Development and characterization of parenteral fat emulsions prepared by dual asymmetric centrifugation

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Abstract

Nano-scaled fat emulsions are well established as a drug delivery system for lipophilic drugs and for the use in parenteral nutrition. Typically the production of nano-scaled fat emulsions requires several formulation steps, including high pressure homogenization and filtration. The applicability of dual asymmetric centrifugation as an alternative technique to produce sub-micron fat emulsions in a short and easy way was investigated. The emulsions could be prepared without substance loss in a closed system within 30 minutes. Formulations with 10 % soybean oil and up to 5 % emulsifier-mixture were produced. The droplet size and droplet size distribution were determined by static light scattering and dynamic light scattering. Stability over six or nine months respectively was shown for different formulations by regular static light scattering measurements and determination of the zeta potential, pH and osmolality. Furthermore, hemolytic activity of the samples was investigated. With the optimized system the possibility of exchanging one emulsifier or the oil phase was examined. Active ingredients were successfully incorporated. Additionally the appearance and structure of the intermediate steps was investigated. For this light microscopy with polarization filter and freeze fracture transmission microscopy, as well as measurements with benchtop nuclear magnetic resonance were carried out. Stability under different conditions was examined. With dual asymmetric centrifugation physiological tolerable emulsions with droplets in the nanometer range could be prepared. This method could be used as a model for screening active pharmaceutical ingredients. Furthermore an observed intermediate step was important for predicting the quality of the final product. Moreover this intermediate step could have the potential to work as new drug delivery system itself.

Kurzfassung

Fettemulsionen mit Partikelgrößen im nm-Bereich sind gut untersucht und bewährt als Träger für lipophile Arzneistoffe und in der parenteralen Ernährung. Typischerweise werden bei der Herstellung von Nanoemulsionen verschieden Schritte durchlaufen, unter anderem werden Hochdruckhomogenisation und Filtration zur Verkleinerung der Tropfengröße eingesetzt. Als eine alternative Technik wurde der Einsatz der dualen asymmetrischen Zentrifuge untersucht, um auf einem schnellen und einfachen Weg Nanoemulsionen herzustellen. Es war möglich Emulsionen innerhalb einer Zeit von 30 Minuten herzustellen. Die Herstellung fand in einem geschlossenen System statt, sodass keine Substanz verloren ging. Die verschiedenen Formulierungen enthielten 10 % Sojaöl und bis zu 5 % Emulgatormischung. Mittels statischer und dynamischer Lichtstreuung wurde die Partikelgröße und Verteilung der Partikel bestimmt. Die Stabilität einiger Formulierungen wurde über 6 bzw. 9 Monate durch regelmäßige Messungen der Partikelgröße, des Zetapotetials, des pH-Wertes und der Osmolalität gezeigt. Desweiteren wurde die hämolytische Aktivität einiger Proben untersucht. Mit dem optimierten System wurden Versuche zum Austausch von Emulgatoren oder der Ölphase unternommen. Außerdem wurde untersucht, ob sich lipophile Arzneistoffe in das System einarbeiten lassen. Zusätzlich wurde das Aussehen und die Struktur der Zwischenschritte näher betrachtet. Verschiedene Methoden wie Polarisationsmikroskopie, Gefrierbruchelektronenmikroskopie oder Benchtop-NMR wurden dazu eingesetzt. Die Stabilität der Zwischenschritte unter verschiedenen Bedingungen wurde getestet. Mithilfe der dualen asymmetrischen Zentrifuge war es möglich physiologisch verträgliche Nanoemulsionen herzustellen. Diese Methode kann in Screening-Prozessen für Arzneistoffe Anwendung finden. Zusätzlich konnte ein Zusammenhang zwischen dem Aussehen der Zwischenschritte und der Qualität des Endproduktes hergestellt werden. Der Zwischenschritt selbst könnte ebenfalls als Trägersystem für lipophile Arzneistoffe fungieren.

Contents

1 Introduction

1.1 Delivery systems for lipophilic drugs intended for intravenous application

The application of lipophilic drugs is a challenging issue. As a large number of newly developed active ingredients have poor aqueous solubility, drug delivery systems for this class of active ingredients are in demand [90]. The oral uptake of such active ingredients causes some problems. Via the oral route the low solubility of these substances in the hydrophilic environment of the gastrointestinal tract leads to an insufficient absorption of the drug and with that to a low concentration of active ingredient at the site of action. Furthermore the stability of the drug in the different pH zones of the gastrointestinal tract, dependence on food intake and a possible early degradation due to the first pass effect in the liver has to be considered. By application with an appropriate drug delivery system via the intravenous route all these points could be avoided. Additionally a faster onset of action compared to the oral application could be achieved. Therefore the development of parenteral delivery systems for this class of drugs is a promising solution. In the following chapters the three main parenteral drug delivery systems, nanoparticles, vesicles and nanoemulsions are introduced with a special focus on nanoemulsions.

1.1.1 Nanoparticles

The development of nanoparticles for delivery of poorly soluble drugs is the youngest compared to vesicles and nanoemulsion. There are many formulations possible for parenteral application e. g. particles from biodegradable polymers or so called solid lipid nanoparticles (SLNs). In the field of biodegradable polymers there is a high number of possible substances. Especially polyglycolic acid, polylactic acid and their copolymer poly(lactic-co-glycolic acid) have been used in research many times [52, 85, 108]. Moreover attempts to form nanoparticles from chitosan or alginate were done [39, 41]. Often nanoparticles from polymers are prepared by the nanoprecipitacion method where the polymer is solved in an organic medium and

then injected dropwise and under stirring conditions to an aqueous surfactant solution [6, 74]. For this technique the problem of large scale production remains until now [135]. In spite of the considerable effort in research there is no drug delivery system based on polymeric nanoparticles available on the market [131].

SLN are composed of triglycerides, hard fat types or waxes that are solid at room temperature and also during administration to the body and at body temperature. They can incorporate lipid drugs and to protect them from the environment. But the drug loading capacity is limited as the solid triglycerides have relatively strict orders and the drug can only be incorporated were the framework of the crystal is disturbed. Mixtures of different substances have better solubility properties. During storage the change of the modification could lead to a higher order and thus a drug could be released from the particles [133]. To overcome these problems nanostructured lipid carriers (NLCs) were developed [103, 119]. As most drugs show a better solubility in fluid lipids in this type of nanoparticles an oil is mixed with a solid lipid. There are some cholesterolesters (e. g. cholesterylmyristate) that are capable of forming thermotrophic liquid crystals which could be prepared as nanoparticles. Those particles remained in their supercooled smectic status over a storage time of several months [81]. A higher drug loading was the reason for development of these formulations. Lyotrophic liquid crystals can also be formed as nanoparticles which are called cubosomes [11]. The incorporation of lipophilic, amphiphilic and hydrophilic drugs is possible. Preparation of SLNs is mostly done by high pressure homogenization (HPH) which means that a scale-up could be possible. Within this technique it has to be differentiated between hot and cold homogenization [96]. Other preparation methods like ultrasound or solvent emulsification/evaporation were also reported for SLNs [114].

1.1.2 Vesicles

Vesicles are basically structures of lipid bilayers in a spherical form in which the lipophilic ends of the molecules are turned to each other and the hydrophilic ends to the surrounding medium. The inner hole of the sphere is filled with the continuous phase medium. Mostly phospholipids are used to prepare vesicles in an aqueous phase which are then called liposomes. Different types of structures were found and can be divided into small unilamellar vesicles (SUV) with about 100 nm size, large unilamellar vesicles (LUV) with a size range from 200–800 nm and multilamellar vesicles (MLV; 500–5000 nm) [122]. Liposomes can be used as carriers for lipophilic, amphiphilic and hydrophilic active ingredients. Within intravenous administration conventional liposomes are cleared from the blood stream relatively fast. Hence, it was taken effort to invent long-circulating liposomes that overcome this effect. Furthermore much work is invested into modification of the surface of liposomes to reach specific targets. For instance in anticancer therapy the focus is not only on tumor cells but also on other cell types supporting tumor growth [109]. Another

example is the application of liposomes in vaccination [27]. Only few drug delivery systems based on vesicles are on the market today. An example is the well known product Caelix® which contains Doxorubicin for mammalian and ovarian cancer treatment.

Different preparation methods are suitable for the formation of liposomes. In many cases the liposome dispersion was spontaneously formed by adding aqueous phase to phospholipids. Mostly before that phospholipids were prepared in a flat layer to allow a faster and more homogeneous swelling of the lecithin and formation of vesicles. Then different methods for reduction of the particle size can be applied. By membrane extrusion and high pressure homogenization mostly MLVs and SUVs are obtained [105]. With the application of ultrasound SUVs could be obtained and freeze/thaw cycles lead mostly to LUVs. For the preparation of liposomes through mixed micelle dialysis, reverse-phase evaporation or solvent injection the structures are formed during the preparation process in the desired particle size.

1.1.3 Nanoemulsions

An emulsion is a thermodynamically unstable system containing at least two immiscible fluids. One of the fluids is dispersed as droplets in the continuous phase of the other fluid. Stabilization of these systems is done by amphiphilic substances known as emulsifiers [33]. For intravenous application there are special requirements. Referring to the European Pharmacopeia emulsions for parenteral application have to be sterile and are not allowed to show phase separation [40]. Particle size has to be in a tolerable range for the intended use. There are no defined limitations for the droplet size for parenteral emulsions [78]. However the droplet size needs to be in the nano-scale range to prevent embolism [20]. Nevertheless there are investigations of nanoemulsions that show that the existence of some particles in a low micrometer range have no negative influence on the safety of the application [46]. If the emulsion is intended to be given as infusion, that means a large volume is applied, the formulation has to be isotonic to the blood. Conservation of the preparation is only allowed when volumes lower than 15 ml are applied [40].

Nanoemulsions for parenteral use can have different functions. There are fat emulsions containing lecithin as emulsifier and vegetable oils as lipophilic phase that have a long tradition in parenteral nutrition [33, 63]. The first market products were Lipomul®, which was approved in the USA, and with the same composition Infonutrol® on the european market [63, 136]. Because of severe side effects both emulsions were removed from the market. The first save commercially available fat emulsion was Intralipid® that came to the european market in 1962 and ten years later to the USA [4]. Until now it can be used for parenteral nutrition of patients that are not able to eat properly [48]. With the positive experiences in parenteral nutrition the use of fat emulsions as drug delivery systems for lipid active ingredients was intensively investigated. Many approved medicines such as Diazepam-Lipuro®,

Lipotalon® or Vitralipid® attest the function of the concept. Until now the incorporation of lipid drugs in fat emulsions is still subject of many studies [5,50,112,137]. Another option is the use of nanoemulsions as adjuvants to improve the immune response of vaccination. Even though there are formulations in approved vaccines on the market effort is made to find new, more save emulsions as adjuvants [38,118,125].

1.1.3.1 Advantages and challenges

Injectable fat emulsions as drug delivery systems have some advantages. They are low immunogenic and biodegredable [55]. With these formulations it is possible to insert a solved lipophilic drug into the blood without having the problem of precipitation. Moreover absorbtion and bioavailability of the drug is increased [30]. Degradation of the drug by hydrolysis or protein binding can be reduced by emulsion formulations [46, 68]. Some studies confirm that side effects like pain, tissue irritation and venous sequelae occurred less when the injection formulation was an emulsion [87, 92, 124]. Nevertheless there are also challenges for nanoemulsions as drug delivery systems. The suitable oil phase for the relevant active ingredients has to be found. Furthermore there is a limitation to the amount of oil phase incorporated in the emulsion. Normally not more than 30 % can be realized [63]. That means that the drug has to be effective within low doses. Furthermore it is important that the active ingredient has no negative influence on the stability of the emulsion formulation. Another point is that only a limited number of emulsifiers can be used in parenteral emulsion formulations [20].

1.1.3.2 Typical ingredients

Emulsions intended for parenteral use consist of different classes of ingredients. They can be divided in the oil phase, the aqueous phase, emulsifier(s) and additional compounds. For the oil component there are different possibilities. Mostly either medium-chain triglycerides (MCTs) or long-chain triglycerides (LCTs) are applied [98]. For some formulations both MCT and LCT are mixed in a certain ratio. LCTs are vegetable oils, for instance soybean oil, safflower oil or sesame oil. For these natural components a high purity is important, as there is a long list of undesirable impurities that have an impact on physiological tolerability and stability of the formulation. MCTs are derived from coconut oil which was hydrolysed, purified and the fatty acids were fractionated. Subsequently defined amounts of different fatty acids with 6 to 12 carbon atoms were esterified with glycerol. This oil component is clearly more soluble in water than LCTs and with that has different drug solubility characteristics [28]. Often α-tocopherol is added as antioxidant to improve storage stability of the lipid phase.

As emulsifier in injectable emulsions mostly lecithin is used. This stabilizer is a mixture of different fatty acid esters of glycerol containing for example phosphatidyl-

choline or phosphatidylethanolamine (more information in chapter 2.1). The advantage of lecithin is that it is a common substance in the environment as is can be found in animals and plants. Therefore it is nontoxic and metabolized like natural fat. Lecithin for parenteral fat emulsions is either obtained from egg yolk or from soybean oil. Many different qualities exist as the grade of purity has an influence on the composition. Even though these substances show good physiological tolerability there are reports about some cases of allergic reaction [128]. Furthermore a hydrolysis of the stabilizer leads for instance to lysophosphatidylcholine that is known to cause hydrolysis of red blood cells. Hence the formation of these degradation products has to be controlled [61]. There are many other possible emulsifiers that were investigated for parenteral fat emulsions, for instance different fatty acid esters of sorbitan (Span®) or polyoxyethylene sorbitan (Tween®), as well as various poloxamer grades (Kolliphor® P-grades), Kolliphor® EL and Kolliphor® HS15 (detailed description in chapter 2.1). Especially for Kolliphor® P 188 good results were obtained. Combinations of lecithin with Kolliphor® P 188 showed improved stability and low hemolysis [69, 126]. Nevertheless it was reported that a long-term administration of formulations containing this stabilizer lead to undesirable side effects [19, 71, 86].

The aqueous phase consists of water for injection. It contains other substances to enhance physiological tolerability. The osmolality is mostly regulated with glycerol, but also sorbitol or xylitol can be used [4]. The pH value of the emulsion can be adjusted by sodium hydroxide to 7–8. A slightly basic value is preferred as the pH value drops during sterilization by autoclaving [63]. In most cases the use of a buffer is not practicable due to negative interaction with the emulsifier system. Some formulations contain sodium oleate or oleic acid as co-stabilizers for an improved stability of the oil droplets. Normally conservation is not applied, but in some formulations substances like EDTA, sodium benzoate or benzyl alkohol were used [56].

1.1.3.3 Preparation methods

One popular method to prepare parenteral fat emulsions is the high pressure homogenization (HPH) which is mostly used in the pharmaceutical industry and research. The preparation includes several manufacturing steps. If necessary a heating step is applied to the lipid phase and the aqueous phase. The aqueous phase contains water for injection and all additional substances that are soluble in water. In the oil the lipid-soluble substances are solved. The emulsifier(s) can be solved in the oil phase or be dispersed in the aqueous phase. After heating the lipid phase is given to the aqueous phase while mixing with a high shear mixer. A coarse emulsion is formed. This step is critical as reported by Collins-Gold *et al.* [33], because a good quality of the coarse emulsion leads to small droplets and a narrow particle size in the final product. Subsequently high pressure homogenization is performed with the emul-

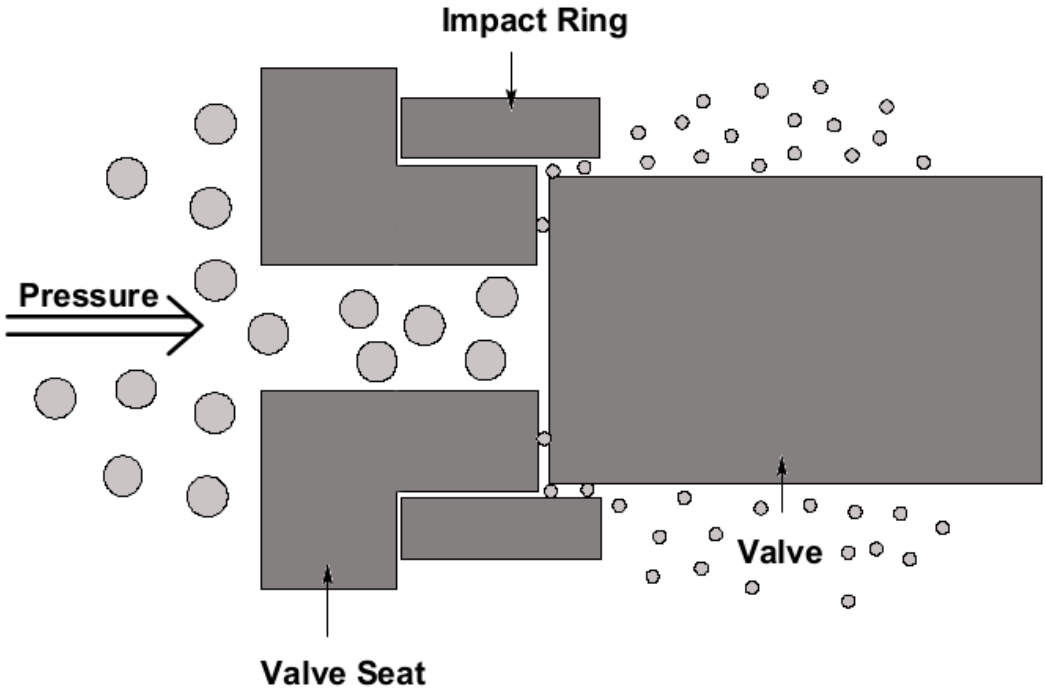

Figure 1.1: Schematic presentation of high pressure homogenization modified after [26]. The coarse emulsion is directed to the valve and pressed through the gap. After the gap passage the fluid is allowed to relax. In this procedure shear forces, impaction and cavitation are reducing the particle size. As main principle for the disruption of oil droplets turbulence is discussed [45].

sions. For this the fluid is given through a small gap under high pressure and after that is allowed to relax. High shear forces, cavitation, impaction and turbulences are mentioned as mechanisms for reducing the particle size (picture 1.1). Several cycles are needed to obtain a product with the desired attributes [127]. Often a filtration step is applied to separate bigger droplets from the emulsion. In the end the pH is adjusted to the desired value. As the emulsion has to be sterile for intravenous application, the use of an autoclave is recommended. If the product is not stable during the sterilization process alternatively an aseptic process with filtration through a 0.22 μm membrane could be applied [89]. For a better stability of oxidation sensitive substances and to confirm aseptic preparation the process should be carried out under nitrogen atmosphere whenever it is possible [20,46]. This effective but labor-intensive procedure has the advantage that it can be used in lab-scale and for industrial processing. Furthermore much research is done on this topic and the proceedings are well established [34]. Nevertheless high shear forces are applied to the product that can be critical with sensitive substances. Furthermore very small sample volumes can not be prepared and the cleaning procedure is time-consuming.

There are also other methods to obtain nanoemulsions for parenteral use. The application of ultrasonics is also a high energy emulsification method. In most studies a coarse emulsion is produced for example by a high shear mixer, then the

pre-emulsion is exposed to ultrasound. The mechanism of nanoemulsion formation is described in two stages. First the surface between oil and aqueous phase is becoming unstable and oil droplets are formed. Second these droplets break up in many fine particles because of a phenomenon that is called acoustic cavitation [1,113]. Not only small batches can be produced, it is also possible to use continuous flow production methods for scale-up [102]. However it was remarked that the final product could be contaminated with metal particles that could occur in high shear techniques due to cavitation erosion [102, 127].

Different methods can be found under the term "membrane emulsification". One is a continuous emulsifying process for small sample volumes [139]. Oil phase and aqueous phase are prepared separately by solving additional substances the respective fluid. Emulsifiers are added to the aqueous phase. Then the aqueous phase is pumped through a membrane device. Subsequently the oil phase is pressed through the membrane to flow rectangular into the stream of aqueous solution and an emulsion is formed. The outlet of the membrane device leads to a beaker where the emulsion is allowed to equilibrate under magnetic stirring [84]. The advantage of this method is that low stress is applied to the product. However all substances have to be soluble in one of the phases as for instance a suspended emulsifier could plug the pores of the membrane. Other workgroups described membrane emulsification as reducing the particle size of a coarse emulsion (mostly prepared by a high shear mixer) by giving it through a porous membrane. Particle reduction could be done with a simple syringe and filter system [51] or with tangential microfiltration [24].

Furthermore it was reported by that injectable emulsions could be formed by spontaneous emulsification or self-emulsification method [42, 138]. For this an ethanolic solution of oil, lecithin and the active ingredient was injected in an aqueous solution, containing isotonic agent and a hydrophilic emulsifier, while stirring with an magnetic stirrer. After equilibration ethanol and excess water were evaporated under reduced pressure [5]. This method can be applied for small batches, but the preparation method is rather complicated and the usage of organic solvent is critical when it comes to physiological tolerability of the emulsion. Because of the lack of high shear forces, this method was characterized as low-energy method [117].

Preparation of emulsions by phase inversion is also classified as low-energy method. Hategekimana *et al.* described that the aqueous phase was added to the organic phase consisting of carrier oil, stabilizer and active ingredient in small amounts. The first existing W/O emulsion was formed in a O/W/O multiple emulsion with a significant increase of the viscosity and then changed to the final O/W system by addition of excess aqueous phase [57]. A similar approach was used by another workgroup to prepare nanoemulsions loaded with different active ingredients [54].

1.2 Dual asymmetric centrifugation (DAC) as a new preparation tool for pharmaceutical drug delivery systems

Dual asymmetric centrifugation is a relatively new technique in the pharmaceutical field. Devices were developed to improve mixing processes which are important for many branches. Especially in the painting industry the technique is used to incorporate pigments in color or lacquer. According to the supplier there are also applications in the manufacturing and formulation with silicone [95], epoxy resins, nanocomposite processing [29] as well as in the cosmetic or dental sector and many more. The use of DAC the mixing is stated to be fast, efficient and low-noise without forming air bubbles.

The first attempt to prepare pharmaceutical drug delivery systems was done by Massing *et al.* with the manufacturing of liposomes by this method [94]. In this study a liposome dispersion with a relatively high amount of lipid was prepared due to the fact that viscous substances are advantageous for the use of DAC. Such formulations are called vesicular phospholipid gels (VPGs) and were first described in the late 1990ies by Brandl *et al.* [23]. The structure of the formulations was discovered as a dense package of vesicles and multilamellar structures. The semisolid appearance of the gels was interpreted as an effect of interaction of vesicles because of the low distance between the structures [22]. VPGs were successfully produced with high pressure homogenization, but for the preparation of small amounts or with the incorporation of shear sensitive drugs DAC was figured out as an alternative method. In first attempts the lipid mixture (40 %) and 0.9 % sodium chloride solution were put together in a glass vial with the same amount of glass beads and immediately treated by DAC. After at least 30 min of mixing the formed VPG appeared to be homogeneous [94]. These formulations could be used for different purposes. VPGs were investigated as a depot structure for the sustained release of human erythropoietin [121]. It was found out that the gels had a high drug entrapment efficiency and that the matrix enables sustained protein release due to diffusion mechanism and erosion of the VPG. In other studies the VPGs were loaded with siRNA and subsequently diluted to suspensions of small unilamellar vesicles (SUV) [2, 64]. The redispersion was achieved by simply adding excess buffer and then mixing for 30 s with DAC. Recent research was done on the VPGs itself. They were loaded with granulocyte-colony stimulating factor (G-CSF) and tested in vitro and in vivo [25]. Besides these promising investigations it must be mentioned that all these attempts have two disadvantages. The first one is the use of glass beads during the mixing process (these were not used to prepare the VPGs with erythropoietin or the G-CSF) that could be critical because of glass fragments in the formulation. The other point is the relatively long mixing time of 30 min or longer. Not only that the device is not designed for long mixing times (the mixing stopped and had

to be started again several times) there is also the problem of uncontrolled warming of the system.

Dual asymmetric centrifugation was also used to prepare liposome formulations for topical treatment. It was found that by incorporation of these liposomes in a hydrogel matrix a higher lipid and also higher drug loading could be achieved compared to the former processing method [66]. Recently a topical liposome dispersion for the treatment of acne was developed by the same group. It was possible to incorporate two active ingredients, benzoyl peroxide and chloramphenicol, simultaneously by using the DAC method. The formulations showed less cytotoxicity in in-vitro cell tests compared to the corresponding drug solutions [65]. Herrmann *et al.* developed a film-forming formulation containing nonivamide by mixing an oil loaded powder with an polymer dispersion in the DAC. This formulation and two others were used to test new methods to determine the amount of formulation removed by skin-to-skin or clothing-to-skin contact [62].

Another attempt using DAC in the pharmaceutical field was done by Kutza *et al.* [82]. The idea was to transfer a fluid lipid to a free flowing powder via adsorption processes and with that enhance the oral bioavailability. Therefore the DAC technique was compared to a mortar/pestle technique. It was found that due to mixing with DAC the oil was adsorbed more homogeneously on the surface of the particles. Moreover the surface was smoother and a smaller amount of particles were destroyed through the mixing process.

1.3 Objective of the thesis

As described above there are many possibilities to obtain nanoemulsions. But especially in the pre-formulation development a new preparation approach could help to improve screening processes in research. Therefore the method has to be fast and easy to handle. For active ingredients that are expensive and/or only available in small amounts the preparation of smaller sample sizes is preferred. In the present study the application of dual asymmetric centrifugation as potential technology for emulsion preparation is investigated. This method combines all attributes mentioned for a successful screening technique. It is designed for lab-scale preparation and the operation is simple. The device is configured for short mixing times. Working with a closed system has the advantage that there is no loss of sample during preparation process. Moreover the contamination of the samples is reduced and it would be possible to use the system under aseptic conditions. As the beakers are intended for single-use only, there is no complicated cleaning of the system necessary after sample preparation. Moreover different sample sizes can be produced because of different beaker sizes.

In this thesis a new drug delivery system based on commercial parenteral fat emulsions was to be developed with DAC. Hence, first a suitable formulation has to be

found and with that a way of preparation of a fluid system with the chosen technique. The critical parameters for emulsion preparation with DAC has to be found out and investigated. An optimized preparation method should be identified by this approach. Obtained emulsions have to be characterized concerning the intravenous application. Therefore determination of particle size and particle size distribution are important. Other methods should include the examination of the pH, osmolality and zeta potential, as well as physiological tolerability by a hemolysis assay and morphology of the samples by using imaging techniques. Furthermore stability over different storage times is important. For emulsions that have to be sterile the suitability for sterilization with an autoclave has to be investigated. With the optimized formulation and method it was tried to replace emulsifiers or the oil phase to investigate into the versatility of the approach. The applicability as screening system has to be proved by the incorporation of different active ingredients. In the end the semisolid intermediate step that was formed during preparation process has to be investigated by different methods. With that more information about the emulsion formation with DAC should be collected.

2 Materials

2.1 Emulsifiers

Different stabilizer mixtures were applied to obtain emulsions with dual asymmetric centrifugation. As a main component a mixture of phospholipids was used in combination with other emulsifiers that are described below.

2.1.1 Lipoid® S75

A mixture of phospholipids and other components like triglycerides, fatty acids, sterols and glycolipids obtained from soybeans or egg is known as lecithin. Many different qualities are available. For parenteral formulations the lecithin should be purified for a better physiological tolerability. Usually mixtures of different phospholipids are used. In this study mostly Lipoid® S75 from Lipoid KG, Ludwigshafen, Germany, was applied. These were yellow-brown agglomerates composed of soybean phospholipids with an amount of 70 % phosphatidylcholine and 10 % phosphatidylethanolamine. Mostly palmitic acid (17–20 %), oleic acid (8–12 %) and linoleic acid (58–65 %) were esters to the glycerol body [91]. An exemplary structure for these two main components is shown in figure 2.1. Lipoid® S75 contained less than 3 % lysophosphatidylcholine (no fatty acid ester at R_2) and less than 0.5 % free fatty acids. A low content of these two substances is important, because the lysolecithin causes hemolysis in parenteral preparations and free fatty acids are known to be irritating [83].

2.1.2 Kolliphor® P 188

Kolliphor® P 188 is a non-ionic block copolymer containing ethylene oxide and propylene oxide. It can be found under the name of poloxamer 188 in the European Pharmacopeia. There are several qualities available that each have defined amounts of the two polymers [15]. The chemical structure of poloxamer 188 is shown in figure 2.2. The substance is a white, waxy, coarse powder with a good solubility both

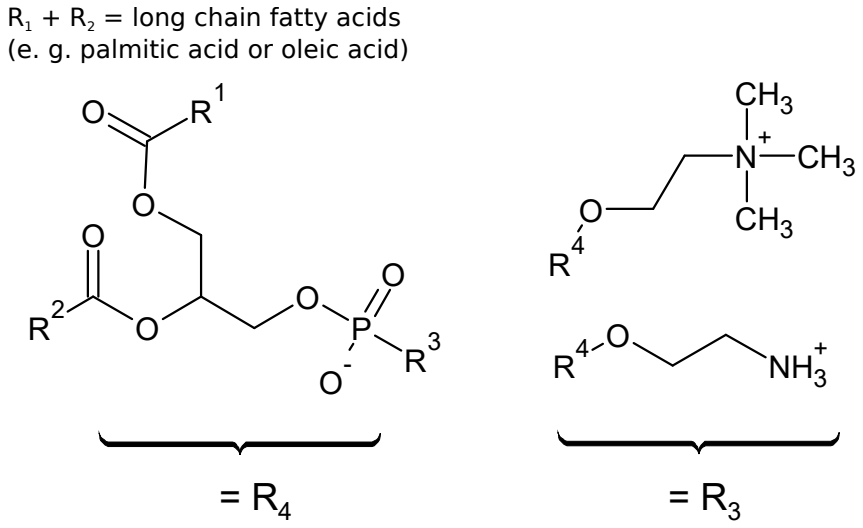

Figure 2.1: Structure of the main components of the phospholipid mixture Lipoid® S75. About 70 % of the phospholipids had a choline group at R_3, 10 % an ethanolamine group.

in water and organic solvents. In emulsions it can be used as co-emulsifier. Commercially available liquid formulations for parenteral use containing poloxamer 188 such as Bemfola® and Gazyvaro® are on the market [44], [104]. Furthermore there is also a great interest in this compound in research [26], [15]. The substance was purchased from BASF, Ludwigshafen, Germany.

2.1.3 Kolliphor® EL

Kolliphor® EL, which was obtained from BASF, Ludwigshafen, Germany, is a liquid non-ionic emulsifier. It consists of different components as the stabilizer is made by reacting castor oil with ethylene oxide in a molar ratio of 1:35 [13]. Most of the reaction products are hydrophobic. Especially glycerol polyethylene glycol ricinoleate and fatty acid esters of polyethylene glycol are formed. In a small amount polyethylene glycols and ethoxylated glycerol are included as hydrophilic substances. The emulsifier is described in the monograph of macogolglycerol ricinoleate in the Eu-

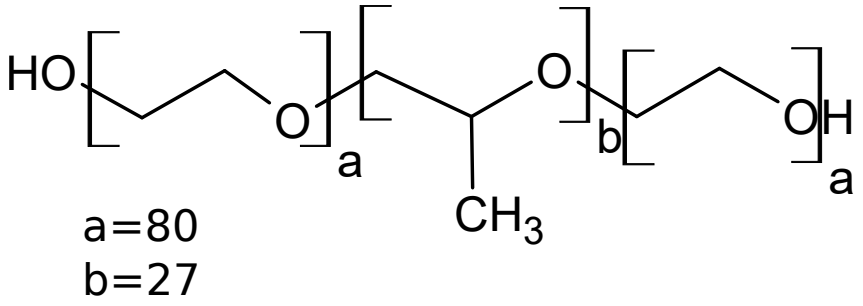

Figure 2.2: Chemical structure of Kolliphor® P 188.

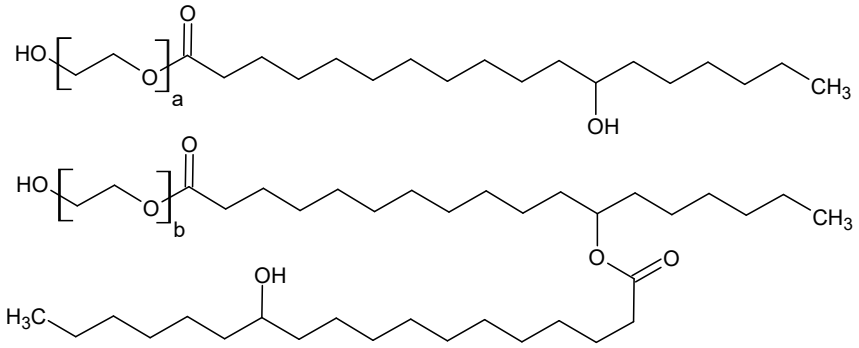

Figure 2.3: Two main structures from the emulsifier mixture Kolliphor® HS15.

ropean Pharmacopeia. Kolliphor® EL is used for instance as a solubilizer in liquid formulations of fat soluble vitamins [13].

2.1.4 Kolliphor® HS15

The white, semisolid emulsifier is known as macrogol 15 hydroxystearate in the European Pharmacopeia. It is produced by a reaction of ethylene oxide with 12-hydroxy stearic acid in a molar ratio of 1:15 [14]. The substance is a mixture of polyglycerol mono- and di-esters of 12-hydroxystearic acid. Exemplary structures of the main components are shown in figure 2.3. As hydrophilic parts free polyethylene glycol (up to 30 %) can be found in the compound. Furthermore some of the 12-hydroxy groups of the 12-hydroxy stearic acid could react with polyethylene glycol and build ethers. Kolliphor® HS15 was purchased from BASF, Ludwigshafen, Germany.

2.1.5 Tween® 80

This non-ionic emulsifier is a polyoxyethylene sorbitan monooleate (structure is shown in figure 2.4). According to the supplier (SERVA Electrophoresis GmbH, Heidelberg, Germany) the viscous, yellowish liquid contained up to 85% oleic acid as esters to the polyoxyethylene sorbitan. In minor concentrations other fatty acids are present. Tween® 80 is used as stabilizer in Imap®, a suspension for long-term treatment of schizophrenic psychoses.

2.2 Commercially available fat emulsions

There are many emulsions for parenteral use on the market. On the one hand injections including active ingredients like Propofol®-Lipuro can be found. On the other hand there are several formulations for intravenous nutrition e. g. Lipofundin® 10 % N or Clinoleic®. As a model emulsion Lipofundin® 10 % N from B. Braun Melsungen AG, Melsungen, Germany, was used. It contained 10 % soybeanoil as lipid phase with α-tocopherol as antioxidant reagent and egg-lecithin with at least

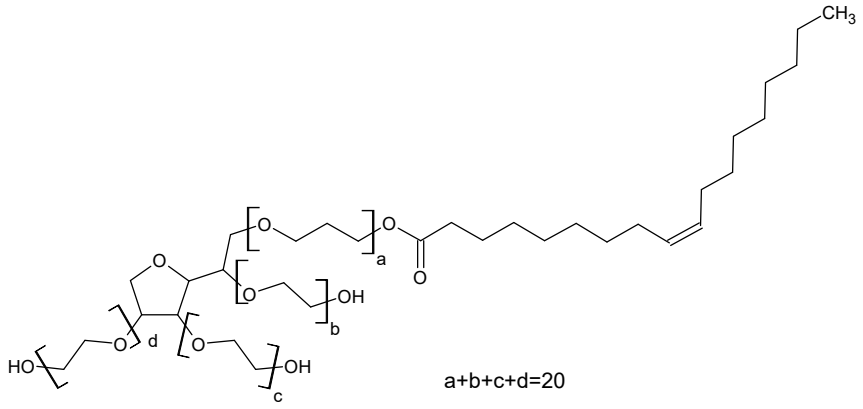

Figure 2.4: Main structure of the emulsifier Tween 80.

75 % (3-sn-phosphatidyl)choline as stabilizer. The aqueous phase consisted of water for injection with glycerol as isotonic substance and sodium oleate for adjusting the pH-value. According to the supplier the emulsion has a pH-value between 6,5 and 8,8 and an osmolality between 290 - 320 mOsmol/kg [7].

2.3 Active ingredients

Several active ingredients were tried to be incorporated in the emulsions prepared by DAC. Propofol (Fresenius Kabi AG, Bad Homburg, Germany) is a clear, slightly yellowish liquid which is used as intravenous anesthetic agent [37]. There are injections containing different amounts of Propofol available on the market (e. g. Propofol®-Lipuro). These formulations are based on parenteral fat emulsions [8]. The structure of Propofol can be found in figure 2.5(b). Another fluid active ingredient, Vitamin E-acetate, was tested (figure 2.5(c)). The clear, yellow fluid is commonly applied in many pharmaceuticals as antioxidant. In higher concentrations it is used to treat vitamin E deficiency for instance in the market product Vitralipid®, which is also intended for parenteral application. It was purchased from Caelo, Hilden, Germany. Probucol (Wako, Japan) is a solid, white powder that was used as third active ingredient. Its structure is shown in figure 2.5(a). There are no finished medicinal drugs with this substance allowed in Germany. Because of its solubility in lipids and its solid state of aggregation the nontoxic substance was a good candidate for a model active ingredient.

2.4 Miscellaneous

Other chemicals used for the experiments are shown in table 2.1.

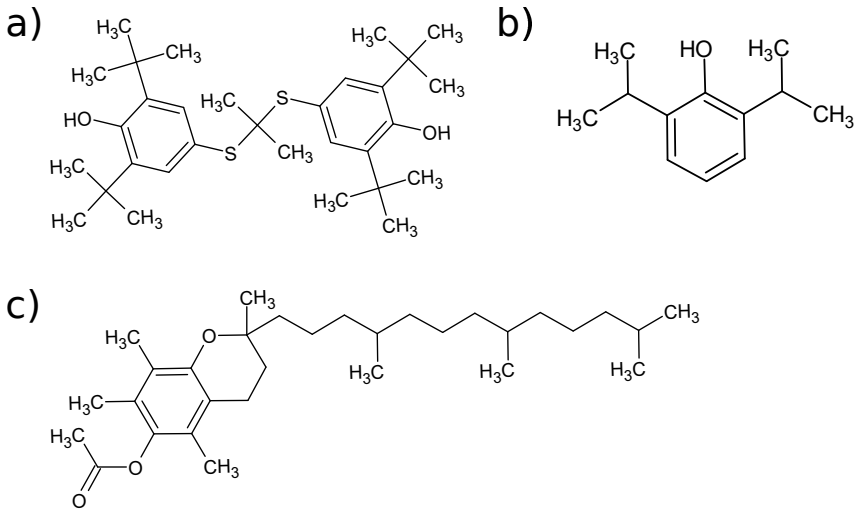

Figure 2.5: Chemical structures of different active ingredients (Probucol (a), Propofol (b) and Vitamin E-acetate (c)) that were given to the emulsions prepared by DAC.

Table 2.1: Further chemicals used for the experiments.

Substance	Synonym / formula	Supplier
Disodium phosphate,	Na_2HPO_4	Roth, Karlsruhe, Germany
Glycerol	$C_3H_8O_3$	Sigma-Aldrich, Steinheim, Germany
Oleum arachidis raffinat.	Peanut oil, refined	Caelo, Hilden, Germany
Oleum Maydis raffinat.	Corn oil, refined	Caelo, Hilden, Germany
Oleum ricini raffinat.	Castor oil, refined	Caelo, Hilden, Germany
Oleum sojae raffinat.	Soy bean oil, refined	Caelo, Hilden, Germany
Pionier MCT	Middle chain triglycerides	Hansen and Rosenthal, Hamburg, Germany
Potassium dihydrogen phosphate	KH_2PO_4	Grüssing, Filsum, Germany
Sodium azide	NaN_3	Sigma-Aldrich, Seelze, Germany
Sodium oleate	$C_{18}H_{33}NaO_2$	Sigma-Aldrich, Steinheim, Germany
Sorbitol	$C_6H_{14}O_6$	Roth, Karlsruhe, Germany
Sudanred G	$C_{22}H_{16}N_4O$	Merck, Darmstadt, Germany

3 Experimental

3.1 Dual asymmetric centrifugation

The SpeedMixer™ DAC 150 SP (Hauschild & Co KG, Hamm (Westf.), Germany) is a piece of benchtop equipment to produce homogenous mixtures within a short time. The mixing method is described as dual asymmetric centrifugation. A schematic representation of the working principle is shown in figure 3.1. In contrast to a common centrifuge there is only one sample holder, which can be modified in size so that different disposable beakers can be inserted. Comparable to a centrifuge the sample holder is attached to a rotating arm at an angle of 40° and rotates clockwise. In addition the holder rotates backwards, which leads to an overlayed agitation of the inward and outward movement of the sample [94], [121]. It is possible to prepare samples up to 150 g (including the weight of holder and beaker) with different rotation velocities from 300 rpm to 3500 rpm [58].

3.2 Dynamic light scattering (DLS) and Zeta potential

Dynamic light scattering and measurement of the zeta potential were both performed with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For dynamic light scattering the samples were diluted 1:100 in bi-distilled, filtered (pore size 0.2 μm) water directly before the measurements. Each sample was measured five times at 25 °C. The scattered light was detected at an angle of 173 °C. By this method an average hydrodynamic diameter (z-average) was obtained for the droplet size. The polydispersity index (PDI) was used to describe the width of the particle size distribution. For the zeta potential measurements the samples were diluted 1:50 in a 0.067 M phosphate buffer pH 7.4. Every sample passed five measurement cycles at 25 °C with a delay of 20 s between each measurement.

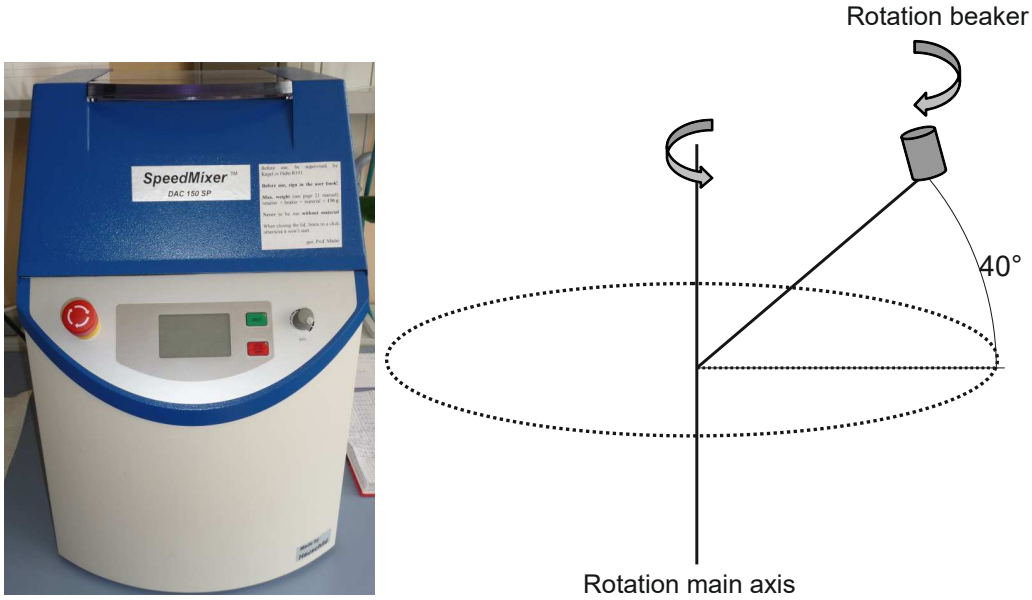

Figure 3.1: Photograph of the used device SpeedMixer™ DAC 150 SP (left) and picture of the functional principle of dual asymmetric centrifugation (right).

3.3 Static light scattering (SLS)

Determination of the particle size distribution and the volume weighted diameters (D[4,3]) of the samples was done using a Mastersizer 2000 with a Hydro 2000 S automatic dispersion unit and sub-micron instrumentation (Malvern Instruments, Worcestershire, UK). The sources of light were a red laser at a wavelength of 633 nm and a blue diode at 450 nm. For each sample five runs were performed with a measurement time of 10 s. Results were calculated employing Mie theory for a refractive index of 1.473 [93] and an absorption of 0.001 [31]. As additional information the D[10], D[50] and D[90] values of the particle size distribution were obtained by this method.

3.4 Osmolality

The osmolality of the samples and of the isotonic solvents was determined by a Semi-Micro Osmometer 4602 or K 7400 from Knauer (Berlin, Germany). The osmometers were calibrated to 0 mOsmol/kg with double distilled water and to 400 mOsmol/kg with a standard sodium chloride solution, which was obtained from Knauer. An amount of 150 μl of the sample was measured. The measurement was repeated at least three times with different samples.

3.5 Measurement of pH-value

The pH-value of the emulsions was determined at room temperature. For the measurements a Portamess® 911(X) pH from Knick (Berlin, Germany) was used.

3.6 Hemolysis assay

For the hemolysis assay human blood charged with EDTA was obtained from the blood donation service of the University Hospital of Halle. The procedure for the hemolysis assay was modified after Weiss *et al.* [130] and Bock *et al.* [21]. After determination of the hematocrit value of the whole blood sample (n=5), the red blood cell fraction was separated from the plasma by centrifugation (5 min at 5000 rpm). The supernatant was replaced by PBS buffer at pH 7.4. This step was performed six times until the supernatant appeared almost clear. The washed red blood cells were diluted with the buffer to obtain a stock dispersion with a red blood cell fraction of 2 %. The stock dispersion was added to the samples 1:1 (v/v) and incubated for 60 min at 37 °C in an end-over-end shaker. After incubation, the samples were centrifuged (5 min at 5000 rpm) to separate the intact red blood cells. 350 μl of the supernatant were added to 1450 μl ethanol/HCl (40+1, v/v) and the solution was measured by UV/VIS spectroscopy (Spekol 1200, Analytik Jena AG, Jena, Germany). Absorption values at a wavelength of 398 nm were used to determine the hemolytic activity of the samples. Control samples with 0 % hemolysis (in PBS buffer) and 100 % hemolysis (in a 2 % sodium dodecyl sulfate (SDS) solution) were employed in all experiments. The hemolytic activity was calculated as the ratio of hemolysis of the sample to hemolysis of the SDS-solution.

3.7 Autoclaving

Selected samples were sterilized in an autoclave KSG 113 (KSG Sterilisatoren GmbH, Olching, Germany) for 20 minutes at 121°C under saturated steam pressure of 2 bar as it is conform to the standard conditions in the European Pharmacopeia [40]. To investigate the stability the samples were characterized before and after the autoclave treatment. As characterization methods the appearance, particle size distribution by static light scattering, osmolality and pH values were determined.

3.8 Asymmetrical flow field-flow fractionation (AF4)

The asymmetrical flow field-flow fractionation was used to take a closer look at the particle size distribution of some emulsions. As a separation tool an Eclipse F (Wyatt, Dernbach, Germany) with an isocratic pump and micro vacuum degasser (Agilent 1100 Series, Agilent Technologies, Böblingen, Germany) was used. The channel

Table 3.1: Method for particle separation with asymmetrical flow field-flow fractionation.

Step	delta t	Mode	x Start (ml/min)	x End (ml/min)	Focus Flow (ml/min)
1	2	Elution	1	1	-
2	1	Focus	-	-	2
3	2	Focus/Injection	-	-	2
4	1	Focus	-	-	2
5	5	Elution	1	0.25	-
6	32	Elution	0.25	0.25	-
7	5	Elution	0.25	0.05	-
8	5	Elution	0.05	-	-
9	5	Elution	0	0	-

with a membrane of regenerated cellulose (MWCO 5 kDa, Microdyn-Nadir, Wiesbaden, Germany) had a trapezoidal shape (length 265 mm, highest width 21 mm, height 350 μm). Intensities were measured with a multiangle light scattering detector DAWN EOS (Wyatt, wavelength of the laser: 690 nm) at 15 different angles. Before injection the samples were diluted 1:200 in bi-distilled water that was filtered (pore size 0.1 μm) and conserved with 0.02 % sodium azide. This water was also used in the separation process. Details of the programme used to separate the particles are shown in table 3.1. The method was modified after Kuntsche *et al.* [80]. After 15 minutes of the program, and then every minute, the fluid leaving the detector was collected and later analyzed by dynamic light scattering.

3.9 Light microscopy with polarization filter

Structure of the intermediate steps was investigated with a light microscope Axiolab from Zeiss, Göttingen, Germany. For detection of birefringent structures polarization filters were used. Pictures were taken with a digital microscopic camera from Olympus, Hamburg, Germany.

3.10 Transmission electron microscopy (TEM)

Emulsions were prepared for negative staining by spreading 2 μl of the dispersion onto a copper grid. After 1–2 min the excess liquid was removed with a filter paper. The grids were washed with water for three times. A droplet of 1 % aqueous uranyl acetate solution was added and drained off after 1 min. The dried negatively stained emulsions were observed with a transmission electron microscope Libra 120 Plus (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) operating at 120 kV. Pictures were taken with a BM-2k-120 Dual-Speed on axis SSCCD-camera (TRS, Moorenweis, Germany). For further investigation of the morphology of the intermediate

steps and the final product the samples were cryofixed using a propane jet-freeze device JFD 030 (BAL-TEC, Balzers, Liechtenstein). Subsequently to that a freeze-fracture/freeze-etching system BAF 060 (BALTEC) was used to freeze-fracture the samples at -150 °C without etching. Once the surface was shadowed with platinum (2 nm layer, shadowing angle 45°) and then carbon (20 nm, 90°), the replica was floated in 4 % sodium chloride, rinsed in distilled water, washed in 30 % acetone and rinsed again in distilled water. Copper grids coated with Formvar® films were used to mount the replica. An identical microscope and camera was used as described above.

3.11 Benchtop nuclear magnetic resonance (Benchtop-NMR)

Investigations of the intermediate steps were carried out with benchtop NMR. A low-field (20 MHz) benchtop ^{1}H NMR spectrometer (MARAN DRX$_2$, Oxford Instruments Molecular Biotools, Oxford, UK) with an included air flow temperature regulation was used. For sample preparation an amount of 1 g intermediate step was weighted into a glass vial with a diameter of 1.3 cm. A short mixing step with DAC (15 s at 3500 rpm) was applied to make sure that the sample was stuck to the bottom of the vial without air wholes inside the mass. The T_2 relaxation of the protons was measured by using the transverse magnetization decay and applying the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [16]. With the use of the WinDXP analysis software (Oxford Instruments Molecular Biotools, Oxford, UK) measurement data was fitted and T_2 relaxation curves were obtained. Measurements of the intermediate steps were carried out with 64 scans. The relaxation delay time was set to 10 s and the number of echoes between two radio frequency pulses was regulated to 8192.

4 Results and discussion

4.1 Development of the system

The goal was to develop a nanoemulsion with the dual asymmetric centrifugation method. As a model the commercially available fat emulsion Lipofundin® 10 % N was used. Lipofundin® 10 % N contains soybean oil as lipid phase and water for injection as aqueous phase. Egg-lecithin with at least 75 % (3-sn-phosphatidyl)cholin is used as emulsifier and glycerol for isotonic reasons. α-tocopherol is added as an antioxidant reagent and sodium oleate for adjusting the pH-value [17]. For preliminary tests the emulsions prepared by the alternative method dual asymmetric centrifugation contained soybean oil as lipid phase, lecithin as emulsifier and an isotonic aqueous phase including double distilled water and glycerol preserved with 0.02 % sodium azide. According to the high pressure homogenization, the standard preparation process for nano-scaled fat emulsions, the oil phase and the aqueous phase were heated in different beakers [120]. The lecithin was put into the oil phase and allowed to swell there during the heating process. The emulsifier could not be added to the aqueous phase, as liposomes would have formed immediately. It was reported by Rydhag and Wilton that if the emulsifier was dispersed in the aqueous phase a significantly higher amount of surfactant was needed to obtain stable emulsions [106]. After heating both phases were united and mixed with the DAC. Different mixing times and velocities were tested. These first attempts to form a stable emulsion probably failed, because the shear forces in the system were too small. If an emulsion was build it was broken after a short time. As DAC is a method that works well for viscous systems [58], the idea was to produce a semisolid intermediate step and obtain an emulsion through a dilution process. The aqueous phase was added to the lipid phase in small amounts to prepare a semisolid phase. After dilution it was possible to form emulsions. It was found that a preparation process with three mixing steps, as shown in figure 4.1, was the most successful one. Two mixing

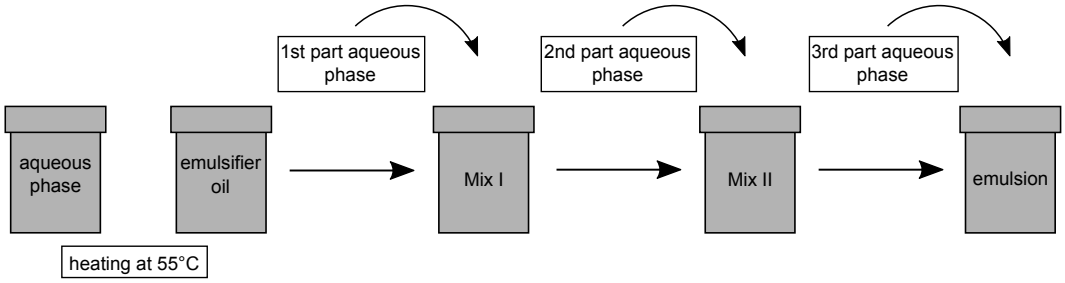

Figure 4.1: Preparation of fat emulsions with dual asymmetric centrifugation.

velocities were used, with the highest (3500 rpm), the intermediate step was stuck to the edge of the beaker. Hence, a lower velocity (1500 rpm) was used in-between. The parameters of the mixing process are shown in table 4.1. Preliminary tests with different lecithin types showed that the emulsifier Lipoid® S75 from soybeans (described in chapter 2.1.1) gave the best results. But the particle size needed to be reduced though. Hence, a second emulsifier was added to the formulation. Sodium oleate was selected, which is used in some commercially available fat emulsions like Lipofundin® 10 % N. Even if the physiological tolerance of this compound is critical in higher amounts, it was found that a second, solid emulsifier improved the emulsion stability and particle size. These parameters seemed to be connected to the appearance of the intermediate step. For further experiments sodium oleate was used as a model emulsifier in order to find an optimized composition to improve particle size and stability. In the improved formulation another emulsifier, which is physiological tolerable, should replace the sodium oleate.

First the influence on the amount of aqueous phase added to the oil phase before the mixing steps was investigated [120]. Formulations with 5 % emulsifier-mixture (lecithin and sodium oleate 1:1, weighted ratio) and 10 % oil phase were prepared. The exact composition of all samples is shown in table 4.2. The influence of adding 10 %, 4 % or 2 % of the aqueous phase to the lipid phase before the first and the second mixing step with DAC was investigated. To build the emulsion the remaining aqueous phase was given to the system before the third mixing step (emulsion LS.2, LS.3 and LS.4). Furthermore, there were samples prepared with the full

Table 4.1: Process of preparing fat emulsions with dual asymmetric centrifugation.

preparation	mixing with DAC
heating aqueous phase	
heating lipid phase	
adding 1st part aq. phase	30 s with 3500 rpm
adding 2nd part aq. phase	30 s with 3500 rpm
	30 s with 1500 rpm
adding 3rd part aq. phase	30 s with 3500 rpm
	30 s with 1500 rpm

Table 4.2: Varying emulsifier compositions (Lipoid® S 75 (S 75), sodium oleate (SoOl) and poloxamer 188 (PX) and varying amounts of aqueous phase for fat emulsions prepared with dual asymmetric centrifugation.

Emulsion	S 75 [%]	SoOl [%]	PX [%]	aq. phase 1st step [%]	aq. phase 2nd step [%]	aq. phase 3rd step [%]
LS.1	2.5	2.5	-	85	-	-
LS.2	2.5	2.5	-	10	10	65
LS.3	2.5	2.5	-	4	4	77
LS.4	2.5	2.5	-	2	2	81
LS.5	2.0	2.0	-	3.2	3.2	77
LS.6	1.5	1.5	-	2.4	2.4	77
LS.7	1.0	1.0	-	1.6	1.6	77
LS.8	0.5	0.5	-	0.8	0.8	77
LPX	2.5	2.5	-	4	4	77
LPX.S.1	2.5	2.47	0.03	4	4	77
LPX.S.2	2.5	2.44	0.06	4	4	77

amount of aqueous solution (85 %) added to the lipid phase before the first mixing step with DAC (emulsion LS.1). Consequently, for these samples the second and third mixing steps were performed directly after the first without addition of any aqueous phase. Not only the final emulsion was characterized by static light scattering measurements, the appearance of the intermediate step was observed as well. The intermediate phase changed depending on the amount of aqueous phase added to the system. A small amount of aqueous solution in the first two steps lead to a semisolid or higher viscous, light yellow and homogenous system. The more aqueous phase was added to the lipid phase before the mixing with DAC, the intermediate step changed to a lower viscous, dark yellow and inhomogeneous phase. Droplet size distributions of the emulsions were determined directly after preparation by static light scattering. The resulting distribution curves are shown in figure 4.2(a). Detailed values of the volume weighted mean diameters are presented in table 4.3. Emulsions with an amount of 2 % aqueous phase in the first two steps had a poor reproducibility (emulsion LS.4), therefore the results are not shown. For the other emulsions there was a trend observed that if a higher amount of aqueous solution is brought into the system in the beginning, bigger droplet sizes will be formed. Emulsions which were prepared with the full amount of aqueous phase in the first step (emulsion LS.1) had an average volume weighted mean diameter of 45.35 ± 2.38 μm. Decreasing the aqueous phase to 10 % added to the lipid phase prior to the first mixing step with DAC lead to a reduction of the particle size and a better reproducibility (emulsion LS.2). Emulsions had an average D[4,3] of 2.11 ± 0.05 μm. According to figure 4.2(a) the addition of the amount of 4 % aqueous solution in the first and second step before mixing with DAC lead to emulsions with the smallest droplet sizes and a monomodal droplet size distribution (emulsion LS.3). This is equivalent to a ratio of 0.8 parts aqueous phase to 1 part emulsifier-mixture (ratio aq/em).

Table 4.3: Average volume weighted mean diameters (D[4,3]) determined by SLS and pH values of fat emulsions. Samples were prepared either with different amounts of aqueous phase added to the lipid phase before mixing with DAC or with different amounts of emulsifier mixture. Details of the composition are shown in table 4.2.

emulsion	D[4,3] [μm]	pH
LS.1	45.35 ± 2.38	10.21 ± 0.05
LS.2	2.11 ± 0.05	10.29 ± 0.09
LS.3	0.87 ± 0.05	10.28 ± 0.13
LS.5	0.99 ± 0.00	10.28 ± 0.04
LS.6	3.58 ± 0.17	10.33 ± 0.08
LS.7	45.16 ± 0.92	10.26 ± 0.04
LS.8	52.80 ± 3.37	10.16 ± 0.06
LPX	0.81 ± 0.06	6.46 ± 0.06
LPX.S.1	0.75 ± 0.16	7.87 ± 0.15
LPX.S.2	0.76 ± 0.08	8.33 ± 0.07

The influence of the amount of emulsifier was tested, too. It was reported before that an increasing emulsifier amount lead to smaller emulsion droplets [47]. Preliminary examinations showed good results for emulsions with the emulsifiers lecithin and sodium oleate in a 1:1 ratio and an emulsifier amount of 5 % (data not shown). Samples with 1 %, 2 %, 3 %, 4 % and 5 % emulsifier-mixture (emulsions LS.8, LS.7, LS.6, LS.5 and LS.3) were prepared with the SpeedMixer^{TM} to investigate if the initial chosen emulsifier-mixture concentration of 5 % could be reduced. The amount of aqueous phase given to the system in the first two steps was calculated by the ratio aq/em determined above of 0.8 parts aqueous phase to 1 part emulsifier-mixture. The appearance of the final emulsions and the intermediate steps as well as the particle size distribution of the emulsions was studied. The prepared emulsions varied in their appearance from white, opaque fluids, comparable to the appearance of the commercially available fat emulsions, to more yellow and transparent samples. This could be connected to the droplet size of the formulations that are presented in figure 4.2(b). Detailed values for the volume weighted mean diameters are given in table 4.3. Samples with 1 % or 2 % emulsifier (LS.8 and LS.7) contained bigger droplets with D[4,3] values of 52.80 ± 3.37 μm and 45.16 ± 0.92 μm. Resulting emulsions had a slight yellow and more transparent appearance. For both formulations the intermediate steps were similar. After the first mixing with DAC dark yellow, viscous, inhomogeneous fluids were formed. The oil fraction was clearly visible. The second intermediate steps were more homogeneous, but stayed viscous. Only the color changed to a lighter yellow. Increasing the amount of emulsifier to 3 % lead to a distinct reduction of the particle size with an average volume weighted mean diameter of 3.58 ± 0.17 μm (LS.6). This came up to a reduction of the particle size close to 15-fold and 13-fold respectively compared to the former D[4,3] values. The final emulsion was a white fluid. The first intermediate step was yellow, not

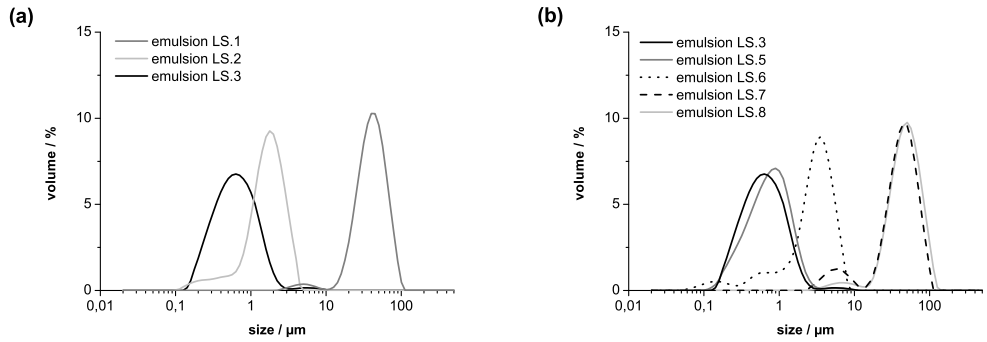

Figure 4.2: Average droplet size distributions determined by static light scattering for emulsions with different amounts of aqueous phase added before the mixing steps with DAC (a) or emulsions with different emulsifier contents (b). Details of the composition of the emulsions are presented in table 4.2.

completely homogeneous and somewhere between a high viscous fluid and semisolid. After the second mixing step with DAC it changed to a more homogeneous, light yellow, semisolid phase. Samples with 4 % emulsifier-mixture showed a 4-fold decreased particle size compared to samples with 3 % emulsifier-mixture, as the D[4,3] value lay at 0.92 ± 0.00 μm. A further increase of the emulsifier content to 5 % generated only a little improvement in the average volume weighted mean diameter (0.87 ± 0.05 μm). With increasing the stabilizers amount over this quantity, no further reduction of the particle size was achieved (data not shown). It was found out before that with a higher emulsifier amounts a multilayer structure of lecithin was formed around the emulsion droplets. This lead to a better stability of the emulsions due to reduced Van der Waals attraction forces and with that a lower probability of particle coalescence [49]. Nevertheless excess emulsifier seemed not to improve the emulsion stability. All of the emulsions LS.5 and LS.3 were white, homogeneous fluids similar to the Lipofundin® 10 % N. The intermediate steps were similar for both emulsifier amounts. The first intermediate step was semisolid and light yellow. Formulations with 5 % emulsifier showed a better homogeneity in this phase compared to formulations with 4 % emulsifier. For both formulations the second intermediate step was homogenous, semisolid and with a shiny surface. The color was similar to the first intermediate step.

These experiments proved that the idea of emulsion formation with DAC worked if an intermediate step was included in the preparation process. Nevertheless the particle size of the droplets had to be in a nanometer range so the process had to be optimized. Before optimizing the attempt was made to replace the sodium oleate with another emulsifier. Sodium oleate is known as lytic agent and relatively high amounts were used [73]. Not only hemolysis could appear but also the pH of the emulsions was rather high with values of 10–11. A good physiological compatibility was not granted. Specification values of commercially available products like

Lipofundin® 10 % N (pH between 6.5 and 8.5, [7]) and ClinOleic® (pH between 6 and 8, [17]) could be used as orientation. In consequence, there was the effort to exchange (eventually only partly) the sodium oleate. As possible alternatives non-ionic surfactants could be used. In recent studies a mixture of phospholipids and non-ionic stabilizers for emulsion preparation by high pressure homogenization were investigated [36, 86]. For example, the interactions of poloxamer 188 with phospholipid monolayers with regard to emulsion stability were examined and an intercalation of the poloxamer 188 in the lecithin bilayer was found [129]. As poloxamer 188 is an emulsifier that has been used for intravenous formulations before in research [26,71,129] and in commercially available formulations (e. g. Norditropin®), it was tested to replace the sodium oleate. Details of the formulation composition can be seen in table 4.2. The average droplet size distribution determined by SLS of emulsion LPX containing 2.5 % lecithin and 2.5 % poloxamer 188 (figure 4.3) was very similar to that of emulsion LS.3 (figure 4.2(b)). Emulsion LPX had a smaller D[4,3] value (table 4.3) compared to emulsion LS.3 and showed a slightly narrower peak. The pH-value was 6.46 ± 0.06 and with that clearly lower than for emulsion LS.3 with 10.28 ± 0.13. For samples with poloxamer 188 the pH value lay close to the lower border of the specifications from commercially available fat emulsions. In order to improve the pH-value it was considered to add a small amount of sodium oleate to the system again. In line with another commercially available fat emulsion Clinoleic®, that contains 0.03 % sodium oleate, two formulations with varying sodium oleate contents (emulsion LPX.S.1 with 0.03 % and emulsion LPX.S.2 with 0.06 %) were prepared by DAC. As expected the pH-values increased but were—with 7.93 ± 0.19 (emulsion LPX.S.1) and 8.44 ± 0,15 (emulsion LPX.S.2)—still in an acceptable range. The droplet size distribution did not change much in comparison to emulsion LPX. Hence, to facilitate the studies for further experiments formulations with poloxamer 188 were used without the addition of sodium oleate, as the droplet size seemed not to be depending on this ingredient.

4.1.1 Parameters of the preparation process

After replacement of sodium oleate as emulsifier with poloxamer 188 the attempt was made to decrease the particle size of the emulsions. There are several parameters listed in table 4.4 that could influence the formation of the emulsion. Some of the parameters were studied more closely in the following chapters.

For the SpeedMixer™ there was the option to use four different beakers. Three beaker types consisted of polypropylene and were available with diameters of 6.8 cm, 4.0 cm and 2.5 cm. Glass vials with a diameter of 1.3 cm could be used additionally. The optimization of the emulsion preparation was performed with the polypropylene beakers that had a diameter of 4.0 cm. It was the easiest to handle for several reasons. The biggest beaker led to a relatively high use of raw material. For the two smaller beakers, especially the glass vial with a diameter of 1.3 cm, the control

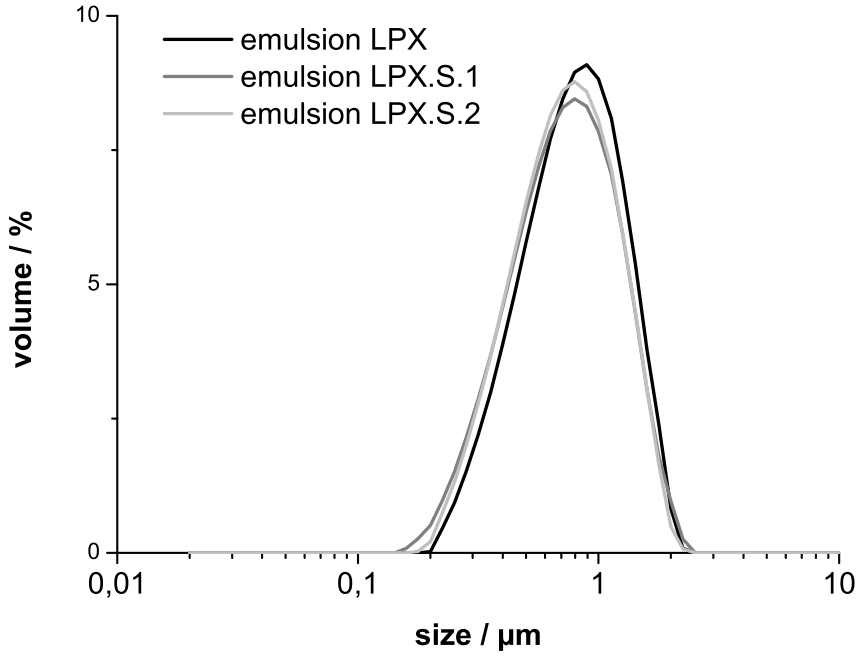

Figure 4.3: Average droplet size distributions determined by static light scattering for emulsions with lecithin, poloxamer 188 and different amounts of sodium oleate. Details of the compositions are shown in table 4.2.

of the formulation composition was more difficult, as only small amounts could be weighted. In preliminary tests the diameter of the beaker appeared to have influence on the formation of the emulsion and the product itself (data not shown). Further work on this issue has still to be done, but is not a topic of this thesis.

Mixing time and velocity were set in the preliminary tests. There is the possibility to either develop programs with 5 different steps (velocity and time can be changed in every step) or control the velocity manually. For the preparation process a combination was found that resulted in small particle sizes (see table 4.1). Some attempts were made to change the mixing parameter, but none of those resulted in significantly smaller particle sizes (data not shown). As there is a great number of possible combinations, the effort to find another combination that could achieve better results is relatively high. Since there were no promising results in the preliminary tests the parameters were not changed after setting them.

The amount of aqueous phase that was added to the lipid phase before the mixing steps was examined in preliminary tests that are described in chapter 4.1. The results showed a large influence of the amount of aqueous phase given to the system on the particle size of the emulsions. This parameter should be optimized to improve the quality of the emulsions and is described more closely in chapter 4.1.3.

While optimizing the production process of the emulsions, the way of adding the aqueous phase to the system was changed. Especially in the first two steps only small amounts were added which is more likely to cause errors. First the heated aqueous phase was added by weighting the solution into the beaker on an analytical balance. Although a pasteur pipette with a fine tip was used, the reproducibility had to be improved. In figure 4.4 the addition of the aqueous phase by weighting is compared to the use of an adjustable eppendorf pipette. Data from weighting the aqueous phase was collected from experiments made before. Hence there were different numbers of experiments. The data for the use of the eppendorf pipette was established by weighting the amount of aqueous phase that was inside the pipette tip. For every amount the weighting was performed 10 times. By only using the balance and not the eppendorf pipette, the error bars are relatively high. Especially with small amounts, like 375 μl, which corresponds to a ratio aq/em of 0.7 (emulsifier-mixture to aqueous solution). With an increasing amount of aqueous phase of at least 600 μl the error bars became smaller. That was due to the inaccuracy of the method for smaller amounts of aqueous phase. Furthermore for higher amounts of aqueous phase the evaporation of the warm solution has a lower influence compared to smaller amounts of aqueous phase. The low error bars for 400 μl were a result of a large number of experiments. Hence, for further studies the eppendorf pipette was used to add the aqueous phase to the system.

Controlling the temperature is a critical point in the system. While the temperature of the aqueous phase is easy to measure and control, the temperature of the lipid phase, which is not homogenous, is not easy to determine. Furthermore the SpeedMixer^{TM} has no option to control the temperature. Hence heated samples cool down during the mixing process. For the production of the emulsions, this cooling process was assumed to be the same for every sample, because the mixing time and velocity did not change. The heating parameters were investigated more closely in chapter 4.1.2 to allow a better control over the production process.

Lecithin is a semisolid material that is stored in the freezer at $-18\,^{\circ}$C. The original batches of lecithin were divided into several parts, so that not the whole amount had to be unfreezed and refrozen every time samples were prepared. Lecithin is a hygroscopic material that absorbs water from the air every time it was taken out of the freezer. This changed the material to be more soft. It was observed that the reproducibility of the results was sometimes difficult even if the same batch of lecithin was used. Therefore it was tested to moisten the lecithin under controlled conditions which is described in chapter 4.1.3.1.

The preparation of the lecithin also seemed to be important. It was found, that the homogeneity of the intermediate steps was improved, if the lecithin was spread as a thin layer on the bottom of the beaker. For this the lecithin was squeezed with a glass rod. That became more easy when the lecithin was allowed to swell 10 min in the oil while heating. In further experiments the second emulsifier poloxamer 188

Table 4.4: Parameters that could be varied in the production of emulsions with dual asymmetric centrifugation and an estimation of the possibility to control these parameter.

Parameter	Possibility of control
Diameter of the beaker	easy
Mixing time and velocity	easy
Amount of aqueous phase	good
Way of adding aqueous phase	good
Temperature of the aqueous phase	good
Temperature of the lipid phase	not precise
Cooling in the SpeedMixer™	not possible
Humidity of the lecithin	not precise
Preparation of the lecithin	not precise

was added to the system directly before the preparation with the glass rod and not directly in the beginning. This modification lead to a better reproducibility of the emulsions (data not shown).

4.1.2 Temperature

The temperature of the aqueous phase as well as the temperature of the lipid phase are critical parameters in the production process. Both were investigated more closely. In a first experiment the influence of the temperature of the aqueous solution on the particle size and particle size distribution of the emulsions was investigated. Therefore emulsions were prepared in the described way (see table 4.1). The amount of aqueous phase given to the system was according to a ratio aq/em of 0.7. This was chosen based on preliminary experiments that resulted in a slightly smaller particle size for this ratio aq/em compared to the former used ratio aq/em 0.8 (data not shown). Formulations contained lecithin 2.5 %, poloxamer 188 2.5 %, soybean oil 10 % and double distilled water isotonized with 2.2 % glycerol and preserved with 0.02 % sodium azide. The temperature of the aqueous phases was varied from 45 °C to 85 °C in 5 °C steps (except 50 °C). The aqueous phase was heated in a bulk on a plate while stirring and controlling the temperature. The temperature of the lipid phase remained stable. It was not measured for this experiment, but every beaker with lipid phase was heated on a waterbath with 55 °C for 30 min. Figure 4.5 shows the volume weighted mean diameters of the emulsions determined by static light scattering. Emulsions were prepared on different days. There were 4 samples produced with aqueous phase heated to 45 °C, 55 °C and 60 °C, 3 samples with 65 °C and only 2 samples with 70 °C to 85 °C. All in all the results for the D[4,3]-values were diverse, except for emulsions with 55 °C, 75 °C and 85 °C. As for the higher temperatures only two samples were prepared, these results could be incidental. For the 55 °C-samples the reproducibility was more significant, because not only the 4 samples of this study confirmed the results, as these parameters were the standard

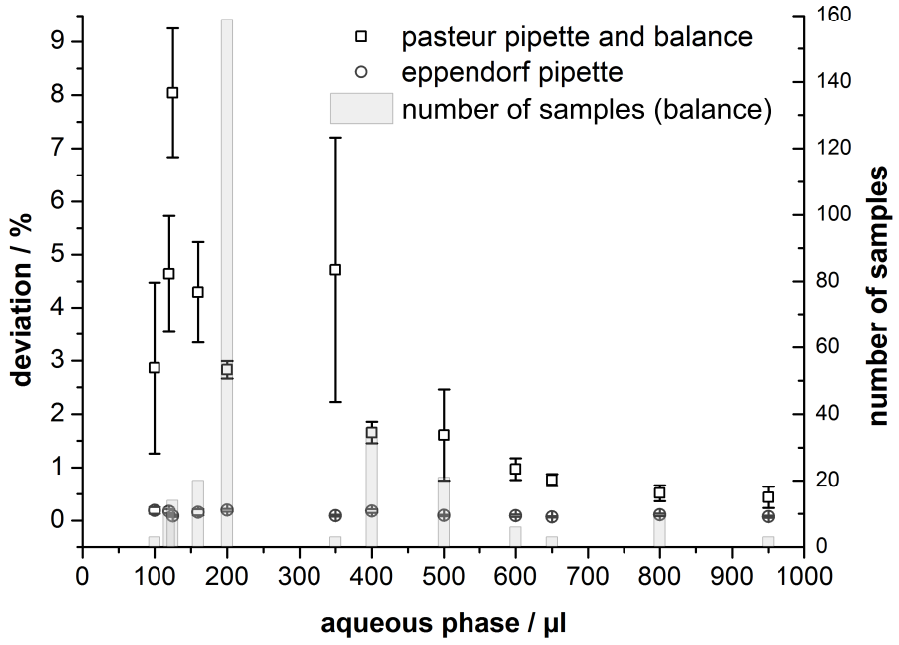

Figure 4.4: Comparison of the standard error of the mean for adding the aqueous phase either with an eppendorf pipette (n=10) or by weighting the amount on a balance (number of samples shown by light gray bars).

production parameters for many samples before. There was a trend of obtaining smaller droplets with a higher temperature of the aqueous solution. A temperature of 70 °C and higher lead to D[4,3]-values of 0.38 μm and lower. Considering not only the D[4,3]-value, but also the particle size distribution, it was observed that some of these emulsions had a broader droplet distribution compared to the samples with lower temperatures. The appearance of the intermediate steps was a homogeneous, light yellow, semisolid phase for all samples but for some samples with higher temperature, the semisolid phase was not spread on the bottom of the beaker but was formed to a sphere. The widest difference was the ”dissolution process” of the intermediate step. Before increasing the temperature, the formation of the emulsion did not take a long time. After the third mixing step with DAC, where the whole amount of the remaining aqueous phase was given to the intermediate step, the emulsion was formed immediately. If there were some pieces left from the intermediate step, the ”dissolution process” needed 10–20 minutes. Higher temperatures of aqueous phase always resulted in bigger pieces of intermediate step that needed longer time to dissolve (about 1–2 hours). This may be explained by the gelling properties of the poloxamer 188. It is known that this substance has a significantly higher viscosity in aqueous solutions that contain more than 20 % poloxamer 188 at 75–80 °C [15]. In the intermediate steps the poloxamer 188 concentration was 41.67 % and 26.32 % respectively based on the concentration of aqueous phase. Furthermore

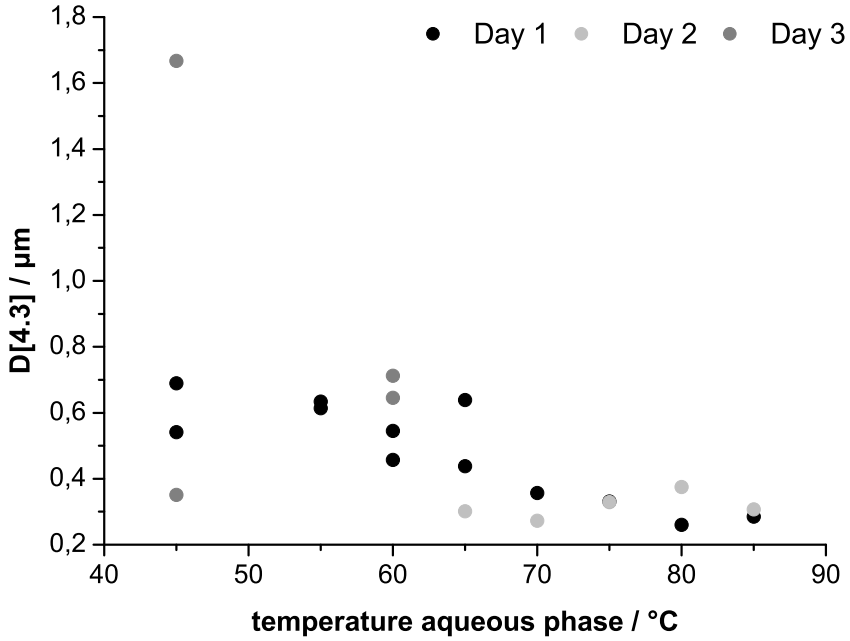

Figure 4.5: Volume weighted mean diameters determined by SLS for emulsions prepared with varying temperatures of the aqueous phase from 45 °C to 85 °C. All formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

high temperatures of the aqueous solution was not easy to handle, as the evaporation was high and could cause errors especially when giving small amounts of aqueous phase to the system. For all these reasons the application of higher temperatures for the aqueous phase was found not solely advantageous. Hence there was the ambition to change other parameters than the temperature of the aqueous phase.

Another attempt was to control the temperature of the lipid phase. This was more challenging, since the temperature of the lipid phase was not easy to measure. On the one hand the lipid phase was not homogeneous and on the other hand, the amount of lipid phase for every sample was rather small. There was only 1.5 g lipid phase, containing 1.0 g soybean oil, 0.25 g lecithin and 0.25 g poloxamer 188, prepared to receive 10 g final product. Aim of the experiment was to find a temperature range for the lipid phase where emulsions with small particle sizes were formed. Therefore the temperature of the waterbath was varied, as well as the heating time with a waterbath temperature of 55 °C. The temperature of the aqueous phase remained at 55 °C. In figure 4.6(a) an overview of the droplet size distributions determined by SLS of all prepared samples is presented. The distribution lines are marked black for samples with acceptable particle size distributions and gray for emulsions that were not in range. It was remarkable that the particle size distributions were similar for many samples and that two stability regions were obtained. Emulsions with

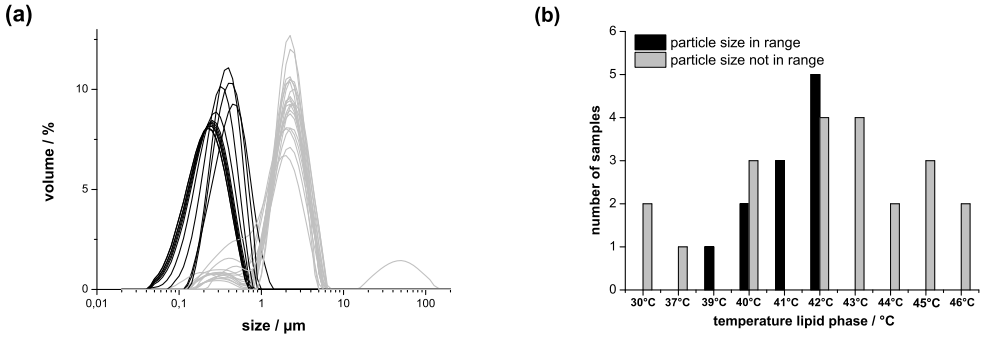

Figure 4.6: Influence of the temperature of the lipid phase on the particle size distribution of the emulsions. An overview of droplet size distributions determined by SLS of all emulsions prepared with different temperatures of the lipid phase is shown on the left (a). Black colored lines show distributions with particle size in the desired range. On the right the correlation of the temperature of the lipid phase in the preparation process and the particle size of the final emulsions is presented (b). Black bars show samples with acceptable particle size. All formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

acceptable droplet sizes had D[4,3] values from 0.25 μm to 0.37 μm and the highest D[90] value lay at 0.61 μm. There was one sample with little higher values (D[4,3]: 0.50 μm and D[90]: 0.81 μm) that was almost in range. Particle size distributions of the other emulsions were more broad. Some of them had two peaks, one had three peaks. All samples had a main peak with D[50] values between 1.97 μm and 2.79 μm in common. Regarding the intermediate steps different appearances could be connected to the resulting particle size distributions. On the one hand the first intermediate step was a light yellow, homogeneous, semisolid phase spread on the bottom of the beaker. After addition of the second amount of aqueous solution and mixing with DAC the intermediate step became more jelly like and had a shiny surface. This appearance of the intermediate steps was used to show up in samples that showed a small particle size distribution in the end. For emulsions with bigger particles the first intermediate step was dark yellow, not homogeneous and viscous but not semisolid. The homogeneity improved in the second mixing step, but the color remained darker yellow. In figure 4.6(b) the results of the static light scattering are combined with the temperatures of the lipid phase directly before the first mixing step. Additionally the number of samples for every temperature is shown. Variable numbers of samples were resulting from the fact that the measured temperature in the beaker differed from the expected temperature. Nevertheless a correlation of the measured particle size with the lipid temperature was found. In a temperature range for the lipid phase between 39 °C and 42 °C all emulsions with acceptable particle size distributions were located. Though the lower limit was arguably because of the low number of samples.

To conclude, there was definitely an influence of the temperature of the aqueous phase as well as the lipid phase. While the temperature of the aqueous phase was easier to control, the temperature of the lipid phase remained more uncertain. The production of the emulsions could be improved by a better control of both parameters. For the lipid phase it was found that desired temperatures could be obtained by the following procedure: The lipid phase, containing lecithin and soybean oil, was heated on a waterbath with a temperature of 55 °C for 8–10 minutes. After homogenizing the lipid phase with a glass rod and addition of the poloxamer 188, the heating on the waterbath was continued to a total heating time of 25 minutes. Then the addition of the aqueous phase and the mixing process with DAC was started. The temperature of the lipid phase was measured shortly before starting the mixing process.

4.1.3 Optimizing the amount of aqueous phase added to the system during preparation

With having a better control over the temperature, the amount of aqueous phase given to the system in the first two steps was optimized. A first attempt was made in chapter 4.1.2 as before emulsions were prepared with a ratio aq/em of 0.8. In preliminary tests after beginning to use the eppendorf pipette it was found that a ratio aq/em of 0.7 had a good influence on the particle size distribution (data not shown). Therefore in the former experiments a ratio aq/em of 0.7 was used. For this study a broader range of ratios aq/em from 0.5 to 1.0 was tested. Intermediate steps of 0.05 were made. In table 4.6 the specific amounts of aqueous phase for different ratios in μl that were given to the system to receive 10 ml emulsion are listed. Additionally detailed volume weighted mean diameters (D[4,3]) are presented. Figure 4.7 gives an overview over resulting D[4,3] values in correlation with the ratio aq/em. Samples were prepared on different days which are marked in the figure as well as in the table. There was one day (day 4) where most of the emulsions had bigger droplet size distributions than expected.

The overview in figure 4.7 clearly shows a correlation between ratio aq/em and the resulting particle size. Up to a ratio aq/em of 0.65 the reproducibility of the samples was poor. Two stability regions for particle size distributions similar as described in chapter 4.1.2 were identified. For the emulsions that had droplets in the acceptable range, the particle size distributions were rather broad compared with samples that were prepared with a ratio aq/em over 0.65. It could be predicted by the appearance of the first and second intermediate step whether the final particle size would be in range or not (description see chapter 4.1.2). But however a reason why the one or the other particle size distribution resulted was not found yet. One hint could be the distribution of the lecithin within the lipid phase, because this was the only component of the lipid phase that was not easy to be homogeneously spread. The oil and the poloxamer 188 as well were fluid (poloxamer 188 after melting) and easy

Table 4.5: Average volume weighted mean diameters (D[4,3]) and standard deviation (SD) of emulsions with different ratios aq/em from 0.7 to 0.95.

Ratio aq/em	average D[4,3] [μm]	SD [μm]	number of samples
0.7	0.28	0.02	9
0.75	0.34	0.05	8
0.8	0.45	0.08	7
0.85	0.52	0.09	7
0.9	0.73	0.10	3
0.95	0.80	0.12	3

to mix. Starting with a ratio aq/em of 0.7 up to 1.0 the correlation between aqueous phase given to the system in the first two steps and the particle size distribution of the resulting emulsions could be displayed (see figure 4.7). A trend to higher droplet sizes with the addition of higher amounts of aqueous solution was clearly shown. The average volume weighted mean diameters of the samples are presented in table 4.5. With a ratio aq/em of 0.7 all of the final emulsions had monomodal particle size distributions and volume weighted mean diameters between 0.26 μm and 0.32 μm, which were with an average D[4,3] of 0.28 μm in a good range for a parenteral application [32], [4]. Only two samples that were produced on day 4 had higher D[4,3] values of 0.47 μm and 0.58 μm and did not fit in the trend. They were not included in the calculation for the average D[4,3] value. In a range between ratios aq/em of 0.7 and 0.85 in every step the particle size of the samples increased about 100 nm regarding the average D[4,3] values, as can be seen in table 4.5. The higher the amount of aqueous phase added to the system, the bigger was the standard deviation. That means that the reproducibility of the emulsion became worse with increasing amount of aqueous solution. But the influence of the number of samples should be regarded, too. Especially emulsions with ratios aq/em of 0.9 and 0.95 have to be considered carefully, as there was only a small number of samples (n=3) made. Hence, average volume weighted mean diameters for these two ratios aq/em were only shown in the table to complete the picture of the trend, but are not as reliable as the other values. Emulsions prepared by adding the same amount of aqueous phase to the lipid phase as emulsifier were included (ratio aq/em 1.0). They followed the trend regarding the D[4,3] values. But as there was only a small number of samples and one of them had a broad particle size distribution with a D[4,3] of 4.52 μm, they were not included in table 4.5. The appearance of the intermediate steps was similar to the description in chapter 4.1. The higher the amount of aqueous solution given to the lipid phase, the more fluid was the intermediate phase. The color changed from light yellow to a darker yellow shade. As mentioned before, emulsions with small particle sizes had semisolid, homogeneous intermediate steps.

All in all the results of the particle size distributions of the emulsions were promising and especially for samples with ratios aq/em 0.7 and 0.75 in a good range for parenteral application. Nevertheless the reproducibility of the emulsions could be

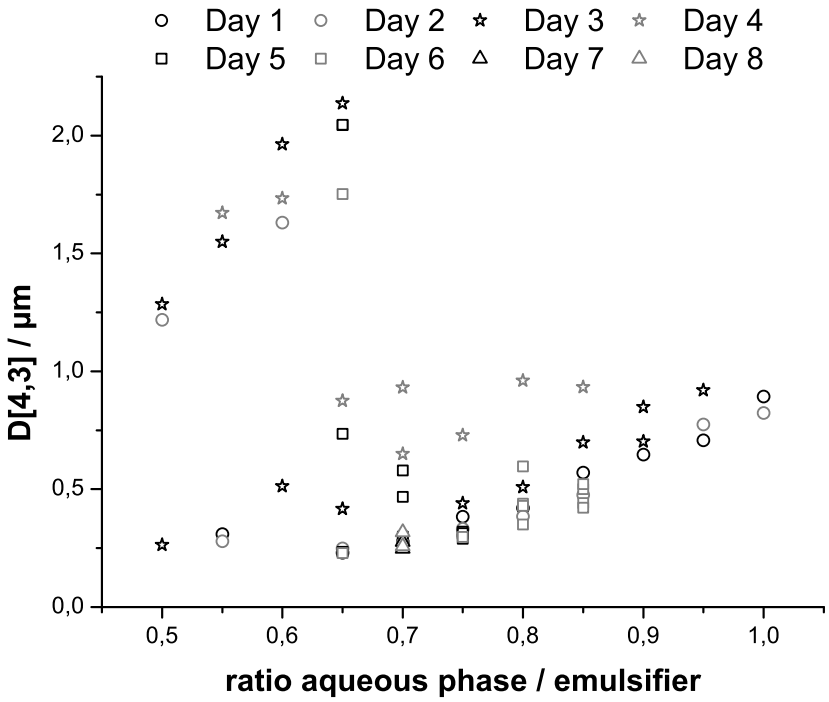

Figure 4.7: Volume weighted mean diameters determined by static light scattering of emulsions prepared with different amounts of water in the first two steps. Samples were prepared on different days. Detailed values for D[4,3] are shown in table 4.6. Formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

improved. As mentioned before in this chapter, the clue could be the homogeneity of the lipid phase and with that the homogenous distribution of the lecithin. Preliminary tests showed that a reduction of the lecithin had a bigger influence on the particle size of the emulsions than a reduction of the second emulsifier (data not shown). As a conclusion the lecithin somehow stabilizes the lipid phase and is important for building the semisolid intermediate step.

4.1.3.1 Moistening of lecithin

Lecithin is a hygroscopic mixture of different phospholipids that, according to the supplier, already contains up to 2% water [91]. Details of the lecithin Lipoid S75 used in the experiments are described in chapter 2.1.1. During the studies it was found that the processability of the lecithin was different depending on the time the lecithin had contact with the air. Normally lecithin is stored in a freezer at −18 °C. The batches used for the studies were divided into smaller parts so that only a small amount had to be unfreezed and refreezed while weighting. For a time saving process the lipid ingredients of several samples were weighted at once before the heating was started. In consequence the lecithin was exposed to the air for some minutes before it was stored in the freezer again. It was observed that

Table 4.6: Amount of aqueous phase given to the system in the first two steps calculated for 10 ml final product for different ratios of aqueous phase (aq. ph.) and emulsifier (ratio aq/em) and resulting volume weighted mean diameters (D[4,3]) of the emulsions. Values marked with * are not shown in figure 4.7. Values marked with † were not included in the calculation of the average volume weighted mean diameters in table 4.5.

ratio aq/em	aq. ph. [μl] step 1 & 2	D[4,3] [μm] Day 1	D[4,3] [μm] Day 2	D[4,3] [μm] Day 3	D[4,3] [μm] Day 4	D[4,3] [μm] Day 5	D[4,3] [μm] Day 6	D[4,3] [μm] Day 7	D[4,3] [μm] Day 8
0.5	250	30.62*	1.22	1.29 0.26	29.21*				
0.55	275	0.31	0.28	1.55	1.67				
0.6	300	5.44*	1.63	1.96 0.51	1.73				
0.65	325	0.23	0.25	2.14 0.42	0.88	0.24 2.04 2.05 0.74	0.23 1.75		
0.7	350	0.28	0.28		0.93† 0.65†	0.47† 0.58† 0.30 0.28	0.26	0.28 0.25	0.32 0.26
0.75	375	0.38	0.33	0.44	0.73†	0.31 0.29 0.32 0.31	0.30		
0.8	400	0.42	0.39	0.51	0.96†		0.35 0.60 0.44 0.43		
0.85	425	0.57	0.48	0.7 0	0.93†		0.44 0.50 0.42 0.52		
0.9	450	0.65		0.70 0.85					
0.95	475	0.71	0.78	0.92					
1.0	500	0.89	0.82	4.52*					

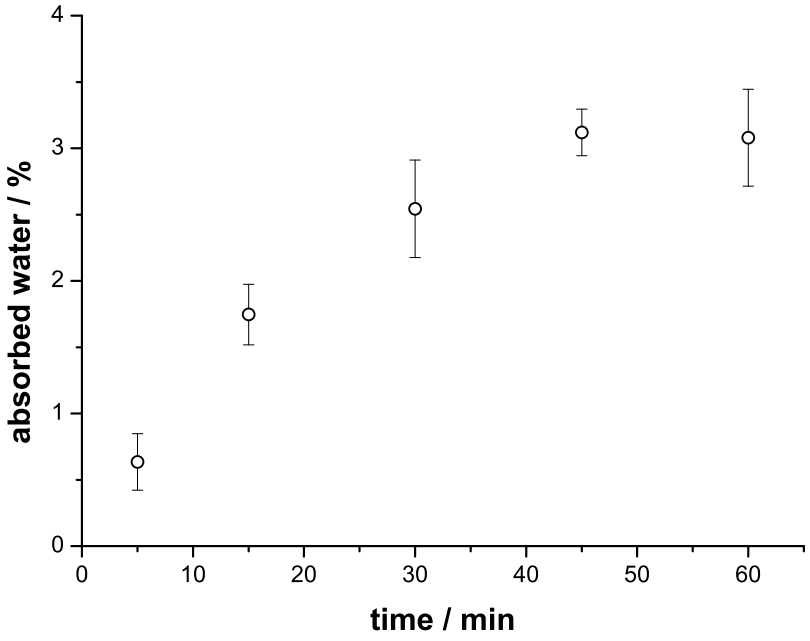

Figure 4.8: Absorbed water from the environment for 2 g and 5 g lecithin at different points of time at 25 °C and 40 % rF.

with longer exposition to the air the lecithin became softer, the color changed to darker yellow and the homogenization of the samples while the production process was easier. Supposedly the lecithin absorbed water from the air and changed the consistence. After storing the changed lecithin in the freezer, the soft and darker yellow appearance stayed. There was the consideration of a controlled moistening of the lecithin to improve the processability of this emulsifier and therefore the reproducibility of the samples. Lipoid S75 was exposed for 5 min, 15 min, 30 min, 45 min or 60 min in a humidifying chamber with 25 °C and 40 % rF. The lecithin was spread in a layer on a glass plate so that water of the environment could easily be absorbed. In figure 4.8 can be seen that the lecithin absorbed the water until a saturation was reached. A stadium of saturation was obtained between 30 min and 60 min and the substance had absorbed 2–3 % water. Considering the fact that lecithin is a mixture of different components the variability of the single values can be explained. It was reported by Shchipunov that soybean lecithin forms a lamellar liquid-crystalline mesophase L_α when a phospholipid absorbs water vapor from the air [111].

Several emulsions were prepared with the moistened lecithin on different days. In figure 4.9 the volume weighted mean diameters of the emulsions are presented for samples with ratios aq/em of 0.7 (figure 4.9(a)) and 0.75 (figure 4.9(b)) in correlation with the absorbed humidity of the lecithin. The ratios aq/em of 0.7 and 0.75 were selected as these showed the smallest droplet sizes in former tests (chap-

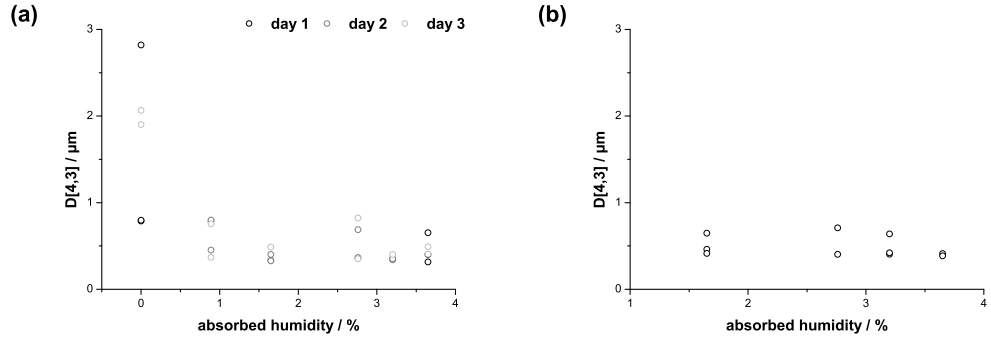

Figure 4.9: Volume weighted mean diameters of emulsions prepared with lecithin that absorbed different amounts of water. Formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase. Samples were prepared either with a ratio aq/em of 0.7 (a) or with a ratio aq/em of 0.75 (b).

ter 4.1.3). Both diagrams show that moistening the lecithin lead to smaller particle sizes and a better reproducibility compared to samples where the lecithin was not allowed to absorb water before the preparation process. Though the range of the absorbed humidity differed between 0.89 % and 3.65 %, the resulting D[4,3] values were all between 0.32 μm and 0.80 μm for emulsions with a ratio aq/em of 0.7 and between 0.39 μm and 0.71 μm for emulsions with a ratio aq/em of 0.75. Note that samples with 1.65 % or 3.2 % from the diagram should be treated cautiously, as there was only a small number of samples. Nevertheless the possibility to moisten the lecithin and with that improve the processability is a further step to obtain a better reproducibility of the samples.

4.1.4 Optimized system

The reproducibility of emulsions prepared by the optimized process was tested. Therefore ten emulsions with the ratio aq/em of 0.7 and moistened lecithin, that absorbed an amount of 3.16 % water, were prepared at the same day. The droplet sizes of the samples were examined by static and dynamic light scattering directly after preparation. All emulsions were white fluids and the appearances of the intermediate steps were similar, too. The first intermediate steps were homogeneous, semisolid and of a light yellow color. The second intermediate steps looked alike, but all had a more shiny surface (see chapter 4.4.1). Figure 4.10 shows the results of the measurements with static light scattering (a) and dynamic light scattering (b). In table 4.7 detailed values of all emulsions are presented. Taken as a whole the samples had similar particles size distributions that lay close together. With volume weighted mean diameters of 0.31 μm (sample 9) to 0.37 μm (sample 2) the emulsions were in the required range for parenteral use [67, 139]. And only 10 % of the particles were above 0.52 μm (sample 9) up to 0.63 μm (sample 2). These findings were confirmed by the dynamic light scattering measurement where z-averages between

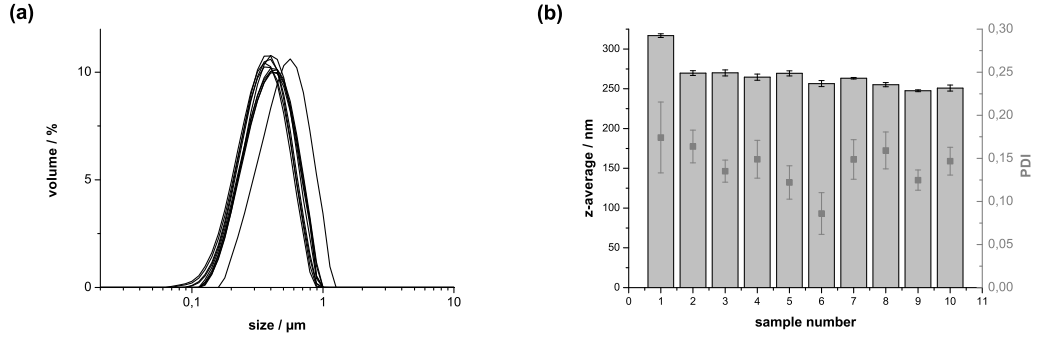

Figure 4.10: Reproducibility of emulsions (n=10) prepared at the same day with the same parameters examined by different methods for particle size determination. Particle size distributions were obtained by static light scattering (a) and Z-average and PDI were determined by dynamic light scattering (b). The formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

248 nm (sample 9) and 270 nm (sample 3) were obtained. PDI-values between 0.09 (sample 6) and 0.16 (sample 2) demonstrated a good quality of the measurements as comparable studies show similar values for emulsions intended for parenteral use [80]. Only one emulsion of the study (sample 1) showed slightly bigger particles with a D[4,3] value of 0.50 μm and a z-average of 317 nm. The particle size distribution itself was similar to the other samples, but was shifted to the right. All in all particle sizes obtained from SLS were about 100 nm bigger than the resulting values from DLS. This is due to the different measurement methods and calculation of the particle size. A more detailed explanation is given in chapter 3.2 and chapter 3.3. Other studies came by with similar values for the volume weighted mean diameter and the z-average of nanoemulsions [3, 71, 99]. After 6 months three of the samples (3,4 and 6) were selected and the particle size was again determined by static and dynamic light scattering. The appearance of all samples was the same as before, a white, homogeneous fluid. The samples showed good stability concerning the particle size, as can be seen in table 4.7. For example sample 4 had changed in the D[4,3] from 0.36 μm to 0.34 μm and in the z-average from 265 nm with a PDI of 0.15 to a z-average of 262 nm with a PDI of 0.15. Particle size distributions did not change (data not shown). Detailed stability studies for other samples are presented in chapter 4.3.3. But as a quick test of the stability the results were promising. Altogether it could be shown that with the optimized parameters the reproducibility of the samples is quite good. Leaving out the time for the SLS and DLS measurements and the preparation of the lecithin it would be possible to produce a lot more samples in a day.

Table 4.7: Results of static and dynamic light scattering for emulsions prepared at the same day with the same parameters directly after preparation and after 6 months for selected samples. The formulations contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

Sample number	Time [months]	D[10] [μm]	D[50] [μm]	D[4,3] [μm]	D[90] [μm]	z-average [nm]	PDI
1	0	0.23	0.46	0.50	0.83	317	0.17
2	0	0.17	0.34	0.37	0.63	270	0.16
3	0	0.16	0.33	0.37	0.62	270	0.14
3	6	0.16	0.34	0.37	0.62	269	0.17
4	0	0.16	0.33	0.36	0.60	265	0.15
4	6	0.14	0.31	0.34	0.60	262	0.15
5	0	0.17	0.33	0.36	0.61	269	0.12
6	0	0.16	0.31	0.33	0.55	257	0.09
6	6	0.13	0.29	0.32	0.56	252	0.16
7	0	0.17	0.33	0.36	0.59	263	0.15
8	0	0.14	0.29	0.32	0.54	255	0.16
9	0	0.14	0.28	0.31	0.52	248	0.13
10	0	0.15	0.30	0.32	0.53	251	0.15

4.2 Flexibility of the system

In chapter 4.1 the development of a formulation to obtain nanoemulsions by preparation with dual asymmetric centrifugation is described. After finding an optimized system the flexibility of this approach should be proved.

4.2.1 Influence of different emulsifiers

For the combination of lecithin and poloxamer 188 (1:1) it was shown that it is possible to form nano-scaled emulsions with DAC. A change of the emulsifiers could lead to more variability of the system. Therefore three emulsifiers commonly used in parenteral formulations were tried to replace the poloxamer 188 [26]. Emulsions were formed with Tween 80, Kolliphor EL and Kolliphor HS15. At least three samples were produced with every emulsifier. First lecithin and soybean oil were prepared in the beaker and heated on the waterbath for 10 min. After that, the second emulsifier was weighted to the system and the beaker was heated on the waterbath for 15 min. Then the addition of the aqueous phase and the mixing steps with DAC followed as described in table 4.1. The emulsions were evaluated by their particle size distribution determined with SLS and the appearance of their intermediate steps and the final product.

In figure 4.11 the droplet size distributions of emulsions with lecithin and Tween 80 (1:1) are presented. The distribution was similar for all samples and showed two peaks with a main peak at about 2 μm. The results for the volume weighted mean diameters were 2.21 μm, 2.10 μm and 2.42 μm and the D[50] values were 2.15 μm,

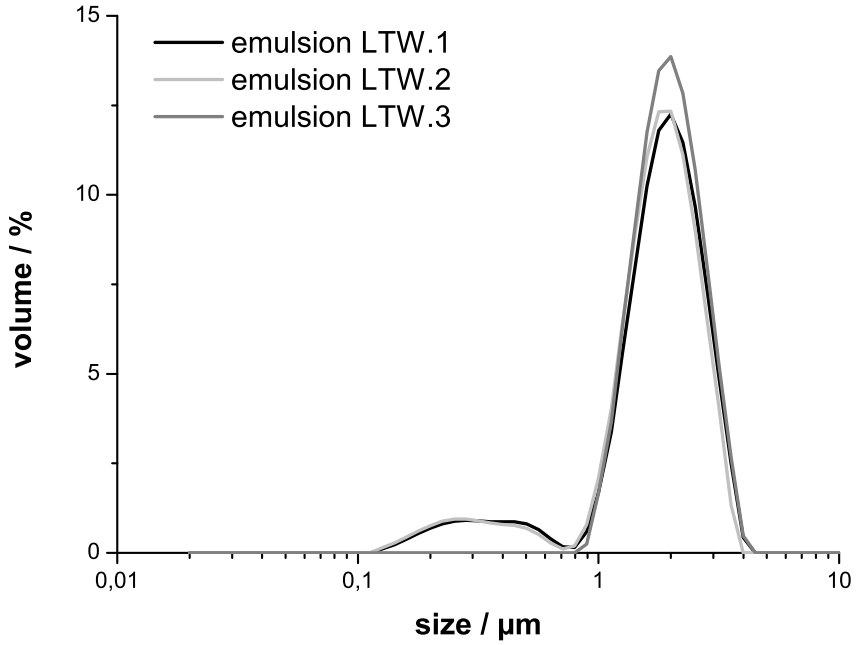

Figure 4.11: Particle size distributions of emulsions produced with 2.5 % lecithin and 2.5 % Tween 80 determined by SLS.

2.05 μm and 2.27 μm. The intermediate steps were different in their appearance compared to emulsions with lecithin and poloxamer 188 where nano-scaled particles were obtained. After the addition of the first part of aqueous phase and mixing with DAC the intermediate step with poloxamer 188 became a homogenous, light yellow, semisolid phase which was stuck to the bottom of the beaker (see figure 4.28). The addition of the second part of aqueous phase and mixing with DAC did not change the system strongly. The intermediate step got a more shiny surface and seemed to be more jelly like, but it remained semisolid. The final product was a homogenous, white fluid. For the formulations with Tween 80 the first intermediate step was not homogenous, dark yellow and semisolid. The phase was not spread over the bottom of the beaker but remained in one edge. Only after the second mixing step it became homogenous and shiny, comparable to the intermediate product with poloxamer 188. The final emulsion was a homogenous white fluid. As the resulting droplet size was not acceptable for an emulsion intended to be applied intravenously, no other attempts to improve this formulation were made.

The droplet size distribution of emulsions with Kolliphor EL is shown in figure 4.12. For the first attempt (figure 4.12(a)) the second emulsifier was added during the heating phase and was not mixed with the lipid phase. Two of the three samples (emulsion LKEL.2 and LKEL.3) had droplet sizes mostly in a range under 1 μm with volume weighted mean diameters of 0.34 μm and 0.31 μm and D[50]

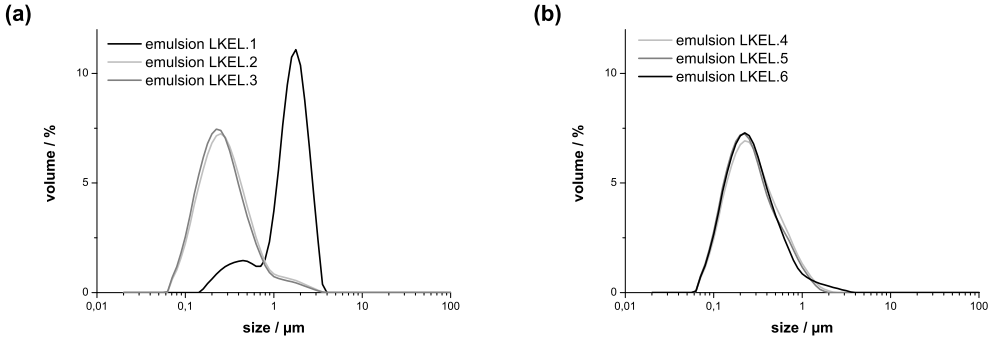

Figure 4.12: Droplet size distributions of emulsions with 2.5 % lecithin and 2.5 % Kolliphor EL as stabilizers determined by SLS. On the left Kolliphor EL was added during the heating process of the lipid phase before the first DAC mixing step. The lipid phase was not homogenized (a). On the right Kolliphor EL was added directly to the lipid phase. The lipid phase was homogenized with a glass rod and heated before the addition of the aqueous phase and mixing with DAC (b).

values of 0.21 μm and 0.19 μm. The third sample emulsion LKEL.1 showed a completely different droplet size distribution with a volume weighted mean diameter of 1.81 μm and a D[50] value of 1.77 μm. An examination of the appearance of the intermediate products showed the differences between the samples before the SLS measurement. Especially the appearance after the first DAC mixing step seemed to be important for the quality of the final product. As for the emulsions with small droplet sizes the first intermediate step was homogenous, light yellow and semisolid. The sample with bigger particles had an inhomogeneous first intermediate step, that was semisolid but with a darker yellow color. The second intermediate step was a homogenous, light yellow, semisolid phase with a shiny surface for all three samples and the final product a white, homogenous fluid. Based on the different appearances of the first intermediate product there was made a slight change in the preparation process to improve the droplet size and the reproducibility of emulsions with Kolliphor EL. The emulsifier was added directly to the lipid phase and was mixed with the lecithin and the soybean oil by using a glass rod. Subsequently the heating process began and afterward the mixing with DAC as described before. The first intermediate step appeared to be a homogenous, light yellow, semisolid phase, as outlined above for emulsions with nano-scaled droplets. As results of SLS measurement smaller particle sizes were presented (figure 4.12(b)) and the reproducibility seemed to be improved. Detailed values can be seen in table 4.8. All samples had smaller volume weighted mean diameters (0.30 μm, 0.28 μm and 0.30 μm) compared to the first method. Additionally the particle size was determined by dynamic light scattering. Z-averages of 209 nm, 212 nm and 210 nm were in line with the SLS measurements. The quality of the DLS measurements was confirmed by PDI values of 0.17 to 0.18 which are in acceptable for emulsion measurements [3, 80]. After 5 months of storage in the refrigerator at 2–8 °C the particle sizes and distributions

Table 4.8: Results of the SLS measurements of emulsions prepared with lecithin and Kolliphor EL (1:1) or lecithin and Kolliphor HS15 (1:1) by the optimized method. The second emulsifier was given to the lipid phase before heating and the lipid phase was mixed with a glass rod. Additionally stability data after 5 months storage at 2–8 °C is shown.

emulsion	D[10] [μm]	D[50] [μm]	D[4,3] [μm]	D[90] [μm]
LKEL.4	0.08	0.20	0.30	0.65
LKEL.5	0.08	0.19	0.28	0.60
LKEL.6	0.08	0.19	0.30	0.59
LKHS.4	0.10	0.27	0.33	0.65
LKHS.5	0.10	0.24	0.27	0.49
LKHS.6	0.11	0.26	0.30	0.54
after 5 months				
LKEL.4	0.08	0.20	0.30	0.64
LKEL.5	0.08	0.19	0.28	0.61
LKEL.6	0.08	0.19	0.31	0.60
LKHS.4	0.09	0.26	0.32	0.64
LKHS.5	0.10	0.23	0.27	0.49
LKHS.6	0.11	0.26	0.29	0.54

did not change (see table 4.8). Furthermore pH and osmolality of the samples were determined to confirm the physiological tolerability of the formulations. With values of 6.35 ± 0.02 for the pH and 305 ± 6 mOsmol/kg both parameters were in an acceptable range compared to formulations that are on the market [7, 17]. As a conclusion from these tests can be derived that Kolliphor EL has the potential to replace poloxamer 188 in the examined formulation and build nanoemulsions by preparation with dual asymmetric centrifugation.

As a third emulsifier Kolliphor HS15 was tested to replace poloxamer 188 in the formulation. When the Kolliphor HS15 was added to the lipid phase during the heating process, emulsions with small droplets were build. The highest D[90] value occurred within emulsion LKHS.1 with 1.39 μm. As shown in figure 4.13(a) the reproducibility was poor. The intermediate steps were similar in their appearance to the intermediate steps from the nanoemulsions described above, but after the first DAC mixing the semisolid phase was not completely homogeneous. To improve the quality of the final emulsions, a slight change in the production process was tried here as well. Kolliphor HS15 was given directly to the lecithin and soybean oil before the heating. The lipid phase was mixed with a glass rod. The production of the emulsion was continued as described before. Results of the SLS measurement are presented in figure 4.13(b). The droplet size distributions of all three emulsions (LKHS.4, LKHS.5 and LKHS.6) were similar and the volume weighted mean diameters were with 0.33 μm, 0.27 μm and 0.30 μm in a good range for emulsions that should be applied on the intravenous route. Measurements with dynamic light scattering were possible here as well and the findings underline the results of the other method. With z-averages of 232 nm, 224 nm and 239 nm the small particle size was confirmed. As

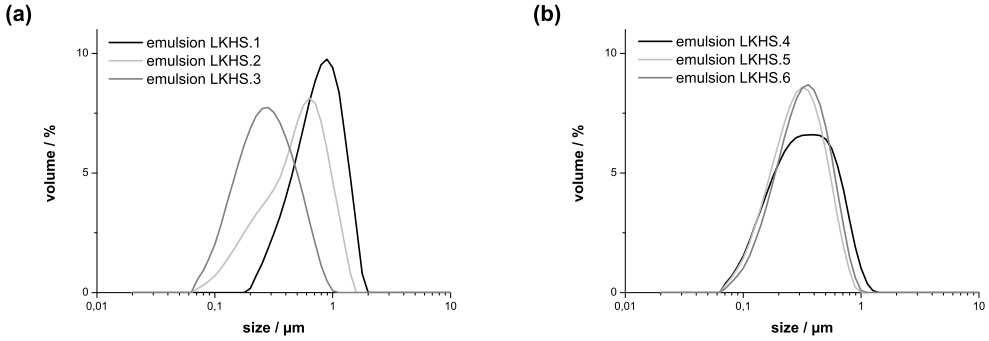

Figure 4.13: Droplet size distributions of emulsions with 2.5 % lecithin and 2.5 % Kolliphor HS15 as stabilizers determined by SLS. On the left Kolliphor HS15 was added during the heating process of the lipid phase before the first DAC mixing step. The lipid phase was not homogenized (a). On the right Kolliphor HS15 was added directly to the lipid phase. The lipid phase was homogenized with a glass rod and heated before the addition of the aqueous phase and mixing with DAC (b).

in emulsions the particle distributions are rather broad the PDI values of 0.18 to 0.20 were in an acceptable range. Determinations of the pH values (5.90 ± 0.02) and the osmolality (324 ± 4 mOsmol/kg) attested a physiological tolerability of the formulations. After 5 months of storage in the refrigerator at 2–8 °C the particle sizes and distributions did not change (see table 4.8). Hence, Kolliphor HS15 could be another possibility to replace poloxamer 188 in the tested formulation.

In summary it was possible to replace poloxamer 188 with another emulsifier in the formulation and still obtaining a nanoemulsion with the optimized preparation process. Due to different characteristics of the stabilizers it was necessary to slightly change the procedure in the preparation of the lipid phase. For the emulsifiers Kolliphor EL and Kolliphor HS15 good results were obtained. Regarding the formulation with the stabilizer Tween 80 rather coarse emulsions were formed with DAC. This result could be anticipated during the preparation process because the intermediate steps were different in their appearance to the intermediate steps before. One reason could be the fluid state of aggregation of the Tween 80. With that it would be more difficult to form a semisolid intermediate step. But in contrast to that the Kolliphor EL was fluid, too. Nevertheless this stabilizer had a higher dynamic viscosity (700–800 mPa s at 25 °C) compared to the Tween 80 (420 mPa s at 25 °C). Another point could be the structure of the emulsifiers. Tween 80 has a typical tenside structure with a hydrophilic head and a lipophilic chain. The other emulsifiers are more complex compounds. Their main components have structures with at least two lipophilic chains (see chapter 2.1.5) that were more similar to the poloxamer 188. Such differences could have lead to varieties in droplet stabilization. The combination of lecithin and Tween 80 was led to droplets about 2 μm.

The other emulsifiers had their stability optimum in combination with lecithin at significantly lower particle sizes.

4.2.2 Influence of the oil

The oil as a solubilizer for the active ingredient is an essential part of the emulsion. With the application of different oils the system could become more flexible for the use of potential drugs. Not only the solubility properties, but also viscosity, sensibility to oxidative substances and physiological tolerability are important points for the decision. In the focus of the investigation were several oils that are currently used in approved parenteral dosage forms, as MCT, olive oil and a mix of MCT/soybean oil 1:1 [7, 17]. For soybean oil, which is also often used in parenteral formulations, the practicability was shown in former experiments. Further sesame oil, corn oil, peanut oil and castor oil were included in the study. Some of them were used before in preparation of fat emulsions because of different polarities of the oils that could enhance the drug uptake [70].

Emulsions were prepared with moistened lecithin (2.5 %) and poloxamer 188 (2.5 %) the way that was worked out as improved way of preparation (see chapter 4.1.4). Every sample contained 10 % oil phase. For each oil or combination of oils at least three emulsions were prepared. The appearance of the intermediate steps was given a closer look and the final products were characterized by SLS. In figure 4.14 the volume weighted mean diameters of the emulsions (part (a)) and the median values of the SLS measurements of the emulsions (part (b)) are compared. In the diagrams the single values are shown. Based on the results olive oil, sesame oil, corn oil and castor oil could be used to form nanoemulsions with DAC. Formulations including MCT, a mixture of MCT/soybean oil (1:1) or peanut oil, resulted in more coarse emulsions. The reproducibility of the particle size of these emulsions was poor. The appearance of the intermediate steps reflected these results. For all oils that formed emulsions with nano-sized droplets, the intermediate steps seemed to be similar, except for the emulsions containing castor oil. After the first mixing step, where the first amount of aqueous phase was given to the oil-emulsifier-mixture, a homogenous, solid, gel like intermediate phase was formed. After the second mixing step the gel became more jelly like with a shiny surface, but also remained solid and homogenous. In the last mixing step the emulsion was formed. For the castor oil emulsions the first intermediate step was also homogenous, solid and gel like, but it was not a flat layer on the ground of the beaker. Instead it formed a sphere in the beaker. That lead to a different second intermediate step, which contained on the one hand the solid, jelly like gel-phase and a bigger part of the former sphere that did not mix with the aqueous phase. Also some time to ”dissolve” the complete intermediate step and obtain a homogenous, white fluid was necessary, but in the end a nanoemulsion was formed without the application of any extra production step. Similar findings were described in chapter 4.1.2 for emulsions that were prepared

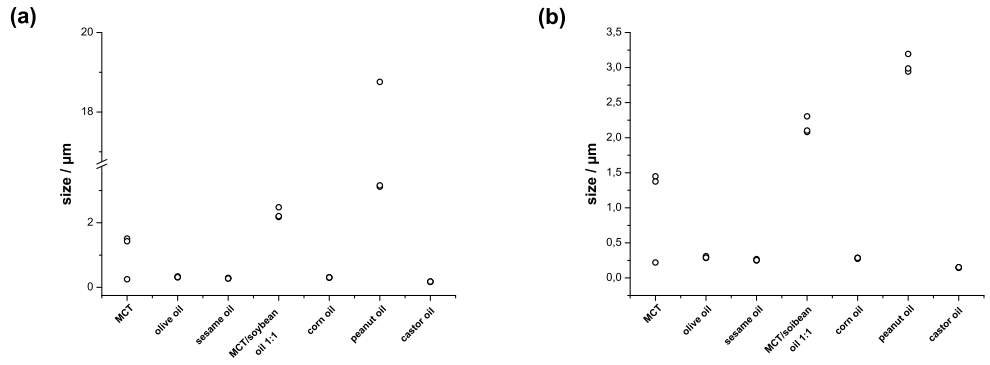

Figure 4.14: Comparison of the volume weighted mean diameters (a) and the D[50] diameters (b) determined by SLS of emulsions containing different oils. All emulsions were composed of 2.5 % lecithin, 2.5 % poloxamer 188, 85 % aqueous phase and 10 % oil phase.

with a higher temperature of the aqueous phase. In contrast to these findings, the formulations with MCT, MCT/soybean oil (1:1) and peanut oil showed different intermediate steps. All of those had an inhomogeneous first intermediate step that was clearly more fluid than for the other formulations. In the second intermediate steps most of the formulations were more homogenous, but here as well the viscosity was lower. In the end a homogenous emulsion was produced, but the particle size was bigger and the distribution broader compared to the other oils (figure 4.14). There was a shift in the D[50]-values and volume weighted mean diameters to higher values as well as non-conformity between the samples. For emulsions containing MCT it became clear within the preparation, that the used lecithin was soluble in the oil. This was proved by the appearance of the oil which normally stayed colorless and clear. In these formulations it became yellow and opaque. Therefore the intermediate step, which seems to be important to create the small particle sizes and the narrow particle size distribution, could not be formed as needed. The peanut oil did not appear to be a good solvent for the lecithin. Nevertheless these formulations did not work out, too. Supposedly the composition of the peanut oil was not optimal to form a stable structure with the applied emulsifiers within this preparation process.

4.2.3 Application of the system

In former chapters a method to prepare nanoemulsions by dual asymmetric centrifugation was found. It was shown that the formulation initially chosen could be varied and the use of different emulsifiers or oil phases also lead to nanoemulsions. As nanoemulsions are used as carrier systems for lipophilic active ingredients it was tested whether a model active ingredient could be included. Three active ingredients with different states of aggregation were used. One was Propofol which is commonly used in parenteral emulsions as intravenous anesthetic agent [35, 37]. Propofol is a clear and slightly yellowish liquid that is only very slightly soluble in water but

soluble in lipids. As another fluid active ingredient Vitamin E-acetate was used. It is a clear, yellow substance which is commonly used in many pharmaceuticals as antioxidant. Nevertheless there are some formulations that are approved to treat vitamin E deficiency. Most of these dosage forms are capsules, but there is also an emulsion containing vitamin E called Vitralipid Adult which is intended for parenteral use [18]. In this study the acetate form was used, as it is more stable than the native form and has a better solubility for lipids [12]. The third model active ingredient was Probucol which has a solid state of aggregation. This active ingredient was developed to treat hyperlipidemia and was available as tablets on the market until 2009. It is barely soluble in water and shows a better solubility in lipids. All active ingredients were incorporated in the formulation described in chapter 4.1.4. Before starting the preparation process the drug was solved in the soybean oil. Then the preparation process was performed as usual.

For Propofol emulsions with different amounts of active ingredient were prepared. On the market several emulsions containing Propofol are available. For example the company B. Braun Melsungen AG distributes the product Propofol®-Lipuro which is a nanoemulsion containing concentrations of 5 mg/ml, 10 mg/ml or 20 mg/ml Propofol [8]. These three concentrations were also used in the following experiments. The active substance was easily miscible with the soybean oil. After preparation the particle size distribution was determined by static light scattering and the results are presented in figure 4.15. The incorporation of Propofol lead to significantly bigger droplet sizes in the emulsions. Droplet size distributions showed one main peak but for all concentrations there were particles in lower regions that lead to a tailing of the peaks. Samples with an amount of Propofol of 5 mg/ml had a main peak at about 2 μm and a smaller peak in the region of 200 nm. The next higher concentration overall showed samples with smaller particle sizes and D[50] values at about 1.3 μm. There was no extra peak but a tailing in the direction of smaller droplets. Formulations with the highest amount of Propofol were rather coarse emulsions. D[50] values at about 20 μm were measured but the distributions showed a tailing to smaller particle sizes as well. Nevertheless all emulsions were not in an acceptable range for parenteral application. This tendency could be expected before the particle size measurement, as the intermediate steps were different to the ones of emulsions with smaller particle sizes described for example in chapter 4.1. Especially for high amounts of active ingredient it could be seen that the mixture of Propofol and soybean oil was able to solve the lecithin. This could be proved by the yellow color of the oil that normally stayed clear and colorless. This phenomenon was described before in chapter 4.2.2. Hence the intermediate steps were more fluid and the shear forces in the DAC were not able to work properly. Another observation was made regarding the poloxamer 188. Normally after heating the molten substance was miscible with the lecithin and the soybean oil. With the addition of Propofol the appearance of lipid phase changed the way that the molten poloxamer 188 was

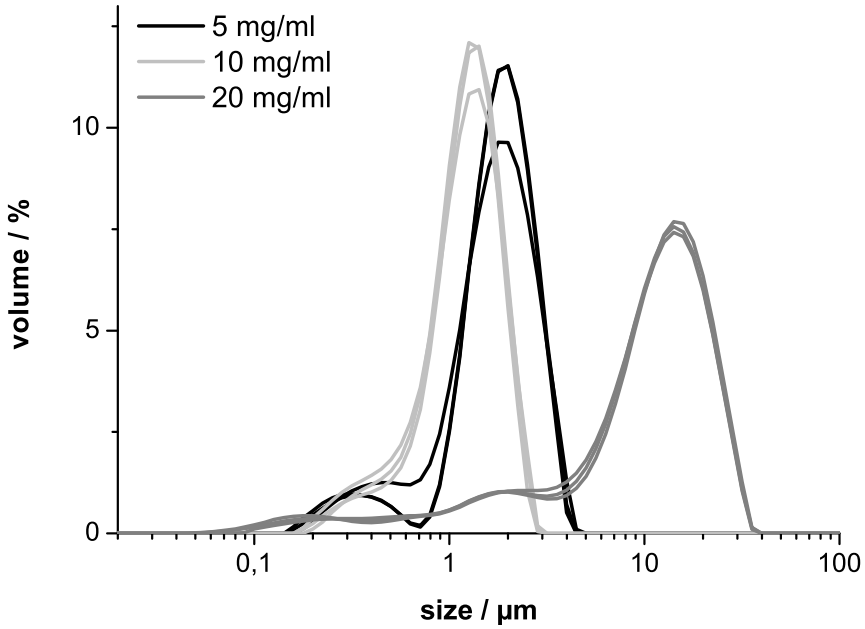

Figure 4.15: Particle size distributions of emulsions containing 5 mg/ml, 10 mg/ml or 20 mg/ml Propofol as model active ingredient determined by SLS.

visible as separate phase. However, the smallest droplet size was formed with the second lowest concentration of active substance.

Emulsions prepared with Vitamin E-acetate had only a small amount of active ingredient incorporated compared to the samples with Propofol. A concentration of 9.1 mg/g was used similar to the product Vitralipid Adult [18]. The substance showed a good miscibility with the soybean oil. Emulsions could be prepared without difficulties. In figure 4.16(a) the results of the static light scattering are presented. Particle size and particle size distribution of the samples were in a good range for parenteral use. There was no difference to emulsions without active ingredient as shown in figure 4.10(a). Each three samples were produced on two different days. All emulsions had volume weighted mean diameters between 0.28 μm and 0.34 μm.

Probucol, the solid active ingredient, was not easily solved in the soybean oil. There were former experiments that suggested a saturation solubility of Probucol of about 90 mg/g in soybean oil [77]. This result was determined by adding the substance to the soybean oil and allowing to equilibrate under gentle rotation for 24 h at 37 °C. For the following tests only 50 % of the saturation solubility was used, as the active ingredient was solved in the oil under gentle rotation at room temperature. The possibility of recrystallization due to temperature differences during the preparation process or storage time should be avoided. Regarding the results of the SLS measurements in figure 4.16(b) there was no difference in particle size and particle size distribution compared to emulsion of the same formulation with-

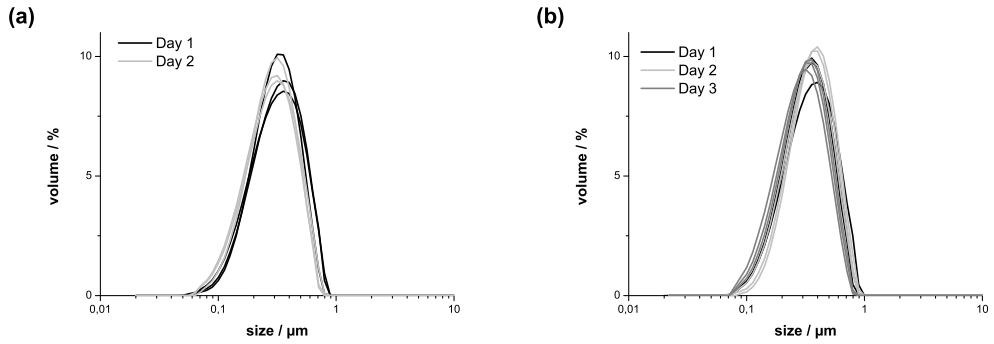

Figure 4.16: Particle size distributions determined by SLS for emulsions containing either Vitamin E acetate (a) or Probucol (b) as model active ingredients.

out Probucol (see figure 4.10(a)). To prove the results each three emulsions with the same amount of Probucol were prepared on three different days. The particle size distributions were all similar and the volume weighted mean diameters of the samples were in a range between 0.26 μm and 0.33 μm. Further characteristics of the samples especially regarding the storage stability are described in chapter 4.3.3.

In conclusion it was possible to show that active ingredients can be incorporated in emulsions prepared by dual asymmetric centrifugation. The emulsions did not change in their appearance compared to emulsions without a model substance. Besides there were no affections on particle size and particle size distribution determined by static light scattering. However one condition was that the active substance was not a solvent for the used emulsifiers, since this prohibits the intermediate step from forming the usual way. Furthermore high amounts of substance as used in the Propofol emulsion with 20 mg/g could lead to difficulties. This was also connected to changes in appearance and viscosity of the intermediate step.

4.3 Further characterization of the system

In chapter 4.1 the development of a method to produce nanoemulsions with dual asymmetric centrifugation was described. The process was optimized to improve the reproducibility. Changes of some emulsion ingredients like oil or emulsifier were done to learn something about the flexibility of the system. Within the development an important characterization method was the particle size of the emulsions determined by static light scattering and later additionally by dynamic light scattering. Furthermore the appearance of the intermediate steps as well as the final product were use to get information about the quality of the sample. Nevertheless for many samples additional characterization steps were carried out. Different methods and results are shown for exemplary studies in this chapter.

Table 4.9: Particle size determined by SLS and DLS before and after centrifugation of emulsions. Samples contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

emulsion	D[10] [μm]	D[50] [μm]	D[4,3] [μm]	D[90] [μm]	z-average [nm]	PDI
A	0.14	0.28	0.30	0.50	235	0.14
A (supernatant)					253	0.14
A (sediment)					186	0.11
B	0.11	0.23	0.26	0.44	216	0.12
B (supernatant)					229	0.12
B (sediment)					190	0.10
C	0.12	0.24	0.27	0.46	222	0.25
C (supernatant)					251	0.14
C (sediment)					188	0.10

4.3.1 Droplet size distribution

Optimized emulsions as described in chapter 4.1.4 were examined with different techniques to get an idea of the distribution of droplet sizes in the formulations. As mentioned above in the discussion of DLS results of these emulsions, it was found that even the emulsions with small particles had a relatively high PDI values. It was shown before that emulsions intended for parenteral application could contain different fractions of droplet sizes [80].

In a first attempt samples were centrifuged to eventually separate a fraction of bigger droplets. Before centrifugation the particle size of the emulsions was determined by SLS and DLS. Detailed values are presented in table 4.9. As expected within SLS measured values for the particle size were higher compared to the values from the DLS technique which was due to the different measurement techniques. Emulsions did not break after the centrifugation process but showed a phase separation which looked like the creaming phenomenon. Creaming describes a reversible floating of oil droplets on the more dense aqueous phase [33]. Hence, it was assumed that the supernatant contained bigger oil droplets than the sediment because of the lower density. Due to the small sample volume of supernatant and the sediment particle size was determined only by DLS (table 4.9). Furthermore all results are summarized in figure 4.17. Oil droplets of the supernatant were, with z-averages between 229 nm and 253 nm, significantly bigger compared to the droplets in the sediment with about 190 nm. Additionally the PDI of the sediment samples was lower which indicated a smaller range in the particle sizes. For the supernatant the PDI did not change in comparison to the complete emulsion. That shows that a strict fracturing was not possible with this centrifugation method as there was a broader range of particles in the supernatant, too.

Another possibility to find out more about the different particle fractions in the emulsion was the application of asymmetrical flow field-flow fractionation. Within

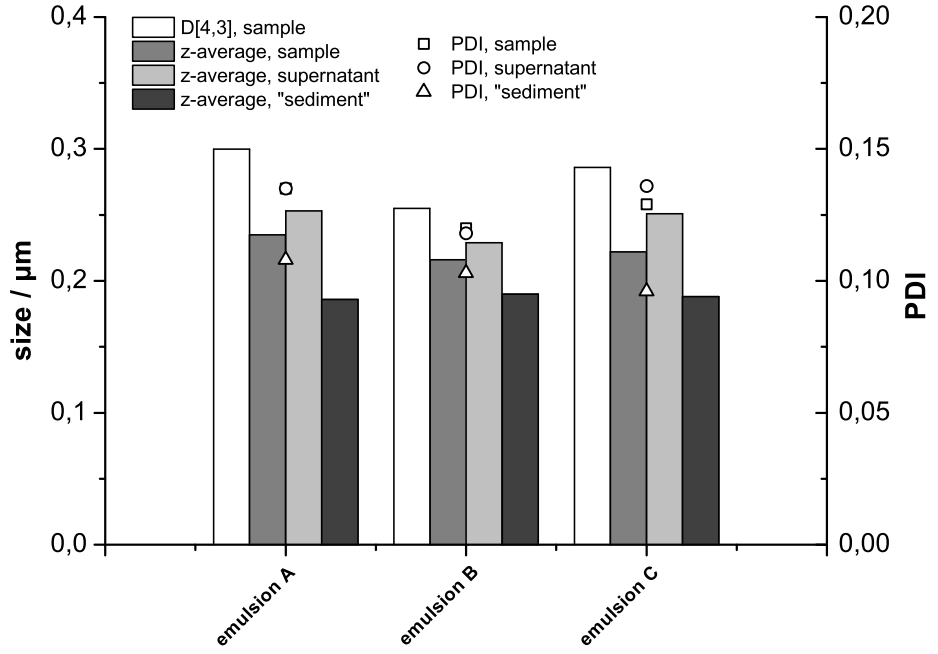

Figure 4.17: Particle size of emulsions and of the centrifugation products determined by static light scattering and dynamic light scattering.

this method different particle fractions can be separated by size [123]. In the beginning a cross flow is applied to concentrate the sample in the membrane. During the elution process small particles are leaving the membrane first as they can diffuse back to the center of the main stream more easily. Bigger particles remain in the pores of the membrane for a longer time and are released later [80]. Detailed information about the used separation method is shown in table 3.1. During separation samples were collected at regular intervals and the particle size of the fractions was determined by dynamic light scattering. The elution profile combined with z-averages of the fractions is presented in figure 4.18. The the geometric diameter values that were derived from the AF4 measurements are presented in this figure as well. They were in good agreement with the values of the hydrodynamic diameter. Two peaks appeared in the elution profile. The first one was the main peak where particles between 200 nm and 400 nm were detected. The highest light scattering detector signal was obtained for a particle size of about 250 nm. The second peak showed a distinctly lower intensity. The highest signal was received at a particle size of about 600 nm. Hence, from this method two statements could be derived. It was possible to detect two particle fractions within the sample. The fraction with the bigger particles was significantly smaller and contained particles mostly under 1 μm. These findings were in good agreement with results for commercially available fat emulsions published before [80].

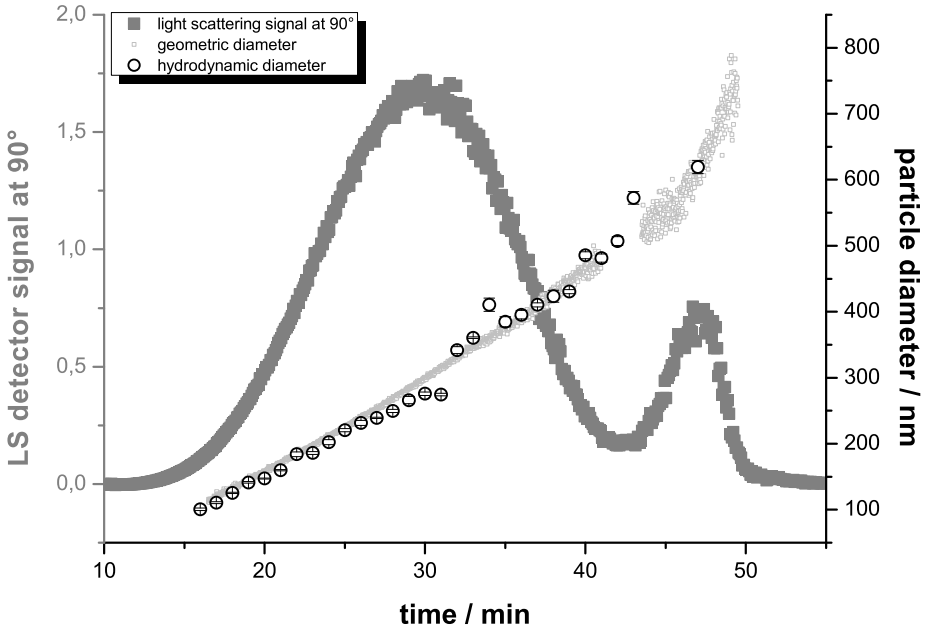

Figure 4.18: Particle size distribution of an optimized emulsion (diluted 1:200 with double distilled water) determined by asymmetrical flow field-flow fractionation. The fractions were collected and measured by dynamic light scattering.

4.3.2 Imaging techniques

Optimized emulsions as described in chapter 4.1.4 were investigated by transmission electron microscopy. As there was used a relatively high stabilizer amount in the formulations it was also searched for structures that were build because of excess emulsifier. First negative staining as preparation method was used. In figure 4.19(a)–(d) typical pictures from this method are presented. Since microscopic pictures only show a small part of the sample statements about the quantity of different particle sizes could not be drawn from this method. Nevertheless fractions of different particle sizes could be detected as described before in chapter 4.3.1. Droplets with diameters of 100 nm to 300 nm which were in good agreement with the results of former particle size measurements were found. Most of them had a spherical shape, but some were also slightly deformed. Besides, spherical particles with significantly smaller diameters than 100 nm were found (figure 4.19(a)-I, (b)-I). However, these very small particles could be an artifact of the preparation method related to the contrast medium uranyl acetate [59, 76]. Furthermore relatively big particles with diameters about 1 μm or even bigger could be found (figure 4.19(a)-II, (d)-II). Interestingly these bigger droplets were of irregular shape though emulsion droplets were expected to be spherical. This phenomenon, too, could be a result of the preparation method. During the application of different solutions as described in chapter 3.10,

the oil droplets could have lost their shape and coalescence could have been occurred. Furthermore some vesicular formed particles were detected as published before for commercially available fat emulsions [9, 43, 88]. These particles, shown in figure 4.19(c)-III for example, could have been formed from excess emulsifier and existed beside the oil droplets.

Another preparation method, the freeze fraction technique, was used to get a second impression of the emulsions. With this method it was possible to confirm the spherical form of the oil droplets (figure 4.19(e)–(h)). Even for bigger droplets no deformation was detected. As mentioned before there appeared to be a rather broad range of particle sizes in the samples. Very small particles under 100 nm could not be found with this method. Hence, these are likely to be uranyl acetate crystals formed as an artifact by the first method. Droplets with diameters of about 100 nm to 200 nm that presented the main fraction of particles according to other measurement methods were found in all samples. Significantly bigger particles with diameters of about 500 nm represented the second fraction. Interestingly droplet size in the fraction of bigger particles seemed to be reduced compared to the negative staining method. This supported the assumption that in the preparation process of the negative staining coalescence of particles occurred. No irregularly shaped particles in the emulsion were found as far as can be derived from the pictures. Few particles were surrounded by shell like layers as shown in figure 4.19(g)-IV. Some others showed a thicker border region that could be formed from an emulsifier bilayer (see figure 4.19(h)-V) [68]. Both forms may be connected with the appearance of vesicles.

4.3.3 Storage stability

An emulsion produced by dual asymmetric centrifugation is intended to include new active substances in a screening process. In addition to the first results, whether it is possible to obtain a stable emulsion or not, there is also the question about the stability of the prepared samples after some days, weeks or even months. Predictions of the stability can only be made if the emulsion itself, without any active ingredient, was examined regarding this issue. First attempts were made with emulsions containing lecithin and sodium oleate or lecithin and poloxamer 188 as emulsifiers. Detailed compositions of the samples are shown in table 4.2. All samples from this study are named with a (2) after their label as it was the second time these emulsions were prepared. For stability studies every emulsion was prepared at least three times and examined by different characterization methods directly after preparation and after 3, 6, and 9 months. Emulsions were stored under cool conditions (2–8 °C) in a refrigerator. Static light scattering measurements gave information about the particle size distribution. Another stability parameter was the zeta potential of the emulsions. For physiological tolerability pH-values and osmolality values were measured. All of the samples had relatively big droplets and were not suitable for

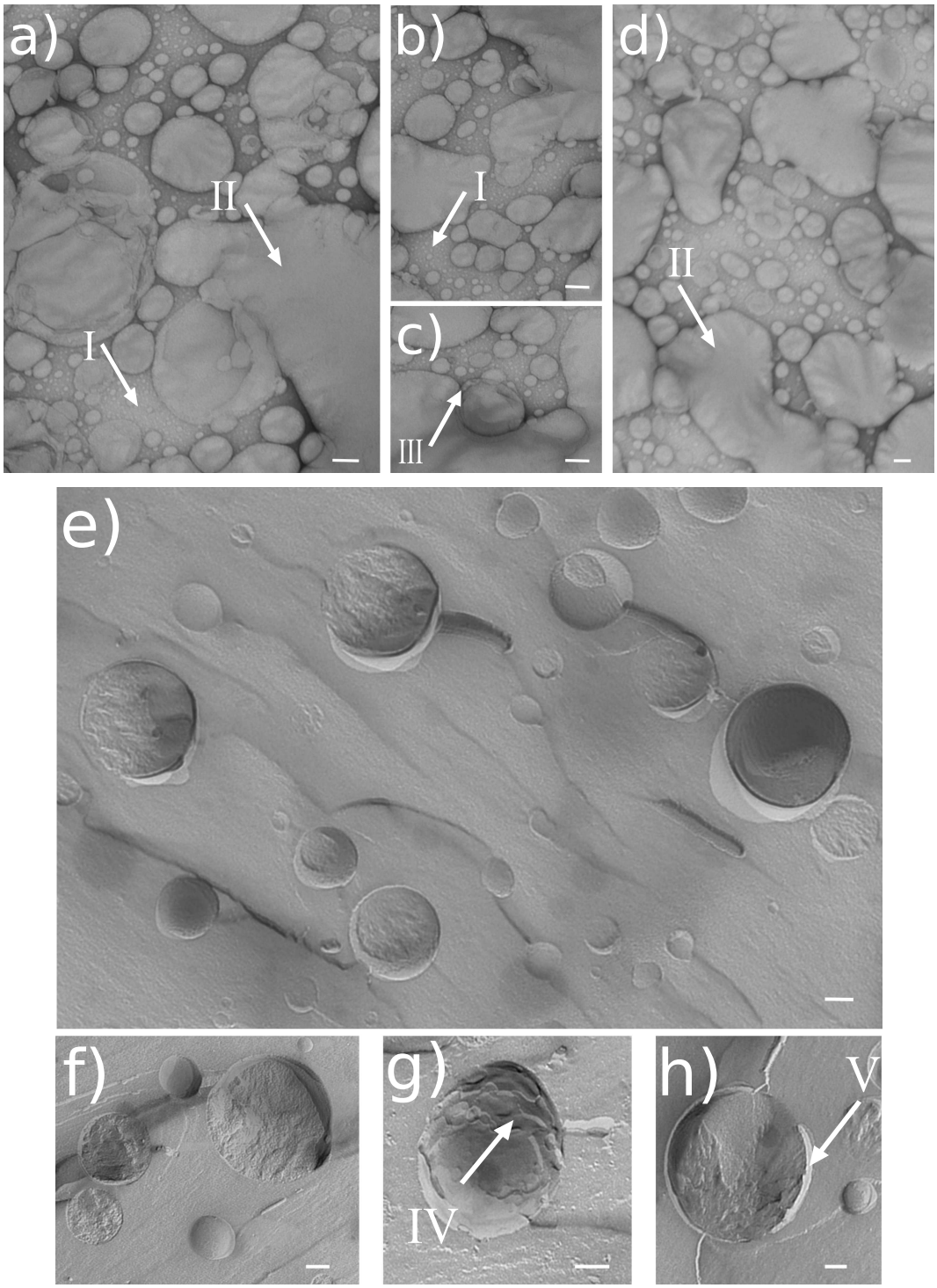

Figure 4.19: TEM-pictures of an optimized emulsion containing 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase. Samples were negatively stained (a - d) or prepared by freeze fracture technique (e - h). White bars indicate 100 nm. Highlighted structures are: I–artifacts assumed to be related to uranyl acetate, II–bigger emulsion particles about 1 μm, III–vesicular formed particles, IV–particles surrounded by shell like layers and V–particles with a thicker border region.

Table 4.10: Average volume weighted mean diameters (D[4,3]) of fat emulsions after different storage periods determined by SLS. Detailed composition of the samples is presented in table 4.2.

emulsion	D[4,3] [μm] after preparation	D[4,3] [μm] after 3 months	D[4,3] [μm] after 6 months	D[4,3] [μm] after 9 months
LS.1(2)	24.03 ± 0.72	24.00 ± 0.62	23.72 ± 0.40	22.27 ± 2.15
LS.2(2)	2.32 ± 0.20	2.28 ± 0.22	2.37 ± 0.26	2.33 ± 0.24
LS.3(2)	0.80 ± 0.05	0.80 ± 0.05	0.80 ± 0.05	0.80 ± 0.06
LS.5(2)	0.75 ± 0.03	0.74 ± 0.04	0.75 ± 0.04	0.72 ± 0.03
LS.6(2)	2.67 ± 0.18	2.65 ± 0.17	2.74 ± 0.14	2.77 ± 0.19
LS.7(2)	37.18 ± 2.13	34.87 ± 2.88	34.59 ± 4.61	35.18 ± 2.72
LS.8(2)	49.70 ± 1.29	not stable	not stable	not stable
LPX(2)	0.81 ± 0.06	0.80 ± 0.06	0.81 ± 0.05	0.79 ± 0.06
LPX.S1(2)	0.75 ± 0.16	0.77 ± 0.16	0.78 ± 0.16	0.77 ± 0.16
LPX.S2(2)	0.76 ± 0.08	0.73 ± 0.08	0.73 ± 0.08	0.72 ± 0.08

parenteral administration. Furthermore a high concentration of sodium oleate is known to be hemolytic. Nevertheless the stability of these emulsions was examined to see, if emulsions prepared by dual asymmetric centrifugation were stable over several months at all. Some emulsions showed a creaming effect after storage which is characterized by Collins-Gold *et al.* [33] as a readily reversible, slow flotation of lipid droplets on the more dense aqueous phase. These samples were shaken gently before the measurement to achieve a homogeneous dispersion. The creaming phenomenon was observed for emulsions LS.1, LS.2, LS.6 and LS.7. Gentle shaking could reverse this effect temporarily. Emulsion LS.8 was not stable over storage time. After 3 months there were visible oil droplets on the surface. Hence the formulation was not investigated any longer. All other emulsions did not change significantly in their appearance.

In table 4.10 and figure 4.20(a) detailed values as well as an overview of the results of the static light scattering are given. Regarding the diagram with the average volume weighted mean diameters the measured values for samples that showed no creaming effect lay close together with a low standard deviation about 0.03 μm up to 0.08 μm, except sample LPX.S1(2) that showed a slightly higher standard deviation with about 0.16 μm for all measurements. Other samples had more variation in the measured volume weighted mean diameters but these were not that high and proportional to their values. For example sample LS.7(2) had a D[4,3] of 37.18 ± 2.13 μm directly after preparation and changed to 35.18 ± 2.72 μm after 9 months. For sample LS.6(2) 2.67 ± 0.18 μm was the D[4,3] directly after preparation and 2.77 ± 0.19 μm after 9 months. With respect to the particle size the stability of most samples could be proved. A distinct growth of particles in the emulsions could not be found.

Considering the pH-value for most of the emulsions, it was found a similar trend for all samples, except the formulation that contained only lecithin and poloxamer 188

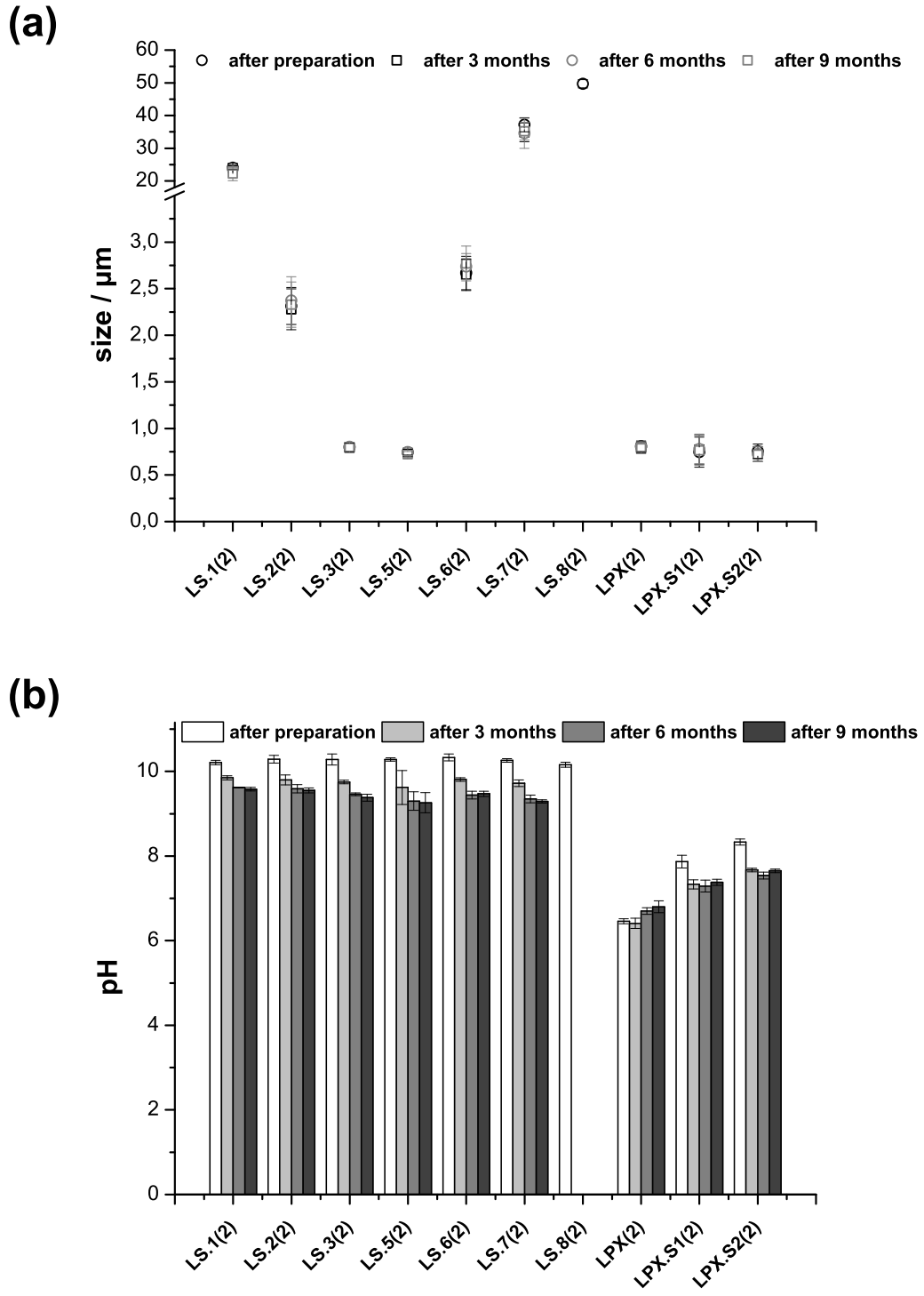

Figure 4.20: Volume weighted mean diameters (a) and pH values (b) of different emulsions with lecithin and sodium oleate as stabilizers stored over 9 months. Detailed composition of the samples is presented in table 4.2.

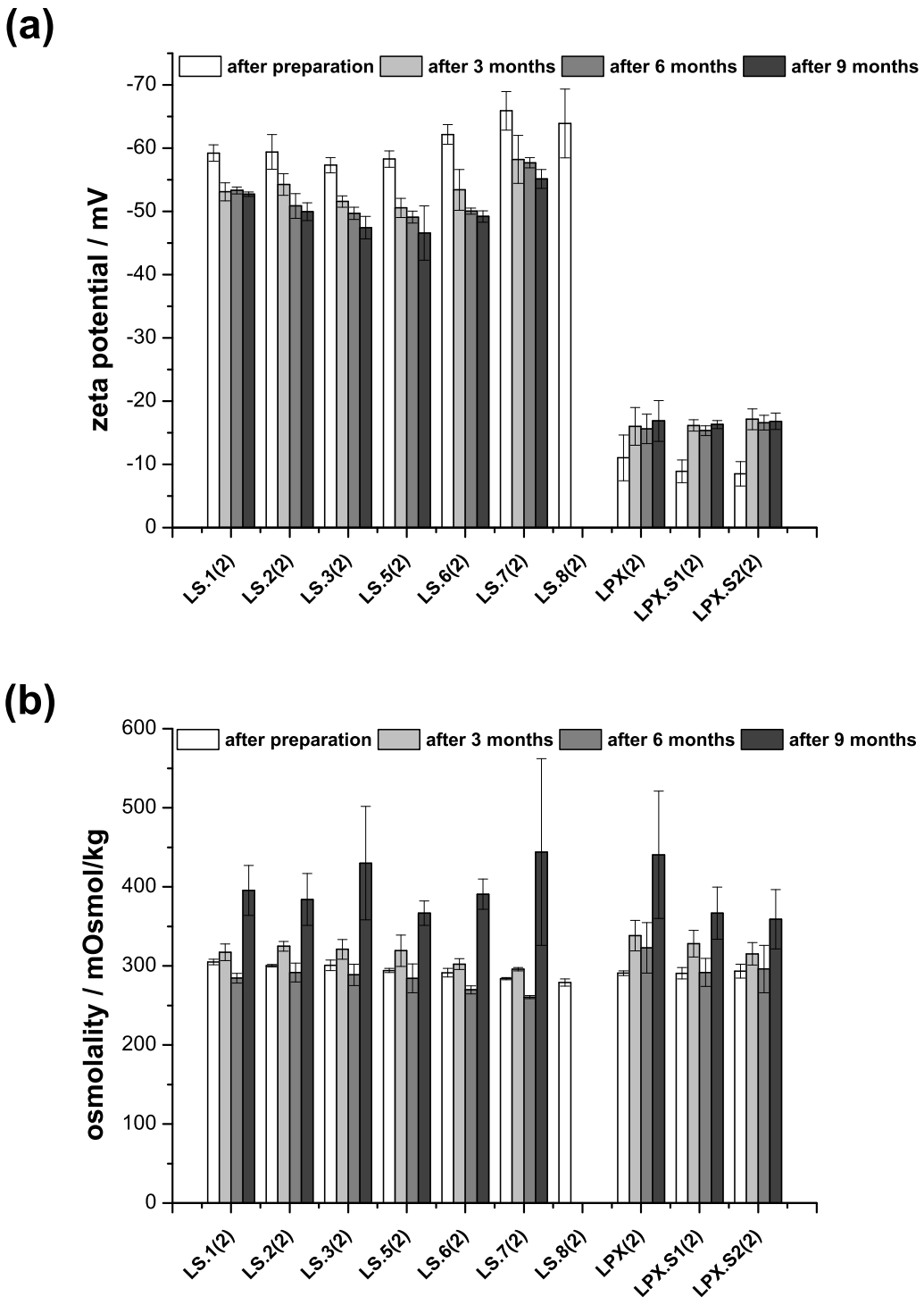

Figure 4.21: Zeta potential (a) and osmolality (b) of different emulsions with lecithin and sodium oleate as stabilizers stored over 9 months. Detailed composition of the samples is presented in table 4.2.

as emulsifiers (sample LPX(2)) (figure 4.20(b)). The first measurement directly after preparation resulted in a higher pH-value compared to the following measurements. Emulsions that contained more than 1 % sodium oleate (sample LS.1(2) - sample LS.7(2)) additionally showed a slight decrease of the pH from 3 months to 6 months. After that the pH did not change significantly. A decrease of the pH could be explained by release of oleic acid that comes from the sodium oleate which was used as stabilizer. Normally the equilibrium state between the protonated oleic acid and its deprotonated form is reached very fast. However in complex systems like emulsions changes can occur over a longer period of time due to the fact that the pK_a-value of oleic acid strongly depends on its surrounding [60]. Different pK_a-values between 4.2 and 9.85 were published before for oleic acid depending on the pH values of the surrounding and the structures in to which the oleic acid was bound [75, 79, 107, 116]. Formulations with only small amounts of sodium oleate of 0.03 % (samlple LPX.S1(2)) or 0.06 % (sample LPX.S2(2)) presented a decrease of the pH likewise. Even though the amount of sodium oleate was clearly lower and with that the pH values of the samples, the trend was similar comparing the values directly after preparation and after 3 months. Over a longer storage time the pH did not change significantly for these samples. Only sample LPX(2) showed another profile, because as emulsifiers only lecithin and poloxamer 188 were used. Over storage time only a slight shift to higher pH values was detected. This could be due to a slight degradation of the emulsifier lecithin which released fatty acids. Another explanation could be that in the flexible system of an emulsion the equilibration of the emulsifiers needed some time. Fatty acids that were first involved in the stabilization of the oil droplets were released over time.

Regarding the zeta potential of the emulsions there was a difference between formulations containing sodium oleate and lecithin as emulsifiers (sample LS.1(2) - sample LS.7(2)) and those that contained poloxamer 188 and lecithin as main stabilizers (sample LPX(2), LPX.S1(2) and LPX.S2(2)). Figure 4.21(a) shows that all emulsions prepared with a relatively high amount of sodium oleate (minimum 1 %) showed a high initial zeta potential of about -60 mV. Over storage time the zeta potential was increasing within these samples. Especially after the first 3 months there was a rise of 5–10 mV. Subsequently the values increased only slightly or remained stable (sample LS.1(2)). This effect could be correlated with the pH values, that showed a similar trend over storage time. As before an explanation could be that the ratio between the stabilizer sodium oleate and its acid changed over storage time because of the sensitivity of the pK_a-value of this compound to its surrounding. Both the sodium oleate and the oleic acid are ionic molecules and hence have an influence on the zeta potential. In contrast samples with poloxamer 188 and lecithin as main emulsifiers showed a clearly higher zeta potential of about -8 mV directly after preparation. After the first 3 months the zeta potential decreased to roughly -16 mV for all samples (sample LPX(2), LPX.S1(2) and LPX.S2(2)) and did not

change significantly over the remaining time. The reason for this decrease of zeta potential could be possibly explained by an equilibration of the emulsifiers during storage time. Another explanation could be the partial hydrolysis of the lecithin in the aqueous phase as described by Grit et al. [53] which results in a stronger charge of the droplet surfaces. Nevertheless a sterical stabilization mechanism for formulations containing poloxamer 188 and lecithin through an intercalation of the poloxamer 188 in the lecithin bilayer has been described [129]. As a consequence stabilization through ionic forces did not play an important role in these formulations and measurements of the zeta potential are of minor importance. Additionally even if the decrease of the zeta potential seemed to be relatively big, changes in the measured values should not be overestimated. The small amount of sodium oleate in sample LPX.S1(2) and sample LPX.S2(2) seemed not to have an influence on the zeta potential.

Last the osmolality of the emulsions was investigated. Figure 4.21(b) shows the results of the measurements. All emulsions had initial osmolality values of about 280–300 mOsmol/kg that were acceptable for parenteral administration [72]. Measurements after 3 months showed a rising of the osmolality values for all samples. Values increased about 20 mOsmol/kg for samples with lecithin and sodium oleate as emulsifiers and about 30–40 mOsmol/kg for emulsions containing poloxamer 188 as second emulsifier. This could be explained by a slow degradation process or by a relocation of emulsifier molecules. After a storage time of 6 months all values decreased again. This was not expected and did not follow the trend, but could be explained by the change of the measurement equipment. At the last measurement time point all samples had clearly higher values for the osmolality of about 350–450 mOsmol/kg and the standard deviations were broad, too. These findings indicated an instability of the samples. The osmolality is only dependent on the number of solved particles in the formulation. If measured values change dramatically the assumption could be made, that some degradation processes started in the samples. That was also the reason, why after 9 months the observation of the emulsions was stopped.

From the presented data it is possible to conclude that emulsions prepared by DAC were stable over some months. Even with particle sizes that were not in an acceptable range for parenteral administration, it was possible to store the emulsions up to 9 months without particle growth. Nevertheless, regarding the osmolality measurements a degradation process could be assumed between 6 months and 9 months storage time for all emulsions. Other changes in the pH and the zeta potential could be connected to the presence of the stabilizer sodium oleate, which was used together with lecithin as a model emulsifier in the development of these formulations. However, emulsions seemed to be stable over 6 months. In further studies sodium oleate was replaced in the formulation with other stabilizers, mostly poloxamer 188.

Table 4.11: Volume weighted mean diameters and z-averages of emulsions with lecithin and poloxamer 188 as stabilizers measured directly after preparation and after 6 months.

sample	D[4,3] [μm]	z-average [nm] after preparation	PDI	D[4,3] [μm]	z-average [nm] after 6 months	PDI
A.1	0.40	282 ± 2	0.14	0.40	270 ± 2	0.18
A.2	0.65	359 ± 4	0.20	0.59	352 ± 2	0.23
A.3	0.32	252 ± 3	0.14	0.29	232 ± 3	0.15
B.1	2.82			2.82		
B.2	0.79			0.80		
B.3	0.80			0.83		

For some selected emulsions containing poloxamer 188 and lecithin as emulsifiers stability data was upraised. These emulsions were prepared by the optimized method described in chapter 4.1.4 and represented the optimized emulsions received by preparation with DAC. All samples contained 2.5 % poloxamer 188, 2.5 % lecithin, 10 % soybean oil and 85 % aqueous phase. In the preparation process a ratio aq/em of 0.7 was used. The formulations can be divided in samples A.1, A.2 and A.3 which were prepared with moistened lecithin (3.65 % absorbed water) and samples B.1, B.2 and B.3 that contained lecithin which was not allowed to absorb water from the environment. Time points of measurement were directly after preparation and after 6 months of storage in the refrigerator at 2–8 °C. As already presented in chapter 4.1.3.1 lecithin that had absorbed water could be processed more easily and therefore emulsions with smaller particles sizes were formed compared to emulsions with unprocessed lecithin. In table 4.11 specific values of the particle size measurements are presented and an overview of the results of all characterization methods is shown in figures 4.22 and 4.23. Regarding the particle size of the samples emulsions A.1 and A.3 have acceptable D[4,3] values for nanoemulsions, sample A.2 showed a higher D[4,3] value. For all these emulsions it was possible to additionally measure the particle size with dynamic light scattering. As expected the z-averages were lower than the volume weighted mean diameters. After 6 months there were only slight changes in particle sizes. Almost the same was found for the particle sizes of emulsions B.1, B.2 and B.3. Even if for sample B.1 particles were clearly bigger than for the other samples. Measurements of the pH and osmolality complemented one another. As the pH decreased slightly over storage time the osmolality values rose. These negligible changes could be explained by a release of free acids from the emulsifier lecithin that entail a lower pH and heighten the number of soluble particles in the formulation resulting in a higher osmolality. The results of the zeta potential measurements were difficult to evaluate. As described before for emulsions with lecithin and poloxamer 188 as stabilizers, values for the zeta potential are not that meaningful. The main stabilizing mechanism is via steric forces. Hence the

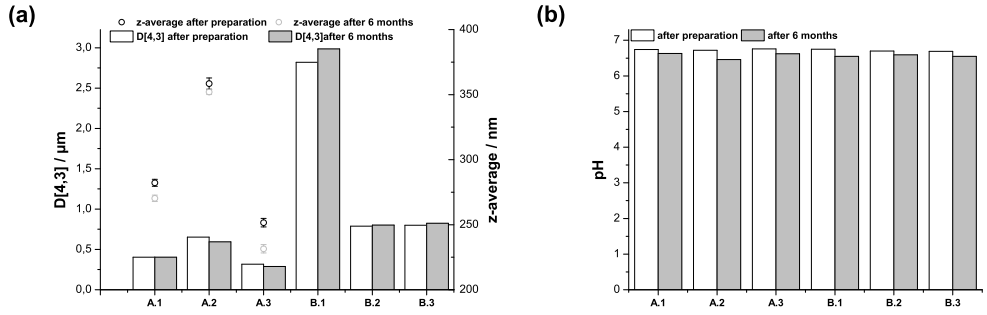

Figure 4.22: Particle size determined by SLS and DLS (a) and pH values (b) of emulsions containing 2.5 % lecithin and 2.5 % poloxamer 188 as emulsifiers stored over 6 months.

increasing of the zeta potential over storage time should not be overestimated, but could also be a result of released acids from the emulsifier. All in all the stability of the emulsions could be shown for several formulations over a storage time of 6 months. As long as the droplet size does not change to critical values and the other parameters stay in a tolerable range, a slight change in some measured values can be accepted as emulsions are complex, thermodynamically unstable systems.

4.3.4 Physiological tolerability of the system

The hemolytic activity is an important parameter for in-vitro evaluation of the physiological tolerability of the emulsions. As nanoemulsions prepared with the DAC should be used as parenteral injections, a low hemolytic activity was desired. Several formulations were investigated: emulsion LS.3, LPX, LPX.S.1 and LPX.S.2 and Lipofundin® 10 % N as a reference. They all contained 2.2 % glycerol (glyc) as isotonic agent. This amount of glycerol was found to suit best the osmotic conditions measured in human blood for the emulsions [134]. Though an aqueous solution of 2.2 % glycerol had slightly lower osmolality values (see table 4.12). Furthermore

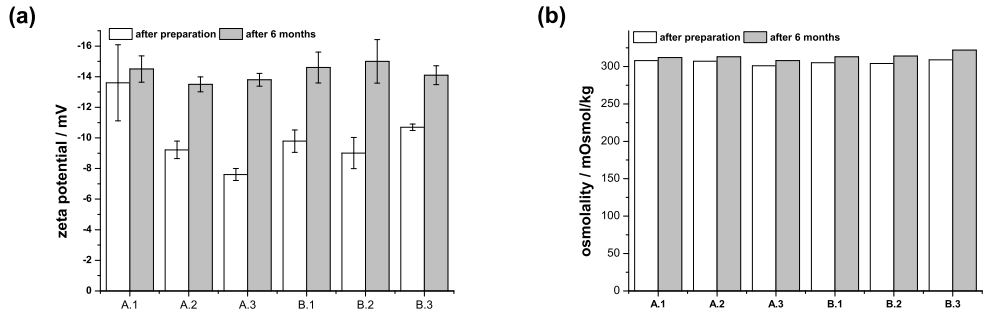

Figure 4.23: Zeta potential (a) and osmolality (b) of emulsions containing 2.5 % lecithin and 2.5 % poloxamer 188 as emulsifiers stored over 6 months.

Table 4.12: Hemolytic activity and osmolality of different emulsion formulations.

emulsion	hemolytic activity [%]	osmolality [mOsmol/kg]
LS.3	47.8 ± 10.8	301 ± 7
LS.3 (sorb)	42.7 ± 1.7	324 ± 8
LPX	14.5 ± 3.4	293 ± 5
LPX (sorb)	3.0 ± 0.4	324 ± 9
LPX.S.1	12.3 ± 1.3	291 ± 7
LPX.S.2	13.2 ± 3.3	293 ± 9
Lipofundin® 10 % N	8.8 ± 1.5	340 ± 2
glycerol 2.2 %	31.6 ± 2.4	242 ± 1
sorbitol 4.5 %	0	259 ± 3
PBS buffer	0	283 ± 2
SDS solution 2 %	100	21 ± 1

all formulations contained 0.02 % sodium azide to prevent microbiological growth. Details of the composition of the investigated emulsions are shown in chapter 4.1 in table 4.2. Additionally two of the samples, emulsion LS.3 and emulsion LPX, were prepared with 4.5 % sorbitol (sorb) as isotonic agent. It was reported before that glycerol as isotonic substance could lead to hemolysis and within this article 4.5 % sorbitol was suggested as an alternative isotonic substance [72]. The authors also showed that emulsion formulations containing glycerol were able to reduce the hemolytic effect compared to aqueous glycerol solution [73]. As a consequence, the hemolytic activity of aqueous solutions containing either 2.2 % glycerol or 4.5 % sorbitol was compared in this study, too. For all formulations at least 3 samples were prepared.

In figure 4.24 the average results of the hemolytic assay are shown. Additionally the osmolality of all samples was determined and is presented in the diagram as well. Detailed values are given in table 4.12. All emulsions had acceptable osmolality values between 291 mOsmol/kg and 340 mOsmol/kg. With respect to hemolysis the samples showed different results. Only two of the emulsions (LS.3 and LS.3(sorb)) had higher hemolytic activities of 47.8 % and 42.7 %. This was expected before as these formulations contained a relatively high amount of sodium oleate as stabilizer. Because of previous pH measurements with values of about 10 to 11 (see chapter 4.1 and chapter 4.3.3) the physiological tolerability for these emulsions was not given. This observation could be explained by the fact that sodium oleate is able to incorporate in the membrane of the erythrocyte cells and with that cause an instability that leads to ruption of the blood cells. Emulsions with poloxamer 188 as stabilizer instead of sodium oleate showed a distinctly lower hemolysis which can be traced back to the replacement of the emulsifiers, as this was the one thing that changed in the formulation. Formulations which contained only small amounts of sodium oleate (emulsions LPX.S1 and LPX.S2) had hemolytic activities similar to emulsion LPX. For all these samples acceptable pH values could be determined (see chapter 4.1

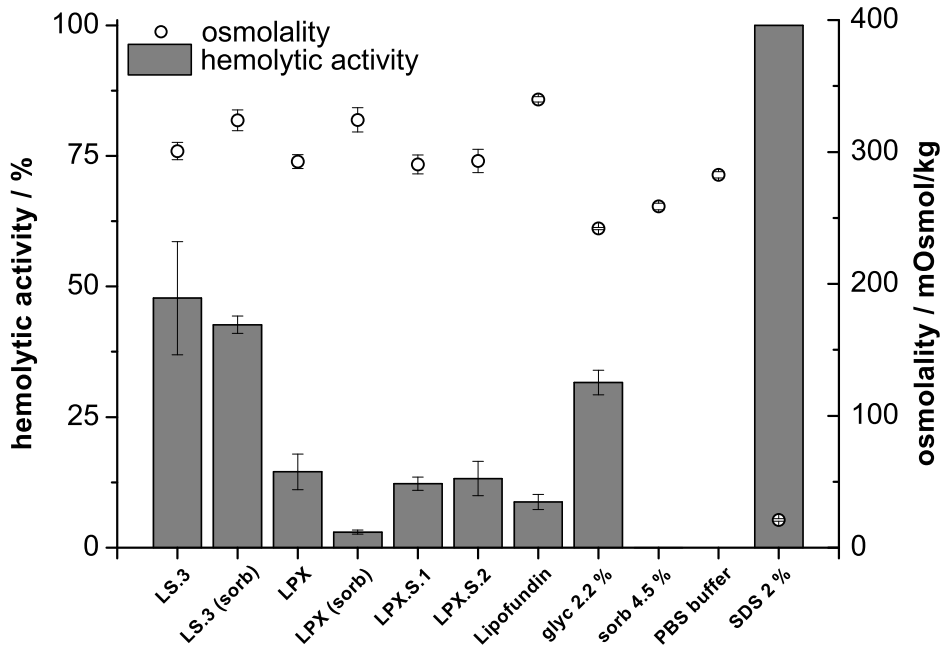

Figure 4.24: Hemolytic activity and osmolality of selected emulsions as well as aqueous solutions of 2.2 % glycerol and 4.5 % sorbitol, Lipofundin® 10 % N, PBS buffer and SDS solution. Composition of the emulsions is shown in table 4.2.

and chapter 4.3.3). The reference Lipofundin® 10 % N also showed hemolysis of about 8 %. Similar results were found before [73]. Although these results seem to be surprising the physiological tolerability of Lipofundin® 10 % N was proved, as this is a formulation that is used in patients a lot and hemolysis events in the clinical application were not reported by now. The in-vivo tolerability was explained by Jumaa and Müller with a slow, drop-wise injection of the emulsions. Hence, the body had time to adapt to the injection and a dissolution process would increase the physiological tolerability [73]. These assumptions could be applied to the examined samples in this study, too.

Different hemolytic values when comparing the isotonic agents were found. Samples with glycerol (emulsion LPX) had higher hemolytic values of about 14 % and in contrast to that emulsions with sorbitol (emulsion LPX(sorb)) showed marginal hemolytic activity of about 3 %. All in all emulsion LPX(sorb) containing 4.5 % sorbitol showed the lowest hemolytic activity and the values were even lower than for the reference. The aqueous solution of 2.2 % glycerol had a high hemolytic activity, about 31 %, but within the emulsion formulations containing glycerol, hemolysis was clearly reduced. An aqueous solution of 4.5 % sorbitol did not show any hemolytic activity. Again these results were surprising but other workgroups also found hints regarding hemolysis in presence of glycerol [73,132]. Nevertheless assumptions about the hemolytic activity of glycerol have to be made carefully as the topic is discussed

critically in literature. Nagasaka and Ishii for example did not find hemolysis in isotonic glycerol solutions of blood cells from three different animals. They also investigated the influence of emulsions to these cells and deduced that hemolysis was caused by excess emulsifier that was not used to stabilize the oil droplets in the formulations [100]. Bakaltcheva and co-workers found out that erythrocytes undergo shape transformation after incubation with glycerol which made the cells more prone to hemolysis [10]. This phenomenon was found to be temperature-dependent as with higher temperatures hemolytic activity increased. The authors explained the changes in the blood cells with an intercalation of the glycerol in their membrane. This is supported by Jumaa and Müller who found out that in presence of an emulsion the hemolysis of glycerol was reduced, maybe due to the glycerol interacting with the oil droplets [73]. Glycerol was also shown for being non-lytic in solutions for which osmolality was created by use of sodium chloride and glucose [115]. All in all the issue is not completely understood. But regarding the practical use of parenteral fat emulsions in vivo hemolysis due to isotonization with glycerol seems not to play an important role.

4.3.5 Autoclaving method

Emulsions for parenteral administration have to be sterile. Several methods to obtain sterile formulations are described in the European Pharmacopeia [40]. As favored way to sterilize aqueous formulations the autoclaving method is mentioned. Not only is an aseptic way of preparation necessary, the formulations also have to be sterilized in their primary packaging. In the following study selected samples were tested for stability in an autoclaving process. Each three samples with a volume of 5 ml containing different emulsifiers or an active ingredient were examined. Poloxamer 188, Kolliphor EL and Kolliphor HS15 were used as stabilizers in combination with lecithin (always 1:1, weighted ratio). More extensive descriptions can be found in chapter 4.2.1. Other emulsions with lecithin and poloxamer 188 as emulsifiers and Probucol as model active ingredient were tested (for more information see chapter 4.2.3). Detailed compositions of all formulations are summarized in table 4.13. Before and after sterilization the particle size distribution of the emulsions was determined by static light scattering. Additionally osmolality, pH values and appearance of the samples were investigated. Samples were stored in glass vials with rubber stoppers sealed with crimped aluminium caps. Emulsions were autoclaved for 20 minutes at 121 °C with a steam pressure of 2 bar.

Before the autoclaving treatment all samples were white, homogeneous fluids. Droplet sizes were in an acceptable range for nanoemulsions as can be seen in figure 4.25. The droplet size distributions were narrow, except for the formulations containing lecithin and Kolliphor EL as stabilizers. These emulsions contained small droplets as the average D[50] value was the lowest compared to the other formulations with 0.19 $\pm$ 0.01 μ (table 4.14), but these emulsions also had a broader

Table 4.13: Composition of formulations treated by the autoclaving method. All emulsions contained 2.5 % lecithin, 10 % soybean oil and 85 % aqueous phase.

emulsion	poloxamer 188	Kolliphor EL	Kolliphor HS15	Active ingredient
LPX	2.5 %	-	-	-
LKEL	-	2.5 %	-	-
LKHS	-	-	2.5 %	-
LPX.AI	2.5 %	-	-	Probucol

distribution peak with the highest D[90] value of 0.62 ± 0.02 μm. Hence, the presence of bigger particles around 1 μm was likely. For all samples pH and osmolality values were determined and in an acceptable range (figure 4.27). For emulsions containing Kolliphor EL and Kolliphor HS15 the osmolality values differed for each sample. Therefore these values were shown in detail and not given as an average value to better judge the changes after the autoclaving process. After the sterilization treatment both formulations with the stabilizer combination of lecithin and poloxamer 188 did not change their appearance. A comparison of the measured particle size before and after the treatment confirms that neither the droplet size nor the droplet distribution changed significantly (figure 4.26(a)). Regarding osmolality and pH, there was no change in the osmolality of the emulsions but a decrease in the pH values from 6.72 ± 0.07 to 6.31 ± 0.04 for formulations without active ingredient and from 6.61 ± 0.03 to 5.60 ± 0.01 for formulations containing the ac-

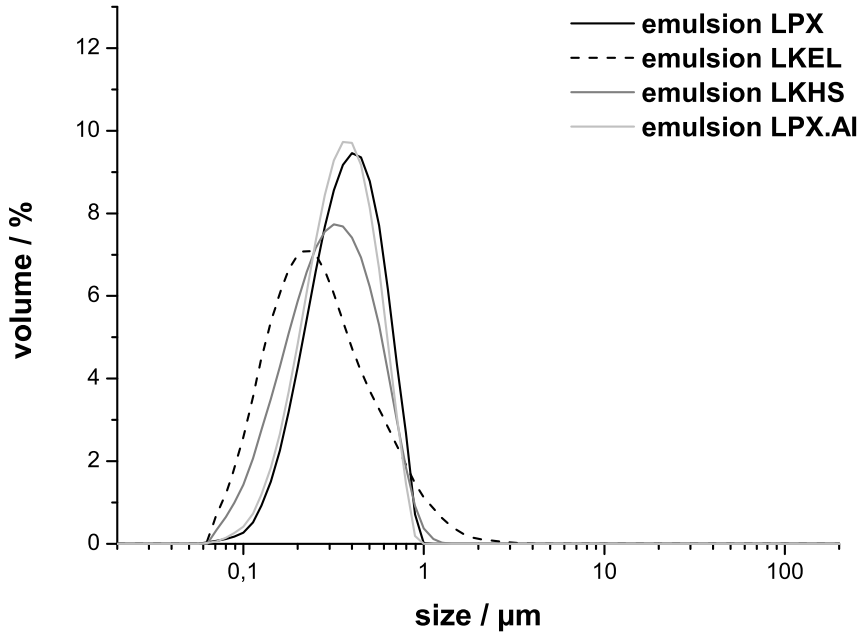

Figure 4.25: Particle size distribution determined by SLS of different emulsions before autoclaving. Detailed composition of the samples is shown in table 4.13.

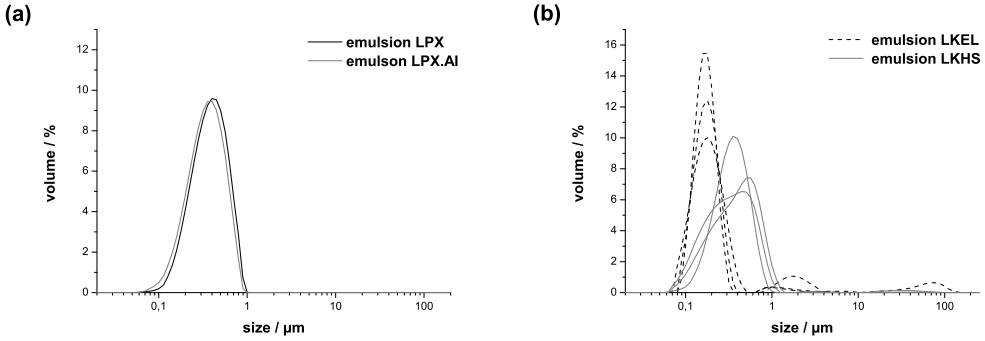

Figure 4.26: Particle size distribution determined by SLS of different emulsions after autoclaving. Detailed composition of the samples is shown in table 4.13.

tive ingredient Probucol (figure 4.27). Hence, the addition of the active ingredient had an influence on the pH value. Supposedly the Probucol was partly destroyed during sterilization process. As the values were lower compared to formulations on the market [7, 17] a pH correction should be considered. The other two formulations with lecithin and either Kolliphor EL or Kolliphor HS15 as emulsifiers showed some changes after the autoclaving. First the appearance of homogeneous white fluids changed to light yellow fluids. For some samples a creaming process could be detected, but gentle shaking removed this phenomenon. Figure 4.27 shows that osmolality values did not change for the single emulsions, even if the values were over a range from about 375 mOsmol/kg to 300 mOsmol/kg. For these samples as well a decrease of the pH values could be detected from 6.54 $\pm$0.10 to 6.19 $\pm$ 0.05 for samples with lecithin and Kolliphor EL and from 6.05 $\pm$ 0.19 to 5.68 $\pm$0.09 for samples with lecithin and Kolliphor HS15. Decrease of the pH after sterilization process was described before for the emulsifier Kolliphor HS15 [110]. Regarding the particle sizes and distributions both formulations reacted different during the sterilization process (figure 4.26(b)). Emulsions containing lecithin and Kolliphor EL changed from an initially broader particle size distribution to a narrower one which was similar for all samples. Droplet size shifted to smaller particles as the values in table 4.14 confirm. The D[50] value was reduced from about 200 nm to 130 nm and the D[90] value from 617 nm to values between 190 nm and 336 nm. But some bigger droplets were also found in the samples with sizes around 2 μm and even a few hundred micrometer. Hence the requirements for parenteral use were not fulfilled anymore. Maybe an additional filtration step could be used for this formulation. The other emulsions containing lecithin and Kolliphor HS15 as stabilizers changed to a broader droplet size distribution during the autoclaving that had in two cases an irregular appearance. All in all the particle size increased.

It could be concluded that not all formulations are stable under the sterilization process. Formulations with Kolliphor HS15 seemed to be the most unstable ones. These should not be sterilized by an autoclaving process. Emulsions containing

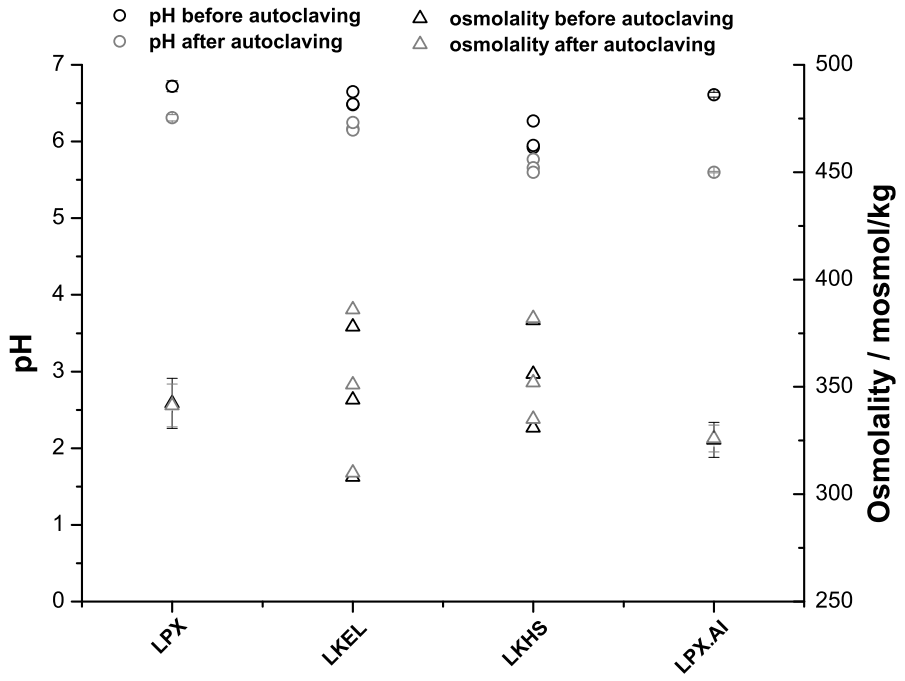

Figure 4.27: Average and detailed pH and osmolality values of different emulsions before and after autoclaving. Detailed composition of the samples is shown in table 4.13.

Kolliphor EL showed some changes in their droplet sizes but eventually an additional filtration step could help to obtain nanoemulsions with clearly smaller particles and a narrow particle size distribution. For samples with poloxamer 188 no significant changes could be detected even if an active ingredient was added. The findings could be explained by the cloud point of the different surfactants. It was found before by Jumaa and Müller that nanoemulsions stabilized with Kolliphor EL or Kolliphor HS15 increased droplet size after sterilization [71]. In this publication the cloud points of aqueous emulsifier solutions were determined and a difference between Kolliphor EL (cloud point at 71.5 °C), Kolliphor HS15 (cloud point at 75.5 °C) and Kolliphor P 188 (cloud point higher than 100 °C) was found. As can be seen for two of the stabilizers the cloud point was found at significantly lower temperatures than the autoclaving temperature. Hence, both emulsifiers undergo dehydration during the sterilization process which means that the surfactant film on the surface of the oil droplets may become unstable and droplet coalescence was more likely to appear.

4.4 Intermediate step

During emulsion preparation with dual asymmetric centrifugation an intermediate step is formed. Preceding work showed that appearance and viscosity were correlated

Table 4.14: Average measurement values of SLS for different formulations before and after the autoclaving procedure. For samples that showed bigger differences single values are shown instead of average values. Detailed composition of the samples is shown in table 4.13.

emulsion	D[10] [μm]	D[50] [μm]	D[4,3] [μm]	D[90] [μm]
LPX	0.14 ± 0.02	0.31 ± 0.02	0.34 ± 0.02	0.60 ± 0.03
LKEL	0.08 ± 0.00	0.19 ± 0.01	0.29 ± 0.02	0.62 ± 0.02
LKHS	0.10 ± 0.01	0.25 ± 0.02	0.29 ± 0.03	0.56 ± 0.08
LPX.AI	0.13 ± 0.01	0.29± 0.02	0.32 ± 0.02	0.54 ± 0.04
after autoclaving				
LPX	0.15 ± 0.02	0.32 ± 0.02	0.35 ± 0.02	0.60 ± 0.03
LKEL	0.07	0.14	0.33	0.34
	0.08	0.12	0.18	0.19
	0.08	0.13	9.29	0.28
LKHS	0.15	0.33	0.38	0.73
	0.13	0.28	1.56	0.56
	0.09	0.25	0.49	0.64
LPX.AI	0.13 ± 0.02	0.29 ± 0.02	0.32 ± 0.02	0.55 ± 0.04

with the particle size of the final product. Further investigations were performed to collect more information about the characteristics of the intermediate step.

4.4.1 Appearance

The appearance of the intermediate step formed during the process of emulsion preparation by dual asymmetric centrifugation was investigated. While testing different parameters and formulations it became clear that in many cases the appearance of the intermediate step was correlated with the quality of the final product. It was frequently used as prediction tool for whether the particle size would be in a nanometer range or not. In figure 4.28 four steps in the emulsion formulation process are presented that were connected with an assumed particle size in the nanometer range. Details about the composition and preparation process are described in chapter 4.1.4. Picture 4.28(a) shows the lipid phase after heating and homogenization with the glass rod. The poloxamer 188 was molten and was mixed with the soybean oil. The lecithin was spread as a thin layer on the bottom of the beaker but was not solved in the lipid phase. After addition of a small amount of aqueous phase and mixing with DAC the first intermediate step was formed (figure 4.28(b)). It was homogeneous, of a yellow color and appeared to be semisolid. The formulation stuck to the bottom of the beaker but it was easily possible to transfer it into another vessel for further characterization steps without changing the outer appearance. The addition of another small part of aqueous phase and again mixing with DAC formed the second intermediate step which is shown in picture 4.28(c). The homogeneous, semisolid formulation changed slightly in color as it became light yellow. Furthermore the intermediate step seemed to be jelly like and had a shiny surface. Again

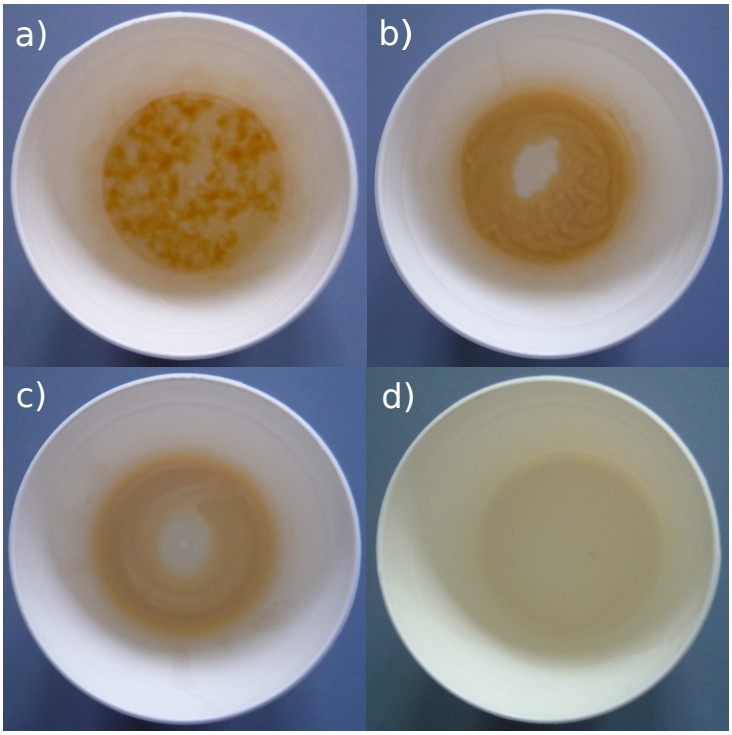

Figure 4.28: Appearance of different steps during the preparation process of emulsions with DAC. The final emulsion contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase.

it was possible to exert mechanical forces on it, e. g. while transferring it to another vessel for further characterization, without changing the outer appearance. Before the last mixing step with DAC the remaining amount of aqueous phase was added. The final product was a white, homogeneous fluid as can be seen in figure 4.28(d). If the appearance of the intermediate steps of the emulsion formation were as described above, there was a good chance that the emulsion which was obtained contained small droplets in a range that would be acceptable for parenteral application.

4.4.2 Light microscopy with polarization filter

In a first attempt to get an idea of the inner structure of the intermediate steps, the samples were investigated by light microscopy. With the normal experimental setup no significant structures were detected. Therefore a polarization filter was used. Exemplary pictures of the two intermediate steps are shown in figure 4.29. Interestingly mostly in the border regions of the samples structures could be identified as bright spots with diameters in the lower μm-region. The inner regions of the samples remained mostly dark. The intermediate steps contained high amounts of lecithin which is known to form birefringence structures [111]. Presumably the bright spots were so called maltese crosses that indicate the presence of vesicles [101]. Some of these structures are highlighted in figure 4.29(d). These findings were in

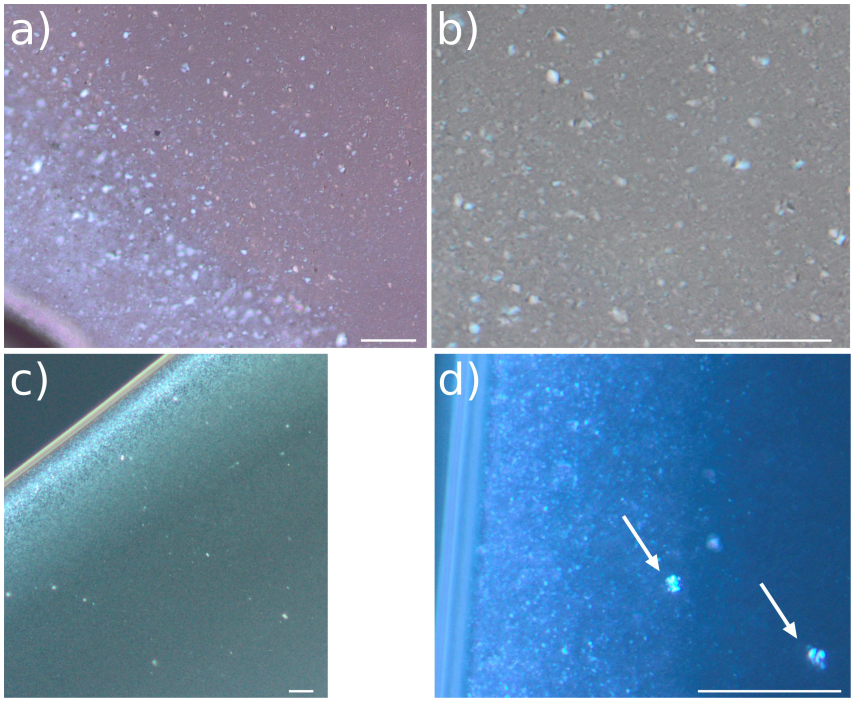

Figure 4.29: Light microscopic images with polarization filter of the first intermediate step (a) and (b) and the second intermediate step (c) and (d) during emulsion formation with DAC. The final emulsion contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase. White bars indicate 50 μm. Highlighted structures (maltese crosses) indicate the presence of vesicles.

good agreement with the structures that were found in the TEM-pictures of the final product (see chapter 4.3.2). The first and the second intermediate step were similar in their appearance in the microscopy study. That is not surprising as the second intermediate step contained only a little more aqueous phase compared to the first intermediate step. As the maltese crosses were rather small for detection under a light microscope other eventually existing structures were maybe to small to be detected by this method. Hence, the application of another imaging method could help to understand the texture of the samples.

4.4.3 Transmission electron microscopy

For a more detailed view on the samples both intermediate steps were investigated by transmission electron microscopy. Attempts of using the negative staining method did not work out but for the freeze fracture method good results were received. An overview of the structure of the first intermediate step is shown in figure 4.30(d). In contrast to the pictures of the emulsion in figure 4.19 many particles were found close to each other. Similar to the emulsion there were particles with diameters of 100 nm to 300 nm and bigger ones with diameters up to 1 μm (see figure 4.30(a)-I) that could be oil droplets stabilized by the emulsifiers. Not all of these particles were spherical, also oval shapes were detected. A deformation of the oil droplets may be

connected with the dense packaging. In every picture multiple layer structures could be found. They appeared as a thick layer around an oil droplet as in figure 4.30(a)-II or a shell like structure as in figure 4.30(b)-III and (c)-III. Layers like these are known as vesicles as described before by Rubas and Schreier [105]. Furthermore some of these structures were also found with the light microscopy technique in chapter 4.4.2. Hence, the emulsifier lecithin has a big influence on the structure of the first intermediate step.

Transmission electron microscopy pictures of the second intermediate step were similar to those of the first intermediate step. Diameters of the particles did not change. In the overview in figure 4.30(f) it can be seen, that most of the particles had a spherical shape. The further addition of aqueous phase could have led to a better distribution of the oil droplets and with that to a relaxation of the deformations. Multiple layer vesicles were found (figure 4.30(e)-II) as well as shell like structures (figure 4.30(g)-III). All in all the inner structure of the intermediate steps seemed to be a kind of pre-emulsion. It can be assumed that with the addition of the remaining aqueous phase and the third mixing step with DAC the final emulsion was formed through a dispersion of the already stabilized oil droplets in the aqueous phase. According to these findings the main droplet forming process happened during the formation of the first intermediate step. This could be the reason why the appearance of the first intermediate step can be used as prediction tool for the quality of the final product.

4.4.4 Benchtop nuclear magnetic resonance relaxometry

For emulsion preparation with dual asymmetric centrifugation the consistency of the intermediate steps was found to be very important. During investigation of different amounts of aqueous phase given to the lipid phase before the first and second mixing step (see chapter 4.1.3) it was noticed that the viscosity of the intermediate step was connected to the quality of the final product. With benchtop nuclear magnetic resonance relaxometry the mobility of protons in the sample and with that the microviscosity can indirectly be estimated [82]. Depending on how strong the protons are connected to the structure the T_2-relaxation time was influenced. Protons that could agitate freely had higher values for the T_2-relaxation time. In contrast a short T_2-relaxation time indicated a stronger binding of the protons to the structure [97]. To get a better idea of the structures surrounding the protons in the intermediate steps the T_2-relaxation times for soybean oil and an emulsion containing 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase were recorded (figure 4.31(a)). For the soybean oil a broad, irregular peak at about 100 ms and two very small peaks at 0.5 ms and 8 ms were found. For the emulsion the main peak, which was thin and had a high intensity, appeared at 2000 ms. A second signal with very low intensity at 100 ms could be connected to the oil fraction of the emulsion. As most of the protons were found in the vicinity of

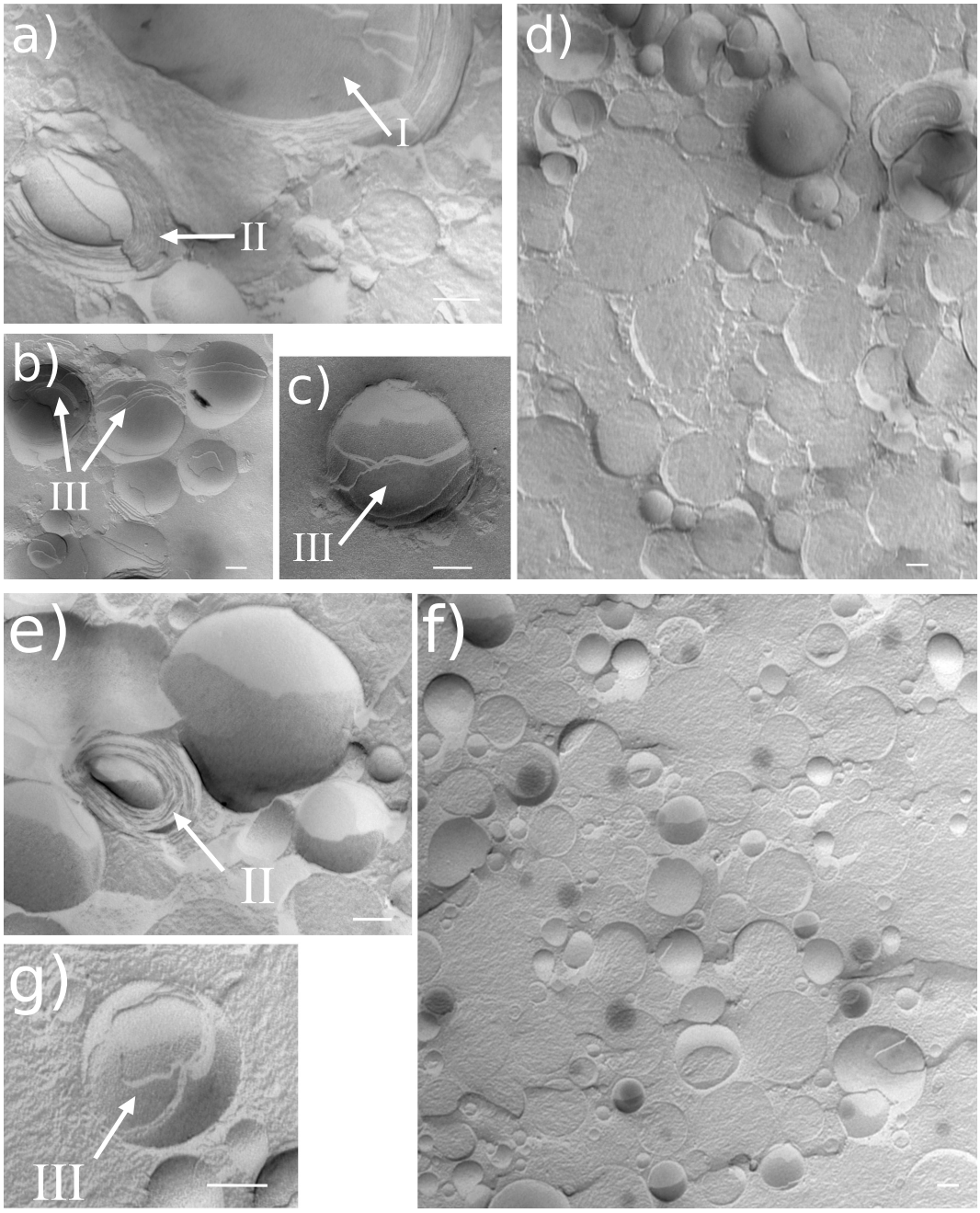

Figure 4.30: TEM pictures of the first intermediate step (a - d) and second intermediate step (e - g) that appeared during emulsion formation. Samples were prepared with the freeze fracture technique. The final emulsion contained 2.5 % lecithin, 2.5 % poloxamer 188, 10 % soybean oil and 85 % aqueous phase. White bars indicate 100 nm. Highlighted structures are: I–bigger particles with diameters up to 1 μm, II–particles surrounded by a thick layer, III–shell like structures.

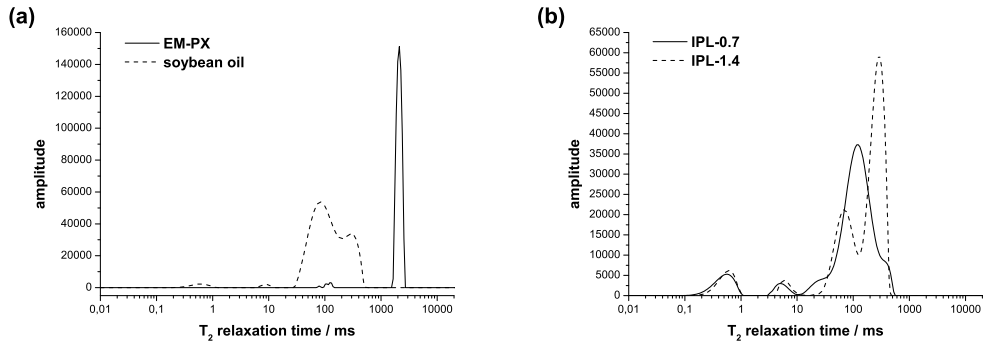

Figure 4.31: T_2-relaxation time profiles of an emulsion with 2.5 % lecithin and 2.5 % poloxamer 188 (EM-PX) and of soybean oil (a) as well as for intermediate steps with lecithin and poloxamer 188 (b).

the oil droplets and had a rather high liberty of action, the amount of more bound protons was relatively low. Other structures could not be detected presumably because of the low frequency.

Samples with different amounts of aqueous phase given to the lipid phase before the mixing step with DAC were prepared. According to chapter 4.1.3 the amounts of aqueous phase were calculated as the ratio of aqueous phase to emulsifier (ratio aq/em). For intermediate steps either with sodium oleate and lecithin as stabilizers (ISL) or with poloxamer 188 and lecithin (IPL) each four different ratios aq/em were examined. Details of the composition are shown in table 4.15. T_2-relaxation time profiles of the intermediate steps are presented in figure 4.32. A comparison of the profiles of the intermediate steps of the two emulsifiers showed that the shape of the curves was similar. The main difference appeared in the curves with a ratio aq/em of 0.4. Here for ISL-0.4 one main peak with a shoulder at 300 ms was found at 100 ms (figure 4.32(a)). Two other signals not fully formed as peaks with lower intensities were detected at 1 ms and 8 ms. The T_2-relaxation time profile was very similar to the profile of the soybean oil (figure 4.31(a)). For IPL-0.4 two peaks were detected (figure 4.32(b)). The main peak appeared at 100 ms with a shoulder at 11 ms. Another smaller peak was found at 2 ms. The influence of the different emulsifiers could be seen in the varying curves.

With the addition of higher amounts of aqueous phase before the mixing with DAC the T_2-relaxation time profiles changed for both formulations in a similar way. The biggest difference was the splitting of the main peak. From sample ISL-0.8to sample ISL-1.6 the signal which was the shoulder of the main peak at 300 ms in sample ISL-0.4 became a single peak with the highest intensity. Furthermore the T_2-relaxation time of this peak increased and had its maximum at 1000 ms in sample ISL-1.6. This peak could represent the protons in the free water. The amount of aqueous phase was duplicated from formulation ISL-0.4 to formulation ISL-1.6 which explained the increase in intensity. Furthermore the shift to higher T_2-relaxation

Table 4.15: Composition of formulations investigated by benchtop nuclear magnetic resonance relaxometry. All intermediate steps contained 0.25 g lecithin and 1.00 g soybean oil.

sample	second emulsifier	aqueous phase	mixing steps with DAC
ISL-0.4	0.25 g sodium oleate	0.20 g	1
ISL-0.8	0.25 g sodium oleate	0.40 g	1
ISL-1.2	0.25 g sodium oleate	0.60 g	1
ISL-1.4	0.25 g sodium oleate	0.80 g	1
IPL-0.4	0.25 g poloxamer 188	0.20 g	1
IPL-0.8	0.25 g poloxamer 188	0.40 g	1
IPL-1.2	0.25 g poloxamer 188	0.60 g	1
IPL-1.4	0.25 g poloxamer 188	0.80 g	1
EM-PX	0.25 g poloxamer 188	3.50 g	3
IPL-0.7	0.25 g poloxamer 188	0.35 g	1
IPL-1.4	0.25 g poloxamer 188	0.70 g	2
IKEL-0.7	0.25 g Kolliphor EL	0.35 g	1
IKEL-1.4	0.25 g Kolliphor EL	0.70 g	2
IKHL-0.7	0.25 g Kolliphor HS15	0.35 g	1
IKHL-1.4	0.25 g Kolliphor HS15	0.70 g	2

times could be interpreted as being caused by the expansion of the space between the structures. This phenomenon was found before in the transmission electron microscopy pictures of the intermediate steps with poloxamer 188 in chapter 4.4.3. Additionally most of the protons in the emulsion had T_2-relaxation times of 2000 ms (see figure 4.31(a)). The second peak remained at 100 ms but developed a shoulder at 11 ms in sample ISL-0.8 and ISL-1.2. This peak was attributed with the protons in an oily surrounding. The two smaller signals at 1 ms and 8 ms grew to bigger intensities from sample ISL-0.4 to sample ISl-1.6. In formulations ISL-1.2 and ISL-1.6 their maxima shifted to 0.7 ms respectively 5 ms an the peaks became more irregular. Both peaks may represent protons surrounded by other structures e. g. vesicles. This would be in good agreement with expectations as for these structures the protons are bound rather strong which leads to low T_2-relaxation times.

Formulations with poloxamer 188 as second emulsifier lead to similar T_2-relaxation time curves (figure 4.32(b))As with the samples with sodium oleate the main peak split up when a higher amount of aqueous phase was included in the formulation. The peak with the highest intensity shifted from a T_2-relaxation time of 100 ms in sample IPL-0.4 to 1000 ms in sample IPL-1.6. The peak that presumably represented the protons in the oil phase developed from the split peak in sample IPL-0.4. For the other three formulations the maximum of this signal was at 70 ms. Other structures surrounding the protons were also found. Two peaks at 11 ms and at 1 ms were found in every sample. The peak at 11 ms sometimes appeared as a shoulder to the ”oil-peak” or stood alone. These structures could not be assigned clearly. From

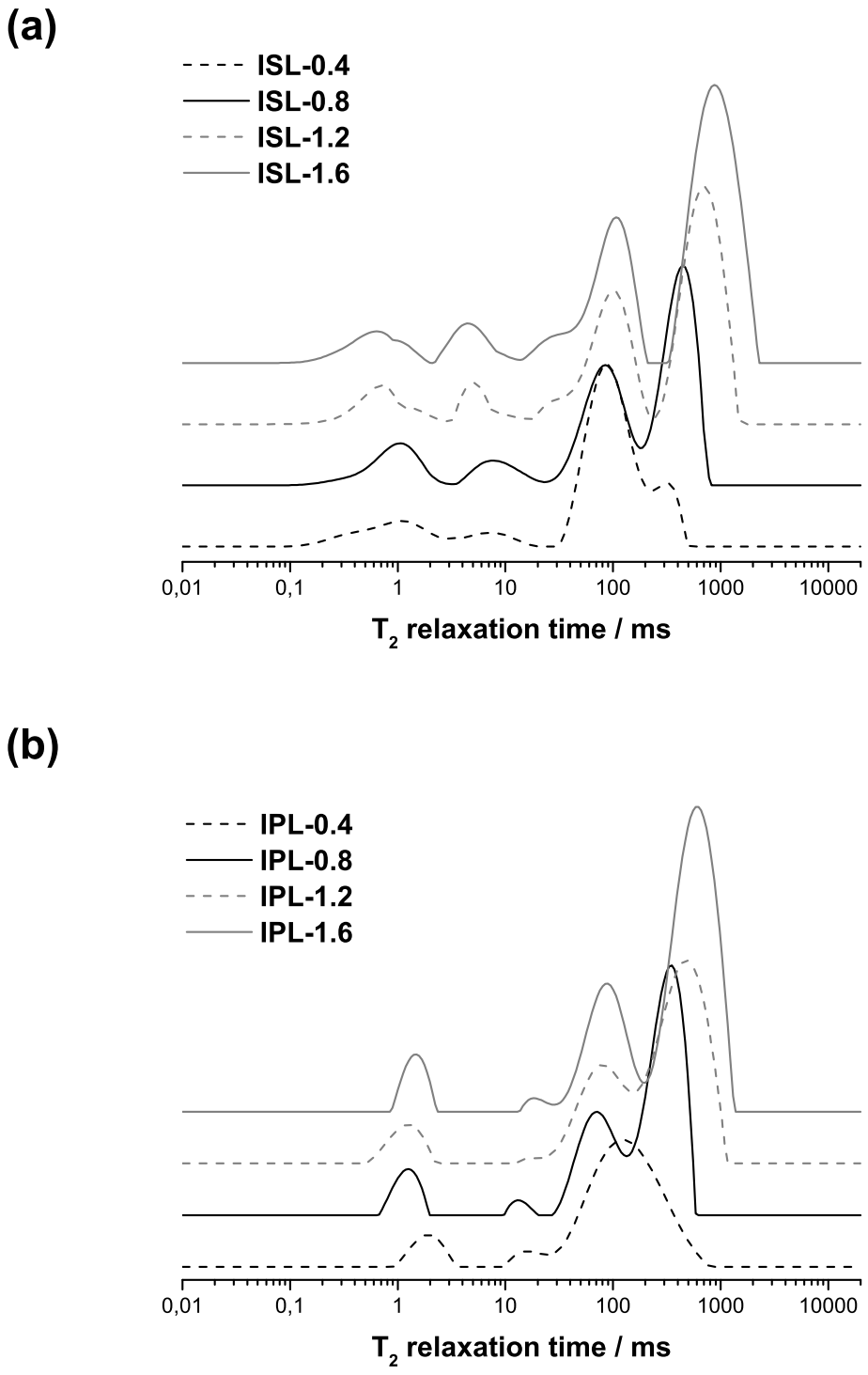

Figure 4.32: T_2-relaxation time profiles of intermediate steps with sodium oleate (a) or poloxamer 188 (b) prepared with different amounts of aqueous phase.

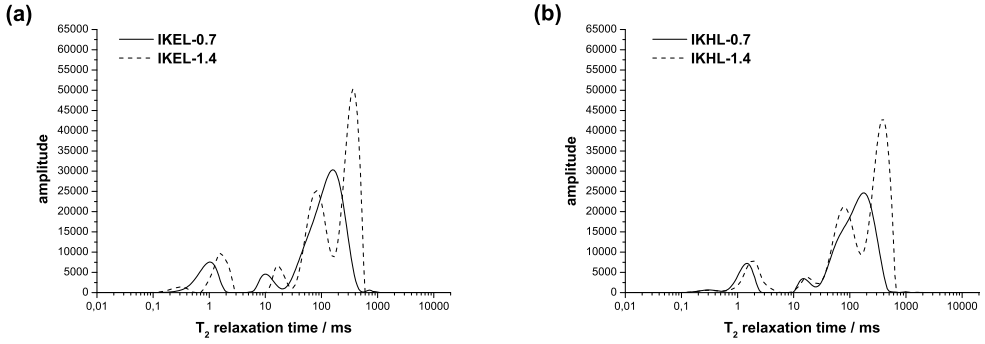

Figure 4.33: T_2-relaxation time profiles of intermediate steps containing either lecithin and Kolliphor EL (a) or lecithin and Kolliphor HS15 (b) as stabilizers.

results of the transmission electron microscopy in chapter 4.4.3 it was deduced that one of the peaks may be connected to the occurrence of multilayer vesicles.

After investigation of the influence of different amounts of aqueous phase given to the lipid phase before mixing with DAC for formulations containing either lecithin and sodium oleate or lecithin and poloxamer 188 as emulsifiers, three other formulations were examined. These were all preliminary stages of nanoemulsions with different emulsifiers. As shown in table 4.15 poloxamer 188, Kolliphor EL or Kolliphor HS15 was used in combination with lecithin. In the optimized preparation process (see chapter 4.1.4) a ratio aq/em of 0.7 was selected for the amount of aqueous solution given to the samples before the first and the second mixing step with DAC. T_2-relaxation time profiles of the two intermediate steps of each formulation are shown in figure 4.31(b) as well as in figure 4.33(a) and (b). All samples appeared to be similar respective to the corresponding intermediate step. For IPL-0.7, IKEL-0.7 and IKHL-0.7 all three peaks were found with a main peak at 100 ms relaxation time that as mentioned before described protons in the soybean oil. In sample IPL-0.7 the peak had two shoulders at 11 ms and at 400 ms. In samples IKEL-0.7 and IKHL-0.7 the main peak was connected with the second peak at 11 ms. The third peak was found at 1 ms for these two formulations, the intermediate step with poloxamer 188 had the third peak at 0.6 ms. For the second intermediate steps (samples IPL-1.4, IKEL-1.4 and IKHL-1.4) the strongest change was the splitting of the main peak into two peaks as described above for the other formulations. Regarding the other peaks there were no significant changes for IPL-1.4 and IKHL-1.4, only for IKEL-1.4 a slight shift from 1 ms to 2 ms for the first peak and from 10 ms to 11 ms for the second peak was found. All in all the T_2-relaxation time profiles were alike the profiles found for the formulations investigated before. Small differences could be attributed to the influence of the different emulsifiers. But because of the similarity of the relaxation profiles it is not unlikely that most of structures that were build in the intermediate steps came from the lecithin.

4.4.5 Storage stability of the intermediate step

The storage stability of the intermediate steps was investigated under different conditions. For this the intermediate steps were prepared as usual. Subsequently the semisolid mass was transferred to a glass vial and then mixed with DAC for 30 s at 3500 rpm. The mixing step was applied to ensure that the mass was stuck to the bottom of the vial and with that reducing the surface of the sample that was exposed to the air. Especially the first intermediate step had a rather high viscosity and without the mixing it would have been distributed all over in the vial. The intermediate steps were stored under different conditions. Three samples each were stored at room temperature (RT), in the refrigerator at 2–8 °C (KS) or at 37 °C (W). All samples were stored in the dark. Directly after preparation (0d), after one week (7d), 3 months (3m) and 6 months (6m) the appearance of the samples was examined. In figure 4.34 photos of the stored samples in comparison to the freshly prepared samples are shown. Pictures of the first intermediate steps are labeled with "I" and those for the second intermediate steps with "II". The storage conditions and storage time can also be derived from the sample labels.

For the first intermediate step the samples were homogeneous, light yellow and semisolid after preparation. The appearance did not change during storage at 2–8 °C and at room temperature. The samples remained homogeneous, light yellow and hat a gel like, viscous texture as the formulations stuck to the bottom of the vial though the vial was turned upside down. Within the samples stored at 37 °C two samples seemed to be stable as their appearance did not change. One sample got a dark yellow color after 3 months of storage and the formulation was more transparent. The viscosity of the sample did not seem to be changed. Similar results were found for the second intermediate step. After preparation the samples were homogeneous, had a lighter yellow color compared to the first intermediate step and had a lower viscosity. During storage at 2–8 °C the formulations did not change. After 6 months of storage at room temperature one of the samples changed as described above to a dark yellow and more transparent system. The same happened during storage at 37 °C with one sample. The slight changes appeared already after 1 week and became stronger after 3 months. One other sample showed only a slightly darker color after 3 months storage. All intermediate steps were also examined by light microscopy with polarization filter. Samples that were stable during storage time regarding their appearance and viscosity showed no difference compared to the samples investigated in chapter 4.4.2. The formulations that changed presented a completely different picture under the microscope with the appearance of coherent birefringent structures that also included maltese crosses.

As a conclusion both intermediate steps were stable under cool conditions up to 6 months regarding their appearance, viscosity and microscopic structure. It must be admitted that for all samples in which changes were detected, it was found that the vial caps were more loose compared to the others. Therefore it was possible that

water could have been evaporated from the samples and had an influence on the appearance. These findings would be in line with the storage temperature, because at higher degrees a loss of water would be noticed earlier. Hence, the results of the storage study have to be interpreted carefully.

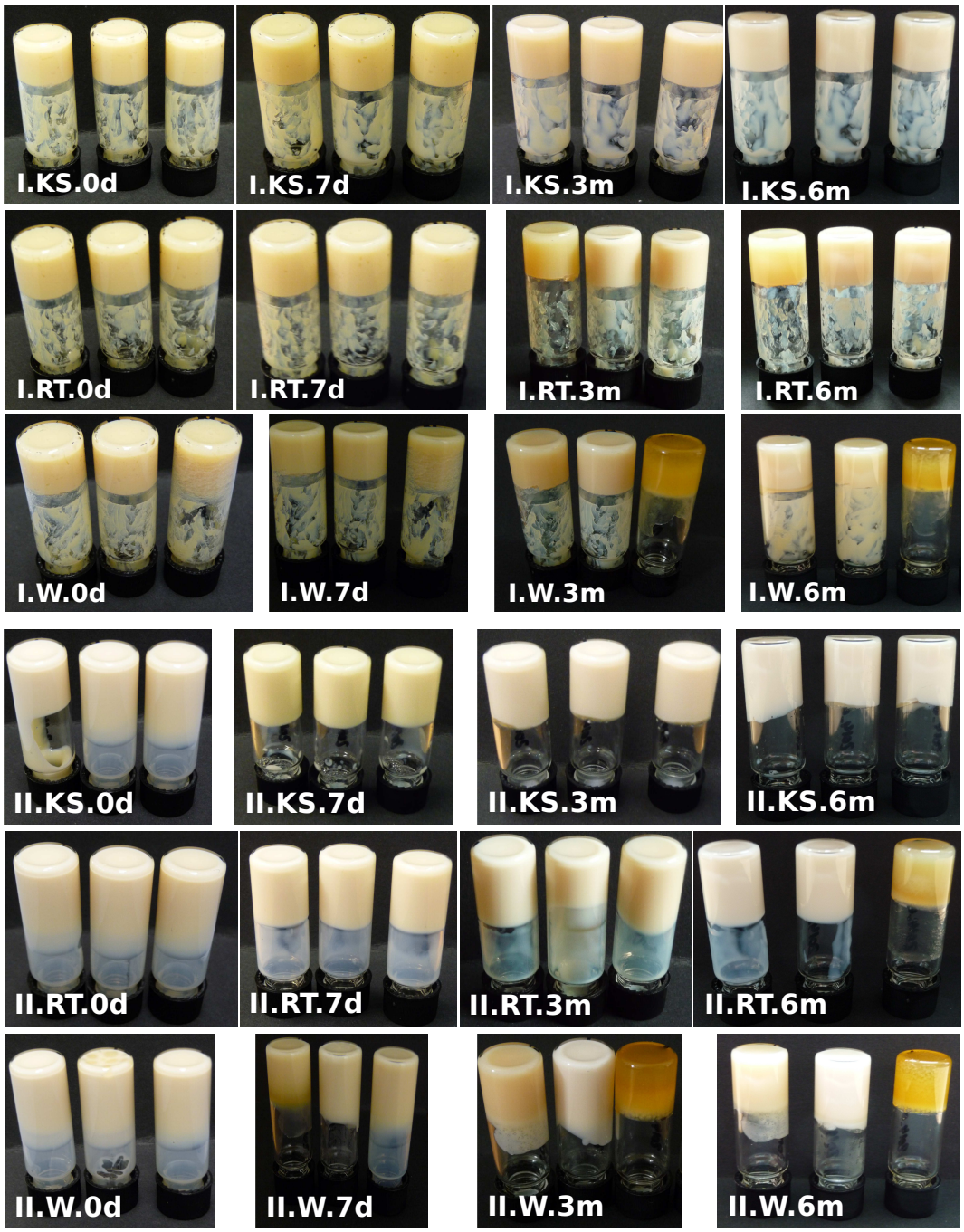

Figure 4.34: Appearance of the first (I) and second (II) intermediate step directly after preparation (0d), after 7 days (7d), 3 months (3m) and 6 months (6m). Different storage conditions were chosen: in the refrigerator at 2–8 °C (KS), at room temperature (RT) and at 37 °C (W).

5

Summary and outlook

The subject of this thesis was to investigate whether the dual asymmetric centrifugation was a suitable method to prepare nanoemulsions. Advantages of preparation with DAC should be small sample amounts and an easy to handle and time saving process. Furthermore different sample volumes could be fabricated because of the possibility to use various beakers. Sample containers were for single-use, saving cleaning time. Nanoemulsions processed by this method were intended to be used in pharmaceutical research for screening processes of new lipophilic active ingredients. Regarding the future application of the emulsions for parenteral use, it was important to develop a formulation that was physiologically tolerable. Therefore commercially available fat emulsions like Lipofundin® 10 % N were used as model emulsions.

Preliminary tests gave an idea of the possibilities to prepare emulsions with DAC. It was found out quickly that two intermediate steps were necessary to form emulsions (see figure 4.1). As in most fat emulsions lecithin was used as emulsifier. Different phospholipids were tried. For samples with Lipoid® S 75 the best results were obtained. To optimize the stability and the particle size a second emulsifier was applied. At first sodium oleate was used as a model emulsifier. Amount of emulsifier mixture and preparation process were developed and emulsion with droplets in a higher nanometer respective lower micrometer range were obtained. Subsequently sodium oleate was replaced by Kolliphor® P 188 (poloxamer 188) because of better physiological tolerability.

Different parameters of the preparation process were studied. For the new formulation some of the parameters were investigated more closely to optimize the emulsions. One parameter that was not examined in detail was the influence of the diameter of the beaker. Although in preliminary tests differences between the varying beakers could be identified, the optimization was done only for one size of beakers with a diameter of 4.0 cm. To utilize the advantage of the different beakers further studies on this topic should be done. One big issue was the temperature of the aqueous phase as well as the lipid phase. The temperature of the aqueous phase

was easier to control but the handling grew more difficult the higher the temperature was. Though an increase of the temperature of the aqueous solution showed a trend to smaller particles in the final product, the difficulties in handling were the reason to leave the temperature at 55 °C. For the lipid phase the temperature was not easy to control. A range of temperatures between 39 °C and 42 °C was identified within which emulsions with good particle size were obtained. Therefore heating times of the lipid phase in the preparation process were adapted. The amount of aqueous phase given to the lipid phase before the first and second mixing step with DAC was investigated carefully. An important optimization was the application of eppendorf pipettes to add the aqueous solution to the lipid phase. Compared to the former used weighting process on a balance errors could be reduced both in the weighted amount of aqueous phase and through the reduction of evaporation. Now it was possible to form emulsions with droplets in a nano-scale range. For the ratios aq/em of 0.7 and 0.75 the best results were obtained. Another attempt was the moistening of the lecithin to improve the processability of the substance. Due to the heterogeneous composition of the lecithin it was not possible to work out a definite amount of water that should be absorbed by the emulsifier. But with an absorption of 2–3 % of water a trend to a better reproducibility due to an improved preparation of the lecithin was shown. Lastly the reproducibility of samples received by the optimized preparation process was confirmed. Ten samples with the same formulation were prepared by the same procedure at one day. Their particle size was investigated by static light scattering as well as dynamic light scattering. For all samples similar results were obtained, except one sample that showed slightly bigger particles. However all emulsions met the required particle size for intravenous application.

With the optimized formulation and preparation process the flexibility of the system was tested. At first different emulsifiers were used in order to replace the poloxamer 188. Though emulsions with polysorbate 80 as second emulsifier were coarse and because of the big particles not practicable for parenteral use, two other emulsifiers, Kolliphor EL and Kolliphor HS15 showed promising results. The different states of aggregation of the stabilizers required a small modification in the preparation process as the emulsifier had to be mixed with the lipid phase before the heating process to improve the reproducibility. The application of different oils was investigated. It was possible to incorporate either olive oil, sesame oil, corn oil or castor oil as lipid phase. A replacement of the soybean oil with MCT or a 1:1 mixture of MCT and soybean oil caused an increase in the droplet size of the final product. Regarding the intermediate step it was found out that MCT was a solvent for the lecithin and the appearance of the intermediate step was changed. Therefore the final emulsion could not be formed properly. With peanut oil also rather coarse emulsions were obtained. This oil phase was not a solvent for the lecithin. Nevertheless the intermediate step that was formed had a different appearance compared to the intermediate steps formed for emulsions with droplets in the nanometer range.

Finally three different active ingredients were tried to be incorporated in the emulsion. All the substances were miscible with soybean oil and the prepared soybean oil was used to form emulsions. Here as well the two active ingredients that did not change the appearance of the intermediate step lead to nanoemulsions similar in particle size to those that were formed without active ingredient. One other substance was able to solve the lecithin and therefore the intermediate step was changed. Hence, more coarse emulsions were obtained. All in all it could be shown that the dual asymmetric centrifugation was suitable for the preparation of nanoemulsions. It was possible to exchange emulsifiers or the oil phase and to incorporate active ingredients.

Further characterization on the system was done. Not only the particle size was confirmed by different methods like static light scattering and dynamic light scattering, also the particle size distribution was investigated more carefully. A centrifugation process and the use of the asymmetrical flow field-flow fractionation helped to collect more information. The existence of two particle fractions was confirmed with both methods. The main fraction involved particles in the recommended size of 200–300 nm the other one was a smaller fraction of bigger particles which were under 1 μm, though. Similar findings were described before by Kuntsche et al. who investigated commercially available fat emulsions [80]. Furthermore transmission electron microscopy was used to examine the morphology of the final product. Samples were prepared either with negative staining or with freeze fracture technique. Besides the different oil droplet fractions some structures indicating vesicles were found. For selected samples the storage stability was proved. Not only differences in the particle size were studied but also pH-values, osmolality and zeta potential. For several formulations stability over 6 respectively 9 months could be claimed. The physiological tolerability of emulsions was shown with a hemolysis assay. Even if slight hemolysis was found for most samples, the values were in good agreement with data found before for other parenteral emulsions and also for commercially available products. In the end selected emulsions were treated in an autoclaving process. It was shown that the optimized formulation was stable regarding particle size and osmolality. Only slight decrease of the pH values was found, therefore it could be advantageous to add a substance for pH correction to the formulation in future. This could for instance be a small amount of sodium oleate as is currently used in commercially available formulations [7]. In summary the detailed characterization of the fat emulsions prepared by DAC showed similar results to investigations of fat emulsions prepared by other techniques. Possibilities to store the emulsions over several months or apply a sterilization method are important for the products regarding their application in screening processes in research.

One big issue was the intermediate step formed during the emulsion preparation process. The appearance and structure of this semisolid, gel like mixture seemed to be important for the quality of the final product. In many cases it was possible

to make predictions about the particle size by investigating the appearance of the intermediate steps. Because of this correlation in a future screening process the preparation could be stopped at an early point if an inadequate intermediate step appears. The intermediate steps were examined more closely. Microscopy techniques gave information about the inner structure that seemed to be similar to the structure of the emulsion but with a more dense packaging of the oil droplets. Especially for the first intermediate step the existence of multi-lamellar vesicles was found by both methods, the light microscope technique using polarization filters and the freeze fracture TEM technique. For a better understanding of the structure and behavior of the intermediate phase the benchtop nuclear magnetic resonance relaxometry was applied. Within this method it was found that the protons in formulations with small amounts of aqueous phase were influenced more strongly by the mixture of oil phase and emulsifier. This could be proved by shorter T_2-relaxation times. With the addition of larger amounts of aqueous phase a higher agitation of the protons was confirmed. The comparison of intermediate steps with different emulsifiers led to similar results. It was found before for all these emulsifier combinations that nanoemulsions were formed by the optimized way of preparation with DAC. Therefore the similarities of the T_2-relaxation time profiles were interpreted as similarities in the structures of the intermediate steps. A confirmation of these assumptions should to be done in further experiments. At last the storage stability of the intermediate steps was examined under different storage conditions. Most of the samples were not influenced in their appearance (complete sample and microscopic investigation) and viscosity during the storage time of 6 months. Changing of the appearance of some samples could be traced back to the fact that some storage vessels were not sealed properly. All in all the intermediate step is an interesting phase that needs further investigations. Not only as intermediate phase of the emulsion preparation process and with that a prediction tool for the quality of the final product, but also maybe as storage form as pre-emulsion concentrate or as a new semisolid drug delivery system.

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List of Abbreviations

AF4	asymmetrical flow field-flow fractionation
BT-NMR	benchtop nuclear magnetic resonance
DAC	dual asymmetric centrifugation
DLS	dynamic light scattering
G-CSF	granulocyte-colony stimulating factor
HPH	high pressure homogenization
LCT	long-chain triglyceride
LUV	large unilamellar vesicles
MCT	middle-chain triglyceride
MLV	multilamellar vesicles
NLC	nanostructured lipid carriers
PDI	polydispersity index
SLN	solid lipid nanoparticles
SLS	static light scattering
SUV	small unilamellar vesicles
TEM	transmission electron microscopy
VPG	vesicular phospholipid gel

Curriculum Vitae

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- ◇ 04/2014 — F. Tenambergen, K. Mäder, Parenteral fat emulsions prepared by dual asymmetric centrifugation, World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Lisbon, Portugal (abstract and poster)
- ◇ 03/2013 — F. Tenambergen, K. Mäder, Alternative preparation of parenteral emulsions - in vitro physiologigal tolerability, CRS Local Chapter Meeting, AbbVie, Ludwigshafen (abstract and poster)
- ◇ 03/2012 — F. Tenambergen, K. Mäder, Preparation of fat emulsions with dual asymmetric centrifugation (DAC), CRS Local Chapter Meeting, Würzburg (abstract and poster)

Publications

- ◇ F. Tenambergen, C. H. Maruiama, K. Mäder, *Dual asymmetric centrifugation as an alternative preparation method for parenteral fat emulsions in preformulation development*, International Journal of Pharmaceutics, DOI: 10.1016/j.ijpharm.2013.02.033, **2013**
- ◇ A. Schädlich, H. Caysa, T. Mueller, F. Tenambergen, C. Rose, A. Göpferich, J. Kuntsche, K. Mäder, *Tumor accumulation of NIR fluorescent PEG-PLA nanoparticles: Impact of particle size and human xenograft tumor model*, ACS Nano, DOI: 10.1021/nn2026353, **2011**

Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die Ergebnisse der vorliegenden Dissertationsarbeit *Development and characterization of parenteral fat emulsions prepared by dual asymmetric centrifugation* am Institut für Pharmazie der Martin-Luther-Universität Halle-Wittenberg unter Anleitung von Herrn Prof. Dr. Karsten Mäder selbstständig erarbeitet habe. Beiträge von Kooperationspartnern zu den Ergebnissen dieser Arbeit habe ich eindeutig gekennzeichnet. Die Dissertation habe ich ohne fremde Hilfe angefertigt und dazu keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt. Die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Ferner erkläre ich, dass ich mich erstmals um die Erlangung eines Doktorgrades bewerbe und die vorliegende Dissertationsschrift keiner anderen in- oder ausländischen Fakultät vorgelegt habe.

Potsdam,

Frederike Tenambergen

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