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Biodegradable Polymer (PLGA) Coatings Featuring Cinnamaldehyde and Carvacrol Mitigate Biofilm Formation

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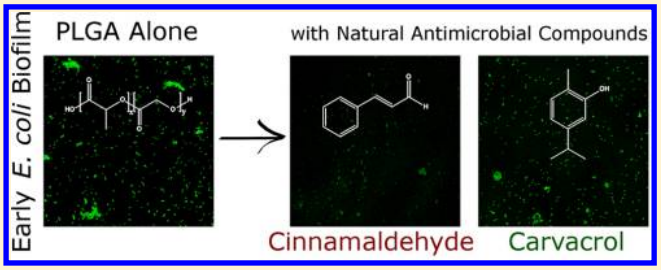
ABSTRACT: Biofilm-associated infections are one of the leading causes of death in the United States. Although infections may be treated with antibiotics, the overuse of antibiotics has led to the spread of antibiotic resistance. Many natural antimicrobial compounds derived from edible plants are safe for human use and target bacteria nonspecifically. Therefore, they may impair biofilm formation with less evolutionary pressure on pathogens. Here, we explore the use of two natural antimicrobial compounds, cinnamaldehyde (CA, from cinnamon) and carvacrol (CARV, from oregano), for biofilm prevention. We have fabricated and characterized films that incorporate CA and CARV into the biodegradable, FDA-approved polymer poly(lactic-*co*-glycolic acid), PLGA. The addition of CA and CARV to PLGA films not only adds antimicrobial activity but also changes the surface properties of the films, making them more hydrophilic and therefore more resistant to bacterial attachment. An addition of 0.1% CA to a PLGA film significantly impairs biofilm development by *Staphylococcus aureus*, and 0.1% CARV in PLGA significantly decreases biofilm formation by both *Escherichia coli* and *S. aureus*. *Pseudomonas aeruginosa*, which is less susceptible to CA and CARV, was not affected by the addition of 0.1% CA or CARV to the PLGA coatings; however, *P. aeruginosa* biofilm was significantly reduced by 1.0% CA. These results indicate that both CA and CARV could potentially be used in low concentrations as natural additives in polymer coatings for indwelling devices to delay colonization by bacteria.



INTRODUCTION

Infectious diseases are one of the leading causes of death in the United States.[1] The development of antimicrobial resistance, globalization, and a decline in antibiotic discovery exacerbate this problem.[2] In the United States, hospital-acquired infections alone cause between 60 000 and 90 000 deaths, amounting to an economic burden of $17 billion to $29 billion annually.[3] It is estimated that as many as 80% of these infections arise from bacterial biofilms. Biofilms are communities of microorganisms that colonize and grow on surfaces, such as indwelling devices or native tissues. Although many biofilms are beneficial, some biofilms cause grave health concerns. Detrimental biofilm formation is problematic because bacteria in a biofilm are more resistant to disinfectants.[4] Many harmful biofilms are associated with indwelling medical devices, and when pathogens form a biofilm on a device, the infection is difficult to eradicate. Often, the device, for example, a catheter, intubation tube, implant, or shunt, must be removed.

One potential way to prevent biofilm formation on indwelling medical devices is to coat them with antimicrobial compounds. For instance, coatings with chlorhexadine and silver sulfadiazine on catheters decreased the occurrence of catheter-related bloodstream infections in intensive care units.[5] However, the use of antibiotics leads to undesired consequences, including the spread of antibiotic-resistant bacteria. For example, 60% of *Staphylococcus aureus* strains isolated from intensive care units were resistant to methicillin.[5] Vancomycin is often used to treat methicillin-resistant *S. aureus* (MRSA) infections; however, a recent increase in vancomycin-resistant strains underscores the need for alternative methods of microbial control.[2] This trend in antibiotic resistance contributes to the popularity of inorganic antimicrobials, including silver. However, the use of silver is also problematic, as silver resistance has been observed in bacteria, notably *Pseudomonas aeruginosa*. It is likely that common use of silver or other heavy metals as antimicrobial agents will lead to increased silver resistance in other microorganisms.[6] Therefore, antimicrobial compounds that will not lead to the spread of resistance genes are in high demand.

The essential oil components cinnamaldehyde (CA) and carvacrol (CARV) are broad-range, natural antimicrobial compounds that can be readily isolated from cinnamon and oregano, respectively (Figure 1). Essential oils have been shown to be effective against a wide range of bacteria. In general, they act by increasing cell membrane permeability, leading to a depletion of the proton gradient and subsequent disruption of ATP synthesis.[7,8] The exact antimicrobial mechanism of essential oils is complex, making the development of resistance by bacteria more difficult.[9] Additionally, some essential oils,

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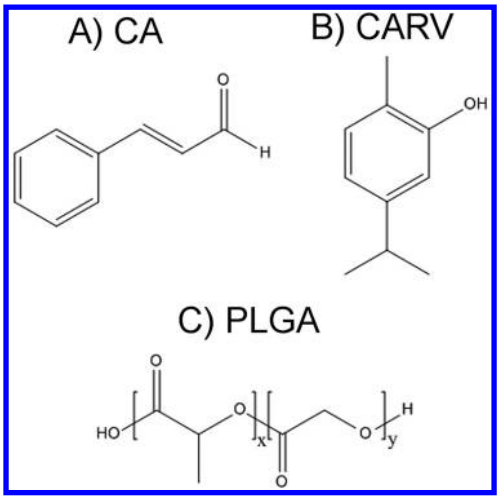

Figure 1. Two naturally occurring antimicrobial compounds (A) cinnamaldehyde (CA), a phenolic aldehyde, and (B) carvacrol (CARV), a monoterpenoid phenol, are incorporated into a (C) biodegradable and biocompatible poly(lactic-*co*-glycolic acid) (PLGA) film.

including CA, inhibit biofilm formation at subinhibitory concentrations.[10,11] Specifically, cinnamon essential oil (of which CA is a major active constituent) decreases metabolic activity and the replication rate of *P. aeruginosa* and *S. aureus* and causes changes in cell morphology.[12] CA inhibits swimming motility in *Escherichia coli*.[13] In *P. aeruginosa*, exposure to cinnamon essential oil leads to a coagulation of cytoplasmic material and an extrusion of intracellular material. In *S. aureus*, fibrous extensions are visible. CA at sublethal concentrations acts as a quorum sensing inhibitor (QSI), interfering with the autoinducer-2 (AI-2) system, and delays biofilm formation by *S. aureus* at concentrations below the minimum inhibitory concentration (MIC).[10,11,14] Therefore, the use of CA at low concentrations could prevent colonization of surfaces by bacteria without exerting evolutionary pressure. Oregano essential oil, of which CARV is a major active component, affects the membrane potential and permeability in *S. aureus*. In *S. aureus*, mesosome-like structures are observed, and in P. *aeruginosa*, coagulated cytoplasm and liberated vesicles are seen.[15] CARV is also known to initiate a response in *E. coli* similar to the response exhibited upon heat stress and to inhibit flagella synthesis.[16]

Many previous studies with CA and CARV focused on applications in food preservation (reviewed by Burt[17]). It has been demonstrated that 10 wt % CARV incorporated into low-density polyethylene films creates slow-release antimicrobial packaging.[18] Suspensions of poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles incorporating CA inhibit the growth of a number of organisms.[19] CARV, 1.0%, has also been added to PLGA particles to treat advanced biofilms, resulting in both a reduction in biofilm and changes in biofilm viscoelastic properties.[20] Coatings, in contrast to free or particle-encapsulated compounds, offer an opportunity to preemptively deliver bioactive compounds to a site at risk for a biofilm-related infection. The activity of CA and CARV in a PLGA film coating for medical devices has not yet been explored.

Here, we investigate the use of natural antimicrobial compounds isolated from plant essential oils and incorporated into a PLGA matrix to prevent biofilm formation on a surface. Three model organisms were tested—*E. coli*, *P. aeruginosa*, and *S. aureus*. These microorganisms are responsible for medically relevant biofilm formation, including biofilms associated with the gastrointestinal tract, lungs, and indwelling medical devices. PLGA coatings containing CA and CARV could potentially be applied to a variety of indwelling medical devices to prevent infections associated with those devices.

MATERIALS AND METHODS

Materials and Chemicals. All compounds were used as received. Cinnamaldehyde (CA) (≥93%, FG, M_w = 132.16 Da), carvacrol (CARV) (≥98%, FCC, M_w = 150.22 Da), poly(D,L-lactide-*co*-glycolide) (PLGA) (85:15, M_w = 50 000−75 000 Da), and chloroform (≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO). Paraformaldehyde was obtained from MP Biomedicals, LLC (Solon, OH). Hydrochloric acid (HCl) and Triton X-100 were purchased from Fisher Scientific, sodium hydroxide (NaOH) and sodium chloride (NaCl) from J.T. Baker (Phillipsburg, NJ), and ethanol (EtOH) (200 proof) from Decon Laboratories, Inc. (King of Prussia, PA). Deionized (DI) water was obtained from a Milli-Q ultrapure water purification system (Millipore, Billerica, MA).

The nucleic acid stain SYTO 9 and mounting oil were purchased from Invitrogen (Eugene, OR). M9 minimal salts and phosphate-buffered saline (PBS) were purchased from Sigma Life Sciences (St. Louis, MO). Difco Luria−Bertani (LB) broth was purchased from BD Life Sciences (Franklin Lakes, NJ), and Müller−Hinton Broth (MHB) from HiMedia Laboratories (Mumbai, India).

Model Bacteria Strains. *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus* (*S. aureus*) were used as the model microorganisms. Kanamycin-resistant *E. coli* BW26437 was obtained from the Yale Coli Genetic Stock Center (New Haven, CT). The *P. aeruginosa* PAO1 strain was described in our previous publication.[21] *S. aureus* RN 6390B was kindly provided by Dr. Naomi Balaban. Overnight cultures were grown in either LB or MHB at 37 °C. These cultures were then diluted in fresh MHB and grown for 2 h to log phase prior to the toxicity and biofilm experiments. Log-phase growth time was determined by a growth curve. Cell density was determined by a plate count. Optical density (OD) was measured at 600 nm to determine cell growth. An OD_{600} of 1.0 corresponds to 10^9 CFU/mL for all three strains tested.

Evaluation of the Biological Effects of Cinnamaldehyde (CA) and Carvacrol (CARV). The minimum inhibitory concentrations (MICs) of both CARV and CA were determined according to the broth dilution method published by Wiegand et al.[22] Overnight cultures of *E. coli*, *P. aeruginosa*, and *S. aureus* were grown in MHB. Cultures were diluted in MHB and grown to log phase (2 h). The culture was diluted in MHB to an optical density at 600 nm (OD_{600}) of 0.1 and subsequently diluted 100×. This corresponds to a concentration of ∼10^6 CFU/mL, as determined by a plate count. Then, 50 μL of the cell suspension was diluted into 50 μL of CARV or CA in MHB in a cell-culture-treated 96-well plate (Falcon Microtest™ 96) and pipetted up and down to mix. The 96-well plate was then placed in an incubator at 37 °C for 17 h. The OD_{600} was measured as an indication of cell growth (SPECTRA max 340PC).

The biofilm that developed on the walls of the polystyrene plate (Falcon Microtest™ 96) was quantified according to the method described by Kiran et al.[23] After the MIC assay, the suspended cells were removed, and the remaining biofilm was gently rinsed with PBS. The biofilms were then fixed with 100% EtOH, and the EtOH was allowed to evaporate at room temperature (25 °C). The biofilm was then stained with 0.1% safranin (LabChem Inc., Pittsburgh, PA) and rinsed three times with PBS. The biofilm cells containing the safranin stain were resuspended with 1% sodium dodecyl sulfate (SDS). The relative amount of biofilm was quantified by an OD_{490} measurement (SPECTRA max 340PC).

Thin Film Fabrication. CA and CARV were incorporated into PLGA thin films. PLGA is used for a variety of biomedical applications and has easily tunable physical properties.[24] PLGA/chloroform (0.4 g in 20 mL corresponding to 2 wt %) solutions were mixed for 24 h using a rotating plate (VWR, Bridgeport, NJ). CARV or CA (0, 0.4, 4.0

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mg, corresponding to 0, 0.1, and 1.0 wt %, respectively) was dispersed into PLGA solutions using a Vortex-Genie (Scientific Industries, Inc., Bohemia, NY). PLGA films were spin-coated (Spin-Coater model SCS P-6708, Specialty Coating Systems Inc., Indianapolis, IN) on 22 × 22 × 1.5 mm glass coverslips (Fisherbrand) with 100 μL of the solution of interest at 200 rpm for 20 s, 300 rpm for 40 s, and 8000 rpm for 5 s; there was an additional 1 s ramp time between hold times.[3] Prior to spin-coating, the coverslips were thoroughly cleaned by an overnight rinse in 2% Liquinox critical-cleaning liquid detergent solution (Jersey City, NJ) at 75 °C, followed by repeat rinsing with DI water. The coverslips were then placed in a UV/ozone chamber for 30 min to oxidize any remaining organic material (UV/Ozone ProCleaner™, BioForce Nanosciences, Ames, IA). Remnant chloroform was allowed to evaporate from the spin-coated PLGA, PLGA−CARV, and PLGA−CA films at room temperature for 3 days.

Characterization of Thin Films. Film thickness was measured using an ellipsometer (Angstrom Advanced, Inc.) positioned at 65° and equipped with a 632.8 nm laser. A Cauchy model was used with a refractive index of 1.45 to determine the film thickness.[3]

Contact angle measurements with DI water, diiodomethane (99+%, Acros Organics), and glycerol (99.9%, J.T. Baker) were carried out using a Goniometer (VCA Video Contact Angle System, AST Products, Billerica, MA). Ten drops of 5 μL on at least two different samples were measured for each film and liquid combination. Surface energy calculations from the averages of the measured contact angles taken with these three liquids were carried out according to a previously reported method.[25,26]

Evaluation of Biofilm Growth on PLGA−CARV and PLGA−CA Films. PLGA−CARV and PLGA−CA films were coated onto the sides and bottom of wells in a glass-coated 96-well plate (Plate+™, SUN Sri, Rockwood, TN). A 150 μL aliquot of 2% PLGA in chloroform with 0.1 and 1.0% CARV or CA was added to the well. The solution evaporated in a chemical hood over 3 days at room temperature, resulting in a visible film in each of the wells. The well plate was then sterilized during 4 h UV treatment (UVP, UVL-28EL series, 365 nm, 8 W). Our tests indicated that 4 h is sufficient for sample sterilization with this lamp. Log-phase *E. coli*, *P. aeruginosa*, and *S. aureus* were added to each well in MHB (5×10^5 CFU/mL), and the bacteria were incubated at 25 °C. After 20 h incubation, the biofilm was quantified according to the method described previously.[23]

Confocal laser scanning microscopy (CLSM, Zeiss LSM 510, and an AXIO Observer Z1 inverted microscope, Carl Zeiss, Inc.) was used to visualize biofilm structure and quantify biofilm biovolume and diffusion distance using COMSTAT2.[27−29] Bacteria were stained with the nucleic acid stain Syto 9 (Invitrogen). Preliminary experiments showed no staining of biofilm by Concanavalin A conjugated with Alexa Fluor 633 (Invitrogen), indicating that extracellular polymeric substances (EPS) production at this stage of development did not contribute to biofilm volume. PLGA films on glass coverslips were affixed to a microscope slide with nail polish to prevent PLGA film detachment. The nail polish was allowed to evaporate for 1 week and was then rinsed with DI water to remove any residual solvent. The thin films were sterilized during 4 h UV treatment (UVP, UVL-28EL series, 365 nm, 8 W) and then placed in sterile Petri dishes. Log-phase bacteria culture was diluted to an OD_{600} of 0.1 in LB broth (10^8 CFU/mL), and then a 0.6 mL culture was added to 30 mL of M9 salts with 0.1% glucose in each of the dishes. Films were grown for 20 h at 25 °C without agitation.

Prior to CLSM visualization, the biofilms were rinsed with PBS and then fixed with 4% paraformaldehyde for 5 min. The remaining paraformaldehyde was removed, and the biofilm was rinsed twice in PBS for 5 min each. The bacteria were then permeabilized with 0.2% Triton X-100 for 5 min and subsequently stained with the nucleic acid stain Syto 9 for 10 min. The biofilms were then dehydrated with an ethanol series (50, 75, and then 100% EtOH) for 5 min each. The slides were mounted using BacLight™ mounting oil prior to visualization with a Plan-Apochromat 20×/0.8 numerical aperture objective. Syto 9 was excited with an argon 488 nm laser, and a BP 500−530 IR emission filter was used. Data was collected with Zen (Carl Zeiss, Inc.). At least 0.45 mm^2 on each sample was used for image analysis. Subsequent image analysis was performed with ImageJ 1.41 software (National Institutes of Health, Bethesda, MD) and COMSTAT2.[27−29]

Statistical Analysis. The data are represented as mean ± standard deviation of at least three samples. Significant differences between samples were determined with a one-way analysis of variance (ANOVA), and post hoc comparisons between groups were made with a Tukey test (*R*, www.r-project.org). Significance ($p \leq 0.05$) is denoted in graphs by an asterisk.

RESULTS AND DISCUSSION

CA and CARV in Solution Inhibit Bacterial Growth and Biofilm Formation. The effects of CA and CARV on cell growth and biofilm formation were evaluated in a solution of MHB. All microorganisms exhibited a dose-dependent response (Figure 2). *S. aureus* was most susceptible to both

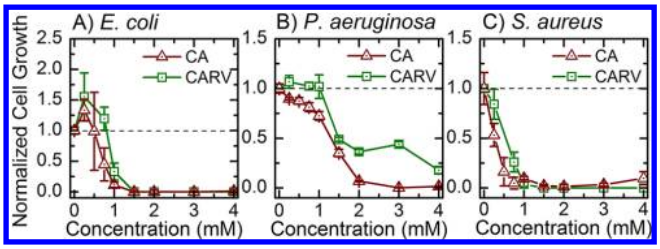

Figure 2. CARV and CA inhibited growth of (A) *E. coli*, (B) *P. aeruginosa*, and (C) *S. aureus*. Bacteria (5×10^5 CFU/mL) in log phase were inoculated into MHB containing CARV and CA. After 17 h, the optical density (600 nm) was measured to determine the amount of cell growth. Cell growth was normalized to the control without CARV or CA (indicated by the dashed line).

CA and CARV, followed by *E. coli* and then *P. aeruginosa*. *E. coli* growth was inhibited by 1.0 mM CA and 1.5 mM CARV (Figure 2a). *P. aeruginosa* was much more resistant to both CA and CARV (Figure 2b). *P. aeruginosa* growth was reduced at 4 mM CARV, the highest concentration tested, and completely inhibited at 3 mM CA. *P. aeruginosa* has been shown to be resistant to the essential oil from *Melaleuca alternifolia* (tea tree), in which terpinen-4-ol is the active component.[7] It is possible that *P. aeruginosa* resistance to both compounds is similar and results from the ability of the bacterium to change its outer membrane composition. Here, the MIC for *P. aeruginosa* is three times the MIC for *E. coli*. No *S. aureus* growth was observed with 0.75 mM CA or 1.0 mM CARV (Figure 2c). For each of the three model bacteria, CA was slightly more toxic than CARV. The strength of the bacteria growth response to both CARV and CA can be generalized as follows: *S. aureus* > *E. coli* ≫ *P. aeruginosa*.

The observed MIC values discussed above for CARV are similar to those reported in the literature. Previously reported MICs for CARV for *E. coli*, *P. aeruginosa*, and *S. aureus* are 1.3−2.7,[30,31] 3.5,[8] and 0.96 mM,[8] respectively. The obtained MIC values for CA are lower than those reported in the literature: 3 mM for *E. coli*,[30] 10 mM for *P. aeruginosa*,[12] and 5−10 mM for *S. aureus*.[10,12] One difference between this study and those previously reported is the lack of stabilizer (e.g., Tween-80,[30] EtOH,[10] or agar[12]) for the CA. The lack of stabilizer may have made more of the CA available to interact with the bacteria, lowering the MIC.[32] We observed the CA to dissolve completely in MHB at the concentrations tested without the use of a stabilizer.

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The biofilm formation observed (Figure 3) with CA and CARV in solution mirrored the bacterial growth (Figure 2),

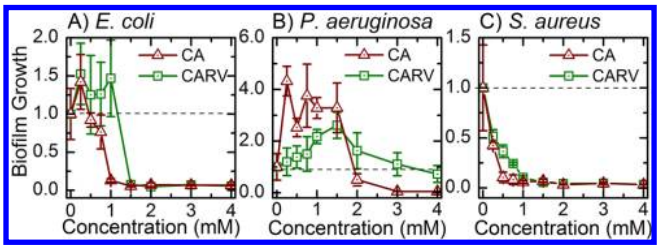

Figure 3. Biofilm formation by (A) *E. coli*, (B) *P. aeruginosa*, and (C) *S. aureus* was inhibited by CA and CARV in solution. Bacteria in the log phase were added to MHB and grown for 17 h. Biofilms were rinsed, fixed, and stained with safranin. Optical density (490 nm) was measured, and the total biofilm was normalized relative to the control without CARV or CA (indicated by the dashed line).

indicating that a lack of bacterial growth caused a lack of biofilm formation for all the bacteria tested. CA inhibited biofilm formation slightly more than CARV. *E. coli* biofilms were eliminated at 1 mM CA and 1.5 mM CARV (Figure 3a). These values directly correspond to the MIC. For *P. aeruginosa*, biofilms were inhibited at 3 mM CA. No inhibition of biofilm formation was observed for CARV (Figure 3b), as the MIC for CARV was above the values tested (Figure 2b). This indicates that with *E. coli* and *P. aeruginosa* there was no growth-independent inhibition of biofilm development. Biofilm formation by *P. aeruginosa*, however, was enhanced significantly at lower concentrations of CA and CARV. The enhancement of biofilm formation upon exposure to subinhibitory concentrations of antibiotics has been observed previously in *P. aeruginosa*.[33] This enhancement does not correspond to an increase in the bacterial growth rate (Figure 2b). A similar, but not statistically significant, enhancement was observed with *E. coli*. No *S. aureus* biofilm formation was observed at 0.5 mM CA or 1 mM CARV (Figure 3c). Although the lack of biofilm formation at 1 mM CARV corresponds to the MIC, the MIC for *S. aureus* and CA is 0.75 mM. Thus, it is possible that the decrease in biofilm formation at 0.5 mM CA is due to a direct inhibition of biofilm formation, also observed by Jia et al.,[10] and not a lack of bacterial growth.

Characteristics of PLGA Films Containing CA and CARV. Spin-coating successfully produced both control PLGA films and films incorporating 0.1 and 1.0% CA or CARV. The average thickness of the films was between 500 and 520 nm, indicating that the addition of 1.0% CA and CARV did not change the properties of the spin-coating solution enough to significantly influence the thickness of the films (Table 1).

Contact angle measurements were performed with three solutions—water, glycerol, and diiodomethane—to quantify the surface energies of the films with CA and CARV. In general, surfaces that are more hydrophilic are less susceptible to biofouling because of lower bacterial adhesion; however, surfaces with lower surface energy show better foulant release.[26] The surface tension (γ) of a film is the sum of two components: nonpolar Lifshitz–van der Waals (LW) forces and polar acid–base (AB) electron donor/acceptor forces. Each molecule of CA adds an electron donor, and each molecule of CARV adds an electron donor and an electron acceptor. The addition of these electron donors and acceptors is reflected in a slight change of the electron donor component of the surface tension (γ^-) for films containing both CA and CARV (Table 1).

Table 1. Properties of Thin Films: PLGA Control, PLGA with 1.0% CARV, and PLGA with 1.0% CA[a]

	PLGA	PLGA–CA	PLGA–CARV
thickness (nm)	510 ± 6.5	519 ± 35	502 ± 13
θ_w (°)	81.7 ± 2.9	76.6 ± 2.9	76.8 ± 3.0
γ^{LW} (mJ/m^2)	36.7	34.8	37.4
γ^+ (mJ/m^2)	0.001	0.067	0.002
γ^- (mJ/m^2)	6.82	9.99	10.0
γ^{AB} (mJ/m^2)	0.166	1.64	0.28
γ^{TOT} (mJ/m^2)	36.8	36.5	37.7
ΔG_{131}^{LW} (mJ/m^2)	−3.85	−3.04	−4.18
ΔG_{131}^{AB} (mJ/m^2)	−22.9	−6.90	−6.31
ΔG_{131}^{TOT} (mJ/m^2)	−26.7	−9.94	−10.5

[a]The thickness, as measured by ellipsometry, and the contact angle of DI water, θ_w, are shown as average ± standard deviation. Surface tension, γ, was calculated from the averages of all contact angle measurements.[26,34] The free energy of cohesion with water, ΔG_{131}, was computed from the calculated surface tension values. (LW is Lifshitz–van der Waals; + is electron acceptor; − is electron donor; AB is acid base; and TOT is the sum of LW and AB.)

However, the overall surface tension of the films is dominated by the nonpolar LW forces, leading to no great difference in surface tension of the films (γ^{TOT} = 36.5–37.7 mJ/m^2).

In contrast to surface tension, the free energy of cohesion (ΔG_{131}^{TOT}) is quite different when CA and CARV are added to PLGA. When $\Delta G_{131}^{TOT} < 0$, the surface is hydrophobic. Therefore, the films with CA and CARV are less hydrophobic than films with PLGA alone. ΔG_{131}^{TOT} is dominated by the AB component, which decreases in magnitude from −22.9 mJ/m^2 for PLGA alone to −6.90 and −6.31 mJ/m^2 with the addition of CA and CARV, respectively (Table 1). This decrease in hydrophobicity may affect bacterial attachment to the CA and CARV films based on physicochemical properties alone.

PLGA Films Containing CA and CARV Inhibit Biofilm Growth. PLGA films with 0.1 and 1.0% CA or CARV were formed in a 96-well plate. PLGA itself is not harmful to *E. coli*,[3] and it acted as a control for the antimicrobial CA and CARV. Bacteria in nutrient-rich MHB were incubated in the coated wells for 20 h, and the remaining biofilm was stained with safranin prior to quantification. No staining of the PLGA with or without CARV and CA was observed.

PLGA films containing the lowest concentration of CA and CARV tested showed promising results. CA (0.1%) lessened biofilm growth by *S. aureus*, and 0.1% CARV impaired biofilm growth by both *E. coli* and *S. aureus* (Figure 4). *P. aeruginosa* was not as susceptible to CA and CARV in the PLGA matrix. However, there was a significant decrease in *P. aeruginosa* biofilm formation on 1.0% CA.

E. coli biofilm formation was inhibited on films with 0.1% CARV (p = 0.01) and 1.0% CA (p = 0.02) (Figure 4a). Therefore, under these particular conditions, there is no benefit in this 10-fold increase of CA. However, under different experimental conditions, a benefit to higher CA concentrations might be observed. No significant decreases in biofilm formation were observed with *P. aeruginosa* on 0.1 or 1.0% CARV (Figure 5b); however, biofilm formation decreased significantly in the 1.0% CA sample. *S. aureus* was the only bacteria affected by both 0.1% CARV (p = 0.02) and 0.1% CA (p = 0.046) (Figure 4c). Thus, PLGA films containing CA and CARV can effectively lessen biofilms formed by different bacterial species.

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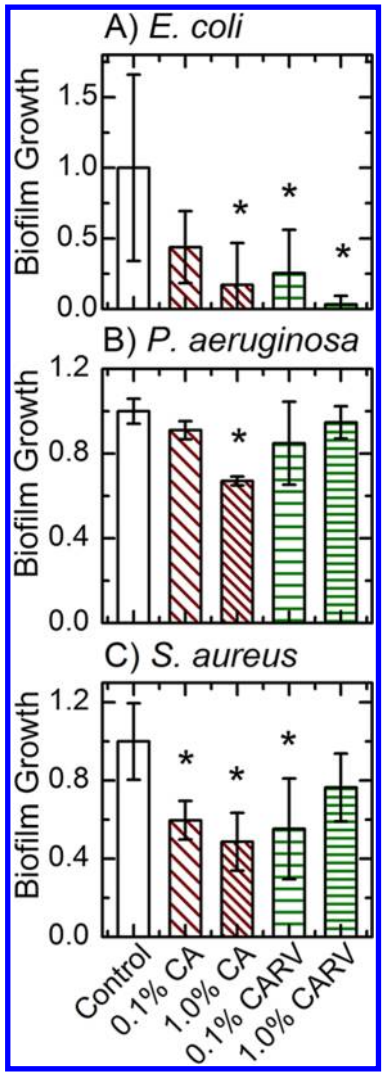

Figure 4. Biofilm growth by (A) *E. coli* was inhibited on PLGA films with 1.0% CA and 0.1% CARV. (B) *P. aeruginosa* biofilms were inhibited by 1.0% CA, and (C) *S. aureus* biofilms were inhibited by both 0.1% CA and 0.1% CARV. PLGA films were formed by evaporation of 2% PLGA in chloroform (with CARV and CA additives) in a glass-coated microtiter plate. Bacteria were incubated in MHB for 20 h. The biofilm was rinsed, fixed with ethanol, and stained with safranin. Absorbance was measured at 490 nm. Data were normalized to the PLGA control for each bacterium. An asterisk denotes that a significant ($p \leq 0.05$) reduction in biofilm growth has occurred between the control PLGA film and a film containing CARV or CA.

Structural Changes in Biofilms on PLGA Films Containing CA and CARV. The structure of the biofilms formed in minimal M9 salts with 0.1% glucose was observed using CLSM. Biofilm formation is a complex process consisting of five stages: (1) conditioning and initial attachment, (2) EPS production and irreversible attachment, (3) cell division and microcolony maturation, (4) further microcolony development, and (5) dispersion.[4] After 20 h, the PLGA control biofilms reached stage 3 (Figure 5a). On 1.0% CA films, some cells were irreversibly attached (stage 2), but overall biofilm formation decreased substantially (Figure 5b). *E. coli* microcolonies were much smaller on the 1.0% CARV films (Figure 5c).

Biofilm biovolume indicates the volume occupied by bacteria per unit area of the substratum ($\mu m^3/\mu m^2$). It is also an indication of the amount of biomass observed.[27] While no significant difference in *E. coli* biovolume was observed on the 1.0% CARV film ($p = 0.59$), a significant decrease in biovolume was observed on the 1.0% CA film ($p = 0.03$). This is a slightly different response than was observed with the 96-well plate assay using MHB. Thus, the response of *E. coli* to these additives in the PLGA depends, in part, on solution composition and/or the quantification method. The slight decrease in *P. aeruginosa* biovolume calculated for both the 1.0% CARV and 1.0% CA films was not statistically significant (Figure 6b). As observed in the 96-well plate assay, *S. aureus* biofilm formation was inhibited on both the 1.0% CARV ($p \ll 0.01$) and 1.0% CA ($p \ll 0.01$) films (Figure 6c). These results emphasize the degree to which *S. aureus* biofilm growth is inhibited by PLGA films that contain either CA or CARV in small amounts.

Diffusion distance is the shortest distance between a small area that contains biomass and an area that does not contain biomass. This distance could also be considered as the distance a nutrient would have to travel to reach an area within a microcolony from the surrounding medium, and, especially in the early stages of colony development, is related to the number of cells in a microcolony.[27] Two diffusion distances were calculated for the image stacks obtained—the maximum diffusion distance (MDD) and the average diffusion distance (ADD) (Figure 7). Together, these distances help characterize the diffusion state of the biofilm.

Significant decreases in ADD was observed on 1.0% CA films with *E. coli* ($p = 0.003$) (Figure 7a). An additional difference in MDD on 1.0% CARV films was observed in the *P. aeruginosa* biofilm ($p \ll 0.001$) (Figure 7b). Significant decreases in both ADD and MDD were observed by *S. aureus* on both 1.0% CA and CARV films ($p < 0.003$ for all) (Figure 7c). We suggest that calculated diffusion distance is related to the number of cells contained within a microcolony, and it is likely that the smaller diffusion distances calculated are due to an inhibition of microcolony growth on the PLGA films with CA and CARV.

The results discussed above emphasize that the effects of both CARV and CA in PLGA are similar to their effects in solution. These compounds are not particularly effective against *P. aeruginosa* but are quite effective against *S. aureus*—the most susceptible bacteria tested here (Figure 2). In general, PLGA films containing CA and CARV are most effective against *S. aureus*, somewhat effective against *E. coli*, and not very effective against *P. aeruginosa.*

■ CONCLUSION

The colonization of medical devices by bacterial biofilms is a problem of increasing concern. Biofilms are difficult to eradicate, and the increased prevalence of antibiotic-resistant organisms increases the risk of bacterial infection. It is possible that the use of broad-range natural antimicrobial compounds in a clinical setting could reduce demand for conventional antibiotics. Here, we verify that cinnamaldehyde (CA) and carvacrol (CARV) in solution significantly impair bacterial growth and, therefore, biofilm formation by *E. coli*, *P. aeruginosa*, and *S. aureus* at millimolar concentrations. We then demonstrate that concentrations up to 1.0% CA and CARV can be incorporated into biocompatible PLGA films. These modified films have a similar thickness but prove to be more hydrophilic due to additional electron donors and acceptors in the CA and CARV. The natural antimicrobial compounds in the films delay biofilm formation by both gram-

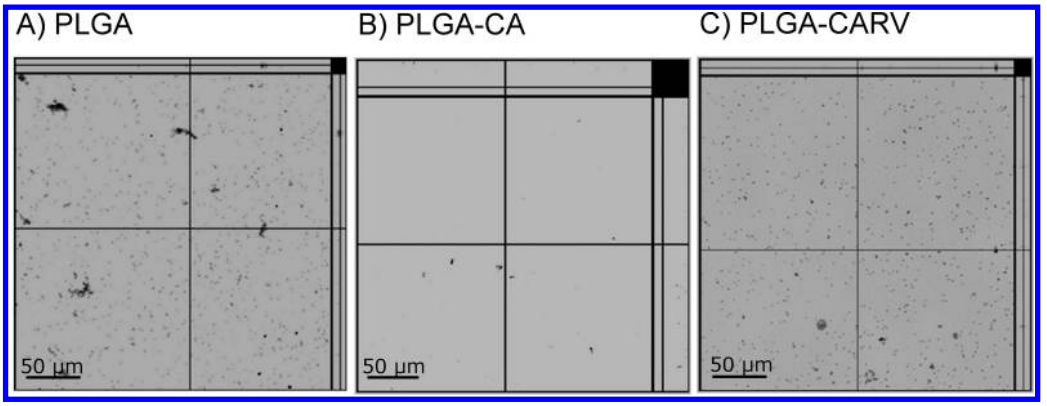

Figure 5. Pictured are orthogonal sections (300 μm × 300 μm) of representative CLSM images formed after 20 h on (A) PLGA control films in M9 salts with 0.1% glucose. CLSM biovolume analysis (Figure 6) showed a significant decrease in biofilm formation by *E. coli* on (B) PLGA with 1.0% CA compared to the (A) PLGA control. No significant difference was observed on PLGA with (C) 1.0% CARV.

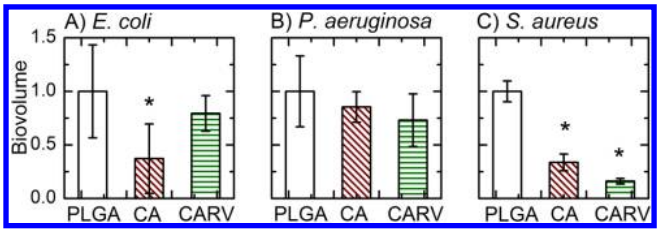

Figure 6. Significantly less (A) *E. coli* biovolume was observed on PLGA films with 1.0% CA. (B) *P. aeruginosa*, the bacterium least susceptible to CA and CARV, was not significantly affected by 1.0% CA or 1.0% CARV. (C) *S. aureus* biovolume on 1.0% CA and 1.0% CARV decreased significantly compared to the PLGA control. An asterisk denotes that a significant ($p \leq 0.05$) reduction in biofilm growth has occurred between the control PLGA film and a film containing CARV or CA. Biofilms were grown in M9 salts and 0.1% glucose for 20 h. The cells were stained with Syto 9. Biovolume was calculated using COMSTAT2.

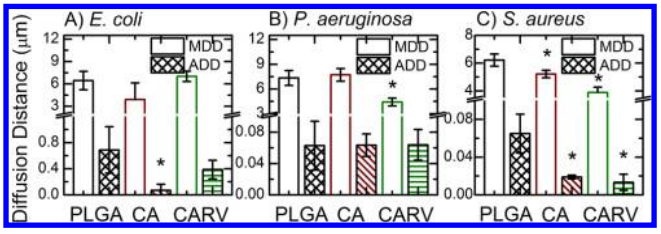

Figure 7. Significantly lower diffusion distances were observed in (A) *E. coli* biofilms grown on 1.0% CA in PLGA, (B) *P. aeruginosa* biofilms grown on 1.0% CARV, and (C) *S. aureus* biofilms grown on both 1.0% CA and 1.0% CARV. An asterisk denotes that a significant ($p \leq 0.05$) reduction in biofilm growth has occurred between the control PLGA film and a film containing CARV or CA. Biofilms were grown in M9 salts and 0.1% glucose for 20 h and stained with Syto 9. Maximum diffusion distance (MDD) and average diffusion distance (ADD) were calculated with COMSTAT2.

positive *S. aureus* and gram-negative *E. coli* on the film surface. These films could potentially be used as natural, plant-based coatings for a number of indwelling devices to safely impede colonization by pathogenic bacteria.

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Notes

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