



# Studying the interactions between therapeutic peptides and absorption enhancers

*Master Thesis in Pharmaceutical Technology, 30hp*

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## Abstract

Therapeutic peptides can often not be formulated as tablets by themselves as they're prone to enzyme degradation the oral route - they also do not present good enough passage through epithelial cells to be viable for this dosage form. But if formulated together with surfactants, e.g., sodium caprate (C10), the absorption through epithelial cells can be increased. When peptides and absorption enhancers are mixed, their interactions can be important to be aware of. This is because unwanted aggregation of peptide could render the therapeutic useless or even dangerous to the patient. Knowing how the peptide affect the aggregation of the absorption enhancer can also be of importance, since different types of aggregates can increase absorption differently, and if the peptides can favour the aggregation of a certain type of structure, this can be useful to be aware of.

This work has demonstrated at which different concentrations and environments C10 and salcaprozate (SNAC) forms different types of aggregates. This work has also indicated that a certain peptide, MEDI7219, might reduce the concentration at which micelles are formed by the formation of mixed micelles. Conformational changes for peptides when interacting with C10 was observed to be correlated with the peptide's degree of lipidation.

Further, this thesis has provided information that a ternary phase diagram for C10, capric acid and water largely contains capric acid crystals at room temperature and indicated that some changes in structure occur when heated to 37°C. The equilibrium time for this system was also observed to be long, which could be useful knowledge in drug formulation.

All results together indicate that there are hydrophobic interactions between MEDI7219 and C10, but more research is needed to determine at what extent their interplay affect both formulation stability and the therapeutic use of the peptides.

## List of abbreviations

$\delta$  – chemical shift

C10 – sodium caprate / sodium decanoate

CD – circular dichroism

CMC – critical micelle concentration

CPP – critical packing parameter

D<sub>2</sub>O – deuterium oxide

DPH – 1,6-diphenylhexarine

MQ-water – Milli-Q water

NMR – nuclear magnetic resonance

SNAC – salcaprozate sodium

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## Introduction

Small molecule drugs have since a long time been the primary choice for treatment of disease. However, the advancement of pharmaceutical development, along with the certain substantial benefits of biologics and synthetic peptides, have drastically increased the relevance of them as preferred treatment of choice. Compared to small molecule drugs, biologics often present better specificity (and thus less side effects) and less probability for adverse immune responses. For pharmaceutical companies, advantageous patent- and approval processes compared to small molecule drugs can further increase their appeal from a financial standpoint (Leader et al., 2008).

Compared to small molecule drugs, the typically complex nature of many biologics and synthetic peptides also result in a greater challenge regarding e.g., characterization, stability, production, and drug delivery (3). Their large size, molecular weight, and susceptibility to degradation limit the available routes of administration, thus requiring them to be administered parentally. For one-time use of a peptide-drug this is generally not problematic, but for patients with chronic diseases which require repeated administration, patient compliance can be negatively affected due to the unpleasant nature of injections. Orally available peptide drugs would in many cases be preferred but peptides in themselves are prone to degraded by enzymes, and the large size of them hinder their passage through epithelial cells (Drucker, 2019). Effort has been made to overcome this both by molecular engineering, to reduce available cleavage sites for enzymes (Pechenov et al., 2021), enteric coatings (Tang et al., 2023) and to use absorption enhancers to increase the passage of peptide through the gastrointestinal epithelium (Pechenov et al., 2021).

When formulating peptide-therapeutics it's important to be aware of the aggregation behaviour of the peptide in different environments (pH, presence of ions, temperature etc), because unwanted aggregation can negatively affect patient safety, whether it be loss in activity, immunogenicity, or increased/induced toxicity (Zapadka et al., 2017). Knowing how the absorption enhancer self-associates is also important because its absorption can be different depending on the structure (Berg et al., 2021). Knowing how the peptides interact with the absorption enhancers is therefore of importance, and this thesis will attempt to provide more knowledge about these interactions. This will be done by studying the interactions between three peptides with different lipidation patterns together with the commonly used absorption enhancer sodium caprate (referred to as C10 for the rest of the report). Worth noting is that peptide aggregation is a complex process with many factors influencing it, and full understanding remains a challenge in the development of peptide-therapeutics (Zapadka et al., 2017).

The main methods used in this paper to investigate the interactions between peptides and absorption enhancers is circular dichroism CD and NMR-spectrometry. NMR will be used to study the aggregation behaviour of C10 and another common absorption enhancer salcaprozate sodium (SNAC), and to study if this differs in the presence of peptides. CD will be used to study possible changes in secondary structure of the peptides at different

concentrations of C10. Hence, for interactions between peptide and C10, NMR will be used to study the effect the peptides have on the aggregation of C10, and CD will be used to study the effect C10 has on the secondary structure of the peptides.

## Aim

The aim is to learn more about the interactions between peptides and absorption enhancers, primarily with respect to self- and co-assembly. This will be done by:

- Creating a partial ternary phase diagram for C10, capric acid and water. The intention is to investigate the extension of the lamellar phase because this indicates around which composition one can expect vesicles (a type of spherical structure consisting of bilayers of surfactant). This is of interest because C10 micelles and vesicles have demonstrated to affect absorption of macromolecules through epithelial cells differently.
- Study the critical micelle concentration (CMC) for C10 and SNAC and to gain insight in which type of self-assembled structures that exist for different chemical environments.
- Study the structure and behaviour of the peptides in buffer and different pH.
- Study systems of C10 and peptides to gain information about the interactions between them – both how the peptides can affect self-association of C10 and how C10 can influence the structure of the peptides.

# Theoretical and experimental background

## Peptides

Peptides are molecules that consist of a series of amino acids, usually in the range of 500-5000 Da, and are connected by amide bonds (Wang et al., 2022). Depending on the amino acid sequence and its size, the peptides can fold into different structures that can be connected by intramolecular interactions such as hydrogen bonds (Koutsopoulos, 2018). Worth noting is that hydrogen bonds is not the sole determinant of peptide secondary structure - other interactions such as charge can also affect the structure (Xing et al., 2018).

The key examples of secondary structures are the  $\alpha$ -helix (coil-structure connected by hydrogen bonds) and the  $\beta$ -sheet ("rows" of peptide chains, also connected by hydrogen bonds) (Koutsopoulos, 2018). See Figure 1 for illustrations.

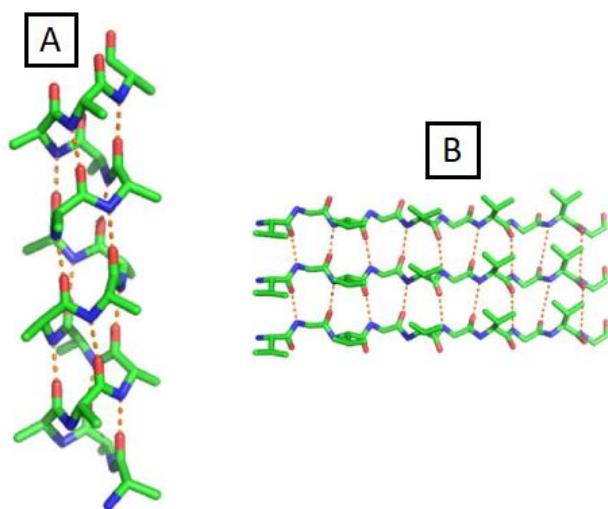


Figure 1: The structure of an  $\alpha$ -helix (A) and a  $\beta$ -sheet (B) (Koutsopoulos, 2018). The orange dotted lines shows intramolecular hydrogen bonds.

## Peptide aggregation

Peptides can form different aggregate structures – key examples include amyloid fibrils and amorphous aggregates. Amyloid fibrils consist of several interacting  $\beta$ -sheets which are stabilized by hydrogen bonds from the peptide backbone (Zapadka et al., 2017), and these structures have been connected to e.g., neurodegenerative diseases (Balasco et al., 2021). Amorphous aggregates are unstructured and can form precipitates under certain conditions which can complicate the development of peptide therapeutics (Zapadka et al., 2017),

Many factors affect both the chemical and physical stability of peptides, examples include concentration, pH, charge, salts, excipients, and temperature. One of the most important

factors for aggregation is often concentration, because concentration have been shown to increase the growth rate of different stages of fibrils-formation. The charge of the peptide also affect aggregation because it affects the electrostatic interactions between individual peptides, which in turn affect their propensity for aggregation. In e.g., buffers, the degree of ionic strength are also influenced by pH, and this have been shown to have a big role in aggregation. In fact, pH alone can influence many of these factors which affect aggregation, which is why pH control is of utmost importance in peptide formulations. Worth noting is that the complex nature of peptide aggregation makes it so that there is no general rule that applies to all peptides, and the physical stability differs between different types of peptides (Zapadka et al., 2017).

## MEDI7219, J211 & J229

Following are the three peptides that was studied in this thesis, MEDI7219, J211 and J229 (see figure 2) – where MEDI7219 was primary peptide that was studied. All these peptides were developed as GLP-1 receptor agonists, where reduction of enzyme cleavage sites and inclusion of lipid groups were made to improve stability and circulation time.

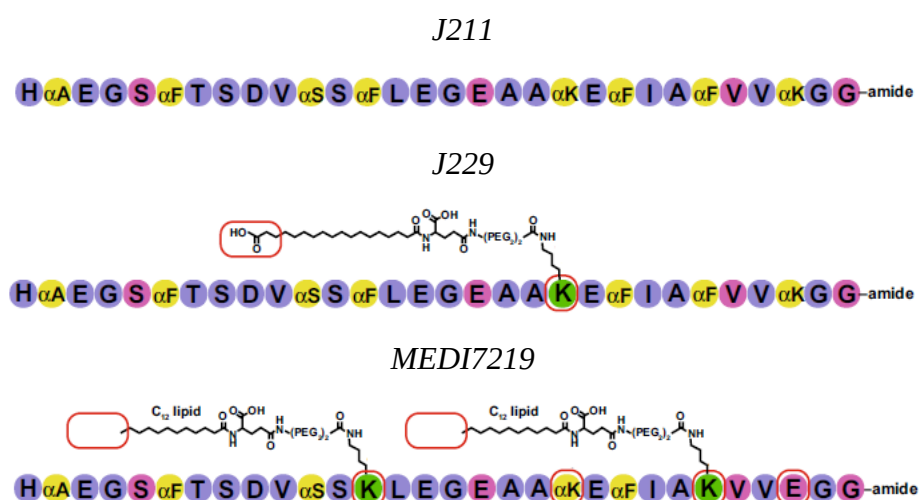


Figure 2: The structures of J211, J229 and MEDI7219. The letters are one-word codes for amino acids where e.g., H is histidine and S is serine (Pechenov et al., 2021).

J211 has no lipidation and eight alpha-methyl amino acids, J229 has mono-lipidation and seven alpha-methyl amino acids and MEDI7219 has bis-lipidation and five alpha-methyl amino acids. The structure of lipid chain also differs between J229 and MEDI7219, where J229 has both a longer hydrophobic chain and a carboxylic acid group at the end of the chain (Pechenov et al., 2021). These three peptides were selected because they are similar but have distinct differences between them, which make them suitable for determining differences in interactions with a surfactant. If one of the peptides behaves very differently compared to the

rest, it can be easier to determine what difference in structure that resulted in the different behaviours.

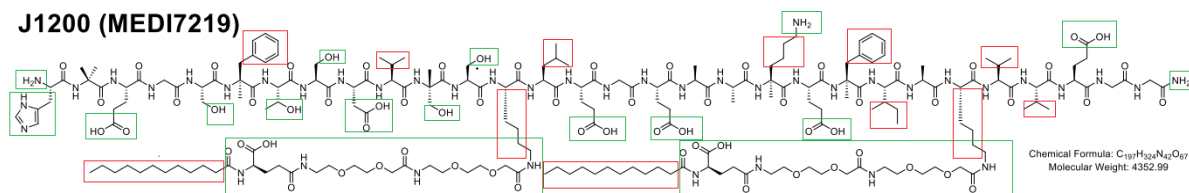


Figure 3: The molecular structure of MEDI7219, where moieties which can participate in different types of interactions (apart from the peptide backbone) are highlighted. Green is for fragments that can take part in hydrogen bonds and red are for hydrophobic moieties.

Since this work is focused on the interactions between peptides and surfactants, an analysis of the different potential parts that could be of interest when interacting with a surfactant was made. Figure 3 shows the molecular structure of MEDI729. The red areas indicate moieties where hydrophobic interactions / pi-pi interactions can take place, and green indicate moieties, apart from the peptide backbone, which can participate in hydrogen bonds. MEDI7219 in solution is expected to carry a negative net charge at pH above  $\sim 4$ .

## Surfactants

Surfactants are molecules that contain both a polar and a non-polar part (Holmberg, 2015) – an example is C10, shown in Figure 4. The polar part is commonly referred to as headgroup, while the non-polar part is referred to as tail. When surfactants are dissolved in water, the polar parts prefer to stay in water and the hydrophobic part prefers to limit their interactions with water. In the example of C10 in water, the polar part is the carboxylate group, and the non-polar part is the nine-carbon chain. The propensity of surfactants to hide their non-polar part from water results in a range of properties, such as them being surface-active and being able to form micelles and other self-assembled structures – the driving force for this is to reduce the free energy of the system. Many ionic surfactants carry a permanent charge. However, surfactants carrying an ionizable head group, such as C10, can depending on the pH become protonated to form capric acid (Figure 5). Important to note is that capric acid is not a surfactant in the same sense as C10 because its less polar head group, but it can still form mixed micelles with C10 where it acts as a cosurfactant (Holmberg et al., 2003:445).

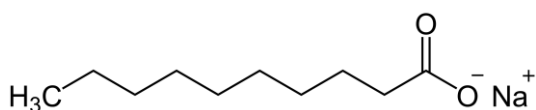


Figure 4: The structure of C10

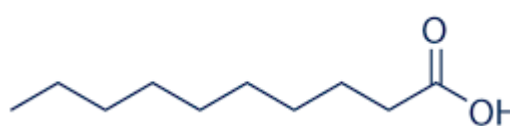


Figure 5: The structure of capric acid

For surfactants unimers there is an equilibrium between staying in solution and adsorbing to interfaces, but at a certain concentration the surfactant starts to form self-assembled structures to, once again, lower the free energy of the system. The CMC (critical micelle concentration) is the point from where on the unimer concentration remains practically unchanged and the surfactants begin to form micelles. Micelles are a type of structure which are a collection of unimers which self-assemble into e.g., spheres where the hydrophobic parts are pointed towards each other, and the hydrophilic parts are facing the aqueous environment, as illustrated in Figure 6. Surfactants can form other self-assembled structures apart from spherical micelles, depending on the environment (Holmberg, 2015). Two examples of relevance to this thesis are vesicles and wormlike micelles. Vesicles consist of bilayers of surfactants as illustrated in Figure 7, A (Holmberg, 2015), and wormlike micelles are long and cylindrical as seen in Figure 7, B (da Silva et al., 2020).

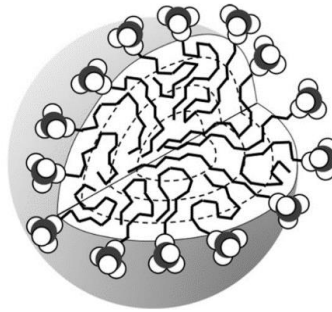


Figure 6: Illustration of a spherical micelle (Holmberg, 2015)

A way to describe the geometry of a surfactant aggregate is to use a dimensionless parameter, based on the curvature at the surface of the aggregate, called the critical packing parameter (CPP). It's defined as following (Equation 1),

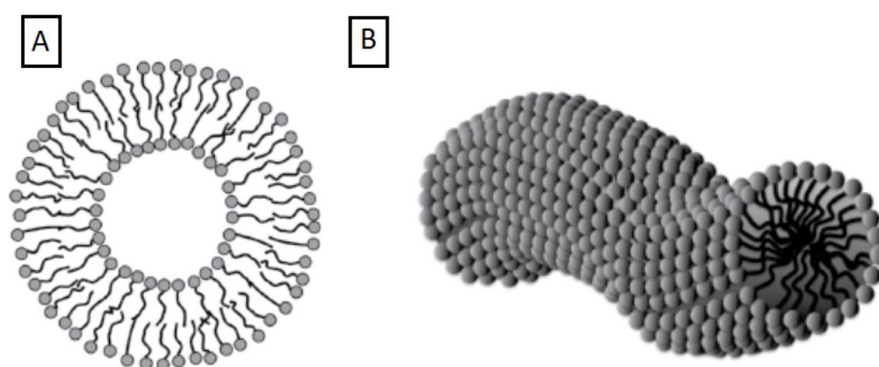
$$CPP = v/a_o l_c \quad (\text{Equation 1})$$

, where  $a_o$  is the area of the head group,  $l_c$  is the chain length (assumed to be fully extended) and  $v$  is the volume of the tail. Spherical micelles have a  $CPP < 1/3$  and vesicles have a CPP between  $1/2$  and  $1$  (Holmberg et al., 2003:92). One factor previously described to affect aggregate formation is the addition of salt, as this screens the electrostatic repulsion between headgroups – thus effectively reducing the area of the head groups. This will in turn increase the CPP. Another example of increasing the CPP is to add non-ionic amphiphiles (such as alcohols) to an anionic surfactant (Holmberg et al., 2003:445).

## Factors influencing surfactant self-assembly.

A range of factors affect the self-assembly of surfactants. For ionic surfactants, like C10, one aspect that typically has significant impact on the CMC and the self-assembly behaviour is addition of salts (Holmberg et al., 2015). This is because salt screens the electrostatic repulsion between the headgroups. Because of this screening a lower concentration of surfactant is needed to form micelles (Bajpai, 2018). Another factor that also affects the CMC is pH. For example, C10 unimers have a pKa of around 4.8 – this means that the pH of the solution dictates the ratio of ionized C10 to non-ionized capric acid (structure shown in Figure 5). Also, the pKa of self-assembled C10 can increase to around 7 (Berg, 2022), which further adds to the complex nature of micelle formation. Studies have shown that at a pH where the majority of C10 is in its ionic form, micelles will be formed (>pH 8.1), but when the pH is lowered (and thus the fraction of non-ionized species is increased), vesicles are formed instead (Berg, 2022). This can be explained by CPP. As pH is reduced, more capric acid is formed, which is an anionic fatty acid. This can act as a cosurfactant which will increase the CPP (Holmberg et al., 2003: 445), which explains the formation of vesicles instead of micelles. At a point where there is a too big fraction of non-ionized species, phase separation of capric acid will occur, which above its melting point presents itself as an oil-phase (Berg, 2022). Temperature also plays an important part in self-assembly of surfactants. The solubility of surfactants can go from very low to high in a relatively slight temperature range – the point at which this happens is known as the Krafft temperature (Holmberg et al., 2015). This can be important to know because if the surfactant is not soluble then it will not participate in self-assembly (Düzgünes, 2009).

Because of the many factors that can affect the CMC there often isn't exact data readily available for the system that one is studying, and the CMC instead need to be experimentally determined.



*Figure 7: A shows the structure of a vesicle and B illustrates the structure of wormlike micelles (Holmberg, 2015).*

## Surfactants as absorption enhancers

The inherent properties of surfactants make them useful in a range of applications. One which is of key relevance to this thesis is their use as absorption enhancers in drug formulation. When e.g., a peptide is formulated with an absorption enhancer, the absorption through the

gastrointestinal epithelium can be increased, both by trans- and paracellular pathways (between cells and through cells) (Berg, 2022). Knowing the self-assembled form of C10 is important in connection to this, since C10 micelles and vesicles have shown to enhance absorption of macromolecules through epithelial cells differently (Berg et al., 2021). As previously described, peptide drugs are often not suitable to be formulated as tablets in part because of their inherent low oral bioavailability, but by using absorption enhancers this may be increased. One example of successful use of permeation enhancers to increase availability of peptides is the oral version of semaglutide developed by Novo Nordisk (Rybelsus). Here semaglutide is formulated together with SNAC (salcaprozate sodium), where SNAC increases the transcellular transport of semaglutide – thus making it possible to formulate the drug as a tablet. Worth noting is that the bioavailability still is relatively low, approximately 1% for Rybelsus (Kim et al., 2022), which is significantly lower than subcutaneous injections of semaglutide which presents a bioavailability of 89% (Abramson et al., 2019).

Notable is the difference in chemical structure when comparing SNAC (Figure 8) to C10 (Figure 4). SNAC contains polar moieties at both ends of the hydrophobic chain, whereas C10 only does so on one side. This indicates that there will be differences in self-assembly behaviour compared to typical surfactants, and that SNAC might not form similar types of micelles as C10 since the left-most moiety of SNAC will not prefer to stay in the hydrophobic centre of a conventional micelle.

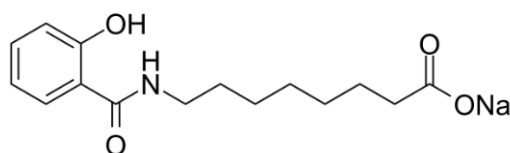


Figure 8: Shows the chemical structure of SNAC.

## Phase diagrams

A phase diagram for a surfactant is a convenient way to describe its phase behaviour. Below is an example of a ternary phase diagram for the system sodium octanoate, octanoic acid and water (Figure 10). Because the molecular structure of sodium decanoate (Figure 3) and sodium octanoate (Figure 9) only differ with two methylene groups, the phase diagram for the latter can be used as reference point when determining one for the former.

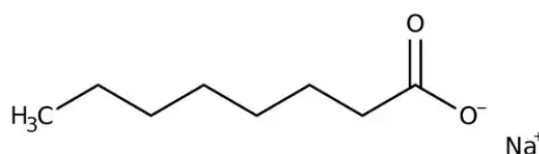


Figure 9: The structure of sodium octanoate

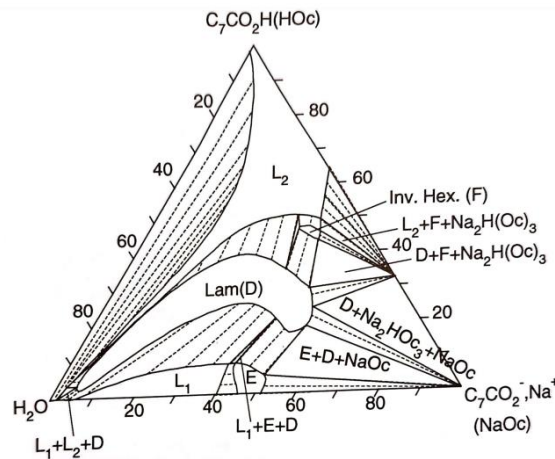


Figure 10: Ternary phase diagram for sodium octanoate, octanoic acid and water at 20°C (Laughlin, 1994).

In the phase diagram illustrated in Figure 10, one can observe several different single phase regions and, in between those, two phase regions. Triangles between two phase regions are three phase regions. Three big phases are  $L_1$  (aqueous region),  $L_2$  (octanoic acid region) and  $L_\alpha$ .  $L_\alpha$  is a lamellar liquid crystalline phase (Fontell & Mandell, 1993), which is of the greatest interest in this thesis. Comparing the structures of vesicles and a lamellar phase, one can identify that they're very similar. In fact, vesicles are a dispersed lamellar phase. The liquid crystals in this region have a one-dimensional lamellar structure (Fontell & Mandell, 1993) (Figure 11), and this can be identified by polarized light microscopy because of the presence of spherical air bubbles and Maltese crosses (see Figure 12, A). A lamellar phase can turn into a lamellar dispersion if there's an excess of water, and this will also result in a different appearance under microscope (see Figure 12, B) (Dutton et al., 2011).

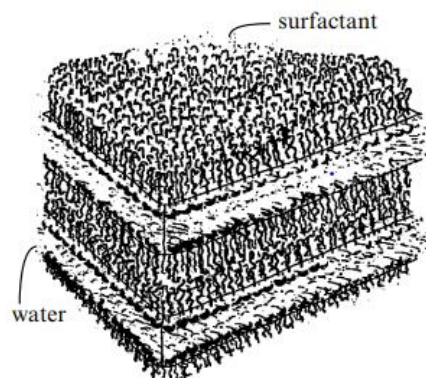


Figure 11: Illustration of a lamellar phase (Holmberg et al., 2003:79).

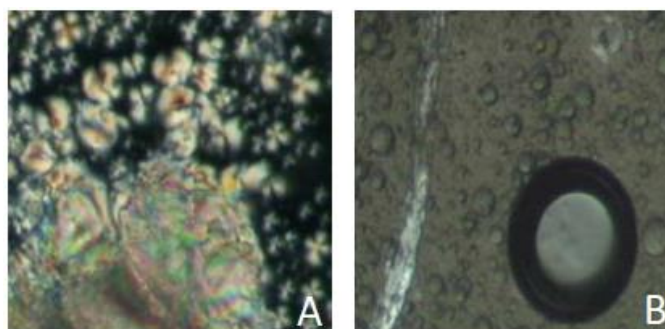


Figure 12: Example of an  $L_{\alpha}$ -phase. In Figure A the characteristic Maltese crosses can be seen, and Figure B shows air bubbles in the lamellar dispersion (Dutton et al., 2011)

No ternary phase diagram for C10, capric acid and water was found in the literature. To locate the expected lamellar phase, a range of solutions with different compositions was created for a part of the diagram where part of the lamellar phase was assumed to be according to the sodium octanoate reference diagram.

## Circular dichroism spectroscopy

Circular dichroism is a phenomenon which can be used to evaluate, e.g., the secondary structure of peptides and proteins, and it's based on the unequal absorption of left- and right polarized light. A basic explanation is that polarized light is directed through a sample of peptide, the light is absorbed by the sample, and then the photons are picked up by a detector on the other side of the sample. When the amide bonds in the peptide backbone are aligned in certain structures, such as  $\alpha$ -helix,  $\beta$ -sheet or unordered – the absorption of polarized light result in different spectra that is picked up by the CD-machine. Examples of typical spectra are illustrated in Figure 13. For example,  $\alpha$ -helices have minima at 208 nm and 222 nm, and a maxima at 195 nm, whereas  $\beta$ -sheets share maxima at the same wavelength but instead have a single minima at 218 nm (Greenfield, 2006).

When running CD, it's important to be aware of the system that one is analysing. For example, it's important to (if possible) minimize optically active components that can interfere with the spectra from the peptide. This means that, e.g., compounds with chloride ions such as NaCl should be avoided in the buffers used. Another aspect that is important to consider is the amount of scattering of the sample. Too high scattering and not enough photons will reach the detector and thus render that data non-reliable (Circular Dichroism Instructions, n.d.).

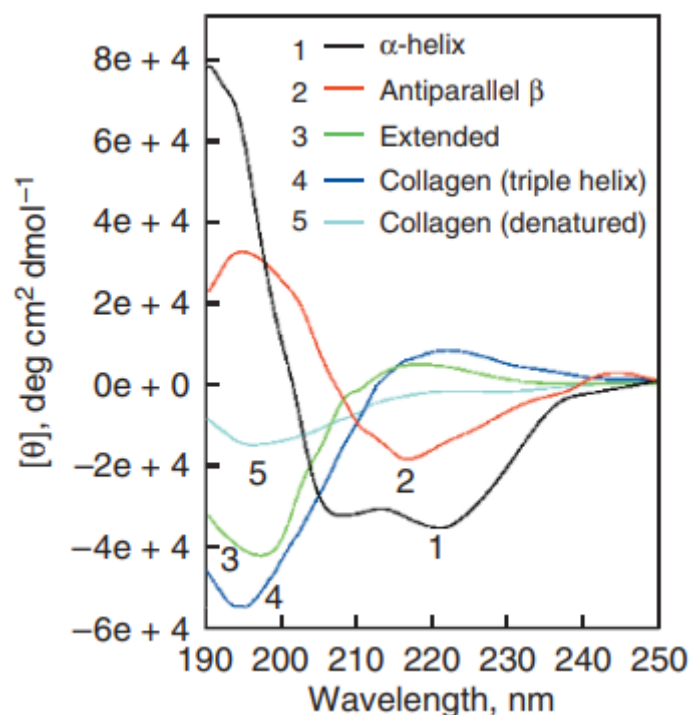


Figure 13: Shows example of how different secondary structures look in CD, where the black line is an  $\alpha$ -helix, and the red line is a  $\beta$ -sheet (Greenfield, 2006).

## NMR-spectroscopy ( $^1\text{H}$ -NMR, $^{13}\text{C}$ -NMR)

NMR-spectroscopy can provide valuable information about the structure of substances. The basic principle is that some atoms have a spin and thus are magnetically active, and when a magnetic field is applied these spins can be aligned either parallel or antiparallel to the external field. An NMR instrument can record the changes in a system after an external magnetic field is applied and gather information about the structure of the molecules analysed (Claridge, 2016).

Below (Figure 14) is an example of an  $^1\text{H}$  spectra for C10 in 20mM phosphate buffer at pH 7.5. The x-axis is the chemical shift (ppm), and the y-axis is intensity. The peaks will have different chemical shift depending on the electronic environment (e.g., proximity to electronegative atoms and conformation) of the different protons.

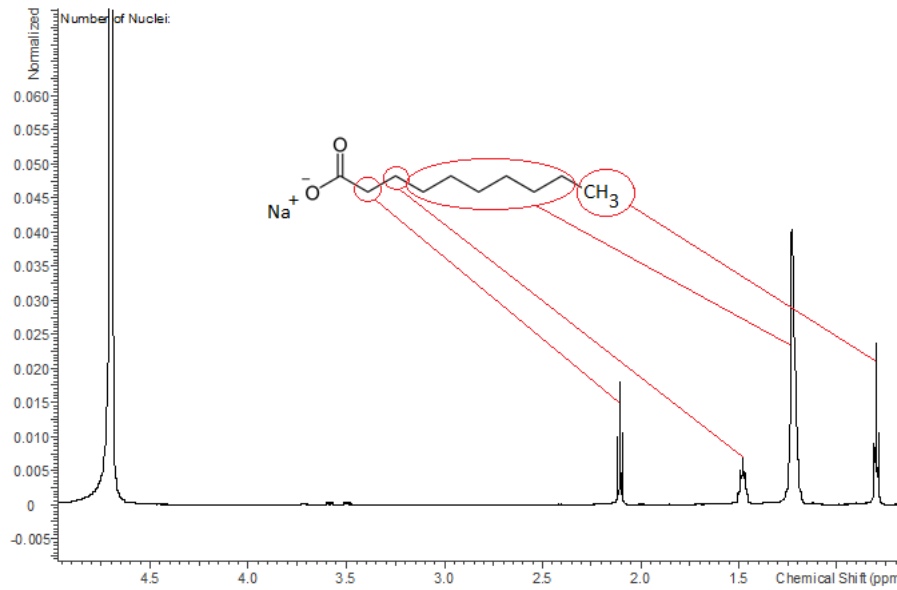


Figure 14: Shows an  $^1\text{H}$  spectra for C10 in 20mM phosphate buffer at pH 7,5. Which peaks belong to which part of the chemical structure have also been identified.

### CMC-determination with NMR

Several NMR-methods can be used to study the self-assembly of surfactants. When a surfactant forms a micelle, the chemical shift of the aggregate differs from the unimers, which can be explained by the conformational changes of the hydrocarbon chains (Lundberg et al., 2008). One method that will be used in this thesis is by recording  $^{13}\text{C}$ -spectra for a concentration series of surfactant and observing changes in chemical shift ( $\delta$ ) for a reference peak. By plotting the differences in chemical shifts in a concentration series against the inverse surfactant concentration, one can be able to observe the CMC as the point where there is a sudden increase in chemical shift.

The observed chemical shift ( $\delta_{obs}$ ) is a weighted average of monomers and micelles, see Equation 2,

$$\delta_{obs} = \frac{c_{mono}}{c} \delta_{mono} + \frac{c_{mic}}{c} \delta_{mic} \quad (\text{Equation 2})$$

, where  $\delta_{mono}$  is the average chemical shift for the monomer,  $\delta_{mic}$  is the average chemical shift for those residing in micelles and  $c$  is the total surfactant concentration (Lundberg et al., 2008).

## Peak broadening

The size of the molecule one's analysing with NMR affects the broadening of the peaks, where larger molecules tend to have broader signals (Wasan et al., 2004). A shift from sharp peaks to broad peaks can therefore indicate that aggregation have occurred, since aggregates (especially vesicles or wormlike micelles) are significantly larger than monomers. Typically, spherical micelles will still result in sharp peaks, partially due to the smaller size compared to e.g., vesicles. This is thus another way, besides noticing a difference in chemical shift, to gain information about the self-assembly behaviour.

## DPH-fluorescence

1,6-diphenylhexarine (DPH) is a hydrophobic molecule which demonstrates fluorescent properties. In hydrophobic environments, the intensity of fluorescence is increased – e.g., when it's solubilized in a C10-micelle. These properties make it possible to determine the CMC by making a concentration series of C10 together with DPH, and the measuring the intensity of fluorescence at different concentrations (Wasan et al., 2004).

## Materials

Peptides MEDI7219, J211 and J229 were present at AstraZeneca, all of which had previously been part of internal drug development projects. All peptides were stored in a fridge at -20°C, and correction factors of 0.86, 0.67 and 0.57 respectively was used during weighing. All water used in this work had been purified by a Milli-Q system. Other materials used was ≥98% capric acid (Sigma Aldrich), sodium phosphate monobasic monohydrate (J.T. Baker), >98% salcaprozate sodium (SynChem), >99.0% sodium caprate (Tokyo Chemical Industry CO., LTD), 1,6-diphenylhexamine (DPH), 2M NaOH (Supleco), 2M HCl (Supleco), 99.0 atom % D deuterium oxide (Aldrich), 35wt% deuterium chloride in D<sub>2</sub>O, 40% sodium deuteroxide in D<sub>2</sub>O, 99.8% anhydrous acetic acid (Sigma-Aldrich).

## Methods

### Sample preparation for determination of partial C10 phase diagram

The samples were created with different compositions in a triangular part of the ternary phase diagram which is limited by points (100% H<sub>2</sub>O), (20% C10, 80% H<sub>2</sub>O) and (20% capric acid, 80% H<sub>2</sub>O). The individual compositions of each sample can be viewed in appendix 2.

All samples were prepared in 10 ml glass vials at compositions indicated in Figure 15. The samples were prepared by first weighing the needed amount decanoic acid, followed by addition of 2M NaOH to produce the desired amount of C10 in situ. MQ-water was then added until a final total weight of 5 grams was reached. The samples were then, without mixing or shaking, allowed to reach equilibrium at room temperature.

The decanoic acid was melted at 37.0° and kept in liquid form during the weighing. The C10 was prepared in-situ to keep the pattern of impurities the same for all samples. If instead C10 would have been added manually, the problem of differences in impurities between samples could arise.

After reaching equilibrium in room temperature, the samples were then placed in a heating cabinet at 37°. This was done both to melt the capric acid crystals and because if a tablet were to be formulated with C10 and ingested in the body, this temperature would be of more relevance than room temperature.

Samples that were believed to have a lamellar phase were analysed with polarized light microscopy.

### Sample preparation for CD-analysis

All samples were prepared in 4 ml glass vials for MEDI719 and in 1ml glass vials for J221 and J229. Stock solutions of peptide were first made by weighing in a weight of peptide to produce a 2mg/ml solution, then adding up to 50% of the final mass with 0.04M sodium

phosphate monobasic monohydrate buffer. MQ was then added until 95% of the final weight was reached. J211 and J229 was not completely soluble at these conditions, and 2M NaOH was added until the solution was completely clear, and then MQ was added for all stock solutions until 100% of the final weight was reached.

The samples for CD-analysis were prepared by weighing in 50% of the final weight with 0.04M sodium phosphate monobasic monohydrate solution. A solution of 8% C10 was then weighed in until the desired concentrations was reached. Peptide stock was then weighed in at a weight which corresponded to  $4,595 \times 10^{-8}$  moles, followed by addition of MQ-water until 95% of the final weight was reached. The samples were then adjusted to the desired pH with a 2M NaOH or 10 wt% acetic acid solution. MQ-water was then added until the 100% of the final weight was reached.

## CD-analysis

Samples were analysed using a Chirascan V100 equipped with a water circulator and a Peltier controller. Only samples with no visible turbidity were analysed. Samples were analysed between 190nm-250nm with 1 step per wavelength, 0,5 seconds per time-point and each measurement was repeated three times.

## Sample preparation for NMR-spectrometry

Samples were first prepared in 1 ml glass vials. A stock solution of 40 mM sodium phosphate monobasic monohydrate was prepared in deuterium oxide D<sub>2</sub>O and was adjusted with sodium deuterioxide until a final pD of 7.5 was reached. A stock solution of 10 wt% C10 was prepared in D<sub>2</sub>O. For the samples with only C10, 50% of the final weight was weighed in with the stock buffer. Then the 10 wt% solution of C10 was weighed until the wanted concentration was reached. Deuterium oxide was then added until 95% of the final weight was reached, the samples were then pD-adjusted (under stirring) with either sodium deuterioxide or deuterium chloride until a pD of 7.5 was reached. Finally, D<sub>2</sub>O was added until 100% of the theoretical final weight was reached and the pD was again checked. For samples where peptide was included, the same preparation was the same as above except that peptide was first weighed in as a powder, and then the theoretical final weight was adjusted until the wanted peptide concentration was reached. When the samples were finished, 500 µl of the samples were transferred to 5 mm NMR tubes.

The density of the final samples were assumed to have the same density as pure D<sub>2</sub>O. Prior to the pD measurements, the pH-electrode was calibrated between pH 7 – pH 10. The electrode was then allowed to sit in D<sub>2</sub>O for 30 minutes. The pK<sub>a</sub> was then measured and the pD was then calculated according to the following formula (Equation 3) (Covington et al., 1968):

$$pD = pH_a + 0,41 \quad \text{(Equation 3)}$$

## NMR-analysis

Standard <sup>1</sup>H and proton-decoupled <sup>13</sup>C NMR spectra were recorded on a 600 MHz Bruker spectrometer at 25°C.

## DPH-fluorescence CMC-measurement

The sample preparation consisted of making a concentration series of C10 in 20 mM phosphate buffer at pH 7.5 where each sample contained 1μM DPH. Each sample was pH adjusted individually with 2M NaOH or 2M HCl until a pH of 7.5 was reached. The general method was the same as for when preparing the concentration series for NMR, except that no deuterated chemicals were used and that these samples contained DPH. The instrument used was a PerkinElmer Multi Plate Reader.

## Results & Discussion

### C10 ternary phase diagram

Several samples were made within a triangle of the ternary phase diagram which is limited by the following points: (100% H<sub>2</sub>O), (20% capric acid, 80% H<sub>2</sub>O) and (20% C10, 80% H<sub>2</sub>O). Figure 15 illustrates the composition range for which samples were prepared, where the black dots represent the samples prepared. For reference, the ternary phase diagram for sodium octanoate, octanoic acid and water is included. When inspected visually, the samples could be placed in different categories, as displayed in Figure 15.

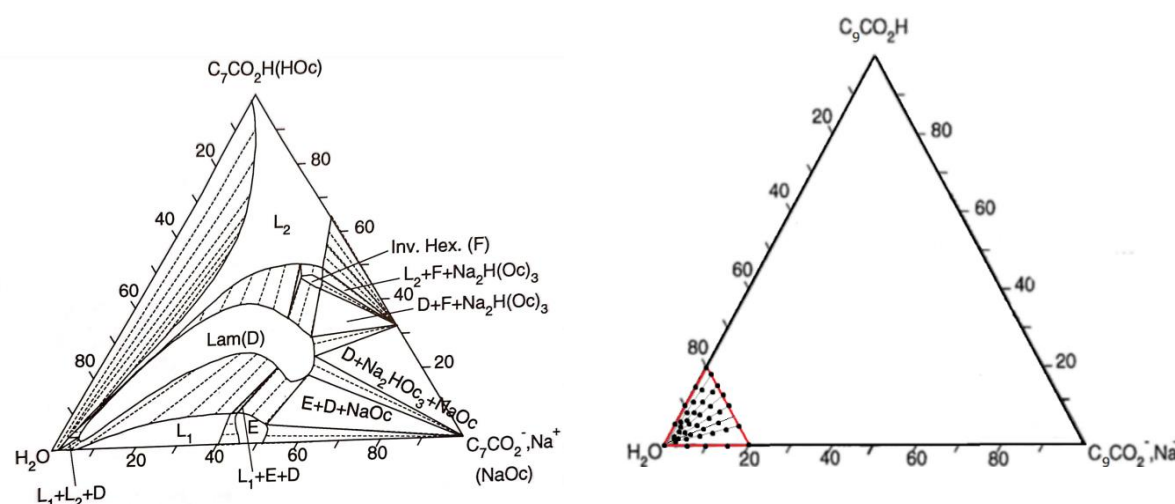


Figure 15: The right figure shows an empty phase diagram for C10, where the red triangle visualises the part in which samples were created. The black dots within the triangle shows which samples that were prepared. The left phase diagram is for sodium octanoate (Laughlin, 1994), and this was used as a reference when creating the phase diagram for sodium decanoate.

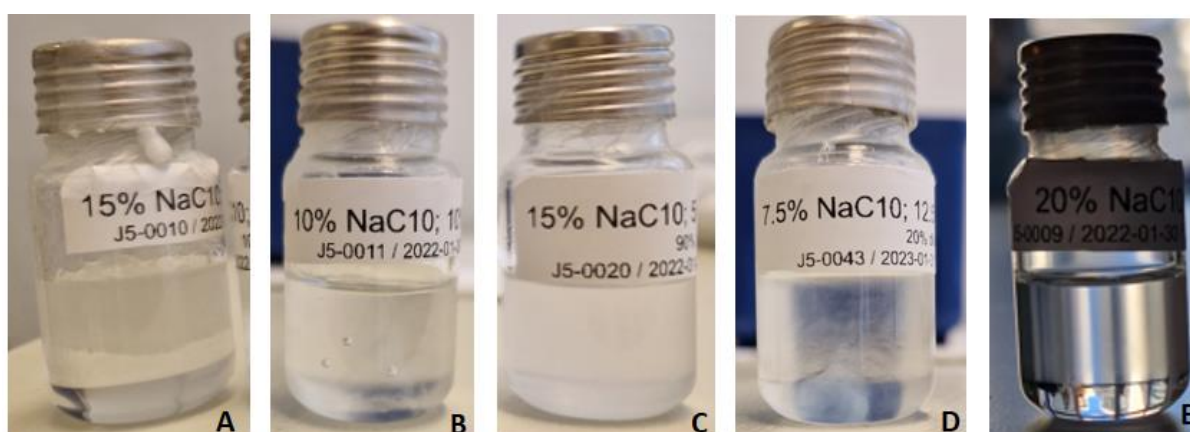


Figure 16: Shows different types of samples that was identified at 37°C. Figure A shows an example of what looks like two phases that have been separated. Figure B shows a viscous liquid. Figure C shows a sample that has similar viscosity to water but is turbid. Figure D has a similar behaviour as sample B, but also has clumps which seem to be of higher viscosity. Sample E shows a completely clear sample – this was only observed when no capric acid was present.

Some samples had what looked like two layers, one completely transparent and one white layer with lower density than the clear one. Other samples looked like gels with varying viscosity, which was believed to be a lamellar phase – or a two-phase region where one of the phases was lamellar. Some of these gels also had more viscous “clumps” present. Some samples were completely clear, but this was only true for those with no fraction capric acid present (Figure 16).

The construction of this phase diagram became more complicated than expected for several reasons. Firstly, while octanoic acid is melted at room temperature, decanoic acid is solid. As such, when the samples of different compositions were prepared, large parts of the phase diagram had solid particles in solution. An example is shown in Figure 17, A, which is a sample with 2,5 wt% sodium decanoate, 17,5 wt% decanoic acid and 80 wt% water. When placed in 37°C the crystals melted and formed droplets of oil which can be seen in Figure 17, B. Changes in those droplets were not noticed for the first days, but after approximately one week the oil had changed appearance and formed a white “phase” which also was separated from the water.



Figure 17: Shows what happened to solid crystals, most obviously observed for samples that contained high fractions capric acid, when they are heated to 37°C. Within a short period of time the crystals melt and form droplets of oil that floats on the top, as shown in Figure B. Figure C shows an example of what those samples could look like after a week at 37°C.

Some of the samples that were viscous gels were analysed with polarized light microscope since they were believed to be a lamellar phase. This is because lamellar structures often have distinct patterns which can become apparent under polarized light (as exemplified in Figure 12) (Dutton et al., 2011). At room temperature, when a sample with 10% C10, 10% capric acid was analysed, none of the typical patterns for lamellar phases could be seen. Instead, solid material and needle shaped crystals could be observed. This was believed to be crystals of capric acid because the melting point of this is 31.6°C. Because they’re not melted, they may not be able to form aggregate structures such as a liquid crystalline lamellar phase. After the samples had been placed at 37°C until visually equilibrated, the same sample was once again analysed with a microscope under polarized light, and some indications of a lamellar phase could be seen (Figure 18). Most indicative is the appearance of Maltese crosses, but the trapped air bubbles might also be indicative of a lamellar phase. For the sodium octanoate system, this sample composition would be in the single lamellar phase region, but it’s not clear that the same is true for corresponding C10 system. It may also be placed in a two-phase region where only a fraction of the sample presents itself as a lamellar structure.

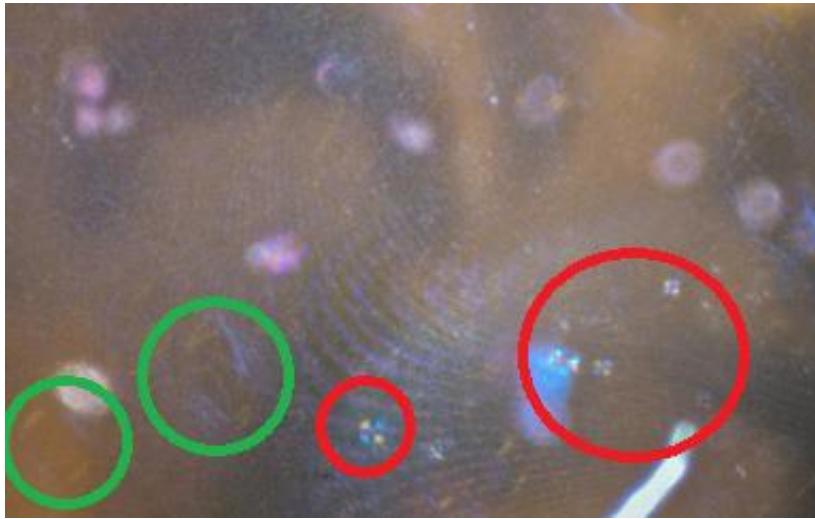


Figure 18: Showing a sample of 10 wt% C10, 10 wt% capric acid and 80 wt% water under polarized light microscopy at 37°C. The red circles highlights the Maltese crosses, and the green circles illustrates what looks like air bubbles.

The following sketch highlights approximately in which different parts of the phase diagram the different appearances of samples could be found (Figure 19) (important to note is that this is a sketch based on the visual appearance of the samples, and phase borders have not been determined). Because of how different samples look visually and based on the polarized light microscopy, the following is likely: The blue area is the lamellar phase, and the grey area is probably the two-phase region between  $L_1$  and  $L_\alpha$  (which makes sense because of the “phase” separation). The red area had much unsolved capric acid at room temperature, which then melted and changed appearance according to what’s described above.

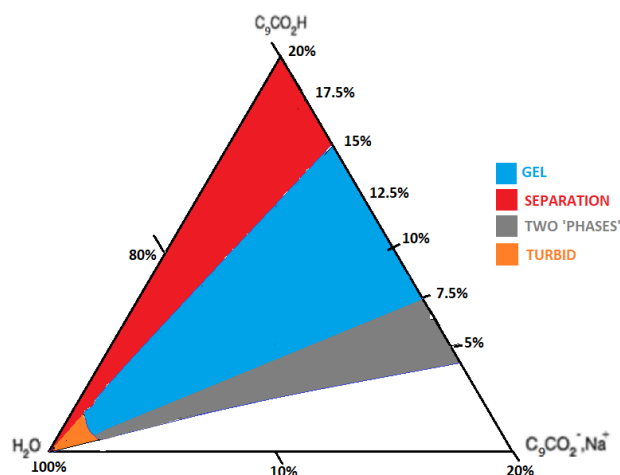


Figure 19: Shows a sketch of approximately where the different types of samples could be located in the ternary phase diagram.

The main objective with creating this phase diagram was to identify the phase borders to the lamellar phase to know in what compositions one can find this. The idea from the beginning

was to add salt to the system to determine this. SAXS (Small Angle X-ray Scattering) was also supposed to be used to better identify different phases. But because of the more than expected solid matter that is present the determination of different phases become complicated - and because of the complex nature of this system and because of time-limitations, neither of this was done. The reason that no ternary phase diagram for this system was found in the literature is probably because of the very complex nature of it. Not only is capric solid at room temperature, the pKa for unimers and aggregates also differs which further adds to the complexity. Despite this, some insight about this system was still obtained that could be of value when working with C10.

- At room temperature, large parts of the constructed phase diagram contain solid material.
- When heated, this material melts, forms oil droplets, which then over time develops into white material of unknown structure.
- The equilibrium time for this system is high (the changes presented in the point above took approximately one week), which potentially could be important information in drug formulation.
- There seem to be structural changes of phases between room temperature and 37°C, most notable is the presence of Maltese crosses which only could be seen at the latter temperature – indicating a lamellar phase. It's not known if samples at room temperature would equilibrate to form a lamellar phase with enough time (and if higher temperature only sped up that process) or if the capric acid crystals need to be melted for the lamellar phase to form. After taking out the equilibrated samples from 37°C to room temperature, the capric acid crystals does not seem to fall out of solution, and the gel seems to be stable. This could mean that it's energetically favourable for the system to stay in its expected lamellar phase and thus the capric acid do not fall out despite that the sample is below the melting point for capric acid.
- The presence of a lamellar phase could not completely be proven but since C10 forms vesicles (and the presence of Maltese crosses could be observed), the expected existence of one is very likely.

## NMR

### C10 & SNAC

The CMC of C10 in deuterium oxide was first established. Fifteen samples of increasing concentrations between 10 mM – 200 mM of C10 was created such that the difference in  $1/C$  between all points was kept approximately the same. Each sample was then analysed with  $^{13}\text{C}$ -NMR. Following is an example of the  $^{13}\text{C}$ -spectrum that was obtained for 20 mM C10 in  $\text{D}_2\text{O}$  (Figure 20).

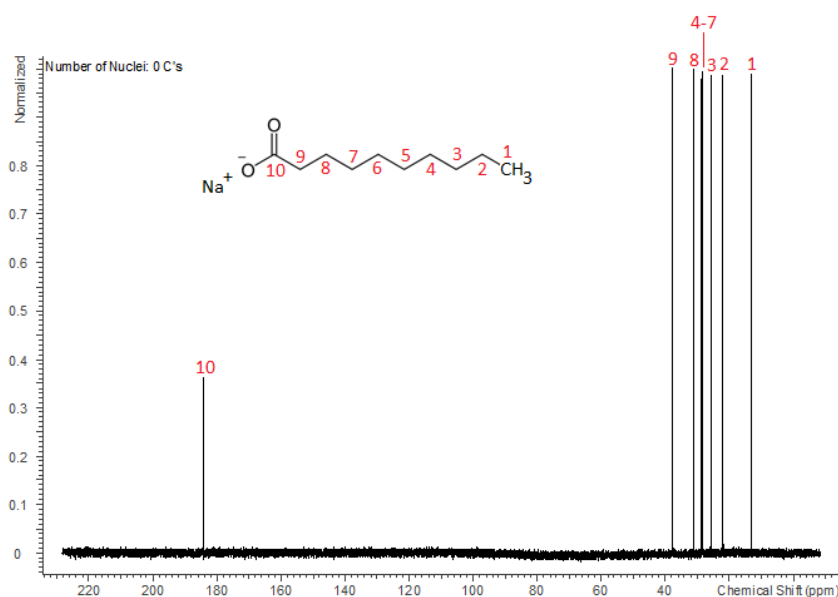


Figure 20: Shows the  $\text{C-}^{13}$  spectra for 20 mM C10 in  $\text{D}_2\text{O}$ . Which peak belongs to which carbon have been labelled in the figure.

To determine the CMC, the chemical shift ( $\delta$ ) for the methyl group was tracked and was plotted against the inverse surfactant concentration, and the graph shown in Figure 22 was obtained. This shows that the chemical shift starts increasing at 88 mM, and the graph shows that the CMC is between 80 mM and 200 mM C10 for this environment. Worth noting is that this was obtained for C10 in pure  $\text{D}_2\text{O}$  – without buffer and without pH-adjustment. The consequence of this is that the pH will be relatively high, to a point where most of the C10 is available in its ionic form. When the pH is lowered, more of the surfactant will be in its non-ionic form. If buffer is included, the CMC will also be lowered because of the screening of electrostatic repulsion between the headgroups from the salt, which effectively reduced the head group area.

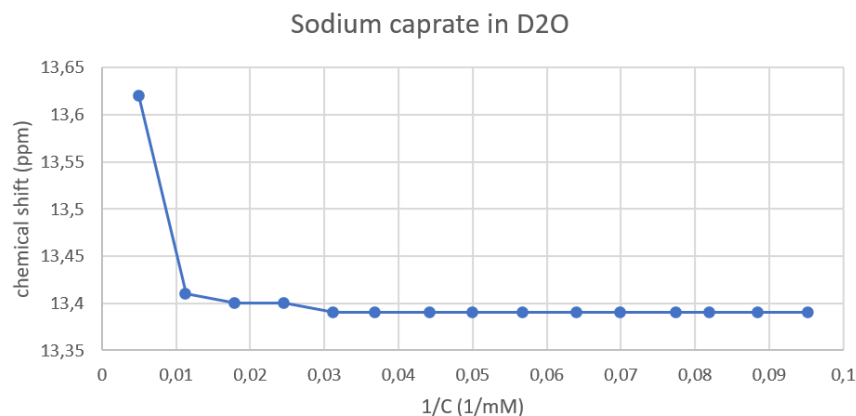


Figure 22: Shows how the chemical shift increases with increasing concentration C10.

The corresponding graph was made with SNAC, shown in Figure 23. An example of an  $^1\text{H}$ -NMR spectra for SNAC is also shown in Figure 26.

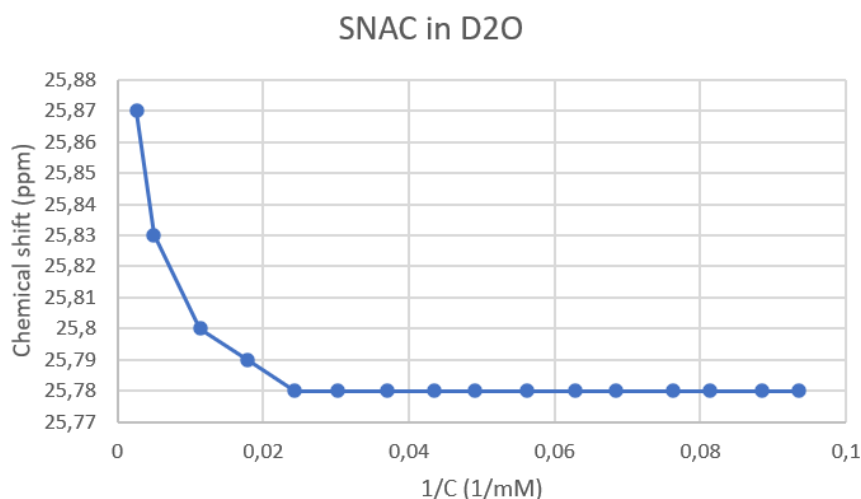


Figure 23: Shows how the chemical shift increases with increasing concentration SNAC.

A concentration series was also made for C10 in phosphate buffer at pD 7.5 (Figure 24), but with a smaller number of samples as they started to get turbid around 30 mM. Structural changes of the aggregates, as indicated by the turbidity, might be the reason for the small reduction in chemical shift for the 30 mM concentration. Still, results show that the chemical shift is increasing starting approximately at 24 mM which indicates conformational changes.

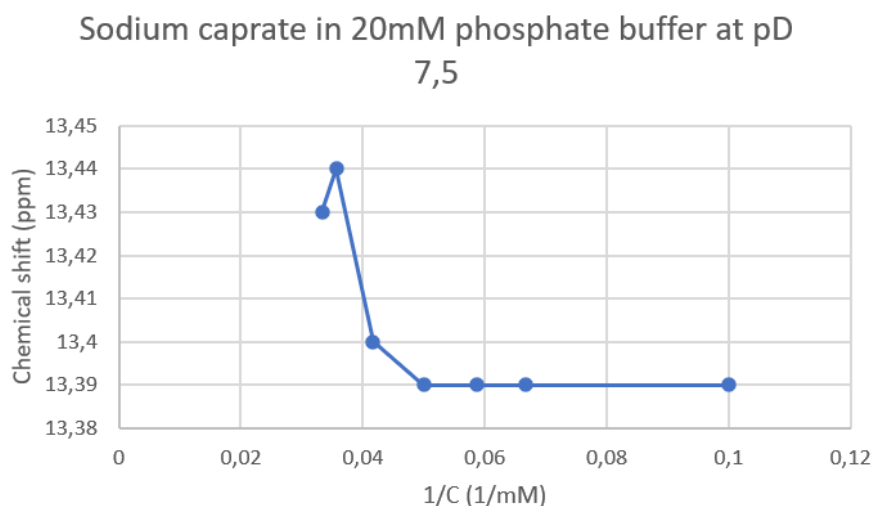


Figure 24: Shows how the chemical shift changes with increasing concentration C10. The concentrations were 10 mM, 15 mM, 17 mM, 20 mM, 24 mM, 28 mM and 30 mM.

What also could be seen for C10 in phosphate buffer is that, when looking at the  $^1\text{H}$ -NMR spectra, there is a gradual broadening of the peaks starting at 24 mM (Figure 25). This could not be seen for C10 in pure  $\text{D}_2\text{O}$ . Generally, as the substances analysed by NMR increases in size, the NMR-signal becomes broader (Wasan et al., 2004) - and therefore a broadening of signal for C10 is indicative of aggregation. But a change in chemical shift for, e.g., the methyl peak, is also indicative of aggregation because it implies a conformational change of the carbon chain. A logical extension of this and the NMR-results above is that C10 in  $\text{D}_2\text{O}$  might form spherical micelles while C10 in phosphate buffer at pH 7.5 forms bigger structures, probably vesicles or wormlike micelles. Spherical micelles are smaller than vesicles and wormlike micelles, so when spherical micelles are formed in pure  $\text{D}_2\text{O}$ , they might not be big enough to cause a broadening of the peaks and only a change in chemical shift is detected. This is also consistent with previous findings that C10 forms micelles when the molecules are primarily in its ionic form. For C10 in  $\text{D}_2\text{O}$ , the pD is above 8.0 which means that most of the C10 is in its ionic form, but at pD 7.5 a greater fraction is in its non-ionic form which is known to favour vesicle formation instead (Namani & Walde, 2005). This is also explained by the CCP. As previously exemplified, as pH (or pD) is reduced, more of C10 will be in its anionic form as the fatty acid capric acid. Capric acid can thus work as a cosurfactant, reduce the effective head group area, increases the CPP, and as such leads to the formation of vesicles or wormlike micelles.

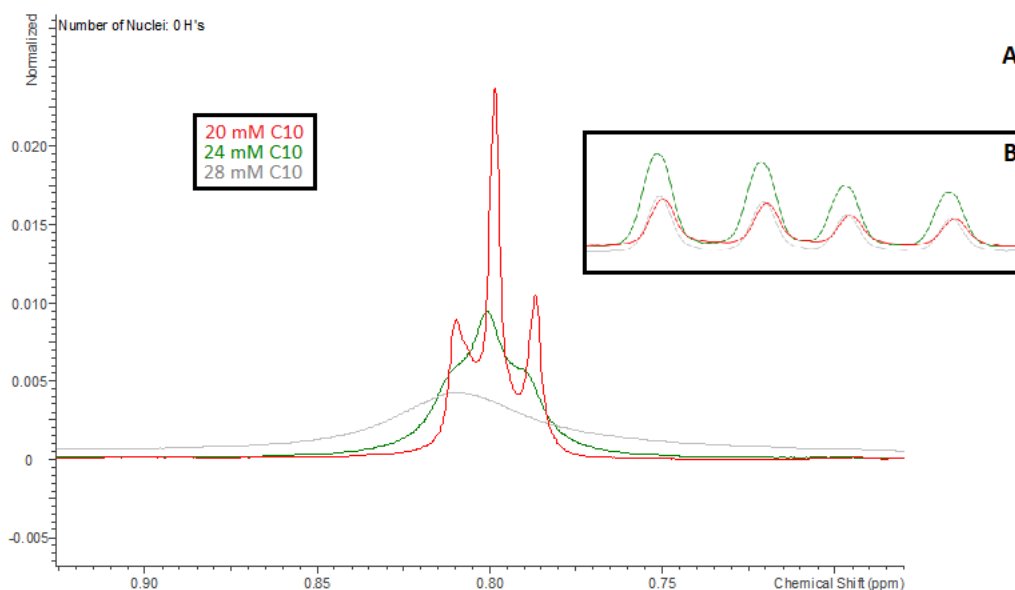
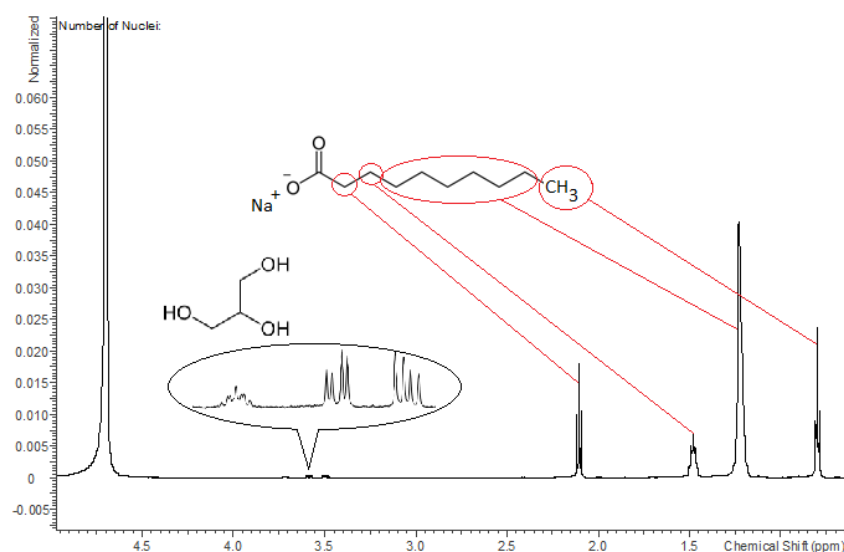
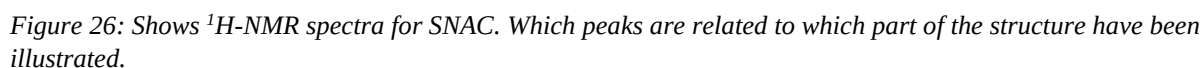


Figure 25: Shows the gradual broadening of the methyl peak with increasing concentrations C10 (A). The red spectra is 20 mM, the green is 24 mM and the grey is 28 mM. Also included is the first peaks from glycerol (B), which shows that their shape remains the same even though the C10 spectra becomes broader.

Before further before further discussion, the following is should be noted. Figure 21 shows a <sup>1</sup>H-NMR spectrum of C10 at 15 mM concentration but in phosphate buffer at pD 7.5. There was found be to trace amounts of glycerol in these samples, which stemmed from the stock phosphate buffer. Initially these samples were supposed to be redone to eliminate the glycerol, but these peaks proved to be useful. As concentration of C10 is increased, a broadening of the peaks was noticeable. Because only the C10 peaks became widened but the glycerol peaks remained sharp, it strengthened the findings that the broadening was from self-association C10, and not something that was affecting the whole system.



Looking at the concentration series for SNAC (Figure 23), as with C10 in pure D<sub>2</sub>O, a broadening of the peaks was not observed for SNAC either. This indicates that neither of the C10-aggregates in phosphate buffer, pure D<sub>2</sub>O or SNAC in pure D<sub>2</sub>O are the same. While SNAC also shows an increase in chemical shift with increasing concentration, the shape of the graph shows that there is a gradual increase in chemical shift instead of a more instantaneous increase. This can indicate there is a gradual change in aggregate structure and that the character of the aggregates formed from SNAC is not typical micelles. This would also make sense when examining its chemical structures. SNAC has moieties of polar character on both sides of the carbon chain (Figure 8), while C10 only has that on one side. All this information could suggest that C10 in pure D<sub>2</sub>O forms more conventional spherical micelles and that C10 in phosphate buffer at pD 7.5 forms wormlike micelles or vesicles. SNAC in pure D<sub>2</sub>O forms some type of aggregate, but they're probably not typical spherical micelles due to its polar moieties and gradual increase in chemical shift.



## MEDI7219 and C10

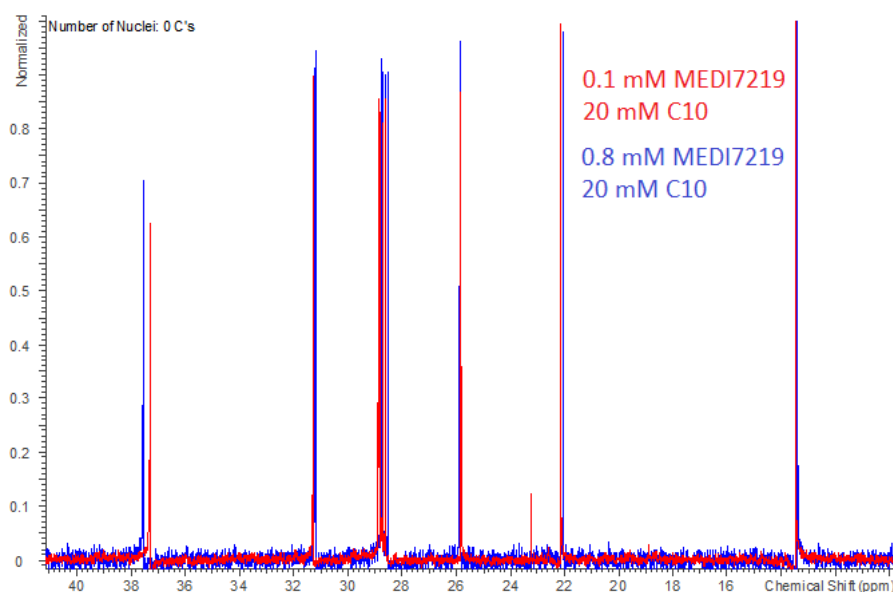


Figure 27: Shows the nine first carbon peaks of a  $^{13}\text{C}$  NMR spectra for MEDI7219 and C10. For the higher concentration MEDI7219, an increase in chemical shift is observed – which also differs between peaks. The red spectra is 0.1 mM MEDI7219 + 20 mM C10 and the blue spectra is 0.8 mM MEDI7219 + 20 mM C10.

Figure 27 shows two different  $^{13}\text{C}$  spectra – the red spectra is for 0.8 mM MEDI7219 + 20 mM C10, and the blue spectra is for 0.1 mM MEDI7219 + 20 mM C10. Both are dissolved in 20 mM phosphate buffer at pD of 7.5. To be noticed is that there is a difference in chemical shift for the higher concentration, which can indicate that the molecular conformation of the carbons has changed. As previously described, the point of aggregation for C10 in these conditions starts at around 24 mM C10, but in this spectrum we see a difference at 20 mM. This may indicate the formation of mixed micelles, where the hydrophobic chains of MEDI7219 might partake in the formation of those. This interaction could reduce the effective head group area which increases the CPP and reduces the CMC – thus resulting in a difference in chemical shift at a lower concentration C10.

## DPH-fluorescence measurement

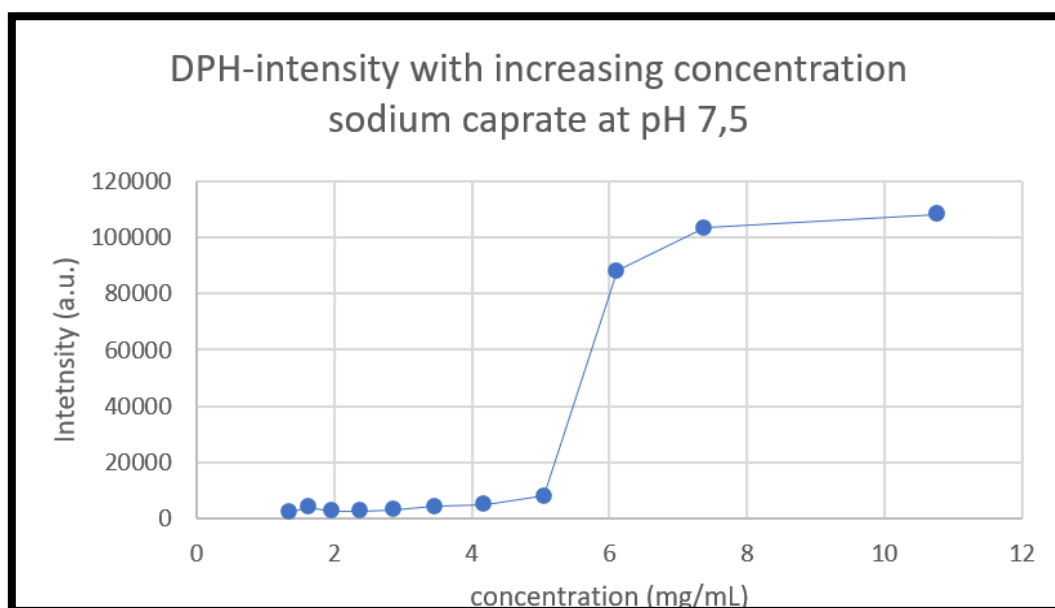


Figure 28: Shows the intensity of DPH-fluorescence as a function of concentration (mg/mL).

The point of aggregation was also measured with DPH fluorescence as a complement to NMR (Figure 28). The point at which the intensity start to increase corresponds to a concentration of 25.7 mM. As previously stated, the florescence for DPH is increased when it's e.g., solubilized in a hydrophobic environment – and if only these results were used one could draw the conclusion that the CMC for C10 in 25.7 mM. But as the NMR results indicated previously, spherical micelles probably are not formed at this environment. Instead, this concentration is probably the point for either vesicle- or wormlike micelle formation. Both NMR and fluorescence resulted in similar values for the point of aggregation.

DPH-fluorescence can work as a complement to verify NMR-results, and this technique is usually quicker than NMR. This is because, usually, a stock solution of surfactant can be diluted several times with buffer to make a concentration series, which then can be analysed in a matter of minutes. But in the case for C10, this wasn't possible, as the pH would change with decreasing concentration C10 despite using buffer at pH 7.5. This was probably because of several reasons. At higher concentrations at pH 7.5, all C10 wasn't soluble and as concentrations decreased, the undissolved C10 could dissolve. The fact that C10 can buffer (in itself), along with the fact that C10 unimers and C10 aggregates differ in pKa, probably resulted in the differences in pH between samples. This made it so that a simple concentration series with constant pH was not possible to do with dilution, and every sample had to be pH adjusted individually. DPH-fluorescence was therefore substantially more time consuming than first expected and determining the CMC with this method wasn't much quicker than NMR.

## CD-analysis

In this section all three peptides J211, J229 and MEDI7219 are studied. They have been analysed to see how different amounts of lipidation affects the secondary structure with increasing concentration C10. The concentration of peptide in the samples are 0.2mg/ml MEDI7219, 0.181mg/ml J229 and 0.148mg/ml J211 (same number of moles).

Looking at the spectra below (figure 29, figure 30, figure 32) it's clear that the secondary structure of all the peptides are  $\alpha$ -helixes, as indicated by the minima at 208 nm and 222 nm. For wavelengths below 200 nm, and at higher concentrations C10, the scattering of the samples would often be too high. This meant that values below 200 nm may not be reliable. Some graphs, referred to as 'normalized', have been adjusted to that all values at 208 nm have the same value. This was done so that changes in the overall shape of the graph could be better visualized.

### J211 & J229

When looking at how to CD-spectra changes with constant peptide concentration and increasing concentration C10 for J211 (Figure A38) and J229 (Figure A39), there is a noticeable increase in signal intensity with increasing concentration C10. As seen in the normalized graphs (Figure 29 & Figure 30) there is a change in spectral appearance as well, but the degree of change does not seem to be correlated with the concentration of C10 (i.e. higher concentrations C10 doesn't necessarily result in greater change in spectral appearance). For J221, the 50 mM concentration has approximately the same shape as pure peptide with no added C10. For J229, all concentrations above 5 mM appear to have very similar conformation.

Figure A36 shows how the conformation for changes at different pH, and to be noted is the relatively large change in shape at pH below 6 for J229. This can indicate that some form of self- or co-aggregation occurs at this pH, and this might be because the peptide is reaching its apparent solubility limit. One possible explanation is that the net charge is positively increased as pH is decreasing, thus reducing the electrostatic repulsion between individual molecules (they are negatively charged at pH 7.5). A corresponding change in structure was not seen for J211 as samples around 6 became turbid and was therefore unsuitable for analysis with CD. Because J221 and J229 have very similar structures and the fact that solutions of J211 became turbid at this pH, this can imply that there is a similar change of spectral appearance also for J211 at this pH.

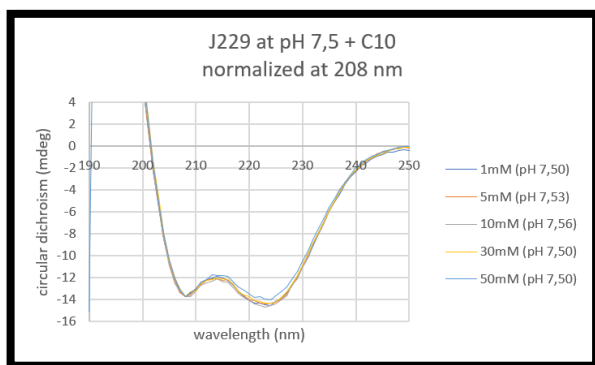


Figure 29: Normalized spectra of J229 at different concentrations C10.

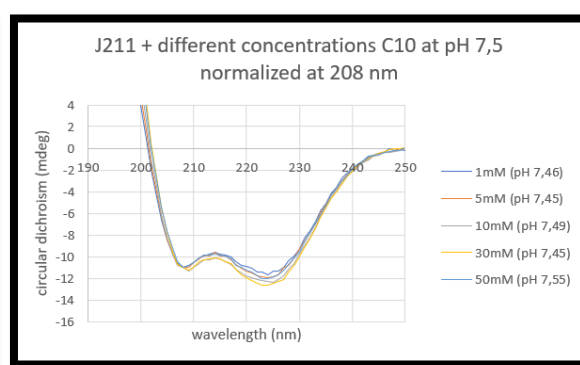


Figure 30: Normalized spectra of J221 at different concentrations C10.

## MEDI7219

For MEDI7219 there is a distinct and apparent change in both signal and shape of the graphs that seem to correlate with a gradual change in concentration of C10, above 10 mM C10 (Figure 31 and Figure 32), as opposed to J221 and J229. A possible explanation is that the two latter lack the lipidation that MEDI7219 has. J211 has no lipidation and J229 has mono-lipidation, but the lipidation of J229 contains a carboxylic acid moiety at the end which could repel the C10 carbon chains and thus have a lower propensity to interact hydrophobically.

Looking at the shape of the graphs for different pH (Figure A37), it's also clear that the pH affects the spectral appearance. Interestingly, the change in shape when lowering pH resembles the change in shape when increasing C10 concentration. The same type of sudden change in spectral appearance around pH 6 that was observed for J229 was not seen for MEDI7219 at pH 6.02. But samples started to get turbid around this pH, so it's not unreasonable that a similar type of change could also be seen for MEDI7219 if the pH was reduced slightly.

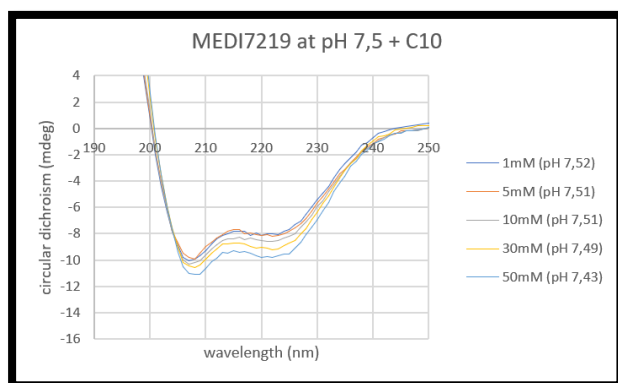


Figure 31: Spectra of MEDI7219 at different concentrations C10.

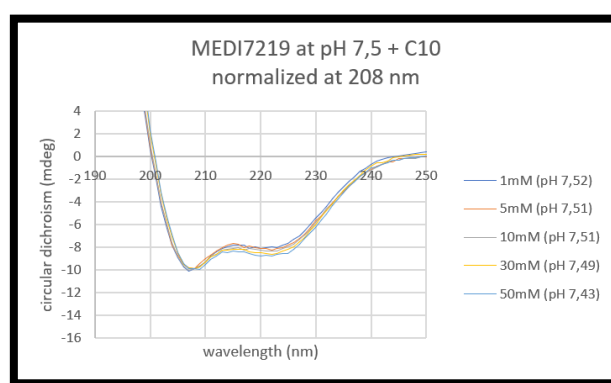


Figure 32: Normalized spectra of MEDI7219 at different concentrations C10

## Comparison between J211, J229 & MEDI7219

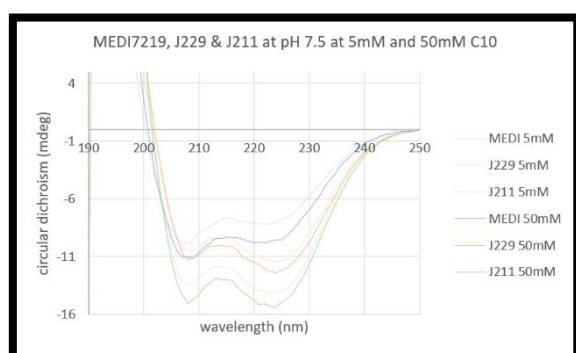


Figure 33: Spectra of MEDI7219, J229 and J211 together with 5mM C10 and 50mM C10

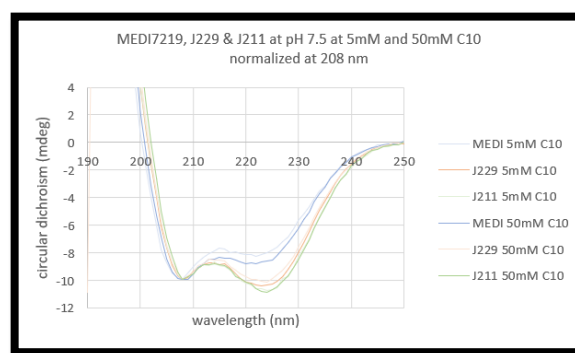


Figure 34: Normalized spectra for MEDI7219, J229 and J211 together with 5mM C10 and 50mM C10

A comparison between how the spectral appearance changed for all individual peptides together with 5 mM C10 and 50 mM C10 was made to see how increased C10 concentration affects them differently. When comparing the graphs for the three different peptides normalized at 208nm, differences in the shape of the CD-spectra become apparent (Figure 34). For J211, there is no visible change in shape when adding 5mM and 50mM C10 respectively. For J229 the minimum point at 222 nm is lower for the 50mM C10 solution and for MEDI7219 the same is true, and the difference in shape is bigger than for J229. This seems to correlate with how lipidated the peptides are - J211 has no lipidation, J229 has mono-lipidation and MEDI7219 has bis-lipidation (Pechenov et al., 2021). A possible explanation is that the hydrophobic chains of C10 interact with the lipidated groups of the peptides, and associated changes in peptide conformation cause the change in shape of the CD-spectra.

When looking at Figure 33, there is an increase in signal strength as lipidation of the peptides increases, with J211 having the highest signal. Increase in signal strength may indicate a more stable alpha-helix structure, and the trend of lowered signal strength with increased lipidation can indicate that the alpha-helix structure becomes less stable/clear with added lipid-groups (or that a lower fraction peptide is present in the  $\alpha$ -helical form. Also, to be observed is that there is an increase in signal with higher C10 concentration between individual peptides, which (if increased signal indicates a more stable structure) could indicate that the C10 helps stabilize the secondary structure, but this is not clear. At 50 mM C10, high scattering for the CD samples surpasses is observed at 200 nm. It is not clear to what (if any) extent the differences in signal strength between 5 mM and 50 mM samples are due to high scattering.

Based on NMR measurements, presented in the NMR section, the formation of C10-aggregates happen around 24 mM and above in phosphate buffer at pH 7.5. There generally doesn't seem to be significant changes in secondary structure at this point, but instead the change seems to be gradual with increasing C10 concentration – where the correlation also increases with increasing amount of lipidation on the peptide. Since the changes seem to be because of hydrophobic interactions between the lipidated part of the peptides, a logical extension is that the carbon-chain of C10 interacts hydrophobically with the lipid groups of MEDI7219, possibly creating a sterical hindrance which affects the secondary structure in some way. The lipid part of the peptides may also participate in the micelle – thus creating a mixed micelle. The most obvious correlation is for MEDI7219, whereas changes for J229 and J211 are not as clear (probably because the more hydrophobic character of the MEDI7219 lipidation).

To further support that the hydrophobic chains of C10 interact with the lipidated groups of the peptide, samples of MEDI7219 in C10 and acetic acid respectively was analysed using CD. This was done because the molecular structure of acetic acid is the same as C10, except that acetic acid lacks the hydrophobic chain that C10 has. When comparing these (Figure A40 and Figure A41) it's obvious that differences in shape and signal strength in samples with 5 mM and 50 mM acetic acid are absent. This implies two things:

- Because acetic acid is in its deprotonated form at this pH, these results indicate that the charged headgroups of C10 aren't causing the changes in spectral appearances at higher concentrations C10.
- Instead, these results indicate that there are interactions between the hydrophobic chain of C10 and the peptide that cause the conformational changes indicated by the CD-spectra.

## Conclusions

In the attempted creation of a ternary phase diagram for C10, insight was gained that large parts of the phase diagram contains solid capric acid at room temperature. When the temperature is increased to 37°C the capric acid melts and polarized light microscopy indicates that this leads to structural changes as Maltese crosses could be seen for this temperature but not at room temperature. This supports the presence of an expected lamellar phase. What was also learned was that there is a high equilibrium time for the samples, and this could potentially be useful information when formulating drugs with C10.

NMR has indicated that C10 forms micelles in pure D<sub>2</sub>O at concentrations between 88 mM and 200 mM. In phosphate buffer at pH 7.5, results indicate instead the formation of larger aggregates – possibly vesicles or wormlike micelles. This is consistent with previous studies that suggest that C10 forms micelles when it's primarily in its ionic form, and vesicles as the fraction of non-ionic species is increased. SNAC forms some type of aggregates starting at around 40 mM in pure D<sub>2</sub>O, but NMR results and analysis of the molecular structure indicate that these are not typical spherical micelles.

CD analysis indicated that there, for MEDI7219, seems to be a gradual change in the secondary structure with increasing concentrations of C10. Minor changes in spectral appearance with C10 could also be seen for J211 and J229, but the correlation is not as strong as for MEDI7219 – this could be because of the more hydrophobic character of the lipidation of MEDI7219. Results also showed that there is a relatively large change in secondary structure around pH 6. Whether this change will have any effect on the formulation stability of the peptide is not known.

The interactions between C10 and MEDI7219 have been investigated by NMR and CD to give insight into how both the peptide might affect self-assembly of C10, and how C10 might affect the conformation of the peptide. There is an observed increase in <sup>13</sup>C-NMR chemical shift for C10 with increasing concentration MEDI7219. This could indicate that the hydrophobic chains of MEDI7219 part-take in the formation of micelles, thus forming mixed micelles and reducing the CMC (by reduction of the head group area which increases the CPP)– but more data is needed to confirm this.

All results together indicate that there are interactions between MEDI7219 and C10, but more studies are needed to confirm what these interactions mean practically and to what extent their interplay affect both stability and the therapeutic use of the peptide.

## Future work

In this work it's clear that there are some interactions between the peptides and C10, but the experiments in this thesis were not enough to determine how this interplay affects drug delivery and the therapeutic use of the peptides. To learn more about this, more research needs to be done. Proposed future work includes identifying the different phases in the phase diagram with SAXS, and to add increasing amounts of salt to investigate e.g., influence on phase boundaries. For CD, the same experiments as were conducted in this thesis could be redone with a smaller cuvette, as this might reduce the scattering of the sample and thus give insight to what extent high scattering is accountable for the observed changes in signal strength. For NMR, the concentration series for determination of CMC should be redone but with the inclusion of a known amount of peptide. This is to make a more detailed study of the influence of the peptide-surfactant interplay on the CMC for the surfactants. Also, cryo-TEM could be used to determine whether there are wormlike micelles or vesicles in phosphate buffer at pH 7.5 and if there's a difference in the micelle-structure of C10 and SNAC at higher pH (if resolution is sufficient for this).

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## Appendix 1

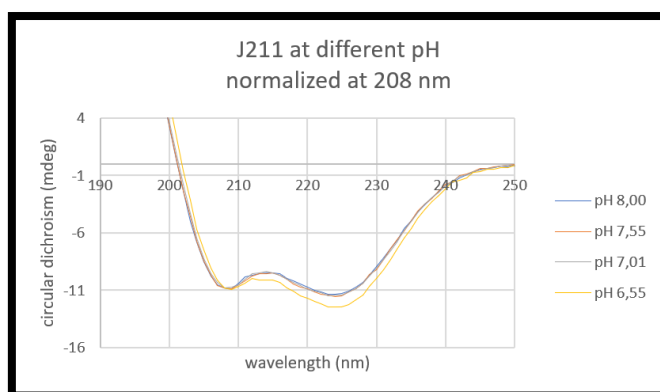


Figure A34: Normalized CD-spectra for J211 at different pH.agg

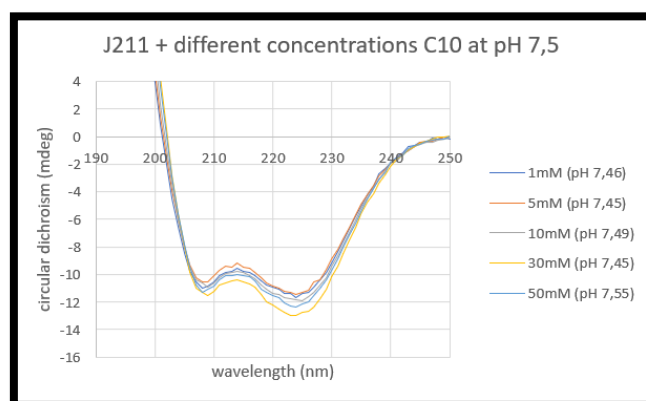


Figure A35: Normalized CD-spectra for J221 at different pH.

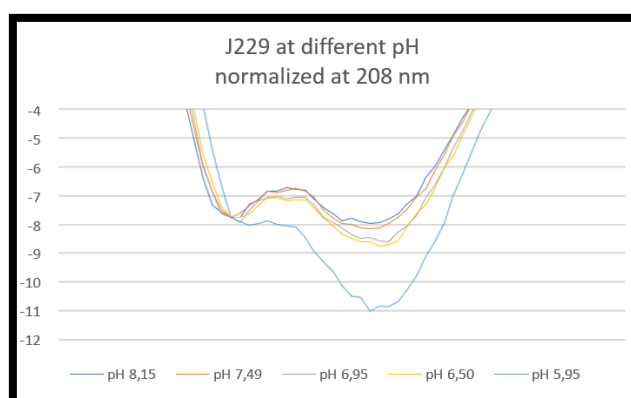


Figure A36: Normalized CD-spectra for J229 at different pH.

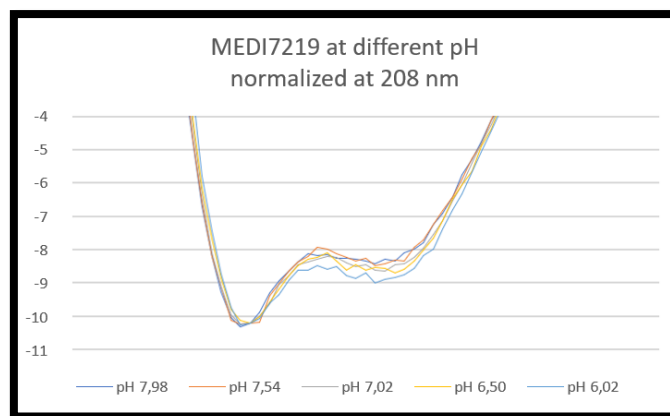


Figure A37: Normalized CD-spectra for MEDI7219 at different pH

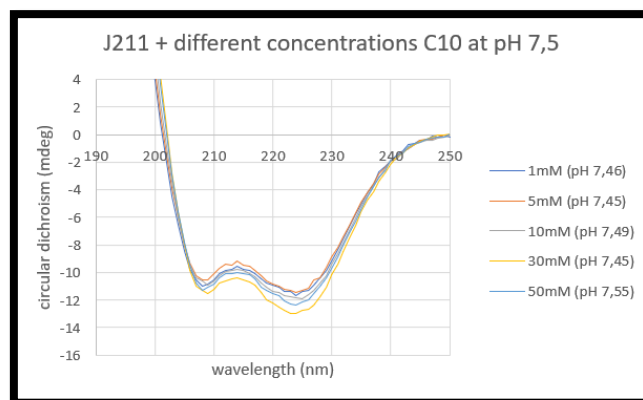


Figure A38: CD-spectra for J211 at different concentrations C10.

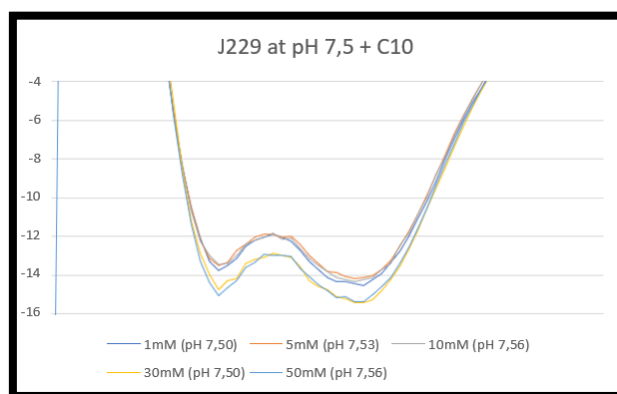


Figure A39: CD-spectra for J229 at different concentrations C10.

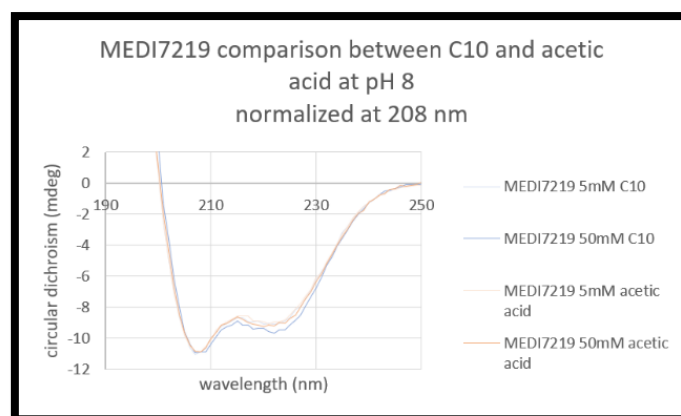


Figure A40: Normalized CD-spectra comparing acetic acid to C10.

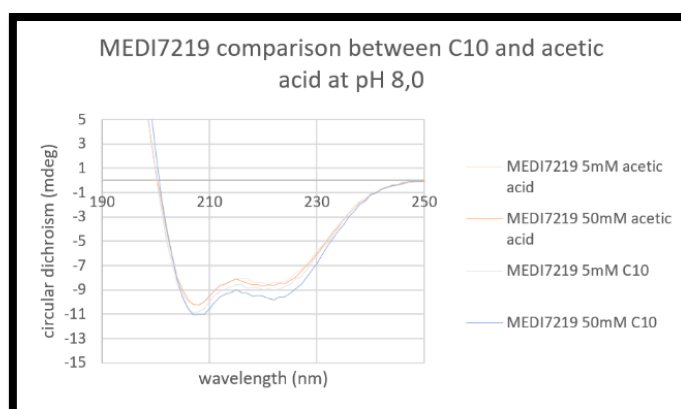


Figure A41: CD-spectra comparing acetic acid to C10.

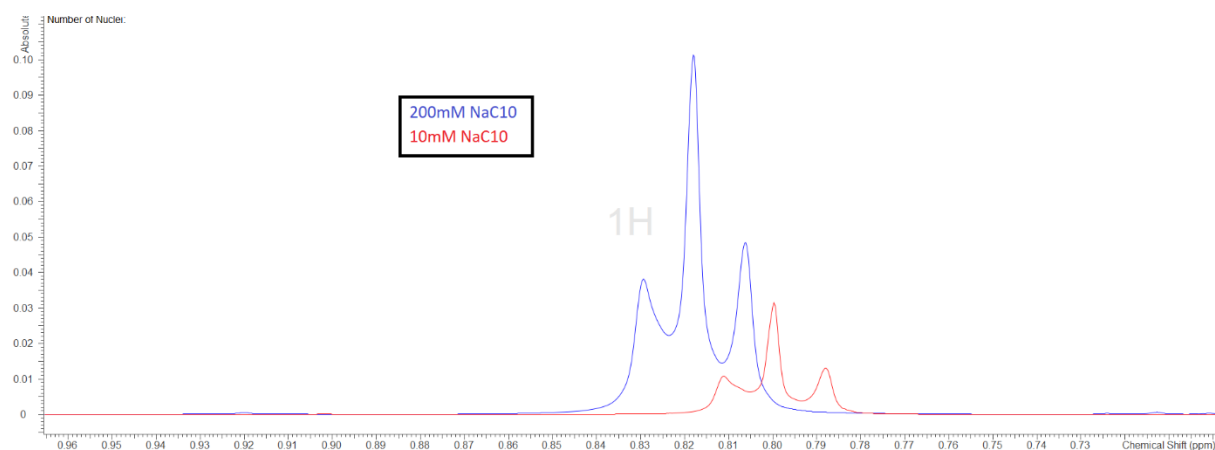


Figure A42: Shows that there is no change in overall shape of the  $^1\text{H}$ -NMR spectra for C10 between the lowest and highest concentration in  $\text{D}_2\text{O}$ .