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

L.E. Rosenberg¹, A.L. Carbone², U. Römling³, K.E. Uhrich² and M.L. Chikindas¹

¹ Department of Food Science, Rutgers University, New Brunswick, NJ, USA
² Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, NJ, USA
³ Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, Stockholm, Sweden

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 signalling 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 sterile environments.

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
Control of *Salmonella* biofilms

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 nonsteroidal anti-inflammatory drugs (NSAID) are known to prevent bacterial adhesion on to medical devices (Arciola *et al.* 1998), although the mechanism has not been identified. PAE consists of NSAID 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 PAE 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. NSAID 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 min of treatment with 0.25 mg ml⁻¹ of 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 SAA 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 millimoles per litre of sodium salicylate combined with 1 μg ml⁻¹ of vancomycin was significantly more effective than either treatment alone. In addition, one-half of 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 suitable for examining 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). *Pseudomonas aeruginosa* is a plant and human pathogen and, as mentioned before, 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).

These 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 PAE 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 this research.

Preliminary data showed that the controlled release of NSAID prevented biofilm formation of *P. 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 NSAID to inhibit biofilm formation. This paper examined the effects of controlled release of salicylic acid-based PAE 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

PAE 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, PDI (polydispersity index): 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 the solvent was cast on to microscope glass coverslips (Fisher Scientific, Fair Lawn, NJ, USA; 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 complete solvent removal. The coverslips were weighed using an analytical balance (Mettler Toledo, AB104-S/FACT, Columbus, OH, USA) before and after coating to determine the mass of polymer applied. The thickness of the coating was determined using a digital micrometer (Fowler ProMax, Newton, MA, USA). Before coating, the coverslips were cleaned using Alconox (Alconox Inc., NY, USA) and H$_2$SO$_4$:H$_2$O$_2$ (10:1, v/v) solutions and stored in ethanol. All reagents and fine chemicals were purchased from Aldrich (Milwaukee, WI, USA). Solvents were purchased from Fisher Scientific (Pittsburg, PA, USA).

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, USA) ultraviolet (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 S. enterica serovar Typhimurium JSG210 (Prouty et al. 2002) were streaked on to brain heart infusion (BHI) plates (37 g l$^{-1}$; BD, Becton, Dickinson & Co., Sparks, MD) containing 1.85% granulated agar (BD) and incubated overnight at 37°C. Glass test tubes (16 x 125 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$^\text{TM}$ 3000 spectrophotometer.

Figure 1 Hydrolysis of salicylic acid-based poly(anhydride ester) (1) to release the bioactive (3) and biocompatible linker molecules (4) via pH-dependent nonenzymatic hydrolytic bond cleavage.
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. The images were recorded by a 6.3 megapixel Fujifilm FinePix digital camera on the macro setting preincubation, postincubation 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 S. enterica JSG210 were removed with sterile forceps and placed into a petri dish containing c. 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 min 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 S. enterica MAE52, the biofilms were removed from the air–liquid interface using a pipette tip and washed gently in a petri dish containing c. 30 ml of saline to remove unattached cells. The entire biofilms were then placed into glass test tubes containing c. 30 (3 mm) glass beads (Scher et al. 2005) and 4.5 ml of saline solution and vortexed strongly for 1 min. 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 (Fig. 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 four. Student’s t-test

Figure 2 Final planktonic and biofilm-associated cell densities for Salmonella enterica serovar Typhimurium MAE52 and JSG210 after 40 h of incubation, plotted in log10 (CFU ml^{-1}) against the initial cell loads of 10^2, 10^3, 10^4, 10^5 and 10^7 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 biofilm cells and MAE52 treatment planktonic cells. The planktonic cell density was very high, yet MAE52 was still unable to form biofilms in the presence of the salicylic acid-based poly(anhydride ester). 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 10^7 CFU ml^{-1} initial cell load due to biofilms being formed only 50% of the time.
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 $1 \times 10^6$ g l$^{-1}$ react irreversibly with the cell, resulting in cell death, but at concentrations below $1 \times 10^5$ 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 the 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 micro-organism growth kinetics (data not shown). The cell densities for the different samples are presented in Fig. 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 *S. enterica* MAE52 and *S. enterica* JSG210. The slight difference seen in the *S. enterica* MAE52 cell densities is not significant (as determined by the Student’s 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 *S. enterica* MAE52 treatment plate at the lower inocula levels. However, there was significant difference between biofilm-associated cell densities of control and treatment *S. 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 ($0.0012 \pm 0.0002$ g l$^{-1}$) were lower than levels with no cells present ($0.0020 \pm 0.0002$ 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. *Salmonella enterica* MAE52 forms biofilms at the air–liquid interface (top-forming); *S. enterica* JSG210 forms biofilms on a tangible surface (bottom-forming). As the salicylic acid-based polymer-coated coverslips lay on the bottom of the wells, if the salicylic acid-based polymer interfered with the attachment, a clear difference in the biofilm-associated cell densities of the control and treatment plates of *S. enterica* JSG210 should be observed. Instead, the treatment wells inoculated with *S. enterica* JSG210 had similar biofilm-associated cell densities as the control wells. However, the treatment wells inoculated with *S. enterica* MAE52 did not form biofilms at all, except at the highest level of the inoculum (Fig. 2). In addition, it appears that this inhibition of biofilm formation was dependant on the initial cell load. The lower inocula ($10^2$–$10^5$ CFU ml$^{-1}$) of *S. 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 the 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 coverslip 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, as 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, NSAID 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.

Acknowledgements

The authors would like to acknowledge Dr John Gunn of the USDA for providing Salmonella enterica serovar Typhimurium JSG210. This research was supported (in part) by the New Jersey Agricultural Experiment Station Project #10152 through U.S. Hatch Act funds. This is publication no. D10550-07-02 of the New Jersey Agricultural Experiment Station supported by State Funds and the Center for Advanced Food Technology (CAFT grant 4-25539). The Center for Advanced Food Technology is an initiative of the New Jersey Commission on Science and Technology Center.

References


