Designing surfaces that kill bacteria on contact

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Poly(4-vinyl-*N*-alkylpyridinium bromide) was covalently attached to glass slides to create a surface that kills airborne bacteria on contact. The antibacterial properties were assessed by spraying aqueous suspensions of bacterial cells on the surface, followed by air drying and counting the number of cells remaining viable (i.e., capable of growing colonies). Amino glass slides were acylated with acryloyl chloride, copolymerized with 4-vinylpyridine, and *N*-alkylated with different alkyl bromides (from propyl to hexadecyl). The resultant surfaces, depending on the alkyl group, were able to kill up to 94 ± 4% of *Staphylococcus aureus* cells sprayed on them. A surface alternatively created by attaching poly(4-vinylpyridine) to a glass slide and alkylating it with hexyl bromide killed 94 ± 3% of the deposited *S. aureus* cells. On surfaces modified with *N*-hexylated poly(4-vinylpyridine), the numbers of viable cells of another Gram-positive bacterium, *Staphylococcus epidermidis*, as well as of the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*, dropped more than 100-fold compared with the original amino glass. In contrast, the number of viable bacterial cells did not decline significantly after spraying on such common materials as ceramics, plastics, metals, and wood.

Because of the ever-growing demand for healthy living, there is a keen interest in materials capable of killing harmful microorganisms. Such materials could be used to coat the surfaces of common objects touched by people in everyday life (e.g., door knobs, children's toys, computer keyboards, telephones, etc.) to render them antiseptic and thus unable to transmit bacterial infections.

Because ordinary materials are not antimicrobial, they require modification. For example, surfaces chemically modified with poly(ethylene glycol) and certain other synthetic polymers can repel (although not kill) microorganisms (1–6). Alternatively, materials can be impregnated with antimicrobial agents, such as antibiotics, quaternary ammonium compounds, silver ions, or iodine, that are released gradually into the surrounding solution over time and kill microorganisms therein (6–9). Although these strategies have been verified in aqueous solutions containing bacteria, they would not be expected to be effective against airborne bacteria in the absence of a liquid medium; this situation is especially true for release-based materials (6), which are also liable to become impotent when the leaching antibacterial agent is exhausted.

It has been reported (10–13) that various polycations possess antibacterial properties in solution, presumably by interacting with and disrupting bacterial cell membranes. However, this antibacterial activity vanishes when these polycations are crosslinked or otherwise insolubilized (12, 14, 15). We have hypothesized that their antibacterial properties can be preserved, even after insolubilization, and expressed in a dry state, if the immobilized polycationic chains are sufficiently long and flexible to be able to penetrate the bacterial cell walls. This approach has been implemented and proven experimentally in the present study. Long chains of *N*-alkylated poly(4-vinylpyridine) (PVP) were attached covalently by different methods to a glass surface. The resultant glass slides kill on contact a number of airborne Gram-positive and Gram-negative bacteria.

Materials and Methods

Materials. Fluorescein (Na salt), 4-vinylpyridine, and NH_2 glass slides (aminopropyltrimethoxysilane-coated microscope slides) were purchased from Sigma. All other chemicals used in this work (analytical grade or purer) were obtained from Aldrich and used without further purification except for 4-vinylpyridine, which was distilled under vacuum before use.

Surface Derivatization. *Method A.* By using a modified literature procedure (16), an NH_2 glass slide was placed in 90 ml of dry dichloromethane containing 1 ml of triethylamine. After cooling to 4°C, 10 ml of acryloyl chloride was added, and the reaction mixture was stirred in a cold room overnight and then was stirred at room temperature for 2 h. The acylated NH_2 glass slides were rinsed with a methanol/triethylamine mixture (1:1, vol/vol) and methanol. As judged from the determination of the NH_2 groups on the glass-slide surface before ($6.6 \pm 0.1 \times 10^{-10}$ mol/cm^2) and after ($3.3 \pm 0.2 \times 10^{-10}$ mol/cm^2) the acryloylation with the picric acid titration (17), approximately one-half of the surface-bound amino groups reacted with acryloyl chloride. The glass-bonded acryloyl moieties were then copolymerized with 4-vinylpyridine following a modified procedure of Frautschi *et al.* (18). Perchloric acid [90 ml of a 20% (vol/vol) solution in water] was degassed, and 30 mg of $Ce(SO_4)_2$ was added under argon. After 1 h of stirring, an acryloylated glass slide was placed in this solution; 15 ml of freshly distilled 4-vinylpyridine was added under argon; and the reaction mixture was stirred at room temperature for 3 h.

PVP that was not chemically attached to the slide was washed off with pyridine, *N*,*N*-dimethylformide, and methanol. Immediately thereafter, the slide with the attached PVP was placed in a 10% (vol/vol) solution of an alkyl bromide in nitromethane. The reaction mixture then was stirred at 75°C for 72 h, after which time more than 90% of the pyridine rings were *N*-alkylated (19). The resultant polycation-derivatized PVP slide was rinsed with methanol and distilled water and air dried.

Method B. An NH_2 glass slide was immersed in a mixture containing 9 ml of 1,4-dibromobutane, 90 ml of dry nitromethane, and 0.1 ml of triethylamine. After stirring at 60°C for 2 h, the slide was removed, thoroughly rinsed with nitromethane, air dried, and placed in a solution of 9 g of PVP (molecular weight of 60,000 or 160,000 g/mol) in 90 ml of nitromethane/hexyl bromide (10:1, vol/vol). After stirring the reaction mixture at 75°C for 24 h, the slide was rinsed with acetone, thoroughly washed with methanol (to remove the nonattached polymer), and air dried. According to the literature (14), more than 96% of the pyridine rings of PVP should be *N*-alkylated under these conditions.

Surface Analysis. A chemically modified glass slide was immersed in a 1% solution of fluorescein (Na salt) in distilled water for 5 min. Under these conditions, the dye binds to quaternary amino groups (20) but not to tertiary or primary ones (we found that PVP-modified- or NH_2 glass slides do not adsorb fluorescein). After rinsing with distilled water, a stained slide was placed in 25

Abbreviations: PVP, poly(4-vinylpyridine); hexyl-PVP, poly(4-vinyl-*N*-hexylpyridinium bromide).

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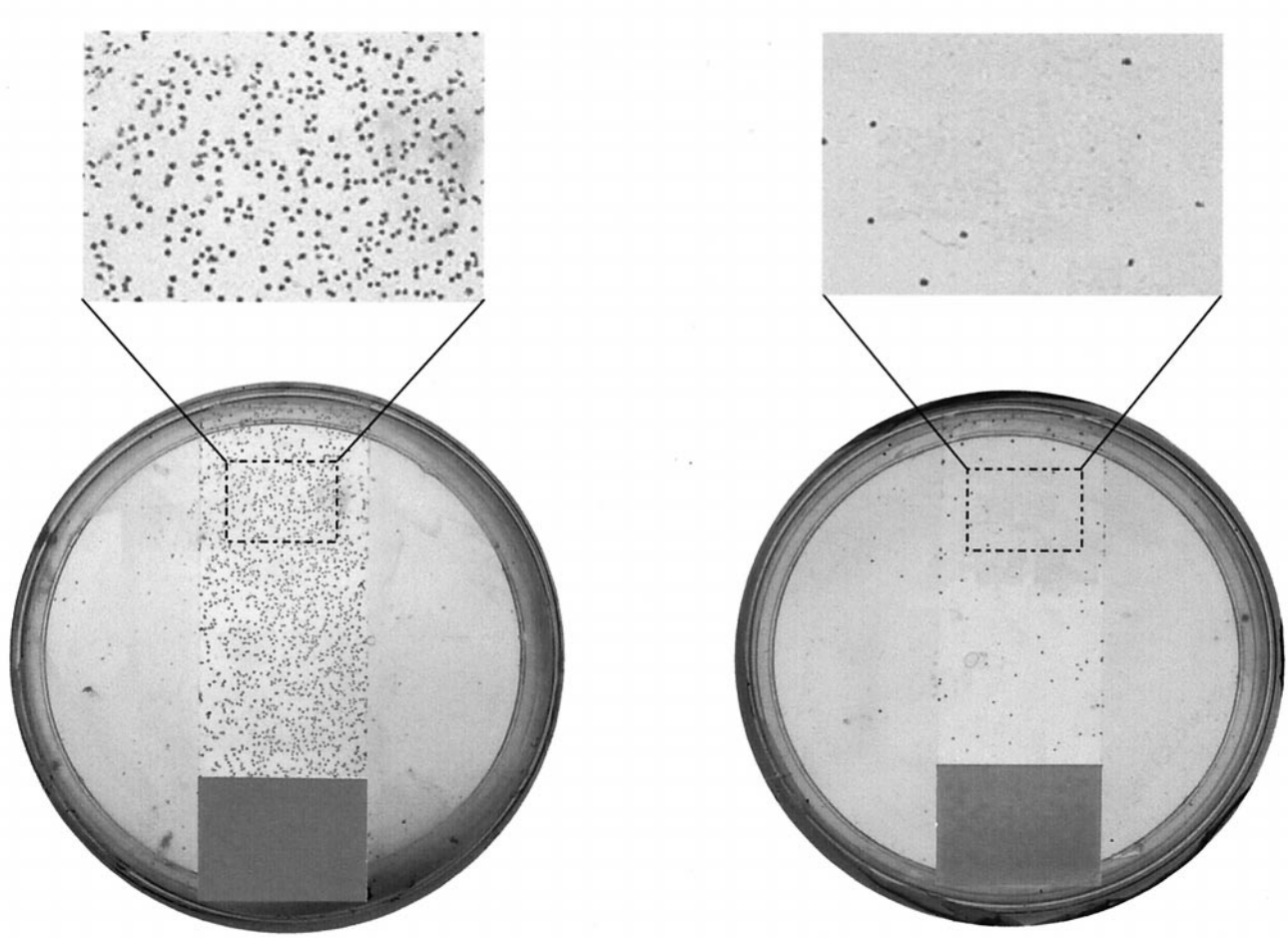

Fig. 1. Photographs of a commercial NH_2 glass slide (*Left*) and a hexyl-PVP-modified slide (*Right*) onto which aqueous suspensions ($\approx 10^6$ cells per ml of distilled water) of *S. aureus* cells were sprayed, air dried for 2 min, and incubated under 0.7% agar in a bacterial growth medium at 37°C overnight.

ml of the 0.1% detergent cetyltrimethyl-ammonium chloride in distilled water and shaken for 10 min to desorb the dye. The absorbance of the resultant aqueous solution was measured at 501 nm (after adding 10% of a 100 mM aqueous phosphate buffer, pH 8.0). The independently determined extinction coefficient of fluorescein in this solution was found to be 77 $mM^{-1} \cdot cm^{-1}$.

From staining different poly(4-vinyl-N-hexylpyridinium bromide) (hexyl-PVP) films (160,000 g/mol, degree of alkylation > 95%) with a known polymer content, the stoichiometry of fluorescein binding was found to be approximately one dye molecule per seven hexyl-PVP monomer units. The following amounts of the attached hexyl-PVP were determined (assuming that more than 90% of the polymer is hexylated): 5.8 ± 3.0 $\mu g/cm^2$ (method A); 2.8 ± 1.0 $\mu g/cm^2$ (method B, PVP with molecular weight = 160,000 g/mol); and 0.4 ± 0.05 $\mu g/cm^2$ (method B, PVP with molecular weight = 60,000 g/mol). In the case of hexyl-PVP immobilized by method A, the minimal chain length of the attached polycation was estimated to be 61 ± 30 monomer units.

Antimicrobial Susceptibility Determination. A suspension (100 μl) of *Staphylococcus aureus* (ATCC, strain 33807), *Staphylococcus epidermidis* (wild type), *Pseudomonas aeruginosa* (wild type), or *Escherichia coli* (ZK 605) in 0.1 M aqueous PBS buffer (pH 7.0, $\approx 10^{11}$ cells per ml) was added to 50 ml of a yeast/dextrose broth [prepared as described by Cunliffe *et al.* (21)] in a sterile Erlenmeyer flask. The suspension was incubated at 37°C with shaking at 200 rpm (G24 Environmental Incubation Shaker, New Brunswick Scientific) for 6–8 h. After centrifugation (2,700 rpm, 10 min; RC-5B Sorvall centrifuge, SS-34 rotor), the bacterial cells were washed with and resuspended in distilled water at a concentration of 10^6 cells per ml (10^7 in the case of *E. coli*).

A bacterial suspension then was sprayed onto a glass slide (or another surface) in a fume hood by using a commercial chromatography sprayer (VWR Scientific) (spray rate of ≈10 ml/min). After drying for 2 min under air, the slide was placed in a Petri dish, and then growth agar (0.7% agar in a yeast/dextrose broth, autoclaved, and cooled to 37°C) was added. The Petri dish was closed, sealed, and incubated at 37°C overnight.

Results and Discussion

Our experimental protocol for assessing the ability of dry surfaces to kill bacteria on contact was as follows: bacteria were suspended in distilled water and were sprayed onto the surface of a slide to simulate the deposition of airborne bacteria—a common method of spreading bacterial infections generated, for example, by talking, sneezing, coughing, or just breathing. To determine the number of viable bacterial cells (the bacteria able to proliferate from countable colonies under these conditions) on the infected surface, the slide, after a 2-min air drying, was incubated under growth agar. The ubiquitous and infectious (22) Gram-positive bacterium *S. aureus* was used for the initial studies.

As seen in Fig. 1 *Left*, numerous colonies of *S. aureus* grown on an NH_2 glass slide (the commercially available starting

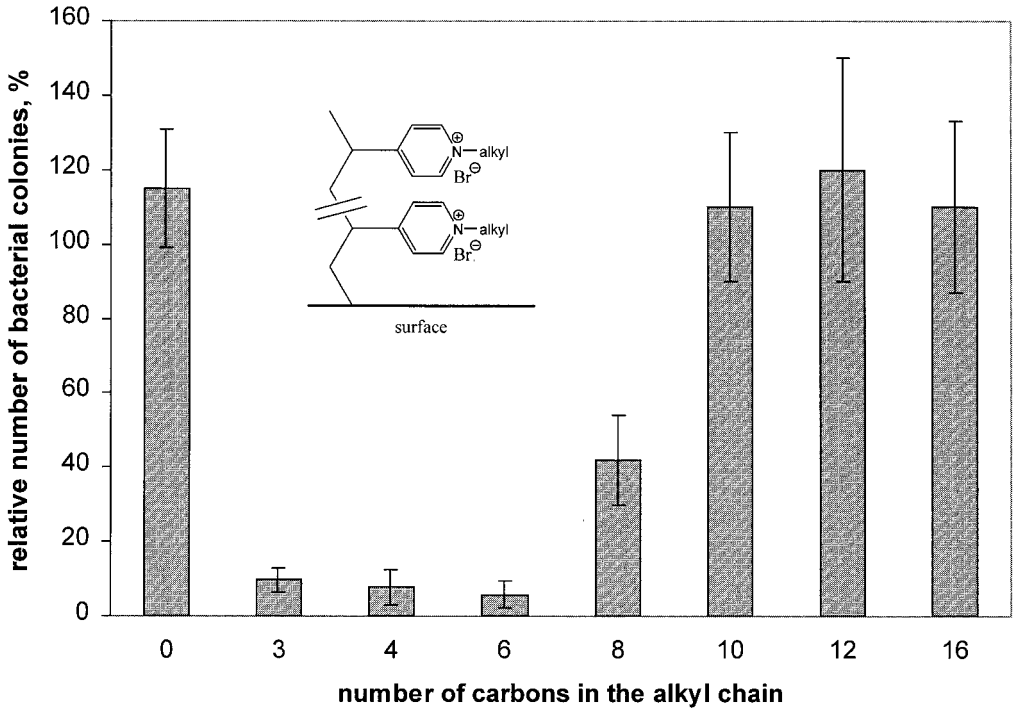

Fig. 2. The percentage of *S. aureus* colonies grown on the infected surfaces of glass slides modified with PVP that was *N*-alkylated with different linear alkyl bromides relative to the number of colonies grown on a commercial NH_2-glass slide (used as a standard). The bacterial cells were sprayed from an aqueous suspension (10^6 cells per ml) onto the surfaces. All experiments were performed at least in quadruplicate, and the error bars indicate the standard deviations from the mean values obtained.

material for subsequent derivatizations) after spraying the bacterial suspension onto its surface are well distinguishable. The number of bacterial colonies formed on NH_2 glass slides was used henceforth as a reference for all other surfaces.

Next, an NH_2 glass slide was acylated with acryloyl chloride to introduce double bonds, followed by copolymerization with 4-vinylpyridine. Such an immobilized PVP was found to afford approximately the same number of viable *S. aureus* cells after spraying the bacterial suspension onto its surface as a plain NH_2 glass slide. The final step was to introduce positive charges into the PVP chains attached to glass. To this end, the polymer's pyridine rings were *N*-alkylated by seven linear alkyl bromides (with chain lengths varying from propyl to hexadecyl). The resultant slides were examined with respect to their ability to kill on contact *S. aureus* cells that were sprayed on them. As seen in Fig. 2, propylated, butylated, hexylated, and octylated immobilized PVP chains were effective in markedly reducing the number of viable bacterial cells, with the most effective hexyl-PVP affording a 94 ± 4% reduction (Fig. 1 *Right*). In contrast, the immobilized PVP *N*-alkylated by decyl through hexadecyl bromides, as well as the nonalkylated chains, were totally ineffective (Fig. 2). This behavior pattern seems to correlate with the visual appearance of the alkylated-PVP slides. Although nonalkylated, decyl-, dodecyl-, and hexadecyl-PVP-modified slides were cloudy (absorbance at 400 nm, A_{400}, of some 0.1), an octyl-PVP-modified slide was much less so ($A_{400} = 0.03$), and propyl-, butyl-, and hexyl-PVP-modified slides were clear ($A_{400} < 0.002$). The cloudiness could reflect polymer aggregates formed on the surface, with such aggregates apparently being unable to interact with bacterial cells. Presumably, nonalkylated immobilized PVP chains, as well as those modified with long alkyl moieties, stick to each other because of hydrophobic interactions; in contrast, in immobilized PVP chains modified with short alkyl groups, such interactions are not strong enough to overcome the electrostatic repulsion of the positively charged polymers.

Having established that the glass slide surface modification leading to the highest antibacterial activity was with hexyl-PVP (henceforth referred to as "that prepared by method A"), we then tested the generality of this phenomenon. To this end, we investigated the bactericidal effect of this surface toward another Gram-positive bacterium, *S. epidermidis*, as well as two Gram-negative bacteria, *E. coli* and *P. aeruginosa*. The first two formed colonies of the same size as *S. aureus* (Fig. 1 *Left*) when sprayed on NH_2 glass slides, whereas the colonies of *P. aeruginosa* were larger but still distinguishable. As seen in Table 1, method A, the number of colonies all three bacteria formed after spraying onto hexyl-PVP slides dropped more than 100-fold compared with the plain NH_2 glass.

Table 1. The ability of hexyl-PVP attached to glass slides by two different methods to kill various airborne bacteria on contact

		Percentage of bacteria killed	
Bacterium	Gram	Method A	Method B
S. aureus	(+)	94 ± 4	94 ± 3
S. epidermidis	(+)	>99	>99
P. aeruginosa	(−)	>99.8	>99.8
E. coli	(−)	>99	>99

Suspensions (10^6 cells per ml for the first three bacteria and 10^7 cells per ml for the last one) of bacteria in distilled water were sprayed on hexyl-PVP-modified glass surfaces, air dried for 2 min, and incubated under 0.7% agar in a bacterial growth medium overnight, and then the colonies were counted. The number of viable cells obtained in the same manner with commercial NH_2-glass slides was used as a standard (i.e., 0% of the bacteria killed). All experiments were performed at least in quadruplicate, and the errors indicate the standard deviations. See *Materials and Methods* for a detailed description of methods A and B.

We also explored antibacterial properties of a PVP-based polycation immobilized onto glass slides in a different way. An NH_2 glass slide was alkylated with 1,4-dibromobutane to introduce reactive bromobutyl groups that were used subsequently for the attachment of PVP. The resultant surface was not able to kill *S. aureus* cells on spraying. To increase the positive charge of the attached PVP chains, we further *N*-alkylated them with hexyl bromide (found optimal in method A, see Fig. 2). After *S. aureus* cells were sprayed, air dried, and cultured, the resultant hexyl-PVP slides (henceforth referred to as ''those prepared by method B'') looked essentially the same as shown in Fig. 1 *Right*. Compared with an NH_2 glass slide, 94 ± 3% of the deposited *S. aureus* cells were killed (first line of method B column in Table 1).

It is worth noting that the molecular weight of the immobilized PVP was found to be important for the antibacterial properties of the surface. A hexyl-PVP slide prepared by method B with shorter PVP chains (60,000 instead of 160,000 g/mol) killed only 62 ± 8% of the deposited *S. aureus* cells.

Inspection of the remainder of the data in Table 1 reveals that method B afforded the slide surface that was as deadly toward *S. epidermidis*, *P. aeruginosa*, and *E. coli* as that obtained by method A.

Described materials (6–9) that kill bacteria by releasing bactericidal agents are eventually depleted of the active substance because of its release into the surrounding solution. To test whether the antibacterial polymers immobilized by our methodology can leach from the glass surface, hexyl-PVP slides prepared by methods A and B were placed into polystyrene Petri dishes, and aqueous suspensions of *S. aureus* cells were sprayed on these slides as well as on the nonantibacterial polystyrene dishes. After air drying and incubation under growth agar, it was observed that only a few (some 3 per cm^2) colonies were growing on both types of the hexyl-PVP-glass slides, whereas a far larger number of colonies (60 ± 10 per cm^2) was growing on the surrounding Petri dishes, even in the immediate proximity of the slides. The lack of inhibition zones around the hexyl-PVP slides, typical of the release of bactericidal agents (23), indicates that the immobilized bactericidal polymer hexyl-PVP does not leach from the slides, i.e., that the bacteria are indeed killed on contact with the slides' surfaces.

To test how unique the ability to kill airborne bacteria is among dry surfaces, an *S. aureus* cell suspension was sprayed on various common materials, including metals, synthetic and natural polymers, and ceramics. The number of colonies remaining viable in all instances was compared with that on NH_2 glass. As seen in Table 2, none of the materials examined significantly lowered the amount of bacterial cells remaining viable after spraying.

Tethered amphipathic polycations described in this study and soluble cationic antimicrobials probably share a similar mechanism of attacking bacteria. Polycations, such as polymyxin B and antimicrobial cationic peptides of animals, displace the divalent cations that hold together the negatively charged surface of the lipopolysaccharide network, thereby disrupting the outer membrane of Gram-negative bacteria like

Table 2. The number of *S. aureus* cells remaining viable after their aqueous suspensions have been sprayed onto various materials

Material	Relative number of viable bacterial cells, %
Plain glass	83 ± 10
Polystyrene	105 ± 15
Polypropylene	97 ± 9
Aluminum	72 ± 7
Steel	95 ± 15
Paper	77 ± 8
Wood (birch)	102 ± 10
Porcelain	85 ± 10
PVP slide	115 ± 16
hexyl-PVP slide (method A)	6 ± 4
hexyl-PVP slide (method B)	6 ± 3

See legend to Table 1 for details.

P. aeruginosa and *E. coli* (24). This effect in itself might be sufficient for a lethal outcome. It is also possible that, having destroyed the outer membrane permeability barrier, the cationic groups of the tethered polymers further penetrate into the inner membrane, producing leakage. Such ''self-promoted penetration'' with the subsequent damage of the inner membrane has been described for polymyxin (24). The action of immobilized polycations against the Gram-positive bacteria *S. aureus* and *S. epidermidis* probably requires penetration of the cationic groups across the thick cell wall to reach the cytoplasmic membrane. Bactericidal action of amphipathic cationic antiseptics, such as benzalkonium chloride or biguanidine chlorhexidine, against Gram-positive bacteria is caused primarily by the disruption of the cytoplasmic membrane (25, 26). The cell wall of *S. aureus* is some 30 nm thick (27); because the estimated average length of the *N*-hexylated PVP (method A) is 19 nm, with some obviously being shorter and others longer, the latter could penetrate the cell wall. Although it is unknown how the initial damage to the outer and/or cytoplasmic membrane ultimately kills bacteria, they may ''actively participate'' by inducing autolysis (28).

In closing, we have found that a glass surface modified by hexyl-PVP either by a graft copolymerization with 4-vinylpyridine and subsequent *N*-hexylation or by the attachment of partially *N*-hexylated PVP kills more than 90% of deposited *S. aureus* cells and more than 99% of deposited *S. epidermidis*, *P. aeruginosa*, and *E. coli* cells in a dry state. Because such surface modifications can be performed readily with a number of other materials, this approach may prove generally useful in coating various consumer and medical products to make their surfaces antibacterial. A simple periodic washing would remove the dead deposited cells and rejuvenate such surfaces.

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