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## Author Manuscript

*Biotechnol Bioeng.* Author manuscript; available in PMC 2012 March 1.

Published in final edited form as:

*Biotechnol Bioeng.* 2011 March ; 108(3): 720–723. doi:10.1002/bit.22967.

## Hydrophobic Polycationic Coatings Disinfect Poliovirus and Rotavirus Solutions

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### Abstract

Coating surfaces with *N*-alkylated polyethylenimines (PEIs), namely branched *N,N*-hexyl,methyl-PEI via covalent attachment to glass or linear *N,N*-dodecyl,methyl-PEI by physical deposition (“painting”) onto polyethylene, enables the resultant materials to quickly and efficiently disinfect aqueous solutions of (non-enveloped) poliovirus and rotavirus.

### Keywords

viruses; polyethylenimine; microbicidal; antiviral surfaces; quaternary ammonium compounds; surface derivatization

More than half a century after the groundbreaking poliovirus vaccine was developed, the virus remains a public health threat in many African and South Asian countries (Minor, 2004). Poliovirus, like other enteric waterborne viruses, mainly stems from contaminated drinking sources in areas of poor sanitation. Once infected, a person can develop poliomyelitis, a debilitating disease causing muscle atrophy and paralysis (Bosch, 1998). Rotavirus, another waterborne human pathogen, also poses a danger in the developing world. It is the chief cause of gastroenteritis and diarrhea in children, causing some 600,000 infant deaths worldwide annually (Parashar et al., 2006). Therefore, stopping transmission of these viruses from potable water supplies would reduce the spread of disease.

Certain hydrophobic polycations, such as some polyethylenimines (PEIs), have been found to confer antimicrobial properties to materials' surfaces (Klibanov, 2007). For example, *N,N*-hexyl,methyl-PEI covalently attached, and *N,N*-dodecyl,methyl-PEI deposited, onto glass have been shown to kill human pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli*, as well as to inactivate multiple influenza virus strains, including drug-resistant ones

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(Haldar et al., 2006, 2007, 2008). The lipid membranes of bacteria or the envelopes of viruses are damaged by the hydrophobic polycationic chains, thus causing death or inactivation of the bacterium or virus, respectively (Klibanov, 2007). Since the foregoing mechanism applies only to viruses containing a lipid envelope, it has been unknown heretofore whether immobilized hydrophobic polycations also can disinfect *non-enveloped*, protein-coated viruses, such as the aforementioned poliovirus and rotavirus (Bosch, 1998). The present study provides an affirmative answer to this question.

To explore the activity of hydrophobic polycationic coatings against poliovirus, a 10  $\mu\text{L}$  droplet of viral solution was sandwiched between a polyethylene slide painted with linear *N,N*-dodecyl,methyl-PEI and an otherwise identical bare (untreated) slide. After a 30 min incubation at room temperature, the slides were separated and washed to recover the viral particles. The washings were twofold serially diluted, and 200  $\mu\text{L}$  of each dilution was used to infect monolayers of HeLa cells in a plaque assay. Following the initial infection period, the viral dilutions were aspirated off the cells and replaced with a nutrient-rich agar solution. Following a 60 h incubation at 37°C to promote the formation of plaques the cells were fixed with formaldehyde, stained with crystal violet dye after the agar overlay was removed, and allowed to dry overnight for plaques to be counted.

Essentially no poliovirus plaques could be detected after such an incubation in contact with a slide painted with *N,N*-dodecyl,methyl-PEI, as compared to a 100% infectivity recovery from a control in which viruses not in contact with any slides were incubated for 30 min under the same conditions (control #1; Fig. 1). To ascertain whether contact with an uncoated polyethylene surface plays a role in the reduction of viral titer observed, an additional control (#2) was carried out in which both slides sandwiching the viruses were bare. No significant decrease in the viral titer was observed for this condition, indicating that the surface-deposited hydrophobic polycations are responsible for disinfecting poliovirus (Fig. 1).

Covalent attachment of hydrophobic polycations to surfaces provides a more robust coating than merely physical deposition by painting. Therefore, to test the generality of the foregoing findings, in addition to painted polyethylene slides, glass slides were covalently modified with branched *N,N*-hexyl,methyl-PEI and tested against poliovirus in the same fashion. Washings from the derivatized glass slides that contacted poliovirus produced no plaques (Fig. 1), indicating high disinfecting potency. In the case of control #2 (bare slides), there is approximately a twofold reduction in recovered infectious viral particles, probably due to a nonspecific adsorption of the virus to the glass surface.

Separately, we found that the covalently coated glass slides that had been used to disinfect polioviruses could be fully regenerated and successfully re-used after a UV irradiation and ultrasonication in methanol.

To shed light on whether the immobilized hydrophobic polycations remove poliovirus from solution by adsorbing it or, alternatively, damage the virus to make it non-infective, we tested the viability of the virus in the presence of the common quaternary ammonium detergent dodecyltrimethylammonium chloride (DDTMAC). Incubating poliovirus for 30 min at room temperature in plain PBS or in the presence of 0.095 M DDTMAC yielded indistinguishable infectivities  $(1.5 \pm 0.37) \times 10^4$  pfu/mL and  $(1.9 \pm 0.23) \times 10^4$  pfu/mL, respectively. The dissolved detergent structurally mimicking our hydrophobic polycations present at a concentration far above DDTMAC's critical micelle concentration (cmc) value of 22 mM (Mehta et al., 2005) fails to adversely affect poliovirus under the same conditions at which immobilized *N,N*-dodecyl,methyl-PEI and *N,N*-hexyl,methyl-PEI completely disinfect it (Fig. 1). This observation points to the coated surface adherence mechanism.

Next, to determine whether our hydrophobic polycationic coatings are effective against other non-enveloped viruses, rotavirus was incubated between a polyethylene slide coated with linear *N,N*-dodecyl,methyl-PEI and its bare counterpart. After a 15 min incubation between the slides, only a single infectious virion could be recovered even from the most concentrated washings (as compared to approximately 100 plaques in control #1 of the same concentration), and none was observed after a 30 min incubation (Fig. 2). In contrast, in the bare sandwiched slide incubation (control #2), only a minimal reduction compared to no slide contact was detected which grew with time (Fig. 2), pointing to sensitivity of the employed strain of rotavirus to contact with surfaces.

The findings reported herein demonstrate for the first time that hydrophobic polycationic coatings can disinfect not only aqueous solutions of enveloped viruses but also those of their protein-coated, non-enveloped counterparts, namely poliovirus and rotavirus.

## Materials and Methods

### Chemicals

Branched PEI (750 kDa), DDTMAC, and all organic solvents and reagents for synthesis were from Sigma–Aldrich Chemical Co. (St. Louis, MO). Linear PEI (217 kDa) was synthesized as described by Thomas and Klibanov (2005). Glass slides were from VWR International (West Chester, PA) and polyethylene sheets from McMaster-Carr (Elmhurst, IL); both were cut into 2.5 cm × 2.5 cm squares. Linear *N,N*-dodecyl,methyl-PEI was synthesized as described by Haldar et al. (2006); the degree of dodecylation in this procedure was determined to be approximately 1.6 mEq by elemental analysis. Branched immobilized *N,N*-hexyl,methyl-PEI was synthesized as described by Lin et al. (2002) after derivatizing plain glass slides with 3-aminopropyltriethoxysilane (Tiller et al., 2002).

### Cells and Viruses

HeLa cells and MA-104 cells (CCL-2 and CRL-2378.1, respectively), were obtained from ATCC (Manassas, VA). Cells were grown at 37°C in a humidified-air atmosphere (5% CO<sub>2</sub>/95% air) in Eagle's minimum essential medium (EMEM, ATCC #30-2003) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 µg/mL streptomycin. Poliovirus strain Chat and rotavirus strain Wa were also obtained from ATCC (VR-1562 and VR-2018, respectively), Manassas, VA. The viruses were stored at –80°C and diluted 20-fold and 2,000-fold in incomplete media for assays with polyethylene and glass slides, respectively.

### Slide Preparation

Polyethylene slides were painted with a 50 mg/mL solution of *N,N*-dodecyl,methyl-PEI in chloroform in triplicate to maximize the slide's coverage (Haldar et al., 2006); this procedure was found to deposit 2.15 mg of the hydrophobic polycation per 2.5 cm × 2.5 cm slide painted on one side. Glass slides covalently derivatized with branched *N,N*-hexyl,methyl-PEI were thoroughly washed with distilled water prior to use (Lin et al., 2002).

### Virucidal Assays

A covalently modified glass slide or painted polyethylene slide was placed treated side up in a polystyrene Petri dish (6.0 cm × 1.5 cm), and 10 µL of a virus solution in incomplete EMEM [(2.2 ± 0.9) × 10<sup>4</sup> pfu/mL of poliovirus in a coated polyethylene slide assay, (1.0 ± 0.5) × 10<sup>3</sup> pfu/mL of poliovirus in a covalently modified slide assay, and (5.1 ± 0.8) × 10<sup>3</sup> pfu/mL of rotavirus in a coated polyethylene slide assay] was placed in the center of the slide. The droplet was sandwiched by a bare slide of the same material as the first. In the case of poliovirus challenged by a coated polyethylene slide, a 1 lb weight was placed on top

of the sandwiched slides to ensure complete spreading of the 10  $\mu\text{L}$  droplet. After a 30 min incubation at room temperature, the sandwiched slides were separated with tweezers and the sides contacting virus were washed thoroughly with 990  $\mu\text{L}$  of incomplete EMEM (Haldar et al., 2006, 2008). Washings were used to assay for infectious viral particles recovered post-incubation with slide surfaces. For poliovirus plaque assays, the washings were twofold serially diluted six times. In the case of rotavirus, 500  $\mu\text{L}$  of the undiluted washings were treated with 500  $\mu\text{L}$  of a 10  $\mu\text{g}/\text{mL}$  solution of porcine trypsin (Sigma–Aldrich) at 37°C for 1 h. Trypsin-treated washings were subsequently twofold serially diluted five times and used in the plaque assay.

In the case of DDTMAC, poliovirus was incubated in 10  $\mu\text{L}$  of a solution of 0.095 M detergent for 30 min. After the incubation, virus and detergent were diluted in 990  $\mu\text{L}$  of EMEM and subsequently diluted further for the plaque assay.

### Poliovirus Plaque Assay

Monolayers of HeLa cells in six-well plates were washed twice with 12 mL of PBS and infected with 200  $\mu\text{L}$  of the twofold serially diluted washings in each well. After 1 h of infection at room temperature, virus solutions were aspirated from the cells and replaced by 3 mL of nutrient-rich agar (incomplete EMEM supplemented with 2% heat-inactivated FBS, 100 units/mL penicillin G, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 0.45% Seakem LE Agarose from Lonza (Walkersville, MD). Plates were then placed in a 37°C incubator with a humidified-air atmosphere (5%  $\text{CO}_2/95\%$  air). Sixty hours post-infection, cells were fixed with 17.5% formaldehyde in distilled  $\text{H}_2\text{O}$  for at least 20 min and then stained with 0.5% crystal violet dye for 1 min. Plates were washed with distilled  $\text{H}_2\text{O}$  to remove excess dye, and plaques were counted the next day (Boone and Albrecht, 1983; Nuanualsuwan and Cliver, 2002).

### Rotavirus Plaque Assay

The rotavirus plaque assay was performed in a similar fashion to poliovirus with minor deviations. Monolayers of MA-104 cells were washed twice with 12 mL of incomplete EMEM lacking phenol red dye supplied by Quality Biological (Gaithersburg, MD) supplemented with 4 mM L-glutamine, 100 units/mL penicillin G, and 100  $\mu\text{g}/\text{mL}$  streptomycin. Cells were infected with 200  $\mu\text{L}$  of the twofold serially diluted washings in each well for 1 h at 37°C. After infection, virus solutions were aspirated, and 4 mL of nutrient-rich agar was placed on top of cells [incomplete EMEM supplemented with 0.01% DEAE–dextran, 100 units/mL penicillin G, 100  $\mu\text{g}/\text{mL}$  streptomycin, 0.5  $\mu\text{g}/\text{mL}$  porcine trypsin, and 0.6% agar (purified agar, L28; Oxoid Co., Hampshire, UK)]. Three to 4 days post-infection, cells were fixed and stained with crystal violet dye as described above (Arnold et al., 2009).

### Acknowledgments

This work was financially supported by the US Army through the Institute of Soldier Nanotechnologies at the Massachusetts Institute of Technology under contract DAAD-19-02-D0002 with the Army Research Office and by Tata Chemicals, Ltd.

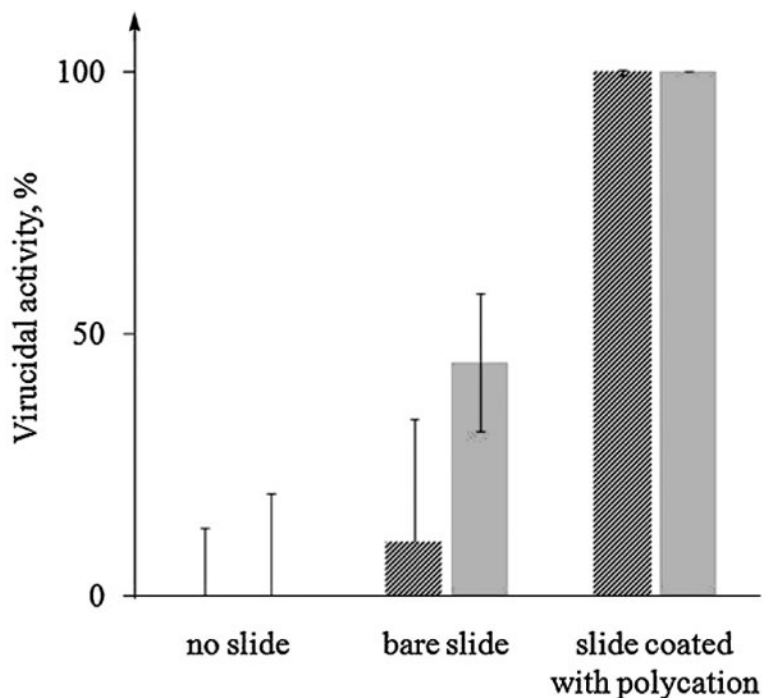
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Contract grant number: DAAD-19-02-D0002

Contract grant sponsor: Tata Chemicals, Ltd

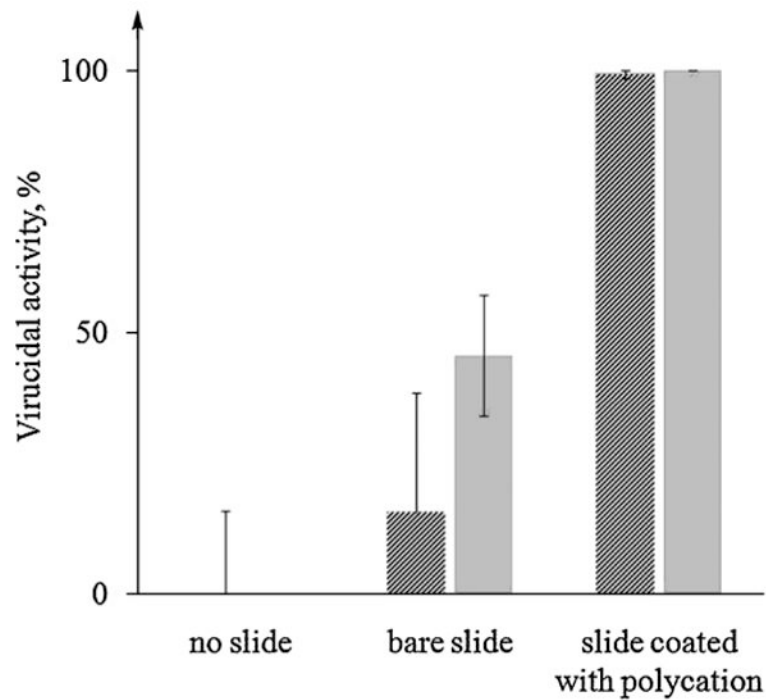
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**Figure 1.**

The virucidal activity of polyethylene slides painted with *N,N*-dodecyl,methyl-PEI and glass slides covalently derivatized with *N,N*-hexyl,methyl-PEI against poliovirus. In each set of two bars, the left one corresponds to polyethylene slides, whereas the right one to glass slides. “No slide” refers to a 30 min incubation at room temperature of poliovirus in aqueous solution and serves as a benchmark for 0% virucidal activity. “Bare slide” shows poliovirus incubated between two untreated polyethylene slides or two underivatized glass slides. “Slide coated with polycation” refers to coatings with *N,N*-dodecyl,methyl-PEI and *N,N*-hexyl,methyl-PEI and shows approximately 100% virucidal activity in the case of incubation with a painted polyethylene slide and 100% virucidal activity in the case of incubation with a covalently modified glass slide. Each experiment was carried out at least in triplicate. For other conditions, see Materials and Methods Section.



**Figure 2.**

The time course of antiviral activity of polyethylene painted with *N,N*-dodecyl,methyl PEI against rotavirus. In each set of two bars, the left one corresponds to a 15 min incubation, whereas the right one a to 30 min incubation between slides. “No slide” depicts a 30 min incubation at room temperature of rotavirus in aqueous solution, and serves as a benchmark for 0% virucidal activity (control #1). “Bare slide” shows rotavirus incubated between two untreated polyethylene slides (control #2). For treated conditions, after 15 min, nearly 100% reduction in infectious particles recovered is shown. After 30 min, no infectious viral particles could be recovered. Each experiment was carried out at least in duplicate. For other conditions, see Materials and Methods Section.