

# Immobilized *N*-Alkylated Polyethylenimine Avidly Kills Bacteria by Rupturing Cell Membranes With No Resistance Developed

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**Abstract:** Several critical mechanistic and phenomenological aspects of the microbicidal surface coatings based on immobilized hydrophobic polycations, previously developed by us, are addressed. Using *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria, remarkable bactericidal action (up to a 10<sup>9</sup>-fold reduction in live bacteria count in the surface-exposed solution and a 100% inactivation of the surface-adhered bacteria) of an amino-glass slide covalently derivatized with *N*-hexylmethylpolyethylenimine (PEI) is found to be due to rupturing bacterial cell membranes by the polymeric chains. The bacteria fail to develop noticeable resistance to this lethal action over the course of many successive generations. Finally, the immobilized *N*-alkyl-PEI, while deadly to bacteria, is determined to be harmless to mammalian (monkey kidney) cells.

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**Keywords:** polycations; immobilization; antimicrobial; microbicidal materials; bactericidal surfaces

## INTRODUCTION

Treating surfaces of common materials and objects to render them sterile is desirable for a host of environmental and medical applications (Arciola et al., 1993; Costerton et al., 1999; Lappin-Scott and Costerton, 1989; McFeters, 1984). A conventional surface treatment, based on incorporation of a leachable antiseptic into a polymeric surface coating (Edge et al., 2001; Nohr and MacDonald, 1994; Nonaka et al., 1996; Schierholz et al., 2001), is time-limited and conducive to development of bacterial resistance. A more promising approach involves a covalent surface functionalization with polymeric antimicrobials that do not leach into solution (Cen et al., 2003; Lee et al., 2004; Lin et al., 2002a, 2003a; Tiller et al., 2001, 2002; Waschinski et al., 2004).

We have found previously that covalent immobilization of certain poly(4-vinyl-*N*-alkylpyridine) and *N*-alkylated polyethylenimine (*N*-alkyl-PEI) onto material surfaces renders the latter permanently microbicidal on contact against both waterborne and airborne bacteria and fungi, including pathogenic and antibiotic-resistant strains (Lin et al., 2002a,b, 2003a,b; Tiller et al., 2001, 2002). We have validated this approach for a variety of diverse materials—organic and inorganic, natural and synthetic, macroscopic and nanoscaled, monolithic and porous—including commercial plastics, textiles, and glass.

In the present work, we address several outstanding issues critical for the mechanistic elucidation of this microbicidal effect and for practical utility of such antimicrobial coatings. To this end, we have kinetically investigated the mode and the time scale of action of immobilized *N*-alkyl-PEI. In addition, we tested the ability of bacteria to develop resistance to such nonleaching polycationic coatings and the latter's potential toxicity to mammalian cells.

## MATERIALS AND METHODS

### Materials

$\gamma$ -Aminopropyltriethoxysilane-modified microscope glass slides (7.5 × 2.5 cm in size, of which the 5.5 × 2.5 cm nonfrosted area was subjected to chemical derivatization) were from Sigma-Aldrich (St. Louis, MO). The Live/Dead BacLight bacterial viability kit and animal cell vitality kit were from Molecular Probes (Eugene, OR). PEI (average  $M_w = 750$  kDa), 6-bromohexane, 4-bromobutyl chloride, KOH, iodomethane, *tert*-amyl alcohol, methanol, and other chemicals and solvents (all from Aldrich, Milwaukee, WI) were used without further purification. The bacteria *Staphylococcus aureus* (strain 33807) and *Escherichia coli* (strain CGSC4401), both wildtype with no known resistances, were obtained from ATCC (Rockville, MD). Phosphate-buffered saline (PBS) and Dulbecco's Modified Eagle's Medium containing glutamine and 10% heat-inactivated fetal bovine

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serum were from Gibco (Grand Island, NY). Amino-glass slides were derivatized with *N*-hexyl,methyl-PEI as described by Lin et al. (2002a).

### Fluorescent Labeling and Microscopy

Ten  $\mu\text{l}$  of a bacterial suspension in water containing  $2 \cdot 10^9$  cells/ml was combined with 10  $\mu\text{l}$  of a fluorescent probe mixture containing 3.0  $\mu\text{M}$  green fluorescent nucleic acid stain SYTO 9 and 15.0  $\mu\text{M}$  red fluorescent nucleic acid stain propidium iodide (Haughland, 1996; Millard and Roth, 1997). The mixture was incubated in the dark for 10 min and a 2- $\mu\text{l}$  aliquot was placed on the polycation-modified amino-glass slide, which was then covered by a coverslip and examined by fluorescence microscopy after different incubation times. The control experiment was conducted the same way, except that the amino-glass slide was unmodified. Fluorescent images were obtained using an upright microscope (Axioskop 2 MAT, Carl Zeiss, Thornwood, NY) equipped with a halogen lamp. A fluorescein bandpass filter was used for visualization of live bacteria and a rhodamine bandpass filter was used for the dead ones (Haughland, 1996). Digital images during a 200-ms exposure were recorded using 1,000-fold magnification. The bacterial cells that appeared green were assumed viable, i.e., alive, whereas red cells were assumed to be with compromised cell membrane integrity, i.e., dead (Millard and Roth, 1997).

### Image Analysis and Kinetic Measurements

For kinetic measurements, each image was taken at a random location on the slide to minimize fluorescence bleaching. Each measurement was done in triplicate. The percentages of live and dead bacteria were obtained by observing and counting at least 200 cells. Each microscope field of view was recorded using the green and the red bandpass filters. Image processing and cell counting were done using Matlab (MathWorks, Natick, MA) software. The plots of the percent of dead bacteria vs. time were fitted to the first-order rate law with SigmaPlot (SSI, Richmond, CA) software.

### Determination of Microbicidal Activity and Testing of Bacterial Resistance

A suspension (100  $\mu\text{l}$ ) of *E. coli* cells in 0.1 M aqueous PBS buffer (pH 7.0,  $10^{11}$  cells per ml) was added to 10 ml of a yeast/dextrose broth (YDB), prepared as described by Cunliffe et al. (1999), in a sterile 15-ml tube. It was shaken at 37°C and 200 rpm (Innova 4200 Incubator Shaker, New Brunswick Scientific, Edison, NJ) overnight. Then 1 ml of this suspension was added to 50 ml of YDB, followed by incubation for another 7 h. After a 10-min centrifugation (8,000 rpm; Sorvall RC-5B centrifuge, SS-34 rotor, DuPont Instruments, Boston, MA), the bacterial cells were washed thrice with, and resuspended in, a PBS solution, to a final concentration of  $10^6$  cells per ml.

The *N*-alkyl-PEI-derivatized slides were each immersed into 40 ml of an *E. coli* suspension in PBS in a 50-ml centri-

fugation tube and incubated at 37°C with gentle shaking at 100 rpm. After a certain period of time, a slide was removed, thoroughly washed from both sides with PBS to remove loosely associated bacterial cells, then dipped into 40 ml of the PBS solution and incubated for 1 h. Thereafter, the slide was taken out, placed in a Petri dish, and immediately covered with a layer of solid growth agar (1.5% agar in the YDB, autoclaved, poured into a Petri dish, and gelled overnight). The slide-containing Petri dish was sealed and incubated at 37°C overnight. The bacterial colonies grown on the slide were counted on a light box.

The number of viable *E. coli* cells remaining in the suspension after removal of the slide was measured by making a series of consecutive 10-fold dilutions. A 20- $\mu\text{l}$  aliquot of each dilution was transferred into a small Petri dish (35  $\times$  10 mm) to which 2 ml of melted agar medium in YDB at 55°C was added. After letting a gel form in a freezer for about 5 min, the Petri dish was incubated at 37°C overnight. The colonies formed in the agar gel were then counted as outlined above.

To assess the resistance of airborne bacteria, we used the assay described by Tiller et al. (2001). After every assay, an inoculum containing surviving cells was withdrawn from a single colony in each sample and treated the same way as the starting colony. All measurements were done in duplicate and the data are reported as the mean value  $\pm$  standard deviation.

### Effect of Antimicrobial Surfaces on Mammalian Cells

COS-7 cells (simian virus 40-transformed kidney cells of an African green monkey) were cultured in Dulbecco's Modified Eagle's Medium containing glutamine supplemented with 10% heat-inactivated FBS (Gibco), sodium pyruvate, and antibiotics (100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin) (Thomas and Klibanov, 2002). The cells were grown at 37°C in humidified air containing 5%  $\text{CO}_2$  and passaged every 3–4 days. At  $\sim$ 80% confluency, the cells were detached by treatment with 0.25% trypsin-EDTA, harvested, washed, and resuspended in Dulbecco's PBS. One hundred  $\mu\text{l}$  of the cell suspension containing  $10^6$  cells/ml was mixed with 1  $\mu\text{l}$  of a 50- $\mu\text{M}$   $\text{C}_{12}$ -resazurin solution and 1  $\mu\text{l}$  of a 1- $\mu\text{M}$  SYTOX Green stain solution, followed by incubation at 37°C for 15 min. A 10- $\mu\text{l}$  aliquot of this mixture was subsequently placed on the polycation-modified amino-glass slide and covered by a coverslip prior to examination by fluorescence microscopy. Fluorescent images were taken as described above, except that the magnification was 100-fold and the exposure time was 500 ms.

## RESULTS AND DISCUSSION

### Mechanism of Bacterial Inactivation

We have previously established that covalent immobilization of certain moderately hydrophobic polycations, such as *N*-hexyl,methyl-PEI, onto solid surfaces renders these surfaces

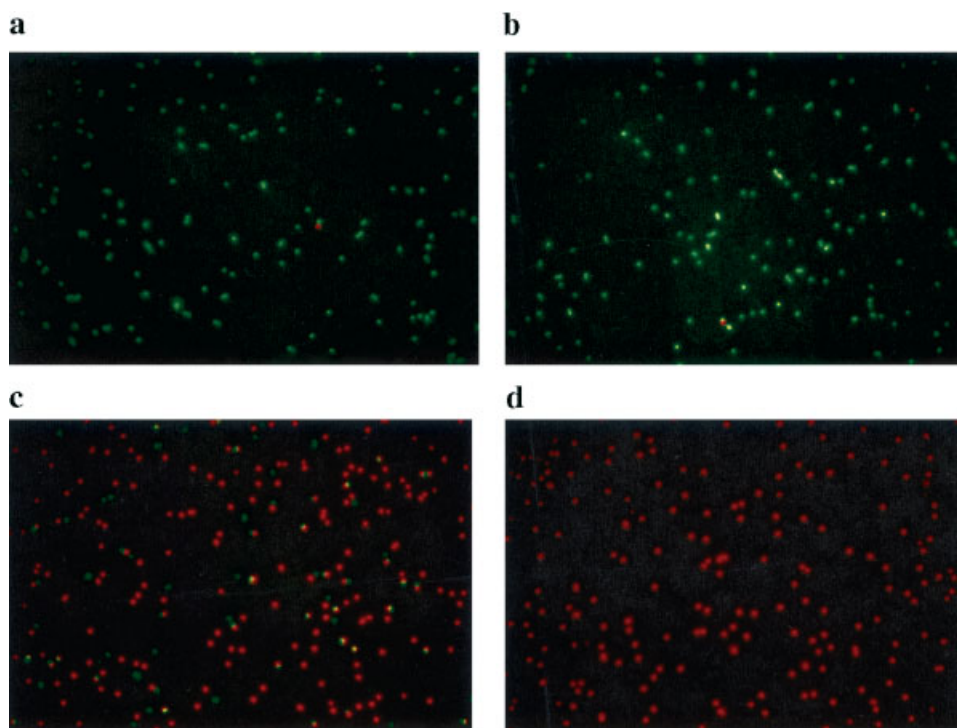
permanently microbicidal (Lin et al., 2002a,b, 2003a; Tiller et al., 2001, 2002). Our goal in the present study was to kinetically investigate the mechanism of this antimicrobial action using *E. coli* and *S. aureus*, common and dissimilar (Gram-negative and Gram-positive, respectively) pathogenic bacteria, as targets. We surmised that this mechanism was likely to be related to that of common water-soluble biocides containing quaternary ammonium groups, which stems from electrostatic/hydrophobic interactions with the bacterial cell membrane/wall undermining its integrity (Fidai et al., 1997; Ikeda et al., 1984; Imazato et al., 1998; Marchisio et al., 1994; Tew et al., 2002; Vaara, 1992). Note that our experiments in this work were aimed at emulating natural, real-life conditions, where a pathogen rapidly propagates in the body at 37°C and is then released into the environment. Accordingly, bacterial cells were grown at 37°C and then tested at room temperature, at which antiseptic biocides, such as membrane-bursting quaternary ammonium compounds, are commonly applied.

To verify this hypothesis, we used the Live/Dead two-color fluorescence method that reports the intactness of bacterial membranes (Haughland, 1996), thus indicating in situ whether the bacterial inactivation is caused by damage to the membrane. This viability assay involves two fluorescent dyes that can bind to nucleic acids—the green stain SYTO 9 and the red stain propidium iodide. In general, the former is membrane-permeable and thus labels all bacteria in the population. In contrast, propidium iodide is unable to penetrate into intact cells and therefore labels only those with

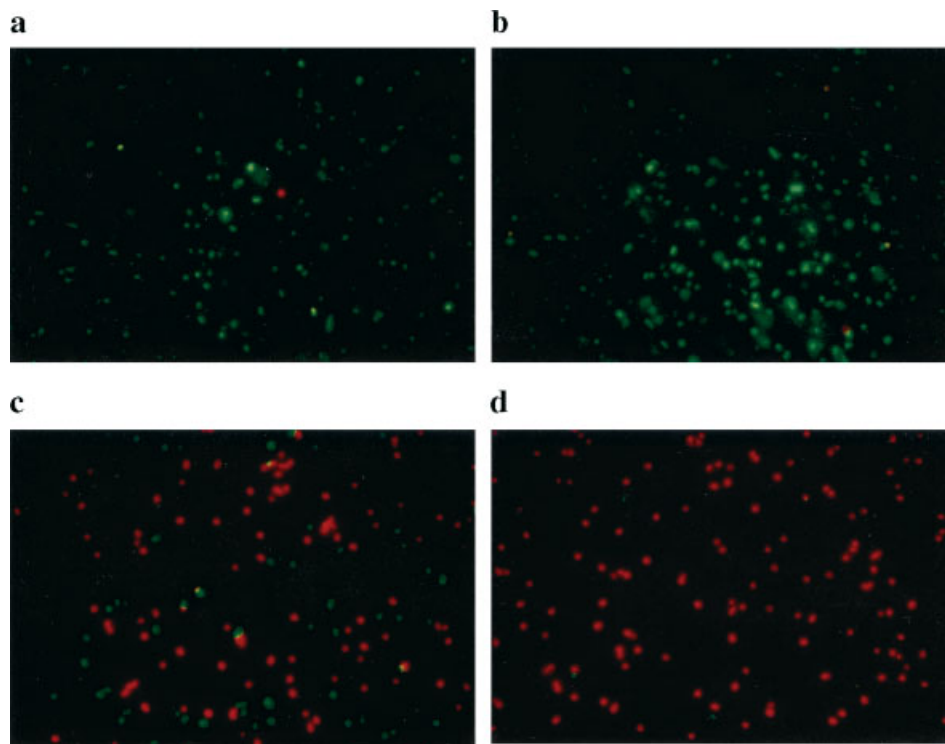
compromised membranes. Moreover, the red propidium iodide abolishes the green fluorescence of SYTO 9 in damaged cells by displacing it from complexes with nucleic acids. As a result, bacteria with intact membrane fluoresce green, while bacteria with damaged cell membrane fluoresce red (Millard and Roth, 1997).

The fluorescence of the suspension of bacterial cells upon incubation with the stains following 20- and 120-min exposures to a control, amino-glass slide was examined under the microscope. As Figures 1a,b and 2a,b show for *S. aureus* and *E. coli*, respectively, almost all bacterial cells fluoresce green, indicating the intactness of their cellular membranes. Therefore, the amino-glass surface has no appreciable effect on the membrane's integrity. Moreover, many bacteria remain planktonic, i.e., float in solution. In sharp contrast, as seen in Figures 1c and 2c, the majority of bacterial cells look red after a 20-min exposure to the surface of an amino-glass slide derivatized with *N*-hexyl,methyl-PEI; furthermore, after a 2-h incubation virtually all cells look red (Figs. 1d, 2d). These data indicate that the contact with the glass-immobilized hydrophobic polycations ruptured bacterial membranes. Also, unlike with the control slides, no floating cells were observed, i.e., once adhered, the cells remained surface-bound.

Polycations dissolved in water act as biocides by damaging bacterial membranes (Chen and Cooper, 2000; Codling et al., 2003; Denyer, 1990; Gelman et al., 2004; Grapski and Cooper, 2001; Ikeda et al., 1984; Imazato et al., 1998; Marchisio et al., 1994; Moon et al., 2003; Tew et al., 2002;



**Figure 1.** Fluorescence microscopy images of *S. aureus* after a 20- and a 120-min room-temperature exposure to the surface of an untreated amino-glass slide (a and b, respectively) and of that covalently derivatized with *N*-hexyl,methyl-PEI (c and d, respectively). The bacterial suspension was preincubated with the cell viability stains; each image is a result of superposition of an image taken by using a green bandpass filter showing intact bacteria, and an image taken by using red bandpass filter showing bacteria with damaged cell membranes. See text for details.



**Figure 2.** Fluorescence microscopy images of *E. coli* after a 20- and a 120-min room-temperature exposure to the surface of an untreated amino-glass slide (a and b, respectively) and of that covalently derivatized with *N*-hexyl,methyl-PEI (c and d, respectively). For other conditions, see the caption to Figure 1.

Vaara, 1992). This antibacterial activity vanishes, however, when the polycations are cross-linked or insolubilized (Imazato et al., 1995, 1998; Kawabata and Nishiguchi, 1988). In contrast, our results in Figures 1 and 2 demonstrate that immobilized *N*-hexyl,methyl-PEI is able to inactivate bacteria by the same mechanism, as hypothesized earlier (Lin et al., 2003b; Tiller et al., 2001). This conclusion underscores that the polycationic chains attached to surfaces must be sufficiently long (Lin et al., 2003b) to reach the adhered bacteria despite a presumably rough surface topography and to penetrate their cell membranes/walls enough to inflict damage. Therefore, various reported microbicidal materials whose surfaces possess merely monomeric positively charged hydrophobic “whiskers” (Isquith et al., 1972; Battice and Hales, 1985; www.carrington-cww.co.uk/permagard. htm; Abel et al., 2002) must act via a fundamentally different mechanism, most likely by gradually releasing a biocide into solution (Clarkson and Evans, 1995; Lin et al., 2003b).

### Kinetics and Efficiency of Bacterial Inactivation

The next question was whether the cellular membrane damage detected by the aforementioned fluorescence assay is indeed sufficient to render the bacteria dead. (Under certain conditions bacteria with compromised membranes may still be able to recover and reproduce (Roszak and Colwell, 1987).) To answer this question, we decided to correlate the foregoing results of fluorescence measurements of *mem-*

*brane integrity* with our previously developed (Tiller et al., 2001) assay of bacterial *viability* as their ability to reproduce in a suitable nutrient medium under favorable conditions (Cunliffe et al., 1999). In the latter method, each surviving cell ideally develops into a distinct colony after an overnight incubation in nutrient-containing agar, thus providing a direct measure of bacterial viability. This test was applied herein to both the floating and surface-bound bacteria.

Table I summarizes the data for the inactivation of *E. coli* after different times of exposure to the polycation-derivatized slides and their amino-glass predecessors. The original bacterial count of  $2.4 \times 10^6$  cfu/ml in the experiment involving the control slides remained unaltered even after 2 h. Hence, virtually all bacteria retained viability following the exposure to the unmodified slide. Moreover, nearly all of those transiently adsorbed onto the slide surface were desorbed during the washing steps, with only a negligible number of viable cells remaining adhered to the surface. The examination of the slides under optical microscope confirmed these observations (data not shown).

In contrast, the *N*-hexyl,methyl-PEI-modified surface had a drastic effect on bacterial viability (second column in Table I). The number of viable bacteria in the solution precipitously declined with time. After 20 min, the cell count dropped to below 1% of the original value. After 2 h, less than 0.01% of the viable bacteria remained in the solution, consistent with the fluorescence microscopy observations (Fig. 2). Moreover, as the third column in Table I shows, no bacteria that adhered to the surface remained viable.

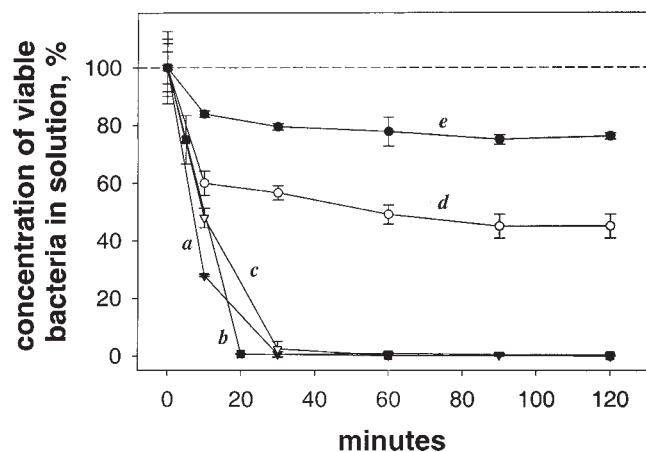
**Table I.** Bactericidal action of *N*-hexyl,methyl-PEI-modified amino-glass slide toward waterborne *E. coli*.

Time of exposure (min)	Cell count for experiments with the modified slide		Cell count for experiments with amino-glass slide (control)	
	In solution (cfu/ml)	On slide (cfu)	In solution (cfu/ml)	On slide (cfu)
0	$(2.1 \pm 0.3) \times 10^6$	1	$(2.4 \pm 0.3) \times 10^6$	0
5	$(1.8 \pm 0.2) \times 10^6$	$1.5 \pm 0.5$	$(2.4 \pm 0.1) \times 10^6$	n/a
20	$(1.6 \pm 0.3) \times 10^4$	0	$(2.4 \pm 0.2) \times 10^6$	4
60	$(1.3 \pm 0.1) \times 10^3$	0	$(2.5 \pm 0.1) \times 10^6$	6
120	$(1.7 \pm 0.2) \times 10^2$	0	$(2.4 \pm 0.1) \times 10^6$	1

The cell counts in solution are expressed in colony-forming units (cfu) per ml, and those on the slide surface are expressed in the total number of cfu.

Clearly, the polycation-derivatized surface exhibits a strong affinity, as well as high capacity (see below), for the bacterial cells.

To quantify the bactericidal prowess of a slide derivatized with *N*-alkyl-PEI, we incubated such a slide in 40 ml of an aqueous suspension of *E. coli* in a concentration range that was varied from  $2.4 \cdot 10^6$  to  $1.8 \cdot 10^8$  cfu/ml. The results, presented in Figure 3, afford several conclusions. First, at the concentration of up to  $2.0 \cdot 10^7$  cfu/ml, virtually all bacteria in solution are killed by a single derivatized slide (curves *a*–*c*). Second, from curves *d* and *e* one can calculate that the number of the bacteria killed by one slide is  $2.2 \cdot 10^9$ . Thus, 1 cm<sup>2</sup> of the derivatized slide is able to kill  $0.8 \cdot 10^8$  cells. Assuming that *E. coli* cells 1) are  $1 \times 2$ - $\mu\text{m}$  rectangles, 2) completely cover the slide's surface and are immediately adjacent to each other, and 3) form a monolayer on the slide's surface, one arrives at the coverage of  $0.5 \cdot 10^8$  cells/cm<sup>2</sup>. That these two numbers are quite similar confirms that all the cells adhered to the surface die. The magnitude of bactericidal action is therefore determined by the surface area available for direct contact with the bacteria. Furthermore, the bactericidal action is absent beyond the first layer of adsorbed bacteria, which confirms our previous observations



**Figure 3.** The reduction in the number of viable *E. coli* in the suspensions containing (a)  $2.4 \cdot 10^6$ ; (b)  $6.1 \cdot 10^6$ ; (c)  $2.0 \cdot 10^7$ ; (d)  $1.2 \cdot 10^8$ ; and (e)  $1.8 \cdot 10^8$  cfu/ml, upon exposure to an amino-glass slide derivatized with *N*-hexyl,methyl-PEI. In the control experiment with the unmodified amino-glass slide, the initial concentrations remained unchanged, as reflected by the dashed line. See text for details.

(Lin et al., 2002a; Tiller et al., 2001) that the bacteria are not killed by the leaching polycation.

The plate-count viability assay thus unambiguously correlates with the fluorescence membrane integrity assay leading to an important conclusion: the *N*-hexyl,methyl-PEI coating indeed severely undermines the integrity of bacterial membranes, thereby rendering the cells nonviable.

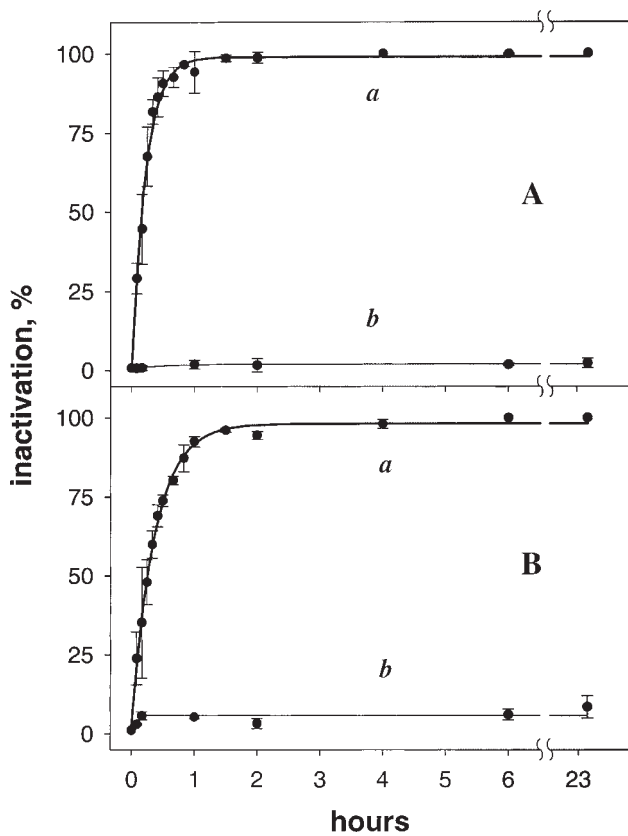
The in situ determination of bacterial viability by the fluorescence assay allows for quantitative time-dependent measurements of bacterial inactivation. The time course of the biocidal action of immobilized *N*-hexyl,methyl-PEI for *S. aureus* and *E. coli* is depicted in Figure 4. The experimental conditions ( $2.0 \mu\text{l}$  of a suspension containing  $1 \cdot 10^9$  cells/ml distributed over  $\sim 1 \text{ cm}^2$ ) provide sub-monolayer bacterial coverage, i.e., the available surface is more than capable of accommodating all the bacteria in the sample. The time course for bacterial inactivation was facily fitted to the first-order rate law process, resulting in half-times of inactivation of 13.5 and 15.1 min, respectively. After 6 h, no viable bacteria were observed. As expected, no significant loss of viable bacteria was detected in the control experiment.

Because the bacteria adhere to the surface without detachment during the course of the experiment, the bactericidal efficiency is physically limited by the space available, as calculated above. We have, however, previously established that the antimicrobial activity can be easily regenerated by simply washing the surface (Lin et al., 2003b). Therefore, the *N*-hexyl,methyl-PEI coatings cause bacterial inactivation which is rapid and efficient, both of which are highly desirable for practical applications.

### Evaluation of Development of Resistance in Airborne Bacteria

A critical issue for the usefulness of our antimicrobial surface coating is whether bacteria can become resistant to it after a prolonged and repeated exposure. We experimentally addressed this issue with airborne *E. coli* and *S. aureus*.

Figure 5 depicts the results of a series of 11 sequential biocidal assays independently carried out with these bacteria. In each assay, the bacterial population originated from a single colony of the survivors from the preceding assay. If these survivors gradually developed resistance to immobilized *N*-alkyl-PEI, the percentage of the bacteria killed should decrease from one cycle to the next. As seen in the

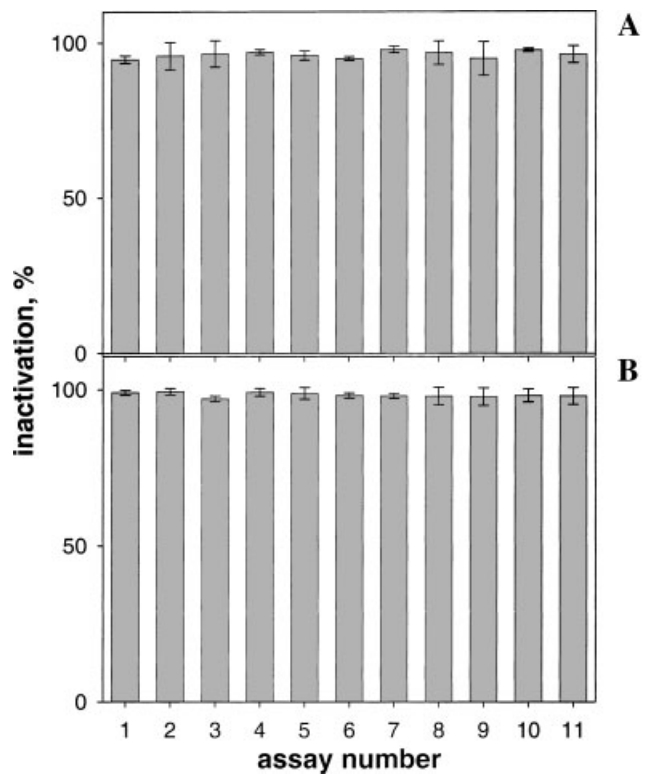


**Figure 4.** The time course of inactivation of *S. aureus* (A) and *E. coli* (B) caused by exposure to the amino-glass surface derivatized with *N*-hexyl,methyl-PEI (a) and nondervatized (b). The data points were obtained from the fluorescence microscopy images as percentages of bacteria that appeared red upon treatment with a Live/Dead green fluorescent stain mixture to the total number of bacteria in a random microscopic field of view. Curve a represents the fitting to the first-order rate law.

figure, however, the bactericidal efficiency in fact did not exhibit even a hint of such a downward trend and remained essentially unchanged throughout the experiments at the levels of  $\sim 99\%$  against *E. coli* and  $96\%$  for *S. aureus*. Specifically, for both bacteria we treated the percentage of inactivation as a linear function of the assay number (Fig. 5), i.e.,  $y = ax + b$ . Using the raw experimental data, we calculated the slope,  $a$ , to be  $0.010 \pm 0.057$  for *S. aureus* (Fig. 5A) and  $-0.022 \pm 0.034$  for *E. coli* (Fig. 5B). Therefore, in both instances the slope is statistically indistinguishable from zero, thereby confirming the absence of any systematic trend. Thus, the polycation-derivatized glass surface retains the original high bactericidal efficiency, i.e., neither microorganism becomes resistant to this coating.

These results reveal that the rare survivors in each cycle are not a fraction of the bacterial population that developed resistance to the antimicrobial coating. Rather, they likely survive by avoiding direct deadly contact with the immobilized polycation, e.g., by forming aggregates with their brethren prior to spraying or due to the presence of “bald” spots on the derivatized slide surface.

The emergence of bacterial resistance to antibiotics is a major therapeutic threat to public health (Levy, 2002;



**Figure 5.** A series of bactericidal activity assays for amino-glass slides covalently coated with *N*-hexyl,methyl-PEI against airborne *S. aureus* (A) and *E. coli* (B). In each assay, the bacterial population originated from a single colony of surviving bacteria from the preceding assay.

McDonnell and Russell, 1999; Russell, 1999). The observed lack of bacterial resistance against *N*-hexyl,methyl-PEI coatings can be explained by the mechanism of its antimicrobial action elucidated above (see Figs. 1, 2). Most biocides disrupt certain specific metabolic processes in bacteria (Levy, 2002; Russell and Chopra, 1996), and bacteria usually counteract by reducing the biocide uptake, degrading it, modifying the biocide’s target site, or over-producing the target molecules (Lewis, 2001). In contrast, the polycation-derivatized antimicrobial surface apparently acts as nonselective, brute-force “permeabilizers” of bacterial membranes causing their fatal damage, rather than by targeting a specific metabolic process.

### Effect of Antimicrobial Surfaces on Mammalian Cells

Our final goal was to determine whether *N*-hexyl,methyl-PEI surfaces are harmful to mammalian cells. Lethal activity against bacteria, but without concurrent detrimental effect on human and animal cells, is required for clinical applications of antimicrobial surfaces, e.g., for implants and medical devices. Such selective toxicity is also needed for environmental applications of biocidal surfaces against biofouling and biocorrosion (Lappin-Scott and Costerton, 1989; McFeters, 1984).

To address this question, we employed monkey kidney cells as a model. To distinguish between live, metabolically active mammalian cells on the one hand and those that are injured or dead on the other, we used another variant of the two-color Live/Dead cell vitality fluorescence assay (Haughland, 1996). This assay, although conceptually similar to the one employed with bacteria and outlined above, uses different dyes and produces a different (opposite) visual end result. Specifically, it involves two reagents: cell-permeable and nonfluorescent C<sub>12</sub>-resazurin, which undergoes enzymatic reduction to the red-fluorescent C<sub>12</sub>-resorufin, and cell-impermeable and green-fluorescent SYTOX Green, which binds to nucleic acids. Healthy, vital cells with intact cell membrane are able to produce the red-fluorescent C<sub>12</sub>-resorufin and avoid the uptake of SYTOX Green. Conversely, dead cells or those with damaged cell membrane are 1) unable to produce C<sub>12</sub>-resorufin, and 2) accumulate SYTOX Green. As a result, healthy mammalian cells emit red fluorescence, whereas injured or dead cells fluoresce green.

Upon mixing and incubation with the fluorescent stains, 10 µl of a suspension containing monkey kidney cells was applied onto a polycation-derivatized glass slide and their fluorescence was monitored for up to 2 h. The original fraction of vital cells of 95% (628 out of 664 cells in the field of vision) remained constant during this time period. No change of fluorescence from red to green, indicative of the loss of vitality, was observed for any of the monitored cells. All of them remained viable and metabolically active, showing no signs of injury from contact with the immobilized polycation. Both the coating of the mammalian cell and its cytoplasmic membrane differ significantly from the envelopes of bacterial cells, as does the cell size. This may account for the lack of activity of immobilized polycations used in this study against mammalian cells. That bactericidal *N*-hexyl,methyl-PEI surface coating has no appreciable detrimental effect on mammalian cells, if general, bodes well for future applications.

The content of this article does not necessarily reflect the position of the US Government, and no official endorsement should be inferred.

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