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Drastically lowering the titer of waterborne bacteriophage PRD1 by exposure to immobilized hydrophobic polycations

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Abstract

Decrease in the titer of bacteriophage PRD1 (a model of animal adenoviruses) in aqueous solutions caused by the presence of systematically chemically derivatized surfaces was kinetically investigated. The greatest loss of infectivity – up to a 4-log reduction in the titer – was observed with immobilized hydrophobic polyethylenimine-based and dendrimer-based polycations.

Introduction

Detoxification of waterborne viruses is of obvious importance for controlling the spread of infectious diseases and for combating bioterrorism. Despite the plethora of approaches to this problem, effective and economical methods, especially those applicable to a large-scale purification of drinking water, are in short supply (Faust & Aly 1998).

Due to severe regulatory restrictions and difficulties of working with pathogenic animal viruses, bacteriophages are routinely used as safe and robust models thereof (Grabow 2001). Bacteriophage PRD1 resembles human adenoviruses causing a number of major illnesses, including gastroenteritis, cystitis, meningitis, and pneumonia (Crabtree *et al.* 1997, Straussberg *et al.* 2001). Although PRD1 infects Gram-negative bacteria while adenoviruses infect vertebrate animals, both are icosohedral viruses containing double-stranded DNA whose coat proteins are quite similar in terms of their folds and coating arrangements, as are their capsids and spikes (Bamford *et al.* 2002).

Removing viruses from water by adsorption is appealing provided that adsorbents with high affinities and capacities are available. Although numerous studies have been carried out concerning adsorption of bacteriophage PRD1 onto different surfaces (Bales *et al.* 1991, Pieper *et al.* 1997, Dowd *et al.* 1998, Ryan *et al.* 1999, 2002, Schijven *et al.* 2002, Harvey & Ryan 2004), the dependence of this phenomenon on the nature of the surface remains unclear, as are rational ways to enhance it. In the present work, we have undertaken a systematic mechanistic investigation of the extent of this adsorption on various surfaces, yielding those that can lower the titer of the bacteriophage in aqueous solution by up to four orders of magnitude.

Materials and methods

Materials

Bacteriophages PRD1 and P1 were obtained and used as described below. Microscope plain glass, as well as amino-glass, 2.5×7.5 cm slides were purchased from Sigma Chemical Co. Polystyrene slides, 2.5×3 cm, were cut out from the bottom of a Petri dish (VWR Corp). Polyethylenimine (PEI) (M_W of 25 kDa), glass powder (mean particle diameter of 9–15 μm), polypropylenimine

dotriacontaamine dendrimer (generation 4.0) (henceforth termed 'dendrimer'), γ-aminopropyltriethoxysilane, 4-bromobutyryl chloride, *N*,*N*-dimethylhexadecylamine, and other chemicals and solvents were purchased from Aldrich Chemical Co. and used without further purification.

Glass slide derivatization

To introduce free carboxyl groups onto the surface, amino-glass slides were acylated [similar to the procedure of Lin *et al.* (2003)] for 5 h with 0.2 M succinic anhydride in chloroform at room temperature with shaking, followed by washing with chloroform and methanol. To produce neutral surfaces of varying hydrophobicities, amino-glass slides were acylated with acetyl and palmitoyl chlorides the same way as with succinylation.

To immobilize PEI, an amino-glass slide was acylated with 4-bromobutyryl chloride, followed by derivatization with PEI, as described previously (Lin *et al.* 2003), except that the concentrations of both reagents were cut by half. Dendrimer was immobilized in the same manner.

Three types of glass slide surfaces that are both positively charged and hydrophobic were prepared. In all, amino-glass slides were initially acylated with 4-bromobutyryl chloride (Lin *et al.* 2003). For the first type, this bromoalkylated slide was derivatized with *N*,*N*-dimethylhexadecylamine using the procedure similar to that of Chen *et al.* (2000), except that the 0.4 M amine was used. The same method was applied to make hydrophobic derivatives of the immobilized PEI and dendrimer prepared above.

Glass powder derivatization

Glass powder was aminated by heating at 90 °C in 10% (v/v) γ-aminopropyltriethoxysilane in dry toluene for 5 h similarly to a literature procedure (Bisse *et al.* 1982). Hexadecylated PEI and dendrimer were subsequently immobilized onto the powder the same way as described above for the slides.

Surface characterization

The slide surface density of immobilized PEI and dendrimer was determined as follows. First, bromoalkyl moieties of the acylated slide (Lin *et al.* 2003) were converted into thiols by shaking overnight with 0.1 M sodium hydrosulfide in dimethylformamide at room temperature (Ichinary *et al.* 1988). The resultant immobilized thiols were titrated spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman 1959). Then the identical protocol was applied to the slide-immobilized polycations. The difference between the results of the two titrations, found to be 0.17 nmol cm^{-2} in both instances, was assumed to correspond to the surface density of immobilized PEI and dendrimer.

Growth and quantification of bacteriophages

All experiments with bacteriophages were performed in 0.1 M Tris/HCl buffer containing 10 mM $MgCl_2$, pH 7.4, referred to in this section as 'the buffer'. Bacteriophage PRD1 and host bacterial cells, *Salmonella typhimurium* LT2, were kindly provided by Dr Joseph N. Ryan (University of Colorado). Bacteriophage P1 was kindly provided by Dr Andrew Wright (Tufts University).

A suspension of PRD1 (0.1 ml) in the buffer was added to 0.5 ml of *S. typhimurium* cells (in the logarithmic-growth phase) in LB medium (containing 10 mM $CaCl_2$), and the mixture was incubated at 37 °C for 15 min, mixed with 2.5 ml melted LB agar (0.6%, containing 2 mM $CaCl_2$), and poured onto LB agar (1.5%, containing 2 mM $CaCl_2$) in a Petri dish. Following an 8-h incubation at 37 °C, the upper agar layer containing the bacteriophage was scraped off into 5 ml of the buffer and shaken at room temperature for 30 min. The agar and bacterial debris were removed by centrifugation at $8000 \times g$ for 30 min, followed by filtration of the supernatant through a 0.45 μm Millipore membrane. Bacteriophage concentration in the supernatant typically ranged from 10^8 to 10^9 pfu ml^{-1}, as determined by the double-agar method (Adams 1959). Bacteriophage P1 was grown according to the same protocol using *E. coli* K-12 cells (ATCC 10798).

Effect of surfaces on the bacteriophage titer

Desired slides (2.5×3 cm) were immersed in a 40 ml polypropylene tube containing 20 ml of a bacteriophage suspension (approximately 2×10^6 pfu ml^{-1}) in the buffer. The tube was shaken at 200 rpm at room temperature for 24 h,

the slide was removed, the solution was diluted 1000 fold, and the bacteriophage concentration was determined as outlined above.

In experiments with powders, a 1.7 ml polypropylene tube containing 1 ml of a PRD1 suspension in the buffer was incubated for 24 h with 20 mg glass powder at room temperature with shaking (200 rpm). The powder was then separated by centrifugation (8000 × *g*, 5 min), and the bacteriophage concentration in the supernatant was determined using sequential 10-, 100-, and 1000-fold dilutions.

Results and discussion

Bacteriophage PRD1 is some 62 nm in diameter and has an isoelectric point of 3–4 (Harvey & Ryan 2004). We found it to not be 'sticky' toward common surfaces. For example, upon a 24-h shaking at room temperature with a 2.5 × 3 cm glass slide immersed into 20 ml of a buffered aqueous solution, pH 7.4, at a 2×10^6 pfu ml^{-1} concentration of the bacteriophage, the reduction in its titer was the same (about one fourth) as without the slide. (The analogous result was obtained with a piece of another material, undyed wool, of the same size.) In contrast, the titer of an unrelated bacteriophage, P1 (Sternberg & Hoess 1983), under the same conditions after just 7 h dropped almost by half. Given its relative recalcitrance toward adsorption, as well as the aforementioned fact that bacteriophage PRD1 is in many respects a realistic model of adenoviruses (Bamford *et al.* 2002), and indeed of other enteric viruses, this bacteriophage was employed as a target virus in the present investigation.

Our goal was to systematically create surfaces capable of maximally reducing the titer of bacteriophage PRD1. As the initial step to this end, we subjected the foregoing glass slide to chemical modifications designed to introduce certain functionalities. In each instance, the 2×10^6 pfu ml^{-1} bacteriophage was shaken with a slide placed in the solution under the conditions outlined above; after 24 h the slide was removed, and the viral titer was determined and compared with that after the same incubation with no slide.

Due to its acidic isoelectric point and a consequent net negative charge at pH 7.4, bacteriophage PRD1 should be electrostatically attracted to positively charged surfaces. To verify this rationale, we replaced a plain glass slide with that whose surface was derivatized with primary amino groups, many of which would be protonated at pH 7.4. As seen in Figure 1, bar *b*, this slide indeed afforded a marginal reduction of the viral titer to 76 ± 5%. Expectedly, when COOH groups were attached instead of the NH_2 groups, the resultant negligible reduction in the titer (to 89 ± 8%, bar *c*) was indistinguishable from that in the case of plain glass (bar *a*).

To boost the surface density of positive charges, we attached *poly*cations to the glass slide – 25 kDa branched polyethylenimine (PEI) and generation 4.0 dendrimer (polypropylenimine dotriacontaamine). Exposure of the bacteriophage solution to these immobilized polycations cut the titer some four (bar *d*) and five (bar *e*) fold, respectively.

In the next series, we instead raised surface hydrophobicity. Acetylation of an NH_2-glass slide

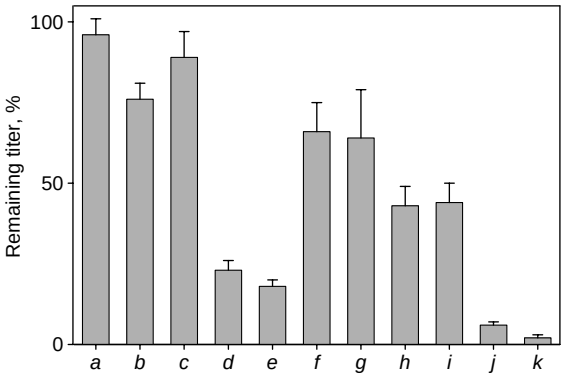

Fig. 1. Percentage of the titer of bacteriophage PRD1 remaining in a buffered aqueous solution (pH 7.4) after a 24-h incubation with various 2.5 × 3 cm slides. Bars: *a*, plain glass (control); *b*, NH_2-glass (i.e., single positive charges); *c*, succinylated NH_2-glass (i.e., single negative charges); *d*, PEI-glass (i.e., multiple positive charges); *e*, dendrimer-glass (i.e., multiple positive charges); *f*, acetylated NH_2-glass (i.e., no charge and minimal hydrophobicity); *g*, polystyrene (i.e., hydrophobic); *h*, palmitoylated NH_2-glass (i.e., no charge and hydrophobic); *i*, 4-bromobutyrylated NH_2-glass to which *N*,*N*-dimethylhexadecylamine was attached (i.e., single positive charges and hydrophobic); *j*, 4-bromobutyrylated PEI-glass to which *N*,*N*-dimethylhexadecylamine was attached (i.e., multiple positive charges and hydrophobic); and *k*, 4-bromobutyrylated dendrimer-glass to which *N*,*N*-dimethylhexadecylamine was attached (i.e., multiple positive charges and hydrophobic). The slides were immersed in 20 ml of a 2×10^6 pfu ml^{-1} bacteriophage PRD1 in 0.1 M Tris/HCl buffer containing 10 mM $MgCl_2$ and shaken at 200 rpm at room temperature; for other conditions, see text.

reduced the viral titer by a third, as did the exposure to a piece of polystyrene of the same size (bars *f* and *g*, respectively, in Figure 1). A more hydrophobic, hexadecanoyl moiety resulted in the remaining titer of 43 ± 6% (bar *h*).

Finally, we prepared glass surfaces containing *both* positive charges *and* hydrophobicity. To this end, first we acylated an amino-glass slide with 4-bromobutyryl chloride (Lin *et al*. 2003), followed by attachment of *N*,*N*-dimethylhexadecylamine. The resultant slide more than halved the viral titer (bar *i*). To raise the positive charge density, we acylated the immobilized polyethylenimine and dendrimer mentioned above with 4-bromobutyryl chloride, followed by alkylation with *N*,*N*-dimethylhexadecylamine (the resulting attached quaternary amine, as in the preceding case, is permanently protonated in addition to being hydrophobic). As seen in Figure 1, bars *j* and *k*, the glass-immobilized hexadecylated PEI and dendrimer slashed the viral titer to 6 ± 1% and 2 ± 1%, respectively. That surfaces combining hydrophobicity and multiple positive charges proved to be far more effective than those with only one of these attributes indicates that the electrostatic attractions and hydrophobic interactions with the bacteriophage are additive and/or that the former are enhanced in hydrophobic environments.

To further explore such both positively charged and hydrophobic surfaces, we examined how the PRD1 titer reduction depends on the time of incubation with the immobilized hexadecyl-PEI and on the latter's available surface. As seen in Figure 2a (the first three bars), the viral titer progressively declined as a function of time over a 24-h period. Interestingly, if the slide was removed after the first 5 h and replaced with a fresh one, a significantly greater titer drop was observed: 99 ± 1% after just a 5-h incubation (the last, dark bar in Figure 2a) as compared to 94 ± 1% after a 17-h incubation with the 'old' slide (the penultimate, slashed slide in Figure 2a). Therefore, we conclude that the surface of immobilized hexadecylated PEI of the size used becomes largely saturated with the bacteriophage and consequently greatly diminished in its capacity to accommodate more.

To confirm this conclusion, we investigated the titer reduction not only with the used heretofore slide size but also with smaller ones. As seen in Figure 2b, the titer reduction was indeed found to be directly proportional to the surface of the hexadecyl-PEI slide. The same phenomenon was observed with hexadecyl-PEI glass *powders* suspended in a bacteriophage solution for 1 h (Figure 2c), whose surfaces were estimated to be roughly an order of magnitude above those of the slides employed in Figure 2b. Thus the PRD1-removing capacity of immobilized hexadecyl-PEI is proportional to the total available surface of that material.

In light of these data, we endeavored to maximize the viral titer reduction afforded by positively charged and hydrophobic surfaces. To this end, bacteriophage PRD1 was shaken in aqueous solutions with 2% glass powders whose surfaces had been derivatized with hexadecyl-PEI and hexadecyl-dendrimers for up to 24 h. As seen in Figure 3, the titer plunge after a 24-h incubation in such conditions approached four orders of magnitude ('logs') – 3.5 for the PEI derivative

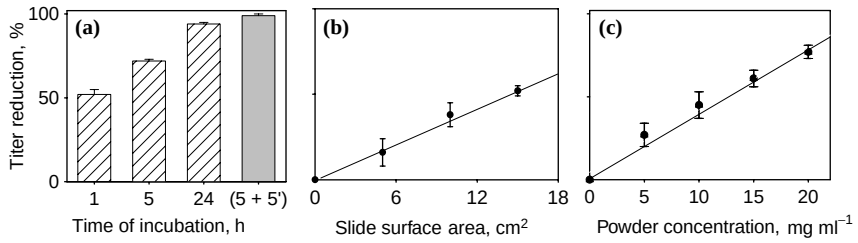

Fig. 2. Percentage of the reduction in the titer of bacteriophage PRD1 in a buffered aqueous solution (pH 7.4) after: (a) an incubation with a hexadecylated PEI-glass slide (see description under bar *j* in Figure 1) for various time periods (slashed bars) or a 5-h incubation with this slide, followed by its removal, replacement with the fresh slide, and another 5-h incubation (dark bar); (b) a 1-h incubation with hexadecylated PEI-glass slides of different sizes (the largest one corresponds to that used in (a); and (c) a 1-h incubation with different concentrations of hexadecylated PEI-glass powder. For the slides, the experimental procedure was the same as outlined in the legend to Figure 1. For the powders, the procedure was identical, except that a 1-ml solution volume was used.

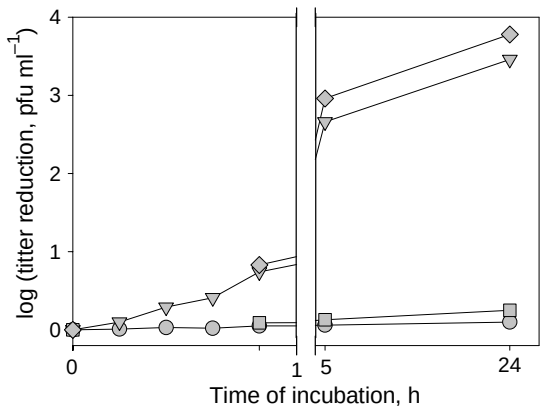

Fig. 3. The time course of the logarithm of the reduction in the titer of bacteriophage PRD1 in a buffered aqueous solution (pH 7.4) containing 2% powders of plain glass (squares), of hexadecylated PEI-glass (see description under bar *j* in Figure 1) (triangles), and of hexadecylated dendrimer-glass (see description under bar *k* in Figure 1) (diamonds), as well as no powder (control) (circles). For other conditions, see the legend to Figure 2.

(diamonds) and 3.8 for the dendrimer derivative (triangles). For comparison, meager titer reductions were observed in analogous circumstances without powder or with plain glass powder (circles and squares, respectively, in Figure 3).

To gain insights into the mechanism of the titer reductions reported above, we addressed the question of whether it was caused by the adsorption of bacteriophage PRD1 to the surfaces or by interactions with the polycations regardless of whether they are immobilized. Since the hexadecylated PEI and dendrimer are practically insoluble in water, in these experiments we employed their non-alkylated predecessors. As shown in Figure 1, those polycations covalently attached to glass slide surfaces markedly reduced viral titers (bars *d* and *e*, respectively), although not as much as their hexadecyl derivatives (bars *j* and *k*, respectively). The first and third columns in Table 1 present the titer reduction data for slide-immobilized PEI and dendrimer. Separately, we determined the concentration of the polycations created when these slides (total surface area of each is approximately 15 cm^2) were immersed in 20 ml of a bacteriophage solution; that concentration of the free polycation was then added to a solution of the bacteriophage and diluted 1000-fold both immediately and after 24-h incubation. The results obtained in this manner with

Table 1. Titer reduction of bacteriophage PRD1 caused by a 24-h exposure to free or glass-slide-immobilized PEI and dendrimer in a buffered (pH 7.4) aqueous solution.[a]

	PEI		Dendrimer	
	Immobilized	Free	Immobilized	Free
Titer reduction, %	77 ± 3	72 ± 7	82 ± 2	93 ± 1

[a]For experimental conditions, see the legend to Figure 1 and text.

PEI and with dendrimer, respectively, are presented in the second and fourth columns in Table 1. It is seen that the free polycations are at least as effective as their corresponding immobilized counterparts. Hence the PRD1 titer reduction is due to inactivation of the bacteriophage brought about by the polycations; the accompanying act of adsorption (in Figures 1, 2, and 3) appears to be merely incidental.

Conclusion

The results of this study demonstrate that surface-immobilized hydrophobic polycations efficiently inactivate waterborne bacteriophage PRD1. This detoxification strategy will be tested next with adenoviruses and other animal viruses.

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