ENGINEERING NANOSTRUCTURED FUNCTIONAL THIN FILMS FOR FROZEN FOODS APPLICATION

By

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ABSTRACT OF THE DISSERTATION

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Nano-foods are becoming more common with the implementation of nanotechnological methods to the production, processing & packaging of foods. Food nanotechnology is bringing a new dimension to product development, by allowing the transfer of functionalities from biological molecules observed in nature to foods. Aim of this study is to transfer the ability of extracellular ice nucleators (ECINs) to trigger ice formation at higher sub-zero temperatures to a food packaging material, by creating a nano-thin ECIN layer on it.

Earlier studies indicated shorter freezing times, higher ice nucleation temperatures and quality improvement when ECINs were mixed into foods. It is hypothesized that this effect can be retained with the nano-thin ECIN films. Our objective is to (1) investigate the biopolymer systems suitable for building food grade multilayers, (2) use these multilayers to immobilize ECINs, and (3) evaluate the ice nucleation activity of these films.
Surface morphology of nano-thin films was investigated by atomic force microscopy (AFM), and the surface hydrophobicity was studied by the water contact angle measurements. Layer thicknesses were determined using the quartz crystal microbalance with dissipation monitoring (QCM-D) technique. A refrigerated water/ethylene glycol bath was used to study the ice nucleation activity of nano-thin layers.

Chitosan, ε-polylysine, carrageenan and pectin were good alternatives to synthetic polyelectrolytes in multilayer formation. Parameter that affected the nanoscale properties of these biogenic multilayer systems were the molecular weight, molecular conformation, charge density and degree of esterification. An average of 1.8°C increase in ice nucleation temperatures and 5.5 minutes decrease in freezing times was observed with high purity deionized water samples frozen in ECIN coated LDPE films. Films retained their activity for up to 50 freeze-thaw cycles.

This dissertation establishes a proof of concept for the application of the LbL deposition technique to engineer functional food grade nano-thin films. Preliminary experiments using our films to freeze milk, fish actomyosin and 20% sucrose solution resulted in shorter freezing times, better quality retention and ice nucleation at higher temperatures versus untreated control films. Implementation of this technology in frozen food packaging applications can actualize the significant energy-saving and quality-improvement potentials of nano-thin ECIN films.
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Dedication

To my dear wife Gigi, and Mu (無).
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1. Introduction

This dissertation summarizes a journey from basic science to applied Food Science. Nanotechnology is regarded as the number one emerging technology in Food Science, however the applications have been very limited to date. This project was initiated with the idea of establishing the proof of concept for the possible use of biopolymers to create food grade nano-thin layers for functionality applications in foods. Layer by layer deposition has been studied extensively as a subject of the Material Science discipline, and many systems of synthetic polymers have been investigated and modeled. This allowed advances and inventions in many fields and industrial applications.

The findings in the basic science part of this project, i.e. nanoscale experimentation which indicated that the bio-polymers are as suitable as synthetic polyelectrolytes for multilayer formation, carried this project directly to a food packaging application. Using a functional proteinaceous substance called the Extracellular Ice Nucleating Matter (ECIN), which has a relatively “concentration-independent” functionality similar to that of an enzyme, we have shown that food grade nano-thin multilayers can be created on a food packaging material in which liquid and semi-solid foods can be frozen faster and at higher temperatures than normal, which can contribute to energy savings in the food industry, especially in flash freezing applications. Thus the following two sub sections will briefly introduce some information about the current status of Food Nanotechnology and freezing, which will be followed by background information about ice nucleators, their food applications, nano-enabled food packaging,
the layer by layer deposition (LbL) technique and the natural biopolymers which are suitable for use with this technique.

1.1. Food Nanotechnology

Nanotechnology is the study of imaging, modeling, control and modification of structures in the size range of 1 to 100 nm. It had found applications in several industries including electronics, space research and pharmaceuticals; which were achieved by the basic and applied scientific studies conducted in the disciplines including material science, chemistry, engineering and physics.

Foods are quite complex systems where many biochemical and biological mechanisms are involved, and the properties of foods change drastically following the processes they go through following harvest (Boyacioglu, 2008). Traditionally, foods are produced using unit operations such as heating, drying, freezing, etc. and changing the composition of the ingredients by pH modification, surfactant, salt or sugar addition. Then textural and sensory qualities are examined in the macro-scale and biological safety is generally evaluated in the micro-scale. With the upcoming demand from customers for healthier foods, the nanoscale modification of foods will become more and more important for new product development (Sanguansri and Augustin, 2006). Therefore, the focus of food innovation is recently shifting to nano-scale evaluation which can allow the control and alteration of food molecules in the nano-scale and to evaluate the performed modifications in the nano and macro-scale. This can involve a range of applications such as addition of nano-additives or ingredients modified using nanotechnology, improvements in the delivery, quality, and safety of foods, and nano-scale modification
of food contact materials, i.e. food packaging (Dudo et al., 2011). The market for these materials is discussed in further detail in Section 2.3. However, the market share of food products developed using nanotechnology is unknown because this information is not generally revealed by producers. This is due to the ethical, legal, and social implications abbreviated as ELSI’s, which have affected many emerging technological in the past (Dudo et al., 2011). According to a study conducted in 2007, consumers are hesitant about consuming nano-modified foods or foods in nano-modified packaging, but they believe using nanotechnology in food packaging would be more beneficial than using it in foods (Siegrist et al., 2007). Another study reveals that in terms of the thematic diversity, frequency of coverage and journalistic expertise level, coverage of food nanotechnology in U.S. newspapers can be considered to be “relatively modest” (Dudo et al., 2011).

1.2. Freezing of Foods

Freezing is a very common food preservation method due to superior retention of sensory and nutritional qualities in comparison to other preservation techniques, such as canning, smoking or drying (Erickson and Hung, 1997). Freezing slows down most chemical deterioration reactions, decreases mobility in the medium and most of the water becomes unavailable for bacterial growth in ice form, all contributing to improved shelf life (Le Bail, 2004). It is considered as the unique preservation method to meet the demand for raw materials off season and also allows their transportation to distant geographical locations (Persson and Londahl, 1993).
Although freezing preservation by natural ice has been used by humans for thousands of years, artificial ice was produced for the first time in 1755 by William Cullen, through the evaporation of ether in partial vacuum (Enochian and Woolrich, 1977). Freezing became a commercial preservation technique around 1870's, but freezing operations were still taking place at very slow rates in chambers (Persson and Londahl, 1993), which causes undesired growth of ice crystals. Ice crystal size is a critical factor in freezing which highly affects the quality of the final product as crystals cause tissue damage and drip loss as they grow bigger (Li and Sun, 2002). Rate of heat removal, i.e. freezing rate, affects the number of nucleation sites. At higher freezing rates, nucleation of ice crystals occur simultaneously at more locations (multiple nucleation sites), and as a result of this ice crystals become more numerous but smaller in size (Erickson and Hung, 1997). This was not well known before 1928, which is regarded as the birth year of the modern frozen food industry with the invention of double belt contact freezer by Clarence Birdseye, allowing the first applications of flash freezing (Persson and Londahl, 1993). Today, frozen foods make a significant portion of the food industry. Global value of frozen food market in 2007 was $100 billion, and 41.1% of this belongs to frozen pizza and ready meals (Datamonitor, 2008). Market value is expected to reach a value of $119.9 billion in 2012, with an increase of 19.9% and 23.8 billion kg of production volume for 2007 is forecasted to reach 27.5 billion kg in 2012 (Datamonitor, 2008). US used to have the highest share in frozen food market with 37.5% in 2005 (Business-Wire, 2007), but in 2007 Asia-Pacific was reported as the largest market with 34.3%. Unilever has the highest share of 4% among all companies, followed by Nestle S.A. and Conagra Foods (Datamonitor, 2008).
Freezing is considered as a method that is free from most quality related drawbacks (except for the minimal nutrient loss with time), as long as the cold chain can be kept intact. However high energy demand of forming and maintaining a cold chain is a major shortcoming, making freezing expensive and even unaffordable in some areas of the world. First, freezing requires the removal of latent heat from water, which is the most energy demanding stage on the freezing curve (Le Bail, 2004). Second, for most applications except for special ones such as frozen dough where slow crystallization is favored to preserve yeast activity (Le Bail, 2004), small crystal size is vital which necessitates the application of extremely low temperatures. Air temperatures of -20°C to -40 °C are required in most commercial air-blast freezers (Erickson and Hung, 1997) and some tunnel and plate freezers are operated at conventional temperatures of -40°C, which is extremely energy inefficient as the same heat removal can be obtained with higher temperatures for some cases (Jul, 1984). For storage, temperature required for the quality retention and microbial safety of frozen foods is -18°C or lower (Barbosa-Cánovas et al., 2005). Evaluating the energy demand of freezing as a whole, storage stage makes a significant portion of the overall energy consumption in the freezing chain (Jul, 1984).

Using ice nucleators as a novel method for rapid freezing has been discussed in several reviews (Li and Sun, 2002; Li and Lee, 1995). Antifreeze proteins and ice nucleators were addressed together as they serve the same function for this instance, they can both be added readily into food products and their interaction with the water molecules affects ice crystal size and ice formation patterns (Li and Sun, 2002). Secondary structure of the protein in ECIN is not well known, but all proposed models agree that the protein acts as a template to orient water into ice (Li and Lee, 1995), which
will be discussed further in the following section. Theory behind the mechanism of ice nucleation activity will also be discussed there in further detail.

Based on this rationale, this study focuses on freezing foods at higher temperatures by minimizing the supercooling stage, using a novel approach to produce nanoscale films which contain significantly lower amounts of ECIN than that required when adding it into a food solution. If proven to be successful, this multilayer fabrication approach can also be used for other charged biopolymers to produce nanofilms of different functionalities.
2. Background Information

Distilled water with no impurities placed in a perfectly smooth container can be supercooled to as low as -40ºC in liquid state (Lundheim, 2002). Formation of ice requires the presence of a nucleus to initiate crystallization of the macroscopic mass. When the nucleus reaches the critical size of seed crystals they can act as sites of ice nucleation far above the temperatures required for homogeneous nucleation (Duman, 2001). In applications where ice nucleation is desired, addition of substances known to have higher ice nucleation activity than naturally present impurities can initiate crystallization at higher temperatures and in shorter times.

2.1. Ice Nucleators

are defined as substances that can initiate nucleation by organizing water molecules into an ice-like pattern, but since this is a very broad definition including all structures that can act as impurities and lead to heterogeneous nucleation at subzero temperatures, this term generally refers to substances which can initiate nucleation at temperatures above -10ºC (Lundheim, 2002). A specific gene, initially discovered in several plant pathogens that are known to cause cold damage, is found to encode for a protein which acts as an ice nucleator, activity of which is enhanced in the presence of sugar and lipid components. This lipoglycoprotein is attached to the cell wall of the bacteria that is producing it. However, it retains its activity when detached from the cell, and this cell free matter is termed ECIN.
Bacterial ice nucleators have been the focus of interest since 1972, which is the year *Pseudomonas syringae* was first identified as an ice nucleator in decaying leaves (Maki et al., 1974). Earlier, researchers believed that nucleation activity is a property of the intact cell, as the activity was lost after chemical treatments or destruction of the cell. Later it was understood that activity is related to a proteinaceous (due to the inhibitory effect of proteases and sulfhydryl modifying chemicals on activity) matter that is bound to the outer cell membrane, and bacteria such as *Erwinia herbicola* shed them to the growth medium in vesicles (Phelps et al., 1986). There is limited scientific information about the structure of extracellular ice nucleating matter for it is hard to study structures bound to the cell wall (Duman, 2001), but chemically, the class of ECINs with the highest nucleation activity, also termed Class A (Turner et al., 1990) are known to be lipoglycoproteins that are covalently anchored to the cell wall via a mannose-phosphatidylinositol (PI) complex (Kozloff et al., 1991), activity of which decreases when the protein is further purified from lipid and carbohydrate components (Muruyoi et al., 2003). For instance, Class B is a glycoprotein with sugar residues such as glucose and mannose attached to the protein core, that has lower ice nucleation activity than Class A, where Class C is composed of aggregates of ice- nucleating protein (INP) with the lowest ice nucleation activity among the three classes (Turner et al., 1991). Class C extracted from *Pseudomonas syringae* has been modeled as a protein with non-repetitive N and C terminal domains (175 and 49 residues respectively), and a repetitive central domain of 48 residues consisting of 3 repeats of 16 residues believed to be the source of nucleation activity (Graether and Jia, 2001). Alternatively, the more hydrophobic N terminal of most ice nucleating proteins of similar primary structure was suggested to make up
approximately 15% of the whole sequence where the highly hydrophilic C terminal makes up 4% with basic residues and the central domain which is also hydrophilic makes up the remaining 81% and dominantly contains alanine, glycine, serine and threonine (Li and Lee, 1995).

In the *Pseudomonas syringae* model, the repetitive region contains a T\(x\)T (\(x\) can be any amino acid in that sequence) motif which is also present on antifreeze proteins (AFP), mutation of which causes a 70% or higher loss of nucleation activity (Graether and Jia, 2001). This supports the proposal that AFP and INP’s have similar \(\beta\)-helical structure and their contrary functions can be related to the size of the protein surface which is interacting with the water or ice (Graether and Jia, 2001).

### 2.1.1 Food Grade Ice Nucleators

*Erwinia herbicola* is a plant pathogen, however there are no reports to date about the human pathogenity. Also, ECIN goes through several procedures to be completely freed from the bacterial cells, and this is verified by plating on agar media to see if there are any remaining viable cells. Still, it may be a challenging procedure to have it approved as a legal food ingredient due to the pythopathogenity of the source bacteria.

*Xanthomonas* species are already approved for food use and there are no reports to indicate human pathogenity (Watanabe et al., 1993). Also, it is considered Biosafety Level 1 according to ATCC, which is described as “not known to cause disease in healthy adult human”. *Xanthomonas campestris* is an important bacteria for its ability to produce xanthan gum through sugar fermentation. Xanthan gum is the first industrially produced biopolymer approved for use in food products by the FDA in 1969 (Rosalam and England, 2006). Since *Xanthomonas* species are currently being used to produce gum for
food applications, it will also be more likely to have FDA approve this bacterium for ECIN production.

Studies on INA positive *Xanthomonas* species is very limited. A novel strain of *Xanthomonas campestris* was isolated from tea shoots for the purpose of food grade ECIN production and named *Xanthomonas campestris* INXC-1 (Watanabe et al., 1993). However, this strain couldn’t be found in common culture collections and is not available through this research group. Another group used a strain with code IFO (Kawahara and Obata, 1998), which stands for Institute for Fermentation in Osaka, Japan. This collection was later transferred to NITE (National Institute of Technology and Evaluation) Biological Resource Center (NBRC) in Kazusa, Japan. IFO 13559 strain was obtained from this collection, and studies on the production and isolation of ECINs from this bacteria were performed.

### 2.1.2 Procedure for the Production and Isolation of ECINs from *Xanthomonas campestris*

The problem with the extraction of ECINs from *Xanthomonas campestris* is that xanthan gum is also being produced simultaneously, and cells are getting entrapped in the gum which makes it hard to extract the ECINs. Addition of lactate into the growth medium was shown to decrease gum production and enhance cell growth for the INXC-1 strain (Watanabe et al., 1993). They used a basal medium with yeast extract, bacto-peptone and magnesium sulfate, and evaluated the effect of other ingredients and also the cultivation time. Glycerol, succinate and sucrose promoted cell growth where salt had an inhibitory effect. Increased incubation time led to higher nucleation temperatures, which
suggests that protein monomers released by the cells accumulate together to form larger clusters possessing higher nucleation activity, thus an incubation time of 3 days was recommended (Watanabe et al., 1993).

Effect of carbon source on cell growth, INA, xanthan gum production and viscosity was studied and lactose was found to decrease xanthan gum production by 5 times when compared to that with glucose in the medium for the IFO 13599 strain as well (Kawahara and Obata, 1998). The gum was also structurally different as indicated by its lower viscosity, and HPLC studies showed that this is due to its defective structure, i.e. missing pyruvic acid residues in the side chain, which is found in commercial xanthan gum and contributes to its high viscosity. Lower viscosity of the xanthan gum is favorable for the separation of ECINs from the solution by centrifugation. Ice nucleation temperature was highest at a whey concentration of 5% (xanthan gum yield is maximized at 6%), and incubation temperature and time of 18°C and 96 hours respectively were the most favorable conditions for ice nucleation activity (Kawahara and Obata, 1998).

These were the starting conditions for trials to see if ECINs can be effectively produced and isolated. Several experiments were conducted by Ke Shi from our group to improve the yield of ECINs produced by *Xanthomonas campestris*. Their work is currently in progress and challenges are present regarding the low ice nucleation activity of this strain, thus throughout this study ECINs from *Erwinia herbicola* were used. This also assured that the results are comparable to previous studies conducted by Dr. Lee’s group in different food solutions where ECINs were directly added into the formulation as an ingredient (Li et al., 1997; Li and Lee, 1995, 1998a; Li and Lee, 1998b; Ryder and Lee, 1987; Van Sleeuw and Lee, 2003; Zasypkin and Lee, 1999; Zhu and Lee, 2007).
2.2. Food Applications of Ice Nucleators

Potential of ice nucleators have been studied in food products by several research groups to increase freeze-thaw resistance thus stability of frozen products, to freeze dry or concentrate food products and modify texture (Hwang et al., 2001; Li-Jung Yin et al., 2005; Li et al., 1997; Li and Lee, 1998a; Ryder and Lee, 1988; Ryder and Lee, 1987; Van Sleeuwen and Lee, 2003; Watanabe et al., 1991; Watanabe and Arai, 1986; Watanabe and Arai, 1994; Watanabe Michiko et al., 1989; Zhu and Lee, 2007).

Whole cells were used first by Watanabe and Arai in 1986, to by-pass the supercooling stage in freeze drying of high salt containing foods. Researchers were able to freeze corn starch, soybean protein isolate and soy sauce in shorter times and produce powdered products more efficiently. They also used Scanning Electron Microscopy (SEM) to observe the effect of adding ice nucleating bacteria on texture of soy sauce and soybean paste and studied the effect of pH on nucleation activity, which was retained in the pH range of 5-10 (Watanabe and Arai, 1986). The same group studied freeze concentration of raw egg white and lemon juice in 1989, using *Erwinia ananas* cells entrapped in calcium alginate to form an ice nucleating gel which allows the positioning of ice nucleus at a desired fixed position in the liquid product. Foaming ability which is the most important function for the egg white product was not affected by the addition of ice nucleating gel and changes in quality attributes for lemon juice were found to be insignificant, suggesting the possible application of ice nucleating bacteria to replace conventional ice crystal seeding method in freeze concentration (Watanabe Michiko et al., 1989). They also conducted experiments on other foods such as fresh milk, and strawberry paste separated into pulp and juice fractions (Watanabe and Arai, 1994). They
were able to produce strawberry jam without heat treatment which was similar in texture but superior to conventional jams in terms of flavor and color, by freeze concentrating the juice and mixing it with the pulp, sugar, pectin and citric acid, followed by high pressure sterilization to kill the added *Erwinia ananas* cells (Watanabe et al., 1991).

Many studies were conducted by Lee’s group on the applications of ice nucleators in food industry, mainly focusing on freezing, freeze drying, concentration and texturizing, as they pointed out as future aspects in a review where results of several unpublished data from earlier trials with salmon muscle (Ryder and Lee, 1987) and other food systems were also referenced (Li and Lee, 1995). They used whole cells of *Erwinia herbicola* and *Pseudomonas syringae* first to study the effect on major food components including sugars, oil/water suspensions and protein solutions (Li et al., 1997). Freezing times were shortened and nucleation temperatures were significantly increased in all systems, including sucrose solution which doesn’t freeze at -6°C without added ice nucleator cells but it does when added. Later they worked on enhancing the production of extracellular ice nucleators from *Erwinia herbicola* (Li and Lee, 1998c), and they applied the cell free ice nucleating matter to a variety of food systems including commercial milk, juices, ice cream, yogurt, soybean curd, ground beef, frankfurter, fish fillet, eggs, lab grade sucrose, egg white protein and starch. Freezing curves verified increased sub-zero temperatures and shortened times for the initiation of ice nucleation for liquid and semi-solid foods, and some foods that wouldn’t freeze at -6°C without ECINs were frozen when ECINs are added, similar to the observations with the added ice nucleation active (INA) cells. Lack of this effect in solid foods can be related to lower thermal conductivity and decreased mobility. Differential scanning calorimetry (DSC) and
cooling stage microscopy confirmed the change in ice formation patterns that leads to changes in the textural properties of food, which can either be favorable or unfavorable depending on the sensory attributes of the food system of interest (Li and Lee, 1998b).

A study was performed to transfer the ice nucleation gene from *Erwinia herbicola* to *Saccharomyces cerevisiae*, a food grade yeast used in baking, to overcome the potential constraints related to the use of plant pathogenic bacteria in food products (Hwang et al., 2001). Although activity wasn’t as high as in the original strain, they were able to obtain INA+ yeast and confirmed the nucleation effect on freezing and texturization of milk, soybean curd and juices. Another strain obtained by the same group by transferring the INA gene of *Erwinia herbicola* to *E.coli* M15 (Hwang and Lee, 1999) was used in Tylose, a meat simulator, and more uniformly distributed, smaller sized crystals were obtained which is desired in freezing for the preservation of quality (Van Sleeuwen and Lee, 2003), as explained earlier. Another important quality for frozen foods, resistance to the freeze-thaw cycles that may be caused by unavoidable breakages in the cold chain, was evaluated using *Erwinia herbicola* ECINs with fish actomyosin obtained from commercial Tilapia (Zhu and Lee, 2007). Gel forming capacity and textural stability which are two most important quality attributes of this product, were preserved significantly better with the addition of ECINs, when compared to control samples.

Another group from China working on biogenic ice nucleators, used *Pseudomonas fluorescens* MACK-4, an INA+ strain obtained from Mackerel surface, to investigate the effect of whole cells and their ECINs on the ice nucleating temperatures of water, fresh full-cream milk, orange juice, 10% soluble starch and mackerel mince (Li-
Jung et al., 2005). They also investigated the stability of ECINs versus pH and confirmed stability in the range of 6-9, with maximal ice nucleation activity at pH 6. Addition of glycerol lowered ice nucleation activity of both ECINs and cells, while maltose, trehalose and sucrose did not have an effect in that pH range. Ice nucleation temperatures were elevated for water, milk and starch solution, however no change was observed for orange juice and mackerel mince. They proposed the lower heat conductivity and limited mobility disallowing ice growth, similar to our proposal above for the same phenomenon observed with other solid foods (Li and Lee, 1998b). For orange juice, authors suggested that this can be related to the suspended solid orange particles acting as ice nucleators during freezing thus a solid food-like freezing behavior is observed (Li-Jung Yin et al., 2005)

In all studies to date, ECIN’s were mixed into the food products, and there is no study so far which investigates the ice nucleating activity of ECIN’s when applied as a nanothin layer.

2.3. Nano-Enabled Packaging Materials

Nano-foods are becoming more and more common, with the increasing use of nanotechnological methods in cultivation, production, processing, or packaging of foods (Tiju and Morrison, 2006). Nano-enabled food and beverage packaging market was worth $ 4.13 billion in 2008 and is forecasted to grow to $7.3 billion by 2014, where the intelligent packaging segment is estimated to grow to $2.47 billion (Plastemart, 2010).

Besides the fundamental functions of food packaging such as the quality and safety improvement, shelf life extension, containment, protection and preservation; it also serves as a marketing and communications aid. Use of polymer nanotechnology is meant
to improve mechanical, barrier and antimicrobial properties of food packaging materials; and they can also help trace and monitor the condition of foods during transport and storage using nano-sensors (Silvestre et al., 2011), thus adding the monitoring dimension to the improvement of safety and quality.

LDPE is a thermoplastic obtained from petroleum and is commonly used to produce plastic bags, containers and bottles. 67% of global demand for LDPE comes from the market of carrying bags, sacks and films, such as agricultural, multi-layer, and shrink films (Ceresana-Research, 2010). Such films are frequently pretreated either to increase their wettability or to modify their surface chemistry, which allows the adhesion paint and ink or formation of composites via lamination (Mathieson and Bradley, 1996). Ultraviolet ozone (UVO) treatment, a photosensitized oxidation method utilizing short wavelength UV radiation to excite and dissociate molecules, has been used earlier in the surface modification of polyethylene films (Mathieson and Bradley, 1996; Peeling and Clark, 1983; Ton-That et al., 1999). Hydrocarbons react with the atomic oxygen that was generated by the dissociation of ozone following UV radiation at 253.7nm, which forms by the dissociation of molecular oxygen by UV radiation at 184.9nm (Ozcam et al., 2009). As the excited groups react with atomic oxygen and form volatile compounds like CO₂, concentration of hydrophilic groups such as carboxyl and aldehyde also increases on the surface, which consequently imparts increased wettability and overall negative charge to the treated surface. A study on the oxidation of polyethylene via UVO treatment, demonstrated through X-ray Photoelectron Spectroscopy (XPS) analysis that the relative atomic percentage of carboxylic acids on the surface increases as the UVO exposure time increase in the range of 0 to 50 minutes (Zander et al., 2009).
2.4. Layer by Layer (LbL) Deposition Technique

Some methods currently being used in nanotechnology were already known before nanoscale instrumentation allowed us to review them with these tools. Layer by Layer deposition is one of these techniques used in the fabrication of thin films on surfaces (Decher, 1997). One of the conventional applications of LbL deposition is a simple technique which relies mainly on the electrostatic interactions of oppositely charged polyelectrolytes for the multilayer self-assembly (Zhang et al., 2007). This enables finer and more robust multilayers than those that can be obtained by physical adsorption, as electrostatic attraction between the layers and with the substrate are stronger (Cai et al., 2005). It is a simple yet powerful way of engineering nano-thin polymer layers, allowing control over the molecular architecture and composition of the surface to be fabricated (Zhang et al., 2007). Application of this method dates back to the multilayer experiments of Iler with charged colloids in 1966, which later was shown by Fromherz in 1980 with protein-polyelectrolyte multilayers (Decher, 1997).

Briefly, the procedure involves dipping of a charged substrate into a solution of molecules with opposite charge followed by dipping into another solution containing molecules of same charge after the rinse step, over-compensating the net charge on the surface in each step which allows the fabrication of multilayers (Decher, 1997). Thus, it is very easy to perform and the only requirement is that the polyelectrolytes are soluble in water. Other advantages that come with the LbL technique are:

1) It is simple and no expensive or complicated instruments are required,

2) Different building blocks can be incorporated into a multilayer by design which allows one to obtain several properties with a film of a targeted molecular structure,
3) It can be applied to any charged substrate of any shape or size (Zhang et al., 2007).

The Langmuir-Blodgett (LB) method was the standard for building nanoscale films during the last 60 years. However, the LbL method has the potential to replace it since the LB method requires special instruments and has limitations, such as substrate size and shape as well as film stability and quality (Decher, 1997).

2.5. Natural Biopolyelectrolytes

Four different types of common food biopolymers are used in this study, two of which are anionic and the other two are cationic. Pectins are commercially extracted from land plants such as citrus peel, apple pomace, lemon and lime peels (Bemiller and Whistler, 1985). Poly epsilon-lysine (ε-PL) is a naturally occurring, water soluble, biodegradable, edible and non-toxic biopolymer which is microbially synthesized and commonly used in the food industry for a variety of functions (Shih et al., 2006). Chitosan and carrageenan are two marine sourced polysaccharides possessing several advantages in addition to being edible, such as biocompatibility, non-cytotoxicity, suitability for trapping and encapsulating biological substances, being renewable and commercial availability at low costs (Bartkowiak and Hunkeler, 2001).

Chitosan is obtained by deacetylation of chitin (an exoskeleton, structure-forming polysaccharide obtained during krill, shrimp, and crab processing as a by-product) under strongly alkaline conditions and it is the second most abundant renewable organic resource after cellulose, as cited in (Shumilina and Shchipunov, 2002). Chitosan is special for being a positively charged biopolyelectrolyte due to the amine groups in its structure (Figure 1). At a deacetylation degree of 85%, chitosan holds one ammonium group for approximately each 1.2 saccharide unit, which allows it to form complexes
with negatively charged polymers (Kujawa et al., 2005). In addition to the advantages summarized above, chitosan can be of use in food industry due to the antiseptic and inhibitory effects on the growth of pathogenic bacteria and molds (Shumilina and Shchipunov, 2002). Currently it has more applications in the pharmaceutical industry for the delivery of drug and delicate macromolecules, antibacterial applications and tissue engineering (Janes et al., 2001). It is also being used as an enterosorbent for weight loss products (Shumilina and Shchipunov, 2002), since chitosan is able to absorb 10-12 times its molecular weight of fat in the digestive track.

![Molecular structure of chitosan repeating unit.](image)

**Figure 1.** Molecular structure of chitosan repeating unit.

ε-PL is a homopolymeric amide formed by the peptide linkages between the ε-amino and α-carboxyl groups of L-lysine monomers (Shih et al., 2006). Lysine is an essential amino acid which is positively charged due to the presence of ε-amino groups in its structure (Figure 2). Its cationic nature makes it a good antimicrobial as it can electrostatically attach to the anionic cell walls of bacteria, yeast and fungi and disintegrating the cell wall they lead to the lysis of the cell (Yoshida and Nagasawa, 2003). Since the isoelectric point of ε-PL is 9 which is relatively high, it can carry its
positive charge in a wider pH range, consequently in a larger group of food products, ideally in the pH range of 5-8 (Curylo et al., 2008). Thus, \( \varepsilon \)-PL is a commonly used food preservative in Japan since 1980’s and has “Generally Recognized as Safe” (GRAS) status since July 2003 in the US for use as an antimicrobial in certain foods in the range of 5-50 ppm (FDA, 2004). It is industrially produced in large scale in Japan by fermentation, using a mutant of \textit{Streptomyces albulus} (Hiraki, 2000). \( \varepsilon \)-PL is also being used as an emulsifying agent, a dietary agent, a drug-delivery carrier, a gene delivery carrier, in hydrogel development, and as a coating material for biochips and bioelectronics, as summarized in (Curylo et al., 2008).

![Figure 2. Molecular structure of \( \varepsilon \)-PL.](image)

Chitosan is an inexpensive and non-toxic biopolymer (Yang et al., 2007), however \( \varepsilon \)-PL has better solubility in water and GRAS status since July 2003 (not self affirmed like chitosan currently is) in the range of 5-50 ppm, which made \( \varepsilon \)-PL the focus of interest for several industrial applications such as food preservation, emulsification and biodegradable fiber applications (Shih et al., 2006). It may also be a good alternative to make nano-enabled food surfaces and packaging materials.
Carrageenan is a high sulfate containing, linear, anionic heteropolysaccharide, commercially extracted from red marine algae (Bartkowiak and Hunkeler, 2001). Carrageenans are used as gelling agents and thickeners in food, cosmetics and pharmaceutical industries (Shumilina and Shchipunov, 2002). Their ability to react with water but more importantly with milk proteins leads to higher viscosities, and stability at low pH values (as the sulphate groups are always ionized), allows them to be used as thickeners for chocolate milk, ice cream, emulsions, infant formulas and whipped cream (Bemiller and Whistler, 1985). This family of sulfated galactans are categorized by their charge densities (Figure 3); and lambda (λ-), iota (ι-), kappa (κ-) carrageenans and furcellaran (aka Danish Agar, a polysaccharide very similar to κ-carrageenans in structure) have 2.07, 1.53, 0.92 and 0.69 sulfate groups per disaccharide unit, respectively (Andreas Hugerth, 2001).

However, these idealized disaccharide units (depicted in Figure 3) for each type are not the homogeneous repeating units of commercially available carrageenans, they contain mixtures of these three types where one type can be dominant but is not necessarily free from other two types. Food scientists and formulators mix carrageenans to obtain the desired properties in terms of syneresis, tenderness, clarity, elasticity, heat stability and thermal irreversibility of the gel (Bemiller and Whistler, 1985). Molecular weights of lambda, iota, kappa carrageenans and furcellaran are reported as 533, 469, 404, 380 kDa, respectively (Hugerth and Sundelöf, 2001).
Figure 3. Idealized disaccharide units of carrageenans of different charge densities. Functional groups (R) seen in the λ and ι-carrageenan images stand for sulfate groups (SO₃).

Pectins are polysaccharides made of D-galacturonic acid units connected via α-(1-4) linkages (Figure 4). Carboxyl groups are commonly esterified as methoxyl groups in nature, and the percentage of esterified groups, a.k.a. degree of esterification, is an important parameter in their classification as either high methoxyl (HM - >50%) or low methoxyl (LM - < 50%) pectins (Worth, 1967). In LM pectins, carboxyl groups can also be amidated, in salt form or unneutralized, depending on the source and extraction procedures (Bemiller and Whistler, 1985). This makes pectic structures a large group of complicated polysaccharides varying in terms of function (Ridley et al., 2001). HM pectins are commonly used in the food industry as thickening agents, especially in jams.
and jellies since they produce spreadable gels by the addition of sugar and acids (Bemiller and Whistler, 1985).

LbL deposition of biopolymers is possible owing to their electrostatic nature, and a substantial amount of research has been conducted in this area, which is attractive due to the potential for use in many technological applications, as cited in (Kujawa et al., 2005). Especially chitosan and carrageenan were forms stable complexes (Bartkowiak and Hunkeler, 2001), and polyelectrolyte complexes of these two polymers were studied by varying parameters such as charge density of carrageenan and degree of acetylation for chitosan (Carlos Peniche, 2001; Hugerth et al., 1997). Coating of meats with carrageenans was suggested as a mechanical barrier and carrier for flavors earlier (Bemiller and Whistler, 1985). Effect of temperature, pH and charge density of carrageenan on microcapsule formation using oligochitosan and carrageenan was studied, and authors pointed out the better stability when iota type carrageenans are used and also the possibility of using those complexes as temperature controlled delivery systems (Bartkowiak and Hunkeler, 2001).

Figure 4. Galactronic acid units of the pectin chain.
Carrageenan microspheres were coated with chitosan using the emulsification/thermal gelation approach to encapsulate Brewer’s yeast (Raymond et al., 2004). LbL deposition of chitosan and κ-carrageenan was studied earlier to fabricate bio-inert, anti-adhesive and antibacterial coatings for biomaterial implant surfaces (Bratskaya et al., 2007). Interaction of chitosan with synthetic polymers to build multilayers using the LbL technique has also attracted attention (Kujawa et al., 2005).

3. **Hypothesis**

1. Food grade nano-thin films can be fabricated using bio-polyelectrolytes.
2. Food grade nano-thin films can be used to anchor ice nucleators onto packaging film surfaces to freeze foods at higher temperatures.
3. ECINs can retain their functions when applied as a nano-thin film.

4. **Objectives**

1. To verify that the ice nucleation activity of ECIN’s can be retained when applied as an ultrathin layer to a surface, using multilayers formed by strong synthetic polyelectrolytes and to evaluate their properties.
2. To study several bio-polymer systems that can be used to fabricate multilayers using the LbL deposition method to act as a foundation for the electrostatic adsorption of ECIN’s on the surface.
3. To study the nanoscale properties of these systems, such as surface morphology of ultrathin polymer multilayers using Atomic Force Microscopy (AFM), determining the layer thickness and absorption kinetics using the Quartz Crystal Microbalance.
with Dissipation (QCM-D) technique, and confirming layer adsorption based on the changes in surface hydrophobicity by measuring water contact angles.

4. To fabricate UVO treated Low Density Polyethylene (LDPE) packaging materials coated with a nano-thin ECIN layer using the LbL technique, i.e. by dipping them in positively and negatively charged polymer solutions consecutively to build ultrathin multilayers, followed by dipping into the ECIN solution to create an ice nucleation active surface.

5. To study the ice nucleation activity and reusability of ECIN coated LDPE films with high purity dionized water and foods, and to evaluate the efficiency of this technology by collecting freezing curves on a cooling bath.
5. Materials and Methods

5.1. Materials

*Erwinia herbicola* subsp. *ananas*, (Cat. No. 11530, ATCC, Rockville, MD) was kindly provided by the group of Prof. T.C. Lee from Rutgers University, Food Science Department. Bacteria with this catalog number was renamed to *Pantoea ananas* after 1995 (Zasypkin and Lee, 1999). *P. ananatis* which is sometimes referred to as *Erwinia urodovara* in literature, is a different species from the same genus but has similar ice nucleation activity (Muryoi et al., 2003). Cationic poly (diallyldimethylammonium chloride) (PDDA) and anionic poly(styrenesulfonate) (PSS) purchased from Sigma-Aldrich (St. Louis, MO), were the synthetic polymers that were used to build multilayers. As natural alternatives, chitosans of 1 kDa and 10 kDa molecular weight were purchased from Kitto Life Co. Ltd. (Seoul, Korea) and 44, 130 and 330 kDa from Kunpoong Bio. Co. Ltd. (South Korea). Degree of deacetylation (DOD) for all chitosans was in the range of 98-99%. ε-PL was purchased from Zhejiang Silver-Elephant Bioengineering Co., Ltd. (China). Pectins with different degrees of esterification and amidation were acquired from Danisco (Grindsted, Denmark) and Sigma Aldrich (St. Louis, MO) (as specified in Section 5.4). Furcellaran was a gift from FMC Biopolymer (Princeton, NJ). Kappa carrageenan (type I, CAS# 9000-07-1), iota carrageenan (type II, CAS# 9062-07-1), lambda carrageenan (type IV, 9064-57-7) were purchased from Sigma Aldrich (St. Louis, MO). LDPE films were obtained by cutting and using the inner surface of sterile TWIRL'EM® sampling bags (EFL-1012) manufactured by Labplas (QC, Canada), in accordance with FDA regulations. H₂O₂, H₂SO₄ were purchased from Sigma-Aldrich (St.
Louis, MO), and all chemicals were used as received without further purification. Silicon wafers were purchased from Montco Silicon Technologies (Spring City, PA). For QCM-D analysis, gold coated quartz crystals with a frequency of 5 MHz manufactured by Q-Sense AB (Sweden) were used. In all procedures, ultrapure water (Milli-Q plus system, Millipore) with a resistivity of 18.2 MΩ·cm was used.

5.2. Production and Isolation of Extracellular Ice Nucleators

ECIN’s were obtained and purified using the procedures developed by Dr. Lee’s group (Li and Lee, 1998b). Erwinia herbicola was grown at 18°C in yeast extract, followed by purification of ECINs using centrifugation, sonication, filtration and ultracentrifugation. Then the pellet was suspended in Tris-buffer, freeze-dried and stored at -20°C (Zhu and Lee, 2007). To evaluate the effect of ultracentrifugation speed on ice nucleators, supernatants were centrifuged using a Beckman ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) equipped with a 60Ti rotor for 1 hour cumulatively, at speeds starting from 10,000 rpm to 50,000 rpm. For instance, the 30,000 rpm sample was centrifuged for a total of 3 hours, starting with 1 hour at 10,000 rpm, another hour at 20,000 rpm with the supernatant removed from the first step, and similarly another hour at 30,000 rpm. When converted into average RCF values for the specific rotor used, centrifugation speeds are 7,100xg, 28,400xg, 63,900xg, 113,600xg and 177,500xg from 10,000 to 50,000 rpm. These samples were used when investigating the effect of centrifuge speed on the molecular morphology/aggregate size and ice nucleation activity, for all other experiments ECINs prepared by the standard extraction procedures were used.
5.3. LbL Film Preparation with Synthetic Polymers

Incremental bilayers of \((\text{PDDA/PSS})_n\) (\(n\) from 1-6) were fabricated on silicon wafers, to serve as a foundation for anchoring ECIN’s. Multilayer films were deposited onto silicon wafers, which were cleaned earlier in a slightly boiled piranha solution (7:3 mixture of 98% \(\text{H}_2\text{SO}_4\) and 30% \(\text{H}_2\text{O}_2\)) for 30 min, then rinsed with copious amounts of Milli-Q water and dried with nitrogen gas. The cleaned silicon wafers cut in 1x1 cm pieces were immersed consecutively in 1mg/mL PDDA and PSS solutions, and finally in either 0.01, 0.1 or 0.5 mg/mL of ECIN solution.

For the fabrication of first layer, wafers were kept in PDDA solution for 1 hour to obtain a strong base of positively charged polyelectrolyte, but for successive layers of PSS, PDDA and ECIN as the top layer, holding time was 20 minutes. Wafers were cleaned with Milli-Q water and flushed with gaseous nitrogen each time before dipping in successive solutions. Wafers to be analyzed for surface morphology were placed in a petri dish covered with parafilm (punched earlier to allow removal of moisture) and vacuum dried overnight prior to AFM measurement.

5.4. LbL Film Preparation with the Biopolymers

After the experiment was successfully performed with the strong synthetic polyelectrolyte system, it was repeated with biopolymers. Different molecular weights (1-330 kDa) of chitosan were used to interact with carrageenans of different charge densities (furcellaran, kappa, iota and lambda, in the respective order of low to high charge density). In the second system, carrageenan was replaced with pectins of different
degrees of esterification (D.E.) and amidation (D.A.) (Danisco Grindsted LA 410-DE:29-33%, DA:20%, LC 950-DE:31-33% and Sigma P9561-DE:90%)

1 mg/mL (0.1%) solutions of chitosans, carrageenans and pectins were prepared in 0.01 M NaCl and 0.01 M acetate buffer of pH 3.74. Carrageenans and pectins were heated at 80°C and stirred at this temperature using an impeller for half an hour. Chitosans were stirred overnight and filtered through a 0.45 µm sized filter. Up to 6 bilayers of chitosan with pectin or carrageenan pairs were fabricated using the same procedure followed for PSS/PDDA, with and without ECIN of different concentrations, as the top surface. ε–PL was used as an alternative to chitosan on the LDPE substrate.

5.5. LbL Film Preparation on the LDPE Surface

As the first step, PE bag was cut into 1x2 cm pieces and UV ozone treatments in the time range of 30 seconds to 10 minutes was applied using a UVO Cleaner (Model No. 42, Jelight Company Inc, Irvine, CA), followed by water contact angle measurements.

A multilayer system of biopolymers was used to provide the positively charged surface for the adsorption of ECINs onto the PE film. ε-PL was used as the positively charged biopolymer where iota carrageenan (i-carr) was used as the negatively charged biopolymer. I-carr solution was prepared in 0.01 M NaCl and 0.01 M acetate buffer of pH 3.74. It was heated at 80°C and stirred at this temperature using an impeller for an hour and diluted 10 fold to obtain a concentration of 1 mg/mL. ECIN @ 0.1 mg/mL and 0.5 mg/mL, and ε-PL@ 1 mg/mL were prepared by stirring in Milli-Q water with no buffer until fully dissolved. Solutions were filtered through 0.45 µm sized syringe filters (Catalog #28143-286, VWR International, West Chester, PA).
Multilayers were prepared by initially dipping the film into the positively charged polymer, ε-PL, for an hour followed by rinsing with Milli-Q water to get rid of the excess solution and drying it with a blow drier using cold air. Then the film is dipped into the iota carrageenan solution for 20 minutes, followed by the same after-procedure, and then procedure is repeated to build the third layer with ε-PL. The final layer is formed by dipping the film into the negatively charged ECIN solution for another 20 minutes, yielding a 2 bilayer system on the polyethylene substrate.

For the initial experiment, coated and control PE films were folded and heat sealed from 2 sides using an Impulse Sealer (Model AIE-300, American International Electric, Whittier, CA), then filled with Milli-Q water and sealed from the top as the last step. Same procedure was followed with the untreated control. Afterwards, both samples were cooled in the water bath to -6°C.

Multilayer films were also deposited onto silicon wafers since the polyethylene film is too rough a substrate to image the polymers with the AFM. Wafers were cleaned earlier in a slightly boiled piranha solution (7:3 mixture of 98% H₂SO₄ and 30% H₂O₂) for 30 min, then rinsed with copious amount of Milli-Q water and flushed with the nitrogen gas. The cleaned silicon wafers cut in 1x1 cm pieces were immersed consecutively into the polyelectrolyte solutions to build the multilayers, as described above for the polyethylene substrate. Wafers were cleaned with Milli-Q water and flushed with gaseous nitrogen each time before dipping in successive solutions. Wafers to be analyzed for surface morphology were placed in a petri dish covered with punched parafilm (Parafilm “M”, Pechiney Plastic Packaging, Chicago, IL), to allow removal of moisture and vacuum dried overnight prior to AFM measurement in an Isotemp Vacuum Oven (Model 282-A,
Fisher Scientific, Waltham, MA). Following day, top ECIN, ε-PL and iota carragenan surfaces were imaged with the AFM and water contact angles were measured to analyze hydrophilicity.

5.6. Verification of Ice Nucleation Activity

Activity of ECIN as an ultrathin layer was confirmed with a refrigerated water/ethylene glycol bath (Model 1156D, VWR International, West Chester, PA), which was used to float blank and ECIN covered wafers, and freezing time of several 10 µL Milli-Q water droplets on those ECIN coated wafers at -8°C was the criteria to observe the heterogeneous nucleation on the surface. Images of droplets have been captured with a digital camera.

Activity of ECIN’s purified from cells was measured quantitatively using Vali’s droplet freezing assay (Vali, 1971) as modified by (Li et al., 1997). This method which allows the determination of ice nucleation activity as a unit using Vali’s formula, is slightly different from how we determined the ice nucleation activity of the thin ECIN layer, but the same temperatures were applied using the same cooling system. Instead of pure 10 µL Milli-Q water droplets being placed on an ultrathin ECIN layer fabricated on a silicon wafer and recording the freezing time and temperature of droplets, same volume of droplets from dilutions of ECIN prepared in Milli-Q water were placed on polished aluminum weighing dishes (Catalog Number 8-732, Size 57 mm, Fisher Scientific Inc., Pittsburg, PA), to minimize the contribution of a potentially rough surface to the ice nucleation temperature of the ECIN droplets. Total number of droplets placed on these dishes was 20. After fixing the temperature to the desired level, dish with the droplets
was kept at this temperature for 3 minutes and number of frozen droplets was counted. The ratio of frozen droplets to the total number of droplets (fraction of frozen droplets) was used as a parameter (f) in Vali’s formula to calculate the ice nucleation activity (INA) at temperature T, where V is the volume of the droplet (10 µL) and D is the dilution which gives a frozen droplet number in the range of 5 to 18, out of the 20 droplets:

\[ INA(T) = -\frac{\ln(1-f)}{V \cdot D} \]

The limitation of this method is that it cannot be used to quantify the ice nucleation activity of a thin film, since such a film cannot be diluted like the solutions of ECIN. However this method was used to study the correlation between the ice nucleation activity and size of ECIN globules measured using the AFM images, as discussed in Section 6.2.

Ice nucleation activity of ECIN coated LDPE films were evaluated using the same cooling bath, which was used to float the PE bags filled with Milli-Q water and to dip the vials in, which are coated on the inside with the PE films. For the experiments with the PE pags, temperature was set to -6°C and then increased to -8°C. Inner walls (not the base) of a cylindrical mini-vial with a base diameter of 2 cm and a height of 4.5 cm was covered with an ECIN coated PE film of ε-PL/Iota/ε-PL/ECIN multilayers. Vial was re-filled with 5 ml of fresh Milli-Q water before each freeze-thaw cycle and 5 refills were done each day for 10 consecutive days, giving a total of 50 freeze-thaw cycles/freezing curves. Another vial which has an untreated film inside was used as a control and tested once each day, just like the ECIN coated vial. Cooling bath was adjusted to a fixed
temperature of -10°C for the testing ECIN coated film and -15°C for the controls, to have a fixed cooling rate. All other parameters such as container type, sample volume and dipping level was carefully checked for consistency in each step so that the cooling rate doesn’t vary among trials. Temperature change was logged vs time every 2 seconds using an Omega OM-CP-TC-4000 datalogger system (Omega Engineering Inc., Stamford, CT).

5.7. Atomic Force Microscopy (AFM)

AFM is a fundamental tool for nanotechnological studies. Use of AFM as a novel tool for Food Science research has recently been the topic of an article in Food Technology Magazine published by the IFT, and its potential in providing insight to the structure, chemical composition, bioactive benefits and functional properties of plant materials were discussed (Gunning et al., 2010). Unlike light microscopes, images in AFM are generated by the changes in force interactions between a probe and the sample, as the sample is moved relative to the probe. It is comparable to how a blind person senses a surface by simply touching it, and similar to how he/she can sense the shape, hardness and stickiness of an object, AFM can generate images for the topography, adhesion forces and elasticity of a surface (Morris et al., 2001). In this study, surface morphology was investigated with Multimode Nanoscope IIIA™ AFM (Digital Instruments, Veeco Metrology, Santa Barbara, CA, USA) which was operated in tapping mode. Silicon tip with an average drive frequency of 265 kHz was used except for the tip used to analyze the ε-PL/Iota/ε-PL/ECIN multilayers, where the drive frequency was 314 kHz. 1x1, 2x2 and 5x5 µm images of the layers of interest were collected. Utility programs already built in the AFM software Nanoscope V5.12r5 (Veeco Metrology, Santa Barbara, CA) were
used to analyze the images. Section analysis tool was used for the measurements of feature sizes, since the measurements on x and y axes might be misleading due to tip broadening effects. All AFM measurements were carried out in the air. Layers were formed by dipping freshly cleaned silicon wafers into the polymer solutions, as described in 5.3 and 5.4. AFM images were analyzed to determine the root-mean-square roughness (RMS) of the surfaces. Calculations were made using the Nanoscope software, according to this formula:

$$RMS = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (z_i - z_{av})^2}$$

Here, $z_i$ is the $z$ value (height) of each pixel where $z_{av}$ is the average of the $z$ values of each pixel in the analyzed scan area, and $N$ is the total number of pixels in this area.

5.8. Water Contact Angle Measurement

Water contact angles were measured using a VCA Optima video contact angle system (Advance Surface Technology, Billerica, MA, USA) to verify the adsorption of multilayers. One condition to be able to use this very practical method to confirm surface modification is that the alternating polymers being used to build the multilayers has different wettabilities, so that the water contact angles of the more hydrophobic top layers will be higher than the more hydrophilic ones, which was not the case for PDDA/PSS multilayers but worked perfectly for carrageenan/chitosan, carrageenan/ε-PL and pectin/chitosan systems. Higher water contact angles indicate higher hydrophobicity, as the droplet tends to get flatter on the hydrophilic surfaces. As an illustrative example,
pictures of droplets on top chitosan and carrageenan layers on the 6th bilayer were provided in Figure 17.

5.9. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

QCM-D was used to monitor the build-up processes of multilayer films. QCM-D is a piezoelectric biosensor which can detect mass changes per unit area with an extremely high sensitivity of 0.4 ng/cm$^2$. Thus it is considered as an inexpensive and very sensitive tool to study the small mass changes which occur adsorption of molecules in a solution to a surface and the visco-elastic properties of films deposited onto gold crystals (Janshoff et al., 1997). Method relies on the change in frequency of a quartz crystal resonator as mass builds on the surface of the acoustic resonator. The crystal oscillates at a constant frequency as the voltage is applied, and any structure or mass changes that occurs on the surface of gold crystal causes the resonant frequency to change. It can either be a positive or negative change, which indicates the absorption or desorption of molecules. Under the assumptions that the film is homogeneous and has enough rigidity, Sauerbrey equation can be simplified to the below form, which suggests a direct correlation between the change in mass ($\Delta m$) and the change in the resonant frequency of the gold crystal (Sauerbrey, 1959):

$$\Delta m = -\frac{C\Delta f}{n}$$

In this formula, C is a constant that is unique for the sensitivity of the quartz crystal to mass changes, it is also a function of the specific density and thickness of the quartz crystal. For a 5 Mhz gold crystal, C is equal to 17.7 ng/(cm$^2$Hz). n is the overtone
order that the resonator is operated at, they are the number of nodal planes that are parallel to the crystal surfaces. Overtone orders are always odd numbers, i.e. \( n = 1, 3, 5, 7, \) and on. Only odd number overtones can be excited electrically, because only odd harmonics can induce charges of opposite sign on the two crystal surfaces. Fundamental frequency of 5 Mhz, 15 Mhz, 25 Mhz and 35 Mhz corresponds to the 1\(^{st}\), 3\(^{rd}\), 5\(^{th}\) and 7\(^{th}\) overtones respectively. Data from the 3\(^{rd}\) overtone, which corresponds to a frequency of 15 Mhz was used throughout this study.

As stated above, assumption of the Sauerbrey equation is that the film has enough rigidity. However when studying soft materials, conventional QCM can overestimate the adsorbed mass in liquid environment. This is because the viscoelastic films do not fully couple to the oscillation of the crystal which causes the oscillation to dampen. In such cases, QCM with Dissipation Monitoring (QCM-D) is used which also takes the energy dissipation factor into account (Q-Sense, 2008). It gives more reliable results by calculating the dissipation factor from the decay rate which is recorded simultaneously with the resonant frequency, and it is a factor of the crystals viscoelastic properties, injected solution and the deposited layer (Rodahl et al., 1995). The rigidity of a deposited layer can be estimated from the equation below:

\[
\Delta D = \frac{E_{dissipated}}{2\pi F_{stored}}
\]

Here, the dissipated energy and stored energy are total energies measured during for one oscillation. When \( \Delta D \) is larger than \( 10^{-6} \), the film is considered an elastic.

Since the polymers of interest in this study are classified as viscoelastic soft materials, a QCM-D apparatus (Q-Sense AB, Sweden) with a Q-Sense D300 electronic
unit was used to detect the frequency changes. Plots of frequency shift versus time were obtained using the built-in Q-Sense software, QSoft401.

The thickness of the layers formed during the LbL deposition process was also determined using the same system. Since the Sauerbrey equation is not valid for viscoelastic films, the alternative Voigt model which takes into account additional variables including the viscosity and density of the injected solution is used when analyzing the data to estimate the layer thickness. A fluid density of 1000 kg/m$^3$, layer density of 1200 kg/m$^3$ and dynamic viscosity of 0.00089 kg/ms was assumed. Validity of the Voigt model is based on three assumptions:

1) Deposited layers cover the surface of the gold crystal evenly.

2) Layers are homogeneous

3) Layers do not contain internal stress, i.e. they are in equilibrium.

The multilayers were fabricated at a temperature of 25.00±0.02℃ which was kept in this narrow range by the built-in controller of the QCM-D instrument. Before multilayer build-up, gold-coated quartz crystals were cleaned in an UV/ozone chamber for 10 minutes, then immersed in a 1:1:5 (v:v:v) mixture of ammonia hydroxide (NH$_4$OH, 25%), hydrogen peroxide (H$_2$O$_2$, 30%), and Milli-Q water for 15 min at 75℃. Then they were rinsed with Milli-Q water, dried with a steam of nitrogen gas, and finally cleaned in an UV/ozone chamber for another 10 min, to remove the possible impurities on the crystal surface.

Afterwards, the crystal is mounted into the QCM-D chamber in a way that the gold surface side will be exposed to the injected solution. The deposition of polymers was recorded by measuring the frequency and dissipation changes over time. When
buffered polymer solutions are being used, the buffer itself was used as the baseline to get a stable frequency before the polymer solutions are injected. After each injection, excess solution containing the non-absorbed polymers was rinsed off/pushed away from the chamber by injecting the buffer once again prior to the injection of the alternating polymer solution. Criteria for deciding when a stable layer thickness is reached and the next solution can be injected, was that the $\Delta f$ is smaller than 1 Hz for a period of 5 minutes.
6. Results and Discussion

6.1. Initial Experiments

In trials, ECINs could be adsorbed onto substrates using the LbL deposition technique as demonstrated by water contact angle variation, AFM images and nucleation activity measurements on the cooling bath. Overall charge of the ECIN matter was determined by the QCM-D analysis, by observing the adsorption of ECINs onto PSS and PDDA top layers. As the frequency change indicates, ECINs do adsorb onto positively charged PDDA but not onto PSS top layer (Figure 5), thus it was concluded that overall charge of ice nucleating matter is negative in a neutral solution which has a final pH of 7 (Table 2). The solution was injected at minute five, which is the reason for the momentary decrease in frequency at that point as the solution was injected into the chamber, and frequency went back to zero and stayed there up to the 20th minute.

Overall charge was also verified by dipping the negatively charged silicon wafer directly into the ECIN solution and imaging by AFM. A smooth surface identical to untreated silicon wafer substrate was observed in the images, similar to the PDDA layer seen in Figure 15 on page 52. AFM images of ECIN on PDDA/PSS bilayer were also smooth with no morphological features at a 50 nm Z scale, demonstrating that the ECIN matter does not absorb onto the negatively charged top PSS layer.

Titration of ECIN matter was performed by Ke Shi, and pI of the ECIN matter was found to be around pH 4 (images not shown). Hence, all solutions of ECIN were prepared in neutral solutions without using buffer, to keep its negative charge at a natural solution pH in the range of 6.5-7.5, depending on the concentration.
Figure 5. Frequency changes versus time when ECINs were adsorbed onto PDDA/PSS/PDDA (a) and (PDDA/PSS)$_2$ (b) multilayers. Decrease in frequency in the left image indicates adsorption onto the positively charged surface, confirming the overall negative charge of ECINs.
Another factor in deciding the pH of the ECIN solution was its effect on ice nucleation activity. We tested two pH levels to analyze this effect, by placing 30 droplets of ECIN in acetate buffer (pH 3.9) and ECIN in neutral MilliQ water (pH 6.8) in polished aluminunm dishes. As temperature was decreased by 0.05°C from -7.05 to -8.2 every 3 minutes, number of frozen droplets was recorded at each temperature point.

Figure 6 shows that the ice nucleation activity was higher with the higher pH ECIN solution.

Figure 6. Ice nucleation activity of ECIN solutions at pH 3.9 and pH 6.8 in the temperature range of 7.05 to 8.2, as determined by the number of frozen droplets.

All droplets of the pH 6.8 ECIN solution did freeze at -8.15°, where 5 droplets of the pH 3.9 ECIN solution remained unfrozen at -8.2°C. The temperature to freeze 10%, 50% and 90% of droplets was 0.38, 0.23 and 0.22°C higher at pH 6.8 than at pH 3.9.
Table 1. Temperatures at which 10, 50 and 90% of ECIN solution droplets were frozen at pH 3.9 and 6.8

<table>
<thead>
<tr>
<th>pH</th>
<th>T 10%</th>
<th>T 50%</th>
<th>T 90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>7.67</td>
<td>8</td>
<td>8.22</td>
</tr>
<tr>
<td>6.8</td>
<td>7.29</td>
<td>7.77</td>
<td>8.00</td>
</tr>
</tbody>
</table>

An earlier study conducted with *Pseudomonas fluorescens* cells concluded that the ice nucleation activity is not affected by the pH in the range of 5.5 to 8 (Obata et al., 1987). Another study was performed with *Pseudomonas syringae* and *Erwinia herbicola* where a pH spectrum of 4 to 9 was studied using acetate, phosphate and Tris buffers. There was not a significant change in the ice nucleation activity of these cells against pH, except for the ~1.5°C increase in freezing temperatures when pH is increased to 6 from 4 (Kozloff et al., 1983). An explanation for why the pH effect was more apparent in our study than in theirs is that they have used whole cells carrying intact ice nucleators on their cell wall, which can provide better protection against an acidic environment. Since ECINs are going through a series of treatments as they are extracted from the cells, they can be more labile and sensitive to low pH.

pH of solutions used throughout the experiments were recorded and are listed in Table 2. Neutral solutions were used when possible, to keep the procedure simple and minimize the use of buffer. ε-PL is known to keep its positive charge in neutral solutions (Yu et al., 2009), however a 0.01M acetate buffer was used when making the carrageenan solution since a low pH is essential to keep the carrageenan anionic enough to initiate the electrostatic interaction (Gu et al., 2005; Hugerth and Sundelöf, 2001).
Table 2. Concentrations and pH levels of the solutions used in the layer by layer deposition experiments.

<table>
<thead>
<tr>
<th>Solution of</th>
<th>Concentration</th>
<th>Buffer?</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.01M NaCl (Acetate)</td>
<td>3.74</td>
<td></td>
</tr>
<tr>
<td>PSS</td>
<td>1 mg/mL</td>
<td>No</td>
<td>5.92</td>
</tr>
<tr>
<td>PDDA</td>
<td>1 mg/mL</td>
<td>No</td>
<td>7.66</td>
</tr>
<tr>
<td>K-Carr</td>
<td>1 mg/mL</td>
<td>No</td>
<td>6.80</td>
</tr>
<tr>
<td>K-Carr</td>
<td>1 mg/mL</td>
<td>Yes</td>
<td>3.70</td>
</tr>
<tr>
<td>ε-PL</td>
<td>1 mg/mL</td>
<td>No</td>
<td>5.69</td>
</tr>
<tr>
<td>Chitosan</td>
<td>1 mg/mL</td>
<td>Yes</td>
<td>4.34</td>
</tr>
<tr>
<td>I-Carr</td>
<td>1 mg/mL</td>
<td>Yes</td>
<td>3.55</td>
</tr>
<tr>
<td>ECIN</td>
<td>0.01 mg/mL</td>
<td>No</td>
<td>6.37</td>
</tr>
<tr>
<td>ECIN</td>
<td>0.1 mg/mL</td>
<td>No</td>
<td>6.96</td>
</tr>
<tr>
<td>ECIN</td>
<td>0.2 mg/mL</td>
<td>No</td>
<td>7.21</td>
</tr>
<tr>
<td>ECIN</td>
<td>0.3 mg/mL</td>
<td>No</td>
<td>7.30</td>
</tr>
<tr>
<td>ECIN</td>
<td>0.5 mg/mL</td>
<td>No</td>
<td>7.48</td>
</tr>
</tbody>
</table>

We verified the necessity of using a buffer by conducting an early trial to compare the multilayers formed using kappa-carrageenan solutions with and without buffer. AFM images indicate that a multilayer system forms via an entangled carrageenan gel network when the acetate buffer is used, where it doesn’t form without the buffer. ECINs adsorption also did not occur when this multilayer system is not in place, as seen in the AFM images (Figure 7). Absence of a top ECIN layer (failure of electrostatic absorption to the surface) was confirmed by the water droplets on this wafer not getting frozen at -8°C, where it did on the wafer made using buffered carrageenan solutions.
Figure 7. 1x1, 2x2 and 5x5 μm AFM images of the top carrageenan and ECIN layers in an ε-PL/ carr/ε-PL/ECIN multilayer system, formed using kappa-carrageenan dipping solutions of pH 3.7 and pH 6.8.

6.2. PDDS/PSS Multilayer System

6.2.1. Verification of ECIN Absorption onto the PDDA Layers via Surface Wettability Detection

After the overall charge of ECINs was confirmed, the first multilayer trial was conducted using PDDA and PSS as the alternating polyelectrolytes and ECIN as the top layer. That is, ECIN layers were built on (PDDA/PSS)$_n$PDDA multilayers where $n$ was varied from 0 to 6. Water contact angles of PDDA and ECIN on successive multilayers of
PSS/PDDA as top surface confirmed that the surface is modified in each step of layer buildup (Figure 8).

**Figure 8.** Variation in water contact angles for top surfaces of PDDA and ECIN during multilayer buildup on silicon wafer.

Water contact angles of PSS are not included in this figure as the values are very close to those of PDDA, i.e. they have similar wettabilities as seen in Figure 9. PDDA film was more hydrophilic than ECIN film as indicated by the decrease in water contact angles each time ECINs were adsorbed onto PDDA layers, starting from 2\textsuperscript{nd} bilayer up to 6\textsuperscript{th}. Inconsistency in water contact angles for ECIN on a single layer of PDDA, for being higher than that of all other ECIN and even PDDA layers, can be attributed to insufficient surface coverage when the multilayer structure underneath isn’t good enough. The water contact angle increase can be due to the partial absorption of a hydrophobic portion of the
ECIN complex, as the ECINs were more hydrophilic than the PDDA on all the following layers (Figure 8).

![Graph showing variation in water contact angles for top surfaces of PDDA and PSS during multilayer buildup on silicon wafer.](image)

**Figure 9.** Variation in water contact angles for top surfaces of PDDA and PSS during multilayer buildup on silicon wafer.

### 6.2.2. AFM Imaging of ECINs

AFM images of ECINs show both globular and thread-like structures (Figure 10). Earlier studies indicate that ice nucleating matter obtained from *Pantoea ananatis* KUIN 3, known to be similar to ECIN’s obtained from *Erwinia herbicola*, has a composition of 43% protein, 35% polysaccharide, 10% lipid and 12% polyamines (Kawahara et al., 1993). Based on this data about composition and common knowledge of the shapes of proteins, lipids and saccharides, globular structures observed should be the aggregates of
ice nucleating protein, and rod shaped structures can be polysaccharides and/or lipids. Images suggest that proteins make up a dominant portion of the ECIN matter.

6.2.3. Effect of Increasing the Number of Underlying Polymer Bilayers on ECIN Morphology

The effect of the number of bilayers was also investigated by AFM. In accordance with the anomaly in water contact angles when there is a single layer of PDDA underneath ECIN, AFM image of the film that makes this single bilayer was different from those in 2-6 bilayers, for the globular features were less concentrated and rod like structures were almost non-existent (Figure 11), which confirms the hypothesis of insufficient surface coverage. With 7 and more polymer layers underneath, a slightly more concentrated ECIN surface is seen. Since the dipping solution concentration was fixed in those samples to 0.1 mg/mL ECIN (Figure 11) this suggests that the increased number of bilayers may be leading to a better foundation for the fabrication of the top ECIN layer, thus allowing the absorption of more ECINs. However this subtle

Figure 10. 1x1 µm height and phase AFM images of extracellular ice nucleators on consecutive layers of PDDA/PSS/PDDA. Z scale of the height image is 50 nm.
observation is not supported by the wettability of these ECIN layers which doesn’t follow an increasing or decreasing trend (Figure 8). Water contact angles do not indicate a significant effect of underlying bilayers on the wettability of the ECIN surface with 2 or more bilayers.

Despite the insufficient surface coverage, pure water droplets on all wafers seen in Figure 11 got frozen on the -8°C cooling bath within 5 minutes (Figure 12). The wafer used as control had 11 layers of consecutive PDDA/PSS polymers which could have acted as ice nuclei if the temperature was low enough, but the water droplets on this wafer remained unfrozen for several hours until the experiment was discontinued. This confirms the significantly higher ice nucleation activity of the thin ECIN nano-coating on the silicon wafer in comparison to other impurities that can cause heterogeneous ice nucleation.

Although a single layer of PDDA was enough for ice nucleation and two bilayers appeared to be satisfactory for layer build-up as suggested by the similarity in contact angles and surface morphology, six bilayers were fabricated in most experiments as ECINs on the 6th bilayer had the highest wettability which is its characteristic in the PDDA/PSS multilayer system, indicating better surface coverage.
Figure 11. 5x5 μm height images of ECINs on 1 to 6 bilayers of PDDA/PSS/PDDA.

Figure 12. Silicon wafers on metal discs in an aluminum container floating on -8°C water bath.
6.2.4. Effect of Solution Concentration on the ECIN Layer Morphology

The concentration of ECIN in the dipping solution hence the amount of ECINs that can absorb to the surface, is likely to have an effect on the ice nucleation probability, due to the increased number of ice nucleators per unit area. AFM images of the samples dipped in ECIN solutions of 0.01, 0.1 and 0.5 mg/mL show a significantly higher concentration of features as the ECIN solution concentration is increased (Figure 13). When these wafers were cooled to -8°C on the cooling bath, pure water droplets on the wafers covered with nanofilms of higher ECIN solution concentration also nucleated faster than the ones with lower concentration.

![Figure 13. 2x2 µm AFM height images of silicon wafers dipped in solutions of 0.01 to 0.1 mg/mL ECIN, after consecutive dipping in PDDA/PSS/PDDA.](image)

6.2.5. Effect of Solution Concentration on the ECIN Layer Thickness

QCM-D analysis of the effect of ECIN solution concentration on layer build-up also confirms the presence of a thicker layer when the concentration is higher (Figure 14). This figure indicates that it takes longer for the surface to come to equilibrium with the solution when the concentration is higher, and the layer obtained is thicker, with a frequency shift of approximately 110 Hz for 0.5 mg/mL, which is almost 10 times higher than that for 0.1 mg/mL. The frequency shift for 0.1 mg/mL is in compliance with the
earlier results from the QCM-D experiment conducted to determine the overall charge of ECINs, where resonant frequency comes to equilibrium at around 13 Hz for this concentration (Figure 5).

![Graph showing frequency shift of the crystal resonant versus time with different concentrations of ECIN solutions](image)

**Figure 14.** Frequency shift of the crystal resonant versus time when different concentrations of ECIN solutions are used to form a layer on top of PDDA/PSS/PDDA.

In addition to the effect of their quantity, size of the protein aggregates is also likely to have an effect on ice nucleation. The gene cloned from *Pseudomonas* species was found to encode for a protein of 180 kDa (Green and Warren, 1985). It was shown earlier that a nucleant mass of 150 kDa can provide ice nucleation at around -12°C while with an aggregate mass of 19,000 kDa, nucleation can be obtained at -2°C (Govindarajan and Lindow, 1988).
To study the effect of the size of globular protein aggregates quantifiable by the section analysis of AFM images and nucleation activity, as well as the correlation between the two, isolation procedure was modified in terms of centrifugation speed as explained in the methods section. As expected, aggregates got smaller with the application of higher cumulative centrifugation speeds; height of the aggregates went down to around 4 nm from 10 nm (Figure 15). Ice nucleation activity of ECINs was measured using a modified version of Vali’s equation (Li, Izquierdo et al. 1997) and expressed as units/ml.

Figure 15. 1x1 and 2x2 μm AFM height images of ECIN as the top layer of 6th PDDA/PSS/PDDA system and the PDDA layer underneath. Captions give the centrifugation speed in average relative centrifugal force (RCF) and revolutions per minute (RPM), and average heights of at least 20 globular features seen in the respective image, in nm ± standard deviation.
Plot of globular feature size versus logarithm of the activity value indicates a critical feature size between 4 to 6 nm that enhances the nucleation activity about 100 times (Figure 16).

Figure 16. Plot of average height of globular features on AFM images in nanometers versus ice nucleation activity expressed as units/ml in logarithmic scale.

6.3. Chitosan / Carrageenan Multilayer System

PDAA and PSS are strong polyelectrolytes and they form robust multilayers, however they are synthetic polymers and not food grade. For food applications, natural polyelectrolytes are required to replace the synthetic polymers.
6.3.1. Effect of the Number of Bilayers Formed on Surface Morphology and Wettability

Chitosan / carrageenan system was the first biopolymer pair studied and water contact angle measurements indicated that they can be used to create uniform food grade multilayers. This conclusion is supported by the steady variation in contact angles as layers are built on top of each other; indicating a better surface coverage (Figure 17).

![Figure 17. Variation in water contact angles as multilayers are fabricated using chitosan of 330kDa molecular weight and iota-carrageenan.](image)

Standard deviation of the 10-15 contact angles measured on each layer was within the range of 1.18-3.12, indicating an evenly formed and homogeneous surface. Chitosan of 330 kDa molecular weight was more hydrophobic than iota-carrageenan, as evidenced by the 10 to 15 degrees of difference in water contact angles as each alternating layer is formed. There is a trend towards increasing contact angles for both polymers with layer build-up, which should be due to the formation of a thicker multilayer system of a higher
nanoscale roughness, which will be discussed in further detail when explaining the variation of water contact angles in Section 6.3.2 in relation to the roughness imparted to the surface by carrageenans that can form three dimensional entangled gel networks.

Effect of building more bilayers on the surface morphology was also investigated, which had an effect on the absorption of ECINs to the PDDA/PSS system (Figure 11). With increasing number of bilayers, AFM images show more concentrated carrageenan gel networks with higher contrast, due to the thicker and more elevated threads on the surface (Figure 18). The double helical structure formation proposed in (Bemiller and Whistler, 1985) as a gelation model for iota carrageenan, is confirmed by the 10\textsuperscript{th} bilayer images that clearly demonstrate those three dimensional networks.
Figure 18. AFM height images of top iota-carrageenan layers, as the top layer of 2 to 10 bilayers of 10kDa chitosan and iota-carrageenan.
6.3.2. Effect of Carrageenan Type (Charge Density) on Surface Morphology and Wettability

Effect of charge density was studied with 10K chitosan as the positively charged polymer to build four different multilayer systems using lambda, iota, kappa carrageenans and furcellaran.

Looking at the surface morphology of carrageenans on the 6th bilayer, iota carrageenan is different from the other three biopolymers for forming a unique more closely packed and entangled gel network, with threads of uniform thickness (Figure 19). In an earlier study, iota-carrageenan was also found to be more suitable for complex formation with oligochitosan leading to more stable capsules (Bartkowiak and Hunkeler, 2001). Kappa carrageenan, which has a slightly lower charge density than iota, also forms a good gel network, both kappa and iota carrageenan led to the formation of a mixture of coiled and double-helical structures as seen in the AFM images collected at around room temperature (20-25°C), which are not seen in the images of lambda carrageenan and furcellaran.
Figure 19. 2x2 and 5x5 μm AFM images of top carrageenan and furcellaran layers on the 6th bilayer, paired with 10kDa chitosan.
These observations were confirmed by the water contact angle measurements which resulted in higher contact angles for the iota and kappa carrageenan surfaces than the lambda carrageenan and furcellaran surfaces (Figure 20). The formation of the entangled gel network leads to a surface which has higher nanoscale roughness. These entangled gels can hold the pure water droplets together on the surface, not letting them spread, making the contact angles of the water droplets higher. Indeed, the root mean square roughness (RMS) calculated from the AFM images was 1.94±0.10, 2.97±0.32, 3.59±0.06 and 1.33±0.14 nm for lambda, iota, kappa carrageenans and furcellaran, respectively. This data is almost parallel to the water contact angle data where the values
are lower for lambda carrageenan and furcellaran than iota and kappa carrageenans, which confirms the proposed correlation between nanoscale surface roughness and surface hydrophobicity.

The specific mechanism of water contact angle variation due to nanoscale surface roughness proposed herein is a different mechanism than when the water contact angle results are used as an indicator of good surface coverage, where the relative wettability of the polymer against the other is imparted when it is covered with the alternating polymer. So for example, it should not be confused with the comparisons of chitosan layers which are characteristically more hydrophobic than carrageenan layers which are relatively hydrophilic. Except for this instance, for the increase in water contact angles of the relatively hydrophobic carrageenan layers as more bilayers are formed (Figure 17), and other cases to follow where this mechanism is specifically addressed, interpretation of the water contact angle experiments is based on such relativity between the wettability of the two polymers making up the system and the top ECIN layers, throughout this study.

AFM images and water contact angles indicate that charge density is not in a direct correlation with surface morphology and wettability, evidenced by the similar properties seen for the lambda carrageenan of the highest charge density and furcellaran of the lowest. Appearance of good entangled networks on the nano-thin carrageenan layers, which also leads to higher surface hydrophobicity, can be attributed to the special gelling mechanism of kappa and iota carrageenans. How the sulfate groups are positioned on the disaccharide units of each carrageenan is another factor which is critical to the gelling mechanism (Bemiller and Whistler, 1985) and as suggested by our results, it is critical to the nano-thin layer formation as well. Although lambda carrageenan has the
highest charge density among all tested which would ideally lead to better electrostatic interactions with the chitosan layers, it cannot form the three dimensional polymer network that helps create an ideal nano-thin multilayer structure.

This is due to the presence of two negatively charged sulfate groups positioned on the opposite sides of the same saccharide unit, which tend to repel each other. As a result, the polymer chain cannot bend and twist to form the three dimensional double helical structures that the iota and kappa carrageenans can form. This makes lambda carrageenan which has the highest change density, almost as unsuitable as the furcellaran which has the lowest charge density, for building nano-thin multilayer systems through electrostatic interactions.

6.3.3. Effect of Chitosan Molecular Weight on Surface Morphology and Wettability

Molecular weight effect of chitosan was varied in the range of 1kDa to 330 kDa to study its effect on multilayer build up. As demonstrated to be more suitable for multilayer fabrication by the experiments on charge density, iota-carrageenan was used as the standard polyelectrolyte of negative charge in all systems.

Figure 21 shows the morphology change when the molecular weight of chitosan is varied in the multilayer system with iota carrageenan. Images of chitosan top layers are almost identical to carrageenan top layers for the 1 kDa and 10 kDa systems, since only carrageenan gels are visible but not the chitosan molecules which are globular features as observed earlier by AFM imaging and reported in (Haas et al., 2005).
Figure 21. 5x5 µm AFM images of top chitosan layers on 5 chitosan/iota carrageenan bilayers where the molecular weight of chitosan was varied between 1 kDa to 330 kDa.

The absence of globular features of chitosan on these images can be explained by the molecular weight being too small to form globules, or the screening effect of carrageenan gel networks. These AFM images have a height (Z scale) of 50 nm, and earlier studies show that the chitosan films are about 20 nm thick (Assis et al., 2002). Thus the image contrast of 50 nm used in a chitosan/carrageenan multilayer system is not small enough to see the chitosans among the carrageenan gels. However, when higher
molecular weight chitosans are used to build the multilayers, chitosan layer covers the carrageenan gels more completely and globular molecules of chitosan become more visible. Increased molecular weight also has an effect to tighten the carrageenan gel chains, giving a more closely packed, denser network. This phenomenon has been observed with the other multilayer systems which will be discussed in the following sections, and the advantages of a more concentrated area of polyelectrolytes will be addressed.

![Water Contact Angles](image)

**Figure 22.** Water contact angles of top chitosan layers on 5 chitosan/iota carrageenan bilayers where the molecular weight of chitosan was varied between 1 kDa to 330 kDa.

Figure 22 shows the water contact angles of the chitosan layers seen in Figure 21. There is a positive correlation between the molecular weight of chitosan used and the hydrophobicity of the layers formed. Variation of water contact angles during layer build-
up is also studied and surface wettability is found to vary more steadily when higher molecular weight chitosan is used (Figure 23).

This suggest a better surface coverage (i.e. more chitosans on the surface than carrageenans), which also explains the linear increase in water contact angles with increased chitosan molecular weight in Figure 22. By covering the surface more completely, higher molecular weight chitosans limit the interpenetration of the more hydrophilic underlying layer of carrageenan, making the chitosan layers more hydrophobic and a bigger variation in water contact angles each time a new layer is added.

Figure 23. Variation of water contact angles with layer build up for the 10kDa and 330kDa chitosan systems with iota carrageenan
6.3.4. Effect of Chitosan Molecular Weight on Surface Morphology in Relation to Solution Concentration

After showing through morphological investigation that more closely packed gel networks of carrageenan can be obtained when higher molecular weight chitosans are used, we attempted to go deeper into this phenomenon and see if this can be due to fixing the concentration of all molecular weights of chitosan to 1 mg/mL. So in the second set of experiments, we used excessive concentrations of low molecular weight chitosans, proportional to the 1 mg/mL solution of 330 kDa chitosan to see if that will allow better surface coverage. Thus, we prepared solutions of 1, 10, 44, 130 and 330 kDa chitosans at concentrations of 330, 33, 7.5, 2.54 and 1 mg/mL, respectively. Solubility of 44 kDa and 130 kDa chitosans were relatively low at these higher concentrations which required stirring for two days coupled with mildly heating to 50°C, where despite the significantly higher concentration, the 1 kDa and 10 kDa chitosan dissolved in less than an hour. After filtering them through 0.45 µm syringe filters, we used these solutions to build up to 6 bilayers with 1 mg/mL iota carrageenan and observe how the surface morphology is affected. Increasing the concentration seems to affect the surface morphology making it more similar to the highest molecular weight system for 44 and 130 kDa chitosans, the surface coverage and density of the layer appears to be almost identical to the 330 kDa system at 1 mg/mL in the AFM images (Figure 24). However, this is not the case for the 1 kDa and 10 kDa chitosan systems. Despite their significantly higher concentrations, carrageenan gels were still as widely spread as when 1 mg/mL solutions were used (Figure 21), indicating weak surface coverage of the chitosan layer. The threads of carrageenan are thicker than they were with the lower concentration for these molecular levels, but there was no improvement in surface coverage. Thickness increase due to
concentration increase, can be explained by the aggregation within the chitosan layer. This is a result of carrageenan sulfate group saturation, since chitosan repeating units are in excess of carrageenan disaccharide units.

Figure 24. 1x1, 2x2 and 5x5 μm AFM images of various molecular weights of chitosans at various concentrations on the 6th bilayers, paired with iota-carrageenan.

Results suggest the presence of a critical molecular weight between 10 to 44kDa, which determines the ability of chitosan to form a layer which is dense and uniform enough to hold the carrageenan gels more closely packed, below which the concentration increase do not have that effect. Above the molecular weight of 44 kDa however,
increasing the solution concentration can be an alternative to using higher molecular weight chitosans to improve the uniformity of nano-thin chitosan layers.

Number of bilayers formed had an effect on the packing of gel networks when studied on the top carrageenan layers with the iota carrageenan and 10 kDa chitosan system, where layer density was improved on the 6\textsuperscript{th} and 10\textsuperscript{th} bilayers in comparison to 2\textsuperscript{nd} and 4\textsuperscript{th} (Figure 18). This phenomenon was observed again on the chitosan layers of the 2\textsuperscript{nd}, 4\textsuperscript{th} and 6\textsuperscript{th} bilayers; using the 10 kDa and 330 kDa chitosans at 33 mg/mL and 1 mg/mL respectively.

Observations in Figure 25 confirm the earlier results seen in Figure 18, where the entanglement of carrageenan gels is improved when more bilayers are built. As opposed to the aggregation that results from the concentration increase of dipping solutions, building more bilayers appears to aid in the formation of more homogeneous layers. Even distribution of repeating units of a layer formed, also helps the formation of a homogeneous layer on top of it, because even charge distribution on the planar surface allows better interaction of oppositely charged groups. Although interpenetration of layers seem to be inevitable since surface coverage isn’t perfect, the interpenetrated groups on the polymer gets an opportunity to be electrostatically attracted and hold by an upper layer that is formed, probability of which is increased when more bilayers are created.
Figure 25. 1x1 and 2x2 µm AFM images of 10 kDa and 330 kDa chitosans making the 2nd, 4th and 6th bilayers, paired with iota-carrageenan.

6.4. ECIN Adsorption onto the Chitosan/Carrageenan Multilayer System

ECIN solutions at concentrations from 0.01 mg/mL to 0.5mg/mL were used to build ice nucleating nanolayers as the top layer of 6th bilayer in the 130 kDa chitosan/iota carrageenan multilayer system, to look into a possible correlation between the concentration of ECIN absorbed onto the surface and ECIN in solution. The reason for using the second highest molecular weight chitosan (despite the finding that higher molecular weight is superior for multilayer fabrication giving more stable water contact angles and better surface coverage) was that the carrageenan gel networks were more clearly visible with 130 kDa in comparison to 330 kDa in AFM images, so that the
globular ECIN matter could still be differentiated from the chitosan molecules using this system. Following 2 sub-sections will summarize the attempts to quantify ECINs on the x and y axis by looking at the nanoscale morphology of the ECIN layers, and on the z scale when the thickness of the layers were estimated via QCM-D analysis.

6.4.1. Studying the Solution Concentration Effect with the AFM

Three nano-thin ECIN layers were fabricated on iota carrageenan/130 kDa chitosan multilayer systems where all parameters were fixed except for the ECIN dipping solution concentration. Globular features which were not seen on the underlying carrageenan and chitosan layers are clearly visible on the AFM images of ECIN top layers at dipping solution concentrations from 0.01 to 0.5 mg/mL (Figure 26). This justifies that these features belong to the ECIN matter and that ECIN was successfully adsorbed onto the bio-multilayer system.

Quantifying the globules on these images is challenging because some of them are embedded between entangled carrageenan networks, and the carrageenan gels crossing one another forms regions looking similar to these globules that can be confused with the ECINs, since they have similar height and shape. Nevermore, images of silicon wafers dipped into the 0.01 and 0.1 mg/mL ECIN solutions appear to have similar concentrations of globular features, where the wafer dipped in the 0.5 mg/mL solution clearly has more ECINs per unit area of silicon wafer.
Figure 26. 2x2 µm images of ECIN’s as the top layer of 6th bilayer of 130kDa-chitosan/iota-carrageenan system. Images below are the biopolymer layers making the background (10th and 11th layers).

Cooling bath experiments were consistent with the AFM images, with respect to the increased nucleation probability due to the presence of more nucleation sites on the surface which is dipped into higher concentration ECIN solution. Droplets on the 0.5 mg/mL ECIN surface got frozen earlier than the other two at -8°C (Figure 27). Nucleation on the 0.01 and 0.1 mg/mL wafers occurred almost simultaneously, which is in line with what the AFM images suggested, since the concentration of ECIN globules on these two wafers were comparable according to the morphological images (Figure 26).
Figure 27. Silicon wafers dipped in different concentrations of ECIN to build 6th bilayer of 130kDa chitosan/iota-carrageenan multilayer system, floating on -8°C water bath. Wafer dipped in 0.5 mg/mL ECIN freezes faster than the other two.

6.4.2. Studying the Solution Concentration Effect with the QCM-D

AFM images indicated that a more concentrated surface which has a higher ice nucleation probability can be obtained with dipping solutions of higher ECIN concentration. But it is quite hard to quantify the amount of ECIN absorbed to the surface for each solution used. It is possible to count the globules on these images which would give a relative measure (similar to how a person would count bacteria on a stained slide under the microscope), but that would require the assumption that ECIN distribution is perfectly homogeneous in the nano-scale. Thus we decided to look into this with the QCM-D using which we are able to determine the thickness of the nano-thin layer from the frequency shift that occurs.

Experiments were conducted where 2 bilayers were created, using iota-carrageenan, 130 kDa chitosan and 6 concentrations of ECIN from 0.001 to 0.5 mg/mL. Only parameter we have changed during the experiments was the concentration of the ECIN dipping solution, which makes up the top layer. Figure 28 is a sample graph which shows the thickness estimated using the Voigt model versus time for the system created using the 0.5 mg/mL of ECIN.
Figure 28. Variation in the thickness of layers as estimated by the Voigt model from the QCM-D data, for the Chitosan/Carrageenan/Chitosan/0.5 mg/mL ECIN system.

At around 8 minutes where the crystal was in equilibrium with the acetate buffer (It is the background, since this buffer was used to make the 1 mg/mL chitosan and carrageenan solutions), chitosan solution was injected and at 28 minutes, it was rinsed out using the acetate buffer to make sure that frequency shift is only due to the layer absorbed onto the gold crystal and not the excess polymers in the solution. Thus the thickness of the first layer was read at 40th minutes, which is when the crystal frequency came to equilibrium following the buffer injection. As seen on the image, y axis reads 1.4x10^-8 meters at that points, meaning the estimated thickness of the first chitosan layer is 14 nm (Figure 28). Iota carrageenan solution was injected at 40th minutes, and the second layer
came to equilibrium at 100 minutes, indicating a thickness increase of 5 nm which is the thickness of the carrageenan layer. This was followed by the third layer of chitosan which showed an 8.5 nm increase, and the top layer of ECIN made using the 0.5 mg/mL solution formed a layer of 100.5 nm thickness.

Experiment was repeated the same way for the 0.001, 0.01, 0.05, 0.1 and 0.3 mg/mL ECIN solutions to make the top layer, where the underlying layers were fixed. The frequency (A) and thickness (B) changes of these systems can be found in Appendix, Section 9.2 (Figure 53 to Figure 58).

**Figure 29.** Thickness of the top ECIN layers formed using dipping solutions of 0.001 to 0.5 mg/mL.
Figure 29 summarizes these figures by plotting the thicknesses of the top ECIN layers versus the concentrations of the ECIN dipping solutions. QCM-D data (thickness of the layer) aligns with the AFM data (concentration/density of the layer) for the 0.5 mg/mL solutions leads to the formation of a significantly thicker layer than that formed using the 0.01 and 0.1 mg/mL solutions, as it did form a denser layer than these, as observed in Figure 26.

Total thickness of the four layers was about 38 nanometers for the multilayers built using 0.1 mg/mL ECIN (Figure 56-B) and 128 nanometers with 0.5 mg/mL ECIN (Figure 58-B). Thickness of the top ECIN layer alone was 16.5 and 100.5 nanometers for the multilayers formed with 0.1 and 0.5 mg/mL ECIN respectively, which indicates the significant increase in layer thickness when the concentration of the ECIN dipping solution is increased. Thicknesses of the underlying iota-carrageenan and chitosan layers built by dipping the substrates into their 1mg/mL solutions, varied in the range of 5-15 nanometers. The observation that the ECIN layers are thicker than pure polyelectrolyte layers is in accordance with the literature information regarding the composition of the extracellular ice nucleating matter, for ECINs has been proposed to be large molecules generally existing as lipoglycoproteins (Muryoi, Matsukawa et al. 2003), and there is a positive correlation between the aggregate size and ice nucleation activity (Muryoi, Kawahara et al. 2003). The nucleant mass required to initiate ice nucleation at -2°C was estimated to be around 19,000 kDa by Govindarajan and Lindow (Govindarajan and Lindow 1988), where the ideal and experimental molecular weights of iota carrageenan are 543 and 482 kDa respectively (Caram-Lelham and Sundelöf 1995), and the actual molecular weight of chitosan used in this study was 130 kDa.
Same gold crystals used for QCM-D analysis were used to study the ice nucleation on these films (Figure 30). At -8°C, the droplets on the gold crystal that was dipped into the 0.5 mg/mL ECIN solutions to make the final layer got frozen in 2 minutes, and it took 14 minutes for those in the range of 0.05 to 0.3 mg/mL. This is consistent with the observations on the silicon wafers where the 0.5 mg/mL froze earlier than the others (Figure 27).

**Figure 30.** Freezing of pure water droplets on ECIN layers formed using 0.001 to 0.5 mg/mL dipping solutions. Labels of crystals with frozen droplets are in bold.

After an hour, temperature was decreased to -9°C and the droplets on the 0.01 mg/mL crystal also got frozen, where the 0.001 mg/mL remained unfrozen for over an hour, proposing that a 1nm thick layer of ECIN is not sufficient to have an ice nucleation activity high enough to freeze water droplets at temperatures higher than -10°C (Figure 30).

6.5. **Chitosan/Pectin/ECIN Multilayer System**

Pectin was used as an alternative poly-anion to replace carrageenan. In food applications, both hydrocolloids are commonly used for their certain functionalities (Bemiller and Whistler, 1985). Pectins are extracted from plants and very commonly from citrus peels, because they are more concentrated on the outer surfaces of the plants.
Our motive behind looking into pectin in addition to carrageenan is that it is naturally present on surfaces, thus our experiments can give direction to the fabrication of nano-thin edible layers built on the food itself. Three different types of pectin were tested, with varied degrees of esterification and one which was amidated.

6.5.1. Effect of Chitosan Molecular Weight on Surface Morphology and Wettability

As with the carrageenan system, chitosan molecular weight was varied first and studied by water contact angles and AFM imaging. Danisco Grindsted LA 410 pectin, with an esterification degree of 29-33% and amidation degree of 20% was used in this part to build the three multilayer systems. LA410 pectin was significantly more hydrophilic than the other biopolymers (Figure 31). As seen earlier with the carrageenan system, chitosan and ECIN showed a trend towards increasing hydrophobicity when higher molecular weight chitosans are used, confirming the better surface coverage. Higher increase of water contact angles with increased molecular weight for the ECIN than that for the chitosan is an indicator of even better attachment of ECINs to the surface when higher molecular weight chitosans are used. As discussed earlier in more detail (Section 6.3.2), increase in nanoscale surface roughness contributes to hydrophobicity by holding the water droplets together, not letting them spread on the surface. Ability of higher molecular weight chitosan to form a more closely packed pectin gel network also makes a contribution, since the number of carboxyl groups per unit area increases and with uniform charge distribution more homogeneous layers are achieved.
The multilayer systems created with the 130kDa and 330kDa chitosans were tested for ice nucleation activity on the cooling bath. Silicon wafers which had either chitosan or pectin of the 6th bilayer on top, remained unfrozen for over an hour at -8°C, where nucleation occurred within five minutes for the wafers which had ECIN on top of (Chitosan/Pectin)5Chitosan. Ice nucleation of droplets occurred almost simultaneously for the 130kDa and 330 kDa systems (images not shown).

AFM images of top chitosan, pectin and ECIN layers were obtained for all three systems (Figure 32). As with the carrageenan/chitosan multilayers, chitosan top layers were not significantly different from top pectin layers due to reasons related to low
contrast, as discussed earlier in Section 6.3.3. On the ECIN top layer images, the globular features are more clearly visible than they were on the iota-carrageenan system, because the gel networks of pectin appear as thinner threads than iota-carrageenan threads on which the globules are easily detected.

Figure 32. 2x2 µm AFM images of top pectin, chitosan and ECIN layers on the 6th bilayer of 10, 130 or 330 kDa chitosan / LA410 pectin multilayer systems.

The shapes of branched pectin chains alone and when they interact with proteins were very similar to how they appeared in AFM images collected by Kirby, MacDougall
et al. in 2008. Using high resolution AFM images (scanned as 1x1 µm and further magnified allowing the use of a 100nm scale bar), they were able to show how pectin threads interact with the protein globules either by surrounding them or getting attached to them through the edges of the chain (Kirby et al., 2008), dominantly through the electrostatic interactions of the oppositely charged groups they possess.

6.5.2. Effect of Pectin Degree of Esterification on Surface Morphology and Wettability

Effect of degree of esterification on layer build-up in terms of wettability and surface morphology were investigated using Grindsted LA410 pectin with 29-33% esterification and 20% amidation and Sigma P9561 citrus pectin with 90% esterification.

As seen in the AFM images, multilayers could not be fabricated successfully with the high esterification pectin system thus images are significantly different from those with LA410 pectin since the surface is relatively smooth with no pectin gel networks but just precipitates and isolated threads (Figure 33). When tested on the cooling bath, the droplets on these wafers did not freeze where those on the low methoxyl pectin systems with and without amidation got frozen at -8°C in five minutes.

This observation serves as another evidence to support the hypothesis that the attachment of polymers to each other during multilayer build-up is mainly driven by electrostatic interaction. The reason is that in pectins with high degree of esterification, most of the carboxylic acid groups on the galacturonic acid backbone are methyl esterified, so they are not available for electrostatic interaction with the amide groups of chitosan. However, low methoxyl pectins have more carboxylic acid groups available as
free acids. In some pectins, these free groups can be amidated as well, effect of which was also investigated and discussed in the following section.

Figure 33. 2x2 AFM images to show the effect of esterification on layer build-up in the 10 kDa chitosan/LA410 (29-33%DE with 20%DA, left row) or P9561 (90%DE- no amidation, right row) pectin multilayer systems.
6.5.3. **Effect of Pectin Degree of Amidation on Surface Morphology and Wettability**

Effect of amidation degree was studied using 130 kDa chitosan and two pectins with similar degrees of esterification where one of them, LC 410 (DE: 29-33%, DA:20%) is amidated; and the other, LA950 (DE: 31-33%, DA:0) is not. Surface morphology of the two systems is not as significantly different as what was observed with the high methoxyl vs. low methoxyl system (Figure 33). Pectin and chitosan top layers look almost identical except for the pectin gels are more clearly visible in the amidated system. However looking at the ECIN images, globular features were more numerous than that observed on the system with the non-amidated pectin (Figure 34).

To test this observation, water contact angles of these surfaces were measured. Contact angles of the similar looking chitosan top layers was very close to each other, where the contact angle of the pectin surface of the amidated system which showed more clearly visible gels in the AFM images had lower contact angles than the non-amidated system (Figure 35).

This suggests the better surface coverage on the amidated system, since earlier contact angle measurements where chitosan molecular weight was varied showed that pectin layers are significantly more hydrophilic than the ECIN and chitosan layers (Figure 31). Thus that characteristic of higher wettability is more apparent with the amidated system. Similarly, the ECIN top layer of the amidated system has higher contact angles than the ECIN on the non-amidated system. It also had higher contact angles in Figure 31 against pectin and the different molecular weights of chitosan. This serves well as an indicator of better ECIN adsorption, and correlates well with the AFM images showing a higher density of ECIN globules.
**Figure 34.** 2x2 µm AFM images top Chitosan, ECIN and Pectin layers with water contact angles ±standard deviation on two multilayers systems with 130 kDa chitosan and either LC950 or LA 410 pectin.
Figure 35. Water contact angles of the top pectin, chitosan and ECIN layers of the 130 kDa Chitosan paired with amidated (20%) and non-amidated pectin of similar esterification.

Although amidated carboxyl groups are no longer available as negatively charged carboxyl groups, amidation is can make a contribution to the multilayer system by allowing stronger electrostatic interactions between the pectin layers that sandwich the chitosan layer. It should be kept in mind that those layers do not have perfect surface coverage over the underlying layers, so there are spots or regions with the lower layers even if the top polymer has optimum surface coverage. Inevitability of this interpenetration explains the role of amidated groups on the pectin galacturonic acid backbone. They allow a stronger interaction with both the chitosan layer and also with the pectin layer under the chitosan, holding both layers together more strongly. This can also be explained by the presence of a threshold for the required degree of esterification.
Having less than 10% of free carboxyl groups is too low to build multilayers, where 50% and 70% is not significantly different, because in both systems there are enough groups to allow electrostatic interactions between the polyelectrolytes.

6.6. Ice Nucleating Polyethylene Films

Commercial low density polyethylene (LDPE) film was treated with UV ozone to modify the surface. Water contact angles of the LDPE films decreased with increasing UVO exposure times, yielding a more hydrophilic surface (Figure 36). This can be explained by the increase in the amount of carboxylic acids on the surface with longer UVO exposure, as illustrated by the XPS data of Zander et al. XPS results indicate an increase in atomic percent oxygen and carboxylic acids with prolonged UV exposure (Zander et al., 2009).

Since carboxylic acids and its derivatives are more hydrophilic than hydroxyl groups, increase in the percentage of carboxyl groups relative to the hydroxyl groups lead to a decrease in water contact angles with prolonged UVO exposure, yielding a more hydrophilic and negatively charged surface (Zander et al., 2009). There was a significant decrease in the average of advancing and receding water contact angles from above 75° to below 45° with longer than 3 minutes of UV-ozone exposure. The increase in wettability was in accordance with Zander et al.’s earlier experiment, where contact angles decreased by approx. 35° following 5 minutes of UV-ozone treatment. Results are in agreement with the earlier observations of (Mathieson and Bradley, 1996) as well, where the contact angles of polyethylene decreased by 30-40° and came to an equilibrium at around 3-5 minutes exposure to UV ozone. Similar results were observed with
polyetheretherketone (Mathieson and Bradley, 1994, 1996) and polypropylene (MacManus et al., 1999).

Figure 36. Variation of water contact angles with standard deviations of the PE film surface against the time of UV-ozone exposure.

AFM images of LDPE films exposed to UV-ozone for 1, 3, 5 and 10 minutes did not indicate a trend or significant change in roughness and morphology (Figure 37). This indicates that the variation in water contact angles is not due to some increase in the nanoscale roughness of the surface that can also contribute to increased wettability. Thus the increase in wettability should be a chemical and not a physical effect, and due to the oxidation of the surface by the UVO treatment, that led to the formation of more hydrophilic groups.
Additionally, different parts of a piece of LDPE film were exposed to 1, 3, 5 and 10 minutes of UVO treatment and PDDA/PSS/PDDA/ECIN multilayers were built on it. After placing 50 microliter water droplets on all four parts, temperature was decreased to -6°C, and ice nucleation occurred almost simultaneously on all parts (Figure 38). Thus the dramatic decrease in surface hydrophobicity with 5 minutes or longer UVO exposure, doesn’t seem to have a significant effect on the quality of layer absorption. One way to explain this is to acknowledge that the thin layers do not form exactly the same way they form on highly smooth substrates such as silicon wafer and gold crystals. We have shown by the nanoscale evaluation of surface morphology, layer thickness and wettability that the layers on these substrates are anchored mainly through the electrostatic interaction to the surface. Physically absorbed molecules are removed quite effectively from these extremely smooth surfaces by rinsing and nitrogen gas flushing. However with the LDPE films which are relatively rough when compared to these substrates, physical absorption
should also be involved in addition to the electrostatic interaction. As a result, more ECINs will be remaining on the surface even after rinsing and blow drying the film. Physical absorption can lead to the absorption of more ECINs than that possible through electrostatic interaction. Such oversaturation can explain the lack of UVO treatment time effect on the ice nucleation activity of the LDPE film. This should also be kept in mind when comparing the ice nucleation temperatures on silicon wafers and gold crystals to LDPE films.

After conducting the initial trials with the PSS/PDDA system, ε-PL/iota carrageenan pair was investigated as a biopolymer alternative. AFM analysis, water contact angle measurements and ice nucleation activity experiments were conducted on

Figure 38. Freezing of 50 µm MilliQ water droplets at -6°C on a LDPE film coated with PDDA/PSS/PDDA/ECIN multilayers following the exposure of its portions to 1, 3, 5 and 10 minutes of UVO treatment.
silicon wafer before replacing this substrate with the LDPE. Figure 39 shows the 1x1, 2x2 and 5x5 µm AFM images of each of the four layers fabricated. Except for the first ε-PL layer, threads which belong to the iota carrageenan are seen in all images. This is because the surface coverage of monolayers is not good enough to completely blot out the underlying polymer layer, hence interpenetration of layers did occur. Globular features seen on the first ε-PL layer (which are easier to see in Figure 40 since the Z-scale was reduced from 50 to 10 nm), become almost invisible as more layers are built (Figure 39).

**Figure 39.** 1x1, 2x2 and 5x5 µm AFM images and root mean square roughness±standard deviation of the four nano-thin layers (Z scale is 50 nm)
This is due to the shielding effect of the iota carrageenan layer, threads of which are thicker than the globules of ε-PL. The globular morphology of a single layer of ε-PL molecules can be seen in Figure 40. At a relatively low Z scale of 10 nm, a dense layer of ε-PL globules can be observed as the lighter (yellowish) areas in contrast to the perfectly smooth silicon wafer surface as the dark (brownish) background (Figure 40).

![Figure 40. 1x1 and 2x2 µm AFM height and phase images of a single layer of ε-PL on silicon wafer (Z scale is 10 nm)](image)

It can be seen that these molecules some of which are hollow (unexpected, since ε-PL is normally a random coil molecule which can shift to either an α-helix or β-sheet
conformation upon pH or temperature increase respectively), are making up a single layer that is very closely packed, and the layer they form is so flat that the individual molecules only become visible when the contrast is increased by lowering the Z scale to 10 nm. Thus the roughness imparted by the iota carrageenan threads, makes it impossible to distinguish ε-PL in the earlier images in Figure 39, unless they are located on those threads due to the attraction to the sulphate groups on the carrageenan backbone. However, the large globules belonging to the ice nucleator proteins of the ECIN matter are clearly visible and numerous on the 4th layer (Figure 39).

In comparison to the multilayer systems of chitosan and iota carrageenan, a more organized gel network of iota carrageenan is observed here, with carrageenan threads almost lining parallel to each other (Figure 39). This provides a better foundation for layer build-up, allowing the even distribution of ε-PL thus the negative charge on the surface. As a result, a more homogeneous layer of ECINs is fabricated, which increases the probability of simultaneous ice nucleation at multiple locations on the surface.

When the ice nucleation activity of top layers ECIN, iota carrageenan and ε-PL layers was evaluated at -7°C using the same silicon wafers used for AFM imaging, only the droplets on the ECIN coated wafers were frozen (Figure 41). Then the temperature was steadily decreased by 1°C every half an hour, and the droplets on the iota carrageenan and ε-PL top layers remained unfrozen until the temperature was dropped to -10°C. This confirms that the ice nucleation observed at -7°C is due to the presence of electrostatically absorbed ECIN matter on the surface and is not a random effect.
Figure 41. Freezing of 10 µL high purity dionized water droplets on ECIN, iota carrageenan and ε-PL top layers at -7°C.

Water contact angles measured on the 6th bilayer indicated that the iota carrageenan layer is more hydrophilic than both ECIN and ε-PL top layers (Figure 42). Wettability of ECIN and ε-PL surfaces is similar. A correlation exists between contact angles and root means square roughness (Rms/rq), since the roughness of ECIN and ε-PL layers are not significantly different and lower than that of the iota carrageenan surface (Figure 39). Roughness of a single layer of ε-PL on silicon wafer was 1.34±0.23 nm. Significant increase in roughness from 2nd to 4th layers indicates the contribution of the carrageenan threads to the overall roughness of the surface.

Figure 42. Water contact angles±standard deviations of top ECIN, iota carrageenan and ε-PL (3rd layer) layers.
After studying the nanoscale properties of this multilayer system and confirming the ice nucleating activity on silicon wafer, we moved on to fabricating these multilayers on a UVO treated LDPE film substrate. When the two bags one made of ECIN coated LDPE and the other with untreated LDPE and filled with Milli-Q water were cooled down to -6°C simultaneously, ice nucleation occurred in the ECIN coated film within 10 seconds, where control stayed unfrozen for over an hour (Figure 43). Picture was taken instantly after both samples were transferred to a dark background desk from the water bath. It should be noted that the ice nucleation temperature was higher than when the nano-thin ECIN layer was tested on silicon wafer with 10 µL water droplets. This shows that when the sample volume increases, the probability of ice nucleation for the whole mass also increases. Although the amount of ECIN per unit area of the film does not change (another important factor that increases the ice nucleation temperature and shortens supercooling time, as demonstrated in Figure 46), contact area of water with the ECIN coated surface increases when the volume gets larger. And once the first ice crystal forms at one of the ice nucleation sites, it grows faster due to the higher mobility of water molecules and the whole mass turns into ice in shorter time.
Figure 43. Images of PE packaging materials filled with milli-Q water and exposed to -6°C, one without any treatment (left) and the other UV-ozone treated on the inner surface followed by LbL deposition to form ε-PL/i-carr/ε-PL/ECIN multilayers (right).

After confirming the hypothesized ice nucleation effect, reusability of the film was investigated to evaluate the stability of the fabricated nano-thin ECIN layer. This allowed us to see how long it takes for it to wear out and lose the activity, which would be an important factor if those films are to be used in the food industry. 50 freeze-thaw cycles were conducted, where the MilliQ water in the vial was thawed and refilled after collecting each freezing curve, under the same cooling rate and volume conditions.

Figure 44 shows the freezing curves for the vial coated with the untreated control film and 1st, 11th, 21st, 31st, 41st and 50th refills for the vial coated with the ECIN coated film. Average ice nucleation temperature in the control vial was 9.26°C, and during the refills of the vial laminated with the ECIN coated LDPE, it varied in the range of -3.46°C (on the 3rd refill) to -9.76°C (on the 50th refill), with an average of -7.52°C (Figure 45).
Figure 44. Freezing curves of 5 mL of pure water in mini-vials coated with the LDPE/ε-PL/i-carr/ε-PL/ECIN multilayer system and with untreated LDPE, upto 50 freeze thaw cycles.
Figure 45. Variation in the ice nucleation temperatures and freezing times (the time it takes to reach -2°C from 2°C) of 5 mL of pure water in mini-vials coated with the LDPE/ε-PL/i-carr/ε-PL/ECIN multilayer system versus the averages with the untreated LDPE, up to 50 freeze thaw cycles.

Average of the 5 refills on the first day was 5.7°C, and it stayed in the range of 7.01°C to 7.93°C for the next 7 days, and then increased to 8.78°C and 9.26°C on the 9th and 10th days respectively, indicating a loss of activity over reuse. Experiment was discontinued at this point since the ice nucleation temperatures of the control and ECIN coated film were very close. For the first 45 freeze thaw cycles, ice nucleation temperatures for the ECIN coated vial were always higher than -9°C, also higher than -8°C during the first 17 refills and higher than -7°C during the first 7 refills.

Time for the water to reach -2°C from 2°C at its geometric center, averaged from the 10 freezing curves of the control vial collected daily was 15.6 minutes; where the
shortest time for the water in ECIN coated PE was 5.66 minutes, and average time from 50 freezing curves was 10.10 minutes (Figure 45). A trend towards increasing ice nucleation times (the time point where freezing starts) with increased number freeze thaw cycles (refills) was observed. A correlation between the ice nucleation time and total freezing time (time it takes for the whole mass to reach the freezer temperature) also exists, which is indicated by the representative freezing curves in Figure 44 as well. This suggests that solely by increasing the ice nucleation temperature which shortens the ice nucleation time, it is possible to save energy by decreasing the total freezing time. Additionally, since the sample volume of 5 mL is relatively small for the high cooling rate of our system, phase change occurred almost instantaneously and temperature continued to decline linearly to come to equilibrium with the water bath temperature. Thus, these experimental freezing curves look different from typical/theoretical freezing curves where an extended phase change stage can be seen as a straight line at the equilibrium freezing temperature until the whole mass turns into ice.

Figure 46 shows the freezing curves of LDPE films prepared by using 0.1 mg/mL (the concentration used for the reusability experiment) and 0.5 mg/mL ECIN solutions to make the top layer. Shorter ice nucleation times are seen for the higher concentration film in both curves, which indicates the higher probability of nucleation when the surface has more ice nucleation sites per unit area. Ice nucleation temperature with the first freezing curve was 4.16°C for the 0.5 mg/mL film and 5.67°C for the 0.1 mg/mL film (Figure 46).
Figure 46. Freezing curves and ice nucleation temperatures of 5 mL of pure water in mini-vials coated with the PE/ε-PL/i-carr/ε-PL/ECIN multilayer system, where ECIN dipping solution concentrations were 0.1 mg/mL and 0.5 mg/mL.

Another test was conducted in triplicate by filling the same vials with the dipping solutions instead with no film, to compare the activity of film versus solution. Dramatically higher ice nucleation temperatures and times were obtained with the 5mL solutions of 0.1 mg/mL and 0.5 mg/mL ECIN, all samples froze at temperatures higher than -1°C and in less than 1.5 minutes (Figure 47). This shows that the activity of nano-thin ECIN layers will be less than the activity observed when they are mixed into the samples which are to be frozen. This is because the film cannot hold the large quantity of ECINs that the solution can, and that with the films ice nucleating sites are present only where the sample contacts the film, whereas in solution they are dispersed into the whole
volume. Still, the use of nano-thin films would be preferable where the ECINs can’t be added into the sample due to safety, quality or sensory concerns.

**Figure 47.** Freezing curves and ice nucleation temperatures of 5 mL of ECIN solutions at concentrations of 0.1 mg/mL and 0.5 mg/mL.
7. Conclusion

Results are promising for the possible application of ice nucleators on packaging materials to obtain higher freezing temperatures and shorter freezing times, which has the potential to provide energy savings. ECIN coated LDPE films retained their ice nucleation activity up to 50 freeze-thaw cycles, indicating the reusability of these films. Higher freezing temperatures (average of 1.8°C) and shorter freezing times (average of 5.5 minutes) were obtained with ECIN nanofilms vs control during the first 50 freeze-thaw cycles. Formation of an ice nucleus is a very subtle phenomena mostly expressed as a probability rate due to highly unpredictable behavior of water molecules making it hard to replicate the freezing curves. However in this study, repeated tests clearly indicated the difference between the freezing of water in ECIN coated films vs non treated films. The reusability studies conducted in New Jersey and Taiwan led to the same conclusions indicating the remarkable reproducibility of the experiment (See Appendices, Section 9.1). This trial is a proof of concept for the possible application of ECIN coated films as a functional packaging material to shorten the supercooling stage when freezing water and reduce the amount of energy required for this purpose. It also suggests the possible use of this approach to produce films of other functionalities (such as antimicrobial activity etc.) via LbL deposition technique, which is simple yet versatile. Future studies will be conducted with 20% sucrose solution and milk to see if the shortened freezing time effect with water can be reproduced with these samples. The effect of ECIN coated LDPE films on the freeze thaw stability of fish actomyosin will also be evaluated.

Globular and thread like structures were observed on the AFM images of ECINs. Effect of layer build-up on ECIN adsorption was investigated and three or more
consecutive layers of PDDA/PSS was found to be satisfactory for the adsorption of ECIN on the surface, based on the fact that surface morphology, nucleation activity and water contact angles did not change significantly when more layers were added. Ice nucleation activity of ECINs when applied as an ultrathin layer was studied for the first time and confirmed that activity is retained. Nucleation occurred earlier when wafers were immersed in solutions of higher ECIN concentration, due to the increased number of nucleation sites available in a unit area.

Biopolymers successfully replaced the synthetic PSS/PDDA pair, which enables the application of this approach to food grade systems. There was no difference in nucleation activity for the biopolymer and synthetic systems and nucleation could be obtained at ECIN dipping solution concentrations of as low as 0.01 mg/mL with the PSS/PDDA and the iota carrageenan/130 kDa chitosan system. With both systems, a big increase was observed in layer thickness when ECIN dipping solution concentration is increased from 0.1 to 0.5 mg/mL.

Iota-carrageenan perform better than others due to the high charge density and ideal positioning of charged groups. Higher MW chitosans provide better surface coverage and lead to the formation of more closely packed gel networks when paired with carrageenans and pectins. Amidated low methoxyl (<50% DE) pectins form optimum multilayer systems for ECIN absorption. Availability of enough free carboxylic acid groups is the main factor when choosing the right pectin (90% DE was too high), where amidation had a secondary effect on building up a better multilayer foundation for ECIN absorption. ε-PL makes a good multilayer pair with iota-carrageenan and ECIN layer formed on this multilayer system had an ice nucleation activity at -7°C, thus it is a
good alternative to chitosan due to its better solubility and for readily having GRAS status for food use at 5-50 ppm levels. Overall, we recommend iota carrageenan and amidated low methoxyl pectins as the negatively charged and high molecular weight chitosan (130 and 330 kDa both performed well) and ε-PL (although it was a much lower molecular weight of 4 kDa) as the positively charged biopolymers for use in food grade multilayer systems.

To sum up, ECIN nanofilms formed using the LbL deposition approach with charged biomaterials, do possess ice nucleation activity. The applicability of LbL deposition for food grade polyelectrolytes itself, has great future potential for the fabrication of ultrathin layers of any protein or polysaccharide with desired functionality, which can be a unique nanotechnological tool especially for but not limited to the food scientists working in the functional foods area.

8. Future Directions

There are many directions that this research can take from this point on. Some common food polymers were already paired for multilayer fabrication in this study and most were found to be effective ingredients in building multilayer systems. However, further investigation on alternative polyelectrolyte multilayer systems can be helpful for specific applications, where there may be certain limitations for the choice of ingredients (ingredient compatibility, sensory concerns, regulatory limitations, etc.). Experimentation food polymers such as starches, gelatin, gum arabic and alginic acid can widen the range of possible food applications.

Final chapter of this study illustrated an example for the transfer of multilayers from experimental substrates such as silicon wafer and gold crystals to commercially
available LDPE packaging material, a practical substrate that can readily be used by the food industry as a nano-enabled film. More research on this aspect will widen the range of possible applications of nano-thin functional films. Potentially, any substrate that is readily charged or can get charged by surface modification can be used to build those multilayers with various functions. However, further experimentation is required to establish the proof of principles.

Thus, a third direction can be to study the alternative functions of those films. We conducted trials on the antimicrobial effect of single layers of chitosan, ε-PL and PDDA on UVO treated LDPE films using the agar diffusion assay. Films did not show a detectable inhibitory effect on a lawn of gram positive *Staphlococcus aureus* and gram negative *Escherichia coli*, which can be due to the high number of organisms making up the lawn and that the layer was too thin (~1-20 nm) to inhibit this population. Nevertheless, using alternative microbiological methods which can provide higher sensitivity with reduced bacterial concentrations, it may be possible to demonstrate the antimicrobial properties of those nano-thin films, which is already well established for chitosan and ε-PL (Shih et al., 2006; Shumilina and Shchipunov, 2002; Yoshida and Nagasawa, 2003).

A final direction that can quickly lead to meaningful and useful results for food applications, is to use the actual films we have fabricated in the industrial freezing of food products. Some research has already been conducted in Taiwan by Hsiang-Chia Hung and Dr. Tung-Ching Lee at the National Taiwan University, using the actual films which we have fabricated at Rutgers University (Hung and Lee, 2009). They have repeated the reusability test we conducted in New Jersey (Figure 45) with up to 30
freeze-thaw cycles, and achieved very similar results where the ice nucleation temperatures were also higher than -7°C for the first 7 refills and higher than -8°C for the first 17 refills (Figure 49).

They conducted another study where they collected the freezing curves of 20% sucrose solution at -8°C. While the solution itself and the same solution in untreated LDPE film remained unfrozen throughout the experiment (for over an hour), putting it into an ECIN coated LDPE film promoted ice nucleation at a temperature of approximately -4°C in about 15 minutes (Figure 50).

They also observed the freezing of milk at -10°C, a study similar to the trial we have conducted with no pulp orange juice (Figure 48). It took about 80 minutes for milk with no packaging and 25 minutes for milk in untreated LDPE film to freeze, and ice nucleation temperatures were about -10°C (Figure 51). In ECIN coated LDPE film, ice nucleation occurred very close to 0°C, almost without a supercooling stage and in about seven minutes. Total freezing time, i.e. the time for the whole the mass to reach the equilibrium temperature of -10°C, was 47.2% shorter than that for milk with no packaging.

Finally, they evaluated the effect of ECIN coated LDPE film use on the quality of fish actomyosin exposed to freeze-thaw cycles. Percent Ca2+-ATPase activity retention of fish actomyosin in ECIN coated LDPE film versus untreated LDPE film was 12-17% higher during the 3 freeze-thaw cycles (Figure 52). These results establish a strong rationale for moving onto larger scale trials with actual food samples.

There are two possible explanations for the fouling of nanothin ECIN layers over freeze thaw cycles, one is the denaturation of the ice nucleating protein and the other is
the physical desorption of ECIN from the surface to the food matrix. A trial conducted with no pulp orange juice indicated that the activity loss can be more significant when the food matrix is acidic, since the ice nucleation temperature was lower and freezing time was longer during the second freeze-thaw cycle than the first (Figure 48).

![Figure 48](image)

**Figure 48.** Freezing curves and ice nucleation temperatures of 5 mL of no pulp orange juice frozen in ECIN coated and untreated LDPE films, during the 1st and 2nd freeze-thaw cycles. ECIN dipping solution concentration was 0.1 mg/mL.

This data suggests but does not provide enough evidence to conclude that ECIN layers will be less stable in acidic environments. As mentioned earlier, ice nucleation is a matter of probability which causes big variations in consecutive freezing curves (as seen
in Figure 45), thus the stability and interaction of the ECIN layer with the actual food systems will require extensive experimentation to make a clear conclusion.
9. Appendices

9.1. Some Food Applications of the Ice Nucleating LDPE Films

These experiments were conducted by Hsiang-Chia Hung from National Taiwan University as a part of his masters study (Hung and Lee, 2009), using the LDPE films coated with nano-thin ECIN layers which were fabricated in Rutgers University, NJ.

Figure 49. Variation in the ice nucleation temperatures of water frozen in ECIN coated LDPE films versus those in untreated LDPE, upto 30 freeze thaw cycles.
Figure 50. Freezing curves of 20% sucrose solution, same solution in untreated LDPE film and in ECIN coated LDPE film, at -8 °C.
**Figure 51.** Freezing of milk, milk in untreated LDPE film and in ECIN coated LDPE film, at -10°C.
Figure 52. Ca2+-ATPase activity retention of fish actomyosin up to 3 freeze-thaw cycles in untreated LDPE film versus that in ECIN coated LDPE film.
9.2. 3rd overtone (15 Mhz) raw QCM-D data and thicknesses (estimated by the Voigt Model) of Different Chitosan/Carrageenan/ECIN Systems

Figure 53. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.001 mg/mL ECIN system.

(A)
Figure 54. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.01 mg/mL ECIN system.
Figure 55. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.05 mg/mL ECIN system.
Figure 56. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.1 mg/mL ECIN system.
Figure 57. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.3 mg/mL ECIN system.
Figure 58. Frequency shifts (A) and estimated thickness (B) of layers absorbed with the Chitosan/Carrageenan/Chitosan/0.5 mg/mL ECIN system.
Bibliography


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