Pea Protein Based Nanoemulsions for Delivery of Vitamin D: Fabrication, Stability and *In vitro* Study using Caco-2 Cells

by

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Abstract

In North America, a significant population is vitamin D deficient due to insufficient sunlight exposure in winters. Increasing research has shown that vitamin D, apart from its skeletal functions, also has potential to lower the risk of chronic diseases such as autoimmune disorders, diabetes mellitus, cardiovascular diseases, and cancers. However, vitamin D exhibits low bioavailability due to its poor solubility in human gut. This research aims to develop pea protein stabilized nanoemulsions as vitamin D delivery systems for improved vitamin absorption.

In the first study, nanoemulsions stabilized by pea protein were developed using a high-energy approach by high-pressure homogenization treatment. The nanoemulsions exhibited controllable sizes ranging from 170 to 350 nm, good stability with zeta-potential value of around -25mV, and high vitamin D encapsulation efficiency of 94-96%. Using Caco-2 cell model, the nanoemulsions were found relatively safe after 6 hours of incubation with cell viability >80%. Cellular uptake efficiency of small sized nanoemulsions (233 nm) was found to be ~ 2.5 folds higher ($p < 0.05$) than large sized nanoemulsions (350 nm). Interestingly, protein based nanoemulsions exhibited significantly higher cellular uptake than emulsions prepared using a combination of protein and lecithin. The transport efficiency of vitamin D across Caco-2 cells for small sized nanoemulsions (233 nm) was ~ 5.3 times greater than free vitamin D suspension.

In the second study, a low-energy approach was used to prepare a complex nanoemulsion formed by a combination of Tween 80 and pea protein by spontaneous emulsification method, which does not rely on high-energy equipment and thus, the processing can be more convenient, cost effective and environmentally sustainable. Tween 80 (3% w/v) was used as a small molecular surfactant to prepare nanoemulsion droplets of 134.8 nm, followed by addition of pea protein (3% w/v), resulting in the formation of complex nanoemulsions with particle size of 207.7 nm. Infrared

spectroscopy was used to investigate the effect of Tween 80 on protein conformation, and Tween 80 showed partial denaturation of pea protein molecules. A "clusters on string" structure was proposed for the complex nanoemulsions, which was supported by transmission electron microscopy and infrared spectroscopy, revealing similar particle size as measured by light scattering, and featuring Tween 80 stabilized micelles clustered together on pea protein polymeric chains. Encapsulation efficiencies of vitamin D were as high as 89.3% and 90.1% for nanoemulsions stabilized by Tween80-pea protein and Tween 80 alone. Such complex nanoemulsions were found to remain physically stable during storage for two weeks. The *in vitro* assays using Caco-2 cells indicated that both nanoemulsions stabilized by Tween 80 alone and the complex nanoemulsion could efficiently improve vitamin D uptake by ≥ 2.5 folds ($p < 0.05$) as compared to free vitamin D suspension. Interestingly, the transport efficiency of vitamin D across Caco-2 cells for complex nanoemulsions was 5.6 folds higher than free vitamin D suspension and 2.3 folds higher than nanoemulsions stabilized by Tween alone.

As a result, these pea protein stabilized nanoemulsions demonstrated efficient encapsulation, cellular uptake and transport of vitamin D using Caco-2 cell model to simulate absorption through intestinal epithelium. Such protein-based nanoemulsions may allow more efficient vitamin D delivery due to its small particle size to improve the status of vitamin deficiencies in aged population and in areas with less sun exposure. Moreover, these nanoemulsions can be developed by both high-energy approach or low-energy approach with the help of natural large molecular surfactants (pea protein) or a combination of pea protein and a low amount of small molecular surfactant (Tween 80), respectively. The former has a "surfactant free" status, and thus appeals more to food industries and gives brand a cleaner label; while the latter is more interesting due to its cost effective and environmentally sustainable way of nanoemulsions production. Moreover,

pea protein is an emerging protein source of plant origin and not regarded as a major allergen, and thus the developed nanoemulsion delivery system of vitamin D has potential applications to create novel non-dairy functional foods and beverages suitable for different populations such as lactose intolerant, vegans or consumers with little preference for dairy.

Preface

This thesis is an original work by Niharika Walia under the supervision of Dr. Lingyun Chen. No part of this thesis has been previously published. Chapter 2 of this thesis is submitted as N. Walia and L. Chen in Food Chemistry journal. Dr. Lingyun Chen will be the supervisory author.

Dedication

This dissertation is dedicated to my beloved family for their motivation, love and support.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

GEO	Genetically engineered organism
GRAS	Generally regarded as safe
pI	Isoelectric point
EDTA	Ethylenediaminetetraaecetic acid
MTT	Thiazolyl blue tetrazolium bromide
DMSO	Dimethyl sulfoxide
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
HBSS	Hank's balanced salt solution
FBS	Fetal bovine serum
NEAA	Non-essential amino acids
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ATCC	American Type Culture Collection
HPLC	High performance liquid chromatography
TEM	Transmission electron microscopy
PBS	Phosphate-buffered saline
CLSM	Confocal laser scanning microscopy
RI	Refractive index
PdI	Polydispersity index
EE	Encapsulation efficiency
WGA	Wheat germ agglutinin
TEER	Transepithelial electrical resistance

ANOVA	Analysis of variance
SDS	Sodium dodecyl sulfate
SOR	Surfactant to oil ratio
FTIR	Fourier-transform infrared
DCL	Deuterium chloride
NaOD	**S**odium deuteroxide
D2O	Deuterium oxide

Chapter 1- Literature Review

1.1 Vitamin D

1.1.1 Vitamin D synthesis

Vitamins are essential micronutrients that play an important rule in the functioning of human body. Vitamin A, D, E and K are classed as lipophilic vitamins (Teleki, Hitzfeld, & Eggersdorfer, 2012). Vitamin D is found in two forms- vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol); vitamin D3 is known to be more bioactive than the former. Vitamin D is an essential vitamin, which is synthesized in the human body upon exposure to sunlight. 7-dehydrocholesterol in skin is first converted to pre-vitamin D3 and later into vitamin D3 (Bikle, 2014). Another source of vitamin D is through dietary intake from natural foods or fortified foods and supplements. After the synthesis of vitamin D in body, it is converted to 25-hydroxy vitamin D metabolite (25(OH)D) in the liver, with further conversion into 1,25-dihydroxycholecalciferol (1,25 $(OH)_2D$) in the kidney. It is known that 25(OH) D is the circulating metabolite and is used as biomarker to determine the vitamin D levels in the human body (Holick, 2009).

1.1.2 Vitamin D functions and sources

Vitamin D is a fat-soluble vitamin that plays important roles in skeletal and non-skeletal body development. Earlier, vitamin D was associated with only bone and teeth development. However, researchers are becoming more aware of the associations between vitamin D and the chronic diseases, indicating the importance of vitamin D for reducing the risks of various conditions including, autoimmune disorders, diabetes mellitus, cardiovascular diseases, celiac disease, cancers, and inflammatory diseases (E.-Q. Chen, Shi, & Tang, 2014; Grossmann & Tangpricha, 2010; Holick, 2009; Muscogiuri et al., 2017; Vatanparast, Calvo, Green, & Whiting, 2010). In a review by Thacher & Clarke, (2011), researchers suggested that most of the vitamin D health

claims are based on observational studies and therefore, conclusion on vitamin D benefits in association with chronic conditions awaits the outcome of clinical trials (Thacher & Clarke, 2011). Vitamin D is naturally found in fish, eggs, mushroom, liver and other organ meats. However, vitamin D levels in naturally occurring foods is inadequate, and thus, the fortification of foods and drinks for higher vitamin D levels is a promising solution to combat vitamin D deficiency.

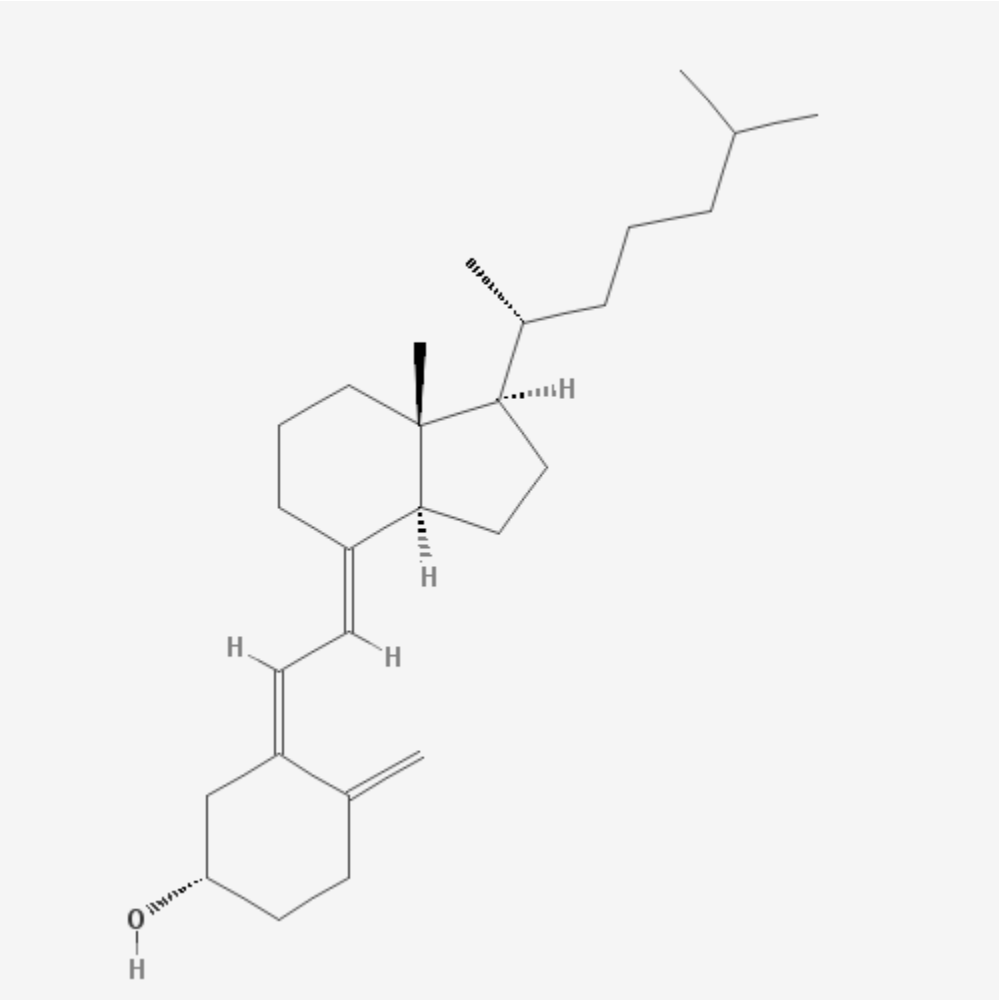

Figure 1.1 Chemical structure of vitamin D3 (cholecalciferol) from PubChem.

Table 1.1 Physicochemical properties of vitamin D_3 (data obtained from PubChem).

Properties	**Vitamin D**
Chemical name	Cholecalciferol
Molecular formula	$C_{27}H_{44}O$
Molecular weight	384.648 g/mol
Solubility	Insoluble in water
Appearance	Fine colorless crystals
Odor	Odorless
Melting point	183 to 185° F
Density	0.969 g/cm^3
Stability	Affected by light and air

1.1.3 Vitamin D status

The lack of adequate sun exposure leads to vitamin D deficiency, that results in bone deformities such as rickets in children and osteomalacia in adults (Holick, 2009). Therefore, health and food agencies started the process of food fortification with vitamin D. Fortification of milk has been a common practice in developed countries like United States and Canada. It is estimated that about one billion people around the world have vitamin D insufficiency (Holick, 2007). About one-third of the United States population is at severe risk of vitamin D inadequacy or deficiency (Kadappan et al., 2018). Population living in areas with minimal sunlight exposure throughout the year is highly prone to vitamin D deficiency or insufficiency. For instance, in North America, extended winters result in limited capacity of vitamin D production in skin. Therefore, a large population nowadays is obtaining vitamin D through food.

Vitamin D insufficiency is highly prevalent in Canadian population, mainly during winters due to limited sunlight exposure. About 32% of Canadians have insufficient vitamin D levels (50 nmol/L) cut-off, with as high as 40% of population below the cut-off levels in winter, and about 25% in summer (Janz, 2015). Another report suggested that more than 70% of Canadians have vitamin D insufficiency (Schwalfenberg, Genuis, & Hiltz, 2010). Due to this, a huge population of Canada relies on dietary intake of vitamin D, including both natural sources and fortified foods to meet the vitamin D recommended dietary allowance (Vatanparast et al., 2010; Vieth, Cole, Hawker, Trang, & Rubin, 2001). A study by Whiting, Green, & Calvo, (2007) reported that despite the fortification, Canadians are struggling to achieve adequate levels of vitamin D. Canadian government has set regulations for mandatory fortification of foods with vitamin D, but is limited to just milk and margarine (Calvo, Whiting, & Barton, 2004). A major disadvantage of limiting vitamin D fortification to staple diet, such as milk, is the failure to reach vulnerable populations with different dietary preferences (Whiting et al., 2007). Therefore, a wider range of foods needs to be fortified with vitamin D so that more people can take advantage of the vitamin D fortification.

1.1.4 Vitamin D Fortification

Food fortification is described as the "deliberate addition of a specific nutrient to a food vehicle which is identified as being widely consumed in adequate quantities by populations at risk of a deficiency" (Liberato & Pinheiro-Sant'ana, 2006). Around the world, it is seen that staple foods are majorly fortified, and this is because they are widely consumed by the population on a regular basis and is affordable, allowing a wider population to benefit and live a nutritious life. One must ensure that the fortified food is stable throughout the processing and transportation, and does not contain toxic levels of bioactives. With more and more consumers realizing the importance of vitamin intake and its relation to reduce the risks of chronic diseases (E.-Q. Chen, Shi, & Tang,

2014; Grossmann & Tangpricha, 2010; Holick, 2009; Muscogiuri et al., 2017; Vatanparast, Calvo, Green, & Whiting, 2010), food fortification industries are witnessing a boom in growth. Now-a-days, food companies are interestingly investing in vitamin fortified products. There has been extensive research on fortification of products with vitamin D, for instance, vitamin D fortification of cheese (Banville, Vuillemard, & Lacroix, 2000; Wagner et al., 2008), yogurt drink (Nikooyeh et al., 2011), ice cream (Kazmi, Vieth, & Rousseau, 2007), and milk (Daly, Bass, & Nowson, 2006). However, there is a lack of focus on vitamin D fortification of non-dairy products.

Vitamin D intake through diet is challenging for vegans as there are not enough plant-based vitamin D sources. A few years back, Health Canada also permitted the addition of calcium, with or without vitamin D to fortify orange juices (Health Canada, 2006). The most commonly consumed vitamin D fortified products are dairy products. However, there are not enough choices for populations with lactose intolerance, vegans or little preference for dairy products. Therefore, more research should be conducted to fortify wider range of products or to develop new products, in order to meet the demands and preferences of our diverse populations. Nowadays, there is a growing interest in plant-based diet, suggesting that plant-based food and beverages may be a good alternative for vitamin D fortification.

Table 1.2 Dietary reference intakes of vitamin D.

Age group	Recommended dietary intake (RDA) per day		Tolerable upper intake levels	
	IU/day	µg/day	IU/day	µg/day
Infants 0-6 months	400 IU	10 µg	1000 IU	25 µg
Infants 7-12 months	400 IU	10 µg	1500 IU	38 µg
Children 1-3 years	600 IU	15 µg	2500 IU	63 µg
Children 4-8 years	600 IU	15 µg	3000 IU	75 µg
Children and adults 9-70 years	600 IU	15 µg	4000 IU	100 µg
Adults above 70 years	800 IU	20 µg	4000 IU	100 µg
Pregnancy and lactation	600 IU	15 µg	4000 IU	100 µg

(Health Canada, 2010)

Although there is an increasing growth of vitamin D fortified food products, vitamin D deficiency is still prevalent (Ganji, Zhang, & Tangpricha, 2012). Increasing vitamin D fortification levels limitlessly is not a practical answer to increasing vitamin D deficiency, because high levels of vitamin D consumption can pose serious health risks to individuals. It has also been estimated that around 1% of US population is already reaching harmful levels of vitamin D (>125 nmol/L) (Kadappan et al., 2018). Hence, a different approach should be considered to meet sufficient vitamin D levels of the population. Methods to improve oral vitamin D bioavailability seem to be a more thoughtful strategy to improve vitamin D status. Since vitamin D is a fat-soluble vitamin, its low solubility in aqueous fluids such as in gastrointestinal tract, leads to low bioavailability of

vitamin D (Kadappan et al., 2018). Therefore, vitamin D is often delivered in the form of oil-in-water emulsion (Grossmann & Tangpricha, 2010), which is specifically formulated to improve its bioavailability by increasing its aqueous solubility.

1.2 Encapsulation technology: Nanoemulsion

Nanoemulsions are colloidal dispersions of two immiscible phases- a mixture of water and oil along with a suitable surfactant required to reduce the interfacial tension between the two phases (Javadzadeh & Azharshekoufeh Bahari, 2017). The particle diameters of nanoemulsions fall below 500 nm (Capek, 2004; Chime, Kenechukwu, & Attama, 2014; Sharma, Mishra, Sharma, Deshpande, & Kumar Sharma, 2013; Walia, Dasgupta, Ranjan, Chen, & Ramalingam, 2017). Nanoemulsions are considered to be an effective way to solubilize and stabilize lipophilic compounds and to improve their bioavailability (Fathi, Mozafari, & Mohebbi, 2012; Hélder Daniel Silva, Cerqueira, & Vicente, 2012). Nanoemulsions with encapsulated bioactives can be incorporated in solid foods as well, with the help of a spray or freeze dryer to form nanoencapsulated bioactive powder (Öztürk, 2017; Teleki et al., 2012). There has been an overwhelming interest in nanotechnology in recent decades (Nishiyama, 2007), and the application of nanoemulsion in food, nutrition and drug field has received great attention (Arora & Jaglan, 2016; McClements & Rao, 2011).

1.2.1 Nanoemulsion production methods

Nanoemulsions are prepared in either oil-in-water (o/w) or water-in-oil (w/o) form, where oil constitutes the dispersed phase in the former and the continuous phase in the latter (Kwasigroch et al., 2016). Nanoemulsions can be prepared using two different approaches i.e, high-energy or low-energy approach. High-energy approach uses mechanical devices like high-pressure homogenizer, microfluidization and ultrasonicators (Öztürk, 2017). A range of particle sizes can be achieved by

controlling the parameters of these devices. This method has several advantages over the low-energy approach as they are more effective in reducing the emulsion droplet size (Maali & Mosavian, 2013), and allows a greater control over the particle sizes produced, which is an important characteristic for emulsion stability and appearance. Also, high- energy methods require shorter processing time for emulsion production, less amount of surfactants, no use of organic solvents and have industrial scalability due to the easy control of homogenization equipment (Maali & Mosavian, 2013). Due to small amount of surfactants required, high-energy methods have low off flavors associated with surfactants and reduced ingredients costs (Ostertag, Weiss, & McClements, 2012). A variety of emulsifiers, including natural polymers such as proteins and polysaccharides can be used to make nanoemulsions by high-energy methods (Öztürk, 2017). However, high-energy methods are less energy efficient as they require a large amount of energy to produce small emulsion droplets.

Nanoemulsions could also be synthesized using low-energy methods, including spontaneous emulsification, phase inversion temperature and phase inversion composition (Kwasigroch et al., 2016). This approach does not require external energy to produce emulsion; instead, it utilizes the stored energy of the system to form emulsion (Chime et al., 2014). Low-energy method depends upon the physicochemical properties of the surfactant and co-surfactants for the production of emulsion droplets (Chime et al., 2014)**.** The most widely used low-energy method is spontaneous emulsification, where a step-by-step addition of water into a solution of surfactant and oil at a constant temperature and stirring, results in spontaneous formation of nanoemulsion droplets. This is due to the movement of a water miscible component (surfactant) from the organic phase into the aqueous phase (Chime et al., 2014; Karthik, Ezhilarasi, & Anandharamakrishnan, 2017). This method requires larger amount of surfactants for emulsion production, which may raise toxicity

concerns mainly in food applications (Öztürk, 2017). However, the major advantage of this method is the low cost of emulsion production (Liu, Sun, Li, Liu, & Xu, 2006), as gentle mixing using magnetic stirrer is sufficient. Since, high-energy input mechanical devices are not used, this method allows encapsulation of heat-sensitive bioactives without causing their degradation (Öztürk, 2017).

1.2.2 Types of surfactants

Surfactants or emulsifiers due to their amphiphilic nature produce stable emulsions by reducing the surface tension between oil and water phases. Emulsifiers adsorb at the oil-water interface and stabilize the emulsion by decreasing the free energy of the system (Berton, Genot, & Ropers, 2011). An effective emulsifier has two important functions- first, it should rapidly adsorb on the interfacial surface, thus reducing the interfacial tension between an aqueous and organic phase, and second, it should be able to form a protective coating around the droplets, and thus, prevent emulsion instabilities, such as coalescence and flocculation. Small-molecular surfactants (e.g. Tween and Span series) and large-molecular surfactants (e.g. polymers like protein and polysaccharides) may stabilize emulsions in different ways. Proteins and polysaccharides can act as emulsifiers due to their hydrophobic and hydrophilic moieties (Adjonu, Doran, Torley, & Agboola, 2014; Chanamai & McClements, 2002; Ozturk & McClements, 2016), whereas, Tween or Span or phospholipids serve as emulsifiers due to their hydrophobic tail and hydrophilic head groups (Kralova & Sjöblom, 2009). Stability of emulsions can be enhanced with repulsive forces that can overcome the attractive forces between the droplets. Repulsive forces could be either in the form of steric interactions, which depend on the type and structure of the emulsifier adsorbed on the oil droplet, or electrostatic interactions, that depend on the electric charge on the surface of oil droplets due to the adsorbed emulsifier.

For instance, gum Arabic (polysaccharide) stabilizes emulsion through steric repulsion by forming a thick interfacial layer around the oil droplets with hydrophobic part adsorbed to the droplet surface, and the hydrophilic part in the aqueous phase, providing stability to droplet aggregation (Chanamai & McClements, 2002; Charoen et al., 2011). Gum Arabic is the most commonly used polysaccharide for emulsion stabilization (Ozturk, Argin, Ozilgen, & McClements, 2015). Interestingly, gum Arabic has a covalently linked hydrophobic protein fraction to its hydrophilic structure, which attributes to its emulsifying activity (Chanamai & McClements, 2002; Ozturk et al., 2015). On the other side, whey protein isolate creates a thin layer of membrane, which causes emulsion stabilization by either electrostatic or steric interactions (Öztürk, 2017; Ozturk et al., 2015).

Smaller emulsion droplets can be achieved with the use of small molecule surfactants as compared to protein or polysaccharide emulsifiers (Qian & McClements, 2011; Teo, Goh, & Lee, 2014). However, protein and polysaccharide based emulsifier are more efficient in improving the stability of emulsion by steric or electrostatic repulsion to prevent flocculation and coalescence (Chu, Ichikawa, Kanafusa, & Nakajima, 2007; David Julian McClements & Rao, 2011). In some cases, a mixture of proteins and polysaccharides is used to stabilize emulsions as they are able to form complexes though various interactions (e.g. electrostatic, hydrophobic and hydrogen bonding) (Aryee & Nickerson, 2012; Weinbreck, de Vries, Schrooyen, & de Kruif, 2003; A. Ye, Flanagan, & Singh, 2006). For instance, Sun et al., 2007 investigated the effect of combining xantam gum and whey protein isolate on emulsion stability, and they observed an increase in emulsion viscosity with the addition of xanthum gum, which resulted in inhibited creaming. Another study tested various other protein-polysaccharide complexes by using whey protein isolate and different kinds of carrageenan, and found that these complexes showed higher emulsion stability as compared to

the emulsions stabilized by protein or polysaccharide alone (Stone & Nickerson, 2012). Lately, natural emulsifiers like proteins and polysaccharides are seeing a growing interest for use in food industries because of the customer demand shift towards "clean label" and natural products worldwide (Öztürk, 2017; Ozturk & McClements, 2016).

1.2.3 Nanoemulsions as delivery systems

1.2.3.1 Benefits

Nanomulsions serve as an efficient delivery systems for a variety of drugs and bioactives. They are found to be effective in encapsulating hydrophobic compounds, and in turn increase their solubility and oral bioavailability (Guttoff, Saberi, & Mcclements, 2015; McClements, 2011).

1) Easy penetration into the cells and better diffusibility:

Nanoparticles are nano-sized colloidal polymeric particles that encapsulate bioactives or drugs of therapeutic interests (Panyam & Labhasetwar, 2003). Nanoparticles are able to penetrate tissues and are easily taken up by the cells due to their relatively small particle size. It is suggested that nanoparticle uptake by intestinal epithelium involves an adhesion process, followed by an internalization process (Gao, Shi, & Freund, 2005). Physicochemical characteristics such as particle size and surface charge play an important role in the cellular uptake of nanoparticles (Alexis, Pridgen, Molnar, & Farokhzad, 2008).

A study by Zhang, Field, Vine, & Chen, 2015, reported that soy protein nanoparticles of particle size 100 nm showed higher cellular uptake as compared to larger particles (180 nm), suggesting the role of reduced particle size in cellular uptake of nanoparticles. Another interesting study by Win & Feng, (2005) also compared the nanoparticle uptake based on varying particle sizes where 100 nm particles showed significantly higher cellular uptake as compared to 50 nm, 500 nm and 1000 nm particles. Win et al. (2005) demonstrated that nanoparticles of approximately 100-200

nm size exhibit best properties for cellular uptake, whereas, a study by Roger, Lagarce, Garcion, & Benoit, 2009 reported that there is no effect of particle size on nanoparticle uptake in the range between 25 and 130 nm. Nano-emulsions with larger particle sizes are known to require additional driving forces and energy for the cellular internalization process (He, Hu, Yin, Tang, & Yin, 2010). Some researchers believe that nanoparticles with positive charge may exhibit stronger interactions with the negatively charged cell membrane, resulting in higher cellular uptake (Gao et al., 2005; He et al., 2010). However, highly positively charged nanoparticles are believed to be toxic and immunogenic, because of their strong interactions with cell membranes and serum proteins (Bajaj, Samanta, Yan, Jerry, & Rotello, 2009). Therefore, mildly positively or negatively charged nanoparticles seem favorable for the purpose of bioactive delivery (Zhang et al., 2015).

Many other studies have shown similar results of relatively higher intracellular uptake of nanoparticles than micro-ranged particles. For instance, Desai et al., demonstrated differences in uptake of nano-sized particles and larger particles by Caco-2 cells and rat *in situ* intestinal loop model, where the uptake efficiency of nanoparticles was 2.5 fold higher in cellular line and over 15 folds higher in intestinal tissues as compared to larger particles. In rat models, they also observed that nanoparticles were able to penetrate into submucosal layer, whereas, larger particles accumulated on the epithelial lining (Desai et al., 1996; Desai, Labhasetwar, Walter, Levy, & Amidon, 1997). Thus, these studies indicate that nano-ranged particles/emulsion show greater uptake efficiency into cells and tissues when compared to larger sized particles.

2) Improved drug delivery:

Among many nano-delivery systems like nano-liposomes, micelles, nanoemulsions, or nanoparticles (Tiwari & Takhistov, 2012), increased attention is given to nanoemulsions for their potential applications in the food industry. Decreased particle size to nanometers result in

increased surface area, which causes an increase in dissolution rate (Tiwari & Takhistov, 2012). It is suggested that physicochemical properties of polymeric nanoparticles can affect its biodistribution and clearance from the blood. For instance, it has been found that smaller nanoparticles show longer blood residence time, twice as slower blood clearance as compared to larger nanoparticles, and higher tissue permeability. Another study demonstrated that smaller particles could cross the blood brain barrier, suggesting therapeutic treatment of complicated brain tumors or disorders using nanoparticles with encapsulated therapeutic drugs (Kroll et al., 1998).

3) High stability:

Due to the small size of nanoemulsions, its molecules follow Brownian motion, which is sufficient to overcome gravitational force and thus, exhibit high resistance to physical instability (Chime et al., 2014; Öztürk, 2017). On the other side, conventional macroemulsions, due to their droplet sizes greater than one micron, are susceptible to gravitational forces. Another interesting feature of nanoemulsion is that they are kinetically metastable, unlike microemulsions *i.e.*, there is no change in the droplet size distribution even on diluting the emulsion (Fernandez, André, Rieger, & Kühnle, 2004).

1.2.3.2 Challenges

Although nanoemulsions have several advantages, there are also some challenges that need to be addressed. High-energy mechanical equipment are energy intensive and expensive for industrial scale nanoemulsion production, whereas, low-energy methods require high surfactant concentration. Therefore, advanced technologies or methods should be developed to overcome these challenges and to achieve large-scale nanoemulsion production. In a study by Kotta, Khan, Ansari, Sharma, & Ali, 2015, nanoemulsions were prepared by both high-energy (high-pressure homogenizer) and low-energy methods (phase inversion composition). They concluded that low-

energy method produced efficient nanoemulsions that were more uniform in particle size distribution as compared to those produced by high-energy method, which was attributed to the coalescence of some droplets because of high-energy inputs. In general, more studies are required to compare the two production approaches in order to have a better understanding of their potential in industrial scale-up.

Nanoemulsions, due to their small particle size can stay stable for impressively long periods, as small particles follow Brownian motion that overcome gravitational forces. However, nanoemulsion stability may get affected by the processing and storage conditions. Lee et al. (2011) compared the oxidative stability of nanoemulsions and conventional emulsions, and they found that nanoemulsions were more stable to droplet aggregation and creaming than emulsions. However, lipid oxidation was faster in nanoemulsions, which was attributed to the increased surface area due to its small particle size as compared to conventional emulsions (Lee et al., 2011). Oxidative stability of nanoemulsions can be improved with the addition of antioxidants in the emulsion as they can delay the oxidation of oils and stabilize them during storage (Naz, Sheikh, Siddiqi, & Sayeed, 2004; Öztürk, 2017). For instance, vitamin E can be used as an antioxidant in these emulsions, which would prevent lipid peroxidation (Traber & Atkinson, 2007) and lead to less degradation of encapsulated bioactives. Better understanding of the stabilization and destabilization mechanisms of nanoemulsions will allow optimization of production techniques and storage conditions of nanoemulsions (Karthik et al., 2017). This suggests the importance of systematic design of emulsions and their utilization in food applications, and the impact of processing parameters and storage conditions on nanoemulsion stability, which should be studied more systematically.

Another challenge of producing nanoemulsions using low-energy approach is that it is only limited to the use of synthetic surfactants. However, due to the growing demand of using natural polymers in industries, it would be interesting to develop new techniques to prepare natural polymers based nanoemulsions with low-energy methods. This could be attempted by either using only natural polymers such as proteins and polysaccharides or by combining natural emulsifiers with food grade synthetic surfactants, which would reduce the requirement of large amount of synthetic surfactants for nanoemulsion production by low-energy approaches.

Overall, nanoemulsions seem to be a potential way of delivering drugs/bioactives into body because of their sub-cellular size, high drug absorption, variety of encapsulating agents and coating compatibility, and controlled and targeted release of bioactives in the body.

1.3 Proteins and the growing interest in plant proteins

Proteins are one of the major components of human diet and constitute high nutritional benefits for the functioning and strengthening of our body. Protein is an amphiphilic polymer, which may possess positive or negative charge depending upon the pH of the protein solution. At its isoelectric point (pI), protein molecules will possess neutral charge and will form a compact structure. At pH higher than its isoelectric point, proteins posses a negative charge, whereas, at pH values lower than pI, it acquires a positive charge (Amine, Dreher, Helgason, & Tadros, 2014). Food products include various proteins with different structural, textural, functional and nutritional properties. The physicochemical properties of proteins contribute to the quality and sensory attributes of food products. The most studied functional properties of proteins in food products are protein solubility, foaming, gelling and emulsifying capacities. These functional properties mainly depend on the interactions of proteins with other proteins, lipids, carbohydrates, water, air and ions (Zayas, 1997b).

There has been a growing interest in the functionalities of plant-based proteins as they are less expensive and abundantly available as compared to animal proteins. To keep up with the growing demand for "consumer-perceived healthier" proteins, researchers are extensively exploring different proteins to replace protein ingredients obtained from animal sources (e.g. whey, casein, and gelatin). The general reasons for this shift in customer demands towards plant-based diet are ethical, religious and health choices (vegetarianism and/or allergies). Also, plant-based diets are considered a more environmentally sustainable option (Karaca, Low, & Nickerson, 2011; Lam, Can Karaca, Tyler, & Nickerson, 2018).

1.3.1 Protein based nanoparticles

Natural polymers such as proteins, polysaccharides, and phospholipids are used for the preparation of nanoparticles that serve as bioactive encapsulation and delivery systems in food industries. Proteins are particularly of great interest to food industries owing to their natural origin, biocompatibility and unique physicochemical properties such as emulsifying and gelling capacities, in addition to their high nutritional value. (Chen, Remondetto, & Subirade, 2006).

Nanoemulsions stabilized by proteins are generally prepared using high-energy techniques such as high-pressure homogenizer, ultrasonication or microfluidization. Multiple component encapsulation systems have also been designed such as protein-polysaccharide (Chen, 2006; Guerin, Vuillemard, & Subirade, 2003; Teng, Luo, & Wang, 2013), protein-phoshopholipid (Bylaite, Nylander, Venskutonis, & Jönsson, 2001; García-Moreno, Horn, & Jacobsen, 2014; Sünder, Scherze, & Muschiolik, 2001), and protein-synthetic polymer (A, A, B, & J.M. Irache a, 2002; Kasper, Kushibiki, Kimura, Mikos, & Tabata, 2005). In a study by Bylaite et al., (2001), lecithin was observed to enhance the stability of oil-in-water emulsion stabilized by beta-lactoglobulin, which was attributed to the interactions between the protein and lipid where the

mixed surfactant layers may have stabilized a larger interfacial area between the oil and aqueous phase, resulting in stable emulsion droplets.

In a study by Teng, Luo, & Wang, (2013), complex nanoparticles were formed using carboxymethyl chitosan (CMCS) and soy protein isolate by ionic gelation method for vitamin D delivery. They observed that the complex was more efficient as it showed higher encapsulation efficiency, reduced release of vitamin in simulated gastric conditions and higher release in simulated intestinal conditions, as compared to CMCS alone or soy protein alone nanoparticles. The complex resulted in larger number of nanoparticles with smaller average sizes. They suggested that the polymeric complex exhibited a more compact structure, with more functional groups involved in polymer-vitamin interaction, resulting in increased encapsulation efficiency. Low release of vitamin in simulated gastric was attributed to the involvement of greater number of nanoparticles in the encapsulation of vitamin D, which might have reduced its release from the polymeric complex. They further suggested that complex nanoparticles exhibits better properties and is an interesting approach for nutraceutical delivery (Teng et al., 2013).

Both animal and plant proteins have been used for nanoemulsion production. Most commonly used animal proteins are gelatin, collagen, albumin, casein and whey protein. In comparison, the efforts to develop plant protein based nanoemulsions are much less. The limited research includes zein and soy protein stabilized nanoemulsions (Tarhini, Greige-Gerges, & Elaissari, 2017; O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004a).

1.3.2 Pea protein as an emerging source of plant proteins

Canada is the world's largest producer and exporter of yellow field pea seeds (Pownall, Udenigwe, & Aluko, 2010). Increasing customer demands for cholesterol-free and low fat food ingredients have led to extensive study of plant-based food sources such as pea seeds (Aluko, Mofolasayo, &

Watts, 2009). Pea is rich in protein and carbohydrates, essential vitamins and minerals, as well as low in fat (Lam et al., 2018). Dry pea grains contain around 20-30% protein, 55-68% starch, 1.5-2 % fat and 6% fiber (Aluko et al., 2009; Lam et al., 2018). Pea protein presents to be a promising alternative to animal proteins due to its low cost, abundance and high nutritional benefits. In addition, it is non-GEO, gluten-free, and not regarded as a major allergen (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Pea proteins are a rich source of lysine as even dry peas consist 20-30% of lysine-rich protein (Zayas, 1997b) but they have lower levels of methionine and tryptophan (Lam, 2016). Pea proteins have two major fractions- globulins (50-60%), subdivided into legumin (11S) and vicilin (7S), and a small amount of convicilin, and albumins (15-25%) (Djoullah, Djemaoune, Husson, & Saurel, 2015). Legumin is a hexameric protein with higher molecular weight (400 kDa) and a sedimentation coefficient of 11S, whereas, vicilin is a trimeric protein of 160-200 kDa and a sedimentation coefficient of 7S. Each legumin monomer consists of an acidic (40 kDa) and basic (20kDa) α-β subunit covalently linked together by a disulphide bond. On the other hand, vicilin monomers have molecular mass of ~50kDa and three subunits- α, β, and γ held together by hydrophobic interactions. Legumin and vicilin possess different functional properties due to the differences in their amino acid composition and structures. For instance, vicilin exhibits better gelling and emulsifying properties as compared to legumin (Lam, 2016). Convicilin is a third category of proteins found in globulins and it has molecular mass of ~70kDa (Lam, 2016). The albumins are water-soluble proteins rich in sulphur amino acids and consist of a larger (26 kDa) and a smaller fraction (3-6 kDa) (Tamm, Herbst, Brodkorb, & Drusch, 2016).

1.3.3 Nutritional/ Amino acid composition of pea protein

The nutritional quality of proteins is determined by their composition of amino acids (J. Boye, Zare, & Pletch, 2010). Legume proteins, including pea protein are generally high in aspartic acid, glutamic acid, leucine, lysine, and arginine but low in methionine, cysteine (sulphur containing amino acids), and tryptophan (J. Boye et al., 2010; Leterme, Monmart, & Baudart, 1990). Therefore, it is suggested that pea protein is consumed with cereal grains, as they are rich in sulphur amino acids (methionine, cysteine) (A. C.Y. Lam et al., 2018).

Table 1.3 Amino Acid composition of pea protein.

Amino acids	(Pownall et al., 2010)	(Fernández-Quintela et al., 1997)	(Gorissen & Witard, 2018)	(Mandy Claessens, 2008)
Essential				
Lysine	7.35	2.4	8.2	8.4
Threonine	3.48	2.7	4.4	4.0
Valine	5.19	-	5.1	3.9
Methionine	1.12	0.7	1.0	0.9
Isoleucine	4.73	5.4	4.6	3.4
Leucine	8.79	8.0	7.4	7.3
Phenylalanine	5.49	5.3	5.0	4.6
Histidine	1.74	2.6	2.5	2.4
Tryptophan	0.83	-	-	0.4
Nonessential				
Arginine	8.6	7.9	10.3	9.8
Aspartic acid	11.81	9.6	11.9	11.7
Serine	5.72	3.7	4.7	5.7
Glutamic acid	16.54	18.6	17.5	19.9
Proline	5.49	-	4.2	4.0
Glycine	4.09	2.4	4.4	4.0
Alanine	4.34	4	4.4	5.4
Cysteine	0.87	-	1.2	0.6
Tyrosine	3.78	4.7	3.0	3.7

1.3.4 Pea protein isolate extraction methods

1) Alkaline/ isoelectric precipitation

Alkaline extraction of pea protein is allowed due to its high solubility in alkaline conditions, followed by the isoelectric precipitation where proteins show little solubility at their isoelectric point between pH 4 and 5 (J. Boye et al., 2010). In brief, pea flour is dispersed in water at a ratio of 1:15 (w/v) and pH is adjusted to 9 (alkaline conditions) using sodium hydroxide, and then left to stir for about 2 hours to ensure high protein solubility at room temperature. The solution is then centrifuged at 4500×g for 20 min at 4 °C to collect the supernatant. The resulting pellet is resuspended in water at a ratio of 1:5 (w/v), followed by centrifugation again to obtain the supernatant. Both supernatants are combined and pH is adjusted to 4.5 (isoelectric point) using hydrochloric acid to precipitate the protein. Later, the solution is centrifuged to collect precipitated protein and then washed, neutralized and dried using freeze dryer (Angie Che Yan Lam, 2016). (J. Boye et al., 2010; Papalamprou, Doxastakis, & Kiosseoglou, 2010).

2) Salt extraction

This method utilizes the salting-in and salting-out phenomena of proteins. In brief, pea flour is dispersed in a salt solution (6.4% KCl) at 1:10 ratio (w/v), adjusted to pH 7 using sodium hydroxide, and allowed to stir for 24 hours at room temperature. This is followed by the removal of insoluble precipitate by centrifugation at 17,700×g for 20 min at 4 °C. The supernatant is collected and desalted by dialyzing for 72 hrs against water to obtain the protein isolate (Karaca et al., 2011). A study found that isoelectric precipitation method yielded higher levels of pea protein as compared to salt extraction method (Karaca et al., 2011).

Other methods include micellar precipitation where defatted pea flour is suspended in NaCl solution at a ratio of 1:10 (w/v) and allowed to stir for 2 hours at room temperature, followed by

centrifugation at 4000 ×g for 20 min at 4 °C. The supernatant is collected and diluted with cold deionized water and then left at 4 °C for 18 hours, followed by centrifugation. The pellet is then collected and freeze dried (Stone et al., 2015). Micellar precipitation results in pea protein isolates comprised of both globulins and albumins, and proteins undergo less degradation in this preparation method as compared to isoelectric precipitation (Stone et al., 2015).

1.3.5 Functionalities of pea protein

1) Protein solubility

Protein solubility is an important attribute for the selection of proteins to be used in beverages or drinks. It is also essential to other protein functionalities like gelling, foaming and emulsifying capacities (Zayas, 1997b). Major factors that influence protein solubility are solvent pH, ionic strength, and temperature (Karaca et al., 2011; Lam et al., 2018). At pH values lower or higher than the isolelectric point (pI) of pea protein, electrostatic repulsion between positive and negative net charges occurs, results in increased solubility of protein (Hall, 1996). Generally, pea protein isolates show lowest solubility between pH 4 to 6 regardless of the extraction method and pea cultivar (Fernández-Quintela et al., 1997; Lam, 2016), and good solubility at neutral pH. One study found protein solubility to be significantly higher for isolates extracted by isoelectric precipitation as compared to those extracted by salt extraction (Karaca et al., 2011).

2) Emulsifying properties

Emulsification is a very common process in the production of formulated foods. Since the last decade, researchers have explored pea protein in its capacity as an emulsifier where pea protein has demonstrated good emulsifying properties (Aluko et al., 2009; Amine et al., 2014; Barac et al., 2010, 2012; Gharsallaoui, Cases, Chambin, & Saurel, 2009; Gharsallaoui, Saurel, Chambin, & Voilley, 2012).

Researchers compared the emulsifying activity of different proteins extracted by alkaline solution followed by isoelectric precipitation, and they found that emulsifying activity of pea protein was similar to those of soy protein and faba bean protein. They also observed that proteins isolated by salt extraction method showed lower emulsifying activity as compared to proteins isolated by isoelectric precipitation, which exhibited higher surface charge and solubility as compared to salt extracted isolates (Karaca et al., 2011). Researchers found that faba bean and chickpea protein isolates produced by micellar precipitation showed higher emulsifying capacities than those produced by alkaline extraction/isoelectric precipitation method, which was attributed to the higher solubility of the isolates extracted by micellar precipitation (Abdel-Aal, Shehata, El-Mahdy, & Youssef, 1986).

At pH value lower or higher than the pI of pea protein, electrostatic repulsive forces between emulsion droplets are higher, which in turn promote stronger interactions between protein and the interface. On the other hand, emulsions are less stable at pH close to the pI of a protein as the electrostatic repulsions are weak relative to the attractive forces between droplets (McClements, 2004). A study by Liang & Tang, 2013, reported poor emulsifying properties at pH close to the pI of pea (around pH 4.5) and improved emulsifying properties at pH 3. They suggested that the emulsifying properties depend on the protein solubility, surface hydrophobicity and ability of protein to adsorb at the oil-water interface.

Another interesting study reported that at acidic pH, the interfacial films formed by proteins were thicker and demonstrated greater elastic properties, resulting in more stabilized emulsions as compared to pH 7. Pea protein was found to adsorb faster at pH 7 and thus, lead to the formation of inhomogeneous films as compared to acidic conditions, which slowed down the adsorption. Further, pea protein stabilized emulsions were found to be more stable with homogenous particle

size distributions at acidic pH. These results were attributed to pea protein solubility, droplet surface charge and globulin dissociation. It was proposed that at acidic pH values, adsorbed dissociated globulins form stronger viscoelastic networks at oil-water interface (Gharsallaoui et al., 2009). A study compared pea protein isolate and soy protein isolate and observed that pea protein isolate was a better emulsifier at pH 7 and a better foaming agent at pH 3 and pH 7 than soy protein isolate (Aluko et al., 2009).

3) Foaming properties

The ability of proteins to form foams is an important characteristic in food applications. In a study by Fernández-Quintela et al., pea protein isolate, faba bean protein isolate and soy protein isolate were compared in terms of their foaming capacities. They observed that pea and faba bean protein showed similar foam expansion (15 %) but pea protein isolate exhibited higher foam stability than both soy protein and faba bean protein. Another study reported foam stability (40%) when foams were stabilized using pea protein isolate, regardless of their method of preparation (alkaline extraction followed by Isoelectric precipitation or ultrafilteration) (Boye et al., 2010). A study suggested that foam stability of a commercial pea protein isolate (Nutri-Pea Ltd.) increased with the increase in pH (4, 7, and 9) from 30% to 80%. This was attributed to the fact that foams were stabilized by electrostatic repulsion, which increased as pH value moved away from isolelectric point (pH 4-5) and prevented rapid coalescence of air bubbles (Adebiyi & Aluko, 2011). It is also suggested that foaming capacity and stability improve at higher protein concentrations (Lam, 2016).

4) Gelling properties

Gelation is a common food process applied to fruit jellies, cake fillings, bread doughs, and meat products. Gels are formed when partially unfolded proteins form a three-dimensional protein

network, as a result of protein-protein and protein-water interactions. Protein gels are induced by heat treatment, pH, salts, pressure or with the help of solvents. However, the most studied method is heat-induced gelation for pea proteins (Lam et al., 2018). Forces that play a role in the formation of gels are hydrogen bonding, ionic bonding, covalent disulphide bonding, hydrophobic interactions, and Van der Waals forces (Zayas, 1997a) . Physicochemical and textural properties of pea protein gels prepared by heat treatment was studied by Shand, Ya, Pietrasik, & Wanasundara, 2007, and the results indicate that both globulins and albumins contribute to gel formation (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004; O'Kane, Vereijken, Gruppen, & Van Boekel, 2005; Shand, Ya, Pietrasik, & Wanasundara, 2008). These studies used pea protein isolates prepared by isoelectric precipitation for gelation, however, Sun & Arntfield, (2010) prepared gels using salt extracted pea protein isolates. They concluded that pea protein extracted by salt extraction showed better gelation ability as compared to commercial pea protein isolate (Nutri-Pea Ltd., Canada) prepared by isoelectric precipitation. This was because of the minimal protein denaturation caused by a mild process of salt extraction as compared to isoelectric precipitation, and it is known that lower the protein denaturation, stronger the gel is formed (X. D. Sun & Arntfield, 2010).

1.4 Research hypotheses and objectives

Vitamin D is essential for human body. A large population around the world is vitamin D deficient or insufficient, and aged population is at a higher risk of vitamin D deficiency. Low aqueous solubility of vitamin D results in its poor bioavailability in human body. Nanotechnology is a growing field in pharmaceutical industry, and its use can be further extended to food applications where nanoemulsions can be used as a delivery system for nutraceuticals to improve their absorption. Currently, many dairy-based vitamin D fortified products are available with only

limited non-dairy products. However, more non-dairy based vitamin D formulations should be developed to suit the needs of lactose intolerant and vegan populations. One way to improve vitamin D absorption is to prepare plant protein based nanoemulsions, resulting in a plant based vitamin D fortified product. Pea protein is a good candidate to prepare plant protein based nanoemulsions due to its good emulsifying capacity, high nutritional value, biocompatibility and GRAS status. Moreover, pea proteins are not regarded as major allergens, which gives brands a cleaner label. However, most current researches focus on developing emulsions using soy protein as an emulsifier (W. He et al., 2011; Li et al., 2018; Walia. et al., 2017; Xu, Mukherjee, & Chang, 2018). The research efforts to make nanoemulsions stabilized by pea protein are still very limited. Recently, Jiang et al., (2019) developed pea protein nanoemulsions to study their ability to protect vitamin D against UV radiation. Moreover, they studied their stability, antioxidant capacity and *in vitro* digestion and thus, suggested use of pea protein nanoemulsions for protection and delivery of such nutraceuticals. Another study prepared oil-in-water nanoemulsions using a combination of sodium caseinate and pea protein isolate to investigate the lipid digestibility and bioaccessibility of curcumin using *in vitro* digestion (Yerramilli, Longmore, & Ghosh, 2018). These studies suggested that pea protein can be used as an emulsifier to prepare nanoemulsions for application in food industries. However, more studies are required to compare pea protein with widely accepted emulsifiers, which will encourage its application in food products. Therefore, this research aims to demonstrate the ability of pea protein to form stable nanoemulsions by high-pressure homogenizer and further, compare it with a commonly used food emulsifier (soy lecithin) in terms of their cellular uptake efficiency using an *in vitro* model (Caco-2). Through this study, we also aim to study the capacity of pea protein and Tween 80 combination in forming and stabilizing nanoemulsions without the use of high-energy equipments.

Literature review of previous studies led to the following hypotheses:

1) Protein based nanoemulsions can be prepared using high-pressure homogenizer and their properties (particle size and stability) can be modulated by controlling the processing and emulsion conditions.

2) Stable nanoemulsions can be prepared with low-energy approach using a combination of emulsifiers (Tween 80 and pea protein).

3) Pea protein nanoemulsions can act as a delivery system for hydrophobic compounds such as vitamin D to improve its absorption in gut.

The overall objective of this research was to develop pea protein based nanoemulsions as delivery systems for hydrophobic nutraceuticals in order to improve their oral bioavailability. Vitamin D was the selected model for hydrophobic nutraceutical.

The specific objectives were:

Objective 1: To develop pea protein stabilized nanoemulsions for efficient encapsulation of vitamin D using high-pressure homogenizer.

Objective 2: To prepare nanoemulsions for vitamin D delivery by spontaneous emulsification (low-energy approach) using Tween 80 and pea protein as emulsifiers.

Objective 3: To investigate the impact of surface properties and nanoemulsions particle size on the *in vitro* cytotoxicity and uptake/transportation efficiency of nanoemulsions using Caco-2 cells.

1.5 Significance of this work

The outcome of this study will lead to the development of nanoemulsions for efficient encapsulation and delivery of hydrophobic nutraceuticals using both high-energy (high-pressure homogenizer) and low-energy (spontaneous emulsification) approaches. This research may demonstrate the possibility of using pea protein as an emulsifier for preparing nanoemulsions

suitable for the food industry, which may allow efficient vitamin D delivery to improve vitamin D status worldwide. The knowledge generated may guide industries to develop non-dairy foods and beverages for vitamin D fortification. Although vitamin D was the selected model in the current study, this research provides methods to develop delivery systems for many other hydrophobic nutraceuticals for improved oral bioavailability.

Chapter 2- Pea protein based nanoemulsions for delivery of vitamin D: Fabrication, stability and *in vitro* study using Caco-2 cells

2.1 Introduction

Vitamin D is one among the essential vitamins that is receiving particular attention owing to its beneficial role for human bone and teeth development. New evidences also suggest the relationship between vitamin D consumption and a reduction of chronic diseases such as autoimmune disorders, diabetes mellitus, cardiovascular diseases, celiac disease, cancers, and inflammatory diseases (E.-Q. Chen, Shi, & Tang, 2014; Grossmann & Tangpricha, 2010; Holick, 2009; Muscogiuri et al., 2017; Vatanparast, Calvo, Green, & Whiting, 2010). However, vitamin D is poorly soluble in water, which makes it difficult to be incorporated in food formulations. Also, crystalline forms of vitamin D are considered to have low bioavailability when it is administered orally due to its low solubility in the human gut (Kadappan et al., 2018; Maurya & Aggarwal, 2017; Öztürk, 2017).

Vitamin D deficiency has become a global issue. The elderly population is at a higher risk of vitamin D deficiency due to reduced cutaneous synthesis, poor dietary intake of vitamin D, and impaired gastrointestinal absorption (Meehan & Penckofer, 2015; Neo & Kong, 2016). It has been suggested to incorporate vitamin D in food products in emulsified form, due to the higher retention as compared to other formulations. For instance, a study by Kazmi et al. (2007) demonstrated higher storage stability of vitamin D_3 in emulsified form as compared to crystalline form when incorporated in cheese and stored for 3 months. Additionally, sensitivity of vitamin D to heat, light, and oxygen further limits its application in food areas. Vitamin D encapsulation in an emulsion based delivery system is an interesting approach to improve its stability during food processing and storage, and promote its absorption in the small intestine (W. He et al., 2011).

Nanoemulsions are colloidal dispersions of two immiscible phases- a mixture of water and oil along with a suitable surfactant required to reduce the interfacial tension between the two phases (Javadzadeh & Azharshekoufeh Bahari, 2017). They exhibit particle diameter below 500 nm (Capek, 2004; Chime et al., 2014; Sharma et al., 2013; Walia et al., 2017), and serve as an efficient encapsulation and delivery system for compounds that have low bioavailability due to poor solubility or permeability in gastrointestinal tract. Nanoemulsions can improve bioavailability of hydrophobic compounds because of its ability to increase their solubility, to protect their degradation in harsh gastric conditions, and to improve their absorption by prolonging its residence time in the intestine due to increased mucoadhesion and direct uptake of nanoparticles through endocytosis (Liu, Zhou, Chen 2019). Therefore, nanoemulsions have been explored for the delivery of bioactive compounds, including beta-carotene, vitamin D and E, curcumin, and lycopene. (Fathi et al., 2012; Odriozola-Serrano, Oms-Oliu, & MartÃ-n-Belloso, 2014; Hélder Daniel Silva et al., 2012). Due to their small size and high stability, nanoemulsions can improved cellular uptake and tissue permeability of the encapsulated compounds (Teng et al., 2013). A large number of studies have formulated nanoemulsions-based delivery systems using synthetic surfactants such as Tweens or Spans, which may give rise to toxicity issues in food, health and environment. It is believed that natural polymer based nanoemulsions possess good biodegradability and low cellular toxicity (Weber, Coester, Kreuter, & Langer, 2000). Among nanoemulsions, those stabilized by food proteins hold specific advantages because they possess high nutritional value, biocompatibility, biodegradability, and are Generally Regarded As Safe (GRAS) ingredients (W. He et al., 2011). Animal proteins such as gelatin, collagen, whey protein and casein, and plant proteins such as gliadin, zein and soy protein have been most explored for nanoparticle synthesis (Tarhini et al., 2017). Both animal and plant proteins have their set of

advantages, however, plant protein based formulations have attracted more interest due to the sustainability and safety considerations and the dietary preference of some consumers such as vegans and vegetarians.

Physicochemical characteristics such as particle size and surface charge play an important role in the cellular uptake and absorption of nanoparticles and nanoemulsions (Alexis et al., 2008; Behzadi et al., 2017). It is suggested that nanoparticle uptake by intestinal epithelium involves an adhesion process, followed by an internalization process (Gao et al., 2005). Some researchers suggest that nanoparticles with high positive charge may exhibit strong interactions with the negatively charged cell membrane, resulting in higher cellular uptake (Gao et al., 2005; He et al., 2010). On the other hand, it has been reported that positively charged nanoparticles depolarize the cellular membrane to a large extent, whereas, negatively or uncharged particles show negligible effect. Such perturbation of the membrane potentially causes cell damage and inhibits the proliferation of cells (Arvizo et al., 2010). In a study by He et al. (2010), the data suggest that negatively charged nanoparticles of 150 nm showed increase in cellular uptake in a surface charge dependent manner; particles with reduced negative charge exhibiting reduced electrostatic repulsion between cells and nanoparticles, and thus higher cellular uptake. Due to the lower negative charge of nanoparticles, weaker electrostatic repulsive forces are generated with the anionic cell membrane, which increases the contact between cells and nanoparticles (Liu, Yang, Wang, Liu, Guan, 2019; He et al., 2010).

Besides, particle size also significantly affect the cellular uptake pathway and efficiency (He et al., 2010). Nanoparticles are reported to enter cell, mainly through endocytosis, however, depending on the particle size, nanoparticles may undergo different uptake mechanisms (Behzadi et al., 2017). Possible mechanisms are 1) paracellular pathway- diffusion of small particles

(particle size < 50nm) through the intercellular space (epithelial cells), 2) transcellular transport by endocytic pathway- particles (size < 500nm) internalized by epithelial cells through passive diffusion or endocytosis, and 3) transcellular transport by lymphatic passage- particles (size < 5μm) adsorbed by M cells of the Peyer's patches (Win & Feng, 2005). In order to develop desirable delivery systems, a fundamental understanding of how surface charge and particle size impact stability, cytotoxicity and uptake of the protein stabilized nanoemulsions is necessary. However, such information has been seldom reported for (plant) protein stabilized nanoemulsions.

Field pea is a leguminous plant that is high in fibre and low in fat (Lam et al., 2018). Like most legumes, pea is a rich source of protein, with a protein content between 20 to 30 % (Aluko et al., 2009; Lam et al., 2018). According to Osborne's classification, pea proteins are mainly globulins, consisting of legumin and vicilin proteins as the major components (Djoullah et al., 2015). Legumins are hexamers of 350-400 kDa consisting of six polypeptide pairs. Each comprises of an acidic (~40 kDa) and a basic subunit (~20 kDa) that are linked by a disulfide bond. Vicilins contain both smaller (~50kDa) and larger (~70 kDa) convicilin subunits, which are associated into heterogeneous trimers of 150-190 kDa (Lam, 2016). Interest in pea protein has recently grown due to their high nutritional value, good emulsifying and foaming properties and their potential use in food product applications. In addition, pea proteins are not regarded as major allergens, and thus give brands a 'cleaner' label. Thus, it would be interesting to develop pea protein based nanoemulsions for the delivery of vitamin D. This would provide an opportunity to develop vitamin D fortification formulation in non-dairy that can be beneficial to lactose intolerant customers, strict vegans or customers with little preference for dairy products, as currently, most vitamin D fortifications have been developed for dairy products. However, research on pea protein based nanoemulsions is still very limited. Researchers have used small molecular surfactant (e.g.

phospholipids) (Heo, Kim, Pan, & Kim, 2016; Khachane et al., 2015) and large molecular surfactant (e.g. proteins) (Fan, Zhang, Yokoyama, & Yi, 2017; Silva et al., 2019) based nanoemulsions to study their cellular uptake separately but comparison of both in terms of absorption is limited or negligible. Therefore, in this study, small molecular surfactant and large molecular surfactant were compared in terms of their cellular uptake using Caco-2 cells.

It should be noted that small particle sizes down to 50 nm can be more readily obtained using synthetic surfactants, whereas, it is still a challenging task to decrease the emulsion particle size to such level using natural polymer based surfactants, like proteins. High-pressure homogenization is an efficient technique used to break down the particle size of emulsion, in order to achieve nano-size with a high level of monodispersity (Yuan et al., 2008a). Homogenization pressure is considered a significant factor dictating particle size and size distribution (Yuan, Gao, Zhao, & Mao, 2008a). In addition, the homogenization temperature, pressure, cycle, and the concentration of emulsifier and oil component can affect the stability and physico-chemical properties of the emulsions (Yuan, Gao, Zhao, & Mao, 2008b). In light of this, pea protein was explored in its capacity to form nanoemulsions using a high-pressure homogenization processing in this study. The effect of homogenization parameters on the physico-chemical properties, such as particle size and the stability of pea-protein nanoemulsions were investigated, with the aim of identifying the optimal conditions with minimum loss of encapsulated vitamin D. Later, caco-2 cells were used as an *in vitro* model to mimic the intestinal epithelium. The impact of emulsion particle sizes and surface properties on the cellular uptake was investigated. Food grade emulsions were prepared for the encapsulation of vitamin D with the aim to improve its solubility, hence, its bioavailability.

2.2 Materials and Methods

2.2.1 Materials

Pea protein concentrate was prepared according to our previous work (Jarpa-Parra et al., 2014) with slight modifications and the protein content was 97.34 % (w/w dry weight) as determined by combustion with a nitrogen analyzer (Leco corporation, St. Joseph, MI, USA), calibrated with analytical reagent grade Ethylenediaminetetraacetic acid (EDTA) using a conversion factor of 6.25. Canola oil was obtained from a local market. Vitamin D3 (Cholecarciferol, >98% purity, MW 384.64 g/mol, analytical grade), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and nile red were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). 4',6-diamidino-2-phenylindole (DAPI) and Alexa flour 488 dye were ordered from Life technologies (Burlington, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), non-essential amino acids (NEAA), HEPES solution, Antibiotic-Antimycotic (Anti-Anti), and trypsin-EDTA were purchased from GIBCO (Burlington, ON, Canada). Human colorectal adenocarcinoma cell line Caco-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All chemicals used were of reagent grade unless otherwise stated.

2.2.2 Preparation of nanoemulsions

Nanoemulsions were prepared using pea protein solution as aqueous phase and canola oil as lipid phase. Specifically, pea protein (1% w/v) was allowed to dissolve in phosphate buffer (pH 7) for two hours using magnetic stirrer. Being a lipophilic compound, vitamin D_3 was dissolved in the lipid phase, prior to the addition into pea protein solution. Concentrated solution of vitamin D (11.7 mg/ml) in canola oil was prepared with the aim of diluting it to appropriate concentrations prior to the addition into the final products. Then, a coarse emulsion was prepared by breaking

down the particles using a high speed mixer (30,000 rpm, PowerGen, Fisher Scientific International, Inc., CA, USA), followed by processing through a high-pressure homogenizer (Nano DeBEE, Bee International, Inc., MA,USA) to form nanoemulsions. Varying processing conditions, including different homogenization pressures (10, 20 and 30 kpsi), oil concentrations (0.5, 1, 2.5, 5% w/v), protein concentrations (1%, 5% and 10% w/v) and number of homogenization cycles (1 to 5) were applied to nanoemulsions to observe their effect on particle size and stability. The prepared nanoemulsions were stored at 4 °C until further use.

2.2.3 Characterization of nanoemulsions

The size and zeta-potential of the prepared nanoemulsions were measured by dynamic light scattering using a Zetasizer Nano S (model ZEN 1600, Malvern Instruments Ltd, UK) at room temperature. Nanoemulsion suspensions were diluted to appropriate concentrations using phosphate buffer (pH 7) before analysis to avoid multiple scattering. The refractive index of the particle and dispersion medium was set at 1.52 and 1.33, respectively (Q. Ye et al., 2016). The analysis was performed in triplicates for each sample and results were described as mean ± SD.

2.2.4 Encapsulation of vitamin D

For estimation of the encapsulation efficiency, vitamin D was extracted from the nanoemulsions by the method adapted from Xue et al. (2008) with slight modifications. Briefly, 10 ml of freshly prepared nanoemulsion were added to the flask and shaken vigorously. Then, 65 ml of methanol were added to the flask to extract vitamin D. The mixture was placed in ultrasonic bath at 40-50 °C for 30 min, and then cooled down to room temperature and centrifuged at 2800 g-force for 10 min. The supernatant was filtered using a 220 nm filter and analyzed using a high performance liquid chromatography (HPLC) for quantification of the total vitamin D. For determination of the free vitamin D in the nanoemulsion suspension, 3 ml of nanoemulsion were mixed with 7 ml of

hexane, vortexed well for 1-2 min and centrifuged at 700 rpm for 5 min, in order to obtain a distinct layer of hexane. The process of vitamin D extraction using hexane was repeated twice. The hexane layers from these three steps were pooled together and free vitamin D was analyzed. The vitamin D quantification was conducted on a HPLC system (1200 series, Agilent Techonologies, Inc., Santa Clara, CA) equipped with an Extend C18 column. The chromatographic conditions were as followed: mobile phase- methanol:water (98:2% v/v), flow rate of 1.5 ml/min, column oven temperature of 35° C, injection volume of 10 µl and detection was carried out at 265 nm with a UV-vis detector. Quantification was achieved using comparison between observed peak area and calibration curve. Chromatographic conditions were the same for standard solutions as well.

The encapsulation efficiency was calculated by using the following equation:

EE (%) = [(total vitamin D - free vitamin D) / total vitamin D)] × 100

2.2.5 Morphology study

The morphology of nanoemulsions was observed using a transmission electron microscope (TEM, Morgagni 268, Philips-FEI, Hillsboro, USA) at 80Kv. One drop of the sample was added to TEM grids coated with carbon films. Then, a drop of sodium phosphotungstate (2% w/v) was placed over the sample droplet as a negative stain. The sample was allowed to dry at room temperature before analysis (W. He et al., 2011; Yang, Zhou, & Chen, 2014).

2.2.6 Caco-2 cell culture model

Caco-2 cell line is derived from human adenocarcinoma and monolayers of Caco-2 cells resemble the properties and structure of human intestinal epithelium as these cells form tight cell junctions and electrical properties similar to intestinal cells (Zhang et al., 2015). For this reason, Caco-2 cell line is a widely accepted *in vitro* model for studying uptake efficiencies and drug deliveries across the small intestinal epithelium (Zhang et al., 2015).

The human adenocarcinoma cell line Caco-2 passages between 20-40 were used for cell culture studies. Cells were cultured in T-75 flasks at 37° C in a humidified incubator 5%CO2/ 95% air in DMEM with sodium pyruvate (high glucose) supplemented with FBS (20% v/v), NEAA (1% v/v), Anti-Anti (1% v/v) and 25 Mm HEPES (Roger, Lagarce, Garcion, & Benoit, 2009; Zhang et al., 2015). Cell morphology was monitored regularly and the culture medium was changed every second day. Cells were harvested at 80% confluence using 0.25% trypsin in 1 mM EDTA solution and seeded onto new culture flasks.

2.2.6.1 Cytotoxicity assay

In order to investigate the safety of the developed nanoemulsions, their cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide viability assay (MTT assay). Caco-2 cells were seeded onto 96 well plates at a density of 8×10^3 cells per well in 100 µl culture medium. After allowing the cells to grow for 24 hrs, nanoemulsions were added to each well at final concentrations of 0.25, 0.125, 0.0625, 0.03125 and 0.0156 mg/ml, respectively with incubation period of 6 hrs at 37° C. After removing nanoemulsions, 10 µl of MTT solvent (5 mg/ml in PBS) were added into each well and incubated for 4 hours at 37°. Upon removing the solvent, 100 µl of DMSO were added to each well, followed by examining the plate at 570 nm using a microplate reader (SpectraMax, Molecular Devices, USA) (Yang et al., 2014). Cell viability was presented as percentage of living cells in the test wells with respect to control wells. (W. He et al., 2011). Different concentrations of samples were evaluated to determine the safe concentration for further analysis.

2.2.6.2 Qualitative and quantitative cell uptake study

The uptake of the nanoemulsions by Caco-2 cells was analyzed with the help of a confocal laser scanning microscopy (CLSM). Pea protein nanoemulsions were labelled with nile red dye (0.025

% w/v in canola oil containing vitamin D), WGA-Alexa Flour 488 (5 µg/mL in HBSS) and DAPI (0.1 µg/mL in PBS) were used to stain cell membrane and nuclei, respectively. Cells were imaged for various samples at different times and concentrations. The cellular uptake study was performed as per the protocol from earlier reports (Yang et al., 2014). Briefly, nile red (0.025 % w/v) was dissolved in canola oil under magnetic stirrer to ensure efficient mixing. This was followed by centrifugation at 12,000 g for 10 min to remove insoluble particles and only supernatant nile red solution was used for nanoemulsions preparation. Cells were seeded onto glass bottom microwell dishes (P35G-1.5-14-C, MatTek Corp., USA) at a density of 10^5 cells per dish and cultured for 2-5 days to form confluent cell monolayer. Once a confluent cell monolayer was formed, uptake experiment was initiated by replacing the media of wells with HBSS buffer and incubated for 30 min at 37 °C. Upon removal of HBSS, nanoemulsion suspensions in HBSS at a concentration of 0.0625 and 0.03125 mg/ml were added to wells, and incubated for two and six hours at 37 °C. After removing the nanoemulsions, cells were gently rinsed with PBS three times before fixing the cells with 4% paraformaldehyde (w/v in PBS pH 7.2) for 15 min. Upon gentle washing of cells, WGA-Alexa Flour 488 conjugate labeling solution (5 µg/mL in HBSS) and DAPI solution (0.1 µg/mL in PBS) were applied to cells for membrane and nucleus staining, respectively. Each staining was performed for 15 min in dark conditions, followed by mounting with Prolong Gold Antifade Reagent. Cells were then observed using CLSM 510 Meta (Carl Zeiss, Jena, Germany) equipped with a diode, an argon laser and a helium/neon laser, providing the excitation at 405 nm, 488 nm and 561 nm respectively. An oil immersion objective (40×) was used for observing and images were processed using ZEN 2009LE software (Carl Zeiss MicroImgaing GmbH, Germany). Cellular uptake of nanoemulsions by Caco-2 cells was quantified by flow cytometry, which estimates the amount of fluorescence labelled nanoemulsions in the cells by measuring the

fluorescence intensity associated with the cells. Flow cytometric analysis was performed using the protocol from a previous study with modifications (Zhang et al., 2015). Briefly, cells were seeded onto 6-well plates at the density of $1x10^5$ cells per well and incubated for 2-5 days to form a confluent layer. Upon removal of media, nanoemulsions suspensions in HBSS at a concentration of 0.0625 and 0.03125 mg/ml were added to wells, and incubated for 0.5, 1, 2, 3 and 6 hours at 37°C. At the end of each time interval, cells were rinsed with PBS to remove any particles of nile red binding to cell surface, and were further trypsinized for 3 min. Trypsinization was stopped by the addition of cell culture media. Cells were removed from the wells by pipetting. After centrifugation, 4% paraformaldehyde (w/v in PBS pH 7.2) was added to cells and incubated for 15 min, followed by resuspension of cells in PBS in FACS tube (VWR). The fluorescence intensity associated with cells was measured using a flow cytometer (B.D. Biosciences FACSort, CA). A 561 nm laser was used to excite nile red and emitted fluorescence was detected at 610 nm. 10000 cells were analyzed for each sample and cellular debris was excluded from the analysis to establish a collection gate using side-scatter versus forward-scatter histogram (Snipstad et al., 2014).

2.2.7 Caco-2 cell monolayers

Caco-2 cells were cultured in 75 cm^2 flasks and trypsinized at 90 % confluence. Cells at passages 20-30 were seeded onto polycarbonate membrane filters (0.4 μm pore size, 1.12 cm^2 grown surface) inside Transwell 12- well plates (Costar, Corning, NY, USA) at a density of $3x10^5$ cells per insert. To apical chambers, 0.5 ml of suspended cell solution were added and basolateral chambers were filled with 1.5 ml DMEM. Cell plates were incubated overnight at 37° C and 5% CO_2 before replacing the media in apical and basolateral chambers. To ensure cell monolayer formation, media was replaced every second day for 21-28 days. To monitor the integrity of monolayers, transepithelial electrical resistance (TEER) values were measured using the epithelial

voltammeter (EVOM^2, World Precision Instruments, Saratosa, FL). Monolayers with TEER values higher than 300 Ωcm2 were used for transport study.

2.2.7.1 Transport efficiency

The transport of vitamin D across Caco-2 cell monolayers was investigated using the method reported by Hubatsch et al., (2007) with minor modifications. In brief, Caco-2 cells were seeded on permeable inserts at a density of 3×10^5 cells/ insert and were allowed to differentiate and polarize to form a continuous monolayer. Caco-2 cells grown in DMEM for 25 days were used to study the transport efficiency of vitamin D. Culture medium in the transport plates was changed 12-24 hrs before the experiment. Filter supports with cell monolayers were gently washed with HBSS and incubated with fresh HBSS for 15-20 min at 37 °C. Meanwhile, donor solutions were prepared with a final vitamin D concentration of 10 µg/ml in the form of P 230, P 350 emulsions and free vitamin D suspension. Then, HBSS buffer was removed and filter supports were transferred to a new plate containing HBSS in the basolateral chamber and 0.5 ml of donor solutions were added to the apical chambers either in the form of emulsion samples or free vitamin D solution. After incubating plates for 2 hrs at 37 °C under an atmosphere containing 5% CO_2, samples from each basolateral chamber were collected into a centrifuge tube. Transport of vitamin D from the apical to the basolateral side of the Caco-2 cell monolayer was determined by HPLC analysis after 2-hour transport study.

Vitamin D from samples was extracted using methanol in the ratio of 1: 6 (v/v) and the mixture was placed in ultrasonic bath for 30 min, and later, centrifuged to obtain the supernatant, which was filtered and used for vitamin D quantification using HPLC. Vitamin D standards were prepared ranging from 10 µg/ml – 500 µg/ml in methanol. Peak areas were determined to estimate

the percent transport of vitamin D, as defined by the ratio of basolateral peak area and initial apical peak area.

2.2.8 Statistical analysis

Experiments were performed in triplicates and results were presented as mean values ± standard deviation. Error bars in figures represent standard deviation. Statistical comparisons were conducted using Student's t-test, one-way ANOVA, two-way ANOVA and Tukey's test. A significance level of $p < 0.05$ was considered significant. Origin 9 software was used for graphing and analysis of numerical data.

2.3 Results and Discussion

2.3.1 Nanoemulsion formation

In this work, protein type (pea protein) was kept constant, while the influence of processing conditions, such as homogenizer pressure and cycles, and protein concentrations were investigated. For comparative studies, nanoemulsions were also prepared using phospholipid alone and in combination with pea protein. Measurements by zetasizer indicate that the nanoemulsions exhibited size ranging from 170 to 350 nm. The polydispersity index (PdI) value is the measurement of particle size distribution and a small PdI value indicates a narrow size distribution (Yuan et al., 2008b). In our studies, all samples exhibited PdI value lower than 0.3, which is an acceptable homogeneity level (Dragicevic-Curic, Gräfe, Gitter, Winter, & Fahr, 2010; W. He et al., 2011). The nanoemulsions were mildly negatively charged with the zeta-potential of around -25mV, which can be favorable for the purpose of bioactive delivery (Zhang et al., 2015). Positively charged nanoparticles can be toxic and immunogenic, because of their strong interactions with cell membranes and serum proteins (Bajaj et al., 2009). The nanoparticles with

or without vitamin D did not show significant difference in particle sizes, suggesting that there is no interference of vitamin D with the nanoemulsion preparation.

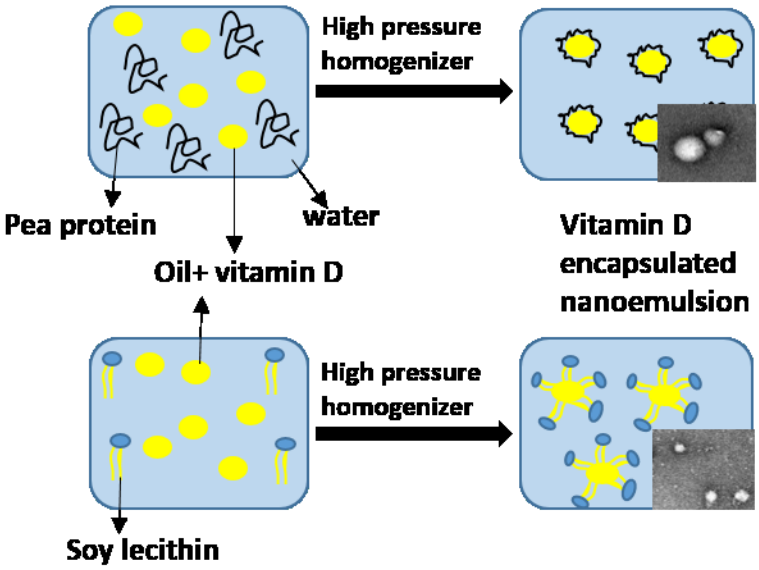

Figure 2.1 Preparation of vitamin D encapsulated nanoemulsions.

2.3.1.1 Influence of high-pressure homogenization pressure

High-pressure homogenizer is a high energy input technique and so, with the increase of homogenizer pressure, a high intensity of shear forces and turbulence are generated that cause the breakdown of emulsion droplets, resulting in smaller particle sizes (Tan & Nakajima, 2005).

In this series of experiments, the effect of homogenization pressure on particle size was determined. The protein concentration and number of homogenization cycles were kept constant at 1% w/v and 2 cycles, respectively, while homogenization pressure was varied. The oil content in the emulsions was 0.5% and 5% w/v. From Figure 2.2a, it can be observed that particle sizes decreased significantly ($p < 0.05$) with increase in the pressure conditions. It is noted that the particle size for both 0.5% and 5% w/v oil samples decreased when pressure was increased from 10 kpsi to 20 kpsi but further increase of pressure to 30 kpsi did not show significant effect on

particle size. For this reason, a further increase in pressure was no longer considered efficient and 20 kpsi was the selected pressure for further experiments. These results are in agreement with the previous findings where increase in homogenization pressure from 1.5 to 11.5 kpsi resulted in significant drop in particle size of whey protein and beta-lactoglobulin nanoemulsions (W. He et al., 2011). They suggested that surfactant adsorption to the surface of emulsion droplets could be enhanced by increasing the homogenization pressure and cycles to a considerable extent.

The trend of decreasing particle size with the increase in pressure was also observed for nanoemulsions stabilized by small molecular surfactant such as Tween 20. Yuan et al., (2008) observed that the particle size decreased from 168 nm to 114 nm as the pressure increased from 8.7 to 20.3 kpsi, with similar trend observed for homogenization cycles. It must be noted that the emulsifier concentration used in their study was much higher (Tween 20- 10%w/w) as compared to this work (1% w/v protein) , which could be one of the factors for a reduced particle size to 114 nm at 20.3 kpsi (Yuan et al., 2008b). Additionally, the use of small molecular surfactants can result in smaller particle sizes as compared to natural polymers, like protein. From Figure 2.2a, it should also be observed that at the same pressure conditions, particle size increased significantly with an increase in oil content in emulsion systems. For further investigation, a series of experiments were later conducted based on varying oil composition.

2.3.1.2 Influence of homogenization cycles

In this experiment, the influence of homogenization cycles on particle size was determined. Based on the previous experiment, 20 kpsi was considered a suitable pressure condition. Here, the number of homogenization cycles were varied from 1 to 5 for nanoemulsions with different oil content (0.5% and 5% w/v). The sample prepared with 0.5% oil showed a drop in size from 257 to ~230 nm from single to three homogenization cycles (Figure 2.2b). Similarly, the sample with 5% oil

composition showed a decrease in size from 377, 350 to 294 nm on passing the emulsion through homogenizer from 1 to 3 homogenizing cycles, respectively. Another study showed similar effect of homogenization cycles as they were successful in bringing the size down to 127 nm from 162 nm, from single to three homogenization cycles, respectively but observed no further reduction in particle size after three passes of emulsion through homogenizer at a pressure of 100 MPa (Yuan et al., 2008a). This demonstrates that increase in the number of homogenization cycles significantly ($p < 0.05$) decreased the particle size of emulsion for both samples (0.5 and 5% w/v oil based). After three passes of emulsion through the homogenizer, an inconsistent trend was observed for both samples with the increase in size at fourth cycle and a dropping trend in particle sizes at the fifth cycle (Figure 2.2b). Meanwhile, a rise in temperature was observed after 3 passes of homogenization cycles. High-energy input may have overly exposed the hydrophobic groups of protein, causing coalescence between emulsion droplets and thus, resulting in increased particle size. It was noticed that samples prepared until two homogenization cycles were found to be more stable for a longer time as compared to the samples prepared with more cycles. In a high-pressure system, thermal energy is produced (Klang & Valenta, 2011), and a rise in temperature in the processing chamber is inevitable. With the increase in temperature, the Brownian motion becomes more rapid, which would lead to more frequent particle collisions and thus, resulting into particle aggregation and emulsion instability (Yuan et al., 2008a). Thus, the low emulsion stability at higher pressures (above 20 kpsi) and cycles (above 2) could be attributed to the increased particle collision, as high collision frequency of the particles is considered as one of the factors responsible for emulsion aggregation (McClements, 2005). It is also known that vitamin D is sensitive to heat, light and oxygen (Walia et al., 2017). Thus, nanoemulsions prepared at 20 kpsi with two cycles were selected for subsequent studies to avoid the degradation of the loaded vitamin D.

2.3.1.3 Influence of oil concentration

Four samples were investigated, consisting of varying oil content (0.5, 1, 2.5 and 5% w/v) for the effect of oil composition on the particle size by maintaining constant homogenization pressure (20 kpsi) and cycles (two). As shown in Figure 2.2c, a significant increase in particle size was observed with an increase in oil content from 0.5 to 5% w/v. As the protein concentration remains the same for all four samples, emulsions containing high oil compositions had lower emulsifier at the oil/water interface, leading to large particle size. In addition, sample with 5% oil was more viscous due to higher volume fraction, which also explain the larger size of the nanoemulsions. Nevertheless, nanoemulsions with 233 and 350 nm both showed unimodal particle size distribution with a satisfactory PdI value (below 0.3). Thus, pea protein based stable emulsions with two distinctive sizes (233 and 350 nm) were prepared and coded as P 230 and P 350, respectively.

In general, high-pressure homogenizer ensures good emulsification as a controllable particle range from 233 to 350 nm was obtained using this study. These results demonstrate that by varying the nanoemulsions formulating conditions, a controllable particle size range could be achieved. In addition, these results play an important contribution in studies that focus on nanoemulsions applications owing to its controllable particle size, such as cellular toxicity, cellular uptake, tissue permeability and controlled release of encapsulated ingredients and drug delivery studies (Lamprecht, Schäfer, & Lehr, 2001; Win & Feng, 2005).

2.3.1.4 Influence of protein concentration

In this experiment, the effect of varying protein concentration (1, 5, 10 % w/v) on particle sizes was studied for 0.5% and 5% w/v oil content samples with constant high pressure homogenization conditions at 20 kpsi for 2 cycles. As shown in Figure 2.2d, it was observed that with the increase in protein concentration, there was a significant ($p < 0.05$) decrease in particle sizes for both

samples. This could be because smaller particles will have larger surface area, and would require more protein to cover and adhere to the interface. Sample with higher oil content showed a consistent trend of reduced particle sizes with increased protein concentration (particle sizes- 1> 5> 10 % w/v), whereas, sample with lower oil content showed significant decrease in size when protein concentration was increased from 1% to 5% w/v (Figure 2.2d). Slight increase in size was observed when further protein concentration was increased to 10%. The reason for the gradual increase in particle size for the latter sample at 5% emulsifier concentration could be that at this particular concentration, the emulsifiers covered all the emulsion droplets, and excess emulsifiers might not be used to cover the interfaces, instead, formed emulsifier layers over the pre-existed emulsion droplets, resulting in larger size droplets. For two reasons, 1% w/v protein concentration was selected for future sample preparation, firstly, economical consideration i.e., it is always better to produce stable emulsions using lower emulsifier concentration, which would result in low cost. Secondly, at 1% emulsifier concentration, a wider range of particle sizes was obtained between two different samples- 0.5% and 5% oil based sample- producing nanoemulsions size of 233 (coded as P230) and 350 nm (coded as P350), respectively. This distinct difference in particle sizes helped in conducting comparative studies in the future experiments. In addition, high concentration of protein at the surface could lead to protein aggregates, affecting emulsion stability. These above results indicate that nanoemulsions of unimodal particle size distribution and satisfactory PdI can be stabilized by pea protein using high-pressure homogenization technique and the particle size can be modulated by varying the processing and formulation parameters. For comparative studies, lecithin nanoemulsion with particle size of 201.8 nm were prepared using the same high-pressure homogenization conditions as those used to prepare pea protein nanoemulsions of 230 nm. In

addition, pea protein was combined with lecithin to prepare protein-lecithin nanoemulsions with particle size of 152.6 nm and coded as PL150.

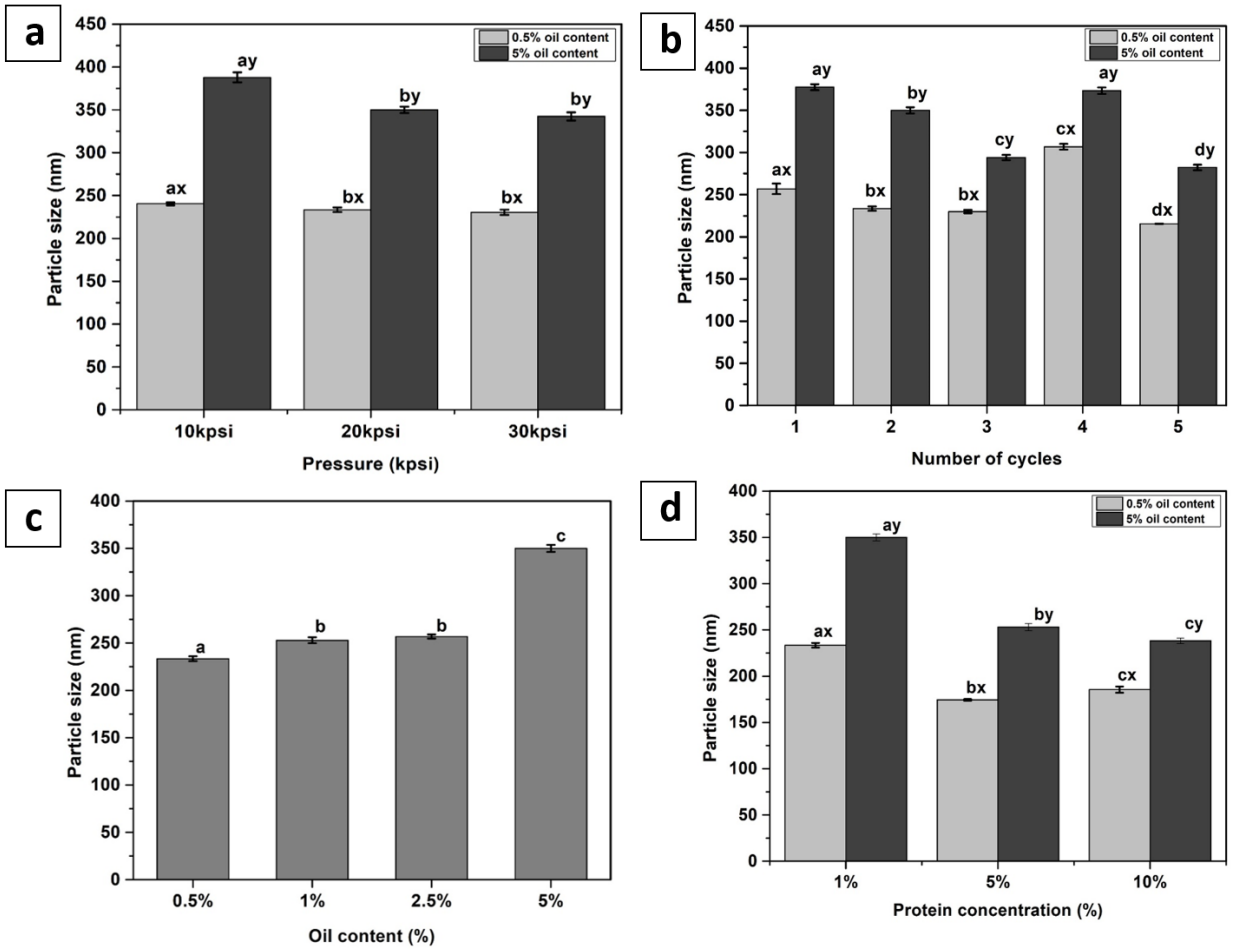

Figure 2.2 a) Effect of homogenization pressure (10kpsi, 20kpsi, 30kpsi), b) number of homogenization cycles (1,2,3,4 or 5), c) varying oil content (0.5, 1, 2.5 and 5 % w/v) d) varying protein content (1, 5, 10 % w/v) on particle size of the emulsions. Letters a,b,c,d denote significant differences within the same sample based on varying parameters and x, y denote significant differences ($p < 0.05$) between the two samples.

2.3.2 Morphology study

TEM observations were made immediately after the nanoemulsions were prepared, and the images are presented in Figure 2.3. Both nanoemulsions stabilized by pea protein and soy lecithin showed spherical shape and smooth surface. Soy lecithin (phospholipids), as a natural small molecule surfactant obtained from the cell membrane of soybeans, is a widely used surfactant/ emulsifier in the food industries (Klang & Valenta, 2011; Ozturk, Argin, Ozilgen, & McClements, 2014; Patel, Schmid, & Lawrence, 2006). The average particle sizes were also determined based on the particle diameter from TEM images (Akbari, Tavandashti, & Zandrahimi, 2011), and they were found to be similar to the size measured by Zetasizer. A slightly smaller particle size observed in TEM images could be due to the shrinking of the particle size when air dried prior to TEM observations (Zhang et al., 2015).

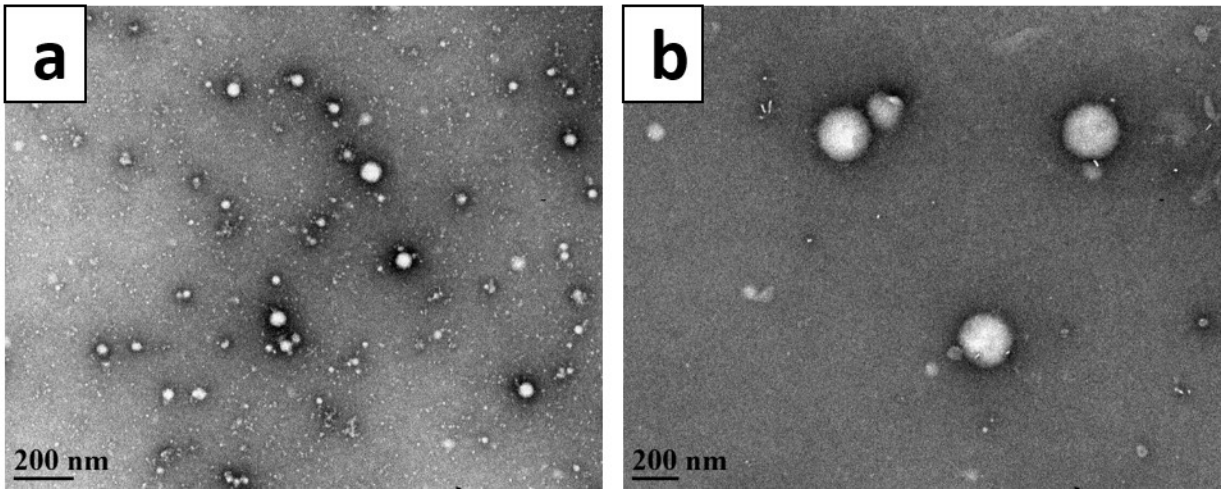

Figure 2.3 Transmission Electron Microscope images for a) Lecithin- based emulsion and b) Protein-based emulsion, showing spherical shape of nanoparticles.

2.3.3 Encapsulation efficiency

Pea-protein nanoemulsions were found to effectively carry vitamin D in a stable integrated form. Encapsulation efficiencies of vitamin D in pea-protein nanoemulsions were 94.8 and 95.3% for nanoemulsions sample P230 and P350, respectively. Pea protein nanoemulsions were considered as an efficient encapsulating system by retaining most of the vitamin D. For the selected nanoemulsions samples, a summarised table of their composition, particle sizes, zeta potential, polydispersity index and encapsulation efficiencies is presented (Table 2.1).

Table 2.1 Characterizations of pea protein stabilized nanoemulsions.

Properties of samples	**Samples**			
	Pea protein 234 nm (P 230)	**Pea protein 350 nm (P 350)**	**Lecithin 202 nm (L 200)**	**Protein and Lecithin 153 nm (PL 150)**
Emulsifier type and concentration (w/v %)	Pea protein (1 % w/v)	Pea protein (1 % w/v)	Soy lecithin (1 % w/v)	Pea protein (1 % w/v) + soy lecithin (1 % w/v)
Oil content (w/v %)	0.5 % w/v	5 % w/v	0.5 % w/v	0.5 % w/v
Size ± SD (nm)	233.6 ± 2.6	350 ± 3.7	201.8 ± 1.9	152.6 ± 1.6
Polydispersity index	0.25 ± 0.005	0.28 ± 0.023	0.24 ± 0.01	0.22 ± 0.005
Zeta potential (mV)	-22.3 ± 1.56	-24.3 ± 1.57	-34.2 ± 0.5	-29.2 ± 1.22
Encapsulation efficiency (%)	94.8	95.3	96	93.6

2.3.4 Cytotoxicity

MTT test was performed to study the cytotoxicity of the nanoemulsion samples. In addition, influence of nanoemulsion (pea protein nanoemulsions - 230 nm particle size) concentration on

cell viability was studied using nanoemulsion concentration range between 0.25 to 0.015 mg/ml at 6 hours. Figure 2.4a shows that nanoemulsions were safe (cellular viability > 80%) at concentrations ≤ 0.0625mg/ml. For *in vitro* cell models, cell viability greater than 80% is acceptable (Piazzini et al., 2019). Cell viability was found to decrease with increasing nanoemulsion concentration. Significant differences in cytotoxicity can be observed between 0.25 mg/ml and ≤ 0.0625mg/ml, as shown in figure 2.4a. Therefore, 0.0625 mg/ml nanoemulsion concentration with incubation time of 6 hrs was tested for all samples, which includes pea protein nanoemulsions (P 230 and P350 nm), lecithin (L 200) and protein-lecithin nanoemulsions (PL 150). As shown in figure 2.4b, all nanoemulsions were relatively safe as they demonstarted cellular viability greater than 80% and no significant difference was found among the samples.

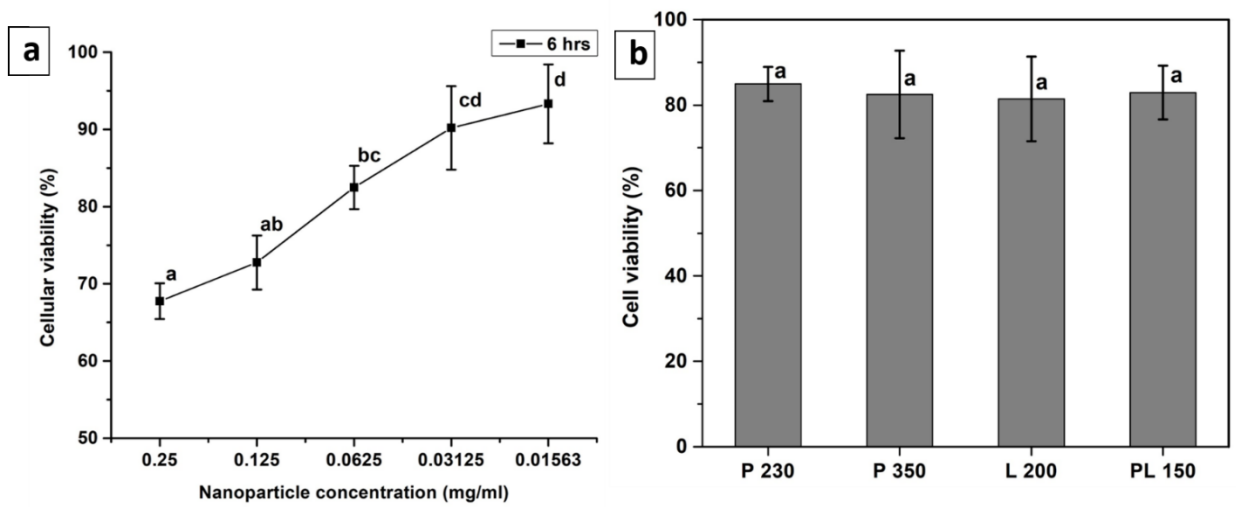

Figure 2.4 a) Influence of pea- protein nanoemulsions (particle size- 230 nm) concentration (250 - 15 µg/ml) after 6 hrs incubation on cellular viability, b) cytotoxicity effect of nanoemulsions at a concentration of 62.5 µg/ml after incubating for 6 hrs using MTT assay. Pea protein-234 nm (P 230), Pea protein-350 nm (P 350), Lecithin-202 nm (L 200) and Protein and Lecithin-153 nm (PL 150).

2.3.5 Cell uptake study

2.3.5.1 Confocal microscopy- Qualitative

As seen in figure 2.5, similar nile red fluorescence can be observed for P230 and L 200, with lower visibility of nile red signals for P 350. Nile red was observed to be distributed throughout the cytosol for P 230 and L 200. Through the confocal images, it was difficult to visualize nile red in all samples; however, a clear qualitative comparison can be made between P 230 and P 350, as P 230 shows higher nile red signals, in comparison to P 350 (Figure 2.5). This indicated that particle size plays an important role in the uptake of pea-protein nanoemulsions by Caco-2 cells as smaller particles of particle diameter 230 nm exhibited higher nile red uptake as compared to larger particles of particle size 350 nm.

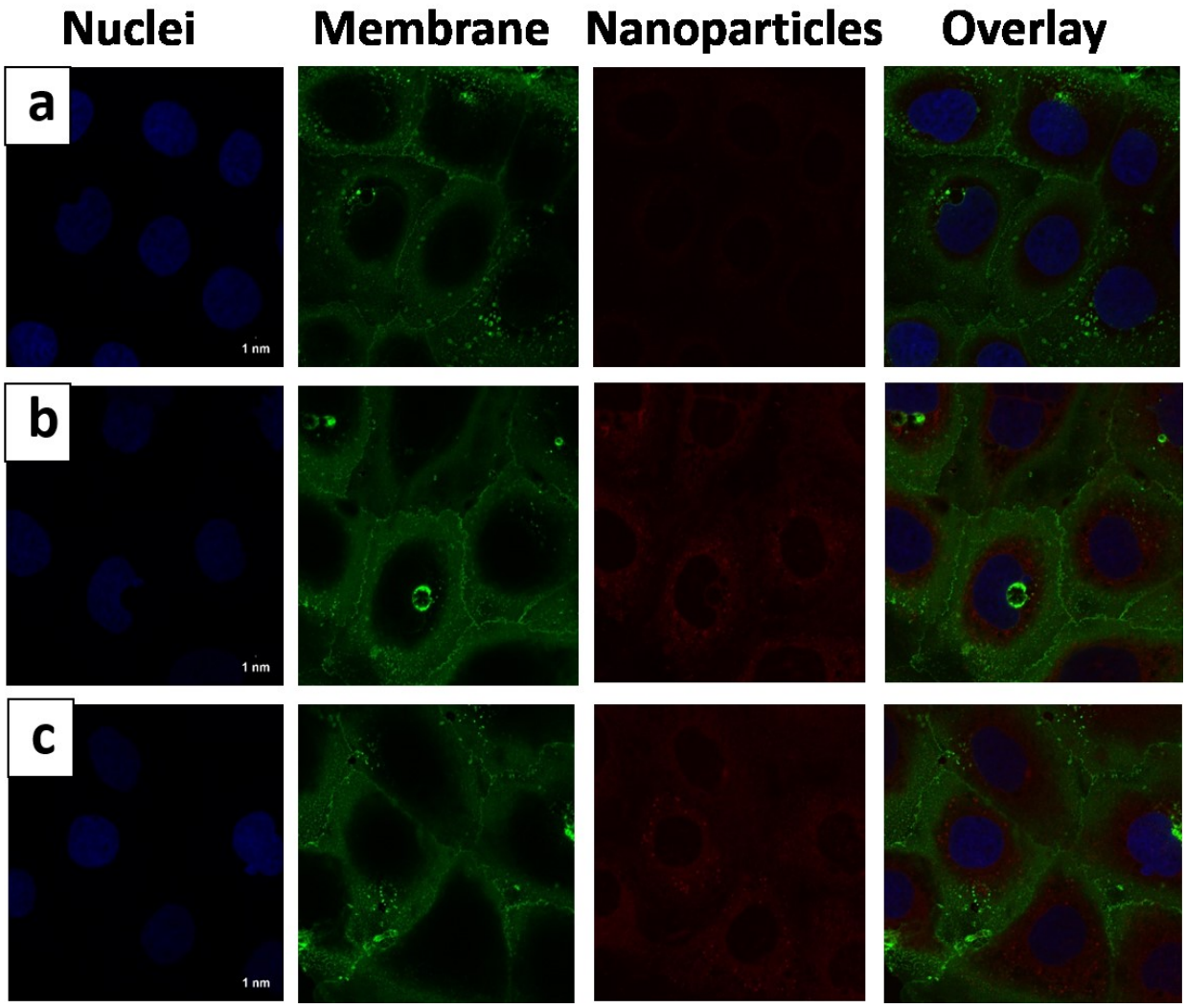

Figure 2.5 Confocal images of Caco-2 cells for a) P 350 b) P 230 and c) L 200 samples at 62.5 µg/ml concentration after incubating for 6 hours. WGA-Alexa Flour 488, DAPI and Nile red dyes were used to stain cell membrane, nuclei and nanoparticles, respectively.

In order to investigate intracellular nanoemulsions distribution, a 3-dimensional analysis of Caco-2 cells was performed as presented in figure 2.6. This 3D confocal image of Caco-2 cells showed red signals throughout the cytoplasm, which confirmed that nanoemulsions were accumulated in the cytosol. No red signals were detected in the cell nuclei, indicating no association of

nanoemulsions into cell nuclei. This was observed favourable because uptake of nanoemulsions into the nucleus may cause a change in gene sequences, resulting into genetic mutation. A previous study involving polymeric nanoparticles with Caco-2 cells has shown similar results in terms of intracellular distribution, which confirmed nanoemulsions distribution throughout the cytoplasm (Snipstad et al., 2014; Zhang et al., 2015).

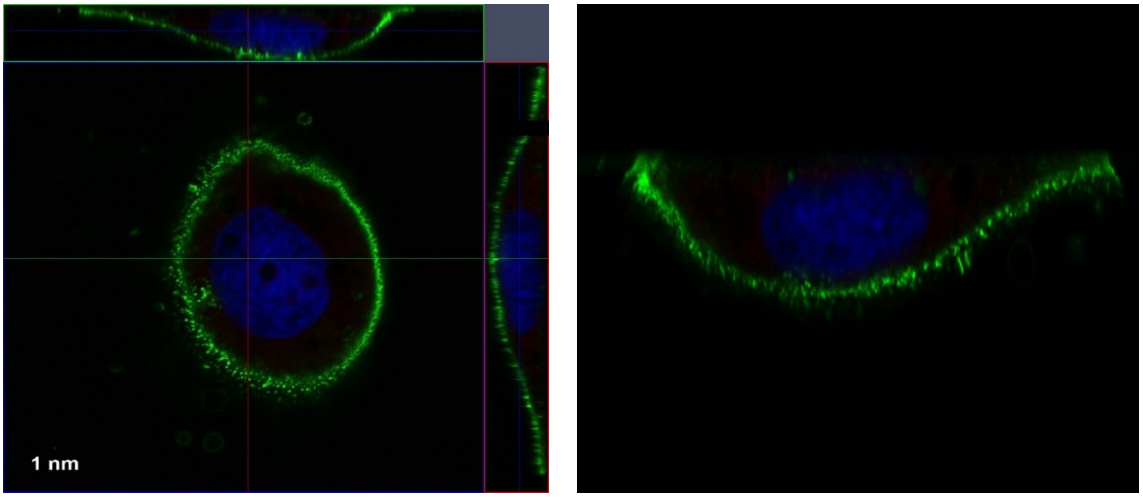

Figure 2.6 Confocal microscopic image of Caco-2 cells after 6 h incubation with Nile red-labeled protein nanoparticles (62.5 µg/ml), presenting three-dimensional analysis of the optical xy-section (center square) with xz- and yz- projections (side panels).

2.3.5.2 Flow cytometry- Quantitative

In order to confirm the uptake efficiency of nanoemulsions into cells, a quantitative analysis was performed for all samples with the use of flow cytometer. The effect of nanoemulsions concentration (0.0625 and 0.03125 mg/ml) and various time intervals (0.5 to 6 hrs) on the cellular uptake was studied. Cells showed an increase in fluorescence gradually and reached a maximum at 2 hours time interval and thereafter, a significant drop in fluorescence intensity to almost half was observed until 6 hours (Figure 2.7a). This observation can be explained by the saturation level

of nile red labelled nanoemulsions into the cells after two hours incubation, which might have led to the reduction in absorption of more nanoemulsions particles into the cells (Win & Feng, 2005). Similar observation was made in another study for polymeric nanoparticles, where nanoparticles uptake increased with the incubation time over a period of 2 hours, and no further increase in uptake was observed after 2 hrs (Snipstad et al., 2014; Win & Feng, 2005). As maximum uptake of nile red was observed at 2 hours, this time period was considered best for further uptake studies, aiming to compare various samples with varying sizes and surface properties.

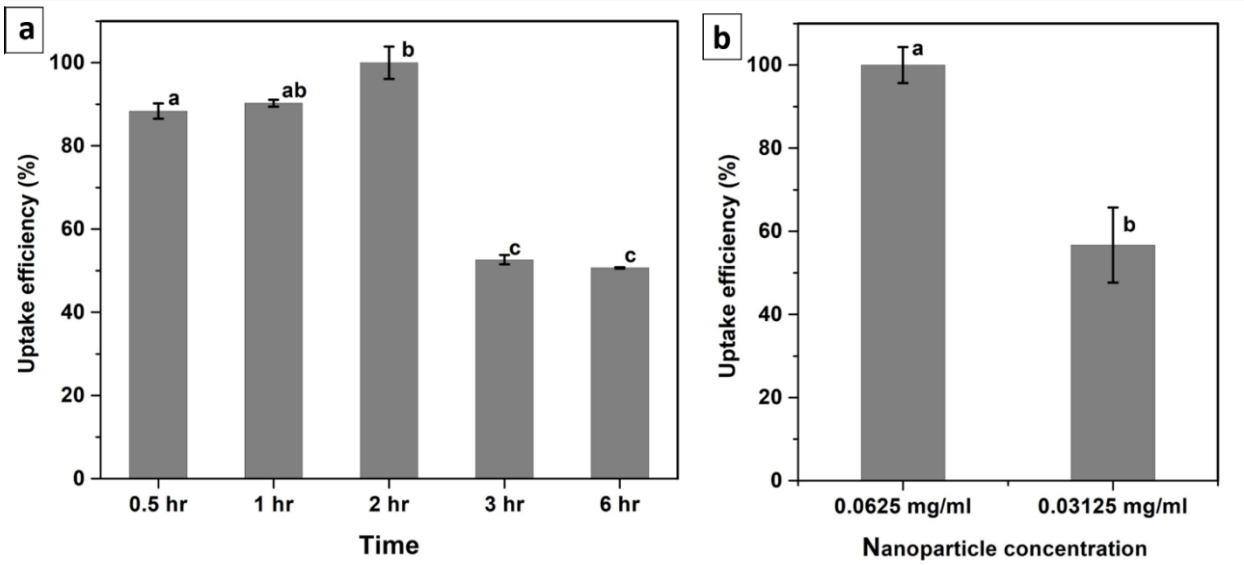

Figure 2.7 Flow cytometric quantitative analysis of cellular uptake of pea protein nanoemulsions by Caco-2 cells, showing a) the effect of time intervals (nanoparticle concentration at 62.5 µg/ml, 37 °C) b) the effect of nanoemulsions concentration at a time interval of 2 hours. Letter a,b,c denote significant difference ($p < 0.05$).

Figure 2.7b shows significant difference in the uptake efficiency of nile red based on different nanoemulsion concentrations, with a decrease in fluorescence at 0.03125 mg/ml to 57% of the fluorescence intensity at 0.0625 mg/ml. Similarly, other study showed increase in the uptake of

nanoparticles with the increase in nanoparticle concentration in the medium when incubated in cells. They found significant increase in nanoparticle uptake by Caco-2 cells from approximately 9% of nanoparticle uptake to 21 % at 100 μg/ml and 500 μg/ml, respectively (Win & Feng, 2005). Another study showed similar results for nanoparticles uptake, indicating nanoparticles uptake is concentration and time dependant (Davda & Labhasetwar, 2002). Similar to our study, Davda & Labhasetwar, 2002 observed that nanoparticle uptake process is saturable, showing decrease in uptake efficiency with higher incubation time and doses. This present study indicates that the nanoemulsions uptake capacities are time-dependant and concentration dependant, which is in agreement with the above results.

Based on the results from cytotoxicity test and concentration-dependent uptake study, 0.0625 mg/ml nanoemulsion concentration was considered for further cellular tests. The difference in cellular uptake between samples with varying properties was confirmed quantitatively by flow cytometry.

Two important factors were evaluated in the current uptake study- a) the impact of surface properties (protein based: P 230, lecithin based: L 200 or protein-lecithin: PL 150 based delivery system) and b) the nanoparticle size on uptake efficiencies of vitamin D encapsulated nanoemulsions. To understand the impact of different surface properties, lecithin-based (L 200) and pea protein- based (P 230) nanoemulsions were prepared with the same emulsifier (1% w/v) and oil concentration (0.5% w/v). Same processing conditions were applied to L 200, and P 230, with the aim to study only surface properties as an influencing parameter.

Phospholipids are often used for bioactive delivery studies, as it has shown to have high efficiency to deliver particles across the cell membrane. For instance, a study by Sessa, concluded that lecithin-based nanoemulsions showed improved transportation through the Caco-2 cell layer by

significantly enhancing their absorption into the cells, followed by improved entrapment in the microvilli due to the similarities in lecithin-based emulsion structure with the phospholipid bilayers of the cell membrane. This is mainly attributed to the fact that cell membrane consists of a phospholipid bilayer, and this may enhance the interaction between cell membrane and phospholipid particles, resulting in higher cellular uptake. A lot of attention is paid to the utilization of phospholipids in emulsion formulations (Lin, Lin, Chen, Yu, & Lee, 2009; Patel et al., 2006). In our study, we compare the uptake efficiency of pea protein based nanoemulsion delivery systems with the well-accepted phospholipid delivery systems. Interestingly, no significant difference existed between the two delivery systems as shown in figure 2.8a, both demonstrating uptake efficiency of more than 80%. This allows more use of protein in the formation of nano-delivery systems to improve bioavailability of bioactives.

Later, protein-lecithin nanoemulsion (PL 150) was prepared using the same processing conditions (0.5% w/v oil concentration, 1% pea-protein and 1% lecithin w/v concentration), in order to compare the synergistic effect of lecithin and protein on nanoemulsions uptake. It was interesting to notice that P 230 showed ~ 2 folds higher uptake efficiency ($p < 0.05$) into Caco-2 cells when compared with PL 150 (Figure 2.8b), which suggests that nanoemulsions stabilized by protein or lecithin alone are better at improving the cellular uptake, even though both samples exhibited similar sizes. Less cellular uptake efficiency of protein-lecithin nanoemulsions, in comparison to protein and lecithin alone, can be attributed to the hydrophobic interactions between protein and lecithin, which may have resulted in less hydrophobic regions exposed to interact with the phospholipid bilayer membrane. The interactions between protein and lecithin was confirmed by Sun et al., 2018, where they studied the effects of soy lecithin on surface hydrophobicity of whey protein using fluorescence spectra, and observed lower fluorescence intensity for whey protein-

soy lecithin combination than protein alone, and attributed the decreased surface hydrophobicity of protein to the hydrophobic interactions between protein and lecithin. The interactions between the two may have promoted aggregation of protein molecules and buried a few hydrophobic regions. Soybean lecithin has both lipophilic and hydrophilic properties, and its hydrophobic groups absorb to the protein surface through hydrophobic interactions (Sun et al., 2018). The interactions between lecithin and proteins may also result in reduced surface activity (displacement of either protein or lecithin from the interface) (Nieuwenhuyzen & Szuhaj, 2002). Cellular uptake efficiency of nanoemulsions was observed in the order of L 200 ≈ P 230 > PL 150. This study suggests that pea-protein nanoemulsions alone have several advantages, such as unique surface composition and properties, which may contribute to their high uptake efficiency.

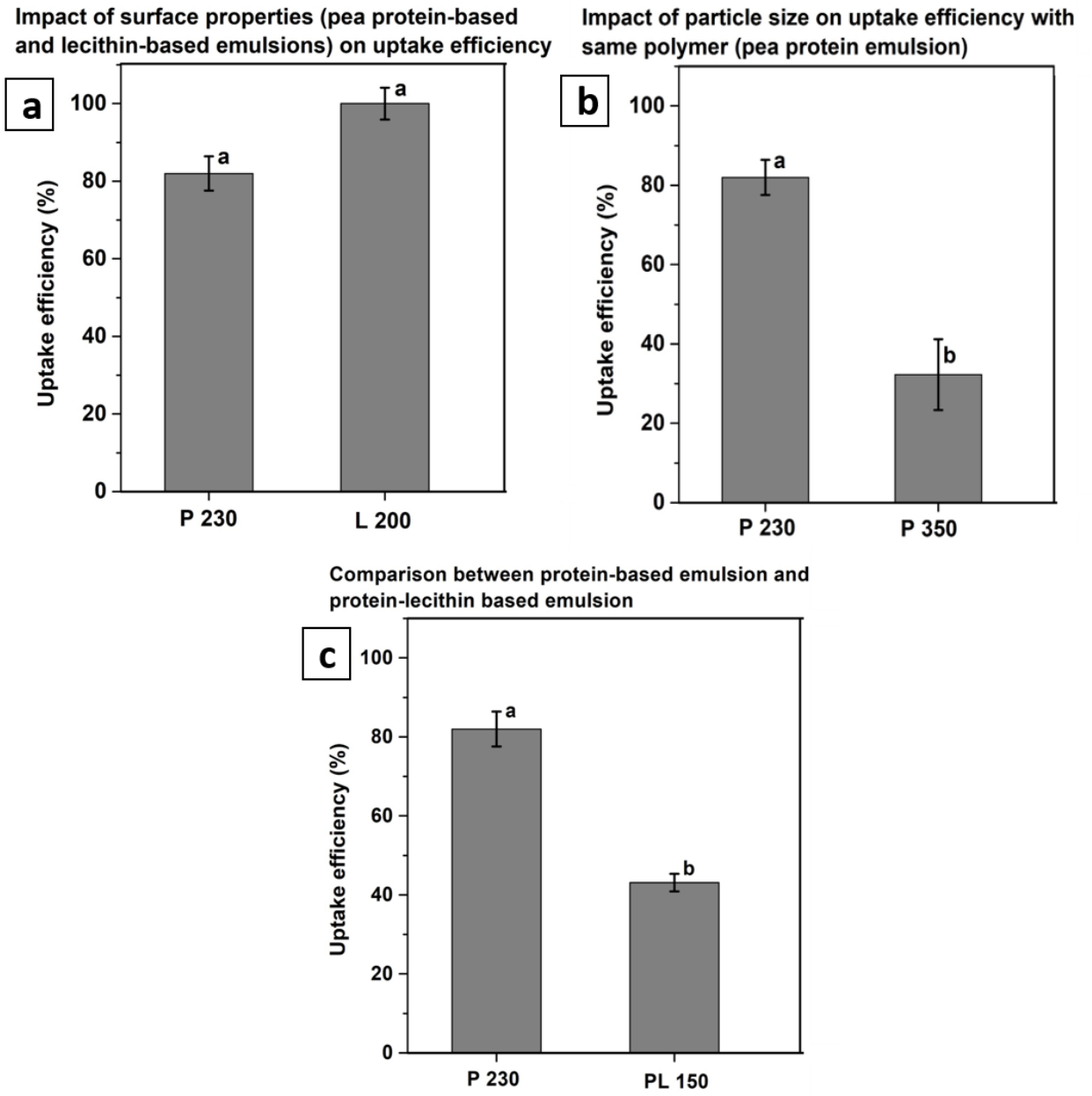

Figure 2.8 Flow cytometric quantitative analysis of cellular uptake of various nanoemulsions by Caco-2 cells, showing a) the impact of surface properties b) nanoemulsion particle size, and c) comparison of protein-alone and protein-lecithin nanoemulsion in terms of uptake intensity by Caco-2 cells (62.5 µg/ml, 37 °C, 2 hrs). Pea protein-234 nm (P 230), Pea protein-350 nm (P 350), Lecithin-202 nm (L 200) and Protein and Lecithin-153 nm (PL 150). Letter a,b denote significant difference ($p < 0.05$).

2.3.5.3 Influence of nanoemulsions size on cellular uptake

Previous studies have shown that nanoparticle size plays an important factor in determining nanoparticle behaviour (Alexis et al., 2008; C. He et al., 2010; Yang et al., 2014). As reported earlier, P 230 and P 350 exhibit particle sizes of 230 nm and 350 nm, respectively. The impact of nanoemulsions size on cellular uptake efficiency was evaluated as shown in figure 2.8a, it was observed that the uptake efficiency of pea-protein nanoemulsions was size-dependent as P 230 showed 2.5 folds higher uptake intensity ($p < 0.05$) as compared to P 350. This result was in agreement with the cellular uptake shown in figure 2.5. This indicates that the size of nanoemulsions is an important parameter to be taken into consideration, in order to achieve high emulsion uptake.

These results are in strong agreement with another study, which reported that soy protein nanoparticles of particle size 100 nm showed higher cellular uptake as compared to larger particles (180 nm), suggesting the role of reduced particle size in cellular uptake of nanoparticles (Zhang et al., 2015). Another interesting study compared the poly(lactic-co-glycolic acid) (PLGA) nanoparticle uptake based on varying particle sizes, where 100 nm particles showed significantly higher cellular uptake as compared to 50 nm, 500 nm and 1000 nm particles. Authors highlighted that 50 nm particles showed lowest uptake, suggesting that there must be a size limit beyond which reduction in particle size no longer influences the extent of uptake. They demonstrated that nanoparticles of approximately 100-200 nm size exhibit best properties for cellular uptake (Win & Feng, 2005). Another reason for low cellular uptake of extremely small nanoparticles (<50 nm) could be their inability to bind with receptors before being engulfed by the membrane (endocytosis) (Behzadi et al., 2017). These results are comparable with our study where nanoemulsions of size 230 nm demonstrated greater cellular uptake than 350 nm particles.

Nanoemulsions with larger particle size are known to require additional driving forces and energy for the cellular internalization process (C. He et al., 2010), and therefore, cellular uptake efficiency decreased significantly ($p < 0.05$) with the increase in particle size as observed for P 350 (Figure 2.8a). The uptake of nanoemulsions was observed in the order of L 200 ≈ P230 > PL 150 > P 350, and the result was in agreement with the cellular uptake efficiency demonstrated through confocal microscopic images as shown in figure 2.5. To conclude, cellular uptake of nanoemulsions was size, time and concentration dependent, which suggest these as important parameters to be taken into consideration to achieve high uptake.

2.3.6 Transport efficiency of vitamin D

It is suggested that the size of nanoparticles is one of the factors that determine the transport efficiency across the small intestinal barrier (C. He et al., 2010). Therefore, the impact of nanoemulsions size on vitamin D transport efficiency was studied. The transport efficiency of vitamin D without encapsulation i.e., free vitamin D suspension was 1.45± 0.42%, whereas, transport efficiency for small sized nanoemulsions (P 230) was 7.77± 0.716 %, which is ~ 5.3 times higher ($p < 0.01$) than free vitamin D (Figure 2.9). Interestingly, in comparison to free vitamin D, no significant difference in transport efficiency was observed when vitamin D was encapsulated in larger particle sized emulsion (P 350). This demonstrated that small sized nanoemulsions significantly improved transportation of vitamin D across Caco-2 cells and has the potential to improve vitamin D bioavailability by increasing its absorption in small intestinal cells. This could be attributed to the small size of nanoemulsions that offers large surface area to interact with the Caco-2 cell monolayers, resulting in enhanced transport efficiency (Rieux et al., 2005; Zhang et al., 2015).

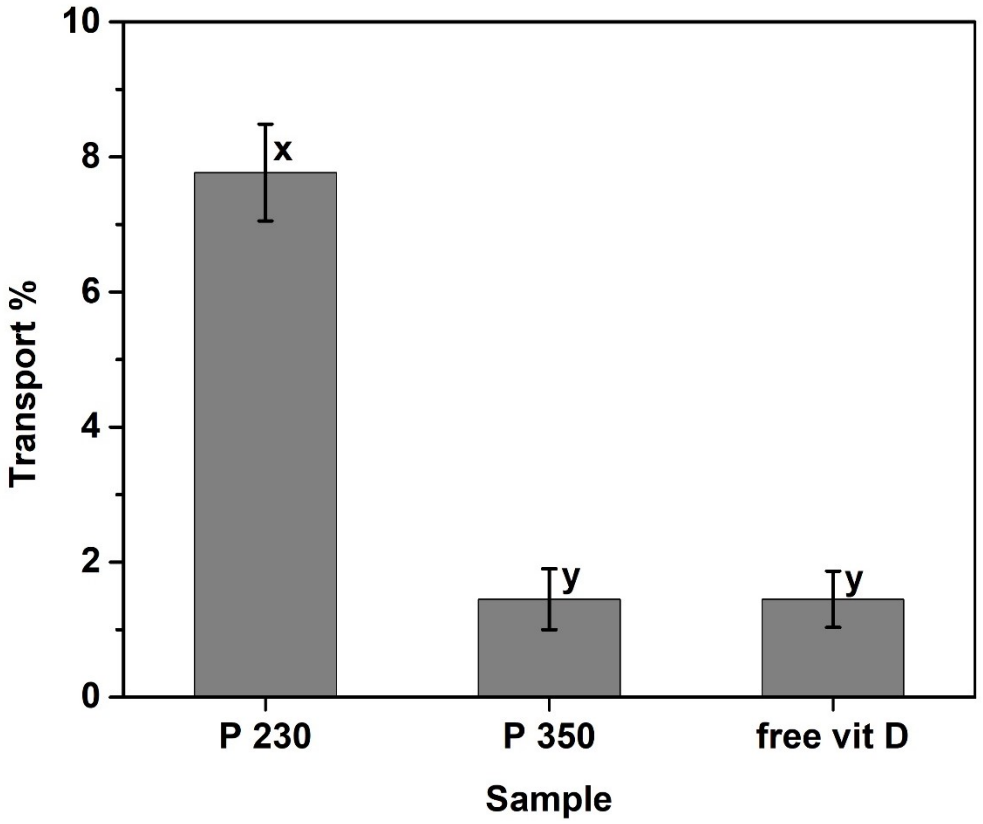

Figure 2.9 Transport efficiency of vitamin D across Caco-2 cell monolayers using small sized pea protein nanoemulsions (P 230), large sized pea protein emulsions (P 350), and free vitamin D suspension at initial apical concentration of 10 µg/ml. The percent transport of the vitamin D across the cell monolayers was calculated from the peak areas observed in the HPLC chromatograms of samples added to the apical chamber and collected from the basolateral chamber after the transport study. Data represented as mean ± SD and significant differences expressed as x,y at $p < 0.05$.

These results were found to be in agreement with previous findings, which demonstrated that small sized nanoparticles improve permeability of compounds across Caco-2 cells (C. He, Yin, Tang, & Yin, 2012; Zhang et al., 2015). This result indicates that the transport efficiency of vitamin D was significantly improved after being encapsulated in small size nanoemulsions.

Nano-sized emulsions are believed to have higher cellular and tissue uptake as compared to macroemulsions. It has been reported that the size of nanoparticles plays an important role in their interaction with the cells (Win & Feng, 2005). This has been demonstrated in our study where nanoemulsions of 230 nm size showed 2.5 times higher cellular uptake than emulsion of 350 nm size in Caco-2 cells. Many other studies have shown similar results of relatively higher intracellular uptake of nanoparticles than micro-ranged particles. For instance, a study by Desai et al., demonstrated differences in uptake of nano- and microparticles by Caco-2 cells and rat in situ intestinal loop model, where the uptake efficiency of nanoparticles was 2.5 fold higher in cellular line and over 15 folds higher in intestinal tissues than microparticles. In rat models, they also observed that nanoparticles were able to penetrate into submucosal layer, whereas, larger size particles accumulated on the epithelial lining (Desai et al., 1996, 1997). Another study demonstrated that smaller particles could cross the blood brain barrier, suggesting therapeutic treatment of complicated brain tumors or disorders using nanoparticles with encapsulated therapeutic drugs (Kroll et al., 1998). Thus, these studies indicated that nano-ranged particles/emulsion show greater uptake intensity into cells and tissues when compared to larger sized particles.

It is important to understand the role of nanoparticle blood residence time, in order to develop an efficient nano-drug delivery system. Over the past two decades, researchers are taking different approaches to improve nanoparticle residence time in the blood, reduce non-specific distribution, and even to target specific cells or tissues to treat specific diseases or conditions (Alexis et al., 2008). It was suggested that physicochemical properties of polymeric nanoparticles can effect its biodistribution and clearance from the blood. For instance, it has been found that smaller nanoparticles show longer blood residence time, twice as slower blood clearance as compared to

larger nanoparticles, and higher tissue permeability. This indicates the importance of particle size for achieving improved drug delivery (Alexis et al., 2008). In our study, it was also observed that serum/ culture medium dissolves emulsion well and does not affect the uptake of nanoemulsions, suggesting that these nanoemulsions can be administered into body circulation without affecting the normal blood circulation, as there will be no particle aggregation.

2.4 Conclusion

In general, it is a challenge to prepare small emulsion droplets using large molecular surfactants such as protein, however, in this study, nanoemulsions of 170 to 350 nm were prepared using high pressure homogenizer. Influences of different surfactants such as pea protein, soy lecithin and protein-lecithin combination on emulsion particle size and cell uptake and transport were studied using Caco-2 cells. Interestingly, no significant difference in the cellular uptake efficiency was observed for nanoemulsions stabilized by pea protein, compared to those stabilized by lecithin. Further, small sized nanoemulsions (233 nm) showed ~ 5.3 times greater transport efficiency of vitamin D across Caco-2 cells than free vitamin D suspension. Overall, this research demonstrated that nanoemulsions stabilized by pea protein have potential to improve vitamin D absorption. The knowledge generated from this research may guide industries to develop non-dairy food and beverage for vitamin D fortification.

Chapter 3- A low energy approach to develop nanoemulsion by combining pea protein and Tween 80 and its application for vitamin D delivery

3.1 Introduction

Nanoemulsions are colloidal dispersions of water and oil along with a surfactant that is required to reduce the interfacial tension between two phases (Javadzadeh & Azharshekoufeh Bahari, 2017). Emulsions with particle size below 500 nm are regarded as nanoemulsions (Capek, 2004; Chime et al., 2014; Sharma et al., 2013; Walia et al., 2017). Nanoemulsions have applications in various industries, including personal care, health care, food and pharmaceuticals (Maali & Mosavian, 2013). Encapsulation of bioactives in nanoemulsions improves their physical and oxidative stability, masks unpleasant odors, increases solubility and bioavailability of hydrophobic compounds and improves permeability through epidermal barriers (Donsì, 2018; Guttoff et al., 2015; McClements, 2011). They can also be used for the modification of product texture (McClements, 2017), and improvement of food shelf-life through encapsulation of natural antimicrobial agents (Donsì & Ferrari, 2016).

In general, two types of surfactants are used to prepare nanoemulsions in food applications including small molecular surfactants; subdivided into non-ionic, ionic or zwitter ionic surfactants, and phospholipids (e.g. soy lecithin), and large molecular surfactants such as proteins from animal and plant resources. Small molecular surfactants such as Tween 80 are more surface active and form smaller emulsion droplets as compared to large molecular surfactants (Qian & McClements, 2011; Teo et al., 2014). Food proteins are widely used emulsifiers due to their amphiphilic nature but are generally less effective to reduce surface tension because of their large size. However, protein-protein interactions at the interface form strong viscoelastic films that prevent emulsion coalescence and flocculation, and thus, offer higher stability (A. N. A. Aryee, Agyei, & Udenigwe,

2017; Sari et al., 2015). Once adsorbed, proteins partially unfold to expose their hydrophobic residues to the oil phase and hydrophilic residues to the aqueous phase, and furthermore, electrostatic and steric interactions function in stabilizing the emulsion system (A. N. A. Aryee et al., 2017). On the other hand, Tween 80 stabilizes emulsion droplets due to steric repulsion caused by large polyoxyethylene (hydrophilic) head groups of Tween molecules (Sari et al., 2015).

Nanoemulsions can be prepared using high energy methods such as high pressure homogenizer, ultrasonication and microfluidization, and low-energy methods such as spontaneous emulsification, phase inversion temperature and phase inversion composition (Kwasigroch et al., 2016; Lee & McClements, 2010; Sugumar, Ghosh, Mukherjee, & Chandrasekaran, 2015). High energy methods rely on mechanical devices that disrupts oil and aqueous phases into small emulsion droplets, whereas, low energy methods use the internal chemical energy of the system to arrange surfactants at oil-water interface by allowing diffusion of surfactant molecules within the emulsion system (Solans & Solé, 2012). The most widely used low-energy method is spontaneous emulsification, where a step-by-step addition of water into a solution of surfactant and oil at a constant temperature and stirring, results in spontaneous formation of nanoemulsion droplets. This is due to the movement of a water miscible component (water-soluble surfactant) from the organic phase into the aqueous phase (Chime et al., 2014; Karthik et al., 2017).

Till now, only small molecular surfactants such as Tweens and Spans have been used to prepare nanoemulsions using low-energy methods, such as spontaneous emulsification. Meanwhile, there is a growing interest in the use of natural emulsifiers such as proteins, however, food protein stabilized nanoemulsions by low-energy method have not been reported. This is due to the large molecular weight of protein emulsifiers that limits their movement to the interface and prevents formation of small emulsion droplets without significant energy. Thus, protein-based

nanoemulsions are usually prepared using high pressure homogenization, microfluidization and ultrasonication (Lee & McClements, 2010; Sari et al., 2015).

However, low energy approaches for nanoemulsion preparation have several advantages, including low preparation costs, ease of method, and sustainability (use of minimal energy) (Goindi, Kaur, Kaur, Kalra, & Chauhan, 2016). Since, high-energy input devices are not used, this method allows encapsulation of heat-sensitive bioactives without causing their degradation (Öztürk, 2017). The major drawback of low energy approach is the limited number of emulsifiers that can be used for nanoemulsions preparation (Komaiko & McClements, 2016).

Until the end of 1990s, many studies have used Sodium Dodecyl Sulfate (SDS) (anionic surfactant) and proteins (Guo, Zhao, Chen, & Teixeira, 1990; Turro, Lei, Ananthapadmanabhan, & Aronson, 1995) to study their interaction in the aqueous phase or at oil/water interface in emulsions. It has been proved that surfactants and proteins interact to form complexes, which are driven by electrostatic and/or hydrophobic interactions (Dimitrova & Leal-Calderon, 1999; Turro et al., 1995). Several structures of protein-surfactant (bovine serum albumin: SDS) complexes were proposed including, a) necklace and bead model, which consists of micelles like structures stabilized by protein molecules, b) rod-like model, with surfactant aggregates aligning as an axis corresponding to surfactant chain length with the protein wrapping around the aggregate and c) helical cylindrical model where protein chains wrap around the surfactant micelles (Turro et al., 1995). However, studies on small molecular surfactant-protein complexes to stabilize emulsions have been very limited thereafter as previous studies suggested that surfactants such as SDS caused protein denaturation due to the surfactant induced unfolding of proteins (Turro et al., 1995), and also resulted in competitive destabilization at the interface. It must be noted that these previous works mainly focused on ionic surfactants, including SDS. Since electrostatic interactions are the

main driving force for binding of ionic surfactants to the protein surface, it is not a surprise that neutral surfactants have negligible/partial effect on protein conformations (Dimitrova & Leal-Calderon, 1999; D. Otzen, 2011; D. E. Otzen, Sehgal, & Westh, 2009). Some recent studies have also prepared nanoemulsions using a combination of Tween 80 surfactants and proteins by high energy approaches (Sari et al., 2015), however, negligible or limited studies used a low energy approach to formulate nanoemulsions with such emulsifier combination.

Pea protein is gaining increased interest for food applications due to its high nutritional value, biocompatibility, good emulsifying properties, non-GEO and GRAS status. Unlike soy protein, pea protein is not regarded as major allergen, which makes it appealing to food industries. On the other hand, Tween 80 was the selected small molecular surfactant for this study because it is reported to form smallest emulsion droplets as compared to other non-ionic surfactants (Hasani, Pezeshki, & Hamishehkar, 2015). Moreover, Tween 80 is commonly used in food and drug formulations and is Generally Recognized As Safe (GRAS). Therefore, in this study, Tween 80, a non-ionic surfactant was used to form emulsion droplets, followed by the addition of pea protein to form complex nanoemulsions stabilized by a combination of small and large molecular surfactants using spontaneous emulsification as a low energy approach. The effects of environmental pH (pH 3,4,5,6 and 7) and the surfactant to oil ratio (SOR=1.0, 3.0, 9.0) on emulsion particle size and stability were investigated, with the aim to identify the optimal conditions for emulsion production. The efficiency of nanoemulsions to encapsulate vitamin D was determined using HPLC, and the morphology and structure of emulsions was studied using Transmission electron microscope (TEM) and Fourier-transform infrared spectroscopy (FTIR). Later, Caco-2 cells were used as an *in vitro* model to simulate the intestinal epithelium. The cellular uptake and transport efficiency of encapsulated vitamin D in nanoemulsion and free vitamin D suspension

were investigated using Caco-2 cells to understand the potential of the complex nanoemulsions as delivery systems to improve vitamin D absorption.

3.2 Materials and Methods

3.2.1 Materials

Pea protein concentrate was prepared according to our previous work (Jarpa-Parra et al., 2014) and the protein content was 97.34 % (w/w dry weight) as determined by combustion with a nitrogen analyzer (Leco corporation, St. Joseph, MI, USA), calibrated with analytical reagent grade Ethylenediaminetetraacetic acid (EDTA) using a conversion factor of 6.25. Canola oil was obtained from a local market. Tween 80, vitamin D3 (Cholecarciferol, >98% purity, MW 384.64 g/mol, analytical grade), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), deuterium oxide and nile red were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM), non-essential amino acids (NEAA), HEPES solution, Antibiotic-Antimycotic (Anti-Anti), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS) and trypsin-EDTA were purchased from GIBCO (Burlington, ON, Canada). Human colorectal adenocarcinoma cell line Caco-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All chemicals used were of reagent grade unless otherwise stated.

3.2.2 Nanoemulsion preparation

Initially, an organic phase was prepared by dissolving vitamin D (1 mg/ml) in canola oil, followed by the addition of Tween 80. The organic phase was slowly titrated into the aqueous phase (distilled water) under constant stirring (800 rpm) by a magnetic bar to form fine emulsion droplets. Varying surfactant-to-oil ratio (SOR=1.0, 3.0, 9.0) were tested to determine the most optimal emulsifying conditions. On the other side, pea protein (3% w/v) was dissolved in water by stirring

with a magnetic bar and then its pH was adjusted to 3 and allowed to stir for an hour. This was followed by the addition of pea protein into the pre-formed emulsions prepared by Tween 80 at the ratio of 1:1 (v/v) under constant mixing, which resulted in the formation of Tween 80-pea protein complex nanoemulsion. Upon investigating the effect of different surfactant to oil ratios, SOR=1 with 3% w/v Tween 80 was observed to be the optimal concentration for emulsion production. Unless otherwise stated, emulsion preparation conditions were as follows: Tween 80 (3% w/v), canola oil (3% w/v), water (94% v/v), pea protein (3% w/v), and magnetic stirring speed of 800 rpm.

3.2.3 Nanoemulsion characterization

The mean particle size and zeta-potential of the nano-emulsions were measured by dynamic light scattering using a Zetasizer Nano S (model ZEN 1600, Malvern Instruments Ltd, UK) at room temperature. To avoid multiple scattering, nanoemulsion suspensions were diluted to appropriate concentrations using double distilled water before analysis. The refractive index of the particle and dispersion medium was set at 1.52 and 1.33, respectively (Q. Ye et al., 2016). All measurements were made three times and results were described as mean ± SD.

3.2.4 Morphology study

The morphology of the nanoemulsions was observed using a transmission electron microscope (TEM, Morgagni 268, Philips-FEI, Hillsboro, USA) at 80Kv as per the previous studies (W. He et al., 2011; Yang et al., 2014). Briefly, one drop of the sample was added to TEM grids coated with carbon films. Then, a drop of sodium phosphotungstate (2% w/v) was placed over the sample droplet as a negative stain for several minutes at room temperature, and dried in air before analysis.

3.2.5 Fourier-transform infrared (FTIR) spectroscopy

Protein conformational changes of pea protein and Tween 80-pea protein complex were characterized using FTIR. The FTIR spectra were recorded at room temperature (22 °C) using a Magna 560 Nicolet spectrometer (Madison, WI, USA). The spectrometer was continuously supplied with nitrogen. Pea protein solution was prepared by dissolving pea protein (3 g/100 ml) in D_2O solution and 0.5 M NaOD or DCl was used to adjust pD. To ensure a complete H/D exchange, samples were prepared 2 days before analysis and kept at 4 °C in a sealed nitrogen environment until used for analysis. Tween 80-pea protein complex nanoemulsion was prepared by the addition of pea protein solution into pre-formed Tween 80 emulsion (prepared in D_2O), which was used to study protein conformation in the emulsion. Samples were placed between two CaF_2 windows separated by a 23 mm polyethylene terephthalate film spacer. Subtraction and Fourier self-deconvolutions were performed using Ominic software to study the amide I region (1700-1600 cm^{-1}) of the protein. Band narrowing was achieved with a full width at half maximum of 24 cm^{-1} and a resolution enhancement factor of 2.5 cm^{-1}.

3.2.6 Encapsulation of vitamin D

For estimation of the encapsulation efficiency, vitamin D was extracted from the nanoemulsions by the method adapted from Xue et al., (2008) with slight modifications. Briefly, 10 ml of freshly prepared nanoemulsion were added to the flask and shaken vigorously. Then, 65 ml of methanol were added to the flask to extract vitamin D. The mixture was placed in ultrasonic bath at 40-50° C for 30 min, and then cooled down to room temperature and centrifuged at 2800 g-force for 10 min. The supernatant was filtered using a 220 nm filter and analyzed using a high performance liquid chromatography (HPLC) for quantification of the total vitamin D. For determination of the free vitamin D in the nanoemulsion suspension, 3 ml of nanoemulsion were mixed with 7 ml of

hexane, vortexed well for 1-2 min and centrifuged at 700 rpm for 5 min, in order to obtain a distinct layer of hexane. The process of vitamin D extraction using hexane was repeated twice. The hexane layers from these three steps were pooled together and free vitamin D was analyzed. The vitamin D quantification was conducted on a HPLC system (1200 series, Agilent Techonologies, Inc., Santa Clara, CA) equipped with an Extend C18 column. The chromatographic conditions were as followed: mobile phase- methanol:water (98:2% v/v), flow rate of 1.5 ml/min, column oven temperature of 35° C, injection volume of 10 µl and detection was carried out at 265 nm with a UV-vis detector. Quantification was achieved using comparison between observed peak area and calibration curve. Chromatographic conditions were the same for standard solutions as well.

The encapsulation efficiency was calculated by using the following equation:

EE (%) = [(total vitamin D - free vitamin D) / total vitamin D)] × 100

3.2.7 Caco-2 cell culture model

Caco-2 cells is a human epithelial colon adenocarcinoma cell line that is a commonly used model to investigate uptake efficiencies and drug deliveries across the small intestinal epithelium (Zhang et al., 2015). The human adenocarcinoma cell line Caco-2 was obtained from ATCC and passages between 20-40 were used for cell culture studies. Cells were cultured in T-75 flasks at 37 °C in a humidified incubator 5%CO2/ 95% air in DMEM with sodium pyruvate (high glucose) supplemented with FBS (20% v/v), NEAA (1% v/v), Anti-Anti (1% v/v) and 25 Mm HEPES (Roger et al., 2009; Zhang et al., 2015). Cell morphology was monitored regularly and the culture medium was changed every alternate day. Cells were harvested at 80% confluency using 0.25% trypsin in 1 mM EDTA solution and seeded onto new culture flasks.

3.2.7.1 Cytotoxicity assay

The potential cytotoxicity of prepared nanoemulsions was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide viability assay (MTT assay) as per the previously published method (Yang et al., 2014). In brief, Caco-2 cells were seeded onto 96 well plates at a density of 8×10^3 cells per well in 100 µl culture medium. After allowing the cells to grow for 24 hrs, nanoemulsions were added to each well at final concentrations of 0.5, 0.125, and 0.03125 mg/ml, respectively with incubation period of 6 hrs at 37° C. After removing nanoemulsions, 10 µl of MTT solvent (5 mg/ml in PBS) were added into each well and incubated for 4 hours at 37 °C. Upon removing the solvent, 100 µl of DMSO were added to each well, followed by examining the plate at 570 nm using a microplate reader (SpectraMax, Molecular Devices, USA) (Yang et al., 2014). Cell viability was presented as percentage of living cells in the test wells with respect to control wells. (W. He et al., 2011). Different concentrations of samples were evaluated to determine the safe concentration for further analysis.

3.2.7.2 Cellular uptake of nanoemulsions

Cellular uptake of nanoemulsions by Caco-2 cells was quantified by flow cytometry, which estimates the amount of fluorescence labelled nanoemulsions in the cells by measuring the fluorescence intensity associated with the cells. Flow cytometric analysis was performed using the protocol from a previous study with modifications (Zhang et al., 2015). Briefly, cells were seeded onto 6-well plates at the density of $1x10^5$ cells per well and incubated for 2-5 days to form a confluent layer. Upon removal of media, nanoemulsion suspensions in HBSS at a concentration of 0.5 mg/ml were added to wells, and incubated for 2 hours at 37 °C. After the incubation period, cells were rinsed with PBS to remove any particles of nile red binding to cell surface, and were further trypsinized for 3 min. Trypsinization was stopped by the addition of cell culture media.

Cell were removed from the wells by pipetting. After centrifugation, 4% paraformaldehyde (w/v in PBS pH 7.2) was added to cells and incubated for 15 min, followed by resuspension of cells in PBS in FACS tube (VWR). The fluorescence intensity associated with cells was measured using a flow cytometer (B.D. Biosciences FACSort, CA). A 561 nm laser was used to excite nile red and emitted fluorescence was detected at 610 nm. 10000 cells were analyzed for each sample and cellular debris was excluded from the analysis to establish a collection gate using side-scatter versus forward-scatter histogram (Snipstad et al., 2014).

3.2.8 Caco-2 cell monolayers

Caco-2 cells were cultured in 75 cm^2 flasks and trypsinized at 90 % confluency. Cells at passages 20-30 were seeded onto polycarbonate membrane filters (0.4 m pore size, 1.12 cm^2 grown surface) inside Transwell 12- well plates (Costar, Corning, NY, USA) at a density of $3x10^5$ cells per insert. To apical chambers, 0.5 ml of suspended cell solution were added and basolateral chambers were filled with 1.5 ml DMEM. Cell plates were incubated overnight at 37° C and 5% CO_2 before replacing the media in apical and basolateral chambers. To ensure cell monolayer formation, media was replaced every second day for 21-28 days. To monitor the integrity of monolayers, transepithelial electrical resistance (TEER) values were measured using the epithelial voltammeter ($EVOM^2$, World Precision Instruments, Saratosa, FL). Monolayers with TEER values higher than 300 Ωcm^2 were used for transport study.

3.2.8.1 Transport efficiency

The transport of vitamin D across Caco-2 cell monolayers in transwells was investigated following a protocol reported by Hubatsch et al., (2007) with slight modifications. In brief, Caco-2 cells were seeded on permeable inserts at a density of 3×10^5 cells/ insert and were allowed to differentiate and polarize to form a continuous monolayer. Caco-2 cells grown in DMEM for 25

days were used to study the transport efficiency of vitamin D. Culture medium in the transport plates was changed 12-24 hrs before the experiment. Filter supports with cell monolayers were gently washed with HBSS and incubated with fresh HBSS for 15-20 min at 37 °C. Meanwhile, donor solutions were prepared with a final vitamin D concentration of 10 µg/ml in the form of Tween 80-pea protein naoemulsion complex, emulsions stabilized by Tween alone and free vitamin D suspension in ethanol. Then, HBSS buffer was removed and filter supports were transferred to a new plate containing HBSS in the basolateral chamber and 0.5 ml of donor solutions were added to the apical chambers either in the form of emulsion samples or free vitamin D suspension. After incubating the plates for 2 hrs at 37 °C under an atmosphere containing 5% CO_2, samples from each basolateral chamber were collected into a centrifuge tube. To confirm efficient uptake of nanoemulsions, transport efficiency of vitamin D from the apical to the basolateral side of the Caco-2 cell monolayer was calculated. After 2-hour transport study, vitamin D quantification was performed using HPLC.

Vitamin D from cell samples was extracted using methanol in the ratio of 1: 6 (vitamin D containing sample: methanol) and the mixture was placed in an ultrasonic bath for 30 min, and later, centrifuged to obtain the supernatant, which was filtered and used for vitamin D quantification by HPLC. Vitamin D standards were prepared ranging from 10 µg/ml – 500 µg/ml in methanol. Samples were analyzed using an Agilent 1200 HPLC system (Agilent Techonologies, Inc., Santa Clara, CA) with an Extend C18 column and the samples were eluted using methanol: water (98: 2% v/v) at 1.5 ml/min flow rate, and 50 µl injection volume. The absorbance was monitored at 265 nm with a UV- Vis detector. Peak areas were determined to estimate the percent transport of vitamin D, as defined by the ratio of basolateral peak area and initial apical peak area.

3.2.9 Statistical analysis

All the experiments were performed in triplicates and the data were expressed as mean values ± standard deviation. Statistical comparisons were conducted using one-way ANOVA, two-way ANOVA and Tukey's test. Differences with p-value below 0.05 were considered statistically significant. Origin 9 software was used for graphing and analysis of numerical data.

3.3 Results and discussion

3.3.1 Nanoemulsion formation

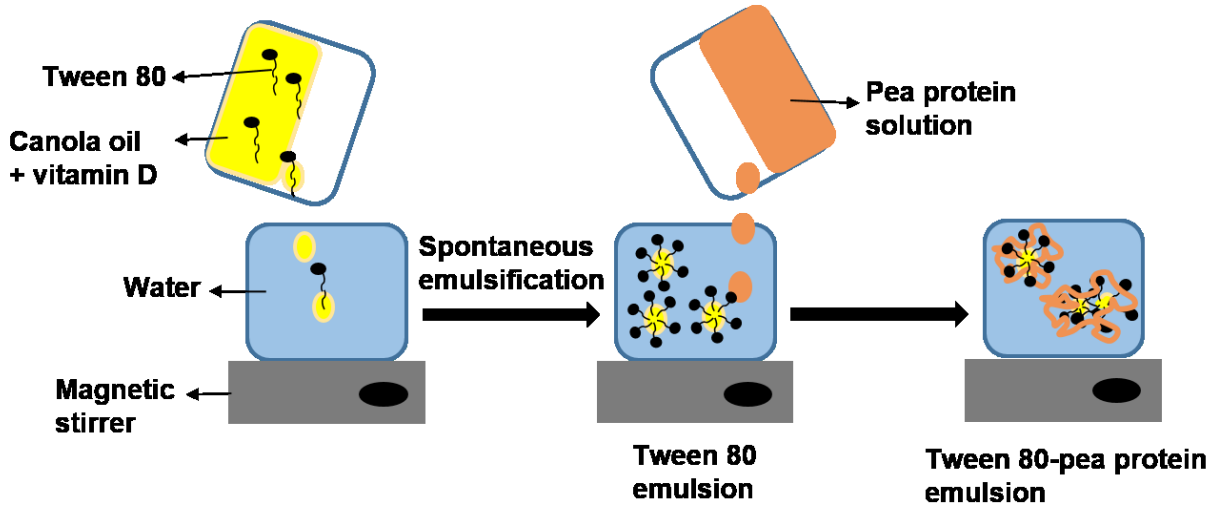

Figure 3.1 Formation of Tween 80-pea protein complex emulsion.

As observed from the TEM images (Figure 3.2) and light scattering measurements (Figure 3.3), emulsions were formed spontaneously when Tween 80 surfactant-oil mixture and water were mixed together, which showed spherical shape with an average particle size of 134.8 nm and a homogenous size distribution as reflected by PDI value of 0.31. The polydispersity index value is the measurement of particle size distribution and a small PdI value indicates a narrow size distribution (Yuan, Gao, Zhao, & Mao, 2008). PDI values ≤ 0.3 is an acceptable homogeneity level (Dragicevic-Curic, Gräfe, Gitter, Winter, & Fahr, 2010; He et al., 2011). Pea protein solution showed spherical shaped particles of 143.8 nm (PDI value: 0.17). Interestingly, after addition of

pea protein to the oil droplets formed by Tween 80, the complex nanoemulsion showed irregular shaped particles with particle size of 207.7 nm (PDI value: 0.31), featuring Tween 80 stabilized micelles clustered together by pea protein polymeric chains (Figure 3.2). Such "clusters on string" structure has some similarity to the necklace and bead model proposed for SDS-protein complex where micelles-like structures are stabilized by proteins (Turro et al., 1995).

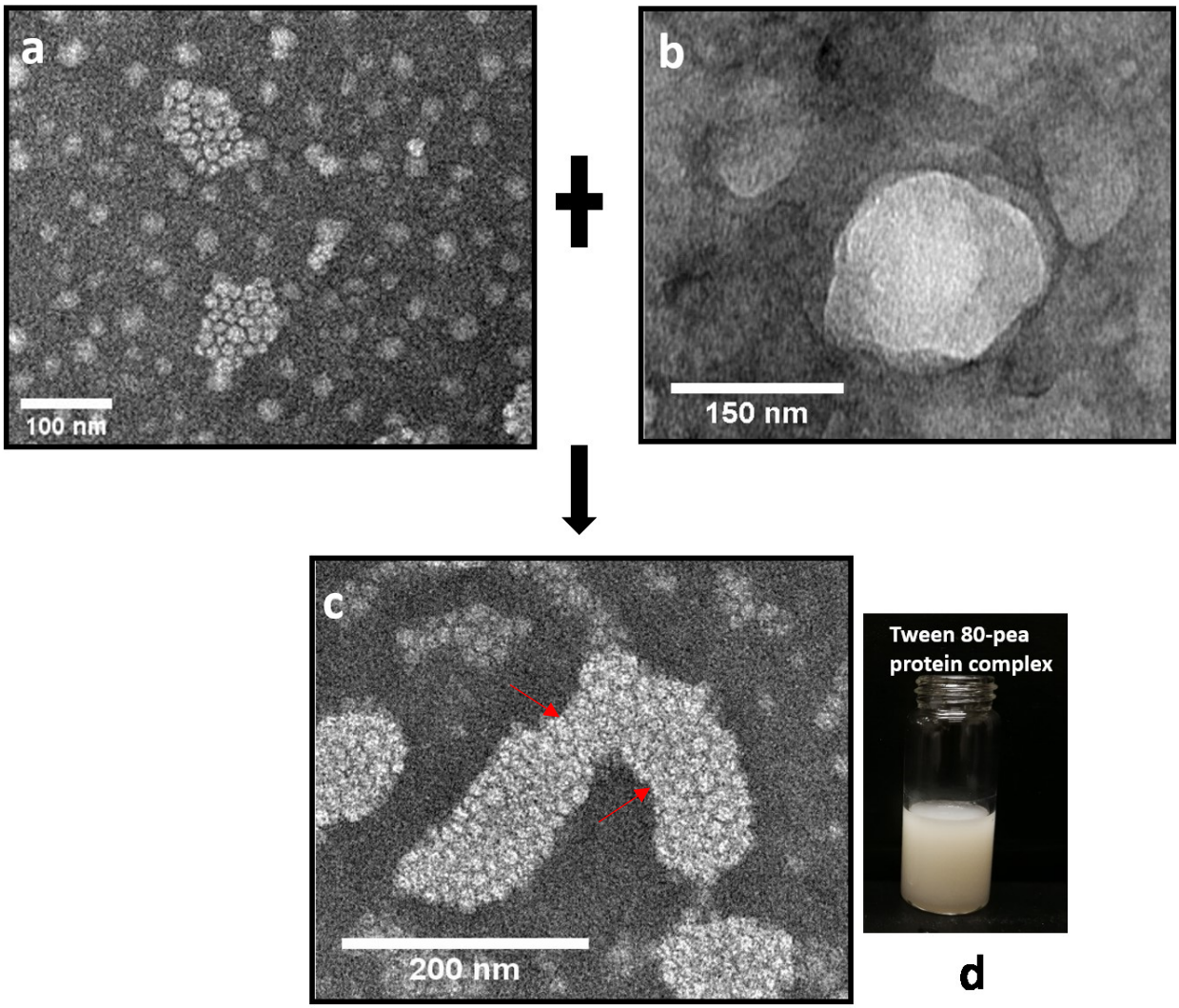

Figure 3.2 Transmission electron microscopic images for a) Tween 80 emulsion, b) Pea protein solution, c) Tween 80-pea protein stabilized emulsion, and d) the formation of stable Tween-protein complex with milky appearance. Arrows show clusters of micelles.

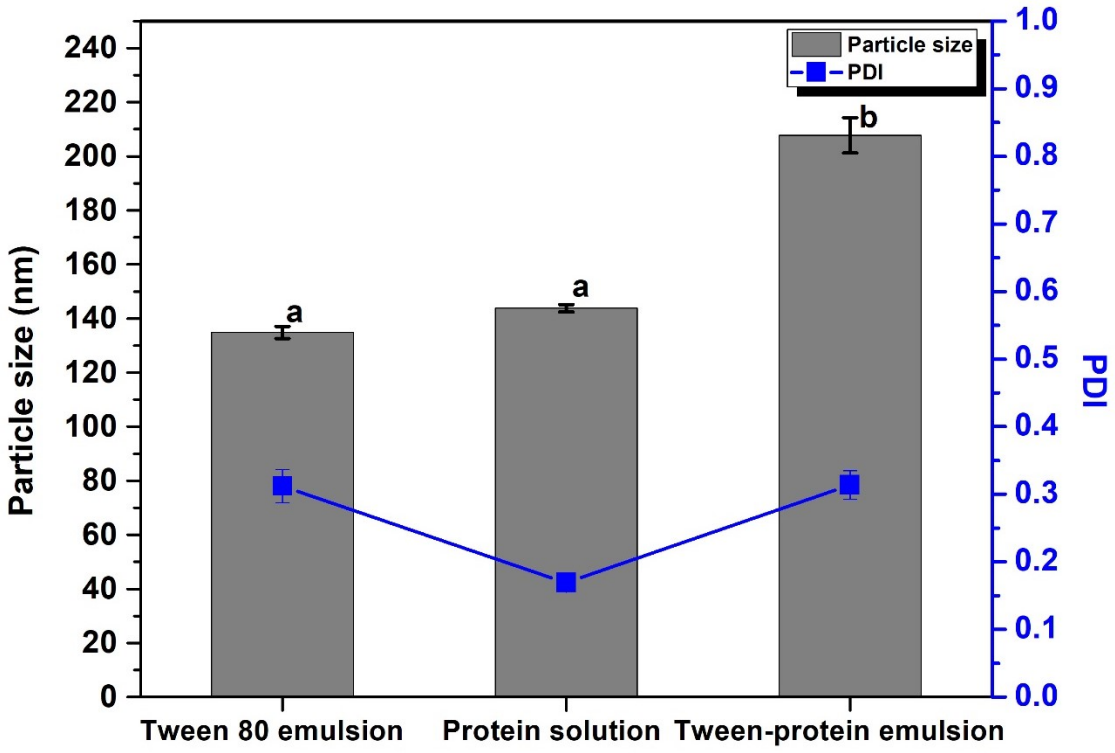

Figure 3.3 Particle sizes and poly dispersity index (PDI) for Tween 80 emulsion, pea protein solution and Tween 80-pea protein stabilized emulsion at Tween 80 (3% w/v), canola oil (3% w/v), and pea protein (3% w/v). Letters a, b denote significant differences between samples.

3.3.1.1 Influence of the surfactant to oil ratio on particle size and stability

The type and concentration of surfactant present at the interface influence the physicochemical properties of emulsion. Surfactant to oil ratio (SOR) is believed to have an impact on the droplet size, size distribution and the stability of nanoemulsions (Zeng, Xin, & Zhang, 2017). Prior to adding protein layer onto Tween emulsion, different surfactant (Tween 80) to oil ratios (SOR= 1, 3, and 9) were tested in order to achieve the most suitable surfactant concentrations. At SOR 9, a large number of surfactant molecules were available to cover oil droplets, which resulted in small nanoemulsion particle size (18.1 ± 0.7 nm) (Figure 3.4). However, to avoid regulatory and safety

concerns related to high surfactant concentration and for economical reasons, SOR=1 was selected for emulsion preparation as it also resulted in relatively stable samples with particle size of 134.8 ± 2.2 nm (Figure 3.4).

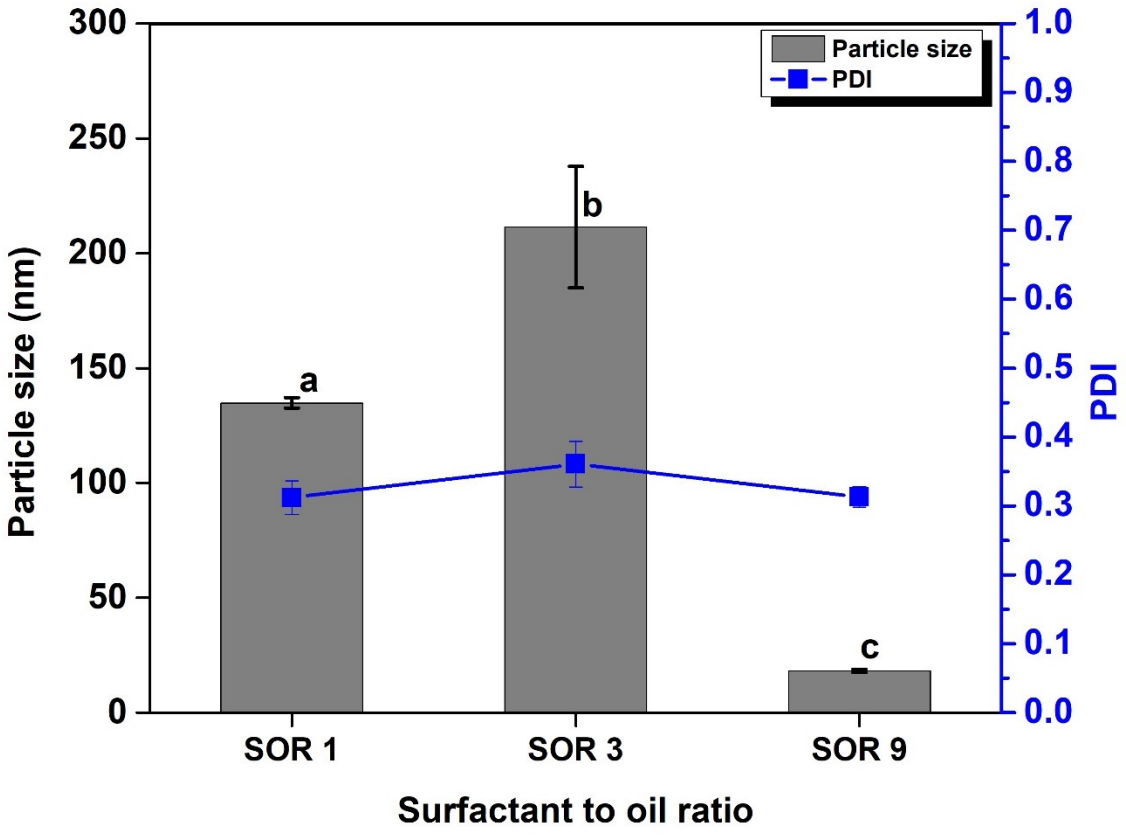

Figure 3.4 Influence of surfactant to oil ratio (SOR) in emulsion system on the particle size of Tween 80 emulsion. Letters a, b, c denote significant differences between samples.

A study by Saberi et al., (2013a) prepared nanoemulsions (55nm) to encapsulate vitamin E, a fat soluble vitamin, by spontaneous emulsification using Tween 80 as a surfactant at SOR=1 and surfactant content of 10 % (Saberi, Fang, & McClements, 2013a). Another work by Hasani, Pezeshki, & Hamishehkar, (2015), studied the influence of surfactant (Tween 80) concentration on the droplet size of emulsion prepared by spontaneous emulsification. The oil content was fixed

at 10% (w/w) with varying surfactant-to-emulsion percentage of 7.5, 10, 15, 17.5, 20 %. They reported that 17.5% w/v surfactant or in other words SOR=1.75 was the most optimal condition that resulted in nanoemulsion particle size below 100 nm and monomodal size distribution. They stated that at lower surfactant concentration (7.5% w/v) or SOR=0.75, surfactant molecules were insufficient to completely cover the surface of droplets, hence, flocculation and coalescence of droplets were observed which resulted in increased particle size. Therefore, increasing the surfactant concentration would allow a greater number of surfactant molecules to emigrate from the oil phase to the aqueous phase of emulsions and hence, to form fine nanoemulsion droplets.

In general, high concentration of surfactants (above 10% wt) are required for the preparation of nanoemulsions using low-energy approach (Chang & McClements, 2014; Maali & Mosavian, 2013; Saberi, Fang, & McClements, 2013b). This is concerning for its application in food industry due to cost, sensory and regulatory reasons (McClements, 2011). Therefore, in the current study, nanoemulsions were produced with relatively low surfactant concentration (Tween 80) of 3% w/v and SOR= 1. Further emulsion stability was enhanced with the addition of pea protein layer onto Tween stabilized emulsion droplets that forms a viscoelastic layer around emulsion droplets, resulting in a Tween-protein complex. The selected Tween-protein emulsion composition for further studies was as followed: 3% w/v Tween 80, 1 mg/ml vitamin D, 3% w/v canola oil, and 3% w/v pea protein. Although the amount of surfactant required to prepare nanoemulsions by low-energy approach is slightly higher than the amount generally used in high-energy approaches, however, it must be noted that low-energy methods such as spontaneous emulsification does not require expensive homogenization equipment, and this allows low cost of emulsion production in a far more sustainable way.

3.3.1.2 Effect of emulsion pH on particle size and stability of Tween80-protein complex emulsion

In this study, the effect of pH on the properties of emulsions stabilized by a combination of Tween 80 and pea protein was investigated. Proteins are known to stabilize emulsion droplets by steric and electrostatic repulsive forces, and the latter makes them more sensitive to pH and ionic strength, whereas, small molecular surfactants such as Tween 80 stabilize emulsions through steric repulsion (Demetriades & McClements, 1998), thus the formed emulsions are less sensitive to changes in pH and ionic strength (Basalious, Shawky, & Badr-Eldin, 2010).

At pH values away from the isoelectric point (pI- 4.5) of pea protein (Barać, Pešić, Stanojević, Kostić, & Čabrilo, 2015), protein droplets exhibited either positive (below pI) or negative charge (above pI), and the strong electrostatic repulsive forces between charged molecules kept the emulsion stable against flocculation. However, at pH values near the pI, the electrostatic repulsive forces were reduced due to neutral charge on droplet surfaces. This explained the increased particle size at pH around 4-6 due to flocculation as shown in figure 3.5. Accordingly, at pH from 4 to 6, polydispersity index was quite high (above 0.6) and creaming in the emulsion appeared. Although small particle size (243 nm) of nanoemulsion was observed at pH 7, it showed physical instability in the form of creaming that was also evident by the increased PDI value to 0.4. This can be attributed to the repulsive forces between negatively charged protein and Tween emulsion when both Tween 80 emulsion (-21.6 ± 0.8 mV) and protein (-24.1 ± 1.2 mV) were negatively charged at pH 7. Nanoemulsions at pH 3 were found to be stable with the particle size of 207.7 nm and PDI value of 0.31 (Figure 3.3 & 3.5), which suggest that the nanoemulsions have relatively homogenous size distribution. The stability of emulsion was indicated by a milky appearance (Figure 3.2d) and the absence of creaming or sedimentation and stable size during storage in refrigerator (4 °C) for 2 weeks. As compared to the particle size measured using Zetasizer (Figure

3.3), smaller size observed in TEM images (Figure 3.2) could be due to the shrinking of emulsion particles when air dried prior to TEM study (Zhang et al., 2015).

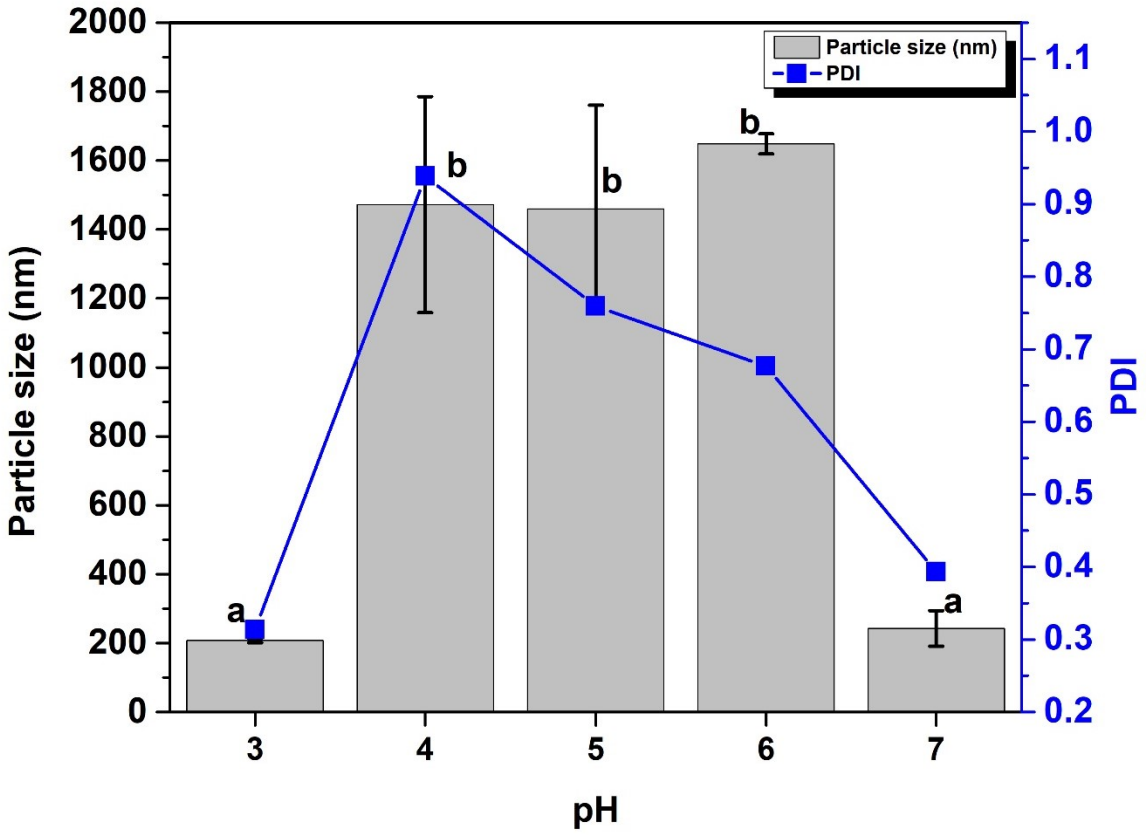

Figure 3.5 Effect of pH on the particle size and poly dispersity index (PDI) of Tween 80-pea protein stabilized emulsion at Tween 80 (3% w/v), canola oil (3% w/v), and pea protein (3% w/v). Letters a, b denote significant differences between samples.

3.3.2 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of protein solution and Tween 80-pea protein nanoemulsions complex were compared to study the effect of Tween 80 on protein conformations. Figure 3.6 demonstrates the FTIR spectra in the amide I region (1600-1700 cm^{-1}). The spectra showed relatively intense peaks at 1631, 1678 and 1691 cm^{-1} in both spectra, which suggests that beta-sheets dominate the protein

secondary structures in pea protein (Figure 3.6). These results are in agreement with previous studies that suggest legume proteins to mainly constitute of beta-sheet structures (Hwang & Damodaran, 1996; Jarpa, 2015; Zhang, 2014a). The peaks observed at 1641 - 1643 cm^{-1} correspond to random coils in both spectrum (Byler & Susi, 1986; Kong & Yu, 2007; Jarpa, 2015; Zhang, 2014a). The peak at 1608 cm^{-1} are known to associate with the beta-structured aggregates (Barth, 2007; Krüger et al., 2019), and the bands at 1678 cm^{-1} represents peptide segments in the turns (Hugonin, Barth, Gräslund, & Perálvarez-Marín, 2008; Zhang, 2014b).

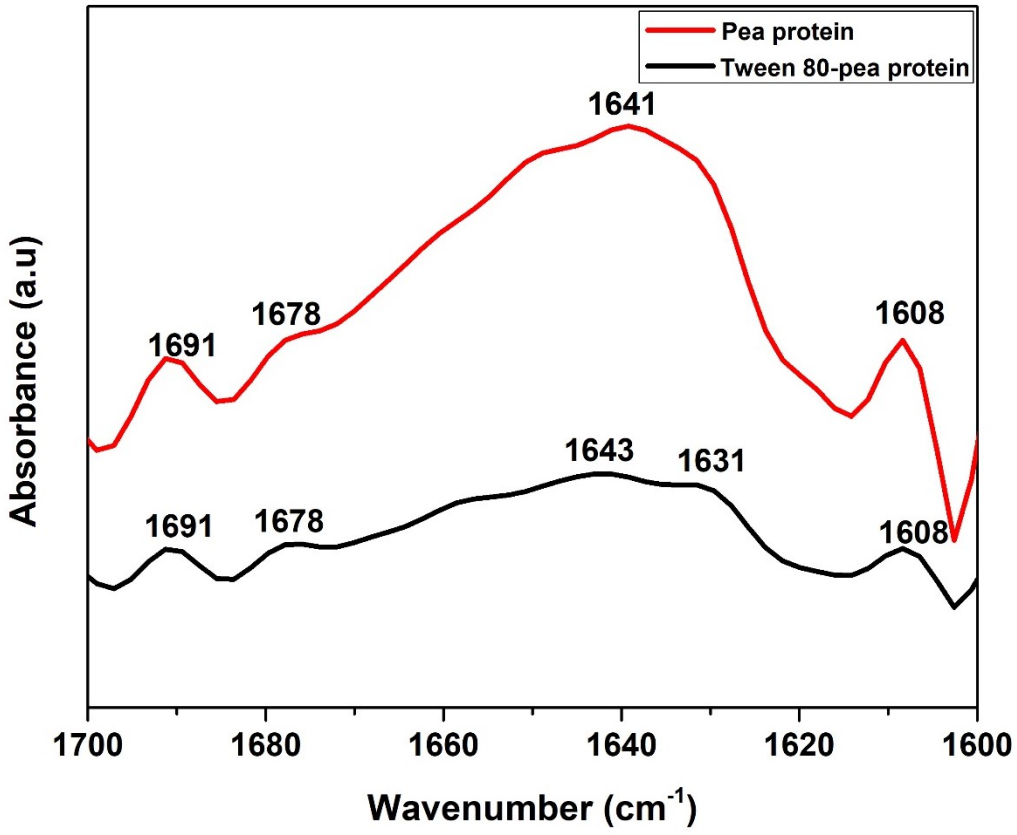

Figure 3.6 Fourier-deconvoluted FTIR spectra of pea protein (3% w/v) solution and Tween 80-pea protein complex (3% w/v each). The samples were prepared with 30 mg/ml protein. (a.u-arbitrary units)

The spectra showed that pea protein maintained beta-sheets upon adsorption at the complex emulsion surface, which is likely to form a viscoelastic film at the emulsion surface. Broaden peaks observed between 1631-1643 cm^{-1} , 1608 cm^{-1} and 1691 cm^{-1} suggest that there was partial unfolding of the protein structure upon pea protein adsorption at the complex emulsion surface, which would have exposed hydrophobic regions of protein, leading to intermolecular hydrophobic interactions between protein molecules and resulting in the formation of film like structure at the complex interface. Partial unfolding of pea protein molecules by Tween 80 to a certain extent resulted in partial denaturation of protein molecules. This may have also resulted in hydrophobic interactions between exposed hydrophobic regions of pea protein and hydrophobic tails of Tween 80, resulting in the formation of a Tween 80-pea protein complex. It was assumed that beta-sheet structures facilitated formation of viscoelastic films, which could be attributed to the beta-sheet aggregates observed at 1608 cm^{-1} that may have led to crosslinking of protein molecules (protein-protein interactions) at the complex emulsion interface, and this film structure is likely to be related to good viscoelastic properties of emulsions stabilized by globular proteins (Lefèvre & Subirade, 2003).

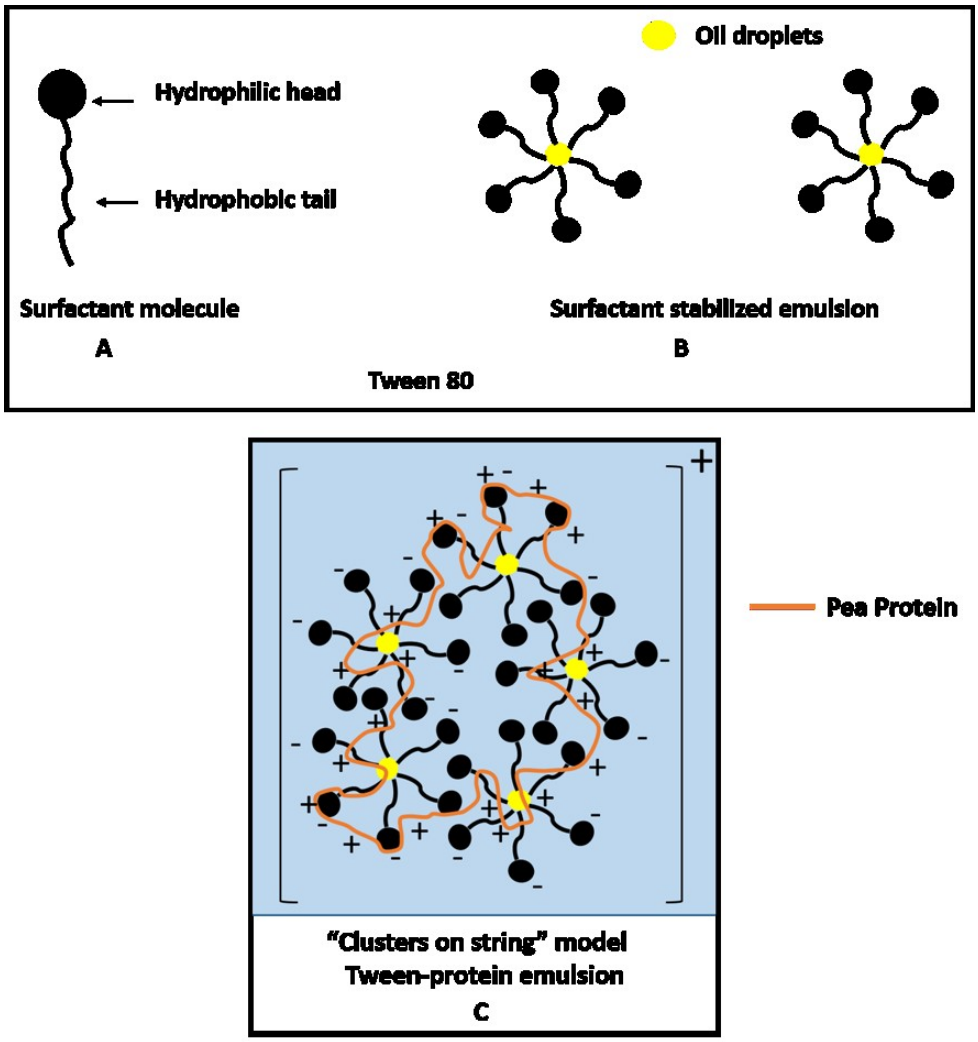

Figure 3.7 This study proposes a "Clusters on string" model for Tween-protein complex nanoemulsion formed due to electrostatic and hydrophobic interactions.

In this study, the concept of necklace model was adapted to explain the complex emulsion formation mechanism. Upon mixing Tween 80 stabilized emulsion and pea protein solution, a complex nanoemulsion was formed due to the electrostatic attractive interactions between negatively charged Tween 80 stabilized emulsion (-21.6 ± 0.8 mV) and positively charged protein molecules at a mild acidic pH of 3 (24.5 ± 0.5 mV). The complex emulsions showed a zeta-

potential of 3.7 ± 0.6 mV due to the residual charge from protein molecules at complex emulsion surface. Therefore, based on the information from FTIR, TEM and emulsion surface charge, it was suggested that both Tween 80 and pea protein surfactants were present at the surface of complex emulsion with a "clusters on string" structure where surfactant micelles are clustered together by pea protein polymeric chains. The clustering of these structures could be further caused by hydrophobic interactions between the hydrophobic tails of Tween 80 and hydrophobic side chains on protein molecules. It was assumed that when protein is added to Tween 80 stabilized emulsion, due to the binding between surfactant and protein, protein molecules opened up to expose more hydrophobic regions and the surfactant micelles clustered around the hydrophobic regions of the protein polymer, resulting in the formation of a complex (Figure 3.7). It is expected that the interactions among proteins, and between proteins and non-ionic surfactants through electrostatic and hydrophobic bond could further strengthen the emulsion system and offer more stability due to the formation of a viscoelastic layer against coalescence (A. N. A. Aryee et al., 2017; Sari et al., 2015; Wilde, Mackie, Husband, Gunning, & Morris, 2004).

3.3.3 Vitamin D encapsulation efficiency

Both nanoemulsions stabilized by Tween 80 alone and Tween 80-pea protein complex demonstrated high vitamin D encapsulation efficiencies of 90.1% and 89.3%, respectively. This indicates that nanoemulsions were effective in retaining most of the encapsulated vitamin D. Similar encapsulation efficiency (90.56%) was found in another study where nanoemulsions were prepared using a combination of whey protein concentrate and Tween 80 by ultrasonification (Sari et al., 2015).

3.3.4 Cytotoxicity

The cytotoxicity of nanoemulsions was evaluated in a Caco-2 cell model with cell viability measured by MTT assay. The emulsions were prepared after dilutions at different concentrations (0.5 mg/ml, 0.125 mg/ml, and 0.03125 mg/ml) with the growth media. Figure 3.8a represents the impact of varying emulsion concentrations on the viability of cells. There were no significant differences in cellular viability between nanoemulsions prepared by Tween alone and complex nanoemulsions at 0.5 mg/ml, 0.125 mg/ml, and 0.03125 mg/ml emulsion concentrations.

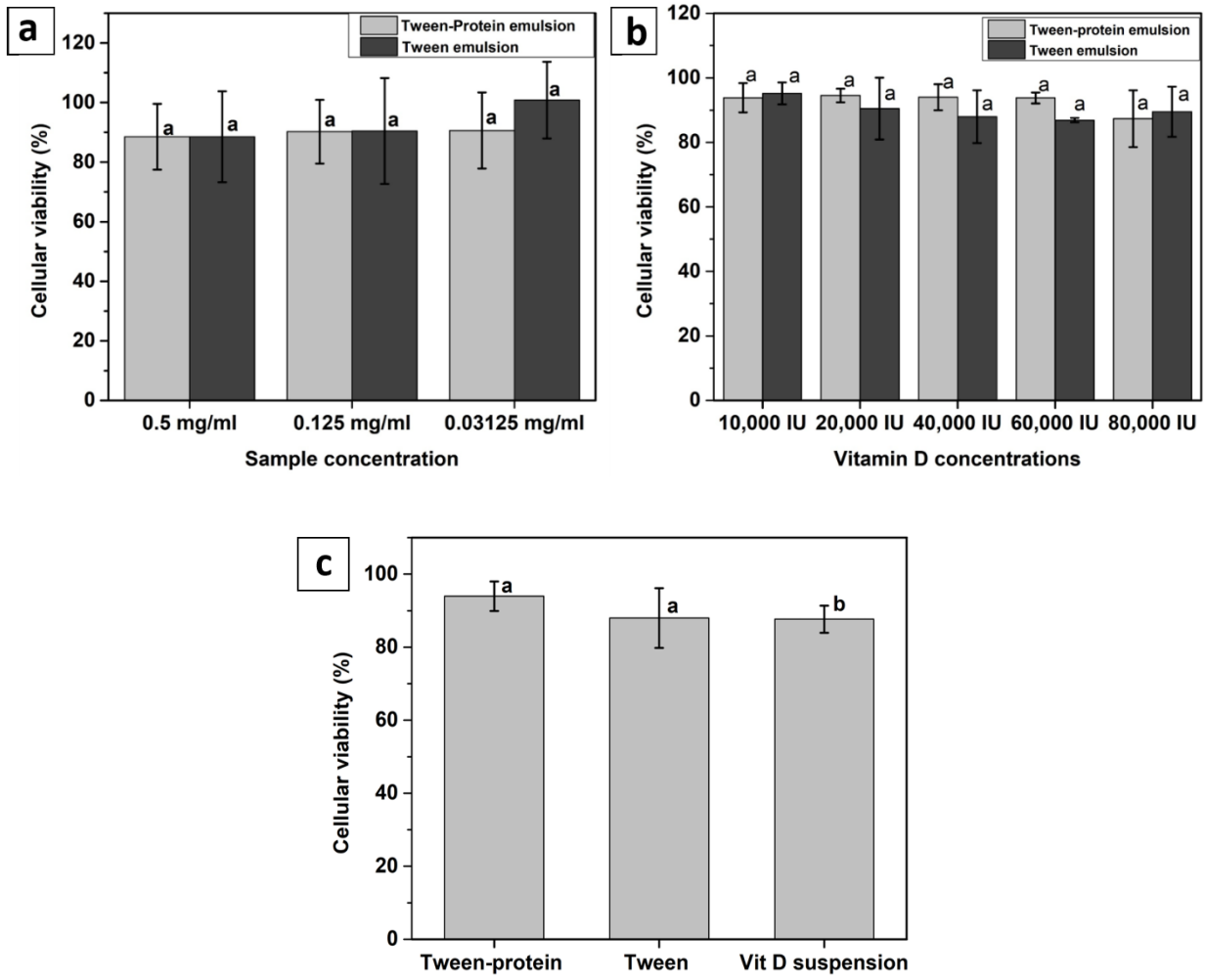

Figure 3.8 Influence of nanoemulsions (without vitamin D) concentration (0.5 mg/ml to 0.03125 mg/ml) after 6 hrs incubation on cellular viability, b) influence of different vitamin D

concentrations (10,000 IUs to 80,000 IUs) in the form of Tween and Tween 80-pea protein emulsions on cellular viability after incubating for 6 hrs using MTT assay, and c) comparison of Tween 80-pea protein emulsion, Tween alone emulsion and vitamin D suspension (nonencapsulated) in terms of cellular viability at 40,000 IU. Letters a,b denote significant differences.

The results indicated that the number of viable cells at all the three tested emulsion concentration were more than 80% of control, suggesting that both Tween emulsions and Tween-protein emulsions were relatively safe to cells. For *in vitro* cell models, cell viability greater than 80% is acceptable (Piazzini et al., 2019). Thus, nanoemulsion concentration of 0.5 mg/ml was selected to further study the cytotoxicity of the nanoemulsions with various vitamin D loading ranging from 10,000 IUs to 80,000 IUs for both emulsion systems. The results showed no significant differences in cellular viability for different vitamin concentrations as all samples showed cellular viability values > 80% (Figure 3.8b.). Therefore, 40,000 IU was the selected vitamin concentration for comparison of encapsulated (Tween 80-protein complex, Tween 80 alone) and non-encapsulated (vitamin D suspension in ethanol) vitamin D formulations in terms of cellular viability (Figure 3.8c). Cellular viability of both complex emulsion and Tween 80 stabilized emulsion was higher than free vitamin D suspension at 0.5 mg/ml nanoemulsions concentration and 40,000 IU vitamin D concentration, which indicates that encapsulating vitamin D in an emulsion demonstrates less toxic effect to cells. Although the difference was not significant, it was observed that Tween-protein complex emulsion showed relatively higher cellular viability than Tween 80 alone emulsion.

3.3.5 Cell uptake study- Flow cytometer

Based on the results of cellular viability tests by MTT assay, 0.5 mg/ml nanoemulsion concentration was selected for uptake studies. Different formulations were compared in terms of their uptake efficiencies using Caco-2 cell model. This experiment evaluates a) the impact of nanoencapsulation and b) the impact of Tween-protein complex nanoemulsion on the cellular uptake of nile red labelled vitamin D into the cells. As compared to the free vitamin D suspension, cellular uptake efficiencies of Tween 80-pea protein nanoemulsions and Tween alone nanoemulsions were ~3 times and ~2.6 folds higher ($p < 0.05$), which could be attributed to efficient encapsulation and improved dispersibility of nile red labelled vitamin D in simulated gut environment with the use of nanoemulsion delivery system (Figure 3.9).

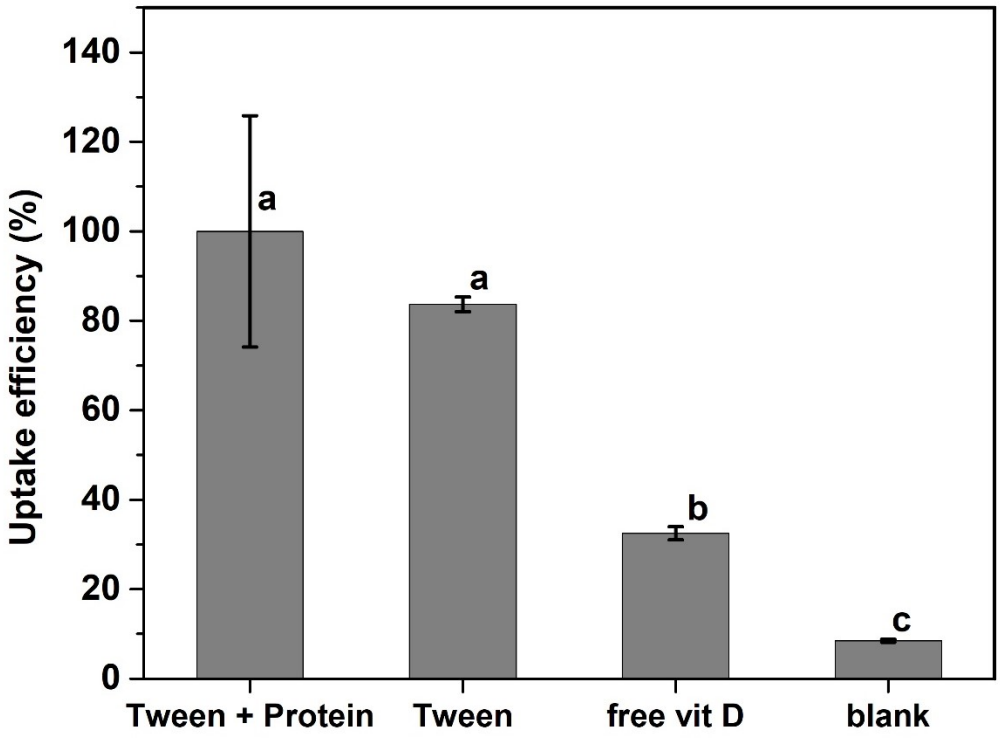

Figure 3.9 Flow cytometric quantitative analysis of cellular uptake efficiency of various vitamin D formulations by Caco-2 cells. Letter a,b,c denote significant difference ($p < 0.05$)

It was interesting to find that Tween 80-pea protein stabilized nanoemulsions had comparable uptake into cells as nanoemulsion stabilized by Tween alone because no significant differences in their uptake efficiencies were observed. This could be attributed to their surface charges as Tween-protein nanoemulsion (+3 mV) and Tween alone nanoemulsion (-21 mV) exhibited mild charges that resulted in less interactions between the nanoemulsion and cell membrane, and thus, resulted in higher cellular uptake. This suggests that nanoencapsulation of vitamin D significantly improved the uptake of nile red labelled vitamin D into the cells.

3.3.6 Transport efficiency of vitamin D

As shown in figure 3.10, transport efficiency of vitamin D encapsulated in nanoemulsion delivery system (Tween-protein nanoemulsion and Tween alone nanoemulsion) was significantly higher than vitamin D without encapsulation (free vitamin D suspension). The transport efficiencies of vitamin D for the three formulations were 8.08 ± 0.32 % for Tween-protein nanoemulsion, 3.58 ± 0.23% for Tween alone nanoemulsion, and 1.45 ± 0.41% for free vitamin D suspension.

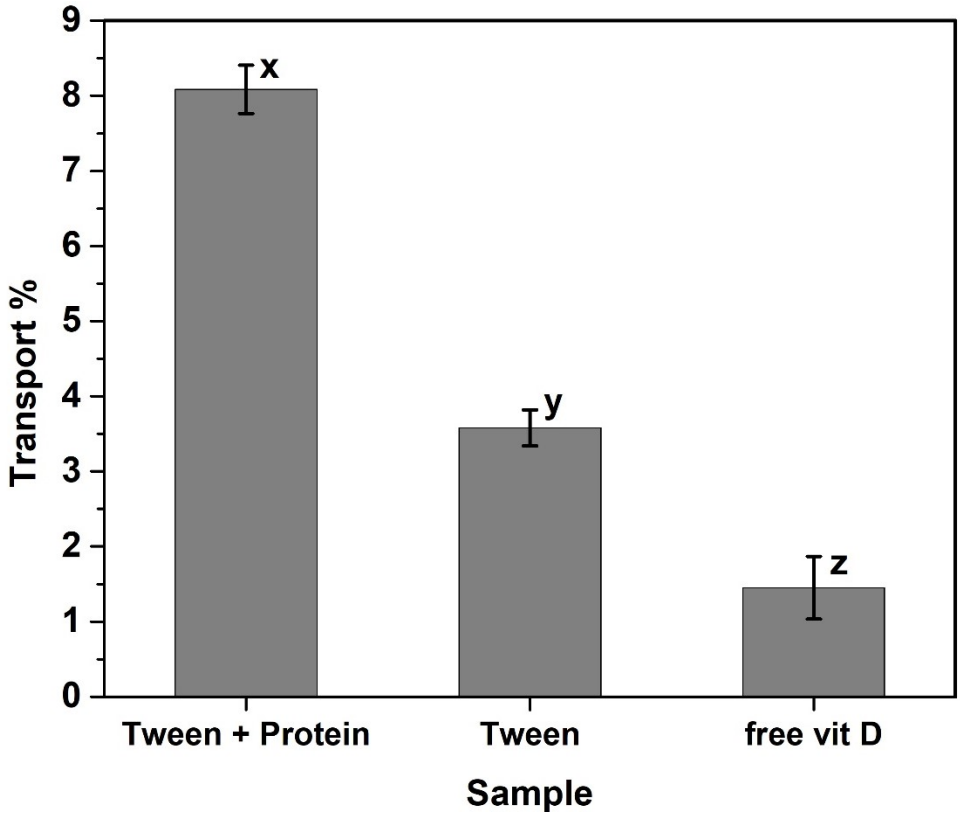

Figure 3.10 Transport efficiency of vitamin D across Caco-2 cell monolayers using Tween 80 emulsion, Tween 80-pea protein emulsion and free vitamin D suspension at initial apical concentration of 10 µg/ml. The percent transport of the vitamin D across the cell monolayers was calculated from the peak areas observed in the HPLC chromatograms of samples added to the apical chamber and collected from the basolateral chamber after the transport study. Data represented as mean ± SD and significant differences expressed as x,y,z at $p < 0.05$.

Tween-protein nanoemulsion greatly increased the transport of vitamin D across basal layer as it showed ~5.6 folds higher transport efficiency as compared to free vitamin D suspension, and 2.3 folds higher than Tween alone nanoemulsion system. In regards to Tween-protein stabilized nanoemulsions, addition of pea protein layer may have promoted interactions with the cell membrane proteins through hydrophobic interactions, which resulted in higher transport

efficiency. Increased surface hydrophobicity of particles may have enhanced their cellular uptake and transport, as hydrophobic surfaces allow easy penetration of nanoparticles into the cells and reside in the hydrophobic region of the cell membrane (Guangyu Liu, Ying Zhao, Lingyun Chen 2019). It was interesting to observe that cellular uptake and transport of vitamin D across Caco-2 cells was significantly higher for complex nanoemulsions and emulsions stabilized by Tween alone as compared to free vitamin D suspension. However, despite similar uptake of emulsions into cells, complex nanoemulsions showed higher transport efficiency as compared to Tween alone stabilized emulsion. This can be attributed to the difference in emulsion surface charges as complex emulsion with weaker charge (3.7 ± 0.6 mV) may have diffused faster as compared to strongly charged Tween 80 emulsion particles (-21.6 ± 0.8 mV), which may have interacted with cell compartments resulting in slower diffusion and hence, lower transport across cell membrane. Overall, this result demonstrated that the transport efficiency of vitamin D was improved significantly after its encapsulation in a nanoemulsion delivery system.

3.4 Conclusion

Generally, high energy techniques are used for developing nanoemulsions using proteins as it is a challenge to produce protein stabilized emulsions by low energy methods. However, this study reports the development of nanoemulsion with the combination of Tween 80 and pea protein by low-energy approach (spontaneous emulsification), resulting in a stable complex nanoemulsion of average diameter of 207.7 nm as supported by data from FTIR, emulsion surface charge and TEM images. TEM and FTIR supported the proposed “clusters on string” model where clusters of surfactant micelles were stabilized by pea protein polymeric chains. Both nanoemulsions stabilized by Tween 80 alone and Tween 80-pea protein complex nanoemulsions showed high encapsulation efficiencies of vitamin D (90.1% and 89.3%). The application of the developed complex

nanoemulsions was demonstrated using an *in vitro* model, Caco-2 cells. Interestingly, complex nanoemulsions improved vitamin D uptake into cells by 3 folds as compared to free vitamin D suspension. Moreover, complex nanoemulsions showed 5.3 folds higher transport efficiency of vitamin D across Caco-2 cells than free vitamin D suspension and 2.3 folds higher than nanoemulsion stabilized by Tween alone. The results revealed that a combination of Tween 80 and pea protein can be effectively used to form complex nanoemulsions that show efficient delivery of bioactives. The ease, low cost and simplicity of producing nanoemulsions by spontaneous emulsification makes it a preferred method for use in food, pharmaceutical and cosmetic applications, which also allows easier industrial scale up for commercial purposes.

Chapter 4- Conclusion and recommendations

4.1 Summary and conclusion

Vitamin D deficiency is a global issue and despite many initiatives to improve vitamin D levels through supplementation and fortification programs, vitamin D deficiency is still prevalent. Furthermore, poor aqueous solubility of vitamin D results in its low bioavailability in human gut. Therefore, new strategies should be developed to improve vitamin D absorption. Nanoemulsions is a widely used technique in pharmaceutical industry to solubilize fat-soluble compounds and to improve their absorption. Mostly, dairy products are vitamin D fortified, however, plant based fortified products are required for lactose intolerant, vegan and vegetarian populations. Therefore, pea protein nanoemulsions were prepared to encapsulate vitamin D and to improve their absorption.

In the first study, protein based nanoemulsions of particle size range from 170 to 350 nm were prepared using high-energy approach by controlling parameters of high-pressure homogenizer and emulsion composition. Nanoemulsions showed high vitamin D encapsulation efficiencies between 94 and 96%. Small sized nanoemulsions showed 2.5 times higher cellular uptake than large sized nanoemulsions. A comparison between protein and lecithin based nanoemulsions in terms of their cellular uptake showed no significant difference in the cellular uptake efficiencies between the two surfactants, which suggests the potential of using pea protein as an emulsifier for nanoemulsions preparation. Lastly, nanoemulsions showed significantly higher (5.3 times) cellular transport of vitamin D than free vitamin D suspension, suggesting the use of nanoemulsions to improve vitamin D absorption.

Although the use of high-pressure homogenizer resulted in stable nanoemulsions with a controlled particle size, high-energy methods are not highly desirable as they are less cost effective and

energy efficient. Therefore, the second study used a low-energy approach (spontaneous emulsification) to prepare complex nanoemulsions using a combination of food grade small molecular (Tween 80) and large molecular surfactants (pea protein). The complex nanoemulsions were proposed to possess a "clusters on string" structure where Tween 80 stabilized micelles were clustered together on pea protein polymeric chains. Light scattering measurements and TEM images revealed that the complex nanoemulsions exhibited particle size of 207.7 nm. Nanoemulsions stabilized by Tween 80 alone and Tween 80-pea protein showed 90.1% and 89.3% vitamin D encapsulation efficiencies. These nanoemulsions were further tested in terms of cellular uptake and transport efficiencies using an *in vitro* Caco-2 cell model. Both nanoemulsions significantly improved vitamin D uptake by ≥ 2.5 folds as compared to free vitamin D suspension. Complex nanoemulsions showed 5.6 folds higher transport efficiency of vitamin D across Caco-2 cells than free vitamin D suspension and 2.3 folds higher than nanoemulsions stabilized by Tween alone. Overall, the results of these studies show that nanoemulsions have a great potential in improving vitamin D absorption.

4.2 Significance of research

1) Particle size is an important characteristic that dictates cellular uptake and drug deliveries, and therefore, the impact of preparation processes on particle size of nanoemulsions was studied. This research demonstrated that nanoemulsions with controlled particle size could be obtained by controlling the parameters of high-pressure homogenizer and emulsion conditions. This will help in optimizing nanoemulsions particle size in order to achieve high uptake and delivery efficiencies.

2) The impact of surface properties and particle size on cellular uptake and transport of nanoemulsions was investigated. No significant differences in cellular uptake were observed between protein and lecithin stabilized nanoemulsions, however, small size nanoemulsions

showed significantly higher uptake as compared to large size emulsions. The results of this study will allow better design of delivery systems in order to achieve optimal drug delivery.

3) Pea protein based nanoemulsions showed significantly higher cellular uptake than emulsions stabilized by a combination of protein and lecithin, and no significant differences were found between pea protein based and lecithin based nanoemulsions. This showed the possibility of using pea protein as an emulsifier for preparing nanoemulsions suitable for the food industry.

4) According to our knowledge, complex nanoemulsions were prepared for the first time using a combination of Tween 80 (small molecular surfactant) and pea protein (large molecular surfactant) by a low energy approach (spontaneous emulsification), which is energy efficient, cost effective and environmentally sustainable. Furthermore, complex nanoemulsions showed great potential in its application as a vitamin D delivery system for improved vitamin D absorption with the help of Caco-2 cell model.

Overall, these nanoemulsions showed efficient encapsulation, cellular uptake and transport of vitamin D using Caco-2 cell model to simulate absorption through intestinal epithelium. Such nanoemulsions may allow more efficient vitamin D delivery to improve vitamin D status in aged population and in areas with less sun exposure. This study may also direct industries to develop novel non-dairy functional foods and beverages to suit the needs of different populations such as lactose intolerant, vegans or consumers with low dietary preference. Although this study used vitamin D as the selected model, the developed methods can be used for the delivery of other hydrophobic nutraceuticals as well.

4.3 Recommendations

In this research, an *in vitro* model was used to study cellular uptake of nanoemulsions for vitamin D delivery, however, animal models should be used in future to evaluate oral bioavailability of

vitamin D in nanoemulsions delivery system. Moreover, *in vivo* models with chronic diseases such as diabetes mellitus, celiac disease and inflammatory disease models should be used to understand the efficacy of developed nanoemulsions for improved vitamin D absorption, and to demonstrate potential benefits of vitamin D in reducing the risks of such chronic conditions. Animal models will also address further safety of the developed nanoemulsions. Further research should be conducted to confirm the potential of nanoemulsion encapsulation system using other hydrophobic nutraceuticals.

This research provided fundamental understanding of Tween 80 and pea protein interactions with the help of FTIR, which showed that Tween 80 does not cause denaturation of pea protein. Moreover, the proposed "clusters on string" model for the structure of complex nanoemulsion laid a foundation for understanding the complexities involved in using a combination of Tween 80 and pea protein emulsifiers. However, further understanding of Tween 80 and protein interactions is required and more studies should be conducted using Electron spin resonance (ESR) and nuclear magnetic resonance spectroscopy (NMR).

Lastly, toxicity and sensory studies should be conducted before utilizing nanoemulsions in food systems. It is also essential to understand how nanoemulsions properties such as stability, particle size and encapsulation efficiencies are affected upon interacting with a food matrix.

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