Antimicrobial Surfaces Hot Paper

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Using Chemoattractants to Lure Bacteria to Contact-Killing Surfaces

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Abstract: *Antimicrobial surfaces with covalently attached biocidal functionalities only kill microbes that come into direct contact with the surfaces (contact-killing surfaces). Herein, the activity of contact-killing surfaces is shown to be enhanced by using gradients in the concentration of soluble chemoattractants (CAs) to attract bacteria to the surfaces. Two natural and nonbiocidal CAs (aspartate and glucose) were used to attract bacteria to model surfaces decorated with quaternary ammonium groups (known to kill bacteria that come into contact with them). These results demonstrate the killing of Escherichia coli and Salmonella typhimurium, two common pathogens, at levels 10- to 20-times greater than that of the native surfaces alone. This approach is general and provides new strategies for the design of active or dynamic contact-killing surfaces with enhanced antimicrobial activities.*

The challenge of minimizing microbial growth on a surface, or in a bulk medium in contact with a surface, occurs in a broad range of industrial, healthcare, and consumer contexts. One common approach used to address this challenge relies on the design of surfaces that release biocides, such as metal ions (for example, silver, lead, mercury, or arsenic) or chemical agents (for example, triclosan, chlorhexidine, peroxides, or chlorine). Limitations of these approaches include the short lifetimes of the released biocides and their potential toxicities.[1] Other approaches have sought to address these and other limitations by developing so-called "contact-killing" antimicrobial surfaces containing covalently attached groups, such as polyionenes or cationic macromolecules containing biguanide, pyridinium, or phosphonium moieties.[1–3] Although these surfaces can kill microbes upon contact (contact-killing surfaces), their effectiveness is inherently limited to cells that come into direct contact with them.

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Herein, we report an approach that increases the effectiveness of these conventional and passive contact-killing surfaces by using concentration gradients of soluble agents to promote the active or directed migration (or chemotaxis) of bacteria toward the surface (Figure 1). This approach exploits the properties of natural and nonbiocidal small-molecule chemoattractants (CAs) and can enhance the cell-killing power of conventional contact-killing surfaces by at least 10- to 20-fold.

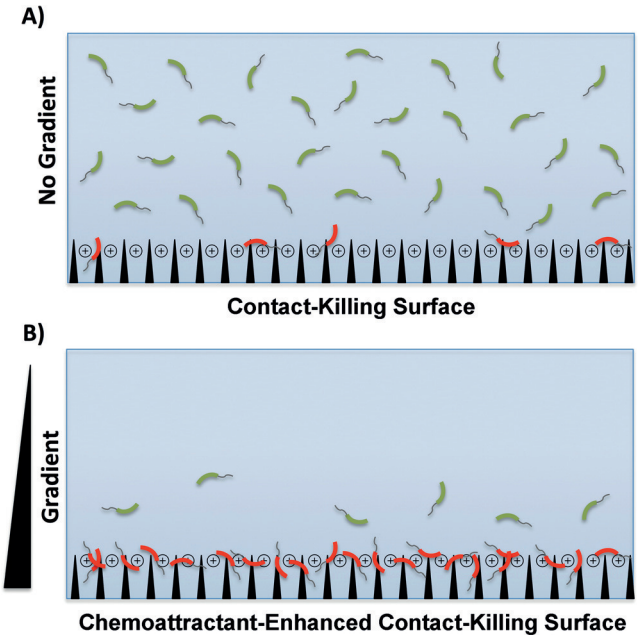

Figure 1. Representation of the concept of CA-enhanced contact-killing of bacteria demonstrated in this paper. Contact-killing of bacteria A) in the absence or B) in the presence of a chemoattractant. Live bacteria are shown in green, dead bacteria are shown in red.

Contact-killing materials can be fabricated by covalently immobilizing a variety of antimicrobial compounds, including quaternary ammonium compounds/polymers, enzymes, or peptides, on a wide range of organic and inorganic surfaces.[4] Immobilized quaternary ammonium salts (QAS), in particular, have been demonstrated to be highly effective against a variety of microbes[5] (both bacteria and fungi).[6] These QASs provide a permanent and pH-independent positive charge coupled with a long, aliphatic hydrocarbon chain. A variety of mechanisms have been proposed to describe the origin of the contact-killing properties of these surfaces,[7] including disruption of the negatively charged outer membranes of bacteria, causing cell leakage and lysis.[3] To demonstrate the proof of concept and to explore the feasibility of our CA-based approach, we selected glass

substrates functionalized by treatment with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride (DMOAP) as a model contact-killing surface.[6,8] We note that DMOAP reacts with hydroxy groups on the surface of glass through formation of covalent oxane bonds[9] and also cross-links laterally to form polymeric films that adhere strongly to surfaces.

Our approach also relies on the use of small-molecule CAs to promote bacterial chemotaxis. Several amino acids (such as aspartate, serine, glutamate, and glycine) and simple sugars (including glucose, fructose, and galactose) are known to function as CAs for bacteria, with the effectiveness of each agent dependent upon the specific species or strain.[10,11] The chemotaxis of dispersions of bacteria in response to concentration gradients of various chemoeffectors has been demonstrated using a variety of experimental approaches, including agar plate assays,[12] two-chamber assays,[13] capillary assays,[14,15] and microfluidics-based assays.[16] For this study, we selected glucose and aspartate as model CAs. These two compounds have been demonstrated to promote chemotaxis in *Escherichia coli* and *Salmonella typhimurium*, two common food pathogens, and are used in studies described below.[10,11]

We hypothesized that the establishment of a concentration gradient of a CA near a contact-killing surface would promote the migration of motile bacterial cells contained in a surrounding bulk medium towards the surface. We envisioned that such active migration would result in an increase in bacterial density at the contact-killing surface and, thus, an increase in the overall antimicrobial activity of the surface. To test these propositions, we used an experimental set-up adapted from a capillary chemotaxis assay,[10] in which a microcapillary containing a small quantity of a dissolved CA is introduced into a bacterial suspension (Figure 2A). As the CA diffuses from the capillary into the bacterial suspension, a concentration gradient of the CA is formed, such that bacteria are attracted toward, and ultimately into, the capillary.[15] We conducted experiments in which the inside walls of the capillary used to administer the soluble CA were coated with DMOAP to kill bacteria upon contact (Figure 2B).

Using previously reported methods,[9] we immobilized DMOAP on the inner surfaces of glass microcapillary tubes and reflective model silicon and verified the formation of a DMOAP coating using ellipsometry and X-ray photoelectron spectroscopy (XPS; see Figure 3A). Relative to untreated samples, the peaks for carbon and nitrogen were prominent in DMOAP-coated samples and the ratio of the C/N atomic percentage (see inset of Figure 3A) was calculated to be circa 27, which is consistent with the molecular formula of DMOAP (with C/N ratio of 26). Characterization of DMOAP coatings using ellipsometry revealed an optical thickness of 20.6 ± 2.2 nm, which is consistent with formation of a multilayer film of DMOAP. To evaluate the contact-killing efficacy of our DMOAP-coated microcapillary tubes, we introduced *E. coli* (10^7 CFU mL^{-1}, circa 0.5 μL) into either DMOAP-functionalized or untreated microcapillaries and

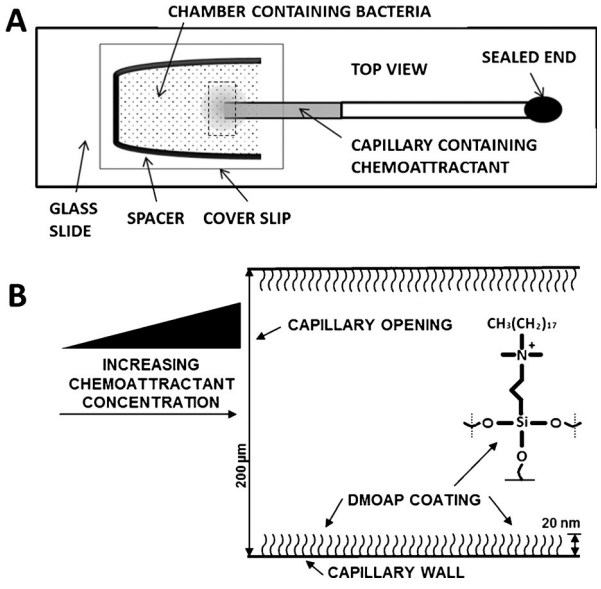

Figure 2. A) Capillary-based experimental set-up used to generate a concentration gradient of chemoattractants. B) Magnified view of the open end of the capillary showing contact-killing surfaces created by reaction with DMOAP.

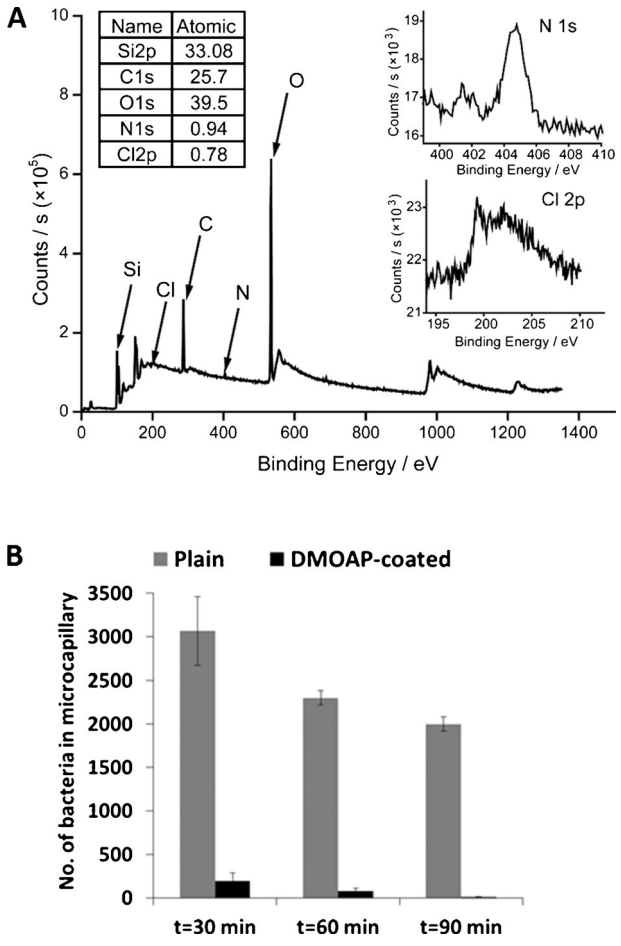

Figure 3. A) XPS spectra of the DMOAP coating on a Si wafer. Insets: The atomic composition of the surface (left), and expanded views of the N1s and Cl2p peaks (right). B) Number of bacteria that survived inside glass microcapillaries (untreated or DMOAP-coated) after different incubation times.

then incubated them at room temperature for 30–90 min. Figure 3B reveals that 95% and 99.99% of bacteria (*E. coli*) were killed within 30 and 90 min, respectively, after introduction into the DMOAP-treated microcapillary tubes. The number of bacterial cells inside untreated microcapillaries also decreased with time (because of the absence of nutrients in the medium (phosphate-buffered saline, pH 7.0) used in these initial experiments), although the effect was far less pronounced than for the DMOAP-treated capillaries and does not impact the conclusions reached in the experiments below. These experiments established that the methods used to coat our microcapillary tubes with DMOAP were sufficient to create effective contact-killing surfaces.

We next evaluated the hypothesis that the antimicrobial activity of a contact-killing surface can be augmented by using a CA to draw the bacterial cells toward the surface. As mentioned above, a small chamber was filled with a bacterial suspension (either *E. coli* K12 or *S. typhimurium* E40; 0.6 mL), into which we then inserted a microcapillary (either DMOAP-treated or untreated) containing a CA of interest (either aspartate or glucose, volume ≈ 0.5 μL; see Figure 2). In an initial series of screening experiments, we screened a range of CA concentrations (aspartate: 0–100 mM; glucose: 0–200 mM) against *E. coli* and *S. typhimurium* to determine the concentrations that yielded maximal chemotactic responses (see Figure S1 in the Supporting Information). Based on the results of those experiments, we selected 10 mM aspartate and 2 mM glucose as CAs for *E. coli*, and 1 mM aspartate for experiments using *S.typhimurium*.[10,11]

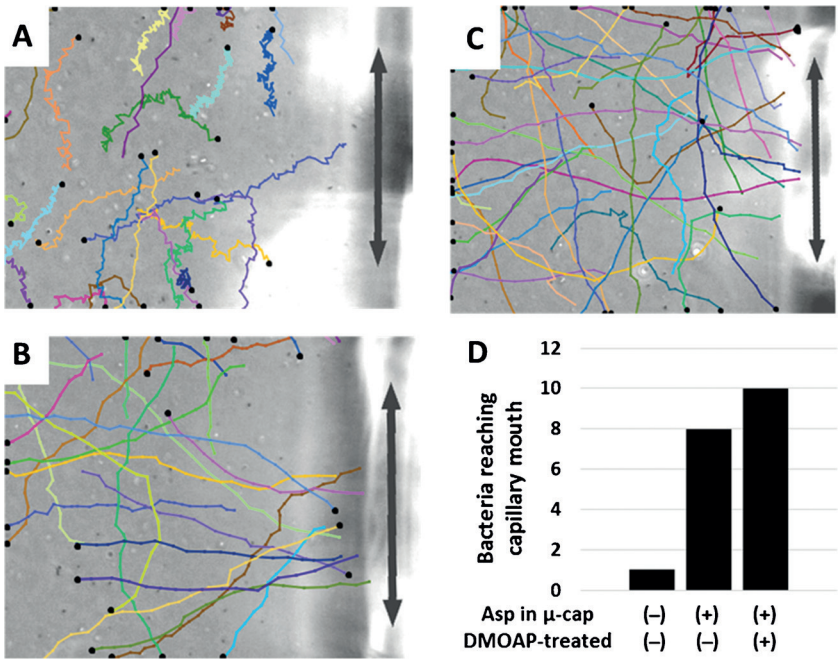

Figure 4. A–C) Images showing bacterial trajectories (strain = *E. Coli;* black dots indicate the start of trajectory) near the capillary entrance (double-headed arrow; size = 200 μm, drawn to scale relative to bacterial pathways) in which A) an untreated microcapillary containing buffer solution was in contact with the bacterial suspension, B) an untreated microcapillary containing CA solution (1 mM aspartate (Asp) in buffer) was in contact with the bacterial suspension, C) and a DMOAP-treated microcapillary containing CA solution (1 mM aspartate in buffer) was in contact with the bacterial suspension. D) Bar chart showing the number of bacteria that reached the capillary entrance in experiments (A–C). See Movies S4–S6 for videos of bacterial trajectory formation. μ-cap = microcapillary.

To quantify the influence of CA on bacterial behaviors, we used imaging software (MtrackJ)[17] to analyze the movement of bacteria (over a duration of 2 min) near the ends of the capillary tubes (see Movies S4–S6 in the Supporting Information). For these studies, a total of 60 bacteria were randomly chosen from six rectangular zones (10 bacteria per zone) in the area near, but outside of, the open ends of the capillaries. The initial and final locations of the bacteria in these experiments were also plotted (Figure S2). In the absence of CA (Figure 4A), only 1 out of the 60 bacteria tracked was observed to reach the entrance of the capillary. A statistical analysis of these bacterial trajectories (see Movie S4) also indicated random movement without directional bias (average speed = $9.5 \pm 2.4\ \mu m\,s^{-1}$).[18] In experiments using CA-filled capillaries, a higher proportion of the bacteria was observed to end their trajectories near the open ends of the capillaries for both untreated (Figure 4B) and DMOAP-coated capillaries (Figure 4C). This behavior is consistent with the directed movement of bacteria in the direction of increasing CA concentration. Moreover, the trajectories of the bacteria recorded (see Movies S5 and S6) in the presence of CA also indicated: i) that bacterial movement was dominated by "runs" over "tumbles", and ii) that a subset of the trajectories was directionally biased toward increasing CA concentrations (regardless of whether the capillary was DMOAP-coated; average speeds were $24.2 \pm 6.1\ \mu m\,s^{-1}$ (untreated) and $28 \pm 5.7\ \mu m\,s^{-1}$ (DMOAP-coated)). Overall, these results demonstrate that bacteria are drawn into both untreated and DMOAP-coated capillaries containing the CA through a mechanism that involves chemotaxis. Figure 4D plots the number of bacteria shown in the images in Figure 4A–C that reach the open ends of the capillaries, and reveals an approximately 8- to 10-fold increase in the number of bacteria drawn to the capillaries when CA is present inside the capillaries.

Next, we incubated capillaries containing CAs in our bacterial chambers for 60 min (a period of time much longer than the 2 min observation times used in the experiments described above). After removing the capillaries from the chambers, we incubated them for an additional 30 min to allow bacteria to interact with the surfaces of the capillaries. We also determined (by plating) the number of bacterial colony forming units (CFU) in the capillaries. Inspection of Figure 5A,B reveals that the presence of 10 mM aspartate or 2 mM glucose in the capillaries (but not in the bulk bacterial dispersions) increased the number of *E. coli* cells drawn into untreated capillaries by 12-fold (for aspartate) and 7-fold (for glucose (Glu)). To determine whether the increased number

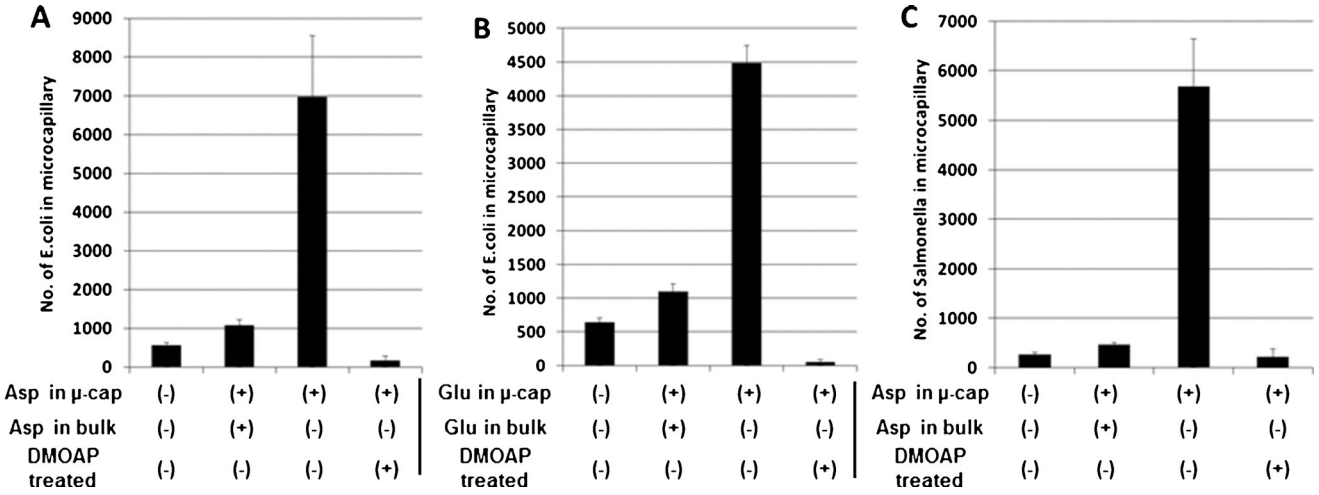

Figure 5. Increase in contact-killing effectiveness of a surface by using CAs. Results of contact-killing assay using A) *E. coli* and Asp (10 mM), B) *E. coli* and Glu (2 mM), and C) *S. typhimurium* and Asp (1 mM). μ-cap = microcapillary.

of bacteria present in untreated capillaries was due to chemotaxis or chemokinesis, we performed additional control experiments in which the CA was dissolved uniformly in both the solution in the capillaries and the bulk bacterial suspension (Figure 5A,B; second bar from left). We observed a small increase in the number of bacteria within the capillaries (2-fold) in this experiment, which we interpret to indicate a small increase in chemokinesis in the presence of a carbon source (aspartate or glucose).[19] These results, when combined, lead us to conclude that the mechanism of action of the CAs localized in the capillaries is largely chemotaxic and not chemokinetic.

When bacterial cells were incubated with DMOAP-functionalized capillaries containing CA, the numbers of live bacterial cells recovered from the capillaries was less than 3% of those recovered from untreated capillaries (Figure 5A and B). These results suggest that *E. coli* is attracted into the capillaries using glucose/aspartate and that the bacterial cells drawn into the capillary are subsequently killed upon contact with the DMOAP-coated surface. Similar results were also obtained using *S.typhimurium* and aspartate as CA: we observed a 20-fold decrease in live bacteria in DMOAP-treated capillaries containing CA relative to control experiments (Figure 5C). Of the cells that entered the capillaries in these experiments, more than 96% of bacteria were killed by the DMOAP-coated surfaces (Figure 5C). We note that the chemotactic response of *S.typhimurium* is greater than *E.coli*, likely as a result of its higher motility.[16]

Overall, our results demonstrate that the antimicrobial activity of a conventional contact-killing surface can be enhanced substantially by using CAs to promote the migration of bacteria toward the surface. By using concentration gradients of glucose or aspartate in a simple, capillary-based model system, we have established that the contact-killing efficiency of a known contact-killing surface can be enhanced by up to 20-fold. We have also established the generality of this approach by conducting experiments using two common Gram-negative food pathogens. We note that a range of materials and technologies have been developed for the controlled (active and passive) release of small-molecule drugs and active compounds.[20] We anticipate that these approaches can be leveraged for the controlled release of CAs, thus generating concentration gradients of CAs near contact-killing surfaces. With future development, this concept could be useful in a range of applications, for example where a contact-killing food packaging material is designed to release a nontoxic CA to attract bacteria to the packaging surface. To explore the feasibility of this concept, we evaluated bacterial chemotaxis in fluid extracted from packaged uncooked chicken using the capillary-based models developed above. The results of these experiments demonstrated that CAs could attract bacteria in the presence of this complex liquid medium containing additional nutrients and other components (see Figure S3), albeit to a lower extent than that observed using nutrient-free media (shown above in Figure 4). Finally, we note that the approaches and principles demonstrated in this proof-of-concept study are not limited to the geometry (glass capillaries), liquid medium (defined buffer or fluid from a chicken package), or specific CAs (glucose and aspartate) used herein. We anticipate that these principles can be applied to a broad range of systems and experimental geometries and thus have the potential to improve the usefulness of existing contact-killing surfaces and, more importantly, guide the design of entirely new classes of active antimicrobial surfaces.

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