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Colloidal Particles as Antimicrobial Carrier Systems

Dustin W. Carnahan
University of Massachusetts Amherst

COLLOIDAL PARTCLES AS ANTIMICROBIAL CARRIER SYSTEMS

A Thesis Presented

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Dustin Carnahan

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DUSTIN CARNAHAN

Approved as to style and content by:

_____
Jochen Weiss, Chair

_____
Julian McClements, Member

_____
Lynne McLandsborough, Member

_____
Fergus M. Clydesdale, Department Head
Food Science

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CHAPTER 1

INTRODUCTION

Over the course of human history, the source of the food that the population relies on for survival has changed dramatically (Jay, 2000). Hunting and gathering have given way to the domestication of plants and livestock. The growing of food for a family or a small region has been replaced by the centralization and globalization of food production. Growing crops in different parts of the country and the world has made fresh produce available at any time of year. Rather than sitting down to a home cooked meal, consumers have turned to meals prepared outside the home or ready-to-eat (RTE) meals that require little to no preparation inside the home. Health consciousness has led to a call for more natural foods, meaning less processing and a reduction in the addition of preservatives to food products. These factors combine to make food safety not only a predominant issue to consumers but also more challenging than ever for those who produce these products.

Food has long been recognized as a potential vector of disease. For example, religious restrictions on the consumption of pork were most likely put into place to protect against diseases associated with consumption of pork products (Hartman, 1997). As a consequence of the centralization of food production, religious and historical doctrine has been replaced by government regulation. The inspection of meat was first introduced in the late 1800's, followed by the Food, Drug, and Cosmetic Act in 1939, and the Food Additives Amendment in 1958 (Jay, 2000). In the 1980s, the importance of food was recognized as a vector for several important pathogens; *Listeria*

monocytogenes, *Escherichia coli* 0157:H7, and *Campylobacter jejuni*. The 1990's thus saw enactment of increased surveillance measures and an increase in research towards the transmission pathways of food pathogens in order to further improve the safety of foods (Allos *et al.*, 2004). The Foodborne Disease Active Surveillance Network (FoodNet), established in 1996, is an effort between the Centers for Disease Control (CDC), the Department of Agriculture (FDA), and state health departments to monitor outbreaks of foodborne diseases (Allos, *et al.*, 2004). PulseNet uses molecular techniques to provide genetic fingerprints for food pathogens associated with contamination and outbreaks (Allos *et al.*, 2004). In 1997, Pathogen Reduction, Hazard Analysis and Critical Control Point (HACCP) were initiated, which calls for the identification and control of points in a process where bacterial contamination can occur (Allos *et al.*, 2004).

The initial 1997 FoodNet estimation for yearly cases of foodborne illness in the United States was 76 million cases, with 325,000 hospitalizations and 5500 deaths (Anonymous, 2006a). Confirmed cases within the 15% of the population that FoodNet surveillance covers for the time period 1996-1998 was 24,511 cases of foodborne illness, with *Campylobacter*, *Salmonella*, and *Shigella* as the leading causative agents (Kennedy *et al.*, 2000). Differences between estimated and confirmed cases are most likely due to the large number of foodborne illnesses that go unreported every year due to mildness of symptoms and short duration of the illness. The initial surveillance results for 2005 shows a general reduction in laboratory confirmed cases of foodborne illness with only 16,614 cases confirmed (Anonymous, 2006c). *Salmonella*, *Campylobacter*, and *Shigella* are still the most commonly reported foodborne pathogens. Though cases have declined

every year since surveillance began, the rates of decline are slowing. The 2005 report shows that *Listeria* levels, though down from 1996-1998, are higher than the lowest reported level in 2002 (Anonymous, 2006c). *Vibrio* levels are actually on the rise.

Though FoodNet data shows that cases of foodborne illness are declining, they still represent a substantial health care burden to the consumers and a monetary burden to the food industry. When contamination is discovered in a product, the manufacturer is expected to voluntarily issue a recall notice. The FDA can only act if the situation is urgent, in which case they request the manufacturer recall the product, typically followed by lawsuits and injunctions if the manufacturer refuses to follow the recommendations (Venugopal *et al.*, 1996). In 1991-1992, there were 230 recalls involving 569 products, 138 (24%) were caused by microbial contamination (Venugopal *et al.*, 1996). Of these 138 recalls, 90 (65%) involved *L. monocytogenes*. Several large food recalls have occurred over the last few years. In 2000, turkey and chicken deli meats were recalled in response to 29 cases of listeriosis which resulted in 4 deaths and 3 miscarriages (Anonymous, 2000). Two years later 46 cases of listeriosis, resulting in 7 deaths and 3 stillbirths prompted a recall of 24.7 million pounds of turkey and deli meats, the largest such recall in history (Anonymous, 2002). Two high profile recalls have occurred in 2006; a recall of 100% carrot juice prompted by 4 cases of botulism following consumption of heat abused juice, and the removal of all fresh bagged spinach from store shelves after 199 people across 26 states became infected with *E. coli* (Anonymous, 2006b). Along with the monetary loss involved with the recall of products, the presence of pathogens in the product also opens up the company to lawsuits by those who became infected. Even more importantly, a loss of trust in the brand name involved in the recall

can permanently eliminate market shares the brand previously held and may even lead to bankruptcy of the producing company.

Though not one of the most commonly found foodborne pathogens, the industry pays a great deal of attention to the Gram positive, non spore forming rod *L. monocytogenes*. Initial FoodNet surveillance found that of the 99 deaths associated with food pathogens, 38 were caused by *L. monocytogenes* (Kennedy *et al.*, 2000). The high mortality rate associated with listeriosis, the name given to a range of symptoms associated with *L. monocytogenes* infection, is due to the severity of the disease. *L. monocytogenes* is an invasive intracellular pathogen. Following consumption, the organisms exit the intestines and are quickly engulfed by immune system macrophages (Rocourt and Cossart, 1997). In individuals with normally functioning immune systems, the intracellular microorganism attracts the attention of cytotoxic T cells, which lyse the infected cell and expose the pathogen to further immune system attention (Parham, 2000). In individuals with suppressed immune systems, the elderly, pregnant women and their unborn children, and those with preexisting conditions that cause immune suppression, the bacteria is able to replicate and spread from cell to cell. Those with active immune systems may suffer mild flu-like symptoms, if they suffer any symptoms at all. Those who are immunocompromised can experience septicemia, bacteremia, meningitis, miscarriages, and stillbirths with a 20-30% mortality rate (Rocourt and Cossart, 1997).

L. monocytogenes was linked to food following several outbreaks in the 1980's. In 1983, 7 adult and 34 perinatal cases of listeriosis in Canada were traced to cole slaw

that was later found to have been made with cabbage grown at a farm with known cases of ovine listeriosis (Schlech *et al.*, 1983). The largest known listeriosis outbreak occurred in Los Angeles in 1985. 142 cases of listeriosis, with 48 ensuing deaths, were traced back to the consumption of Mexican-style soft cheese tainted with unpasteurized milk (Linnan *et al.*, 1988). The second largest outbreak, 108 cases, with 14 associated deaths and 4 miscarriages/stillbirths, occurred in 1998-1999 and was linked to hot dogs from a facility in which the removal of a contaminated air conditioning unit caused post-process contamination (Mead *et al.*, 2005). The outbreak ended with the recall of 35 million pounds of product. In response to these outbreaks, the United States adopted a zero tolerance policy for *L. monocytogenes* in ready to eat foods, instituted an emphasis on Good Manufacturing Practices (GMP), HACCP, recommendations for facility sanitation and safe handling of processed foods, and education for populations at risk for listeriosis (Shank *et al.*, 1996).

Controlling the growth of *L. monocytogenes* in a food production facility is challenging for manufacturers. Many of the unprocessed products that come into the facility carry *L. monocytogenes*. Franco *et al.* (1995) took samples from a poultry processing plant and found high levels of contamination, particularly on drumsticks, 96% of which showed counts exceeding 3 log CFU/g. Beef and pork also come into the processing facilities carrying *L. monocytogenes*, with pork showing higher levels (Vandenelzen and Snijders, 1993). Though the bacteria are often carried in the intestines (Thevenot *et al.*, 2006), Autio *et al.* (2000) found that the tongue and tonsils are often the sources of carcass and facility contamination. In sampling pork products processed in stores and in centralized facilities, the cuts that originated in the store and required the

most handling showed the most microbial contamination (Duffy *et al.*, 2001). *Listeria*, in this case, was the most common source of contamination (26.7%). Raw vegetables can also bring *L. monocytogenes* into a processing facility from any of a variety of sources, including decaying vegetation and feces used as fertilizer, the soil the plants grow in, or the water source for the crops (Beuchat, 1996).

With nearly every raw product coming into a facility being a potential carrier of *L. monocytogenes*, it is up to the food processors to insure that all RTE products leave the facility free of contamination. *L. monocytogenes* coming into the plant contaminates areas where raw products are processed (Franco *et al.*, 1995; Thevenot *et al.*, 2005). Frequent cleaning is used to control this contamination, but *L. monocytogenes* has shown resistance to some commonly used cleaning solutions and sanitizers (Taormina and Beuchat, 2002). Resistance to sanitizers, including quaternary ammonium compounds, has been linked to the presence of *mdrL*, a gene that encodes an efflux pump (Romanova *et al.*, 2002). The inefficieny of cleaning procedures to remove *L. monocytogenes* has also been associated with its ability to adhere to stainless steel and form biofilms (Vatanyoopaisarn *et al.*, 2000; Thevenot *et al.*, 2006). The complexity of the machinery used in production can also hinder the cleaning process, with bacteria growing in corners and crevices that are difficult to clean (Lunden *et al.*, 2002).

The difficulty in controlling *L. monocytogenes* levels in RTE food products lies in its resistance to many commonly used preservation methods. Proper heating and pasteurization insures a *Listeria*-free product. Improper heating, however, allows survival of the pathogen, which will continue to grow once the product is refrigerated

(Samelis and Metaxopoulus, 1999). Most *L. monocytogenes* contamination occurs following processing (Linnan *et al.*, 1988; Reij and Den Aantrekker., 2004; Mead *et al.*, 2005). Common routes of recontamination include improper packaging, unsanitary equipment, improper handling and storage, and the introduction of contaminated ingredients, like spices or flavorants (Reij and Den Aantrekker, 2004). *L. monocytogenes* is psychrotrophic, meaning it can grow at low temperatures, making refrigeration selective for the growth of the organism. It can also grow at high salt concentrations and in the presence of nitrates (Cole *et al.*, 1990). A survey of vacuum packaged processed meat showed that 53% of those surveyed were contaminated with *L. monocytogenes*, due to its ability to grow at low oxygen levels (Grau *et al.*, 1992). Antimicrobials, several of which are strong inhibitors of *L. monocytogenes* growth, are also used to control microbial growth in food products (Geise, 1994; Sofos *et al.*, 1998). Combinations of these preservation methods often have a synergistic effect, commonly referred to as the hurdle effect (Leistner *et al.*, 2000).

Recent consumer trends show an increased interest in more natural, less processed foods, which has turned research attention towards antimicrobial compounds that are found naturally in foods or produced naturally by microorganisms involved in food processing (Sofos *et al.*, 1998). The antimicrobials nisin and lysozyme are examples of such naturally produced compounds. Bacteriocins are a class of peptides produced by lactic acid bacteria to inhibit the growth of similar bacteria, like *L. monocytogenes,* that compete for resources (Harris *et al.*, 1989). Nisin, a Class IIa bacteriocin, has been shown to be a strong inhibitor of *L. monocytogenes* growth (Ukuku and Shelef, 1997; Ennahar *et al.*, 2000; Mota-Meira *et al.*, 2000). Nisin binds to the bacterial membrane

via interaction between positively charged lysine residues in the nisin molecule and negatively charged phospholipid headgroups in the membrane (Abee, 1994; Moll *et al.*, 1997). Following insertion into the membrane, transient pores are formed via a hydrophilic interaction between nisin molecules (Winkowski *et al.*, 1996; Moll *et al.*, 1997). Potassium ions are lost via these pores which causes depolarization of the membrane and loss of proton motive force (Abee *et al.*, 1994). Lysozyme, an enzyme found in eggs and milk, is a strong inhibitor of *L. monocytogenes* growth (Hughey and Johnson, 1987). Lysozyme breaks down the peptidoglycan layer of Gram positive bacteria by hydrolyzing the 1,4 ß-D-linkage between N-acetylhexosamines (Proctor and Cunningham, 1988).

The leveling of the rate of decrease in foodborne illnesses may suggest that the current technology available to prevent growth of pathogens in food products has reached its limit. New technology may be needed to continue reducing the number of illnesses and recalls caused by foodborne pathogens. Though antimicrobials like nisin and lysozyme are effective in inhibiting the growth of *L. monocytogenes* in laboratory media, their effectiveness is greatly reduced in actual food products due to interactions between the antimicrobial and the food constituents. Lysozyme was less effective in reducing the numbers of *L. monocytogenes* in cheese and sausage compared to in fresh fruits and vegetables, likely due to interaction with fats in the cheese and sausage (Hughey *et al.*, 1989). Nisin activity was shown to be greatly reduced in meat products due to interaction with glutathione in raw meats and nitrates and fats in bacon (Ghalfi *et al.*, 2006; Stergiou *et al.*, 2006).

The ***objective*** of this thesis is to develop a method by which antimicrobials are delivered into a food product as a concentrated dose to the specific area in which the microorganism is growing without interference from the food matrix. More specifically, we plan to achieve this by delivering the antimicrobials nisin and lysozyme attached to nanoparticles and emulsion droplets. We hypothesize that (a) the attachment to a delivery vessel may increase the local concentration of the antimicrobial in the vicinity of the bacterial pathogens and (b) that the size and charge of the nanoparticle following attachment of the antimicrobials will be critical to its efficacy against pathogens. This thesis is designed to test this hypothesis using silver nanoparticles with well defined sizes and surface chemistry that allow control over the loading of the particle and oil droplets to which nisin is a secondary layer attached to pork gelatin which acts as the primary emulsifier.

CHAPTER 2

LITERATURE REVIEW

Food Antimicrobials

The traditional function of food antimicrobials or preservatives is to prolong shelf life and preserve quality of food through inhibition of spoilage microorganisms. While food antimicrobials have been in use since ancient times, few are used exclusively to control the growth of specific foodborne pathogens. An exception is nitrite, which has been used, in association with salt, ascorbate and erythorbate, and low pH, for hundreds of years to inhibit growth and toxin production of *Clostridium botulinum* in cured meats. More recently, other antimicrobials have been applied to foods against foodborne pathogens. For instance, organic acids (e.g., lactic acid, acetic acid) have been employed as spray sanitizers against pathogens on beef carcasses. Organic acid salts (e.g., sodium lactate, sodium diacetate) have been added to processed meats to inactivate pathogens (primarily *L. monocytogenes*). Finally, nisin and lysozyme are approved for use in pasteurized processed cheese as a safeguard against growth and toxin production by *C. botulinum* and lactoferrin was recently approved to control *E. coli* O157:H7 in meats. In most instances, the antimicrobial is part of a multiple intervention system that involves the chemical along with environmental (extrinsic) and food related (intrinsic) stresses and processing steps. This has been termed "hurdle technology" or multiple interventions (Leistner and Gorris, 1995, Leistner, 2000).

There are two arbitrary classifications of food antimicrobials, traditional or "regulatory approved" and naturally occurring (Davidson, 2001). The former includes

organic acids (acetic, lactic, propionic), sorbic acid, nitrites, sulfites, alkyl esters of *p*-hydroxybenzoic acids (parabens) and some natural antimicrobials including lysozyme, nisin, natamycin and lactoferrin. The latter includes compounds from microbial, plant and animal sources. Using naturally occurring antimicrobials is desirable to the food industry for several reasons: (a) it is highly unlikely that new synthetic compounds will be approved for use as food antimicrobials due to the expense of toxicological testing, (b) there exists a significant need for expanded antimicrobial activity both in terms of spectrum of activity and of broad food application, (c) food processors are interested in producing "green" labels, i.e., ones without chemical names that apparently confuse consumers, and (d) there are potential health benefits that come with the consumption of some naturally occurring antimicrobials.

Some of the most effective natural antimicrobials are extracted from spices and herbs, including *Amaryllidaceae* (e.g. garlic, onion) and members of the Cruciferae family (mustard, horseradish) (Davidson and Naidu, 2000; Sofos et al., 1998). The chemical compositions of the active ingredients in these plants are diverse. One commonality is that most of these compounds are oil-soluble and often derivatives of phenolic compounds. The use of phenolic compounds is increasing because these components exhibit antimicrobial properties and have therapeutic and pharmaceutical applications as well. Extracts from dietary herb species belonging to the family *Lamiaceae* (mint family), including thyme, have been used as sources of medicine and food preservatives for over 4000 years. Recently, some of the bioactive components linked to these medicinal and preservative functions have been determined to be phenolic metabolites (Peake et al, l991; Deighton et al, 1993; Deighton et al, l994). Specific

phenolic metabolites from *Lamiaceae,* like rosmarinic acid (from rosemary, spearmint, thyme and oregano) and thymol (from thyme and oregano), have both anti-inflammatory (Peake et al, l991) and antioxidant properties (Kuhnt et al, l995; Shapiro and Guggenheim, l995) in addition to their antimicrobial function. These pharmacological functions may contribute to long-term cancer prevention due to their effectiveness as antioxidants.

In the context of foodborne pathogens, the antimicrobial activity of the key metabolites of thyme, thymol and carvacrol, have been reported to inhibit the growth of *Salmonella enteritidis, Staphylococcus aureus, E. coli* and *Vibrio parahaemolyticus* (Katayama and Nagai, 1960; Beuchat, 1976). A second class of natural antimicrobials, derived from animal sources, are the polypeptide antimicrobials (Sofos et al., 1998). Lysozyme, a lytic enzyme extracted from egg whites, is an example of this class of antimicrobials. Other animal source enzymes, such as peroxidases and oxidases, and chelators, such as transferrins and lactoferrin, can inhibit microorganism growth as well (Payne et al., 1994; Branen and Davidson, 2000). Microorganisms themselves are also an abundant source of naturally occurring peptides that effectively inhibit the growth of a wide variety of foodborne pathogens and spoilage microorganisms. The most promising of these are produced by members of the *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Propionibacterium* species.

Limitations of Available Food Antimicrobial Systems

A report published by Sofos et al. (1998) identified the following factors as major reason for the failure to implement widespread use of antimicrobials: (a) the efficacy of

currently available antimicrobials in foods is low, (b) suitable antimicrobial delivery systems for commercial product formulations have not been developed, (c) large-scale extraction, isolation and production processes have not been pursued and (d) the toxicology and safety in food formulations has not been properly evaluated. Low antimicrobial efficacy in actual food systems is the primary restriction on the extensive use of these substances by the food production industry. At present, large quantities of traditional and naturally occurring food antimicrobials are needed to achieve even moderate reductions in growth rates of pathogenic organisms in food products (Davidson, 2001). Large quantities do not only increase production costs considerably, they also negatively affect the sensory properties of the product (Sofos et al., 1998).

The effectiveness of traditional antimicrobials is limited by pH and food component interactions. For example, organic acids are only effective at low pH due to the fact that their antimicrobial activity is attributed to the undissociated acid form (Davidson, 2001). Many of the compounds are also partially hydrophobic which causes them to interact with food lipids, making them less available for inhibition of microorganisms. A limitation of phenolic phytochemicals from food-grade herbs is the wide variation in their water-solubility characteristics. Thymol, the major antimicrobial in thyme, is primarily hydrophobic and therefore insoluble in water, unlike simple hydroxylated phenolic acids which are primarily hydrophilic and therefore soluble in water. Rosmarinic acid, another major phytochemical compound in thyme, is only partially soluble in cold water, but is fully soluble in a solution of hot water and ethanol. The plant and animal tissue from which we derive our food contains a variety of different microenvironments. These microenvironments may be polar (*e.g.,* aqueous solutions),

non-polar (*e.g.,* fat cells) or amphiphilic (*e.g.,* membranes). Thus, for a phenolic compound to exhibit beneficial properties in a plant, it must be located in a microenvironment where it is chemically active. The phenolic compound, for example, may need to be located at the interface between the oil and water regions to demonstrate antioxidant or antimicrobial activity (Coupland and McClements, 1996). Many of the key phenolic metabolites from herbs are ineffective as antimicrobials or antioxidants in foods because they have yet to be successfully incorporated into the appropriate microenvironment.

Similar limitations are observed when using protein or phospholipid based antimicrobials such as lysozyme and nisin. These compounds may also readily absorb at interfaces that are present in multiphase, multicomponent food systems, making them unable to interact with target microorganisms. Furthermore, chemical interactions may take place that lead to structural and functional changes in the antimicrobials that render them inactive. This chemical and physical instability, as well as the thermodynamically driven accumulation of the compounds in certain regions of the food product are key problems that reduce the efficacy of these otherwise potent compounds in foods.

Encapsulation and Carrier Systems To Overcome Antimicrobial Deficiencies

For many years the pharmaceutical industry has studied the encapsulation or surface adsorption of a variety of chemical compounds as a way of overcoming the difficulties involved in their delivery and their potential for causing unwanted side effects. By encapsulating or surface adsorbing the compound, it can be protected from degradation and release a stronger dosage in a site specific manner that increases the

potency of the drug while decreasing the amount needed. The use of a carrier/encapsulation systems allows for breakdown of the particles and release of the encapsulated compound in a time dependent manner without the production of toxic breakdown products.

The use and delivery of many compounds in food products have the same inherent difficulties. Many compounds that give desirable flavors or structural properties to a product are unstable in the conditions that exist within the product or processing and storage conditions to which the product is exposed. These compounds can undergo undesirable reactions with other ingredients or breakdown at high temperatures, high or low pH, or undergo hydrolytic breakdown via moisture in the product. The use of antimicrobials that inhibit the growth of unwanted microorganisms in foods is also difficult due to the nature of the product and the antimicrobial. Food antimicrobials, like nisin and lysozyme, are hydrophobic and as such end up sequestered in the lipid component of the food. This drastically increases the amount of antimicrobial needed to show growth inhibition levels exhibited in laboratory growth medium. Encapsulation of these compounds in nano-sized particles or adsorption to their surfaces may allow some of these difficulties to be overcome.

General Use of Encapsulation in the Food Industry

Though the encapsulation of antimicrobial compounds for delivery in a food product has not yet been fully examined, the encapsulation procedure itself has been used in the food industry for many years. Ingredient encapsulation has been examined to protect food constituents against damaging reactions like hydrolysis and oxidation, to

enhance flavors by prolonging or controlling their release, to protect ingredients from damaging processing or cooking temperatures, and to mask the odor and flavor of nutritionally desirable compounds that have a less than desirable flavor profile (Dziezak, 1988). Food applications for these encapsulated ingredients encompass a wide variety of products, including but not limited to such varied products as dehydrated fruit juices, baked goods, meat products, and chewing gum. For example, a major concern in the production of fruit juice powders and concentrates is the loss of volatile flavor components during the dehydration process. By producing a cold pressed citrus oil encapsulated in a modified lipophilic starch, maltodextrin, or sugar, the desired flavor can be reintroduced into the powdered juice and give the resulting reconstituted juice a high percentage of the flavor found in fresh fruit juice (Bangs and Reineccius, 1990; Kopelman *et al.*, 1977a; Anandaraman and Reineccius, 1986; Andres, 1977; Bhandari *et al.*, 1992; Schulz *et al.* 1956). Kopelman *et al.*, (1977b) produced a reconstituted orange juice that retained 75% of the initial volatiles and water soluble aroma essences of the fresh juice by encapsulating them in a maltodextrin sucrose carrier. Besides enhancing juice flavor, encapsulation also increases the shelf life of the volatile flavor components by protecting them against hydrolysis and oxidation (Anandarman and Reineccius, 1986; Bhandari *et al.* 1992).

The beneficial effects of ingredient encapsulation are not limited to flavor constituents. Ingredients desired for their functional properties or nutritional value can often lead to instability in the food product in which they are used. Food acids are an important food constituent due to their use as flavoring agents, acidulants, preservatives, and texture modifiers. The use of food acids is problematic, however, due to their

hygroscopic nature, which causes caking in powdered products, and their undesired reactivity with flavor and color components. The outer coating provided via encapsulation sequesters the acid, protecting it from water absorption and unwanted reactions with other ingredients, while protecting the beneficial aspects of the acid by releasing it into the product when it is most desired (Werner, 1980; Ciliberto *et al.*, 1981). Vitamin C, when used in baked goods, can react with other compounds, including iron and cinnamon, producing unwanted reaction products, and can cause yeast to begin producing carbon dioxide earlier than desired for proper product rising. By encapsulating the vitamin C, unwanted reactions are avoided and the vitamin can be released when desired via time, heat, pH, or enzyme activity (Andres, 1977). Encapsulation can be used to protect the taste and appearance of a product, like meat, that would normally be altered by cooking or processing (Hoashi, 1986; Shahidi and Pegg, 1991). Hoashi (1986) attempted to replace the meat flavors lost in juices that escape during the cooking of meat products through the addition of calcium alginate gel encapsulated meat stocks and soups that burst and release flavor when the product is chewed. Shahidi and Pegg (1991) stabilized the color of freshly cut meat via treatment with cooked-cured meat pigments encapsulated in food grade carbohydrates. The color of the treated meat was comparable to meat treated with nitrite, but without the fear of the production of carcinogenic nitrosamines. Encapsulation can also be used to protect against some unwanted flavors that are associated with the use of nutritionally valuable ingredients. Thiamine, which gives food products a fishy, yeasty odor, was successfully encapsulated in a matrix of cellulose derivatives and hydroxylated lipids which allowed

use of the nutrient in a bioavailable form without the less than desirable flavor and odor (Hall *et al.*, 1980).

In some cases, one food product will receive a great deal of attention from those who encapsulate ingredients, as is the case with chewing gum. The goal of using encapsulation in chewing gum is twofold; providing stability to the relatively unstable sweeteners used in the gum whilst prolonging their sweet flavor (Cea *et al.*, 1983; Wei *et al.* 1986; Yang *et al.*, 1988; Schobel *et al.*, 1986; Cherukuri *et al.*, 1989; Cherukuri *et al.*, 1990; Hoashi, 1989; Levine *et al.*, 1992). Aspartame, the non-caloric sweetener of choice in many chewing gums due to the lack of a bitter aftertaste often associated with the use of certain artificial sweeteners, is unstable at high temperatures and prone to hydrolytic breakdown. Several encapsulation methods have been proposed to overcome these shortcomings. Cea, *et al.* (1983) proposed a variety of wall materials including cellulose ethers or esters, starches, gums, gelatin, vinyl polymers, and zeins to protect the aspartame. Both low and high molecular weight polyvinyl acetate in combination with a hydrophobic plasticizer or an emulsifier have been proposed as encapsulation medium (Yang, 1988; Cherukuri *et al.*, 1989). The addition of the emulsifier is hoped to further protect the encapsulated sweetener by retarding the hydrolysis of the shell material (Cherukuri *et al.*, 1989). Schobel *et al.* (1986), on the other hand, proposed a hydrophobic polymer and hydrophobic plasticizer for similar reasons. Wie *et al.* (1986) proposed a matrix of encapsulated sweetener in an elastomer, elastomer solvent, and wax shell would be best to prolong sweetness. A second encapsulation step, which would provide an outer coating to the layer in which the aspartame is encapsulated, was

suggested to improve stability and flavor release characteristics (Cherukuri *et al.*, 1990; Levine *et al.*, 1992).

Metal Nanoparticles as Novel Carrier Systems

Metal nanoparticles, constructed from metals such as gold, silver, and magnetic metals like iron oxide, have many potential uses in the biomedical field. The antimicrobial activity of silver has long been utilized in the treatment of wounds and during surgical procedures to prevent bacterial infections (Fox Jr. *et al.*, 1974; Bosetti *et al.*, 2002; Alt *et al.*, 2004). The antimicrobial activity of silver is dependent upon it being present in its ionic form. Silver ions interact with the cell membrane, competing with other compounds for binding sites, and internal compounds, particularly sulfur and phosphorous containing compounds, like DNA, which becomes condensed, preventing replication (Brown and Anderson, 1968; Doyle *et al.*, 1980; Feng *et al.*, 2000; Morones *et al.*, 2005). Silver nanoparticles able to produce ionic silver also demonstrate antimicrobial activity (Lee and Jeong, 2004; Morones *et al.*, 2005; Cho *et al.*, 2005; Sondi and Salopek-Sondi, 2004). The apparent lack of toxicity of silver and silver ions combined with this antimicrobial activity makes incorporation of silver nanoparticles in bone cement and surgical devices a tool in combating nosocomial infections (Berger *et al.*, 1976; Bosetti *et al.*, 2002; Alt *et al.*, 2004; Lee and Jeong, 2004). Silver nanoparticles have also shown antimicrobial activity against HIV-1 via post infection reduction in T-cell apoptosis through an unknown mechanism (Sun *et al.*, 2005).

Gold nanoparticles also have a variety of potential uses, most dealing with the interaction between the functional group attached to the nanoparticle and another

molecule. Gold nanoparticles can be used gain insight into the mechanism by which a biochemical system functions, as seen with the binding of flavin, a cofactor in the flavoenzyme system (Bayir *et al.*, 2006). Binding the cofactor reduces it reduction potential, altering activity in the system. IgG molecules attached to gold nanoparticles aid in the characterization of the interaction between the molecule and a target pathogen (Ho *et al.*, 2004). Attaching a molecule to a gold nanoparticle can alter its stability. Cytochrome c, when bound to mecapto-undecanoic acid functionalized gold nanoparticles increases its susceptibility to proteolysis (Worrall *et al.*, 2006). Attachment can also serve to strengthen a molecule, as in the case of stabilizing chymotrypsin at air-water interfaces, protecting DNA from physical and enzymatic degradation, and stabilizing tetraaspartate peptide in water (Verma *et al.*, 2004; Han *et al.*, 2006a; Jordan *et al.*, 2006). The ability to bind DNA to functionalized gold nanoparticles may provide a vector for gene delivery for the purpose of gene therapy (Han *et al.*, 2006a; Han *et al.*, 2006b; Goodman *et al.*, 2006). Han *et al*. (2006) showed that following binding, DNA can subsequently be released from the nanoparticle, in this case with light, and achieve a high level of transcription. Gold nanoparticles can be used to exert control over enzymatic systems, whether by competing with the substrate for a binding site or by controlling the mechanism by which the enzyme regulates itself (You *et al.*, 2006; Bayraktor *et al.*, 2006).

Several useful applications have also been devised for magnetic nanoparticles. The use of small magnetic particles has been examined for the cancer treatment known as hyperthermia, the raising of the temperature of cancerous cells to increase their susceptibility to chemotherapeutic agents. A magnetic field can be used to cause the

nanoparticles to oscillate, causing them to generate heat. Smaller particles are able to generate greater heat in a given amount of time than larger ones (Bonder *et al.*, 2006). Magnetic nanoparticles show potential as recoverable and reusable enzyme carriers (Tsang *et al.*, 2006). Attachment of β lactamase to iron oxide nanoparticles was achieved without blocking the enzyme active site, allowing the attached enzyme to be as accessible as a free enzyme, a trait that is lacking when enzymes are attached to other solid substrates (Tsang *et al.*, 2006). Magnetic nanoparticles can also be used as contrast agents in magnetic resonance imaging (MRI), a powerful medical diagnostic tool (Mornet *et al.*, 2006).

Nanoparticle Applications

Nanoparticles as Antimicrobial Carrier Systems

Nanoparticle systems can be used to overcome some of the difficulties inherent in using antimicrobials to treat bacterial and parasitic infection. By encapsulating the antimicrobial, the need to flood the body with the compound while trying to maintain the delicate balance between minimum effective dose against the infection and maximum safe dose for the infected organism is removed (Langar and Peppas, 1981). Nanoparticles can be designed to deliver the antimicrobial compound to the site of the infection. This is particularly useful in the case of intracellular pathogens such as *L. monocytogenes* and *Salmonella* Typhimurium. Encapsulated antibiotics can be endocytosed by the cell, allowing for the delivery of antibiotics to the interior of phagocytic cells, a place most could normally not reach on their own.

Nanoparticles synthesized using the polymer polyacrylamide have shown promise in the treatment of intracellular pathogens. Polyacrylamide nanoparticles show a high binding capacity to several known antibiotics and are easily taken up by phagocytic cells (Courvreur *et al.*, 1979). This compound shows no toxicity at the cellular or whole organism level, only causing cell damage at levels above 1% (~$2x10^4$ particles/cell) (Kante *et al.*, 1982). Once introduced into an organism, polyacrylamide nanoparticles are quickly cleared from the bloodstream and accumulate in the liver (42.9%), the kidneys (5.42%) and the bone marrow (1.96%) (Krause *et al.*, 1985). The majority of the particles in the liver accumulate in the Kuppfer cells (Lenaerts *et al.*, 1984). Thus, this polymer meets the criteria set forth by Oppenheim (1981), they are non-toxic, biodegradable, and accumulate at the desired site of action.

The ability of the nanoparticle to enter cells in which intracellular microorganisms have taken up residence allows for the release of antibiotics into areas that were formerly off limits to them. By encapsulating ampicillin in polyisohexylcyanoacrylate (PIHCA) nanoparticles, Youssef *et al.* (1988) increased its therapeutic index 20 fold against a chronic *L. monocytogenes* infection in athymic nude mice. The liver was completely sterilized after two 0.8 mg injections of PIHCA encapsulated ampicillin. A 0.8 mg dose of ampicillin bound in PIHCA nanoparticles suppressed mortality in mice with acute fatal salmonellosis (Fattal *et al.*, 1989). This represents a 120-fold increase in the therapeutic index of ampicillin. For unbound ampicillin, three 32 mg doses were needed to achieve the same results. Complete sterilization of the liver was not achieved in the case of the *Salmonella* infection. Other

beta lactam antibiotics, including benzathine penicillin G, have also been encapsulated in nanoparticles and shown *in vitro* antimicrobial activity (Santos-Magalhaes *et al.*, 2000).

The cytotoxicity of the compound being used must be taken into affect when designing a nanoparticle system. Fawaz *et al.* (1997) encapsulated the synthetic drug ciprofloxacin in polyisobutylcyanoacrylate (PIBCA) nanoparticles. Ciprofloxacin is a member of the fluoroquinolones, antimicrobial agents believed to work via the inhibition of DNA gyrase. The hydrophobicity of the compounds prevents their effectiveness against intracellular pathogens. When testing these nanoparticles against a *Mycobacterium avium* infection in a human macrophage culture, it was found that though nanoparticle associated ciprofloxacin was more effective than unbound ciprofloxacin, it was much less so than anticipated (Fawaz *et al.*, 1998). This was most likely due to the cyotoxicity of the PIBCA itself at ciprofloxacin concentrations greater than 8 μg/ml (80 μg/ml PIBCA) (Fawaz *et al.*, 1998).

Nanoparticles as Vaccine Carriers

To overcome the difficulties and limitations of parental vaccine delivery, micro- and nanoparticles have received extensive study as an alternate method of vaccine delivery. Delivery of vaccine loaded nanoparticles to the mucosa or skin are viable alternatives to intramuscular injection. By encapsulating the vaccine in a biodegradable polymer, it is possible to protect it from the harsh conditions in the stomach and thus deliver a vaccine orally. Kofler *et al.* (1997) orally administered 800 nm PLGA nanoparticles loaded with LW 50020, an immunomodulator containing lysates of 7 common respiratory pathogens, to BALB/c mice. The particles were taken up by the

Peyer's Patches and mesenteric lymph nodes that line the small intestine. The resulting immune response, consisting of increased levels of IgA and IgG, was significantly higher than the immune response following administration of comparable levels of free LW 50020. The uptake of orally administered chitosan nanoparticles by Peyer's Patches has also been demonstrated (van der Lubben, 2001). Oral delivery of the vaccines allows boosting of the immune response in the mucosa, the immune systems first line of defense against infection.

The ability to encapsulate and deliver protein and DNA vaccines has also been demonstrated. Radio labeled bovine serum albumin (BSA) was used to demonstrate the uptake of protein from nanoparticles in the small intestine (van der Lubben, 2001). Bivas-Benita *et al.* (2003) administered chitosan encapsulated *Toxoplasma gondii* GRA1 protein and DNA vaccines orally to mice. The mice showed increased production of IgG1 and IgG2a, immunoglobulins associated with an immune boosting TH2 response, as opposed to a protective TH1 response. Oral administration of a plasmid encoding 8 epitopes from *Mycobacterium tuberculosis* caused increased IFN-γ secretion in mice (Bivas-Benita *et al.*, 2004). The sub-400 nm particles elicited a stronger response than intramuscular vaccination with the plasmid. In order to encapsulate DNA in PLGA nanoparticles, it is necessary to alter the charge of the polymer to avoid the use of harsh organic acids in the encapsulation procedure (Kumar *et al.*, 2004). A blend of chitosan and PVAL was used to give a positive charge to the normally negatively charged nanoparticles.

Nanoparticles have also proved useful in the relatively new area of vaccine delivery through the skin. Chitosan nanoparticles (200nm) with internally encapsulated or surface attached plasmid DNA (pDNA) were topically applied to mice (Cui *et al.*, 2001). The mice showed increased production of luciferase, which is encoded on the plasmid, after 24 hours at a higher level than in mice treated with naked pDNA. The IgG titer of the nanoparticle treated mice increased after 28 days, significantly (32 fold) more than in mice treated with naked pDNA. Kohli and Alpar (2004) determined that the charge density determined the ability of nanoparticles to move through the skin. Particles with a large surface area (50 nm) or a large number of charged groups (500 nm) were successful in crossing the skin.

Nanoparticles as Cancer Treament Vehicles

Paclitaxel is a strong anti-tumor agent. Its cytotoxicity is due to its ability to promote the formation of microtubules in cells (Rowinsky *et al.*, 1990). By disrupting the cells ability to control the growth and placement of microtubules, paclitaxel disrupts, among other things, the shape, motility, and ability to undergo mitosis (Rowinsky *et al.*, 1990). The low water solubility of this compound has led to its delivery as Taxol®, a nonaqueous formulation containing the compound and the dehydrated alcohol Cremophor EL®. There have been several documented cases of hypersensitivity to this compound. Many researchers have turned to nanoparticles to overcome this limitation.

Several research groups have produced nanoparticle encapsulated paclitaxel. Mu and Feng (2002, 2003) produced paclitaxel nanoparticles using the polymer PLGA. The resultant nanoparticles showed a biphasic release pattern, with a small burst release

followed by a steady release (11% released after 30 days). Burt *et al.* (1995) encapsulated the drug in a 50:50 mixture of PLA and ethelyene-vinyl acetate (EVA). This copolymer formulation had a high loading capacity (95-100% loading at 100 and 1000 μg) and a slow release rate (15% released after 50 days). Drug loaded PLA:EVA nanoparticles (6μg drug/ml polymer) showed localized activity in a chick chorioallantoic membrane (CAM) model. The effectiveness of these nanoparticles *in vivo* would be lowered, however by the fact that they would be quickly removed from the blood stream as has been documented repeatedly with nanoparticles made with PLA as the polymer. In order to maintain a high level of nanoparticles in the bloodstream, paclitaxel was also encapsulated in PLA-MPEG nanoparticles (Dong *et al.*, 2004). The addition of MPEG to the nanoparticle surface increased the release rate dramatically (90% released over 14 days), however.

In vitro studies of PLGA encapsulated paclitaxel have been undertaken. When tested against a human small cell lung cancer cell line (NCI-H69) showed that paclitaxel loaded PLGA nanoparticles showed a higher rate of activity than paclitaxel alone or as Taxol® (Fonseca *et al.*, 2002). A 25 μg/ml dose of Taxol® reduced the cancer cell viability 100%. Paclitaxel loaded nanoparticles showed the same results with a dose of 2.5 μg/ml, which represents a ten fold increase in efficacy. The same amount of free paclitaxel only reduces the viability by 70%.

Summary of Literature Review

There are a wide range of antimicrobials available to control the growth of pathogenic bacteria, many of which are naturally found in foods or produced by

microorganisms utilized in food production. There exists an inherent difficulty in applying these compounds to food, however. Nanoparticles, produced using compounds ranging from food grade chemicals to biodegradable polymers to metals, have long been used by the pharmaceutical industry to overcome similar difficulties in the delivery of therapeutic agents. Though nanoparticles as a delivery vessel have been used to some extent to deliver or protect food ingredients, no work has been done to test their viability as delivery agents for food antimicrobials. By using nanoparticles to deliver the antimicrobial compound into the food product, it may be possible to overcome the limitations that have deterred the use of such compounds in the past.

CHAPTER 3

MATERIALS AND METHODS

Materials

Carboxyl-terminated silver nanoparticles were purchased from Nanohorizons (University City, PA). Nisin, corn oil, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Sigma Chemical Co. (St. Louis, MO). 200 Bloom Porkskin 8 mesh gelatine was donated by Gelita (Souix City, IA). *L. monocytogenes* strains J1-225, J2-020, J1-177, and C11-115 were obtained from the International Life Sciences Institute North America *Listeria monocytogenes* Strain Collection (Fugett *et al.*, 2006). *Escherichia coli* strains 35150, 43895, 51685, and 700599 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Tryptic soy broth (TSB), tryptic soy agar (TSA), and yeast extract were purchased from BD Diagnostics (Franklin Lakes, NJ) Double distilled and deionized water was used in all experiments.

Attachment of Antimicrobials to Silver Nanoparticles

Nisin and lysozyme were attached to the nanoparticles according to the method developed by Fischer *et al.* (2003). Briefly, the antimicrobial and the silver nanoparticles were incubated together in distilled water at room temperature for 16 hours. For the purpose of antimicrobial testing, the concentration ratio (in μg/ml) of nisin or lysozyme to silver was varied (1:0.167, 1:0.25, 1:0.5, 1:1, and 2:1) during the drug loading step. The concentration of nisin (5 μg/mL) or lysozyme (4 μg/mL) remained constant in each ratio while the concentration of silver nanoparticles varied. Following antimicrobial loading, the size and charge of the antimicrobial/nanoparticle combination was measured

using a Nano Series Zetasizer (Malvern Instruments). Attachment of the antimicrobials to the gold nanoparticles will be attempted using the same protocol.

ζ-Potential

The ζ-potential was determined by placing 1 ml samples in a disposable cuvette. The cuvette was inserted into the measurement chamber of a particle electrophoresis instrument (Nano Series Zetasizer, Malvern Instruments, Worcestershire, UK). The ζ-potential was then determined by measuring the direction and velocity that the particles moved in the applied electric field. The Smoluchowsky mathematical model was used by the software to convert the electrophoretic mobility measurements into ζ-potential values.

Particle Size Determination

The particle size distribution of nanoparticles was determined using a dynamic light scattering technique (Nano Series Zetasizer, Malvern Instruments, Worcestershire, UK). 1.0 ml samples, were placed in disposable cuvettes and inserted into the measurement chamber. The Sauter mean diameter (d_{32}) of the samples was determined from the particle size distribution as:

$$d_{32} = \frac{\sum d_i^3 N_i}{\sum d_i^2 N_i} \quad (1)$$

where N is the number of particles that had a particle diameter of d.

Bacterial Growth Conditions

Four strains of *E. coli* (F4546, H1730, E0019, and 932) and four strains of *L. monocytogenes* (Scott A, 310, 108, and 101) obtained from the University of Tennessee Department of Food Science and Technology culture collection were maintained at 4°C on tryptic soy agar (TSA) or tryptic soy agar with yeast extract (TSAYE) respectively. Tryptic soy broth (TSB) or tryptic soy broth with yeast extract (TSBYE) was inoculated with *E. coli* or *L. monocytogenes* and incubated at 32°C for 24 hours and subcultured for an additional 18 hours. Cultures were diluted to approximately 10^4 CFU/mL prior to use.

Antimicrobial Assay

The antimicrobial activity of the nanoparticles and nanoparticle/antimicrobial combinations was determined via a microdilution assay in 96 well microtiter plates. 120 µL of the bacteria in double strength TSB or double strength TSBYE and 120 µL of the silver nanoparticle solution were added to the wells. Antimicrobial only controls and silver nanoparticles were tested via a 12 fold 1:1 dilution which allowed testing at concentration ranging from 215 µg/mL to 0.1µg/mL. Silver nanoparticle/antimicrobial combinations were tested via a 5 fold 1:1 dilution series. Microtiter plates were incubated at 32°C and 20°C. Optical density readings were taken at 630 nm (OD_{630}) at hours 0, 3, 6, 12, 24, and 48 using a BIO-TEK Automated Plate Reader. Minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no bacterial growth was observed.

Emulsion Preparation

An emulsifier solution was prepared by dispersing 0.5 wt % pork gelatin into water and gently heating to insure gelatin went into solution. The solution was stirred overnight to insure hydration of the protein. A primary emulsion was prepared by homogenizing 2 wt % corn oil with 98 wt % aqueous emulsifier solution in a blender followed by three passes through a microfludizer (Microfluidics 110L, Microfluidics Corp., Newton MA) at 9000 psi. The pH of the emulsion was adjusted to pH 7 using NaOH and HCl solutions. An aqueous nisin solution was prepared at a concentration of 5120 μg/ml and the pH was adjusted to pH 7 using an NaOH solution. The primary emulsion was diluted with the aqueous nisin solution to form secondary emulsions over a range of nisin concentrations.

Antimicrobial Assay

Four strains of *E. coli* (35150, 43895, 51685, and 700599) and four strains of *L. monocytogenes* (J1-225, J2-020, J1-177, and C11-115) were maintained at 4°C on TSA or tryptic soy agar with yeast extract (TSAYE) respectively. TSB or tryptic soy broth with yeast extract (TSBYE) was inoculated with *E. coli* or *L. monocytogenes* and incubated at 32°C for 24 hours and subcultured for an additional 18 hours. Cultures were diluted to approximately 10^4 CFU/mL prior to use. The minimum inhibitory concentration (MIC) of the double layered emulsion was determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 1990) agar dilution method. TSAYE was autoclaved and allowed to cool in a water bath until it reached a temperature of 50 ºC. The double layered emulsion, the primary emulsion, and free nisin were added to the tempered agar at concentrations ranging from 128 μg/ml to 0.5 μg/ml.

Bacteria were applied to the plate via spot inoculation. Briefly, plates were inoculated with three 10 μL drops containing approximately 10^4 CFU/ml of the strains being tested. The plates were incubated at 32 ºC for 24 hours. The MIC was recorded as the lowest concentration at which no growth was observed.

CHAPTER 4

ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES CARRYING NISIN AND LYSOZYME AGAINST ESCHERICHIA COLI O157:H7 AND LISTERIA MONOCYTOGENES

Abstract

The efficacy of nisin and lysozyme attached to silver nanoparticles against strains of *Listeria monocytogenes* (Scott A, 310, 108, and 101) and *Escherichia coli* (F4546, H1730, E0019, and 932) was investigated. The effects of varying the antimicrobial to nanoparticle ratio and the effect of temperature on efficacy were tested. Silver nanoparticles, which have been shown to have antimicrobial activities elsewhere, did not inhibit growth of any strain tested at any temperature. The efficacy of nisin, a strong inhibitor of *L. monocytogenes* growth, was increased when attached to the nanoparticles. The antimicrobial/nanoparticle combination showed an increased efficacy at lower temperatures compared to growth at 32°C. Lysozyme, also a strong inhibitor of *L. monocytogenes* growth, showed no antimicrobial activity against any strain when attached to the nanoparticles. Neither antimicrobial inhibited *E. coli* growth when attached to the nanoparticles. The change in antimicrobial activity of both nisin and lysozyme when attached to nanoparticles is attributed to the mechanism by which the antimicrobial inhibits growth.

Introduction

Food borne pathogens pose a significant health risk to consumers and a potential for lost capital to the food industry. Though food producers go to great lengths to prevent

microbial contamination of their products, the nature of the product and the growth capabilities of the organisms often defeat even their best efforts. In 2005, nearly 17,000 cases of food borne illness were identified in the FoodNet surveillance population (roughly 15% of the US population) (Anonymous, 2006c). *Listeria* infections, though lower than during the period lasting from 1996-1998, are up compared to the lowest known number of cases four years ago (Anonymous, 2006c). Recent massive recalls of deli meats due to *L. monocytogenes* contamination (Anonymous, 2002) and fresh bagged spinach due to *E. coli* contamination (Anonymous, 2006d) show that continuing efforts are needed to insure the safety of the food supply.

There are a number of antimicrobials available that inhibit the growth of food borne pathogens. Several of these antimicrobials, like nisin and lysozyme, have GRAS (generally recognized as safe) status that allows for their use in certain food products. Unfortunately, these antimicrobials are far less effective in heterogeneous food products than they are in homogenous microbiological laboratory media. Nisin activity, for example, has been shown to be greatly reduced in several meat products (Murray and Richard, 1997; Aasen *et al.*, 2003; Grisi and Gorlach-Lira, 2005; Ghalfi *et al*., 2006; Stergiou *et al.*, 2006). Interfering food constituents include glutathione (Stergiou *et al.*, 2006), high salt concentration (Boziaris and Nychas, 2006), nitrates (Ghalfi *et al.*, 2006), fat content (Benech *et al.*, 2002b; Ghalfi *et al.*, 2006), and proteolytic enzymes (Murray and Richard, 1997; Aasen *et al.*, 2003).

In order to effectively use these antimicrobials in a food product, it is necessary to deliver them in a manner by which the antimicrobial can interact with the microorganism

without interference from the product itself. Several novel delivery systems have been studied for this purpose. The first type of delivery system involves the incorporation of antimicrobials into films that are then applied to a food surface. Nisin has been incorporated into edible cellulose films (Coma *et al.*, 2001), corn zein films (Hoffman *et al.*, 2001), and polyethylene based plastic films (Siragusa *et al.*, 1999). Antimicrobial activity was maintained in each case. The second delivery method involves the use of additional compounds as carriers of the antimicrobials. Encapsulation in liposomes has been shown to increase the efficacy of nisin in laboratory media (Were *et al.*, 2004) and in cheddar cheese (Benech *et al.*, 2002a). Surfactant micelles have also been shown to increase the antimicrobial efficacy of the essential oil components eugenol and carvacrol over a range of temperatures and pH (Gaysinsky *et al.*, 2005a; Gaysinsky *et al.*, 2005b).

The pharmaceutical industry faces similar challenges in the delivery of antimicrobial compounds into their patients. Compounds must be delivered in a dose potent enough to kill the microorganism, but mild enough to cause no harm to the patient (Langar and Peppas, 1981). One strategy used to overcome this difficulty is the attachment of therapeutic agents to nanoparticles (Langer and Peppas, 1981; Oppenheim, 1981; Soppimath *et al.*, 2001). A wide range of starting materials are used to construct the nanoparticles, from biodegradable polymers like polylactic acid (PLA) (Soppimath *et al.*, 2001) to metals, like silver or gold (Sondi and Salopek-Sondi, 2004; Aymonier *et al.*, 2002).

Nanoparticles have seen limited use in the food industry as a means to deliver ingredients into food products (Shahidi and Han, 1993). Encapsulation methods can be

used to protect volatile flavor components that are often lost when a product is heated or dried (Kopelman *et al.*, 1997) or to allow an ingredient, like artificial sweeteners in gum, to be released slowly over time (Cherukuri *et al.*, 1989). It is the goal of this study to develop a nanoparticle delivery system for the food antimicrobials nisin and lysozyme. To this end, we will use nanoparticles made from silver, which in and of its self is known to have antimicrobial properties (Brown and Anderson, 1968; Zhao and Stevens, 1998; Sondi and Salopak-Sondi, 2004; Morones *et al.*, 2005)

Materials and Methods

Attachment of antimicrobials to silver nanoparticles

Carboxyl-terminated silver nanoparticles were purchased from Nanohorizons (University City, PA). Nisin and lysozyme (MP Biomedicals, Aurora, OH) were attached to the nanoparticles according to the method developed by Fischer *et al.* (2003). Briefly, the antimicrobial and the silver nanoparticles were incubated together in distilled water at room temperature for 16 hours. For the purpose of antimicrobial testing, the concentration ratio (in μg/ml) of nisin or lysozyme to silver was varied (1:0.167, 1:0.25, 1:0.5, 1:1, and 2:1) during the drug loading step. The concentration of nisin (5 μg/mL) or lysozyme (4 μg/mL) remained constant in each ratio while the silver concentration was varied. Following antimicrobial loading, the size of the antimicrobial/nanoparticle combination was measured using a Nano Series Zetasizer (Malvern Instruments).

Bacterial Growth Conditions.

Four strains of *E. coli* (F4546, H1730, E0019, and 932) and four strains of *L. monocytogenes* (Scott A, 310, 108, and 101) obtained from the University of Tennessee

Department of Food Science and Technology culture collection were maintained at 4°C on tryptic soy agar (TSA) or tryptic soy agar with yeast extract (TSAYE) (Difco, Detroit, MI) respectively. Tryptic soy broth (TSB) or tryptic soy broth with yeast extract (TSBYE) (Difco, Detroit, MI) was inoculated with *E. coli* or *L. monocytogenes* and incubated at 32°C for 24 hours and subcultured for an additional 18 hours. Cultures were diluted to approximately 10^4 CFU/mL prior to use.

Antimicrobial Assays

The antimicrobial activity of the nanoparticles and nanoparticle/antimicrobial combinations was determined via a microdilution assay in 96 well microtiter plates. 120 µL of the bacteria in double strength TSB or double strength TSBYE and 120 µL of the silver nanoparticle solution were added to the wells. Antimicrobial only controls and silver nanoparticles were tested via a 12 fold 1:1 dilution which allowed testing at concentration ranging from 215 µg/mL to 0.1µg/mL. Silver nanoparticle/antimicrobial combinations were tested via a 5 fold 1:1 dilution series. Microtiter plates were incubated at 32°C and 20°C. Optical density readings were taken at 630 nm (OD_{630}) at hours 0, 3, 6, 12, 24, and 48 using a BIO-TEK Automated Plate Reader. Minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no bacterial growth was observed. All assays were run in duplicate.

Results

Size of Silver Nanoparticles with Attached Antimicrobials.

The sizes of the silver nanoparticles with attached nisin and lysozyme are shown in Table 1. The silver nanoparticles without the attached antimicrobial range in size from

20-30 nm. The nanoparticles with attached nisin ranged in size from around 280-340 nm. The nanoparticles with attached lysozyme were smaller, ranging in size from 45-160 nm. Both antimicrobials showed an increase in size as the concentration ratio decreased from 6:1 to 2:1 (290 to 337 nm with nisin, 45 to 160 nm with lysozyme), a decrease in size from 2:1 to 1:1 (337 to 283 nm with nisin, 160 to 60 nm with lysozyme), and an increase in size from 1:1 to 1:2 (283 to 329 nm with nisin, 60 to 140 nm with lysyozyme).

Antimicrobial Activity of Silver Nanoparticles

Figure 1 shows the growth of *L. monocytogenes* Scott A (A) and *E. coli* strain H1730 (B) in the presence of silver nanoparticles at 32°C. No growth inhibition was observed in either strain at silver concentrations up to 215 μg/mL. Both strains showed growth levels comparable to bacteria only controls (data not shown). These results are typical for all strains tested at either temperature.

Antimicrobial Activity of Nisin Attached to Silver Nanoparticles

Table 2 shows the MIC's obtained in the antimicrobial assays performed with nisin and nisin attached to silver nanoparticles. Nisin alone inhibited growth of three strains of *L. monocytogenes* at a concentration of 21 μg/mL, with Scott A being the most resistant strain with an MIC of 41 μg/mL. When loaded, the silver nanoparticles, at the highest concentration, were only loaded with a 5 μg/ml dose of nisin. Any inhibition would therefore be seen as an increase in efficacy. The efficacy of the silver nanoparticle/nisin combination increased against three strains, 101 (Figure 2A), 310 (Figure 2B), and 108. Scott A (Figure 2C) was again the most resistant strain. Nisin is not a strong inhibitor of *E. coli* growth and attachment did not increase its ability to

inhibit growth. There was, however, a reduction in strength of growth seen in strain F4546 (Figure 2D). Reduction of the antimicrobial to nanoparticle ratio caused a decrease in efficacy against three of the four *L. monocytogenes* strains. Only strain 101 did not show decreased efficacy when the nisin to silver ratio was decreased. There was a marked increase in antimicrobial activity of the silver nanoparticles with attached nisin at 20°C against both *L. monocytogenes* and *E. coli* when compared to the activity at 32°C (Figure 3).

Antimicrobial Activity of Lysozyme Attached to Silver Nanoparticles

Lysozyme did not inhibit the growth of *L. monocytogenes* and *E. coli* at high or low temperature when attached to the silver nanoparticles. Lysozyme is a strong inhibitor of *L. monocytogenes* growth. The typical MIC of lysozyme against *L. monocytogenes* was found to be 2.5 μg/mL. Strain 101 was the most susceptible to lysozyme, with an MIC of 0.5 μg/ml. Nanoparticles were prepared with a starting lysozyme concentration of 4 μg/mL, a concentration high enough to inhibit growth in all strains testd. When attached to silver nanoparticles, lysozyme lost the ability to inhibit the growth of these organisms (data not shown). Temperature did not effect the efficacy of the lysozyme/nanoparticle combinations. Lysozyme, which is not a strong inhibitor of *E. coli* growth, did not inhibit growth of any strain of *E. coli* at any antimicrobial to silver nanoparticle ratio tested at either temperature.

Discussion

Silver nanoparticles were chosen as a delivery system for food antimicrobials due to the long known antimicrobial activity of silver (Russell and Hugo, 1994). This

antimicrobial activity has been exploited in the medical profession to prevent infection on burns wounds and in association with surgical implants (Fox *et al.*, 1974; Bosetti *et al.*, 2002; Alt *et al.*, 2004) and has been examined as a packaging implement to reduce bacterial growth in apple juice (Nobile *et al*., 2004). The silver nanoparticles in our study showed no antimicrobial activity against *L. monocytogenes* or *E. coli*.

The antimicrobial activity of silver is dependent on its state. Though the mechanism of action is not completely understood, the antimicrobial activity of silver is attributed to it being in its ionized state. Silver ions being positively charged have been shown to bind to negatively charged compounds such as peptidoglycan, techoic acid, and protein thiol groups, disrupting membrane and protein activity in the cell (Doyle *et al.*, 1980; Beveridge and Murray, 1980; Feng *et al.*, 2000). The silver nanoparticles used in this study, with attached carboxyl group, were not used at a pH which would allow them to exist in an ionic state. Thus the absence of any antimicrobial activity is not a surprise.

Interestingly, Sondi and Salopek-Sondi (2004) showed that silver nanoparticles, regardless of charge, inhibited *E. coli* growth. This activity was limited to solid media. Nanoparticles in liquid media only delayed growth, which resumed as nanoparticles were removed from the media via interaction with dead cellular material. Though no such growth delay was noted in this case, solid media was not used to determine if the liquid media was in fact reducing antimicrobial activity. Panàček *et al.* (2006) demonstrated that silver nanoparticle size effects antimicrobial activity. The relative uniform size of the nanoparticles used in this study did not allow this to be examined.

Lysozyme, an enzyme that occurs naturally in many food products, inhibits the growth of Gram positive bacteria by hydrolyzing the 1, 4 ß-D-linkage between N-acetylhexosamines in the peptidoglycan layer (Proctor and Cunningham, 1988). Lysozyme inhibited *L. monocytogenes* at low concentrations. *E. coli* growth was not inhibited by lysozyme, which is not an effective inhibitor of Gram negative bacterial growth due to the protection provided by lipopolysaccharide in the outer membrane (Ohno and Morrison, 1989). When attached to silver nanoparticles, lysozyme showed no inhibitory activity against any strain of *L. monocytogenes* or *E. coli* at any concentration ratio or temperature tested. This is likely due to the interaction between the enzyme and the nanoparticle blocking or altering the active site of the enzyme in such a way that the enzyme activity is lost. Fischer *et al.* (2002) found that similarly binding the enzyme α-chemotrypsin to gold nanoparticles led to the eventual denaturation of the enzyme.

Like lysozyme, nisin, which is a strong inhibitor of Gram positive bacterial growth, is not effective in controlling the growth of Gram negative bacteria. Herein, nisin did not inhibit the growth of any *E. coli* strain. Attachment of nisin to sliver nanoparticles did not enhance it efficacy against *E. coli* at 32°C. Two strains, E0019 and F4546, were, however inhibited at lower temperatures. A marked increase in efficacy was observed against *L. monocytogenes*. Growth inhibition was observed when silver nanoparticles loaded with as little as 5 μg/mL of nisin, a 4 fold increase in efficacy when compared to nisin alone.

This increased efficacy may also be explainable via the mechanism by which nisin inhibits growth. Nisin inhibits bacterial growth by forming pores in the bacterial

membrane, which causes ATP efflux, reduced intracellular ATP concentration, and a dissipated proton motive force (Winkowski *et al.*, 1996). Pore formation is accomplished via the barrel-stave method (Ojcious *et al.*, 1991). Briefly, the amphiphilic nisin molecule attaches to the membrane through interactions between the negatively charged phospholipid head groups and the positive arginine residues in the nisin molecule. The nisin molecules are pulled into the membrane where they float around until contact with other nisin molecules via hydrophobic interaction causes formation of transient pores (Winkowski *et al.*, 1996). The assumed ability of the silver nanoparticles to deliver a more concentrated dose of nisin molecules to a smaller area of the membrane increases the likelihood of nisin-nisin interaction and thus pore formation in the membrane. Figure 4 demonstrates this proposed mechanism of action. This increased interaction would allow increased efficacy against the bacterium with a smaller initial dose.

Increased efficacy of nisin and nisin/nanoparticle combinations was observed at lower temperatures. This is a commonly observed phenomenon when antimicrobials are used to inhibit *L. monocytogenes* growth at low temperatures. Membrane fluidity and phospholipids content have been shown to affect the efficiency of nisin (Mazzotta and Montville, 1997; Crandall and Montville, 1998; Ming and Daeschel, 1993; Li *et al.*, 2002). Juneja and Davidson (1993) observed similar results with propyl paraben. The ability of *L. monocytogenes* to grow at low temperatures is due, at least in part, to the ability to change the fatty acid composition of the membrane of fluidity, in effect increasing the membrane fluidity (Annous *et al.*, 1997).

Varying antimicrobial to nanoparticle concentrations were used to determine the effect of the nanoparticle charge on antimicrobial activity. Some decrease in efficacy was observed when nanoparticle to antimicrobial concentration increased. It is thought that this is due to repulsion between the negatively charged head groups in the membrane. More experiments will be needed to determine how the antimicrobial is loading on the nanoparticle and in turn interacting with the membrane to fully understand the effects of the nanoparticle delivery. Further studies will also be needed to determine if the increased efficacy demonstrated in this study will occur in an actual food product.

Conclusions

Silver nanoparticles were examined for their ability to deliver the food antimicrobials nisin and lysozyme. Attaching nisin to the nanoparticles increased its efficacy, suggesting that silver nanoparticles are a potential delivery mechanism for this antimicrobial. Lysozyme, however, lost its ability to inhibit microbial growth when attached to the nanoparticles. Further research is needed to characterize the interaction between the antimicrobial, nanoparticle, and microorganism. The ability of this increased efficacy to be duplicated in an actual food product will also be examined.

CHAPTER 5

DOUBLE LAYER EMULSION DROPLETS AS A POTENTIAL DELIVERY VESSEL FOR THE FOOD ANTIMICROBIAL NISIN

Abstract

Layer-by-layer deposition is the process by which alternating layers of positively and negatively charged particles are deposited on a charged surface to form thin film structures. This technique has also proven useful in the building of layers of charged emulsifiers on the surface of emulsion droplets, conferring added protection against aggregation in the face of changes in temperature, pH, salt concentration, and moisture content, as well as improved protection against lipid oxidation. Using the layer-by-layer deposition technique, we created a double layered emulsion in which the second layer was composed of the food antimicrobial nisin. This antimicrobial, though a potent inhibitor of the foodborne pathogen *Listeria monocytogenes* in laboratory media, often proves ineffective in food products due to interactions with food constituents reducing the antimicrobial activity of the molecule. The goal of this project was to use the double-layered emulsion as a delivery system to overcome this deficiency. Binding the nisin to the emulsion droplet, however, decreased its antimicrobial activity substantially, most likely due to strong interactions with the primary protein emulsifier or a reduction in the charge of the nisin molecule itself as part of the attachment process. The addition of EDTA, which has been shown to increase effectiveness of certain antimicrobials against Gram-negative bacteria, had no effect on the efficacy of the nisin loaded emulsion droplets.

Introduction

Decher and Hong (1991a) described layer-by-layer deposition as a technique by which thin films of alternating layers of positively and negatively charged molecules are deposited onto a charged surface via electrostatic interactions. These molecules, whether bipolar amphiphiles or multipolar electrolytes, can be brought onto the charged surface by dipping the surface into aqueous solutions containing the charged molecules (Decher and Hong, 1991a; Decher and Hong, 1991b). The layer-by-layer deposition process has been used to build multilayer films on solid micro- and nanosized particles as well as flat surfaces (Pommersheim *et al.*, 1994; Caruso *et al.*, 1998; Sukorukov *et al.*, 1998; Caruso and Möhwald, 1999a; Caruso and Möhwald, 1999b). This method allows for the building of functionalized surfaces on particles with nanometer-level control over the thickness of the deposited layer (Pommersheim *et al.*, 1994; Caruso and Möhwald, 1999a). One key use of this technique is to design particles that exhibit biological activity, including attachment of antibodies like immunoglobulin G (IgG) and fluorescent labels for immunoassays or the attachment of active enzymes for variety of enymatic reactions (Pommersheim *et al.*, 1994; Yang *et al.*, 2001).

The application of the layer-by-layer technique to emulsion droplets makes this technique potentially useful to the food industry (Guzey and McClements, 2006). In the formation of multiple layered emulsions, a primary emulsion is first created by homogenizing oil, water, and a charged emulsifier, which acts as the first layer. The second layer is added by diluting the primary emulsion in an aqueous solution containing oppositely charged molecules that will attach to the primary charged emulsifier (Ogawa *et al.*, 2003a). The addition of two or more layers to the oil droplet conveys stability

against aggregation in the presence of environmental stresses encountered during the processing of the food product, including changes in temperature, salt concentration, pH, and moisture content (Aoki *et al.*, 2005; Klinkersorn *et al.*, 2005a; Surh *et al.*, 2005). Coating the oil droplets with multiple layers of emulsion droplets has also been shown to slow lipid oxidation (Ogawa *et al.*, 2003; Klinkerson *et al.*, 2005a). The goal of this study was to use the layer-by-layer deposition technique to assemble emulsion droplets with nisin, a generally recognized as safe (GRAS) antimicrobial, as the secondary emulsifier for the purpose of antimicrobial delivery.

Nisin is a bacteriocin, a peptide produced by lactic acid bacteria to inhibit the growth of similar bacteria that compete for their resources, such as the foodborne pathogen *Listeria monocytogenes* (Harris *et al.*, 1989; Ukuku and Shelef, 1997; Ennahar *et al.*, 2000; Mota-Meira *et al.*, 2000). Ethylenediaminetetraacetic acid (EDTA), used in conjunction with several antimicrobials, including nisin, has been shown to have a synergistic effect in regard to the efficacy of the antimicrobial against foodborne Gram-negative pathogens like *E.* coli via disruption of the outer membrane (Jay, 2000; Branen and Davidson, 2004; Lambert *et al.*, 2004). *L. monocytogenes* is problematic to food manufacturers in that it can grow at low temperatures, at high salt concentrations, and at low oxygen levels, nullifying many of the methods by which manufacturers limit the growth of microorganisms in their products (Cole *et al.*, 1990; Grau *et al.*, 1992; Samelis and Metaxopoulus, 1999). Though nisin inhibits the growth of this organism, its use in food products is limited due to the loss of efficacy caused by interactions between nisin and the constituents of the food matrix into which it is introduced (Murray and Richard,

1997; Aasen *et al.*, 2003; Grisi and Gorlach-Lira, 2005; Boziaus and Nychas, 2006; Ghalfi *et al.*, 2006; Stergiou *et al.*, 2006).

To overcome the loss of antimicrobial activity due to the interference of the food product itself, research has turned to the development of delivery systems to deliver the antimicrobial into the product in a way that it still remains active against the target pathogens. Several novel delivery systems have been studied for this purpose. The first type of delivery system involves the incorporation of antimicrobials into films that are then applied to a food surface. Nisin has been incorporated into edible cellulose films (Coma *et al.*, 2001), corn zein films (Hoffman *et al.*, 2001), and polyethylene based plastic films (Siragusa *et al.*, 1999). Antimicrobial activity was maintained in each case. The second delivery method involves the use of additional compounds as carriers of the antimicrobial. Encapsulation in liposomes has been shown to increase the efficacy of nisin in laboratory media (Were *et al.*, 2004) and in cheddar cheese (Benech *et al.*, 2002). Surfactant micelles have also been shown to increase the antimicrobial efficacy of the essential oil eugenol over a range of temperatures and pH (Gaysinsky *et al.*, 2005a; Gaysinsky *et al.*, 2005b). By attaching nisin to a primary emulsifier, in this case the protein pork gelatin, we hypothesized that emulsion droplets may act as a delivery vehicle for nisin and thus overcome the difficulties inherent in the use of this antimicrobial in a food system.

Materials and Methods

Materials

Nisin, corn oil, sodium hydroxide (NaOH), hydrochloric acid (HCl), and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). 200 Bloom Porkskin 8 mesh gelatine was donated by Gelita (Souix City, IA). Distilled and deionized water was used for the preparation of all solutions. *L. monocytogenes* strains J1-225, J2-020, J1-177, and C11-115 were obtained from the International Life Sciences Institute North America *Listeria monocytogenes* Strain Collection (Fugett *et al.*, 2006). *Escherichia coli* strains 35150, 43895, 51685, and 700599 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Tryptic soy broth (TSB), tryptic soy agar (TSA), and yeast extract were purchased from BD Diagnostics (Franklin Lakes, NJ).

Emulsion Preparation

An emulsifier solution was prepared by dispersing 0.5 wt % pork gelatin into water and gently heating to insure gelatin went into solution. The solution was stirred overnight to insure hydration of the protein. A primary emulsion was prepared by homogenizing 2 wt % corn oil with 98 wt % aqueous emulsifier solution in a blender followed by three passes through a microfludizer (Microfluidics 110L, Microfluidics Corp., Newton MA) at 9000 psi. The pH of the emulsion was adjusted to pH 7 using NaOH and HCl solutions. An aqueous nisin solution was prepared at a concentration of 5120 μg/ml and the pH was adjusted to pH 7 using NaOH and HCl solutions. The primary emulsion was diluted with the aqueous nisin solution to form secondary emulsions over a range of nisin concentrations.

Particle Size and ζ -Potential Measurement

Particle size and ζ- potential measurements were conducted using a Nano Series Zetasizer (Malvern Instruments). Concentrated emulsions were diluted to a concentration of approximately 0.005 wt % prior to measurement. Mean particle diameters were calculated as the average of three measurements with standard deviation. The ζ- potential measurements are reported as the average and standard deviation of three measurements.

Antimicrobial Assay

Four strains of *E. coli* (35150, 43895, 51685, and 700599) and four strains of *L. monocytogenes* (J1-225, J2-020, J1-177, and C11-115) were maintained at 4°C on TSA or tryptic soy agar with yeast extract (TSAYE) respectively. TSB or tryptic soy broth with yeast extract (TSBYE) was inoculated with *E. coli* or *L. monocytogenes* and incubated at 32°C for 24 hours and subcultured for an additional 18 hours. Cultures were diluted to approximately 10^4 CFU/mL prior to use. The minimum inhibitory concentration (MIC) of the double layered emulsion was determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 1990) agar dilution method. TSAYE was autoclaved and allowed to cool in a water bath until it reached a temperature of 50 ºC. The double layered emulsion, the primary emulsion, and free nisin were added to the tempered agar at concentrations ranging from 128 μg/ml to 0.5 μg/ml. EDTA was added to plates at concentrations ranging from 2048 μg/ml to 8 μg/ml. EDTA was added to the double layered emulsion dilution series at 512 μg/ml and 256 μg/ml.

Bacteria were applied to the plate via spot inoculation. Briefly, plates were inoculated with three 10 μL drops containing approximately 10^4 CFU/ml of the strains being tested. The plates were incubated at 32 ºC for 24 hours. The MIC was recorded as the lowest concentration at which no growth was observed.

Results

Formation of double-layered emulsions

Double layered emulsions were produced using the layer-by-layer depositioning technique. The attachment of the second layer, the nisin, to the primary emulsifier pork gelatin was determined via ζ- potential measurements. When an oppositely charged polymer is attached to the first layer, a reversal in the surface charge can typically be observed (Caruso *et al.* 1998). Figure 5 shows ζ- potential measurements taken across a pH range for the primary emulsion (A) and the aqueous nisin solution (B). In order to attach positively to charged emulsion droplets stabilized by gelatin, nisin in the aqueous solution must carry a negative charge. Figure 5(B) indicates that the isoelectric point (pI) of nisin is approximately pH 5. Thus nisin was added to the pork gelatin emulsions at pH 7 to obtain dual layered emulsion droplets. At pH 7 pork gelatin carruer a strongly positive charge while nisin carries a slightly negative charge. The ζ- potential emulsion droplets stabilized by pork gelatin as a function of added nisin is shown in Figure 6. The addition of the negatively charged nisin caused a strong decrease in the positive charge of the emulsion droplet suggesting at least a partial, if not full coverage of the positively charged primary emulsion layer. A slight increase in droplet size was also observed

following addition of the nisin solution, from an average diameter of 322.33 ± 8.32 nm to 338.33 ± 13.05 nm, which is characteristic after adsorption of a secondary layer.

Antimicrobial Assays

The results of the agar dilution assay are shown in Table 3. Dilutions were prepared from an initial stock solution containing the emulsion plus nisin at a concentration of 1024 μg/ml, representing the concentration of nisin which caused the greatest amount of charge reduction in the primary protein emulsion layer (Figure 5). Nisin, not normally a strong inhibitor of *E. coli* growth, did not inhibit the growth of any strain tested at either pH whether alone or as the secondary layer on the corn oil droplet. Nisin alone inhibited three of the four *L. monocytogenes* strains tested, with strain J2-020 showing resistance to the highest nisin concentration tested. The results in Table 3 show, however, that corn oil droplets with a positively charged protein as the primary emulsifier did not act as a delivery vessel of antimicrobially active nisin. The highest concentration of nisin in combination with the emulsion droplets, 128 μg/ml, only inhibited one strain, J1-177, at pH 6. All other strains of *L. monocytogenes* tested grew at the highest nisin concentration, which is two to four times the MIC of the free nisin. The addition of EDTA to the emulsion had no effect on the efficacy of the nisin against any of the strains tested (Table 4).

Discussion

The reduction in charge of the primary emulsifier demonstrated in Figure 5 suggests that the emulsion droplets were successfully coated with the nisin as a

secondary emulsifier. Caruso and Möhwald (1999) reported a complete charge reversal, as measured by ζ- potential, followed the addition of each oppositely charged layer on latex particles. Klinkesorn *et al.* (2005a), Klindersorn *et al.* (2005b) and Aoki *et al.* (2005) showed similar changes in ζ- potential when adding layers to emulsion droplets. Ogawa *et al.* (2003) found a small increase in particle diameter following addition of the secondary layer similar to the size increase of emulsion droplets upon addition of nisin in this study.

The attachment of the nisin to the protein emulsifier layer greatly decreased its antimicrobial efficacy, however. As previously stated, nisin activity is greatly reduced in food products due to the interaction of the antimicrobial with the constituents of the product, including lipids (Benech *et al.*, 2002; Ghalfi *et al.*, 2006), salts (Boziaris and Nychas, 2006), nitrates (Ghalfi *et al.*, 2006), proteolytic enzymes (Murray and Richard, 1997; Aasen *et al.*, 2003), and proteins (Aasen *et al.*, 2003; Stergiou *et al.*, 2006). Aasen *et al.* (2003) showed that more than 80% of the bacteriocins nisin and sakacin P added to chicken cold cuts and smoked salmon were absorbed into the protein matrix after 10 minutes, making them unavailable for antimicrobial activity. Stergiou *et al.* (2006) showed that lost nisin activity in meat products was due to the formation of bonds between nisin and glutathione in proteins. Though the formation of a secondary nisin emulsion layer may have been successful, the binding of the nisin to the pork gelatin emulsifier may have caused the loss of antimicrobial activity.

The loss of nisin activity may also be attributed to the reduction in charge. Nisin activity is most likely charge dependent, with positive lysine residues in the nisin molecule interaction with the negatively charged phospholipids head groups in the

membrane (Abee, 1994; Moll *et al.*, 1997). The nisin molecules used in this study were either negatively charged when used at pH 7 (Figure 5 (B)) or have an approximate charge of zero when attached to the oil droplets (Figure 6). Nisin susceptibility tests conducted at pH 7 showed no efficacy difference compared to nisin susceptibility at an unaltered pH (data not shown). The net zero charge of the nisin molecule attached to emulsion droplet may have reduced the ability of the nisin molecule to interact with the bacterial membrane, thus reducing its ability to inhibit the growth of the *L. monocytogenes* strains. The ability of EDTA to disrupt the outer membrane of Gram-negative bacteria did not increase the efficacy of the nisin either, further supporting the molecules inability to interact with the bacterial membrane.

Conclusions

A double layer emulsion was successfully constructed using pork gelatin as a cationic primary emulsifier and the food antimicrobial nisin as an anionic secondary emulsifier. The construction of this emulsion, however, greatly reduced the antimicrobial efficacy of the nisin, rendering the emulsion unsuitable as a deliver vessel for nisin.

CHAPTER 6

CONCLUSIONS

Microbiological contamination is a serious problem for both producers and consumers of food products. *L. monocytogenes*, in particular, is problematic due to its ability to grow under a wide range of conditions and the high mortality rate associated with listeriosis. Though antimicrobials like nisin and lysozyme effectively inhibit the growth of this organism, and are approved for food use, the application of these compounds to actual food products is problematic. Constituents of the food interferes with the activity of the antimicrobial, greatly decreasing its efficacy. The focus of this study was to devise a method by which these antimicrobials can be delivered into a food product without losing activity. To this end, nisin and lysozyme were attached to silver nanoparticles.

The attachment of lysozyme, an enzyme whose antimicrobial activity is dependent upon its enzymatic activity, to the nanoparticles greatly decreased its antimicrobial efficacy, most likely due to a conformational change or blocking of the active site. Nisin activity, on the other hand, was significantly increased when attached to the metal nanoparticles, suggesting that metal nanoparticles are a viable candidate for food antimicrobial delivery. The increase in efficacy is likely due to the ability of the nanoparticles to deliver a concentrated dose of the antimicrobial to the bacterial membrane. Further characterization of the interaction between the antimicrobial nisin and the silver nanoparticle and between the loaded silver nanoparticles and the bacteria is needed before this increase in efficacy can be fully explained. Further testing is also

needed to determine if this increased efficacy will still exist when applied to a food product. Since silver is not likely to be an acceptable food additive, a method by which these nanoparticles can be used with a food product will need to be determined; addition to packaging may be one such option.

The use of a double layered emulsion as the delivery method for nisin is advantageous due to the importance of emulsions in many food products and the all natural status of the constituents of the emulsion, in this case corn oil and gelatin. Using gelatin as an emulsifier, however, has proven problematic due to the lose of activity observed in nisin when it has bound a protein. The results of this study support the inactivation of nisin by proteins in a food product. Addition of EDTA to the emulsion proved ineffective in the restoration of antimicrobial activity to the molecule. The ability to use a wide array of non-protein emulsifiers, allowing for the testing of numerous primary emulsifiers, in the production of emulsions makes this a promising delivery method.

CHAPTER 7

TABLES AND FIGURES

Tables

Table 1. Particle size of nanoparticle/antimicrobial combinations.

Concentration Ratio	Particle size (nm)
Nisin	
1:0.167†	290±35
1:0.25†	324±132
1:0.5†	337±132
1:1†	283±61
2:1†	329±101
Lysozyme	
1:0.167†	45±4
1:0.25†	60±10
1:0.5†	160±37
1:1†	60±9
2:1†	140±15

†concentration ratio of antimicrobial to nanoparticles in μg/mL

Table 2. MIC's (µg/ml) of each silver/nisin concentration against strains of *L. monocytogenes* and *E. coli* at 32°C after 48 hours.

	Nisin		Silver		Silver + Nisin*									
					1:0.167†		1:0.25†		1:0.5†		1:1†		2:1†	
Pathogen	32°C	20°C	32°C	20°C	32°C	20°C	32°C	20°C	32°C	20°C	32°C	20°C	32°C	20°C
L. monocytogenes														
Scott A	41	21	>215	>215	5	1.25	5	1.25	>5	1.25	>5	1.25	>5	1.25
310	21	21	>215	>215	1.25	1.25	1.25	1.25	2.5	1.25	>5	1.25	5	1.25
108	21	12	>215	>215	2.5	1.25	5	1.25	1.25	1.25	5	1.25	2.5	1.25
101	21	12	>215	>215	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
E. coli														
H1730	>215	>215	>215	>215	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
EOO19	>215	>215	>215	>215	>5	2.5	>5	2.5	>5	2.5	>5	5	>5	1.25
F4546	>215	>215	>215	>215	>5	5	>5	2.5	>5	5	>5	5	>5	2.5
932	>215	>215	>215	>215	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5

* MIC data for nisin:silver concentration ratios with starting nisin concentration of 5 µg/mL and corresponding level of silver nanoparticles

†concentration ratio of antimicrobial to nanoparticles in µg/mL

>: MIC greater than highest concentration tested

Table 3. MIC (μg/mL) of primary and secondary emulsions against strains of *L. monocytogenes* and *E. coli*

MIC (μg/mL)				
Pathogen	Nisin	Emulsion	Emulsion/Nisin	
			pH 6	pH 7
L. monocytogenes				
J1-125	32	>128	>128	>128
J2-020	>128	>128	>128	>128
J1-177	64	>128	128	>128
C11-115	32	>128	>128	>128
E. coli				
35150	>128	>128	>128	>128
43895	>128	>128	>128	>128
51685	>128	>128	>128	>128
700599	>128	>128	>128	>128

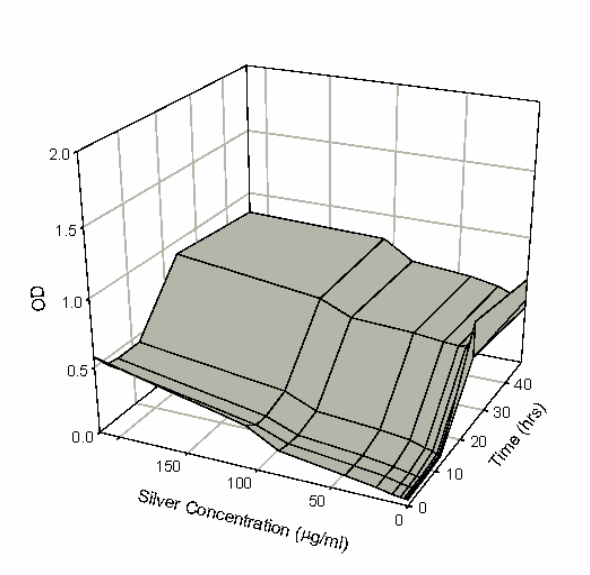
Table 4. MICs (µg/mL) of secondary emulsions with added EDTA against strains of L. monocytogenes and E. coli.

MIC (µg/mL)			
Pathogen	EDTA	Emulsion/Nisin+EDTA	
		512 µg/mL*	256 µg/mL*
L. monocytogenes			
J1-125	>2048	>128	>128
J2-020	>2048	>128	>128
J1-177	>2048	128	>128
C11-115	>2048	>128	>128
E. coli			
35150	>2048	>128	>128
43895	>2048	>128	>128
51685	>2048	>128	>128
700599	>2048	>128	>128

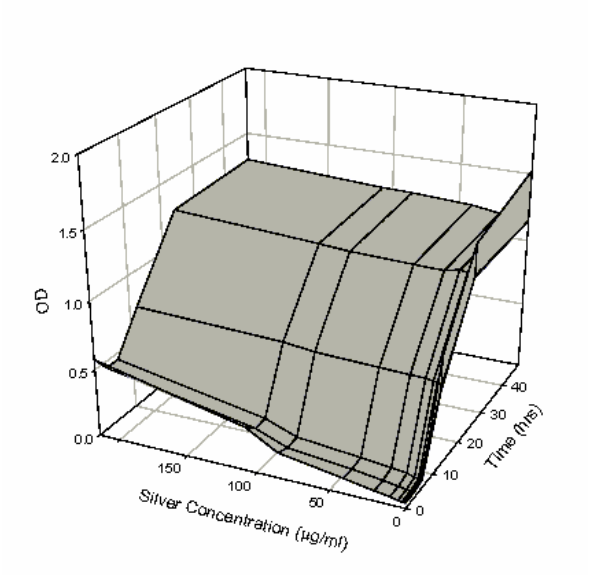
*amount of EDTA in µg/mL added to emulsion containing various concentrations of nisin

Figures

Figure 1. Growth as measured by OD_{630} of L. monocytogenes strain Scott A (A) and E. coli strain H1730 (B) in the presence of silver nanoparticles at 32°C for 48 hours.

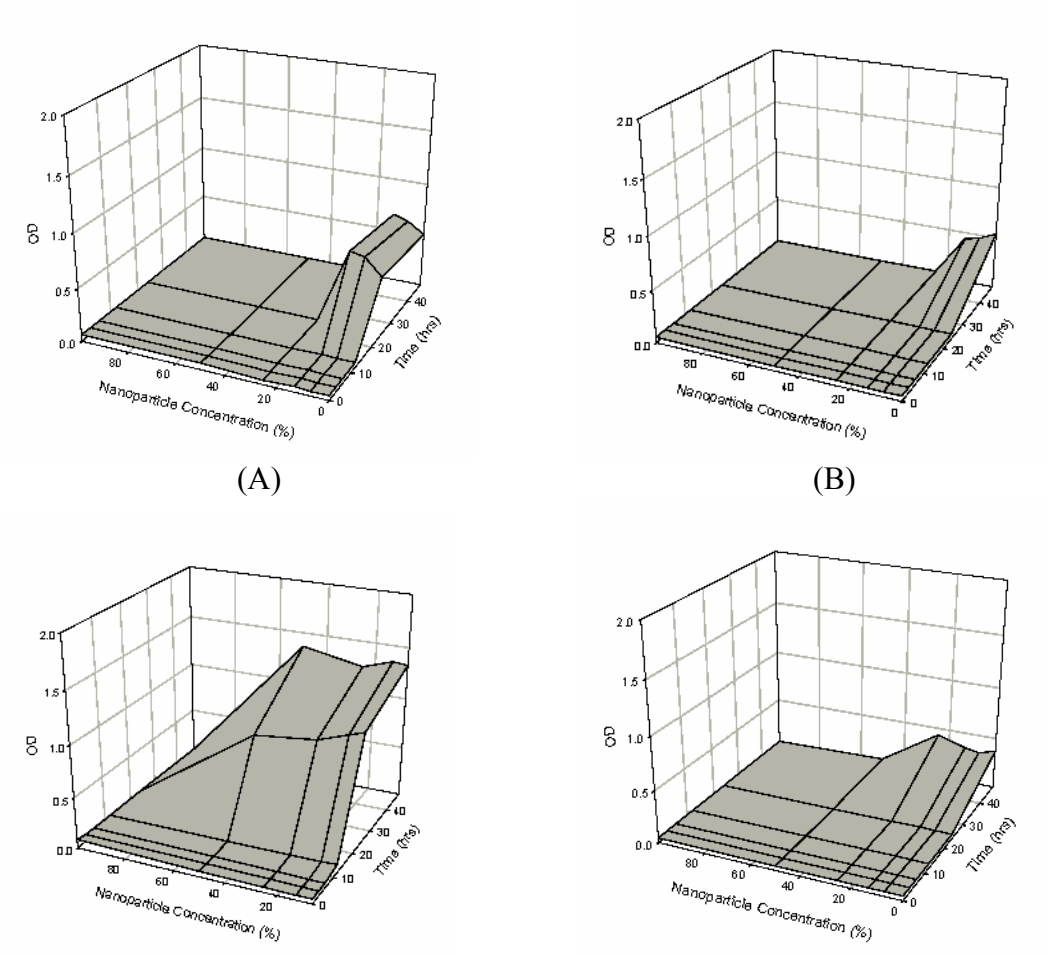


(A)



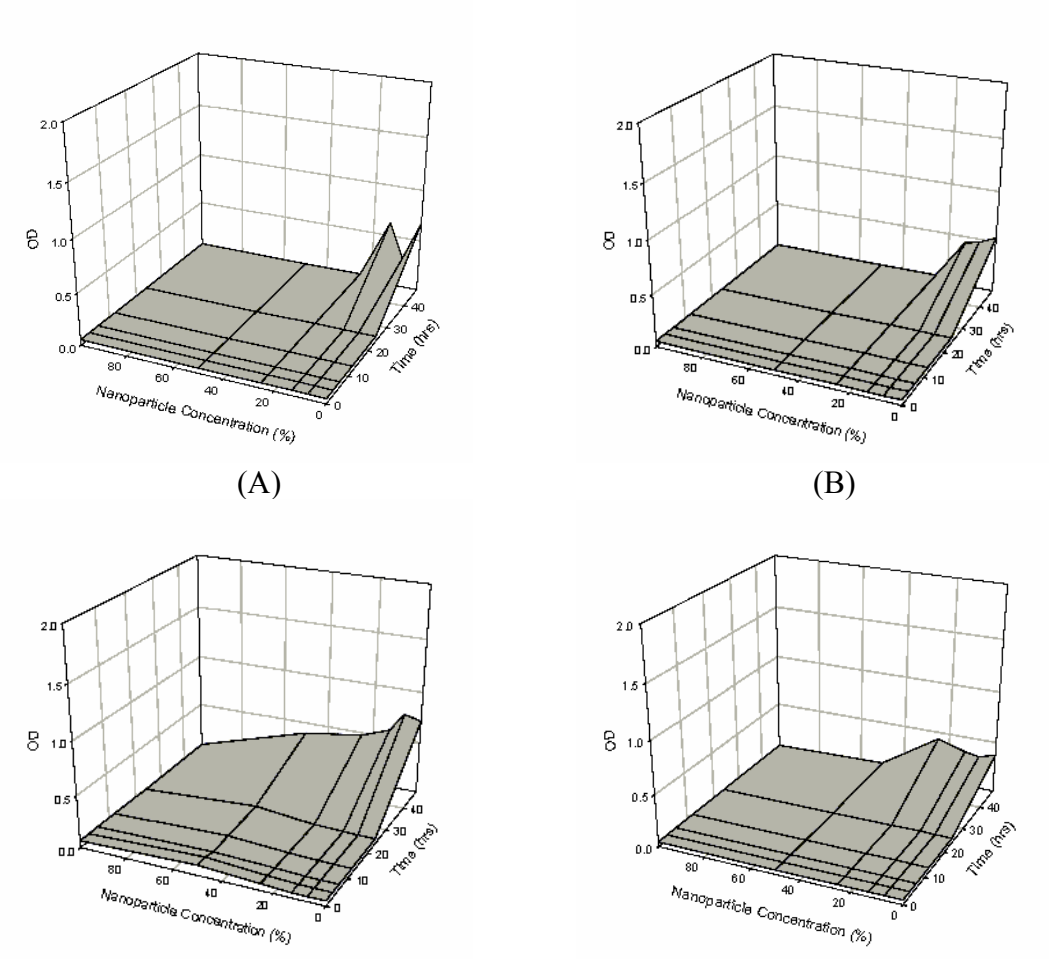
(B)

Figure 2. Growth as measured by OD_{630} of *L. monocytogenes* and *E. coli* in the presence of nisin attached to silver nanoparticles at 32°C for 48 hours.



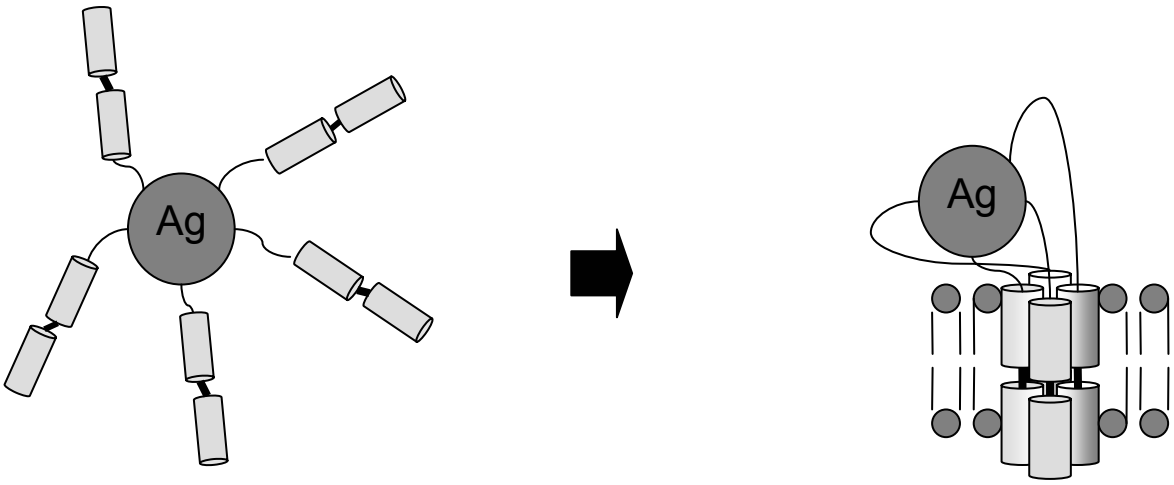
(A) Strain 101 in the presence of nisin attached to silver nanoparticles at a ratio of 6:1. (B) Strain 310 in the presence of nisin attached to silver nanoparticles at a ratio of 6:1. (C) Strain Scott A in the presence of nisin attached to silver nanoparticles at a ratio of 4:1. (D) Strain F4546 in the presence of nisin attached to silver nanoparticles at a ratio of 6:1.

Figure 3. Growth as measured by OD_{630} of *L. monocytogenes* and *E. coli* in the presence of nisin attached to silver nanoparticles at 20°C for 48 hours.



(A) Strain Scott A in the presence of nisin attached to silver nanoparticles at a ratio of 4:1. (B) Strain 310 in the presence of nisin attached to silver nanoparticles at a ratio of 6:1. (C) Strain 310 in the presence of nisin attached to silver nanoparticles at a ratio of 2:1. (D) Strain F4546 in the presence of nisin attached to silver nanoparticles at a ratio of 6:1.

Figure 4. Proposed mode of action for increased efficacy of nisin when attached to silver nanoparticles.

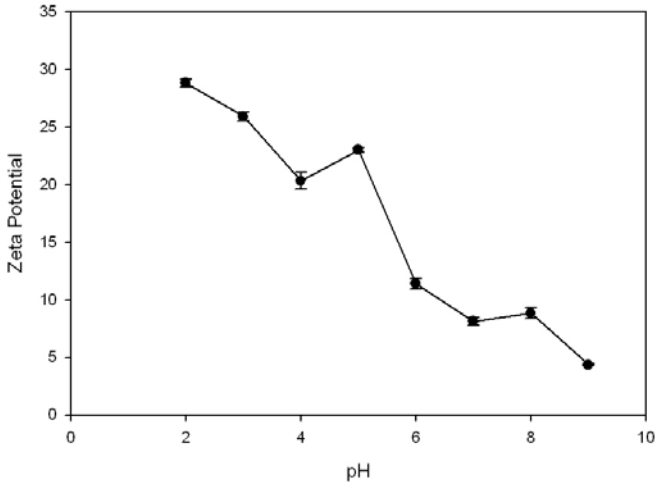


Nisin molecules attached to silver nanoparticle

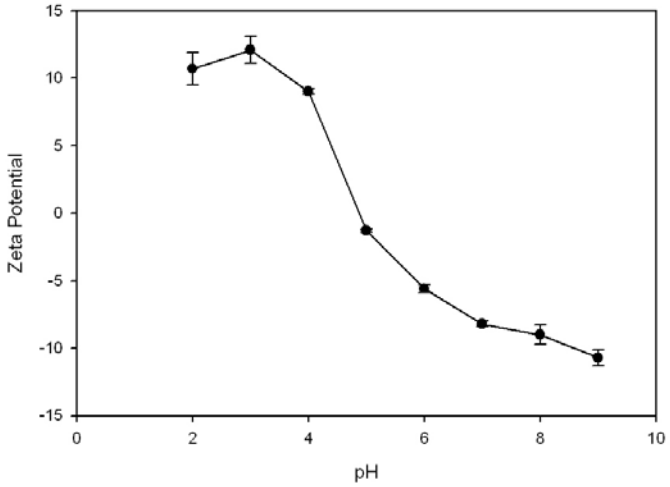
Nisin molecule interaction in bacterial membrane

Nisin molecule structure and hypothesized mode of action adapted from Abee *et al.* (1995)

Figure 5. ζ-potential as a function of pH for an emulsion and a nisin solution.



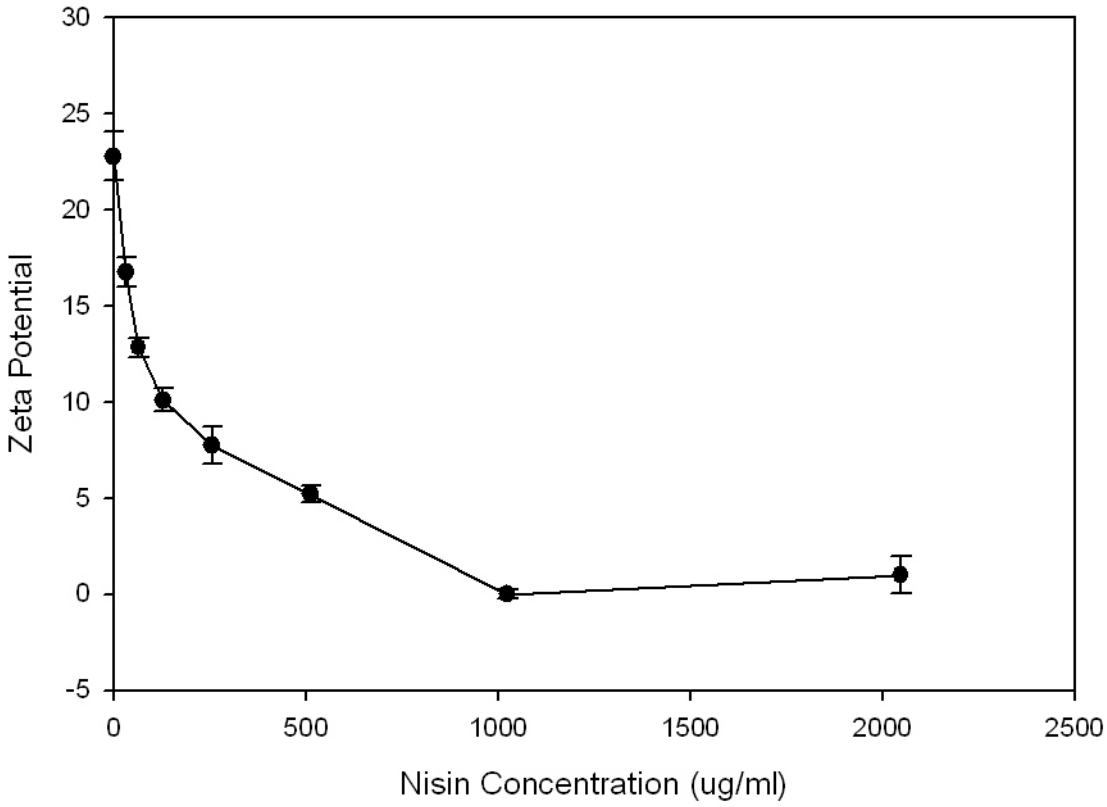
(A)



(B)

(A) ζ- potential of the primary emulsion at pH 5 to pH 8. (B) ζ- potential of a nisin solution at pH 2 to pH 9.

Figure 6. ζ-Potential of the primary gelatin stabilized emulsion with increasing addition of nisin.



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