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# Practical Aspects of Hydrophobic Polycationic Bactericidal "Paints"

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**Abstract** We previously discovered that coating solid surfaces with long-chained linear *N*-dodecyl,*N*-methyl-polyethylenimine makes them bactericidal and virucidal. In the present study, focusing on the use of this microbicidal paint to kill airborne *Escherichia coli* and *Staphylococcus aureus*, we have systematically investigated the dependence of this effect on the concentration and mode of application of the hydrophobic polycation, the number of coats, the nature of the solvent, and the presence of a dye in such paint. In addition, the latter's ability to be regenerated after use, stability upon repeated washings, and mammalian toxicity has been evaluated.

**Keywords** Polyethylenimine · Antibacterial · Coating · Toxicity · *Escherichia coli* · *Staphylococcus aureus* · Biocidal materials

## Introduction

One way to reduce encounters with potentially harmful microbes is to make objects and things touched by people in the course of everyday activities microbicidal. Conventionally, this is achieved by impregnating materials with biocides which gradually leach out over time [1, 2]. Due to inherent drawbacks of this approach, we have been developing an alternative which entails applying to solid surfaces permanently microbicidal hydrophobic polycationic coatings [3]. Two types of such coatings able to kill on contact diverse microbial pathogens have been created [3]: covalently attached [4] and physically deposited [5].

Coatings of the latter type ("microbicidal paints") are particularly attractive for large-scale uses because they involve simply painting solutions of hydrophobic derivatives of polyethylenimine (PEI), notably long-chained linear *N*-dodecyl,*N*-methyl-PEI in an organic solvent, onto materials [5–7]. Such microbicidally painted surfaces have been found to be

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lethal to pathogenic bacteria by damaging their cellular membranes [6, 7], as well as to both wild-type and drug-resistant strains of human and avian influenza virus [7, 8].

Due to their novelty, however, a number of practical aspects of the aforementioned microbicidal paints have not been characterized yet. The present study begins to fill that void.

## Materials and Methods

### Materials

Poly(2-ethyl-2-oxazoline) ($M_w$ of 500 kDa), 1-bromododecane, iodomethane, *tert*-amyl alcohol, synthetic indigo, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals and solvents were from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Polypropylene and nylon were supplied by Polymer Plastics (Reno, NV, USA). Aircraft wool carpet fabric was provided by Boeing.

Linear *N*-dodecyl,*N*-methyl-polyethylenimine was synthesized as previously described [7]. Briefly, deprotected linear polyethylenimine ($M_w$ of 217 kDa) was obtained by deacylation of poly(2-ethyl-2-oxazoline) [9], followed by dissolution of the resultant protonated PEI in water and precipitation with aqueous KOH. The precipitate was filtered and washed repeatedly with water. This deprotected PEI was alkylated with bromododecane and then with methyl iodide to give *N*-dodecyl,*N*-methyl-PEI. $^1$H NMR in $CDCl_3$ ($\delta$, ppm): 5.5–3.0 [$NCH_2CH_2(CH_2)_9CH_3$, $NCH_2CH_2N$, $NCH_3$], 1.80 [$NCH_2CH_2(CH_2)_9CH_3$], 1.6–1.0 [$NCH_2CH_2(CH_2)_9CH_3$], 0.88 [$NCH_2CH_2(CH_2)_9CH_3$]. When needed, the resultant solid was dissolved in butanol or another organic solvent, typically at 50 mg/ml, and a clear colorless solution thus obtained was used as a "bactericidal paint".

The hydrophobic polycation prepared as outlined above is yellowish. This off-color could be eliminated, if desired, without affecting the bactericidal potency as follows: to a 50-mg/ml solution of *N*-dodecyl,*N*-methyl-PEI in butanol, $NaBH_4$ was added (to its final concentration of 100 mg/ml), followed by a stirring for 1 h at room temperature; the remaining insolubles were filtered off, and the filtrate was concentrated to form a white solid. The latter was further refined by redissolving in chloroform and washing with water, and then the solvent was evaporated again. The de-colorized bactericidal paint was found to be 100% antibacterial at 50 mg/ml, just like its yellowish predecessor.

### Bacteria and Media

The bacteria employed in this work were *Staphylococcus aureus* (ATCC 33807) and *Escherichia coli* (*E. coli* genetic stock center, CGSC4401). Phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) containing glutamine and 10% heat-inactivated fetal bovine serum were from Gibco (Grand Island, NY, USA). The yeast-dextrose broth contained (per liter of deionized water): 10 g of peptone, 8 g of beef extract, 5 g of NaCl, 5 g of glucose, and 3 g of yeast extract. The PBS solution contained 8.2 g of NaCl and 1.2 g of $NaH_2PO_4{\cdot}H_2O$ per liter of deionized water; its pH was adjusted to 7.0 with 1 M aqueous NaOH. Both solutions were autoclaved for 30 min prior to use.

### Preparation of Painted Slides and Carpet Fabric

Commercially available glass slides (VWR microscope 2.5×7.5 cm slides), as well as polypropylene and nylon ones (cut by us to a 2.5×7.5 cm size), were brush-coated with a

solution of 50 mg of *N*-dodecyl,*N*-methyl-PEI in 1 ml of butanol using a cotton swab, followed by air drying. By measuring the weight difference between a painted and a non-painted glass slide and assuming that the density of the dry polymeric paint exceeds 1 g/cm$^3$, we calculated that the average thickness of a paint layer on a glass slide is less than ~1 micron.

The hydrophobic polycation was dissolved not only in butanol but also in other organic solvents. It was also readily soluble in chloroform, dichloromethane, and cyclohexanone upon vortexing for 5–10 min at both 10 and 50 mg/ml. In the case of dimethyformamide, tetrahydrofuran, and dimethylsulfoxide, 10 mg/ml *N*-dodecyl,*N*-methyl-PEI was vortexed and then kept in a water sonication bath for 20 min to obtain a clear solution; however, at 50 mg/ml, it was not fully soluble in these solvents. Various mixtures of *N*-dodecyl,*N*-methyl-PEI with indigo (the total concentration of 100 mg/ml) were dissolved in chloroform.

The airplane wool carpet fabric (5.0×2.5 cm) was immersed in a 50-mg/ml solution of *N*-dodecyl,*N*-methyl-PEI in butanol for 5 min and then air-dried overnight.

## Washing the Coated Carpet Fabric

A laundry wash cycle consisted of stirring the coated carpet fabric in soapy (Soft soap, Colgate-Palmolive) water at 50 °C for 12 h, followed by thorough rinsing with distilled water. The fabric was then left to dry overnight at room temperature.

## Determination of Bactericidal Efficiency

A 100-μl suspension of *S. aureus* or *E. coli* in 0.1 M PBS (approximately $10^{11}$ cells per milliliter) was added to 20 ml of the yeast-dextrose broth in a 50-ml sterile centrifuge tube, followed by shaking at 200 rpm and 37 °C overnight (Innova 4200 Incubator Shaker, New Brunswick Scientific, Edison, NJ, USA). The bacterial cells were harvested by centrifugation at 6,000 rpm for 10 min (Sorvall RC-5B, DuPont Instruments, Wilmington, DE, USA), washed twice with PBS, and diluted to $5\times10^6$ cells per milliliter for *S. aureus* and to $5\times10^7$ cells per milliliter for *E. coli*. These bacterial suspensions were sprayed (each spraying was very brief, lasting about 1 s) onto slides at a rate of approximately 10 ml/min in a fume hood. After a 2-min drying at room temperature under air, the resultant slides were 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 allowed to gel at room temperature overnight). The Petri dish was sealed and incubated at 37 °C overnight, and the bacterial colonies grown on the slide surface were counted on a light box or under a microscope.

## Cytotoxicity Experiments

COS-7 cells (simian virus 40 transformed kidney cells of an African green monkey) were cultured in DMEM containing glutamine supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown at 37 °C in humidified air containing 5% $CO_2$ and passaged every 3–4 days. At 95% confluency, the medium from the flask containing the cells was removed, followed by washing with PBS twice and careful removal of the PBS. The cells were then detached by treatment with 0.25% trypsin-EDTA.

Cytotoxicity of surfaces painted with *N*-dodecyl,*N*-methyl-PEI was evaluated by measuring the metabolic activity of the cells exposed to such surfaces. Briefly, 2 μl of a 50-mg/ml solution of the hydrophobic polycation in butanol was added into wells of a 96-well polystyrene plate. The solvent was allowed to evaporate, and the plate was kept in a tissue culture hood under an ultraviolet light for 3 min. Three sets of coated and uncoated (control) wells were used for each experiment, with 20,000 COS-7 cells in DMEM added to each well. After incubation for 3, 6, 9, or 12 h, the culture medium was removed, and 200 μl of the fresh medium and 50 μl of MTT were added to each well. The 96-well plate was wrapped in an aluminum foil and incubated at 37 °C for 4 h. The liquid was then removed from each well, followed by addition of 200 μl of dimethyl sulfoxide to dissolve the formazan crystals formed from the reduction of MTT by viable cells. After 15 min incubation, the absorbance at 570 nm was measured using a Molecular Devices plate reader.

### Atomic Force Microscopy

A Nanoscope IV/Dimension D3000 Scanning Probe Microscope (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA, USA) used in a tapping mode was employed for all atomic force microscopy (AFM) experiments [10, 11]. Images were obtained in air at room temperature and humidity. New clean phosphorus-doped silicon cantilevers with a nominal spring constant of 40 N/m, a nominal tip radius of <10 nm, and front and back angles of 15° were used. The drive frequency for tapping mode was set near the resonance frequency of the cantilevers (nominally ~300 kHz). The images were obtained by tapping the cantilever tip across the sample surface (of uncoated or coated glass slides), while keeping the oscillation amplitude fixed at the specified set point. To ensure proper tracing, the integral and proportional gains of the feedback loop [11] were slightly adjusted in each case to improve the trace and retrace. When increasing these gains, visual inspection of the trace and retrace lines was crucial to prevent introducing high-frequency noise to the system. Typical values for these gains were in the 0.20–0.35 range. When small increases in the integral and proportional gain induced no change, the amplitude set point was slightly decreased to improve surface tracking, while being careful to not exert unnecessary tapping forces on the sample. Adjustments were made with each sample until their trace and retrace lines looked similar. Images were recorded in a 5×5 μm scan size window at a scan rate of 1 Hz and a resolution of 256×256 pixels. The Veeco Nanoscope software (version 5.30r3sr3) was used to obtain the three-dimensional image representations and quantitative measurements of topological features.

## Results and Discussion

The overall experimental design employed herein, detailed in "Materials and Methods", was similar to that as in our previous studies [3, 5]. Namely, an aqueous solution containing a representative human pathogenic bacterium, *E. coli* (Gram negative) or *S. aureus* (Gram positive), was sprayed onto a solid surface of a glass slide (unless stated otherwise)—either uncoated (control) or painted with *N*-dodecyl,*N*-methyl-PEI—to simulate landing of airborne bacteria generated by coughing or sneezing. The cells remaining viable (i.e., able to multiply), following a 2-min air drying and an overnight incubation under growth agar, were quantified by counting the bacterial colonies formed. The bactericidal efficiency of our hydrophobic polycationic coating was calculated as the number of colonies per square centimeter on the coated material divided by that in the control (done side by side), times 100%.

In our previous studies [5–8], the microbicidal paint always comprised a 50-mg/ml (i.e., 5% *w*/*v*) solution of *N*-dodecyl,*N*-methyl-PEI in butanol. Herein, we examined the dependence of the bactericidal activity on both the polycation concentration and the nature of the organic solvent.

Inspection of Fig. 1 reveals that the concentration of *N*-dodecyl,*N*-methyl-PEI in butanol can be at least halved from 50 mg/ml before the bactericidal activity—toward either *E. coli* or *S. aureus*—of glass slides painted with this solution starts appreciably decreasing from essentially a 100% level. Moreover, a 10-mg/ml hydrophobic polycation solution affords a 86–87% bactericidal activity, and even for a 2-mg/ml solution it is still around 50%. Thus, the concentration of the microbicidal paint can be drastically cut without sacrificing most of its bactericidal activity.

Why is the bactericidal activity lower for dilute paints? A plausible explanation is that they leave microscopic "bald spots" (i.e., gaps or voids) on the glass surface where the bacterial cells can survive. We tested this hypothesis experimentally. First, glass slides were repeatedly coated with a dilute, 4 mg/ml, *N*-dodecyl,*N*-methyl-PEI solution in butanol. As seen in Fig. 2a, the activity against both bacteria indeed jumps from around 60% for a single coat of the microbicidal paint to 96–98% for two dozen coats. The relatively small increase per coat may be in part due to our admittedly imperfect paint application technique with a cotton swab. Therefore, in the second set of experiments, we instead applied successive paint coats by repeatedly immersing glass slides in a 4-mg/ml *N*-dodecyl,*N*-methyl-PEI solution in butanol. Roughly the same jump in bactericidal activity in this case was attained at 16 coats (Fig. 2b). Third, since adhesion of the microbicidal paint may depend on the nature of the treated surface, we repeatedly painted nylon and polypropylene, instead of glass (Fig. 2a), slides. As seen in Fig. 2c, qualitatively similar data were obtained. All these results (Figs. 2a–c) support the hypothesis that a dilute paint does not completely cover the treated surface, thus reducing the observed bactericidal efficiencies.

These views were independently verified when we imaged uncoated and various coated glass slides by means of AFM. As seen in Fig. 3a, there are no large gaps on the surface of uncoated (control) slides. Upon painting with a 50-mg/ml *N*-dodecyl,*N*-methyl-PEI solution in butanol (resulting in essentially a 100% bactericidal activity, see Fig. 1), the surface becomes rougher (Fig. 3b) but, importantly, no gaps are observed comparable in size with bacterial cells, i.e., ~1 μm. In contrast, when a glass slide is painted with a 2-mg/ml hydrophobic polycation solution (resulting in only some 50% bactericidal activity, see

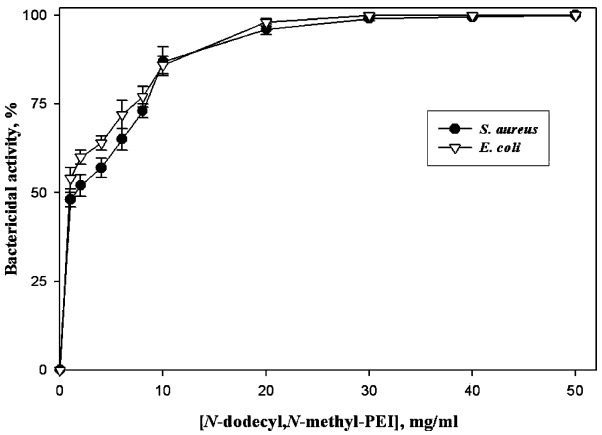


**Fig. 1** Effect of the concentration of *N*-dodecyl,*N*-methyl-PEI in butanol on the bactericidal activity against airborne *S. aureus* and *E. coli* of glass slides painted with this solution. See "Materials and Methods" and text for further experimental details

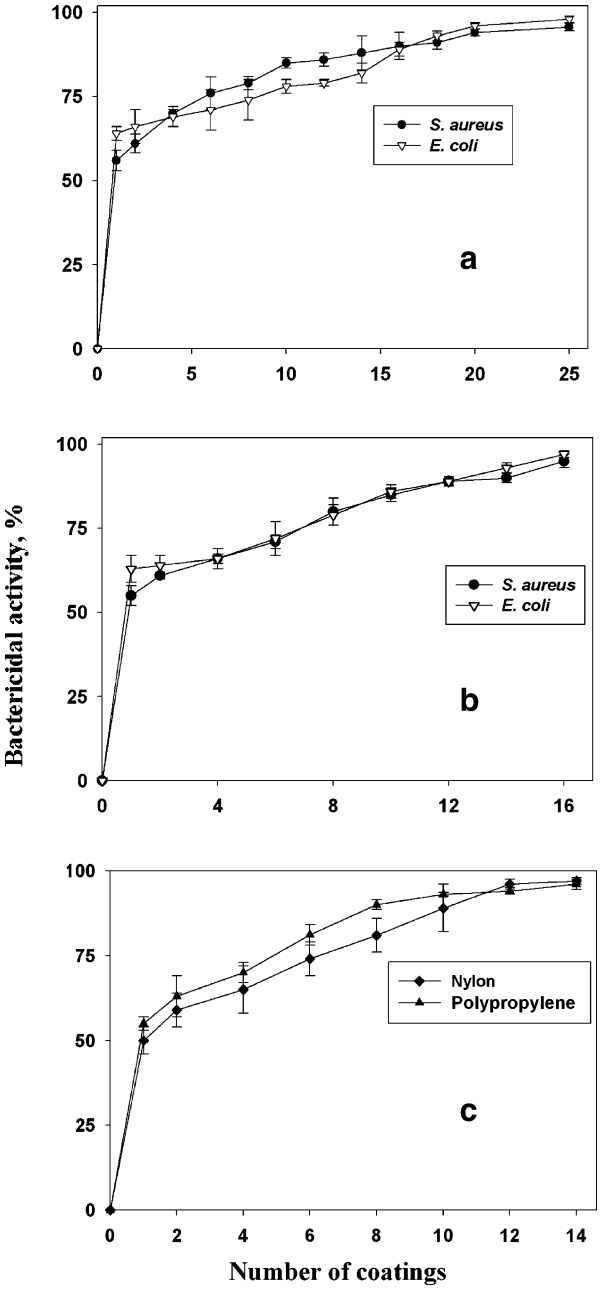


**Fig. 2** Dependence of the bactericidal activity against airborne *S. aureus* and *E. coli* of glass (**a** and **b**), as well as of nylon and polypropylene (against airborne *S. aureus*, **c**), slides coated by with a 4-mg/ml solution of *N*-dodecyl,*N*-methyl-PEI in butanol on the number of times this coating was successively applied. The coating was carried out by painting either with a cotton swab (**a** and **c**) or by dipping (**b**). See "Materials and Methods" and text for further experimental details

Fig. 1), the resultant surface (Fig. 3c) has multiple gaps or "craters" of over 1 μm in diameter, i.e., large enough to situate a bacterial cell with little direct contact with the bactericidal paint. However, repeated coating with a 2-mg/ml *N*-dodecyl,*N*-methyl-PEI solution eliminates these craters (and also boosts bactericidal activity)—as one can see in Fig. 3d, a smooth surface results after painting with this dilute hydrophobic polycation 15

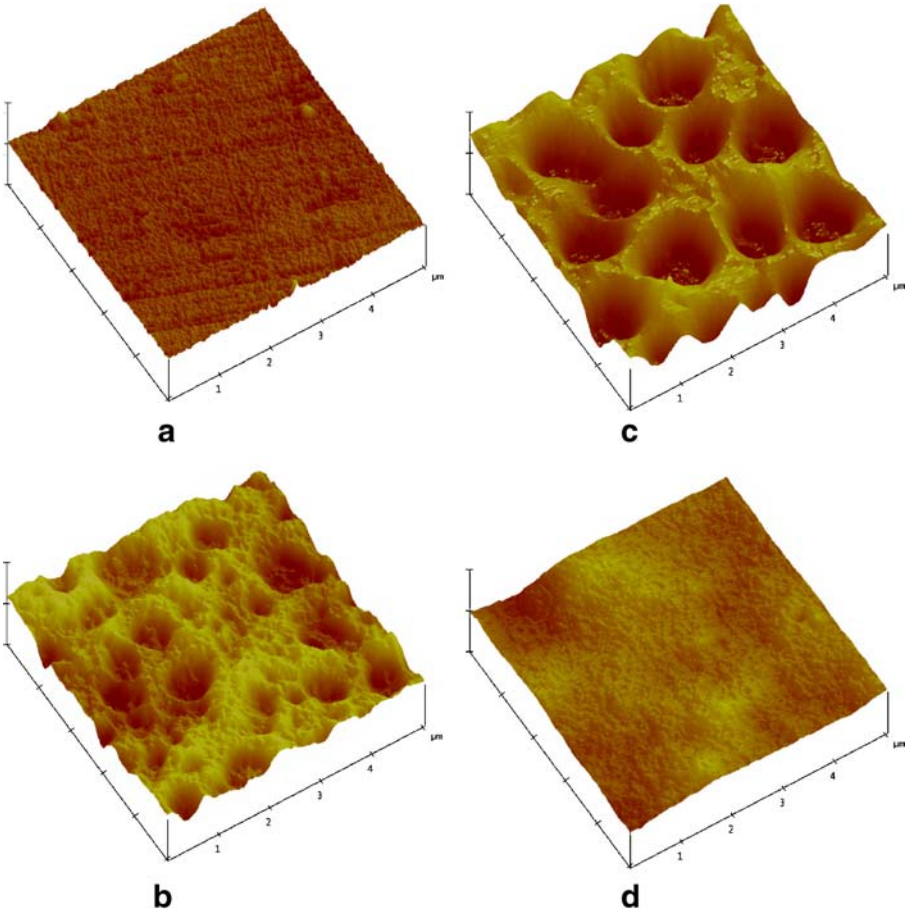


**Fig. 3** Atomic force microscopy images of an **a** uncoated glass slide surface; **b** glass slide surface coated once with a 50-mg/ml *N*-dodecyl,*N*-methyl-PEI solution in butanol; **c** glass slide surface coated once with a 2-mg/ml *N*-dodecyl,*N*-methyl-PEI solution in butanol; and **d** glass slide surface coated 15 times with a 2-mg/ml *N*-dodecyl,*N*-methyl-PEI solution in butanol (one has to expect a similar, smooth surface topology with a 4-mg/ml hydrophobic polycation solution employed for repeated coating in Fig. 2a). In every case (**a**–**d**), five different 5×5 μm regions were imaged, and very similar pictures were observed within each case. See "Materials and Methods" and text for further experimental details

times. These AFM observations are consistent with the bactericidal efficiency data depicted in Figs. 1 and 2.

The foregoing conclusions were further supported by experiments with a different, unrelated to glass material, namely commercial wool carpets used. When a piece of such a carpet was immersed in a 50-mg/ml *N*-dodecyl,*N*-methyl-PEI in butanol once and the solvent was allowed to evaporate, the resultant activity against airborne *S. aureus* was approximately 85%. When this painting procedure was repeated, however, the bactericidal activity jumped to some 98% (also see below).

Next, we examined whether the nature of the organic solvent in which *N*-dodecyl,*N*-methyl-PEI is dissolved (note that it is essentially insoluble in water, which is critical for its non-leaching mechanism of action) [6] affects the activity against *S. aureus* of the painted glass slide surface. To this end, we deliberately used a sub-optimal, 10 mg/ml concentration

**Table 1** Bactericidal activity against airborne *S. aureus* of glass slides painted with a 10-mg/ml solution of *N*-dodecyl,*N*-methyl-PEI in different organic solvents.[a]

| Solvent | Bactericidal activity (%) |
|---|---|
| Butanol | 88±2 |
| Cyclohexanone | 79±3 |
| Tetrahydrofuran | 80±2 |
| Dimethylsulfoxide | 85±2 |
| Dimethylformamide | 75±6 |
| Dichloromethane | 80±4 |
| Chloroform | 86±1 |

[a] See "Materials and Methods" and text for further experimental conditions.

of this hydrophobic polycation to magnify the putative difference among the seven solvents tested. The results presented in Table 1 show that the solvent plays only a minor role, with the bactericidal efficiency always varying in roughly a 75–90% range, perhaps by slightly influencing how snugly the paint adheres to the surface and hence the number/size of the putative bold spots.

Since *N*-dodecyl,*N*-methyl-PEI is yellowish (and, if desired, this yellowish off-color can be eliminated by a $NaBH_4$ treatment without losing antibacterial activity, see "Materials and Methods"), one might want to have a dye present in the microbicidal paint to expand its functionality. To explore this possibility, we co-dissolved in chloroform (one of the Table 1 solvents) both the hydrophobic polycation and the blue pigment indigo in different ratios, with their combined concentration always totaling 100 mg/ml. One can see in Fig. 4 that adding indigo to *N*-dodecyl,*N*-methyl-PEI in a 1:1 (*w*/*w*) ratio has little, if any, detrimental effect on the ability of the painted glass surface to kill airborne *S. aureus*. However, at higher proportions of the dye, the bactericidal activity decreases, presumably because indigo competes with the hydrophobic polycation for the available slide surface space. Nevertheless, the data in Fig. 4 show that coating with such binary solutions can provide a dual purpose of bactericidal action and coloring.

One would expect that, as more bacterial cells land and die on a painted surface, the bactericidal activity of the painted surface should decline because the killed bacteria should shield their later-to-arrive brethren from the damage inflicted by the hydrophobic polycation. Indeed, it is seen in Fig. 5 that, while the first batch of *S. aureus* sprayed onto the wool carpeting repeatedly (thrice) immersed in a 50-mg/ml *N*-dodecyl,*N*-methyl-PEI is killed with a 98±1% efficiency, for the next batch, the bactericidal efficiency declines to 87±3% and for the third and fourth batches to 72±2% and 58±7%, respectively.

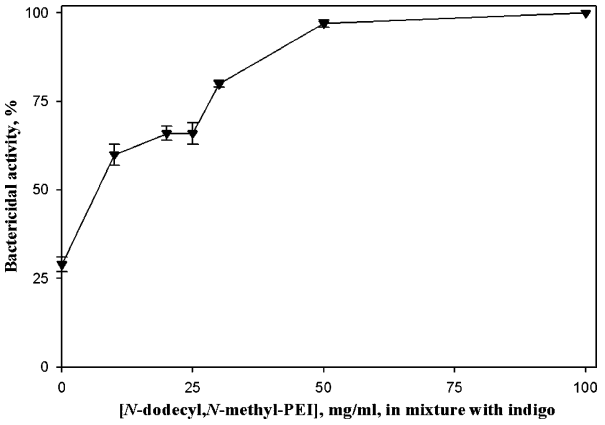


**Fig. 4** Bactericidal activity against airborne *S. aureus* of glass slides painted with various mixtures of *N*-dodecyl,*N*-methyl-PEI with the blue dye indigo in chloroform, with the combined concentration of the two being 100 mg/ml. See "Materials and Methods" and text for further experimental details

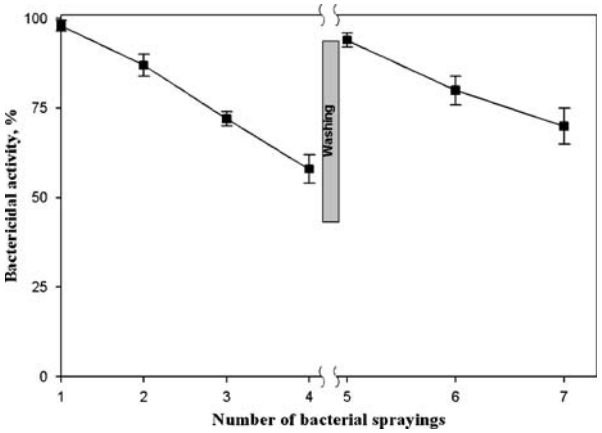


**Fig. 5** Rejuvenation by washing of a wool carpet fabric coated with *N*-dodecyl,*N*-methyl-PEI after repeatedly spraying it with *S. aureus* in water. See "Materials and Methods" and text for experimental details

Gratifyingly, however, when the carpet sprayed with bacteria four times was subsequently washed in soapy water, followed by rinsing, the bactericidal activity returned to almost the original level (Fig. 5). Thus, although the surface of a microbicidally painted carpet becomes partially blocked by the accumulating dead bacteria, it can be readily rejuvenated by a simple act of washing.

We then tested the durability of a painted fabric with respect to such repeated washings with soap. To this end, the same wool carpeting as mentioned in the preceding paragraph, except immersed in the microbicidal paint only once to make the subsequent effects more noticeable, was subjected to seven consecutive thorough laundry wash cycles. One can see in Table 2 that the washing reduces the bactericidal activity only slightly—on average about 2% per laundry cycle.

Finally, we addressed the important problem of safety of our microbicidal paints by gauging their mammalian toxicity in the following experiments. Some wells of a commercial polystyrene 96-well plate were coated with *N*-dodecyl,*N*-methyl-PEI, while others, used as controls, were left uncoated. To each well, 20,000 African green monkey kidney cells (COS-7) in a DMEM medium containing glutamine supplemented with heat-inactivated FBS, sodium pyruvate, and antibiotics were added, followed by a 3-h incubation at 37 °C in a humidified $CO_2$-enriched air, aspirating the liquid out of three painted and three control wells, and replacing it with the fresh medium containing MTT to assess the level of metabolic activity of the cells adhered to the wells' walls [12]. After a subsequent

**Table 2** Effect of repeated washings with warm soapy water on the bactericidal activity against *S. aureus* of an aircraft wool carpet fabric coated with *N*-dodecyl,*N*-methyl-PEI.[a]

| Number of washing cycles | Bactericidal activity (%) |
|---|---|
| 0 | 85±1 |
| 1 | 83±2 |
| 2 | 82±3 |
| 3 | 79±2 |
| 4 | 77±3 |
| 7 | 70±2 |

[a] Coating was carried out by immersing the fabric once in a 50-mg/ml solution of *N*- dodecyl,*N*-methyl-PEI in butanol. Each washing cycle consisted of a vigorous agitation in soapy water at 50 °C for 12 h, followed by thorough rinsing with distilled water. See "Materials and Methods" and text for further experimental details.

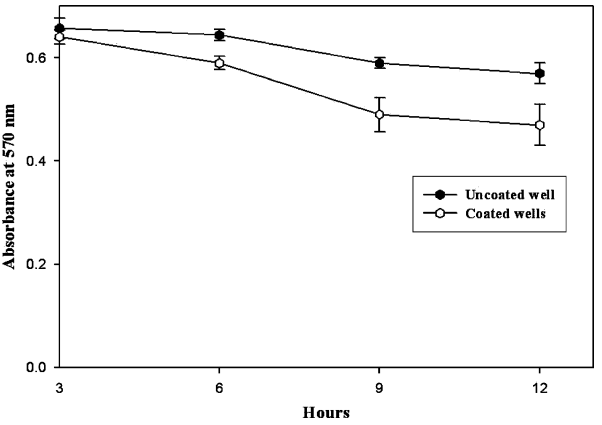


**Fig. 6** Relative level of metabolic activity of African green monkey kidney (COS-7) cells as a function of time of their incubation while adhered to the well walls, either uncoated (control) or coated with *N*-dodecyl,*N*-methyl-PEI, of a 96-well polystyrene plate. The metabolic activity was assessed by the MTT assay. See "Materials and Methods" and text for further experimental details

4-h incubation, the liquid was pipetted out again, replaced with dimethylsulfoxide to dissolve the formazan crystals formed, and the absorbance of the resultant purple solution was measured at 570 nm. The same procedure was separately carried out with additional pairs of painted and control cell-containing wells after 6, 9, and 12 h. The results obtained, depicted in Fig. 6, show that the absorbance (reflecting the cell viability [13]) moderately decreases for both control and painted wells, with the decline for the latter wells being somewhat greater. We conclude, therefore, that even uncoated polystyrene surface is slightly toxic to the mammalian cells, and the painting makes it a little more so. While these observations may be encouraging, a thorough evaluation of *N*-dodecyl,*N*-methyl-PEI's toxicity, both acute and chronic, would need to be conducted prior its practical use.

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