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GHENT UNIVERSITY
FACULTY OF PHARMACEUTICAL SCIENCES
Department of Pharmaceutics
Laboratory of General Biochemistry and Physical Pharmacy

PHYSICOCHEMICAL CHARACTERIZATION OF A NANODISPERSSION,
OBTAINED WITH A PHASE INVERSION TEMPERATURE TECHNIQUE

Justin VANOVENBERGHE
First master of Drug Development

Promoter
Prof. dr. S. De Smedt

Co-promoter
Prof. dr. K. Mäder

Commissioners
Prof. dr. K. Braeckmans
dr. I. Lentacker

Master thesis performed at:
MARTIN-LUTHER-UNIVERSITÄT
HALLE-WITTMENBERG
INSTITUT FÜR PHARMAZIE
Department of Pharmaceutical Technology and Biopharmaceutics
Laboratory of Pharmaceutical Technology
Abstract

Numerous kinds of nanosystems for drug delivery exist. These can be made through high-energy or low-energy methods. Low-energy methods have the obvious advantage that they require less energy. They often have the disadvantage however that they require organic solvents. These can be toxic and can’t be used for therapeutic applications. Nanodispersions can be made with high amounts of surfactants. Some of these nanosystems are sensitive to dilution, as hydrophilic surfactants can leach out of nanodispersion upon dilution. This can negatively affect the nanodispersion’s structure.

Heurtault et al. proposed a phase inversion temperature technique for nanodispersion creation. This low-energy method has the benefit that it only requires surfactants and temperature changes to obtain nanodispersions. This technique was successfully established in the pharmaceutical technology group of professor dr. Mäder. It gave rise to a reproducible translucent nano dispersion in the lower nano range (±25-30nm).

The structure of the nanodispersion was investigated with $^1$H NMR experiments. This gave indications about the mobility of the used excipients, but was not sufficient to explain the exact structure. The formulation was tried to be visualised with electron microscopy and negative staining. This appeared to be a bad sample preparation for this formulation, as aggregates were easily formed.

Samples were stored under different circumstances over a long period of time. The obtained formulation appeared to be stable in daylight at room temperature and fridge temperature (4°C). A precipitation could be seen in the sample after some time. This occurred faster when the formulation was stored at 37°C. It also appeared to be stable against dilution, as the nanodispersion’s size remained more or less the same when the samples were diluted.

In further research, the formulation can be visualised with electron microscopy, however another sample preparation must be chosen then (for example cryofixation). The structure of the nanodispersion can also be further investigated with differential scanning calorimetry and atomic force microscopy.
Samenvatting


De structuur van de nanodispersie werd onderzocht met $^1$H NMR technieken. Dit gaf indicaties over de mobiliteit van de bestanddelen, maar het was niet voldoende om hiermee de exacte structuur te bepalen. Er werd ook geprobeerd de formulatie te visualiseren met elektron microscopie en negatieve kleuring. Deze laatste bleek een slechte voorbereidingstechniek te zijn voor deze formulatie, aangezien aggregaten werden gevormd.

Stalen werden voor een lange periode onder verschillende condities bewaard. De formulatie was stabiel bij kamer- en koelkasttemperatuur (4°C) en in het daglicht. Na een tijd verscheen echter een bezinksel in alle stalen. Dit verscheen sneller als de formulatie bewaard werd bij verhoogde temperatuur (37°C). Ook bleef het system stabiel als de formulatie werd verdund en wijzigde de deeltjesgrootte nagenoeg niet.

De formulatie kan gevisualiseerd worden bij verder onderzoek met behulp van elektron microscopie. Dan moet wel een andere voorbereidingstechniek gebruikt worden. (bijvoorbeeld cryofixatie). De structuur kan ook verder onderzocht worden met differential scanning calorimetry en atomic force microscopie.
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H-NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>Kcps</td>
<td>Kilo counts per second</td>
</tr>
<tr>
<td>MCT</td>
<td>Medium chain triglycerides</td>
</tr>
<tr>
<td>NIBS</td>
<td>Non-Invasive Backscatter Detection</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil in water</td>
</tr>
<tr>
<td>OTCS</td>
<td>Original temperature cycled sample</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PIT</td>
<td>phase inversion temperature</td>
</tr>
<tr>
<td>Ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SLS</td>
<td>Static light scattering</td>
</tr>
<tr>
<td>TC</td>
<td>Temperature cycling</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>W/O</td>
<td>water in oil</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Nanoemulsions

Nanoemulsions are O/W or W/O emulsions in the submicron range. They are made out of oil, water and surfactant. They have benefits over systems with larger particles. They usually are more stable against gravitational separation for example. The small particles also scatter light waves more weakly, therefore they can be used in products which must be only slightly turbid or optically clear. They can also have novel rheological properties. Another advantage is the large surface area, it increases drug transport and their delivery. Hydrophobic drugs can be dissolved in the nanoemulsion droplet (in case of an O/W emulsion) and be protected against hydrolysis and enzymatic degradation. (1) When some drugs and cosolvents are injected intravenously, they can lead to local irritation of the veins. Nanoemulsions avoid this direct contact and also avoid the use of cosolvents. (1-3)

Colloidal dispersions with very small particles (diameter<200nm) have numerous benefits over larger particles. These include: better stability to particle aggregation and gravitational separation, optical clearer, they may also increase the bioavailability of some lipophilic substances encapsulated within them. (4)

1.2 NANOEMULSIONS VERSUS MICROEMULSIONS

In literature confusion exists between the terms “nanoemulsions” and “microemulsions”. These are different types colloidal dispersions. The structure is fairly similar. A hydrophobic core, consisting of oil and the hydrophobic parts of the surfactant, is surrounded by a hydrophilic shell of surfactant head groups. The main difference is that a microemulsion is thermodynamically stable and a nanoemulsion is not. Nanoemulsions can however also be kinetic stable and stay in a metastable state over a longer period of time. Microemulsions can be formed in theory by simply bringing the oil, water and surfactant together. In practice however, it is often necessary to apply external energy to overcome kinetic energy barriers or mass transport limitations and facilitate the process. Nanoemulsions on the other hand always require external energy
to bring the separate components together to a colloidal dispersion. The terms “micro” and “nano” can be misleading, because they usually mean respectively $10^{-6}$ and $10^{-9}$. This suggests that nanoemulsions are smaller than microemulsions, however it is usually the other way around in practice. The historical development of the field of colloid science is the reason for this confusing terminology. These concepts were searched for in an online literature in the “Web of Knowledge” (Thomson Reuters) in October 2011. The term “microemulsion” appeared first in a publication from 1961. The expression “nanoemulsion” was seen a lot later in a publication, dating from 1996. Nanoemulsions were already used before then, but they were referred to by other names like: ultrafine emulsions, submicron emulsions or mini-emulsions. The term “microemulsion” was already commonly used in research before the term “nanoemulsions” was introduced. That is why sometimes confusion exists when using these 2 terms. (4) The question can arise: 'Why are nanoemulsions made, when microemulsions, which are thermodynamically stable and are easier to make, can be made as well?' Nanoemulsions can however withstand dilution, whereas microemulsions often break up (5). Another advantage is that nanoemulsions usually require less surfactant than microemulsions. (2)

1.3 DISTINCTION FROM COMMON EMULSIONS

There is a lot of disagreement about the upper particle size limit of “nano”emulsions: ranging from 100 nm to 500 nm. Some authors also don’t specify if they describe particle radius or diameter. When a W/O emulsion droplet size is decreased from the micrometer to the nanometer range, no distinct change in physicochemical or thermodynamic properties occur. Some differences in system properties can however be potentially used to define nano- and conventional emulsions. These include: optical properties and gravitational stability. A colloidal dispersion becomes more translucent/transparent if the particle radius is smaller than ±30nm. The stability concerning gravitational separation (creaming/sedimentation) increases when the radius is lower than ±90nm, because the Brownian motion overcomes gravitational forces then. This illustrates that there is no precise cut-off size between conventional emulsions and nanoemulsions, based on the physicochemical properties of the system. (4)
1.4 HIGH- AND LOW ENERGY METHODS FOR NANOEMULSION CREATION

1.4.1 General

As said before, nanoemulsions are a non-equilibrium system and therefore their preparation requires energy, surfactants or a combination of both. High- and low-energy methods can be used in the preparation. High-energy methods include mechanical devices, which create intensely disruptive forces to create nanoemulsions. The size will depend on the kind of device used and the respective operating conditions. A good control of particle size and big choice of composition are possible with this method. Examples of high-energy methods are: high-pressure homogenization, ultrasonication and microfluidization. They may however not be fit for thermolabile drugs. Low-energy methods use stored energy of the system to form nanoemulsions by changing parameters, which influence the hydrophilic lipophilic balance of the system. Examples are: the phase inversion temperature method and the solvent displacement method.

1.4.2 Phase inversion temperature (PIT) technique

Shinoda et al. introduced the phase inversion temperature method in 1969. The temperature dependent solubility of non-ionic surfactants is the key to this method, for example polyethoxylated surfactants. The affinities of these surfactants to water and oil vary in function of the temperature. The polyoxyethylene groups become dehydrated at higher temperatures and become more lipophilic. When the water, oil and surfactants are mixed together this leads initially to an O/W macroemulsion. When the sample is heated, the surfactants become more lipophilic and get completely solubilized in the oily phase. The O/W emulsion is destabilised and undergoes a phase inversion from an O/W- to a W/O emulsion. When it is cooled down, it gives rise to an O/W emulsion again. When this process is repeated and the emulsion is heated and cooled quickly around the PIT, nanoemulsions may be formed. A main advantage of this method is the absence of organic solvents (unlike the solvent displacement method for example). When thermolabile drugs are used this “temperature cycling” can be a downside. It may be avoided by changing other parameters to change surfactant affinities, instead of the temperature: for example the salt concentration. These changes can however be done only in one direction (3, 6-8)
2 Recent indications for toxicity risks of nanocarriers.

Recent studies indicate the accumulation of nanoparticles, nanocapsules and nanosized lipid emulsions in the ovaries and adrenals. This however does not imply risk for all nanocarriers used in medicine, as the accumulation was only seen in special regions of the rodent’s ovaries. The toxic effect can be rather low in humans. It should however be investigated as well in other species to explain the mechanism of accumulation. This may have possible applications for future ovarian targeted therapy. (9, 10)
2.1 USED EXCIPIENTS

2.1.1 SURFACTANTS

2.1.1.1 Macrogol-15 Hydroxystearate.

![Chemical structure of Macrogol-15 Hydroxystearate](image)

Figure 2.1 Main components of Kollirophor HS15 (BASF): PEG mono- and di-esters of 12-hydroxystearic acid. A part of the 12-hydroxy groups can be etherified with PEG too. There is also about 30% free PEG.

Macrogol-15 hydroxystearate (European Pharmacopoeia), also known as polyoxyl 15 hydroxystearate (United States Pharmacopeia), is a nonionic surfactant. It is a mixture of especially mono- and di-esters of 12-hydroxystearic acid and a smaller fraction of free polyethylene glycol. It is formed by the ethoxylation of 12-hydroxystearic acid. 1 mole 12-hydroxystearic acid reacts with 15 moles ethylene oxide.

It is commercially made by BASF as “Kollirophor HS15” (it used to name: “Solutol HS15”). It consists out 70% mono- and diesters and 30% free polyethylene glycol. The micelles are about 10 nm. (11) It is semisolid at room temperature and becomes liquid at approximately 30°C. It has a critical micelle concentration between 0,005 and 0,02%. (13)(http://www.pharma-ingredients.basf.com/Statements/Technical%20Informations/EN/Pharma%20Solutions/03_030748e_Solutol%20HS%2015.pdf (12))
2.1.1.2 Phospholipids

![Phospholipid Structure](image)

Figure 2.2 Phosphatidylcholine. The "R" groups represent aliphatic chains.

Phospholipids are surface active, amphiphilic structures. They can be from natural origin (egg yolk or soybean) or synthesized. Both are used in pharmaceutical applications. They can be used in many formulations, for example: suspensions, emulsions, mixed micelles and liposomal preparations. Phosphatidylcholine is the most common phospholipid and is a major component of lecithin. (14, 15)

According to the United States Pharmacopoeia (USP), "Lecithin is a complex mixture of acetone-insoluble phosphatides, which consist chiefly of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol, combined with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates, as separated from the crude vegetable oil source. It contains not less than 50.0 percent of acetone-insoluble matter." (16)
2.1.2 Oil

Medium chain triglycerides (MCT) are commonly used as an oily phase. According to the European Pharmacopoeia 5.0, the definition of medium-chain triglycerides is “Mixture of triglycerides of saturated fatty acids, mainly of caprylic acid (octanoic acid, $C_8H_{16}O_2$) and of capric acid (decanoic acid, $C_{10}H_{20}O_2$). Medium-chain triglycerides are obtained from the oil extracted from the hard, dried fraction of the endosperm of Cocos nucifera L. or from the dried endosperm of Elaeis guineensis Jacq.” It has a relative density of 0.93-0.96 (13)
2.2 ARTICLES PUBLISHED BY HEURTAULT ET AL.

The articles: "The influence of lipid nanocapsule composition on their size distribution" and "A novel phase inversion-based process for the preparation of lipid nanocarriers" published by Heurtault et al, were the foundation for the research in this thesis. (17, 18) They developed this method to avoid the use of organic solvents, because of their potential toxicity after human administration. The process is based on the PIT technique, explained in 1.4.2. The method went as following: Solutol® HS15, Lipoid® S75-3 (hydrogenated lecithin) and Labrafac® WL 1349 (MCT) were mixed together with de-ionised H₂O and NaCl. The mixture was then heated from room temperature to 85°C at 4°C/min. Next, it was cooled down until 60°C at 4°C/min. This was repeated another 2 times (so it was heated as following: ±25-85-60-85-60°C). This process will be further referred to as "temperature cycling". This temperature range, necessary to reach the inversion process, depends on the composition of the mixture.

An irreversible shock was done by diluting the mixture with ice-cold de-ionised water (±0°C) around 1-3°C under the beginning of the beginning of the phase inversion zone. The mixture was slowly magnetically stirred afterwards for 5 minutes.

![Figure 2.4 Ternary diagram of the relative compositions of the excipients, leading to the formation of nanostructures after the temperature cycling process. The diagram was obtained from "The influence of lipid nanocapsule composition on their size distribution" by Heurault et al. (18)](image-url)
They made 41 formulations with different compositions. The concentration of the salted water, hydrophilic surfactant and oil varied in these formulations. The concentrations of NaCl and lipoid remained constant at 1.75m/m% and 1.50m/m% respectively. The NaCl concentration influenced the PIT: the higher the concentration, the lower the PIT. Lipoid on the other hand had no effect on particle size distribution up to 5 m/m%. These formulations were tested in triplicate and the resulting structures can be seen in the ternary diagram in figure 2.4. The zone with compositions, which led to nanostructures, was called the ‘feasibility domain’. 13 different compositions were made in the feasibility domain. The resulting particle sizes were measured with DLS, using an Autosizer® 4700 (Malvern Instruments, Worcestershire, UK). The sizes varied in function of the relative composition in a range from 20 to 100 nm (diameter), as can be seen in Table 2.1. They described the resulting nanostructures as a “hybrid between polymeric nanocapsules and liposomes” and refer to it as “nanocapsules”. (18)

Table 2.1 Obtained particle sizes with DLS from the different compositions, shown in Figure 1.2. The table was obtained and adapted from "The influence of lipid nanocapsule composition on their size distribution" by Heurault et al. (18)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Weight fraction of exocipient (%)</th>
<th>Measured response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Oil</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>0.100</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>0.250</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>10</td>
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<td>4</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>10</td>
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<td>7</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>17</td>
</tr>
</tbody>
</table>

Figure 2.5 Ternary diagram of the relative compositions of the 13 different samples in the feasibility domain, analyzed with DLS. The diagram was obtained from "The influence of lipid nanocapsule composition on their size distribution" by Heurault et al. (18)
3 OBJECTIVES

The method proposed by Heurtault et al. has interesting advantages. It is a low-energy method and it uses no organic solvents. No advanced equipment is necessary to obtain the nanodispersion. High amounts of surfactants (10-40%) are used however. As described earlier it is possible that hydrophilic surfactants leach out of the nanodispersion as the system gets diluted.

No phase inversion temperature techniques for the formation of nanodispersions were used yet in the group of pharmaceutical technology of prof. dr. Mäder. The main goal in this thesis is to introduce this method in the group and to see if the resulting nanodispersion is reproducible. Another goal is to characterise the dispersion’s structure and to have an understanding of the stability in function of time and upon dilution.

It is tried to reproduce the nanodispersion in a first stage with the temperature cycling technique, proposed by Heurtault. The resulting sample is measured with static light scattering to have a first impression of the size range of the nanodispersion. A sample without MCT is measured as well to see the differences between the samples.

Dynamic light scattering is further used to analyse the nanodispersion’s size and is a key technique in this thesis. Good parameters must be chosen to obtain correct sizes with DLS. Viscosity has a big influence on the obtained particle size and is measured for each different sample. Other parameters are assumed.

The stability of the resulting nanodispersion will be further examined, after the technique is established. This can be done by measuring the particle sizes with DLS after storage under different conditions and after dilution.

The nanodispersion’s structure is also further investigated. Transmission electron microscopy is used to see the appearance of the structure. $^1$H NMR techniques can be used to see how the excipients interact with each other within the structure.
4 METHODS AND MATERIALS

4.1 GENERAL PROCEDURE

Samples were made with Labrafac CC (caprylic/capric triglycerides, Gattefossé, Saint-Priest, France, lot: 450701007), Solutol HS15 (BASF, Ludwigshafen, Germany, lot: 10151888Q0, Lipoid S75-3 (hydrogenated lecithin, Lipoid, Ludwigshafen, Germany, lot: 252159-1), double distilled H₂O and NaCl (Grüssing, Filsum, Germany, lot: 0040). These first three were further referred to as respectively MCT, solutol and lecithin. Solutol is in a semi-solid state at room temperature. It was molten at 90 °C and stirred before processing.

Table 4.1 Composition (m/m%) of the temperature cycled mixture.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Composition (m/m %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase (1,75% NaCl in H₂O)</td>
<td>56,70</td>
</tr>
<tr>
<td>Solutol</td>
<td>24,87</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1,52</td>
</tr>
<tr>
<td>MCT</td>
<td>16,91</td>
</tr>
</tbody>
</table>

A sample was made by mixing the individual components as described in Table 4.1. This composition corresponds to the one in the middle of the feasibility domain (trial number 9 in Table 2.1), described by Heurtault et al. in 2.2. It was then submitted to 3 continuous heating and cooling cycles. The mixture was heated until 85°C on a Yellow Line MSC basis C (IKA®) heating plate. It was cooled back down until 60°C afterwards at room temperature. This process was further referred to as “temperature cycling (TC)”. The sample was magnetically stirred at 450 rpm during this process. After the third cycle, an irreversible cooling shock was done by adding ice-cold double distilled water to the sample at 60°C. The sample was then magnetically stirred for 5 min at 300 rpm.

A sample was made by adding 25g of ice-cold water to 20g of the temperature cycled sample. Another sample was made by adding 50 mL of ice-cold water to 25g of cycled sample without MCT.
Table 4.2 Composition (m/m%) of the temperature cycled sample without MCT

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Composition (m/m %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous phase (1,75% NaCl in H₂O)</td>
<td>73,60</td>
</tr>
<tr>
<td>Solutol</td>
<td>24,88</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1,52</td>
</tr>
<tr>
<td>MCT</td>
<td>0</td>
</tr>
</tbody>
</table>

4.2 LIGHT SCATTERING TECHNIQUES FOR SIZE DETERMINATION

4.2.1 Static light scattering

The particle size of each sample was measured 5 times with static light scattering (Mastersizer 2000, Malvern instruments, UK) with a laser obscuration of 1-2%. The data was averaged and analysed with Mastersizer 2000 software. A volume weighted size distribution was obtained by applying the Mie theory. A spherical particle with a refractive index of 1,450; an absorption of 0,001 and a dispersant refractive index of 1,330 were assumed.

4.2.2 Dynamic light scattering

4.2.2.1 General

Samples were appropriately diluted with double distilled water, filtered (pore size 0,22 µm) and measured with DLS (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) at 25°C. The concentration of each sample after the dilutions, when measured with DLS, was expressed as m/m% of the original temperature cycled sample (m/m% OTCS). These dilutions included the dilution caused by adding the ice-cold water and the other dilution, which was done immediately before DLS measurement. Scattered light was detected at 173° in the backscattering mode. The following assumptions were made: a particle RI of 1,45; a material absorption of 0,01 and a dispersant RI of 1,330. Each dilution had a specific viscosity (measured in 4.2.2.2), which was used together with the other assumed parameters in the calculation of the corresponding particle size.
These parameters were always used when measuring with DLS, unless it was specified otherwise. 5 measurement runs were done to analyse a sample. Each of those runs comprised more than 10 individual measurements. The data was averaged and processed with Zetasizer software (version 7.03, Malvern Instruments, Worcestershire, UK). Z-averages and PDI’s were obtained in this manner.

4.2.2.2 Viscosity measurement

A sample was made as described in 4.1. Different amounts of ice-cold double distilled H$_2$O were added to the sample, which had undergone temperature cycling. Different dilutions with therefore different viscosities were achieved in this manner. These viscosities corresponded with those of the different samples, used throughout this thesis.

Viscosities were obtained by measuring the efflux times of the different dilutions and using Equation 4.1. Capillary number I (type number 53010, $k = 0.010192$ mm$^2$.s$^{-2}$) and capillary number II (type number 50120, $k = 0.10008$ mm$^2$.s$^{-2}$) ubbelohde viscometers (Schott Instruments, Mainz, Germany) were used to measure efflux times. The efflux time of each sample was measured 3 times and the average was used. The Hagenbach correction for each efflux time was derived from the table in the appendix.

Densities were measured using a 20 mL pycnometer and an analytical balance (SBA 31, Scaltec, Heiligenstadt, Germany).

$$\eta = k \times (t - F) \times \rho$$

Equation 4.1

$\eta$ = Dynamic viscosity (mPa.s)

$k$ = Device constant (mm$^2$/s$^2$)

$t$ = Efflux time (s)

$F$ = Hagenbach correction (s)

$\rho$ = Fluid density (g/cm$^3$)
4.3 CONDUCTIVITY MEASUREMENT

The conductivity was measured during the temperature cycling (as described in 4.1), using a SevenCompact\textsuperscript{TM} S230 conductivity meter (Mettler Toledo\textsuperscript{®}). The conductivity meter was calibrated with a 12.88 mS/cm conductivity standard (Lot: 1Y053C, Mettler Toledo, Gießen, Germany). The conductivity was measured every 10 seconds. The system was equipped with linear temperature correction and the conductivity was corrected according to Equation 4.2. A reference temperature of 25°C was selected and a linear compensation coefficient $\alpha$ was calculated according to Equation 4.3. (HANDLEIDING referentie)

\[
\kappa_{T_{ref}} = \frac{\kappa_T}{1 + \frac{\alpha}{100\%} \times (T - T_{ref})}
\]

Equation 4.2

- $\kappa_{T_{ref}}$ = Conductivity at reference temperature (mS/cm)
- $\kappa_T$ = Conductivity at sample temperature (mS/cm)
- $T_{ref}$ = Reference temperature (°C)
- $T$ = Sample temperature (°C)
- $\alpha$ = Linear compensation coefficient (%/°C)
\[ \alpha = (\kappa_{T1} - \kappa_{T2}) \times \frac{100\%}{(T_1 - T_2)/\kappa_{T2}} \]

Equation 4.3

\( \kappa_{T1} \) = Conductivity at typical sample temperature (mS/cm)

\( \kappa_{T2} \) = Conductivity at reference temperature (mS/cm)

\( T_1 \) = Typical sample temperature (°C)

\( T_2 \) = Reference temperature (°C)

\( \alpha \) = Linear compensation coefficient (%/°C)

Table 4.3 Used temperatures and respective conductivities for the calculation of \( \alpha \), using Equation 4.3. The compositions of the mixtures are described in Table 4.1 and Table 4.2

<table>
<thead>
<tr>
<th>Mixture</th>
<th>( T_1 ) (°C)</th>
<th>( \kappa_{T1} ) (mS/cm)</th>
<th>( T_2 ) (°C)</th>
<th>( \kappa_{T2} ) (mS/cm)</th>
<th>( \alpha ) (%/°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture without MCT</td>
<td>28,4</td>
<td>9,79</td>
<td>40,9</td>
<td>12,98</td>
<td>1,9661</td>
</tr>
<tr>
<td>Mixture without MCT</td>
<td>31,4</td>
<td>15,77</td>
<td>43,5</td>
<td>19,51</td>
<td>1,5843</td>
</tr>
</tbody>
</table>

4.4 INFLUENCE OF THE COOLING SHOCK

A sample was made in the same way as described in 4.1. It was diluted by adding different amounts of ice-cold double distilled water, after the temperature cycling. The samples were stirred and afterwards diluted to a same concentration of 12,5 m/m % OTCS with double distilled water at room temperature. Like this, the only difference between the samples was the cooling rate. The size was measured with DLS (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK), including a viscosity of 1,247 mPa.s.
4.5 INFLUENCE OF DILUTION

A sample was made as in 4.1. Varying amounts of ice-cold double distilled water were added to the sample after the temperature cycling process. Different dilutions were obtained in this way. The several dilutions were consecutively measured with DLS at 25°C.

4.6 STORAGE

A sample was made as described in 4.1, by adding 20g of ice-cold water to 25g of the temperature cycled sample. It was stored at room temperature in daylight and measured at different times with DLS (with a viscosity of 1,185 mPa.s). The sample was diluted to 11.1 m/m% OTCS, just before each DLS measurement.

Another sample was made as described in 4.1. The water in this temperature cycled sample however, contained 0.02% NaN₃ (Merck-Schuchardt, Hohenbrunn, Germany). 20g ice cold water was added to 25g of the temperature cycled sample. It was stored at 4°C, 37°C and 25°C (dark+daylight). The macroscopic appearance and the size, which was obtained with DLS (with a viscosity of 1,913 mPa.s), were checked on different times. The sample was diluted to 27.8 m/m% OTCS, just before each DLS measurement.

Precipitated material was further examined with a light microscope (Axio Lab.A1, Carl Zeiss, Germany) with 20x and 50x objectives. Pictures were taken with an Olympus UC 30 camera (Olympus soft imaging solutions, Germany), which was equipped on top of the microscope. Stream motion 1.7 software (Olympus soft imaging solutions, Germany) was used to process the images.
4.7 $^1$H-NMR SPECTROSCOPY

4.7.1 Full spectrum $^1$H-NMR
4.7.1.1 Obtaining temperature cycled mixture spectrum

A sample was made in the same way as in 4.1, except heavy water (99.9\% atom\% deuterium, Sigma-Aldrich, St. Louis, USA, lot: MKBB2073) was used instead of normal water. The heavy water contained 0.75\% 3-(trimethylsilyl)propionic-2,2,3,3-d$_4$ acid sodium salt. 1.75 mL ice-cold heavy water was added to 0.25 mL of the mixture, which had undergone temperature cycling. The sample was mixed and 0.8 mL was used to obtain $^1$H NMR spectra. All spectra were obtained with a Gemini 2000 spectrometer (Varian, Les Ullis, France), operated at 27°C.

4.7.1.2 Obtaining solutol and lecithin reference spectra

Dispersing the molten solutol in heavy water created solutol micelles. In this manner a 25 m/m\% solutol reference sample was obtained.

0.125g lecithin was dissolved in 4 mL dichloromethane (Carl Roth, Karlsruhe, Germany, lot: 23896449). The organic solvent was evaporated with a Rotavapor-RE (Büchi, Flawil, Switzerland). The solution was revolving in a warm water bath, held at 40°C, whilst the pressure was gradually decreased from 800 mbar to 200 mbar. After this procedure a thin layer of lecithin remained in the flask. 4.5 mL heavy water was added to the layer and the flask was consecutively shaken at 60 °C until the complete layer was dispersed. After this procedure lecithin liposomes were formed. The size and appearance of the liposomes were checked with a light microscope (Axio Lab.A1, Carl Zeiss, Germany) and static light scattering (Mastersizer 2000, Malvern instruments, UK). 0.8 mL of the lecithin and solutol samples were analysed with $^1$H NMR and reference spectra were obtained.

Predicted solutol and lecithin spectra were obtained with MestReNova software (version 6.0.2-5475, Mestrelab Research S.L., Santiago de Compostela, Spain). Chloroform was selected as solvent in the software, since no other solvents were
available. The obtained predicted spectra, the solutol and lecithin reference spectra, and the mixture spectrum were then compared.

4.7.2 $^1$H-NMR relaxometry

A low-field (20MHz) benchtop NMR spectrometer (Maran DRX2, Oxford Instruments Molecular Biotools, Oxfordshire, UK) was used to measure $^1$H NMR transverse relaxation times ($T_2$). It was equipped with airflow temperature regulation. Carr-Purcell-Meiboom-Gill pulse sequences were applied to obtain transverse magnetization decays at 25°C. Following samples were analyzed: pure MCT; 25% solutol (in $^2$H$_2$O); 2,70% lecithin (in $^2$H$_2$O), prepared as described in 4.7.1.2; the temperature cycled mixture (in $^2$H$_2$O), prepared as described in 4.1.

Each pulse sequence was detecting 12k echoes with a relaxation delay time of 20 s and 256 repetitions. 2000 repetitions were done for the lecithin sample. WinDXP analysis software (Oxford Instruments, Abingdon, UK) was used to fit the transverse magnetization decays. An inverse Laplace transformation method was used to analyse the relaxation decays. $T_2$ distributions were calculated with 256 points. About 1 cm$^3$ sample was necessary to achieve an appropriate signal-to-noise ratio. For a sufficient signal-to-noise ratio a long acquisition time of about 110 minutes was necessary. Graphs were obtained with Origin® software (OriginPro 8 v8.0891, OriginLab, Northampton, USA)
4.8 TRANSMISSION ELECTRON MICROSCOPY

A sample was made as described in 4.1. Different amounts of ice-cold water were added after the temperature cycling. Samples were obtained with the following concentrations of OTCS, after the dilution: 100 m/m%, 50 m/m%, 5 m/m%, 0,5 m/m% and 0,05 m/m%. Before analysing the samples with transmission electron microscopy, they were prepared by negative staining. Drops (±10 µL) of each dilution were pipetted on parafilm. 200 mesh Cu grids, covered with a formvar film, were laid on the drops.

The grids were removed after one minute and excess solvent was removed by gently touching the side of the grid with filter paper. Consecutively, this procedure was repeated in the same way but now the grids were laid in 1% uranyl acetate drops (manufacturer:/ batch:/) instead of the dilution drops. After the excess uranyl acetate solution was removed with a filter paper, the grids were dried at room temperature. The grids were analysed under a transmission electron microscope when they were dry (TEM 900 series, Zeiss, Germany).
5 RESULTS

5.1 CHANGES DURING THE TEMPERATURE CYCLING PROCESS

Macroscopic changes can be seen in the mixture during the temperature cycling. This is illustrated in Picture 5.1. These changes are also reflected by conductivity variations in function of the temperature in Figure 5.1 and Figure 5.2. The mixture, before TC, showed creaming when standing still after a short period of time. The mixture remained translucent after the TC and no creaming occurred anymore.

Picture 5.1 Macroscopic change of the sample during the temperature cycling process, described in 4.1. LEFT: the mixture before temperature cycling; MIDDLE: the mixture in the first heating cycle at 80°C; RIGHT: the mixture after the 3 temperature cycles.
Figure 5.1 Conductivity in function of heating in the first heating-cooling cycle. The conductivity under ±62°C remains high and steady (o/w emulsion). At ±62°C the conductivity drops. A conductivity peak at ±73°C can be seen but then drops again to a steady low conductivity.

Figure 5.2 Conductivity in function of cooling in the first heating-cooling cycle. The conductivity increases gradually at ±78°C and remains stable at ±68°C.
Figure 5.3 Conductivity in function of heating during the first heating-cooling cycle of the TC mixture without MCT. The conductivity decreases gradually at 63°C.

Figure 5.4 Conductivity in function of cooling during the first heating-cooling cycle of the TC mixture without MCT. The conductivity gradually increased.
5.2 MEASUREMENT PARTICLE SIZES

5.2.1 Difference in particle sizes between the temperature cycled mixtures with and without MCT

The mixtures with and without MCT, described in Table 4.1 and Table 4.2 respectively, were measured with static light scattering and compared in Figure 5.5.

![Figure 5.5 Volume weighted size distribution of a temperature cycled mixture containing: solutol, NaCl, H2O, lecithin and MCT (RED) and a temperature cycled mixture without MCT (GREEN). The graph was obtained with static light scattering and Mastersizer 2000 software](image-url)
5.2.2 Viscosity

The obtained densities of the 27.8 m/m% OTCS, measured in triplicate at 21°C, are: 1.0078 g/ml; 1.0020 g/ml and 1.0016 g/ml. It is challenging to obtain reproducible results, as bubbles form easily in the pycnometer because of the high surfactant concentration. As the OCTS gets more diluted, the density of the dilution approaches 0.998 g/mL (density of water at 20°C). A simplification is done therefore by assuming a sample density of 1 g/mL in all dilutions for the calculation of corresponding viscosity. The obtained viscosities in Table 5.1, are used in the Zetasizer software to obtain correct particle sizes with DLS.

Table 5.1 Viscosity of the different dilutions at 25°C, as described in 4.2.2.2.

<table>
<thead>
<tr>
<th>Concentration OTCS (m/m%)</th>
<th>Efflux time (s)</th>
<th>Hagenbach correction (s)</th>
<th>Viscosity(^c) (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>91(^a)</td>
<td>1.197</td>
<td>0.9153</td>
</tr>
<tr>
<td>0.1</td>
<td>91(^a)</td>
<td>1.197</td>
<td>0.9153</td>
</tr>
<tr>
<td>0.25</td>
<td>91(^a)</td>
<td>1.197</td>
<td>0.9153</td>
</tr>
<tr>
<td>0.5</td>
<td>91(^a)</td>
<td>1.197</td>
<td>0.9153</td>
</tr>
<tr>
<td>2.5</td>
<td>95(^a)</td>
<td>1.105</td>
<td>0.9570</td>
</tr>
<tr>
<td>5.0</td>
<td>100(^a)</td>
<td>0.990</td>
<td>1.009</td>
</tr>
<tr>
<td>10.0</td>
<td>112(^a)</td>
<td>0.794</td>
<td>1.133</td>
</tr>
<tr>
<td>11.1</td>
<td>117(^a)</td>
<td>0.729</td>
<td>1.185</td>
</tr>
<tr>
<td>12.5</td>
<td>123(^a)</td>
<td>0.660</td>
<td>1.247</td>
</tr>
<tr>
<td>25.0</td>
<td>164(^a)</td>
<td>0.372</td>
<td>1.664</td>
</tr>
<tr>
<td>27.8</td>
<td>188(^a)</td>
<td>0.284</td>
<td>1.913</td>
</tr>
<tr>
<td>100.0</td>
<td>872(^b)</td>
<td>&lt;0.01</td>
<td>87.24</td>
</tr>
</tbody>
</table>

\(^a\) Efflux time measured with an ubbelohde capillary viscometer type I (type: 53010)
\(^b\) Efflux time measured with an ubbelohde capillary viscometer type II (type: 50120)
\(^c\) Densities were assumed to be 1g/mL in the calculation of the viscosity
5.2.3 Particle size measurement with dynamic light scattering

A sample is made by adding 20g ice-cold water to 25g temperature cycled sample. Before DLS measurement it is diluted 1:4 to obtain the particle sizes. 2 samples are made in this way. The concentration after these dilutions correspond to 11,1 m/m% OTCS. The obtained particle sizes can be seen in Table 5.3.

Table 5.2 Concentration (m/m %) of the individual substances in 11,1m/m% OTCS when measured with DLS.

<table>
<thead>
<tr>
<th>substance</th>
<th>Concentration (m/m %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1091</td>
</tr>
<tr>
<td>Labrafac</td>
<td>1.8606</td>
</tr>
<tr>
<td>Solutol</td>
<td>2.7361</td>
</tr>
<tr>
<td>Lipoid</td>
<td>0.1666</td>
</tr>
</tbody>
</table>

Table 5.3 Sizes (z-average, diameter) and PDI's of 2 different samples 11,1 m/m% OTCS, obtained with DLS.

<table>
<thead>
<tr>
<th>11,1 m/m% OTCS</th>
<th>Size (z-average, nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>25,49</td>
<td>0,084</td>
</tr>
<tr>
<td>Sample 2</td>
<td>25,18</td>
<td>0,046</td>
</tr>
</tbody>
</table>
5.2.4 Transmission electron microscopy

Nothing could be seen on the images of the 100m/m% and 0,05m/m% OTCS, the image were respectively completely black and completely white. The other obtained pictures can be seen below.

![Picture 5.2 50m/m% OTCS, obtained with TEM after negative staining with uranyl acetate](image1)

![Picture 5.3 5m/m% OTCS, obtained with TEM after negative staining with uranyl acetate](image2)
5.3 STABILITY

5.3.1 Storage

Picture 5.4 0.5 m/m% OTCS, obtained with TEM after negative staining with uranyl acetate

Picture 5.5 Pictures of the observed precipitation in the 11.1 m/m% OTCS (left) and the preserved 27.8 m/m% OTCS (stored at 25°C in daylight)(right). The black bar represents 50 µm. Enlarged pictures can be found in annex.
2 different samples were stored as described in 4.6: one in daylight at room temperature and another one, which was preserved, under different conditions. The sizes in function of time can be seen in Table 5.4 and Table 5.5. A precipitation can be seen in both samples when they are stored. It can be easily dispersed again when shaken. However, it always reappears again. The precipitation can be seen a lot faster in the sample stored at 37°C. The sedimentation was further investigated under a light microscope, as can be seen in Picture 5.5.

Table 5.4 Particle size and PDI of a 11,1 m/m% OTCS in function of time. Data was obtained with DLS at 25°C with viscosity of 1,1850 mPa.s. The sample was stored in daylight and at room temperature.

<table>
<thead>
<tr>
<th>Amount of days after the TC</th>
<th>PDI</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.084</td>
<td>25.49</td>
</tr>
<tr>
<td>4</td>
<td>0.092</td>
<td>26.77</td>
</tr>
<tr>
<td>7</td>
<td>0.052</td>
<td>25.12</td>
</tr>
<tr>
<td>10</td>
<td>0.053</td>
<td>25.09</td>
</tr>
<tr>
<td>16</td>
<td>0.046</td>
<td>25.44</td>
</tr>
<tr>
<td>21</td>
<td>0.060</td>
<td>25.34</td>
</tr>
<tr>
<td>28</td>
<td>0.057</td>
<td>25.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>0.061</td>
<td>25.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> a precipitation was seen in the sample, which could be easily dispersed when shaken

Table 5.5 Particle size and PDI of the preserved sample in function of time, under different storage conditions. The samples were measured at 26,8 m/m% OTCS with DLS (at 25°C and a viscosity of 1,913 mPa.s).

<table>
<thead>
<tr>
<th>Amount of days after the TC</th>
<th>25°C daylight</th>
<th>25°C dark</th>
<th>37°C</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDI Size (nm)</td>
<td>PDI Size (nm)</td>
<td>PDI Size (nm)</td>
<td>PDI Size (nm)</td>
</tr>
<tr>
<td>0</td>
<td>0.128 16.68</td>
<td>0.128 16.68</td>
<td>0.128 16.68</td>
<td>0.128 16.68</td>
</tr>
<tr>
<td>3</td>
<td>0.128 17.29</td>
<td>0.131 17.43</td>
<td>0.131 17.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.121 17.37</td>
</tr>
<tr>
<td>9</td>
<td>0.142 18.07</td>
<td>0.137 18.07</td>
<td>0.151 19.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.138 18.05</td>
</tr>
<tr>
<td>14</td>
<td>0.139 18.29</td>
<td>0.140 18.32</td>
<td>0.158 20.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.147 18.31</td>
</tr>
<tr>
<td>21</td>
<td>0.162 18.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.151 18.66</td>
<td>0.158 21.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.159 18.77</td>
</tr>
<tr>
<td>42</td>
<td>0.169 19.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.165 19.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.212 29.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.167 19.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> a precipitation was seen in the sample, which could be easily dispersed when shaken
<sup>b</sup> the macroscopic appearance of the sample was totally changed as can be seen in Picture 5.6
The macroscopic appearance of the preserved sample, stored at 37°C, looks totally different after 42 days of storage. This can be seen in Picture 5.6. This macroscopic change is also reflected by its high PDI (0.212) in the DLS measurement in Figure 5.6 Evolution of particle size over time. Data obtained from Table 5.5.

Table 5.5. The volume weighted size distribution of this measurement shows a bimodal distribution, which can be seen in the annex (annex 6)
5.3.2 Influence of dilution on particle size

Table 5.6 Particle sizes (z-average, diameter) and PDI’s of several dilutions of a same temperature cycled sample. Sizes were obtained with DLS at 25°C. The corresponding viscosity of each dilution was used from 5.2.2.

<table>
<thead>
<tr>
<th>m/m% OTCS</th>
<th>TC Sample (mL)</th>
<th>H₂O added (mL)</th>
<th>PDI</th>
<th>Particle size (nm)</th>
<th>Count rate (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2,030</td>
</tr>
<tr>
<td>B</td>
<td>75</td>
<td>1.5</td>
<td>0.5</td>
<td>0,524</td>
<td>/</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>1</td>
<td>1</td>
<td>0,258</td>
<td>/</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>0.5</td>
<td>1.5</td>
<td>0,141</td>
<td>19,77</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>0.2</td>
<td>1.8</td>
<td>0,079</td>
<td>27,14</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>0.1</td>
<td>1.9</td>
<td>0,050</td>
<td>29,94</td>
</tr>
<tr>
<td>G</td>
<td>2.5</td>
<td>0.05</td>
<td>1.95</td>
<td>0,045</td>
<td>31,33</td>
</tr>
<tr>
<td>H</td>
<td>0.5</td>
<td>0.01</td>
<td>1.99</td>
<td>0,041</td>
<td>32,59</td>
</tr>
<tr>
<td>I</td>
<td>0.25</td>
<td>0.005</td>
<td>1.995</td>
<td>0,049</td>
<td>33,05</td>
</tr>
<tr>
<td>J</td>
<td>0.1</td>
<td>0.002</td>
<td>1.998</td>
<td>0,053</td>
<td>33,02</td>
</tr>
</tbody>
</table>

5.4 INFLUENCE TEMPERATURE

5.4.1 Influence cooling shock on particle size

Table 5.7 Influence of the cooling rate after the temperature cycling on the particle size. Different amount of ice-cold water were added to the TC sample. They were all diluted to a concentration of 12.5 m/m% OTCS afterwards, before DLS measurement. Sizes (z-average, diameter) were obtained with DLS at 25°C and a viscosity of 1,247 mPa.s (obtained from Table 5.1). This experiment was done 2 times.

<table>
<thead>
<tr>
<th>1st attempt</th>
<th>2nd attempt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature cycled sample (mL)</td>
<td>Ice-cold water added (mL)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

a These sizes correspond to the sample which didn’t receive a cooling shock (0mL ice-cold water added)
5.4.2 Influence of heating on particle size

The same preserved sample (see 4.6), which was made for the stability experiment, is also used to observe the influence of a higher temperature on particle size. The sample, which was stored at 37°C for 21 days, is now measured at 37°C instead of 25°C with DLS. The obtained average size is now 16,84 nm (z-average, diameter) and PDI: 0,174. The obtained size at 25°C is 21,58 nm and PDI: 0,158, as can be seen in in Table 5.5. After the measurement at 37°C with DLS, a sort of phase separation can be seen in the cuvette. The sample is mixed again in the cuvette and measured again. This phase separation reappears again after each DLS measurement. This is attempted 3 times. The individual runs of the DLS measurement are shown in Table 5.8.

Table 5.8 Particle size obtained with DLS at 37°C (with a viscosity of 1,913 mPa.s) of a 26,8 m/m% OTCS, 21 days after temperature cycling.

<table>
<thead>
<tr>
<th></th>
<th>1st attempt</th>
<th></th>
<th>2nd attempt</th>
<th></th>
<th>3th attempt</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>PDI</td>
<td>Size (nm)</td>
<td>PDI</td>
<td>Size (nm)</td>
<td>PDI</td>
<td></td>
</tr>
<tr>
<td>1st run</td>
<td>17,16</td>
<td>0,174</td>
<td>17,38</td>
<td>0,175</td>
<td>17,00</td>
<td>0,144</td>
</tr>
<tr>
<td>2nd run</td>
<td>16,90</td>
<td>0,173</td>
<td>16,96</td>
<td>0,189</td>
<td>16,79</td>
<td>0,153</td>
</tr>
<tr>
<td>3rd run</td>
<td>16,76</td>
<td>0,180</td>
<td>16,98</td>
<td>0,177</td>
<td>16,61</td>
<td>0,164</td>
</tr>
<tr>
<td>4th run</td>
<td>16,75</td>
<td>0,159</td>
<td>16,82</td>
<td>0,187</td>
<td>16,72</td>
<td>0,164</td>
</tr>
<tr>
<td>5th run</td>
<td>16,62</td>
<td>0,182</td>
<td>16,83</td>
<td>0,175</td>
<td>16,56</td>
<td>0,153</td>
</tr>
</tbody>
</table>

After this observation, a temperature cycled sample is made again as in 4.1. Different amounts of ice-cold double distilled water are added after the temperature cycling, therefore different dilutions are obtained. The several dilutions are held in a 37°C water bath for 20 minutes.

Table 5.9 m/m% OTCS of every dilution, after adding ice-cold double distilled water to the temperature cycled sample

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/m% OTCS</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>10</td>
<td>5</td>
<td>2,5</td>
<td>0,5</td>
</tr>
</tbody>
</table>
The same kind of phase separation can be seen in the different dilutions after 20 minutes, as in the cuvette after DLS at 37°C. This experiment is not reproducible however and the separation doesn’t occur each time in every dilution.

5.5 INTERACTION OF THE EXCIPIENTS

5.5.1 Formation liposomes

Lecithin liposomes are formed in the process, described in 4.7.1.2. This is shown with light microscopy and static light scattering in respectively Figure 5.7 and Figure 5.8

**Figure 5.7** Volume weighted size distribution of lecithin liposomes. The data was obtained with static light scattering.

**Figure 5.8** Lecithin liposomes, viewed under a light microscope. The black bar represents 50 µm.
5.5.2 Full spectrum $^1$H NMR

Figure 5.9 $^1$H NMR spectrum of the temperature cycled mixture (containing: solutol, lecithin, MCT, NaCl and H$_2$O) and solutol + lecithin reference spectra
5.5.3 $^1$H NMR relaxometry

Transverse relaxation times $T_2$ (ms) of individual compounds and the mixture, which underwent temperature cycling, can be seen on the left. A comparison between the temperature cycled mixture and the theoretical sum of the individual components can be seen on the right. (A separate, more zoomed in, graph for lecithin can be found in ANNEX).
6 DISCUSSION
6.1 COMPARISON WITH THE ARTICLES BY HEURTAULT ET AL.

A translucent nanodispersion in the lower nano-range was obtained, as in the articles by Heurtault et al. They maintained a constant heating and cooling rate of 4 °C/min during the temperature cycles. This was not manageable however in this research. This rate didn’t appear to have a big influence however, as the sizes of the dispersions appeared to be reproducible in this research.

The importance of a fast cooling rate for nanoemulsion creation is emphasised in literature (7). Heurtault et al, always did this cooling shock as well. The results in this thesis however only showed a very small difference in particle size when this cooling shock was not done. The particle size only rose ±1 nm and ±0,020 PDI units when it cooled down normally (at room temperature), without receiving a cooling shock. A similar result was seen when this experiment was repeated. This suggests that the cooling rate, after the temperature cycling, only has a minor influence on the resulting particle size in this formulation. It could also be possible that the cooling rate has an influence, but that the batch already cooled down quick enough at room temperature to form the nanodispersion. This may be possible because a small badge of only 20g was used. When bigger batches would be made for industrial purposes, they would cool down a lot slower at room temperature. Bigger differences in particle size could possibly be seen then. These results should be interpreted cautiously, because only 5 samples were made each attempt and the experiment was only repeated once. This experiment should be repeated with bigger and a lot more samples, so better conclusions can be made. The temperature should also be measure before and after the ice-cold water was added, so the precise cooling rate can be known.

Heurtault et al. obtained: 35,2 nm; 34,0 nm and 32,7 nm particles for the same composition of excipients, which was used throughout this thesis (described in Table 2.1). This corresponds to the sizes of the most diluted samples in the dilution experiment, described in table 5.6. Heurtault et al. didn’t describe any parameters for the dynamic light scattering. The used parameters (assumptions and measurements) in this thesis can differ from the parameters that they used and therefore the obtained sizes with DLS.
6.2 PARAMETER DISCUSSION

6.2.1 Assumed parameters

When using DLS and SLS, it should be kept in mind that the acquired distributions and values are the result of a calculation. Parameters, specific for each kind of sample, contribute in this calculation. Some of the parameters are assumed. This implies that the acquired sizes aren’t the real sizes. These parameters include: material refractive index and absorption, dispersant refractive index and viscosity. (Assumed particle refractive index: 1,45; absorption: 0,01; dispersant refractive index: 1,330; viscosity: described in 4.2.2.2)

6.2.2 Refractive index

![Particle Size Distribution](image)

**Figure 6.1 Influence of refractive index on the volume weighted size distribution of the TC mixture without MCT.**

The used particle refractive index was 1,450. This was not measured exactly, but assumed. Refractive indexes for solutol HS15, phospholipids and MCT were found to be respectively: 1,468 (11); 1,450 (http://www.materials-talks.com/blog/2014/08/05/faq-how-important-are-refractive-index-absorption-for-nanoparticles/(19)) and 1,440-1,452 (16) in literature. When higher assumed RI’s were used to plot the same data, bigger volume shares were shifted from the lower to the higher sizes of the volume distribution of the sample. This can be seen in the TC mixture without MCT in Figure 6.1 (the composition is described in Table 4.2). The changes in RI only have a minor influence on the volume weighted distributed of the complete TC mixture (this can be seen in ANNEX 1).
6.2.3 Viscosity

The measured experimental viscosity of water was 0.915 mPa.s at 25°C in the viscosity experiment (Table 5.1). In the literature (20) a viscosity of 0.891 mPa.s at 25 °C is described for water. This difference can be caused by an imperfection in the ubbelohde viscometer. This must be kept in mind when the experimental viscosity values are used for calculating the particle size and therefore the sizes obtained with DLS. The viscosity has a big influence on the particle size, obtained with DLS. This can be illustrated as following: the used viscosity for the calculation of the particle size was changed with the DLS software of a same sample (27.8 m/m% OTCS). The resulting change in particle size was then observed. A sample viscosity of 0.890 mPa.s (corresponding with viscosity of water in literature) gave a z-average of 33.97 nm. 0.915 mPa.s (experimental viscosity of water, obtained from Table 5.1) gave 33nm. 1.185 mPa.s (Experimental viscosity of the 11.1 m/m% OTCS, obtained from Table 5.1) gave 25.49 nm. The simplification of the density, which was assumed to be 1 g/mL in the calculation of the viscosities, must also be kept in mind.

6.3 TEMPERATURE CYCLING PROCESS

During the temperature cycles, a macroscopic change can be seen in the sample in function of the temperature. This is caused by a phase inversion. Lecithin and the dipole-dipole interactions between the water and solutol’s PEG chains, stabilize the O/W emulsion under the PIT. The non-covalent interactions start to break at higher temperatures. When the temperature reaches the PIT, enough dipole-dipole interactions are broken and the system is destabilized. A W/O emulsion is formed then.
6.4 CONDUCTIVITY

6.4.1 Temperature cycling of the mixture

The conductivity measurements prove this phase inversion. The conductivity remains high at low temperatures, as water is the outer phase (O/W emulsion). The phase inversion occurs at 62°C and oil becomes the outer phase (W/O emulsion), as can be seen in Picture 5.1. The conductivity decreases quickly, as oil has a lower conductivity than water. A peak in conductivity can be seen in the phase inversion zone at 73°C. This appeared in the other heating cycles as well. A similar peak was seen too in the literature (17). An explanation was proposed by stating that the peak corresponded to a liquid crystalline lamellar phase. This allowed some surface conductivity.

6.4.2 Temperature cycling of the mixture without MCT

The conductivity was also measured during the temperature cycling of a sample without MCT. A stable high conductivity was expected because an oily phase was absent to perform phase inversion with and therefore a jump in conductivity. In contrary to expectation, the conductivity decreased gradually in function of a rising temperature and increased again when cooled down. This can be seen in Figure 5.3. The decreasing began at approximately the same temperature (±62°C) as the sample with MCT. A possible theory for this phenomenon is that solutol becomes more lipophilic at higher temperatures and the solubility in water decreases. Less soluble solutol attaches to the probe of the conductivity meter and the conductivity decreases.

6.4.3 Temperature correction

Temperature has a big influence on conductivity measurements (about 2% variation per °C). That is why the conductivity in function of temperature was corrected. A slight tendency of a rising conductivity can be seen in Figure 5.1. The calculation of the temperature correction coefficient is only based on 2 measurements. This can give errors especially when conductivities are extrapolated at higher temperatures. The used sample temperatures for the correction coefficient (40,9°C and 43,5°C) were too low. It
would have been better to measure the typical sample temperatures (Equation 4.3) at 80°C. The measured conductivities for the reference temperature (normally 25°C) were also too high (28.4°C for the mixture and 31.4°C for the mixture without MCT). This tendency however doesn’t affect the interpretation of the conductivity experiments, because a quick de- and increase of conductivity can be seen.

6.5 INTERPRETATION FIGURE 5.5, STATIC LIGHT SCATTERING

Figure 5.5 shows multiple broad peaks over a broad size range. These can be possibly caused by solutol micelles and mixed micelles, containing solutol and lecithin, in the lower size range. Lecithin liposomes can cause the peaks in the higher size range. When MCT was added to the mixture a single narrow peak can be seen. This suggests the association of the components. It should be kept in mind when interpreting these size distributions that a general purpose algorithm was used to process the data. Multiple individual peaks over a small size range can be hidden by a big broad peak because of the used algorithm. This can give misleading peaks and therefore wrong conclusions. These measurements were done to get a first impression of the size range of the formed dispersion. Further size measurements were done with dynamic light scattering.
6.6 STABILITY

6.6.1 Storage

The sample, which was diluted to 11,1 m/m% OTCS for stability measurements, was not initially made for stability measurements. That’s why no preservative was added. For the sample, which was, diluted to 27,8 m/m% OTCS, this do was the case and preservative was added. Literature (21) suggests preserving at 0,02 % m/m NaN₃ (Merck-Schuchardt, Hohenbrunn, Germany). The temperature cycled original sample contained this concentration, however after the cycling there was a cooling step so it was further diluted. So it was actually stored at 0,0088 m/m % NaN₃ instead of the recommended 0,02 m/m %. A sedimentation could be seen in both samples (Picture 5.5). This sedimentation could be easily dispersed again when shaken. The comparison between the two samples under a light microscope doesn’t show a difference in the appearance of the sedimentation. The 2 samples were made in the exact same way, only the dilution immediately before DLS measurement was different. The size and PDI in the sample without preservative remained the same, even when a sedimentation was seen.

The same sedimentation was seen in the preserved sample. In contrary with the other sample, the sizes became bigger over time. The sedimentation occurred in all the samples, stored under different conditions. The sedimentation occurred faster when it was stored at 37°C and the sizes rose also faster. Light didn’t appear to have an influence on stability, because the particle sizes and PDI remained the same for the samples stored in the light and dark at 25 °C. After 42 days the sample looked completely different, this was also reflected in the DLS measurement with a bimodal distribution (ANNEX 6).

It is interesting that the sedimentation is not reflected in the DLS data. A possible reason is that the sedimentation is formed by some kind of aggregation between the particles. The aggregation breaks then down again in particles, if the sample is shaken. This theory is however not completely closing, because the sedimentation appears again after a couple of days.
6.6.2 Higher temperature

When the sample was measured at 37°C, the calculated particle sizes became slightly smaller throughout the measurement runs. After the measurement a slight phase separation could be seen in the cuvette. When this was tested by different dilutions in a warm water bath this gave this phase separation occurred as well, however it was not reproducible each attempt. A possible reason for this is that solutol becomes less hydrophilic at higher temperatures, phase separation occurs.

6.6.3 Dilution

The sizes gradually increase at higher dilutions at first sight and then remain constant at ±33nm. The PDI should be noticed however, as the PDI is an overall quality parameter for the obtained sizes. The PDI is too high (>0,1) in the samples A-D in Table 5.6 and no over-conclusion should be made because of these high PDI’s. These high PDI’s are caused by the main limitation of DLS: a too high concentration in the cuvette. This can lead to multiple scattering and the particles also have a decreased path length, which intervenes with the Brownian motion. These phenomena’s can lead to obtaining wrong particle sizes. The obtained sizes of these dilutions (A-D) are therefore not trustworthy. The size becomes only slightly bigger first and then remains more or less the same in the samples E-J in Table 5.6. DLS measures the hydrodynamic radius of a particle. A possible reason may be that the solutol can attract more water when more water is added, therefore the hydrodynamic diameter rises slightly. It rises until a maximum amount of water is non-covalently bound by the solutol, the hydrodynamic size remains the same then.

The contrary was expected. Solutol HS15 is a mixture of free polyethylene glycol (=hydrophilic) and PEG mono- and di-esters of 12-hydroxystearic acid (= lipophilic part). The free polyethylene glycol, the mono-ester and the di-ester of 12-hydroxystearic acid were suspected to be respectively: totally, partly and partly in the aqueous phase because of the hydrophilic polyethylene glycol groups. As the sample gets more diluted, it could be possible that more solutol (especially the mono-ester) leaches out of the particle and the particle size decreases. Fortunately, this is not the case, as this can give substantial problems when this would be used for therapeutic applications.
The peaks at 0 ppm are caused by the 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt. The heavy water contained this and it was used as a standard to determine chemical shifts of the compounds in the ¹H NMR spectrum. (0ppm= δTMS)

A triplet around 0.9 ppm and a big peak around 1.4 ppm, together with smaller peaks, can be seen in the TC mixture and solutol reference spectrum. These peaks are caused by the methyl end groups and the methylene groups respectively of the fatty acid chains. The peaks are less defined however than the theoretically obtained spectra with MestReNova software. This may indicate an interaction of the fatty acid chains. In the solutol sample, this can be explained by the interaction of the fatty acid chains in the micelles, resulting in a decreased mobility. In the TC mixture, this can be explained by a decreased mobility because of the interaction between the fatty acid chains of the surfactants and the MCT. These peaks can’t be seen in the lecithin reference spectrum. The fatty acid chains in lecithin are immobile because of the rigid conformation of the chains in the liposomes and therefore can’t be seen in the ¹H NMR spectrum.

Peaks around 2.3 ppm can be seen in the solutol and the mixture spectra. These peaks are caused by the methylene groups immediately next to the carbonyl groups in the fatty acid chains. In the lecithin sample however, a big peak can be seen in this area. This is not caused by the respective methylene group alone. It may be caused by the trimethylamine head group of phosphatidylcholine. When a prediction of the phosphatidylcholine is made with the MestReNova software, a similar trimethylamine peak can be seen at 3.3 ppm. This implies that, if the peak at 2.3 ppm in the lecithin reference sample is indeed derived from the trimethylamine group, it is shifted 1.0 ppm upfield.

The big peak at 3.7 ppm and the peaks around it in the mixture can also be seen in the reference solutol spectrum. It is caused by the PEG part of solutol. The peaks are not symmetrical, which indicates that not all PEG chains have the same mobility. Some PEG parts, which are closer to the oily core, are probably more restricted in mobility. Other PEG parts, which are further away from oily core, are more mobile. Around 30% of Solutol HS15 consists out free PEG. This makes the interpretation of the spectra
harder, as the free PEG is very mobile and can dominate the spectrum over less mobile PEG parts.

At around 4.8 ppm, big peaks can be seen in all samples. This is caused by normal water in the samples. Although D$_2$O ($^2$H$_2$O) was used as a solvent, a small fraction of $^1$H$_2$O was present in the samples. This was the result of an exchange of water molecules between the air and the deuterium solvent.

6.8 $^1$H NMR RELAXOMETRY

The graphs, obtained with $^1$H NMR relaxometry show a shorter transverse relaxation time $T_2$ for the mixture, which underwent temperature cycling, than the theoretical summation of the individual components of the mixture. This shows that there is indeed an interaction between the different components. Most likely, the interaction is caused by the hydrophobic fatty acid chains of solutol and lecithin with the fatty acid chains of MCT. The PEG chains of solutol on the other hand are stabilised by the interaction with water. The exact configuration of the components can’t be deduced however from this experiment. Small peaks with short transverse relaxation times can be seen for every sample. In the literature (22), it was shown that these small peaks were related to the relaxation of water protons at the glass surface.
6.9 TRANSMISSION ELECTRON MICROSCOPY

Well-defined structures can be seen in the picture obtained from the 50m/m% original sample. The size is about 100 nm, which is not in line with the DLS measurements (±25nm). Bright polydisperse structures can be seen in the 0.5 m/m% sample, varying from 20 to 100 nm in size. These are probably artefacts, which were formed during the negative staining. The surfactants and the hydration shell normally stabilize the particles. This hydration shell could have been affected during the drying process. The stability of the particle and the specific configuration could therefore have been lost. NaCl also could have been crystalized and interfere with the interpretation of the images. Negative staining has some substantial benefits: easy, fast, very high contrast and requires no advanced equipment unlike cryofixation for example. However, (as here was likely the case) a major downside is the possibility of the aggregation of substances). A possible alternative for the sample preparation is cryofixation, which is technically more demanding however. (http://web.path.ox.ac.uk/~bioimaging/bitm/instructions_and_information/em/neg_stain.pdf (23))

6.10 SOLID OR LIQUID?

Further research should investigate if the nanodispersion is solid or liquid. The $^1$H NMR experiments indicate an interaction between the substances (and the hydrophobic chains in particular). Heurtault et al. described this system as a nanoparticle. However, these current experiments are not sufficient to conclude that it is totally/partly rigid or not. Therefore, more experiments should be done. Possible methods are: differential scanning calorimetry or atomic force microscopy.
7 Conclusion

The phase inversion temperature technique, proposed by Heurtault et al, was successfully established in the pharmaceutical technology group of professor Dr. Mäder. The technique and the used excipients (oil, water, surfactants), gave rise to a reproducible translucent dispersion in the lower nano-range with a very low PDI. The nanodispersion may be loaded with hydrophobic drugs in a next stadium.

The nanodispersion is stable in daylight at room temperature and in the fridge. It is also stable against dilution. A sedimentation is formed however after a while. When it is stored at higher temperatures, this sedimentation can be seen more quickly. In general, high temperatures must be avoided. This is the biggest downside of the system.

It appears that the cooling shock, proposed by Heurtault et al, only had a questionable influence. This should be repeated however with more and bigger samples to give a better conclusion.

\(^1\)H NMR experiments (Full spectrum+relaxometry) indicate that the excipients interact with each other. It appears that the hydrophobic chains are less mobile and that the PEG chains are mobile. It can be further examined how rigid the shell and core are of the nanodispersion. This can be done with atomic force microscopy for example.

Electron microscopy is a possible way to visualize the formed nanodispersion. The method of sample preparation is important in this case and it must be kept in mind that the dispersion has a hydrated shell. This implies that the system should not be dried. When negative staining was used, aggregates were formed which prevented interpretation of the obtained images. A better sample preparation would have been cryofixation, this is however technically more demanding.
8 Used literature

12. Medicines EDftQo, Commission EP. European pharmacopoeia: Council of Europe; 2009.

Websites:

23. http://web.path.ox.ac.uk/~bioimaging/bitm/instructions_and_information/em/neg_sustain.pdf [20/05/2015].
ANNEX 1 Influence of refractive index on volume weighted size distribution of TC mixture (The composition is described in Table 4.1.) Data was obtained with Mastersizer 2000 software.

ANNEX 2 Enlarged transverse relaxation time of the lecithin, obtained with NMR relaxometry.
### ANNEX 3 Table with Hagenbach correction times.

ANNEX 4 enlarged Picture 5.5 11,1m/m% OTCS

ANNEX 5 Enlarged Picture 5.5 (right) 27,8 m/m% OTCS
ANNEX 6 Average volume weighted size distribution of the sample described in Table 5.6, 42 days of the temperature cycling. A bimodal distribution can be seen. Graph was obtained with DLS at 25°C.