ABSTRACT

Title of Document: **OPTIMIZATION OF XANTHAN CHITOSAN POLYELECTROLYTIC HYDROGELS FOR MICROENCAPSULATION OF PROBIOTIC BACTERIA**

Pavan Kumar Soma, Doctor of Philosophy, 2011

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The effectiveness of microencapsulation system for targeted delivery of probiotics depends on its ability to protect cells from harsh gastrointestinal conditions of stomach followed by effectively releasing the cells in intestinal conditions. Oppositely charged xanthan and chitosan form stable polyelectrolytic hydrogels capable of encapsulating enzymes and cells. The present study aims at developing an effective microencapsulation system for probiotics by screening and optimizing the factors critical to xanthan-chitosan hydrogel (XCH) capsule formation. The changes in the core pH of the hydrogel capsule in response to simulated gastric juice (SGJ) were characterized. Increase in xanthan concentration and chitosan molecular weight improved the barrier properties, however, increasing complexation time beyond 40 min had the opposite effect. Increase in molecular weight of chitosan resulted in improved viability of probiotic bacteria, *Lactobacillus acidophilus*, after SGJ treatment, which could be attributed to the differences in hydrogel membrane

thickness at the surface of capsule, as evidenced by scanning electron micrographs (SEM). Introducing XCH capsules made with high molecular weight (HMW) chitosan into xanthan solution resulted in the formation of xanthan-chitosan-xanthan hydrogel (XCXH) capsules. Unlike HMW and medium molecular weight (MMW) chitosan, low molecular weight (LMW) chitosan did not form the outer layer beyond XCH, suggesting the significance of chitosan molecular weight in the formation of XCXH. The increased hydrogel thickness of XCXH capsules formed with HMW chitosan compared to XCH capsules rendered better retention of cells in SGJ treatment for a longer period of time, further suggesting the importance of membrane thickness on the hydrogel stability and its barrier properties. Furthermore, complete release of cells from XCXH in simulated intestinal fluid (SIF) was extended by approximately an hour compared to XCH capsules. Smaller, nozzle-sprayed XCXH capsules using HMW chitosan protected probiotic bacteria in SGJ albeit one-log reduction in its protective efficacy compared to syringe extruded capsules. When incorporated into stirred yogurt, XCXH microcapsules improved the viability of *L. acidophilus* by ~1 log CFU/ml between 15 and 30 days of storage. The stability of bacteria against bile salts was significantly improved, enabling the delivery of prescribed number of cells to attain the claimed health benefits.

OPTIMIZATION OF XANTHAN CHITOSAN POLYELECTROLYTIC HYDROGELS FOR MICROENCAPSULATION OF PROBIOTIC BACTERIA

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Chapter 1: Introduction

1.1 Introduction

Gastrointestinal tract is a host for numerous bacteria that work along with the digestive system in delivering nutrients and chemicals that affect our body in a positive way (Dunne 2001; Weinbreck et al. 2010). The microorganisms can be either beneficial or detrimental. Health of an individual depends on the balance between the number of health beneficial and health deteriorating microorganisms colonizing in the intestine. Many factors such as age, diet and diseases can cause an imbalance in the intestinal microbiota (Fooks et al. 2001). Dietary supplements of live bacteria that could re-establish this balance are termed as probiotics. Probiotics have become popular due to their contribution to good health by improving immune system and providing antipathogenic effects when consumed at large numbers (Scheinbach 1998; Kumar et al. 2010). However, ingestion of these bacteria will have little consequence unless enough probiotic bacteria reach beyond small intestine and adhere to the intestinal epithelium (Salminen et al. 2002). Harsh gastrointestinal conditions followed by high bile salt concentration often reduce the viability to levels where no health benefits are achieved.

Microencapsulation is considered one of the effective methods that can provide protection against the harsh gastrointestinal conditions. Over the years, many researchers have developed microencapsulation techniques to protect bacteria. These differ in the kind of coating material used or technique of encapsulation. Used mainly for microencapsulation of flavors and other sensitive chemicals in the food industry, polysaccharides such as starch and modified starches, alginate, chitosan have been applied to microencapsulate probiotic bacteria by various researchers (Mortazavian et al.,

2007; Hansen et al., 2002; Crittendon et al., 2001; Lee et al., 2004). Although most of these encapsulation techniques have improved viability of probiotic bacteria in gastrointestinal conditions, they suffer from limitations like susceptibility to ions, low mechanical strength, and inability to release specifically in the intestine resulting in poor final effects (Kailasapathy, 2002). Therefore, there is a pressing need for a better microencapsulation system to protect and deliver bacteria in intestine.

Xanthan and chitosan being natural biopolymers are biocompatible and non-toxic and are good candidates for microencapsulation of probiotics. Xanthan, a negatively charged polysaccharide, forms instant hydrogels when its aqueous solution comes in contact with that of chitosan, a positively charged polysaccharide. Xanthan-Chitosan hydrogels have been used previously for immobilization of xylanase enzyme (Dumitriu et al., 1994) and *Corynebacterium glutamicum* (Chu et al., 1997). Xanthan in combination with chitosan has been demonstrated by our research group to be effective in protecting and releasing probiotic bacteria in gastrointestinal conditions (Argin-Soysal, 2007). However, reduction in capsule size showed a drastic drop in protective effects leading to low viability in gastrointestinal conditions. Therefore, there exists a need to optimize and improve the xanthan-chitosan microencapsulation system to effectively protect and deliver probiotic bacteria in the intestine.

1.2 Research Objectives

The ultimate goal of this research was to develop and optimize the microencapsulation system based on xanthan-chitosan polyelectrolyte hydrogel capsules to effectively maintain the viability of probiotic bacteria in order to confer the claimed health benefits. The emphasis was placed on improving the ability of the hydrogel in retarding gastric fluid from diffusing to the core. The effectiveness of an extra layer of

thickness on the capsules' ability to protect probiotic bacteria was evaluated. Process parameters required to make small capsules to enable incorporation into foods were optimized. Finally the efficacy of the microencapsulation system in sustaining the probiotic bacteria in stirred yogurt during refrigerated storage was evaluated. The specific objectives directed towards this research are listed below. The subsequent chapters following literature review, aimed at these objectives, are presented as separate manuscripts.

Objective 1: Evaluate the factors that affect the ability of hydrogel to retard gastric juices from entering the hydrogel.

Objective 2: Investigate the effect of molecular weight of chitosan on the protective effects of hydrogel on probiotic bacteria against gastrointestinal conditions.

Objective 3: Compare the effectiveness of xanthan-chitosan-xanthan hydrogel capsules in effectively protecting and releasing probiotic bacteria in gastrointestinal conditions to xanthan-chitosan hydrogel capsules. Optimize the formation of smaller capsules and study the effect of size on protective effects.

Objective 4: Study the efficacy of xanthan-chitosan-xanthan hydrogel microcapsules in stirred yogurt.

Chapter 2: Literature Review

2.1 Human Intestinal microbiota

The human gastrointestinal tract is a host for numerous bacteria. There is a considerable variability in the number of bacteria in stomach, small intestine and large intestine. These differences are primarily due to the availability or lack of optimum growth conditions that suit bacterial growth. Due to the low pH environment, the stomach has the lowest bacterial count usually below 10^3 CFU/ml of gastric contents followed by small intestine ranging from 10^3 to 10^4 CFU/ml. Whereas, the large intestine is heavily colonized with 10^{11}-10^{12} CFU/g of gut contents (Gorbach 1967). Gut microbiota is by far the most complex and diverse ecosystem in our gastrointestinal tract with 300-400 different species and thousands of strains (Guarner and Malagelada 2003; Aureli et al. 2011). Favorable growth conditions such as pH of around 5.5 to 7 along with abundance in availability of undigested substrates lead to such high number of bacteria (Fooks et al. 1999).

Gut microbiota predominantly consists of anaerobes and include members of genera *Bacteriosides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus* among others (Simon and Gorbach 1984; Guarner and Malagelada 2003). *Bacteroides* and *Bifidobacteria* account for 30 and 25% of the total anaerobic counts respectively (Salminen et al. 1998). While *Bacteroides* like *Bacteroides ovatus* and *Bacteroides fragilis*, and *Bifidobacteria* such as *B. bifidum* and *B. infantis* predominate in the colon, *Lactobacillus* such as *L. acidophilus*, *L. plantarum*, *L. casei*, and *L. rhamnosus* are also found in the small intestine (Salminen et al. 1998).

The principal role of the intestinal microbiota is to metabolize carbohydrates not digested in the upper gut, through fermentation. The major substrates for fermentation are the dietary carbohydrates that have bypassed digestion. These include resistant starch, non-digestible oligosaccharides, cellulose, hemicelluloses, pectins, gums, and sugar alcohols. In addition, some proteins like elastin, collagen, albumin as well as bacterial proteins released after cell lysis act as effective growth substrates for colonic bacteria. (Fooks et al. 1999; Salminen et al. 1998; Simon and Gorbach, 1984). Metabolism of these substrates result in short chain fatty acids (SCFA) and vitamin synthesis. Among other functions, production of SCFA also plays part in enhanced absorption of ions such as calcium, magnesium and iron. However, along with the beneficial chemicals, a series of potentially toxic substances are also produced. These include ammonia, amines, phenols, thiols, and indols (Guarner and Malagelada 2003).

The major functions of microbiota in the human intestine include providing barrier function by participation in the formation of the intestinal wall, immune system stimulation, maintenance of mucosa nutrition and circulation, production of nutrients and improving bioavailability, and resistance to colonization of pathogenic bacteria (Holzapfel and Schillinger 2002; Aureli et al. 2011).

Intestinal microbiota of each individual has a distinctly different bacterial fingerprint from other individuals in a particular combination of predominant species (Salminen et al. 1998; Guarner 2003). The composition of the intestinal microbiota is influenced by factors such as use of antibiotics, administration of drugs for treatment of diseases, surgical operations of the stomach or small intestine, immune disorders, stress, poor diet and aging (Luchansky et al. 1999). Such factors often cause an imbalance

between the health beneficial and health detrimental microbiota. This delicate balance may be restored to normal by oral administration of probiotic bacteria.

2.2 Probiotics

Probiotics have been defined as "live microbial feed supplements that have beneficial effects on the host by improving their intestinal microbial balance" (Fuller, 1989). The Food and Agriculture organization (FAO) of the United Nations and the World Health Organization (WHO) define probiotics as "Live micro organisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host" (FAO/WHO, 2001).

The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria, improves digestion and reinforces the body's natural defense mechanisms (Fooks et al. 1999; Tsuji et al. 2008; Aureli et al. 2011;). Other efficacies of probiotic bacteria include prevention of diarrhea and constipation diseases (Davidosn et al. 2000; Szajewska et al. 2006), improvement of lactose utilization by producing β-galactosidase (Montalto et al. 2006), nutrient synthesis and their bioavailability (Scheinbach 1998; Saavedra and Tschernia 2002). In a recent study, administration of infant formula enriched with prebiotics, non-digestible food ingredients that can be utilized by microbes and stimulate their growth, like galacto- and fructo-oligosaccharides have shown to reduce the number of diarrheal episodes, lower the incidence of respiratory infections, gastroenteritis, and increase the height and weight of children at 3 and 6 months as compared to children fed with standard infant formula (Bruzzese et al. 2009). In another study involving anti-*Helicobacter pylori* therapy, adult patients showed significantly lower number of incidences of nausea, diarrhea and taste disturbances when supplemented with *Lactobacillus* GG (Armuzzi et al. 2001). The

probiotic bacteria regulates the number of potentially harmful bacteria by several different mechanisms like decreasing the luminal pH by the production of short chain fatty acids such as acetic acid, lactic acid, or propionic acid, rendering vital nutrients unavailable to pathogens, altering the redox potential of the environment, producing hydrogen peroxide or producing bacteriocins or other inhibitory substances (Kailasapathy & Chin 2000; Gismondo et al. 1999). Compelling evidence for probiotic efficacy in the areas of improved digestion and reduced incidences of diarrhea and possibility of other health benefits including no risk associated with probiotic bacteria have made them an attractive option for improved health benefits (Sanders, 1999).

Lactic acid bacteria (LAB) are the most important probiotic bacteria typically associated with the human gastrointestinal tract. Other common microorganisms are *Bifidobacteria*, *Streptococcus* and *Enterococcus*. Table 1 gives the list of species of bacteria used as human probiotics. The benefits of probiotics are related to the microorganisms which live naturally in its digestive tract (Abe et al. 1995). The natural adaptation of many LAB to the environment in the gastrointestinal tract and the antimicrobial substances produced by them (organic acids and bacteriocins) have provided these organisms a competitive advantage over other microorganisms to be used as probiotics (Salminen et al. 1998).

The ability of probiotic microorganisms to survive and multiply in the host strongly influences their probiotic benefits. The bacteria should be metabolically stable and active in the product, survive passage through the upper digestive tract in large numbers and have beneficial effects when in the intestine of the host (Gilliland 1989).

Table 2.1 Lactic acid bacteria (LAB) used as human probiotics

Lactobacillus species	*acidophilus* *gallinarum* *reuteri*	*amylovorous* *gasseri* *rhamnous*	*casei* *johnsoni* *salivariusi*	*crispatus* *plantrium*
Bifidobacterium species	*animalis* *longum*	*bifidum* *lactis (animalis)*	*breve*	*infantis*
Streptococcus species	*thermophilus*			
Enterococcus species	*faecium*			

Table adapted from Klaenhammer and Kullen, 1999

The standard for any food sold with health claims from the addition of probiotics is that it must contain per gram at least 10^6 to 10^7 CFU of viable probiotic bacteria (FAO/WHO 2001). Several factors of the food that affect the viability of bacteria include the pH, titratable acidity, dissolved oxygen content, and storage temperature, among others. Furthermore, probiotic bacteria has to survive the harsh acidic conditions of stomach followed by a high pH and a high concentration of bile salts in the small intestine before it can reach the large intestine inorder to provide health benefits. (Dave and Shah 1997).

Different approaches that increase the resistance of these sensitive microorganisms against adverse conditions have been proposed, including appropriate selection of acid- and bile-resistant strain, use of oxygen impermeable containers, two-step fermentation, and stress adaptation, incorporation of micronutrients such as peptides and amino acids, and microencapsulation (Gismondo et al. 1999). While selection of acid and bile resistant strains, stress adaptation directly enhances the resistance of the bacteria, they may not be feasible to all the organisms. Microencaspulation has gained a significant attention due to its applicability to a wide variety of microorganisms and has

been a proven technology in terms of protecting other sensitive biomaterials like enzymes, flavors etc.

2.3 Microencapsulation:

Microencapsulation is the process by which a pure material or a mixture is coated or entrapped into another material called wall material, membrane, carrier or shell to produce capsules in the micrometer to millimeter range known as microcapsules. The purpose of microencapsulation is to protect the functional core ingredient to be separated from the surrounding destructive environment until its release is desired (Anal and Stevens 2005). Natural examples of encapsulation include egg shells, plant seeds, bacterial spores (Gibbs et al. 1999).

Microencapsulation has been used for a variety of functional materials like cells, enzymes, and pharmaceutical drugs, food ingredients like oleoresins, oxidation-sensitive vitamins, sweeteners, minerals, antioxidants and proteins (Gibbs et al. 1999; Bansode et al. 2010). Food and pharmaceutical industries often rely on microencapsulation technology for controlled release applications, enhanced stability, flavor masking, protection against harsh conditions, and improved nutrition (Shahidi et al. 1993; Schrooyen et al. 2001; Obeidat 2009). Release of the core material can be triggered by temperature, pH changes, osmotic shock or a combination of factors (Bansode et al. 2010). Wall material for capsule formation utilizes a combination of one or more of sugars, proteins, natural and modified polysaccharides, lipids and synthetic polymers. Different techniques used for encapsulation include spray drying, spray chilling or cooling, extrusion coating, fluidized bed coating, liposome entrapment, coacervation, inclusion complexation, centrifugal extrusion (Gibbs et al., 1999).

Microencapsulation of probiotic bacteria is a relatively newer technology developed based on cell immobilization technologies. These two technologies are different as they address different challenges. Immobilization of cells is applied mainly for bio-technological applications that require diffusion of small molecules like nutrients and spent molecules through the matrix. Whereas probiotics need to be isolated from the environment during processing/storage for better shelf life, for protection against high acidic stomach conditions upon ingestion and also release the bacteria in the gastrointestinal tract. Survival of probiotic bacteria during processing and storage can be affected by a range of factors including pH, post-acidification in fermented products, hydrogen peroxide formation, oxygen toxicity and storage temperature (Brunner et al. 1993; Kailasapathy 2002; Anal and Singh 2007). Thus, providing probiotic bacteria with a physical barrier is an effective approach currently followed by many researchers.

Table 2.2 summarizes some of the techniques used by researchers for encapsulating probiotic bacteria. It is evident that the ability of each system in protecting and delivering the bacteria is varying and each system has its own disadvantages. The most common system used by researchers has been alginate gum as it is very biocompatible, non-toxic and forms gels instantly in the presences of calcium ions and has high mechanical strength (Sheu et al. 1993). However, alginate gels are not stable in the presence of phosphate and lactate ions as these ions displace the calcium ions that are responsible for gelation. Hence, use of alginate has limitations considering microencapsulation of lactic acid bacteria and in fermented products (Kailasapathy 2002). Also, these hydrogels may be permeable to cells (Lacroix et al. 1990). Different coatings and combinations of alginate gels with other polymers like starch, whey protein, and chitosan were also used to overcome some of the aforementioned disadvantages.

However, each approach has its own limitations, it is important to consider the mechanical stability, processing conditions, storage and scale up factors along with protection and release of probiotics when choosing a system for microencapsulation. While each technology has its pros and cons, electrostatic method has gained attention because of the ease of making capsules using simple biocompatible and biodegradable polymers.

Table 2.2 Comparison of microencapsulation systems developed for probiotic bacteria.

Carrier/strain	Method	Size (μm)	Purpose/Remarks	Reference
Alginate				
Bifidobacteria	Emulsification	19 and 67 μm	-Acid Stable -Better control over gelation and size. Bacteria more densely packed.	Hansen et al. 2002
Bifidobacterium lactis	Emulsification	~20 μm	Storage; application in ice cream	Homayouni et al. 2008
L. acidophilus ATCC 4356	Direct compression	N/A (Tablets)	Storage; Storage stability increased 10 times compared to freeze- dried cells	Chan and Zhang 2002
L. acidophilus CSCC 2409	Spraying method-Gel beads	450-1000μm	-Acid and bile stable -3-log cycles improvement upon encapsulation at pH2 for 3 h.	Chandramouli et al. 2004
Alginate/chitosan				
L. bulgaricus KFRI 673	Gel beads(Chitosan coated alginate beads)	40-80 μm	-Acid and bile stable -3-log cycles improvement at pH-2 for 3h upon high mol. wt. chitosan coating.	Lee et al. 2004
Bifidobacterium breve	Extrusion	>1 μm	-Acid stability	Cook et al. 2011
Alginate/pectin/whey protein				
B. bifidum	Gel beads	2-3 mm	-Acid stable -<2.5 log reduction at pH-2.5 for 2h. Free cell did not survive.	Guerin et al. 2003
Alginate/starch				

Lactobacillus Bifidobacterium	Gel beads (Starch coated alginate beads)	0.5-1 mm	-Acid stable/storage - Improved acid stability and viability due to synergy in gelling between starch and alginate.	Sultana et al. 2000, Kailasapathy and Chin 2000, Dothindi 2010
Cellulose acetate phthalate				
B. pseudolongum	Phase separation-coacervation method-gel beads	1000 μm	-Acid and bile stable -2 log cycles decrease during encapsulation process. 3 log difference after 3 h at pH 1.33	Rao et al. 1989
Resistant starch				
Bifidobacterium PL1	Gel beads	_	-Acid stable/storage - Limited to strains adhering to resistant starch	Crittendon et al. 2001
Waxy maize starch				
Bifidobacterium	Spray drying	5 μm	-Poor acid stability. No survival of bacteria after 3 h at pH 2.8	O'Riordan et al. 2001
Whey protein				
B. longum R03 *B. breve* R07	Micronization and spray drying	10-50 μm	-Acid stable -Suitable for dairy fermented products. Micronized cultures less thermo-resistant. 2.6-log cycles improvement upon encapsulation.	Picot and Lacroix 2003a, 2003b, 2004
Lactobacillus rhamnosus GG	Extrusion	200 μm	Acid stable	Doherty et al. 2011

2.4 Polyionic Complexation Interactions (Electrostatic method)

Microencapsulation by polyionic complexation involves two oppositely charged polymers. One acts as a core material and other acts as a coating material. When two polymer solutions are mixed they form an irreversible polyionic hydrogel because of the electrostatic interactions between the oppositely charged polymers (Anal and Singh 2007). Hydrogels could be water soluble or water insoluble based on the polymers used. Two oppositely charged polymers that are used in this project are xanthan and chitosan.

2.5 Xanthan

Xanthan gum is a high-molecular weight extracellular polysaccharide produced by the bacterium *Xanthomonas campestris.* As shown in figure 2.1, xanthan gum is a heteropolysaccharide with a cellulosic backbone and a trisaccharide side chain linked to O-3 position of every other β-D-glucose residue. Trisaccharide side chain consists of one D-glucuronic acid unit between two D-mannose units as shown in the figure. Presence of acetic acid linked to the mannose residue connecting the main chain and pyruvic acid linked to the terminal mannose residue makes xanthan gum an anionic polysaccharide. Presence of pyruvic acid at various terminal mannose residues is dependent on the *Xanthomonas campsetris* strain.

Shear thinning behavior of xanthan is explained by association of xanthan chains stabilized by hydrogen bonds resulting in higher viscosity at low shear and reduced aggregation hence lower viscosity at high shear (Cuvelier and Launay 1986). Along with the marked shear thinning property, ordered helical conformation of xanthan gum is believed to be responsible for its extraordinary stability. Xanthan gum

is biodegradable, stable at wide range of pH and temperatures, resistant to enzymatic breakdown, shows synergistic properties with other natural polymers like guar gum, locust bean gum and galactomannans. Along with food and pharmaceutical industries, xanthan gum has seen applications in oil drilling, printing pastes, paints, colors, texture coatings and adhesives (Katzbauer 1987).

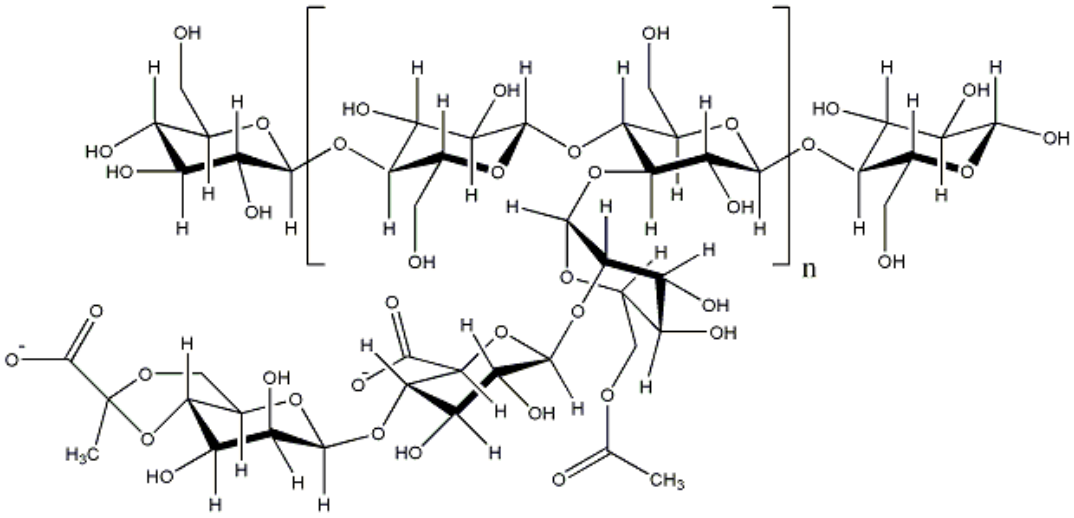


Figure 2.1 Structure of xanthan gum

Based on extensive research on toxicological properties and safety of xanthan gum for food and pharmaceutical applications, United States Food and Drug Administration has given xanthan a GRAS (generally regarded as safe) status without any specific quantity limitations. In 1980, the European Economic Community has added xanthan to the food emulsifier/stabilizer list, as item E-415 (Garcia et al. 2000).

Xanthan has been used in combination with other polymers like gellan for immobilization of cells (Wenrong and Griffiths 2000), alginate for encapsulation of urease enzyme (Elcin 1995), chitosan for immobilization of xylanase (Dumitriu

1997). Xanthan with its excellent properties is a good candidate for microencapsulation of probiotics.

2.6 Chitosan

Chitosan is the deacetylated form of chitin, the most abundant natural biopolymer after cellulose. Chitin is a copolymer of glucosamine and N-acetyl-d-glucosamine linked together by β(1,4) glycosidic bonds (Figure 2.2). Chitin is the major structural component of invertebrate exoskeletons and the cell walls of fungi (Shahidi et al. 1999). Chitosan is a primary aliphatic amine with a pK of 6.3 and can be protonated by selected acids. Chitosan is a biocompatible polymer and does not result in adverse reactions when in contact with human cells. Chitosan has seen many applications in food industry including edible film industry owing to the anti fungal properties of chitosan (Ghaouth et al. 1992), water purification as a chelation ion exchange polymer (Jeuniaux 1986), clarification and deacidification of fruit juices (Soto-Perlata 1996). Chitosan being a cationic polymer has pharmaceutical applications like nasal drug delivery agent due to its bioadhesive nature. Chitosan also interferes with the metabolic process of cholesterol (and other neutral lipids) by binding them with hydrophobic bonds (Kumar et al. 2004).

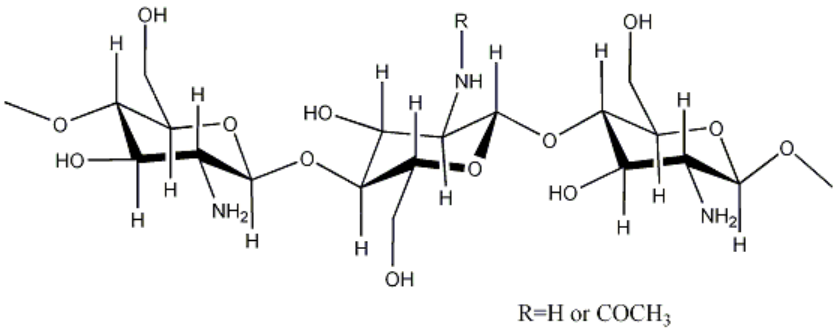


Figure 2.2 Structure of chitosan

2.7 Xanthan-Chitosan Hydrogel

2.7.1 Hydrogel structure & properties:

Xanthan and chitosan being oppositely charged polysaccharides readily form stable hydrogels by simple complexation between amine (chitosan) and carboxylic (xanthan) groups in aqueous phase within wide pH range of 3.6 to 8.0 (Dumitriu et al. 1994). pH and degree of deacetylation of chitosan are the major factors influencing the ratio of xanthan to chitosan content in the hydrogel (Dumitriu et al. 1994). These hydrogels have open fibrillar structure with characteristic pore dimension of 100-1000 nm and fibril diameter between 50 and 100 nm (Dumitriu et al. 1994). Chitosan-xanthan hydrogel have a very elastic texture and high rupture strength (Mitchell 1976).

Xanthan chitosan hydrogel as polyelectrolyte complexes shows pH-sensitive swelling behavior. pH sensitive swelling is a result of an increase in osmotic pressure. When hydrogel is immersed in alkaline solution, electrostatic linkage between the two functional groups disappears due to deionization of amino groups resulting in an increase in counterions (Na^+) thus increasing osmotic pressure (Chu et al. 1995). While concentrations of xanthan, pH and molecular weight of chitosan were reported to be important factors influencing the swelling of chitosan (Argin et al. 2009; Dumitriu et al. 1994), complexation time was related to the permeability of surface membrane of the hydrogel beads, decrease in permeability was reported with increasing complexation time due to increase in chitosan thickness on the surface of the beads (Severian & Esteban 1997).

2.7.2 Application of xanthan chitosan hydrogels

Polyionic hydrogels formed by xanthan and chitosan have the advantage of creating a favorable ionic microsystem for stabilizing enzymes and proteins by interacting with the free acid and base functions (Chellat et al. 2000). In one study, use of xanthan chitosan hydrogel to immobilize xylanase showed over 50% increase in enzyme activity and workable temperatures of 80-95^{0}C as compared to 50-60^{0}C for the free enzymes (Dumitriu and Chornet 2001). High yields of immobilization 85-98% were also achieved for xylanase enzyme (Dumitriu et al. 1994). Immobilzation of *Corynebacterium glutamicum* resulted in a 5-fold increase in its fumarase activity as compared to free cells and higher stability at high electrolyte concentrations for longer periods.

2.7.3 Degradation

Polymer degradation is a chain scission process that breaks polymer chains down to oligomers and into monomers. Degradation of polymers is an important topic of concern as it not only affects the release rate of the active component but also determines its applications based on chemical nature of the degradation products (Park et al. 1993). Owing to the stability of xanthan polymer in extreme pH and temperature conditions, along with enzyme resistance, xanthan chitosan hydrogel follows degradation pathway of chitosan leaving xanthan intact. Chitosan can be easily degraded by enzymes like lysozymes, N-acetyl-D-glucosaminidases and lipases (Kumar et al. 2004). One of the key factors affecting degradation is degree of deacetylation, with increase in degree of deacetylation degradation rate decreased (Ren et al. 2005). The major degradation products of xanthan chitosan hydrogel are

Glucosamine (GA) and N-Acetyl-Glucosamine (NAc-GA) (Chellat et al. 2000). Both these products are non toxic and in vivo studies show good tolerance of these hydrogels (Chellat et al. 1999, 2000).

Chapter 3: Assessment of xanthan-chitosan hydrogel composition on its internal pH stability against gastric acidity using fluorescence-based technique

3.1 Introduction

Known to destroy most viable probiotics upon digestion, the highly acidic gastric conditions (pH 1.5-4.0) remain a critical challenge for the food industry, since the desired probiotic benefits could not be achieved without delivery of sufficient viable cells into the small intestine (Annan et al. 2008; Gbassi et al. 2011; Shah 2002). It is thus crucial to develop a system capable of shielding probiotics from gastric acidity in order to maintain a suitable pH environment for the cells (Del Piano et al. 2011). Equally noteworthy is that the system needs to properly release the cells upon reaching the small intestine in order for probiotics to colonize and modify the microbiota. Numerous efforts have been attempted to improve the viability of probiotics in food products and in gastrointestinal conditions by microencapsulating probiotics in different wall materials, predominately starch-based (Donthidi et al. 2010; Homayouni et al. 2008), protein-based (Doherty et al. 2011; Heidebach et al. 2009), lipid-based (Del Piano et al. 2011; Maillard & Landuyt 2008) and alginate-based carriers with (Chan and Zhang 2002) or without compression (Cook et al. 2011; Chandramouli et al. 2004; Hansen et al. 2002; Lee et al. 2004; Mokarram et al. 2009). While most research reported protective effects on probiotics against gastric conditions or some during storage (Adhikari et al. 2000; Heidebach et al. 2010),

evidence in the literature remains scarce on whether the cells could be properly released in intestinal conditions.

Immobilization of microbial cells using polyelectrolyte complex gel composed of xanthan and chitosan was first reported by Chu et al. (1996) to evaluate the enzymatic activity of the immobilized bacteria. In our previous research (Argin-Soysal 2007), it was demonstrated that microencapsulation of probiotic cells using xanthan-chitosan hydrogel complex significantly improved their viability in gastric conditions and the cells could be released under intestinal conditions. The oppositely charged nature of xanthan (-) and chitosan (+) due to the respective carboxylic and amine groups involved in the complex formed stable hydrogel immediately upon contact, and stayed stable under gastric conditions. The effect of complexation conditions on swelling characteristics of the hydrogel has also been reported (Argin-Soysal et al. 2009). The maximal swelling degree of the hydrogel was reached under intestinal conditions, indicating its pH-sensitive nature. It was also pointed out that pH and concentration effects on the xanthan–chitosan network properties are dependent on each other. Therefore, it is important to optimize the hydrogel composition in order to provide the most stable pH environment for the encapsulated cells.

It is generally recognized that the dense network structure provided by alginate or other hydrogels should extend the time for the acidic media to diffuse into the core and reach equilibrium with external pH (Amsden 1998; Anal and Singh 2007). However, the majority of research has been focused on the diffusion of molecules such as sugars (Tanaka et al. 1984; White and Dorion 1961), proteins

(Hennink et al. 1996; Tong and Anderson 1996), and sodium and calcium ions (Chu et al. 1996; Graham et al. 1988) into hydrogel to provide nutrients for immobilized cells. Nevertheless, only a few studies addressed the diffusion of hydrogen ion but were limited to gastric mucous in the stomach (Lucas 1984; Sarosiek et al. 1983). In the present study, a fluorescence technique was employed to indicate the changes in pH inside the hydrogel with time when subjected to harsh acidic conditions. Eosin Y, a water-soluble acidic dye responsive to pH changes, was used to investigate factors that are crucial in ushering the diffusion of acidic media into the hydrogel.

3.2 Materials & Methods

3.2.1. Reagents and chemicals

Xanthan gum and chitosan, including low molecular weight (LMW, 20-200 cP), medium molecular weight (MMW, 200-800 cP), and high molecular weight (HMW, 1200-1600 cP), at 75-85% deacetylation was purchased from Sigma-Aldrich Chemicals (St Louis, MO). Eosin Y was purchased from Acros Organics (Morris Plains, NJ). Xanthan solution was prepared by dissolving predetermined amount of xanthan in deionized (DI) water under constant stirring. Chitosan solution was prepared by dissolving known amount of chitosan in 1 N hydrochloric acid with heating (65-70°C for 15 min) and agitation. The pH of the clear solution was adjusted to 6.0 using 1 N NaOH solution and DI water was added to bring the solution to the final volume.

3.2.2. Fluorescence technique

3.2.2.1 Effect of xanthan concentration on Eosin Y EI signal

To assess the effect of xanthan concentration on emission intensity (EI) signal of Eosin Y, solutions with different xanthan concentrations (0.7%, 1.0%, 1.2% w/v) with 0.01% Eosin Y were prepared. Control was 0.01% Eosin Y in DI water. Aliquots (150 µl) of sample were transferred into 96-well plate and EI was measured on a fluorescence microplate reader (FLUOstar, BMG Labtech, Offenburg, Germany) with the excitation and emission wavelengths set respectively at 485 and 510 nm. Same wavelengths are used in all the following experiments.

3.2.2.2. Optimal pH for eosin Y as a fluorescence indicator

Solutions of 0.01% (w/v) eosin Y at different pH were prepared using DI water to identify the most acidic pH value (to simulate the gastric conditions) under which eosin Y could emit steady fluorescence signals. Each eosin Y solution (150 µl) was added to at least 3 wells in a 96-well microplate and the EI was measured.

3.2.2.3. Emission intensity at different hydrogel internal pH

To assess the changes in internal pH of the hydrogel over time, various xanthan solutions containing 0.01% (w/v) eosin Y were prepared. Aliquots of this mixture (150 µl) were added to at least 3 wells in a 96-well plate before adding 100 µl of chitosan solution. After a predetermined complexation time, excess chitosan was removed by inverting the plate and tapping gently. To get rid of residual chitosan, each well was washed twice with pH 4.0 HCl solution to minimize swelling and leaching of the dye, followed by addition of 100 µl HCl solution (pH 2.75). The

fluorescence intensity was measured under aforementioned excitation and emission wavelengths.

For experiments conducted in custom-made tubes (Figure 3.1), standard 1-mL pipettes were cut into 3 identical hollow tubes, each at 4 cm long, and filed to smooth out the edges. Each tube was dipped into xanthan solution (1.0% w/v) until 0.02 mL of xanthan was inside the tube, which was then thumb-sealed at the top end before it was removed from the solution. The bottom end of the tube was parafilm-sealed before adding chitosan solution from the other end using a Pasteur pipette to form the hydrogel. After a complexation time of 40 min, unbound chitosan solution was removed. The top section was replenished with 0.1 mL SGJ and parafilm-sealed, followed by opening of the bottom seal before the tube was attached to the side of a 50-mL beaker containing 20 mL of 0.01% eosin Y. After 90 min of SGJ diffusion, small aliquots (150 μL) of eosin Y solution were taken for fluorescence measurements. The experiments were conducted in triplicates.

Figure 3.1 Example of custom-made tube with xanthan-chitosan hydrogel (XCH) formed at the bottom for gastric juice diffusion experiments.

3.2.3 Scanning Electron Microscope (SEM)

To provide visual confirmation of the hydrogel structure, XCH samples, after the prescribed complexation time, was separated from the free polymer solution and rinsed thoroughly with pH 4.0 solution, followed by freeze-drying for 24 hrs. Dried hydrogel samples were mounted on a metal holder using double-sided tape and coated with Au/Pd mixture before taking SEM photographs using the SU-70 Ultra High Resolution Analytical SEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 3 kV.

3.2.4. Statistical Analysis

Emission intensity measurements were statistically analyzed using SAS version 9.2 (SAS Institute, Cary, NC). Factorial analysis of variance was used to assess the effects of xanthan concentration, chitosan molecular weight, complexation time, and diffusion time on the EI of eosin Y. Differences in least square means were used for pair-wise comparison of means. Mixed procedure of SAS was used for analysis.

3.3 Results & Discussion

3.3.1 Effect of xanthan concentration on Eosin Y signal

As seen in Figure 3.2, the xanthan concentrations investigated did not significantly affect the EI of eosin Y. Eosin Y, a negatively charged pH sensitive dye has been used as a histological stain for decades (Waheed et al. 2000; Xie et al. 2001) and has recently found applications as a reagent for protein assays (Gao et al. 2007; Heltweg and Jung 2002; Hong et al. 1999) and as a model anionic dye in adsorption studies (Chatterjee et al. 2005; Du et al. 2008). The fluorescence intensity was

reported to be dependent on solvent mixtures as well as viscosity of the solvent (Deshpande and Iyer 1989, 1990). However, increase in xanthan concentration from 0.7% to 1.2% did not result in any significant differences ($P>0.05$) in emission intensity of eosin Y.

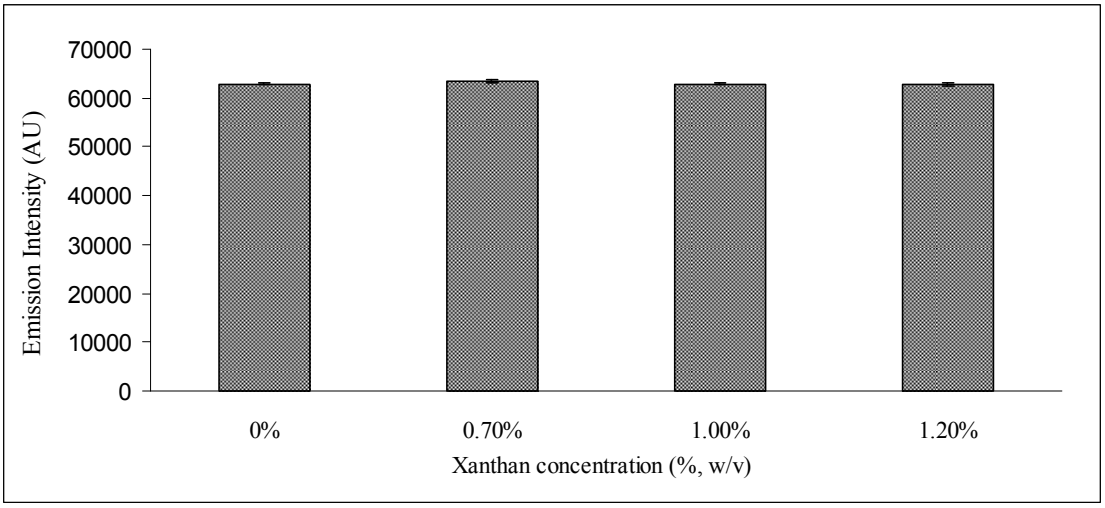


Figure 3.2 Effect of xanthan concentration on eosin Y emission intensity (EI) (n = 3)

3.3.2 Determination of optimal eosin Y pH

Since the typical gastric pH ranges between 1.5 and 4, it was critical to first profile the fluorescence of eosin Y under such conditions. As seen in Figure 3.3, eosin Y fluorescence intensity (AU, arbitrary unit) between pH 2 and 4 was a function of pH, as the EI of eosin Y increased with increasing pH. At $pH<2$, eosin Y was not completely soluble. Experiments with pH 2.5 solution resulted in slightly less transparent hydrogel after 90 min, which could be attributed to possible phase separation due to chitosan degradation (Chellat et al. 2000). Moreover, at such a low pH, strong protonation of xanthan's carboxylic group could take place, which impeded the reaction with the amine groups in chitosan. According to Dumitriu et al.

(1994), the yield of polyionic hydrogels obtained by complexation between xanthan and chitosan was significantly reduced when pH dropped to less than 2.5. On the other hand, the EI changed dramatically between pH 3 and 4, rendering eosin Y too sensitive to pH variations within that range and thus prone to errors. Hence, to simulate conditions close to gastric acidity, pH 2.75 was selected for subsequent experiments.

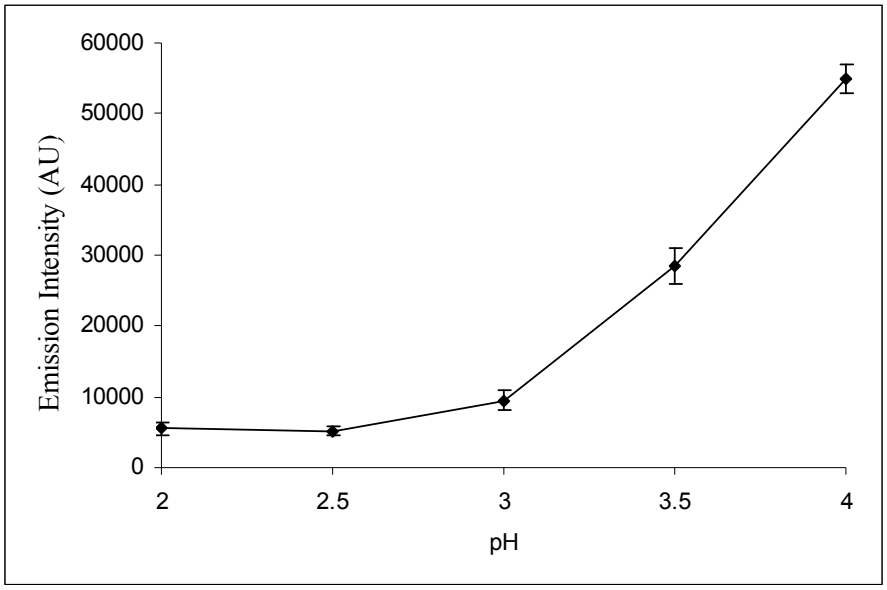


Figure 3.3 Fluorescence emission intensity (EI) of eosin Y (0.01% w/v) under different pH values (n=3)

3.3.3 Changes in EI of eosin Y in hydrogel

3.3.3.1. Effect of xanthan concentration and chitosan molecular weight

The effect of xanthan concentration and chitosan molecular weight on the diffusion of acidic media was investigated over time (Table 3.1). The EI was found to decrease with time until it reached a lower limit, followed by a steady period in all cases with no significant difference (P>0.05) between adjoining values. Decrease in

EI was more pronounced in the case of 0.7% xanthan compared to other concentrations investigated. Xanthan at 1.2% (w/v) reached a constant level of EI in 60 min, whereas 1.0% and 0.7% xanthan plateaued at 45 and 30 min, respectively. This could be attributed to the formation of less permeable hydrogel membrane due to elevated cross-linking density (Abubakr et al. 2010). It could be postulated that the higher xanthan polymer concentration resulted in higher charge density at the point of contact between the two polymers, leading to improved cross-linking density.

While the effect of chitosan molecular weight on EI of eosin Y was insignificant (P<0.05) in the 96-well setup (Table 3.1), it is important to note that such measurements were indicative of pH changes in the well. It is known that LMW chitosan, when acting alone, allows for higher permeability than HMW chitosan under same degree of deacetylation when spread and oven dried to form a membrane (Chen and Hwa 1996). The authors further supported the results by measuring membrane crystallinity, which was found to increase with increasing chitosan molecular weight due possibly to higher degrees of entanglement. Nonetheless, the swelling degree of hydrogels made with xanthan and LMW chitosan was significantly higher than that of xanthan-HMW chitosan hydrogels (Dumitriu et al. 1994), making it difficult to prevent eosin Y from leaking away from the hydrogel when rinsing off unbound chitosan.

To further characterize the effect of chitosan molecular weight on SGJ diffusion, a set of custom-made pipes was employed (Figure 3.1). Significant differences were found among chitosan with different molecular weights. HMW chitosan was the best barrier in preventing SGJ diffusion, as indicated by the least

Table 3.1 Variation in emission intensity (EI) of eosin Y with changes in xanthan concentration, chitosan molecular weight (low, medium, and high), and time (n = 3).

Chitosan	Time (min)	Xanthan Concentration 0.7%	1.0%	1.2%
0.7% LMW	0	48635^{ed}	51843^{bdc}	54873^{abc}
	12	33387^{ij}	42639^{gf}	50974^{dc}
	30	20047^{uvtrsq}	25381^{knlm}	34276^{i}
	45	18331^{uvtrws}	17004^{uvtw}	25295^{oknlm}
	60	17284^{uvtws}	16075^{vw}	20682^{uptrsq}
	90	16542^{uvtw}	15871^{vw}	$18852^{uvtrqsw}$
	120	16704^{uvtw}	15178^{w}	17309^{uvtws}
0.7% MMW	0	48964^{ed}	50007^{d}	55846^{ab}
	12	35195^{ih}	44836^{ef}	51526^{bdc}
	30	22551^{opnrmq}	26501^{klm}	35395^{ih}
	45	20002^{uvtrsq}	20652^{uptrsq}	26426^{klm}
	60	17877^{uvtws}	17575^{uvtws}	22895^{opnlmq}
	90	17306^{uvtws}	17270^{uvtws}	19933^{uvtrsq}
	120	16437^{uvw}	16624^{uvtw}	18274^{uvtrws}
0.7% HMW	0	52303^{bdc}	55526^{ab}	55997^{a}
	12	35703^{ih}	45579^{ef}	52331^{bdc}
	30	22602^{opnrmq}	27298^{kl}	39250^{gh}
	45	$19214^{uvtrwsq}$	20918^{optrsq}	29488^{kj}
	60	17568^{uvtws}	$18755^{uvtrwsq}$	24722^{oklmnq}
	90	17081^{uvtw}	17446^{uvtws}	21555^{opnrsq}
	120	16308^{uvw}	16378^{uvw}	$19275^{uvtrwsq}$

Means with the same superscript are not significantly different ($P > 0.05$)

change in EI compared with the control (Figure 3.4). MMW chitosan formed a weaker barrier against SGJ than HMW chitosan based on the statistically significant difference ($P<0.05$) in EI, whereas LMW chitosan seemed to provide a better shield than MMW. However, further increase in LMW chitosan concentration in fact decreased the barrier property of the hydrogel against SGJ, indicating a weakened boundary between SGJ and eosin Y solution. Therefore, HMW chitosan was used for subsequent studies.

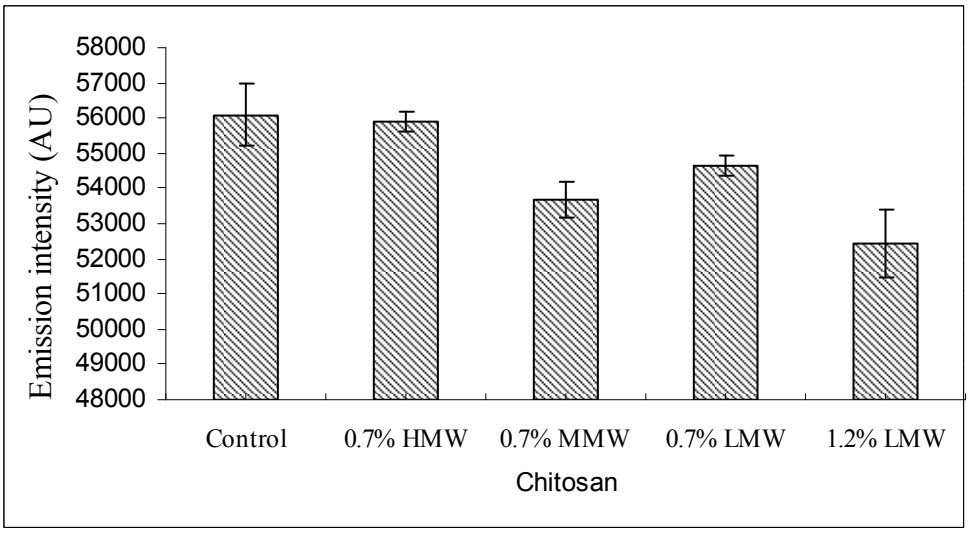


Figure 3.4 Diffusion of simulated gastric juice through xanthan-chitosan hydrogel (XCH) in tube (n=3)

3.3.3.2. Effect of complexation time

The effect of complexation time on the hydrogel ability to shield internal pH from external acidity variations was characterized using eosin-containing hydrogels formed by 1.2% xanthan and 0.7% HMW chitosan. As seen in Figure 3.5, the EI of eosin Y (in percentage compared with the original EI) decreased with time in all cases with significant differences ($p<0.05$) between complexation times of 40 and 80 min. Further increase in complexation time to 120 min did not change the barrier property.

It is noteworthy that complexation time of 40 min appeared to be the most desirable profile due to the delay in the decrease in EI compared to 80 and 120 min. This may be counterintuitive as longer complexation time leads to formation of thicker polyeletrolyte hydrogels, which results in lower permeability (Dumitriu & Chornet 1997). However, in the present study, with increase in complexation time, the hydrogel was found to shrink due possibly to the higher osmotic pressure exerted by the higher molar concentration of chitosan in the external solution than the xanthan molar concentration in the core, consequently resulting in migration of water from the hydrogel to the external chitosan solution.

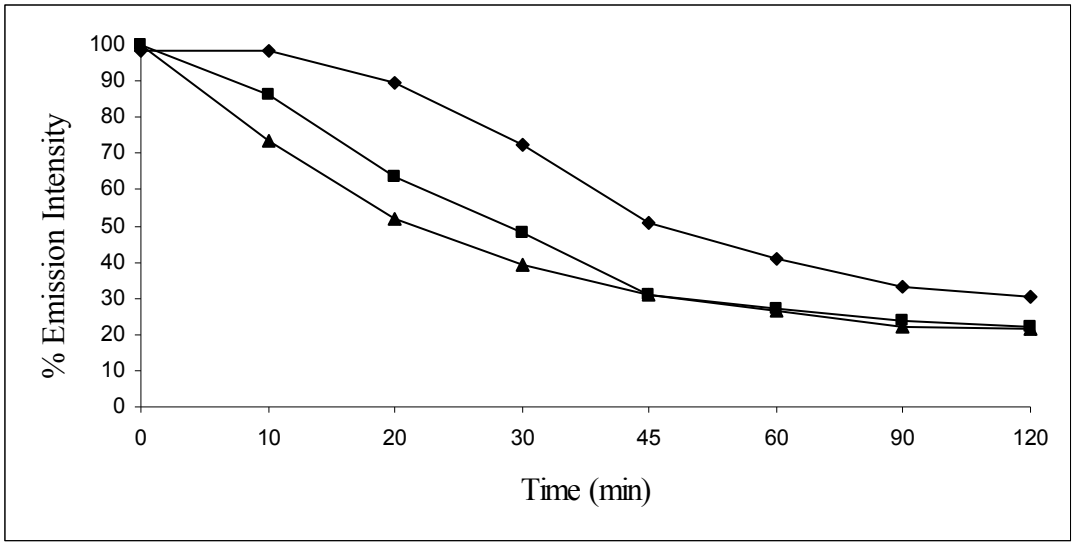
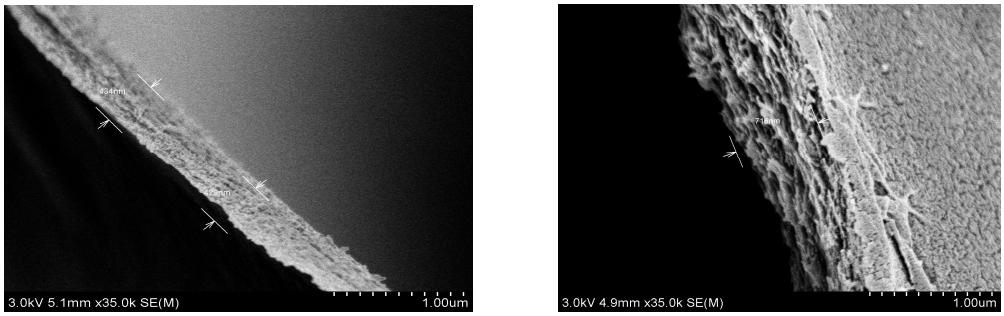


Figure 3.5 Diffusion profiles of eosin-containing xanthan-chitosan hydrogels (XCH) formed by 1.2% xanthan and 0.7% high molecular weight (HMW) chitosan at complexation times 40 (♦) , 80 (■), and 120 min (▲) (n = 3)

3.3.4 SEM

SEM was used to examine the cross-section of the hydrogel. As seen in the scanning electron micrographs (Fig. 3.6), the hydrogel formed using 1.0% xanthan and 0.7% HMW was thicker (approx. 716 nm) than the one formed with 1.2%

xanthan and 0.7% HMW (approx. 420 nm), indicating that a longer distance existed in the hydrogel formed by 1.0% xanthan for acidic media (SGJ) to diffuse through. However, hydrogel with 1.2% xanthan showed a much more densely packed structure compared to 1.0% xanthan. Increase in concentration of xanthan means increased number of biopolymer molecules per unit solution volume, hence the binding sites for chitosan also increased. Similar mechanism was suggested in case of alginate gels formed by reacting with $CaCl_2$. Increase in alginate concentration formed a thinner membrane compared to lower alginate concentration (Blandino et al. 2001). Furthermore, according to Chu et al. (1996), the hydrogel formed by xanthan and chitosan is considered as a porous body, and the average distance between neighboring crosslinks in the xanthan/chitosan gel increased as the swelling ratio increased. This was further supported by Argin-Soysal et al. (2009) that xanthan concentration has a pronounced effect on swelling degree of xanthan/chitosan hydrogel. Therefore, 1.2% xanthan contributed more barrier power to the hydrogel against acid diffusion than 1.0% xanthan.



A B

Figure 3.6 Xanthan-chitosan hydrogel (XCH) formed by high molecular weight (HMW) chitosan with 1.2% (A) and 1.0% (B) xanthan at complexation time of 60 min

3.4 Conclusion

A fluorescence method using a pH-dependent fluorescent dye eosin Y was developed to study the dependency of the internal pH of xanthan-chitosan hydrogel on external acidity variation such as gastric acidity. Critical factors affecting the formation of the hydrogel were investigated, including complexation time, molecular weight of chitosan, and xanthan concentration. At 0.7% (w/v), HMW chitosan provided better shielding power than MMW against SGJ, while LMW chitosan formed a weak barrier even at elevated concentration (1.2%). Complexation time had significant effect on the hydrogel, with 40 min being the most effective in delaying acid diffusion. Moreover, xanthan concentration was found to be the most significant factor affecting the barrier property of the hydrogel with minimum pH changes for the period of time investigated. Such shielding power against external acidity (SGJ) could be attributed to the formation of a thin but dense layer at the xanthan/chitosan interface as visualized by SEM.

Chapter 4: Effect of chitosan molecular weight on the survival of probiotics encapsulated in xanthan-chitosan hydrogel complex

4.1 Introduction

Increased concerns on whether products containing probiotics could deliver sufficient numbers of viable cells past harsh gastric acidity (Robinson 1991; Dave and Shah 1997; Ouwehand and Salminen 1998; Weese and Martin 2011) have led to vast movements in identifying effective mechanisms to protect the cells against processing conditions, storage, and through gastrointestinal tract (Shah et al. 1995; Sun and Griffiths 2000; Weinbreck et al. 2010) in order to exert desirable health benefits, including establishing balance in intestinal microbiota (Fuller 1989; Shah 2007), alleviating lactose intolerance (Vrese et al. 2001; He et al. 2008), providing anticarcinogenic effects (Wollowski et al. 2001; Kumar et al. 2010), inhibiting pathogenic bacteria (Chenoll et al. 2011; Vanderpool et al. 2008), and improving host immune system (Kailasapathy et al. 2000; El-Nezami et al. 2000; Dunne 2001). To date, microencapsulation of probiotic bacteria has been demonstrated to provide various degrees of protection (Anil and Harjinder 2007) using carriers made of starch (Homayouni et al. 2008; Donthidi et al. 2010), protein (Heidebach et al. 2009; Doherty et al. 2011), lipid (Del Piano et al. 2011; Maillard and Landuyt 2008) and alginate with (Chan and Zhang 2002) or without compression (Hansen et al. 2002; Chandramouli et al. 2004; Lee et al. 2009; Cook et al. 2011). However, most carriers suffer from one or more limitations such as susceptibility to ions (Smidsrod and

Skjak-Braek 1990), low mechanical strength (Buchhloz et al. 1980; Roy et al. 1987), and most critically inability to release probiotic bacteria in the intestine (Kailasapathy 2002; Lee et al. 2004), resulting in poor final effects.

Hydrogels formed by polyelectrolytic complexation between xanthan and chitosan have been employed to immobilize *Corynebacterium glutamicum* with a fivefold increase in enzyme activity compared to that of free cells (Chu et al. 1996), to encapsulate xylanase and increase its enzymatic activity by 50% (Dumitriu and Chornet 1997), and to protect probiotics from gastrointestinal conditions (Argin-Soysal 2007). The oppositely charged nature of xanthan (-) and chitosan (+) due to the respective carboxylic and amine groups involved in the complex formed stable hydrogel structure immediately upon contact, and stayed stable under gastric conditions. At the intestinal pH (ca. 6.8), swelling of the hydrogel allows for the enzymes and bile salt in the intestinal fluid to penetrate into the gel, repelling the polymers, and releasing the encapsulated cells (Argin-Soysal 2007). While xanthan-chitosan hydrogel appears to be a promising carrier for probiotics, it must be noted that, besides processing conditions such as pH, polymer concentration, and complexation time, the hydrogel structure is dependent on inherent polymer parameters such as the pyruvic acid content of xanthan and the degree of deacetylation and molecular weight of chitosan (Dumitriu et al. 1994; Chu et al. 1996; Dumitriu and Chornet, 1997; Magnin et al. 2004; Argin-Soysal et al. 2009).

Known to affect the crystallinity of chitosan when forming a homopolymeric membrane (Chen and Hwa 1996), the molecular weight of chitosan plays an important role in the resulting structure porosity when interacting with other polymers

(Polk et al. 1994; Ribeiro et al. 1999; Lee et al. 2004). Gåserød Sannes, and Skjåk-Bræk (1999) reported that low molecular weight chitosan forms a porous and stable gel structure with negatively charged alginate. The permeability of the chitosan-alginate capsule could be minimized by using high molecular weight chitosan. It has been demonstrated that the rigid, stereo-regular structure of chitosan can induce a conformational change of the other non-rigid polyelectrolyte when forming a polyelectrolytic gel complex with α-keratose (Park 1996), poly acrylic acid (Cerrai et al. 1996), xylan (Gabrielii et al. 2000) or collagen (Taravel and Domard, 1995). Intriguingly, when compressed into layered matrix tablets, low-molecular-weight chitosan (70 kDa) in combination with xanthan was found to prolong the release of drug extensively than using a single polymer (Phaechamud and Ritthidej, 2007), with visuals demonstrating the mechanical interlocking of the fibrous polymers. The tablets were able to retain their gel network during dissolution, with swelling being the mode for releasing the core, in agreement with the findings reported by Argin-Soysal et al. (2009). However, the influence of chitosan molecular weight on the changes of hydrogel properties has not yet been elucidated.

The primary goal of this study was to characterize the effect of chitosan molecular weight on the protective effects of XCH hydrogel on probiotic bacteria against gastrointestinal conditions. The corresponding mechanical properties as well as the microstructure of the XCH hydrogel were also elucidated.

4.2 Materials and Methods:

4.2.1 Materials

Xanthan gum, chitosan at different molecular weights (high [1200-1600 cP], medium [200-800 cP], and low [20-200 cP]) with 75-85% deacetylation, pancreatin (1x USP grade) were purchased from Sigma-Aldrich Chemicals (St Louis, MO). Artificial gastric juice (SGJ) and simulated intestinal fluid without pancreatin were purchased from Fisher Scientific (Rochester, NY) and Ricca Chemicals (Arlington, Texas), respectively. *L. acidophilus* ATCC 43121 was obtained from ATCC (Manassas, VA). Simulated intestinal fluid (SIF) was prepared by adding a sterile concentrated solution of pancreatin (sterility achieved by filtering through 0.22 μm sterile syringe filter) to autoclaved solution of simulated intestinal fluid to get a final concentration of 0.1% pancreatin.

4.2.2 Preparation of xanthan and chitosan solutions

To prepare 0.7% and 1.2% (w/v) chitosan solutions, 7 and 12 g of chitosan were dissolved respectively in 125 ml of 1 N HCL by heating and agitation. The pH of the solution was adjusted to 6.0 by drop wise addition of 1 M NaOH. Deionized (DI) water was used to bring the final volume to 1 L. Xanthan solution was prepared by adding known quantity of xanthan to DI water under room temperature with constant stirring. Air bubbles were removed by centrifuging at 2200 rpm using Beckman Tj-6 centrifuge (Beckman Coulter, Fullerton, CA) (750 × *g*) for 10 min. Both solutions were autoclaved before use in encapsulation procedures.

4.2.3 Preparation of probiotic bacteria

Active culture of *L. acidophilus* ATCC 43121 was maintained throughout the course of the experiments. *L. acidophilus* ATCC 43121 was transferred twice in MRS broth at 37^{o}C under anaerobic conditions (Anaerogen, Oxoid, Hampshire, England). Culture cells were harvested after 17 hrs of incubation at 37^{o}C by centrifugation at 10000 rpm (7000 x *g*) for 20 min at 4^{o}C using Beckman L7-65 ultracentrifuge (Beckman Coulter, Palo Alto, CA). Centrifuged cells were washed twice and re-suspended using DI water.

4.2.4 Encapsulation Procedure:

Harvested and washed cell suspension was added to xanthan solution to reach the final concentration of 1% xanthan solution containing live cells (~10^{9} CFU/ml). Twenty ml of xanthan solution was extruded into 100 ml of chitosan solution by drop wise addition using manually operated syringe with a 0.7 mm cannula (Becton-Dickinson, Franklin Lakes, NJ). The capsules formed by xanthan and chitosan upon contact were kept in chitosan solution for 40 min at room temperature with continuous stirring to allow cross-linking and to avoid coalescence. Capsules were filtered using a 160 μm Millipore nylon filter (Carrigtwohill, Ireland) before being washed using DI water. Four sets of hydrogel capsules were prepared following the same procedure using 0.7% high molecular weight (HMW), 0.7% medium molecular weight (MMW), 0.7% low molecular weight (LMW), and 1.2% LMW chitosan.

For freeze-dried capsules, freshly made capsules were transferred to a freeze drying flask and frozen using dry ice for 24 h before connecting to a freeze dryer (Thermosavant, Holbrook, NY). Freeze drying process took around 36-48 h

depending on the amount of capsules. The mean weight of 10 freeze dried capsules was reported. Experiments were replicated three times.

4.2.5 Viability of probiotic bacteria

4.2.5.1 Effect of chitosan on free cells

Centrifuged and washed cell suspension was added to autoclaved chitosan solution (0.7% HMW, 0.7% MMW, 0.7% LMW, and 1.2% LMW) in a test tube and incubated at 37^{o}C. Samples were drawn at 0, 10, 20 and 40 min, diluted and plated on MRS agar for total plate count (CFU/ml). Cells suspended in the same volume of DI water were used as the control.

4.2.5.2 Effect of SIF on free cells

Since SIF treatment is the critical step in releasing probiotic bacteria from the hydrogel, it was essential to determine its effect on the cells. Centrifuged and washed cell suspension was added to SIF and incubated at 37^{o}C for 5 h. Samples were taken at 0, 1.5, 3 and 5 h, diluted and plated on MRS agar.

4.2.5.3 Effect of encapsulation procedure

To study the effect of encapsulation on *L. acidophilus* ATCC 43121, initial count of the cells and final cell count of encapsulated bacteria were determined. Initial cell count was obtained by diluting and plating cells immediately after centrifugation and washing. Cell count after encapsulation procedure was obtained by releasing the cells from hydrogel by suspending in SIF for 5 h.

4.2.5.4 Simulated gastrointestinal conditions

Filtered and washed capsules were suspended in 50 ml SGJ at pH 1.5 (pH adjusted using 1N NaOH) and incubated at 37^{o}C for 1.5 h with agitation at 150 rpm.

Capsules were filtered, washed, and transferred into SIF solution and incubated at 37^{o}C for 5 h with agitation at 150 rpm. Sample from the SIF solution was used to determine the total plate count (CFU/ml) of the viable bacteria after serial dilutions. Experiments were replicated 4 times. Free cells were subjected only to gastric juice at pH 1.5 for 1.5 h under 37^{o}C.

4.2.6 Mechanical resistance of capsules

Mechanical resistance of capsules was measured using TA-XT2i texture analyzer (Texture Technologies Corporation, New York, USA). Mechanical deformation tests of the xanthan-chitosan hydrogel capsules were performed by placing a freshly made capsule under the probe (5 Kg load cell) and measuring the mean bursting force at a probe speed of 0.05 mm/s until bursting was observed. Average bursting force of 15 capsules was reported.

4.2.7 Cryofracturing and scanning electron microscopy

Freshly prepared capsules were quickly frozen using liquid nitrogen. Frozen capsules were fractured mechanically using a mortar and pestle under liquid nitrogen conditions. Fractured frozen capsules were then freeze-dried for 24 h. Freeze dried capsule particles were carefully mounted onto aluminum stub using a double sided tape and gold sputtered for analysis. The SEM analysis was performed using a SU-70 Ultra High Resolution Analytical SEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV.

4.2.8 Statistical Analysis

Tests for statistical significance of differences were compared by general linear model (GLM) procedure of SAS. Analysis of variance and t-tests were performed using SAS v.9.2 (SAS Institute Inc., Cary, N.C., USA)

4.3 Results and Discussion

4.3.1 Viability of *L. acidophilus* ATCC 43121

4.3.1.1 Effect of chitosan on free cells

Application of chitosan as a constituent of a carrier for microbial encapsulation might remain doubtful because, when acting alone, chitosan does show antimicrobial effect against spoilage organisms (Shahidi et al. 1999; Rhodes and Roller 2000). Without complexation with xanthan, chitosan at 0.7% (w/v) showed significant inhibition on the viability of *L. acidophilus* ATCC 43121 (Figure 4.1), with up to 4 log reduction after 40 min. After 10 min of contacting time, 0.7% LMW chitosan showed the highest antimicrobial activity as evidenced by the significantly lower probiotic viability compared to other treatments. The viability of *L. acidophilus* continued to decrease with increasing contacting time, and the differences among treatments also became less significant. Various mechanisms have been proposed in the literature on how chitosan works against specific components of microorganisms (Rabea et al. 2003), the most widely accepted mode is its interaction with cell membrane that alters cell permeability, which results in leakage of proteinaceous and other intracellular constituents critical for cell survival (Raafat et al. 2008). Dependence of antimicrobial activity on the molecular weight of chitosan was also reported (Qin et al. 2006; Mendel and Juneja, 2010). Tokura et al.

(1997) found that, when using fluorescence dye to label chitosan, high molecular weight of chitosan oligomers tend to stack to the cell wall and inhibit cell growth, whereas low molecular weight chitosan permeates through the cell wall and accelerates cell growth. Zheng and Zhu (2003) reported that, the molecular weight and concentration of chitosan affects its antimicrobial activity against *E. coli* and *S. aureus*. Increase in molecular weight of chitosan strengthened the antimicrobial effect against *S. aureus* whereas the antimicrobial effect weakened against *E. coli*. However, increase in concentration of chitosan resulted in increased antimicrobial activity in both bacteria irrespective of molecular weight. Although Park et al. (2004) further indicated that increase in molecular weight of chitosan-oligosaccharides (COS) increased antimicrobial activity against five gram negative and five gram positive bacteria, it is reasonable to suggest that the molecular weight of chitosan in conjunction with chitosan concentration is characteristic to its antimicrobial effects regardless of species specificity.

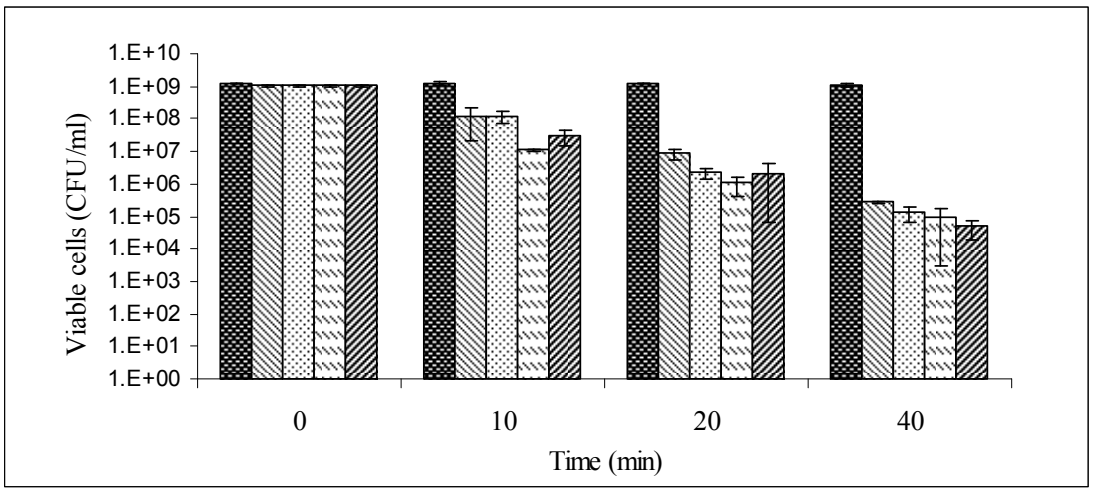


Figure 4.1 Effect of molecular weight of chitosan on viability of non-encapsulated *L. acidophilus* ATCC 43121 cells. Control (▩), 0.7% high molecular weight (▧), 0.7% Medium molecular weight (▨), 0.7% Low molecular weight (▧), and 1.2% Low molecular weight (▨) chitosan (n=3)

4.3.1.2 Effect of encapsulation on L. acidophilus *cells*

The effect of the encapsulation procedure on the viability of *L. acidophilus* ATCC 43121 is shown in Table 4.1. Although there was a significant difference ($P < 0.05$) between the initial and final cell counts after encapsulation, the decrease in viability was less than 1 log compared to a 4-log reduction shown with free chitosan solution (Figure 4.1). The reduction in cell viability could be attributed to the effect of SIF during release. As seen in Figure 4.2, *L. acidophilus ATCC* 43121 were fairly stable and resulted in less than 1-log reduction over 5 h of SIF treatment. Significant difference in viability of *L. acidophilus* was found after 1.5 h of exposure to SIF.

Table 4.1 Effect of xanthan chitosan (XCH) encapsulation procedure on viability of *L. acidophilus* ATCC 43121. Bacteria released using simulated intestinal fluid treatment for 5 h. (n=3)

	Viability of probiotic bacteria ($\log_{10}$ CFU/ml)	
Encapsulation type	Initial	Final
0.7% HMW	9.26 ± 0.17	8.80 ± 0.08
0.7% MMW	9.16 ± 0.15	8.75 ± 0.19
0.7% LMW	9.19 ± 0.09	8.74 ± 0.10
1.2% LMW	9.21 ± 0.10	8.79 ± 0.14

These results were consistent with data from other researchers (Kim et al. 2008; Chou and Weimer 1999). Moreover, since the polyelectrolytic complexation between xanthan and chitosan is due to the interaction between their respective negative and positive charges, during the complexation process, the positive charges on chitosan were exhausted, thereby curbing its antimicrobial properties against cells.

Therefore, the antimicrobial activity of chitosan was not a concern during encapsulation of *L. acidophilus* cells.

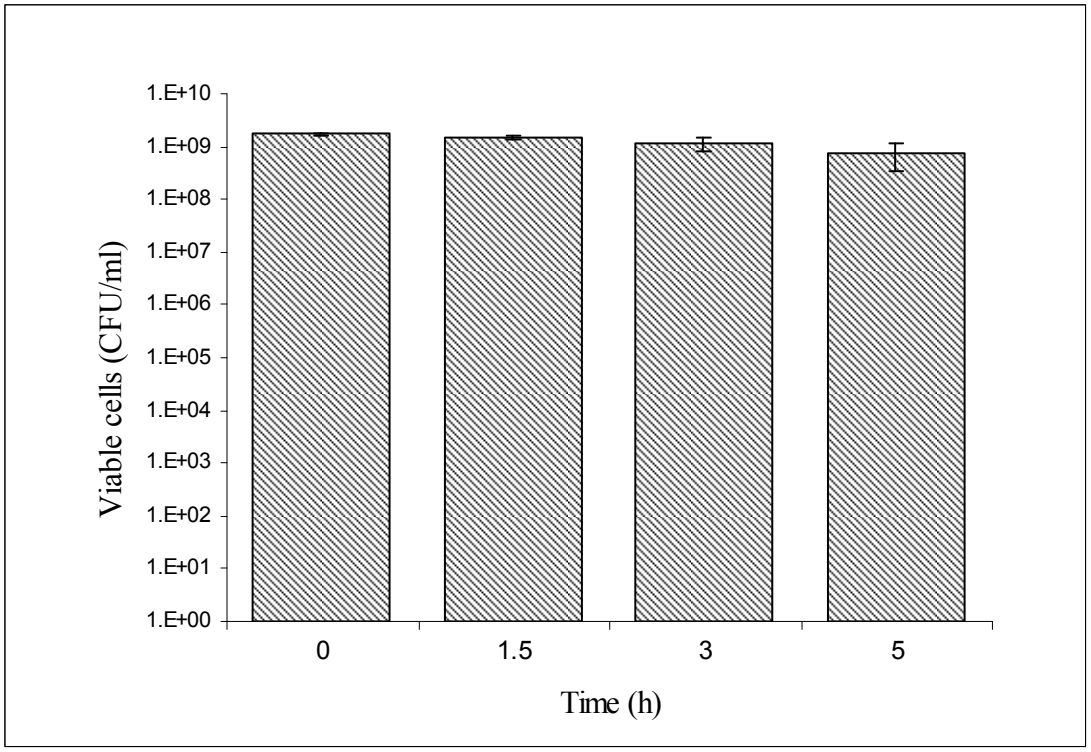


Figure 4.2 Viability of *L. acidophilus* ATCC 43121 in simulated intestinal fluid (SIF) (n=3)

4.3.1. Effect of simulated gastrointestinal conditions on free and encapsulated cells

The effect of gastric conditions on viability of encapsulated and non-encapsulated *L. acidophilus* ATCC 4312 is shown in Figure 4.3. The initial cell count of harvested cells was approximately 10^9 CFU/ml. It is noteworthy that throughout the experiments the spent chitosan solution after filtration of capsules and wash water showed no viability of *L. acidophilus ATCC* 43121, suggesting no leakage of cells during encapsulation. Under gastric conditions at pH 1.5 for 1.5 h, non-encapsulated free cells suffered approximately 5-log reduction. On the other hand, encapsulated cells retained significantly higher viability in all cases. Capsules

made from 0.7% HMW resulted in highest viability (>10^6 CFU/ml). This level of viability is critical as it is considered a therapeutic minimum dose to confer health benefits to the consumer (Robinson 1991; Ouwehand and Salminen 1998).

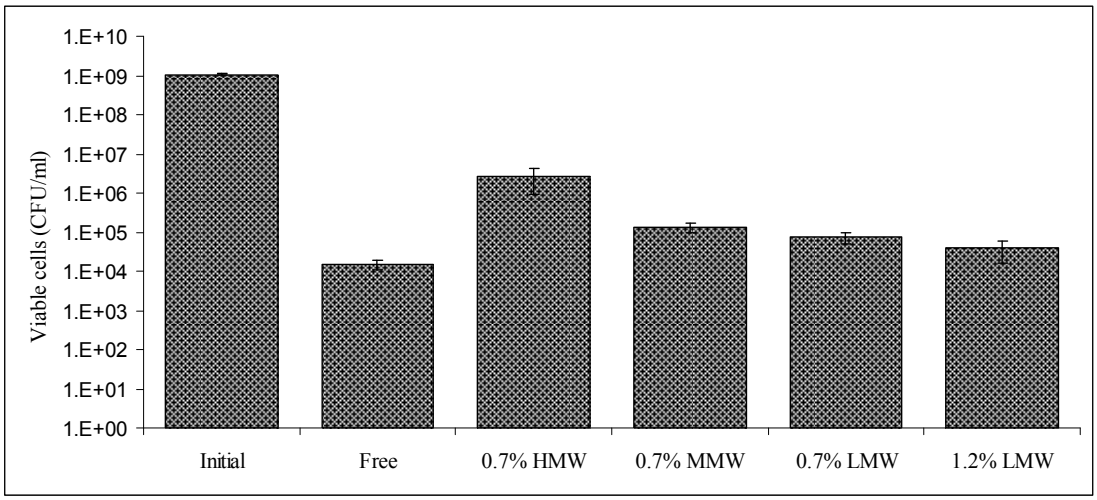


Figure 4.3 Viability of non-encapsulated and xanthan-chitosan encapsulated *L. acidophilus* ATCC 43121 in simulated gastric juice at pH 1.5 for 1.5 h (n=4)

Although hydrogels formed with 0.7% MMW, 0.7% LMW and 1.2% LMW gave statistically higher viability than free cells; substantial improvement in viability as compared to commonly used alginate-based microencapsulation systems was not achieved (Hansen et al. 2002; Mandal et al. 2006; Ding and Shah 2009). Among all the combinations, 1.2% LMW resulted in least protection against SGJ followed by 0.7% LMW. These results are consistent with those found by Lee et al. (2004), where alginate beads with HMW chitosan gave higher protection against gastric environment compared to LMW chitosan. The authors attributed the effect to thicker membranes formed by HMW chitosan with alginate. A similar study by Polk et al. (1994) found that delayed release of albumin from HMW coated alginate microcapsules was due to formation of thicker and less permeable membrane

compared to those formed by LMW chitosan. In another study, alginate microspheres coated with HMW chitosan reached higher encapsulation yield of lipophilic drugs compared to LMW chitosan (Ribeiro et al. 1999).

4.3.2 Mechanical characterization:

The effects of MW of chitosan on mechanical property of XCH at a constant complexation time of 40 min are shown in Figure 4.4. Capsules made with 1.2% LMW chitosan resulted in the highest mechanical strength. While 0.7% MMW chitosan formed stronger capsules than 0.7% HMW chitosan, capsules made with 0.7% LMW chitosan showed the least resistance. Swelling resulted in capsule size increase (data not shown), significantly lowered its mechanical strength due to reduced cross-linking density compared to fresh capsules (Kim et al. 2005).

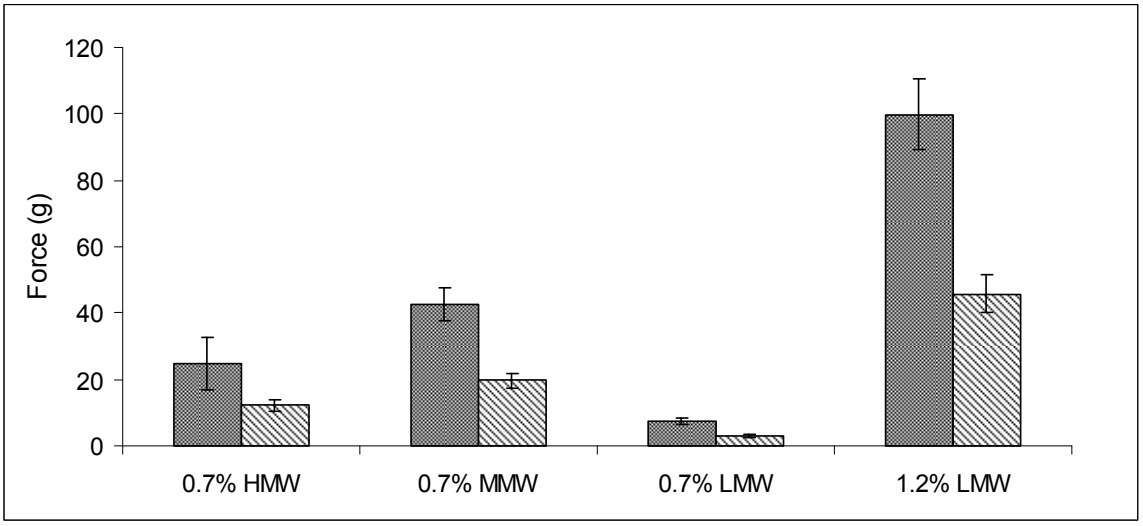


Figure 4.4 Mechanical resistance of fresh (▨) and swollen (▧) capsules with 0.7% high, 0.7% medium, 0.7% low and 1.2% low molecular weight of chitosan (n=3)

The mechanical strength of polyelectrolytic capsules is dependent on factors such as membrane thickness (Rehor et al. 2001), membrane permeability to core contents (Sun and Zhang 2002), and capsule volume (Kim et al. 2005). The membrane

thickness depends on the extent of diffusion of one polymer into the capsule core made of the oppositely charged polymer (Rehor et al. 2001). Therefore, membrane thickness can depend on factors affecting diffusion, namely molecular weight, complexation time, and concentration of polymers (Bartkowiak and Hunkeler 1999; Gao et al. 2001).

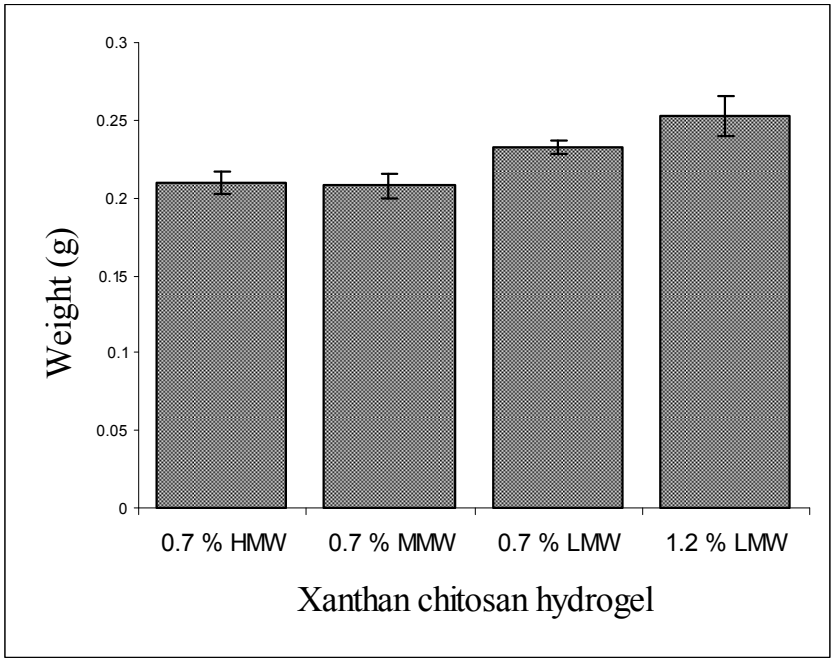


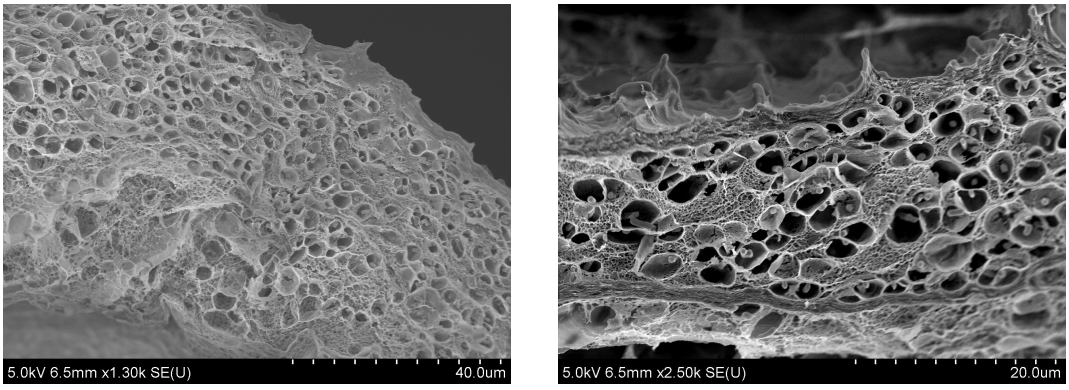
Figure 4.5 Weight of freeze dried hydrogel capsules made with 0.7% high, 0.7% medium, 0.7% low, 1.2% low molecular weight of chitosan (n=3)

Consistent with this theory, as seen in Figure 4.5, at constant xanthan concentration, decreasing molecular weight of chitosan resulted in an increase in the weight of the hydrogel, which suggested the presence of more chitosan in the hydrogel capsule possibly due to increased diffusion of chitosan into the xanthan core. Furthermore, an increase in concentration of LMW chitosan from 0.7% and 1.2% increased the hydrogel weight, indicating more diffusion of chitosan. However, 0.7% LMW chitosan resulted in lower mechanical strength compared to 0.7% HMW

and 0.7% MMW chitosan. This could be due to the formation of a very thick hydrogel membrane by 0.7% LMW chitosan as shown in Figure 4.6 compared to hydrogel membrane formed by 1.2% LMW chitosan, in agreement with study reported by Rehor et al. (2001) in which capsules with thicker membranes resulted in lower mechanical strength because the opposite membranes of the capsule touched before the bursting event happened.

4.3.3 Scanning electron microscopy

In order to study the internal structure of the XCH hydrogel capsule, the interior was exposed by fracturing the capsules under frozen conditions. The external surface of the capsule did not show any pores (data not shown). SEM images of the cross-section of the capsules showed a dense outer membrane in all combinations with different MW of chitosan (Figure 4.6). The outer membrane could be the result of the initial polyanion/polycation interaction. In the formation of a heteropolymeric capsule involving two oppositely charged polymers, it is suggested that the reaction in the first few minutes forms a thin outer membrane, followed by diffusion of polymer into the core of the capsule that forms the overall thickness of the hydrogel capsule membrane (Gugerli et al. 2002). Similar explanation was given by other researchers in case of alginate/polylysine capsules (Bruheim et al. 1996; Gugerili et al. 2002), and alginate/chitosan beads and films (Ribeiro et al. 1999; Yan et al. 2001).



(a) **(b)**

Figure 4.6 Scanning electron microscope images of the porous structure formed by (a) 0.7% low molecular weight chitosan and (b) 1.2% low molecular weight chitosan

In the present study, a porous structure was only observed in case of LMW chitosan, indicating diffusion of chitosan chains into the capsule after the formation of the initial outer layer. Furthermore, the porous structure formed by 1.2 % LMW appeared to be sandwiched between an outer membrane and a second dense membrane (Figure 4.6), whereas porous structure formed by 0.7% LMW did not have the second dense membrane and continued deep into the capsule. The higher osmotic pressure in case of 1.2% LMW may have resulted in the formation of the sandwich structure. The osmotic pressure difference could be attributed to the difference in molar concentrations of chitosan and xanthan solutions. The thickness of the outer membrane correlated directly with the molecular weight of chitosan. Outer membrane with highest thickness was formed with 0.7% HMW and least thickness with 0.7 and 1.2% LMW chitosan. This is consistent with one study, in which thickness of films formed by mixing chitosan and alginate was affected by molecular weight of chitosan where higher molecular weight resulted in thicker film than medium and low molecular weight chitosan (Yan et al. 2001). Therefore, based on the results acquired

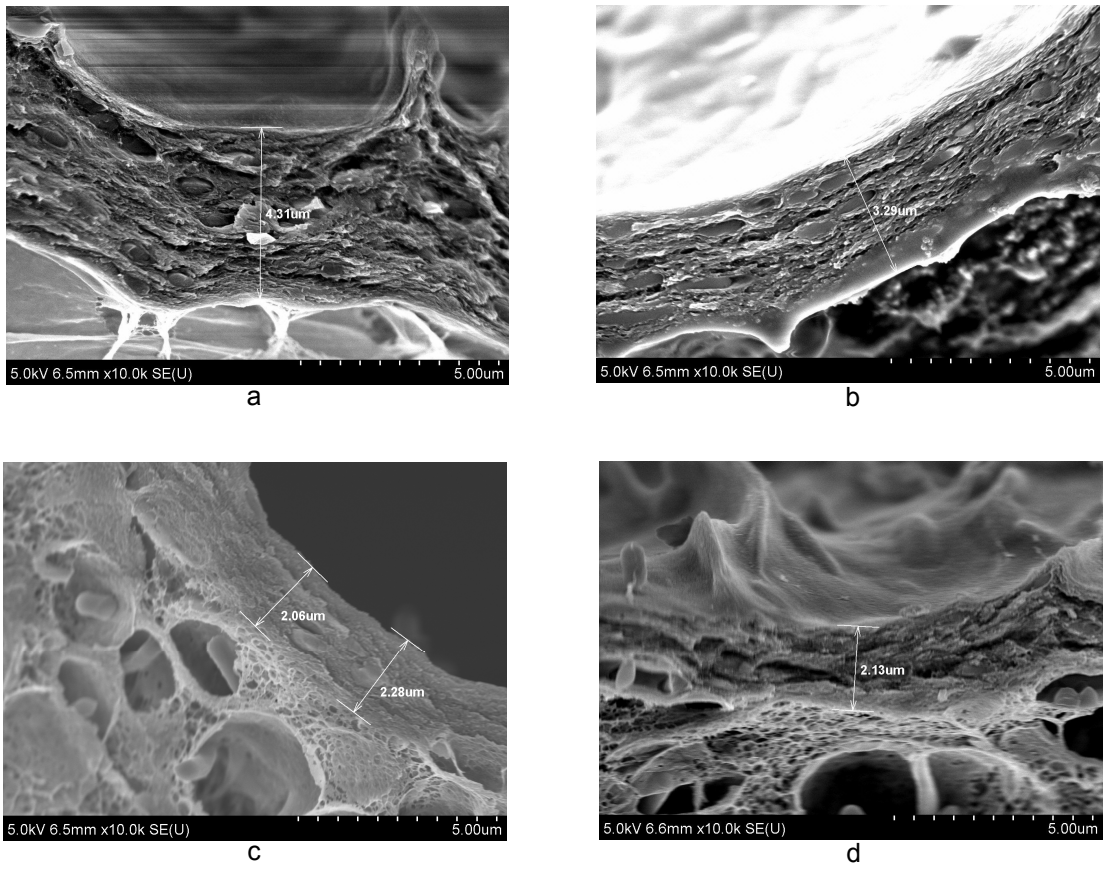


Figure 4.7 Scanning electron microscope images of cross section of freeze dried hydrogels. Hydrogel formed with (a) 0.7% high molecular weight chitosan, (b) 0.7% medium molecular weight chitosan, (c) 0.7% low molecular weight chitosan, (d) 1.2% low molecular weight chitosan

on the protective effects of XCH hydrogels and the SEM cross-sectional observation of the hydrogel containing encapsulated *L. acidophilus* cells, it can be summarized that the outer membrane (Figure 6) may be critical in providing the barrier properties to the capsule, resulting in enhanced viability of *L. acidophilus* ATCC 43121 against harsh gastrointestinal solutions.

4.4 Conclusion

Microencapsulation of probiotics using XCH hydrogels significantly improved the viability of *L. acidophilus* ATCC 43121 in simulated gastrointestinal conditions. Although chitosan showed strong antimicrobial properties and reduced the

viability of free cells, it did not have any effect on encapsulated bacteria after forming the hydrogel with xanthan. Mechanical resistance of hydrogel capsules varied significantly with chitosan molecular weight, with 1.2% LMW chitosan forming the strongest capsules. SEM images revealed major differences in hydrogel structure in response to different chitosan molecular weight and concentration. LMW chitosan formed a porous structure and a sandwich structure at elevated concentration. A positive correlation between molecular weight of chitosan and the outer membrane of the capsules was observed. The thickness of outer membrane may be the key in protecting *L. acidophilus* ATCC 43121 against simulated gastric juice and can be a critical factor when further optimizing XCH hydrogels for effective microencapsulation of probiotics bacteria.

4.5 Acknowledgements

We acknowledge the support of the Maryland NanoCenter and its NispLab. The NispLab is supported in part by the NSF as a MRSEC Shared Experimental Facility.

Chapter 5: Effect of xanthan/chitosan/xanthan encapsulation and capsule size on survival of *Lactobacillus acidophilus* ATCC 43121 in simulated gastrointestinal conditions

5.1 Introduction

Probiotics are live microorganisms proven to provide number of health benefits like improving immune system, reducing lactose intolerance and providing anti-pathogenic effects when consumed at a therapeutic minimum of 10^6 per gram of food. However, probiotics are susceptible to various factors like processing conditions, storage, and most importantly the harsh gastrointestinal conditions. Microencapsulation of probiotics has received a great deal of attention in the recent past due to its ability to protect probiotic cells in gastrointestinal conditions. Many microencapsulation systems based on various carriers made of alginate, starch, protein, lipid have been developed (Hansen et al. 2002; Chandramouli et al. 2004; Lee et al. 2004; Mokarram et al. 2009; Cook et al. 2011; Heidebach et al. 2009; Del Piano et al. 2011; Doherty et al. 2011). However, most carriers suffer from one or more limitations such as susceptibility to ions (Smidsrod and Skjak-Braek 1990), low mechanical strength (Buchhloz et al. 1980; Roy et al. 1987), and most critically inability to release probiotic bacteria in the intestine (Kailasapathy 2002; Lee et al. 2004), resulting in poor final effects.

Hydrogels formed by xanthan and chitosan by polyelectrolytic complexation have been applied to immobilize *Corynebacterium glutamicum* (Chu et al. 1996), to encapsulate xylanase (Dumitriu amd Chornet 1997), and to protect probiotics from

gastrointestinal conditions (Argin-Soysal 2007). The oppositely charged nature of xanthan (-) and chitosan (+) form polyelectrolytic hydrogel structure immediately upon contact and remained stayed stable under gastric conditions (pH 2.0) and effectively released at intestinal pH (ca. 6.8) (Argin-Soysal 2007) While xanthan-chitosan hydrogel (XCH) has effectively protected probiotic bacteria in gastrointestinal conditions, reduction is capsule size showed drastic fall in protective effects leading to low viability in gastrointestinal conditions. The protective effects of XCH could possibly be improved if the thickness of the capsule is increased by formation of an extra layer of hydrogel.

The formation of successive layers of hydrogel to increase the thickness of capsules and to fine tune properties of capsule has been done with various natural polymer systems like chitosan/chitosan sulfate (Berth et al. 2002), poly(DL-lactic acid)/poly(DL-lactic-co-glycolic acid) (Shenoy et al. 2003), chondrotin sulfate/poly(L-arginine) (Shchukin et al. 2004), dextran sulfate/protamine (Balabushevich et al. 2003), poly(L-lysine)/poly(glutamic acid) (Yu et al. 2005). The driving forces for formation of extra layers are the electrostatic interaction between adjacent layers and entropy increase upon capsule formation (Antipov and Sukhorukov, 2004). In one study, the multilayer capsules made by Schneider et al. (2001) for encapsulating pancreatic islets were found advantageous in protecting the core over capsules made by a single-step procedure. Namely, the multilayer capsules made by suspending sodium alginate beads in a series of polyanion and polycation solutions resulted in high mechanical stability and physical integrity with precise permeability while preserving the biocompatibility of capsules. In another study, Mokarram et al. (2009)

further improved the alginate beads containing probiotic bacteria by coating them with alginate to form a second and third layers that resulted in better protection against gastric conditions, suggesting that the permeability of a capsule is dependent on the thickness, porosity, and structure of the layers present (Antipov and Sukhorukov 2004, Johnston et al. 2006). While the protective effects of XCH on probiotic cells have been characterized in Chapter 4, the effect of an extra layer of hydrogel outside XCH remained to be explored.

The primary goal of this study was to characterize the protective effects of xanthan-chitosan-xanthan (XCXH) at different sizes compared to xanthan-chitosan hydrogel (XCH) on probiotic cells against gastrointestinal conditions. The release properties of XCXH and XCX and the corresponding microstructure of the hydrogels were also elucidated.

5.2 Materials and Methods:

5.2.1 Materials

Xanthan gum, chitosan at different molecular weights (high [1200-1600 cP], medium [200-800 cP], and low [20-200 cP]), pancreatin (1x USP grade) were purchased from Sigma-Aldrich Chemicals (St Louis, MO). Simulated gastric juice (SGJ) and simulated intestinal fluid without pancreatin were purchased from Fisher Scientific (Rochester, NY) and Ricca Chemicals (Arlington, TX), respectively. *L. acidophilus* ATCC 43121 was obtained from ATCC (Manassas, VA). Simulated intestinal fluid (SIF) was prepared by adding a sterile concentrated solution of pancreatin (sterility achieved by filtering through 0.22 μm sterile syringe filter) to

autoclaved solution of simulated intestinal fluid to get a final concentration of 0.1% pancreatin.

5.2.2 Preparation of xanthan and chitosan solutions

Chitosan solution was prepared by dissolving known quantity of chitosan in 125 ml of 1 N HCL by heating and agitation. The pH of the solution was adjusted to 6.0 by drop wise addition of 1 M NaOH. Deionized (DI) water was used to bring the final volume to 1 L. Xanthan solution was prepared by adding known quantity of xanthan to DI water under room temperature with constant stirring. Air bubbles were removed by centrifuging at 2200 rpm using Beckman Tj-6 centrifuge (Beckman Coulter, Fullerton, CA) (750 ×*g*) for 10 min. Xanthan and chitosan solutions were autoclaved before encapsulation procedures.

5.2.3 Preparation of probiotic bacteria

Active culture of *L. acidophilus* ATCC 43121 was maintained throughout the course of the experiments. *L. acidophilus* ATCC 43121 was transferred twice in MRS broth at 37^{o}C under anaerobic conditions (Anaerogen, Oxoid, Hampshire, England). Culture cells were harvested after 17 hrs of incubation at 37^{o}C by centrifugation at 10000 rpm (7000 x *g*) for 20 min at 4^{o}C using Beckman L7-65 ultracentrifuge (Beckman Coulter, Palo Alto, CA). Centrifuged cells were washed twice and re-suspended using DI water.

5.2.4 Encapsulation Procedure

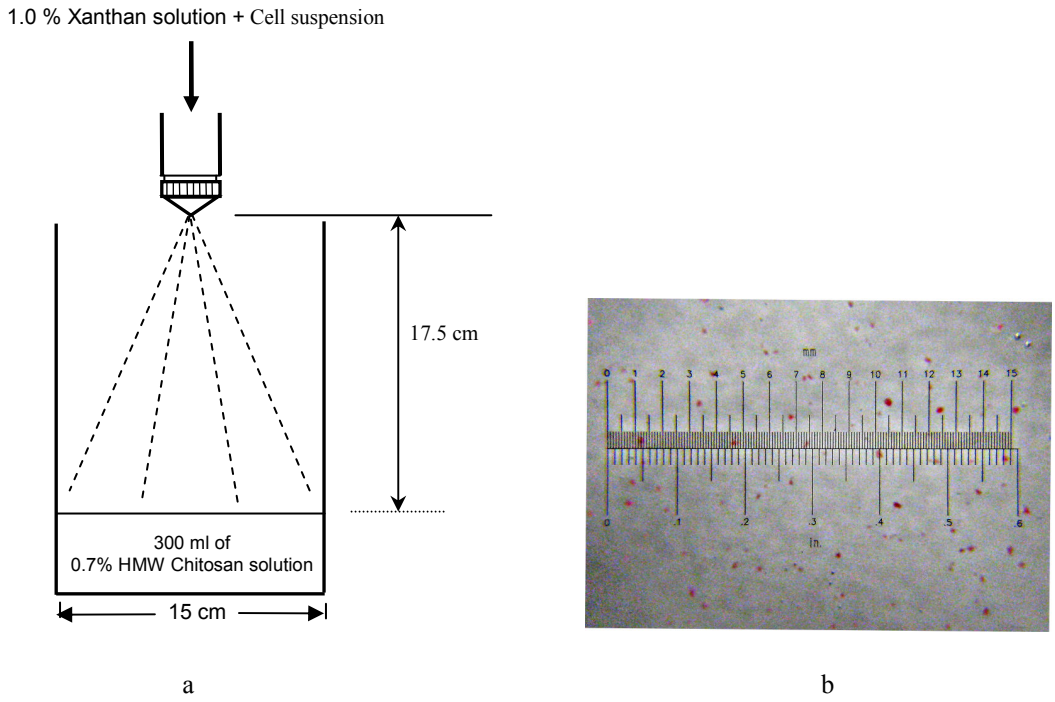
5.2.4.1 Syringe extruded capsules

Harvested and washed *L. acidophilus* cell suspension was added to xanthan solution to reach the final concentration of 1% xanthan solution containing ~10^{9}

CFU/ml live cells. The solution was then extruded into 0.7% HMW chitosan solution by dropwise addition using manually operated syringe with a 0.7 mm cannula (Becton-Dickinson, Franklin Lakes, NJ). The capsules formed by xanthan and chitosan (XCH) upon contact were kept in chitosan solution for 40 min at room temperature with continuous stirring to allow cross-linking and to avoid coalescence. Capsules were filtered using a 160 μm Millipore nylon filter (Carrigtwohill, Ireland) before being washed using DI water. XCXH capsules were made by suspending the XCH capsules into 0.1% xanthan solution for 30 min with continuous agitation followed by filtration and washing using DI water.

5.2.4.2 Spray-nozzle extruded capsules

Xanthan solution (1%, w/v) containing viable cells (~10^9 CFU/ml) was extruded into 0.7% HMW chitosan solution through a 0.7 mm nozzle using a Büchi B-290 mini spray dryer (Büchi, Flawil, Switzerland). Specifications of the encapsulation conditions are shown in Figure 1a. Air flow rate of 246 L/h and the feed pump rate of 3.6 ml xanthan/min into chitosan solution (300 ml) with constant stirring resulted in well defined hydrogel capsules (<500 μm) with no coalescence (Figure 1b). The capsules were kept in chitosan solution for 40 min at room temperature with continuous stirring to allow cross-linking. Capsules were filtered using a 100 μm Millipore nylon filter (Carrigtwohill, Ireland) before being washed using DI water. Washed and filtered capsules were dispersed into 0.1% xanthan solution with continuous stirring for 30 min followed by washing and filtering.



a b

Figure 5.1 (a) Microencapsulation setup using spray nozzle. (b) XCXH microcapsules (dyed with eosin to visualize) formed by spray nozzle (The ruler shown is at the scale of 15 mm or 0.6 inch).

5.2.5 Viability of probiotic bacteria in gastrointestinal conditions

Filtered and washed capsules were suspended in 50 ml SGJ at pH 1.5 (pH adjusted using 1N NaOH) and incubated at 37^{o}C for 1.5 h with agitation at 150 rpm. Capsules were filtered, washed, and transferred into SIF solution and incubated at 37^{o}C for 5 h with agitation at 150 rpm. Sample from the SIF solution was used for total plate count (CFU/ml) of viable bacteria after serial dilutions. Experiments were conducted in quadruplicates.

5.2.6 Release of probiotic bacteria in gastrointestinal conditions

Freshly prepared capsules were suspended in 50 ml SGJ for 1.5 h followed by 150 ml SIF for 5 h. The cell concentration was monitored over time by periodical sampling with optical density (OD) measurements taken at 600 nm using a Helios

spectrophotometer (Thermospectronic, Rochester, NY). Optical density was converted into corresponding concentration (g/l) based on a calibration curve. To construct a calibration curve, optical densities of various dilutions of *L. acidophilus* cells were measured and plotted against the corresponding dry weight of the cells obtained after oven drying the samples for 24 h.

5.2.7 Cryofracturing and scanning electron microscopy

Four sets of syringe-extruded XCXH capsules were prepared following the same procedure using 0.7% high molecular weight (HMW), 0.7% medium molecular weight (MMW), 0.7% low molecular weight (LMW), and 1.2% LMW chitosan. Freshly prepared capsules were quickly frozen using liquid nitrogen. Cryofracturing of the frozen capsules were conducted mechanically using a mortar and pestle under liquid nitrogen conditions. Fractured frozen capsules were then freeze-dried for 24 h before carefully mounted onto an aluminum stub using a double sided tape and gold sputtered prior to analysis. The SEM analysis was performed using a SU-70 Ultra High Resolution Analytical SEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV.

5.2.8 Statistical Analysis

Tests for statistical significance of differences between cell counts were compared by general linear model (GLM) procedure of SAS. Analysis of variance and t-tests were performed using SAS v.9.2 (SAS Institute Inc., Cary, N.C., USA)

5.3 Results and Discussion

5.3.1 Calibration curve

As seen in figure 5.2, the optical density of *L. acidophilus* ATCC 43121 cells was found to be related to cell concentration (g/l) by a factor of 3.6574.

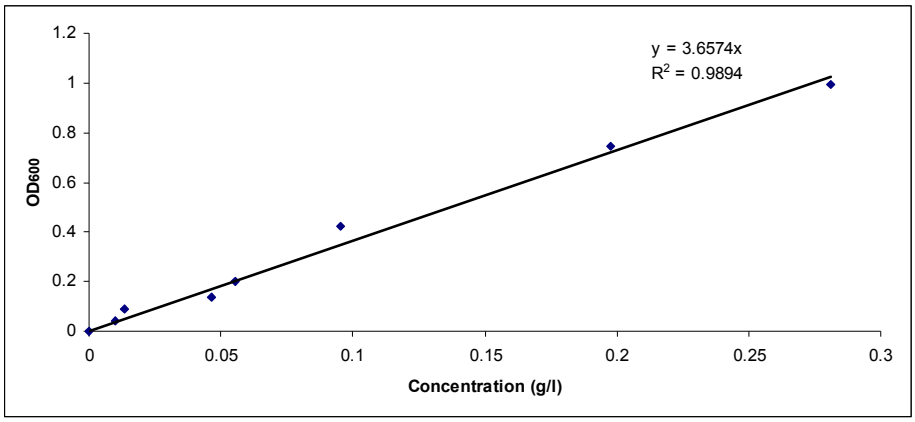


Figure 5.2 Calibration curve of L. acidophilus ATCC 43121

5.3.1 Release of *L. acidophilus* in gastrointestinal conditions

Figure 5.3 shows the amount of cells released from XCH- and XCXH-encapsulated probiotics after SGJ (pH 1.5) treatment. While XCXH capsules remained stable and retained bacteria for approximately 3 h without any release, XCH capsules started releasing cells at approximately 40 min. This could be due to damage of the XCH hydrogel membrane in harsh gastric environment. It should be noted that xanthan is a resilient molecule known to possess ordered conformation and stabilizing forces between the strands (Katzbauer 1998). However, on the other hand, chitosan is very susceptible to depolymerization due possibly to hydrolysis of the *O*-glycosidic linkages and the *N*-acetyl linkages as seen in dilute and concentrated HCl solutions (Vårum et al. 2001). The differences in the stability of XCX and XCXH capsules in

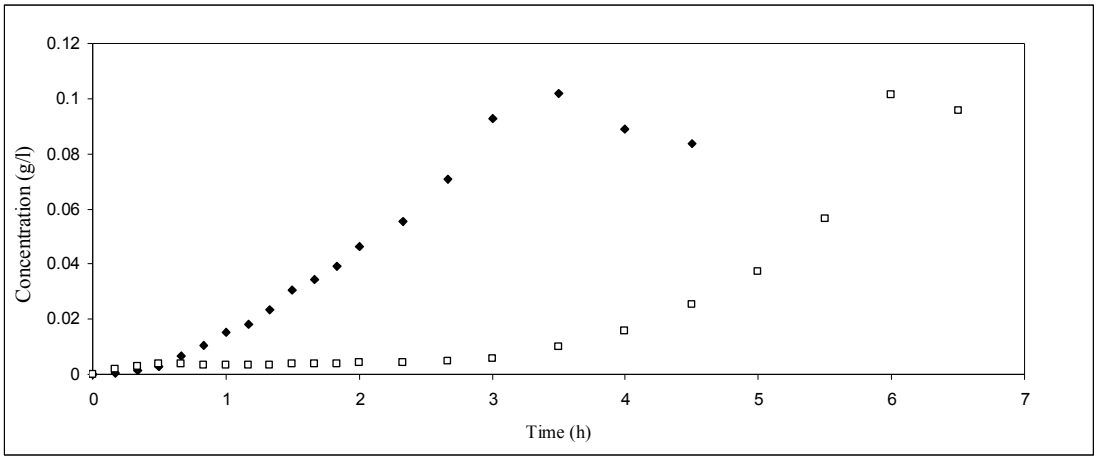


Figure 5.3 Release of *L. acidophilus* ATCC 43121 cells from xanthan-chitosan hydrogel (XCH, ♦) and xanthan-chitosan-xanthan hydrogel (XCXH, □) capsules in simulated gastric juice at pH 1.5 (n = 2)

harsh SGJ conditions could be attributed to the presence of acid-susceptible chitosan at the surface of XCX, unlike acid-resistant xanthan at the surface of XCXH.

The ability of a microencapsulation system to release the probiotic bacteria in intestinal conditions is critical in ushering the efficacy of probiotic bacteria to confer desirable health benefits. Figure 5.4 shows the fractional release of probiotic bacteria subjected to sequential SGJ and SIF treatments. After SGJ treatment at pH 1.5 for 1.5 h, both XCH and XCXH were able to release the encapsulated cells shortly after transfer to SIF. While XCH reached the maximum release at 5 h, XCXH took up to 6 h. This difference could be due to the presence of already damaged XCH capsules in SGJ that led to faster release in SIF compared to XCXH. Furthermore, it could also be due to the differences in hydrogel membrane thickness between XCH and XCXH. XCH are known to exhibit diffusion-controlled release of its core contents under intestinal conditions due to swelling (Argin-Soysal 2007). It is also noteworthy to

mention that chitosan is susceptible to enzymetic depolymerization (Muzzarelli 1997) with the enzymes present in SIF, further aiding the release of probiotic cells.

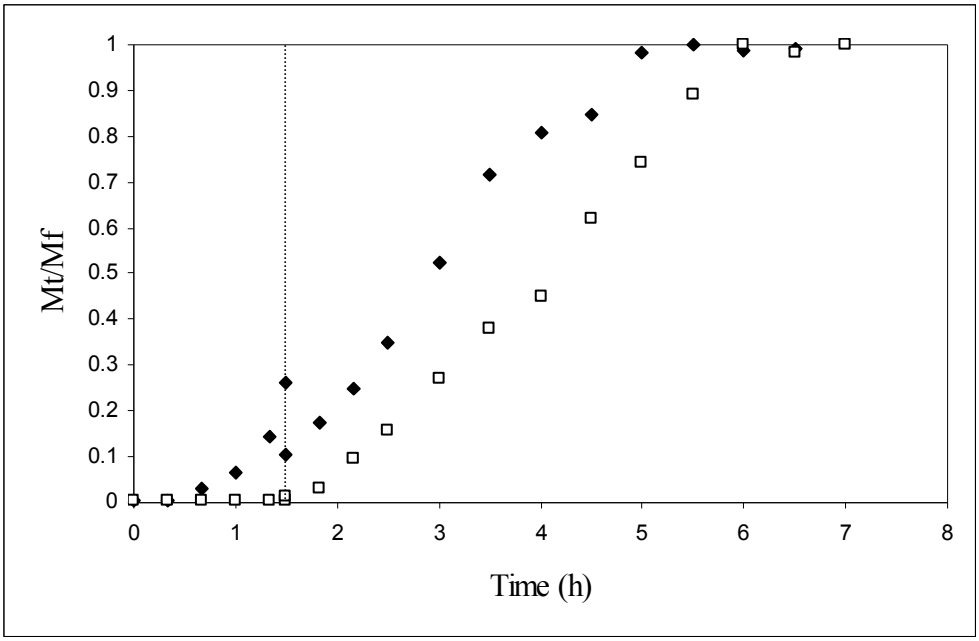


Figure 5.4 Fractional release of *Lactobacillus acidophilus* ATCC 43121 cells from xanthan-chitosan hydrogel (XCH, ♦) and xanthan-chitosan-xanthan hydrogel (XCXH, □) in simulated gastric juice (SGJ) at pH 1.5 and in simulated intestinal fluid (SIF) at pH 6.8 (n = 3)

5.3.2 Effect of simulated gastrointestinal conditions on encapsulated cells

The effect of simulated gastric conditions on the viability of encapsulated and non-encapsulated *L. acidophilus* ATCC 4312 is shown in Figure 5.5. The initial cell count of harvested cells was approximately 10^9 CFU/ml. Under gastric conditions at pH 1.5 for 1.5 h, non-encapsulated free cells suffered ca. 5-log reduction. On the other hand, encapsulated cells retained significantly higher viability in all cases. Syringe extruded XCXH capsules (2-3 mm) resulted in at least 1.5 log CFU/ml more viable cells than those in XCH capsules. Smaller capsules (<500 μm) produced using a spray nozzle resulted in significantly lower viability than syringe extruded XCXH

capsules and was not significantly different from those in XCH capsules. The differences in the protective effects of XCXH and XCH could be a combination of two effects. First, the ability of XCXH to retain probiotic bacteria in SGJ at pH 1.5 for 1.5 h without any leakage was superior to XCH capsules, which released probiotic bacteria after only 40 min of SGJ treatment. Second, the extra layer of xanthan coated on the surface of XCXH means formation of thicker hydrogel membrane, which corresponds to more time it takes for the core of the capsule to reach pH equilibrium with SGJ.

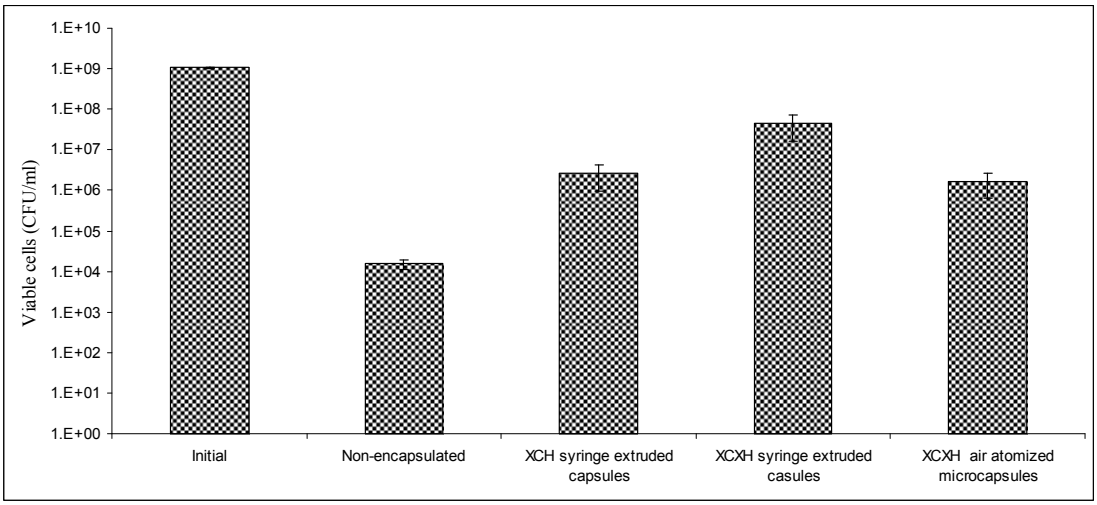


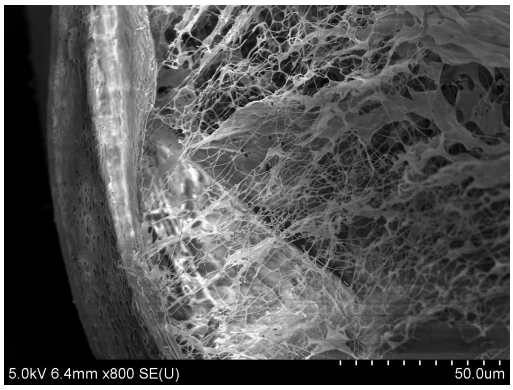
Figure 5.5 Viability of non-encapsulated and encapsulated (syringe extruded XCH, XCXH and air atomized XCXH) *Lactobacillus acidophilus* ATCC 43121 in simulated gastric juice at pH 1.5 for 1.5 h followed by releasing in simulated intestinal fluid for 5 h (n=4).

Although XCXH syringe-extruded capsules gave better protection than XCH capsules in SGJ (Figure 5.5), the protective effects decreased when the size of the capsules decreased. Many researchers have reported similar findings (Sheu et al. 1993; Lee and Heo 2000; Argin-Soysal 2007). In one such study, Chandramouli et al. (2004) reported that the viability of encapsulated *Lactobacillus* in simulated gastric conditions increased with increasing alginate capsule size. As retarding

permeation of gastric fluid into the hydrogels is the primary purpose of microencapsulation for probiotics (Anal and Singh 2007), the smaller sized capsules may have resulted in lower protective effects due to the reduced time it takes for the core of the capsule to reach gastric pH compared to bigger capsules. Nevertheless, smaller XCXH capsules resulted in a level of viability (ca. 10^6 CFU/ml) that is critical to be considered a therapeutic minimum dose to confer health benefits to the consumer (Robinson 1991; Ouwehand and Salminen 1998).

5.3.3 Scanning electron microscopy

The internal structure of the XCXH hydrogel was exposed by fracturing the capsules under frozen conditions for observation using SEM. XCXH capsules formed with 0.7 % HMW chitosan showed a very thick shell formation (Figure 5.6 a). Probiotic cells were observed in the core of the capsule adhered to xanthan polymer (Figure 5.6 b) and also entrapped inside the capsule wall (Figure 5.6 c). The thickness of the XCXH hydrogel shell was considerably increased compared to thickness of XCH hydrogel membrane (Figure 4.7a). While thickness of XCXH formed by 0.7% MMW chitosan (Figure 5.6 d) also increased considerably, it formed a more porous structure than 0.7% HMW chitosan. XCXH capsules formed with 0.7% LMW chitosan (Figure 5.6 e) did not show any changes compared to XCH (Figure 4.7c) and retained a highly porous structure (Figure 5.6 f). However, XCXH using 1.2% LMW chitosan (Figure 5.6 g) showed a loosely attached hydrogel layer possibly due to the formation of hydrogel between xanthan and residual unattached chitosan on the capsule surface. Internal structure remained porous, similar to XCXH formed with 0.7% LMW. The absence of extra layer of hydrogel and presence of only a loosely bound outer layer at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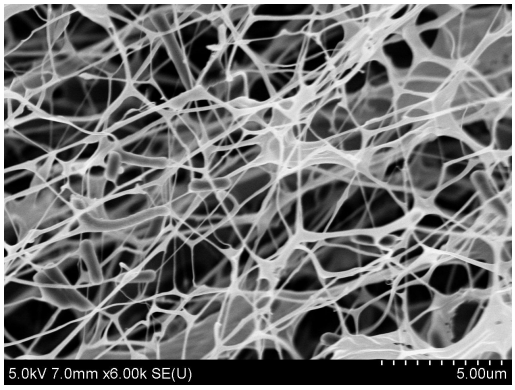
(a)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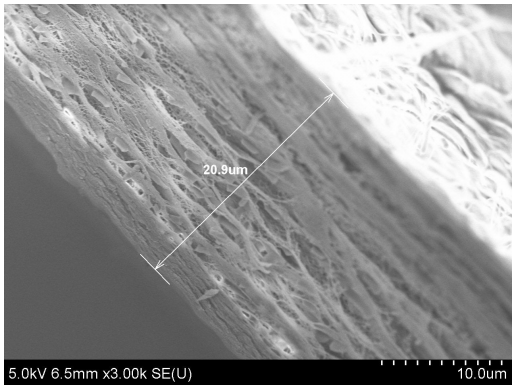
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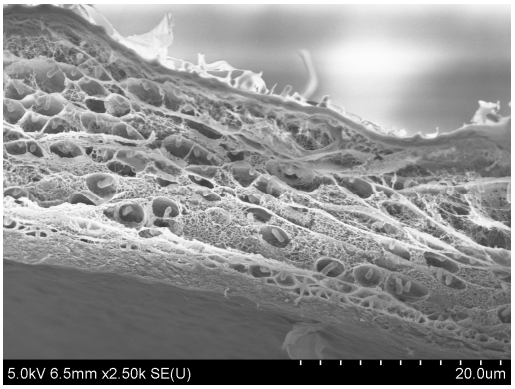
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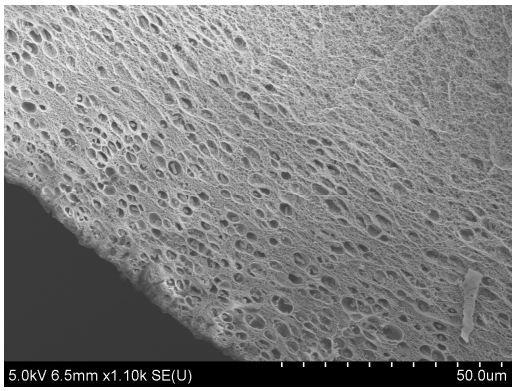
(d)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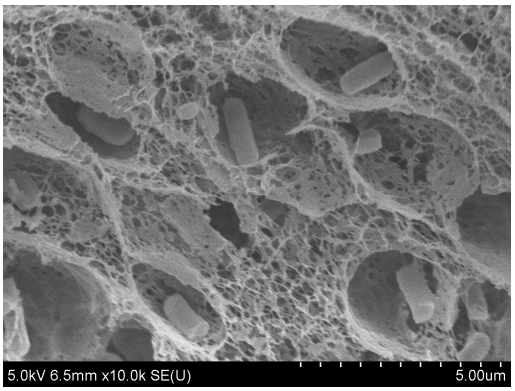
(e)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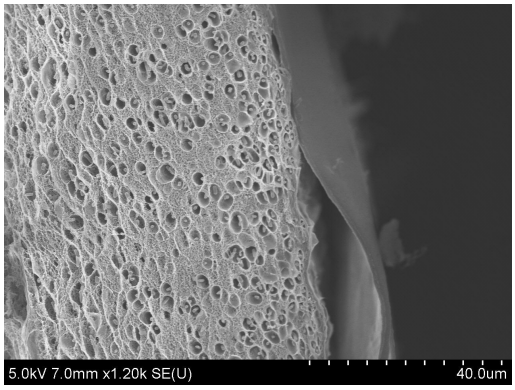
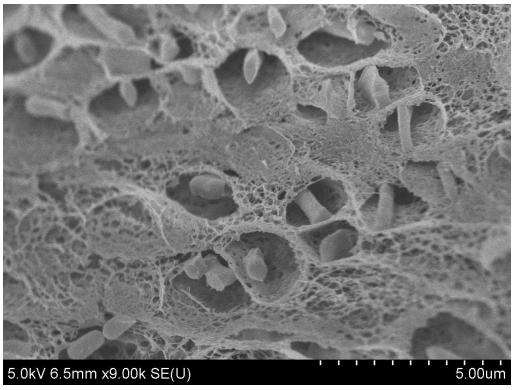
(f)





(g) (h)

Figure 5.6 Scanning electron microscope images of cross section of freeze dried hydrogels. Xanthan-chitosan-xanthan hydrogel (XCXH) formed with 0.7% high molecular weight chitosan: (a) 800×, (b) core at 6000×, and (c) shell at 3000×; with 0.7% medium molecular weight chitosan: (d) 2500×; with 0.7% low molecular weight chitosan: (e) 1100× showing no distinct outer layer and (f) 10000×; and with 1.2% low molecular weight chitosan: (g) 1200× showing a loose outer layer and (h) 9000×.

the surface of XCXH formed by 0.7% LMW and 1.2% LMW, respectively, suggests that there is not enough chitosan on the surface after formation of XCH to bind to surrounding xanthan for the second layer to form. This might expose more pores on the surface of the capsule, hindering the hydrogel's ability in retarding gastric fluid, and consequently affecting the protective effect of the capsules adversely.

5.4 Conclusion

Microencapsulation of probiotics using XCXH hydrogels significantly improved the viability of *L. acidophilus* ATCC 43121 in SGJ at pH 1.5 for 1.5 h compared to XCH capsules. Reduction in the size of XCXH capsules by air atomization resulted in significantly lower viability compared to syringe extruded capsules; however the viability was significantly higher than free cells in SGJ. XCXH capsules retained cells for significantly longer period of time (up to 3h), whereas

XCH capsule lost its integrity after 40 min. XCXH capsules took longer (approximately 1h) to release all cells in SIF compared to XCH. Scanning electron micrographs revealed that HMW and MMW chitosan formed thicker hydrogel membranes compared to XCH, while LMW chitosan did not form a firm extra layer. This suggests that the key parameter, namely chitosan molecular weight that affects XCH cross-linking during capsule formation is also critical for the formation of XCXH capsules.

5.5 Acknowledgements

We acknowledge the support of the Maryland NanoCenter and its NispLab. The NispLab is supported in part by the NSF as a MRSEC Shared Experimental Facility.

Chapter 6: Survival of xanthan-chitosan-xanthan microencapsulated *Lactobacillus acidophilus* ATCC 43121 in stirred yogurt during refrigerated storage

6.1 Introduction

Yogurt is one of the widely consumed foods throughout the world with an established market as a functional therapeutic food (Samona and Robinson 1994; Sarkar, 2008). Yogurt has been the simplest form of preservation for milk since long ages. Elie Metchinkoff, a scientist from early 20th century was the first to postulate the health benefits of yogurt related to the extraordinary longevity of Bulgarians (Metchinkoff, 1908). The normal yogurt cultures, *Lactobacillus delbrueckii ssp. Bulgaricus* and *Streptococcus thermophilus*, cannot survive and grow in the intestinal tract due to poor bile salt tolerance. Many different microorganisms are added to dairy products including yogurt to enhance their probiotic potential (Fuller, 1997; Bhadoria and Mahaputra, 2011). However, retaining the viability of probiotic bacteria has long been a challenge and studies have shown that a lot of the commercial products do not contain the prescribed number of live probiotic bacteria at the time of consumption. In one study, Shah (2000) studied the viability of *L. casei* content in commercial yogurts and found no traces of live microorganisms in 3 of 6 products tested and only low concentrations in two others. In another study, Huges and Hoover (1991) analyzed 11 products claiming to contain *L. acidophilus*, of those only two products contained live bacteria. The reduction in viability of probiotic bacteria is due to one or more factors including storage temperature, pH, post-

acidification due to residual fermentation during storage, stability in dried or frozen form, and incompatibility with traditional starter culture during fermentation (Anal et al. 2007; Kailasapathy 2002; Brunner et al. 1993; Shah 2000). In addition, the harsh gastrointestinal conditions, namely high acidity of gastric juices in stomach followed by high bile concentration further reduces the number of probiotic bacteria before they can reach the large intestine and colonize.

Microencapsulation is the most commonly used method by researchers to improve viability of probiotic bacteria during storage and during transit through gastrointestinal tract. Numerous efforts have been attempted to improve the viability of probiotics in yogurt and other fermented and non-fermented dairy products by microencapsulating probiotics in various wall materials, predominately alginate-based (Sheu and Marshall 1993; Kebary et al. 1998; Chandramouli, 2004; Brinques 2011), cellulose acetate phthalate (Rao et al. 1989), carrageenan (Adhikari et al 2003), gelatin (Hyndman 1993), and whey protein based (Picot and Lacroix 2004) encapsulation. While most research reported protective effects on probiotics during storage conditions and some during gastric transit, they suffer from one or more limitations including low stability in the presence of chelating agents such as phosphate, lactate and citrate (Smidsrod and Skjak-Braek 1990). Furthermore, evidence in the literature remains scarce on whether the cells could be properly released in intestinal conditions (Kailasapathy 2002; Lee et al. 2004).

In our previous research (Argin-Soysal, 2007), it was demonstrated that microencapsulation of probiotic cells using xanthan-chitosan hydrogel complex significantly improved their viability in gastric conditions and the cells could be

released under intestinal conditions. The oppositely charged nature of xanthan (-) and chitosan (+) due to the respective carboxylic and amine groups formed xanthan – chitosan polyelectrolytic hydrogel complex immediately upon contact, and stayed stable under gastric conditions. However, the efficacy of these hydrogels has not been evaluated in food systems. Therefore, the main objective of this study was to study the effect of xanthan chitosan-xanthan hydrogel encapsulated *L. acidophilus* ATCC 43121 in stirred yogurt at refrigerated storage.

6.2 Materials & Methods

6.2.1. Reagents and chemicals

Xanthan gum and high molecular weight (HMW, 1200-1600 cP), at 75% deacetylation was purchased from Sigma-Aldrich Chemicals (St Louis, MO). Yogourmet (Lyo-San Inc., Lachute, Cabada), a commercial freeze dried yogurt starter (*L. bulgaricus, S. thermophilus, L. acidophilus*), was purchased. Artificial intestinal fluid without pancreatin was purchased from Fisher Ricca Chemicals (Arlington, Texas), respectively. *L. acidophilus* ATCC 43121 was obtained from ATCC (Manassas, VA). Simulated intestinal fluid (SIF) was prepared by adding a sterile concentrated solution of pancreatin (sterility achieved by filtering through 0.22 μm sterile syringe filter) to autoclaved solution containing artificial intestinal fluid to reach a final concentration of 0.1% pancreatin.

6.2.2. Microencapsulation procedure

Lactobacillus acidophilus ATCC 43121 cells were encapsulated in triple layer spray-nozzle extruded capsules as described in Chapter 5.

6.2.3 Stirred yogurt preparation

Two percent skimmed milk was pasteurized using a water bath at 85^{o}C for 30 min. It was cooled to 43^{o}C and inoculated with 2.5 g of starter culture already suspended in 10 ml of pasteurized 2% skimmed milk pre-cooled to 43^{o}C. The mixture was shaken thoroughly and incubated for 4 h at 43^{o}C until the pH of yogurt reached 4.6. Yogurt was stirred at 700 rpm for 10 min to obtain a smooth liquid like consistency followed by pasteurization (85^{o}C for 30 min) and cooling. Stirred yogurts containing either free or encapsulated *L. acidophilus* cells were prepared by respectively adding the free cell suspension or encapsulated *L. acidophilus* to stirred yogurt respectively. Yogurts were then stirred at 500 rpm for 10 min to disperse free cells or capsules uniformly. A fixed volume (10 ml) of stirred yogurts (containing free cells and encapsulated cells) were dispensed into several sterilized test tubes and stored at 4^{o}C for storage studies. For pH measurements, 200 ml of stirred yogurt was stored in sterilized beaker at 4^{o}C. Experiments were conducted in triplicates.

6.2.4 Release and enumeration of bacteria

Two 10 ml yogurt samples from each batch (encapsulated and non-encapsulated *L. acidopjhilus*) were analyzed for viability of probiotic bacteria every 5 days during the 45 days of storage period. For stirred yogurt containing microcapsules, sample was added to 100 ml of SIF containing 0.1% pancreatin for 5 hours at 35^{o}C to release cells from microcapsules, followed by enumeration by serial dilution and plating on MRS agar. For stirred yogurt with non-encapsulated cells, sample was directly diluted using DI water and plated on MRS agar for plate count. To observe the effect of SIF on non-encapsulated cells in stirred yogurt, sample was added to 100 ml of SIF and incubated at 35^{o}C, followed by serial dilution and plating

on MRS agar. The number of viable cells was obtained after incubation of plates for 48 hours in anaerobic conditions.

6.2.5. Statistical Analysis

Tests for statistical significance of differences were compared by general linear model (GLM) procedure of SAS. Analysis of variance and t-tests were performed using SAS v.9.2 (SAS Institute Inc., Cary, N.C., USA)

6.3. Results & Discussion

The pH of yogurt (Figure 6.1) with free *L. acidophilus* cells was slightly higher than pH of yogurt with encapsulated cells possibly due to differences in the pH of free cell suspension and microcapsule suspension before addition to yogurt. Yogurt with free cells had significantly higher pH until 5 days of storage and the differences subsided after 10 days indicating post-acidification in case of yogurt containing free cells.

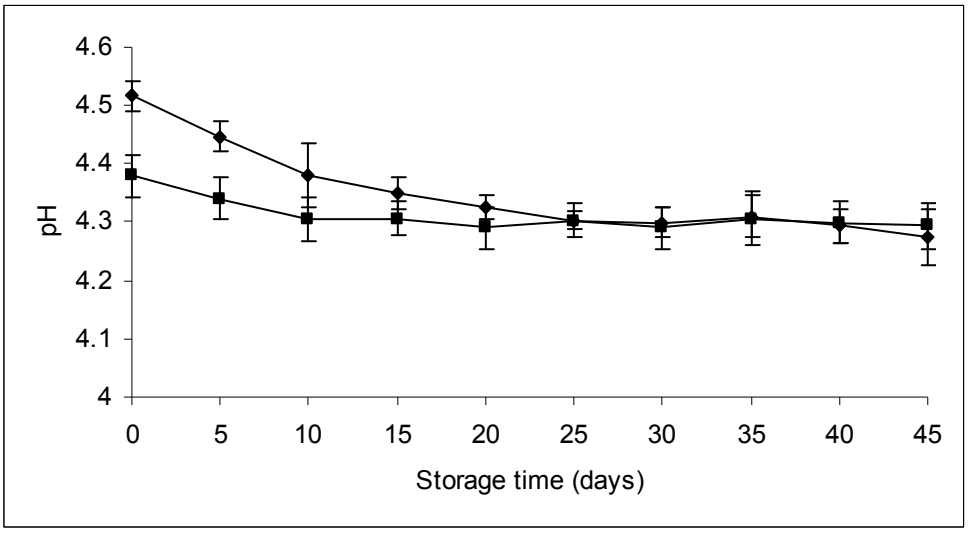


Figure 6.1 Changes in pH of yogurt with free (♦) and xanthan-chitosan-xanthan hydrogel (XCXH) microencapsulated (■) *Lactobacillus acidophilus* ATCC

The number of *L. acidophilus* ATCC 43121 cells, both encapsulated and non-encapsulated, in stirred yogurt declined with storage time at refrigerated temperature (Figure 2).

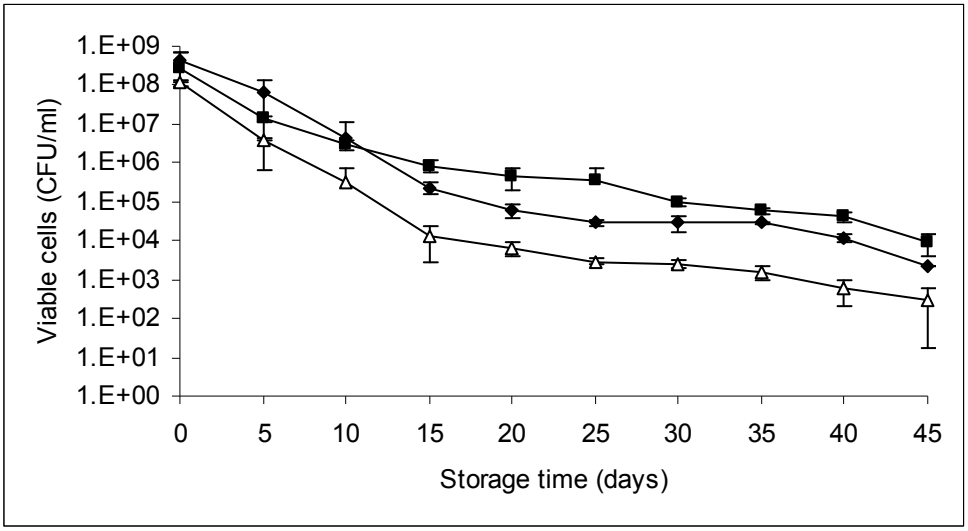


Figure 6.2 Effect of refrigerated storage time on viability of *Lactobacillus acidophilus* ATCC. Xanthan-chitosan-xanthan hydrogel (XCXH) microencapsulated cells released by 5 h simulated intestinal fluid (SIF) treatment (■); Free cells (♦); Free cells after 5 h SIF treatment (△)

Encapsulated *L. acidophilus* ATCC 43121 retained significantly higher viability starting from day 15 until day 45 except day 35. However, it must be emphasized that the encapsulated cells were subjected to a 5 h SIF treatment to release them from XCXH microcapsules. When compared to free cells subjected to 5 h SIF treatment, encapsulated cells retained significantly higher viability (1 to 2 log CFU/ml) during the entire storage period. Free cells from yogurt subjected to SIF resulted in less than 10^6 CFU/ml after only 10 days of storage. However encapsulation maintained the viability of 10^6 CFU/ml, a therapeutic minimum to achieve health benefits, until 25 days of storage.

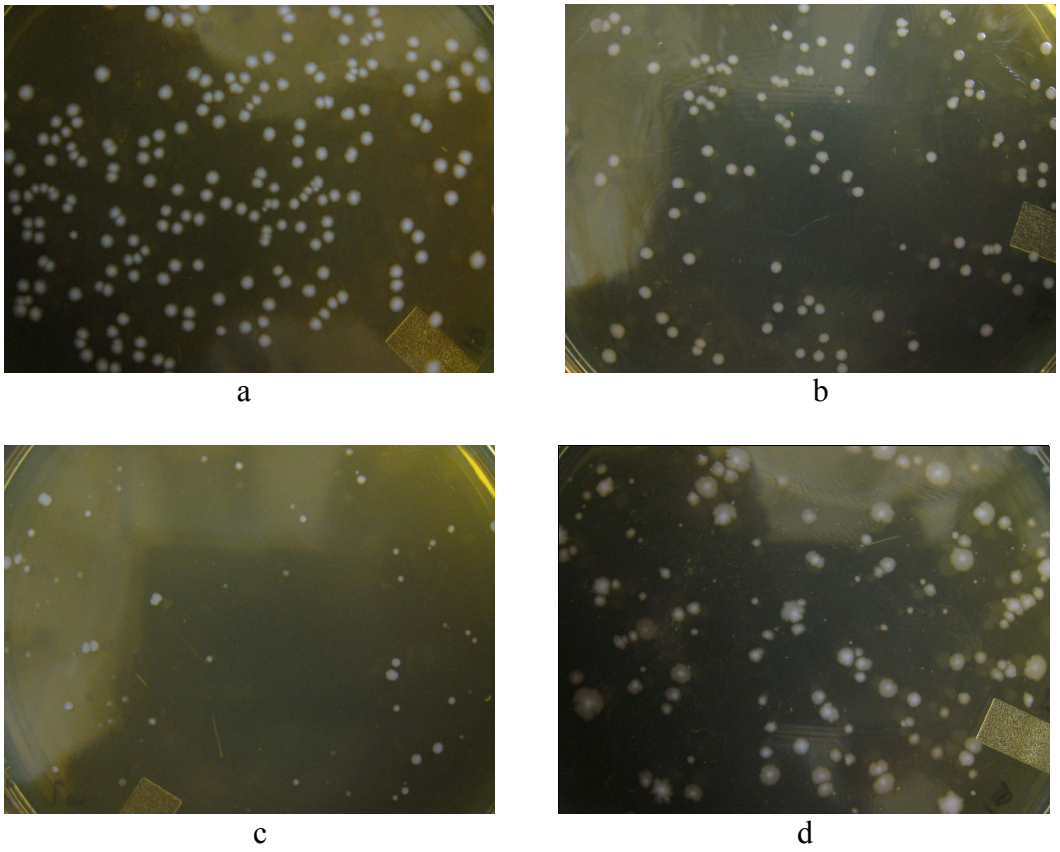


Figure 6.3 MRS agar plates with cells incubated for 48 hrs at 35°C. Freshly harvested cells from MRS broth (a), cells released from xanthan-chitosan-xanthan hydrogel (XCXH) capsules in yogurt after 5h simulated intestinal fluid (SIF) treatment (b), free cells from yogurt (c), free cells from yogurt after 5 h SIF treatment (d). Dilutions made before plating are different for each of the treatments.

The colonies formed by *L. acidophilus* ATCC 4312 are shown in Figure 6.3. Encapsulated cells after release formed circular, smooth edged colonies similar to those from freshly harvested cells after fermentation. While free cells from yogurt did not show healthy growth of colonies indicating cell damage, free cells after 5 h SIF treatment formed two different types of colonies one similar to encapsulated cell but smaller in size and other with large, irregular, rough edged colonies. The difference in morphology of colony could be an indication of the change in the acid

or bile resistance of the bacteria. Klaenhammer and Kleeman (1981) reported that the differences in morphology of *Lactobacillus acidophilus* colonies were not a result of genetic variability but a phenotypic variability caused by environmental factors. According to Suskovi et al. (2000), differences in bile resistance of *Lactobacillus acidophilus* M92 resulted in two distinct types of colonies with different morphological characteristics. It is worth mentioning that *L. acidophilus* ATCC 43121 cells are bile resistant as evidenced by only a slight decrease in viability after 5 h of SIF treatment (Figure 4.2). However, a decreased bile resistance is noticed when added to yogurt with a loss of at least 2 logs CFU/ml during refrigerated storage indicating a change in bile resistance possibly due to extended exposure to low pH of yogurt.

4. Conclusion

The numbers of both encapsulated and non-encapsulated *Lactobacillus acidophilus* ATCC 43131 bacteria added to yogurt declined with storage time at refrigerated conditions. Xanthan-chitosan-xanthan hydrogels resulted in significant improvement in probiotic viability compared to non-encapsulated cells. Non-encapsulated cells in yogurt when subject to SIF declined in viability indicating a loss in bile resistance supported by changes in morphological characteristics of cell colonies. Encapsulated bacteria formed regular colonies and resulted in viability above the therapeutic minimum ($>10^6$ CFU/g) thus promising to deliver enough number of probiotic bacteria in the intestine to confer health benefits.

Chapter 7: Conclusions and Recommendations

Xanthan chitosan hydrogel formed stable hydrogels capable of protecting probiotic bacteria against gastric conditions. Factors affecting the protective effects of XCH were characterized by studying the changes in pH of the core of XCH on external pH variation such as gastric acidity. The barrier properties were found to depend on the factors affecting the hydrogel structure such as molecular weight of chitosan, complexation time, and xanthan concentration. Formation of a dense hydrogel membrane was found to be critical in retarding gastric fluid diffusion.

Viability studies revealed that despite strong antimicrobial behavior of chitosan when acting alone, no antimicrobial effect on probiotic *L. acidophilus* was observed when combined with xanthan. A thicker hydrogel outer layer formed by HMW chitosan resulted in best protective effects on the viability of *L. acidophilus* against SGJ compared to porous hydrogel formed by LMW chitosan, indicating the importance of molecular weight dependent membrane structure. The protective effects of probiotics against SGJ were further improved with the formation of XCXH hydrogels compared to XCH due to the formation of a thicker hydrogel layer, resulting in enhanced stability in SGJ. Reduced XCXH capsule size had adverse effect on the protective effects, though still significantly higher than free cells in SGJ.

Moreover, application of XCXH capsules in stirred yogurt resulted in higher viability during refrigerated storage conditions and retained the bile resistance of *L. acidophilus* ATCC 43121. This indicates that, with XCXH encapsulation, it is possible to achieve the delivery of a therapeutic minimum number of probiotic bacteria that could confer the claimed health benefits. It is highly recommended that

future studies focus on evaluation of XCXH capsules in other food systems such as non-fermented dairy beverages and dry food products. Developing the technology required in manufacturing small and uniformly sized capsules will be crucial in expanding its application in various foods.

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