STABILITY, RHEOLOGY AND THE EFFECT OF VARIOUS ENVIRONMENTAL FACTORS ON THE FORMATION OF FOOD PROTEIN-STABILIZED NANOGELS FROM NANOEMULSIONS

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ABSTRACT

Nanoemulsions (NEs) typically have average droplet size less than 200 nm which provides them many characteristics advantages over conventional emulsions. NEs have higher stability and form nanogels at a lower oil volume fraction than conventional emulsions. However, so far, all nanogels were formed with small molecular weight surfactants, such as sodium dodecyl sulfate (SDS). The primary objective of this thesis was to achieve gelation in oil-in-water (O/W) NEs stabilized with sodium caseinate (SC) and whey protein isolate (WPI) and to investigate their gelation behavior as influenced by long-term storage, addition of salt, change in pH and heat treatment.

SC and WPI-stabilized NEs with different protein concentration (2 - 5 wt%) and different oil concentrations (30 & 40 wt%) were prepared by multiple cycles of high-pressure homogenization. Only SC-stabilized NEs (SC NEs) formed strong elastic gel at 5% protein and 40% oil. All the other SC NEs at 40% oil showed weak gel behavior while no gelation behavior was observed for any of the WPI-stabilized NEs (WPI NEs). The droplet interaction potential calculation indicated that the longer steric layer of SC, in combination with the strong electrostatic barrier provided a thicker interfacial layer in SC-stabilized droplets compared to WPI. This increase in interfacial layer thickness led to an increase in effective volume fraction ($\phi_{\text{eff}}$) towards ~0.7 for SC NEs, which caused a close packing of droplets leading to repulsive gelation in SC NEs. All the NEs showed stability in droplet size, while their viscosity and gel strength remained unchanged over a period of 3 months.

Next, selected NEs, which showed weak to no gelation behavior (2 & 4% SC or WPI and 30 & 40% oil) were further investigated by the addition of salt, change in pH and heat-treatment to induce attractive gelation. WPI NEs showed gelation upon addition of salt due to a screening of charge which led to attractive interaction between the droplets. However, SC NEs did not show any gelation due to their longer hydrophilic tail which provided a strong steric barrier against salt-induced attractive interaction. NEs with both proteins showed gelation at pH near the isoelectric point which was contributed to the charge neutralization. Heat-treatment did not cause any gelation in any of the NEs, despite WPI being a heat-labile protein. This was attributed to the lack of protein in the continuous phase to cause any heat-induced gelation.
Overall, the study showed that it is possible to develop repulsive gels from SC NEs due to its high interfacial layer thickness and smaller droplet size even at an oil volume fraction of 0.4, which was much lower compared to the oil volume fraction of greater than 0.64 for conventional emulsion. Addition of salt or change in pH towards the protein’s isoelectric point may lead to attractive gelation in certain NEs, provided the interfacial steric barrier prevent close interaction of the droplets. However, heat-treatment could not induce gelation in the NEs due to a lack of protein in the continuous phase.
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DEDICATION

To,

My beloved parents

Mr. Dilipkumar Shankarlal Patel and Mrs. Sunita Dilipkumar Patel
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LIST OF ABBREVIATIONS AND SYMBOLS

\( a \)  
Radius of the droplets

\( A_s \)  
Total interfacial area

BLG  
\( \beta \)-lactoglobulin

BSA  
Bovine serum albumin

\( C_{\text{excess}} \)  
Concentration of excess protein

\( d_{32} \)  
Volume surface mean droplet diameter

\( d_{43} \)  
Surface average droplet diameter

DLVO  
Derjaguin, Landau, Verwey and Overweek

\( e \)  
Charge of an electron

\( G'' \)  
Loss modulus

\( G' \)  
Storage modulus

\( k_B \)  
Boltzmann constant

\( L \)  
depleting protein species size

LVR  
Linear viscoelastic region

NE  
Nanoemulsion

O/W  
Oil-in-water emulsion

pI  
Isoelectric point

PIC  
Phase inversion composition

PIT  
Phase inversion temperature

\( P_L \)  
Laplace pressure

\( r_{sm} \)  
Size of sub-micelle

\( r_{\text{eff}} \)  
Effective droplet radius

SC  
Sodium caseinate

SC NE  
Sodium caseinate-stabilized nanoemulsion

SDS  
Sodium dodecyl sulphate

SEM  
Scanning electron microscopy

\( T \)  
Absolute temperature

TEM  
Transmission electron microscopy
W/O Water-in-oil emulsions
$W_{dep}$ Depletion attraction forces
$W_{ele}$ Electrostatic repulsion forces
WPC Whey protein concentrate
WPI NE Whey protein isolate-stabilized nanoemulsion
WPI Whey protein isolate
$W_{vdw}$ van der Waals attraction forces
$z_i$ Valency of ion $i$
$\delta$ Thickness of repulsive barrier
$\Delta$ Thickness of the steric layer
$\varepsilon_0$ Permittivity of the air
$\varepsilon$ Permittivity of the medium
$\phi$ Volume fraction
$\phi_{core}$ Initial volume fraction
$\phi_{eff}$ Effective volume fraction
$\phi_{MRJ}$ Volume fraction for Maximum random jamming
$\gamma$ Interfacial tension
$\Gamma_s$ Surface load
$\kappa^{-1}$ Debye length
$\Pi$ Depletion osmotic pressure
$\psi_0$ Surface potential
$\rho_{sm}$ Submicelle density
CHAPTER 1: INTRODUCTION

1.1 Overview

Nanoemulsions (NEs) are by definition emulsions with average droplet size less than 200 nm (Mason et al., 2007). NEs have several distinct properties compared to the conventional emulsions, e.g., improved stability, different optical and rheological properties. Previous research has shown an improved gelation properties of NEs at lower oil concentration compared to the conventional emulsion with synthetic small molecular weight surfactants (Erramreddy & Ghosh, 2014; Wilking & Mason, 2007). In this case, the nanodroplets along with their charge cloud repulsively jammed to give a behavior similar to highly concentrated conventional emulsions. Moreover, NEs have been found to have good bioavailability of encapsulated bioactives in the dispersed phase which opens the possibility of its application in various food and pharmaceutical products (Rao & McClements, 2011). NEs can also be used in foods to get high viscosity or gelation at a lower dispersed phase volume fraction, however, this aspect of NEs has not been investigated in depth.

The emulsifier is an important component in the preparation of NEs as it reduces the interfacial tension between the two phases and eases the process of emulsification by reducing the energy required to break up droplets. Sodium dodecyl sulfate (SDS), a small molecular weight surfactant has been successfully used in NE preparation. However, SDS can not be used in food formulation. Food proteins are ideal options as food-based emulsifier for this application. Proteins from bovine milk (casein and whey) have good emulsifying properties and have been widely used as emulsifiers in food industry (McClements, 2005). Moreover, both the proteins have a different structure, casein have a random coil-like structure while whey consists of a predominant globular structure. The interfacial layer formed by casein is also known to be five times thicker than that of whey (Atkinson et al., 1995; Dalgleish, 1993; Dickinson et al., 1993; Nylander et al., 1999). These differences will enable to understand the effect of protein structure and interfacial layer thickness on the gelation in protein-stabilized NEs. In the present research sodium salt of casein (sodium caseinate (SC)) and whey protein isolate (WPI) was used due to their good emulsification property and higher protein content.

Apart from repulsive gelation, the present research also investigated how NE can be transformed into gel by inducing attractive interaction among the nanodroplets. Attractive gelation
by the addition of salt in NE has been observed by Fryd and Mason (2012). Addition of salt leads to screening of charge from the protein surface which could induce attraction between the droplets (Dickinson et al., 1998; Marangoni et al., 2000). Moreover, at pH near the isoelectric point, the charge on the protein is canceled which also causes attractive gelation of protein covered droplets (Chen et al., 1999). Whey protein emulsions have also shown gelation upon heating due to heat-denaturation of whey protein leading to a gelation in the continuous phase (Dickinson & Chen, 1999). Apart from the gelation behavior, the study of these factors will also help us understand the effects of salt, change in pH and heat treatment on food protein-stabilized NEs as there are various changes in environmental factors like, salt concentration, pH and temperature in foods during processing.

The overall goal of this research is to develop nanogels from food protein-stabilized NEs with lower oil volume fraction and to study the effect of addition of salt, change in pH and heat treatment on their gelation behavior.

1.2 Objectives

To reach the above-discussed research goal, the following objectives were developed:

1. To understand the effect of dispersed phase volume fraction, protein type and concentration on the formation, rheology and long-term stability of food-grade nanogels.

2. To develop nanogels by inducing attractive interactions among the nanodroplets using change in pH, temperature and ionic strength of the NEs and investigate their stability and rheological behavior.

Objective 1 is investigated in chapter 3, while in objective 2 is studied in chapter 4.

1.3 Hypotheses

The below-listed hypotheses were tested in the above objectives. The first four hypotheses are tested in objective 1 and the rest of the hypotheses are tested in objective 2:

1. Reduction in droplet size will lead to random jamming due to the increased effect of interfacial charge cloud and protein steric-layer thickness on effective droplet size and volume fraction leading to repulsive gelation in NEs.

2. The increase in protein concentration will cause a decrease in droplet size and an increase in gel strength of NEs.
3. The excess unadsorbed protein in the continuous phase may lead to attractive depletion interaction causing stronger gel compared to the repulsive one.

4. Sodium caseinate will form thicker interfacial layer compared to whey protein isolate, which will also contribute to the increased gel strength.

5. Change in pH to the pI of protein will lead to protein aggregation in the continuous phase with protein coated oil droplets entrapped, which would significantly improve gel strength of the NEs by acting as active fillers.

6. Heating whey protein-stabilized NEs will lead to temperature-induced gelation thereby improve the gel strength of the corresponding nanogels.

7. Addition of salt will screen the electrostatic charge on protein-coated droplets, leading to reduction in effective droplet size thereby decreasing the gel strength of repulsive nanogel.

8. At higher salt concentration, due to complete charge screening, attractive inter-droplet interactions will dominate which would lead to the development of network of aggregated nanodroplets thereby forming attractive nanogels.
CHAPTER 2: LITERATURE REVIEW

2.1 Emulsion and nanoemulsions

2.1.1 Definition

Emulsions are mixtures of two or more immiscible liquids, namely, oil and water, in which one or more liquids are dispersed as droplets in the other. The liquid present as droplets is called the dispersed phase, whereas the liquid in which the droplets are dispersed is called the continuous phase. Emulsions can be divided into two types: oil-in-water (O/W) and water-in-oil (W/O) (Figure 2.1). In O/W emulsion, the oil phase is dispersed in the continuous aqueous phase, while in W/O emulsion the aqueous phase is dispersed in the continuous oil phase. Emulsions are an integral part of food as many commonly used food products consist of various forms of emulsions. For example, mayonnaise, milk, and cream are O/W emulsions, whereas butter and margarine are W/O emulsions. Emulsions widely affect shelf-life and stability of food products as destabilization of the emulsion may significantly reduce shelf-life and quality of many food products (McClements, 2005).

![Diagram of emulsions](image)

**Figure 2.1** Schematic diagram of two different types of emulsions.

Normally, the droplet size in emulsions is in the micrometer range. In recent years, NEs have taken attention due to their distinctive properties. NEs are emulsions where the average droplet diameter of less than 200 nanometers (Mason et al., 2007; Tadros et al., 2004). NEs have more surface area to volume ratio compared to micron-scale conventional emulsions because of their smaller droplet size. Due to smaller droplet size, nanodroplets may exhibit very little light scattering, which could make them translucent compared to micron-scale emulsions and improves their application in clear beverages (Mason et al., 2007). Increased interest in NEs and study of their characteristics could also be due to the greater bioavailability of bioactives encapsulated in
nanodroplets (Huang et al., 2010). Extremely small droplet size also makes NEs highly stable against gravitational separation leading to higher stability and extended shelf-life compared to micron-scale emulsions (Tadros et al., 2004). Nevertheless, NEs require a higher concentration of emulsifiers compared to micron-scaled emulsions. Therefore, it is important to utilize natural bio-based emulsifiers for NE preparation in order to reduce any adverse effect on health.

2.1.2 Composition – emulsifier, oil, water and other

Emulsions in foods are made of certain essential components irrespective of the type of emulsions (O/W or W/O). The three most important components are oil for the lipid phase, water for the aqueous phase and emulsifiers at the oil-water interface. Apart from oil and water, these phases consist of different other ingredients like thickening agent, salts, colorants, flavors, preservatives, antioxidants, bioactives and sweeteners depending on their hydrophilic or lipophilic nature (McClements, 2005). The amount of these components and their distribution in different phases defines various physicochemical and organoleptic properties of food (Malone et al., 2003).

Lipid phase in food emulsion is important to provide texture, nutrients and certain physicochemical properties to the food. Presence of dispersed oil phase affects textural properties and provides rich mouthfeel and creamy texture to the food (McClements, 2005). The lipid phase also carries oil-soluble vitamins (vitamins A, D, E and K), flavors, nutrients that contribute to organoleptic and nutritional properties of food emulsions (McClements, 2011). The dispersed oil droplets are also responsible for optical properties of O/W emulsions due to scattering of light. Moreover, changes in oil concentration affects rheological properties of emulsion providing higher viscosity with an increase in oil concentration and generating elastic gels at a very high oil concentration due to close packing of droplets (Erramreddy & Ghosh, 2015; Pal, 1996).

Similar to lipid phase, the aqueous phase of an emulsion is also important to deliver various water-soluble and dispersible components, e.g., vitamins, proteins, preservatives, carbohydrates, etc. (McClements, 2005). These ingredients are responsible for functional, nutritional and physicochemical properties of food. Moreover, the viscosity of aqueous phase is also responsible for droplet size and rheology of an emulsion. It has been reported that the droplet size is affected by the change in viscosity during homogenizations (Meleson et al., 2004). The aqueous phase composition also affects the freeze-thaw stability of emulsions which is important for foods with emulsions that undergo freezing during processing or storage (i.e., ice-cream).
Figure 2.2 Emulsion stabilization by different types of emulsifiers. (A) small molecule emulsifier, (B) flexible protein (casein), (C) globular protein (whey protein) and (D) particles (Pickering)

Apart from the two phases, the interface is also important in formation and stability of emulsions. The emulsifiers are amphiphilic surface-active substances normally present at the interface. They are responsible for reducing the interfacial tension between a lipid and an aqueous phase (McClements, 2005). This aids in the reduction of energy needed for the formation of emulsions. Apart from the creation of emulsion, the emulsifiers also act as a barrier and provide stability to oil droplets (Walstra, 1993). Emulsifiers can be classified into three types: 1) small molecular weight surfactants, 2) biopolymers and, 3) solid particles. Small molecular weight surfactants could be ionic (sodium dodecyl sulfate (SDS), sodium stearoyl lactate) or non-ionic (monoglycerides, Tween 20®). The small molecular weight surfactants stabilize emulsions due to their hydrophilic head and hydrophobic tail. Biopolymers can also be divided into types based on their structures, i.e., globular protein (whey protein) or flexible protein (caseins). The hydrophobic and hydrophilic amino acids provide amphiphilic nature to proteins (Dickinson, 1998). Solid particles (i.e., silica nanoparticles) yields a unique type of emulsion called ‘Pickering emulsions’ (Dickinson, 2012b). Orientation and arrangement of various types of emulsifiers at interface is shown in Figure 2.2.

### 2.1.3 Formation of emulsion and nanoemulsion

During preparation of the emulsion, external energy is required to disperse one phase as droplets into other. This energy causes rupture of larger droplets into smaller ones by acting on the internal droplet pressure, also known as Laplace pressure, which can be calculated by,
\[ P_L = \frac{2\gamma}{r} \]  

where, \( \gamma \) is interfacial tension (N/m) between the two phases and \( r \) is droplet radius (Walstra, 1993). It is essential to decrease Laplace pressure in order to break the droplets easily into smaller size. From Eq. (2.1), we can conclude that reduction in interfacial tension can reduce Laplace pressure, which eases the process of droplet disruption. Surface active emulsifiers that decrease interfacial tension between oil and water are added to reduce Laplace pressure and the amount of energy required to prepare emulsions. The emulsifier also develops a protective barrier around the droplets that prevents inter-droplet interactions and coalescence by various mechanisms discussed below (McClements, 2005).

Methods for preparation of NEs are primarily divided into two types: low energy and high energy methods. Low energy methods include spontaneous creation of small droplets due to a change in solution composition or temperature, e.g., phase inversion temperature (PIT), phase inversion composition (PIC) and spontaneous emulsification methods (Fryd & Mason, 2012; McClements, 2011). Nevertheless, some of the low energy methods are still not suitable for the development of NE at large scale and with natural protein emulsifiers (Lee et al., 2011; McClements & Rao, 2011). For example, in PIT method, it is still very difficult to achieve sudden temperature change for a large volume of materials (Solans & Sole, 2012). Also, proteins and other polymeric emulsifiers cannot form oil or water droplets as a function of solution conditions, which is an important requirement for the low-energy method. High energy method, on the other hand, includes the use of mechanical energy to create disruptive forces, which rupture the droplet into very small size, e.g., high-pressure homogenization, microfluidization and ultrasonic emulsification. The process of emulsion preparation by homogenization consists of the preparation of a premix of oil & water and homogenization of the premix to disperse one phase into the other as small droplets. During homogenization, droplets are ruptured and covered by emulsifier where, delay in emulsifier adherence on the droplet surface can lead to coalescence (McClements, 2007). According to Eq. (2.1) as the droplet size decreases, Laplace pressure of droplet increases which leads to the requirement of more energy to further break the droplets into even smaller nanodroplets (McClements, 2011). Therefore, the formation of NE requires very high energy compared to micron-scale emulsions. To prepare NE with high-energy methods, multiple passes of emulsions through the homogenizer are required so that all the droplets experience the highest shearing to
achieve small and uniform droplet size (Mason et al., 2007). Microfluidization is another method of emulsion preparation where pumping a dispersion through the different micro-channels of the device at very high velocity leads to a collision at the end of the channels and generation of very small droplets (McClements, 2005).

As discussed above, the presence of surface active emulsifier is an important aspect of emulsion preparation. Various types of small molecular weight emulsifiers and amphiphilic biopolymer are used for emulsion formation and stabilization, for example, SDS as small molecular weight emulsifiers and surface-active milk proteins as amphiphilic biopolymers (Dickinson, 1999; Erramreddy & Ghosh, 2014; Nespolo et al., 2001). Emulsion stabilization mechanisms (section 2.1.2) vary depending on the type of emulsifier used and the different intermolecular forces that act on the emulsifiers. These intermolecular forces can be a combination of covalent, electrostatic, van der Waals, depletion interactions, steric repulsion and various other colloidal interactions (Israelachvili, 2011; McClements, 2005).

2.1.4 Characterization of nanoemulsions and emulsion

Droplet size, appearance, rheology and microstructure of NEs are some of the factors that give them distinct advantages compared to micron-scale emulsions. For example, their use in transparent cosmetics, as a carrier for readily adsorbed pharmaceutical drugs (Mason et al., 2007). Droplet size is one of the primary aspects that differentiate NEs from micron-scale emulsions. There are various ways average droplet size of emulsions, and NEs can be expressed, e.g., number average diameter ($d_{10}$), surface area average diameter ($d_{32}$) and volume average diameter ($d_{43}$) (McClements, 2005; Walstra, 2003). Among these, $d_{43}$ is more influenced by large droplets than $d_{32}$, as a few large droplets would take more volume than many small droplets (McClements, 2005). This is the main reason $d_{32}$ is preferable for NE as a few large droplets can give a false picture of the entire droplet size distribution with $d_{43}$.

As NEs have droplet size smaller than the visible light wavelength, light scattering by nanodroplets is normally low (Mason et al., 2007), which can make them translucent or even transparent based on droplet volume fraction and refractive indices of the phases (Fryd & Mason, 2012). Depending on the molecular characteristics and environmental conditions, emulsifiers on droplet surface may display anionic, cationic or nonionic characteristics (McClements, 2005). Droplets stabilized with ionic emulsifiers have repulsive interactions, which prevents them from
coming close to each other and thus, they resist coalescence. Electrostatic charge on droplets also has a significant effect on the inter-droplet separation, which in turn may influence their rheological behavior when the droplets with their associated charge clouds, fill up the whole space & are packed in a random jamming state. It has been shown that upon reduction in droplet size by high-pressure homogenization NEs stabilized with charged emulsifier display increased elastic modulus (Wilking & Mason, 2007). As the droplet size decrease, the distance between two droplet reaches towards Debye screening length, which is the measure of how far the effect of electrical properties sensed. This causes an increase in the effective droplet size & their repulsive jamming, which leads to the development of elastic modulus (Wilking & Mason, 2007).

As the characteristics of any material depend on its internal structure, it is essential to study the micro and nanostructure of NEs. Several microscopic techniques can be used to visualize extremely small droplets of NEs. Optical microscopy, electron microscopy, and atomic force microscopy are few techniques that can be used to study the structure of emulsions (McClements, 2005). However, optical microscopy is not very effective to study NEs due to its inability to detect nanodroplets. Nevertheless, techniques like dynamic light scattering, X-ray or neutron scattering, atomic force microscopy, and transmission electron microscopy (i.e., negative straining TEM, Freeze-fracture TEM and Cryo-TEM) have been successfully used to record nanostructure of NEs (Fryd & Mason, 2012; Mason et al., 2007). Confocal laser scanning can also be used to examine the structure of NEs, which uses staining of lipid or protein with fluorescent dyes for a distinct observation of dispersed droplets and associated structures (Blonk & Van Aalst, 1993; Yerramilli et al., 2017). Freeze-fracture Cryo-SEM has also been used previously to study the nanostructure of NEs (Eskandar et al., 2009).

Flow behavior and gelation in emulsion are measured using a rheometer and based on range of different flow behavior or gelation properties; the emulsions can be characterized based on them. Depending on their flow behavior, emulsions can be classified in a broad range from low viscosity liquids (i.e., milk, low-fat cream) and high viscosity liquids (Mayonnaise, high-fat cream) to viscoelastic solids (margarine, butter and other solid-like soft materials) (McClements, 2005; Rao, 2007).
2.1.5 Stability of emulsion vs. nanoemulsion

As the contact between oil and water phases is thermodynamically unfavorable, both of these phases are immiscible (McClements, 2007), which leads to emulsion destabilization and separation of oil and water phases to minimize the contact area. There are various mechanisms that lead to emulsion destabilization, e.g., gravitational separation, flocculation, coalescence, partial coalescence, Ostwald ripening and phase inversion (McClements, 2005, 2007).

2.1.5.1 Gravitational separation (creaming / sedimentation)

Due to the density difference between the two phases, phase separation due to gravitational forces is a primary destabilization mechanism in emulsions. If the density of the dispersed phase droplets is lower than the continuous phase, the droplets move upwards due to gravitational effect, which is called creaming (Figure 2.3B). On the contrary, if the density of the droplets is higher than continuous phase, it moves downwards, which is called sedimentation (Figure 2.3C). The rate of creaming in a dilute emulsion can be determined by Stoke’s formula,

\[ \nu = \frac{2g\Delta\rho r^2}{9\eta} \]  

(2.2)

Where \( \nu \) is velocity of droplet, \( \Delta\rho \) is the density difference between the two phases, \( r \) is droplet size, \( \eta \) is viscosity of continuous phase and \( g \) is acceleration due to gravity (McClements, 2005). From Eq. (2.2), reduction in droplet size leads to reduction in the rate of creaming/sedimentation, hence, NEs are more stable against gravitational separation due to their small droplet size. Along with this, the presence of Brownian motion in nanodroplets, which is the random movement of small particles in suspended fluid, make nanodroplets very stable against gravitational separation (Tadros et al., 2004).

2.1.5.2 Flocculation

Flocculation is the process where two or more droplets adhere to each other but do not merge and retain their individual identity (Figure 2.3D). Flocculated droplets act like one large droplet altogether, which leads to increased gravitational separation (McClements, 2007). Droplet collision required for flocculation mainly depends on the attractive forces between them and for the systems containing smaller droplets, mainly on the Brownian motion of droplets (McClements, 2007). Higher attractive forces increase the rate of flocculation, while increased Brownian motion
reduces it. As the droplet size decreases the range of attractive forces also decreases, whereas steric repulsion created by physical barrier due to presence of emulsifier at interface, is not much affected by droplet size. This phenomenon provides good stability to NE against aggregation and flocculation (Silva et al., 2012; Tadros et al., 2004). A higher emulsifier concentration may lead to attractive depletion flocculation, which is caused by increased attractive interaction due to osmotic pressure difference between inter-droplet region and bulk continuous phase created by non-adsorbed emulsifier micelles or biopolymers in the continuous aqueous phase (McClements, 2005).

2.1.5.3 Coalescence

Coalescence is the process in which two or more droplets merge to make one large droplet (McClements, 2007) (Figure 2.3E). For this to happen, two or more droplets must come in close contact with each other followed by thinning of the interdroplet film and eventual rupturing (van Aken, 2004). Stability of emulsion against coalescence is largely influenced by the type of emulsifier. Coalescence can be prevented by emulsifier that makes thick interfacial layer, which is difficult to rupture. Another way to prevent coalescence is to use electrically charged emulsifier that produces repulsive forces among the droplets.

When the droplet radius \( r \) is in nanometers, the thickness of the interfacial layer \( \delta \) may become significant compared to droplet size. Tadros et al. (2004) showed that it is thermodynamically unfavorable for the droplets to come together in NE (Tadros et al., 2004). Thus, smaller droplet size and thickness of the interfacial layer prevents thinning and rupture of the interdroplet film, which makes NEs stable against coalescence.

Figure 2.3 Schematic diagram of the mechanism of destabilization in emulsions. A) stable emulsions B) creaming, C) sedimentation, D) flocculation. E) coalescence and F) Ostwald ripening.
2.1.5.4 Ostwald ripening

In Ostwald ripening, larger droplets grow larger at the expense of smaller droplets due to mass transport of dispersed phase from smaller to larger droplets through the continuous phase (McClements, 2007) (Figure 2.3F). Ostwald ripening is significantly affected by the solubility of the dispersed phase in the continuous phase and permeability of the interfacial membrane. (McClements, 2005; Weiss & McClements, 2000; Wooster et al., 2008). In NEs, the Laplace pressure of the droplets are very high, so they are more prone to Ostwald Ripening due to increased solubility of the dispersed phase materials in the continuous phase. Moreover, a large difference between the range of droplet radii increases the rate of Ostwald Ripening (Tadros et al., 2004), which can affect NEs with a high degree of polydispersity. However, by using a dispersed phase that is insoluble in the continuous phase and selection of appropriate emulsifier can lead to the prevention of Ostwald ripening in NEs. For example, as vegetable oils are essentially insoluble in water, food-grade NEs are generally stable to Ostwald ripening (Wooster et al., 2008).

2.2 Food proteins in the formation & stabilization of emulsions and nanoemulsions

Emulsifier eases the process of emulsification due to their amphiphilic nature and prevents coalescence by creating a barrier between the two dispersed droplets. Small molecular weight emulsifiers are better at forming nanodroplets as they reduce oil-water interfacial tension to a great extent compared to proteins (McClements, 2005). However, proteins are better at providing long-term stability due to the formation of the thick viscoelastic membrane. Food proteins are also a more attractive choice as emulsifier due to their natural origin and health-promoting factors.

2.2.1 Sources of protein as food emulsifier

Due to their amphiphilic nature, proteins adhere to the surface of dispersed droplets and create a thick viscoelastic membrane between the two immiscible phases thereby improving emulsion stability (McClements, 2004). Food proteins for emulsion stabilization are obtained from various sources, for example, milk proteins, meat and fish proteins, egg proteins and plant proteins (McClements, 2005). Milk protein can be divided into two categories: casein and whey proteins. Casein is available in different forms, for example, sodium caseinate, calcium caseinate, rennet casein and acid casein. Among them, sodium caseinate is a readily soluble form of casein and widely used as food emulsifier (Fox & McSweeney, 1998). Whey protein is used in the form of whey protein isolate (WPI) and whey protein concentrate (WPC) as an emulsifier. Gelatin, myosin,
actomyosin, sarcoplasmic protein, and actin are some of the surface active proteins obtained from meat and fish (McClements, 2005). Gelatin has been found to have some interfacial and gelling property, which helps in forming and stabilizing emulsions. Still, gelatin alone is not much effective in forming small droplets, so it is hydrophobically modified by attaching nonpolar side groups (e.g., by reacting N-hydroxysuccinimide fatty acid ester with a primary amine group on lysine of gelatin increases the emulsifying capacity of gelatin) (Toledano & Magdassi, 1998). Protein from both egg white and egg yolk can also be used as an emulsifier (Azzam & Omari, 2002; Mine, 1998). Anton and Gardener studied effect of pH on egg yolk stabilized emulsions. Average droplet diameter for emulsions stabilized with egg yolk protein was found to be smallest at pH 6, which is the best pH for the emulsifying capacity of egg yolk. More protein (all the proteins except phosvitin) were adsorbed at interface compared pH 3 or pH 9 (Anton & Gardener, 1999).

Plant proteins obtained from different cereals and legumes can also be used in their native or modified form as surface-active compounds to form and stabilize emulsions. Proteins from soy, pea, lentil, and chickpea have been used in the form of isolates as emulsifier (Ducel et al., 2004; Karaca et al., 2011; Liu et al., 1999; Papalamprou et al., 2010). Legume proteins contributes to nutritional value and have low cost but many of them need modifications (using isoelectric precipitation or high-pressure homogenization) to improve their emulsion forming and stabilizing capacity (Dong et al., 2011; Ducel et al., 2004; Karaca et al., 2011; Papalamprou et al., 2010). Oilseed proteins also have emulsifying properties. For example, sunflower protein isolate, sunflower globulins (helianthinin) and sunflower albumins have been used as an emulsifier in which droplet aggregation has been observed at different pH (González-Pérez et al., 2005). Another oilseed protein from canola has been used to make emulsion as canola protein isolate by Chang et al. (2015) and found that canola protein isolate provides emulsions with smaller droplet size at lower pH (pH = 3).

2.2.2 Formation of protein stabilized nanoemulsions

Preparation of NEs with very small droplets using protein as an emulsifier is relatively difficult compared to small molecular weight emulsifiers due to various reasons. The adsorption rate of proteins on droplet during homogenization is very slow (McClements, 2011), which limits their capacity to cover the small droplets immediately after their formation (Qian & McClements, 2011). Proteins are not very effective in reducing interfacial tension compared to small molecular weight emulsifiers.
weight emulsifiers (Dickinson, 1997). Unlike small molecular weight emulsifiers, proteins are prone to microbial deterioration that can lead to destabilization of the protein-stabilized emulsions. Therefore, it is essential to add antimicrobial agent and to store NEs at a lower temperature to prevent the destabilization due to microbial activity. Nevertheless, proteins do form a thick viscoelastic membrane at the droplet surface. Therefore, once droplets are formed with proteins, their long-term stability significantly improved compared to small molecular weight emulsifiers. Hence, it is important to implement various protein modifications to enhance their emulsification properties. It has been seen that emulsion stability of whey protein concentrate can be improved by a pre-heat treatment at high temperatures (80 - 95 °C) (Dybowska, 2011). On the other hand, some finishing techniques can also be used to further reduce the droplet size of protein-stabilized emulsions. Solvent evaporation has been used to reduce the droplet size of O/W NEs by evaporating the solvent from dispersed droplets containing a mixture of oil and a volatile solvent (Fryd & Mason, 2010). A similar technique of solvent displacement can also be used, in which a solvent is selected such that it is soluble in both the dispersed and continuous phases of emulsions. Upon dilution of the continuous phase, the solvent is displaced from the droplets to the continuous phase due to concentration difference, leading to a decrease in droplet size (McClements, 2011). These two techniques can be used to produce food-proteins stabilized NEs, although the presence of solvent may not be most suitable for food applications. However, some researchers have recently produced protein stabilized NEs by multiple cycles of high-pressure homogenization. Donsi et al. has obtained pea protein stabilized NEs by high-pressure homogenization for delivery of nutraceuticals (Donsi et al., 2010). In recent years, Yerramilli et al. and Primozic et al. have produced sodium caseinate, pea protein and lentil protein stabilized NEs with similar approach of high-pressure homogenization (Primozic et al., 2017; Yerramilli & Ghosh, 2017; Yerramilli et al., 2017).

2.2.3 Advantages and applications of protein stabilized emulsions and nanoemulsions

Protein-stabilized NEs are different in several ways compared to small molecular weight emulsifier-stabilized NEs. Apart from electrostatic repulsion, one of the most important mechanisms of protein-stabilized droplets is steric stabilization. Thick viscoelastic interfacial protein layer on the droplets prevents them from coming in close proximity and avoid coalescence (McClements, 2005).
Apart from acting as an emulsifier, protein also acts as a gelling, stabilizing, or thickening agent (Dickinson, 2012a). This also makes the continuous phase more viscous due to the presence of unabsorbed proteins. An increase in the continuous phase viscosity causes a reduction in droplet movement that helps to prevent coalescence, flocculation, and gravitational separation. However, excess protein molecules in the continuous phase can also lead to attractive depletion interactions between the droplets (Dickinson & Golding, 1997). However, very high amounts of protein in the continuous phase can also prevent droplets from flocculation by occupying all the spaces between the droplets, a phenomenon termed as depletion stabilization (Rajagopalan & Hiemenz, 1997).

The protein forms a thick steric layer at the interface which makes emulsions more stable against coalescence by providing a good physical barrier at the interface. The knowledge of this interfacial protein layer thickness helps in understanding its contribution to different properties of the emulsion. Various methods have been used to measure the adsorbed protein layer thickness, for example, ellipsometry, specular neutron reflectance, small angle X-ray scattering, and dynamic light scattering (Atkinson et al., 1995; Dalgleish, 1990; Mackie et al., 1991; Malmsten, 1994). Using ellipsometry, the adsorbed layer thickness of whey protein at the oil/water interface was measured at around 2 nm (Nylander et al., 1999). The adsorbed layer thickness of β-casein and β-lactoglobulin measured by neutron reflectance spectroscopy were 7.4 ± 0.5 nm and 3.1 ± 0.3 nm, respectively (Atkinson et al., 1995; Dickinson et al., 1993). On the other hand, using dynamic light scattering, the hydrodynamic thickness of β-casein at the interface was found to be 10-15 nm (Dalgleish, 1993; Dickinson, 1992). The high values obtained from dynamic light scattering technique were ascribed to the very high sensitivity of hydrodynamic thickness towards the long tails of adsorbed casein molecule (Dickinson, 1992).

Use of food protein as an emulsifier in NE makes them highly acceptable for use in many edible products. These edible NEs can be used in food or pharmaceutical industry to encapsulate and deliver oil-soluble flavors, drugs, or nutrients (McClements, 2011). The characteristic of proteins to form gels by heat, acid, or enzyme treatment creates an opportunity to make emulsion gels (Dickinson, 2012a). These NE gels with droplets entrapped in protein gel structure will have no chance of coalescence or gravitational separation, thus providing NEs with longer shelf-life. Also, at very high dispersed phase volume fraction, droplets of emulsions do not have any space to move due to close packing leading to gel formation by random jamming. Nanodroplets in a close-packed state do not rupture due to the thick interfacial protein layer and also high Laplace
pressure generated by very small oil droplets (Mason et al., 2007). These gel-like viscoelastic NEs could be used in various capacities in food, cosmetics and pharmaceutical industries.

2.3 Influence of interdroplet interactions on emulsion physicochemical properties

The physicochemical properties of emulsions depend on many factors like dispersed phase concentration, droplet size, viscosity of the continuous and dispersed phases. Another significant factor that influences the physicochemical properties of emulsions is interdroplet interaction (Friberg et al., 2004). These interdroplet interactions are influenced by factors like thickness of the interfacial layer, type of emulsifier, the charge on emulsifier coated droplets, etc. These factors are altered in food emulsions by the change in pH, ionic strength or temperature. As discussed above, these interdroplet interactions can be determined by calculating the forces between two interacting droplets. The various interaction forces that are responsible for interdroplet interactions are covalent, electrostatic, van der Waals, depletion interactions, steric repulsion or a combination of them (Israelachvili, 2011; McClements, 2005). These interdroplet forces and their effect on the attractive or repulsive interaction between the two droplets can be calculated to understand the interdroplet interaction in any particular emulsion system.

2.3.1 DLVO interdroplet interaction

As discussed above, droplet interactions are important to determine if droplets will remain separate or form aggregates (Friberg et al., 2004). Droplet interactions are expressed in terms of the interdroplet pair potential, which is the energy required to bring two droplets from an infinite distance to a close separation (Ghosh & Rousseau, 2010). The DLVO calculation is the calculation of all individual forces acting on the droplets. This theory of interdroplet potential was first proposed by Derjaguin, Landau, Verwey and Overweek and is known as DLVO theory (Ghosh & Rousseau, 2010). The van der Waal forces (attractive in nature) increases as the droplets approach each other. The electrostatic repulsion increases exponentially with a decrease in distance between the droplets with same droplet charge. Other forces like, depletion force (attractive) also plays a certain role depending on the presence of dispersed particles in the aqueous phase and steric barrier that provides a physical barrier (repulsion) to prevent interaction between two droplets act depending on emulsion composition and choice of emulsifier. The total interaction potential is the sum of all these forces. To simplify the explanation, we will consider electrostatic repulsive component and van der Waals attractive component. The total interaction potential decreases as
the particles come near, and the droplets form loose aggregates when the interaction potential drops to a secondary minimum as shown in Figure 2.4. Upon further droplet approach, the electrostatic repulsion dominates over the van der Waal interaction and if the thermal energy of the droplets is not enough to overcome the repulsive energy barrier, they return back to initial non-aggregated state (termed as reversible flocculation). On the other hand, if the thermal energy of the droplet pair is more than the repulsive energy barrier, the interaction potential passes through the energy barrier and the droplets form strong irreversible aggregates (also known as coagulation) at the primary energy minimum. The original DLVO theory only took electrostatic and van der Waals interaction. However, steric repulsions, depletion forces, hydrophobic and hydration interactions can be included in a modified DLVO theory that could be used to predict inter-droplet interactions and determine emulsion stability (Israelachvili, 2011; McClements, 2005). The interdroplet pair potential shown in Figure 2.4, is the sum of van der Waal and electrostatic interactions.

![Figure 2.4 DLVO interdroplet pair potential between two droplets (adapted from Ghosh and Rousseau, 2010). The interdroplet interaction pair potential is shown on the Y-axis, and the distance of separation between two droplets is represented by the X-axis.](image)

2.3.2 Effect of pH

As electrostatic repulsive forces are an important factor in inter-droplet interactions, change in electrostatic interactions can have a significant effect on emulsion stability. Change in pH could
affect the electrostatic repulsive forces and cause attractive interactions between the droplets. Change in pH leads to canceling or increasing the charge on the droplets depending on the isoelectric point (pI) of emulsifier covering the droplet. When pH is at the isoelectric point, the droplets do not have any charge which leads to decrease/ cancellation of electrostatic repulsive forces. Tcholakova et al. have observed the increase in droplet size at pH near isoelectric point which was attributed to the flocculation or coalescence due to increased interdroplet attraction (Tcholakova et al., 2005). The group has also confirmed the absence of any electrostatic charge from the zeta potential measurement at the isoelectric point. Thus, pH could be an important factor to control the interdroplet interaction to either make more stable emulsion or achieve desired interactions (attractive or repulsive) in food emulsions.

2.3.3 **Effect of ionic strength**

Electrolytes in continuous phase can have a significant impact on interdroplet interaction. Researchers have reported that an increase in electrolyte concentration in the continuous phase leads to a decrease in electrostatic repulsion (Berli et al., 2002; Tcholakova et al., 2005). This ultimately leads to a smaller or no potential energy barrier compared to the thermal energy of the system and causes the droplets to aggregate easily. Berli et al. showed the decrease in total interaction potential between sample without salt and with 12.5 mM salt by DLVO interaction potential calculations for emulsion stabilized with sodium caseinate. A similar effect of NaCl on β-lactoglobulin stabilized emulsion was also observed by Kim et al., where the repulsive barrier was found to reduce with increase in NaCl concentration from 50 mM to 250 mM. The peak before secondary minimum gradually decreased and became nearly zero for emulsion with 250 mM NaCl.

2.4 **Gelation in emulsion & nanoemulsion**

2.4.1 **Types of gels**

Gels can be classified in different types based on various factors of gel structure or gelation. (Nishinari, 2009) has discussed the classification of gels based on the constitutes responsible gel network structure in two types: Polymer gels and Particulate / Colloidal gels. Polymer molecules in the continuous phase are responsible for network structure. Some of the examples of polymer gels are, alginate gels, agarose & gelatin. Polymers could be both natural and synthetic. When colloidal particles present in continuous phase are responsible for the gel network formation, the
gel can be classified as particulate gel. Cottage cheese is one of the example of particulate induced by network of casein particles (Fox & McSweeney, 1998).

Gels can also be classified base on their transition temperature and reversibility at varying temperatures (Banerjee & Bhattacharya, 2012). Based on temperature of transition from sol to gel gels can be classified in two types: Cold setting gels and Heat-setting gels. When gelation occurs upon decrease of temperature, it is called cold-setting gels, whereas when gelation happens upon heating, it is heat-setting gel. Gels could also be thermoreversible or thermoireversible based on the reversibility of gelation (Banerjee & Bhattacharya, 2012; McClements, 2005). Gelatin is an example of thermoreversible cold-setting gel, which achieves gelation when cooled below certain temperature and melts upon reheating. Well-known example of thermoireversible heat-setting gel is egg-white as heating of egg white gives characteristic white gel which does not change structure upon cooling (McClements, 2005).

Another classification is based on types of bonds in the structure of gel network. The gels can be classified in two types: Chemical gels and physical gels (Katsuyoshi, 2009). Chemical gels are gels where chemically crosslinked covalent bonds are responsible for forming the gels network structure whereas in physical gels, secondary molecular forces like hydrogen bond and ionic bond are responsible for gel network structure formation. Hydrogel formed with β-lactoglobulin monomers formed by di-sulfide covalent bonds is an example of chemical gel (Jost, 1993). Gelatin jellies formed by hydrogen bonds between helical structure of gelatin molecules is an example of physical gel (Banerjee & Bhattacharya, 2012). Another example is heat-induced gelation of protein where heat causes unfolding of the native protein and enable it to react with neighboring proteins with hydrophobic interactions which is essential for sol to gel transformation (Clark et al., 2001).

2.4.2 Rheological characterization of emulsion gels

Rheology is the science of flow and deformation. To measure rheological properties, the relationship between applied force and resulting flow/deformation behavior is recorded. Rheological properties of materials are ranged from the pure liquid (e.g., water), whose viscosity (ability to resist flow) does not change with shear, to elastic solid which can instantly recover its original form upon removal of the deforming force. In between these two scenarios, viscoelastic materials possess properties of both viscous liquid and elastic solid (e.g., gels, yogurt, and mayonnaise). These materials have the ability to retain their original structure, however, upon
application of force, may deform and yield due to significant changes in structural behavior (Rao, 2007). Rheological measurement of viscoelastic gels is done using the oscillatory test, where a sinusoidal stress is applied and the resultant sinusoidal strain is measured (Rao, 2007). From these dynamic tests, storage (G') and loss moduli (G'”) of materials can be determined which provides quantitative information on gel strength and the critical force required for gel-sol breakdown. Storage modulus is a measure of the magnitude of the energy stored in a material upon application of strain while loss modulus is a measure of energy lost as viscous dissipation and can be taken as the liquid-like behavior of viscoelastic materials (Rao, 2013).

Frequency and amplitude (strain) of oscillation are important aspects of oscillatory rheological testing. Four types of tests can be carried out to obtain rheological properties of gels: 1) frequency sweep, in which G' and G'” are obtained as a function of frequency while keeping the amplitude of oscillation constant, 2) strain or amplitude sweep, in which G' and G'” are obtained as a function of strain while keeping the frequency of oscillation constant, 3) time sweep, in which G' and G'” are obtained as a function of time at constant frequency and amplitude of oscillation and, 4) temperature sweep, in which G' and G'” are obtained as a function of temperature at constant frequency and amplitude of oscillation (Rao, 2007). It is essential that oscillatory tests are conducted in the linear viscoelastic region (LVR), where storage modulus is independent with respect to the controlling strain or frequency of oscillation.

2.4.3 Attractive emulsion gels

Emulsions have been found to form attractive gels. These gels are mainly formed due to attractive interactions between the droplets. There are different factors that reduce repulsive forces or increases attractive forces leading to interactions between the droplets. In attractive emulsion gels, the droplets an aggregated structure in which the continuous phase is trapped leading to a self-supporting gel that does not flow under gravity. Attractive interactions between the droplets can be brought about in many different ways. Of importance for the present work are depletion interaction and salt-induced charge-screening on droplets.

2.4.3.1 Depletion attraction

Depletion attraction in emulsion occurs when emulsion droplets are surrounded by smaller particles or emulsifier micelles. These small particles or micelles are called depletants as they induce depletion attraction between droplets (Tuinier et al., 2000). A schematic of depletion
attraction is given in Figure 2.5, where two droplets surrounded by depletants approach each other. The depletion zones are considered as excluded volumes by the expulsion of depletants due to steric and electrostatic repulsion (Bibette et al., 1992). When the droplets come closer such that their depletion zone overlap, the depletants are expelled into the outer continuous phase from the inter-droplet area as shown in Figure 2.5. This phenomenon causes an osmotic pressure difference between the surrounding continuous phase and the inter-droplet gap created due to the expulsion of depletant. This osmotic pressure difference induces the attractive interaction between the droplets, leading to aggregation (Lekkerkerker & Tuinier, 2011).

Asakura-Oosawa first modeled depletion interaction forces (Asakura & Oosawa, 1954). Many other researchers have also studied depletion forces since then (Berli et al., 2003; Bibette et al., 1990; Furusawa et al., 2002; Mondain-Monval et al., 1995). Depletants can be ionic-micelles or unadsorbed biopolymers which induces depletion interactions, the strength of which also depends on the size of the depletants and the ionic strength of the solvent. Researchers have observed that altering the ionic strength of solvent screens the electrostatic charge on particles or droplets and lead to the lower effective size of micelle or droplets (Mondain-Monval et al., 1996; Petsev et al., 1995). The depletion flocculation has also been observed in SC-stabilized emulsions which was induced due to the presence of un-adsorbed casein sub-micelles in the continuous phase by acting as depletants (Dickinson & Golding, 1997).

Figure 2.5 Schematic representation of aggregation of emulsion droplets induced by depletion forces due to presence of excess free proteins in the continuous phase. Arrows in Figure A indicates expulsion of depletants and arrows in figure B shows aggregation of droplet due to osmotic pressure difference.

The depletion induced attraction has been observed in NEs as well in recent years with various types of depletants. Erramreddy and Ghosh have observed depletion flocculation of NE
droplets due to excess SDS micelles (Erramreddy & Ghosh, 2015). Similar depletion flocculation has been observed in sodium caseinate stabilized NEs, where casein micelles induced depletion attraction which led to depletion flocculation and creaming in NEs (Yerramilli & Ghosh, 2017).

2.4.3.2 Attraction due to change in ionic strength

Attractive gelation in emulsion can be induced by the changes in ionic strength of the continuous phase. As discussed in section 2.3.3, the addition of electrolytes to the continuous phase screens the charge on the droplets. This leads to a decrease in electrostatic repulsion and could cause attractive interactions between the droplets. These attractive interactions could lead to an attractive gelation in emulsions if appropriate amount of electrolyte is added. As discussed above (Section 2.3), screening of the charge results in the reduction of repulsive barrier between the droplets and also lowering the secondary minima of interdroplet interaction potential (Datta et al., 2011; Fryd & Mason, 2012; McClements, 2005). Addition of salt in excess concentration could lead to complete destabilization of emulsions due to coalescence (McClements, 2005). However, emulsions stabilized with an emulsifier with good steric barrier are less prone to coalescence even upon complete screening of charge. Protein stabilized emulsions are an ideal example of this. Researchers have seen gelation of protein-stabilized emulsion upon complete screening of charge. For example, the addition of NaCl has also shown to induce gelation in whey protein stabilized emulsions (Line et al., 2005; Rosa et al., 2006). Salt-induced gelation of protein stabilized emulsions was also termed as ‘Cold gelation’. Apart from NaCl, Marangoni et al. (2000) and Maltais et al. (2005) have shown gelation in whey or soy protein stabilized emulsions, respectively by the addition of CaCl₂ to screen the charge and reduce electrostatic repulsive forces among the droplets.

2.4.3.3 Attraction due to change in pH

As discussed in section 2.3.2, change in pH to pI induce strong attraction between the protein-stabilized emulsion droplets. The electrostatic charge on the droplets with pH near isoelectric point gets canceled and causes a decrease in the repulsive barrier and a very low secondary minimum. Chen et al. showed emulsion gel formation at pH near the isoelectric point of casein (4.6) (Chen et al., 1999). They also reported that the elastic modulus of emulsion with pH 4.6 was higher than emulsion at any other pHs. They observed no gelation at pHs higher than 5.8 and lower than 3.2. Perrechil and Cunha (2010) SC-stabilized emulsions have found to have higher
stability at pH near pI due to gelation induced by attractive interactions compared to emulsions with pH away from pI. Another common example of pH-induced attractive gelation is cottage cheese where cheese formation is achieved by the addition of acid to milk.

2.4.4 Repulsive emulsion gels

Repulsive gelation in conventional emulsion is influenced by dispersed phase volume fraction, droplet size and charge (McClements, 2005). The critical droplet volume for this to happen is commonly known as volume fraction for maximal random jamming ($\phi_{MRJ}$) (Wilking & Mason, 2007). Using theoretical calculation, it can be showed that for a monodispersed emulsion $\phi_{MRJ}$ would be 0.64 while for polydisperse emulsion it would be even higher as the smaller droplets can fit into the interstices of larger droplets (Groot & Stoyanov, 2011). Repulsive gelation in NEs is also influenced by similar properties as in conventional emulsions, although in this case, gelation can occur at a much lower oil volume fraction due to the additional effect of repulsive shell layer around nanodroplets (Wilking & Mason, 2007). It was proposed that with a decrease in droplet size, the charge cloud thickness around each droplet significantly influenced the overall size of the droplets, leading to a substantial increase in the effective volume fraction ($\phi_{eff}$) of the dispersed phase. When $\phi_{eff}$ reaches a critical level where the droplet surface touch each other due to close packing, the NEs form a solid-like gelled structure. The $\phi_{eff}$ of NEs can be calculated from the effective radius of the droplets ($r_{eff}$) which is the sum of actual droplet radius ($r$) plus the shell layer thickness due to the charge cloud ($\delta$) (Weiss & McClements, 2000; Wilking & Mason, 2007):

$$\phi_{eff} = \phi_{core} \left(1 + \frac{\delta}{r}\right)^3$$  \hspace{1cm} (2.3)

Where $\phi_{core}$ is the actual oil volume fraction. To understand the effect of shell layer thickness on $\phi_{eff}$, Eq. (2.3) is plotted with various droplet size in Figure 2.6 for different shell layer thickness where $\phi_{core}$ is 0.4. It can be observed that as the droplet size decreased below 100 nm, $\phi_{eff}$ rapidly increased. It even reaches a value of 0.81 for a shell layer thickness of 20 nm at droplet size of 175 nm. This shows that decrease in droplet size increases the effect of shell layer thickness on $\phi_{eff}$ that may lead to close packing of droplets and gelation in NEs at a lower actual oil volume fraction. In an electrostatically-stabilized emulsion, the shell layer thickness can be estimated from the thickness of the electrical double layer created by the charge cloud around the droplets (Erramreddy & Ghosh, 2015; Erramreddy & Ghosh, 2014; Wilking & Mason, 2007). As there are repulsive interactions among the droplets, these nanogels are called repulsive nanogels (Datta et
It was also found that addition of salt to electrostatically stabilized nanogels causes gel breakdown because salt reduces the thickness of the electrical double layer, which results in a decreased effective radius of the droplet and ultimately weakening the gel structure (Fryd & Mason, 2012).

In sterically-stabilized NEs (e.g., droplets coated with proteins), the steric barrier also acts as a shell layer around the droplets. In protein-stabilized NEs, various factors can act in combination to make a nanogel. These factors can be protein’s electrostatic charge as well as steric barrier, the related increase in effective volume fraction due to this barrier assisted by the presence of protein-stabilized droplets (Dickinson, 2012a; Mason et al., 2007; Wilking & Mason, 2007).

**Figure 2.6** Calculated values of $\phi_{\text{eff}}$ for emulsion at different droplet sizes and droplet interfacial shell layer thickness of 2, 10 and 20 nm at 40 wt% ($\phi_{\text{core}}$) oil volume fraction using equation 2.3.

### 2.4.5 Gelation in protein stabilized emulsions and nanoemulsions

Emulsions with gelled texture are termed as emulsion gels (Dickinson, 2012a). Gels formed in protein-stabilized emulsions can be divided into two types: emulsion-filled protein gel and protein-stabilized emulsion gel (Dickinson, 2012a). In emulsion-filled protein gels, the emulsion droplets are entrapped in a protein gel structure (Figure 2.7A). Whereas protein-stabilized emulsion gels are particulate gels formed by the network of aggregated protein-coated droplets (Figure 2.7B) (Dickinson, 2012a). Emulsion gels commonly found in food applications are a combination of both of these two types, e.g., yogurt prepared from homogenized milk, where both casein gel in the...
continuous phase and milk fat globules coated with proteins take part in forming the network structure (Dickinson, 2013).

In emulsion-filled protein gels certain processing steps are required in order to induce protein gelation in the continuous phase, e.g., heat-induced gelation in whey protein-stabilized emulsions (Dickinson & Yamamoto, 1996), lowering of pH during yogurt manufacturing, and enzyme treatment in cheese making (Dickinson, 2012a). In protein-stabilized emulsions, protein covered droplets can also act as active fillers, which mechanically connect with the gel matrix to increase the gel strength (Dickinson, 2012a, 2013). At a higher dispersed phase volume fraction, gel formation may occur due to jamming of droplets and depletion attraction by excess protein in the continuous phase. Moreover, if the protein is highly charged and the counterion cloud around the droplets become significant compared to the droplet radius (for NEs), the effective droplet size and volume fraction rapidly increased, which can lead to jamming and gelation at much lower droplet volume fraction. This type of gelation in NE ($\phi = 0.4$) was observed for ionic small molecular weight emulsifiers (Erramreddy & Ghosh, 2014; Wilking & Mason, 2007). A gelation behavior has also been observed for highly concentrated conventional protein-stabilized emulsions ($\phi > 0.7$) with droplet size around 0.5 µm (Dimitrova & Leal-Calderon, 2001). Moreover, it was found that longer hydrophobic tail of polymeric surfactants can also significantly contribute to the viscoelasticity of emulsions (Wulff-Pérez et al., 2013) Polymeric surfactants with different lengths of hydrophilic tail and hydrophobic chain were used. The polymeric surfactant with longer hydrophilic tail showed better gel strength compared to ones with shorter tail. In the proposed research similar approach of using combined steric and electrostatic barrier will be taken to develop protein-stabilized NE gels.
2.4.6 Application of emulsion gels & nanogels in food & related soft matter

Many foods are made of emulsions gels (weak or strong) including mayonnaise, yogurt, salad dressing, cottage cheese etc. Mayonnaise is a good example of a food with a high viscosity and gel strength achieved mainly due to high dispersed phase volume fraction (~0.8). The ability to achieve gel or retain gel properties even after significantly decreasing dispersed phase oil volume would help in the development of low-fat products with no other additives and same texture/flow behavior as regular product. Other than food, emulsion gels have also been used in pharmaceutical industry to deliver drugs and other medicinal substances. Anti-inflammatory gel-like Voltaren® Emulgel is already available in North American market indicating the significance of emulsion gels in the pharmaceutical industry (MediResource). Nanogels are also being used for anti-inflammatory creams in India as Volini Nanogel with claims of quick absorption of cream upon application.

2.5 Choice of materials

2.5.1 Emulsifier

As discussed before, gelation in NEs have previously been obtained by small molecular weight emulsifiers. However, the nanogels made with small molecular weight emulsifiers are not edible. For application in food or nutraceutical industry, it is essential to use food-grade emulsifiers. Food proteins can be ideal emulsifiers for this purpose due to their amphiphilic nature provided by hydrophilic and hydrophobic amino acids. Milk proteins: casein and whey protein, were chosen

Figure 2.7 Types of gelation in protein stabilized emulsions. A) emulsion-filled protein gel and B) protein-stabilized emulsion gel.
for this research. They are widely used as emulsifying agents in a variety of food products including beverages, ice creams, sports supplements, infant formula and coffee creamer (McClements, 2005). Despite obtained from same source (Milk), casein and whey protein have different structures. Casein has random coil-like structure whereas whey protein has globular structure. Moreover, the thickness of the interfacial layer for casein is found to be 10 nm while for whey, it is 2 nm. This difference in interfacial layer thickness will be ideal to study the effect on gelation behavior due to change in effective volume fraction. Sodium caseinate was used as emulsifier due to the ease of its solubility and good emulsification properties and whey protein isolate was used due to its higher whey protein content compared to whey protein concentrate.

2.5.2 Oil phase

Vegetable oils are ideal to use in edible formulation of NEs/nanogels. Canola oil was utilized as the oil phase as Saskatchewan is the largest producers of canola in Canada. Canola oil is also widely used as cooking oil in Canada. It has also been used in the development of nanogels from NEs by Erramreddy and Ghosh (2014).
CHAPTER 3: EFFECT OF PROTEIN TYPE, CONCENTRATION AND OIL VOLUME FRACTION ON THE FORMATION AND LONG-TERM STABILITY OF NANOGELS

3.1 Abstract

Gelation behavior of sodium caseinate (SC) and whey protein isolate (WPI)-stabilized nanoemulsions was investigated as a function of protein (2 – 5 wt%) and oil (30 and 40 wt%) concentration and storage time. Nanoemulsions (NEs) were prepared by multiple cycles of high-pressure homogenization at 20,000 psi and characterized by droplet size distribution and rheology. Only SC was able to form nanogel where gel strength increased with increase in protein and oil concentration and decrease in droplet size. When plotted as a function of droplet size, storage moduli (G') of all nanoemulsions with a constant oil concentration merged into a single line, confirming the critical importance of droplet size on nanogelation. Surprisingly, WPI-stabilized nanoemulsions did not form nanogels and no effect of droplet size on gelation behavior was observed, although their droplet sizes were similar to those with SC. DLVO inter-droplet interaction potential for both the nanoemulsions were calculated. SC-stabilized nanodroplets showed significantly higher repulsive interaction and inter-droplet separation due to a larger steric barrier, which led to a higher effective oil volume fraction (\(\phi_{\text{eff}}\)) and random jamming of nanodroplets giving rise to a strong gelation behavior. Repulsive interaction among the WPI-stabilized nanodroplets and their inter-droplet separation was much lower due to a smaller steric layer, leading to a lower \(\phi_{\text{eff}}\) and much lower gel strength. Re-plotting the G' data with \(\phi_{\text{eff}}\) merged all data points for SC NEs with different protein and oil concentration into a single line, confirming the driving force of inter-droplet separation and increased \(\phi_{\text{eff}}\) behind the nanogelation process. The NEs also showed stability of their viscosity and gelation behavior as a function of time for an experimental period of 3 months. The protein-stabilized nanogels developed from NEs could be used as a novel soft material for various food and related applications where a lower oil phase volume fraction and long-term stability is required.
3.2 Introduction

Emulsions are a mixture of two immiscible liquids (oil and water) in which one phase is dispersed as droplets into the other. Oil-in-water (O/W) emulsion is an integral part of many foods, such as, milk, coffee creamer, and mayonnaise. Generally, dispersed oil droplet size in various foods are in the range of 1-100 µm. If average droplet diameter of an emulsion is less than 200 nm, it is termed as nanoemulsion (NE) (Mason et al., 2007; Tadros et al., 2004). NEs have displayed unique optical properties (Fryd & Mason, 2012) and rheological behavior (Erramreddy & Ghosh, 2015; Erramreddy & Ghosh, 2014), higher stability (Tadros et al., 2004; Yerramilli & Ghosh, 2017), and improved bioavailability of bioactives dissolved in the oil phase during digestion (Huang et al., 2010). Previous research has also shown that a reduction of average droplet size in the emulsion to less than 200 nm converts them to a gel, the so-called nanogel (Erramreddy & Ghosh, 2014; Wilking & Mason, 2007). Wilking & Mason (2007) showed the effect of droplet size on the rheology of sodium dodecyl sulfate (SDS)-stabilized silicone oil-in-water emulsions with 40% oil. They observed that decrease in droplet size below 150 nm led to a significant increase in storage moduli $G'$ of monodisperse nanoemulsions yielding close-packed gels (nanogels). SDS is a small molecular surfactant which primarily gives stable emulsions due to their electrostatic charge and formation of a repulsive barrier around the droplets. They attributed the gelation to an increase in the effective volume fraction ($\phi_{eff}$) of the dispersed phase due to the presence of repulsive charge cloud that forms an interfacial layer around the nanodroplets leading to a close packing and gelation (Wilking & Mason, 2007). Similar findings have also been reported by Erramreddy & Ghosh (2015) for polydisperse SDS-stabilized NEs made with canola oil and had surface average droplet size ($d_{32}$) less than 200 nm. They observed that reduction in droplet size led to gelation in NEs despite having same oil and emulsifier concentration. Depending on SDS concentration, these were repulsive gels formed due to close packing of droplets or attractive gels formed by micelle-induced depletion interaction (Erramreddy & Ghosh, 2015).

The influence of charge cloud-induced interfacial layer thickness on the $\phi_{eff}$ can be calculated by the equation (Weiss & McClements, 2000):

$$\phi_{eff} = \phi_{core} \left(1 + \frac{\delta}{r}\right)^3$$

(3.1)

where $\phi_{core}$ is the actual dispersed phase volume fraction, $r$ is the droplet radius, and $\delta$ is the thickness of the interfacial layer. From Eq. 3.1, it can be derived that the decrease in droplet
size or a significant increase in the interfacial layer thickness could increase the value of $\phi_{\text{eff}}$, such that the droplets along with their charge cloud may make a close-packed structure by random jamming which may provide gel-like properties to an emulsion. For monodisperse emulsions, the critical volume fraction that induces this maximum random jamming (MRJ) is 0.64, also known as $\phi_{\text{MRJ}}$. For a polydisperse emulsion, $\phi_{\text{MRJ}}$ is higher than 0.64, as smaller droplets can fit in the interstices of larger droplets. Even if the $\phi_{\text{core}}$ of a NE is much lower than the $\phi_{\text{MRJ}}$, the increase in $\phi_{\text{eff}}$ beyond $\phi_{\text{MRJ}}$ would lead to a close-packed structure, converting them into a nanogel. For example, Erramreddy & Ghosh (2014) have reported that due to the repulsive interfacial layer around the nanodroplets, the $\phi_{\text{eff}}$ of their NEs (with a $\phi_{\text{core}}$ of 0.4) reached above 0.7 due to the additional volume of the repulsive interfacial charge cloud around the nanodroplets, thereby inducing repulsive gelation in the NEs.

So far, researchers have shown nanogel formation by the reduction of droplet size in SDS stabilized NEs. However, SDS is not edible which hinders the application of these nanogels in foods and pharmaceuticals. Hence, there is a need to investigate gelation behavior in NEs prepared with an edible emulsifier. Therefore, the objective of this research is to investigate the gelation behavior in food protein-stabilized NEs and to understand the effect of protein type, concentration and oil volume fraction on the formation and long-term stability of the nanogels. To our knowledge, no study so far has investigated formation and stabilization of nanogels from protein-stabilized NEs. In the present work, we have used two dairy proteins, sodium caseinate (SC) and whey protein isolate (WPI) as emulsifiers as they have very different structures in solution as well as at the oil droplet surface (Dickinson, 2001). It is well known that SC has a disordered flexible random coil structure in solution, whereas whey proteins have a predominant globular structure (Dickinson, 1992, 1998). SC forms a thick interfacial layer around oil droplets with a portion of the molecule extended into the aqueous phase, while whey proteins form a thinner but stronger interfacial layer due to their compact globular structure (Færgemand & Murray, 1998; Hu et al., 2003). It is expected that the difference in interfacial structure of these two proteins will significantly influence the gelation behavior of their NEs.
3.3 Materials and methods

3.3.1 Materials

Canola oil was purchased from local grocery store. Milli-Q™ water (Millipore Corporation, MA, USA) was used for the preparation of continuous aqueous phase. SC was purchased from Sigma Aldrich, ON, Canada. Whey protein isolate (WPI) was a gift from Fonterra (USA) Inc., IL, USA. Sodium dodecyl sulfate (SDS) was purchased from Fisher Scientific (Nepean, ON, Canada). All the other chemicals were purchased from Sigma Aldrich (ON, Canada).

3.3.2 Preparation of nanoemulsions

Solution with different concentrations (2 – 5 % w/w) of either SC or WPI were prepared by stirring overnight on a benchtop stirrer. All quantities mentioned in this research were taken by weight percent. For simplification, we will mention it as % instead of % w/w. Sodium azide was added to solution at the rate of 0.02% to inhibit any microbial growth. A coarse oil-in-water emulsion was initially prepared using a rotor/stator mixer (Polytron, Brinkmann Instruments, ON, Canada) for 1 minute at 20,000 rpm after adding a respective quantity of oil (30 or 40%) to the protein solutions. NEs were then prepared by passing these coarse emulsions through a high-pressure homogenizer (EmulsiFlex-C3, Avestin Inc., Ottawa, ON, Canada) at 20,000 psi (137.9 MPa) pressure for 8 cycles. The homogenization was carried out for 8 cycles to reduce the droplet size into the nanoscale (average diameter less than 200 nm). The maximum number of passes were limited to 8th cycle as the viscosity of some NEs after this cycle were too high to flow through the homogenizer. Homogenization was carried out at room temperature, however, during homogenization temperature of the emulsions reached 55 - 60 °C towards the final cycle. All emulsions were transferred into 120 ml glass bottles (VWR International, Edmonton, AB) and stored at room temperature (25 ± 2 °C) for 3 months.

3.3.3 Droplet size distribution

The droplet size distribution and the surface mean diameter ($d_{32}$) of the emulsions collected during each homogenization cycle were determined using a static laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada) with a relative refractive index of the dispersed to continuous phases of 1.465. Samples were gently mixed before addition to sampling cell to take a uniform sample. A few drops of samples were added to the cell, mixed
and pumped through the analysis chamber to get a proper dilution with de-ionized water for diffraction analysis before starting the measurement.

3.3.4 Determination of viscosity

Viscosity of the emulsions was measured by a AR-G2 rheometer (TA Instruments, Montreal, QC, Canada). A 40mm cross-hatched parallel plate geometry was used for viscosity analysis to eliminate any wall-sleep during analysis. Liquid samples were loaded on the Peltier plate of the rheometer with a pipette and the nanogel samples were loaded with a spatula. Viscosity analysis was carried out by rotational shear between the two parallel plates at 25°C with a gap of 1000 µm and as a function of increasing shear rate from 0.01 to 1000 s⁻¹. A pre-shear was applied at 2 s⁻¹ shear rate for 10 seconds before the viscosity analysis to eliminate the effect of sample history before loading.

3.3.5 Determination of viscoelasticity

Viscoelastic behavior of all the samples were analyzed by oscillatory measurement on the same AR-G2 rheometer (TA Instruments, Montreal, QC, Canada). Geometry and sample loading procedure was similar to the viscosity measurement. Oscillatory strain sweep was applied at a constant frequency of 1 Hz and an increasing strain from 0.01% to 100% at 25°C. The storage ($G'$) and loss modulus ($G''$) of the samples were recorded with the Rheology Advantage Software (TA Instruments, Montreal, QC, Canada). A pre-shear was also applied at 2 s⁻¹ shear rate for 10 seconds before the strain sweep analysis to eliminate any sample history.

3.3.6 Visual observation of gelation

Samples of emulsions after the final cycle of homogenization were filled in 40 ml glass vials. The vials were put horizontally to visually observe and record the flow behavior of strong and weak gels and liquid NEs with a digital camera. The picture was taken after putting the vials horizontal for 30 seconds.

3.3.7 Freeze-Fracture Cryo-Scanning Electron Microscopy (FESEM)

Nanostructure of the nanogels and NEs were recorded using a FESEM at the University of British Columbia (UBC) Bioimaging Facility. Cryogenic imaging of the selected NE sample was done with a Hitachi S-4700 field emission scanning electron microscope (FESEM) equipped with an Emitech K1250x cryo-preparation unit. Samples were pipetted in quantity of 10-20 µL either a)
into a fracture rivet or b) directly into a slot of a copper sample holder, covered with a high-pressure freezing (HPF) hat, then plunged into sub-cooled liquid nitrogen. After freezing the holder was transferred at liquid nitrogen temperatures to the preparation stage of the Emitech K1250x, where the hat was popped off before transferring to the SEM cryo-stage. All steps of sample preparation post-freezing were done under high vacuum. When required, samples were sublimed at -90°C on the cryo-stage of the FESEM, until structures of interest were seen by direct observation. Samples were sputter coated with gold (10mA, 5e^{-1} mbar) before imaging. Images were taken at different magnification to get best results.

3.3.8 Zeta potential

Zeta potential measurements were carried out by Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). The sample was diluted (2 drops of sample in 50 ml DI water) and filled in the measurement cell, which was loaded to the Zetasizer for analysis. The zeta-potential was measured by the electrophoretic mobility of the protein-coated droplets towards an oppositely charged electrodes in an electric field under a certain applied voltage.

3.3.9 Long-term stability of the nanogels

The nanogels in glass bottles were stored for 3 months (90 days) at room temperature. Droplet size, viscosity, and viscoelasticity of the samples were measured at an interval of 1 month over the period of 3 months. The droplet size was observed to check the stability of emulsion against coalescence and flocculation. The rheological analysis (viscosity and viscoelasticity) was carried out to investigate any change in flow behavior and gel strength of the nanogels over time.

3.3.10 Statistics

All samples were prepared, and experiments were performed with at least three replicates (n=3) and the statistical significance of the data was analyzed to determine the statistical significance at a 95% confidence level using single factor ANOVA function available in Microsoft Excel (Microsoft Canada Co, Mississauga, ON, Canada).
3.4 Results and discussion

3.4.1 Average droplet diameter and size distribution

Figure 3.1 shows the effect of homogenization cycles on surface average mean diameter ($d_{32}$) of NEs. A decrease in average droplet size was observed with an increase in homogenization cycle except for the 2% SC NEs with 30% oil and 2% WPI NEs with 40% oil. This indicates that repeated homogenization cycles further rupture the larger droplets into smaller ones until a limit of homogenizer and emulsifier efficiency was reached. The increase in droplet size beyond 5th cycles for the emulsions mentioned above indicates lack of enough proteins to cover smaller droplets as the interfacial area increased with a decrease in their size. This might lead to either
coalescence or bridging flocculation which was reflected in an increased droplet size as the droplet aggregates are perceived as one large droplet by the particle size analyzer. The types of droplet destabilization were confirmed by measuring the droplet size after the NEs were diluted with SDS solution, which would replace the proteins from the oil droplet surface. Therefore, a reduction in droplet size would indicate breakdown of droplet aggregates and the presence of bridging flocculation. However, no change in droplet size upon dilution with SDS would imply re-coalescence of the droplets during homogenization. Based on the droplet size analysis results with SDS, it was found that the increase in droplet size with the number of homogenization cycles for SC NEs (Figure 3.1B) was due to re-coalescence, while for WPI (Figure 3.1C) it was due to droplet aggregation (more discussion on this can be found under Figure 3.3).

**Figure 3.2** Effect of protein concentration on the final emulsion mean droplet size ($d_{32}$) after 8 cycles of homogenization with (A) SC with 40% (circle) and 30% (triangle) oil concentrations or with (B) WPI with 40% (square) and 30% (diamond).

Figure 3.2 shows the effect of protein concentration on the average droplet size of the final NEs homogenized for eight cycles. For both SC and WPI, droplet size decreased with an increase in protein concentration. In SC NEs, the droplet size decreased with an increase in SC concentration for both 30 and 40% oil from $290 \pm 15.6$ nm and $310 \pm 103.2$ nm, respectively at 2% SC to $154 \pm 2.8$ nm and $186 \pm 4.2$ nm, respectively at 4% SC, thereafter no such decrease was observed at 5% SC (Figure 3.2A). For WPI NEs with 40% oil, a much larger size ($644 \pm 103.2$ nm) was observed at 2% protein, followed by steep decrease to $241.5 \pm 6.4$ nm at 3% WPI, and a slow but steady decrease to $169 \pm 4.2$ nm at 5% WPI (Figure 3.2B). The WPI NEs with 30% oil, however, showed
a much smaller droplet size even at 2% protein (209.5 ± 4.9 nm) which decreased to 145 ± 0.0 nm at 5% WPI (Figure 3.2B). As emulsions with average droplet diameter less than 200 nm are considered as NEs, it can be said that the emulsions with 30% oil produced NEs at higher than 3% proteins, while for 40% oil it started with 4% proteins for both SC and WPI. The droplet size distributions of the final NEs (after 8 cycles of homogenization) with different protein and oil concentrations are shown in Figure 3.3.

Figure 3.3 Droplet size distribution for SC and WPI NEs with different protein (2 – 5%) and oil concentrations (30 & 40%). SC NEs with (A) 40% and (B) 30% oil, and WPI NEs with (C) 40% and (D) 30% oil are shown.
3.4.2 Viscosity as a function of protein type, concentration and oil volume fraction

![Graphs showing viscosity as a function of shear rate for SC NEs and WPI NEs with different oil concentrations.]

**Figure 3.4** Effect of shear rate on the viscosity of SC NEs with (A) 40% and (B) 30% oil, and WPI NEs with (C) 40% and (D) 30% oil with various aqueous phase protein concentrations 2 (circle), 3 (triangle), 4 (rectangle) and 5 (diamond)%. Error bars are removed for clarity. Note: the range of y-axis is different for SC and WPI NEs.

Change in viscosity of the final emulsions with different protein and oil concentration as a function shear rate is shown in Figure 3.4. All SC NEs shows clear shear-thinning behavior irrespective of their oil concentration. Viscosity is increased with increase in SC concentration and for any specific protein concentration viscosity of 40% oil NEs was higher than the 30% oil NEs. A high-shear plateau can be observed for 2% SC NEs with both 30 and 40% oils (Figure 3.4A,
3.4B), indicating Newtonian fluid-like behavior and loss of structural features at higher shear rates. On the other hand, viscosity of emulsions with 3 – 5% SC concentration showed a continual decrease even at a high shear rate, signifying the existence of structural features that are still broken down. These emulsions with 3 – 5% SC also showed a higher shear thinning behavior (larger drop in viscosity with shear) compared to 2% SC emulsions.

Contrary to SC NEs, WPI NEs showed a much less viscosity and shear-thinning behavior which is visible from the Y-axis scale difference in Figure 3.4C, and 3.4D and change in magnitude of viscosity at a lower shear rate. Among the WPI NEs, higher shear thinning behavior was observed for 40% oil, while for 30% oil, except for low shear range below 1 s\(^{-1}\), virtually no shear thinning was observed, and the emulsions behaved like Newtonian fluid. At that lower shear range there was a noticeable difference in viscosities of the WPI NEs with difference in WPI concentration. However, in the higher shear range (greater than 1 s\(^{-1}\)) the difference became negligible which might be attributed to complete break-up of structure yielding similar viscosity.

To better compare the effect of protein type and concentration on viscosity, values of viscosities at 1 s\(^{-1}\) shear rate from Figure 3.4 was re-plotted in Figure 3.5. The viscosity values were compared at 1 s\(^{-1}\) because at very low shear, the data showed significant variation due to rheometer limitation, whereas, at higher shear rates, the values could not represent ideal viscosity behavior of the NEs due to breakdown of original structure. From Figure 3.5, it can be seen that viscosity of the SC NEs increased with an increase in SC concentration for both the oil concentrations. However, very little change in viscosities of the WPI NEs was observed with an increase in WPI concentration. For both the proteins, NEs with 40% oil have higher viscosity compared to the ones with 30% oil at the same protein concentrations which must be due to the presence of higher dispersed phase volume fraction preventing the flow of emulsions. Similar increase in viscosity with oil volume fraction was also observed by many researchers. For example, Quemada and Berli have discussed the modeling of an increase in viscosity of emulsions with an increase in the volume fraction of charged dispersed phase (Quemada & Berli, 2002). Pal (1996) has shown increased yield stress in petroleum oil-in-water emulsion stabilized by Triton X-100 (non-ionic surfactant) upon increase in the volume fraction from 0.55 or 0.65 to 0.76.
Figure 3.5 Effect of protein concentration on the apparent viscosity at 1 s\(^{-1}\) shear rate for NEs made with SC (filled symbols) containing 40% (circle) and 30% (triangle) oil concentrations and with WPI (open symbols) containing 40% (square) and 30% (diamond) oil concentrations.

To understand the effect of emulsion droplet size on their viscosity data from Figure 3.5 (viscosities at 1 s\(^{-1}\) shear rate) were re-plotted as a function of \(d_{32}\) for all samples collected during every homogenization cycles (Figure 3.6). Viscosity of all SC emulsions \((d_{32} < 500 \text{ nm})\) with different droplet sizes and protein concentrations merged on a single line for each oil concentration and increased with a decrease in droplet size, indicating the importance of droplet size on viscosity (Figure 3.6A). The plotted data for emulsions with different oil concentrations followed a power law relationship with a correlation of 0.83-0.85 between droplet size and the NE viscosity. The viscosity of emulsions with 40% oil remained more than an order of magnitude higher than the 30% oil containing emulsions at all droplet size below 500 nm. Viscosity of SC emulsions with average droplet size greater than 500 nm were from the 1\(^{\text{st}}\) and 2\(^{\text{nd}}\) pass of homogenization. Their droplet size distribution was highly polydisperse and contained multimodal peaks (data not shown), which significantly differs from the main trend in viscosity and gelation behavior as a function of droplet size. Contrary to SC emulsions, no such effect of droplet size on viscosity was observed for WPI emulsions (Figure 3.6B). Viscosity varied between 0.001 to 1 Pa.s for all samples with different oil and protein concentrations when the droplet size ranged from 800 to less than 200 nm.
Figure 3.6. Effect of average droplet size ($d_{32}$) on the viscosities (at $1\text{s}^{-1}$ shear rate) of all NEs made with (A) SC with 40% (circle) and 30% (triangle) oil concentrations or with (B) WPI with 40% (square) and 30% (diamond) oil concentrations for different cycles of homogenization. The line represents a power law model fit to the experimental data for SC NEs with $d_{32} < 500$ nm ($R^2$ is 0.82 and 0.90 for 40% oil and 30% oil, respectively).

Increase in viscosity and shear thinning behavior with a decrease in droplet size was also observed for concentrated ($\phi = 0.745$) petroleum oil-in-water emulsions stabilized by a non-ionic small molecule surfactant (Pal, 1996). It was proposed that viscosity of concentrated emulsion depends on the electrical double layer and adsorbed stabilizing layer around the droplets, hydration or solvation effect of the droplets and their Brownian motion (if the droplet size is considerably less than 1 $\mu$m). As the extent of these various phenomena depends on droplet size, viscosity of concentrated emulsions would also be significantly affected. In a subsequent study using the same emulsion composition, Pal (2000) also showed that the effect of droplet size on emulsion viscosity depends on their oil volume fraction. At $\phi < 0.6$, when the emulsion showed Newtonian behavior droplet size did not have any effect of viscosity, however, for $\phi > 0.6$, for non-Newtonian emulsions, viscosity significantly increased when the droplet size decreases from 21.4 $\mu$m to 4.6 $\mu$m. In the present case, a maximum $\phi = 0.4$ was used, however, the creation of droplets less than 0.5 $\mu$m significantly influenced emulsion viscosity only when a flexible random coil protein (SC) was used as emulsifier compared to a globular compact protein (WPI).
3.4.3 Viscoelasticity

Oscillatory strain sweep measurements were carried out on all the final emulsion samples after 8 passes of homogenization to understand the effect of oil volume fraction, protein type and concentration on their viscoelastic behavior (Figure 3.7). The graphs show measured $G'$ (storage modulus) and $G''$ (loss modulus) plotted against % strain. Values of $G' > G''$ indicates gelation in emulsion, whereas $G'' > G'$ indicates no gelation or a broken-down gel due to high shear. A common behavior shown by most the graphs is a near-linear or increasing region of $G'$ at lower strains which suddenly declined at a certain strain known as yield strain. The values of $G'$ continuously dropped after yield strain where $G''$ showed a peak followed by a crossover of $G'$ and $G''$ indicating gel breakdown and liquid-like behavior at higher strain. Other researchers have also observed this peak in $G''$ and attributed it to the relaxation of gel structure made by random jamming of nanodroplets before strain-induced breakdown (Datta et al., 2011; Koumakis & Petekidis, 2011).

NEs with 2-3% SC and 40% oil (Figure 3.7A, 3.7B) showed $G'$ higher than $G''$ in the low strain regime indicating gel formation, but a lack of clear linear viscoelastic region (LVR) reveals weak gel-like behavior. On the other hand, the NEs with 4 and 5% SC (Figure 3.7C, 3.7D) showed higher $G'$ and the presence of LVR below 10% strain, indicating stronger gel formation (the so-called nanogel). All SC NEs with 30% oil showed weak gel behavior due to a lack of LVR.

Contrary to SC, all NEs made with WPI showed very weak (for 2-3% WPI) to no gelation at all for 4 and 5% WPI with 40% oil. Moreover, for the WPI NEs with 30% oil, the instrument could not measure the values of $G'$ at lower strain indicating no structure formation. The available results for these NEs showed $G''$ being higher than $G'$ which indicates no gel formation.
Figure 3.7 Effect of Protein concentration (2-5%) on the strain-sweep viscoelasticity of NEs stabilized by SC with 40% (circle, A - D) and 30% (triangle, E - H) oil or WPI with 40% (square, I - L) and 30% (diamond, M - P) oil for final NEs after 8 passes of homogenization. G' (filled) and G'' (open) were measured as function of % strain at a constant frequency of 1 Hz.

For better presentation and ease of understanding, the values of G' and G'' data at 0.15% strain from Figure 3.7 was re-plotted as a function of protein concentration in Figure 3.8. The G' values at 0.15% strain falls in the linear viscoelastic region before the yield strain so these values are representative of an un-disturbed gel structure. Moreover, 0.15% strain is significantly higher to avoid any variation occurring in the G' values due to the sensitivity of the rheometer at a lower strain region. For all SC NEs, G' was higher than G'' (Figure 3.8A). For SC NEs with 40% oil, G' and G'' increased with an increase in SC concentration. However, for SC NEs with 30% oil, G' and G'' increased with protein concentration until 4% SC, thereafter decreased for 5% SC. This
indicates that SC concentration has a key role in NE gelation. In contrast, WPI NEs with 40% oil showed a decrease in G' with increase in WPI concentration. The values of G'' was lower than G' for 2 and 3% WPI, at 4% WPI G' and G'' were almost equal to each other and 5% G'' was higher than G' signifying no gelation behavior of the WPI NEs at high protein concentration. For WPI NEs at 30% oil no values of G' could be recorded except for 2% WPI, which showed G' = G''. For these NEs the values of G'' continuously decreased with an increase in WPI concentration. Comparing the NEs with two different oil concentrations, we can also notice that G' and G'' are higher for NEs with 40% oil than that for 30% oil at all protein concentrations.

**Figure 3.8.** Effect of protein concentration on storage (G') (filled) and loss (G'') (open) moduli at 0.15% strain for the NEs made with (A) SC for 40% (circle) and 30% (triangle) oil and (B) WPI with 40% (square) and 30% (diamond) oil (Note: different Y-axis scales for SC and WPI). No G' data could be determined for WPI NEs with 30% oil except 2% WPI.

To understand how the droplet size of the protein-stabilized NE influences their gelation behavior, values of G' at 0.15% strain for all NEs collected from all cycle of homogenization was plotted against their $d_{32}$ values in Figure 3.9. For SC NEs with both 40 and 30% oil, an increase in G' can be noticed with a decrease in droplet size below 500 nm which also followed a simple power law relationship (Figure 3.9A). However, there was no significant change in G' for all NEs made with WPI (Figure 3.9B). Similar behavior was also observed for the change in viscosity as a function of droplet size (Figure 3.6). The observation that G' of different SC NEs with different droplet size follows a single line reveals that the governing factor for gelation of SC NEs are their droplet size. Nevertheless, a few SC emulsions with droplet size > 500 nm did not follow the trend. These emulsions were obtained after the first cycle of homogenization and were not very stable.
Perhaps emulsion breakdown during a few hours of storage and during strain sweep analysis led to inconsistent results of G' and G".

**Figure 3.9** Effect of droplet size ($d_{32}$) on storage modulus (G') at 0.15% strain for all NEs made with SC with 40% (circle) and 30% (triangle) or WPI with 40% (square) and 30% (diamond) oil. Data for all samples from different homogenization passes are plotted. The line represents a power law model fit to the experimental data for SC NEs with $d_{32} < 500$ nm ($R^2$ is 0.78 and 0.68 for 40% oil and 30% oil, respectively).

Dimitrova and Leal-Calderon (2001) studied elasticity of highly concentrated protein-stabilized emulsions (average droplet diameter 0.5 µm). At an oil volume fraction of ~0.7, the emulsions stabilized with β-casein, β-lactoglobulin (BLG) and bovine serum albumin (BSA) showed highly elastic behavior with a G' values ranging from 4,000 to 20,000 Pa, higher than what we have recorded for our nanogels. However, at a sufficiently high strain when the value of G' dropped, the emulsions did not flow, but exhibited shear-induced coalescence (Dimitrova & Leal-Calderon, 2001). In the present case, much less oil volume fraction was used and the nanodroplets were more resistant to coalescence due to the less external pressure from the lack of actual close packing and very high internal Laplace pressure of nanoscale droplet size. The nanodroplets’ surface didn’t touch even when they were repulsively jammed (for SC NEs) as the charge cloud and interfacial protein layer around the droplets were enough to prevent close interaction among the SC-stabilized nanodroplets. Comparing the elasticity of the different protein-stabilized emulsions, Dimitrova and Leal-Calderon (2001) found that the bulk elasticity of the emulsions
correlated with the interfacial elasticity. As BSA and BLG formed a much stronger interface compared to casein, their ability to prevent droplet deformation is also much higher, hence the bulk elasticity of BSA and BLG-stabilized emulsions was also higher than the casein-stabilized emulsions. This observation is in contrast with our data, where SC NEs formed stronger gels, while the NEs stabilized with WPI (made of BSA and BLG) did not provide any elasticity. This points out that the interfacial elasticity is not a critical factor in our protein-stabilized nanogel formation, rather the combined effect of decrease in droplet size and increased interfacial repulsive barrier are important, the calculation for which is shown in 3.4.7.

3.4.4 Visual observation of flow behavior

![Visual observation of flow behavior](image)

**Figure 3.10** Visual observation of flow behavior of NEs with 40% oil and (A) 3%SC, (B) 4% SC, (C) 5% SC and (D) 5% WPI after keeping the vials horizontal for 30 seconds.

The visual observation of selected NEs with different flow behavior was carried out and shown in Figure 3.10. The NEs selected were SC NEs with 3, 4 and 5% SC and WPI NE with 5% WPI (all with 40% oil) to show difference in flow behavior with protein concentration and type. SC NE with 3% SC showed liquid-like flow behavior and flowed completely covering the whole length of the vials when laid horizontally (Figure 3.10A). SC NEs with 30% oil and the ones with
2% SC and 40% oil showed similar flow behavior as in SC NE with 3% SC. SC NE with 4% SC showed limited flow behavior where it flowed to only a certain distance and tried to retain its original shape (Figure 3.10B), which indicates a weak gel behavior. However, SC NE with 5% SC did not show any flow and retained its shape in the vial exhibiting a strong gel behavior (Figure 3.10C). Compared to this gelled NE with 5% SC, NE with 5% WPI exhibited liquid-like flow behavior despite similar protein and oil concentration (Figure 3.10D). It flowed freely to cover the complete length of the vial. All other WPI NEs showed similar flow behavior shown in Figure 3.10D. The visual observation of the NEs’ flow behavior gave us an important understanding of their gelation, but it is also essential to understand the microstructure that is responsible for this drastic difference of flow behavior with different protein concentrations and protein types.

3.4.5 Nanostructure of nanoemulsions and nanogels

To understand the difference in the gel structure, NEs used towards the visual observation was also used for Cryo-SEM analysis. SEM image of 3% SC NEs (Figure 3.11A) shows droplets scattered at distance in the continuous phase which show lack of any type of close packing in the microstructure. The thin layer covering some of the droplets in the Figure 3.11A is a solid phase material that has separated during the freezing process and agglomerated into sheets. It could be due to some droplet coalescence as the interface was not fully covered with SC. SEM images of SC NEs with 4% SC shows loosely packed droplets with different droplet size. This loosely packed microstructure could be responsible for its weak gel behavior (Figure 3.7C) and some resistance to flow as reported in visual observation (Figure 3.10B). 5% SC NEs (Figure 3.7D) showed droplets arranged in a highly close-packed structure resembling the packing of corn kernels on a cob, which could be the reason behind the strong gel behavior of this NE. The strong close packing of droplet provides a firm structure to the gel and stops any movement of droplets or continuous phase. This justifies the lack of any flow for 5% SC NEs (Figure 3.10C). Contrary to NEs with 5% SC, 5% WPI NEs showed droplets distributed at a distance in the continuous phase (Figure 3.11D) similar to the 3% SC NEs. This led to a flow behavior similar to the NEs with 3% SC due to the lack of close packing and the ability of the droplets to freely move in the continuous phase.
Long-term stability of the nanogels

The long-term gel stability of the protein-stabilized nanogels were investigated by measuring their rheological behavior as a function of time for up to 3 months. Droplet size of the nanogels were also determined in that time to ensure their emulsion stability. For this purpose, all SC NEs with different protein and oil concentrations were used, while only the WPI NEs with 40% oil was used as a comparison. WPI NEs with 30% oil were not tested for long-term gel stability analysis as these NEs did not form any gel structure.

Figure 3.11 Cryo-SEM images of NEs with (A) 3% SC, (B) 4% SC, (C) 5% SC and (D) 5% WPI with 40% oil. All the samples are sublimed and sputter coated with gold (10mA, 5e⁻¹ mbar). The images with ideal magnification are presented to show proper nanostructure where A, B & D are at 10kx magnification and C is at 25kx magnification.

3.4.6 Long-term stability of the nanogels

The long-term gel stability of the protein-stabilized nanogels were investigated by measuring their rheological behavior as a function of time for up to 3 months. Droplet size of the nanogels were also determined in that time to ensure their emulsion stability. For this purpose, all SC NEs with different protein and oil concentrations were used, while only the WPI NEs with 40% oil was used as a comparison. WPI NEs with 30% oil were not tested for long-term gel stability analysis as these NEs did not form any gel structure.
**Figure 3.12** Change in average droplet size ($d_{32}$) (A - C), viscosity (D – F) and $G'$ (H - J) of SC NEs with 40% oil (A, D, H), 30% oil (B, E, I) and WPI NEs with 40% oil (C, F, J) with increase in storage time for protein concentrations of 2 (circle), 3 (triangle), 4 (square) and 5 (diamond)% over the period of 3 months at an interval of 1 month.

### 3.4.6.1 Droplet size of the nanogels as a function of time.

The average droplet size ($d_{32}$) of the NEs with different protein and different oil concentrations are plotted against time in Figure 3.12. SC NEs with 40% oil and 2% SC showed an increase in droplet size with time from $333.7 \pm 29.5$ nm for the fresh NEs to $470.6 \pm 56.0$ nm after 1 month ($p < 0.05$) but did not change after 1st month ($p > 0.05$). (Figure 3.12A). SC NE with 3% SC also showed a slight increase in droplet size over the span of 3 months from $199.3 \pm 13.6$ to $263.3 \pm 62.0$ which was statistically insignificant ($p > 0.05$), whereas NEs with 4 and 5% SC did not show any change in average droplet size over the period of 3 months (Figure 3.12A). Similar
to SC NEs with 40% oil, 2% SC NEs with 30% oil also showed increase in droplet size from 217.0 ± 5.3 nm for fresh NEs to 301.6 ± 24.6 nm after 3 months (p < 0.05) (Figure 3.12B). For SC NEs with 3, 4 and 5% SC no significant change in droplet size with an increase in storage time was observed (p > 0.05) (Figure 3.12B). A common observation is that the increase in droplet size over time was only seen in the emulsions with droplet size of 200 nm or above, demonstrating the significance of nanoscale droplet size on the long-term stability of the NEs. For WPI NEs with 2% protein an increase in average droplet size was observed from 470.7 ± 55.5 on day 0 to 518.7 ± 37.5 after 3 months (p > 0.05) (Figure 3.12C). All other WPI NEs with higher protein concentration did not show any significant change in average droplet size as a function of time (p > 0.05) even though for 3 and 4% WPI NEs average droplet size was above 200 nm (Figure 3.12C).

3.4.6.2 Effect of long-term storage on the rheological behavior of the nanoemulsions

The viscosity and viscoelasticity of the NEs were measured to record the change in rheology and gel strength as a function of time. To compare the change in flow behavior, viscosity values of the NEs at 1 s⁻¹ shear rate were plotted against time for the NEs with different oil and protein concentrations in Figure 3.12 D-F. The viscosity of all the SC NEs with 40% and 30% and WPI NEs with 40% oil did not show any significant change over the period of 3 months (p > 0.05) (Figure 3.12 D-F).

The change in gel strength of the NEs was recorded by plotting the G' values at 0.15% strain from the strain-sweep data as a function of time (Figure 3.12 G-I). Similar to viscosity, gel strength of all the SC NEs with both 40% and 30% oil and all WPI NEs with 40% oil did not change significantly with time (p > 0.05), except for the NEs with 3% SC for 30% oil concentrations, which showed a decrease over time (p < 0.05).

From the long-term stability data, it can be said that most of the NEs and nanogels (especially with 4 & 5% protein) showed remarkable stability in viscosity and gel strength as a function of time over 3 months. This behavior, however, is in stark contrast with the rapid loss of gel strength for SDS-stabilized nanogels observed by Erramreddy et al. (2017). The authors proposed that canola oil oxidation in the NEs led to the generation of surface active components which altered the interfacial composition and reduced the thickness of the charge cloud and corresponding effective droplet volume fraction. This resulted in a reduction in G’ loss of gel strength. To prove this, they prepared SDS-stabilized nanogels with mineral oil instead of canola
oil and showed that using a non-oxidizable oil prevented loss of gel strength with time (Erramreddy et al., 2017). In the present case, nanogels were prepared with canola oil, but stabilized with proteins instead of SDS. SDS is a non-ionic small molecule emulsifier and are known to attract pro-oxidant metal cations from the aqueous phase thereby emulsions stabilized with SDS are susceptible towards lipid oxidation (Mei et al., 1998). On the other hand, milk proteins SC and WPI are known to prevent lipid oxidation in emulsion (Coupland & McClements, 1996). SC is regarded as the most effective antioxidant protein in milk due to its ability to form thick protective layer (Allen & Wrieden, 1982) around oil droplets. Oxidative stability of O/W emulsion stabilized by proteins is also influenced by the presence of specific amino acids in SC and WPI which are known to act as antioxidants (Hu et al., 2003). Moreover, proteins can also scavenge pro-oxidant metal ions from the aqueous phase thereby help prevent lipid oxidations (Tong et al., 2000). These stabilization effect of proteins against lipid oxidation might be the principle reason behind the long-term stability of the nanogels observed in the present research. It is also notable that NEs were visibly more stable at refrigeration temperature for even longer period compared to room temperature.

3.4.7 Mechanism of gelation in protein-stabilized nanoemulsions

Gelation in NEs could be originated from either repulsive or attractive interactions among the nanodroplets. In repulsive nanogels such an increase in viscosity and gel strength with reduction of droplet size has been ascribed to the influence of effective volume fraction ($\phi_{\text{eff}}$), which increased due to the increase in the ratio of the thickness of the interfacial layer ($\delta$) to the droplet radius ($r$) according to Eq. 2.1 (Weiss & McClements, 2000). For ionic emulsifiers, such as SDS, the interfacial shell layer thickness around the nanodroplets was associated with a charge cloud around them which prevented their close interaction, thereby increasing the effective size and volume fraction (Erramreddy & Ghosh, 2014).

In the present case, the NEs with different types of protein showed vastly different rheological properties. Here the interface is made of either SC or WPI, which are known for their ability to stabilize droplets by both electrostatic and steric stabilization mechanisms (Berli et al., 2002). The steric barrier of the proteins plays an important role in forming the interfacial layer and keeping the nanodroplets away from each other thereby further increasing the repulsive interaction and interfacial layer thickness. Nevertheless, the presence of excess of SC or WPI in the continuous
phase of the emulsions are also known to induce depletion attraction among the oil droplets leading to a rapid creaming or gelation behavior (Dickinson, 2010; Dickinson & Golding, 1997; Dickinson et al., 1997; Rosa et al., 2006; Yerramilli & Ghosh, 2017). The concentration of excess protein ($C_{\text{excess}}$) in our emulsions was determined by calculating the amount of protein required to saturate the total interfacial area ($A_s$) and subtracting from the total protein ($C_{\text{total}}$) according to the following equation:

$$C_{\text{excess}} = C_{\text{total}} - \Gamma_s A_s \quad (3.2)$$

Where $\Gamma_s$ is the surface load of proteins which are the quantities needed to fully cover a surface. Surface load of SC and WPI has been well documented in literature as 3 mg/m$^2$ and 2 mg/m$^2$, respectively (Berton-Carabin et al., 2014; Dickinson & Golding, 1997). $A_s$ was calculated from $6\phi/d_{32}$ (McClements, 2005; McClements & Dungan, 1993). Our calculation showed that SC NEs with both 40 and 30% oil did not have any excess protein in the continuous phase at any protein concentration. Therefore, all SC molecules were utilized to cover the high surface area of the nanoscale droplets. This lack of excess protein indicates no possibility of depletion interaction in all SC NEs. However, for WPI NEs, positive values of $C_{\text{excess}}$ was observed with 2 and 5% protein and 40% oil and 4 and 5% protein with 30% oil (Table 3.1). Excess proteins in the continuous phase of WPI NEs at higher protein concentrations could be due to the lower surface load of WPI compared to SC. For 40% oil and 2% WPI, the calculation of excess protein would not indicate the actual value as the average droplet size taken in the calculation as it does not give representative result due to bridging flocculation occurring in this particular emulsion (discussed in 3.4.1). The droplet aggregates are perceived as one large droplet in the size analysis which gave an under-estimation of interfacial area and protein needed for surface coverage. Hence the excess protein concentration was over-estimated. For the values of excess protein in the continuous phase, depletion osmotic pressure ($\Pi$) in the bulk phase was calculated to determine the effect of excess protein on droplet interaction according to (Berli et al., 2002):

$$\Pi = n k_B T \left(1 + 2 \frac{C_{\text{excess}}}{\rho_{sm}} \right) \quad (3.3)$$

where $n$ is number density of the macromolecules; $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $C_{\text{excess}}$ is concentration of protein in the aqueous phase and $\rho_{sm}$ is submicelle
density. \( n \) was calculated from \( n = \frac{C_{\text{excess}} \cdot N_A}{M} \), where \( N_A \) is the Avogadro number and \( M \) is the molecular weight of the submicelle. The values of \( \Pi \) was further used to calculate the depletion forces and its effect on the overall inter-droplet interaction according to Eq. 3.6 given below. As most NEs didn't have any excess protein (hence no depletion attraction between the droplets), they are expected to have strong repulsive interaction.

Table 3.1 Calculated values of excess protein concentration in the aqueous phase \( (C_{\text{excess}}) \) of WPI-stabilized NEs (from Eq. 3.2) and bulk osmotic pressure \( (\Pi) \) due to the presence of excess free proteins in the aqueous phase (from Eq. 3.3) as a function of protein and oil concentration. No excess protein was obtained in the calculation for SC-stabilized NEs.

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The repulsive steric layer thickness for SC and WPI at the oil-water interface has been documented by several researchers using various techniques (Dalgleish, 1993; Dickinson, 1992; Nylander et al., 1999). Random coil SC molecule is known to form a thick interfacial steric barrier where the tail part of the molecule extends towards the aqueous phase for 8 - 10 nm length (Atkinson et al., 1995; Dalgleish, 1993; Dickinson et al., 1993), which combined with its electrostatic repulsive barrier could be responsible for their enhanced \( \phi_{\text{eff}} \) and increase in viscosity and gel strength with a decrease in droplet size. However, for WPI NEs, no such increase in viscosity and gel strength \( (G') \) with decrease in droplet size was observed, indicating no significant contribution of the interfacial layer and droplet size towards the \( \phi_{\text{eff}} \). Previous research has reported that thickness of globular WPI at the interface was about 2 nm (Nylander et al., 1999). It was hypothesized that lower interfacial layer thickness of WPI could be responsible for its lower
effective droplet size and $\phi_{\text{eff}}$ even though the electrostatic repulsive barriers still exist, leading to a considerably lower viscosity and gel strength of the WPI NEs compared to SC NEs. 

To prove these hypotheses, we calculated the overall DLVO inter-droplet interaction potential beyond the steric protein layer and determined how that influence the overall repulsive barrier among the droplets stabilized by SC or WPI. It was assumed that the van der Waals, electrostatic and depletion interactions begin at the outer surface of the adsorbed protein layer around the droplets. Steric interactions due to adsorbed protein, on the other hand, starts from the oil droplet surface and its effect was taken into consideration by known values of thickness as described above. A schematic diagram of two droplets approaching each other with their steric layer and electrostatic is shown in Figure 3.13. The DLVO interaction potential as a function of distance between two droplets was calculated by combining the electrostatic repulsion ($W_{\text{ele}}$), van der Waals attraction ($W_{\text{vdw}}$) and depletion attraction ($W_{\text{dep}}$) forces according to the following equations (Berli et al., 2002):

\begin{align}
W_{\text{ele}} &= 2\pi\varepsilon\psi_0^2 a \ln\{1 + \exp[-\kappa (R - 2a)]\} \quad (3.4) \\
W_{\text{vdw}} &= -\frac{A_H}{6} \left[ \frac{2a^2}{R^2-4a^2} + \frac{2a^2}{R^2} + \ln \left( \frac{R^2-4a^2}{R^2} \right) \right] \quad (3.5) \\
W_{\text{dep}} &= -\frac{4\pi}{3} (a + L)^3 \left[ 1 - \frac{3R}{4(a+L)} + \frac{R^3}{16(a+L)^3} \right] \Pi; \quad \text{for} \ R < 2(a + L) \\
&= 0; \quad \text{for} \ R \geq 2(a + L) \quad (3.6)
\end{align}

Eq. 3.4 gives the electrostatic repulsion as a function of the distance between the centers of two droplets, $R$, where $\varepsilon$ is the electric permittivity of the medium ($5.404 \times 10^{-10} C^2 N m^2$), $\psi_0$ is the surface potential, $\kappa$ is the inverse Debye length. $a$ is the radius of the droplets including the steric layer thickness of the adsorbed protein, calculated by $a = \frac{d_{z2}}{2} + \Delta$, where, $\Delta$ is thickness of the steric layer. A similar approach was also used by Berli et al., (2002), where the authors assumed that the surface charge of the droplets is placed at the onset of the steric wall. In fact, the values of zeta potential at the stern layer measured by electrophoresis for droplets under infinite dilution was assumed to be equivalent to their surface potential, $\psi_0$. The value of $\psi_0$ have been taken from
previously calculated interdroplet interactions in emulsions for droplets coated with sodium caseinate (-40 mV) (Berli et al., 2002) and β-lactoglobulin (-42 mV) (Kim et al., 2002).

The Debye length ($\kappa^{-1}$) was calculated from the following equation (Ghosh, 2009):

$$
\kappa^{-1} = \left[ \frac{N_A e^2}{\epsilon_0 \varepsilon_0 k_B T} \sum_i z_i^2 c_i^{\infty} \right]^{-1/2}
$$

(3.7)

where, $\varepsilon_0$ is the permittivity of air ($8.854 \times 10^{-12} \text{C}^2 \text{J}^{-1} \text{m}^{-1}$), $e$ is the charge of an electron ($1.602 \times 10^{-19} \text{C}$), $z_i$ is valency of ion $i$ and $c_i^{\infty}$ is the bulk concentration of ion $i$ (mol/m$^3$). In aqueous medium, at 298 K, $\frac{N_A e^2}{\epsilon_0 \varepsilon_0 k_B T}$ can be simplified into $5.404 \times 10^{15} \text{m}$. Therefore, Eq. 3.7 can be written in a simplified form as:

$$
\kappa^{-1} = 1.38 \times 10^{-8} \left[ \sum_i z_i^2 c_i^{\infty} \right]^{-1/2}
$$

(3.8)

To calculate the term within the parenthesis type, valency and concentration of counterions in the aqueous solution of the proteins are required. For SC, all the counter ions are in the form of sodium and its concentration is supplied by the manufacturer (10 mg sodium/g protein powder). It was assumed that all the sodium ions would be free in the continuous phase of the emulsion. In actual case, some sodium ions may be bound to the protein molecule and attached to the droplet surface, thereby will not take part in Debye screening. However, due to lack of data on SC counter ion dissociation factor, we are underestimating the values of $\kappa^{-1}$. The counterion concentration (mol/m$^3$) at various $\phi$ and protein concentration and the values of corresponding Debye screening length ($\kappa^{-1}$) are given in Table 3.2. For WPI, the supplier specification indicated the presence of various ions (Na$^+$, K$^+$, Ca$^{2+}$, Cl$^-$ and PO$_4^{3-}$) at different concentrations (Table 3.3). From this data, the values of $\sum_i z_i^2 c_i^{\infty}$ was estimated for various $\phi$ and protein concentration and the Debye screening length ($\kappa^{-1}$) was calculated using Eq. 3.8 (Table 3.3). The calculated values of $\kappa^{-1}$ were utilized in determining the electrostatic repulsive forces as a function of distance between two droplets using Eq. 3.4.

Eq. 3.5 gives the van der Waal interactions between the droplet, where $A_H$ is Hamaker constant taken as $4 \times 10^{-21} \text{J}$ for oil droplets dispersed in aqueous phase without considering the effect of protein layer at the oil droplet surface (Berli et al., 2002). Although the presence of protein
at the droplet surface can modify the van der Waals interaction at short distances, it was assumed to be insignificant for repulsive barrier calculation in the present case.

Figure 3.13 Schematic diagram of oil droplets covered with SC and WPI indicating parameters used in calculation of interdroplet interaction potential and effective volume fraction. \( r \) = droplet radius, \( \Delta \) = thickness of Steric Barrier, \( x \) = thickness of Repulsive charge cloud, \( \delta \) = total interfacial thickness, \( R \) = distance between centres of two droplets, \( L \) = depleting protein species size.
Table 3.2 Parameters for the calculations of Debye screening length ($\kappa^{-1}$) and effective oil volume fraction ($\phi_{eff}$) of SC NEs. Only the final emulsions prepared after 8 passes of homogenization was considered for this calculation.

<table>
<thead>
<tr>
<th>Oil conc. $\phi_{core}$ (wt%)</th>
<th>Protein conc. (mol/m³) *</th>
<th>Counterion conc. (mol/m³) *</th>
<th>$\kappa^{-1}$ (nm)</th>
<th>Inter-droplet distance at $1 \ k_B T$ ** (nm)</th>
<th>$\delta^+$ (nm)</th>
<th>$\phi_{eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
<td>5.5</td>
<td>25</td>
<td>22.5</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.1</td>
<td>4.5</td>
<td>19.6</td>
<td>19.8</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.2</td>
<td>3.9</td>
<td>16.6</td>
<td>18.3</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15.2</td>
<td>3.5</td>
<td>14.8</td>
<td>17.4</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.2</td>
<td>5.9</td>
<td>26.8</td>
<td>23.4</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.8</td>
<td>4.9</td>
<td>20.2</td>
<td>20.1</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.4</td>
<td>4.2</td>
<td>17.2</td>
<td>18.6</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>13.0</td>
<td>3.8</td>
<td>15</td>
<td>17.5</td>
<td>0.72</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from quantity of ions present and their valency (Eq. 3.6)
** Calculated as distance from overall DLVO potential at $1 \ k_B T$ (Eq. 3.9)
$^+\delta = \text{Thickness of Steric Barrier (}\Delta\text{)} + \text{Thickness of Repulsive charge cloud (}x\text{)}$
$\Delta = 10 \text{ nm for SC}$
$x = \text{half of total inter-droplet distance at } 1 \ k_B T$

Eq. 3.6 is for the calculation of depletion interaction between two droplets when excess proteins in the continuous phase act as depleting species. In the present case in the depleting species are present only for globular whey proteins which themselves carry an electrostatic charge and hence surrounded by an electric double layer ($\kappa^{-1}$). The size of the protein species is denoted as L, which is calculated from $L \approx r_{sm} + bk^{-1}$, where $r_{sm}$ is size of sub-micelle and b is a constant (Berli et al., 2002). From Figure 3.13, depletion interaction would be active and the depleting species are excluded from the inter-droplet region if $R < 2(a + L)$. L is also considered to be the effective exclusion thickness around the droplets. The difference in osmotic pressure between the depleted zone and the bulk solution is denoted by $\Pi$, which was calculated by Eq. 3.3 (Berli et al., 2002).
Table 3.3 Type and concentration of ions present in WPI for the calculations of Debye screening length ($\kappa^{-1}$) and effective oil volume fraction ($\phi_{eff}$) of WPI NEs. Only the final emulsions prepared after 8 passes of homogenization was considered for this calculation.

<table>
<thead>
<tr>
<th>Ions in WPI</th>
<th>Emulsion composition</th>
<th>Counterion conc. (mol/m³) * $\sum z_i^2 c_i^\infty$</th>
<th>$\kappa^{-1}$ (nm)</th>
<th>Inter-droplet distance at 1 $k_B T^{**}$ (nm)</th>
<th>$\delta^+$ (nm)</th>
<th>$\phi_{eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>5.86</td>
<td>2 7.2 5.08 23.2 13.6 0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>0.57</td>
<td>3 10.7 4.15 17.8 10.9 0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.8</td>
<td>4 14.3 3.59 16.4 10.2 0.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>0.12</td>
<td>5 17.9 3.21 14.7 9.4 0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>0.55</td>
<td>4 12.3 3.88 15.4 9.7 0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from quantity of ions present and their valency (Eq. 3.6)
** Calculated as distance from overall DLVO potential at 1 $k_B T$ (Eq. 3.9)
$\delta^+$ = Thickness of Steric Barrier ($\Delta$) + Thickness of Repulsive charge cloud ($x$)

The overall interaction potential as a function of distance between two droplets ($R$) was calculated by adding Eq. 3.4, 3.5 and 3.6 as:

$$W = W_{ele} + W_{vdw} + W_{dep} \quad (3.9)$$

$W$ was divided by $k_B T$ to obtain a dimensionless energy parameter $W/k_B T$. All NEs showed significant repulsive interaction within a certain distance between the droplet. As the droplets approach each other, the strength of the repulsion increased. It was assumed that a value of repulsive interaction of one times the $k_B T$ would be enough to prevent close interaction between the droplets and stop them from touching their steric wall. The inter-droplet distance at one times $k_B T$ is calculated from Eq. 3.9 and reported in Table 3.2. Half of this value was taken as the thickness of charge cloud ($x$) around each droplet. It can be seen that the thickness of the charge cloud around a droplet at a repulsive barrier of one-time $k_B T$ decreased with increase in protein concentration in the emulsions. The values are also comparable for SC and WPI. The repulsive charge cloud length calculated here is a little more than twice their Debye length. Although, by
definition, Debye length is the distance for the electrostatic repulsion to reduce to 1/e of their surface potential, the repulsive interaction can still be significant at a distance greater than the Debye length (Weiss and McClements 2000). In fact, Mondain Monval et al. (Mondain-Monval et al., 1996) showed that for SDS-stabilized emulsions, the repulsive interaction between the droplets could be significant at a distance 2.4 times the Debye length.

The overall repulsive barrier ($\delta$) around a droplet is therefore a combined effect of interfacial protein steric layer ($\Delta$) and electrostatic charge cloud ($x$):

$$\delta = \Delta + x$$  \hspace{1cm} (3.10)

The steric layer thickness ($\Delta$) was taken as a constant value, which is 10 nm for SC, and 2 nm for WPI as discussed above. Finally, the effective oil droplet volume fraction ($\phi_{\text{eff}}$) was calculated from Eq. 3.1 using the values of $\delta$ reported in Table 3.2 and 3.3.

From Table 3.2, for SC NEs $\phi_{\text{eff}}$ increased to 0.54 – 0.58 when the $\phi_{\text{core}}$ was 0.3 at various protein concentrations. However, as these values are still significantly lower than the $\phi_{MRJ}=0.64$ for monodispersed emulsions, 30% oil containing emulsions did not show any sign of strong gel formation. It should be noted that our emulsions were polydisperse, therefore, it is expected that the critical volume fraction of random jamming of oil droplets would be even higher than 0.64. For $\phi_{\text{core}} = 0.4$, calculated values of $\phi_{\text{eff}}$ increased with an increase in protein concentration, and for 3, 4 and 5% SC, reached 0.69, 0.70 and 0.72, respectively. Such a high value of $\phi_{\text{eff}}$ would certainly be close to or may even be higher than the critical volume fraction required for random jamming of polydisperse droplets. This explains why these NEs had a high value of $G'$ and form nanogels. In contrast, the maximum value of $\phi_{\text{eff}}$ for WPI NEs reached only 0.53, due to the lower values of the WPI steric layer leading to a lower overall repulsive barrier ($\delta$) compared to SC NEs. This effective volume fraction is insufficient to induce droplet jamming and corresponding gelation behavior of the NEs.
**Figure 3.14** Effect of effective volume fraction on storage modulus of NEs stabilized with SC (filled circle) and WPI (open triangle). Inset shows schematic diagram of droplet close packing in nanogels stabilized with SC (on the right) and corresponding liquid-like WPI-stabilized NEs (on the left).

The calculated values of $\phi_{\text{eff}}$ from all replicates were plotted against $G'$ to understand the driving force for gelation in the NEs (Figure 3.14). For SC NEs, an increase in $G'$ was observed with an increase in $\phi_{\text{eff}}$. A gradual increase in $G'$ could be observed between 0.63 to 0.68 $\phi_{\text{eff}}$ followed by a sudden increase after 0.68 indicating transition into nanogel. WPI NEs were unable to achieve that high $\phi_{\text{eff}}$ due to the lower thickness of the interfacial layer, which explains lower values of $G'$ and the lack of gel formation. From these findings, we can derive that the formation of thicker steric interfacial layer of SC around the nanodroplets was the main factor behind the difference in gelation behavior between these two protein-stabilized NEs. A similar behavior of efficient steric barrier of polymeric emulsifiers in increasing emulsion viscoelasticity was also reported by Wulff-Pérez et al. (2013). Using synthetic copolymers, made of polyethylene oxide-polypropylene oxide-polyethylene oxide blocks, the authors showed that the as the length of the hydrophilic polyethylene oxide tail increased the viscosity and $G'$ of the oil-in-water emulsions.
(with 65% oil) also increased. However, importance of oil droplet size was not investigated as all the emulsions had a very similar average droplet size (~300 nm).

3.5 Conclusion

In conclusion, an increase in viscosity and gelation behavior was observed with an increase in protein and oil concentration and a decrease in droplet size for SC NEs, while no such change in rheology was observed for WPI NEs. All SC NEs with 30% and 40% oil with at 2-4% protein showed weak gelation behavior, while at 5% protein, the NEs transformed into strong gel which did not flow under gravity. In contrast, all WPI NEs showed very weak to no gelation behavior at all protein and oil concentrations. The SEM micrographs of the 5% SC NEs with 40% oil showed a close-packed droplet structure like corn kernels on a cob, while for all other NEs the nanodroplets were loosely packed and far apart from each other. Most of the NEs showed remarkable stability of their viscosity and viscoelasticity as a function of time for an experimental period of 3 months. Considerable higher gel strength of SC NEs compared to WPI NEs and the increase in their viscosity and gelation behavior as a function of decrease in droplet size was attributed to an increased effective volume fraction due to a higher shell layer thickness originated from the combined repulsive steric barrier and charge cloud. The DLVO interdroplet potential was used to calculate the shell layer thickness and the effective oil droplet volume fraction ($\phi_{\text{eff}}$) at an overall repulsive interaction of $1 \ k_BT$. It was found that the gel strength of SC NEs increased with an increase in $\phi_{\text{eff}}$ and for the nanogels reached a value above 0.7, which could be enough to induce random jamming of the nanodroplets and repulsive gelation. In contrast, $\phi_{\text{eff}}$ for WPI NEs remained below 0.55 due to the lower value of the WPI steric barrier which could be responsible for weak to no gelation behavior even when the droplet size was less than 200 nm. The protein-stabilized nanogels developed from NEs could be used as a novel soft material for various food and related applications where a lower oil phase volume fraction and long-term stability is required.
CHAPTER 4: EFFECT OF SALT CONCENTRATION, PH AND HEAT TREATMENT ON THE GELATION IN FOOD-PROTEIN STABILIZED NANOEMULSIONS

4.1 Abstract

The aim of this study was to investigate the possibility of forming nano-colloidal gelation by inducing attractive interactions among the nanodroplets. The effect of salt concentration, changes in pH and heat treatment on the stability and gelation behavior of 2 and 4% sodium caseinate (SC) and whey protein isolate (WPI)-stabilized 30 and 40% canola oil-in-water nanoemulsions (NEs) were investigated. For effect of salt, sodium chloride was added in a concentration of 0.1, 0.5 and 1 M in the continuous phase of the NEs at neutral pH, while to study the effect of change in pHs, the NEs were adjusted to pH values below, at or above the pI of the proteins. For the effect of heat treatment, the samples were heated at 85°C for 30 minutes and cooled to room temperature. The addition of salt led to attractive gelation in WPI NEs due to a screening of charge, but no such effect was seen in SC NEs due to stronger repulsive steric interactions. Moreover, reduction in gel strength of 4% SC-stabilized nanogels was observed which can be attributed to the reduction close packing of droplets and their surrounding repulsive barriers due to screening of charge. All the NEs with pH at the pI of respective proteins transformed into strong attractive gels made of droplet aggregates irrespective of type or concentration of protein due to the complete charge neutralization. Heating the NEs did not induce any gelation despite WPI being a heat-labile protein, which was attributed to the lack of excess protein to induce gelation in the continuous phase and the presence of strong repulsive charge at the droplet surface. Overall, research on the effect of different environmental factors on the stability and gelation behavior of protein-stabilized NEs could be useful in possible application of these materials in various food systems.
4.2 Introduction

Emulsion is an integral part of many food and related soft materials. Milk, coffee creamer, and mayonnaise are some of the common examples of oil-in-water (O/W) emulsions. In food systems, dispersed phase droplet size are normally in the range of 1-100 µm. However, nanoemulsions (NEs), whose average droplet size is less than 200 nm have recently caught attention of researchers due to their several advantages over conventional emulsions. (Mason et al., 2007; Tadros et al., 2004). NEs are unique due to their significantly higher stability, different rheology, and decreased opacity.

Previous researchers have also shown that merely a reduction of average droplet size in the emulsion to nanoscale (< 200 nm) converted them to a gel, the so-called nanogel where gelation happened at a lower oil droplet volume fraction than that for a conventional emulsion (Erramreddy & Ghosh, 2014; Wilking & Mason, 2007). Wilking & Mason showed the effect of droplet size on the rheology of sodium dodecyl sulfate (SDS)-stabilized emulsions/nanoemulsions with silicone oil. They observed that a decrease in droplet radius below ~75 nm led to the formation of strong gel in monodisperse NEs. The authors attributed the gelation to an increase in effective volume fraction of the dispersed phase that led to a close packing of droplets due to their surrounding repulsive charge clouds (Wilking & Mason, 2007). Similar findings have also been reported by Erramreddy & Ghosh for polydisperse SDS-stabilized NEs made with canola oil that showed increase in viscosity and gel formation when surface average droplet size \(d_{32}\) was reduced to less than 200 nm. Depending on SDS concentration, these were repulsive nanogels formed due to close packing of droplets or attractive nanogels formed by micelle-induced depletion attraction (Erramreddy & Ghosh, 2015).

In the previous part of this research project, we have also shown that even protein can be used to form nanogels. For example, sodium caseinate (SC)-stabilized emulsions have shown an increase in G' with a decrease in average droplet size. The SC NEs prepared with 5% SC and 40% oil transformed into a nanogel at an average droplet size of 153 ± 13.23 nm (Chapter 3). The gelation at a dispersed phase of 40% is significantly lower than 64% needed for close packing in monodisperse emulsions (Berryman, 1983). The gelation in this SC NE at a lower oil volume fraction was attributed to the combined contribution of the thickness of the protein steric layer and the repulsive charge cloud around them that led to an increase in effective droplet volume fraction to more than 0.7. However, we could not form nanogel in the NEs with less than 40% oil and less
than 4% SC, presumably due to the inability to sufficiently reduce the oil droplet size. Moreover, when the NEs were formed with whey protein isolate (WPI), no gelation was observed at both 30 and 40% oil and 2 – 5% WPI, even if the average droplet size was less than 200 nm. The interdroplet interaction potential and effective droplet volume fraction calculation for these SC and WPI NEs, with weak to lack of gelation behavior, showed an effective volume fraction ranging from 0.69 – 0.54 and 0.53 – 0.42, respectively, which was not enough to induce gelation by repulsive jamming for polydisperse emulsions. The higher effective volume fraction for SC NEs was ascribed to the significantly higher steric layer of the flexible casein molecule compared to the compact globular structure of WPI. The lack of higher effective volume fraction was responsible for lack of droplet close packing and resulted in no or weak gelation in most of the NEs.

This led us to the primary aim of this study, to investigate the possibility of forming nanocolloidal gelation by inducing attractive interactions at a lower oil volume fraction and protein concentration by modifying the inter-droplet interactions than that obtained in Chapter 3. Therefore, the NEs that showed liquid-like or weak gel-like properties were selected for this study. SC NEs with 2 and 4% protein with 30 and 40% oil were selected as they showed weak gel behavior. Similar concentrations of WPI NEs were also selected for comparison between the two different proteins with different structures. These WPI NEs showed very weak to no gelation behavior at all. Three different approaches were used to induce attractive interactions among the nanodroplets: addition of salt, altering the pH and heat treatment and their influence on nanocolloidal gelation in SC and WPI NEs were investigated to achieve gelation in these NEs.

4.3 Materials and methods

4.3.1 Materials

Canola oil was purchased from local grocery store. Milli-Q™ water (Millipore Corporation, MA, USA) was used for the preparation of continuous aqueous phase. SC was purchased from Sigma-Aldrich, ON, Canada. WPI was a gift from Fonterra (USA) Inc., IL, USA. Sodium dodecyl sulfate (SDS) was purchased from Fisher Scientific (Nepean, ON, Canada). All the other chemicals were purchased from Sigma-Aldrich (ON, Canada).
4.3.2 Preparation of nanoemulsions

Protein solution with two different concentrations (2 and 4% w/w) of either SC or WPI were prepared on a benchtop stirrer by stirring overnight. Sodium azide was added to solution at the rate of 0.02% of aqueous phase to inhibit the microbial growth. The protein solutions were mixed with either 30 or 40% canola oil and a coarse oil-in-water emulsion was prepared using a rotor/stator mixer (Polytron, Brinkmann Instruments, ON, Canada) for 1 minute at 20,000 rpm. NEs were then prepared by passing these coarse emulsions through a high-pressure homogenizer (EmulsiFlex-C3, Avestin Inc., Ottawa, ON, Canada) at 20,000 psi (137.9 MPa) pressure for 8 cycles. The homogenization was carried out for 8 cycles to reduce the droplet size smaller than 200 nm. Homogenization was carried out at room temperature (24 ± 1 ºC), however, due to pressurized treatment during homogenization temperature of emulsions reached 55 - 60 ºC towards the final cycle. Emulsions were stored at room temperature. All quantities discussed in the paper are by weight basis and will be termed with ‘%’ instead of ‘% w/w’.

4.3.2.1 Preparation of samples to study the effect of Salt

10 g of NEs were transferred to plastic centrifuge tubes. Different quantities of salt (NaCl) were directly added to the vials to obtain a final salt concentration of 0.1, 0.5 or 1 M in the aqueous phase of the NEs. Samples were stirred with a vortex mixer to properly mix salt in the NEs. The samples were analyzed for droplet size, zeta potential, rheology and visual observation after overnight storage at room temperature.

4.3.2.2 Preparation of samples to study the effect of change in pH

10 g of NEs were transferred to plastic vials. Different quantities of HCl solutions (1 M) were added to the vials to obtain NEs with pH below and at the isoelectric points (pI) of the proteins used. For pI, the pH of the NEs was set to the pH to 4.6 for SC and 5.0 for WPI. For the samples with the pH below the pI it was set to 3.6 for SC and 4.0 for WPI. The original NEs at pH 7 were also equally diluted with de-ionized water to prepare the samples with pH above the pI. The final oil concentrations after dilution for 30% and 40% oil containing NEs were 29% and 39%, respectively. The samples were analyzed for droplet size, zeta potential, rheology and visual observation after overnight storage at room temperature.
4.3.2.3 Preparation of samples to study the effect of temperature

25 g of the sample was taken in 50 ml glass beakers. The samples were put in a heated water bath at 85°C for 30 minutes. The beakers were removed from the water bath and kept at room temperature overnight (~15 hr) before analyzing them for droplet size and rheology.

4.3.3 Droplet size distribution

The droplet size distribution and the surface mean diameter \(d_{32}\) of the NEs were determined using a static laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada) with a relative refractive index 1.465 for canola oil and 1.33 for water. Samples were gently mixed before addition to sampling cell to obtain a uniform sample and few drops were added to the measuring cell, mixed to get the desired dilution for proper laser diffraction before starting the analysis.

4.3.4 Zeta potential

Zeta potential measurements were carried out by Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA) by determining the electrophoretic mobility of the protein-coated droplets under a certain electric field. The sample was diluted (2 drops of sample in 50 ml DI water/ salt solution/ pH buffer) and filled in the electrode cell, which was loaded into the Zetasizer for analysis. For salt added NEs, samples were diluted with respective salt solutions. For the NEs at different pH values, dilution was done with specific pH buffer solution.

4.3.5 Viscoelasticity Analysis

Viscoelastic behavior of the NEs was analyzed using a AR-G2 rheometer (TA Instruments, Montreal, QC, Canada). A 40mm cross-hatched parallel plate geometry was used to avoid wall-sleep. Viscoelasticity was determined by applying oscillatory strain sweep from 0.01% to 100% strain at a constant frequency of 1 Hz at 25 °C. The analysis provided storage modulus \(G'\) and loss modulus \(G''\) as a function of applied strain. To eliminate any effect of sample history and loading, a pre-shear was applied at 2 s\(^{-1}\) shear rate for 10 seconds before the strain sweep analysis.

4.3.6 Visual observation of gelation

For visual observation, samples of NEs with different ionic strength and pH were prepared in 30 ml glass beakers. The beakers were tilted at a 45° angle and the flow behavior of the samples were recorded with a digital camera.
4.3.7 Compression analysis

Compression analysis was carried out for some pH-adjusted NEs (at pH = pI of the protein) with a firm gel texture, which could not be analyzed by the rheometer. Compression analysis was carried out by TA-XT plus Texture Analyzer (Stable Micro System, England) at room temperature with a cylindrical probe with ½ inch diameter. Samples were placed in a beaker (20 mm in height and 15 mm in diameter) and the probe was set to penetrate 5 mm from the surface of the gel at a rate of 10 mm/second to measure the peak force of gel compression.

4.3.8 Statistics

All samples were prepared, and experiments were performed with at least two replicates (n ≥ 2). Statistical significance of the data was analyzed at a 95% confidence level using a single factor ANOVA function available in Microsoft Excel (Microsoft Canada Co, Mississauga, ON, Canada).
4.4 Results and discussion

4.4.1 Effect of salt concentration on the gelation in nanoemulsions

4.4.1.1 Influence of salt concentration on droplet size and charge

Figure 4.1 shows the average droplet size of all the NEs at different concentration of salt in the aqueous phase. In all cases, average droplet size was smaller for 4% protein-stabilized emulsions, compared to 2% protein, which could be due to greater lowering of interfacial tension at higher emulsifier concentration and availability of more protein to cover increased surface area of smaller droplets. A similar effect of salt on the NEs with both, 30 and 40% oil can be seen, where a noticeable increase in the average droplet size with the addition of salt for the WPI NEs. However, the SC NEs did not show any significant change with an increase in salt concentration. These results indicate that the addition of salt has a profound effect on WPI NEs whereas it is virtually ineffective on SC NEs in the present concentration range of salt used in the aqueous phase.
This variation of droplet size also resonates with the droplet size distributions of the salt added NEs (Figure 4.2).

**Figure 4.2** Effect of addition of different concentrations of salt on the droplet size distribution of various NEs prepared with 40 and 30% oil and 2 and 4% SC and WPI.
In SC NEs, the addition of salt does not cause much change in droplet size distributions irrespective of protein and oil concentration (Figure 4.2 A-D). A slight increase in the peak above 1 µm was observed with an increase in salt concentration, while the peak below 1 µm showed a slight decrease. Such a change in droplet size distribution did not significantly influence surface average droplet diameter ($d_{32}$), while the volume average droplet diameter ($d_{43}$) was significantly affected (data not shown). For WPI NEs with 2% protein, droplet size distribution moved toward bigger size range even upon addition of 0.1 M salt, which led to a decrease of peak height at smaller droplet size and increase at larger droplet size upon increasing salt concentration (Figure 4.2E, 4.2F). For NEs with 4% WPI, addition of salt at 0.1 M did not significantly change the droplet size distribution, but 0.5 and 1 M salt led to multimodal droplet size distribution with a couple of large peaks at higher droplet sizes (1 – 1000 µm) while the peak below 1µm was significantly reduced (Figure 4.2G, 4.2H). The increase in droplet size for WPI NEs could be due to coalescence or flocculation. Flocculated droplets act as one large droplet in the laser diffraction particle size analyzer. To determine the exact mechanism of the increase in droplet size, 0.5% SDS solution was gently mixed with the emulsion in 1:5 ratio to break any flocs before droplet size analysis. As shown in Figure 4.3, the results demonstrated a significant shift in droplet size distribution to smaller sizes upon SDS addition, which indicates that the increase in droplet size was due to extensive droplet aggregation upon addition of salt.

![Figure 4.3](image-url)

**Figure 4.3** Effect of SDS addition on droplet size distribution of WPI NEs containing 40% oil and (A) 2% WPI and (B) 4% WPI. Data for the sample without SDS (dotted line) and after the addition of SDS (straight line) are shown.
The effect of salt addition on the screening of droplet charge was confirmed by measuring zeta potential (measure of charge on droplets) of the NEs. The results shown in Figure 4.4 supports the proposed hypothesis about the decrease in zeta potential of emulsions with the addition of salt irrespective of protein or oil concentration. The control NEs (NEs without any salt) had a zeta potential ranging from -50 to -70 mV. With the addition of only 0.1 M salt the zeta potential decreased to values ranging from -10 to -30 mV. With further increase in salt concentration up to 1M, only a slight decrease in zeta potential was observed and the minimum zeta potential was reported to be about -10 mV. From Figure 4.4, almost similar values of zeta potential for SC and WPI-stabilized NEs was observed at high salt concentrations, although extensive droplet aggregation was only observed for WPI NEs. This could be due to the difference in the structure of the proteins. As for SC, even after screening of charge by the addition of salt, the strong steric barrier by the hydrophilic tail of SC (which extends towards the aqueous phase for about 10 nm) would create enough steric repulsion between the droplets to stop them from causing attractive aggregation (Dickinson 1999). Stability of SC-stabilized emulsions against salt-induced aggregation has also been reported and explained by Dickinson in late 1990s (Casanova & Dickinson, 1998; Dickinson et al., 1998). Casanova and Dickinson (1998) showed excellent stability of mixed $\alpha_{s1}$-casein and $\beta$-casein stabilized emulsion against flocculation at an ionic strength as high as 2 M NaCl when about 40% of the adsorbed protein consisted of $\beta$-casein and

**Figure 4.4** Change in zeta potential of NEs made with 2 (open) & 4% (filled) protein, SC (circle), WPI (triangle), containing (A) 40% oil and (B) 30% oil as a function of increase in salt concentration in the aqueous phase of NEs.
up to 1 M NaCl (highest salt conc. in our samples) when 30% of the adsorbed protein consisted of \( \beta \)-casein. It was also showed that the emulsion stabilized with only \( \alpha_{s1} \)-casein was extensively flocculated above 0.1 M NaCl, while \( \beta \)-casein-stabilized droplets was not affected. It was ascribed to the mechanism of stabilization of the two casein proteins. While \( \alpha_{s1} \)-casein stabilized emulsions were stabilized by high surface charge density, the main stabilization mechanism of \( \beta \)-casein is steric repulsion. Hence, in presence of salt a net attraction prevails for \( \alpha_{s1} \)-casein-stabilized emulsions, while strong steric repulsion prevented flocculation among the \( \beta \)-casein-stabilized droplets. Sodium caseinate is known to contain roughly equal amount of both \( \alpha_{s1} \) and \( \beta \)-casein, hence the NEs in present research were stable against salt-induced flocculation (Dickinson et al., 1998). WPI, on the other hand, forms a compact globular structure at the oil droplet surface, extending only about 2 nm in the aqueous phase, which is probably not enough to prevent them from aggregation (Nylander et al., 1999). Increase in average droplet size of WPI-stabilized conventional emulsions due to the addition of salt and neutral pH has also been reported by many researchers which was attributed to the inability of WPI to prevent close approach of droplet due salt-induced charge screening (Demetriades et al., 1997; Kim et al., 2005).

A better understanding of the effect of salt concentration on the droplet aggregation behavior can be obtained from their DLVO interdroplet pair potential. To calculate it was assumed that the van der Waals attraction \( W_{vdw} \), electrostatic repulsion \( W_{ele} \) and depletion interaction \( W_{dep} \) begin at the outer surface of the adsorbed protein layer. The overall interaction potential as a function of distance between the two droplets along with their interfacial protein layer \( R \) was calculated from the following equations (Berli et al., 2002):

\[
W_{ele} = 2\pi\varepsilon\psi_0^2 a \ln\{1 + \exp[-\kappa (R - 2a)]\} \quad (4.1)
\]

\[
W_{vdw} = -\frac{A_H}{6} \left[ \frac{2a^2}{R^2-4a^2} + \frac{2a^2}{R^2} + \ln\left( \frac{R^2-4a^2}{R^2} \right) \right] \quad (4.2)
\]

\[
W_{dep} = -\frac{4\pi}{3} (a + L)^3 \left[ 1 - \frac{3R}{4(a + L)} + \frac{R^3}{16(a + L)^3} \right] \Pi; \text{ for } R < 2(a + L) \]

\[
= 0; \text{ for } R \geq 2(a + L) \quad (4.3)
\]

\[
W = W_{ele} + W_{vdw} + W_{dep} \quad (4.4)
\]
where \( \varepsilon \) is the electrical permittivity of the medium \((5.404 \times 10^{-10} C^2 N m^2)\), \( \psi_0 \) is the surface potential, \( \kappa \) is the inverse Debye length. \( a \) is the radius of the droplets including the steric layer thickness of the adsorbed protein, calculated by \( a = \frac{d_{32}}{2} + \Delta \), where \( \Delta \) is thickness of the steric layer, taken as 10 nm for SC and 2 nm for WPI (Atkinson et al., 1995; Dalgleish, 1993; Dickinson et al., 1993; Nylander et al., 1999). The value of \( \psi_0 \) have been taken as -40 and -42 mV for SC (Berli et al., 2002) and WPI (Kim et al., 2002), respectively. The Debye length \((\kappa^{-1})\) was calculated from the following equation (Ghosh, 2009):

\[
\kappa^{-1} = \left[ \frac{N_A e^2}{\varepsilon \varepsilon_0 k_B T} \sum_i Z_i^2 c_i^\infty \right]^{-1/2} \quad (4.5)
\]

where, \( \varepsilon_0 \) is the permittivity of air \((8.854 \times 10^{-12} C^2 J^{-1} m^{-1})\), \( e \) is the charge of an electron \((1.602 \times 10^{-19} C)\), \( Z_i \) is valency of ion \( i \) and \( c_i^\infty \) is the bulk concentration of ion \( i \) \((\text{mol/m}^3)\). In aqueous medium, at 298 K, \( \frac{N_A e^2}{\varepsilon \varepsilon_0 k_B T} \) can be simplified into \( 5.404 \times 10^{15} \) m. Therefore, Eq. 4.5 can be written in a simplified form as:

\[
\kappa^{-1} = 1.38 \times 10^{-8} [\sum_i Z_i^2 c_i^\infty]^{-1/2} \quad (4.6)
\]

To calculate the term within the parenthesis type, valency and concentration of the electrolytes in the aqueous solution of the emulsion are required. The electrolytes present in the proteins were calculated from the supplier provided data and the concentration of amount of salt \((\text{Na}^+ \text{ and Cl}^-)\) added in the NEs. Finally, the overall interaction potential, \( W \) in Eq. 4.4 was divided by \( k_B T \) to obtain a dimensionless energy parameter, \( W/k_B T \), and the values for all NEs at all salt concentration are plotted in Figure 4.5. All NEs without salt showed significant repulsive interaction within a certain distance between the droplet. As the droplets approach each other, the strength of the repulsion increased. For all NEs, addition of just 0.1 M salt significantly shifted the interdroplet potential from strongly repulsive to weakly attractive with the curve going through secondary minima in attractive interaction. The depth of the minima and the strength of the attractive interaction increased with increase in salt concentration, leading to droplet aggregation as seen in Figure 4.2. Beyond the secondary minima, on further approach, the droplets face a strong electrostatic repulsion, but at a much shorter interdroplet separation compared to no salt added emulsions. From the droplet size distribution in Figure 4.2, it can be seen that the SC NEs with 2 % protein showed a large second peak which further increased with the addition of salt.
Figure 4.5 DLVO interdroplet pair potential for all NEs at two different oil and protein concentration as a function of distance between the adsorbed protein layers on the droplets.
This change in droplet size could be due to higher attractive interaction at the secondary minima for the both 2% SC NEs (Figure 4.5A & 4.5E) compared to 4% SC NEs. Among the four WPI NEs, 2% WPI showed a large increase in droplet aggregation upon addition of just 0.1 M salt, which matches with their higher attractive secondary minima as shown in Figure 4.5C & 4.5G. Of all the NEs at all salt concentrations, attractive interaction was maximum for 2% WPI NEs followed by 2% SC NEs, which could be due to their larger initial droplet size compared to NEs with 4% protein. Attractive interaction was minimum for 4% protein containing NEs and no significant difference was observed between SC and WPI NEs, which also could be due to their similar and smaller droplet size. Initial droplet size of the NEs plays an important role in salt-induced aggregation where larger droplets (NEs with 2 % proteins) led to more depth in the secondary minima and higher probability of droplet aggregation.

### 4.4.1.2 Visual observation of flow behavior

![Figure 4.6](image)

**Figure 4.6** Visual observation of flow behavior of (A) SC NEs and (B) WPI NEs at different salt concentrations. The samples were placed in 30 ml beakers which were tilted at 45° angle to demonstrate gelation and flow of the NEs. (Dotted lines indicate liquid meniscus or gel surface)
From the photographs of visual observation (Figure 4.6) it can be seen that most of the SC NEs with salt were flowable as they were weak gel. In contrast, all WPI NEs with 0.5 M or higher salt, form non-flowable gel with self-supporting structure. Similar to droplet aggregation behavior, flow behavior of the NEs was also significantly affected by the addition of salt only in case of WPI NEs.

**4.4.1.3 Influence of salt concentration on viscosity**

![Graphs showing viscosity changes](image)

**Figure 4.7** The viscosity of as a function of shear rate for NEs with different salt concentration prepared with SC (A, B) or WPI (C, D) with various oil (40% and 30%) and protein concentration 2% (open) and 4% (filled). Salt concentrations are indicated by different symbols: 0 M (circle), 0.1 M (triangle), 0.5 M (square) and 1 M (diamond). Error bars are removed for clarity.

Figure 4.7 shows the change in viscosity as a function of shear rate for all NEs with different concentration of salt. All NEs exhibited shear thinning behavior, where viscosity decreased with
an increased in shear rate. For SC NEs with both 40 & 30% oil and 4% SC, the samples without salt showed linear shear thinning behavior where viscosity decreases steadily with an increase in shear rate (Figure 4.7A, 4.7B). All other 4% SC NEs with added salt showed lower viscosity than the ones without any salt. With 2% SC, the viscosity profiles of the NEs did not show much difference with the addition of salt. However, in contrast to the NEs without salt, the salt added 4% SC NEs showed plateau in viscosity at high shear, indicating complete breakdown of any structure and free movement of droplets. In contrast to SC NEs, viscosity profiles of WPI NEs showed an opposite trend, where low shear viscosity increased with increase in salt concentration. For all WPI NEs with 0.1M salt, the low-shear viscosities were higher than the ones with no salt, while at a higher shear rate the viscosities were similar (Figure 4.7C & 4.7D). All WPI NEs with 0.5 and 1 M salt showed a clear shear thinning behavior with an increase in shear rate and these NEs also showed significantly higher viscosities compared to the NEs with 0 or 0.1M salt at all shear rates.

![Figure 4.8 Viscosity at 1 s⁻¹ shear rate at different salt concentrations for NEs made with SC (circle) and WPI (triangle) with an oil concentration of 40% and 30% and protein concentration of 2% (open) and 4% (filled).](image)

To better compare the low-shear viscosity of the various NEs, data from Figure 4.4 at 1 s⁻¹ shear rate has been re-plotted in Figure 4.8. The viscosity of the NEs with 4% SC and 40% oil (filled black circles in Figure 4.8A) had significantly (p < 0.05) dropped upon the addition of 0.1 M salt, after that with increasing salt no further change in viscosity was observed (p > 0.05). This phenomenon of the initial decrease in gel strength with the addition of 0.1 M salt was called
‘melting’ of gel structure and observed in case of SDS-stabilized nanogels (Fryd & Mason 2012). This effect can be attributed to the weakening of repulsive gel structure by the screening of droplet charge which led to a reduced effective volume fraction and elimination of close-packed structure. With increasing salt concentration, the repulsive interaction decreased further, but no significant increase in viscosity of the NEs was observed due to the ability of SC to prevent droplet aggregation (p > 0.05).

A vastly different viscosity behavior was observed for WPI NEs as a function of salt which matches closely with the average droplet size data (Figure 4.1). For both 40 and 30% oil NEs a steady increase in viscosity was observed up to 0.5 M salt and thereafter no significant (p >0.05) change in viscosity was observed till 1 M salt (Figure 4.8). The viscosities of 0.5 M salt added WPI NEs were about 3 to 4 orders of magnitude higher than the original NEs without any salt. For example, viscosity of 30% oil and 4 % WPI NEs increased from 0.02 ± 0.00 Pa.s without any salt to 327.85 ± 170.06 in presence of 0.5 M salt. The lowest increase in viscosity was observed for WPI NEs with 40% oil and 2% protein, where viscosity increased from 0.12 ± 0.03 Pa.s without salt to 26.42 ± 3.21 Pa.s with 0.5 M salt, which could be ascribed to their larger initial droplet size (Figure 4.1). For WPI NEs with 2% protein and 30% oil a rapid increase in viscosity was observed with just 0.1 M salt, which could be ascribed to the presence of less protein and lower droplet surface area of larger droplets so that less salt would be needed to cause droplet aggregation and increase in viscosity. This effect can also be confirmed from droplet size distribution, where unlike others the NE with 2% WPI and 30% oil showed a large shift in droplet size upon addition on 0.1 M salt (Figure 4.2F).

**4.4.1.4 Influence of salt concentration on viscoelasticity**

Viscoelasticity of the NEs was measured as a function of strain at a constant frequency to understand the effect of salt on their gel strength. Figure 4.9 shows the $G'$ (storage modulus) & $G''$ (loss modulus) of SC NEs with 40% and 30% oil and 2 and 4% protein at different concentration of salt. Figure 4.10 shows similar data for WPI NEs. Higher $G'$ compared to $G''$ indicates presence of gel structure and a clear linear viscoelastic region (LVR) in $G'$ shows strong gelation. Most of the SC NEs (Figure 4.9) didn't show any LVR in the low strain regime indicating weak gelation, except the NE with 40% oil and 4% SC (Figure 4.9E). For all NEs, $G'$ and $G''$ increased with strain until a critical yield strain where $G'$ dropped, which was followed by a crossover of $G'$ and $G''$. The
crossover indicates breakdown of gel structure, beyond which both the G' and G'' dropped linearly and G'' remain higher than G' indicating a liquid-like behavior. For the samples without any salt G'' showed a peak at the crossover, which indicates structural relaxation of the repulsive gel where the close-packed nanodroplets break away from the cage. However, with the addition of salt, specifically with at 0.5 M salt and higher, the peak in G'' disappeared. It should be noted that with the addition of salt, the repulsive electrostatic interactions among the nanodroplets were screened and the Debye screening lengths were decreased, leading to a lowering of the repulsive barrier around the nanodroplets (shown in Figure 4.5). As discussed in Chapter 3, the gelation in SC NEs was due to an increased effective oil droplet volume fraction resulted from both the steric layer of

Figure 4.9 Effect of salt concentration on the storage (G', filled) and loss (G'', open) moduli as a function of % strain for SC-stabilized NEs with 40% oil stabilized with 2% (circle, A-D), 4% (triangle, E-H) protein; and 30% oil stabilized with 2% (square, I-L) and 4% (diamond, M-P) protein.
SC and the Debye screening length. With an increase in salt concentration, the electrostatic Debye screening length decreased which lowered the effective oil droplet volume fraction and diminished the close-packed structure. Hence application of strain did not induce a peak in $G''$ for the NEs with high concentration salt.

The NE with 40% oil and 4% SC (Figure 4.9E) showed a strong LVR in the low strain regime, indicating strong gelation. However, upon addition of 0.1 M salt a significant change in the rheology of the NE was observed (similar to their change in viscosity as reported in Figure 4.8). The LVR disappeared (Figure 4.9F) indicating a breakdown of gel structure with salt-induced

**Figure 4.10** Effect of salt concentration on the storage ($G'$, filled) and loss ($G''$, open) moduli as a function of % strain for WPI-stabilized NEs with 40% oil stabilized with 2% (circle, A-D), 4% (triangle, E-H) protein; and 30% oil stabilized with 2% (square, I-L) and 4% (diamond, M-P) protein.

The NE with 40% oil and 4% SC (Figure 4.9E) showed a strong LVR in the low strain regime, indicating strong gelation. However, upon addition of 0.1 M salt a significant change in the rheology of the NE was observed (similar to their change in viscosity as reported in Figure 4.8). The LVR disappeared (Figure 4.9F) indicating a breakdown of gel structure with salt-induced
lowering of Debye screening length and effective oil volume fraction. Similar salt-induced repulsive gel breakdown was also reported by Fryd & Mason (2012). With increase in salt concentration, the repulsive interaction further decreased, but as SC-stabilized droplets were stable against aggregation no significant change in viscoelasticity was observed (Figure 4.9G).

Contrary to SC NEs where not much change in viscoelastic behavior upon addition of salt was observed for most oil and protein concentration, a clear LVR appeared for WPI NEs at a higher salt concentration which was absent in all the WPI NEs without any added salt thereby converting a weak gel and liquid-like NEs into strong gel (Figure 4.10). There is also a gradual increase in the magnitude of G' and G'' with increasing salt concentration for all WPI NEs.

In order to better compare the effect of salt concentration on the gel strength of the various SC and WPI NEs, their G' values at 0.15% strain (at a strain below yield point) from the strain sweep data from Figure 4.9 and 4.10 were re-plotted against salt concentration (Figure 4.11A & 4.11B). The G' and G'' crossover strain is the force required to break a gel, and hence gives further indication of the strength of a gel to withstand applied force before breakdown. For comparison, crossover strain of the all NEs were also plotted from Figure 4.9 and 4.10 into Figure 4.11C & 4.11D as a function of salt concentration. It can be seen that for the SC NEs with 40% and 30% oil, no notable change in G' values (p > 0.05) was observed, however, the SC NE with 4% SC and 40% oil showed a drop in G' from 77.6 ± 24.8 with no salt to 23.7 ± 6.2 Pa upon addition of 0.1 M salt (p > 0.05), which indicates the gel-melting behavior as discussed before. However, for WPI NEs (at both 30 and 40% oil) similar to viscosity, G' increased drastically with salt concentration till 0.5 M salt followed by a plateau to 1 M. The values of gel strength with 0.5 M salt and higher were much higher for WPI NEs compared to SC NEs. This behavior indicates that addition of salt affects WPI NEs more profoundly and leads to stronger gel formation at higher salt concentration whereas, SC NEs are quite unaffected by the presence of salt in the aqueous phase. From zeta potential measurement, we have seen that the reduction in droplet charge with the addition of salt was very similar for all NEs. Therefore, strong steric repulsion from the SC molecules on the oil droplet surface which extends towards the aqueous phase for about 10 nm (Atkinson et al., 1995; Dalgleish, 1993; Dickinson et al., 1993) prevented aggregation of the oil droplets (Casanova & Dickinson, 1998). For WPI, the steric barrier is much smaller, reported to be about 2 nm in length (Nylander et al., 1999), hence the droplets, in the absence of a strong electrostatic repulsion (as shown in Figure 4.5) would be able to form stronger aggregates, as evident from their droplet size.
distribution reported in Figure 4.2. The strong attractive aggregates of WPI-stabilized nanodroplets in the presence of salt led to a substantial increase in gel strength compared to the SC NEs. Cold gelation in WPI-stabilized conventional emulsion (average droplet size $> 1 \mu$m) has previously been reported by many authors (Line et al., 2005; Rosa et al., 2006). However, in most cases a heat-denatured protein was used to make emulsions, followed by the addition of salt to induce gelation. Heat denaturation opened up whey protein molecules and improved the formation of intermolecular and interdroplet interactions which led to strong gelation. An excess amount of proteins (9.5 wt%) was also used which facilitated network formation in the continuous phase and the protein-covered oil droplets acted as active filler thereby increasing the overall gel strength. (Line et al., 2005). In the present case, no heat treatment was used, and the formation of extremely small nanodroplets (and the corresponding high surface area) led to lower amount of excess WPI.

**Figure 4.11** (A & B) Storage modulus ($G'$) at 0.15% strain and (C & D) crossover strain for all NEs with different salt concentrations prepared with SC (circle) or WPI (triangle) with (A & C) 40% and (B & D) 30% oil concentration and 2% (open) and 4% (filled) protein concentration.
in the continuous phase. Together these two factors led to a lower values of gel strength compared to the Line et al. (2005) and Rosa et al. (2006). Nevertheless, formation of self-supporting nanogels from WPI NEs in the presence of salt and without using any heat treatment and acidification could be novel way to utilize these materials in food and related application.

Unlike G' values, the crossover strain values of the NEs showed different behavior for 40% in comparison to 30% oil concentrations. For the SC NEs with 40% oil (Figure 4.11C) a drop in crossover strain was observed with an increase in salt concentration, indicating less force would be needed to break the weak-gel structure. While for the SC NEs with 4% SC and 30% oil (Figure 4.11D), an increase in crossover strain was observed with an increase in salt concentration (p < 0.05). For WPI NEs with both 40% (Figure 4.11C) and 30% oil (Figure 4.11D), a substantial increase in crossover strain was observed with an increase in salt concentration. Without any salt, low values of crossover strain were observed with 4% WPI NEs, while for 2% WPI NEs no crossover was observed. In most cases (except WPI NEs with 0.1 M salt) the crossover strain values were significantly higher for WPI NEs than SC NEs. However, unlike G’, the values of crossover strain for 30% oil WPI NEs were significantly higher compared to the 40% oil NEs. A high value of crossover strain indicates the gels were stretchy under applied shear, which could be due to the presence of excess WPI in the continuous phase. More research would be needed in order to understand the mechanism behind this phenomenon.

4.4.2 Effect of pH on the gelation in nanoemulsions

4.4.2.1 Droplet size of the nanoemulsions at different pH

The droplet size analysis of NEs was carried out at different pH values for all different NEs. As shown in Figure 4.12, a steep increase in surface average droplet diameter (d_{32}) was observed at pH = pI of the protein compared to pH > pI (pH 7) for all NEs with both oil concentration irrespective of their protein type and concentration. All NEs with pH < pI also showed an increase in droplet size compared to pH > pI but the increase was lower than that when the pH = pI. The droplet size distribution is also essential to understand the shift in droplet size upon a change in pH. Figure 4.13 shows droplet size distribution at different pH values for different SC and WPI NEs. For all the NEs at pH > pI, a single significant peak at droplet size lower than 1µm could be seen. For SC and WPI NEs at pH = pI, droplet distribution shifted to significantly large multimodal
peaks at droplet size ranging from 10 to more than 1000 µm. This change in droplet size distribution justifies the change in average droplet size upon changing the pH to pI, where the droplets lost their charge leading to strong aggregation due to hydrophobic attraction among the protein-coated droplets. However, for pH < pI the droplet size distribution became multimodal in the case of all SC NEs and retained a small peak at lower droplet size and a broad peak at higher droplet size. An increase in SC-stabilized emulsion droplet size at a pH below the protein’s isoelectric point was also reported by other researchers (Surh, 2009; Surh et al., 2006). It was proposed that when the emulsion pH was reduced from 7 to 3, it went through the pI of SC which resulted in extensive flocculation and some droplet coalescence leading to an increased droplet size even at pH 3.

In contrast, 4% WPI NEs at pH < pI did not show any change in droplet size distribution compared at pH > pI. However, for 2% WPI NEs an increase in droplet size at pH < pI was also observed. Similar observation of increase in droplet size at pH lower than pI was also observed by Kim et al. (2004) in case of 1wt% β-lactoglobulin-stabilized 10 wt% n-hexadecane oil-in-water emulsions where the average droplet size of 0.46 µm at pH 7 increased to 1.3 µm at pH 3. When the flocculated droplets were treated with mercaptoethanol (a reagent to break disulfide bonds), no further decrease in droplet size was observed, indicating that the increase in droplet size was not due to interfacial protein crosslinking. The authors attributed this to increase in protein surface hydrophobicity at the droplet surface at pH 3 which led to more droplet aggregation compared to pH 7 (Kim et al., 2004). β-lactoglobulin is a major protein present in WPI and in the present case, increase in droplet size was observed at 2% protein concentration, but not at 4%. Perhaps, the

Figure 4.12 Average droplet size ($d_{32}$) of NEs containing different protein and oil concentrations as a function of change in pH.
presence of significantly smaller droplets (average droplet size less than 200 nm for 4% WPI NEs) improved their stability against flocculation.

**Figure 4.13** Droplet size distribution of NEs with different protein and oil concentrations at different pHs in the aqueous phase of NEs.
4.4.2.2 Zeta potential as a function of pH

Figure 4.14 Change in zeta potential of NEs containing 40% oil with an increase in salt concentration in the aqueous phase of NEs.

The zeta potential of the NEs at different pH values was measured to confirm the change in droplet charge and understand the mechanism of droplet aggregation or gelation in NEs. Figure 4.14 shows that all NEs had a high negative zeta potential at pH 7 in which values were below -60 to -70 mV for all SC NEs and below -58 to -60 mV for WPI NEs. This zeta potential reached near zero for NEs with pH = pI indicating the cancellation of charge upon bringing pH to the isoelectric point of the respective protein. Upon further decreasing the pH below pI, the zeta potential of all NEs became positive and reached in the range of +40 mV to +45 mV for SC NEs and +35 mV to +45 mV for WPI NEs. The results indicate the presence of significant repulsive charge at pHs away from pI whereas repulsive charge on droplets was canceled at pH near pI. The cancellation of repulsive charge over droplet makes them susceptible to aggregation which could be crucial for attractive gelation in NEs.

4.4.2.3 Visual observation of the nanoemulsions at different pH values

The visual observation of the NEs were recorded to understand their gelation and flow behavior. Both SC and WPI NEs at pH values lower and higher than pI flowed like a viscous liquid upon tilting the beakers at a certain angle except 4% SC NE with 40% oil which showed a soft gel-like behavior (Figure 4.15). However, when the pH of the NEs reached the pI of the proteins, a strong gel was observed which did not flow upon tilting the beakers. All samples at pH = pI showed
a hard and grainy texture, while the samples at pH away from pI showed a viscous liquid-like flow behavior.

Figure 4.15 Visual observation of flow behavior of (A) SC NEs and (B) WPI NEs at different pHs. The samples were placed in 30 ml beakers which were tilted at 45° angle to demonstrate gelation and flow of the NEs. (Dotted lines indicate liquid meniscus or gel surface)

4.4.2.4 Viscoelasticity of the nanoemulsions as a function of pH

Figure 4.16 An example of attractive aggregate gel formed at pH = pI for SC NE with 40% oil and 4% SC. Note: Other NEs also showed similar attractive gel at pH = pI.

The viscoelasticity of the NEs at different pH values was measured to understand the effect of pH on the gelation behavior. The viscoelasticity of the NEs at pH = pI could not be measured as there was an operational difficulty in to analyze the sample on rheometer. The strongly aggregated
attractive gel at pH = pI could not be compressed to the required gap between the two plates in rheometer. An example of aggregated gel at pH = pI is shown in Figure 4.16. The hard and grainy gels were self-standing and non-flowing which made it difficult to achieve the desired gap for the rheological measurement. Instead we have performed compression analysis for these samples using a texture analyzer to compare the strength of the materials.

**Figure 4.17** G’ at 0.15% strain for both SC and WPI at different protein concentration at pH > pI and pH < pI. Note: Rheometer was unable to provide any G’ data due to lack of gel properties for NEs with 30% oil and 4% WPI at pH > pI (Figure D).

To compare the gel strength of the NEs at two different pH values (less than and greater than the pI of the protein used), the values of the storage moduli (G’) at 0.15% strain were plotted in Figure 4.17 from the oscillatory strain sweep rheology data. For SC NEs, an increase in gel strength was observed with a change in pH from 7 to 3.6, except the SC NEs with 4% protein and 40% oil (Figure 4.17A, 4.17B). Increase in elastic modulus with a decrease in pH for SC-stabilized emulsions was also reported by J. S. Chen et al. (1999). The researchers prepared 30% oil-in-water
conventional emulsions with 2% SC solution and observed that $G'$ reached a maximum between 4 to 5, thereafter a drop in $G'$ was observed with a lowering of pH below 4. However, the $G'$ value at pH $\sim 3.6$ was much higher compared to the values at pH 6. The decrease in gel strength upon lowering the pH for 4% SC 40% oil NEs was similar to what was observed upon addition of 0.1 M salt into this NE. This particular NE formed a repulsive nanogel at pH 7 due to charge cloud and steric barrier-induced increase in effective volume fraction. Upon lowering the pH, absolute value of zeta potential significantly decreased (Figure 4.14), which led to a drop in the thickness of the charge cloud and subsequent reduction in gel strength due to loss of close-packing of droplets.

WPI NEs with 40% oil also showed similar behavior to most of the SC NEs, where an increase in $G'$ was seen with lowering of pH from 7 to 4 (Figure 4.17C), however, the increase in gel strength was statistically significant only for 2% WPI NEs ($p < 0.05$). WPI NEs with 30% oil, while no $G'$ could be recorded during rheological analysis at pH 7 (liquid-like behavior), at pH 4, the $G'$ values increased to $\sim 5$ Pa, which could be attributed to the droplet aggregation as evident from the droplet size data in Figure 4.12.

### 4.4.2.5 Compression analysis of the nanoemulsions at the protein’s isoelectric point

The compression analysis was carried out to understand the firmness of the aggregated gels made by both the SC and WPI NEs at pH values equal to the pI of the protein. As mentioned before, it was not possible to measure these samples using the rheometer. The results of compression analysis indicate the firmness of respective NEs gels by the maximum force needed to compress the aggregated gel structure up to certain distance. In general, the average results of peak force for all WPI NEs were higher than their SC counterparts ($p < 0.05$). For SC NEs, no significant difference ($p > 0.05$) in peak force was observed for all samples, irrespective of protein and oil concentration. However, WPI NEs with 4% protein showed the highest peak force of compression irrespective of oil concentration among all NEs, which could be attributed to their significantly lower initial droplet size (recorded at pH 7) compared to 2% WPI NEs. Formation of many smaller droplets would lead to a better aggregated network compared to a lesser number of large oil droplets (R. A. Mantovani et al., 2016). Presence of high amount of WPI could also be responsible for more attractive interactions between the droplets (Rosa et al., 2006). Firmness of 4% WPI 40% oil NE was much higher compared to the 30% oil containing sample due to the presence of excess oil droplets leading to enhanced gel network. However, firmness of 2% WPI NEs at pI was similar for
both 30 and 40% oil, which could be attributed to the significantly higher initial droplet size of 2% WPI 40% oil NE thereby weakening the gel structure making it similar in firmness to the 30% oil NE.

Compression analysis of acid-induced WPI emulsion gel has been studied before (Mantovani et al., 2016; Rosa et al., 2006). Rosa et al. (2006) investigated the formation and rheology of emulsion-filled gels by first preparing a heat-induced whey protein aggregates followed by mixing with whey protein-stabilized emulsions, which was prepared with native whey proteins at room temperature, and finally acidification to induce gelation at protein isoelectric point. It was found that heating the whey protein solution was essential to form a self-supporting gel. Recently, Mantovani et al. (2016) reported that acidification of a 5% WPI-stabilized 30% oil-in-water emulsion did not form gel, while mixing with a heat treated 5% WPI prior to acidification led to self-supporting gel. In contrast, Ye and Taylor (2009) was able to form whey protein-stabilized emulsion gel by heating an emulsion followed by acidification, without the need for adding additional heat treated protein to form an emulsion-filled gel. In the present work, no heat treatment was used, still a strong self-supporting gel was formed by acidification of WPI NEs to pH 4. The improvement in firmness of our gel could be attributed to the nanoscale droplet size of our NEs compared to microscale droplets of both Rosa, et al. (2006) and Mantovani, et al. (2016)’s emulsions. Ye and Taylor (2009) showed that storage modulus of a WPI emulsion gel, made by

![Figure 4.18 Peak compression force of NEs with different protein and oil concentration at pH = pI.](image-url)

**Figure 4.18** Peak compression force of NEs with different protein and oil concentration at pH = pI.
acidification of a pre-heated WPI emulsion, increased with a decrease in average droplet size. Perhaps the large surface area of a greater number of smaller droplets was better able to stabilize a three-dimensional structure necessary for gelation.

4.4.3 **Effect of heat treatment of nanoemulsion gelation**

4.4.3.1 **Influence of heat treatment on the droplet size of the nanoemulsions**

![Figure 4.19](image) Average droplet size ($d_{32}$) of (A) SC and (B) WPI NEs with different protein and oil concentration before and after the heat treatment at 85 °C for 30 min.

All NEs were heat treated at 85 °C for 30 minutes, cooled down to room temperature before measuring their droplet size and rheology. The aim was to induce gelation in WPI NEs as WPI is known for their thermal denaturation above 85 °C, thereby opening up their inner hydrophobic region and upon cooling leading to the formation of a hydrogel by forming extensive intermolecular interactions (Jost et al., 1986). SC, on the other hand, are stable against thermal denaturation and was expected to show unchanged physicochemical properties after heat treatment. The droplet size of the NEs with and without heat treatment is compared in Figure 4.19 to identify any aggregation between the protein-coated droplets. The droplet size of SC and WPI NEs showed similar trend upon heating for a specific protein concentration. All NEs with 2% protein showed an increase in average droplet size ($p < 0.05$) upon heating except WPI NEs with 40% oil, for which no significant change in $d_{32}$ was observed after heat treatment ($p > 0.05$). In Chapter 3 we have shown that at 2% concentration, none of the protein was able to fully cover the droplet surface, thereby heating may induce some droplet destabilization by coalescence. The emulsion with 2% WPI and 40% oil had the largest average droplet size of all samples due to bridging flocculation (Chapter 3), and upon
heating it also showed a further increase in droplet size distribution (p > 0.05). However, with 4% protein both SC & WPI NEs did not show any change in droplet size after heat treatment. This could be due to the higher stability of the oil droplets with 4% protein as the droplets in these NEs have enough protein to cover their surface and will not be affected by heat-induced aggregation or coalescence, the lack of which was probably the reason behind the increase in droplet size for 2% protein containing NEs. The average droplet size of the untreated samples with 4% protein was significantly lower ($d_{32} < 200\text{nm}$) than the samples with 2% protein and has shown improved stability against flocculation due to greater electrostatic repulsion by higher amount of adsorbed protein and against coalescence due to higher internal Laplace pressure, which could also be a factor in their improved stability against heat-induced aggregation. Contrary to the hypothesis that heat-induced denaturation of WPI on the oil droplet surface would lead to extensive aggregation, these NEs did not show any significant increase in droplet size, which indicates that heat-induced denaturation of WPI was not enough to produce inter-droplet attraction by hydrophobic or disulfide bonds. The repulsive electrostatic force of WPI stabilized droplets was enough to prevent close interactions of the droplets.

### 4.4.3.2 Influence of heat treatment on the Viscoelasticity of the nanoemulsions

Determining viscoelasticity of the NEs would indicate any change in rheological properties upon heat treatment. From strain sweep viscoelasticity results, $G'$ values at 0.15% strain collected before and after the heat treatment were plotted in Figure 4.20. NEs with 2% SC did not show any significant change in $G'$ after the heat treatment irrespective of their oil concentration (p > 0.05) (Figure 4.20A), which shows that a slight increase in their average droplets size did not significantly influence gelation behavior. However, NEs with 4% SC showed a decrease in $G'$ after heat treatment, inspite of their unchanged droplet size upon heat-treatment. Gelation in untreated 4% SC NEs was due to an increase in effective oil droplet volume fraction near close-packing originated from the combined thickness of repulsive steric barrier and electrostatic charge cloud. Any changes in the interfacial protein composition leading to a decrease in steric layer and interfacial charge would reduce the effective droplet volume fraction and consequently gel strength of the emulsions. It has been reported that heating milk or SC-stabilized emulsions at neutral pH would lead to dissociation of $\kappa$-casein fraction and the dissociation was found to be higher for interfacial casein compared to casein in solution (Singh et al., 1993; Srinivasan et al., 2002).
heating at 90 ºC led to mostly generation of smaller peptides due to protein degradation (Singh et al., 1993), heating at 120 ºC (retorting temperature) resulted both degradation in smaller peptides and polymerization of casein molecules (Srinivasan et al., 2002). In the present case, the NEs were heated to 85 ºC, and therefore dissociation of κ-casein fraction would more likely to occur. As κ-casein fraction is highly charged and hydrophilic, loss of it would generate droplets with less repulsive barrier, which could be significant in reducing effective droplet size and volume fraction leading to a drop in gel strength of the heated SC NEs.

All WPI NEs with measurable gel strength did not show any significant change in G' values after the heat treatment. It should be noted that for these NEs although G' > G" in the low-strain regime, the lack of any LVR meant that they were weak gels. The 4% WPI and 30% oil NEs did not give any values of G' both before and after the heat treatment indicating liquid-like flow behavior. It is well known that WPI is a heat labile protein (Fox & McSweeney, 1998) and WPI solutions are known to form heat-induced gels (Dickinson & Chen, 1999). However, in the present case, heating of WPI NEs did not induce any further gelation. This could be attributed to two factors: presence of enough repulsive interaction to prevent droplet aggregation and the lack of excess protein that may induce network formation in the continuous phase. Hunt and Dalgleish (1995) reported that WPI-stabilized emulsions were stable to heating at 90 º and 121 ºC at pH 7, when no salt was present. However, either decreasing the pH near the protein’s pI or presence of

Figure 4.20 G' at 0.15% strain for (A) SC and (B) WPI NEs with different protein and oil concentration before and after the heat treatment at 85 ºC for 30 min. All samples were analyzed at 25 ºC on a rheometer using an oscillatory strain sweep test from 0.01 to 100% at a constant frequency of 1 Hz. No G' values were recorded for the 4% WPI NEs with 30% oil.

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more than 40 mM KCl led to heat-induced aggregation of the droplets and gelation of the emulsions. Both decreasing pH towards pI or addition of salt, significantly reduced repulsive interaction among the droplets, allowed them to interact at closer separation, which facilitated strong droplet aggregation and gelation by heat denaturation of interfacial protein. Similar behavior of heat-induced aggregation in the presence of salt was also observed by other authors (Guo et al., 2013; Kim et al., 2004). From the calculation of excess protein in the continuous phase of WPI NEs (Chapter 3), it was shown that among all the WPI NEs with measurable gel strength, only the NEs with 2% WPI and 40% oil showed the presence of excess protein (0.5 wt%) in the continuous phase. This lack of WPI in the aqueous phase hinders the denatured WPI matrix formation in the continuous phase upon heating and the development of gel structure. Euston et al. (2000) have reported that the unadsorbed protein in continuous phase acts as a ‘glue’ in the aggregation of protein-coated droplets where the droplet may act as active filler particles. Therefore, the lack of free protein in the aqueous phase led to no change in weak gelation behavior of WPI NEs after heat-treatment.

4.5 Conclusion

In this work, effect of different levels of salt, pH and heat treatment was used to investigate the possibility of converting a weak gel and liquid-like NEs into elastic nanogel. The addition of salt did not show a significant effect on SC NEs except a slight decrease in gel strength for 4% SC and 40% oil which was due to the screening of surface charge that caused a decrease in effective droplet volume fraction and close packing. WPI NEs showed gelation upon addition of salt and increasing gel strength with increase in salt concentration. DLVO calculation showed that, screening of charge can be attributed as a key factor in altering droplet-droplet interaction in WPI NEs which induced flocculation and led to droplet aggregate gel. In contrast, SC NEs did not show the aggregation or formation of attractive gel despite of similar screening of charge which was attributed to the stronger steric repulsion among the SC-coated droplets.

Changing pH to the pI of the protein resulted in the formation of aggregated gels for both SC an WPI NEs irrespective of their oil concentration due to a complete loss of repulsive interaction among the droplets. However, NEs pH < pI showed certain aggregation and increase in gel strength which was attributed to droplet coalescence and aggregation during pH transition through the pI of protein. Similar to salt, pH-induced gelation without the aid of any heat treatment.
could be attributed to the smaller droplets responsible for forming a stringer three-dimensional structure.

Heating the NEs resulted in an increase in droplet size for 2% protein containing NEs, which was attributed to the lack of enough protein to prevent droplet aggregation and coalescence. At 4% protein, oil droplets were better covered, and no change in droplet size was observed after heat treatment. However, despite no change in droplet size, the gel strength of the 4% SC NEs significantly decreased, which was attributed to dissociation of \( \kappa \)-casein from the droplet surface upon heat treatment of SC NEs resulting in decrease of repulsive charge and weakening of droplet close-packing. Despite being heat labile, heating WPI NEs was unable form gel. This inability of gel formation was attributed to the strong repulsive charge on droplets which prevented attractive interaction and also a lack of WPI in the aqueous phase needed to form a gel matrix. Overall, research on the effect of different environmental factors on the stability and gelation behavior of protein-stabilized NEs could also be useful in food and related materials application as changes in these conditions are frequently observed during processing, storage and consumption of these materials.
CHAPTER 5: GENERAL DISCUSSION

Emulsions are an important part of food systems and contributes to structure, rheology, opacity and sensory properties. In recent years, nanoemulsions, where the average droplet size is defined as less than 200 nm, have been a hot topic in emulsion science due to their distinct advantages over conventional emulsions. One of the important properties of NEs is the formation of gel (the so-called ‘nanogel’) upon reduction in droplet size, which has so far been studied with small molecular surfactants. However, no research was done to study the use of food protein as emulsifier to make nanogel mainly due to the challenges faced with food proteins, e.g., slow adsorption at the interface compared to small molecular surfactants, large size of protein molecules and lesser degree of lowering of interfacial tension. The purpose of this study was to understand the effect of protein type and concentration as well as oil volume fraction on the gelation in protein-stabilized NEs.

The two food proteins selected for the study were milk proteins: sodium caseinate (SC) and whey proteins isolate (WPI). The selection of these proteins was primarily because they are edible food-grade proteins/emulsifiers and have two different type of structures, flexible random coil for SC and predominantly globular for WPI. These milk proteins are also widely used as emulsifiers in the food industry for beverages, ice creams, sports supplements, infant formula and coffee creamer (McClements, 2005). Another major difference between these two proteins is the thickness of their interfacial layer around oil droplets in an emulsion, which are calculated as 10 nm for casein due the presence of flexible hydrophilic chain of the molecules (Atkinson et al., 1995; Dalgleish, 1993; Dickinson et al., 1993) and 2 nm for whey due to its compact globular structure at the interface (Nylander et al., 1999). These two different properties would be important to important to understand the importance of protein structure and interfacial layer thickness in NE gelation.

The results of the first study showed that NEs with both SC and WPI showed a decrease in droplet size with an increase in protein concentration. SC NEs showed a significant increase in viscosity as well as $G'$ with a decrease in droplet size. Despite same oil and protein concentration SC NEs samples taken from different cycles of homogenization showed an increase in viscosity and $G'$ upon decrease in droplet size with each cycle, and also converting in to a strong gel for the NEs with 5% SC and 40% oil when the droplet size was reduced below 200 nm. When the droplet
size was plotted against $G'$ or viscosity, data for all different NEs with same oil, but different protein concentration merged into a single line where the trend of increase in $G'$ and viscosity with decrease in droplet size followed a power law relationship. Similar increase $G'$ and viscosity with decrease in droplet size has also been observed by previous researchers with small molecular surfactants (Erramreddy & Ghosh, 2015; Wilking & Mason, 2007). To confirm that the mechanism of gelation is due to repulsive close packing of droplets, the total interfacial layer thickness was calculated by the addition of steric barrier and repulsive charge cloud calculated with DLVO interdroplet potential. This interfacial layer thickness at a repulsive interaction of $1 \ k_B T$ was used to calculate the change in effective volume fraction ($\phi_{eff}$) with a decrease in the droplet size. When the calculated values of ($\phi_{eff}$) was plotted against $G'$ for all SC NEs, they merged into a single line irrespective of oil concentration where the values of $G'$ increased with an increase in $\phi_{eff}$, indicating close packing of nanodroplets along with their interfacial “shell” layer led to gelation by repulsive random jamming. Similar study with small molecular surfactant has been carried by Erramreddy and Ghosh (2014) with SDS and Tween 20 where SDS NEs showed gelation at lower droplet size due to strong repulsive interfacial layer but Tween 20 NEs did not show any gelation due to the lack of interfacial repulsive layer from the non-ionic emulsifier. Wulff-Pérez et al. (2013) also reported similar gelation behavior in emulsions made with polymeric emulsifiers, where the gel strength increased with an increase in emulsifier hydrophilic chain length leading to an increased effective droplet size and oil volume fraction. However, the authors did not investigate the influence of oil droplet size.

In contrast, WPI NEs showed no significant change in $G'$ and viscosity with a reduction in droplet size. It was hypothesized that the difference in rheology of SC NEs and WPI NEs could be due to the difference in the interfacial layer thickness. The DLVO calculation showed that the repulsive barrier in the NEs with both proteins was not very different yet the huge difference of steric layer thickness (five times thicker for SC compared to WPI gave the distinct behavior in SC NEs. The $\phi_{eff}$ calculations for WPI NEs showed an increase with a decrease in droplet size, but the maximum $\phi_{eff}$ was 0.53, which is well below the random jamming volume fraction, which explains the lack of gelation.

A notable outcome from this study is the absence of depletion from SC NEs which was observed by many previous researchers who used SC to make emulsions or NEs (Dickinson & Golding, 1997; Dickinson et al., 1997; Yerramilli & Ghosh, 2017). The absence of depletion in
SC NEs is due to the lack any excess protein in the continuous phase. This lack of excess protein was due to the increased surface area of smaller size of the nanodroplets compared to the conventional emulsions made by Dickinson and Golding (1997). Despite similar droplet size range, the presence of excess protein in the NEs made by (Yerramilli & Ghosh, 2017) was due to the lower dispersed phase volume fraction (5%) in their NEs compared to 30 & 40% in the present case.

The long-term stability analysis showed that the NEs are stable for a 3 months period even if stored at room temperature. Certain key factors for higher stability of these NEs could be their smaller droplet size and stronger interface. The smaller droplet size has higher Laplace pressure which avoids coalescence and it also slows down the gravitational separation compared to bigger size droplet size conventional emulsions. Moreover, the interfacial steric layer of protein resists coalescence and the repulsive charge cloud beyond this steric layer stops the droplets from coming close together and avoids flocculation. The lack of excess protein in aqueous phase also led to the absence of depletion flocculation in the NEs. Erramreddy et al. (2017) investigated long-term stability of SDS-stabilized nanogels and found that although the droplet size did not change as a function of time, the gel strength of the nanogels significantly dropped converting the elastic gels into viscoelastic liquid. It was proposed that extensive lipid oxidation in the NEs led to the generation of surface-active compounds which significantly influenced the interfacial dynamics and altered the repulsive electrostatic barriers and loss of charge cloud-induced $\phi_{eff}$. In the present case, the droplets were stabilized by SC and WPI, which are known to provide stability against lipid oxidation in emulsions (Hu et al., 2003), and hence prevented any loss of gel strength as observed by Erramreddy et al (2017).

The NEs that did not show gelation by just reduction of droplet size were further treated in the second study to change pH and ionic strength or were given heat-treatment to investigate if it would be possible to induce gelation in these NEs. Contrary to repulsive gelation in the first study, all the gels obtained in the second study were attractive gels. These gels were formed due to droplet interactions resulted from the screening or cancellation of charge by the addition of salt or changing pHs to pI, respectively. Results of heat-treated NEs showed that WPI NEs did not show any gelation after heating despite WPI being heat-labile protein, which could be due to the lack of WPI in the continuous phase to form denatured WPI gel network. SC NEs with stronger repulsive gel properties showed a decrease in gel strength upon addition of salt or by changing the pH due to
possible elimination of repulsive close packing caused by screening or cancellation of charge. More research with a combination of these factors can improve our understanding of effect of many environmental factors and can give nanogels with higher strength and stability.

An interesting outcome from this study showed that despite using these parameters individually, some NEs showed gelation whereas the previous researchers have showed that use of multiple factors like heat and salt or heat and pH is necessary to achieve gelation in emulsions stabilized by WPI (Line et al., 2005; Rosa et al., 2006). The gel network formation was achieved by heating the WPI solution prior to emulsion preparation and either change in pH (Rosa et al., 2006) or addition of salt (Line et al., 2005). The gelation was attributed to excess protein in aqueous phase for forming gelation upon heating and protein covered droplets acted as active filler strengthening the gel structure. The heating opened up whey protein molecules and changing interdroplet interaction to more attractive by either change in pH or addition of salt, led to strong gelation. Raphaela Araujo Mantovani et al. (2016) also reported that presence of whey protein in continuous phase is essential in forming gel structure upon change in pH. The smaller droplet size in NEs could be one the distinct factor responsible for gelation in WPI NEs by change in pH or addition of salt without any heat-treatment.

To understand the difference in gel strength between attractive gels from second study and repulsive gel from first study (Chapter 3), the compression analysis shown in second study (Chapter 4), was also carried out on the repulsive gels from the first study which was formed in NEs with 5% SC and 40% oil. Both the gels had visually drastic difference where attractive gels had grainy aggregate structure whereas repulsive gel had cream like structure. From Figure 4.18 the peak force for gel compression was 30.9 ± 6.4 g for 4% SC NEs with 40% oil at pH = pI, however, the gel compression force for the repulsive nanogel was found to be only ~9 g under similar experiment conditions as attractive gels. The compression force needed for the attractive gels at pH = pI showed more than three times higher firmness compared to the repulsive gels obtained from the same emulsifier. This indicates that the attractive gel network formed by the network of droplets give more firmer gel structure compared to the jammed pack structure of repulsive droplets.

The cream like repulsive nanogel has various application as soft-materials in food, medicine, cosmetics, and nutraceutical drug delivery (Erramreddy & Ghosh, 2016). Gelation at as lower oil volume fraction as 0.4 opens the possibility of application in high-fat foods like mayonnaise and salad dressings, where these gels/NEs can be used to maintain the rheological
properties of food products despite of low-fat content and without adding additives to enhance rheology. Huang et al. (2010) has discussed improved bioavailability of NEs in delivery of nutraceuticals, which opens the possibility of using these nanogels in for drug delivery. Further research of digestibility of these NEs can give us better idea for food and pharmaceuticals application. Apart from the rheological properties, these NEs are very stable at room temperature even after 3 months at higher protein concentrations. This puts them at an advantage over conventional emulsions which destabilizes faster.
CHAPTER 6: OVERALL CONCLUSION

Overall, this research project developed food protein-stabilized nanogels from oil-in-water nanoemulsions and investigated their stability, rheology, and the effect of various environmental factors on their gelation behavior. Nanogels are novel soft materials where the principle building block of the gels are nanodroplets, either in a repulsively jammed-state or aggregated due to attractive interactions. The major advantages of nanogels compared to conventional emulsion gels are their ability to gel at a much lower dispersed phase volume fraction and stability under shear and long-term storage. In spite these advantages, formation of food-grade nanogels has not been reported in literature.

The overall goal of the development of food-grade nanogels was divided into two distinct, but related objectives. The first objective was to study the effect of protein type and concentration and oil concentration on the rheology and long-term stability of NEs. Two different dairy proteins, sodium caseinate (SC) and whey protein isolates (WPI), was utilized at a range of concentrations (2 – 5% in the aqueous phase) to develop NEs with 30 and 40% canola oil. It was observed that average droplet size decreased with an increase in protein concentration. For the emulsions with 30% oil, droplet size decreased below 200 nm (NE by definition) with a protein concentration higher than 3%, while for 40% oil, 4% proteins was required to develop NEs for both SC and WPI. All the SC NEs showed shear-thinning behavior, where an increase in shear rate caused decrease in viscosity. WPI NEs, on the other hand, although showed some shear thinning at lower shear rate but exhibited a plateau at high shear rates indicating formation of Newtonian fluid-like behavior due to complete breakdown of any structure. The increase in protein concentration also led to an increase in viscosity of SC NEs, however, no such increase was observed for WPI NEs. Similar to viscosity, an increase in gel strength (G') was observed with an increase in protein and oil concentration and a decrease in droplet size for SC NEs, while no such change in G' was observed for WPI NEs. This effect of concentration of SC on gelation approved the second hypothesis. All SC NEs with 30% and 40% oil with at 2-4% protein showed weak gelation behavior, while at 5% protein, the NEs transformed into strong gel which did not flow under gravity. In contrast, all WPI NEs showed very weak to no gelation behavior at all protein and oil concentrations. Nevertheless, both the SC and WPI NEs showed extreme stability during the three-month storage study where their droplet size, viscosity and viscoelastic behavior remain unchanged.
The excess protein calculation showed that due to high surface area of smaller droplet size, all added protein was used to cover droplets which led to absence of excess protein in aqueous phase. This did not satisfy the third hypothesis for these NEs by eliminating the possibility of depletion attraction due to lack of excess protein. It was hypothesized that the difference in gelation behavior could be attributed to the different interfacial repulsive barriers among the SC and WPI-stabilized droplets. The interdroplet separation at an overall repulsion of $1 \, k_B T$ was calculated from the DLVO interdroplet interaction potential by combining the electrostatic, van der Waals and depletion interactions beyond the steric barrier. It was found that SC had significantly higher interfacial repulsive layer thickness compared to WPI mainly due to their higher steric barrier (10 nm) compared to WPI (2 nm). The interfacial repulsive layer thickness was added to the droplet size to calculate the effective oil volume fraction ($\phi_{eff}$) for NEs which when plotted against their $G'$ showed an increase in gel strength with an increase in $\phi_{eff}$, indicating repulsive gelation in SC NEs at an $\phi_{eff}$ greater than 0.7 due to the random jamming of droplets along with their repulsive barriers. No such gelation was observed for WPI NEs as their $\phi_{eff}$ values of about 0.5 remain well below random jamming. The increase gel properties upon reduction in droplet size in SC NEs approves first hypothesis about the effect of droplet size on gelation. Additionally, the difference in gelation behaviour of SC and WPI caused due to difference in interfacial thickness approved the fourth hypothesis.

The second objective of the research was to develop nanogels by inducing attractive interactions among the nanodroplets using a change in pH, ionic strength and subjecting the NEs to heat treatment. For this purpose, the NEs, which did not form gel strong repulsive nanogel, were used to induce attractive gelation by changing various environmental conditions. It was observed that the SC NEs did not show any significant effect upon addition of salt with an exception of the NE with 4% SC and 40% oil which lost its gel strength upon addition of even 0.1 M salt due to the screening of charge that led to the elimination of close-packed gel structure which approved the seventh hypothesis regarding melting of gel structure by salt addition. However, WPI NEs were affected significantly with the addition of salt where an increase in gel strength was observed with an increase in salt concentration turning the weak gel into strong aggregate gels. This drastic change in rheology was attributed to screening of droplet surface charge as observed in DLVO interdroplet interaction calculation where the repulsive barrier significantly reduced with the presence of salt in the continuous phase, leading to an increased droplet aggregation and the formation attractive
nanogels approving the eighth hypothesis regarding salt induced attractive gelation by charge screening. In contrast, SC NEs did not show much change in rheology upon salt addition, which was attributed to the presence of strong steric barrier in SC-stabilized nanodroplets that prevented droplet aggregation even after the screening of droplet charge. The change of pH to the isoelectric point of the protein resulted in the formation of strong attractive gels in the NEs with both the proteins irrespective of their oil concentration which approved hypothesis 5 regarding attractive gelation at isoelectric point. The NEs with pH > pI and pH < pI did not show any gelation due to the presence of negative and positive repulsive charge, respectively, on the protein covered droplets.

The heat treatment applied in the present research decreased the gel strength of the 4% SC NEs, which was attributed to the dissociation of κ-casein upon heat treatment resulting in a decrease of repulsive charge and weakening of droplet close-packing. Contrary to the sixth hypothesis regarding heat-induced gelation in WPI NEs (WPI being heat labile), we were unable get any increase in gel strength, which was ascribed to both the strong repulsive charge on droplets preventing attractive interaction and also on the lack of enough free unabsorbed WPI in the continuous phase needed to form a gel matrix. Therefore, further research on the combination of pH, salt and heating could be useful in the formation of nanogels from attractive interactions among the nanodroplets. In conclusion, the knowledge developed from this research could lead the way in developing the nanogels made from food-grade ingredients for many novel applications in food, cosmetics and pharmaceutical industries.
CHAPTER 7: FUTURE STUDIES

The present research has increased our understanding of the effect of reduction in droplet size in food protein stabilized NEs and their gelation behavior. The nanogels formed have endless possibilities for further understanding beyond this research. An important outcome from this research was that the reduction in droplet size significantly affected the rheology of the emulsions. In future projects, further reduction of droplet size could be carried out by various techniques. Previous researchers have used evaporative ripening to reduce droplet size of emulsion to nanodroplet range. In this process, a part of the dispersed phase is mixed with an evaporative solvent which is evaporated after making the emulsion and causes the droplets to shrink leading to a reduction in droplet size Fryd and Mason (2012). However, this method could have different results on protein stabilized emulsions based on the interfacial layer, the thickness of the interface and the heat-induced aggregation of protein. This technique have been previously used by Lee and McClements (2010) to make nanoemulsion with WPI using ethyl acetate as evaporative solvent to reduce droplet size by solvent evaporation. Viscosity modification of continuous phase could also be used to decrease droplet size by increasing the viscosity of the continuous phase which leads to an increased turbulence during homogenization (Fryd & Mason, 2012). It would be interesting to see if making NEs with lower droplet size than that obtained in this research can help achieve gelation at lower oil concentration or further increase the gel strength at similar oil concentration.

Apart from reduction in droplet size, thickness of interfacial layer was found to make a significant effect on the difference in gel strength of between SC & WPI NEs. To increase the thickness of interfacial thickness multilayer interface could be formed with layer-by-layer deposition of emulsifiers and biopolymers (polysaccharides, proteins etc.). This increased steric barrier as well as modulation of interfacial charge may significantly increase the effective volume fraction, which could lead possible gelation at a further lower dispersed phase volume fraction. Finally, a combination of the above-mentioned techniques could be applied to achieve a synergistic effect on $\phi_{eff}$ and nanogelation at a very low actual oil volume fraction.

The second study of the present research showed gelation upon addition of salt or by changing pH to pI. However, SC NEs did not show any gelation upon salt addition. As mentioned in the chapter 5, other researchers have studied the effect of a combination of multiple factors (pH, salt addition and heat treatment) on the gelation in emulsions. It would be interesting to understand
the effect of these combined factors on the protein stabilized NEs as well as to achieve gelation in certain NEs which did not form gels in this study by modifying just one factor.

Apart from the experiments for fundamentals of nanogelation, application trials should also be a critical part of future studies to understand the possibility of practical uses of the nanogel in the food as well as pharmaceutical industry. Production of mayonnaise, shortening or drug delivery topical creams using the nanogels could be key in evaluating the practical applications of the nanogels. Moreover, research on digestion, delivery and bioavailability of encapsulated substances dissolved in the dispersed nanodroplets would enhance our understanding for application of these nanogels in food and pharmaceutical industries.
REFERENCES


