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Development of Structured Delivery Systems Using Nanolaminated Biopolymer Layers

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|---------------|---|
| Item Type | dissertation |
| Authors | Cho, Young-Hee |
| DOI | 10.7275/p5gt-0p24 |
| Download date | 2024-12-04 21:45:11 |
| Link to Item | https://hdl.handle.net/20.500.14394/39312 |

**DEVELOPMENT OF STRUCTURED DELIVERY SYSTEMS USING
NANOLAMINATED BIOPOLYMER LAYERS**

A Dissertation Presented

by

YOUNG-HEE CHO

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2009

The Department of Food Science

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NANOLAMINATED BIOPOLYMER LAYERS**

A Dissertation Presented

by

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DEDICATION

To my dear parents, brother and sisters, who make me believing I'm a much better person than I am and have been devoted supporters

ACKNOWLEDGMENTS

First of all, I would like to thank Julian, who has been an excellent role model as a scientist and an advisor. I have accomplished many things beyond my ability during my Ph.D. due to his help. I'm privileged to have been his student. I owe thanks to Dr. Decker who has given me a lot of opportunities and done many favors despite being busy. I am also grateful to have Dr. Xiao and Dr. Dubin as my committee members and thankful for their time.

I am deeply thankful to have Jean during my Ph.D. I can't imagine my Ph.D. without her and she has made my life in the lab easier for taking care of all problems and listening to my complaints.

I'm grateful to have Ricard (Dr. Bou), Hanna (Dr. Saleminen), Thrandur, and Christina as my friends and share good times together in Amherst. They have brightened up my life in Amherst with their cheer and help even from Europe. I give thanks to Sandra (Dr. Po) and Seung-Jun (Dr. Choi) for making Rm 430 in Chenoweth lab a pleasant place to work and spending a lot of hard times together. Specially, I'd like to give heartfelt thanks to Owen (Dr. Jones) for being an excellent companion during my Ph.D. and sharing all good times and bad times together.

Finally, I'd like to sincerely thank to my family for their endless support and help, who have waited for a long time and been patient to finish my Ph.D.. My Dad and Mom have inspired me to be a better person and proud daughter. My sisters and brother always have been best friends in my life. I hope I will pay them back for their love in the future.

ABSTRACT

DEVELOPMENT OF STRUCTURED DELIVERY SYSTEMS USING NANOLAMINATED BIOPOLYMER LAYERS

SEPTMEBER 2009

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The objectives of this study were to carry out research to better understand of the formation, stability and properties of multilayer emulsions containing nano-laminated biopolymer coatings, and to utilize this information to develop food-grade delivery systems.

The effect of various preparation parameters on the formation and stability of multilayer emulsions was investigated: droplet concentration; mean droplet diameter; droplet charge; biopolymer concentration. β -lactoglobulin (β -Lg) stabilized emulsions (0.5 – 10 wt% oil) containing different pectin concentrations (0 to 0.5 wt%) were prepared at pH 7 (where lipid droplets and pectin molecules were both anionic) and pH 3.5 (where lipid droplets were cationic and pectin molecules anionic) and “stability maps” were constructed. At pH 3.5, pectin adsorbed to the droplet surfaces, and the emulsions were unstable to bridging flocculation at intermediate pectin concentrations and unstable to depletion flocculation at high pectin concentrations. At certain droplet

and pectin concentrations stable multilayer emulsions could be formed consisting of protein-coated lipid droplets surrounded by a pectin layer.

An *in situ* electro-acoustic (EA) technique was introduced to monitor the adsorption of charged polysaccharides onto oppositely charged protein-coated lipid droplets. The possibility of controlling interfacial and functional characteristics of multilayer emulsions by using mixed polysaccharides (pectin/carrageenan or pectin/gum arabic) was then examined. Emulsions containing different types of polysaccharides had different interfacial characteristics and aggregation stabilities: carrageenan had the highest charge density and affinity for the protein-coated lipid droplets, but gave the poorest emulsion stability.

The possibility of assembling protein-rich coatings around lipid droplets was examined using the electrostatic deposition method, with the aim of producing emulsions with novel functionality. Protein-rich biopolymer coatings consisting of β -Lg and pectin were formed around lipid droplets using the electrostatic deposition method. The composite particles formed had relatively small diameters ($d < 500$ nm) and were stable to gravitational separation. They also remained stable after they were heated above the thermal denaturation temperature of the globular protein and had better stability to aggregation at high salt concentrations (50 – 200 mM NaCl) than conventional emulsions stabilized by only protein.

The effect of a polysaccharide coating on the displacement of adsorbed globular proteins by non-ionic surfactants from lipid droplet surfaces was examined to simulate situations where competitive adsorption occurs. Oil-in-water emulsions stabilized by β -Lg were prepared containing either no pectin (1^o emulsions) or different amounts of pectin (2^o emulsions). At pH 3.5, where pectin forms a coating around the β -Lg stabilized lipid droplets, the amount of desorbed protein was much less for the 2^o emulsion (3%) than for the 1^o emulsion (39%), which indicated that the pectin coating inhibited protein desorption by surface active agents.

Knowledge gained from this research will provide guidelines for rationally designing emulsion-based delivery systems that are resistant to environmental stresses or with controlled release properties. These delivery systems could be used to encapsulate, protect and release functional components in various industrial products, such as foods, pharmaceuticals, cosmetics, and personal care products.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

There is a growing demand for healthy foods by consumers. The incorporation and delivery of functional food components into food systems have been a major issue for researchers and food producers. Among functional foods, developing a delivery system of bioactive lipids has been a major challenge in the food industry due to their being highly susceptible to oxidation and water-insoluble (1). Encapsulation using emulsion systems has been the most successful way of preparing food products containing them with good oxidative stability (2-6).

Many food grade emulsifiers can be found in the food industry. For two decades, the replacement of synthetic emulsifiers with lecithin, protein and polysaccharide have been extensively tried in an effort to produce natural food products (“all natural” labeled) (7). Meanwhile, another issue in food emulsions has been to make an emulsion, which can maintain its quality without any off-flavors and phase separation during processing, storage, and delivery until digestion in the body. Until now, there is no single emulsifier that can be used as an all-round emulsifier for products with different compositions and that experience different environmental stresses. Protein and polysaccharide are one of the most popular ingredients used for producing various types of food products in the food industry. This is partly because they are perceived as being natural and partly because of their range of different functional properties (1), *i.e.* emulsifiers, stabilizers, texture modifiers, gelling agents, and foaming agents.

Recently, multilayer emulsion technology has been introduced to form structured delivery systems consisting of lipid droplets coated by nanolaminated biopolymer layers formed through electrostatic deposition. Biopolymer coatings around lipid droplets can be produced using a simple procedure (8). Initially, a “primary emulsion” is produced by homogenizing oil with an emulsifier solution. An ionic biopolymer is then added into the primary emulsion and the pH of the system is adjusted to where the droplets and biopolymer are oppositely charged. It has been shown that multilayer emulsions have better functionality than conventional emulsions, such as prevention of lipid oxidation and increased stability to environmental stresses. Nevertheless, there are still a number of scientific problems that need to be overcome before this technology can be successfully utilized by the food industry. In particular there is currently a poor understanding of the relationship between the stability and performance and the interfacial properties of the nanolaminated coatings.

The digestibility and bioavailability of food lipids have recently been of greater interest with respect to their impact on obesity and disease (9). The bioavailability of dietary fats is dependent on food source, processing and preparation conditions (10, 11). There are different forms of dietary fats including bulk, structural, emulsified and interfacial (1). During the digestion of dietary fats in the body, however, most fats are broken down into oil-in-water emulsions as they pass through the mouth and gastrointestinal tract due to the mechanical stresses and the presence of a number of surface-active components and stabilizing components from the food and the body: small molecule surfactants, bile salts, phospholipids, proteins, polysaccharides, their derivatives, and enzymes (12). Therefore, a variety of compositional and structural

changes occur after ingestion of emulsified lipids. *e.g.* lipid droplet size, interfacial composition, and droplet aggregation state (13). In principle, digestion of dietary lipids can be modulated by designing structured delivery systems using biopolymer coatings with different molecular structures and interfacial properties.

Competitive adsorption among surface-active components within food products and during digestion may play an important role in designing emulsion-based structured delivery systems. It has been proposed that the adsorption of pancreatic lipase is facilitated by the presence of bile salts, which are surface-active lipids with anionic and hydrophobic parts and it promotes lipid digestion by pancreatic lipase (14, 15). Hence, the composition, structure, and physicochemical properties of the interfacial layer surrounding the fat droplets should play an important role in determining the extent of enzyme binding to the emulsion surface and therefore the extent of lipolysis (16).

There are few studies that deal with the use of fundamental interfacial measurements to investigate the molecular changes at interfaces under digestion conditions. The main advantage of the nanolaminated biopolymer coatings would be the modulation of the interfacial composition with different types of biopolymers (17-22). Therefore, understanding changes in interfacial properties during digestion would be of importance in designing biopolymer coatings to control the kinetics of lipid digestion.

1.2 Objectives

The ultimate objective of this study was to carry out research that would lead to a better fundamental understanding of the major factors that determine the formation, stability and properties of multilayer emulsions, and then to utilize this information to

develop food-grade delivery systems. This study was carried out with four specific objectives:

- To examine the influence of droplet concentration, droplet size, droplet charge, and polysaccharide concentration on the formation and stability of multilayer emulsions consisting of lipid droplets coated with β -lactoglobulin/pectin layers,
- To develop an electroacoustic (EA) measurement system as an analytical method of characterizing the *in situ* interfacial properties of multilayer emulsions and study the interactive or competitive adsorption behavior of mixed polysaccharides (pectin/carrageenan or pectin/gum arabic) on protein-coated lipid droplet surfaces,
- To produce protein-rich coatings around lipid droplets using the electrostatic deposition method for potential practical applications of multilayer emulsions in the food industry *e.g.*, to protect ω -3 oils from oxidation or to develop natural weighting agents,
- To simulate the digestion of emulsions containing lipid droplets coated with protein-polysaccharide layers in various pH conditions.

CHAPTER 2 LITERATURE REVIEW

2.1 Food emulsions

2.1.1 Food emulsions

A significant number of natural and processed foods exist in an emulsified state. Food products such as milk, cream, soft drinks, salad dressings, mayonnaise, sauces, ice cream, coffee whitener, spreads, butter, and margarine are all food emulsions (23). A system consisting of two immiscible liquids (usually oil and water) is called an emulsion, where one of the liquids is dispersed as small droplets referred to as the dispersed phase in the other referred to as the continuous phase (23). Two-phase emulsions are usually either *oil-in-water* (O/W) or *water-in-oil* (W/O) emulsions (24). O/W emulsions consist of oil droplets dispersed in an aqueous phase, *e.g.*, milk, cream, dressings, mayonnaise, beverages, soups, and sauces (23). On the other hand, W/O emulsions consist of water droplets dispersed in an oil phase, *e.g.*, margarine and butter (23). Multiple emulsions consist of more than two phase and they are emulsions of emulsions, *e.g.*, water droplets-in-oil droplets-in-water (*water-in-oil-in-water*, W/O/W) or oil droplets-in-water droplets-in-oil (*oil-in-water-in-oil*, O/W/O) (25-29). Mixed biopolymer solutions may also form “emulsions”, *i.e.* water-in-water (W/W) or oil-in-water-in-water (O/W/W) emulsions (30-32).

2.1.2 Food emulsifiers

Emulsions are thermodynamically unstable due to the unfavorable contact between the two immiscible liquids (33). Therefore they are prone to break down over

time in the absence of a stabilizer. Destabilization of the emulsions involves a variety of physicochemical mechanisms such as gravitational separation, flocculation, coalescence, and Ostwald ripening (23). It is possible to make a kinetically stable emulsion with the addition of stabilizers including an emulsifier and a texture modifier. Emulsifiers are surface-active compounds that work at the oil-water interface by lowering the interfacial tension through their amphiphilic nature (7, 23). Food grade emulsifiers used commonly in the food industry include Tween series, Span series, diacetyltartaric esters of monoglycerides (DATEM), lecithin, mono- and diglycerides, proteins, and polysaccharides (7). For two decades, the replacement of synthetic emulsifiers with lecithin, protein and polysaccharide have been extensively studied in an effort to produce natural food products (“all natural” labeled) (7).

Protein is one of the heavily used emulsifier in the food industry. It has been regarded as a good emulsifier due to their ability to fast enough adsorb to the surfaces of the oil droplets produced and lowering the interfacial tension (23). The protein film around the lipid droplets is highly viscoelastic preventing the rupture or displacement by other surface active component (34). They can also provide emulsion good stability to droplet aggregation by generating an electrostatic repulsion between droplets (17). Emulsions stabilized by proteins, however, are sensitive to pH, ionic strength and thermal treatment due to changes in their charge and/or conformation (35). Adsorbed proteins lose their net charge around the isoelectric point thereby promoting extensive droplet aggregation. Conformation changes of proteins occur above their thermal denaturation temperature (T_m) and after interfacial adsorption, which exposes non-polar and sulfhydryl groups (36).

Polysaccharides, such as gum arabic and modified starch, are good at providing emulsion stability to aggregation and environmental stresses due to the formation of a thick interfacial layer, increasing the steric repulsion and decreasing the van der Waals attraction (23). Emulsions stabilized by polysaccharides, however, contain a considerable higher amount of polysaccharide increasing the manufacturing cost due to their low efficiency for lowering surface tension. Hence, it is possible to make an improved emulsifier by combining protein and polysaccharides.

The use of protein-polysaccharide complexes as emulsifiers has been extensively studied. The most common method for making protein-polysaccharide complexes is the dry heating of a mixture of protein and polysaccharide to form Maillard conjugates (37). Conjugation by the dry heating method is based on the Amadori rearrangement in the Maillard reaction of terminal amines of the protein and the reducing end of the polysaccharide (38). Different pairs of protein and polysaccharide have been used including whey protein-dextran (37, 39), β -Lg-dextran (40), whey protein-pectin (41-43), whey protein-maltodextrin (44), sodium caseinate-pectin (41, 45), casein-dextran (46), BSA-galactomann (47), soy protein isolate (SPI)-carboxymethyl cellulose (CMC) (48), and SPI-dextran (49, 50). All of them showed better emulsion stability than protein alone, but this technique involves the preparation time for producing a conjugate varying from 2 hr to 16 days and the emulsion stabilized by a conjugate produces relatively large droplets. Recently, a multilayer emulsion system has been developed in our laboratory as another effort to producing stable emulsions using mixed biopolymer systems. It has been shown that multilayer emulsions have better functionality than conventional emulsions, such as prevention of lipid oxidation and increased stability to environmental stresses.

2.2 Multilayer emulsions

2.2.1 Formation of multilayer emulsions

Multilayer emulsions are based on the electrostatic deposition of charged polymers onto oppositely charged colloidal particles (51-54), in this case charged polysaccharides onto protein-coated lipid droplets. Multilayered interfacial layers can be formed around lipid droplets using a simple procedure which is well-established in the literature (8) (Fig. 2.1). Polysaccharide coatings are typically formed around protein-coated lipid droplets using either a one-step or two-step process. Initially, an oil-in-water emulsion is formed containing protein-coated droplets with a known isoelectric point (pI).

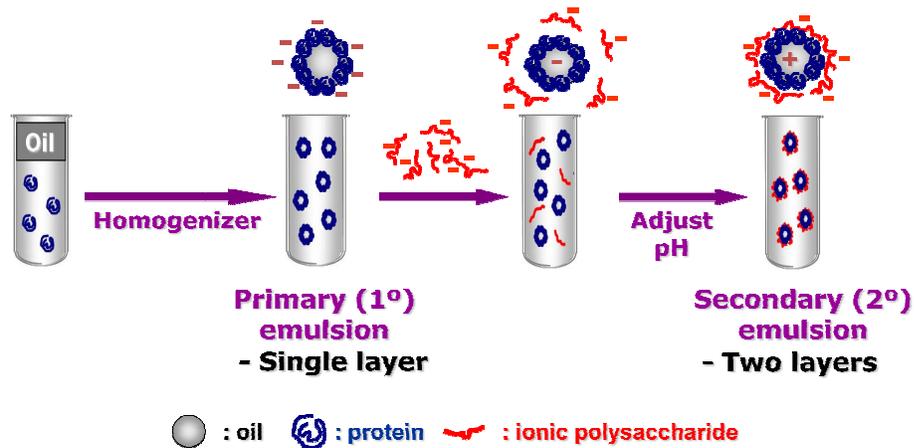


Figure 2.1. Formation of the multilayer emulsion using protein and polysaccharide interactions

In the one step process, this emulsion is mixed with a polysaccharide solution at a pH where the polysaccharide molecules directly adsorb to the droplet surfaces (usually $\text{pH} \leq \text{pI}$). At this pH, the polysaccharide molecules are negatively charged and the protein-coated droplets are positively charged (or have appreciable positive patches on

their surfaces). In the two step process, the emulsion and polysaccharide solution are initially mixed together at a pH where the polysaccharide molecules do not adsorb to the droplet surfaces ($\text{pH} > \text{pI}$) because the droplets and polysaccharide molecules are both negatively charged. The pH is then decreased so that the protein-coated droplets become positively charged and the anionic polysaccharide molecules adsorb to their surfaces. In either case, it is also important to ensure that the polysaccharide-coated droplets are stable once formed, *i.e.*, there is sufficient electrostatic and steric repulsion between them to avoid droplet flocculation (55). The two step method generally produces more stable polysaccharide-coated droplets because there is a uniform concentration of polysaccharide molecules around the droplets when adsorption occurs (8).

2.2.2 Properties of multilayer emulsions

The surface properties and stability of a multilayer emulsion depend on both the emulsifier forming the primary layer and the polymer type forming the secondary layer. The electrical charge of a primary emulsion stabilized by protein would change from negative above the isoelectric point (IEP) to positive below the IEP, however, the electrical charge of a primary emulsion stabilized by ionic emulsifiers such as SDS and lecithin or non-ionic emulsifier such as Tween 20 would not change over a range of pH values (23). Therefore, the most common combinations of primary and secondary layers studied are milk proteins, especially β -Lg and sodium caseinate with anionic polysaccharides such as pectin (17, 56-59), carrageenan (60, 61), alginate (62); and SDS with chitosan (20, 63) or fish gelatin (64); and, lecithin with chitosan (19, 65-68).

The stability and electrical charge of the emulsions stabilized by different types of proteins and the same polysaccharide depend on the proteins adsorbed onto the lipid

droplets. For example, ζ -potential of primary emulsions coated by different proteins was around to be +44 mV for β -Lg (17, 58) and +21 mV for sodium caseinate (59) at pH 3.5 and the emulsion stabilized by β -Lg was more stable to aggregation than the emulsion stabilized by sodium caseinate. The electrical charge of the secondary emulsions coated with protein-anionic polysaccharide layer changed from positive to negative: -18 mV for β -Lg-HM pectin and -12 mV for sodium caseinate-HM pectin at pH 3.5, respectively. The secondary emulsion stabilized by β -Lg-pectin layers was also more stable to aggregation ($d_{43} \approx 0.44 \mu\text{m}$) than those stabilized by sodium caseinate-pectin layers ($d_{43} < 10 \mu\text{m}$).

The magnitude of the electrical charge and stability of secondary emulsions strongly depends on the types of polysaccharide used in the case of primary emulsions stabilized by the same protein. Emulsions stabilized adsorption of carrageenan (60, 61) or alginate (69) onto oil droplets stabilized by β -Lg were more negatively charged than those stabilized by pectin (17, 58) due to their higher charge density. However, the particle size coated with carrageenan or alginate was larger and the particles also showed more creaming than with pectin.

Primary emulsions stabilized by SDS were highly negatively charged at pH 3 (~-50 mV) and charge reversal occurred when cationic polymers were added: +50 mV for SDS-chitosan (63) and +30 mV for SDS-fish gelatin (64), respectively. Both the emulsions stabilized by SDS-chitosan and SDS-fish gelatin were stable to droplet aggregation, but fish gelatin needed a higher concentration to completely saturate the lipid droplets. i.e. C_{sat} of fish gelatin was 1.6 wt% for 10 % corn oil, but C_{sat} of chitosan was 0.024 wt% for 1 % corn oil. This effect may be attributed to the fact that the

competitive adsorption between SDS and fish gelatin could occur as well as the electrostatic interaction (23).

Lecithin-stabilized emulsions are highly negatively charged (-50.9 ± 2.7 mV) and the electrical charge changes from negative to positive (54.5 ± 0.5 mV) with increasing chitosan concentration (19, 65-68). The concentration of chitosan (C_{sat}) to completely saturate lipid droplet surfaces (1 wt%) coated with lecithin was 0.04 wt% regardless of oil types (corn oil (67, 70) or tuna oil (66, 71)).

2.2.3 Stability of multilayer emulsions to environmental stresses

Numerous studies have shown that an electrostatic layer-by-layer (LbL) deposition technique can be used to improve the stability of protein-coated lipid droplets to a variety of environmental stresses, such as pH extremes, ionic strength, thermal processing, freezing and dehydration.

2.2.3.1 pH and salt stability

Food emulsions are exposed to variations in pH and ionic strength during food processing, storage, distribution, consumption, and digestion. Many food-grade emulsifiers are not pH sensitive, but proteins are highly susceptible to pH changes. For example, emulsions stabilized by whey proteins or caseins showed extensive aggregation around their isoelectric point (pI) due to the loss of their charges and therefore the lack of electrostatic repulsion to prevent flocculation. The addition of another layer onto lipid droplets stabilized by a protein layer or an emulsifier layer could provide stability of the emulsions to pH (Figure 2.2). For example, a primary emulsion stabilized by b-Lg was highly unstable to droplet aggregation near pI of the adsorbed β -Lg molecules, however,

a secondary emulsion stabilized by β -Lg/pectin layer showed a homogeneous stable emulsion over a whole pH range studied. Several different types of polysaccharides have been used to form a second layer or a third layer around lipid droplets coated with proteins or/and polysaccharide layers. The effect of an additional polymer layer on the emulsion stability to aggregation at various pH depended on the types of polysaccharides used.

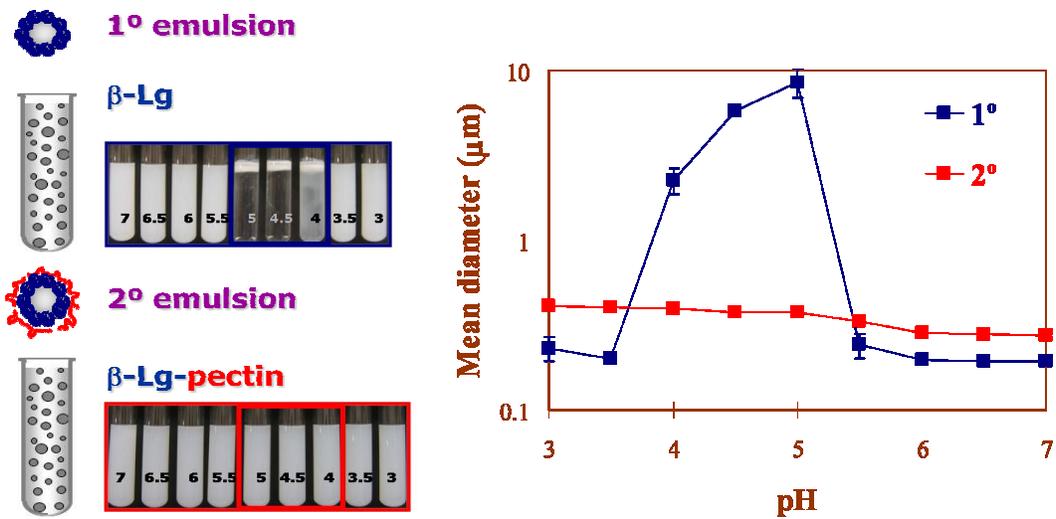


Figure 2.2. Dependence of the emulsion stability to droplet aggregation of a primary emulsion coated β -Lg layer and a secondary emulsion coated with β -Lg/pectin layers on pH: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 0.1 wt% pectin in 5 mM Na phosphate buffer

Electrostatic interactions between milk proteins and anionic polysaccharides such as pectin, gum arabic, and carrageenan have been extensively studied in solutions and emulsions and they revealed that they interact strongly around and below the pI of the protein (72-74). The addition of pectin to an emulsion stabilized by β -Lg improved the stability to aggregation around the pI of β -Lg (pH 4-5) (75). The effect of the addition of polysaccharide to form a secondary emulsion on emulsion stability to aggregation

depended on the nature of the primary emulsifier layer. A secondary emulsion containing sodium caseinate-pectin layers was stable to flocculation only at pH 5 and there were extensive aggregations at pH 3-4 different from the results from β -Lg-pectin layers systems due to its relatively low droplet charge of sodium caseinate leading to weak interaction with pectin unlike the electrostatic interaction between β -Lg and pectin (59). On the other hand, a secondary emulsion coated with sodium caseinate and alginate showed better stability to aggregation at higher salt concentration and around the pI of the protein than a primary emulsion stabilized by sodium caseinate alone due to its higher binding affinity of alginate for the sodium caseinate-coated lipid droplets (76).

The effect of molecular weight and charge density of chitosan on the emulsion stability containing the emulsion droplets stabilized by Tween 20 or SDS was studied: Mw 15 KDa~200 KDa and degree of deacetylation (DDA) 40~92 % (77). The emulsion stabilized by low molecular weight chitosan showed the higher magnitude of ζ -potential than other emulsions stabilized medium and high molecular weight chitosan, but showed the least stability to droplet aggregation. It could be explained by the difference in the thickness of an interfacial layer formed by different molecular weight chitosans. *i.e.* Low molecular chitosan formed a thinner interfacial membrane than other chitosans.

The addition of pectin to form soybean oil bodies coated with a polysaccharide layer improved physical stability over a wide range of pH (78). The electrical charge of the emulsion stabilized by soybean oil bodies changed from negative to positive with decreasing the pH of the emulsion from 8 to 2 and showed extensive aggregation around pH 5.5-3. On the other hand, the emulsion stabilized by soybean oil bodies-pectin layers was negatively charged and was stable to aggregation over all pH values studied. The

emulsion containing the lipid droplets coated with additional layers around an emulsifier layer showed the better stability in different types of emulsifiers and polysaccharides. Considering the variations of the environment of product experienced and increasing incidents of blends of mixing different types of components in a product, this technology would provide crucial information of producing products with different functionalities in the food industry.

The major colloidal interactions are van der Waals, electrostatic, steric, depletion, hydrophobic and those interactions should be considered when designing a stable emulsion to aggregation (23). Van der Waals and electrostatic interactions should be taken into account in emulsion systems consisting of oil droplets or water droplets, polymer molecules, and salt. Since the strength of the van der Waals and electrostatic interaction decreases in the presence of salt due to the reduction of the Hamaker function (A_{212} , Equation 2.2.1) and the electrostatic screening effects (the Debye screening length (κ^{-1}), Equation 2.2.2).

$$w_{VDW}(h) = -\frac{A_{212}r}{12h} \quad (2.2.1)$$

$$\kappa^{-1} = \sqrt{\frac{\epsilon_0 \epsilon_R kT}{e^2 \sum n_{oi} z_i^2}} \quad (2.2.2)$$

The influence of order of addition on the extent of droplet flocculation has been studied. It also has shown in the secondary emulsion stabilized by β -Lg/pectin layers. The emulsion coated with β -Lg/pectin layers was stable to aggregation in the presence of salt (0-50 mM NaCl), provided the salt was added after pectin adsorbed onto the droplets stabilized by β -Lg (79). The formation of multilayer around the lipid droplets is highly efficient in the absence of salt and the strong electrostatic interaction would occur

between oppositely charged protein-coated lipid droplets and polysaccharides. In the presence of salt, the electrostatic interaction between the protein-coated lipid droplets and polysaccharide molecules would be weakened leading to less dense packing of interfacial layers around the lipid droplets. The addition of salt before a deposition of polysaccharide layer, it would prevent the formation of multilayer around the lipid droplets due to the electrostatic screening effects.

The cross-linking of secondary adsorbed layers can improve emulsion stability to environmental stresses. The main disadvantage of this multilayered emulsion can be a possibility of the detachment of the adsorbed protein or polysaccharide layer from the droplet surfaces at pH, where the adsorbed layer has the same charge to the emulsifier-coated lipid droplets. The cross-linking of adsorbed layers could be carried out using a cross-linking enzyme like laccase or heat treatment of the adsorbed layer above their thermal denaturation temperature. Sugar beet pectin layers cross-linked by an enzyme (laccase) in emulsions stabilized by β -Lg/sugar beet pectin layers showed better stability to environmental stresses such as pH and NaCl (0-500 mM) than primary emulsions and non-cross-linked secondary emulsions (80). It can be attributed the fact that the adsorbed sugar beet pectin layer remained attached on the protein-coated lipid droplets due to the cross-linking by the laccase. The electrical charge and the mean particle diameter of the secondary emulsion with laccase treatment did not change after cross-linking of sugar beet pectin by laccase.

2.2.3.2 Lipid oxidation

An effective way to prevent lipid oxidation in oil-in-water emulsions has been to form a cationic charge on the droplet surface, e.g. using dodecyl trimethylammonium

bromide (DTAB) (81, 82) or proteins (5, 6, 83-85). This improved oxidative stability is thought to be due to repulsion of prooxidative metals from the droplet surface. Lipid oxidation was also significantly inhibited in the presence of proteins in the continuous phase of menhaden oil-in-water emulsions, which may have been due to their metal chelation or radical scavenging ability (85, 86).

Cationic emulsion droplets can be formed using the multilayered emulsion technique by deposition of a cationic polysaccharide layer onto anionic lipid droplets. The most widely studied polysaccharide for this strategy is chitosan, which is a cationic polysaccharide ($pK_a \approx 6.3-7$) and is composed primarily of glucosamine, (1→4)-linked 2-amino-2-deoxy- β -D-glucan [49-50]. Spray dried tuna oil (71) and ω -3 fatty acids (65) stabilized by lecithin and chitosan layers were more oxidatively stable than bulk oils. The oxidative deterioration of citral and limonene stabilized by SDS-chitosan layers was inhibited (87). Oxidative stability of multilayer emulsions depends on the types of polysaccharides used to form a secondary layer on the lipid droplets. A menhaden oil-in-water emulsion stabilized by β -Lg-citrus pectin showed better stability than β -Lg-sugar beet pectin (57). Even though it contains an antioxidant ferulic acid, it can be explained by the fact that sugar beet pectin contains higher mineral concentrations such as iron and copper than citrus pectin.

2.2.3.3 Freeze-thaw stability

One potential application of the multilayer emulsion technique is the ability to form thick protective layers around droplets that prevent rupture by oil or fat crystals during freeze-thaw cycling (8, 23). There have been several studies of the freeze-thaw stability of multilayer emulsions and their stability was characterized by ζ -potential,

particle size, microstructure, and creaming index. A tertiary emulsion consisting of β -Lg- ι -carrageenan-gelatin showed a better freeze-thaw stability during three freeze-thaw cycles (-20 °C for 22 hrs, 40 °C for 2 hrs) than a primary (β -Lg) or secondary (β -Lg- ι -carrageenan) emulsion, which can be explained by the formation of a thick interfacial layer around the tertiary emulsion (88).

2.3 Competitive adsorption at oil-water interface

Competitive adsorption between proteins and surfactants or polysaccharides at oil-water interfaces has been widely studied with increasing usages of the combinations of surface-active ingredients. Competitive adsorption in the presence of more than two emulsifiers or surface active ingredients depends on their concentration, surface activity, and saturation concentration. For decades, the most common systems studied for the competitive adsorption at interface are milk proteins and surfactants, milk proteins and lecithins, and between two different proteins.

2.3.1 Milk protein and surfactant

Protein is more surface active than small molecule surfactants at low concentrations, but surfactant is more efficient at lowering free energy (Π_{SAT}) at higher concentration (23). Therefore, one would expect that protein would dominate the adsorption at the oil-water interface at lower concentrations and the adsorbed protein layer would be displaced by the surfactant at higher concentrations. Ice cream is a good example for the displacement of protein by small molecule surfactants from fat globule

surfaces (23, 89). This displacement promotes partial coalescence of the fat globule helping structure formation during the freezing/agitation process (23, 89).

Other explanation of the displacement of the adsorbed protein layer from the interface by a small molecule surfactant could be that the differences in molecular structure causing the different adsorption behavior (34, 90). Surfactants, simple amphiphilic molecules, form a compact adsorbed layer than protein a viscoelastic layer and adsorb onto the interface through Gibbs-Maragoni mechanism. On the other hand, protein, complex macromolecules, has the property of poly-ionic and adsorb to the droplet surface through hydrogen, electrostatic, and hydrophobic interactions. Incompatible interface stabilization mechanism of protein and surfactant would lead to the displacement of one molecules from the other molecules form the interface when they exist together.

There have been extensive studies of the displacement of proteins (mainly β -Lg, α -casein (α -CN), and β -casein (β -CN)) by anionic surfactant SDS (91, 92), non ionic surfactants such as Tween series (93-106) and Span series (107-109), oil-soluble surfactants $C_{12}E_2$ (diethylene glycol n-dodecyl ether) (102, 103), and monoglyceride, diglyceride (110) from oil-water interfaces.

Competitive adsorption experiments using mixed solutions of β -casein or β -Lg with Tween 20 showed that Tween 20 completely (95-99) or partially (100) displaced proteins from an oil-water interface. It also suggested that both the displacement of the proteins by surfactants could modify the conformational change of the adsorbed proteins.

Anionic surfactants have relatively low surface activities compared to non-ionic surfactants and tend to interact with proteins at interfaces forming complexes and

therefore it needs a higher surfactant concentration than non-ionic surfactant for complete displacement of protein from an interface (111). When the amount of surfactant required for competitive displacement of protein at oil-water interfaces was compared between non-ionic surfactant (C₁₂E₅, hexaoxyethylene n-dodecyl ether) and anionic surfactant (SDS), the non-ionic surfactant was more effective at displacing the protein from the interface than the anionic surfactant. This difference was also clearly shown in a displacement experiment using β -Lg or gelatin (type A) and Tween 20 or sodium lauryl ether sulphate (SLES 2EO) (112). It revealed that the displacement of protein (whey protein isolate) by SDS in soy oil-in-water emulsion improved the stability of the emulsions by preventing flocculation (92).

Partial displacement of protein (β -casein) by glycerol monolinoleate (oil-soluble) and Tween 20 (water-soluble) surfactants in oil-in-water emulsions took place (104). The displacement performance by an oil soluble emulsifier, Span 80, was not as efficient as a water soluble emulsifier in an O/W emulsion stabilized by β -Lg (107, 109). It has been shown that displacement of β -CN by oil soluble emulsifier from an oil-water interface took place more readily than β -Lg. It could be explained by the fact that β -Lg forms a stronger viscoelastic film and therefore is more resistant to displacement than β -CN from the lipid droplet surfaces due to its compact globular structure and extensive protein-protein interactions.

The age of the adsorbed layer may also affect the competitive adsorption of protein and surfactants at oil-water interfaces. Macromolecular changes such as rearrangement, unfolding, and polymerization of free sulfhydryl groups of the adsorbed protein film occur over time leading to the formation of viscoelastic interfacial films

around droplets in emulsions. The aged adsorbed protein layer (β -Lg) was less susceptible to displacement by non-ionic surfactants (tween 20 or $C_{12}E_2$) in n-hexadecane oil-in-water emulsions (102). The presence of oil-soluble surfactant during emulsification improved the engagement of β -Lg with oil droplets in the emulsion over time.

The addition of another protein (gelatin type A) into a protein-stabilized emulsion allowed the adsorbed protein layer to be displaced by non-ionic surfactant (Tween 20) and anionic sodium lauryl ether sulphate (SLES 2EO). It suggests that a second layer of gelatin was formed around the lipid droplets coated with protein through an electrostatic interaction between β -Lg and gelatin. But it didn't provide the protective membrane for the displacement of the adsorbed protein layer by surfactants (112).

2.3.2 Protein and lecithin

Lecithin is one of the most widely used natural emulsifiers in the food industry and the main sources are from soybean oil and egg yolk (113). There are several different types of lecithins (*e.g.*, phosphatidylcholine, PC) and they form a lamellar mesophase in water (7).

The partial displacement of β -casein by lecithin has been shown at soya or tetradecane oil-water interfaces, when lecithin was added into emulsions before emulsification (95, 114). The surface viscosity of the adsorbed protein layer was decreased with the displacement of protein by lecithin at the oil-water interface (115). The greater solubility of lecithin in the continuous phase than in the discontinuous phase was observed and it can be attributed to the fact that lecithin can form lamellar mesophases and vesicles in aqueous media and produce lipid-protein complexes with excellent emulsifying properties (116). The degree of displacement of casein by egg

lecithin at oil-water interface depended on the solubility of lecithin in the discontinuous phase. Di-oleyl phosphatidylcholine (DOPC) showed the most effective competitor to casein at soya oil-in-water emulsions followed by phosphatidylcholines (PC) and dipalmitoyl phosphatidylcholine (DPPC) (117). On the other hand, protein (β -casein) was completely displaced by dioleoylphosphatidylcholine (DOPC) at oil-water interface, which also modified the hydrodynamic thickness of the adsorbed casein layer (118).

2.3.3 Different types of polysaccharide

Previously, researchers have examined the adsorption of single types of polysaccharide molecules onto protein-coated lipid droplet surfaces, such as carrageenan (60, 61, 119-121), pectin (17, 79, 122-125), alginate (126, 127), gum arabic (127) and soy soluble polysaccharide (128). Each type of polysaccharide has its own unique molecular characteristics, *e.g.*, molecular weight, electrical charge, branching, hydrophobicity, and conformational flexibility (129). Consequently, the interfacial coatings formed by different polysaccharide types will have different physicochemical properties (such as thickness, charge, permeability, and environmental responsiveness), which in turn will lead to different emulsion functional properties (such as stability, rheology, and delivery). It should therefore be possible to create polysaccharide-coated droplets with controllable functional properties by rational selection of different types of polysaccharides. Alternatively, it may be possible to control the functional properties of these systems by using polysaccharide blends, *i.e.*, assembling polysaccharides coatings using two or more polysaccharides with different molecular characteristics. However, there is little information of the competitive adsorption between different types of polysaccharide at an interface.

Recently, the competitive interaction between two different polysaccharides, soy soluble polysaccharide and pectin at oil-in-water interface was studied using sodium caseinate-stabilized O/W emulsion (130). The emulsion in the presence of both polysaccharides was stable to aggregation during acidification through diffusing wave spectroscopy (DWS) and ultrasonic spectroscopy (US). In our laboratory, the competitive adsorption between pectin and carrageenan, both anionic polysaccharides having different charge densities, was studied using an electroacoustic technique. In the presence of both polysaccharide, carrageenan dominated the adsorption onto the lipid droplets coated with β -Lg due to its higher charge density (22).

2.3.4 Techniques of characterization of the competitive adsorption

Traditionally, the competitive adsorption between protein and small molecules surfactants have been studied through particle size measurements by light scattering, ζ -potential measurements by micro-electrophoresis, and surface composition measurements by SDS-PAGE using oil-in-water emulsions containing more than one surface active ingredient (101, 117, 131-135). The technique of neutron reflectivity has also been used to study the structure and composition of the interface in the presence of both protein and surfactant (136-139).

Recently, the visualization of the competitive adsorption at a planar interface has been carried out using atomic force microscopy (AFM) (140-145) and Brewster angle microscopy (BAM) (146-149). Protein films can be observed on mica (140), graphite surfaces (140), air-water interfaces and oil-water interfaces (141). The combined use of AFM with studies of surface tension and surface rheology have revealed the mechanism of protein desorption from the air/water interface (140, 141). The surfactant is found to

adsorb at defects in the protein network and grow after nucleating sites followed by compressing the protein network, disrupting the protein network at higher surface pressure, and releasing proteins from the interface. This mechanism has been called orogenic displacement. The surfactant domain at the interface was different on protein types forming protein networks at the interface (140). For example, globular proteins such as β -Lg and α -La form stronger networks and the surfactant domain is irregular due to heterogeneous surface pressure. Random coil proteins such as casein form weaker networks and produce homogenous surface pressures. The mechanism of orogenic displacement explains the competitive adsorption between anionic surfactant and protein at oil-water interface by AFM (142). AFM and BAM have been widely used to characterize the properties of interfaces in the presence of mixed surface-active molecules. The most common systems studied by BAM are the competitive adsorption between milk protein and monoglyceride at an interface. This technique also has supported the orogenic displacement mechanism between milk proteins and monoglycerides (146-149). It is interesting to note that the study of competitive adsorption between adsorbed β -Lg layer on the droplet surfaces and bile salt was carried out using a AFM and they successfully found out the displacement of the adsorbed protein layer from the interface by bile salt (9). It could provide a crucial implication in understanding the digestion mechanism of the emulsified fats in the small intestine and fundamental information behind the digestion of dietary fat by lipase in the intestine.

2.4 Characterization of the surface properties of emulsions

2.4.1 Electroacoustic techniques

Over the past two decades, electroacoustic (EA) techniques have emerged as a powerful means of monitoring droplet charge in concentrated emulsions. EA techniques use a combination of electrical and acoustic phenomenon to determine the ζ -potential of emulsion droplets. EA measurements can be carried out in one of two ways (150, 151): (i) electrokinetic sonic amplitude (ESA), in which an electric signal is applied to an emulsion and the resulting acoustic field generated by the oscillating particles and generated dipoles is recorded, or (ii) colloid vibration potential (CVP), in which an acoustic field is applied to an emulsion and the resulting electric field generated by the oscillating particles and generated dipoles is recorded, provided that there is a density difference between the particles and the solvent. EA spectroscopy can be also be used to provide information about the droplet size distribution of emulsions, however, the droplet size range is usually rather limited (~ 0.1 - $10 \mu\text{m}$). The major advantage of the EA technique over more conventional micro-electrophoretic (ME) techniques based on light scattering is that it is capable of analyzing emulsions with high droplet concentration ($< 50\%$) without any sample dilution. EA techniques have been used to measure particle size distributions and ζ -potentials in a wide variety of different model and industrial oil-in-water (O/W) and water-in-oil (W/O) emulsions.

The figure below shows a typical electroacoustic measurement cell of a CVP or an ESA measurement. It consists of a transducer, delay rod, electrode, and high frequency voltage supply (Figure 2.3). The process of CVP and ESA are different within the the same electroacoustic spectroscopy. For CVP, an alternating voltage is applied to

the transducer and the resulting sound wave travels down the delay rod and passes into the colloidal dispersion (150, 152). The resulting potential difference is measured between the electrodes. For ESA, an electric field is applied between the electrodes, a sound wave is generated, and this travels out of the colloidal dispersion and down the delay rod where it is detected by the transducer (150, 152).

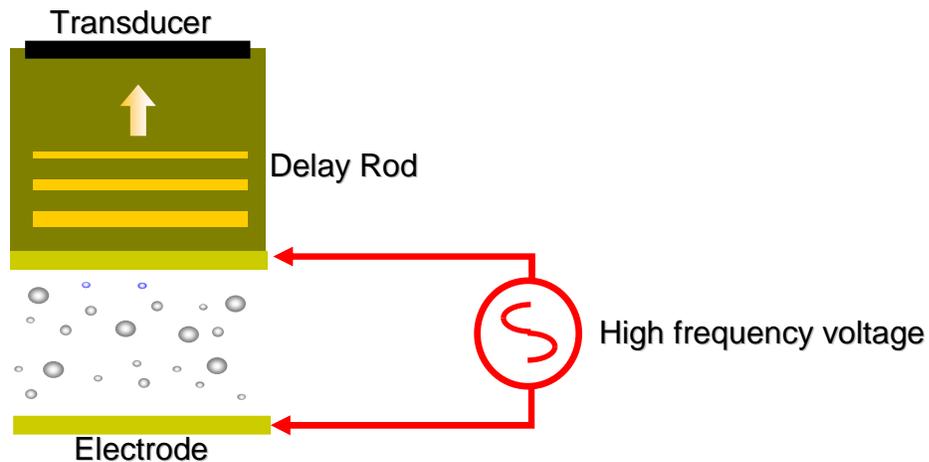


Figure 2.3. Diagram of a typical electroacoustic measurement cell of a CVP or an ESA measurement

2.4.2 Dynamic mobility

Electroacoustic spectroscopy requires an appropriate theory to interpret the experimental data. The dynamic mobility of the particles, the electric field and the pressure gradient are both alternating and, in general, the particle mobility lags behind the applied field, so that μ_d is a complex quantity with a magnitude and phase lag (152). Dynamic mobility measures the velocity of the particle motion per unit field strength. The phase angle measures the time lag between the applied field and the subsequent motion of the particle and this is zero at low frequencies and rises with increasing in

frequency and particle size (150, 153). The derivation of a dynamic mobility from the ESA effect has described in detail in Hunter's book (152) as well as the determination of particle size distribution and electrical charge from the equation of the dynamic mobility. Smoluchowski suggested the formula (2.4.1) for a colloidal particle whose double layer is thinner than its radius ($ka \gg 1$):

$$\mu_E = \frac{\varepsilon\zeta}{\eta} \quad (2.4.1)$$

Where ε is the permittivity of the liquid and η is the viscosity. O'Brien (154, 155) has shown that the dynamic mobility, μ_d , of the particles can be derived from the ESA signal:

$$ESA = A\phi \frac{\rho_p - \rho}{\rho} \mu_d \quad (2.4.2)$$

Where A is an instrument factor determined by calibration of the instrument, ϕ is the volume fraction of particles, ρ is a density difference of the particle and the solvent, and μ_d is a dynamic mobility. The magnitude of the ESA signal is proportional to the dynamic mobility. O'Brien (154, 155) has shown that the dynamic mobility would be defined:

$$\mu_d = \frac{2\varepsilon\zeta}{3\eta} G\left(\frac{\omega a^2}{\nu}\right)(1 + f) \quad (2.4.3)$$

Where G is an inertia factor and ε represents a permittivity of the liquid; ω is the frequency of the applied field; ν is the kinematic viscosity. The magnitude of the ESA signal is proportional to the dynamic mobility and therefore the zeta potential and particle size can be obtained from the equation of the dynamic mobility. It is clear that if

permittivity (particle) is small compared to the permittivity and the surface conduction parameter is not large, the function $f \approx 0.5$ and then (150):

$$\mu_d = \left(\frac{\varepsilon\zeta}{\eta}\right)G(\alpha) \quad (2.4.4)$$

So the dynamic mobility is given by a modified Smoluchowski equation, in which the G function accounts for the particle size. Dukhin et al. (156) introduced a new cell model theory (157, 158) and completely characterized a dispersion of particle size distribution and ζ -potential with a volume fraction up to 40%.

2.4.3 Particle size distribution and ζ -potential in O/W emulsions

The major advantage of the EA technique over more conventional micro-electrophoretic (ME) techniques based on light scattering is that is capable of analyzing colloidal dispersions with high droplet concentration (<50%) without any sample dilution. There are many studies of the electroacoustic characterization of the colloidal particles (156, 159-180). Meanwhile, there have been growing introductions and applications of the EA techniques to electro-surface characterization of the particles in emulsions. The surface property of an undiluted intravenous emulsion consisting of purified soybean oil, egg yolk, and phospholipids (181) and three different types of fat globule (182) was successfully characterized by EA techniques. Recently, Kong et al. (183-185) prepared sunflower and *n*-hexadecane oil-in-water emulsions stabilized by SDS and the particle size and electrical charge of the emulsions were measured by the EA techniques as a function of homogenization conditions, temperature, surfactant concentrations, and volume fractions of oil from 2 to 50 %. At higher volume fraction (> 10%), the new modified formulae with a concentration correction was introduced to

calculate the particle size and electrical charge from the measured dynamic mobility and it was valid for volume fractions up to 50%, provided the particles were not too small. For the formulae for the concentration correction, a modified formulae for near-neutrally buoyant systems has derived (179) and later O'Brien (186) has made a correction which be valid for volume fractions up to 60% with a assumption that only interaction with nearest neighbors are important and that these are pairwise additive.

The possibility has been investigated of using the EA techniques to determine the saturation concentration of ionic salts and polymers onto the electrically charged surface and it has been proven that the EA techniques would be a convenient tool for studying cmc or saturation concentration in non-diluted systems. For a colloidal dispersion, kaolin slurries was used (159); For emulsions, n-hexadecane emulsions stabilized by SDS (185); soybean O/W emulsions stabilized by Tween series (187); soybean O/W emulsion stabilized by BSA or lecithin (188); corn oil O/W emulsion stabilized by β -Lg (189). The effect of different types of ions on the formation of the emulsions such as monovalent cations, divalent cations, and trivalent cations was investigated by the combination of acoustic and electroacoustic devices in Hsu et al. (187) study. The influence of the addition of SDS on the formation of the O/W emulsions stabilized by BSA or lecithin was studied by the dynamic mobility from the ESA signal and it suggested that SDS micelles may forms a bridge between the adsorbed protein molecules (188). The adsorption of pectin onto the droplets stabilized by β -Lg was monitored by the ESA (189). The critical pectin concentration where the droplet surfaces became saturated with pectin was directly determined and the pH profile of the primary and secondary emulsion was successfully constructed in undiluted emulsion by the ESA.

2.4.4 Particle aggregation and growth

The aggregation of particles causes a change in the density difference depending on the degree of compaction of the floc (190). Therefore, it would affect the distribution of the electric field around the particle. The looser the floc, the more the field lines will be able to penetrate, thereby reducing the tangential electric field at the outer surface of the floc. Since this field drives the electrophoretic motion, the results will be a reduced mobility. On the other hand, the decrease in density associated with the open structure will tend to increase the mobility, since the inertial effect is smaller. The most common physical phenomena indicating instability of dispersions is the evolution of particle size leading to change in particle size distribution (23, 191). Particle size evolution as a function of pH and storage time was studied with Alumina and silica ludox and successfully traced the particle size evolution by the EA techniques (176). The shear stress caused by stirring during the pH titration may disrupt the aggregates and this effect should be considered when measuring particle size distributions using EA techniques.

Ostwald ripening (OR) is observed in emulsions containing oils with relatively highly solubility in water. The OR growth rate of droplets in emulsions can be measured by the EA techniques in the changes of attenuation or phase angle (191). The EA technique has been used to monitor Ostwald ripening over time in tetraline O/W emulsion (192) and octane and decane O/W emulsions (193). Depletion flocculation of tetraline O/W emulsions stabilized by SDS was observed over time with ESA (192). A large positive phase angle observed due to the formation of flocs by depletion flocculation of a polydisperse emulsion. The Ostwald ripening rate has also been

measured in octane or decane O/W emulsions stabilized by SDS using acoustic attenuation measurements (193).

2.4.5 Interfacial layer thickness

Another interesting application of electroacoustic techniques is the possible estimation of the thickness of the adsorbed layer onto the particles in colloidal systems. The influence of an addition of polymer or surfactant on the electroacoustic signal can be studied by the dynamic mobility (194). In other words, the ratio of dynamic mobility in the presence and absence of polymer or surfactant ($\Delta\mu_D(\text{coated})/\Delta\mu_D(\text{uncoated})$) can be calculated (194). It can be used to estimate the layer thickness using polymer gel theory (195). The effect of adsorbed layers on the electroacoustic signal has been carried out with silica (196) and titania (197) particles. In both silica with PEO and titania with PVA, the ESA signal was altered by the presence of the adsorbed layer with a reduction of the magnitude. Carasso et al (195) showed that the outer layer in the presence of PVA and nonyl phenol ethoxylate became thicker with increasing concentration and molecular weight by the ESA technique. There is a growing trend of using mixtures of surfactants or polymers to provide new or better functionality in colloidal dispersions. Therefore, the study of the adsorption of polymers onto particles coated with another layer of polymer provides important information for designing systems containing more than one component. The adsorption of non-ionic surfactant (nonyl phenol ethoxylate) (185) on SDS stabilized hexadecane O/W emulsion or neutral polymer (PEO) (198) on SDS stabilized sunflower O/W emulsions were studied by EA techniques based on the gel theory. The overall layer thickness increased with the surfactant concentration reaching a plateau at the higher concentrations.

2.4.6 Crystallization

The crystallization behavior of emulsified substances such as fats in O/W emulsions and water in W/O emulsions changes the density of the particles and the volume fraction of the suspension (23). Ultrasound has been used to study the crystallization behavior in emulsions (23, 199-206). The only one trial of the investigation of the crystallization by the EA techniques was made with a n-eicosane O/W emulsion stabilized by SDS (207). Hysteresis in the freezing-melting cycle in the crystallization of n-alkane emulsions allows us to use the EA techniques for monitoring the particle size and ζ -potential on both liquid and solid dispersions during the phase transition of particles from liquid to solid. The formula for particle size and ζ -potential from the dynamic mobility was independent to the state of particle, e.g. solid or liquid. The reduction in the magnitude due to the viscosity increase and the change in the phase angle due to the density difference were clearly shown as the evidences of the crystallization. Therefore, the EA technique would provide the opportunity to monitor the crystallization behavior on both liquid and solid dispersions of the same material under nearly identical conditions.

2.4.7 W/O emulsion

The main advantage of the EA techniques over ME techniques is that the electro-surface properties of W/O emulsions can be characterized. The characterization of the electrical surface properties of water droplets in W/O emulsions stabilized by dodecylbenzene sulphonic acid (HDBS) (208) and electrically charged micelles in hydrocarbon media (209) have been studied. A new theory for concentrated colloids including non-aqueous colloids was presented by Shilov et al. (174). It was interesting to note that

Kerosene W/O emulsion stabilized with SPAN was used to study a transition from an emulsion to mini-emulsion (210). It would open a new way to characterize the phase transition and the evolution of interfacial property along the phase transition of micro-emulsions without any possible defects along with sample dilution step necessary for the ME techniques.

CHAPTER 3

THEORETICAL STABILITY MAPS FOR GUIDING PREPARATION OF MULTILAYER EMULSIONS STABILIZED BY PROTEIN- POLYSACCHARIDE INTERFACIAL COMPLEXES

3.1 Abstract

The purpose of this study was to evaluate the usefulness of a simple theoretical model at predicting the stability of emulsions containing lipid droplets and polysaccharides. The influence of droplet concentration, mean droplet diameter, droplet charge and pectin concentration on the aggregation stability of the emulsions was examined. β -lactoglobulin stabilized emulsions (0.5 – 10 wt% oil) containing different pectin concentrations (0 to 0.5 wt%) were prepared at pH 7 (where lipid droplets and pectin molecules were both anionic) and pH 3.5 (where lipid droplets were cationic and pectin molecules anionic). The particle charge, size and creaming stability of the emulsions were then measured, and stability maps were constructed at pH 3.5 and 7. At pH 7, there was no evidence of pectin adsorption to droplet surfaces and the emulsions were stable to bridging flocculation, but depletion flocculation occurred when the pectin concentration exceeded about 0.1 wt% (independent of droplet concentration). At pH 3.5, pectin adsorbed to the droplet surfaces, and the emulsions were unstable to bridging flocculation at intermediate pectin concentrations (dependent on droplet concentration) and unstable to depletion flocculation at high pectin concentrations. At certain droplet and pectin concentrations stable multilayer emulsions could be formed consisting of protein-coated lipid droplets surrounded by a pectin layer. The information gained from

this study would be useful for optimizing the production of multilayer emulsions stabilized by protein-polysaccharide interfacial complexes.

3.2 Introduction

Recently, it has been shown that an electrostatic layer-by-layer (LbL) deposition technique can be used to improve the stability of protein-coated lipid droplets to a variety of environmental stresses, such as pH extremes, ionic strength, thermal processing, freezing and dehydration (8, 17, 18, 73, 79, 119, 123, 127, 211-213). This technique is based on the electrostatic deposition of charged polymers onto oppositely charged colloidal particles (51-54), in this case charged polysaccharides onto protein-coated lipid droplets. Multilayered interfacial layers can be formed around lipid droplets using a simple procedure which is well-established in the literature (8). Initially, a “primary emulsion” containing lipid droplets coated with a layer of protein molecules is prepared by homogenization. Subsequently, a “secondary emulsion” is formed by adsorbing an oppositely charged polysaccharide onto the droplet surfaces so that each droplet is covered by a protein-polysaccharide coating. These multi-component interfacial coatings often provide lipid droplets with improved stability to environmental stresses such as thermal processing, ionic strength, pH, freezing and dehydration than single-component coatings (8, 18, 60, 79, 127, 214-216). In addition, the ability to systematically control the properties of the interfacial coatings can be used to develop delivery systems with novel controlled or triggered release properties (53). A major challenge associated with utilizing these multilayer emulsions industrially is that they are highly susceptible to

flocculation and therefore it is important to establish the optimum conditions required for their preparation (60).

The purpose of the present study was to examine some of the major factors that impact the preparation of stable oil-in-water emulsions containing lipid droplets coated with globular protein-anionic polysaccharide interfacial layers. For this reason, we examined the influence of droplet concentration, droplet size, droplet charge, and polysaccharide concentration on the formation and stability of these multilayer emulsions. A special emphasis was placed on comparing the experimentally determined stability maps with theoretical stability maps calculated using a recently developed theory (60).

3.3 Theoretical Prediction of Multilayer Emulsion Stability Maps

A simple theoretical model was recently developed to predict the influence of various factors on the stability of multilayer emulsions prepared by mixing spherical droplets with oppositely charged polyelectrolytes (60). This model predicts that the stability of an emulsion can be divided into a number of different regimes depending on the polyelectrolyte concentration (C).

(I). $C = 0$. In the absence of polyelectrolyte, the stability of the droplets to aggregation is governed by the relative strength of the attractive (usually van der Waals and hydrophobic) and repulsive (usually electrostatic and steric) interactions between them. If the attractive interactions dominate, then the droplets will aggregate, but if the repulsive interactions dominate, then the droplets will remain as individual entities.

(II). $0 < C < C_{Sat}$. Bridging flocculation occurs when the polyelectrolyte concentration is insufficient to completely saturate the particle surfaces (C_{Sat}). This is because there are both positive and negative patches on the droplet surfaces, which promotes bridging flocculation due to sharing of single polyelectrolyte molecules between neighboring droplets. These aggregates cannot subsequently be disrupted by the application of mechanical stresses since there is always insufficient polyelectrolyte available to coat all of the droplets.

(III). $C_{Sat} < C < C_{Ads}$. Bridging flocculation also occurs when the polyelectrolyte concentration is sufficient to completely saturate the droplets surfaces, but it is too low to ensure that the droplets are saturated with polyelectrolyte before a droplet collision occurs (C_{Ads}). In principle, it should be possible to disrupt these aggregates by the application of mechanical stresses because there is sufficient polyelectrolyte present to saturate all the droplet surfaces once the flocs have been broken.

(IV). $C_{Ads} < C < C_{Dep}$. Multilayer emulsions containing droplets coated by a polyelectrolyte layer can be formed when their surfaces are rapidly and completely saturated with polyelectrolyte, and when there is not enough free polyelectrolyte present in the continuous phase to promote depletion flocculation. Under these circumstances it should be possible to prepare stable multilayer particles consisting of particles completely surrounded by a polyelectrolyte layer (provided the net droplet repulsion overcomes the net droplet attraction).

(V). $C > C_{Dep}$. When the concentration of free polyelectrolyte exceeds some critical value (C_{Dep}) depletion flocculation occurs because the depletion forces make the

overall attractive forces strong enough to overcome the overall repulsive forces (*e.g.*, electrostatic and steric).

The following expressions were derived for the critical polyelectrolyte concentrations mentioned above (60):

$$C_{Sat} = \frac{3\phi\Gamma_{Sat}}{r} \quad (3.1)$$

$$C_{Ads} = \sqrt{\frac{60\Gamma_{Sat}^2 r_{PE}\phi}{r^3}} \quad (3.2)$$

$$C_{Dep} = \frac{M}{N_A} \left(\frac{-1 + \sqrt{1 - 8vX}}{4v} \right) \quad (3.3)$$

Where:

$$X = \left(\frac{w_{Dep}}{k_B T} \right)_{Crit} \frac{1}{2\pi r_{PE}^2 \left(r + \frac{2}{3} r_{PE} \right)}$$

where ϕ is the volume fraction of the particles, Γ_{Sat} is the surface load of the polyelectrolyte at saturation (in kg m⁻²), r is the radius of the spherical particles (in m), r_{PE} is the radius of the polyelectrolyte molecules in solution (in m), M is the molecular weight of the polyelectrolyte (in kg mol⁻¹), v ($=4\pi r_{PE}^3/3$) is the effective molar volume of the polyelectrolyte in solution (in m³), N_A is Avogadro's number, w_{Dep} is the strength of the depletion attraction at droplet contact, which depends on droplet size and non adsorbing polyelectrolyte concentration, k_B is the Boltzmann's constant, and T is the absolute temperature.

These equations can be used to develop “stability maps”, which indicate the region where stable multilayer emulsions can potentially be created . To produce a

colloidal dispersion that is stable to flocculation it is necessary to ensure that (C_{Sat} & C_{Ads}) $< C < C_{\text{Dep.}}$, *i.e.*, that there is enough polyelectrolyte to completely saturate the surfaces of the particles, but not too much free polyelectrolyte to promote depletion flocculation. Using the various equations derived above for C_{Sat} , C_{Dep} and C_{Ads} it is possible to generate stability maps for formation of multilayer droplets without promoting bridging flocculation and depletion flocculation. Further details about the construction of stability maps are given in an earlier reference . Stability maps predicted using the above expressions will be compared with experimental data on a system consisting of protein-coated droplets and pectin (see below).

3.4 Materials and Methods

3.4.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Pectin extracted from citrus fruit was purchased from Sigma Chemical Company (Lot # 016K0713, St. Louis, MO). The degree of esterification (DE) of the pectin was reported to be 60% by the supplier. The average molecular weight of the pectin was determined to be 310 kDa by static light scattering (NanoZS, Malvern Instruments, Worcs., UK). Corn oil was purchased from a local supermarket and used without further purification. Analytical

grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from the Sigma Chemical Company (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Iowa) was used for the preparation of all solutions.

3.4.2 Solution preparation

An emulsifier solution containing 1 wt% protein was prepared by dispersing powdered β -Lg into 5 mM phosphate buffer (pH 7.0) and stirring for at least two hours to ensure complete hydration. A 1 wt% pectin solution was prepared by dispersing powdered pectin into the same phosphate buffer and stirring for at least four hours to ensure complete hydration.

3.4.3 Emulsion preparation

A stock emulsion was prepared by homogenizing 20 wt% corn oil with 80 wt% aqueous emulsifier solution (1 wt% β -Lg, pH 7.0) with a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min followed by 5 passes through a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA): the first stage at 3000 psi, the second stage at 300 psi. Emulsions containing different droplet concentrations (0.5 to 10 wt%) and pectin concentrations (0 to 0.5 wt%) were prepared by mixing different proportions of stock emulsion, pectin solution (1 wt%, 5 mM phosphate, pH 7) and buffer solution (5 mM phosphate, pH 7). The pH of the resulting emulsions was then adjusted to either pH 7.0 or pH 3.5 using HCl. To investigate the disruption of flocs formed during preparation, some of the emulsions were subjected to an ultrasound treatment using a high-intensity ultrasonic

generator (model 500, sonic Disembrator, Fisher Scientific, Pittsburgh, PA) with a titanium alloy horn.

3.4.4 ζ -Potential measurements

To determine the electrical charge on lipid droplets, emulsions were diluted to a droplet concentration of 0.05 wt% using an appropriate buffer solution (at the same pH as the sample) and placed into the measurement chamber of a micro-electrophoresis instrument (ZEM 5300, Zetamaster, Malvern Instruments, Worcs., UK). This instrument determines the electrical charge (ζ -potential) on the particles in an emulsion by measuring the direction and velocity of particle movement in an applied electric field. The ζ -potential measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with five readings made per sample.

3.4.5 Particle size analysis

Emulsion samples were diluted to a droplet concentration of approximately 0.005 wt% using an appropriate buffer solution (at the same pH as the sample) and placed into the measurement chamber of the laser diffraction instrument (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). This instrument finds the particle size distribution of an emulsion that gives the best fit between the experimental measurements and predictions made using light scattering theory (*i.e.* Mie theory). A refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions. Measurements are reported as the volume weighted mean diameter: $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$ or as the volume-surface mean diameter: $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of droplets of diameter

d_i . The particle size measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with two readings made per sample.

3.4.6 Creaming stability measurements

Ten grams of each emulsion were transferred into a test tube (with 15 mm internal diameter and 125 mm height), and then stored for 24 hours at room temperature. After storage a number of emulsions separated into an optically opaque “cream layer” at the top and a transparent (or turbid) “serum layer” at the bottom. The total height of the emulsions (H_E) and the height of the cream layer (H_C) were measured. The extent of creaming was characterized by the Cream Layer Thickness = $(H_C/H_E) \times 100$. The cream layer thickness provided indirect information about the extent of droplet aggregation in the emulsions: the more aggregation, the larger the particles, the faster the creaming and the thicker the cream layer. All measurements were made on at least two freshly prepared samples.

3.4.7 Microstructure measurements

Emulsion microstructure was examined by optical microscopy (Nikon microscope Eclipse E 400, Nikon Corporation, Japan). Emulsions were mixed in a glass test tube using a vortexer to prepare a homogeneous sample and then a drop of emulsion was placed on a microscope slide and covered by a cover slip. Microstructure images of emulsions were then obtained using a CCD camera (CCD-300-RC, DAGE-MTI, Michigan City, IN) and the images were processed by Digital Image Processing Software (Micro Video Instruments Inc., Avon, MA).

3.4.8 Statistical analysis

Experiments were performed twice using freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements.

3.5 Results and Discussions

3.5.1 Stability map at pH 7

The droplet size, electrical charge and creaming stability of β -Lg stabilized oil-in-water emulsions was measured at pH 7.0 as a function of droplet concentration (0.5 to 10 wt%) and pectin concentration (0 to 0.5 wt%) and an experimental stability map was constructed (Table 3.1). This stability map was based on observations of the creaming stability of the emulsions after 24 hours storage at room temperature. The creaming stability of the emulsions was characterized according to the degree of visible phase separation that had occurred: *stable* (S) – emulsions appeared homogeneous throughout; *partially unstable* (U*) – a cream layer was observed at the top of the tubes, and a highly turbid or opaque serum layer was observed at the bottom; *highly unstable* (U) - a cream layer was observed at the top of the tubes, and either a transparent or slightly turbid serum layer was observed at the bottom. All the emulsions were stable to creaming when the pectin concentration was ≤ 0.06 wt%, were partially unstable from 0.08 to 0.1 wt% pectin, and were highly unstable at 0.3 and 0.5 wt% pectin, irrespective of the initial droplet concentration. This data indicated that there was a critical flocculation concentration (CFC) for the emulsions somewhere between 0.08 and 0.1 wt% pectin, which did not depend strongly on droplet concentration. We attribute the creaming

instability of the emulsions at high pectin concentrations to *depletion flocculation* caused by the presence of non-adsorbed pectin molecules in the aqueous phase, as has been reported by many other workers (73, 217-222).

Table 3.1. Stability map of β -Lg stabilized oil-in-water emulsions as a function of droplet and pectin concentration (pH 7.0). The stability was defined in terms of their creaming stability: S = stable (no visible separation); U* = (visible cream layer, highly turbid or opaque serum layer); U = (visible cream layer, clear or slightly turbid serum layer).

| Pectin Concentration (wt%) | Droplet Concentration (wt%) | | | | | |
|----------------------------|-----------------------------|----|----|----|----|----|
| | 0.5 | 1 | 3 | 5 | 8 | 10 |
| 0.00 | S | S | S | S | S | S |
| 0.01 | S | S | S | S | S | S |
| 0.02 | S | S | S | S | S | S |
| 0.04 | S | S | S | S | S | S |
| 0.06 | S | S | S | S | S | S |
| 0.08 | U* | U* | U* | U* | U* | U* |
| 0.10 | U* | U* | U* | U* | U* | U* |
| 0.30 | U | U | U | U | U | U |
| 0.50 | U | U | U | U | U | U |

At pH 7, the electrical charge on both the pectin molecules ($\zeta = -44.1 \pm 0.6$ mV) and the protein-coated lipid droplets ($\zeta = -61.1 \pm 0.4$ mV) was negative, and therefore one would not expect the pectin molecules to adsorb to the droplet surfaces because of a strong electrostatic repulsion. The non-adsorbed pectin will therefore be excluded from a narrow region surrounding each droplet, which is approximately equal to the radius of

hydration of the pectin molecules. Consequently, there will be a pectin concentration gradient between the “exclusion zone” surrounding each droplet ($C_{\text{pectin}} \approx 0$) and the bulk aqueous phase ($C_{\text{pectin}} \approx C_{\text{bulk}}$) (55). This concentration gradient leads to the generation of an osmotic pressure that tends to drive the droplets into close proximity so as to reduce the total volume of the region from where pectin molecules are excluded. The magnitude of this osmotic pressure increases with increasing pectin concentration, until the depletion attraction is sufficiently strong to overcome the repulsive interactions operating between the droplets, and so the droplets flocculate. The minimum pectin concentration where flocculation is first observed is the CFC.

Measurements of the creaming stability of the emulsions showed that the thickness of the cream layer at the top of the unstable emulsions increased with decreasing droplet concentration, and with increasing pectin concentration above the CFC (Figure 3.1). This result can be attributed to differences in the concentration and packing of the droplets within the cream layer. At higher initial droplet concentrations, there are more droplets available to pack into the creamed layer (55). In addition, at higher initial droplet concentrations flocculation is more likely to lead to the formation of a three-dimensional particle network that extends throughout the volume of the container and therefore restricts further creaming (55). As the pectin concentration increases the strength of the depletion attraction between the droplets increases, which means that they are more likely to stick strongly together and form an open structured particle network (223). In addition, the viscosity of the continuous phase increases with increasing pectin concentration, which would slow down droplet movement.

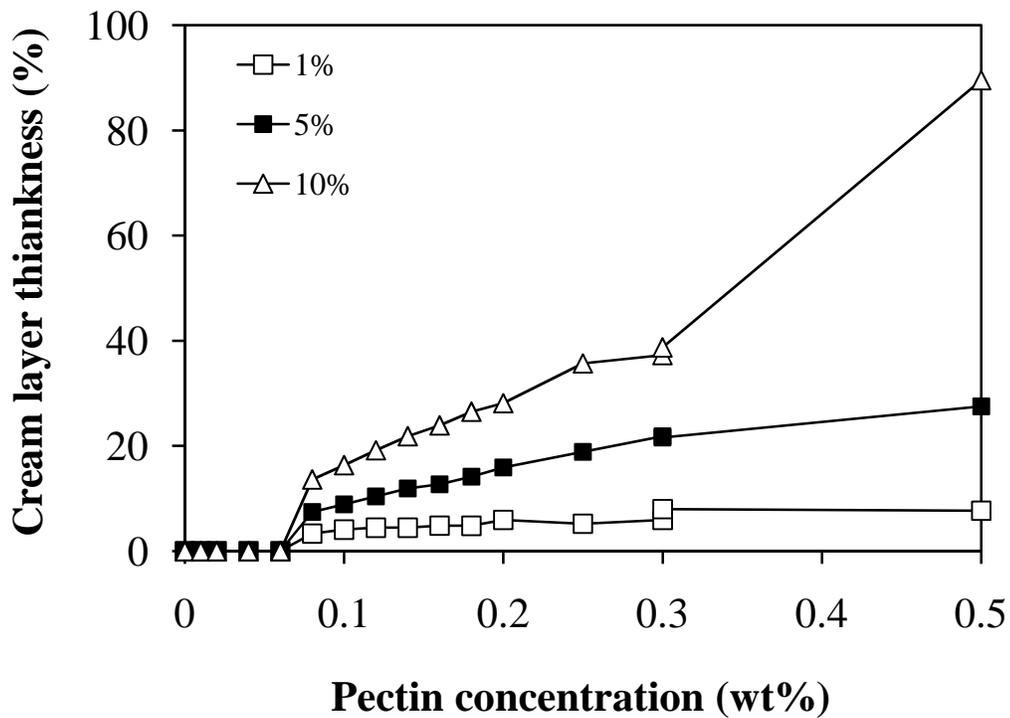


Figure 3.1. Influence of droplet and pectin concentrations on the thickness of the creamed layer of β -Lg stabilized oil-in-water emulsions (pH 7).

After storage, the measured mean particle diameter of all the emulsions was the same as the initial value ($d_{32} = 0.27 \pm 0.01 \mu\text{m}$; $d_{43} = 0.37 \pm 0.01 \mu\text{m}$), which indicated that the emulsions were stable to droplet coalescence, and that flocs formed in the emulsions above the CFC were disrupted when the emulsions were diluted for particle size measurements. The disruption of flocs upon dilution is commonly seen with emulsions that have been flocculated through a depletion mechanism, and can be attributed to the fact that the concentration of non-adsorbed biopolymer in the continuous phase is reduced to below the CFC when the emulsion is diluted for the particle size measurements. At pH 7 there should not be any bridging flocculation because the pectin does not adsorb to the droplet surfaces ($C_{Sat} = 0$), hence C_{Sat} and C_{Ads} do not need to be

calculated. Consequently, a relatively simple theoretical stability map can be calculated using the equations given above, since only C_{Dep} needs to be determined. Theoretical calculations of the dependence of C_{Dep} (Equation 3.3) on droplet concentration were made using experimentally determined parameters for the pectin molecules and oil droplets used in this study (Figure 3.2). Three droplet radii ($r = 150, 200$ and 250 nm) were used in these calculations to examine the effects of emulsion polydispersity. The median droplet radii of the emulsions used in this study was 180 nm, with 50 vol% of the particles being within the range 120 and 290 nm. The mean molecular weight of pectin (310 kDa) was measured by static light scattering in this study, and the radius of gyration of pectin ($r_{PE} = 20$ nm) was taken from a previous study of a number of pectins (224). The theoretical predictions are in reasonable agreement with the experimental measurements, indicating that depletion flocculation should occur when the pectin concentration exceeds about 0.2 wt% (depending on droplet diameter and concentration). Nevertheless, the theoretical model predicts that the critical flocculation concentration (CFC) for depletion should slightly decrease with increasing droplet concentration (Figure 3.2). We did not observe this phenomenon in our study (Figure 3.1), possibly because of the polydispersity of both the emulsion droplets and the pectin molecules (see below) The predictions were made assuming a polymer molecular weight of 310 kDa and a radius of gyration of 20 nm.

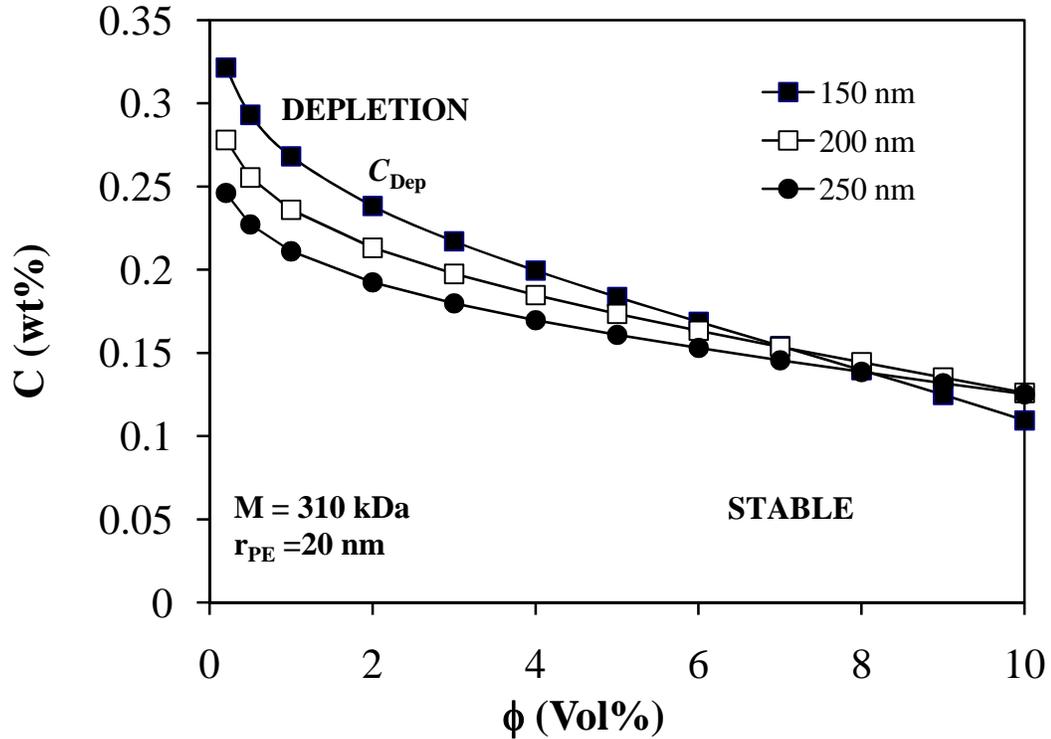


Figure 3.2. Predicted stability map of oil-in-water emulsions with difference droplets sizes and concentrations due to depletion flocculation (pH7). The stability map shows phase boundaries plotted as polymer concentration (C) versus droplets concentration (ϕ).

The experimental measurements indicated that there was not a distinct transition at a particular pectin concentration from a stable emulsion (*i.e.*, homogeneous whitish appearance throughout) to an unstable emulsion (*i.e.*, a white cream layer on top of a transparent serum layer). Instead, there was a range of pectin concentrations where a cream layer was observed on top of a turbid serum layer, with the turbidity of the serum layer decreasing with increasing pectin concentration (Figure 3.3). This phenomenon can largely be attributed to the polydispersity of both the emulsion droplets and the pectin molecules. Theory and experiment have shown that the CFC for depletion flocculation increases with decreasing droplet size and increasing polymer molecular weight (225-

228). Hence, in a polydisperse emulsion a greater amount of pectin is required to induce depletion flocculation (and therefore creaming instability) for the larger droplets than the smaller ones. This effect is seen in Figure 3.2, which predicts the dependence of C_{Dep} on droplet concentration and size for emulsions similar to the ones used in this study. The three values of the particle radius used in these predictions are in the range of the values for the emulsion used in this study: 50 vol% of the droplets in the emulsions had radii between 120 and 290 nm. These predictions show that there should actually be a range of pectin concentrations where depletion flocculation and creaming should occur, which is what we observed experimentally (Figure 3.3).

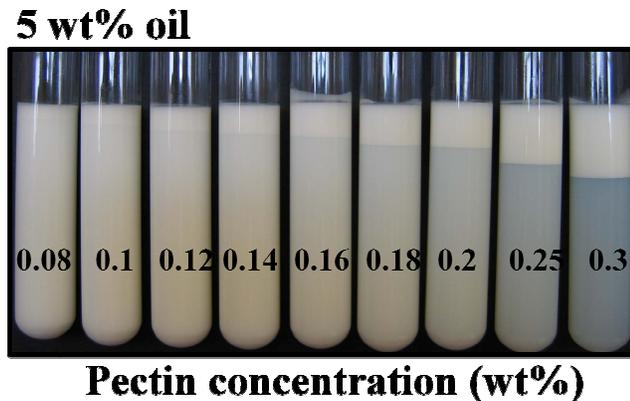


Figure 3.3. Photograph of a 5 wt% oil-in-water emulsion containing different pectin concentrations (pH 7). Some emulsions separated into an opaque white cream layer and a turbid serum layer, which was attributed to depletion flocculation.

Theoretical calculations of the effect of pectin radius of gyration on the critical flocculation concentration ($M = 310$ kDa, $r = 200$ nm) indicated that the CFC decreased from about 0.34 wt% to 0.1 wt% as r_{PE} was increased from 10 to 100 nm for a 1 wt% oil-in-water emulsion. Consequently, if there was a range of pectin molecules with different chain lengths one might also expect a range of CFC values.

3.5.2 Stability map at pH 3.5

The droplet size, electrical charge and creaming stability of β -lactoglobulin stabilized oil-in-water emulsions was also measured as a function of droplet concentration (0.5 to 10 wt%) and pectin concentration (0 to 0.5 wt%) at pH 3.5 (Figures 3.4 to 3.6). This information was used to construct an experimental stability map (Table 3.2).

Table 3.2. Stability map of β -Lg stabilized oil-in-water emulsions as a function of droplet and pectin concentration. The stability was defined in terms of their creaming stability: S = stable (no visible separation); U* = (visible cream layer, highly turbid or opaque serum layer); U = (visible cream layer, clear or slightly turbid serum layer).

| Pectin Concentration (wt%) | Droplet Concentration (wt%) | | | | | |
|----------------------------|-----------------------------|----|----|----|---|----|
| | 0.5 | 1 | 3 | 5 | 8 | 10 |
| 0.00 | S | S | S | S | S | S |
| 0.01 | U* | U | U | U* | S | S |
| 0.02 | S | S | U | U | S | U* |
| 0.04 | S | S | U | U | U | U* |
| 0.06 | S | S | U | U | U | U |
| 0.08 | S | S | S | U | U | U |
| 0.10 | S | S | S | U* | U | U |
| 0.30 | U* | U* | U* | S | S | S |
| 0.50 | U* | U* | U* | S | S | S |

At this pH, the electrical charge on the pectin molecules is negative ($\zeta = -25.5 \pm 0.9$ mV) and on the protein-coated lipid droplets is positive ($\zeta = +54.6 \pm 0.5$ mV), and

therefore one would expect the pectin molecules to adsorb to the droplet surfaces because of a strong electrostatic attraction (8, 79). The emulsions were stable to creaming in the absence of pectin, which indicated that the electrical charge on the protein-coated droplets was high enough to generate a strong electrostatic repulsion that prevented droplet flocculation.

In the presence of pectin, the emulsions were either stable or unstable to creaming depending on the amount of pectin added and the droplet concentration. For example, the 3 wt% oil-in-water emulsion was stable to creaming at 0 wt% pectin, unstable at 0.01 to 0.06 wt% pectin, stable from 0.08 to 0.10 wt% pectin, and unstable again at 0.30 and 0.50 wt% pectin (Table 3.2, Figures 3.5 & 3.6). We attribute this fairly complicated dependence of emulsion creaming stability on pectin concentration to a combination of bridging and depletion flocculation:

(I). *No Pectin*. In the absence of pectin, the emulsions were stable to creaming because the relatively strong electrostatic repulsion between the droplets prevented them from coming into close proximity and aggregating.

(II). *Low Pectin Concentrations (Bridging Flocculation)*. The poor creaming stability observed at relatively low pectin concentrations (*e.g.*, 0.01 to 0.04 wt% pectin for the 3 wt% oil emulsion) can be attributed to charge neutralization and bridging flocculation caused by sharing of anionic pectin molecules between two or more cationic protein-coated droplets. The range of pectin concentrations where this kind of creaming instability was observed increased with increasing droplet concentration (Table 3.2), as would be expected because the amount of polysaccharide needed to completely cover all

the droplet surfaces present should increase with increasing droplet concentration (Equation 3.1).

(III). *Intermediate Pectin Concentrations (Stable Multilayer Formation)*. The good creaming stability observed at intermediate pectin concentrations (*e.g.*, 0.08 to 0.1 wt% for the 3 wt% oil emulsion) can be attributed to the fact that the lipid droplets were completely coated with pectin molecules, and there was a strong electrostatic and steric repulsion between the coated droplets. Thus stable multilayer emulsions can be formed in this range of pectin concentrations.

(IV). *Relatively High Pectin Concentrations (Depletion Flocculation)*. The poor creaming stability observed at relatively high pectin concentrations (*e.g.*, 0.3 and 0.5 wt% for the 3 wt% oil emulsion) can be attributed to depletion flocculation caused by the presence of a large quantity of non-adsorbed pectin in the aqueous phase. No creaming instability was observed in the pH 3.5 emulsions with the highest droplet concentrations (5 to 10 wt%) at relatively high pectin concentrations, even though creaming instability was observed in pH 7.0 emulsions with the same droplet concentrations. The most likely reason for this observation is that there was insufficient non-adsorbed pectin to promote depletion flocculation in the pH 3.5 emulsions because an appreciable fraction was adsorbed to the droplet surfaces (rather than free in the aqueous phase). Alternatively, the droplet concentration in these emulsions may have been sufficient to prevent droplet creaming due to network formation.

Evidence for adsorption of pectin molecules to the protein-coated droplet surfaces at pH 3.5 was obtained from ζ -potential *versus* pectin concentration measurements (Figure 3.4). In the absence of pectin, the protein-coated droplets had a ζ -

potential of around +55 mV because the adsorbed proteins were below their isoelectric point (pI = 5). The ζ -potential of the droplets became increasingly less positive and then became negative as the pectin concentration was increased, eventually reaching a plateau value at around 0.1 wt% pectin. This data indicates that anionic pectin molecules adsorbed to cationic protein-coated droplets until the droplet surfaces became saturated with pectin. The critical polysaccharide concentration where the droplet surfaces became saturated with pectin was established by modeling the ζ -potential *versus* polysaccharide concentration curves using an empirical equation to model the data (79):

$$\frac{\zeta(c) - \zeta_{Sat}}{\zeta_0 - \zeta_{Sat}} = \exp\left(-\frac{c}{3C_{Sat}}\right) \quad (3.4)$$

where $\zeta(c)$ is the ζ -potential of the emulsion droplets at polysaccharide concentration c , ζ_0 is the ζ -potential in the absence of polysaccharide, ζ_{Sat} is the ζ -potential when the droplets are saturated with polysaccharide, and C_{Sat} is the minimum amount of polysaccharide required to completely cover the droplet surfaces. The binding of a polysaccharide to the droplet surfaces can therefore be characterized by ζ_{Sat} ($= \zeta_0 - \zeta_{Sat}$) and C_{Sat} . Values for ζ_{Sat} and C_{Sat} were calculated by fitting the above equation to the experimental ζ -potential *versus* pectin concentration curve for the 3 wt% oil-in-water emulsion: $\zeta_{Sat} = +71$ mV and $C_{Sat} = 0.069$ wt% pectin. The good agreement between the model and experiment is shown in Figure 3.4.

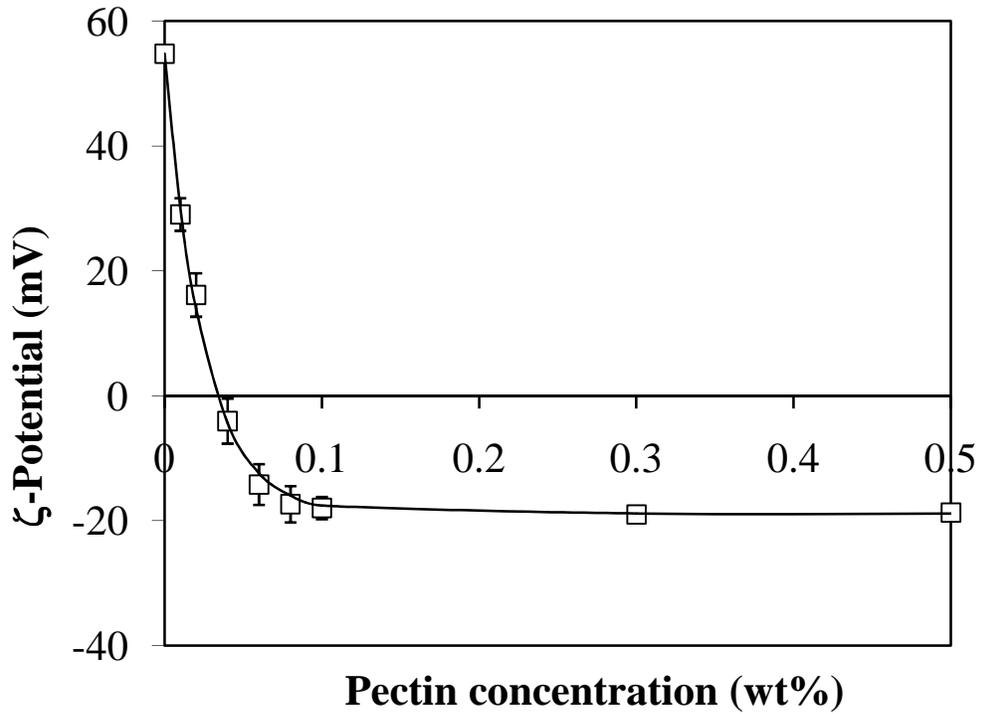


Figure 3.4. Influence of pectin concentration on the ζ -potential of β -Lg stabilized 3 wt% oil-in-water emulsions (pH 3.5). The initial d_{32} of the primary emulsion was 270 nm. The symbols represent the experimental data points, and the line presents the best-fit to the data using Equation 3.4.

The saturation concentration depends on the size and concentration of emulsion droplets used (since this determines the exposed surface area), and therefore it is useful to calculate a more system-independent quantity for the amount of adsorbed pectin. The surface load at saturation can be calculated using the following expression:

$$\Gamma_{Sat} = \frac{C_{Sat} d_{32}}{6\phi} \quad (3.5)$$

Here, C_{Sat} is defined as the mass of polyelectrolyte (pectin) adsorbed to the surface of the droplets per unit volume of emulsion (kg m^{-3}), d_{32} is the volume-surface mean droplet diameter, and ϕ is the droplet volume fraction. For this study: $d_{32} = 0.27$

μm , $\phi = 0.03$ (3%) and $C_{Sat} \approx 0.69 \text{ kg m}^{-3}$ (0.069 wt%), hence $C_{Sat} = 1.03 \text{ mg m}^{-2}$. This value is in good agreement with those found for the surface loads of other anionic polysaccharides adsorbed to the surfaces of protein-coated oil droplets: $C_{Sat} \sim 1.3, 1.6, 2.1$ and 5.1 mg m^{-2} for alginate, pectin, carrageenan and gum arabic adsorbed to β -Lg coated oil droplets, respectively (8, 21, 62).

The influence of droplet concentration on particle aggregation and creaming stability of the emulsions was measured (Table 3.2, Figures 3.5 and 3.6). The increase in mean particle diameter and creaming instability due to bridging flocculation at intermediate pectin concentrations are clearly seen in Figures 3.5 and 3.6. As the droplet concentration in the emulsions increased, the range of pectin concentrations where bridging flocculation was observed increased, which can be attributed to the increase in the total droplet surface area that needs to be covered by pectin (Equation 3.1.). Using the information for the saturation concentration given above we calculated $C_{Sat} = 0.012, 0.023, 0.069, 0.12, 0.18, 0.23 \text{ wt\%}$ pectin for the 0.5, 1, 3, 5, 8 and 10 wt% emulsions. This would account for the fact that emulsions stable to bridging flocculation could only be formed when the pectin concentration exceeded these critical values *e.g.*, 0.01, 0.02, 0.06, >0.1, >0.1 and >0.1 wt% pectin for the for the 0.5, 1, 3, 5, 8 and 10 wt% emulsions, respectively (Table 3.2, Figures 3.5 and 3.6). At higher droplet concentrations, the mean particle diameter ($d_{32} < 0.4 \mu\text{m}$) of the emulsions was fairly similar to the initial values, which indicated that the emulsions were stable to droplet coalescence, and that any flocs formed in the emulsions were disrupted when the emulsions were diluted for particle size measurements. Nevertheless, the formation of a thin creamed layer was observed on top of some of the emulsions at higher pectin concentrations (Table 3.2), which indicated that

flocculation did occur. This kind of droplet aggregation can be attributed to depletion flocculation due to the presence of relatively high concentrations of non-adsorbed pectin in the continuous phase, as discussed in the previous section.

As mentioned above, at pH 3.5 the droplets are positively charged while the pectin is negatively charged and so one would expect both bridging and depletion flocculation to occur. The theoretical approach described earlier was used to construct a stability map based on calculations of the C_{sat} , C_{ads} and C_{dep} values given in Equations 3.1 to 3.3 and the following experimentally determined parameters: $r = 180 \text{ nm}$, $r_{\text{PE}} = 20 \text{ nm}$, $M = 310 \text{ kg mol}^{-1}$ and $C_{\text{Sat}} = 1.03 \times 10^{-6} \text{ kg m}^{-2}$ (Figure 3.7).

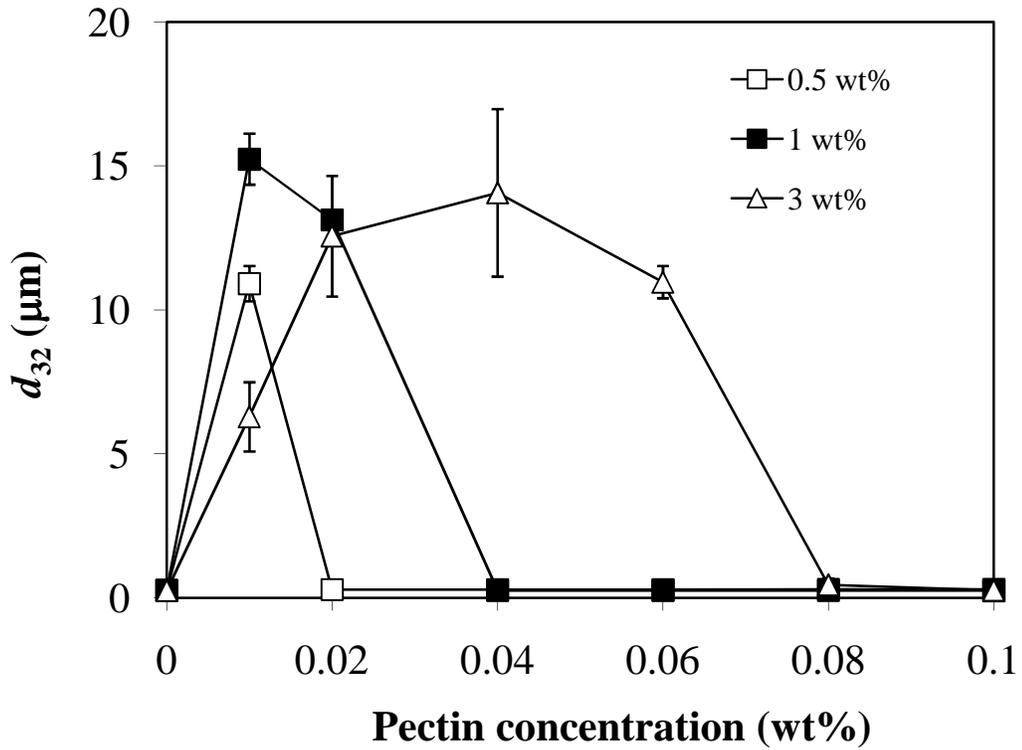


Figure 3.5. Influence of pectin and droplet concentrations on the mean particle diameters of β -Lg stabilized oil-in-water emulsions (pH 3.5).

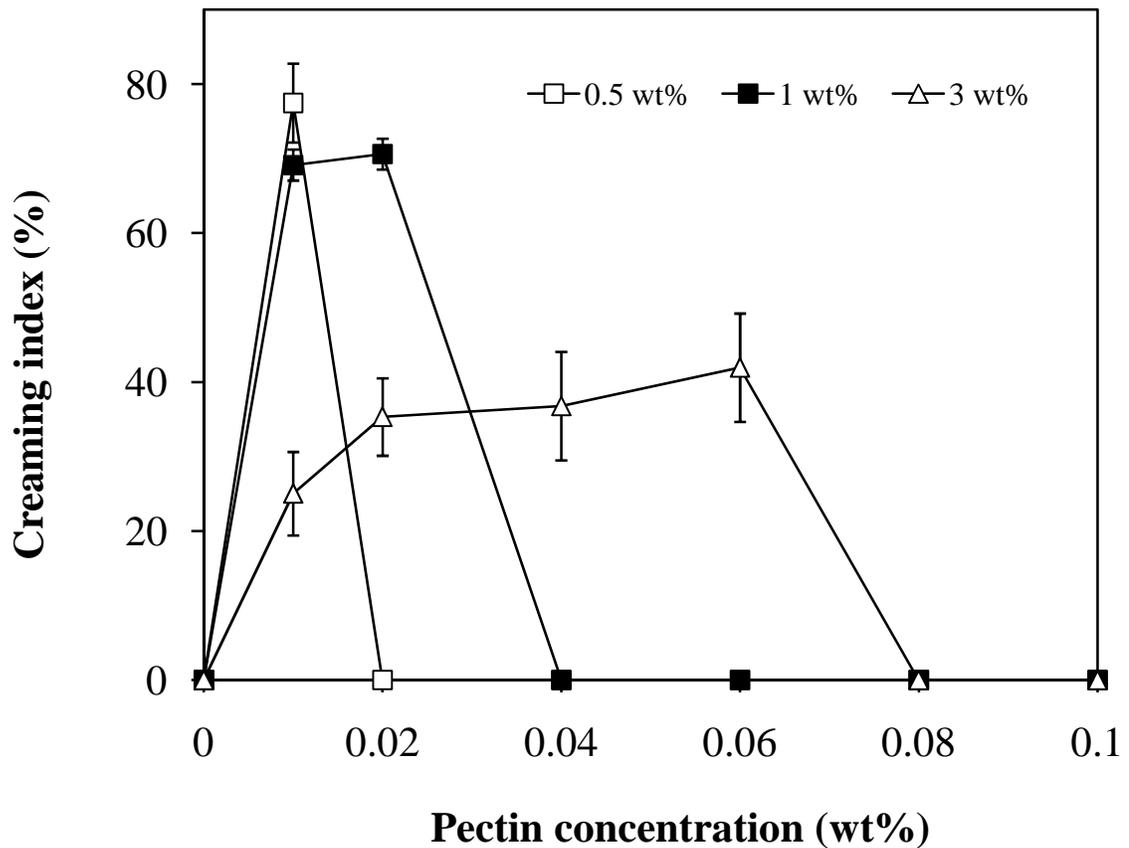


Figure 3.6. Influence of pectin and droplet concentrations on the creaming stability in β -Lg stabilized oil-in-water emulsions (pH 3.5).

The theoretical stability map predicts that stable multilayer emulsions can only be produced over a narrow range of droplet and pectin concentrations. In particular, the stability map suggests that stable multilayer emulsions can only be produced at droplet concentrations less than about 3 wt%, with the range of pectin concentrations where stable emulsions can be produced increasing with decreasing droplet concentration (Figure 3.7). For example, for 1 wt% emulsions it should only be possible to create stable multilayer emulsions at pectin concentrations between about 0.15 and 0.26 wt%. Below 0.02 wt% pectin one would expect to get irreversible bridging flocculation

because there was insufficient pectin to cover the droplet surfaces, between 0.02 and 0.14 wt% one might expect to get reversible bridging flocculation because the pectin did not adsorb rapidly enough to the droplet surfaces (even though there was sufficient present to saturate the surfaces), and above 0.26 wt% one would expect to get depletion flocculation.

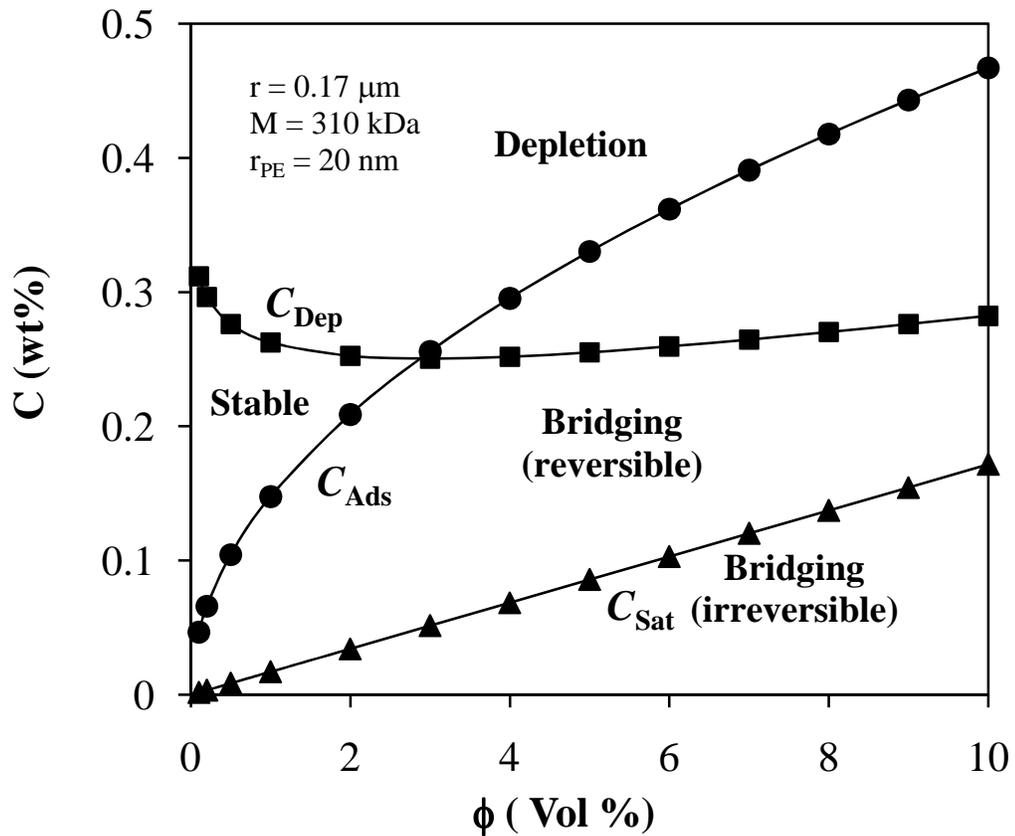


Figure 3.7. Predicted stability map of oil-in-water emulsions with difference droplet and pectin concentrations (pH 3.5). The stability map shows phase boundaries plotted as polymer concentration (C) versus droplet concentration (ϕ). The predictions were made assuming a polymer molecular weight of 310 kDa and a radius of gyration of 20 nm, and a droplet radius of 180 nm.

The theoretical predictions are in fairly good qualitative agreement with the experimental measurements (Table 3.2, Figures 3.5 and 3.6). For example, the experimental stability map shows: (i) bridging flocculation occurs over a range of intermediate pectin concentrations; (ii) depletion flocculation occurs at high pectin concentrations in some of the samples; (iii) the range of pectin concentrations where emulsions stable to bridging flocculation can be formed increases with decreasing droplet concentration. The theoretical stability maps therefore seem to provide a good qualitative prediction of the stability of the emulsions, which may prove useful when designing optimum conditions for preparing multilayer emulsions. Nevertheless, there are quantitative differences between the experimental measurements and theoretical predictions, which can be attributed to limitations in the assumptions underlying the theory, as well as the effects of droplet and pectin polydispersity.

Microscopic images of the emulsions indicated that different kinds of aggregate structures were formed in the emulsions in the intermediate pectin range (bridging flocculation) and the high pectin range (depletion flocculation) (Figure 3.8).

At intermediate pectin concentrations all of the droplets appeared to be present in the form of large aggregates, which appeared to contain some coalesced droplets. At high pectin concentrations a smaller fraction of the droplets appeared to be aggregated, and the aggregates formed were much smaller. These differences in aggregate structure can be attributed to the different mechanisms responsible for droplet flocculation: bridging flocculation (strong, irreversible) and depletion flocculation (weak, reversible).

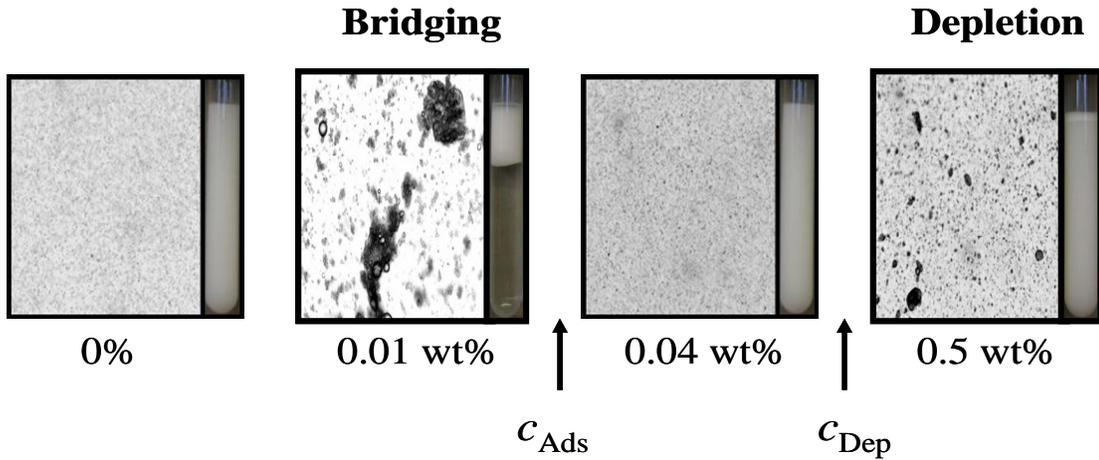


Figure 3.8. Photographs and microscopy images of 1 wt% oil-in-water emulsions containing different pectin concentrations (shown beneath each image) at pH 3.5. The microscopy images were taken after 7 day storage at a magnification of $\times 100$ (the scale bar is shown in the 0 wt% pectin figure). The creaming measurements were made after emulsions were stored in glass test tubes (15 mm internal diameter, 125 mm height) for 24 hours.

3.5.3 Influence of initial droplet size

The expressions for C_{Sat} , C_{Ads} , and C_{Dep} given above (Equation 3.1 to 3.3) indicate that the stability map of an emulsion containing oppositely charged droplets and polymer molecules should depend on the mean droplet diameter. We therefore investigated the influence of initial droplet diameter on the properties of emulsions as a function of pectin concentration (Figures 3.9 to 3.11). Three 1 wt% oil-in-water emulsions (pH 3.5) with different mean droplet diameters ($d_{32} = 0.26 \pm 0.01$, 0.47 ± 0.02 , $0.62 \pm 0.04 \mu\text{m}$) and pectin concentrations (0 to 0.5 wt%) were prepared using the approach described earlier.

Initially, we calculated the dependence of C_{Sat} , C_{Ads} , and C_{Dep} on droplet diameter for a 1% oil-in-water emulsion using Equations 3.1 to 3.3 and the values of the

mean molecular weight (310 kDa) and radius of gyration ($r_{PE} = 20$ nm) of pectin given earlier (Figure 3.12). The value of C_{Sat} should be inversely proportional to d_{32} . Nevertheless, we did not observe an appreciable difference in the pectin-dependence of the ζ -potential of the three emulsions with different mean droplet diameters (Figure 3.9). Indeed, the surface loads of the pectin molecules calculated from the ζ -potential *versus* pectin profiles using Equations 4 and 5 were $C_{SAT} = 1.1, 1.6$ and 2.0 mg m⁻² for the emulsions with $d_{32} = 0.26, 0.47,$ and 0.62 μm , respectively. These values suggest that the amount of pectin adsorbed to the droplet surfaces decreased with decreasing mean droplet diameter. This phenomenon may be due to the effects of droplet curvature on pectin adsorption, since theoretical calculations indicate that adsorption of a charged polyelectrolyte to an oppositely charged surface becomes less favorable as the curvature of the surface increases (229). Alternatively, it may be due to differences in the type of pectin molecules that adsorb to the droplet surfaces, *e.g.*, the molecular weight and packing of pectin molecules may be different on small and large droplets. Further research is needed to identify the origin of the effects of droplet size on pectin surface load.

There were some differences between the stability of the three emulsions to aggregation and creaming (Figures 3.10 and 3.11). Extensive droplet aggregation and creaming instability were observed in all three emulsions at 0.01 and 0.02 wt% pectin concentrations, which can be attributed to bridging flocculation. Nevertheless, the extent of droplet aggregation was greatest in the emulsion with the smallest droplets ($d_{32} = 0.26$ μm) in this pectin range (Figure 3.10). This effect can be accounted for by the fact that C_{Ads} is expected to increase rapidly with decreasing droplet size (Figure 3.12), which

occurs because the time between droplet collisions decreases more rapidly than the time required for the droplet surfaces to be saturated with pectin, leading to more bridging flocculation.

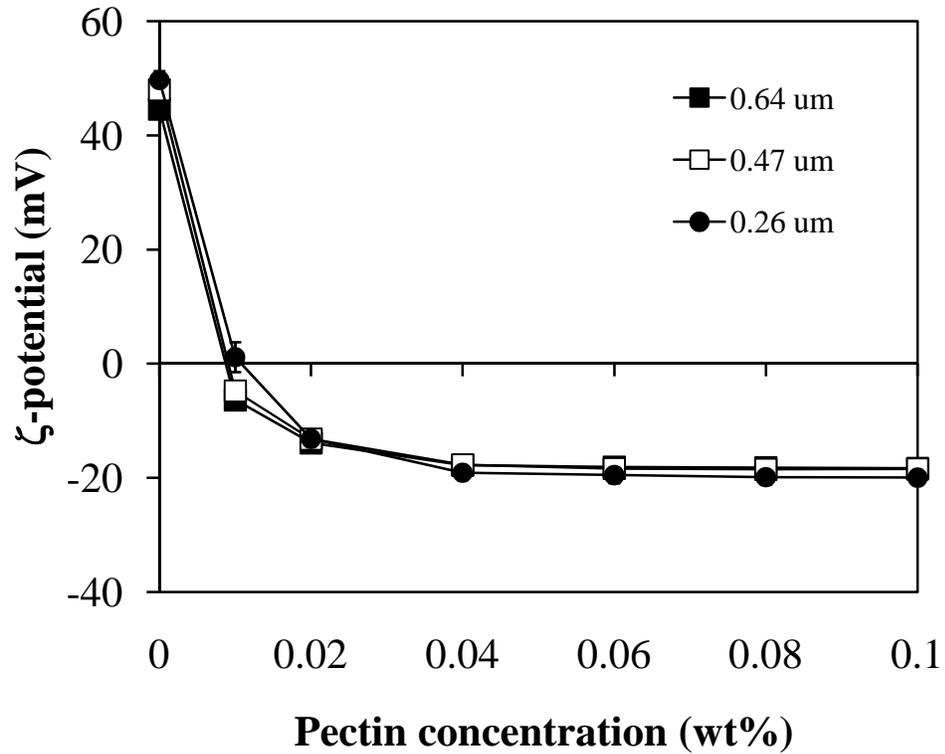


Figure 3.9. Influence of pectin concentration on electrical charge of β -Lg stabilized 1 wt% oil-in-water emulsions with difference mean droplet diameters (pH3.5).

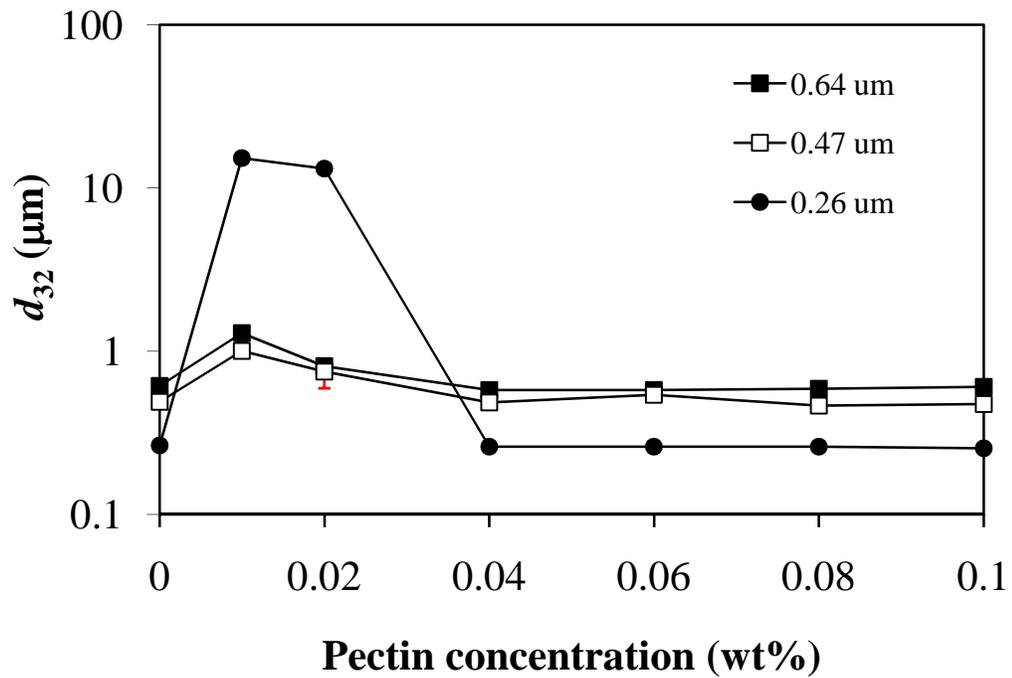


Figure 3.10. Influence of pectin concentration on mean particle diameter of β -Lg stabilized 1 wt% oil-in-water emulsions with difference mean droplet diameters (pH3.5).

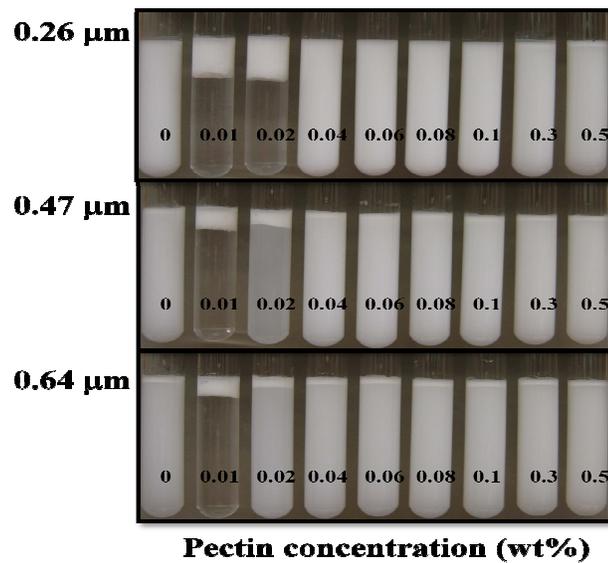


Figure 3.11. Influence of pectin concentration on appearance of β -Lg stabilized 1 wt% oil-in-water emulsions with difference mean droplet diameters (pH 3.5).

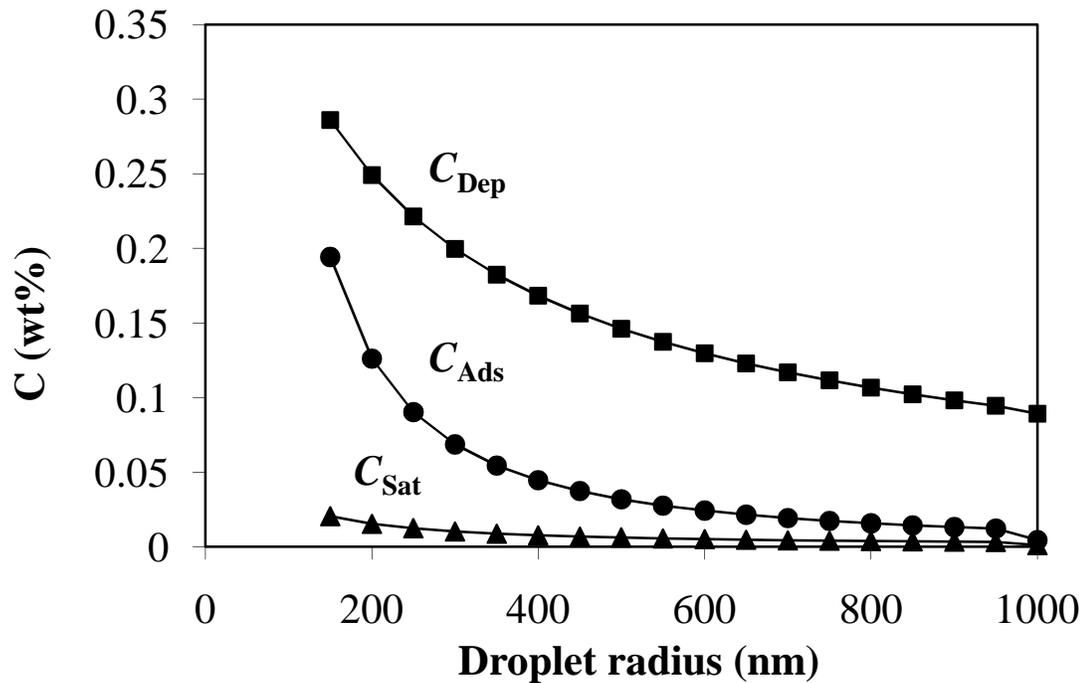


Figure 3.12. Predicted dependence of critical flocculation concentration (C_{Sat} , C_{Dep} and C_{Ads}) on droplet size for oil-in-water emulsions. The predictions were made assuming a polymer molecular weight of 310 kDa and a radius of gyration of 20 nm, and a droplet concentration of 1 wt%.

3.5.4 Distinguishing irreversible and reversible bridging flocculation

The theoretical model predicts that bridging flocculation may have two potential origins: (i) insufficient pectin present to cover all of the droplet surfaces present; (ii) relatively slow adsorption of pectin molecules to droplet surfaces compared to the time between droplet collisions. In the first case, bridging flocculation should be irreversible since there will never be enough pectin to entirely cover the droplet surfaces. In the second case, it may be possible to disrupt flocs once they have formed by applying mechanical agitation, since there should be sufficient pectin present in the system to cover all the droplet surfaces. Previous studies have shown that mechanical treatment,

such as homogenization, high-speed blending or high-intensity ultrasound, can be used to disrupt the flocs formed during preparation of multilayer emulsions under some circumstances (17, 63, 67). In these studies, it was suggested that this improvement in emulsion stability was due to the presence of sufficient polymer to adsorb to the droplet surfaces and provide a strong repulsion between the droplets after the flocs had been disrupted. The data presented in the previous section showed that there was only a narrow range of pectin concentrations where stable multilayer emulsions could be produced at pH 3.5, which was attributed to bridging flocculation caused by the two mechanisms described above. We postulated that we might be able to broaden this range by applying ultrasound treatment to breakdown any flocs formed due to slow pectin adsorption during the preparation of the multilayer emulsions.

The influence of sonication on floc disruption in 1 wt% oil-in-water emulsions containing different levels of pectin is shown in Figure 3.13. In the absence of sonication extensive droplet flocculation occurred at 0.01 and 0.02 wt% pectin, as demonstrated by an increase in mean particle diameter and creaming index. After the application of sonication, there appeared to be a slight improvement in stability of the emulsions to droplet aggregation at 0.02 wt% pectin, although a visible creaming layer was still observed. This result suggests that applying mechanical agitation to emulsions may be able to improve their stability to bridging flocculation under certain conditions, but not under conditions where there is insufficient pectin to saturate the droplet surfaces.

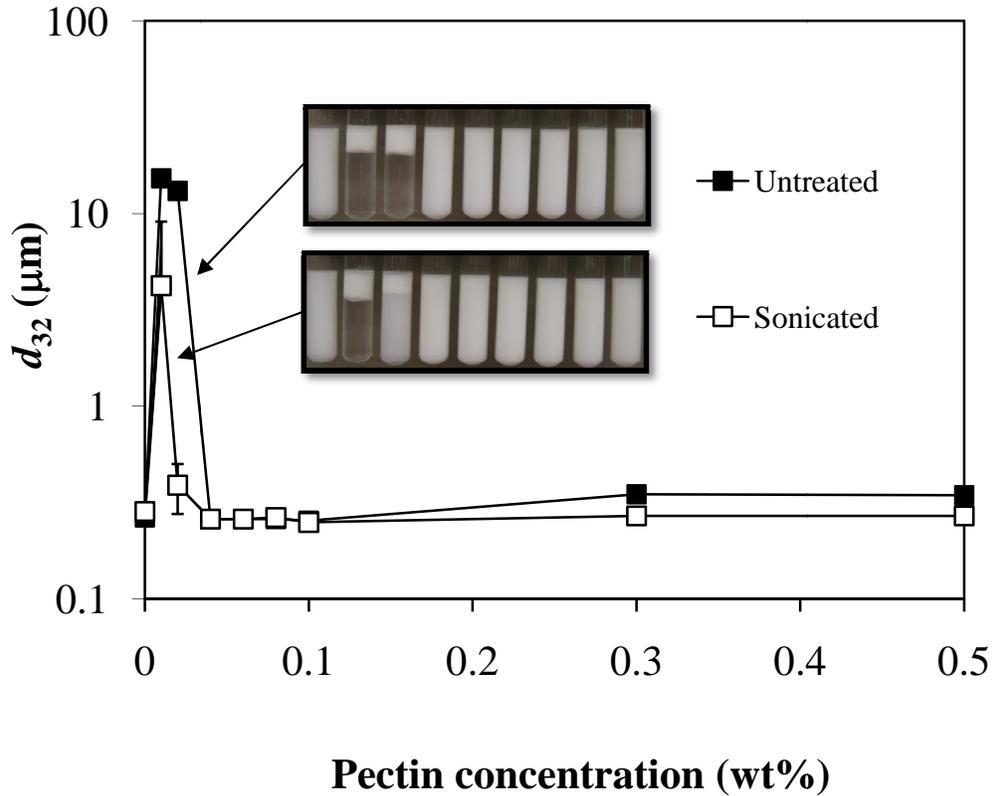


Figure 3.13. Influence of sonication on the mean particle diameter and appearance of β -Lg stabilized 1 wt% oil-in-water emulsions containing different levels of pectin (pH 3.5).

3.6 Conclusions

This study has shown that a simple theoretical model can be used to predict the stability of protein-stabilized oil-in-water emulsions containing charged polysaccharides. Experimental stability maps of the influence of droplet and pectin concentrations on emulsion stability were established at pH 7 (where pectin does not adsorb to the droplet surfaces) and at pH 3.5 (where pectin does adsorb). At pH 7, droplet flocculation and creaming were observed when the pectin concentration exceeded a particular level, which was attributed to depletion flocculation. At pH 3.5, the stability of the emulsions was

much more complex, going from stable, to unstable, to stable, to unstable with increasing pectin concentration. In these systems, the instability at low pectin concentrations was attributed to bridging flocculation, whereas the stability at high pectin concentrations was attributed to depletion flocculation. At intermediate pectin concentrations stable multilayer emulsions could be formed, which consisted of lipid droplets coated with a pectin layer. The simple theoretical model used in this work was able to qualitatively predict this behavior and may therefore prove useful for optimizing the preparation conditions for forming stable multilayer emulsions.

CHAPTER 4

ELECTRO-ACOUSTIC MONITORING OF POLYELECTROLYTE /PROTEIN COATED OIL DROPLETS

4.1 Abstract

The purpose of this study was to compare the ability of electro-acoustic (EA) and micro-electrophoresis (ME) techniques for monitoring the adsorption of a charged polysaccharide onto the oppositely charged surfaces of protein-coated oil droplets. The ζ -potential values determined by the EA technique were in excellent agreement with those determined by the ME technique. Both techniques were able to monitor the adsorption of pectin onto the surfaces of β -lactoglobulin coated droplets as a function of polysaccharide concentration and pH. The major advantage of the EA technique was that it could be carried out in situ without having to dilute the emulsions, so that the equilibrium between adsorbed and non-adsorbed polyelectrolyte would not have been disturbed by dilution. Nevertheless, the good agreement between the ζ -potential values determined by the EA and ME techniques suggested that emulsion dilution did not cause an appreciable change in polysaccharide partitioning for the system used in this study. In summary, the electro-acoustic technique appears to be a powerful means of monitoring polyelectrolyte adsorption in concentrated colloidal dispersions.

4.2 Introduction

Over the past two decades, electro-acoustic techniques have emerged as a powerful means of monitoring droplet charge (ζ -potential) in concentrated emulsions.

(150, 153, 156, 181, 183, 230-233) Electro-acoustic techniques use a combination of electrical and acoustic phenomenon to determine the zeta potential of emulsion droplets. Electro-acoustic measurements can be carried out in one of two ways: (i) Electro-Sonic Amplitude (ESA) - an electric signal is applied to an emulsion and the resulting acoustic signal generated by the oscillating particles is recorded; (ii) Colloid Vibration Potential (CVP) - an acoustic signal is applied to an emulsion and the resulting electric signal generated by the oscillating particles is recorded. Electro-acoustic spectroscopy can also be used to provide information about the droplet size distribution of emulsions, however the droplet size range is usually rather limited ($\sim 0.1 - 10 \mu\text{m}$). The major advantage of the electro-acoustic technique over more conventional micro-electrophoretic techniques based on light scattering is that it is capable of analyzing emulsions with high droplet concentrations ($< 50\%$) without any sample dilution (173, 230, 234). Electro-acoustic techniques have been used to measure particle size distributions and ζ -potentials in a wide variety of different model and industrial oil-in-water (O/W) and water-in-oil (W/O) emulsions (181, 183-185, 187, 188, 194, 198, 207, 210, 235-238). Nevertheless, there are some limitations of the electro-acoustic technique for certain applications. For example, the droplets must have an electrical charge, there must be a significant density contrast between the droplets and the surrounding liquid, and the viscosity of the continuous phase must be known at the measurement frequency (which is not always the same as that measured in a conventional viscometer).

In the present study, we examine the possibility of using electro-acoustic measurements to monitor the adsorption of charged polysaccharides onto the surfaces of oppositely charged protein-coated droplets in relatively concentrated emulsions *in situ*.

In previous studies, we used micro-electrophoresis techniques based on light scattering to monitor this process, but it was usually necessary to dilute the emulsions considerably prior to analysis to avoid multiple scattering effects (17, 56, 61, 71, 121, 214, 239). Potentially, this dilution step could affect the partitioning of polysaccharide molecules between the droplet surfaces and the surrounding continuous phase, thereby altering the composition of the interfacial layer and thus the droplet ζ -potential. In this study, we therefore compared the ζ -potential measurements made using an electro-acoustic method with those made using a micro-electrophoresis method. The emulsion system selected for study was one that we have previously characterized by micro-electrophoresis measurements, *i.e.* adsorption of an anionic polysaccharide (pectin) onto the surfaces of oil droplets coated by a globular protein (β -lactoglobulin). (17, 56) Bovine β -lactoglobulin (β -Lg) is obtained from cow's milk, where it is the major protein in the whey fraction (240). Structurally, it is a compact globular protein (molecular mass = 18.3 kDa) containing 162 amino acid residues with one thiol group and two disulfide bonds. The isoelectric point of β -Lg has been reported to be around 4.7 to 5.2 (241-243). The term "pectin" describes a complex group of oligosaccharides and polysaccharides that have some common features, *i.e.*, they are all rich in galacturonic acid (244). The conventional structural model for pectin consists of a poly-galacturonic backbone that contains some "smooth regions" (non-branched) and some "hairy regions" (branched) (129, 245). The backbone is anionic with the linear charge density depending on the degree of esterification of the carboxyl groups of the galacturonic acids, whereas the branches are largely comprised of non-ionic monosaccharides. Pectin molecules are negatively charged at high pH values, but lose their charge at lower pH values ($\text{pK}_a \sim$

3.5), whereas β -Lg is negatively charged at high pH values, but becomes positively charged below its isoelectric point (pI \sim 5). Consequently, pectin molecules tend to adsorb to the surfaces of β -Lg coated droplets at pH values around and below the pI (17, 56).

4.3 Materials and methods

4.3.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Pectin extracted from citrus fruit was purchased from Sigma Chemical Company (Lot # 016K0713, St. Louis, MO). The degree of esterification (DE) of the pectin was reported to be 60% by the supplier. Corn oil was purchased from a local supermarket and used without further purification. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from the Sigma Chemical Company (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Iowa) was used for the preparation of all solutions.

4.3.2 Solution preparation

An emulsifier solution containing 0.5 wt% protein was prepared by dispersing powdered β -Lg into 5 mM phosphate buffer (pH 3.5 or pH 7.0) and stirring for at least

two hours to ensure complete hydration. A 1 wt% pectin solution was prepared by dispersing powdered pectin into the same phosphate buffer at pH 3.5 or pH 7.0 and stirring for at least two hours to ensure complete hydration.

4.3.3 Emulsion preparation

A primary emulsion was prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.5 wt% β -Lg, pH 3.5 or pH 7.0) with a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min followed by 5 passes through a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA): 3000 psi the first stage, 300 psi the second stage. Primary emulsions were diluted with phosphate buffer solution (pH 3.5 or pH 7.0) to obtain a final composition of 5 wt% corn oil and 0.225 wt% β -Lg. These emulsions were then analyzed using the electro-acoustic, micro-electrophoresis or laser diffraction methods described below.

4.3.4 Electro-acoustic measurements

The electrical charge (ζ -potential) of the particles in undiluted emulsions was determined using a commercial instrument (Field ESA, PA Partikel-Analytik-Meßgerate GmbH, Frechen, Germany) capable of electro-acoustic measurements, based on the ESA principle. Two different types of experiments were carried out:

4.3.4.1 Concentration titration

In these experiments, the change in ζ -potential was measured when anionic pectin was titrated into an emulsion containing cationic β -Lg coated droplets. A primary emulsion containing 5 wt% oil droplets coated by β -Lg (pH 3.5) was placed into the

measurement chamber of the instrument, then a 1 wt% pectin solution (pH 3.5) was titrated into the measurement chamber. Thirty 1 mL aliquots of pectin solution were titrated into the emulsion, with a delay time of 60 seconds between each aliquot.

4.3.4.2 Potentiometric titration

In these experiments, the change in ζ -potential was measured as a function of pH in primary and secondary emulsions. Initially, a 5 wt% oil-in-water emulsion stabilized by β -Lg containing either 0 wt% pectin (primary) or 0.1 wt% pectin (secondary) was placed into the measurement chamber of the instrument at pH 7.0. At this pH the pectin and droplets have the same charge and so there should be no adsorption. A 0.2 N HCl solution was then titrated into the measurement chamber with 0.2 pH increment and 60 sec equilibrium delay time and the change of ζ -potential with pH was measured.

4.3.5 Micro-electrophoresis measurements

Aliquots of emulsion (1 mL) were collected from the measurement chamber of the electro-acoustic instrument during concentration and potentiometric titration. These emulsion samples were then diluted 1:100 using an appropriate buffer solution (at the same pH as the sample) and placed into the measurement chamber of a micro-electrophoresis instrument (ZEM 5300, Zetamaster, Malvern Instruments, Worcs., UK). This instrument determines the electrical charge (ζ -potential) of the particles in an emulsion by measuring the direction and velocity of particle movement in an applied electric field. The ζ -potential measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with five readings made per sample.

4.3.6 Particle size analysis

Aliquots of emulsion (1 mL) were collected from the measurement chamber of the electro-acoustic instrument. These emulsion samples were then diluted 1:1000 using an appropriate buffer solution (at the same pH as the sample) and placed into the measurement chamber of the laser diffraction instrument (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). This instrument finds the particle size distribution of an emulsion that gives the best fit between the experimental measurements and predictions made using light scattering theory (*i.e.* Mie theory). A refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions. Measurements are reported as the volume weighted mean diameter: $d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$, where n_i is the number of droplets of diameter d_i . The particle size measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with two readings made per sample.

4.3.7 Statistical analysis

Experiments were performed twice using freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements.

4.4 Results and discussion

4.4.1 Monitoring pectin adsorption to protein-coated droplets at pH 3.5

Initially, we compared the ζ -potential measurements made using electro-acoustics (EA) and micro-electrophoresis (ME) techniques when a pectin solution (1 wt% pectin, pH 3.5) was titrated into an emulsion containing oppositely charged droplets (5

wt% corn oil, 0.225 wt% β -Lg, pH 3.5) (Figure 4.1). As mentioned earlier, the electroacoustic measurements were made directly on the emulsions, whereas the microelectrophoresis measurements were made after the emulsions had been diluted (1:100). There was excellent agreement between the ζ -potential measurements made using the two analytical techniques, despite the differences in operating principles and in droplet concentration. These results suggested that the pectin molecules adsorbed strongly to the surfaces of the protein-coated droplets at pH 3.5, and that they were not detached by emulsion dilution.

In the absence of pectin, the electrical charge on the emulsion droplets was around +54 mV, which was due to the fact that β -Lg was below its isoelectric point (pI \sim 5) at pH 3.5 and therefore had a net positive charge. The electrical charge on the droplets changed from positive to negative as the pectin concentration in the emulsions was increased (Figure 4.1). There was no net charge on the droplets when the pectin concentration was around 0.062 wt%, which corresponded to a pectin to β -Lg mass ratio (R) of about 0.1 gram/gram.

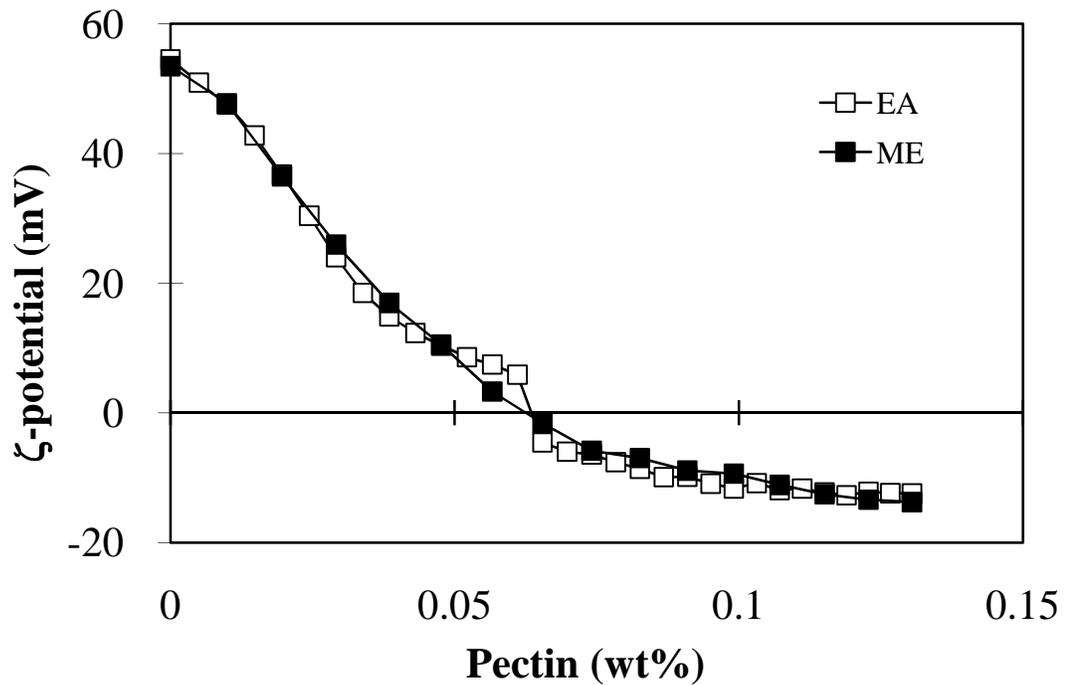


Figure 4.1. Dependence of droplet ζ -potential of protein stabilized oil-in-water emulsions on pectin concentration: 5 wt% corn oil, 0.225 wt% β -Lg, 5 mM Na-phosphate buffer, pH 3.5. The EA measurements were made on undiluted emulsions, whereas the ME measurements were made on diluted emulsions.

This value is similar to that reported in earlier studies.⁽⁵⁶⁾ The negative charge on the droplets reached a constant value ($\zeta \approx -13 \pm 1$ mV) when the pectin concentration exceeded about 0.1 wt% ($R \approx 0.22$ gram/gram). These measurements indicated that negatively charged pectin adsorbed to the surface of the positively charged β -Lg stabilized emulsion droplets. The ability of charged polyelectrolytes to adsorb to the surface of oppositely charged colloidal particles and cause charge reversal is well established in the literature. (52, 246, 247)

The critical polysaccharide concentration where the droplet surfaces became saturated with pectin was established by modeling the ζ -potential *versus* polysaccharide concentration curves using the following empirical equation (79):

$$\frac{\zeta(c) - \zeta_{Sat}}{\zeta_0 - \zeta_{Sat}} = \exp\left(-\frac{c}{3C_{Sat}}\right) \quad (4.1)$$

where $\zeta(c)$ is the ζ -potential of the emulsion droplets at polysaccharide concentration c , ζ_0 is the ζ -potential in the absence of polysaccharide, ζ_{Sat} is the ζ -potential when the droplets are saturated with polysaccharide, and C_{Sat} is the minimum amount of polysaccharide required to completely cover the droplet surfaces. The binding of a polysaccharide to the droplet surfaces can therefore be characterized by ζ_{Sat} ($= \zeta_0 - \zeta_{Sat}$) and C_{Sat} . Values for ζ_{Sat} and C_{Sat} were calculated by fitting the above equation to the experimental ζ -potential *versus* pectin concentration curve: $\zeta_{Sat} = +67$ mV and $C_{Sat} = 0.12$ wt% pectin for the EA instrument, and $\zeta_{Sat} = +72$ mV and $C_{Sat} = 0.15$ wt% pectin for the ME instrument. The saturation concentration depends on the size and concentration of emulsion droplets used (since this determines the exposed surface area), and therefore it is useful to calculate a more system-independent quantity for the amount of adsorbed pectin. The surface load at saturation can be calculated using the following expression:

$$\Gamma_{Sat} = \frac{C_{Sat} d_{32}}{6\phi} \quad (4.2)$$

Here, C_{Sat} is defined as the mass of material adsorbed to the surface of the droplets per unit volume of emulsion ($C_a / \text{kg m}^{-3}$), d_{32} is the volume-surface mean

droplet diameter, and ϕ is the droplet volume fraction. For this study: $d_{32} = 0.32 \mu\text{m}$, $\phi = 0.05$ (5%) and $C_a \approx 1.2$ or 0.15 kg m^{-3} (0.12 or 0.15 wt%), hence $C_{\text{Sat}} = 1.5$ or 1.8 mg m^{-2} , for the EA and ME instruments, respectively. These values are similar to each other, and are close to those found for the surface loads of other anionic polysaccharides adsorbed to the surfaces of protein-coated oil droplets: $C_{\text{Sat}} \sim 1.3, 1.6, 2.1$ and 5.1 mg m^{-2} for alginate, pectin, carrageenan and gum arabic adsorbed to β -Lg coated oil droplets, respectively. (21, 79)

We also measured the mean particle diameter (d_{43}) of the emulsions as the pectin concentration was increased to provide some insight into droplet aggregation (Figure 4.2). As the pectin concentration was increased there was evidence of strong flocculation in the emulsions (that was not disrupted when the emulsions were diluted for the light scattering experiments). This type of flocculation can be attributed to bridging flocculation, *i.e.*, the ability of an anionic pectin molecule to bind to the surfaces of more than one cationic protein-coated droplet (63). These results indicate that even though there was appreciable flocculation in the emulsions, the ME and EA techniques were still capable of providing similar values for the ζ -potential.

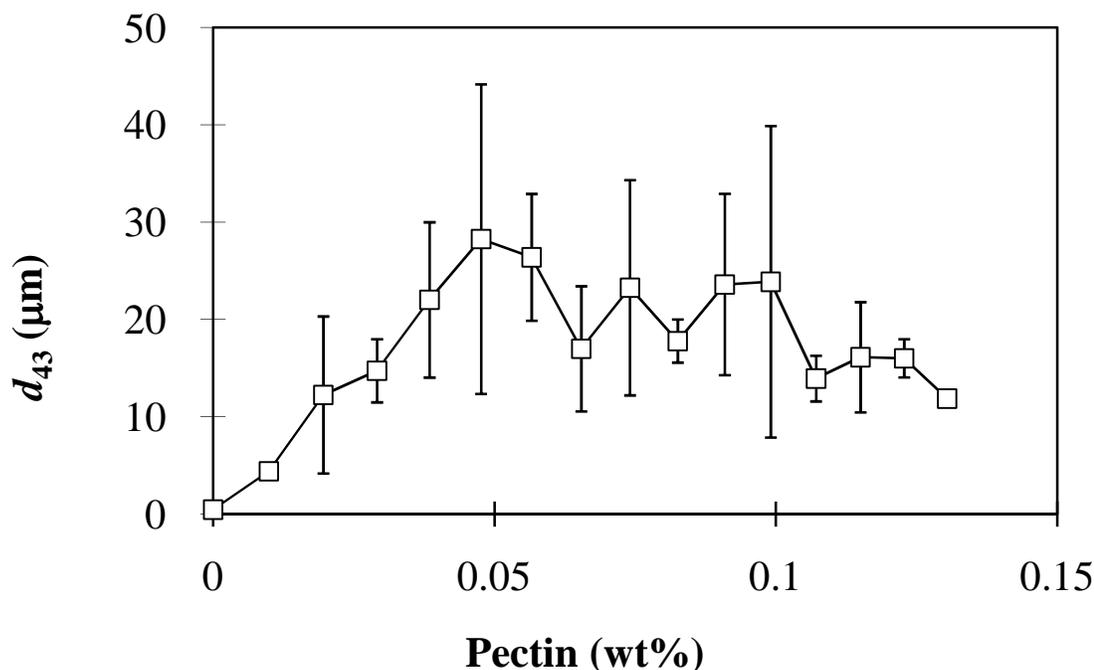


Figure 4.2. Dependence of mean particle diameter (d_{43}) of protein stabilized oil-in-water emulsions on pectin concentration: 5 wt% corn oil, 0.225 wt% β -Lg, 5 mM Na-phosphate buffer, pH 3.5. The measurements were made by laser diffraction on diluted emulsions.

4.4.2 Monitoring pH dependence of pectin adsorption to protein-coated droplets

In this series of experiments we compared the ζ -potential measurements made using electro-acoustics (EA) and micro-electrophoresis (ME) when the pH of an emulsion containing β -Lg coated oil droplets and pectin was reduced from around 7 to 3.5 (Figure 4.3). At pH 7, the pectin molecules and protein-coated droplets are both strongly anionic, so that no adsorption would be expected to occur. Indeed, a measurement of the ζ -potential *versus* pectin concentration (0 to 1 wt%) using the EA instrument at pH 7 showed only a slight change in droplet charge from -71.5 mV in the absence of pectin to -69.3 mV in the presence of 1 wt% pectin. As the pH is reduced, the

proteins develop positive amino groups on their surfaces ($-\text{NH}_3^+$) to which the anionic pectin molecules can bind. Consequently, pectin molecules should start to adsorb to the droplet surfaces at pH values slightly above the isoelectric point (pI) of the adsorbed proteins.

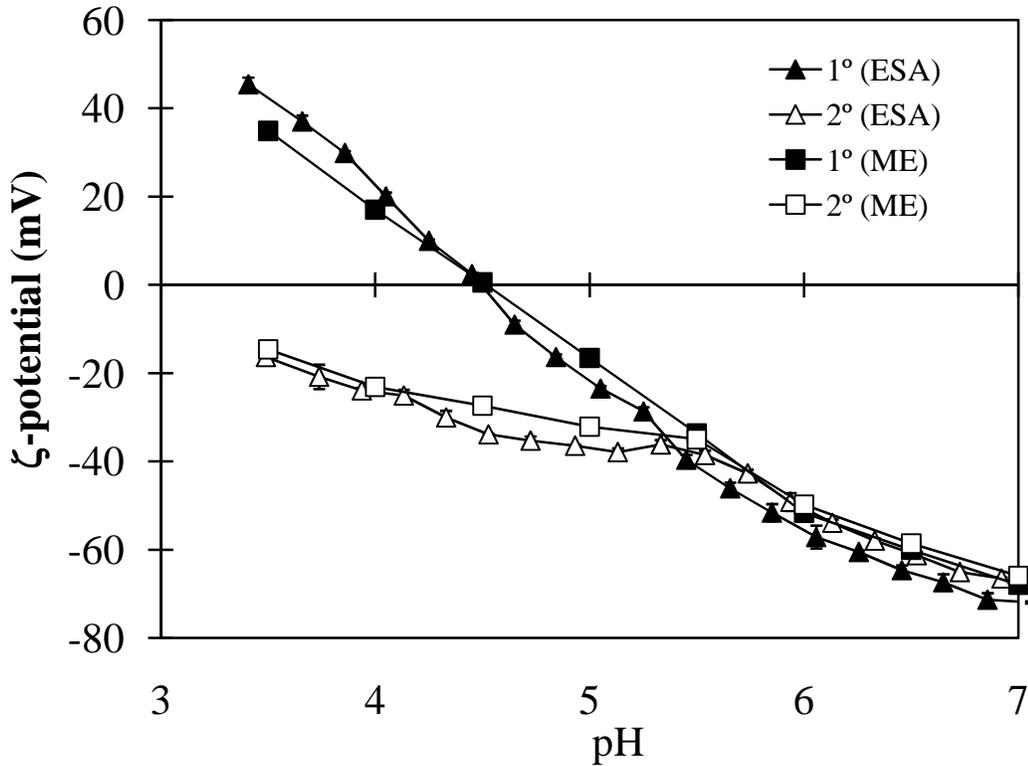


Figure 4.3. Dependence of ζ -potential of primary (0 wt% pectin) and secondary (0.1 wt% pectin) emulsions on pH: 5 wt% corn oil, 0.225 wt% β -Lg, 5 mM Na-phosphate buffer. The EA measurements were made on undiluted emulsions, whereas the ME measurements were made on diluted emulsions.

Again we found excellent agreement between the ζ -potential-pH profiles determined by EA and ME (Figure 4.3). In the primary emulsions, the ζ -potential changed from highly positive to highly negative as the pH was adjusted from 7 to 3.5, which can be attributed to the fact that the solution pH was adjusted from above to below the pI of the adsorbed protein molecules (pI ~ 4.8). The ζ -potential crossed the pH axis

at about 4.5 for both the EA and ME instruments, indicating that the measured pI was close to the expected one. The ζ -potential of the secondary emulsions was close to that of the primary emulsions at pH 6 and 7, which indicated that the pectin molecules did not adsorb to the droplet surfaces. This should be expected because there would be a relatively strong electrostatic repulsion between the anionic polysaccharide molecules and anionic protein-coated droplets, and there would be few positive patches on the surfaces of the adsorbed proteins. At pH 5 and below the ζ -potential of the secondary emulsions was appreciably more negative than that of the primary emulsions, which indicated that pectin molecules adsorbed to the surfaces of the protein-coated droplets. This effect has been observed for a number of different anionic polysaccharides to protein-coated droplets, *e.g.*, pectin, carrageenan, alginate and gum arabic to β -Lg-coated droplets (21, 60, 61, 79) and pectin to caseinate-coated droplets.(59)

The influence of pH on the mean particle diameter (d_{43}) of the emulsions was also measured to provide information about droplet aggregation (Figure 4.4). From pH 7 to 5.5 the mean particle diameter was relatively small in both the primary and secondary emulsions, indicating that there was little flocculation. The good stability of the emulsions to flocculation in this pH range can be attributed to the relatively high electrical charge on the droplets, which prevented them from coming into close proximity due to a relatively strong electrostatic repulsion. At lower pH values, there was an appreciable increase in mean particle diameter in both the primary and secondary emulsions. In the primary emulsion this flocculation can be attributed to the fact that the electrical charge on the droplets was relatively small around the isoelectric point of the adsorbed protein (pI \sim 5), so that the electrostatic repulsion was not sufficiently high to

overcome the various attractive interactions between the droplets. In the secondary emulsions, the particle charge was still relatively high ($> |20 \text{ mV}|$) so one would have expected the electrostatic repulsion to be relatively strong between the droplets. Consequently, one can attribute the droplet aggregation observed in these emulsions to bridging flocculation, *i.e.*, sharing of a single anionic pectin molecule between two or more cationic protein-coated droplets. (63) Again, this data indicates that even though there was appreciable flocculation in the emulsions, the ME and EA techniques gave similar values for the ζ -potential.

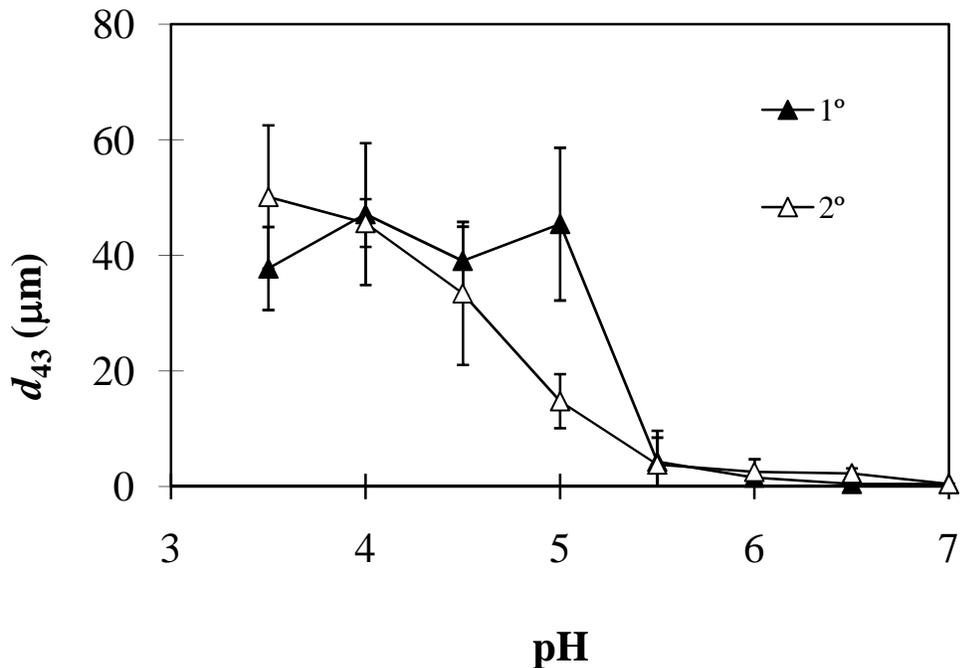


Figure 4.4. Dependence of mean particle diameter of primary (0 wt% pectin) and secondary (0.1 wt% pectin) emulsions on pH: 5 wt% corn oil, 0.225 wt% β -Lg, 5 mM Na-phosphate buffer. The measurements were made by laser diffraction on diluted emulsions.

4.4.3 Influence of stirring speed on ζ -potential measurements

In Electro-Acoustic Technique, One potential problem that we noted with the electro-acoustic technique was the fact that the measurements may be unreliable on highly flocculated systems when the samples are not stirred properly. The variation in ζ -potential with pH is shown in Figure 4.5 for secondary emulsions when the pH is varied from 7 to 3 at relatively low and high stirring speeds. At high stirring speeds the pH dependence of the ζ -potential is as expected. On the other hand, at low stirring speeds the ζ -potential remained relatively constant at -30 mV when the pH was reduced from pH 5 to 4.25, but then it rapidly increased to around $+37$ mV when the pH was further reduced from pH 4.25 to 4.0. This phenomenon can be attributed to the formation of a highly viscous or gelled layer of emulsion on the surface of the electro-acoustic transducer that restricts the movement of continuous phase (and thus H^+ ions) into its interior. This gelled layer formed because the emulsion was near the isoelectric point of the adsorbed protein molecules so that the droplets became strongly flocculated. Consequently, the solution pH and droplet ζ -potential in the interior of the gelled layer was different from that in the bulk of the solution. This problem could be avoided by ensuring that the emulsions were stirred sufficiently vigorously to prevent the formation of this gelled layer at the surface of the electro-acoustic transducer.

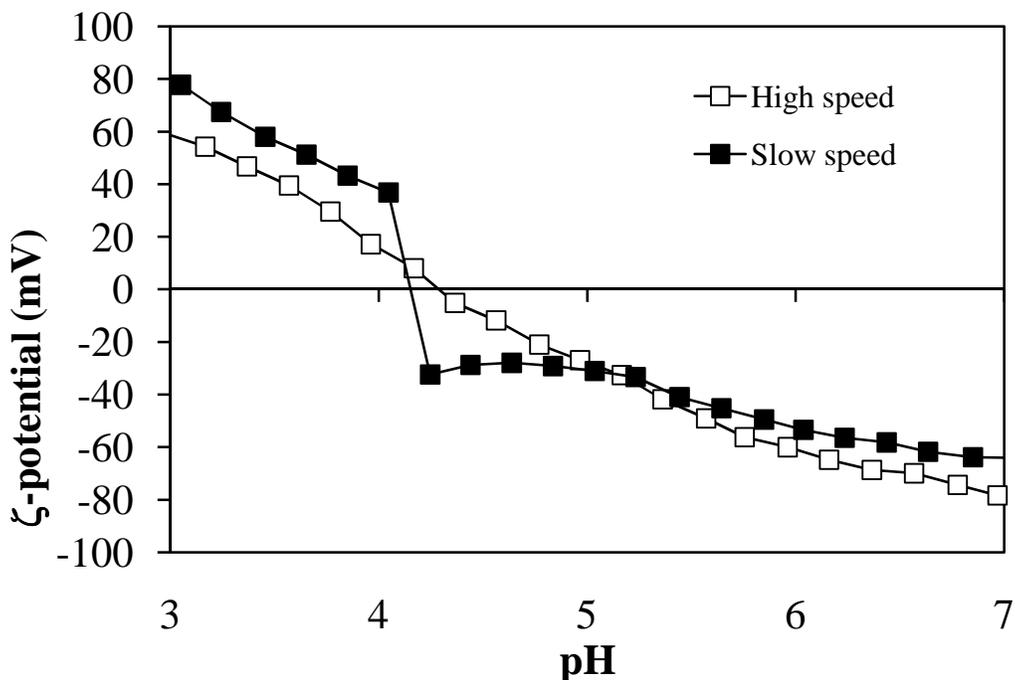


Figure 4.5. Influence of agitation speed on the ζ -potential *versus* pH dependence of undiluted primary emulsions measured using the EA technique: 5 wt% corn oil, 0.225 wt% β -Lg, and 5 mM Na-phosphate buffer.

4.5 Conclusions

In this manuscript we have shown that an electro-acoustic technique can be used to monitor the adsorption of anionic polysaccharide molecules onto the surfaces of cationic protein-coated droplets. The ζ -potential measurements determined by the electro-acoustic (EA) technique were in excellent agreement with those determined by a more traditional micro-electrophoresis (ME) technique. The major advantage of the EA technique is that it can be carried out *in situ* without having to dilute the emulsions. In principle, emulsion dilution may change the partitioning of a polyelectrolyte between the droplet surfaces and the surrounding continuous phase. Consequently, the EA technique

should have a major advantage over the ME technique for the study of concentrated emulsions. Nevertheless, the good agreement between the ζ -potentials determined by the EA and ME techniques in this study suggested that emulsion dilution did not cause an appreciable change in polysaccharide partitioning for the system we used. Another important finding of this study was the importance of ensuring that highly flocculated emulsions were well-stirred during the measurements to enable any acids or bases in the continuous phase to penetrate. Otherwise, the pH of the sample at the transducer surface, and hence its ζ -potential, would be different from that in the rest of the sample.

CHAPTER 5

COMPETITIVE ADSORPTION OF MIXED ANIONIC POLYSACCHARIDES AT THE SURFACES OF PROTEIN-COATED LIPID DROPLET

5.1 Abstract

Charged polysaccharides can improve the stability of protein-coated lipid droplets by forming a protective coating around them. Potentially, the interfacial characteristics of these coatings can be controlled by assembling them from mixed polysaccharides with different molecular characteristics. The purpose of this study was to examine the competitive adsorption of two anionic polysaccharides (carrageenan and pectin) to β -lactoglobulin coated-lipid droplets. Carrageenan has a higher charge density than pectin, and carrageenan has a linear backbone whereas pectin has a linear backbone with branches. Emulsions ($\phi = 1$ wt% oil, $d_{32} = 0.28 \pm 0.02$ μm) were mixed with polysaccharide solutions (0 or 0.04 wt%) at pH 7, then the pH was decreased to promote polysaccharide adsorption. The adsorption of the polysaccharide molecules to the droplet surfaces occurred at a higher pH for carrageenan (pH ≈ 5.85) than for pectin (pH ≈ 5.45). When polysaccharide mixtures were added at pH 7, the carrageenan molecules preferentially adsorbed to the droplet surfaces when the pH was reduced. At pH 3.5, carrageenan coated droplets had a higher negative charge ($\zeta = -38.5 \pm 3.1$ mV) than pectin-coated droplets ($\zeta = -17.9 \pm 2.0$ mV). Carrageenan was effective at displacing pectin from the surfaces of pectin-coated droplets, but pectin was less effective at displacing carrageenan from carrageenan-coated droplets. The stability of pectin-coated droplets was better than carrageenan-coated droplets, which was attributed to steric

hindrance effects. These results have important implications for the design of delivery systems based on polysaccharide/protein-coated droplets.

5.2 Introduction

Numerous studies have shown that electrostatic deposition of anionic polysaccharides onto the surfaces of protein-coated lipid droplets can be used to improve their stability to environmental stresses, such as pH, ionic strength, thermal processing, freezing and dehydration (21, 60, 69, 75, 79, 130, 214, 219, 248-250). In principle, these polysaccharide coatings could also be used to design novel functional attributes into emulsion-based delivery systems, such as burst, sustained, or targeted release (251-254). Polysaccharide coatings are typically formed around protein-coated lipid droplets using either a one-step or two-step process. Initially, an oil-in-water emulsion is formed containing protein-coated droplets with a known isoelectric point (pI). In the one step process, this emulsion is mixed with a polysaccharide solution at a pH where the polysaccharide molecules directly adsorb to the droplet surfaces (usually $\text{pH} \leq \text{pI}$). At this pH, the polysaccharide molecules are negatively charged and the protein-coated droplets are positively charged (or have appreciable positive patches on their surfaces). In the two step process, the emulsion and polysaccharide solution are initially mixed together at a pH where the polysaccharide molecules do not adsorb to the droplet surfaces ($\text{pH} > \text{pI}$) because the droplets and polysaccharide molecules are both negatively charged. The pH is then decreased so that the protein-coated droplets become positively charged and the anionic polysaccharide molecules adsorb to their surfaces. The two step method generally produces more stable polysaccharide-coated droplets because there is a uniform

concentration of polysaccharide molecules around the droplets when adsorption occurs (8). In either case, it is important to carefully control the lipid droplet and polysaccharide concentration to avoid bridging or depletion flocculation (23). It is also important to ensure that the polysaccharide-coated droplets are stable once formed, *i.e.*, there is sufficient electrostatic and steric repulsion between them to avoid droplet flocculation (23).

Previously, researchers have examined the adsorption of single types of polysaccharide molecules onto protein-coated lipid droplet surfaces, such as carrageenan (60, 61, 119-121), pectin (17, 79, 122, 123, 125, 255), alginate (256), gum arabic (21) and soy soluble polysaccharide (257). Each type of polysaccharide has its own unique molecular characteristics, *e.g.*, molecular weight, electrical charge, branching, hydrophobicity, and conformational flexibility (258). Consequently, the interfacial coatings formed by different polysaccharide types will have different physicochemical properties (such as thickness, charge, permeability, and environmental responsiveness), which in turn will lead to different emulsion functional properties (such as stability, rheology, and delivery). It should therefore be possible to create polysaccharide-coated droplets with controllable functional properties by rational selection of different types of polysaccharides. Alternatively, it may be possible to control the functional properties of these systems by using polysaccharide blends, *i.e.*, assembling polysaccharides coatings using two or more polysaccharides with different molecular characteristics.

The purpose of the present study was to examine the competitive adsorption of polysaccharides onto protein-coated lipid droplets, and to determine the impact of this process on subsequent droplet properties. Lipid droplets were formed by homogenizing

an oil phase with an aqueous solution containing a globular protein (β -lactoglobulin, β -Lg). β -Lg was used because its molecular and emulsification properties are well documented (259-261). The isoelectric point of β -Lg has been reported to be around 4.7 to 5.2 (243, 262, 263). Two anionic polysaccharides with different molecular characteristics were selected for this study: citrus pectin and ι -carrageenan. Citrus pectin can be considered to be a block copolymer with anionic “linear regions” and neutral “hairy regions” (264, 265), whereas carrageenan can be considered to be an anionic linear polymer (258, 266, 267). The negative charges on the polysaccharides are due to carboxylic acid groups on pectin ($pK_a \approx 3.5$) and sulfate groups on carrageenan ($pK_a \approx 2.0$). In this study, we examined the competitive absorption of pectin and carrageenan molecules from mixed solutions, as well as the possible displacement of one type of polysaccharide from a droplet initially coated by the other type of polysaccharide. The results from this study should provide information that would be useful for the rational design of delivery systems containing mixed polysaccharide-coated droplets with improved or novel functional properties.

5.3 Materials and methods

5.3.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product

are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Pectin extracted from citrus fruit was purchased from Sigma Chemical Company (Lot # 016K0713, St. Louis, MO). The degree of esterification (DE) of the pectin was reported to be 60% by the supplier. Carrageenan was kindly donated by FMC Biopolymers (Viscarin ® SD 389, Philadelphia, PA) and used without further purification. Corn oil was purchased from a food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN) and used without further purification. The manufacturer reported that the corn oil contained approximately, 14.3, 28.6 and 57.1 wt% of saturated, monounsaturated and polyunsaturated fats, respectively. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from the Sigma Chemical Company (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, IW) was used for the preparation of all solutions.

5.3.2 Solution preparation

An emulsifier solution containing 0.5 wt% protein was prepared by dispersing powdered β -Lg into 5 mM Na phosphate buffer (pH 7.0) and stirring for at least two hours to ensure complete hydration. Pectin and carrageenan stock solutions were prepared by dispersing 1 w% pectin or 1 wt% carrageenan in 5 mM Na phosphate buffer and stirring for at least four hours to ensure complete hydration.

5.3.3 Emulsion preparation

A stock emulsion was prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.5 wt% β -Lg, pH 7.0) with a high-speed blender (Tissue Tearor, Biospec Products, Inc., Bartlesville, OK) for 2 min followed by 5 passes through

a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA): the first stage at 3000 psi, the second stage at 300 psi. The stock emulsion was diluted with 5 mM Na phosphate buffer solution (pH 7, primary emulsion) or polysaccharide solution in 5 mM Na phosphate buffer (pH 7, secondary emulsion) to obtain a final composition of 1 wt% corn oil, 0.045 wt% β -Lg, and varied concentration of polysaccharides (0-0.04 wt%).

5.3.4 Electroacoustic measurements

The electrical charge (ζ -potential) of the particles in undiluted emulsions was determined using an electro-acoustic instrument (Field ESA, Partikel Analytik GmbH, Frechen, Germany) capable of electro-acoustic measurements, based on the electrosonic analysis (ESA) principle. The frequency of the ESA signal applied to the samples ranged between 0.85 and 1.15 MHz. The theory used to calculate the ζ -potentials from the measured particle mobility data was the Smoluchowski equation. This equation is applicable under conditions where the electrical double layer around the particle is thin compared to the particle radius. At the electrical conductivities of our samples, the double layer was only a few nanometers thick, so the Smoluchowski equation is applicable for both polymers and particles studied. The ESA instrument was calibrated using a standard colloidal suspension of known charge characteristics (10% Ludox, Sigma Chemical Co., St Louis, MO) immediately before running each set of experiments, then a phase calibration was carried out for each sample prior to starting a titration. The measurement of ζ -potential by the ESA was repeated at least three times using freshly prepared samples for each repetition. All experiments were carried out at ambient temperature (24.5 ± 0.8 °C).

Preliminary studies were carried out to determine the kinetics of adsorption of pectin to the β -Lg coated lipid droplet surfaces in the emulsions. The ζ -potential signal reached a constant value about 10~20 s after injecting a pectin solution into the emulsion, indicating that adsorption had occurred within this timescale. In addition, we found no significant difference between ζ -potentials measured using delay times of either 30 or 60 s during titration experiments, again indicating that pectin adsorption was complete within this period. For this reason, a delay time of 60 s was used for the remainder of the ESA measurements.

5.3.4.1 Concentration titration

In these experiments, the change in electrical charge of the emulsions was measured when anionic polysaccharides (pectin or carrageenan) were titrated into a primary emulsion (1 wt% of β -Lg coated oil droplets). A primary emulsion (pH 4) was placed into the measurement chamber of the instrument, then thirty 1 mL aliquots of polymer solution (1 wt% pectin or 1 wt% carrageenan, pH 4) were titrated with computer controlled auto-titration into the emulsion, with a delay time of 60 seconds between each aliquot. For displacement experiments, a secondary emulsion containing the lipid droplets (1 wt%) stabilized by β -Lg and polysaccharide (0.04 wt% pectin or carrageenan) was prepared at pH 7 followed by adjusting the pH of the secondary emulsion to 4.5. The secondary emulsion (pH 4.5) was placed into the measurement chamber of the ESA, and then thirty 1 mL aliquots of the other polysaccharide solution (1 wt% carrageenan or 1 wt% pectin) were titrated with a delay time of 60 seconds between each aliquot. In other words, a carrageenan solution was titrated into a secondary emulsion containing β -

Lg/pectin coated droplets, or a pectin solution was titrated into a secondary emulsion containing β -Lg/carrageenan coated droplets.

5.3.4.2 Potentiometric titration

In these experiments, the change in ζ -potential was measured as a function of pH in primary and secondary emulsions. A primary emulsion (1 wt% oil droplets, 0.045 wt% β -Lg) and secondary emulsion (1 wt% oil droplets, 0.045 wt% β -Lg, 0.04 wt% polysaccharide) were placed into the measurement chamber of the instrument at pH 7.0. A 0.2 N HCl solution was then automatically titrated into the measurement chamber with 60 sec equilibrium delay time between each 0.1 pH decrement and the change of ζ -potential with pH was measured. To investigate the impact of mixed polysaccharides on adsorption to the lipid droplets, the mixing ratios (MR) of pectin and carrageenan solution were varied at a fixed total polymer concentration (0.04 wt%) as shown in Table 5.1.

Table 5.1. Composition of secondary emulsions containing the droplets stabilized by β -Lg (0.045 wt%) and polymer (0.04 wt%)

| Mixing ratio | Pectin (wt%) | Carrageenan (wt%) |
|---------------------|---------------------|--------------------------|
| P10: C0 | 0.04 | 0 |
| P9: C1 | 0.036 | 0.004 |
| P5: C5 | 0.02 | 0.02 |
| P1: C9 | 0.004 | 0.036 |
| P0: C10 | 0 | 0.04 |

The calculation of ζ -potential using the ESA method relies on knowledge of the size and density of the particles. We used densities of 920, 1000 and 1200 kg m⁻³ for the oil phase, aqueous phase, and hydrated polysaccharide molecules (268), respectively. We used particle diameters of 320 and 30 nm for the lipid droplets (measured) and hydrated polysaccharide molecules (literature values (224, 269)), respectively. We found no major differences in the ζ -potentials calculated from the measured electrophoretic mobilities when the input particle diameter used in the calculations was varied between 10 and 500 nm, but the magnitude of the calculated ζ -potentials increased appreciably at higher particle diameters. It should be noted that the size of the particles in the ESA instrument will increase when droplet aggregation occurs. It was not possible to measure particle size changes directly during the ESA measurements, and hence there may have been some errors in the calculated ζ -potentials of highly aggregated systems. The reported ζ -potential values on these systems should therefore be treated with some

caution, and mainly used as a guide to differences between samples, rather than absolute values.

5.3.5 Particle size analysis

Aliquots of emulsion (1 ml) were collected from the measurement chamber of the electroacoustic instrument every two injection steps or every 0.5 pH unit, during concentration titration and potentiometric titration, respectively. These emulsion samples were then diluted to 1:100 using an appropriate buffer solution (at the same pH as the sample) and placed into the measurement chamber of the diffraction instrument (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). This instrument finds the particle size distribution of an emulsion that gives the best fit between the experimental measurements and predictions made using light scattering theory (*i.e.* Mie theory). A refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions. Measurements are reported as the volume weighted mean diameter: $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$ or as the volume-surface mean diameter: $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of droplets of diameter d_i . The particle size measurements are reported as the average and standard deviation of measurements made on three freshly prepared samples, with two readings made per sample.

5.3.6 Statistical analysis

Experiments were performed at least three times using freshly prepared samples. Averages and standard deviations were calculated from these triplet measurements.

5.4 Results and discussions

5.4.1 Charge characteristics of protein-coated lipid droplets and polysaccharide molecules

Initially, we characterized the pH-dependence of the ζ -potential of each of the three electrically charged species used in this study: protein-coated lipid droplets; carrageenan; and, pectin (Figure 5.1). As observed in previous studies, the ζ -potential of the protein-coated lipid droplets changed from negative to positive as the pH of the emulsion was decreased from pH 7 to 3, with a point of zero charge around pH 4.5 (60, 79, 216). This type of ζ -pH profile can be attributed to the fact that the electrical charge of the adsorbed β -Lg molecules goes from negative to positive as the pH moves from above to below their isoelectric point ($pI \approx 5$) (55). The carrageenan molecules were highly negatively charged across the entire pH range, which can be attributed to the fact that they have a high linear charge density and a low pK_a value (≈ 2.0) (270, 271). The pectin molecules had a lower negative charge than the carrageenan molecules whose value decreased at acidic pH values, which can be attributed to its lower charge density and higher pK_a value (≈ 3.5) (245, 272). The linear charge density of carrageenan has been reported to be around 0.7 to 1.38 (270, 273), which is appreciably higher than the linear charge density of pectin which has been reported to be around 0.35 to 0.4 (270, 274). This difference has been used to account for the fact that proteins typically interact more strongly with carrageenan than pectin in aqueous solutions (275-277). We hypothesized that these differences in electrical characteristics, as well as differences in other molecular characteristics, would influence the affinity of the anionic

polysaccharides for the surfaces of protein-coated lipid droplets. We therefore examined the impact of polysaccharide type on their adsorption and competitive adsorption to lipid droplet surfaces, as well as on emulsion stability.

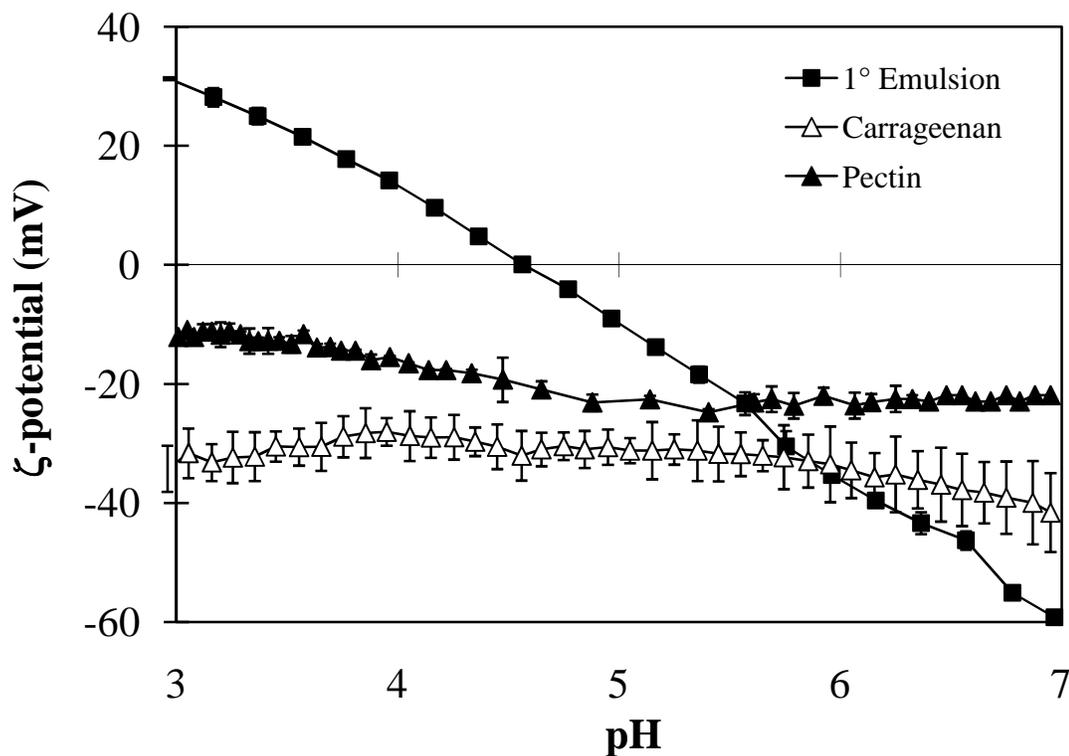


Figure 5.1. Dependence of the electrical charge of protein-stabilized O/W emulsions (5 wt% corn oil, 0.225 wt% β -Lg, 5 mM Na phosphate buffer), pectin solution (0.5 wt% in 5 mM Na phosphate buffer) and carrageenan solution (0.5 wt% in 5 mM Na phosphate buffer) on pH.

5.4.2 Adsorption of individual polysaccharides to droplet surfaces: Concentration titration

Initially, we compared the adsorption of pectin and carrageenan to protein-coated lipid droplets under conditions where the polysaccharides and proteins had opposite electrical charges. Each polysaccharide was titrated into a primary emulsion containing lipid droplets (1 wt%) stabilized by β -Lg (0.045 wt%) at pH 4 (Figure 5.2). At this pH,

the protein-coated droplets were positively charged and the polysaccharide molecules were negatively charged (Figure 5.1). The droplet ζ -potential changed from positive to negative in both systems with increasing polysaccharide concentration, which indicated that the anionic polysaccharides adsorbed to the surfaces of the cationic protein-coated lipid droplets. Nevertheless, there were distinct differences between the emulsions containing the two different types of polysaccharide. The initial decrease in ζ -potential with increasing polysaccharide concentration was steeper for carrageenan than for pectin, and the final ζ -potential (ζ_{final}) attained was more negative for carrageenan than for pectin. These effects can be attributed to the higher charge density of carrageenan, which increases its affinity for the lipid droplet surfaces and the final surface charge density of the coated droplets. It should be noted that the ζ -potential of the emulsions titrated with carrageenan became less negatively charged with increasing polysaccharide concentration after the lipid droplets were saturated with carrageenan (Figure 5.2). This effect may have been because the non-adsorbed carrageenan molecules contributed to the overall ESA signal, because the ionic strength of the aqueous solution increased, or because there was a change in interfacial properties at higher carrageenan concentrations. It should be noted that we observed an increase in pH (from 4.0 to 4.7) during the titration of the primary emulsion with carrageenan, which has previously been attributed to H^+ uptake in the presence of high carrageenan concentrations (277). These measurements demonstrate that the electrical characteristics of the interfacial layers formed when pectin and carrageenan molecules adsorb to the surfaces of protein-coated lipid droplets are appreciably different, which would be expected to influence their (competitive-) adsorption to the protein-coated lipid droplet surfaces.

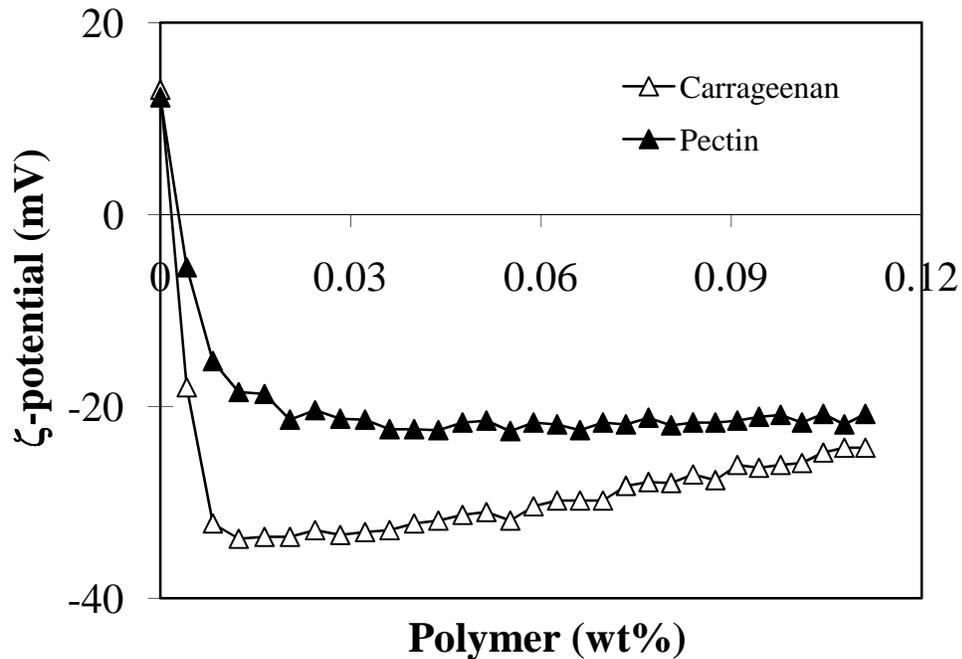


Figure 5.2. Dependence of the droplet ζ -potential of protein-stabilized O/W emulsions on polysaccharide concentration: 1 wt% corn oil, 0.045wt% β -Lg, 5 mM phosphate buffer at pH 4. Either pectin or carrageenan solution (1 wt% in 5 mM Na phosphate buffer, pH 4) was titrated into the primary emulsion.

5.4.3 Adsorption of individual polysaccharides to droplet Surfaces: Potentiometric titration

The pH dependence of polysaccharide adsorption to the protein-coated lipid droplet surfaces was studied by measuring changes in ζ -potential. A pH-titration from pH 7 to 3.5 was carried out for emulsions containing individual polysaccharides at a concentration known to be above the critical saturation level determined in the previous section (Figure 5.3), i.e., 0.04 wt%. This experiment was carried out to determine the critical pH where the polysaccharides first adsorbed to the lipid droplet surfaces: pH_c . As discussed earlier, the ζ -potential of the protein-coated lipid droplets changed from

negative to positive as the pH of the emulsion was decreased from above to below the pI of the adsorbed β -Lg molecules (Figure 5.3a) (79, 189). In the presence of the polysaccharides, the ζ -potential on the droplets remained negative across the entire pH range when the solution was reduced from pH 7 to 3.5, which is due to the adsorption of anionic polysaccharides onto positively charged patches on the droplet surfaces when the pH falls below pH_c (79). The ζ -pH profiles of the emulsions were fairly similar at relatively high pH values ($\text{pH} > 6$) in the absence and presence of anionic polysaccharides, suggesting that the polysaccharides did not adsorb to the droplet surfaces (Figure 5.3a). This lack of adsorption can be attributed to the relatively strong electrostatic repulsion between the anionic polysaccharides and the anionic droplets, as well as to the fact that there were few positively charged patches on the protein surfaces in this pH range (278). Nevertheless, the ζ -pH profiles of the emulsions containing polysaccharides diverged from that containing no polysaccharide when the pH was lowered below the critical value (pH_c). This value was higher for carrageenan ($\text{pH}_c \approx 5.85$) than for pectin ($\text{pH}_c \approx 5.45$), which can be attributed to the fact that there was a stronger electrostatic attraction between the positively charged patches on the protein and carrageenan (higher charge density) than pectin (lower charge density) (276, 279). At relatively low pH values, where the adsorbed polysaccharides had saturated the droplet surfaces, the ζ -potential was considerably more negative for carrageenan ($\zeta = -35$ mV at pH 3.5) than for pectin ($\zeta = -20$ mV at pH 3.5). Again this can be attributed to the higher charge density of the carrageenan molecules compared to the pectin molecules, so that they were able to bring more charge per unit mass of adsorbed polysaccharide to the droplet surfaces (280, 281).

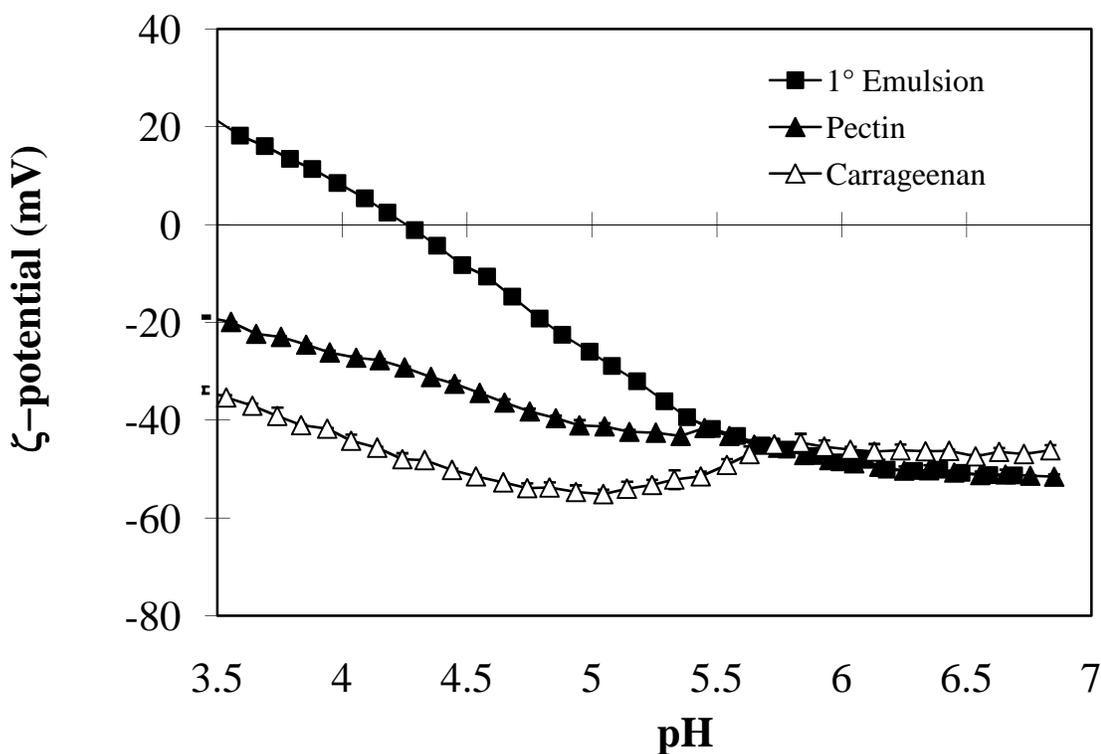


Figure 5.3a. pH-dependence of the ζ -potential of primary emulsions (0 wt% polysaccharide) and secondary emulsions (0.04 wt% polysaccharide) containing either pectin or carrageenan titrated from pH 7 to 3.5.

The mean particle diameter (d_{43}) of the emulsions as a function of pH was also measured to provide information about droplet aggregation (Figure 5.3b). In the absence of polysaccharide, there was a large increase in mean particle diameter around the pI of the adsorbed proteins, which can be attributed to extensive droplet flocculation associated with the low net charge on the droplets, i.e., the attractive interactions (mainly van der Waals) outweighed the repulsive interactions (mainly electrostatic and steric). The high particle diameters observed at pH values less than the pI can be attributed to the fact that the aggregates formed were pH-irreversible on the experimental timescale.

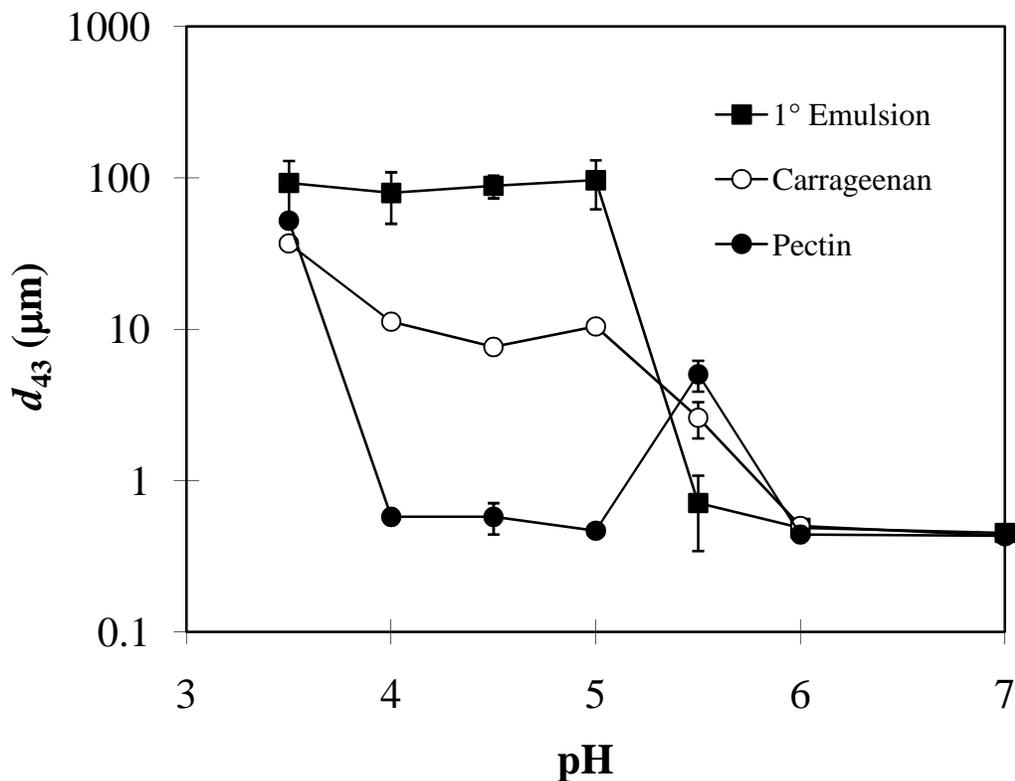


Figure 5.3b. pH-dependence of the mean particle diameter (d_{43}) of primary emulsions (0 wt% polysaccharide) and secondary emulsions (0.04 wt% polysaccharide) containing either pectin or carrageenan titrated from pH 7 to 3.5.

In the presence of pectin, the emulsion stability to aggregation depended on the solution pH relative to the critical pH for polymer adsorption ($\text{pH}_c \approx 5.45$). At pH 7 and 6, the mean particle diameter of the emulsions containing pectin was similar to that of the primary emulsion, which can be attributed to the fact the pectin did not adsorb to the droplets surfaces and there was insufficient free pectin to promote depletion flocculation (Figure 5.3b)(79). At $\text{pH} = 5.5$, extensive aggregation was observed in the pectin containing emulsion (Figure 5.3.2). The origin of this effect can be attributed to the fact that the aggregation of the protein-coated droplets began at a fairly similar pH to the one where pectin adsorption began (Figure 5.3a). Consequently, there may have been some

aggregation before the protein-coated droplets could be fully coated with pectin molecules, i.e., due to charge neutralization or bridging flocculation. At $4 \leq \text{pH} \leq 5$, the mean particle diameter was relatively small suggesting that the droplets were stable to aggregation because they were fully coated by a relatively thick layer of negatively charged pectin molecules. These pectin molecules would increase the steric and electrostatic repulsion between the droplets, as well as reducing the van der Waals attraction (79). At $\text{pH} < 4$, the pectin containing emulsions exhibited extensive droplet aggregation, which can be attributed to the fact that the pectin molecules lost some of their negative charge ($\text{pK}_a \approx 3.5$) (Figure 5.1), hence the electrostatic repulsion between the molecules was reduced (Figure 5.3a).

In the presence of carrageenan, the emulsion stability to aggregation also depended on the solution pH relative to the critical pH for polymer adsorption ($\text{pH}_c \approx 5.85$). At $\text{pH} \geq 6$, the carrageenan containing emulsions were stable to droplet aggregation, but when the pH was reduced below this value extensive droplet aggregation was observed (Figure 5.3b). It is notable that the emulsions containing carrageenan were unstable to aggregation at all $\text{pH} < \text{pH}_c$, whereas the pectin containing emulsions were stable over a range of pH values, even though the droplets coated by carrageenan had a high negative charge than those coated by pectin (Figure 5.3a). This effect may be attributed to the differences in molecular structure and again in charge density of the two types of polysaccharide. The linear carrageenan molecules may lie flat against the lipid droplet surfaces forming a thin interfacial layer, whereas the non-ionic side chains on the pectin molecules may extend into the aqueous phase thereby forming a thick interfacial layer that increases the steric repulsion between the droplets. In addition, there may me

differences in the nature and extent of bridging flocculation in the two different polysaccharide systems. It seems likely that the highly charged carrageenan molecules will promote stronger (more irreversible) polymer bridges than the weakly charged pectin molecules. Our results therefore support previous studies, which have also shown that adsorption of polysaccharides onto protein-coated lipid droplets may either stabilize or destabilize then depending on their molecular structure (21, 61, 79, 121, 126). For example, pectin was found to increase the stability of protein-coated droplets near the protein's pI (79), whereas carrageenan was found to promote extensive droplet aggregation (60, 61). However, previous measurements were conducted with microelectrophoresis and it was usually necessary to dilute the emulsions considerably prior to analysis. This dilution step could affect the partitioning of polysaccharide molecules between the droplet surfaces and the surrounding continuous phase, thereby altering the composition of the interfacial layer and thus the droplet ζ -potential. The ESA instrument used in this work should be less prone to errors associated with changes in interfacial properties induced by sample dilution because it can measure the ζ -potential in concentrated systems *in situ*.

5.4.4 Adsorption of mixed polysaccharides to droplet surfaces: pH-titration

In principle, the interfacial properties of adsorbed polysaccharide layers can be controlled by using mixtures of polysaccharides with different molecular characteristics. For example, one polysaccharide could be used to provide a high charge, whereas another could be used to form a thick layer. We therefore investigated the impact of mixed polysaccharides on the interfacial properties and physical stability of protein-coated lipid droplets. The adsorption of mixed polysaccharides to the lipid droplet surfaces was

measured when the pH was reduced from 7.0 to 3.5. The total polysaccharide concentration was held constant (0.04 wt%), while the mass mixing ratio (MR) of pectin-to-carrageenan in the aqueous phase was varied (10:0 to 0:10). This total polysaccharide concentration was chosen so that it was above the level required for the droplet surfaces to be completely saturated with polysaccharide molecules (Figure 5.2).

When the pH of the emulsions in the presence of mixed polysaccharides was reduced from pH 7 to 3.5, the electrical charge on the droplets remained negative across the entire pH range, indicating that polysaccharides adsorbed to the droplet surfaces (Figure 5.4a). The magnitude of the ζ -potential depended on the MR of pectin-to-carrageenan used. In the presence of relatively low carrageenan concentrations (0.004 wt%, MR = 9:1), the ζ -pH profile was similar to the pectin-coated droplets (MR = 10:0) at high (pH 7 - 5.5) and low (pH 4 - 3.5) pH values, but was closer to the carrageenan-coated droplets (MR = 0:10) at intermediate pH values (pH 5.5 - 4). This effect can be attributed to the fact that carrageenan adsorbs to the lipid droplet surfaces at a higher critical pH than pectin (Figure 5.3a), but that it was displaced from the droplet surfaces by pectin at lower pH values due to the much higher concentrations of pectin present compared to carrageenan. In the presence of higher carrageenan concentrations (MR = 5:5 or 1:9), the ζ -pH profile was similar to the carrageenan-coated droplets across the entire pH range studied, which suggested that the carrageenan molecules preferentially adsorbed to the protein-coated lipid droplets and remained attached. This phenomenon can be attributed to the higher affinity of the carrageenan molecules for the droplet surfaces because of their higher charge density compared to pectin.

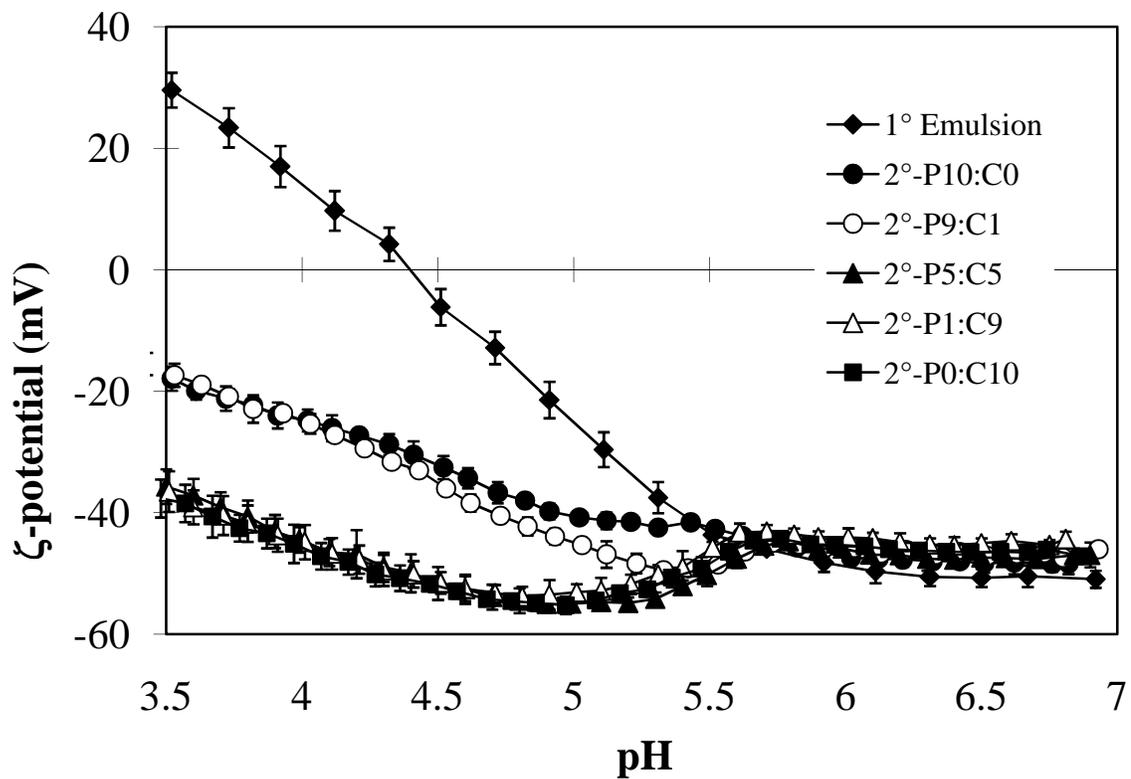


Figure 5.4a. Influence of the mass mixing ratio (MR) of pectin to carrageenan on the ζ -potential of β -Lg stabilized emulsions as a function of pH. The total polysaccharide concentration was held constant (0.04 wt%), while the mixing ratio was varied (10:0 to 0:10).

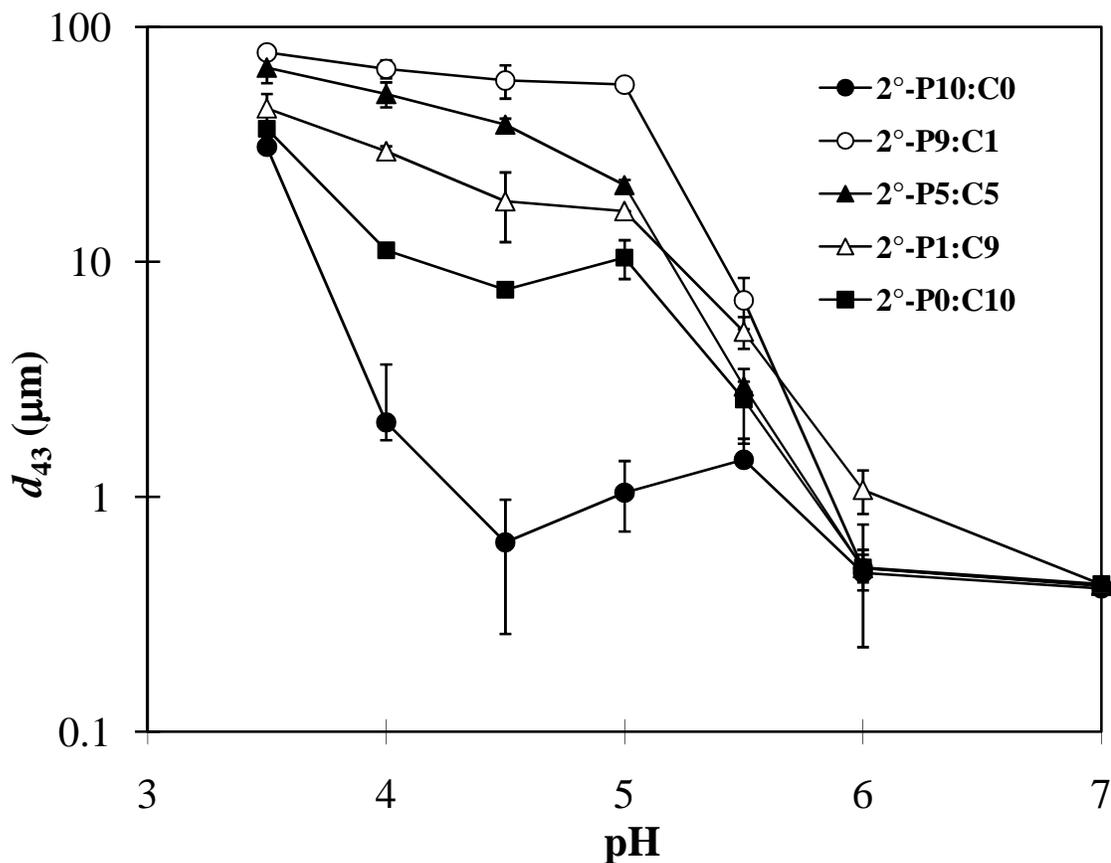


Figure 5.4b. Influence of the mass mixing ratio (MR) of pectin to carrageenan on the mean particle diameter (d_{43}) of β -Lg stabilized emulsions as a function of pH.

The change in mean particle diameter as a function of pH and MR was also measured to provide some insight into the emulsion stability to aggregation (Figure 5.4b). Extensive aggregation occurred in all the emulsions containing mixed polysaccharides. As discussed earlier, the emulsion containing only pectin-coated droplets (MR = 10:0) was relatively stable to droplet aggregation at lower pH values (pH 4 to 6), whereas the one containing only carrageenan-coated droplets (MR = 0:10) was highly unstable. The presence of even small quantities of carrageenan (MR = 9:1) in the emulsions promoted extensive droplet aggregation when the pH was reduced from neutral. This effect can be

attributed to the fact that carrageenan molecules adsorbed to the protein-coated lipid droplets at a higher critical pH than pectin due to their higher charge density. However, there was insufficient carrageenan present to completely saturate the droplet surfaces with a polysaccharide layer (Figure 5.2), and so extensive bridging flocculation occurred (8, 79). At higher carrageenan concentrations, the emulsions were not stable to droplet aggregation because this polysaccharide forms a relatively thin layer at the droplet surfaces that does not generate a strong steric repulsion.

5.4.5 Displacement of previously adsorbed polysaccharide coatings

In this study, we examined the possibility of displacing a previously adsorbed polysaccharide layer from the surfaces of protein-coated lipid droplets using another polysaccharide. An emulsion containing polysaccharide-protein-coated droplets was prepared by mixing polysaccharide (0.04 wt%) and protein-coated lipid droplets (1 wt%) at pH 7, and then adjusting the emulsion to pH 4.5. The resulting emulsion was then titrated with a solution containing a different polysaccharide (1 wt%) and the change in ζ -potential was measured (Figure 5.5a). Thus, a carrageenan solution was titrated into a secondary emulsion initially containing β -Lg/pectin coated droplets, or a pectin solution was titrated into a secondary emulsion initially containing β -Lg/carrageenan coated droplets.

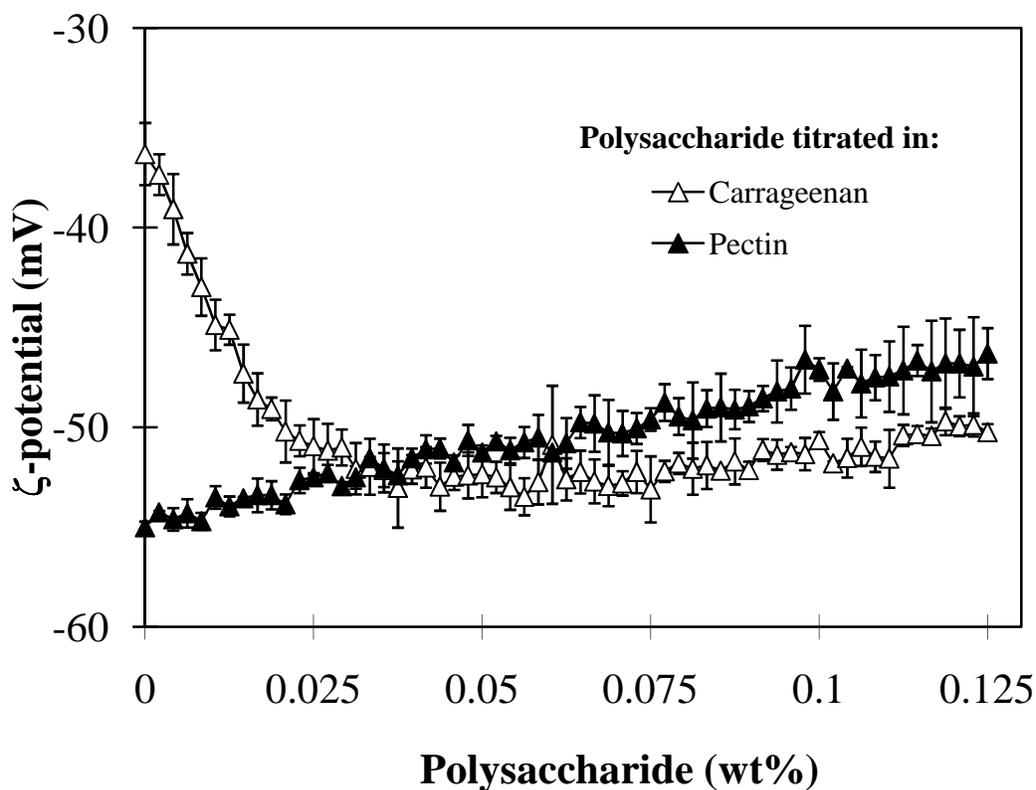


Figure 5.5a. Dependence of the ζ -potential of secondary emulsions initially coated by one kind of polysaccharide when an increasing concentration of a different kind of polysaccharide is added. The composition of the initial secondary emulsions was 1 wt% corn oil, 0.045 wt% β -Lg, 0.04 wt% pectin or carrageenan (5 mM phosphate buffer, pH 4.5).

When pectin (0 to 0.12 wt%) was titrated into the emulsion initially containing carrageenan coated droplets there was a gradual decrease in ζ -potential (Figure 5.5a). This decrease in z-potential may have been due to some displacement of carrageenan molecules from the droplet surfaces by pectin (since pectin-coated droplets are less negatively charged than carrageenan-coated droplets). At the highest pectin concentration added (0.12 wt%) the ζ -potential was -46 mV, whereas the ζ -potential of fully pectin-saturated droplets should be around -36 mV, suggesting that only limited carrageenan displacement could have occurred. On the other hand, when carrageenan was titrated into

an emulsion initially containing pectin-coated droplets the ζ -potential became more negative and eventually reached the value for carrageenan-coated droplets (- 51 mV). This result suggests that carrageenan displaced pectin from the droplet surfaces. Spectroscopy studies have shown that synthetic polyelectrolytes with a higher linear charge density will displace those with a lower linear charge density from oppositely charged solid surfaces (282). However, the reversibility of the polyelectrolyte adsorption/desorption processes depends on the strength of the initial attraction between the polyelectrolyte molecules and the oppositely charged surfaces. When the electrostatic attraction is very strong there is a large kinetic energy barrier to subsequent desorption of the polyelectrolyte. This may explain why pectin was unable to displace carrageenan from the droplet surfaces even at relatively high concentrations, whereas carrageenan was able to displace the pectin.

The emulsion initially containing β -Lg-pectin coated droplets was relatively stable to aggregation in the absence of carrageenan molecules ($d < 1 \mu\text{m}$), but the addition of even small amounts of carrageenan ($\geq 0.012 \text{ wt}\%$) promoted extensive droplet aggregation (Figure 5.5b). This effect can be attributed to the displacement of pectin from the droplet surfaces by carrageenan, and the inability of carrageenan to stabilize droplets against aggregation on its own. The emulsion initially containing β -Lg-carrageenan coated droplets was unstable to aggregation in the absence of pectin molecules ($d < 1 \mu\text{m}$), and became even more unstable upon the addition of pectin molecules (Figure 5.5.2). This result suggests that pectin was unable to displace carrageenan from the droplet surfaces and form a more stable system. The increase in mean particle diameter may have been due to some depletion flocculation in the

emulsions caused by the presence of additional non-adsorbed polymer. There were some differences in the mean particle diameters reported in Figures 5.3b/5.4b and Figure 5.5b, which can be attributed to differences in preparation procedures. In figures 5.3b/5.4b the pH was adjusted automatically with constant stirring using the ESA instrument, whereas in Figures 5.5b the initial emulsion were prepared by manually adjusting the pH to 4.5.

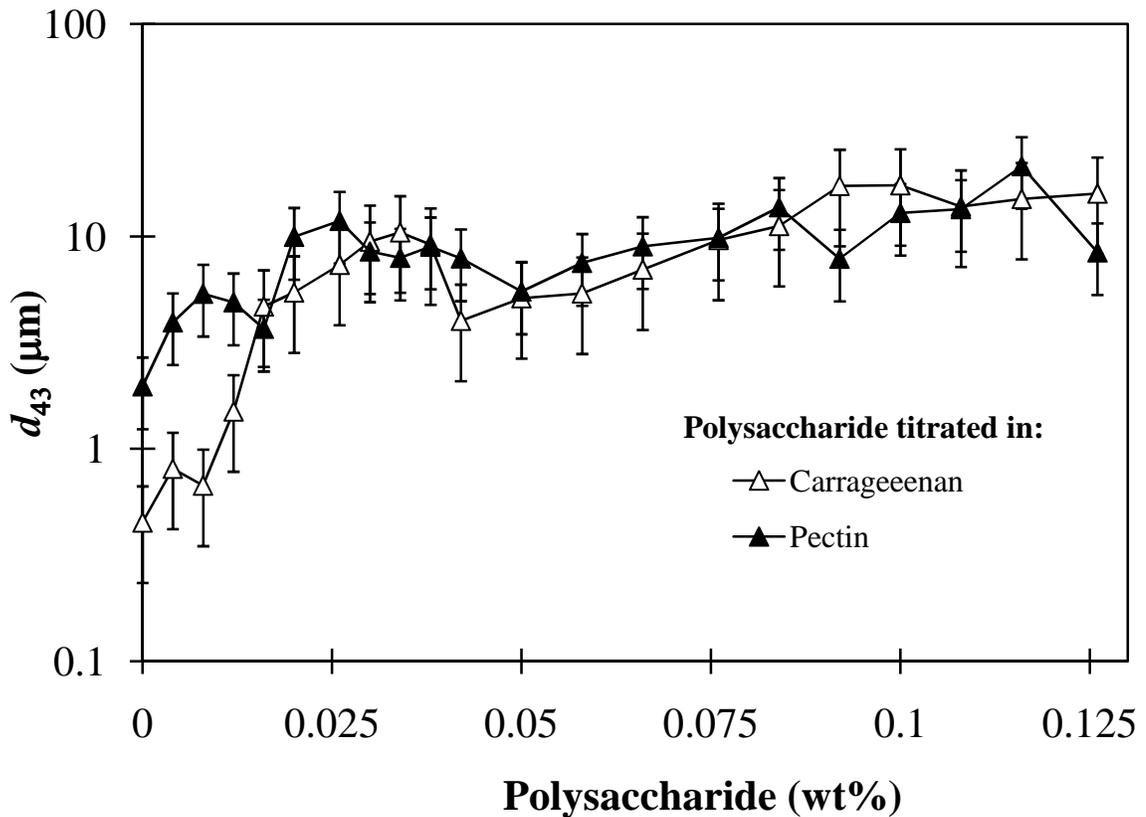


Figure 5.5b. Dependence of the mean particle diameter (d_{43}) of secondary emulsions initially coated by one kind of polysaccharide when an increasing concentration of a different kind of polysaccharide is added. The composition of the initial secondary emulsions was 1 wt% corn oil, 0.045 wt% β -Lg, 0.04 wt% pectin or carrageenan (5 mM phosphate buffer, pH 4.5).

5.5 Conclusion

This study has shown that carrageenan adsorbs more strongly to protein-coated lipid droplets than pectin, and that it produces a higher negative charge on the droplets at saturation. This effect can be attributed to the higher linear charge density of carrageenan, since electrostatic attraction is the major driving force for polysaccharide adsorption. As a consequence of its higher affinity for the lipid droplets it preferentially adsorbs to their surfaces from mixed polysaccharide solutions, and it is able to displace pectin from the droplet surfaces. On the other hand, pectin molecules are much less effective at displacing the more highly charged carrageenan molecules. These results may have important implications for the design of emulsion-based delivery systems with novel or improved properties. For example, polysaccharide blends may be used to control the charge, thickness or environmental responsiveness of the polysaccharide-coatings around lipid droplets, which may be useful for controlling their stability or release characteristics. In this study, it appeared that the stability of protein-coated droplets could not be improved by adding combinations of pectin and carrageenan. However, other combinations of polysaccharides with different molecular characteristics need to be investigated.

CHAPTER 6

MONITORING THE ADSORPTION OF MIXED ANIONIC POLYSACCHARIDES AT THE SURFACES OF PROTEIN-COATED LIPID DROPLETS

6.1 Abstract

The purpose of the present study was to monitor the adsorption of two anionic polysaccharides (pectin and gum arabic) onto protein-coated lipid droplets to examine whether a stable emulsion to droplet aggregations could be prepared using the mixed polysaccharide with the reduced amounts of each polysaccharide. Pectin and gum arabic molecules have similar electrical characteristics at lower pH (pH 3.5). Emulsions ($\phi = 1$ wt% oil, $d_{32} = 0.26 \pm 0.02 \mu\text{m}$) were mixed with polysaccharide solutions (0 or 0.05 wt%) at pH 7, then the pH was decreased to 3.0 to promote polysaccharide adsorption. The adsorption of the polysaccharide molecules to the droplet surfaces occurred at a higher pH for pectin (pH ≈ 5.2) than for gum arabic (pH ≈ 4.8). When polysaccharide mixtures were added at pH 7, the pectin molecules preferentially adsorbed to the droplet surfaces and the emulsion stability to aggregations was dependent on the concentration of pectin molecules when the pH was reduced. In the presence of the same amount of pectin and gum arabic, the emulsion was stable to the droplet aggregation at pH 4.5 through co-adsorption of pectin and gum arabic molecules. At higher concentrations of gum arabic, the emulsions showed extensive droplets aggregations. These results have important implications for designing the structured delivery systems in the food industry, especially beverage emulsions.

6.2 Introduction

The electrostatic interaction between protein and polysaccharide have been utilized to develop an emulsion-based structured delivery system with easily tunable and improved stability in various processing, storage, delivery and digestion conditions (17, 18, 21, 60, 62, 69, 73, 75, 79, 119, 123, 130, 214, 219, 248-252, 254, 256, 283-285). In the emulsion formed with an electrostatic deposition technique, multiple layers of biopolymers, mainly protein and polysaccharide, are built onto the lipid droplets coated with ionic emulsifiers (55). In this case, the emulsion stability to aggregation or environmental stresses is highly dependent on molecular structure, concentration, and electrical characteristic of biopolymers used (55).

Gum Arabic has been widely used for encapsulating flavor oils and stabilizing beverage emulsions (83, 286-296). Gum Arabic consists of a complex mixture of different biopolymer fractions including polysaccharide, protein and arabinogalactan protein species (297). The structure of gum arabic has been considered as the so-called 'wattle-blossom' model, which carbohydrate blocks link to a polypeptide chain. The emulsifying properties of gum arabic is believed to the presence of a high molecular weight fraction, which consists of arabinogalactan protein component (297). It has been postulated that the hydrophobic polypeptide chain adsorbs onto the droplet surface, whereas the hydrophilic arabinogalactan blocks extend into the solution, providing steric stabilization(297). The presence of charged amino acids such as aspartic and glutamic acids, minor amounts of arginine on the molecules is believed to provide the droplet stability to aggregation through electrostatic repulsion (21, 297, 298). The main advantage of using gum arabic as an emulsifier would be its low-viscosity even at

relatively high gum concentrations due to the highly branched gum structure, whereas it has to be used at relatively high concentration compared to other biopolymer emulsifiers leading to problems including cost and fluctuations in the quality (21, 44). Therefore, there have been a lot of efforts in replacing existing gum arabic with other biopolymer emulsifiers (44, 87, 299-301).

Previously, we have examined the competitive adsorption of two anionic polysaccharides (carrageenan and pectin) to protein-coated lipid droplets and found that the interfacial characteristics and emulsion stability can be controlled by assembling them from mixed polysaccharides with different molecular characteristics (22). We hypothesize that this competitive adsorption could be used to overcome the limitations of gum arabic in utilizing in emulsions.

The purpose of the present study was to monitor the adsorption of polysaccharides onto protein-coated lipid droplets, to determine the impact of this process on subsequent droplet properties, and eventually to see whether the stable emulsion to droplet aggregations could be prepared using the mixed polysaccharide with the reduced amounts of each polysaccharide. Lipid droplets were formed by homogenizing an oil phase with an aqueous solution containing a globular protein (β -lactoglobulin, β -Lg). β -Lg was used because its molecular and emulsification properties are well documented (240, 260, 261, 302). The isoelectric point of β -Lg has been reported to be around 4.7 to 5.2 (242, 243, 262). Two anionic polysaccharides with different molecular characteristics were selected for this study: citrus pectin and gum arabic. Both citrus pectin and gum arabic are anionic polysaccharides and highly branched polysaccharides. Numerous studies have shown that citrus pectin provides the excellent emulsion stability to aggregation and environmental

stresses at a relatively low concentration (22, 57, 58, 79, 303). Therefore, a pair of citrus pectin and gum arabic would be a good system to examine the impact of the mixed polysaccharides on emulsion properties to prepare an emulsion with a good physical stability (pectin) and low viscosity (gum arabic). In this study, we monitored the adsorption of pectin and carrageenan molecules from mixed solutions, as well as the possible displacement of one type of polysaccharide from a droplet initially coated by the other type of polysaccharide. The results from this study should provide useful information for designing the structured delivery system in the food industry, especially beverage emulsions.

6.3 Material and Methods

6.3.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Pectin was kindly provided by CPKelco (TS#1781, DE=54%, Mw160 KDa, Denmark). Gum arabic was kindly donated by TIC gum (TIC pretested[®] gum arabic spray dry FCC powder, Belcamp, MD) and used without further purification. Corn oil was purchased from a food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN) and used without further purification. The manufacturer reported that the corn oil contained approximately,

14.3, 28.6 and 57.1 wt% of saturated, monounsaturated and polyunsaturated fats, respectively. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from the Sigma Chemical Company (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, IW) was used for the preparation of all solutions.

6.3.2 Solution preparation

An emulsifier solution containing 0.5 wt% protein was prepared by dispersing powdered β -Lg into 5 mM Na phosphate buffer (pH 7.0) and stirring for at least two hours to ensure complete hydration. Pectin and gum arabic stock solutions were prepared by dispersing 1 w% pectin or 1 wt% gum arabic in 5 mM Na phosphate buffer and stirring overnight.

6.3.3 Emulsion preparation

A stock emulsion was prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.5 wt% β -Lg, pH 7.0) with a high-speed blender (Tissue Tearor, Biospec Products, Inc., Bartlesville, OK) for 2 min followed by 5 passes through a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA): the first stage at 3000 psi, the second stage at 300 psi. The stock emulsion was diluted with 5 mM Na phosphate buffer solution (pH 7, primary emulsion) or polysaccharide solution in 5 mM phosphate buffer (pH 7, secondary emulsion) to obtain a final composition of 1 wt% corn oil, 0.045 wt% β -Lg, and varied concentration of polysaccharides (0-0.05 wt%).

6.3.4 Electroacoustic measurements

Electrical charge (ζ -potential) of the particles in undiluted emulsions was determined with an electro-acoustic instrument (Field ESA, Partikel Analytik GmbH, Frechen, Germany), based on the electrosonic analysis (ESA) principle. The frequency of the ESA signal applied to the samples ranged between 0.85 and 1.15 MHz. The Smoluchowski equation was used to relate ζ -potentials of the emulsion droplets to the measured ESA signal:

$$\zeta = \frac{A (\text{ESA})\eta_s}{G \varepsilon_s \phi \Delta \rho c_s} \quad (6.1)$$

where A is an instrument constant determined by calibration with a standard suspension (see below), ESA is the signal measured by the instrument, η_s is the dynamic viscosity of the solvent surrounding the droplets, ε_s is the relative dielectric permittivity of the solvent, ϕ is the disperse-phase volume fraction, $\Delta \rho$ is the density difference between the particles and solvent ($\rho_P - \rho_S$), and $G [= f(\rho_P, \rho_S, \eta_s, r)]$ is a calculated instrument parameter that depends on density, viscosity, and particle radius (r). This equation is applicable under conditions where the electrical double layer around the particle is thin compared to the particle radius. At the electrical conductivities of our samples, the double layer was only a few nanometers thick, so the Smoluchowski equation is applicable for both polymers and particles studied. The ESA instrument was calibrated by use of a standard colloidal suspension of known charge characteristics (10% Ludox, Sigma Chemical Co., St Louis, MO) immediately before each set of experiments was run, and then a phase calibration was carried out for each sample before a titration was started. Measurement of ζ -potential by the ESA was repeated at least three times

with freshly prepared samples for each repetition. Two different types of experiments were carried out:

(1). *Concentration Titration.* In these experiments, the change in electrical charge of the emulsions was measured when anionic polysaccharides (pectin or gum arabic) were titrated into a primary emulsion (1 wt% of β -Lg coated oil droplets). A primary emulsion (pH 3.5) was placed into the measurement chamber of the instrument, then thirty 1 mL aliquots of polymer solution (1 wt% pectin or 1 wt% gum arabic, pH 3.5) were titrated with computer controlled auto-titration into the emulsion, with a delay time of 60 seconds between each aliquot. For displacement experiments, a secondary emulsion containing the lipid droplets (1 wt%) stabilized by β -Lg and polysaccharide (0.05 wt% pectin or gum arabic) was prepared at pH 7 followed by adjusting the pH of the secondary emulsion to 4.5. The secondary emulsion (pH 4.5) was placed into the measurement chamber of the ESA, and then thirty 1 mL aliquots of the other polysaccharide solution (1 wt% gum arabic or 1 wt% pectin) were titrated with a delay time of 60 seconds between each aliquot. i.e. a gum arabic solution was titrated into a secondary emulsion containing β -Lg/pectin coated droplets, or a pectin solution was titrated into a secondary emulsion containing β -Lg/gum arabic coated droplets.

(2). *Potentiometric Titration.* In these experiments, the change in ζ -potential was measured as a function of pH in primary and secondary emulsions. A primary emulsion (1 wt% oil droplets, 0.045 wt% β -Lg) and secondary emulsion (1 wt% oil droplets, 0.045 wt% β -Lg, 0.05 wt% polysaccharide) were placed into the measurement chamber of the instrument at pH 7.0. A 0.2 N HCl solution was then automatically titrated into the measurement chamber with 60 sec equilibrium delay time between each 0.1 pH

decrement and the change of ζ -potential with pH was measured. To investigate the impact of mixed polysaccharides on adsorption to the lipid droplets, the mixing ratios (MR) of pectin and carrageenan solution were varied at a fixed total polymer concentration (0.05 wt%) as shown in Table 6.1.

Table 6.1. Composition of secondary emulsions containing the droplets stabilized by β -Lg (0.045 wt%) and polymer (0.05 wt%)

| Mixing ratio | Pectin (wt%) | Gum arabic (wt%) |
|--------------|--------------|------------------|
| P10 | 0.05 | 0 |
| P9: G1 | 0.045 | 0.005 |
| P5: G5 | 0.025 | 0.025 |
| P1: G9 | 0.005 | 0.045 |
| G10 | 0 | 0.05 |

6.3.5 Particle size analysis

Aliquots of emulsion (1 ml) were collected from the measurement chamber of the electroacoustic instrument every two injection steps or every 0.5 pH unit, during concentration titration and potentiometric titration, respectively. These emulsion samples were diluted in the measurement chamber of the diffraction instrument (Mastersizer X, Malvern Instruments Ltd., Malvern, UK) to 0.01 wt% droplet concentration using an appropriate buffer solution (at the same pH as the sample). This instrument finds the particle size distribution of an emulsion that gives the best fit between the experimental measurements and predictions made using light scattering theory (*i.e.* Mie theory). A

refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions. Measurements are reported as the volume weighted mean diameter: $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$ or as the volume-surface mean diameter: $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of droplets of diameter d_i .

6.3.6 Statistical analysis

Experiments were performed at least three times using freshly prepared samples. Averages and standard deviations were calculated from these triplet measurements.

6.4 Results and Discussion

6.4.1 Adsorption of individual polysaccharides to droplet surfaces: Concentration titration

The main stabilization mechanism for the colloidal particles is known as the electrostatic and steric and steric for pectin and gum arabic molecules, respectively (23, 292, 297, 298, 304). To examine the electrical property of each polysaccharide, the pH titration of pure pectin (0.5 wt%) and gum arabic (0.5 wt%) solution in 5 mM phosphate buffer was conducted with the ESA to investigate the pH- ζ profile of polysaccharide and both polysaccharides were negatively charged over a whole pH (7~3) range studied (data not shown). The ζ -potential of pectin and gum arabic at pH 3.5 was -12.45 ± 0.21 and -23.93 ± 0.19 mV, respectively. Pectin molecules had a lower negative charge than gum arabic molecules at this pH, where it is close to its pK_a value (245, 272). The surface charge of gum arabic molecules mainly be contributed by the presence of amino acid groups of the arabinogalactan branches including arginine, aspartic and glutamic acids:

its pK_a value of 1.8-2.2 (α -carboxyl group)(298). It suggests that gum arabic molecules could provide the emulsion stability to aggregation through both steric and electrostatic repulsion. Therefore, we compared the adsorption of pectin and gum arabic to protein-coated lipid droplets under conditions where the polysaccharides and proteins had opposite electrical charges. Each polysaccharide was titrated into a primary emulsion containing the lipid droplets (1 wt%) stabilized by β -Lg (0.045 wt%) at pH 3.5 (Figure 6.1a). At this pH, the protein-coated droplets were positively charged due to the adsorption of β -Lg molecules on the lipid droplets (pI of β -Lg \sim 4.8-5.2) (242, 243, 262) and each polysaccharide was negatively charged mentioned above. Therefore, one might expect that the electrostatic interaction would take place between oppositely charged protein-coated lipid droplets and polysaccharide molecules at this pH.

The droplet ζ -potential changed from positive to negative in both systems with increasing polysaccharide concentration, which indicated that the anionic polysaccharides adsorbed to the cationic protein coated lipid droplets (17, 20-22, 58). The electrical charge of the emulsions to which pectin or gum arabic was titrated changed from positive to negative with increasing polysaccharide concentration and reached a plateau value due to the adsorption of oppositely charged polysaccharide on the droplets surface coated with protein molecules. This charge reversal has been found in many literatures (17, 21, 22, 58, 61, 62, 67, 305). At relatively low concentration of polysaccharide, pectin showed higher binding affinity for the protein-coated droplets than gum arabic with the initial steep decrease in ζ -potential of the emulsion titrated with pectin. The ζ -potential of the emulsions titrated with gum arabic was dramatically decreased and the charge reversal from positive to negative of the emulsion took place around 0.03 wt%. The

magnitude of ζ -potential became similar in both the emulsions titrated with pectin and gum arabic around 0.035 wt% and reached a plateau value around 0.05 wt% in both the emulsions containing pectin and gum arabic molecules. This type of ζ -potential-polysaccharide concentration profile of the emulsion to which gum arabic was added may be the fact that the droplet aggregations may affect the ESA signal during the concentration titration leading to less difference in density of the droplets and continuous phase. Therefore, the particle size of the emulsion titrated with gum arabic was measured *in-situ* during the ESA measurement using a static light scattering and then we corrected the ζ -potential with the measured particle diameter. There was no difference in ζ -potential profile after correction except the magnitude of ζ -potential (higher for the larger particles). The ζ -potential of the emulsion was measured with a dynamic light scattering and the magnitude of ζ -potential was gradually decreased with increasing of gum arabic concentration (Figure 6.1b). These measurements demonstrate that the electrical characteristics of the interfacial layers formed when pectin and gum arabic molecules adsorb to the surfaces of protein-coated lipid droplets are appreciably similar including the polysaccharide concentration to completely saturate the protein-coated lipid droplets (~0.05 wt%). The pH dependence of polysaccharide adsorption to the protein-coated lipid droplet surfaces was studied by measuring changes in ζ -potential to examine whether these similar behaviors of pectin and gum arabic molecules at the protein-coated droplet surfaces would influence the affinity of anionic polysaccharide for the protein coated lipid droplets and provide the similar emulsion stability to droplet aggregations as

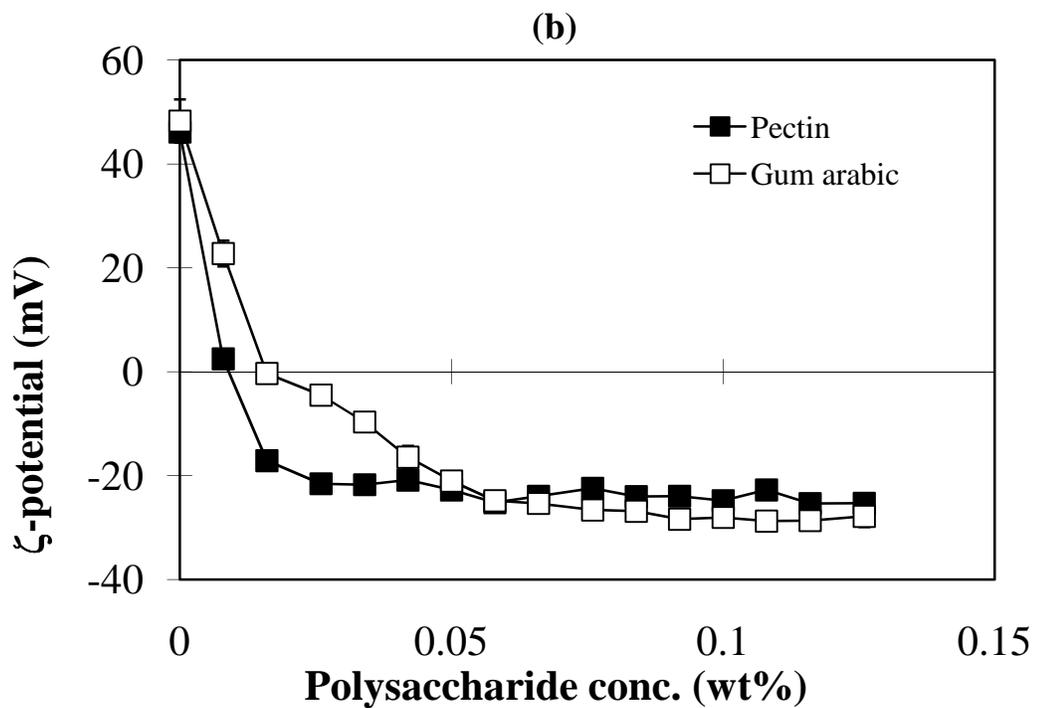
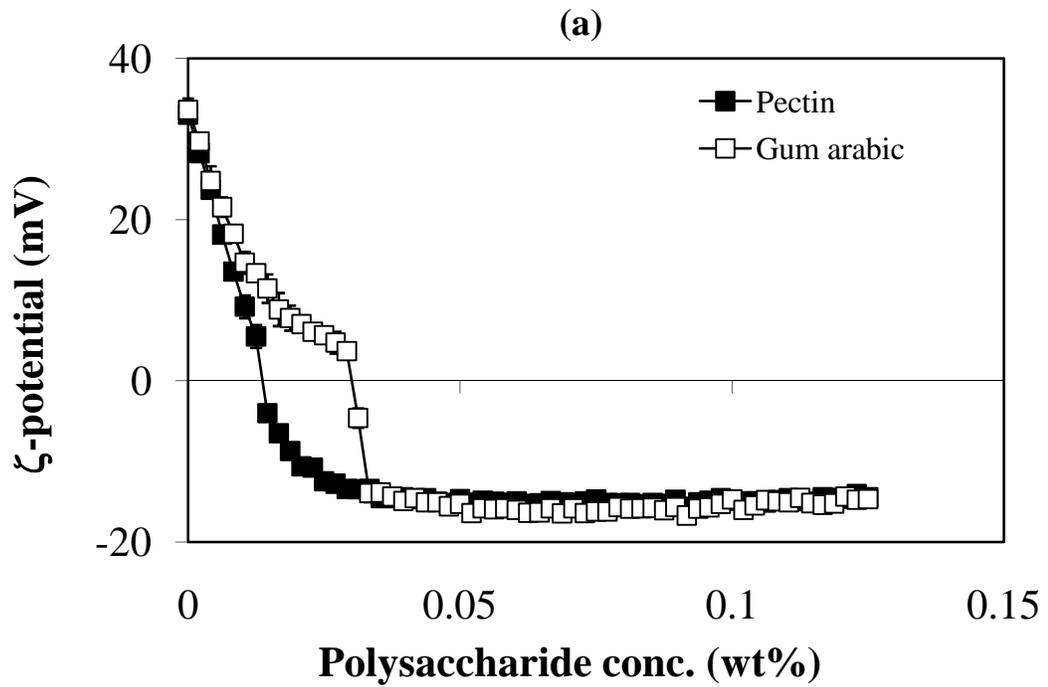


Figure 6.1a-b. Dependence of the droplet ζ -potential of protein-stabilized O/W emulsions on polysaccharide concentration: 1 wt% corn oil, 0.045wt% β -Lg, 5 mM phosphate buffer at pH 3.5. Either pectin or gum arabic solution (1 wt% in 5 mM Na phosphate buffer, pH 3.5) was titrated into the primary emulsion. The ζ -potential measurement was carried out using the ESA *in situ* (a) and a dynamic light scattering (b).

a function of pH. It also should be interesting to examine whether the mixed polysaccharide with a fixed total polymer concentration would have similar electrical characteristics to individual polysaccharide molecules. Since the saturation concentration of pectin and gum arabic molecules to completely saturate the protein coated lipid droplets was similar at pH 3.5 (~0.05 wt%).

6.4.2 Adsorption of mixed polysaccharides to droplet surfaces: Potentiometric titration

The pH dependence of polysaccharide adsorption to the protein-coated lipid droplet surfaces was studied by measuring changes in ζ -potential to investigate the impact of mixed polysaccharides on the interfacial properties and physical stability of protein-coated lipid droplets. A pH-titration from pH 7 to 3 was carried out for emulsions containing individual polysaccharides as well as mixed polysaccharides at a concentration known to be above the critical saturation level determined in the previous section (Figure 6.2a) *i.e.*, 0.05wt%. The adsorption of mixed polysaccharides to the lipid droplet surfaces was measured when the pH was reduced from 7.0 to 3.0. The total polysaccharide concentration was held constant (0.05 wt%), while the mass mixing ratio (MR) of pectin-to-gum arabic in the aqueous phase was varied (10:0 to 0:10). This total polysaccharide concentration was chosen so that it was above the level required for the droplet surfaces to be completely saturated with polysaccharide molecules (Figure 6.2a). First, the pH-titration was carried out for the emulsions containing gum arabic molecules without/with heat treatment of the primary emulsion at 85 °C for 20 min to examine whether the displacement of the adsorbed protein layer from the interface could occur in the presence of gum arabic due to its surface activity. Both emulsions showed a similar ζ -

pH profile irrespective of the heat treatment of the primary emulsion with the same critical pH (pH_c below) except for the magnitude of ζ -potential (data not shown). Therefore, unheated primary emulsions were used in the remainder of experiments.

This experiment was carried out to determine the critical pH where the polysaccharides first adsorbed to the lipid droplet surfaces: pH_c . The ζ -potential of the protein-coated lipid droplets changed from negative to positive as the pH of the emulsion was decreased from above to below the pI of the adsorbed β -Lg molecules (Figure 6.2a) (22, 58, 79). This type of ζ -pH profile can be attributed to the fact that the electrical charge of the adsorbed β -Lg molecules goes from negative to positive as the pH moves from above to below their isoelectric point ($\text{pI} \approx 5$) (55). In the presence of the polysaccharides, the ζ -potential on the droplets remained negative across the entire pH range when the solution was reduced from pH 7 to 3, which is due to the adsorption of anionic polysaccharides onto positively charged patches on the droplet surfaces when the pH falls below pH_c (79). The ζ -pH profiles of the emulsions were fairly similar at relatively high pH values ($\text{pH} > 5.2$) in the absence and presence of anionic polysaccharides, suggesting that the polysaccharides did not adsorb to the droplet surfaces (Figure 6.2a). This lack of adsorption can be attributed to the relatively strong electrostatic repulsion between the anionic polysaccharides and the anionic droplets, as well as to the fact that there were few positively charged patches on the protein surfaces in this pH range (278). Nevertheless, the ζ -pH profiles of the emulsions containing polysaccharides diverged from that containing no polysaccharide when the pH was lowered below the critical value (pH_c). This value was higher for pectin ($\text{pH}_c \approx 5.2$) than for gum arabic ($\text{pH}_c \approx 4.8$), which can be attributed to the fact that there was a stronger

electrostatic attraction between the positively charged patches on the protein and pectin than gum arabic (276, 279). At relatively low pH values ($< \text{pH } 4.2$), where the adsorbed polysaccharides had saturated the droplet surfaces, the ζ -potential was fairly similar in the emulsion containing pectin or gum arabic. Again this similarity in the ζ -potential between pectin and gum arabic molecules had shown in previous section.

The magnitude of the ζ -potential depended on the MR of pectin-to-carrageenan used. In the presence of relatively fair amount of gum arabic (0.004~0.025 wt%, MR = 9:1~5:5), the ζ -pH profile was similar to the pectin-coated droplets across the entire pH range studied. This effect can be attributed to the fact that pectin adsorbs to the lipid droplet surfaces at a higher critical pH than gum arabic (Figure 6.2a), and the electrostatic interaction between the protein-coated lipid droplets and pectin layer already adsorbed was strong enough to prevent the displacement of the adsorbed polysaccharide molecules from the lipid droplet surfaces even in the presence of the same amount of gum arabic and pectin molecules. It suggested that pectin molecules preferentially adsorbed to the protein-coated lipid droplets and remained attached.

In the presence of higher gum arabic concentrations (MR = 1:9), the ζ -pH profile was similar to that of gum arabic coated droplets at higher pH ($> \text{pH } 5$), but the magnitude of ζ -potential was decreased with decreasing the pH at intermediate pH values (pH 5~ 4.5). At lower pH values ($< \text{pH } 4.5$), it became similar to that of gum arabic coated droplets. It suggested that pectin molecules had the higher affinity for the protein coated lipid droplets and therefore the magnitude of ζ -potential of the emulsion containing small amounts of pectin molecule was higher than that of gum arabic coated droplets. It is interesting to note that the electrical charge of pectin and gum arabic

solution was fairly similar, but the electrical charge of the lipid droplets containing pectin or gum arabic was different at the intermediate pH ranges (pH 5.2~4.2). The structure of gum arabic has been known as the wattle-blossom model, which the arabinogalactan blocks are linked into the polypeptide chain and gum arabic is a higher molecular weight molecule compared to pectin molecules (292, 297, 298). On the other hand, the structure of pectin molecule has proposed to be a block copolymer (264, 304). We proposed that this difference in molecular structure between gum arabic and pectin may influence the affinity of each polysaccharide for the protein coated lipid droplets. *i.e.* Less flexible molecular structure of gum arabic would restrict the electrostatic interaction between the protein coated lipid droplets and gum arabic molecules or the presence of amino acids on the gum arabic molecules would promote the electrostatic repulsion between the protein adsorbed onto the lipid droplets and gum arabic molecules. These measurements demonstrate that the electrical characteristics of interfacial layers formed when pectin and gum arabic molecules adsorb to the surfaces of protein coated lipid droplets are appreciable different. In the presence of mixed polysaccharide, pectin molecules had a higher affinity for the protein coated lipid droplets than gum arabic molecules due to its more flexible molecular structure.

The evolution of the mean particle diameter (d_{43}) of the emulsions as a function of pH was also measured to provide information about droplet aggregation (Figure 6.2b). To examine kinetic changes of the particle size, the emulsions during the pH-titration were

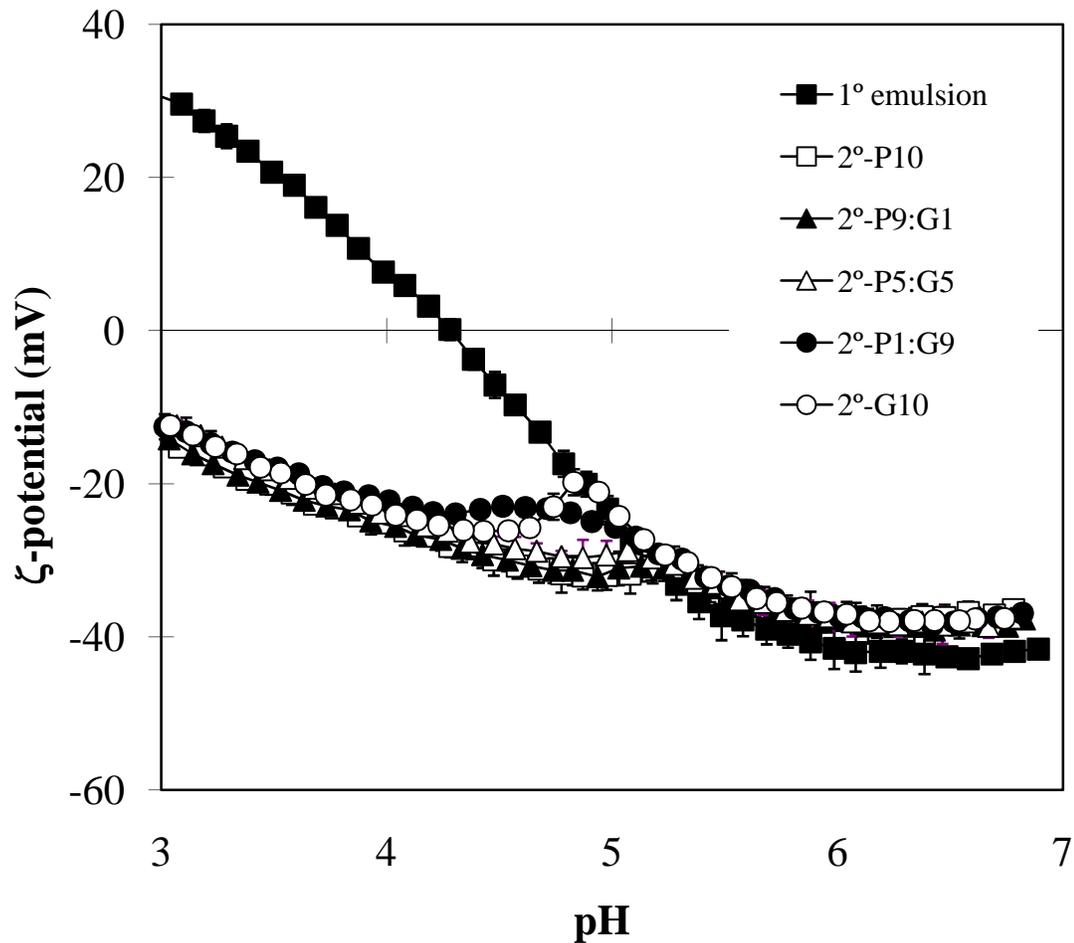


Figure 6.2a. Influence of the mass mixing ratio (MR) of pectin to gum arabic on the ζ -potential of β -Lg stabilized emulsions as a function of pH. The total polysaccharide concentration was held constant (0.05wt%), while the mixing ratio was varied (10:0 to 0:10)

taken at every 0.5 pH unit and the mean particle diameter was measured using a static light scattering *in situ*. The emulsions taken kept at ambient temperature and the mean particle diameter was measured after 24 hr. In the absence of polysaccharide, there was a large increase in mean particle diameter around the pI of the adsorbed proteins, which can be attributed to extensive droplet flocculation associated with the low net charge on the droplets, *i.e.*, the attractive interactions (mainly van der Waals) outweighed the

repulsive interactions (mainly electrostatic and steric). At pH 5.5, the primary emulsion was stable to aggregation *in situ*, but there were extensive droplet aggregations after 24 hr. It has been shown that unfolding and the development of new protein-protein interaction of the adsorbed protein layer took place over time (102). This could be the case for the growth of particles over time in the emulsion at pH 5.5, where a lot of conformation changes could occur for the protein molecules adsorbed onto the lipid droplets near pI of the protein. The high particle diameters observed at pH values less than the pI can be attributed to the fact that the aggregates formed were pH-irreversible.

In the presence of polysaccharide, the emulsion stability to aggregation was strongly dependent to the types of polysaccharide used. In the presence of high amounts of pectin, the emulsions (2°-P10 and 2°-P9:G1) showed the good stability to droplet aggregation *in situ*, but there was an increase in the particle size at pH 5.5 and below pH 3.5 after 24 hr. At pH 5.5, the instability of pectin-coated emulsions could be due to the instability of the primary emulsion and only few electrostatic interactions presented between positive patches of protein molecules and negatively charged pectin molecules at this pH (pH_c for pectin around pH 5.2). At below pH 3.5, pectin molecules started losing their charge due to its pK_a value (~pH 3.5) (245, 272) and it may promote the charge neutralization of protein-coated lipid droplets over time leading to the droplet aggregations. In the presence of the same amount of pectin and gum arabic (2°-P5:G5), the emulsion was stable to the droplet aggregation over the whole pH range studied *in-situ* except at pH 3. The growth of the lipid droplets occurred almost all lower pH values except pH 4.5. At pH 5, the droplet growth could be the destabilized primary emulsion over time with increasing conformational and interaction changes mentioned above. In

addition, the pectin concentration was too low to completely saturate the protein-coated lipid droplets and gum arabic didn't adsorb onto the lipid droplets at this pH (pH_c for gum arabic around 4.8). At pH 4.5, the emulsion containing the same amount of pectin and gum arabic molecules (total polymer concentration: 0.05 wt%) was stable to aggregations *in situ* and after 24 hr. It suggested that co-adsorption of pectin and gum arabic molecules could take place at this pH, where, at below their pH_c, pectin and gum arabic molecules started to adsorb onto the protein-coated lipid droplets and completely saturate the droplets. It supported by the fact that the emulsion became unstable at below pH 4. It could be explained by the fact that the pectin molecules started approaching to its pK_a value and less negatively charged at below pH 4. This electrical property of pectin molecules showed a significant impact on the emulsion stability in the presence of the same amount of pectin and gum arabic. In the presence of higher amounts of gum arabic (2°-P1:G9 and 2°-G10), the emulsion was highly unstable to the droplet aggregations at lower pH (pH 5-3) both *in situ* and after 24 hr. Surprisingly, the emulsions containing the higher concentration of gum arabic didn't show instability at pH 5.5. The electrical charge of the primary emulsion (1° emulsion) was similar to secondary emulsion containing the higher amount of gum arabic (2°-P1G9 and 2°-G10) at this pH. It may be the reason that the protein-protein interaction could occur between protein molecules adsorbed to the lipid droplets and amino acids on gum arabic molecules leading to form a viscoelastic film (102, 103, 292, 297, 298). These set of experiments have shown that pectin molecules provided a good stability to the droplet aggregations through electrostatic and steric repulsion. However, gum arabic molecules poorly provided the emulsion stability, even though the magnitude of ζ-potential was similar to that of pectin.

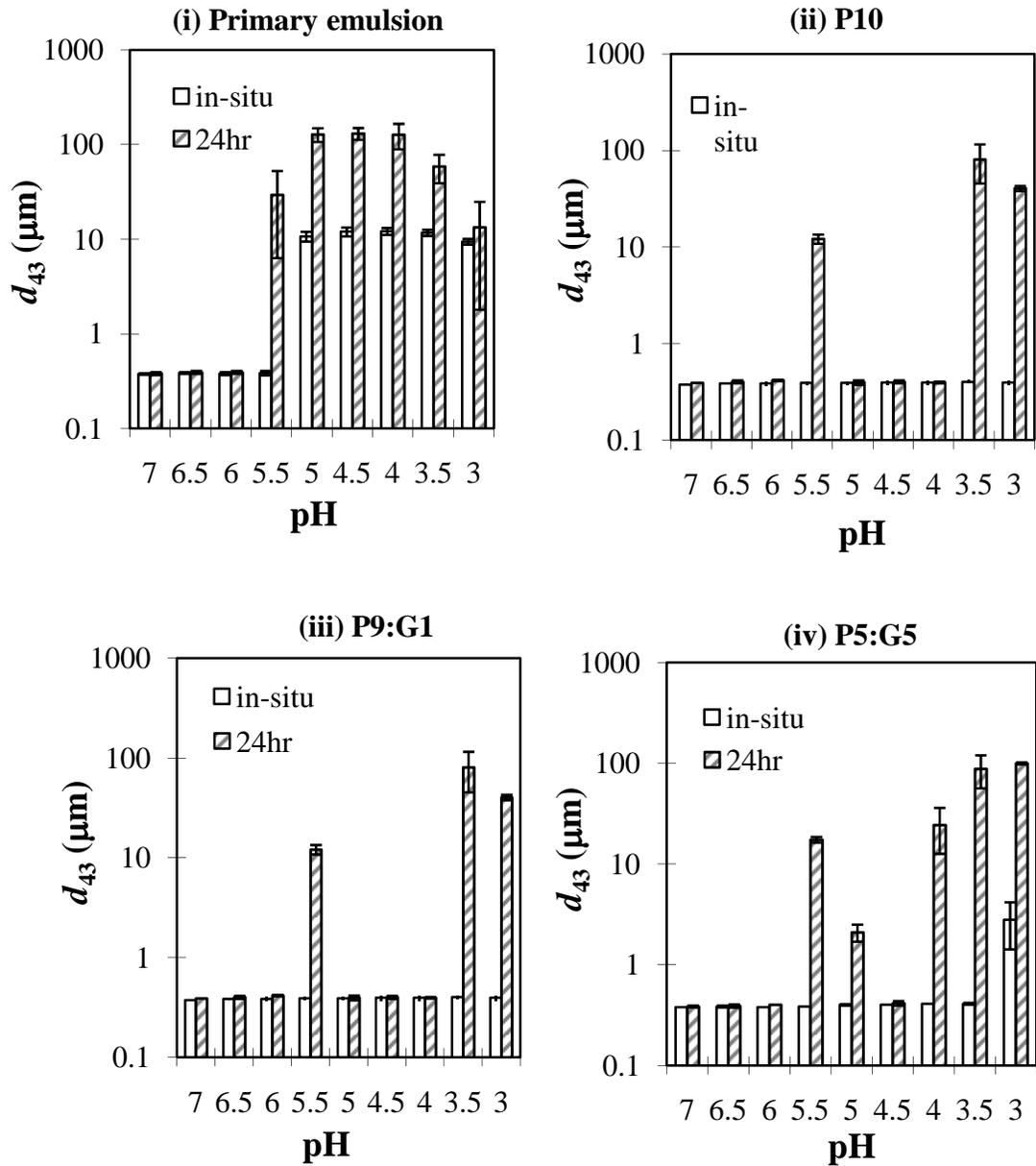


Figure 6.2b. Influence of the mass mixing ratio (MR) of pectin to gum arabic on the mean particle diameter (d_{43}) of β -Lg stabilized emulsion (i) primary emulsion (1°); (ii) secondary emulsion containing only pectin (0.05 wt%) (2° -P10); (iii) secondary emulsion containing pectin (0.045 wt%) and gum arabic (0.005 wt%) (2° -P9:G1); and (iv) secondary emulsion containing pectin (0.025 wt%) and gum arabic (0.025 wt%) (2° -P5:G5) as a function of pH *in situ* and after 24 hr.

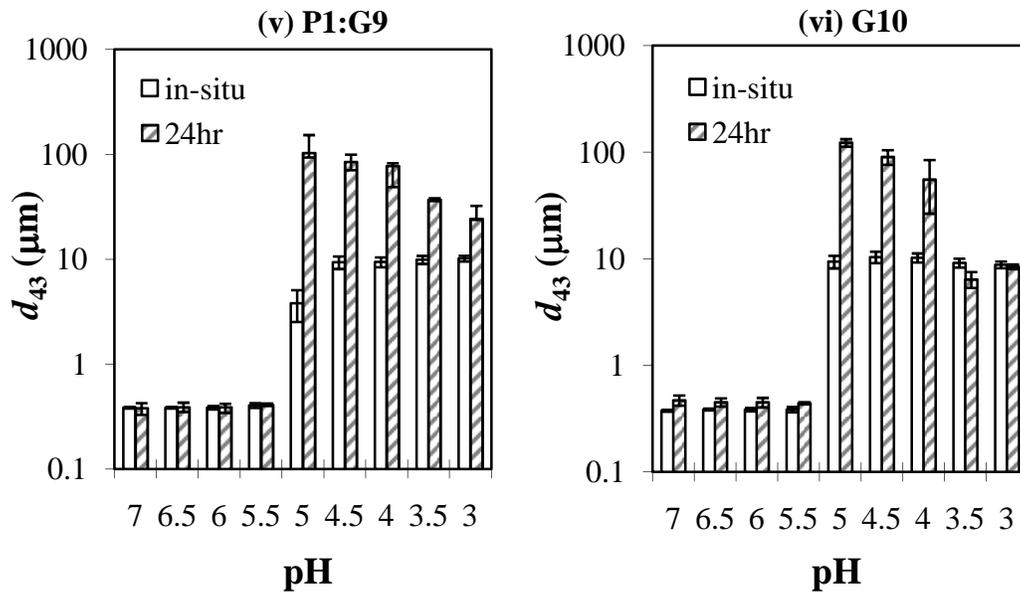


Figure 6.2b. Influence of the mass mixing ratio (MR) of pectin to gum arabic on the mean particle diameter (d_{43}) of β -Lg stabilized emulsion (v) secondary emulsion containing pectin (0.005 wt%) and gum arabic (0.045 wt%) (2°-P1:G9); (vi) secondary emulsion containing only gum arabic (0.05 wt%) (2°-G10) as a function of pH *in situ* and after 24 hr (continued figure).

6.4.3 Displacement of previously adsorbed polysaccharide coatings

In this study, we examined the possibility of displacing a previously adsorbed polysaccharide layer from the surfaces of protein-coated lipid droplets using another polysaccharide. An emulsion containing polysaccharide-protein-coated droplets was prepared by mixing polysaccharide (0.05 wt%) and protein-coated lipid droplets (1 wt%) at pH 7, and then adjusting the emulsion to pH 4.5. We selected this pH, where the emulsion containing the same amount of pectin and gum arabic was stable to droplet aggregation both *in situ* and after 24 hr in previous section. The resulting emulsion was then titrated with a solution containing a different polysaccharide (1 wt%) and the change in ζ -potential was measured (Figure 6.3a). Thus, a gum arabic solution was titrated into a secondary emulsion initially containing β -Lg/pectin coated droplets, or a pectin solution

was titrated into a secondary emulsion initially containing β -Lg/gum arabic coated droplets.

When pectin (0-0.12 wt%) was titrated into emulsions initially containing gum arabic-coated droplets, the ζ -potential became gradually less negative with increasing the amounts of pectin added. This decrease in ζ -potential may have been due to some desorption of gum arabic molecules from the droplet surfaces by pectin. Since both pectin molecules and gum arabic molecules were highly negatively charged the magnitude of ζ -potential was similar in the emulsions containing pectin or gum arabic molecules. On the other hand, when gum arabic was titrated into an emulsion initially containing pectin-coated droplets the ζ -potential became less negative similar to that of titration with pectin. In this case, however, the change in ζ -potential was less appreciable than pectin. In previous study (22), we found that the displacement of adsorbed pectin layer from the droplet surfaces took place by carrageenan molecules due to its higher charge density than pectin. That is not the case in this system probably due to their similar electrical characteristics of pectin and gum arabic molecules.

The emulsion initially containing β -Lg-pectin coated droplets was relatively stable to aggregation in the absence of gum arabic molecules ($d < 0.5 \mu\text{m}$) and the mean particle diameter remained the same with increasing the concentrations of gum arabic (Figure 6.3b). On the other hand, the emulsion initially containing β -Lg-gum arabic coated droplets was unstable to aggregation in the absence of pectin molecules ($d < 1 \mu\text{m}$), and the mean particle diameter remained the same upon the addition of pectin molecules (Figure 6.3b). When the mean particle diameter of the emulsions was measured after 24 hr, the emulsion containing the pectin-coated lipid droplets in the presence of gum arabic

remained the small droplet size like only pectin-coated droplets (Figure 6.3c). On the other hand, the emulsion containing the gum arabic-coated lipid droplet in the presence of pectin showed extensive droplet aggregations like only gum arabic-coated droplets (Figure 6.3c). It suggested that the displacement of the adsorbed polysaccharide layer from the droplet surfaces has not happened in these systems.

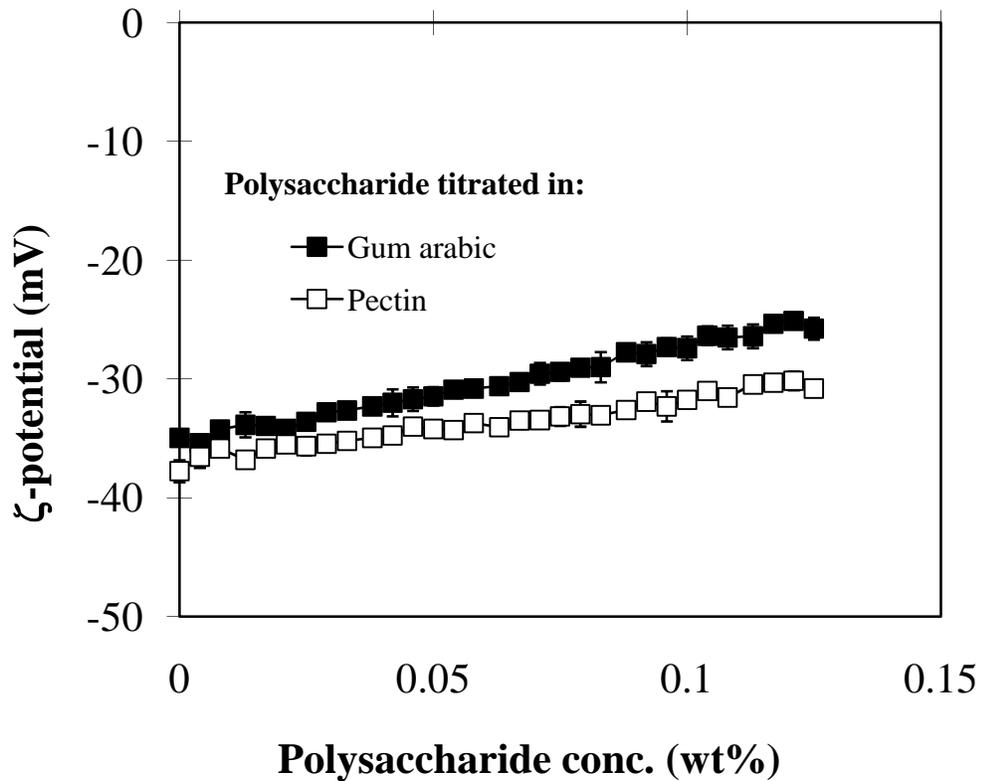


Figure 6.3a. Dependence of the ζ -potential of secondary emulsions initially coated by one kind of polysaccharide when an increasing concentration of a different kind of polysaccharide is added. The composition of the initial secondary emulsions was 1 wt% corn oil, 0.045 wt% β -Lg, 0.05 wt% pectin or gum arabic (5 mM phosphate buffer, pH 4.5).

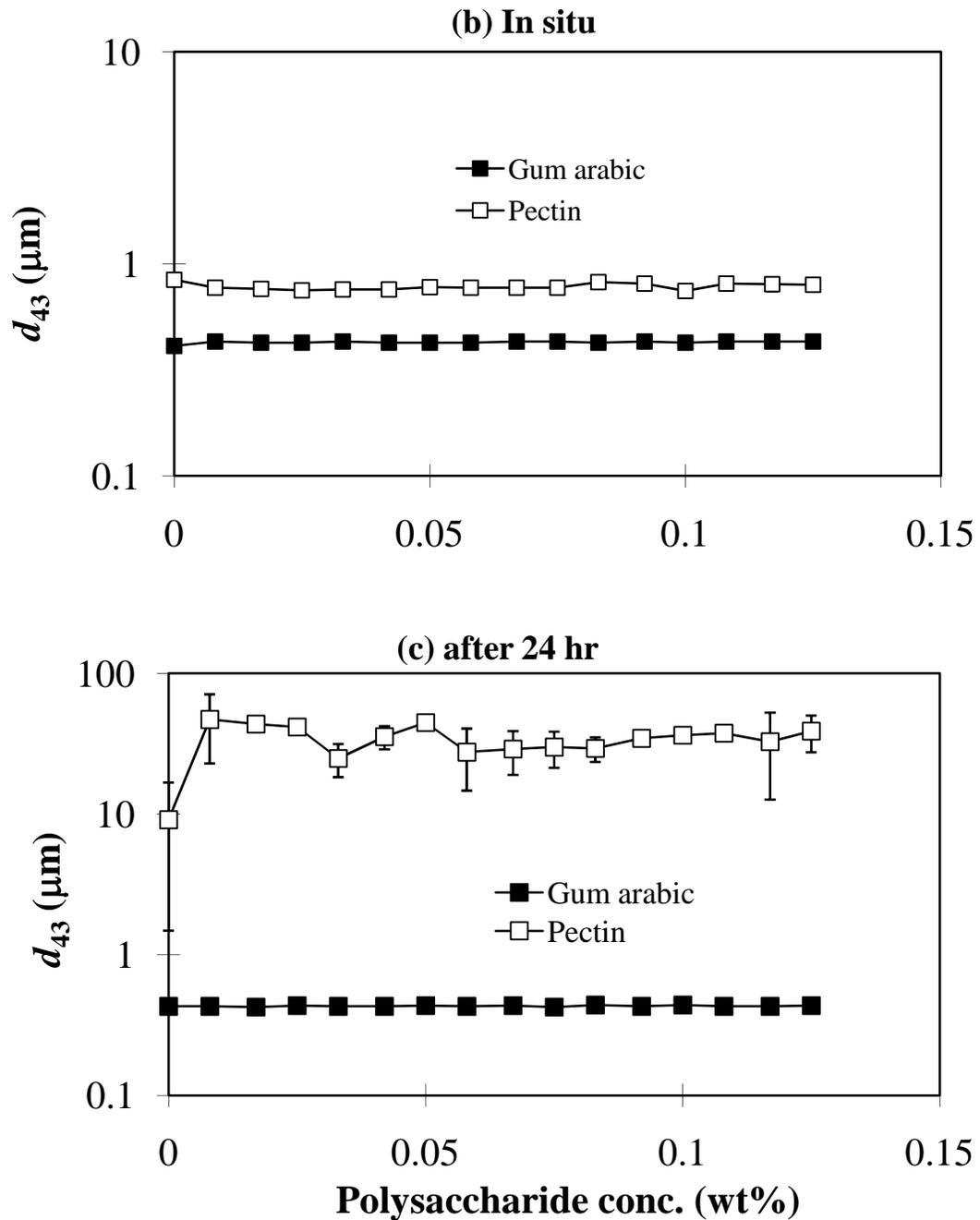


Figure 6.3b-c. Dependence of the mean particle diameter (d_{43}) (b) *in situ* and (c) after 24 hr of secondary emulsions initially coated by one kind of polysaccharide when an increasing concentration of a different kind of polysaccharide is added. The composition of the initial secondary emulsions was 1 wt% corn oil, 0.045 wt% β -Lg, 0.05 wt% pectin or gum arabic (5 mM phosphate buffer, pH 4.5).

6.5 Conclusions

This study has shown that pectin adsorbs more strongly to protein-coated lipid droplets than gum arabic, and that it produces a more stable emulsion to the droplet aggregations. The emulsions containing the same amounts of polysaccharides showed a good stability to aggregations at pH 4.5, where the lipid droplets were completely saturated with both pectin and gum arabic molecules (total polymer concentration 0.05 wt%) through co-adsorption. The electrical characteristics of pectin and gum arabic were fairly similar and consequently the displacement of the adsorbed polysaccharide layer from the droplet surfaces by other polysaccharide had not take place. The molecular structure of pectin and gum arabic would provide the good emulsion stability to aggregation due to the combination of electrostatic and steric repulsion. Emulsion stability tests with different salt concentrations and oxidative stability tests need to be investigated using the same condition in this study (0.025wt% pectin and 0.025wt% gum arabic at pH 4.5). These results may have important implications for the design of emulsion-based delivery systems with improved properties in the food industry.

CHAPTER 7

FORMATION OF PROTEIN-RICH COATINGS AROUND LIPID DROPLETS USING THE ELECTROSTATIC DEPOSITION METHOD

7.1 Abstract

The purpose of this study was to determine whether protein-rich coatings could be formed around lipid droplets using an electrostatic deposition method. These coatings were assembled using two different methods: (i) β -lactoglobulin was adsorbed to β -lactoglobulin-pectin coated lipid droplets; (ii) β -lactoglobulin-pectin complexes were adsorbed to β -lactoglobulin-coated lipid droplets. We showed that composite particles, consisting of lipid-droplets with protein-rich biopolymer coatings, could be formed using both approaches. These composite particles had relatively small diameters ($d < 500$ nm) and were stable to gravitational separation. The composite particles remained stable after they were heated above the thermal denaturation temperature of the globular proteins. When the heated composite particles were adjusted to pH 7, where the β -lactoglobulin and pectin are both negatively charged, some of the pectin and β -lactoglobulin became detached from the droplet surfaces but there was an increase in the protein concentration in the coating. These composite particles may be useful for increasing the protein concentration in biopolymer coatings surrounding lipid droplets, which potentially has practical applications in the food industry *e.g.*, to protect ω -3 oils from oxidation or to develop natural weighting agents.

7.2 Introduction

The stability of protein-coated lipid droplets to environmental stresses, such as pH extremes, high ionic strength, thermal processing, freezing and dehydration, can be improved by coating them with an additional layer of charged polysaccharides (17, 18, 21, 60, 69, 73, 75, 79, 119, 214, 219, 256). These polysaccharides form relatively thick charged layers around lipid droplets that can improve emulsion stability by modulating the colloidal interactions in the system, *e.g.*, increasing electrostatic and steric repulsion while decreasing van der Waals attraction (79). This approach is based on the layer-by-layer (LbL) method of depositing charged polymers onto oppositely charged colloidal particles (51, 53, 54, 247). In this case, the charged polymers are polysaccharides and the charged colloidal particles are protein-coated lipid droplets. Emulsions containing protein-polysaccharide coated lipid-droplets can be prepared using a simple procedure (8). A “primary emulsion” that contains lipid droplets stabilized by a layer of protein molecules is formed by homogenizing an oil phase and an aqueous phase containing protein. A “secondary emulsion” is then formed by adsorbing an oppositely charged polysaccharide onto the protein-stabilized droplet surfaces so that each droplet is covered by a protein-polysaccharide coating. As mentioned earlier, these two-component interfacial coatings can provide lipid droplets with improved stability to environmental stresses such as thermal processing, ionic strength, pH, freezing and dehydration (8, 63, 127, 214, 216). In addition, the ability to rationally control the composition and physicochemical characteristics of the coatings can be used to develop smart delivery systems with controlled or triggered release properties (53). One of the major challenges facing the widespread utilization of this approach by industry is that these emulsions are

highly susceptible to flocculation during their preparation, and it is important to establish the optimum formulation and preparation conditions in order to prepare stable systems (8, 306).

The purpose of the present study was to examine the possibility of increasing the amount of protein present within protein-polysaccharide coatings surrounding the lipid droplets, which has a number of potential practical benefits. First, it has been shown that many proteins are highly effective at protecting emulsified lipids against oxidation, particularly when they are in close proximity to the lipids (5, 84-86, 307-309). Consequently, increasing the protein load within the interfacial coating may be an effective way of protecting emulsified lipids (such as ω -3 oils) from oxidation. Second, it may be possible to reduce the density contrast between protein-polysaccharide coated particles and the surrounding aqueous phase thereby reducing the creaming velocity, since proteins have a higher density than oil, *i.e.*, the protein would act as a natural weighting agent. In this study, we compared two methods based on electrostatic deposition for increasing the protein concentration within the coatings surrounding lipid droplets: (i) adsorbing protein-polysaccharide complexes (rather than pure polysaccharides) to protein-coated lipid droplets; (ii) adsorbing additional protein to preformed protein-polysaccharide-coated lipid droplets. We also examined the possibility of cross-linking the globular proteins in the adsorbed layer by thermal treatment, so as to increase the stability of the coatings to dissociation when the electrostatic attraction between the protein and polysaccharide molecules is weakened. It is well known that β -lactoglobulin molecules will unfold and aggregate through

hydrophobic and disulfide bonds when they are heated above their thermal denaturation temperature ($T_m \approx 70\text{ }^\circ\text{C}$).

7.3 Materials and Methods

7.3.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Pectin extracted from citrus fruit was purchased from Sigma Chemical Company (Lot # 016K0713, St. Louis, MO). The degree of esterification (DE) of the pectin was reported to be 60% by the supplier. Corn oil was purchased from a local supermarket and used without further purification. Corn oil was purchased from a food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN, USA) and used without further purification. The manufacturer reported that the corn oil contained approximately, 14.3, 28.6 and 57.1 wt% of saturated, monounsaturated and polyunsaturated fats, respectively. Other chemicals used in this study were analytical grade and purchased from the Sigma Chemical Co. (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Iowa) was used for the preparation of all solutions. All the experiments were carried out at ambient temperature ($24.1 \pm 8\text{ }^\circ\text{C}$).

7.3.2 Solution Preparation

An emulsifier solution containing 0.5 wt% β -Lg was prepared by dispersing powdered β -Lg into 5 mM Na phosphate buffer (pH 7.0) and stirring for at least two hours to ensure complete hydration. A 1 wt% pectin solution and a 1 wt% β -Lg solution were prepared by dispersing powdered pectin or β -Lg into 5 mM Na phosphate buffer and stirring for at least four hours to ensure complete hydration.

7.3.3 B-Lg/pectin solution preparation

A 0.1 wt% β -Lg solution (5 mM Na phosphate buffer), a 0.1 wt% pectin solution (5 mM Na phosphate buffer), and a mixture of β -Lg solution (0.1 wt% in 5 mM Na phosphate buffer) and pectin solution (0.1 wt% in 5 mM phosphate buffer) were prepared at pH 7. The mixture of β -Lg solution and pectin solution (BP solution) was stirred for 1 hr before potentiometric titration (pH titration). The pH of three solutions was decreased from pH 7 to 3, while 3 mL aliquot of the solutions was taken every 0.5 pH unit for further turbidity and ζ -potential measurements.

7.3.4 Emulsion Preparation

A stock emulsion was prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.25 wt% β -Lg, pH 7.0) with a high-speed blender (Tissue tearor, Biospec products Inc., Bartlesville, OK) for 2 min followed by 5 passes through a microfluidizer (Model M-110L, Microfluidics Co., Newton, MA) at 9,000 psi. The stock emulsion was diluted with pectin solution (pH 7, 5 mM Na phosphate buffer) or buffer solutions (pH 7, 5 mM Na phosphate buffer) to prepare the following samples at pH 7.

1° - The stock emulsion was diluted with buffer solution and then the pH of the emulsion was adjusted to pH 4 or 3.5 using HCl.

1° -P: The stock emulsion was diluted with pectin solution and then the pH of the emulsion was adjusted to pH 4 or 3.5 using HCl.

1° -BP: The stock emulsion was diluted with BP solution stirred for 1 hr and then the pH of the emulsion was adjusted to pH 4 or 3.5 using HCl.

Titration method: The pH of the secondary emulsion containing the droplets (0.2 wt%) stabilized by β -Lg and pectin (0.2 wt%) referred to 1°-P was adjusted to pH 4 or 3.5 using HCl and then the β -Lg solution (pH 4 or 3.5 5 mM Na phosphate buffer) was added to the secondary emulsion to give the final compositions of the droplet, pectin, and β -Lg concentration, 0.1 wt%, 0.1 wt%, and 0~0.5 wt%, respectively.

For heated samples, the resulting emulsions (1°, 1°-P, and 1°-BP) were transferred to screw-capped test tubes and heated at 85 °C for 20 min followed by cooling to room temperature.

7.3.5 Turbidity measurement

The turbidity of β -Lg solutions, pectin solutions, and BP solutions taken during pH titration was measured after 24 hr with a UV/visible spectrophotometer at 600 nm (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK). 5 mM Na phosphate buffer was used a blank.

7.3.6 Determination of free protein and polysaccharide concentrations

After 24 hr storage, emulsions were transferred to centrifugal filters (Amicon ultra, 100,000 Mw, Millipore corp., Billerica, MA) and centrifuged (Centrifuge centrifuge,

Fisher Scientific, Dubuque, Iowa) at 1000 g for 20 min. The serum phases were collected and then used for further analysis of the free protein and polysaccharide concentration.

The free protein concentration in the serum phase was determined by a modified Lowry method (310). The reaction solution (The mixture of Solution A-0.5 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1 % $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, and Solution B- 2% Na_2CO_3 and 0.4% NaOH) was added to the serum phase (0.5 mL) of each sample followed by the addition of the Folin-Ciocalteu reagent. The absorbance of the solutions including standard solutions (β -Lg) was measured at 650 nm by a UV/visible spectrophotometer (Shimadzu UV-2101, Shimadzu Scientific Instruments, Columbia, MD).

The free polysaccharide concentration in the serum phase was obtained by a phenol-sulfuric acid method (311). The reaction solutions (5% phenol solution and sulfuric acid) were added to the serum phase (1 mL) followed by the mixing and standing. The absorbance was measured at 485 nm in a UV/visible spectrophotometer (Shimadzu UV-2101, Shimadzu Scientific Instruments, Columbia, MD). A calibration curve was produced using citrus pectin as the standard.

7.3.7 ζ -Potential measurement and particle size analysis

To determine the electrical charge on lipid droplets and particle mean diameter of emulsions, the undiluted samples were placed into the disposable capillary cell of a dynamic light scattering (DLS, Zetasizer NanoZS, Malvern Instruments, Worcs., UK). This instrument determines the electrical charge (ζ -potential) on the particles in an emulsion by measuring the direction and velocity of particle movement in an applied electric field. The ζ -potential measurements are reported as the average and standard deviation of measurements made on two freshly prepared samples, with twelve readings

made per sample. The Smoluchowsky mathematical model was used to calculate the electrophoretic mobility measurement into ζ -potential values. The mean particle diameter measured by DLS reported as Z-average size, which are calculated from the signal intensity and converted to a size using the dispersant viscosity and some instrument constants.

7.3.8 Statistical Analysis

Experiments were performed twice or triple freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements.

7.4 Results and Discussions

7.4.1 Characterization of protein, polysaccharide, and protein/polysaccharide solutions

Initially, we characterized the pH-dependence of the ζ -potential and turbidity of each of three different solutions: β -Lg solution (0.1 wt%); pectin solution (0.1 wt%); and, β -Lg/pectin (BP) solutions (0.1 wt% β -Lg and 0.1 wt% pectin) (Figure 7.1a and 7b). The measurement of both the ζ -potential and turbidity was carried out after 24 hr storage. The electrical charge of β -Lg solution changed from negative to positive as the pH of protein solution was decreased from 7 to 3 with a zero charge around pH 4.7. The isoelectric point (pI) of β -Lg has been reported to be around 4.7 to 5.2 (242, 243, 262) It can be explained by the fact that the pH moved from above to below the pI of β -Lg molecules. Pectin was negatively charged over the whole pH ranges studied due to its low pKa value (≈ 3.5) (245). One might expect that the electrostatic interaction between oppositely

charged protein and polysaccharide would occur over the pH ranges of 5 to 3.5 leading to the formation of protein/polysaccharide complex. The possibility of the formation of protein/polysaccharide complex was examined by mixing a mass ratio of 1:1 of β -Lg solution (0.1 wt%) with pectin solution (0.1 wt%). The electrical charge of BP solution became less negative with decreasing the pH from 7 to 3 and the magnitude of the electrical charge of BP solution was between β -Lg solution alone and pectin solution alone indicating of the formation of soluble or insoluble complexes between protein and polysaccharide. The electrical charge of BP solution was less negatively charged than pectin solution at pH ranges between pH 6 and 4 and became the similar values as pectin solution at around pH 4 and 3.5. It is well known that that the formation of soluble complexes occurs when β -Lg and pectin carry the same net charge, near the pI of the protein due to the existence of small patches onto the protein and its ability of charge regulation of the protein (72, 312-314). An appreciable decrease in ζ -potential was observed at pH 3, which can be attributed that pectin molecules approach their pKa and start losing negative charge at this pH leading to neared neutralization of pectin molecules with β -Lg molecules.

Turbidity measurements of the solutions were also carried out to follow the formation of the protein/polysaccharide complex observed the ζ -potential measurement and get information of phase transition of BP solution. Turbidity of the β -Lg solution and the pectin solution remained the almost same values over a whole pH ranges studied except a little increase of turbidity at pH 4.5 of the β -Lg solution. It can be the fact that the pI of the β -Lg solution was around pH 4.7 as observed with the ζ -potential measurement and therefore β -Lg molecules have low net charge at this pH promoting

aggregations between β -Lg molecules. However, there was no visible change of the turbidity at this pH, it may be due to the low concentration of the protein solution (0.1 wt%). Turbidity of the BP solution increased as the pH of the solutions was decreased from pH 7 to 3 and the transition of the solution from transparent (pH 7-5) to opaque (pH 3) through turbid (pH 4.5-3.5) was clearly observed. There was no phase separation of the BP solution even at pH 3 after 24 hr. It can be explained by the fact that complete charge neutralization didn't occur at this pH and a mass ratio of 1:1 of β -Lg and pectin (Figure 7.1a). Turbidity of the BP solution increased as the pH of the solutions was decreased from pH 7 to 3 and the transition of the solution from transparent (pH 7-5) to opaque (pH 3) through turbid (pH 4.5-3.5) was clearly observed. There was no phase separation of the BP solution even at pH 3 after 24 hr. It can be explained by the fact that complete charge neutralization didn't occur at this pH and a mass ratio of 1:1 of β -Lg and pectin (Figure 7.1a).

There were distinctive differences in the electrical charge, phase transition, and turbidity of BP solution with the changes of pH. Therefore, we investigated the possibility of the deposition of BP solution to an emulsion containing the lipid droplets coated with β -Lg and the improvement of the resulting emulsion stability to aggregation.

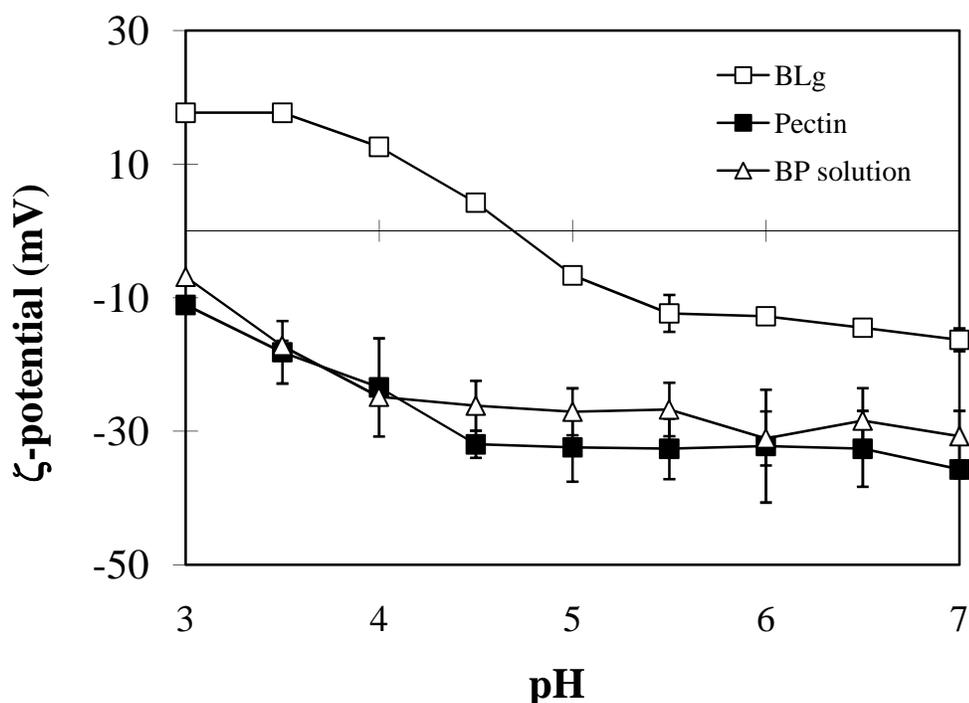


Figure 7.1a. Dependence of the electrical charge of β -Lg solution (0.1 wt% in 5 mM Na phosphate buffer), pectin solution (0.1 wt% in 5 mM Na phosphate buffer) and β -Lg/pectin solution (BP solution, 0.2 wt% in 5 mM Na phosphate buffer) on pH.

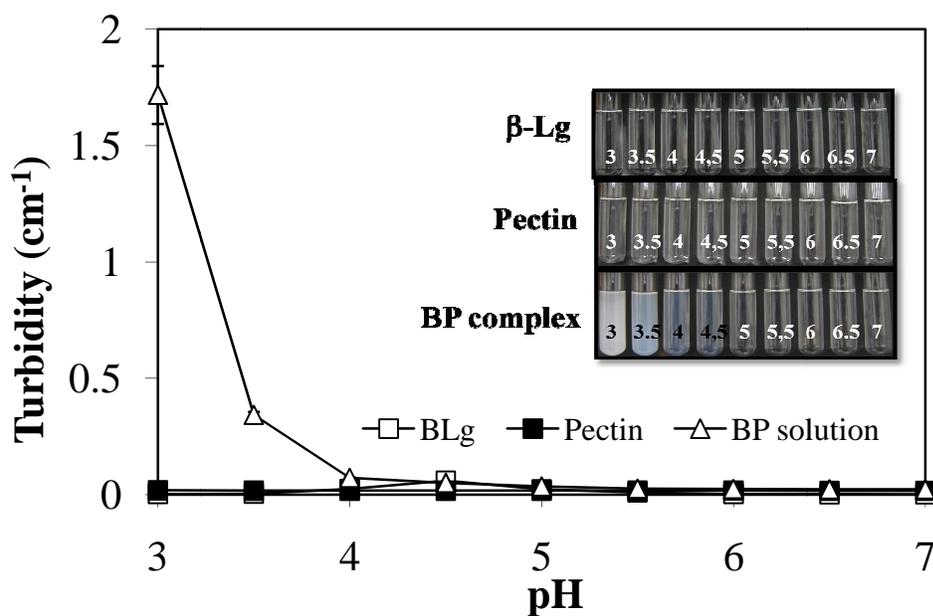


Figure 7.1b. Dependence of the turbidity of β -Lg solution (0.1 wt% in 5 mM Na phosphate buffer), pectin solution (0.1 wt% in 5 mM Na phosphate buffer) and β -Lg/pectin solution (BP solution, 0.2 wt% in 5 mM Na phosphate buffer) on pH.

7.4.2 Adsorption of pectin or pectin/ β -Lg to protein-coated lipid droplets

We examined the impact of pH on the adsorption of polysaccharides or BP solutions to the surfaces of protein-coated lipid droplets. The electrical charge, particle size, and gravitational separation of β -Lg stabilized oil-in-water emulsions (0.0045 wt% β -Lg; 0.1 wt% oil) was measured when the pH was adjusted from 7 to 3 for three systems: (1°) no additional biopolymer; (1°-P) 0.1 wt% pectin; (1°-BP) BP solution (0.1 wt% pectin + 0.1 wt% β -Lg). We selected the droplet concentration of an emulsion in this study to 0.1 wt%, since the electrical charge and droplet diameter of the 0.1 wt% emulsion can be measured without any further dilution. The electrical charge on the protein-coated droplets (1°) went from highly negative at pH 7 to highly positive at pH 3, with a point of zero charge around pH 5 (Figure 7.2a), which is due to the fact that pI of the adsorbed β -Lg is around pH 5. The particle charge in the emulsions containing 0.1 wt% pectin (1°-P) was negative at high pH values, which can be attributed to the fact that both pectin and β -Lg coated lipid droplets are negative around neutral pH. The particles remained negatively charged across the entire pH range studied when the pH was reduced, which can be attributed to adsorption of anionic pectin molecules to cationic the protein-coated lipid droplets. The pH-dependence of the particle charge on the emulsions containing BP solution (1°-BP) was fairly similar to that containing only 0.1 wt% pectin, but it was slightly less negative (Figure 7.2a). We postulate that polysaccharide-protein complexes adsorbed to the lipid droplet surfaces in this case, rather than just polysaccharide molecules.

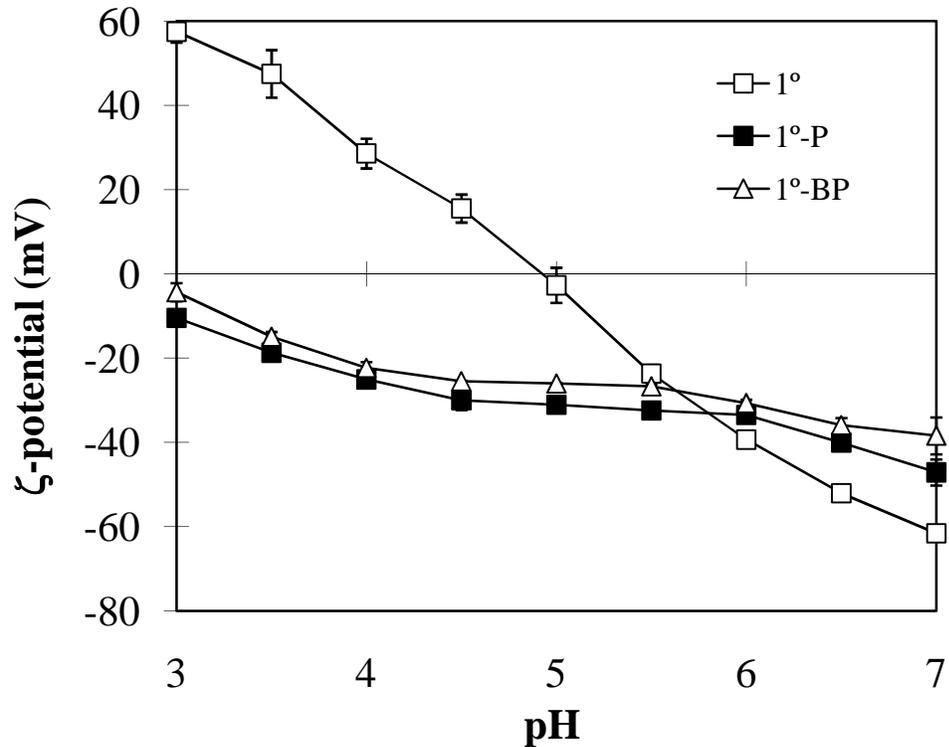


Figure 7.2a. Dependence of the ζ -potential of primary emulsion (1°, 0 wt% polysaccharide), primary emulsion containing pectin molecules (1°-P, 0.1 wt% polysaccharide), and primary emulsion containing β -Lg/pectin (BP) solution (1°-BP, 0.2 wt% polymer solution) on pH: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

The stability of the emulsions to particle aggregation and gravitational separation was highly dependent on solution pH (Figures 7.2b and 7.2c). The primary emulsion (1°) containing no additional biopolymer was highly unstable to droplet aggregation and creaming near the isoelectric point of the adsorbed proteins (pH \approx 5) due to the relatively weak electrostatic repulsion between the droplets. The emulsion containing only added pectin (1°-P) was stable to droplet aggregation and creaming across the entire pH range studied. It can be attributed to the ability of the adsorbed pectin layer to increase the electrostatic and steric repulsion between the droplets, as well as reducing the van der Waals attraction due to its molecular structure of a block copolymer with anionic “linear

regions” and neutral “hairy regions” (79, 264, 265). The emulsion containing BP solution (1°-BP) was stable to droplet aggregation and creaming from pH 7 to 3.5, but was unstable at pH 3.0 (Figures 7.2b and 7.2c). A likely reason for the instability of the droplets to aggregation at pH 3 is that their electrical charge was relatively low (Figure 7.2a), so that the electrostatic repulsion may have been insufficient to prevent droplet aggregation. There was no phase separation of the BP solution at pH 3, but interestingly the 1°-BP emulsions exhibited sedimentation rather than creaming at pH 3.0. It indicated that the protein-polysaccharide coated lipid particles were heavier than the surrounding aqueous phase. This result demonstrates the potential of creating protein-polysaccharide coated lipid particles that are density matched to the surrounding aqueous phase.

7.4.3 Adsorption of additional protein onto protein-polysaccharide coated droplets

In the previous section we showed that additional protein could be incorporated into biopolymer coatings by adsorbing protein-polysaccharide complexes onto protein-coated lipid droplets. In this section, we examine the possibility of directly adsorbing protein to the surfaces of preformed protein-polysaccharide coated droplets. Emulsions containing lipid droplets coated by protein-polysaccharide layers were formed by mixing an oil-in-water emulsion (0.045 wt% β -Lg; 1 wt% corn oil) with a pectin solution (0.1 wt%) at pH 7, and then reducing the pH to either 3.5 or 4.0 to promote pectin adsorption. The electrical charge, particle size, and gravitational separation stability of the emulsions was then measured when increasing amounts of β -Lg were titrated into these emulsions (Figure 7.3).

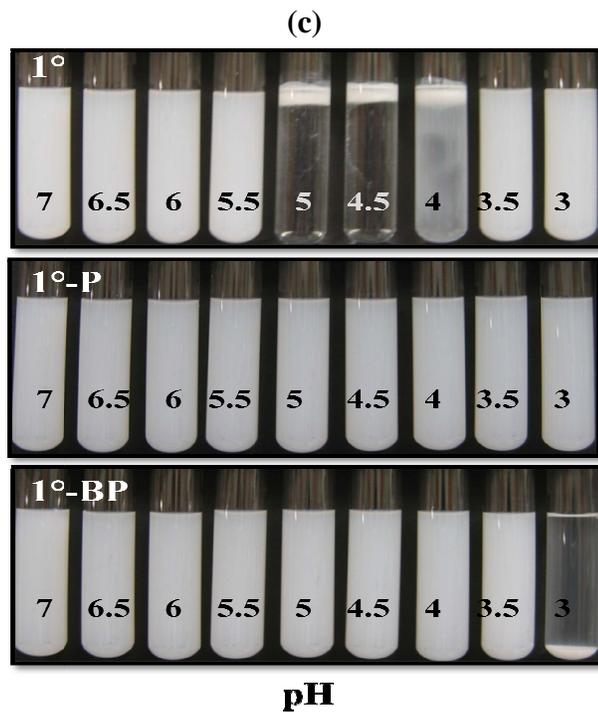
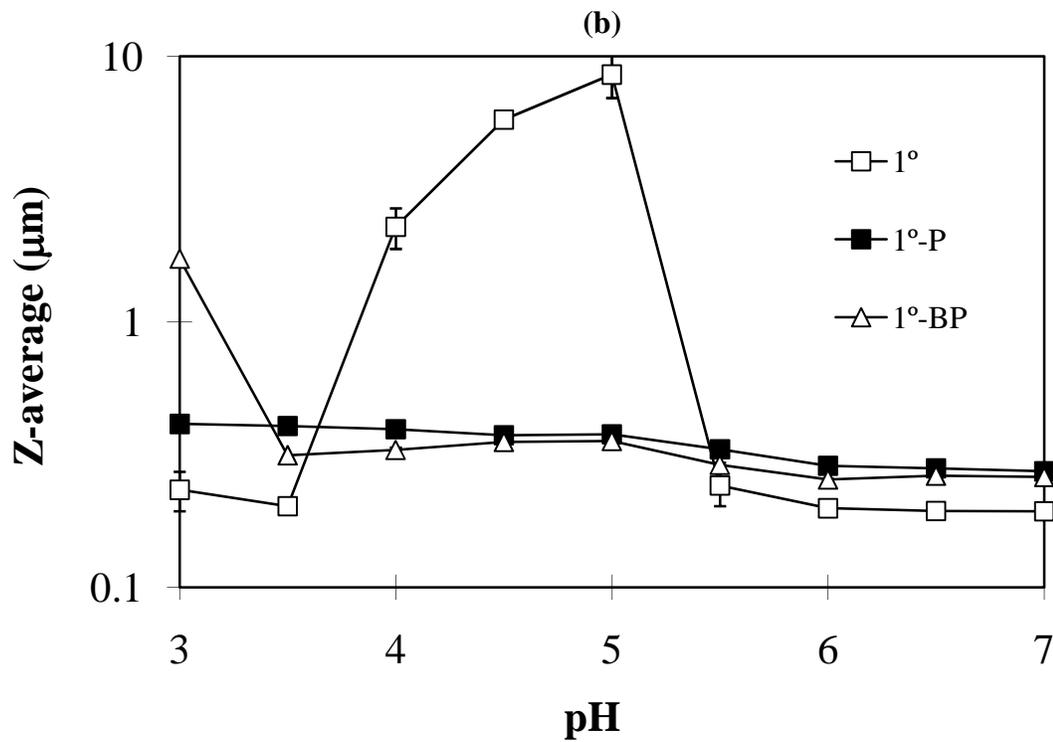


Figure 7.2b-c. Dependence of the mean diameter (b) and photographs (c) of primary emulsion (1°, 0 wt% polymer), primary emulsion containing pectin molecules (1°-P, 0.1 wt% pectin), and primary emulsion containing β -Lg/pectin (BP) solution (1°-BP, 0.2 wt% polymer solution) on pH: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

In the absence of additional protein, the ζ -potential of the emulsions (1°-P) were -19 and -26 mV at pH 3.5 and 4.0, respectively, reflecting the fact that an anionic pectin layer was adsorbed around the cationic protein-coated lipid droplets. The electrical charge was initially less negative at pH 3.5 than pH 4.0 because pectin loses some negative charge ($pK_a \approx 3.5$) and β -Lg ($pI \approx 5$) gains some positive charge as the pH is decreased. There was a progressive decrease in the negative charge on the droplets at both pH values when the β -Lg concentration was increased, indicating that protein adsorbed to the droplet surfaces (Figure 7.3a). Eventually the ζ -potential reached a value that was close to zero, with the amount of protein required to reach this value being greater at pH 4 than pH 3.5. This observation can be attributed to the fact that there were more anionic groups present on the pectin molecules and less cationic groups present on the proteins at pH 4, and so a greater amount of protein was required to reach charge neutralization.

The emulsions were relatively stable to droplet aggregation and gravitational separation when the added protein concentration remained below a critical value, which was around 0.08 and 0.16 wt% β -Lg at pH 3.5 and 4.0, respectively (Figures 7.3b and 7.3c). The emulsions containing additional protein molecules up to 0.3 wt% (pH 3.5) and 0.4 wt% (pH4.0) showed visible sedimentation with clear serum layer and white precipitate layer, but a little sedimentation was observed at the bottom of turbid serum layer in the emulsions containing < 0.08 wt% β -Lg and < 0.16 wt% β -Lg at pH 3.5 and 4.0, respectively. We hypothesize that extensive droplet aggregation occurred in these systems when the electrical charge on the composite particles was insufficient to provide electrostatic stabilization. At high protein concentrations the emulsions were unstable to

sedimentation rather than creaming (Figure 7.3c), which again indicates that the density of the composite particles was greater than that of the surrounding aqueous phase.

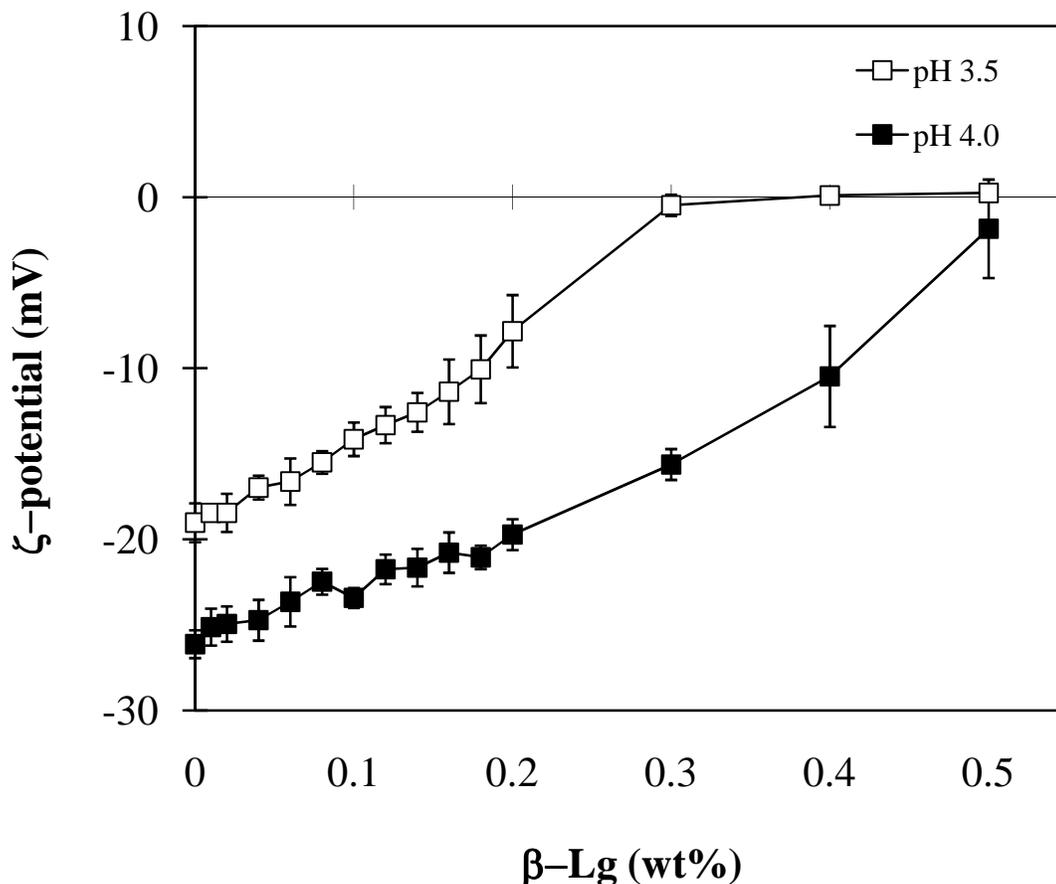


Figure 7.3a. Influence of the amount of added β -Lg molecules on the electrical charge of secondary emulsion containing the 0.1 wt% corn oil coated with 0.0045 wt% β -Lg and 0.1 wt% pectin molecules in 5 mM Na phosphate buffer at pH 3.5 and pH 4.

These results indicate that the protein load in biopolymer coatings can be increased by adding protein to an emulsion containing lipid droplets already coated by a protein-polysaccharide layer. The amount of protein that can be incorporated into the coatings, while still keeping the emulsions stable, depends on the initial charge characteristics of the droplets. We found that the protein concentration could be

increased from around 0.0045 wt% β -Lg per 0.1 wt% oil, to around 0.08 or 0.16 wt% β -Lg at pH 3.5 and 4.0, respectively. This corresponds to around 18- to 36-fold increase in protein concentration.

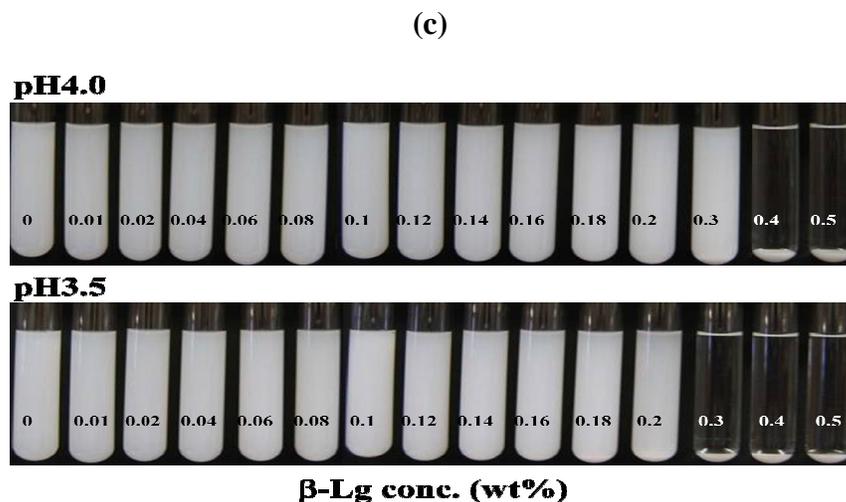
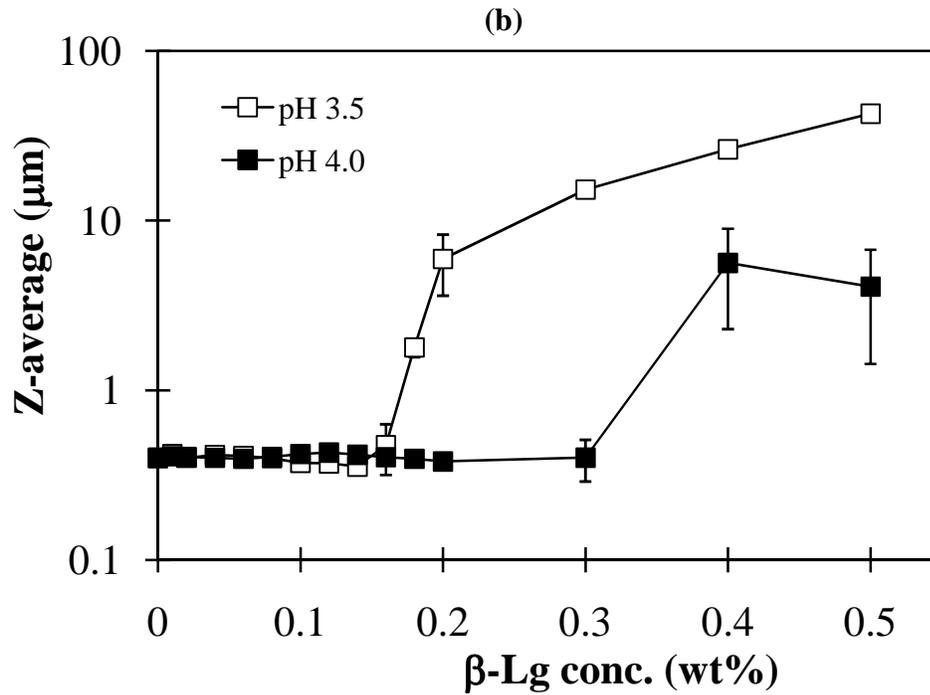


Figure 7.3b-c. Influence of the amount of added β -Lg molecules on the mean diameter (b) and photographs (c) of secondary emulsion containing the 0.1 wt% corn oil coated with 0.0045 wt% β -Lg and 0.1 wt% pectin molecules in 5 mM Na phosphate buffer at pH 3.5 and pH 4.

7.4.4 Thermal treatment of adsorbed proteins

A potential limitation of lipid droplets surrounded by protein-polysaccharide complexes is that these complexes are held together by electrostatic interactions. Consequently, any changes in solution conditions that weaken these interactions (*e.g.*, pH or ionic strength) may lead to dissociation and detachment of the adsorbed biopolymer coatings. We therefore examined the possibility of cross-linking the proteins in the adsorbed layer by thermal treatment. It is well known that β -Lg molecules unfold when they are heated above their thermal denaturation temperature ($T_m \approx 70$ °C), which can lead to extensive protein aggregation through hydrophobic and disulfide bonds (315-318). Two different methods were used to prepare the emulsions:

- (i) *Protein-titration method.* An emulsion containing β -Lg stabilized droplets and pectin was prepared at pH 7, then the pH was reduced to pH 4 to induce pectin adsorption. Finally, β -Lg was titrated into this emulsion to a level of either 0 or 0.04 wt%.
- (ii) *Complex adsorption method.* An emulsion containing β -Lg stabilized droplets, pectin and additional β -Lg (0 or 0.04 wt%.) was prepared at pH 7, then the pH was reduced to pH 4 to induce complex adsorption.

These emulsions were then heated (85 °C, 20 min) to denature and aggregate the proteins in the coatings. We found that neither the preparation method nor the heat treatment had any appreciable effect on the particle charge, particle aggregation or gravitational separation of the emulsions (Table 7.1). These experiments showed that

stable composite particles could be formed that contained increased levels of thermally denatured proteins in the interfacial layer.

Table 7.1. Impact of preparation method, additional β -Lg and heat treatment (85 °C for 20 min) on the particle charge and mean diameter of emulsions stabilized by protein-polysaccharide complexes at pH 4. No creaming was observed after one day in any of the emulsions.

| Added β -Lg (wt%) | ζ (mV) | Mean diameter (μm) |
|----------------------------------|-----------------|---------------------------------|
| <i>Titration Method</i> | | |
| 0% (unheated) | -26.9 ± 0.9 | 0.400 ± 0.020 |
| 0% (heated) | -28.4 ± 0.4 | 0.386 ± 0.002 |
| 0.04% (unheated) | -25.3 ± 0.3 | 0.391 ± 0.004 |
| 0.04% (heated) | -26.4 ± 0.3 | 0.389 ± 0.002 |
| <i>Complex Adsorption Method</i> | | |
| 0% (unheated) | -26.9 ± 0.9 | 0.400 ± 0.020 |
| 0% (heated) | -28.4 ± 0.4 | 0.386 ± 0.002 |
| 0.04% (unheated) | -27.4 ± 0.1 | 0.372 ± 0.011 |
| 0.04% (heated) | -26.7 ± 0.4 | 0.376 ± 0.010 |

We therefore prepared oil-in-water emulsions containing protein-stabilized lipid droplets (0.0045 wt% β -Lg; 0.1 wt% oil) surrounded by either pectin alone (0.1 wt%) or pectin (0.1 wt%) + β -Lg (0.1 wt%) with a complex adsorption method in the rest of this study. We also selected this higher level of β -Lg (0.1 wt%) than previous one (0.04 wt%) to prepare a protein-rich layer around the lipid droplets coated with β -Lg and pectin

layers, where the electrical charge on the droplets coated with pectin and an additional β -Lg layer was enough negatively charged ($>|20|$ mV) to provide the electrostatic repulsion and stable to sedimentation or creaming (23). The electrical charge and mean diameter of all of emulsions in the absence of pectin or pectin/ β -Lg (1°) and in the presence of pectin (1° -P) or pectin/ β -Lg (1° -BP) was similar after heat treatment at pH4 (Figure 7.4a and 7.4b) as the result above (Table 7.1). The droplets in the primary emulsion was positively charged at pH 4 due to the fact that the adsorbed β -Lg molecules was below its isoelectric point ($pI \approx 5.2$) (319). The unheated and heated primary emulsion were both slightly unstable to aggregation because the pH was fairly close to the isoelectric point of the adsorbed β -Lg so that there was not a sufficient strong electrostatic repulsion between the droplets to prevent aggregation. It is interesting to note that the mean particle diameter of the primary emulsion with heat treatment was smaller than unheated one. It is likely that the electrical charge of the primary emulsion was slightly above $|20|$ mV and it may still provide some electrostatic repulsion between the droplets coated with β -Lg and filamentous aggregates of the adsorbed β -Lg molecules on the lipid droplets (317). It is well known that filamentous aggregates formed during the heating of β -Lg molecules are tend to be smaller than particulate aggregates (315, 318, 320-322). On the other hand, all of the emulsions containing pectin and pectin/ β -Lg were stable to droplet aggregation and they were negatively charged due to the adsorption of the pectin molecules or pectin/ β -Lg molecules onto the protein-coated droplets providing the steric and electrostatic repulsion between the droplets by increasing the thickness and charge of the interfaces.

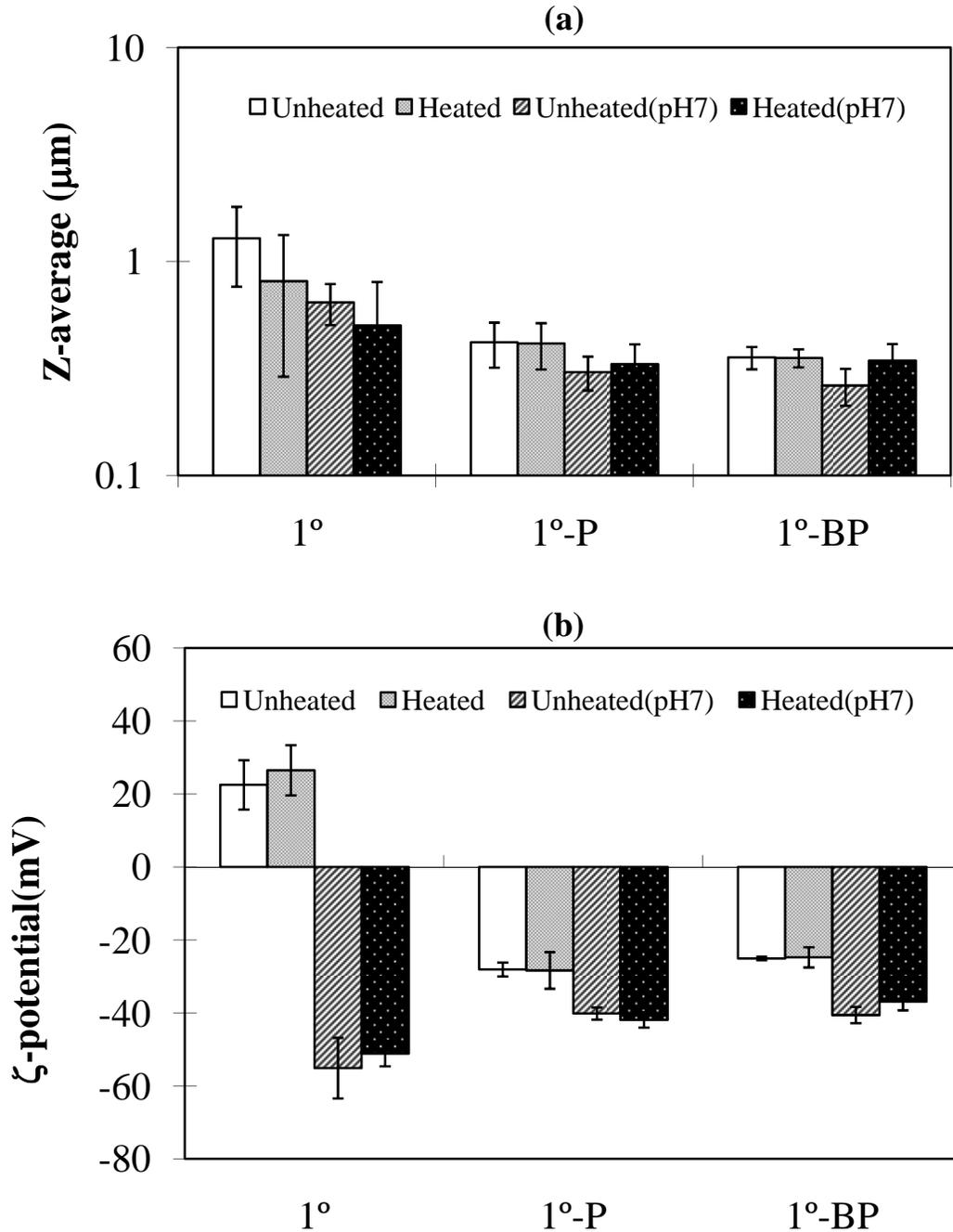


Figure 7.4a-b. Dependence of the mean diameter (a) and ζ -potential (b) of primary emulsion (1°, 0 wt% polymer), primary emulsion containing pectin molecules (1°-P, 0.1 wt% pectin), and primary emulsion containing β -Lg/pectin (BP) solution (1°-BP, 0.2 wt% polymer solution) on thermal treatment (85 °C, 20 min) : 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

There were no significant differences in the mean particle diameter and ζ -potential with heating at pH 4. It suggests that heating did not cause the detachment of the adsorbed layers of pectin or pectin/ β -Lg from the droplet surfaces.

We hypothesized that thermal treatment of the emulsions would cross link the protein molecules within the coatings around the droplets, which would prevent the coating from dissociating when the pH was changed. Therefore, the pH of all emulsion was adjusted to pH 7 to examine whether the adsorbed polysaccharide or polysaccharide/protein layers remained undetached from the lipid droplets. All of emulsions were negatively charged including the primary emulsion due to the fact that the adsorbed β -Lg molecules onto the lipid droplets were at above its isoelectric point. There was no significant difference in the electrical charge in three different emulsions with heating. The mean diameter of the primary emulsion was much bigger than initial primary emulsion at pH 7 ($0.194 \pm 0.01 \mu\text{m}$ in previous section). It suggests that there were still droplet aggregations in the primary emulsion in both unheated and heated primary emulsions. At pH 7, one might expect that electrostatic interaction would not take place between negatively charged the protein-coated lipid droplet and pectin or pectin/ β -Lg. There was a slightly increase in the particle size ($0.3 \mu\text{m}$) of the unheated primary emulsion containing pectin (1°-P) compared to the initial emulsion ($0.274 \mu\text{m}$). On other hand, there was a decrease in the mean diameter of unheated primary emulsion pectin/ β -Lg (1°-BP) ($0.360 \mu\text{m}$) after increasing the pH to 7 ($0.260 \mu\text{m}$), however, it was fairly similar to initial emulsions ($0.261 \mu\text{m}$). It indicates that the adsorbed pectin/ β -Lg layers onto the protein-coated lipid droplets were detached from the droplet surfaces. The mean diameter of heated emulsions containing polysaccharide/protein complex remained the

same after adjusting the pH to pH 7. We propose that the cross-linking of adsorbed protein layers by heating prevents the coating from dissociating when the pH was changed.

7.4.5 Influence of thermal treatment of the emulsions on the free protein and polysaccharide concentration.

To support a proposition above, we measured the concentration of free protein and polysaccharide in the aqueous phase when the emulsions were prepared at pH 4 and the emulsions were adjusted from pH 4 to pH 7.

Little free protein was detected in the primary emulsions (1°) or the emulsions containing only pectin (1°-P) either before or after heat treatment at pH 4, which indicated that the most of the initial protein added prior to homogenization was located at the oil-water interface. Free protein was detected in the aqueous phase when the emulsions contained an additional 0.1 wt% β -Lg (1°-BP) (Figure 7.5a). We found \approx 0.0018 wt% (18 μ g/mL) and \approx 0.0012 wt% (12 μ g/mL) β -Lg in the aqueous phase for the unheated and heated emulsions, respectively. The decrease in free protein after heating suggests that there was an increase in the amount of protein present within the biopolymer coatings, which suggests that most of protein molecules may adsorb onto the lipid droplets coated with protein and polysaccharide layers.

There was dramatic increase in free protein when the emulsions containing an additional protein were adjusted to pH 7 (Figure 7.5a). We found \approx 0.0404 wt% (404 μ g/mL) and \approx 0.0369 wt% (369 μ g/mL) β -Lg in the aqueous phase for the unheated and heated emulsions, respectively. Only 60% of protein molecules remained adsorbing onto the surface of the lipid droplets. The slightly decrease in free protein after heating

suggests that there was an increase in the amount of protein present within the biopolymer coatings, which suggests that protein cross-linking may have decreased its detachment at neutral pH.

As expected, no pectin was detected in the aqueous phase of the primary emulsions (1°) since they contain no added polysaccharide (Figure 7.5b). The emulsions containing added pectin (1°-P) and added pectin/ β -Lg (1°-BP) both had relatively high concentrations of non-adsorbed pectin at pH 4 (Figure 7.5b) irrespective of heat treatment. The free polysaccharide amount of the emulsions containing pectin (1°-P) was ≈ 0.0196 wt% (196 $\mu\text{g}/\text{mL}$) and ≈ 0.0163 wt% (163 $\mu\text{g}/\text{mL}$) for the unheated and heated emulsions, respectively. Surprisingly, there was only slight increase in the free polysaccharide amount for the unheated and heated emulsion when the emulsions (1°-P) were adjusted to pH 7. It suggests that the adsorbed pectin layer onto the protein-coated lipid droplets was stable to environmental stresses such as pH changes and thermal treatment. The emulsions containing added pectin/ β -Lg (1°-BP) both unheated and heated emulsions had relatively high concentrations of non-adsorbed pectin at both pH 4 and 7 (Figure 5b). The free polysaccharide amount of the emulsions containing added pectin/ β -Lg (1°-BP) was ≈ 0.0120 wt% (120 $\mu\text{g}/\text{mL}$) and ≈ 0.0196 wt% (196 $\mu\text{g}/\text{mL}$) for the unheated and heated emulsions, respectively. The free pectin concentration in heated emulsion was higher than in unheated emulsion and the same as the unheated emulsion containing added pectin (1°-P). It suggests that the polysaccharide/protein complex may be dissociated from the lipid droplets or added protein molecules may be dissociated from the polysaccharide/protein complex and interact with the protein-coated lipid droplets with hydrophobic interaction due to the conformational change of adsorbed β -Lg

layer by heating (318, 322, 323). It can be supported by the fact that the free protein concentration of the heated emulsion containing added pectin/ β -Lg was lower than the unheated emulsion suggesting that the most of the initial protein was located at the oil-water interface. Unlike at alkaline pH, heat treatment of β -Lg molecules at acidic pH promotes the unfolding and changes in the surface hydrophobicity through non-covalent interactions leading to monomer-dimer transitions and aggregations, not through sulphhydryl-disulphide interactions (324-326). At pH 4, it's likely that the denatured β -Lg molecules by heat treatment have been held together around the lipid droplets coated by β -Lg and pectin molecules through non-covalent interactions.

The free pectin concentration in the emulsion containing added polysaccharide/protein complex both unheated and heated emulsion was similar when the emulsions were adjusted to pH 7. Considering both the free protein/polysaccharide concentration, it suggests that pectin and β -Lg molecules became detached from the droplet surfaces at neutral pH due to the electrostatic repulsion between the anionic pectin molecules and anionic protein-coated lipid droplets at this pH. Thermal treatment of the proteins slightly improved the detachment of the adsorbed polysaccharide/protein layers. The increase pH of the emulsion to pH 7 would promote the electrostatic repulsion between the denatured β -Lg molecules and pectin molecules. In consequence, it could lead to the rearrangement of molecules around the lipid droplets coated by β -Lg molecules after dissociating of pectin molecules and protein-protein interactions could take place the protein-protein interaction between β -Lg molecules on the surface of lipid droplets and β -Lg molecules in the continuous phase due to the increased hydrophobicity of heat denatured β -Lg molecules.

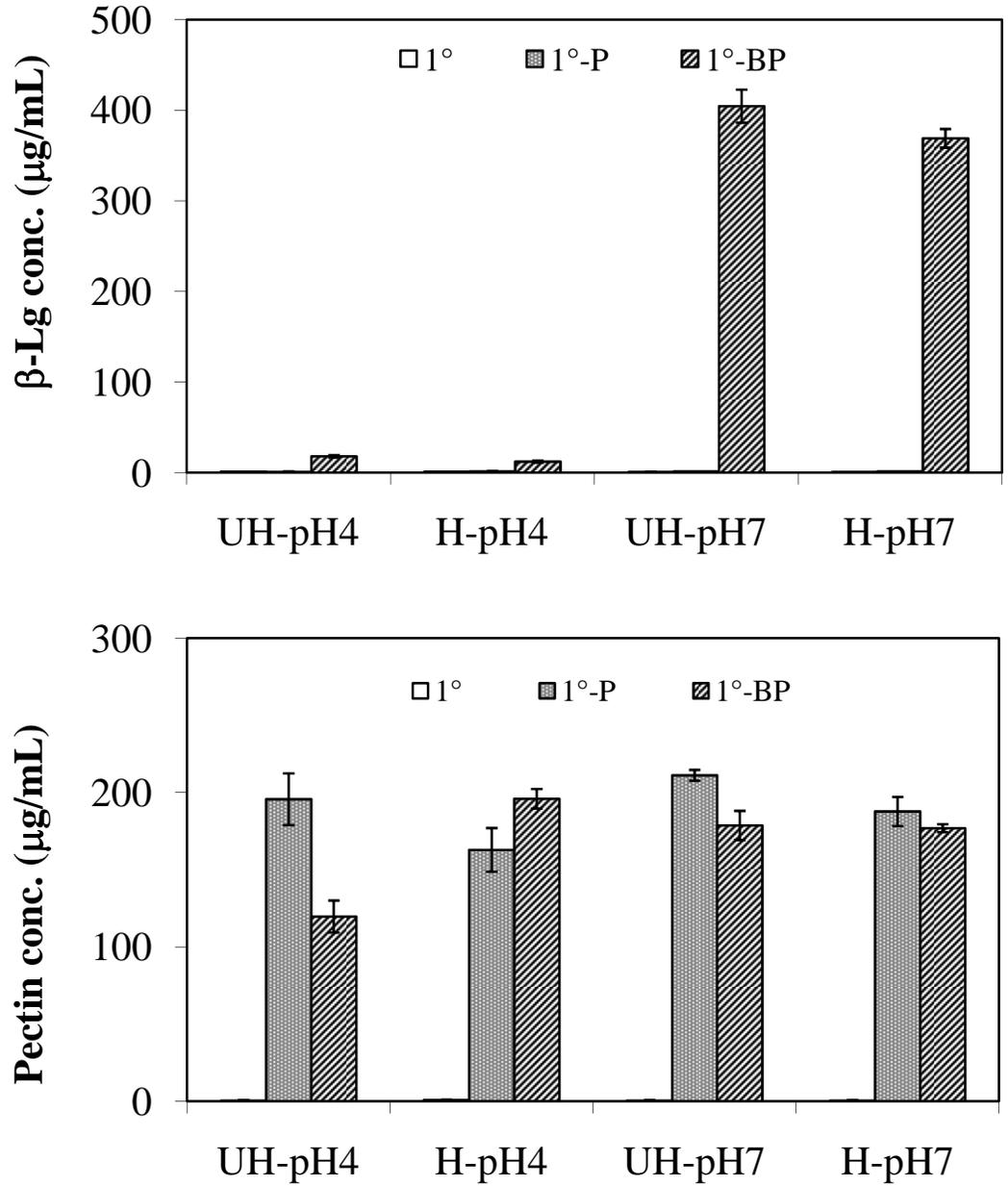


Figure 7.5a-b. Influence of thermal heat treatment (85 °C, 20 min) on free pectin concentration (a) and polysaccharide (b) in aqueous phase of primary emulsion (1° , 0 wt% polymer), primary emulsion containing pectin molecules (1°-P , 0.1 wt% pectin), and primary emulsion containing β -Lg/pectin (BP) solution (1°-BP , 0.1 wt% pectin and 0.1 wt% β -Lg solution) at pH 7: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

7.4.6 Salt stability of emulsions containing added protein and polysaccharide molecules

It is well established that the presence of protein in continuous phase of the emulsion can inhibit the lipid oxidation through radical scavenging by aromatic and sulfur-containing amino acids (327-331). It has shown that the antioxidant activity of protein can be improved by thermal treatment (328, 329, 332). Therefore, protein-rich coating formed onto the protein or protein/polysaccharide-coated lipid droplets by heating in this study would be an ideal system for encapsulating bioactive lipids.

We found that the emulsion containing added pectin or pectin/ β -Lg molecules was stable to droplet aggregation at pH 4 and pH 7 in previous section. However, the free protein and polysaccharide concentration in aqueous phase was fairly high. Therefore, we examined the influence of salt addition on the stability of the emulsions to determine whether the cross-linking of adsorbed β -Lg layer by heat treatment would provide better stability to aggregation than unheated emulsions. Three stock emulsions (0.2 wt% droplet concentration) were prepared and then diluted with 5 mM phosphate buffer or 500 mM NaCl solution in 5 mM phosphate buffer at pH4 and 7 to give final concentration of the primary emulsion (1°), the primary emulsion containing added pectin (1°-P), and added pectin/ β -Lg (1°-BP) to 0.1 wt% droplet concentration, 0.1 wt% pectin, and 0.1 wt% β -Lg with different salt concentration (0-200 mM). Some of resulting emulsions showed the change in the pH, so the pH of the emulsions was readjusted to H 4 or 7 with adding 0.1 M NaOH or 0.1 N HCl solution. The mean diameter of the resulting emulsions was measured by dynamic light scattering and volume mean diameter was obtained (Figure 7.6a-c) as well as a visual observation of creaming (data not shown).

The primary emulsion was highly unstable to droplet aggregation at all salt concentrations at pH4 and 7 and clear creaming on the top of emulsion and phase separation at higher levels of salt (100 and 200 mM) were observed after 24 hr storage.

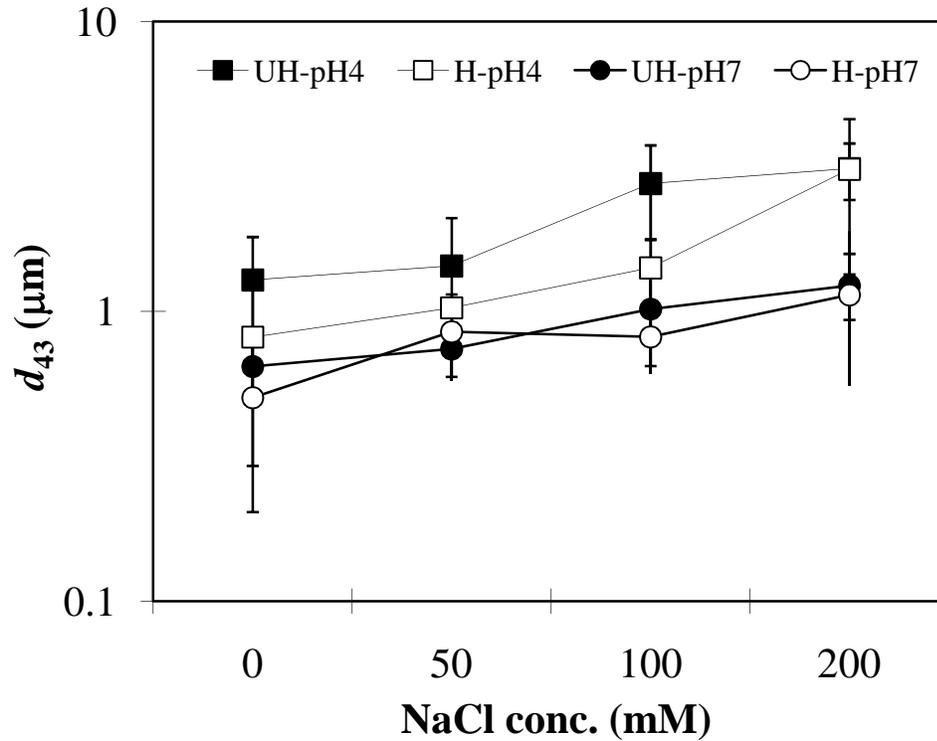


Figure 7.6a. Influence of salt addition on the volume mean diameter of primary emulsion (1° , 0 wt% polymer) at pH 4 and pH 7 in both heated (H) and unheated (UH) emulsions: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

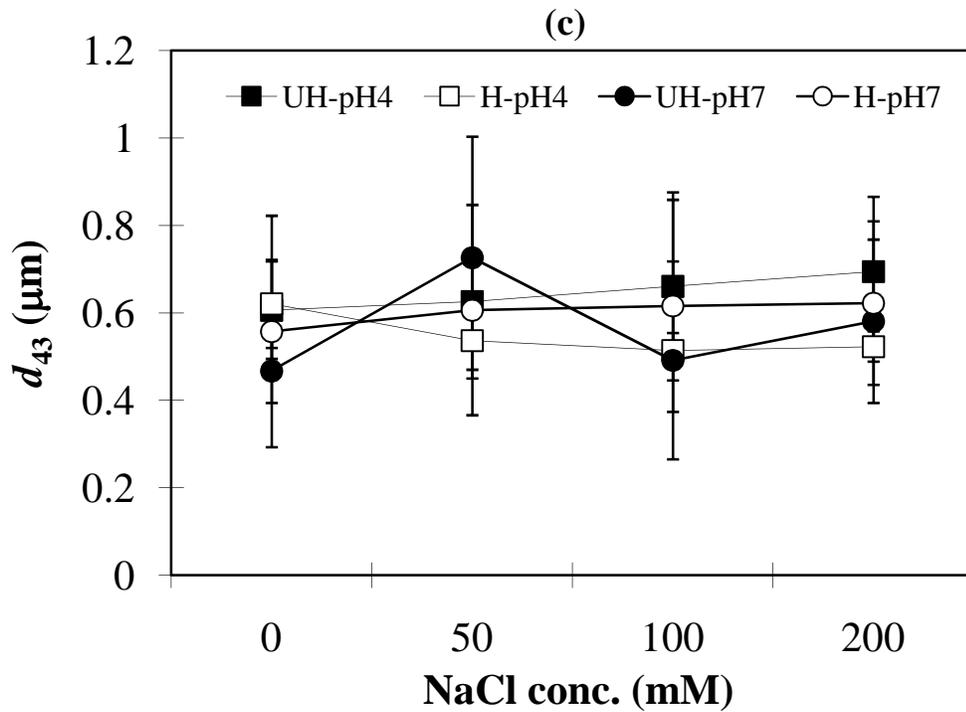
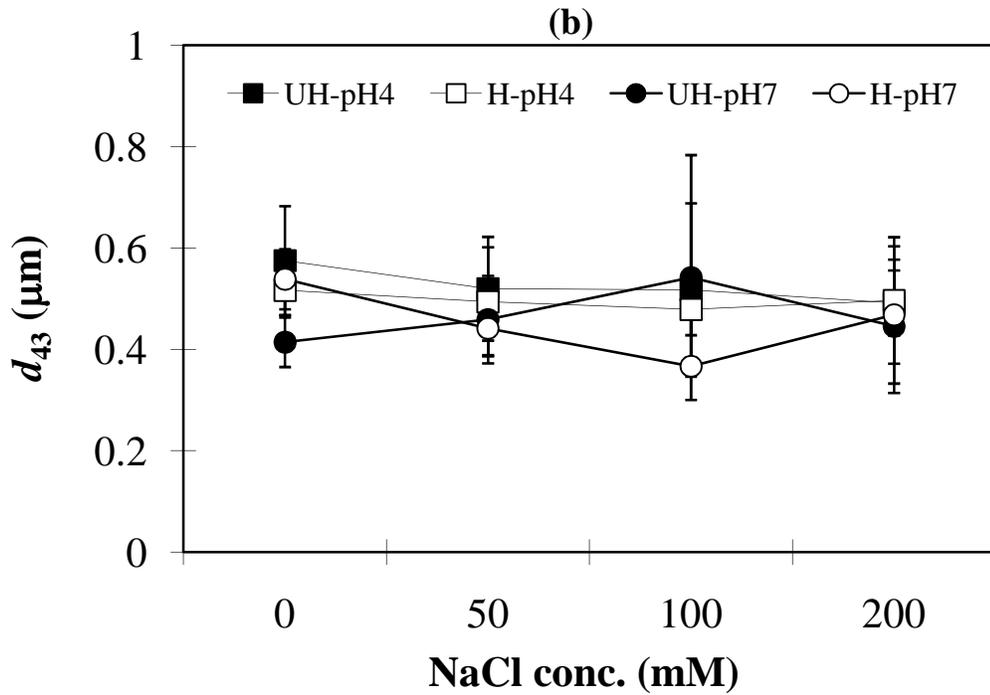


Figure 7.6b-c. Influence of salt addition on the volume mean diameter of primary emulsion containing (b) pectin solution (1°-P, 0.1 wt% pectin); (c) β -Lg/pectin (BP) solution (1°-BP, 0.1 wt% pectin and 0.1 wt% β -Lg solution) at pH 4 and pH 7 in both heated (H) and unheated (UH) emulsions: 0.1 wt% corn oil, 0.0045 wt% β -Lg, 5 mM Na phosphate buffer.

The droplet aggregations were increased gradually with increasing salt concentration due to the electrostatic screening effects of salt leading to weak electrostatic repulsion between the lipid droplets (Figure 7.6a) (23, 79, 300). The emulsion containing pectin (1°-P) or pectin and additional β -Lg (1°-BP) were highly stable to aggregation and there were no creaming and sedimentation after 24 hr in the emulsions (data not shown) at over salt concentrations studied at both pH 4 and 7 (Figure 7.6b and 7.6c). It is interesting to note that the mean diameter of the emulsion containing added pectin (1°-P) was decreased with increasing salt concentration at pH 4 in both unheated and heated emulsion. It can be explained by the fact that pectin is more densely packed on the protein coated lipid droplets due to the decreased electrostatic repulsion with increasing salt concentration (79, 333). However, the emulsions (1°-P) showed the different behavior with increasing salt levels when the pH of the emulsions was adjusted to pH 7. The particle size of the unheated emulsion was increased with increasing levels of salt, however, the heated emulsion showed the same behavior as the emulsions at pH 4. It suggests that partial or full desorption of the polysaccharide molecules may occur due to the decrease in electrostatic attraction between the lipid droplet and the polysaccharide in the presence of salt (21, 23, 300). The unheated and heated emulsions containing added pectin/ β -Lg molecules (1°-BP) was highly stable to the droplet aggregation in the presence of salt at pH 4 (Figure 7.6c). The heated emulsions showed the similar behavior to the emulsions containing added pectin molecules that the mean diameter was decreased with increasing the levels of salt. It can be explained by the same reason mentioned above of the close packing of pectin and β -Lg by heating. The unheated emulsion showed marginally aggregations in the presence of 50 mM salt when

the pH of the emulsion was adjusted to pH 7. It suggests that the desorption of adsorbed β -Lg molecules may occur at this pH due to less strong electrostatic repulsion between pectin and β -Lg molecules in the presence of salt. It already revealed in the previous section that the free protein concentration was dramatically increased when the pH of the emulsion was adjusted at pH 7. Interestingly, the mean diameter of the unheated emulsions in the presence of above 100 mM salt was decreased to the similar value as the emulsion in the absence of salt. It has been shown that the primary emulsion in the presence of free protein in the continuous phase showed the stability to droplet aggregations in the presence of 150 mM salt at pH 7 (334). It suggests that addition of high level of salt to the emulsion would reduce the interaction between the protein coated lipid droplets and free protein in the continuous phase lead to less possibility of hydrophobic interaction. Therefore, it may provide the stability to droplet aggregation in the emulsions again (334). Heated emulsions showed a good stability to droplet aggregation in the presence of various levels of salt when the pH of the emulsions was adjusted to pH 7 due to remain cross-linking of the adsorbed protein layer by heating intact.

7.5 Conclusions

This study has shown that protein-rich interfacial coatings can be formed around lipid droplets using an electrostatic deposition method. The proteins can be assembled into the coatings using two different methods: (i) proteins can be adsorbed to preformed protein-polysaccharide coated lipid droplets; (ii) protein-polysaccharide complexes can be adsorbed to protein-coated lipid droplets. Our results suggest that heating these

systems above the thermal denaturation temperature of the proteins leads to cross-linking of the proteins within the coatings, which helps prevent them desorbing at high pH. On the other hand, thermal treatment did not completely prevent the polysaccharide molecules from detaching from the droplet surfaces at neutral pH. The systems developed in this study may be useful for increasing the local protein concentration in biopolymer coatings around lipid droplets, which may have important practical applications *e.g.*, to protect ω -3 oils from oxidation or to develop natural weighting agents.

CHAPTER 8
THE ROLE OF POLYSACCHARIDE COATINGS ON DISPLACEMENT OF
PROTEIN FILMS FROM OIL-WATER INTERFACES BY NON-IONIC
SURFACTANT

8.1 Abstract

The effect of a polysaccharide coating on the displacement of adsorbed globular proteins by non-ionic surfactants from lipid droplet surfaces was examined. Oil-in-water emulsions stabilized by β -lactoglobulin (β -Lg) were prepared containing either no pectin (1° emulsions) or different amounts of pectin (2° emulsions). Protein displacement was then determined by measuring changes in droplet ζ -potential and non-adsorbed protein as the mass ratio of Tween 20 to β -lactoglobulin (r) was increased. At pH 7, where pectin remains in the aqueous phase, the amount of desorbed protein was fairly similar for the 1° and 2° emulsions. At pH 3.5, where pectin forms a coating around the β -Lg stabilized lipid droplets, the amount of desorbed protein was much less for the 2° emulsion (3%) than for the 1° emulsion (39%), which indicated that the pectin coating inhibited protein desorption by surface active agents. The insights gained from this study are important for understanding and designing emulsion-based delivery systems in which various types of surface active components may compete for the droplet surfaces, *e.g.*, multi-component industrial products (foods, pharmaceuticals, cosmetics, and personal care products) or products passing through the human body.

8.2 Introduction

Lipids are an important nutrient for the human diet and are important ingredients for providing functional properties in foods (335). Meanwhile, the digestibility and bioavailability of lipids have been of interest with respect to obesity and disease (9). The bioavailability of dietary fats is dependent on food source, processing and preparation conditions (10, 11). There are different forms of dietary fats including bulk, structural, emulsified and interfacial (1). During the digestion of dietary fats in the body, however, most fats are broken down into oil-in-water emulsions as they pass through the mouth and gastrointestinal tract due to the mechanical stresses and the presence of a number of surface-active components and stabilizing components from the food and the body: small molecule surfactants, bile salts, phospholipids, proteins, polysaccharides, their derivatives, and enzymes (12). Therefore, it would result in a variety of compositional and structural changes of ingested emulsified lipids. e.g. lipid droplet size, interfacial composition, and droplet aggregation state (13). Consequently, the digestion of dietary fats would be modulated by designing structured delivery systems using mixed biopolymers with different molecular structures and interfacial properties.

Competitive adsorption among surface-active components within food products and during digestion may play an important role in designing the structured delivery system. Competitive adsorption in the presence of more than two emulsifiers or surface active ingredients depends on their concentration, surface activity, and saturation concentration. Protein is more surface active than small molecule surfactants at low concentrations, but surfactant is more efficient at lowering free energy (Π_{SAT}) at higher concentration (23). Therefore, one would expect that protein would dominate the

adsorption at the oil-water interface at lower concentrations and the adsorbed protein layer would be displaced by the surfactant at higher concentrations. There have been extensive studies of the displacement of proteins (mainly β -Lg, α -casein (α -CN), and β -casein (β -CN)) by anionic surfactant SDS (91, 92), non ionic surfactants such as Tween series (93-106) and Span series (107-109), oil-soluble surfactants $C_{12}E_2$ (diethylene glycol n-dodecyl ether) (102, 103), and monoglyceride, diglyceride (110) from oil-water interfaces. Recently, The combined use of AFM with studies of surface tension and surface rheology have revealed the mechanism of protein desorption from the air/water interface (140, 141). The surfactant is found to adsorb at defects in the protein network and grow after nucleating sites followed by compressing the protein network, disrupting the protein network at higher surface pressure, and releasing proteins from the interface. This mechanism has been called orogenic displacement. The surfactant domain at the interface was different on protein types forming protein networks at the interface (140).

It has been proposed that the adsorption of pancreatic lipase is facilitated by the presence of bile salts, which are surface-active lipids with anionic and hydrophobic parts and it promotes the lipid digestion by the pancreatic lipase (14, 15). Hence, the composition, structure, and physicochemical properties of the interfacial layer surrounding the fat droplets should play an important role in determining the extent of enzyme binding to the emulsion surface and therefore the extent of lipolysis (16).

Combining the competitive adsorption of surface active components at oil-water interface and the displacement of an emulsifier layer from the interface by bile salts, the interfacial composition of the emulsion before and after digestion could be extremely important for the stability and digestibility of the emulsion (9, 16, 336-338).

There are only few studies which deal with the use of fundamental interfacial measurements to investigate the molecular changes at interfaces under digestion conditions. The main advantage of the multilayer emulsion would be the modulation of the interfacial composition with different types of biopolymers (17-22). Therefore, the fundamental interfacial mechanism during digestion would be of importance to investigate the kinetic of digestion of the lipids emulsified by multilayer interfacial membrane technique.

The purpose of the present study was to examine the competitive displacement of the adsorbed protein layer by a non-ionic surfactant at the oil-water interface and determine the impact of this process on subsequent the interfacial composition and emulsion stability to the droplet aggregations. A primary emulsion containing the protein coated lipids droplets was formed by homogenizing a corn oil with a β -Lg solution as an emulsifier. For the impact of polysaccharide layer around the protein coated lipid droplets on the desorption, a secondary emulsion was formed by simply mixing the primary emulsion with a pectin solution at pH 7 and 3.5. The formation of a secondary emulsion is dependent on the electrostatic interaction between protein and polysaccharide molecules. Therefore, the pH adjustment could cause the detachment of the adsorbed polysaccharide layer from the protein coated lipid droplets at pH, where both the protein coated lipid droplet and polysaccharide molecules are negatively charged like pH 7 (80). To simulate the digestion of the emulsion containing the lipid droplet coated with protein-polysaccharide layers in various pH conditions and the impact of polysaccharide coating around the protein coated lipid droplets on the digestibility, we chose a non-ionic small surfactant as a replacement of bile salts. With the small molecule surfactant, we

could simulate the changes of the interfacial composition of the emulsion at pH 7, where the electrostatic interaction between the protein coated lipid droplets and polysaccharide molecules would not take place, and pH 3.5, where multilayered interfacial membrane containing protein-polysaccharide layers would be formed due to the electrostatic interaction between oppositely charged lipid droplets and polysaccharide molecules (58). The results from this study should provide information that would be useful for designing the structured delivery system for functional food components.

8.3 Materials and Methods

8.3.1 Materials

Powdered β -Lg was obtained from Davisco Foods International (Lot # JE 001-1-922, Le Sueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with β -Lg making up 95.5 % of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and lactose contents of this product are reported to be 0.3 ± 0.1 , 2.5 ± 0.2 and < 0.5 wt%, respectively. Tween 20 was purchased from Sigma Chemical Co. (St. Louis, MO) Pectin was kindly donated from CPKelco (TS#1781, Denmark). The degree of esterification (DE) of the pectin was reported to be 54% by the supplier. Corn oil was purchased from a local supermarket and used without further purification. Corn oil was purchased from a food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN, USA) and used without further purification. The manufacturer reported that the corn oil contained approximately, 14.3, 28.6 and 57.1 wt% of saturated, monounsaturated and polyunsaturated fats, respectively. Other

chemicals used in this study were analytical grade and purchased from the Sigma Chemical Co. (St. Louis, MO). Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Iowa) was used for the preparation of all solutions.

8.3.2 Solution preparation

An emulsifier solution containing 0.5 wt% β -Lg was prepared by dispersing powdered β -Lg into 5 mM Na phosphate buffer (pH 7.0) and stirring for at least two hours to ensure complete hydration. A 1 wt% pectin solution was prepared by dispersing powdered pectin into 5 mM Na phosphate buffer (pH 7.0) overnight. The pH of pectin solution was then adjusted to 7 by adding 2N NaOH solution. A 2 wt% tween 20 solution was prepared by dispersing tween 20 into 5 mM Na phosphate buffer (pH 7 or 3.5) for at least two hours and the pH of tween solution was adjusted to pH 7 or 3.5 using 2N NaOH.

8.3.3 Emulsion preparation

A stock emulsion was prepared by homogenizing 10 wt% corn oil with 90 wt% aqueous emulsifier solution (0.25 wt% β -Lg, pH 7.0) with a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min followed by 5 passes through a microfluidizer (Model M-110L, Microfluidics Co., Newton, MA) at 9,000 psi. The stock emulsion was diluted with a buffer solution (pH 7, 5 mM Na phosphate buffer) to prepare a primary emulsion (1^o) and the final composition of the droplet and β -Lg was 1 wt% and 0.045 wt%, respectively. For a secondary emulsion (2^o), the stock emulsion was diluted with buffer and pectin solution (pH 7, 5 mM Na phosphate buffer) to give the final compositions of the droplet, β -Lg, and pectin to 1

wt%, 0.045 wt%, and 0.05 wt%, respectively. The pH of the resulting emulsions (1° and 2°) was adjusted to 7 or 3.5 using 1N HCl solution. For a heated primary emulsion (Heated 1°), the primary emulsions was then transferred to a pyrex media bottle (Fisher scientific, PA) and heated in a water bath (85 °C) for 20 min followed by cooling in ice water until reaching ambient temperature.

8.3.4 Electroacoustic measurements.

Electrical charge (ζ -potential) of the particles in undiluted emulsions was determined with an electro-acoustic instrument (Field ESA, Partikel Analytik GmbH, Frechen, Germany), based on the electrosonic analysis (ESA) principle. The frequency of the ESA signal applied to the samples ranged between 0.85 and 1.15 MHz. The Smoluchowski equation was used to relate ζ -potentials of the emulsion droplets to the measured ESA signal:

$$\zeta = \frac{A (\text{ESA}) \eta_s}{G \varepsilon_s \phi \Delta \rho c_s} \quad (8.1)$$

where A is an instrument constant determined by calibration with a standard suspension (see below), ESA is the signal measured by the instrument, η_s is the dynamic viscosity of the solvent surrounding the droplets, ε_s is the relative dielectric permittivity of the solvent, ϕ is the disperse-phase volume fraction, $\Delta \rho$ is the density difference between the particles and solvent ($\rho_P - \rho_S$), and $G [= f(\rho_P, \rho_S, \eta_s, r)]$ is a calculated instrument parameter that depends on density, viscosity, and particle radius (r). This equation is applicable under conditions where the electrical double layer around the particle is thin compared to the particle radius. At the electrical conductivities of our samples, the double layer was only a few nanometers thick, so the Smoluchowski

equation is applicable for both polymers and particles studied. The ESA instrument was calibrated by use of a standard colloidal suspension of known charge characteristics (10% Ludox, Sigma Chemical Co., St Louis, MO) immediately before each set of experiments was run, and then a phase calibration was carried out for each sample before a titration was started. Measurement of ζ -potential by the ESA was repeated at least three times with freshly prepared samples for each repetition. All measurement with the ESA were carried out in a water bath (37 ± 0.5 °C) and the samples in the chamber was incubated in a water bath until reaching 37 °C before a titrated was started.

A concentration titration was carried out using the ESA to examine the ζ -tween 20 concentration profile. In these experiments, the change in electrical charge of the emulsions was measured when tween 20 (2 wt% in pH 7 or 3.5 5 mM Na phosphate buffer) was titrated into emulsions: primary emulsion (1°); heated primary emulsion (Heated 1°); and secondary emulsion (2°). The emulsion (pH 7 or 3.5) was placed into the measurement chamber of the instrument, and then 30 1-mL aliquots of 2 wt% tween 20 solution were titrated with computer-controlled autotitration into the emulsion, with a delay time of 60 s between each aliquot.

8.3.5 Particle size analysis

Aliquots of emulsion (0.5 mL) were collected from the measurement chamber of the electroacoustic instrument every two injection steps during concentration titration. These emulsion samples collected were diluted with a 5 mM Na phosphate buffer (pH 7 or 3.5) to give a final droplet concentration to 0.1 wt%. The diluted samples were placed into the disposable capillary cell of a dynamic light scattering (DLS, Zetasizer NanoZS, Malvern Instruments, Worcs., UK). The mean particle diameter measured by DLS

reported as Z-average size, which are calculated from the signal intensity and converted to a size using the dispersant viscosity and some instrument constants.

8.3.6 Determination of free protein concentrations

The free protein concentration in the aqueous phase of the emulsions was determined using a modified Lowry method (310). Emulsions were placed in centrifuge tubes (Lot # 21915, Thermo Scientific, NC) and centrifuged at 146,550g for 1 hr using a ultracentrifuge (Sorvall[®] Ultra 80[®], Kendro Laboratory Products, CT) to separate the emulsions into a creamed layer and a serum layer. The serum layer was carefully collected from the tubes using a pipet (BD glass disposable pasteur pipet, 9 in, Fisher scientific, PA) after removing the upper cream layer. The collected serum phases were filtered using a syringe filter (0.45 μm , PTFE, Millipore Millex[®] syringe filters, Fisher scientific, PA) to remove any remaining oil droplets. The reaction solution (The mixture of Solution A (0.5 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1 % $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and Solution B (2% Na_2CO_3 and 0.4% NaOH)) was added to the serum phase (0.5 mL) of each sample (10 min) followed by the addition of the Folin-Ciocalteu reagent (30 min). The absorbance of the solutions including standard solutions (β -Lg) was measured at 600 nm by a UV/visible spectrophotometer (Shimadzu UV-2101, Shimadzu Scientific Instruments, Columbia, MD).

8.3.7 Statistical analysis

Experiments were performed in duplicate or triplicate with freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements.

8.4 Results and Discussions

The aim of this research was to establish the effect of an anionic polysaccharide (either adsorbed or non-adsorbed) on the displacement of globular proteins from β -Lg-coated lipid droplets by small molecule surfactants. 1 wt% corn oil-in-water emulsions stabilized by β -Lg were prepared in the absence (1° emulsions) or presence (2° emulsions) of pectin. Displacement experiments were then performed at pH 7 (where the polysaccharide remains in the aqueous phase) and pH 3.5 (where the polysaccharide forms a coating around the lipid droplets).

8.4.1 Effect of non-adsorbed polysaccharide on protein displacement

Initially, we examined the effect of non-adsorbed pectin on the ability of Tween 20 to displace protein from β -Lg-stabilized lipid droplets. These experiments were therefore carried out at pH 7, where both pectin and β -Lg are negatively charged, so that the pectin did not adsorb to the droplet surfaces. Insights into changes in interfacial composition were obtained by measuring changes in droplet ζ -potential with increasing non-ionic surfactant concentration (Tween 20) using ESA (Figure 8.1). The Tween 20 concentration was increased from 0 to 0.22 wt%, which corresponded to a surfactant-to-protein mass ratio (r) of 0 to 5.6. In the absence of Tween 20, the two emulsions contained highly negatively charged droplets because the adsorbed β -Lg layer was well above its isoelectric point ($pI \approx 4.7\sim 5.2$) (242, 243, 262). The ζ -potential of the primary emulsion was slightly less negative than the secondary emulsion, which may have been because of some contribution of the pectin to the ESA signal. The change in ζ -potential ($\Delta\zeta$) due to the presence of Tween 20 was calculated: $\Delta\zeta = \zeta - \zeta_0$, where ζ_0 and ζ are the

ζ -potential measurements in the absence and presence of Tween 20, respectively (Figure 8.1).

The two emulsions had similar $\Delta\zeta$ *versus* surfactant concentration profiles, with $\Delta\zeta$ increasing steeply from 0 to 0.1 wt% Tween 20, and then reaching a more constant value at higher surfactant concentrations (Figure 8.1). The observed change in $\Delta\zeta$ with surfactant concentration suggests that there was a change in interfacial composition, which can be attributed to displacement of protein by surfactant. Indeed, it is well established that water-soluble small molecule surfactants displace adsorbed protein layers from oil-water interfaces (93-106, 111, 339-343). The presence of pectin in the emulsions did not affect the displacement of the adsorbed protein layer from the interface (Figure 8.1). This observation can be attributed to the fact that pectin should not be adsorbed to the droplet surfaces at this pH because of the strong electrostatic repulsion between the anionic polysaccharide molecules and the anionic protein-coated droplets.

The amount of protein desorbed from the droplet interfaces by Tween 20 was quantified by measuring the free (non-adsorbed) protein concentration in the emulsions at the beginning and end of the surfactant titration experiments (Figure 8.2). Both emulsions (1° and 2°) contained the same total amount of β -Lg (450 $\mu\text{g}/\text{mL}$ emulsion).

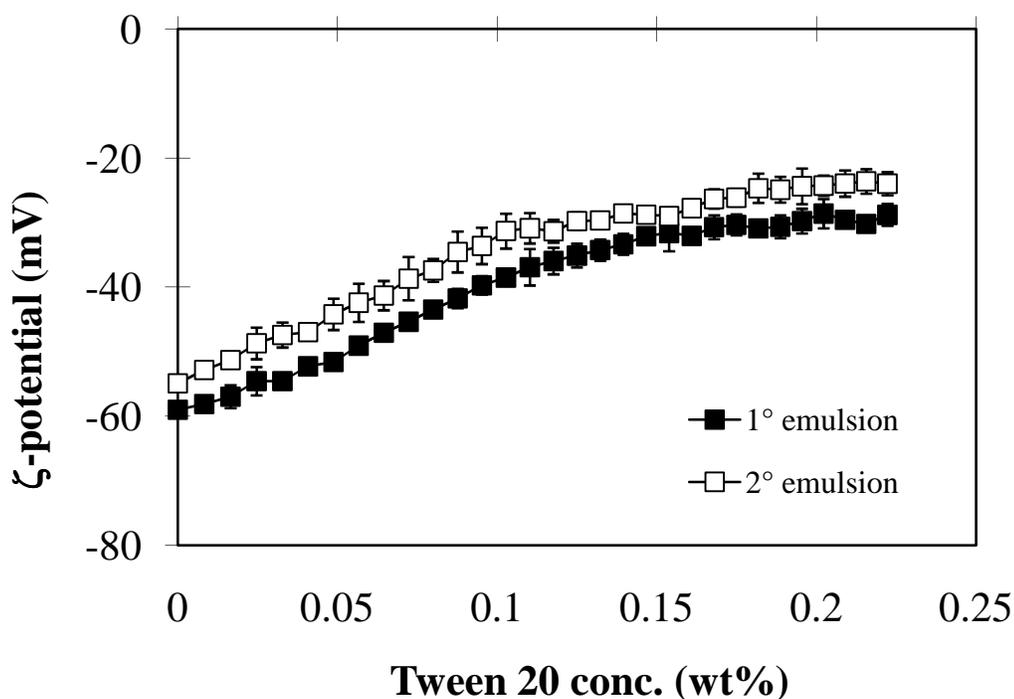


Figure 8.1. Dependence of the droplet ζ -potential of protein stabilized O/W emulsions on Tween 20 concentration: primary emulsion (1° emulsion: 1 wt% corn oil and 0.045 wt% β -Lg in 5 mM phosphate buffer, pH7) and secondary emulsion (2° emulsion: 1 wt% corn oil, 0.045 wt% β -Lg and 0.05 wt% pectin in 5 mM phosphate buffer, pH 7) . Tween 20 (2 wt% in 5 mM phosphate buffer, pH7) was titrated into the emulsions.

Initially, the free protein concentration in the aqueous phase was 77 ± 1 and 70 ± 2 $\mu\text{g/mL}$ for the 1° and 2° emulsions, respectively. The majority (>83~85%) of the protein was therefore adsorbed to the droplet surfaces, however there was some free protein remaining in the aqueous phase (<15~17 %). After the addition of 0.22 wt% Tween 20 ($r = 5.6$), the free protein concentration increased to 193 ± 5 and 180 ± 3 $\mu\text{g/mL}$ for the 1° and 2° emulsion, respectively. These measurements supported the fact that adsorbed β -Lg was displaced from the droplet surfaces by the small molecule surfactant, which accounts for the observed changes in ζ -potential (Figure 8.1). It should be noted that only partial

displacement of the adsorbed β -Lg layer from the droplet interface by Tween 20 would be expected at the surfactant-to-protein ratio used.

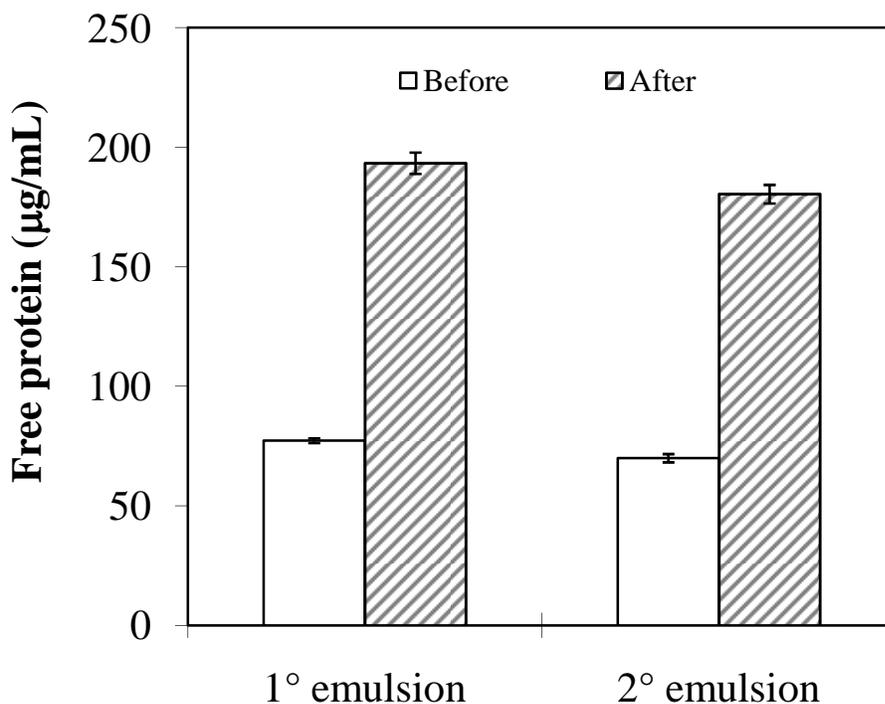


Figure 8.2. The free protein concentration in aqueous phase before and after the completion of the concentration titration of the emulsions with Tween 20 by the ESA at pH 7: primary emulsion (1° emulsion) and secondary emulsion (2° emulsion)

To determine whether protein displacement from the interface would affect emulsion stability to aggregation, the particle size was also measured as a function of Tween 20 concentration using dynamic light scattering. In the absence of Tween 20, the mean particle diameter of the emulsions was $0.198 \pm 0.003 \mu\text{m}$ and $0.199 \pm 0.001 \mu\text{m}$ for the 1° and 2° emulsions, respectively. The fact that these droplet diameters were so similar indicates that the pectin molecules did not adsorb to the droplet surfaces. There were no appreciable changes in the mean particle diameter of the two emulsions when

Tween 20 was added (Figure 8.3), which indicates that the emulsions were stable to droplet aggregation even though some of the protein layer had been replaced with non-ionic surfactant.

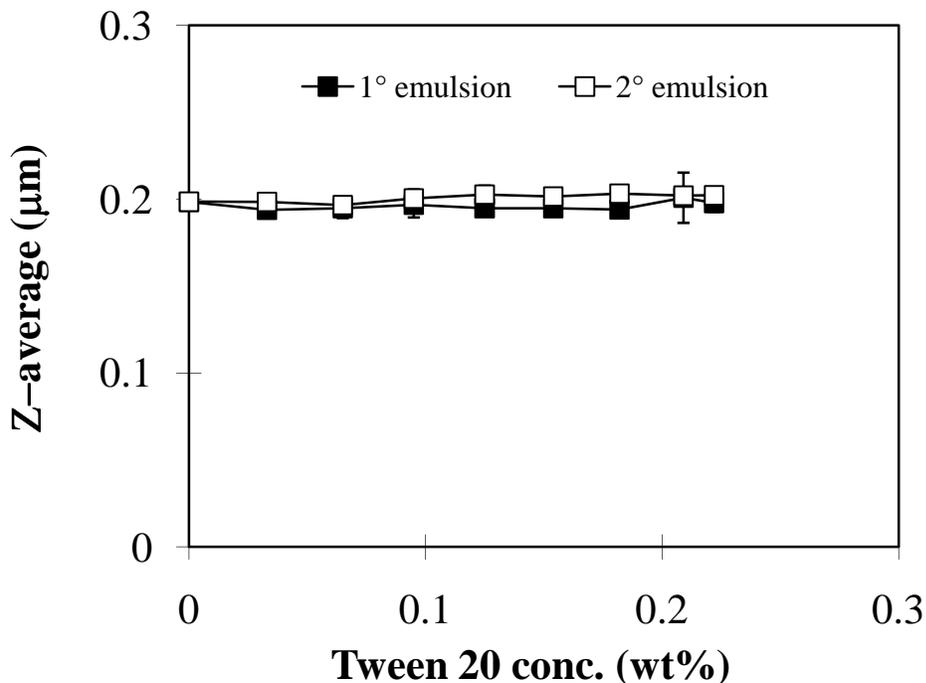


Figure 8.3. Dependence of the mean particle diameter of protein stabilized O/W emulsions on Tween 20 concentration at pH 7: primary emulsion (1° emulsion) and secondary emulsion (2° emulsion). Tween 20 (2 wt% in 5 mM phosphate buffer, pH7) was titrated into the emulsions.

8.4.2 Effect of polysaccharide coating on protein displacement

The effect of a polysaccharide coating on the displacement of adsorbed globular proteins by surfactant was examined by repeating the above experiments at pH 3.5. At this pH the pectin adsorbs to the droplet surfaces due to the electrostatic attraction

between the anionic pectin molecules and the cationic protein-stabilized droplets (58). All the emulsions were initially prepared at pH 7 and then adjusted to pH 3.5.

Again, insights into changes in interfacial composition were obtained by measuring changes in ζ -potential and $\Delta\zeta$ with increasing surfactant concentration using ESA (Figure 8.4). In the absence of surfactant, the droplets in the 1° emulsion were positively charged because the pH was below the pI of the adsorbed β -Lg layer (242, 243, 262). On the other hand, the droplets in the 2° emulsion were negatively charged due to the presence of an anionic pectin coating around the cationic protein-stabilized droplets (58). When increasing amounts of Tween 20 were added, the 1° emulsions became less positively charged, while the 2° emulsions became less negatively charged, indicating that the surfactant altered the interfacial composition. The ζ -potential of lipid droplets stabilized by Tween 20 alone at pH 3.5 was measured by ESA to be -11.7 mV, which suggested that the surfactant did not completely displace the adsorbed biopolymer layers in either 1° or 2° emulsions. The negative charge on the lipid droplets stabilized by non-ionic surfactants has previously been attributed to adsorption of OH^- species from water or cationic impurities in oil (*e.g.*, free fatty acids) to the oil–water interface (23). The changes in ζ -potential with increasing surfactant concentration were appreciably larger for the 1° emulsions than for the 2° emulsions, suggesting that more protein was desorbed in the former case. This hypothesis was supported by measurements of the non-adsorbed protein concentration in the emulsions before and after Tween 20 was added (Figure 8.5).

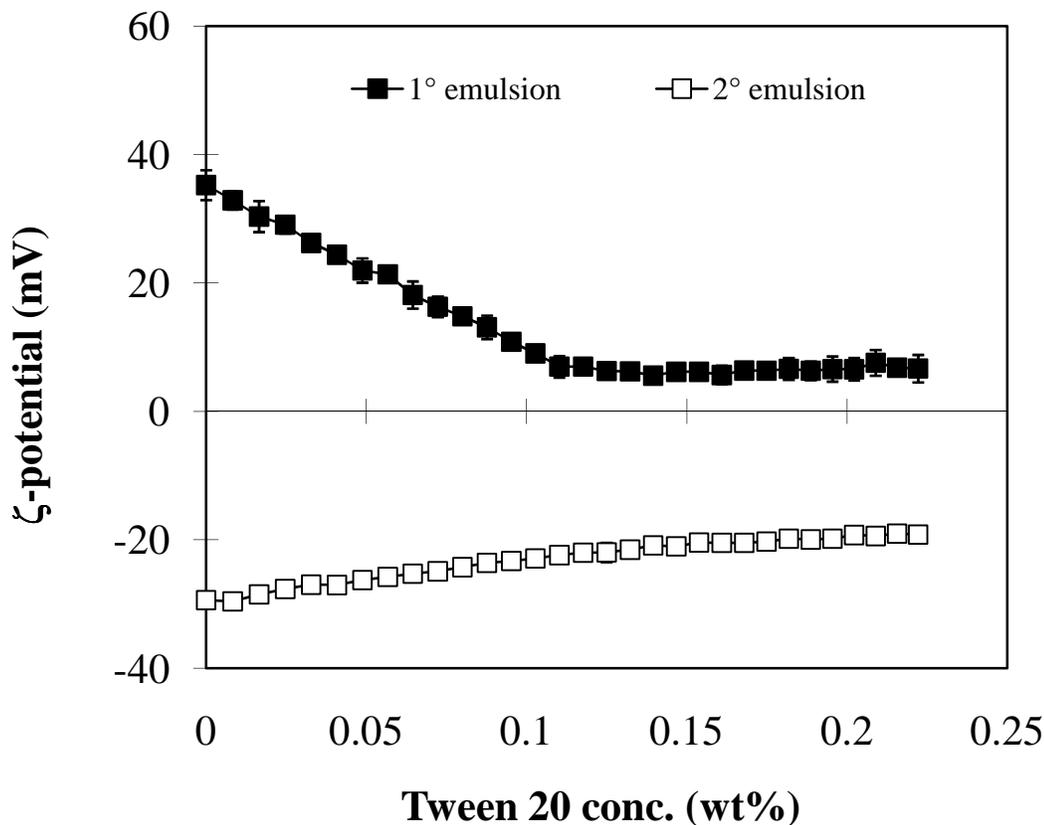


Figure 8.4. Dependence of the droplet ζ -potential of protein stabilized O/W emulsions on Tween 20 concentration: primary emulsion (1° emulsion: 1 wt% corn oil and 0.045 wt% β -Lg in 5 mM phosphate buffer, pH3.5) and secondary emulsion (2° emulsion: 1 wt% corn oil, 0.045 wt% b-Lg and 0.05 wt% pectin in 5 mM phosphate buffer, pH 3.5). Tween 20 (2 wt% in 5 mM phosphate buffer, pH3.5) was titrated into the emulsions.

In the absence of Tween 20, the free protein concentration in aqueous phase was 8 ± 2 and 32 ± 1 $\mu\text{g/mL}$ for the 1° and 2° emulsions, respectively. Thus, almost all of the protein was adsorbed to the droplet surfaces (>93%) for both emulsions, with little free protein remaining in the aqueous phase. Surprisingly, the free protein concentration in the aqueous phase was determined to be slightly higher for the secondary emulsion than for the primary emulsion (Figure 8.5). The origin of this effect is currently unknown: it may have been due to some protein associated with the pectin ingredient used, or because

some of the adsorbed protein was displaced by pectin. After the addition of Tween 20 ($r = 5.6$), the free protein concentration increased to 182 ± 1 and 46 ± 1 $\mu\text{g/mL}$ for the 1° and 2° emulsions, corresponding to a decrease in interfacial protein of 39% and 3%, respectively.

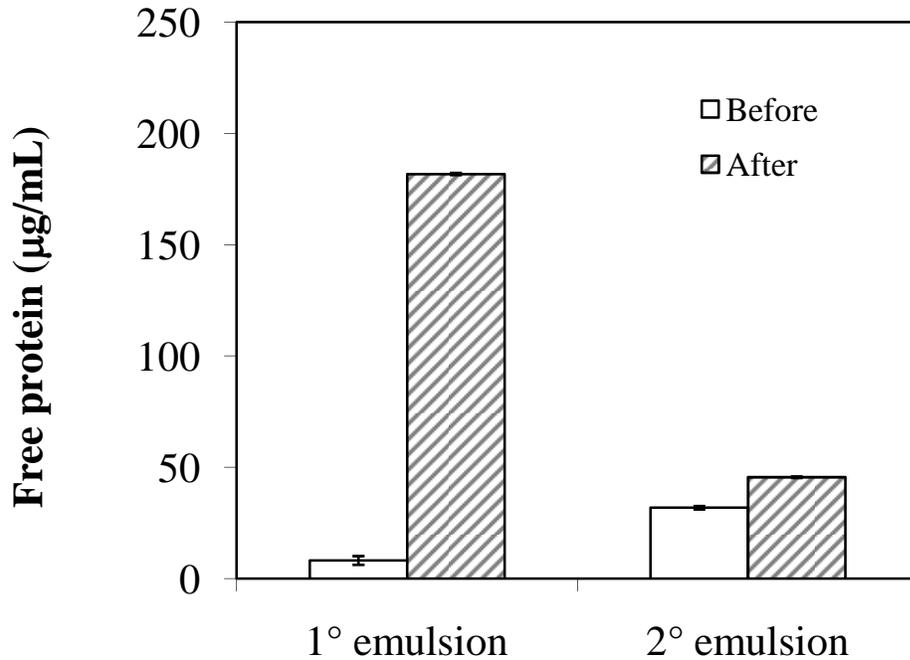


Figure 8.5. The free protein concentration in aqueous phase before and after the completion of the concentration titration of the emulsions with Tween 20 by the ESA at pH 3.5: primary emulsion (1° emulsion) and secondary emulsion (2° emulsion)

The presence of a pectin coating around the lipid droplets therefore appeared to have a major effect on the ability of surfactant to desorb an adsorbed protein layer. This effect may be due to the ability of the polysaccharide coating to limit the access of the surfactant to the protein layer beneath, or to the ability of the polysaccharide molecules to hold the proteins together at the interface. Further studies using methods such as Brewster angle microscopy and AFM would be useful to elucidate this mechanism.

The effect of surfactant addition on the stability of the 1° and 2° emulsions to aggregation was determined by measuring the mean particle diameter using dynamic light scattering (Figure 8.6). In the absence of Tween 20, the mean particle diameter of the emulsions was 0.246 ± 0.004 and 0.390 ± 0.009 μm for the 1° and 2° emulsions, respectively. The initial mean particle diameter of the secondary emulsion was considerably larger than the primary emulsion due to the formation of a polysaccharide coating around the protein-coated lipid droplets. There was a slight decrease in the mean particle diameter of the two emulsions upon addition of Tween 20, which may be due to reorganization or displacement of the biopolymers at the droplet surfaces. Interestingly, the secondary emulsions did not return to the initial size of the primary emulsions, which would have indicated that the pectin coating had become detached. It should be noted that the electrical charge of the primary emulsion in the presence of Tween 20 was around +6 mV, which should not have been large enough to provide stability through electrostatic repulsion alone. Nevertheless, the primary emulsion was stable to droplet aggregation, which can be attributed to some steric repulsion by the Tween 20 head groups.

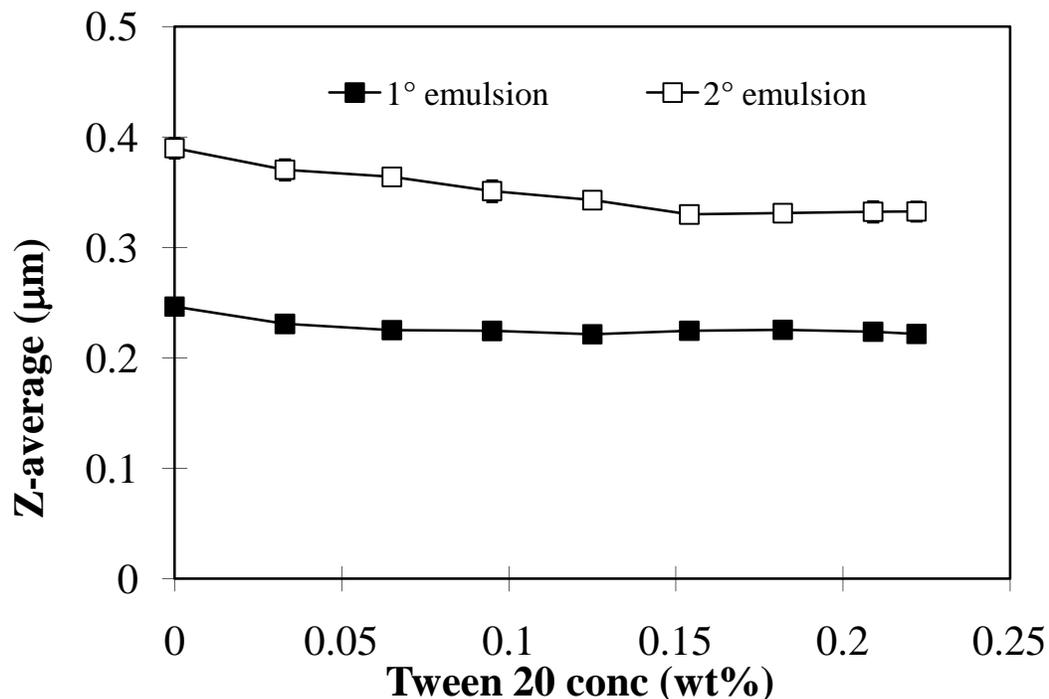


Figure 8.6. Dependence of the mean particle diameter of protein stabilized O/W emulsions on Tween 20 concentration at pH 3.5: primary emulsion (1° emulsion) and secondary emulsion (2° emulsion). Tween 20 (2 wt% in 5 mM phosphate buffer, pH7) was titrated into the emulsions.

8.5 Conclusions

This study has shown that a polysaccharide coating formed around globular protein-stabilized lipid droplets restricts the displacement of the adsorbed protein by small molecule surfactants. On the other hand, the presence of free polysaccharide in the aqueous phase has little effect on protein displacement by surfactant. The polysaccharide layer may restrict access of the surfactant to the proteins, or it may act as a glue holding the protein molecules together at the surface. Further studies are needed to elucidate the precise physicochemical mechanisms involved. The knowledge gained from this study is useful for understanding and designing emulsion-based delivery systems in which various

types of surface active components compete for the droplet surfaces, *e.g.*, in multi-component industrial products (foods, pharmaceuticals, cosmetics, and personal care products) or in products that must pass through the human body.

CHAPTER 9 CONCLUSION

9.1 Major findings

The ultimate objective of this study was to carry out research that would lead to a better fundamental understanding of the major factors that determine the formation, stability and properties of multilayer emulsions. These emulsions consist of lipid droplets coated with nanolaminated biopolymer layers formed using an electrostatic deposition method. In this study, a β -Lg stabilized emulsion was employed as a primary emulsion and pectin was used as the major polysaccharide to assemble nanolaminated biopolymer layers around lipid droplets.

First, the major factors that impact the preparation of stable oil-in-water emulsions containing lipid droplets coated with globular protein-anionic polysaccharide interfacial layers was examined, *i.e.* droplet concentration, droplet size, droplet charge, pH, and polysaccharide concentration on the formation and stability of these multilayer emulsions. Experimental stability maps of the influence of droplet and pectin concentrations on emulsion stability were established at pH 7 (where pectin does not adsorb to the droplet surfaces) and at pH 3.5 (where pectin does adsorb). At pH 7, droplet flocculation and creaming were observed when the pectin concentration exceeded a particular level, which was attributed to depletion flocculation. At pH 3.5, the stability of the emulsions was much more complex, going from stable, to unstable, to stable, to unstable with increasing pectin concentration. In these systems, the instability at low pectin concentrations was attributed to bridging flocculation, whereas the instability at high pectin concentrations was attributed to depletion flocculation. At intermediate

pectin concentrations stable multilayer emulsions could be formed, which consisted of lipid droplets coated with a pectin layer.

Secondly, an electro-acoustic technique was introduced to characterize the formation of multilayer emulsions *in situ*. Concentrated emulsions need to be diluted to measure the electrical charge using the traditional micro-electrophoresis (ME) technique and this dilution may change the interfacial composition through partitioning of a polyelectrolyte between the droplet surfaces and the surrounding continuous phase. The EA technique was successfully able to monitor the adsorption of pectin onto the surfaces of β -Lg coated droplets as a function of polysaccharide concentration and pH. Multilayer emulsions with different interfacial and physicochemical properties were then prepared by assembling them from mixed polysaccharides with different molecular characteristics: pectin/carrageenan or gum arabic/pectin. Carrageenan showed the highest binding affinity for the β -Lg coated droplet surfaces due to its high charge density, however the emulsions formed were unstable to aggregation. Emulsions containing pectin molecules showed the best stability to aggregation and at certain pH, co-adsorption of pectin and gum arabic promoted better emulsion stability to aggregation than emulsions containing only gum arabic or pectin.

Thirdly, protein-rich coatings around lipid droplets were assembled using the electrostatic deposition method: β -Lg-pectin complexes were adsorbed to β -Lg-coated lipid droplets. These composite particles had relatively small diameters ($d < 500$ nm) and were stable to gravitational separation. The composite particles remained stable after they were heated above the thermal denaturation temperature of the globular proteins. When the heated composite particles were adjusted to pH 7, where the β -Lg and pectin

are both negatively charged, some of the pectin and β -Lg became detached from the droplet surfaces but some protein remained in the coating.

Lastly, the effect of a polysaccharide coating on the displacement of adsorbed globular proteins by non-ionic surfactants from lipid droplet surfaces was examined. A primary emulsion stabilized by β -Lg and secondary emulsion stabilized by β -Lg/pectin was prepared at pH 7 and 3.5 and protein displacement was then determined by measuring changes in droplet ζ -potential with the EA technique. At pH 7, where pectin remains in the aqueous phase, the amount of desorbed protein was fairly similar for the primary and secondary emulsions. At pH 3.5, where pectin forms a coating around the β -Lg stabilized lipid droplets, the amount of desorbed protein was much less for the secondary emulsion (3%) than for the primary emulsion (39%), which indicated that the pectin coating inhibited protein desorption by surface active agents.

To summarize the major findings from this study:

- *Designing a nanolaminated biopolymer coating:* Several factors should be considered including mixing pH, electrical characteristics of protein and polysaccharide, and concentration of ingredients (oil, emulsifier, and polysaccharide). For example, at a pH where an electrostatic attraction between lipid droplets and biopolymer does not take place, the amount of biopolymer concentration should not exceed the level required to promote depletion flocculation. At a pH where an electrostatic attraction between lipid droplets and biopolymer does take place, several instability mechanisms as a function of biopolymer concentration should be taken into account, e.g.

bridging flocculation at lower biopolymer concentrations and depletion flocculation at higher biopolymer concentrations.

- *Characterization of interfacial property of emulsions coated with structured biopolymer layers:* Biopolymer adsorption onto lipid droplets is a kinetic process that leads to changes in interfacial properties, particle size and ζ -potential. In this study, the changes of ζ -potential in emulsions containing different types of polysaccharides were successfully monitored *in situ* using a novel electroacoustic technique. The adsorption of polysaccharides onto protein-coated lipid droplets and the subsequent emulsion stability depended strongly on the electrical characteristics of the polysaccharides used, as well as their molecular structure. Hence, when you select biopolymers to design a multilayered emulsion, you must consider both the electrical characteristics and molecular structure of the biopolymers used.
- *New assembled composites onto protein-coated droplets.* Protein-rich coatings were assembled onto β -Lg coated lipid droplets by thermal treatment of adsorbed β -Lg/pectin complexes. These composites were stable to gravitational separation and high salt concentrations. The concentration of biopolymer added was critical to assemble stable composites onto lipid droplets.
 - *Protective interfacial membrane.* A polysaccharide coating formed around globular protein-stabilized lipid droplets restricts the displacement of the adsorbed protein by small molecule surfactants. One limitation of nanolaminated biopolymer coatings is a possibility that the adsorbed

biopolymer layers could be desorbed from the interface with pH changes and heating. The pH of the final target of the product by this electrostatic deposition technique should be considered for the best performance of a protective interfacial membrane.

9.2 Future work

This study research has shown that nanolaminated biopolymer coatings around the lipid droplets can be used to form structured delivery systems. To utilize this technology in the food industry, more research is required on the design and application of multilayer emulsions:

- *Different oil types.* Different emulsifiers have different affinities for different oil types. Therefore, the impact of oil type on the formation of multilayer emulsions should be examined, *e.g.* flavor oils and ω -3 fatty acids. Especially, protein-rich coatings will give different stability to gravitational separation due to the differences in densities of different oils.
- *Environmental stresses test using the EA technique.* The EA technique is a powerful tool for monitoring the interfacial composition changes without any sample dilution. The study of interfacial changes with the EA in various salt concentrations and accelerated oxidation conditions will provide direct information of the stability of emulsions coated with different biopolymers without any changes in interfacial composition and any possible partitioning.
- *Simulation of digestion using the EA technique.* To mimic the digestion system, the pH of the emulsion may be adjusted to the similar pH values

of the body and the interfacial changes of the emulsion can be monitored using the EA *in situ*. The differences in the electrical characteristics of biopolymer coating such as net charge or charge density would influence the displacement of the adsorbed biopolymer coating by any possible surface-active components in the body. Therefore, the investigation of biopolymer coating displacement with different electrical charge would be needed to clarify the fundamental mechanism of the displacement of the adsorbed interfacial layer by several different types of enzyme or surface active components.

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