CONTROLLED-RELEASE ANTIMICROBIALS FOR PREVENTING BIOFILM FORMATION IN FOOD AND MEDICAL APPLICATIONS

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ABSTRACT OF THE THESIS

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Bacterial biofilms generally are more resistant to stresses as compared to free planktonic cells. Therefore, the discovery of antimicrobial stress factors that have strong inhibitory effects on bacterial biofilm formation would have great impact on the food, personal care, and medical industries. Biofilm formation can be prevented through controlled release of nature-derived antimicrobials formulated into polymer systems, especially those featuring multiple stresses as hurdles for the bacteria to overcome to prevent the development of resistance. Salicylate-based poly(anhydride-esters) have previously been shown to inhibit biofilm formation. Our research evaluated the effect of salicylate-based poly(anhydride-esters) on biofilm forming *Salmonella enterica* serovar Typhimurium. The results indicate that the salicylic acid-based polymers do interfere with biofilm formation through a combination of pH effects and a thus-far-unknown mechanism. Systems combining pH stress with the controlled release of natural antimicrobials have great potential to prevent biofilm development in many environments to prevent adverse outcomes, especially implant contamination and foodborne illness.

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INTRODUCTION

Bacterial contamination represents one of the major concerns of the food and medical industries. Bacteria predominantly grow throughout many environments in the form of biofilms, associated communities of cells within a complex polymeric matrix that exhibit increased resistance to environmental stresses. Biofilm structure can vary greatly from species to species, depending not only on strain characteristics but also the character of the material on which the biofilm forms. This diversity is epitomized by *Salmonella enterica*, an organism that causes numerous human health issues including food-borne illness and typhoid fever. Controlled release of active substances has been in existence for many years as a method of releasing functional compounds at specific rates into the environment. Controlled delivery of antimicrobials *via* degradable polymers has the potential to reduce microbial contamination through the sustained release of antimicrobials that specifically act against biofilms. Salicylic acid and its derivatives are appealing for use in medical, food and personal care applications because they are antiseptic, anti-infective but not antimicrobial, hence there is little long-term possibility of creating microbial resistance. Salicylic acid is a compound produced by many plants as part of their defense against microbial invasion and the controlled release of SA from a poly(anhydride-ester) or PAE has been proven to prevent biofilm formation in *S. typhimuirum* MAE52, a mutant strain that forms biofilms at the air-liquid interface. Stresses can alter the actual gene expression and structure within biofilms, so a thourough investigation of the effects of salicylic acid and pH on *Salmonella* biofilms will lead to innovative methods of controlling the growth of biofilms in many environments.

Controlled Release and Active Packaging: Potential vs. Reality – A Review [1]

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Running title: Active packaging: potential vs. reality

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INTRODUCTION

The safety and quality of the food supply has gained significant attention in recent years (Burt 2004), yet increasing concerns over recalls and ingredient sourcing trouble many consumers. These concerns lead to a need for more effective safety regulations as well as better systems for maintaining food quality. However, there is also an increase in consumer desire for natural, local and organic products (de Kruijf et al. 2002; Suppakul et al. 2003; Burt 2004) which creates new challenges in providing efficient food preservation, especially in the area of microbial safety. Many current methods to maintain and enhance food quality do not satisfy consumer demand for products from natural sources, so the food industry has begun to investigate alternatives to synthetic substances. Numerous natural substances have been tested, from grapefruit extract to bacterially-synthesized antimicrobial peptides known as bacteriocins to mustard oil (Chikindas et al. 1997; Han 2000; Kim, Paik, and Lee 2002; Burt 2004; Cha and Chinnan 2004). A good number of substances, including antimicrobials and antioxidants, from natural sources had proven effective in laboratory settings, but the real test is consumer products. Can their effectiveness be maintained in complex, real life applications?

In addition to the desire for effective natural compounds that enhance quality, there is a need to maintain the concentration of the active substance a level that is effective but not dangerous for human consumption. However, when substances are added as part of the formulation, the amount can gradually decrease due to complex interactions with the food matrix or by natural degradation over time (Ouattara et al. 2000; Kim et al. 2002; Chi-Zhang, Yam, and Chikindas 2004). A method of maintaining concentrations at active

levels that has achieved increased interest is controlled release. Controlled release, also known as time-release and slow-release, of active substances has been in existence for many years now both as a concept and as a marketable method of utilizing drugs, antimicrobials and many other compounds in various applications. It is defined as a system that releases active compounds at specific controlled rates into its environment (Lacoste et al. 2005). It can be utilized to release antibiotics, prescription medications, vitamins, antioxidants and many other compounds into a variety of environment. This approach has applications in many fields, including medicine, personal care and of course, the food industry.

Active packaging is defined as an intelligent system that alters the state of the packaged food system or headspace to improve quality through extension of shelf-life, maintenance of microbial safety or enhancement of sensory qualities (Han 2000; de Kruijf et al. 2002; Quintavalla and Vicini 2002; Suppakul et al. 2003). It skillfully applies the principles of controlled release to the world of packaging science and engineering. Active packaging is quickly attracting interest, in the scientific world as well as in the consumer consciousness. Increased desire for high-quality, natural, safe and fresh products are some of the reasons why active packaging has gained such popularity (Han 2000; Cha and Chinnan 2004; Ozdemir and Floros 2004). Previous reviews have dealt mainly with the biodegradable polymers themselves, the compatibility of certain active substance with certain polymers, or the regulations made and needed to deal with emerging active and intelligent packaging (Han 2000; de Kruijf et al. 2002; Vermeiren, Devlieghere, and Debevere 2002; Cha and Chinnan 2004; Ozdemir and Floros 2004). Little has been made

of the comparative benefits and drawbacks of these active packaging concepts and applications. While this review is mainly geared towards the use of controlled release as it relates to microbial food safety and quality, important advances in medicinal and personal care uses will also be evaluated. Most importantly, the relative advantages and disadvantages of the different applications, and their effectiveness in real word situations, will be discussed.

CONTROLLED RELEASE

The concept of controlled release has existed for some time now. Simply taking medicine in small doses over a period of time can be considered a method of controlling the release of the medicine and its effects. Of course, technology has allowed for significant advancement to the concept. Not only can drugs be taken over a period of time, they can also release their active compounds over time or only when certain conditions are reached; for instance, pH-based release can be initiated by exposure to stomach acid or intestinal pH levels (Risbud et al. 2000).

A variety of devices are currently used in the medical area to enable continuous release over a prolonged period, including microparticles, gels, osmotic minipumps and adhesive patches (Machluf, Orsola, and Atala 2000). Often, the fine-tuning of the details is where controlled release systems can differ the most. Different polymer compositions can affect the release rate of the active compound(s); in addition, incorporation of active substances into the polymer can change polymer chemistry, which in turn can affect characteristics like oxygen permeability, tensile strength, release rates and brittleness (Amass, Amass,

and Tighe 1998; Putney and Burke 1998; Suppakul et al. 2003; Schmeltzer and Uhrich 2004; Whitaker-Brothers and Uhrich 2004; Lacoste et al. 2005; Schmeltzer, Schmalenberg, and Uhrich 2005).

The control of release rates has large implications for the medical and personal care industries. Many diseases or conditions are the result of persistent infections or hormonal imbalances (Chikindas et al. 1997; Machluf, Orsola, and Atala 2000; Costerton et al. 2003). The controlled release of treatments for those conditions can more effectively treat the cause of the disease, rather than just easing the symptoms. In particular, implants are a major concern for the medical industry due to their susceptibility to contamination, leading to infection in the host and ultimate rejection or replacement of the implant (Costerton et al.2003; Darouiche 2003; Etienne et al. 2004; Bryers et al. 2006). To prevent biomaterial-related infections, the current strategy is to treat patients systemically with antibiotics at high concentrations. Sometimes, this approach alleviates the infection, but often it leads to rejection of the implant and a subsequent second surgery to remove and replace it in the patient. The controlled release of antimicrobials and antiseptics from the implant itself can greatly decrease the rejection rates of implants like heart stents and knee replacements, for example (Benoit et al. 1997; Bower et al. 2002; Costerton et al. 2003; Darouiche 2003; Huneault et al. 2004; Bryers et al. 2006). In addition, many skin conditions and external wounds could benefit from dressings or patches that release treatment compounds in a controlled manner, eliminating the need for constant reapplication of creams and bandages (Jenning, Schafer-Korting, and Gohla 2000; Baar et al. 2001). Another facet of medical treatment that can be aided by controlled-release

applications is protein therapeutics. Proteins have low oral bioavailability and are particularly susceptible to degradation by metabolic activities or temperature, which can be solved by encapsulation or incorporation into a polymer. Release rates can be adjusted by modifying polymer composition and up to fourfold less drug may be required when using sustained release as opposed to immediate delivery (Putney and Burke 1998).

ACTIVE PACKAGING

In recent years, the incorporation of functionality through polymer properties or functional ingredients into packaging materials has become known as ‘active packaging’. Active packaging can be classified into two main categories: scavenging systems and emitting systems. Scavenging systems absorbs harmful compounds from the surface of the food or the headspace, while emitting systems release compounds to act at the surface of the food or within the headspace (de Kruijf et al. 2002; Quintavalla and Vicini 2002). Many examples of this category of material have come to the market in Japan and the US. Some types of active packaging systems include oxygen scavengers, antioxidant systems, ethylene absorbance and antimicrobial release (Han 2000; de Kruijf et al. 2002; Cha and Chinnan 2004; Ozdemir and Floros 2004). These systems are attractive to the consumer for a number of reasons; they frequently are composed of substances considered ‘natural’ and the controlled release nature of their composition prevents large amounts of the functional compounds from being present in the food at the time of consumption, while still maintaining the desired activity (Vermeiren, Devlieghere, and Debevere 2002). In addition, the concentration of active compound in the packaging must

be calculated based upon the amount needed in or regulated for the final product (Nerin et al. 2006).

The point of active packaging is to maintain, or even enhance, the safety and quality of the food held within in the package. Direct application of active compounds has limited benefits in foods due to inactivation by food components or diffusion into the bulk and away from the surface where changes are actually taking place (Ouattara et al. 2000). Controlled-release of the substances from packaging materials will extend the effect of the active compounds. To attain the goals that this packaging fulfills, changes can be made in the materials used, the functional compounds, the engineering of the application and combinations of compounds with complementary activities (Han 2000; Duan et al. 2007). The active compounds can modify the atmospheric proportions in the headspace, lower water activity on the surface, prevent oxidation of key nutrients and prevent bacterial growth (Suppakul et al. 2003).

MATERIALS

Plastics have been the polymer of choice for packaging in recent years, due to low cost and high performance (Liu et al. 2007). The cost of plastics has been reduced due to innovations in the packaging field as well as the inherent properties of the barrier material (Cha and Chinnan 2004). Biodegradable polymers, defined as polymers created from raw materials of agricultural or marine sources and broken down through biological or chemical reactions, have been manufactured for biomedical purposes for over 30 years and are gaining in popularity over plastics (Amass, Amass, and Tighe 1998; Ikada and

Tsuji 2000; Cha and Chinnan 2004). Although most commercial active packaging systems currently available are made from plastics, tests of those made from biodegradable polymers show comparable efficacy (Cha and Chinnan 2004).

The limitations of specific plastics and biodegradable polymers lead to the combination of different polymers to accomplish a particular packaging or coating goal (Amass, Amass, and Tighe 1998; Cha and Chinnan 2004; Lopez-Rubio et al. 2004; Lacoste et al. 2005). Different polymers can be formed into single films, combining the characteristics of the varying structures to deliver unique features appropriate for the particular application. Incorporation of functional substances can be achieved by optimizing the concentrations of the different polymers to achieve the desired release rates, sometimes by erosion release and sometimes by simple diffusion (Amass, Amass, and Tighe 1998; Kim et al. 2002; Cha and Chinnan 2004; Lacoste et al.2005). Many studies have been done to determine release rates of compounds from these polymers in addition to assays to determine activity of the compounds following the release (Kim et al. 2002; Grower, Cooksey, and Getty 2004). Some results have proved promising while others are quite discouraging, including total loss of activity and activity at released levels for *in vitro* tests but not in simulations of real systems, as will be discussed in detail further on in this review.

However, the increased use of plastics in packaging materials can lead to larger amounts of waste and concerns about unintended migration of polymer components into food items, especially those given to children (Amass, Amass, and Tighe 1998; Quintavalla

and Vicini 2002; Cha and Chinnan 2004; Lopez-Rubio et al. 2004). Biodegradable films address some of these issues. Edible films and coatings made from natural sources are also an attractive alternative to plastics that addresses those consumer concerns. These films often have the added benefit of being made from otherwise unused byproducts of industrial processes, further increasing their attractiveness to concerned consumers (Ouattara et al. 2000; Cha and Chinnan 2004).

Edible films and coatings can be classified into several categories: carbohydrate-based (also known as hydrocolloids), protein-based, lipid-based and composites (Cha and Chinnan 2004). Some hydrocolloid polymers being researched include cross-linked starch films, cellulose-based films, agar and carrageenan which are both galactose polymers, and chitosan films (Cha and Chinnan 2004; Grower, Cooksey, and Getty 2004; Huneault et al. 2004; Seydim and Sarikus 2006). Cross-linked starches have been used in food applications for years, and have properties that extend well into controlled-release applications. Release rates increase with increased cross-linking of the amylose and the resulting release profiles are not greatly affected by manufacturing conditions (Lenaerts et al. 1998). Cellulose-based films are popular because they are very water-soluble, therefore making it easier for additives to be released into foods with a high water content (Grower, Cooksey, and Getty 2004). Chitosan is especially of interest to the packaging and biomaterials industries because it is cationic and naturally antimicrobial, mainly due to its protonated amino group which interferes with negatively charged membrane components (Coma, Deschamps, and Martial-Gros 2003; Cha and Chinnan 2004; Duan et al. 2007). Proteins that are being investigated for use in packaging films and coatings

include zein, soy protein, gluten, collagen and whey protein as well as other milk proteins(Cha and Chinnan 2004; Seydim and Sarikus 2006). Whey protein films especially have gained attention in recent years, due to their mechanical strength and excellent barrier properties (Huneault et al. 2004; Seydim and Sarikus 2006). Lipid-based films are mostly used as moisture barriers, especially for fresh fruits and vegetables (Cha and Chinnan 2004).

A novel concept for films and coatings is the use of the active compound itself as the polymer matrix. As the polymer degrades, it releases one or more substances into the environment. This approach to active packaging is easily expanded into multi-purpose packaging systems to enhance food quality. Polyanhydrides are a class of biodegradable polymers gaining popularity in biomedical application, including drug delivery, implant coatings and tissue scaffolds. They frequently contain hydrophobic compounds bound by hydrolytically labile anhydride bonds, and the degradation rate can be controlled by changing the composition of the polymer (Schmeltzer and Uhrich 2004; Schmeltzer, Schmalenberg, and Uhrich 2005). Anhydrides added to films made of other polymers have proven antimicrobial activity (Suppakul et al.2003), and films made of these active anhydrides can act as prodrugs, breaking down to release the compound in a controlled manner in the desired area. Poly(anhydride-ester) or PAE polymers can controllably release salicylic acid as they undergoes hydrolytic degradation (Erdmann and Uhrich 2000; Erdmann, Macedo, and Uhrich 2000). Salicylates and other non-steroidal anti-inflammatory drugs (NSAIDs) are known to prevent bacterial adhesion onto medical devices (Arciola et al. 1998). PAEs comprised of NSAIDs such as salicylic acid, like

other polyanhydrides, predominately undergo surface erosion (Tamada and Langer 1993; von Burkersroda, Schedl, and Gopferich 2002; Whitaker-Brothers and Uhrich 2004). PAEs have been shown to prevent biofilm formation (Bryers et al. 2006), and could be used in combination with other active substances for food packaging to enhance food safety. Polyanhydride films are easily manipulated into many functional forms and are currently among the few biodegradable systems approved by the FDA for use in humans (Anastasiou and Uhrich 2003), though use in foods has yet to be determined. These films, which have great potential for the food safety industry, will be discussed in more detail in the following section.

CONTROLLED RELEASE SYSTEMS

Spheres

Microspheres and nanospheres are among the most common applications of controlled release, especially in medical and personal care applications (Jenning, Schafer-Korting, and Gohla 2000). The area of the sphere has an effect on the release rate and can change as the active substance is release from or through the surface of the sphere. In addition, larger particles have the potential to absorb more water from the surrounding environment (Lim et al. 2000).

Due to their theoretical usefulness in the human body, many micro- or nanosphere applications have been researched (Mathiowitz et al. 1997; Machluf, Orsola, and Atala 2000; Jenning, Schafer-Korting, and Gohla 2000; Lim et al. 2000). Solid lipid nanoparticles, for example, are capable of controlled release of drugs, vitamins and other

lipid or emulsified active substances. They can be used as a drug, an additive or suspended in semi-solid hydrogels or emulsified creams for topical applications (Jenning, Schafer-Korting, and Gohla 2000). Other substances have been investigated for *in vitro* drug release, including novel microspheres made from hyaluronic acid and chitosan, with the intent on enhancing drug absorption through mucoadhesion. The spheres display a characteristic burst release, and though the majority of the micropheres dissolved within 1 hour, release of the active compound was not complete after 5 hours. The remaining compound retained activity, lengthening the effectiveness of these microspheres for drug release (Lim et al.2000).

Another novel application of controlled release is the encapsulation of living cells in microspheres for testosterone-replacement therapy. Cell encapsulation by a biocompatible, semipermeable polymeric barrier helps protect the cells from attack by the host immune system and prevents the metabolic inactivation of the hormone which occurs during oral administration (Machluf, Orsola, and Atala 2000). Other microspheres, made from fast-degrading hydrophobic polymers, are good bioadhesives and enhance the uptake of drugs that are inefficiently absorbed by the body (Mathiowitz et al. 1997). The same principle can be applied to microencapsulation of plasmid DNA, another disease treatment that suffers from low efficiency when applied on its own, due to degradation below pH 8 and lack of response from the immune system. Microencapsulation in poly(DL-lactide-co-plycolide) polymers (PLGA) resulted in increased stability and release rates characterized by an initial burst followed by slow release due to polymer

degradation. DNA released during the burst phase had much higher activity than that released during the second phase (Walter et al. 1999).

Films

Films and coatings are very popular and effective systems for the controlled release of substances into an environment. They are especially effective in food systems, where changes often take place on the surface of foods or in the headspace of the package interior (Han 2000; Kim et al. 2002; Quintavalla and Vicini 2002). The amount of active compound can be changed by percentage incorporated into the polymer matrix as well as the thickness of the coating. Films on the inside of bottles can prevent the loss of important vitamins and nutrients, like ascorbic acid, during storage (Baiano et al. 2004). Another application for films and coatings is for release of antimicrobials into food systems to prevent contamination and prolong shelf-life (Han 2000; Franke, Wijma, and Bouma 2002; Kim et al. 2002; Chi-Zhang, Yam, and Chikindas 2004; Duan et al. 2007).

As mentioned above, active compounds can either be released onto the surface or within the headspace between the food and the packaging. Compounds released into the gaseous environment within the package can change the atmosphere to prevent ethylene production, lipid oxidation or growth of microorganisms (Lopez-Rubio et al. 2004). Utilizing these require the optimization of analysis techniques for determining release rates and effectiveness of volatile active compounds in the vapor phase (Han 2000; Becerril et al. 2007). The increasing interest in the use of essential oils with antimicrobial activity makes these techniques crucial (Burt 2004; Becerril et al. 2007). Essential oils

(EOs) are lipid fractions obtained from plant materials through a variety of methods, including extraction and steam distillation. They are mostly used in flavors and fragrances, but recent scientific studies are supporting their possible health and safety benefits for the food and medical industries. Some EOs have shown antimicrobial properties, while others exhibit antiviral, antimycotic, antioxidant and other functional effects (Burt 2004). Frequently, the EOs with the highest antimicrobial properties contain high concentrations of phenolic compounds (Seydim and Sarikus 2006). Direct addition of EOs with antimicrobial or antioxidant activity would change the flavor profile of the product, while controlled release of the same EOs over time would maintain the food's characteristics while enhancing safety and quality.

An alternative aspect of active packaging would be the use of enzyme immobilized onto the packaging material. This technology is currently used in production lines to catalyze enzymatic reactions without losing the enzyme itself along the way. However, in order for this packaging concept to work, the packaging needs to be in contact with the food surface. Unlike volatile or diffusible compounds that can migrate through headspace, enzymes can only work when the substrate is in close proximity. It is also possible to immobilize antimicrobial compounds, like silver ions or covalently-linked peptides and organic acids. Some polymers, like chitosan, are inherently antimicrobial, but it is also possibly to make plastic surfaces themselves antimicrobial using UV radiation. A UV excimer laser can convert nylon amides to antimicrobial amine groups (Lopez-Rubio et al. 2004).

As mentioned above, polyanhydrides are a promising new area of research for medical, food and personal care controlled release systems. The polymers degrade at different rates depending on the active compounds and linkers that are incorporated into the backbone, and other functional materials like antibiotics or antioxidants can be physically admixed into the polymer and manipulated into many forms, including fibers, films, pellets and microspheres (Anastasiou and Uhrich 2003; Whitaker-Brothers and Uhrich 2004). Recent studies investigated the effects of polyanhydride polymers on biofilm formation and discovered that the release of salicylic acid from polymer films can prevent the formation of *Salmonella typhimurium* biofilms. The results are extremely promising, especially considering biofilms are extremely resistant to conventional concentrations of antibiotics and antimicrobials (Rosenberg, et al 2008). Eradicating persistent biofilms is difficult because they require both antimicrobials and physical removal; preventing their formation in the first place is crucial to food safety. The potential for producing polymers that release antimicrobials of natural origins that can prevent biofilm formation and enhance food quality is very attractive to industry as well as to consumers, and the results of this research has generated a great deal of excitement in the media and scientific publications. The mechanism of action is currently under investigation, as is the utilization of other natural antimicrobials in these polymer films for future use as food packaging technology. This especially has implications for use as multiple hurdle technology, which will be discussed in more detail later in this review, due to the ability to formulate polymers with multiple active components, whether they are integrated into the backbone itself or physically admixed and co-extruded into packaging materials.

Other application methods

Sometimes, low levels of antibiotic or antimicrobial are desired or pre-coated items are not available and treatment is required quickly. At those times, rather than applying a film of known quantity, a device may merely be placed in contact or soaked in a solution containing the involved substance. Standardizing the time of the contact and the concentration of active compound in the solution replaces coating in known quantities of film containing specific concentrations of the active compound (Bower et al. 2002; Darouiche 2003). For example, collagen patches impregnated with antimicrobials are in use as biomaterials. Antimicrobial efficiency and adhesive strength as a biomaterial have been evaluated, but release rates or profiles were unavailable (Baar et al.2001). Another application using a less controlled method of application is the soaking of plaster of Paris implants in antibiotic solution. Antibiotic effect was retained both in vitro and in vivo; slow release on the antibiotic did not even approach 50% of the initial concentration within 3 weeks (Benoit et al. 1997).

Such results have potential for clinical uses, where weeks of antibiotic treatment may be necessary to treat persistent infections (Bower et al. 2002; Costerton et al.2003; Darouiche 2003), and personal care applications that can be reapplied. In addition, dental caries and periodontal diseases are the most common bacterial diseases in the world, and systemic antimicrobials are recommended for treatment (Dashper, Liu, and Reynolds 2007). Controlled-release antimicrobial systems are very attractive both to consumers and to the industry since they have the potential to treat chronic dental problems as well as

satisfying the consumer's desire for naturally-derived products. For foods, however, the more standardized films/coatings have far more potential.

EFFECTIVENESS IN REAL-LIFE SITUATIONS

The main concern with active packaging is whether lab results can translate effectively into the complexity of the real world. Often, lab tests are done with food simulants that are far less complex than actual food systems (Kim, Paik, and Lee 2002). Real foods will have more nutrients, lower water activity, higher salt contents and fats or proteins which may interact with the active compounds (Vermeiren, Devlieghere, and Debevere 2002; Burt 2004;Baiano et al. 2004; Grower, Cooksey, and Getty 2004; Mauriello et al. 2005). In addition, the conditions in which the foods are transported and stored have a great effect on its characteristics (Franke, Wijma, and Bouma 2002; Kim et al. 2002; Burt 2004; Cha and Chinnan 2004; Lee et al. 2004). Temperature and moisture content can have a large effect on release rates of compounds and their effectiveness (Ouattara et al. 2000; Suppakul et al. 2003). Release rates are a big issue, since they determine how much of the compound will emerge from the packaging and how long it will take to saturate the area. When simple diffusion in employed, active compounds diffuse across a gradient, but as the food surface or headspace becomes saturated, the release may slow to a stop (Han 2000). In addition, many substances undergo an initial burst effect, releasing a large quantity all at once and ceasing release until it is all consumed (Huneault et al. 2004). Often, the release of the active substance is not governed solely by diffusion, generally due to swelling and water uptake of the film (Ouattara et al. 2000).

Microbiological, chemical and analytical tests can determine where the active compound goes, in what quantities and the method of action in experiments that simulate food systems (Suppakul et al. 2003; Becerril et al. 2007), but establishing these same results in real foods is far more difficult. When tests using real foods are reported, they often reveal that the developed active packaging system is less effective than it was in previous lab experiments (Kim, Paik, and Lee 2002; Duan et al. 2007). Tests with essential oils have revealed that much higher levels of EOs than are used for *in vitro* tests are needed to achieve an effect in foods, including milk and cheese applications (Burt 2004). Some proposed reasons for this phenomenon are greater availability of nutrients for cellular repair, higher organic acid and trace metal content, and interactions with compounds in the food that may complex with or inactivate the active substance (Burt 2004; Lee et al. 2004).

With medical applications, clinical trials reveal whether the developed controlled release system is effective. For example, catheter tubes containing a bacterially-synthesized antimicrobial protein active against closely related species (also known as a bacteriocin) called nisin were proven effective at reducing bacterial infection for 24 h (at which time the nisin activity drops significantly) without any adverse effects to the animals used in the testing for up to a week. The authors of this study suggest further experiments using replacement of the nisin over time to enhance its effects, as well as further tests to retain nisin activity for longer periods of time and ascertain nisin's effects on tissues and bacterial cells over longer periods of time (Bower et al. 2002). Another study used chitosan for wound dressings loaded with antibiotics to inhibit wound infection,

especially of wounds from armed conflict where extensive medical care is often not immediately available. The incorporation of silver sulfadiazine, an antimicrobial commonly used in burn wounds, reduced cell counts by 7-logs in 7 days, a rather impressive result. Sulfadiazone release showed a burst effect followed by slower release, while the silver ions were released very slowly over the time period (Mi et al. 2002). This dual-action film proved very effective in lab tests, and demonstrated suitability for real situations where a slow, sustained delivery of antimicrobials can mean the difference between life and death.

Often, conclusions that are reached by the results of a study may not be applicable for the desired field. For example, packaging containing triclosan was investigated with regard to food applications. No triclosan was released in pure water, but even in 10% ethanol solution less than 2% was released. Approximately 2-log reduction of *Enterococcus faecalis* was achieved at this amount (Chung, Papadakis, and Yam 2003). While the reduction is impressive for such a small amount of antimicrobial, due to the high minimum inhibitory concentration (MIC) of triclosan, it might not be sufficient to prevent illness in humans. In addition, the majority of foods do not contain a high enough proportion of ethanol or other hydrophobic substances on their surface, so the triclosan may not even be released from the packaging material. Another study using triclosan-containing films showed some inhibition in laboratory conditions but none when applied to chicken breasts vacuum-packed and stored at 7°C (Vermeiren, Devlieghere, and Debevere 2002). Due to suspected correlation between resistance to triclosan and resistance to other antimicrobials and antibiotics (Suller and Russell 2000; Schweizer

2001), the widespread use of triclosan should not be encouraged, especially not in food systems. Perhaps a more efficient conclusion from these results would be the use of triclosan in low amounts can be effective in combination with natural antimicrobials or other stresses to the bacterial system to provide a preservation system with multiple modes of action. This would prevent resistance from becoming more widespread, as will be discussed in more detail later in this review.

A frequent deficiency on the part of some experiments is the lack of proper study design, which should include measurements of essential parameters including pH, released substance vs. amount retained in the packaging, kinetics of growth and effects of the active substances on resistance. For example, conclusions in a study focusing on nisin release from cellulose-based films attributed the failure of nisin to inhibit *Listeria monocytogenes* to neutralization of the bacteriocin by the pH of the peptone water used as a food stimulant. Yet pH was not measured over time, and no pH data was given. In addition, no zones of inhibition were seen between the first 30 minutes and 4 days but at day 8 the inhibition reappeared. This was attributed to the possibility that the nisin released in the first 30 minutes was neutralized by 24 h, but it took until the time between 4 days and 8 days for the remaining amount of nisin to be released to act against the microorganism and overcome the pH neutralization (Grower, Cooksey, and Getty 2004). Again, there were no data to confirm this assumption besides the fact that at 8 days there was inhibition while at 4 days there was none. This lag in release can allow bacteria not only to overcome the stress of the antimicrobial but also for survivors of the first wave of release to develop resistance.

Sometimes, the results using liquid media or lab simulants are not very impressive to begin with, and the application of these systems to real life applications also results in further unimpressive results. In one study, researchers tested a nisin-containing film against *Micrococcus luteus*, a common reference organism, in TSB media and milk. At 4°C, less than 1-log reduction was seen over 50 hours as an effect of nisin; at 25°C, the nisin-treated cells reached, over 30 hours, the same concentration as untreated cells took approximately 5 hours to reach. While the slowed growth is promising, the cells still reached the same concentration in a relatively short period of time. When the coating was tested in raw milk, pasteurized milk and UHT milk, the largest decrease in cell numbers was 2-logs, in pasteurized milk. Only in raw milk was a pH decrease seen, which seems to be an effect of the native lactic acid bacteria present in raw milk rather than an effect of the action of nisin on the *M. luteus*. In the release rate studies, it was shown that a back-absorption mechanism, in which the released nisin is reabsorbed by the film, resulting in an alternation between high inhibition and low inhibition (Mauriello et al. 2005). While the results of this study alone do not lead to a fully developed packaging concept, the data obtained from these experiments can aid in the development of a combination system, with a second antimicrobial to supplement nisin.

However, the attempt to validate active packaging systems in real food products leads to increased knowledge as to the potentials and limitations of the systems – as long as the results are accurately reported and not inflated in significance. Chitosan films have been tested for antimicrobial activity for a variety of foods, sometimes in combination with

other substances and sometimes as the sole antimicrobial. When tested as a coating on *Pseudomonas*-challenged Emmental cheese samples, chitosan films reduced cell counts by more than 2 logs (Coma, Deschamps, and Martial-Gros 2003). Though not an impressive reduction, the results could lead to further development of the films using additional antimicrobials to further stress the cells. Indeed, chitosan films containing lysozyme, a lytic enzyme used in cheese manufacturing to prevent growth of *Clostridia* ssp., were also tested against cheese following preliminary laboratory tests, this time of the Mozzarella variety. The films reduced cell counts of *P. fluorescens* by slightly more than 2 logs and all other microorganisms tested by less than 2 logs, yet the significance is marred by the fact that the control cell counts dropped approximately one log over the two week test period. This drop is attributed to lactic acid produced by the native bacteria in cheese, which makes the actual effect of the chitosan and lysozyme difficult to determine. In addition, only one strain of yeast was evaluated, *Candida inconspicua*, and the results showed almost no inhibition by the coatings. When tested against molds, less than 2-log reduction was seen, yet the authors called it 'complete inhibition' (Duan et al. 2007). Considering yeast and molds are the most common colonizers of cheese products, testing more species would have been prudent and a more realistic view of the results is necessary so as not to over-inflate the effects of systems that still require more research and fine-tuning.

Another example of a system tested in various real-life applications is that of the ethanol-emitter. Ethanol, when emitted from a sachet or packaging material, lowers the water activity of the system and acts as an antimicrobial (de Kruijf et al. 2002; Franke, Wijma,

and Bouma 2002). As bakery products are particularly susceptible to yeast and mold contamination, since they do not contain the preservatives present in commercial baked goods, the absorption of ethanol from the headspace could extend the shelf-life significantly. At the two highest doses of ethanol tested, the buns remained yeast- and mold-free for almost 20 days as opposed to the 4 days seen in untreated control buns. However, bacterial counts were too high, due mainly to the growth of *Bacillus cereus* in the center of the buns (Franke, Wijma, and Bouma 2002). Further research should be dedicated to reducing the bacterial count through other methods in combination with the ethanol.

Many active packaging concepts utilizing nisin as an antimicrobial have been evaluated, especially in combination with other stresses or antimicrobials to provide a multiple hurdle approach. This approach utilizes multiple stresses on the cells with different modes of action in the pursuit of maintaining food safety without causing resistance to any one stress factor. Many of these combinatorial studies utilize nisin, an antibacterial peptide produced by *Lactococcus lactis* that has GRAS status as a food preservative for the inhibition of *Clostridium botulinum* and other bacteria for certain food applications (US Food and Drug Administration, 2007). One such study, assessing antimicrobial packaging materials made from acrylic and vinyl acetate-ethylene polymers, was aimed specifically at highly liquid foods and beverages. The polymer caused a 6-log reduction of total aerobic counts from the control samples. The authors speculated that the targeted cells were sensitized to nisin by additional stress factors, specifically sublethal injury to the cells from pasteurization, so further research was required to check the effects of

pasteurization levels and possible post-process contamination on initial cell counts as well as nisin activity (Kim et al. 2002). Another study along the same lines examined low-density polyethylene films containing nisin and lactocin for fresh oyster and ground beef packaging. The goal was to extend the shelf life of real food systems beyond their current limits. Unfortunately, the promising laboratory results did not translate into an extension of the shelf-life in this case, with less than 2-log reduction in total aerobic and coliform counts at 10°C for both oysters and ground beef (Kim, Paik, and Lee 2002). The authors deemed this reduction significant, but in foods that are so often involved with recalls and pathogen outbreaks like ground beef (Food Safety and Inspection Service, 2006, 2007, 2008), more research needs to be done into further preventing cell growth through higher concentrations or combinations of antimicrobials.

Errors in judgment, like the above-mentioned, are occasionally seen when assessing results, but sometimes erroneous assumptions plague a paper from start to finish. One such paper involves evaluation of composite films formed from poly(lactic acid), also called PLA, and pectin for use in antimicrobial packaging (Liu et al. 2007). First, assumptions are made regarding the antimicrobial activity of PLA polymer based on prior results from PLA oligomers in solution or PLA monomers used in conjunction with antimicrobials. Polymers are not the same as solutions and do not behave the same at all, and certainly antimicrobial activity cannot be assumed for one component in a mixture when other components in the solution possess their own antimicrobial activities. Then, when the polymers are formed, they are loaded with nisin before being evaluated for inherent antimicrobial activity. If the polymer is expected to have its own antimicrobial

activity, why not test it before loading nisin to ascertain its activity and get a baseline activity? The choice of bacterial strain was not justified, and the application for which this polymer was being researched was also not clearly outlined from the beginning. The chemical and physical evaluations of the polymer were detailed and well-outlined, but the antimicrobial data were given very little attention or thought. An image of the control and nisin-loaded polymer samples tested for antimicrobial activity claims that samples without nisin have no zones of inhibition. However, even a cursory examination of the image shows that the pectin/PLA polymer without nisin has a zone of inhibition. There is no diffusion of antimicrobial, since it is a result of the polymer itself and not a diffusible compound, but the zone is clearly apparent. There are no measurements of the zone given, no comparison to nisin solution or diluent as additional controls – simply a denotation of the zone size as 'significant inhibition'. How can significance be determined without comparison? A line chart comparing cell growth in the presence of polymer samples fails to elaborate on the control sample, simply writing 'a control'; since the previous image showed the PLA/pectin control contained its own antimicrobial effects, the distinction of which polymer was used as the control becomes of utmost importance to prevent skewing of the results. The time marks on the chart are not equidistant, leading to an altered and inaccurate view of the results. Overall, the paper started with a good idea but as a result of all the misjudgments that occurred, the results can't be considered reliable or accurate.

Another such problematic study involved cellulose casings for frankfurters coated with nisin (Luchansky and Call 2004). The concept of nisin resistance developing in cells from

prolonged exposure to the antimicrobial is not a novel one, yet it is introduced at the beginning but never pursued in the rest of the research. The difference in *L. monocytogenes* growth between control casings and casings coated with nisin was about 0.2-logs, a very insignificant number; the authors referred to it in rather unscientific terms as 'decreased somewhat'. In addition, tests in which lactic acid bacteria were used showed higher levels of cells in nisin-coated casings than in the controls. After 90 days (a typical shelf-life for refrigerated frankfurters), *L. monocytogenes* reached the same cell density in control and nisin-coated samples. Therefore, what was the effect of the nisin in this case? The cell growth in nisin-coated frankfurters decreased until 15 days, then rose to the same levels as the control. There is no indication that testing was done to determine if the survivors had nisin resistance following the treatment, as was alluded to in the beginning of the paper. If the cells had become resistant, than using the antimicrobial packaging could theoretically be worse for food safety than not using antimicrobials. The authors maintain in the discussion that resistance is unlikely because three strains of *L. monocytogenes* were used, yet why is it unreasonable to consider that under the same conditions, three closely related strains of the same microorganism might react in the same manner? Rather than just making assumptions, actual tests should have been done to determine whether or not resistance was acquired by the strains. The authors also maintain that the manufacturing process did not contribute to the lack of activity of the nisin coatings because a second set of samples from a similar manufacturing process was used. Why wasn't a comparison done with products using a different manufacturing process, to see if differences really occur as a result? Using all similarly-produced samples and then claiming that because the results were the same, it can't be the

manufacturing process, is not a valid scientific method. Further on, the possibility of comparing the coatings on frankfurters with different ingredients and processing techniques is mentioned, yet it would have been fairly simple to have done it together with the currently-published data, and the results would have been far more robust as a result. Towards the end, further research is mentioned, aiming to use higher levels of nisin (to overcome the diminishing effect) and 'variants of nisin' either alone or in combination with other substances. A better explanation of such statements is required, and assays to determine if resistance occurs should have been the first thing planned for future research.

A sad and avoidable result of unsatisfactory experimental designs and poor analysis of the obtained data in the aforementioned and many other published papers are frequently due to the fact that the study of controlled delivery systems require multi-disciplinary teams comprised of food microbiologists, chemists and engineers. Each field contains nuances which may not be fully explored by many current studies, such as cases where the packaging structure and chemistry are fully realized but microbiological tests are underdeveloped. Only through the combined efforts of all aspects of food science can successful active packaging concepts be developed

IMPLICATIONS FOR THE FUTURE

Consumers today expect more from their foods. They prefer fresh or local foods and search for claims of health benefits, antioxidant content, probiotic and prebiotic content, and fiber. The development of packaging that keeps their food safe by releasing

compounds that maintain food safety and enhance food quality and also utilizes substances of natural origin was an intuitive extension of current trends in the marketplace. As mentioned previously, hurdle technology, the use of multiple preservation techniques in the same product, is especially desirable today with the current trend towards shelf-stable convenience foods (Vermeiren, Devlieghere, and Debevere 2002; Rao, Chander, and Sharma 2005). The use of multiple types of stresses can extend the shelf-life of many food products without exceeding the legal limit for many antimicrobial compounds or changing the sensory qualities of the food. Research geared towards the use of antimicrobials that work synergistically with other stresses (including temperature, osmotic, pH and antimicrobial compounds) has the potential to greatly enhance the area of food safety.

A further extension of the active packaging trend would be multi-purpose films or other examples of hurdle technology. Films containing nisin and α-tocopherol have been studied in milk cream applications in order to deliver antimicrobial and antioxidant functionality within the same packaging concept (Lee et al. 2004). Films containing multiple antimicrobials with different mechanisms of action, like bacteriocins together with chitosan or essential oils, are another method of maintaining food safety and quality while still upholding a natural viewpoint. One such film used chitosan as the base material and irradiated it to increase its antioxidant capabilities. When applied to fresh meat products, fungal and bacterial growth were inhibited for up to 28 days of storage at ambient temperatures (Rao, Chander, and Sharma 2005). Another novel hurdle technology concept is the combination of modified atmosphere packaging (MAP) with

active packaging. The application, aimed mostly at preventing mold and yeast growth on bread, utilized volatile mustard EO together with different levels of CO_2 and O_2 gases in the atmosphere of the package (Suhr and Nielsen 2005). The inhibitory effect could be overcome with time and/or high inoculum levels, but the validity of the concept holds true and the potential for future use is high. MAP alone can lead to elevated levels of psychrotrophic and anaerobic pathogens, which can lead to food safety issues (Suppakul et al.2003). Engineering active packaging that specifically counters this tendency has many possibilities.

Hurdle technology is an extremely important tactic in the battle against current food safety and human health issues. Biofilms have the most defensive mechanisms against hostile environments and stresses found in prokaryotic life, including low pH (Li et al. 2001; Marsh 2004) and limited oxygen diffusion (Walters et al. 2003). These biofilms consist of bacterial cells networked in an extracellular matrix that communicate through releasing and responding to signaling molecules (Costerton et al. 2003). In order to eradicate bacteria, which preferentially grow in a biofilm, the concentration of antimicrobial or antibiotic must be many times higher than the minimum inhibitory concentration, or MIC (Benoit et al. 1997). It has been discovered that not only do sublethal concentrations of antimicrobials fail to eliminate pathogenic or spoilage organisms, they can cause resistance to other antimicrobials and even promote biofilm formation (O'Toole and Stewart 2005).

The option of using bacteriocins, especially in a controlled release application, may offer a way to treat resistant bacterial strains (Bower et al. 2002; Chi-Zhang, Yam, and Chikindas 2004). Studies have shown that while instantaneous release of nisin can inhibit cell growth, the survivors will undergo mutations to develop resistance to nisin. On the other hand, merely releasing nisin from packaging without any nisin added directly into the formulation did not reduce cell counts. A combination of the two resulted both in reduced cell counts and lack of mutation; instead, the cells adapted and regained their sensitivity to nisin following one passage through nisin-free medium (Chi-Zhang, Yam, and Chikindas 2004). Since the main target of antimicrobial peptides is the bacterial membrane, resistance to them would require a restructuring of the membrane structure. In addition, preventing the initial attachment of bacteria to prevent biofilm formation greatly reduces the inherent resistance to stresses within the cell.

Utilizing a combination approach, similar to the way other organisms combat bacterial infection, would result in more effective treatments that prevent the development of resistant strains as best as possible (Etienne et al. 2004). Chitosan films containing other antimicrobials are one example of hurdle technology as applied to active packaging. The GRAS status of certain bacteriocins, like nisin and pediocin, in certain foods and proven safety in clinical trials (Bower et al. 2002; Lee et al. 2003) allow them to be used either as an alternative to or in conjunction with other antimicrobials for a hurdle effect (Grower, Cooksey, and Getty 2004). Specifically, nisin works extremely well against Gram-positive organisms while chitosan inhibits growth of a wide range of microorganisms, including yeasts and molds (Lee et al. 2003). Essential oils have been investigated for the

same purposes, due to mechanisms of action different from common antimicrobials and the GRAS status some of them possess (Becerril et al. 2007).

While many papers regarding controlled release applications for the medical field discuss the possibility of resistant strains developing (Etienne et al. 2004; Huneault et al. 2004), almost no studies involving food systems investigate the development of resistance as a result of the antimicrobials involved in the research. When they do, it is often brief and without experimental data from an investigation of whether resistance occurs or how it could be avoided. The researchers emphasize extended lag phases of growth, with the resulting cells growing to lower cell counts (Lee et al. 2003). However, tests for mutations or adaptations to the antimicrobial are not done or discussed, and neither are the implications of such a recovery on shelf-life.

Active packaging has the potential to enhance food safety and help prevent the formation of resistant strains of bacteria, but in addition to testing for the occurrence of resistance in survivors of the treatments, safety evaluations for the active compounds and packaging materials are also required. Although many materials used in active packaging systems are safe for use in foods on their own, the mere act of incorporating them into a new packaging system changes the regulatory rules. Some essential oil components are considered flavorings in the EU and have GRAS status in the US, while others are specifically prohibited for toxicological reasons. Some compounds can cause irritation due to cytotoxic effects, while others can cause allergic or spasmodic reactions. In addition, organoleptic changes may occur due to the release of some active compounds,

like EOs or their main components (Burt 2004). In the EU, no specific regulations exist for active packaging systems. Compounds released into the food would fall under the category of food additives and be subject to those particular laws, but active compounds that remain in the packaging materials would be considered food-contact material constituents. Regulations for food-contact materials are very strict to prevent migration of undesirable components into the food (de Kruijf et al. 2002). As of 2003, a limit of 10 mg/dm^2 has been set for migration of active materials from packaging polymers (Vartiainen et al. 2003). Assurance that the compounds utilized are safe for use in humans, especially through research evaluating their safety specifically from the standpoint of an active packaging system, will greatly influence the ongoing legal debate. As active packaging research addresses the mentioned issues, including the possibility of resistance, the safety of the packaging and functional materials, and the synergistic effects of different types of compounds, the number of commercial systems should expand even more in the consumer consciousness and the market.

CONCLUSIONS

A comprehensive review of the available information on controlled release and active packaging systems presents an interesting and as-yet-incomplete picture. The majority of active packaging systems are aimed at improving food quality and safety through the reduction of bacterial growth and the extension of product shelf-life. While some preliminary results of the research are promising, the vast majority are limited in scope. A realistic view of the actual efficacies of the main components is required to expand the formulations into a complete active packaging system. Synergy between functional

substances with different modes of action will assuredly develop the systems into safe, efficient packaging concepts that serve to extend shelf-life, maintain food quality and prevent adverse conditions like biofilm formation and resistance promotion in bacteria.

Salicylic acid-based poly(anhydride-esters) for control of biofilm formation in *Salmonella enterica* serovar Typhimurium

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Running title: Control of *Salmonella* biofilms

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ABSTRACT

Aims: Bacterial biofilms generally are more resistant to stresses as compared to free planktonic cells. Therefore, the discovery of antimicrobial stress factors that have strong inhibitory effects on bacterial biofilm formation would have great impact on the food, personal care, and medical industries.

Methods and Results: Salicylate-based poly(anhydride-esters) have previously been shown to inhibit biofilm formation, possibly by affecting surface attachment. Our research evaluated the effect of salicylate-based poly(anhydride-esters) on biofilm forming *Salmonella enterica* serovar Typhimurium. To remove factors associated with surface physical and chemical parameters, we utilized a strain that forms biofilms at the air-liquid interface. Surface properties can influence biofilm characteristics, so the lack of attachment to a solid surface eliminates those constraints. The results indicate that the salicylic acid-based polymers do interfere with biofilm formation, as a clear difference was seen between bacterial strains that form biofilms at the air-liquid interface (top-forming) and those that form at the surface-liquid interface (bottom-forming).

Conclusion: These results lead to the conclusion that the polymers may not interfere with attachment; rather, the polymers likely affect another mechanism essential for biofilm formation in *Salmonella*.

Significance and Impact: Biofilm formation can be prevented through controlled release of nature-derived antimicrobials formulated into polymer systems.

Keywords: biofilm, poly(anhydride-esters), *Salmonella*, salicylic acid, antimicrobials

INTRODUCTION

Bacterial contamination, especially in the form of biofilms, represents one of the major concerns of the food, personal care and medical industries today. Biofilms have the most defensive mechanisms against hostile environments and stresses found in prokaryotic life, including low pH (Li *et al.* 2001; Marsh 2004) and limited oxygen diffusion (Walters *et al.* 2003). These biofilms consist of bacterial cells networked in an extracellular matrix that communicate through releasing and responding to signaling molecules (Costerton *et al.* 2003). As attachment or adsorption of bacteria to a surface typically is the first step in the formation of biofilms (Costerton *et al.* 2003; Marsh 2004; O'Toole and Stewart 2005; Parsek and Greenberg 2005), control of attachment of bacteria to surfaces and subsequent formation of biofilm structures will significantly improve the ability to control biofilm formation in environments required to be sterile.

Biofilms have the ability to grow on many surfaces (Branda *et al.* 2005) and lead to many problems throughout the food industry, including biofouling of dairy and food processing equipment and spoilage in meat and poultry applications (Kumar and Anand 1998). Biofilms are also known to be associated with a high infection rate in medical implants, often leading to removal of the device (Costerton *et al.* 2003; Bryers *et al.* 2006). Another medical problem related to biofilms is chronic infections, such as those associated with cystic fibrosis (Costerton *et al.* 2003) or dental plaque (Marsh 2004).

Controlled delivery of antimicrobials *via* degradable polymers has the potential to reduce microbial contamination through the sustained release of antimicrobial substances that specifically act against biofilms. The development of a poly(anhydride-ester) or PAE resulted in polymers that yield salicylic acid (Erdmann and Uhrich 2000), the active

component of aspirin, as it undergoes hydrolytic degradation (Erdmann *et al.* 2000). Salicylates and other non-steroidal anti-inflammatory drugs (NSAIDs) are known to prevent bacterial adhesion onto medical devices (Arciola *et al.* 1998), though the mechanism has not been identified. PAEs comprised of NSAIDs such as salicylic acid, like other polyanhydrides (Tamada and Langer 1993; von Burkersroda *et al.* 2002), predominately undergo surface erosion (Whitaker-Brothers *et al.* 2004). The PAEs are unique because polymer degradation directly controls the release of salicylic acid and antimicrobial(s) if previously admixed (Johnson and Uhrich 2006).

Salicylic acid and its derivatives are appealing for use in medical, food and personal care applications because they are antiseptic, anti-infective but not antimicrobial, hence there is little long-term possibility of creating microbial resistance. NSAIDs do not induce antibiotic resistance in bacteria, as shown with some antimicrobial substances (Gilbert *et al.* 2002; Prithiviraj *et al.* 2005). Salicylic acid is a compound produced by many plants as part of their defense against microbial invasion. Salicylic acid has also proven useful for treatment of human ailments. Aspirin (acetylsalicylic acid), a commonly used NSAID, is broken down by the body into salicylic acid (Paterson *et al.* 2006) to inhibit production of cyclo-oxygenase (COX), which is the key to the body's inflammatory response.

Biofilms have been identified as an issue of concern in food processing environments (Kumar and Anand 1998; Wong 1998), eye care (Perilli *et al.* 2000), oral care (Phan *et al.* 2000), chronic infections and implant contamination (Muller *et al.* 1998; Polonio *et al.* 2001). In many of these cases, salicylic acid or similar molecules have been investigated for efficacy against biofilm-forming bacteria. In the case of contact lens

biofilms formed by *Staphylococcus epidermidis*, salicylate is not available in common eye care products; sodium diclofenac, an NSAID with a similar mode of action, was substituted. For strains that did not contain slime-associated antigen (SAA), 15 minutes of treatment with 0.25 mg ml^{-1} sodium diclofenac significantly reduced the optical density of biofilms adhered to microplate wells (Perilli *et al.* 2000). In a different study, 5 mmol l^{-1} of salicylic acid was seen to inhibit production of SAAs in *Staph. epidermidis*, as well as affecting cation chelation (Muller *et al.* 1998).

In an attempt to address catheter-related infections common to hospitals, a combination of sodium salicylate and vancomycin were examined as a treatment against *Staph. epidermidis* biofilms. Five mmol l^{-1} of sodium salicylate combined with 1 μg ml^{-1} of vancomycin was significantly more effective than either treatment alone. In addition, one-half the minimum biofilm eradication concentration (MBEC) combined with sodium salicylate reduced the number of viable biofilm cells >99.9 %, while neither treatment alone had any significant effect (Polonio *et al.* 2001).

The dental plaque organisms *Actinomyces naeslundii* and *Streptococcus sanguis* are relatively acid-sensitive compared with other oral pathogens, which made them suited to examine the effects of weak acids like fluoride, salicylate and sorbate on dental biofilms. Fluoride was proven to enhance acid killing of these cells; so did benzoate, sorbate and salicylate to a lesser degree. They also enhanced proton permeability, indicating that the membrane is partially responsible for resistance to acid killing by increasing F-ATPase activity to pump out protons and maintain pH (Phan *et al.* 2000).

Other experiments focused on the use of salicylic acid in combating serious risks to human health. *Pseudomonas aeruginosa* is an opportunistic pathogen in humans,

especially immuno-compromised individuals. It exhibits numerous cell-associated and excreted virulence factors, with intrinsic and acquired resistance to a wide array of antimicrobial treatments (Prithiviraj *et al.* 2005). *Ps. aeruginosa* is a plant and human pathogen and, as mentioned above, salicylic acid is a plant defense hormone (Prithiviraj *et al.* 2005, Paterson *et al.* 2006). It has been shown that endogenous as well as added salicylic acid can affect bacterial attachment, biofilm formation and exoenzyme production, resulting in reduction of virulence and pathogenicity (Prithiviraj *et al.* 2005).

The above reports are based on salicylic acid molecules added instantaneously, rather than controlled release treatments. The objective of this study was to investigate the activity of salicylic acid-based PAEs against pathogenic bacteria that form biofilms. The increase in recognition of food borne pathogens and nosocomial infections as triggered by biofilm forming bacteria (Costerton *et al.* 2003) and the increased resistance to stresses have led to investigation into treatments specifically for biofilms (Marsh 2004). The utilization of many kinds of biofilm forming bacteria is extremely important to widen the scope of the research.

Preliminary data showed that the controlled release of NSAIDs prevented biofilm formation of *Ps. aeruginosa* on polymer surfaces (Bryers *et al.* 2006). Therefore, elucidation of the mechanism of biofilm prevention *via* localized NSAID release should lead to effective methods of controlled release of NSAIDs to inhibit biofilm formation. This paper examined the effects of controlled release of salicylic acid-based PAEs on biofilm formation and growth. If successful, the elimination of bacterial biofilms should certainly improve methods of controlling bacterial contamination for the food, medical and personal care industries.

MATERIALS AND METHODS

Poly(anhydride-ester) synthesis and formation of polymer-coated glass coverslips

Poly[1,6-bis(o-carboxyphenoxy)-hexanoate] (Fig 1) (1) was prepared using previously described methods (Schmeltzer et al. 2003; Prudencio et al. 2005). Salicylic acid (3) was coupled to adipoyl chloride using tetrahydrofuran in the presence of base, to yield salicylic acid-based monomer precursor or diacid, 2. The diacid (2) was activated by acetylation, which was polymerized via melt-condensation to yield the polymer, 1. The characteristics of the polymer used in this study are: M_w: 16,000, polydispersity index (PDI): 1.3; T_g: 59°C, T_d: 292°C; Contact Angle: 77°.

Polymer **1** was dissolved in methylene chloride (10 g l^{-1}) and solvent-cast onto microscope glass coverslips (Fisher Scientific, Fair Lawn, NJ; 12 mm diameter, 0.15 mm thickness). The coated coverslips were allowed to dry at room temperature for 12 h, and under vacuum at room temperature for 12 h, to ensure full solvent removal. The coverslips were weighed using an analytical balance (Mettler Toledo, AB104-S/FACT, Columbus, OH) before and after coating to determine mass of polymer applied. The thickness of the coating was determined using a digital micrometer (Fowler ProMax, Newton, MA). Before coating, the coverslips were cleaned using Alconox (Alconox Inc., NY) and H_2SO_4:H_2O_2 (10:1, v/v) solutions and stored in ethanol. All reagents and fine chemicals were purchased from Aldrich (Milwaukee, WI). Solvents were purchased from Fisher Scientific (Pittsburg, PA).

Preparation of salicylic acid polymer-coated glass coverslips for microbiological assay

The glass coverslips coated with polymer and uncoated glass coverslips used as controls were placed into sterile plastic Petri dishes (Fisher). The Fotodyne Incorporated (New Berlin, WI) UV light device surface was sterilized with 70 % ethanol, glass coverslips were placed on the surface, and it was activated for 120 s. The coverslips were immediately transferred to sterile Petri dishes using sterile forceps (Fisher).

Preparation and incubation of the biofilm plates

Salmonella enterica serovar Typhimurium MAE52 (Scher et al. 2005) and Salmonella enterica serovar Typhimurium JSG210 (Prouty et al. 2002) were streaked onto Brain Heart Infusion (BHI) plates (37 g l-1; Becton, Dickinson and Company [BD]; Sparks, MD, USA) containing 1.85 % granulated agar (BD) and incubated overnight at 37 °C. Glass test tubes (16x125 mm, Fisher) containing 4.5 ml of BHI broth were inoculated with a colony isolated from the streak plate and incubated overnight at 37 °C. The optical density at 600 nm (OD_{600}) of the overnight culture was measured using a Bio-Rad SmartSpec™ 3000 spectrophotometer. The cultures were then serially diluted from the overnight culture (10^8 CFU ml^{-1}) to 10^2-10^5 CFU ml^{-1} for inoculation. Each well contained 1.8 ml of BHI broth, except the media control wells, which contained 2.0 ml of BHI broth. Then 200 µl of either a diluted inoculum or the original overnight culture was aliquoted into the test wells according to experimental design. The sterile uncoated glass coverslips (control) or polymer-coated coverslips (treatment) were placed into each well with sterile forceps, and the plates were incubated at 37 °C with aeration for 40 h. Images were recorded by a 6.3 megapixel Fujifilm FinePix digital camera on the Macro setting

pre-incubation, post-incubation and at intervals during the incubation process (data not shown).

Biofilm-associated and free cell enumeration

After 40 h, the plates were removed from the incubator, at which time the coverslips from wells containing *Salm. enterica* JSG210 were removed with sterile forceps and placed into a Petri dish containing ~30 ml of 0.85 % saline solution. The coverslips were held between the forceps and washed gently in the saline with back-and-forth motion for 2 minutes to remove all unattached cells. The discs were placed into glass test tubes containing 4.5 ml of saline and vortexed (Fischer Vortex Genie 2™) strongly for a minute at room temperature to remove all attached biofilm cells, which were then serially diluted and plated to enumerate control cells and survivors of salicylic acid treatment. For control wells of *Salm. enterica* MAE52, the biofilms were removed from the air-liquid interface using a pipette tip and washed gently in a Petri dish containing ~30 ml of saline to remove unattached cells. The whole biofilms were then placed into glass test tubes containing ~30 (3 mm) glass beads (Scher *et al.* 2005) and 4.5 ml of saline solution and vortexed strongly for 1 minute. The disrupted biofilms were serially diluted and plated to enumerate viable cells. A final OD_{600} was measured to determine the cell density of the planktonic cells in each well, from which cell counts were mathematically determined (Figure 2). UV/vis measurements of cell-free supernatants were taken at 213 nm to determine final concentrations of salicylic acid released as the polymer degraded.

Statistics

Each experiment was carried out in duplicate at least twice for a minimal sample size of 4. Student's t-test was used to compare replicates within the experiment and to determine if there was significant difference between control biofilms and treatment biofilm cells ($p<0.01$).

RESULTS

High salicylic acid concentration kills free planktonic cells

Data were collected to determine a working concentration of salicylic acid that would inhibit biofilm formation without killing cells. To eliminate lethality as the mechanism of action, experiments were performed at a series of salicylic acid concentrations: concentrations above 1.0 g l^{-1} react irreversibly with the cell, resulting in cell death, but at concentrations below 1.0 g l^{-1}, no effect on cell density was observed (data not shown). Salicylic acid kills bacterial cells at high concentrations which may interfere with determination of biofilm inhibition, so the concentration of salicylic acid-based polymer in further experiments was kept at sublethal concentrations.

Biofilm forming ability of cells, but not cell density, is affected by salicylic acid-based polymer-coated coverslips

Thus far, it was in question whether the lack of biofilm formation of organisms exposed to salicylic acid-based polymers was the result of interference with biofilm forming ability of the cells or reduction of viable cells to numbers sufficiently low such that biofilm formation could not be initiated. The biofilm-associated cell numbers were determined through plating of the biofilm cells and the planktonic cell numbers were determined by plotting the OD_{600} against the calibration curve for the microorganism

growth kinetics (data not shown). The cell densities for the different samples are presented in Figure 2 as a compilation of biofilm cells and planktonic cells at the different starter inocula levels. Planktonic cell density was high in both control and treatment wells of *Salm. enterica* MAE52 and *Salm. enterica* JSG210. The slight difference seen in the *Salm. enterica* MAE52 cell densities is not significant (as determined by the Students t-test) and can be attributed to the formation of the biofilm, which only occurred in the treatment plate at the highest level of inoculum (10^7 CFU ml^{-1}). The data clearly suggest that low cell density is not the reason for lack of biofilm formation in the *Salm. enterica* MAE52 treatment plate at the lower inocula levels. However, there was significant difference between biofilm-associated cell densities of control and treatment *Salm. enterica* MAE52, with p= 0.004086. The final concentrations of salicylic acid remaining at the end of the experiment for each of the variables were determined using UV/vis analysis. The amounts detected (0.0012 ± 0.0002 g l^{-1}) were lower than levels with no cells present (0.0020 ± 0.0002 g l^{-1}). Instead of more salicylic acid being released due to enzymes secreted by the bacterial cells, the amounts were very low, indicating that the inhibition of the biofilm was caused by irreversible interaction of salicylic acid molecules with the cells.

Attachment of cells is not affected by salicylic acid-based polymer-coated coverslips

In addition, it was hypothesized that salicylic acid-based polymers hindered biofilm formation through interference with the attachment mechanism. Our results indicate that although biofilm formation was inhibited, it may be through some mechanism other than attachment. *Salm. enterica* MAE52 forms biofilms at the air-liquid interface (top-forming); *Salm. enterica* JSG210 forms biofilm on a tangible surface (bottom-forming).

Since the salicylic acid-based polymer-coated coverslips lay on the bottom of the wells, if the salicylic acid-based polymer interfered with attachment, a clear difference in the biofilm-associated cell densities of the control and treatment plates of *Salm. enterica* JSG210 should be observed. Instead, the treatment wells inoculated with *Salm. enterica* JSG210 had similar biofilm-associated cell densities as the control wells. However, the treatment wells inoculated with *Salm. enterica* MAE52 did not form biofilms at all, except at the highest level of inoculum (Figure 2). In addition, it appears that this inhibition of biofilm formation was dependant upon the initial cell load. The lower inocula (10^2 –10^5 CFU ml^{-1}) of *Salm. enterica* MAE52 grew to high cell density but could not form biofilms. Only at the highest initial cell load (10^7 CFU ml^{-1}) did biofilms begin to form in the presence of the salicylic acid-based PAE. These results suggest that salicylic acid-based polymers interfere with biofilm forming abilities of bacteria, but not through the attachment mechanism. The cell load at the end of experiment was more than sufficient to form biofilms, eliminating cell death as a cause of biofilm prevention in this study. The release of the salicylic acid from the polymer might interfere with some other genetic or functional mechanism(s). Further studies of the gene expression in the presence and absence of the antimicrobial, as well as experiments to isolate and identify the active ingredient produced by the cells to break down the salicylic acid-based polymer, will shed light on this process and are in progress.

DISCUSSION

The difference between various biofilm-forming strains of bacteria are clear: distinct structural, physical and functional diversity is observed (Marsh 2004). The approach of using different types of biofilm-formers in the same experimental conditions

can shed light on the processes that underlie biofilm formation and resistance to stresses that kill or inhibit planktonic cells. Bottom forming biofilms are significantly influenced by the surface upon which they grow. Top forming biofilms grow at the air-liquid interface, having no contact with the surface of the polymer, the glass cover slip or the tissue culture plate used in the experiment. The decision to use a top biofilm-forming mutant removed the variable of surface structure and chemistry, allowing the antimicrobial to work on biofilms that would be structurally consistent throughout the experiment. In addition, it removed the attachment mechanism as a factor, since the bacteria aggregate at the air-liquid interface as opposed to a solid surface. The release of salicylic acid from polymer-coated coverslips affects biofilm formation in top-forming films but not bottom-forming films; these results inspire further examination of genes and/or metabolic processes that are affected in the former, but not the latter. In addition, our results suggest that salicylic acid-based polymers may not affect surface attachment, as previously suggested (Bryers *et al.* 2006), and that salicylic acid reacts irreversibly with bacterial cells.

Our future work aims to determine kinetics of the antimicrobial release from the polymeric film, the antimicrobial's interaction with the targeted bacteria, and the exact mode of action of salicylic acid in biofilm inhibition. We plan to isolate the compounds produced by the bacterial cells that release the salicylic acid from its polymer backbone. We also intend to look into how gene expression and transcription are affected by salicylic acid, especially in genes known to be part of biofilm formation and cell communication. Further experiments will look at other natural antimicrobial substances known to be active against bacteria that are safe for human consumption.

Ultimately, the NSAID-containing polymers can be assessed as controlled delivery systems that potentially release two biologically active compounds, NSAIDs generated upon hydrolysis of the polymer backbone and admixed antimicrobials. The polymer as well as the admixed substance can be fabricated into cleaning systems for processing equipment, or into films that can line packaging of food products. The polymer itself can be used to coat or fabricate medical implants and devices, in order to reduce biofilm infections.

Figure Legend:

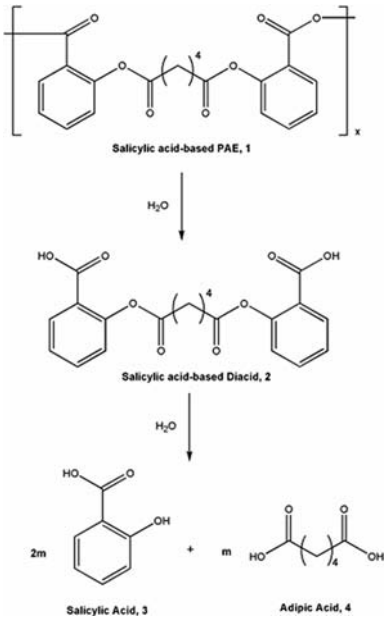

Figure 1. Hydrolysis of salicylic acid-based poly(anhydride-ester) (**1**) to release the bioactive (**3**) and biocompatible linker molecules (**4**) *via* pH-dependent non-enzymatic hydrolytic bond cleavage.

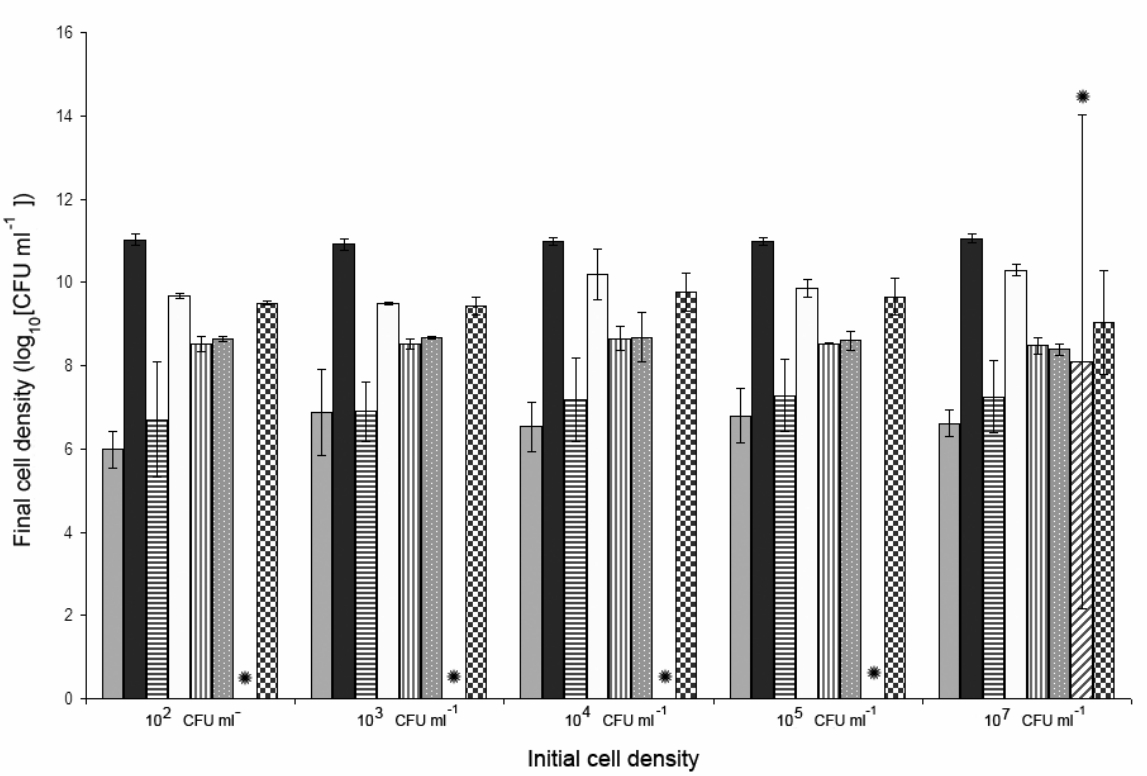

Figure 2. Final planktonic and biofilm-associated cell densities for Salm. enterica serovar Typhimurium MAE52 and JSG210 after 40 h incubation, plotted in log10 (CFU ml-1) against the initial cell loads of 102, 103, 104, 105 and 107 CFU ml-1. At each initial cell load, the bars from left to right represent: JSG210 control biofilm cells, JSG210 control planktonic cells, JSG210 treatment biofilm cells, JSG210 treatment planktonic cells, MAE52 control biofilm cells, MAE52 control planktonic cells, MAE52 treatment biofilms cells, MAE52 treatment planktonic cells. Planktonic cell density was very high, yet MAE52 was still unable to form biofilms in the presence of the salycilic acid-based PAE. Student's t-test was used to compare replicates within the experiment and to determine if there was significant difference between control biofilms and treatment biofilm cells ($p<0.01$). Asterisks mark significant difference in biofilm formation from the control, with the large variation in the 107 CFU ml-1 initial cell load due to biofilms being formed only 50% of the time

Salicylic acid and pH affect biofilm formation by *Salmonella typhimurium* [2]

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Running title: pH effects in *Salmonella* biofilms

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[2] This section of the thesis is being prepared for publication.

ABSTRACT

Aims: The changes that occur during cell growth and biofilm development are not well characterized. In order to identify new targets for antimicrobial systems, the effect of pH changes on biofilms and antimicrobials requires investigation.

Methods and Results: Salicylate-based poly(anhydride-esters) have previously been shown to inhibit biofilm formation. Our research evaluated the pH changes during biofilm forming *Salmonella typhimurium* MAE52. Applying the information thus generated, experiments were done testing the effects of different initial pH levels on biofilm formation and the release of salicylic acid from its polymer form. The results indicate that lower pH alone can inhibit biofilm formation, as can controlled-release salicylic acid. The salicylic acid release, while pH-dependant, continues even as pH drops during cell growth, leading to the conclusion that the release is attained by more than just pH.

Conclusion: These results lead to the conclusion that pH has a significant effect on gene expression, metabolic products and biofilm development. The combination of pH and antimicrobials prevent biofilm formation by *S. typhimurium* MAE52. In addition, lowering the initial pH may prevent metabolic reactions or gene expression that contribute to biofilm formation.

Significance and Impact: Further investigation into the effects of pH, alone and in combination with salicylic acid, on biofilm formation can lead to more efficient systems to prevent contamination by biofilms in many environments.

Keywords: Salmonella, biofilms, pH, salicylic acid, antimicrobial

INTRODUCTION

In their natural environments, bacteria predominantly grow in the form of biofilms, associated communities of cells within a complex polymeric matrix that exhibit increased resistance to environmental stresses. In most of the cases, biofilms started to form when the cells are present in the environment in a high number and the growth phase has passed from exponential to stationary (Li et al. 2001; Marsh 2004; Scher, Romling, and Yaron 2005). The tenacity of these biofilms, including their ability to establish on many different surfaces (Branda et al. 2005), can lead to numerous problems in human health, including persistent infections, rejection of implants and contamination that can lead to food-borne illnesses (Costerton et al. 2003; Kumar and Anand 1998).

Biofilm structure can vary from species to species, depending not only on strain characteristics but also on the nature of the material on which the biofilm forms. This type of diversity is exemplified by *Salmonella enterica*, an organism that causes numerous human health issues including food-borne illness and typhoid fever. Salmonella is one of the most common and widely distributed causes of foodborne diseases, with over 2500 known serotypes (World Health Organization, 2005). *S. enteric*a serovars Typhimurium and Typhi can form biofilms on gallstones, causing the infected to become chronic carriers of the organism. These microorganisms also have the ability to form biofilms on glass, which provides a method of examination in research studies. However, the presence of different materials can cause different genes to be expressed during the biofilm formation, changing the composition of the exo-polysaccharides that form the biofilm matrix (Prouty and Gunn 2003). The effect of surface chemistry and

physical characteristics on gene expression can have major implications for biofilm formation, since most bacteria form biofilms at the interface between a solid surface and a liquid or air environment (Scher, Romling, and Yaron 2005). *S. enterica* serovars are capable of colonizing the interface between the air and liquid phases, primarily due to overproduction of cellulose polymers. The extracellular matrix enhances the resistance to stresses inherent in stationary phase cells, and multiple mechanisms have been proposed, including delayed diffusion due to the matrix and slow growth rate (Scher, Romling, and Yaron 2005).

The effects of external applications of acid and pH stresses have been studied in many biofilm-forming microorganisms (Scher, Romling, and Yaron 2005; Li et al. 2001). However, there is little known about the inherent pH changes in the environment during biofilm formation. Biofilm-associated cells differ in many of their genes' expression from planktonic stationary cells, yet there is limited information about the differences in metabolic products. *S. enterica* serovar Typhimurium (from here on referred to as *S. typhimurium*) produces mainly acetic acid, which in its undissociated state acts as a self-limiting mechanism to halt growth (Wilson et al. 2003). Organic acids have bacteriocidal activity beyond merely decreasing the pH of a solution (Phan, Reidmiller, and Marquis 2000). When *S. typhimurium* was grown in media adjusted to different pH levels, the total amount of acetic acid produced changed due to differing metabolic activities at lower pH. However, there were no significant differences between both the final pH level and amount of undissociated acetic acid resulting from the different initial pH levels (Wilson et al. 2003). While observing the changes in pH during microbial growth, it was

revealed that at initial pH of 4.4 - 5.0, a preliminary rise in pH preceded a rapid decline to a pH of 4.5 – 4.9. This was assumed to be caused by oxidation of amino acids before glucose consumption began. This was not seen when the initial pH of the media was 7.0, though the same final pH range and amount of undissociated acetic acid were attained (Wilson et al. 2003). These results, though very informative as to general metabolic and growth properties of *S. typhimurium*, were determined in planktonic cells. As mentioned previously, cells in a biofilm express different genes than planktonic cells, but the results of this study provided a solid starting point for the investigation into the production of acid and the subsequent effect of pH on biofilm development.

Salicylic acid (SA) is a compound produced by plants as part of their defense against microbial invasion (Paterson et al. 2006) which has proven useful for treatment of human ailments. SA and its derivatives have been investigated for efficacy against biofilm-forming bacteria in food processing environments (Kumar and Anand 1998), eye care (Perilli et al. 2000), oral care (Phan, Reidmiller, and Marquis 2000), chronic infections and implant contamination (Muller et al. 1998; Polonio et al. 2001; Bryers et al. 2006). The controlled release of salicylic acid from a poly(anhydride-ester) or PAE backbone has been proven to prevent biofilm formation in *S. typhimuirum* MAE52, a mutant strain that forms biofilms at the air-liquid interface (Rosenberg et al. 2008). The SA-based PAE undergoes hydrolytic degradation based upon the composition of the polymer, and the rate of hydrolysis of the ester and anhydride bonds directly controls the amount of salicylic acid released (Erdmann, Macedo, and Uhrich 2000; Prudencio, Schmeltzer and Uhrich 2005). This degradation is heavily influenced by the pH of the surrounding

environment. At pH 10, the polymer can degrade completely within 40 hours, while at pH 3.5 the polymer does not degrade significantly over 90 days. Most laboratory media, along with many food and physiological environments, maintain a pH around 7.0; at this pH, the polymer degrades ~50% within 4 days and then the remaining salicylic acid is slowly released until depleted after 1 week (Erdmann and Uhrich 2000; Prudencio, Schmeltzer and Uhrich 2005).

Results from a previous study utilizing *Pseudomonas aeruginosa* biofilms suggested that pH decreases in the media were mainly due to metabolic byproducts of growth rather than the effect of SA. In addition, this decrease in pH should have slowed the degradation of the PAE; instead the polymer degraded more rapidly in the presence of growing cells than it did in cell-free medium (Bryers et al. 2006). Another observation made from the application of SA PAEs to biofilms was that the SA might be interacting irreversibly with the *S. typhimurium* cells (Rosenberg et al. 2008). Since there was little information as to the progression in pH during the growth of *S. typhimurium* and biofilm formation, it was difficult to calculate the amount of SA that should be released from the polymer based on pH alone. Therefore, it was rather challenging to determine the actual amount of SA released from the polymer, especially if the cells are consuming the SA as they grow. Additionally, Bryers et al. (2006) speculated that since SA release progressed faster rather than slower as pH decreased during cell growth, bacteria may release enzymes that enhance the degradation of the polymer. To further study the phenomenon, experiments were conducted to measure pH over the growth of the biofilm to determine if changes occur in the presence of the SA PAE. Here, we report on the changes in pH during the

growth of *S. typhimurium* MAE52. Following the initial experiments outlining the changes in pH resulting from biofilm formation, the initial pH of the media was altered to ascertain the role of pH in biofilm formation as well as salicylic acid release. In order to discover if metabolites produced during cell growth are at all responsible for the release of salicylic acid from its polymer form and subsequent prevention of biofilm formation, the effect of cell-free supernatants was compared to pH-adjusted media.

MATERIALS AND METHODS

Poly(anhydride-ester) synthesis and formation of polymer-coated glass coverslips

Poly[1,6-bis(o-carboxyphenoxy)-hexanoate] was prepared using previously described methods (Prudencio, Schmeltzer and Uhrich 2005; Rosenberg et al. 2008).

Preparation of salicylic acid polymer-coated glass coverslips for microbiological assay

The glass coverslips coated with polymer and uncoated control coverslips were placed in sterile Petri dishes (Fisher Scientific; Fair Lawn, NJ, USA). The Fotodyne Incorporated (New Berlin, WI) UV light device surface was sterilized with 70 % ethanol, coverslips were placed on the surface, and it was activated for 120 s. The coverslips were immediately transferred to sterile Petri dishes using sterile forceps (Fisher).

Preparation and incubation of the biofilm plates for pH measurements during biofilm growth

Salmonella typhimurium MAE52 (Scher *et al.* 2005) was streaked onto Brain Heart Infusion (BHI) plates (37 g l^{-1}; Becton, Dickinson and Company [Becton Dickinson Labware, Franklin Lakes, NJ]; Sparks, MD, USA) containing 1.85 % granulated agar (BD) and incubated overnight at 37 °C. Glass test tubes (16x125 mm, Fisher) containing 4.5 ml of BHI broth (pH 7.2 – 7.3) were inoculated with a colony isolated from streak plates and incubated overnight at 37 °C. The optical density at 600 nm (OD_{600}) of the overnight culture was measured using a Bio-Rad SmartSpec™ 3000 spectrophotometer. The cells were diluted from the overnight culture (10^8 CFU ml^{-1}) to 10^5 CFU ml^{-1} in BHI broth. In each well, 1.8 ml of BHI broth either at pH 7.2 – 7.3 or adjusted to pH 6.0 with 1M HCl (Sigma Aldrich; St. Louis, MO, USA) and 200 µl of the diluted inoculum was aliquoted into the test wells in a 24 well Falcon Multiwell 24 tissue culture plate (Fisher). Controls consisted of 2 ml of BHI broth either at pH 7.2 – 7.3 or adjusted to pH 6.0. The sterile uncoated glass coverslips (control) or polymer-coated coverslips (treatment) were placed into each well with sterile forceps. The plates were incubated at 37 °C with aeration and sampled at 5, 8, 12, 24, and 48 h.

Preparation and incubation of filtered supernatants and pH-adjusted media for pH measurements

Salmonella typhimurium MAE52 was grown in a 24 well plate as described above for 24 h. The growing cells suspensions were collected at 5, 8, 12, and 24 h and the cells were removed by centrifugation at 4500 *g* for 15 min in 15 ml Falcon polypropylene centrifuge tubes (BD) and then filter-sterilized (0.45 µm pore filter, Fisher). The pH was measured

for each time point and aliquots of BHI broth were adjusted to match the pH produced at each time point using 1M HCl or 30% acetic acid (Sigma). Then 24 well plates were filled with either 2 ml of filtered supernatant, 2ml of BHI adjusted with HCl or 2 ml BHI adjusted with acetic acid. Sterile uncoated glass coverslips (control) or polymer-coated coverslips (treatment) were placed into each well with sterile forceps, and the plates were incubated at 37 °C with aeration and sampled at 5, 8, 12, 24, and 48 h. An OD_{600} and pH values were recorded at each time point.

Determination of salicylic acid concentration

Both HPLC and ELISA techniques were used to determine the concentrations of SA in the cell-free samples.

A Salicylates Direct ELISA 96 well kit was obtained from Immunalysis Corporation (Pomona, CA, USA). The samples and calibration standards (0 – 100 μg ml^{-1}) were prepared according to the manufacturer's procedure. The plate was incubated for 1 h in the dark at room temperature, washed with distilled water, inverted and dried on absorbent paper, after which 100 μl of substrate reagent was added to each dried, empty well. The plate was again incubated in the dark at room temperature. After 30 min, 100 μl of stop solution was added to each well and absorbance was read at a dual wavelength of 450 nm and 650 nm.

High performance liquid chromatography (HPLC) was conducted as previously described (Schmeltzer et al. 2008), using a Waters 2695 Separations Module equipped with a Water

2487 Dual Wavelength Absorbance Detector. The wavelength used was 210 nm (for salicylic acid). Samples were filtered through 0.45 micron poly(tetrafluoroethylene) (PTFE) syringe filters (Whatman, Clifton, NJ) before autoinjection. A Gemini reverse phase C18 column (150 x 4.6 mm; Phenomenex, Torrance, CA) was used with 75% phosphate buffer solution (20 mM, pH 2.5) and 25% acetonitrile as the isocratic mobile phase. Sample automation and data processing was done using an IBM ThinkCentre computer running Waters Empower 2 software Build 2154.

Statistics

Each experiment was carried out in triplicate. Student's t-test was used to compare replicates within the experiment and to determine if there was significant difference between control biofilms and treatment biofilm cells ($p<0.01$).

RESULTS

The pH changes over growth of *S. typhimurium* MAE52 cells in a distinctive pattern

The changes in pH over the period of microbial growth and the final values reached were significantly different from data for other *S. typhimurium* serovars (Wilson et al. 2003). The planktonic cells of strain LT2 in those experiments reached a final pH of 4.5 – 5.0, whereas the biofilms of MAE52 lowered the pH of the medium from 7.3 to 5.5 within 5 – 8 h, followed by a rise to 6.0 at 12 h and finally reaching pH 8.5 after 24 h (Fig 1, open squares), at which time biofilms were fully formed.

Lowering the pH of the medium affects biofilm formation and salicylic acid release

The natural pH of BHI broth is between 7.2 and 7.3, well within the optimal range for growth of *Salmonella*, though the cells can also grow at lower pH. In order to clarify the effect of initial pH on biofilm development, experiments were conducted to track the change in pH over the growth of biofilms at two different initial pH media. The optical densities of cells grown in BHI media at pH 7.3 and pH 6.0 were comparable at each time point, indicating little difference in growth. The pH progression of MAE52 cells grown in BHI broth at pH 7.3 and pH 6.0 are presented in Fig 1 as a comparison of different pH levels as well as the presence or absence of the SA-based polymer. At initial pH 7.3, there was marked difference between the pH progressions of the media when cells were grown with and without the polymer. Throughout the first 12 h of growth, the two were almost identical. However, as the biofilms began to form (between 12 and 24 h), cells grown without SA-based polymer reached a pH of 8.5 while cells grown in the presence of SA reached a pH of about 6.0. This difference was minimized by 48 h of growth, at which time cells grown in the presence of the SA-based polymer reached a pH of approximately 8.0.

The pH dependence of the release of SA from the polymer form is depicted in Fig 2. Less than 0.15 $\mu g\ ml^{-1}$ of SA was present in the medium when no polymer was present at both initial pH values. However, SA release from the polymer was greatly influenced by the initial pH in which cells were incubated. When MAE52 was grown over 48 h in BHI with

initial pH 6.0, only 1.5 μg ml^{-1} of SA was released; in contrast, when grown in BHI with initial pH 7.3, 4.5 μg ml^{-1} of SA was released.

The nature of the substance used to attain pH levels does not influence final pH or salicylic acid release from the polymer

To determine if release of SA from the polymer is solely regulated by pH or if other substances produced extracellularly enhanced the release, an experiment was devised using cell-free supernatants. The pH of supernatants from different growth time points of MAE52 were determined, and BHI media were adjusted to the same pH using two different methods. HCl was utilized in order to ascertain if it was merely an effect of pH and acetic acid was utilized to determine if organic acids, specifically, has additional effects. If release was purely pH-dependent, all three samples would release the same amount of SA from the polymer. If organic acids particularly were causing increased release of SA, then the supernatant and acetic-adjusted samples would release more than the HCl-adjusted samples.

DISCUSSION

There is little known about the effects of pH on development of biofilms. These microbial communities appear in stressful environments, yet stresses can alter the actual gene expression and structure within biofilms. In our previous research (Rosenberg et al. 2008), we applied the stress of salicylic acid to *S. typhimurium* biofilms and observed the inability of cells to form biofilms in the presence of the polymer. In further experiments, it was determined that polymer-released SA does not kill cells or disturb the extracellular

matrix in pre-existing biofilms, showing the SA polymer to be prevention of biofilm formation rather than treatment for extant biofilms (data not shown). In our search to discover the method in which the released salicylic acid affected the formation of biofilms, pH measurements were taken. SA is released more rapidly at higher pH levels (Erdmann and Uhrich 2000), so release was expected to slow down or cease as production of organic acids by cells lowered the pH of the surrounding environment. Since the release of SA continued even at this point, it was assumed that the cells must produce some substance that promote the SA's release from the polymer more rapidly than pH alone (Bryers et al. 2006).

The understanding of the biological meaning behind the pH values that occur as *S. typhimurium* MAE52 cells grow and form biofilms is, alone, a significant finding. As mentioned before, little information is available regarding the pH values attained in the surrounding media as biofilms form or effects of different pH on the development of the biofilm. What little is known suggests that changes in external pH can affect many of the cell's physiological functions, which in turn can trigger secondary effects like changes in signaling molecules and metabolic products (Li et al. 2001). Since our research suggested that pH and metabolic products may combine to release salicylic acid and prevent biofilm formation in *S. typhimurium* MAE52, further elucidation of these effects is of utmost importance.

The most interesting finding is the occurrence of a bimodal pH curve; that is, pH decreased for the first 8 h, rose slightly by 12 h and then increased to above the initial pH

by 24 h. The rise in pH coincided with formation of the biofilm, and it has been speculated that the genes that are expressed to cause biofilm development also result in the pH increase. In fact, the experiments composed of adjusting pH of the media to 6.0 demonstrate that if the pH rise does not coincide with those time points, biofilm formation does not occur. This was clearly illustrated as the pH rose to 8.5 after 48 h in the presence of SA, yet despite the high cell density, biofilms didn't develop. This is a significant observation that could lead to innovative methods of controlling and preventing the growth of biofilms in many environments. Further experiments utilizing gene expression studies are under development, and the identification of novel targets involved in biofilm formation can lead to innovative multiple hurdle systems that prevent biofilms and reduce resistance to stresses at the same time as other stresses inhibit bacterial growth and reproduction.

Another investigated assumption was the possibility that more SA was being released than the amount determined by the employed analytical methods. This question arose as we investigated whether irreversible interaction of SA with the bacterial cells occurred. If the SA was consumed by the cells, then measurements of the amount of SA released from the polymer at the end of experiments may be underestimated. This triggered the use of cell-free supernatants from certain time points in the growth to determine if SA release was solely influenced by pH or if some other mechanism contributed to the release. If pH alone caused the release, there should have been no difference between pH-adjusted media and the filtered supernatant containing metabolic byproducts produced during the growth period.

Figure Legend

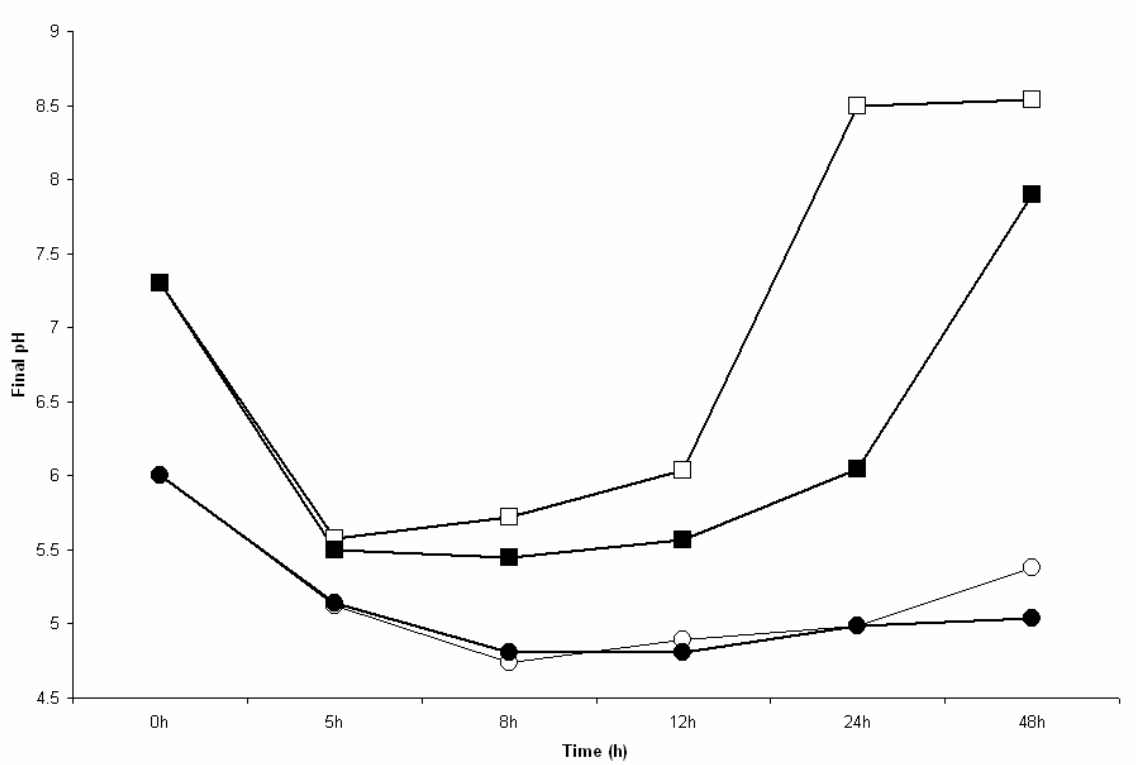

Figure 1. The pH profile of *S. typhimurium* MAE52 grown in different initial pH media. Cells grown at initial pH 7.3 without any salicylic acid (□) reach a final pH of 8.5 within 24 h while cells grown at pH 7.3 in the presence of salicylic acid (■) only reach a pH pf 6.0 in the same time. Cells grown at pH 6.0 in the presence and absence of salicylic acid (● and ○, respectively) had similar profiles; neither reached a final pH above 5.5 even at 48 h and neither formed biofilms at all.

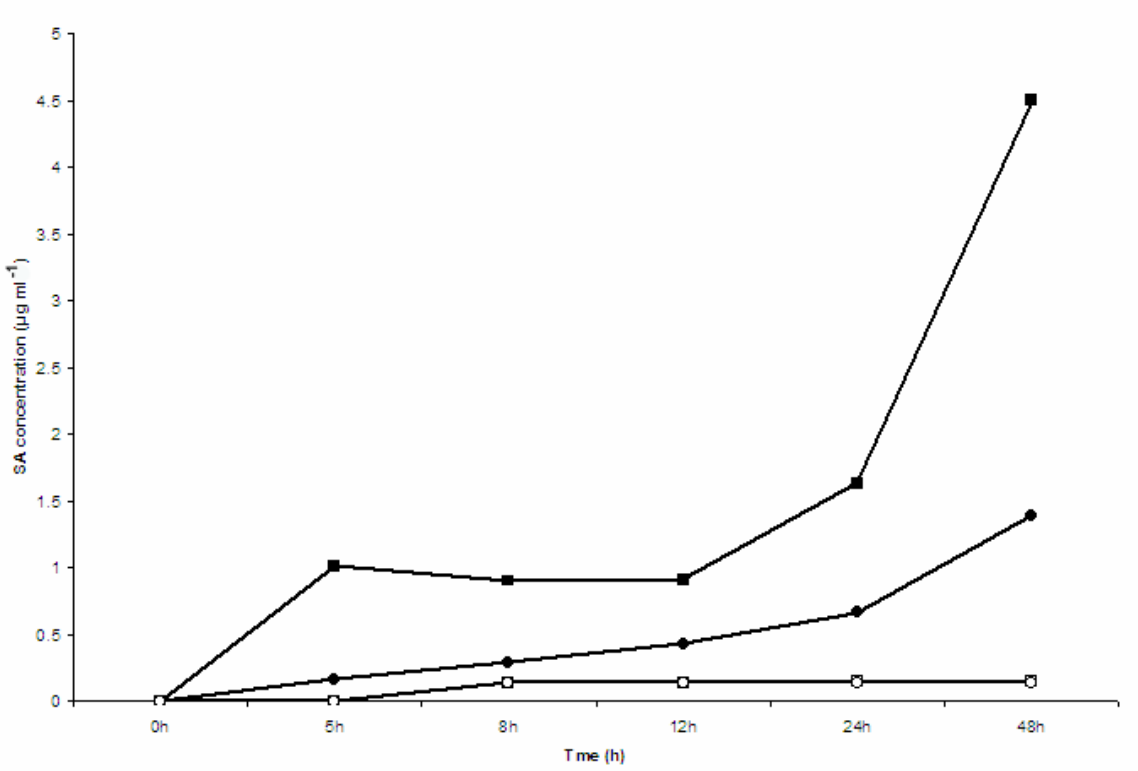

Figure 2. The release of salicylic acid, though pH-dependant, does not follow a true pH-response pattern. When salicylic acid polymer is incubated with MAE52 cells in media of pH 7.3 (■), approximately 1 µg ml^{-1} was release as the pH dropped from 7.3 to 5.5 over the first 5 h. The amount of salicylic acid remained the same as pH rose to 6.0 at 12 h, then increased along with pH to about 1.8 µg ml^{-1} at 24 h when biofilms were fully formed. At the end of 48 h, the concentration of salicylic acid present in the media increased to about 4.5 µg ml^{-1}. When the polymer was incubated with cells in media of pH 6.0 (●), the release was much slower and did not even reach a concentration of 1.5 µg ml^{-1} after 48 h. When cells of MAE52 were incubated without salicylic acid at pH 7.3 and 6.0 (□ and ○, respectively, which overlap), less than 0.15 µg ml^{-1} was released in 48 h.

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APPENDIX A

PUBLICATIONS AND POSTERS PRODUCED BY COLLABORATORS BASED ON THE REEARCH USED FOR THIS MASTERS THESIS

1. Carbone, AL; Rosenberg, LE; Chikindas, ML and Uhrich, KE. Design and synthesis of biodegradable polyanhydrides based on plant and food-derived antimicrobials for biofilm prevention, (pre-print), ACS national meeting, Boston, MA, August 2007.

2. Carbone, AL; Rosenberg, LE; Chikindas, ML and Uhrich, KE. Design and synthesis of biodegradable polyanhydrides based on plant and food-derived antimicrobials for biofilm prevention, (poster), ACS national meeting, Boston, MA, August 2007.

DESIGN AND SYNTHESIS OF BIODEGRADABLE POLYANHYDRIDES BASED ON PLANT AND FOOD-DERIVED NATURAL ANTIMICROBIALS FOR BIOFILM PREVENTION

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Introduction

Biofilms are currently a major problem in many industries including food, medicine and personal care. In essentially every field relating to hygiene, prevention of biofilms is a primary concern.[1] Biofilm prevention is particularly important in the food processing industry. The attachment of bacteria and development of biofilms is a major cause of food spoilage and contamination.[2]

Slow, controlled release of antimicrobials can be achieved using biodegradable polymers. Namely, poly(anhydride-esters) (PAE) composed of salicylic acid have been proven effective in preventing biofilm formation on different strains of *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium.[3,4] Salicylic acid-derived poly(anhydride-esters) (**Figure 1**) predominately undergo surface erosion[5], have high drug loading capabilities and the ability to control the degradation rate based upon polymer composition.[6]

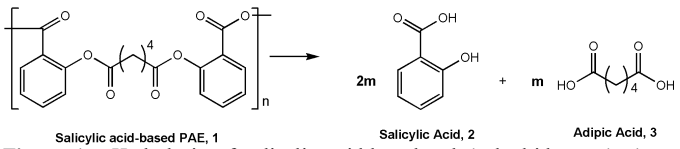

Figure 1. Hydrolysis of salicylic acid-based poly(anhydride-ester), **1**, to release the bioactive compound (**2**) and the biocompatible linker molecule (**3**).

Previously, polyanhydrides prepared from antimicrobials derived from natural sources such as spices and plant extracts were designed and synthesized (**Figures 2** and **3**).[7,8] One application of these polymers is their formulation into micro- or nanospheres, which can then be mixed with foods. The polymers could also be used to coat food processing equipment. Alternatively, other bioactive agents can be physically admixed into the antimicrobial-based polyanhydrides to result in a dual action delivery device.

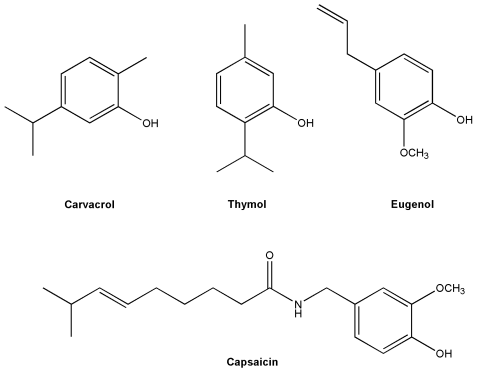

Figure 2. Natural antimicrobial agents incorporated into polyanhydrides.

Experimental

Instrumentation. All polymers and intermediates were characterized by proton nuclear magnetic resonance (^{1}H NMR) and Fourier transform infrared spectroscopy (FTIR). Polymer properties were determined using gel permeation chromatography (GPC) for molecular weights and poldispersity, thermogravimetric analysis (TGA) for decomposition temperatures (T_d), and differential scanning calorimetry (DSC) for glass transition (T_g) and melting (T_m) temperatures.

Synthesis of Polyanhydrides. Polyanhydrides were synthesized using solution polymerization (**Figure 3**).[9-11] In brief, the diacid (**6**) was prepared by a ring-opening of pyromellitic anhydride (**5;** 7.5 mmol) with the mono-functional antimicrobial compound (**4**; 15 mmol) in the presence of a base (e.g., triethylamine; 53 mmol) and in an appropriate solvent (e.g., THF; 40 mL). After the reaction was stirred for 2 h under nitrogen, it was poured over water (~ 400 mL) and acidified using concentrated HCl. The solid formed (**6**) was vacuum filtered, washed with water (3 x 100 mL) and dried under vacuum at room temperature. Diacid (**6**) (4.6 mmol) was dissolved 20 % (w/v) CH_2Cl_2 and triethylamine (20 mmol). The reaction was cooled to 0 °C. The coupling reagent, triphosgene (5.1 mmol), dissolved in CH_2Cl_2 (5 mL) was added drop-wise to the reaction mixture. The reaction was allowed to stir for 2 h at 0 °C under nitrogen. It was then poured over diethyl ether (~ 100 mL), and the polymer formed (**7**) was vacuum filtered, washed with acidified water (3 x 100 mL; pH 2 using concentrated HCl) and dried under vacuum at room temperature.

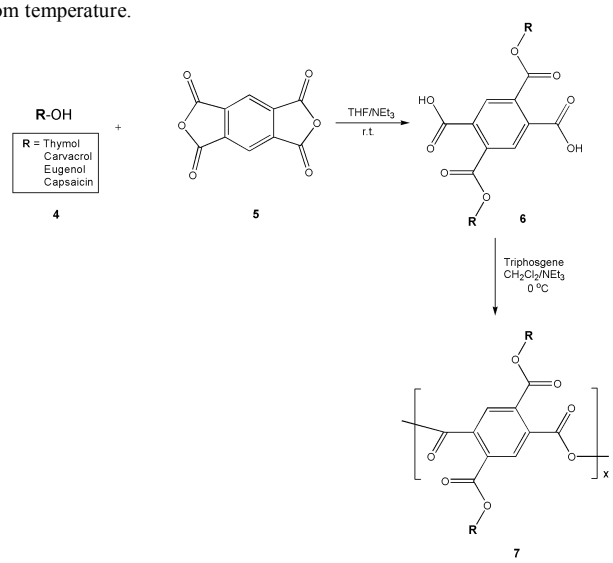

Figure 3. Synthetic scheme for the chemical incorporation of a mono-functional antimicrobial (**4**) into the backbone of a polyanhydride (**7**).

Results and Discussion

Antimicrobial-based polyanhydrides have been successfully synthesized by incorporating the bioactive agent into the polymer backbone *via* an ester linkage. Due to the instability of the anhydride and ester bonds, these polymers should completely degrade to release the active antimicrobial compounds.

Polymers based on plant and spice extracts are unique as their components are not synthetic, but natural, antimicrobials. This aspect is extremely desirable if the polyanhydrides are to be mixed with foods, used in food processing and/or packaging materials.

Conclusions

Biodegradable food-based polyanhydrides with natural antimicrobial properties were synthesized and characterized to be used to inhibit/prevent biofilm formation. Since the polymers can fully degrade to release the bioactives, it is anticipated that they will be very useful in controlled delivery applications for the food industry and other such areas.

Acknowledgements

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DESIGN AND SYNTHESIS OF BIODEGRADABLE POLYANHYDRIDES BASED ON PLANT AND FOOD-DERIVED NATURAL ANTIMICROBIALS FOR BIOFILM PREVENTION

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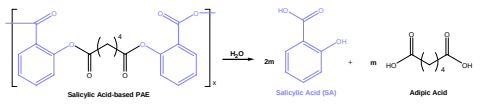
Abstract

Polyanhydrides were designed and prepared from antimicrobials derived from natural sources such as spices and plant extracts. Incorporation of these bioactive molecules into polymeric backbones should result in biodegradable polyanhydrides that slowly release the bioactive agents to reduce or prevent biofilm formation when incorporated into food and food packaging materials.

Salicylic Acid-derived Poly(anhydride-esters) (PAE)

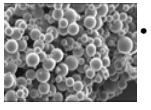
- Hydrolytically degradable biomaterials that release salicylic acid



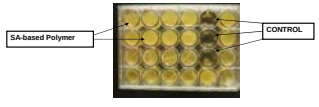
- Predominantly undergo surface erosion[1]
- High drug loading capabilities (60-75 % wt)[2]
- Ability to control degradation rate based upon polymer composition[2]

Biofilm Prevention

- SA-based PAE proven to prevent biofilm formation on strains of *Pseudomonas aeruginosa*[3] and *Salmonella enterica* serovar Typhimurium[4]



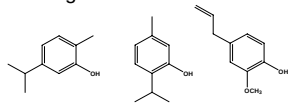
- Good processing capabilites: fabrication into micro- or nanoparticles to be mixed with foods



- *S. typhimurium* MAE52 was used to study inhibition of biofilm formation
- Results indicate that SA-based PAE interferes with biofilm formation but not with cell growth at non-lethal concentrations
- The polymer may not interfere with attachment but rather, affect another mechanism(s) essential for biofilm formation in *Salmonella*

Food-based Polymers: Choice of Bioactive Molecules

- Food and food extract-based polymers were designed using natural antimicrobials:



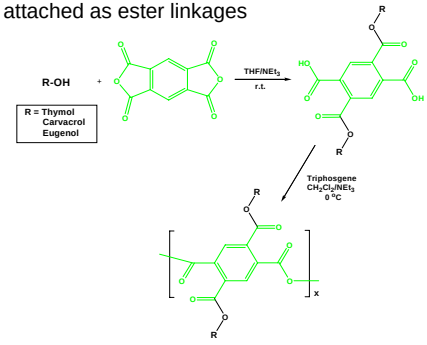
- Polymers derived from these bioactive molecules may prevent biofilm formation if formulated into food products, packaging materials and processing equipment

Synthetic Approaches

- These bioactives contain 1 reactive functional group
- The monofunctional bioactive is therefore attached to the polymeric repeat unit as a pendant *prior* to polymerization

➢Pyromellitic Acid Linker

- Aromatic "linker" molecule for easy identification during degradation studies
- Ring-opening with symmetrical cyclic pyromellitic dianhydride to form diacids with the bioactives attached as ester linkages

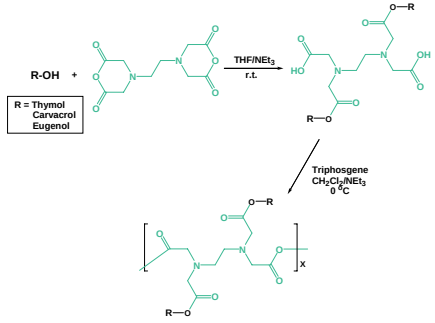


Bioactive	M_w	PDI	T_d (°C)	T_g (°C)
Thymol	38,000	1.0	179	37
Carvacrol	21,700	1.0	182	27
Eugenol	19,900	1.0	171	58

- Polymer drug loadings 58-60 % by weight
- Nearly uniform polymer chain lengths
- T_g near body temperature

➢Ethylenediaminetetraacetic Acid (EDTA) Linker

- Biocompatible food-grade chelating EDTA "linker" molecule
- Ring-opening with symmetrical EDTA dianhydride to form diacids with the bioactives attached as ester linkages
- EDTA widely used as preservative in packaged foods, vitamins, and personal care products



Bioactive	M_w	PDI	T_d (°C)	T_g (°C)
Thymol	23,200	1.0	223	77
Carvacrol	19,500	1.1	221	65
Eugenol	11,100	1.5	229	86

- Polymer drug loadings 54-56 % by weight
- Nearly uniform polymer chain lengths
- T_g well above body temperature

Biofilm Assays on Polymer Surfaces

- Use of *S. typhimurium* MAE52 to study inhibition of biofilm formation on polymer-coated glass coverslips

➢Pyromellitic Acid Linker

Bioactive	Observations
Thymol	Prevented cell growth almost completely
Carvacrol	Formed weak biofilms after 24 h
Eugenol	Formed full biofilms after 24 h

➢EDTA Linker

Bioactive	Observations
Thymol	Formed weak biofilms after 24 h
Carvacrol	Completely prevented biofilm formation
Eugenol	Formed cell aggregates after 32 h

Future Work: Polymer Degradation and Biofilm Studies

Hydrolytic degradation of the polymers will be studied to determine the rate of release of the natural antimicrobial from the polymer backbone. Polymers will be incubated in PBS (pH 7.4) at 37 °C. At predetermined time intervals, the media will be replaced with fresh media, and the spent media will be analyzed by HPLC.

Future studies include identification of the mechanism(s) involved in controlling biofilm formation in targeted microorganisms by polymer-based antimicrobials of natural origin.

Conclusions

Two alternative synthetic procedures for incorporating bioactive molecules with one reactive functional group into polymeric backbones have been established. In these methods, the natural antimicrobial is attached *prior* to polymerization, so that the number of bioactive molecules attached is well defined. These polymers should fully degrade, releasing the incorporated antimicrobials, which may prevent the formation of biofilms. These biodegradable, natural polymers were designed for formulation into food products and various active packaging materials. In addition, these polymers may be effective in medical devices, oral care and personal care products.

Acknowledgements

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