

Biodegradation of poly(anhydride-esters) into non-steroidal anti-inflammatory drugs and their effect on *Pseudomonas aeruginosa* biofilms in vitro and on the foreign-body response in vivo

James D. Bryers^{a,*}, Rebecca A. Jarvis^b, Jason Lebo^b, Almudena Prudencio^c, Themis R. Kyriakides^d, Kathryn Uhrich^c

^aDepartment of Bioengineering, University of Washington, Box 355061, Seattle, WA 98195, USA

^bThe Center for Biomaterials, University of Connecticut Health Center, Farmington, CT 06030, USA

^cDepartment of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

^dDepartments of Pathology and Biomedical Engineering, Yale University, New Haven, CT 06519, USA

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Abstract

The ability of poly(anhydride-esters) composed of non-steroidal anti-inflammatory drugs that biodegrade to salicylic acid (SA) and adipic acid to prevent colonization by *Pseudomonas aeruginosa* and their effects on the foreign-body response were studied in vitro and in vivo, respectively. Soluble SA in bacterial medium at concentrations up to 300 mg/L did not affect the growth rate or viability of *P. aeruginosa*, indicating that SA does not exhibit a direct toxicity effect on the bacterium. Batch degradation rates of the salicylate-based polymer in the presence of an actively growing bacterial culture only marginally (14%) increased relative to polymer degradation rates in sterile medium. Short-term (3 h) bacterial adhesion studies in agitated batch systems indicated a 47% reduction in the rate of *P. aeruginosa* adhesion relative to a control polymer that does not release SA upon biodegradation. Long-term (3-day) biofilm accumulation studies indicated a dramatic reduction in biofilm formation on salicylate-based polymer versus controls. A recombinant *P. aeruginosa* pMHLAS, containing a fluorescent reporter gene prior to the *las* regulon, was employed to determine whether salicylate-based polymer prevents biofilm formation by the released SA inhibiting quorum sensing pathways. Long-term biofilm accumulation studies with *P. aeruginosa* pMHLAS insinuate that *salicylate-based polymer* prevents biofilm accumulation by inhibiting the *las* quorum sensing system. Furthermore, unlike control polymer, salicylate-based polymer implanted subcutaneously for a period of 4 weeks-resisted cell-mediated degradation and remained intact. Histological and immunohistochemical analysis indicated a reduction in overall encapsulation and paucity of macrophages in the area of the salicylate-based polymer implant.

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1. Introduction

It is estimated that over 3 million artificial or prosthetic parts are implanted per annum in the United States alone [1,2]. Bacterial colonization and biofilm formation on any substratum proceeds by a series of complex physical and biological processes. The body reacts to prosthetic implants by first coating them with a film composed of various proteins (e.g., fibronectin, laminin, fibrin, collagen,

immunoglobulins), some or all of which can serve as receptors for colonizing bacteria. Bacteria, transported to the substratum by either molecular diffusion or convective transport, can adhere by either a nonspecific adhesion mechanism governed by electrostatic forces acting between the cell and surface or by a specific adhesion binding reaction. Once attached to the substratum, bacteria can secrete copious amounts of insoluble extracellular polymers (e.g., polysaccharides, DNA), forming a tenacious three-dimensional matrix, termed a “biofilm” [3,4]. These bacterial polymers can mix with the extracellular polymers of other species, host cells, or blood platelets to

*Corresponding author. Tel.: +1 206 221 5876; fax: +1 206 616 9763.

E-mail address: jbryers@u.washington.edu (J.D. Bryers).

form a mixed-cell line biofilm that is highly resistant to host immune defenses and rigorous antibiotic challenges [5,6].

To prevent biomaterial-related infections, current clinical strategy is to treat patients with antibiotics at high systemic concentrations. Since this approach is only marginally successful [7,8], the inevitable recourse is the surgical removal of the infected implant. A better approach is to provide a specific, localized anti-biofilm therapy, immediately upon implantation and directly at the site where it is most required—the material/body interface. Here, we address this significant scientific and social issue by designing implantable, self-healing biomaterials that biodegrade into bioactive molecules (i.e., non-steroidal anti-inflammatory drugs (NSAIDs)) that locally inhibit bacterial colonization and minimize inflammation. Specifically, poly(anhydride-esters) that biodegrade into salicylic acid (SA) [9,10], the hydrolysis product of aspirin [11], were evaluated for their ability to prevent bacteria adhesion and reduce foreign-body response. This series of polymers was originally designed to minimize pain and inflammation by locally releasing SA following implantation [12,13]. This manuscript describes how these polymers that biodegrade into SA—referred to as salicylate-based polymers—serendipitously prevent biofilm accumulation.

2. Materials and methods

2.1. Disk preparation

Synthesis of SA-derived poly(anhydride-ester) [14,15] and the inactive polyanhydride (control) [16] are detailed elsewhere [14–16]. These papers describe at length the synthesis of a poly(anhydride-ester) composed of alkyl chains linked by ester bonds to aromatic moieties of SA. The polymer, by design, undergoes hydrolytic degradation to release SA and adipic acid. Pellets of inactive (control) and active salicylate-based polymer (Fig. 1) were prepared by pressing ground polymers ($\sim 181 \pm 23$ mg) into 13 mm diameter \times 1 mm thick disks for the in vitro studies and 6 mm diameter \times 3 mm thick disks (106 ± 11 mg) for the in vivo studies in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver model M, Wabash, IN). A pressure of 5000 psi was applied for 5 min at room temperature. No change in polymer color was observed upon applying pressure.

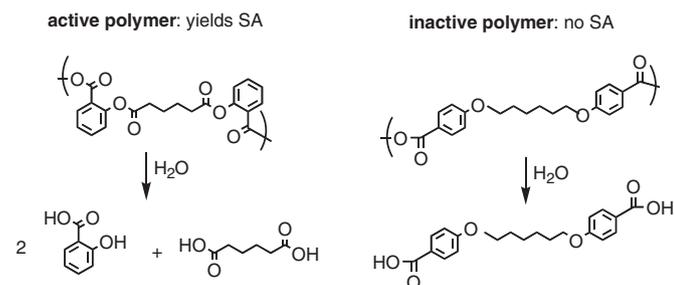


Fig. 1. Biodegradation of active poly(anhydride-esters), referred to as salicylate-based polymers, releases salicylic acid (SA) and adipic acid compared with the biodegradation of inactive, control polyanhydride.

2.2. Bacterial strains and media

Wild-type *Pseudomonas aeruginosa* PAO1 (ATCC number BAA-47) was purchased from ATCC. A recombinant *P. aeruginosa* pMHLAS strain was obtained (courtesy of S. Mølin, Technical University Copenhagen, Denmark) and previously described [17]. The quorum sensing reporter system, pMHLAS, was constructed by a two-step cloning procedure. The *PlasB_{gfp}*(ASV) translational fusion was made by amplifying a 348 bp PCR product starting 345 bp upstream of the *lasB* initiation codon, using the primers *lasB* fwd and *lasB* rev and chromosomal DNA of *P. aeruginosa* PAO1 as template. The PCR fragment was subsequently digested with *Xba*I and *Sph*I and inserted into the corresponding site of pMH391 [17]. This gave rise to the plasmid pMHLB, which carries the translational *PlasB-gfp*(ASV) followed by translational stop codons in all three reading frames and two strong transcriptional terminators [18]. According to Henzter [17], the reporter cassette was inserted at random positions in the chromosomes of *P. aeruginosa* PAO1 by triparental mating. The selected transconjugants with random insertion of the mini-Tn5 elements showed no sign of phenotypic changes compared to the parental strains when cultivated in liquid medium or in biofilms. Should the *las* regulon be activated by homoserine lactones, then the activated cells would also express GFP and fluoresce.

Stock cultures of *P. aeruginosa* strains were kept at 4 °C on trypticase soy broth (TSB; Gibco, Co.) agar slants (for pMHLAS strains, agar contained 60 µg/mL gentamicin and 100 µg/mL ampicillin). Colonies were subcultured every 2–4 weeks to maintain culture viability. Before a bacterial challenge to the salicylate-based polymer samples, microorganisms were grown in pure culture overnight in 1 mL of trypticase soy broth (TSB, 10 g/L), started via loop inoculation from TSB plates. These cultures were used to subsequently inoculate a sterile 250 mL Erlenmeyer flask containing 50 mL of 10 g/L TSB that was then placed in a temperature-controlled rotary shake incubator at 37 °C. Typical growth curves for each bacterial strain were essentially undistinguishable and are available upon request.

Effects of soluble SA on suspended cultures of *P. aeruginosa* PAO1 were carried out as above but with culture media augmented with SA (Sigma, St. Louis, MO) with concentrations ranging from 10 to 300 mg/L (Fig. 2). Based upon the size and mass of the salicylate-based polymer disks, the active polymers contain 139.9 ± 2.1 mg of SA. Based on measured degradation rates, bacterial suspended cultures exposed to salicylate-based polymers did not experience ambient SA concentrations ≥ 200 mg/L. Therefore, 300 mg/L is a concentration far in excess of any level attainable by the degradation of a single salicylate-based polymer disk in 10 mL fluid over the length of these experiments. Suspended cell concentrations were determined by direct cell count on samples withdrawn periodically from the suspended cultures. Samples were instantly filtered through 0.1 µm black polycarbonate membrane (Nuclepore, Whatman, Chicago, IL), stained using the Live/Dead Bac-Light™ assay (Molecular

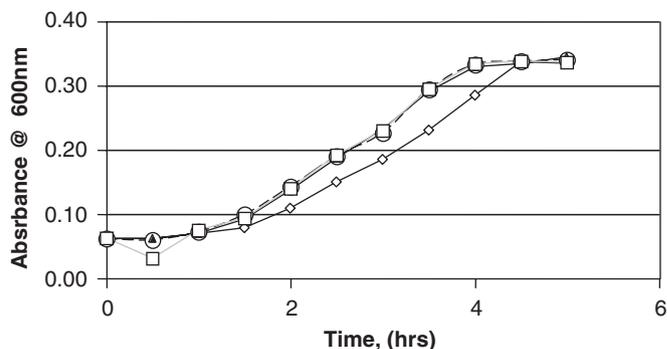


Fig. 2. Suspended growth of *P. aeruginosa* wild-type in culture medium with various levels of soluble salicylic acid. ◇ = medium + 300 mg/L salicylic acid, ○ = medium + 30.0 mg/L salicylic acid, ▲ = medium + 3.0 mg/L salicylic acid, and □ = medium alone.

Probes, Salem OR). Live and dead cells were counted as per manufacturer's instructions using Zeiss Axophot epi-fluorescence microscope with a 100× oil immersion objective. Live cells fluoresce green while any dead cells fluoresce red.

To ascertain whether bacterial growth in the fluid phase would affect salicylate-based polymer degradation rates, release rates of SA from salicylate-based polymer were also quantified in the presence of actively growing suspended bacterial cultures of *P. aeruginosa*.

2.3. Bacterial challenges

2.3.1. Bacterial adhesion kinetics

For bacterial *adhesion-only* studies, salicylate-based polymer disks (1 mm thick, 13 mm diameter) in 10 mL scintillation vials were submerged with a suspension of *P. aeruginosa* PAO1 in buffered saline at a concentration of 1×10^6 cells/mL for time periods less than 3 h. At a designated time, contents of the vial were infinitely diluted with a stream of sterile buffered saline and polymer disks removed. Disks were placed into sterile buffer (5 mL) and sonicated for 30 s to suspend any attached bacterial cells. Resultant cell suspensions were vacuum-filtered through a 0.10 μm pore diameter black polycarbonate membrane and stained using the Live/Dead Bac-Light™ assay (Molecular Probes, Salem OR), and living and dead cells counted as per manufacturer's instructions using Zeiss Axophot epi-fluorescence microscope with a 100× oil immersion objective. Live cells fluoresce green while any dead cells fluoresce red. All experiments were repeated at least in triplicate.

2.3.2. Biofilm formation

Salicylate-based polymer disks (1 mm thick × 13 mm diameter disc) were submerged in sterile medium and inoculated with *P. aeruginosa* PAO1. Every 8 h over a 3-day period, spent medium was replaced with sterile fresh medium (5 mL), thus cultivating any adherent cultures in a semi-continuous fashion. The number of live/dead/total bacterial cells was measured daily as described above, in solution as well as directly on the salicylate-based polymer disk surfaces. The pH was periodically measured by micro-pH probe (Lazar Research Laboratories, Los Angeles, CA). Soluble SA concentration in the medium, released from the tablets, was also measured by collecting 0.5 mL samples for spectrophotometer readings at 235 nm. All experiments were repeated at least in triplicate.

2.3.3. Quorum sensing regulation

Increasing evidence indicates that cell-to-cell communication plays a crucial role for the maturation of biofilms, i.e., for the development of characteristic three-dimensional biofilm architecture. Although several signal molecule families have been identified in Gram-negative bacteria, the most intensively studied and best understood are those that belong to the N-acylhomoserine lactone (AHL) family. AHLs contain a homoserine lactone ring attached via an amide bond to an acyl side chain containing from 4 to 14 carbons. Two AHL-mediated quorum sensing circuits have been identified in *P. aeruginosa*. The *las* system consists of *lasI*, an AHL synthase gene responsible for the synthesis of OdDHL [*N*-(3-oxo-dodecanoyl)-L-homoserine lactone; 3-oxo-C12-HSL; PAI-1] [19], and *lasR* which encodes a LuxR-type transcriptional regulator protein [20,21]. The *las* system has been shown to regulate the expression of several virulence factors, such as extracellular enzymes (LasB elastase, LasA protease, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdinin), toxins (exotoxin A) and *lasI* itself. In the *rhl* system, the *rhlI* gene product directs the synthesis of BHL (*N*-butanoyl-L-homoserine lactone; C4-HSL; PAI-2), which, in conjunction with the *rhlR* gene product, activates transcription of the *rhlAB* rhamnolipid biosynthesis genes and the *rhlI* gene itself. The *rhl* system is also involved in modulating the expression of several of the virulence factors controlled by the *las* system [22,23]. Several studies using homoserine lactone-like analogs (furanones) have successfully negated biofilm formation [17].

The similarity of (a) the molecular structure of SA to certain furanones and (b) reported biofilm inhibition by SA without toxicity suggests that SA may inhibit biofilm formation by also interfering in homoserine lactone

quorum sensing. Biofilm formation studies were repeated as described in Section 2.3.2 using the recombinant *P. aeruginosa* pMHLAS strain. Should SA inhibit the *las* regulon system, then SA will also affect the expression of the GFP reporter gene.

2.4. Foreign-body response studies

Two polymer systems were compared—the active polymer (i.e., salicylate-based polymer) and an inactive (inert) polyanhydride as our polymer control. For the following studies, a total of ten, 3–4 months of age, control mice (C57/Bl6 genetic background) were used for implantations. Each mouse received two implants (6 mm diameter, 3 mm thick) allowing for 10 implants per material. Polymer pellets (both active and inert) were sterilized under UV light and implanted subcutaneously in the dorsal region, through a midline incision. After 4 weeks, the animals were sacrificed and the polymers were retrieved and fixed in 10% zinc-buffered formalin for 24 h. Following processing and embedding, 6-μm sections were stained with Masson's trichrome. Immunolocalization of macrophages was performed with the Mac3 antibody as described previously [24]. All secondary peroxidase-conjugated antibodies were used at a dilution of 1:200. Controls included sections treated with pre-immune sera.

3. Results

3.1. Soluble salicylic acid effects on bacterial growth rates

Effects of soluble SA on suspended cultures of *P. aeruginosa* PAO1 were carried out with culture media augmented with SA at concentrations ranging from 10 to 300 mg/L. Representative results, summarized in Fig. 2, indicate that at concentrations below ~300 mg/L, SA does not exhibit any detrimental effects on the growth of *P. aeruginosa*. Note that 300 mg/L is a concentration far in excess of any level attainable by the degradation of a single salicylate-based polymer tablet in 10 mL fluid. Consequently, any retardation in *P. aeruginosa* colonization on salicylate-based polymer materials cannot be attributed to a growth-inhibitory or antibiotic effect of SA alone.

3.2. Polymer degradation rates

To ascertain whether bacterial growth in the fluid phase would affect salicylate-based polymer degradation rates, release rates of SA were also quantified in the presence of actively growing suspended bacterial cultures of *P. aeruginosa*. Samples of the liquid phase in which the salicylate-based polymer wafer was submerged were collected periodically, and SA concentrations, bacterial cell concentrations, and pH of the media were determined. Representative release rates of SA in both the absence and presence of actively growing bacterial suspensions are depicted in Fig. 3. Release rates of SA were only slightly higher in the presence of actively growing suspended bacteria cultures versus sterile medium. The corresponding difference in pH seen in these experiments may not be entirely due to the release of salicylic and adipic acids but rather due to active microbial growth producing CO₂ in a weakly buffered growth medium.

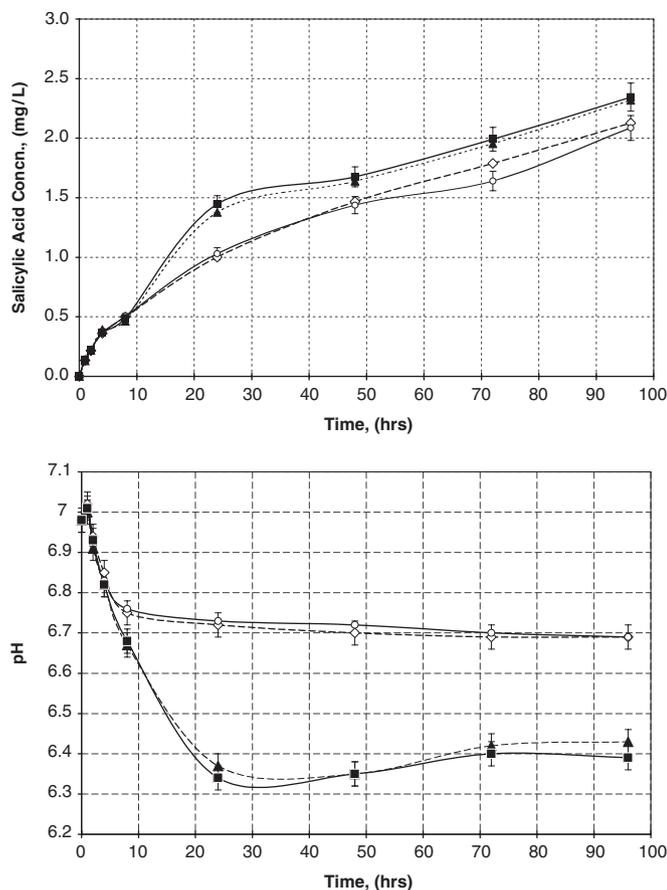


Fig. 3. Salicylic acid concentration (top graph) and pH changes (bottom graph) resulting from the hydrolytic degradation of active salicylate-based polymers in growing bacterial culture (closed symbols; two replicate experiments ■ and ▲) and in sterile medium (open symbols; two replicate experiments ◇ and ○). Each data point is an average of three separate samples.

3.3. Bacterial adhesion on salicylate-based polymer disks

For bacterial *adhesion-only* studies, polymer disks (1 mm thick, 13 mm diameter) in 15 mL scintillation vials were submerged (10 mL total liquid volume) with a suspension of *P. aeruginosa* PAO1 in buffered saline at a concentration of 1×10^6 cells/mL for time periods *less than 3 h*. To minimize cell growth and replication, no carbon source was provided in the medium. Results of *P. aeruginosa* PAO1 adhesion, to both salicylate-based polymer releasing SA and the biodegradable inactive control are shown in Fig. 4.

Fig. 4 illustrates adherent cell areal concentrations as a function of time for *P. aeruginosa* on either the biodegradable inactive controls or active salicylate-based polymer. Initial net accumulation rates (number of cells per time per area) were determined as per Poelstra et al. [25] and Busscher et al. [26], as the slope of the time course of adherent cell numbers per area at time = 0,

$$J_0 = \left(\frac{dX(t)}{dt} \right)_{t=0}, \quad (1)$$

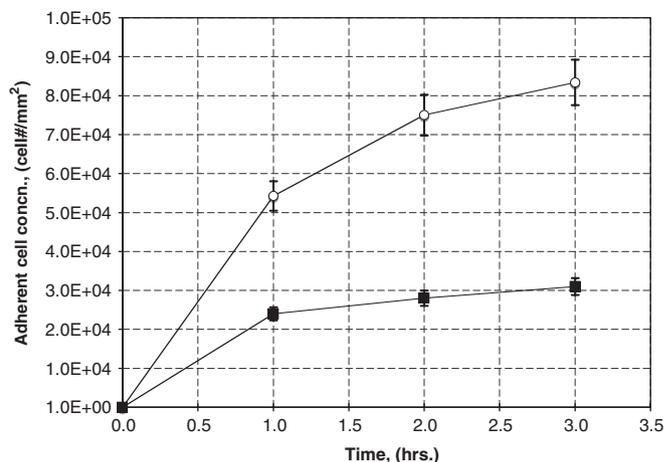


Fig. 4. *P. aeruginosa* short-term adhesion-only studies with active salicylate-based polymer disks (■) relative to inactive control polymers that biodegrade but do not release SA (○).

where J_0 (cell Nr mm⁻² s⁻¹) is the initial rate of bacteria accumulation; $X(t)$ (cell Nr mm⁻²) is the number of adherent cells per area at time t ; and t (s) is the time. Ideally one would like to employ several early data points to estimate J_0 . Here, we were able to use only 3–4 initial data points, thus introducing some error in the estimation of J_0 . Consequently, these calculations can only be regarded as a qualitative comparison of initial accumulation. Initial net accumulation rates as per Eq. (1) were calculated as 14.7 and 6.9 cell/mm²/s, for control and salicylate-based polymer materials, respectively.

3.4. Biofilm formation on salicylate-based polymer disks

Salicylate-based polymer disks (1 mm thick × 13 mm diameter disc) were submerged in sterile medium and inoculated with *P. aeruginosa* PAO1. Experiments were carried out in triplicate. Every 8 h over a 3-day period, spent medium was replaced with sterile fresh medium, thus cultivating any adherent cultures in a semi-continuous fashion. The number of live/dead/total bacterial cells was measured, as described above, daily in solution as well as on the salicylate-based polymer disk surfaces. The pH and SA concentration in the medium were also measured.

Fig. 5 summarizes a series of bacterial accumulation experiments on salicylate-based polymer and control biomaterials for *P. aeruginosa* PAO1 wild type. Photomicrographs of biofilm accumulation on salicylate-based polymer and control materials taken over the same 3-day period are shown in Fig. 6. Both results indicate a dramatic decrease in the overall amount of biofilm accumulation on the salicylate-based polymer versus the biodegradable, inactive control polymers.

3.5. Salicylate-based polymer effects on quorum sensing

Studies using homoserine lactone-like analogs (furanones) have successfully negated biofilm formation [17].

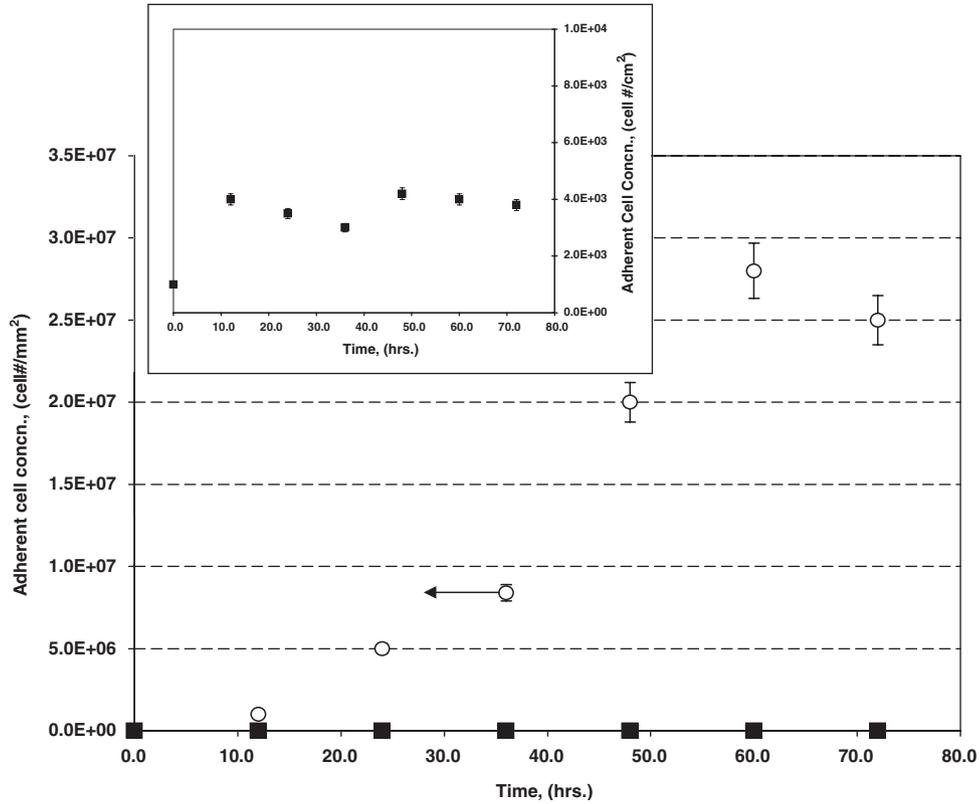


Fig. 5. Adherent cell accumulation by *P. aeruginosa* in long-term biofilm experiments performed in stirred batch systems comparing (major figure) active salicylate-based polymer disks (■) relative to inactive control polymers that biodegrade but do not release SA (○). Inset provides the same adherent cell data for the salicylate-based polymers (■) on an expanded scale.

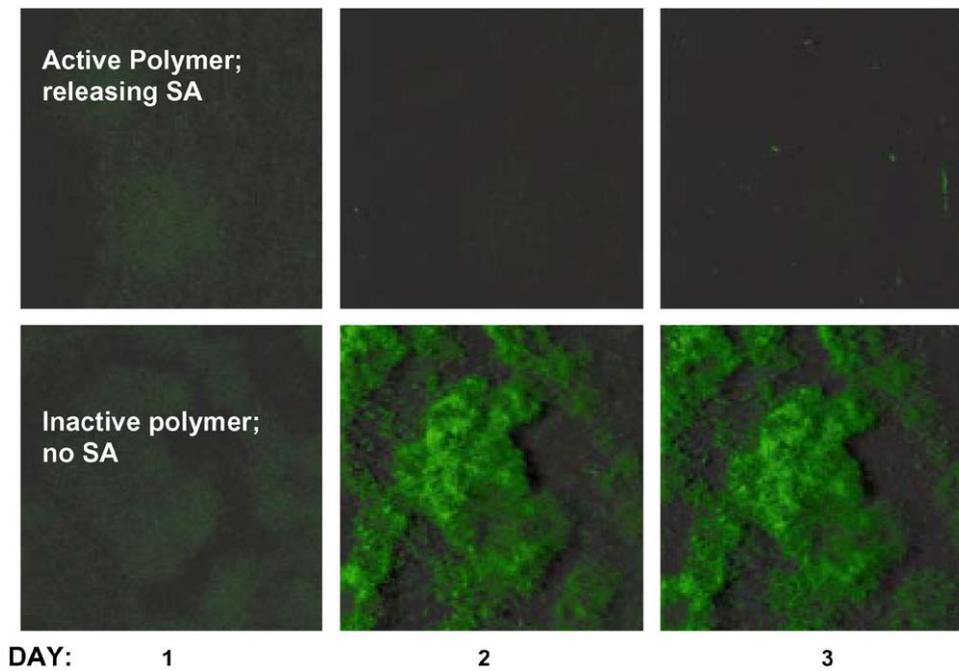


Fig. 6. Time course photographs of biofilm formation on active salicylate-based polymers and inactive control polymer pellets.

Biofilm formation studies were repeated using the recombinant *P. aeruginosa* pMHLAS strain. Fig. 7 illustrates that *P. aeruginosa* pMHLAS accumulates to a much lesser

degree on the SA-releasing polymers versus inactive, control polymers. Further, *las* regulon expression (as indicated by the GFP reporter gene expression) is clearly

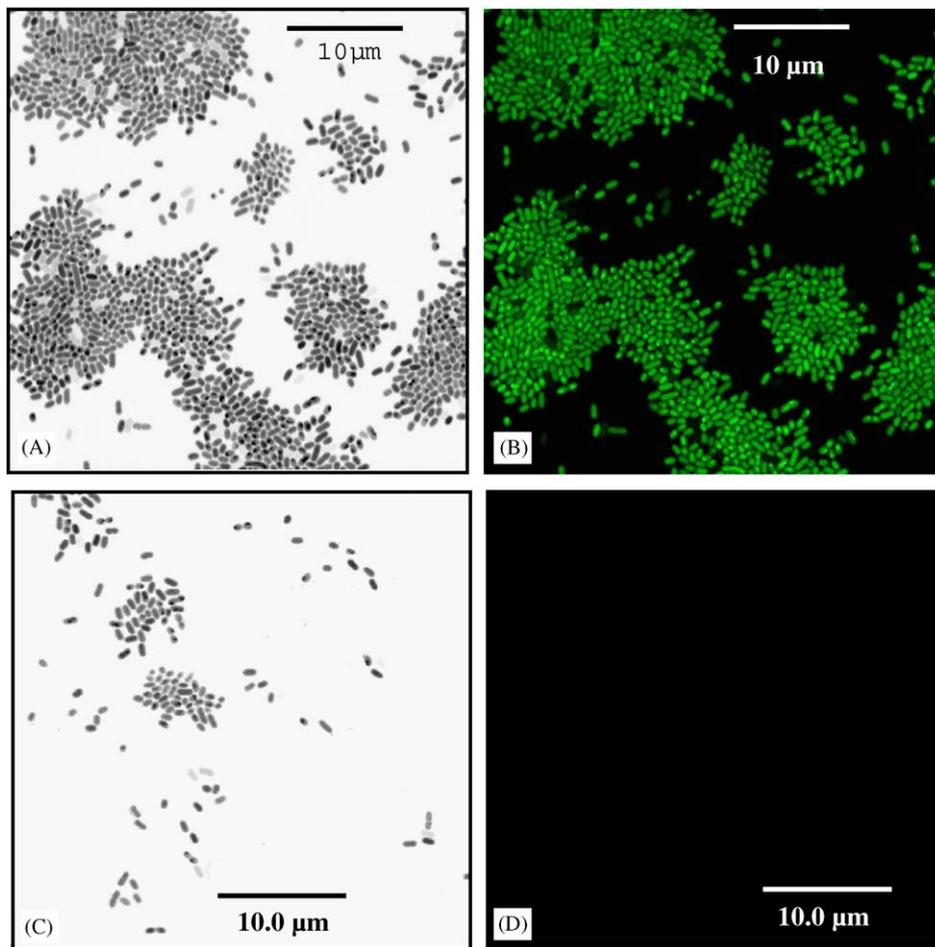


Fig. 7. Influence of biodegrading polymers on *las* regulon activity in *P. aeruginosa* pMHLAS mutant: (A) Transmitted light micrograph of *P. aeruginosa* pMHLAS during the attachment phase of biofilm development on the inactive, control polymer; and (B) the same image under UV illumination indicating activation of the *las* regulon. (C) Transmitted light micrograph of *P. aeruginosa* pMHLAS during the attachment phase of biofilm development on active polymer, salicylate-based polymer that slowly releases salicylic acid upon biodegradation; and (D) the same image under UV illumination indicating inactivation of the *las* regulon.

seen for the strain accumulating on the control biomaterials, while no such expression is seen in those few cells adsorbed to the salicylate-based polymer materials.

3.6. *In vivo* foreign-body response

According to observation of Masson's trichrome-stained histological sections (Fig. 8), salicylate-based polymers remained almost completely intact and allowed very low cell invasion (arrows in D). On the contrary, the inactive control polymer was extensively degraded and induced a large cellular influx (arrows in C). Semi-quantitative analysis indicated that approximately 80% of salicylate-based polymer was still intact, whereas only 20% of the control polymer remained after 4 weeks. In addition, the capsule surrounding salicylate-based polymer implants was loose and vascularized (Fig. 9B). Both observations suggest an alteration of the foreign-body response, an inflammation-driven process. Control implants displayed normal encapsulation with dense and largely avascular collagenous

capsules (see Fig. 9A). Immunohistochemical analysis of sections with a macrophage-specific antibody revealed the presence of numerous macrophages in the capsule of control implants with many at the interface between the implantation site and the newly formed tissue (Fig. 9C). In contrast, macrophages were present at a significant distance from salicylate-based polymers, mostly at the outer edges of the foreign capsule (Fig. 9D).

4. Discussion

SA or salicylate is the major *in vivo* metabolite of acetylsalicylic acid, i.e., aspirin [11]. Farber et al. reports that SA inhibited adherence (55%), growth, and biofilm production of *Staphylococcus epidermidis* [27]. In a second study, Farber et al. further illustrated that NSAIDs, including sodium salicylate, inhibit bacterial biofilm production [28]. The authors studied the effect of soluble sodium salicylate on bacterial adherence and biofilm formation on contact lenses, lens cases, and commonly

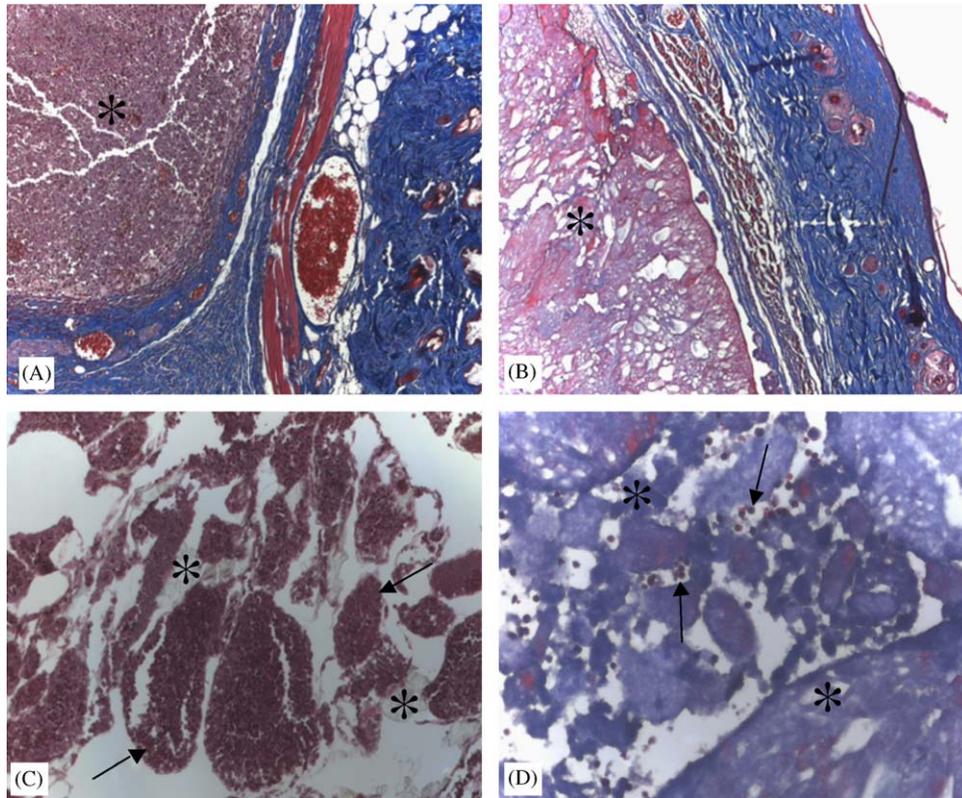


Fig. 8. Salicylate-based polymer resists degradation and induces a limited foreign-body response. Area of initial polymer implantation is identified (*) in inactive control (A) and salicylate-based (B) polymer. Skin and capsule collagen stains blue and muscle tissue appears red. Higher-power images from the center of the implant indicate the degradation of the polymer (*) and invasion of cells (arrows) in inactive polymer (C) and salicylate-based polymer (D). Original magnification $100\times$ (A, B) and $400\times$ (C, D).

used medical polymers and observed that biofilm formation for *S. epidermidis* and *P. aeruginosa* was inhibited in a dose-related manner with sodium salicylate. Related studies also demonstrated that SA and other NSAIDs decreased bacterial colonization on contact lenses in a dose-dependent manner [29,30].

Stepanovic et al. explored the effect of aspirin on the biofilm-producing capacity of various *Candida* species [31]. Concentrations of soluble aspirin, which induced statistically significant decreases in yeast biofilm formation, ranged from 0.43 to 1.73 mM (59–239 mg/L SA) and depended on the tested yeast strain. Muller et al. reports that the inclusion of 5 mM (691 mg/L) SA inhibited both growth and biofilm production by *S. epidermidis* by up to 55% [32].

Our results in Fig. 2 indicate that soluble SA below 300 mg/L had little effect on bacterial growth rate but concentrations ≥ 300 mg/L decreased *P. aeruginosa* growth. Based upon the size and mass of the salicylate-based polymer disks used in this study, we estimate that the active polymers contain 139.9 ± 2.1 mg of SA. If this amount were instantly dispersed throughout in the 10 mL test volume, it would affect a concentration of ~ 14.0 gm/L, which—according to our batch growth curves—would be toxic to these bacterial cultures. However, based on the rates of polymer degradation, bacterial suspended cultures

were never exposed to ambient SA concentrations ≥ 100 mg/L, which were found not to be toxic. These results suggest that SA must prevent biofilms by a mechanism other than simply killing suspended bacterial cells.

Presence of enzymes secreted by growing bacteria and the acidic environment caused by bacterial generation of carbon dioxide could increase salicylate-based polymer degradation rates. Based upon the release of SA as shown in Fig. 3 (top), polymer degradation slightly increased when placed in the active, bacterial suspended culture. Degradation rates of polyanhydrides such as the salicylate-based polymer are typically slowed in acidic environments [9,25,26]. The combination of data shown in Fig. 3 is compelling: the presence of bacteria decreases the overall pH of the solution, which should slow the hydrolysis of the polymers, yet the polymers degraded slightly faster with bacteria present, suggesting that the bacteria may secrete enzymes (e.g., esterases) that are slightly enhancing polymer hydrolysis.

Polymer disks submerged in a *P. aeruginosa* suspension for up to 3 h also displayed differences in bacterial adhesion (Fig. 4). Both the inactive control and salicylate-releasing polymer disks displayed similar degradation profiles and generate degradation products with similar pKa values, thus the observed effects are due to the release

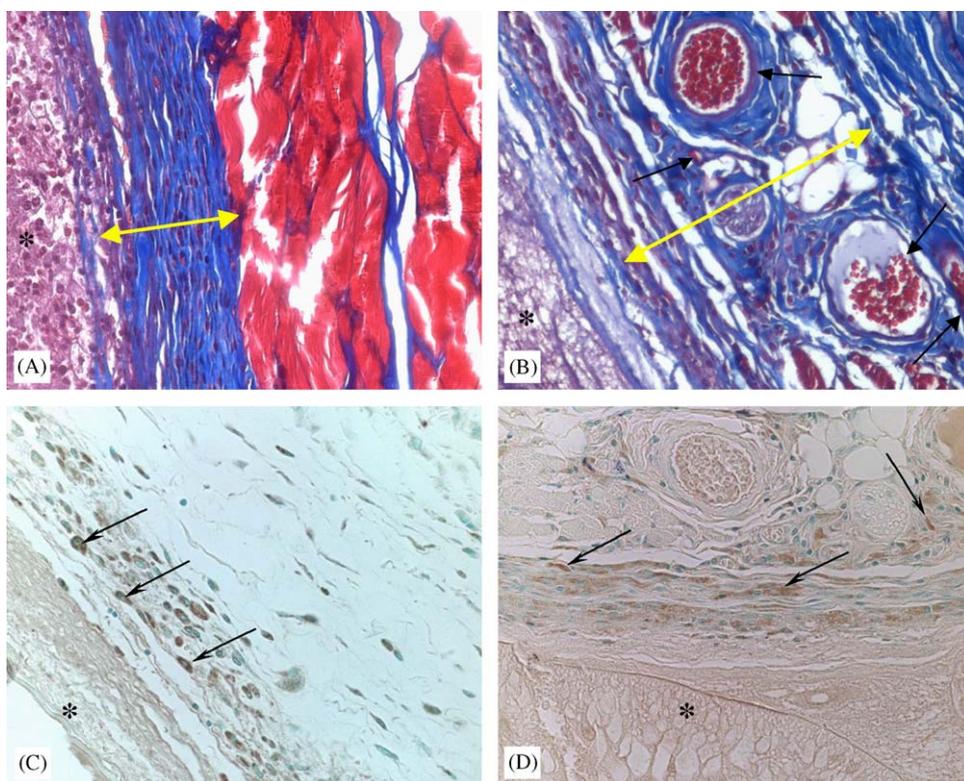


Fig. 9. Salicylate-based polymer is encapsulated by vascularized capsules that are void of macrophages. Area of foreign-body capsule is identified by yellow bidirectional arrow (A, inactive control; B, salicylate-based polymers). The presence of blood vessels in the capsule surrounding Salicylate-based polymer is evident (arrows in B). Immunolocalization of macrophages by the peroxidase reaction, brown color (arrows in C, D), indicate their presence in close proximity to the inactive control (*) and at a distance from the salicylate-based polymer.

of SA and not to dissimilar degradation rates or localized pH effects.

As shown in the long-term biofilm experiments (Figs. 5 and 6), the number of adherent cells accumulating on the inactive control polymer disks is five orders-of-magnitude more than on the salicylate-releasing polymers. Again, the slow release of SA resulting from salicylate-based polymer degradation significantly prevents biofilm formation, even relative to a polymer control undergoing an equivalent degradation rate. Local interfacial concentrations of SA either globally inhibit pathways leading to biofilm formation or have a more directed effect on a particular biofilm formation process.

Suspecting that SA may inhibit biofilm formation by interrupting quorum sensing control of polysaccharide secretion and subsequent biofilm formation, we repeated biofilm experiments employing a recombinant strain of *P. aeruginosa*. This strain carries a gene marker upstream of the *las* regulon quorum-sensing pathway. Should bacteria be activated by homo-serine lactone signals and begin polysaccharide secretions, the *gfp* gene would also be up-regulated and fluoresce. Results for the inactive control polymer disks (Fig. 7) indicate that was indeed the case. However, for recombinant cells adherent to salicylate-based polymers no GFP fluorescence was observed insinuating SA negates the *las* regulon system. This observation could mean that either SA acts as an analog

competing with the homo-serine lactones or that SA merely inhibited some portion of the *las* pathway. Prithiviraj et al. [33] report that SA, at a concentration that did not inhibit *P. aeruginosa* growth was sufficient to significantly affect the ability of the bacteria to attach and form biofilm communities on abiotic surfaces. Furthermore, SA down regulated three known virulence factors of *P. aeruginosa*: pyocyanin, protease, and elastase. Using microarray technology to identify SA target genes, Prithiviraj et al. [33] reports that SA treatment affected the expression of 331 genes. In essence, that the *las* system appears influenced by SA in our work may not be a “quorum sensing” analog effect but rather a global effect on multiple metabolic pathways. A series of similar experiments were carried out with the bacterium, *S. epidermidis* that resulted in a similar reduction in adhesion and long-term biofilm formation (data available upon requested). The mode of interaction of released SA on *S. epidermidis* is unknown. Research has shown that Gram-positive bacteria do not employ homo-serine lactones as signal molecules; rather they use small peptides. Thus, since SA does exhibit similar effects on *S. epidermidis*, a species that does not employ the *las* pathway, it is most likely that SAs effects on *P. aeruginosa* could be far more global than simply inhibiting quorum signaling. More work is underway to determine SA’s role in the inhibition of biofilm formation.

5. Conclusion

Polymers of NSAIDs that degrade to produce salicylic acid and adipic acid were evaluated for their ability to reduce or eliminate bacterial biofilm accumulation. Results indicate that the wild-type *P. aeruginosa* exhibited slower adhesion rates and significantly less biofilm formation on salicylate-based polymer than on a degrading polymer that does not release salicylic acid.

Slow release of salicylic acid that occurs with the hydrolytic degradation of salicylate-based polymers has a significant impact on biofilm formation. In vitro, the salicylate-based polymers and inactive control polymers have similar degradation rates yet their in vivo degradation characteristics are significantly different in this animal model. After 4 weeks, the majority of salicylate-based polymers remained intact and exhibited significantly less infiltration of inflammation-associated cells such as macrophages and neutrophils. We have previously observed that a dampened inflammatory response, such as reduced foreign-body giant cell formation, can reduce the degradation rate of biodegradable implants [24]. Our current observations suggest that the released salicylic acid is modulating the inflammatory response and thus, leads to protection of the implant. The ability of salicylate-based polymers to modulate the macrophage response is also obvious by the lack of macrophages in the immediate proximity of the implantation site. Salicylic acid at therapeutic concentrations has been shown to suppress the expression of inflammatory molecules in macrophages such as cyclo-oxygenase (COX)-2, induced nitric oxide synthase (iNOS), and interleukin-4 [34]. At suprapharmacological concentrations it can also interfere with NF-kappaB binding. It is possible that in our implantation model, salicylate is influencing the expression of all these molecules and exerts a complex effect. Nevertheless, our in vivo studies demonstrate the ability of salicylate-based polymers implants to modulate the foreign-body response.

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