Bactericidal Properties of Flat Surfaces and Nanoparticles Derivatized with Alkylated Polyethylenimines

Jian Lin,† Shuyi Qiu,† Kim Lewis,‡ and Alexander M. Klibanov*,†,§

Department of Chemistry and Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Department of Biology, Northeastern University, Boston, Massachusetts 02115

We previously discovered that covalently coating glass and plastic slides with certain long poly(vinyl-N-alkylpyridinium) chains enables the resultant surfaces to kill a variety of airborne and waterborne bacteria on contact. In the present study, these findings have been extended to an unrelated polymer class, polyethylenimines (PEIs). Alkylated PEIs attached to flat macroscopic surfaces and to those of nanoparticles make these materials highly bactericidal toward both Gram-positive and Gram-negative pathogenic bacteria. Systematic chemical modifications of the immobilized PEI conducted herein shed light on the relationship between the structure of the polymer and the antibacterial efficiency of the resultant coating.

Introduction

One of the thrusts of our ongoing studies is developing novel bactericidal materials whose mechanism of action, in contrast to conventional approaches, is not based on releasing an antiseptic into the environment (Tiller et al., 2001). We have discovered that covalently attaching certain long poly(4-vinyl-N-alkylpyridinium) chains to various surfaces renders the latter highly bactericidal: the resultant coated materials efficiently kill on contact both Gram-positive and Gram-negative bacteria, whether airborne or waterborne (Tiller et al., 2001, 2002). This strategy has been found effective against not only wild-type but also antibiotic-resistant mutant strains of the common human pathogen Staphylococcus aureus regardless of its growth phase (Lin et al., 2002).

In the present work, we have demonstrated that N-alkylated polyvinylpyridines are not a unique bactericidal coating; the structurally unrelated family of N-alkylated polyethylenimines are equally lethal to bacteria. The structure–activity relationship analysis conducted has revealed that in order to create bactericidal surfaces immobilized long polymeric chains have to be hydrophobic (but not excessively so) and positively charged. This design strategy has been validated not only with macroscopic surfaces but also with nanoparticles.

Materials and Methods

Immobilization of Alkylated PEI onto Glass Slides. An NH2-glass slide was ultrasonicated in isopropyl alcohol for 5 min, dried at 80 °C, and placed in a solution of 5 mL of 4-bromobutyril chloride in 95 mL of dry chloroform, followed by stirring at room temperature for 5 h. The acylated NH2-glass slide (note that the acylation in this system is far more facile than the alkylation and thus should predominate) was rinsed with chloroform and methanol and immersed in a mixture containing 20 g of PEI and 0.5 g of KOH in 80 mL of tert-amy alcohol. After stirring at 90 °C for 9 h, the PEI-modified slide was removed, rinsed with methanol, air-dried, and placed in a solution of 0.5 g of KOH and 10 mL of an alkyl bromide in 90 mL of tert-amy alcohol (Noding and Heitz, 1998). After stirring at 90 °C overnight, the slide was removed, rinsed with methanal, and further methylated in a solution of 20 mL of iodomethane in 80 mL of tert-amy alcohol at 60 °C for 9 h in a sealed bottle (Masson et al., 1995; Myatt and Scarpa, 1990, 1992; Nagaya et al., 1993).

Immobilization of Hexyl-PEI and Hexyl-PVP onto Nanoparticles. Magnetic Fe3O4 nanoparticles containing NH2 groups were prepared as described in the literature (Chen and Liao, 2002; Koneracka et al., 1999; Mehta et al., 1997). Solutions of 27 g of FeCl3·6H2O and 14 g of FeSO4·7H2O each in 50 mL of distilled water were mixed at room temperature, followed by a gradual addition of a 29.6% NH4OH solution, resulting in a precipitate. The pH was maintained at approximately 10 throughout. The suspension was then heated at 80 °C for 30 min, and the precipitate was recovered using a horseshoe magnet, washed with distilled water and ethanol, and dried at 80 °C under vacuum overnight.

For the attachment of hexyl-PEI, 5 g of dry Fe3O4 nanoparticles was placed in a mixture of 5 mL of 4-bromobutyryl chloride and 95 mL of dry chloroform. The suspension was stirred at room temperature for 5 h, and the acylated magnetic nanoparticles were recovered by placing the reaction vessel on a permanent magnet. The nanoparticles settled within 10 min. After washing with 60 mL of methanal, the acylated nanoparticles were suspended in a solution of 10 g of PEI and...
0.5 g of KOH in 90 mL of tert-amyl alcohol, and the mixture was stirred at 90 °C overnight. The PEI-modified nanoparticles were collected using a horseshoe magnet, the precipitate was washed with 60 mL of methanol, immersed in a solution of 0.5 g of KOH and 10 mL of 1-bromohexane in 90 mL of tert-amyl alcohol, and the mixture was stirred in 90 °C overnight. After recovery using a horseshoe magnet and washing with 60 mL of methanol, the hexyl-PEI-modified nanoparticles were further methylated in a solution of 10 mL of iodomethane in 90 mL of tert-amyl alcohol at 60 °C overnight in a sealed bottle.

For the binding of hexyl-PVP, 5 g of dry Fe3O4 nanoparticles was immersed in a mixture of 9 mL of 1,4-dibromobutane, 90 mL of dry nitromethane, and 0.1 mL of triethylamine. After 2 h of stirring at 60 °C, the nanoparticles were recovered from the mixture using a magnet, rinsed with 60 mL of nitromethane, and placed in a solution of 0% of PVP in 90 mL of nitromethane/tert-butyl alcohol (10:1, v/v). The reaction mixture was stirred at 75 °C for 24 h, and the nanoparticles were recovered using a magnet, rinsed with 50 mL of acetone, washed with methanol, and air-dried.

### Analysis of Quaternary Amino Groups

An alkyl-PEI-modified glass slide (5.5 × 2.5 cm) was dipped in a 1% solution of fluorescein (Na salt) in distilled water for 5 min, rinsed with distilled water, placed in 20 mL of 0.1% cetyltrimethylammonium chloride in distilled water, and shaken for 10 min to desorb the dye (Ledbetter and Bowen, 1969). The absorbance of the resultant aqueous solution was measured at 510 nm (after adding 10% (v/v) 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 cm⁻¹. For the nanoparticles derivatized with N-hexylated PEI and PVP, 0.1 g of the nanoparticles was immersed in a 1% solution of fluorescein in distilled water and ultrasonicated for 5 min. The nanoparticles were recovered using a permanent magnet and washed with distilled water, and the absorbed fluorescein was desorbed by adding 20 mL of 0.1% cetyltrimethylammonium chloride in distilled water and shaking for 10 min. After removal of the nanoparticles, the absorbance of the solution was measured at 510 nm.

### Determination of Bactericidal Activity

Bacteria were grown in yeast-dextrose broth (Cunliffe et al., 1999) at 37 °C with aeration and agitation at 200 rpm for 6–8 h. The inoculum from an overnight culture was transferred into 0.1 M PBS (approximately 10¹² cells/mL) and introduced into the growth medium at a 1:500 dilution.

The airborne bacteria were produced as described by Tiller et al. (2001, 2002). The bacterial cells were centrifuged at 5160 × g for 10 min and washed with distilled water twice. A 10⁶ cells/mL bacterial suspension in distilled water was sprayed at a rate of some 10 mL/hr onto the slide surface in a fume hood. After a 2-min drying under air (after which time period no traces of water were any longer visible on the slide surface), the slide was placed in a Petri dish and 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 Petri dish was sealed and incubated at 37 °C overnight. The grown bacterial colonies were counted on a light box.

### Results and Discussion

To test the generality of our strategy for creating bactericidal coatings, instead of poly(vinyl-N-alkylpyridinium) (alkyl-PVP) used thus far (Tiller et al., 2001, 2002; Lin et al., 2002), herein we employed another, unrelated, common, and commercially available polycation, polyethylenimine (PEI) (Rivas and Geckeler, 1992), as the backbone. To this end, commercially available NH₂-glass slides (Tiller et al., 2001) were N-acetylated with 4-bromobutyryl chloride, followed by addition of high-molecular-weight PEI (the first two steps in Scheme 1). When an aqueous suspension of Staphylococcus aureus was sprayed on the resultant PEI-derivatized glass, followed by air-drying, covering with growth agar, and cultivation, the number of grown bacterial colonies on the slide surface was found to be 58 ± 2 colonies/cm². This number is similar to that observed in the case of the original, untreated NH₂-glass slide: 68 ± 10 colonies/cm². Thus covalent coating with PEI is not effective in making the glass surface bactericidal.

From our experience with PVP-based polyelectrolytes (Tiller et al., 2001) the most likely reason for PEI coating's impotence is its insufficient hydrophobicity and/or positive charge. Therefore we decided to increase both by alkylating the immobilized PEI with linear alkyl bromides of various lengths. This N-alkylation (step 3 in Scheme 1) should not only make the polymer more hydrophobic but also raise its positive charge by converting PEI's primary, secondary, and tertiary amino groups (some of them presumably already positively charged) as a result of the protonation at neutral pH (Rivas and Geckeler, 1992) into permanently cationic quaternary amino groups. As an assay, we directly titrated the latter groups in all subsequent experiments.

One can see in the first seven entries in Table 1 that, expectedly, the alkylation of the PEI coating of a glass slide in all cases introduces quaternary amino groups absent in PEI. The alkylation also gives rise to a pronounced bactericidal activity (the last column for the first seven entries in Table 1). Interestingly, as the alkyl chain length increases, the bactericidal efficiency first also rises to 80% at C₆ but then declines to some half of that at C₁₆. Analysis of the effect of the alkyl chain length on the density of the resultant quaternary amino groups, which gradually drops from some 10 nmol/cm² at C₃ and C₅ to just one tenth of that at C₁₆ (Table 1), provides a possible clue as to why. When the alkyl chain length is raised, the hydrophobicity of the N-alkylated immobilized
Thus by using the foregoing alkylation for the three longest alkyl moieties to the 90\% range. Indeed, as seen in the last five lines of Table 1, the methylation following the initial alkylation elevates the bactericidal efficiency of the three longest alkyl moieties to the 90–93\% range. Thus by using the foregoing alkylation + methylation methodology the bactericidal efficiency achieved with the immobilized PEI backbone is the same as previously obtained with the optimal alkyl-PVP coating (Tiller et al., 2001).

If this hypothesis is true, then one should be able to boost the quaternary amino group density even for longer alkyl groups, and thus hopefully enhance the overall bactericidal efficiency, by N-methyllating the N-alkylated immobilized PEI (step 4 in Scheme 1). Indeed as seen in the last five lines of Table 1, the methylation following the initial alkylation elevates the bactericidal efficiency for the three longest alkyl moieties to the 90–93% range. Thus by using the foregoing alkylation + methylation methodology the bactericidal efficiency achieved with the immobilized PEI backbone is the same as previously obtained with the optimal alkyl-PVP coating (Tiller et al., 2001).

Scheme 1

Table 1. Bactericidal Activity against Airborne S. aureus of Glass Slides Derivatized with N-Alkylated PEs\(\text{a}\)

<table>
<thead>
<tr>
<th>alkylation</th>
<th>subsequent methylation</th>
<th>quaternary amino group density (nmol/cm(^2))</th>
<th>bactericidal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>no</td>
<td>~0</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>methyl</td>
<td>n.a.</td>
<td>10.2 ± 3.0</td>
<td>18 ± 20</td>
</tr>
<tr>
<td>ethyl</td>
<td>no</td>
<td>9.4 ± 3.9</td>
<td>15 ± 16</td>
</tr>
<tr>
<td>butyl</td>
<td>no</td>
<td>6.9 ± 2.0</td>
<td>62 ± 16</td>
</tr>
<tr>
<td>hexyl</td>
<td>no</td>
<td>6.1 ± 2.4</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>dodecyl</td>
<td>no</td>
<td>3.3 ± 1.2</td>
<td>66 ± 13</td>
</tr>
<tr>
<td>octadecyl</td>
<td>no</td>
<td>1.1 ± 0.6</td>
<td>44 ± 22</td>
</tr>
<tr>
<td>ethyl</td>
<td>yes</td>
<td>9.2 ± 3.5</td>
<td>22 ± 15</td>
</tr>
<tr>
<td>butyl</td>
<td>yes</td>
<td>7.7 ± 2.7</td>
<td>56 ± 13</td>
</tr>
<tr>
<td>hexyl</td>
<td>yes</td>
<td>6.8 ± 1.9</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>dodecyl</td>
<td>yes</td>
<td>4.8 ± 1.5</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>octadecyl</td>
<td>yes</td>
<td>4.2 ± 2.1</td>
<td>93 ± 5</td>
</tr>
</tbody>
</table>

\(\text{a}\) All experiments were carried out at least in duplicate, and the mean values and experimental errors were calculated as described by Harris (1995). Bactericidal activity/efficiency is defined as the number of bacterial colonies/cm\(^2\) observed following cultivation on a derivatized slide divided by that in the case of the original NH\(_2\)-glass slide, times 100\%. See text and Scheme 1 for further details.

Inspection of the data in Table 1 reveals that effective PEI-based coatings are all hydrophobic polycations. To verify the importance of the charge, we varied it while keeping the hydrophobicity roughly similar. To this end, instead of N-alkylating the immobilized PEI with hexyl bromide (the first route in Scheme 2), we either N-acylated it with hexanoyl chloride or N-alkylated it with 6-bromohexanoic acid (the last two routes in Scheme 2). Whereas the N-hexylation introduces positive charges due to the quaternization, the N-hexanoylation does not, and the carboxyhexylation introduces a zwitter-ion. It was found that while the first route resulted in the bactericidal efficiency of 80\%, which rose to 90\% after the subsequent methylation (the fifth entry in Table 1), the other two yielded nonbactericidal materials, even following the final methylation. These findings underscore the importance of positively charged polymeric chains, presumably because of their ability to stretch perpendicularly to the glass surface to which they are attached, thus forming a “brush” (Halperin et al., 1992).

Next, our hexyl-PEI coatings were tested against other bacteria, both Gram-positive and Gram-negative. As seen in Table 2, the pattern of behavior observed with airborne S. epidermidis, P. aeruginosa, and E. coli was similar to that with S. aureus: bactericidal efficiency in the 70–80% range, which was further boosted to the 90–100% level by additionally methyllating the material (step 4 in Scheme 1).

It was found that alkylated PEI coatings were even more lethal to waterborne than to airborne S. aureus: the bactericidal activity of a dodecyl-PEI-derivatized glass slide was 98 ± 1\% against the former, while only 66 ± 13\% (Table 1) against the latter.

In all our studies to date, flat macroscopic material surfaces have been derivatized to render them bactericidal. Given a strong current interest in nanotechnology (Vaia et al., 2001; Sergeev, 2001), we addressed the question of whether the same can be achieved with nanosize particles. In addition, we decided to work with magnetic nanoparticles (Lopez-Quintela and Rivas, 1996) because they can be easily recovered and separated simply by applying magnetic field.

Using a recent literature procedure (Chen and Liao, 2002), we heated a mixture of ferric and ferrous salts,
followed by addition of NH4OH. The resultant precipitate consists of Fe3O4 nanoparticles containing covalently bound NH2 groups (Chen and Liao, 2002). The latter were directly titrated with picrylsulfonic acid to reveal 120 (10 mol of NH2 groups per 1 g of dried nanoparticles. The presence of the primary amino groups enabled us to employ the same polymer attachment methodology as with NH2-glass slides (Tiller et al., 2001, and Scheme 1).

First, we attached hexyl-PVP to the magnetic nanoparticles in the same way as previously to macroscopic surfaces (Tiller et al., 2002). After washing, these coated nanoparticles were suspended in distilled water at 0.05 mg/mL, and 10^6 S. aureus cells/mL was added. Following gentle agitation for 5 min at room temperature, the nanoparticles were pulled out using a permanent magnet. An aliquot of the remaining bacterial suspension was sprayed onto an NH2-glass slide, and the number of viable bacteria was subsequently determined as described above. In this experiment, we also measured whether a contact with derivatized nanoparticles affects the viability of S. aureus, only 3 ± 2 bacterial colonies/cm² were eventually observed, as compared to 80 ± 3 colonies/cm² in a control experiment where the cells were shaken for 3 min without any particles. These data indicate that some 96% of the cells were killed on a 5-min contact with hexyl-PVP-derivatized Fe3O4 nanoparticles under these conditions. (Note that while we cannot rule out the possibility that the derivatized nanoparticles removed live bacterial cells from the suspension by adsorption, this scenario seems unlikely because the cells were killed by the same polycations immobilized onto macroscopic surfaces.)

We carried out two additional controls. In one, 0.05 mg/mL of the nonderivatized nanoparticles was used instead of their coated counterparts with all else being identical. This experiment yielded 77 ± 10 S. aureus colonies/cm², i.e., virtually the same number as without nanoparticles. Thus the latter by themselves, at least at this low concentration, are not toxic to the bacterium. In another control, we tested a possible leakage of the polymer from the coated nanoparticles. To this end, 0.05 mg/mL of hexyl-PVP-derivatized nanoparticles was shaken in distilled water for 10 min (i.e., twice as long as in contact with S. aureus to be on a conservative side) and then removed by centrifugation, and 10^6 of S. aureus cells was added, followed by a 5-min agitation, spraying onto an NH2-glass slide, and incubation as before. In this experiment, 56 ± 7 bacterial colonies/cm² was observed, i.e., some 80% of those detected with no nanoparticles ever encountered. Therefore, while a minor fraction of S. aureus cells may have been killed by the leached hexyl-PVP (although even this could be an artifact due to the remaining debris of the nanoparticles not removed by centrifugation), the majority were killed by a contact with coated nanoparticles.

We next extended this study to the Fe3O4 nanoparticles derivatized with hexyl-PEI as outlined in Scheme 1. As seen in the first line of Table 3, 0.05 mg/mL of these nanoparticles killed about half of the S. aureus cells, with the bactericidal efficiency jumping to 92% if the immobilized polymer was also methylated (step 3 in Scheme 1). Similar experiments conducted with three other
bacteria (Table 3) revealed the same behavior: significant bactericidal activity in all cases even without methylation and almost complete activity with it (note that for all bacteria the nonderivatized Fe3O4 nanoparticles presented no or little toxicity; the penultimate column in Table 3). Finally, the last column in the table shows that for each bacterium tested the observed bactericidal activity was not caused by the leached hexyl/methyl-PEI.

In closing, this investigation further expands the scope of our bactericidal strategy with respect to both new polymeric coatings and novel core materials structures. It is likely that any sufficiently long, hydrophobic, and positively charged polymeric coatings may be suitable. Bactericidal nanoparticles, on the other hand, are conducing to a range of potential applications, e.g., antibacterial paints and fillers.

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References and Notes


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