1-1-2011

Development and stability of microemulsions as carriers for nutraceuticals

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DEVELOPMENT AND STABILITY OF MICROEMULSIONS AS CARRIERS FOR NUTRACEUTICALS

by

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A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the Program of
Molecular Science

Toronto, Ontario, Canada, 2011

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DEVELOPMENT AND STABILITY OF MICROEMULSIONS AS CARRIERS FOR NUTRACEUTICALS

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Abstract

The formulation and characterisation of food-grade water-in-oil (w/o) microemulsions as carriers for bioactive molecules were studied. The microemulsions consisted of deionised water, polysorbate 80, soybean oil, glycerol monooleate and sodium chloride (as a model marker).

The formulated microemulsions were studied for droplet size via dynamic light scattering and transmission electron microscopy, conductance and viscosity. Phase behaviour was studied along two dilution lines. Along these dilution lines, microemulsions were loaded with NaCl at their maximum solubilisation capacity. The stability of the microemulsions with and without NaCl was studied visually and through droplet size determination. Using a conductivity meter, sodium chloride-containing microemulsions were studied for release along one of the dilution lines. The release mechanism was established based on visual observation and using confocal laser scanning microscopy. The release of sodium chloride upon de-stabilisation of the microemulsion resulted from phase inversion. This phase inversion was brought about by the dilution of the microemulsion upon ingress of water as a result of osmosis.

Overall, this study demonstrated that microemulsions have the potential to solubilise hydrophilic food additives such as NaCl and the solubilised additive (sodium chloride) could be released following microemulsion de-stabilisation.

Acknowledgements

I would like to thank my supervisor, Dr. Dérick Rousseau, for sharing with me his expertise, research insight and wisdom. With his enthusiasm, patience and great efforts to explain things clearly and simply, he helped me through the course of my Master's. Throughout experiments as well as thesis-writing, he provided encouragement and intellectual advice to help me work to the best of my ability. I would also like to express my gratitude to Drs. Darrick Heyd and Stephen Wylie, my committee members, whose thoughtful advice often served to give me a further sense of direction during my Masters studies. I would like to thank Dr. Daniel Foucher for his useful advice during earlier involvement with the project.

I am grateful to Dr. Khursigara for the TEM work carried out at the University of Guelph. I would like to individually thank all of my friends and members of the lab, Dr. Supratim Ghosh, Shane Hodge, Dr. Lanny Sapei, Tu Tran, Moumita Ray, Muhammad Ali Naqvi, Renuka, Samira Haj-Shafiei and Dr. Tejas Patel.

Finally, I would like to thank my family. Particular thanks to my parents, Vijay Kumar Berry and Ritu Berry for their unconditional love, support and guidance. A special thanks to my sisters, Michelle and Priyanka for being my pillar of strength. Lastly, I would like to thank Jatin for his support and friendship that I truly cherish. To them I dedicate this thesis.

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Chapter 1

Introduction

The food industry is often faced with challenges such as the solubilisation of oil-insoluble additives in hydrophobic products or the protection of labile additives from oxidation during storage. In this regard, the potential to improve the solubility of hydrophilic vitamins, flavours and other additives in water-in-oil (w/o) microemulsions is of immense interest, as these can help protect such ingredients from degradation.[1, 2] Little progress has been made in the formation of microemulsions using only food-grade materials.[3] Microemulsions offer improved stability and solubilisation of compounds encapsulated within their nano-sized dispersed phase domains. Also, the use of non-ionic surfactants negates the influence of pH on formation and stability of microemulsions. In terms of release mechanisms, *in-vivo* w/o microemulsions undergo phase inversion, releasing the encapsulated drug/nutraceutical.[4, 5]

In the pharmaceutical industry, the use of microemulsions for oral delivery is primarily centered on their potential for the delivery of peptides/proteins. For example, a formulation of cyclosporin (a cyclic peptide) is administered as a soft capsule that contains an oil solution of the drug and surfactants. It converts into an o/w microemulsion in the aqueous environment of the stomach or the small intestine. As it mimics bile salt micelles, which play an important role in the adsorption of poorly-soluble drugs, its absorption can be significantly enhanced.[6] Similarly, oil-insoluble food additives such as vitamins can be dispensed in hydrophobic foods by entrapping them in the nano-sized water domains of w/o microemulsions. The enclosed environment offers protection to

either poorly-soluble or labile compounds against oxidation, enzymatic or pH degradation. This novel application offers an exciting opportunity for the food industry where shelf-life of food products is a continual concern.

As part of a long-term strategy, this project intends to develop and test the stability of nano-scale liquid delivery systems (microemulsions) as carriers for nutraceuticals. Sodium chloride will be used as a hydrophilic marker. These novel systems can be used to encapsulate hydrophilic food additives in the aqueous domains of water-in-oil microemulsions and offer them protection. Ultimately, these systems will be tested for sodium chloride release and the microemulsion de-stabilisation pathway will be established. The reasons for choosing microemulsions as delivery vehicles were as follows:

1) Easy formulation without high-energy input;

2) Thermodynamically-stable, suggesting the possibility of release only when triggered;

3) Nano-scale systems have higher solubilisation capacity; hence more salt can be solubilised within the water droplets.

1.1 What are microemulsions?

Microemulsions are thermodynamically stable dispersions of water and an apolar (oil) phase, stabilized by a surfactant, usually in conjunction with a co-surfactant and/or short chain alcohol. Diverse self-assembled, well-defined and non-birefringent structures, such as droplets [oil-in-water (o/w) or water-in-oil (w/o)] and bicontinuous phases can exist within microemulsions (Fig. 1.1).

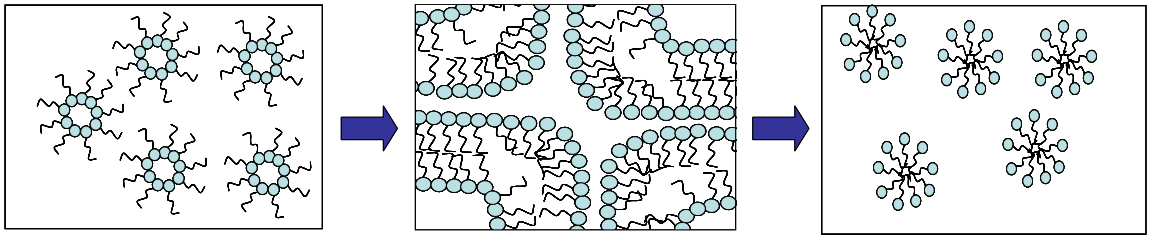


Figure 1.1: Possible nanostructures present within microemulsions: a) w/o; b) bicontinuous, and; c) o/w (adapted from ref.[7]).

Microemulsion dispersed domains are normally well-below 100 nanometres (nm) in diameter. They have a number of advantages for use in food, pharma and other applications (*e.g.*, cosmetics and petrochemicals), including ease of preparation, a clear appearance promoting consumer acceptance and patient compliance, the improved bioavailability of incorporated compounds and a possible shelf life of many years.[8, 9] As a result, microemulsions have been investigated as delivery systems for pharmaceuticals, nutraceuticals and ingredients as they can improve the solubility of poorly-soluble compounds (*e.g.*, sterols[10]), increase bioavailability (*e.g.*, of oil-soluble vitamins[11]) and protect labile compounds against light and/or enzyme-induced breakdown (*e.g.*, oxidation of omega-3 fatty acids)[12-14]. Given their high surface-to-volume ratio[15], microemulsions have also been used as nano-scale 'bioreactors' for the efficient synthesis of numerous compounds, including lysophospholipids, free fatty acids[16] and 2-furfurylthiol, a key product associated with Maillard browning.[17]

The preparation of microemulsions requires the delicate admixture of an aqueous phase, an oil phase and a surfactant phase. Often, co-surfactants are added to aid in

formation and to extend the range of compositions leading to microemulsion formation for a given set of ingredients.

1.2 Microemulsions for salt encapsulation

Developing microemulsions for food applications poses a challenge. The solubilisation of long-chain triglycerides, *e.g*., soybean oil, is more difficult to achieve than the solubilisation of short- or medium-chain triglycerides. Microemulsions that do solubilise long-chain triglycerides are usually formulated using short-chain alcohols (such as C3–C5); making them inappropriate for use in foods.[12] Easily available and low price vegetable oil (soybean oil) was used as the organic phase in microemulsion formulation.

The aim of the thesis was to develop food-grade matrices that can easily be incorporated within processed food products. Sodium chloride was used as a hydrophilic marker to model release study.

1.3 Thesis objectives

The overall objective of this thesis was to develop stable salt-containing w/o microemulsions for possible release applications. The specific objectives were:

1. To prepare and optimise food-grade w/o microemulsions using combinations of food-grade surfactants, organic and aqueous phases and to characterise the resulting microemulsions along two dilution lines within the monophasic region in ternary phase diagrams;
2. To incorporate a model hydrophilic guest molecule (sodium chloride) into the water domains of oil-continuous microemulsions and to characterise these salt-

containing microemulsions along the two dilution lines within the monophasic region in the developed ternary phase diagrams;

3. To test the efficiency of selected salt-containing microemulsion compositions for salt-release using conductivity and establish the mechanism of release.

1.4 Hypotheses

The following two hypotheses were tested:

Stable food-grade w/o microemulsions can be formulated using soybean oil, a combination of non-ionic surfactants (polysorbate 80 and glycerol monooleate) and deionised water.

The formulated microemulsions are capable of being utilised as carriers for the water-soluble compounds such as sodium chloride.

1.5 Thesis organisation

The thesis is divided into seven chapters as follows:

Chapter 1: Overall summary and thesis background, objectives and hypotheses.

Chapter 2: Literature review, with background information on microemulsions and their use as delivery systems and applications.

Chapter 3: Material and methods.

Chapter 4: Results and discussion pertaining to the formulation and optimisation of food-grade microemulsions with and without sodium chloride as well as the characterisation of microemulsion structure along two dilution lines.

Chapter 5: Results and discussion on the release of the sodium chloride from microemulsions.

Chapter 6: Conclusions.

Chapter 7: Possible future studies.

Chapter 2

Literature Review

The literature review is divided in two sections. The first serves as an introduction to microemulsions, their classification/phase behaviour and composition. The second part highlights the use of w/o microemulsions as carriers for hydrophilic molecules (*e.g.*, sodium chloride).

Microemulsions were first recognized in the 1940s with the pioneering work of Hoar and Schulman[18-20], who referred to them as oleopathic hydro-micelles. In 1959, the term microemulsion was first coined by Schulman *et al.*[19, 21] Macroscopically, microemulsions are homogeneous mixtures of water, oil, surfactant and/or co-surfactants. However, on a microscopic level, water-rich and oil-rich domains exist within these mixtures, which are separated from the continuous phase by an interfacial amphiphilic film.[22]

2.1 Microemulsions, emulsions and nano-emulsions

Microemulsions are self-assembled nano-scale entities.[3, 12, 19, 20, 23, 24] On the basis of size alone, however, the term microemulsion is a misnomer, and has led to confusion. *Vis-à-vis* standard emulsions, which normally consist of dispersed droplet sizes in the low micron range (1- 20 μm), microemulsion dispersed domains are normally well-below 100 nm in diameter. More recently, nano-emulsions have gained attention as vehicles for controlled drug release and delivery. Despite the fact that these also consist of nano-scale droplets (typically 20-200 nm), they lack the thermodynamic stability of microemulsions.[12]

2.2 Winsor classification/phase behaviour

In 1947, Winsor developed an approach to classify equilibrium systems consisting of mixed water, oil and surfactants (Fig. 2.1)[25], which is still used to this day. The four categories are:

A) Type I: o/w microemulsion in equilibrium with excess oil;

B) Type II: w/o microemulsion in equilibrium with excess water;

C) Type III: bicontinuous structure in equilibrium with excess oil and water;

D) Type IV: single phase microemulsion (o/w or w/o) with no excess oil or water.

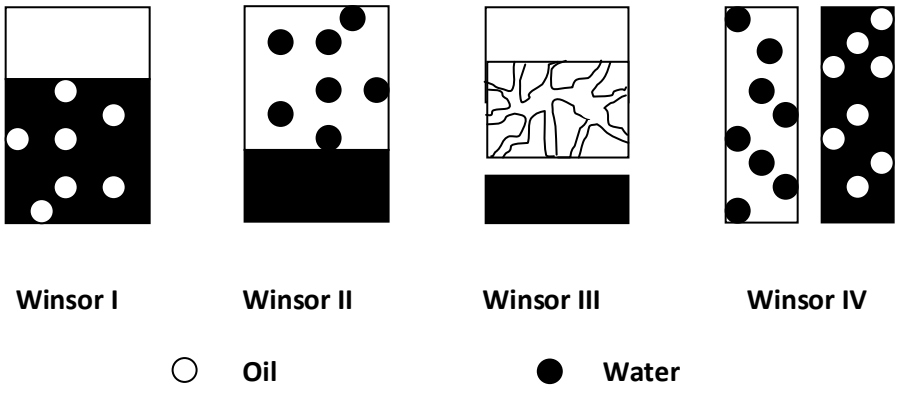


Figure 2.1: Mixed water-oil-surfactant structures as defined by Winsor (adapted from refs.[23-25]).

Winsor type IV systems are usually used as drug delivery systems.[26]

2.3 What makes microemulsions thermodynamically stable and emulsions unstable?

Every molecular assembly theoretically organizes itself such that the Gibbs free energy (ΔG) of the system is minimised.[27] For any process to occur spontaneously, ΔG

of the system should be negative.[28] ΔG depends on interfacial energy ($\gamma \Delta A$) and the change in configurational entropy of the system upon droplet formation ($T\Delta S$):

$$\Delta G = \gamma \Delta A - T\Delta S \quad 2.1$$

where γ is the interfacial tension, ΔA is the change in interfacial area on emulsification of the droplets, T is the temperature and ΔS is the configurational entropy of the system. Configurational entropy is the entropy of mixing of microemulsions corresponding to the number of arrangements that can be generated by the water and oil domains while conserving their respective volumes and interfacial area.[29] Upon microemulsion formation, ΔA is very large owing to the formation of large number of very small (nano-sized) droplets. Initially, it was believed that a negative γ was required for microemulsion formation.[26] However, it is now recognised that even though γ is small, it is always positive.[26] Interfacial tensions as low as 10^{-4} mN/m have been achieved.[30] According to Kumar and Mittal[31], microemulsions will form when the oil-water interfacial tension is below 10^{-2} mN/m. The component that dominates is the entropy of formation that arises from the dispersion of one phase into another in the form of a large number of tiny droplets.[26] Hence, a large reduction in interfacial tension along with favourable entropic change results in a negative free energy for the system. As food-grade surfactants alone cannot normally lower γ to such low levels, co-surfactants such as alcohols are often used for further reduction.

The configurational entropy is much smaller than the interfacial energy in most food emulsions and thus can be neglected. For instance, a 10 vol % o/w emulsion containing droplets of 1 μm (with γ of 0.01N/m) has an interfacial energy of ~ 3 kJ/m^3

while the configuration entropy term ($T\Delta S$) is ~ 3 x 10^{-7} kJ/ m^3.[32] Hence the expression for ΔG is usually reduced to:

$$\Delta G = \gamma \Delta A \qquad 2.2$$

Since the change in interfacial energy ($\gamma \Delta A$) is always positive in an emulsion due to the increase in contact area between the oil and water phases after emulsification[32], ΔG in Equation 2.2 will be positive and hence an input of energy will be required to form stable emulsions.

2.4 Methods of microemulsion formation

There are numerous ways to generate microemulsions, including the low-energy and phase inversion methods.[12] With the former, microemulsions can be formed *via*: 1) dilution of an oil-surfactant mixture with water; 2) dilution of a water-surfactant mixture with oil; or 3) mixing all components at once. In some systems, the order of ingredient addition may determine whether a microemulsion forms or not. For instance, Flanagan *et al.*[33] established that the order of ingredient addition was crucial in systems with soybean oil, ethoxylated mono- and di-glycerides as surfactants and a mixture of sucrose and ethanol as the aqueous phase. Transparent microemulsions resulted from dilution of the oil-surfactant mixtures with water along several regions in the pseudo-ternary phase diagram. However, upon dilution of aqueous-surfactant mixtures with oil, microemulsions only resulted when the aqueous phase:surfactant ratio was 3:2. The authors did not explain the reason for such behaviour and only mentioned the existence of a kinetic energy barrier that inhibited microemulsion formation.

Phase reversal can also occur at the Phase Inversion Temperature (PIT), *i.e.*, the temperature range in which an o/w microemulsion inverts to a w/o type or *vice versa*.[12, 19, 34, 35] Microemulsions formulated using non-ionic surfactants, especially those based on polyoxyethylene are very susceptible to temperature since surfactant solubility (in oil or water) strongly depends on temperature. With increasing temperature, the polyoxyethylene group becomes dehydrated, altering the critical packing parameter (section 2.8.2), which results in phase inversion.[26] Many surfactants do not readily undergo phase inversion with this method as they form a rigid lamellar phase at the PIT rather than phase-inverting.[5] For ionic surfactants, increasing temperatures increase the electrostatic repulsion between the surfactant headgroups thus causing reversal of film curvature (from w/o to o/w microemulsion). Hence the effect of temperature is opposite to the effect seen with non-ionic surfactants.[36]

Changes in composition can also result in phase inversion, which is known as the phase inversion composition (PIC) method. For example, upon dilution with water, a w/o microemulsion can invert to an o/w emulsion. As discussed later, this was the method by which the developed w/o microemulsions transformed into o/w emulsions during the NaCl release study.

2.5 Polydispersity

Polydispersity is a measure of the uniformity of a droplet size distribution and typically varies from 0.0 to 1.0 (unitless)[37], where values of 0.000 to 0.02 indicate a monodisperse or nearly monodisperse distribution, values of 0.02 to 0.08 are common for narrowly-distributed droplet sizes and values higher than 0.08 indicate broad

distributions. Despite microemulsions being polydisperse, they are frequently interpreted as being monodisperse to simplify analysis.[23] Ideally, microemulsions should be monodisperse, with a narrow size distribution centred around a mean value well below 100 nm. An increase in breadth usually implies reduced stability, and is often observed when the composition of a microemulsion system is altered, *e.g.*, with the addition of a bioactive compound to the microemulsion formulation or with changes in environmental (pH, temperature, ionic strength, *etc.*) conditions.[23]

2.6 Composition

Most microemulsions consist of an organic phase (usually oil), an aqueous phase, surfactant and a co-surfactant. However, in some studies, co-surfactants can be eliminated by the judicious combination of high and low-HLB (hydrophilic lipophilic balance) surfactants that are structurally complementary.[3, 38] For instance, surfactants with bulkier head groups [*e.g.*, polysorbates or polyoxyethylene lauryl ether[39]] will tend to form o/w microemulsions whereas surfactants with bulkier tails [*e.g.*, AOT (sodium bis(2-ethylhexyl) sulfosuccinate)] will tend towards w/o microemulsion formation.[15] Thus, combining such surfactants can promote a wide range of compositions leading to microemulsion formation.

2.6.1 Organic phase

This phase usually consists of a hydrophobic substance mixed with short-chain alcohols. From a food industry perspective, an ideal candidate for the formation of microemulsions is vegetable oil (due to its low cost and abundance). However, the most common organic phase used in microemulsions is mineral oil, which is a mixture of aliphatic chains with chain lengths normally varying from 8 to 30 carbons[40, 41]). Mineral

oil has been used in microemulsions as it is effective at generating well-defined dispersed phases.[15]

Many food-based oils including mineral, soy, corn, cottonseed and sunflower have been used to generate microemulsions, although not necessarily for food applications.[15, 33, 40-42] Long-chain triglycerides (*e.g.*, with 18 carbons) are difficult to solubilise given their high molecular weight which limits their flexibility at the interface and restricts film penetration and formation.[15] These have been often substituted by medium-chain triglycerides (*e.g.*, tricaprylin), where the smaller fatty acid chain lengths permit greater flexibility at the interface.[38-43] Also, medium-chain triglycerides have been used to enhance absorption and bioavailability of water-soluble compounds such as calcein and peptides.[44-46]

Soybean oil was used as the continuous phase in the microemulsions. It is an abundant, low-cost vegetable oil that consist of ~80 % unsaturated fatty acids (Table 2.1).[47]

Table 2.1: Key fatty acids found in soybean oil

Fatty acid	**% Composition**
1) Palmitic acid (C16:0)	7 - 14%
2) Oleic acid (C18:1)	19 - 30 %
3) Linoleic acid (C18:2)	44 - 62 %
4) Alpha-linolenic acid (C18:3)	4 - 11%

2.6.2 Aqueous phase

Water is an essential component of microemulsions. In o/w microemulsions, water acts as the continuous phase. Water's immiscibility with hydrocarbon oils combined with its ability to hydrogen bond with surfactants results in the formation of oil droplets when it is present as the major component (although this depends on the properties of the surfactant). Formation of microemulsions usually requires the presence of a water-soluble co-surfactant (*e.g.*, short chain polyols such as propylene glycol), as discussed later.

2.6.3 Surfactants

Surfactants are surface-active compounds that consist of a hydrophilic headgroup attached to a hydrophobic tail (usually a long alkyl chain). They have a high affinity for water or oil depending on the dominant moiety.[32, 48] When present in sufficiently high concentrations, surfactants form a monolayer at the interface between the oil and water, with the hydrophobic tails of the surfactant oriented towards the oil phase and the hydrophilic head groups towards the aqueous phase. There are four categories of surfactants available: ionic, non-ionic, zwitterionic and cationic.

2.6.3.1 Ionic surfactants

Ionic surfactants have been used to formulate microemulsions, however their toxicity at concentrations normally encountered in microemulsions limits their application in foods.[33, 49]

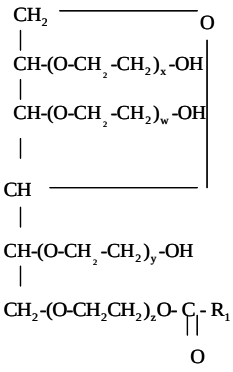
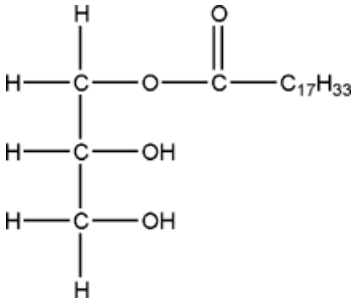
2.6.3.2 Non-ionic surfactants

Surfactants from this group are commonly used to formulate microemulsions, due to their low toxicity, lack of irritation and capacity to easily form microemulsions.[50] Examples include sugar ester surfactants (*e.g.*, sorbitan monooleate), polyoxyethylene ether surfactants such as Brij 96 (used in the detergent industry)[15, 40] and ethoxylated sorbitan esters (polysorbates) such as Tween 60 (polyoxyethylene sorbitan monostearate) and Tween 80 (polyoxyethylene sorbitan monooleate)[15, 33, 40]. Mixtures of non-ionic surfactants are far more effective than the other class of surfactants for microemulsion formation as they potentially eliminate the need for a co-surfactant.[19]

The water-soluble surfactant polysorbate 80 was used in this study. The polysorbate family contains a sorbitan ring and four polyoxyethylene head groups, one of which is esterified with fatty acids.[49, 51, 52] The fatty acid chain length differentiates the various members of this family. The fatty acid found in polysorbate 80 is oleic acid (C18:1). Glycerol monooleate (GMO) was used as the oil-soluble surfactant and, along with polysorbate 80, was used to aid in the formation of the w/o microemulsions. This unique combination of a hydrophilic and hydrophobic surfactant enabled the elimination of alcohol (co-surfactant) from the system. A huge difference in HLBs of the used surfactants leads to a greater increase in the solubilisation of water or oil in the microemulsions.[53] In the system being studied, glycerol monooleate had an HLB of 3.8 while polysorbate 80 had an HLB of 15.

The chemical name, formula and HLB of polysorbate 80 and glycerol monooleate are shown in Table 2.2.

Table 2.2: Formula, chemical structure and HLB values of the polysorbate 80 and glycerol monooleate used in this study[54-56]

Surfactant	**Chemical name**	**Formula**	**HLB**
Polysorbate 80	Polyoxyethylene 20 sorbitan monooleate	 R_1=Oleic acid x+y+z+w=20	15
Glycerol monooleate	1-Glycerol oleate		3.8

2.6.3.3 Zwitterionic surfactants

These surfactants can be anionic, cationic or non-ionic, depending on the acidity of the solution and the presence of other components.[57] Phospholipids are perhaps the most common zwitterionic surfactants used in microemulsions. However, they have a fairly high critical packing parameter, making their incorporation into microemulsions difficult.[58] If used, co-surfactants are also required to increase the fluidity of the interface.[15, 59]

2.6.3.4 Cationic surfactants

Presently, no cationic surfactants (*e.g.*, cetyl trimethylammonium bromide) are used in the manufacture of food-grade microemulsions. This class of surfactants is mostly used in industrial chemistry applications.[60, 61]

2.6.4 Co-surfactants

A co-surfactant [*e.g.*, propylene glycol or ethanol] can play many roles in microemulsion formation: a) by reducing the interfacial tension[50, 51, 62-65]; b) by increasing the flexibility and fluidity of the interface by positioning itself between the surfactant tails[62, 63, 66] which alters the solvent properties of both the dispersed and continuous microemulsion phases; c) by lowering overall viscosity, which restricts formation of more rigid structures such as gels and liquid crystals[62, 67-72]; d) by being often soluble in both organic and aqueous phases, co-surfactants help solubilise poorly-soluble compounds (*e.g.*, peptides, vitamins, *etc.*)[40, 63, 64, 73, 74]; e) use of co-surfactants can result in fully dilutable microemulsions[53, 64, 68, 75], and; f) they aid in 'connecting'

the w/o and o/w regions *via* a bicontinuous region[72, 76], hence the transition from one regime to another occurs without phase separation.

The inherent properties of alcohol co-surfactants will impact on usage. In particular, alcohol chain length has a significant effect on interfacial disorder, with shorter-chained alcohols increasing interfacial disorder (flexibility).[77, 78] However, toxicity concerns limit the use of effective co-surfactants such as 1-butanol, 2-butanol and *tert*-butanol.[65] The most commonly used food-safe co-surfactant is ethanol, though its use in foods limits product marketing due to regulatory requirements, these differing from country to country.

Though commonly used to aid in microemulsion formation, no co-surfactant was used in the present study since the use of two surfactants (polysorbate 80 and glycerol monooleate) were sufficient to increase the flexibility of the interface and decrease the interfacial tension to a desired low value to generate stable w/o microemulsions.

2.7 Factors affecting phase behaviour

Microemulsion phase behaviour and microstructure depend on two interfacial parameters, namely the spontaneous film curvature (the optimal curvature that the surfactant film attains) and the elasticity (bending modulus) of the surfactant film.[15, 65, 73] The free energy per unit area for curvature deformation is[22, 73, 79]:

$$f = 2k(H - H_0)^2 + \bar{k}K \qquad 2.3$$

where $H = 1/2(c_1 + c_2)$ is the mean film curvature, in which c_1 and c_2 are the principal curvatures of a regular surface at each point, K is the Gaussian curvature, H_0 is the

spontaneous film curvature, and k and $\bar{k}$ describe the elastic properties of the surfactant film (bending rigidity and saddle splay rigidity respectively), which refers to the deformation of the surfactant film from the preferred mean curvature.[22, 30] The spontaneous curvature is the curvature formed by the surfactant film when the system consists of equal amounts of oil and water where there is no constraint on the film and it can adopt a state of lowest free energy. When one of the two phases dominates, the curvature deviates from the spontaneous curvature. Every point on a surface has two radii of curvature (R_1 and R_2) and their corresponding principal curvatures are $c_1 = 1/R_1$ and $c_2 = 1/R_2$. The mean and Gaussian curvatures account for the bending of surfaces.[80] For $H_0 > 0$, the surfactant film is convex toward water (o/w microemulsion) whereas with H_0 values near zero, bicontinuous microemulsions or lamellar liquid crystalline phases are formed.[22, 73, 30] If the surfactant film is convex toward the oil phase, $H_0 < 0$, and a w/o microemulsion will form. Values of H_0 may be affected by the nature of the surfactant and the composition of the polar and apolar phases.[15, 73, 79] Co-surfactants are also used as 'tuning parameters' to modify H_0 and to increase the flexibility of the surfactant film. These compounds may affect the spontaneous curvature of the interface by changing the polarity of the polar and apolar phases.

2.8 Parameters that modify microemulsion structure

In developing controlled release matrices, the solubilisation capacity of microemulsions should be optimized, which depends on various factors such as the critical micelle concentration, the surfactant's HLB value, the critical packing parameter and molecular compatibility between the oil, surfactant and co-surfactant.[39, 76, 81]

2.8.1 Critical micelle concentration (CMC)

Surfactants at low concentrations in solution are predominantly dispersed as monomers. Above the CMC, they spontaneously associate into (reverse) micelles, either through strong interaction of their hydrophobic tails (resulting in reverse micelles, w/o) or by hydrophilic interaction of the polar headgroups (micelles, o/w). Most micellar solutions consist of particles in the 2-5 nm range. Surfactant monomers have the highest surface activity, yet this activity is greatly diminished once micelles are formed, given that either the tails or heads are buried and thus unavailable to participate in surface/interfacial tension reduction.[12, 48, 82]

2.8.2 Critical packing parameter (CPP)

The CPP is a measure of the preferred geometry adopted by a surfactant in solution:

$$p = v / la_o \qquad 2.4$$

where p is the packing parameter, v and l are the volume and length of the hydrophobic tail and a is the optimal headgroup area. In solution, when the hydrophilic headgroup volume is greater than that of its hydrophobic tail, o/w microemulsions will form. When the headgroup has a smaller volume than its hydrophobic tail, w/o microemulsions are preferentially formed. Bicontinuous structures will prevail when these volumes are similar, resulting in $p \approx 1$[83, 84] (Fig. 2.2). When surfactants have a great headgroup volume, it is difficult for these to be constrained within the confined core of an o/w

microemulsion droplet. Likewise, for surfactants with large hydrophobic tails, these tails can be widely spread in the oil, thus facilitating formation of a w/o microemulsion.[85]

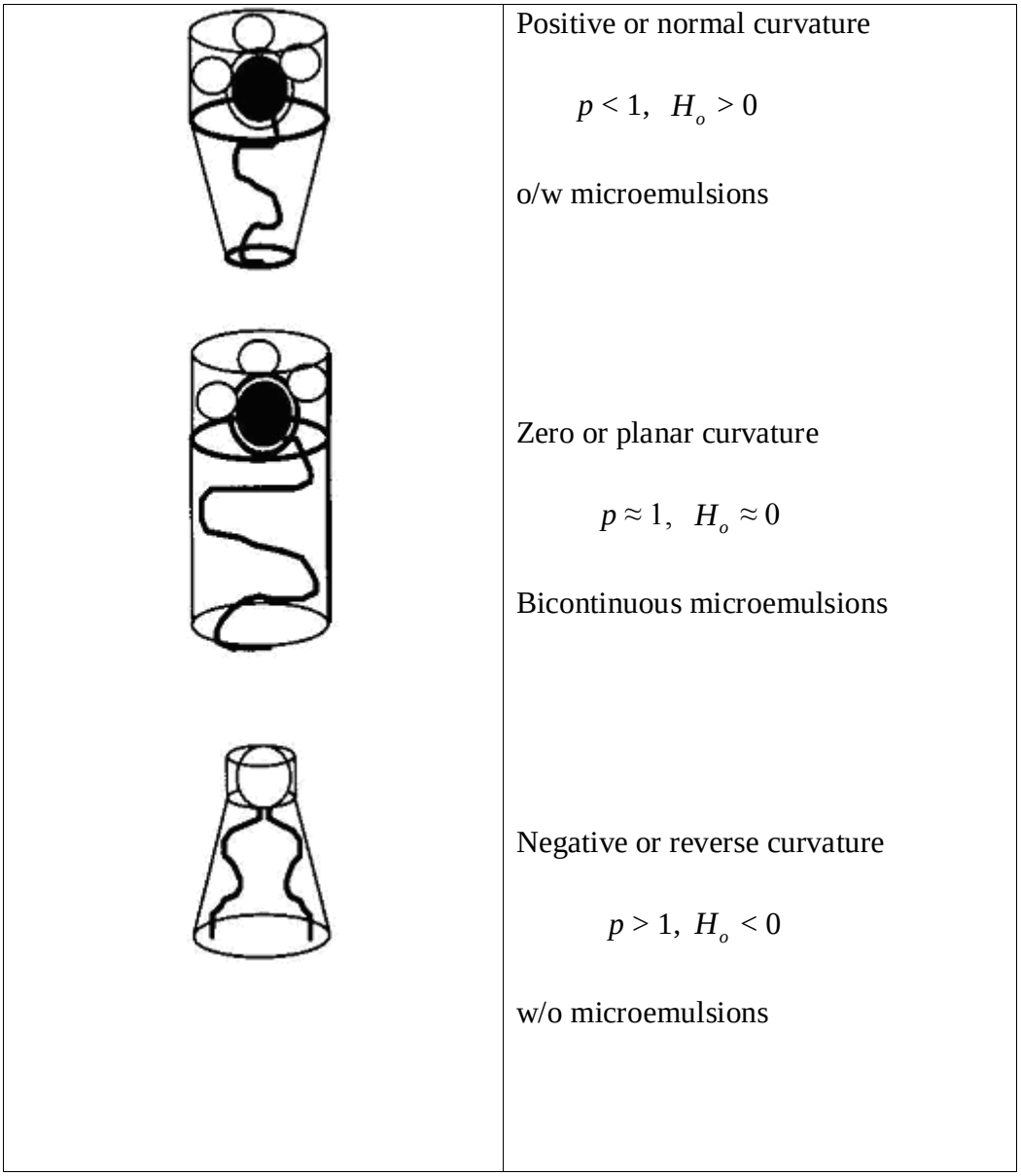


Figure 2.2: Formation of w/o, bicontinuous and o/w microemulsions, and their relationship to p and H_0 (adapted from refs.[26, 32]).

2.8.3 Hydrophile-lipophile balance (HLB)

The HLB of a surfactant typically ranges from 1-20 and is an indication of the relative affinity of a surfactant molecule for the oil or aqueous phase. A widely used semi-empirical method of calculating the HLB value is:

$$\text{HLB} = 7 + \sum(\text{hydrophilic group numbers}) - \sum(\text{hydrophobic group numbers}) \qquad 2.5$$

Each surfactant has a specific HLB value, which depends on its chemical structure, with a higher HLB number indicating a higher ratio of hydrophilic to hydrophobic groups and *vice-versa*.[22, 32] Surfactants with a low HLB values (ca. 3-8) promote w/o microemulsion formation whereas o/w microemulsions will be formed by surfactants with higher HLB values (ca. 8-18).

2.8.4 Ingredient Compatibility

There is a strong relationship between microemulsion monophasic total area, molecular complementarity and the interface's flexibility. Compatibility between the alkyl chains of the surfactant and oil will influence microemulsion formation by strongly affecting the bending modulus of the interfacial layer[48, 64], with increased molecular compatibility reducing the bending modulus of the interfacial layer.[64]

An increase in the alkyl chain length of the compound in the oil will typically decrease its solubility in water[27], and consequently reduce the total area of the microemulsion monophasic region. Using the BSO (Bansal-Shah-O'Connell) equation, the maximum solubilisation for ionic microemulsions occurs when[62, 64, 86-88]:

$$l_o + l_a = l_s \qquad 2.6$$

Where:

l_o = oil alkyl chain length

l_a = alcohol alkyl chain length

l_s = surfactant alkyl chain length

However, this equation should be used with caution. Shiao *et al.*[87] reported that the BSO equation failed if the microemulsion contained components with branched alkyl chains, as these decreased the packing order of the hydrocarbons in the interface compared to linear alkyl chains. Similarly, Li *et al.*[38] found that the validity of this equation was limited to hydrophobic molecules with a linear alkyl chain structure. Garti *et al.*[86-89] showed that the BSO equation was valid for non-ionic surfactants in five-component microemulsions only when the surfactant (alcohol) exhibited limited solubility in both the aqueous and oil phases.

2.9 Delivery systems

In microemulsions, the location of an incorporated bioactive compound, whether in the oil, water or interface, will affect its release pattern.[90] As delivery systems, microemulsions offer several advantages over emulsions, namely,[26, 91-98]:

a) The possibility of tailored absorption (rate and predictability) and enhanced bioavailability of poorly-soluble bioactive compounds;

b) Higher solubilisation capacity and/or entrapment rate of bioactives;

c) Aqueous dosage form for hydrophobic compounds, given the much smaller domain size;

d) Better protection of labile compounds (*e.g.*, against oxidation) and flavours/aromas;

e) Improved transdermal penetration compared to other matrices, given the much smaller domain size;

f) Straightforward preparation and scaling up for industrial production, though this also applies to emulsions.

The poor aqueous solubility of most drugs and nutraceuticals can result in low absorption after *in vivo* administration, thus limiting biological efficacy. Enhancement in biological efficacy and lowering of toxicity may be achieved through judicious encapsulation and delivery of the bioactive compound in aqueous- or oil-based microemulsion delivery systems.[91]

2.10 Microemulsions as delivery systems for nutraceuticals and pharmaceuticals

Microemulsions have the potential to protect labile compounds by encapsulating them in the dispersed phase domains. The dispersal of a drug as a solution in nano-sized droplets increases its dissolution rate and possibly its bioavailability. The preferred sites of incorporation of a hydrophobic, water-insoluble moiety into an o/w microemulsion will be the dispersed oil phase and/or the tail region of the surfactant. Conversely, a hydrophilic material will most likely be incorporated into the dispersed aqueous phase of a w/o microemulsion. As delivery systems, o/w systems are preferred over w/o systems

as the droplet structure is often retained upon dilution within the body. In contrast, w/o microemulsions are destabilised to a greater extent upon dilution with an aqueous phase. This dilution can result in either phase separation or phase inversion. However, w/o microemulsions are very promising for the delivery of labile drugs since many of the new drugs/nutraceuticals are hydrophilic (peptides or oligonucleotides). Encapsulation of these hydrophilic materials in the aqueous droplets of w/o microemulsion offers them protection from enzymatic degradation.[26]

Overall, microemulsions with desirable characteristics can be generated by modifying a number of parameters (spontaneous curvature, bending rigidity etc.) and the resulting systems can be used for the delivery of nutraceuticals.

Chapter 3

Experimental Methods

The following chapter introduces each of the experimental methods used to formulate and characterise the microemulsions. Specific measurements associated with each experiment are addressed within the experimental sections of the subsequent chapters. Unless otherwise indicated, all experiments within this thesis were performed in triplicate, as were their respective measurements.

A number of oils and surfactants were investigated in order to maximise the monophasic region present within the ternary phase diagrams without the use of ethanol.

3.1 Microemulsion preparation

Soybean oil was purchased at a local grocery store and used without further treatment (acid value < 0.2). Polysorbate 80 (>95% purity) was purchased from ACROS (Ottawa, ON, Canada) and glycerol monooleate was obtained from Danisco (Dimodan® MO 90/D, 90% purity, New Century, KS, USA). Crystalline sodium chloride was obtained from Fischer Scientific (Nepean, ON, Canada). Deionised water was used for all experiments. All components were used without further purification.

Microemulsions were generated *via* dilution of the oil:surfactant mixture with the aqueous phase. Initially, stock solutions of soybean oil and molten GMO at a 3:1 weight ratio were prepared. These soybean oil and GMO mixtures were then combined with polysorbate 80 at pre-determined concentrations, followed by 30 min of mixing with a magnetic stirrer to achieve a visually homogeneous solution. These mixtures were kept in

Pyrex bottles (250 ml) sealed with screw caps. Microemulsions were formed by titrating the oil-surfactant phase with the aqueous phase (deionised water or saline solution) to the desired composition. All samples were prepared in screw-capped test tubes, vortexed for 30 s and allowed to equilibrate at 40-50˚C in a water bath for 24h before analysis. Equilibration was necessary to eliminate metastable states.[26] The different phases within the ternary phase diagrams were determined using cross polarisers and visual inspection. Dynamic light scattering (DLS) was used to examine all samples that remained transparent and homogeneous after vigorous vortexing. Compositions within the monophasic region of the ternary phase diagrams with mean particle sizes < 100nm were considered microemulsions.

Pseudo-ternary phase diagrams (section 3.2.1) with the four components (soybean oil, glycerol monooleate, polysorbate 80 and deionised water/saline) were constructed to study their phase behaviour. Phase transitions were examined visually by the appearance of cloudiness or sharply defined separated phases. The phase diagrams were constructed at 25 ˚C.

3.2 Characterisation techniques

As a first approximation, the presence of microemulsions was gauged using a ternary phase diagram (TPD) and visual observation. Further characterisation was performed using DLS, transmission electron microscopy (TEM), rheometer, confocal laser scanning microscopy (CLSM) and conductivity meter. Since the aim of the thesis was to develop food-grade systems, microemulsions with low amounts of surfactant were chosen for further study (dilution lines 28 and 37 with a fixed surfactant:oil ratio).

Dilution line (DL) 28 and 37 represented 20% polysorbate 80 and 80% oil phase (soybean oil:glycerol monooleate(3:1)) and 30% polysorbate 80 and 70% oil phase (soybean oil:glycerol monooleate(3:1)). Along these dilution lines (DL 28 and DL 37), compositions with 6% water/saline were selected for further characterisation. The composition of these microemulsions is given in Table 3.1.

Table 3.1: Microemulsion composition.

	Dilution line 28	
Polysorbate 80 (wt%)	Soybean oil:glycerol monooleate (3:1) (wt%)	Water/Saline (wt%)
18.8	75.2	6.0

	Dilution line 37	
Polysorbate 80 (wt%)	Soybean oil:glycerol monooleate (3:1) (wt%)	Water/Saline (wt%)
28.2	65.8	6.0

3.2.1 Ternary phase diagrams

TPDs are used to identify and characterise microemulsion regions (Fig. 3.1). The three components (an aqueous phase, an organic phase and a surfactant phase) that compose the system are located at the triangle's apexes, where their corresponding volume fraction is 100%. Moving away from that apex reduces its volume fraction and increases the volume fraction of one or both of the other two components. Thus, each point within the triangle represents a unique combination of the three components, unless

positioned along an axis where the mixture is binary. Varying the composition leads to the determination of phase boundaries, and hence phase behaviour. As with the surfactant phase, the aqueous and organic phases may also be mixtures of multiple compounds. Where four or more components are used, pseudo-ternary phase diagrams (PTPDs) are used with one or more apexes represent a defined mixture of two or more components.[12]

Within a putative TPD (Fig. 3.1), any point within the triangle represents a 3-component mixture of the apexes. For point 'X', the proportion of water (A) can be calculated by drawing a line through 'X', parallel to the surfactant-oil (BC) axis. As apex A in the TPD represents 100% water, point X shows a water concentration of 70%. By applying the same logic, the proportions of the surfactant (B) (10%) and oil phases (C) (20%) can be determined. Any point along the axes consists of two components only.

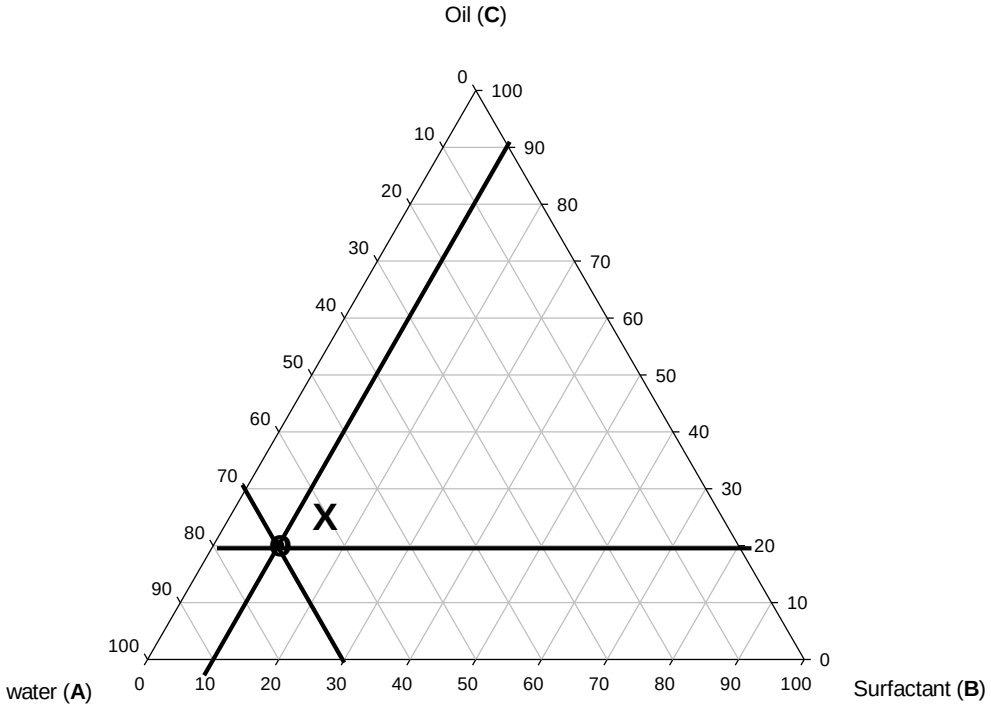


Figure 3.1: Putative ternary phase diagram.

3.2.2 Dynamic Light Scattering (DLS)

DLS is used to determine droplet size and polydispersity in microemulsion dispersed domains. When a coherent light beam interacts with colloidal particles undergoing Brownian motion, the particles scatter light. The original measurement is a time correlation function of the scattered intensity of the particles within the microemulsion. The decrease of this correlation function with time (lag time) is used to extract the diffusion coefficient of a particle or droplet in solution.[99] The measured diffusion coefficient can be used to calculate a hydrodynamic radius (R_h) of the droplet using the Stokes-Einstein equation:

$$R_h = kT / 6\pi\eta D \qquad 3.1$$

where k is the Boltzmann constant, T is the absolute temperature in Kelvins; η is the viscosity (cP) of the continuous phase, and D (cm^2/s) is the diffusion coefficient.[99, 100] For proper interpretation of results, it is very important to establish whether the R_h values are based on unimodal or multimodal distributions.

A particle size analyzer (Brookhaven 90 Plus with BI-MAS-multi angle particle sizing option, Brookhaven Instruments Corporation, New York, USA) was used to measure the sample mean particle diameter, particle size distribution and conductance as well as polydispersity. Particle sizing was key to optimising formulation and overall microemulsion characterisation. Measurements were carried out at a scattering angle of 90° at 25 °C.

A typical DLS system comprises six main components.[101] A laser (1) provides a light source to illuminate the sample contained in a cell (2). For dilute concentrations,

most of the laser passes through the sample, but some is scattered by the particles within the sample at all angles. A detector (3) is used to measure the scattered light at a 90° angle. The attenuator (4) reduces the scattered light intensity to within a certain range so that the detector does not become saturated. For samples that do not scatter much light, such as very small particles or samples at low concentration, the amount of scattered light can be increased. In this situation, the attenuator allows a higher laser intensity to pass through to the sample. For samples that scatter more light (*e.g.*, large particles or samples at higher concentration), the intensity of scattered light must be decreased. The scattered light signal is then passed to a digital processing board (5), which compares the scattering intensity at successive time intervals and then to a computer (6), where the software will analyze the data and derive size information[101] (Figure 3.2).

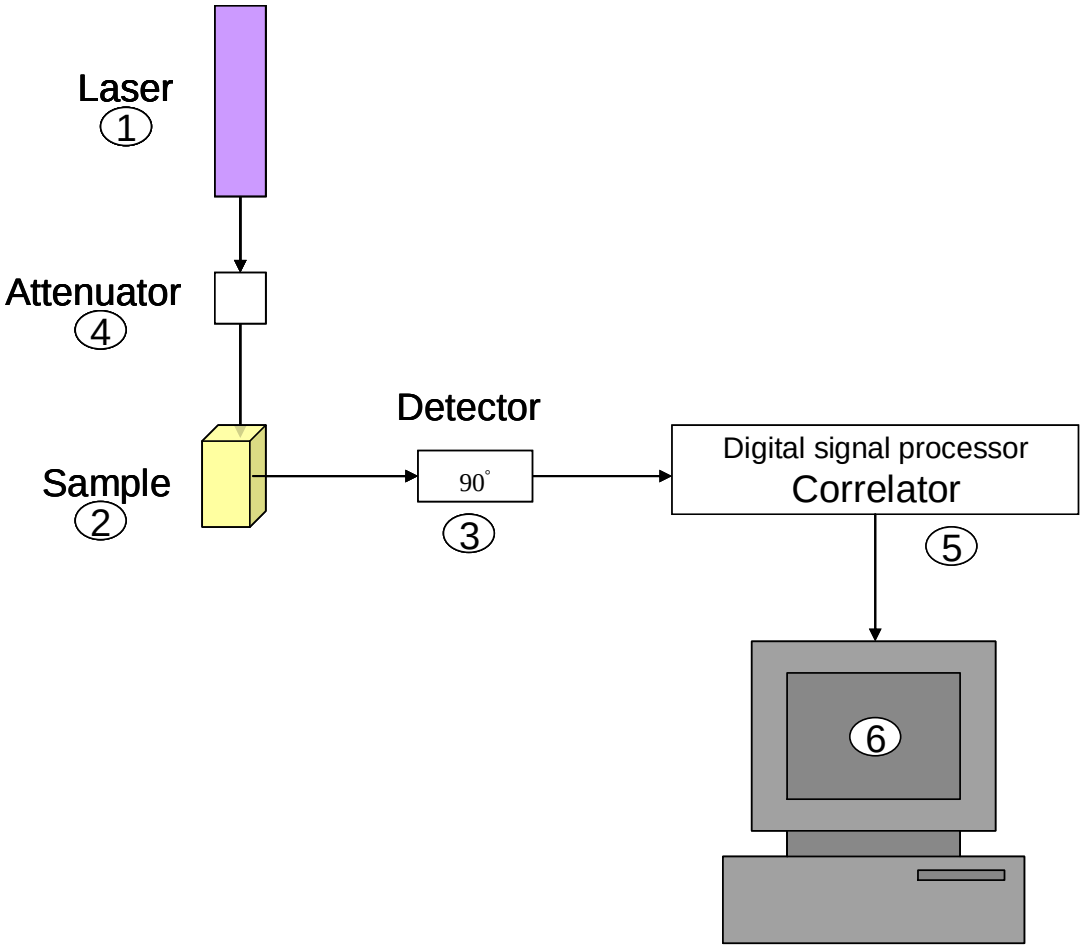


Figure 3.2: A schematic diagram of the dynamic light scattering used to measure particle size (adapted from reference[101]).

Conductance measurements, which were also obtained with the same instrument, using the zeta potential mode, are a measure of how easily electrical current flows through a sample. The SI unit of conductance is the siemens (S).

$$Conductance = 1/resistance \qquad 3.2$$

Conductance in water is usually much less than 1 S and is usually reported in microsiemens (μS) [102]. Conductance is strongly correlated with composition. As oil has

very poor or no conductance, it was possible to establish whether a microemulsion was oil or water-continuous.

3.2.3 Viscosity measurements

The dynamic viscosity of the microemulsions along dilution lines DL28 and DL37 was investigated at a shear rate of 30-125 s^{-1} with a rotational rheometer (MCR 301 rheo-microscope, Anton Paar Physica, Ostfildern, Germany), operating with a cone (CP25-1/TG) and plate (P-PTD 200/TG) geometry (25 mm diameter with 1° angle) and a gap of 0.048 mm. All measurements were performed at 25 ± 0.5°C.

3.2.4. Transmission electron microscopy (TEM)

In a TEM, an electron beam is focused and directed through a sample by several magnetic lenses, with part of the beam adsorbed or scattered by the sample while the remaining is transmitted. The transmitted electron beam is magnified and then projected onto a screen to generate an image of the specimen. Images are typically 100-500,000 times larger than the portion of the examined specimen. The fraction of electrons transmitted depends on the specimen's electron density. Substances of different density appear as regions of different intensity on the image.[32] Using the TEM for characterisation requires the use of samples that are 20 -100 nm thick so that they are transparent to electrons.

Microemulsion samples were prepared by floating 200-mesh formvar/carbon-coated copper electron microscopy grids (Canemco, Inc., Quebec, Canada), film side down, onto a 20 μL drop of microemulsion. Grids were applied to two successive 20 μL drops of 1% phosphotungstic acid (w/v) and incubated for 1 minute each. The excess

liquid was removed by wicking the grids with filter paper (Whatman no. 1) and the grids were allowed to air dry. Stained samples were loaded into a FEI Tecnai 20 TEM operating at 200 keV and equipped with a field emission gun and a 4k x 4k CCD camera.

3.2.5 Confocal laser scanning microscopy (CLSM)

CLSM is a light microscopy technique where the incident light source consists of a laser that can be tuned to set wavelengths and focussed by the objective lens onto a single point in the specimen plane. A subsequent X/Y raster scan of that plane produces an image. Through computer control, scans of a specific focal plane can be produced at set heights within the sample. Reflected and fluorescent light (if the samples autofluoresces or if such stains are used) return *via* the illumination path and are focussed at the confocal point located within a pinhole. Since the spot on the pinhole and the spot on the specimen are both located in the focal plane of the imaging lens, they are said to be confocal. These pinhole apertures limit the specimen focal plane to a confined volume of ~1 μm and blocks light from planes other than the focal plane. Relatively thick specimens (i.e., 100 μm) can thus be imaged by successively acquiring a series of thin sections (< 1 μm) along the optical (z) axis of the microscope.

A Zeiss Axioplan-2 microscope equipped with a LSM 510 confocal module and the associated software (version 3.2; Zeiss Instruments, Toronto, ON, Canada) was used together with a 40× Achroplan objective to look at the structural changes resulting from phase inversion of w/o microemulsion upon dilution with water. The fluorescent stain Rhodamine B at 0.001% (v/v) (Acros, NJ, USA) was added to the water phase of microemulsion. Fluorol Yellow 088 at 0.1% (w/w) (Sigma-Aldrich, St. Louis, USA) was

added to the water phase of the phase-inverted sample. Rhodamine B was excited at 543 nm and Fluorol Yellow 088 at 488 nm, with the emitted light passing through an LP 560 and LP 505 filter for detection respectively. Glass slides were used to load samples for microscopy.

3.2.6 Release study

Salt release from the microemulsions was evaluated *via* dialysis. A dialysis membrane (7Spectra/Por®, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) was used to separate the microemulsion sample from deionised water. The membrane was made of regenerated cellulose with a molecular weight cut-off of 1000 Daltons and had a diameter of 24 mm. The membrane was first hydrated in deionised water for 24 hours. Microemulsion samples (10 g) to be tested for sodium chloride release were loaded into the dialysis tubing and immersed into 400 g of deionised water. The water was stirred on a stir plate at 100 rpm. Microemulsion along DL-37 with 10% (w/w) saline was chosen to test the release of sodium chloride while microemulsion along DL-37 without salt served as the control. The change in the conductivity of the dialysis fluid was used to determine sodium chloride release from the microemulsions.

3.2.7 Conductivity

A Hanna conductivity meter HI 98188 (Hanna instruments, Laval, QC, Canada) with a conductivity range from 0.001 µS/cm to 400 mS/cm and a HI 76313 4-ring EC probe with built-in temperature sensor were used to measure NaCl release from the microemulsions. The data was collected using the associated software (HI 92000; Hanna instruments, Laval, QC, Canada). The conductivity meter was calibrated using a 4-point

calibration before and after every use. The electrical conductivity was measured every half an hour for four consecutive days.

3.2.8 Data analysis

Results are reported in all experiments as arithmetic means ± standard deviation. Statistical analysis was performed using a two-tailed Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Chapter 4

Results and discussion – microemulsion characterisation

4.1 Pseudo-ternary phase diagrams (PTPDs)

The three apices of the PTPD are represented by water/saline, the mixture of soybean oil and GMO (3:1 ratio) and polysorbate 80, respectively. Characterisation of the microemulsions was performed along two dilution lines (DLs) found within the PTPDs (DL28 and DL37) in which water or saline was added dropwise to the oil-surfactant mixture until the samples became cloudy. DL 28 represents 20% polysorbate 80 and 80% oil phase [soybean oil and glycerol monooleate (3:1)] while DL 37 stands for 30% polysorbate 80 and 70% oil phase [soybean oil and glycerol monooleate (3:1)]. The effect of aqueous phase concentration on the structure of the microemulsions was observed visually and using cross polarisers. The goal was to identify the w/o microemulsions region in the PTPD. We also wanted to determine whether replacement of water by saline had an effect on microemulsion area within the PTPD. Along both the dilution lines, a maximum of 6 wt% water or saline could be incorporated.

The pseudo-ternary phase diagrams of the soybean oil:glycerol monooleate (3:1)/polysorbate 80/water and soybean oil:glycerol monooleate (3:1)/polysorbate 80/1% (w/w) saline (Figs. 4.1 and 4.2, respectively) at 25˚C resulted in large isotropic microemulsion regions (region A). The remainder of the phase diagram (region B) consisted of turbid, liquid crystalline or multiphase regions, based on visual

identification. The pseudo-ternary phase diagrams for other saline solutions (0.5, 1.5, 2, 5 and 10% (w/w)) were only partially developed (along DL-28 and DL-37)). For these remaining compositions, 6 wt% saline could be incorporated along both dilution lines.

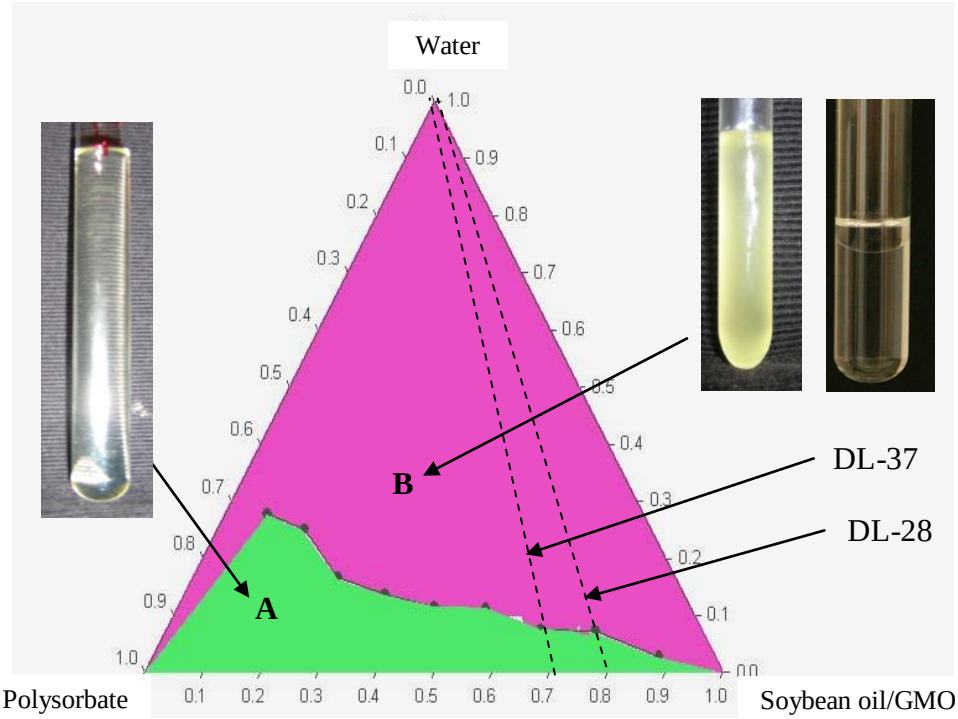


Figure 4.1: Pseudo-ternary phase diagram at 25 ˚C for the soybean oil:GMO(3:1)/polysorbate 80/water system, showing the one-phase microemulsion area (A) and multiphasic/liquid crystalline area (B).

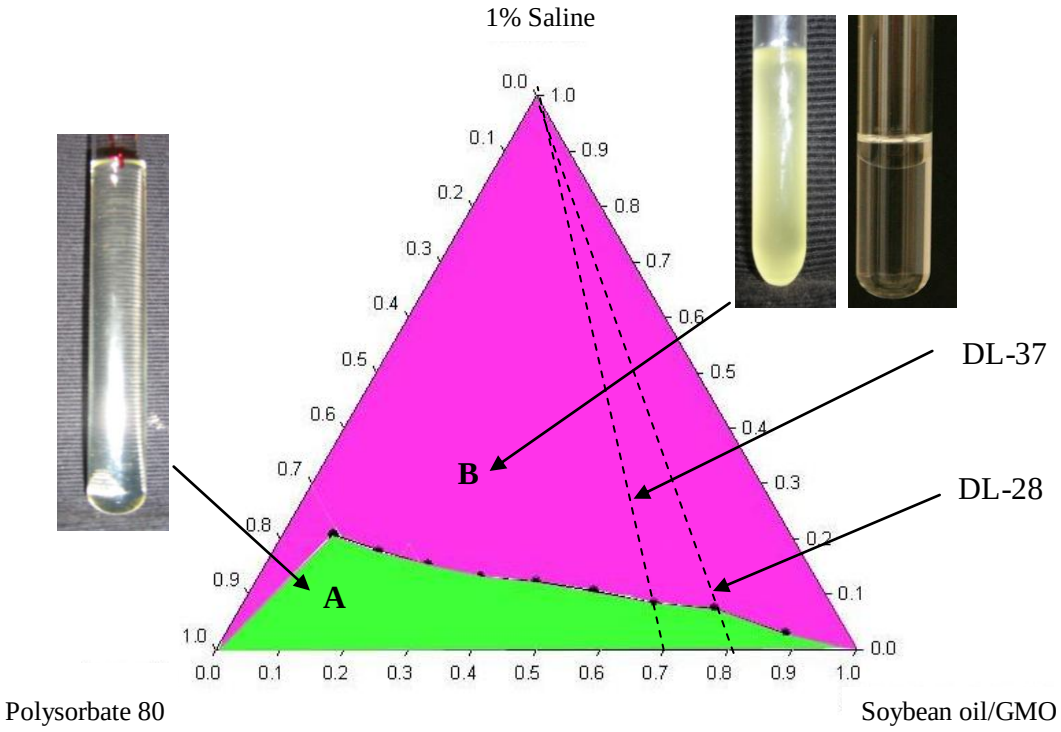


Figure 4.2: Pseudo-ternary phase diagram at 25 ˚C for the soybean oil:GMO(3:1)/ polysorbate 80/1% (w/w) saline system, showing the one phase microemulsion area (A) and multiphasic/liquid crystalline area (B).

Comparison of the microemulsion area (region A) in the two PTPDs showed that addition of saline solution (at 1% w/w NaCl) to the system reduced the microemulsion area from 25% to 23%, though the microemulsion region remained largely unchanged with only slight changes along the boundary.[38] Though the addition of an electrolyte can influence the phase behaviour of microemulsions[26], the addition of NaCl did not influence phase behaviour as the surfactants used were non-ionic. In systems with non-ionic surfactants, the influence of salt will depend on its impact on the degree of solvation of the surfactant's hydrophilic group. When salt enhances the structure of water (increases organization of water molecules through addition of ions), the introduction of

surfactant into the interface requires additional amount of work to overcome the added structural energy. This can lead to salting-out of the surfactant from the interface.[103] Surfactants like polysorbate 80, which exhibit high hydrogen bonding with water (due to the presence of polyoxyethylene chains) usually do not show significant salting-out effects unless salt concentration has reached to the level where it affects the water activity. There might be a significant difference in the monophasic area upon increasing the salt concentration form 1% w/w to 10% w/w. However the phase diagram for the latter was only partially developed and the change in monophasic area was not calculated.[103]

4.2 Droplet size and polydispersity

Microemulsions along both dilution lines (DL-28 and DL-37) were characterised for droplet size using DLS (Table 4.1). All average droplet sizes were < 20 nm, which is typical of microemulsions. The addition of a saline solution (irrespective of NaCl concentration) to the microemulsion system along both dilution lines had no impact on droplet size ($p>0.05$). Also, the difference in microemulsion droplet size for similar compositions along DL-28 and DL-37 was not significant ($p>0.05$).

Table 4.1: Microemulsion average droplet size (nm) (control, 0.5%, 1%, 1.5%, 2.5% and 10% saline (w/w)) along dilution lines 28 and 37 during 8 months of study. All data are means ± standard deviations (SD) for n = 3 replicates.

Microemulsion (%w/w)	Droplet size (nm) (DL-37)	Droplet size (nm) (DL-28)
Water (control)	16.1 ± 3.3	17.4 ± 0.6
0.5% Saline	10.1 ± 1.9	15.5 ± 3.5
1% Saline	13.6 ± 2.6	14.4 ± 0.5
1.5% Saline	10.8 ± 4.5	16.3 ± 3.5
2% Saline	12.8 ± 2.6	13.5 ± 2.5
5% Saline	10.5 ± 2.2	12.2 ±1.3
10% Saline	12.3 ± 4.3	20.1 ± 4.9

Polydipersity values (Table 4.2) along DL-28 and DL-37 for each microemulsion composition were not statistically significantly different ($p>0.05$). The difference in polydispersity values of microemulsions for similar composition along DL-28 and DL-37 was not significant ($p>0.05$).

Table 4.2: Polydispersity of microemulsion samples (control, 0.5%, 1%, 1.5%, 2.5% and 10% saline (w/w)) along dilution lines 28 and 37. All data are means ± standard deviations for n = 3 replicates.

Microemulsion (%w/w)	Polydispersity (DL-37)	Polydispersity (DL-28)
Water (control)	0.28 ± 0.02	0.21 ± 0.06
0.5% Saline	0.24 ± 0.04	0.11 ± 0.09
1% Saline	0.29 ± 0.02	0.25 ± 0.03
1.5% Saline	0.25 ± 0.04	0.23 ± 0.08
2% Saline	0.30 ±0.03	0.20 ± 0.07
5% Saline	0.27 ± 0.02	0.17 ± 0.08
10% Saline	0.30 ± 0.03	0.24 ± 0.04

4.3 Microemulsion stability

Microemulsion stability (with and without sodium chloride) was tested visually along both dilution lines (DL-28 and DL-37) at 25 ˚C, as well as through droplet size determination. The stability study was conducted for 8 months. The microemulsions were tested for any signs of destabilisation (phase separation, turbidity, *etc.*). The average droplet size of the microemulsions along both dilution lines was plotted as a function of storage time (Fig. 4.3 and Fig. 4.4).

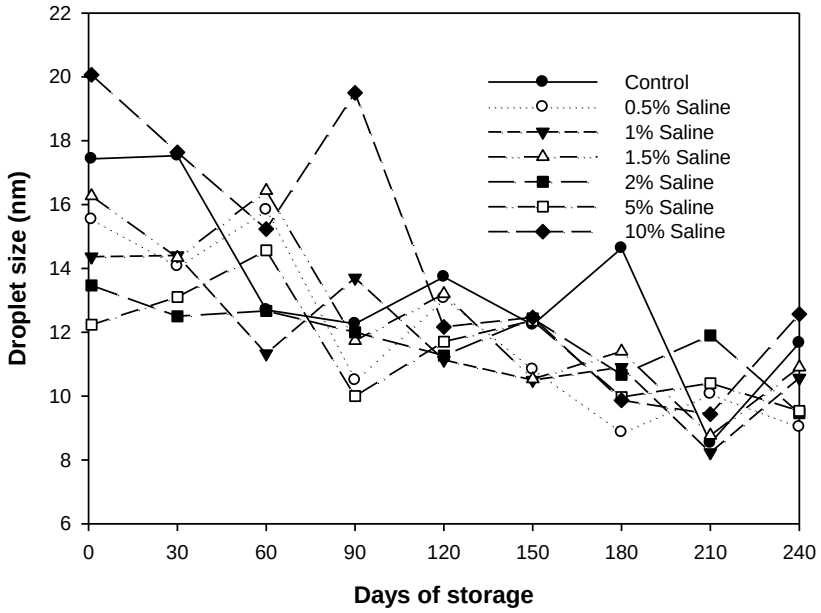


Figure 4.3: Microemulsion average droplet size as a function of storage time along DL-28. All data are means for n = 3 replicates (error bars have been removed for clarity).

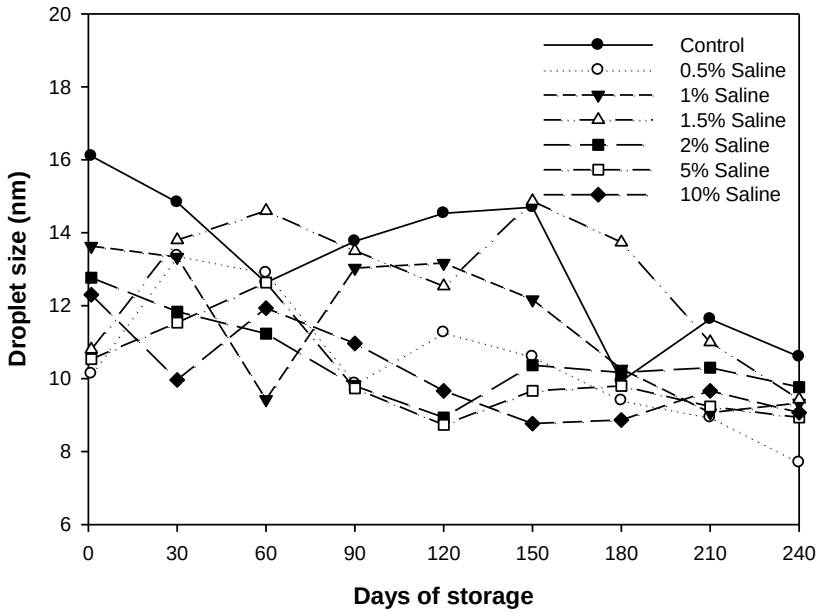


Figure 4.4: Microemulsion average droplet size as a function of storage time along DL-37. All data are means for n = 3 replicates (error bars have been removed for clarity).

All systems (with and without sodium chloride) remained transparent and monophasic after 8 months of storage with no signs of phase inversion. From the droplet size data along both dilution lines (Figs. 4.3 Fig. 4.4), the day 1 and 8 month droplet size for all compositions were not significantly different ($p > 0.05$). Similar results were found by Cilek *et al.*[104] during a 6-month study on lecithin-based microemulsions intended for oral delivery.

4.4 Conductance

Conductance measured along both dilution lines was very low, as is typical for w/o microemulsions when the water content is minimal (<20%) (Fig 4.5).[105, 106]

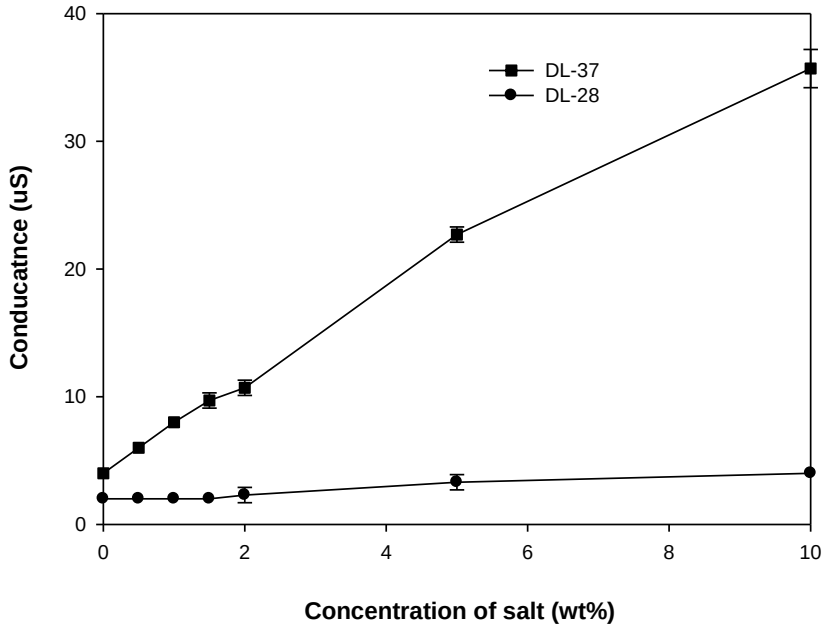


Figure 4.5: Conductance *vs.* concentration of salt (wt%) for w/o microemulsions along dilution lines 28 and 37. All data are mean ± standard deviations for n = 3 replicates.

Along DL-28, the conductance increased from 2 μS to 4 μS with the addition of 10 wt% salt into the water phase of the microemulsion. However, along DL-37 this

increase was from 4 μS to 36 μS. The higher conductance of the microemulsions along DL-37 was likely due to the greater water wt. fraction (~51%) compared to the water wt. fraction in microemulsions along DL-28 (~44%).

4.5 Viscosity

It is well documented in the literature that microemulsions exhibit Newtonian behaviour and that their viscosity is structure-dependent.[51, 72, 107, 108] The viscosity was studied as a function of shear rate for all measurements along both dilution lines and the result confirmed the Newtonian behaviour of microemulsions with and without sodium chloride (Fig. 4.6 and 4.7). The addition of any given concentration of saline had no influence on the Newtonian behaviour of the microemulsions (along both dilution lines) ($p > 0.05$).

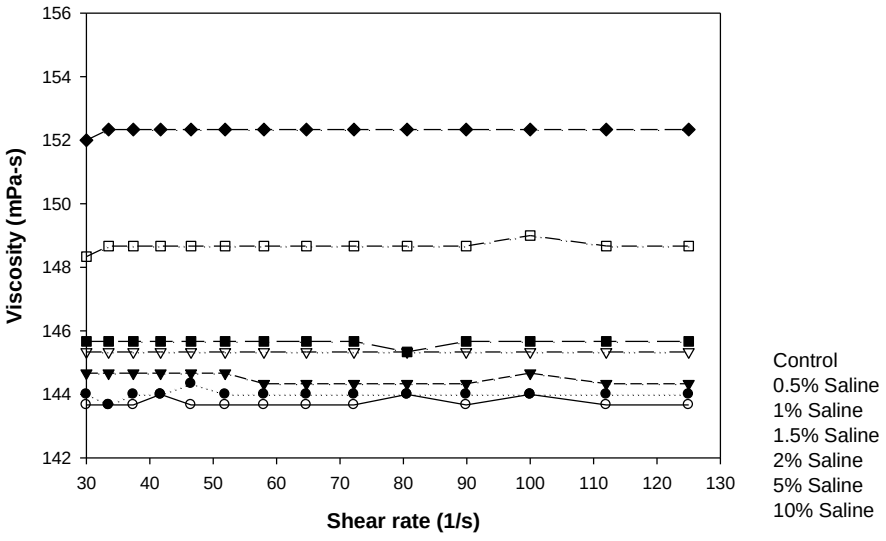


Figure 4.6: Viscosity *vs.* shear rate for w/o microemulsions along dilution line 28. All data are mean for n = 3 replicates (error bars have been removed for clarity).

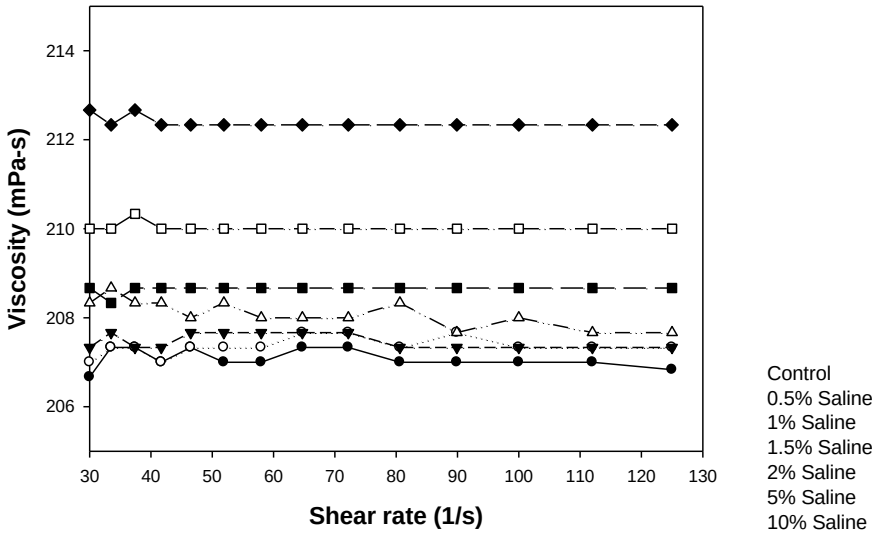


Figure 4.7: Viscosity *vs.* shear rate for w/o microemulsions along dilution line 37. All data are mean for n = 3 replicates (error bars have been removed for clarity).

To understand the change in viscosity upon addition of NaCl to microemulsions, the viscosity of the microemulsions (with and without salt) along DL-28 and DL-37 was plotted as a function of salt concentration in the microemulsion (Fig. 4.8 and Fig. 4.9).

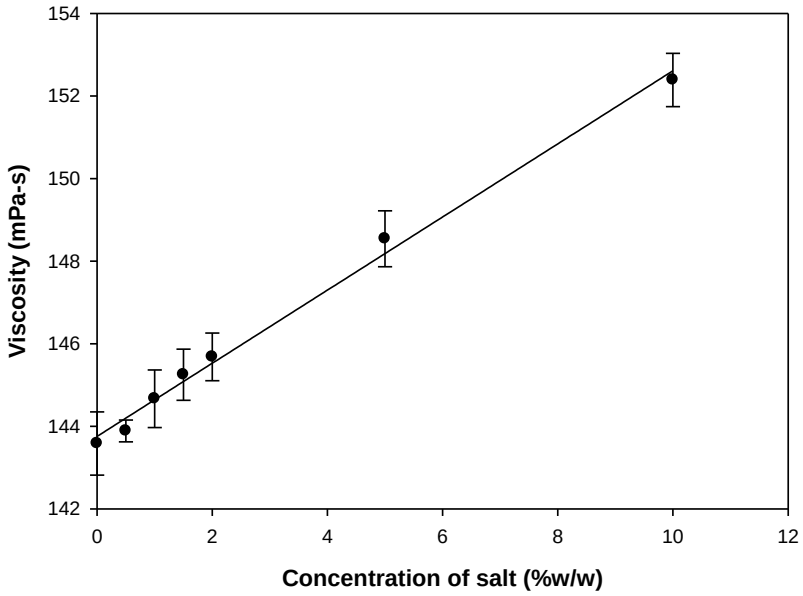


Figure 4.8: Viscosity of the microemulsions vs. concentration of salt (0, 0.5, 1, 1.5, 2, 5 and 10 (wt%)) in mPa·s along DL-28. The black solid line represents linearity with an R^2 of 0.994. All data are means ± standard deviations for n = 3 replicates.

It can be seen from Fig. 4.8 that microemulsion viscosity linearly increased with an increase in salt concentration. The linearity in viscosity with the concentration of NaCl along DL28 indicated that sodium chloride concentration had an impact on viscosity.

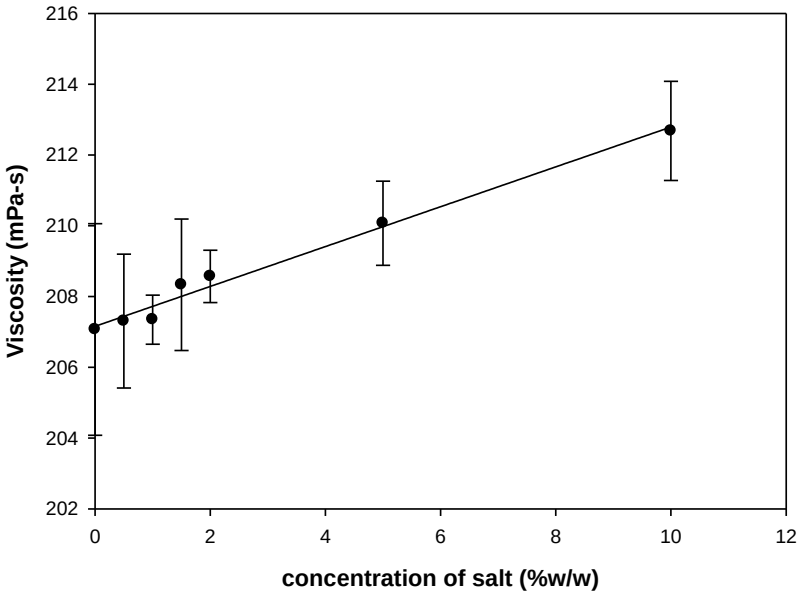


Figure 4.9: Viscosity of microemulsions vs. concentration of salt (0, 0.5, 1, 1.5, 2, 5 and 10 (%w/w)) in mPa·s along DL-37. The black solid line represents linearity with an R^2 of 0.984. All data are means ± standard deviations for n = 3 replicates.

Similarly, an increase in viscosity with increasing sodium chloride concentration was observed for microemulsions along DL-37 (Fig. 4.9). Again, the linearity of the increase in viscosity with salt concentration (R^2 = 0.984) confirmed the positive contribution of NaCl to viscosity.

In order to understand whether the increase in the viscosity of salt-containing microemulsions was due to salt interactions with the water or perhaps the oil phase, a control viscosity study was performed. Various solutions of sodium chloride in water were prepared. The concentration of sodium chloride used was the same as that found in the microemulsions (0.5, 1, 1.5, 2, 5 and 10 (wt%)). The viscosity of water and of the NaCl solutions was plotted against salt concentration (Fig. 4.10).

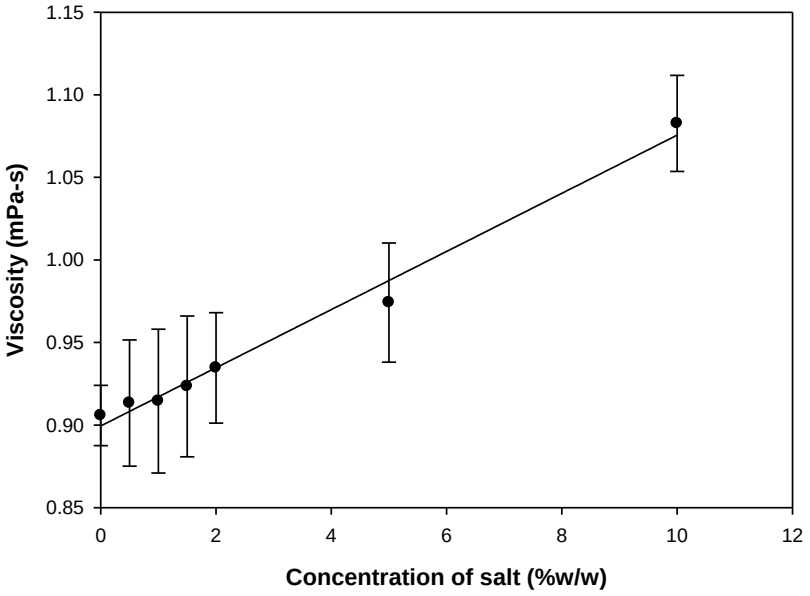


Fig. 4.10: Viscosity of NaCl solutions in water vs. concentration of salt (0, 0.5, 1, 1.5, 2, 5 and 10 (% w/w)) in mPa·s. The black solid line represents linearity with an R^2 of 0.987. All data are means ± standard deviations for n = 3 replicates.

The existence of a similar trend (increase in viscosity with increasing salt concentration) in salt-containing microemulsions (Fig. 4.8 and Fig. 4.9) as well as in aqueous solutions of sodium chloride (Fig. 4.10) confirmed that the increase in viscosity due to the interaction of sodium chloride with water was also present in w/o microemulsions. Microemulsion viscosity increased from 143.6 to 152.4 mPa·s in the DL-28 microemulsions, 207.1 to 212.7 mPa·s in the DL-37 microemulsions and from 0.906 to 1.083 mPa·s in the salt solutions.

4.6 Transmission electron microscopy

Using TEM, spherical water droplets in a continuous oil phase were observed. The size distribution obtained from TEM closely matched that obtained with DLS (Fig. 4.11). The droplet size obtained via TEM varied from ~20 to 60 nm whereas the DLS size distribution ranged from ~25 to 45 nm. The TEM micrograph also confirmed the polydisperse nature of the microemulsions as per Table 4.1.

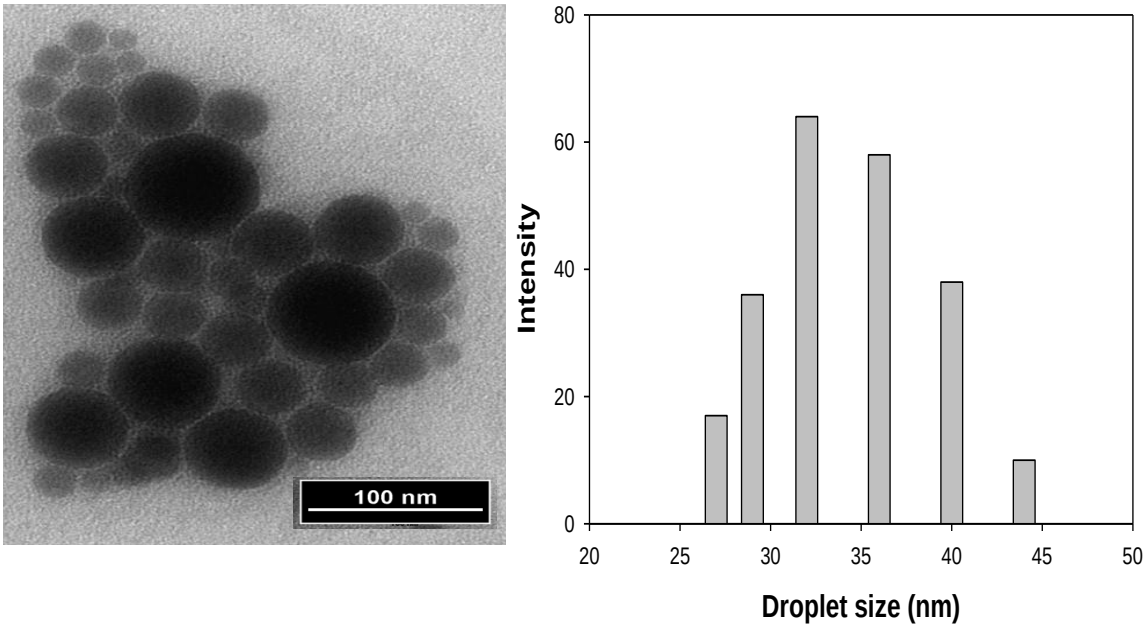


Figure 4.11: TEM of microemulsion along DL-37 with 1% w/w salt (left) and the corresponding droplet size distribution obtained via DLS (right).

Overall, stable w/o microemulsions consisting of soybean oil, glycerol monooleate, polysorbate 80, deionised water or saline (different % w/w), were formulated and characterised. The size of the microemulsions was well below 100 nm, as detected by DLS and confirmed by TEM imaging. The microemulsions were stable for over 8 months, based on the lack of change in dispersed droplet size. The presence of w/o

microemulsions was confirmed from TEM, DLS and conductance results. Addition of salt to the microemulsion did not influence average droplet size, polydispersity, stability of microemulsions as well as their Newtonian behaviour. However, an increase in viscosity was observed upon addition of salt to the microemulsions.

Chapter 5

Results and discussion - NaCl release from microemulsion

5.1 Release study

Fig. 5.1 shows the setup for sodium chloride release from the microemulsions. Microemulsion-loaded dialysis tubing was immersed into a beaker with deionised water. The external deionised water was continuously stirred and the conductivity meter immersed into the water for conductivity measurements.

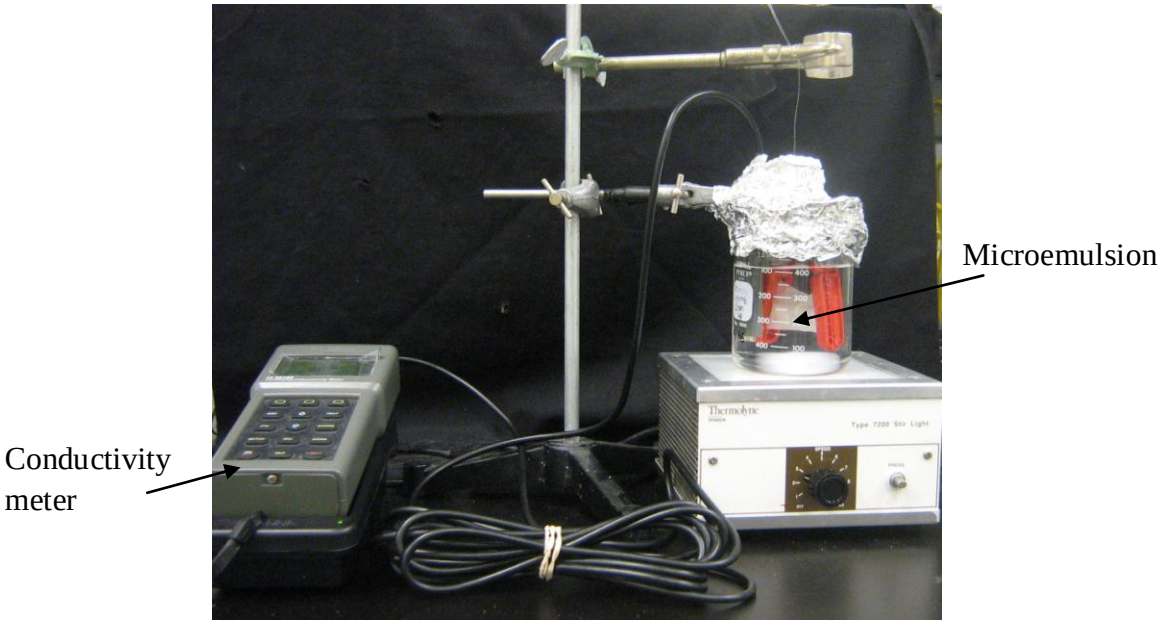


Figure 5.1: Sodium chloride release set-up for a 4day study on microemulsion with 10 % w/w saline along DL-37.

The net electrical conductivity of the external aqueous solution was plotted as a function of hours of release (Fig. 5.2) to establish the sodium chloride release pattern.

During the first 30 min, the conductivity sharply increased from ~10 to 18 μS/cm. The initial sharp increase in conductivity was likely the result of the high concentration gradient across the membrane arising from the presence of sodium chloride in the dispersed domains of the microemulsion and its absence in the outer dialysis fluid. Within another 30 min, an increase of ~3 μS/cm was observed. Beyond this point and until 4 days of release, the increase in conductivity was slow (~ <2 μS for every 30 minutes). After the first 30 min, the two limiting factors responsible for slow salt release were the increase in the amount of inner water due to osmosis and the decrease in the concentration of salt in the inner water due to its diffusion. Only ~4% of the NaCl present in the microemulsion was released after 4 days and the release did not plateau.

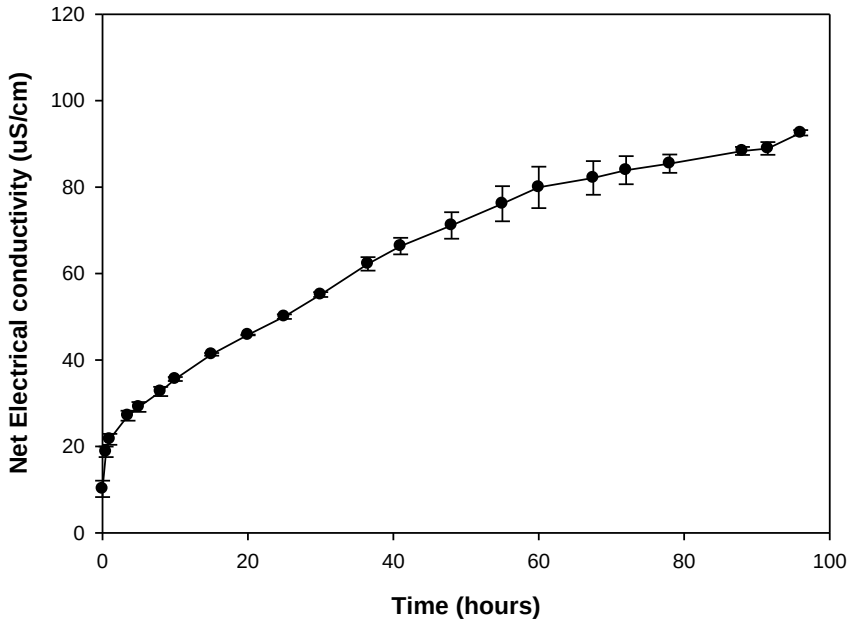


Figure 5.2: Sodium chloride release profile from microemulsions along DL-37 with 10 % w/w NaCl. All data are means ± standard deviations for n = 3 replicates.

A schematic showing the possible sodium chloride release mechanisms from the w/o microemulsions is shown in Fig. 5.3. Diffusion-controlled release of sodium chloride

was assumed to take place from the inner water to the outer dialysis fluid (deionised water) and simultaneously osmosis (movement of water molecules) occurred from outer water (w_2) to inner water (w_1). Upon ingress of water, the w/o microemulsion broke down leading to a bi-phasic system (Fig. 5.4). Similar results have been reported by Sonneville-Aubrun and others[5] where upon dilution with water, a w/o microemulsion underwent phase inversion forming an o/w emulsion. The authors reported the transition of the w/o microemulsion into an o/w emulsion through the formation of a lamellar phase based upon visual observations and small angle neutron scattering results. The anisotropic interference pattern obtained using SANS confirmed the existence of lamellar phase.

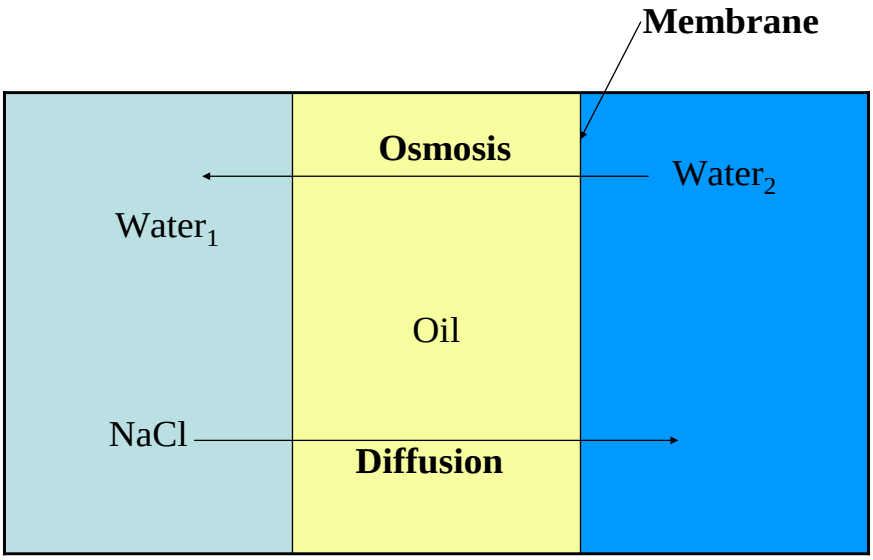


Figure 5.3: Schematic of sodium chloride release from a microemulsion with 10% (w/w) saline.

The phases (Fig 5.4) observed during sodium chloride release from the microemulsion along DL-37 with 10% (w/w) NaCl were as follows:

(1) Initially, there existed the microemulsion that was non-birefringent and transparent. It held 6 % saline (10% w/w) dispersed as nano-scale domains.

(2) Within minutes of starting the release experiments, a bi-phasic system with a lower white phase in equilibrium with upper phase (excess oil) appeared. The white phase corresponded to an o/w emulsion.

(3) After 4 days of release and ingress of water, the aqueous weight fraction of the destabilised microemulsion increased from 6% to 49%.

Figure 5.4: Microemulsion images before (A) and after (B) release. Image (A) is the initial, optically-clear w/o microemulsion and (B) is the phase-separated o/w emulsion (with excess oil) after day 4 of release.

5.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to study the structural changes occurring upon NaCl release from the microemulsions. Using a dual staining technique, the existence of an o/w emulsion resulting from phase inversion of w/o microemulsion was observed (Fig. 5.5). Nano-sized water droplets (Fig. 4.11) transitioned into micron-sized oil droplets within minutes of being exposed to an external fluid (Fig. 5.5). Phase inversion in microemulsions can result from either a change in temperature or composition (section 2.4).[5] A change in composition was brought about by dilution of the microemulsion with water due to osmosis.

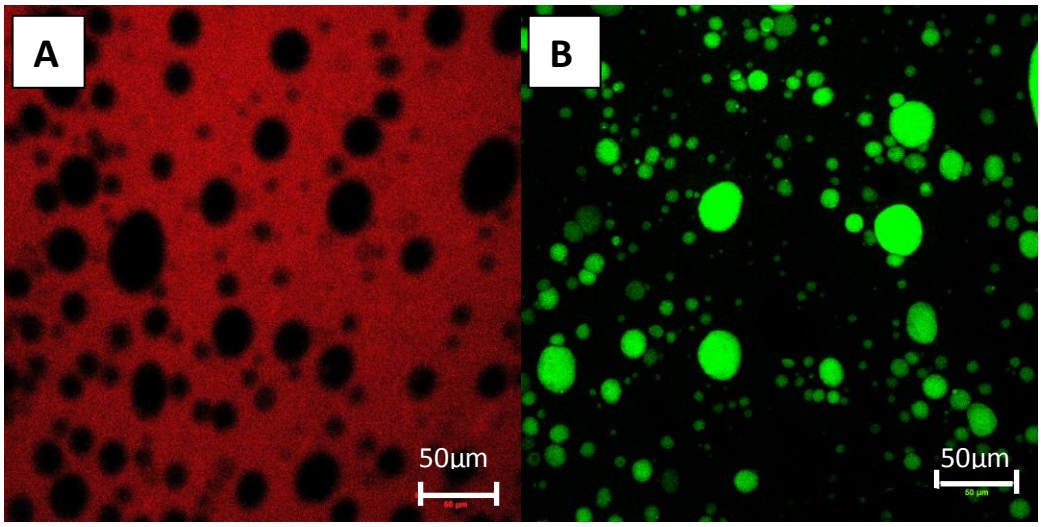


Figure 5.5: CLSM images of o/w emulsion (resulting from phase inversion) following 4 days of release. (A) is the fluorescence image of the emulsion stained with 0.001% v/v Rhodamine B in the continuous water phase, and; (B) is the emulsion stained with 0.1% w/w Fluorol Yellow 088 in the dispersed oil droplets.

As these systems were being studied as potential carriers for nutraceuticals, it was important to establish how fast microemulsion destabilisation occurred. To do so, a time-

controlled CLSM study was performed (Fig. 5.6) whereby a microemulsion of similar composition (DL 37 and 10% w/w saline) was subjected to similar release conditions as outlined in section 5.1. An aliquot of sample was removed after 5, 10, 30, 60 and 120 mins of release. The removed sample was immediately stained with Fluorol Yellow 088 and studied under CLSM to observe how fast structural changes occurred. Following the start of the experiments, it took less than 5 min for phase inversion to occur, corresponding to an ingress of <1.3 g of water, which increased the aqueous weight fraction from 6% to 17%. In Fig. 5.6A, the w/o microemulsion is shown converting into an o/w emulsion as a result of water ingress.

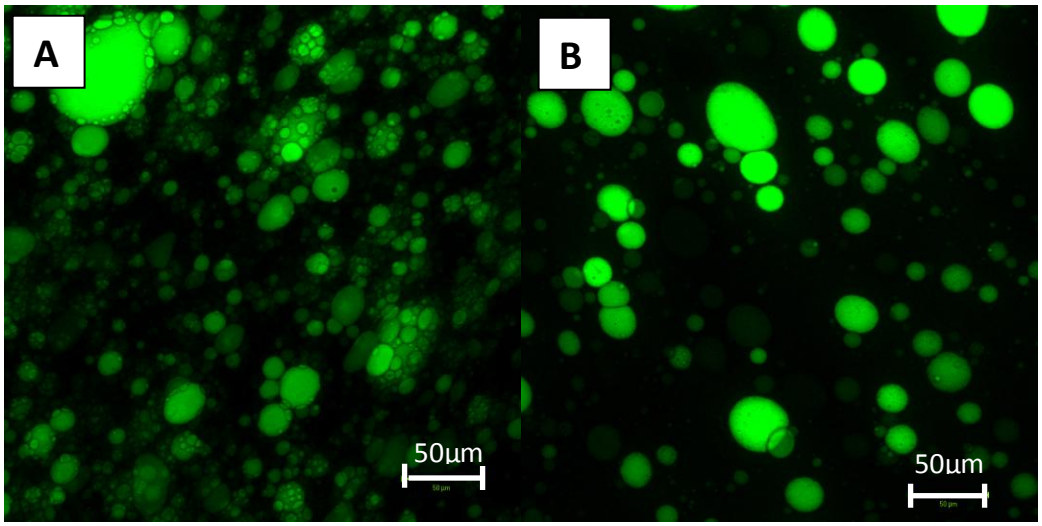


Figure 5.6: CLSM images of o/w emulsions (resulting from phase inversion), after (A) 5 min and (B) 10 min.

After 10 min of release (Fig. 5.6B), phase inversion was complete, with the presence of more sparsely-packed oil droplets in a continuous aqueous phase. This probably results from ingress of more amount of water in an additional 5 min. To determine the weight gain for phase inversion of microemulsion, water was added drop-

wise to microemulsion (10 wt% NaCl, DL-37) and phase-inversion was observed visually. The microemulsion turned turbid with the aqueous weight fraction increasing from 6% to 14.5%.

5.3 Salt release and transport mechanisms

When the microemulsion under study was placed in a release medium (w_2), osmosis of water occurred from w_2 to w_1. Such water transport is influenced by the magnitude of the osmotic pressure gradients between the two water phases, the concentration of surfactants used as well as the nature and viscosity of the oil phase.[109] In our system, the osmotic pressure gradient was created due to the presence and the absence of sodium chloride (solute) in w_1 and w_2 respectively. As a result of ingress of water, the microemulsion underwent phase inversion and released the entrapped sodium chloride, which then diffused across the oil phase and into the outer water.

The release of hydrophilic compounds from w/o/w double emulsions is comparable to that from a w/o microemulsion into an external aqueous phase separated by a membrane. In the case of a w/o/w double emulsion, hydrophilic additives solubilised in the inner aqueous phase will migrate from the internal to the external aqueous phase without film rupture, the driving force being the difference in concentration.[110] Various diffusion mechanisms are possible[111] including (i) the direct solubilisation and diffusion of salt into the oil, (ii) permeation through the oil layer and (iii) the solubilisation of the encapsulated compounds into surfactant reverse micelles and diffusion of the micelles in oil. In reverse micelle ion transport, reverse micelles (w/o) with entrapped ions and water can be formed in oil. The direction of ion transport depends upon the direction of the

osmotic pressure gradient across the oil layer. Similar to our w/o microemulsion system, Cheng *et al.*[111] reported ion transport *via* reverse micelles in double emulsions occurred when the oil layer was thick and the two water phases were non-contacting. This would suggest that salt release from the internal to the external aqueous phases occurred as a result of micellar transport.

Overall, salt release from the microemulsions occurred as a result of the microemulsions' phase inversion upon dilution, as confirmed by confocal laser scanning microscopy. The release of sodium chloride into the outer aqueous phase was confirmed from the increase in its conductivity. At this point in time, the NaCl release mechanism has not been confirmed.

Chapter 6

Overall conclusions

Stable w/o microemulsions consisting of soybean oil, glycerol monooleate, polysorbate 80, deionised water or saline (different % w/w), were formulated and characterised. The phase behaviour along two dilution lines permitted the characterisation of a portion of the monophasic w/o region. The microemulsion with the highest content of NaCl was chosen for a release study.

Microemulsions were characterised for droplet size, polydispersity, viscosity, conductance and sodium chloride release. The size of the microemulsions was well below 100 nm, as detected by DLS and confirmed by TEM imaging. The microemulsions were stable for over 8 months, based on the lack of change in dispersed droplet size. The presence of w/o microemulsions was confirmed from TEM, DLS and conductance results. Overall, TEM images were in a good agreement with DLS results. Salt release from the microemulsions occurred as a result of the microemulsions' phase inversion upon dilution, as confirmed by confocal laser scanning microscopy. The release of sodium chloride into the outer aqueous phase was confirmed from the increase in its conductivity.

Overall, this study demonstrated that it is possible to develop stable food-grade w/o microemulsions for encapsulation of hydrophilic food additives. This serves as an important first step towards development of novel microemulsion-based food products for the encapsulation of labile food additives.

Chapter 7

Future studies

1) Given that this system has been optimised, the exploration of similar microemulsions with hydrophilic bioactive compounds (*e.g.*, vitamins) should be considered.

2) The release of sodium chloride from microemulsions should be studied beyond 4 days of study until release reaches a plateau. Also, modelling the release will be essential to understand the release kinetics.

3) The precise mechanism of salt release from the microemulsions should be established.

4) Future work should include microemulsion characterisation through small angle X-ray scattering (SAXS) as it would enable differentiation of various structures present within the microemulsion PTPD (w/o, o/w, bi-continuous *etc.*).

5) SAXS would also be helpful in understanding the phase inversion of microemulsions and establishing the intermediate pathways through which the formation of a biphasic system occurs.

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