

## Insights into bactericidal action of surface-attached poly(vinyl-*N*-hexylpyridinium) chains

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### Abstract

The surface of polyethylene slides nanocoated with silica and derivatized with long-chain poly(vinyl-*N*-hexylpyridinium) becomes permanently bactericidal: it kills 90–99% of (both airborne and waterborne) wild-type and antibiotic-resistant strains of the human pathogen *Staphylococcus aureus*. The material created was similarly lethal to strains expressing multidrug resistance pumps, the only known mechanism of resistance to cationic antiseptics.

### Introduction

Creating inherently bactericidal materials that could be used to make common objects would be a major step toward a healthier living. Recently, we (Tiller *et al.* 2001, 2002) have discovered that when certain long-chain, hydrophobic polycations are immobilized onto surfaces of glass or plastics, the latter acquire the ability to efficiently kill bacteria on contact. Specifically, various surfaces covalently coated with poly(vinyl-*N*-hexylpyridinium) (hexyl-PVP) have been found to kill the vast majority of bacteria, both Gram-positive and Gram-negative, either deposited onto them through aerosols (followed by drying) or allowed to attach from aqueous solution.

In the present study, we have further explored this potentially useful phenomenon. In particular, it has been ascertained that polyethylene surface-derivatized with hexyl-PVP is equally lethal against wild-type and mutant, including antibiotic-resistant, strains of the ubiquitous pathogenic bacterium *Staphylococcus aureus*. The dead cells can be readily removed – and the surface fully rejuvenated – by washing with a detergent. *S. aureus* cells collected during the logarithmic growth phase are as susceptible to the bactericidal action as those in the stationary phase.

### Materials and methods

#### Materials

The list of *Staphylococcus aureus* strains used in this study is given in Table 1. A hand-held burner containing 0.6% (v/v) tetramethylsilane in a propane/butane mixture (7:3, v/v) (Pyrosil) was from SurA Instruments GmbH (Jena, Germany). High-density polyethylene sheets were purchased from Polymer Plastic Co. Poly(4-vinylpyridine) (PVP) (Mw 160 000 g mol), 3-aminopropyltriethoxysilane, 1,4-dibromobutane, 1-bromohexane, and all other chemicals and solvents were obtained from Aldrich Chemical Co. and used without further purification.

#### Surface modification

A polyethylene slide (7.5 × 2.5 cm) was ultrasonicated in 2-propanol for 5 min and dried at 80 °C. The front (oxidizing) part of the flame of a hand-held burner was fanned over the slide surface for 15 s, and the slide was stored under air overnight. The SiO<sub>2</sub>-coated slide was aminated with a 20% (w/v) of 3-aminopropyltriethoxysilane in dry toluene at room temperature for 3 h. The aminated slide was immersed

Table 1. Strains of *Staphylococcus aureus* used in this study.

Strain	Description	Reference
8325-4	Wild-type parent of 1758	Kaatz <i>et al.</i> (1999)
1758	<i>norA</i>	Kaatz <i>et al.</i> (1999)
982	Wild-type parent of 2355	Rouch <i>et al.</i> (1990)
2355	QacA <sup>+</sup> , Kan <sup>r</sup>	Rouch <i>et al.</i> (1990)
ATCC 700698	Methicillin-resistant strain	Hiramatsu (1997)
ATCC BAA-38	Methicillin-resistant strain	De Lencastre (2000)
ATCC BAA-39	Methicillin-resistant strain	De Lencastre (1997)
ATCC 33807	No pyrogenic exotoxin C <sup>a</sup>	

<sup>a</sup>Obtained from ATCC.

in a solution containing 9 ml 1,4-dibromobutane, 90 ml dry nitromethane, and 0.1 ml triethylamine with stirring at 60 °C for 5 h, followed by placing in a freshly prepared solution of 9 g PVP, 10 ml 1-bromohexane, and 81 ml nitromethane. After stirring at 75 °C for 9 h, the slide was thoroughly rinsed with methanol and distilled water, and dried under air.

#### Antibacterial efficiency determination

Bacteria were grown in yeast/dextrose broth (Cunliffe *et al.* 1999) at 37 °C with aeration at 200 rpm for 6–8 h. The inoculum from an overnight culture was transferred into 0.1 M PBS (approximately 10<sup>11</sup> cells ml<sup>-1</sup>) and then introduced into the growth medium at a 1:500 dilution.

The airborne bacterial suspension was prepared as described earlier (Tiller *et al.* 2001). The bacterial cells were centrifuged at 5160 × *g* for 10 min and washed with distilled water twice. A bacterial suspension at a concentration of 10<sup>6</sup> cells ml<sup>-1</sup> in distilled water was sprayed at a rate of approximately 10 ml min<sup>-1</sup> onto the surface of a slide in a fume hood. After drying for 2 min under air, the slide was placed in a Petri dish, and growth agar (0.7% agar in the yeast/dextrose broth, autoclaved, and cooled to 37 °C) was added. The Petri dish was sealed and incubated at 37 °C overnight. The grown bacterial colonies were counted on a light box.

The waterborne bacterial suspension was prepared as follows: bacterial cells were centrifuged at 5160 × *g* for 10 min, washed twice with PBS at pH 7, re-suspended in the same buffer, and diluted to 2 × 10<sup>6</sup> cells ml<sup>-1</sup>. A slide was immersed in 45 ml of the suspension and incubated with shaking at 200 rpm at 37 °C for 2 h, then rinsed three times with sterile PBS, and incubated in it for 1 h. The slide was immediately covered with a layer of solid growth agar (1.5%

agar in the yeast/dextrose broth, autoclaved, poured into a Petri dish, and dried under reduced pressure at room temperature overnight). The bacterial colonies were then counted.

#### Synthesis of pyridinium-containing monomer and polymer

Pyridine (10 ml), hexyl bromide (18 ml), and triethylamine (0.1 ml) were dissolved in 122 ml of toluene, and the solution was stirred at 75 °C for 9 h. The solvent was then removed under reduced pressure. *N*-Hexylpyridinium bromide thus obtained was washed with hexane four times and dried overnight under vacuum.

Poly(4-vinylpyridine) (5.25 g), methyl iodide (13 ml), and triethylamine (0.1 ml) were dissolved in 87 ml nitromethane, and the solution was stirred at 75 °C for 9 h. After cooling to room temperature, the solvent was removed and 100 ml toluene was added to the residue. Poly(4-vinyl-*N*-methylpyridinium iodide), insoluble in toluene, was recovered by filtration, washed with toluene and acetone, and dried.

#### Results and discussion

The objective of this work was to address some previously unanswered questions concerning the mechanism and practicalities of the bactericidal effect of surfaces derivatized with poly(vinyl-*N*-alkylpyridinium) chains (Tiller *et al.* 2001, 2002). To this end, 7.5 × 2.5 cm slides cut out of a large sheet of commercial high-density polyethylene were coated with a nanolayer of silica, followed by the covalent attachment of 160 000 g mol<sup>-1</sup> hexyl-PVP, as previously described (Tiller *et al.* 2002). The antibacterial activity of the resultant slides was tested against the common pathogen, *S. aureus*, in two distinct modalities (referred to as 'airborne' and 'waterborne'). In the airborne case, an aqueous suspension of the bacterial cells was sprayed onto a slide, followed by drying, overlaying with growth agar, incubation at 37 °C, and counting the number of bacterial colonies. In the waterborne case, a slide was immersed in an aqueous suspension of the bacterial cells, incubated there at 37 °C, washed, covered with solid growth agar, and incubated at 37 °C again, followed by counting the number of bacterial colonies.

When wild-type *S. aureus* cells were deposited onto the surface of an unmodified high-density polyethylene slide via the airborne method, followed by

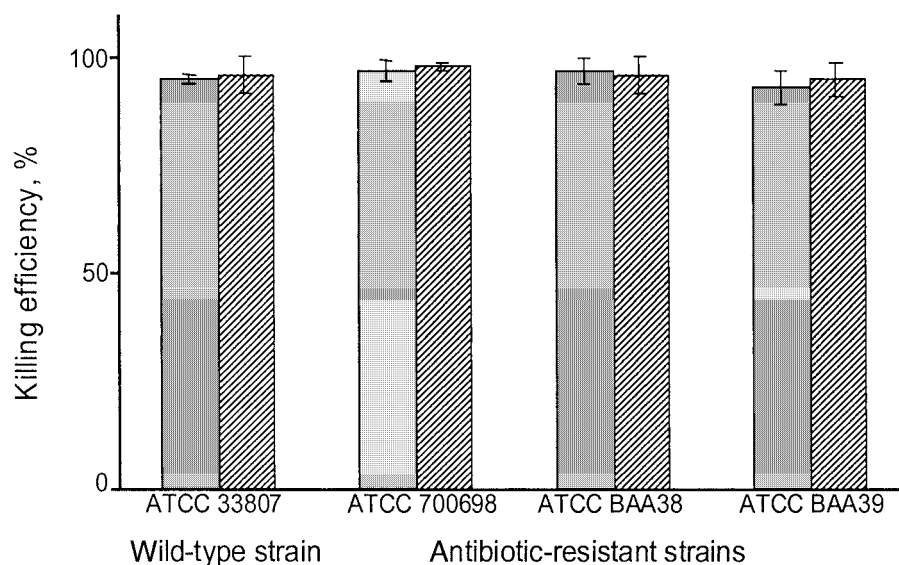


Fig. 1. Bactericidal activity of polyethylene slides covalently coated with hexyl-PVP against wild-type and various antibiotic-resistant strains of *S. aureus*. Either airborne (shaded bars) or waterborne (dashed bars) bacterial suspensions were deposited onto the slide surface; see text for details. All measurements were done in duplicate; the standard deviations from the mean killing efficiency values are represented by error bars.

Table 2. The minimal inhibitory concentration (MIC) for quaternary ammonium containing monomers and polymer.

Antiseptic	MIC, $\mu\text{g ml}^{-1}$							
	ATCC 33807	ATCC 700698	ATCC BAA-38	ATCC BAA-39	8325-4	<i>norA</i>	982	QacA <sup>+</sup>
Benzalkonium chloride	2.5	2.5	2.5	2.5	2.5	0.6	2.5	5
<i>N</i> -Hexylpyridinium bromide	4,000	>4,000	>4,000	4,000	4,000	500	4,000	>4,000
Poly(vinyl- <i>N</i> -methyl-pyridinium iodide)	75	75	75	75	75	75	75	75

cultivation as described above,  $308 \pm 16$  colonies were subsequently detected in a  $3.75 \text{ cm}^2$  frontal area. The same experiment was performed with the slide coated with silica and with that also aminated; the corresponding numbers of bacterial colonies were  $339 \pm 2$  and  $228 \pm 2$ , respectively. Thus the bacterial cells deposited onto all three different surfaces remain highly viable. In contrast, when the identical procedure was applied to the hexyl-PVP-derivatized slide, only  $14 \pm 1$  bacterial colonies were observed in a  $3.75\text{-cm}^2$  frontal area, i.e.,  $5 \pm 1\%$  compared to the unmodified slide. Likewise, with waterborne *S. aureus* cells, only  $4 \pm 2\%$  of colonies were counted on the immobilized hexyl-PVP surface compared to the original polyethylene slide. These 95%+ killing efficiencies are analogous to those discovered previously (Tiller *et al.* 2001, 2002) for surfaces modified with hexyl-PVP.

Next, we addressed the question of the durability of the aforementioned hexyl-PVP surface protection.

To this end, after the colonies grown from the surviving *S. aureus* cells were counted, they were removed by thoroughly washing the hexyl-PVP-derivatized slides with 0.1 M cetyltrimethylammonium chloride in water, followed by rinsing with distilled water. The resultant washed slides were re-used for the deposition of either airborne or waterborne *S. aureus*. The percentages of the colonies formed, as compared to those on the identically washed unmodified polyethylene slides, were  $4 \pm 1\%$  and  $2 \pm 1\%$ , respectively. Thus washing with the detergent has no effect on the bactericidal potency of immobilized hexyl-PVP.

In our studies thus far, both herein and elsewhere (Tiller *et al.* 2001, 2002), bacterial cells used in all experiments were in the stationary phase of their growth curve. It was of interest to establish whether the cells in the logarithmic phase of growth would be equally susceptible to the antibacterial action of the surface-attached hexyl-PVP chains. Consequently, we carried out a fermentation of *S. aureus* whereby the cell con-

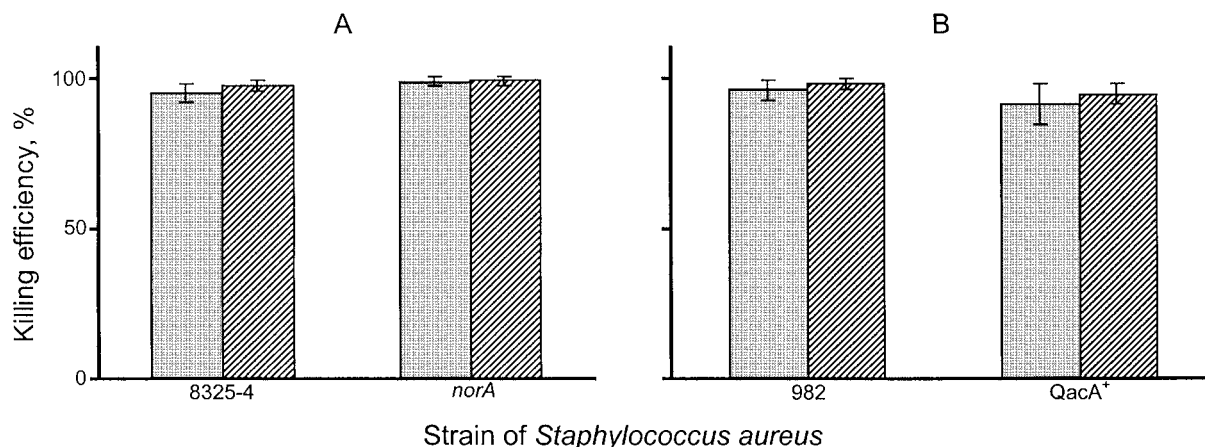


Fig. 2. Bactericidal activity of polyethylene slides covalently coated with hexyl-PVP against two parent/mutant pairs of *S. aureus* strains. In the first pair (A), the mutant is missing the MDR pump encoded by the *norA* gene (which had been knocked out), while its otherwise identical parent has it. In the second pair (B), the parent lacks the MDR pump encoded by the *qacA* gene, whereas the plasmid-bearing strain has it. For other details, see the legend to Figure 1.

centration was monitored as a function of time. The resultant data yielded a classical sigmoidal curve (Ingraham *et al.* 1983), with the stationary phase fully reached after 6 h under our conditions (see Materials and methods). Instead of collecting the bacterial cells after 6–8 h as before, we did so after just 4 h, i.e., during their logarithmic growth phase. When these cells, airborne or waterborne, were deposited onto hexyl-PVP-derivatized slides, the killing efficiencies obtained (compared to the same cells on the unmodified polyethylene slides) were  $5 \pm 2\%$  and  $2 \pm 1\%$ , respectively, i.e., identical to the values observed for the cells in their stationary phase (see above).

Multidrug resistant (MDR) bacterial strains pose a major threat to human health (Levy 1998, Lewis *et al.* 2001). With this in mind, we tested whether immobilized hexyl-PVP would be effective against such strains. Toward this end, in addition to the heretofore used wild strain of *S. aureus* (ATCC 33807), we explored three different antibiotic-resistant strains (Kluytmans *et al.* 1997) – ATCC 700698 (resistant to methicillin), ATCC BAA-38 (resistant to methicillin, penicillin, streptomycin, and tetracycline), and ATCC BAA-39 (resistant to penicillin, tetracycline, imipenem, cefaclor, oxacillin, tobramycin, cephalexin, cefuroxime, gentamicins, amoxicillin, clindamycin, erythromycin, and cephamandole). As seen in Figure 1, polyethylene slides coated with hexyl-PVP were similarly lethal to these bacterial strains, whether airborne or waterborne – the killing efficiencies in all instances well exceeded 90%. Immobilized hexyl-PVP chains likely exert their bactericidal

effect by penetrating the bacterial cell wall/membrane and perhaps causing autolysis (Tiller *et al.* 2001).

Like all other bacteria studied (Lewis 1994, Lewis & Lomovskaya 2001), *S. aureus* possesses several MDR pumps that expel various toxic compounds from the cell. For example, the NorA MDR pump protects the cells from numerous amphipathic cations including the common disinfectant benzalkonium chloride (Ng *et al.* 1994). Consequently, the mutant of *S. aureus* with a knockout in the *norA* gene coding for the MDR pump has a substantially greater sensitivity to such compounds (Hsieh *et al.* 1998). Since hexyl-PVP, like benzalkonium, is a hydrophobic quaternary ammonium cation, we decided to test the sensitivity of the *norA* mutant strain to immobilized hexyl-PVP. The results of this experiment are depicted in Figure 2A. One can see that the killing efficiencies against the pump-lacking mutant were somewhat higher than of its pump-competent parent –  $99 \pm 1\%$  for both airborne and waterborne cells *vs.*  $95 \pm 2\%$  and  $97 \pm 1\%$ , respectively.

We carried out similar experiments with a *S. aureus* strain with an additional MDR pump (denoted *QacA*<sup>+</sup>) expressed from a natural transmissible plasmid compared to its parent. This strain had a slightly higher resistance to immobilized hexyl-PVP than its pump-deficient parent (Figure 2B) –  $92 \pm 3\%$  and  $94 \pm 3\%$  for the airborne and waterborne mutant, respectively, *vs.*  $96 \pm 2\%$  and  $98 \pm 1\%$  for its parent.

The very small difference in observed susceptibilities to hexyl-PVP between strains lacking/overexpressing MDRs suggest that the polymeric

form of the antiseptic is not effectively extruded by the pump. Alternatively, *N*-hexylpyridinium may not be a substrate for MDRs. In order to distinguish between these possibilities, cells were treated with an aqueous solution of *N*-hexylpyridinium bromide, and the minimal inhibitory concentration (MIC) was determined. That compound turned out to be a very weak antimicrobial, with an MIC of 4 mg ml<sup>-1</sup> against wild-type *S. aureus* (Table 2). This value is more than 1000 times above that of the conventional antiseptic benzalkonium chloride (Table 2). There was a difference in strain susceptibilities to *N*-hexylpyridinium depending on their MDR status. Thus the MIC of the *norA* strain was 0.5 mg ml<sup>-1</sup>, and that of the *QacA*<sup>+</sup> strain was above 4 mg ml<sup>-1</sup>. This qualitatively resembles the difference in susceptibilities of these strains to benzalkonium chloride and shows that while *N*-hexyl-pyridinium is a weak antimicrobial, it is a reasonable substrate for MDRs. When water-soluble poly(vinyl-*N*-methylpyridinium iodide) was tested instead (note that hexyl-PVP itself, used in its immobilized form in our studies thus far, is not soluble in water and therefore could not be used in MIC studies), the MIC value was found to be 75 µg ml<sup>-1</sup>, i.e., considerably lower than that of the monomeric precursor *N*-hexylpyridinium bromide. There was no difference in the MIC values of this compound among the strains tested. It thus appears that polymerization of an antiseptic has two consequences for its properties – increases its potency and makes it insensitive to the action of MDR pumps.

Development of a resistance is a major concern in introducing any new antimicrobial. Extrusion by MDRs is the only known mechanism of resistance to hydrophobic cationic antiseptics (Lewis 2001, Severina *et al.* 2002). Our findings suggest that resistance to surface-attached hexyl-PVP is unlikely to develop through this mechanism.

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