Chitin nanocrystal stabilized emulsions: stability, interactions with beta-lactoglobulin, digestion behavior and cytotoxicity

by

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ABSTRACT

CHITIN NANOCRYSTAL STABILIZED EMULSIONS: STABILITY, INTERACTIONS WITH BETA-LACTOglobulin, DIGESTION BEHAVIOR AND CYTOTOXICITY

Chandni Chandran Advisor
University of Guelph, 2015 Dr Loong Tak Lim

Solid particle stabilized emulsions have gained attention due to their surfactant free nature and high stability. However, insights on the interactions between solid particles stabilizing emulsions and surface active species found in food are important in formulation and use of such emulsions in real food applications. The properties of chitin nanocrystal (ChN) stabilized emulsions in the presence of beta-lactoglobulin (β-Lg), a commonly used emulsifier protein were studied as a function of pH. SDS-PAGE established that the protein co-existing with ChN at the interface. In vitro digestion studies demonstrated that, addition of β-Lg did not alter the rate of lipolysis observed with ChN stabilized emulsions. Electrophoresis on digestion end products also showed evidence for some protection offered by ChN to β-Lg at the interface against proteolysis. The results of cytotoxicity studies using Caco-2 cells, indicate that ChN dispersions do not have a detrimental effect on the proliferation of cells.
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# TABLE OF CONTENTS

Acknowledgements............................................................................................................iii

Table of contents..................................................................................................................iv

List of tables.........................................................................................................................vi

List of figures.........................................................................................................................vii

List of abbreviations............................................................................................................x

## CHAPTER 1: GENERAL INTRODUCTION AND OBJECTIVES...............................................1

## CHAPTER 2: LITERATURE REVIEW......................................................................................7

2.1 Emulsions.......................................................................................................................7

2.2 Emulsifiers......................................................................................................................10

2.3 Emulsion stability..........................................................................................................14

2.4 Particle stabilized emulsions.........................................................................................18

2.4.1 Chitin........................................................................................................................25

2.4.2 Chitin nanocrystals.................................................................................................26

2.5 Chitin nanocrystal stabilized emulsions......................................................................26

2.5.1 Emulsion digestion.................................................................................................27

2.5.2 Cytotoxicity studies...............................................................................................32

2.6 ChN emulsion mixed systems.....................................................................................33
CHAPTER 3: EXPERIMENTAL DETAILS

3.1 Preparation of chitin nanocrystals ................................................................. 36
3.1.1 Transmission Electron Microscopy ............................................................. 37
3.2 Emulsion preparation ...................................................................................... 37
3.3 Characterisation of ChN stabilized emulsions ................................................. 38
3.3.1 Creaming stability ...................................................................................... 38
3.3.2 Scanning Electron Microscopy Imaging ...................................................... 38
3.3.3 Emulsion droplet characterisation ............................................................... 39
3.3.3.1 Static Light Scattering ........................................................................ 39
3.3.3.2 Dynamic Light Scattering .................................................................. 40
3.4 Digestion ........................................................................................................ 41
3.4.1 In vitro digestion model for rate of lipolysis .............................................. 41
3.4.2 Cytotoxicity study ..................................................................................... 42
3.5 SDS-PAGE electrophoresis ............................................................................ 43
3.6 Statistical analysis .......................................................................................... 44

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Chitin nanocrystals morphology ................................................................. 45
4.2 Characterisation of ChN stabilized emulsions .............................................. 45
4.2.1 Microstructure of emulsion droplets ....................................................... 45
4.2.2 Creaming stability of emulsions on storage ............................................. 48
4.2.3 Emulsion droplet size distribution as studied using static light scattering.........................50

4.3 Effect of addition of $\beta$-lactoglobulin to ChN stabilized emulsions...........................................52

4.3.1 Droplet size distribution........................................................................................................52

4.3.2 Emulsion stability as a function of pH.......................................................................................53

4.4 In vitro lipid digestion of ChN stabilized emulsions....................................................................56

4.4.1 Size of lipid droplets before and after digestion.......................................................................60

4.5 SDS-PAGE behaviour of ChN stabilized emulsions mixed with $\beta$-Lg before and after digestion........................................................................................................................................63

4.6 Cytotoxicity studies.........................................................................................................................65

CHAPTER 5: CONCLUSIONS..............................................................................................................70

REFERENCES.........................................................................................................................................72
Table 4.1 Free fatty acids(FFA) released after 2 hr intestinal digestion at 370C at pH 7 from 1% ChN stabilized emulsion alone and mixed with 1% β-Lg solution (oil concentration of 0.5%(w/w)) [same superscript letters indicate no significant difference at p< 0.05].

..........59
LIST OF FIGURES

Figure 2.1: Forces acting on molecules at the interface ................................................................. 9

Figure 2.2: Schematic representation of an oil droplet emulsified using different emulsifiers … 11

Figure 2.3: (Upper) Position of a small spherical particle at a planar oil–water interface for a contact angle (measured through the aqueous phase) less than 90° (left) and greater than 90° (right). (Lower) For Θ < 90°, o/w emulsions may form (left). For Θ > 90°, w/o emulsions may form (right) ........................................................................................................................................ 20

Figure 2.4: The increase in energy of detachment with size for a single spherical particle assuming contact angle 90°, oil–water interfacial tension 27 mN/m ................................................................. 22

Figure 2.5: Diagrammatic representation of the proposed mechanism of lipid digestion in ChN stabilized o/w emulsions .............................................................................................................. 30

Figure 4.1: TEM images of chitin nanocrystals (bar size-1µm). (A) Diluted 10 times and (B) Diluted 100 times with 10 mM sodium acetate buffer (pH 3) ........................................................................ 46

Figure 4.2: Representative images obtained using Cryo-SEM of oil-in-water emulsions (oil content 5% w/w) stabilized with chitin nanocrystals (A) 0.5% ChN, (B) 1% ChN, (C) 2% ChN..47

Figure 4.3: Effect of increasing concentration of ChN [ A (0.5%), B (1.0%) and C (2.0 %)w/w] on the stability of 5% oil in sodium acetate buffer emulsion at pH 3 on storage at 40°C for a period of one month .................................................................................................................................................. 49

Figure 4.4: Mean droplet size diameter as D4,3 of ChN stabilized emulsions made with different concentrations of ChN and measured over a 30 day period from the day of preparation (day 0). Superscript letters indicate mean diameters are not significantly different (p < 0.05)..................... 51

Figure 4.5: Droplet size distribution of emulsions (A-0.5% ChN, B-1.0% ChN, C- 2.0% ChN) with (filled symbols) and without β-Lg (empty symbols). Representative runs ........................................... 54

Figure 4.6: Changes in hydrodynamic diameter measured with DLS (A) and zeta potential (B) as a function of pH for 1% ChN emulsion with (filled symbols) and without (empty symbols) β-Lg. Data are the average of three independent measurements with bars representing standard deviation .................................................................................................................................. 55
Figure 4.7: Time dependent free fatty acid (% FFA) release from 1% ChN stabilized emulsion alone and mixed with 1% β-Lg solution (oil concentration of 0.5%(w/w), pH 7 at 37°C)……..58

Figure 4.8: Mean droplet diameter (D (4, 3) µm) of 1% ChN stabilized emulsions alone and mixed with 1% β-Lg solution (1:1) before and at the end of gastric and intestinal digestion……62

Figure 4.9: SDS-PAGE electrophoresis of 1% ChN emulsion mixed with β-Lg and its gastric digest (Lane 1- emulsion with β-Lg, Lane 2& 5 cream phase of emulsion mixture and gastric digest, Lane 3& 6 serum phase of emulsion mixture and gastric digest, Lane 4- gastric digest of the emulsion mixture, Lane 7- pure β-Lg solution 1%)………………………………………………………………64

Figure 4.10: Cell viability (%) of Caco-2 cells after 2 hrs (A) and 24 hrs (B) incubation of 1% ChN dispersion (1% ChN D), 1% ChN emulsion(1% ChN E), 1% ChN with 1% β-Lg(1% ChN + B-Lg) and 1% β-Lg emulsion(1% B-Lg E) before digestion. Control wells contain only medium and considered to have 100% proliferation……………………………………………………67

Figure 4.11: Cell viability (%) of Caco-2 cells after 2 hrs (A) and 24 hrs (B) incubation of 1% ChN dispersion (1% ChN D), 1% ChN emulsion(1% ChN E), 1% ChN with 1% β-Lg(1% ChN + B-Lg) after digestion ……………………………………………………………………………………………..68
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>β –Lg</td>
<td>Beta lactoglobulin</td>
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<tr>
<td>γ</td>
<td>Interfacial tension</td>
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<tr>
<td>ΔA</td>
<td>Interfacial area</td>
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<tr>
<td>ζ</td>
<td>Zeta potential</td>
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<tr>
<td>θ</td>
<td>Contact angle</td>
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<td>ChN</td>
<td>Chitin Nanocrystal</td>
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<tr>
<td>D&lt;sub&gt;4,3&lt;/sub&gt;</td>
<td>Volume weighted mean diameter</td>
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<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin, Landau, Verwey and Overbeek</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
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<tr>
<td>HLB</td>
<td>Hydrophile Lipophile Balance</td>
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<tr>
<td>mM</td>
<td>milli Molar</td>
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<tr>
<td>mN/m</td>
<td>milli Newton/meter</td>
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<tr>
<td>o/w</td>
<td>Oil-in-water</td>
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<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phopholipase A2</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SGF</td>
<td>Simulated gastric fluid</td>
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<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
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<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<td>w/o</td>
<td>Water-in-oil</td>
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CHAPTER 1
GENERAL INTRODUCTION AND OBJECTIVES

Emulsions are defined as a mixture of at least two immiscible phases (e.g., oil and water). One of the phases exists as discrete droplets (known as the dispersed phase) suspended in the other phase (known as continuous phase). Emulsions are broadly classified based on the relative spatial distribution of the oil and aqueous phases as oil-in-water emulsions (oil droplets dispersed in aqueous phase, for example milk, cream, mayonnaise and soups) and water-in-oil emulsions (water droplets dispersed in oil, for example butter and margarine) (Dickinson & Rodriguez Patino, 1999). Emulsions are thermodynamically unstable systems as oil and water do not coexist favourably because of the surface energy (Gibbs free energy) at the oil-water interface (Israelachvili, 1992). However, emulsions that are kinetically stable for a reasonable period of time (ranging from days to years) can be formed by including stabilizers and emulsifiers. An emulsifier is defined as a substance that reduces surface tension between oil-water or any two immiscible phases, thus enhancing emulsification and helping in emulsion stability. Emulsifier molecules are amphiphilic with a hydrophobic and hydrophilic domain. The hydrophobic part interacts with the oil phase while the hydrophilic portion dissolves in the aqueous phase. The surfactant molecules forms a layer on the surface of the emulsion droplet (Mc Clements, 2005). Often surfactants may provide a charge around the oil droplet or protrude into the water phase causing an increase in the electrostatic and steric repulsion forces, providing colloidal stabilization. In food formulations for emulsions such as butter, mayonnaise, and ice cream, the industry takes advantage of natural emulsifiers such as lecithin in egg yolk and proteins in milk. The most commonly used emulsifiers in food industry are small molecule surfactants (lecithin, fatty acid salts, monoglycerides and polysorbates), phospholipids, proteins, and polysaccharides (Hansenhuetl, 2008).
Emulsion stability refers to the ability of an emulsion to maintain its properties over time. Colloidal scientists have been constantly working on finding new and improved stabilizers to increase emulsion stability. In 1907, S.U. Pickering first reported about o/w emulsions stabilized by solid particles adsorbed at the surface of oil droplets. Such emulsions stabilized using solid particles came to be known as Pickering emulsions. In his paper, he studied the formation of stable emulsions of paraffin oil stabilized by solid particles adsorbed at the oil droplet surface. He presented evidence for the adsorption of solid particles, and demonstrated that pickering emulsions provide superior stability with respect to surfactant-based emulsions.

In particle stabilized emulsions, the type of emulsion formed depends on the particle wettability, expressed in terms of the contact angle (θ) that the particle makes at the oil-water interface (see Fig 2.3). Accordingly, hydrophilic particles that make a contact angle < 90° stabilize oil-in-water emulsions and hydrophobic particles that makes a contact angle > 90° stabilizes water-in-oil emulsions (Binks & Lumsdon, 2000). Efficient adsorption of solid particles at the oil–water interface requires the partial wetting of the particle by both phases. Particle surfaces are often chemically modified to achieve optimum wetting and adsorption properties (Dickinson, 2012).

Even though it was made clear that solid particle stabilized emulsions had definite advantages in comparison to surfactant-based emulsions (Ramsden, 1903; Pickering, 1907), they did not receive great attention for a long period. However, there has been a recent upsurge in interest, which has been driven by the increasing availability of nanoparticles that are suitable for the formation of particle stabilized emulsions. By using nanoparticles, the thickness of the adsorbed particle layer is reduced, and the total particle loading for a given emulsion formulation is reduced. It is yet not clear how nanoparticles stabilized emulsions will be accepted by the consumer, in view of the public concerns regarding the safety of the nanoparticles (Frith et al. 2008).
However, it is important to point out that in food systems, natural nanoparticles and particle stabilized emulsions do exist. Hence, prior to this, people worked with Pickering emulsions without acknowledging it, especially in the field of food emulsions where crystals of solid fats adsorb at the surface of emulsion droplets (Rousseau, 2000). Pickering emulsions started receiving scientific attention due to problems faced by the petroleum industry with regards to the difficult demulsification of crude oil in the field of oil recovery (Tambe & Sharma, 1994). Since then, many types of particles which could be successfully used as pickering stabilizers have been reported in literature. A recent review (Dickinson, 2012) on the use of nanoparticles and microparticles in the formation and stabilization of food emulsions describes the developments in food grade emulsion systems based on traditional edible dispersed particles (fat crystals), commercial nanoparticles (silica nanoparticles) and novel particles of biological origin (starch microparticles and chitin nanocrystals).

Oil-in-water emulsions stabilized using chitin nanocrystals (ChN) have been studied (Tzoumaki et al., 2011b). The emulsions did not show any change in $d_{4,3}$ over a period of one month, indicating limited coalescence of the oil droplets. These emulsions have been characterized with respect to changes in ChN concentration, temperature, ionic strength and pH. It has been demonstrated that, in addition to the adsorption of the crystals at the interface, an inter-droplet network forms in the continuous phase, causing improved stabilization. The same group of researchers in another study looked into the rheological behaviour of aqueous dispersions containing ChN and whey protein isolate, demonstrating that the mixtures can form a gel (Tzoumaki et al, 2011a). But these studies with mixed systems were carried out on dispersions (particles in aqueous medium) and not emulsions (particles at interface).

Proteins are surface active due to their amphiphilic nature and are commonly used as emulsifiers in food. Protein molecules unfold and interact with each other on adsorption at the interface. In some cases, they form a viscoelastic layer that provides stability to the emulsion.
This depends on the size, shape, net charge, surface hydrophobicity, stability, flexibility, amino acid composition and structure of proteins (Kim et al., 2005). β-lactoglobulin is a relatively small globular protein (18,400 Da) whose molecular and functional properties are well characterized. At pH 3, β-Lg carries positive charge, similar to ChN particles.

Drop tensiometry has been employed to study the interactions occurring between β-Lg and ChN, and it was demonstrated that the interfacial elasticity was enhanced by the presence of small amounts of protein, and that ChN once adsorbed at the interface cannot be displaced by the protein (Gulseren & Corredig, 2013). But the study of the effect of the presence of proteins in a ChN stabilized emulsion is an area yet to be explored, and it is of importance, as relevant models for food systems are needed. This thesis further studied ChN stabilized emulsions, and the effect of addition of β-lactoglobulin to ChN stabilized emulsions. Indeed the utilization of such particle stabilized emulsions will be possible only after sufficient knowledge has been gathered on the interactions of ChN with other components in milk.

There have been studies on the applications of chitin nanofibers and researchers have successfully produced optically transparent chitin nanofiber acrylic resin composites with significant increase in tensile strength and decrease in thermal expansion capacity due to the reinforcement effect of chitin. Chitin has also been shown to improve clinical symptoms in acute ulcerative colitis in a mouse model of dextran sulfate sodium induced colitis (Ifuku & Saimoto, 2012). Other reported applications of chitin or chitosan include tissue engineering scaffolds, drug delivery and wound dressing (Muzzarelli et.al, 2007)

The use of nanoparticles in food has remained an area of speculation. It might be attributed to the knowledge gap that exists regarding the effect of ingestion of such particles and its cytotoxicity. Studies on the in vitro lipid digestion of chitin nanocrystals stabilized o/w emulsions demonstrated that ChN stabilized emulsions showed higher stability against lipolysis as compared to whey protein isolate and sodium caseinate stabilized emulsions, and this is
attributed to the ChN particles at the interface forming a ChN network in the continuous phase (Tzoumaki et al., 2013). This provides an opportunity for the use of such emulsions in the development of products aimed at reducing calorie intake and combating obesity while promoting satiety.

This thesis also looks into the digestion behaviour of ChN stabilized emulsions in the presence of the interface active protein β-Lg using an in vitro digestion model to evaluate the free fatty acid release, and the physical properties of the emulsion during digestion. Studies on the cytotoxicity of ChN particles were also conducted. Although there have been a few studies on the cytotoxicity of chitin and chitosan nanoparticles (Huang et al., 2004, Jayakumar et al., 2010, Wongpanit et al., 2007), these studies mainly looked into chitosan derivatives. This thesis attempts to look into the cytotoxicity of ChN particles in aqueous dispersion, in emulsions and also in emulsion systems in the presence of β-Lg. Digestion end products subjected to cytotoxicity studies will give insights about the nature of ChN particles used as emulsion stabilizers.

This research focuses on understanding the interactions between ChN stabilized emulsions and β-Lg solution in a mixed system, with the following specific objectives:

1. Investigate the droplet size distribution and stability of ChN emulsions.
2. Measure the change in droplet size distribution of ChN stabilized emulsions on addition of β-Lg.
3. Study the changes in size and zeta potential of ChN emulsions and β-Lg mixed systems with changes in pH.
4. Investigate the stability of ChN emulsions mixed with β-Lg and the presence of the protein at the interface.
5. Compare ChN emulsions (1% ChN) and β-Lg (1%) mixed systems (1:1) during digestion.
6. Investigate the cytotoxicity of whole emulsions and digestion end products.

The results of this study will support further development and utilization of ChN stabilized emulsions in dairy food systems.
CHAPTER 2

LITERATURE REVIEW

2.1 Emulsions

An emulsion is a suspension of two or more immiscible phases, with an interfacial layer between the two phases occupied by surfactant molecules (Dalgleish, 2004). Formation of emulsions is a thermodynamically unfavourable event due to the increase in the surface area between the oil and water phases leading to an increase in the interfacial free energy which opposes emulsion formation. Therefore, it requires utilization of surface-active agents (emulsifiers) and/or amphiphilic polymers with the input of energy (Sakai, 2008). Simple emulsions consists of oil droplets dispersed in water (o/w) or water droplets dispersed in oil (w/o). Some emulsions contain smaller droplets of the continuous phase dispersed within each droplet of the dispersed phase and they are known as multiple emulsions (Bibette et al., 1999).

Milk, cream, mayonnaise, salad dressings, butter, sauces and soups are commonly occurring emulsions in the food industry. Emulsions are used to encapsulate, stabilize and deliver functional food components like flavors, bioactive lipids, enzymes, peptides, antimicrobials and antioxidants (Guzey & McClements, 2006a). The ability of an emulsion to resist changes in its properties over time is referred to as emulsion stability. Food emulsions are more complex due to the presence of other interacting components in food like proteins, polysaccharides, vitamins, glycerol components and minerals.

Creaming, sedimentation, flocculation, coalescence, Ostwald ripening and phase inversion are different forms of physical instability while oxidation and hydrolysis causes chemical instability encountered in food emulsions (McClements, 2005, Fennema, 1996). The major changes that occur during emulsification include deformation of droplets and their eventual break down, transportation of the surfactants to the newly formed interface and their adsorption, and the
coalescence of droplets while they collide against each other. The process of combining two immiscible liquids to form an emulsion is known as homogenization. Homogenization can be classified into two types – primary homogenization involves formation of an emulsion by mixing two separate liquids whereas secondary homogenization refers to reduction of droplet size of an already prepared emulsion (McClements, 1999). Homogenizers provide the mechanical energy needed for droplet disruption. The disruptive forces that the droplets experience during homogenization depend on the flow conditions (laminar, turbulent or cavitational) occurring in the type of homogenizer used. Emulsions can also be prepared by ultrasonication, but in this case cavitation is the main effect that leads to droplet break up. Other factors that influence emulsification include viscosity of the dispersed and continuous phases, interfacial tension, power density and residence time of the emulsion in the dispersing zone (Schubert et al., 2003, Walstra, 1993).

The minimum amount of energy ($E_{\text{min}}$) that has to be applied to form an emulsion is equal to the free energy required to increase the interfacial area between the oil and water phases:

$$E_{\text{min}} = \Delta A \gamma$$  \hspace{1cm} \text{Eq. 2.1}

where $\Delta A$ is the interfacial area and $\gamma$ is the interfacial tension.

Since oil and water phases are molecularly incompatible, at the interface between these two phases the molecules experience an imbalance in the attractive forces as shown in Figure 2.1. Interfacial tension at the oil-water interface originates from this imbalance in the attractive forces acting on the molecules at the interface and is defined as a measure of the free energy required to increase the area of the interface by an unit amount (Jm$^{-2}$).
Figure 2.1: Forces acting on molecules at the interface (Adapted from McClements, 2005)
In order to reduce the interfacial tension, emulsion droplets shrink in volume to minimize the unfavourable thermodynamic contact area between the water and oil phases (Everett, 1988, Jonsson et al., 1998). This causes the droplets to develop an internal pressure, and this difference in pressure across the interface is known as the Laplace pressure.

This pressure difference is represented by the Young-Laplace equation:

$$\Delta P = \frac{2\gamma}{r}$$  \hspace{1cm} Eq. 2.2

where $\gamma$ is the interfacial tension and $r$ is the droplet radius (Adamson, 1990).

To achieve efficient disruption of droplets during homogenisation, the external force applied should be significantly larger than the interfacial force. Droplet disruption proceeds at greater ease when interfacial tension decreases and this can be achieved by the addition of suitable surfactants.

### 2.2 Emulsifiers

Emulsifiers can be defined as the broad group of substances that are capable of adsorbing at an oil-water interface and provide protection to droplets against aggregation. Low molecular weight amphiphiles are only defined as “emulsifiers” in food industry. Amphiphilic molecules are characterized by the presence of a hydrophilic head group and a lipophilic tail group which enables them to adsorb at the oil-water interface (Dickinson, 2003). Figure 2.2 compares attachment of solid particles at the interface with commonly used emulsifiers in food. Bancroft’s rule describes the type of emulsion that can be formed with a particular surfactant. It states that the phase in which the surfactant is more soluble becomes the continuous phase (Davis, 1996).

Another concept used for classifying small molecular weight surfactants is the HLB (hydrophile-lipophile balance) number. Based on the chemical structure every surfactant has an HLB number. A molecule with more hydrophilic groups has a higher HLB number compared to one
Figure 2.2: Schematic representation of an oil droplet emulsified using different emulsifiers
(Adapted from Hasenhuettl, 2008)
with more lipophilic groups. A surfactant with high HLB number stabilizes oil-in-water emulsions while those with lower HLB numbers form water-in-oil emulsions (Davis, 1994, Bergensthal, 1997). A surface active molecule prefers to exist at the interface rather than in the bulk phase. Adsorption of a surfactant molecule at the interface is based on its ability to orient itself with the hydrophilic part in water and the lipophilic part in the oil phase. This forms a barrier at the interface minimizing the contact area between hydrophilic and lipophilic regions leading to reduction in interfacial tension. The major groups of surfactants used in the food industry are low molecular mass surfactants (fatty acids, glycolipids and fatty alcohols) and macromolecular emulsifiers extracted from natural sources, such as proteins and polysaccharides. The mechanisms and kinetics of adsorption vary greatly with the type of compound used (Kralova & Sjoblom, 2009, McClements 2005).

Low molecular weight surfactants have much faster adsorption kinetics than macromolecules, and they are more effective in reducing the interfacial tension. They rapidly coat the newly formed oil-water interface during emulsification and prevent droplet coalescence (Wilde, 2000). These surfactants are most effective when they form a fluid adsorbed layer that allows them to migrate to regions of lower surfactant concentration during the homogenization process. This is known as the Gibbs-Marangoni effect and causes the droplets to be more stable against coalescence (Walstra & Smulders, 1998).

Proteins exhibit surface activity due to the presence of lipophilic amino acids such as phenylalanine, leucine and isoleucine. They modify their conformation at the oil-water interface to decrease the free energy of the system, and may penetrate into the lipid phase to various degrees (Nnanna & Xia, 2001). The adsorption of proteins at the interface proceeds in different steps and includes, diffusion of the protein molecules from the bulk phase to the vicinity of the interface, the actual adsorption and the reorganization of the adsorbed proteins (Graham & Phillips, 1979). The unfolding of proteins at the interface is influenced by its structure in solution,
flexible proteins unfold quickly and lower the interfacial tension more rapidly than globular proteins. Intermolecular interactions may also occur between the adsorbed proteins leading to the formation of a viscoelastic film. The protein residues that remain in the aqueous phase provide steric stabilization against flocculation and coalescence (Dickinson, 1999, Wilde et al., 2004, Bouyer et al., 2012). Most proteins decrease the oil–water interfacial tension by about 15–20 mN/m in a saturated monolayer coverage, compared to 30–40 mN/m for small synthetic surfactants (Razumovsky & Damodaran, 1999).

Whey proteins represent about 20% of the total milk proteins, with β-Lactoglobulin (β-Lg) accounting for about 60% of the whey proteins in cow milk. Whey proteins have been widely used to stabilize emulsions in food systems. Bovine β-Lg exists as a dimer in the pH range of 5.5–7.5 and as a monomer below pH 3.5 and above pH 7.5. It has 162 amino acid residues per monomer with a molecular weight of approximately 18kDa. It has two disulphide bonds and a single free thiol group, and shows a calyx type structure under native conditions. The iso-electric point of β-Lg is around pH 5.2 (Sawyer, 2003, O’ Mahony & Fox, 2013).

Globular whey proteins from milk are used as emulsifiers in a great range of applications because of their ability to facilitate emulsion formation and improve emulsion stability. These proteins adsorb to the surfaces of oil droplets formed during homogenization, they also facilitate further droplet disruption by lowering the interfacial tension and retarding droplet coalescence by acting as a protective covering around the droplets (Kim et al., 2004). Proteins exhibit surface activity due to their molecular flexibility and the amphiphilicity of their polypeptide chains. The type and number of contacts the protein makes with the interface decides its effectiveness in lowering the surface tension. Based on the reported surface activity of different milk proteins, β-casein is the most surface active while β-lactoglobulin exhibits the least surface activity (Dickinson, 1999, Mulvihill, D. M. & Fox, P F, 1989).
On adsorption at the interface, proteins reorient their hydrophobic groups towards the oil phase and the hydrophilic groups towards the aqueous phase. This causes β-lactoglobulin to lose its native tertiary structure and assume non-native secondary structures. The proteins tend to aggregate at the interface forming a viscoelastic film around the droplets (Lam & Nickerson, 2014, Dickinson & Leser, 2013). These electrically charged membranes, stabilize droplets by electrostatic repulsion and to some extent steric hindrances. This makes them more sensitive to changes in pH, at pH values close to the isoelectric point of the protein they tend to flocculate (McClements, 2004)

2.3 Emulsion stability

The ability of an emulsion to maintain its physical and chemical properties over time is referred to as emulsion stability. Emulsion formation is always thermodynamically unfavourable due to the increase in the interfacial area after homogenization (McClements, 2005). In the presence of surfactants, kinetically stable emulsions can be formed.

Every system consists of molecules at high energy and low energy states. Systems with lowest free energy are considered to be thermodynamically favourable. The difference in these energy level (ΔE) is the energy barrier that the molecules should overcome to change from a high energy state to a low energy one. When this energy barrier is quite high, systems tend to remain thermodynamically unstable for longer periods of time and this is known as a kinetically metastable state (Atkins, 1994).

Emulsion stability is determined by the sum of attractive and repulsive colloidal interactions, determining whether emulsion droplets aggregate or remain as separate entities. The major forces that act on emulsion droplets include van der Waals interactions, electrostatic interactions, steric interactions and depletion interactions. Nonpolar molecules exhibit attraction via van der Waals forces. These forces arise from the electrical dipole due to the instantaneous
distribution of electrons, as this leads to some polarity. This electrical field polarizes adjacent groups creating a weak attraction between the transient dipole and the resultant induced dipole. Van der Waals are strong, attractive long range forces (Coupland, 2007). In addition, electrostatic interactions arise from the electrical characteristics of the droplet surface. The electrical charge on droplet surfaces depends on the type and concentration of the emulsifier at the interface and the conditions of the environment including pH, temperature and ionic strength. This electrical charge affects the distribution of ions in the electrolyte surrounding the droplets. Counter ions (oppositely charged) are preferentially attracted towards the surface and co-ions (similarly charged) are repelled. The combination of the charged surface and the region of unequal concentration of counter and co-ions near the charged surface is called the electrical double layer or the diffuse layer (Dickinson & Stainsby, 1982, Claesson et al., 2004).

When two charged droplets are close enough that their electrical double layers overlap, repulsive forces prevent them from further approach. This repulsion can arise from the difference in osmotic pressure: the excess counter ion concentration in the double layer causes a higher osmotic pressure between the surfaces than in the bulk. The solvent molecules tend to diffuse into the overlap region to reduce the osmotic pressure difference creating a repulsive interaction that prevents droplet coalescence. The energy barrier preventing the coalescence of charged droplets is estimated by the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory that takes into account the attractive van der Waals forces and the repulsive double layer forces (Dickinson, 1992, Ichikawa, 2007).

Steric interactions are observed in emulsions stabilized by macromolecules. The adsorbed macromolecular layer prevents the close approach of droplets contributing to emulsion stability. These forces act at short range and the main contributors to this mechanism are the volume exclusion and the osmotic effects (Coupland, 2007). Depletion interactions arise from the presence of non-adsorbing colloidal particles like biopolymers or surfactant micelles in the
continuous phase that increases the attraction between droplets due to an osmotic pressure gradient due to the exclusion of polymer molecules from the immediate vicinity of the droplets (Dickinson 1992, McClements, 2005).

The major forms of instability that cause an increase in droplet size are Ostwald ripening and coalescence. Ostwald ripening is more evident in emulsions where the dispersed phase has a high solubility in the continuous phase, for example, in an oil-in-water emulsion with a continuous phase containing ethanol. The driving force for this process is the difference in the Laplace pressure between droplets of different radii. This causes the dispersed phase to diffuse from the smaller to the larger droplets (Leal-Calderon & Poulin, 1999). The process of Ostwald ripening is explained by the Lifshitz, Slezov and Wagner (LSW) theory, according to which the rate of ripening depends on the solubility and diffusion coefficient of the dispersed phase and the interfacial tension between the droplets and the continuous phase. The solubility of the dispersed phase in the bulk phase increases with decreasing radius of the droplets. Thus the smaller droplets tend to dissolve and their material diffuses and re-deposits on to larger droplets resulting in an overall increase in the average radius of the emulsion (Taylor, 1995).

In the absence of surfactants, coalescence is the primary mechanism of instability in emulsions. The process involves rupture of the thin film that forms between droplets causing the two droplets to fuse and form a single droplet (Kabalnov, 1998). Droplet coalescence is initiated by the close distance between droplets, for example during collision or creaming. The sum of the interactive forces (both attractive and repulsive) determines whether colliding droplets coalesce, aggregate or rebound. Some collisions result in the deformation of the droplets and the contact zone gets flattened. This zone of contact between droplets is a thin film of liquid and the stability of droplets depends on the properties of this film. When attractive forces are predominant, the film will be unstable, liquid drains and the film thins. On reaching the critical thickness of film, it
ruptures and the droplets fuse together. When repulsive forces dominate, the droplets repel each other (Danner & Schubert, 2001).

Gravity plays a major role in the instability of emulsions. Creaming (or, the inverse case, sedimentation) involves the upward movement of oil droplets under gravity to form a concentrated layer at the top. The driving force for this gravitational separation is the difference in densities of the continuous and dispersed phases. Sedimentation may occur, for example, in water in oil emulsions. The rate at which an oil drop of radius $r$ moves through the continuous phase of density $\rho_0$ and viscosity $\eta_0$ is given by the Stokes equation:

$$V_s = \frac{2r^2 (\rho_0 - \rho) g}{9\eta_0}$$

Eq. 2.3

where $g$ is acceleration due to gravity and $\rho$ is the density of the dispersed phase (Binks, 1998). This form of instability does not lead to a change in drop size distribution and can be reversed by mechanical agitation of the sample (McClements, 2005).

The collision of emulsion drops also gives rise to another form of instability in emulsions, known as flocculation, where drops aggregate without losing their individual integrity. The aggregation can be strong or weak depending on the intensity of the interdroplet forces (Dickinson, 1992). Flocculation can increase the rate of creaming since flocs cause an increase in effective radius of droplets. The driving forces for aggregation of droplets include Brownian motion and shear flow (Binks, 1998). It may also happen in emulsions stabilized by biopolymers when there is an insufficient amount of surfactant for complete surface coverage, this leads to sharing of the macromolecules between adjacent droplets or when the emulsion droplets and the biopolymer carries opposite charges. This is known as bridging flocculation (McClements, 2005).

The presence of non-adsorbing polymers in the continuous phase of an emulsion increases the attraction between emulsion droplets leading to depletion flocculation. This attractive force
arises when the droplets approach each other and the separation between them is less than the diameter of the free polymer. The local osmotic pressure gradient effect associated with the exclusion of polymer molecules from the narrow space surrounding the droplets causes a depletion zone in the immediate vicinity of the droplets (Jenkins & Snowden, 1996). The strength of this depletion interaction depends on the osmotic pressure gradient and the thickness of the depletion layer (Dickinson, 2014). The different structural and functional characteristics of the non-adsorbing species in emulsions has been a well-studied area as it is critical to improve emulsion stability.

Studies over the years have looked into ways of improving emulsion stability and methods to overcome common instability mechanisms. Emulsions stabilized by particles have gained considerable research interest due to their improved stability and the ability to replace conventional surfactants especially in pharmaceutical applications where some surfactants act as possible irritants (Rayner et al., 2014).

2.4 Particle stabilized emulsions

Emulsions stabilized by solid particles are also known as Pickering emulsions. They are either oil-in-water (o/w), water-in-oil (w/o), or even multiple phase emulsions, and they are characterized by solid particles adsorbed at the interface, in place of surfactants (Chevalier & Bolzinger, 2013). The history of particle stabilized emulsions as a separate class dates back to 1900s, when studies on emulsions stabilized by solid particles were reported by Ramsden (1903) and Pickering (1907). The adsorption of solid particles at the interface is different from surfactant adsorption in that, the basic requirement for a surfactant to be amphiphilic is not applicable for solid particles. However, the adsorbed particles should remain intact during the lifetime of the emulsion to be considered a Pickering emulsifier/stabilizer (Dickinson, 2012).
Particles adsorb at the oil-water interface based on their partial dual wettability, if they are completely wetted by either of the phases, they remain dispersed and cannot form stable emulsions. The relationship between particle wettability and the type of emulsion stabilized was first described by Finkle et al., 1923, which came to be known as the Finkle’s rule. According to it, the partial wettability expressed in terms of the contact angle $\Theta$ that the particle makes with the oil-water interface measured through the water phase determines whether an oil-in-water or water-in-oil emulsion is favoured (Rayner et al., 2014). The most wetting liquid becomes the continuous phase. Similar to the HLB concept used for surfactants, the Finkle’s rule determines the type of emulsions stabilized by different particles. The angle between the tangent to the solid-water interface and the tangent to the oil-water interface at the point where the three phases meet is defined as the contact angle ($\Theta$) of the particle (Leal-Calderon & Schmitt, 2008).

Figure 2.3 depicts the positioning of hydrophilic and hydrophobic particles at the oil-water interface favouring the formation of oil-in-water and water-in-oil emulsions, respectively. Relatively hydrophilic particles show a contact angle measured into the aqueous phase $< 90^\circ$. These particles will have a larger fraction of the surface residing in water than in the nonpolar phase, stabilizing oil-in-water emulsions. On the other hand, hydrophobic particles with contact angles $> 90^\circ$ have the larger portion of surface area residing in the nonpolar phase and such particles will stabilize water-in-oil emulsions.

It has been shown that the free energy change associated with the attachment of particles at the interface is negative at all contact angles (except at the extremes $0^\circ$ and $180^\circ$) for colloidal sized particles with smooth chemically homogenous surfaces. Therefore, attachment of particles is thermodynamically favourable leading to the accumulation of particles at the oil-water interface (Rayner et al., 2014).
Figure 2.3: (Upper) Position of a small spherical particle at a planar oil–water interface for a contact angle (measured through the aqueous phase) less than $90^\circ$ (left) and greater than $90^\circ$ (right). (Lower) For $\Theta <90^\circ$, o/w emulsions may form (left). For $\Theta >90^\circ$, w/o emulsions may form (right). (Adapted from Schmitt et.al, 2014)
The strength of attachment of the particles at the interface is described in terms of the free energy of detachment. The energy of desorption of a spherical particle from the interface follows the relationship:

\[ \mathcal{E} = \gamma \pi R^2 (1 - \cos\theta)^2 \]  

Eq. 2.4

Where \( \gamma \) is the oil-water interfacial tension, \( R \) is the particle radius and \( \theta \) is the contact angle. The desorption energy of a surfactant molecule will be around 106 kT, where \( k \) is Boltzmann’s constant and \( T \) is the absolute temperature (300K), assuming typical values of \( \gamma \) (10-40 mN/m) and particle radius of 1 \( \mu \)m.

Therefore even for relatively small nanoparticles in the range of 5-10 nm in radius, the adsorption can be considered irreversible as the energy required to remove the particle from the interface is far greater than 10 \( kT \), when \( \theta \) is not too far away from 90\(^\circ\) (Leal-Calderon & Schmitt, 2008, Dickinson, 2012). Figure 2.4 illustrates the relationship between the energy of detachment and the size of a spherical particle.

This high energy of detachment makes the particle adsorption at the interface effectively an irreversible process. In surfactant stabilized emulsions the attachment and detachment of surfactant molecules at the interface happens continuously at a relatively fast timescale (Binks, 2002). This is one of the major differences that confer solid particle stabilized emulsions greater stability compared to surfactant stabilized emulsions.

The influence of particle shape on its ability to stabilize emulsions has been studied by Madivala et al., 2009. Using ellipsoidal and spindle shaped particles with aspect ratios of 1 to 9, it was possible to demonstrate that emulsions can be formed with particles having sufficiently large aspect ratios when spherical, while less elongated particles of similar wettability cannot form a stable emulsion. This has been attributed to the effect of particle aspect ratio on the surface coverage and shape induced attractive capillary interactions at the oil-water interface.
Figure 2.4: The increase in energy of detachment with size for a single spherical particle assuming contact angle 90°, oil–water interfacial tension 27 mN/m (Adapted from Rayner et al., 2014).
Similar findings were also reported for ellipsoidal particles (Loudet et al., 2005). In this case, ellipsoidal particles at the oil-water interface were attracted to one another with long range interactions, and the interaction energies are very large compared to the thermal energy $k_BT$ and interaction energies of spherical particles with similar surface chemistry.

The mechanisms of stabilization of emulsions by particles can then be summarized as follows. First the particles adsorb at the interface and form a monolayer or multilayer surrounding the droplets preventing coalescence. The second mechanism involves particle-particle interactions like a three dimensional network in the continuous phase that may provide additional stability to the emulsions (Binks, 2002).

A major hurdle in stabilizing emulsions using particles is the kinetics of adsorption of the particles at the newly formed interfaces. Compared to low molecular weight surfactants and macromolecules, particles have slow diffusion coefficients that makes them take longer to reach the interface. In dense emulsion systems where the rate of droplet collision is high, this will lead to smaller droplets colliding and coalescing before full surface coverage can be achieved (Ettelaie & Murray, 2014).

It has been reported that at liquid-liquid interfaces, on adsorption of particles the interfacial tension decreases with time and finally approaches equilibrium (Kutuzov et al., 2007). In their work, they showed that larger particles (about 6 nm in diameter) cause more reduction in interfacial tension compared to smaller particles (2.3 nm of diameter). At the early stage of homogenisation the interface is considered empty and all colliding particles will freely adsorb. Then a concentration gradient driven diffusion occurs from the bulk to the interface till the concentration is equalized in the bulk and the sublayer surrounding the interface.

The adsorption kinetics of these systems have been studied by drop tensiometry (Stocco et al., 2009; Du et al., 2010). The rate of adsorption is not only diffusion controlled but also affected by
an energy barrier which leads to slow rates of adsorption, typically around $10^3$ seconds before equilibrium interfacial tension values are observed. An energy barrier to adsorption due to steric or electrostatic interactions of the particles of size <10 nm at the oil water interface has also been reported (Kutuzov et al., 2007).

Coalescence is a major reason for emulsion destabilization. In Pickering emulsions the adsorbed layer of solid particles acts like a rigid covering, a mechanical barrier that prevents coalescence (Chevalier & Bolzinger, 2013). There is not enough experimental evidence to support the theory of high stability of particle stabilized emulsions to drop-drop coalescence when compared with surfactant or protein stabilized emulsions. However, particle stabilized emulsions have been proven to completely arrest the process of Ostwald ripening due to the high energy of desorption and the absence of driving forces for compression and collapse of the droplets: the apparent interfacial tension and drop capillary pressure approaches zero at close distance in particle stabilized droplets (Tcholakova et.al, 2008). It has also been reported that the diffusion of oil from smaller droplets to the larger ones in the initial phase of Ostwald ripening causes compression of the adsorbed particle layer, which either buckles or resists further reduction in area halting the process of ripening. The excess particles present in the continuous phase get adsorbed during swelling of larger droplets, causing overall increase in stability to prevent coalescence (Binks, 2002).

In a recent study on the disproportionation process in Pickering stabilized bubbles, it was reported that the stabilization of bubbles against ripening requires the formation of a particle network at the interface. This network formation takes time to develop because of a finite rate of adsorption of particles at the interface and during this time period significant shrinkage of bubbles can occur, especially in the case of smaller bubbles with higher dissolution rates. But a similar problem does not occur in Pickering stabilized oil emulsions because the rate of
adsorption of particles is much faster compared to the rate of Ostwald ripening in such systems (Ettelaie & Murray, 2014).

A wide variety of solid particles used as emulsion stabilizers has been reported in the literature. These include organic and inorganic materials like laponite clay (Ashby & Binks, 2000), polystyrene latex particles (Binks & Lumsdon, 2001, Amalvy et.al, 2003) and fumed silica (Sugita et.al, 2008). Some food compliant hydrocolloids that can act as Pickering stabilizers include hydrophobically modified starch granules (Yusoff & Murray, 2011), fat crystals (Rousseau, 2013), flavonoid particles (Luo et al., 2011), bacterial cellulose nanocrystals (Kalashnikova et al., 2011) and chitin nanocrystals (Tzoumaki et al., 2011).

2.4.1 Chitin

Chitin is the second most abundant natural polysaccharide, occurring in shell fish exoskeleton complexed with mineral salts, cell wall of fungi, insects and marine algae. It was first discovered in mushrooms and later isolated from insects in the 1830's. Marine shellfish like lobster, crab and shrimp are the richest sources of chitin in the biosphere. The majority of commercially available chitin is derived from crustacean shell wastes that are available in high volumes at low cost from seafood processing plants. It is a cationic linear polysaccharide composed of β-1, 4 linked N-acetyl-D-glucosamine units, structurally similar to cellulose (Venugopal, 2011). Traditional methods for the commercial preparation of chitin from crustacean shells involves deproteinisation by alkali treatment and demineralisation by acidic treatment under high temperature followed by a bleaching step to obtain a colourless product. The use of such harsh conditions has always been an area of concern. Recent studies have reported that bacteria that produce organic acids and enzymes can be utilized for the demineralisation and deproteinisation of crustacean shells. This appears to be promising as it is less harmful to the environment and can preserve the characteristics of the final product (Arbia et al., 2013).
2.4.2 Chitin nanocrystals

Early references about the presence of rod like particles on acid hydrolysis of chitin appeared in the 1900s. Marchessault et al. (1959) studied liquid crystal systems from fibrillar polysaccharides, and reported that chitin formed rod like particles similar to cellulose crystallites on acid hydrolysis followed by strong mechanical treatment. These particles formed colloidal suspensions in water after dialysis to neutral conditions. The study of electrophoretic mobility of chitin crystallite particles obtained from crab shell chitin during different stages of hydrolysis revealed that at the beginning of hydrolysis, the particles carry a small negative charge and after 15 minutes of hydrolysis there is a reversal to positively charged particles. This positive charge arises from the protonation of the amino groups in chitin and also those that are formed by deacetylation during the hydrolysis process. These amino groups (NH$_3^+$) and their counter ions form an electrical double layer around the crystallites which prevents flocculation leading to a stable colloidal suspension.

Electron micrographs of chitin nanoparticles showed some degree of aggregation, but the majority of nanocrystals seem to be in the 100-200 nm range (Revol & Marchessault, 1993). When extracted from α-chitin of shrimp shells, their lateral dimensions range from 200-500 nm and individual nanocrystals have a transverse dimension of 10-15 nm (Goodrich & Winter, 2007). Different sources and different hydrolysis times result in nanoparticles with widths ranging from 10-50 nm, and lengths varying from 150-2200 nm. This has been attributed to the different original sizes of the chitin particles and the diffusion controlled nature of the acid hydrolysis (Zeng et al., 2012).

2.5 Chitin nanocrystals stabilized emulsions

The role of chitin nanocrystals as oil-in-water emulsion stabilizers has been described in detail by Tzoumaki et al., 2011. Corn oil in water emulsions prepared using ultrasonic homogenisation
for 2 min can be stabilized with chitin nanocrystals. The influence of chitin nanocrystals concentration, pH, temperature and ionic strength on the elastic behavior of such emulsions has been reported (Tzoumaki et al., 2011). The elastic behaviour is enhanced with increase in pH (3.0 to 6.7), NaCl concentration (up to 200 mM) and temperature (from 20 to 74°C). Chitin nanocrystals are capable of forming highly stable emulsions with a dispersed phase volume fraction of 0.74 or greater (Perrin et al., 2014). The texture of these emulsion gels formed at high concentration of oil phase can be modulated by changing the ionic strength, pH and concentration of chitin nanocrystals. Such emulsion gels can be utilised in food preparation, oil recovery and cosmetics.

Emulsions made with different concentrations of chitin nanocrystals (0.01 to 1.0%) did not show any change in $D_{4,3}$ values on storage for a period of one month. The size of the emulsions droplets was reported to be around 100 µm and decreased with increase in ChN concentration (Tzoumaki et al., 2011). The increased stability of such emulsions was attributed to the adsorption of ChN at the interface, build-up of an inter-droplet network and a ChN network in the continuous phase. In a similar study using bacterial cellulose nanocrystals (Kalashinikova et al., 2011), it was shown irreversible adsorption of particles, and the high stability was attributed to the steric hindrance from a 2-D network at the interface at high concentration of added nanocrystals.

2.5.1 Emulsion Digestion

Emulsions form a major class of food products that act as a lipid food source. Advances in research techniques have led to improved understanding of the mechanisms of lipid digestion and bioavailability. This has paved the way for researchers to investigate how food material properties can be manipulated to regulate physiological breakdown and uptake of lipids in the gastrointestinal system (Singh et al., 2009). The process of digestion is studied both by using *in vivo* methods (involves actual feeding to animals or humans), or more commonly *by in vitro* methods.
models (studies that mimic the digestion process). It is very challenging to relate *in vitro* studies to those obtained from *in vivo* models; however, it is important to carry out *in vitro* studies, to be able to derive a mechanistic understanding of the processes of breakdown in food, and also, for ethical reasons. Nonetheless, it is very difficult to accurately simulate the different biochemical and physiological changes that food undergoes in the human or animal gastro intestinal system (Hur et al., 2011). Researchers have been continuously working on developing more sophisticated *in vitro* models to improve the representation of the physiological conditions both in terms of the biochemical environment and the biophysics associated with the human gastrointestinal system reducing the costs associated with expensive human trials (Golding & Wooster, 2010).

*In vitro* digestion models commonly used to study digestion can be divided into static and dynamic models. In static models, the products of digestion are not removed during the course of digestion. Furthermore, static models do not mimic the physical processes that take place *in vivo*. They are useful to study limited digestion (eg: gastric phase) stages, and the digestion behaviour of simple foods and isolated nutrients. Dynamic models may or may not have the products of digestion removed during the process but incorporate the physical processing of food in the gut and can be used to study the digestion of complex food materials (Wickham et al., 2009).

The models can differ, for example, in the number of steps included in the digestion process (e.g mouth, stomach, small intestine and large intestine), the composition of the digestive juices used and the mechanical stresses applied during the process (Hur et al., 2011). The most commonly used enzymes and biological molecules in different *in vitro* models include α-amylase, pepsin, pancreatin, trypsin, chymotrypsin, peptidase, lipase, bile salts, phospholipids and mucin. These enzymes may be obtained from human, animal or plant sources. The significant influencing factors in *in vitro* digestion includes sample characteristics, enzyme
activity, ionic environment, mechanical stresses, incubation temperature regulation of the equipment used and digestion time (Boisen & Eggum, 1991).

The differences observed among *in vitro* digestion models make it difficult to compare results between research teams. A network of more than 200 researchers from 32 countries working in the field of digestion recently proposed a static model for *in vitro* digestion suitable for food. It includes a frameset of parameters for oral, gastric and small intestinal phases of digestion. The paper provides a starting point with exact step by step guidelines including composition of digestive juices used, sampling during digestion and sample preservation for further analysis. It also compares the proposed model with available *in vivo* data to justify and state the limitations associated with the model (Minekus et al., 2014).

In the only available study on *in vitro* lipid digestion of ChN stabilized emulsions, researchers compared the kinetics of fatty acid release and the change in droplet size distribution during lipid digestion in these emulsions with sodium caseinate and whey protein isolate stabilized emulsions. It was observed that ChN stabilized emulsion droplets resist lipolysis, as there was a lower percentage of free fatty acid release when compared with milk protein stabilized emulsions, under the *in vitro* conditions used. The droplet size distribution did not change considerably indicating relative stability of the emulsions towards coalescence during the initial stages of digestion. This presents a clear opportunity for design of functional food emulsions.

The irreversible adsorption of ChN particles at the interface as well as the resulting ChN network seem to protect the lipid droplets from adsorption of bile salts, somewhat hindering lipase digestion (Tzoumaki et al., 2013). Figure 2.5 summarizes the mechanism underlying the lipolysis inhibition (Tzoumaki et al., 2013). However, more work needs to be carried out to better understand if these observations may also be confirmed in more complex matrices, and under more complex *in vitro* or *in vivo* conditions.
Figure 2.5: Diagrammatic representation of the proposed mechanism of lipid digestion in ChN stabilized o/w emulsions (Adapted from Tzoumaki et al., 2013).
Pickering emulsions may show great potential to modulate lipid digestion. Silica nanoparticle stabilized emulsions with encapsulated curcumin upon digestion retained more than 80% of curcumin during the gastric phase and 60% of curcumin was released during intestinal digestion, indicating their ability to act as bioactive carriers (Tikekar et.al, 2013). However, the digestion behaviour of nanoparticle stabilized emulsions vary significantly with the type of nanoparticle used to stabilize emulsions and their interactions with other components present in the food matrix.

Proteins in food are exposed to a great range of pH, minerals and enzymes during their course through the human gastrointestinal tract. The hydrolysis of proteins begins with pepsin in the stomach and continues with the action of proteolytic enzymes in the pancreas including trypsin, chymotrypsin and membrane peptidases (Tome & Debabbi, 1998). Early research has shown that native β-Lg shows significant resistance to peptic hydrolysis at pH 2.0. This was attributed to the structural integrity and conformational stability of the protein (Reddy et.al, 1988). Until recently there has been limited knowledge about the physicochemical and structural changes occurring during the digestion of emulsions and proteins at oil-water interface. Studies (Macierzanka et al., 2009) reported that β-Lg is more susceptible to proteolysis by pepsin when present at the interface, and this is explained by the conformational changes occurring during adsorption, which render the protein sites more prone to peptic cleavage.

Emulsions undergo considerable changes in physical properties during gastrointestinal transit. The proteolysis usually plays a significant role before lipolysis can be effectively achieved (Singh & Ye, 2013). Soy oil (20%) emulsions stabilized with β-Lg (1%) were subjected to in vitro digestion, incubating at 37°C for 2 h with simulated gastric fluid (protein: enzyme ratio 3:1 (w/w)). The particle size of the emulsion varied throughout the digestion, from a monomodal distribution following homogenisation to bimodal after 1 h and multimodal at 2 h of gastric digestion (Sarkar et al., 2009). These changes reflect modifications of the interfacial layer during
digestion. In another study to differentiate the digestive behaviour of whey protein isolates (WPI) in solution and while adsorbed at an oil-water interface, it was observed that pure β-Lg in solution resisted pepsin hydrolysis whereas protein at the interface did undergo hydrolysis. SDS-PAGE electrophoresis confirmed the presence of a β-Lg band even after incubation with simulated gastric fluid (SGF) for 60 min whereas both the cream and serum phases of the WPI stabilized emulsions did not show any residual bands for β-Lg indicating a higher rate of hydrolysis for both the adsorbed and unadsorbed proteins in emulsion compared to solution (Malaki Nik et al., 2010).

2.5.2 Cytotoxicity studies

With the emergence of nanoparticle utilization in food, cosmetic and pharmaceutical industries, the safety of such particles has been an area of concern. Size is the defining characteristic of nanoparticles, which can modify the physicochemical properties of the material that can result in increased uptake and interaction with biological tissues. In addition to size, surface charge, solubility, shape and aggregation are the major factors that determine the biokinetics of nanoparticles. These determine the cellular uptake, protein binding, translocation from site of entry to the target and possible tissue injury (Nel et al., 2006, Oberdorster et al., 2005).

In a study aimed at improving the stability of silk fibroin for tissue engineering applications, chitin whiskers from shrimp shells were incorporated as nanofillers, and it was demonstrated that the sponges with and without chitin whiskers were cytocompatible with L929 cells (mouse connective tissue) (Wongpanit et al., 2007). In addition, the incorporation of chitin whiskers into the silk fibroin matrix significantly increased cell spreading. In a similar study, α-chitin whiskers from crab shells were used to reinforce hyaluronan-gelatin nanocomposite scaffolds. These scaffolds are used in tissue engineering as supportive framework. The cytotoxicity and cell proliferation studies were carried out on human osteosarcoma cells (SaOS-2). The results indicated that 10% chitin whisker incorporated scaffolds supported cell proliferation while at
higher levels of chitin whisker incorporation (30%) the scaffolds exhibited adverse effect on cell viability (Hariraksapitak & Supaphol, 2010).

Chitosan and chitosan nanoparticles from chitin have been assessed for cytotoxicity behaviour with respect to changes in molecular weight and degree of deacetylation. It was observed that the transformation of chitosan into nanoparticles caused a change in its cellular uptake but did not change its cytotoxicity towards A549 cells (human lung carcinoma cell line). The cytotoxicity of both chitosan and chitosan nanoparticles was shown to decrease with a lower degree of deacetylation. Lowering the degree of deacetylation lowers the zeta potential of the nanoparticles contributing to their uptake and cytotoxicity behaviour to a greater degree than the molecular weight. Indeed, uptake by cells is affected by the electrostatic interactions between chitosan nanoparticles and the cell membrane (Huang et.al, 2004).

Although these studies indicate the cytocompatibility of chitin derived nanoparticles, cytotoxicity assessment of chitin nanocrystals as Pickering stabilizers in an oil-in-water emulsion system before and after digestion is an area that has not been explored.

2.6 ChN emulsion mixed systems

There is no available data on chitin nanocrystals stabilized emulsions used in a system with other surface active components present. A considerable number of studies are available on the interactions between chitosan and milk proteins (Lee et al., 2012, Campina et al., 2010, Vilma et al., 2007, Guzey & McClements, 2006b, Anal et al., 2008). Chitosan, being a cationic polysaccharide also derived from chitin has been the subject of numerous studies, as it may be used as an unique ingredient in the food industry. Being cationic, it readily interacts with negatively charged proteins, anionic polysaccharides, fatty acids, bile acids and phospholipids (Agulló, et al., 2003).
The interactions between soluble chitosan and β-Lg in aqueous solutions have also been reported as a function of pH (Guzey & McClements, 2006b). At pH 3 when the protein and chitosan have similar charges there is little interaction and at pH 6 when they carry opposite charges strong interactions are present. This was confirmed by turbidity and solubility data that showed insoluble complex formation at pH 6.0 and 7.0. Similar results were also reported by others (de Souza et al., 2009). Spherical nanoparticles form between sodium caseinate and chitosan, with size ranging between 200-300 nm (Anal et al., 2008). It was observed that the ability to form soluble or insoluble chitosan-caseinate complexes depends on the pH and that they form larger particles that result in phase separation in the pH range of 4.0–4.5 or >6.5. At a lower pH of 3.0–3.8, chitosan and sodium caseinate carry similar charges and they may dissociate from each other and become solubilized in solution.

The interactions between β-Lg and chitosan at a solid-liquid interface were also studied (Campina et al., 2010) by using electrostatically bound ultrathin films of chitosan on polycrystalline gold as the solid phase. The study confirmed that interactions occur at both pH above and below the protein’s isoelectric point. All these studies indicated the dependence of chitosan interactions with proteins on the pH and concentration of chitosan in the system.

The presence of protein in a chitin nanocrystals stabilized emulsion system is an area that has not been much explored. The interactions of β-Lg with chitin nanocrystals were measured using drop shape tensiometry as a model to study oil-water interfaces (Gülseren & Corredig, 2013). On addition of β-Lg to ChN dispersions the interfacial tension was significantly lowered, compared to that of ChN alone and there was a slightly higher interfacial tension in the mixture than in a pure β-Lg solution. It was shown that protein molecules may co-exist with ChN at the interface resulting in lower interfacial tension and higher film elasticity by packing in regions between ChN until a certain concentration of protein beyond which the available surface...
becomes saturated. However, the ChN adsorbed at the interface cannot be displaced by the protein (Gülseren & Corredig, 2013).

This thesis attempts to study the changes in a ChN stabilized oil-in-water emulsion in the presence of β-Lg, a surface active milk protein.
CHAPTER 3

EXPERIMENTAL DETAILS

3.1 Preparation of chitin nanocrystals

Crude chitin from shrimp shells was obtained from Sigma (St. Louis, 132 MO, USA). This crude chitin was used to prepare aqueous stock dispersions of chitin nanocrystals (ChN) based on a published method (Tzoumaki et al., 2010).

Powdered chitin (30 g) was dispersed in 700 mL of 5% KOH solution and boiled for 6 h under stirring to remove contaminating proteins. This mixture was stirred overnight at room temperature (23°C), filtered (Whatman filters, 934-AHTM148, Florham Park, NJ, USA) and washed with distilled water. The filtrate was bleached with 1.7% sodium chlorite solution prepared in sodium acetate buffer (0.3 M, pH 4.0) at 80°C for 6 h in a temperature controlled water bath. The bleaching solution was changed every 2 h along with rinsing the sample with distilled water. After 6 h, the filtrate was dispersed and kept in 700 mL of 5% KOH solution at room temperature for 48 h to ensure removal of proteins. At the end of 48 h, the dispersion was centrifuged at 5000 x g for 15 min (J2-21 rotor, Beckman-Coulter, Mississauga, Ontario, Canada). The precipitate was suspended in 3N HCl and acid hydrolysis was carried out for 90 min under boiling conditions. The supernatant was then removed by the same centrifugation conditions. The precipitate was placed in a dialysis bag and kept under running tap water for 1 h followed by extensive dialysis overnight in distilled water. The final pH of the dispersion was adjusted to pH 3 and was subjected to high shearing using a shear mixer (Power Gen 125, Fisher Scientific, Ontario, Canada) in order to break the large ChN aggregates in the dispersion. The final dispersion was stored at 4°C after adding a small amount of sodium azide (0.02% by
wt) (Fisher Scientific, Fair Lawn, NJ, USA) to prevent bacterial growth. The stocks were diluted with 10 mM sodium acetate buffer (pH 3.0) for further analysis.

The chitin content of the stock dispersion was determined gravimetrically by drying the samples at 50 °C until a constant weight was obtained; the total solids content of the stock dispersion was approximately 7.3 % (w/w).

3.1.1 Transmission electron microscopy

Transmission electron microscopy (TEM) uses an accelerated electron beam from an electron gun (source used to emit electrons), that can interact with thin specimens and generate variable signals. The electron beam that emerges from the specimen carries information about the specimen and forms an electronically magnified image via electromagnetic lenses. The importance of TEM in the study of nanoscale particles is that it provides information about the morphology (microstructure) and symmetry of the crystals (structure) (Lei, 2012).

In our study, TEM with negative staining was employed to observe the morphology of the aqueous ChN dispersions. A drop (10 µL) of ChN dispersion was floated on a formvar-carbon coated copper grid and any excess liquid was eliminated using filter paper. The grids were then quickly touched to a drop of deionized water and the excess wicked off as a quick wash. Grids with sample on were then floated on a drop of 0.5% aqueous uranyl acetate for 10 s. The excess was wicked off using filter paper and allowed to dry. Micrographs were taken on a transmission electron microscope (CM-10, Philips, Eindhoven, Netherlands) at 80 kV.

3.2 Emulsion preparation

Oil-in-water emulsions were prepared by mixing appropriate quantities of ChN stock dispersion, soy oil ( from Sigma without further purification) and sodium acetate buffer adjusted to pH 3.0, with a high-speed blender (PowerGen 125, Fisher Scientific, Mississauga, ON, Canada) and passing them through a homogenizer (Emulsiflex C5, Avestin, Ottawa, Canada). The emulsions
were homogenized with six passes at 500 bar. The soy oil concentration was 5% w/w, while the ChN concentration varied from 0.5, 1 and 2 % (in the final product).

ChN emulsions were mixed with β-Lg solution (1%) at 1:1 ratio. Purified β-Lg was obtained from whey protein isolate (New Zealand Dairy Products, NZMP) using preparative ion chromatography on Q Sepharose (GE Healthcare) as previously reported (Andrew et al., 1985). Milli-Q water was used to prepare all reagents.

3.3 Characterisation of ChN stabilized emulsions

3.3.1 Creaming stability

Freshly prepared emulsions were poured into 15 mL centrifuge tubes immediately after preparation. These emulsion samples were stored under refrigeration (4 °C) and the creaming stability was monitored by visual observation of the height of the aqueous phase formed at the bottom of the tubes. The samples were photographed on day zero (day of preparation) and 15 and 30 days after preparation.

3.3.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) uses a focused beam of electrons produced from a gun inside the microscope that scans the surface of the sample. It is capable of producing 3D images resulting in analysis of the surface morphology and structural changes (Bucak & Rende, 2014).

The emulsions were studied using cryo-scanning electron microscopy (Cryo-SEM). Samples (3 mm³), were mounted on a copper holder designed for the Emitech 1250x Cryo- preparation unit (Ashford, Kent, UK). Tissue-Tek, a cryo-mounting gel, was used to ensure that the samples were affixed to the holder. The copper holders were plunged into liquid nitrogen slush (-207 °C), obtained by pulling a vacuum on the liquid nitrogen. The copper holders were withdrawn from
the freezing chamber through argon to prevent frost forming on the surface of the samples and placed under vaccum. They were transferred into the preparation chamber of the cryo unit where the frozen samples were fractured to provide a fresh surface free of frost. The samples were coated with < 30 nm of gold in the cryo-preparation system (Model K550, Emitech, Ashford, Kent, England) at < -135°C. The holder was then transferred, and frozen under vacuum, onto the cold stage in the SEM (S-570 Hitachi High Technologies Corporation, Tokyo, Japan). The temperature was maintained at below -150 °C. Images were captured digitally using Quartz PCI imaging software (Quartz Imaging Corp. Vancouver, BC).

3.3.3 Emulsion droplet characterization

3.3.3.1 Static light scattering

Emulsions with droplet diameters between about 100 nm and 1000 μm can be analysed using static light scattering technique. A beam of light illuminating a suspension of particles causes some of the light to be scattered and this depends on the size of the particles, shape, refractive index, wavelength of light and the angle of observation (McClements, 2007).

Droplet-size distributions of the emulsions were determined by using a Mastersizer 2000S (Malvern, Southborough, MA). Emulsion droplets were measured under high dilution conditions by dispersing the sample in the sampling chamber containing 10 mM sodium acetate buffer at pH 3.0. The refractive indices of water and soy oil were taken as 1.333 and 1.47, respectively.

The particle size measurements are reported as the volume weighted mean diameter $D_{4,3} = \Sigma n_i d_i^4/\Sigma n_i d_i^3$, where $n_i$ is the number of droplets of diameter $d_i$. The volume weighted mean diameter is considered more sensitive to the presence of large particles as compared to other mean particle size values (McClements, 2007). Each individual measurement was determined from the average of three readings made on the same sample. In this study, ChN stabilized
emulsions before and after mixing with β-Lg were measured. The emulsion samples before and during different stages of digestion were also analysed for particle size distribution.

### 3.3.3.2 Dynamic light scattering

Dynamic light scattering (DLS) measurements depend on light scattered by moving particles. When particles are in constant Brownian motion, the intensity of light scattered varies with their relative spatial location. The frequency of intensity fluctuations depends on the speed at which the particles move and therefore, on their size. The particle size distribution is measured from the change in intensity of light scattered over time at a particular scattering angle using a suitable mathematical model. DLS can measure particle diameters in the range between 3 nm to 5 μm (McClements, 2007).

Zeta potential is the electrical potential at the ‘shear plane’, that is defined as the distance away from the droplet surface below which the counter ions remains strongly attached to the droplet when it moves in an electrical field. Droplet charges are usually characterized in terms of zeta potential (ζ) as it inherently accounts for the adsorption of charged counter ions on droplet surfaces (Hunter, 1986).

In this study, DLS was employed to determine the change in average hydrodynamic radius and the zeta potential of the emulsion droplets with change in pH. Zeta-potential was calculated from the particle mobility values by applying the Smoluchowski model. The zeta-potential changes over pH (from pH 3.0 to 6.0 with an increment of 0.5 units) were measured using a Zetasizer Nano ZS (Malvern Instruments Inc., Southborough, MA) at room temperature. Emulsions were diluted 1:1000 with sodium acetate buffer (pH 3.0). The samples were filled in DTS-1070 clear disposable zeta cells and the hydrodynamic radius and zeta potential changes at each point of pH was measured when titrated using an autotitrator (MPT-2, Malvern Instruments Inc., Southborough, MA). The titrants used were 0.01 N HCl, 0.01 N NaOH and 0.1 N NaOH.
3.4 Digestion

3.4.1 In vitro lipid digestion model for rate of lipolysis

Pepsin from porcine gastric mucosa (1064 units/mg), pancreatin from porcine pancreas (4xUSP, contains amylase, lipase, trypsin, chymotrypsin, and ribonuclease), porcine bile extract (contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts), Trizma® (tris maleate salt), PLA₂, analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma–Aldrich Chemical Co (MO, USA). Alcolec® PC 75 (fat-free soybean phospholipids with 70% phosphatidyl choline) was purchased from American lecithin company, Connecticut, USA.

A pH stat digestion model was used to measure the rate of lipolysis from the amount of free fatty acid released (Tzoumaki et al., 2013, Pool et al., 2013, Malaki Nik et al., 2010). The emulsions were diluted with sodium acetate buffer (10mM, pH 3) to a final oil concentration of 0.5% w/w. Simulated gastric and intestinal fluids were prepared as described previously (Malaki Nik et al., 2010). Diluted samples (10 mL) were mixed with 15 mL simulated gastric fluid (SGF) with a pepsin content of 3.2 mg/mL (1064 units/mg) of the whole digestion volume in a heated jacketed reaction vessel of the pH-stat auto-titration unit (Titrando 902, Metrohm, USA Inc) under constant stirring for the gastric phase of digestion. At the end of 30 min of gastric digestion, bile extract (20 mg/mL of whole digestion volume) and CaCl₂ (0.11 g/mL of whole digestion volume) dissolved in Trizma® buffer (2mM Tris maleate containing 150mM NaCl) was added to the digestion vessel. Pancreatin (2.4 mg/mL) was added to the digestion mixture after raising the pH to 7.0 for optimal enzyme activity (McClements & Li, 2010). The amount of free fatty acids (FFA) released was calculated from the volume of NaOH used to neutralize the FFA formed during digestion. The amount of NaOH consumed from the automatic burette was recorded using the equipment software (Tiamo® 2.0, Metrohm).
The percent of FFA released was calculated from the formula:

$$\%\, FFA = 100 \left( \frac{V_{NaOH} \times M_{NaOH} \times M_w}{W \times 2} \right)$$

Eq. 3.1

where $V_{NaOH}$ is the volume of NaOH used (mL), $M_{NaOH}$ the molarity of NaOH, $M_w$ the molecular weight of soy oil and $W$ the weight of lipid (g) in the digestion sample (Tzoumaki et al., 2013). The average molecular weight of soybean oil was 874.63 g/mol (Shrestha & Gerpen, 2010).

### 3.4.2 Cytotoxicity study

A shaking jar digestion model was used for cytotoxicity studies as the emulsion samples could be used without dilution and sample collection for gastric phase was possible without affecting the digestion volume. The stock simulated gastric and intestinal fluids were prepared at 1.25 x concentration (4 parts of stock electrolyte to 1 part of water) to allow for correct electrolyte concentration in the final digestion mixture while making pH adjustments and addition of enzyme solutions as described in literature (Minekus et al., 2014).

Aliquots (10 mL) of emulsions were mixed with 10 mL of SGF (6.4 mL stock SGF + 1.6 mL pepsin (concentration of 2000 U/mL of the final mixture) +5 µL CaCl$_2$ (0.3mM) +1.995 mL water) in dark glass bottles and incubated at 37°C for 30 min in a shaking water bath (VWR International, USA) at 4 Hz. The pH of the mixture was found to be around 3.7 - 3.8, so no further adjustments were carried out. At the end of gastric digestion, 20 mL of simulated intestinal fluid (SIF) (11 mL stock SIF + 5 mL pancreatin solution (40 mg/mL) +1.305 mL water + 40 µL CaCl$_2$ (0.3 mM) + 2.5 mL bile salt solution (0.4 mM) +0.03104 g phospholipids (1 mM) + 5 µg of PLA$_2$ (903 U/mg) + 150 µL NaOH) was added to the gastric digest and the digestion was allowed to proceed for another 120 min. Separate bottles were used to obtain the gastric and
intestinal digests for SDS-PAGE analysis. The proteolysis in the gastric digest was stopped by raising the pH to 6.5 with 1 M NaHCO₃ (Malaki Nik et al., 2010).

Sulforhodamine B (SRB) assay was used to evaluate the effect of emulsion samples before and after digestion on the proliferation rate of Caco-2 cancer cells (Vichai & Kirtikara 2006). Caco-2 cells at a concentration of 4x10³ cells/well were seeded on 96 well plates (Fisher Scientific, Mississauga, Ontario, Canada) and allowed to adhere for 24 h. Afterwards, freshly prepared samples of ChN emulsion, were added to the cells in a final dilution 1:5.6 in medium (sample: media) (v/v) and incubated for 24 h. Control samples (cell with media only) along with blank samples (media only) were also tested. The optical density of plates was measured using an automated 96-well plate reader (Multi detector Microplate Reader, Biotek Synergy HT Model, Vermont, USA) at a wavelength of 570 nm. Results were expressed as percentage proliferation with respect to control wells grown under regular conditions.

3.5 SDS-PAGE electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) separates proteins in a sample based on their molecular weight and surface charge. In this study SDS-PAGE was utilized to study the presence of β-Lg at the interface of ChN stabilized emulsions and also the hydrolysis behaviour of proteins at the interface during digestion.

Samples of ChN stabilized emulsions mixed with β-Lg solution, before and after digestion, were subjected to SDS–PAGE analysis under reducing conditions as adapted from the literature (Malaki Nik et al., 2010). A vertical slab gel of 1.5 mm thickness with 15% acrylamide running gel and 4% stacking gel at a constant voltage of 175 V was used in a Bio-Rad mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, CA).

ChN emulsions mixed with 1% β-Lg solution(1:1) and the digested aliquots were centrifuged at 13,000 g for 30 min (Eppendorf centrifuge- 5415D, Hamburg, Germany) to separate the cream
and serum phases. 100 µL samples of both cream and serum phases and whole mixture as such were mixed with 400 µL of electrophoresis sample buffer (125 mM Tris–HCl, 5 M Urea, 1% SDS, 20% glycerol and 1% bromophenol blue). Aliquots from after digestion samples were mixed in the ratio of 200 µL to 300 µL of electrophoresis buffer. The solutions were heated at 95°C for 5 min and centrifuged at 5000 g for 4 min using the Eppendorf centrifuge. Samples (10 µL) were then loaded onto the gels. Pure β-Lg solution was used as the standard.

At the end of each run, the gels were stained using Coomassie blue R-250 for 30 min followed by destaining with a destaining solution composed of 45% milliQ water, 45% methanol and 10% acetic acid, for 2 h. The destaining step was done twice and the gels were left overnight in a destaining solution containing 22.5% methanol and 5% acetic acid. Gels were scanned using a scanner (Bio Rad Gel DocTM Ez Imager, ON, Canada) and the bands were analyzed using image analysis software (ImageMaster 1D, Version 2.0, Amersham Biosciences).

3.6 Statistical analysis

All measurements were carried out in triplicate and presented as averages. Statistical analysis was carried out using Minitab Express software (State College, PA, USA) and Microsoft Excel 2013 (Redmond, WA, USA). The differences between means were determined using Tukey’s multiple comparison test. Differences between means at p< 0.05 are considered to be significant.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Morphology of Chitin nanocrystals

Aqueous ChN dispersions at pH 3.0 were analyzed using TEM, to observe the morphology of the chitin nanocrystals, as shown in Figure 4.1. The images show the presence of rod shaped chitin fragments of varying lengths between 200-500 nm. Chitin nanocrystal length varies with the origin of chitin and the extent of hydrolysis (Zeng et al., 2012); the size of the nanocrystals in this study was in line with previous reports. For example, Goodrich & Winter (2007) studied chitin nanocrystals prepared from shrimp shells and reported lateral dimensions ranging from 200-400 nm. Average lengths of 240 nm have also been reported by Tzoumaki et al. (2010) in chitin nanocrystals from crab shells.

TEM images clearly showed that the crystals were mostly present in aggregated form. The rod-like chitin nanocrystals tend to align themselves along their longitudinal axis to form bundles via van der Waals and electrostatic interactions, and have been shown to be capable of forming stable gelled networks in aqueous conditions (Tzoumaki et al. 2010).

4.2 Characterisation of ChN stabilized emulsions

4.2.1 Microstructure of emulsion droplets.

Oil-in-water emulsions were prepared using 5% oil (w/w) and sodium acetate buffer (pH 3.0, 10 mM) with varying concentrations of ChN [0.5, 1.0 and 2.0% (w/w)]. The emulsion droplets were observed using Cryo SEM, as shown in Figure 4.2.
Figure 4.1: TEM images of chitin nanocrystals (bar size-1 µm). (A) Diluted 10 times and (B) Diluted 100 times with 10 mM sodium acetate buffer (pH 3).
**Figure 4.2:** Representative images obtained using Cryo-SEM of 5% oil-in-water emulsions prepared with (A) 0.5%, (B) 1%, (C) 2% chitin nanocrystals.
Microstructural observations confirmed the presence of ChN nanocrystals at the oil-water interface. Emulsions prepared with 0.5% ChN (Fig. 4.2A) showed extensive aggregation. At higher concentrations of ChN (1 and 2% of ChN) (Fig. 4.2B and C), the emulsion droplets maintained their identity and showed diameters < 1 µm. Results would suggest that 1% ChN was sufficient in stabilizing the oil droplets. At the highest concentration tested (2% ChN) the emulsion droplets appeared to be embedded in a ChN network, indicating an excess of ChN in the continuous phase. These observations were in line with previous observations of continuous networks formed by ChN nanocrystals when present in sufficient concentrations (Tzoumaki et al., 2011).

4.2.2 Creaming stability of emulsions on storage

The emulsions were stored at 4°C and visually monitored over time to determine their creaming stability. Figure 4.3 illustrates the appearance of emulsions made with 0.5, 1.0 and 2% ChN concentration at pH 3, immediately after preparation, as well as after 15 days and one month of storage at 4°C. The emulsions made with 1 and 2% ChN concentration did not show any creaming even after one month of storage whereas 0.5% ChN emulsions showed serum separation after a few days. These results confirmed the microscopy observations (Figure 4.2), which showed larger oil droplets at 0.5% ChN compared to those prepared with a higher concentration of nanocrystals. Earlier studies have suggested that the increase in the creaming stability of emulsions containing higher concentration of ChN may be due to the high viscosity of the continuous phase (Zinoviadou et al., 2012).

In a similar study conducted on oil-in–water emulsions stabilized by kaolinite clay particles, it was demonstrated that the creaming velocity decreases rapidly with increasing clay concentration (Yan & Masliyah, 1997). The presence of crystals in the continuous phase creates a network which entraps the oil droplets and would allow for an improved stability to creaming of these emulsions.
Figure 4.3: Effect of increasing concentration of ChN [A (0.5%), B (1.0%) and C (2.0 %) w/w] on the stability of 5% oil in sodium acetate buffer emulsion at pH 3 during storage at 4°C.
The present results are in agreement with data reported by Touzmaki et al. (2011). Also in this study, emulsions stabilized with 1% ChN concentration did not exhibit creaming. It is clear that when the concentration of ChN is insufficient to cover the interface, aggregation will occur, with visible separation after a few days. Bridging flocculation and extensive coalescence were observed under these conditions, as clearly indicated by the cryo-SEM images (Figure 4.2A).

### 4.2.3 Emulsion droplet size distribution as studied using static light scattering

The size distribution of the emulsion droplets was measured using static light scattering. The average droplet size (measured as $D_{4,3}$) as a function of time during storage at 4°C and pH 3.0 is shown in Figure 4.4. The average size decreased with concentration of ChN. At the lowest concentration of ChN used (0.5%), the diameter of the droplets was around 3.0 µm and at the highest concentration of ChN (2.0%) it was less than 1 µm, confirming Cryo-SEM observations (Figure 4.2). It is also important to note that the average droplet size decreased with increasing concentration of ChN, indicating that while 1% was already sufficient to stabilize the emulsion, the amount of ChN present was still limiting at this concentration.

The droplet sizes did not significantly increase during the 30 day storage period. The ChN nanocrystals adsorbed at the interface, and formed a strong interface which resisted coalescence. Creaming did occur at the lowest concentration of ChN, but it was reversible upon mixing. It was concluded that the emulsions were stabilized against creaming by the presence of excess ChN in the dispersed phase, forming a highly viscous continuous phase, or a nanocrystal network.

A previous study (Touzmaki et al., 2011) also demonstrated that the average diameter of ChN stabilized oil-in-water emulsions decreases with increasing concentration of ChN; however, in this case, at concentrations > 0.7% ChN, the size of the droplets was found to increase. The
Figure 4.4: Mean droplet size diameter ($D_{4.3}$) of ChN stabilized emulsions made with different concentrations of ChN and measured over a 30 day period from the day of preparation (day 0). Superscript letters indicate mean diameters are not significantly different ($p < 0.05$).
increase in size was attributed to an increase in the viscosity of the continuous phase during emulsification leading to slower adsorption kinetics. In the present work, up to 2% ChN, there was a decrease in size with increasing concentration of nanocrystals.

The difference in the processing history of the emulsions is likely the cause of the discrepancy between the present results and those reported in the literature. In the present research, high pressure homogenisation was employed (using 6 passes at 500 bar), while earlier work used ultrasonic homogenisation (Touzmaki et al., 2011). This may explain the continuous reduction in size, as well as the lack of aggregation at 2% ChN. As previously mentioned, the results showed in Figures 4.3 and 4.4 clearly demonstrated that higher concentrations of ChN, allowed for more nanocrystals to adsorb at the interface, further decreasing the size of the droplets, but that the high viscosity of the continuous phase was responsible for limiting creaming of the emulsions.

Researchers working on silica particle-stabilized emulsions have reported a decrease in droplet sizes with an increase in particle concentration until a plateau is reached, after which further reduction in size does not occur (Chevalier et al., 2010). It has also been reported in the literature that the plateau in droplet size reduction observed could be changed with changes in the emulsification process, because of further disruption for the size of the particles aggregates, allowing for better adsorption at the interface. Furthermore, at high levels of particle concentration a network can form in the continuous phase embedding the oil droplets (Midmore, 1998).

4.3 Effect of addition of β-lactoglobulin to ChN stabilized emulsions

4.3.1 Droplet size distribution

To be able to employ ChN stabilized emulsions in food, nutraceutical and cosmetic applications, it is important to understand how these emulsions, stabilized by a Pickering stabilization mechanism, would behave in the presence of other surface active ingredients. In this research,
ChN stabilized emulsions (with 0.5, 1.0 and 2.0% ChN) were prepared and subsequently mixed with a 1% β-lactoglobulin (β-Lg) solution, in a 1:1 ratio (volume of solution). This mixture was allowed to stand for 5 minutes under constant stirring. Figure 4.5 illustrates the particle size distribution of ChN stabilized droplets before and after addition of β-Lg. At pH 3.0 both ChN and β-Lg are positively charged, minimizing electrostatic attraction forces. In general, the addition of β-Lg did not cause changes in the particle size distribution of the emulsions, and there was no significant change (p<0.05) in the corresponding volume weighted mean diameters (D_{4,3}). SDS-PAGE electrophoresis of the cream phase demonstrated that the protein was present at the interface (data not shown). Although because of charge repulsion, the adsorption of β-Lg was not expected.

It has been previously demonstrated that when chitosan is added to a β-Lg stabilized emulsions at pH ≤ 4.5 or pH ≥ 6.0 there is no droplet aggregation. In this case, the stability of the emulsion droplets was also attributed to charge repulsion (Hong & McClements, 2007). In addition, it has been reported that whey proteins may interact with chitin nanocrystals at acidic pH, and the mixtures exhibit a gel like behaviour (Touzmaki et al., 2010). However, the gel-like behaviour was attributed to structuring of the polysaccharide due to the thermodynamic incompatibility of the polymers. The present work would suggest that at acidic pH, in spite of the positive charge on both polymers, there was adsorption of β-Lg at the interface. This would also confirm the results published by drop tensiometry on a similar system (Gülseren & Corredig, 2013).

**4.3.2 Emulsion stability as a function of pH**

To determine the effect of pH on the stability of the emulsions in the presence of protein, emulsions stabilized with 1% ChN were tested with and without the addition of β-Lg. The apparent size of the emulsion droplets and the zeta potential were determined as a function of pH using dynamic light scattering (Figure 4.6).
Figure 4.5 Droplet size distribution of emulsions (A-0.5% ChN, B-1.0% ChN, C-2.0% ChN) with (filled symbols) and without β-Lg (empty symbols). Representative runs.
Figure 4.6: Changes in hydrodynamic diameter measured with DLS (A) and zeta potential (B) as a function of pH for 1% ChN emulsion with (filled symbols) and without (empty symbols) β-Lg. Data are the average of three independent measurements with bars representing standard deviation.
The average hydrodynamic diameter remained constant until pH 4.2 for ChN emulsions without β-Lg. At pH 5.0 there was visual aggregation of the emulsions, and it was reflected in a sudden decrease in the average size, due to precipitation. At this pH, ChN particles are close to their isoelectric point and electrostatic repulsion between the droplets is minimized. The pKₐ values for chitin is around 6.3 (Revol & Marchessault, 1993).

Destabilization occurred at a pH close to 5.0, where the zeta potential of the emulsion droplets was close to 0, as shown in Figure 4.6B. In the presence of β-Lg, there was a different behaviour. At pH >4.5, the emulsion droplets showed an increase in size, caused by bridging between the droplets. The zeta potential values were also significantly different (p < 0.05) in the presence of β-Lg mixture. For example, at pH 5.0, emulsions containing β-Lg still showed a positive charge, improving the stability of the emulsion at that pH compared to the emulsions stabilized with ChN alone. This difference supported the conclusion that β-Lg was also present at the interface. Complexes between the nanocrystals and β-Lg may form at the interface at pH of about 5.0. Previous studies indeed reported that insoluble complexes form between chitosan and β-Lg in aqueous solutions at a pH of above 5.0 (Guzey & McClements, 2006).

4.4 In vitro lipid digestion of ChN stabilized emulsions

Oil in water emulsions stabilized with 1% ChN and with or without 1% β-Lg were subjected to in vitro digestion. It was hypothesized that the presence of proteins at the interface would affect the accessibility of lipase to the interface. The extent of lipolysis was estimated by measuring the free fatty acids released, as previously described in the literature (Tzoumaki et al., 2013). During lipid digestion, lipases break down triglycerides into easily absorbable fatty acids and glycerols. Interfacial composition is a major factor affecting lipase action (Reis et al., 2009). Bile salts play a major role in lipolysis, by displacing adsorbed material and products of lipolysis from the oil droplet surface (Maldonado-Valderrama, et.al, 2011). Calcium ions too play a similar role in lipid digestion by forming insoluble complexes with the free fatty acids formed and facilitating
their removal from the oil droplet surface. The time dependent release of free fatty acids from the emulsions stabilized with 1% ChN is summarized in Figure 4.7. Emulsions containing 1% ChN emulsions and those mixed with 1% β-Lg were also subjected to a 30 min gastric digestion before intestinal digestion to account for the additional protein presence in the sample.

Lipolysis proceeded very rapidly in the first 15 min of digestion, reaching a plateau at the end of the 2 h reaction. The presence of β-Lg in the emulsion stabilized with ChN did not significantly (p <0.05) affect the digestion behaviour in terms of % FFA released (Table 4.1). The initial increase in rate of FFA release and the subsequent plateauing off was attributed to the increasing hindrance to the access of pancreatic lipase to the oil droplet surface. The initial presence of high concentration of calcium ions causes significant increase in the rate of lipolysis during the initial digestion period as calcium ions reduce charge repulsion between lipase and the surface of the emulsion droplet. As digestion progressed there was an accumulation of lipolysis products (e.g fatty acids and monoglycerides) on the droplet surface, and these products are known to inhibits lipase activity (Ye et al., 2013).

Another reason for the decreased digestion could be the change in surface charge of ChN particles from positive to negative under intestinal pH conditions. The $pK_a$ for ChN is reported to be around 6.3 (Revol & Marchessault, 1993). The change in charge density promotes phase transition from isotropic to nematic and formation of a gel network structure as suggested by earlier researchers (Touzmaki et al., 2010, Revol & Marchessault, 1993). Such a strong network formation by ChN (see for example, Figure 2.5), may lead to slower diffusion rates of the lipolysis products, bile salts and lipase.
Figure 4.7 Time dependent free fatty acid (% FFA) release from 1% ChN stabilized emulsion alone and mixed with 1% β-Lg solution (oil concentration of 0.5%(w/w), pH 7 at 37°C).
Table 4.1 Free fatty acids(% FFA) released after 2 hr intestinal digestion at 37°C at pH 7 from 1% ChN stabilized emulsion alone and mixed with 1% β-Lg solution (oil concentration of 0.5%(w/w)) [ same superscript letters indicate no significant difference at p< 0.05 ].

<table>
<thead>
<tr>
<th>Digestion sample</th>
<th>% FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% ChN emulsion</td>
<td>11.78 ± 1.11a</td>
</tr>
<tr>
<td>1% ChN emulsion + 1% β-Lg solution</td>
<td>13.08 ± 0.75a</td>
</tr>
</tbody>
</table>
Another reason for the reduced rate of lipolysis could be the low extent of ChN displacement from the interface by bile salts when compared to milk proteins, as these nanocrystals stabilize the emulsions by Pickering stabilization, and desorption would be unfavourable.

Comparative studies on emulsions stabilized with ChN, sodium caseinate and whey protein isolate (0.5% w/w) have shown that % FFA released from protein stabilized emulsions were more than twice that was released from ChN stabilized emulsions (Touzmaki et.al, 2013). This clearly demonstrates that ChN resists lipolysis when present at the interface. The inhibitory effect of chitin and chitosan on pancreatic lipase has also been established in high fat diet fed rats (Han et al., 1999).

It is interesting to note that in the present study, with a 1% ChN emulsion, the final amount of % FFA detected was around 12% whereas 0.5% ChN stabilized emulsions were reported to have around 33% FFA after 1 h intestinal digestion (Touzmaki et al., 2010). Although the conditions of digestion had some differences, it may be possible to hypothesize that a higher concentration of ChN leads to greater reduction in rate of lipolysis. This could be due to the higher amount of particles providing better coverage to the droplets, even though smaller droplets at higher ChN concentration leads to increased surface area available for lipase action. Li and McClements (2010) in their study on the effect of droplet characteristics on in vitro digestibility of β-Lg stabilized emulsions, reported that the rate of FFA release was appreciably higher for emulsions with smaller droplet size. The results of this study indicates that it might not be the case with solid particle stabilized emulsions.

4.4.1 Size of lipid droplets before and after digestion

Emulsions with ChN 1% concentration and those mixed with 1% β-Lg were measured before and at the end of gastric and intestinal phases of digestion to understand the impact of lipid digestion on the droplet size distribution. The mean volume diameter expressed as $D_{4,3}$ is
summarized in Figure 4.8. It is clear from the graph that particle sizes increased as digestion progressed with the intestinal digestate having an average diameter around 20-30 µm. These results are quite different from what is observed in the case of emulsions stabilized with commonly used emulsifiers like Tween 20, lysolecithin, caseinate or whey protein. In those emulsions considerable reduction in droplet size occurs when they move from gastric to intestinal phase. This reduction in size is attributed to the digestion of emulsified lipids by lipase and the incorporation of digested products within micelles and vesicles (McClements et.al, 2009).

The different behaviour of droplet size observed in the case of ChN stabilized emulsion can be explained by the decreased rate of lipolysis observed in particle stabilized emulsions. The type of emulsifier used affects the susceptibility of lipid droplets to coalescence and break up during digestion thereby altering the extent of surface area exposed to lipase action. Numerous studies have established the influence of composition (molecular structure of lipids and droplet size) and structure of interfacial layer (type of emulsifier like protein, lecithin, Tween 20) on the extent of lipid digestion (Armand, 2007, Fave et al., 2004, Mun et al., 2007).

The increase in droplet size of ChN emulsions at the end of intestinal phase may be attributed to the increased flocculation of ChN covered droplets at pH 7.0. ChN emulsions and mixtures with β-Lg undergo a change in surface charge from positive to negative values at a pH above their isoelectric points. This lead to increased aggregation of ChN particles. Since the displacement of ChN from the droplet interface is an energy intensive process, coalescence of droplets may not occur in particle stabilized emulsions.

Lipid digestion does not proceed rapidly as the access for lipase to the interface is prevented by the presence of ChN and also the entrapment of digestion products and bile salts in the network created by ChN in the continuous phase at higher pH.
Figure 4.8 Mean droplet diameter ($D_{4,3}$ µm) of 1% ChN stabilized emulsions alone and mixed with 1% β-Lg solution (1:1) before and at the end of gastric and intestinal digestion.
The increase in $D_{4,3}$ observed at the end of intestinal digestion therefore could be due to flocculated oil droplets that had not undergone total lipolysis. The addition of $\beta$-Lg solution did not show any significant change in the pattern of digestion or the droplet size distribution. Since $\beta$-Lg is added to an emulsion already stabilized with ChN, it does not form the primary interfacial layer.

In a similar study on *in vitro* digestion of chitin nanocrystal stabilized oil-in-water emulsions, it was observed that the increase in droplet size after digestion was significantly lower in ChN stabilized emulsions when compared to sodium caseinate and whey protein isolate stabilized emulsions. In protein stabilized emulsions, extensive droplet coalescence occurs, and proteins are easily displaced by bile salts and lipase components that promote the action of lipases at the interface (Touzmaki et al., 2013).

### 4.5 SDS-PAGE behaviour of ChN stabilized emulsions mixed with $\beta$-Lg before and after digestion.

ChN emulsions made with 1% ChN and mixed with 1% $\beta$-Lg solution (at a ratio of 1:1) were analyzed by SDS-PAGE to further determine if the protein was present at the interface at pH 3.0, when both polysaccharide and the protein are positively charged. Figure 4.9 shows the electrophoretic migration of emulsions containing ChN and $\beta$-Lg, as well as the corresponding serum and cream phase before and after gastric digestion. It is clear from SDS-PAGE results that, there was substantial adsorption of $\beta$-Lg on the surface of the emulsion droplets (Lane 2, Figure 4.9). Gülseren & Corredig, 2013 in their study on the interactions of chitin nanocrystals with $\beta$-lactoglobulin at the oil-water interface found that the films containing ChN and 0.1% $\beta$-Lg, showed a comparable behaviour with that of the protein alone; but the film elasticity was found to be different, leading to the conclusion that ChN and $\beta$-Lg co-existed at the interface.
Figure 4.9 SDS-PAGE electrophoresis of 1% ChN emulsion mixed with β-Lg and its gastric digest (Lane 1 - emulsion with β-Lg, Lanes 2 & 5 cream phase of emulsion mixture and gastric digest, Lanes 3 & 6 serum phase of emulsion mixture and gastric digest, Lane 4 - gastric digest of the emulsion mixture, Lane 7 - pure β-Lg solution 1%).
SDS-PAGE (Figure 4.9, Lanes 4, 5 & 6) also showed that β-Lg was still present even after gastric digestion. The three dimensional structure of β-Lg consisting of an eight stranded antiparallel β-hydrophobic barrel unit with α helix is responsible for its resistance towards digestive enzymes in the gastrointestinal tract in its native state (Reddy et al., 1988, Schmidt & Poll, 1991). However, it was shown that β-Lg present at the interface of oil droplets undergoes hydrolysis by pepsin due to the change in conformation that leads to unfolding of the protein exposing the peptic cleavage sites making it susceptible to hydrolysis (Sarkar et al., 2009).

From the results shown in Figure 4.9, it could be assumed that ChN present at the interface provided protection to β-Lg from pepsin hydrolysis. Both in before and after digestion samples the amount of β-Lg in the interface is higher compared to that present in the continuous phase.

The presence of β-Lg at the interface is consistent with the theory that in Pickering stabilized systems, the solid particles do not need to completely cover the interface for emulsions to be stable. Kalashnikova et.al, 2013 had reported that at 44% dispersed phase coverage, cellulose nanocrystals from green algae were able to stabilize an oil-in-water emulsion with an oil/water ratio of 30/70. Similarly Capron & Cathala, 2013 showed that cellulose nanocrystals can form gel-like high internal phase oil-in-water emulsions with more than 90% of hydrophobic phase stabilized with less than 0.1% wt of cellulose nanocrystals. It was concluded that the presence of rigid fibril shaped chitin nanocrystals prevented complete coverage of the interfacial surface between the oil droplets and the continuous phase, providing enough opportunity for a surface active protein like β-Lg to access the interface.

4.6 Cytotoxicity studies

The safety of nanocrystals from natural sources is an important issue in the development and use of polysaccharide nanocrystals in a food matrix. Earlier studies have suggested that polysaccharide nanocrystals affect cell activity or proliferation; hence their penetration into the
cell membrane and the viability of the cells are aspects that need to be emphasized while utilizing such nanocrystals (Lin, et al., 2012). A cell proliferation assay was used to determine cytotoxicity of the ChN dispersions and emulsions on Caco-2 cancer cells. For the purpose of comparison a 1% β-Lg emulsion (with 1% β-Lg (w/w) in place of ChN) was prepared for this experiment.

Figure 4.10 compares the proliferation of cells exposed to various dilutions of a 1% ChN dispersion, 1% ChN emulsion, 1% ChN emulsion mixed with 1% β-Lg solution (1:1) and 1% β-Lg emulsion at 2 and 24 h of incubation. Control wells contained only medium and were used as a comparison for full cell proliferation. ChN stabilized emulsions showed a different trend from ChN dispersion with very low cell viability at lower dilutions and improved viability at higher dilution. However, this trend was not only found for ChN emulsions, but also for 1% β-Lg emulsions and 1% ChN emulsions mixed with β-Lg.

Figure 4.11 depicts the % cell proliferation for digested emulsions. ChN dispersions after digestion appeared to promote proliferation of cells at 2 h of incubation even at the lowest dilution tested. Digestion did not significantly change the pattern of cell proliferation in the case of emulsions tested except for 1% ChN emulsions mixed with β-Lg showing slight improvement at lower dilutions too.

The results of this study (Figures 4.10 and 4.11) demonstrated that ChN dispersions do not have a detrimental effect on the proliferation of cells. To further understand the behaviour of cell proliferation observed with emulsions we did digestion of an oil-water mixture (5% w/w soy oil) with and without enzymes and the cells were exposed to the digestion end products for 2 and 24 hrs. It was observed that the digestion end products with enzymes showed very low proliferation rates at the lower rates of dilution whereas those without enzymes (only buffer solutions) appeared to promote cell proliferation even at low dilution rates.
Figure 4.10 Cell viability (%) of Caco-2 cells after 2 hrs (A) and 24 hrs (B) incubation of 1% ChN dispersion (1% ChN D), 1% ChN emulsion(1% ChN E), 1% ChN with 1% β-Lg(1% ChN + B-Lg) and 1% β-Lg emulsion(1% B-Lg E) before digestion. Control wells contain only medium and considered to have 100% proliferation.
Figure 4.11 Cell viability (%) of Caco-2 cells after 2 hrs (A) and 24 hrs (B) incubation of 1% ChN dispersion (1% ChN D), 1% ChN emulsion(1% ChN E), 1% ChN with 1% β-Lg(1% ChN + B-Lg) after digestion.
The lower viability showed by all emulsion samples compared to dispersions clearly suggested that lipids and their digestion end products affect the viability of the culture cells, and not ChN.
CHAPTER 5
CONCLUSIONS

Emulsions stabilized by particles are considered a separate class of emulsions, and have been defined first by Pickering and Ramsden at the beginning of the 1900’s. There are a very few studies on the applications of Pickering emulsions in foods, due to the challenges in making food grade nanoparticles that are effective and safe for use. The surfactant free nature and exceptional stability of Pickering emulsions, however, make them very attractive for use in food.

In real life food systems particle stabilized emulsions will exist in an environment containing other surface active molecules; hence, it is important to understand the interaction mechanisms among such species and particles at the interface.

This study improved the understanding of the behaviour of chitin nanocrystal stabilized emulsions in the presence of a protein commonly used as emulsifier, β-Lg. An initial characterisation of ChN stabilized emulsions indicated that the emulsions prepared with ChN were in full agreement with published literature reports. The presence of β-Lg in ChN emulsions at all levels of ChN tested did not cause any significant change in the droplet size distribution, suggesting that there was no destabilization by bridging, or significant displacement of the particles by protein. SDS-PAGE analysis confirmed that at low pH, both protein and nanocrystals were present at the interface, in spite of their positive charge. At a pH of about 5, aggregation of the ChN stabilized emulsions occurred in the presence of protein. In the absence of β-Lg, the emulsions showed 0 charge at pH of about 5, and phase separation.

Digestion studies on ChN stabilized emulsions demonstrated that increasing the ChN concentration in the emulsions decreased free fatty release during in vitro lipolysis. This decrease in lipolysis observed may suggest that the use of chitin nanoparticles presents an opportunity for the design of encapsulated bioactives with targeted release and structured
emulsions with controlled lipid digestibility. The presence of protein did not significantly affect the percent of free fatty released indicating that the interfacial protection provided by the nanocrystals was not altered.

SDS-PAGE results confirmed the presence of β-Lg at the interface even when added to a previously particle covered interface at a pH where both ChN and protein carry the same charge. Also analysis of the protein adsorbed at the interface after in vitro digestion demonstrated that the presence of ChN offered some protection to β-Lg at the interface against hydrolysis by pepsin.

This study for the first time also looked at the cytotoxicity of ChN stabilized emulsions and their digestion end products on intestinal cell cultures. Earlier studies had only looked into the cytotoxicity of chitin, chitosan or chitin nanocrystal solutions. The cell proliferation data indicates that ChN dispersions promote proliferation by enriching the media. The lower viability observed with ChN emulsions can be attributed to the presence of lipids and its digestion end products and not due to the chitin nanocrystals.

This work demonstrated the potential for the use of chitin nanocrystals in encapsulation systems that require some protection during digestion. However, it remains clear that for a successful utilization of ChN stabilized pickering emulsions in a food system it is important to better understand the interactions occurring between the emulsion droplets and the rest of the components present in the system.
REFERENCES


