THERMAL STABILITY STUDY OF AQUEOUS OXYTOCIN FORMULATION FOR INTRANASAL DELIVERY

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(*B.Eng.(Hons.), NUS*)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF ENGINEERING

DEPARTMENT OF CHEMICAL & BIOMOLECULAR ENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2015

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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6 August 2015

ACKNOWLEDGEMENTS

I would like to deeply thank my supervisor Professor Reginald Tan for supporting me in this research work through the invaluable advice that he has given me and also setting me in the proper research direction.

I would also like to express my gratitude to Dr. Sanggu Kim for inspiring me in the area of liquid formulation and helping me organize my research focus for this project. I would also like to thank Dr. Kim on the advice that he has provided me with that is relevant to my research work.

I would also like to thank Dr. Chen Ye for the fruitful discussions that we have had which I benefitted a lot from. Finally, I want to thank Mr. Frederick Toh, senior laboratory officer from the Institute of Chemical and Engineering Sciences, for his support on any assistance that I needed in the laboratory.

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SUMMARY

Post-partum haemorrhage has been highlighted as one of the most common causes of maternal deaths in third world countries. Oxytocin, a pharmaceutical neuropeptide, was identified as the drug of first choice to treat post-partum haemorrhage. It is currently available in the form of intravenous administration and requires cold-chain supply and storage condition as oxytocin degrades rapidly at 30 ^{o}C and above. Most third world countries experience tropical climate where temperature frequently hovers above 30 ^{o}C. Cold-chain supply and storage are often inadequate in these places. Additionally, in rural areas, most women give birth at home and intravenous injection requires skilled medical personnel to administer which are often not immediately available. In the present studies, intranasal delivery of oxytocin in aqueous micellar formulation is explored. Intranasal delivery of oxytocin eliminates the requirement of medical personnel to perform the injection. Furthermore, the neuroepithelium in the olfactory region of the nasal cavity facilitates the delivery of brain drug through the central nervous system, making intranasal delivery of oxytocin the preferred route of administration. Here, we developed surfactant micellar formulations using Tween 80, Cremophor RH40 and sucrose laurate for intranasal delivery of oxytocin in aqueous medium. Thermal stability of these formulations was evaluated and micellar formulation with sucrose laurate demonstrated enhanced stability of oxytocin at storage temperature of 40 ^{o}C while those with Tween 80 and Cremophor RH40 adversely affected the thermal stability of oxytocin.

Addition of a mucoadhesive, carboxylmethylcellulose, also negatively influenced the thermal stability of oxytocin in aqueous formulations.

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LIST OF SYMBOLS

d_H	hydrodynamic diameter
D	diffusion coefficient
γ	surface tension
k_B	Boltzmann constant
λ	wavelength
η	viscosity
T	absolute temperature

LIST OF ABBREVIATIONS

Asn	Asparagine
Cys	Cysteine
Gln	Glutamine
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Pro	Proline
Tyr	Tyrosine
CMC	Critical Micelle Concentration
DLS	Dynamic Light Scattering
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
MS	Mass spectrometry
PDI	Polydispersity Index
VWD	Variable Wavelength Detector

CHAPTER 1 INTRODUCTION

1.1 Overview

Peptides are short chain amino acids with at least two amino acids linked by an amide or disulfide bond. The number of amino acids that makes up a peptide is arbitrarily defined to be less than 50 to distinguish peptide from protein (Kaspar *et al*., 2013). Peptides are attractive therapeutic drugs due to their high selectivity in binding with their in vivo targets at specific cell surface receptors, thus resulting in high efficacy with minimal side effects. Currently, more than 7000 naturally occurring peptides were identified and these often serve important biological functions in human physiology (Fosgerau *et al*., 2015). However, these natural peptides are usually not readily suitable for therapeutic purposes because of their poor chemical and physical stabilities as well as difficulties in crossing physiological barriers. Therefore, formulation strategies targeted at overcoming these intrinsic weaknesses of peptides are necessary before they can be approved pharmaceutics.

Postpartum haemorrhage is defined as more than 500 ml or 1000 ml of blood loss for vaginal delivery and cesarean delivery respectively, within 24 hours following childbirth. In general, it is normal for bleeding to occur following a delivery due to open blood vessels after the separation of placenta from the uterus. The uterus typically continues to contract and these blood vessels will be closed off. However, some women may experience excessive blood loss and would require special treatment to stop the bleeding. Oxytocin, a

pharmaceutical peptide, is usually the first choice drug to be administered for the treatment of postpartum haemorrhage (Dyer *et al*., 2010).

One of the Millennium Development Goals established by the United Nations is to reduce the maternal mortality ratio by 75%, between 1990 and 2015. Between 1990 and 2013, the maternal mortality ratio was decreased by 45% (United Nations). Clearly, progress was made but at a slower pace, and interventions are required to accelerate the reduction. Postpartum haemorrhage accounts for nearly 20% of maternal deaths worldwide. In addition, more than 99% of maternal deaths due to postpartum haemorrhage happened in the developing countries (Say *et al*., 2014).

Despite the success of oxytocin in treating postpartum haemorrhage thereby leading to a reduction in the maternal mortality rates in developed countries, postpartum haemorrhage is still the main cause of maternal death in many developing countries. This is largely due to the poor thermal stability of oxytocin in aqueous formulation, which requires a cold chain to maintain its drug integrity. Many developing countries experience tropical climate which is unfavorable to the stability of oxytocin and cold chain is usually lacking in these countries, especially in rural or remote areas. Additionally, current commercial oxytocin drug is administered intravenously by skilled medical personnel who may not be readily available in these remote places. It is therefore important to develop an oxytocin formulation that can be easily administered, and with improved heat stability in aqueous medium. The motivation of this work is to further bring down the maternal mortality rate,

especially in developing countries, by designing an oxytocin liquid formulation with enhanced thermal stability, suitable for intranasal administration, which would make cold chain unnecessary.

1.2 Objectives and Scope of Study

The objective of the present work is to design and develop a heat-stable formulation for intranasal delivery of oxytocin. Nonionic surfactants, sucrose laurate, Cremophor RH40 and Tween 80, and the concentrations used were chosen based on availability of toxicological data for intranasal administration (Kürti *et al*., 2012).

More specifically, the scope of this work encompasses the following:

1. Studying the effect of salts on the critical micelle concentration of sucrose laurate, Cremophor RH40 and Tween 80.
2. Studying the physical stability of oxytocin between 20 to 60 ^{o}C by monitoring the change in their droplet size and polydispersity index in various aqueous micellar formulations.
3. Monitoring the thermal stability of oxytocin in various aqueous micellar formulations on storage temperature at 40 ^{o}C for three weeks by measuring the change in oxytocin concentration over time using HPLC.
4. Investigating the effect of surfactant concentration on the thermal stability of oxytocin in aqueous micellar formulation.
5. Investigating the effect of addition of mucoadhesives on the thermal stability of oxytocin in aqueous micellar formulation.

1.3 Organization of the Thesis

Chapter 1 is the introduction of this thesis which provides an overview of this project. The objectives and scope of this work are outlined. Chapter 2 is the literature review section, providing an in-depth discussion on oxytocin, intranasal drug delivery and various strategies to formulate oxytocin for effective intranasal drug delivery. Chapter 3 describes the materials and experimental procedures employed in the study of oxytocin stability in the various formulations. Chapter 4 is a discussion on the results obtained from the experiments. Chapter 5 is the outlines the conclusions of the study. Chapter 6 highlights the limitations of this project and proposes future studies to better enhance the thermal stability of oxytocin in aqueous formulation for intranasal drug delivery.

CHAPTER 2 LITERATURE REVIEW

2.1 Oxytocin

Oxytocin is a cyclic nonapeptide mammalian neurohypophyseal hormone, with molecular weight of 1,007.19 g/mol, produced by the hypothalamus and secreted by the posterior pituitary gland. It consists of a 20-membered cyclic link connected by a disulfide bridge between two cysteine amino acid groups. Its use is associated with uterine contraction and lactation simulation (Nachtmann *et al*., 1981; Chaibva *et al*., 2007). Oxytocin is used primarily in labor induction and augmentation, postpartum haemorrhage control and uterine hypotonoicity management in the third stage of labor (Chaibva *et al*., 2007).

2.1.1 Stability of oxytocin

Oxytocin comprises a six-membered ring, $Cys^1 – Tyr^2 – Ile^3 – Gln^4 – Asn^5 – Cys^6$, and a three-membered tail, $Pro^7 – Leu^8 – Gly^9 – NH_2$, linked at Cys^1 and Cys^6 by a disulfide bridge. Asn^5 is responsible for oxytocin to serve its biological function at the active site of the uterine receptor while Ile^3, Gln^4, Pro^7 and Leu^8 are required for receptor binding (Walter *et al.*, 1971). Degradation of oxytocin that resulted in structural changes could lead to loss of affinity to the receptor and/or modification of biological activities.

Without special treatment, freeze-dried oxytocin acetate may remain stable for several years, with minimal loss of oxytocic activity, under refrigerated (2 – 6^{o}C) conditions (Nachtmann *et al*., 1981). However, the main problem with oxytocin stability occurs when it is incorporated in aqueous formulation at temperature 30 ^{o}C and above (Groot *et al.*, 1994). Furthermore, the stability of aqueous oxytocin formulation is greatly dependent on the pH with highest stability at pH 4.5 (Hawe *et al.*, 2009). The degradation products and mechanisms have been extensively studied and shall be discussed next.

2.1.2 Degradation of oxytocin

Peptide degradation in an aqueous solution may take place chemically and/or physically. Chemical degradation involves the modification of the peptide by covalent bond formation or covalent bond cleavage, resulting in a new chemical structure. For instance, peptide degradation by hydrolysis, oxidation, deamidation, disulfide exchange and β-elimination. In contrast, physical degradation occurs when the peptide undergoes higher order structural change such as dimerization.

Oxytocin undergoes various degradation pathways, as shown in **Figure 2.1**, depending on target residues, pH and temperature. One of the most common forms of chemical degradations is deamidation. For oxytocin, under physiological conditions, Gln^{4} and Asn^{5} are susceptible to instantaneous deamidation to form aspartic acid, Asp, and glutamic acid, Glu residues respectively. Deamidation of Gln^{4} and Asn^{5} are mainly under acidic

conditions via direct acid catalyzed hydrolysis. Under neutral or alkaline conditions, deamidation occurs by the formation of cyclic imide intermediates, though the deamidation of Asn is more prevalent than that of Gln residues (Hawe *et al.*, 2009). Additionally, the terminal Gly^9 is also a likely target for deamidation in a similar manner to Gln^4 and Asn^5.

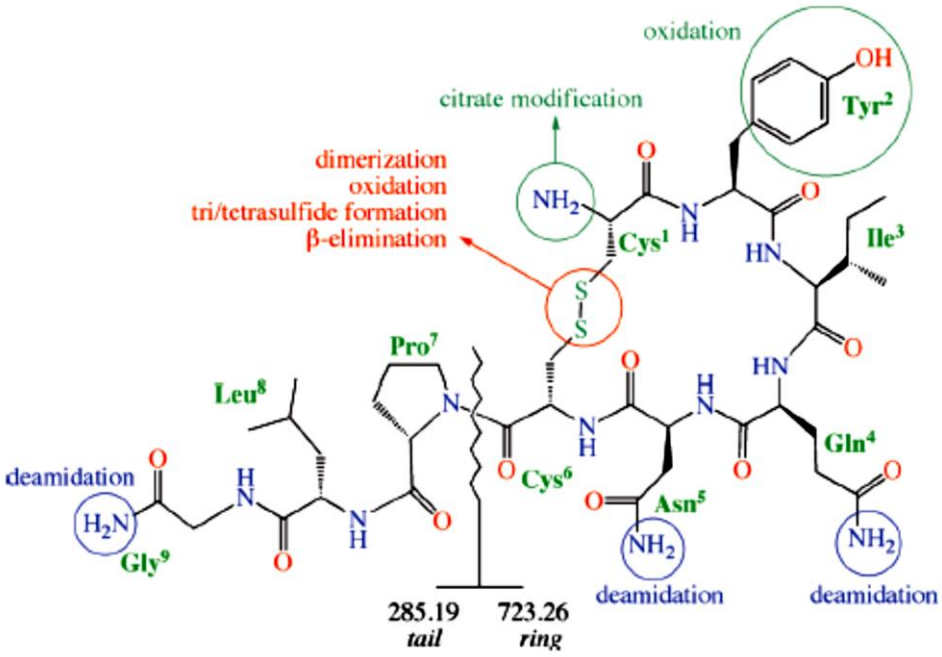


Figure 2.1 Molecular structure of oxytocin with its ring and tail fragment ions formed upon MS/MS fragmentation (Avanti *et al.*, 2012a).

Peptides containing Cysteine (Cys) and Tyrosine (Tyr) are predisposed to oxidative degradation. Oxidation of Cys^1, Cys^6 and Tyr^2 in oxytocin may occur in the presence of light and metal ions, and the process may be influenced by pH, temperature and buffer composition. Cys is especially sensitive to oxidative damage (Avanti *et al.*, 2012a).

The presence of Cys^1 - Cys^6 disulfide bridge may also be a site for potential chemical degradation. This can take place via β-elimination or disulfide

exchange reaction. Under alkaline condition or at elevated temperature, β-elimination may occur, resulting in the destruction of the disulfide bridge to form free thiol groups. Under acidic condition, protonation of the disulfide bridge results in disulfide exchange reaction via the sulfenium cation. Disulfide exchange reactions could further lead to the formation of dimers or larger aggregates, and therefore destabilizing the formulation (Avanti *et al.*, 2012a).

Physical degradation of oxytocin can be a result of dimerization, which in turn leads to aggregation. When more aggregates are brought together, precipitation is likely to occur. As discussed previously, dimerization can be brought about by disulfide exchange reaction. Additionally, dimers may also be formed due to light or metal induced oxidation. Also, heating, freezing or agitation may induced aggregation (Avanti *et al.*, 2012a).

2.1.3 Limitation and formulation challenges

Several publications highlighted postpartum haemorrhage as one of the most common causes of maternal deaths in third world countries (Royston *et al.*, 1989; Van Dongen *et al.*, 1991). Currently, oxytocin is available commercially under the trade name Syntocinon® as an aqueous solution for intravenous delivery. There are several problems associated with the use of Syntocinon in third world countries. First, Syntocinon has to be stored under refrigerated condition and cold-chain supply and storage are often inadequate in third world countries. Second, in rural areas where it is common for women to give

birth at home, medical provisions for intravenous delivery are often lacking which makes the use of Syntocinon even more challenging. Additionally, intravenous delivery of oxytocin is impractical in third world countries as it requires skilled medical personnel who are often not easily or immediately accessible to for the administration of the injection.

Pulmonary delivery of oxytocin was explored in which ultrafine (1 – 5 μm) oxytocin powder was formulated by spray-drying and aerosolized oxytocin has demonstrated potential for the design of a safe and inexpensive formulation for effective treatment of postpartum haemorrhage in resource-poor settings (Prankerd *et al.*, 2013). However, pulmonary delivery systems often encounter challenges with poor deposition of the drug as it has to overcome an efficient aerodynamic filter in the lung as well as to prevent deposited drugs from clearing back up the throat by the mucous lining in the pulmonary airway. Additionally, the stability of dry oxytocin is more sensitive to humidity than temperature (Groot *et al.*, 1994). A lot of developing countries experience high humidity, suggesting the need for extra measures to ensure that the solid formulation is always kept away from moisture.

Therefore, in this work, intranasal delivery of oxytocin is proposed as an alternative delivery route of administration. Furthermore, since powder sprays are more likely to trigger nasal mucosa irritation than solution or suspension sprays (Afzelius, 1997), formulation of oxytocin in an aqueous solution is explored.

2.2 Intranasal drug delivery of oxytocin

Intranasal drug delivery has been used for treating and relieving a variety of nasal conditions and disorders for a long time. It has increasingly been recognized as a useful and reliable alternative to oral or parenteral systemic drug delivery. One of the advantages of intranasal drug delivery is that the drug can evade the blood-brain barrier, allowing more direct interaction of the drug with the central nervous system. This makes intranasal delivery of oxytocin, a neuropeptide, an attractive route of administration. Additionally, intranasal delivery circumvents the gastrointestinal and hepatic presystemic metabolism, thereby enhancing the bioavailability of oxytocin.

2.2.1 Nasal cavity

Breathing and smelling seems to be the more apparent functions of the nasal cavity. More importantly, the nasal cavity is part of the respiratory system whereby inhaled air is pretreated for proper respiratory function. Inhaled air is heated or cooled, filtered and humidified in the nasal cavity before entering the lungs. Mucus and hairs lining the nasal cavity help to trap inhaled particles and pathogens, preventing them from entering the lungs (Mygind *et al.*, 1998).

A human nasal cavity has an estimated total volume and total surface area of 15 ml and 150 cm^2 (Mygind *et al.*, 1998). The nasal cavity is divided into four main areas which include the nasal vestibule, atrium, respiratory region and olfactory region as shown in **Figure 2.2**.

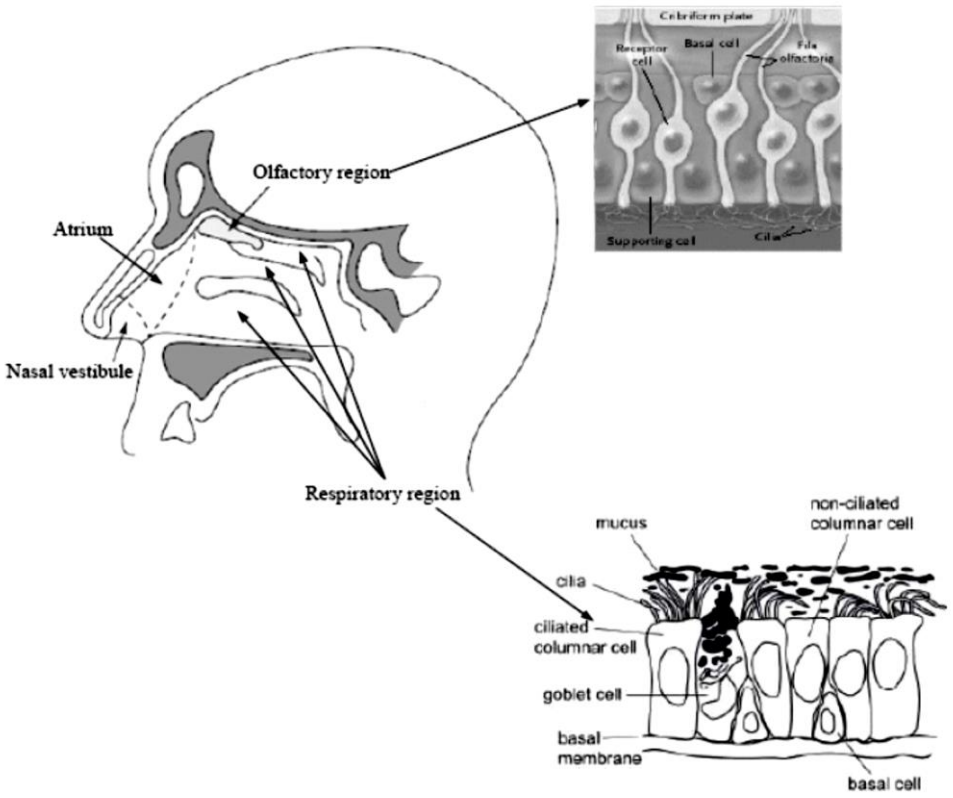


Figure 2.2 Anatomy and histology of human nasal cavity (Pires *et al.*, 2009).

Just inside the nostrils is the nasal vestibule, which consists of nasal hairs, that filters inhaled particles from the air that we breathe in. This region is lined with stratified squamous and keratinized epithelium, mainly to provide protection against toxic environmental substances. It is likely that the protection against toxic substances is due to poor permeability in the nasal vestibule, which suggests that drug absorption in the nasal vestibule is low (Illum, L., 2003; Probst *et al.*, 2011; Kimbell *et al.*, 1997).

Inhaled air crosses the nasal vestibule into the atrium, which comprises the stratified squamous in the anterior portion and the pseudostratified columnar cells with microvilli, before entering the respiratory region. The atrium does not offer much absorption activity.

Inhaled air from the atrium subsequently enters the respiratory region which is the largest section of the nasal cavity, with a total surface area of approximately 130 cm^2. The large surface area can be attributed to the presences of mucosa-covered folds, which are the superior, middle and inferior turbinates, projected from the lateral wall. Pseudostraified columnar epithelial cells with microvilli line the wall of the nasal cavity also help enhance the respiratory surface area. The large surface area in the respiratory region is where the humidification and temperature regulation of inhaled air is carried out in the nasal cavity. Secretory glands and goblet cells present in the epithelium in the respiratory region are responsible for production of nasal mucous which thinly coat the nasal epithelium. The nasal mucous is approximately 95% water, 2.5-3% mucin and 2% electrolytes, proteins, lipids, enzymes, antibodies, sloughed epithelial cells and bacterial products (Dondeti *et al.*, 1996; Verdugo, 1990; Lethem, 1993). The nasal mucous is important for several reasons. First, it is required for the humidification and temperature adjustment of inhaled air. Next, the adhesive behavior of the mucous helps to trap inhaled particulates and pathogens. The nasal mucociliary clearance then function to transport these trapped particles to the mouth towards the nasopharynx, to be removed by swallowing or coughing (Kim, 2008). Pathogen binds with mucin, found in the nasal mucous produced by epithelial tissue, to form part of the human immune system. Additionally, mucin can bind with large molecules, such as peptide drugs, for drug delivery (Constantino *et al.*, 2007). Furthermore, the provision of high surface area in the nasal respiratory region, together with the high vascularization beneath the epithelium with good permeability, is important for drug delivery.

The olfactory region is the only region that is part of the central nervous system that provides opportunity for direct contact to the external environment. It is located on the roof of the nasal cavity near the septum. Its epithelial structure is similar to that in the respiratory region except that a special olfactory receptor cells is also present. These receptor cells binds to odor molecules and are important for the detection of smell.

2.2.2 Advantages of intranasal drug delivery

Intranasal delivery of oxytocin offer several advantages compared to other route of delivery. In comparison with parenteral delivery, the most apparent advantages would be comfort and ease of administration. Intranasal delivery is non-intrusive and can be self-administered easily, eliminating the need of skilled medical personnel. Next, deposition of oxytocin is more effective in the respiratory and olfactory region of the nasal cavity than that in the lungs. This may be due to the relative difference in distance the drug has to travel to the target sites as well as the resistance present in the pulmonary airway. In addition, Oral delivery is irrelevant as peptides generally have poor stability in the gastrointestinal environment, poor intestinal absorption and extensive hepatic first-pass elimination (Illum, L., 2003). Hence, the gastrointestinal region can be avoided for intrasanal delivery. Furthermore, the activity of oxytocin targets the brain. The neuroepithelium in the olfactory region facilitates the delivery of brain drug through the central nervous system, making intranasal delivery of oxytocin the preferred route of administration.

2.2.3 Limitations of intranasal drug delivery

Inside the nasal cavity, the central nervous system is separated from the outside environment by the mucous layer and the epithelial membrane. The efficacy of intranasal drug delivery may be influenced by a number of factors and these can be divided into three categories, namely the nasal physiology, the drug physicochemical properties and the drug formulation.

2.2.3.1 Nasal physiology

For nasal physiology, blood flow in the nasal mucosa, mucociliary clearance, enzyme degradation in nasal tissue and the presence of drug resistance transporter in the nasal tissue. First, absorption of drug is influenced by the blood flow in the nasal mucosa. The main mechanism for drug absorption is diffusion. Drugs may exhibit vasodilatation or vasoconstriction effect depending on vascular body which affects the flow of blood which in turn influences the drug diffusion. However, in a recent study, Ludwig *et. al.* suggested that intranasal administration of oxytocin in rats did not substantially affect the heart rate and arterial pressure, implying negligible vasodilatation or vasoconstriction (Ludwig *et al.*, 2014).

Next, the complexity of the mucociliary clearance can affect nasal drug absorption in several ways. For instance, abnormal transport rate of mucus and its residence time can impair the mucociliary clearance (Marttin *et al.*, 1998). A slower mucociliary clearance results in longer residence time of the drug in

the nasal mucosa thereby increasing drug permeation. On the contrary, a faster mucociliary clearance leads to shorter residence time, implying premature discharge of administered drugs from nasal cavity and resulting in poor absorption. Additionally, site of drug deposition affects drug absorption as well, which is highly affected by dosage form. For nasal spray, drugs are deposited more anteriorly compared to drugs administered by nasal drops (Illum, L., 2003). Mucociliary clearance is more active in the posterior part of the nasal cavity than the anterior part (Marttin *et al.*, 1998). Hence, nasal drops have faster mucociliary clearance than nasal spray. Also, polar drugs solubilize easily in the mucus but permeate slowly across the membrane. Therefore, polar drugs suffer from poor absorption due to high mucociliary clearance. Furthermore, drug absorption can be influenced by external factors that affect the mucociliary clearance which includes temperature, presence of sulphur dioxide, cigarette smoking, drug formulation and variability in mucociliary clearance between different people.

Enzymatic degradation may also have a negative impact on nasal drug absorption. Numerous metabolic enzymes are found in the nasal cavity and these may affect the pharmacokinetic and pharmacodynamics profile of the administered drug. More specifically, proteolytic enzymes found in the nasal cavity can be detrimental to the absorption of peptide drugs, such as oxytocin. However, nasal first-pass metabolism is normally less destructive than hepatic or intestinal metabolism. Nevertheless, it cannot be ignored and requires further investigation (Pires *et al.*, 2009).

Another aspect of nasal physiology that influences drug delivery is the presence of transporter systems in nasal tissue. Several studies (Graff *et al.*, 2005; Westin *et al.*, 2005; Kandimalla *et al.*, 2005) have identified drug resistance transporter molecules present in the human nasal respiratory and olfactory mucosa, which hinder the absorption of drug across the nasal membrane into the central nervous system.

2.2.3.2 Drug physicochemical properties

The physicochemical properties of the drug may affect nasal drug absorption. First, hydrophobicity and molecular weight of the drug. Hydrophobic drugs 1kDa or smaller can be readily absorbed across the nasal membrane. Drugs with molecules larger than 1 kDa may exhibit reduced absorption. In contrast, hydrophilic drugs show poor absorption and are highly dependent on their molecular weight. This is due to the lipophilic nature of the nasal membrane which has greater compatibility with increased lipophilicity. However, drugs that are too hydrophobic are unable to dissolve in the mucus readily and will be removed by mucociliary clearance, thereby reducing the permeation through nasal mucosa. For hydrophilic drugs, such as oxytocin, absorption through the nasal membrane is further influenced by their ionization strength and the pH of the nasal mucosa which is between 5.0 and 6.5. It was found that largest permeation through the nasal mucosa is for non-ionized species and decreased with increased degree of ionization (Corbo *et al.*, 1989; Hirai *et al.*, 1981; Zaki *et al.*, 2006).

Additionally, nasal drug absorption is also affected by the intrinsic biological, chemical and physical drug stability. This is especially relevant to oxytocin with poor thermal stability in the aqueous environment as previously discussed.

Drug absorption is influenced by its water solubility as it is required to dissolve in the fluid of the nasal cavity for contact with the nasal mucosa and posterior absorption (Zaki *et al.*, 2006). Furthermore, the volume of drug solution administered is constrained by the small size of the nasal cavity. Hence, the drug solubility in the delivery medium has to be sufficiently high to be effective.

2.2.3.3 Drug formulation

Drug formulation is likely to influence the nasal drug absorption as well. First, viscosity of the drug affects the residence time of the drug in the nasal cavity. In general, formulation with high viscosity may increase drug absorption due to the increased in contact time between the drug and the nasal mucosa. However, increased in formulation viscosity does not always imply an increased in drug absorption. Zaki and coworkers reported that enhanced viscosity of formulation with metoclopramide hydrochloride in the nasal cavity resulted in the reduction in drug absorption (Zaki *et al.*, 2006). This is likely the result of slow diffusion which leads to poorer drug absorption.

The pH of drug formulation may also have an impact on nasal drug absorption. It is essential to select a formulation pH that would minimize nasal mucosa

irritation, at the same time maintaining drug stability. The pH of the human nasal mucosa is between 5 and 6.5 (Washington *et al.,* 2000). Potential nasal irritation can be avoided by formulating the drug delivery system between pH 4.5 and 6.5. Additionally, at this pH condition bacteria growth may be impeded. (Arora *et al.*, 2002).

Furthermore, the pharmaceutical dosage forms and the types of pharmaceutical excipients used may also affect the nasal drug absorption. Nasal drops are often the most convenient form but delivering the required dose accurate is a challenge. Solution or suspension sprays are often preferred over nasal drops. Pharmaceutical excipients present in nasal formulation, such as buffer components, gelling agents, and solubilizers, may also alter the drug absorption profile.

2.2.4 Methods to improve nasal drug absorption

As previously discussed, oxytocin is a relatively hydrophilic peptide and it is likely that absorption through the lipophilic membrane of the nasal cavity is poor. Furthermore, the mucociliary clearance adversely affects the absorption of polar drug in the nasal cavity. Hence, intranasal formulation design needs to take into consideration these two factors to ensure sufficient bioavailability.

There are many ways to improve the absorption of hydrophilic drug in the nasal cavity. For instance, absorption enhancer may be added to assist the crossing of hydrophilic drugs through the nasal epithelium. It is likely that the

addition of absorption enhancer induces reversible structural change to the epithelial barrier, thereby increasing its compatibility with hydrophilic drug leading to better absorption. Absorption enhancer for intranasal delivery may be surfactants, bile salts, fatty acids or polymeric enhancers, such as gelatin, cyclodextrins, chitosan and poly-L-arginine (Charlton *et al.*, 2007; Wang *et al.*, 2006; Hersey *et al.*, 1987; Mishima *et al.*, 1987; Baglioni *et al.*, 1990; Illum *et al.*, 1994; Ekelund *et al.*, 2005). Depending on the type of absorption enhancer used, the epithelial cells may be affected in different ways. In general, the phospholipidic layer may be modified, the membrane fluidity may be increased or the tight junctions between epithelial cells may be opened, resulting in increased in paracellular transport (Karasulu *et al.*, 2008). Selection of absorption enhancers need to take into consideration their intranasal toxicity for safe administration of the formulation.

Additionally, the effect of mucociliary clearance on the absorption of polar drug may be minimized by the addition of mucoadhesives. Mucoadhesives are synthetic or natural polymeric compound, such as chitosan, alginate, cellulose and their derivates, added to formulation to interact with mucin in the nasal mucosa. Upon contact with the mucus layer, the formulation becomes wet and swells. The mucoadhesives then penetrate into the mucus, thereby creating a localization of the formulation in the nasal cavity, which lead to an increased concentration gradient across the epithelium (Ugwoke *et al.*, 2005).

2.3 Formulation strategies for intranasal delivery of oxytocin in aqueous medium

Developing a stable oxytocin formulation in aqueous medium at elevated temperature is challenging due to its poor thermal stability. On top of that, the hydrophilic nature of oxytocin may adversely impact its bioavaibility in the nasal cavity. Therefore, it is important that these are taken in consideration in formulation design. Formulation components with acceptable toxicity tolerance have to be thoughtfully selected for safe administration in the human nasal cavity.

2.3.1 Oxytocin stabilization

The degradation kinetics of oxytocin in aqueous environment were studied by Hawe and coworkers and they discovered that the degradation profile of oxytocin is greatly dependent on the pH of the formulation. They concluded that oxytocin is most stable in formulation at pH 4.5 (Hawe *et al.*, 2009). Therefore, designing oxytocin formulation with pH 4.5, thermal degradation by oxidation and β-elimination at Cys^1 and Cys^6 is more prevalent than the other degradation processes at other sites.

In another study by Avanti and coworkers, they found out that use of citrate buffer at pH 4.5 greatly enhances the thermal stability of oxytocin in aqueous formulation for parenteral delivery. Additionally, the presence of divalent metal ions, such as Mg^{2+}, Ca^{2+} and Zn^{2+}, significantly impact the stability of oxytocin favorably. They suggested that a synergistic effect exists when citrate

buffer and the divalent salts are added into the aqueous oxytocin formulation (Avanti *et al*., 2011).

In a separate study by Avanti and coworkers, the mechanism of stabilizing oxytocin in aqueous formulation with a citrate buffer and divalent metal ions were investigated. It was shown that degradation processes at Cys^{1} and Cys^{6} were suppressed when a citrate buffer was used in the presence of divalent metal ions. A complex is likely formed in the ring structure of oxytocin thereby suppressing intermolecular reactions. There were no observable differences when either Mg^{2+}, Ca^{2+} or Zn^{2+} were used. Hence, they concluded that divalency is essential for stabilization of oxytocin in aqueous formulation (Avanti *et al*., 2012b).

2.3.2 Formulation components

Components for design of oxytocin formulation need be safe for intranasal administration in human beings. In addition, the components need to be used at the appropriate concentrations while maintaining its desired function.

Non-ionic surfactants are in general less toxic to biological membranes than ionic surfactants (Arechabala *et al*., 1999). Both Cremophor RH40 and Tween 80 are non-ionic surfactants used to increase cell membrane fluidity or augmenting paracellular absorption (MacDonald *et al*., 2010). Sucrose esters are non-ionic surfactants that are biodegradable and have shown to be non-

toxic and non-irritant to the skin. There is evidence that show the use of sucrose esters as permeation enhancers through the skin (Csoka *et al*., 2007).

In 2012, a study by Kürti and coworkers evaluated the non-toxic doses of sucrose laurate, Cremophor RH40 and Tween 80 to the human nasal epithelial cells. They have found that sucrose laurate, Cremophor RH40 and Tween 80 can safely be administered intranasally at 0.1 mg/ml, 5 mg/ml and 1 mg/ml. Furthermore, they also concluded that these non-ionic surfactants can potentially be used as permeation enhancers in nasal formulations (Kürti *et al*., 2012).

In addition to absorption enhancers, mucoadhesives may also be added into aqueous oxytocin formulation to increase its bioavailability. Ugwoke and coworkers have proven that Carbopol 971® and carboxylmethylcellulose can be used to increase the residence time of the formulation in the nasal cavity (Ugwoke *et al*., 2000a). Toxicological studies on Carbopol 971® and carboxylmethylcellulose to determine if they can be used in nasal formulations were carried out. In an in vivo intranasal administration of Carbopol 971® in rabbits, it was found that Carbopol 971® resulted in severe inflammation in the nasal mucosa in rabbits (Ugwoke *et al*., 2000b). On the other hand, 0.25% (w/v) of carboxylmethylcellulose was well tolerated, showing no inhibitory effect on the ciliary beat frequency in the human nasal epithelial cell culture, implying that it can be used for acute drug administration (Ugwoke *et al*., 2000c).

Absorption enhancers and mucoadhesives are selected based on available toxicity data. Therefore, in this work, aqueous oxytocin formulations with absorption enhancers, sucrose laurate, Cremophor RH40 or Tween 80, and mucoadhesives, carboxylmethylcellulose, at their safe doses were developed and their long term stability at elevated temperature condition was studied.

CHAPTER 3 METHODOLOGY

3.1 Materials

Oxytocin acetate was purchased from Sinoway Industrial Co., Ltd. (Xiamen, China). Food grade sucrose laurate [L-1695, HLB = 16, average molecular weight = 560 g/mol] consisting of not less than 80% of mono-, di-, and tri-esters was provided by Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). Sorbitan monooleate (Tween 80), Polyoxyl 40 hydrogenated castor oil (Cremophor® RH40), Carboxymethylcellulose sodium salt (low viscosity) and sodium citrate dehydrate were purchased from Sigma-Aldrich. Citric acid monohydrate and zinc chloride were obtained from Merck KGaA. Potassium dihydrogen phosphate was purchased from Alfa Aesar. Sodium hydroxide pellets was obtained from Schedelco Pte Ltd. Chemicals used for HPLC assay were HPLC grade supplied by J.T. Baker. All other chemicals were used as received without further purification. Ultra-pure water (Millipore-Q® Gradient A10™ ultra-pure water system, Millipore, France) was used throughout the study.

3.2 Critical micelle concentration determination

The critical micelle concentration (CMC) of sucrose monolaurate, Cremophor RH40 and Tween 80 in pure water were published as shown in **Table 3.1**. In this study, sucrose laurate containing a mixture mono-, di-, and tri-laurate was used. Deviation of the CMC from published value is possible. The CMC of the

sucrose laurate mixture was evaluated by surface tensiometry in pure water at 25 ^{o}C. Sucrose laurate in concentration ranging from 0.0005 mg/ml to 10 mg/ml in ultra-pure water were prepared and their surface tensions (γ, mN/m) were measured by Attension Sigma 700 Force Tensiometer using a standard Du Noüy ring [R(ring) = 9.58 mm, R(wire) = 0.185mm]. The surface tension was measured at fixed interval for ten minutes until the values agreed within 0.1 mN/m of each other for three consecutive readings. The final surface tension was recorded. All samples were prepared in triplicate and the mean values were taken.

Table 3.1 Critical micelle concentration of sucrose monolaurate, Cremophor RH40 and Tween 80 in pure water at 25 ^{o}C

Surfactant	**CMC [mg/ml]**	**Reference**
Sucrose monolaurate	0.18	Youan *et al.*, 2003
Cremophor RH40	0.1	Matsaridou *et al.*, 2012
Tween 80	0.017	Kerwin, 2007

A plot of surface tension vs logarithm of concentration was obtained for the determination of CMC values. At very low surfactant concentration, the surface tension decreased with increased in surfactant concentration. The CMC therefore corresponds to the concentration at which the surface tension does not continue to change significantly with further increase in surfactant concentration.

Additionally, the formulations were prepared with citrate buffer containing Zn^{2+} metal ions as the aqueous phase. As the CMC of the surfactants can be affected by the addition of buffer and divalent salts, the operative CMC of the respective surfactants were also determined in the aqueous phase in the same manner. Solutions of sucrose laurate, Cremophor RH40 and Tween 80 were prepared in the aqueous citrate buffer with Zn^{2+} metal ions at different concentrations, ranging from 0.0001 mg/ml to 10 mg/ml and their CMC values were estimated based on surface tensions measurement.

3.3 Formulation development and stability study

Sucrose laurate, Cremophor RH40 and Tween 80 were selected for the development formulation of oxytocin in 10 mM citrate buffer pH 4.5 consisting of 2 mM Zn^{2+} divalent metal ions. The formulation development process is outlined in **Figure 3.1**. 10 mM citrate buffer at pH 4.5 with of 2 mM Zn^{2+} divalent metal ions was prepared as aqueous phase and used as the control experiment. Sucrose laurate, Cremophor RH40 and Tween 80 were solubilized in the aqueous phase at 0.1 mg/ml each. 0.1 mg/ml of oxytocin was then solubilized in each of the micellar formulations. The formulations were stored at $40^{o}C$ for three months and the recovery of oxytocin was analyzed using High-Performance Liquid Chromatography (HPLC) to compare the thermal stability of oxytocin in the different micellar formulations against the control. Subsequently, the concentrations of surfactants used were increased up to their maximum tolerable limit for intranasal delivery, i.e. 5 mg/ml and 1 mg/ml for Cremophor RH40 and Tween 80 respectively, and the thermal

stability of oxytocin in these formulations were monitored. For sucrose laurate, 0.1 mg/ml was already the maximum non-toxic dose and was therefore excluded for this part of the study. Lastly, mucoadhesives, carboxymethylcellulose was added to the formulations that demonstrated enhanced thermal stability of oxytocin in aqueous formulations and its effect on thermal stability of oxytocin was evaluated.

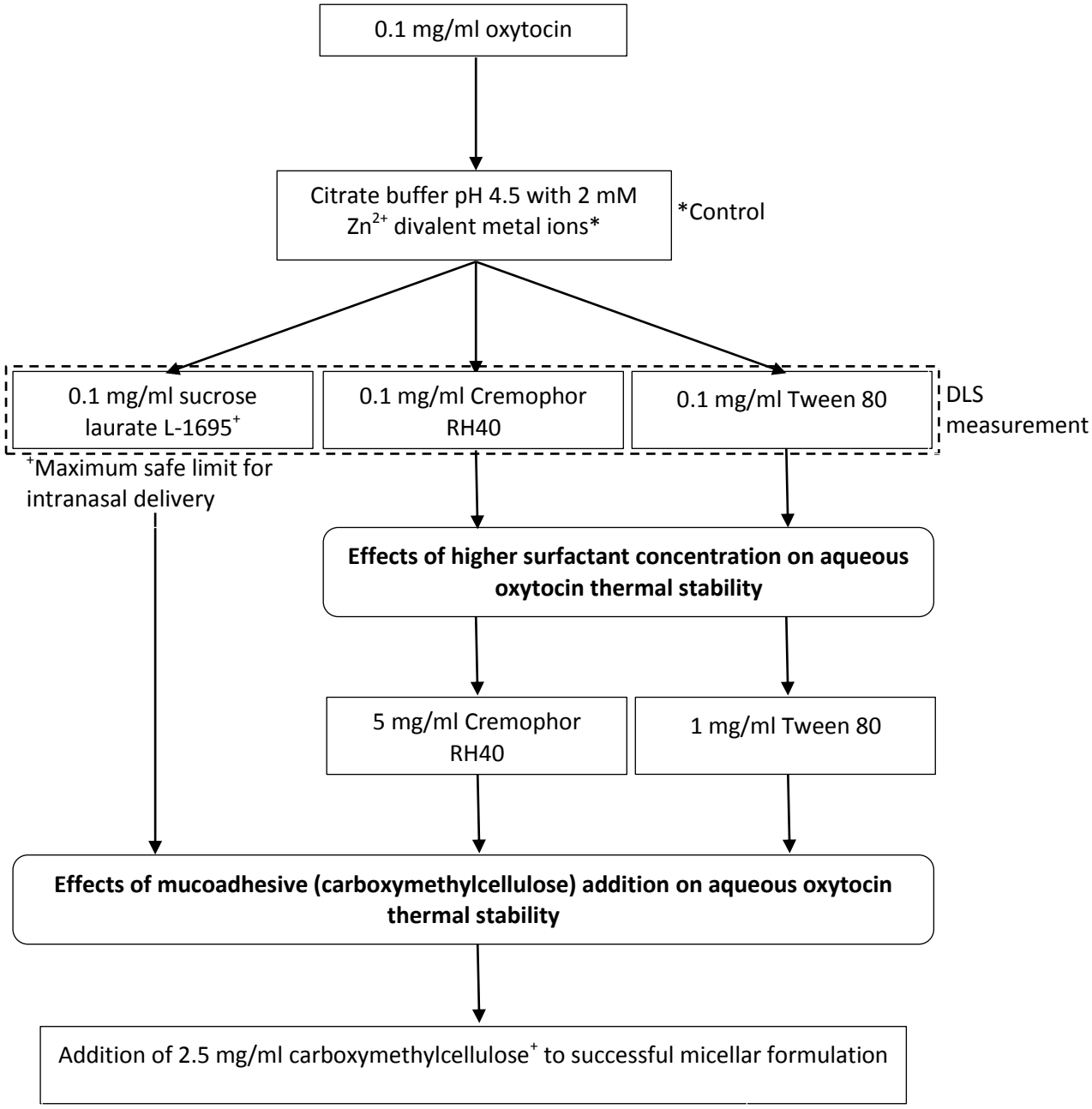


Figure 3.1 Formulation design procedure

3.4 Micellar droplet size and polydispersity index measurement by Dynamic Light Scattering

The micellar droplet size and polydispersity index of 0.1 mg/ml of sucrose laurate, Cremophor RH40 and Tween 80 formulations with 0.1 mg/ml oxytocin were measured, for the temperature range between 20 and 60 ^{o}C, by the Dynamic Light Scattering (DLS) technique. The Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., UK) was used, which consists of a 4mW He-Ne laser (λ = 633 nm) with detection angle 173°. The change in intensity pattern of light scattered by the droplets in the micellar formulation is tracked and this is use for the determination of diffusion coefficient. The Stokes-Einstein equation (**Equation 3.1**) is utilized to calculate the hydrodynamic diameter, d_H, of the droplet based on the diffusion coefficient, assuming the droplets are spherical. The detection range of the instrument is between 0.6 nm to 6 μm.

$$D = \frac{k_B T}{3\pi\eta d_H} \quad (\textbf{Equation 3.1})$$

where k_B is the Boltzmann constant; T is absolute temperature; η is viscosity and D is diffusion coefficient. Samples were measured undiluted. Three independent experiments (N = 3) were performed and each experiment was conducted with five replicates (n = 5).

3.5 Quantitative analysis by High-Performance Liquid Chromatography

The concentration of oxytocin in the aqueous formulations were quantified using an Agilent 1100 series HPLC system which consists of a standard quaternary pump, a variable wavelength detector (VWD), an auto-sampler and a vacuum degasser (Model G1311A) controlled by Chemstation software. Chromatographic column, ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 micron packing size, Agilent Technologies, was used for separation of oxytocin from the rest of the excipients in the formulation. The mobile phase is a mixture of 80 vol. % 80 mM Phosphate Buffer pH 5 and 20 vol. % acetonitrile. The elution flow rate was maintained at 1 ml/min with a sample injection volume of 20 μl. The UV detection wavelength was fixed at 220 nm. The analysis was carried out at 25 ^{o}C. The retention time of oxytocin was approximately 7 minutes.

The initial amounts of oxytocin were measured in each of the formulations using HPLC in terms of peak absorbance area. Oxytocin concentrations in the formulations were monitored over three months. The recovery of oxytocin was calculated as percentage of the initial peak absorbance area. For each formulation, three same samples were prepared for statistical evaluation of mean and standard deviation.

3.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the results performed in **Section 3.5**. The results were deemed statistically different when $p < 0.05$.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Critical micelle concentration determination of micellar formulation

Based on surface tension measurement as shown in **Figure 4.1**, the CMC of sucrose laurate in pure water at 25 ^{o}C is estimated at 0.205 mg/ml. This value is similar to published data which was displayed in **Table 3.1**. Even though sucrose laurate is a mixture of sucrose mono-, di- and tri-laurate, the measured CMC is approximately the same as that of pure sucrose monolaurate in water.

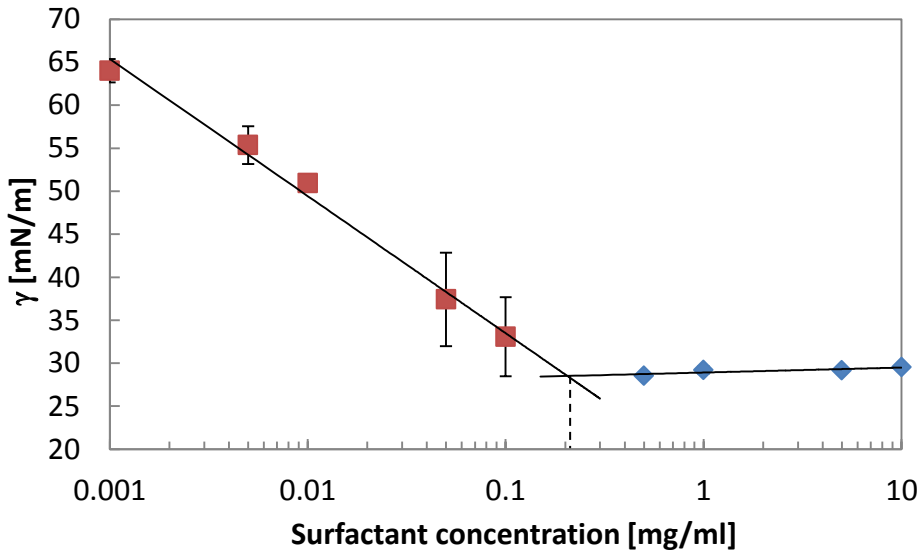


Figure 4.1 Surface tension as a function of sucrose laurate L-1695 concentration in pure water for determination of critical micelle concentration.

For nonionic surfactant, the CMC value may be influenced by the addition of salt which can either increase or decrease the CMC depending on the type of surfactant and salt used (Kronberg *et al.*, 2014). Furthermore, sucrose laurate was used at a concentration less than its CMC value in pure water. It is

therefore important to evaluate the operative CMC value of sucrose laurate in the aqueous citrate buffer solution with Zn^{2+} ions to confirm if micellization takes place.

The surface tension measurement of sucrose laurate L-1695, Cremophor RH40 and Tween 80 in citrate buffer pH 4.5 with Zn^{2+} ions are plotted as shown in **Figure 4.2-4.4**. The CMC at 25 ^{o}C were tabulated in **Table 4.1**. It can be observed that the presence of inorganic salt resulted in an order of magnitude reduction in CMC values for sucrose laurate and Cremophor RH40 solutions but an increased in CMC value for Tween 80. In general, a change in CMC is largely related to the salting effect by electrolyte addition (Carale et al., 1994; Ray et al., 1971; Ruiz, C.C., 2008). CMC decreases due to salting out effect while CMC increase because of salting in effect. Salts are either 'water-structure-making' or 'water-structure-breaking' in nature (Nishikido et al., 1977; Ruiz, C.C., 2008). The presence of salts in the aqueous environment can either promote hydration or dehydration of the surfactant molecules. The salting out effect occurs when the addition of water-structure-making salts resulted in attraction of water molecules around the ions, reducing the water molecules in the bulk, causing the dehydration of the surfactant molecules. The solubility of the surfactant is decreases, resulting in greater driving force for micellization, therefore reducing the CMC. On the other hand, water-structure-breaking salts increase the amount of water molecules in the bulk solutions, allowing better hydration of the surfactant molecules which is the salting in effect. Solubility of the surfactant is increased and CMC is increased. In addition, Zn^{2+} ions can form complex with ether linkages in

polyoxyethylene, which imparts a positive charge to the polar head group (Schott, 1973; Schott et al., 1976). The nonionic surfactant becomes cationic and the resultant electrostatic repulsion between cationic molecules raises the CMC, implying salting in.

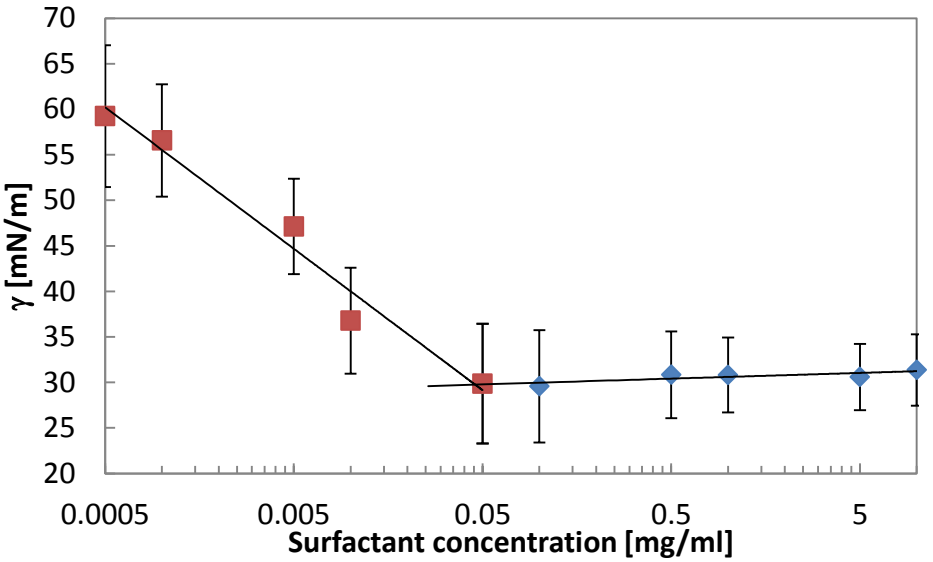


Figure 4.2 Surface tension as a function of sucrose laurate concentration in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions for determination of critical micelle concentration.

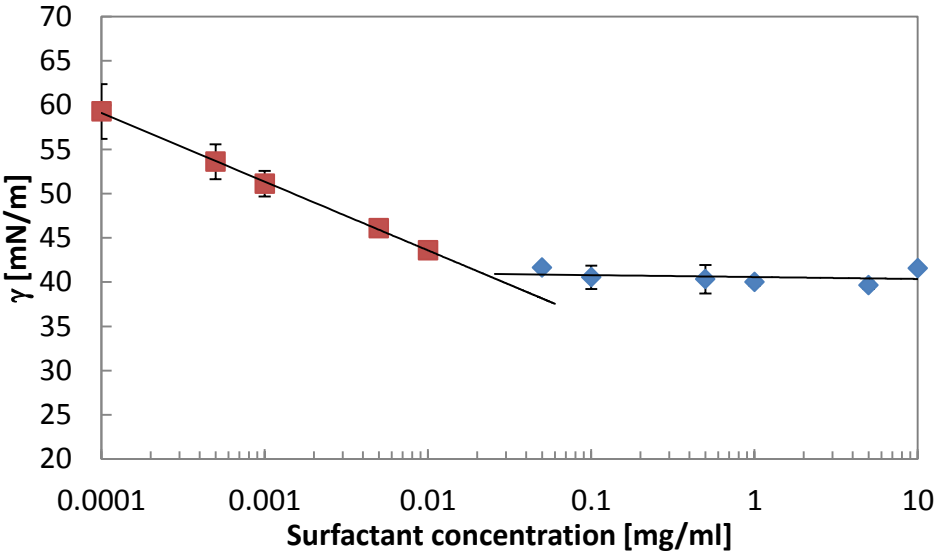


Figure 4.3 Surface tension as a function of Cremophor RH40 concentration in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions for determination of critical micelle concentration.

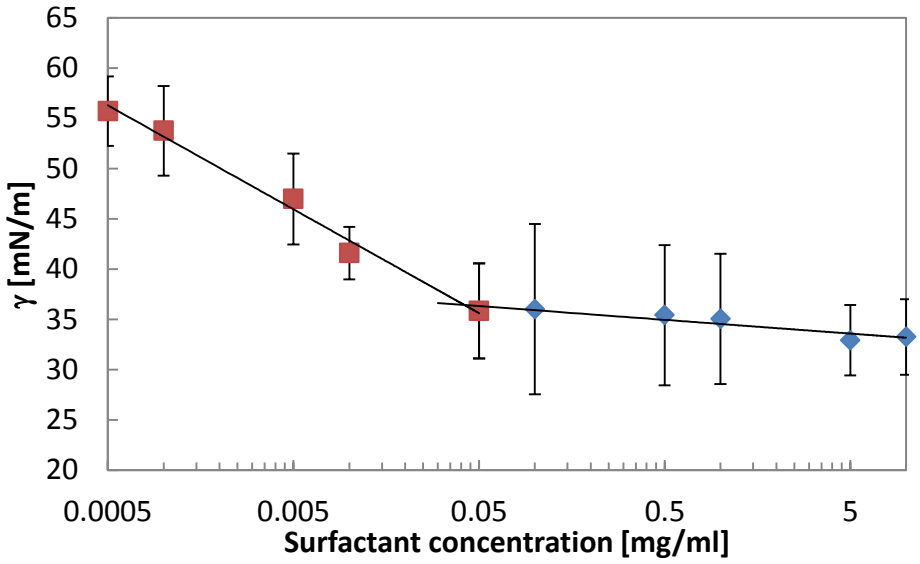


Figure 4.4 Surface tension as a function of Tween 80 concentration in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions for determination of critical micelle concentration.

Table 4.1 Critical micelle concentration of sucrose laurate L-1695, Cremophor RH40 and Tween 80 in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions at 25 ^{o}C.

Surfactant	CMC in pure water [mg/ml]	Measured CMC in citrate buffer with Zn^{2+} [mg/ml]
Sucrose laurate L-1695	0.205	0.046
Cremophor RH40	0.1	0.022
Tween 80	0.017	0.042

In this case, sucrose laurate does not contain polyoxyethylene group and hence, salting out by dehydration of surfactant molecules is probable. For Cremophor RH40 micellar formulation, complex formation with Zn^{2+} may be impeded due to steric interference. Dehydration effect of the surfactant molecules is likely to be more dominant compared to complexation with Zn^{2+}. Therefore,

salting out of the surfactant leads to reduction in CMC. For Tween 80, it may be possible that salting in effect due to complex formation with Zn^{2+} is more dominant than salting out effect due to dehydration of surfactant molecules. Therefore, CMC is increased.

For all the formulations prepared for this study, the surfactants are used at concentration above CMC. Hence, it is reasonable to regard them as micellar formulations. More specifically, these formulations are normal micellar systems since the disperse phase is the aqueous environment.

4.2 Physical stability of oxytocin in micellar formulation

The change in the hydrodynamic droplet diameter of the micelle in the formulation was measured by DLS at temperature ranging from 20 to 60 ^{o}C. **Figures 4.5-4.7** show the results obtained from DLS measurement. Based on **Figures 4.5(i)**, there is a general increased in the hydrodynamic droplet diameter with increased in temperature, demonstrating the temperature dependence of the micellar formulations of sucrose laurate. The droplet size for sucrose laurate micellar formulation increased from 259.4 to 482.7 nm when the temperature is increased from 20 to 60 ^{o}C. On the contrary, with reference to **Figure 4.6(i)** and **4.7(i),** the micellar droplet sizes for the Cremophor RH40 and Tween 80 formulations were not significantly affected by temperature. The mean hydrodynamic diameter fluctuates between 14.18 and 15.19 nm for Cremophor RH40 and 10.92 and 24.47 nm, implying that

micellar formulation of Cremophor RH40 and Tween 80 has better thermal stability up to 60 ^{o}C as compared to that of sucrose laurate.

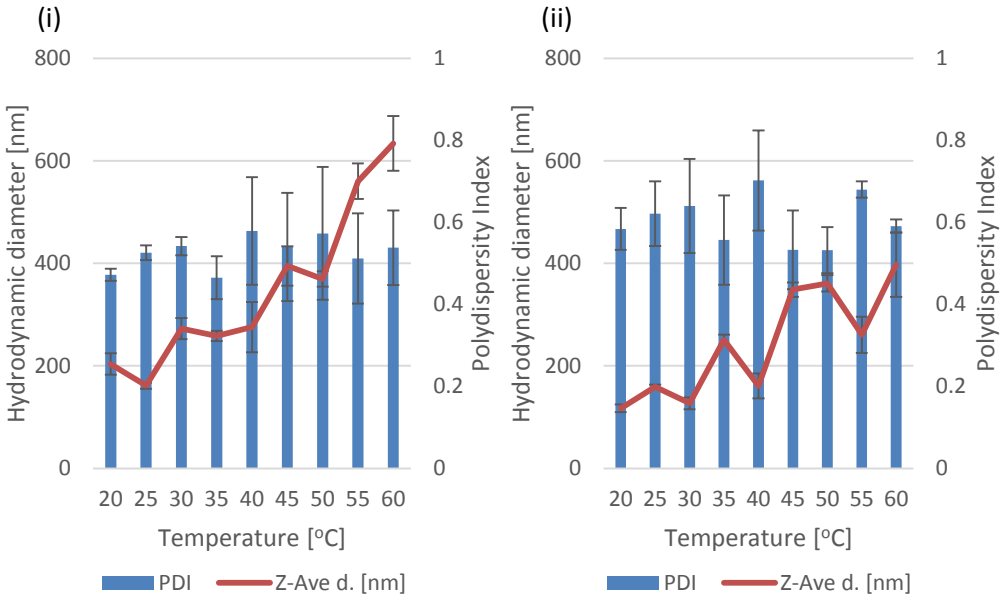


Figure 4.5 Hydrodynamic diameter (d_H) and polydispersity index of droplets in **(i)** 0.1 mg/ml sucrose laurate **(ii)** 0.1 mg/ml sucrose laurate with 0.1 mg/ml oxytocin formulations. (n = 5)

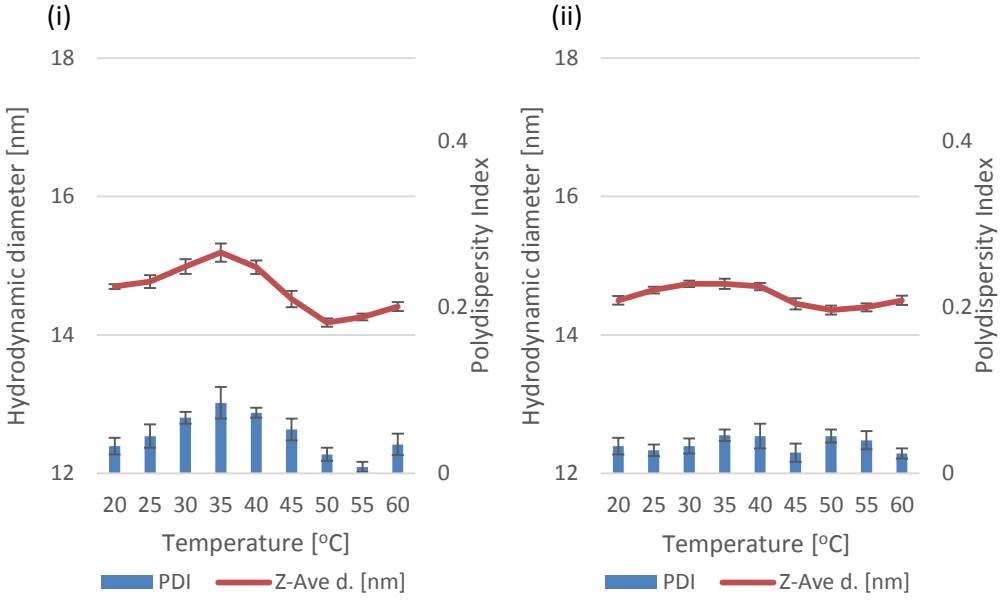


Figure 4.6 Hydrodynamic diameter (d_H) and polydispersity index of droplets in **(i)** 0.1 mg/ml Cremophor RH40 **(ii)** 0.1 mg/ml Cremophor RH40 with 0.1 mg/ml oxytocin formulations. (n = 5)

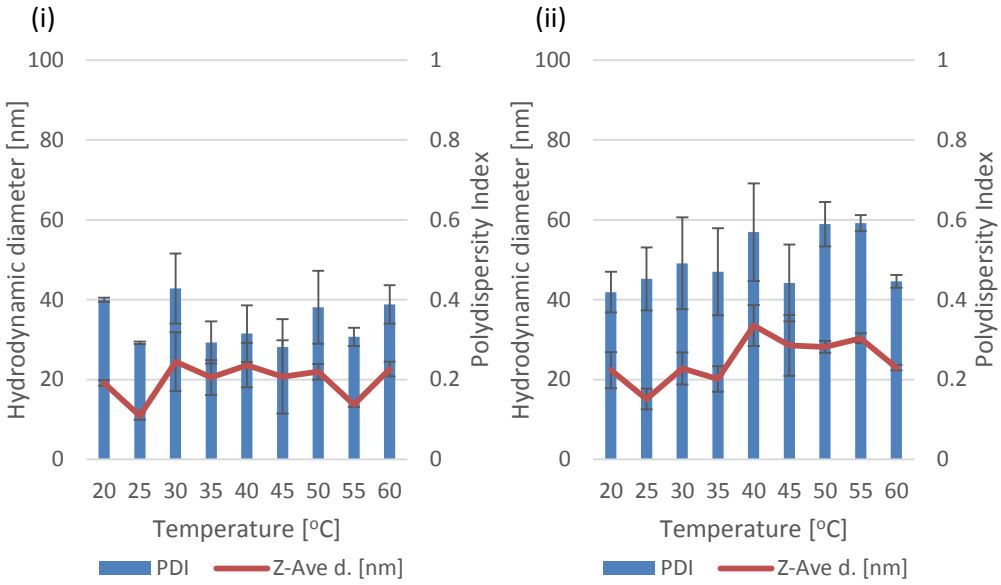


Figure 4.7 Hydrodynamic diameter (d_H) and polydispersity index of droplets in **(i)** 0.1 mg/ml Tween 80 **(ii)** 0.1 mg/ml Tween 80 with 0.1 mg/ml oxytocin formulations. (n = 5)

Large micelles tend to be very polydisperse (Kronberg *et al.*, 2014). Sucrose laurate micellar droplets are larger than those of Cremophor RH40 and Tween 80. The mean polydispersity index of the Cremophor RH40 and Tween 80 micellar formulations are both less than 0.5 between 20 and 60 ^{o}C while that for the sucrose laurate micellar formulation fluctuates between 0.465 and 0.579 in the same temperature range. This shows that the micellar droplet of the Cremophor RH40 and Tween 80 formulations are relatively more homogeneous than that of the sucrose laurate formulation. For the Cremophor RH40 formulation it is the most homogeneous, with polydispersity index of less than 0.1 at the given temperature range depicting monodispersed sample. However, for Tween 80 and sucrose laurate formulations, PDI are above 0.2, implying broad particle size distribution or bimodal size distribution.

Figure 4.5(ii), 4.6(ii) and **4.7(ii)** show the effect of temperature on the droplet size and polydispersity index of the micellar formulations with the addition of oxytocin. It can be seen that the micellar droplet size for sucrose laurate formulation is reduced after oxytocin is added. In addition, a slight increase in the mean polydispersity index of the sample across the temperature range was detected. Conversely, the droplet size and polydispersity index for Cremophor RH40 micellar solution did not change significantly with the introduction of oxytocin into the formulation. For Tween 80 formulation, oxytocin addition resulted in a slight increase in the average droplet size of the micellar formulation, ranging between 15.13 and 33.54 nm in the temperature 20 to 60 ^{o}C. Additionally, the formulation is slightly more polydispersed with polydispersity index increasing to above 0.5 between 20 and 60 ^{o}C.

It is evident that oxytocin showed negligible interaction with the base Cremophor RH40 micellar formulation since the hydrodynamic diameter and polydispersity index is relatively unaffected by the addition of oxytocin. However, interactions of oxytocin with sucrose laurate and Tween 80 micelles are clearly present, resulting in changes in the droplet sizes and polydispersity indices.

For sucrose laurate micelles, the reduction in droplet size by the addition of oxytocin is possibly due to oxytocin behaving like a co-surfactant. A co-surfactant is amphiphilic consisting of terminal hydroxyl group. It interacts with the micelle at the interface which then affects the micellar curvature and interfacial energy (Alany *et al.,* 2000). This results in the increase in

interfacial fluidity due to the penetration of co-surfactant into the surfactant monolayer and creates void spaces among surfactant molecules in the monolayer (Constantinides *et al.*, 1997). The bending rigidity of the monolayer is then reduced (Kozlov *et al.*, 1992) which favors the formation of smaller micelles (El Maghrab, 2008). Oxytocin is made up of amino acids that are both polar and nonpolar as simplified in **Figure 4.8**. The three-membered tail, Pro^{7} – Leu^{8} – Gly^{9}, are all nonpolar amino acids while the six-membered ring, Cys^{1} – Tyr^{2} – Ile^{3} – Gln^{4} – Asn^{5} – Cys^{6}, consists of all polar amino acids except Ile^{3} which is nonpolar. Based the structural analysis, it seems that oxytocin exhibits amphiphilic behavior. In addition, the tyrosine amino acid has a terminal hydroxyl group. Therefore, it can be postulated that addition of oxytocin reduces the droplet size of the sucrose laurate micelle due to co-surfactant behavior of oxytocin.

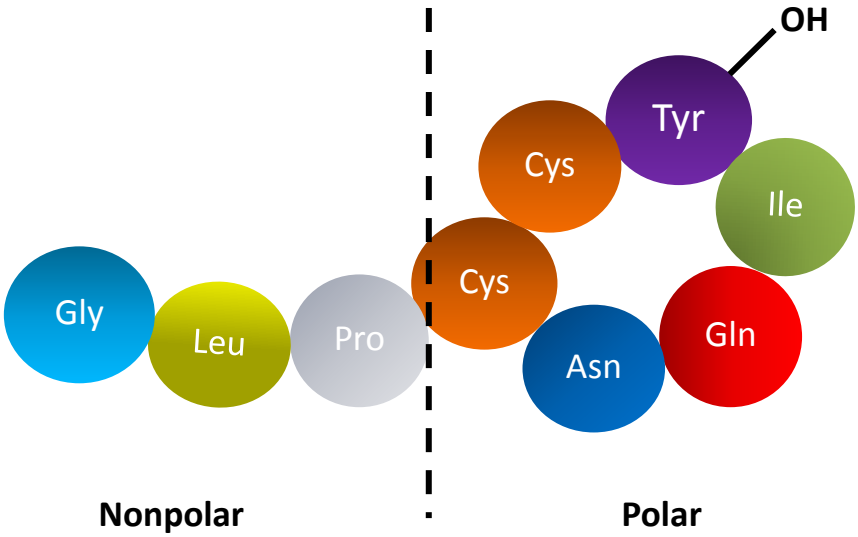


Figure 4.8 Simplified chemical structure of oxytocin showing potential amphiphilc behavior of the peptide.

For Tween 80, it may be possible that the oxytocin molecules interact with the interface of the Tween 80 micelles, therefore resulting in slightly larger droplet size. In general, there are several possible site where micellar solubilization can take place in a normal micellar system depending on the hydrophilicity/lipophilicity of the solubilizate. With reference to **Figure 4.9** (Tadros, 2005), lipophilic solubilisate is incorporated in the non-polar core while hydrophilic solubilisate can either be located within the core, short or deep penetration, or be adsorbed on the micellar surface. In addition, for surfactants with polyoxyethylene chain in the polar head group, hydrophilic drug can also be trapped within the polyoxyethylene chains of the micelle. The slight increase in the droplet size of Tween 80 micelles by the addition of oxytocin is probably due to the solubilization of oxytocin by the Tween 80 micelles at the surface or shallow penetration into the polar head group.

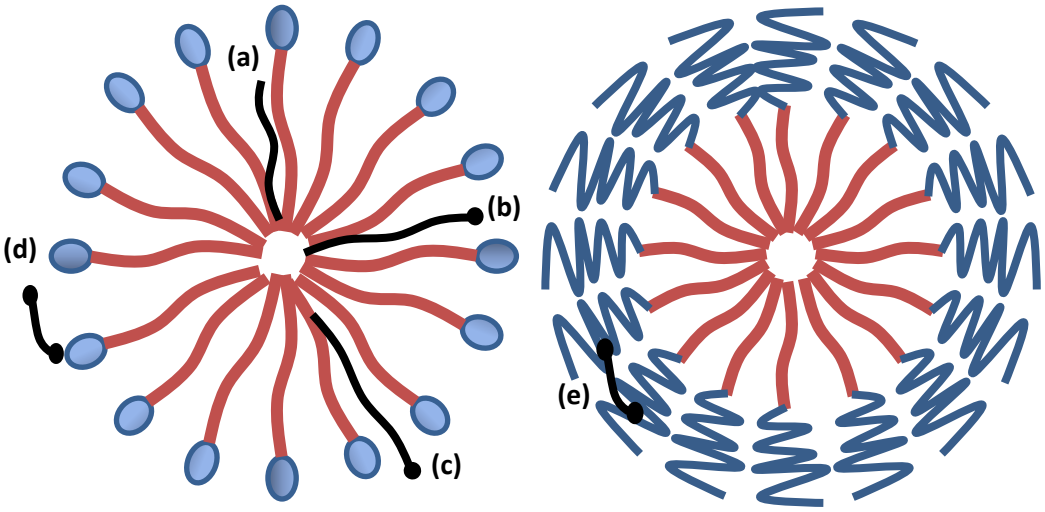


Figure 4.9 Site of incorporation of solubilisate: (a) in the hydrocarbon core; (b) deep penetration; (c) short penetration; (d) adsorption; (e) in the polyoxyethylene chain (Tadros, 2005).

4.3 Thermal stability of oxytocin in micellar formulation

In this section, the thermal stability of oxytocin in micellar formulations was studied by quantitatively analyzing the amount of oxytocin recovered from the micellar formulations on storage at 40 ^{o}C for three months. The recovery of oxytocin over time at storage temperature of 40 ^{o}C was influenced by the choice of surfactant used in the aqueous solution. The concentration of sucrose laurate L1695, Tween 80 and Cremophor RH40 were fixed at 0.1 mg/ml and the stability of oxytocin in these solutions were compared with that in control which is the citrate buffer with Zn^{2+} divalent metal ions as shown in **Figure 4.10**. After three months, the average oxytocin recovery in the control sample is 73.1%. In comparison, average recovery of oxytocin in 0.1 mg/ml each of Tween 80 and Cremophor RH40 micellar formulations are lower at 70.7% and 72.4% respectively ($p > 0.05$), but is not statistically significant. Conversely, for the micellar formulation with 0.1 mg/ml sucrose laurate L1695, average oxytocin recovery is 76.5%, demonstrating enhanced thermal stability ($p < 0.05$) and is statistically significant.

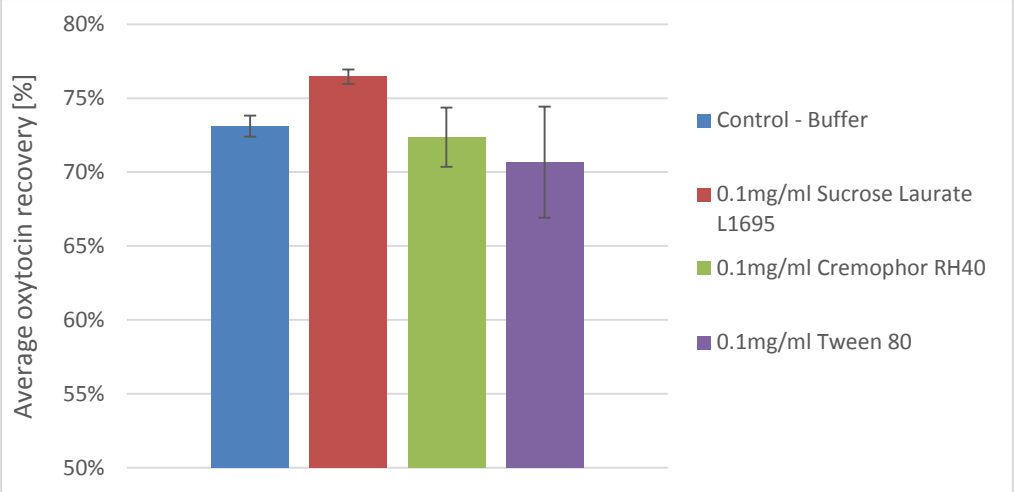


Figure 4.10 Average oxytocin recovery in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions with 0.1 mg/ml of sucrose laurate L-1695, Cremophor RH40 and Tween 80 on storage at 40 ^{o}C for three months.

In order to discuss the difference in the stabilization or destabilization of oxytocin in the various aqueous micellar formulations, it is essential to identify the interactions that may be present. Since the surfactants are used at concentrations above their CMC values, it is likely that micellization took place in the citrate buffer with Zn^{2+} metal ions. Additionally, CMC decreases with increased in temperature for nonionic surfactant (Kronberg *et al.*, 2014). Therefore at storage temperature of 40 ^{o}C, micelle structure was preserved.

In general, these aqueous micellar formulations consists of micelles, salts and a peptide (oxytocin). Since oxytocin is a hydrophilic peptide, it is possible that it is localized in the dispersed phase or at the micellar interface as shown in **Figure 4.11**. In the dispersed phase, the stability of oxytocin may be influenced by the interaction with salt or metal ions present in the aqueous buffer solution. At the interface, the peptide may interact with the polar head groups of the surfactants. Additionally, change in properties of the surfactant micelles due to the presence of salt or metal ions may affect the thermal

stability of oxytocin in aqueous formulations. Furthermore, any interactions with the micelle is likely to occur at the interface. It is therefore useful to analyze the chemical structures of the surfactants and identify the functional group that made up the polar interface of the micelles as shown in **Figure 4.12**. For sucrose laurate, the hydrophilic polar head group is made up of sucrose functional group while that for Tween 80 consists of polyoxyethylene groups. For Cremophor RH40, the polar head group is made up of polyethylene glycols and glycerol ethoxylate.

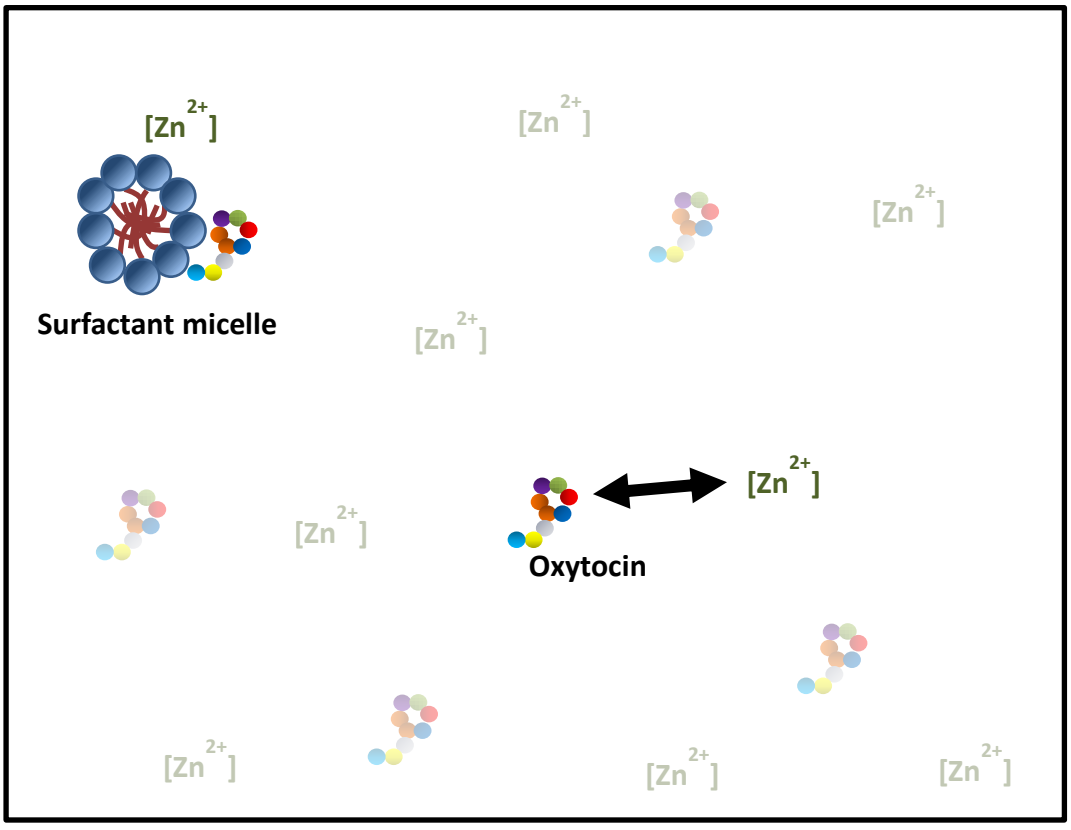
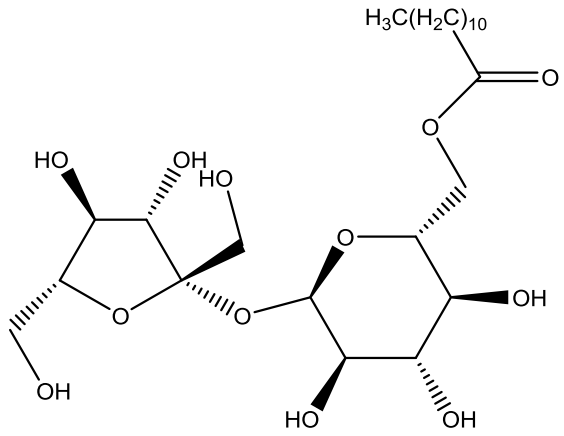
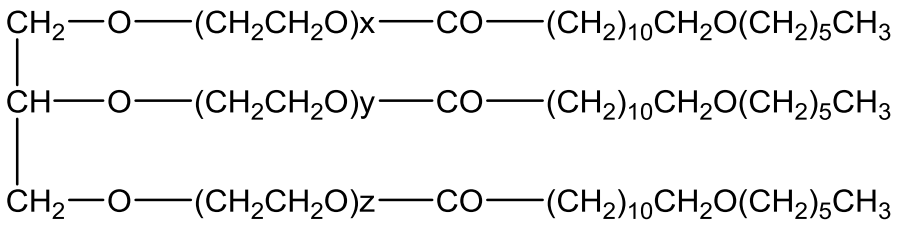


Figure 4.11 Illustration of the interactions that are possible in an aqueous environment with surfactant, salt and oxytocin.

(i)

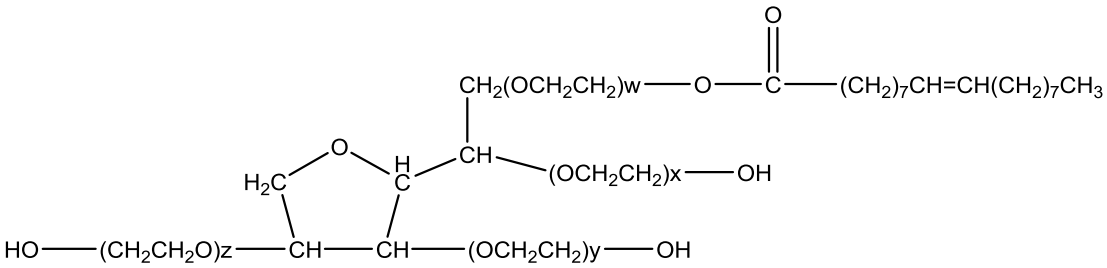


(ii)



where x + y + z = 40

(iii)



where w + x + y + z = 20

Figure 4.12 Chemical structure of (i) sucrose monolaurate (ii) Cremophor RH40 and (iii) Tween 80.

As discussed previously, micelles of Cremophor RH40 showed negligible interaction with the added oxytocin. Hence, oxytocin largely remains in the dispersed phase. Complex formation of Zn^{2+} ions with the ether of polyoxyethylene group may be hindered by steric interference. Inorganic counterions are loosely associated to the micelle and are very mobile (Kronberg *et al.*, 2014). With reference to **Figure 4.13**, it is likely that bulk Zn^{2+} ions remain in the dispersed phase with small amount of Zn^{2+} ions weakly interacting with the interface of the Cremophor RH40 micelles. Oxytocin stabilization by divalent metal ions with citrate buffer is still dominant with the presence of Cremophor RH40 micelles. Since a small amount of Zn^{2+} interacts with the Cremophor RH40 micelles, it is possible that oxytocin stabilization effect by Zn^{2+} ions is reduced. Therefore, the average oxytocin recovery is slightly lower in the Cremophor RH40 formulation than that in the control citrate buffer with Zn^{2+} ions.

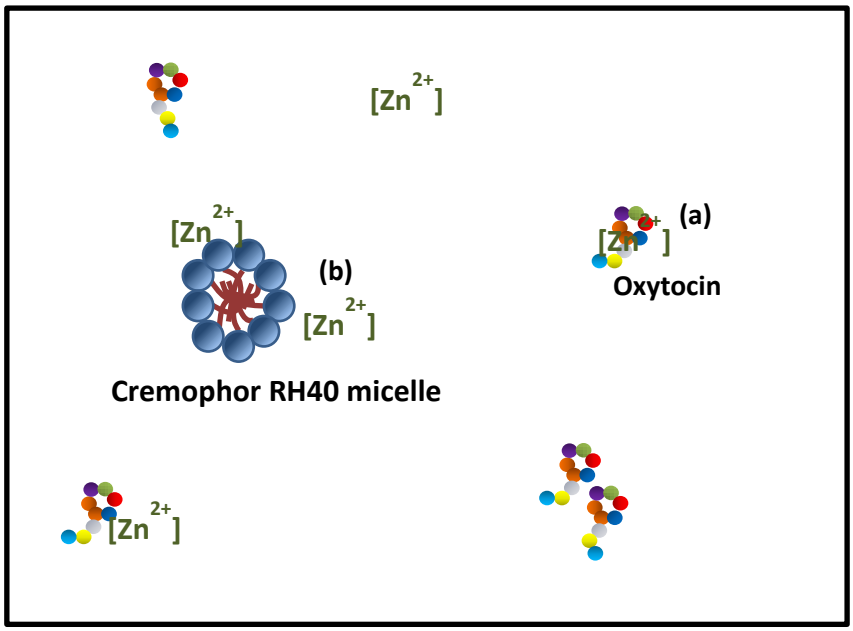


Figure 4.13 Illustration of the (a) complex formation of Zn^{2+} metal ions with oxytocin, and (b) loose association between Cremophor RH40 micelle and Zn^{2+} metal ions in a system with Cremophor RH40 micelles, oxytocin and Zn^{2+} divalent metal ions.

Oxytocin stability in aqueous formulation was negatively influenced by the presence of Tween 80 micelles. In this system, both stabilization and destabilization effects may occur. As illustrated in **Figure 4.14**, first, there exists a stabilization effect of oxytocin by the Tween 80 micelle due to interaction of oxytocin molecules with the polar head group at the interface of the micelles as identified during the thermal stability analysis by DLS. The interaction may prevent the degradation of oxytocin due to dimerization and aggregation by restricting solvent accessibility. However, the very slight increase in the micellar droplet size with the addition of oxytocin may imply that oxytocin interaction at the Tween 80 micellar surface is small and that most of the oxytocin molecules may still remain in the dispersed phase. In the

dispersed phase, some of the Zn^{2+} ions may have formed complex with the ether at the polar head group of Tween 80 micelles. This implies that there is a competition for interaction with Zn^{2+} ions between the Tween 80 micelles for complexation and oxytocin for stabilization. As such, the amount of Zn^{2+} ions remaining in the dispersed phase for oxytocin stabilization is substantially reduced which resulted in poorer thermal stability. In this system, it seems that the destabilization effect may be stronger than the stabilization effect which explains the poorer thermal stability of oxytocin in the Tween 80 aqueous formulation.

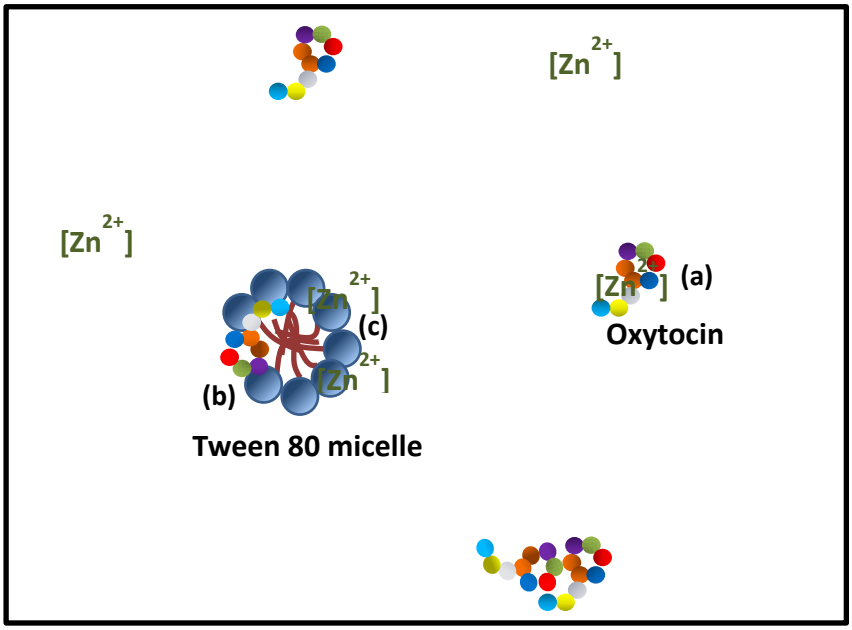


Figure 4.14 Illustration of the (a) complex formation of Zn^{2+} metal ions with oxytocin, (b) micellar interaction between Tween 80 micelle and oxytocin at the interface, and (c) complex formation between Zn^{2+} metal ions and the ether functional group in the polar head group of Tween 80 surfactant, in a system with Tween 80 micelles, oxytocin and Zn^{2+} divalent metal ions.

In addition to interactions of oxytocin with the Tween 80 micelles and the Zn^{2+} ions, impurities present in Tween 80 surfactant is also likely to affect the thermal stability of oxytocin in aqueous phase. It is well known that low level of residual peroxide are found in Tween surfactants which may accumulate

during long-term storage. This can potentially result in direct degradation of pharmaceutical actives or changes to the physico-chemical properties of the surfactant (Chou *et al.*, 2005; Ha *et al.*, 2002). Therefore, this may be unfavorable to the thermal stability of oxytocin in aqueous formulation as well.

For sucrose laurate formulation, oxytocin may be stabilized in the dispersed phased and/or at the interface of the sucrose laurate micelle. As shown in **Figure 4.15**, oxytocin in the aqueous phase is stabilized by the Zn^{2+} ions that are present. At the interface, oxytocin exhibits co-surfactant-liked behavior and is likely to penetrate into the sucrose laurate monolayer of the micelle. This may prevents the oxytocin from contact with the aqueous environment, thereby suppressing disulfide exchange reaction due to protonation of the disulfide bridge. Furthermore, aggregation of oxytocin molecules can also be avoided since they are held within the micellar interface.

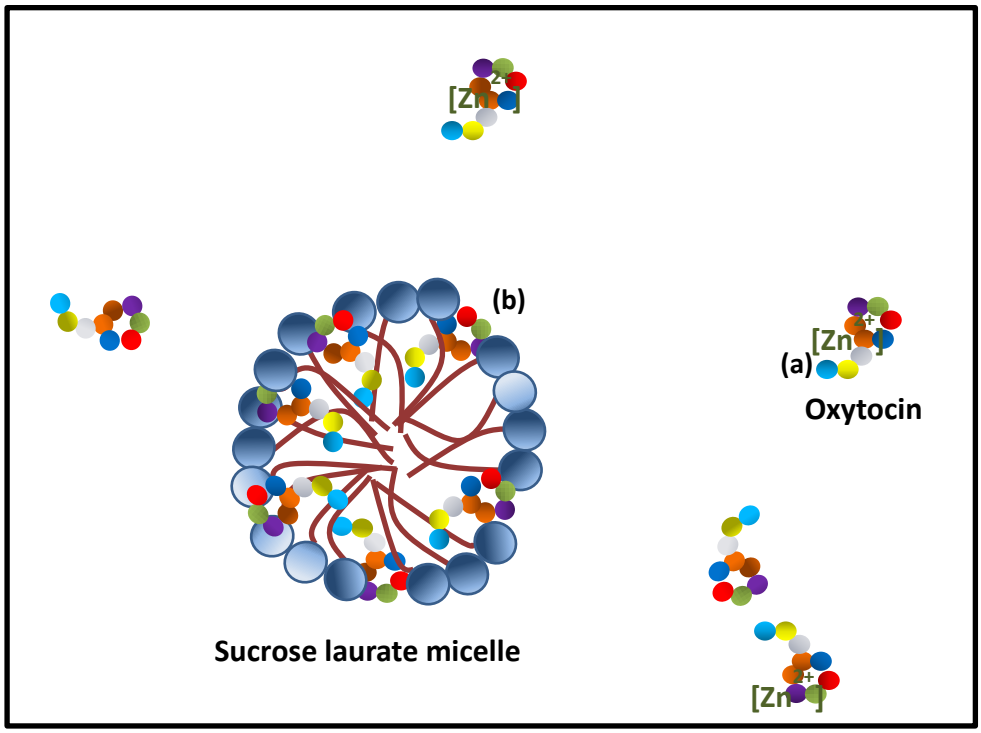


Figure 4.15 Illustration of the (a) complex formation of Zn^{2+} metal ions with oxytocin, (b) micellar interaction between sucrose laurate micelle and oxytocin at the interface, where oxytocin exhibits co-surfactant-liked behavior, in a system with sucrose laurate micelles, oxytocin and Zn^{2+} divalent metal ions.

There have been studies showing that sucrose addition in aqueous formulation exhibits stabilizing effect on peptide and protein against aggregation, deamidation and oxidation (Kamberi *et al.*, 2005; Ueda *et al.*, 2001). Kamberi and coworkers studied mechanism for the stabilization of two peptides, a human brain natriuretic peptide [hBNP(1–32)] and a human parathyroid hormone [hPTH(1–34)], using sucrose (2001). They conclude in their work that the presence of sucrose aid in preserving the native conformation of the peptides, thus demonstrating the stabilizing effects. In the sucrose laurate micellar formulation with oxytocin, the sucrose functional group of the surfactant is exposed to the aqueous environment at the micelle interface. This

may also help to keep oxytocin in its native form, thereby providing additional stabilization against chemical and physical degradation.

On top of examining the different interactions that may stabilize/destabilize oxytocin in aqueous formulations, the temperature effect on the different surfactants may influence the average oxytocin recovery as well. Generally, nonionic surfactants that contain polyoxyethylene group are affected by clouding. When temperature is increased, the polar head group of the surfactant shrinks. This leads to weaken interaction between the polyoxethylene and water. As such, the surfactant hydration is reduced and becomes less water-soluble (Kronberg *et al.*, 2014). Above a specific temperature, which is the cloud point, the surfactant becomes insoluble in the aqueous phase and a cloudy solution results. Both Cremophor RH40 and Tween 80 contain polyoxyethylene functional group and are therefore be susceptible to clouding at elevated temperature. However, sucrose ester surfactants are unaffected by clouding and are not significantly affected by temperature increase (Kunieda *et al.*, 1993; Angeles Pes *et al.*, 1996). This is because, comparing with the long polyoxyethylene polar head group, sucrose polar head group is more compact and does not undergo large conformational changes by hydration (Angeles Pes *et al.*, 1996). In the Cremophor RH40 formulation, at storage temperature of 40 ^{o}C, since oxytocin interaction with the Cremphor RH40 micelle is minimal, the stability of oxytocin may not be substantially affected by cloud point. However, for Tween 80 formulation, micelle stabilization effect on oxytocin may be reduced due to the weaken interaction with the polar head group at storage temperature of 40 ^{o}C. This

may also contribute to the poor thermal stability of oxytocin in aqueous Tween 80 micellar formulation.

4.4 Effects of surfactant concentration on thermal stability of oxytocin in micellar formulation

In this section, emphasis is placed on determining the effect of concentration on the thermal stability of oxytocin for Cremophor RH40 and Tween 80. Sucrose laurate at 0.1 mg/ml exhibits positive thermal stabilization effect of oxytocin in aqueous formulation and is already the maximum non-toxic dose for intranasal delivery. Hence, it is interesting to investigate if Cremophor RH40 and Tween 80 at higher concentrations would enhance the thermal stability. Therefore, thermal stability monitoring was performed at 40 ^{o}C for Cremophor RH40 and Tween 80 at their maximum concentrations, 5 mg/ml and 1 mg/ml respectively, which can be safely administered intranasally. The results are shown in **Figure 4.16**. It seems that a higher concentration of surfactant does not correspond to better thermal stability of oxytocin in aqueous medium. Instead, the destabilization effects are more pronounced with increased in surfactant concentrations.

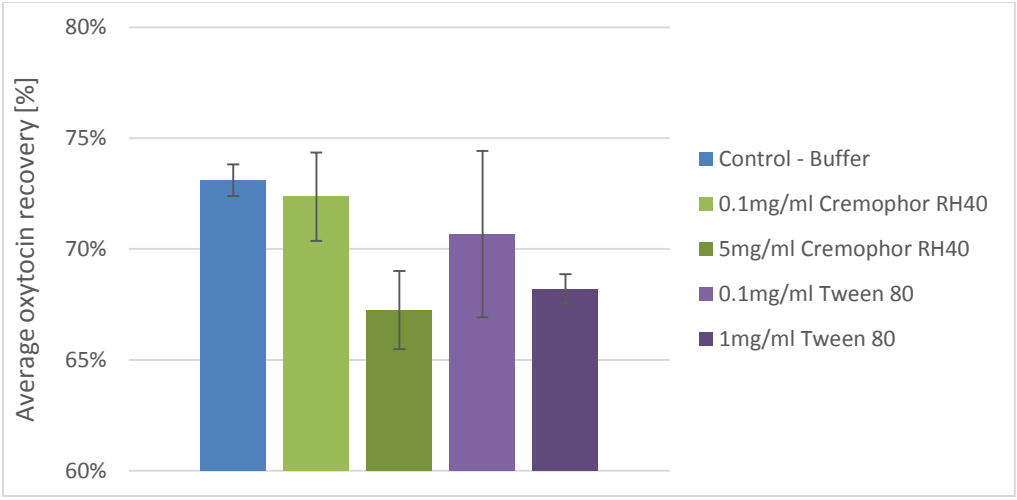


Figure 4.16 Average oxytocin recovery in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions with 0.1 mg/ml and 5 mg/ml of Cremophor RH40, and 0.1 mg/ml and 1 mg/ml Tween 80 on storage at 40 ^{o}C for three months.

For Cremophor RH40, higher surfactant concentration resulted in lower average oxytocin recovery ($p < 0.05$). With increased in surfactant concentration, the surface area of the micelles increased and creates more opportunity for Zn^{2+} to be weakly associated to the micellar interface. As a result, the amount of Zn^{2+} ions remaining in the dispersed phase to stabilize oxytocin is further reduced.

Conversely, for Tween 80, an increased in surfactant concentration of Tween 80 did not significantly affect the average oxytocin recovery ($p > 0.05$). Interaction between Zn^{2+} ions is likely more dominant compared with that between Zn^{2+} ions and the Tween 80 micelles.

4.5 Effects of addition of mucoadhesives on thermal stability of oxytocin in micellar formulation

In order to enhance the bioavailability of oxytocin, mucoadhesives can be added into the micellar formulations. It has been shown that sucrose laurate is the most suitable surfactant for development of intranasal delivery system for oxytocin in aqueous solutions as compared to Cremophor RH40 and Tween 80. Therefore, the effect of mucoadhesives addition on the thermal stability of oxytocin in aqueous solution will be investigated for the sucrose laurate micellar formulation. In this case, carboxymethycellulose sodium was chosen as the mucoadhesive use at 2.5 mg/ml which is the maximum tolerable dosage for intranasal delivery. The average oxytocin recovered from the formulations with mucoadhesives stored at 40 ^{o}C after three months were quantified as displayed in **Figure 4.17**.

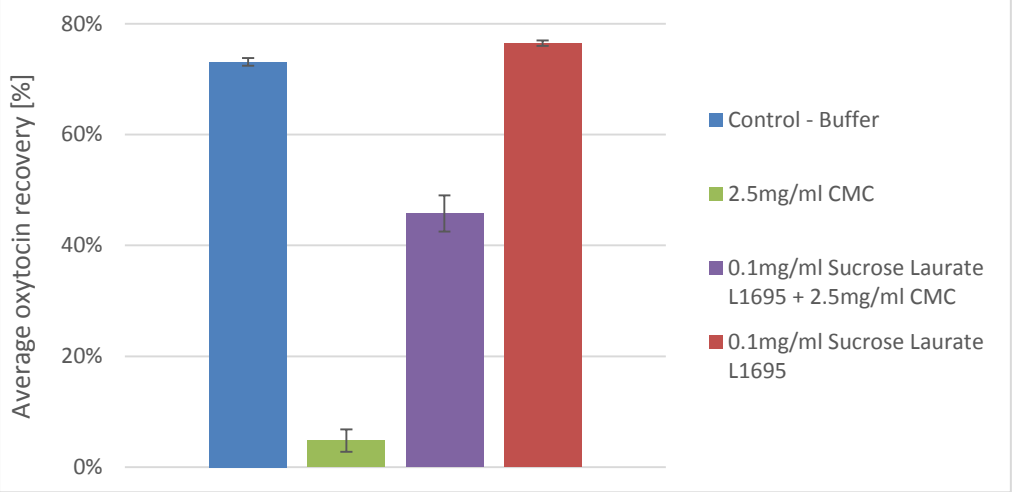


Figure 4.17 Average oxytocin recovery in citrate buffer pH 4.5 with 2 mM Zn^{2+} divalent metal ions with 2.5 mg/ml carboxylmethylcellulose, with and without 0.1 mg/ml of sucrose laurate on storage at 40 ^{o}C for three months.

Clearly, the addition of carboxymethylcellulose sodium as mucoadhesives is detrimental to the thermal stability of oxytocin in liquid formulation ($p < 0.05$). In a system with only carboxymethylcellulose sodium and oxytocin in the citrate buffer with Zn^{2+} metal ions, the average recovery of oxytocin from the formulation is only 4.8% after storage at 40 ^{o}C for three months. If sucrose laurate micelles were mixed with carboxymethylcellulose, the average oxytocin recovery was increased to 45.8%. Thermal stability of oxytocin was enhanced after sucrose laurate addition ($p < 0.05$).

Carboxylmethylcellulose is an anionic natural polymer. When it is dissolved in citrate buffer consisting of Zn^{2+} ions, it may interact with the Zn^{2+} ionically as shown in **Figure 4.18**. Ionic interaction between Zn^{2+} ions and carboxymethylcellulose is stronger than complex formation between Zn^{2+} ions and oxytocin. As such, oxytocin stabilization effect by the Zn^{2+} ions was drastically reduced. In **Figure 4.19**, the presence of sucrose laurate, some oxytocin molecules may be stabilized by the sucrose laurate micelles as discussed previously. Therefore, oxytocin recovery was increased. However, as compared to the micellar formulation of sucrose laurate without carboxymethylcellulose, the thermal stability of oxytocin is significantly poorer in the presence of carboxymethylcellulose. The use of carboxymethylcellulose as mucoadhesives needs to be reduced such that its influence on oxytocin thermal stability is kept minimum.

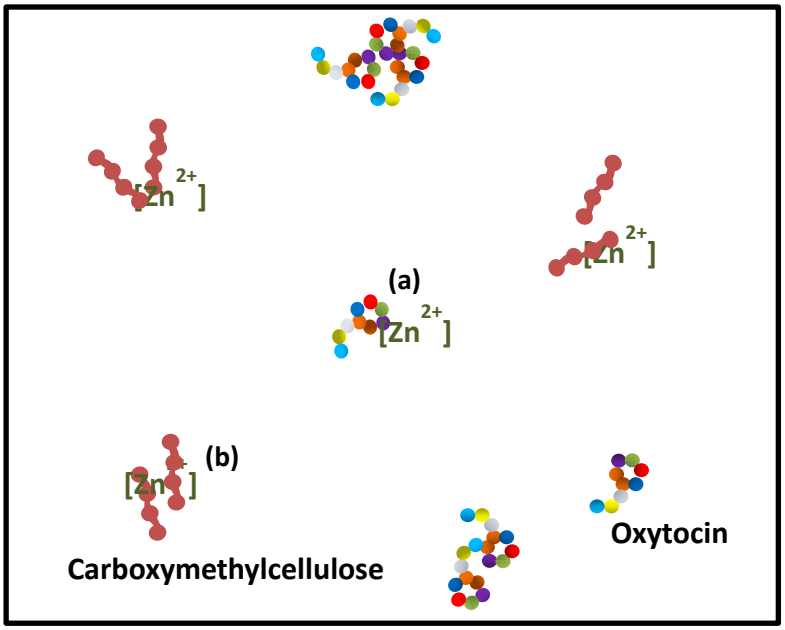


Figure 4.18 Illustration of the (a) complex formation of Zn^{2+} metal ions with oxytocin, and (b) ionic interaction between carboxymethylcellulose and Zn^{2+} metal ions at the interface, in a system with carboxymethylcellulose, oxytocin and Zn^{2+} divalent metal ions.

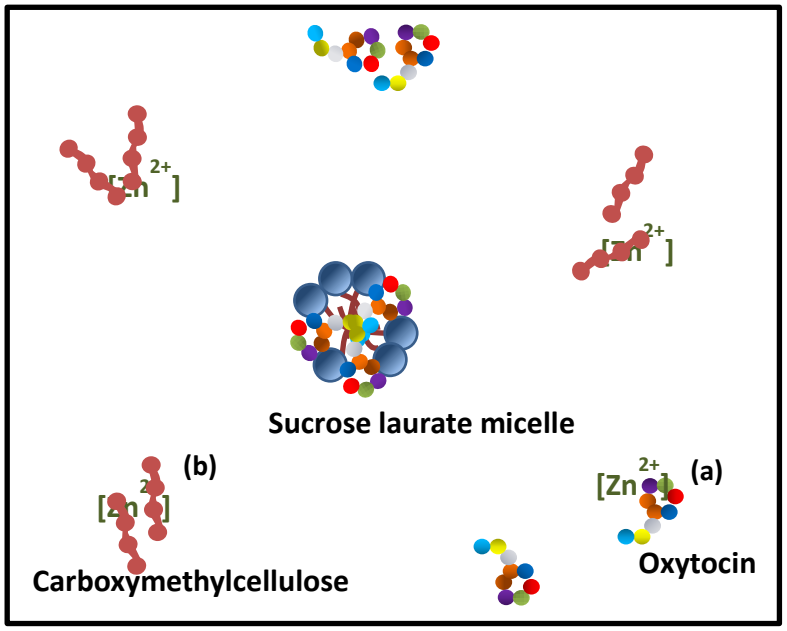


Figure 4.19 Illustration of the (a) complex formation of Zn^{2+} metal ions with oxytocin, (b) ionic interaction between carboxymethylcellulose and Zn^{2+} metal ions at the interface, and (c) micellar interaction between sucrose laurate micelle and oxytocin at the interface, where oxytocin exhibits co-surfactant-liked behavior, in a system with sucrose laurate micelles, carboxymethylcellulose, oxytocin and Zn^{2+} divalent metal ions.

CHAPTER 5 CONCLUSIONS

In this study, micellar formulations with oxytocin were developed. These formulations were made up of surfactants and oxytocin in a buffer containing a divalent and the potential interactions were studied. First, it was demonstrated that the salts present in the formulations are likely water-structure-making which led to the reduced CMC value for sucrose laurate and Cremophor RH40. Zn^{2+} ions in the system may form complex with the ether group found in Tween 80 polyoxyethylene chains which resulted in the increased in CMC.

Next, it was discovered that oxytocin exhibits co-surfactant-liked behavior which caused the reduction in the droplet size in the micellar formulation. Cremophor RH40 micellar formulation was not significantly affected by the presence of oxytocin. For Tween 80 system, the slight increase in droplet size may be due to micellar solubilization of oxytocin by at the surface or shallow penetration into the polar head group.

Subsequently, it was found that the co-surfactant-liked behavior of oxytocin in the micellar formulations helped to improve its thermal stability by reducing the contact with the aqueous environment, thereby suppressing the disulfide exchange reaction that destabilize oxytocin in aqueous formulation. Additionally, the presence of sucrose functional group at the micellar interface may also contributed to the enhanced thermal stability by preserving oxytocin

in its native conformation and provide additional stabilization against chemical and physical degradation.

In contrast, the thermal stability of oxytocin was slightly poorer in Cremophor RH40 formulation due loss of Zn^{2+} ions to stabilize oxytocin in the aqueous phase, because some of the Zn^{2+} ions may weakly interact with the Cremophor RH40 micelle surface. Tween 80 formulations showed the poorest oxytocin thermal stability due to the loss of Zn^{2+} ions in the aqueous phase by complex formation at the Tween 80 micelle interface. Hence, the amount of Zn^{2+} ions remaining in the aqueous phases for oxytocin stabilization is reduced. Furthermore, Tween 80 surfactant is likely to be affected by clouding at elevated temperature, leading to poorer stabilization interaction between oxytocin and the polar head group on the Tween 80 micellar surface. For Cremophor RH40, higher surfactant concentration led to stronger destabilization interaction with oxytocin. Hence, the poorer thermal stability of oxytocin in aqueous formulations. Finally, it was found that ionic interaction between Zn^{2+} ions and carboxymethylcellulose adversely affected the thermal stability of oxytocin.

In conclusion, a micellar formulation of oxytocin using 0.1 mg/ml of sucrose laurate in 10 mM citrate buffer at pH 4.5 with 2 mM Zn^{2+} divalent metal ions was successfully formulated with enhanced thermal stability when compared to the control solution.

CHAPTER 6 FUTURE WORK

Following success in formulating oxytocin in aqueous medium with enhanced thermal stability, further investigation is necessary to incorporate an appropriate mucoadhesives to enhance the bioavailability without contributing to the degradation of oxytocin. In this study, addition of carboxymethycellulose into aqueous formulation showed adverse effect on the recovery of oxytocin on storage at 40 ^{o}C. In order to use carboxymethycellulose as mucoadhesives in the micellar formulation, it is important to reduce its concentration such that the impact on oxytocin stability is controlled at an acceptable level. Besides carboxymethylcellulose, it would be desirable to look into other type of mucoadhesives. One particular compound of interest is hyaluronan. Even though hyaluronan is deemed a non-toxic molecule, information on the safe dosage for intranasal delivery was not found. It is therefore valuable to evaluate the potential toxicity and safe operating concentration on the human nasal cavity before formulation development can take place.

Additionally, it may also be interesting to study the effects of using Ca^{2+} ions instead of Zn^{2+} ions in the aqueous micellar formulations since it also show stabilization effect of oxytocin in aqueous medium (Avanti *et al.*, 2011).

After formulation development, the efficacy of the formulation may be assessed by in vitro monitoring the contractile response of myometrial strips obtained from pregnant women following cesarean delivery (Balki *et al.*,

2014). The myometrium is the layer of uterine smooth muscle cells in the middle of the uterine wall, primarily responsible for the induction of uterine contractions. These strips were immersed in Krebs' bicarbonate solution at 37 ^{o}C pH = 7.4, equilibrated with 5 % carbon dioxide and 95 % oxygen until base contraction pattern was established. The contractile response was examined based on the effect of dosing the bath with the oxytocin micellar formulation, on the contraction pattern.

Furthermore, in vivo animal study may be performed to more accurately quantify the efficacy of the micellar formulation. The micellar formulation can be intranasally administered at the target delivery site of rats, using a micropipette connected to a low density polyethylene tubing (Seju *et al.*, 2011). Subsequently, a balloon-tipped and water-filled cannula can be placed into one uterine horn at the ovarian end to track contractile responses from the uterus (Park *et al.*, 2000).

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APPENDIX I

Hydrodynamic droplet size distribution

The hydrodynamic droplet size distributions measured by Dynamic Light Scattering using Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., UK) between 20 – 60 ^{o}C are shown in in **Figures I.1 – I.6**. As shown in **Figures 4.5 and 4.7,** formulations with sucrose laurate L-1695 and Tween 80 micelles have polydispersity indices greater than 0.2, implying the presence of bimodal or broad size distribution. The droplet size distributions for micellar formulations with 0.1 mg/ml sucrose laurate, with and without 0.1 mg/ml oxytocin, are shown in **Figures I.1 and I.2** respectively. **Figures I.3 and I.4** show the droplet size distributions for micellar formulations with 0.1 mg/ml Tween 80, with and without 0.1 mg/ml oxytocin.

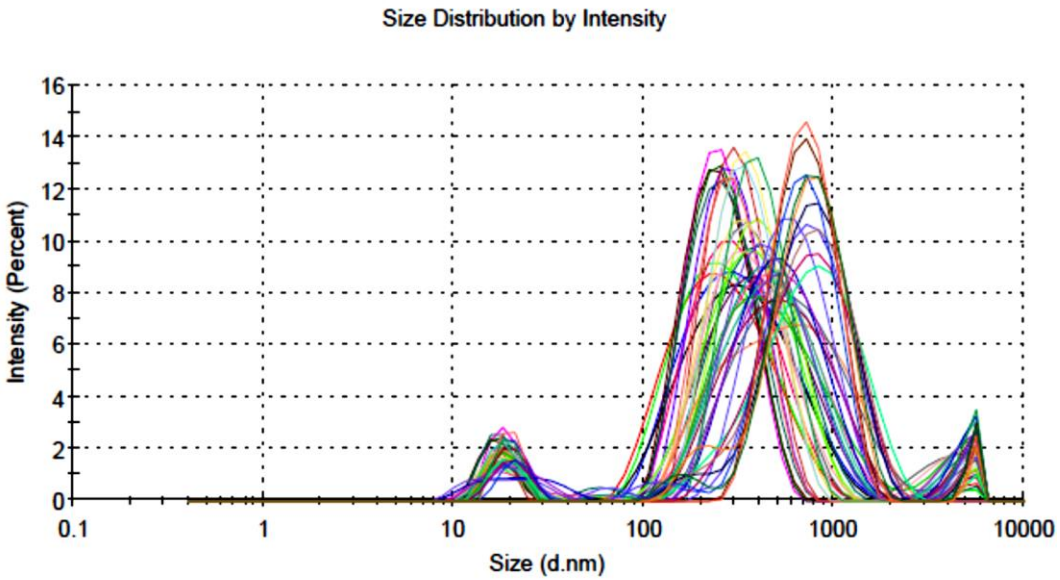

Figure I.1 Hydrodynamic droplet size against intensity for 0.1 mg/ml sucrose laurate micellar formulation, without oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.

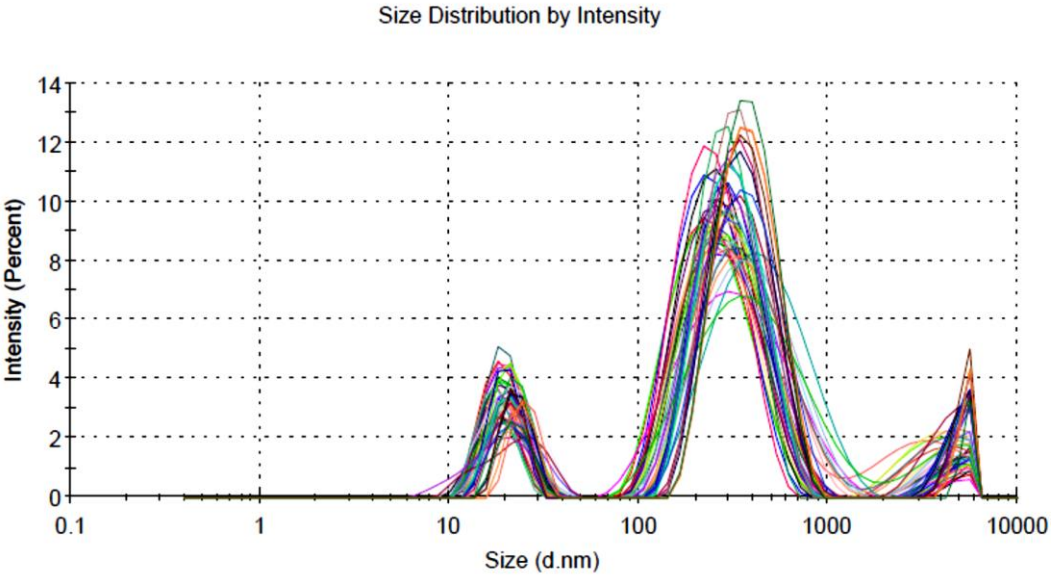

Figure I.2 Hydrodynamic droplet size against intensity for 0.1 mg/ml sucrose laurate micellar formulation, with 0.1 mg/ml oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.

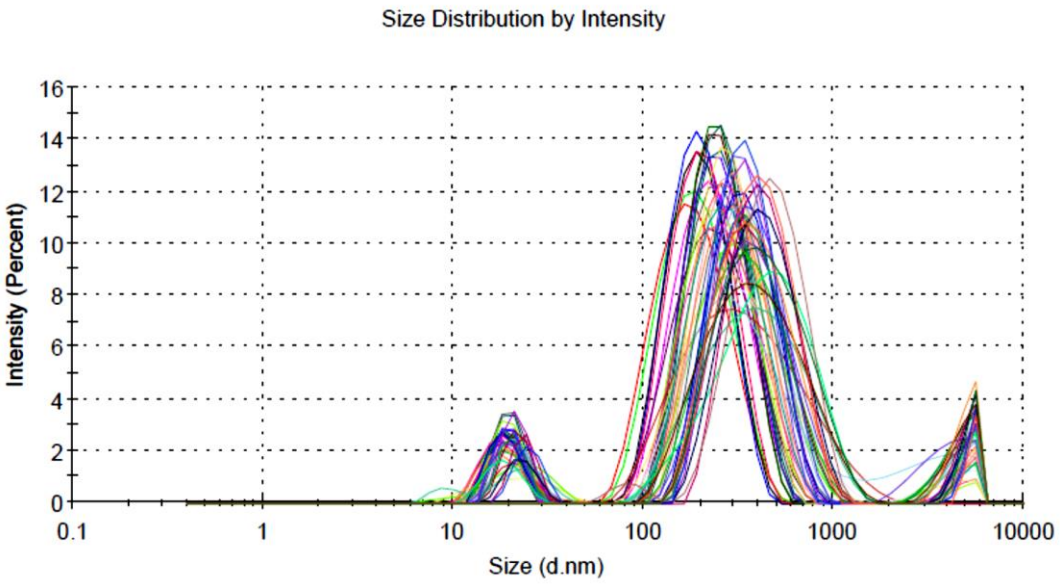

Figure I.3 Hydrodynamic droplet size against intensity for 0.1 mg/ml Tween 80 micellar formulation, without oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.

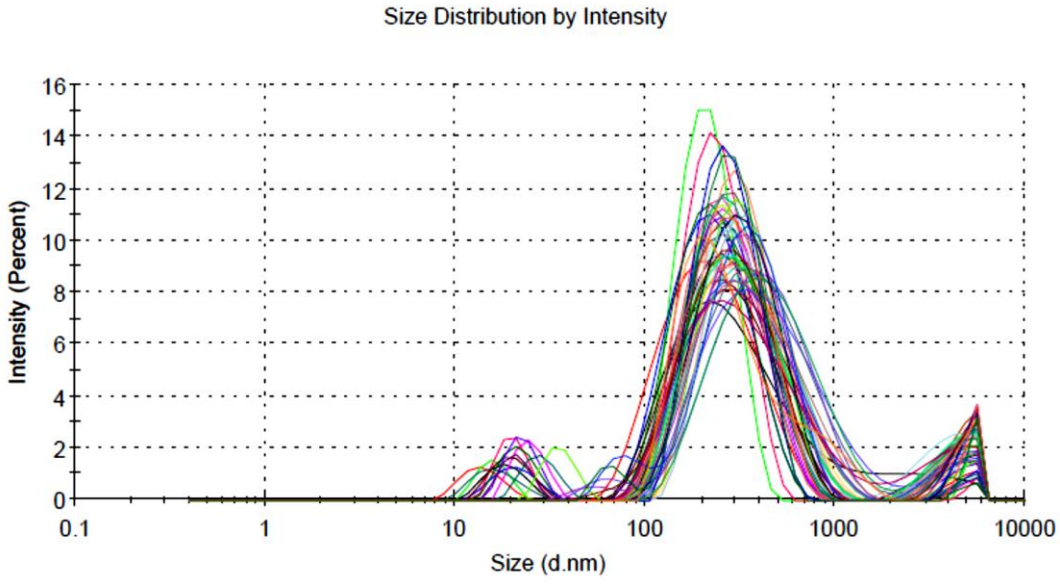

Figure I.4 Hydrodynamic droplet size against intensity for 0.1 mg/ml Tween 80 micellar formulation, with 0.1 mg/ml oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.

As shown in **Figure 4.6,** formulations with Cremophor RH40 micelles have polydispersity indices less than 0.2, implying a monodisperse sample. The droplet size distributions for micellar formulations with 0.1 mg/ml Cremophor RH40, with and without 0.1 mg/ml oxytocin, are shown in **Figures I.5 and I.6** respectively.

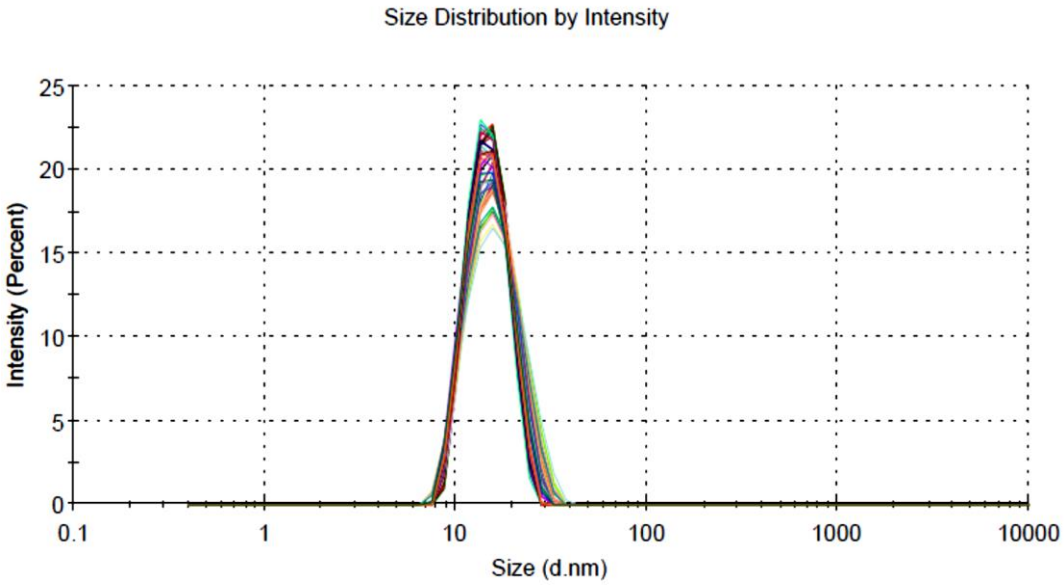

Figure I.5 Hydrodynamic droplet size against intensity for 0.1 mg/ml Cremophor RH40 micellar formulation, without oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.

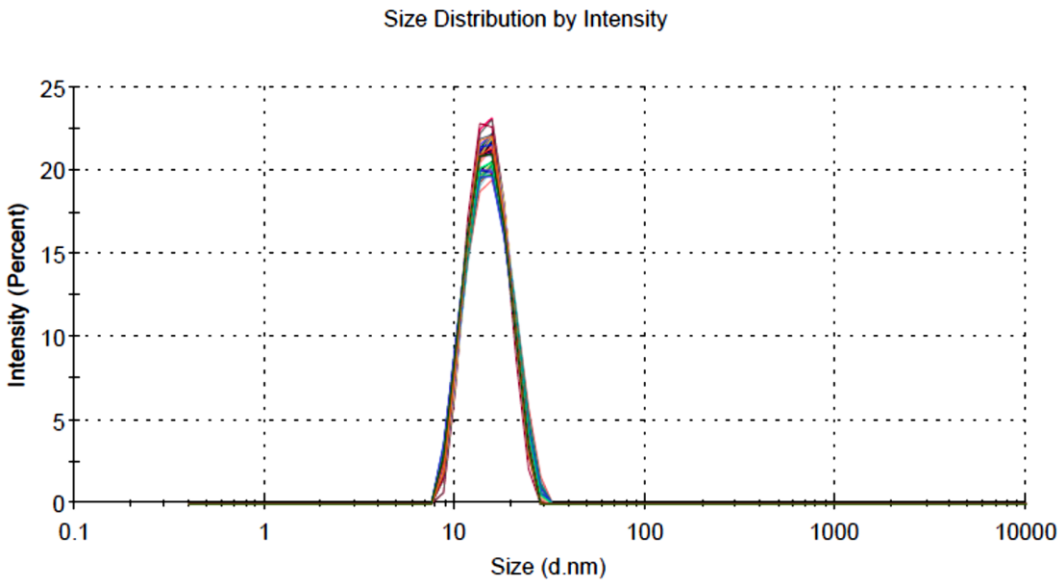

Figure I.6 Hydrodynamic droplet size against intensity for 0.1 mg/ml Cremophor RH40 micellar formulation, with 0.1 mg/ml oxytocin. Data combined for all temperature points between 20 and 60 ^{o}C.