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ORIGINAL RESEARCH PAPER

On structural damage incurred by bacteria upon exposure to hydrophobic polycationic coatings

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Abstract Hydrophobic polycations previously developed by us efficiently kill *E. coli* and *Staphylococcus aureus* on contact. As visualized by electron microscopy herein, these pathogenic bacteria incur marked morphological damage from the exposure to these *N*-alkylated-polyethylenimine "paints" which results in the leakage of an appreciable fraction of the total cellular protein. The quantity and composition of that leaked protein is similar to that released upon traditional lysozyme/EDTA treatment, thus providing insights into the mechanism of action of our microbicidal coatings.

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Introduction

Pathogenic microbes continue to exert their deleterious effects on human health despite numerous antiseptics and antibiotics currently in use. Our strategy of addressing this critical public health problem is to prevent infection by inactivating microbes during transmission. To this end, we have developed hydrophobic polycationic surface treatments with potent, on-contact, broad-spectrum efficacy against pathogenic bacteria, fungi, and viruses (Klibanov 2007).

Further innovation in this area would benefit from understanding the mechanism by which our coatings inactivate microbes. We have previously demonstrated that upon exposure to certain *N*-alkylated-PEIs (PEI = polyethylenimine) both Gram-negative and Gram-positive bacteria are killed because the integrity of their cellular membranes is compromised (Milovic et al. 2005; Park et al. 2006). Herein we report further progress in elucidating how these hydrophobic polycations kill bacteria. In particular, we have determined by scanning electron microscopy that bacteria suffer severe structural damage upon contact with the *N,N*-Dodecyl,methyl-PEI surface

coatings. This damage, in turn, results in the leakage of significant quantities of proteins disproportionately from the periplasmic, as opposed to cytoplasmic, cellular space. Side-by-side comparison of these observations with those upon traditional lysozyme treatment in the presence of the chelating agent EDTA reveals striking similarities.

Materials and methods

Materials

All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich. Silicon (Si) wafers were from Silicon Quest International.

Linear PEI was synthesized as previously described (Thomas et al. 2005). Briefly, 20 g of poly(2-ethyl-2-oxazoline) ($M_W = 500$ kDa) was refluxed in 800 ml of 24% (v/v) HCl at 125°C for 96 h. The formed precipitate was filtered off, dissolved in water, and neutralized with excess KOH until re-precipitation. Following filtration and washing with water to a neutral pH, the precipitate was dried under vacuum to give 7.6 g of 217 kDa linear PEI.

Synthesis of *N,N*-Dodecyl,methyl-PEI was performed similarly to a previously described method (Haldar et al. 2006). Briefly, a mixture of 4 g of linear PEI, 15.4 g of KOH, 60 ml 1-bromododecane, and 50 ml *tert*-amyl alcohol was stirred at 95°C for 96 h, after which the solids were removed by centrifugation at 6,000×*g* for 30 min at room temperature. The supernatant was stirred with 15 ml iodomethane in a sealed flask at 60°C for 24 h. The resulting polymer was extracted by precipitation with an excess of ethyl acetate, followed by filtering and drying under vacuum. The yield was 12.2 g.

N,N-Dodecyl,methyl-PEI coatings were prepared by painting the surfaces with a 50 mg/ml polycation solution in chloroform with a 3/8 inch nylon-bristled paint brush (Loew-Cornell) and then allowing the solvent to evaporate. Coating and drying was performed in quadruplicate.

Scanning electron microscopy

Staphylococcous aureus (ATCC 33807) was grown overnight at 37°C in cation-adjusted Mueller-Hinton Broth II (CMHB) (Difco, BD), washed, and prepared as previously described (Haldar et al. 2007), then diluted to 5×10^6 cells/ml and sprayed onto surfaces using a chromatography sprayer (VWR International) at ~10 ml/min. Preparation and spraying of *Escherichia coli* (*E. coli*) K12 (the Coli Genetic Stock Center, CGSC4401) was performed similarly, except that it was cultured in LB-Miller broth (VWR) and diluted to 5×10^7 cells/ml.

Sprayed samples of both plain and *N,N*-Dodecyl,methyl-PEI-coated Si wafers were incubated at room temperature for 30 min, and then fixed using the Karnovsky's Fixative kit (Polysciences) according to manufacturer's instructions. Briefly, samples were incubated in a fixing solution (2% paraformaldehyde plus 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for 2 h and rinsed for 10 min in phosphate buffer. They were then incubated in 1% osmium tetroxide solution for 1 h in darkness, rinsed with the phosphate buffer, and serially dehydrated in 35, 50, 70, and 95% (v/v) ethanol solutions for 10 min each, before final dehydration (repeated thrice) in pure anhydrous ethanol for 10 min. Samples were then freeze-dried in liquid N_2 and sputter-coated before imaging with a JEOL JSM-6060 scanning electron microscope at a 11,000× magnification.

Bacterial lysis

E. coli K12 and *S. aureus* were cultured and prepared as described previously (Haldar et al. 2007). Multi-drug-resistant, extended-spectrum β-lactamase *E. coli* (ATCC, BAA-196) was grown overnight in tryptic soy medium (BD) with 10 μg ceftazidime/ml (Sigma-Aldrich) at 37°C (Haldar et al. 2007).

Lysozyme treatment of bacterial cells, based on previously described procedures (Yamato et al. 1975), comprised incubating 10^8 cells/ml *E. coli* K12 in phosphate-buffered saline (PBS), 20% (w/v) sucrose, 1 μg/ml hen egg white lysozyme (Sigma-Aldrich, ~50,000 units of activity per mg), and 10 mm EDTA (Na salt) at 4°C for 2 h. French press treatment consisted of two passes at 16,000 psi using a French pressure cell press (Spectronic Instruments).

Exposure to surfaces painted by *N,N*-Dodecyl,methyl-PEI consisted of shaking 10^8 bacteria/ml in polypropylene conical centrifuge tubes at 250 rpm. For *E. coli*, 40 ml bacterial suspension was incubated in 50 ml tubes (BD), with an inner surface area of 94 cm^2, for 6 h at 37°C. For *S. aureus*, 30 ml

bacterial suspension was incubated in a 50 ml tube with another 13 ml culture tube (BD) inserted in it (with an inner surface area of 143 cm^2) for 8 h at room temperature. Bacterial viability was assessed by plating serial dilutions of the bacterial suspension onto yeast-dextrose broth (YDB)-agar plates and incubating overnight at 37°C. The bactericidal efficiency was calculated by comparing the numbers of colony-forming units (CFU) of suspensions incubated in plain tubes with those in the case of *N,N*-Dodecyl,methyl-PEI-coated tubes.

Protein purification and enzymatic assays

Protein extracts from the aforementioned treatments were obtained by centrifugation of the treated bacterial suspensions at 4,000×*g* at 4°C for 10 min and subsequent concentration of the supernatant to 1.5 ml using Amicon Ultracel 3 k Centrifugal Filters (Millipore). The resultant solutions were analyzed with a standard BSA Bradford assay or further concentrated to 0.1 ml using Amicon Ultracel 10 k Centrifugal Filters (Millipore) for *β*-lactamase and *β*-galactosidase assays.

The *β*-lactamase assay was executed using the chromogenic reagent, Nitrocefin (EMD Chemicals), according to manufacturer's instructions. A mixture of 100 µl each of a concentrated sample and of 50 µg Nitrocefin/ml in PBS was incubated at 37°C for 10 min, and the change in absorbance was measured at 486 nm. Sample solutions were referenced to standard curves constructed from purified *Enterobacter cloacae* *β*-lactamase (Sigma-Aldrich) in PBS.

The *β*-galactosidase assay employed the *β*-Gal Assay Kit (Invitrogen) according to manufacturer's instructions. After combining 30 µl of a concentrated sample with 70 µl 4 mg/ml *ortho*-nitrophenyl-*β*-galactoside (ONPG) and 200 µl of phosphate buffer, pH 7.0, supplemented with KCl, $MgSO_4$, and *β*-mercaptoethanol at 37°C for 30 min, the reaction was stopped with 500 µl 1 M Na_2CO_3. The absorbance measured at 420 nm was referenced to standard curves based on purified *E. coli* *β*-galactosidase (Sigma-Aldrich) in PBS.

Results and discussion

Our previous bactericidal studies have demonstrated that both waterborne and airborne bacteria are killed upon contact with either covalently attached *N,N*-hexyl, methyl-PEI or physically deposited *N,N*-Dodecyl,methyl-PEI (Lin et al. 2002; Park et al. 2006). These observations, however, provided only limited insights into *how* bacteria were killed. In particular, a fluorescent assay that assessed viability of bacteria by their ability to accumulate a membrane-impermeable dye not only confirmed the potent bactericidal action results independently but also indicated that the integrity of the bacterial membrane was compromised by the hydrophobic polycations (Milovic et al. 2005; Park et al. 2006).

To elucidate the magnitude of the imparted damage, we used scanning electron microscopy (SEM) to visually characterize the morphology of bacteria upon contact with microbicidally painted surfaces. The micrographs presented in Fig. 1a and b for *S. aureus* and *E. coli*, respectively, on a bare silicon wafer show that they retain a well-defined morphology and surface smoothness characteristic of unperturbed bacteria, as previously demonstrated (Belaaouaj et al. 2000). In contrast, after contact with *N,N*-Dodecyl,methyl-PEI-coated Si, both *S. aureus* and *E. coli* (Figs. 1c, d, respectively) exhibit profound morphological deformations. The shrunken appearance of

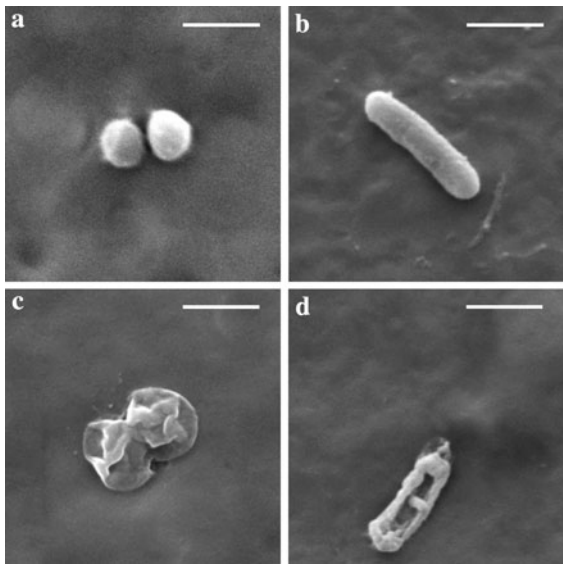

Fig. 1 Scanning electron microscopy images of *S. aureus* and *E. coli* K12 in contact with bare silicon wafers (**a** and **b**, respectively) and with those coated with *N,N*-Dodecyl,methyl-PEI (**c** and **d**, respectively). The SEM micrographs shown are representative for their respective bacterial conditions; the *scale bars* 1 µm

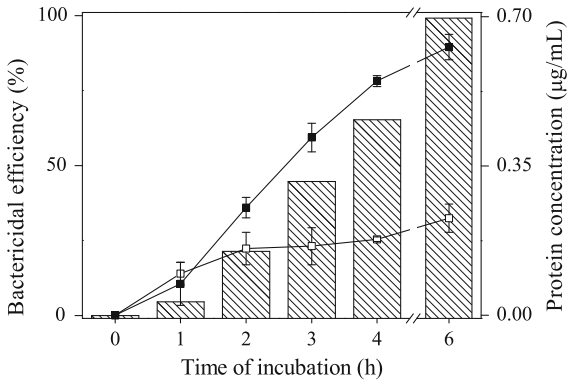

Fig. 2 The effect of the *N,N*-Dodecyl,methyl-PEI coating on the viability of *E. coli* K12 and on the bacterial protein concentration released into solution (see text for details). The *shaded bars* represent bactericidal efficiencies; *error bars* were omitted for clarity. Total protein in solution after incubation with plain (*empty square*) and *N,N*-Dodecyl,methyl-PEI-coated (*filled square*) polypropylene tubes are shown by lines. All values were obtained in duplicate, and are shown as averages ± standard deviations

these impacted bacteria indicates a loss of structural integrity, which is predominately maintained by the cellular wall (Vollmer et al. 2008), thereby suggesting a specific and undermining interaction with the bacteria's peptidoglycan layer by the hydrophobic polycations. While these SEM images allow us to visually confirm the damage to the bacteria, its specific details remained obscure. To illuminate them, we examined the solution for leaked intracellular proteins and compared the findings with those obtained upon the destruction of bacterial cells using standard microbiological lysis techniques.

To this end, we first incubated *E. coli* K12 suspensions in plain and *N,N*-Dodecyl,methyl-PEI-coated polypropylene tubes and monitored both their bacterial viability and protein leakage over time. As seen in Fig. 2, incubation of *E. coli* in bare tubes gives rise to small amounts of protein in solution over 6 h, which can be attributed to secreted extracellular proteins (Glenn 1976). However, when the bacterial cells were incubated in the bactericide- painted tubes under the same conditions, a dramatic increase (e.g., 170% after 6 h) in protein concentration over these baseline levels was observed (Fig. 2). In parallel with the protein leakage, the bactericidal efficiency also rises with time of incubation (to reach 99 ± 1% after 6 h, see Fig. 2). Therefore, in addition to the bacterial membranes being permeabilized enough for small-molecule dyes to move across the membrane (Milovic et al. 2005; Park et al. 2006), the degree of the damage inflicted by the hydrophobic polycations is so extensive that even intracellular proteins can escape into solution.

The generality of these findings was confirmed when we similarly tested the drug-resistant *E. coli* BAA-196, as well as *S. aureus*, and found 300 and 160%, respectively, more protein in solution compared with the bare polypropylene tube experiments (the first two lines in Table 1).

To calibrate the protein released upon incubation in a polycation-coated tube, we separately subjected *E. coli* K12 to a traditional lysozyme/EDTA treatment, which is known to permeate the outer membrane due to the presence of the chelator EDTA, and to enzymatically degrade the cell wall, shedding the cell's periplasmic constituents (Malamy and Horecker 1964). As seen in Table 1, there is a 140% increase in protein concentration over the baseline, i.e., similar to that seen with the polycation treatment.

To put the quantity of the leaked protein into perspective vis-à-vis the total amount of protein in the cell, we separately employed the French press treatment commonly used for extensive cell lysis (Jorgensen et al. 1995; Schmitt 1976). One can see in

Table 1 The protein concentrations released into solution by *E. coli* and *S. aureus* after various treatments

Treatment	Total protein concentrations (μg/ml)		
	E. coli K12	*E. coli* BAA-196[a]	*S. aureus*
None	0.23 ± 0.03	0.23 ± 0.03	0.20 ± 0.04
N,N-Dodecyl,methyl-PEI coating	0.63 ± 0.03	0.93 ± 0.01	0.53 ± 0.01
Lysozyme/EDTA	0.56 ± 0.05		
French press	15.6 ± 0.6	14.8 ± 0.7	3.2 ± 0.3

Each measurement was performed in duplicate, and the values are given as averages ± standard deviations

[a] The same conditions as with the K12 strain

Table 1 that for the three tested kinds of bacteria, there is far more protein generated by the French press disintegration than released by polycation or lysozyme/EDTA treatments. Specifically, after correction for the baseline extracellular proteins, the 6-h exposure to *N,N*-Dodecyl,methyl-PEI coatings yields 2.6, 4.6, and 2.1% of the proteins extracted in the French press treatment for *E. coli* K12, *E. coli* BAA-196, and *S. aureus*, respectively (Table 1). That these amounts, however, are comparable with those produced in the lysozyme/EDTA treatment (2.1% for *E. coli* K12) suggests that mechanistically these two dissimilar modes of lysis may nevertheless target the same bacterial components.

Since the lysozyme/EDTA treatment is known to release the periplasmic proteins and leave the cytoplasm relatively intact (Malamy and Horecker 1964), we focused on determining the origins of the leaked proteins resulting from exposure to the *N,N*-Dodecyl,methyl-PEI coating. To this end, we measured the concentrations of the β-lactamase and β-galactosidase enzymes leaked into solution from *E. coli* as indicators of the polycationic chain's penetration into the periplasm and cytoplasm, respectively.

As seen in Table 2, upon contact with the hydrophobic polycation *E. coli* gave 83 and 420% above the baseline quantities of β-lactamase and β-galactosidase, respectively. Normalization to the total proteins yielded by French press shows that for *E. coli* 8.1% of β-lactamase is leaked from the cells, as compared to 4.6% of the total protein, or a disproportionately large fraction of this periplasmic enzyme (Table 2). With respect to β-galactosidase, 0.5% of it is leaked from *E. coli*, as compared to 2.6% of the total protein, or a disproportionately small fraction of the cytoplasmic enzyme (Table 2). Taken together, these results indicate that relatively larger quantities of periplasmic proteins are leaked when compared to cytoplasmic ones, which suggests that the periplasm is breeched by the polycationic chains significantly more than the cytoplasm. In other words, there appears to be a considerable damage to the outer membrane and relatively minor damage to the cytoplasmic membrane. Since both the quantity of proteins leaked and the nature of the damage by the *N,N*-Dodecyl,methyl-PEI coating are very similar to those imparted by a traditional lysozyme/EDTA treatment, the two treatments may target the same bacterial structures, namely the outer membrane and the peptidoglycan cell wall.

Table 2 The concentrations of periplasmic and cytoplasmic enzymes (β-lactamase and β-galactosidase, respectively) released into solution by *E. coli* after various treatments

Treatment	*E. coli* enzyme concentrations (ng/ml)	
	β-Lactamase[a]	β-Galactosidase[a]
None	0.060 ± 0.001	1.9 ± 0.2
N,N-Dodecyl,methyl-PEI coating	0.11 ± 0.01	9.9 ± 0.6
French press	0.68 ± 0.04	1700 ± 100

Each measurement was performed in duplicate, and the values are given as averages ± standard deviations

[a] Since *E. coli* K12 did not produce measurable levels of β-lactamase, *E. coli* BAA-196 was employed to assay for leakage of β-lactamase, while the former strain to assay for leakage of β-galactosidase

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