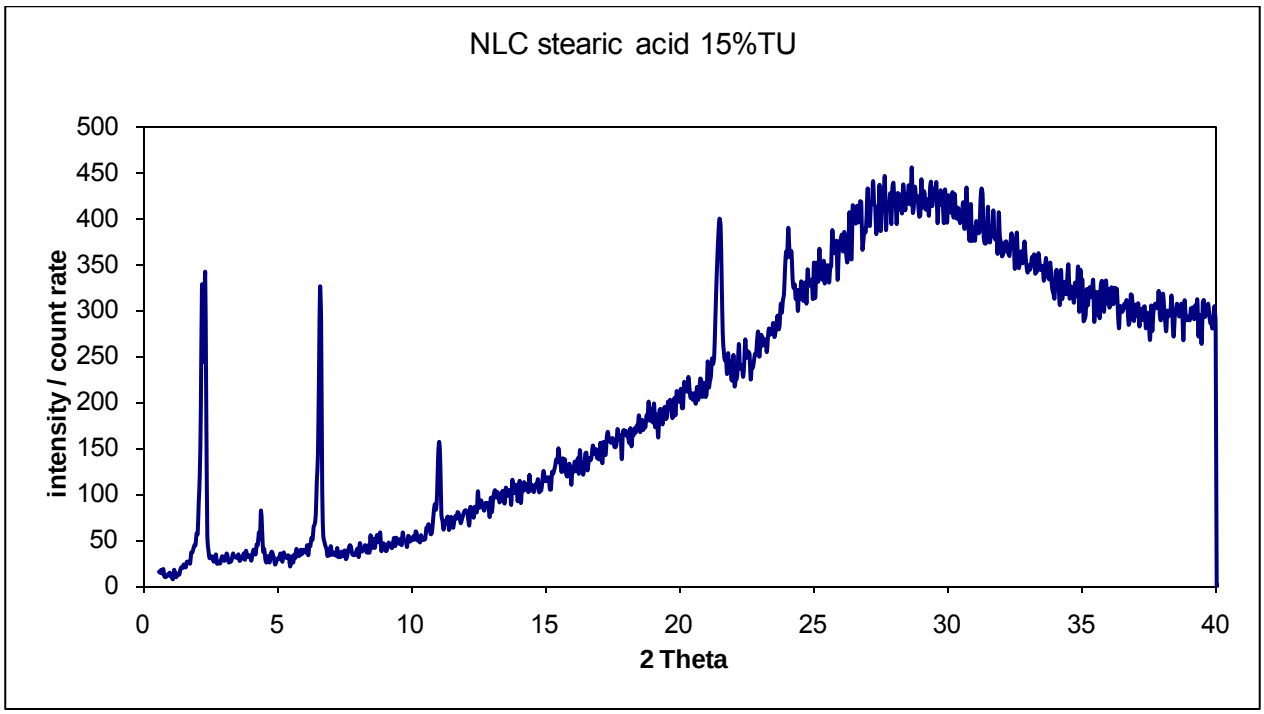
Bulk material: Stearic acid/oleic acid melt (50/50) (m/m)



bulk material: Stearic acid, oleic acid, TU (42,5/42,5/15) (m/m)



NLC 10 % lipid phase (0 % TU, 50 % stearic acid, 50 % oleic acid), 2 % Tween 80



NLC 10 % lipid phase (15 % TU, 42.5 % stearic acid, 42.5 % oleic acid), 2 % Tween 80

7 Published articles

7.1 Omega-3 fatty acids-loaded lipid nanoparticles for patient-convenient oral bioavailability enhancement

ORIGINAL ARTICLES

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Omega-3 fatty acids-loaded lipid nanoparticles for patient-convenient oral bioavailability enhancement

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Omega-3 fatty acids are commonly used as food supplements not only for their positive effects on the blood lipid profile but also for their cardioprotective properties. The majority of the commercially available products is made out of fish oil. Apart from the unpleasant side effects, up to 10 capsules per day have to be taken by the patients. This article describes the development and characterisation of an alternative lipid nanoparticle delivery system, which has the potential to reduce side effects and enhance bioavailability.

1. Introduction

The nutritional status of cancer patients is important; maintaining them in a good nutritional condition will support their battle against the disease (Chapkin et al. 2007; Dupertuis et al. 2007). Therefore various nutrition supplements for cancer patients are on the market, e.g. NT24 [Orcapharm, Pensberg/Germany]. The additional oral nutrition supply improves the health condition and prolongs the time until the necessity of parenteral nutrition.

Omega-3 fatty acids are important for the body because of various aspects:

- Synthesis by the body of omega-3 fatty acids eicosapentaenic acid (EPA) and docosahexanic acid (DHA) from the essential alpha-linoleic acid is very limited. Nevertheless, EPA and DHA are considered the most valuable omega-3 fatty acids: providing them with the nutrition is recommended (Harris, 2004; Hansen & Harris, 2007).
- Omega-3 fatty acids are known to be cardioprotective (Lemaitre et al. 2003; Hansen and Harris 2007) and have a positive effect both on the blood lipid profile (Anil 2007) and on inflammatory processes (Kelley et al. 1999; Belluzzi 2004).

There are a number of products on the market for additional supply with omega-3 fatty acids by oral administration. In general these products are soft gelatine capsules filled with fish oil. Most of the commercial products contain 500 mg of fish oil per capsule with an omega-3 fatty acid content of approximately 20–25% [Lipiscor®, Sanum-Kehlbeck, Hoya, Germany], [Ameu®, Lichtwer Pharma, Germany], [Eicosan®, Stada, Germany] (Lichtwer Pharma GmbH 2007). Disadvantage of this dosage form is that the patients actually need to swallow 3–6 capsules. Such a high number of capsules is necessary to meet the required dose considering the oral bioavailability of the omega-3 fatty acids. Cansell et al. have found a 72 ± 6% oral bioavailability in a rat model, which was improved to 98% using a very diluted liposome suspension (Cansell et al. 2003). Increasing the oral bioavailability without enlarging the volume of the dosage form decreases the total dose to be administered, which is patient-friendly.

In special conditions, e.g. for the treatment of high triglyceride blood levels, the dose is even higher; in this case 10–20 capsules per day (which is equivalent to 1200–2400 mg omega-3 fatty acids) may have to be administered. At a certain stage, especially cancer patients develop the problem of swallowing capsules and tablets. Therefore a liquid or semi-solid dosage form will be definitely more appropriate for such patients. In addition, such a dosage form would be very convenient for elderly patients.

The soft gelatine capsules disintegrate in the stomach releasing the fish oil. Reported undesired side effects are a fishy odour in the breath and undesired regurgitation leaving an unpleasant fishy taste in the mouth (Lichtwer Pharma GmbH, 2007). Enteric-coated fish oil capsules have also been developed for studies but are not commercially available (Belluzzi et al. 1996).

Based on these considerations, a novel semi-solid, paste-like formulation was developed. It consists of a highly concentrated lipid nanoparticle dispersion (70% w/w). This paste can be administered via a teaspoon and swallowed directly; alternatively the paste can be dispersed in soft drinks or water, avoiding swallowing problems with solid dosage forms. The omega-3 fatty acids were incorporated in particles with a solid matrix, nanostructured lipid carriers (NLC®) (Müller et al. 2000). The solid matrix has a taste masking effect minimizing odour and taste problems. This report describes the development and characterization of this semi-solid oral formulation.

2. Investigations, results and discussion

2.1. Rationale of development

The price of omega-3 fatty acids as a pure substance is fairly high, for example 340 Euro for 1 gram of purified docosahexaenoic acid. Therefore fish oils with a high percentage of unsaturated omega-3 fatty acids and plants oils (like flax seed oil) are used as nutrition supplements. The fish oil used for preparation of the nanostructured lipid carriers (NLC®) had a relatively high content of 38% omega-3 fatty acids (Schmitt). For chemical stabilisation, inclusion of chemically labile compounds in a solid matrix can be protective. Therefore creation of a solid particle matrix in form of NLC® was chosen to stabilise the fatty acids. In addition, a solid matrix can mask unpleasant taste and smell to a certain extent. Further reduction of the unpleasant smell was achieved by the solid-in-water dispersion system since the aqueous phase may additionally act as a barrier with the external environment. The fish oil-loaded NLC® are surrounded by a water phase, which has a very low solubility for the lipophilic compounds, *thus* further reduces evaporation. This is an old principal used in many pharmacopoeiae for taste masking, e.g. by producing oleum jecoris emulsions.

NLC® are prepared by mixing a solid lipid with a liquid lipid (oil). In this case the oil compound is the fish oil. Admixing of the oil reduces the melting point of the solid lipid. To minimize unpleasant side effects from the stomach, the NLC® should be solid at body temperature to slow down the degradation, that means degradation should mainly take place in the gut. Therefore a number of lipids was screened. The mixture should still melt above 40 °C but nevertheless contain the highest possible percentage of fish oil. This should minimise the total amount of NLC® to be administered in a single dose. It was found that a lipid blend containing 20% Dynasan 118 and 80% fish oil was still solid at 40 °C.

To promote absorption, the NLC® paste should be dispersible easily to yield a fine NLC® suspension taking into account that aggregation reduces the bioavailability. The gut contains many electrolytes able to destabilise dispersions due to zeta potential reduction. Therefore a combination of electrostatic and steric stabilisation was chosen to minimize aggregation. Sodium dodecyl sulfate (SDS) is well known as an efficient dispersing agent; in addition it creates highly negative zeta potentials after adsorption to the surface (Lucks et al. 1990). As steric stabilisers TPGS and PVP were also selected. Both SDS and TPGS are known absorption enhancers. TPGS was used primarily as steric stabilizer. It is also known as absorption enhancer mainly due to inhibition of P-glycoprotein (Rege et al. 2002). The SDS was used in the first formulation attempts because it is an efficient surfactant for dispersing oils. In addition it was a challenge to produce a 70% concentrated NLC® suspension, which is fine in size and not too viscous. Generally, the SDS concentration in pharmaceutical preparations is up to 2%, whereas 3% were used in this formulation approach. However, we have also shown that is was possible to replace SDS by Poloxamer 188, which can be used also at higher concentrations (data not shown).

The solid matrix of the NLC® is chemically protective but additionally Vitamin E can be added as anti-oxidant. It is also frequently used in commercial fish oil soft gelatine capsules (e.g. Ameu®).

2.2. Particle size and charge

PCS covers a size range of approximately 3 nm – 3 µm, thus it was employed to determine the size of the bulk population. The PCS diameter was 243 nm and the polydisperity index 0.064. Polydispersity indices around 0.1 indicate a relatively narrow particle size distribution. The NLC® with 243 nm represent a highly dispersed ultra fine system compared to orally administered oils. Once administered, an oil is emulsified by the surfactants present in the gut and dispersed by gut movement resulting in oil droplets typically in the size of 1–50 µm (Patton and Carey 1979; Armand et al. 1996). The degree of dispersity affects bioavailability as known from the first cyclosporin A product Sandimmu (Meinzer et al. 1998). With decreasing droplet/particle size, the absorption increases.

Laser diffractometry (LD) was performed to assess the potential presence of particles in the low micrometer range (measuring range of LD: 0.04–2000 µm). The LD yields a volume distribution, thus is very sensitive to detect even low amounts of particles larger than the bulk population. The 50% diameter was 0.313 µm, 90% diameter 0.950 µm and 99% diameter 1.240 µm. This indicates a product with particles almost completely in the nanometer range with a low amount of micrometer particles. Figure 1 shows the LD size distribution curve.

The zeta potential measurement is a tool to foresee the physical stability of colloidal suspensions. Stability is

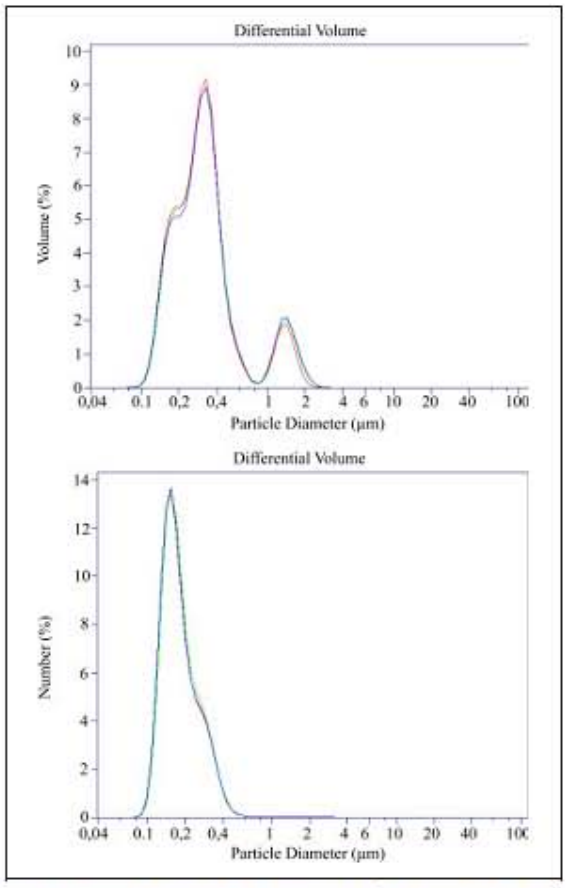

Fig. 1: LD size analysis of fish oil-loaded NLC® paste: Volume distribution curve (upper) and transfer to the number distribution curve (lower)

higher in case of high electrostatic repulsion. The measurements in ultrapure water (conductivity adjusted to 50 µs/cm) yielded a value of −20 mV. This is a characteristic value for combined electrostatic (SDS) and steric stabilisation (TPGS, PVP). In addition, zeta potential measurements were performed in the original dispersion medium (water with 3.0% SDS, 1.0% TPGS and 0.1% PVP). Measurement in the original dispersion medium provides information about the stability in the original dispersion. The high electrolyte concentration leads to compression of the diffuse double layer and subsequently to a reduction in the measured zeta potential. The zeta potential of −15 mV is relatively low but the rather high viscosity of the system additionally stabilises the dispersion. Furthermore, the high fraction of inner phase (70%) promotes the formation of a pearl-like particle network also stabilising the system. This is well described for highly concentrated NLC® dispersions. Considering the SDS adsorption onto the NLC® surface (electrostatic stabilisation), the additional steric barrier by TPGS and PVP in combination with the pearl network structure, the NLC® pastes are predicted to be physically stable.

2.3. Crystalline status

The NLC® paste was analysed by differential scanning calorimetry (DSC) and Fig. 2 shows the DSC graph. Compared to lipid Dynasan 118 bulk material, the peak onset decreased from 72.7 °C to 55.1 °C (Fig. 3).

Dynasan 118 as a triacylglycerol can crystallise in three different modifications: α, β′ and β (most thermodynamically stable). Running a DSC with two cycles reveals the three modifications. The heating curve of the first cycle shows the β modification with a peak maximum at 72.7 °C whereas cooling leads to cristallisation into the α modification (51.7 °C). Furthermore, the second cycle shows the β′ modification with its melting point of 62.9 °C.

The melting enthalpy of the lipid blend in NLC® (fish-oil + Dynasan 118) was 41.47 J/g, compared to 201.04 J/g of the bulk lipid. Taking into account that only 20% of the NLC® consists of Dynasan, the melting enthalpy increased to 207.35 J/g, which means that all the Dynasan in the particles is of crystalline status.

Cooling of the melted lipid dispersion lead to a delay in recrystallisation with a temperature shift from 61.1 °C to 41.4 °C (peak maximum). In addition, the melting enthalpy was distinctly reduced (6.26 J/g) indicating a delayed solidification process and increased α modification of the Dynasan.

The recorded x-ray diffraction spectrum of the bulk material Dynasan 118 (Fig. 4) shows the three expected peaks

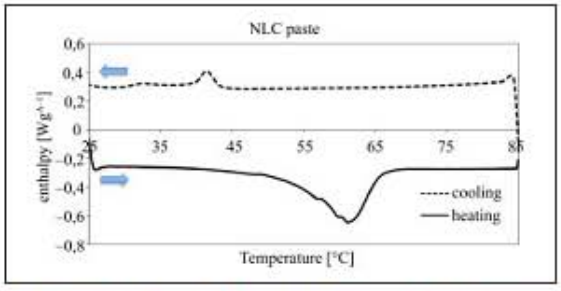

Fig. 2: DSC graph of the highly viscous 70% lipid nanoparticle dispersion (=NLC® paste), heating from 25 °C to 75 °C and subsequent cooling to 25 °C

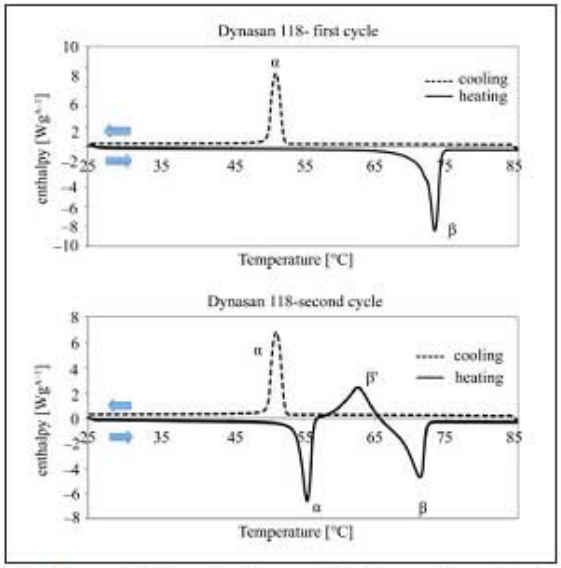

Fig. 3: Two-cycle DSC graph of Dynasan 118 bulk material revealing the three different crystalline modifications of the lipid

for triacylglycerols representing the three modifications of the lipid: 16.4 (2θ) for the α modification, 19.3 (2θ) for β and 23.1 (2θ) and 24.1 (2θ) for the β′ modification (Hagemann 1988).

The fish oil spectrum shows no significant peaks, which is also expected for a liquid.

The x-ray diffraction pattern of the paste exhibit mainly the peaks of the β′ and β modifications of Dynasan 118. The peaks indicate the solid, crystalline character of the particles (Fig. 5).

2.4. Re-dispersion properties

From the concept the fish oil NLC® paste can be dosed via a spoon and swallowed directly. For this, the paste

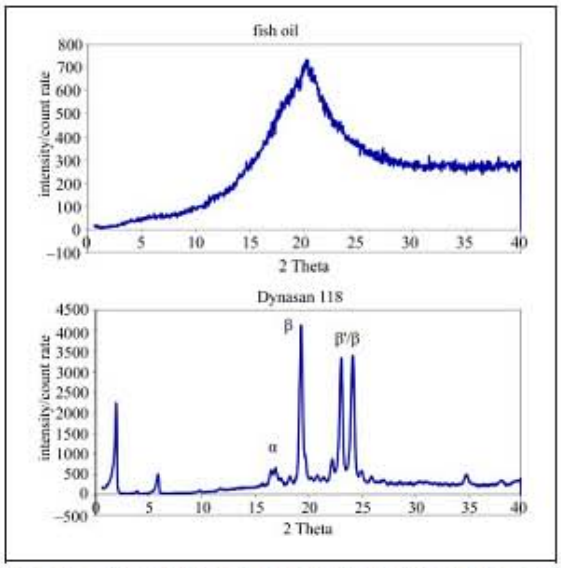

Fig. 4: X-ray diffraction pattern of the bulk materials fish oil and Dynasan 118 (explanations in the text)

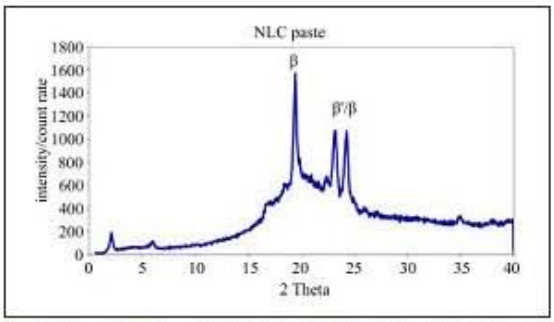

Fig. 5: X-ray diffraction pattern of the NLC® paste with the β' and β modification peaks of Dynasan 118

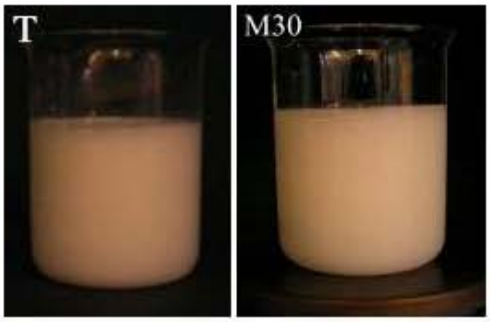

Fig. 6: Appearance after 10 seconds of stirring with a teaspoon (T) and after magnetic stirring at 50 rpm for 30 s (M30) following administration of 5 cm NLC® paste (squeezed out of a tube) in 200 ml water

requires appropriate flavouring. Alternatively the non-flavoured paste can be dispersed for example in soft drinks. In both cases the paste should re-disperse easily, either in the gut fluids or in water. A 500 mg capsule of fish oil (Ameu®) contains approximately 125 mg of omega-3 fatty acids. Considering the higher omega-3 fatty acid content in the fish oil used for preparation of the NLC® paste, despite addition of Dynasan 118 and water, 500 mg paste will contain approximately 110 mg omega-3 fatty acids. That means for a dose of approximately 500 mg omega-3 fatty acids a total of about 2.5g NLC® paste should be taken (equals 3–4 capsules of the commercial product).

The two grams of paste represent approximately one level teaspoon or alternatively an approximately 5 cm long strand squeezed out of a standard tube. To test the redispersibility, 5 cm paste were given into 200 ml of water (approximately equivalent to a typical volume of a soft drink). The paste was either dispersed by manual stirring with a spoon (Fig. 6 left) or alternatively with a slow moving magnetic stirrer to imitate gut forces (Fig. 6 right).

The formulation is well dispersible by spoon stirring and shows also good redispersibility when applying weak forces. To admix the NLC® paste to soft drinks instead of using a spoon, a tiny eggbeater can be used which eases even further dispersion.

2.5. *Absorption mechanism*

It is well documented in the literature that the presence of lipids can promote the absorption of drugs (Charman et al. 1997; Porter and Charman, 2001). To obtain maximum effect, the drug or active needs to be closely associated with the lipid, that means preferentially the active

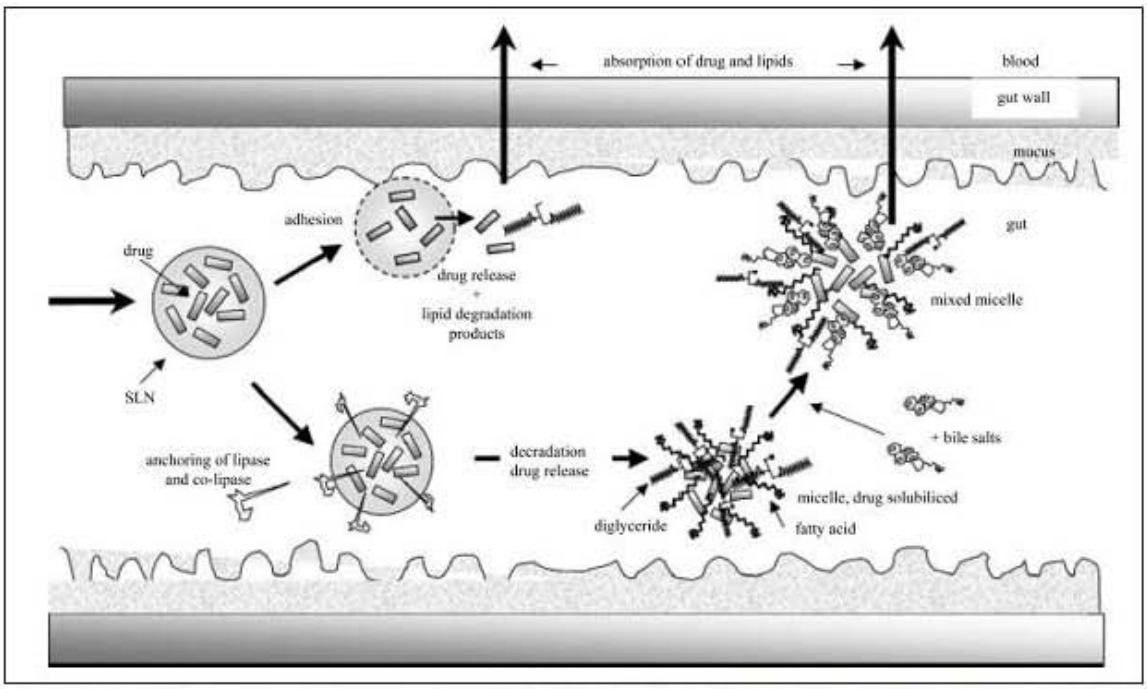

Fig. 7: Degradation of drug loaded lipid nanoparticles (SLN) in the gut and absorption mechanism by formation of micelles and subsequently mixed micelles with bile salts (reprinted from Journal of Biotechnology (113), Müller RH, Keck CM (2004), Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles, J Biotechnol 113, 151–170, Copyright with permission from Elsevier)

to be absorbed should be incorporated or dissolved in the lipid.

It was also found that the length of the fatty acid chains has an effect on the absorption. Fatty acids with a chain length of C14 to C18 promote absorption by lymphatic uptake (Porter and Charman, 2001). In addition, it is beneficial if the lipid is presented as finely dispersed as possible. The variations in bioavailability of the first cyclosporin A product, Sandimmune®, were explained by individual variation in surface active bile salts: indeed, less bile salts, less effective dispersion of the oil, less bioavailability and *vice versa*. Based on these considerations, the omega-3 fatty acids were incorporated into the solid matrix (close association). The triglyceride chosen (Dynasan 118 = glyceryl tristearate) contains C18 fatty acids and the size of the particles was as small as possible, that means in the nanometre range. Fulfilling all these three requirements should provide an optimal formulation, whereas of course the bioavailability enhancement has to be investigated in *in vivo* studies, which are currently in preparation.

It could be shown that there is no major difference in the mechanism of degradation between oil droplets and solid lipid particles. The solid lipid particles are degraded by lipases, the degradation velocity is a function of the chemical composition of the particle matrix and the stabilisers used (Müller and Olbrich 1999; Olbrich et al. 2002).

Figure 7 shows a model of the degradation and the subsequent absorption promoting mechanism. The lipid nanoparticles enter the gut, lipase adsorbs onto the particle surface whereas the adsorption is promoted by the presence of co-lipase. Enzymatic degradation of the lipid particles leads to the formation of surface-active di- and monoglycerides forming micelles. In case of drug-loaded lipid nanoparticles, these micelles contain solubilised drug. In case of the fish oil-loaded NLC® they consist of glycerides with various fatty acids including the omega-3 fatty acids. In the next step mixed micelles will be formed with bile salts leading finally to the lipid absorption.

2.6. Flavouring and colouring

Dilution of the fish oil in the solid particle matrix and surrounding the particles by a poorly diffusible medium (water) for the lipophilic fish oil components could not completely eliminate the unpleasant smell: A slight fishy smell remained which could irritate sensitive noses. Therefore various flavours were tested for masking: Orange, lemon, strawberry and mango. The flavours were applied in different concentrations. The most efficient one was orange flavour in a concentration of 1.25%. The flavour was incorporated by adding it to the molten lipid blend just prior to homogenisation. Evaporation of the volatile flavour does not seem to be a problem because the processing time is relatively short. In addition, at large scale production, homogenization will take place in closed containers avoiding any relevant flavour loss.

A colour appropriate to the flavour was chosen for colouring the product: Food colour yellow ZLT3 in a concentration of 0.24% and 0.013% food colour red ZLT2.

2.7. Conclusions

The fish oil-loaded NLC® paste possesses an ultra fine particle size, which is favourable for absorption in the gut. It is easily dispersible in fluid media. The formulation represents an alternative for persons, especially cancer patients, who have problems swallowing solid dosage forms. Encapsulation in the particle matrix and formulation as O/W system minimized undesired odour and taste.

3. Experimental

3.1. Materials

Dynasan 118 (glyceryl tristearate) used as matrix lipid for the production of NLC® was obtained from Condea (Witten, Germany). Sodium dodecyl sulfate (SDS) and polyvinyl pyrrolidon were purchased from Fluka Chemie GmbH (Buchs, Switzerland) and from Merck (Darmstadt, Germany), respectively. Tween 80 was bought from Uniqema (Eversberg, Belgium), whereas TPGS (D-alpha tocopheryl polyethylene glycol 1000 succinate) was a kind gift from Eastman (Anglesey, U.K.). All excipients were used as received. The water used was produced by a MilliQ system (Millipore, Billerica, MA, United States). The fish oil was obtained as a gift from Pharmacol GmbH (Berlin, Germany). It contained 38% omega-3 fatty acids including 19% EPA (eicosapentaenoic acid) and 13% DHA (docosahexaenoic acid) according to its analysis certificate.

For taste masking, aroma concentrates (lemon, orange, mango and strawberry flavour) from Symrise (Holzminden, Germany) were used which were a kind gift of the company. For colouring, Sicovit food colours (BASF AG, Ludwigshafen, Germany) were used.

3.2. Methods

The NLC® were prepared by high pressure homogenisation. The solid lipid Dynasan 118 was molten at 70 °C and the fish oil was then added. Both lipids where mixed at 70 °C at a ratio of 20% Dynasan 118 and 80% fish oil. Then a stabilizer solution containing 3.0% (w/w) SDS, 0.1% polyvinylpyrrolidon (PVP), 0.1% Tween 80 and 1% vitamin E TPGS at equivalent temperature was added under stirring (30% stabiliser solution, 70% oil phase). Alternatively a SDS-free stabiliser mixture containing Poloxamer 188 was used. Stirring (9000 rpm) was performed for 30 s using an Ultra-Turrax with a T-25 head (IKA Janke und Kunkel, Stauffen, Germany.) The obtained pre-emulsion was then homogenized using a Micron LAB 40 (APV Deutschland GmbH, Unna, Germany) at 500 bar and one homogenization cycle at 70 °C. The LAB 40 was equipped with a temperature control jacket; the temperature controlling fluid (water) was heated to 85 °C.

For determination of the bulk population, photon correlation spectroscopy (PCS) was applied using a Malvern Zetasizer 4 (Malvern Instruments, Malvern, UK). PCS yields a mean diameter (z-average) and a polydispersity index (PI) as a measure for the width of the size distribution. A PI of 0 is theoretically indicating a monodisperse population, 0.10–0.20 indicates a relatively narrow distribution and values > 0.50 indicate a very broad distribution. For the detection of microparticles, a Coulter LS 230 laser diffractometry (LD) was applied (Beckmann-Coulter, Krefeld, Germany). LD analysis was performed applying the Mie-theory, the real refractive index was 1.456 and the imaginary refractive index 0.001. The values were previously assessed to be valid for lipid nanoparticle dispersions (unpublished data). There are slight variations in the indices depending on the nature of matrix lipid and stabilizer used (Keck, 2006) but for the envisaged development of an oral formulation (not an intravenous formulation) these effects can be neglected.

Zeta potential was also determined using the Zetasizer 4. The measurements were performed in two different media: MilliQ water (with a conductivity adjusted to 50 µS/cm by adding sodium chloride solution) and the original dispersion medium. The pH was in the range of 5.8–6.3 during all measurements. Applied field strength was 20 V/cm. The obtained electrophoretic mobility was converted to the zeta potential by applying the Helmholtz-Smoluchowski equation.

Differential scanning calorimetry (DSC) was performed using a Mettler Toledo DSC 821e (Mettler Toledo, Gießen, Germany). The heating rate was 5 K/min. The peaks were analysed using the provided "Star" software by Mettler Toledo.

X-ray analysis was used to determine the crystalline status of the lipid nanoparticles, in addition to the DSC measurements. Diffraction patterns were measured using a Philips X-ray generator PW 1830 equipped with a copper cathode ($\lambda = 1.5418$ Å, 40 kV, 20 mA) coupled to a computer-interfaced Philips PW 1710 diffractometer control unit. The scattered radiation was measured with a vertical goniometer (Philips PW 1820) (Philips Industrial & Electro-Acoustic Systems Division, Almelo, The Netherlands). The system is a powder diffractometer – it can only analyse particles in suspensions if the viscosity of the dispersion medium is sufficiently enhanced. For analysis of highly fluid suspensions a viscosity enhancer is added (e.g. xanthan gum). In the case of the paste this was not necessary because it had already a sufficiently high viscosity.

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7.2 Lipid Nanoparticles with a Solid Matrix (SLN, NLC, LDC) for Oral Drug Delivery, Drug Development and Industrial Pharmacy

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Lipid Nanoparticles with a Solid Matrix (SLN®, NLC®, LDC®) for Oral Drug Delivery

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Solid lipid nanoparticles (SLN®), nanostructured lipid carriers (NLC®), and lipid–drug conjugates (LDC®), commonly produced by high-pressure homogenization, are interesting vectors for oral delivery of lipophilic and, to a certain extent, hydrophilic substances. Their production can be done without the use of organic solvents. Techniques to make them a physically stable delivery system have been developed. Scaling up of the production process from lab-size to large-scale dimensions using high-pressure homogenization can be easily achieved by using a different type of homogenizer. The machines used for large-scale production often yield an even better product quality than the lab-scale types. This review article covers the methods of production, characterization, mechanisms of oral bioavailability enhancement, scale-up, final oral dosage forms, and regulatory aspects of lipid nanoparticles for oral drug delivery. It focuses mainly on high-pressure homogenization production methods.

Keywords SLN; NLC; LDC; drug nanocrystals; nanosuspensions; oral drug delivery; high-pressure homogenization

INTRODUCTION

Lipid-based drug delivery systems gained increased attention during the last years (AAPS Lipid-Based Drug Delivery Systems Focus Group: www.aaps.org/inside/focus_groups/Lipid). Lipids are known to promote oral absorption of drugs (Charman et al., 1992; Charman, 2000; Charman, Porter, Mithani, & Dressman, 1997; Holm, Porter, Mullertz, Kristensen, & Charman, 2002; Porter & Charmann, 2001a; Stuchlik & Zak, 2001). Vitamins such as vitamin A and E are better absorbed in the presence of fats/lipids (Kuksis, 1987). There are quite a number of drugs for which an increased oral bioavailability is reported when they are administered in the presence of fat-rich food. Examples are testosterone and halofantrine (Charman & Porter, 1996; Holm et al., 2002; Khoo, Porter, & Charman, 2000; Khoo, Shackleford, Porter, Edwards, & Charman, 2003; Porter, Charman, Humberstone, & Charman, 1996; Porter & Charmann, 2001b; Shackleford et al., 2003). An excellent model drug to demonstrate this—which is at the same time of high commercial interest—is cyclosporin A. Delivery of cyclosporin A in the form of a microemulsion (Sandimmun® Optoral/Neoral), the second generation product by Novartis, distinctly reduced the variation in bioavailability which was a major problem of the classic Sandimmun® emulsion. However, an undesired plasma peak occurred, which is being held responsible for side effects such as nephrotoxicity (Martindale, 1989).

In a comparative in vivo study, cyclosporin A was administered as a drug nanocrystal suspension (so called "nanosuspension") and incorporated into solid lipid nanoparticles (SLN®). Nanocrystals consist of 100% drug without any matrix material. The increases in oral bioavailability reported for drug nanocrystals are really impressive for certain drugs. An example is the increase in absolute bioavailability for the drug danazol from 5.1 ± 1.9% as a normal suspension to 82.3 ± 10.1% as a drug nanosuspension (Liversidge & Cundy, 1995). In contrast to this literature data, the results of the cyclosporin A

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nanosuspension study were disappointing; the oral bioavailability was very low and far away from the required pharmacological levels (Penkler, Müller, Runge, & Ravelli, 1999, 2003; Runge, 1998; Müller et al., 2006; Müller, Runge, & Ravelli, 1998).

However, this first in vivo animal study of cyclosporin A-loaded SLN® confirmed the theory of an absorption-enhancing effect of lipids. Instead of using an oil as in Sandimmun® Optoral/Neoral, the oil was replaced by a solid lipid. The lipid particles were already administered in the nanosize range (in contrast to in situ generation of a nanosized microemulsion by breaking in the stomach as in the commercial formulation). The cyclosporin A-loaded SLN® showed a sufficiently high oral bioavailability; they remained within the therapeutic window equally long as the Sandimmun® Optoral/Neoral microemulsion formulation. However, they avoided the nephrotoxic peak above 1000 ng/mL due to controlled release from the solid lipid (Müller et al., 2006; Runge, 1998). This in vivo study demonstrates that lipids can enhance oral bioavailability and that drug nanocrystals without lipids being present can potentially be of limited effect.

SLN® were developed in the beginning of the 1990s. Their characteristic property is that they are made from a solid lipid only (Gasco, 1993; Müller & Lucks, 1996; zur Mühlen, Schwarz, Mehnert, & Müller, 1993). At the turn of the millennium, the second generation, nanostructured lipid carriers (NLC®), was developed (Müller, Mäder, Lippacher, & Jenning, 2000a). Identical to SLN®, the particle matrix is still solid at body temperature, but is produced from a blend of a solid lipid with a liquid lipid (= oil) leading to certain advantages compared to SLN®.

SLN® and NLC® can incorporate only lipophilic molecules efficiently. Loading with hydrophilic molecules can only be achieved to a very low extent by solubilization in the lipid melt. This limits the use of SLN® and NLC® to very potent/low-dose lipophilic drugs such as erythropoietin (EPO) or other potent peptides. Lysozyme was successfully incorporated as "model peptide" (Almeida, Runge, & Müller, 1997). From this point, there was a definite need to create a lipid nano-delivery system for highly dosed hydrophilic actives having a substantial loading capacity (e.g., up to 30%) and exploiting the oral absorption enhancing effect of solid lipids.

An approach for improved oral drug delivery of hydrophilic molecules is the formation of lipid–drug conjugates (LDC®). Many hydrophilic drugs show a poor oral bioavailability due to their explicit hydrophilic character. The lipophilicity of the molecules is not high enough to sufficiently pass the gastrointestinal wall. Transferring them to a LDC® and forming a nanoparticle has three major effects:

1. In case of chemically labile drugs, transfer to an insoluble molecule/particle reduces distinctly the gastrointestinal enzymatic/nonenzymatic degradation.
2. Due to the increase in lipophilicity, the drug shows an improved permeation through the gastrointestinal wall.
3. Promotion of oral absorption takes place by the matrix lipids present in the LDC® nanoparticles.

The presence of lipids in the absorption process (SLN®, NLC®, LDC®) or transfer of hydrophilic molecules to more lipophilic molecules by conjugation (LDC®) can generally increase the oral absorption. The lipid absorption enhancing effect can even be more pronounced when combining lipid technology with nanotechnology, which is creating lipidic solid nanoparticles. This article deals with the production, characterization, and performance of various types of lipid nanoparticles with a solid matrix. Which are as follows:

1. SLN®,
2. NLC®, and the
3. LDC® nanoparticles.

DEFINITIONS

There are basically three different types of lipid nanoparticles with a solid matrix: SLN®, NLC®, and LDC® nanoparticles.

SLN

SLN® are particles made from a lipid being solid at room temperature and also at body temperature. The starting material is solely a solid lipid, e.g., Dynasan 112 (zur Mühlen, Schwarz, & Mehnert, 1998) or other lipids such as Compritol 888 ATO (Souto, Mehnert, & Müller, 2006) or Imwitor 900 (Müller et al., 2006). There are two main production methods for SLN®: the high-pressure homogenization method (Mehnert & Mäder, 2001; Mehnert, zur Mühlen, Dingler, Weyhers, & Müller, 1997; Müller, Dingler, Weyhers, zur Mühlen, & Mehnert, 1997a; Müller & Lucks, 1996; Müller, Mäder, & Gohla, 2000b; Müller et al., 1995; Müller, Schwarz, zur Mühlen, & Mehnert, 1994; Müller, Weyhers, zur Mühlen, Dingler, & Mehnert, 1997b; zur Mühlen et al., 1998;) and the mircoemulsion method (Cavalli, Caputo, & Gasco, 1993; Gasco, 1993). The homogenization method is described in detail in Section 4 of this article. The microemulsion method after Gasco utilizes a basic mechanism of microemulsions: They transform into an ultrafine emulsion by breaking (i.e., after addition of, e.g., water). During production of the microemulsion the solid lipid is melted, the drug is dissolved in the melted solid lipid. Surfactant, cosurfactant and water are then added until a hot microemulsion is formed. This hot microemulsion is subsequently poured into cold water. The microemulsion breaks and ultrafine emulsion droplets develop, which immediately crystallize to form SLN® (Gasco, 1993). Disadvantages of the microemulsion method are the partially required relatively high concentration of surfactants, the organic solvents used to form the microemulsion, and the strong dilution of the particle suspension by pouring the microemulsion into water. This is not very production friendly concerning the subsequent processing to solid oral

dosage forms (e.g., tablets, pellets) as a lot of water needs to be removed to end up with a dry dosage form. However, both production processes—homogenization and microemulsion method—yield an identical product: solid lipid particles dispersed in an aqueous dispersion medium. As described in the patent (Müller & Lucks, 1996), SLN® prepared by high-pressure homogenization can also be produced in nonaqueous dispersion media as long as the dispersion medium does not dissolve the lipid, e.g., liquid polyethylene glycol (PEG) or oils (e.g., mineral oil).

SLN® are an interesting delivery system. However, there are some potential limitations: the relatively low loading capacity for a number of drugs and potential expulsion of the drug during storage. Especially SLN® prepared from one highly purified lipid can crystallize in a more or less perfect crystalline lattice. Such a perfect crystalline structure leaves little room for the incorporation of drugs. Usually, drugs are incorporated between the fatty acid chains, alternatively in between lipid layers or in amorphous clusters in crystal imperfections. The more perfect densely packed the crystal is, the fewer drug can be incorporated.

After production, SLN® crystallize in high-energetic lipid modifications such as α and β′. During storage, the lipid molecules undergo a time-dependent restructuring process leading to formation of the low-energetic modifications βi and β. Consequently, the more perfect lipid crystalline structure leads to expulsion of drug (Westesen, Bunjes, & Koch, 1997). This phenomenon has been well known for a long time from suppositories (Müller, 1986).

NLC

To overcome these limitations, NLC® have been developed (Müller et al., 2000a). Characteristic feature of the NLC® is a controlled nanostructuring of the lipid particle matrix, i.e., creation of a lipid particle matrix as imperfect as possible. To achieve this, spatially very different molecules are mixed. In general a solid lipid is mixed with a liquid lipid (oil). This blend is used to produce the lipid particles that are still solid at temperatures up to about 40°C.

It can be summarized that SLN® are particles produced from a solid lipid only and NLC® are particles produced from a blend of solid lipid with a liquid lipid (oil). This results in differences of the particle matrix structure. NLC® posses many imperfections increasing drug loading capacity and minimizing or avoiding drug expulsion during storage.

LDC

Based on their lipophilic character, SLN® and NLC® have only a limited loading capacity for hydrophilic drugs. This is not a problem in case of highly potent hydrophilic drugs such as EPO: they can be solubilized in the melted lipid matrix using surfactant mixtures or just simply by the mono- and diglycerides present in the lipid anyway (e.g., Imwitor 900). However, a higher loading capacity was not achievable with this kind of lipid nanoparticles. To solve this problem, LDC® particles were developed. The hydrophilic drug is transformed to a more lipophilic, insoluble molecule by conjugation with a lipidic compound. The conjugation can be performed by covalent linkage or simply by formation of a salt with a fatty acid (in case of drugs having for example protonizable functional groups).

The LDC® are poorly water soluble; they typically have a melting range of approximately 50–100°C and can be transformed to nanoparticles using a high-pressure homogenization method similar to the one described for SLN® and NLC®. Considering the molecular weight of the two fractions in the conjugate molecule, i.e., of the drug itself and the lipid part, a drug loading of approximately 30–50% is achievable (e.g., as reported for diminazene of about 33% formulated as diminazene diaceturate–acid conjugate with palmitic acid/stearic acid (Olbrich, Geßner, Kayser, & Müller, 2002a)). LDC® nanoparticles can be made from the conjugated drug only or solid lipids can be additionally admixed to form a mixed matrix of LDC® and lipid.

PRODUCTION ON LAB SCALE

The production method of first choice for the three types of lipid nanoparticles—SLN®, NLC®, and LDC®—is high-pressure homogenization. In case of SLN®, the drug is dissolved or dispersed in the melt of a solid lipid, typically approximately 5–10°C above the lipid's melting point. In case of NLC®, the drug is dissolved in a melted blend of a solid lipid with a liquid lipid (oil), again slightly above the melting point of the lipid blend. LDC® nanoparticles are commonly produced at room temperature. In the next production step, the drug-containing lipid melt or LDC® is dispersed in a surfactant/stabilizer solution of identical temperature by high-speed stirring. This yields an aqueous oil-in-water "pre-emulsion" (SLN® and NLC®) or suspension (LDC®). To achieve a high dispersity in the subsequent homogenization process, it is recommended to use dispersion-efficient, fast diffusing, electrostatically stabilizing, low molecular weight surfactants (e.g., sodium dodecylsulfate [SDS]). They diffuse very fast into newly formed interfacial layers and stabilize the formed small droplets efficiently minimizing subsequent coalescence phenomena. To achieve highest physical stability in gastrointestinal medium, the combination with a steric stabilizer is recommended (e.g., Tween 80 or Poloxamer 188). Steric stabilization is little or less impaired by the presence of electrolytes compared with electrostatic stabilization (high zeta potential). The pre-emulsion/suspension is passed through a temperature-controlled high-pressure homogenizer, either from the piston-gap type (APV Gaulin, APV Deutschland GmbH, Unna, Germany; Avestin Europe, Mannheim, Germany) or from the jet-stream type (microfluidization principle, Microfluidizer®, Microfluidics Inc., Newton, MA, USA). Homogenization

temperature for SLN® and NLC® is again typically about 5°C above the melting point of the lipid phase and room temperature for LDC®. In principle for SLN® and NLC® production, one homogenization cycle at 500 bar is sufficient to yield a hot oil/water emulsion with a particle size of approximately 250–300 nm. Cooling of the nanoemulsion during their production leads to crystallization of the lipid and formation of solid nanoparticles.

Typical homogenization parameters reported in the literature are 500 bar and up to three homogenization cycles. The two additional cycles lead to a further slight reduction in the particle size to approximately 220 nm and a narrowing of the width of the distribution (reduction in the polydispersity index of photon correlation spectroscopy, PCS). However, for oral drug delivery, such a small difference as 30 or 40 nm does not matter. The same is valid for the homogeneity in the particle size distribution. Hence, one cycle at 500 bar is considered as being sufficient for particles intended for oral administration.

LOADING CAPACITY AND DRUG INCORPORATION MECHANISMS

It has to be differentiated between *loading capacity* and *entrapment efficiency*. *Entrapment efficiency* is defined as the percentage of drug incorporated into the lipid nanoparticles relative to the total drug added. It specifies how many percent of drug are included in the particles and how many percent of free drug are still present in the dispersion medium.

Loading capacity refers to the percentage of drug incorporated into the lipid nanoparticles relative to the total weight of the lipidic phase (i.e., lipid + drug). To give an example, we assume a 10% lipid nanoparticle dispersion (i.e., 10% lipid, about 1% surfactant, and 89% water). In this case, the particle mass is equivalent to 10%, the particle mass is defined as the sum of lipid and drug (= 100%). If the drug had a *loading capacity* of 10% in the lipid phase, this would correspond to 1% in the total aqueous lipid nanoparticle dispersion.

Entrapment efficiencies in the literature are relatively high. They range from 80% for the drug tetracaine to about 99% for example for prednisolone (Schwarz, 1995). Of course, *entrapment efficiency* figures might look nice in case only very little drug is added. The more important point is the *loading capacity* of the lipid particles themselves. How many percent of drug can be incorporated into the total lipidic mass of the particles? The loading capacity depends on the solubility of the drug in the solid lipid (in case of SLN®) or the lipid blend (in case of NLC®). In case of LDC® nanoparticles, the *loading capacity* is determined from the ratio of the molecular fractions in the conjugate (e.g., drug and conjugated fatty acid). Examples of loading *capacities* reported for SLN® are 1% for prednisolone (Schwarz, 1995), 10% for tetracaine (Schwarz, 1995), and 20% for cyclosporin A by Runge (Runge, 1998), and even 25% for cyclosporin A by Radtke (Radtke, 2003). In case of very lipophilic compounds, a good solubility in the lipid loading capacities up to 50% can be achieved (e.g., vitamin E) (Dingler, Blum, Niehus, Müller, & Gohla, 1999).

What are the mechanisms of drug incorporation? There are different models described in the literature. The major work has been done by the research group of Mehnert and coworkers (Mehnert & Mäder, 2001; Mehnert et al., 1997; Müller et al., 1995, 1997a; Schwarz, 1995; zur Mühlen, 1996; zur Mühlen & Mehnert, 1998). Basically, there are three incorporation models (Figure 1):

1. Homogenous matrix of solid solution
2. Core-shell model with drug enriched in the shell (= drug-enriched shell)
3. Core-shell model with drug enriched in the core (= drug-enriched core)

In case of a homogenous matrix (model 1, Figure 1, left), the drug is molecularly dispersed evenly in the particle matrix. Drug release takes place by diffusion from the solid lipid matrix and additionally by lipid nanoparticle degradation in the gut.

In model 2 (Figure 1, middle), the drug is enriched in the shell. This can be explained by a lipid precipitation mechanism occurring during particle production. After homogenization, there is a mixture of drug and lipid in each droplet. It is then being cooled. Depending on the TX solubility diagram, the lipid can precipitate earlier than the drug to form a drug-free core or at least a core with reduced drug content (TX diagram: a two-dimensional graphical representation, with temperature and concentration coordinates, of the isobaric phase relationship in a binary system). Reaching the eutectic temperature and composition, lipid and drug precipitate simultaneously in the outer shell of the particles. Examples are coenzyme Q10 SLN. They possess a soft Q10-rich outer shell as proven by atomic force microscopy (AFM) (Dingler, Lukowski, Gohla, & Müller, 1997; Lukowski, Hoell, Dingler, Kranold, & Pflegel, 1998; Lukowski, Hoell, Kranold, Gehrke, & Pflegel, 1997). Drug enrichment in the shell is also a function of the solubility of the drug in the water-surfactant mixture at increased temperature during the production process. The drug partially leaves the lipid particle and dissolves in the aqueous phase during hot homogenization. Reason for this is the increased solubility for many drugs in the outer phase (surfactant solution) at elevated temperatures. Cooling of the oil/water

FIGURE 1. Incorporation models for the three types of SLN® (Modified after Müller et al., 2000b).

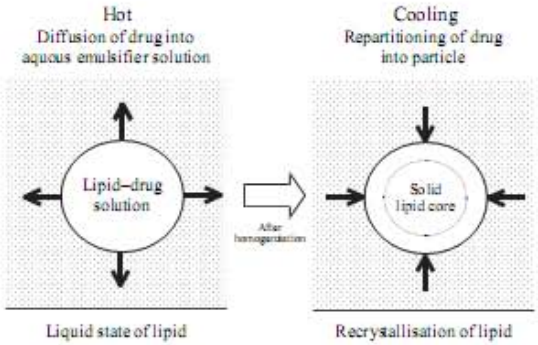

FIGURE 2. Forming of SLN® with drug-enriched shell: during production drug leaves the liquid particle and diffuses into the outer phase, having an increased solubility for the drug Because Of the high temperature. During cooling, drug repartitions into the particle shell. The core of the particle is less/not accessible because lipid crystallization has started. (Modified after Müller et al., 2000b).

nanoemulsion reduces the drug solubility in the aqueous phase, drug tries to repartition into the lipid particles leading to enrichment in the particle shell in case the particle core already started to solidify (Figure 2). Such particles are known to lead to a burst release (Müller et al., 1995). In the case of the oral cyclosporin A formulation, this was a desired effect. The dissolution rate needed to be sufficiently high to reach the therapeutic drug level but not too high to avoid nephrotoxic levels. In case of other applications, it might be necessary to have a prolonged release formulation which can be achieved by varying the production conditions during the particle production (zur Mühlen & Mehnert, 1998). Such conditions are low production temperature (preferably "cold homogenization") and low surfactant concentration.

Enrichment in the shell (model 2) takes place when particles are produced by hot homogenization method and the drug used shows a distinctly increased solubility in the aqueous surfactant phase at production temperature. By contrast, the drug-enriched core (model 3, Figure 1, right) is formed in case cooling of the hot oil/water emulsion leads to precipitation of the drug first. This takes place preferentially in lipid solutions with drug dissolved at its saturation solubility in the lipid at production temperature. During cooling, a super saturation and subsequent drug precipitation are achieved. The prolonged release of prednisolone is explained by this model (zur Mühlen, 1996; zur Mühlen & Mehnert, 1998).

ORAL DELIVERY OF DRUGS USING LIPID NANOPARTICLES

The first drug intensively investigated to be formulated as SLN® dispersion was cyclosporin A (Müller et al., 1998, 2006; Müller, Runge, Ravelli, Thünemann, Mehnert, & Souto, 2007b; Penkler et al., 1999, 2003; Runge, 1998). As mentioned above, it was known from the literature that the "old" Sandimmun® emulsion showed variations in the oral bioavailability ranging from 10 to 60%. The second generation product, the microemulsion Sandimmun® Optoral/Neoral, avoids this strong variation in bioavailability but possesses potential nephrotoxicity as an undesired side effect due to plasma peaks well above 1000 ng/mL (Meinzer, Müller, & Vonderscher, 1998). The aim of formulating a cyclosporin A-loaded SLN® formulation was to avoid the undesired plasma peak and to achieve a similarly high reproducible oral bioavailability in the therapeutic window. Hence, combination of the advantages of the "old" Sandimmun® (no nephrotoxic plasma peak) and the "new" Sandimmun® Optoral/Neoral (little variation in bioavailability) should be achieved by the SLN® formulation. Three formulations were used in the study: cyclosporin A-loaded SLN® suspension, cyclosporin A drug nanocrystals, and Sandimmun® Optoral/Neoral microemulsion as a reference. The SLN® blood profile did not exhibit the undesired plasma peak and remained over a similar time period time in the therapeutic window as the microemulsion (Figure 3).

At the first glance, it was surprising that the cyclosporin A nanosuspension had such a low bioavailability. This is in contrast to increases in bioavailability reported for a number of drugs by the scientists of the company Nanosystems (nowadays Élan): as discussed above, the bioavailability of danazol could be increased from 5 to 82% by transferring the drug to a nanosuspension (Liversidge & Cundy, 1995). The reasons for the low bioavailability of cyclosporin A nanocrystals might be multifactorial. Cyclosporin A is a substrate of *p*-glycoprotein; thus the absorption is limited a priori. In addition, it cannot be excluded that the drug nanocrystals aggregated in the gastrointestinal fluid. It is known that nanocrystal aggregation can

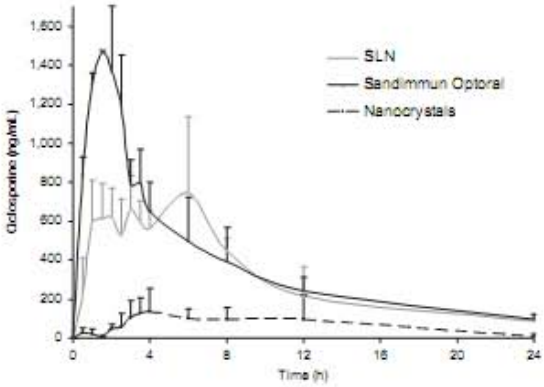

FIGURE 3. Blood profiles of cyclosporin-loaded SLN® suspension, drug nanocrystals, and sandimmun® microemulsion lipids. (Reprinted from Müller, R. H., Keck, C. M. (2004). Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles. *J. Biotech., 113*, 151–170, Copyright (2004), with Permission from Elsevier).

reduce dissolution rate (Keck, Fichtinger, Viernstein, & Müller, 2004; Keck, 2006) and subsequently oral bioavailability. Furthermore, it might be that the dissolution rate of cyclosporin A from the crystals was not as high as from the lipid nanoparticles. The cyclosporin A was located—at least to a certain extent—in the outer shell of the SLN® as indicated by the fast release observed in in vitro release studies (Runge, 1998).

In sum, drug nanocrystals, often seen as the ultimate, universal formulation approach for poorly soluble drugs, do not work in any case. For a number of drugs, it seems to be beneficial to have lipids present during the absorption process. This is well in agreement with publications describing the positive effect of lipids on oral drug absorption (Charman, 2000; Charman et al., 1997; Holm et al., 2002; Porter et al., 1996; Porter & Charmann, 2001a). Therefore, the first choice in screening might be drug nanocrystals, but lipid nanoparticles are the essential back up formulation.

MECHANISM OF ORAL ABSORPTION ENHANCEMENT

There are different mechanisms discussed that lead to an absorption enhancement and also to a reproducible bioavailability (little variation in bioavailability). These mechanisms are

1. a general adhesiveness of nanoparticles;
2. reproducibility of adhesion; and
3. an absorption enhancing effect of lipids.

It is a general property of nanoparticles that they are adhesive (Tarr & Yalkowsky, 1989). The adhesiveness of particles to a surface increases with the surface area of the particles; the interaction forces can be calculated (Stieß, 1995). This is a general behavior of all nanoparticles, not specific for lipid nanoparticles. After adhesion to the gut wall, the drug is exactly released at its place of absorption (Liversidge & Cundy, 1995).

This adhesion process proved to be very reproducible. In vivo data obtained with drug nanocrystals show that there is little variability between the fasted and fed state of rats (Liversidge & Conzentino, 1995). The same is considered as valid for the lipid nanoparticles. The low variation in bioavailability observed with cyclosporin A-loaded SLN® suspension was similar to the Sandimmun® Optoral/Neoral microemulsion (Runge, 1998).

It is known that lipids can promote the absorption of active compounds; examples are lipid soluble vitamins such as vitamin A, D, E, and K (Kuksis, 1987). The body absorbs the fat and simultaneously the drug is taken up; it can be considered as a kind of "Trojan Horse" effect.

The lipid absorption enhancing effect can be explained more specifically by the studies performed by W. Charman and coworkers (Charman, 2000; Charman et al., 1992, 1997; Porter & Charmann, 2001a). In sum, the lipids are degraded by enzymes in the gut leading to the formation of surface active mono- and diglycerides on the surface of the lipid droplets or solid lipid particles. These molecules detach and form micelles. During the detachment and micelle forming process, the drug dissolved in the lipid is taken up in the micelle (solubilized). Solubilization is a well-known principle for solubility enhancement in pharmaceutical technology. The formed micelles interact with surface-active bile salts (e.g., sodium cholate) leading to the formation of so-called "mixed micelles." In the subsequent absorption process of the lipid degradation product, the drug is simultaneously absorbed (Figure 4). Charman and co-workers could show that the absorption enhancing effect differs from one lipid to the other. For example, it was observed that long-chain triglycerides (LCTs) are more effective in promoting absorption of the drug halofantrine compared with medium-chain triglycerides (MCTs) (Khoo et al., 2003).

Additionally, the length of the fatty acid chains affects the primary place of absorption. Fatty acids with C-14 chains to C-18 chains promote lymphatic absorption (Porter & Charmann, 2001a). This is of interest for drugs such as testosterone, which undergo a strong first-pass metabolism when they are absorbed through the gut wall and passing the liver.

Lymphatic absorption avoids this first-pass metabolism to a certain degree and can be used to increase the oral absorption of testosterone or testosterone undecanoate. A commercial formulation exploiting this concept is Andriol® capsules with testosterone undecanoate dissolved in oleic acid (Shackleford et al., 2003). Oleic acid as a C-18 fatty acid is ideally suited as oil compound in capsules. The bioavailability of testosterone undecanoate is reported to be $3.25 \pm 0.48\%$ in dogs (Shackleford et al., 2003). As a kind of "second generation formulation" with improved chemical stability, Andriol Testocaps® were launched by the company Organon. The capsules can be stored at room temperature, whereas the "first generation" needs to be stored below 8°C. Andriol Testocaps® contain a blend of oleic acid and castor oil (which major component is the C-18 fatty acid ricinoleic acid) as oil component and additionally a surfactant lauroglycol/FCC (=propylene glycol monolaurate) (Shackleford et al., 2003). The bioavailability reported is about $2.88 \pm 0.88\%$ (Shackleford et al., 2003). It can be imagined that the surfactant being present in this formulation will contribute or promote the formation of mixed micelles. Adding the surfactant moves this formulation to the direction of the self-emulsifying drug delivery systems (SEDDS) (Charman et al., 1992; Pouton, 2000).

Another important point is that for reaching the maximum absorption enhancing effect, the drug needs to be closely associated with the lipid. It is less efficient when drug and lipid are given separately (as it happens during normal food intake and simultaneous drug administration). If a separate administration of lipid and drug worked with similar efficiency, the easiest way to exploit the absorption enhancing effect would be simultaneous administration of a lipid-filled capsule with drug intake (Olbrich, Mehnert, & Müller, 1998). This required very close association of lipid and drug has been realized with the three

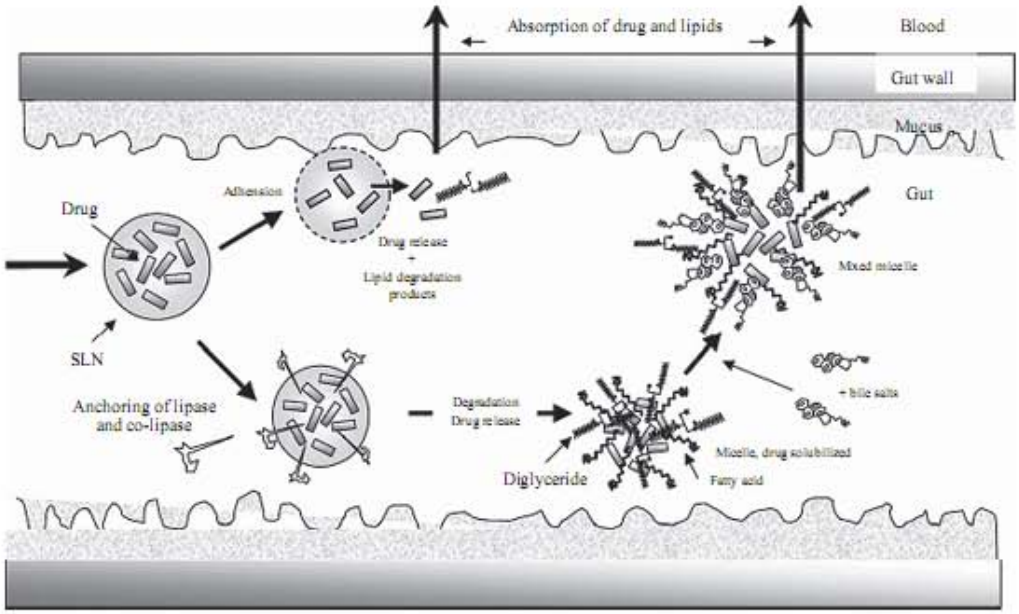

FIGURE 4. Mechanisms of absorption promoting effect of lipids being formulated as a lipid nanoparticle (reprinted from Müller and Keck (2004), Copyright (2004), with Permission from Elsevier).

types of lipid nanoparticles. The drug can be incorporated in between the fatty acid chains or the lipid lamella (Runge, 1998) (Figure 5) or alternatively be present as amorphous clusters in imperfections of the lipid crystal lattice. The surface active compounds in the gut disperse lipids from food to relatively fine droplets. Their diameter is typically in the range of 1–50 µm (Armand et al., 1996; Patton & Carey, 1979). Lipid nanoparticles represent an ultrafine dispersion with typical diameters of about 250 nm (= 0.25µm). Solubilization can take place very fast and efficiently due to the large surface area of the particles. In case a slower degradation is required (e.g., for prolonged release), a fraction of the lipid nanoparticles can be stabilized with a high molecular weight steric stabilizer (e.g., Poloxamer). It could be shown that to some extent the nature of the lipid matrix but dominantly the type of stabilizer affects the enzymatic degradation velocity (Olbrich, 2002; Olbrich, Mehnert, & Müller, 1998; Olbrich, Kayser, & Müller, 2002b).

Stabilization of the lipid particles with a mixture of sodium cholate and lecithin promotes the anchoring of the lipase/co-lipase complex and thus accelerates the degradation (Olbrich et al., 2002b; Olbrich & Müller, 1999). The use of high molecular weight stabilizers such as Poloxamer 407 leads to a steric hindrance of the anchoring of the complex, thus delaying the lipid degradation. It is also possible to adjust the degradation time between "fast" and "slow" by using mixtures of degradation accelerating and degradation delaying stabilizers (Olbrich & Müller, 1999).

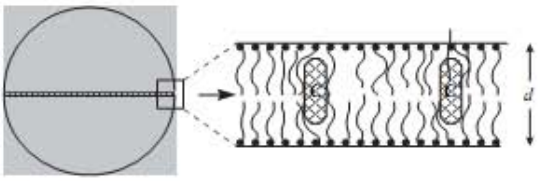

FIGURE 5. Proposed incorporation of cyclosporin A (c) in the lipid matrix of inwitor 900. (Reprinted from Müller et al., 2008, Copyright (2007), with permission from Elsevier).

LARGE-SCALE PRODUCTION

In most cases, scaling up of a process encounters problems. This is different for the production of lipid nanoparticles based on high-pressure homogenization. On the contrary, using larger scale machines leads to an even better quality of the product with regard to a smaller mean particle size and its homogeneity (width of the size distribution). Basic advantage is that high-pressure homogenization is a production technique widely used, e.g., in pharmaceutical industry for the production of emulsions for parenteral nutrition (Klang, Parnas, & Benita, 1998). High-pressure homogenization is also used in nonpharmaceutical areas, e.g., in food production, where homogenizers

are running with a capacity of up to several tons of homogenization volume per hour (e.g., for homogenized milk).

Typical machines for lab-scale production are the Micron LAB 40 (batch size: 20–40 mL, APV Deutschland GmbH) and the Avestin C5 (batch size: 7 mL to 1 L, capacity: 5 L/h, Avestin Europe). In case of very expensive drugs or if there is a limited supply (e.g., new chemical entities), it is favorable to reduce the batch size (scaling down capability). Batch size reduction can be done by using the Avestin B3 (Avestin Europe). The batch volume is 0.5–3.2 mL.

A first scaling up step to 500 mL to 1 L can be realized by using the continuous version of the Micron LAB 40. It uses the same homogenization tower as the discontinuous version with only slight modifications. Essential parts such as cylinder, piston, and geometry of the homogenization valve are identical. The continuous version is equipped with product containers. The product is pumped through the valve by a number of piston movements (e.g., for 400 mL homogenization volume, 10 piston movements with 40 mL each are required). In case of the continuous LAB 40 version, the homogenization tower and the two product containers are equipped with temperature control jackets. In theory, one could enlarge the product containers (e.g., to 2 kg); however, the machine is originally not designed to run larger volumes in continuous mode.

The next scaling up step with a minimum batch size of 2 kg and a maximum of 10 kg was achieved using the LAB 60 (APV Deutschland GmbH). The LAB 60 has a homogenization capacity of 60 L/h.

The two product containers of the LAB 60 are equipped with powerful high-speed stirring units. The feeding container has a dissolver disc built in to prepare the pre-emulsion in it. For the production of a 2-kg batch, the pre-emulsion is passed through the homogenization unit, and then it circulates back to the feeding container. It is a continuous homogenization process in a loop. It can be calculated that it takes 15 min to ensure that—statistically—99.9% of the droplets have passed the homogenization valve at least once (Leviton & Pallansch, 1959; Müller, Wissing, & Radomska, 2001). For such a relatively small production volume of 2 kg, it is not sensible to run the homogenization with the LAB 60 in a discontinuous process. The void volume is relatively large (~250 mL). Running discontinuous cycles would leave about 10–15% of the batch each time in the void volume being nonhomogenized. Running a 10-kg batch in continuous circulating required more than 90 min to ensure that 99.9% of the droplets have passed the homogenization gap at least once (Leviton & Pallansch, 1959). Therefore, a discontinuous production is recommended. The 10 kg pre-emulsion is prepared in the feeding container, passed through the homogenizing unit, and then collected in the second product container. When the pre-emulsion has passed the homogenizer, it will be fed back from the second container by gravity via a temperature-controlled tube to the first container. Then the second homogenization cycle starts. In general, two homogenization cycles at 500 bar are fully sufficient to yield a relatively monodisperse product; the total homogenization time for 10 kg is only 20 min (10 min per cycle). As already mentioned, for orally administered lipid nanoparticles, even one single homogenization cycle is considered to be sufficient. In that case, it does not make any difference if the particle size is 260 or 230 nm.

The LAB 60 unit was designed for a pharmaceutical company; that is why it can be used in a GMP area for clinical batch production. As pointed out above, the homogenization results obtained with large-scale machines is typically better than with the lab-scale LAB 40. There are several reasons for this: First of all, transfer of the process from a smaller capacity machine to a larger capacity machine is relatively easy because of the identical or similar geometry of the homogenization valves. Secondly, the larger machines have two or three plungers leading to less fluctuation in the actual homogenization pressure. The LAB 40 has only one plunger; therefore, the pressure needs to build up; the first small fraction of the pre-emulsion is less efficiently homogenized than the rest. This effect is distinctly reduced when a homogenizer is running with three plungers smoothing the pressure fluctuation profile. Furthermore, the temperature control of the larger machines is much more effective. Machines can be ordered equipped with a temperature control unit for the homogenization unit itself. Also, all required tubes and containers can be ordered double-walled for temperature control with a temperature controlling liquid. In addition, these homogenizers are equipped with two homogenization valves in series, main (first) valve and a second valve. When the homogenized droplets leave the first homogenization valve, they possess a relatively high kinetic energy. Their surface might not yet be fully covered with stabilizer. Therefore, limited coalescence can occur. A part of the coalesced or flocculated droplets is immediately re-dispersed when they pass the subsequent homogenization valve. Typically, the second valve operates at one tenth of the pressure of the first valve, e.g., 500 bar for the first valve and 50 bar for the second valve.

The next step in scale-up was the use of a Gaulin 5.5 (APV Deutschland GmbH) with a homogenization capacity of 150 L/h (~150 kg). The pre-emulsion is prepared in larger product containers. Product containers and homogenizer are made from material of pharmaceutical grade. The product containers can be sterilized by autoclaving; preparation of the pre-emulsion under protective gas is possible. They also have the features of cleaning-in-place (CIP) and sterilization-in-place (SIP). For the production, the lipid can be melted in the feeding container. Surfactant and sterile water from a sterile water supply system are then added. Advantageous is a hot storage supply system providing sterile water of 80°C, which is ideal for the production process. This way of production leads also to a very low microbiological load. For oral administration, one homogenization cycle with the Gaulin 5.5 is sufficient. The product is collected in the second product container and cooled in a controlled way under stirring. A batch size of about half a ton

can be produced in approximately 3-h homogenization time with the Gaulin 5.5. For many products, this is already a typical batch size. This way of production was used by the company "Chemisches Laboratorium Dr. Richter GmbH (CLR)" in Berlin to produce the first large-scale batch of NLC® for cosmetic industry to realize the first cosmetic NLC® products (Müller, Rimpler, Petersen, Hommoss and Schwabe, 2007a).

For even larger scales, an Avestin EmulsiFlex C1000 (Avestin Europe) or a Rannie 118 (APV Deutschland GmbH) can be employed. Their capacity is 1, 000/2, 000 L/h at the low pressure required for lipid nanoparticle production.

In this case, it is not sensible any more to prepare a 2-ton batch in a discontinuous way, i.e., preparing the pre-emulsion in a container. The heat exchange times are too long giving a too high temperature burden on the active (cosmetic active or drug). For production lines of this size, static blenders are recommended. The drug containing melted lipid is admixed in a static blender to the hot surfactant/stabilizer solution (e.g., blenders from Sulzer Chemtech, Winterthur, Switzerland). The homogenized product needs to be cooled; the temperature-controlled container will then be replaced by a heat exchanger to remove at least most of the heat. Figure 6 shows a design for such an arrangement.

To sum up, the larger machines are more effective in dispersing and more sophisticated regarding their features to control the process. As a result, the product quality is in general better when moving to larger scale machines.

PRODUCTION OF FINAL ORAL DOSAGE FORMS

In principle, the aqueous lipid nanoparticle suspensions can be used as an oral dosage form. Using optimal stabilizer formulations, a physical stability of up to 3 years has been reported (Müller et al., 1995). Liquid dosage forms might be convenient for certain groups of patients (e.g., children and elderly patients). However, the dosage forms of first choice for delivery are dry forms: tablets, capsules, or fast-dissolving drug delivery systems (FDDS) for the oral cavity. In some countries, sachets are also accepted. Pellets with incorporated SLN® have been produced by Pinto and Müller (1996). The aqueous SLN® dispersion was used as wetting agent in the production process of the pellets. It could be shown that the pellets released the SLN® completely and without or very little aggregation. Such pellets can be filled into hard gelatine capsules or can be compressed to tablets (similar to pellet-containing tablets such as Beloc ZOK®).

Alternatively, the aqueous SLN® dispersion can be used as granulation fluid in the production process of tablets or the SLN® can be spray-dried (Freitas, 1998; Freitas & Müller, 1998) and the obtained powder added to the tabletting mixture (Direct-Compress® technology) (Müller, 1997). As mentioned above, when producing oral dosage forms, the loading capacity of each single dose needs to be considered. In case too much lipid mass is required to dissolve the required drug dose, the excipients necessary to produce pellets or tablets might lead to an unacceptable large volume. In such cases, it might be considered to produce a spray-dried powder for oral administration.

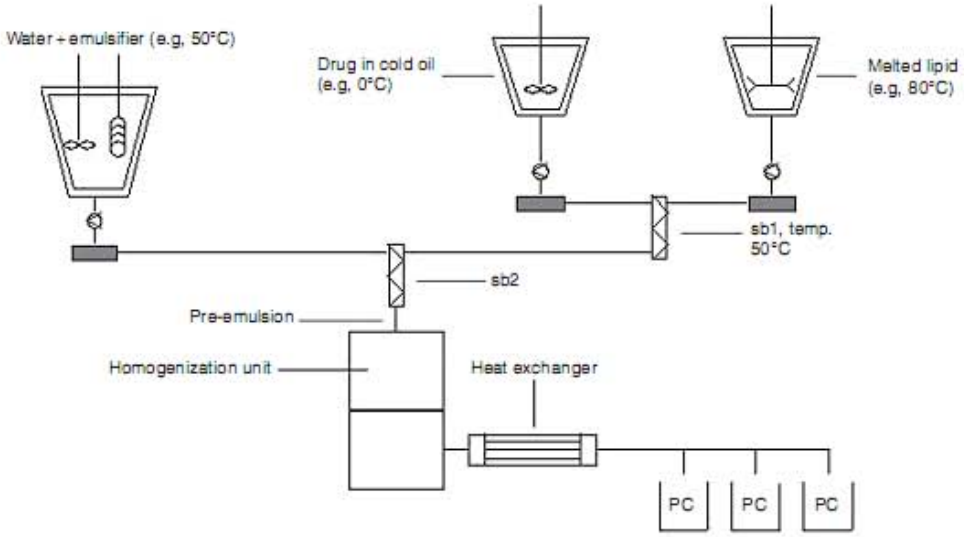

FIGURE 6. Production flow chart for a production capacity of 1–2 tons per hour. First cold drug solution (drug dissolved in oil) and "overheated" melted lipid (e.g., 80°C) are mixed in the first static blender (sb1). Mixing leads to a temperature of the melt of, for example, 50°C, which is still above the melting point of the solid–liquid lipid blend. Then the hot surfactant solution and melted drug-containing lipid are mixed in static blender 2 (sb2) the pre-emulsion passes the homogenization unit, the nanoemulsion is cooled by passing a heat exchanger, and final cooling is performed in the product container (PC).

Another interesting approach is the production of FDDS. The adhesive properties of lipid nanoparticles could be exploited for delivery to the mouth cavity. Incorporation into such an FDDS would be relatively easy by simply lyophilizing the lipid nanoparticle suspension under addition of cryoprotective agents such as mannitol or trehalose.

REGULATORY ASPECTS

One of the key pre-requisites for introducing a new technology and new products to the market is meeting regulatory requirements, not only with regard to excipients but also qualification and validation of production lines. In general, for the production of lipid nanoparticles, only excipients accepted by the regulatory authorities are used, which means excipients with a GRAS status (FDA Summary of GRAS Notices: http://vm. cfsan.fda.gov/~rdb/opagras.html [retrieved 10/07]) or excipients which are already used in products on the pharmaceutical market. In the latter case, these excipients need to be used in their commonly applied, regulatorily accepted concentrations. If distinctly higher concentrations are used, a limited toxicity study might be necessary to prove the safety of the excipient in this concentration. All lipids, surfactants, and stabilizers used in the production of capsules, pellets, and tablets can be fully exploited for the production of oral lipid suspensions. There is definitely no lack of accepted excipients. In addition, all lipids, surfactants, and polymeric stabilizers used in food industry can be employed. However, they need a registration for pharmaceutical purposes. One can refer to the toxicity data collected for registration as food or food additive. To sum up, a rich variety of excipients is available for oral lipid nanoparticles.

Another important point is the qualification of production lines within the general quality management (QM) (Müller, Dingler, Schneppe, & Gohla, 2000). The production lines need to be made out of materials and compounds allowing a qualification of the production line. In addition, other QM steps such as validation need to be performed. Also each compound of the line needs to be capable of validation. Unfortunately, many experimental production lines developed in academic research labs cannot meet these criteria. The production lines for lipid nanoparticles can be validated; for example, a clinical batch production unit is available (Müller, Dingler, Schneppe, & Gohla, 2000). A very important point is that the lipid nanoparticle technology uses production lines already established and existing in the pharmaceutical industry. The regulatory accepted production lines for emulsions can be used for lipid nanoparticle production because many are temperature-controlled anyway. Therefore, it is possible to use the lines of parenteral emulsions for the production of lipid particle suspensions. The only "disadvantage" is that a certain minimum batch size will be required, which is approximately half a ton.

CONCLUSION AND PERSPECTIVES

The three different types of lipid nanoparticles—SLN®, NLC®, and LDC®—represent a promising tool box for the oral delivery of lipophilic but also hydrophilic drugs, especially for hydrophilic drugs showing a reduced stability in the gut and a limited bioavailability. Lipid nanoparticles exploit the absorption enhancing properties of lipids, which are now commonly used for new improved delivery systems and oral dosage forms. Lipid nanoparticles fulfill essential prerequisites for entering the market with a new formulation. Such prerequisites are low cost production, clinical and large-scale production facilities, and accepted status of excipients. In contrast to liposomes, they are not only a low cost system but also physically more stable. Interesting for commercial exploitation is the exclusivity of the various lipid particle technologies; they are protected by issued patens or by patent applications in the major countries. By acquisition of the SLN® technology by SkyePharma PLC in 1999 the technology of SLN® has meanwhile entered pharmaceutical industry. The second generation technology of NLC® is meanwhile present on the cosmetic market in more than 20 products world wide (Müller et al., 2007). The cosmetic products proved the feasibility of NLC® products in general. Also large-scale production lines were established for these products in industry (e.g., Rimpler GmbH, www.rimpler.de), which can also be used for pharmaceutical products. In November 2007, the US pharmaceutical company Brookwood acquired the worldwide exclusive rights for the NLC® technology from Pharmasol to turn NLC® into pharmaceutical products. The future will show if this is successful.

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8 Curriculum vitae

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9 Publications

9.1 Peer reviewed articles

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9.2 Proceedings

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2. **Muchow, M., Shegokar, R., Maincent, P., Müller, R.H., Keck, C.M.** (2009). Testosterone undecanoate-loaded nanostructured lipid carriers (NLC) for oral bioavailability enhancement, Annual Meeting of American Association of Pharmaceutical Scientists (AAPS), Los Angeles, USA, November 8-12, 2009
3. **Muchow, M., Shegokar, R., Maincent, P., Müller, R.H., Keck, C.M.** (2009). Nanosuspensions of testosterone and testosterone undecanoate: oral bioavailability, Annual Meeting of American Association of Pharmaceutical Scientists (AAPS), Los Angeles/USA, November 8-12, 2009
4. **Muchow, M., Maincent, P., Müller, R.H.** (2009). Testosterone and testosterone undecanoate – increased oral bioavailability by nanocarriers, Pre-Satellite Meeting of the Pharmaceutical Sciences Fair & Exhibition, Nice, France, 2009
5. **Muchow, M., Sapin, A., Kobierski, S., Maincent, P., Müller, R.H.** (2008). In Vivo Evaluation of Different Nanostructured Lipid Carriers for Enhanced Oral Testosterone Delivery, Annual Meeting of American Association of Pharmaceutical Scientists (AAPS), Atlanta, USA, 2008
6. **Muchow, M., Sapin, A., Kobierski, S., Maincent, P., Müller, R.H.** (2008). Oral Absorption Enhancement by Nanocarrier Technology, European Workshop on Particulate Systems (EWPS), Berlin, Germany, 2008
7. **Muchow, M., Ubrich, N., Maincent, P., Müller, R.H.** (2007). Nanostructured Carriers for Oral Testosterone Delivery, Pre-Satellite Meeting of the 3rd Pharmaceutical Sciences World Congress, Amsterdam, The Netherlands, 2007
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9. **Muchow, M., Despatova, N., Schmitz, E.I., Müller, R.H.** (2005). Omega-3 Fatty Acid-loaded Lipid Nanoparticles as Oral Food Supplement, Annual Meeting of American Association of Pharmaceutical Scientists (AAPS), Nashville, USA, 2005

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11 Summary of the thesis

11.1 English

The overall goal of this work consisted in ameliorating the bioavailability of drugs known for their poor hydrosolubility (testosterone) or for their lipidic character (omega-3 fatty acids). This was achieved using lipid nanoparticle systems and nanocrystals

In case of testosterone the work consisted of the development of an oral dosage form with superior properties compared to the currently commercially available oral system (Andriol Testocaps®). The other part of this work was the design of a lipid nanoparticle-based omega-3 fatty acid formulation, which, despite the use of cheap fish oil as source of omega-3 fatty acids, has low smell and taste properties while nevertheless being stable.

The development of the oral testosterone drug delivery system was accomplished on the basis of lipid nanoparticles technology and also using drug nanocrystal technology.

In both cases, systems could be developed that met the requirements with regards to drug loading (NLC) and stability (NLC and drug nanocrystals). Up to 30 % of testosterone undecanoate could be incorporated into the lipid phase of the NLC. Furthermore, the production of particles with different lipids, which are supposed to promote lymphatic absorption and hence the bioavailability of the hormone. Drug nanocrystals of testosterone (T) and testosterone undecanoate (TU) were prepared with a mean size of about 470 nm (TU) and 860 nm (T). Also with this system, an enhanced lymphatic absorption was expected.

The bioavailabilites of the developed NLC based drug delivery systems were all higher than the bioavailability of the product on the market when no additional lipid was supplied. This gives reason to believe, that the influence of co-administered food on the bioavailability of the systems is less pronounced than with Andriol Testocaps®.

Based on the findings that lipid nanoparticles can improve oral bioavailability, the development of an omega-3 fatty acids nanoparticulate system (NLC) out of cheap fish oil was a logic step. The oral bioavailability of the omega-3 fatty acids is much higher than the one of TU (about 70 %). Through the use of NLC technology, the taste and smell is even more reduced. It was rather unexpected that we achieved to have a formulation that consisted of 70 % lipid phase (and 30 % water) but still was paste-like and easy to redisperse. This makes the use of the paste as an additive in food and beverages possible to better supply the patient with essential omega-3 fatty acids.

Keywords: Drug delivery system, testosterone, pharmacokinetics, nanoparticles, SLN, NLC, nanosuspensions

11.2 Deutsch

Das generelle Ziel dieser Arbeit lag in der der Verbesserung der Bioverfügbarkeit von Arzneistoffen, die für Ihren schwer wasserlöslichen (Testosteorne) oder lipidartigen Charakter (Omega-3 Fettsäuren) bekannt sind. Im Fall von Testosteron gehört dazu das Entwickeln einer peroralen Arzneiform, die dem kommerziell verfügbaren, oralen System (Andriol Testocaps®) überlegen ist. Ein weiterer Aufgabenbereich war das Design einer auf Lipidnanopartikeln basierender Omega-3-Fettsäure-Formulierung, die trotz der Verwendung des preiswerten Fischöls als Omega-3-Quelle möglichst geringe Geruchs- und Geschmackseffekte aufweist und trotzdem stabil ist.

Die Entwicklung von oralen Testosteronträgersystemen gelang sowohl auf Basis der Lipidnanopartikeltechnologie (hier Nanostructured Lipid Carriers, NLC), als auch auf Basis von Nanosuspensionen.

In beiden Fällen konnten Systeme entwickelt werden, die den Anforderungen in Bezug auf Beladung (NLC) und Stabilität (NLC und Nanosuspensionen) entsprachen. Die Herstellung von NLC gelang in den gewünschten und für die später durchgeführten *in vivo*-Untersuchungen als geeignet erachteten Größen von rund 200 nm und 600 nm. Dabei konnten bis zu 30 % Testosteronundecanoat in die Lipidphase eingearbeitet werden. Auch gelang die Produktion mit verschiedenen Lipiden, die die lymphatische Absorption und damit auch die Bioverfügbarkeit des Hormons erhöhen sollten. Es konnten Nanosuspensionen sowohl aus Testosteron (T) als auch aus Testosteronundecanoat (TU) hergestellt werden, die im Schnitt ca. 470 nm (TU) und 860 nm (T) groß waren. Auch hier kann eine erhöhte Aufnahme des Wirkstoffs durch die Lymphe erwartet worden.

Die Bioverfügbarkeiten der entwickelten Trägersysteme auf Basis der NLC waren dabei durchweg höher als die der kommerziellen Arzneiform, wenn sie ohne zusätzliches Fett gegeben wurde. Im Vergleich zum Handelspräparat mit zusätzlicher Fettverabreichung waren sie gleichwertig (aber ohne, dass sie mit zusätzlichem Fett verabreicht wurden). Dies gibt Anlass zur Annahme, dass der Einfluss der gleichzeitigen Nahrungsaufnahme auf die Bioverfügbarkeit weniger ausgeprägt sein kann, als bei Andriol Testocaps®.

Ausgehend von den gezeigten Ergebnissen, dass Lipidnanopartikel die orale Bioverfügbarkeit erhöhen können, war die Entwicklung eines Omega-3-Fettsäuren-Nanopartikelsystems aus preisgünstigem Fischöl ein logischer Schritt. Die orale Bioverfügbarkeit von Omega-3-Fettsäuren ist wesentlich höher als die von TU (ungefähr 70 %). Durch die Verwendung der NLC-Technologie wurde der Eigengeruch und -geschmack noch weiter reduziert. Die Formulierung hatte einen bemerkenswert hohen Lipidanteil von 70 % und war trotzdem noch pastös und gut redispergierbar. Dies ermöglicht die Verwendung als Zusatz in Getränken oder Speisen und so die verbesserte Versorgung von Patienten mit essentiellen Omega-3-Fettsäuren.

Schlüsselwörter: Arzenistoffträger, Testosteron, Pharmakokinetik, Nanopartikel, SLN, NLC, Nanosuspensionen

11.3 Français

Le but général de ce travail correspond à l'amélioration de la biodisponibilité de principes actifs connus pour leur faible biodisponibilité (testostérone) ou pour leur caractère lipidique (acides gras oméga 3). Des systèmes nanoparticulaires à base de lipides et des systèmes nanocristaux ont été développés notamment dans le cas de la testostérone, afin d'obtenir une biodisponibilité supérieure au système oral actuellement commercialisé, Andriol Testocaps®.
L'autre partie de ce travail consistait en la conception d'une formule d'acides gras omega-3 dans des nanoparticules lipidiques, susceptibles malgré l'utilisation d'une huile de poisson bon marché comme source d'acides gras omega-3, de n'avoir que peu d'effets sur le goût et l'odorat tout en s'avérant stable.

Le développement de systèmes oraux à base de testostérone a été possible autant sur la base de la technologie des nanoparticules lipidiques (Nanostructured Lipid Carriers, NLC), que sur celle des nanocristaux. Dans les deux cas, les systèmes développés ont permis de répondre aux exigences en matière d'incorporation (NLC) et de stabilité (NLC et suspensions Nano). Les NLC ont permis d'incorporer jusqu'à 30% d'undecanoate de testostérone en phase lipidique. La formulation a également été possible avec différents lipides, susceptibles, d'augmenter l'absorption lymphatique et de ce fait également la biodisponibilité de l'hormone. Les nanocristaux ont pu être produits à partir de la testostérone (T) ainsi que d'undecanoate de testostérone (TU), avec des tailles moyennes respectives d'environ 470 nm (TU) et 860 nm (T). Cette taille particulaire doit permettre une absorption lymphatique accrue.
Les biodisponibilités des systèmes développés à base de NLC, se sont avérées, chez le rat Wistar, toujours plus élevées que la formulation commerciale, quand ils ont été administrés sans lipides additionnels ce qui permet de supposer, que l'influence simultanée de l'absorption de nourriture sur la biodisponibilité devrait être moins prononcée qu'elle ne l'est pour l'Andriol Testocaps®.
En partant de ces résultats, à savoir l'augmentation de la biodisponibilité orale avec les nanoparticules lipidiques, le développement d'un système de nanoparticules avec les acides gras oméga-3 d'huile de poisson bon marché était un pas logique. La biodisponibilité orale des acides gras oméga-3 est largement supérieure à celle du TU (environ 70 %). L'utilisation de la technologie NLC a ainsi permis la réduction de l'odeur et du goût du produit. La formulation avait un pourcentage remarquablement élevé de 70 % en phase lipidique tout en restant pâteuse et redispersible. Ceci permet d'envisager son utilisation dans des boissons et dans la nutrition ce qui facilitera l'assistance des patients (et donc la biodisponibilité) avec les acides gras omega-3 essentiels.

Mots-clés : Vecteurs de médicaments, Testostérone, Pharmacocinétique, Nanoparticules, SLN, NLC, nanosuspensions